Marine Biology (2004) 144: 1151–1155
DOI 10.1007/s00227-003-1285-3

RESEARCH ARTICLE

B. W. Jones · M. K. Nishiguchi

Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda)

Received: 27 May 2003 / Accepted: 24 November 2003 / Published online: 10 January 2004

Abstract The mutualism between the Hawaiian bobtail squid *Euprymna scolopes* and the luminescent symbiont *Vibrio fischeri* has been used extensively as a model system for studies ranging from co-speciation and biogeography to gene regulation and the evolution of pathogenesis. In this association, the luminescent bacterium *V. fischeri* is housed in a complex light organ within the mantle cavity of *E. scolopes*. Prior hypotheses have assumed that sepiolid squids in general utilize the bioluminescence produced by their *V. fischeri* symbionts for counterillumination, a behavior that helps squid camouflage themselves by matching down-welling moonlight via silhouette reduction. This assumption, based solely on the morphology of the squid light organ, has never been empirically tested for *Euprymna* in the laboratory. Here, we present data demonstrating that *E. scolopes* can modify the intensity of light produced by *V. fischeri* in the light organ as down-welling light intensity changes. Bacterial bioluminescence from the light organ is directly correlated with down-welling light intensity, suggesting that *E. scolopes* individuals utilize and control *V. fischeri* luminescence for counterillumination.

Introduction

Marine organisms use bioluminescence in a variety of behaviors, including intraspecific communication (Herring 2000), prey attraction (Munk 1999; Johnson et al. 1999), predator evasion (Hartline et al. 1999), and counterillumination, an antipredatory behavior common to many midwater cephalopods, decapod crustaceans, and fishes (Young 1977; Harper and Case 1999; Lindsay et al. 1999). Animals exhibiting counterillumination reduce their silhouette by producing bioluminescence in an attempt to match the intensity and wavelength of down-welling light (Young and Roper 1977), providing a mechanism that allows them to evade predators by camouflage. The light produced can either be autogenic (luminescence produced intrinsically by the animal itself), or bacteriogenic (produced by bacterial symbionts).

Establishing a morphological design for efficient counterillumination has resulted in the evolution of a variety of complex organs and photoreceptors found in many families of cephalopods and fishes (Nesis 1982; Mangold and Boletzky 1988; McFall-Ngai 1990). For ventral bioluminescent counterillumination to be effective in these animals, most individuals must live at a depth where bioluminescence is feasible (Young 1977). Light levels cannot be too low (where counterillumination is unnecessary) or too bright (where animals cannot efficiently reduce their shadow). The bioluminescence must shield opaque structures in the animal when viewed from below, and match the characteristics of down-welling light. Light must also be produced for extended periods of time and adjusted with animal movement or body position in the water column (Young 1977). Many examples exist in cephalopod and fish species that have light organs presumably for counterillumination (Young and Roper 1976; Young 1977; McFall-Ngai 1989, 1990; Lindsay et al. 1999). Although many of the morphological features of these taxa are similar, few species have been tested empirically (Young and Roper 1976).

There are a number of other photophore or light-sensitive organs that also allow animals to monitor light for counterillumination. For example, some squids contain extra-ocular photosensitive vesicles that work in conjunction with their eyes to monitor both down-welling light and light created for counterillumination

Communicated by P.W. Sammarco, Chauvin

B. W. Jones · M. K. Nishiguchi (✉)
Department of Biology, New Mexico State University,
Box 30001, MSC 3AF, Las Cruces,
NM 88003-8001, USA
E-mail: nish@nmsu.edu
Tel.: +1-505-6463721
Fax: +1-505-6465665

(Young et al. 1979). Many cephalopods also have a photoreceptive nuchal organ (Parry 2000). This organ is found in the mantle cavity (a conserved morphological trait), which differs from other photosensitive vesicles found in many decapod cephalopods (Parry 2000). Although it has the morphology of a photoreceptive organ, the function of the nuchal organ is unclear. The pineal complex also functions as a light receptor in the lanternfish, *Triphoturus mexicanus* (McNulty and Nafpaktitis 1976). This organ contains a number of receptor cells behind a translucent window, enabling photoreception. Both organ types enable the animal to detect light and respond in a manner that will help it conform to its surroundings (in this case, down-welling light).

Sepiolid squids (Cephalopoda: Sepiolidae) utilize bacteriogenic light from a variety of luminescent symbionts, mostly in the genera *Vibrio* and *Photobacterium* (Ruby and Nealson 1976; Nealson and Hastings 1991). The bacteria are housed in a bilobed light organ that is located within the mantle cavity of the squid (Fig. 1A). This light organ contains a number of unique tissue layers, including the mesodermally derived reflective tissue and lens (Fig. 1B), which reflects (reflector) and focus (lens) light ventrally (Montgomery and McFall-Ngai 1992); the ectodermally derived crypts (Fig. 1B), which invaginate to form cavities where the bacteria reside (Montgomery and McFall-Ngai 1994); and an ink sac which enables the squid to control the intensity of light produced by functioning as a diaphragm around the light organ complex (McFall-Ngai and Montgomery 1990). Together, these tissues form a dynamic and complex organ that allows the squid to focus light ventral (Fig. 1A) to its body cavity while swimming (Montgomery and McFall-Ngai 1993). The distinct and unique morphology of the bacteriogenic light organ has led researchers to hypothesize that it is involved in counterillumination. Since sepiolid squids are nocturnal, they are known to be active predators only after dusk. During the day, squids bury themselves in the sand to evade diurnal predators. Previous research has demonstrated that 95% of the bacteria in the light organ are vented daily with the onset of dawn (Boettcher et al. 1996), presumably to reduce the metabolic costs of maintaining a full bacterial population in the light organ (Nyholm and McFall-Ngai 1998). In an adult sepiolid squid, this approximates to 10^{12} bacteria in one entire light organ (Ruby and Asato 1993). During daytime hours, numbers of bacteria slowly increase from the remaining 5%, until the full complement of bacteria is attained by dusk when bioluminescence is needed for predator evasion (Boettcher et al. 1996; Nishiguchi et al. 1996; McFall-Ngai 1999; Nishiguchi 2001). Morphological and physiological evidence have led researchers to assume that the light organ is used for counterillumination. At this time, no studies have shown whether *E. scolopes* responds to changes in down-welling light with respect to immediate changes in its surrounding environment. Therefore, we have chosen to test this hypothesis by monitoring *E. scolopes* behavior with respect to changes in artificially produced down-welling light.

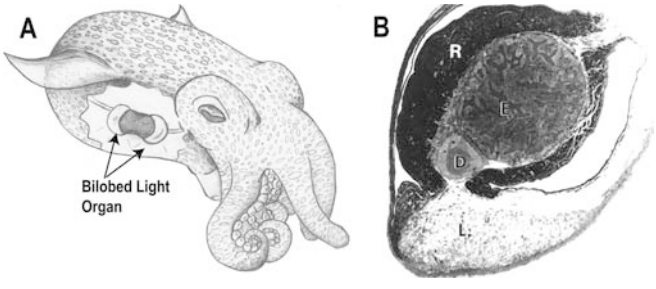


Fig. 1 A Cartoon diagram of the Hawaiian bobtail squid, *Euprymna scolopes*, with a schematic of the light organ exposed within the mantle cavity. Drawing by G. Williams. **B** A saggital section of the light organ, which contains a variety of tissues, including dorsal reflective tissue (*R*), the epithellia-lined crypts (*E*) housing *Vibrio fischeri* symbionts, diverticulum (*D*) and lens (*L*). Photo by M.J. McFall-Ngai

Materials and methods

Thirty-two *E. scolopes* individuals were collected from Paiko Beach (Honolulu, Hawaii) for use in the experiments. Animals were collected at low tide after sunset near the shoreline using a flashlight and handheld dip net. Animals were brought back to the University of Hawaii Kewalo Marine Laboratory and acclimated in running seawater tanks until ready for transport. After 4–5 days, animals were then transported to our squid facilities at New Mexico State University and maintained in a 379-L re-circulating tank held at 22°C in 34 ppt seawater (Instant Ocean) with a 12:12 h light:dark cycle. All counterillumination measurements were made approximately 2–4 h after the room lights were turned off for the evening.

Each squid was placed in a small, seawater filled, clear plastic chamber that was approximately the length and width of the animal in order to restrict any movement. This chamber was then placed within an opaque compartment that inhibited horizontally directed light and enabled down-welling light to be detected by the animal. A counterillumination device was used to measure both overhead light and luminescence that was produced by each squid (Fig. 2). This apparatus consisted of a 3.2-mm fiber-optic probe connected to an EMI 9789 photomultiplier tube. Light intensity

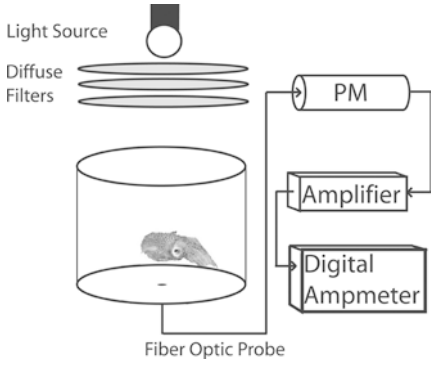


Fig. 2 The luminescence apparatus. Diffuse filters in front of the light source modified the intensity of light reaching *E. scolopes*. Bioluminescence was detected by the fiber-optic probe connected to the photomultiplier tube (PM) and intensity recordings were read from the ampmeter. Ambient light intensity measurements were taken in the identical apparatus not containing a specimen

values were displayed on a digital Keithley Instruments Model 480 picoampmeter. A diffuse light source was used and projected in the room for each of the experimental trials, and intensity measurements (in microamperes) were recorded.

To quantify down-welling light intensities, the fiber-optic probe was placed directly below an identical chamber to that previously described, without any squid present. To measure bioluminescence, the probe was placed directly beneath the light organ of each squid in the chamber after the animal had acclimated to a particular light level for 5 min. Since the mantle of *Euprymna* is somewhat translucent, it was impossible to measure bioluminescence directly while the lights were on without overhead light contamination. For this reason, bioluminescence intensity measurements were taken immediately as the overhead light was turned off. Animals were subjected to three different types of experiments, which consisted of measuring bioluminescence at (1) periods of 5-min intervals of increased down-welling light followed by 5-min intervals of no light; (2) acclimation to light at high levels (approximately 0.09 μA) for 5 min, followed by 15-s intervals with no down-welling light source; and (3) varying down-welling light intensities, including intensities where squids could not efficiently countershade. Six squids were tested for each type of light trial. Statistical analyses of light intensity and squid bioluminescence were used to estimate degree of correlation between the two.

Results

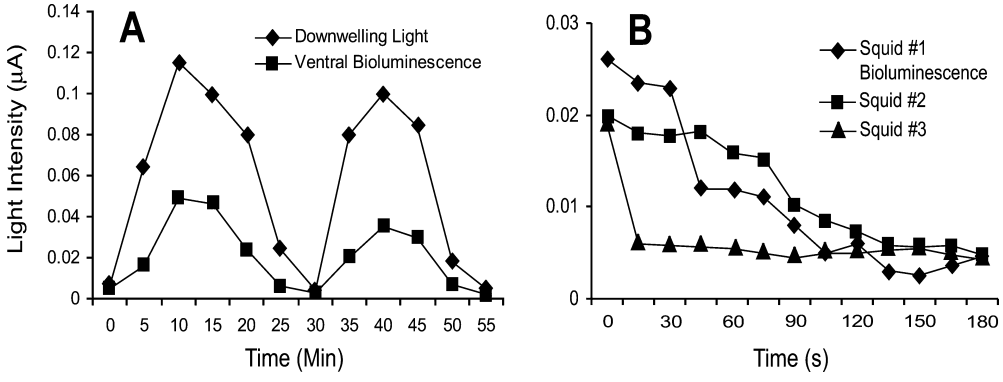
Figure 3 demonstrates the amount of light produced by each squid as down-welling light intensity changed during each trial. As the artificial light source was increased or decreased to different levels, each squid was able to adjust luminescence accordingly, even when light levels increased or decreased over multiple cycles (Fig. 3A). Squids only produced a fraction (about one-third) of bioluminescence at higher intensities (Fig. 3A). During consecutive trials, squid bioluminescence was also monitored after the artificial light source was terminated for light-acclimated animals. Two responses were observed for those trials; first, either squids terminated bioluminescence from their light organs within about 15 s (squid 3, Fig. 3B), or light production gradually decreased over a period of 2–3 min (squids 1 and 2, Fig. 3B). Some animals did not emit any luminescence when multiple trials were attempted. Those animals seemed to be perturbed by the manipulations and were not used for the remainder of the experiments.

Overhead light intensity was strongly correlated with bioluminescence of *E. scolopes* individuals when tested for all types of light fluctuations (Fig. 4A; $r^2 = 0.89$–0.96). We found that animals were able to increase their light emission up to tenfold. Ventral bioluminescence decreased in intensity after squids were exposed to increased light levels. This is shown in Fig. 4B, where bioluminescence and down-welling light were highly correlated ($r^2 = .90$) except at high light intensities, where squids reduced their bioluminescence. We also observed that if animals were perturbed during any point in the trial (sudden movement of the investigator), they would ink in the chamber or reduce or eliminate their luminescence, which terminated the experimental trial and measurements.

Discussion and conclusions

This is the first reported study that demonstrates counterillumination by *E. scolopes*. Even though counterillumination has been shown in other related cephalopod species, this information provides a number of insights for studying the interactions between hosts that contain light organs and their luminescent symbionts. *E. scolopes* appears to control light emission in a rapid, practical manner in response to down-welling light (Fig. 3A). Each squid was able to appropriately adjust its own inherent bioluminescence intensity over a period of increasing or decreasing down-welling light. As previously noted in other cephalopods by Young et al. (1980), test animals would increase their light production with increasing down-welling light to a certain intensity, then decrease bioluminescence at higher light levels (Fig. 4B). This may be due to the need to decrease the amount of energy required to support the metabolically expensive bacterial light production reactions at high light levels where counterillumination is no longer effective (Ruby 1999; Ruby and McFall-Ngai 1999). Also, the amount of bacteria in an adult sepiolid squid is approximately 10^{12} cells (McFall-Ngai and Ruby 1991) and the light limi-

Fig. 3 **A** Changes in the intensity of light produced by *E. scolopes* during one trial (1 h) in which down-welling light levels were slowly increased, decreased, and repeated. **B** Two types of responses by *E. scolopes* when overhead light (approximately 0.09 μA) was terminated after 5 min of light exposure. Squids 1 and 2 slowly decreased light production whereas squid 3 stopped light production within 15 s



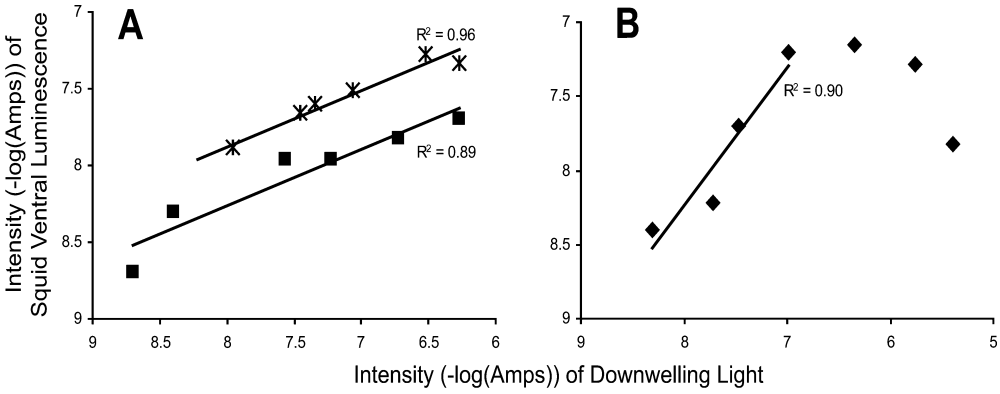


Fig. 4 **A** Two examples of the correlation between overhead light intensity and *E. scolopes* ventral bioluminescence. **B** Reduction of ventral bioluminescence at increased down-welling light intensities. The regression was performed on the first four data points

tations on that concentration of bacteria may also limit the bioluminescence produced by an individual squid. Finally, in the natural environment of sepiolids, light levels probably do not exceed the levels to which squids produce bioluminescence (Young 1977).

As mentioned, *E. scolopes* has two behavioral responses that reduce light production after down-welling light has been terminated. In the first response, bioluminescence from the squid decreases rapidly, due to either (1) the ink sac diverticula that act as an iris to control the intensity of emission (McFall-Ngai 1999), or (2) yellow filters that are present over the ventral surface that may act to shift the wavelength of luminescence (McFall-Ngai 1999). The second response is a slow decline in light production over a period of 2–3 min (Fig. 3B). It has been previously suggested that oxygen limitation in the light organ crypts by the host may be used to control bacterial light production since oxygen is a necessary component of the luminescence reactions (Hastings et al. 1987; Ruby 1996; Small and McFall-Ngai 1998; Ruby and McFall-Ngai 1999). The effect of oxygen limitation on light production has also been explored in the leiognathid fishes, which have a light organ that is separated from the gas bladder by an oxygen-permeable membrane (McFall-Ngai 1989). When oxygen concentrations within the gas bladder are experimentally increased, there is a concurrent increase in light production (McFall-Ngai 1989). This phenomenon is presumably responsible for controlling light produced by the bacterial symbionts. Accordingly, a source of oxygen for the light organ in sepiolid squids has not been identified, although several attempts have been made to discover what sources are responsible for the oxygen needed for the luciferase reactions (Small and McFall-Ngai 1998; Ruby and McFall-Ngai 1999; Visick et al. 2000). In our present study, it was not possible to examine whether each individual squid was decreasing luminescence by covering its light organ very slowly with its ink sac. The idea of oxygen limitation in the crypts leading to decreased luminescence, however, is an intriguing possibility to pursue in the future.

While it is clear that *E. scolopes* is attempting counterillumination, whether this behavior confers a fitness advantage has yet to be investigated. To date, only one study using the plainfish midshipman *Porichthys notatus* has successfully attempted to quantify the reduction in predation by counterilluminating animals (Harper and Case 1999). In this study, both juvenile non-luminous and luminous *P. notatus* were exposed to predation by conspecific adults. In dim light, luminous juveniles were preyed upon significantly less than their non-luminous counterparts. Similar experiments presenting both aposymbiotic and symbiotic *E. scolopes* to an appropriate predator may verify whether counterillumination does increase survival (and therefore, fitness). Work by Nishiguchi et al. (1998) and by Nishiguchi (2002) has shown the association between sepiolid squids and their bacterial endosymbionts to be highly specific for bacteria that are capable of producing luminescence in squid light organs. Other experiments have shown that once bacterial genes responsible for light production are removed, there is a decrease in colonization efficiency and induction of cell swelling in the host epithelia (Visick et al. 2000). Future experiments examining the quality of light produced by non-native symbionts in *E. scolopes* and whether a better adapted symbiont affects host fitness will provide interesting and valuable information about the nature of local adaptation and specificity among symbiotic partners.

Acknowledgements We would like to thank R.E. Young for donating the counterillumination apparatus and assisting in the initial stages of this project. Drs. M.J. McFall-Ngai and E.G. Ruby from the Kewalo Marine Laboratory at the University of Hawaii have been generous with space and tanks during collection of *Euprymna* on the island of Oahu, Hawaii. The authors greatly appreciate the help of J. Bitsoie and E. Amezquita for their assistance during midnight hours for all counterillumination experiments. We would also like to acknowledge two anonymous reviewers and members of the Nishiguchi lab for their suggestions and comments during the project and on the manuscript. Funding was provided by NSF DEB-0316516, NSF DBI-0079820, NSF SBE-0123690, and NIH SO6-GM08136-26 grants to M.K.N., and NIH-RISE GM61222-01, NIH-MARC GM0766724, and NIH-BRIDGES GM48998 for support of minority undergraduates (Amezquita and Bitsoie) at NMSU. All experiments comply with the current laws of the country in which the experiments were performed (U.S.A.).

References

Boettcher KJ, Ruby EG, McFall-Ngai MJ (1996) Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. J Comp Physiol B 179:65–73

Harper RD, Case JF (1999) Counterillumination and its antipredatory value in the plainfin midshipman fish *Porichthys notatus*. Mar Biol 134:529–540

Hartline DK, Buskey EJ, Lenz PH (1999) Rapid jumps and bioluminescence elicited by controlled hydrodynamic stimuli in a mesopelagic copepod, *Pleuromamma xiphias*. Biol Bull 197:132–143

Hastings JW, Makemson JC, Dunlap PV (1987) How are growth and luminescence regulated independently in light organ symbionts? Symbiosis 4:3–24

Herring PJ (2000) Species abundance, sexual encounter and bioluminescent signaling in the deep sea. Philos Trans R Soc Lond B 355:1273–1276

Johnson S, Balser EJ, Fisher EC, Widder EA (1999) Bioluminescence in the deep-sea cirrate octopod *Stauroteuthis syrtensis* Verrill (Mollusca: Cephalopoda). Biol Bull 197:26–39

Lindsay SM, Frank TM, Kent J, Partridge JC, Latz MI (1999) Spectral sensitivity of vision and bioluminescence in the midwater shrimp, *Sergestes similis*. Biol Bull 197:348–360

Mangold K, Boletzky S (1988) Mediterranean cephalopod fauna. In: Clarke MR, Trueman ER (eds) The Mollusca. Academic Press, San Diego, Calif., pp 315–330

McFall-Ngai MJ (1989) Luminous bacterial symbiosis in fish evolution: adaptive radiation among the Leiognathid fishes. In: Margulis L, Fester R (eds) Symbiosis as a source of evolutionary innovation. MIT Press, Cambridge, Mass., pp 381–409

McFall-Ngai MJ (1990) Crypsis in the pelagic environment. Am Zool 30:175–188

McFall-Ngai MJ (1999) Consequences of evolving with bacterial symbionts: lessons from the squid-vibrio association. Annu Rev Ecol Syst 30:235–256

McFall-Ngai MJ, Montgomery MK (1990) The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). Biol Bull 179:332–339

McFall-Ngai MJ, Ruby EG (1991) Symbiont recognition and subsequent morphogenesis as early events in an animal–bacterial symbiosis. Science 254:1491–1494

McNulty JA, Nafpaktitis BG (1976) The structure and development of the pineal complex in the lanternfish *Triphoturus mexicanus* (family Myctophidae). J Morphol 150:579–605

Montgomery MK, McFall-Ngai MJ (1992) The muscle-derived lens of a squid bioluminescent organ is biochemically convergent with the ocular lens. Evidence for recruitment of aldehyde dehydrogenase as a predominant structural protein. J Biol Chem 267:20999–21003

Montgomery MK, McFall-Ngai M (1993) Embryonic development of the light organ of the Sepiolid squid *Euprymna scolopes* Berry. Biol Bull 184:296–308

Montgomery MK, McFall-Ngai MJ (1994) Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Eupyrmna scolopes*. Development 120:1719–1729

Munk O (1999) The escal photophore of ceratioids (Pisces: Ceratioidei): a review of structure and function. Acta Zool (Stockholm) 80:265–284

Nealson KH, Hastings JW (1991) The luminous bacteria. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH (eds) The Prokaryotes, a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Springer, Berlin Heidelberg New York, pp 625–639

Nesis KN (1982) Cephalopods of the world. TFH Publications, Neptune City, N.J.

Nishiguchi MK (2001) Co-evolution of symbionts and hosts: The sepiolid-*Vibrio* model. In: Seckbach J (ed) Symbiosis: mechanisms and model systems. Cole-Kluwer Academic, Dordrecht, The Netherlands, pp 757–774

Nishiguchi MK (2002) Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. Microb Ecol 44:10–18

Nishiguchi MK, Lamarcq LH, Ruby EG, McFall-Ngai MJ (1996) Competitive dominance of native strain symbiotic bacteria measured by colonization and *in situ* hybridization. Am Zool 36:41A

Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-vibrio symbioses. Appl Environ Microbiol 64:3209–3213

Nyholm SV, McFall-Ngai MJ (1998) Sampling the light-organ microenvironment of *Euprymna scolopes*: description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri*. Biol Bull 195:89–97

Parry M (2000) A description of the nuchal organ, a possible photoreceptor, in *Euprymna scolopes* and other cephalopods. J Zool (Lond) 252:163–177

Ruby EG (1996) Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri–Euprymna scolopes* light organ symbiosis. Annu Rev Microbiol 50:591–624

Ruby EG (1999) Ecology of a benign "infection": colonization of the squid luminous organ by *Vibrio fischeri*. American Society of Microbiology, Washington, D.C.

Ruby EG, Asato LM (1993) Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch Microbiol 159:160–167

Ruby EG, McFall-Ngai MJ (1999) Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends Microbiol 7:414–420

Ruby EG, Nealson KH (1976) Symbiotic associations of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*: a model of symbiosis based on bacterial studies. Biol Bull 151:574–586

Small AL, McFall-Ngai MJ (1998) A halide peroxidase in tissues interacting with bacteria in the squids *Euprymna scolopes*. J Cell Biochem 72:445–457

Visick KL, Foster JS, Doino JA, McFall-Ngai MJ, Ruby EG (2000) *Vibrio fischeri lux* genes play and important role in colonization and development of the host light organ. J Bacteriol 182:4578–4586

Young RE (1977) Ventral bioluminescent countershading in midwater cephalopods. Symp Zool Soc Lond 38:161–190

Young RE, Roper CFE (1976) Bioluminescent countershading in mid-water animals: evidence from living squid. Science 191:1046–1048

Young RE, Roper CFE (1977) Intensity regulation of bioluminescence during countershading in living midwater animals. Fish Bull U S:239–252

Young RE, Roper CFE, Walters JF (1979) Eyes and extraocular photoreceptors in midwater cephalopods and fishes: their roles in detecting down-welling light for counterillumination. Mar Biol 51:371–380

Young RE, Kampa EM, Maynard SD, Mencher FM, Roper CFE (1980) Counterillumination and the upper depth limits of midwater animals. Deep-Sea Res 27A:671–691

Blackwell Publishing

Cladistics 20 (2004) 454–486

Cladistics

www.blackwell-synergy.com

A combined approach to the phylogeny of Cephalopoda (Mollusca)

A. R. Lindgren[1,†], G. Giribet[2] and M. K. Nishiguchi[1,*]

[1]*Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA;* [2]*Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 16 Divinity Ave., Cambridge, MA 02138, USA*

Accepted 21 September 2004

Abstract

Cephalopoda represents a highly diverse group of molluscs, ranging in habitat from coastal regions to deep benthic waters. While cephalopods remain at the forefront of modern biology, in providing insight into fields such as neurobiology and population genetics, little is known about the relationships within the group. This study provides a comprehensive phylogenetic analysis of Cephalopoda (Mollusca) using a combination of molecular and morphological data. Four loci (three nuclear 18S rRNA, fragments of 28S rRNA and histone H3 and one mitochondrial cytochrome *c* oxidase subunit I) were combined with 101 morphological characters to test the relationships of 60 species of cephalopods, with emphasis within Decabrachia (squids and cuttlefishes). Individual and combined data sets were analyzed using the direct optimization method, with parsimony as the optimality criterion. Analyses were repeated for 12 different parameter sets accounting for a range of indel/change and transversion/transition cost ratios. Most analyses support the monophyly of Cephalopoda, Nautiloidea, Coleoidea and Decabrachia, however, the monophyly of Octobrachia was refuted due to the lack of support for a Cirroctopoda + Octopoda group. When analyzing all molecular evidence in combination and for total evidence analyses, Vampyromorpha formed the sister group to Decabrachia under the majority of parameters, while morphological data and some individual data sets supported a sister relationship between Vampyromorpha and Octobrachia. Within Decabrachia, a relationship between the sepioids Idiosepiida, Sepiida, Sepiolida and the teuthid Loliginidae was supported. Spirulida fell within the teuthid group in most analyses, further rendering Teuthida paraphyletic. Relationships within Decabrachia and specifically Oegopsida were found to be highly parameter-dependent.

Cephalopoda Cuvier, 1797 is the third largest molluscan class (after gastropods and bivalves), and comprises more than 800 marine species, inhabiting a variety of ecosystems, ranging from coastal to abyssal depths. Cephalopods exhibit many unique characteristics that distinguish them from other molluscs such as horny beaks, complex eyes with a lens, a closed circulatory system, a highly centralized nervous system, modification of the foot into circum-oral appendages, and a funnel apparatus that allowed them to become active swimmers, mostly independent of the ancestral benthic lifestyle of other molluscs. While cephalopods exhibit major morphological and physiological divergence from other molluscan classes, a great deal of diversity also exists within the group. Size-wise, cephalopods range from about 10 millimeters in mantle size in *Idiosepius,* to several meters in the giant squid, *Architeuthis*.

Cephalopoda is subdivided into Nautiloidea and Coleoidea. Nautiloidea consists of a single taxon, Nautilidae, which possesses a coiled, chambered, calcified, external shell, hypothesized to be plesiomorphic (Young et al., 1998). Coleoidea contains all other extant taxa, where the characteristic shell has been internalized and reduced, or completely lost. The extant Coleoidea can be divided into two subgroups (*sensu* Boletzky, 2003); Decabrachia (the squids and cuttlefish) and Vampyropoda. Within Vampyropoda (e.g., Boletzky, 2003), three lineages have been recognized, Vampyromorpha (monotypic), Cirroctopoda (finned octopods) and Octopoda (all non-finned octopods). Octobrachia was also used to delineate a close relationship between

*Corresponding author.
E-mail address: nish@nmsu.edu
†Present address: Department of Evolution, Ecology and Organismal Biology, The Ohio State University, 300 Aronoff Labs, Columbus, OH 43210, USA.

Octopoda and Cirroctopoda. Initially, Vampyroteuthidae was placed within the finned octopods; however, Pickford (1939) elevated the family Vampyroteuthidae to the ordinal level, Vampyromorpha, based on the presence of a broad gladius and 10 arms (two of which were identified as retractile filaments). The position of Vampyromorpha has been highly controversial because it contains several autapomorphic characters which are not shared with octobrachians nor decabrachians. Young et al. (1998) considered it to be an intermediate form, but more closely related to octobrachians, and sperm morphology also suggests a relationship to octobrachians (Healy, 1989).

Decabrachian classification

Within Decabrachia, Boletzky (2003) proposed five orders, Spirulida (monogeneric), Sepiida, Sepiolida, Idiosepiida and Teuthida. The so-called sepioid orders, Sepiida, Sepiolida, Idiosepiida, Spirulida, have traditionally been placed in a single order, Sepioidea (*sensu* Naef, 1921/23) based on shared characters such as simple funnel locking apparatus, rounded fins, conservative embryonic development and progressively reduced shells. Spriulida and Sepiida have calcified shells, Sepiolidae has an uncalcified, reduced gladius, with a proostracum still present, while Idiosepiida has a very thin, uncalcified remnant shell that went unnoticed in older descriptions. Spirulida was placed within the sepioid order by Chun (1914), a position further investigated by Naef (1921/23). Although the shells of Spirulida and Sepiida appear to be vastly different, Naef believed that they could have arisen from a common ancestor due to their similar phragmocone morphology. However, the position of Spirulida has remained questionable (e.g., Bonnaud et al., 1997; Carlini and Graves, 1999). Two suborders were established within Teuthida; Myopsida (containing a single family, Loliginidae [now also including Pickfordiateuthidae]) and Oegopsida (all other squid families). Myopsida was distinguished by the presence of a corneal cover over the eye, whereas the oegopsid eye has no covering and is in direct contact with the water.

The interfamilial relationships within Teuthida have remained problematic, partly because many characters uniting the suborders remain untested in a phylogenetic study. Myopsids and oegopsids share a similar gladius, branchial canal structure and tentacular club, as well as having a generally "similar" appearance (Young et al., 1998). However, several characters suggest that myopsids may be more closely related to sepiolid squids rather than the oegopsids, such as the presence of a corneal covering, benthic eggs, a similar position of the seminal vesicle, accessory nidamental glands and the presence of suckers on the buccal crown (Young et al., 1998). Traditionally, the Decabrachia has been divided into two orders, Sepioidea (comprising Spirulida, Sepiida, Sepiolida and Idiosepiida) and Teuthoidea, comprising all other squids (e.g., Young and Vecchione, 1996; Beesley et al., 1998). However, Boletzky's (2003) classification accounts for the variability within Decabrachia by establishing separate orders for divergent groups (whose interrelationships remain unknown) while conserving the hypothesized sister relationship between Vampyromorpha and Octobrachia.

Phylogenetic relationships

Although most phylogenetic relationships among the recognized families of Cephalopoda remain ambiguous, morphologically based studies have provided valuable information for higher-level relationships. A recent study by Young and Vecchione (1996) used 25 characters to delineate the interfamilial relationships among 17 families of cephalopods. Their findings provided support for the monophyly of Decabrachia and Octobrachia, respectively, and placed Vampyromorpha as the sister group to Octobrachia. Although resolution was proposed for taxa closely associated with the family Enoploteuthidae (Young and Harman, 1998), little resolution was achieved within the remainder of the Decabrachia. Other morphological studies (Roper et al., 1969, 1984; Toll, 1982; Hess, 1987; Nesis, 1987) also provided characters useful for classification essential but remained untested in any large-scale phylogenetic study.

Molecular studies have recently provided information regarding relationships within cephalopods (Bonnaud et al., 1997; Carlini and Graves, 1999; Carlini et al., 2000, 2001). Bonnaud et al. (1997) generated the first molecular cephalopod study using data from the mitochondrial 16S rRNA locus for 16 species. While this study supported many higher-level relationships hypothesized in morphologically based studies, it did not include many exemplars pertinent for determining lower level relationships. Subsequently, a more comprehensive study by Carlini and Graves (1999) used the cytochrome *c* oxidase subunit I (COI) locus for 48 cephalopod species to examine higher-level relationships. Their results confirmed previously supported morphological data in some areas, but left the relationship of *Vampyroteuthis* questionable, and did not resolve many interfamilial relationships within Decabrachia. A second study (Carlini et al., 2000) using several actin gene loci provided additional data; however, due to the presence of multiple gene copies, results of the analyses were not easy to interpret. Consequently, the first study to analyze both morphological and molecular data in concert (Carlini

et al., 2001) focused on relationships within Octobrachia, but due to a lack of agreement between morphological and molecular data, no new hypotheses were presented. Several recent studies have provided further data on families within Octopoda (Voight, 1997; Carlini et al., 2001; Piertney et al., 2003), but little information has been presented regarding relationships among many of the major groups within Decabrachia.

Given the many discrepancies among defining characters for cephalopods and that their evolution has likely proceeded with large variations in rates among different groups, it is impossible to construct a non-contradictory system based on a single organ or system (Nesis, 1998). The use of combined analyses has provided increased resolution within other "problematic" metazoan clades, particularly within arthropods (e.g., Giribet et al., 2001; Edgecombe et al., 2002), but also for other molluscan classes (e.g., Giribet and Wheeler, 2002). Due to the diverse nature of Cephalopoda, a combined approach is likely to provide further insight into both higher and lower-level relationships. It is the aim of this study to further refine the relationships within Cephalopoda and particularly Decabrachia by incorporating a combination of 101 morphological characters and DNA sequence data from four molecular loci, including two nuclear ribosomal genes, one nuclear protein coding gene and one mitochondrial protein coding gene. By analyzing all data simultaneously, a new hypotheses will be presented for the relationships within Cephalopoda.

Methods

Taxon sampling

Molecular and morphological data from five molluscan classes were analyzed (Tables 1, 2 and 3; Appendices 1 and 2 for voucher information): Caudofoveata (1 sp.), Solenogastres (2 spp.), Polyplacophora (4 spp.), Gastropoda (4 spp.), Bivalvia (4 spp.), Scaphopoda (3 spp.) and Cephalopoda (60 spp.). Cephalopod taxa were sampled from 34 taxonomically recognized families, representing all eight major orders (Tables 2 and 3). Samples from nine cephalopod families were not available for this study due to a lack of specimen availability. Preserved specimens used for molecular analysis were obtained from a number of sources (for collection data and repository institutions see Appendix 1). Specimens for morphological study are listed in Appendix 2.

Morphological characters

Morphological data were scored via the direct observation of cephalopod specimens, and in cases where specimens were unavailable, information was taken from the primary literature (Naef, 1921/23; Roper et al., 1969; Salvini-Plawen and Steiner, 1996; Young and Vecchione, 1996; Young and Harman, 1998), which resulted in 101 characters, described in Appendix 3 and coded in Table 3. Sperm characters were coded entirely from literature sources (Franzén, 1955, 1958; Maxwell, 1974, 1975; Healy, 1990a,b, 1993, 1996). Primary

Table 1
Outgroup taxa and accession numbers for each locus used in this study

	18S rRNA	28S rRNA	Histone H3	COI
Aplacophora				
Chaetoderma nitidulum	AY377658	AY377692	AY377763	AY377726
Heliocoradomenia sp.	AY21210	AY377688	AY377764	AY377725
Epimenia azuri	AY377657	AY377691	AY377765	AY377723
Polyplacophora				
Leptochiton asellus	AY377631	AY377662	AY377734	
Stenoplax alata	AY377644	AY377675	AY377748	AY377711
Chiton olivaceus	AY377651	AY377682	AY377755	AY377716
Acanthochitona crinita	AF120503	AF120566	AY377759	AF120627
Gastropoda				
Theodoxus fluviatilis	AF120515	AF120573		AF120633
Haliotis tuberculata	AF120511	AF120570	AY377775	AY377729
Crepidula fornicata	AY377660	AY377625	AY377778	AF353154
Siphonaria pectinata	X91973	AF120578	AY377627	AF120638
Bivalvia				
Yoldia limatula	AY070111	AF120585	AY377768	AF120642
Arca imbricata	AY654986	AY654987	AY654989	AY654988
Neotrigonia margaritacea	AF411690	AF411689	AY070155	AF56850
Cardita calyculata	AF120549	AF120610	AY070156	AF120660
Scaphopoda				
Rhabdus rectius	AF120523	AF120580	AY377772	AF120640
Antalis pilsbryi	AF120522	AF120579		AF120639
Entalina tetragona	AF490598			

Table 2
Cephalopod taxa and GenBank accession numbers for each locus used in this study. Classification based on Boletzky (1999). Sequences with an asterisk indicate those not obtained by the author

			18S rRNA	28S rRNA	Histone H3	COI
Nautiloidea (2 spp.)						
Nautilida	Nautilidae	*Nautilus pompilius*	AY557452	AF311688*		AY557514
		Nautilus scrobiculatus	AF120504*	AF120567*	AF033704*	
Coleoidea (58 spp.)						
Octobrachia						
Octopoda	Allopsidae	*Haliphron atlanticus*	AY557460	AY557549	AY557409	AY557516
		Haliphron sp.	AY557461	AY557550	AY557410	
	Argonautidae	*Argonauta nodosa*	AY557462	AY557551	AY557411	AY557517
	Bolitaenidae	*Japetella diaphana*	AY557463	AY557552		AY557518
	Ocythoidae	*Ocythoe tuberculata*	AY557464	AY557553		AY557519
	Octopodidae	*Bathypolypus arcticus*	AY557465	AY557554		*AF000029
		Benthoctopus sp.	AY557466	AY557555	AY557412	
		Eledone cirrosa	AY557467	AY557556		AY557520
		Grandeledone verrucosa	AY557468	AY557557	AY557413	*AF000042
		Thaumeledone guntheri	AY557469	AY557558	AY557414	AY557521
Cirroctopoda	Cirroteuthidae	*Cirrothauma murrayi*	AY557456	AY557545		*AF000034
		Stauroteuthis syrtensis	AY557457	AY557546	AY557406	*AF000067
	Opisthoteuthidae	*Opisthoteuthis* sp.	AY557458	AY557547	AY557407	AY557515
Vampyromorpha						
Vampyromorpha	Vampyroteuthidae	*Vampyroteuthis infernalis*	AY557459	AY557548	AY557408	*AF000071
Decabrachia						
Sepiolida	Sepiolidae	*Heteroteuthis hawaiiensis*	AY557472	AY293703	AY557416	*AF000044
		Stoloteuthis leucoptera	AY557475	AY293704	AY557419	*AF000068
		Sepiola affinis	AY557474	AY557562	AY557418	AY557523
		Rossia palpebrosa	AY557473	AY557561	AY557417	*AF000061
Sepiida	Sepiidae	*Sepia officinalis*	AY557471	AY557560	AY557415	*AF000062
		Sepiella inermis	AY557470	AY557559		AY557522
Spirulida	Spriulidae	*Spirula spirula*	AY557476	AY557563	AY557420	*AF000066
Idiosepiida	Idiosepiidae	*Idiosepius pygmaeus*	AY557477	AY293684	AY557421	*AF000046
Teuthida Myopsida	Loliginidae	*Loligo formosana*	AY557478	AY557564	AY557422	AY557524
		Loligo pealei	AY557479	AY557565	AY557423	*AF000052
		Sepioteuthis lessoniana	AY557480	AY557566	AY557424	AY557525
Teuthida Oegopsida	Ancistrocheiridae	*Ancistrocheirus lesueuri*	AY557491	AY557575		*AF000026
	Architeuthidae	*Architeuthis dux*	AY557482	AY557567	AY557426	*AF000027
	Bathyteuthidae	*Bathyteuthis abyssicola*	AY557483	AY557568	AY557427	*AF000030
	Batoteuthidae	*Batoteuthis skolops*	AY557484	AY557569	AY557428	AY557527
	Brachioteuthidae	*Brachioteuthis* sp.	AY557485	AY557570	AY557429	AY557528
	Chiroteuthidae	*Chiroteuthis veranyi*	AY557486			AY557529
	Chtenopterygidae	*Chtenopteryx sicula*	AY557481	AY293698	AY557425	AY557526
	Cranchiidae	*Cranchia scabra*	AY557487	AY557571	AY557430	*AF000035
		Leachia atlantica	AY557488	AY557572	AY557431	AY557530
	Cycloteuthidae	*Cycloteuthis syrventi*	AY557489	AY557573	AY557432	*AF000036
		Discoteuthis laciniosa	AY557490	AY557574	AY557433	*AF000037
	Enoploteuthidae	*Abraliopsis pfefferi*	AY557492	AY557576	AY557434	AY557531
		Enoploteuthis leptura	AY557493	AY557577	AY557435	AY557532
		Ornithoteuthis antillarum	AY557494	AY557578	AY557436	AY557533
	Gonatidae	*Gonatus antarcticus*	AY557497	AY557581	AY557439	AY557536
		Gonatus fabricii	AY557498	AY557582	AY557440	AY557537
	Histioteuthidae	*Histioteuthis corona*	AY557499	AY557583	AY557441	
		Histioteuthis hoylei	AY577500	AY557584	AY557442	*AF000045
		Histioteuthis reversa	AY577501	AY557585	AY557443	
	Joubiniteuthidae	*Joubiniteuthis portieri*	AY577502	AY557586	AY557444	*AF000048
	Lepidoteuthidae	*Lepidoteuthis grimaldii*	AY577503	AY557587	AY557445	*AF000049
	Mastigoteuthidae	*Mastigoteuthis agassizii*	AY577504	AY557588	AY557446	AY557538
		Mastigoteuthis magna	AY577505	AY557589	AY557447	AY557539
	Neoteuthidae	*Neoteuthis thielei*	AY577506	AY557590	AY557448	AY557540
	Octopoteuthidae	*Octopoteuthis nielseni*	AY557507	AY557591		*AF000055
		Octopoteuthis sicula	AY557508	AY557592	AY557449	AY557541
	Ommastrephidae	*Illex coindeti*	AY557509	AY557593	AY557450	AY557542
		Ommastrephes bartrami	AY557510	AY557594	AY557451	*AF000057
		Sthenoteuthis oualeniensis	AY557511	AY557595	AY557452	*AF000069
	Onychoteuthidae	*Moroteuthis knipovitchi*	AY557512	AY557596	AY557453	AY557543
	Psychroteuthidae	*Psychroteuthis* sp.	AY557513	AY557597	AY557454	AY557544
	Pyroteuthidae	*Pyroteuthis margaretifera*	AY557496	AY557580	AY557438	AY557535
		Pterygioteuthis gemmata	AY557495	AY557579	AY557437	AY557534

Table 3
Morphological data matrix of 101 characters

	1	11	21	31	41	51	61	71	81	91	
Chaetoderma nitidulum	000--0--0- --010----0	----------	-0-0----00	0000-0----	---1000---	---0-00100	1000-010-0	00000?0000	00000??000	0
Helicoradomenia sp.	000--0--0- --010----0	----------	-0-0----00	0000-0----	---0--0---	---0-?????	????-010-0	00000?0000	00000??000	0
Epimenia azuri	000--0--0- --010----0	----------	-0-0----00	0000-0----	---0--0---	---0-0?0??	1000-010-0	00000?0000	00000??000	0
Leptochiton asellus	000--0--0- --010----0	----------	-0-0----00	0000-0----	---1020---	---0-00000	1000-110-0	0000000000	0000001100	0
Stenoplax alata	000--0--0- --010----0	----------	-0-0----00	0000-0----	---1020---	---0-?????	????-110-0	0000000000	0000001100	0
Chiton olivaceus	000--0--0- --010----0	----------	-0-0----00	0000-0----	---1020---	---0-10000	1001-110-0	0000000000	0000001100	0
Acanthochitona crinita	000--0--0- --010----0	----------	-0-0----00	0000-0----	---1020---	---0-10000	1001-110-0	0000000000	0000001100	0
Theodoxus fluviatilis	100--0--0- --010----0	----------	-0-0----00	1001-0----	---1030---	---0-?????	????000111	0110101000	0101011000	0
Haliotis tuberculata	100--0--0- --010----0	----------	-0-0----00	1001-0----	---1000---	---0-00000	1000000111	0110101000	0101011000	0
Crepidula fornicata	100--0--0- --010----0	----------	-0-0----00	1001-0----	---1030---	---0-?????	????000111	0110101000	0101011000	0
Siphonaria pectinata	100--0--0- --010----0	----------	-0-0----00	1001-0----	---10?0---	---0-00000	100?000111	0110101000	0101011000	0
Yoldia limatula	100--0--0- --000----0	----------	-0-0----00	1001-0----	---1000---	---0-?????	????200000	1001-00010	1010121000	0
Arca imbricata	100--0--0- --000----0	----------	-0-0----00	1001-0----	---1000---	---0-00000	1000200000	1001-10011	1010121000	0
Neotrigonia margaritacea	100--0--0- --000----0	----------	-0-0----00	1001-0----	---1000---	---0-00000	1000200000	1001-10011	1010121000	0
Cardita calyculata	100--0--0- --000----0	----------	-0-0----00	1001-0----	---1000---	---0-00000	1000200000	1001-10011	1010121000	0
Rhabdus rectius	100--0--0- --010----0	----------	-0-0----00	1001-0----	---0--0---	---0-?????	????100000	0000001010	1100011001	1
Antalis pilsbryi	100--0--0- --010----0	----------	-0-0----00	1001-0----	---0--0---	---0-00000	1000100000	0000001010	1100011001	1
Entalina tetragona	100--0--0- --010----0	----------	-0-0----00	1001-0----	---0--0---	---0-?????	????100000	0000001010	1100011001	1
Nautilus pompilius	110--0–01- -111??000-	---0-----0	001-0-1111	01100??00-	001?11220-	--12000110	00?0000010	0000?11001	1000101100	0
Nautilus scrobiculatus	110--0–01- -111??000-	---0-----0	001-0-1111	01100??00-	001?11220-	--12??????	???0000010	0000?11001	1000101100	0
Cirrothauma murrayi	0012-10010	--10120111	00020-----	000110-010	1011112212	1?01100001	?0011?????	????-001-1	00000?1100	1100010100	1
Stauroteuthis syrtensis	0012-10010	--10120111	00020-----	000110-010	1011112212	1201100001	?0011?????	????-001-1	00000?1100	1100010100	1
Opisthoteuthis sp.	0012-10010	--10120111	00020-----	000110-010	101111?212	1001100001	?001100010	1000-001-1	00000?1100	1100010100	1
Haliphron sp.	0013-00010	--11120001	00000-----	000101-011	121111?21?	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Haliphron atlanticus	0013-00010	--11120001	00000-----	000101-011	121111?21?	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Argonauta nodosa	001--00010	--11120001	00000-----	0001?1?011	1211112211	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Japetella diaphana	001--00010	--11120?01	00020-----	000100-011	1211112211	1101100201	00010?????	????-001-1	00000?1100	1100010100	1
Ocythoe tuberculata	0013-00010	--11120001	00000-----	0001?1?011	1211112211	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Bathypolypus arcticus	0013-00010	--11120001	00000-----	000100-010	1211112211	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Benthoctopus sp.	0013-00010	--11120001	00000-----	000100-010	1211112211	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Eledone cirrosa	0013-00010	--11120001	00020-----	000100-011	1211112211	1101100201	0101000000	1000-001-1	00000?1100	1100010100	1
Graneledone verrucosa	0013-00010	--11120001	00020-----	000100-010	1211112211	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Thaumeledone guntheri	0013-00010	--11120001	00020-----	000100-010	1211112211	1101100201	01010?????	????-001-1	00000?1100	1100010100	1
Vampyroteuthis infernalis	0?11011210	--11110011	1?020-----	000100-111	101111111?1	1101000220	0101001000	1000-001-1	00000?1100	1100010100	1
Sepiella inermis	0110010111	1011101001	2110010000	0001110111	1111010000	0101001010	10110?????	????-001-1	00000?1100	1100010100	1
Sepia officinalis	0110-10111	1011101001	2110010000	0001110111	1111010000	0101001010	1011000110	0101-001-1	00000?1100	1100010100	1
Heteroteuthis hawaiiensis	0014010011	1011101001	2110000000	1001110111	1111010000	0101001010	1001000000	0010-001-1	00000?1100	1100010100	1
Rossia palpebrosa	0011010011	1011101001	2110000000	0001110111	1111010000	0101001010	1001000110	0101-001-1	00000?1100	1100010100	1
Sepiola affinis	001?010011	1011101001	2110000000	1001110111	1111010000	0101001010	10010?????	????-001-1	00000?1100	1100010100	1
Stoloteuthis leucoptera	0014010111	1011101001	2110000000	1001110111	1111010000	0101001010	10010?????	????-001-1	00000?1100	1100010100	1
Spirula spirula	0110-10011	1011101001	2111100000	0001110111	1011010000	0101001010	0011000000	0010-001-1	00000?1100	1100010100	1
Idiosepius pygmaeus	0014010010	?011101001	2110000000	000111?111	1111010000	0101001110	00110?????	????-001-1	00000?1100	1100010100	1
Loligo formosana	0011010111	1111101001	2110010000	0001110111	1111010001	0101001010	0011000110	0101-001-1	00000?1100	1100010100	1
Loligo pealei	0011010111	1111101001	2110010000	0001110111	1111010001	0101001010	0011000110	0101-001-1	00000?1100	1100010100	1
Sepioteuthis lessoniana	0011010111	1111101001	21100?0000	0001110111	1111010001	0101001010	00110?????	????-001-1	00000?1100	1100010100	1
Chtenopteryx sicula	0011010111	1111101001	2111110000	0001110111	1011010001	0101001?10	00010?????	????-001-1	00000?1100	1100010100	1
Bathyteuthis abyssicola	0011010111	0111101001	2111110000	0001110111	1011010001	0101001210	00010?????	????-001-1	00000?1100	1100010100	1

Architeuthis dux	00111101111 0?11101001 21100000002 00011101111 10111010001 01010010210 00110????? ????-001-1 00000?1100 11000101001
Batoteuthis skolops	00112111111 1?11101001 21100100000 00011131111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Brachioteuthis sp.	01131021111 11111010012 11010000020 00111101111 01101000010 10100121100 00102????? ????-001-1 00000?1100 11000101001
Chiroteuthis veranyi	00112111111 11111010001 21100000000 00011131111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Cranchia scabra	00113100111 11111010001 21100000002 000120-111 10111010001 01110011210 00110????? ????-001-1 00000?1100 11000101001
Leachia atlantica	00113100111 11111010001 21100000002 000120-111 10111010001 01110011210 00110????? ????-001-1 00000?1100 11000101001
Cycloteuthis sirventi	00113111111 11111010001 21100000002 00011111111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Discoteuthis laciniosa	00113111111 11111010001 21100000002 00011111111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Ancistrocheirus lesueuri	00111101111 02111010001 21100001101 00011101111 10111010001 01010010210 00110????? ????-001-1 00000?1100 11000101001
Abraliopsis pfefferi	00111101111 02111010001 21100001101 00111101111 10111010001 01010000210 00110????? ????-001-1 00000?1100 11000101001
Enoploteuthis leptura	00111101111 02111010001 21100001101 00111101111 10111010001 01010000210 00110????? ????-001-1 00000?1100 11000101001
Pterygioteuthis gemmata	00111101111 02111010001 21100001101 00011101111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Pyroteuthis margaretifera	00113101111 02111010001 211?001101 00111101111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Gonatus antarcticus	00111101111 11111010001 21111011102 00011101111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Gonatus fabricii	00111101111 11111010001 2111-011102 00011101111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Histioteuthis corona	00110101111 01111010001 21101100002 01011101111 10111010001 01010011210 10010????? ????-001-1 00000?1100 11000101001
Histioteuthis hoylei	00110101111 01111010001 21101100002 01011101111 10111010001 01010011210 10010????? ????-001-1 00000?1100 11000101001
Histioteuthis reversa	00110101111 01111010001 21101100002 01011101111 10111010001 01010011210 10010????? ????-001-1 00000?1100 11000101001
Joubiniteuthis portieri	00112111111 11111010001 21111100000 00011113111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Lepidoteuthis grimaldii	00112101111 11111010001 2110?000010 00011101111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Mastigoteuthis agassizii	00112111111 11111010001 21101100000 00011113111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Mastigoteuthis magna	00112111111 11111010001 21101100000 00011113111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Neoteuthis thielei	00111101111 01111010001 2110A00002 00011101111 10111010001 01010011211 00?10????? ????-001-1 00000?1100 11000101001
Octopoteuthis nielseni	00112100111 10111010001 2110-01-1- 00?1110111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Octopoteuthis sicula	00112100111 10111010001 2110-01-1- 00?1110111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Illex coindeti	00111101111 01111010001 21101100002 00011121111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Ommastrephes bartrami	00111101111 01111010001 21100000002 00011121111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Ornithoteuthis antillarum	00111101111 01111010001 21100000002 00011121111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Sthenoteuthis oualaniensis	00111101111 01111010001 21100000002 00011121111 10111010001 01010011210 00110????? ????-001-1 00000?1100 11000101001
Moroteuthis knipovitchi	00111101111 11111010001 21100000102 00?1110111 10111010001 01010011210 00010????? ????-001-1 00000?1100 11000101001
Psychroteuthis sp.	00113102?11 00111010001 21101000002 00011102?11 10111010001 01010011210 00?10????? ????-001-1 00000?1100 11000101001

literature sources were also used to score outgroup characters (Giribet and Wheeler, 2002; Salvini-Plawen and Steiner, 1996; Ponder and Lindberg, 1997; Waller, 1998; Haszprunar, 2000; Haszprunar and Wanniger, 2000). Morphological character data were summarized for each terminal taxon where possible using MacClade (Madison and Madison, 2000); in a few cases where codings were based on related species or primary literature, notations were made in the character description section. Multiple specimens for each family were examined in an attempt to eliminate coding irregularities. Irregularity in specimen morphology could arise genetically via mutation events or perhaps as a result of damage during collection, making it important to establish the character states by examining multiple organisms.

Molecular loci

PCR amplification and sequencing.

DNA was isolated from small pieces of mantle, gill, gonad, or arm tissue of previously identified specimens. DNA extraction was performed using the Qiagen DNeasy Tissue Kit (Qiagen©, Valencia, CA). Upon isolation, the purified total DNA template was used for PCR amplification of four molecular loci: nuclear 18S rRNA (1900–2800 bp), the D3 expansion fragment of 28S rRNA (400–600 bp) and histone H3 (327 bp), as well as a 679 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI). Several primer sequences, described in Table 4, were obtained from primary literature (Folmer et al., 1994; Giribet et al., 1996; Whiting et al., 1997; Colgan et al., 1998) or designed specifically for this study. The complete 18S rRNA (1.8–2.8 kb) was amplified and sequenced in three overlapping fragments of approximately 800–1000 bp in length using primer pairs: 1F/4R, 3bf/18Sbi, 18sa2.0/9R. Additional primers (4bf, 5bf, 5br, 18Sa2.0R, 7F, 7R) were used in samples that were difficult to amplify. PCR amplification, cleanup and sequencing were performed as described in Nishiguchi et al. (2004).

Sequence editing and fragmentation.

Resulting chromatograms were edited and joined into contiguous sequences using Sequencher v. 4.1 (Gene Codes™, Ann Arbor, MI). Complete sequences were visualized and partitioned using the Genetic Data Environment (GDE) software (Smith et al., 1994). External primers (1F/9R for 18S rRNA and standard primer sequences for all other loci) were excluded from the analyses. For non-coding genes (18S rRNA, 28S rRNA), sequences were initially partitioned in GDE using secondary structure models, unambiguous regions and internal primers as described in Giribet and Wheeler (2001). GDE was further used to examine individual

Table 4
Primer sequences obtained from literature; 18S rRNA (Giribet et al., 1996; Whiting et al. 1997), 28S rRNA (Whiting et al., 1997), COI (Folmer et al., 1994) and H3 (Colgan et al., 1998). Primers marked with an asterisk indicate cephalopod-specific primers designed for this study by the authors. Annealing temperature indicates a range over which successful loci were amplified. See Nishiguchi et al. (2004) and Giribet and Wheeler (2002) for a further description of PCR amplification

Primer		Annealing temperature
18S rRNA (Primer pairs commonly used: 1F/4R; 3bf/18Sbi; 18Sa2.0/9R). Other primers listed were used in hypervariable internal regions.		35–49 °C
1F	5′- TAC CTG GTT GAT CCT GCC AGT AG -3′	
3R	5′- AGG CTC CCT CTC CGG AAT CGA AC -3′	
4R	5′- GAA TTA CCG CGG CTG CTG G -3′	
3bf*	5′- GGG TCC GCC CTA TCA ACT G -3′	
4bf*	5′- CCG CGA TCG GAA TGA GTA CAC -3′	
5bf*	5′- GCA TTC CCG GCC CTT -3′	
5br*	5′- GAC CAC CCT TGG AGG AGA AA -3′	
18Sbi	5′- GAG TCT CGT TCG TTA TCG GA -3′	
7R	5′- GCA TCA CAG ACC TGT TAT TGC -3′	
18Sa2.0rev*	5′- GTT TCA GCT TTG CAA CCA T -3′	
18Sa2.0	5′- ATG GTT GCA AAG CTG AAA C -3′	
7F	5′- GCA ATA ACA GGT CTG TGA TGC CC -3′	
9R	5′- GAT CCT TCC GCA GGT TCA CCT AC -3′	
28S rRNA		37–40 °C
28Sa	5′- GAC CCG TCT TGA AAC ACG GA -3′	
28Sb	5′- TCG GAA GGA ACC AGC TAC -3′	
Cytochrome *c* oxidase subunit I (COI)	35–39 °C	
LCO1490	5′- GGT CAA CAA ATC ATA AAG ATA TTG G -3′	
HCO2198	5′- TAA ACT TCA GGG TGA CCA AAA AAT CA -3′	
Histone H3		37–42 °C
H3a F	5′- ATG GCT CGT ACC AAG CAG AC(ACG) GC -3′	
H3a R	5′- ATA TCC TT(AG) GGC AT(AG) AT(AG) GTG AC -3′	

sequences to identify regions with large insertions or deletions. To account for the high degree of variability in sequence length (with indels up to 500 bp in some cases) and the large size of the nuclear genes, 18S rRNA was partitioned into 30 fragments and 28S rRNA in three fragments. For the protein-coding gene COI, sequences were partitioned into four sections due to the presence of length variability in some species. Histone H3 (also protein-coding) was not fragmented since no sequence length variation was present and therefore was treated as "prealigned" (command -prealigned) in the analysis. Sequences with no length variation can be treated as prealigned because they require no insertion of gaps during alignment. A number of hypervariable regions within the ribosomal genes (18S rRNA, 28S rRNA) were excluded from the analyses because they are extremely difficult to align, can be uninformative and may introduce conflict into the analyses (Giribet et al., 2000). These fragments may show considerable variation even among members of the same species. Fragmented sequences, as well as a list of those fragments removed are available at http://biology-web.nmsu.edu/Faculty&Staff/Nishiguchi/Nishiguchi.htm.

Phylogenetic analysis

Morphological data analysis.

Morphological data were analyzed with parsimony in NONA v. 2.0 (Goloboff, 1998), with 1000 random addition sequence replicates (RAS) followed by tree bisection and reconnection (TBR) branch swapping. In order to avoid spending too much time searching tree space in suboptimal islands, the number of trees held per replicate was limited to 10. Strict consensus calculations and character optimization were completed using Winclada v. 1.00.08 (Nixon, 2002). Character optimizations calculated in Winclada only show unambiguous changes. Nodal support was determined using jackknifing (Farris et al., 1996; Farris, 1997), where jackknife proportions were calculated from 1000 replicates using 10 RAS + TBR in Winclada/Nona.

Molecular and combined analysis.

Molecular and combined data were analyzed with the computer program POY (Wheeler et al., 2002) using the direct optimization method (Wheeler, 1996) with parsimony as the optimality criterion. Independent sets of analyses were executed in POY for each of the following data sets: COI, H3, ribosomal (18S rRNA + 28S rRNA), and for all molecules simultaneously (COI, H3, 18S, 28S). Although COI and H3 are protein-coding genes, fragments were analyzed at the DNA level. Lastly, all molecular and morphological data were analyzed simultaneously, referred to in the text as total evidence and this is taken as our preferred hypothesis for explaining the evolution of all characters simultaneously. Nodal support was calculated in POY using Farris's parsimony jackknifing procedure (Farris et al., 1996) for 100 replicates (using the commands -jackboot -replicates 100).

Tree searches were conducted in parallel at Harvard University on a 19 dual-processor cluster (darwin.oeb.harvard.edu) using pvm (parallel virtual machine). Commands for load balancing of spawned jobs were used to optimize parallelization procedures (-parallel -dpm -jobspernode 2). Trees were built via a random addition sequence procedure (10 replicates) followed by a combination of branch-swapping steps (SPR "subtree pruning and regrafting" and TBR "tree bisection and reconnection") and tree fusing (Goloboff, 1999) in order to further improve on tree length minimization. Discrepancies between heuristic and actual tree length calculations were addressed by adjusting slop values (-slop5 -checkslop10).

Each one of the five partitions was analyzed under 12 parameter sets for a variety of indel/change costs and transversion/transition ratios, where change costs refer to the highest nucleotide transformation (as in Wheeler, 1995). Gap/transversion ratios of 1 and 2 as well as transversion/transition ratios of 1, 2 and 4 were explored, although the extension of gaps was also downweighted with respect to the first occurrence of an indel event. These 12 parameter sets were considered a starting point for testing the stability of phylogenetic hypotheses (Giribet, 2003). Further increasing the weight ratios for transformation and indels would generate topologies with higher amounts of incongruence, are uninformative and computationally expensive.

For this study we chose to do sensitivity analysis (Wheeler, 1995) and stability analysis (Giribet, 2003). A sensitivity analysis was conducted to determine the degree of character incongruence among different parameter sets; the parameter that minimized incongruence was then chosen as the optimal parameter (similar to identifying the shortest tree). Character incongruence was measured using a modified version (Wheeler and Hayashi, 1998) of Incongruence Length Difference (ILD) metric (Mickevich and Farris, 1981; Farris et al., 1995). The ILD value was calculated by subtracting the sum of individual trees from the length of the combined data tree and dividing the result by the length of the combined data:

$$\mathrm{ILD} = (\mathrm{Length}_{\mathrm{Combined}} - \mathrm{Sum\ Length}_{\mathrm{Individual\ Data\ Sets}})/\mathrm{Length}_{\mathrm{Combined}}$$

Results

Morphological analyses

The search adopted in NONA yielded 665 trees of shortest tree length (190 steps; CI = 0.668; RI =

0.935; RC = 0.625), which was found in 13.4% of the replicates performed. These trees were subjected to a subsequent round of TBR with a total of 727 retained. The strict consensus of the morphological cladograms (Fig. 1) shows monophyly for all molluscan classes represented, and monophyly of Cephalopoda is furthermore supported in 99% of jackknife replicates. Within Cephalopoda, Nautiloidea and Coleoidea were also supported as monophyletic, but relationships within Coleoidea remain unresolved to a large degree, except for Vampyromorpha + Octobrachia (79% jackknife support). A Vampyromorpha + Octobrachia clade was supported by several characters, such as the presence of unmodified arms IV (Appendix 3, character 17), outer statocyst capsules (character 45), radial sucker symmetry (character 23) (although assumptions do exist within some of these characters, see Appendix 3). Other ordinal relationships supported were Cirroctopoda + Octopoda (found in 65% of jackknife replicates), but Decabrachia was not found to be monophyletic due to lack of resolution in basal nodes (Fig. 1).

While further resolution was found in Decabrachia, none of the fundamental trees supported monophyly of Teuthida, or Oegopsida. Furthermore, sepioids (Sepiolidae, Sepiidae, *Spirula* and Idiosepiidae) were paraphyletic with respect to Loliginidae. None of the relationships involving sepioid taxa received jackknife values above 50%. Within Oegopsida, several clades suggested relationships among oceanic cephalopods. One such clade is the enoploteuthid family complex proposed by Young and Harman (1998), comprised here of Ancistrocheiridae + *Pterygioteuthis* + *Pyroteuthis* + Enoploteuthidae. Characters supporting this relationship include buccal membrane attachment (character 11), buccal lappet number (character 12) and the presence of a tentacle locking apparatus (character 30). The position of *Pyroteuthis* + Enoploteuthidae was supported by the presence of photophores containing collagen light guides (character 33).

Other interesting clades within decabrachians included Bathyteuthidae + Chtenopterygidae (both exhibit suckers on buccal membrane; Appendix 3; character 26) and Chiroteuthidae + Mastigoteuthidae + Batoteuthidae + Joubiniteuthidae (these families have an oval funnel locking apparatus with projecting knobs; character 36). Lastly, several decabrachians were united by the presence of a primary conus (character 5): Architeuthidae + Neoteuthidae + Ommastrephidae + Onychoteuthidae + Gonatidae + Enoploteuthidae (except for Pyroteuthidae, which has a pseudoconus).

Congruence analysis

The parameter set that minimized overall character incongruence for the simultaneous analysis of all data consisted of an opening gap cost of 2 (extension gap was fixed at 1) and any other transformation costs set to 2 (parameter set 2221). This resulted in an ILD value of 0.0416 (Table 5). A second parameter set with gap opening cost of 2 (extension gap of 1) and any other changes receiving a cost of 1 had a similar ILD value of 0.0439 (parameter set 2111). The lowest ILD value for the molecular-only analysis consisted of an opening gap cost of 4 (extension gap of 1) and any other changes receiving a cost of 1 with an ILD value of 0.0304 (parameter set 4111).

Partitioned analyses

COI.

The COI tree for the overall optimal parameter set (2221) provided a single tree of 10 671 weighted steps, after tree fusing (Fig. 2). This tree does not provide support for the monophyly of any molluscan classes investigated. Within cephalopods, monophyly was shown for Cirroctopoda and Decabrachia. Very few relationships were supported in the jackknife analysis; those with jackknife values greater than 50% were primarily associated with closely related genera, but Decabrachia were monophyletic under all explored parameter sets. Sepioids were not monophyletic, forming a clade with ommastrephids and loliginids. Previous investigations with COI (Carlini and Graves, 1999) have shown that this gene may be too variable to provide a great deal of useful information alone.

Histone H3.

Analyses of the overall optimal parameter set for histone H3 yielded eight trees of 2240 weighted steps; the best tree length was found in three replicates and not improved after tree fusing. The strict consensus of these eight trees did not show monophyly for any classes investigated (Fig. 3). In the case of cephalopods, *Nautilus* clustered within a clade containing a gastropod and two aplacophorans. However, monophyly was shown for Coleoidea and Decabrachia. Again, sepioids were not monophyletic because Spirulida formed a clade with Bathyteuthidae and Chtenopterygidae. Jackknife support for the monophyly of Lepidoteuthidae + Octopoteuthidae + Neoteuthidae + Cycloteuthidae + Batoteuthidae + Histioteuthidae was 94%. A Gonatidae + Ommastrephidae clade was also supported in the histone analysis (78% jackknife support). The strict consensus of all parameter sets for the histone H3 data set supports few deep relationships within cephalopods, except for Cirroctopoda.

Combined ribosomal data.

The optimal parameter set for the combined ribosomal data (18S rRNA, 28S rRNA) yielded 100 trees of 4029 weighted steps. The best tree length was obtained

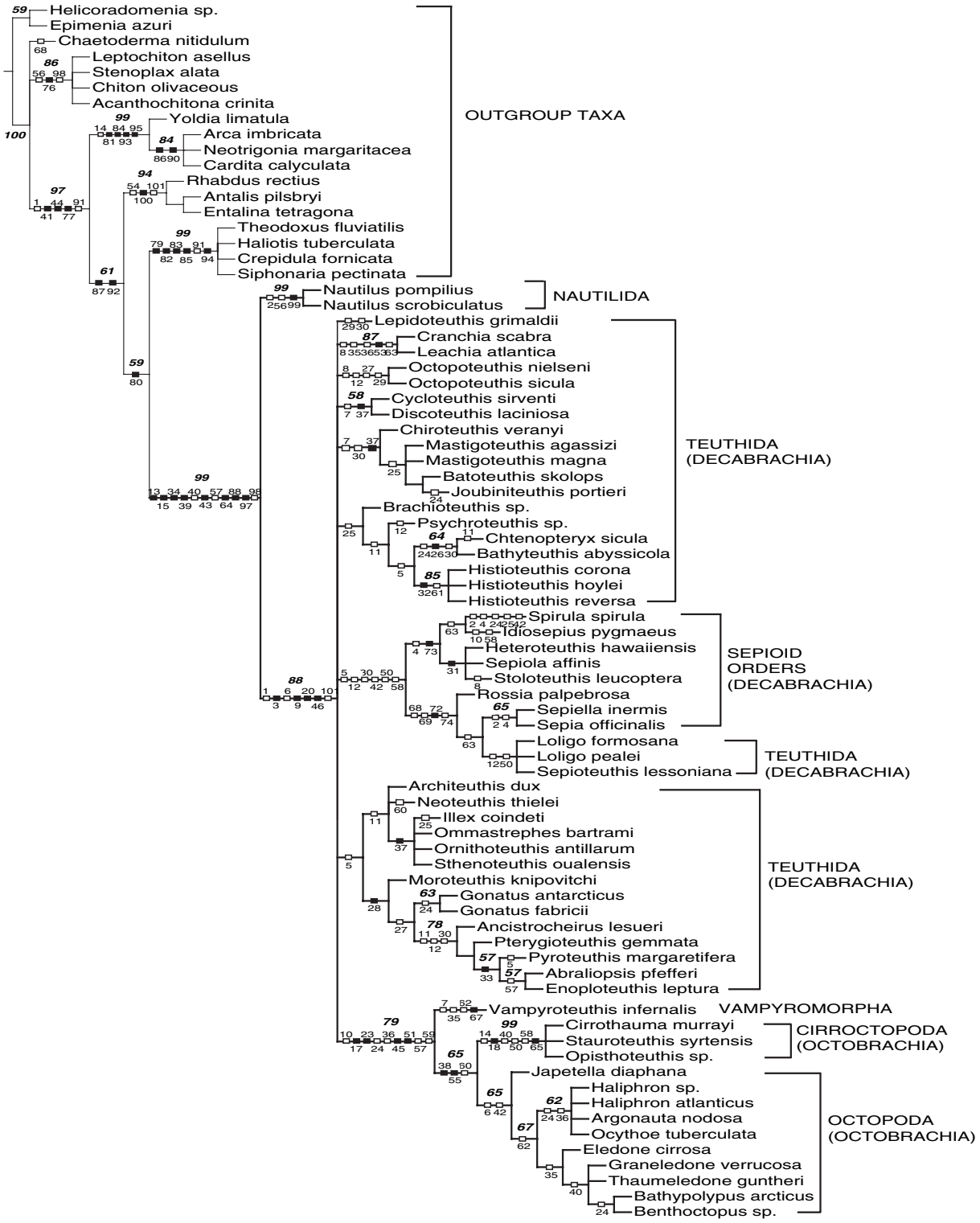


Fig. 1. Strict consensus of 665 trees (190 steps; CI = 0.668; RI = 0.935; RC = 0.625). Bold italic numbers above branches indicate jackknife support values greater than 50% calculated in Winclada/Nona. Unambiguous character optimizations calculated in Winclada are shown at each node. Black boxes on branches indicate character states present only in a given clade (hypothetical synapomorphies); white boxes indicate homoplastic character states.

Table 5
Weighted tree lengths for the individual and combined analyses at different gap/tv and tv/ts cost ratios and ILD values for the combined molecular (mol) and total evidence (total) data sets. Other abbreviations: rib (= 18S rRNA +28S rRNA), mor (= morphology)

			Individual			Combined		ILD values	
gap/tv	tv/ts	rib	COI	H3	mor	mol	total	mol	total
1	8	1673	4840	740	380	7548	8084	0.0391	0.0558
1	1	4029	10671	2240	380	17504	18071	0.0322	**0.0416**
1	2	2949	7882	1514	380	12749	13309	0.0317	0.0439
1	4	4459	12696	2275	760	20103	21176	0.0335	0.0466
2	8	1051	2452	370	380	4035	4529	0.0401	0.0609
2	1	2279	5393	1120	380	9069	9607	0.0305	0.0453
2	2	3228	7943	1514	760	13098	14140	0.0315	0.0492
2	4	4989	12824	2275	1520	20812	22840	0.0348	0.0539
4	8	1236	2471	370	760	4243	5157	0.0391	0.0621
4	1	2489	5414	1120	760	9306	10317	**0.0304**	0.0518
4	2	3603	7957	1514	1520	13533	15439	0.0339	0.0547
4	4	5736	12911	2275	3040	21727	25433	0.0371	0.0578

in three replicates and although the number of trees (100) was the buffer limit, we used the command -fitchtrees, which stores more trees than set by the limit and selects the 100 most diverse ones. No shorter trees were found after a final round of tree fusing. Strict consensus of these 100 trees (Fig. 4) illustrates monophyly for Bivalvia, Scaphopoda, Polyplacophora and Cephalopoda, but not Gastropoda. Of all individual analyses, the combined ribosomal tree provided the least amount of backbone resolution. Coleoidea was monophyletic, with Nautiloidea as its sister group. Two cirroctopod species were sister to all other coleoids, but the third cirroctopod (*Opisthoteuthis*) nested within the Decabrachia + Vampyromorpha clade. Family level resolution was minimal, except for the clade that formed *Moroteuthis* + *Neoteuthis* + *Architeuthis*. When results from all the parameter sets are combined, the consensus tree had no resolution.

Combined molecular data.

The optimal parameter set for the combined molecular data (4111) yielded 12 trees with a minimal length of 9306 weighted steps after tree fusing. The strict consensus of these trees (Fig. 5) illustrates monophyly for Cephalopoda, Bivalvia, Polyplacophora and Scaphopoda. The cephalopods were divided into Nautiloidea and Coleoidea, the latter clade divided into Octobrachia and Decabrachia + Vampyromorpha (rather than Vampyromorpha + Octobrachia). Teuthida as well as Oegopsida were polyphyletic. However, Myopsida did not form a clade with the sepioid orders, grouping with Cranchiidae, Ancistrocheridae and Onychoteuthidae. This relationship was supported in less than 50% jackknife replicates. Sepioids (except Spirulida) were monophyletic and sister to an Enoploteuthidae + Onychoteuthidae clade. Spirulida was found sister to the oegopsid clade containing Mastigoteuthidae + Joubiniteuthidae. The enoploteuthid families proposed by Young and Harman (1998) were not monophyletic; however, a close relationship between *Pterygioteuthis* and *Pyroteuthis* was supported in 79% of jackknife replicates. Jackknife support for the deepest divergences within Cephalopoda show values above 70%, and these divergences correlated with stable relationships when evaluating all parameter sets explored thus far. It is especially interesting to note the stability of a relationship between *Vampyroteuthis* and Decabrachia (also with a jackknife frequency of 74%). Other groups supported under all analytical parameter sets were Octopoda + *Opisthoteuthis*, Decabrachia, or Bathyteuthidae + Chtenopterygidae.

Total evidence.

When all morphological and molecular data were combined, the most congruent data set (ILD = 0.0416) was where all parameter sets received equal weights, with the exception of extension gaps (parameter set 2221). Under such a parameter scheme, three replicates generated trees of length 18 073 but after tree fusing, two trees of 18 071 weighted steps were saved. The strict consensus of the optimal parameter set is shown in Fig. 6. With respect to outgroups, Scaphopoda, Solenogastres, Polyplacophora and Bivalvia were monophyletic. However, no solid conclusion between outgroups and cephalopods can be reached at this point.

Cephalopoda, Coleoidea and Nautiloidea were found to be monophyletic under all parameter sets and in 100% jackknife replicates. Within Coleoidea, a monophyletic Octopoda + *Opisthoteuthis* and Decabrachia were also supported. In the optimal parameter tree, cirroctopods were not nested within octopods (except for *Opisthoteuthis*); instead they formed a sister group to Vampyromorpha + Decabrachia. Sepioids (except Spirulida) formed a clade sister to the myopsid Loliginidae. Spirulida formed a clade with Bathyteuthidae and Chtenopterygidae. With the exception of a clade formed

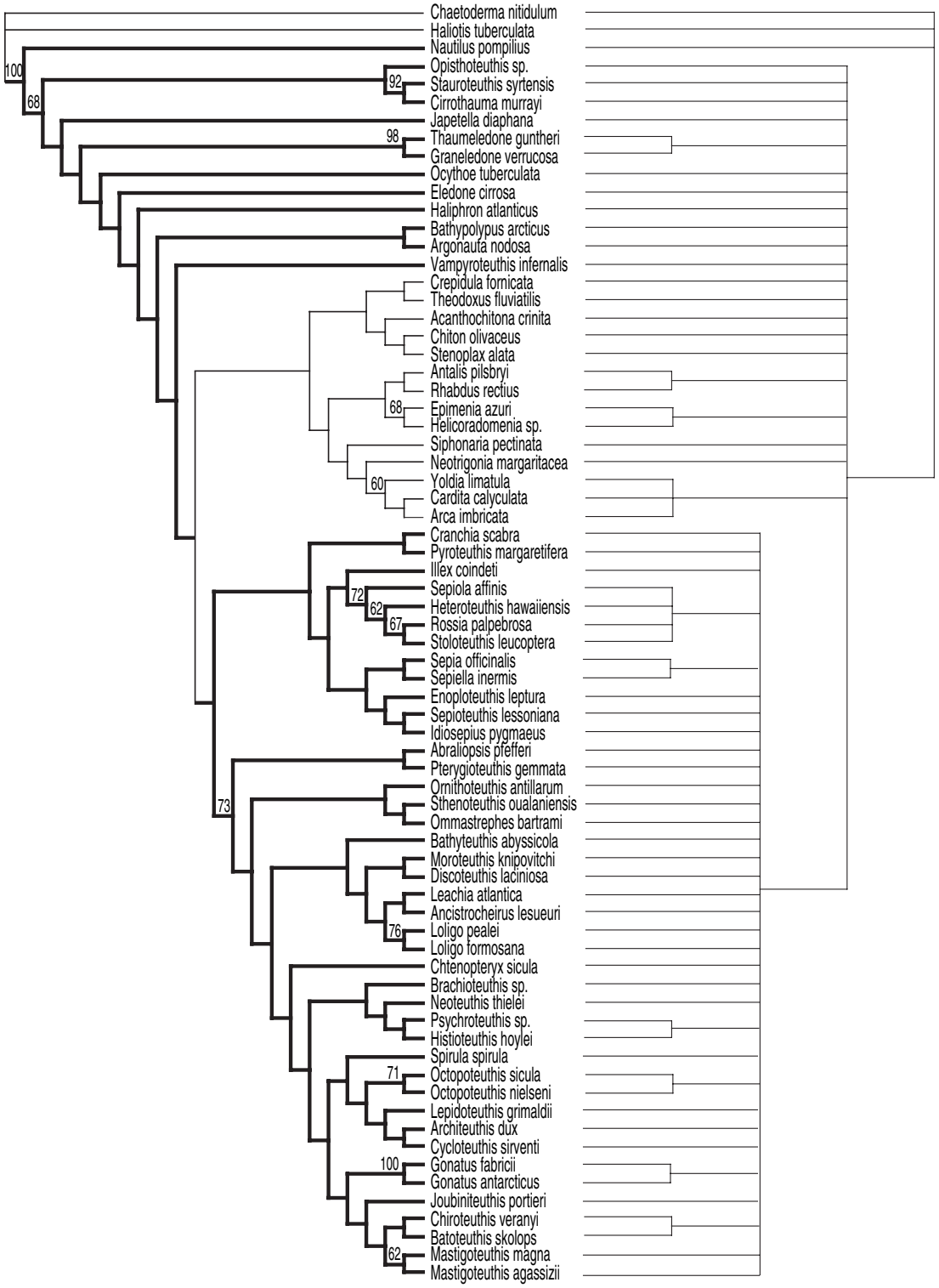


Fig. 2. Tree on the left represents the single tree of 10 671 weighted steps for the COI data set obtained under the optimal parameter set (2221). Branches in bold indicate cephalopod taxa. Numbers above branches indicate jackknife support values above 50%. Right cladogram is a strict consensus of all trees obtained for the 12 parameter sets explored.

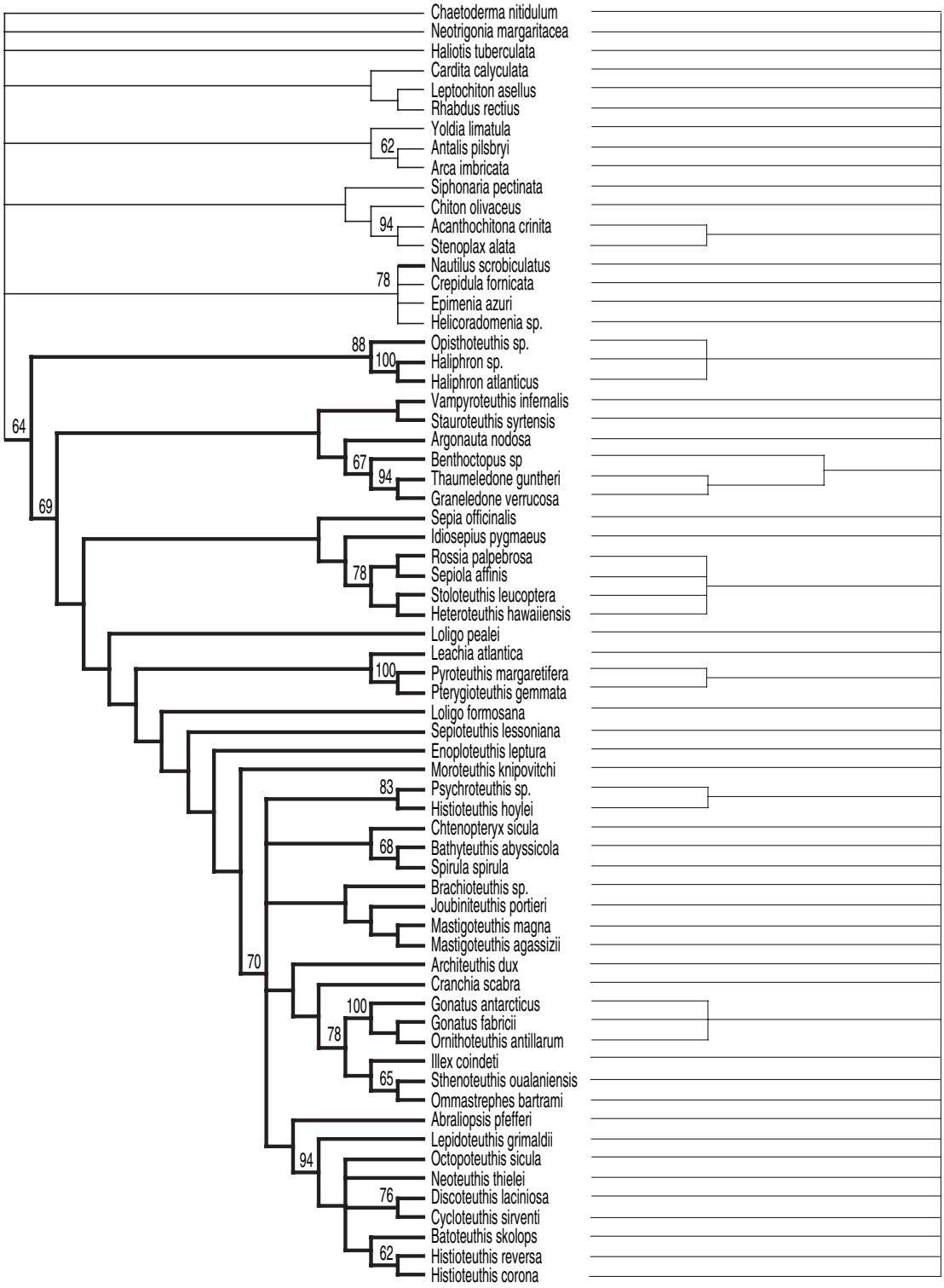


Fig. 3. Left tree shows the strict consensus of eight trees of 2240 weighted steps for the H3 data set yielded by the optimal parameter set (2221). Branches in bold indicate cephalopod taxa. Numbers above branches indicate jackknife support values above 50%. Right cladogram is a strict consensus of all trees obtained for the 12 parameter sets explored.

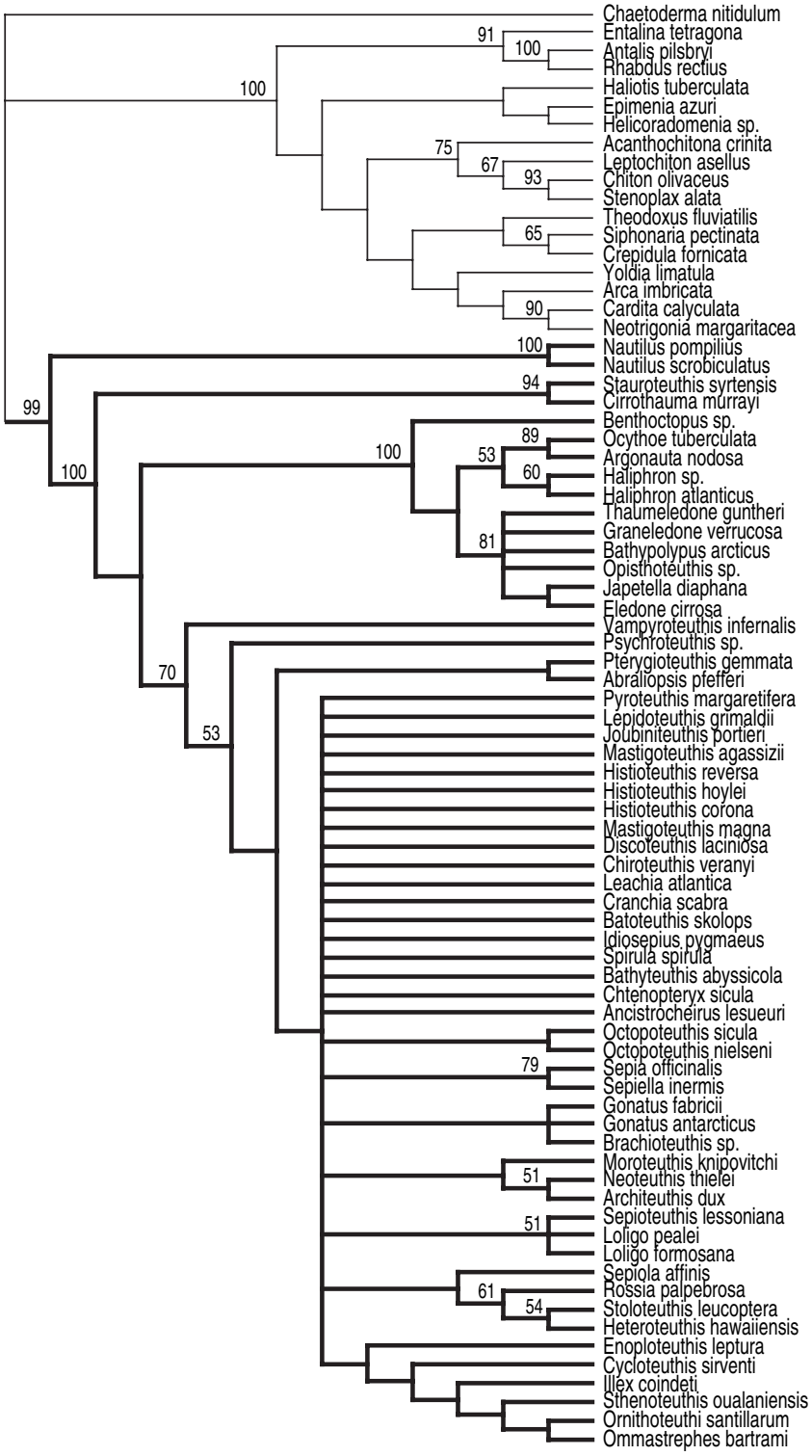


Fig. 4. Strict consensus of 101 trees at 4029 weighted steps for the combined ribosomal (18S rRNA and 28S rRNA) data yielded by the optimal parameter set (2221). Branches in bold indicate cephalopod taxa. Numbers above branches indicate jackknife support values above 50%. No resolution was found for the strict consensus of 12 parameters explored.

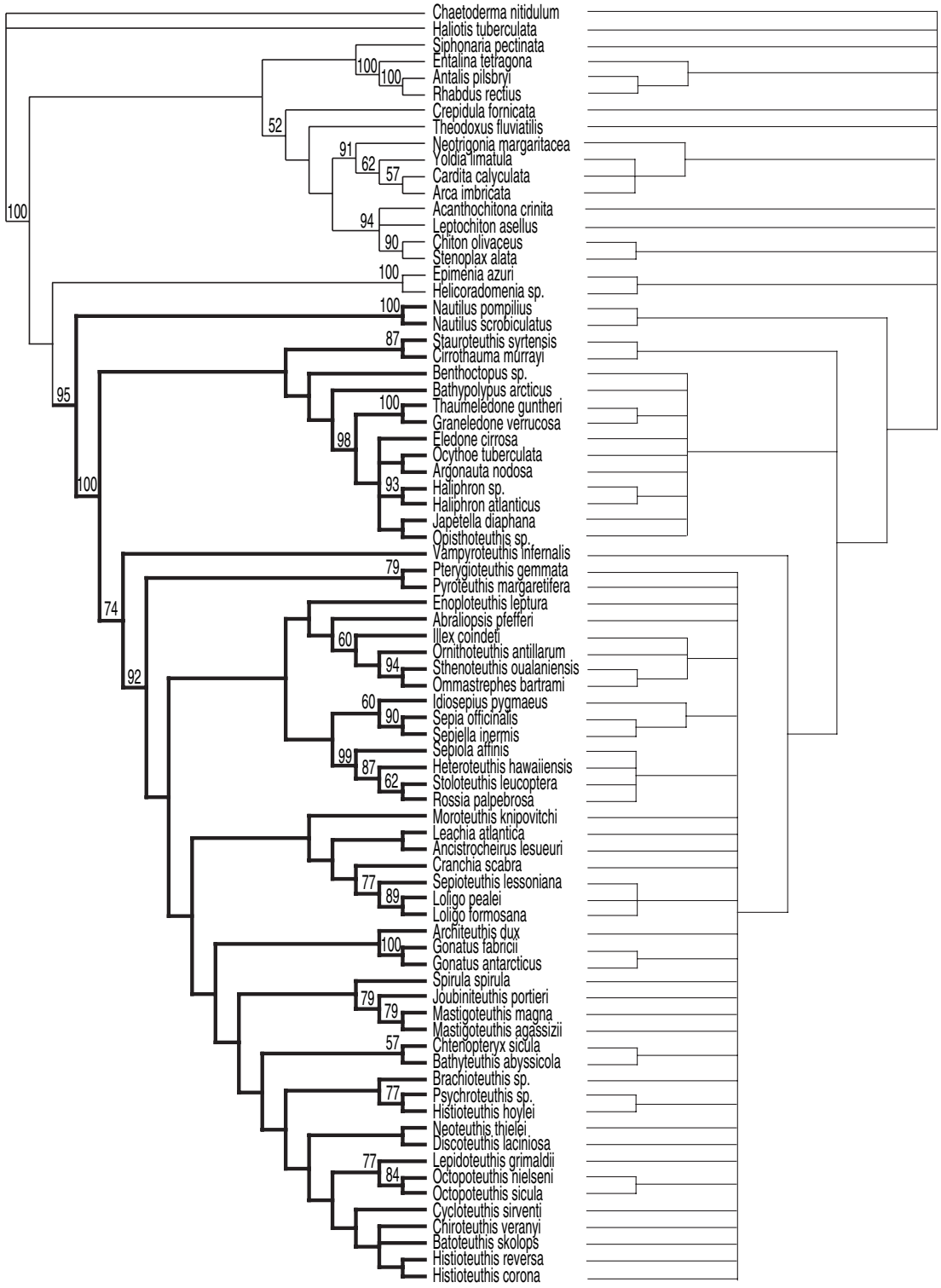


Fig. 5. Left tree illustrates the strict consensus of 12 trees at 9306 weighted steps for the combined molecular data (18S rRNA, 28S rRNA, COI, H3) yielded by the optimal parameter set (4111). Branches in bold indicate cephalopod taxa. Numbers above branches indicate jackknife support values above 50%. Right cladogram is a strict consensus of all trees obtained for the 12 parameter sets explored.

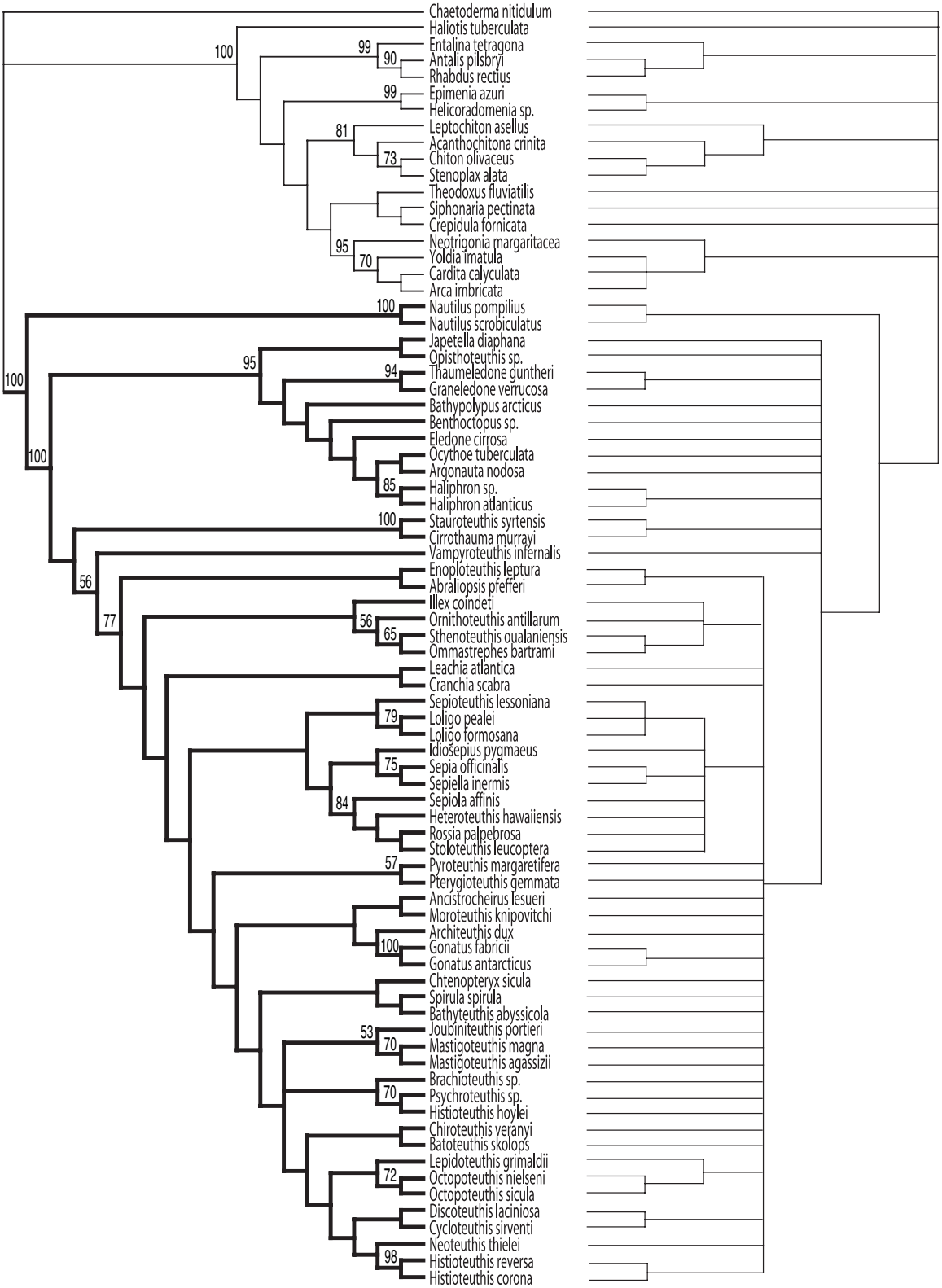


Fig. 6. Tree on the left illustrates the strict consensus of two trees at 18 071 weighted steps for the combined morphological and molecular sequence data for the optimal parameter set (2221). Branches in bold indicate cephalopod taxa. Numbers above branches indicate jackknife support values above 50%. Right cladogram is a strict consensus of all trees obtained for the 12 parameter sets explored.

by the sepioid orders + Myopsida, most other decabrachian relationships were only supported under certain parameter schemes. Joubiniteuthidae + Mastigoteuthidae was found in the optimal parameter set (2221; 53% jackknife support, Fig. 6). Ancistrocheiridae and Onychoteuthidae formed a clade sister to Architeuthidae + Gonatidae, although this relationship was not stable to parameter set variation. All parameter sets supported a close relationship between Lepidoteuthidae and Octopoteuthidae. That clade was sister to Cycloteuthidae + Neoteuthidae + Histioteuthidae under the optimal parameter set (2221). A suggested clade of Enoploteuthidae + Ancistrocheiridae + *Pterygioteuthis* + *Pyroteuthis* (e.g., Young and Harman, 1998) was not found in our analyses; however, a relationship between *Pterygioteuthis* and *Pyroteuthis* was supported by the data.

Discussion

This study provides the most comprehensive analysis published to date of internal relationships among a large number of cephalopod species by simultaneously analyzing information from their morphology and multiple molecular loci. Individual data sets did not show large agreement at most nodes and in fact showed a large disagreement with morphological-based hypotheses. Furthermore, results for the individual partitions were highly parameter-dependent and only when combining all molecular evidence or molecules + morphology was stability achieved in the hypotheses of cephalopod monophyly as well as in the major divisions within the Cephalopoda. As argued by proponents of the total evidence approach (see Kluge, 1989), only by combining all available evidence can reliable interpretation regarding the phylogenetic history of a group be attained (*sensu* Giribet, 2002). The addition of multiple genes indeed contributed to different but overlapping levels of resolution. However, decabrachian relationships still require major improvement in terms of stability and nodal support. Morphological data provided a higher degree of resolution among decabrachians than was previously observed, but there was less information regarding basal relationships among those sampled, while molecular data provided more resolution at both familial and ordinal levels. Given this, a simultaneous analysis of all data established more overall support for resolved clades at both ordinal and family levels better than any individual data set alone.

Ordinal relationships

Several findings in this study supported previous hypotheses of cephalopod relationships (e.g., Naef, 1921/23; Engeser and Bandel, 1988; Young and Vecchione, 1996; Bonnaud et al., 1997; Carlini and Graves, 1999; Boletzky, 2003), such as the monophyletic nature of cephalopods (Fig. 7, node 1) and their subdivision into Nautiloidea and Coleoidea (Fig. 7, node 2). Other more conflicting relationships, such as the position of Vampyromorpha, disagreed with previous hypotheses (Fig. 7, nodes 4, 5). Historically, Vampyromorpha and Octobrachia had been treated as sister taxa based on embryological and developmental data (Boletzky, 2003; Naef, 1928; Young and Vecchione, 1996), as well as morphological characters such as the presence of radial sucker symmetry (Appendix 3, character 23), similar sperm morphology (e.g., character 67; Healy, 1989) and outer statocyst capsules (character 45), although vampyromorph gladius morphology is similar to that of decabrachians (character 4; Toll, 1982; Toll, 1998). Alternatively, octobrachian gladii have been lost (character 4) or reduced to form fin supports (Cirroctopoda) or stylets (Octopoda). The position of Vampyromorpha has remained questionable, particularly in light of past molecular evidence (Bonnaud et al., 1997; Carlini and Graves, 1999). For example, in Bonnaud et al. (1997) the position of Vampyromorpha varied with outgroups used to generate the cladogram; analyses using the chiton *Katharina tunicata* as an outgroup placed Vampryomorpha sister to octobrachians, but when *K. tunicata* was not included, Vampyromorpha was found sister to the decabrachians. In this study, Vampyromorpha + Decabrachia was supported (Fig. 7, node 4) in the combined analysis under the best parameter set, plus six additional parameter sets. An alternative resolution of Vampyromorpha as sister group to Octobrachia (Fig. 7, node 5) was found under five analytical parameter sets. The origin of conflict regarding Vampyromopha in the present study was difficult to determine. Morphological data supported Vampyromorpha + Octobrachia (Fig. 1), while molecular data supported Vampyromorpha + Octobrachia as well as Vampyromorpha + Decabrachia, depending on the parameter and data set (Fig. 7). Ribosomal, combined molecular and simultaneous analysis of all data provided overall support for Vampyromorpha + Decabrachia. The two most variable loci, H3 and COI disagreed, placing *Vampyroteuthis* sister to the cirroctopod *Stauroteuthis* (Fig. 3), or placing it in a more basal position (Fig. 2). Vampyromorpha exhibited many autapomorphic features found in neither octobrachians nor decabrachians (Young, 1964), which makes this taxon difficult to place using morphological data alone. Disagreement among morphological and molecular data is not uncommon among metazoans (e.g., Giribet, 2003) and has been previously established for Octobrachia (Carlini et al., 2001). A second problem that needs consideration in determining the position of Vampyromorpha relative to other coleoids is the possibility that rampant extinction may obscure the affinities of *Vampyroteuthis*. While

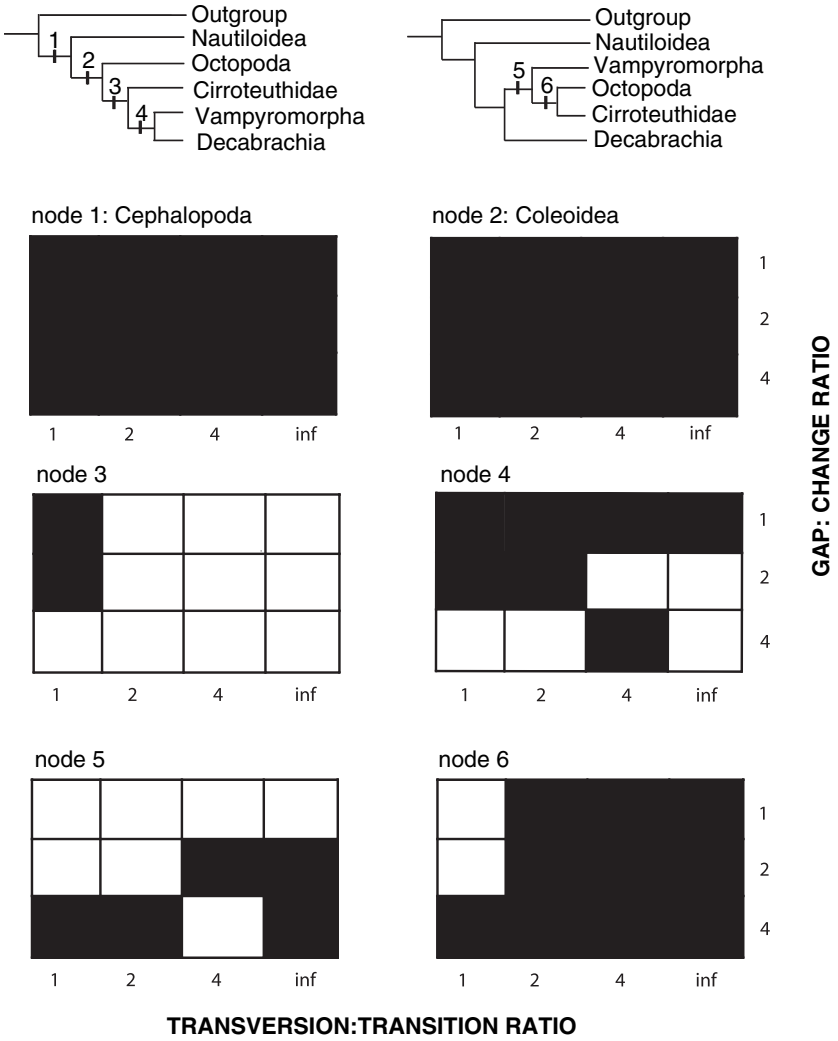


Fig. 7. Higher-level relationships among Cephalopoda as derived from analyses of combined morphology and molecular data for the optimal parameter set (2221). Two alternative topologies generated from different parameter sets, are illustrated above. The bottom squares illustrate the congruence plots (Navajo rugs) for selected nodes on cladograms above. Black squares indicate monophyly for a given parameter set, while white squares indicate non-monophyly.

some fossil evidence exists for cephalopods, many of the fossils are difficult to interpret and could be placed with either Octobrachia or Decabrachia (Young et al., 1998). Due to the difficulty of homologizing characters between fossil and extant taxa, fossil evidence was not included in the present study, although fossils may have a fundamental role in elucidating cephalopod relationships, as shown in other metazoan groups (Gauthier et al., 1988; Donoghue et al., 1989; Eernisse and Kluge, 1993; Giribet et al., 2002; Wheeler et al., 2004). Therefore, we caution the reader to interpret our results and conclusions in the absence of fossils.

A close relationship has been hypothesized to exist between cirroctopods and octopods (Carlini et al., 2001; Naef, 1921/23; Chun, 1914; Nesis, 1987; Engeser and Bandel, 1988; Voight, 1997). The morphological analyses provided the only cladogram to support a Cirroctopoda + Octopoda relationship (Fig. 1). Furthermore, the monophyly of Cirroctopoda was not established; COI was the only data set to support monophyly of the three species of cirroctopods (Fig. 2). Histone H3 (only available for two species of cirroctopods) placed *Opisthoteuthis* as sister group to the octopod *Haliphron* and *Stauroteuthis* as sister group to *Vampyroteuthis*. All other cladograms that included ribosomal data placed *Opisthoteuthis* within Octopoda (Figs 4, 5 and 6), which could be due to the use of a partial 18S rRNA sequence in the analyses. Despite several attempts to complete the *Opisthoteuthis* 18S rRNA fragment, we were not able to do so. However, Carlini et al. (2001) also questioned the monophyly of cirroctopods. While the optimal parameter set for this study supported Cirroteuthidae +

Vampyromorpha + Decabrachia, this result was not corroborated by other parameters (Fig. 7, nodes 3 and 6) or by individual trees. The instability of these nodes could be due to a disagreement between morphological and molecular data; previous molecular analyses found cirroctopods to be polyphyletic (Carlini et al., 2001), while morphological data suggested that cirroctopods are monophyletic (Young and Vecchione, 1996). Incongruence between molecular and morphological data is not uncommon in cephalopods (Carlini et al., 2001) and in order to resolve this issue, further sampling of cirroctopod species and analyses need to be conducted.

Decabrachian relationships

Previous investigations have consistently disagreed on decabrachian relationships, citing gene choice, taxon sampling, or a rapid radiation as reasons for unresolved phylogenies (Young and Vecchione, 1996; Bonnaud et al., 1997; Carlini and Graves, 1999; Carlini et al., 2000). While this study cannot address all questions pertaining to decabrachian relationships, certain hypotheses were tested (Fig. 8). Naef (1921/23) placed *Spirula* with Sepiidae, Sepiolidae, Idiosepiidae and Sepiadariidae in the suborder Sepioidea, with all other families in Teuthoidea. Naef initially placed *Spirula* sister to Sepiidae based on shared characteristics in shell development, stating that the differences between the two shells were secondary (Naef, 1921/23). In this study, monophyly of the sepioids was supported, with the exception of *Spirula*, which consistently grouped with oegopsids, and not sepioids (Figs 5, 6 and 8). However, monophyly of sepioids was not found in the morphological analyses, because their clade also included the loliginid squids (Fig. 1). In the simultaneous analyses of all data, the monophyly of sepioids + loliginids without *Spirula* was supported under all analytical parameters, suggesting a close relationship between sepioids (except Spirulida) and Loliginidae (Myopsida). Such a relationship of sepioids and loliginids was previously discussed by Naef (1921/23), although as previously discussed, Naef also considered *Spirula* within this clade. Naef described a Myopsida group consisting of Sepiidae, Sepiolidae, Loliginidae and Idiosepiidae, but later removed Loliginidae from this group citing drastic differences in shell morphology and development. This study found support for the reunification of Naef's original myopsid group (but excluding *Spirula*) based on both morphological and molecular data (Figs 1, 6 and 8). Several unusual morphological characteristics are shared among these families; all have accessory nidamental glands (character 57; but this seems to be plesiomorphic), benthic eggs with embryos containing an external yolk sac (not present in most oegopsids) and a cornea, which permanently covers the pupil (Naef, 1921/23; character 42). While the position of Loliginidae relative to sepioids and other teuthids has been debatable, evidence here suggested that Loliginidae is in fact sister to sepioids (except *Spirula*) and therefore not true teuthids, corroborating previous findings based on molecular data (Bonnaud et al., 1997; Carlini and Graves, 1999; Nishiguchi et al., 2004). The placement of Sepiolida, Sepiida and Idiosepiida with relation to Teuthida remains somewhat debatable, particularly due to their apparent relatedness to Loliginidae, but not to other teuthids. The position of *Spirula* remained unclear due to disagreement between morphological and individual molecular loci; individual and combined molecular trees placed *Spirula* with oegopsids, while morphological evidence placed it with sepioids.

Families within Oegopsida (Teuthida) did not form a monophyletic group. However, our results suggested closer relationships among several oegopsids than in previous cases (e.g., Bonnaud et al., 1997; Carlini and Graves, 1999). Many of the tested relationships were supported by previous monographs and general classifications of cephalopod taxonomy (Chun, 1914; Naef, 1921/23; Joubin, 1825/1924; Roper et al., 1969). For example, Chtenopterygidae and Bathyteuthidae were considered closely related by Pfeffer (1912) based on the presence of a long narrow gladius, subterminal fin position, presence of suckers of buccal lappet and quadraserial suckers on the arms. Only the combined analyses and histone H3 cladograms supported a close association among *Chtenopteryx*, *Spirula* and *Bathyteuthis* (Figs 3 and 7), although *Bathyteuthis* + *Chtenopteryx* was supported by morphology (Fig. 1) and the simultaneous analysis of molecules (Fig. 5).

The "Enoploteuthid families" proposed by Young and Harman (1998) consisted of Enoploteuthidae, Ancistrocheiridae, Pyroteuthidae and Lycoteuthidae, where the authors found a (Ancistrocheiridae (Enoploteuthidae (Lycoteuthidae + Pyroteuthidae))) relationship. Naef (1921/23) proposed a slightly different scenario, placing Enoploteuthidae and Pyroteuthidae in a single family, Enoploteuthidae, while grouping Ancistrocheiridae with Onychoteuthidae in a single family Onychoteuthidae. The present study supported the latter relationship to some extent (Fig. 8). Morphological data placed Enoploteuthidae, *Pterygioteuthis*, *Pyroteuthis* and Ancistrocheiridae in a single clade (Fig. 1), which was further supported by their many shared characters, such as the presence of hooks on arms (character 27) and tentacles (except for *Pterygioteuthis*, character 28), armature in two series of suckers (character 24), eight buccal supports (character 12), dorsal buccal attachment to arms V (character 11) and the presence of a conus (character 5). None of the individual molecular loci found the four families to be monophyletic. Combined data illustrated that *Ancistrocheirus* and *Moroteuthis* (Onychoteuthidae) clustered together with *Architeuthis* and *Gonatus*, while *Enoploteuthis* + *Abralilopsis* formed

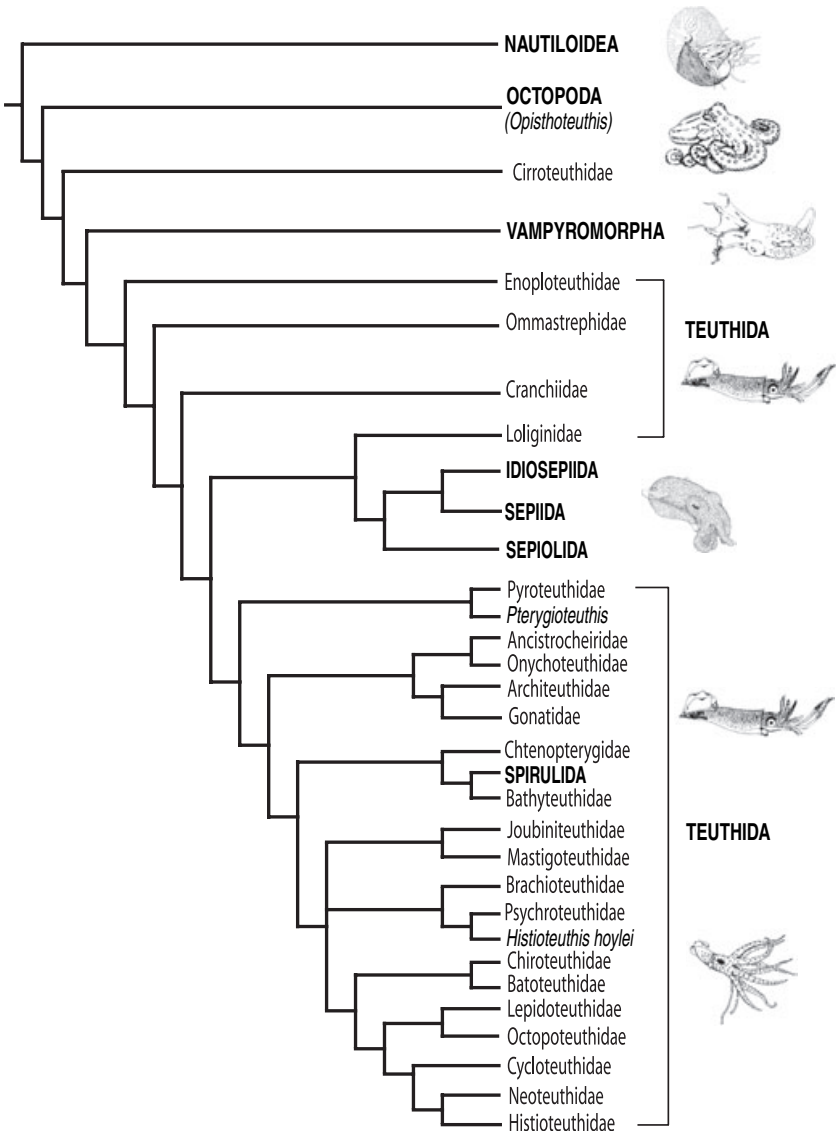


Fig. 8. Schematic representation of cephalopod relationships based on the optimal parameter set for the combined analysis of morphological and molecular data. Taxa in bold represent orders of cephalopods that appeared monophyletic in the analysis. Drawings by G. Williams.

the outermost branch on the decabrachian clade (Fig. 8), implying polyphyly for the enoploteuthid families. The position of *Pterygioteuthis* was unclear, the combined molecular data suggesting a close relationship to *Abraliopsis* (Enoploteuthidae, Fig. 6). However, our combined analysis of all data placed *Pterygioteuthis* + *Pyroteuthis* (Fig. 7) separate from other "enoploteuthids". Histone H3, the simultaneous analysis of the molecules and the simultaneous analysis of all data supported a sister relationship between *Pterygioteuthis* and *Pyroteuthis,* thus corroborating previous findings (e.g., Nesis, 1987).

Other interesting relationships were observed within four recognized oegopsid families: Joubiniteuthidae, Mastigoteuthidae, Batoteuthidae and Chiroteuthidae. While morphology and COI data supported the monophyly of these four families (Fig. 2), the combined analysis of all data found support for separate *Joubiniteuthis* + *Mastigoteuthis* and *Batoteuthis* + *Chiroteuthis* (Figs 7 and 8) clades. The polyphyletic nature of these four families was difficult to explain, partially because of their morphological similarity (Table 3, Fig. 1). All four families lack hectocotylization (characters 61–63), exhibit ventral buccal membrane attachment on arms V (character 11), an oval funnel locking apparatus (character 37) and a secondary conus (character 5). One possible reason for the apparent polyphyly was that the family Promachoteuthidae, commonly believed to be closely related to

the Mastigoeuthidae (e.g., Roper et al., 1969), was not included in this study, due to a lack of available specimens. Therefore, further sampling of these four families, as well as Promachoteuthidae, is needed in order to fully understand their relationships.

Decabrachian relationships supported in this study which were not identified by previous studies include *Brachioteuthis* + *Psychroteuthis* (+ *Histioteuthis hoylei*) and *Cycloteuthis* + *Neoteuthis* + *Histioteuthis* (Fig. 8). However, both Brachioteuthidae and Psychroteuthidae are monotypic and their taxonomy is poorly understood. The two families have several morphological characters in common, such as biserial arm suckers (character 24), simple funnel locking apparatus (character 37) and rhomboidal fins (Nesis, 1987). The grouping of *H. hoylei* with the family Psychroteuthidae was not entirely understood, although previous morphological data have suggested a close relationship between Psychroteuthidae and Histioteuthidae (Toll, 1998). However, this does not explain the polyphyletic nature of Histioteuthidae, nor does it explain why one species would be sister to *Psychroteuthis* and all others would form a clade on a different region of the tree.

The families Neoteuthidae and Histioteuthidae have several characteristics in common such as dorsal attachment on arms V (character 11), simple funnel locking apparatus (character 37) and biserial sucker arrangement on arms (character 24). Cycloteuthidae is distinct, sharing only the biserial sucker arrangement with both Histioteuthidae and Neoteuthidae. The characteristics that these three families share may be plesiomorphic; they are fairly common throughout decabrachians (Table 3, Fig. 1) and therefore may not provide additional information pertaining to relatedness. Hence, further investigation needs to be completed in order to thoroughly understand this relationship.

Conclusion

This study supports the monophyly of Cephalopoda, with Nautiloidea sister to a monophyletic Coleoidea. While the relationships between Cirroctopoda and Octopoda are somewhat unclear, the data support a sister relationship between Vampyromorpha and Decabrachia. Within Decabrachia, support was found for several intrafamilial relationships. It is clear that Sepiolida, Sepiida and Idiosepiida form a monophyletic group not related to Spirulida, which is instead nested within oegopsids. The analyses also indicate that the family Loliginidae is more closely related to sepioids, rather than oegopsids. The order Teuthida is consistently paraphyletic under all parameters and analyses and will need further clarification. Teuthida is comprised of oceanic decabrachians from a variety of habitats and locations around the world and with such diversity it is not surprising that it would be paraphyletic. Intensive sampling needs to be conducted on teuthid families to determine if re-organization is warranted. Furthermore, the ecology of these oceanic cephalopods could perhaps explain why many interfamilial relationships are not supported. Only families found in more coastal regions, such as the sepioids (except *Spirula*) and loliginids, are consistently resolved across data sets and parameters. Due to the position of Loliginidae, it seems likely that some taxonomic revisions are needed within Decabrachia. However, many of the more basal relationships within Decabrachia are not corroborated and further investigations will be needed before taxonomic re-organization can be undertaken.

While morphological and molecular data do not agree on all nodes in all cladograms, when evaluated in concert, the five matrices complemented each other, providing support and resolution for cephalopods at many levels. Molecular loci did not agree at all nodes, possibly due to differing degrees of variability; for example, COI may not have been as informative at basal nodes, but provided more information regarding terminal relationships. Alternatively, ribosomal genes are not able to resolve terminal nodes in many cases, but provide support for more basal relationships. When data are not in agreement it provides researchers with more questions and therefore more hypotheses to investigate: What information is in greatest disagreement? Is there a biological explanation? In order to address such questions, further morphological characters should be examined in order to evaluate basal relationships; other relevant species and more genetic loci (such as developmental genes) could also be included to provide further support and resolution at all taxonomic levels.

Acknowledgments

The authors gratefully acknowledge Dr Sigurd von Boletzky for his integral input regarding cephalopod evolution, development, biology and morphology. Thanks are also due to Dr Eric Hochberg and Mike Sweeney for assistance and support with the morphological data collection. We would also like to thank M. Vecchione, D. Carlini, F. G. Hochberg, S. Piertney, M. Collins, R. Young, W. K. Macy, T. Kubodera and S. v. Boletzky for providing us with cephalopod tissue for this study. Many thanks to M. Sweeney, S. v. Boletzky, T. Hartley and J. E. Lopez for reviewing the manuscript prior to submission and to five anonymous reviewers and Arnold Kluge for comments on our submitted manuscript, some of which we chose not to follow and for which we take full responsibility. Drawings were rendered by G. Williams. Lastly, thanks to members of the Nishiguchi laboratory for input and

support on this project. This project was funded in part by NIH SO6-GM08136-26, NIH-RISE GM61222, NIH-MARC GMO766726, NSF DEB0316516, NSF DBI007982 and NSF SBE0123690 to M.K.N. A.R.L was funded in part by AMNH Lerner-Gray Fellowship, NMSU Biology Department Summer Fellowship, NMSU Graduate Teaching Assistantship, NMSU CHE Fellowship and the Hennig Society Rosen Award.

References

Arnold, J.M., Williams-Arnold, L.D., 1978. Spermiogenesis of *Nautilus pompilius*. J. Exp Zool. 205, 13–26.

Bartolomaeus, T., 1989. Larvale Nierenorgane bei *Lepidochiton cinereus* (Polyplacophora) und *Aeolidia papillosa* (Gastropoda). Zoomorphology, 108, 297–307.

Beesley, P.L., Ross, G.J.B., Wells, A. (Eds.), 1998. Mollusca: The Southern Synthesis. Fauna of Australia. CSIRO Publishing, Melbourne.

Boletzky, S.V., 1982. Developmental aspects of the mantle complex in coleoid cephalopods. Malacologia, 23, 165–175.

Boletzky, S.V., 1987. Ontogenetic and phylogenetic aspects of the cephalopod circulatory system. Experientia, 43, 478–483.

Boletzky, S.V., 1999. Breve mise au point sur la classification des cephalopodes actuels. Bull. Soc. Zool. Fr. 124, 272–278.

Boletzky, S.V., 2003. Biology of early life stages in cephalopod molluscs. Adv. Mar. Biol. 44, 144–184.

Bonnaud, L., Boucher-Rodoni, R., Monnerott, M., 1997. Phylogeny of cephalopods inferred from mitochondrial DNA sequences. Mol. Phylogenetics Evol. 7, 44–54.

Budelmann, B.U.R., Schipp, Boletzky, 1997. Cephalopoda. In: Harrison, F.W., Kohn, A.J. (Eds.), Microscopic Anatomy of Invertebrates. Wiley-Liss, New York, pp. 119–414.

Carlini, D.B., Graves, J.E., 1999. Phylogenetic analysis of cytochrome *c* oxidase I sequences to determine higher-level relationships within the coleoid cephalopods. Bull. Mar. Sci. 64, 57–76.

Carlini, D.B., Reece, K.S., Graves, J.E., 2000. Actin gene family evolution and the phylogeny of coleoid cephalopods (Mollusca: Cephalopoda). Mol. Biol. Evol. 17, 1353–1370.

Carlini, D.B., Young, R.E., Vecchione, M.V., 2001. A molecular phylogeny of the Octopoda (Mollusca: Cephalopoda) evaluated in light of morphological evidence. Mol. Phylogenetics Evol. 21, 388–397.

Chun, C., 1914. The Cephalopoda. Keter Publishing House, Jerusalem.

Colgan, D.J., McLaughlan, A., Wilson, G.D.F., Livingston, S.P., Edgecombe, G.D., Macaranas, J., Cassis, G., Gray, M.R., 1998. Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. Aust. J. Zool. 46, 419–437.

Denton, E.J., Gilpin-Brown, J.B., 1973. Flotation mechanisms in modern and fossil cephalopods. Adv. Mar. Biol. 11, 197–268.

Donoghue, M.J., Doyle, J.J., Gauthier, J., Kluge, A.G., Rowe, T., 1989. The importance of fossils in phylogeny reconstruction. Annu. Rev. Ecol. Syst, 20, 431–460.

Edgecombe, G.D., Giribet, G.D., Wheeler, W., 2002. Phylogeny of the Henicopidae (Chilopoda: Lithobiomorpha): a combined analysis of morphology and five molecular loci. Syst. Entomol. 27, 31–64.

Eernisse, D.J., Kluge, A.G., 1993. Taxonomic congruence versus total evidence, and amniote phylogeny inferred from fossils, molecules, and morphology. Mol. Biol. Evol. 10, 1170–1195.

Engeser, T., Bandel, 1988. Phylogenetic classification of coleoid cephalopods. In: Weidmann, J., Kullmann, J. (Eds.), Cephalopods – Present and Past. Schweizerbart'sche-Verlagsbuchhandlung, Stuttgart, pp. 105–115.

Farris, J.S., 1997. The future of phylogeny reconstruction. Zool. Scripta, 26, 303–311.

Farris, J.S., Albert, V.A., Källersjö, M., Lipscomb, D., Kluge, A.G., 1996. Parsimony jackknifing outperforms neighbor-joining. Cladistics, 12, 99–124.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1995. Testing significance of incongruence. Cladistics, 10, 315–319.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R.C., 1994. DNA primers for amplification of mitochondrial cyctochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar. Biol. Biotechnol. 3, 294–299.

Franzén, A., 1955. Comparative Morphological Investigations into the spermiogenesis among Mollusca. Zool. Bidrag Fran Uppsala, 30, 399–456.

Franzén, A., 1958. On sperm morphology and acrosome filament formation in some Annelida, Echiuroidea and Tunicata. Zool. Bidrag Fran Uppsala, 33, 1–29.

Gauthier, J., Kluge, A.G., Rowe, T., 1988. Amniote phylogeny and the importance of fossils. Cladistics, 4, 105–209.

Giribet, G., 2002. Current advances in the phylogenetic reconstruction of metazoan evolution. A new paradigm for the Cambrian Explosion? Mol. Phylogenetics Evol. 23, 345–357.

Giribet, G., 2003. Stability in phylogenetic formulations, and its relationship to nodal support. Syst. Biol. 52, 554–564.

Giribet, G., Edgecombe, G.D., Wheeler, W.C., Babbitt, C., 2002. Phylogeny and systematic position of Opiliones: a combined analysis of chelicerate relationships using morphological and molecular data. Cladistics, 18, 5–70.

Giribet, G.D., Edgecombe, G.D., Wheeler, W.C., 2001. Arthropod phylogeny based on eight molecular loci and morphology. Nature, 413, 157–161.

Giribet, G., Carranza, S., Baguñà, J., Ribera, C., 1996. First molecular evidence for the existence of a Tardigrada + Arthropoda clade. Mol. Biol. Evol. 13, 76–84.

Giribet, G.D., Distel, M., Polz, M., Sterrer, W., Wheeler, W., 2000. Triploblastic relationships with emphasis on the acoelomates and the position of Gnathostomulida, Cycliophora, Plathelminthes, and Chaetognatha: a combined approach of 18S rDNA sequences and morphology. Syst. Biol. 49, 539–562.

Giribet, G., Wheeler, W.C., 2001. Some unusual small-subunit ribosomal RNA sequences of metazoans. Am. Museum Novitates, 3337, 1–14.

Giribet, G.D., Wheeler, W.C., 2002. On bivalve phylogeny: a high-level analysis of the Bivalvia (Mollusca) based on combined morphology and DNA sequence data. Invertebrate Biology, 121, 271–324.

Goloboff, P.A., 1998. Nona, Version 2.0. Program available at http://www.cladistics.com.

Goloboff, P.A., 1999. Analyzing large data sets in reasonable times: solutions for composite optima. Cladistics, 15, S26–S34.

Haszprunar, G., 1988. On the origin and evolution of major gastropod groups, with special reference to the Streptoneura. J. Molluscan Studies, 54, 367–441.

Haszprunar, G., 2000. Is the Aplacophora monophyletic? A cladistic point of view. Am. Malacal. Bull. 15, 115–130.

Haszprunar, G., Wanninger, A., 2000. Molluscan muscle systems in development and evolution. J. Syst. Evol. Res. 38, 157–163.

Healy, J.M., 1989. Spermatozoa of the deep-sea cephalopod *Vampyroteuthis infernalis* Chun: ultrastructure and possible phylogenetic significance. Phil. Trans. R. Soc. 323, 589–600.

Healy, J.M., 1990a. Ultrastructure of spermatozoa in *Spirula spirula* – Systematic importance and comparison with other cephalopods. Helgoländer Meeresuntersuchungen, 44, 109–123.

Healy, J.M., 1990b. Ultrastructure of spermiogenesis in *Vampyroteuthis infernalis* Chun – A relict cephalopod mollusc. Helgoländer Meeresuntersuchungen, 44, 95–108.

Healy, J.M., 1993. Sperm and spermiogenesis in *Opisthoteuthis persephone* (Octopoda: Cirrata): ultrastructure, comparison with

other cephalopods and evolutionary significance. J. Molluscan Studies, 59, 105–115.
Healy, J.M., 1996. Molluscan sperm ultrastructure: correlation with taxonomic units within Gastropoda, Cephalopoda and Bivalvia. In: Taylor, J.D. (Ed.), Molluscan Sperm Ultrastructure: Correlation with Taxonomic Units Within Gastropoda, Cephalopoda and Bivalvia. Oxford University Press, Oxford, pp. 99–113.
Herring, P.J., 1988. Luminescent organs. In: Wilbur, K.M. (Ed.), The Mollusca, Vol. 11. Academic Press, San Diego, pp. 449–485.
Hess, H.C., 1987. Comparative morphology, variability, and systematic applications of cephalopod spermatophores (Teuthoidea and Vampyromorpha). PhD Dissertation. University of Miami, FL.
Jeletzky, J.A., 1966. Comparative Morphology, Phylogeny, and Classification of Fossil Coleoidea, Paleontological Contributions: University of Kansas, Vol. 7, pp. 1–162.
Joubin, L., 1825/1924. Cephalopods from the Scientific Expedition of Prince Albert I of Monaco. Amerind Publishing, New Dehli.
Kluge, A.G., 1989. A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). Syst Zool. 38, 7–25.
Lee, P., Callerts, P., d. Couet, H.G., Martindale, M.Q., 2003. Cephalopod *Hox* genes and the origin of morphological novelties. Nature, 424, 1061–1065.
Madison, D.R., Madison, W.P., 2000. MacClade 4: Interactive Analysis of Phylogeny and Character Evolution, Version. 4.01. Sinauer Associates, MA.
Maxwell, W.L., 1974. Spermiogenesis of *Eledone cirrhosa*, Lamark (Cephalopoda: Octopoda). Proc. R. Soc. Lond. 186, 181–190.
Maxwell, W.L., 1975. Spermiogenesis of *Eusepia officinalis* (L.), *Loligo forbesi* (Steenstrup) and *Alloteuthis subulata* (L.) (Cephalopoda). Proc. R. Soc. Lond. 191, 527–535.
McFall-Ngai, M.J., Ruby, E.G., 1998. Sepiolids and Vibrios: when they first meet. Bioscience, 48, 257–265.
Mickevich, M.F., Farris, J.S., 1981. The implications of congruence in *Menidia*. Syst Zool. 27, 143–158.
Montgomery, M.K., McFall-Ngai, M., 1992. Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. Biol. Bull. 184, 296–308.
Naef, A., 1921/23. Fauna and Flora of the Bay of Naples. Keter Press, Jerusalem.
Naef, A., 1928. Cephalopoda Embryology. R. Friedlander & Sons, Berlin.
Nesis, K.N., 1987. Cephalopods of the World. T.F.H. Publications, Neptune City, Canada.
Nesis, K.N., 1998. Biodiversity and systematics in cephalopods: Unresolved problems require an integrated approach. Cephalopod biodiversity, ecology and evolution. S. Afr. J. Mar. Sci. 20, 165–173.
Nishiguchi, M.K., Lopez, J.E., Boletzky, S.V., 2004. Enlightenment of old ideas from new investigations: The evolution of bacteriogenic light organs in squids. Evol. Dev. 6, 41–49.
Nixon, K.C., 2002. Winclada, Version 1.00.08. Program available at http://www.cladistics.com.
Nixon, M., Young, J.Z., 2003. The Brains and Lives of Cephalopods. Oxford University Press, Oxford.
Okusu, A., Schwabe, E., Eernisse, D.J., Giribet, G., 2003. Towards a phylogeny of chitons (Mollusca, Polyplacophora) based on combined analysis of five molecular loci. Org. Diversity Evol. 3, 281–302.
Pfeffer, G., 1912. Die Cephalopoden der Plankton-Expedition. Pauls Press, New Dehli.
Pickford, G.E., 1939. A re-examination of the types of *Melanoteuthis lucens* Joubin. Bull. Inst. Océanographique, Monaco, 777, 1–12.
Piertney, S.B., Hudelot, C., Hochberg, F.G., Collins, M.A., 2003. Phylogenetic relationships among cirrate octopods (Mollusca: Cephalopoda) resolved using mitochondrial 16S ribosomal DNA sequences. Mol. Phylogenetics Evol. 27, 348–353.
Ponder, W.F., Lindberg, D.R., 1997. Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. Zool. J. Linnean Soc. 119, 83–265.
Reynolds, P.D., 2002. The Scaphopoda. Adv. Mar. Biol. 42, 139–204.
Ribes, E., Giménez-Bonafe, P., Zamora, M.J., González, A., Kasinsky, H., Chiva, M., 2002. Evolution of octopod sperm II: Comparison of acrosomal morphogenesis in *Eledone* and *Octopus*. Mol. Reprod. Dev. 62, 363–367.
Roper, C.F.E., 1969. Systematics and zoogeography of the worldwide bathypelagic squid *Bathyteuthis* (Cephalopoda: Oegopsida). Bull. US Natl. Mus. 291, 1–210.
Roper, C.F.E. Sweeney, M.J., Nauen, C.A., 1984. FAO Species Catalog: Cephalopods of the World. An Annotated and Illustrated Catalog of Species of Interest to Fisheries. FAO Fisheries Synopsis, Rome.
Roper, C.F.E., Young, R.E., Voss, G.L., 1969. An illustrated key to the families of the order Teuthoidea (Cephalopoda). Smithsonian Contrib. Zool. 13, 1–32.
Salvini-Plawen, L., Steiner, 1996. Synapomorphies and Plesiomorphies in higher classification of Mollusca. In: Taylor J.T. (Ed.), Origin and Evolutionary Radiation of the Mollusca. Oxford University Press, Oxford, pp. 29–51.
Shimek, R.L., Steiner, 1997. Scaphopoda. In: Harrison, F.W., Kohn, A.J. (Eds.), Microscopic Anatomy of Invertebrates, Vol. 6B: Mollusca 11. John Wiley and Sons, New York, pp. 13–54.
Smith, S.W., Overbeek, R., Woese, C.R., Gilbert, W., Gillivet, P.M., 1994. The genetic data environment: an expandable GUI for multiple sequence analysis. Comput. Appl. Biosci. 10, 671–675.
Toll, R.B., 1982. The comparative morphology of the gladius in the order Teuthoidea (Mollusca: Cephalopoda) in relation to systematics and phylogeny. PhD Dissertation, University of Miami, FL.
Toll, R.B., 1998. The gladius in teuthoid systematics. In: International Workshop on the Systematics and Biogeography of Cephalopods. Smithsonian Institution Press, Washington, DC, pp. 55–68.
Voight, J.R., 1997. Cladistic analysis of the octopods based on anatomical characters. J. Molluscan Studies, 63, 311–325.
Waller, T.R., 1998. Origin of the molluscan class Bivalvia and a phylogeny of major groups. In: Johnston, P.A., Haggart, J.W. (Eds.), Origin of the Molluscan Class Bivalvia and a Phylogeny of Major Groups. University of Calgary Press, Calgary, pp. 1–45.
Wanninger, A., Haszprunar, G., 2002. Chiton myogenesis: perspectives for the development and evolution of larval and adult muscle systems in molluscs. J. Morph, 251, 103–113.
Wheeler, W., 1995. Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. Syst Biol. 44, 321–331.
Wheeler, W., 1996. Optimization alignment: The end of multiple sequence alignment in phylogenetics? Cladistics, 12, 1–9.
Wheeler, W.C., Gladstein, D. DeLaet, J., 2002. POY, Version 3.0. Program and documentation available at ftp://ftp.amnh.org/pub/molecular. American Museum of Natural History.
Wheeler, W.C., Giribet, G., Edgecombe, G.D., 2004. Arthropod systematics: The comparative study of genomic, anatomical, and paleontological information. In: Cracraft, J., Donoghue, M.J. (Eds.), Assembling the Tree of Life. Oxford University Press, New York, pp. 281–295.
Wheeler, W.C., Hayashi, C.Y., 1998. The phylogeny of extant chelicerate orders. Cladistics, 14, 173–192.
Whiting, M.F., Carpenter, J.M., Wheeler, Q.D., Wheeler, W.C., 1997. The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Syst Biol. 46, 1–68.
Young, R.E., 1964. The anatomy of the vampire squid. MSc Thesis. University of Southern California, Los Angeles, CA.
Young, R.E., 1977. Ventral bioluminescent countershading in midwater cephalopods. Symp. Zool. Soc. Lond. 38, 168–190.
Young, R.E., Harman, R.F., 1998. Phylogeny of the 'Enoploteuthid' Families. In: International Workshop on Systematics and Bioge-

ography of Cephalopods. Smithsonian Institution Press, Washington, DC, pp. 257–271.

Young, R.E., Vecchione, M., Donovan, D.T., 1998. The evolution of coleoid cephalopods and their present biodiversity and ecology. In: Payne, A.I.L, Lipinski, M.R., Clarke, M.R., Roeleveld, M.A.C. (Eds.), Cephalopod Biodiversity, Ecology and Evolution. National Book Printers, Cape Town, pp. 393–420.

Young, R.E., Vecchione, M., 1996. Analysis of morphology to determine primary sister-taxon relationships within coleoid cephalopods. Am. Malacal. Bull. 12, 91–112.

Appendix 1

Voucher information for cephalopod specimens used for DNA extraction in this study. Information for outgroups listed in Giribet and Wheeler (2002) and Okusu et al. (2003).

Classification			Source	Collection data
Nautiloidea				
Nautilida	Nautilidae	*Nautilus pompilius* Linnaeus, 1758	GG	AMNH; 2003
		Nautilus scrobiculatus Lightfoot, 1786	GG	AMNH; 2003
Coleoidea				
Octobrachia				
Octopoda	Allopsidae	*Haliphron atlanticus* Steenstrup, 1861	MV	DE0304 (Sta. 2), 2003; NMNH
		Haliphron sp.	SP	UA; 2003
	Argonautidae	*Argonauta nodosa* Lightfoot, 1786	MV	DE0304 (Sta. 2), 2003; NMNH
	Bolitaenidae	*Japetella diaphana* Hoyle, 1885	MV	DE0304 (Sta. 5), 2003; NMNH
	Octopodidae	*Bathypolypus arcticus* (Prosch, 1847)	FGH	Saquatucket Harbor; SBMNH
		Benthoctopus sp.	MC	NMNH; 2003
		Eledone cirrosa (Lamarck, 1798)	SvB&AL	Banyuls-sur-Mer, 2001; NMSU
		Grandeledone verrucosa (Verril, 1881)	DC (MV&RY)	F/V Contender, 1994; NMNH
		Ocythoe tuberculata Rafinesque, 1814	MV	NMNH; 2003
		Thaumeledone guntheri Robson, 1930	MC	South Georgia; BAS
Cirroctopoda	Cirroteuthidae	*Cirrothauma murryae* (Chun, 1911)	MC	Porcupine Seabright; BAS
		Stauroteuthis syrtensis Verrill, 1884	DC (MV)	F/V Contender, 1995; NMNH
	Opisthoteuthidae	*Opisthoteuthis* sp. 1 (18S, 28S, H3)	FGH	Santa Barbara; SBMNH
		Opisthoteuthis sp. 2 (COI)	FGH	Santa Barbara; SBMNH
Vampyromorpha				
	Vampyroteuthidae	*Vampyroteuthis infernalis* Chun, 1903	DC	Hokusei Maru, 1996
Decabrachia				
Sepiolida	Sepiolidae	*Heteroteuthis hawaiiensis* (Berry, 1909)	DC	Hokusei Maru, 1996
		Stoloteuthis leucoptera (Verril, 1878)	DC (MV)	ALB9402.14.18; NMNH
		Rossia palpebrosa Owen, 1834	DC (MV)	ALB9402.19.27; NMNH
		Sepiola affinis Naef, 1912	MKN	Banyuls-sur-Mer, 2002; NMSU
Sepiida	Sepiidae	*Sepiella inermis* (Van Hasselt, 1835)	MKN	Banyuls-sur-Mer, 2002; NMSU
		Sepia officinalis Linnaeus, 1758	MKN	Banyuls-sur-Mer, 2002; NMSU
Spirulida	Spirulidae	*Spirula spirula* Linnaeus, 1758	DC (MK&RY)	NMNH; 1999
Idiosepiida	Idiosepiidae	*Idiosepius pygmaeus* Steenstrup, 1881	MKN	Botany Bay, AU, 2000; NMSU
Teuthida	Loliginidae	*Loligo pealei* LeSueur, 1821	WKM	Northern Atlantic, 2003; NMSU
		Loligo formosana Sasaki, 1929	MKN	Rayong, Thailand, 2001; NMSU
		Sepioteuthis lessoniana Férussac, 1830	MKN	Rayong, Thailand, 2001; NMSU
	Ancistrocheiridae	*Ancistrocheirus lesueuri* (Orbigny, 1842)	DC (RY)	Hokusei Maru, 1994
	Architeuthidae	*Architeuthis dux* Steenstrup, 1857	DC (MV)	NMNH; 1999
	Bathyteuthidae	*Bathyteuthis abyssicola* Hoyle, 1885	DC	Hokusei Maru, 1996
	Batoteuthidae	*Batoteuthis skolops* Young & Roper, 1968	MC	South Georgia; BAS
	Brachioteuthidae	*Brachioteuthis* sp.	MV	DE0304, 2003; NMNH
	Chiroteuthidae	*Chiroteuthis veranyi* (Férussac, 1835)	MV	DE0304 (Sta. 4), 2003; NMNH
	Cranchiidae	*Cranchia scabra* Leach, 1817	DC (RY)	Hokusei Maru, 1994; NMNH
		Leachia atlantica (Degner, 1925)	MV	NMNH; 2003
	Chtenopterygidae	*Chtenopteryx sicula* (Vérany, 1851)	TK	NSMT; 1999
	Cycloteuthidae	*Cycloteuthis sirventyi* (Joubin, 1919)	DC (RY)	Hokusei Maru, 1994
		Discoteuthis laciniosa Young & Roper, 1969	DC (RY)	Hokusei Maru, 1994
	Enoploteuthidae	*Abraliopsis pfefferi* Joubin, 1896	MV	DE0304 (Sta. 12), 2003; NMNH
		Enoploteuthis leptura (Leach, 1817)	MV	DE0304 (Sta. 15), 2003; NMNH
	Gonatidae	*Gonatus antarcticus* Lönnberg, 1898	MC	South Georgia, BAS
		Gonatus fabricii (Lichtenstein, 1818)	MV	DE0304 (Sta. 3), 2003; NMNH
	Histioteuthidae	*Histioteuthis corona* (Voss & Voss, 1962)	TK	NSMT; 1999
		Histioteuthis hoylei (Goodrich, 1896)	DC	Hokusei Maru, 1996
		Histioteuthis reversa (Verrill, 1880)	MV	DE0304 (Sta. 3), 2003; NMNH

Appendix 1
Continued

Classification			Source	Collection data
	Joubiniteuthidae	*Joubiniteuthis portieri* (Joubin, 1912)	MV	DE0304 (Sta. 14), 2003; NMNH
	Lepidoteuthidae	*Lepidoteuthis grimaldii* Joubin, 1859	DC (RY)	Hokusei Maru, 1994
	Mastigoteuthidae	*Mastigoteuthis agassizii* Verril, 1881	MV	DE0304 (Sta. 3), 2003; NMNH
		Mastigoteuthis magna Joubin, 1913	MV	DE0304 (Sta. 1), 2003; NMNH
	Neoteuthidae	*Neoteuthis thielei* Naef, 1921	MV	DE0304 (Sta. 4), 2003; NMNH
	Octopodeuthidae	*Octopoteuthis nielseni* Robson, 1948	DC (RY)	Hokusei Maru, 1994
		Octopoteuthis sicula Rüppel, 1844	TK	NSMT;1999
	Ommastrephidae	*Illex coindeti* (Vérany, 1837)	SvB	Banyuls-sur-Mer, 2001; NMSU
		Ommastrephes bartramii (LeSueur, 1821)	DC	Hokusei Maru, 1996
		Ornithoteuthis antillarum Adam, 1957	MV	DE0304 (Sta. 14), 2003; NMNH
		Sthenoteuthis oualaniensis (Lesson, 1830)	DC (RY)	Hokusei Maru, 1994
	Onychoteuthidae	*Moroteuthis knipovitchi* Filippova, 1972	MC	South Georgia; BAS
	Psychroteuthidae	*Psychroteuthis* sp.	MC	South Georgia; BAS
	Pyroteuthidae	*Pyroteuthis margaretifera* (Rüppel, 1844)	MV	DE0304 (Sta. 3), 2003
		Pterygioteuthis gemmata Chun, 1908	MV	DE0304 (Sta. 2), 2003; NMNH

Source Abbreviations: AL, Annie Lindgren; DC, David Carlini; FGH, Eric Hochberg; GG, Gonzalo Giribet; RY, Richard Young; MC, Martin Collins; MKN, Michele Nishiguchi; MV, Michael Vecchione; SP, Stuart Piertney, TK, Tsunemi Kubodera, WKM, William Macy, (MV), tissue sample collected originally by Michael Vecchione. Repository institutions: AMNH, American Museum of Natural History, New York; NMNH, National Museum of Natural History, Washington D.C.; NMSU, New Mexico State University, Las Cruces; UA, University of Aberdeen, Scotland; BAS, British Antarctic Survey, United Kingdom; NSMT, National Science Museum, Tokyo. Where collection information is not available, repository and date sent to NMSU are listed.

Appendix 2
List of cephalopod specimens used in morphological character coding.

Classification			Repository/catalog number*	Sex
Nautiloidea				
Nautilida	Nautilidae	*Nautilus pompilius* Linnaeus, 1758	literature	
		Nautilus scrobiculatus Lightfoot, 1786	literature	
Coleoidea				
Octobrachia				
Octopoda	Alloposidae	*Haliphron atlanticus* Steenstrup, 1861	SBMNH	f
	Argonautidae	*Argonauta arago* Linnaeus, 1758	MCZ	f
		Argonauta nodosa Lightfoot, 1786	literature	
	Bolitaenidae	*Japetella diaphana* Hoyle, 1885	SBMNH #45791	
		Japetella heathi (Berry, 1911)	SBMNH #63008	f
		Japetella sp.	SBMNH #63086	m
		Japetella sp.	SBMNH #63072	f
	Octopodidae	*Bathypolypus arcticus* (Prosh, 1847)	SBMNH; Falkland Islands	f
		Bathypolypus arcticus (Prosh, 1847)	SBMNH; Sea Scallop Dredge	m
		Benthoctopus hokkaidensis (Berry, 1921)	SBMNH #45787	m
		Eledone cirrosa (Lamarck, 1798)	SBMNH #142574	f
		Grandeledone verrucosa (Verrill, 1881)	literature	
		Octopus rubescens Berry, 1953	SBMNH #41962	f
		Octopus vulgaris Cuvier, 1797	SBMNH #OV-90-17	f
		Octopus vulgaris Cuvier, 1797	SBMNH #OV-90-16	m
		Thaumeledone guntheri Robson, 1930	literature	
Cirroctopoda	Cirroteuthidae	*Cirrothauma murrayi* (Chun, 1911)	literature	
		Stauroteuthis syrtensis Verrill, 1884	literature	
	Opisthoteuthidae	*Opisthoteuthis massyae* Grimpe, 1920	SBMNH #45973	m
		Opisthoteuthis sp.	SBMNH	f
		Opisthoteuthis sp.	SBMNH	m
		**Opisthoteuthis* sp. 1	SBMNH	
Vampyromorpha				
	Vampyroteuthidae	*Vampyroteuthis infernalis* Chun, 1903	SBMNH #62500	f
Decabrachia				
Sepiolida	Sepiolidae	*Heteroteuthis hawaiiensis* (Berry, 1909)	SBMNH; Hokusei Maru, Sta. 1C	f, m
		Stoloteuthis leucoptera (Verril, 1878)	literature	
		Rossia palpebrosa Owen, 1834	literature	
		**Sepiola affinis* Naef, 1912	NMSU;Banyuls-sur-Mer, 2002	f, m

Appendix 2
Continued

Classification			Repository/catalog number*	Sex
Sepiida	Sepiidae	*Sepiella inermis* (Van Hasselt, 1835)	literature	
		**Sepia officinalis* Linnaeus, 1758	NMSU;Banyuls-sur-Mer, 2002	f, m
Spirulida	Spirulidae	*Spirula spirula* Linnaeus, 1758	MCZ #093798	
Idiosepida	Idiosepiidae	*Idiosepius pygmaeus* Steenstrup, 1881	NMSU	
Teuthida	Loliginidae	**Loligo pealei* LeSueur, 1821	NMSU	f, m
		Loligo formosana Sasaki, 1929	literature	
		Sepioteuthis lessoniana Férussac, 1830	SBMNH; Philippines,Zambango;1948	
		Sepioteuthis lessoniana Férussac, 1830	SBMNH #USC1204	
	Ancistrocheiridae	*Ancistrocheirus lesueuri* (Orbigny, 1842)	SBMNH;NH2-93 Hawaii	f
	Architeuthidae	*Architeuthis dux* Steenstrup, 1857	literature	
	Bathyteuthidae	*Bathyteuthis abyssicola* Hoyle, 1885	SBMNH #49331	f
	Batoteuthidae	*Batoteuthis* sp.	SBMNH;NH2-93 Hawaii	
	Brachiteuthidae	*Brachioteuthis* sp. Verrill, 1881	SBMNH #60131	
	Chiroteuthidae	*Chiroteuthis calyx* Young, 1972	SBMNH #45799	m
		Chiroteuthis sp.	FMNH #296689	m
		Chiroteuthis veranyi (Férussac, 1830)	literature	
	Cranchiidae	*Cranchia scabra* Leach, 1817	SBMNH #45727	f
		Leachia atlantica (Degner, 1925)	literature	
	Chtenopterygidae	*Chtenopteryx sicula* (Vérany, 1851)	MCZ #278566, 278657	
	Cycloteuthidae	*Cycloteuthis sirventyi* (Joubin, 1919)	literature	
		Discoteuthis laciniosa Young and Roper, 1969	SBMNH #142131	f
	Enoploteuthidae	*Abraliopsis affinis* (Pfeffer, 1912)	SBMNH #49436	f
		Abraliopsis pfefferi Joubin, 1919	literature	
		Enoploteuthis sp.	SBMNH #51695	f
		Enoploteuthis leptura (Leach, 1817)	literature	
	Gonatidae	*Gonatus antarcticus* Lönnberg, 1898	literature	
		Gonatus fabricii (Lichtenstein, 1818)	SBMNH #00011	
		Gonatus onyx Young, 1972	SBMNH #60597	f
	Histioteuthidae	*Histioteuthis* sp.	MCZ #277836	
		Histioteuthis sp.	SBMNH # 890909	f
		Histioteuthis corona (Voss & Voss, 1962)	literature	
		Histioteuthis heteropsis (Berry, 1913)	SBMNH #61158	f
		Histioteuthis hoylei (Goodrich, 1896)	literature	
	Joubiniteuthidae	*Joubiniteuthis portieri* (Joubin, 1912)	FMNH #278105	m
		Joubiniteuthis sp.	SBMNH; NH2-93 Hawaii	f
	Lepidoteuthidae	*Lepidoteuthis* sp.	SBMNH #51304	
	Mastigoteuthidae	*Mastigoteuthis* sp.	FMNH #78309	
		Mastigoteuthis pyrodes Young, 1972	SBMNH;Trawl #14 San Clemente, 2003	m
	Neoteuthidae	*Neoteuthis* sp.	SBMNH #11308	f
	Octopodeuthidae	*Octopoteuthis* sp.	SBMNH #61554	m
		Octopoteuthis sp.	SBMNH #61563	f
	Ommastrephidae	*Illex coindeti* (Vérany, 1837)	SBMNH;Bay of Naples, 1959	
		Ommastrephes bartramii (LeSueur, 1821)	MCZ #338290	m
		Sthenoteuthis oualaniensis Lesson, 1830	SBMNH #64394	m
		Ornithoteuthis antillarum Adam, 1857	literature	
	Onychoteuthidae	*Moroteuthis* sp.	SBMNH;British Antarctic Survey, 1988	m
		Onychoteuthis banskii (Leach, 1817)	MCZ #293703	m
	Pyroteuthidae	*Pyroteuthis margaretifera* (Rüppel, 1884)	FMNH #78300	f
		Pyroteuthis sp.	SBMNH;NH-2 93, Hawaii	f
		Ptergioteuthis gemmata Chun, 1908	SBMNH #64434	m
		Ptergioteuthis sp.	FMNH #28690	m

Taxa listed below also include specimens not used in analysis, merely to confirm character states. Asterisk indicates voucher specimen for DNA analysis. Source abbreviations; MCZ, Museum of Comparative Zoology, Harvard University; NMSU, New Mexico State University; FMNH, Field Museum of Natural History; SBMNH, Santa Barbara Museum of Natural History. Where catalog number not available, collection information has been listed.

Appendix 3. Character descriptions

Cephalopod characters were scored in Table 3 via direct specimen observation. When specimens were not available, or characters were difficult to measure, primary literature was used (Naef, 1921/23; Roper et al., 1969; Salvini-Plawen and Steiner, 1996; Young and Vecchione, 1996; Young and Harman, 1998). Characters coded in Table 3 as not applicable (–) indicate that a particular character could not be scored across all taxa. Characters 1–65 are primarily specific to cephalopods and most were therefore coded as inapplicable in other molluscs. In cases where a particular state could not be identified, it was coded as "?".

1. Calcified outer shell: (0) absent; (1) present. A calcified outer shell is no longer present in extant cephalopods except for species within Nautliloidea.

2. Siphuncle: (0) absent; (1) present (Young and Vecchione, 1996). The presence of a siphuncle is a synapomorphy of all cephalopods (Salvini-Plawen and Steiner, 1996).

3. Inner shell sac: (0) absent; (1) present. All coleoid cephalopods have an internal shell sac, which secretes the internal shell. In Octopoda an embryonic shell sac/gland is present during embryonic development but shell material is not always secreted such as in the case of Argonautidae (Naef, 1928; Boletzky, 1982).

4. Inner shell morphology: (0) chambered with siphuncle; (1) uncalcified gladius; (2) uncalcified fin supports; (3) uncalcified stylets. Due to the variability among coleoid internal shells, separate states have been identified (Toll, 1982, 1998). All character states are included as a single character because the origins of each shell type are likely homologous due to the presence of a shell gland (see character 3). Only those taxa that have a shell sac that secretes shell material are considered. Sepiidae exhibit a chambered internal shell while *Spirula* has an internal, calcified, chambered shell with a siphuncle. The teuthid gladius differs greatly from other internalized shells within Coleoidea, but is the most common (Toll, 1982). While Octopoda does not have an uncalcified inner shell, stylets are present in many families (with a shell sac in embryonic stage). Alternatively, Cirroctopoda has a gladius modified to act as fin supports. However, the fin supports in Cirroctopoda differ greatly from both the gladius as well as the stylets, so separate character states are provided for each.

5. Conus morphology: (0) conus absent (1) primary conus present; (2) secondary conus present; (3) pseudoconus present. (Toll, 1982). The primary conus is small and cuplike or sub-triangular in outline and exhibits a cone field and a rostrum, located at the apical tip of the gladius. The ventral rim forms a broad U-shaped border or is completely transverse. The primary conus is considered homologous to the phragmocone portion of the ancestral shell (Jeletzky, 1966). The secondary conus is considered a more derived state, formed by ventral curvature and midventral fusion of the posterolateral edges of the vanes (Toll, 1982). Because it is formed from the vanes, the secondary conus is presumed to be derived from the proostracum portion of the ancestral shell and is also never found in association with a rostrum. The pseudoconus state occurs when the posterolateral edges of the vanes overlap but no fusion occurs. Pseudoconus morphology has been expanded to include all conuses formed by the in-folding of the posterolateral edges of the vanes with or without fusion (this state is applicable only to some genera of cranchiids) (Toll, 1982).

6. One pair of fins: (0) absent; (1) present. At least one pair of fins is present in most cephalopods (Salvini-Plawen and Steiner, 1996). The fins are attached to the cartilage-enforced shell epithelium forming an articulated capsule adjacent to the shell sac (Naef, 1921/23).

7. Additional fins (with postembryonic fin developing second and posterior to adult fin): (0) absent; (1) present at some stage in life cycle. In decabrachians the fins typically insert on a flattened cartilage (which attaches to the shell sac) with a straight medial ridge. During development a juvenile fin develops first, followed by an adult fin. The juvenile fin is subsequently reabsorbed during growth while the adult fins enlarge (Naef, 1928; Boletzky, 1982). However, in some cases, two sets of fins remain, such as in Vampyroteuthidae (separated by light organs) and some teuthids (although the second fin is often broken off). Within Teuthida, Chiroteuthidae, Grimpoteuthidae, Batoteuthidae, Joubiniteuthidae and Mastigoteuthidae all species possess some form of additional fins.

8. Nuchal cartilage: (0) absent; (1) present and exposed; (2) present but not exposed (Young and Vecchione, 1996). The nuchal cartilage supports the head component of the nuchal locking apparatus; the muscles of the collar, head and shell sac attach to the cartilage. The head of cuttlefishes and squids is well separated from the body by a neck (nuchal construction), believed to be the plesiomorphic state (Young and Vecchione, 1996). In some sepiolids the mantle is dorsally fused with the head and ventrally connected by a narrow or wide cutaneous nuchal b and such as in Sepiolinae, *Sepiolina*, *Stoloteuthis*, *Iridioteuthis* and Sepiadariidae. In Idiosepiidae the mantle is not fused with the head, but no nuchal cartilage is present. All remaining squids and cuttlefish have nuchal cartilage connecting the mantle to the head. Nuchal cartilage is present in Vampyroteuthidae but no longer supports a locking apparatus, instead providing a site for muscle attachment. The lack of exposure in *Vampyroteuthis* is likely to be apomorphic and was therefore coded as a separate state.

9. Chromatophores: (0) absent; (1) present. Chromatophores are vesicular cells that expand due to contractile radiating fibers, found only in coleoid cephalopods (Naef, 1921/23; Salvini-Plawen and Steiner, 1996).

10. Buccal crown: (0) absent; (1) present (Young and Vecchione, 1996). The buccal crown consists of muscular buccal supports and connective membranes that surround the lips and mouth. In *Idiosepius*, the buccal crown is apparent in dissected animals, just barely intercalated within the arms of the animal. A buccal crown is absent in octobrachians and not applicable in *Nautilus* because the homologous structure is unknown.

11. Buccal membrane connective attachment to arms V (see character 15 for explanation of arm numbering): (0) dorsal; (1) ventral (Roper et al., 1969; Roper, 1969; Young and Harman, 1998; Young et al., 1998). The arms of squids and cuttlefish are attached to the outer membrane surrounding the mouth by a cutaneous and muscular buccal membrane attachment. The major function of the buccal attachment is to hold the arms together in a cone during swimming (Naef, 1921/23). Vampyromorpha, Cirroctopoda and Octopoda have no buccal attachments; the arms are muscular hydrostats. The buccal membrane is attached to the dorsal side of arms I and II, ventral side of arms III and either dorsal or ventral to arms V.

12. Buccal lappet number: (0) 6; (1) 7; (2) 8 (Roper et al., 1969). The buccal membrane is star shaped and consists of 6, 7, or 8 rays. Initially eight lappets are present, though those extending to the first and fourth set of arms may merge together.

13. Beak: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996; Waller, 1998). A beak is present both in the coleoid and nautiloid cephalopods (with calcified additions to the edge in the latter).

14. Radular apparatus: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996; Haszprunar, 2000). While a true radula is lost in some molluscs such as the cirroctopods, a radular apparatus, consisting of a radular sac and odontophore is still present in cephalopods.

15. Circumoral appendages (arms): (0) absent; (1) present (Waller, 1998). The cephalopod head bears an outer circle of at least eight arms that are believed to be derived from the molluscan foot (Naef, 1921/23).

Note on arm numbering:

Octobrachia	Vampyromorpha/Decabrachia
1	I
lost?	II
2	III?
3	IV
4	V

Embryological and developmental studies indicate that arms II (rather than arms III) are the pair likely lost by Octobrachia (Naef, 1928). In this case, arms I, II, III, IV and V will be used to describe individual arm pairs.

16. Arms II: (0) unmodified; (1) filaments; (2) absent (Young and Vecchione, 1996). Arms II are present in decabrachians, absent in octobrachians and modified into filaments in *Vampyroteuthis*. Early growth stages provide evidence that vampyroteuthid filaments are homologous to arms II (Naef, 1921/23; Boletzky, 1982).

17. Arms IV: (0) unmodified; (1) tentacles (Young and Vecchione, 1996). Modification of Arms IV is one of the significant characters used to separate decabrachians from octobrachians. Arms IV are unmodified in octobrachians and vampyromorphs and modified into tentacles in decabrachians.

18. Horizontal arm septa inserted in the arm muscles: (0) absent; (1) present (Young and Vecchione, 1996). Cirroctopoda possess a horizontal septum that inserts into the circular muscle layer that forms the outer and thinner portion of the cylindrical muscular wall of the arm. The septum is orally concave in cross section and divides the muscular tube within each arm into oral and aboral regions. *Japetella diaphana* was coded as "?" because similar septa are present and are inserted as two membranes, extending in an oral/aboral plane internal to arm muscles. It is unclear whether the two states evolved independently. Due to the difficulty of coding fixed specimens, this character was taken directly from Young and Vecchione (1996).

19. Cirri on arms: (0) absent; (1) present (Young and Vecchione, 1996). Cirri are elongate, fleshy, finger-like papillae or palps located along the lateral edges of the oral surface of the arms, particularly in cirrate octobrachians. However, the cirri on cirroctopod arms may not be homologous to trabeculae found in some decabrachians. Therefore the presence of cirri is considered an independent character state.

20. Suckers: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996). While present in all coleoids, suckers of decabrachians are thought to be homologous with the octobrachian form, which is considered the more primitive state (Naef, 1921/23).

21. Acetabulum composition lining suckers: (0) cuticular rings; (1) neither cuticular nor horny rings; (2) horny rings (Young and Vecchione, 1996). An acetabulum lines the inside of the sucker ring on all coleoids. Decabrachians have horny rings, octobrachians exhibit cuticular rings and vampyromorphs have neither.

22. Sucker stalk: (0) absent; (1) present (Young and Vecchione, 1996). Decabrachian suckers are not attached directly to the arm, but connected by a flexible stalk, while octobrachian suckers are attached directly to the arm (Naef, 1921/23). The suckers of *Vampyroteuthis* are not attached directly to the arm but do not have "decabrachian-like" stalks and were therefore coded as "?". This character was modified from Young and Vecchione (1996; character 9) to account for the unknown state of *Vampyroteuthis*.

23. Sucker symmetry; (0) radial; (1) bilateral (Young and Vecchione, 1996). Decabrachian suckers are bilateral while those of *Vampyroteuthis* and the Octobrachia exhibit radial symmetry.

24. Armature I–III series: (0) in two rows; (1) in more than two rows; (2) in one row (Young and Vecchione, 1996). Sucker or hook series refers to that in the midarm, not at the tip where numerous rows of suckers can occasionally be observed.

25. Tentacle sucker series: (0) in up to four rows; (1) in greater than four rows (Roper et al., 1969). This state refers to rows of either hooks or suckers on the midportion of the tentacle club. This character is only applicable to decabrachians and subsequently coded as n/a in all other taxa (tentacles absent in all other coleoids).

26. Suckers on buccal membrane: (0) absent; (1) present (Roper, 1969). Small suckers are located on the oral region of the buccal crown in several decabrachians: Chtenopterygidae, Bathyteuthidae, Loliginidae and Sepiidae. This character is only applicable to decabrachians because the buccal membrane is absent in Nautiloidea, Vampyromorpha and Octobrachia.

27. Hooks on arms I–III: (0) absent; (1) present (Roper et al., 1969; Young and Harman, 1998). Hooks are modified suckers found on the arms of several decabrachians.

28. Hooks on tentacles (arms IV); (0) absent; (1) present (Roper et al., 1969; Young and Harman, 1998). This character is only applicable to decabrachians, because tentacles are absent in all other extant cephalopods.

29. Tentacles (arms IV) in adults: (0) absent; (1) present. Tentacle absence refers to taxa in which tentacles were present during development but are autotomized prior to or upon maturation. In the case of Gonatidae, some females autotomize their tentacles during reproduction, however, this is not synapomorphic for the family. This character is only applicable to decabrachians.

30. Tentacle locking apparatus: (0) absent; (1) present on carpus only; (2) present on manus and carpus (Young and Harman, 1998). The locking apparatus on the tentacle stalk consists of several suckers with smooth rings and tubercles (knobs) present on the carpal region of the club, which correspond to alternating rings and knobs on the opposite tentacle. The apparatus is applicable only in decabrachians and is highly variable in structure. Young and Harman (1998) used the presence of a tentacle locking apparatus to further investigate the relationships among enoploteuthid-like families.

31. Luminous bacteriogenic, round, bilobed organ located ventrally on ink sac: (0) absent; (1) present (Herring, 1988; Montgomery and McFall-Ngai, 1992; McFall-Ngai and Ruby, 1998). Bacteriogenic light organs are found in two families, Sepiolidae and Loliginidae (Young, 1977). As loliginid light organs are more elongated than that of sepiolids, it is unclear whether the presence of a bacteriogenic light organ is a synapomorphy, therefore only genera within Sepiolidae are coded as "present".

32. Luminous autogenic organs with a centrally situated luminous body distributed across mantle and arms: (0) absent; (1) present (Chun, 1914; Herring, 1988). Luminescent organs are found in almost all decabrachians, however, they are morphologically and biochemically diverse (Herring, 1988). The presence of light organs across the mantle and arms is specific for the members of Histioteuthidae.

33. Photophores containing collagen light guides: (0) absent; (1) present (Young and Harman, 1998). Collagen light guides are found only in the photophores of Enoploteuthidae, Lycoteuthidae and Pyroteuthidae.

34. Funnel: (0) absent; (1) present (Waller, 1998). The presence of a funnel (called hyponome in nautiloids) is a synapomorphy of Cephalopoda (Salvini-Plawen and Steiner, 1996).

35. Funnel: (0) attached to ventral mantle; (1) not attached to ventral mantle; (2) fused to mantle (Young and Vecchione, 1996). Funnel-mantle fusion is present in Cranchiidae and absent in all other decabrachians. In most octopods, cirroctopods and *Vampyroteuthis* the funnel and ventral mantle are attached but a narrow ventral slit remains (complete fusion does not exist). While the mantle-funnel attachment in Vampyromorpha is thought to be reminiscent of the funnel-mantle locking cartilage of decabrachians, it was treated as a separate character state.

36. Funnel locking apparatus: (0) absent; (1) present (Roper et al., 1969; Young and Vecchione, 1996). The funnel locking apparatus is a lock and key structure used to keep the mantle from inverting during rapid movement. Most often, individuals that do not exhibit mantle/funnel attachment possess a funnel locking apparatus. However, there are some cases in which there is no funnel/mantle attachment and no funnel locking apparatus, such as in cirroctopods.

37. Funnel locking apparatus morphology: (0) simple, straight; (1) triangular, round; (2) inverted T or -| shaped; (3) oval with projecting knobs (Roper et al., 1969; Nesis, 1987). The morphology varies greatly, particularly among decabrachians. The most common type is the simple, straight found in many oegopsids, sepiolids and sepiids.

38. Funnel valve; (0) absent; (1) present (Young and Vecchione, 1996). The funnel valve is a one-way muscular flap located on the inner dorsal wall of the funnel.

39. Closed circulatory system: (0) absent; (1) present (Waller, 1998; Haszprunar, 2000). A closed circulatory system is synapomorphic for cephalopods (Boletzky, 1987; Budelmann et al., 1997).

40. Ink sac: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996). The presence of an ink sac is unique to coleoids (although secondarily absent in some octobrachians).

41. Cerebral (pretrochal) eyes: (0) absent; (1) present (Haszprunar, 2000). Synapomorphic character for Monoplacophora, Scaphopoda, Bivalvia, Gastropoda and Cephalopoda.

42. Cornea: (0) absent; (1) one-part cornea present; (2) two-part cornea present (Young and Vecchione, 1996). The one-part cornea is the transparent protective outer membrane covering the eye in so-called myopsid cephalopods as well as Sepiidae and Sepiolidae while all other decabrachians lack a cornea (the eye is in direct contact with the environment). Octopods and cirroctopods have a fully closed, or two-part cornea.

43. Extra-ocular eye muscles: (0) absent; (1) present (Haszprunar and Wanninger, 2000). Extra-ocular eye muscles are autapomorphic for cephalopods although distinct differences occur between nautiloids, decabrachians and octobrachians (Budelmann et al., 1997).

44. Paired statocysts: (0) present; (1) absent (Salvini-Plawen and Steiner, 1996; Haszprunar and Wanninger, 2000). In Mollusca paired statocysts are restricted to conchiferans. Codings taken directly from primary literature sources (Nesis, 1987; Salvini-Plawen and Steiner, 1996).

45. Statocyst outer capsule: (0) absent; (1) present (Young and Vecchione, 1996). Coleoid cephalopods have one pair of statocysts situated in the occipital region of the head capsule, which allow for orientation and balance relative to gravitational direction (Nesis, 1987). An outer fluid-filled sac is present in *V. infernalis*, octopods and cirroctopods. A single sac embedded in cartilage is present in all other coleoids. Codings for this character were taken directly from Young and Vecchione (1996).

46. Stellate ganglia: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996). Stellate ganglia are present in all cephalopods.

47. Photosensitive vesicles: (0) within cephalic cartilage; (1) above funnel; (2) on stellate ganglia (Young and Vecchione, 1996). Photosensitive vesicles function in the detection of light but vary in location across cephalopods.

48. Inferior frontal lobe system of the brain: (0) absent; (1) partially present; (2) present (Young and Vecchione, 1996; Nixon and Young, 2003). An inferior frontal lobe system is present in Octobrachia. Due to difficulty in coding brain morphology in fixed specimens, this character was coded directly from literature (Young and Vecchione, 1996).

49. Superior buccal lobe: (0) widely separated from brain; (1) adjacent to brain; (2) fused to brain (Young and Vecchione, 1996). The position of the buccal lobe relative to the supraesophageal mass varies among cephalopods depending on the distance between the buccal mass and brain. This character was coded directly from literature (Young and Vecchione, 1996).

50. Branchial canal: (0) absent; (1) present; (2) secondary reduction of canal (Young and Vecchione, 1996). The branchial canal allows for the passage of seawater between gill lamellae and is present in all coleoids except for Sepiolidae, Sepiidae and Spriulidae (Young and Vecchione, 1996). This character was coded directly from Young and Vecchione (1996).

51. Relative position of digestive gland duct appendages: (0) lies in nephridial coelom; (1) not in nephridial coelom (Young and Vecchione, 1996). Digestive gland duct appendages are present in all coleoid cephalopods although their location is variable.

52. Posterior salivary gland: (0) absent; (1) posterior to brain; (2) proximal to buccal mass (Young and Vecchione, 1996). The primitive location of the posterior salivary gland is posterior to the cephalic cartilage; however, in Cirroctopoda it is located proximal to the buccal mass (Young and Vecchione, 1996).

53. Enlarged coelomic cavity with large amounts of ammonium chloride (0) absent; (1) present. Many cephalopods possess ammonium chloride in their mantle, which is used for buoyancy. However, Cranchiidae is the only group to exhibit a modified coelomic cavity to house large amounts of ammonium chloride. This character was coded from primary literature (Denton and Gilpin-Brown, 1973).

54. Ctenidia: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996; Waller, 1998; Haszprunar and Wanninger, 2000). Gills with filaments or leaflets are present in all molluscan classes except for Scaphopoda and Solenogastres (Giribet and Wheeler, 2002; Reynolds, 2002).

55. Gill lamellae attachment: (0) free; (1) sessile (Young, 1964). Gill lamellae hang free in *V. infernalis* and decabrachians whereas the lamellae of octopods are sessile or attached. Young (1964) believed that the combination of gill lamellae attachment and branchial canal (character 50) morphology could indicate the primitive nature of the vampyromorph gill.

56. Gill number: (0) one pair; (1) two pairs; (2) more than two pairs; (3) single post-torsional left (Haszprunar, 2000). Coleoid cephalopods have a single pair of gills, while *Nautilus* has two pairs. Other molluscs such as Polyplacophora have more than two pairs, while some gastropods have a single post-torsional left gill (Haszprunar, 2000).

57. Nidamental glands: (0) absent; (1) present (Young and Vecchione, 1996). Nidamental glands are large, paired organs that are involved in secreting a layer of coating on eggs or egg masses and found in most decabrachians and Nautiloidea.

58. Right oviduct: (0) absent; (1) present (functional or non-functional) (Young and Vecchione, 1996). In coleoid cephalopods a left oviduct is always present, however, the right is not. This character was modified from Young and Vecchione (1996; character 30) to consider Idiosepiidae, in which both oviducts are present, but the right is non-functional (Nesis, 1987).

59. Oviducal gland symmetry: (0) radial; (1) bilateral; (2) asymmetrical (Young and Vecchione, 1996). The oviducal glands surround the oviducts and provide a layer of coating on eggs or egg masses. Decabrachian oviducal glands are bilateral whereas cirroctopods and octopods exhibit radial symmetry. *Vampyroteuthis* appears to exhibit neither radial nor bilateral symmetry.

60. Oviducal gland position: (0) gland terminal (located at end of oviduct); (1) gland subterminal (Young and Vecchione, 1996). The oviducal gland can be positioned at the end of the oviduct (in decabrachians and Nautiloidea), or midway along the oviduct (in octobrachians).

61. Arm I hectocotylization: (0) absent; (1) present. Hectocotylization refers to the modification of one of the arms in male cephalopods for the transfer of sperm to the female (Young and Vecchione, 1996). Hectocotylization can occur on different arm pairs, but is not thought to be homologous and is therefore coded independently. Arms I are hectocotylized only in Histioteuthidae and Sepiolidae.

62. Arm IV hectocotylization: (0) absent; (1) present (Young and Vecchione, 1996). As the homology of Arms III in octopods and Arms IV in decabrachians is only hypothesized, hectocotylization was coded as present in taxa with an unmodified arm IV (Octobrachia).

63. Arm V hectocotylization: (0) absent; (1) present (Young and Vecchione, 1996). Arm V is hectocotylized in several decabrachian families.

64. Yolky, meroblastic egg, with non-spiral cleavage and direct development: (0) absent; (1) present (Waller, 1998; Boletzky, 2003). Most molluscs exhibit spiral cleavage and some form of a larval stage, except for the cephalopods, which have direct development and non-spiral cleavage (coded from primary literature, Boletzky, 2003; Waller, 1998).

65. Spermatophores with an ejaculatory apparatus: (0) encapsulated coil; (1) present; (2) absent (modified from Young and Vecchione, 1996). A complex ejaculatory apparatus is present in all coleoid cephalopods except Cirroctopoda, which produce sperm packets. Nautiloidea and other molluscs lack an ejaculatory apparatus. This character was coded directly from Young and Vecchione (1996).

Sperm characters

Sperm morphology has been studied in a wide range of cephalopods (see summary in Healy, 1996) such as: *Nautilus pompilius* (Arnolds and Williams-Arnold, 1978), *Vampyroteuthis infernalis* (Healy, 1989, 1990a), *Spirula spirula* (Healy, 1990a), *Opisthoteuthis persephone* (Healy, 1993), *Eledone cirrhosa* (Maxwell, 1974; Ribes et al., 2002), *Sepia officinalis* (Maxwell, 1975), *Loligo forbesi* (Maxwell, 1975) and *Alloteuthis subulata* (Maxwell, 1975). Due to the difficulty of directly examining sperm, as well as the lack of availability, characters were coded entirely from literature sources (Franzén, 1955, 1958; Maxwell, 1974, 1975; Healy, 1990a,b, 1993, 1996).

66. Acrosomal vesicle: (0) present; (1) absent (Healy, 1990a,b, 1996; Ribes et al., 2002).

67. Large, dense plug within nuclear fossa (= extracellular rod): (0) absent; (1) present (Healy, 1993, 1996). A large, dense plug within the nuclear fossa is shared among *Vampyroteuthis infernalis* and *Octopus* spp. According to Healy (1993, p. 113) "the plug is so distinctive in its ultrastructure that there seems little chance of it having evolved independently in *Vampyroteuthis* and *Octopus*."

68. Curved nucleus: (0) absent; (1) present (Healy, 1990b). A curved nucleus is present in Sepiidae, Loliginidae, and *Rossia* (but not *Heteroteuthis*).

69. Membrane skirt: (0) absent; (1) present (Healy, 1996). A membrane skirt is present in Sepiidae, Loliginidae and *Rossia*.

70. Two longitudinal furrows in the nucleus, each accommodating an elongate mitochondrion: (0) absent; (1) present (Healy, 1996). The presence of such a structure is considered autapomorphic for Nautiloidea.

71. Mitochondrial midpiece: (0) absent; (1) present (Healy, 1990a, 1996). Present in all molluscan classes, but not all cephalopods.

72. Mitochondrial spur: (0) absent; (1) present (Healy, 1990a). Mid-piece formation occurs late in spermiogenesis in all cephalopods; however, the spur varies morphologically. The mitochondrial spur occurs in Sepiida, Teuthida and *Rossia* (Maxwell, 1975; Healy, 1990a,b).

73. Periflagellar mitochondrial sleeve: (0) absent; (1) present (Healy, 1990a). A periflagellar mitochondrial sleeve is present in *Spirula* and *Heteroteuthis* and forms the midpiece.

74. Nucleus with eccentrically positioned flagellum: (0) absent; (1) present (Healy, 1996). An eccentrically positioned or offset flagellum is found in *Rossia*, Loliginidae and Sepiidae.

Outgroup characters

Several large-scale molluscan studies were evaluated to determine informative outgroup characters for the Cephalopoda as well as previously identified synapomorphies (Salvini-Plawen and Steiner, 1996; Ponder and Lindberg, 1997; Waller, 1998; Haszprunar and

Wanninger, 2000; Haszprunar, 2000; Giribet and Wheeler, 2002; Reynolds, 2002; Wanninger and Haszprunar, 2002). Codings for outgroups were taken directly from primary literature sources listed for each character. More detailed descriptions for each character can be found in those sources.

75. Type of outer shell: (0) univalve with one aperture present; (1) univalve with two apertures present; (2) bivalve shell (Giribet and Wheeler, 2002). One aperture is present in gastropods and nautiloids, two in scaphopods.

76. Eight external shell plates: (0) absent; (1) present (Giribet and Wheeler, 2002). An autapormophy for Polyplacophora.

77. Cuticle with spicules: (0) absent; (1) present (Giribet and Wheeler, 2002). Found in Caudofoveata, Solenogastres and Polyplacophora.

78. Mantle covering dorsal surface: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996; Giribet and Wheeler, 2002; Lee et al., 2003). The dorsal surface of the mantle is covered in gastropods and cephalopods.

79. Tubular protoconch: (0) absent; (1) present (Giribet and Wheeler, 2002; Ponder and Lindberg, 1997). The presence of a tubular protoconch is an autapomorphic character for Gastropoda.

80. Specific head retractor: (0) absent; (1) present (Haszprunar, 2000). Gastropoda and Cephalopoda exhibit a free head that is retractable by a separate head retractor. Haszprunar (2000) described the state in gastropods, limpets in particular, as having "a distinct insertion scar of the head retractor" while in cephalopods he called them the anterior pair of the "depressors infundibuli". In Scaphopoda, only the buccal cone is free, while the cerebral and buccal masses remain fixed.

81. Lateral body compression: (0) absent; (1) present (Giribet and Wheeler, 2002). Bivalvia exhibits a body form that has been laterally compressed.

82. Torsion: (0) absent; (1) present (Ponder and Lindberg, 1997; Giribet and Wheeler, 2002). Gastropods are the only class to exhibit body torsion.

83. Operculum: (0) absent; (1) present (Giribet and Wheeler, 2002; Ponder and Lindberg, 1997). An operculum is present in all Gastropoda in the larval stage but is secondarily lost in some adults.

84. Differentiated head: (0) present; (1) absent (Ponder and Lindberg, 1997; Giribet and Wheeler, 2002). A differentiated head is present in all molluscs except for Bivalvia.

85. Snout: (0) absent; (1) present (Ponder and Lindberg, 1997). This character refers to only those molluscs with a differentiated head (Bivalvia coded as inapplicable), particularly Gastropoda.

86. Ventral surface of foot: (0) present; (1) absent (Giribet and Wheeler, 2002). The cephalopods are coded as "?" because it is unclear where the ventral surface of the foot is located.

87. Position of anus: (0) opposite oral opening; (1) near mouth opening at ventral side (Haszprunar, 2000). An "ano-pedal flexure" is shared among Scaphopoda, Gastropoda and Cephalopoda whereas anterior-posterior axis predominates the rest of the mollusca (Ponder and Lindberg, 1997; Waller, 1998).

88. Cartilagenous cranium: (0) absent; (1) present (Waller, 1998). The cartilaginous cranium is formed to accommodate an extensive fusion of ganglia and is unique to Cephalopoda.

89. Mantle lobes: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996; Giribet and Wheeler, 2002). Mantle lobes are found only in Scaphopoda and Bivalvia.

90. Posterior pedal gland: (0) absent; (1) present (Giribet and Wheeler, 2002). All bivalves have a posterior pedal gland in the juvenile state, which is commonly absent in adults.

91. True pedal ganglia: (0) absent; (1) present (Haszprunar, 2000). True pedal ganglia are found in Bivalvia, Scaphopoda and Cephalopoda whereas elongate, pedal cords are found in Gastropoda and Polyplacophora.

92. Hydrostatic muscular system: (0) absent; (1) present (Haszprunar, 2000). Gastropods and cephalopods share a "hydrostatic muscular system" (Haszprunar, 1988: 405), wherein the extension of body parts occurs via muscle contraction rather than hemolymphatic pressure. Shimek and Steiner (1997) believe the same is true for the dentalid scaphopod foot, which can be extended and utilized rapidly.

93. Adductor muscles: (0) absent; (1) present (Giribet and Wheeler, 2002). Adductor muscles are present in Bivalvia.

94. Cephalic tentacles: (0) absent; (1) present (Salvini-Plawen and Steiner, 1996; Ponder and Lindberg, 1997; Giribet and Wheeler, 2002). Cephalic tentacles are likely a synapomorphy for Gastropoda. Ponder and Lindberg (1997) and Giribet and Wheeler (2002) did not consider the innervated structures of other molluscs as true cephalic tentacles and these were therefore coded as (1) in the present study only for gastropods.

95. Labial palps: (0) absent; (1) present (Giribet and Wheeler, 2002). Labial palps are present in Bivalvia.

96. Kidneys: (0) tubular; (1) sac-shaped; (2) U-shaped (Giribet and Wheeler, 2002). Kidneys are present throughout the Mollusca but vary morphologically.

97. Protonephridia: (0) absent; (1) present (Haszprunar, 2000). The presence of protonephridia in molluscan larvae has previously been established for several molluscs (Bartolomaeus, 1989; Haszprunar and Wanninger, 2000; Haszprunar, 2000). However, no such protonephridia have been observed in Cephalopoda (Haszprunar, 2000).

98. True gonoducts: (0) absent; (1) present (Haszprunar, 2000). True gonoducts are present only in Cephalopoda and Polyplacophora, although a secondary form does occur in the other molluscs.

99. Number of coelomoducts: (0) one; (1) two (Haszprunar, 2000). *Nautilus* is the only mollusc to exhibit two coelomoducts.

100. Captacula: (0) absent; (1) present (Giribet and Wheeler, 2002; Reynolds, 2002). Captacula are retractile feeding tentacles unique to Scaphopoda.

101. Osphradia: (0) present; (1) absent (Giribet and Wheeler, 2002). Osphradia are present in all molluscan classes except for Scaphopoda and Monoplacophora. Osphradia are absent in coleoid cephalopods including *Nautilus*, where they are also referred to as ''interbranchial papillae'' (Naef, 1921/23).

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Phylogenetics and Evolution 36 (2005) 101–111
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Evolutionary relationships among squids of the family Gonatidae (Mollusca: Cephalopoda) inferred from three mitochondrial loci

A.R. Lindgren[a,1], O.N. Katugin[b], E. Amezquita[a], M.K. Nishiguchi[a,*]

[a] *Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA*
[b] *Pacific Research Fisheries Centre (TINRO-Centre), Vladivostok, Russia*

Received 2 September 2004; revised 10 December 2004
Available online 1 February 2005

Abstract

The oceanic squid family Gonatidae (Mollusca: Cephalopoda) is widely distributed in subpolar and temperate waters, exhibiting behavioral and physiological specializations associated with reproduction. Females of several species undergo muscular degeneration upon maturation; origins of this complex morphogenic change are unknown, hindering our understanding of ecological and morpho-physiological adaptations within the family. To provide further information regarding the evolutionary relationships within Gonatidae, three mitochondrial loci (12S rRNA, 16S rRNA, and cytochrome *c* oxidase subunit I) were analyzed for 39 individuals representing fourteen gonatid and six outgroup cephalopod species. In addition to elucidating relationships among gonatids, molecular data provided more information than morphological data for problematic specimens. Although some data sets are incongruent or have low nodal support values, combined molecular analysis confirms the presence of gonatid groups previously established by morphological characteristics (i.e., possessing radular teeth in seven longitudinal rows and muscular mantle tissue). These characteristics are basal to taxa possessing radular teeth in five longitudinal rows and less muscular mantle tissue, indicating that the derived forms are those species exhibiting physiological adaptation such as tissue degeneration upon maturation and egg brooding.

Keywords: Cephalopoda; Gonatidae; Phylogeny; Systematics; Brooding; Speciation

1. Introduction

Gonatidae (Mollusca: Cephalopoda) is a family of oegopsid oceanic squid characterized by quadriserial armature on the arms, with most species possessing hooks on arms I–III (in two central rows) and a rhomboidal or heart-shaped fin (Nesis, 1973, 1987). The family is composed of 17–19 species (Nesis, 1982, 1985; Sweeney and Roper, 1998) with three recognized genera: *Gonatus*, *Gonatopsis*, and *Berryteuthis*, which are distinguished from each other by tentacle and radular morphology (Nesis, 1973). *Gonatus* possesses complex tentacle fixing apparatus, and a radula consisting of five longitudinal rows of teeth. *Gonatopsis* is distinguished by the absence of tentacles in adults, and presence of either seven or five longitudinal rows of teeth in the radula. Members of *Berryteuthis* have no hooks on the tentacle club, weakly differentiated fixing apparatus on tentacles, and a radula with seven longitudinal rows of teeth. Nesis (1971, 1973) suggested that the ancestral gonatid was an oceanic shallow water squid, with a powerful and muscular mantle, rhomboid or oval fin, radula with seven rows of longitudinal teeth, arms and tentacles equipped with only suckers, and a poorly developed tentacular fixing apparatus. Thus, more derived forms were species that migrated to deeper waters, exhibiting less muscular mantle and arm

* Corresponding author. Fax: +1 505 646 5665.
E-mail address: nish@nmsu.edu (M.K. Nishiguchi).
[1] Present address: The Ohio State University, Department of Evolution, Ecology and Organismal Biology, 318 West 12th Avenue, Columbus, OH 43210, USA.

1055-7903/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2004.12.009

crown tissues, and possessing five longitudinal rows of radular teeth.

Several species of gonatid exhibit a characteristic previously identified only with octopods: post-spawning egg care or brooding, where the female cares for the eggs until hatching (Hunt and Seibel, 2000; Katugin, 2003; Okutani et al., 1995; Seibel et al., 2000). All other extant decabrachian cephalopods freely spawn with or without egg-masses, or lay their eggs on substratum, but none are known to exhibit parental care. During brooding, gonatid females do not feed; instead, they utilize the digestive gland weight and lipid energy conserved during maturation. Gonatid females known to brood also exhibit morphological change upon maturation, which is believed to be related to post-spawning egg-care (e.g., Hunt and Seibel, 2000; Katugin, 2003; Katugin and Merzlyakov, 2002; Seibel et al., 2000). During brooding, muscle tissue degrades, tentacles are autotomized, and mantle and arms become water logged (Katugin, 2003; Katugin and Merzlyakov, 2002). To date, seven gonatid species have been recorded to exhibit this change upon maturation, which include *Gonatus berryi*, *Gonatus madokai*, *Gonatus onyx*, *Gonatus pyros*, *Gonatus tinro*, *Gonatus fabricii*, and *Gonatopsis octopedatus* (Arkhipkin and Bjorke, 1999; Katugin, 2003; Katugin and Merzlyakov, 2002; Nesis, 1993; Seibel et al., 2000; Young, 1973).

Although the reproductive strategies of all gonatid species have yet to be determined, brooding appears to occur in more derived forms, those that already exhibit fragile arm and mantle tissue and are usually found at greater depths (Nesis, 1973). Although brooding is present in Octopoda, gonatids are distantly related (e.g., Carlini and Graves, 1999; Lindgren et al., 2004) and exhibit different physiology and behavior during brooding (Boletzky, 1992), indicating that brooding has evolved independently in Gonatidae. Brooding octopods glue their eggs together into "long strait or branched strings" (Norman, 2000), which are either attached to substrate or carried within their arm webs, keeping them oxygenated and free of debris (Boletzky, 1992; Cosgrove, 1993; Norman, 2000). In gonatids, females that brood their eggs hold them between their arms, floating midwater until hatching (Katugin and Merzlyakov, 2002; Okutani et al., 1995; Seibel et al., 2000), and evidently do not exhibit such invested care as octopuses. Male and immature female members of brooding species have been recorded as having delicate arm and mantle tissue, implying that at least in some species, such as in *Gonatus madokai* and *Gonatus tinro* (Katugin, 2003), morphogenic change in tissue precedes or coincides with maturation. Therefore, it is possible that muscular degeneration may be a species character influenced by physiology and environment. Whether or not tissue degeneration and associated egg brooding is a synapomorphy of Gonatidae can be tested using an independent non-morphological approach, such as a molecular phylogenetic analysis.

No combined sequence-based phylogenetic hypothesis exists for Gonatidae, although an allozyme-based phylogeny has been recently generated (Katugin, 2004). Previous molecular work has been completed using the cytochrome *c* oxidase subunit I locus (COI; Seibel et al., 2000) to identify juvenile gonatids from southern California waters as well as incorporating two species of *Gonatus* in a COI based, higher-level phylogeny to investigate relationships between coleoid cephalopods (Carlini and Graves, 1999). In the present study comparisons between brooding strategies, morphology, and physiology were made to the molecular phylogeny of three mitochondrial loci for 14 gonatid species and analyzed to determine whether these life history characteristics were important in the radiation of this family of squids.

2. Methods

2.1. PCR amplification and sequencing analysis

DNA was extracted from gill or mantle tissues of 39 individuals representing 14 gonatid species (13 nominal species and one undescribed species) as well as six outgroup species (Table 1) using Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA). PCR amplification was carried out in 50 μL reactions using Promega *Taq* Polymerase (see Nishiguchi et al., 2004 for further descriptions of PCR methods) for fragments of three mitochondrial loci: 12S RNA (404 bp), 16S rRNA (528 bp), and cytochrome *c* oxidase subunit I, COI (658 bp) (see Nishiguchi et al., 2004 for primer information and annealing temperatures). Although nuclear data has proven useful in higher-level analyses (e.g., Lindgren et al., 2004), nuclear loci have been determined to be too conserved for discerning species-level relationships among cephalopods (e.g., Nishiguchi et al., 2004). Sequences were obtained using an ABI PRISM 3100 Genetic Analyzer (Foster City, CA). External primers were excluded from resulting forward and reverse chromatograms which were assembled and edited using either Sequencher v. 4.1 or v. 4.2 (Gene Codes, Ann Arbor, MI). Resulting sequences were compiled and partitioned in Genetic Data Environment (GDE, Smith et al., 1994). For non-coding genes (12S and 16S), sequences were partitioned in GDE using secondary structure models and unambiguous regions; 12S was partitioned into five fragments and 16S was partitioned into eight. The protein-coding gene COI was tested as one entire fragment for the analysis.

2.2. Phylogenetic analysis

Individual and combined sets of molecular data were analyzed using the direct optimization program POY (Wheeler et al., 2002), with parsimony as the optimality

Table 1
Accession numbers for cephalopod taxa used in the present study

		Collection data	12S rRNA	16S rRNA	COI
Nautiloidea (1 sp.)					
Nautilidae	*Nautilus pompilius*	Voucher at AMNH		AY686585	AY557514
Coleoidea (19 spp.)					
Vamproteuthidae	*Vampyroteuthis infernalis*	Hokusei Maru (DC)		AY686586	* AF000071
Sepiolidae	*Sepiola affinis*	Banyuls sur Mer, France		AY686588	AY557523
Spirulidae	*Spirula spirula*	DC (voucher at NMNH)		AY686589	* AF000066
Loliginidae	*Loligo pealei*	Northern Atlantic (WKM)		AY686590	* AF000052
Architeuthidae	*Architeuthis dux*	DC (voucher at NMNH)		AY686587	* AF000027
Gonatidae (14 spp.)	*Gonatopsis japonicus 01*	Sea of Japan		AY681019	AY681051
	Gonatopsis japonicus 02	Sea of Japan	AY680997	AY681020	AY681052
	Gonatopsis japonicus 03	Sea of Japan	AY680998	AY681021	AY681053
	Gonatopsis japonicus 05	Sea of Japan		AY681022	AY681054
	Gonatopsis japonicus 07	Sea of Japan		AY681023	AY681055
	Gonatopsis octopedatus 02	Sea of Okhotsk	AY680999	AY681024	AY681056
	Gonatopsis borealis (L) 01	Sea of Okhotsk	AY681000	AY681025	AY681057
	Gonatopsis borealis (L) 02	Sea of Okhotsk		AY681026	AY681058
	Gonatopsis borealis (S) 02	Sea of Okhotsk	AY681001	AY681027	AY681059
	Gonatopsis borealis (S) 06	Sea of Okhotsk	AY681002	AY681028	AY681060
	Gonatopsis borealis (S) 07	Sea of Okhotsk	AY681003	AY681029	AY681061
	Gonatopsis borealis (S) 09	Sea of Okhotsk	AY681004	AY681030	AY681062
	Gonatopsis sp. *01*	Sea of Okhotsk	AY681005	AY681031	AY681063
	Gonatus antarcticus 01	South Georgia (MC)		AY681032	AY681064
	Gonatus fabricii 01	DE0304 (Sta. 3), 2003 (MV)	AY681006	AY681033	AY681065
	Gonatus berryi 02	Pacific off Kuril Islands		AY681034	
	Gonatus tinro 01	Sea of Okhotsk	AY681007	AY681035	AY681066
	Gonatus tinro 08	Sea of Okhotsk	AY681008	AY681036	AY681067
	Gonatus tinro 09	Sea of Okhotsk	AY681009	AY681037	AY681068
	Gonatus kamtschaticus 01	Sea of Okhotsk	AY681010	AY681038	AY681069
	Gonatus kamtschaticus 04	Sea of Okhotsk		AY681039	AY681070
	Gonatus kamtschaticus sp. *03*	Sea of Okhotsk	AY681011	AY681040	AY681071
	Gonatus kamtschaticus sp. *06*	Sea of Okhotsk	AY681012	AY681041	AY681072
	Gonatus kamtschaticus sp. *07*	Sea of Okhotsk		AY681042	AY681073
	Gonatus cf. *onyx 01*	Sea of Okhotsk	AY681013	AY681043	AY681074
	Gonatus pyros 02	Pacific off Kuril Islands		AY681044	AY681075
	Gonatus madokai 06	Sea of Okhotsk	AY681014	AY681045	AY681076
	Gonatus madokai 10	Sea of Okhotsk	AY681015	AY681046	AY681077
	Gonatus madokai 19	Sea of Okhotsk	AY681016	AY681047	AY681078
	Berryteuthis magister 02	Sea of Japan (GG)	AY681017	AY681048	AY681079
	Berryteuthis magister 01	Sea of Okhotsk (GG)		AY681049	
	Berryteuthis magister 01	Pacific off Vancouver (GG)		AY681050	AY681080
	Berryteuthis anonychus 11	Central North Pacific (SS)	AY681018		AY681081

All ingroup taxa (Gonatidae) collected by the authors, except where noted (DC, Dr. David Carlini; GG, Dr. Graham Gillespie; MC, Dr. Martin Collins; MV, Dr. Michael Vecchione; SS, Dr. Satoshi Suyama; WKM, Dr. William Macy; AMNH, American Museum of Natural History, New York; NMNH, National Museum of Natural History, Washington DC).

* Indicates sequences obtained from GenBank.

criterion (for a discussion of POY, see Giribet, 2001). All molecular data were analyzed at the nucleotide level, including the protein-coding gene, COI. In cases where sequences were unavailable for analysis, the data were treated as "missing." Tree searches in POY were conducted in parallel using a 19 dual processor cluster at Harvard University (darwin.oeb.harvard.edu) with commands described in Lindgren et al. (2004). Each of the four data sets (individual 12S, 16S, COI, and combined molecules) was analyzed under 12 parameter sets with different transition/transversion and indel cost ratios. Gap/transversion ratios of 1 and 2 and transversion/transition ratios of 1, 2, and 4 were explored. In all cases, gap extension was down-weighted with respect to the first occurrence of an indel event. To determine the optimal parameter set, a sensitivity analysis was conducted (Wheeler, 1995). In sensitivity analysis, character congruence is treated as an extension of parsimony in that the parameter set minimizing overall character incongruence is the "optimal parameter set." Lastly, nodal support for each analysis under the optimal parameter was assessed using Farris's parsimony jackknifing procedure (Farris et al., 1995) on 100 replicates (commands -jackboot -replicates 100).

3. Results

Our sensitivity analyses indicated the parameter set that minimized overall character incongruence for the combined molecular analysis was that which weighted the ratio of gap/transversion = 2 and transversion/transition = 2 (referred to as 221, Table 2). Therefore, 221 is considered the optimal parameter set for both combined and individual data sets to better facilitate comparisons among data sets.

3.1. 12S rRNA

Due to amplification difficulties and specimen availability, outgroup taxa (*Nautilus pompilius*, *Vampyroteuthis infernalis*), as well as several gonatid taxa (Table 1) were not included in the 12S rRNA individual analysis. Under the optimal parameter set (221), monophyly was shown for Gonatidae (77%, Fig. 1) with *Spirula spirula* as the closest outgroup relative. No genera were recovered as monophyletic. One clade contained representatives of all genera investigated; *Berryteuthis magister* + *Gonatus kamtschaticus* 03, was sister to *Gonatopsis octopedatus*, with *Gonatopsis japonicus* as the outgroup. Within a second clade, *Gonatopsis borealis* was monophyletic (57%), *Gonatus tinro* 01 + *Gonatus* cf. *onyx* was supported in 100% of jackknife replicates, and all other *Gonatus tinro* individuals fell into a clade with *G. kamtschaticus* 01 and *Gonatus madokai* + *Gonatus kamtschaticus* 06. Although 12S provided additional information regarding genus-level dynamics, many of the nodes remained poorly supported, indicating that 12S alone does not provide enough information to resolve family level relationships.

3.2. COI

Under the optimal parameter set (221), monophyly was supported for Gonatidae (with 100% jackknife support), with a polytomy of other decabrachians sister to the clade (Fig. 2). Within Gonatidae, little generic resolution was found; however, several interspecies clades were observed. *Gonatopsis octopedatus* formed a clade with specimens of *Gonatopsis japonicus* (80% jackknife support), except for *G. japonicus* 04, which fell within a clade comprised of several *Gonatus kamtschaticus* individuals and two individuals of *Gonatus madokai*. However, this was the only clade that was supported in fewer than 50% of jackknife replicates. Lastly, *Berryteuthis magister* individuals were sister to *Gonatopsis borealis*. Although *G. borealis* includes two forms, one small and one large size, the species is monophyletic (71% jackknife support). Furthermore, the small and large individuals each formed a clade with 100% jackknife support. Thus, COI provided support at the individual and species level, but was not able to resolve genus-level relationships.

3.3. 16S rRNA

The optimal parameter set (221) supported the monophyly of Gonatidae, with *Architeuthis dux* as its sister (54%, Fig. 3). Individuals of *Berryteuthis magister* formed a clade sister to the rest of Gonatidae, indicating monophyly for this species. However, due to amplification difficulties, *B. anonychus* could not be included in the 16S analysis, and therefore the monophyly of *Berryteuthis* could not be tested. Monophyly could not be established for *Gonatopsis* or *Gonatus*, as species from both genera were found throughout several clades. *Gonatopsis borealis* was paraphyletic due to the presence of *Gonatus fabricii* within the clade of small *G. borealis* individuals (86% jackknife support). *Gonatopsis* sp. was sister to all remaining species, although the support values were less than 50%. Individuals from *Gonatus tinro*, *G. pyros*, *G. kamtschaticus*, and *G.* cf. *onyx* formed a single clade, indicating potential evidence for some intergeneric relationships. *G. kamtschaticus* individuals fell in several different clades, with *G. kamtschaticus* 06 forming the outermost branch of a *Gonatopsis* clade (*Gonatopsis japonicus* and *Gonatopsis octopedatus*). Consequently, the 16S topology provides some support for genus-level groups, however, overall nodal support values remain fairly low.

Table 2
Weighted tree lengths for the individual and combined analyses at different gap/tv and tv/ts cost ratios and ILD values

Gap/tv	tv/ts	COI	12S	16S	Combined	ILD values
1	∞	338	109	230	693	0.0231
1	1	1026	227	438	1736	0.0259
1	2	1383	342	675	2455	0.0224
1	4	2061	561	1141	3851	0.0229
2	∞	338	163	321	845	0.0272
2	1	1026	290	539	1904	0.0257
2	2	1383	460	866	2768	**0.0213**
2	4	2061	788	1508	4476	0.0266
4	∞	338	268	478	1117	0.0295
4	1	1026	399	696	2191	0.0319
4	2	1383	668	1172	3321	0.0295
4	4	2061	1204	2121	5551	0.0297

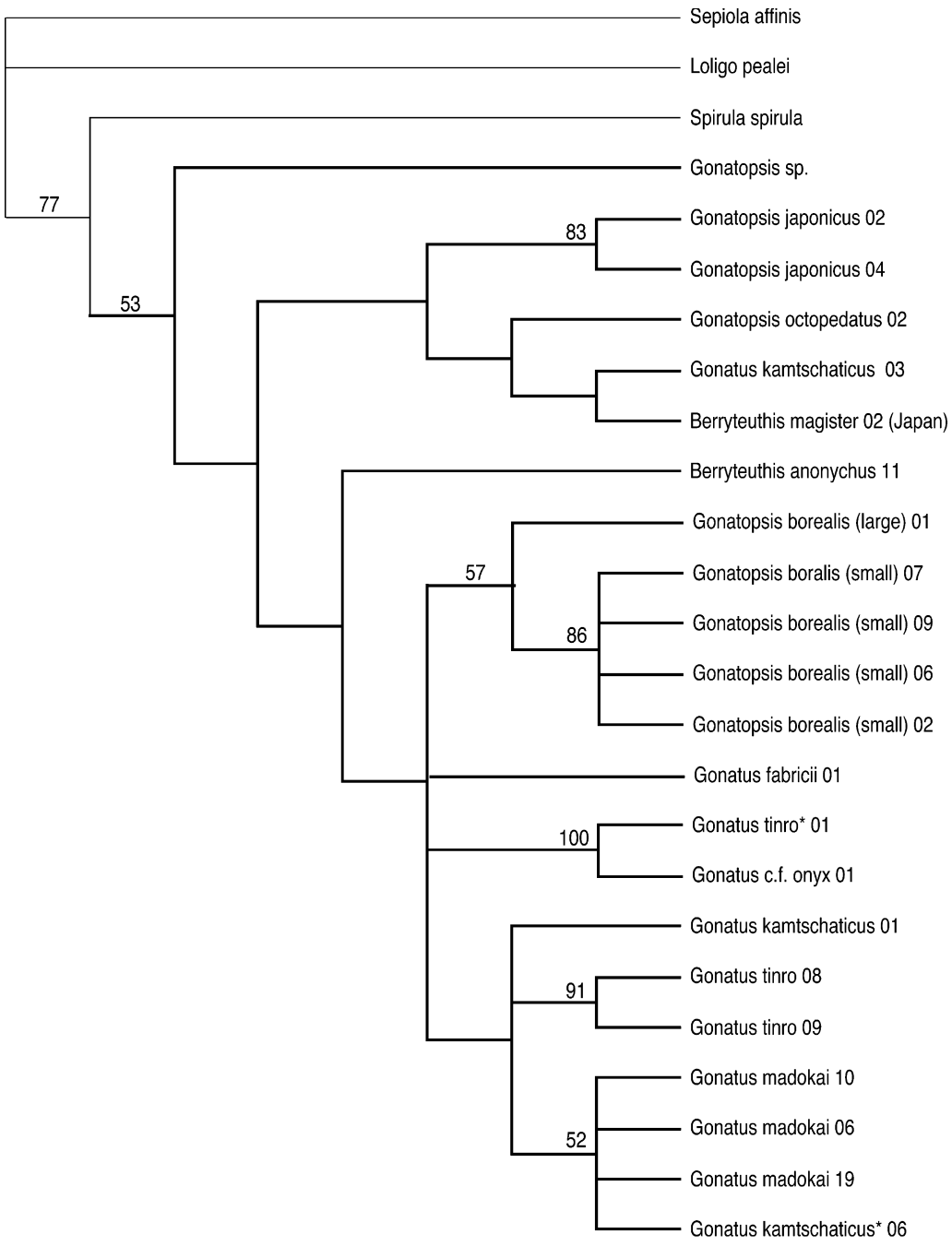


Fig. 1. 12S rRNA cladogram generated from a eight equally parsimonious trees, each of 460 equally weighted steps under the optimal parameter set (221). Lines in bold indicate individuals from the family Gonatidae. Asterisk (*) refers to specimens that may have been misidentified. Jackknife support values greater than 50% are noted above corresponding nodes.

3.4. Combined molecules

The optimal parameter set (221) for the combined molecular data analysis (Fig. 4) recovers monophyly of Gonatidae (100% jackknife support), as well as monophyly of Decabrachia (90%). All gonatid species, with the exception of *Berryteuthis magister*, formed a single clade (58% jackknife support). Within this primary gonatid clade, *Gonatopsis* sp. forms the outermost branch, with a *Gonatopsis borealis* clade (89% jackknife support) basal to all other gonatids. Within the *Gonatopsis borealis* clade, small and large individuals formed two clades, each with 100% jackknife support. *Berryteuthis anonychus* is embedded within *Gonatopsis* and *Gonatus*, recovering non-monophyly of all gonatid genera. However, several *Gonatus* and *Gonatopsis* clades are present. *Gonatus tinro*, *Gonatus* cf. *onyx*, *Gonatus pyros*, and several individuals from *G. kamtschaticus* form a single clade, with *G. tinro* + *G.* cf. *onyx* (100%), and *G. kamtschaticus* 07 sister to *G. kamtschaticus* 03 + *G. pyros* (95%). All *Gonatopsis japonicus* individuals

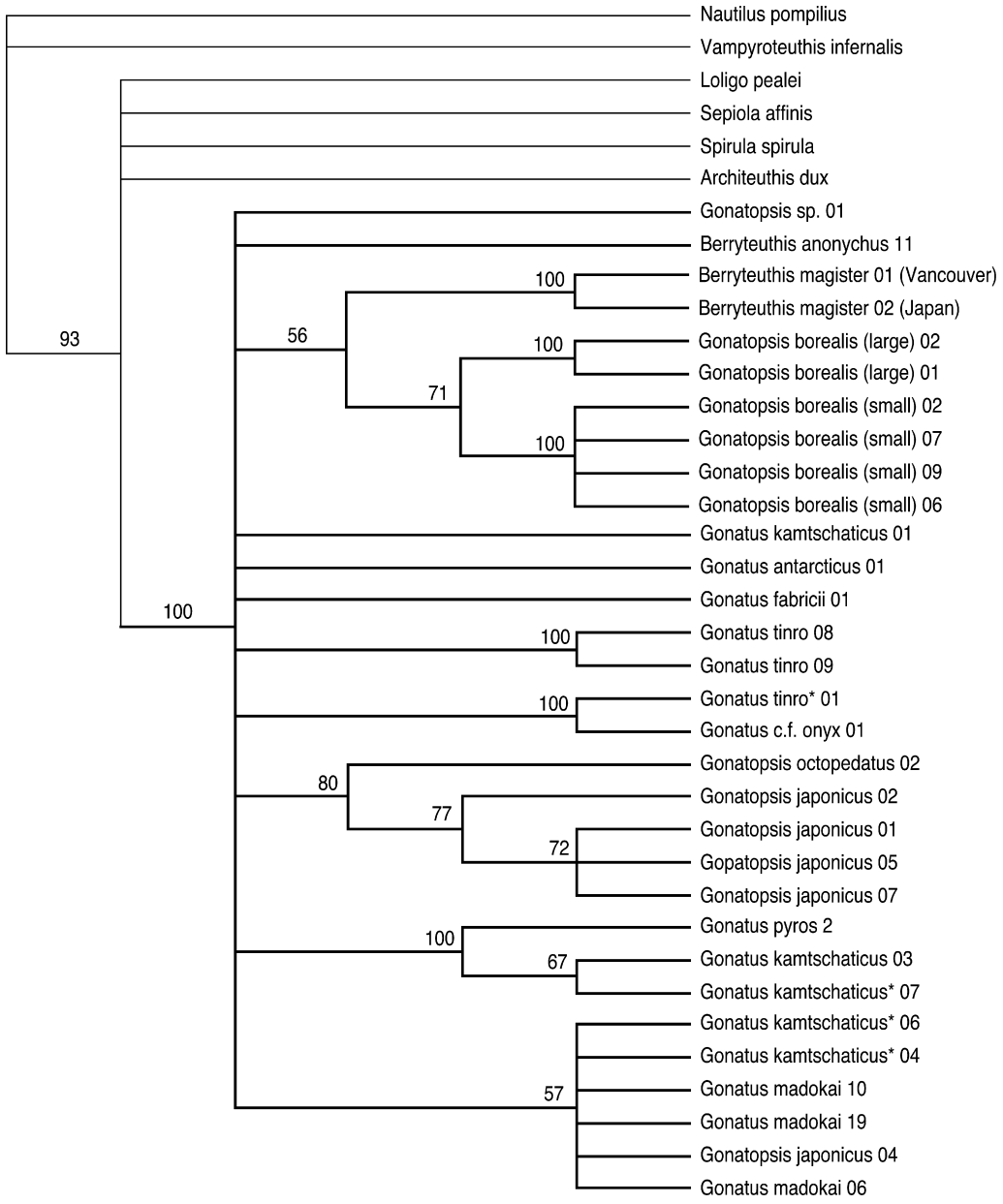


Fig. 2. COI consensus tree generated from 20 equally parsimonious trees of 1383 equally weighted steps under the optimal parameter set (221). Individuals from Gonatidae are indicated in bold. Asterisk (*) refers to specimens that may have been misidentified. Jackknife support values greater than 50% are listed.

form a clade with *Gonatopsis octopedatus* (96%); this clade is sister to a crown *Gonatus* clade (supported in 54% of replicates) consisting of *G. berryi*, *G. antarcticus*, *G. kamtschaticus* (01, 04), *G. kamtschaticus* (06), and *G. madokai*. Within the crown clade, *Gonatus berryi* and *Gonatus antarcticus* are sister to *G. kamtschaticus* 01 + (*G. madokai* + *G. kamtschaticus* 06 + *G. kamtschaticus* 04).

4. Discussion

Although individual data sets disagreed to some extent, the combined molecular analyses provided the highest degree of resolution and nodal support (Fig. 4). The topology of the combined analysis is congruent with morphological characteristics such as the number of teeth in a transverse row of a radula, tissue degeneration upon sexual maturation (Fig. 4), and allozyme data (Katugin, 2004).

There is a clear separation between two major evolutionary lineages: rather primitive, muscular gonatids with seven longitudinal rows of radular teeth and more advanced, less muscular (at least in late ontogenetic stages) gonatids with five rows of radular teeth. The radula of *Nautilus* contains thirteen elements (nine longitudinal rows of teeth and four rows of plates) whereas

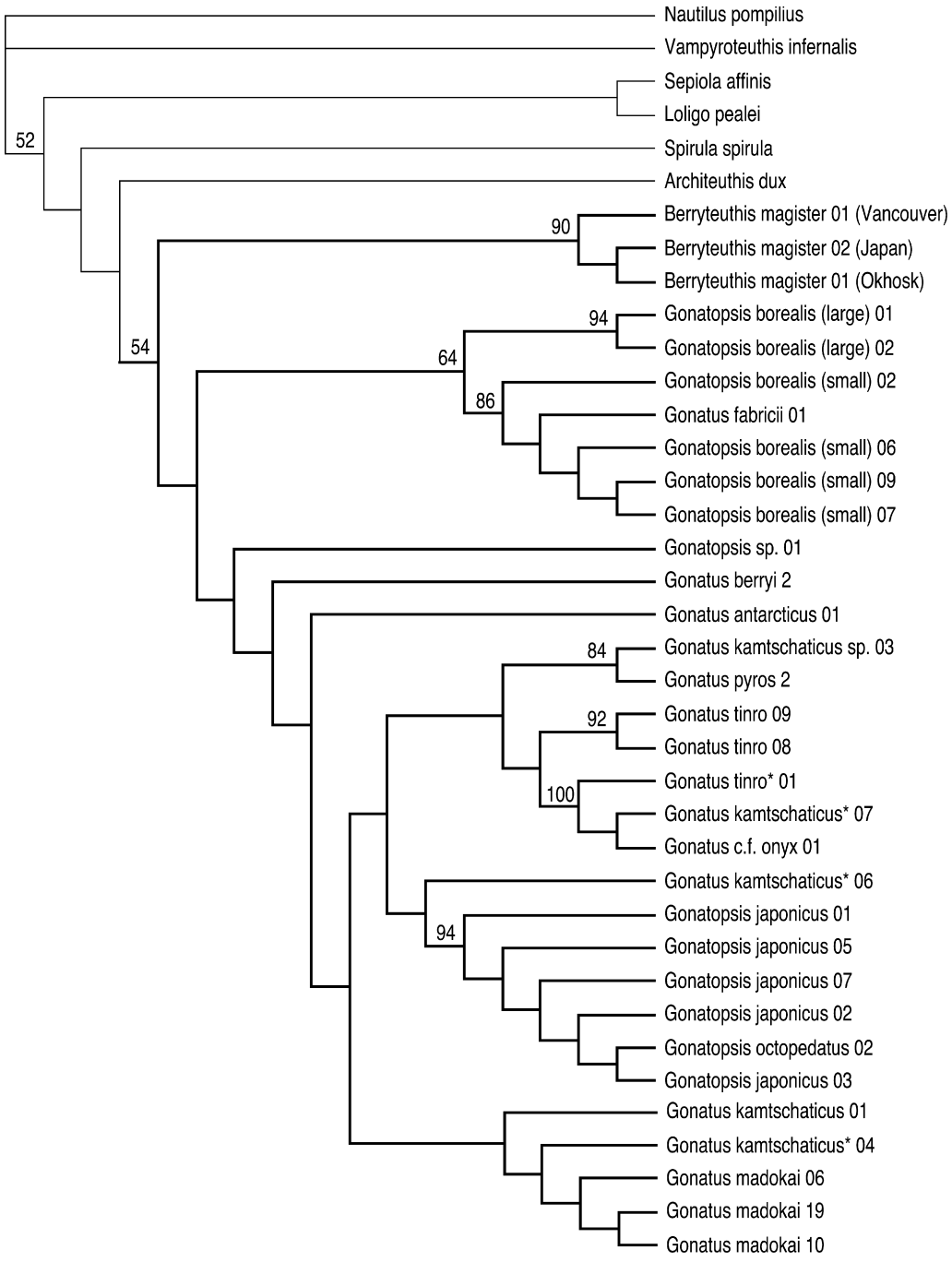


Fig. 3. 16S rRNA cladogram generated from a single tree of 866 equally weighted steps under the optimal parameter set (221). Gonatid taxa are illustrated in bold. Asterisk (*) refers to specimens that may have been misidentified. Jackknife support values greater than 50% are noted above corresponding nodes.

most decabrachians contain nine (seven rows of longitudinal teeth and two rows of plates; Nixon, 1998). Our findings suggest the more basal gonatid group is comprised of the 7-toothed recent taxa: two paraphyletic *Berryteuthis* species (*B. magister* and *B. anonychus*), and several representatives of *Gonatopsis* (large and small sized *G. borealis* and *Gonatopsis* sp., partly conforming to the description of *Gonatopsis makko* (Okutani and Nemoto, 1964)). The more derived 5-toothed recent species are monophyletic with 7-toothed *B. anonychus* as sister.

The combined analysis establishes several other relationships that can be compared to earlier morphological investigations. For example, Nesis (1973) believed that *Berryteuthis anonychus* was the more ancestral form within *Berryteuthis*, and hence the closest exemplar of the family prototype. However, our findings suggest that *B. anonychus* may be the derived form (sister to all taxa

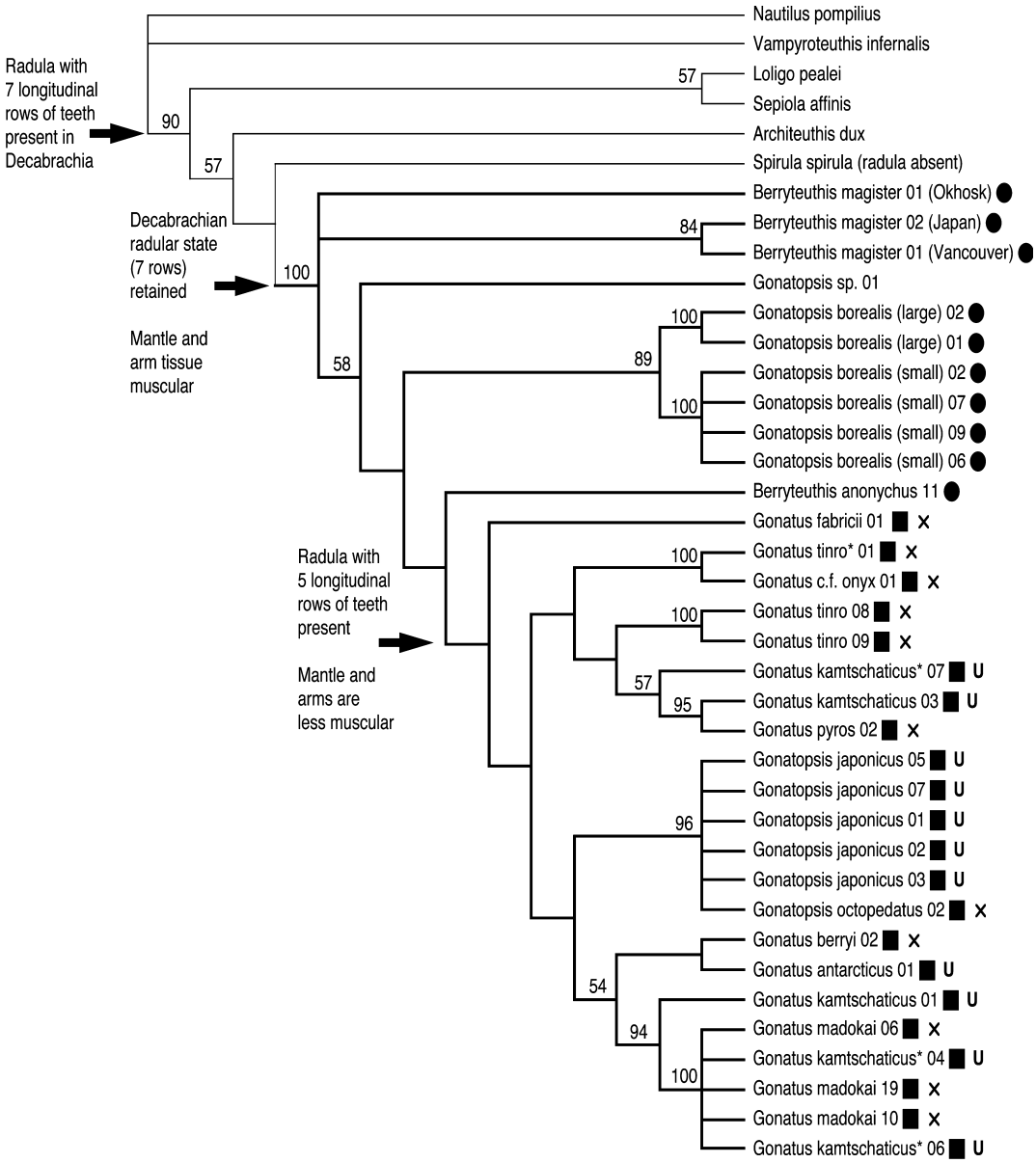


Fig. 4. Combined molecules phylogeny generated from five equally parsimonious trees, each of 2768 steps under the optimal parameter set (221). Bold lines indicate species within the family Gonatidae. Jackknife values greater than 50% are listed above corresponding nodes. Asterisk (*) indicates specimens that may have been misidentified. Circles indicate gonatid species known to have muscular mantle and arm tissues (no degeneration). Black squares indicate species that may have some mantle and arm tissue with a higher water content level (noted from Nesis, 1973). Females recorded to have watery arm and mantle tissue and brood their eggs are indicated with an X. Species with no information on degeneration are indicated with a U. Although *G. japonicus* may undergo degeneration, further analysis is warranted and was therefore coded as "U."

with 5 radular rows of teeth), while *Berryteuthis magister* is most basal of all gonatid species sampled. This outcome is in concert with Jefferts (1983), who considered *B. magister* to be the most primitive type within the family based on morphology alone. Our findings, as well as previous allozyme analysis (Katugin, 2004) confirm this result, suggesting that *B. magister* is more basal, whereas *B. anonychus* is more derived. Furthermore, monophyly of *B. magister* did not receive high nodal support, and is congruent with the fact that the species is polymorphic with geographical (Katugin, 2000) and presumably ecological (Okutani et al., 1987) subspecies. Further studies into the population dynamics of *B. magister* are needed to better understand the evolutionary dynamics within this species.

One of the most geographically distinct species, *Gonatus fabricii*, which inhabits Arctic and North Atlantic waters, was found as the sister to all other *Gonatus* spp., *Gonatopsis japonicus*, and *Gonatopsis octopedatus*, demonstrating that it is the most basal of the taxa with five longitudinal rows of radular teeth. The position of *Gonatus fabricii* suggests that morphological change to a 5-rowed state may have occurred prior to geographic radiation.

Gonatus antarcticus is also geographically distinct from other gonatids, and is distributed sub-polar in notal waters close to Antarctica, migrating as far north as South Africa, Cook Strait and north Peru (Nesis, 1982). However, *G. antarcticus* nested within a *Gonatus* clade, sister to *Gonatus berryi*, which is native to the North Pacific. This may be indirect evidence that gonatid squids migrated to the Southern Ocean not from North Atlantic, but rather via a Pacific route, as was suggested by Nesis (1985, 1997).

We have also demonstrated that individuals conforming to the description of *Gonatus kamtschaticus* represent a heterogeneous group, comprised of at least two paraphyletic taxa. This species was originally described by Middendorff (1849) from a specimen caught in the oceanic waters off the Kuril Islands; later, the same species was re-described under the new name *Gonatus middendorffi* (Kubodera and Okutani, 1981), with holotype and paratype specimens also obtained from the oceanic area off the Kuril Islands. Our study contains both large- and medium-sized squid that conformed to the species description. The most readily identifiable specimen was *G. kamtschaticus* 01 (= *G. middendorffi*), a fairly large, still maturing female with dorsal mantle length (ML) of 403 mm, which fell within a *Gonatus madokai* group with jackknife support of 94% (Fig. 4). A much smaller individual, listed as *G. kamtschaticus* 07, a maturing female with ML of only 132 mm, was recovered within a separate clade, closely related to the *Gonatus* group represented by *G. tinro*, *G. pyros*, *G.* cf. *onyx*, and *G. kamtschaticus* 03. Additionally, in the 16S analysis *G. kamtschaticus* 07 formed a clade with *G. tinro* 01 and *G.* c.f. *onyx* 01. It is possible that either *G. kamtschaticus* 07 was misidentified or that there was sequence contamination. To further resolve this issue, additional data needs to be collected. From our analyses, we can draw several conclusions regarding *Gonatus kamtschaticus*: *G. kamtschaticus* (04 and 06) may be *G. madokai.* The 04 specimen was an immature female of ML 313 mm with both *G. madokai* and *G. kamtschaticus* features, with its mantle consistency similar to *G. madokai.* The specimen had a rather slim body (mantle width index of 0.22, more typical of *G. kamtschaticus*), and fin proportions similar to those of *G. kamtschaticus* (the fin was as wide as long, with fin length index of 0.44, more typical of *G. kamtschaticus*). However, it lacked the tentacles for valid identification. The *G. kamtschaticus* 06 specimen also lacked tentacles, and was initially questionable whether it represented *G. madokai* or *G. kamtschaticus. Gonatus madokai* and *G. kamtschaticus* can be hard to distinguish morphologically when specimens are damaged, such as when tentacles or fins are missing, and *G. kamtschaticus* is as yet an unresolved group of closely related species without evident differences in morphology (= sibling species). This situation indicates that morphologically based identification can sometimes be erroneous and lead to misinterpretation of the results, resulting in an incorrect classification, providing additional support that molecular data is useful not only for phylogenetics, but also for species identification in the Gonatidae.

Gonatus cf. *onyx* and *Gonatus tinro* 01 were recovered as sister taxa, with all other *G. tinro* individuals forming a clade with *G. kamtschaticus* individuals and *G. pyros.* Further investigation of the *G. tinro* 01 specimen indicated that it was most likely misidentified. This specimen, identified as a maturing female with ML of approximately 160 mm (onset of sexual maturation in *G. tinro* females occurs at around 210 mm ML), was collected from a trawl net and was in extremely bad condition, with arms significantly damaged, tentacles torn and missing, and fins absent. Therefore, we infer that *G. tinro* 01 was actually a specimen of *G. onyx*; however, further sampling of *G. onyx* would provide more support of this finding.

Within *Gonatopsis*, a sister relationship between *Gonatopsis japonicus* and *Gonatopsis octopedatus* is further supported by the presence of a 5-toothed radula, as well as their location in deep waters of the Japan Sea (this species being much rarer in the Okhotsk Sea). Furthermore, there is indirect evidence that in *G. japonicus,* musculature degenerates upon maturation; parts of the arm crown (only eight arms, no trace of tentacles), presumably belonging to *G. japonicus*, have been occasionally observed floating on the surface in the Japan Sea (Katugin and Mokrin, personal observation).

The possibility of a recent speciation event may be evident in *Gonatopsis borealis*, which was divided into two subgroups (large and small), with 100% nodal support for each clade. Large-sized squids mature at mantle length over 200 mm in males and over 250 mm in females, while mature small-sized are 130–150 and 150–170 mm, respectively (Nesis, 1989). Distribution ranges of these two groups are different, with the small group distributed predominantly in northern areas, while the large group inhabits mostly southerly offshore oceanic areas, although they are found in sympatry along the Kuril Islands and in south Okhotsk Sea (Nesis, 1997; Nesis and Nezlin, 1993). Our findings also support preliminary results from allozyme electrophoretic comparisons of *G. borealis* groups (Katugin, unpublished), where fixed differences at a number of protein loci have been observed between the two groups, suggesting their reproductive isolation from each other. Although no morphological traits other than size allows discrimination between small- and large-sized groups of *G. borealis*, two independent sources of genetic evidence (allozyme and DNA) along with geographical patterns suggest that they are taxonomically distinct. Whether they represent two subspecies within a polymorphic species, or they have already diverged at a specific level within a superspecies remains unclear, and further research into the problem is warranted.

Physiological transitions within this family raise several pertinent questions. If weaker musculature exists in males and immature females, is muscular degeneration in brooding females the result of physiological or hormonal cues? Can female change be considered a synapomorphy supporting the monophyly of five-toothed gonatids? Was morphological change and egg brooding a cause for speciation and radiation, or vice versa? At the present time, it is difficult to fully answer these questions since the reproductive characteristics of several key taxa remain unknown. No mature females have been recorded for *G. kamtschaticus*, *G. antarcticus*, *G. californiensis*, or *G. steenstrupi*, and although *G. japonicus* may undergo muscular degeneration (Nesis, 1997; Katugin and Mokrin, personal observation), further investigation is needed. Nesis (1971) believed that all deep-water taxa were also those with water-rich tissue and the ability to degenerate during maturation, but he was not able to substantiate his beliefs due to the lack of specimen availability. Okutani et al. (1995) refuted Nesis "deep water rule" in observing an unidentified species of *Gonatopsis* in the Okhotsk Sea near the Hokkaido coast (later it was suggested by Tsuchiya et al. (2002) that the species in fact belongs to the genus *Gonatus*), which was found to release juveniles from an egg-mass between the arms near in-shore shallower waters. Observations on spent females of *Gonatus madokai* also suggest that prevailing currents largely influence their distribution patterns in the Okhotsk Sea, as they are frequently found in shallow depths (Katugin and Merzlyakov, 2002; Katugin et al., 2004).

Gonatids remain an elusive group, due in part to the lack of available mature specimens, but also because considerable ontogenetic change is seen in at least several species. While this study was able to provide further insight into the evolutionary dynamics within Gonatidae and support previous morphological hypotheses, future studies of this complex group of decabrachians will provide additional information as to how reproductive strategies, ecological factors, biogeography, and physiological modifications may affect speciation in pelagic marine invertebrates.

Acknowledgments

The authors would like to thank Dr. Gonzalo Giribet for his assistance in phylogenetic analysis, as well as David Carlini, William Macy, and Martin Collins for loaning us outgroup tissue for molecular analyses. Much thanks to Dr. Satoshi Suyama, Graham E. Gillespie, Dr. Gennadyi A. Shevtsov, and M.A. Zuev for assisting in providing specimens within Gonatidae. The authors would also like to thank the comments of two anonymous reviewers. This project was funded in part to NSF SBE-0123690, NSF DBI-0079820, NSF DEB-0316516, and NIH S06-GM08136-26 to M.K.N. and NIH-MARC GM07667-26 for support of E.A.

References

Arkhipkin, A.I., Bjorke, H., 1999. Ontogenetic changes in morphometric and reproductive indices of the squid *Gonatus fabricii* (Oegopsida: Gonatidae) in the Norwegian Sea. Polar Biology 22, 357–365.

Boletzky, S.V., 1992. Evolutionary aspects of development, life style, and reproductive mode in cirrate octopods (Mollusca: Cephalopoda). Revue Suisse Zool 99, 755–770.

Carlini, D.B., Graves, J.E., 1999. Phylogenetic analysis of cytochrome c oxidase sequences to determine higher-level relationships within the coleoid cephalopods. Bulletin of Marine Science 64 (1), 57–76.

Cosgrove, J.A., 1993. In situ observations of nesting female *Octopus dofleini* (Wulker, 1910). Journal of Cephalopod Biology 2 (2), 1–15.

Farris, J.A., Albert, V.A., Källersjö, M., Lipscomb, D., Kluge, A.G., 1995. Parsimony jackknifing outperforms neighbor-joining. Cladistics 12, 99–124.

Giribet, G., 2001. Exploring the behavior of POY, a program for direct optimization of molecular data. Cladistics 17, S60–S70.

Hunt, J.C., Seibel, B.A., 2000. Life history of *Gonatus onyx* (Cephalopoda): ontogenetic changes in habitat, behavior and physiology. Marine Biology 136, 543–552.

Jefferts, K., 1983. Zoogeography and Systematics of Cephalopods of the Northeastern Pacific Ocean. Ph.D. Thesis, Oregon State University, Corvallis, OR, 291 p.

Katugin, O.N., 2003. The Gonatid squids—Why do some of them become watery upon maturation? CIAC 2003: Biology, Recruitment and Culture of Cephalopods. Programme and Abstracts, 46.

Katugin, O.N., 2004. Squids of the family Gonatidae from the North Pacific Ocean and their genetic differentiation: controversial issues in the systematics and phylogeny. Ruthenica 14 (1), 73–87 (In Russian with English summary).

Katugin, O.N., Merzlyakov, A.Y., 2002. Spent females of *Gonatus madokai* (Teuthida: Oegopsida) from the Okhotsk Sea. In: Proceedings from the 1st International Workshop of Squids, 2nd International Symposium of Pacific Squids. Abstracts. La Paz, Baja California Sur, Mexico, pp. 39–40.

Katugin, O.N., Merzlyakov, A.Y., Vanin, N.S., Volkov A.F., 2004. Distribution patterns of the gonatid squid *Gonatus madokai* in the Okhotsk Sea in spring 2002. In: McKinnel, S. (Ed.), Proceedings of the Third Workshop on the Okhotsk Sea and Adjacent Areas, PICES Scientific Report (26), pp. 158–161.

Kubodera, T., Okutani, T., 1981. *Gonatus middendorffi*, a new species of gonatid squid from the northern North Pacific, with notes on morphological changes with growth and distribution in immature stages (Cephalopoda, Oegopsida). Bulletin of the National Science Museum, Tokyo, ser A 7(1), 7–26.

Lindgren, A.R., Giribet, G., Nishiguchi, M.K., 2004. A combined approach of the phylogeny of Cephalopoda (Molllusca). Cladistics 20 (5), 454–486.

Middendorff, A.T., 1849. Beiträge zu einer Malacozoologia rossica. II. Aufzählung und Beschreibung der zur Meeresfauna Russlands gehörigen Einschaler, Memoires de L'Academie Imperiale des Sciences de Siant-Petersbourg, 6 serie, 6, 329–610.

Nesis, K.N., 1971. The family Gonatidae—abundant squids of the North Pacific (their distribution, ecology, system and phylogeny). In: Likharev, I.M. (Ed.), Molluscs. Trends, Methods and Some Results of Their Investigation. Abstracts from the Fourth Meeting on Molluscan Research. Nauka leningrad, pp. 63–65.

Nesis, K.N., 1973. Taxonomy, phylogeny, and evolution of squids of the family Gonatidae (Cephalopoda). Zoologicheskii Zhurnal (Zoological Journal) 52 (11), 1626–1638 (In Russian, with English summary; translated into English).

Nesis, K.N., 1982. Short Guide to Cephalopod Molluscs of the World Ocean. Moscow: Legkaya I Pischevaya Prmyshlennost. 360 p. (In Russian).

Nesis, K.N., 1985. Oceanic Cephalopods: Distribution, Life Forms, Evolution. Nauka Publ., Moscow (In Russian).

Nesis, K.N., 1987. Cephalopods of the World. T.F.H Publications, Neptune City. 351 p.

Nesis, K.N., 1989. Teuthofauna of the Okhotsk Sea. Biology of squids *Berryteuthis magister* and *Gonatopsis borealis* (Gonatidae). Zoologicheskii Zhurnal (Zoological Journal) 68 (9), 45–56 (In Russian, with English summary).

Nesis, K.N., 1993. Spent females of deep-water squid *Gonatopsis octopedatus* Sasaki, 1920 (Gonatidae) in the epipelagic layer of the Okhotsk and Japan seas. Ruthenica 3 (2), 153–158.

Nesis, K.N., 1997. Gonatid squids in the Subarctic North Pacific: ecology, biogeography, niche diversity and role in ecosystem. Adv. Mar. Biol. 32, 243–324.

Nesis, K.N., Nezlin, N.P., 1993. Intraspecific groupings in gonatid squids. Russian Journal of Aquatic Ecology 2 (2), 91–102.

Nishiguchi, M.K., Lopez, J.E., Boletzky, S.V., 2004. Enlightenment of old ideas from new investigations: the evolution of bacteriogenic light organs in squids. Evolution and Development 6, 41–49.

Nixon, M., 1998. The Radulae of Cephalopoda. In: International Workshop on Systematics and Biogeography of Cephalopods. Smithsonian Institution Press, Washington DC, pp. 39–54.

Norman, M., 2000. Cephalopods, A World Guide. ConchBooks, Hackenheim. 320 p.

Okutani, T., Nemoto, T., 1964. Squids as the food of sperm whales in the Bering Sea and Alaskan Gulf. Scientific Reports of the Whales Research Institute 18, 111–122.

Okutani, T., Tagawa, M., Horikawa, H., 1987. Cephalopods from Continental Shelf and Slope around Japan. Japan Fisheries Resource Conservation Association, Tokyo.

Okutani, T., Nakamura, I., Seki, K., 1995. An unusual egg-brooding behavior of an oceanic squid in the Okhotsk Sea. Venus 54, 237–239.

Seibel, B.A., Hochberg, F.G., Carlini, D.B., 2000. Life history of *Gonatus onyx* (Cephalopoda: Teuthoidea): deep-sea spawning and post-spawning egg care. Marine Biology 137, 519–526.

Sweeney, M.J., Roper, C.F.E., 1998. Classification, type localities, and type repositories of recent Cephalopoda. In: Voss, N.A., Vecchione, M., Toll, R.B., Sweeney, M.J. (Eds.), Systematics and Biogeogrpahy of Cephalopods, Volume II. Smithsonian Contributions to Zoology, vol. 586., pp. 561–595.

Smith, S.W., Overbeek, R., Woese, C.R., Gilbert, W., Gillivet, P.M, 1994. The Genetic Data Environment: an expandable GUI for multiple sequence analysis. Computational Applications in Bioscience 10, 671–675.

Tsuchiya, K., Yamamoto, N., Abe, H., 2002. Cephalopods in Japanese Waters. TBS-Britannica Co., Ltd, 1-24-12 Meguro, Meguro-Ku, Tokyo 153-8940, Japan (In Japanese).

Wheeler, W., 1995. Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. Systematic Biology 44, 321–331.

Wheeler, W.C., Gladstein, D., DeLaet, J., 2002. POY version 3.0. Program and documentation available at ftp.amnh.org/pub/molecular. American Museum of Natural History.

Young, R.E., 1973. Evidence for spawning by *Gonatus* sp. (Cephalopoda: Teuthoidea) in the high Artic Ocean. Nautilus 87 (2), 53–58.

Phuket mar. biol. Cent. Res. Bull. **66:** 11-22 (2005)

IDIOSEPIUS: ECOLOGY, BIOLOGY AND BIOGEOGRAPHY OF A MINI-MAXIMALIST

Convenor:	Sigurd v. Boletzky	boletzky@obs-banyuls.fr
Rapporteur:	Michele K. Nishiguchi	nish@nmsu.edu
Coordinators:	Jaruwat Nabhitabhata	rcas@loxinfo.co.th
	Jintana Nugranad	jinna@cscoms.com

Registered workshop participants:

Laure Bonnaud	bonnaud@mailhost.ccr.jussieu.fr
Ruth A. Byrne	a9303751@unet.univie.ac.at
Ke-Yang Chang	ky_chang@pchome.com.tw
Jorgen Hylleberg	hylleberg@biology.au.dk
Jutamas Jivaluk	Jutamasj@fisheries.go.th
Takashi Kasugai	t-kasugai@nagoyaaqua.or.jp
Mary T. Lucero	mary.lucero@m.cc.utah.edu
Jennifer A. Mather	mather@uleth.ca
Volker Christian Miske	volker.miske@teuthis.de
Delphine Pichon	pichon.del@canamail.com
Shuichi Shigeno	shigeno@cdb.riken.go.jp
Arie A. Sitthichai	arieml@yahoo.com
Mike A. Steer	masteer@utas.edu.au
Sean R. Tracey	sean.tracey@dpiwe.tas.gov.au
Toshie Wakabayashi	toshiew@fra.affrc.go.jp
Kerstin Warnke	warnke@zedat.fu-berlin.de

Rationale

The original call for participation announced the following outline:

<<The seven known species of the pygmy squid genus *Idiosepius* [*I. pygmaeus* Steenstrup, 1881, *I. paradoxus* Ortmann, 1888, *I. picteti* (Joubin, 1894), *I. notoides* Berry, 1921, *I. biserialis* Voss, 1962, *I. macrocheir* Voss, 1962, *I. thailandicus* Chotiyaputta, Okutani and Chaitiamvong, 1991] are among the smallest living cephalopods. Recent studies revealed that at least some of these miniature squids are extremely short-lived, while they are "maximalists" in terms of their reproductive output.

The small adult size and the special sedentary adult mode of life of *Idiosepius* raise questions about lifetime mobility: is the post-hatching phase of planktonic life the only means of dispersal, or do adult migrations also support dispersal ? How much of the biogeography is a consequence of biological and ecological constraints, and how much is due to evolutionary contingencies ?

The systematics of the genus and species and the phylogenetic background of this monogeneric family may be studied under different viewpoints, using analytical approaches that range from embryological, morphological and behavioural studies, to biochemical phenetics and molecular phylogeny.

Ultimately, laboratory cultures starting out from planktonic juveniles will be necessary to study post-hatching behaviour of *Idiosepius*, and in particular to find out (1) how the tentacles, which grow out only after hatching, are functionally integrated into the arm crown for prey capture, and (2) how the dorsal attachment behaviour is established when young animals switch to the adult life style.

The aim of this workshop is to study live *Idiosepius*, to discuss their biology and the related ecological and bio-geographic aspects, and to collect all the available information on these unique cephalopods. The local coordinators will provide live animals in aquaria, so that the workshop participants may get first-hand experience in observing some of the peculiar behaviour patterns of these animals. If spawning animals are available, or eggs can be collected from the field, a hands-on session for the study of living embryos will be organized. Some emphasis will be placed on the procurement and circulation of unpublished reports on *Idiosepius*; therefore, anyone having access to such documents is kindly requested to bring at least one copy of each relevant item to the workshop. The results of this workshop are intended to be published as a group report.>>

Workshop Format and Group Report

During the first day of the workshop, a series of presentations and open discussions set the stage for a wider survey during the second day, with a series of parallel, in-depth reflections by sub-groups, and a final presentation of the conclusions.

17 February 2003:

Jaruwat Nabhitabhata opened the session with a welcome address and a first presentation of some live animals producing egg masses in their tanks: *Idiosepius pygmaeus* and *I. biserialis*.

Sigurd Boletzky recalled the historical background and rationale of this workshop (as outlined in the introductory text) and pointed out that renewed interest in pygmy squid arose when Voss (1962) described two new species of *Idiosepius* (*I. biserialis*, *I. macrocheir*) – four decades after the description of *I. notoides* by Berry (1921). Voss' work brought the number of recognized species to 6 – three decades before *I. thailandicus* was described by Chottiyaputta *et al.* (1991). This work, along with studies by Hylleberg and Nateewathana (1991a, b), confirmed the presence of a gladius in *Idiosepius* spp., a fact that had been previously disputed. Another important turn was the first detailed description of *Idiosepius* embryos, especially the peculiar morphogenetic retardation of the tentacle rudiments, by Natsukari (1970) – nearly two decades before complementary information and a staging system were published by Yamamoto (1988). In the same period Adam (1986) drew attention to some very peculiar aspects of the radula and beaks that were first described by Appellöf (1898). The rather slow progress in morphological and systematic studies with *Idiosepius* was finally overtaken by a much more rapid development in recent years. Biological studies increasingly drew attention to the "maximalist" performance of pygmy squid, especially with regard to fecundity (Lewis and Choat, 1993).

Jaruwat Nabhitabhata gave an overview of the biology of *I. thailandicus*. This is the smallest species of the genus and is characterized by strong sexual dimorphism, the female being 3–4 times larger than the male. Colour patterns also differ between the two sexes, the females being light brown, whereas the males are dark brown (substrate colour matching notwithstanding). Likewise, orientation in space differs in resting individuals, with females attaching themselves in a head up position, whereas males position themselves head down. During mating, males approach females, apparently using the tentacles (not the hectocotylus!) for spermatophore transfer to the buccal area of the female. Females then spread out their arm crown for acceptance of spermatophores. Mating in attached males and females may be induced by females enticing males to move down to them on the substrate. Females explore the substrate for egg attachment with arms stretched to a sharp point. Eggs are laid on seaweed using the arms. During pauses in egg laying, females undergo mating. There is no mate guarding by males.

Freshly laid eggs are surrounded by many jelly layers. Subsequent developmental events were described up to hatching after 2 weeks. As in other species, no tentacles are developed at hatching.

Main differences between the three species of *Idiosepius* found in Thailand (*I. thailandicus*, *I. pygmaeus*, *I. biserialis*) and reproductive strategies were discussed.

Finally, culture of pygmy squid was considered in comparison to cultures of loliginid

squids and sepiid cuttlefish performed at temperatures up to 28°C (Nabhitabhata, 1994a,b, 1998). *I. pygmaeus* was reared from hatching, and two individuals survived to 30 days. They fed on wild copepods and mysids (*Mesopodopsis orientalis*). Adhering behaviour was not observed. One of them spent its time lying on the bottom as well as swimming. Final mantle lengths were 3.67 and 4.92 mm.

Sean Tracey presented a paper entitled "Life history traits of the temperate mini-maximalist *Idiosepius notoides*" (Tracey *et al.*, 2003). Wild-caught individuals were aged by interpreting the increment structure on transverse sections of statoliths (growth rings counted at 40x magnification from natal ring to dorsal dome surface). Histological preparations established sexual maturation stages. Strong sexual dimorphism was displayed in the age at onset of maturation, females mature at 88 days and males at 69 days, with growth rates being similar in both sexes. Maximum age of around 107 days was observed in one female.

Intraspecific variability in growth rates was detected, it was proposed that this be due to the influence of SST on metabolic rate. *I. notoides* occurs from New South Wales to Tasmania, surviving temperatures as low as 10°C. In contrast, *I. pygmaeus* occurs from N. Australia to NSW and grows faster at the higher temperatures, which range from 16 to 30°C. Overlap of geographic distributions in NSW and differences between life history strategies under tropical and temperate conditions were discussed.

Toshie Wakabayashi presented some distribution data on *Idiosepius pygmaeus* based on plankton net catches (night samples) taken from NW Australia to S Australia. Temperatures ranged from 26 to 30°C, salinities from 34.2 to 35.2 psu. Animals were mostly found in coastal waters (0–100 m) and close to shore. Specimens were identified by tentacle and arm structures, with emphasis on early hectocotylization in males. Arm crown and beaks were studied by SEM (oral view of arm crown).

Takashi Kasugai summarized his poster presentation "Life cycle of the Japanese pygmy cuttlefish *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) in the *Zostera* bed at the temperate coast of central Honshu, Japan" (Kasugai and Segawa, 2003).

I. paradoxus is the northernmost species, occurring from Japan to S. Korea and E. China. Sexual dimorphism was pronounced, females being much larger than males. There was an alternation of generations with smaller and larger individuals correlating with warmer and cool seasons, respectively. *Zostera* beds were affected by changes in temperature, and also typhoon conditions. Temperatures ranged from 30°C (August 1998) to 6.0°C (February 1999). Mature specimens appeared throughout cool and warm seasons (both males and females). Males matured earlier than females. Sex ratio changed between seasons. If *Idiosepius* permanently resides in *Zostera* beds, it might be subject to degradation of the habitat.

Behavior in captivity: Copulation was observed not only when the female was at rest (*i.e.* not spawning) but also in course of laying eggs. Copulation was initiated by males. Males approached slowly, then grasped females quickly and attached spermatophores close to the female's buccal mass. Males finding egg laying females grasped females at the base of their arms and attached spermatophores between phases of egg laying. Up to 3 males may remain in stand-by position awaiting an opportunity to copulate with one and the same female. Both ventral arms of males were hectocotylized (left arm IV has 2 small lobes at the tip; it is raised while the right arm IV, which is devoid of such lobes, is inserted into the female's arm crown).

After copulation outside of egg-laying, spermatangia implanted in the arm crown were picked up by females (using her buccal mass, which is protruded) and might have been moved to the seminal receptacle below the mouth. On the contrary, in copulation with egg-laying females, spermatangia just implanted by males seemed to be used to fertilize spawned eggs, whereas spermatozoa in the seminal receptacle were left where they were as a reserve for subsequent egg laying. The observation of buccal mass protrusion raised the question of which parts were actually being pushed out, the buccal mass alone, or the

buccal mass along with surrounding integumental tissues. Probably the homologue of the (undifferentiated) buccal crown was also pushed out, thus forming a proboscis (NB: no differentiated buccal lappets exist in *Idiosepius*).

Egg laying females used all arms to attach eggs to the surface of a substrate. Egg laying lasted up to 80 seconds for a single egg. Uninterrupted terminal spawning continued during more than two weeks. During egg laying the oviducal jelly surrounded the chorion of each egg and was immediately covered by nidamental jelly, which subsequently "fused" with the added nidamental jelly of the next following eggs.

Rearing of juveniles: juveniles preferred mysids over *Artemia*. The empty exoskeleton of the prey was discarded after removal of the flesh by the protruded buccal mass. Only 3 individuals were raised to the age of 19 days, 1 individual reached the age of 26 days. At hatching, there were no tentacles. After 2 weeks, individuals began to adhere to a substrate using the mantle tip (remainder of Hoyle's organ ?). After 3 weeks, the tentacles were visible within the arm crown. The oldest specimen (26 days) had a mantle length of 3 mm, the tentacles had a length of about 2/3 arm length. At that stage, the short tentacles were probably used like arms (fast musculature developing?).

Mary Lucero gave an informal presentation of the cephalopod olfactory organ and its physiology, recalling the definitions of taste (contact chemoreception in suckers, lips, fins, around eyes) which involves solitary chemoreceptor neurons and smell or olfaction (distance chemoreception in olfactory pit/olfactory organ, situated ventral and posterior to the eye). Benthic organisms have better chemosensory systems than pelagic organisms. Solitary organisms use distance chemoreception. Therefore, since *Idiosepius* is both benthic and solitary, it should have a better chemosensory system than other types of squid.

Behaviorally relevant odors for cephalopods were identified from the use of bait with amino acids (bait accepted) or bait with betaine (bait rejected)(Lee *et al*., 1994). Additional behavioral studies which mapped the chemosensory regions of the squid showed that the region of highest chemical sensitivity was the olfactory organ (Gilly and Lucero, 1992).

The cephalopod olfactory organ is composed of sensory neurons and ciliated support cells that generate a whirlpool to pull the water towards the receptor cells. Neurons within the olfactory organ send fibers to the olfactory lobe of the brain and to the optic gland (supposed importance for gonadal maturation). Its anatomy suggests both olfactory function and pheromonal function (possibly triggering egg laying). Cross sections of squid olfactory organs exhibit an epithelium with pseudo-stratified layers of support cells and five different morphological subtypes of olfactory neurons (Emery, 1975).

Electrophysiological recordings from squid olfactory neurons showed that odors activate either excitatory or inhibitory receptor potentials.

It will be interesting to look at different morphotypes of cephalopod olfactory neurons and map onto them the functional responses to different chemicals.

Shuichi Shigeno presented his observations on nervous system development in *Idiosepius* in relation to behavioural changes. An SEM study of embryonic development illustrated some peculiar features, such as the presence of so-called pseudoarms (ostensible duplication of arm rudiment) in the early stage of brachial morphogenesis, or tuft type cilia in the mantle cavity, similar to those described in sepiolids. Toothed beaks were differentiated in the hatchlings. Cup-shape of the olfactory organ was unique to *Idiosepius*, also its morphogenetic "migration" to the definitive ventral posterior position in relation to the eye. Other peculiarities existed in Hoyle's organ, in the lateral line homologue and in ciliary distribution in general.

An atlas of the embryonic brain in *Idiosepius paradoxus* is now available (Yamamoto *et al*., 2003). A description of the neural network formation using confocal microscopy was given. Comprehensive gene cloning from the *Idiosepius* brain was performed to recognize brain elaboration processes. Expression of neuron specific glycoprotein mRNA is now being studied.

Adult *Idiosepius* had disruptive chromatophore patterns (similar to young loliginids). Is this a

synapomorphy suggesting a monophyletic group uniting pygmy squids with loliginids, or is the brain structure/anatomy more closely similar to the Sepiolidae?

Overall, in the phylogenetic context, where does *Idiosepius* have its place? Does dwarfism play an important role in the processes of development, life history strategy, and reproduction? Reduction of tentacles perhaps is less important than a similarity to ommastrephid squid brain development.

Mike Steer and **Jennifer Mather** led an open discussion of behavior, from aquarium studies to the questions of natural *in situ* behaviour of *Idiosepius* compared to other cephalopods, focusing on questions of species' ecology and possible implications for biogeography. Emphasis was placed on the links between ecology, neurophysiology, and physiology when behavior was analyzed. The ultimate requirements were that an individual must eat, avoid predation, and reproduce. The following questions asked how this is achieved by the animals.

Foraging: possible options were sit and wait, or actively hunt. Related questions were: if they sit and wait, for how long? At what time of the day do they hunt? What range of species do they take? What cues are used for prey capture? Do they return home after hunting?

Predator avoidance: possibilities included passive camouflage, a sequence of strategy changes, and deception by inking.

Reproduction: how does an individual of a sessile species find a mate? Males are supposed to play the first active part. Chemical cues could be involved in mate finding.

But females may be selective as well. Who initiates, who ends mating? How does a female choose a substrate for egg laying? How many eggs are laid at one site?

Habitat selection : to what extent is juvenile settlement selective? If there is selectivity, what are the basic cues?

Activity cycles: are the settled individuals permanently adhering to their chosen substrate, or only during a certain time of day? In other words, are there well-defined periods of activity during a 24 hour cycle?

Ecological implications of *Idiosepius* behavior led to addressing growth in relation to food availability (crustaceans being the main prey), perhaps involving chemo-reception under dim light conditions. Possible alternative modes of "grazing" (Moynihan, 1983) may result in various requirements for different growth stages. Growth also needs to be viewed with regard to sexual dimorphism in adult size and related effects on social interactions before and during reproduction.

The general poor knowledge about biogeography of pygmy squid is due to the virtual absence of empirical data on natural dispersal, in terms of juvenile and adult displacement (including rafting), but also regarding eggs that might be attached to drift weed. Aquarium studies can address these questions, but will provide much less than field studies.

Jutamas Jivaluk summarized her poster presentation "Description of hatchling of Thai pygmy squid, *Idiosepius thailandicus* Chotiyaputta, Okutani and Chaitiamvong, 1991" (Jivaluk *et al*., 2003). The mantle of hatchlings was firm and cylindrical, bell-shaped with round blunt end. Fins were separated, small, sub-terminal, oval in outline. Dorsal mantle length was 0.85-0.90 mm.

The head was nearly square in outline, with large eyes situated in the anterior corners. The arms were short and stout with blunt tips, suckers were in 2 rows on arms II and III. Arm formula: II, III, I, IV. There were no tentacles.

Up to 30 chromatophores covered the dorsal side of the mantle, 15-20 the ventral side. Arms had 1-2 chromatophores on the aboral side.

All these characters allowed differentiation between hatchlings of *I. thailandicus* and those of *I. pygmaeus* and *I. paradoxus*.

18 February 2003

The essential insights gained during the first day were briefly surveyed. Some open questions were: How many species are there really in the genus *Idiosepius*? (cf. English, 1981). How are they related to each other? Are *I. thailandicus* and *I. biseralis* sister taxa? In mature females, what is the function of the right oviducal gland, how much does it contribute to the entire jelly mass during spawning? Where is the lower limit of temperature tolerance in different species of *Idiosepius* ?

Laure Bonnaud and **Michele Nishiguchi** then led an open discussion of the placement of *Idiosepius* in the system of coleoid cephalopods. The following questions were approached: What are the phylogenetic hypotheses of the group? What are the important sister groups to the Idiosepiidae? What are the phylogenetic relationships between different species? There was general agreement that formalization of relationships must be sought by using cladistic analysis in the sense of phylogenetic systematics.

This led to the following questions: What are likely plesiomorphic characters (widely common ancestral characters) and what are likely apomorphic characters (common derived characters in species that share a more recent common ancestor)? How do we as scientists first obtain a "phylogenetic framework" to then address more comparative questions regarding ecology/ physiology/biogeography? Can similar patterns (morphological, physiological, ecological, behavioral) be mapped onto the phylogenetic framework to give us clues as to the relationships, evolution and radiation of this unique group of cephalopods? What characters are likely homoplasic (convergent characters) that might obscure the phylogenetic relationships between *Idiosepius* and other Decabrachia? It is advisable to preface an investigation with a phylogenetic framework, to generate and orientate questions regarding the physiological, morphological and biochemical aspects that may further improve the entire analysis. An essential question in this type of analysis is: What is a good character? Can molecular and morphological characters provide « mutual elucidation » ?

Molecular data are not as static as morphological data. It is harder to resolve these characters for analyses because the ancestral state of the nucleotide is unknown. How many transitions/transversions/gaps occur, what type of cost can one assign each one?

Phenetic methods cannot be expected to refine a phylogenetic hypothesis.

In-depth reflections by sub-groups

The following items are fair copies of the handwritten notes collected at the end of the session.

1. - Behaviour

- Why study behaviour ? Necessary to know if the species considered is a model species useful for form-function relationships needed for keeping in the laboratory.
- Why *Idiosepius* ? We need to understand the extreme to better know the usual (extreme in size, attachment and sedentary lifestyle)

Habitat selection. See adhesion as an energy conservation strategy needed because of the small size.
Why ? Refuging, similar to the octopus den, notice also there is 'opposite' countershading, camouflage patterns.
Adhesion is not selective – any surface will do.

Social organization. What male/female proportion, probably near 50:50.
Are they clumped ? Solitary ? Distribution may be dictated by shelter availability – distribution has not yet been studied.

Reproduction. Note the high fecundity of females, eggs laid in batches over time – necessity here for energy maximization to manage to produce all those eggs.
- Mating strategies – how do sperm get selected: active selection, selection by the female, sperm interaction, sperm removal.
- Initiative can be both by male and female – there are a lot of males mating, and they may select 'targets' by size.
- Recognition by female postures, size, would there be chemical cues ?
- There are color patterns – males can be dark brown, dark/pale halves, side stripe, also flashing 'corner'.

Feeding. The animals can forage by ambush, out and catch, maybe even 'grazing', the usual cephalopod variety.
Back to our energy conservation theme –> any way that works.
Why can't cephalopods be smaller than *Idiosepius* – and what do you do to be good at being small ?

Predator Avoidance. Note hiding and camouflage, inking as a 'last resort', can be a 'mimic' ink blob.

Notice the habitat is close to but never on the bottom.

===

Keeping *Idiosepius*:
They tolerate a wide range of salinity and a reasonable O2 level, temperature range is wide but species-specific, *I. paradoxus* 10°–25°, *I. thailandicus* 22°–32°.
Food: they eat crustaceans, a variety is fine, size isn't a big problem and *I. paradoxus* doesn't eat fish.
They need an attachment surface but it doesn't have to be *Zostera*.
Light ? Maybe dim – but undersurfaces are dim anyway.
Very small *Idiosepius* are a problem for food – NOT *Artemia* or crab zoeae, maybe newly hatched mysids and zooplankton.
If they live in the plankton, we should go collect food species there.

2. - Morphology and Physiology

Morphology
(A) Hatchling
- (1) Chromatophore patterns ? Only smaller numbers related to hatchling size ? *I. thailandicus* < *I. pygmaeus* ?
- (2) Smaller suckers ? Hatchling size *I. thailandicus* < *I. pygmaeus*
- (3) The brain is very immature but all lobes are differentiated (check olfactory lobe ?)

(B) Development
- egg size 1 mm or smaller (laid in layer, unlike *e. g.* ommastrephid spawn)
- embryonic period very short
- pseudo-arms, as are typical in sepioids and sepiolids
- beak structure: toothed beaks
- cilia: no smooth type cilia
- heterochronic retardation of tentacles (anlage appears in the embryo): 26 days after hatching tentacles become similar in length to arms
- cup-shaped olfactory organ – relation to benthic life ?
- jelly morphology: nidamental jelly fusing into one coat
- gland-like organ at the lip (base of arm crown)
- hatchling lives as paralarva (at least up to the age of 16 days)

Physiology

Very few physiological studies have been performed in *Idiosepius* spp.

A mostly anatomical study described the digestive system of *Idiosepius*. Unlike most cephalopods, *Idiosepius* utilizes external digestion. Several unique morphological characteristics are associated with external digestion including an "s-shaped" esophagus, a ciliated stomach lining instead of the usual chitinous lining, and absorptive capabilities in the intestine and caecum. There was no evidence for energy storage in the form of glycogen or lipid in digestive gland cells. In general, the digestive system of *Idiosepius* shows similarities to loliginid species, especially paralarval forms, and differs from *Sepia*.

3. - Systematics

Observations of selected specimens under a dissecting microscope showed the following characteristics:

I.biserialis from Phuket
- rounded body end
- olfactory organ near eye
- pale in fix – no chromatophores
- clear cornea in fix
- back fins attached oblique to body
- rectangular adhesion organ

I.biserialis from South Africa
- pointed body end
- olfactory organ near eye
- pale in fix – many small chromatophores
- opaque cornea in fix
- back fins attached vertical to body
- oval adhesion organ

I. thailandicus
- pointed body end
- olfactory organ farther from eye
- dark in fix – many small chromatophores
- clear cornea in fix
- back fins attached vertical to body
- oval adhesion organ

I. picteti found around Indonesia.

4. - Phylogeny

It was discussed that both an internal phylogeny for all 7 species of *Idiosepius* be pursued in order to better understand sister taxa relationships, particularly for those species which have habitat/niche overlap. In particular, *I. biserialis* from both S. Africa and Thailand should be investigated thoroughly in order to establish if they are indeed the same species (the same holds for *I. pygmaeus* from Australia and Thailand), or are separate subspecies/populations. Also, the overlap between *I. pygmaeus* and *I. notoides*. This can be accomplished quite easily with a molecular phylogenetic analysis combined with morphological data from each of the species and their respective populations.

As for the position of *Idiosepius* within the Decabrachia, it was determined that more representative taxa for related "in-groups" and "out-groups" were needed to ensure the placement of *Idiosepius* with the squids. Although some preliminary evidence suggests that the family Idiosepiidae is sister to the Loliginidae, more data are needed to support their placement and to also answer the question of which families are sister to this unique group of squids. According to earlier data by Bonnaud *et al*. (1996, 1997, 2002), it appears that other squid families than Loliginidae, such as the oegopsid squid families Ommastrephidae and Enoploteuthidae, are possible candidates for sister taxa to the Idiosepiidae. There was general agreement that removal of the Idiosepiidae from the order Sepiolida was appropriate for placement of this family.

CONCLUSION

The survey of established knowledge and open questions relating to *Idiosepius* as achieved during the workshop convinced all the participants and other people interested in the subject (see Pecl, 1994; Roberts, 1997; Semmens, 1993; van Camp, 1997; Pecl and Moltschaniwskyj, 2003) that the time was ripe for a synthesis. Therefore it was decided to assemble available data, especially from unpublished dissertations, in a monograph of the genus *Idiosepius*, which should be published before the CIAC2006 symposium.

REFERENCES

[with translation of title where appropriate, and other indications]

Adam, W. 1986. La radule et les mandibules de quelques espèces d'*Idiosepius* Steenstrup, 1881 (Mollusca Cephalopoda Decapoda). Bull. Inst. R. Sci. Nat. Belg. : Biologie, **56**: 149–154. [The radula and the beaks of some species of *Idiosepius* Steenstrup, 1881].

Appellöf, A. 1898. Cephalopoden von Ternate. Abhdl. Senckenberg. Natf. Ges., **24**: 561-637, pls 32–34. [Cephalopods of Ternate; pp. 562–563, 572–593, plates XXXII-XXXIV: *Idiosepius pygmaeus* Steenstrup, with remarks on the genus *Microteuthis* Ortmann; p. 623: creation of family Idiosepiidae].

Berry, S.S. 1912. A Catalogue of Japanese Cephalopods. Proc. Acad. Nat. Sci. Philadelphia, July, **1912**: 380–444. [p. 405: *Idiosepius paradoxa* (Ortmann, 1888)]

Berry, S.S. 1921. A review of the cephalopod genera *Sepioloidea*, *Sepiadarium* and *Idiosepius*. Rec. S. Austr. Mus., **1**: 347–364. [pp. 361–362: key to the species of *Idiosepius*, description of a new species *Idiosepius notoides*].

Berry, S.S. 1932. Cephalopods of the genera *Sepioloidea*, *Sepiadarium* and *Idiosepius*. Philippine J. Sci., **47**: 39–55.

Berthold, Th. and Th. Engeser. 1987. Phylogenetic analysis and systematisation of the Cephalopoda (Mollusca). Verh. Naturwiss. Ver. Hamburg (NF) **29**: 187–220.

Boletzky, S.v. 1977. Post-hatching behaviour and mode of life in cephalopods. **In**: M. Nixon and J. B. Messenger (eds.). The Biology of Cephalopods Symp. Zool. Soc. Lond., **38**: 557–567. [Juvenile lifestyle switching in Idiosepiidae and Octopodidae].

Boletzky, S.v. 1995. The systematic position of the Sepiolidae (Mollusca : Cephalopoda). Bull. Inst. Océanogr. Monaco, n° spéc. **16**: 99–104. [Sister group relationship of Idiosepiidae and Sepiolidae considered unlikely].

Boletzky, S.v. 1996. Développement retardé des tentacules chez *Idiosepius*: une hétérochronie originale dans la morphogenèse brachiale d'un Céphalopode nain. Bull. Soc. zool. Fr., **121**(1): 135–136. [Delayed tentacle development in *Idiosepius*: An original heterochronism in the brachial morphogenesis of a pygmy cephalopod].

Boletzky, S.v. 1999. Dwarf cephalopods : conditions of reproduction at small size (Abstract; "Cephalopods – Present and Past V", Vienna). Ber. Geol. Bundesanst., **46**: 24.

Boletzky, S.v. 2001. The impact of gamete protection on biodiversity : reflections on a moderately diversified marine molluscan class (Cephalopoda). Océanis, **24**(4)[1998]: 137–157. [p. 151: likely relation between secondary reduction of adult size and partial suppression of the right oviduct in *Idiosepius*].

Boletzky, S.v. 2002. How small is « very small » in coleoid cephalopods ? (Abstract; "Coleoid cephalopods through time", Berlin). Berliner Paläobiol. Abh., **1**: 14–15. [Different adaptive solutions under fecundity limitations at extremely small adult size: comparison between Idiosepiidae and Octopodidae].

Bonnaud, L., R. Boucher-Rodoni and M. Monnerot. 1996. Relationship of some coleoid cephalopods established by 3' end of the 16S rDNA and cytochrome oxidase III gene sequence comparison. Amer. Malac. Bull., **12**: 87–90.

Bonnaud, L., Boucher-Rodoni, R. and Monnerot, M. 1997. Phylogeny of cephalopods inferred from mitochondrial DNA sequences. Mol. Phylogen. Evol., **7**: 44–54.

Bonnaud, L., D. Pichon and R. Boucher-Rodoni. 2003. Molecular approach of cephalopod phylogeny : the case of *Idiosepius*. CIAC2003: Biology, Recruitment and Culture of Cephalopods (17–21 February 2003). Programme and Abstracts: p. 9.

Bonnaud, L., A. Saihi and R. Boucher-Rodoni. 2002. Are 28s rDNA and 18s rDNA informative for cephalopod phylogeny ? Bull. Mar. Sci., **71**(1): 197–208.

Carlini, D.B. and J. E. Graves. 1999. Phylogenetic analysis of Cytochrome *C* Oxidase I sequences to determine higher-level relationships within the coleoid cephalopods. Bull. Mar. Sci., **64**: 57–76.

Carlini, D.B., K.S. Reece and J.E. Graves. 2000. Actin gene family evolution and the phylogeny of coleoid cephalopods (Mollusca: Cephalopoda). Mol. Biol. Evol., **17**: 1353–1370.

Chotiyaputta, C., T. Okutani and S. Chaitiamvong. 1991. A new pygmy cuttlefish from the Gulf of Thailand *Idiosepius thailandicus* n. sp. Venus (Jap. Jour. Malac.), **50**: 165–174. [Detailed description of gladius].

Clarke, M.R. 1988. Evolution of recent cephalopods - a brief review. **In**: M. R. Clarke and E.R. Trueman (eds.). The Mollusca 12, Paleontology and Neontology of Cephalopods. Academic Press, London, pp.331–340.

Day, J. H. 1974. The ecology of Morrumbene estuary, Moçambique. Trans. roy. Soc. S. Afr., **41**(1): 43–97. [p. 94: *Idiosepius biserialis* Voss, *I. macrocheir* Voss].

Emery, D.G. 1975. The histology and fine structure of the olfactory organ of the squid *Lolliguncula brevis* Blainville. Tissue and Cell, **7**(2): 357–367.

English, S. 1981. The biology of two species of estuarine cephalopods from the Sydney region. M. Sc. thesis, School Biol. Sci., Univ. Sydney. 125 pp. [*Idiosepius* sp., possibly *I. notoides*; specimens conserved in the Australian Museum, catalogue number 126511].

Fioroni, P. 1981. Die Sonderstellung der Sepioliden, ein Vergleich der Ordnungen der rezenten Cephalopoden. Zool. Jb. Syst., **108**: 178–228. [The special position of the Sepiolidae, a comparison of the orders of recent cephalopods].

Gilly, W.F. and M.T. Lucero. 1992. Behavioral responses to chemical stimulation of the olfactory organ in the squid *Loligo opalescens*. J. exp. Biol., **162**: 209–229.

Grimpe, G. 1931. Teuthologische Mitteilungen XIII. Über die Cephalopoden der Sunda-Expedition Rensch. Zool. Anz., **95**: 149–174. [Teuthological notes XIII. On the cephalopods collected by the Sunda expedition Rensch; pp. 165–174: new subspecies *Idiosepius pygmaeus hebereri*, in addition to *I. pygmaeus pygmaeus* and *I. pygmaeus paradoxus*, with remarks on *I. (Naefidium) picteti* Joubin and *I. (Berryidium) notoides* Berry].

Hylleberg, J. and A. Nateewathana. 1991a. Redescription of *Idiosepius pygmaeus* Steenstrup, 1881 (Cephalopoda: Idiosepiidae), with mention of additional morphological characters. Phuket mar. biol. Cent. Res. Bull. **55**: 33–42. [Aspect of gladius in sagittal and cross sections].

Hylleberg, J. and A. Nateewathana. 1991b. Morphology, internal anatomy, and biometrics of the cephalopod *Idiosepius biserialis* Voss, 1962. A new record for the Andaman Sea. Phuket mar. biol. Cent. Res. Bull. **56**: 1–9.

Jackson, G.D. 1988. The use of statolith microstructures to analyse life-history events in the small tropical cephalopod *Idiosepius pygmaeus*. Fish. Bull. **87**: 265–272.

Jackson, G. D. 1992. Seasonal abundance of the small tropical sepioid *Idiosepius pygmaeus* (Cephalopoda: Idiosepiidae) at two localities off Townsville, North Queensland, Australia. Veliger. **35**: 396–397.

Jackson, G.D. 1993. Seasonal variation in reproductive investment in the tropical loliginid squid *Loligo chinensis* and the small tropical sepioid *Idiosepius pygmaeus*. Fish. Bull. **91**: 260–270.

Jackson, G.D. 1994. Application and future potential of statolith increment analysis in squids and sepioids. Can. J. Fish. Aquat. Sci. **51**: 2612–2625.

Jivaluk, J., J. Nabhitabhata and A. Nateewathana. 2003. Description of hatchling of Thai pygmy squid, *Idiosepius thailandicus*, Chotiyaputta, Okutani and Chaitiamvong, 1991. CIAC2003: Biology, Recruitment and Culture of Cephalopods (17–21 February 2003). Programme and Abstracts: p. 40.

Joubin, L. 1894. Céphalopodes d'Amboine. Rev. Suisse Zool. **2**: 23–64. [Cephalopods from Ambon; pp. 60-64, plates III, IV : *Loligo picteti* n. sp. = *Idiosepius picteti*].

Joubin, L. 1902. Révision des Sepiolidae. Mém. Soc. Zool. Fr. **15**: 80–145. [Revision of the Sepiolidae ; pp. 105, 106: *Microteuthis paradoxa*].

Kasugai, T. 2000. Reproductive behavior of the pygmy cuttlefish *Idiosepius paradoxus* in an aquarium. Venus (Jap. Jour. Malac.), **59**(1): 37–44.

Kasugai, T. 2001. Feeding behaviour of the Japanese pygmy cuttlefish *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) in captivity: evidence for external digestion? J. Mar. Biol. Ass. U. K. **81**: 979–981.

Kasugai, T. and Y. Ikeda. 2003. Description of the egg mass of the pygmy cuttlefish, *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae), with special reference to its multiple gelatinous layers. Veliger. **46**: 105–110.

Kasugai, T. and S. Segawa. 2003. Life cycle of the Japanese pygmy cuttlefish *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) in the *Zostera* bed at the temperate coast of central Honshu, Japan. CIAC2003: Biology, Recruitment and Culture of Cephalopods (17–21 February 2003). Programme and Abstracts: p. 45.

Lee, P.G., F.P. DiMarco and R.T. Hanlon. 1994. Chemoreception and feeding behavior in cephalopods (Abstract). CIAC International Conference on "The Behaviour and Natural History of Cephalopods", Vico Equense (Napoli) Italy, 5–11 June 1994.

Lewis, A.R. and J.H. Choat. 1993. Spawning mode and reproductive output of the tropical cephalopod *Idiosepius pygmaeus*. Can. J. Fish. Aquat. Sci. **50**: 20–28.

Lu, C.C., A. Guerra, F. Palumbo and W.C. Summers. 1992. Order SEPIOIDEA Naef, 1916. **In**: M. Sweeney, *et al*. (eds.). "Larval" and Juvenile Cephalopods: A Manual for Their Identification Smiths. Contr. Zool. **513**: 21–36.

Moynihan, M. 1983. Notes on the behaviour of *Idiosepius pygmaeus* (Cephalopoda: Idiosepiidae). Behaviour, **85**: 42–57.

Nabhitabhata, J. 1994a. Rearing of Thai Pygmy Cuttlefish, Idiosepius thailandicus Chot., Okut. and Chai., I: Some Biological Aspects. Technical Paper No. 23/1994, Rayong Coastal Aquaculture Station. 19pp. (In Thai, with English Abstract).

Nabhitabhata, J. 1994b. Rearing of Thai Pygmy Cuttlefish, Idiosepius thailandicus Chot., Okut. and Chai., II: Mating and Spawning Behavior. Technical Paper No. 25/1994, Rayong Coastal Aquaculture Station. 19pp. (In Thai, with English Abstract).

Nabhitabhata, J. 1998. Distinctive behaviour of Thai pygmy squid, *Idiosepius thailandicus* Chotiyaputta, Okutani and Chaitiamvong, 1991. Phuket Mar. Biol. Center Spec. Publ., **18**: 25–40.

Naef, A. 1923. Die Cephalopoden (Systematik). Fauna Flora Golf. Napoli. 35th monogr. (I-1), 863 pp. [The Cephalopoda (Systematics); English translation available from the Smithsonian Institution Libraries, Washington, D. C., USA].

Natsukari, Y. 1970. Egg-laying behavior, embryonic development and hatched larva of the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann. Bull. Fac. Fish. Nagasaki Univ., **30**: 15–29.

Nesis, K.N. 1987. Cephalopods of the world. T. F. J. Publications. 351 pp.

Nesis, K.N., O.N. Katugin and A.V. Ratnikov. 2003a. Pygmy cuttlefish *Idiosepius paradoxus* (Ortmann, 1888)(Cephalopoda) - first record of Idiosepiidae in Russian seas. Ruthenica, **12**(1): 81–84.

Nesis, K.N., O.N. Katugin and A.V. Ratnikov. 2003b. Pygmy cuttlefish *Idiosepius paradoxus* (Ortmann, 1888)(Cephalopoda) - first record of Idiosepiidae in Russian seas. CIAC2003: Biology, Recruitment and Culture of Cephalopods (17–21 February 2003). Programme and Abstracts: p. 65.

Norman, M. 2000 Cephalopods - A World Guide. ConchBooks, 320 pp.

Norman, M. and A. Reid. 2000. A Guide to Squid, Cuttlefish and Octopuses of Australasia. CSIRO Publishing, 96 pp.

Ortmann, A.S. 1888. Japanische Cephalopoden. Zool. Jahrb., Abt. Syst. **3**: 639–670. [Japanese cephalopods ; pp. 648, 649, plate XII : *Microteuthis paradoxa*].

Pecl, G.T. 1994. Muscle structure and dynamics of *Idiosepius pygmaeus*, a small tropical sepioid. B. Sc. Thesis, Dept. Mar. Biol., James Cook Univ. North Queensland. 95 pp.

Pecl, G.T. and N.A. Moltschaniwskyi. 1997. Changes in muscle structure associated with somatic growth in *Idiosepius pygmaeus*, a small tropical cephalopod. J. Zool., Lond., **242**(4): 751–764.

Pecl, G.T. and N. A. Moltschaniwskyj. 2003. The cephalopod mantle as an energy store: using the 'mini-maximalist' *Idiosepius* as a model. CIAC2003: Biology, Recruitment and Culture of Cephalopods (17–21 February 2003). Programme and Abstracts: p. 71.

Pruvot-Fol, A. 1926. Le bulbe buccal et la symétrie des Mollusques. - I. La radula. Thèse d'Etat Fac. Sci. Univ. Paris. [The buccal mass of molluscs. - I. The radula. Doctoral dissertation].

Roberts, D.A. 1997. Factors affecting the predatory behaviour of *Idiosepius pygmaeus*. B. Sc. Thesis, Dept. Mar. Biol., James Cook Univ. North Queensland. 76 pp.

Sasaki, M. 1923. On an adhering habit of a pygmy cuttlefish, *Idiosepius pygmaeus* Steenstrup. Annot. Zool. Japonens., **10**(21): 209–213. [Observations dealing with *Idiosepius paradoxus*]

Sasaki, M. 1929. A monograph of the dibranchiate Cephalopods of the Japanese and adjacent waters. J. Coll. Agricult., Hokkaido Imperial Univ. **20**: 1–357 [pp. 132–134: *Idiosepius paradoxa*].

Semmens, J.M. 1993. Structure and function of the cephalopod digestive gland and experimental study on *Idiosepius pygmaeus* (Family Idiosepiidae). B. Sc. Thesis, Dept. Mar. Biol., James Cook Univ. North Queensland. 80 pp.

Semmens, J.M., Moltschaniwskyj, N. A. and C. G. Alexander. 1995. Effect of feeding on the structure of the digestive gland of the tropical sepioid *Idiosepius pygmaeus*. J. mar. biol. Ass. U. K. **75**: 885–897.

Shigeno, S. and M. Yamamoto. 2002. Organization of the nervous system in the pygmy cuttlefish, *Idiosepius paradoxus* Ortmann (Idiosepiidae, Cephalopoda). J. Morph., **254**: 65–80.

Shigeno, S. and M. Yamamoto. 2002. A useful marker for the study of neural development in cephalopods. Veliger, **45**: 171–172.

Steenstrup, J. 1881. *Sepiadarium* og *Idiosepius* to nye Slaegter af Sepiernes Familie. Med Bemaekninger om de to beslaegtede Former *Sepioloidea* D'Orb. og *Spirula* Lmk. Vidensk. Selsk. Skr., 6. Raekke, naturvidenskabelig og mathematisk Afd. I. **3**: 235–242, Tab. I [*Sepiadarium* and *Idiosepius*, two new genera of the family of Sepia. With remarks on the two related forms *Sepioloidea* d'Orb. And *Spirula* Lmk.; English translation in: "The cephalopod papers of Japetus Steenstrup" by Volsoe, A., Knudsen, J. and Rees, W., 1962, Danish Science Press Ltd.].

Tracey, S., Steer, M. and G. Pecl. 2003. Life history traits of the temperate mini-maximalist *Idiosepius notoides*. CIAC2003: Biology, Recruitment and Culture of Cephalopods (17–21 February 2003). Programme and Abstracts: p. 111.

Tsuchiya, K., N. Yamamoto and H. Abe. 2002. Cephalopods in Japanese Waters. TBS-Britannica Co., Ltd., Meguro-ku, Tokyo. 141 pp.

van Camp, L.M. 1997. The effects of temperature and food availability on growth and reproductive output: using a cephalopod model. B. Sc. Thesis, Dept. Mar. Biol., James Cook Univ. North Queensland. 81 pp.

Voss, G.L. 1962. South African cephalopods. Trans. Roy. Soc. S. Afr. **36**: 245-272. [pp. 258–261: description of new species *Idiosepius biserialis*, *I. macrocheir*].

Voss, G.L. 1963. Cephalopods of the Philippine Islands. U. S. National Mus. Bull. **234**: 1–180. [pp. 63–67: *Idiosepius pygmaeus*].

Yamamoto, M. 1988. Normal embryonic stages of the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann. Zool. Sci. **5**: 989–998.

Yamamoto, M., Y. Shimazaki and S. Shigeno. 2003. Atlas of the embryonic brain in the pygmy squid, *Idiosepius paradoxus*. Zool. Sci. **20**: 163–179.

Evidence for a clade composed of molluscs with serially repeated structures: Monoplacophorans are related to chitons

Gonzalo Giribet*†, Akiko Okusu*, Annie R. Lindgren‡§, Stephanie W. Huff*, Michael Schrödl¶, and Michele K. Nishiguchi‡

*Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 16 Divinity Avenue, BioLabs 1119, Cambridge, MA 02138; ‡Department of Biology, New Mexico State University, P.O. Box 30001, Las Cruces, NM 88003; and ¶Zoologische Staatssammlung München, Münchhausenstrasse 21, 81247 München, Germany

Communicated by James W. Valentine, University of California, Berkeley, CA, April 3, 2006 (received for review December 5, 2005)

Monoplacophorans are among the rarest members of the phylum Mollusca. Previously only known from fossils since the Cambrian, the first living monoplacophoran was discovered during the famous second *Galathea* deep-sea expedition. The anatomy of these molluscs shocked the zoological community for presenting serially repeated gills, nephridia, and eight sets of dorsoventral pedal retractor muscles. Seriality of organs in supposedly independent molluscan lineages, i.e., in chitons and the deep-sea living fossil monoplacophorans, was assumed to be a relict of ancestral molluscan segmentation and was commonly accepted to support a direct relationship with annelids. We were able to obtain one specimen of a monoplacophoran Antarctic deep-sea species for molecular study. The first molecular data on monoplacophorans, analyzed together with the largest data set of molluscs ever assembled, clearly illustrate that monoplacophorans and chitons form a clade. This ''Serialia'' concept may revolutionize molluscan systematics and may have important implications for metazoan evolution as it allows for new interpretations for primitive segmentation in molluscs.

Antarctica | deep sea | Mollusca | Monoplacophora | phylogeny

Molluscs (snails, slugs, clams, mussels, squids, octopuses, chitons, etc.) exhibit the largest disparity of all animal phyla and rank second behind arthropods in species diversity. Although the majority of species still remain in the oceans, where they inhabit all types of ecosystems from the upper littoral to the abyss, they are also major components of freshwater and terrestrial habitats. Molluscan diversity can be extraordinary in tropical and temperate regions (1) but can be found at all latitudes.

The phylogenetic position of molluscs within Spiralia is supported by the presence of spiral cleavage and a trochophore larva (2, 3), although their immediate sister group remains uncertain. Although some have proposed a relationship to sipunculans (peanut worms) (4) or entoprocts (5), most researchers still consider molluscs closely related to annelids, in part because of the assumption that they retain traces of segmentation (3). The removal of arthropods and their relatives from the clade Spiralia (6) and the evolutionary importance given to segmentation in annelids have contributed to reengaging the debate about ancestral segmentation in other spiralian clades such as molluscs. This supposed segmentation in molluscs is often justified by the presence of eight sets of pedal retractor muscles and serially repeated gills in both chitons (Polyplacophora) (7) and members of the living fossil class Monoplacophora (8–10), based on the assumption that both groups are basal within their distinct lineages. Certain bivalves also exhibit multiple pedal retractor muscles (11), and caudofoveate larvae show seven transverse rows of calcareous spicules on the dorsal side (3).

Monoplacophorans are perhaps the least known members of the phylum Mollusca. They have been thought to be "primitive" forms based on their rich fossil record, which dates back to Cambrian–Devonian periods (8). After the recent discovery of the first living monoplacophoran, *Neopilina galatheae*, during the second Danish *Galathea* expedition (8), it was suggested that its dorsal uncoiled cap-like shell (Fig. 1) fit the prevalent *HAM* (hypothetical ancestor mollusc) theories (12). This idea positioned monoplacophorans at the base of "Conchifera," a clade that includes all molluscs with a true dorsal shell (the classes Monoplacophora, Gastropoda, Cephalopoda, Bivalvia, and Scaphopoda). *Neopilina*'s newly discovered anatomy [with serially repeated gills and eight sets of dorsoventral pedal retractor muscles, as those found in chitons, and serially repeated nephridia (8, 10)] suggested that serial homology was present at least in two extant molluscan lineages, Aculifera (molluscs with spicules) and Conchifera (molluscs with a true shell).

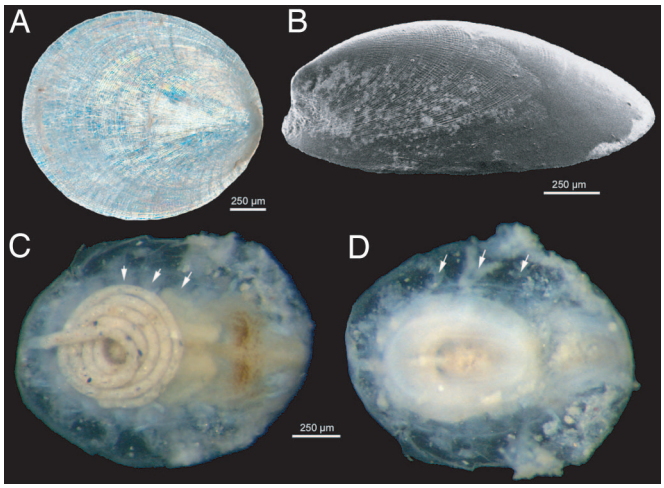


Fig. 1. Details of *L. antarctica* Warén & Hain, 1992. (*A*) Shell, dorsal view. Note the limpet-like shape with anterior apex and light reflection caused by prismatic and inner nacreous layers. (*B*) Scanning electron micrograph of the shell (dorsolateral view from left side). (*C*) Soft body (shell removed) (dorsal view). Note the characteristic spiral intestine (left) filled with mineral particles, brown-dotted esophageal pouches (right), and serial shell muscles (arrows). (*D*) Soft body, ventral view. Note the round sucker-like foot (central), serial gills (arrows), and mouth area with tentacles (right).

Although a generalized mollusc is portrayed as a limpet-like form with a creeping foot and a dorsal shell made of calcium carbonate

Conflict of interest statement: No conflicts declared.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ279932–DQ280054).

†To whom correspondence should be addressed. E-mail: ggiribet@oeb.harvard.edu.

§Present address: Department of Evolution, Ecology, and Organismal Biology, Ohio State University, 1315 Kinnear Road, Columbus, OH 43215.

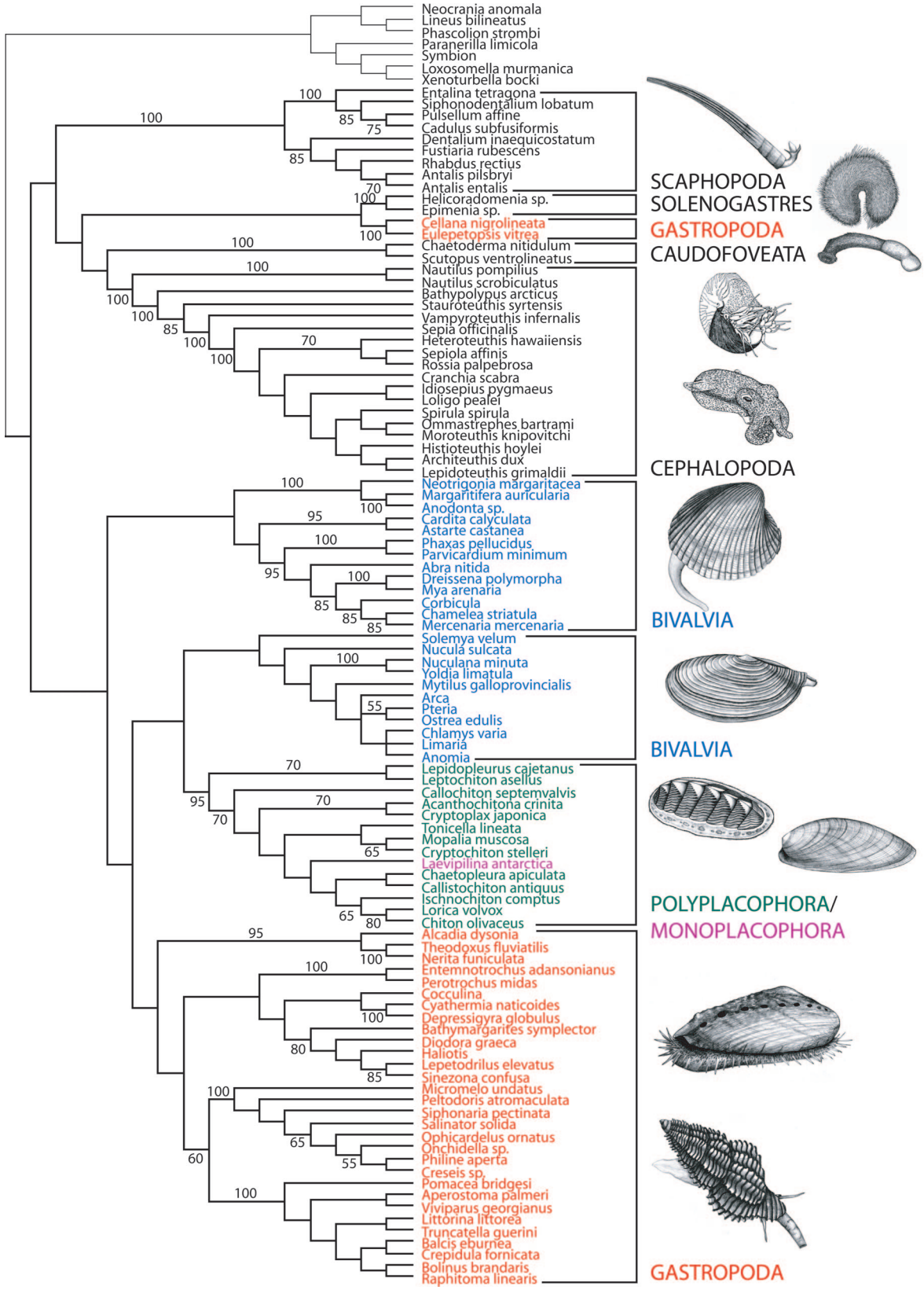


Fig. 2. Phylogenetic tree depicting the relationships of Monoplacophora to other molluscs based on the combined analysis of all molecular loci. Shown is strict consensus of two most parsimonious trees at 64,679 weighted steps (gap opening cost of 3, gap extension cost of 1, all base transformations cost 2) for the analysis of all data under direct optimization with tree fusing. Numbers on branches indicate jackknife support values. Gastropods (in red) and bivalves (in blue) appear diphyletic. Polyplacophora and Monoplacophora form a well supported clade (95% jackknife support). The monoplacophoran species (purple) appears nested within chitons (dark green), but nodal support for its exact position is low. The tree shows monophyly of molluscs, as well as that of Scaphopoda, Cephalopoda, Caudofoveata, and Solenogastres.

(as in the class Monoplacophora), other body plans such as those of the worm-like, shell-less fossorial chaetodermomorphs (class Caudofoveata) and neomeniomorphs (class Solenogastres), or the bentho-pelagic cephalopods (class Cephalopoda) differ radically from this prototype. Mussels, clams and their kind (class Bivalvia) are also quite divergent from this model. Furthermore, modern chitons (class Polyplacophora) have a distinct dorsal "shell" formed by eight interlocking plates. In fact, the disparity of mollusc body plans is so great that it is quite difficult to find a single trait shared by all seven classes of molluscs (13).

Our understanding of relationships among the major molluscan lineages is still in its infancy. Recent attempts to resolve their relationships by using morphological data found limitations in character homology definitions and polarization because of uncertainty regarding the molluscan sister group (4, 5, 14). Molecular attempts have not been conclusive, but they have aided to refute the "Diasoma" hypothesis (a clade uniting bivalves and scaphopods). Most recent molecular analyses suggest a relationship of scaphopods to cephalopods and gastropods (15–17), further corroborated through morphological and developmental studies (5, 18). To date, the phylogenetic position of monoplacophorans remained untested using molecular data because of difficulties in collecting live samples of these enigmatic animals.

Results and Discussion

An Antarctic Benthic Deep-Sea Biodiversity oceanographic campaign (ANDEEP III) with the RV *Polarstern* to the Weddell Sea (Antarctica), 3 km southwest of Wegener Canyon at ≈3,100-m depth, yielded one small specimen (1.7-mm shell length) of the monoplacophoran *Laevipilina antarctica* Warén & Hain, 1992 (19), one of the 26 known species of this group of molluscs (9, 20). The single specimen was obtained from an epibenthic sledge sample that had been fixed with precooled 96% EtOH for molecular studies and stored at −20°C for 48 h. The shell (ZSM Moll 20050866; Fig. 1 *A* and *B*) was removed for gross anatomy and SEM examination, the soft body was photographed (Fig. 1 *C* and *D*), and half of the specimen was used for molecular work.

Although monoplacophoran DNA was highly degraded, perhaps because of bulk fixation of the sediment performed in the vessel, we were able to amplify and sequence a 1.2-kb fragment of the large nuclear ribosomal subunit (28S rRNA). This gene has proven to be highly informative in recent studies on metazoan and molluscan evolution (17, 21).

Analysis of the data using a single-step phylogenetic approach with direct optimization (Fig. 2) and a two-step approach using Bayesian phylogenetics (Fig. 4, which is published as supporting information on the PNAS web site) exhibited congruent results suggesting monophyly of molluscs as well as that of the molluscan classes Caudofoveata, Solenogastres, Scaphopoda, and Cephalopoda. Resolution with high jackknife support is found mostly within the main clades of Scaphopoda (Dentaliida and Gadilida), Cephalopoda (Nautiloidea, Coleoidea, and the sister group relationship of the vampire squid to decabrachians, which include the giant squid *Architeuthis dux* and the pygmy squid *Idiosepius pygmaeus*), as well as within Bivalvia (Palaeoheterodonta and Euheterodonta) and Gastropoda (Patellogastropoda, Neritopsina, Caenogastropoda, and Heterobranchia). However, the available sequence data do not recover monophyly of Gastropoda or Bivalvia, which are both diphyletic, with patellogastropods separated from the other gastropods and heteroconchs separated from the remainder of the bivalves (protobranchs and pteriomorphians).

Nodal support for interclass relationships or for the relationships of the two clades of bivalves and gastropods is low in general, but a clade containing Monoplacophora and Polyplacophora received strong nodal support (90–100% jackknife support value depending on the analysis, as well as 1.0 posterior probability). Interestingly, this clade, which we name "Serialia," contains the two classes whose members present a variable number of serially repeated gills and eight sets of dorsoventral pedal retractor muscles. This result clearly contrasts with previous cladistic hypotheses suggesting that Monoplacophora constitute the sister group to the remainder of the conchiferans (4, 5, 14), those molluscs with a true shell unlike that of chitons or the vermiform aplacophorans, although it finds no clear support for the exact position of Serialia. To our knowledge, this is also the first published analysis that demonstrates monophyly of the phylum Mollusca using a range of appropriate outgroups, but we caution the reader to consider that jackknife support for molluscan monophyly is low. The results further support a previous study (22) that indicates that *Xenoturbella* is not a bivalve mollusc.

Fig. 3. Alignment of one of the regions of 28S rRNA illustrating that *L. antarctica* does not share unique chiton synapomorphies (asterisks).

All analyses (including different optimality criteria and alternative models of indel and base substitutions) support a Polyplacophora plus Monoplacophora clade. However, *L. antarctica* appears nested within the chiton tree in some analyses, a result that may look suspicious at first. Evidence for including Monoplacophora within Polyplacophora is restricted to one node, which groups nonlepidopleurid chitons with the monoplacophoran species (70% jackknife support; Fig. 1), but this is not the case when considering only the 1.2-kb region of 28S rRNA amplified for *Laevipilina* (tree not shown). Furthermore, detailed examination of the DNA sequences clearly illustrates that chitons share unambiguous positions in the alignment not found in *L. antarctica* (Fig. 3). This fact eliminates the possibility of contaminant DNA in our analysis.

Evidence for a clade of serialian molluscs is important for our current understanding of molluscan relationships and may have implications for deeper metazoan evolution. This new evidence may imply that serially repeated structures (e.g., gills and pedal retractor muscles in both monoplacophorans and chitons) are not primitive for molluscs, as was previously thought (9). However, it is fair to mention that additional types of serial repetition of dorsoventral musculature have been reported in other molluscan groups (23), including the eight sets of pedal retractors of the Ordovician lucinoid bivalve *Babinka* (11), the serially repeated rows of spicules in caudofoveate larvae (3), or the two pairs of gills and nephridia in cephalopods (3). Whether these represent true seriality or not may have profound implications in reconstructing the molluscan common ancestor, but it does not contradict the evidence of our Serialia clade.

The classical hypothesis for the position of monoplacophorans as basal conchiferans relies heavily on the presence of a true dorsal shell with similar mineralogical composition to that of many basal members of each conchiferan class. However, the mode of shell deposition by the mantle edge and the microstructure and composition of the chitinous organic layer in monoplacophorans differ from those of higher conchiferans or polyplacophorans (9, 24, 25), which makes monoplacophorans apomorphic (derived) in the form of shell deposition. The rejection of conchiferan monophyly based on shell deposition would be consistent with our findings, which suggest that serial repetition of anatomical structures such as gills and muscles may have evolved once in the common ancestor of chitons and monoplacophorans. Therefore, serial repetition of these structures could constitute a derived feature that would not

EVOLUTION

Table 1. Taxon sampling and GenBank accession numbers employed in this study

Phylum/class	Species	GenBank accession nos. 18S rRNA	28S rRNA	H3	COI	16S rRNA
Nemertea	*Lineus bilineatus*	DQ279932	DQ279947	DQ279996	DQ280014	DQ280022
Annelida	*Paranerilla limicola*	DQ279933	DQ279948			DQ280023
Brachiopoda	*Neocrania anomala*	DQ279934	DQ279949	DQ279997		DQ280024
Entoprocta	*Loxosomella murmanica*	AY218100	DQ279950	AY218150		
Xenoturbellida	*Xenoturbella bocki*	AY291292	DQ279951			
Sipuncula	*Phascolion strombi*	DQ299984	AY210468	DQ279998		
Cycliophora	*Symbion americanus*	AY218107	AY210472	AY218153	AY218085	DQ280025
Mollusca						
Caudofoveata	*Chaetoderma nitidulum*	AY377658	AY145397	AY377763	AY377726	AY377612
	Scutopus ventrolineatus	X91977				
Solenogastres	*Helicoradomenia* sp.	AY21210		AY377764	AY377725	AY377613
	Epimenia cinerea	AY377657	AY377691	AY377765	AY377723	AY377615
Polyplacophora	*Lepidopleurus cajetanus*	AF120502	AF120565	AY377735	AF120626	AY377585
	Leptochiton asellus	AY377631	AY145414	AY377734		AY377586
	Callochiton septemvalvis	AY377632	DQ279952	AY377736	AY377700	
	Chaetopleura apiculata	AY377636	AY145398	AY377741	AY377704	AY377590
	Ischnochiton comptus	AY377639	AY145412	AY377744	AY377709	AY377593
	Callistochiton antiquus	AY377645	DQ279953	AY377749	AY377712	AY377599
	Lorica volvox	AY377647	DQ279954	AY377751		AY377601
	Chiton olivaceus	AY377651	DQ279955	AY377755	AY377716	AY377605
	Mopalia muscosa	AY377648	DQ279956	AY377752	AY377713	AY377602
	Tonicella lineata	AY377635	AY377665	AY377739	AY377702	AY377588
	Acanthochitona crinita	AF120503	DQ279957	AY377759	AF120627	AY377609
	Cryptochiton stelleri	AY377655	AY377686	AY377760	AY377720	AY377610
	Cryptoplax japonica	AY377656	AY145402	AY377761		AY377611
Monoplacophora	*Laevipilina antarctica*		DQ279958			
Scaphopoda	*Dentalium inaequicostatum*	DQ279935	DQ279959	DQ279999	DQ280015	DQ280026
	Rhabdus rectius	AF120523	AF120580	AY377772	AF120640	AY377619
	Antalis pilsbryi	AF120522	AF120579		AF120639	
	Antalis entalis	DQ279936	AY145388	DQ280000	DQ280016	DQ280027
	Fustiaria rubescens	AF490597				
	Entalina tetragona	AF490598				
	Pulsellum affine	AF490600				
	Siphonodentalium lobatum	AF490601				
	Cadulus subfusiformis	AF490603				
Bivalvia	*Solemya velum*	AF120524	AY145421	AY070146	U56852	DQ280028
	Nucula sulcata	DQ279937	DQ279960	DQ280001	DQ280017	DQ280029
	Nuculana minuta	DQ279938	DQ279961	DQ280002	DQ280018	DQ280030
	Yoldia limatula	AF120528	AY145424	AY070149	AF120642	
	Mytilus galloprovincialis	L33452	AB103129	AY267748	AY497292	AY497292
	Arca imbricata/A. ventricosa	AY654986	AB101612	AY654989	AY654988	
	Pteria hirundo/P. loveni	AF120532	AB102767 AF137047/		AF120647	DQ280031
	Ostrea edulis	L49052	AF120596	AY070151	AF120651	DQ280032
	Limaria hians/L. fragilis	AF120534	AB102742	AY070152	AF120650	
	Anomia ephippium/A. sinensis	AF120535	AB102739			
	Chlamys varia	DQ279939	DQ279962	DQ280003		DQ280033
	Neotrigonia margaritacea	AF411690	DQ279963	AY070155	U56850	DQ280034
	Margaritifera auricularia	AY579097	AY579113	AY579137	AY579125	DQ280035
	Anodonta sp.	AY579090	DQ279964	AY579132	AY579122	
	Cardita calyculata	AF120549	AF120610	AY070156	AF120660	
	Astarte castanea	AF120551	AF131001	DQ280004	AF120662	
	Abra nitida	DQ279940	DQ279965	DQ280005		
	Phaxas pellucidus	DQ279941	AY145420	DQ280006	DQ280019	DQ280036
	Parvicardium minimum	DQ279942	DQ279966	DQ280007		DQ280037

support the hypothesis of a segmented ancestral mollusc. Again, other interpretations may exist if the pedal scars of *Bibankia* were the result of muscles homologous to the serialian dorsoventral pedal muscles.

Molluscs are undoubtedly one of the animal phyla with the largest disparity. Numerous Cambrian forms such as *Wiwaxia* and *Halkieria* or the Silurian *Acaenoplax* have been more or less ambiguously assigned to this animal phylum (26–28). *Kimberella* is another putative mollusc extending the age of the group back to the Neoproterozoic (29). Although chitons were once thought to have changed little since their first appearance in the Late Cambrian period (30), recent discoveries of articulated polyplacophorans and multiplacophorans from the Ordovician to the Carboniferous (31, 32) suggest that a much larger disparity evolved during the Paleozoic. Perhaps such an episode of diversification is responsible for the two modern anatomies of molluscs with conspicuous serial repetition of organs, but no explanation for their divergent evolution of shell morphologies can be provided at this point. Recognition of a serialian clade comprised of chitons and monoplacophorans broadens our perspective toward new interpretations of molluscan anatomy and once more questions preconceived ideas on molluscan relationships that rely almost entirely on shell morphology.

Here we provide the first molecular test for the phylogenetic position of Monoplacophora by using sequence data from a deep-

Table 1. (continued)

Phylum/class	Species	GenBank accession nos. 18S rRNA	28S rRNA	H3	COI	16S rRNA
	Dreissena polymorpha	AF120552	AF131006	AY070165	AF120663	DQ280038
	Corbicula fluminea/C. japonica	AF120557	AB126330	AY070161	AF120666	DQ280039
	Mercenaria mercenaria	AF120559	AF131019	DQ280008	AF120668	DQ280040
	Chamelea striatula	DQ279943	DQ279967	DQ280009		DQ280041
	Mya arenaria	AF120560	AB126332	AY377770	AY070140	AY377618
Cephalopoda	*Nautilus pompilius*	AY557452	AY145417		AY557514	AY377628
	Nautilus scrobiculatus	AF120504	AF120567	AF033704		U11606
	Stauroteuthis syrtensis	AY557457	DQ279968	AY557406	AF000067	DQ280042
	Vampyroteuthis infernalis	AY557459	AH012197	AY557408	AF000071	DQ280043
	Bathypolypus arcticus	AY557465	AY557554		AF000029	DQ280044
	Sepia officinalis	AY557471	AY557560	AY557415	AF000062	DQ093491
	Sepiola affinis	AY557474	AY557562	AY557418	AY557523	AY293667
	Heteroteuthis hawaiiensis	AY557472	DQ279969	AY557416	AF000044	AY293680
	Rossia palpebrosa	AY557473	AY557561	AY557417	AF000061	DQ280045
	Spirula spirula	AY557476	AY557563	AY557420	AY293709	AY293659
	Idiosepius pygmaeus	AY557477	AY293684	AY557421	AY293708	AY293658
	Loligo pealei	AT557479	AH012196	AY557423	AF120629	AF110079
	Architeuthis dux	AY557482	DQ279970	AY557426	AF000027	AY377629
	Cranchia scabra	AY557487	AY557571	AY557430	AF000035	DQ280046
	Histioteuthis hoylei	AY557500	AY557584	AY557442	AF000045	DQ280047
	Lepidoteuthis grimaldii	AY577503	AY557587	AY557445	AF000049	DQ280048
	Ommastrephes bartrami	AY557510	AY557594	AY557451	AF000057	DQ280049
	Moroteuthis knipovitchi	AY557512	AY557596	AY557453	AY557543	DQ280050
Gastropoda	*Cellana* sp.	DQ093425	DQ279971	DQ093493	DQ093515	DQ093467
	Eulepetopsis vitrea	DQ093427	DQ279972	DQ093495	DQ093516	DQ093468
	Cocculina messingi/Cocculina sp.	AF120508	DQ279973	AY377777	AY377731	AY377624
	Alcadia dysonia	DQ093428	DQ279974	DQ093496		DQ093469
	Theodoxus fluviatilis	AF120515	DQ279975		AF120633	DQ093470
	Nerita funiculata	DQ093429	DQ279976	DQ093497	DQ093517	DQ093471
	Cyathermia naticoides	DQ093430	DQ279977	DQ093498	DQ093518	DQ093472
	Depressigyra globulus	DQ093431	DQ279978	DQ093499	DQ093519	DQ093473
	Perotrochus midas	AF120510	DQ093453	DQ093500	AY296820	DQ093474
	Entemnotrochus adansonianus	AF120509	DQ279979	AY377774		AY377621
	Lepetodrilus elevatus	DQ093432	AY145413	DQ093501	DQ093520	DQ093475
	Diodora graeca	AF120513	DQ279980	DQ093502	AF120632	DQ093476
	Haliotis tuberculata/H. discus	AF120511	AY145418	AY070145	AY377729	AY377622
	Sinezona confusa	AF120512	DQ279981	AY377773	AF120631	
	Bathymargarites symplector	DQ093433	DQ279982	DQ093503	DQ093521	DQ093477
	Aperostoma palmeri	DQ093435	DQ279983	DQ093505	DQ093523	DQ093479
	Pomacea bridgesi	DQ093436	DQ279984	DQ093506	DQ093524	DQ093480
	Viviparus georginaus	AF120516	AF120574	AY377779	AF120634	AY377626
	Balcis eburnea	AF120519	AF120576		AF120636	DQ280051
	Crepidula fornicata	AY377660	AY145406	AY377778	AF353154	AY377625
	Littorina littorea	DQ093437	DQ279985	DQ093507	DQ093525	DQ093481
	Truncatella guerini	AF120518	AF120575		AF120635	
	Bolinus brandaris	DQ279944	DQ279986	DQ280010	DQ280020	DQ280052
	Raphitoma linearis	DQ279945	DQ279987	DQ280011		DQ280053
	Philine aperta	DQ093438	DQ279988	DQ093508		DQ093482
	Creseis sp.	DQ279946	DQ279989	DQ280012	DQ280021	
	Peltodoris atromaculata	AF120521	DQ279990	DQ280013	AF120637	DQ280054
	Salinator solida	DQ093440	DQ279991	DQ093510	DQ093528	DQ093484
	Onchidella sp.	DQ093441	DQ279992	DQ093511	DQ093529	DQ093485
	Siphonaria pectinata	X91973	DQ279993	AY377780	AF120638	AY377627
	Ophicardelus ornatus	DQ093442	DQ279994	DQ093512	DQ093530	DQ093486
	Micromelo undatus	DQ093443	DQ279995	DQ093513		DQ093487

sea monoplacophoran species from Antarctica. Contrary to all previously published accounts, which placed monoplacophorans as a sister group to higher, i.e., shelled, molluscs, our data strongly support a clade including Monoplacophora and Polyplacophora. This rather surprising result from a conchological perspective is congruent with soft anatomy data. It furthermore reopens the debate about the putative ancestral segmentation of molluscs (3), because serial repetition of gills and pedal retractor muscles may be derived and not primitive features within molluscs. If this were the case, little evidence would remain for the case of homology of segmentation in annelids and serial repetition in molluscs (33), as confirmed in part by recent reevaluation of their early development (34, 35).

Materials and Methods

Species Sampling. Taxon sampling was carefully designed following original and published work on the internal phylogeny of chitons, bivalves, cephalopods, gastropods, and scaphopods (15, 16, 36–38). Outgroups were selected among other spiralian protostomes (lophotrochozoans) (39). The enigmatic *Xenoturbella* was also included because it was once postulated to be a derived mollusc, although more recent data consider it to be an ancestral deuter-

ostome (22). In total, we analyzed 101 molluscs including 2 Caudofoveata, 2 Solenogastres, 13 Polyplacophora, 1 Monoplacophora, 9 Scaphopoda, 32 Gastropoda, 24 Bivalvia, and 18 Cephalopoda (see Table 1).

Molecular Data. Molecular data were obtained from ethanol-preserved specimens following standard protocols for molluscan samples (15, 37, 38, 40). Monoplacophoran DNA samples were extracted from the half specimen preserved in 96% EtOH. DNA from preserved tissues was extracted by using the Qiagen DNeasy tissue kit. Data include complete sequences of 18S rRNA, a 3-kb fragment of 28S rRNA, the protein-coding nuclear gene histone H3, and two mitochondrial gene fragments for cytochrome *c* oxidase subunit I and 16S rRNA, totaling ≈6.5 kb per complete taxon (see Table 1). The amplified samples were purified by using the QIAquick PCR purification kit (Qiagen), labeled by using BigDye Terminator 3.0 (Applied Biosystems), and sequenced with an ABI 3730 genetic analyzer (Applied Biosystems) following the manufacturer's protocols. Chromatograms obtained from the automatic sequencer were read, and "contig sequences" were assembled by using the editing software SEQUENCHER 4.0 and further manipulated in GDE 2.2 (41).

From the five different molecular loci chosen for this study, only one yielded positive amplification for the monoplacophoran specimen. This fragment corresponds to a 1.2-kb segment of 28S rRNA obtained by amplifying two overlapping fragments using primer pairs 28Sa and 28S rd5b (5′-GACCCGTCTTGAAGCACG-3′ and 5′-CCACAGCGCCAGTTCTGCTTAC-3′) and 28S rd4.8a and 28S rd7b1 (5′-ACCTATTCTCAAACTTTAAATGG-3′ and 5′-GACTTCCCTTACCTACAT-3′).

Data Analyses. DNA sequence data were analyzed following two approaches. First, a dynamic homology approach ("single-step phylogenetics") using parsimony as an optimality criterion for direct optimization (42) was undertaken in the computer package POY 3.0.11 (43). Second, a static homology approach ("two-step phylogenetics") using a model-based approach was executed under Bayesian phylogenetics in MRBAYES 3.1.1 (44).

For the direct optimization analysis, tree searches were conducted by a combination of random addition sequences with multiple rounds of tree fusing (45) on a small 50-processor cluster assembled at Harvard University. Support measures were estimated by using jackknifing with a character probability of deletion of e^{-1} (46). The data were analyzed for all genes in combination as well as restricted to the 28S rRNA fragment sequenced for *L. antarctica* under different analytical parameter sets (47, 48).

Bayesian posterior probabilities were calculated by using a general time-reversible model with corrections for the proportion of invariant sites and a discrete gamma distribution, as selected in MODELTEST 3.7 (49) under the Akaike Information Criterion (50). Two runs of 10^6 generations were performed, storing 1/100th visited trees. Results from MRBAYES 3.1.1 were visualized in the program TRACER 1.3 (51), which served to determine the burnin, which differed considerably in the two runs. Aligned data were obtained from the implied alignment (52) generated in POY 3.0.11 for the analyses presented in Fig. 2.

We are indebted to the numerous colleagues who supplied tissue samples, without whom this work would not have been possible. Rebecca Budinoff assisted with laboratory work. Angelika Brandt organized the ANDEEP III expedition. Katrin Linse, Enrico Schwabe, and the onboard sorting team provided the monoplacophoran specimen. Greg Edgecombe, Andy Knoll, Sigurd von Boletzky, Claus Nielsen, Jim Valentine, and an anonymous reviewer provided comments that helped to improve this article. Enrico Schwabe provided pictures for Fig. 1 *A* and *B*. This material is based on work supported by the National Science Foundation Assembling the Tree of Life Program (Grant 0334932 to G.G.) and Population Biology Program (Grant 0316516 to M.K.N.). Field activities of M.S. and his team were supported by the GeoBioCenterLMU. This article is ANDEEP contribution no. 58.

1. Bouchet, P., Lozouet, P., Maestrati, P. & Heros, V. (2002) *Biol. J. Linn. Soc.* **75,** 421–436.
2. Valentine, J. W. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8001–8005.
3. Nielsen, C. (2001) *Animal Evolution: Interrelationships of the Living Phyla* (Oxford Univ. Press, Oxford), 2nd Ed.
4. Scheltema, A. H. (1993) *Biol. Bull.* **184,** 57–78.
5. Haszprunar, G. (2000) *Am. Malacol. Bull.* **15,** 115–130.
6. Aguinaldo, A. M. A., Turbeville, J. M., Lindford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. & Lake, J. A. (1997) *Nature* **387,** 489–493.
7. Eernisse, D. J. & Reynolds, P. D. (1994) in *Microscopic Anatomy of Invertebrates*, eds. Harrison, F. W. & Kohn, A. J. (Wiley-Liss, New York), Vol. 5, pp. 55–110.
8. Lemche, H. (1957) *Nature* **179,** 413–416.
9. Haszprunar, G. & Schaefer, K. (1997) in *Microscopic Anatomy of Invertebrates*, eds. Harrison, F. W. & Kohn, A. J. (Wiley-Liss, New York), Vol. 6B, pp. 415–157.
10. Lemche, H. & Wingstrand, K. G. (1959) *Galathea Rep.* **3,** 9–71.
11. McAlester, A. L. (1965) *Paleontology* **8,** 231–246.
12. Lindberg, D. R. & Ghiselin, M. T. (2003) *Proc. Calif. Acad. Sci.* **54,** 663–686.
13. Valentine, J. W. (2004) *On the Origin of Phyla* (Univ. of Chicago Press, Chicago).
14. Salvini-Plawen, L. V. & Steiner, G. (1996) in *Origin and Evolutionary Radiation of the Mollusca*, ed. Taylor, J. D. (Oxford Univ. Press, Oxford), pp. 29–51.
15. Giribet, G. & Wheeler, W. C. (2002) *Invertebr. Biol.* **121,** 271–324.
16. Steiner, G. & Dreyer, H. (2003) *Zool. Scripta* **32,** 343–356.
17. Passamaneck, Y. J., Schander, C. & Halanych, K. M. (2004) *Mol. Phylogenet. Evol.* **32,** 25–38.
18. Wanninger, A. & Haszprunar, G. (2002) *J. Morphol.* **254,** 53–64.
19. Schrödl, M., Linse, K. & Schwabe, E. (2006) *Polar Biol.*, in press.
20. Schrödl, M. (2006) *Spixiana*, in press.
21. Mallatt, J. & Winchell, C. J. (2002) *Mol. Biol. Evol.* **19,** 289–301.
22. Bourlat, S. J., Nielsen, C., Lockyer, A. E., Littlewood, D. T. & Telford, M. J. (2003) *Nature* **424,** 925–928.
23. Salvini-Plawen, L. V. (1972) *Z. Wiss. Zool. (Leipzig)* **184,** 205–394.
24. Schaefer, K. & Haszprunar, G. (1997) *Zool. Anz.* **236,** 13–23.
25. Haas, W. (1981) *Biominer. Res. Rep.* **6,** 1–52.
26. Scheltema, A. H., Kerth, K. & Kuzirian, A. M. (2003) *J. Morphol.* **257,** 219–245.
27. Vinther, J. & Nielsen, C. (2005) *Zool. Scripta* **34,** 81–89.
28. Sutton, M. D., Briggs, D. E. G., Siveter, D. J. & Siveter, D. J. (2004) *Palaeontology* **47,** 293–318.
29. Martin, M. W., Grazhdankin, D. V., Bowring, S. A., Evans, D. A., Fedonkin, M. A. & Kirschvink, J. L. (2000) *Science* **288,** 841–845.
30. Runnegar, B., Pojeta, J., Jr., Taylor, M. E. & Collins, D. (1979) *J. Paleontol.* **53,** 1374–1394.
31. Pojeta, J., Jr., Eernisse, D. J., Hoare, R. D. & Henderson, M. D. (2003) *J. Paleontol.* **77,** 646–654.
32. Vendrasco, M. J., Wood, T. E. & Runnegar, B. N. (2004) *Nature* **429,** 288–291.
33. Haszprunar, G. & Schaefer, K. (1996) *Acta Zool. (Stockholm)* **77,** 315–334.
34. Nielsen, C. (2004) *J. Exp. Zool. B* **302,** 35–68.
35. Wanninger, A. & Haszprunar, G. (2002) *J. Morphol.* **251,** 103–113.
36. Giribet, G. & Distel, D. L. (2003) in *Molecular Systematics and Phylogeography of Mollusks*, eds. Lydeard, C. & Lindberg, D. R. (Smithsonian Books, Washington, DC), pp. 45–90.
37. Okusu, A., Schwabe, E., Eernisse, D. J. & Giribet, G. (2003) *Org. Divers. Evol.* **3,** 281–302.
38. Lindgren, A. R., Giribet, G. & Nishiguchi, M. K. (2004) *Cladistics* **20,** 454–486.
39. Giribet, G. (2002) *Mol. Phylogenet. Evol.* **24,** 345–357.
40. Okusu, A. & Giribet, G. (2003) *J. Moll. Stud.* **69,** 385–387.
41. Linton, E. W. (2005) MACGDE: Genetic Data Environment for Mac OS X (Michigan State Univ., East Lansing).
42. Wheeler, W. C. (1996) *Cladistics* **12,** 1–9.
43. Wheeler, W. C., Gladstein, D. & De Laet, J. (2004) POY (Am. Museum of Natural History, New York), Version 3.0.
44. Ronquist, F. & Huelsenbeck, J. P. (2005) MRBAYES: Bayesian Analysis of Phylogeny (Florida State Univ., Tallahassee), Version 3.1.1.
45. Goloboff, P. A. (1999) *Cladistics* **15,** 415–428.
46. Farris, J. S., Albert, V. A., Källersjö, M., Lipscomb, D. & Kluge, A. G. (1996) *Cladistics* **12,** 99–124.
47. Wheeler, W. C. (1995) *Syst. Biol.* **44,** 321–331.
48. Giribet, G. (2003) *Syst. Biol.* **52,** 554–564.
49. Posada, D. (2005) MODELTEST (Univ. of Vigo, Vigo, Spain), Version 3.7.
50. Posada, D. & Buckley, T. (2004) *Syst. Biol.* **53,** 793–808.
51. Rambaut, A. & Drummond, A. (2003) TRACER: MCMC Trace Analysis Tool (University of Oxford, Oxford), Version 1.3.
52. Wheeler, W. C. (2003) *Cladistics* **19,** 261–268.

Molecular Ecology (2006) **15**, 4317–4329 doi: 10.1111/j.1365-294X.2006.03073.x

Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis

B. W. JONES, J. E. LOPEZ, J. HUTTENBURG and M. K. NISHIGUCHI
Department of Biology, MSC 3AF, New Mexico State University, PO Box 30001, Las Cruces, NM 88003-8001, USA

Abstract

Squids from the genus *Euprymna* (Cephalopoda: Sepiolidae) and their symbiotic bacteria *Vibrio fischeri* form a mutualism in which vibrios inhabit a complex light organ within the squid host. A host-mediated daily expulsion event seeds surrounding seawater with symbiotically capable *V. fischeri* that environmentally colonize newly hatched axenic *Euprymna* juveniles. Competition experiments using native and non-native *Vibrio* have shown that this expulsion/re-colonization phenomenon has led to cospeciation in this system in the Pacific Ocean; however, the genetic architecture of these symbiotic populations has not been determined. Using genetic diversity and nested clade analyses we have examined the variation and history of three allopatric *Euprymna* squid species (*E. scolopes* of Hawaii, *E. hyllebergi* of Thailand, and *E. tasmanica* from Australia) and their respective *Vibrio* symbionts. *Euprymna* populations appear to be very genetically distinct from each other, exhibiting little or no migration over large geographical distances. In contrast, *Vibrio* symbiont populations contain more diverse haplotypes, suggesting both host presence and unidentified factors facilitating long-distance migration structure in Pacific *Vibrio* populations. Findings from this study highlight the importance of how interactions between symbiotic organisms can unexpectedly shape population structure in phylogeographical studies.

Keywords: Cephalopoda, Indo-West Pacific, nested clade analysis, phylogeography, Sepiolidae, *Vibrio fischeri*

Received 7 March 2006; revision received 9 April 2006; accepted 15 April 2006

Introduction

Non-random mating, the precursor to genetic subdivision, can be selected by several biological (mating systems, dispersal methods, etc.) and/or abiotic factors (geological processes, distribution of suitable habitat; (Achmann *et al*. 2004; Cherry 2004; Marko 2004; Miller & Ayre 2004; Ovenden *et al*. 2004; Palo *et al*. 2004; Whiteley *et al*. 2004). Interestingly, while much work has been completed to identify co-evolving host/symbiont assemblages through studies of parallel cladogenesis (Munson *et al*. 1991, 1992; Brooks 1997; Nishiguchi *et al*. 1998; Nishiguchi 2001, 2002), few studies have examined how host population structure may dictate symbiont genetic distribution in these intimate associations. This being so, there is a need to better understand the influences of host population structure and abiotic factors affecting distribution between these populations on symbiont phylogeography and subsequently, speciation.

Correspondence: M. K. Nishiguchi, Fax: (505) 646–5665; E-mail: nish@nmsu.edu

The association between bobtail squids (Cephalopoda: Sepiolidae) and the luminescent bacterium *Vibrio fischeri* has proved to be an innovative and successful model for examining the population biology of cospeciating organisms according to their phylogeographical distribution (Nishiguchi *et al*. 1998; Kimbell *et al*. 2002). In this environmentally transmitted mutualism, axenic juvenile *Euprymna* hatch and soon obtain their *Vibrio* symbionts from surrounding waters (Ruby & McFall-Ngai 1992; McFall-Ngai & Ruby 1998). Upon acquisition of these bacteria, both host and symbiont undergo a number of structural and physiological changes (Montgomery & McFall-Ngai 1993; Foster & McFall-Ngai 1998; Visick *et al*. 2000), the result of which is a highly specific association where *Vibrio*-produced light is emitted from the host light organ in a camouflage behaviour termed counterillumination (Nishiguchi *et al*. 1998; Nishiguchi 2002; Jones & Nishiguchi 2004). In the mature association, the light organ is inhabited by a monoculture of *Vibrio fischeri* in Pacific populations (Ruby & McFall-Ngai 1992).

Despite being an environmentally transmitted association, a high degree of specificity has arisen in Pacific *Euprymna*/

Vibrio assemblages (Nishiguchi *et al.* 1998; Nishiguchi 2002). This is likely due to the biology of this association, where vibrios are cycled through hosts within local populations. This cycling is mediated by a daily host behaviour where approximately 90–95% of the symbiont population is vented into the environment (Ruby & Lee 1998), seeding vibrios into local populations (Lee & Ruby 1994; Jones *et al.*, in review). Expelled vibrios subsequently become an available inoculum for newly hatched juveniles in the same areas, permitting the opportunity for the evolution of specificity in these populations. Although these 'viable' free-living vibrios are capable of colonizing naïve juvenile hosts, they are also subject to abiotic factors such as currents, which may provide an opportunity for free-living vibrios to invade areas where other populations occur, as well as colonize hosts from distantly related host species.

This present study seeks to examine this unique intraspecific association by employing a population genetics approach to examine evolutionary relationships among host squids and their associated *Vibrio* symbionts. Specifically, we quantified population structure and patterns of gene flow among populations in three *Euprymna* species found in the Indo-West Pacific region (Table 1; Fig. 1) by examining the cytochrome *c* oxidase subunit I (COI) locus. These three species were chosen based on distributions of their populations, which represent three distinct geographical areas that may lead to unique patterns of gene flow and population structure. Importantly, no other species of *Euprymna* exist sympatrically with the three species examined here, eliminating the possibility of horizontal transfer of *Vibrio* between host species. *Euprymna scolopes*, which is found along the coastal waters of the Hawaiian archipelago, represents a species where populations are in relatively close proximity to each other, allowing for the possibility of panmictic associations between populations (Fig. 1). Conversely, *Euprymna hyllebergi* (Thailand) and *Euprymna tasmanica* (Australia) represent two species whose populations are more geographically isolated (Fig. 1). *Euprymna hyllebergi* is the more extreme case of the two; populations in this species are separated by a geographical barrier (Malaysian peninsula) making it virtually impossible for migration of individuals between the two populations.

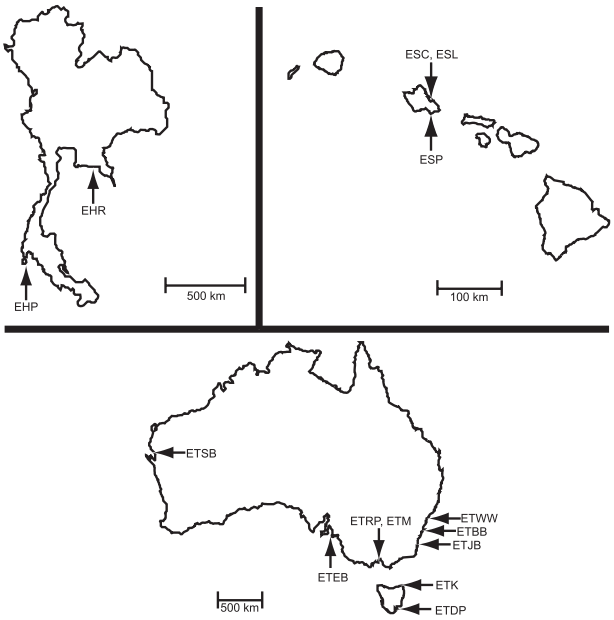


Fig. 1 Graphical representation of Hawaii, Australia, and Thailand sites sampled in this study. Refer to Table 1 for specific site information.

Table 1 Populations and collection site information for all sites in this study. The measurements of within population genetic diversity theta (per-base-pair) are listed for both *Euprymna* host and *Vibrio* symbiont at each location. Refer to Fig. 1 for mapped locations

Host	Name	Population location	Host *N*	Latitude	Longitude	Host theta	*Vibrio* theta
Euprymna	ESP	Hawai'i Kai, Honolulu, Hawai'i, USA	15	N 21°16′	W 157°45′	0.0023	0.0033
scolopes	ESL	Lilli'puna Pier, Kane'ohe Bay, Hawai'i, USA	10	N 21°25′	W 157°47′	0.0016	0.0027
	ESC	Coconut Island, Kane'ohe Bay, Hawai'i, USA	11	N 21°26′	W 157°47′	0.0041	0.0021
Euprymna	ETWW	Woywoy, NSW, Australia	1	S 33°29′6″	E 151°19′47″	0.0000	0.0052
tasmanica	ETBB	Kurnell, Botany Bay, Sydney, NSW, Australia	29	S 34°00′	E 151°12′	0.0236	0.0093
	ETJB	Sanctuary Point, Jervis Bay, NSW, Australia	2	S 35°06′	E 150°39′	0.0015	0.0022
	ETM	St. Leonard's Pier, Melbourne, VIC, Australia	10	S 38°10′14″	E 144°43′8″	0.0005	0.0087
	ETRP	Rye Pier, Melbourne, VIC, Australia	7	S 38°22′10″	E 144°49′20″	0.0006	0.0058
	ETNO	Narooma, NSW, Australia	1	S 33°29′05″	E 150°06′54″	0.0000	NA*
	ETEB	Adelaide, SA, Australia	1	S 34°58′	E 138°16′	0.0000	0.0000
	ETK	Kelso Point, TAS, Australia	13	S 41°3′24″	E 146°47′52″	0.0005	0.0132
	ETDP	Drew Point, TAS, Australia	9	S 43°01′38″	E 147°16′45″	0.0011	0.0079
	ETSB	Shark Bay, WA, Australia	3	S 25°56′	E 113°30′	0.0860	0.0007
Euprymna	EHR	Rayong, Thailand	13	N 12°38′	E 101°14′	0.0010	0.0027
hyllebergi	EHP	Phuket, Thailand	14	N 7°38′	E 98°24′	0.0033	0.0052

*, no *Vibrio* was obtained from ETNO hosts.

To examine *Vibrio* phylogeographical structure, an 891-bp fragment of the glyceraldehyde phosphate dehydrogenase (*gap*A) was examined. This gene was chosen because previous studies have shown this locus to be variable enough to discriminate between *Vibrio* strains (Nishiguchi & Nair 2003). We hypothesized that local cycling of *Vibrio* through *Euprymna* hosts has led to the specificity seen in Pacific populations, and this cycling has created concordant phylogenies that can be detected through the examination of haplotype networks. If, however, *Vibrio* genetic distribution is random, or if genetically distinct *Vibrio* strains not directly involved in local cycling are able to environmentally colonize juvenile hosts, the structure of host and symbiont haplotype networks will be markedly different. This study therefore hopes to expand our understanding of the intraspecies relationships within the sepiolid squid–*Vibrio* mutualism and provide a unique view into the phylogeography of environmentally transmitted symbiosis.

Materials and methods

Specimen collection

Table 1 lists all sites sampled in this study. Thailand populations were collected from coastal waters near Rayong and Phuket (Fig. 1). Rayong is located on the northern shoreline of the Gulf of Thailand, while Phuket is an island located in the Andaman Sea that is connected to Thailand's southwestern coast by means of a land bridge. Both Rayong and Phuket populations are separated from each other by approximately 2100 km following the coastline around the Malaysian peninsula (see Fig. 1). A total of 10 populations were sampled in Australia, encompassing multiple locations along southeastern coast ranging from Woy Woy to Adelaide, Tasmania, and Shark Bay in Western Australia (Fig. 1). In Hawaii, three populations were sampled around the island of O'ahu. Two of the populations (Coconut and Lili-puna) are located in Kanéohe Bay. The Lili-puna population is located on the southern coastline of Kanéohe Bay and the Coconut population is located around Coconut Island, which is about 480 m (offshore) in Kanéohe Bay. The third Hawaiian population is located on the southeastern tip of O'ahu and is approximately 49 km southeast of Kanéohe Bay in Nui Valley (Paiko; Fig. 1).

DNA isolation and sequencing

Individual specimens collected from *Euprymna hyllebergi*, *Euprymna tasmanica*, and *Euprymna scolopes* populations were preserved in 95% ethanol after dissection of the light organs. To isolate *Vibrio* from each host, the light organ was homogenized in sterile seawater, serially diluted, and plated on 15% agar-supplemented seawater tryptone (SWT; 0.5% tryptone, 0.3% yeast extract, 0.3% glycerol, 70% seawater). After 12–18 h of growth, 10–20 *Vibrio fischeri* colonies were isolated from each plate and transferred to 5 mL SWT for 12 h. These clones were then frozen as stocks at −80 °C in SWT in 20% glycerol.

Vibrio fischeri DNA was extracted from each strain using a rapid DNA isolation protocol. One and a half millilitres of 8- to 12-h culture grown in SWT was centrifuged at 14 000 × *g* for 5 min. The resulting pellet was re-suspended in sterile TE, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) and placed at 100 °C for 3 min, followed by a 2-min centrifugation at 6000 *g*. One microlitre of the DNA-containing supernatant was then used as a template in the polymerase chain reaction (PCR; see below for protocol) to amplify an 891-bp fragment of the glyceraldehyde phosphate dehydrogenase (*gap*A) locus using *V. fischeri* specific primers (Table S1, Supplementary material). These primers were different from previous *gap*A primers in that both primer sets are internal to those used [16 bp (*gap*AF1) and 20 bp (*gap*AR1)] in prior studies (Lee & Ruby 1994; Nishiguchi & Nair 2003). These primer sets were created due to the difficulty in amplification using the original primers for the wide variety of strains tested.

To isolate *Euprymna* DNA, approximately 25 mg of tissue was removed from either the mantle or gill inside the body cavity of each squid. DNA was then isolated from the tissue using the QIAGEN DNeasy Isolation Kit (QIAGEN). Following DNA extraction, 1–10 ng of each DNA sample was used as template for PCR to amplify the 658-bp cytochrome *c* oxidase subunit I (COI; Lopez 2004; Nishiguchi *et al*. 2004).

All loci were amplified using a 50-μL PCR containing 1–10 ng of DNA template, 0.2 μM of each forward and reverse primer, 2.5 mM of $MgCl_2$, 200 μM of each dNTP, 1× buffer (10 mM Tris-HCl, pH 9.0 50 mM KCl, and 0.1% Triton X-100), and 0.2 U of Promega *Taq* polymerase. All PCR amplifications were performed with an MJ Research Dyad thermocycler. PCR products of all loci were purified using the GENECLEAN II DNA purification kit (BIO 101). PCR products were then presequenced using Applied Biosystems BigDye Terminator version 3.1. Excess fluorescent dNTPs were removed from the presequencing reactions using DTR V3 96-well short spin plates (Edge Biosystems). All samples were sequenced using an Applied Biosystems 3100 Automated Capillary Sequencer. Forward and reverse sequences of each individual were combined into contigs using SEQUENCHER 4.1. Sequences were then examined for base miscalls and edited using the GENETIC DATA ENVIRONMENT (GDE) program (Tulio de Oliveria, University of Natal, South Africa).

Analysis of molecular variance

Analysis of molecular variance (AMOVA) was calculated using the population genetics program ARLEQUIN version

2.0 (Schneider *et al*. 2000) to examine population structure with variation partitioned among each country (Australia, US, and Thailand), among populations within each country, and within populations. Within-population genetic diversity was estimated with theta, the per-base-pair nucleotide polymorphism.

Nested clade analysis and secondary contact analysis

Several programs were used to test and analyse genetic variation among individuals in the three species being studied. For both host and symbiont populations, haplotype networks were created using a statistical parsimony procedure (Templeton *et al*. 1992; Crandall *et al*. 1994) performed by the program TCS version 1.12 (Clement *et al*. 2000). Haplotype network ambiguities (closed loops) were resolved using the empirical predictions derived from the frequency, topological, and geographical coalescent theory criterion outlined by Pfenninger & Posada (2002). The haplotype network was then nested and ambiguities were resolved using the nesting procedures given by Templeton (Templeton *et al*. 1987; Templeton & Sing 1993; Templeton *et al*. 1995). The nested clade information was input into GEODIS version 2.0 (Posada *et al*. 2000) which performed a contingency analysis of the nested clades in the haplotype network, and provided an analysis of the categorical variation for the association between clades and their geographical locations.

To determine if secondary contact had occurred for *Vibrio* lineages that displayed fragmentation, the procedures of Templeton (2001) were followed. Briefly, the average pairwise distance from the geographical centres of all haplotypes and clades was calculated for each population and nesting level. Theoretically, panmictic populations would display near-identical or decreasing average distances for increasing clade levels, while haplotypes that were previously fragmented and occurring in the parent population would have high or increasing distances with increasing clade level (Templeton 2001). An unpublished program by David Posada (GEOLOC version 3.1) was used to implement the above algorithm of Templeton (2001). This program calculates the statistical significance of the calculated clade distance values through 10 000 random permutations of each clade against geographical location (Pfenninger & Posada 2002).

Tests for recombination

Given the commonality of bacterial recombination in nature (Feil *et al*. 2000; Feil *et al*. 2001; Spratt *et al*. 2001; Mu *et al*. 2005), the programs PAIRWISE and PERMUTE in the LDHAT software package were used to investigate linkage disequilibrium in *Vibrio* populations (McVean *et al*. 2002). These programs estimated the population-scaled recombination rate ($2N_e r$) using a modified version of the composite likelihood method of Hudson (2001). In addition, these programs were used to implement a nonparametric permutation test (using 1000 permutations) for recombination by computing the linkage disequilibrium between pairs of sites through calculating the correlation of both summary statistics r^2 and $|D'|$ with physical distance. These observed statistics are compared to null distributions containing randomized single nucleotide polymorphisms to detect nonzero recombination (McVean *et al*. 2002). Finally, the minimum number of recombination events (R_m) was calculated in PAIRWISE using the algorithm of Hudson & Kaplan (1985).

Phylogenetic analysis

Phylogenetic analysis of distinct *Vibrio* haplotypes was conducted using maximum likelihood as an optimality criterion, as implemented in PAUP* (Swofford 2002). The best-fit model of sequence evolution was selected under the Akaike Information Criterion (AIC) as implemented in MODELTEST version 3.06 (Posada & Crandall 1998; Posada & Crandall 2001), and that model was used to infer the phylogenetic tree via a heuristic search of 100 replicates of random addition sequence followed by tree-bisection–reconnection (TBR) branch swapping. Nodal support was assessed with nonparametric bootstrapping obtained from 1000 heuristic replicates under TBR branch swapping.

Results

Genetic variation and nested clade analysis

For each host, a 658-bp region of COI was amplified, while an 891-bp region of the *gap*A gene was amplified for 200 *Vibrio* symbionts isolated directly from 139 field-caught sepiolid squid hosts. A total of 46 different symbiont haplotypes (GenBank Accession nos DQ646741–DQ646786), and 38 different host haplotypes were observed (GenBank Accession nos DQ646703–DQ646740). Non-synonymous mutations were identified in 18 of the 68 *Vibrio* polymorphic sites (Table S2, Supplementary material), and seven *Euprymna* polymorphic sites (Table S3, Supplementary material). There were no nonsynonymous mutations in Hawaiian *Euprymna scolopes* sequences. For *Vibrio* populations, the Thailand, Hawaiian, and Australian populations contained 7, 6, and 32 distinct haplotypes, respectively. One distinct haplotype was found within both Hawaiian and Australian populations (Figs 2 and 3). *Euprymna* populations consisted of 10 Hawaiian, 18 Australian, and 10 Thailand haplotypes (Fig. 4).

Vibrio sequence data yielded theta ranging from 0 to 0.0132 between all sites (Table 1). *Euprymna* theta ranged from 0.00 to 0.0860 between all sites. For hosts, the small number of samples from the sites containing no genetic

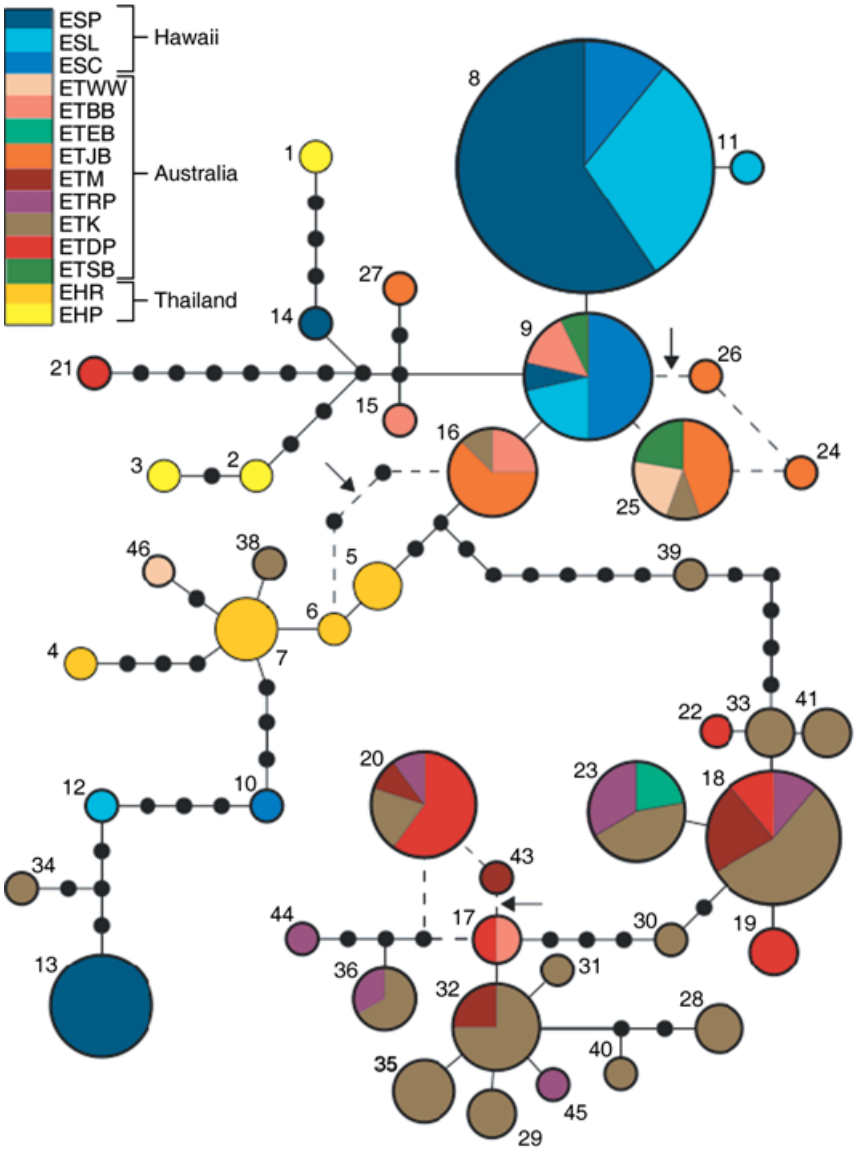


Fig. 2 Haplotype network for Indo-west-Pacific symbiotic vibrios. Black circles represent unsampled haplotypes, while colours in each circle represent the proportion of locations having that specific haplotype. Each line represents one mutational step. Loop structures are represented with a dashed line, and the point at which they were resolved is marked with an arrow. The size of each circle is proportional to the number of samples with that haplotype. Haplotype numbers are listed beside each haplotype.

variation (ETEB, ETWW, and ETNO) resulted in values of 0.00 for theta. Interestingly, within-population genetic divergence had a much greater range in the Australian populations than Hawaiian and Thailand populations. The extraordinary high values of theta for ETSB hosts are from two very distinct haplotypes found at this location (see below).

Vibrio Pacific-wide haplotype distribution contains a southern network of haplotypes exclusive to Australia (the lower right-hand portion of Fig. 2). However, a number of haplotypes more closely related to haplotypes from Hawaii and Thailand were also found in Australia. These included almost all of the Northern sites of Botany Bay, Jervis Bay, and Woy Woy (Fig. 2). For *Vibrio* haplotype networks, nesting clade 1-12 with 2-7 provided more parsimonious inference for population-level processes. This is because clade 1-12 borders a major split between clades at the total cladogram level. Preliminary analysis demonstrated that if clade 1-12 was nested within 2-6 instead of 2-7, a secondary colonization event of Thailand would have been inferred, which is clearly not the case since all EHR strains are closely related (Figs 2 and 3).

The original *Vibrio* haplotype network contained three closed-loop structures, which were resolved according to Templeton & Sing (1993). The points of resolution have been marked with an arrow on Fig. 2. The final nested network is displayed in Fig. 3. The fourth level nesting displays differentiation of southern Australian haplotypes (clade 4-3), which contain no Hawaiian or Thailand haplotypes. The null hypothesis of no geographical structure within a single clade was rejected for seven clades and at the total cladogram level (Table 2). Clade 1-10 was an internal clade exhibiting geographical structure. Since the

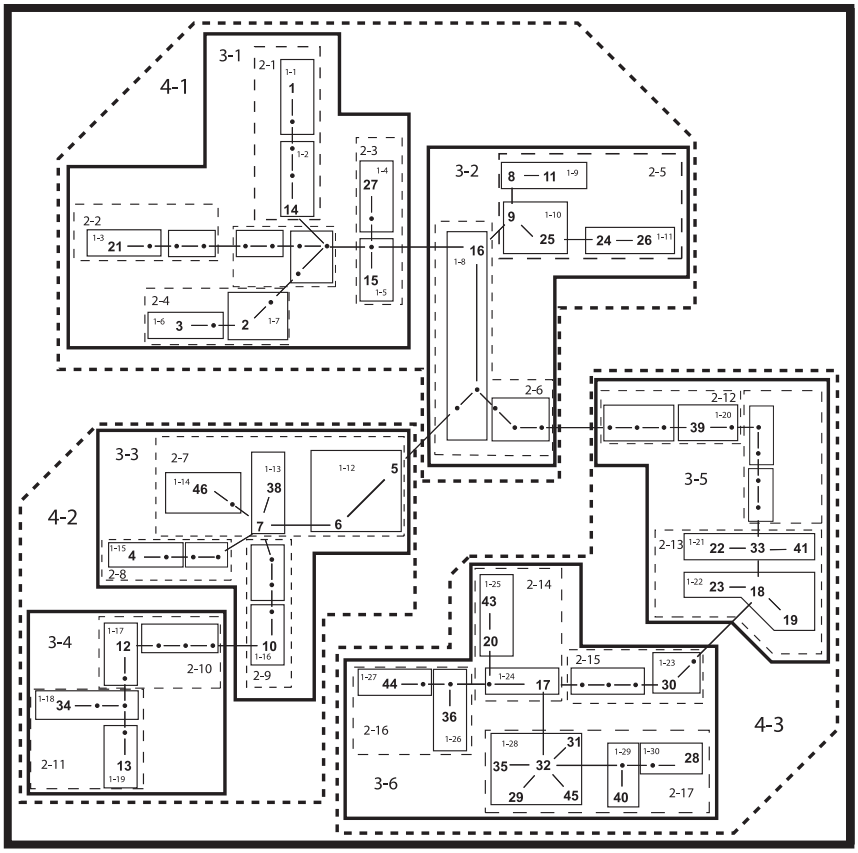


Fig. 3 Nesting diagram for the haplotype network in Fig. 2. The network has been simplified, using only haplotype numbers. Ambiguities were resolved using the rules of Templeton & Sing (1993). Clades are designated with two digit identifiers within each boxed clade. As in Fig. 2, one line represents one mutational step, but the length of the line has been varied for illustrative purposes.

interior-tip distinction of nested groups could not be determined for this clade, no inference could be made about its population structure and history. Clades 1-22 and 3-2 exhibited contiguous range expansion, while 3-6, 4-1, and the total cladogram illustrate restricted gene flow with isolation by distance. Clade 4-2 demonstrates past fragmentation followed by range expansion.

Secondary analysis shows significantly large population distances for populations at Woy Woy and Botany Bay from the 2-step to 4-step clades. Significantly, large distances were observed for Jervis Bay at the 2-step clade level. The Paiko population in Hawaii also had a significantly large population distance at the 3-step clade level (Fig. 5).

AMOVA for *Vibrio* populations failed to reveal significant genetic structure among continents, but did so among populations within continents and within populations. Sixty-four percent of the genetic variation was within populations, while 33% of the variation was explained between populations (Table 3).

Euprymna genetic data resulted in five different haplotype networks and three haplotypes that did not fit into any network (Fig. 4). Two Thailand networks contained haplotypes exclusive to Rayong and Phuket. Since there was no geographical variation in these networks, they were not included in NCA. The three networks that did contain phylogenetically informative information were exclusive to Hawaii (clades identified with Hx-x), eastern Australia (EAx-x), and southern Australia (Sax-x). The southern Australia network contained one closed-loop ambiguity, which used the criterion described above. In addition, three Botany Bay haplotypes (23, 24, and 25) grouped with the southern Australia network.

In examining the *Euprymna* haplotype network, the null hypothesis of no genetic structure was rejected for the Hawaiian clades H1-1 and H2-1, which both illustrated isolation by distance. In addition, the EA2-1 exhibited restricted gene flow with isolation by distance as well. SA1-1 showed contiguous range expansion, but there was insufficient genetic resolution to differentiate between range expansion/colonization and restricted dispersal/gene flow in clade SA2-1 (Table 4). AMOVA for *Euprymna* populations showed 67.22% genetic variation between continents and 25.44% genetic variation between populations. Within populations, 7.34% of total variation was observed (Table 3).

Tests for recombination

For *Vibrio* populations, the estimated population mutation rate (θ) was 0.012, and the population rate of recombination ($2N_e r$) was found to be 5.0. The estimated number of recombination events (assuming absence of homoplasies

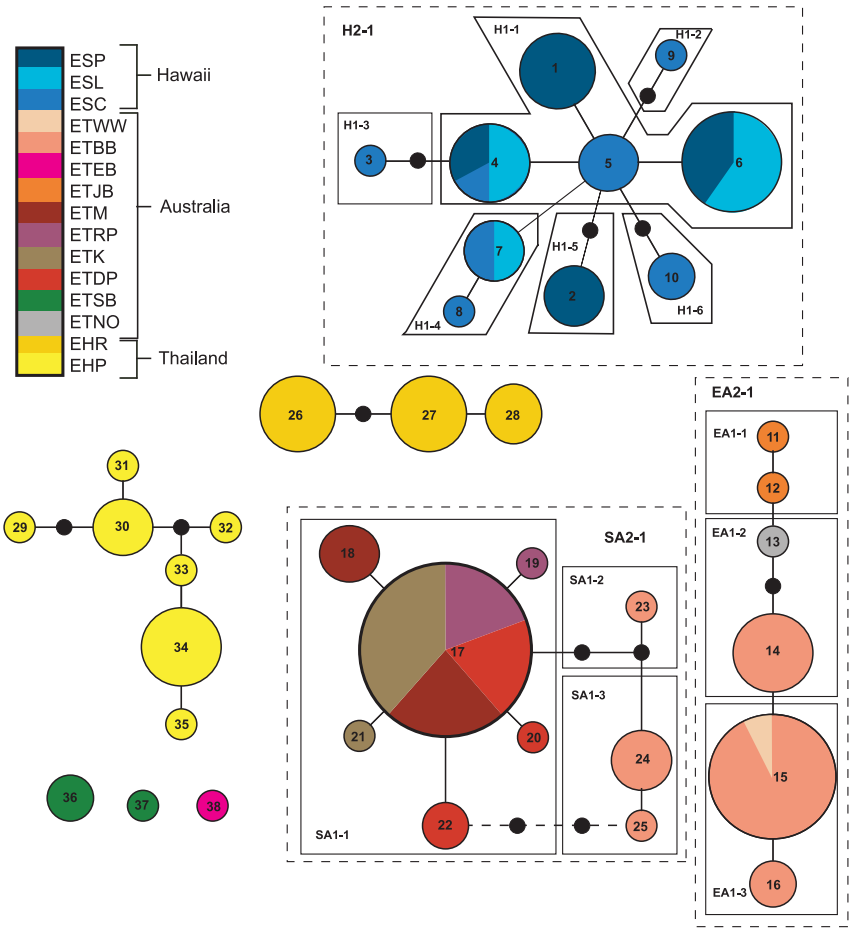


Fig. 4 Haplotype network for *Euprymna* hosts. Hawaii (H), East Australia (EA), and South Australia (SA) networks have been nested as in Fig. 3.

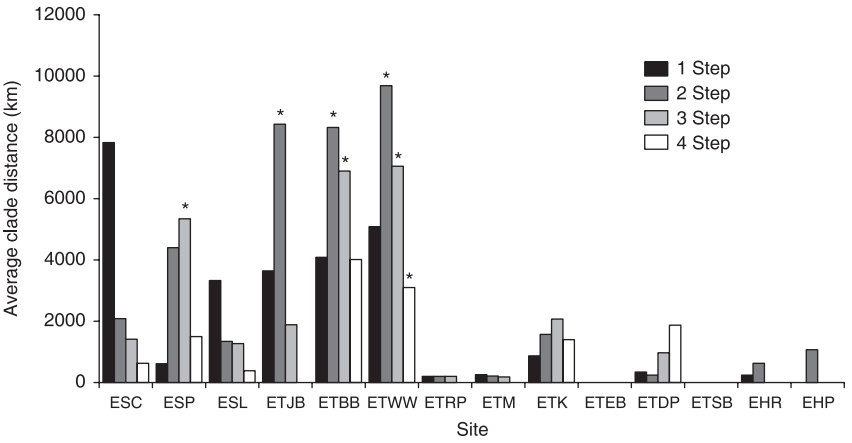


Fig. 5 Secondary contact analysis for the nested clade analysis data set. Bars represent the average clade distance for each clade level. Each asterisk represents significantly large clade distances ($P < 0.01$).

in the data), R_m, was 13. Both the r^2 and $|D'|$ measures of linkage disequilibrium provided evidence of recombination ($r^2 = 0.12$, $P = 0.004$; $|D'| = -0.135$, $P = 0.001$).

Phylogenetic analysis

The best-fit model under AIC was the Hasegawa–Kishino–Yano (HKY) model with a correction for invariant sites and a discrete gamma distribution (HKY + I + G) for *Vibrio* sequence data. The HKY model assumes two substitution types, one for transversions and one for transitions, and unequal base frequencies. The substitution model assumes unequal base frequencies, which are estimated as follows: $\pi_A = 0.3024$, $\pi_C = 0.\ 1880$, $\pi_G = 0.\ 1906$, $\pi_T = 0.3190$. The proportion of invariant sites was set to 0.8220. The α parameter of the discrete gamma distribution (four rate categories) was estimated to 0.5992. 80% of the 100 replicates yield a single unrooted tree of –ln L = 1628.87297 (Fig. 6).

Table 2 *Vibrio* significant clades and inference for population genetic data. Listed are clades (or haplotypes) exhibiting significant distributions, whether the significant values are within the clade (D_c) or nested clade (D_n), and the *P* value associated with the observed data

Clade	Nested clade	Dist.	Value (S or L)	*P*	Inference key steps	Inference
1-10	9	D_c	15083 L	0.0059	1, 2	Inconclusive
		D_n	9982 L	0.0151		
	25	D_c	1176 S	0.0008		
		D_n	6140 S	0.0252		
1-22	18 (I)	D_c	206 S	0.0167	1, 2, 11, 12, No	Contiguous range expansion
		D_n	228 S	0.0008		
	19 (T)	D_c	0.0 S	0.0000		
		D_n	536 L	0.0679		
	I-T	D_c	−296 S	0.0021		
2-5	1-9 (T)	D_c	8.2 S	0.0000	1, 2, 3, 5, 6, 7, 8, No	Sampling inadequate to discriminate between isolation by distance and long-distance dispersal
		D_n	16098 L	0.0000		
	1-11 (T)	D_c	0.0 S	0.0337		
	I-T	D_c	7787 L	0.0000		
		D_n	−1403 S	0.0063		
3-2	I-T	D_c	−14999 S	0.0161	1, 2, 11, 12, No	Contiguous range expansion
3-6	2-14 (I)	D_n	376 L	0.0458	1, 2, 3, 4, No	Restricted gene flow with isolation by distance
	2-17 (T)	D_c	179 S	0.0136		
		D_n	184 S	0.0001		
	I-T	D_c	183 L	0.0065		
		D_n	173 L	0.0016		
4-1	3-1 (T)	D_n	9471 S	0.0002	1, 2, 3, 4, No	Restricted gene flow with isolation by distance
	3-2 (I)	D_c	15201 L	0.0001		
		D_n	14698 L	0.0001		
	I-T	D_n	4956 L	0.0001		
4-2	3-3 (I)	D_c	3018 S	0.0079	1, 2, 11, 12, 13, Yes	Past fragmentation followed by range Expansion
	3-4 (T)	D_c	3474 S	0.0104		
		D_n	14149 L	0.0000		
	I-T	D_n	−8118 S	0.0000		
Total	4-1 (I)	D_c	13043 L	0.0000	1, 2, 3, 4, No	Restricted gene flow with isolation by distance
		D_n	9044 L	0.0073		
	4-3 (T)	D_c	352 S	0.0000		
		D_n	7281 S	0.0058		
	I-T	D_n	11046 L	0.0000		
		D_c	1809 L	0.0018		

Source of *Vibrio* variation	d.f.	Variance components	% Variation
Among continents	2	0.01^{NS}	1.80
Among pops. within continents	11	0.17*	33.33
Within populations	186	0.32*	64.86
Overall F_{ST}		0.35*	
Source of *Euprymna* Variation	d.f.	Variance components	% Variation
Among continents	2	30.44*	67.22
Among pops. within continents	12	11.52*	25.44
Within populations	124	3.32*	7.34
Overall F_{ST}		0.93*	

Table 3 AMOVA results for *Vibrio fischeri gap*A and *Euprymna* COI genetic subdivision between populations from Hawaii, Thailand, and Australia

*, $P < 0.001$; NS, not significant.

Table 4 *Euprymna* significant clades and inference for population genetic data

Clade	Nested clade	Dist.	Value (S or L)	*P*	Inference key steps	Inference
H1-1	1 (T)	D_c	0 S	0.0240	1, 2, 3, 4, No	Restricted gene flow with IBD
H2-1	1-2 (T)	D_c	0 S	0.0340	1, 2, 3, 4, No	Restricted gene flow with IBD
		D_n	11.95 L	0.0270		
	I-T	D_n	7.993 L	0.0020		
EA2-1	I-T	D_n	77.84 L	0.0170	1, 2, 11, 17, 4, No	Restricted gene flow with IBD
SA1-1	21 (T)	D_c	338 L	0.0474	1, 2, 11, 12, No	Contiguous range expansion
	I-T	D_n	222 L	0.0245		
SA2-1	1-1 (T)	D_c	231 S	0.0000	1, 2, 3, 5, 6, Too	Insufficient genetic resolution to
		D_n	275 S	0.0000	few clades	discriminate between range
	1-3 (T)	D_c	0 S	0.0011		expansion/colonization and
		D_n	665 L	0.0000		restricted dispersal/gene flow
	I-T	D_c	–205 L	0.0000		
		D_n	345 L	0.0000		

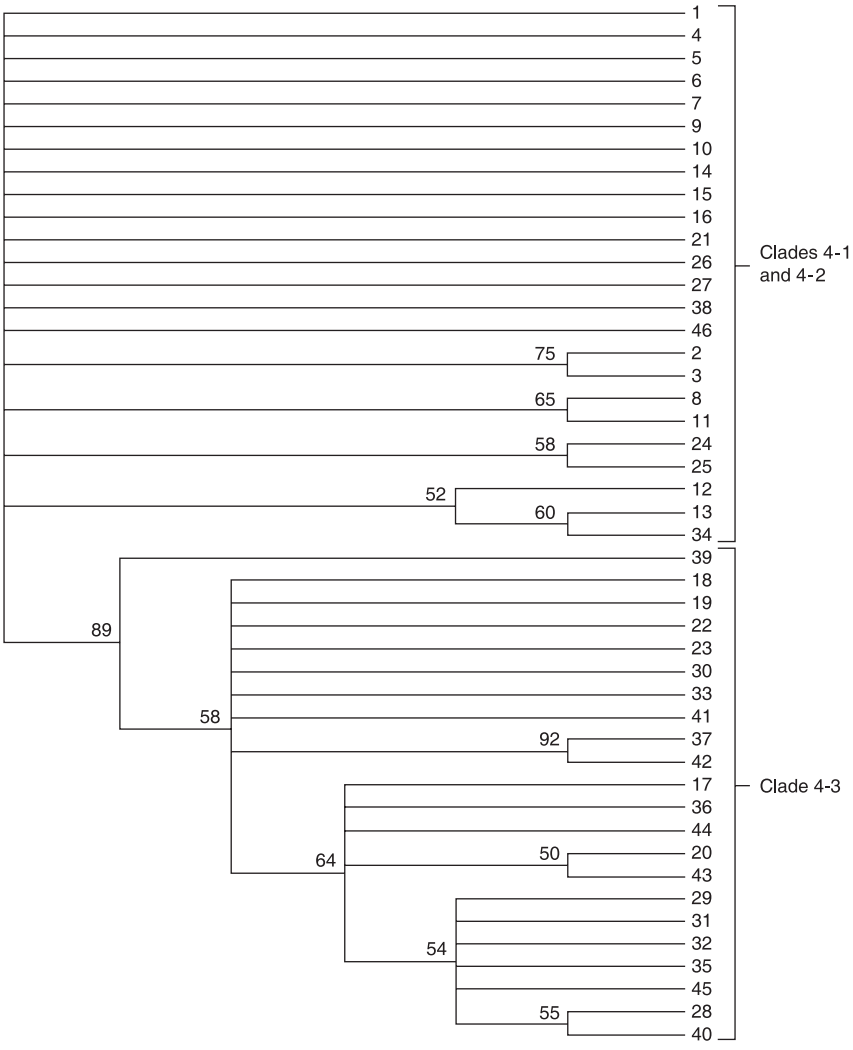


Fig. 6 Phylogenetic analysis of *Vibrio fischeri* haplotypes. Refer to Fig. 2 for haplotype designations. There was not sufficient resolution to distinguish between closely related haplotypes, but the tree supports the separation between clades 4-3 and 4-1/4-–2.

Discussion

Sampling

Sampling strategies for *Euprymna scolopes* in O'ahu were more exhaustive than *Euprymna hyllebergi* and *Euprymna tasmanica* populations. This was due to the heavy sampling of *E. scolopes* populations over the past 20 years. Sampling efforts have searched for other populations between Kanéohe Bay and Nui Valley (Paiko), but no specimens have been found due to inappropriate habitat with rough surf in these areas.

Results of the analysis for Australian *E. tasmanica* and Thailand *E. hyllebergi* yielded two distinct haplotype networks between populations in each country that cannot be connected due to the large number of mutational steps between populations. Further sampling between Rayong and Phuket should allow connection between the two Thailand networks. Likewise, future sampling between Shark Bay, Adelaide, and Melbourne may also connect the Australian networks, providing a more clear view of gene flow within and between these distant populations.

Euprymna population structure

In Hawaii, host networks differ dramatically from the *Vibrio* network. The host network shows restricted gene flow with isolation by distance in both clades H1-1 and H2-1 (Fig. 4). Previous work examining *E. scolopes* geographical structure supports these results as Paiko (ESP) and Lilli-puna, O'ahu (ESL, ESC) were also shown to be genetically distinct (Kimbell *et al*. 2002) based on allozyme data. Since little is known regarding active dispersal of benthic *Euprymna* species, the probability of introgression between relatively close populations may be less frequent compared to other invertebrate species, and may be affected more by abiotic factors such as currents and tidal changes.

In contrast to the relatively simple Hawaiian network, NCA and AMOVA revealed some interesting trends in *E. tasmanica* host populations. First, Melbourne and Tasmania populations share nearly all haplotypes (clade SA1-1). This suggests that a large amount of gene flow is occurring between these two populations, and NCA corroborates this by inferring contiguous range expansion in clade SA1-1. Being that *E. tasmanica* is also benthic and is capable of only swimming short distances, the probability that individuals migrate between the populations (290 km across the Bass Straight) is more likely a phenomenon of currents than active dispersal. Oceanographic data suggests that currents in the Bass Straight are turbulent, characterized by many underwater cascades and eddy or vortex patterns due to the convergence of several oceanic currents at this geographical region (Fandry *et al*. 1997). In addition, the Bass Straight is part of the Australian continental shelf with shallow waters of 50–70 m in depth. This combination of turbulent current and shallow depth may account for mixing between Melbourne and Tasmania populations via juvenile migrants carried in these cascade and eddy/vortex currents across the Bass Straight.

The second interesting aspect of the Australian haplotype networks is that individuals from Botany Bay (ETBB) were represented in both the SA and EA haplotype networks (haplotypes 14, 15, 16, 23, 24, 25). As demonstrated from phylogenetic inference, there was insufficient genetic resolution to determine what processes have led to this variation (Table 4). While the individuals found in Botany Bay with South Australian-like haplotypes could represent a small number of migrants, it is clear that some sort of barrier exists between the South Australian and East Australian networks.

Symbiotic Vibrio *population structure*

A small amount of recombination within the *Vibrio* data set was detected using three different methods. Recombination could potentially disrupt inference of NCA due to construction of an incorrect network. In TCS, homoplasies created by recombination are displayed as closed loops, and existing procedures were used to resolve these ambiguities (Templeton & Sing 1993). Even so, inference based on any network should be treated with caution due to the fact that no estimated network is completely correct. Strengthening the data set will include future analysis examining other informative loci in addition to *gap*A.

Potential caveats aside, the *Vibrio* network illustrates Hawaii *Vibrio fischeri* grouping together with East Australian congeners (Fig. 2). In addition, clade 4-2 in the *Vibrio* network contains a number of Hawaiian strains that are more closely related to the Rayong strains than the other Hawaiian strains found in clade 2-5 (Fig. 2). The inference provided here is past fragmentation, followed by range expansion. Result of this historical process therefore may be a secondary colonization event in Hawaii at the Paiko population. This can be visualized on the cladogram (Fig. 2) by the large sample from Paiko (ESP, dark blue) that is more closely related to Rayong isolates (EHR, orange-yellow). Indeed, secondary contact analysis supports this position as 3-step clade population distances are significantly high when compared to randomly distributed populations (Fig. 5).

Given that there is no connection between the Hawaii and Thailand networks based on COI data (Fig. 4) and that *Euprymna* hosts are different species in Thailand, Hawaii, and Australia, it is important to consider how *Vibrio* symbionts may have migrated between host populations. *Vibrio* may conceivably travel by oceanographic currents through the South China Sea, ride the Kuroshio current North past Japan, where it would follow the North Equatorial current North of Hawaii, and then down to the

Hawaiian archipelago. A more plausible hypothesis suggests that *Vibrio* strains were moved by ballast water from large tankers. One problem with this hypothesis, however, is that secondary contact occurs deep at higher nesting levels in the cladogram, suggesting that the event happened long ago. Additionally, there may be populations of *Euprymna* that exist along many of the South Pacific Islands, allowing *Vibrio* bacteria to leap frog from one population to the next. Examination of both additional *Euprymna* populations within these areas, as well as *V. fischeri* haplotypes from both hosts and surrounding seawater may support either of these hypotheses, and would also explain why Australian strains appear scattered throughout the cladogram.

Unlike the COI host networks, the complex *gap*A network allows inference at higher clade levels. At the total cladogram level of the *Vibrio* network, analysis suggests restricted gene flow with isolation by distance between clades 4-1 and 4-3 (Fig. 3, Table 3). This inference is mainly due to the presence of only Australian haplotypes in clade 4-3 and the mixture of Australian and Hawaiian haplotypes in clade 4-1. Interestingly, northeast Australian strains (with the exception of one ETBB strain) group with the Hawaiian or Thailand strains (Fig. 2). Significantly large population clade distances for Jervis Bay, Woy Woy, and Botany Bay are the results of isolates from these populations grouping with Hawaiian strains. Results of the phylogenetic analysis on individual haplotypes supported the observed high-level division between clades 4-3 and 4-2/4-2 (Fig. 6), despite little or no resolution between other haplotypes. Since *Vibrio* moving with a host is not a possibility at these high clade levels, the observed structure may be due to current activity and/or ballast water.

Population structure and cospeciation

Studies in other systems suggest there is a genetic barrier between Sydney and Melbourne for other types of invertebrates. Work with the asteroid sea-star *Coscinasterias muricata* identified a split between northern and southern haplotypes in Mallacoota, Victoria, which is approximately 425 km east of Melbourne and 425 km south of Sydney on the Southeast Coast of Australia (Waters & Roy 2003). This site corresponds to the eastern-most edge of the Maugean marine biogeographical province proposed by Bennett & Pope (1953). The Maugaen biogrographical zone extends from Mallacoota to Adelaide, and includes all of Tasmania, which corresponds to the observed data for *V. fischeri*, as these populations are almost exclusively contained in clade 4-3 (Fig. 3). Ecological factors that have structured the establishment of this biogeographical zone are still under contention, but the zone appears to be the result of temperature gradients and ocean currents (O'Hara & Poore 2000). Given the data at hand, the structure of *V. fischeri* populations in clade 4-3 and the EA and SA host networks raises a number of interesting questions regarding the biogeography of this mutualism. For example, if temperature is keeping these strains mostly restricted to the Maugaen biogeographical zone, is adaptation to these colder waters reflected in *V. fischeri* host-associated growth rates of these strains? If so, how might this cold-water adaptation affect squid-host colonization in warmer waters where *V. fischeri* must compete with other strains?

At first glance, our results seem to contradict previous studies demonstrating competitive dominance of native over non-native *V. fischeri* strains in *E. scolopes*. In Nishiguchi *et al*. (1998), however, *V. fischeri* strains used in colonization studies were from Melbourne, Australia and Lillipuna, O'ahu, Hawaii. Although the present study did not examine the original strains used in the competition studies, the haplotype network presented here show no shared haplotypes between the ETM and ESL populations (Fig. 2). Nonetheless, results of colonization studies using strains collected in this study (particularly between those from Australia and Hawaii that share identical haplotypes) will provide unique insight to the nature of competitive dominance and local host adaptation by *V. fischeri*. Future studies that include not only allopatric but sympatric populations of sepiolid squids and their *Vibrio* symbionts will shed light on how quickly adaptation occurs between co-occurring partners, and which factors predominate in selection and the overall evolution of this dynamic mutualism.

Acknowledgements

Funding was provided by NSF SBE-0123690, NSF DBI-0079820, NSF DEB-0316516, and NIH S06-GM08136-26 to M. K. N. and NIH-RISE GM 61222–01 in support of J. E. L. We would like to thank Sandra Duran, David Posada, and Tom Parchman for all their assistance with the population genetics programs. Finally, we would like to thank the Ruby and McFall-Ngai laboratories, Natalie Moltschaniwskyj, Geoffrey Woolcott, Jarawat Nabhitabhata, and Anuwat Nateewathana for all their help in collecting the squid specimens.

Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC3073/MEC3073sm.htm

Table S1 Bacterial *gap*A, eukaryotic COI primer sequences

Table S2 DNA sequence variation in *Vibrio* haplotypes. Refer to Figure 2 for haplotype number designation. Bases in bold represent nonsynonymous mutations

Table S3 DNA sequence variation in *Euprymna* haplotypes. Due to the large amount of variation in COI, haplotypes are divided into Hawaiian, East Australian, South Australian, Phuket, and Rayong populations to examine variation. Listed is

each polymorphic site with associated base pair location for each haplotype. Haplotypes from Adelaide and Shark Bay, Australia are not included due to their high level of variation from all other sequences. Nonsynonymous sites are in bold. Refer to Figure 4 for haplotype number designation

References

Achmann R, Curik I, Dovc P *et al.* (2004) Microsatellite diversity, population subdivision and gene flow in the Lipizzan horse. *Animal Genetics*, **35**, 285–292.

Bennett I, Pope EC (1953) Intertidal zonation of the exposed rocky shores of Tasmania and its relationship with the rest of Australia. *Australian Journal of Marine and Freshwater Research*, **11**, 182–221.

Brooks D (1997) Testing the context and extent of host-parasite co-evolution. *Systematic Zoology*, **28**, 229–249.

Cherry JL (2004) Selection, subdivision and extinction and recolonization. *Genetics*, **166**, 1105–1114.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Crandall KA, Templeton AR, Sing CF (1994) *Intraspecific Cladogram Estimation: Problems and Solutions*. Clarendon Press, Oxford.

Fandry C, Cresswell G, Baines PG, Edwards RJ (1997) *The Facts: Ocean Currents Around Tasmania. Three Oceans Influence the Coastal Environment of the Island State, its Climate and Wealth and Distribution of its Natural Life*. CSIRO, Australia.

Feil EJ, Smith JM, Enright MC, Spratt BG (2000) Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. *Genetics*, **154**, 1439–1450.

Feil EJ, Holmes EC, Bessen DE *et al.* (2001) Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. *Proceedings of the National Academy of Sciences, USA*, **98**, 182–187.

Foster JS, McFall-Ngai MJ (1998) Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Development Genes and Evolution*, **208**, 295–303.

Hudson RR (2001) Two-locus sampling distributions and their application. *Genetics*, **159**, 1805–1817.

Hudson RR, Kaplan N (1985) Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics*, **111**, 147–164.

Jones BW, Nishiguchi MK (2004) Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Marine Biology*, **144**, 1151–1155.

Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK (in review) Development and application of phylogenetic probes for the *in situ* identification of symbiotic *Vibrio fischeri* and *Vibrio logei* (Proteobacteria: Vibrionaceae), in review.

Kimbell JR, McFall-Ngai MJ, Roderick GK (2002) Two genetically distinct populations of bobtail squid, *Eyprymna scolpes*, exist on the island of O'ahu. *Pacific Science*, **56**, 347–355.

Lee K-H, Ruby EG (1994) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Applied and Environmental Microbiology*, **60**, 1565–1571.

Lopez JE (2004) *Symbiosis among sepiolid squid: origins of the symbiotic light organ and examination of host population structure at the intraspecies level*, MS Thesis, New Mexico State University, New Mexico.

Marko PB (2004) 'What's larvae got to do with it?' Disparate patterns of post-glacial population structure in two benthic marine gastropods with identical dispersal potential. *Molecular Ecology*, **13**, 597–611.

McFall-Ngai MJ, Ruby EG (1998) Sepiolids and vibrios: when first they meet. *Bioscience*, **48**, 257–265.

McVean G, Awadalla P, Fearnhead P (2002) A coalescent-based method for detecting and estimating recombination from gene sequences. *Genetics*, **160**, 1231–1241.

Miller KJ, Ayre DJ (2004) The role of sexual and asexual reproduction in structuring high latitude populations of the reef coral *Pocillopora damicornis*. *Heredity*, **92**, 557–568.

Montgomery MK, McFall-Ngai M (1993) Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. *Biology Bulletin*, **184**, 296–308.

Mu J, Awadalla P, Duan J *et al.* (2005) Recombination hotspots and population structure in *Plasmodium falciparum*. *PLoS Biology*, **3**, e335.

Munson MA, Baumann P, Clark MA *et al.* (1991) Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *Journal of Bacteriology*, **173**, 6321–6324.

Munson MA, Baumann P, Moran NA (1992) Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera: Pseudococcidae) based on 16S rDNA sequences. *Molecular Phylogenetics and Evolution*, **1**, 26–30.

Nishiguchi MK (2001) Co-evolution of symbionts and hosts: the sepiolid–*Vibrio* model. In: *Symbiosis: Mechanisms and Model Systems* (ed. Seckbach J), pp. 757–774. Cole-Kluwer Academic Publishers, Dordrecht, The Netherlands.

Nishiguchi MK (2002) Host-symbiont recognition in the environmentally transmitted sepiolid squid–*Vibrio* mutualism. *Microbial Ecology*, **44**, 10–18.

Nishiguchi MK, Nair VS (2003) Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. *International Journal of Systemic Bacteriology*, **53**, 2019–2026.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid–*Vibrio* symbioses. *Applied and Environmental Microbiology*, **64**, 3209–3213.

Nishiguchi MK, Lopez JE, Boletzky Sv (2004) Enlightenment of old ideas from new investigations: more questions regarding the evolution of the bacteriogenic light organs in squids. *Evolution & Development*, **6**, 41–49.

O'Hara TD, Poore GCB (2000) Patterns of distribution for southern Australian marine echinoderms and decapods. *Journal of Biogeography*, **27**, 1321–1335.

Ovenden JR, Salini J, O'Connor S, Street R (2004) Pronounced genetic population structure in a potentially vagile fish species (*Pristipomoides multidens*, Teleostei; Perciformes; Lutjanidae) from the East Indies triangle. *Molecular Ecology*, **13**, 1991–1999.

Palo JU, Schmeller DS, Laurila A *et al.* (2004) High degree of population subdivision in a widespread amphibian. *Molecular Ecology*, **13**, 2631–2644.

Pfenninger M, Posada D (2002) Phylogeographic history of the land snail *Candidula unifasciata* (Helicellinae, Stylommatophora): fragmentation, corridor migrations, and secondary contact. *Evolution*, **56**, 1776–1788.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Posada D, Crandall KA (2001) Evaluation of methods for detecting recombination from DNA sequences: computer simulations.

Proceedings of the National Academy of Sciences USA, **98**, 13751–13756.

Ruby EG, Lee K-H (1998) The *Vibrio fischeri-Euprymna scolopes* light organ association: current ecological paradigms. *Applied and Environmental Microbiology*, **64**, 805–812.

Ruby EG, McFall-Ngai MJ (1992) A squid that glows in the night: development of an animal-bacterial mutualism. *Journal of Bacteriology*, **174**, 4865–4870.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN, version 2.000: A Sofware for Population Genetics Data Analysis*. University of Geneva, Switzerland.

Spratt BG, Hanage WP, Feil EJ (2001) The relative contributions of recombination and point mutation to the diversification of bacterial clones. *Current Opinion in Microbiology*, **4**, 602–606.

Swofford DL (2002) *PAUP*v4.08b Phylogenetic analysis using parsimony (*and other methods)*. Sinauer Associates: Sunderland MA.

Templeton AR (2001) Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology*, **10**, 779–791.

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659–669.

Templeton AR, Beorwinkle E, Sing CF (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 343–351.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. III. Cladogram estimation. *Genetics*, **132**, 619–633.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Abystoma tigrunum*. *Genetics*, **140**, 767–782.

Visick KL, Foster J, Doino J, McFall-Ngai M, Ruby EG (2000) *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. *Journal of Bacteriology*, **182**, 4578–4586.

Waters JM, Roy MS (2003) Marine biogeography of southern Australia: phylogeographical structure in a temperate sea-star. *Journal of Biogeography*, **30**, 1787–1796.

Whiteley AR, Spruell P, Allendorf FW (2004) Ecological and life history characteristics predict population genetic divergence of two salmonids in the same landscape. *Molecular Ecology*, **13**, 3675–3688.

B.W. Jones is currently a postdoctoral research associate at University of Calgary Medical School, and is interested in the evolution of pathogenesis. J.E. Lopez is a medical student at the University of New Mexico, and has interests in eukaryotic-bacterial interactions. J. Huttenberg is a PhD student at the University of Wisconsin, Madison, and has interests in microbial symbiosis. M.K. Nishiguchi is an associate professor of Biology at New Mexico State University, whose research encompasses the evolution of symbiotic associations.

Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism

B.W. Jones and M.K. Nishiguchi

Abstract: A major force driving in the innovation of mutualistic symbioses is the number of adaptations that both organisms must acquire to provide overall increased fitness for a successful partnership. Many of these symbioses are relatively dependent on the ability of the symbiont to locate a host (specificity), as well as provide some novel capability upon colonization. The mutualism between sepiolid squids and members of the *Vibrionaceae* is a unique system in which development of the symbiotic partnership has been studied in detail, but much remains unknown about the genetics of symbiont colonization and persistence within the host. Using a method that captures exclusively expressed transcripts in either free-living or host-associated strains of *Vibrio fischeri*, we identified and verified expression of genes differentially expressed in both states from two symbiotic strains of *V. fischeri*. These genes provide a glimpse into the microhabitat *V. fischeri* encounters in both free-living seawater and symbiotic host light organ-associated habitats, providing insight into the elements necessary for local adaptation and the evolution of host specificity in this unique mutualism.

Key words: Vibrionaceae, gene expression, Sepiolidae, *Euprymna*, SCOTS.

Résumé : La principale force motrice régissant les nouvelles symbioses est le nombre d'événements adaptatifs que les deux organismes doivent acquérir en vue d'une aptitude accrue à établir un partenariat fructueux. Plusieurs de ces symbioses sont relativement dépendantes de la capacité des symbiotes à s'établir dans un hôte (spécificité), et à lui fournir de nouvelles caractéristiques lors de la colonisation. Le mutualisme entre les pieuvres de l'ordre des Sépiolidées et les membres de la famille des Vibrionacées constitue un système unique dans lequel le développement du partenariat symbiotique a été étudié en détail, mais il reste encore beaucoup de zones inconnues quant à la génétique de la colonisation du symbiote et sa persistance dans l'hôte. Grâce à une méthode qui permet de détecter des transcrits exclusivement exprimés, soit dans des souches de *Vibrio fischeri* libres, soit associées à l'hôte, nous avons identifié et vérifié l'expression de gènes exprimés de façon différentielle dans les deux formes de souches symbiotiques de *V. fischeri*. Ces gènes donnent un aperçu du micro-habitat que *V. fischeri* rencontre en eau libre ou associé aux organes de l'hôte symbiotique, donnant une idée des éléments nécessaires à l'adaptation locale et à l'évolution de la spécificité pour l'hôte dans ce mutualisme unique.

Mots clés : Vibrionacées, expression génique, Sépiolidées, *Euprymna*, SCOTS.

[Traduit par la Rédaction]

Introduction

While much recent work on mutualistic associations has focused on symbiont evolution through identification of colonization factors or identification of coevolving host–symbiont associations and their specificity to closely related hosts (parallel cladogenesis) (Hafner et al. 1994; Parker et al. 2004), few studies have examined how both ecology (abiotic) and host (biotic) factors dictate symbiont fitness (Thompson 1999; Thompson and Cunningham 2002; Barneah et al. 2004). In particular, the means of transmission (vertical, horizontal, or environmental) can determine whether bacteria are selected solely for a host species or whether there are certain trade-offs for viability in both the environment and host (Sicard et al. 2003; Stewart et al. 2005). These trade-offs between two different sets of phenotypes are crucial for determining how quickly bacterial species can adapt to novel hosts and their surroundings. Determining which adaptations are important for driving specialization between host and environmental niches can help predict such forces affecting microbial diversity through speciation (Rainey and Travisano 1998; DeLong and Karl 2005).

Symbiosis between bobtail squids (Cephalopoda: Sepiolidae) and luminous bacteria (γ-Proteobacteria: *Vibrionaceae*) has been previously studied to examine the evolution and specificity of environmentally transmitted symbioses (Nishiguchi 2002). Upon colonization of the squid host light organ, symbiotic bacteria (*Vibrio fischeri*) benefit from high growth rates compared with those free-living in seawater, while the host uses bioluminescence produced by *V. fischeri* for counter-illumination (Ruby and Asato 1993; Jones and Nishiguchi 2004). The initiation of this association has been well studied, and colonization occurs in a highly specific manner, resulting

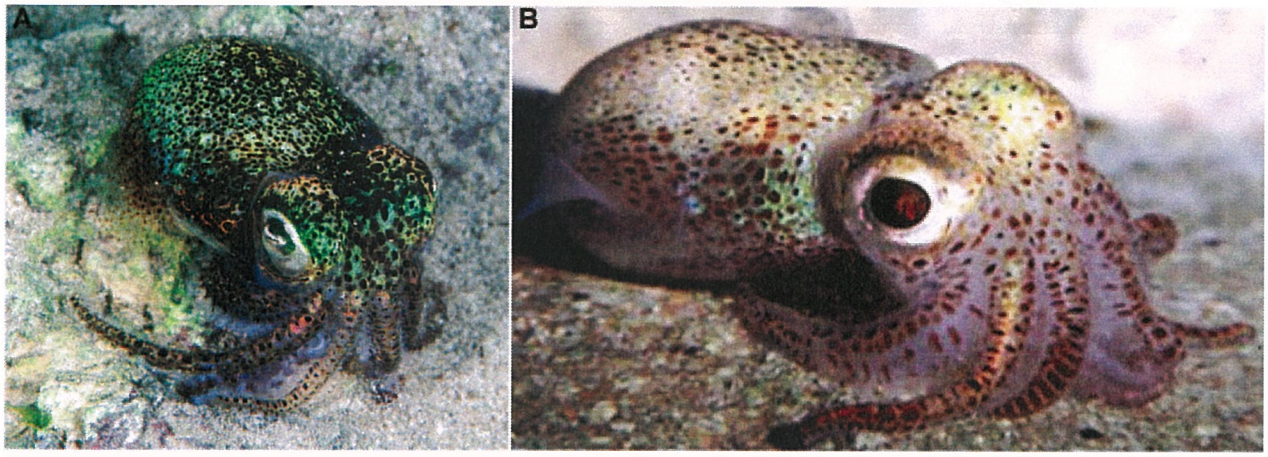
Received 24 May 2006. Revision received 24 July 2006. Accepted 25 August 2006. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 18 January 2007.

B.W. Jones and M.K. Nishiguchi.[1] Department of Biology, MSC 3AF, New Mexico State University, P.O. Box 30001, Las Cruces, NM 88003-8001, USA.

[1]Corresponding author (e-mail: nish@nmsu.edu).

 doi:10.1139/W06-088

Fig. 1. Two allopatric species of bobtail squid from the Indo-west Pacific used in this study. (A) *Euprymna tasmanica* (reprinted from *Cephalopods, A World Guide* with the kind permission of ConchBooks, D-Hackenheim, Germany, and Mark Norman). (B) *Euprymna scolopes* (photograph provided courtesy of Ron Holcolm).



in a number of physiological and morphological changes in both the symbiont and host (McFall-Ngai and Ruby 1991, 1998; Nyholm et al. 2000; McFall-Ngai 2002).

In vivo competition studies with the Hawaiian bobtail squid *Euprymna scolopes* have demonstrated that strains of *V. fischeri* are highly adapted to their native hosts (Nishiguchi et al. 1998; Nishiguchi 2002). In these experiments, *V. fischeri* strains isolated directly from a number of *Euprymna* hosts (including *Euprymna tasmanica* from Australia and *Euprymna morsei* from Japan) were used to inoculate newly hatched axenic *E. scolopes* individuals. While each strain was able to colonize *E. scolopes*, the native *E. scolopes* strain was always found to dominate the light organ contents after 48 h of infection (Nishiguchi et al. 1998). For example, after 48 h of infection, the ratio of the Hawaiian strain *V. fischeri* ES114 to the Australian strain ET101 was 95:5 (Nishiguchi et al. 1998), suggesting the native ES114 is able to out compete non-native strains despite their high degree of relatedness (96% sequence identity between ES114 and ET101 *gapA* sequences; Nishiguchi et al. 1998). Subsequent work has not yet revealed the underlying factors that confer this fitness advantage to local strains of *V. fischeri*.

The mode of symbiont transmission provides clues to how local adaptation has developed in this system. Environmental transmission occurs through a host-controlled daily ritual of venting excess vibrios into local waters, creating an inoculum from which naïve (axenic) juvenile squids can obtain their symbionts (McFall-Ngai and Ruby 1991; Jones et al. 2007). This cycling of local symbionts has likely set the stage for the evolution of host specificity observed in Indo-west Pacific *Euprymna* – *V. fischeri* populations (Nishiguchi et al. 1998). Since *Vibrio* fitness is higher within the light organ (doubling time of the population is approximately 4 h (Ruby and Asato 1993)) compared with seawater, selection should favor strains that are highly adapted to the light organ environment, as seen in competitive studies (Nishiguchi et al. 1998; Nishiguchi 2002). Consequently, native light organ strains should out-compete other less fit strains and potentially evade daily host expulsion. Expelled strains must also face selection for persistence in the environment until they encounter and colonize another light organ in a new host squid. These opposing selective pressures are thus likely to be common forces underlying the evolution of environmental transmission.

To better understand these selective pressures involved in environmental persistence and infection by bacteria, we have identified genes expressed exclusively either in seawater or during host infection by *V. fischeri*, using selective capture of transcribed sequences (SCOTS; Graham and Clark-Curtiss 1999), a method used to compare gene expression of the same bacterium in two distinct environments. SCOTS has the advantage over other methods, such as microarray analysis, of identifying strain-specific genes that may not be present in the genomes of sequenced conspecifics (Daigle et al. 2002). This method has successfully been implemented to identify genes expressed by *Listeria monocytogenes* growing at low temperature (Liu et al. 2002), *Mycobacterium avium*, *Mycobacterium tuberculosis*, and *Salmonella typhi* genes expressed in human macrophages (Graham and Clark-Curtiss 1999; Daigle et al. 2001; Hou et al. 2002), and *Helicobacter pylori* genes expressed in human gastric mucosa (Graham et al. 2002). Until now, SCOTS has been used to compare in vivo expression with expression of broth-grown bacteria, mostly because of the limitations of the different systems being studied. Here, we present results of experiments examining environmental (grown in seawater) and host-associated (grown in the light organ) gene expression by two strains of *V. fischeri*, each isolated directly from field-captured adult hosts *E. scolopes* (of Hawaii) and *E. tasmanica* (of Australia; Fig. 1).

Materials and methods

Bacterial growth conditions and RNA extraction

Gene expression from the identical *V. fischeri* strain grown in both the light organ of its host and in seawater was examined with SCOTS. To obtain host-associated *V. fischeri* RNA, strains were obtained directly from the light organ of *E. scolopes* (from Hawai'i-Kai Bay, O'ahu, Hawaii) or *E. tasmanica* (from Jervis Bay, New South Wales, Australia). The light

Table 1. Primers used in selective capture of transcribed sequences (SCOTS) and verification procedures.

Name	Sequence (5′→3′)	Use
Xba random*	TGCTCTAGACGTCCTGATGGTT9(N)	cDNA library construction
Xba*	TGCTCTAGACGTCCTGATGGTT	Amplification of cDNA
Sal random*	ATATGTCGACTGAATTCCGTAGG9(N)	cDNA library construction
Sal*	ATATGTCGACTGAATTCCGTAGG	Amplification of cDNA
16S forward	AGAGTTTGATCMTGGCTCAG	Ribosomal operon cloning
23S reverse	ATGGTTAAGCCTCACGGGCA	Ribosomal operon cloning
flrA sense	AGGCGGCAATCAAAGTATTCGTGC	RT–PCR verification
flrA antisense	CAGCTAATGCCGCTTGTTCTTGCT	RT–PCR verification
Arginine decarboxylase sense	GTTAAACCACGTCTTGGCTTGCGT	RT–PCR verification
Arginine decarboxylase sense	ACCACCACCCACATCGAGGTATTT	RT–PCR verification
mshA sense	TAAACCTGCAATCTGATGCCGTGC	RT–PCR verification
mshA antisense	GATAGACCAACTACCGCTTCAGCA	RT–PCR verification

*Graham and Clark-Curtiss 1999.

organs were dissected immediately after capture of the animals during the evening. One-half of the light organ was plated on Luria–Bertani high-salt plates (Ruby 1993) to isolate the strain present in the host, and the other half of the light organ was placed in RNAlater® (Ambion, Austin, Tex.) until RNA extraction. The strains from *E. tasmanica* and *E. scolopes* hosts were designated ETJB1A and ESP915, respectively. Prior to RNA extraction, the organs were homogenized in RNAlater® (Ambion) and then centrifuged at 12 000*g* for 2 min to pellet eukaryotic tissue. Bacteria in the supernatant was then pelleted at 16 000*g* for 10 min.

To obtain RNA from seawater-grown *V. fischeri*, both ETJB1A and ESP915 were grown for 48 h to OD_{600} of ~0.5 (approximately 2.5×10^8 cells/mL) in sterile, filtered (0.2 μm pore size) seawater supplemented with 0.1% (*m/v*) chitin at 28 °C. Prior to RNA extractions, the sample was filtered through an 8 μm filter to remove excess chitin and was pelleted at 16 000*g* for 10 min.

RNA extractions were performed using a protocol modified from Mangan et al. (1997). Bacterial pellets were washed in 0.5% Tween-80 prior to resuspension in 200 μL of diethylpyrocarbonate-treated water. The cleaned cells were then homogenized in a bead beater at 4200 r/min (1 r = 2πrad) for 50 s in a suspension of 1 g zirconium–silica beads (0.1 mm diameter), 600 μL of an acid-equilibrated phenol – chloroform – isoamyl alcohol mixture (25:24:1 (by volume) pH 4.7), and 500 μL of STET buffer (8% (*m/v*) sucrose, 5% (*v/v*) Triton X-100, 50 mmol/L ethylene diamine tetraacedic acid (EDTA), and 50 mmol/L Tris (pH 6.80)). After being equilibrated at 4 °C for 5 min, each sample was centrifuged for 5 min at 16 000*g* to remove cellular debris and then chloroform extracted. RNA was then precipitated in 1× volume of alcohol solution (0.3 mL 3 mol/L sodium acetate and 49.7 mL 2-propanol) at –20 °C for 1 h. RNA was pelleted at 13 000*g* for 15 min at 4 °C and resuspended in diethylpyrocarbonate-treated water and 0.1% SDS (sodium dodecyl sulfate).

Prior to cDNA library construction for seawater and light-organ-derived RNA, contaminating DNA was removed using DNA-*free*™ (Ambion), as per manufacturer's directions. After this, removal of rRNA with MICROB*Express*™ (Ambion), following the manufacturer's protocol, was necessary to eliminate contaminating rRNA-derived cDNAs during the SCOTS procedure.

cDNA library construction

All primers used in this study are listed in Table 1. Random cDNA libraries were constructed using identical primers and the methodology of Graham and Clark-Curtiss (1999, 2000). Importantly, the seawater-derived and the light-organ-derived *V. fischeri* cDNA libraries each contained linkers enabling specific polymerase chain reaction (PCR) amplification of each library (the Xba primer was used for light organ libraries, and the Sal primer was used for seawater libraries). First-strand cDNA was synthesized with Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, Calif.), according to manufacturer's directions, using 1 μg of each primer with a known 5′ end and random 9-mer at the 3′ end (Table 1). Second-strand cDNA was synthesized using the Klenow fragment of DNA polymerase (New England Biolabs, Beverly, Mass.), as per instructions. Double-stranded cDNA was then passed over a PCR purification column (Qiagen, Valencia, Calif.) to remove remaining salts, enzyme, and unincorporated nucleotides.

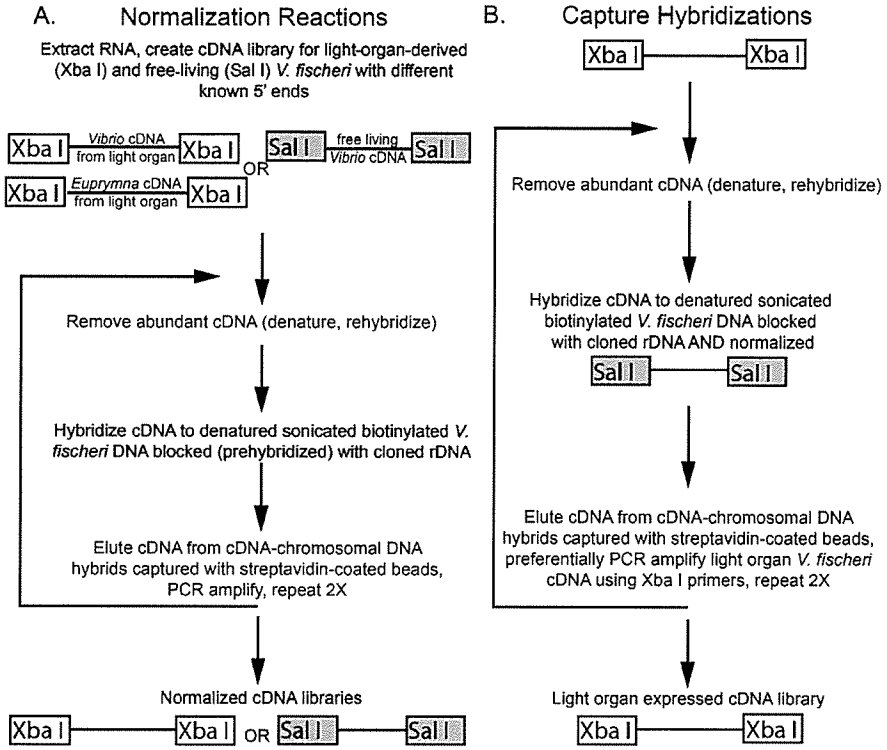
Isolation of rRNA operon

To block binding of rRNA-derived cDNA during SCOTS, the *V. fischeri* ESP915 ribosomal operon was PCR amplified in a 50 μL reaction containing 2.5 mmol/L magnesium chloride, 1× buffer, 0.2 mmol/L each dNTP, 2 U *Taq* polymerase (Continental Lab Products, San Diego, Calif.), and 0.2 μmol/L each primer (16S forward and 23S reverse; Table 1) with an annealing temperature of 48 °C and 5 min extension for 35 cycles. The ~5 kb product was excised from a 1% agarose gel and cloned using the PCR-XL TOPO cloning kit (Invitrogen), following manufacturer's directions.

SCOTS

SCOTS was performed in an identical manner for both ESP915 and ETJB1A, hybridizing the cDNA of each strain against its own DNA. In addition, subtractions were completed against both the seawater and light-organ-derived cDNA libraries to identify genes expressed in each environment by both strains. Three rounds of normalization hybridizations were used to normalize the cDNA libraries and to eliminate host cDNA contamination (Fig. 2A). Before initial normalization hybridizations, 10 μL of each cDNA library was amplified via PCR in 10 separate reactions for each library,

Fig. 2. The selective capture of transcribed sequences (SCOTS) procedure (modified from Graham and Clark-Curtiss 1999). To identify genes expressed by *Vibrio fischeri* exclusively in the light organ, cDNA libraries for light-organ-derived and seawater-derived *V. fischeri* were created. Following a series of three normalization reactions (A), seawater-derived *V. fischeri* cDNAs were used to block during capture hybridizations (B), followed by preferential amplification of the light-organ-derived *V. fischeri* cDNA library. The equivalent procedure was used to identify *V. fischeri* genes expressed exclusively in seawater.



using *Taq* polymerase with an annealing temperature of 52 °C. These PCR reactions were then pooled, and 2.5 initial PCR reactions (125 μL PCR product) were used in the first round of capture hybridizations (Fig. 2B; Graham and Clark-Curtiss 1999).

Thirty micrograms of sonicated, biotinylated, genomic *V. fischeri* ESP915 or ETJB1A DNA and 100 μg of sonicated, cloned, *V. fischeri* ESP915 rRNA operon-containing plasmids (isolated above) were suspended in 40 μL of 10 mmol/L 3-[4-(2-hydroxyethyl)-1-peperazinyl] propanesulfonic acid (EPPS; Sigma, St. Louis, Mo.) and 1 mmol/L ethylene diamine tetraacetic acid (EDTA) and were heated to 98 °C for 2 min. Similarly, 2.5 50 μL PCR reactions (125 μL total) for each library were precipitated and resuspended in 40 μL of the same buffer. Each mixture was then incubated for 30 min at 60 °C. During this time, all rDNA sites were blocked by hybridization of the cloned rRNA operon in the tubes containing genomic DNA, and common cDNAs were allowed to self-hybridize in each tube containing cDNA. After this, 10 μL of 1 mol/L sodium chloride was added to each tube. All mixtures were incubated for an additional 30 min at 50 °C, after which the genomic DNA–rRNA hybrids were added to each cDNA library for overnight hybridization at 50 °C. After hybridizations, 100 μL of water was added to each sample and placed in ice.

Biotinylated genomic DNA–cDNA hybrids were recovered with streptavidin-coated beads and a magnetic separator (Dynal, Oslo, Norway). Briefly, washed beads were resuspended in 100 μL of 10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, and 2 mol/L NaCl, and then the hybridized sample was added. cDNA was then eluted from genomic DNA with 100 μL each of 0.5 mol/L NaOH and 0.1 mol/L NaCl. Eluted cDNAs were then amplified in 10 parallel PCR reactions (using the same conditions as above). After PCR, the cDNA was once again passed over a PCR purification column (Qiagen). Rounds two and three of normalization hybridizations used the cDNA from the previous round, but all volumes were one-tenth of those from the first round.

The cDNA capture procedure was performed in a nearly identical manner to the normalization hybridizations, with the exception that 25 μL of the third round normalized cDNA was added to the sonicated, cloned rRNA plasmid and sonicated, biotinylated, genomic DNA mixture. When identifying cDNAs exclusively from the light organ, normalized cDNA from the seawater library was used, blocking each site expressed in seawater. Similar to the normalization hybridizations, this sample, along with the third round normalized cDNA (the light-organ-derived cDNA library when identifying light-organ-expressed genes), was denatured and allowed to partially hybridize before the addition of 1 μL of 1 mol/L NaCl. These two mixtures were then combined and allowed to hybridize overnight. The hybrids were collected with streptavidin-coated beads, and cDNA was eluted and cleaned as described above. After PCR amplification with

the primer appropriate for the differentially expressed sequences of interest, the products were cloned with the TOPO Cloning kit (Invitrogen), as per manufacturer's directions.

Primary verification using Southern hybridizations and sequencing

To eliminate false-positive cDNA sequences from recovered seawater or light-organ-derived clones, Southern hybridizations were employed. Clone libraries were denatured by heating each sample to 98 °C for 10 min in denaturing solution (0.4 mol/L NaOH, 10 mmol/L EDTA, final concentration), blotted onto a positively charged nylon membrane using a 96 well blotting apparatus (Bio-Rad Laboratories, Hercules, Calif.), and cross-linked using a UV Stratalinker (Stratagene, La Jolla, Calif.). The clones were then hybridized against a digoxigenin-labeled probe created from the third round captured sequences used to block during the enrichment reactions. In doing so, only the clones expressed in the final enrichment reactions that were expressed in both capture hybridizations would bind during this procedure, allowing elimination of false-positive sequences that escaped the subtraction process.

To perform Southern hybridizations, the membranes were hybridized overnight at 42 °C with 25 ng of probe in 3.5 mL DIG EasyHyb hybridization solution that was added to the membrane after prehybridization in 10 mL hybridization solution for 30 min at 42 °C. The membranes were then washed twice for 5 min each in low stringency buffer (2× SSC, 0.1% SDS), followed by two 15 min washes at 65 °C in high stringency buffer (0.5× SSC, 0.1% SDS). Hybridizations were detected using the chemiluminescent substrate CSPD-Star (Roche Diagnostics Corporation, Indianapolis, Ind.) and a ChemiDoc XRS System (Bio-Rad Laboratories).

Hybridization signals indicated a false-positive result, since not all sequences were exclusive to one library. No hybridization signal indicated a gene expressed exclusively in the light organ or seawater. Distinct clones were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, Calif.) and previous protocols (Nishiguchi and Nair 2003). The identity of the sequences was determined by using the National Center for Biotechnology Information BLAST function to search GenBank for sequences similar to those discovered using SCOTS. In addition, the newly published *V. fischeri* genome (Ruby et al. 2005) was searched to find identical sequences. Clones were placed in one of five categories, including metabolic genes, stress-related genes, regulatory genes, membrane-associated genes, and genes of unknown function. Among the genes of unknown function, genes were categorized as hypothetical proteins and those having no known homology.

Secondary verification using reverse transcriptase PCR

Based on the function of discovered clones, three clones were picked for secondary verification. These included the seawater-expressed mannose-sensitive hemagglutin (*mshA*), the light-organ-expressed arginine decarboxylase, and the flagella regulator *flrA*. Based on the *V. fischeri* genomic sequence of each of these genes, primers were developed (Table 1) to amplify 428 bp (*flrA*), 294 bp (arginine decarboxylase), and 145 bp (*mshA*) fragments by reverse transcriptase (RT)–PCR using the SuperScript™ III One-Step RT–PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen).

Each 25 μL of reaction mixture contained 12.5 μL of 2× buffer (supplied by manufacturer), sense and anti-sense primer final concentrations of 4 μmol/L, 1 μL of SuperScript™ III Reverse Transcriptase and Platinum® *Taq* DNA polymerase mixture, and either 100 or 1 ng of seawater or light-organ-derived RNA. The reaction consisted of 30 min of incubation at 55 °C for first strand synthesis, denaturation at 94 °C for 2 min, 40 cycles each of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 30 s, followed by a final extension of 68 °C for 7 min.

Results and discussion

SCOTS has been used to identify exclusively expressed cDNA of bacteria cultivated in a natural environment and in broth (Graham and Clark-Curtiss 1999, 2000; Daigle et al. 2001, 2002; Graham et al. 2002). These experiments have a minor limitation in that bacteria growing in broth undoubtedly express many genes identical to those of bacteria in the natural environment, resulting in a cDNA library that does not completely describe the ecological state being studied. Here, we have applied SCOTS to examine gene expression in two natural environments: *V. fischeri* grown in the light organs of field-caught sepiolid squid and the same strain isolated from these hosts grown in chitin-supplemented seawater. This being so, the expressed genes identified in this study provide a unique chance to examine the microhabitats to which *V. fischeri* must adapt (Tables 2 and 3).

Primary verification using Southern hybridizations provided evidence that there was no rRNA contamination after three rounds of capture hybridizations. Furthermore, using this procedure, a number of clones were eliminated from the libraries (Fig. 3). After elimination of the clones that escaped the subtraction procedure, a total of 53 clones were sequenced from seawater-expressed libraries. Nineteen of these belonged to *V. fischeri* derived from *E. scolopes*, whereas the other 34 were from *V. fischeri* derived from *E. tasmanica*. Among the 54 light-organ-derived clones, 33 were from *V. fischeri* of *E. tasmanica*, and 21 were from *V. fischeri* of *E. scolopes* (Tables 2 and 3). Interestingly, host-associated *V. fischeri* gene expression was very consistent between hosts. Southern hybridizations using the enriched cDNA from *E. scolopes* light-organ-derived *V. fischeri* as a probe against the analogous *V. fischeri* library from the light organ of *E. tasmanica* indicated that each clone was expressed in both hosts (data not shown). The remarkable similarity of *V. fischeri* expression between hosts indicates that local adaptation is not due to exclusive expression of single or a few genes but is likely due to changes in levels of expression of different genetic pathways coupled with small mutational changes; a view of adaptation that is prevalent in the literature (Johnson and Porter 2001; Johannesen and Hansen 2002; West-Eberhard 2005). Many of the genes and pathways identified here represent a starting point for future studies that will identify the specifics of local adaptation by *V. fischeri* at the genetic level.

Vibrio fischeri that colonize host tissues are at an advantage over their environmental counterparts for a number of reasons. Bacteriovory is limited, the light organ is nutrient

Table 2. Genes expressed by *Vibrio fischeri* in the light organs of *Euprymna scolopes* and *Euprymna tasmanica*.

Class	Clone name*	ES114 open reading frame	Genes coding for protein
Metabolism	etx17	VFA0839	Arginine decarboxylase
	etx20	VF1740	3-Oxoacyl-[acyl-carrier protein] reductase
	etx35	VF1932	Acyl-CoA dehydrogenase
	etx38	VF0772	Pyruvate dehydrogenase E1 component
	etx42	VF2440	Oligopeptide transport ATP-binding protein (OppD)
	etx46	VF1262	Glutaredoxin
	etx52	VF1590	Formate acetyltransferase
	etx57	VF1363	Formate hydrogenlyase (FdhF)
	etx60	VFA0004	Peptidase T
	etx65	VF2251	GatB/Yqey domain protein
	etx67	VF0060	3-Polyprenyl-4-hydroxybenzoate decarboxylase
	etx68	VF1302	Cytochrome-*c* oxidase, diheme subunit
	etx19	VF0439	*S*-Adenosylmethionine synthetase
	eslo4	VF0198	UDP glucose 6-dehydrogenase
	eslo5	VF0480	Dihydropteroate synthase (FolP)
	eslo15	VF2402	Uroporphyrinogen decarboxylase (HemE)
	eslo18	VF2340	Allosteric 6-phosphofructokinase
	eslo20	VF1590	Formate acetyltransferase
	eslo34	VF2258	Pyruvate kinase
	eslo7	VFA1078	Acetyltransferase and (or) hydrolase family protein
Stress	etx24	VF2539	Sporulation-control protein
	etx66	VF1263	Mercuric reductase
Membrane	etx29	VFA0971	Vitamin B12 transport ATP-binding protein (BtuD)
	eslo78	VFA1047	Mg^{2+} transporter MgtE
	etx47	VFA0189	Cytochrome-*c*-type protein TorC
	etx23	VF1065	Integral membrane protein with TRKA (tyrosine kinase *a*) domains
	eslo3	VF1856	Transcriptional activator FlrA
	eslo31	VF1193	Mechanosensitive ion channel
	eslo61	VFA0736	ABC transporter permease protein
	etx16	VF2108	Mechanosensitive ion channel
Regulatory	etx22	VF0551	tRNA (Guanine-N(1)-)-methyltransferase
	etx26	VF2477	33 kDa chaperonin
	etx49	VF0241	Large subunit ribosomal protein L22P
	etx27	VF1784	tRNA methyltransferase
	etx63	VF1759	Small subunit ribosomal protein S1P
	etx64	VF0074	DNA polymerase I
	etx31	VF0231	Small subunit ribosomal protein S7P
	eslo17	VF1689	Hnr protein
	eslo23	VF1748	Ribonuclease E
	eslo28, etx37, etx40	All VF2412	DNA-directed RNA polymerase beta chain
	eslo33	VF1891	Cell division protein ZipA
	eslo38	VF1204	DNA gyrase subunit A
Unknown	etx28, etx43, eslo30	VF0882, VF1653, VF0882	Hypothetical proteins
	etx51, etx62	Both VFA1018	Hypothetical cytosolic proteins
	eslo75	VF2401	Hypothetical protein YhgL
	eslo8, eslo9, etx45	Not applicable	No known homology
	eslo66	pES213 OR5	*V. fischeri* plasmid open reading frame

*Clones eslo and etx were derived from *Euprymna scolopes* and *Euprymna tasmanica*, respectively.

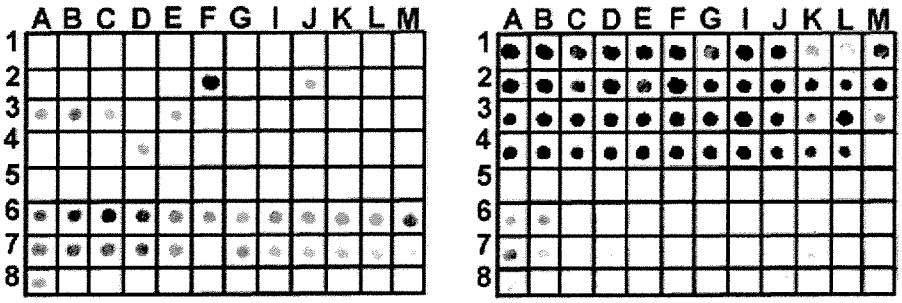
rich (Graf and Ruby 1998), and growth rates are much higher (Ruby and Asato 1993). For these reasons, environmental expression of genes necessary for adhesion during the initiation of colonization is critical. The seawater-expressed pilin synthesis gene, *pilM*, and a mannose-sensitive hemagglutin, *mshA*, are two genes that are likely important to the initial steps of colonization. Significantly, *V. fischeri* with *pilA* deletions have a decreased competitive ability against parent wild-type strains when both strains are introduced to uninnoculated *E. scolopes* juveniles (Stabb and Ruby 2003). In addition, comparative genetic studies of *pilA* (which is in the same operon as *pilM*) suggest this gene may be involved in local adaptation to host species (Brown-Silva and Nishiguchi in review), creating the competitive advantage

Table 3. Genes exclusively expressed by *Vibrio fischeri* strains isolated from *Euprymna tasmanica* and *Euprymna scolopes* in seawater.

Class	Clone name*	ES114 open reading frame	Genes coding for protein
Metabolism	essw35	VF2554	1-Acyl-*sn*-glycerol-3-phosphate acyltransferase
	essw11	VF2537	Phosphoribosylaminoimidazole carboxylase
	essw7	VF2383	Acetyl-CoA synthetase
	essw37	VF0480	Phosphoglucomutase and (or) phosphomannomutase family protein MrsA
	essw36	VF0214	Phosphoribulokinase
	essw40	VF2461	Thioredoxin
	ets05	VF1743	Fatty acid and (or) phospholipid synthesis protein PlsX
	ets10	VF0758	PhoH-like protein
	ets13	VF0543	Glutamate–cysteine ligase
	ets18	VF2271	Maltose operon periplasmic protein precursor
	ets27	VF0531	Cytosine deaminase
	ets38	VF0772	2-Dehydro-3-deoxyphosphooctonate aldolase
	ets39	VF0063	HemY protein
	ets52	VF1972	Isocitrate lyase
	ets56	VFA0099	Glutamate synthase (NADPH) small chain
	ets53	VF2234	D-Glycero-D-manno-heptose-7-phosphate 1-kinase
	ets61	VF0406	Aspartate carbamoyltransferase
	ets66	VF1070	CDP-4-dehydro-6-deoxy-D-glucose 3-dehydratase
	ets71	VF0077	2-Polyprenyl-3-methyl-5-hydroxy-6-metoxy-1,4-benzoquinol methylase
Stress	essw16	VFA0916	Na^+ driven multidrug efflux pump
	essw29	VF2341	Cobalt–zinc–cadmium resistance protein CzcD
	essw30	VF0720	*ampG* protein
Membrane	essw23	VF0031	Chloride channel protein
	essw24	VF2518	Dipeptide-binding protein
	essw38	VF0322	Transporter
	ets75	VF1304	Copper-exporting ATPase
	ets14	VFA1022	Transporter
	ets40	VF2440	Na^+ driven multidrug efflux pump
	essw17	VF2297	Pili assembly protein PilM
	essw28	VF1137	Putative lipoprotein
	ets16	VF0436	Surface protein
	ets20	VF0366	Mannose-sensitive hemagglutin MshA
	ets36	VF0367	Type II secretory pathway pseudopilin PulG
Regulatory	essw39	VF0454	Transcriptional regulator
	essw20	VF1707	Ribonuclease D
	ets03	VFA0750	Ribosomal-protein-alanine acetyltransferase
	ets17	VF0548	Signal recognition particle, subunit FFH/SRP54
	ets22	VF2345	23S rRNA methyltransferase
	ets19	VF2545	16S rRNA m(5)C 967 methyltransferase
	ets23	VF0425	Xanthosine triphosphate pyrophosphatase
	ets67	VF0971	GTP-binding protein HflX
Unknown	essw18	VF1638	Nif3 family protein
	essw19, ets12, ets15, ets63	VF0898, VFA0908, VF1166, VF2500	Hypothetical proteins
	essw33	VF2071	Hypothetical cytosolic protein
	ets24	VFA0834	*nirV* precursor
	ets64, ets65	Not applicable	No known homology
	ets76, ets70	VFA0756, VF1495	Conserved hypothetical protein

*Clones ets and essw were derived from *Euprymna tasmanica* and *Euprymna scolopes*, respectively.

Fig. 3. Primary verification of all *Vibrio fischeri* clones derived from *Euprymna scolopes*. Lanes 1A-4L are light organ expressed cloned cDNAs (right panel), while lanes 6A–8A are seawater expressed cloned cDNAs (left panel). In the left panel, a probe created from the normalized seawater libraries shows a number of light organ clones that escaped the selection procedure (2F, 2J, 3A–3C, 3E, 4D), whereas every seawater-exclusive clone gives signal. In the right panel, a probe created from normalized light organ libraries produces a signal in every light-organ-exclusive library as well as six clones that escaped the selection procedure (6A, 6B, 7A, 7B, 7K, 8A). The analogous procedure was performed for clones derived from *Euprymna tasmanica*.

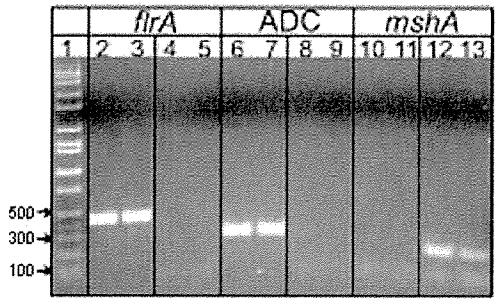


over non-natives seen in colonization experiments (Nishiguchi et al. 1998). The contribution of *mshA* to the ability of *V. fischeri* to colonize *E. scolopes* has yet to be examined, but it is known that in *V. cholerae*, *mshA* is necessary for biofilm formation (Watnick and Kolter 1999; Watnick et al. 1999; Moorthy and Watnick 2004), suggesting a role in adherence to host cells. Secondary verification using RT–PCR further supported expression of *mshA* (Fig. 4).

In addition to *pilA* and *mshA*, the functional identity of a number of other genes found in this study is supported by previous work on *E. scolopes* and *V. fischeri* ES114 (a native Hawaiian strain). For example, Arora et al. (1997, 1998) demonstrated that the expression of *flrA*, a motility master regulator, is necessary for mucin adhesion during *Pseudomonas aeruginosa* infection. Mucin adhesion is also critical to *V. fischeri* because it must adhere to a string of mucus from the host ciliated epithelia appendages before moving to the light organ pores upon initial host colonization (Nyholm et al. 2000, 2002; Nyholm and McFall-Ngai 2003). Previous infection studies of *E. scolopes* using *V. fischeri flrA* mutants have shown that expression is essential to normal colonization in the symbiosis (Millikan and Ruby 2004). Since *flrA* was also found to be expressed in adult light organs (further confirmed using RT–PCR; Fig. 4), it can be hypothesized that flagellar expression may be necessary to avoid daily expulsion by the host. Interestingly, previous studies have shown that *V. fischeri* lose their flagella upon colonization (Ruby and Asato 1993). It is not known, however, if flagellation in the light organ may differ depending on symbiont spatial orientation (associated with host epithelial cells or in the middle of the host crypt space) or time of day (growth rates are increased as the light organ is repopulated during the day). It is worth noting that hosts were collected during the evening when *Vibrio* growth rates are near their maximum rate. Further examination of flagellation within the host light organ is necessary to understand the expression of this regulator.

Colonization of host tissues also appears to be accompanied by a shift in metabolism. In contrast to genes for aerobic respiration found exclusively in seawater (Table 3), *V. fischeri* expresses a number of genes involved in anaerobic pathways, respiration, and oxidative metabolism inside the light

Fig. 4. Secondary verification of *flrA* (lanes 2–5), arginine decarboxylase (ADC, lanes 6–9), and *mshA* (lanes 10–13) transcripts using reverse transcriptase polymerase chain reaction. Lane 1: 1 kb ladder. Lane pairs 2–3, 6–7, and 10–11, 1× and 1:100× dilutions, respectively, of *Euprymna tasmanica* light-organ-derived *Vibrio fischeri* RNA. Lane pairs 4–5, 8–9, and 12–13, 1× and 1:100× dilutions, respectively, of seawater-derived *V. fischeri* RNA. *flrA* and ADC are expressed exclusively in the light organ, while *mshA* is expressed only in seawater. Controls using DNA polymerase verified the absence of DNA contamination in the RNA samples (not shown).



organs of both hosts (Table 2). This diverse metabolism by *V. fischeri* inside the host may be due to microhabitat differences within the light organ and the fact that bioluminescence reactions consume large amounts of oxygen. This could result in anaerobic microenvironments in which *V. fischeri* must persist.

If fermentative processes are common in the light organ, the process can be potentially hazardous for *Vibrio* bacteria, since acidic by-products excreted into the local environment may accumulate, lowering the pH of the light organ. It appears that *Vibrio* may tolerate this acidity through the expression of arginine decarboxylase, an enzyme involved in pH regulation as part of homeostasis (Park et al. 1996). As with *mshA*, secondary verification by RT–PCR of arginine decarboxylase confirmed exclusive expression in the light organ (Fig. 4). The potential reduction of the light organ pH may also have unidentified effects in terms of persistence of the symbiont. For example, expression of *toxR*, a virulence

regulator in *V. cholerae*, changes dramatically with pH (Skorupski and Taylor 1997). This implies that the shift to fermentation may be a signal for bacteria to express genes, exploiting a novel environment during infection.

Along with shifting metabolic activities upon host contact, *Vibrio* must also adapt to dramatic changes in the physical architecture of their environment and to the presence of dense populations of con-specifics during symbiotic colonization. uridine diphospho (UDP)-glucose-6-dehydrogenase, which is involved in colanic acid biosynthesis during capsular formation in addition to starch and sugar metabolism, has been demonstrated to be expressed when *Pseudomonas aeruginosa* or *Escherichia coli* contact surfaces, suggesting its importance for bacterial adhesion (Davies et al. 1993). Colanic acid also appears to play a role in maintaining the three-dimensional architecture of biofilms (Hanna et al. 2003). Given this, light organ expression of colanic acid may be of importance in the localization and three dimensional structure of clonal *Vibrio* communities.

This study provides evidence to support the importance of differential gene expression for the evolutionary ecology of a bacterium that inhabits two distinct environmental niches. Our results indicate that *V. fischeri* displays radically different expression patterns in seawater versus in the light organs of their adult squid hosts, and expression is remarkably consistent in two different strains from *E. tasmanica* of Australia and *E. scolopes* of Hawaii. Future studies will examine the extent to which each discovered gene and associated pathway is important for persistence in these ecological habitats, providing insight into the genetics to host colonization by environmentally transmitted bacteria. In addition, studies using these genes will hopefully identify the elements necessary for local adaptation and the evolution of host specificity as research further determines the ecological genetics of these two interacting species.

Acknowledgements

We thank N. Moltschaniwskyj, G. Woolcott, and M. McGrouther for assistance collecting *E. tasmanica*; M.Q. Martindale for space at the Kewalo Marine Laboratory; University of Hawaii for *E. scolopes* collections; J. Graham for helpful suggestions with the SCOTS technique; and E.G. Ruby, M.J. McFall-Ngai, and G. Giribet for their insightful discussions and advice. The data are based on work supported by the National Science Foundation Population Biology program to M.K.N.

References

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal, R. 1997. A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. J. Bacteriol. **179**: 5574–5581.

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal, R. 1998. The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. Infect. Immun. **66**: 1000–1007.

Barneah, O., Weis, V.M., Perez, S., and Benayahu, Y. 2004. Diversity of dinoflagellate symbionts in Red Sea soft corals: mode of symbiont acquisition matters. Mar. Ecol. Prog. Ser. **275**: 89–95.

Brown-Silva, J., and Nishiguchi, M.K. Variation in pilin gene arrangement may have consequences for specificity in a squid-*Vibrio* mutualism. FEMS Microbiol. In review.

Daigle, F., Graham, J.E., and Curtiss, R., III. 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Mol. Microbiol. **41**: 1211–1222.

Daigle, F., Hou, J.Y., and Clark-Curtiss, J.E. 2002. Microbial gene expression elucidated by selective capture of transcribed sequences (SCOTS). Methods Enzymol. **358**: 108–122.

Davies, D.G., Chakrabarty, A.M., and Geesey, G.G. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **59**: 1181–1186.

DeLong, E.F., and Karl, D.M. 2005. Genomic perspectives in microbial oceanography. Nature (London, U.K.), **437**: 336–342.

Graf, J., and Ruby, E.G. 1998. Host-derived amino acids support the proliferation of symbiotic bacteria. Proc. Natl. Acad. Sci. U.S.A. **95**: 1818–1822.

Graham, J.E., and Clark-Curtiss, J.E. 1999. Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). Proc. Natl. Acad. Sci. U.S.A. **96**: 11554–11559.

Graham, J.E., and Clark-Curtiss, J.E. 2000. Identifying *Mycobacterium tuberculosis* virulence determinants — new technologies for a difficult problem: response. Trends Microbiol. **8**: 100.

Graham, J.E., Peek, R.M., Jr., Krishna, U., and Cover, T.L. 2002. Global analysis of *Helicobacter pylori* gene expression in human gastric mucosa. Gastroenterology, **123**: 1637–1648.

Hafner, M.S., Sudman, P.D., Villablanca, F.X., Spradling, T.A., Demastes, J.W., and Nadler, S.A. 1994. Disparate rates of molecular evolution in cospeciating hosts and parasites. Science (Washington, D.C.), **265**: 1087–1090.

Hanna, A., Berg, M., Stout, V., and Razatos, A. 2003. Role of capsular colanic acid in adhesion of uropathogenic *Escherichia coli*. Appl. Environ. Microbiol. **69**: 4474–4481.

Hou, J.Y., Graham, J.E., and Clark-Curtiss, J.E. 2002. *Mycobacterium avium* genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect. Immun. **70**: 3714–3726.

Johannesen, P.F., and Hansen, J. 2002. Differential transcriptional regulation of sulfur assimilation gene homologues in the *Saccharomyces carlsbergensis* yeast species hybrid. FEMS Yeast Res. **1**: 315–322.

Johnson, N.A., and Porter, A.H. 2001. Toward a new synthesis: population genetics and evolutionary developmental biology. Genetica, **112–113**: 45–58.

Jones, B.W., and Nishiguchi, M.K. 2004. Counterillumination in the Hawaiian Bobtail Squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Mar. Biol. **144**: 1151–1155.

Jones, B.W., Maruyama, A., Cuverney, C.C., and Nishiguchi, M.K. 2007. Spatial and temporal distribution of the *Vibrionaceae* in coastal waters of Hawaii, Australia, and France. Environ. Microbiol. In press.

Liu, S., Graham, J.E., Bigelow, L., Morse, P.D., II, and Wilkinson, B.J. 2002. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. Appl. Environ. Microbiol. **68**: 1697–1705.

Mangan, J.A., Sole, K.M., Mitchison, D.A., and Butcher, P.D. 1997. An effective method of RNA extraction from bacteria refractory to disruption, including mycobacteria. Nucleic Acids Res. **25**: 675–676.

McFall-Ngai, M.J. 2002. Unseen forces: the influence of bacteria on animal development. Dev. Biol. **242**: 1–14.

McFall-Ngai, M.J., and Ruby, E.G. 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science (Washington, D.C.), **254**: 1491–1494.

McFall-Ngai, M.J., and Ruby, E.G. 1998. Sepiolids and Vibrios: when first they meet. BioScience, **48**: 257–265.

Millikan, D.S., and Ruby, E.G. 2004. FlrA, a σ^{54}-dependent transcriptional activator in *Vibrio fischeri*, is required for motility and symbiotic light-organ colonization. J. Bacteriol. **185**: 3547–3557.

Moorthy, S., and Watnick, P.I. 2004. Genetic evidence that the *Vibrio cholerae* monolayer is a distinct stage in biofilm development. Mol. Microbiol. **52**: 573–587.

Nishiguchi, M.K. 2002. Host–symbiont recognition in the environmentally transmitted sepiolid squid–*Vibrio* mutualism. Microb. Ecol. **44**: 10–18.

Nishiguchi, M.K., and Nair, V.S. 2003. Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. Int. J. Syst. Bacteriol. **53**: 2019–2026.

Nishiguchi, M.K., Ruby, E.G., and McFall-Ngai, M.J. 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in Sepiolid squid–vibrio symbioses. Appl. Environ. Microbiol. **64**: 3209–3213.

Nyholm, S.V., and McFall-Ngai, M.J. 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. Appl. Environ. Microbiol. **69**: 3932–3937.

Nyholm, S.V., Stabb, E.V., Ruby, E.G., and McFall-Ngai, M.J. 2000. Establishment of an animal–bacterial association: recruiting symbiotic vibrios from the environment. Proc. Natl. Acad. Sci. U.S.A. **97**: 10231–10235.

Nyholm, S.V., Deplancke, B., Gaskins, H.R., Apicella, M.A., and McFall-Ngai, M.J. 2002. Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. Appl. Environ. Microbiol. **68**: 5113–5122.

Park, Y.K., Bearson, B., Bang, S.H., Bang, I.S., and Foster, J.W. 1996. Internal pH crisis, lysine decarboxylase and the acid tolerance response of *Salmonella typhimurium*. Mol. Microbiol. **20**: 605–611.

Parker, M.A., Doyle, J.L., and Doyle, J.J. 2004. Comparative phylogeography of *Amphicarpaea* legumes and their root-nodule symbionts in Japan and North America. J. Biogeogr. **31**: 425–434.

Rainey, P.B., and Travisano, M. 1998. Adaptive radiation in a heterogeneouos environment. Nature (London, U.K.), **394**: 69–72.

Ruby, E.G., and Asato, L.M. 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch. Microbiol. **159**: 160–167.

Ruby, E.G., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., Lostroh, P., Lupp, C., McCann, J., Millikan, D., Schaefer, A., Stabb, E., Stevens, A., Visick, K., Whistler, C., and Greenberg, E.P. 2005. Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. Proc. Natl. Acad. Sci. U.S.A. **102**: 3004–3009.

Sicard, M., Le Brun, N., Pages, S., Godelle, B., Beoemare, N., and Moulia, C. 2003. Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interaction. Parasitol. Res. **91**: 520–524.

Skorupski, K., and Taylor, R.K. 1997. Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. Mol. Microbiol. **25**: 1003–1009.

Stabb, E.V., and Ruby, E.G. 2003. Contribution of pilA to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. Appl. Environ. Microbiol. **69**: 820–826.

Stewart, A.D., Logsdon, J.M., Jr., and Kelley, S.E. 2005. An empirical study of the evolution of virulence under both horizontal and vertical transmission. Evol. Int. J. Org. Evol. **59**: 730–739.

Thompson, J.N. 1999. The evolution of species interactions. Science (Washington, D.C.), **284**: 2116–2118.

Thompson, J.N., and Cunningham, B.M. 2002. Geographic structure and dynamics of coevolutionary selection. Nature (London, U.K.), **417**: 735–738.

Watnick, P.I., and Kolter, R. 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. Mol. Microbiol. **34**: 586–595.

Watnick, P.I., Fullner, K.J., and Kolter, R. 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. J. Bacteriol. **181**: 3606–3609.

West-Eberhard, M.J. 2005. Developmental plasticity and the origin of species differences. Proc. Natl. Acad. Sci. U.S.A. **102**(Suppl. 1): 6543–6549.

Microbial Ecology

Spatial and Temporal Distribution of the *Vibrionaceae* in Coastal Waters of Hawaii, Australia, and France

B.W. Jones[1], A. Maruyama[2], C.C. Ouverney[3] and M.K. Nishiguchi[1]

(1) Department of Biology, New Mexico State University, P. O. Box 30001 MSC 3AF, Las Cruces, NM 88003-8001, USA
(2) Biological Resources and Functions, National Institute of Advanced and Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, 305-8566, Japan
(3) Department of Biology, San Jose State University, San Jose, CA 95192-0100, USA

Received: 21 December 2006 / Accepted: 27 December 2006 / Online publication: 8 March 2007

Abstract

Relatively little is known about large-scale spatial and temporal fluctuations in bacterioplankton, especially within the bacterial families. In general, however, a number of abiotic factors (namely, nutrients and temperature) appear to influence distribution. Community dynamics within the Vibrionaceae are of particular interest to biologists because this family contains a number of important pathogenic, commensal, and mutualist species. Of special interest to this study is the mutualism between sepiolid squids and *Vibrio fischeri* and *Vibrio logei*, where host squids seed surrounding waters daily with their bacterial partners. This study seeks to examine the spatial and temporal distribution of the Vibrionaceae with respect to *V. fischeri* and *V. logei* in Hawaii, southeastern Australia, and southern France sampling sites. In particular, we examine how the presence of sepiolid squid hosts influences community population structure within the *Vibrionaceae*. We found that abiotic (temperature) and biotic (host distribution) factors both influence population dynamics. In Hawaii, three sites within squid host habitat contained communities of *Vibrionaceae* with higher proportions of *V. fischeri*. In Australia, *V. fischeri* numbers at host collection sites were greater than other populations; however, there were no spatial or temporal patterns seen at other sample sites. In France, host presence did not appear to influence *Vibrio* communities, although sampled populations were significantly greater in the winter than summer sampling periods. Results of this study demonstrate the importance of understanding how both abiotic and biotic factors interact to influence bacterial community structure within the Vibrionaceae.

Correspondence to: M.K. Nishiguchi; E-mail: nish@nmsu.edu

Introduction

Recent work in marine microbial ecology has uncovered the importance of bacterioplankton in many ecological processes [4, 15, 24, 27, 31]. Given the ecological significance of these communities, there is a need to understand the dynamic forces shaping their structure. Changes in abiotic factors, such as temperature, salinity, and nutrients, have been shown to be important causes of community changes. More recently biotic factors, including bacterivory, bacteriophage parasitism, and mutualistic relationships, have been shown to affect bacterioplankton [22, 34]. One mutualism that appears to influence bacterial community structure is the relationship between sepiolid squids (Cephalopoda: Sepiolidae) and their *Vibrio* symbionts.

In the association between sepiolid squids and *Vibrio fischeri* and/or *Vibrio logei*, the squid host benefits from *Vibrio*-produced light that is used in counterillumination (an antipredation mechanism), whereas vibrios benefit from a nutrient-rich habitat in which near-maximum growth rates can be achieved [5, 17]. In this environmentally transmitted symbiosis, juvenile squids acquire their bacterial symbionts from the surrounding seawater upon hatching. After colonization, the host exhibits a daily venting behavior in which 90–95% of the symbionts are released into the surrounding seawater [19, 32]. Because bacterial populations in mature squids can reach levels of approximately 10^8–10^{11} cells within the light organ [33], the daily flux of *Vibrio* into the environment can potentially influence local bacterioplankton community structure.

DOI: 10.1007/s00248-006-9204-z • Volume 54, 314–323 (2007)

On the island of O'ahu, Hawaii, studies using *lux* gene probes [20, 21] have demonstrated that the presence of the squid host *Euprymna scolopes* does appear to influence community structure within the *Vibrionaceae*. However, Hawaiian waters represent a relatively simple system to study bacterioplankton community dynamics because of the relatively constant water temperature and lack of a predominant thermocline within host habitats. It appears that in different locations where this symbiosis occurs, other factors may influence community dynamics within the *Vibrionaceae*. For example, at sites in Banyuls-sur-mer, France, studies using plate counts of *V. fischeri*, a warm-adapted symbiont, and *V. logei*, a cold-adapted symbiont, have demonstrated that the presence of a thermocline presents a barrier to mixing between these two species [28]. The profound impact on the spatial distribution of *Vibrio* species at this location suggests that there may be additional factors capable of influencing local bacterioplankton community structure. Further adding to this complexity are a number of different strains of *V. fischeri* in nature, many of which cannot colonize at all (nonsymbiotic) or can only partially colonize the light organ of sepiolid hosts [21].

The goal of this study was to examine the distribution of *V. fischeri* and *V. logei* in reference to host location using fluorescence *in situ* hybridization (FISH) with a nested set of probes (i.e., γ-Proteobacteria > *Vibrionaceae* > *V. fischeri* and *V. logei*) in an attempt to understand the complex spatiotemporal patterns of members of the *Vibrionaceae*. Using a modification of traditional FISH, which incorporates direct counts (DC) on filters [23], we have accumulated an extensive dataset examining the *Vibrionaceae*, *V. fischeri* and *V. logei*, at field sites in southern France and along the east coast of Australia to obtain a more definitive picture of how various factors influence population structure within the *Vibrionaceae*.

Methods

Probe Construction and Optimization. Probes used in this study are listed in Table 1. To design the Vib749 probe (*Vibrionaceae*), 16S rRNA sequences of representative members of the *Vibrionaceae* (obtained from GenBank; http://www.ncbi.nlm.nih.gov/) and *Escherichia coli* K-12 (as the outgroup; GenBank accession number U00096) were aligned in MacVector™ 6.5 using the ClustalW alignment algorithm. Conserved sequences of 18–23 base pairs in length within the family *Vibrionaceae* that contained at least one base difference from *E. coli* K-12 were screened visually. Sequence specificity of each candidate probe sequence was determined using the Probe Match function in the Ribosomal Database Project II [6], and the probe that represented the most species within the *Vibrionaceae* was found at *E. coli* K-12 base position 749. The analogous procedure was implemented for designing Vfsh84a and Vlog251 probes. In these cases, a number of GenBank 16S rRNA sequences from *V. fischeri* strains [29] (accession numbers AY292941, AY292938, AY292922, AY292921, AY292920, and AY292919) were aligned against two *V. logei* strains (accession numbers X74708 and AY292934), and probe sequences were determined as above.

Stringency conditions for *in situ* hybridization of each probe were empirically optimized to allow the probe to bind only to target sequences containing zero mismatches (Table 1). To do so, probes were hybridized against bacterial strains with zero, one, and two mismatches in hybridization solutions containing various formamide concentrations between 5–50%. The Vib749 probe was tested against *V. fischeri* ES114 (zero mismatches), *Photobacterium damselae* (one mismatch; accession number X78105), and *E. coli* K-12 (two mismatches). For Vfsh84a, test strains were *V. fischeri* ES114 (0 mismatches), *P. damselae* (1 mismatch), and *Vibrio parahaemolyticus* strain 113 (two mismatches; accession number AY527396). Vlog251 was tested against *V. logei* (zero mismatches; accession number X74708) and *V. fischeri* ES114 (1 mismatch). All *Vibrio* and *Photobacterium* strains were grown overnight at 28°C in Luria–Bertani high salt solution (1% tryptone, 0.5% yeast extract, 2% NaCl, 0.3% glycerol, and 50 mM Tris–HCl at

Table 1. Probes used in this study

Probe	*Target group*	*Test strain*	*Sequence*	*Percent formamide*
Gam42a[a]	γ-Proteobacteria	N/A	5′GCCTTCCCACATCGTTT	35
Vib749	*Vibrionaceae*	*V. fischeri* ES114	5′TCGCATCTGAGTGTCAGT	35
		P. damselae	3′AGCGUAGACUCGCAGUCA	
		E. coli K12	3′AGCGUGGACUCGCAGUCA	
Vfsh84a	*V. fischeri*	*V. fischeri* ES114	5′ACGCCCTTAACGTTCCCCG	50
		P. damselae	3′GGCGGGAAUUGCAAGGGGC	
		V. parahaemolyticus	3′UGCGGCAAUAGCAAGGGGC	
Vlog251	*V. logei*	*V. logei*	5′CCTTGGTGAGCTCTTACCCC	40
		V. fischeri ES114	3′GGAACCACUCGAGAAUGGAG	

The optimal concentration of formamide was empirically derived for Vib749, Vfsh84a, and Vlog251. Each probe was hybridized at varying concentrations of formamide ranging from 0–50% against test strains containing zero, one, or two mismatches against the probe. The target ribosomal sequences were listed from 3′ to 5′ for test strains containing one or two mismatches to clearly identify the location of each underlined mismatch.
[a][2]

pH 7.4). *Escherichia coli* was grown overnight at 37°C in Luria broth (Invitrogen, Carlsbad, CA, USA).

For probe optimization hybridizations, overnight cultures were fixed in 3% paraformaldehyde and 5 μL spots of individual test strains were allowed to dry in each of 10 wells (5 mm diameter) on a Teflon-treated slide (Cel-Line Associates, Inc., Newfield, NJ, USA). Wells were treated with 20 μL of hybridization solution (using Cy-3-labeled probes; see below for recipe) containing increasing concentrations of formamide at 5% intervals from 5–50%. Wash steps and viewing are described below. To determine the optimal formamide concentration for each probe, each test strain was examined at every formamide concentration. The optimal concentration was that at which the one and two mismatch strains showed no visible fluorescence, but the zero mismatch strain was strongly fluorescent.

Field Sampling. Water samples were collected from field sites in O'ahu, Hawaii, on the SE coast of Australia, and southern France for sampling. In Hawaii, eight sites were sampled around O'ahu in March 2003 (Fig. 1). At these sites, samples were collected during the morning low tides from waist-deep water. In Australia, samples were collected predawn and postsunset from waist-deep water at ten sites along the east coast between Sydney and Melbourne in March and September 2004 (Fig. 2). For logistical reasons, not every site was sampled during both months. In France, samples were collected at various depths at two research sites (Bay of Banyuls and Bay of Elmes in Southern France; Fig. 3) in November 2003 and August 2004. Access to conductivity–temperature–depth recorder (CTD) and research vessels at these sites enabled a more complete examination of the bacterioplankton. At each site, samples were taken in triplicate.

Upon collection of 100 mL of seawater, the samples were immediately fixed with paraformaldehyde (3% final concentration) at 4°C for 4–12 h. After incubation, various volumes of the sample (5 mL for the γ-Proteobacteria, 10 mL for the *Vibrionaceae*, and 20 mL for *V. fischeri* and *V. logei*) were filtered onto 0.2-μm pore size nucleopore filters (Whatman, Clifton, NJ, USA) coated with 0.1% poly-L-lysine, and backed with 8-μm pore size filters. Filtration took place using a vacuum below 40 kPa. Dried filters were stored at −20°C until hybridization.

In Situ Hybridization. Fluorescence *in situ* hybridization incorporating DC was performed on each filter according to previous methods with minor modifications [23]. Each filter was aseptically cut into a square, which fit in a 15 × 15-mm Frame-Seal Incubation Chamber (MJ Research, Waltham, MA, USA) and placed on a microscope slide. The sample was then covered with 40 μL of hybridization solution (0.9 M of NaCl, 5 mM of EDTA, 0.5% of sodium dodecyl sulfate [SDS], 50 mM of sodium phosphate buffer [pH 7], 10× Denhardt's solution (Sigma), 1 μg/μL of poly(A), and 1 ng/μL of Cy3 or Cy5-labeled oligonucleotide) supplemented with the appropriate formamide concentration depending on the probe used (Table 1). The *V. logei* probe was labeled with Cy-5, whereas the *Vibrionaceae* and *V. fischeri* probes contained Cy-3. Each chamber was sealed and placed in a DNA Engine Dyad® Peltier Thermal Cycler equipped with a slide chamber Alpha Unit (MJ Research) at 42°C for 4 h.

After 4 h of hybridization, each filter was washed (0.9 M of NaCl, 0.1% of SDS, and 50 mM of sodium phosphate buffer at pH 7) for 30 min at 46°C. Filters were rinsed in 0.2 μm of filtered distilled water and air-

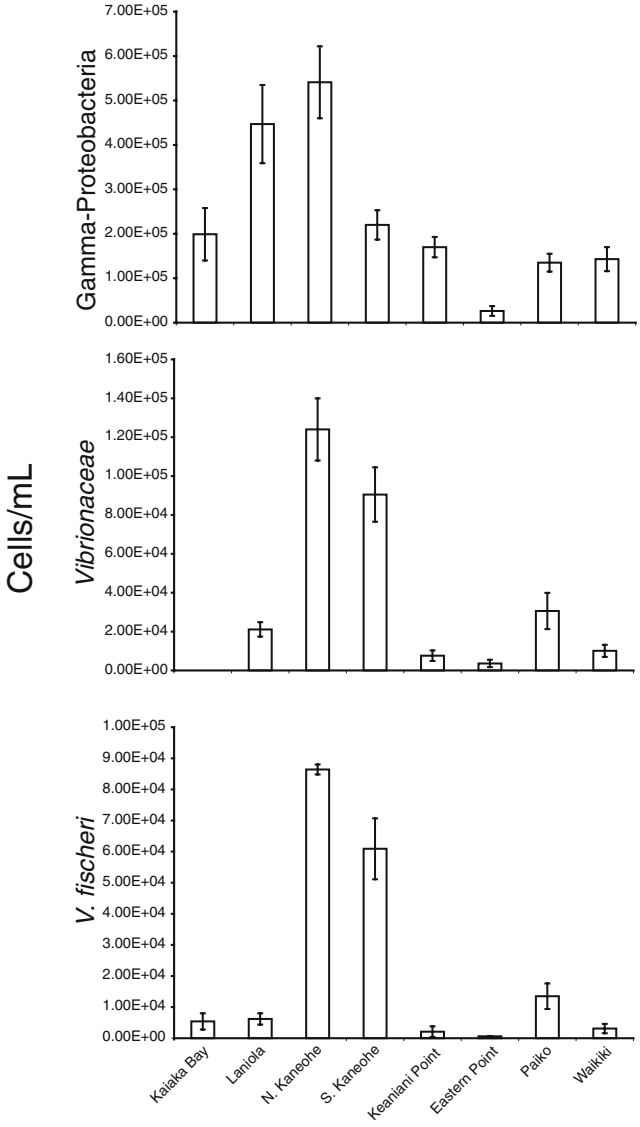


Figure 1. Numbers of γ-Proteobacteria, *Vibrionaceae*, and *V. fischeri* at the eight sampling sites in O'ahu, Hawaii (see Table 3). Error bars represent one standard deviation. There are no data available for the *Vibrionaceae* estimate in Kaiaka Bay.

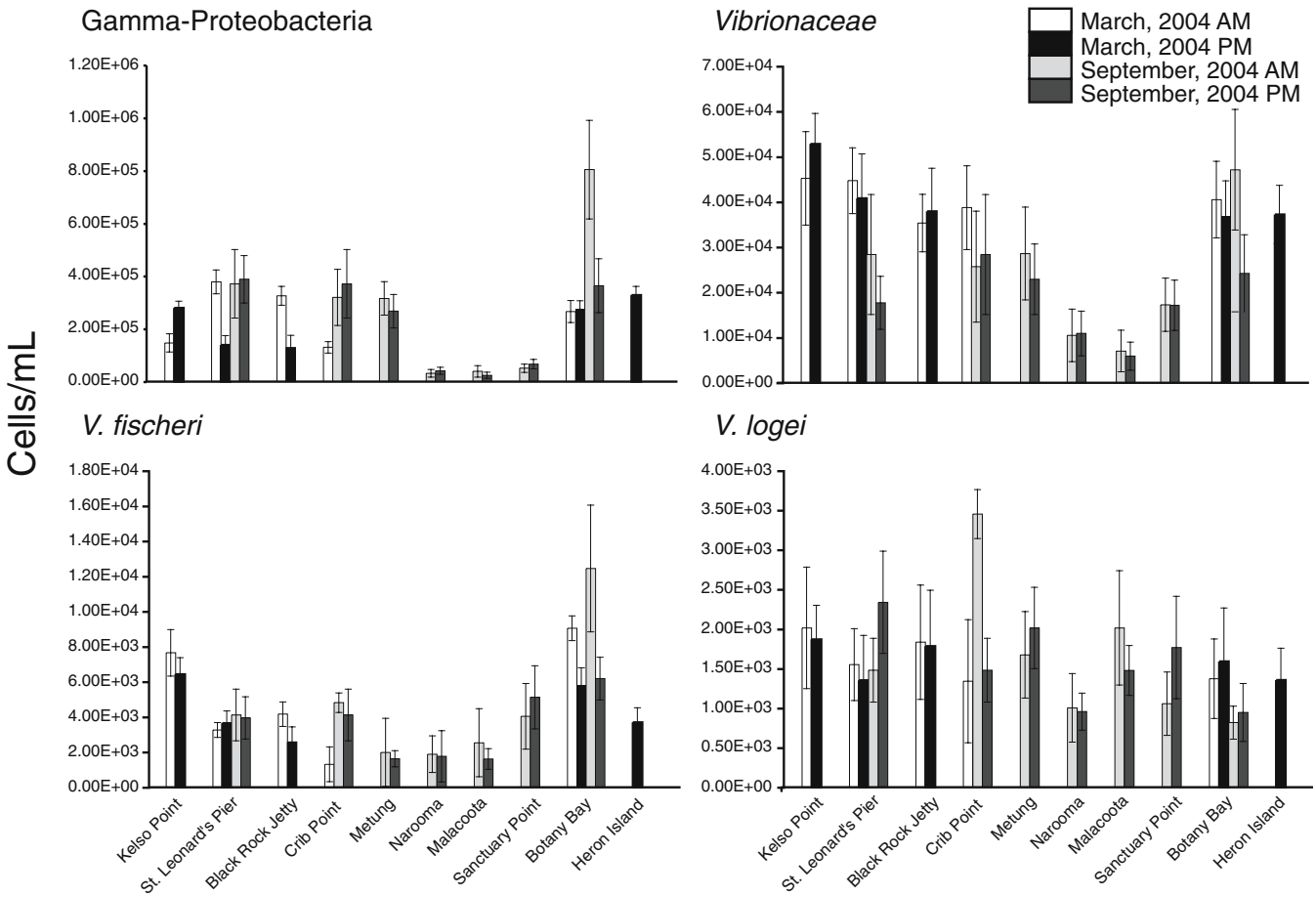


Figure 2. Numbers of γ-Proteobacteria, *Vibrionaceae*, *V. fischeri*, and *V. logei* at ten different sites during March and September 2004 in Australia. The *Y*-axis represents numbers of bacteria at each site. Samples were taken in the morning at sunrise and in the evening after sunset. Only the St. Leonard's Pier and Botany Bay sites were sampled in the morning and evening both years. Error bars represent one standard deviation.

dried. Immediately before viewing, filters were mounted with ProLong Antifade (Molecular Probes, Eugene, OR, USA). Samples were viewed with a Nikon E800 epifluorescence microscope equipped with excitation/emission filters of 546/565 for Cy3 and 620/700 for Cy5. Samples were counted manually and a total of 30 random fields and at least 400 cells were counted for each probe in every sample. When Cy5 was used, samples were enumerated by counting from pictures taken with a CCD camera.

Results

Probe Development and Optimization. The *Vibrionaceae*, *V. fischeri*, and *V. logei* probes were found to have optimal formamide concentrations of 35, 50, and 45%, respectively. Database searches and sequences from our own laboratory strains found the *V. fischeri* probe to be specific for all known symbiotic *V. fischeri* strains. Database searches also found that the *V. fischeri* probe also hybridizes to *Vibrio orientalis*, *Vibrio hepatarius*, and *Vibrio agarivorans*. The *V. logei* probe also hybridizes to an uncultured marine γ-Proteobacterium, *Aquicella lusitana*, and *Oscillatoria limnetica*. Given the lack of 100% specificity to only *V. fischeri* and *V. logei* by these probes, the numbers presented in this study may be slight overestimates of actual populations.

The *Vibrionaceae* probe outperformed two previously constructed probes by Amann et al. [2] and Nishimura et al. [30] for estimating total number of species hybridized (Table 2). Whereas the probe of Amann et al. recognized amore species of *Enterovibrio* and *Photobacterium*, the probe developed in this study recognized more species of *Vibrio*, *Listonella*, and unclassified species of the *Vibrionaceae*.

Field Test. In Hawaii, total numbers of *Vibrionaceae* ranged from 3.6×10^3 to 1.2×10^5 cells/mL, and the percentage of *Vibrionaceae* that was *V. fischeri* ranged from 17 to 31% at sites not known to harbor large numbers of *E. scolopes* (Kaiaka Bay, Laniola, Keaniani

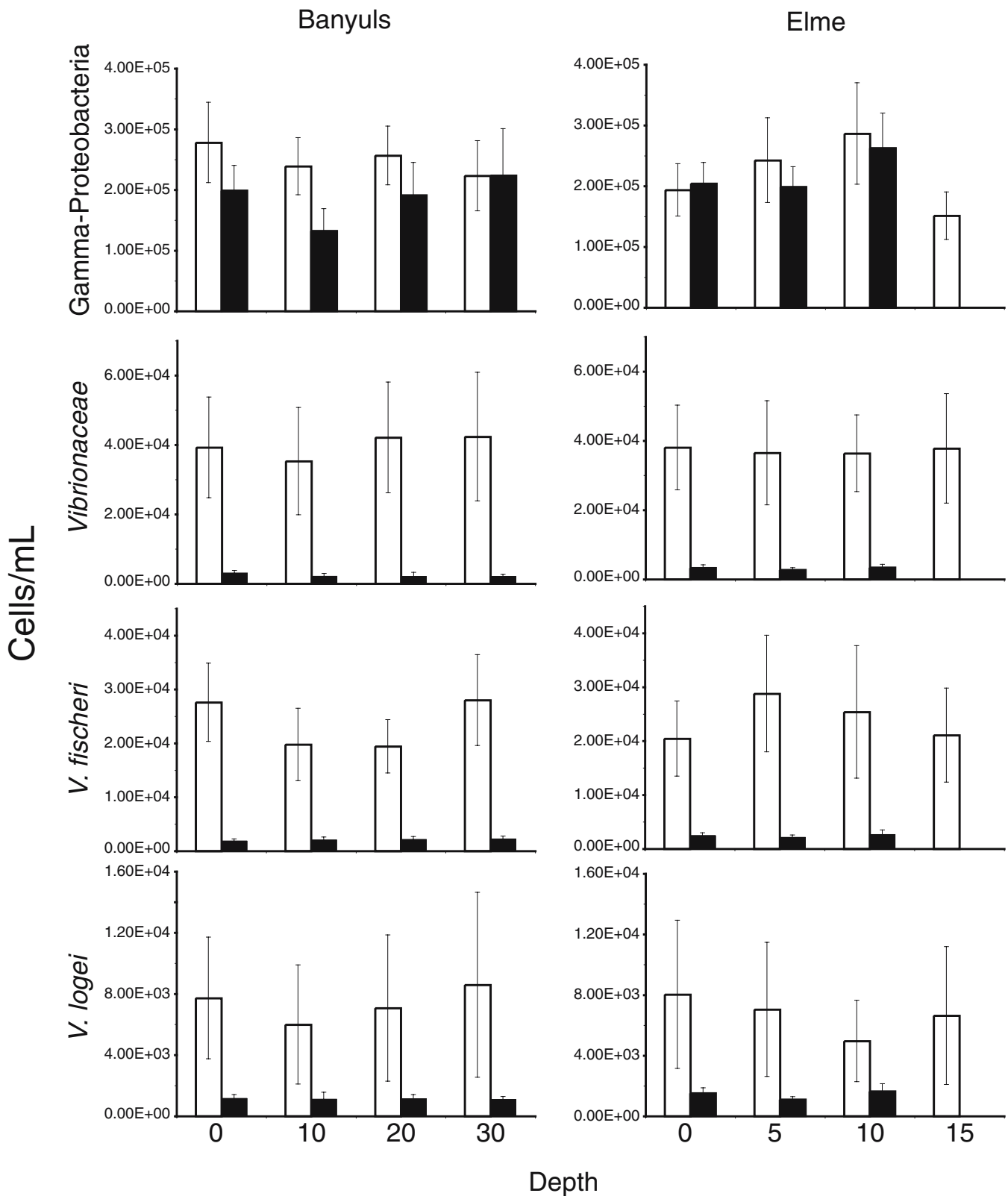


Figure 3. Community numbers at Banyuls and Elmes field sites in France. White colored bars are from the field season in November 2003, whereas black bars are from August 2004. There was no sampling at 15 m at the Elmes field site in August 2004. Error bars represent one standard deviation.

Table 2. Comparison of published *Vibrionaceae* family specific probes

	Vibrio	*Photobacterium*	*Listonella*	*Enterovibrio*	*Salinovibrio*	Unclassified
VIB749	**53.5**	4.4	**51.0**	0.0	0.0	**22.3**
GV[a]	30.8	**26.6**	44.5	**1.6**	0.0	21.2
Vir1[b]	48.2	4.4	30.3	0.0	0.0	15.7

Each cell lists the percentage of sequences in the five genera that are 100% complimentary to the three probes. Sequences were gathered from the Ribosomal Database Project II, release 9.34. Numbers in bold indicate the highest percentage within each genus.
[a][2].
[b][30].

Point, Eastern Point, and Waikiki; Fig. 1 and Table 3). Sites containing stable populations of *E. scolopes* (N. Kaneohe, S. Kaneohe, and Paiko) had approximately 44 to 70% of the *Vibrionaceae* that were identified as *V. fischeri*. The total number of *V. fischeri* ranged from 0.6×10^3 to 8.6×10^4 cells/mL among the different sites sampled in Hawaii (Fig. 1). *Vibrio logei* was not detected in Hawaiian waters.

In Australia, bacterial populations were relatively constant between sites and between morning and evening sampling times (Fig. 2), with two notable exceptions being the September 2004 AM samples for Botany Bay and Crib Point. Within the γ-Proteobacteria, populations were similar to France sites, with the exception of Narooma, Malacoota, and Sanctuary Point, which had very low numbers. Comparatively low numbers of *Vibrionaceae* were also observed at these sites. *Vibrio fischeri* distribution exhibited pronounced variation in cell density, with the highest numbers enumerated from Botany Bay. *Vibrio logei* numbers were lower than *V. fischeri*, and exhibited no apparent trends between seasons (Fig. 2).

Conductivity–temperature–depth recorder sampling in France enabled depth profiling of communities at different temperatures and salinities (Figs. 3 and 4). The summer thermocline generally resulted in a decrease from about 21 to 14°C. One-way analysis of variance (ANOVA) failed to reject the null hypothesis of no difference in cell numbers at different depths for each nested group of bacteria at both France sites (p values ranging from 0.29 to 0.64), which meant there was no apparent reduction or increase in community numbers across the thermocline. Numbers did, however, differ significantly between the summer and winter sampling times, as the null hypothesis of no difference in cell numbers between seasons was rejected for *Vibrionaceae*, *V. fischeri*, and *V. logei* using ANOVA ($p < 0.001$ for each sample, except for the γ-Proteobacteria where $p = 0.007$). The most notable difference in cell numbers was within the *Vibrionaceae*, *V. fischeri*, and *V. logei*. There was

Table 3. Sites sampled in this study

	Site name	*Latitude*	*Longitude*
Hawaii	Kaiaka Bay	N 21°34′56″	W 158°07′46″
	Laniola	N 21°38′31″	W 157°55′02″
	Keaniani Point	N 21°33′50″	W 157°52′23″
	N. Kaneohe	N 21°29′31″	W 157°50′47″
	S. Kaneohe	N 21°25′45″	W 157°47′32″
	Eastern Point	N 21°19′01″	W 157°39′51″
	Paiko	N 21°16′49″	W 157°43′49″
	Waikiki	N 21°16′10″	W 157°49′23″
Australia	Kelso Point	S 41°3′24″	E 146°47′52″
	St. Leonard's Pier	S 38°10′14″	E 144°43′8″
	Black Rock Jetty	S 38°13′37″	E 145°01′29″
	Crib Point	S 38°21′01″	E 145°13′11″
	Metung	S 37°53′02″	E 147°58′35″
	Narooma	S 33°29′05″	E 150°06′54″
	Malacoota	S 37°33′59″	E 149°45′59″
	Sanctuary Point	S 35°06′	E 150°39′
	Botany Bay	S 34°00′	E 151°12′
	Heron Island	S 23°26′53″	E 151°55′49″
France	Banyuls	N 42°35′29″	E 3°02′57″
	Elmes	N 42°37′35″	E 3°02′22″

In Hawaii and Australia samples were taken from surface waters approximately 20 m offshore. In France samples were taken at various depths from boat using a CTD.

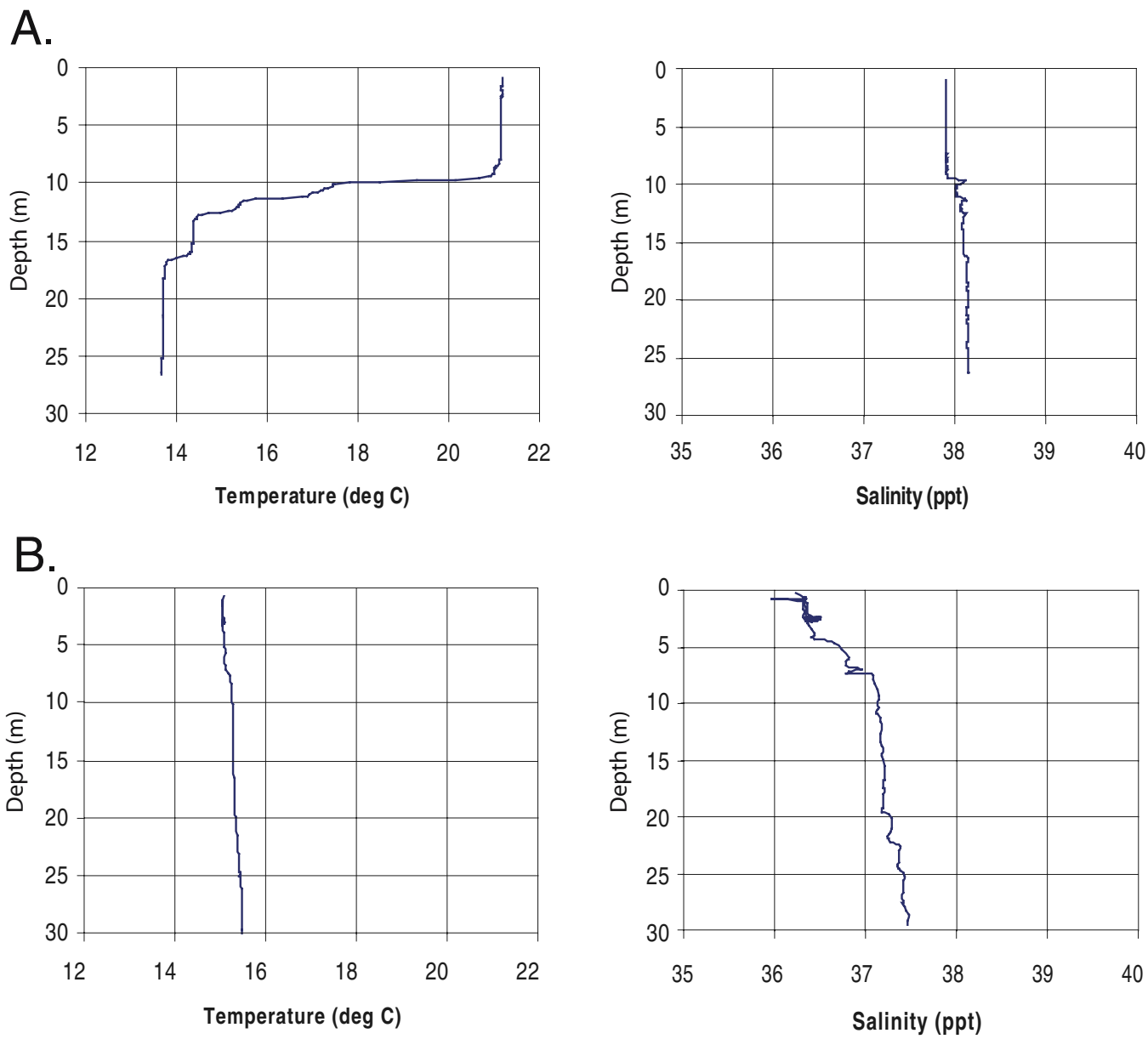


Figure 4. Representative salinity and temperature profiles from France study sites during the summer sample period (A) and the winter sample period (B). Note the lack of the thermocline during the winter.

approximately a tenfold difference in total counts of these groups of bacteria at both sites between seasons.

Discussion

Fluorescence *in situ* hybridization, a technique commonly used to examine microbial communities [1, 2, 7–9, 23], has provided a wealth of information to researchers through "snapshots" of microbial populations. Fluorescence *in situ* hybridization has been successfully applied to oceanographic studies examining bacterioplankton, and has provided tremendous insight as to how these communities are structured [10–14]. Although past studies have mainly focused on the α-Proteobacteria, it is interesting to note that members of the *Vibrionaceae* still constitute a large proportion of the bacterioplankton community in the areas we were able to sample, with or without the presence of squid hosts. This strengthens the fact that multiple factors (abiotic and biotic) are probably responsible for trends in the distribution of any division of bacteria, particularly vibrios.

Results from the Hawaiian dataset confirm previous reports [20, 21], which exhibited greater numbers of *V. fischeri* in the water column within the Kaneohe Bay study sites (Fig. 1). This bay is known to harbor large

numbers of *E. scolopes*, and daily expulsion of *V. fischeri* may explain the elevated levels. In addition, this location also contains a large number nutrient inputs from streams entering the Bay, which may allow certain species of bacteria to flourish in these waters [16]. The Paiko site also had statistically significant numbers of *V. fischeri*. This site is also a common *E. scolopes* collection location, but because Paiko is not a bay it is more open to ocean currents than Kaneohe. These currents may flush out excess vibrios, preventing the increased accumulation observed in North and South Kaneohe Bay.

The results presented in this study also demonstrate a marked increase in numbers of *V. fischeri* detected in Hawaiian waters compared to previous studies [20, 21]. Specifically, Lee and Ruby [21] found the abundance of *V. fischeri* to be between 276–400 cells/mL based on *luxA* quantitative DNA–DNA hybridization and between 130–1680 cells/mL based on most-probable-number analysis using *luxA*-specific primers for polymerase chain reaction from cell lysates. Indeed, the lowest estimate of *V. fischeri* abundance at Eastern Point (600 ± 100 cells/mL) falls into the high end of the estimates of Lee and Ruby [21]. This could be because of binding of the probe to complementary sequences from bacterial species other than *V. fischeri* that were not catalogued in the Ribosomal Database Project.

Australian total numbers of all bacterial samples probed compared similarly to Hawaiian estimates of γ-Proteobacteria, *Vibrionaceae*, and *V. fischeri* presented in this study (Fig. 2). Within the *Vibrionaceae*, however, the percentage of the *Vibrionaceae* that was *V. fischeri* was much lower than Hawaiian estimates. Reasons for this discrepancy are unclear, but because Australian and Hawaiian habitats are geographically separated and ecologically distinct, there is no reason to expect that the bacterial communities in these areas should be structured in a similar manner, nor follow patterns that link *Vibrio* concentration to host squid number.

Numbers of *V. fischeri* in Botany Bay are of particular interest because of the aims of this study. Although specific population size of the sepiolid host is unknown in this area, Botany Bay is a common collection site because of the abundance of *Euprymna tasmanica*. Much like Kaneohe Bay in Hawaii (but on a much greater scale), Botany Bay is a large enclosed body of water in which numbers of vented *V. fischeri* could accumulate. In this population, numbers of *V. fischeri* are greater than every other site, with the exception of Kelso Point (another site where *E. tasmanica* is commonly collected) in Tasmania. In addition, the morning *V. fischeri* numbers are greater than those collected in the evenings at these sites. At these locations, the presence of *E. tasmanica* may be the reason for this phenomenon, although no causal relationship can be inferred with the present data.

In France, there were greater numbers of all groups of bacteria detected in the November sampling season (Fig. 3). In general, the greatest discrepancies between years were observed when comparing *Vibrionaceae*, *V. fischeri*, and *V. logei*, whose November populations were much higher than the summer populations. Greater availability of nutrients during the winter when the thermocline is absent and less competition from other bacteria during this time may account for this general increase in *Vibrio* numbers.

Population structure of the symbiotic bacteria *V. fischeri* and *V. logei* was not affected by depth at Banyuls-sur-mer or Bay of Elmes study sites during the two seasons samples were collected. This is counterintuitive for a number of reasons. First, it is known that light organs of sepiolid squids from greater depths tend to have a higher proportion of the cold-adapted *V. logei* than the more warm-adapted *V. fischeri* [28]. Second, there is a predominant thermocline in summer months, which prevents mixing of deep, cold waters with warm surface waters at both study sites (Fig. 4). In late autumn, the thermocline disappears, and waters become a homogeneous mixture. Given this information, it was hypothesized that *V. logei* would be more abundant than *V. fischeri* in deep waters below the thermocline in summer months because of increased expulsion of *V. logei* by deep water sepiolid squids and increased growth rates over *V. fischeri* in these colder waters. The analogous hypothesis was made for V. *fischeri* distribution in warmer waters above the thermocline. Given the data, the hypotheses were clearly rejected, as no depth-related differences were observed.

There are several factors that may explain why these differences were observed. First, during the summer months in Southern France, periods of periodic winds known as the mistral can produce heavy gusts that can potentially disrupt the thermocline for short periods of time (1–4 days). If the thermocline is continually disrupted throughout the sampling period, bacterial temperature-related distribution patterns would disappear or at least be transient. The second possibility is that there is no temperature-dependent distribution of *V. fischeri* and *V. logei* associated with the thermocline. Given that sepiolid squid light organs are environmentally colonized upon hatching, this may infer that initial colonization might be equally parsimonious for either *V. fischeri* or *V. logei*, and subsequent dominance in the organ could be because of *V. logei* outcompeting *V. fischeri* at colder temperatures in a high nutrient environment where the squid resides. This was demonstrated *in vitro* with two species of Mediterranean sepiolids, *Sepiola affinis* and *Sepiola ligulata* with both *V. fischeri* and V. *logei* [28]. Thus, the symbiosis is probably more specific once infection has occurred by one of the two symbiont species available in the Mediterranean.

In conclusion, there appears to be multiple factors that structure bacterioplankton community composition

in natural environments. Whereas overall bacterial numbers were similar between Australia, France, and Hawaii, fine-scale processes appear to influence bacterial community structure in both space and time. Our hypothesis that sepiolid squid presence influences bacterial community structure was supported to an extent, but future sampling efforts will need to take into account as many abiotic and biotic factors (including temperature, salinity, dissolved organic matter, blooms of other species, bacterivory, etc.) for an increased understanding of what determines microbial community structure. Other studies with molluscan and vertebrate hosts have demonstrated that salinity and temperature influence a number of factors [3, 18, 25, 26], which not only influence colonization, but also the physiological state of *Vibrio* species in the water column. Thus, deciphering the mechanisms of how the ecology of squid hosts and their *Vibrio* symbionts affect their surrounding habitat and whether they are influential players in the overall microbial community structure are important aspects that still need to be addressed in future studies.

Acknowledgments

We are grateful to M. Sunamura and C. Miyako from the Maruyama Laboratory for advice with developing FISH probes. We also thank T. Henderson for help with FISH probe optimization. We would also like to thank the crew of the Neres and especially Laurent Zudaire for help with the CTD collection at the Laboratoire Arago. This work was supported by NSF DEB0316516, SBE0123690, and DBI007982, and NIH-SO6 GM08136-26 to M.K.N., and NIH-RISE GM61222-01 and NIH BRIDGES GM48998 for support of T. Henderson. B.W.J. was partially supported by an NSF Summer in Japan fellowship.

References

1. Amann, RI, Krumholz, L, Stahl, DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172: 762–770
2. Amann, RI, Ludwig, W, Schleifer, KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59: 143–169
3. Amaro, C, Biosca, EG, Fouz, B, Alcaide, E, Esteve, C (1995) Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. Appl Environ Microbiol 61: 1133–1137
4. Anesio, AM, Abreu, PC, de Assis Esteves, F (1997) Influence of the hydrological cycle on the bacterioplankton of an impacted clear water Amazonian Lake. Microb Ecol 34: 66–73
5. Boettcher, KJ, Ruby, EG (1990) Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J Bacteriol 172: 3701–3706
6. Cole, JR, Chai B, Marsh, TL, Farris, RJ, Wang, Q, Kulam, SA, Chandra, S, McGarrell, DM, Schmidt, TM, Garrity, GM, Tiedje, JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31: 442–443
7. DeLong, EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89: 5685–5689
8. DeLong, EF (1997) Marine microbial diversity: the tip of the iceberg. Trends Biotechnol 15: 203–207
9. DeLong, EF (2001) Microbial seascapes revisited. Curr Opin Microbiol 4:290–295
10. DeLong, EF, Pace, NR (2001) Environmental diversity of bacteria and archaea. Syst Biol 50:470–478
11. DeLong, EF, Wu, KY, Prezelin, BB, Jovine, RV (1994) High abundance of Archaea in Antarctic marine picoplankton. Nature 371:695–697
12. DeLong, EF, Taylor, LT, Marsh, TL, Preston, CM (1999) Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. Appl Environ Microbiol 65: 5554–5563
13. Glockner, FO, Amann, RI, Alfreider, A, Pernthaler, J, Psenner, R, Trebesius, K, Schleifer, KH (1996) An in situ hybridization protocol for detection and identification of planktonic bacteria. Syst Appl Microbiol 19: 403–406
14. Glockner, FO, Fuchs, BM, Amann, RI (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl Environ Microbiol 65: 3721–3726
15. Herndl, GJ, Reinthaler, T, Teira, E, van Aken, H, Veth, C, Pernthaler, A, Pernthaler, J (2005) Contribution of Archaea to total prokaryotic production in the deep Atlantic Ocean. Appl Environ Microbiol 71: 2303–2309
16. Hunter, CL, Stephenson, MD, Tjeerdema, RS, Crosby, DG, Ichikawa, GS, Goetzl, JD, Paulson, KS, Crane, DB, Martin, M, Newmans, JW (1995) Contaminants in oysters in Kaneohe Bay, Hawaii. Mar Poll Bull 30: 646–654
17. Jones, BW, Nishiguchi, MK (2004) Counterillumination in the Hawaiian Bobtail Squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). Marine Biology 144: 1151–1155
18. Kaspar, CW, Tamplin, ML (1993) Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl Environ Microbiol 59: 2425–2429
19. Lee, K-H, Ruby, EG (1992) Detection of the light organ symbiont, *Vibrio fischeri*, in Hawaiian seawater by using *lux* gene probes. Appl Environ Microbiol 58: 942–947
20. Lee, K-H, Ruby, EG (1994) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl Environ Microbiol 60: 1565–1571
21. Lee, K-H, Ruby, EG (1995) Symbiotic role of the viable but nonculturable state of *Vibrio fischeri* in Hawaiian coastal seawater. Appl Environ Microbiol 61: 278–283
22. Lindstrom, ES (2001) Investigating influential factors on bacterioplankton community composition: results from a field study of five mesotrophic lakes. Microb Ecol 42: 598–605
23. Maruyama, A, Sunamura, M (2000) Simultaneous direct counting of total and specific microbial cells in seawater, using a deep-sea microbe as target. Appl Environ Microbiol 66: 2211–2215
24. Massana, R, Pedros-Alio, C (1994) Role of anaerobic ciliates in planktonic food webs: abundance, feeding, and impact on bacteria in the field. Appl Environ Microbiol 60: 1325–1334
25. Morris, RM, Rappe, MS, Connon, SA, Vergin, KL, Siebold, WA, Carlson, CA, Giovannoni, SJ (2002) SAR11 clade dominates ocean surface bacterioplankton communities. Nature 420: 806–810
26. Motes, ML, DePaola, A, Cook, DW, Veazey, JE, Hunsucker, JC, Garthright, WE, Blodgett, RJ, Chirtel, SJ (1998) Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Appl Environ Microbiol 64: 1459–1465
27. Murray, RE, Hodson, RE (1985) Annual cycle of bacterial secondary production in five aquatic habitats of the Okefenokee swamp ecosystem. Appl Environ Microbiol 49: 650–655

28. Nishiguchi, MK (2000) Temperature affects species distribution in symbiotic populations of *Vibrio* spp. Appl Environ Microbiol 66: 3550–3555
29. Nishiguchi, MK, Nair, VS (2003) Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. Int J Syst Bacteriol 53: 2019–2026
30. Nishimura, M, Kogure, K, Kita-Tsukamoto, K, Ohwada, K (1995) Detection and direct count of specific bacteria in natural seawater using 16S rRNA oligonucleotide probe. Bull Jap Soc Microb Ecol 10: 109–113
31. Reinthaler, T, Winter, C, Herndl, GJ (2005) Relationship between bacterioplankton richness, respiration, and production in the Southern North Sea. Appl Environ Microbiol 71:2260–2266
32. Ruby, EG, Lee, K-H (1998) The *Vibrio fischeri–Euprymna scolopes* light organ association: current ecological paradigms. Appl Environ Microbiol 64: 805–812
33. Ruby, EG, McFall-Ngai, MJ (1992) A squid that glows in the night: development of an animal–bacterial mutualism. J Bacteriol 174: 4865–4870
34. Winter, C, Smit, A, Szoeke-Denes, T, Herndl, GJ, Weinbauer, MG (2005) Modelling viral impact on bacterioplankton in the North Sea using artificial neural networks. Environ Microbiol 7: 881–893

Rev Fish Biol Fisheries (2007) 17:455–476
DOI 10.1007/s11160-007-9056-8

RESEARCH PAPER

Ethical and welfare considerations when using cephalopods as experimental animals

N. A. Moltschaniwskyj · K. Hall · Marek R. Lipinski · J. E. A. R. Marian · M. Nishiguchi · M. Sakai · D. J. Shulman · B. Sinclair · D. L. Sinn · M. Staudinger · R. Van Gelderen · R. Villanueva · K. Warnke

Received: 3 January 2007 / Accepted: 26 February 2007 / Published online: 7 April 2007

Abstract When using cephalopods as experimental animals, a number of factors, including morality, quality of information derived from experiments, and public perception, drives the motivation to consider welfare issues. Refinement of methods and techniques is a major step in ensuring protection of cephalopod welfare in both laboratory and field studies. To this end, existing literature that provides details of methods used in the collection, handling, maintenance, and culture of a range of cephalopods is a useful starting point when refining and justifying decisions about animal welfare. This review collates recent literature in which authors have used cephalopods as experimental animals, revealing the extent of use and diversity of cephalopod species and techniques. It also highlights several major

N. A. Moltschaniwskyj (✉) · R. Van Gelderen
School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Locked Bag 1370, Launceston, Tasmania 7250, Australia
e-mail: Natalie.Moltschaniwskyj@utas.edu.au

K. Hall
Murray-Darling Freshwater Research Centre, Albury, Australia

M. R. Lipinski
Marine and Coastal Management, DEAT, Cape Town, South Africa

J. E. A. R. Marian
Department of Zoology, Institute of Biosciences, University of São Paulo, Sao Paulo, Brazil

M. Nishiguchi
Department of Biology, New Mexico State University, Las Cruces, USA

M. Sakai
National Research Institute of Far Seas Fisheries, Shizuoka, Japan

D. J. Shulman
Hopkins Marine Station of Stanford University, Pacific Grove, USA

B. Sinclair
School of Biological and Environmental Sciences, Central Queensland University, Rockhampton, Australia

D. L. Sinn
School of Zoology, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Hobart, Australia

M. Staudinger
Department of Natural Resources Conservation, University of Massachusetts Amherst, Amherst, USA

R. Villanueva
Institut de Ciencies del Mar (CSIC), Barcelona, Spain

K. Warnke
Paläontologie, Freie Universität Berlin, Berlin, Germany

issues when considering cephalopod welfare; how little is known about disease in cephalopods and its relationship to senescence and also how to define objective endpoints when animals are stressed or dying as a result of the experiment.

Keywords Animal welfare · Animal ethics · Capture · Cephalopods · Cuttlefish · Handling · Housing · *Nautilus* · Octopus · Squid

Introduction

There is a long tradition of using cephalopods as experimental animals, particularly squid and octopus, in the field of neurophysiology (e.g. Young 1971; Wells 1978). However, more recently, the use of a range of cephalopod species for field and laboratory experiments has increased, largely due to their importance in fisheries and their potential in aquaculture. Experiments using cephalopods range from use of neural and optic material (e.g. Eyman et al. 2003), to tank experiments that manipulate biotic and abiotic growth conditions (e.g. Sykes et al. 2003), to tagging individuals and releasing them into the wild to obtain information about their ecology, biology, and behaviour (e.g. Nagasawa et al. 1993; Gilly et al. 2006). These experiments all seek to obtain information that will contribute to an accurate and informative picture of biology and/or ecology of the animal.

When considering the welfare of vertebrate animals in experiments it is recommended that the three R's (reduction, replacement, and refinement) be considered. This involves ensuring that the number of animals used in the experiments is valid (reduction), considering alternatives to live animals in experiments (replacement), and adoption of experimental methods that minimize distress to the animals (refinement). We recommend that the three R's should be a major consideration when using cephalopods in experiments. When addressing the question of whole animal biology there are limited opportunities to replace animals with alternatives, such as computer simulations and cell cultures. However, replacement is worth considering for species groups which have juveniles that are currently impossible to rear (e.g. ommastrephids and Idiosepiidae). Given the 100% mortality rates of these juveniles in culture conditions, it may be worth considering using alternative cephalopod species, for which we have developed culture techniques, as ''model species''.

Reduction and refinement are important areas to address when planning and designing experiments. Given the need to provide good water quality and live food when maintaining many cephalopods (Table 1), it is typically difficult to hold large numbers of adult cephalopods. As a result logistics will restrict the numbers of animals, however, smaller individuals (small adults and juveniles) can be potentially held in larger numbers. Therefore, reduction in the number of animals can be addressed a number of ways including, ensuring that animals in research facilities are held to answer direct biological results and not only to see if they can be held. For many commercially important species details of maintenance and culture are readily available (Tables 2–8). The number of animals needed in an experiment to determine differences among treatments is a function the variability among replicate animals. Such information is often difficult to obtain, however, an increasing number of publications (Tables 2–8) are presenting results of rigorous experiments. These studies provide estimates of inter-animal variability that is readily usable as a guide for future experimental designs. Refining the methods associated with all aspects of using cephalopods as experimental animals is one of the three R's that experimenters can readily address and should be given full consideration. Refinement based on past experience (as reported in the literature) is the major consideration of this paper.

We have identified existing expertise and literature that outlines techniques for handling and holding cephalopods in different life stages, from embryo to adults (e.g. Teuthoids and Sepioids in Boletzky and Hanlon 1983; Hanlon 1990). This depth of knowledge provides an informed basis upon which cephalopod biologists can justify decisions about the welfare of animals used in scientific endeavours. However, this realization needs to be tempered with an understanding and recognition that cephalopods have unique biological characteristics that need consideration (Table 1). In particular the very short lifespan of most species means that death in captivity

Table 1 General biological characteristics of cephalopod of particular relevance when handling and holding

Biological characteristics	Special consideration
External protection	No protection (except for Nautilus) and the delicate skin is readily damaged by physical contact e.g. handling and contact with the side of tanks. All species are marine and most have limited capacity to tolerate changes to salinity changes.
Mobility	Active species e.g. pelagic species will need to swim constantly, may jet out of tank, and repeatedly hit side of tank. Some benthic species need shelter or hides.
Response to stress e.g. attack, toxic substances, disease	Cephalopods will display a startle and escape response. The copious volumes of black ink should be removed. Self damage can occur through eating of arms and repeated contacting with the side of tanks. Lesions will be evident where damage to the skin has occurred, these lesions may be an early sign of senescence.
Food	All species are carnivores and most require live prey, especially during early life history. Artificial food suitable for cephalopod is not yet available. Cannibalism can occur when insufficient food provided. Prey size is limited by body size rather than mouth size. Prey items up to 150% larger than the juvenile can be captured and eaten.
Life span	Many inshore cephalopod species live for 12 months or less, so adults brought into the laboratory are likely to die in captivity due to natural senescence. Some species will die shortly after egg production or after hatching of juveniles.
Reproductive biology	Range of reproductive traits, with some species producing a single batch of eggs at end of life while others will produce numerous batches. Most cephalopod species produce external eggs that are sheathed and well protected by a mucilaginous coat. A suitable substrate or the presence of an egg mass often needs to be provided to encourage egg deposition. Egg size varies with species, with large egg species being easier to culture in captivity. Larger eggs have longer development times, but can be vulnerable to fungal infections the longer they are held. Removal of the parents is not necessary and in the case of *Octopus* species the females must be left in the tank to care for eggs. Eggs with no maternal care will need to be oxygenated using a gentle flow of water across the eggs.
Respiration	Oxygen is taken up via the gills and skin.
Social behaviour	Some species naturally school, particularly squid, and space and water quality needs to be carefully monitored. Some species are non-aggregative and keeping high densities of mixed genders can cause problems with constant interactions resulting in death.
Early life history	Cephalopods have a short (ca. 5 days) endogenous feeding period during which they must learn to capture prey. Many species suffer extremely high juvenile mortality in a laboratory setting, possibly due to starvation when predation behaviour fails to develop. There is variation in the size and life style of cephalopod hatchlings; small-egged species usually produce planktonic young while large-egged species produce benthic young. The latter have been easier to rear, because the juveniles look and behave as miniature adults capable of jetting, inking and prey capture.
Behaviour	Complex and diverse (Hanlon and Messenger 1996). There will need to an awareness of ''typical'' behaviours for the species being held in captivity. In particular the need for objects to hide under or in, and the interaction among individuals held in the same tanks.

(not initiated by the experimenter) is the norm not the exception, particularly following spawning (e.g. octopus species).

In most countries ethical guidelines for the use of animals in experiments are restricted to vertebrate species. As invertebrates, ethical guidelines for the use and handling of animals in science do not include cephalopods. However, cephalopods have a well developed nervous system (Young 1971; Wells 1962; Budelmann 1995) and display advanced behaviours (Hanlon and Messenger 1996), suggesting that welfare guidelines for these animals are needed. The presence of free nerve endings in the skin suggests that perception of pain is possible and behavioural responses suggest that many cephalopods do respond to pain (Mather and Anderson in press). The concept of pain can be extended to include psychological suffering, and the appropriateness of culture conditions, including behavioural enrichment should be considered (Mather 1986, 2001). Currently, there is no universal standard or legislation concerning the welfare of cephalopods, and the adoption of

Table 2 Examples of recent literature that has used Nautilidae as experimental animals

Species	Collection, Handling, Transport	Housing	Food and Feeding	Eggs, embryos and juveniles	Anaesthetics and Euthanasia	Behaviour	Treatment of Diseases	Tagging and Tracking
N. macromphalus	1, 2, 3	2, 4	2, 5, 6, 7	2, 6, 7		1, 2		3, 4
N. belauensis	2	2	2	2, 8, 9		1, 10, 11		
N. pompilius	1, 2, 12, 13, 14	2, 4, 12, 15, 16, 17, 18	2	2, 17, 19		1, 12, 15, 17		4, 12, 20
Unnamed N. species	12, 16	12, 16, 21		22		12, 22, 23, 24		12, 22, 24

(1) Ward (1987); (2) Hamada et al. (1980); (3) Saunders (1983); (4) Hamada (1987); (5) Ward and Wicksten (1980); (6) Ward (1983); (7) Mikami and Okutani (1977); (8) Okubo et al. (1995); (9) Arnold et al. (1990); (10) Carlson et al. (1984); (11) Saunders (1984); (12) Saunders and Landman (1987); (13) Saunders and Spinosa (1978); (14) Wells and Wells (1985); (15) Zann (1984); (16) Carlson (1987); (17) Fields (2006); (18) Westermann et al. (2004); (19) Arnold et al. (1993); (20) O'Dor et al. (1990a); (21) Spinosa (1987); 22. Boyle and Rodhouse (2005); (23) O'Dor et al. (1993); (24) Ward et al. (1984)

Table 3 Examples of recent literature that has used Sepiida as experimental animals

Life Stage	Collection, handling, and transport	Housing	Feeding	Reproduction	Behaviour	Anaesthetics	Health, disease, and treatment	Tagging and tracking
Eggs	1, 2, 3, 4, 5	6, 7, 4, 8					2, 3	
Juveniles		4, 9, 10, 11, 12, 13, 14	14, 15, 16	17, 18, 19, 20, 21	22			
Adults	23	9, 10, 22, 24, 25, 26, 27	28, 29	5, 9, 19, 30, 31, 32, 33	19, 34, 35, 36, 37, 38	39	21, 24, 40, 41, 42, 43,	44, 45, 46

(1) Blanc and Daguzan (1998); (2) Boletzky (1998); (3) D'Aniello et al. (1990); (4) Minton et al. (2001); (5) Oka (1993); (6) Bouchaud and Daguzan (1989); (7) Cronin and Seymour (2000); (8) Paulij et al. (1991); (9) Correia et al. (2005); (10) Forsythe et al. (2002); (11) Hanley et al. (1998); (12) Koueta and Boucaud-Camou (1999); (13) Sykes et al. (2003); (14) Domingues et al. (2003); (15) Blanc et al. (1998); (16) Moltschaniwskyj and Martínez (1998); (17) Boal and Ni (1996); (18) Boletzky and Roeleveld (2000); (19) Crook et al. (2002); (20) Hanlon and Messenger (1996); (21) Warnke (1994); (22) Sherrill et al. (2000); (23). Forsythe et al. (1991); (24) Hanley et al. (1999); (25) Loi and Tublitz (1999); (26) Sykes et al. (2006); (27) Domingues et al. (2002); (28) Castro and Lee (1994); (29) Domingues et al. (2005); (30) Boletzky (1987); (31) Corner and Moore (1980); (32) Hall and Hanlon (2002); (33) Nabhitabhata and Nilaphat (1999); (34) Adamo et al. (2000); (35) Boal et al. (1999); (36) Castro and Guerra (1989); (37) Lipiński et al. (1991); (38) Quintela and Andrade (2002); (39) Aitken et al. (2005); (40) Castro et al. (1992); (41) Halm et al. (2000)l; (42) Sangster and Smolowitz (2003); (43) Reimschuessel et al. (1990); (44) Ezzedine-Najai et al. (1995); (45) Jackson et al. (2005); (46) Watanuki and Iwashita (1993)

Table 4 Examples of recent literature that has used Sepiolida as experimental animals

Species	Collection, handling, and transport	Housing	Feeding	Eggs, embryos and juveniles	Behaviour
Sepietta spp, *Rossia* spp, *Sepiola* spp, *Euprymna scolopes*	1, 2, 3	1, 2, 3, 4	2, 3, 4, 5	1, 2, 6, 7, 8, 9, 10	2, 5, 11, 12

(1) Summers (1985); (2) Summers and Colvin (1989); (3) Hanlon et al. (1997); (4) Claes and Dunlap (2000); (5) Bergström (1985); (6) Salman (1998); (7) Anderson and Shimek (1994); (8) Boletzky (1975); (9) Yau and Boyle (1996); (10) Arnold et al. (1972); (11) Moynihan (1982); (12) Shears (1988)

Table 5 Examples of recent literature that has used Idiosepiidae as experimental animals

Species	Capture, Handling, Transport	Housing	Feeding	Eggs, embryos and juveniles	Behaviour
Idiosepius biserialis			1		
I. notoides	2, 3	3	2, 3, 4		
I. macrocheir					
I. paradoxus			5	6, 7, 8, 9	
I. picteti					
I. pygmaeus	5, 10, 11		12, 13	6, 8, 14, 15	17, 18
I. thailandicus	19, 20	19, 20	19, 20	20	

(1) Hylleberg and Nateewathana (1991); (2) English (1981); (3) Tracey et al. (2003); (4) Eyster and Van Camp (2003); (5) Kasugai (2001); (6) Kasugai (2000); (7) Kasugai and Ikeda (2003); (8) Natsukari (1970); (9) Yamamoto (1988); (10) Jackson (1992); (11) Moynihan (1983); (12) Jackson (1989); (13) Semmens (1993); (14) Jackson (1993); (15) Lewis and Choat (1993); (16) Van Camp (1997); (17) Roberts (1997); (18) Sasaki (1923); (19) Nabhitabhata (1994); (20) Nabhitabhata (1998)

legislation by scientists is patchy. For example, in the UK *Octopus vulgaris* is included in the legislation, Canada includes all cephalopods, and in Australia and USA legislation is in place for some research institutes in some states. The future implementation of legislation for the ethical use of cephalopods is unclear and research scientists will be responsible for determining the legal requirements for their country and/or research institute when using cephalopods in experiments. For example, the status of cephalopods in the EU legislation for animal ethics is currently under review, and one recommendation is that all cephalopods are included in the legislation.

Legislation aside, there are a suite of reasons that cephalopod research scientists may need to consider the welfare of their study animals. These include moral and ethical issues associated with experimental manipulations and the need to ensure that obtaining information in experiments is with minimal distress to the animals. This becomes important for scientists with limited experience or restricted access to experienced researchers, who may have difficulty obtaining guidance about what may be the ''right'' or suitable method to manage cephalopods, and is critical to maximising the scientific value of animals used in experiments.

On a pragmatic level, experiments obtain information about the biology and ecology of cephalopods that cannot be obtained directly from wild animals (e.g. factors affecting growth and reproduction, movement, and behaviour), by either holding animals in captivity or handling animals prior to release into the wild. In these cases, it is essential that experiments accurately reflect the biology of the species, as there is often an explicit interest in extrapolating these results to wild populations, or at least understanding the processes that shape wild populations. To do this it is vital that the handling and maintenance conditions of these experiments are as close to ''natural'' as possible, therefore animal welfare will be an important element of these experiments. However, cephalopod researchers must also be aware of, and sensitive to, the public perception of their experiments and the animals upon which these experiments are conducted. The natural charisma of cephalopods draws significant public interest (e.g. Anderson 2000), and their complex behaviours are subject to frequent anthropomorphism.

Table 6 Examples of recent literature that has used Loliginidae as experimental animals

Life stage	Capture Handling Transport	Housing	Feeding	Anaesthetics	Treatment of diseases	Artificial fertilization	Tagging and tracking	Behaviour
Eggs/ Embryos	1, 2, 3, 4	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 42, 44, 46, 47			1, 4, 20	21		
Hatchlings/ Paralarvae		1, 2, 4, 5, 6, 9, 10, 16, 17, 18, 19, 24	1, 2, 4, 6, 9, 10, 16, 17, 19, 24, 25, 26, 27, 28, 43	18, 19				
Juveniles/ Adults	29, 30, 31, 32	1, 2, 4, 10, 29, 31, 32, 33, 34, 45	1, 2, 4, 10, 27, 31, 32, 33, 35, 43	1, 31, 36, 41	20, 31, 37, 38		29, 39, 40	32, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58

(1) Lee et al. (1994); (2) Lee et al. (1998); (3) Steer et al. (2003); (4) Walsh et al. (2002); (5) Nabhitabhata et al. (2001); (6) Cardoso et al. (2005); (7) D'Aniello et al. (1989); (8) Gowland et al. (2003); (9) Ikeda et al. (2005); (10) Nabhitabhata (1996); (11) Omar et al. (2001); (12) Oosthuizen et al. (2002a); (13) Oosthuizen et al. (2002b); (14) Paulij et al. (1990); (15) Sen (2004a); (16) Vidal et al. (2002a); (17) Vidal et al. (2002b); (18) Villanueva (2000a); (19) Villanueva (2000b); (20) Forsythe et al. (1990); (21) Crawford (2002); (22) Ikeda et al. (2004a); (23) Fagundez and Robaina (1992); (24) Mladineo et al. (2003); (25) Akiyama et al. (1997); (26) Navarro and Villanueva (2000); (27) Segawa (1993); (28) Villanueva (1994); (29) Gonçalves et al. (1995); (30) Ikeda et al. (2004b); (31) Oestmann et al. (1997); (32) Porteiro et al. (1990); (33) Hanlon et al. (1991); (34) Nabhitabhata and Nilaphat (2000); (35) DiMarco et al. (1993); (36) Garcia Franco (1992); (37) Hanlon and Forsythe (1990); (38) Hanlon et al. (1988); (39) Estacio et al. (1999); (40) Sauer et al. (2000); (41) Messenger et al. (1985); (42) Sen (2005); (43) Segawa (1990); (44) Ito and Sakurai (2001); (45) Segawa (1995); (46) Sen (2004b); (47) Sen (2004c); (48) King et al. (2003); (49) Dimarco and Hanlon (1997); (50) Sauer et al. (1997); (51) Jantzen and Havenhand (2003a); 52. Boal and González (1998); (53) Buresch et al. (2004); (54) Hanlon et al. (2002); (55) Hanlon et al. (1999); (56) Cornwell et al. (1997); (57) Jantzen and Havenhand (2003b)

Table 7 Examples of recent literature that has used Ommastrephidae as experimental animals

Species	Collection, handling, transport	Housing	Feeding	Eggs, embryos and juveniles	Anaesthetics and Euthanasia	Treatment of Disease	Tagging and Tracking
Dosidicus gigas				1			2,3,4
Illex spp (I. argentinus, I. coindetii, I. illecebrosus)	5	5, 6, 7, 8, 9	5, 10	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21	8, 22		23, 24, 25, 26, 27, 28
Nototodarus spp (N. sloanii and gouldi, hawaiiensis)					29		30, 31
Ommastrephes bartramii				32			33, 34, 35, 36
Sthenoteuthis oualaniensis				32			
Todarodes pacificus	37, 38	39, 40, 41	42	43, 44	45		46

(1) Yatsu et al. (1999a); (2) Bazzino et al. (2005); (3) Markaida et al. (2005); (4) Yatsu et al. (1999b); (5) O'Dor et al. (1977); (6) Bradbury and Aldrich (1969a); (7) Bradbury and Aldrich (1969b); (8) Portner et al. (1993); (9) O'Dor et al. (1982a); 10. O'Dor et al. (1980); 11. Sakai and Brunetti (1997); (12) Sakai et al. (1997); (13) Sakai et al. (1998); (14) Sakai et al. (2004); (15) Sakai et al. (1999); (16) Boletzky et al. (1973); (17) O'Dor et al. (1982b); (18) Durward et al. (1980); (19) O'Dor et al. (1985); (20) Balch et al. (1985); (21) O'Dor et al. (1986); (22) O'Dor et al. (1990b); (23) Brunetti et al. (1996); (24) Brunetti et al. (1997); (25) Dawe et al. (1981); (26) O'Dor et al. (1979); (27) Webber and O'Dor (1985); (28) Webber and O'Dor (1986); (29) Lykkeboe and Johansen (1982); (30) Sato (1985); (31) Yamada and Kattho (1987); (32) Sakurai et al. (1995); (33) Nakamura (1991); (34) Nakamura (1993); (35) Tanaka (2000); (36) Tanaka (2001); (37) Bower et al. (1999); (38) Flores et al. (1976); (39) Flores et al. (1977); (40) Mikulich and Kozak (1971a); (41) Mikulich and Kozak (1971b); (42) Soichi (1976); (43) Ikeda et al. (1993); (44) Ikeda and Shimazaki (1995); (45) Sakurai et al. (1993); (46) Mori and Nakamura (2001)

Table 8 Examples of recent literature that has used Octopodidae and other octopods as experimental animals

	Collection, handling and transport	Housing	Feeding	Eggs, embryos and planktonic stages	Anaesthetics and Euthanasia	Health, disease, and treatment	Tagging and tracking
Coastal octopods	1, 2, 3	4, 5, 6, 7, 8, 9, 10, 11, 12	5, 7, 12, 13, 14, 15	16, 17, 18, 19, 20, 21, 22, 23, 24	10, 25, 26, 27, 28, 29	30, 31, 32, 33, 34	35, 36, 37
Deep-sea and cold-adapted octopods	38, 39, 40	38, 39, 40	38, 39, 40, 41	38, 42			
Pelagic octopods	43	44, 45, 46, 47	44, 48				

(1) Voight (1988); (2) Sánchez and Obarti (1993); (3) Smale and Buchan (1981); (4) Van Heukelem (1977); (5) Forsythe and Hanlon (1980); (6) Boyle (1981); (7) Forsythe (1984); (8) Hanlon and Forsythe (1985); (9) Forsythe and Hanlon (1988); (10) Anderson (1996); (11) Mather and Anderson (1999); (12) Anderson and Wood (2001); (13) Joll (1977); (14) Garcia Garcia and Cerezo Valverde (2006); (15) Segawa and Nomoto (2002); (16) Itami et al. (1963); (17) Marliave (1981); (18) Snyder (1986); (19) Boletzky (1989); (20) Forsythe and Toll (1991); (21) Villanueva (1995); (22) Iglesias et al. (2004); (23) Villanueva et al (2004); (24) Okumura et al (2005); (25) Batham (1957); (26) Dew (1959); (27) Brough (1965); (28) Forsythe and Hanlon (1985); (29) Seol et al. (2007); (30) Hanlon et al. (1984); (31) Budelmann (1988); (32) Adams et al (1989); (33) Forsythe et al (1990); (34) Pascual et al. (2006); (35) Robinson and Hartwick (1986); (36) Anderson and Babcock (1999); (37) Domain et al. (2002); (38) Wood et al. (1998); (39) Daly and Peck (2000); (40) Hunt (1999); (41) Collins and Villanueva (2006); (42) Boletzky (1994); (43) Seibel et al. (1997); (44) Lacaze-Duthiers (1892); (45) Boletzky (1983b); (46) Bello and Rizzi (1990); (47) Packard and Würtz (1994); (48) Young (1960)

On a moral basis, as scientists and biologists using these animals in experiments, we have a responsibility to be proactive about minimizing the distress of individuals in our care and to better understand how we can care for welfare of the animals that we are using in experiments. We recommend that collecting cephalopods humanely as possible, e.g. basket traps for cuttlefish, jigs for squid, and pots for octopuses. An anaesthetic that is commonly and successfully used on cephalopods is magnesium chloride (Messenger et al. 1985; Scimeca and Forsythe 1999). The best method for euthanising cephalopods varies among species, but the pool of information is limited. Boyle (1991) recommends decapitation; however, this is not always easy with very small or large animals, or onboard on a rolling ship. Decapitation is completely unsuitable when intact specimens are need for museum collections or collection of statoliths, as these sit at the back of the head. Chilling is suitable for tropical and warm temperate species (Anderson 1996; Moltschaniwskyj and Semmens 2000), but is not useful for cold-water species. Recent studies on fish demonstrate that live chilling is stressful (Lambooij et al. 2006). Over-anaesthesia is useful when decapitation or chilling are not logistically possible. Several anaesthetic agents have been used with success, including an isotonic solution of $MgCl_2$ (e.g. Messenger et al. 1985; Cornwell et al. 1997; Bartol 2001; Thompson and Kier 2001), although this does not work on some *Octopus* species (Anderson 1996). An alternative is ethanol with increasing concentrations from 1 to 5% over a period of hours (Anderson 1996), or clove oil (Seol et al. 2007). Whatever the end use of the animals, euthanising cephalopods should be done as rapidly as possible and decapitation and overdose of anaesthetics are suitable methods.

Given that welfare considerations of cephalopods is relatively new, particularly in relation to capture and culture (this includes handling, housing, maintenance, and rearing), this review has compiled literature that provides details of using cephalopods as experimental animals. Cephalopods are a diverse group of animals and it is not possible to provide a single set of guidelines or rules with respect to capture and culture of cephalopods as a group, or even across all life stages. Furthermore, it is not possible to provide strict guidelines about techniques or methods that minimize distress and maximize welfare, especially since limited knowledge is available or published. Published work provides a starting point, with techniques and approaches that have worked, but these techniques may be further refined especially as more information about a species comes to light. The aim of this paper is to identify welfare issues that researchers using cephalopods in experiments should consider (Table 1), and to provide sources of information that can help in making the best possible decision (Tables 2–8). The information is designed to help those scientists with limited experience or with little access to experienced researchers. It will also help research scientists needing to apply for permission from ethics committees to use cephalopods and be able to provide evidence to justify welfare decisions.

Nautilidae

Three species of *Nautilus* have been maintained in captivity over the past 30–40 years (Table 2). As a result there is a reasonable volume of information about collection, handling, and housing of individuals, as well as basic aspects of their biology (Table 2). Saunders and Landman (1987) provide the best overview of collection, maintenance, and rearing of *Nautilus* in aquariums, including housing systems. A newer publication that provides detailed specifics on the conditions and infrastructural requirements for optimum rearing in aquarium settings and for general aspects of *Nautilis pompilius* husbandry is provided by Fields (2006). Boyle and Rodhouse (2005) provide a good general summary of many facets of *Nautilus* species biology, covering form and function, ecology, predation, growth, reproduction, activity, and fisheries.

Natural growth rates of *Nautilus* are available for both wild and lab/aquarium held animals (e.g. Saunders 1983). Juvenile *N. belauensis* have the capacity to add 0.1 mm of shell per day at the ventral circumference, but this rate decreases rapidly as individuals approach maturity. Individual chamber formation is estimated to take >100 days to >1 year. Similarly, the period required for an individual animal to reach sexual maturity varies across species and individuals with a range of 2.5–15 years. Other studies by Ward (1983) and Westermann et al. (2004) review laboratory growth rates for *N. macromphalus*

and *N. pompilius*, respectively and relate them to wild caught animals for age estimation.

A clear gap in our knowledge of maintaining *Nautilus* is assessment of health, besides using discoloration of the mantle and loss of ability to maintain buoyancy little is known about disease and health issues. Research is required on the best methods for euthanizing individuals in apparent poor health.

Sepiida

Due to their benthic ecology and tolerance of handling cuttlefish adapt well to life in captivity (Hanlon 1990). Methods of transport, housing, and culture of cuttlefish are reasonably well established (Forsythe et al. 1991, Table 3). One species in particular, the European common cuttlefish *Sepia officinalis*, has been studied and maintained in captivity for many years and cultured through multiple generations (Schröder 1966; Pascual 1978; Boletzky 1979; Forsythe et al. 1994). More recently, several other species have also been cultured through multiple generations, including the needle cuttlefish *Sepiella inermis* (Nabhitabhata 1997) and pharoah cuttlefish *Sepia pharaonis* (Minton et al. 2001).

Due to the ease of maintaining cuttlefish in captivity, much information on sepiid growth and behaviour has been obtained from captive individuals (Boal et al. 1999; Domingues et al. 2006). Unfortunately, few studies have related their captive conditions to the likely field environments experienced by wild individuals and this remains a significant area needing further investigation to ensure that laboratory studies are relevant to natural ecology and life history of species. Boletzky (1983a) provides a thorough review of the biology and ecology of *S. officinalis* and more recent studies on wild species include juvenile growth rates (Challier et al. 2002, 2005; Minton 2004), reproductive biology (e.g. Gabr et al. 1998), life cycles (e.g Guerra and Castro 1988), and behaviour (e.g. Aitken et al. 2005). Other areas of research specifically related to ethics that require further investigation include a critical examination of appropriate euthanasia, anaesthesia and disease treatment methods and non-invasive ways to accurately assess condition, well-being, and stress levels of individuals.

Aspects of sepiid biology that are of particular relevance to maintaining captive individuals include: their social behaviour and habitat requirements, as many species show dominance hierarchies, cannibalism or territoriality especially when reproductively active or in crowded conditions (Boal et al. 1999); dietary requirements, which are poorly understood, particularly during the early life stages when individuals require live food and are voracious feeders (Sykes et al. 2006); and water quality requirements, as cuttlefish are susceptible to skin ulceration and buoyancy malfunction in response to poor water quality and bacterial infection (Forsythe et al. 1991; Sherrill et al. 2000).

Sepiolida

The sepiolids have attracted a lot of attention from a number of research teams because of the symbiotic relationship between these animals and light-producing *Vibrio* spp. hosted within the light-organ (see review McFall-Ngai 1999). Given their relatively solitary nature and benthic mode of life, large eggs and large benthic juveniles, it has been relatively easy to culture a number of sepiolid species through several generations (Sinn et al. 2006). Detailed descriptions of culture methods, including collection, transport, housing and reproduction, are available for *Euprymna scolopes* (Hanlon et al. 1997), *Rossia pacifica* (Summers and Colvin 1989), and five *Sepiola* spp. (Bolezky et al. 1975). With careful handling and correct packing live adults and eggs of many sepiolids have been flown half-way around the world with excellent results.

Details of the biology and growth of wild animals are limited, largely due to their nocturnal and cryptic behaviour. The well-hidden eggs are rarely seen in the natural environment. Estimates of growth from natural populations have yet to be obtained due to the lack of daily growth increments on the statoliths and absence of other hard structures (Moltschaniwskyj and Cappo, in press). As much of our knowledge of the biology of these animals is being obtained from captive populations it is critical to take careful consideration of culture conditions and husbandry is made if we are to extrapolate information to wild populations (Table 4).

There are several issues to consider when culturing these species. Every 24 h, at first light, sepiolids release a high number of *Vibrio* from the light organ into the seawater. As a result, in recirculating seawater systems, the *Vibrio* levels in the seawater can reach reasonably high concentrations. It is not clear what the consequences for the health of the sepiolids are, but there is no evidence in the literature that this impacts negatively on their growth and survival. If the high densities of *Vibrio* are of concern then it is possible to reduce the density of *Vibrio* in the water using UV sterilisers. However, use UV sterilisation with caution and for limited periods, as there is the possibility of killing the biofilm in the biofilter.

Another issue that needs to be considered is that these species use a sand coat adhered to the outside of the animal and bury themselves into the soft substrate during daylight hours. Although providing sand for burying may not be essential for successful culture, the lack of available burying substrate could potentially cause increased stress (e.g. Mather 1986). The addition of sand to the tanks can cause problems with maintaining water quality and cleanliness, especially from rotting debris from prey items. On the other hand, bacteria flora building up in the sand may effectively provide a type of ''biofilter' in the tank.

Idiosepiidae

There are no papers that explicitly describe handling or care of the species of *Idiosepius*. Most of the following information is from papers that have used these species in their research; to date five of the seven described species have been collected and held in aquaria. Research has focussed on aspects of the biology of both wild and captive animals, in particular growth (Jackson 1989; Pecl and Moltschaniwskyj 1997), reproduction (Table 5), and behaviour (Table 5). For the other two species, only species descriptions are available (*I. macrocheir* and *I. picteti*). A review of the biology and ecology of this genus considers the issues and specialisation of this mini-maximalist, the smallest of the cephalopod species (Boletzky et al. 2005).

It is worth noting that adults of these species are extremely amenable to collection and maintenance, and will readily mate and deposit eggs in captivity. However, the juveniles have so far proven impossible to hold for more than five days; therefore the lifecycle has not been closed for any of the species in this group.

Loliginidae

Due to their economic value to inshore fisheries and the relative ease of capture and holding of a range of life history stages, there have been a very large number of studies that have used loliginid squids as experimental animals. Three substantial overviews of maintenance of loliginid squid have been produced (Boletzky and Hanlon 1983; Hanlon 1987, 1990). These documents are a valuable starting point to anyone wishing an overview of techniques and information about handling and holding these species. The literature in Table 6 focuses on publications since 1990 that provide additional information that complements and extends the knowledge in these earlier reviews.

Over the last six years there has been an increase in the number of species that are used for experimental work. Species which have been used extensively in experiments include: *Alloteuthis subulata, Doryteuthis gahi, D. opalescens, D. pealeii, D. plei, D. sanpaulensis, Heterololigo bleekeri, Loligo forbesi, L. reynaudii, L. vulgaris, Lolliguncula brevis, Sepioteuthis australis, S. lessoniana, S. sepioidea, Uroteuthis chinensis, U. duvauceli, U. noctiluca*, and *Uroteuthis* sp. There is extensive information available on holding and maintaining loliginid squid in captivity during all life history stages. Further, as more research teams are setting up or using land-based facilities to conduct experiments involving loliginid squids, it is evident that a diversity of seawater systems, flow through and recirculating, may be successfully used (Table 6).

Research over the last decade has provided more valuable biological detail of particular value when rearing these animals. Some examples include, the role of temperature (e.g. Oosthuizen et al. 2002a), light intensity (e.g. Ikeda et al. 2004a), salinity (e.g. Sen 2005), ionic composition of seawater on embryo growth and viability (e.g. D'Aniello et al. 1989); and rates and causes of embryonic abnormalities (e.g. Oosthuizen et al. 2002b; Gowland et al. 2003). There

has also been a significant increase in the knowledge of diet and nutrition of juvenile loliginids (e.g. Vidal et al. 2002a and b).

The increase in tagging and tracking of wild loliginids means that issues of how to handle individuals are going to be critical to the success of these projects (Table 6). Such research techniques need to maximise the survival of animals upon release and ensure that data retrieved realistically represents what occurs in the wild. For example, the use of antibiotics (2–4 ml of 6 mg/ml tetracycline) at the time of tagging may benefit survival and tag retention (Moltschaniwskyj and Pecl this issue). Types of tags used include spaghetti tags inserted in the mantle or fin (Sauer et al. 2000; Moltschaniwskyj and Pecl this issue), and acoustic tags (Pecl et al. 2006).

A considerable volume of work on wild populations and individuals provides a strong base upon which experimenters can assess the performance of captive-reared animals, especially growth rates and behaviours. There is an extensive body of work on estimating growth rates for a range of loliginid squids (Jackson 2004), including measures of spatial and temporal variability. This provides a useful basis for determining what growth rates in captivity may be expected. The importance of considering captive conditions is highlighted when validation of statolith incremental structure has been conducted using captive reared animals (Jackson 2004). The use of both tagging and photographic information of wild squid has allowed an assessment of the behaviours or absence of certain behaviours in captive individuals. Hanlon and Messenger (1996) provide descriptions of behaviours associated with feeding, reproduction, and general inter-individual interactions. More recent work that is relevant to captive animals is provided in Table 6.

Ommastrephidae

The ommastrephid squids are large, oceanic species that undertake migrations between feeding and spawning grounds over thousands of kilometres. Their significant commercial importance has resulted in considerable interest in their biology and ecology (e.g. *Illex* sp.). However, their large size and continuous, active swimming make them one of the hardest groups of cephalopods to maintain in captivity. They do not hover in the water column as many sepiids and loliginids do and thus they require much larger holding tanks. Many ommastrephids also have strong cannibalistic tendencies.

Ommastrephids have not yet been successfully reared from eggs. Females produce large, gelatinous, neutrally buoyant egg masses that are difficult to find in the wild. However, techniques of artificial fertilization have allowed hatchlings of a number of ommastrephid species to be produced. Unlike many of the cephalopods discussed earlier, newly hatched ommastrephids are not functional adults; hatchlings are very small paralarvae referred to as rhynchoteuthions. Their feeding tentacles are fused into a proboscis, resulting in a diet and mode of food capture that is probably unique among cephalopods during these early stages. Feeding of these early stages has never been successful, and thus the life cycle has yet to be closed for any species.

Nevertheless, there is a significant amount of information available about certain aspects of ommastrephid life that will be of value when optimising captive conditions and conducting experiments (Table 7). This information includes details of the capture and transport of adults, the holding of eggs and observations of the rhynchotheution paralarvae, and maintenance of adults in tanks (Table 1). Researchers have successfully tagged and tracked of a number of species; details of these techniques are readily available (Table 7). For 19 of the 21 Ommastrephidae species growth rates of wild individuals has been estimated using statoliths (Arkhipkin 2004), providing a strong basis for assessing the growth rates of captive animals.

Octopodidae and other octopods

There is a long history of using coastal octopus species of the family Octopodidae for experiments in behavioural, physiological, and ecological studies, probably because this group of cephalopods is best adapted to laboratory conditions. This is due, in part, to their benthic mode of life, reclusive behaviour, and reduced swimming activity in comparison with other cephalopod groups. Recent interest in octopus as

biomedical and aquaculture species has resulted in a refinement of handling and culture methods (Table 8). Studies of wild octopus populations, exploring aspects of biology, ecology, and fisheries, use traditional fishing methods, *in-situ* observations, and mark-recapture techniques with internal and external tags. This is currently the only group of cephalopods covered by Ethic Guidelines in the UK.

Reviews of information about how to maintain, rear, and culture inshore large-egged octopus species of the family Octopodidae with benthic juveniles is a function of research done during the 1980s (Boletzky and Hanlon 1983; Forsythe 1984; Forsythe and Hanlon 1988). Inshore octopus species with small eggs have a delicate planktonic stage ranging from three weeks to six months depending on temperature and species, which requires special handling methods (Table 8). Rearing benthic juveniles to adult stages for all inshore species of Octopodidae are similar for small- and large-egg species. There are species-specific requirements, as preference for temperature ranges, diurnal or nocturnal activity patterns, size and quality of food, and breeding behaviour.

In comparison with the coastal species of the family Octopodidae, little information exists for the deep-sea, polar, and pelagic octopod species represented by more than 10 octopod families. Considerably less is known about the cirrate octopods as well as families of deep-sea and cold-adapted incirrate octopods due to the difficulties of collecting and holding these cephalopods. They have very specific requirements for food, low temperature, and light levels, and high water pressure (Table 8). Pelagic octopod species have been maintained for very short periods of time, less than two weeks, and their rearing requirements are practically unknown (Table 8).

Deep-sea cephalopods

There is relatively little information on the use of deep-sea cephalopods in laboratory studies, due to difficulties in collecting and maintaining them under suitable conditions of light and temperature. Deep-sea sepiolids have been reared (Summers and Colvin 1989); however, no deep-sea squid species has been maintained for extended periods in captivity. Methods for collection and laboratory maintenance of deep-sea octopods, under suitable conditions of low light and temperature levels are available for the cirrate (Hunt 1999) and incirrate (Wood et al. 1998) octopods. Most deep-sea cephalopods seem to be long-lived species with low fecundity rates making their populations particularly vulnerable to deep-sea fishing activities (Collins and Villanueva 2006).

Concluding thoughts

It is evident that over the past 10 years an extensive volume of work done has used cephalopods as experimental animals (Tables 2–8). As a result of this research, a strong knowledge base is developing about a diversity of species and techniques. This information should guide scientists in refining their methodologies and approaches when using cephalopods in experimental systems. Refinement of experimental techniques reduces the stress of the investigation on the animals. Such refinement is achievable through careful consideration of the experimental design and procedures, housing conditions, and handling. Experiments should also be planned keeping in mind how you will monitor, assess, and manage impacts; and what procedures can be used to identify and respond to unforeseen complications. Adequate and suitable methods of euthanasia will also be points for consideration, especially when collecting animals.

Explicitly identifying how to assess welfare, the exact cause of stress, and when to terminate experiments will pose the greatest challenge to biologists using cephalopods as experimental animals. As a result of work to date with cephalopods we are gaining significant insights into their capacity to respond in adverse ways that suggests a capacity to perceive pain, suffering, and stress (Mather and Anderson in press). In a recent review of issues in fish welfare, Huntingford et al. (2006) highlighted the fact that wild fish experience stress and will suffer damage in the natural environment. Consequently, there is a conflict between what animals experience in the wild and the assessment of welfare and condition when caring for animals in captivity; we can say the same for cephalopods. Wild animals will suffer damage and stress associated with natural processes, e.g. large scale migrations, predation, interspecific

and intraspecific interactions. However, in experimental conditions we expose animals to a different set of stressors and factors causing damage, which need to be minimised. One resource currently available to scientists for use as an assessment tool is a body of work on cephalopod behaviour derived from both wild and captive studies (reviewed by Hanlon and Messenger 1996, recent work in Tables 2–8). Therefore, while we continue to build upon other areas of knowledge which we are lacking, normal behaviours associated with locomotion, feeding and reproduction provide a powerful tool in the assessment of cephalopod welfare.

While disease does not cause senescence, disease is often associated with senescence (Anderson et al. 2002). Given the short life span of cephalopods a persistent question is how we identify and separate natural processes that result in senescence from health problems associated with captive conditions and handling. Tables 2–8 clearly highlight just how little we know about the health of and diseases states in these animals. There is some work on the octopus *Eledone cirrhosa* that suggests a linkage between stress and health in cephalopods (Malham et al. 2002). Furthermore, the interaction of stress and health in cephalopods is likely to be as complex as it is for fish (Huntingford et al. 2006). Evidence of disease is rarely seen in captive populations and because of this, some cephalopod species are thought to be resistant to disease (e.g. *Sepia pharaonis*, Minton et al. 2001). As a result, we know very little about causes of disease, disease progression, and the capacity of immune systems to deal with disease in cephalopods. There is anecdotal information about the occurrence of extensive skin lesions in squid by fishers, but is not clear what association the presence of these lesions has with natural senescence processes.

Given that cephalopods are invertebrates, their inclusion in animal welfare legislation is the exception not the rule; however this situation is seriously being re-considered by a number of countries. Once challenged by animal welfare legislation, cephalopod biologists will find it difficult to provide evidence and standards for techniques to collect, hold, and kill animals. Few studies that have used cephalopods as experimental animals have had to justify their decisions about animal welfare to an independent body. This is starting to change and it will be increasingly important that biologists can develop a set of welfare indicators against which we can justify decisions about experimental protocols and methods.

Not addressed in this review, but of equal importance is the issue of ecological or environmental ethics; this includes the taking of sustainable numbers of eggs, juvenile, and adults from wild populations in a way that minimizes impact to the ecosystem. This is an issue, given the collateral damage associated with the use of trawl gear to collect or sample cephalopods. It is estimated that 60–100% of cephalopods that manage to escape otter trawls will die (Broadhurst et al. 2006). Again there are no standards or guidelines for collection currently available and it is possible that in the future, scientists will have to argue that the numbers they plan to remove from the wild and the methods of collection not only have minimized impact on the animals, but also have minimal impact on the populations and environments. We recommend that targeted collection methods are preferentially used e.g. pots for octopus, jigs for squid, and hand nets for sepioids. These will ensure that both the stress on the animals is minimized and that there is limited damage to the environment. Estimating the population size of the most accessible of cephalopods (loliginids) is difficult, even when there is an established fishery (e.g. Lipinski and Soule this issue). Collection from populations that may be at risk of over exploitation e.g. giant cuttlefish in Australia or the mimic octopus in Indonesia, highlight the need to balance protection of wild populations and the benefits of developing culture techniques. Without a doubt it will be necessary to use existing studies and knowledge base as the starting point in justifying how biologists address issues of welfare and ethics (animal and environmental) when using cephalopods as experimental animals.

Acknowledgements This paper was produced as a result of a workshop held as part of CIAC '06 on Feb 2–3 2006, in which we discussed issues associated with using cephalopods as experimental animals. We would like to thank the School of Aquaculture, University of Tasmania for hosting the workshop. JEARM's participation in the CIAC 2006 Symposium was funded by the Provost's Office for Graduate Studies (USP), FAPESP, and CAPES/PROAP. RV was supported by the Centre de Referència de Recerca i Desenvolupament en Aqüicultura, CIRIT, Generalitat de Catalunya and the Programa para Movilidad de Investigadores of the Spanish Ministry of Science.

References

Adamo SA, Brown WM, King AJ, Mather DL, Mather JA, Shoemaker KL, Wood JB (2000) Agonistic and reproductive behaviours of the cuttlefish *Sepia officinalis* in a semi-natural environment. J Molluscan Stud 66: 417–419

Adams PM, Hanlon RT, Forsythe JW (1989) Toxic exposure to ethylene dibromide and mercuric chloride: effects on laboratory-reared octopuses. Neurotoxicol Teratol 10:519–523

Aitken JP, O'Dor RK, Jackson GD (2005) The secret life of the giant Australian cuttlefish *Sepia apama* (Cephalopoda): behaviour and energetics in nature revealed through radio acoustic positioning and telemetry (RAPT). J Exp Mar Biol Ecol 320:77–91

Akiyama N, Ueno S, Nagai A (1997) Effectiveness of *Neomysis japonica* as an incipient food for the oval squid, *Sepioteuthis lessoniana*. Bull Inst Oceanic Res Develop Tokai Uni 18:83–88

Anderson R, Shimek RL (1994) Field observations of *Rossia pacifica* (Berry, 1911) egg masses. Veliger 37:117–119

Anderson RC (1996) Sedating and euthanizing octopuses. Drum and Croaker 27:7–8

Anderson RC (2000) The release of a captive-reared giant Pacific octopus. Drum and Croaker 31:7–10

Anderson RC, Wood JB (2001) Enrichment for giant Pacific octopuses: happy as a clam? J Appl Anim Welfare Sci 4:157–168

Anderson RC, Wood JB, Byrne RA (2002) Octopus senescence: the beginning of the end. J Appl Anim Welfare Sci 5:275–283

Anderson TJ, Babcock CB (1999) Subcutaneous electromagnetic tagging of benthic *Octopus*: a preliminary evaluation. Mar Freshwat Res 50:225–227

Arkhipkin AI (2004) Diversity in growth and longevity in short-lived animals: squid of the suborder Oegopsina. Mar Freshwat Res 55:341–355

Arnold JM, Awai M, Carlson B (1990) Hatching of *Nautilus* embryos in the Waikiki Aquarium. J Cephalopod Biol 1:117

Arnold JM, Awai M, Carlson BA (1993) *Nautilus* embryology: egg capsule formation, deposition, and hatching. J Cephalopod Biol 2:51–56

Arnold JM, Singley CT, Williams-Arnold LD (1972) Embryonic development and post-hatching survival of the sepiolid squid *Euprymna scolopes* under laboratory conditions. Veliger 14:361–364

Balch N, O'Dor RK, Helm P (1985) Laboratory rearing of rhynchoteuthions of the ommastrephid squid *Illex illecebrosus* (Mollusca: Cephalopoda). Vie Milieu 35:243–246

Bartol IK (2001) Role of aerobic and anaerobic circular mantle muscle fibers in swimming squid: electromyography. Biol Bull 200:59–66

Batham EJ (1957) Care of eggs by *Octopus maorum*. Trans R Soc NZ 84:629–638

Bazzino G, Gilly WF, Markaida U, Salinas CS (2005) Habitat selection of jumbo squid (*Dosidicus gigas*) in the Gulf of California through satellite tagging. Libro de Resumenes, III Simposio Internacional sobre Calamares del Pacifico (23–30 November 2005) y II Taller Internacional en Calamares (01–02 December 2005)

Bello G, Rizzi E (1990) Comportamento di tre femmine di *Argonauta argo* in acquario (Cephalopoda: Argonautidae). Atti Soc ital sci nat mus civ stor nat Milano 131:450–451

Bergström BI (1985) Aspects of natural foraging by *Sepietta oweniana* (Mollusca: Cephalopoda). Ophelia 24:65–74

Blanc A, Daguzan J (1998) Artificial surfaces for cuttlefish eggs (*Sepia officinalis* L.) in Morbihan Bay, France. Fish Res 38:225–231

Blanc A, Du Sel GP, Daguzan J (1998) Habitat and diet of early stages of *Sepia officinalis* L. (Cephalopoda) in Morbihan Bay, France. J Molluscan Stud 64:263–274

Boal JG, Gonzales SA (1998) The social behavior of individual oval squids (*Sepioteuthis lessoniana*) within a captive school. Ethology 104:161–178

Boal JG, Hylton RA, Gonzalez SA, Hanlon RT (1999) Effects of crowding on the social behavior of cuttlefish (*Sepia officinalis*). Contemp Topics Lab Anim Sci 38:49–55

Boal JG, Ni JN (1996) Ventilation rate of cuttlefish, *Sepia officinalis*, in response to visual stimuli. Veliger 39: 342–347

Boletzky Sv (1975) The reproductive cycle of Sepiolinae (Mollusca: Cephalopoda). Pubbl Staz Zool Napoli 1:84–95

Boletzky Sv (1979) Growth and life span of *Sepia officinalis* under artificial conditions. Rapp Comm Int Mer Medit 25/ 26:159–168

Boletzky Sv (1983a) *Sepia officinalis*. In: Boyle PR (ed) Cephalopod Life Cycles, Vol I Species Accounts. Academic Press. pp 31–52

Boletzky Sv (1983b) Laboratory observations on a female *Argonauta argo* (Mollusca: Cephalopoda). Rapp Comm Int Mer Medit 28:289–290

Boletzky Sv (1987) Fecundity variation in relation to intermittent or chronic spawning in the cuttlefish, *Sepia officinalis* L. (Mollusca: Cephalopoda). Bull Mar Sci 40: 382–388

Boletzky Sv (1989) Recent studies on spawning, embryonic development, and hatching in the Cephalopoda. Adv Mar Biol 25:86–109

Boletzky Sv (1994) Embryonic development of cephalopods at low temperatures. Antarc Sci 6:139–142

Boletzky Sv (1998) Cephalopod eggs and egg masses. Oceanogr Mar Biol Annu Rev 36:341–371

Boletzky Sv, Hanlon RT (1983) A review of the laboratory maintenance, rearing and culture of cephalopod molluscs. Mem Natl Mus Vic 44:147–187

Boletzky Sv, Nishiguchi MK, Nabhitabhata J (2005) *Idiosepius*: ecology, biology, and biogeography of a mini-maximalist. Phuket Mar Biol Cent Res Bull 66:11–22

Boletzky Sv, Roeleveld MAC (2000) ''Ventral adhesion'' to hard substrates: a thigmotactic response in sepiid cuttlefish (Mollusca: Cephalopoda). Vie Milieu 50:59–64

Boletzky Sv, Rowe L, Aroles L (1973) Spawning and development of the eggs, in the laboratory, of *Illex coindetii* (Mollusca: Cephalopoda). Veliger 15:257–258

Bouchaud O, Daguzan J (1989) Etude du développement de l'oeuf de *Sepia officinalis* L. (Céphalopode: Sepiidae) en conditions expérimentales. Haliotis 19:189–200

Bower JR, Sakurai Y, Yamamoto J, Ishii H (1999) Transport of the ommastrephid squid *Todarodes pacificus* under cold-water anesthesia. Aquaculture 170:127–130

Boyle PR (1981) Methods for the aquarium maintenance of the common octopus of British waters, *Eledone cirrhosa*. Lab Anim 15:327–331

Boyle PR (1991) The UFAW Handbook on the care and management of cephalopods in the laboratory. Universities Federation for Animal Welfare, 64 pp

Boyle PR, Rodhouse P (2005) Cephalopods: Ecology and Fisheries. Blackwell Publishing, 452 pp

Bradbury HE, Aldrich FA (1969a) Observations on feeding of the squid *Illex illecebrosus illecebrosus* (Lesueur 1821) in captivity. Can J Zool 47:913–915

Bradbury HE, Aldrich FA (1969b) Observations on locomotion on the short-finned squid, *Illex illecebrosus illecebrosus* (Lesueur, 1821), in captivity. Can J Zool 47:741–744

Broadhurst MK, Suuronen P, Hulme A (2006) Estimating collateral mortality from towed fishing gear. Fish Fish 7:180–218

Brough EJ (1965) Egg-care, eggs and larvae in the midget octopus, *Robsonella australis* (Hoyle). Trans R Soc NZ 6:7–19

Brunetti N, Ivanovic M, Rossi G, Elena B, Benavides H, Guerrero R, Blanco G, Marchetti C, Pinero R, Harada S (1997) Report of JAMARC-INIDEP joint research cruise on Argentine short-finned squid *Illex argentinus*, January - March 1997. 39 pp

Brunetti N, Rossi G, Ivanovic M, Elena B, Guerrero R, Benavides H, Blanco G, Marchetti C, Suekane RN, Kuroiwa M, Murai T (1996) Final report of the JAMARC-INIDEP joint research cruise on Argentine short-finned squid (*Illex argentinus*) during January and February 1996. 42 pp

Budelmann BU (1995) The cephalopod nervous system: what evolution has made of the molluscan design. In: Breidback O, Kutsch W (eds) The nervous system of invertebrates. An evolutionary and comparative approach. Birkhauser Verlag, pp 115–138

Budelmann BU (1998) Autophagy in *Octopus*. Sth Afr J Mar Sci 20:101–108

Buresch KC, Boal JG, Nagle GT, Knowles J, Nobuhara R, Sweeney K, Hanlon RT (2004) Experimental evidence that ovary and oviducal gland extracts influence male agonistic behavior in squids. Bio Bull 206:1–3

Cardoso F, Baltazar P, Bautista J (2005) The early development of the Patagonian squid *Loligo gahi* D'Orbigny, 1835 in Peruvian waters (Cephalopoda: Loliginidae). Rev Peru Biol 12:369–376

Carlson BA (1987) Collection and aquarium maintenance of *Nautilus*. In: Saunders WB, Landman NH (eds) Nautilus: the biology and paleobiology of a living fossil. Plenum Press, pp 563–578

Carlson BA, McKibben JN, DeGruy MV (1984) Telemetric investigation of vertical migration of *Nautilus belauensis* in Palau. Pac Sci 38:183–188

Castro BG, Garrido JL, Sotelo CG (1992) Changes in composition of digestive gland and mantle muscle of the cuttlefish *Sepia officinalis* during starvation. Mar Biol 114:11–20

Castro BG, Guerra A (1989) Feeding pattern of *Sepia officinalis* (Cephalopoda: Sepiodidea) in the Ria de Vigo (NW Spain). J Mar Biol Assoc UK 69:545–553

Castro BG, Lee PG (1994) The effects of semi-purified diets on growth and condition of *Sepia officinalis* L. (Mollusca: Cephalopoda). Comp Biochem Physiol A: Mol Integr Physiol 109:1007–1016

Challier L, Dunn MR, Robin J-P (2005) Trends in age-at-recruitment and juvenile growth of cuttlefish, *Sepia officinalis*, from the English Channel. ICES J Mar Sci 62:1671–1682

Challier L, Royer J, Robin JP (2002) Variability in age-at-recruitment and early growth in English Channel *Sepia officinalis* described with statolith analysis. Aquat Living Resour 15:303–311

Claes MF, Dunlap PV (2000) Aposymbiotic culture of the sepiolid squid *Euprymna scolopes*: role of the symbiotic bacterium *Vibrio fischeri* in host animal growth, development, and light organ morphogenesis. J Exp Zool 286:280–296

Collins MA, Villanueva R (2006) Taxonomy, ecology and behaviour of the cirrate octopods. Oceanography and Marine Biology: An Annual Review 44:277–322

Corner BD, Moore HT (1980) Field observations on the reproductive behaviour of *Sepia latimanus*. Micronesica 16:235–260

Cornwell CJ, Messenger JB, Hanlon RT (1997) Chromatophores and body patterning in the squid *Alloteuthis subulata*. J Mar Biol Assoc UK 77:1243–1246

Correia M, Domingues PM, Sykes A, Andrade JP (2005) Effects of culture density on growth and broodstock management of the cuttlefish, *Sepia officinalis* (Linnaeus, 1758). Aquaculture 245:163–173

Crawford K (2002) Culture method for *in vitro* fertilization to hatching of the squid, *Loligo pealeii*. Biol Bull 203: 216–217

Cronin ER, Seymour RS (2000) Respiration of the eggs of the giant cuttlefish *Sepia apama*. Mar Biol 136:863–870

Crook AC, Baddeley R, Osorio D (2002) Identifying the structure in cuttlefish visual signals. Philos Trans R Soc Lond, Ser B: Biol Sci 357:1617–1624

Daly HI, Peck LS (2000) Energy balance and cold adaptation in the octopus *Pareledone charcoti*. J Exp Mar Biol Ecol 245:197–214

D'Aniello A, D'Onofrio G, Pischetola M, Denuce JM (1989) Effect of pH, salinity and Ca^{2+}, Mg^{2+}, K^{+} and SO_4^{2+} ions on hatching and viability of *Loligo vulgaris* embryo. Comp Biochem Physiol, A: Mol Integr Physiol 94A:477–481

D'Aniello A, Pischetola M, Vanacore F, De Nicola M, Denuce M (1990) Effect of mercury, cadmium and copper on the development and viability of *Loligo vulgaris* and *Sepia officinalis* embryos. Italian J Biochem 39:130A–132A

Dawe EG, Beck PC, Drew HJ, Winters H (1981) Long-distance migration of a shortifinned squid, *Illex illecebrosus*. J Northw Atl Fish Sci 2:75–76

Dew B (1959) Some observations on the development of two Australian octopuses. Proc R Zool Soc NSW 1957–1958:44–52

DiMarco FP, Hanlon RT (1997) Agonistic behavior in the squid *Loligo plei* (Loliginidae, Teuthoidea): fighting tac-

tics and the effects of size and resource value. Ethology 103:89–108

DiMarco FP, Turk PE, Scimeca JM, Browning WJ, Lee PG (1993) Laboratory survival, growth, and digestive gland histologic features of squids reared on living and nonliving fish diets. Lab Anim Sci 43:226–231

Domain F, Caverivière A, Fall M, Jouffre D (2002) Expériences de marquages du poulpe *Octopus vulgaris* au Sénégal. In: Caverevière A, Thiam M, Jouffre D (ed) Le poulpe *Octopus vulgaris* Sénégal et côtes nord-ouest africaines. IRD Èditions, pp 41–58

Domingues P, Poirier R, Dickel L, Almansa E, Sykes A, Andrade JP (2003) Effects of culture density and live prey on growth and survival of juvenile cuttlefish, *Sepia officinalis*. Aquacult Int 11:225–242

Domingues PM, Bettencourt V, Guerra A (2006) Growth of *Sepia officinalis* in captivity and in nature. Vie Milieu 56:109–120

Domingues PM, Dimarco FP, Andrade JP, Lee PG (2005) Effect of artificial diets on growth, survival and condition of adult cuttlefish, *Sepia officinalis* Linnaeus, 1758. Aquacult Int 13:423–440

Domingues PM, Sykes A, Andrade JP (2002) The effects of temperature in the life cycle of two consecutive generations of the cuttlefish *Sepia officinalis* (Linnaeus, 1758), cultured in the Algarve (South Portugal). Aquacult Int 10:207–220

Durward RD, Vessey E, O'Dor RK, Amaratunga T (1980) Reproduction in the squid, *Illex illecebrosus*: first observations in captivity and implications for the life cycle. Int Comm Northwest Atl Fish, Sel Pap 6:7–13

English S (1981) The biology of two species of estuarine cephalopods from the Sydney region. M.Sc. dissertation, University of Sydney, Sydney, p 125

Estacio S, Gonçalves J, Porteiro F, Andrade JP (1999) Comparison of fluorescent bands during staining of statoliths of the squid *Loligo forbesi* (Cephalopoda: Loliginidae). J Molluscan Stud 65:374–377

Eyman M, Crispino M, Kaplan BB, Giuditta A (2003) Squid photoreceptor terminals synthesize calexcitin, a learning related protein. Neurosci Lett 347:21–24

Eyster LS, van Camp LM (2003) Extracellular lipid droplets in *Idiosepius notoides*, the southern pygmy squid. Biol Bull 205:47–53

Ezzedine-Najai S, Ros J, Guerra A (1995) Tagging of the cuttlefish, *Sepia officinalis* L. (Cephalopoda: Decapoda), in the Gulf of Tunis. Sci Mar 61:59–65

Fagundez SB, Robaina G (1992) Effects of temperature, salinity and photoperiod on the embryonic development of the squid *Sepioteuthis sepioidea* (Blainville, 1823). Mem Soc Cien Nat La Salle 52:93–103

Fields RI (2006) Hatching of *Nautilus pompilius* in a closed system using artificial seawater. Drum and Croaker 37:42–47

Flores EEC, Igarashi S, Mikami T (1977) Studies on squid behavior in relation to fishing part 2. On the survival of squid *Todarodes pacificus* in experimental aquarium. Bull Fac Fish Hokkaido Univ 28:137–142

Flores EEC, Igarashi S, Mikami T, Kobayashi K (1976) Studies on squid behavior in relation to fishing I On the handling of squid *Todarodes pacificus* for behavioral study. Bull Fac Fish Hokkaido Univ 27:145–151

Forsythe JW (1984) *Octopus joubini* (Mollusca: Cephalopoda) - a detailed study of growth through the full life-cycle in a closed seawater system. J Zool 202:393–417

Forsythe JW, DeRusha RH, Hanlon RT (1994) Growth, reproduction and life span of *Sepia officinalis* (Cephalopoda: Mollusca) cultured through seven consecutive generations. J Zool 233:175–192

Forsythe JW, Hanlon RT (1980) A closed marine culture system for rearing *Octopus joubini* and other large-egged benthic octopods. Lab Anim 14:137–142

Forsythe JW, Hanlon RT (1985) Aspects of egg development, post-hatching behaviour, growth and reproductive biology of *Octopus burryi* Voss, 1950 (Mollusca: Cephalopoda). Vie Milieu 35:273–282

Forsythe JW, Hanlon RT (1988) Effect of temperature on laboratory growth, reproduction and life span of *Octopus bimaculoides*. Mar Biol 98:369–379

Forsythe JW, Hanlon RT, DeRusha R (1991) Pilot large-scale culture of *Sepia* in biomedical research. In: Boucaud-Camou E (ed) The Cuttlefish, 1st International Symposium on the Cuttlefish, *Sepia*. Centre de Publications de l'Université de Caen, pp 313–323

Forsythe JW, Hanlon RT, Lee PG (1990) A formulary for treating cephalopod mollusc diseases. In: Perkins FO, Cheng TC (eds) Pathology in Marine Science. Academic Press, pp 51–63

Forsythe JW, Lee PG, Walsh LS, Clark T (2002) The effects of crowding on growth of the European cuttlefish, *Sepia officinalis* Linnaeus, 1758 reared at two temperatures. J Exp Mar Biol Ecol 269:173–185

Forsythe JW, Toll RB (1991) Clarification of the Western Atlantic Ocean pygmy octopus complex - the identity and life-history of *Octopus joubini* (Cephalopoda: Octopodinae). Bull Mar Sci 49:88–97

Gabr HR, Hanlon RT, Hanafy MH, El-Etreby SG (1998) Maturation, fecundity and seasonality of reproduction of two commercially valuable cuttlefish, *Sepia pharaonis* and *S. dollfusi*, in the Suez Canal. Fish Res 36:99–115

Garcia Franco M (1992) Anesthetics for the squid *Sepioteuthis sepioidea* (Mollusca: Cephalopoda). Comp Biochem Physiol, C: Toxicol Pharmacol 103:121–123

Garcia Garcia B, Cerezo Valverde J (2006) Optimal proportions of crabs and fish in diet for common octopus (*Octopus vulgaris*) ongrowing. Aquaculture 253:502–511

Gilly WF, Markaida U, Baxter CH, Block BA, Boustany A, Zeidberg L, Reisenbichler K, Robison B, Bazzino G, Salinas C (2006) Vertical and horizontal migrations by the jumbo squid *Dosidicus gigas* revealed by electronic tagging. Mar Ecol Prog Ser 324:1–17

Gonçalves J, Porteiro F, Cardigos F, Martins H (1995) The Azorean adult squid *Loligo forbesi* (Cephalopoda: Loliginidae) in captivity: transport, handling, maintenance, tagging and survival. International Council for the Exploration of the Sea (ICES) / Mariculture Committee (MC). 15 pp

Gowland FC, Boyle PR, Noble LR (2003) Asymmetry in the embryonic chromatophore pattern of the squid *Loligo forbesi* (Mollusca: Cephalopoda): a proxy for development instability. J Mar Biol Assoc UK 83:1101–1105

Guerra A, Castro BG (1988) On the life cycle of *Sepia officinalis* (Cephalopoda: Sepioidea) in the ria de Vigo (NW Spain). Cah Biol Mar 29:395–405

Hall KC, Hanlon RT (2002) Principal features of the mating system of a large spawning aggregation of the giant Australian cuttlefish *Sepia apama* (Mollusca: Cephalopoda). Mar Biol 140:533–545

Halm MP, Agin V, Chichery MP, Chichery R (2000) Effect of aging on manipulative behavior in the cuttlefish, *Sepia*. Physiol Behav 68:543–547

Hamada T (1987) Experience with aquarium rearing of *Nautilus* in Japan. In: Saunders WB, Landman NH (eds) *Nautilus*: The Biology and Paleobiology of a Living Fossil. Plenum Press, pp 579–584

Hamada T, Obata I, Okutani T (1980) *Nautilus macromphalus* in captivity. Japanese Expert Consultation on Living *Nautilus*, (JECOLN). Tokai University Press. 80 pp

Hanley JS, Shashar N, Smolowitz R, Bullis RA, Mebane WN, Gabr HR, Hanlon RT (1998) Modified laboratory culture techniques for the European cuttlefish *Sepia officinalis*. Biol Bull 195:223–225

Hanley JS, Shashar N, Smolowitz R, Mebane W, Hanlon RT (1999) Soft-sided tanks improve long-term health of cultured cuttlefish. Biol Bull 197:237–238

Hanlon RT (1987) Mariculture. In: Boyle PR (ed) Cephalopod Life Cycles Vol II Comparative Reviews. Academic Press, pp 291–305

Hanlon RT (1990) Maintenance, rearing, and culture of teuthoid and sepioid squids. In: Gilbert DL, Adelman WJ, Arnold JM (eds) Squid as experimental animals. Plenum Press, pp 35–62

Hanlon RT, Claes MF, Ashcraft SE, Dunlap PV (1997) Laboratory culture of the sepiolid squid *Euprymna scolopes*: a model system for bacteria-animal symbiosis. Biol Bull 192:364–374

Hanlon RT, Forsythe JW (1985) Advances in the laboratory culture of octopuses for biomedical research. Lab Anim Sci 35:33–40

Hanlon RT, Forsythe JW (1990) Diseases of Mollusca: Cephalopoda. In: Kinne O (ed) Diseases of marine animals III: Cephalopoda to Urochardata. Biologische Anstalt, Helgoland, pp 23–46

Hanlon RT, Forsythe JW, Cooper KM, Dinuzzo AR, Folse DS, Kelly MT (1984) Fatal penetrating skin ulcers in laboratory reared octopuses. J Invertebr Pathol 44:67–83

Hanlon RT, Forsythe JW, Lee PG (1988) External pathologies of cephalopods in captivity. In: Perkins FO, Cheng TC (eds) Pathology in marine science. Academic Press, pp 17–18

Hanlon RT, Maxwell MR, Shashar N, Loew ER, Boyle K-L (1999) An ethogram of body patterning behavior in the biomedically and commercially valuable squid *Loligo pealei* off Cape Cod, Massachusetts. Biol Bull 197:49–62

Hanlon RT, Messenger JB (1996) Cephalopod behaviour. Cambridge University Press, 232 pp

Hanlon RT, Smale MJ, Sauer WHH (2002) The mating system of the squid *Loligo vulgaris reynaudii* (Cephalopoda, Mollusca) off South Africa: Fighting, guarding, sneaking, mating and egg laying behavior. Bull Mar Sci 71:331–345

Hanlon RT, Turk PE, Lee PG (1991) Squid and cuttlefish mariculture: an updated perspective. J Cephalopod Biol 2:31–40

Hunt JC (1999) Laboratory observations of the feeding behavior of the cirrate octopod, *Grimpoteuthis* sp.: one use of cirri. Veliger 42:152–156

Huntingford FA, Adams C, Braithwaite VA, Kadri S, Pottinger TG, Sandøe P, Turnbull JF (2006) Current issues in fish welfare. J Fish Biol 68:332–372

Hylleberg J, Nateewathana A (1991) Morphology, internal anatomy, and biometrics of the cephalopod *Idiosepius biserialis* Voss, 1962. A new record for the Andaman Sea. Phuket Mar Biol Cent Res Bull 56:1–9

Iglesias J, Otero JJ, Moxica C, Fuentes L, Sánchez FJ (2004) The completed life cycle of the octopus (*Octopus vulgaris*, Cuvier) under culture conditions: paralarval rearing using *Artemia* and zoeae, and first data on juvenile growth up to 8 months of age. Aquacult Int 12:481–487

Ikeda Y, Ito K, Matsumoto G (2004a) Does light intensity affect embryonic development of squid (*Heterololigo bleekeri*)? J Mar Biol Assoc UK 84:1215–1219

Ikeda Y, Sakurai Y, Shimazaki K (1993) Fertilizing capacity of squid (*Todarodes pacificus*) spermatozoa collected from various sperm storage sites, with special reference to the role of gelatinous substance from oviducal gland in fertilization and embryonic development. Invertebr Reprod Dev 23:39–44

Ikeda Y, Sakurazawa I, Ito K, Sakurai Y, Matsumoto G (2005) Rearing of squid hatchlings, *Heterololigo bleekeri* (Keferstein 1866) up to 2 months in a closed seawater system. Aquacult Res 36:409–412

Ikeda Y, Shimazaki K (1995) Does nidamental gland jelly induce the formation of perivitelline space at fertilization in the squid *Todarodes pacificus*? J Mar Biol Assoc UK 75:495–497

Ikeda Y, Ueta Y, Sakurazawa I, Matsumoto G (2004b) Transport of the oval squid *Sepioteuthis lessoniana* Ferussac, 1831 in Lesson 1830–1831 (Cephalopoda: Loliginidae) for up to 24 h and subsequent transfer to an aquarium. Fish Sci 70:21–27

Itami K, Izawa Y, Maeda S, Nakai K (1963) Notes on the laboratory culture of octopus larvae. Bull Jap Soc Sci Fish 29:514–520

Ito K, Sakurai Y (2001) Effect of low temperature on embryonic development of *Loligo bleekeri* (Cephalopoda: Loliginidae). Bull Aomori Prefect Fish Exp Stn 1:1–8

Jackson GD (1989) The use of statolith microstructures to analyze life history events in the small tropical cephalopod *Idiosepius pygmaeus*. Fish Bull 87:265–272

Jackson GD (1992) Seasonal abundance of the small tropical sepioid *Idiosepius pygmaeus* (Cephalopoda: Idiosepiidae) at two localitites off Townsville, North Queensland, Australia. Veliger 35:396–397

Jackson GD (1993) Seasonal variation in reproductive investment in the tropical loliginid squid *Loligo chinensis* and the small tropical sepioid *Idiosepius pygmaeus*. Fish Bull 91:260–270

Jackson GD (2004) Advances in defining the life histories of myopsid squid. Mar Freshwat Res 55:357–365

Jackson GD, O'Dor RK, Andrade Y (2005) First tests of hybrid acoustic/archival tags on squid and cuttlefish. Mar Freshwat Res 56:425–430

Jantzen TM, Havenhand JN (2003a) Reproductive behavior in the squid *Sepioteuthis australis* from South Australia: Ethogram of reproductive body patterns. Biol Bull 204:290–304

Jantzen TM, Havenhand JN (2003b) Reproductive behavior in the squid Sepioteuthis australis from South Australia: Interactions on the spawning grounds. Biol Bull 204: 305–317

Joll LM (1977) Growth and food intake of *Octopus tetricus* (Mollusca: Cephalopoda) in aquaria. Aust J Mar Freshw Res 28:45–56

Kasugai T (2000) Reproductive behavior of the pygmy cuttlefish*Idiosepius paradoxus* in an aquarium. Venus Jap J Malacol 59:37–44

Kasugai T (2001) Feeding behavior of the Japanese pygmy cuttlefish *Idiosepius paradoxus* (Cephalopoda: Idiosepidae) in captivity: evidence for external digestion? J Mar Biol Assoc UK 81:979–981

Kasugai T, Ikeda Y (2003) Description of the egg mass of the pygmy cuttlefish, *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) with special reference to its multiple gelatinous layers. Veliger 46:105–110

King AJ, Adamo SA, Hanlon RT (2003) Squid egg mops provide sensory cues for increased agonistic behavior between male squid. Anim Behav 66:49–58

Koueta N, Boucaud-Camou E (1999) Food intake and growth in reared early juvenile cuttlefish *Sepia officinalis* L. (Mollusca: Cephalopoda). J Exp Mar Biol Ecol 240: 93–109

Lacaze-Duthiers H (1892) Observation d'un Argonaute de la Méditerranée. Arch Zool Exp Gén 10:37–56

Lambooij B, Kloosterboer K, Gerritzen MA, André G, Veldman M, Van De Vis H (2006) Electrical stunning followed by decapitation or chilling of African catfish (*Clarias gariepinus*): Assessment of behavioural and neural parameters and product quality. Aquacult Res 37:61–70

Lee PG, Turk PE, Forsythe JW, DiMarco FP (1998) Cephalopod culture: physiological, behavioral and environmental requirements. Suisanzoshoku 46:417–422

Lee PG, Turk PE, Yang WT, Hanlon RT (1994) Biological characteristics and biomedical applications of the squid *Sepioteuthis lessoniana* cultured through multiple generations. Biol Bull 186:328–341

Lewis AR, Choat JH (1993) Spawning mode and reproductive output of the tropical cephalopod *Idiosepius pygmaeus*. Can J Fish Aquat Sci 50:20–28

Lipiński MR, Roeleveld MAC, Augustyn CJ (1991) Feeding studies on *Sepia australis* with an assessment of its significance in the Benguela ecosystem. The Cuttlefish, 1st International Symposium on the Cuttlefish, *Sepia* 117–129

Lipinski MR, Soule (this issue) A new direct method of stock assessment of the loliginid squid. Rev Fish Biol Fisheries, doi: 10.1007/s11160-007-9050-1

Loi PK, Tublitz NJ (1999) Long term rearing of cuttlefish in a small scale facility. Aqua Sci Conserv 2:135–143

Lykkeboe G, Johansen K (1982) A cephalopod approach to rethinking about the importance of the Bohr and Haldane effects. Pac Sci 36:305–313

Malham SK, Lacoste A, Gélébart F, Cueff A, Poulet SA (2002) A first insight into stress-induced neuroendocrine and immune changes in the octopus *Eledone cirrhosa*. Aquat Living Resour 15:187–192

Markaida U, Rosenthal JJC, Gilly WF (2005) Tagging studies on the jumbo squid (*Dosidicus gigas*) in the Gulf of California, Mexico. Fish Bull 103:219–226

Marliave JB (1981) Neustonic feeding in early larvae of *Octopus dofleini* (Wülker). Veliger 23:350–351

Mather J (1986) Sand digging in *Sepia officinalis*: assessment of a cephalopod mollusc's ''fixed'' behavior pattern. J Comp Psychol 100:315–320

Mather JA (2001) Animal Suffering: An Invertebrate Perspective. J Appl Anim Welfare Sci 4:151–156

Mather JA, Anderson RC (1999) Exploration, play, and habituation in octopuses (*Octopus dofleini*). J Comp Psychol 113:333–338

Mather JA, Anderson RC (in press) Ethics and invertebrates: a cephalopod perspective. Dis Aquat Organ

McFall-Ngai MJ (1999) Consequences of evolving with bacterial symbionts: insights from the squid-*Vibrio* associations. Annu Rev Ecol Syst 30:235–256

Messenger JB, Nixon M, Ryan KP (1985) Magnesium chloride as an anesthetic for cephalopods. Comp Biochem Physiol, C: Toxicol Pharmacol Endocrinol 82C:203–205

Mikami S, Okutani T (1977) Preliminary observations on maneuvering, feeding, copulating and spawning behaviors of *Nautilus macromphalus* in captivity. Venus Jap J Malacol 36:29–41

Mikulich LV, Kozak LP (1971a) Experiment in the keeping of the pacific squid under artificial conditions. Russ J Ecol 2:94–96

Mikulich LV, Kozak LP (1971b) Experimental rearing of pacific ocean squid under artificial conditions. Russ J Ecol 2:266–268

Minton JW (2004) The pattern of growth in the early life cycle of individual *Sepia pharaonis*. Mar Freshwat Res 55: 415–422

Minton JW, Walsh LS, Lee PG, Forsythe JW (2001) First multi-generation culture of the tropical cuttlefish *Sepia pharaonis* Ehrenberg, 1831. Aquacult Int 9:379–392

Mladineo I, Valic D, Jozic M (2003) Spawning and early development of *Loligo vulgaris* Lamarck,1798, under experimental conditions. Acta Adriat 44:77–83

Moltschaniwskyj NA, Cappo M (in press) Non-otolith ageing techniques: the use of other techniques to derive estimates of age and growth. In: Green B, Begg G, Carlos G, Mapstone B (eds) Gathering information from Otoliths of tropical fishes

Moltschaniwskyj NA, Martínez P (1998) Effect of temperature and food levels on the growth and condition of juvenile *Sepia elliptica* (Hoyle 1885): an experimental approach. J Exp Mar Biol Ecol 229:289–302

Moltschaniwskyj NA, Pecl GT (this issue) Spawning aggregations of squid (*Sepioteuthis australis*) populations: a continuum of 'microcohorts. Rev Fish Biol Fisheries, doi: 10.1007/s11160-006-9025-7

Moltschaniwskyj NA, Semmens JM (2000) Limited use of stored energy reserves for reproduction by the tropical loliginid squid *Photololigo* sp. J Zool 251:307–313

Mori K, Nakamura Y (2001) Migration patterns of the pacific subpopulation of Japanese common squid *Todarodes pacificus*, estimated from tagging experiments. Bull Hokkaido Natl Fish Res Inst 65:21–44

Moynihan M (1982) Notes on the behavior of *Euprymna scolopes* (Cephalopoda: Sepiolidae). Behaviour 85:25–41

Moynihan M (1983) Notes on the behavior of *Idiosepius pygmaeus* (Cephalopoda: Idiosepiidae). Behaviour 85: 42–57

Nabhitabhata J (1994) Rearing of Thai pygmy cuttlefish, I*diosepius thailandicus* Chotiyaputta, Okutani, and Chaitiamvong. I: Some biological aspects. Rayong Coastal Aquaculture Station, Rayong Thailand, p 19

Nabhitabhata J (1996) Life cycle of cultured big fin squid, *Sepiotheutis lessoniana* Lesson. Phuket Mar Biol Cent Spec Publ 16:83–95

Nabhitabhata J (1997) Life cycle of three cultured generations of spineless cuttlefish, *Sepiella inermis* (Férussac and D'Orbigny, 1848). Phuket Mar Biol Cent Spec Publ 17:289–298

Nabhitabhata J (1998) Distinctive behavior of Thai pygmy squid,*Idiosepius thailandicu*s Chotiyaputta, Okutani, and Chaitiamvong, 1991. Phuket Mar Biol Cent Spec Publ 18:25–40

Nabhitabhata J, Asawangkune P, Amornjaruchit S, Promboon P (2001) Tolerance of eggs and hatchlings of neritic cephalopods to salinity changes. Phuket Mar Biol Cent Spec Publ 25:91–99

Nabhitabhata J, Nilaphat P (1999) Life cycle of cultured pharaoh cuttlefish, *Sepia pharaonis* Ehrenberg, 1831. Phuket Mar Biol Cent Spec Publ 19:25–40

Nabhitabhata J, Nilaphat P (2000) Behavior of juvenile cephalopods: preference for texture and brightness of substrata. Phuket Mar Biol Cent Spec Publ 21:103–112

Nagasawa K, Takayanagi S, Takami T (1993) Cephalopod tagging and marking in Japan: a review. In: Okutani T, O'Dor RK, Kubodera T (eds) Recent advances in Cephalopod fisheries biology. Tokai University Press, pp 313–329

Nakamura Y (1991) Tracking of the mature female of flying squid, *Ommastrephes bartramii*, by an ultrasonic transmitter. Bull Hokkaido Natl Fish Res Inst 55: 205–208

Nakamura Y (1993) Vertical and horizontal movements of mature females of *Ommastrephes bartramii* observed by ultrasonic telemetry. In: Okutani T, O'Dor RK, Kubodera T (eds) Recent advances in Cephalopod fisheries biology. Tokai University Press, pp 331–333

Natsukari Y (1970) Egg-laying behavior, embryonic development and hatched larva of the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann. Bull Fac Fish Nagasaki Univ 30:15–29

Navarro JC, Villanueva R (2000) Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture 183:161–177

O'Dor RK, Balch N (1982a) Laboratory observations of midwater spawning by *Illex illecebrosus*. Northwest Atl Fish Organ Sci Counc Res Doc 82/VI/5:8 pp

O'Dor RK, Balch N, Foy EA, Hirtle RWM, Johnston DA, Amaratunga T (1982b) Embryonic development of the squid, *Illex illecebrosus*, and effect of temperature on development rates. J Northw Atl Fish Sci 3:41–45

O'Dor RK, Durward RD, Balch N (1977) Maintenance and maturation of squid (*Illex illecebrosus*) in a 15 metre circular pool. Biol Bull 153:322–335

O'Dor RK, Durward RD, Hirtle RWM, Amaratunga T (1979) Tagging trials on captive squid (*Illex illecebrosus*). ICNAF Res Doc 79/II/12:10 pp

O'Dor RK, Durward RD, Vessey E, Amaratunga T (1980) Feeding and growth in captive squid, *Illex illecebrosus*, and the influence of food availability on growth in the natural population. Int Comm Northwest Atl Fish, Sel Pap 6:15–21

O'Dor RK, Forsythe J, Webber DM, Wells J, Wells MJ (1993) Activity levels of *Nautilus* in the wild. Nature 362: 626–628

O'Dor RK, Foy EA, Helm PL, Balch N (1986) The locomotion and energetics of hatchling squid, *Illex illecebrosus*. Am Malacol Bull 4:55–60

O'Dor RK, Helm P, Balch N (1985) Can rhynchoteuthions suspension feed? (Mollusca: Cephalopoda). Vie Milieu 35:267–271

O'Dor RK, Portner HO, Shadwick RE (1990b) Squid as elite athletes: locomotory, respiratory and circulatory integration. In: Gilbert DL, Adelman WJ, Arnold JM (eds) Squid as experimental animals. Plenum Press, pp 481–503

O'Dor RK, Wells J, Wells MJ (1990a) Speed, jet pressure and oxygen consumption relationships in free-swimming *Nautilus*. J Exp Biol 154:383–396

Oestmann DJ, Scimeca JM, Forsythe JW, Hanlon RT, Lee PG (1997) Special considerations for keeping cephalopods in laboratory facilities. Contemp Topics Lab Anim Sci 36:89–93

Oka M (1993) Methods to collect eggs of giant cuttlefish in an aquarium. In: Okutani T, O'Dor RK, Kubodera T (eds) Recent advances in cephalopod fisheries biology. Tokai University Press, pp 397–402

Okubo S, Tsujii T, Watabe N, Williams DF (1995) Hatching of *Nautilus belauensis* Saunders, 1981, in captivity: culture, growth and stable isotope compositions of shells, and histology and immunohistochemistry of the mantle epithelium of the juveniles. Veliger 38:192–202

Okumura S, Kurihara A, Iwamoto A, Takeuchi T (2005) Improved survival and growth in *Octopus vulgaris* paralarvae by feeding large type *Artemia* and Pacific sandeel, *Ammodytes personatus*—improved survival and growth of common octopus paralarvae. Aquaculture 244:147–157

Omar S, Danakusumah E, Rani C, Siswanto E, Hade A (2001) Incubation period and hatching rate of bigfin squid, *Sepioteuthis lessoniana*, in 24 to 38 (per mill) salinity. Phuket Mar Biol Cent Spec Publ 25:139–143

Oosthuizen A, Roberts MJ, Sauer WHH (2002a) Temperature effects on the embryonic development and hatching success of the squid *Loligo vulgaris reynaudii*. Bull Mar Sci 71:619–632

Oosthuizen A, Roberts MJ, Sauer WHH (2002b) Early postcleavage stages and abnormalities identified in the embryonic development of chokka squid eggs *Loligo vulgaris reynaudii*. Sth Afr J Mar Sci 24:379–382

Packard A, Wurtz M (1994) An octopus, *Ocythoe*, with a swimbladder and triple jets. Philos Trans R Soc Lond, Ser B: Biol Sci 344:261–275

Pascual E (1978) Crecimiento y alimentación de tres generaciones de *Sepia officinalis* en cultivo. Invest Pesq 42:421–441

Pascual S, González AF, Guerra A (2006) Unusual sites of *Aggregata octopiana* infection in octopus cultured in floating cages. Aquaculture 254:21–23

Paulij WP, Herman PMJ, Roozen MEF, Denucé JM (1991) The influence of photoperiodicity on hatching of *Sepia officinalis*. J Mar Biol Assoc UK 71:665–678

Paulij WP, Herman PMJ, Van Hannen EJ, Denucé JM (1990) The impact of photoperiodicity on hatching of *Loligo vulgaris* and *Loligo forbesi*. J Mar Biol Assoc UK 70: 597–610

Pecl GT, Moltschaniwskyj NA (1997) Changes in muscle structure associated with somatic growth in *Idiosepius pygmaeus*, a small tropical cephalopod. J Zool 242: 751–764

Pecl GT, Tracey SR, Semmens JM, Jackson GD (2006) Use of acoustic telemetry for spatial management of southern calamary, *Sepioteuthis australis*, a highly mobile inshore squid species. Mar Ecol Prog Ser 328:1–15

Porteiro FM, Martins HR, Hanlon RT (1990) Some observations on the behaviour of adult squids, *Loligo forbesi*, in captivity. J Mar Biol Assoc UK 70:459–472

Portner HO, Webber DM, O'Dor RK, Boutilier RG (1993) Metabolism and energetics in squid (*Illex illecebrosus*, *Loligo pealei*) during muscular fatigue and recovery. Am J Physiol Regul Integr Comp Physiol 265:R157–165

Quintela J, Andrade JP (2002) Diel feeding rhythms, daily ration and gastric evacuation rates of *Sepia officinalis* in the Ria Formosa lagoon (South Portugal). Bull Mar Sci 71:665–680

Reimschuessel R, Stoskopf MK, Bennett RO (1990) Myocarditis in the common cuttlefish (*Sepia officinalis*). J Comp Pathol 102:291–297

Roberts D (1997) Factors affecting predatory behaviour of Idiosepius pygmaeus. BSc(Hons) dissertation, James Cook University, Townsville

Robinson SMC, Hartwick EB (1986) Analysis of growth based on tag-recapture of the giant Pacific octopus *Octopus dofleini martini*. J Zool 209:559–572

Sakai M, Brunetti N, Ivanovic M, Elena B, Nakamura Y (2004) Interpretation of statolith microstructure in reared hatchling paralarvae of the squid *Illex argentinus*. Mar Freshwat Res 55:403–413

Sakai M, Brunetti NE (1997) Preliminary experiments on artificial insemination of the Argentine shortfin squid *Illex argentinus*. Fish Sci 63:664–667

Sakai M, Brunetti NE, Elena B (1997) Artificial insemination of *Illex argentinus*. VII Colacmar Congresso Latino-Americano sobre Ciências do Mar, 380–382

Sakai M, Brunetti NE, Elena B, Sakurai Y (1998) Embryonic development and hatchlings of *Illex argentinus* derived from artificial fertilization. Sth Afr J Mar Sci 20:255–265

Sakai M, Brunetti NE, Ivanovic M, Elena B (1999) Artificial fertilization (*Illex argentinus*). *In* Seninario Final del Proyecto: Avances en Metodos y Tecnologia Aplicados a la Investigacion Pesquera. (ed.), INIDEP-JICA: Mar del Planta, pp 31–33

Sakurai Y, Ikeda Y, Shimizu M, Shimazaki K (1993) Feeding and growth of captive adult Japanese common squid (*Todarodes pacificus*) measuring initial body size by cold anesthesia. In: Okutani T, O'Dor RK, Kubodera T (eds) Recent advances in Cephalopod fisheries biology. Tokai University Press, pp 467–476

Sakurai Y, Young RE, Hirota J, Mangold K, Vecchione M, Clarke MR, Bower J (1995) Artificial fertilization and development through hatching in the oceanic squids *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis* (Cephalopoda: Ommastrephidae). Veliger 38:185–191

Salman A (1998) Reproductive biology of *Sepietta oweniana* (Pfeffer, 1908) (Sepiolidae: Cephalopoda) in the Aegean Sea. Sci Mar 62:379–383

Sánchez P, Obarti R (1993) The biology and fishery of *Octopus vulgaris* caught with clay pots on the Spanish Mediterranean coast. In: Okutani T, O'Dor RK, Kubodera T (eds) Recent advances in Cephalopod fisheries biology. Tokai University Press, pp 477–487

Sangster CR, Smolowitz RM (2003) Description of *Vibrio alginolyticus* infection in cultured *Sepia officinalis*, *Sepia apama*, and *Sepia pharaonis*. Biol Bull 205:233–234

Sasaki M (1923) On an adhering habit of a pygmy cuttlefish, *Idiosepius pygmaeus* Steenstrup. Annot Zool Jpn 10: 209–213

Sato T (1985) Reports on the squid taggings in New Zealand waters, Part 1 and Part 2. National Research Institute of Far Seas Fisheries Fishery Agency, Shimizu, p 72

Sauer WHH, Lipinski MR, Augustyn CJ (2000) Tag recapture studies of the chokka squid *Loligo vulgaris reynaudii* d'Orbigny, 1845 on inshore spawning grounds on the south-east coast of South Africa. Fish Res 45:283–289

Sauer WHH, Roberts MJ, Lipinski MR, Smale MJ, Hanlon RT, Webber DM, O'Dor RK (1997) Choreography of the squid's 'nuptial dance'. Biol Bull 192:203–207

Saunders WB (1983) Natural rates of growth and longevity of *Nautilus belauensis*. Paleobiology 9:280–288

Saunders WB (1984) The role and status of *Nautilus* in its natural habitat: evidence from deep-water remote camera photosequences. Paleobiology 10:469–486

Saunders WB, Landman NH (1987) *Nautilus*: The biology and paleobiology of a living fossil. Plenum Press, 632 pp

Saunders WB, Spinosa C (1978) Sexual dimorphism in *Nautilus* from Palau. Paleobiology 4:349–358

Schröder W (1966) Beobachtungen bei der zuchtvon Tintefischen (*Sepia officinalis*). Sber Ges Naturf Freunde 6: 101–107

Scimeca JM, Forsythe JW (1999) The use of anesthetic agents in cephalopods. Proc Int Assoc Aquat Anim Med 30:94

Segawa S (1990) Food consumption, food conversion and growth rates of the oval squid *Sepioteuthis lessoniana* by laboratory experiments. Nippon Suisan Gakkasihi 56:217–222

Segawa S (1993) Field and laboratory feeding studies of the-Japanese oval squid, *Sepioteuthis lessoniana*. In: Okutani T, O'Dor RK, Kubodera T (eds) Recent advances in Cephalopod fisheries biology. Tokai University Press, pp 499–511

Segawa S (1995) Effect of temperature on oxygen consumption of juvenile oval squid *Sepioteuthis lessoniana*. Fish Sci 61:743–746

Segawa S, Nomoto A (2002) Laboratory growth, feeding, oxygen consumption and ammonia excretion of *Octopus ocellatus*. Bull Mar Sci 71:801–813

Seibel BA, Thuesen EV, Childress JJ, Gorodezky LA (1997) Decline in pelagic cephalopod metabolism with habitat depth reflects differences in locomotory efficiency. Biol Bull 192:262–278

Semmens JM (1993) Structure and function of the cephalopod digestive gland and experimental study on *Idiosepius pygmaeus* (Family Idiosepiidae). B.Sc. dissertation, James Cook University, Townsville p 80

Sen H (2004a) A preliminary study on the effects of different light intensities on hatching of European squid (*Loligo vulgaris* Lamarck, 1798) eggs. Turk J Fish Aquat Sci 4: 1–4

Sen H (2004b) A preliminary study on the effects of salinity on egg development of European squid (*Loligo vulgaris* Lamarck, 1798). Isr J Aquacult 56:95–101

Sen H (2004c) Effects of photoperiodicity on development and incubation of squid (*Loligo vulgaris* Lamarck, 1798) eggs. Su Urun Derg 21:211–214

Sen H (2005) Incubation of European Squid (*Loligo vulgaris* Lamarck, 1798) eggs at different salinities. Aquacult Res 36:876–881

Seol DW, Lee J, Im SY, Park IS (2007) Clove oil as an anaesthetic for common octopus (*Octopus minor*, Sasaki). Aquacult Res 38:45–49

Shears J (1988) The use of a sand-coat in relation to feeding and diel activity in the sepiolid squid *Euprymna scolopes*. Malacologia 29:121–133

Sherrill J, Spelman LH, Reidel CL, Montali RJ (2000) Common cuttlefish (*Sepia officinalis*) mortality at the National Zoological Park: implications for clinical management. J Zoo Wildlife Med 31:523–531

Sinn DL, Apiolaza LA, Moltschaniwskyj NA (2006) Heritability and fitness-related consequences of squid personality traits. J Evol Biol 19:1437–1447

Smale MJ, Buchan PR (1981) Biology of *Octopus vulgaris* off the East coast of South Africa. Mar Biol 65:1–12

Snyder S (1986) Successful rearing of *Octopus dofleini* from hatchling to settlement. American Association of Zoological Parks and Aquariums 1986 Annual Conference Proceedings, pp 781–783

Soichi M (1976) On the growth and food quantity of a young common squid, *Todarodes pacificus*, in captivity. Dosuishi 13:79–82

Spinosa C (1987) A small, closed aquarium system for *Nautilus*. In: Saunders WB, Landman NH (eds) *Nautilus*: The biology and paleobiology of a living fossil. Plenum Press, pp 585–594

Steer MA, Moltschaniwskyj NA, Jordan AR (2003) Embryonic development of southern calamary (*Sepioteuthis australis*) within the constraints of an aggregated egg mass. Mar Freshwat Res 54:217–226

Summers WC (1985) Ecological implications of life stage timing determined from the cultivation of *Rossia pacifica* (Mollusca: Cephalopoda). Vie Milieu 35:249–254

Summers WC, Colvin L (1989) On the cultivation of *Rossia pacifica* (Berry, 1911). J Cephalopod Biol 1:21–32

Sykes AV, Domingues PM, Correia M, Andrade JP (2006) Cuttlefish culture - state of the art and future trends. Vie Milieu 56:129–137

Sykes AV, Domingues PM, Loyd M, Sommerfield A, Andrade JP (2003) The influence of culture density and enriched environments on the first stage culture of young cuttlefish, *Sepia officinalis* (Linnaeus, 1758). Aquacult Int 11: 531–544

Tanaka H (2000) Application of biolelemetry for tracking and resource estimation of *Ommastrephes bartramii*. National Research Institute of Far Seas Fisheries Fishery Agency, Shimizu, pp 81–98

Tanaka H (2001) Tracking the neon flying squid by the biotelemetry system in the central North Pacific Ocean. Aquabiology 137:533–539

Thompson JT, Kier WM (2001) Ontogeny of mantle musculature and implications for jet locomotion in oval squid *Sepioteuthis lessoniana*. J Exp Biol 209:433–443

Tracey SR, Steer MA, Pecl GT (2003) Life history traits of the temperate mini-maximalist *Idiosepius notoides*, (Cephalopoda: Sepioidea). J Mar Biol Assoc UK 83:1297–1300

van Camp LM (1997) The effects of temperature and food availability on growth and reproductive output: using a cephalopod model. B.Sc. dissertation, James Cook University, Townsville, 81 pp

van Heukelem WF (1977) Laboratory maintenance, breeding, rearing, and biomedical research potential of Yucatan octopus (*Octopus maya*). Lab Anim Sci 27:852–859

Vidal EAG, DiMarco FP, Wormuth JH, Lee PG (2002a) Optimizing rearing conditions of hatchling loliginid squid. Mar Biol 140:117–127

Vidal EAG, DiMarco FP, Wormuth JH, Lee PG (2002b) Influence of temperature and food availability on survival, growth and yolk utilization in hatchling squid. Bull Mar Sci 71:915–931

Villanueva R (1994) Decapod crab zoeae as food for rearing cephalopod paralarvae. Aquaculture 128:143–152

Villanueva R (1995) Experimental rearing and growth of planktonic *Octopus vulgaris* from hatching to settlement. Can J Fish Aquat Sci 52:2639–2650

Villanueva R (2000a) Differential increment-deposition rate in embryonic statoliths of the loliginid squid *Loligo vulgaris*. Mar Biol 137:161–168

Villanueva R (2000b) Effect of temperature on statolith growth of the European squid *Loligo vulgaris* during early life. Mar Biol 136:449–460

Villanueva R, Riba J, Ruiz-Capillas C, Gonzalez AV, Baeta M (2004) Amino acid composition of early stages of cephalopods and effect of amino acid dietary treatments on *Octopus vulgaris* paralarvae. Aquaculture 242:455–478

Voight JR (1988) A technique for trapping sandflat octopuses. Am Malacol Bull 6:45–48

Walsh LS, Turk PE, Forsythe JW, Lee PG (2002) Mariculture of the lolignid squid *Sepioteuthis lessoniana* through seven successive generations. Aquaculture 212:245–262

Ward P, Carlson B, Weekly M, Brumbaugh B (1984) Remote telemetry of daily vertical and horizontal movement of *Nautilus* in Palau. Nature 309:248–250

Ward P, Wicksten MK (1980) Food sources and feeding behaviour of *Nautilus macromphalus*. Veliger 23:119–124

Ward PD (1983) *Nautilus macromphalus*. In: Boyle PR (ed) Cephalopod life cycles, Vol I species accounts. Academic Press, pp 11–28

Ward PD (1987) The natural history of *Nautilus*. Allen and Unwin. 267 pp

Warnke K (1994) Some aspects of social interaction during feeding in *Sepia officinalis* (Mollusca: Cephalopoda) hatched and reared in the laboratory. Vie Milieu 44: 125–131

Watanuki N, Iwashita T (1993) Tags for cuttlefish *Sepia esculenta*. In: Okutani T, O'Dor RK, Kubodera T (eds) Recent advances in Cephalopod fisheries biology. Tokai University Press, pp 619–625

Webber DM, O'Dor RK (1985) Respiration and swimming performance of short-finned squid (*Illex illecebrosus*). NAFO Sci Coun Studies 9:133–138

Webber DM, O'Dor RK (1986) Monitoring the metabolic rate and activity of free-swimming squid with telemetered jet pressure. J Exp Biol 126:205–224

Wells MJ (1962) Brain and behaviour in cephalopods. Heinemann. 171 pp

Wells MJ (1978) Octopus: physiology and behaviour of an advanced invertebrate. Chapman and Hall. 417 pp

Wells MJ, Wells J (1985) Ventilation and oxygen uptake by *Nautilus*. J Exp Biol 118:297–312

Westermann B, Beck-Schildwachter I, Beuerlein K, Kaleta EF, Schipp R (2004) Shell growth and chamber formation of aquarium-reared *Nautilus pompilius* (Mollusca: Cephalopoda) by X-ray analysis. J Exp Zool, A: Comp Exp Biol 301A:930–937

Wood JB, Kenchington E, O'Dor RK (1998) Reproduction and embryonic development time of *Bathypolypus arcticus*, a deep-sea octopod (Cephalopoda: Octopoda). Malacologia 39:11–19

Yamada H, Kattho K (1987) Report on the arrow squid tagging survey in the New Zealand waters in December 1985–January 1986. Far Seas Fisheries Laboratory S Series 15:47

Yamamoto M (1988) Normal embryonic stages of the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann. Zool Sci 5:989–998

Yatsu A, Tafur R, Maravi C (1999a) Embryos and rhynchoteuthion paralarvae of the jumbo flying squid *Dosidicus gigas* (Cephalopoda) obtained through artificial fertilization from Peruvian waters. Fish Sci 65:904–908

Yatsu A, Yamanaka K, Yamashiro C (1999b) Tracking experiments of the jumbo flying squid, *Dosidicus gigas*, with an ultrasonic telemetry system in the Eastern Pacific Ocean. Bull Nat Res Inst Far Seas Fish 36:55–60

Yau C, Boyle PR (1996) Ecology of *Sepiola atlantica* (Mollusca: Cephalopoda) in the shallow sublittoral zone. J Mar Biol Assoc UK 76:733–748

Young JZ (1960) Observations on *Argonauta* and especially its method of feeding. Proc Zool Soc Lond 133:471–479

Young JZ (1971) The anatomy of the nervous system of *Octopus vulgaris*. Clarendon Press, 690 pp

Zann LP (1984) The rhythmic activity of *Nautilus pompilius*, with notes on its ecology and behavior in Fiji. Veliger 27:19–28

Blackwell Publishing

Cladistics 23 (2007) 497–506

Cladistics

10.1111/j.1096-0031.2007.00155.x

Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis*, *Loliolus* and *Euprymna* (Mollusca: Cephalopoda)

R. C. Guerrero-Ferreira and M. K. Nishiguchi*

Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA

Accepted 22 February 2007

Abstract

Luminescent bacteria in the family Vibrionaceae (Bacteria: γ-Proteobacteria) are commonly found in complex, bilobed light organs of sepiolid and loliginid squids. Although morphology of these organs in both families of squid is similar, the species of bacteria that inhabit each host has yet to be verified. We utilized sequences of 16S ribosomal RNA, luciferase α-subunit (*luxA*) and the glyceraldehyde-3-phosphate dehydrogenase (*gapA*) genes to determine phylogenetic relationships between 63 strains of *Vibrio* bacteria, which included representatives from different environments as well as unidentified luminescent isolates from loliginid and sepiolid squid from Thailand. A combined phylogenetic analysis was used including biochemical data such as carbon use, growth and luminescence. Results demonstrated that certain symbiotic Thai isolates found in the same geographic area were included in a clade containing bacterial species phenotypically suitable to colonize light organs. Moreover, multiple strains isolated from a single squid host were identified as more than one bacteria species in our phylogeny. This research presents evidence of species of luminescent bacteria that have not been previously described as symbiotic strains colonizing light organs of Indo-West Pacific loliginid and sepiolid squids, and supports the hypothesis of a non-species-specific association between certain sepiolid and loliginid squids and marine luminescent bacteria.

Members of the families Loliginidae and Sepiolidae (Mollusca: Cephalopoda) have been previously shown to contain luminous bacteria that reside in specialized light organ complexes. These light organs vary in structure and complexity, from simple, spherically shaped structures [*Rondeletiola* and *Sepiolina*; (Naef, 1912a; Nesis, 1982)], to complex, bilobed organs (Naef, 1912b; McFall-Ngai and Montgomery, 1990; Foster et al., 2002; Nishiguchi et al., 2004).

Squid use the light produced from the bacteria for a behavior known as counterillumination (Young and Roper, 1977; Young et al., 1980; Jones and Nishiguchi, 2004). Luminescence emitted from the light organ reduces the squid's silhouette to match the intensity and wavelength of down-welling light (Young and Roper, 1977). This provides squid with a mechanism that allows them to evade predators by camouflage. All bacteria housed in the light organs are able to produce light via the *lux* operon both inside the light organs and in their free-living state, although intensity of light and differences between strains of bacteria have never been thoroughly investigated. Likewise, regulation of the *lux* operon inside the light organ of squid has only been extensively studied in the *Euprymna scolopes–Vibrio fischeri* symbiosis, where reduction in the amount of light produced affects symbiotic competence (Visick et al., 2000).

Previous investigations have demonstrated that two genera, *Vibrio* and *Photobacterium*, are prevalent in both sepiolid and loliginid squid (Fidopiastis et al., 1998; Nishiguchi et al., 2004). Sepiolid squid primarily contain two species of *Vibrio*, *V. fischeri* and *V. logei* (Fidopiastis et al., 1998; Nishiguchi, 2002), although the genera *Rondeletiola* and *Sepiolina* have been shown to contain *Photobacterium leiognathi* in their light organs (Nishiguchi and Nair, 2003; Nishiguchi et al., 2004). Photobacteria are more commonly found in the genera

*Corresponding author:
E-mail address: nish@nmsu.edu

Uroteuthis and *Loliolus* (Nishiguchi, 2002), with no cases of *Vibrio* species detected in bacteriogenic light organ of loliginids. Because there is geographic overlap between several species of *Uroteuthis*, *Loliolus* and *Euprymna* in the Indo-West Pacific, we were interested in isolating and identifying bacteria from these squid, determining whether these strains were phylogenetically related to one another, and whether there was any biogeographical or host-specific patterns evident between the symbionts. We used the complete 16S rRNA sequence from each isolate in a combined analysis with the luciferase α-subunit gene (*luxA*) and the glyceraldehyde phosphate dehydrogenase gene (*gapA*) sequences to determine phylogenetic relationships among closely related species. In addition, we measured both growth and luminescence to determine whether substantial differences occurred between these isolates. Previously isolated squid symbionts from host species collected in close or distant geographic locations were also compared in order to evaluate whether luminous bacteria that have been environmentally transmitted are promiscuous across hosts, or alternatively, whether symbionts exhibit squid family specificity. These comparisons would then allow us to determine if geographic location of the species investigated was a determining factor in predicting the associations between light organ symbionts and loliginid squid.

Materials and methods

Collection of squid and isolation of bacteria from light organs

Squid were collected from either fishermen in the local Thai markets, or by trawl net and brought into the lab for identification. Squid were collected from two geographically separated locations: Rayong, Thailand, which is located on the Gulf of Thailand, and the island of Phuket, in southern Thailand, which is located in the Andaman Sea (Table 1, in bold). Animals collected in Rayong were brought back to the Rayong Coastal Aquaculture and Fisheries Research Center for confirmation of identity. Specimens collected in Phuket were identified at the Phuket Marine Biological Station. Host specimens were used for isolation of symbiotic bacteria according to previous protocols (Nishiguchi, 2002; Nishiguchi and Nair, 2003). Mantle cavities were opened and the entire light organ complex was removed and placed in sterile seawater (700 μL). Adult light organs were homogenized, serially diluted to 10^{-5} and plated on seawater tryptone (SWT) agar media (Ruby and Asato, 1993). Cultures were incubated overnight at room temperature, and kept at 5 °C upon return to New Mexico State University. Approximately 20–30 light-producing colonies from each plate were subsequently grown overnight in SWT medium at 20–28 °C, and cultures were either frozen in a glycerol stock for further biological assays or bacterial DNA was extracted for isolate identification and phylogenetic analyses.

Determination of bacterial growth and light production

Samples of symbiotic strains were recovered from each stock by streaking onto SWT agar plates and growing for 16 h at 28 °C. One CFU from this culture was used to inoculate 5 mL of seawater tryptone (SWT) broth and grown for 16 h on a shaking incubator (250 r.p.m.) at the same temperature. Ten microliters of this culture was then re-inoculated in 5 mL of SWT broth and incubated for 3 h on a shaking incubator (250 r.p.m.) at 28 °C. The starting aliquot for inoculation of the growth flask containing 50 mL of SWT was taken from this culture. Optical density was measured at 600 nm to guarantee that all strains tested would have the same density at time T_0 by adjusting the volume of each culture. Flasks were maintained in a shaking incubator (250 r.p.m.) at 28 °C for the entire 8- or 16-h period.

Measurements for growth and light production were obtained simultaneously every 30 min for both 8- and 16-h incubation assays. For growth curves, OD_{600} was determined using 1 mL of the initial culture transferred to a 1000 μL cuvette. Optical density was measured using an Uvikon XL spectrophotometer (Bio-tek Instruments, San Diego, CA, USA) and transferred to a PC using the Laboratory Power Junior 2.06 software. Sterile SWT broth was used as the blank.

For measurements of light emitted by cell suspensions (bioluminescence), 100 μL of the liquid culture was transferred to a 20 mL disposable scintillation vial and placed in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Light production was measured in light units per milliliter and also indicated as luminescence per cell (luminescence per OD600). The volume of culture was reduced whenever light production exceeded the maximum reading allowed by the luminometer (9999 light units). Light production values were adjusted using dilution factors corresponding to each volume reduction.

Carbon source utilization for phylogenetic analysis

Strains from frozen stock were plated on SWT agar plates and grown overnight at 28 °C. Single colony forming units (CFU) of each strain were recovered after incubation and grown in 10 mL of SWT medium to an OD_{600} of 0.5 (Nishiguchi and Nair, 2003). Bacterial cells were harvested by centrifugation at 5000 r.p.m. (2655 ***g***) for 10 min after being washed three times using 5 mL of PBS buffer (pH 7.3). The pellet was finally resuspended in 10 mL of PBS. A 100 μL aliquot of the cell

Table 1
Bacterial strain, location, host, 16S ribosomal DNA, gapA and *luxA* accession number. Strains in bold were isolated for this study

Bacterial strains	Source	Location	Accession numbers		
			16S rRNA	*gapA*	*luxA*
Vibrio fischeri CG101	*Cleidopus gloriamaris*	Australia	AY292939	AY292958	DQ026810
Vibrio fischeri ES 114	*Euprymna scolopes*	Hawaii	AY292919	AF034845	DQ026811
Vibrio fischeri ES915	*Euprymna scolopes*	Hawaii	AY292920	AY292953	DQ026812
Vibrio fischeri ET101	*Euprymna tasmanica*	Australia	AY292923	AF034847	DQ026813
Vibrio fischeri MJ101	*Monocentrus japonicus*	Japan	AY292946		DQ026814
Vibrio fischeri SL518	*Sepiola ligulata*	France	AY292950	AY292962	DQ026815
Vibrio fischeri SR5	*Sepiola robusta*	France	AY292926	AF034851	DQ026816
Vibrio fischeri WH1	Free-living	USA	AY292930	AY292955	DQ026817
Photobacterium leiognathi LN101	*Uroteuthis noctiluca*	Australia	AY292944	AF034849	DQ026808
Photobacterium leiognathi RM1	*Rondeletiola minor*	France	AY292947		DQ026809
EHR1	*Euprymna hyllebergi*	Thailand	AY332401		
LAR1	*Loliolus affinis*	Thailand	AY332398		
EHP8	*Euprymna hyllebergi*	Thailand	AY332400		
UCP6	*Uroteuthis chinensis*	Thailand	AY332404		
UDP1	*Uroteuthis duvauceli*	Thailand	AY332407		
Photobacterium phosphoreum ATCC 11040	Chlorophthalmid fishes		AY341437		AY341063
Photobacterium leiognathi ATCC 25521	*Leiognathus splendens*	Thailand	D25309		M63594
Vibrio fischeri ET401	*Euprymna tasmanica*	Australia	AY292943	AY292960	
Vibrio fischeri SA1G	*Sepiola affinis*	France	AY292924	AF034848	
Vibrio fischeri SI1D	*Sepiola intermedia*	France	AY292948	AY292961	
Vibrio logei ATCC15382	*Gadus macrocephalus*	USA	AY292932	AY292956	
Vibrio logei SR181	*Sepiola robusta*	France	AY292934	AY292957	
Vibrio anguillarum ATCC 19264	*Gadus morhua*	Denmark	X16895		
Vibrio alginolyticus ATCC 17749	*Trachurus trachurus*	Japan	X56576		
Vibrio campbellii ATCC 25920	Seawater		X56575		
Vibrio harveyi EH701	*Euprymna hyllebergi*	Thailand	AY292941		
Vibrio cholerae N16961	Cholera patient	Bangladesh	AE004096	AE004274	
Vibrio fischeri EB12	*Euprymna berryi*	Japan	AY292921	AY292954	
Vibrio fischeri EM17	*Euprymna morsei*	Japan	AY292922	AF034846	
Vibrio fischeri ET301	*Euprymna tasmanica*	Australia	AY292942	AY292959	
Vibrio vulnificus YJ016	Clinical isolate	Taiwan	BA000037	BA000037	
Vibrio parahaemolyticus RIMD 2210633			BA000031	BA000031	
Vibrio salmonicida NCMB 2262	Fanned *Salmo salar*	Norway	X70643		AF452135
Vibrio pelagius ATCC 25916T	Succinate enriched seawater		X74722		
Salinivibrio costicola ATCC 33508T	Bacon curing brine		X74699		
Photobacterium damselae ATCC 33539T	*Chromis punctipinnis*	USA	X74700		
Escherichia coli W3110		USA	AC_000091	AP009048	
Photobacterium damselae damselae ATCC 33539	*Chromis punctipinnis*	USA	AB032015		
Vibrio splendidus ATCC 33125	Marine fish		X74724		
Vibrio lentus 40M4T CECT 5110	Oysters	Spain	AJ278881		
Vibrio orientalis ATCC 33934	Seawater	China	X74719		
Vibrio tasmaniensis LMG 20012	*Salmo salar*	Australia	AJ316192		
Vibrio rumoiensis S 1FERM P-14531	Fish product processing plant	Japan			AB013297
Vibrio fischeri ES 191	*Euprymna scolopes*	Hawaii	DQ026825		DQ026819
Vibrio fischeri PP3	Free-living	Hawaii	DQ026824		DQ026818
Vibrio fischeri PP42	Free-living	Hawaii	DQ026826		DQ026820
Vibrio fischeri VLS2	Free-living	Hawaii	DQ026827		DQ026821
EHP1	*Euprymna hyllebergi*	Thailand	DQ530284	DQ530298	
EHP2	*Euprymna hyllebergi*	Thailand	DQ530285	DQ520299	
EHP3	*Euprymna hyllebergi*	Thailand	DQ530286	DQ520300	
EHP4	*Euprymna hyllebergi*	Thailand	DQ530287	DQ520301	
EHP5	*Euprymna hyllebergi*	Thailand	DQ530288	DQ520302	
EHR2	*Euprymna hyllebergi*	Thailand	DQ530289	DQ520303	
EHR3	*Euprymna hyllebergi*	Thailand	DQ530290	DQ520304	
EHR4	*Euprymna hyllebergi*	Thailand	AY332402	DQ520305	
LAR2	*Loliolus affinis*	Thailand	DQ530291	DQ520306	
LAR3	*Loliolus affinis*	Thailand	DQ530292	DQ520307	
LARS	*Loliolus affinis*	Thailand	DQ530293	DQ520308	
UCP4	*Uroteuthis chinensis*	Thailand	DQ530294	DQ520309	

Table 1
Continued

Bacterial strains	Source	Location	Accession numbers		
			16S rRNA	*gapA*	*luxA*
UCR4	*Uroteuthis chinensis*	Thailand	DQ530295	DQ520310	
UCR6	*Uroteuthis chinensis*	Thailand	DQ530296	DQ520311	
UCR7	*Uroteuthis chinensis*	Thailand	DQ530297	DQ520312	
Vibrio harveyi ATCC 14126	Amphipod *Talorchestia* sp.	USA	X74706		AF147084

ATCC: American type culture collection (USA)
LMG: Belgian Coordinated Collections of Microorganisms (BCCM). Division of Bacteria: BCCM/LMG (Belgium) CECT: Coleccion espanola de cultivos tipo (Spanish type culture collection) (Spain)
FERM: Patent and Bio-Resource Center (Japan)
NCMB: National Collections of Industrial and Marine Bacteria Ltd (United Kingdom)
RIMD: Research Institute for Microbial Diseases (Japan)

suspension was added to each of the 96 wells of a GN2 Micro Plate™ (Biolog, Hayward, CA, USA) plate and incubated overnight at 28 °C. Carbon use was determined using an ELx800 absorbance microplate reader (Bio-Tek instruments, Winooski, VT, USA). Each strain was analyzed in duplicate plates. Biolog data were converted into binary information giving a value of 1 for positive results (i.e., use of carbon source) and 0 to the negative ones (i.e., carbon source not utilized). These phenotypic values were then converted into Hennig 86/Nona input data to be used in a combined analysis with the nucleotide data.

DNA extraction, amplification and sequencing of bacterial genes

To extract DNA from isolates, bacteria were grown overnight at 28 °C at 250 r.p.m. in 5 mL of SWT. 2 mL of each culture was centrifuged for one minute, and media removed. Bacterial DNA was isolated using the DNAeasy kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions. One to 10 ng of template DNA was used for polymerase chain reaction (PCR) amplifications. For the 16S rRNA locus, four sets of primers (each amplifying approximately 420 bp) were used to determine the entire 16S rRNA sequence (~1600 bp). Primer sequences were obtained from Nishiguchi and Nair (2003) and are shown in Table 2. A single PCR reaction (50 μL) contained 2.5 mM of $MgCl_2$, 0.5 mM dNTPs (25 μM each; Promega, Madison, WI, USA), 0.2 μM of forward and reverse primer, 10 × reaction buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100) and 0.05 U/μL of Taq DNA Polymerase (Promega; Madison, WI), Amplitaq Gold® or AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA, USA).

The *luxA* gene was used as a second molecular marker for additional phylogenetic analyses. A group of primers was used for the amplification of this locus that codes for the α-subunit of luciferase. PCR protocol and conditions for each of these sets of primers are shown in Table 2. PCR products were cleaned with Gene Clean II DNA purification kit (Q-biogene, Irvine, CA, USA).

gapA is a gene that codes for the glyceraldehyde phosphate dehydrogenase enzyme, and was used as the third phylogenetic marker in the analysis. For its amplification, primers *gapA* F and *gapA* R were used. Primer sequences and conditions for amplification are shown in Table 2.

PCR products were sequenced using Applied Biosystems Big Dye© (v.3.1). Excess fluorescently labeled dNTPs were removed by spin column or plate (Edge Biosystems, Gaithersburg, MD, USA) according to manufacturer instructions. Samples were sequenced in an Applied Biosystems 3100 automated capillary sequencer. Independent forward and reverse sequences obtained were combined and edited using Sequencher v. 4.6. (Gene Codes™, Ann Arbor, MI, USA). Edited sequences were imported to BBEdit v.7.0.3 and converted into UNIX format to be revised, prealigned, and compared through the Genetic Data Environment v.2.2. (Smith et al., 1994).

Phylogenetic analysis

Sequences were analyzed using the direct optimization method described by Wheeler (Wheeler, 1996, 1998) and implemented in the computer program POY (Wheeler, 1995; Wheeler et al., 2002). The method assesses the number of transformations in DNA sequences required by a phylogenetic topology without using pair-wise optimization through multiple sequence alignment. POY treats insertions and deletions as processes, which place it apart from multiple sequence alignment (Wheeler, 1998).

Several analyses were implemented with character transformations weighted differently to determine how different phylogenetic hypotheses were affected (sensitivity analysis *sensu* Wheeler, 1995). Final analysis was performed with characters equally weighted, using a

Table 2
Oligonucleotide primers and PCR conditions used in this study

Primer name and amplified region	Primer sequence	Cycles	Hot start	Cycle			Final extension
				Denaturation	Annealing	Extension	
16S rRNA**	IF 5′-AGA GTT TGA TCM TGG CTC AG-3′		94 °C	94 °C	49 °C	72 °C	72 °C
	4R 5′-AGG CCT TCT TCA TAC ACG CG-3′	25	2 min	15 s	15 s	15 s	7 min
16S rRNA**	2F 5′-GCA AGC CTG ATG CAG CCA TG-3′		94 °C	94 °C	49 °C	72 °C	72 °C
	3R 5′-ATC GTT TAC GGC GTG GAC TA-3′	25	2 min	15 s	15 s	15 s	7 min
16S rRNA**	3F 5′-AAA CAG GAT TAG ATA CCC TG-3′		94 °C	94 °C	49 °C	72 °C	72 °C
	2R 5′-CTG GTC GTA AGG GCC ATG AT-3′	25	2 min	15 s	15 s	15 s	7 min
16S rRNA**	4F 5′-AGG TGG GGA TGA CGT CAA GT-3′		94 °C	94 °C	49 °C	72 °C	72 °C
	1R 5′-AAG GAG GTG WTC CAR CC-3′	25	2 min	15 s	15 s	15 s	7 min
luxA-specific*	F 5′-GTTCTTAGTTGGATTATTGG-3’		94 °C	94 °C	44 °C	72 °C	72 °C
Positions 568–995	R 5′-TCAGTTCCCATTAGCTTCAAATCC-3’	29–33	2 min	15 s	30 s	30 s	7 min
1st *luxA*†	F 5′-CAGAATCACCAAAAAGGAATAGGT-3’	30	94 °C	94 °C	55 °C	72 °C	72 °C
Positions 1–590	R 5′-CAATTTCATTATAGAGTTCCATCTGTG-3’		2 min	15 s	30 s	30 s	7 min
2nd *luxA*†	F 5′-GTTCTTAGTTGGATTATTGGTA-3’	26–30	94 °C	94 °C	50 °C	72 °C	72 °C
Positions 450–1068	R 5′-AACTTGTTCCTTATTATCTCTAGTAT-3’		2 min	15 s	30 s	30 s	7 min
luxA 1–569†	F 5′-GCGAAGATTAAGAAATAAAA-3’	25–30	94 °C	94 °C	47 °C	72 °C	72 °C
Positions 1–569	R 5′-TTCTTTAGAGTACTTTGTTGG-3’		2 min	15 s	30 s	30 s	7 min
luxA 400–1168†	F 5′-ATTTAATTTTGGTGTTGTTA-3’	25–30	94 °C	94 °C	43 °C	72 °C	72 °C
Positions (400–1168)	R 5′-TTATTTTTGAGGTTCTTTTA-3’		2 min	15 s	30 s	30 s	7 min
luxA 401–1168[6]	F 5′-TTTAATTTTGGTGTTGTT-3’	25–30	94 °C	94 °C	43 °C	72 °C	72 °C
Positions 401–1168	R 5′-TTATTTTTGAGGTTCTTTT-3’		2 min	15 s	30 s	30 s	7 min
luxA ext†	F 5′-AATTTATTTAGGTTCTTTTAAG-3’	28	94 °C.	94 °C.	42.5 °C	72 °C	72 °C
	R 5′-AAYATTTRTTTTTCGTATCA-3’		2 min	15 sec	30 s	30 s	7 min
luxA intl†	F 5′-CTATAATCAACACGTCGATTS-3’	28	94 °C	94 °C	48.4 °C	72 °C	72 °C
	R 5′-CAATGGTWCTTAGTTGGATTAT-3’		2 min	15 s	30 s	30 s	7 min
luxA int2†	F 5′-CCATYTGTGYTTTTTTYTCATT-3’	28	94 °C.	94 °C	48.5 °C.	72 °C	72 °C
	R 5′-KTTTGATACATATTGGACSTT-3’		2 min	15 s	30 sec	30 s	7 min
luxA int3†	F 5′-TGATAATCRTAACCWCGAGT-3’	28	94 °C	94 °C	47.5 °C	72 °C	72 °C
	R 5′-AAGGTCGWTTTAATTTTGG-3’		2 min	15 s	30 s	30 s	7 min
Pleiogleiog A‡	F 5′-GTTTAAGATCAACTGTCTAAAGGRCG-3’	25–30	94 °C	94 °C	56 °C	72 °C	72 °C
	R 5′-TCAGAACCATTCGCTTCAAATCCAAC-3’		2 min	15 s	30 s	30 s	7 min
Pleiogleiog AB‡	F 5′-CGTCGCCGACTTGATTACAGTAACG-3’	25–30	94 °C	94 °C	60 °C	72 °C	72 °C
	R 5′-ATCCTTTTCTTGCTGCCCATTCAACC-3’		2 min	15 s	30 s	30 s	7 min
Pphosp A‡	F 5′-CTTTTAGATCCAATGTCAAAAGGCCG-3’	25–30	94 °C	94 °C	58 °C	72 °C	72 °C
	R 5′-TCAGAGCCATTTGCTTCGAAACCAAG-3’		2 min	15 s	30 s	30 s	7 min
Pphosp AB‡	F 5′-AAACGTCGTGTTGATTATAGCAACG-3’	25–30	94 °C	94 °C	55.4 °C	72 °C	72 °C
	R 5′-TCCAACGATATGTTAGTGGAAGC-3’		2 min	15 s	30 s	30 s	7 min
luxAl27§	F 5′-GANCANCANTTNACNGAGTTTGG-3’	30	94 °C	94 °C	58 °C	72 °C	72 °C
	R 5′-ATTTCNTCTTCAGNNCCATTNGCTTCAAANCC-3’		5 min	30 s	30 s	90 s	7 min
*luxA*f Wimpee¶	F 5′-CTACTGGATCAAATGTCAAAAGGA-3’	30	94 °C	94 °C	56 °C	72 °C	72 °C
	R 5′-TCAGAACCGTTTGCTTCAAAACC-3’		5 min	1.5 min	30s	2 min	7 min
*gapA***	F 5′-GTG TAC TTC GAG CGT TAT AC-3′	29	95 °C	95 °C	40 °C	70 °C	70 °C
	5′-GCC CAT TAC TCA CCC TTG TT-3′		5 min	15 s	15 s	1 min	7 min

* Lee and Ruby, 1995.
† Oligonucleotide primers designed in this study.
‡Ast and Dunlap, 2004.
§Modified from Budsberg *et al.*, 2003.
¶Wimpee *et al.*, 1991.
** Nishiguchi and Nair, 2003.

molecular matrix in which a value of 1 was given to each transition, transversion, insertion, deletion and gap. Combined phylogenetic analysis produced a total of 54 875 156 trees examined in TBR after the initial construction of a total of 3948 trees. In total, there were 54 879 104 trees examined in all branch swapping strategies. This process took a total of 102.8 h. A total of 241 838 974 alignments were performed by direct optimization during this time.

Tree search was implemented by TBR (tree bisection and reconnection) branch-swapping on the best of 100 random addition replicates, holding 1000 trees per round and performing one round of tree-fusing (Goloboff, 1999). At the same time, the command "exact" was used to guarantee a more accurate calculation of tree costs for direct optimization on the downpass; it also performs a complete Sankoff optimization (Sankoff and Rousseau, 1975). Binary trees were plotted in TreeView 0.4.1 and the consensus tree was calculated using PAUP*4.0.10 (Swofford, 2002). Analysis was executed in the NetBSD biology computer system at New Mexico State University.

All newly sequenced 16S rRNA, *gapA* and *luxA* loci have been deposited in GenBank with the accession numbers shown in Table 1 (strains isolated from this study are in bold type in Table 1). Previously determined gene sequences were obtained from GenBank.

Table 3
Light emission of bacterial isolates

Bacterial isolate	Maximum luminescence (light units/mL)	Bacterial isolate luminescence (luminescence/cell)	Time (min) of maximum luminescence
Vibrio fischeri ES114	0.045	0.023	600
Vibrio fischeri ET101	0.59	0.52	420
Vibrio harveyi EH701	3490	7759.6	420
Vibrio fischeri WH1	159.1	158.9	480
Vibrio fischeri CG101	4.25	3.24	540
Vibrio fischeri VLS2	136.9	91.6	450
Photobacterium leiognathi RM1	123.4	227.1	300
Vibrio fischeri PP3	2.05	1.39	330
Vibrio fischeri PP42	0.27	0.17	330
UCP1	119.8	68.25	420
UCR1	134.9	91.74	300
UCR4	195.2	107.4	240
UCR6	3011	1347.6	420
UCR7	9998	4489.3	270
EHR1	44.14	19.48	300
EHR2	16.13	7.61	270
EHR3	29.1	13.06	330

Results and discussion

Quantification of bacterial numbers of loliginid and sepiolid light organ symbionts from Thailand

Colonies isolated from light organ homogenates of either *Uroteuthis*, *Loliolus* or *Euprymna* hosts were luminescent on SWT plates. In average, 10 light organs were extracted from this location in Thailand and used for strain isolation. A total of 40 isolates from each host specimen were extracted, with one to five isolates randomly selected for analyses (Table 1). Colony forming units (CFUs) from each light organ homogenate were enumerated to calculate the total number of CFUs present in each squid light organ. Adult light organs from both *U. chinensis* and *U. duvauceli* ranged from 10^{11} to 10^{12} CFU/light organ, whereas *E. hyllebergi* light organs contained approximately 10^{9}–10^{10} CFU/light organ.

Bacterial growth and light production

Results of the luminescence assays are shown in Table 3. Growth and light production clearly demonstrated that peak luminescence (the point of time in which the amount of light units per milliliter reaches its maximum), coincides in most cases with a period at which bacteria are in stationary phase. Strains in Table 3 showed a unique peak of maximum luminescence occurring in culture. From this group of strains, symbionts from *Euprymna* (ES114 and ET101) exhibited the lowest light production, registering between 0.045 and 0.6 light units per milliliter. Strains PP3 and PP42, both free-living *V. fischeri* strains from Hawaii, emitted their peak luminescence simultaneously at 330 min, but their intensities varied slightly, with PP3 higher (~2 light units per milliliter). It is noticeable that strains with the lowest light production grouped together in the combined phylogenetic tree (Fig. 1), in a clade containing other sepiolid squid symbionts, such as *V. fischeri* ES191, ES915 and EM17.

The remaining free-living strains VLS2 and WH1 exhibited similar values of light production (Table 3). Both strains produced peak luminescence after 450 min and production of light was similar: 140 and 160 light units per milliliter for VLS2 and WH1, respectively.

Even though UCP1 and UCR1 displayed relatively similar luminescence, their peak luminescence occurred at different times during growth. UCP1 isolated from *Uroteuthis chinensis* from Phuket, emitted its peak

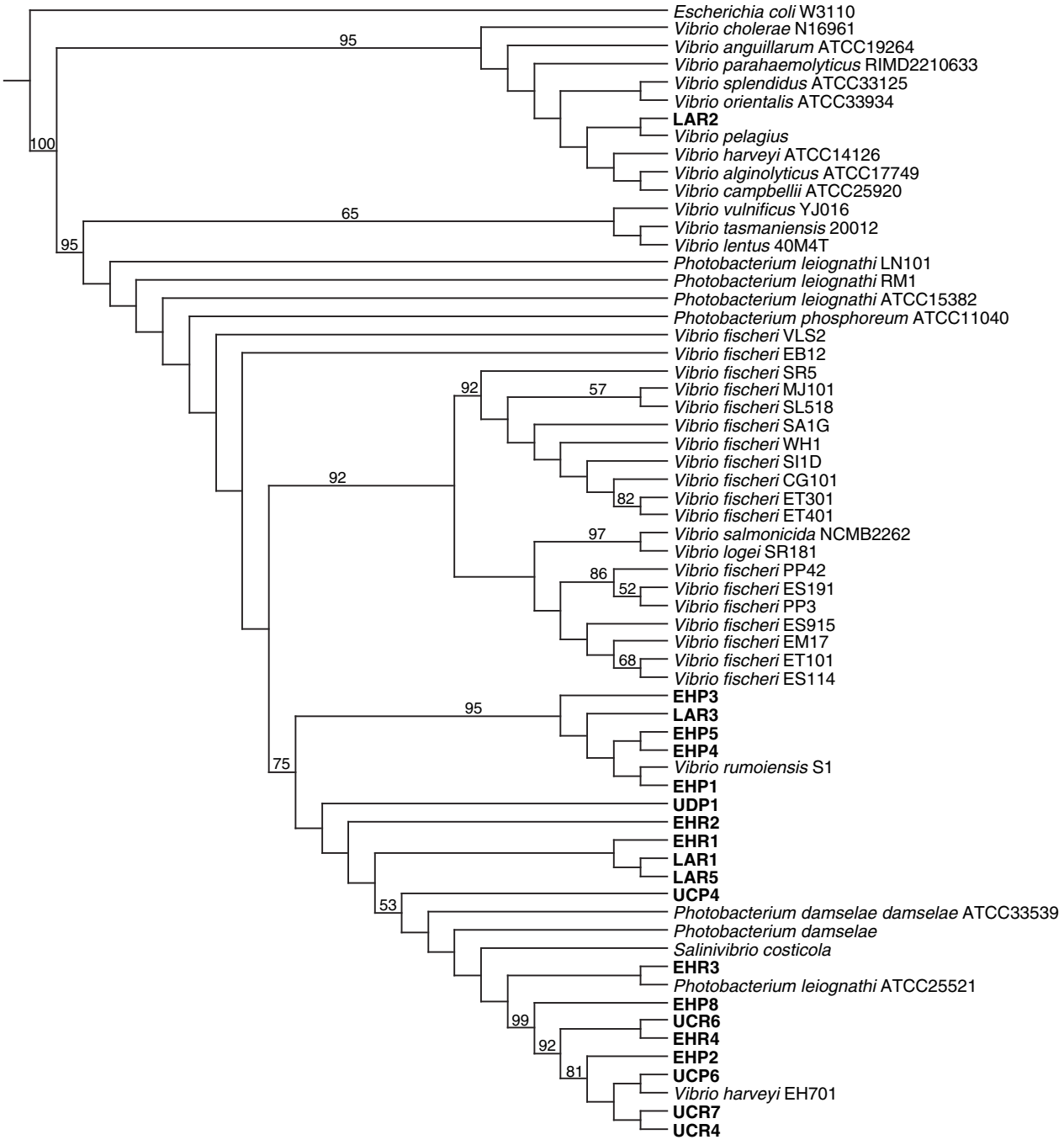


Fig. 1. Phylogenetic tree of *Vibrionaceae* species and isolates, based on DNA sequences of three loci and 95 biochemical characters. The tree was obtained with insertions, deletions, transitions and transversions equally weighed. Jackknife percentages of more than 50% are shown as numbers on nodes. Strains isolated from this study are in bold.

luminescence at 420 min, whereas UCR1, also from the same squid species but collected in a different location (Rayong), emitted higher luminescence at 300 min. UCR4, an isolate from *U. chinensis* (Rayong), displayed 195.2 light units/mL (specific luminescence of 107.4). This value is close in range to ones emitted by other isolates from the same host, but peaked earlier in the assay. Also, light production values from Thailand strains were comparatively higher than levels exhibited by symbiotic *V. fischeri* strains ES114, ET101, CG101 and free-living *V. fischeri* strains PP3 and PP42. High values of light produced by VLS2 are due to the very

luminous phenotype of this *V. fischeri* strain isolated from Hawaii (Lee and Ruby, 1995).

Thailand strains showing high levels of bioluminescence are found forming a group that also includes *Vibrio harveyi* EH701. Previous research has shown that luminescence in *V. harveyi* strain B392 reaches a higher intensity than *V. fischeri* strain ATCC 7744 (Meighen, 1999). These results indicate that Thailand strains are comparable with *V. harveyi* in that their luminescence is greater than *V. fischeri*. Measurements of light intensity from this study may indicate that Thailand symbiotic strains are more closely related to *V. harveyi* than to *V. fischeri*, which is confirmed by the phylogenetic data (see next section) in Fig. 1.

Combined phylogenetic analysis of 16S rRNA, gapA, luxA sequences and biological data

Using several loci and combining molecular information with biochemical attributes has been described as an appropriate approach to understanding relationships among members of a group of taxa (Giribet and Wheeler, 2002; Nishiguchi and Nair, 2003). By exploring our data set using a sensitivity analysis it was possible to generate hypotheses of relationships and at the same time evaluate their stability.

Direct optimization produces more parsimonious and congruent explanations of sequence variation than multiple sequence alignment (Giribet et al., 2001). Without the use of multiple sequence alignment, the method assesses the number of transformations in DNA sequence required by a phylogenetic topology (Giribet and Wheeler, 2002). Therefore, it is clear that the most parsimonious hypothesis is represented by the phylogeny reported here.

The most parsimonious tree (Fig. 1) produced from the analyses using all parameter sets that minimized overall incongruence among the 16S rRNA, *gapA*, *luxA* genes and phenotypic data, demonstrates that the clade containing symbiotic *Vibrio fischeri* is not monophyletic. A number of internal clades contain symbiotic isolates, irrespective of host or geographic location, although support for some internal nodes is below the 50% threshold. This group also includes free-living bacteria, as well as both fish and squid symbionts, indicating that symbiosis in these *V. fischeri* strains has arisen multiple times. *Vibrio salmonicida* and *V. logei* SR181 group in a single clade for both 16S rRNA analysis (data not shown) and the combined phylogenetic analysis (Fig. 1). Previous molecular and biochemical data (Fidopiastis et al., 1998; Nishiguchi and Nair, 2003) has demonstrated that *V. salmonicida* is a close relative (and possible sibling species) to *V. logei*, which provides additional evidence that these species of bacteria may share a common ancestor that was psychrophilic in nature.

Our analysis also resolved the placement of *V. fischeri* fish isolates CG101 and MJ101 within a more uniform group with strains from France (SR5 and SL518) and Australia (ET401 and ET301) as well as the free-living strain WH1. Interestingly, symbiotic strain LAR2, isolated from *Loliolus affinis* (Loliginidae), did not group with clades containing the remaining squid strains. This clade contains only *Vibrio* species, including recently described species found either associated with or causing disease to marine organisms (Banatvala et al., 1997; Thompson et al., 2001, 2003). Additionally the node was well supported, with a jackknife value of 95% (Fig. 1). Species *V. vulnificus*, *V. tasmaniensis* and *V. lentus* also grouped in one clade with 65% support. Thompson et al. (2004) reported a close relationship between *V. lentus* and *V. tasmaniensis*, which coincides with the phylogenetic hypotheses presented here that these species are sister taxa.

All Thailand strains isolated from either *Uroteuthis, Loliola* or *Euprymna* squid hosts (with the aforementioned exception of isolate LAR2) grouped in one separate clade with *Vibrio harveyi* EH701 (previously described by Nishiguchi and Nair, 2003), *Vibrio rumoiensis*, some *Photobacterium* species and *Salinivibrio costicola* (Fig. 1). This result suggests that Thailand molecular variants, found colonizing light organs in loliginid and sepiolid squid are neither *Vibrio fischeri* nor *Vibrio logei*, which are the bacterial species commonly found as light organ symbionts. This is a relationship not previously reported in squid.

Our combined analysis produced a phylogeny that was able to resolve Thailand isolates with a relatively high bootstrap value (75%). Within this clade, there is one node (95% jackknife support), containing isolates EHP3, LAR3, EHP5, EHP4 and EHP1, which includes the recently described species *Vibrio rumoiensis* (Yumoto et al., 1999). This facultative psychrophilic bacterium is known to exhibit high catalase activity, which may explain why Thailand squid symbionts exhibit a closer relationship to identified strains of *V. rumoiensis*. Previous studies with *E. scolopes* light organs have demonstrated high levels of halide peroxidase present in this organ (Small and McFall-Ngai, 1998). This enzyme produces potent microbicides in response to pathogens by generating hypohalous acids from hydrogen peroxide. In bacteria, catalase is used to detoxify any host generated hydrogen peroxide (Ruby and McFall-Ngai, 1999) therefore giving an enormous ecological advantage to those bacteria exhibiting high catalase activity in the light organ. Production of this enzyme would allow bacteria to combat any immunological response, and successfully colonize light organ tissue. Any host-generated hydrogen peroxide would therefore be neutralized before it has the chance to enter the bacterium. Ecological variants related to *V. rumoiensis* and possibly

sharing this beneficial defensive phenotype will easily colonize squid light organs.

With respect to isolates UDP1, EHR2, EHR1, LAR1, LAR5 and UCP4, there is no clear resolution in the phylogenetic tree. However, they are clearly part of the clade containing the Thailand isolates, which separate them from the *V. fischeri* group.

In addition, *Vibrio harveyi* EH701 is also included in the Thailand clade, indicating a possible relationship between luminescent symbionts and *V. harveyi*. *V. harveyi* has been found to produce much higher luminescence in culture than *V. fischeri* (Meighen, 1999), which may increase its ecological advantage over potential but low light producing symbionts. *V. harveyi* has been recognized as a cause of disease in marine invertebrates, particularly crustaceans used for aquacultural farms. Similarly, a *V. harveyi*-like organism, classified as *Vibrio carchariae* has been isolated from sharks (Austin and Zhang, 2006). Thus, *V. harveyi* is classified as a pathogen of invertebrates and vertebrates. Furthermore, Pizzutto and Hirst (1995) reported the high diversity within the species by genetic fingerprinting and protein profiling in an attempt to differentiate virulent from avirulent strains of *V. harveyi*. The authors concluded that virulence and phylogenetic history are not associated, which led them to consider that virulence was acquired by association with mobile genetic elements, such as plasmids or transposons. It is then possible that the Vibrionaceae strains found colonizing light organs in Thai squid correspond to a non-virulent form of *V. harveyi* with the conserved ecological advantage of being highly luminescent.

Within this clade other species such as *Salinivibrio costicola*, *Photobacterium damselae*, and *Photobacterium leioghathi* are also found, supporting previous tested hypotheses proposed by Kita-Tsukamoto et al. (1993), Alsina and Blanch (1994) and Ruimy et al. (1994).

Of importance to this study is the fact that isolates obtained from a common host found in the same geographic area are embedded within a large clade that contains a variety of bacterial species with characteristics that make them excellent candidates as bacterial symbionts. However, strains isolated from a particular squid host are not found to be phylogenetically related to a unique bacterial species. This result indicates a possible non-specific association between Thai squid with marine luminescent bacteria. The results presented here clearly suggest that symbioses between Vibrionaceae bacteria and their squid hosts are family specific and not strain specific. This is in contrast to earlier studies of sepiolid squid symbionts (Nishiguchi et al., 1998), where parallel cladogenesis occurred between native strains and their host squid. More recently, better taxon sampling among strains within and between populations of host squid has provided a more complex picture of *Vibrio*-sepiolid squid dynamics than previously thought (Jones et al., 2006). Introgression of shared haplotypes among strains isolated from different host species seems to be more common than previously thought, with genetic breaks observed at locations where abiotic factors (i.e., temperature) seem to be a major influence. As sepiolid and loliginid squid in Thailand are colonized by more than one species from the Vibrionaceae, this suggests that specificity for genera of this family still exists, but is influenced by additional factors besides host species. Future studies to determine whether homologous genes are co-opted for similar functions between these bacteria will help determine the level of specificity that is required for symbiosis within the Vibrionaceae, and whether abiotic or biotic factors have an effect for determining species level colonization in this environmentally transmitted association.

Acknowledgments

The authors would like to thank Dr Jaruwat Nabhitabhata, Dr Renu Yashiro, Pitiporn Nilaphat, and Pichitra Promboon from the Rayong Coastal Aquaculture Station for help collecting in Rayong, Thailand, and Dr Praulai Nootmorn and her students from the Andaman Sea Fisheries Development Center for help collecting in Phuket, Thailand. We would also like to thank G. Giribet for his advice with the phylogenetic analyses. William Soto, Serena Rogers, Latoya Dale and Clayton Gorman contributed with sequencing bacterial loci. Special thanks to Co Thai Ngo for her assistance with the NMSU cluster. This work was supported in part by NSF DEB-01316516, DBI-0079820 and SBE-123690, and NIH S06-GM08136-26 to M.K.N.

References

Alsina, M., Blanch, A.R., 1994. Improvement and update of a set of keys for biochemical identification of *Vibrio* species. J. Appl. Bacteriol. 77, 719–721.

Ast, J.C., Dunlap, P.V., 2004. Phylogenetic analysis of the lux operon distinguishes two evolutionarily distinct clades of *Photobacterium leiognathi*. Arch. Microbiol. 181, 352–361.

Austin, B., Zhang, X.H., 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett. Appl. Microbiol. 43, 119–124.

Banatvala, N., Hlady, W.G., Ray, B.J., McFarland, L.M., Thompson, S., Tauxe, R.V., 1997. *Vibrio vulnificus* infection reporting on death certificates: the invisible impact of an often fatal infection. Epidemiol. Infect. 118, 221–225.

Budsberg, K.J., Wimpee, C.F., Braddock, J.F., 2003. Isolation and identification of *Photobacterium phosphoreum* from an unexpected niche: migrating salmon. Appl. Environ. Microbiol. 69, 6938–6942.

Fidopiastis, P.M., Boletzky, S.V., Ruby, E.G., 1998. A new niche for *Vibrio logei,* the predominant light organ symbiont of squids in the genus *Sepiola*. J. Bacteriol. 180, 59–64.

Foster, J.S., Boletzky, S.V., McFall-Ngai, M.J., 2002. A comparison of the light organ development of *Sepiola robusta* Naef and *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). Bull. Mar. Sci. 70, 141–153.

Giribet, G., Wheeler, W.C., 2002. On bivalve phylogeny: a high-level analysis of the Bivalvia (Mollusca) based on combined morphology and DNA sequence data. Invert. Zool. 121, 271–324.

Giribet, G., Edgecombe, G.D., Wheeler, W.C., 2001. Arthropod phylogeny based on eight molecular loci and morphology. Nature 413, 157–161.

Goloboff, P.A., 1999. Analyzing large data sets in reasonable times: solutions for composite optima. Cladistics 15, 415–428.

Jones, B.W., Nishiguchi, M.K., 2004. Counterillumination in the bobtail squid, *Euprymna scolopes*. Mar. Biol. 144, 1151–1155.

Jones, B.W., Lopez, J.E., Huttenburg, J., Nishiguchi, M.K., 2006. Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. Mol. Ecol. 15, 4317–4329.

Kita-Tsukamoto, K., Oyaizu, H., Nanba, K., Simidu, U., 1993. Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of 16S rRNA sequences. Int. J. Syst. Bacteriol. 43, 8–19.

Lee, K.-H., Ruby, E.G., 1995. Symbiotic role of the viable but nonculturable state of *Vibrio fischeri* in Hawaiian coastal seawater. Appl. Environ. Microbiol. 61, 278–283.

McFall-Ngai, M.J., Montgomery, M.K., 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). Biol. Bull. 179, 332–339.

Meighen, E.A., 1999. Autoinduction of light emission in different species of bioluminescent bacteria. Luminescence 14, 3–9.

Naef, A., 1912a. Teuthologische Notizen. 2. Gattungen Sepioliden. Zool. Anzeiger, 39, 244–248.

Naef, A., 1912b. Teuthologische notizen. 3. Die Arten der Gattungen *Sepiola* und *Sepietta*. Zool. Anzeiger, 39, 262–271.

Nesis, K.N., 1982. Cephalopods of the World. T.F.H. Publications, Neptune City, NJ.

Nishiguchi, M.K., 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. Microbiol. Ecol. 44, 10–18.

Nishiguchi, M.K., Nair, V.S., 2003. Evolution of symbioses in the Vibrionaceae: a combined approach using molecules and physiology. Int. J. Syst. Evol. Microbiol. 53, 2019–2026.

Nishiguchi, M.K., Lopez, J.E., Boletzky, S.V., 2004. Enlightenment of old ideas from new investigations: The evolution of cephalopod bacteriogenic light organs. Evol. Dev. 6, 41–49.

Nishiguchi, M.K., Ruby, E.G., McFall-Ngai, M.J., 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-Vibrio symbiases. Appl. Environ. Microbiol. 64, 3209–3213.

Pizzutto, M., Hirst, R.G., 1995. Classification of isolates of *Vibrio harveyi* virulent to *Penaeus monodon* larvae by protein profile analysis and M13 DNA fingerprinting. Dis. Aquat. Organ. 21, 61–68.

Ruby, E.G., Asato, L.M., 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch. Microbiol. 159, 160–167.

Ruby, E.G., McFall-Ngai, M.J., 1999. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends Microbiol. 7, 414–420.

Ruimy, R., Breittmayer, V., Elbaze, P., Lafay, B., Boussemart, O., Gauthier, M., Christen, R., 1994. Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. Int. J. Syst. Bacteriol. 44, 416–426.

Sankoff, D., Rousseau, P., 1975. Locating the vertices of a Steiner tree in arbitrary space. Math. Program. 9, 240–246.

Small, A.L., McFall-Ngai, M.J., 1998. A halide peroxidase in tissues interacting with bacteria in the squids *Euprymna scolopes*. J. Cell. Biochem. 72, 445–457.

Smith, S.W., Overbeek, R., Woese, C.R., Gilbert, W., Gillevet, P.M., 1994. The genetic data environment an expandable GUI for multiple sequence analysis. Comput. Appl. Biosci. 10, 671–675.

Swofford, D.L., 2002. PAUP* Phylogenetic Analysis Using Parsimony (*and other methods), Version 4.08b8. Sinauer Associates, Sunderland, MA.

Thompson, C.C., Thompson, F.L., Vandemeulebroecke, K., Hoste, B., Dawyndt, P., Swings, J., 2004. Use of *recA* as an alternative phylogenetic marker in the family Vibrionaceae. Int. J. Syst. Evol Microbiol. 54, 919–924.

Thompson, F.L., Hoste, B., Vandemeulebroecke, K., Swings, J., 2001. Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. Syst. Appl. Microbiol. 24, 520–538.

Thompson, F.L., Thompson, C.C., Vicente, A.C., Theophilo, G.N., Hofer, E., Swings, J., 2003. Genomic diversity of clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 2001 as revealed by fluorescent amplified fragment length polymorphism analysis. J. Clin. Microbiol. 41, 1946–1950.

Visick, K.L., Foster, J.S., Doino, J.A., McFall-Ngai, M.J., Ruby, E.G., 2000. *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J. Bacteriol. 182, 4578–4586.

Wheeler, W.C., 1995. Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. Syst. Biol. 44, 321–331.

Wheeler, W.C., 1996. Optimization alignment: the end of multiple sequence alignment in phylogenetics? Cladistics 12, 1–9.

Wheeler, W.C., 1998. Alignment characters, dynamic programming and heuristic solutions. In: DeSalle, R., Schierwater, B. (Eds.), Alignment Characters, Dynamic Programming and Heuristic Solutions. Birkhauser-Verlag, Basel, pp. 243–251.

Wheeler, W.C., Gladstein, D., DeLaet, J., 2002. POY v. 3.0. American Museum of Natural History, New York.

Wimpee, C.F., Nadeau, T.L., Nealson, K.H., 1991. Development of species-specific hybridization probes for marine luminous bacteria by using in vitro DNA amplification. Appl. Environ. Microbiol. 57, 1319–1324.

Young, R.E., Kampa, E.M., Maynard, S.D., Mencher, F.M., Roper, C.F.E., 1980. Counterillumination and the upper depth limits of midwater animals. Deep Sea Res. 27A, 671–691.

Young, R.E., Roper, C.F.E., 1977. Intensity regulation of bioluminescence during countershading in living midwater animals. Fish. Bull. 75, 239–252.

Yumoto, I.I., Iwata, H., Sawabe, T., Ueno, K., Ichise, N., Matsuyama, H., Okuyama, H., Kawasaki, K., 1999. Characterization of a facultatively psychrophilic bacterium, *Vibrio rumoiensis* sp. nov., that exhibits high catalase activity. Appl. Environ. Microbiol. 65, 67–72.

MOLECULAR PHYLOGENY OF COLEOID CEPHALOPODS (MOLLUSCA: CEPHALOPODA) INFERRED FROM THREE MITOCHONDRIAL AND SIX NUCLEAR LOCI: A COMPARISON OF ALIGNMENT, IMPLIED ALIGNMENT AND ANALYSIS METHODS

JAN STRUGNELL[1,2] AND MICHELE K. NISHIGUCHI[3]

[1]*School of Biology and Biochemistry, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK;*
[2]*British Antarctic Survey, Natural Environmental Research Council, High Cross, Madingley Road, Cambridge CB3 0ET, UK;*
[3]*Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA*

(Received 15 December 2006; accepted 1 September 2007)

ABSTRACT

Recent molecular studies investigating higher-level phylogenetics of coleoid cephalopods (octopuses, squids and cuttlefishes) have produced conflicting results. A wide range of sequence alignment and analysis methods are used in cephalopod phylogenetic studies. The present study investigated the effect of commonly used alignment and analysis methods on higher-level cephalopod phylogenetics. Two sequence homology methods: (1) eye alignment, (2) implied alignment, and three analysis methods: (1) parsimony, (2) maximum likelihood, (3) Bayesian methodologies, were employed on the longest sequence dataset available for the coleoid cephalopods, comprising three mitochondrial and six nuclear loci. The data were also tested for base composition heterogeneity, which was detected in three genes and resolved using RY coding. The Octopoda, Argonautoidea, Oegopsida and Ommastrephidae are monophyletic in the phylogenies resulting from each of the alignment and analysis combinations. Furthermore, the Bathyteuthidae are the sister taxon of the Oegopsida in each case. However many relationships within the Coleoidea differed depending upon the alignment and analysis method used. This study demonstrates how differences in alignment and analysis methods commonly used in cephalopod phylogenetics can lead to different, but often highly supported, relationships.

INTRODUCTION

The class Cephalopoda comprises two extant subclasses, Nautiloidea (*Nautilus* and *Allonautilus*) and the Coleoidea. The Coleoidea contains two subdivisions, the Belemnoidea, which became extinct at the end of the Cretaceous, and the Neocoleoidea, which contains the octopuses, squids and cuttlefishes. Neocoleoid cephalopods are characterized by the reduction and internalization, or complete loss, of the shell and, as a result, they very rarely fossilize well. Therefore, very little information regarding the origins and relationships of extant coleoid cephalopods can be gleaned from the fossil record (Nishiguchi & Mapes, 2007).

Morphological studies have proved to be useful in classifying species within subfamilies and/or genera (e.g. Berthold & Engeser, 1987; Clarke, 1988; Khromov, 1990; Voight, 1993a,b; Young & Vecchione, 1996; Voss, 1988), but less so in determining higher-level relationships. Morphological studies attempting to resolve these relationships have been constrained by the number of characters used with confidence due to "problems primarily involving character independence, apomorphic 'loss', or assessment of homology/homoplasy" (Young & Vecchione, 1996).

Presently, extant coleoids are divided into two superorders, Decapodiformes and Octopodiformes (Berthold & Engeser, 1987). In his website 'The Fossil Coleoidea Page' (http://userpage.fu-berlin.de/~palaeont/fossilcoleoidea/welcome.html), Engeser draws attention to the fact that the term Octopodiformes is in use elsewhere and suggests the use of Vampyropoda (Boletzky, 1992) instead. The Decapodiformes (Decembrachiata Winckworth *sensu* Engeser, loc. cit.) contains the orders Teuthoidea [suborders Myopsida (closed-eye squids) and Oegopsida (open-eye squids) and Sepioidea (families Idiosepiidae (pygmy squid), Sepiidae (cuttlefishes), Spirulidae (ram's horn squid), Sepiolidae (bobtail squids) and Sepiadariidae (bottletail squids)]. Current debate exists on the validity of the ordinal level of classification (Naef, 1921–1923; Voss, 1977; Berthold & Engeser, 1987; Young & Vecchione, 1996). Furthermore, Lindgren, Giribet & Nishiguchi (2004) question whether the suborder Oegopsida is monophyletic.

The Octopodiformes contains the orders Vampyromorpha (vampire 'squid') and Octopoda (pelagic and benthic octopuses), hence the name Vampyropoda (Boletzky, 1992). A sister-taxon relationship between these two orders is accepted primarily based on morphology (Pickford, 1939; Boletzky, 1992; Young & Vecchione, 1996; Engeser, 1997; Young, Vecchione & Donovan, 1998; Carlini, Reece & Graves, 2000), but combined analysis using molecular and morphological data suggests a sister-taxon relationship between the Decapodiformes and Vampyromorpha (Lindgren *et al.*, 2004). The Octopoda comprises the suborders Cirrata (deep-sea finned octopuses) and Incirrata (benthic octopuses and pelagic octopuses, including the argonautoids and blanket octopuses. A sister-taxon relationship between these suborders is also widely accepted (Grimpe, 1921; Naef, 1921–1923; Young & Vecchione, 1996; Voight, 1997). Phylogenetic relationships between the nine Incirrata families remain unresolved and have been debated in the literature (Naef, 1921–1923; Robson, 1929, 1931; Voss, 1977; Young & Vecchione, 1996; Voight, 1997).

In the mid 1990s the first studies using DNA sequence data to estimate phylogenetic relationships within cephalopods were reported (Bonnaud, Boucher-Rodoni & Monnerot, 1994, 1996, 1997; Boucher-Rodoni & Bonnaud, 1996). These studies sequenced portions of 16S rDNA, COII and COIII from 8 to 28 cephalopod taxa. These authors aligned their sequences by

Correspondence: J. Strugnell; e-mail: jmst@bas.ac.uk

eye (with the aid of the secondary structure where possible) and analysed the data using neighbour-joining (NJ) and parsimony methods. Although the genes proved useful in helping resolve intrafamilial relationships, little resolution of higher-level relationships was recovered. Subsequently, molecular studies investigating higher-level phylogenetic relationships of cephalopods have sequenced additional mitochondrial genes (Carlini & Graves, 1999; Piertney *et al*., 2003; Nishiguchi, Lopez & Boletzky, 2004; Zheng *et al*., 2004; Guzik *et al*., 2005) including whole mitochondrial genomes (Yokobori *et al*., 2004; Akasaki *et al*., 2006) and also nuclear genes (Carlini *et al*., 2000; Warnke *et al*., 2003; Strugnell *et al*., 2004; Guzik *et al*., 2005; Strugnell *et al*., 2005) often from a greater number of taxa (Carlini & Graves, 1999; Anderson, 2000a, b; Carlini *et al*., 2000; Lindgren *et al*., 2004; Strugnell *et al*., 2005) (Table 1).

Furthermore, since these first studies of cephalopod molecular phylogenetics, the range of sequence alignment and analysis methods available to phylogeneticists has increased (Table 1), and debate concerning the best methods to use has flourished (e.g. Wheeler, 1995, Kjer, Gillespie & Ober, 2007). Studies investigating cephalopod phylogenetics have aligned sequences by eye (Carlini & Graves, 1999; Carlini *et al*., 2000; Strugnell *et al*., 2004, 2005) or with the aid of alignment packages (Piertney *et al*., 2003; Yokobori *et al*., 2004; Zheng *et al*., 2004; Guzik *et al*., 2005) and have employed a variety of methods of analysis, including neighbour-joining (Allcock & Piertney, 2002; Warnke *et al*., 2003; Yokobori *et al*., 2004; Zheng *et al*., 2004), parsimony (Carlini & Graves, 1999; Anderson, 2000a,b; Carlini *et al*., 2000; Carlini, Young & Vecchione, 2001; Allcock & Piertney, 2002; Warnke *et al*., 2003; Lindgren *et al*., 2004, 2005; Nishiguchi *et al*., 2004; Zheng *et al*., 2004; Guzik *et al*., 2005), maximum likelihood (ML) (Anderson, 2000a,b; Carlini *et al*., 2000, 2001; Allcock & Piertney, 2002; Warnke *et al*., 2003; Strugnell *et al*., 2004; Yokobori *et al*., 2004; Guzik *et al*., 2005), Bayesian (Strugnell *et al*., 2004, 2005; Guzik *et al*., 2005) and LogDet (Anderson, 2000b; Strugnell *et al*., 2005). Recently, some studies have employed direct optimization where alignment is coupled with tree estimation in a dynamic procedure (Nishiguchi *et al*., 2004; Lindgren *et al*., 2004, 2005) (Table 1).

Although providing some insights [e.g. sister taxon relationships between the suborder Oegospida and family Bathyteuthidae (Strugnell *et al*., 2005)] none of these studies have conclusively resolved all higher-level cephalopod phylogenetic relationships and in many cases the results have been conflicting (see Akasaki *et al*., 2006; Nishiguchi & Mapes, 2007 for review of conflicting decapodiform relationships).

A number of reasons have been suggested for these varying and unresolved relationships. These include the early divergence of taxa, saturated sequence data, insufficient data, insufficient taxa and gene duplication (see Bonnaud *et al*., 1994, 1996; Carlini & Graves, 1999; Carlini *et al*., 2000; Lindgren *et al*., 2004; Strugnell *et al*., 2005 for discussion).

The large molecular data sets generated by Lindgren *et al*. (2004) (four genes) and Strugnell *et al*. (2004, 2005) (six genes) contained 18 of the same species (including 6 Octopodiformes and 11 Decapodiformes). Together, these provide the single largest dataset (with regard to sequence length) available for investigating higher-level phylogenetic relationships within

Table 1. Summary of studies of the molecular phylogenetics of coleoid cephalopods.

Reference	Focal taxa	Genes used	No. of species	Sequence alignment method	Analysis method(s)
Bonnaud *et al.* (1994)	Decapodiformes	16S	28	eye (2° structure)	NJ, P
Bonnaud *et al.* (1996)	Decapodiformes	16S, COIII	8	eye	NJ, P
Boucher-Rodoni & Bonnaud (1996)*	Coleoidea	16S	10		NJ, P
Bonnaud *et al.* (1997)	Coleoidea	COIII, COII	17	eye	NJ, P
Bonnaud *et al.* (1998)	Onychoteuthidae	16	14	eye	NJ, P
Carlini & Graves (1999)	Coleoidea	COI	48	eye	P
Anderson (2000)	Loliginidae	16S, COI	~30	Clustal and eye	P, ML, LogDet
Anderson (2000)*	Loliginidae	16S, COI	53	Clustal and eye	P, ML
Carlini *et al.* (2000)	Coleoidea	actin	44	eye	P, ML
Carlini *et al.* (2001)	Octopoda	COI	29	eye	P, ML
Allcock & Piertney (2002)	Octopodidae	16S	9	Clustal X and eye	NJ, P, ML
Piertney *et al.* (2003)	Cirrata	16S	27	Clustal X and eye	NJ, P, ML
Warnke *et al.* (2003)	Decapodiformes	complete 18S	8	Clustal V, MegAlign, checked by eye	NJ, P, ML
Bonnaud *et al.* (2004)	Nautilus	complete 18S	3	eye	2° structure
Lindgren *et al.* (2004)*	Coleoidea	complete 18S, 28S, hist. COI	60	POY	P
Nishiguchi *et al.* (2004)	Sepiolidae	12S, 16S, COI, 28S	30	POY	P
Strugnell *et al.* (2004)	Octopodiformes	16S, 12S, COI, rhod, pax-6, ODH		eye	ML, Bayesian
Yokobori *et al.* (2004)	Coleoidea	whole mitochondrial genome	3	ClustalX	NJ, ML
Zheng *et al.* (2004)	Decapodiformes	COI, 16S	13	ClustalX v1.8	NJ, P
Guzik *et al.* (2005)	Octopodinae	COIII, cyt b, ef-1α	30	Sequencher 3.1	P, ML, Bayesian
Lindgren *et al.* (2005)	Gonatidae	12S, 16S, COI	39	POY	P
Strugnell *et al.* (2005)	Coleoidea	16S, 12S, COI, rhod, pax-6, ODH	35	eye	Bayesian, LogDet
Takumiya *et al.* (2005)	Coleoidea	12S, 16S, COI	36	SeqPup v. 0.9, ClustalX ver1.83	NJ, P, ML
Akasaki *et al.* (2006)	Coleoidea	whole mitochondrial genome	5	–	ML

*note these studies also used further information in some analyses in addition to gene sequences, e.g. morphology, allozymes, immunology etc.
Abbreviations: cyt b, *cytochrome b apoenzyme*, COI, *cytochrome c oxidase subunit I*; 16S, 16S rDNA; 12S, 12S rDNA; 28S, 28S rDNA; 18S, 18S rDNA; ODH, *octopine dehydrogenase*; rhod, *rhodopsin*; hist, *histone* H3; ef-1α, elongation factor-1α; All sequences were of partial fragments unless otherwise stated.
NJ, neighbour-joining; P, parsimony; ML, maximum likelihood.

the subclass Coleoidea. In the present study we used two methods to align these data: by eye and implied alignment using POY; and also three methods of analysis: parsimony, ML and Bayesian, to investigate the effect of these analyses on the resulting phylogeny. The effect of base composition heterogeneity upon coleoid phylogenetic relationships was also investigated.

MATERIAL AND METHODS

Eighteen species were used in the present study, including representatives from each higher-level taxon within the subclass Coleoidea (Table 2). Portions of nine genes were included, three mitochondrial genes (12S rDNA, 16S, rDNA, COI) and six nuclear genes (28S rDNA, 18S rDNA, *histone*, *octopine*

Table 2. Accession numbers of each of the genes used in this study.

	Mitochondrial genes			Nuclear genes					
	12S rDNA	16S rDNA	COI	28S rDNA	18S rDNA	hist.	ODH	pax-6	rhod.
Nautiloidea									
Nautilida									
Nautilidae									
Nautilus pompilius	AY616965	AY377628	AY557514	AF311688	AY557452			AY617039	
Coleoidea									
Octopodiformes									
Vampyromorpha									
Vamyroteuthidae									
Vampyroteuthis infernalis	AY545077	AY545101	AF000071	AY557548	AY557459	AY557408	AY545114	AY545139	AY545163
Octopoda									
Allopsidae									
Haliphron atlanticus	AY616942	AY616971	AY557516	AY557549	AY557460	AY557409	AY616910	AY617016	AY617040
Argonautidae									
Argonauta nodosa	AY545080	AY545104	AY557517	AY557551	AY557462	AY557411	AY545117	AY545142	AY545166
Bolitaenidae									
Japetella diaphana	AY545093	A252766	AY545192	AY557552	AY557463		AY545130	AY545155	AY545179
Octopodidae									
Eledone cirrhosa	AY616946	AY616973	AY557520	AY557556	AY557467		AY616992	AY617020	AY617043
Graneledone verrucosa	AY545091	AY545111	AF000042	AY557557	AY557468	AY557413	AY545129	AY545153	AY545177
Decapodiformes									
Sepiolida									
Sepiolidae									
Heteroteuthis hawaiiensis	AY616873	AY616884	AF000044	AY293703	AY557472	AY557416	AY616906	AY616937	AY616922
Sepiida									
Sepiidae									
Sepia officinalis	AY545098	X9570	AJ583491	AY557560	AY557471	AY557415	AY545135	AY545160	AF000947
Idiosepiida									
Idiosepiidae									
Idiosepius pygmaeus	AY545095	AJ001647	AY545193	AY293684	AY557477	AY557421	AY545132	AY5157	AY545181
Spirulida									
Spirulidae									
Spirula spirula	AY545097	AY293659	AY293709	AY557563	AY557476	AY557420	AY545134	AY545159	AY545183
Teuthida Myopsdia									
Loliginidae									
Sepioteuthis lessoniana	AY616869	AJ001649	AY131036	AY557566	AY557480	AY557424	AY616902	AY616933	AY616918
Teuthida Myopsdia									
Bathyteuthidae									
Bathyteuthis abyssicola	AY616958	AJ000104	AF000030	AY557568	AY557483	AY557427	AY617002	AY617032	AY617057
Octopoteuthidae									
Octopoteuthis nielseni	AY616957	AY616983	AF000055	AY557591	AY557507		AY617011–AY617013	AY617031	AY617056
Cranchiidae									
Cranchia scabra	AY616962	DQ280046	AF000035	AY557571	AY557487	AY557430	AY617014 AY617015	AY617036	AY617061
Ommastrephidae									
Illex coindetii	AY616963	AY616985	AY617065	AY557593	AY557509	AY557450	AY617008 AY617015	AY617037	AY617062
Sthenoteuthis oualaniensis	AY545100	X79582	AF000069	AY557595	AY557511	AY557452	AY545137	AY545162	AY545185
Ommastrephes batramii	AY616866	AY616880	AF000057	AY557594	AY557510	AY557451	AY616899	AY616930	AY616915

dehydrogenase [ODH], *pax*-6 and *rhodopsin*). Sample details and methodologies used to obtain DNA sequences from these species are outlined in Lindgren *et al*. (2004) and Strugnell *et al*. (2005). Accession numbers for these sequences are listed in Table 2.

Sequence alignment and homology assessment

Two methods of sequence alignment were used within this study (1) by eye, and (2) using implied alignment using the homology scheme via POY (Wheeler, 2003; Giribet, 2005).

Aligned by eye

DNA sequences were compiled and aligned by eye in Se-Al v2.0a11 Carbon (Rambaut, 2002). Gaps were inserted where necessary to allow sequences to be aligned. Sequence data that were not alignable using this method were removed prior to analyses. Sequence alignment files are available on request. The total concatenated sequence length was 5,651 bp, of which 2,219 bp were variable.

Dynamic homology and implied alignments

Sequence data were analysed by using the direct optimization method described by Wheeler (1996) and implemented in the computer program POY. This method directly assesses the number of DNA sequence transformations (evolutionary events) required by a phylogenetic topology without the use of multiple sequence alignment. This is accomplished by generalization of existing character optimization procedures, including insertion and deletion events (indels) in addition to base substitutions. This method treats indels as processes, as opposed to the patterns implied by multiple sequence alignment (Wheeler, 1995). It is claimed that this method generates more efficient (and therefore simpler) explanations of sequence variation than multiple sequence alignment (Wheeler, 1996). Direct optimization, although computationally intense, is much less demanding than parsimony-based multiple sequence alignments when congruence among partitions is used as a criterion (Wheeler & Hayashi, 1998). The implied alignments produced via POY were used for both ML and Bayesian analyses. These sequences were concatenated for ML and Bayesian analysis (6,377 bp, of which 2,330 bp were variable).

Base composition heterogeneity

PAUP*4.0b10 (Swofford, 1998) was used for χ^2 tests of composition homogeneity of the sequence data aligned by eye. Tests of base homogeneity were based on variable sites only. Where base composition heterogeneity was detected it was RY coded to remove base composition heterogeneity.

The three sequence data sets, (1) implied alignments, (2) aligned by eye, (3) aligned by eye and RY coded were analysed using three methods, (a) parsimony, (b) maximum likelihood, (c) Bayesian analysis. It is important to note that the sequence data aligned by eye were analysed using parsimony analyses in PAUP rather than POY.

Dynamic homology under parsimony

Molecular data were analysed with the computer program POY (Wheeler *et al*., 1996–2003) using the direct optimization method (Wheeler, 1996), with parsimony as the optimality criterion. Nodal support was calculated in POY using Farris's parsimony jackknifing procedure (Farris *et al*., 1996) for 100 replicates (using the commands: jackboot; replicates 100). Tree searches were conducted in parallel at Harvard University on a 19 dual-processor cluster (Darwin.oeb.harvard.edu) using pvm (parallel virtual machine). Commands for lad balancing of spawned jobs were used to optimize parallelization procedures (-parallel–dpm–jobspernode 2). Trees were built via a random-addition sequence procedure (10 replicates) followed by a combination of branch-swapping steps [SPR (subtree pruning and regrafting) and TBR (tree bisection and reconnection)] and tree fusing (Goloboff, 1999) in order to further improve on tree length minimization. Discrepancies between heuristic and actual tree length calculations were addressed by adjusting slop values (-slop5–checkslop10). Phylogenetic trees were obtained using parsimony with a gap/ts/tv cost of various weighting. Several analyses were implemented with character transformations weighted differently to determine how various phylogenetic hypotheses were affected (sensitivity analysis *sensu* Wheeler, 1995). Each gene was analysed separately, using character transformations (indels/ts/tv) of equal weighting (111), and unequal weighting (121, 141, 211, 221, 241, 411, 421, 441). The parameter set that optimized the least amount of character incongruence was the equal weighted transformation (111) for all genes. *Histone* H3 and *pax-6* were the two exceptions that also had similar character incongruence values for the 211 and 411 transformations. The final tree was drawn with Tree View (Win32) and consensus trees were analysed in PAUP version 4.02b (Swofford, 1998). To determine nodal support all jackknife calculations were performed in POY using the procedure described in Nishiguchi *et al*. (2004).

Implied alignment under parsimony

PAUP*4.0b10 (Swofford, 1998) was used to perform maximum parsimony analyses on the sequence data that were aligned by eye. All parsimony searches were performed with 1,000 random sequence-addition replicated and TBR (tree bisection-reconnection) branch swapping. All characters were unordered and equally weighted. One thousand bootstrap replicates were performed to measure the support for each clade on the phylogenetic trees.

Alignment by eye and implied alignment under maximum likelihood

PAUP*4.0b10 (Swofford, 1998) was used to perform 100 full heuristic searches. Starting trees were generated by the neighbour-joining method (NJ) (Saitou & Nei, 1987). A $GTR + I + \Gamma$ likelihood model incorporating rate heterogeneity was used. Branch swapping was performed using TBR (tree-bisection-reconnection). Parameters were then re-estimated, and final branch swapping was performed using NNI (nearest-neighbour-interchange). ML bootstrap values of clade support were generated using the parameters estimated in the analysis, but with starting trees generated by the neighbour-joining method.

Alignment by eye and implied alignment under Bayesian analyses

MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) was used to calculate marginal posterior probabilities using the $GTR + I + \Gamma$ model of nucleotide substitution. Model parameter values were treated as unknown and were estimated in each analysis. Random starting trees were used for the analyses and were run between 1 and 500,000 generations, sampling the Markov chain every 100 generations.

Three strategies were used to ensure that analyses were not trapped in local optima: (1) analysis was performed twice, starting with a different random tree and log-likelihood values at stationarity were compared for convergence

(Huelsenbeck & Bolback, 2001); (2) the topologies and clade posterior probabilities from each of the two analyses were compared for congruence (Huelsenbeck & Imennov, 2002); and (3) Metropolis-coupled Markov chain Monte Carlo (MCMCMC) was used with one cold and three incrementally heated Markov chains run simultaneously (default Mr Bayes heating values) to allow a more extensive exploration of parameter space (Huelsenbeck & Ronquist, 2001).

Stationarity was deemed to be reached when the average standard deviation of split frequencies, shown in MrBayes 3.1.2 was less than 0.01 (Ronquist & Huelsenbeck, 2003).

Tracer v1.3 (Rambaut & Drummond, 2003) was used to determine the correct 'burnin-in' for the analysis (i.e. the number of initial generations that must be discarded before stationarity is reached).

RESULTS

Sequence alignment

Alignment of the ODH, *pax*-6, COI and *histone* sequences required no insertion/deletion events (indels). Indels were introduced into aligned sequences of 12S rDNA, 16S rDNA, 28S rDNA, 18S rDNA and *rhodopsin* both by eye and 'dynamically' during the analysis using POY. The alignments of these genes where indels were required differed notably depending upon the alignment method (Table 3) (alignments available on request). A greater number of gaps were inserted using POY than by eye for the 12S rDNA, 16S rDNA and 28S rDNA genes (Table 3), whereas a greater number of insertions was used aligning by eye than by using POY for *rhodopsin* and 18S rDNA (Table 3). For each of these five genes requiring indels, regions that were deemed to be unalignable with confidence by eye were removed prior to analysis. In contrast, no sequence was removed from the POY analysis due to the fact that sequences are aligned simultaneously during analysis.

Base composition heterogeneity

Chi-squared homogeneity tests of each of the genes shows that third positions of ODH, *rhodopsin* and COI have significant base frequency heterogeneity (Table 4). RY-coding the third positions of these genes was used to resolve base composition heterogeneity (Table 4). RY coding pools purines (adenine and guanine:R) and pyrimidines (cytosine and thymine:Y) into two-state categories (R,Y), and helps resolve bias resulting from differences in the relative frequency of either the two purines or pyrimidines (Phillips *et al.*, 2001).

A number of taxonomic groupings are robust to the different methods of coding, alignment and analysis. The following taxa are always monophyletic: Octopoda, Argonautoidea, Ommastrephidae and Oegopsida (Figs 1–9). Furthermore, in each topology the Bathyteuthoida is the sister taxon to the Oegopsida (Figs 1–9). Bayesian posterior probabilities provide the highest support for each of these clades (Figs 3, 6, 9).

Vampyromorpha and the Decapodiformes are sister taxa in the phylogenies resulting from parsimony, ML and Bayesian analyses of the sequences aligned using POY and also by eye (no RY) with variable levels of support (Figs 1–6). This relationship is also recovered from parsimony analysis of RY coded sequence (BS = 99) (Fig. 7). In contrast, ML and Bayesian analysis of RY coded sequence aligned by eye recovered a sister-taxon relationship between Vampyromorpha and Octopoda, i.e. the Octopodiformes (Figs 8, 9). However, these relationships are not highly supported by bootstraps or posterior probabilities (Figs 8, 9).

The placement of *Eledone* within the Octopoda differs depending upon alignment and analysis method. *Eledone* is the sister

Table 4. Chi-squared homogeneity test for base composition across all genes and codon positions.

Gene	Codon position	$\chi^2(P)$
12S rDNA	–	0.998
16S rDNA	–	0.997
18S rDNA	–	0.963
28S rDNA	–	1.000
COI	1st	1.000
COI	2nd	1.000
COI	3rd	**0.000**
COI (RY)	3rd	0.938
histone H3	1st	1.000
histone H3	2nd	1.000
histone H3	3rd	0.560
ODH	1st	1.000
ODH	2nd	1.000
ODH	3rd	**0.000**
ODH (RY)	3rd	0.999
pax-6	1st	1.000
pax-6	2nd	1.000
pax-6	3rd	0.945
rhodopsin	1st	0.994
rhodopsin	2nd	0.962
rhodopsin	3rd	**0.003**
rhodopsin (RY)	3rd	0.721

Tests were performed on variable sites only. $\chi^2(P) < 0.05$ are in bold.

Table 3. Comparison of alignment length of genes.

	Gene	Total base pairs in gene sequenced (no gaps) (bp)	Alignment method		
			POY (bp)	Eye (total alignment length) (bp)	Eye (unalignables removed, in analysis) (bp)
Mitochondrial	12S rDNA	417	573 (417)*	486 (417)*	283
	16S rDNA	528	627 (528)*	554 (528)*	427
Nuclear	18S rDNA	2,845	1,893 (1,842)*	3,202 (2,845)*	1,943
	28S rDNA	661	198 (191)*	166 (166)*	166
	rhodopsin	1,040	1,022 (991)*	1,032 (954)*	765

*Number in brackets indicates the starting sequence length without gaps. The portion of available sequence able to be aligned by eye was less for 28S and *rhodopsin* than by POY. A larger sequence fragment of 18S was attempted for alignment by eye, however a large proportion was unalignable and was removed prior to analysis.

Table 5. Phylogenetic relationships recovered by two alignment methods (by eye, dynamic homology/implied alignment using POY) and three analysis methods (P, parsimony; ML, maximum likelihood; Bayes, Bayesian).

Alignment method	POY			By eye					
				No RY			RY		
Analysis method	POY	ML	Bayes	POY	ML	Bayes	POY	ML	Bayes
Vampyromorpha(Decapodiformes)	√	√	√	√	√	√	√	X	X
Vampyromorpha(Octopoda)	X	X	X	X	X	X	X	√	√
Octopoda	√	√	√	√	√	√	√	√	√
Argonautoidea	√	√	√	√	√	√	√	√	√
((*Japetella*, *Graneledone*)*Eledone)*	√	√	√	√	X	X	√	X	X
(*Eledone*((*Japetella,Graneledone*)(*Haliphron,Argonauta*))	X	X	X	X	√	√	X	√	√
Decapodiformes	√	√	√	√	√	√	√	√	√
(Oegopsida)(remaining Decapodiformes)	X	X	X	X	√	√	X	√	√
Polyphyletic Sepioidea	√	√	√	√	√	√	√	√	√
Ommastrephidae	√	√	√	√	√	√	√	√	√
Oegopsida	√	√	√	√	√	√	√	√	√
Bathyteuthoida(Oegopisda)	√	√	√	√	√	√	√	√	√
Spirulida(Bathyteuthoida(Oegopisda))	√	√	√	√	X	X	X	X	X
Idiosepiidae(Sepioidea(Myopsida(Spirulida(Bathyteuthoida(Oegopisda)))))	√	X	X	X	X	X	X	X	X
Sepioidea(Myopsida(Spirulida(Bathyteuthoida(Oegopisda))))	√	√	√	X	X	X	X	X	X
Myopsida(Spirulida(Bathyteuthoida(Oegopisda)))	√	√	√	X	X	X	X	X	X
(*Heteroteuthis, Idiosepius*)	X	√	√	X	X	X	X	X	X
(*Sepioteuthis*, *Idiosepius*)	X	X	X	√	X	X	X	X	X
(*Sepia, Idiosepius*)	X	X	X	X	√	√	√	√	√
((*Sepia, Idiosepius*)*Sepioeuthis*)	X	X	X	X	√	√	√	X	√
(((*Sepia, Idiosepius*)*Sepioeuthis*)*Spirula*)	X	X	X	X	√	√	√	X	√
((((*Sepia, Idiosepius*)*Sepioeuthis*)*Spirula*)*Heteroteuthis*)	X	X	X	X	√	√	√	X	√
(Oegopsida,Bathyteuthoidea)(Sepioidea, Myopsida*)	X	X	X	X	√	√	X	√	√

*Myopsida falls within Sepioidea in this topology.
The data aligned by eye have been analysed for both nucleotide data and RY coded data. √, the relationship is supported, X, the relationship is not supported.

taxon to a clade containing *Japetella* and *Graneledone* in each of the phylogenies resulting from the POY alignment, and also parsimony analysis of the sequence data aligned by eye, both RY coded and not RY coded (Figs 1–4,7). High support for this relationship is evident on each of these phylogenies. However, *Eledone* is highly supported as being basal within the Octopoda in analysis of sequence data aligned by eye (both RY coded and not RY coded) and analysed using ML (no RY, BS = 100; RY, BS = 100) and Bayesian analyses (no RY, PP = 1.00; RY, PP = 1.00) (Figs 5, 6, 8, 9).

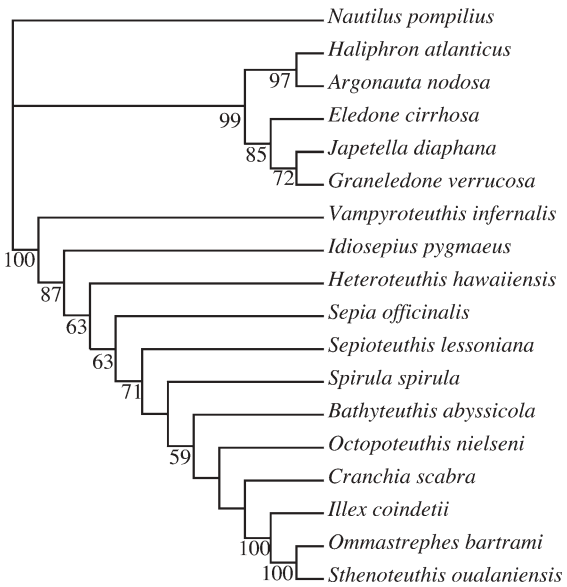


Figure 1. Parsimony topology of coleoid cephalopod relationships obtained using direct optimization using POY. Jackknife support values are indicated beneath each node.

Higher-level decapodiform relationships differ markedly between the various methods of alignment, coding, and analysis (Figs 1–9). Phylogenies generated from ML and Bayesian analyses of sequences aligned by eye (both RY coded and not RY coded) demonstrate Decapodiformes to be divided into two monophyletic groups, one containing the Oegopsida, and the second containing the remaining decapodiforms (i.e. Myopsida, Spirulidae, Sepiidae, Sepiolidae and Idiosepiidae) (Figs 5, 6,8,9). This division is highly supported by bootstrap support (no RY, BS = 98; RY, BS = 98) and posterior probabilities (no RY, PP = 0.99; RY, PP = 0.97) (Figs 5, 6,8,9). Within these topologies *Sepia* and *Idiosepius* are sister taxa, thereby rendering 'Sepioidea' (including Sepiidae, Sepiadariidae and Sepiolidae) polyphyletic (Figs 5, 6, 8, 9).

In contrast, a clade containing *Heteroteuthis* and *Idiosepius* is basal within decapodiforms in ML and Bayesian analysis (PP = 0.90) of sequence data aligned using POY (Figs 2, 3). *Heteroteuthis* alone is basal in phylogenies resulting from parsimony analysis of sequences aligned by eye, both RY coded (BS = 100) and not RY coded (BS = 100) (Figs 4, 7).

The position of Spirulidae within the Decapodiformes is highly dependent upon the method of alignment and analysis. Spirulidae are the sister taxon to a clade containing the Oegopsida and Bathyteuthoidea in all three analyses where sequences were aligned using POY, although support was only obtained

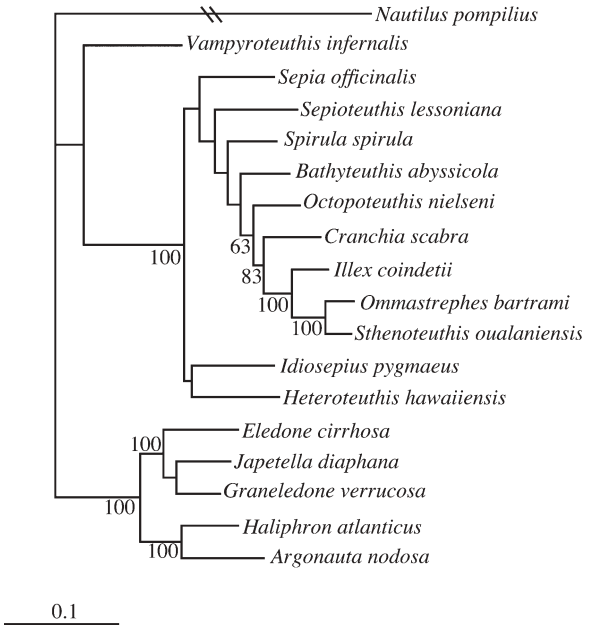


Figure 2. ML topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were obtained from implied alignments using POY. Bootstrap support values are indicated beneath each node.

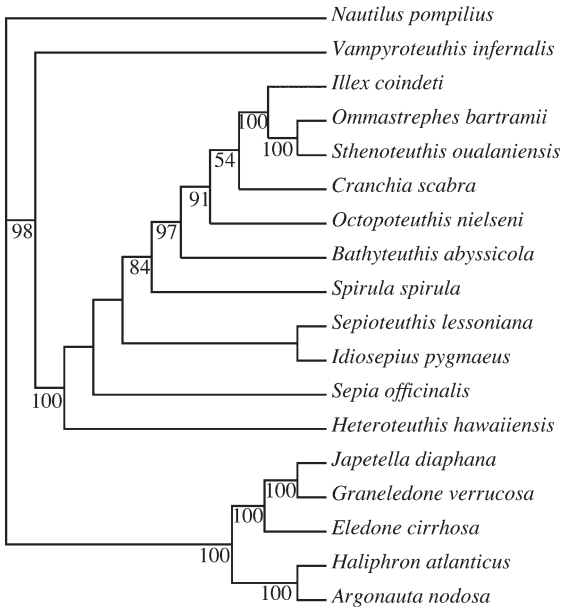


Figure 4. Parsimony topology of coleoid cephalopod relationships. Sequences were aligned by eye. Boostrap support values are indicated beneath each node.

for this relationship from the Bayesian analysis (PP = 1.00) (Figs 1–3). Interestingly, this same arrangement results from parsimony analysis of sequence data aligned by eye, not RY coded (BS = 84) (Fig. 4). In contrast, Spirulidae are the sister taxon to a clade containing Idiosepiidae, Sepiidae and Myopsida in the topologies resulting from ML and Bayesian analysis (PP = 0.99) of data aligned by eye (not RY coded) (Figs 5, 6) and in parsimony and Bayesian analysis (PP = 0.92) of RY coded data aligned by eye (Figs 8, 9).

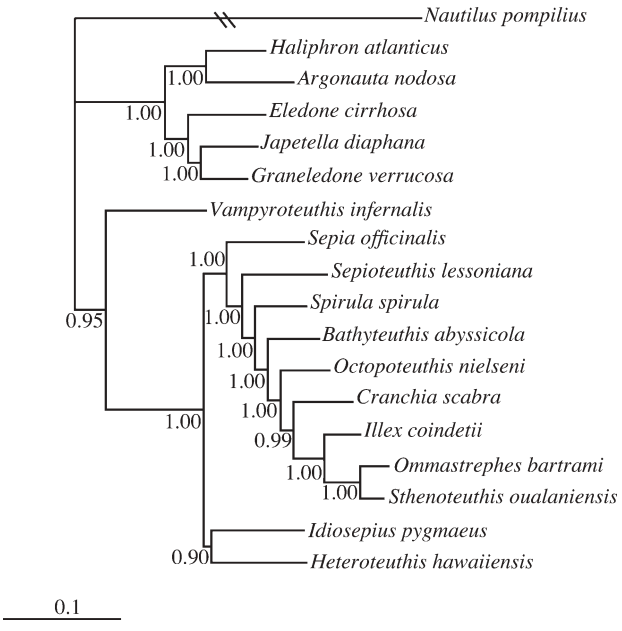


Figure 3. Bayesian topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were obtained from implied alignments using POY. Bayesian posterior probabilities are indicated beneath each node.

DISCUSSION

The present study is the largest molecular analysis of cephalopod phylogeny to date, with regard to sequence length, and provides a thorough comparison of the effect of commonly used alignment and analysis methodologies on the resulting higher-level phylogenetic relationships.

The different alignment, analysis and coding methods used within this study produced a range of considerably different topologies. Only the clades Octopoda, Argonautoidea, Decapodiformes, Oegopsida, Ommastrephidae and a sister-taxon

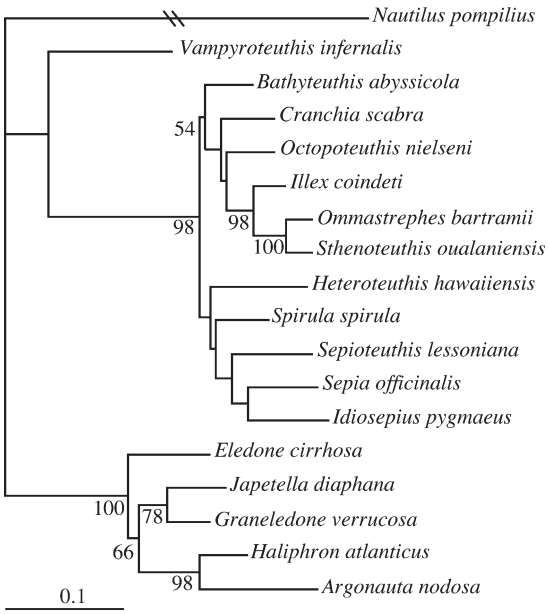


Figure 5. ML topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye. Bootstrap support values are indicated beneath each node.

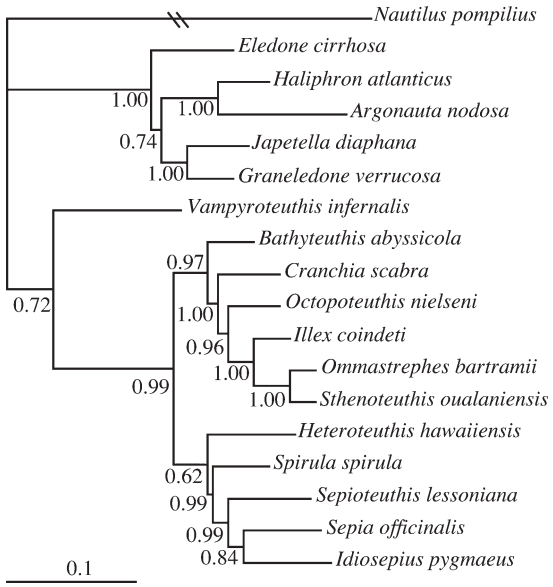


Figure 6. Bayesian topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye. Bayesian posterior probabilities are indicated beneath each node.

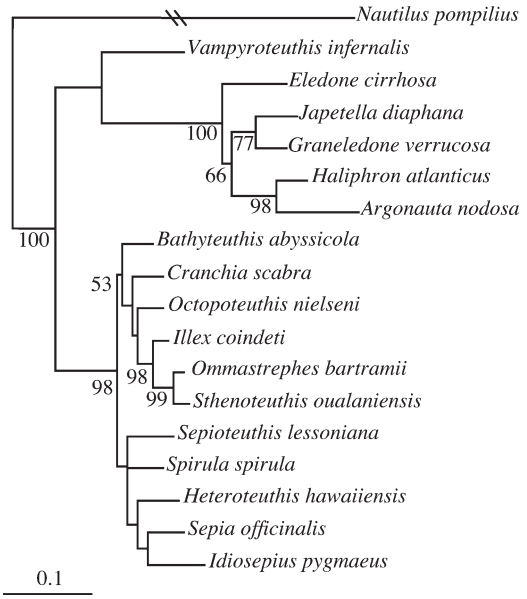


Figure 8. ML topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye, and third positions of *rhodopsin*, COI and ODH were RY coded. Boostrap support values are indicated beneath each node.

relationship between Bathyteuthidae and Oegopsida are robust to the alignment and analysis methods used.

Alignment methods

It is not surprising that different alignments can affect the resulting phylogeny, as the process of alignment aims to recover the evolutionary history of the sequences and therefore provides the very data upon which the algorithm performs (Giribet, Desalle & Wheller, 2002). For protein coding genes, the method of sequence alignment is usually insignificant, since in theory they should all produce the same alignment, i.e. an alignment without indels. However, as we demonstrate here, alignment methods for rDNAs, and both coding (i.e. *rhodopsin*) and non-coding genes can differ in their resulting sequence alignment and phylogenies.

There is debate in the literature regarding the best method of sequence alignment. Proponents of aligning sequences by eye (using secondary structural information) claim that

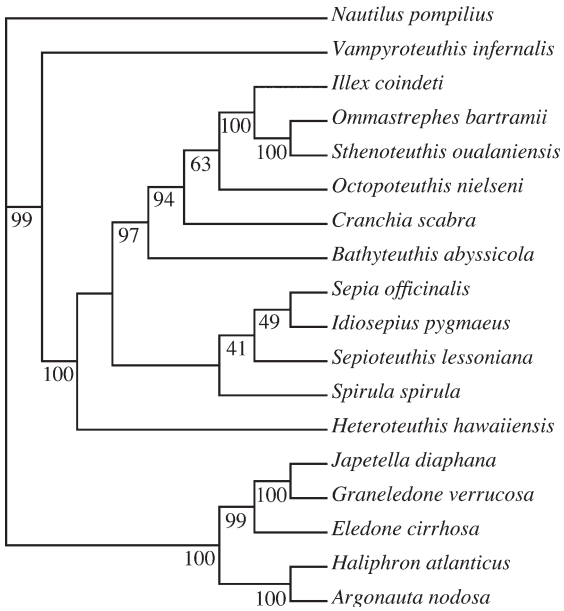


Figure 7. Parsimony topology of coleoid cephalopod relationships. Sequences were aligned by eye, and third positions of *rhodopsin*, COI and ODH were RY coded. Bootstrap support values are indicated beneath each node.

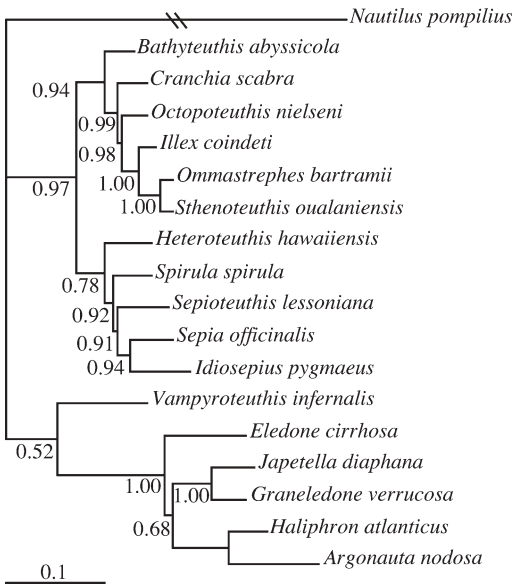


Figure 9. Bayesian topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye, and third positions of *rhodopsin*, COI and ODH were RY coded. Bayesian posterior probabilities are indicated beneath each node.

they are 'both philosophically and operationally superior' (Kjer *et al*., 2007), whereas proponents of computational methods claim that alignments performed by eye are subjective and therefore not repeatable (Giribet & Wheeler, 1999).

Proponents of the method of direct optimization using POY claim that it avoids the problem of alignment by generalizing phylogenetic character analysis to include insertion/deletion events (indels), with the sequence data proceeding directly to phylogenetic reconstruction, obviating the necessity to create gap characters. Indels do not appear as states, but as transformations linking ancestral to descendent nucleotide sequences (Giribet & Wheeler, 1999; Giribet *et al*., 2002). POY assumes that shorter trees are better trees and that aligning nucleotides together based on state is parsimonious and algorithmically less costly. Kjer *et al*. (2007) argues that this is not justified in structurally conserved molecules such as rDNAs, where conserved structures in the molecules are more important than the states of the nucleotides.

There has been intense disagreement over the relative merits of manual alignment and direct optimization (Kjer, 1995; Wheeler, 1995; Shull *et al*., 2001; Belshaw & Quicke, 2002; Gillespie, Yoder & Wharton, 2005) but few rigorous comparisons of these methods. Recently, Kjer *et al*. (2007) compared the phylogenies obtained by three phylogeneticists who independently aligned and analysed the same 16S rDNA datset by eye (using rDNA secondary structure and analysed by parsimony) and using direct optimization within POY. Interestingly, although all three alignments by eye differed at some positions, each alignment produced nearly identical topologies. In contrast, when using POY, none of the three phylogeneticists converged on the same parameters or the same tree. Kjer *et al*. (2007) suggest that the reason for this is that gap cost to change ratios (used within POY) are arbitrary, and this allows different researchers to obtain different results.

Sequence alignments resulting from POY have been reported to be 'gappy' in some studies (Pons & Vogler, 2006) with the program inserting a greater number of indels than other methods when utilizing indel costs of 1. Similarly, in the present study POY inserted a greater number of gaps in the 12S rDNA, 16S rDNA and 28S rDNA alignments, even though several parameters were explored using POY (costs of 1, 2 and 4 for indels, transitions and transversions, respectively). In addition, no sequence data were removed from the POY alignments used in the analysis. In contrast, a notable proportion of the sequence alignments of 12S rDNA, 16S rDNA, 18S rDNA and *rhodopsin* was removed after alignment by eye as it was deemed to be unalignable and would contribute noisy signal to the analysis. Therefore the starting information present in both datasets differed. The sequence data that were deemed 'unalignable' when aligning by eye are by their nature 'variable' and would therefore have an important contribution in the POY alignments in determining the resulting phylogenetic relationships. Differences in phylogenetic relationships observed in this study between the two alignment methods is largely due to the deleted sequences. Over 60% of the sequence information in both the datasets was constant (i.e. not variable) further demonstrating the significance of these variable sites. Despite these obvious differences in output, both of these methods of sequence alignment are widely accepted and appear in the cephalopod (Table 1) and wider literature today. It is likely that debate will continue regarding the best method of sequence alignment and while this continues to be the case, it may be beneficial to employ more than one method of alignment in phylogenetic studies.

Analysis methods

There is considerable debate in the literature regarding methods of phylogenetic analysis (e.g. Giribet, 2003). Parsimony methods have the benefit of being relatively easy to understand and require few assumptions about the evolutionary process (Page & Holmes, 1998). However they have been shown to produce the wrong topology under the most realistic models of evolution (e.g. long branch attraction; Huelsenbeck & Hillis, 1993).

ML methods allow the incorporation of sophisticated models of sequence evolution and allow statistical tests of different evolutionary hypotheses (i.e. likelihood ratio testing Felsenstein, 1981) yet require very large computational resources. Furthermore ML methods have been shown to be susceptible to long branch repulsion and long branch attraction under some circumstances (Pol & Siddall, 2001).

Bayesian methodologies (differing from likelihood methods only in the use of a prior distribution of the quantity being inferred, which is typically the tree) have the advantage over ML methods of being computationally efficient. They allow very complex models of sequence evolution to be implemented and also can efficiently analyse large datasets. Bayesian methods have been criticized however, for producing unrealistically high posterior probability support (Suzuki, Glazko & Nei, 2002; Simmons, Pickett & Miya, 2004).

In the present study, the majority of topologies resulting from the three analysis methods on the implied aligned data (from POY) are very similar. The exception to this is the position of *Idiosepius*. In contrast, the method of analysis had a greater effect on the data aligned by eye. In many cases ML and Bayesian methods of analysis produced the same or very similar topology for both RY coded and non-RY coded data, while the parsimony analysis produced a different topology. This is the case for the relationships of octopod taxa, and the relationship between the Oegopsida and the rest of the decapodiforms. It is unsurprising that ML and Bayesian analysis methods produce more similar topologies than parsimony analysis, because both are based on the same probabilistic model of evolution. In contrast, parsimony analysis is based on the idea that the preferred phylogenetic tree is the one that requires the fewest evolutionary changes.

Discussion of phylogenetic relationships

Order Vampyromorpha: *Vampyroteuthis infernalis* is the only species within the order Vampyromorpha. It possesses a number of unusual characteristics including two pairs of fins in juveniles (one pair in adults) and a second pair of arms modified into retractile filaments. Traditionally Vampyromorpha and Octopoda have been suggested to be sister taxa due to embryological, developmental (Naef, 1928; Young & Vecchione, 1996; Boletzky, 2003) and morphological similarities, such as sperm morphology (Healy, 1989) and the presence of radial sucker symmetry (Lindgren *et al*., 2004). However, the vampyromorph gladius is known to be morphologically similar to that of decapodiforms (Toll, 1982, 1998). Previous molecular studies have found support for both a sister taxon relationship between Vampyromorpha and Octopoda (Bonnaud *et al*., 1997; Carlini & Graves, 1999; Lindgren *et al*., 2004; Strugnell *et al*., 2004,2005) and Vampyromorpha and the Decapodiformes (Bonnaud *et al*., 1997; Lindgren *et al*., 2004). This present study found support for both of these relationships. The majority of alignment and analysis combinations support a sister-taxon relationship between Vampyromorpha and Decapodiformes. Only ML and Bayesian analysis of the 'by eye' alignment of RY coded data support a sister-taxon relationship between Vampyromorpha and Octopoda. RY coding rectified the base composition heterogeneity identified in the third positions of

COI, *rhodopsin* and ODH and thus is possible that this contributed to the Vampyromorpha and Octopoda sister-taxon relationship. RY coding also would have aided in reducing the effect of saturation (Phillips & Penny, 2003). However, parsimony analysis of the same dataset recovered a vampyromorph and decapodiform sister-taxon relationship. These results suggest that this relationship is unstable. The lineage Vampyromorpha is supposed to be at least 162 Myr from fossil evidence (Fischer & Riou, 2002) and has been estimated from fossil and molecular data to be potentially 252 Myr (Strugnell *et al*., 2006). The ancient diversification of this lineage provides support for the supposition that the molecular data used within this study are likely to be saturated at this level (Strugnell *et al*., 2005). Furthermore, the numerous extinction events throughout the Coleoidea during this time may contribute to the obscuring of affinities of Vampyromorpha (Lindgren *et al*., 2004).

Order Octopoda: *Eledone* was traditionally placed within the subfamily Eledoninae because it possesses an ink sac, a single row of suckers and large eggs (Robson, 1929). The taxonomic value of these characters has been debated; the presence of an ink sac has been suggested to be a function of depth (Robson, 1931; Voss, 1988; Allcock & Piertney, 2002) and sucker arrangement has been suggested to be a plastic character (Naef, 1921–1923; Voight, 1993a; Allcock & Piertney, 2002). Allcock & Piertney, (2002) suggested that sub-familial level assignment within the Octopodidae is 'a totally artificial classification with no evolutionary basis.' *Eledone* has been included in relatively few molecular studies (Bonnaud *et al*., 1997; Lindgren *et al*., 2004; Warnke *et al*., 2004). The present study recovered two differing placements for *Eledone*. All parsimony analyses, and also ML and Bayesian analyses of the POY alignment, show a sister-taxon relationship between *Eledone* and a clade containing *Japetella* and *Graneledone*, thus grouping together all species with a single row of suckers. In contrast ML and Bayesian analyses of data aligned by eye show *Eledone* to be basal within the Octopoda. This relationship was also recovered by Strugnell (2004), using a subset of the genes used within the present study, but with additional octopod species. *Eledone* possesses a number of morphological features supporting a basal position within the Octopoda, including the absence of a ligula (Naef, 1921–1923). It must be noted that there are relatively few octopod taxa included within the present study. The inclusion of additional taxa such as *Benthoctopus*, *Bathypolypus* and members of the suborder Cirrata would likely improve stability and resolution of octopod relationships.

Suborder Oegopsida and the family Bathyteuthidae: The suborder Oegospida contains squids that possess a gladius and lack a cornea. Molecular studies by Bonnaud *et al*. (1994, 1997), Carlini & Graves (1999), Carlini *et al*. (2000) and Lindgren *et al*. (2004) have suggested that the suborder may be polyphyletic, the later three studies reporting *Spirula* to fall within the Oegopsida. In contrast, Strugnell *et al*. (2005) supported a monophyletic Oegopsida. The present study also strongly supports a monophyletic Oegospida, since all alignment and analysis combinations supported this grouping. It is possible that the datasets in the previous studies that suggested a polyphyletic Oegopsida have been too small, and thus contained insufficient information to recover this relationship. All alignment and analysis combinations also support a sister-taxon relationship between the Oegopsida and the family Bathyteuthidae. This supports previous molecular studies by Carlini *et al*. (2000) and Strugnell *et al*. (2005) and also agrees with Naef's (1921–1923) suggestion that the Bathyteuthidae possess 'primitive characters for all Oegopsida'.

Suborder Myopsida and Sepioidea: Traditionally Spirulidae, Sepiidae, Idiosepiidae and Sepiadariiae/Sepiolidae have been grouped together in the suborder Sepioidea (Naef, 1921–1923), while the suborder Myopsida was grouped with the suborder Oegopsida in the order Teuthoidea on the basis of similar gladii and tentacular clubs (Naef, 1916, 1921–1923). However, the Myopsida has also been suggested to be derived from the 'Sepioidea' line based on a number of characteristics including possession of a cornea, suckers with circularis muscle, beak without angle point and a vena cava ventral to the intestine (d'Orbigny, 1845; Berthold & Engeser, 1987; Engeser, 1997; Haas, 1997, see Young *et al*., 1998, for a more detailed discussion). Molecular studies have suggested a close relationship between the Myopsida and some or all members of the Sepioidea (Carlini *et al*., 2000; Lindgren *et al*., 2004; Strugnell *et al*., 2005), although the precise relationship has varied depending upon the genes and analyses used. The present study also suggests a closer relationship between the Myopsida and the Sepioidea than the Myopsida and the Oegopsida, although the exact configuration of this is dependent upon the alignment method and analysis employed. In the phylogenies resulting from data aligned using POY, Myopsida was consistently the sister taxon to a clade containing Spirulida, Bathyteuthoidea and Oegopsida, with the remaining Sepioidea taxa falling outside this clade. However, in the phylogenies resulting from ML and Bayesian analyses of data aligned by eye (RY coded and not RY coded) the Myopsida fell within Sepioidea, together forming a sister taxon to a clade containing the Oegopsida and Bathyteuthidae.

These results clearly show that differing alignment and analysis strategies commonly used in coleoid cephalopod phylogenetics can produce notably different phylogenetic relationships. Researchers are far from agreeing on a single 'best' strategy of phylogenetic analysis, because the advantages and disadvantages of competing strategies are not yet clear. Until such a time, we advocate the use of a variety of different alignment and analysis strategies in phylogenetic analysis.

ACKNOWLEDGEMENTS

We thanks the following people for their generosity in donating tissue samples: S. von Boletzky, David Carlini, Martin Collins, Stephen Craig, Eileen Dillane, F.G. Hochberg, T. Kubodera, W.K. Macy, Mark Norman, S. Piertney, Richard Stride, Michael Vecchione, Kerstin Warnke and Richard Young. We are also grateful to G. Giribet for running the POY analysis on the Darwin cluster. J.S. is supported by a Natural Environment Research Council Antarctic Funding Initiative grant (NE/C506321/1). M.K.N. is partially supported by NIH SO6 GM008136-32S2 and NSF DEB-0316516.

REFERENCES

AKASAKI, T., NIKAIDO, M., TSUCHIYA, K., SEGAWA, S., HASEGAW, M. & OKADA, N. 2006. Extensive mitochondrial gene arrangements in coleoid Cephalopoda and their phylogenetic implications. *Molecular Phylogenetics and Evolution*, **38**: 648–658.

ALLCOCK, A.L. & PIERTNEY, S.B. 2002. Evolutionary relationships of Southern Ocean Octopodidae (Cephalopoda: Octopda) and a new diagnosis of *Pareledone*. *Marine Biology*, **140**: 129–135.

ANDERSON, F.E. 2000a. Phylogenetic relationships among loliginid squids (Cephalopoda: Myopsida) based on analyses of multiple data sets. *Zoological Journal of the Linnean Society*, **130**: 603–633.

ANDERSON, F.E. 2000b. Phylogeny and historical biogeography of the Loliginid squids (Mollusca: Cephalopoda) based on mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution*, **15**: 191–214.

BELSHAW, R. & QUICKIE, D.L.J. 2002. Robustness of ancestral state estimates: Evolution of life strategy in ichneumonoid parasitoids. *Systematic Biology*, **51**: 450–477.

BERTHOLD, T. & ENGESER, T. 1987. Phylogenetic analysis and systematization of the Cephalopoda (*Mollusca*). *Verhandlungen des naturwissenschaftlichen Vereins Hamburg*, **29**: 187–220.

BOLETZKY, S.von 1992. Evolutionary aspects of development, life style, and reproductive mode in incirrate octopods (Mollusca, Cephalopoda). *Revue suisse de Zoologie*, **99**: 755–770.

BOLETZKY, S.von 2003. Biology of early life stages in cephalopod molluscs. *Advances in Marine Biology*, **44:** 144–184.

BONNAUD, L., BOUCHER-RODONI, R. & MONNEROT, M. 1994. Phylogeny of decapod Cephalopods based on partial 16S rDNA nucleotide sequences. *Comptes Rendus de l'Académie des Sciences*, **317**: 575–580.

BONNAUD, L., BOUCHER-RODONI, R. & MONNEROT, M. 1996. Relationship of some coleoid cephalopods established by 3' end of the 16S rRNA and cytochrome oxidase III gene sequence comparison. *American Malacological Bulletin*, **12**: 87–90.

BONNAUD, L., BOUCHER-RODONI, R. & MONNEROTT, M. 1997. Phylogeny of cephalopods inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, **7**: 44–54.

BONNAUD, L., RODHOUSE, P.G. & BOUCHER-RODONI, R. 1998. A phylogenetic study of the squid family Onychoteuthidae (Cephalopoda: Oegopsida). *Proceedings of the Royal Society of London. Series B*, **265**: 1761–1770.

BOUCHER-RODONI, R. & BONNAUD, L. 1996. Biochemical and molecular approach to cephalopod phylogeny. *American Malacological Bulletin*, **12**: 79–85.

CARLINI, D.B. & GRAVES, J.E. 1999. Phylogenetic analysis of cytochrome c oxidase I sequences to determine higher-level relationships within the coleoid cephalopods. *Bulletin of Marine Science*, **64**: 57–76.

CARLINI, D.B., REECE, K.S. & GRAVES, J.E. 2000. Actin gene family evolution and the phylogeny of coleoid cephalopods (Mollusca: Cephalopoda). *Molecular Biology and Evolution*, **17**: 1353–1370.

CARLINI, D.B., YOUNG, R.E. & VECCHIONE, M., 2001. A molecular phylogeny of the octopoda (Mollusca:Cephalopoda) evaluated in light of morphological evidence. *Molecular Phylogenetics and Evolution* **21**: 388–397.

CLARKE, M.R. 1988. Evolution of recent cephalopods-A brief review. In: *The Mollusca. Paleontology and neontology of cephalopods*. Vol. 12 (M.R. Clarke & E.R. Trueman, eds), 1–355. Academic Press, San Diego.

D'ORBIGNY, A. 1845. *Mollusques vivants et fossiles*, **2**. Paris.

ENGESER, T. 1997. The fossil Coleoidea page. http://userpage.fu-berlin.de/ ~ palaeont/fosscol.html.

FARRIS, J.S., ALBERT, V.A., KÄLLERSJÖ, M., LIPSCOMB, D. & KLUGE, A.G. 1996. Parsimony jacknifing outperforms neighbor-joining. *Cladistics*, **12**: 99–124.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, **17**: 368–376.

FISCHER, J.C. & RIOU, B. 2002. *Vampyronassa rhodanica* nov. gen. nov sp., vampyromorphe (Cephalopoda, Coleoidea) du Callovien inférieur de la Voulte-sur-Rhône (Ardèche, France). *Annales de Paléontologie*, **88**: 1–17.

GILLESPIE, J.J., YODER, M.J. & WHARTON, R.A. 2005. Predicted secondary structures for 28S and 18S rRNA from Ichneumonoidea (Insecta: Hymenoptera: Apocrita): impact on sequence alignment and phylogeny estimation. *Journal of Molecular Evolution*, **61**:114–137.

GIRIBET, G. 2003. Stability in phylogenetic formulations, and its relationship to nodal support. *Systematic Biology*, **52**: 554–564.

GIRIBET, G. 2005. Generating implied alignments under direct optimization using POY. *Cladistics*, **21**: 396–402.

GIRIBET, G., DESALLE, R. & WHEELER, W.C. 2002. 'Pluralism' and the aims of phylogenetic research. In: *Molecular systematics and evolution: theory and practice* (R. DeSalle, G. Giribet, & W.C. Wheeler, eds), 141–146. Birkhauser, Basel.

GIRIBET, G. & WHEELER, W.C. 1999. On gaps. *Molecular Phylogenetic and Evolution*, **13**: 132–143.

GOLOBOFF, P.A. 1999. Analyzing large data sets in reasonable times: solutions for composite optima. *Cladistics*, **15**: S26–S34.

GRIMPE, G. 1921. Teuthologische Mitteilungen. VII:Systematiche Ubersicht der Nordsee-Cephalopoden. *Zoologischer Anzeiger*. **52**: 297–305.

GUZIK, M.T., NORMAN, M.D. & CROZIER, R.H. 2005. Molecular phylogeny of the benthic shallow-water octopuses (Cephalopoda: Octopodinae). *Molecular Phylogenetic and Evolution*, **37**: 235–248.

HAAS, W. 1997. Der Ablauf der Entwicklungsgeschichte der Decabrachia (Cephalopoda, Coleoidea). *Palaeontographica*, **245A**: 63–81.

HEALY, J.M. 1989. Spermatozoa of the deep-sea cephalopod *Vampyroteuthis infernalis* Chun: ultrastructure and possible phylogenetic significance. *Philosophical Transactions of the Royal Society, Series B*, **323**: 589–600.

HUELSENBECK, J.P. & BOLBACK, J.P. 2001. Empirical and hierchical Bayesian estimation of ancestral states. *Systematic Biology*, **50**: 351–366.

HUELSENBECK, J.P. & HILLIS, D.M. 1993. Success of phylogenetic methods in the four-taxon case. *Systematic Biology*, **40**: 247–264.

HUELSENBECK, J.P. & IMENNOV, N.S. 2002. Geographic origin of mitochondrial DNA: accomodating phylogenetic uncertainty and model comparison. *Systematic Biology*, **51**: 155–165.

HUELSENBECK, J.P. & RONQUIST, F. 2001. MrBayes:Bayesian inference of phylogeny. *Bioinformatics*, **17**: 754–755.

KHROMOV, D.N. 1990. Cuttlefishes in the systematics and phylogeny of Cephalopoda. *Zoologicheskii Zhurnal*, **69**: 12–20.

KJER, K.M. 1995. Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. *Molecular Phylogenetics and Evolution*, **4**: 314–330.

KJER, K.M., GILLESPIE, J.J., & OBER, K.A. 2007. Opinions on multiple sequence alignment, and an empirical comparison of repeatability and accuracy between POY and structural alignment. *Systematic Biology*, **56**: 133–146.

LINDGREN, A.R., GIRIBET, G. & NISHIGUCHI, M.K. 2004. A combined approach to the phylogeny of Cephalopoda (Mollusca). *Cladistics*, **20**: 454–486.

LINDGREN, A.R., KATUGIN, O.N., AMEZQUITA, E. & NISHIGUCHI, M.K. 2005. Evolutionary relationships among squids of the family Gonatidae (Mollusca: Cephalopoda) inferred from three mitochondrial loci. *Molecular Phylogenetics and Evolution*, **26**: 101–111.

NAEF, A. 1916. Uber neue Sepioliden aus dem Golf von Neapel. *Pubblicazioni della Stazione Zooloica di Napoli*, **1**: 1–10.

NAEF, A. 1921–1923. *Cephalopoda*. Fauna e flora del Golfo di Napoli, Monograph. (translated from German by the Israel program for Scientific translations, Jerusalem 1972).

NAEF, A. 1928. *Die Cephalopoden. Vol. 2. Embryologie* Fauna e Flora del Golfo di Napoli, Monographie 35.

NISHIGUCHI, M.K. & MAPES, R. 2007. Cephalopoda. In: *Molluscan evolution* (W. Ponder & D. Lindberg, eds), 161–197. University of California Press, Berkeley, California.

NISHIGUCHI, M.K., LOPEZ, J.E. & BOLETZKY, S.von 2004. Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. *Evolution and Development*, **6**: 41–49.

PAGE, R.D.M. & HOLMES, E.C. 1998. *Molecular evolution. A phylogenetic approach*. Blackwell Science, Oxford.

PHILLIPS, M.J., LIN, Y.-H., HARRISON, G.L. & PENNY, D. 2001. Mitochondrial genomes of a bandicoot and a brushtail possum confirm the monophyly of australidelphian marsupials. *Proceedings of the Royal Society of London, Series B*, **268**: 1533–1538.

PHILLIPS, M.J. & PENNY, D. 2003. The root of the mammalian tree inferred from whole mitochondrial genomes. *Molecular Phylogenetics and Evolution*, **28**: 171–185.

PICKFORD, G.E. 1939. The Vampyromorpha. A new order of dibranchiate Cephalopoda. *Věstník Československé zoologické společnosti*, **6–7**: 346–358.

PIERTNEY, S.B., HUDELOT, C., HOCHBERG, F.G. & COLLINS, M.A. 2003. Phylogenetic relationships among cirrate octopods (Mollusca: Cephalopoda) resolved using mitochondrial 16S ribosomal DNA sequences. *Molecular Phylogenetics and Evolution*, **27**: 348–353.

POL, D. & SIDDALL, M.E. 2001. Biases in maximum likelihood and parsimony: a simulation approach to a 10-taxon case. *Cladistics*, **17**: 266–281.

PONS, J. & VOGLER, A.P. 2006. Size, frequency, and phylogenetic signal of multiple-residue indels in sequence alignment of introns. *Cladistics*, **22**: 144–156.

RAMBAUT, A. 2002. SE-AL. Ver. v2.0a11 Carbon. Oxford University.

RAMBAUT, A. & DRUMMOND, A.J. 2003. TRACER. v1.0.1. Oxford University.

ROBSON, G.C. 1929. *A monograph of Recent Cephalopoda. Part I. Octopodinae*. British Museum (Natural History), London.

ROBSON, G.C. 1931. *A monograph of the Recent Cephalopoda. Part II. The Octopoda (excluding the Octopodinae)*. British Museum (Natural History), London.

RONQUIST, F. & HUELSENBECK, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**: 1572–1574.

SAITOU, N. & NEI, M. 1987. The neighbor-joining method. a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**: 406–425.

SHULL, V.L., VOGLER, A.P., BAKER, M.D., MADDISON, D.R. & HAMMOND, P.M. 2001. Sequence alignment of 18S ribosomal RNA and the basal relationships of adephagan beetles: evidence for monophyly of aquatic families and the placement of Trachypachidae. *Systematic Biology*, **50**: 945–969.

SIMMONS, M.P., PICKETT, K.M. & MIYA, M. 2004. How meaningful are Bayesian support values? *Molecular Biology and Evolution*, **21**: 188–199.

STRUGNELL, J. 2004. *The molecular evolutionary history of the Class Cephalopoda (Phylum Mollusca)*. DPhil thesis, Department of Zoology, University of Oxford.

STRUGNELL, J., JACKSON, J., DRUMMOND, A.J. & COOPER, A. 2006. Divergence time estimates for major cephalopod groups: evidence from multiple genes. *Cladistics*, **22**: 89–96.

STRUGNELL, J., NORMAN, M., DRUMMOND, A.J., JACKSON, J. & COOPER, A. 2005. Molecular phylogeny of coleoid cephalopods (Mollusca: Cephalopoda) using a multigene approach; the effect of data partitioning on resolving phylogenies in a Bayesian framework. *Molecular Phylogenetics and Evolution*, **37**: 426–441.

STRUGNELL, J.M., NORMAN, M.D., DRUMMOND, A.J. & COOPER, A. 2004. The octopuses that never came back to earth: neotenous origins for pelagic octopuses. *Current Biology*, **18**: R300–R301.

SUZUKI, Y., GLAZKO, G.V. & NEI, M. 2002. Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. *Proceedings of the National Academy of Science of the USA*, **99**: 16138–16143.

SWOFFORD, D.L. 1998. *PAUP*4.0-Phylogenetic Analysis Using Parsimony (*and other methods)*. Sinauer, Sunderland, MA.

TAKUMIYA, M., KOBAYASHI, M., TSUNEKI, K. & FURUYA, H. 2005. Phylogenetic relationships among major species of Japanese coleoid cephalopods (Mollusca: Cephalopoda) using three mitochondrial DNA sequences. *Zoological Science*, **22**: 147–155.

TOLL, R.B. 1982. *The comparative morphology of the gladius in the order Teuthodea (Mollusca: Cephalopoda) in relation to systematics and phylogeny*. PhD thesis, University of Miami.

TOLL, R.B. 1998. The gladius in teuthoid systematics. In: *International workshop on the systematics and biogeography of cephalopods*, 65–68. Smithsonian Institution Press, Washington D.C.

VOIGHT, J.R. 1993a. The arrangement of suckers on octopodid arms as a continuous character. *Malacologia*, **35**: 351–359.

VOIGHT, J.R. 1993b. A cladistic reassessment of Octopodid classification. *Malacologia*, **35**: 343–349.

VOIGHT, J.R. 1997. Cladistic analysis of the octopods based on anatomical characters. *Journal of Molluscan Studies*, **63**: 311–325.

VOSS, G.L. 1977. Present status and new trends in cephalopod systematics. In: *The biology of cephalopods* (M. Nixon & J.B. Messenger, eds), 49–60. Academic Press, London.

VOSS, G.L. 1988. Evolution and phylogenetic relationships of deep-sea octopods (Cirrata and Incirrata). In: *The Mollusca*, **12**: *Paleontology and neontology of cephalopods* (M.R. Clarke & E.R. Trueman, eds), 253–276. Academic Press, San Diego.

WARNKE, K., PLÖTNER, J., SANTANA, J.I., RUEDA, M.J. & LLINÀS, O. 2003. Reflections on the phylogenetic position of *Spirula* (Cephalopoda): preliminary evidence from the 18S ribosomal RNA gene. *Berliner Paläobiologisch Abhandlungen*, **3**: 253–260.

WARNKE, K., SOLLER, R., BLOHM, D. & SAINT-PAUL, U. 2004. A new look at geographic and phylogenetic relationships within the species group surrounding *Octopus vulgaris* (Mollusca, Cephalopoda): indications of very wide distribution from mitochondrial DNA sequences. *Journal of Zoological Systematics and Evolutionary Research*, **42**: 306–312.

WHEELER, W.C. 1995. Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. *Systematic Biology*, **44**: 321–331.

WHEELER, W.C. 1996. Optimization alignment: the end of multiple sequence alignment in phylogenetics? *Cladistics*, **12**: 1–9.

WHEELER, W.C. 2003. Implied alignment: a synapomorphy-based multiple-sequence alignment method and its use in cladogram search. *Cladistics*, **19**: 261–268.

WHEELER, W.C. & HAYASHI, C.Y. 1998. The phylogeny of extant chelicerate orders. *Cladistics*, **14**: 173–192.

WHEELER, W.C., GLADSTEIN, D.S. & LAET, J.D. 1996–2003. POY, v.3.0.11 ftp.amnh.org/pub/molecular/poy Wheeler.

YOKOBORI, S.-I., FUKUDA, N., NAKAMURA, M., AOYAMA, T. & OSHIMA, T. 2004. Long-term conservation of six duplicated structural genes in cephalopod mitochondrial genomes. *Molecular Biology and Evolution*, **22**: 2034–2046.

YOUNG, R.E. & VECCHIONE, M. 1996. Analysis of morphology to determine primary sister-taxon relationships within celeoid cephalopods. *American Malacological Bulletin*, **12**: 91–112.

YOUNG, R.E., VECCHIONE, M. & DONOVAN, D.T. 1998. The evolution of coleoid cephalopods and their present biodiversity and ecology. *South African Journal of Marine Science*, **20**: 393–420.

ZHENG, X., YANG, J., LIN., X. & WANG, R. 2004. Phylogenetic relationships among the decabrachia cephalopods inferred from mitochondrial DNA sequences. *Journal of Shellfish Research*, **23**: 881–886.

International Journal of Systematic and Evolutionary Microbiology (2008), **58**, 1292–1299 **DOI** 10.1099/ijs.0.65370-0

Gene sequences of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*

J. Browne-Silva and M. K. Nishiguchi

Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA

Correspondence
M. K. Nishiguchi
nish@nmsu.edu

Symbiosis between the bobtail squid *Euprymna scolopes* (Mollusca: Cephalopoda) and *Vibrio fischeri* bacteria has been a well-studied model for understanding the molecular mechanisms of colonization and adherence to host cells. For example, pilin expression has been observed to cause subtle variation in colonization for a number of Gram-negative bacteria with eukaryotic hosts. To investigate variation amongst *pil* genes of closely related strains of vibrios, we amplified *pil* genes *A*, *B*, *C* and *D* to determine orientation and sequence similarity to other symbiotic vibrios. The *pilA* gene was found to be upstream from all other *pil* genes, and not contiguous with the rest of the operon. The *pilB*, *pilC* and *pilD* loci were flanked at the 3′ end by *yacE*, followed by a conserved hypothetical gene. DNA sequences of each *pil* gene were aligned and analysed phylogenetically using parsimony for both individual and combined gene trees. Results demonstrate that certain *pil* loci (*pilB* and *pilD*) are conserved among strains of *V. fischeri*, but *pilC* differs in sequence between symbiotic and free-living strains. Phylogenetic analysis of all *pil* genes gives better resolution of Indo-west Pacific *V. fischeri* symbionts compared with analysis of the 16S rRNA gene. Hawaiian and Australian symbiotic strains form one monophyletic tree, supporting the hypothesis that *V. fischeri* strain specificity is selected by the geographical location of their hosts and is not related to specific squid species.

INTRODUCTION

The occurrence of bacterial symbionts with host partners is quite extensive and has been a topic for many evolutionary and developmental microbiologists (Nyholm & McFall-Ngai, 2004; Visick & Ruby, 2006). *Vibrio fischeri* belongs to the highly diverse family *Vibrionaceae* of the *Gammaproteobacteria* (Nishiguchi & Nair, 2003; Thompson *et al.*, 2004a). These luminous bacteria specifically colonize the light organ of sepiolid squids and monocentrid fishes (McFall-Ngai, 1994; McFall-Ngai & Ruby, 1998; Jones & Nishiguchi, 2004). The association begins after hatching, where bacteria are acquired environmentally via the light organ pores, which are continuously exposed to surrounding seawater. Although all environmental bacteria are in contact with the pores, only specific strains of *V. fischeri* can colonize and persist therein, out-competing non-native strain even during direct competition (Nishiguchi *et al.*, 1998; Nishiguchi, 2002).

Features known to play significant roles in success of the squid–symbiont relationship include chemotaxis, specific adherence, colonization, persistence and out-competing other bacterial strains (Graf *et al.*, 1994; Millikan & Ruby, 2004; Nyholm & McFall-Ngai, 2004). Likewise, competition studies have determined that both ecological and environmental factors may be responsible for the evolutionary history of specificity between the partners (Nishiguchi *et al.*, 1998; Nishiguchi, 2000, 2002). Although a number of biochemical and molecular mechanisms are found to increase the ability of *V. fischeri* to colonize and persist in the light organ, phenotypic characteristics such as pili have been observed to increase the surface area between bacterium and host (Nair, 2006). Pili are long, filamentous projections on the surface of both Gram-positive and Gram-negative bacteria, and are known to be important in both colonization and twitching motility (Darzins & Russell, 1997). They also mediate adherence or attachment to both abiotic and biotic surfaces (Stabb & Ruby, 2003). Pili are formed by a number of proteins encoded by genes found in the *pil* operon, and are grouped as types I, II, III and IV based on the pathway by which they are secreted (Soto & Hultgren, 1999). For example, *V. fischeri* expresses type IV pili, which are secreted by a pathway homologous to the type II secretion apparatus (Nunn, 1999; Sandkvist, 2001). The *pilA* gene codes for the major structural pilus component and *pilB* is involved in nucleotide binding, which controls the extension of type IV pili by the process of polymerization of the pilin subunit (Villar *et al.*, 2001). *pilC* codes for a transmembrane protein that belongs to the

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are detailed in Table 1.

GspF family of proteins and is involved in pilus assembly and adherence (Winther-Larsen *et al.*, 2001), while *pilD* serves as a leader peptidase for processing PilA precursors and *N*-methylation of the first amino acid of the mature protein. Previous observations of different wild-type *V. fischeri* strains isolated from both seawater and squid light organs exhibited differences in pili (both *in vitro* and *in vivo*; Nair, 2006), which may influence the specificity between sepiolid squids and their *Vibrio* symbionts and may be useful loci to determine subtle strain differences when determining the phylogenetic relatedness between symbiotic and free-living strains of *V. fischeri*. Therefore, we sequenced individual *pil* genes from strains of *V. fischeri* isolated from different geographical locations from both *Euprymna* and *Sepiola* squid hosts and two monocentrid fishes, as well as some free-living isolates. All sequences were used to construct individual and combined phylogenetic trees to determine the relatedness amongst *pil* genes and to determine whether these loci can reveal whether host specificity via pili is a common character between phylogenetically distinct *V. fischeri* strains. In addition, we established the gene order in each of the strains tested to determine whether differences existed in the *pil* operons of free-living and symbiotic *V. fischeri*.

METHODS

Bacterial strains. Bacteria used in this study were isolated previously from a number of sepiolid squids and monocentrid fishes, as well as free-living strains from seawater (Table 1) (Nishiguchi & Nair, 2003). Bacteria were initially plated from a frozen glycerol stock onto Luria–Bertani high-salt (32 ‰) (LBS) agar and grown at their optimum temperature (28 °C). A single colony-forming unit (c.f.u.) was then used to inoculate 5 ml LBS, grown for 12 h overnight at the optimum growth temperature and used subsequently for DNA isolation. Chromosomal DNA was extracted using the DNeasy kit (Qiagen) as described by the manufacturer. DNA was determined to be of PCR quality by electrophoresis on 1.0 % agarose gels.

PCR amplifications. Individual loci of pilin genes were amplified by PCR in 25 µl reaction volumes. Primers were designed on the basis of the *V. fischeri* ES114 genome sequence (Ruby *et al.*, 2005) (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&Cmd=Retrieve&list_uids=12986). Primers used in these experiments are listed in Table 2. All PCRs contained 2.5 mM $MgCl_2$, 1 × reaction buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 0.1 % Triton X-100), 2.5 mM each dNTP (Promega), 0.2 µM of each primer and 0.2 U *Taq* polymerase (Promega) or AmpliTaq (Applied Biosystems). PCR conditions are described in Table 3. All reactions included a hot start for 2 min at 94 °C and a final termination for 7 min at 72 °C using a DNA Engine Dyad (MJ Research). Amplified products were visualized via gel electrophoresis on a 1 % agarose gel stained with ethidium bromide.

Southern blotting. Southern blotting was used to test whether strains of *V. fischeri* that had unsuccessful PCR amplification of *pil* genes still possessed homologous regions of the *pil* operon. Genomic DNA was digested with *Hin*dIII, run on a 1 % agarose gel and transferred to nylon blotting paper (Millipore) by a standard method (Sambrook *et al.*, 1989). Each blot was probed with ESP915pilA (designed from the *V. fischeri* ES114 genome) and labelled with ^{32}P. Reactions contained 10 pmol oligonucleotide (5.0 µl), 2.0 µl kinase buffer (50 mM Tris/HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT,

Table 1. Bacterial isolates used in this study

Accession numbers respectively represent 16S rRNA gene and *pilB*, *pilC* and *pilD* sequences unless indicated. –, Not sequenced.

Strain	Host	Source	GenBank accession numbers
V. fischeri ATCC 7744	Free living	Not known	AY292938, –, –, DQ207634
V. fischeri ESP915	*Euprymna scolopes*	USA (Paiko, HI)	AY292920, DQ093340 (*B*, *C*, *D*)
V. fischeri ES114	*Euprymna scolopes*	USA (Kaneohe Bay, HI)	AY292919, DQ093345 (*B*, *C*, *D*)
V. fischeri EM17	*Euprymna morsei*	Japan (Tokyo Bay)	AY292922, DQ093341 (*B*, *C*, *D*)
V. fischeri WH1	Free living	USA (Woods Hole, MA)	AY292930, DQ093344 (*B*, *C*, *D*)
V. fischeri CG101	*Cleidopus gloriamaris*	Australia (Townsville)	AY292939, DQ207619, DQ207627, DQ207636
V. fischeri ET101	*Euprymna tasmanica*	Australia (Crib Point)	AY292923, DQ093342 (*B*, *C*, *D*)
V. fischeri ET301	*Euprymna tasmanica*	Australia (Botany Bay)	–, DQ207620, –, –
V. fischeri ET401	*Euprymna tasmanica*	Australia (Magnetic Island)	AY292943, DQ207621, DQ207628, DQ207637
V. fischeri ETJB5	*Euprymna tasmanica*	Australia (Jervis Bay)	–, DQ207622, DQ207629, DQ207638
V. fischeri SR5	*Sepiola robusta*	France (Banyuls sur mer)	AY292926, DQ093343 (*B*, *C*, *D*)
V. fischeri SI1D	*Sepiola intermedia*	France (Banyuls sur mer)	AY292948, DQ207625, DQ207632, DQ207635
V. fischeri SL518	*Sepiola ligulata*	France (Banyuls sur mer)	AY292950, DQ207626, DQ207633, DQ207641
V. fischeri MJ101	*Monocentrus japonicus*	Japan	AY292946, DQ207624, DQ207631, DQ207640
V. fischeri MDR7	Free living	USA (Marina del Rey, CA)	AY292945, DQ207623, DQ207630, DQ207639
Photobacterium leiognathi RM1	*Rondeletiola minor*	France (Banyuls sur mer)	AY292947, –, –, –

Table 2. Primers used in this study for amplification of *pil* loci

Primers designed in this study were based on the *V. fischeri* ES114 genome sequence, as described in the text.

Primer	Sequence (5′–3′)	T_m (°C)	Reference
ESpilA47	GCAGAGCTCATATTAAAGCTCCTTCGTG	64.6	Stabb & Ruby (2003)
ESpilA48	CGTCGTATTGAGGCGATGCGTGAG	70.7	Stabb & Ruby (2003)
PilC2	CCAACTTGAGGGCTTGCACAATGG	66.3	This study
PilC3	GTAACGAGTGTACGGGCGGCTATT	66.3	This study
JBSpilB F	GCACACCAACCTCGCCTC	59.3	This study
JBSpilB R	GAGCACGGTTTAGTTCTTG	51.9	This study
JBSpilC F	GCTTTCAATGGCGTGGC	55.6	This study
JBSpilC R	GCCAATAACACTCATAAG	45.7	This study
JBSpilD F	GATTATTACCCTTGGCTT	41.7	This study
JBSpilD R	GTGGCAAACCTAGAATG	48.0	This study
pilC 3′1	CCGCCGGTAATCTTTACTATCAAGAAGCCA	63.7	This study
5′1pilC	TGAACAACWGGAACACCTGAGGCGAGC	64.1	This study
pilB1-175	GAACTCCACTAGTTGCACTGAGTTAAAACT	60.7	This study
pilD1-3860	GCTATTGCTTGGGTTTGCCTATTATGG	65.1	This study
AP1	GTAATACGACTCACTATAGGC	59.0	BD Biosciences

0.1 mM spermidine), 2.0 µl sterile distilled water, 0.5 U polynucleotide kinase (1.0 µl) and 10 µl [γ-^{32}P]ATP (100 µCi; Amersham Quebec). Labelling reactions were incubated at 37 °C for 30 min. Membranes were hybridized overnight individually with each probe at the specific activity of 5×10^5 µCi ml^{-1} at 48 °C. The hybridization solution was discarded and the membranes were washed twice for 15 min each with washing buffer at 48 °C. Membranes were then exposed to X-ray film for 3 h to determine the presence of the *pil* operon.

Cloning and sequencing. Reactions that produced the desired PCR product were purified with the Geneclean II kit (Bio 101) and sequenced using Applied Biosystems Big Dye (version 3.1). PCR products that proved difficult to sequence directly were cloned into pGEM-T Easy vector (Promega) using the manufacturer's protocol. Plasmid DNA from positive clones was extracted using the Qiagen miniprep kit and sequenced. Excess fluorescently labelled dNTPs were removed via spin columns or plates (Edge Biosystems) and DNA was subsequently sequenced using an Applied Biosystems 3100 automated sequencer. Accession numbers for all sequences generated are listed in Table 1.

Gene walking to determine gene order. New primers corresponding to the 5′ and 3′ ends of each *pil* locus were designed using the derived sequences to link and determine the order of individual *pil* loci. Using the Universal Genome Walker kit (BD Biosciences), chromosomal DNA was digested separately with four restriction enzymes, *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. The reactions were purified with 100 % saturated phenol (pH 8.0), with the upper aqueous layer re-extracted with chloroform. DNA was precipitated with 2 vols cold 95 % ethanol, 20 µg glycogen and 0.1 vols 3 M sodium acetate. The mixture was centrifuged at 12 000 ***g*** for 10 min and dissolved in TE (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5).

Ligation reactions included 4 µl digested DNA, 25 µM GenomeWalker adaptor, 1.6 µl ligation buffer and 0.5 µl (3 U) T4 DNA ligase. Reactions were performed overnight at 16 °C in a DNA Engine Dyad (MJ Research). Samples were diluted 1 : 100 with sterile distilled water and used for PCRs. PCR was performed using Herculase *Taq* DNA polymerase (Stratagene), AP1 primer and individual *pilC* primers (Table 2). The PCR was completed in 25 µl reaction volumes containing 2.5 µl 10× reaction buffer, 0.2 µl dNTPs (25 mM each), 0.5 µg each primer and 1.25 U Herculase *Taq* polymerase. The following conditions were followed: seven cycles at 94 °C for 25 s and the primer T_m for 3 min; 32 cycles of 94 °C for 25 s and the primer T_m minus 3 °C for 3 min and a final extension at 61 °C for 7 min. PCR products were purified using Geneclean II and sequenced directly (ABI Big Dye v. 3.1) or cloned into pGEM-T Easy vector as described above. Fluorescently labelled products were purified using Edge DTR columns (Edge Biosystems) and analysed on an ABI 3100 automated sequencer.

Sequence alignment and phylogenetic analyses. Double-stranded DNA sequences were assembled and edited using the program Sequencher version 4.0 (Gene Codes). Sequences were

Table 3. PCR conditions used in this study

Gene target	Cycles (*n*)	Temperature (°C)		
		Denaturing	Annealing	Extension
pilA	35	94	58	72
pilB	30	94	49	72
pilC (primers PilC2 and PilC3)	30	94	53	72
pilC (other primers)	30	94	43	72
pilD	30	94	43	72

translated to determine the correct reading frame and promoter region to establish whether the genes were in one continuous operon. Comparison between strains was essential, since the arrangement of *pil* genes was not universal among all *V. fischeri* strains tested, as well as the presence of all loci. Each sequence was compared with the NCBI BLASTX search engine to determine similarity to other available *pil* sequences.

Molecular data were analysed with the computer program POY (Wheeler *et al.*, 2002) using the direct optimization method, with parsimony as the optimality criterion (Wheeler, 1996). This method assesses the number of DNA sequence transformations (evolutionary events) required by a phylogenetic topology directly, without the use of multiple sequence alignment. This is accomplished by generalization of existing character optimization procedures, including insertion and deletion events (indels) in addition to base substitutions. This method treats indels as processes, as opposed to the patterns implied by multiple sequence alignment (Wheeler, 1995).

Nodal support was calculated in POY using Farris's parsimony jackknifing procedure (Farris *et al.*, 1996) for 100 replicates (Using the commands –jackboot –replicates 100). Tree searches were conducted in parallel at Harvard University on a 19 dual-processor cluster using pvm (parallel virtual machine). Commands for load balancing of spawned jobs were used to optimize parallelization procedures (–parallel –dpm –jobspernode 2). Trees were built via a random addition sequence procedure (10 replicates) followed by a combination of branch-swapping steps [SPR (subtree pruning and regrafting) and TBR (tree bisection and reconnection)] and tree fusing (Goloboff, 1999) in order to improve tree length minimization further. Discrepancies between heuristic and actual tree length calculations were addressed by adjusting slop values (–slop5 –checkslop10). Phylogenetic trees were obtained using parsimony with a gap/ts/tv cost of equal weighting. The final tree was drawn with TreeView (Win32) and consensus trees were analysed in PAUP version 4.02b (Swofford, 2002). Nodal support for all jackknife calculations was performed in POY by the procedure described in Nishiguchi & Nair (2003). Individual gene trees for each of the *pil* loci were produced, as well as total molecular trees combining all *pil* loci and all *pil* loci with the 16S rRNA gene.

RESULTS AND DISCUSSION

Analysis of *pil* gene order and content

The amplified *pilA* gene from *V. fischeri* ESP915 was used as a probe (designed in this study) and hybridized to 2.5 kb fragments from *V. fischeri* strains EM17, ES114, ET101, SA1G and SR5 using Southern blot verification. This demonstrated the presence of a homologous *pilA* gene in those strains. In addition, a 4.5–5.0 kb band was observed in *V. fischeri* strains EB12 and WH1 and *Vibrio logei* strain SR18-1 (Fig. 1). This is expected, since previous evidence had demonstrated that the *V. fischeri* genome contains ten individual clusters of *pil* genes, of which two are *pilA* homologues (Ruby *et al.*, 2005). Although similarities existed among the symbiotic strains of *V. fischeri* probed, no hybridization occurred with DNA from either *Vibrio harveyi* ATCC 14126^T or *Vibrio cholerae* RGELP40. This may be due to the fact that both *V. cholerae* and *V. harveyi* are pathogenic bacteria, and may have evolved a different pilin structure necessary for the infection of their respective host tissues. In a phylogenetic context, *V. cholerae* and *V. harveyi* are not closely related to one another or to *V. fischeri*, which again provides evidence that colonization and infection mechanisms have evolved multiple times within the *Vibrionaceae* (Nishiguchi & Nair, 2003; Thompson *et al.*, 2004a).

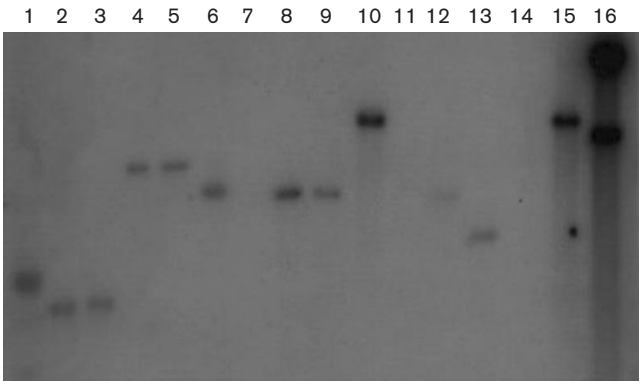


Fig. 1. Southern blot of *Hin*dIII digests probed with the *pilA* gene from *V. fischeri* ESP915. Lanes: 1, 1 kb ladder; 2, *V. fischeri* EB12; 3, *V. fischeri* EM17; 4, *V. fischeri* ES114; 5, *V. fischeri* ET101; 6, *V. fischeri* SA1G; 7, *V. logei* SR18-1; 8, *V. fischeri* SR5; 9, *V. fischeri* ESP915; 10, *V. harveyi* ATCC 14126^T; 11, *Photobacterium leiognathi* RM1; 12, *V. fischeri* WH1; 13, *V. parahaemolyticus* ATCC 17802^T; 14, *V. logei* ATCC 35077; 15, *E. coli* O157 : H7-84/24; 16, *V. cholerae* RGELP40.

Availability of the *V. fischeri* ES114 genome sequence also enabled the design of primers for the *pilB*, *pilC* and *pilD* loci. Targeted genes were amplified as well as an additional 350 bp from *V. fischeri* strains ET101, ESP915 and WH1. *V. fischeri* EB12 produced an additional 650 bp fragment, while *V. fischeri* strains ET101, SA1G, SA1J and SI1E produced an additional 550 bp band with the JBSpilB primers (Table 2). All *V. fischeri* strains except for ATCC 7744, a free-living isolate, were successfully amplified with the JBSpilC primers. *Vibrio parahaemolyticus* ATCC 17802^T, *Photobacterium phosphoreum* PP21 and *V. fischeri* ATCC 7744 yielded a smaller 850 bp fragment, with sequence similarity to other *pilB* sequences. All of these vibrios as well as *V. fischeri* ATCC 7744 cannot colonize squid light organs (M. K. Nishiguchi, unpublished) and may therefore be missing an important functional group of the *pil* gene cluster that enables symbiotic bacteria to colonize animal tissues successfully.

When comparing all *V. fischeri* DNA sequences from this study, it was noted that all sequences were A+T rich (61 mol%). For the *pilA* locus, a 740 bp fragment was amplified and sequenced from all bacteria. BLASTX demonstrated that the initial 502 bp was related to *pilA* and the remaining 309 bp was homologous to the polar flagellar sequence of other vibrios, with the only exception being *V. harveyi*. CLUSTAL W alignment demonstrated that *pilA* was 86–95 % conserved in nucleotide sequence among all strains examined in this study. A multiple alignment of

the in-frame amino acid sequences exhibited the same conservation. Results for the *pilB* comparisons gave ⩾93 % similarity. This was not surprising, since previous evidence has demonstrated that *pilA* can be replaced by corresponding genes from non-transformable species (Graupner *et al.*, 2000). The *pilA* locus is a pre-pilin gene that is flanked by other *pil*-like genes in numerous Gram-negative bacteria but, in the *V. fischeri* strains examined, no other *pil*-like genes were found in the vicinity (Stabb & Ruby, 2003). From our genome-walking experiments, only proteins whose functions are unknown (as determined for the *V. fischeri* ES114 genome; Ruby *et al.*, 2005) were within the 1000 bp region examined. Previous studies in other Gram-negative bacteria have indicated that the *pilA* and *pilD* genes are 100 % conserved, with variation observed most widely at the *pilC* locus (Fullner & Mekalanos, 1999). We found that the *pilC* genes sequenced in this study were 94–97 % similar, with the exception of *V. fischeri* WH1 and *V. fischeri* MDR7, both of which are free-living isolates (100 % sequence similarity to each other at the *pilC* locus, but less than 90 % similarity to symbiotic isolates). *pilC* has been one of the *pil* loci identified as critical for adhesin in the binding of *Neisseria gonorrhoeae* to human epithelial cells (Rudel *et al.*, 1995b). Likewise, a *pilC* gene homologue has also been observed in *Pseudomonas aeruginosa* and has been linked to virulence in this species (Rudel *et al.*, 1995a; Alm *et al.*, 1996), suggesting that the *pilC* gene is important for the infectivity of these pathogenic bacteria. Interestingly, neither of our free-living strains (WH1 and MDR7) can colonize or persist inside squid light organs, and the 10 % sequence difference observed at this locus may account for a loss of symbiotic competence. Future experiments to complement free-living *V. fischeri* strains such as WH1 and MDR7 with *pil* genes may help to determine whether improved colonization can be achieved with this operon alone, or whether other factors may be responsible for colonization and persistence in squid light organs.

The order of *pil* genes in the *V. fischeri* strains examined also differed from pathogenic *V. cholerae* and *V. parahaemolyticus*. *pilA* was determined to be monocistronic in *V. fischeri* and was separated from the other *pil* loci, which were present downstream and not in one continuous reading frame (Fig. 2). This is different from *pilA* found in both *V. cholerae* and *V. parahaemolyticus*. *pilB* (1674 bp) and *pilC* (1239 bp) were both translated in the second reading frame, while *pilD* (933 bp) was translated in the first reading frame. There were 19 bases between *pilB* and *pilC* (the pilin assembly/biogenesis protein), 59 bases between *pilC* and *pilD* (the pilin peptidase), a 24 base overlap of the dephospho-CoA kinase (CoA, or *yacE* in *V. cholerae*) and 6 bases between *yacE* and a conserved hypothetical protein (HP) when compared to the *V. fischeri* ES114 genome. Using PsortB (http://psort.nibb.ac.jp/form.html), the following predictions were made for the *V. fischeri* strains examined in this study: (i) PilB has the conformation of a cytoplasmic protein, (ii) PilC is similar to an internal membrane protein that contains four internal helices and (iii) PilD has the conformation of an inner-membrane protein (Pepe & Lory, 1998). Additionally, a fragment which proved to be homologous to the membrane-bound phosphotransferase system (*pts*), which phosphorylates the *manXYZ* operon (Erni & Zanolari, 1985), was found to follow the hypothetical protein (not shown).

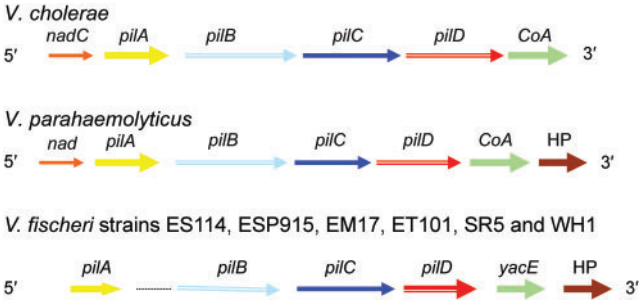


Fig. 2. Gene order of each of the *pil* operons from five symbiotic (ES114, ES915, EM17, ET101 and SR5) and one free-living (WH1) strain of *V. fischeri* compared with *V. cholerae* RGELP40 and *V. parahaemolyticus* ATCC 14126^T. Sequences of ~5 kb were utilized to construct the gene maps for all strains.

Phylogeny of symbiotic vibrios using the *pil* operon

All individual and total molecular gene trees were outgrouped with *V. fischeri* WH1 and MDR7, both of which are free-living isolates. We did not use *V. fischeri* ATCC 7744 as a free-living outgroup, since earlier studies suggested that this strain was divergent from the other *V. fischeri* strains used in this study (Nishiguchi & Nair, 2003). Phylogenetic trees based on individual *pil* loci produced phylogenies that were similar to each other, with most Indo-west Pacific vibrios ('E' strains) grouping together (Fig. 3). This was expected from our sequence analysis, since individual sequences for each locus were highly conserved with little variation. The two symbiotic fish strains (MJ101 and CG101) did not group together in all three individual gene analyses, or in the combined molecular analysis of all *pil* loci (Fig. 4). The three Mediterranean strains ('S' strains) analysed in our study were also included in clades containing fish and squid 'E' strains (Fig. 3). Thus, no geographical or host separation was made evident by any single gene phylogeny or the combined phylogenetic analysis of the entire *pil* gene operon (Fig. 4). The combined *pil* gene analysis (*pilB*, *pilC* and *pilD*) did group all ET and ES strains into a single clade, but this clade also included *V. fischeri* SR5 (Mediterranean) and ATCC 7744 (Fig. 4). A combined analysis of *pilB*, *pilC*, *pilD* and the 16S rRNA locus also indicated that all ET and ES strains were grouped within one clade (again including SR5 and ATCC 7744; Fig. 5).

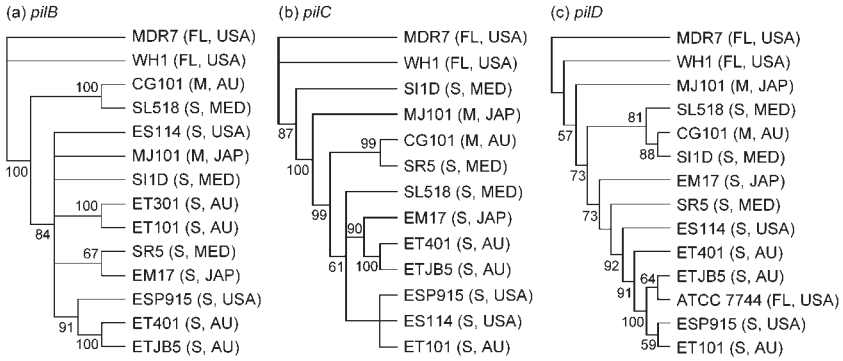


Fig. 3. Gene trees for *pilB* (a), *pilC* (b) and *pilD* (c) for equal-weighted parameter sets (indels/is/tv) that minimized overall incongruence using POY with parsimony as the criterion. Jacknife values >50 % are shown at nodes. Outgroups are *V. fischeri* strains MDR7 and WH1 (free-living). FL, Free-living; S, sepiolid symbiont; M, monocentrid symbiont; USA, United States isolate; AU, Australian isolate; MED, Mediterranean Sea isolate; JAP, Japanese isolate. See Table 1 for sequence accession numbers.

Both combined tree analyses verify previous work using haplotype comparisons and nested clade analysis that demonstrated that ET and ES strains are closely related to one another and occupy populations of *Euprymna* that span a large geographical range (Hawaii, Australia and Thailand; Jones *et al.*, 2006). *Vibrio* strains did not cluster together based on geographical location or host specificity, as hypothesized previously based on competition experiments (Nishiguchi *et al.*, 1998; Nishiguchi, 2002). However, these earlier studies were based on a single clone isolated from an individual squid host and were not representative of the large genetic breadth exhibited by *Vibrio* (Thompson *et al.*, 2004b; Thompson & Swings, 2006). Likewise, with more *Vibrio* genomes becoming available, there is growing evidence that horizontal gene transfer events and the number of increased mutation events (particularly those *Vibrio* species that are subject to host control, such as *V. fischeri*) are responsible for the rapid radiation of this dynamic group of bacteria. Recent work has demonstrated that vibrios have the capability to expand their environmental niches (both free-living and symbiotic; Jones *et al.*, 2006), which may explain why so many symbiotic *V. fischeri* strains have the capability to infect other species of host squid (Nishiguchi, 2002). In addition, recent work has demonstrated that *V. harveyi* is also capable of infecting and colonizing the light organs of loliginid squids (Guerrero-Ferreira & Nishiguchi, 2007), which exemplifies how *Vibrio* bacteria are adept at expanding into new ecological niches. The fact that *V. fischeri* and *V. logei* had previously been identified as the only two species of light-organ-inhabiting bacteria illustrates how little we know about the vast number of ecological niches that are available to vibrios and whether they are more susceptible to environmental selection or horizontal gene transfer events that may lead to incongruence in phylogenetic topologies (Thompson *et al.*, 2004b). Although this study was focused mainly on determining whether pilin gene sequences could distinguish between closely related *V. fischeri* strains, further studies need to be completed that will determine whether changes in any of the *pil* genes influence colonization specificity observed among closely related *Vibrio* sym-

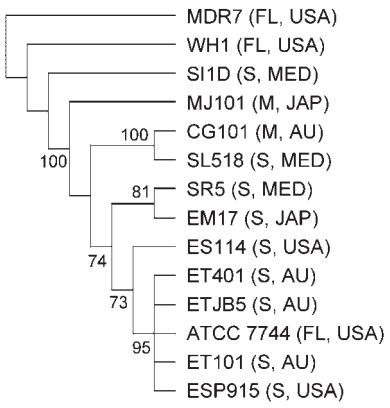


Fig. 4. Total combined *pil* loci (*B*, *C* and *D*) tree for equal-weighted parameter set (indels/ts/tv) that minimized overall incongruence of the three genes using POY with parsimony as the criterion. See legend to Fig. 3 for further details.

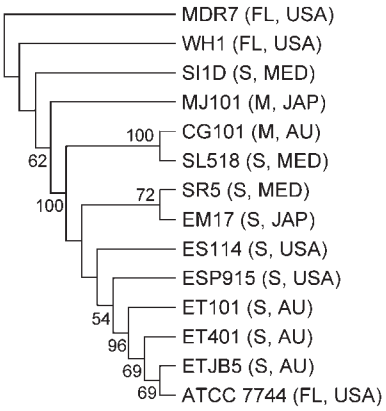


Fig. 5. Total molecular tree combining all *pil* loci (*B*, *C* and *D*) and the 16S rRNA locus for equal-weighted parameter set (indels/ts/tv) that minimized the overall incongruence of the four genes using POY with parsimony as the criterion. See legend to Fig. 3 for further details.

bionts. This would include constructing mutations within the *pil* genes to determine how deletions will affect adhesion and colonization specificity. Also, *in vivo* adherence assays of mutant *V. fischeri* strains to light organ epithelia cells would determine whether a change in colonization efficiency between wild-type and mutant vibrios is a main factor for driving recognition among closely related host–symbiont pairs. Whether variability between these genes is caused by rapid evolution, host selection or other gene transfer events remains to be determined and will provide yet another window into the evolution of this interesting group of gammaproteobacteria.

ACKNOWLEDGEMENTS

The authors would like to thank E. Stabb for advice on *pilA* primers in this project. R. DeVinney provided our *E. coli* strains used as controls in this project. This research was supported by grant SO6-GM0813626 from the National Institutes of Health and NSF SBE-0123690 and DEB-0316516 to M. K. N. We would like to thank the support of undergraduates Kitty Brown, Rebecca Lopez, Jonathan Clyde and Charity Watching, who were supported by either NIH-MARC (GM0766724) or NIH-BRIDGES (GM48998).

REFERENCES

Alm, R. A., Hallinan, J. P., Watson, A. A. & Mattick, J. S. (1996). Fimbrial biogenesis genes of *Pseudomonas aeruginosa*: *pilW* and *pilX* increase the similarity of type 4 fimbriae to the GSP protein-secretion systems and pilY1 encodes a gonococcal PilC homologue. *Mol Microbiol* **22**, 161–173.

Darzins, A. & Russell, M. A. (1997). Molecular genetic analysis of type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system – a review. *Gene* **192**, 109–115.

Erni, B. & Zanolari, B. (1985). The mannose-permease of the bacterial phosphotransferase system. Gene cloning and purification of the enzyme IIMan/IIIMan complex of *Escherichia coli*. *J Biol Chem* **260**, 15495–15503.

Farris, J. S., Albert, V. A., Källersjö, M., Lipscomb, D. & Kluge, A. G. (1996). Parsimony jackknifing outperforms neighbor-joining. *Cladistics* **12**, 99–124.

Fullner, K. J. & Mekalanos, J. J. (1999). Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae*. *Infect Immun* **67**, 1393–1404.

Goloboff, P. A. (1999). Analyzing large data sets in reasonable times: solutions for composite optima. *Cladistics* **15**, 415–428.

Graf, J., Dunlap, P. V. & Ruby, E. G. (1994). Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. *J Bacteriol* **176**, 6986–6991.

Graupner, S., Frey, V., Hashemi, R., Lorenz, M. G., Brandes, G. & Wackernagel, W. (2000). Type IV pilus genes *pilA* and *pilC* of *Pseudomonas stutzeri* are required for natural genetic transformation, and *pilA* can be replaced by corresponding genes from nontransformable species. *J Bacteriol* **182**, 2184–2190.

Guerrero-Ferreira, R. C. & Nishiguchi, M. K. (2007). Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis*, *Loliolus* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* **23**, 497–506.

Jones, B. & Nishiguchi, M. (2004). Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* **144**, 1151–1155.

Jones, B. W., Huttenberg, J., Lopez, J. E. & Nishiguchi, M. K. (2006). Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol Ecol* **15**, 4317–4329.

McFall-Ngai, M. J. (1994). Animal-bacterial interactions in the early life history of marine invertebrates: the *Euprymna scolopes*/*Vibrio fischeri* symbiosis. *Am Zool* **34**, 554–561.

McFall-Ngai, M. J. & Ruby, E. G. (1998). Sepiolids and vibrios: when first they meet. *Bioscience* **48**, 257–265.

Millikan, D. S. & Ruby, E. G. (2004). *Vibrio fischeri* flagellin A is essential for normal motility and for symbiotic competence during initial squid light organ colonization. *J Bacteriol* **186**, 4315–4325.

Nair, V. S. (2006). *The evolution of symbiosis in Vibrionaceae and factors influencing host colonization in a squid-Vibrio mutualism*. PhD dissertation, New Mexico State University, Las Cruces, NM, USA.

Nishiguchi, M. K. (2000). Temperature affects species distribution in symbiotic populations of *Vibrio* spp. *Appl Environ Microbiol* **66**, 3550–3555.

Nishiguchi, M. K. (2002). Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. *Microb Ecol* **44**, 10–18.

Nishiguchi, M. K. & Nair, V. S. (2003). Evolution of symbiosis in the *Vibrionaceae*: a combined approach using molecules and physiology. *Int J Syst Evol Microbiol* **53**, 2019–2026.

Nishiguchi, M. K., Ruby, E. G. & McFall-Ngai, M. J. (1998). Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-*Vibrio* symbioses. *Appl Environ Microbiol* **64**, 3209–3213.

Nunn, D. (1999). Bacterial type II protein export and pilus biogenesis: more than just homologies? *Trends Cell Biol* **9**, 402–408.

Nyholm, S. V. & McFall-Ngai, M. J. (2004). The winnowing: establishing the squid-*Vibrio* symbiosis. *Nat Rev Microbiol* **2**, 632–642.

Pepe, J. C. & Lory, S. (1998). Amino acid substitutions in PilD, a bifunctional enzyme of *Pseudomonas aeruginosa*. Effect on leader peptidase and N-methyltransferase activities *in vitro* and *in vivo*. *J Biol Chem* **273**, 19120–19129.

Ruby, E. G., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., Lostroh, P., Lupp, C., McCann, J. & other authors (2005). Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc Natl Acad Sci U S A* **102**, 3004–3009.

Rudel, T., Boxberger, H. J. & Meyer, T. F. (1995a). Pilus biogenesis and epithelial cell adherence of *Neisseria gonorrhoeae pilC* double knock-out mutants. *Mol Microbiol* **17**, 1057–1071.

Rudel, T., Facius, D., Barten, R., Scheuerpflug, I., Nonnenmacher, E. & Meyer, T. F. (1995b). Role of pili and the phase-variable PilC protein in natural competence for transformation of *Neisseria gonorrhoeae*. *Proc Natl Acad Sci U S A* **92**, 7986–7990.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sandkvist, M. (2001). Biology of type II secretion. *Mol Microbiol* **40**, 271–283.

Soto, G. E. & Hultgren, S. J. (1999). Bacterial adhesins: common themes and variations in architecture and assembly. *J Bacteriol* **181**, 1059–1071.

Stabb, E. V. & Ruby, E. G. (2003). Contribution of *pilA* to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. *Appl Environ Microbiol* **69**, 820–826.

Swofford, D. L. (2002). PAUP*: Phylogenetic analysis using parsimony (and other methods), version 4. Sunderland, MA: Sinauer Associates.

Thompson, F. L. & Swings, J. (2006). Taxonomy of the vibrios. In *The Biology of Vibrios*, pp. 29–43. Edited by F. L. Thompson, B. Austin & J. Swings. Washington, DC: American Society for Microbiology.

Thompson, C. C., Thompson, F. L., Vandemeulebroecke, K., Hoste, B., Dawyndt, P. & Swings, J. (2004a). Use of *recA* as an alternative phylogenetic marker in the family *Vibrionaceae*. *Int J Syst Evol Microbiol* **54**, 919–924.

Thompson, F. L., Iida, T. & Swings, J. (2004b). Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**, 403–431.

Villar, M. T., Hirschberg, R. L. & Schaefer, M. R. (2001). Role of the *Eikenella corrodens pilA* locus in pilus function and phase variation. *J Bacteriol* **183**, 55–62.

Visick, K. L. & Ruby, E. G. (2006). *Vibrio fischeri* and its host: it takes two to tango. *Curr Opin Microbiol* **9**, 632–638.

Wheeler, W. C. (1995). Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. *Syst Biol* **44**, 321–331.

Wheeler, W. (1996). Optimization alignment: the end of multiple sequence alignment in phylogenetics? *Cladistics* **12**, 1–9.

Wheeler, W. C., Gladstein, D. S. & DeLaet, J. (2002). POY: the optimization of alignment characters. New York: American Museum of Natural History.

Winther-Larsen, H. C., Hegge, F. T., Wolfgang, M., Hayes, S. F., van Putten, J. P. & Koomey, M. (2001). *Neisseria gonorrhoeae* PilV, a type IV pilus-associated protein essential to human epithelial cell adherence. *Proc Natl Acad Sci U S A* **98**, 15276–15281.

VIE ET MILIEU - LIFE AND ENVIRONMENT, 2008, 58 (2) : 87-106

DECIPHERING EVOLUTIONARY MECHANISMS BETWEEN MUTUALISTIC AND PATHOGENIC SYMBIOSES

M.K. NISHIGUCHI[1], A. M. HIRSCH[2], R. DEVINNEY[3], G. VEDANTAM[4], M.A. RILEY[5], L.M. MANSKY[6]*

[1]*Department of Biology, New Mexico State University, Box 30001 MSC 3AF, Las Cruces, NM 88003-8001, USA*
[2]*Department of Molecular, Cell and Developmental Biology, University of California, 405 Hilgard Ave., Los Angeles, CA 90095-1606, USA*
[3]*Department of Microbiology and Infectious Disease, University of Calgary, 3330 Hospital Dr. NW, Calgary, AB, T2N 4N1, Canada*
[4]*Department of Medicine, Section of Infectious Diseases, Loyola University Medical Center, 2160 S. First Ave., Maywood, IL, 60153, USA*
[5]*Department of Biology, University of Massachusetts Amherst, Amherst, MA 01003, USA*
[6]*Institute for Molecular Virology, University of Minnesota, 18-242 Moos Tower, 515 Delaware St. SE Minneapolis, MN 55455, USA*
[*]*Corresponding author: nish@nmsu.edu*

SYMBIOSIS
MUTUALISTIC
PATHOGENIC
EVOLUTION

ABSTRACT. – The continuum between mutualistic and pathogenic symbioses has been an underlying theme for understanding the evolution of infection and disease in a number of eukaryotic-microbe associations. The ability to monitor and then predict the spread of infectious diseases may depend upon our knowledge and capabilities of anticipating the behavior of virulent pathogens by studying related, benign symbioses. For instance, the ability of a symbiotic species to infect, colonize, and proliferate efficiently in a susceptible host will depend on a number of factors that influence both partners during the infection. Levels of virulence are not only affected by the genetic and phenotypic composite of the symbiont, but also the life history, mode(s) of transmission, and environmental factors that influence colonization, such as antibiotic treatment. Population dynamics of both host and symbiont, including densities, migration, as well as competition between symbionts will also affect infection rates of the pathogen as well as change the evolutionary dynamics between host and symbiont. It is therefore important to be able to compare the evolution of virulence between a wide range of mutualistic and pathogenic systems in order to determine when and where new infections might occur, and what conditions will render the pathogen ineffective. This perspective focuses on several symbiotic models that compare mutualistic associations to pathogenic forms and the questions posed regarding their evolution and radiation. A common theme among these systems is the prevailing concept of how heritable mutations can eventually lead to novel phenotypes and eventually new species.

INTRODUCTION

Because symbiotic systems exhibit a variety of behaviors ranging from mutualistic to pathogenic associations, general evolutionary principles can be expected to emerge from well-studied systems that can address fundamental mechanisms of specificity and recognition (Hirsch *et al*. 2003, Hirsch & McFall-Ngai 2000, Wilkinson & Parker 1996). Additionally, many animal-bacterial mutualisms have been used as models to study colonization and co-evolution, without the interference of tissue necrosis or cell mediated death due to virulence factors that are found in pathogenic associations (McFall-Ngai 2002, Wilkinson *et al*. 1996). Comparing closely related symbiotic bacteria, which have a wide range of host preference, specificity, and virulence, can infer the evolutionary relatedness of each bacterium/virus, as well as origins of pathogenicity islands, horizontal gene transfer of virulence factors, and colonization mechanisms that are shared features among each taxon (Andre *et al*. 2003, Nishiguchi & Nair 2003). Similarities that are associated with both benign and pathogenic associations can provide necessary information contributing to the basic knowledge of infectious associations, rather than specific disease entities. Therefore, once we understand how shared colonization strategies are used between closely related organisms, we may be able to determine whether virulence is derived from either a benign or more pathogenic form of symbiosis (Cooper *et al*. 2002).

Most symbiosis research has focused on specificity involved in each type of association. Three major stages are recognized that distinguish how each part of the symbiosis is unfolded: (1) the convergence of both host and symbiont, (2) infection and colonization, and finally (3) persistence. The initial stage involves the actual encounter between host and symbiont. Environmentally transmitted (that is, the symbiont is obtained from the environment where the host lives) symbioses entail a multitude of factors that have a major influence upon the actual infectivity of the symbiont and the susceptibility of the host. Initially, the host must be poised to accept the symbiont in its present state. Depending on the type of asso-

ciation, the age of the host (McFall-Ngai 1999), fitness, host size (Bates 2000), behavior (Secord 2001), and other additional factors can drastically change the dynamics of the association. At this point, abiotic factors also play an important role in determining whether the conditions for the actual infection can occur during contact. Such factors may include temperature, salinity, pH, ion concentration, geography, and other related micro-climate factors (Hentschel *et al*. 2000, Hirsch *et al*. 2003, McFadden *et al*. 1997, Nishiguchi 2000, Olafsen *et al*. 1993, Secord 2001, Soto *et al*. 2008a, b, Soto & Nishiguchi 2008). Finally, the symbiont has to be in an "infectious state"; if there are other biotic factors that prevent the symbiont from obtaining access to the host or inhibiting the symbiont (such as competition from other bacteria, inability to access the host, or repression of necessary "symbiotic factors" that are required for colonization), then the symbiosis cannot commence (Millikan & Ruby 2002, Nyholm & McFall-Ngai 2004, Silver *et al*. 2007b).

Once the partners have been united, then the onset of the association can begin. Whether it is a mutualistic or pathogenic association, there must be some specificity involved, which targets particular sites of infection or colonization. Most symbiotic associations with prokaryotes have very specific sites where the bacteria either enter the host or subsequently colonize tissue that harbor the symbiotic bacteria (Hirsch *et al*. 2001, Nyholm & McFall-Ngai 2004, Visick & McFall-Ngai 2000). There are also biotic factors that may induce the symbiosis to occur, such as interactions among other bacteria (Nyholm *et al*. 2002, Nyholm & McFall-Ngai 2003), specific chemical signals that may induce the bacteria to aggregate or adhere to specific sites on or around the host (Bassler 1999, Hirsch *et al*. 2003), and the induction of effector molecules that may interfere with host function (Finlay & Falkow 1997, Foster *et al*. 2000, Hueck 1998, Thomas & Finlay 2003). These types of interactions may be the initiation of what becomes either a "beneficial" association, or a deleterious one.

After infection and colonization has occurred, a number of host-mediated responses oftentimes follow. Whether the symbiosis is mutualistic or pathogenic, the partnerships have very similar routes of interplay. For example, many mutualistic and pathogenic bacteria have virulence or symbiosis factors that are only expressed when colonization has successfully occurred. These factors may include specific gene products that enable the symbiont to exploit host nutrients, metabolites or enzymes (produced by the symbiont that are beneficial to the host), or the production of toxins that enable symbiont transfer of nutrients to and from the host (Hentschel & Felbeck 1993, Pak & Jeon 1997, Sandstrom *et al*. 2000, Stabb *et al*. 2001). Genes induced by infection may also be expressed during initial stages to increase the interactions between host and symbiont (Braschler *et al*. 2003, Chun *et al*. 2006, Handfield *et al*. 2000, Lee & Camilli 2000, Smith 1998). Recently, many of the genes expressed upon symbiosis have been shown to contain regulatory elements that are only expressed when the bacteria have infected the host (Girardin *et al*. 2003, Lee *et al*. 1999, Millikan & Ruby 2003, Young *et al*. 1999). Similarly, the host has a multitude of responses, which are activated upon infection that either select which symbiont maintains the association (Koropatnick *et al*. 2007, Nishiguchi 2002, Silver *et al*. 2007a), or causes morphological or physiological alterations that enhance the partnership (Ben-Haim *et al*. 2003, Downie & Walker 1999, Koropatnick *et al*. 2004, Montgomery & McFall-Ngai 1994, 1995). In the most extreme cases, hosts can be detrimentally affected, with tissue necrosis or death as the end result of the infection. It is the carefully balanced liaison between maintaining a mutualistic association (beneficial), or extension into a pathogenic one that has intrigued scientists to study the similarities/differences between these symbioses, and whether they have independently co-evolved similar mechanisms. Deciphering similar infection mechanisms is also relevant to understanding how organisms can adapt to a specific host environment rapidly, and whether mechanisms such as horizontal gene transfer has some influence on the chimeric nature of such organisms. This perspective will attempt to bring together some common themes of symbiosis in relation to the evolution, radiation, and speciation among different groups of benign and pathogenic microbes. By comparing a number of well studied model systems, we hope to further our understanding of how complex interactions evolve, and whether these "evolutionary innovations (Margulis 1989, Sapp 1989)" can be thought of as a continuum of speciation.

Maintaining balance: Mutualistic associations between sepiolid squid and luminescent bacteria

Mutualistic associations between animals and their bacterial partners have been long studied in a number of model systems. A large portion of these studies focus on the evolutionary or ecological effects of how the association initially began, the specificity between host and symbiont, and whether this state of "even exchange" is a peaceful truce or one that requires constant "en garde" between the players. Sepiolid and loliginid squids (Cephalopoda: Sepiolidae and Loliginidae) are unique model hosts in that most species within the family have a monoculture of symbionts; that is, they usually maintain one phylotype or strain of luminescent bacteria (McFall-Ngai 1999, Ruby & McFall-Ngai 1999). These strains are of the family Vibrionaceae (Nishiguchi & Nair 2003, Ruimy *et al*. 1994) and have species that form symbiotic niches (pathogenic and mutualistic) with many eukaryotic partners (Colwell 1984, Guerrero & Nishiguchi 2007, Nishiguchi & Jones 2004). Generally, mutualisms involving *Vibrio* bacteria include the production of luminescence generated from the symbionts; this involves a series

of reactions through genes that are located in the *lux* operon (Nealson *et al*. 1981, Nealson & Hastings 1979, Nealson *et al*. 1970). In squid-*Vibrio* mutualisms, symbionts are housed in a bi-lobed or round light organ (Nishiguchi *et al*. 2004) and luminescence production is controlled by the host in a behavior known as counterillumination (Jones & Nishiguchi 2004). Both loliginid and sepiolid squids are known to contain bacteriogenic light organs (those that contain bacteria that produce bioluminescence), and have evolved a highly regulated sequence of events that produce a tightly coupled symbiosis, which allows both host and symbiont a means of increased fitness (Nishiguchi 2001).

In normal seawater, *Vibrio* bacteria number approximately 1 X 10^3-10^4/ml of seawater, and specifically, symbiotic *V. fischeri* comprise about 8% of total vibrios present (Jones *et al*. 2007, Lee & Ruby 1992, 1994b). Initially, symbiotic *Vibrio* bacteria must first locate and find the entrance to the light organ. During this time, squid hatchlings are induced by gram-negative bacteria to secrete mucus from this area, which are then recruited from the environment to the sites of infection (DeLoney-Marino *et al*. 2003, Nyholm *et al*. 2002, Nyholm & McFall-Ngai 2003). Once the bacteria are in contact with the mucus, they amass in dense aggregations, and only symbiotic *V. fischeri* dominate the population of bacteria found in the mucus aggregate which eventually infects the host (Nyholm & McFall-Ngai 2003, Nyholm *et al*. 2000). Symbiotic *V. fischeri* begin to increase in population size, and are able to out-compete any competitor (non-native symbiont) during the first 48 hours of colonization (Lee & Ruby 1994a, Nishiguchi *et al*.1998). It is yet undetermined whether symbiotic vibrios are better adapted to their specific hosts by unique recognition factors (Graf *et al*. 1994, Graf & Ruby 2000, Millikan & Ruby 2002, 2003, Visick *et al*. 2000) or are influenced by abiotic factors such as temperature or nutrient limitation (DeLoney-Marino *et al*. 2003, Graf & Ruby 1998, Nishiguchi 2000, 2002, Soto *et al*. 2008a, b, Soto & Nishiguchi 2008). Such factors have played an important role in related pathogens, such as *Vibrio cholerae*, where outbreaks have been linked to increased water temperatures, or pollution (Colwell 1984).

Recognition and specificity

Since both pathogenic and mutualistic vibrios are found amongst a myriad of other Vibrionaceae genotypes, there are a number of mechanisms that may be responsible for the evolution of recognition and specificity in environmentally transmitted symbiosis (Visick & McFall-Ngai 2000). Theoretical predictions state that most symbiotic associations evolve between two or more competing strains, with partial ordering imposed based on the virulence of a dominant (native) strain being more virulent than a suppressed (non-native) strain (Frank 1996). It has also been observed that similar mechanisms exist between mutualistic and pathogenic associations, which facilitate successful colonization of the eukaryotic host (Hentschel *et al*. 2000, Reich & Schoolnik 1994, 1996). It has been commonly observed that both mutualistic and pathogenic bacteria "acclimatize the symbiotic niche", where they have evolved regulatory mechanisms which induce the host to be more accommodating to their arrival. This is important to the selection of more and more "adapted" strains, since those that cannot colonize or persist in the symbiosis will not be successful. Mechanisms which govern this adaptability in the squid-*Vibrio* mutualism include quorum sensing (Gilson *et al*. 1995, Lupp *et al*. 2003), two component regulatory mechanisms (Darnell *et al*. 2008, Geszvain & Visick 2008, Hussa *et al*. 2007, Visick & Skoufos 2001), cell signaling (Stabb *et al*. 2001), and the ability of differential adhesion to a particular host light organ (Hensey & McFall-Ngai 1992, Stabb & Ruby 2003). These mechanisms can also be found in closely related *Vibrio* pathogens, such as *V. cholerae* and *V. parahaemolyticus*, which have similarly related mechanisms that enable strains to infect and colonize their eukaryotic hosts (Colwell 1984, Mekalanos 1985, Nishibuchi & Kaper 1995, Reich *et al*. 1997, Reich & Schoolnik 1994, 1996, Sechi *et al*. 2000). Obviously, there are many more genes that are regulated both at onset and during colonization (Crookes *et al*. 2004, Davidson *et al*. 2004, Doino Lemus & McFall-Ngai 2000, Kimbell *et al*. 2006), and future research will aid in understanding how those mechanisms are similar among various symbiotic strains, whether they are pathogenic or mutualistic in origin.

Evolutionary consequences of environmentally transmitted symbiosis

Earlier studies of *Vibrio* symbionts and their sepiolid squid hosts have indicated that phylogenetic patterns of cospeciation exist among allopatric populations residing in the Indo-West Pacific (Kimbell *et al*. 2002, Nishiguchi 2001, Nishiguchi *et al*. 1998). Along with this, a competitive hierarchy was observed among symbionts that was congruent to host and symbiont phylogenies (Nishiguchi 2002, Nishiguchi *et al*. 1998). These congruencies demonstrated that native symbionts had a competitive advantage over non-native symbionts. It also suggests that all light organ symbionts tested had evolved independently from a free-living *Vibrio* strain, not from other host taxa living in the same environment. Since these allopatric populations have shown strain specificity (and possible speciation) among the Vibrionaceae found in environmental seawater, this brings to question whether sympatric populations are able to evolve the same specific mechanisms for recognition. More recent evidence has suggested that Vibrio strains are adapting at a much faster rate than their host squids, and are able to migrate large

distances via "leap frogging" between host populations (Jones *et al*. 2006). This generates doubt as to whether environmentally transmitted symbioses are strictly evolving with each other, but rather have ecological factors that also drive the symbiosis (Dunlap *et al*. 2007). Since sympatric symbionts oftentimes lack host fidelity or display host fidelity but use multiple hosts (Berlocher 1998, Lynch 1989, Nishiguchi 2000), examining patterns of cospeciation may help us understand whether speciation among prokaryotic partners is influenced by their direct environment (their host) or other extrinsic factors (Boucher & Stokes 2006). Previous work determining whether pathogenic *Vibrio* strains have a common ancestor with mutualistic strains show no clear pattern of a single radiation within the group (Nishiguchi & Nair 2003, Ruimy *et al*. 1994). Consequently, some patterns exist between symbiont strains that have common or related host species (Browne-Silva & Nishiguchi 2008, Nishiguchi & Nair 2003). Although similar mechanisms exist between mutualistic and pathogenic *Vibrio* strains, many of those mechanisms have been co-opted for other functions that may not induce tissue necrosis or other pathogenic interactions which result in damage or death of the host (Colwell 1984, Reich & Schoolnik 1994). Future studies will hope to enlighten whether multiple lineages of pathogenic and mutualistic genotypes of *Vibrio* have evolved under specific conditions, or, if they have been able to horizontally transmit any of these genes for infection and colonization. This would enable multiple strains of *Vibrio* to have similar genotypic mechanisms of infection, but because they are in a different genetic background, are incapable of expressing complete virulence (similar to *E. coli*, see below in later section). Determining if these chimeras exist, and whether this is another mechanism of pathogen evolution, still needs further attention.

Plant Symbioses: Altered States

Associations between eukaryotes and microbes exist as commensalisms, mutualisms, and parasitisms, with some well-known human pathogenic organisms, such as *Vibrio cholerae* or *Helicobacter pylori*, living as either commensals or parasites depending on the host (Lipp *et al*. 2002, Merrell & Falkow 2004). Rhizobia, some of the best-studied plant-associated bacteria, live as commensals on non-host plants and as mutualists with their legume hosts (Foster *et al*. 1983, Schwieger & Tebbe 2000). As mutualists, they induce, when soil nitrogen is in short supply, the formation of nitrogen-fixing nodules on the roots of their legume host (Lum & Hirsch 2003). This interaction involves signal exchange between the symbiont partners to ensure recognition and specificity. Rhizobia may also live as parasites in nodules (Denison & Kiers 2004, Kiers *et al*. 2003) although Timmers and coworkers (Timmers *et al*. 2000) defined this mildly parasitic interaction as a saphrophytic state. In any case, rhizobia appear to traverse the commensal-mutualist-parasitic continuum (Denison & Kiers 2004, Hirsch 2004).

Does understanding rhizobia's ability to exhibit alternative life styles (Denison & Kiers 2004) help us better understand rhizobial evolution? Rhizobia have been portrayed as "refined parasites", nodulation being described as a "beneficial plant disease" (Djordjevic *et al*. 1987, Vance 1983). Although many of the early stages of rhizobial entry into legume host cells resemble pathogenic events on plant hosts see references in McKhann & Hirsch 1994, Mithöfer 2002, rhizobial species are not related to plant pathogenic bacteria other than *Agrobacterium*. Indeed, except for *Sinorhizobium meliloti*, most rhizobial species are only distantly related to *Agrobacterium* (Goodner *et al*. 2001, Wood *et al*. 2001).

Moreover, *Agrobacterium* is an atypical plant pathogen; it does not elicit a disease in the classic sense, but rather a transformed tumor. In general, only a minimal amount of cellular damage occurs in tumors or in aborted or senescent nodules in contrast to most plant pathogen-elicited host defense responses (McKhann & Hirsch 1994, Mithöfer 2002). Nevertheless, several investigators have proposed that when the nitrogen-fixing symbiosis fails, rhizobia elicit a hypersensitive response (HR), the classic symptom of disease resistance.

Rhizobia *is not a pathogen*

Although rhizobia may initially live as parasites (or saprophytes) upon host entry or within ineffective nodules, they differ significantly from plant pathogenic bacteria, particularly those pathogens involved in cultivar-dependent host resistance, where the parallels to rhizobia-legume association are often drawn. In gene-for-gene resistance, the product of a dominant *R* (resistance) gene from the host was originally defined as a protein interacting with the product of the corresponding *avr* (avirulence) gene of the pathogen. The complementary combination of *R* and *avr* genetic backgrounds, i.e. recognition, would then bring about resistance via the HR, which is characterized by rapid, localized host cell death thereby limiting the pathogen's growth. In contrast, non-recognition brings about the disease. This is the exact opposite of what happens in the *Rhizobium*-legume symbiosis (Table I). However, the gene-for-gene model is much more complicated than when it was originally proposed and subsequently applied to the rhizobial-legume interaction. The *R* and *avr* players are constantly evolving-the pathogen to avoid detection and the plant to recognize the pathogen. Moreover, numerous proteins are involved in resistance, associating in multi-protein resistance systems that can yield new complexes as a result of rearrangement, recombination, transposon activity, and genome shuffling (see references in Friedman & Baker 2007). In addition, the *avr* gene products, also known as pathogen effectors, are now

believed to suppress basal resistance or PAMP-mediated immunity (PMI) and elicit effector-triggered immunity (ETI), i.e. the HR (Jones & Dangl 2006, Zipfel 2008).

Basal resistance, also known as cultivar-independent, or non-host resistance (Jones & Dangl 2006, Thordal-Christensen 2003, Zipfel 2008), commonly occurs between plants and pathogenic fungi or bacteria. Molecules derived from infectious microbes, such as flagellin, chitin, small peptides, glycans, and lipopolysaccharide (LPS) ("elicitors" in the plant pathology literature), are now known as Pathogen-Associated Molecular Patterns (PAMPs) or Microbe-Associated Molecular Patterns (MAMPs) and have been shown to interact with proteins named Pattern Recognition Receptors (PRRs) to generate innate immunity and disease resistance (Janeway 1989). Leucine-rich repeat receptor-like kinases (LRR-RLKs) in plants resemble the animal PRRs, e.g., Drosophila TOLL. Similar to animal innate immunity, a PRR (FLS2 protein from *Arabidopsis*) interacts with a PAMP (a 22 amino-acid epitope from bacterial flagellin) (Gómez-Gómez & Boller 2000), triggering a MAP kinase signaling cascade with similarity to signal transduction pathways in insects and mammals (Asai *et al*. 2002). Recognition between PRRs and PAMPs enables plants to develop resistance to many pathogens. Interestingly, although a peptide derived from flagellin from a pathogenic bacterium is recognized by FLS2 and triggers a host response, the conserved peptide, if derived from *Rhizobium*, does not bind to FLS2 or activate flagellin recognition (Felix *et al*. 1999). This indicates that the host perceives pathogenic versus symbiotic signal molecules (Symbiont-Associated Molecular Patterns; Hirsch 2004) differently. It further argues for the existence of PRR-type proteins that recognize these SAMPs. However, very few have been identified.

An important exception is Nod factor and the proteins that recognize it. Whereas plant pathogen-produced chitin, an *N*-acetylglucosamine polymer, serves as an elicitor of host defense. Nod factor, a substituted *N*-acetylglucosamine oligomer produced by rhizobia is vital for the establishment of the legume-rhizobial symbiosis (Table I). Recognition of the cognate Nod factor occurs via receptor proteins that interact with this SAMP in some yet unknown way. These proteins are essential for nodule development. NORK/SymRK (Nodule Receptor Kinase/ Symbiotic Receptor Kinase) was one of the first receptor proteins in the nodulation pathway to be identified and cloned (Endre *et al*. 2002, Stracke *et al*. 2002), but so far there is no evidence for Nod factor binding to NORK/ SymRK. Putative Nod factor binding proteins, predicted on the basis of genetics have been identified (Limpens *et al*. 2003, Madsen *et al*. 2003, Radutoiu *et al*. 2003). These proteins are serine/threonine RLKs with extracellular LysM domains and could be considered PRRs (Zipfel 2008). LysM domains are found in proteins involved in peptidoglycan and chitin binding, suggesting that these proteins bind Nod factor, but the critical experiments have not yet been performed. Additional downstream genes in the Nod factor-signaling pathway have been cloned and are the focus of many studies concerning nodule development.

In any case, rhizobia are not perceived as pathogens even when they lack the appropriate Nod factor to be recognized by plant receptor proteins. Moreover, if either cheaters or the wrong rhizobia enter the nodule, they elicit neither a disease nor an HR.

What happens when the symbiosis fails?

When the *Rhizobium*-legume symbiosis fails, an ineffective (Fix^-) nodule is the result. It is usually smaller than an effective (Fix^+) nodule, and white or green in color due to either the lack or degradation of leghemoglobin (Hirsch *et al*. 1992). Depending on the stage where the symbiosis fails, the nodule may show a histological organization similar to an effective nodule, but the bacteroid zone (zone III) of the nodule may be reduced in size.

Table I. - Similarities and differences between selected symbiotic and pathogenic bacteria-host interactions.

	***Rhizobium*-legume cell**	**Plant pathogen-host cell (cultivar dependent)**	***Brucella*-mammalian cell**
Recognition	Invagination of bacteria into membrane-bound compartment. Nodule development. Sustained infection.	Symptoms of host defense response, including localized accumulation of phenolics, PR proteins (a hypersensitive response). Lack of sustained infection.	Invagination of bacteria into membrane-bound compartment. Sustained Infection. Disease.
Non-recognition	No response or minimal symptoms of a host defense response. Lack of sustained infection.	Sustained infection. Disease.	Lack of sustained infection. Little or no disease.

Some nodules completely lack differentiated bacteroids (see Perotto *et al*. 1994), and in the indeterminate nodule type, a discrete, persistent nodule meristem may not be maintained (Hirsch *et al*. 1992).

Earlier, we had suggested that rhizobial entry into the plant cell was mechanistically more similar to how a pathogen such as *Yersinia* invades a mammalian cell (McKhann & Hirsch 1994). What is the evidence to support this hypothesis? There are surprising parallels between rhizobial entry into its host and that of a mammalian pathogen, such as *Salmonella* and *Yersinia*, which enter their hosts by membrane-mediated endocytosis. *Sinorhizobium meliloti*, a nitrogen-fixing endosymbiont, and *Brucella abortus*, which affects a broad spectrum of mammals, are both enclosed in acidified, host-derived membrane compartments, yielding either a populated nodule cell or a chronically infected mammalian cell. To establish this membrane-compartmentalized state, both bacteria employ the products of *bacA* genes, which are 68.2% identical between them (LeVier *et al*. 2000). The BacA protein has been shown to affect lipid-A fatty acids, which make up LPS (Ferguson *et al*. 2004). In a *bacA* mutant-elicited infection, the membrane compartments are not maintained, most likely because lipid A is altered. This results in the subsequent collapse of the infection in either the legume or mammalian cell (Table I). In this instance, the defective LPS functions as a PAMP, which is recognized by a specific PRR in the host (Ferguson *et al*. 2004). Recognition brings about innate immunity in the infected animal, or the legume version thereof in the case of the *Rhizobium bacA* mutant. The identity of a PRR that recognizes the defective LPS in either the animal or plant cell remains unknown.

By contrast, wild-type LPS must be recognized as a SAMP, which interacts with its corresponding PRR (Hirsch 2004). Alternatively, the SAMP and the PAMP could bind to the same receptor in an agonist/antagonist type of interaction (Mithöfer 2002), but trigger different downstream signal transduction pathways. Thus, when the nitrogen fixing symbiosis fails, because of the production of defective LPS or other PAMPs, a type of defense reaction akin to that observed in type I non-host resistance or in the mammalian cell may ensue. In any case, the plant response is not an HR, which requires a much higher threshold of defense response (Jones & Dangl 2006).

How do legumes differentiate friend (Rhizobium) from foe (pathogen) or cheater rhizobia (non-nitrogen fixers)?

For cheaters that enter nodules but do not fix nitrogen, recognition signals may protect against rhizobia that are not host-capable, but they do not protect the host from rhizobia that produce the correct Nod factor but are mutated in *nif* (nitrogenase) or other critical metabolic genes. Host-induced sanctions are proposed to select against freeloaders or cheater rhizobia, and experimental evidence supports this (Denison & Kiers 2004). However, many field nodules are likely to have multiple inhabitants, both friends that fix nitrogen and cheaters that do not. Under these conditions, host sanctions are unlikely because the plant cannot discriminate between the nitrogen fixer and non-fixer, especially if a sufficient level of fixed nitrogen is produced for the plant's survival.

In general, recognition of friend versus foe is still incompletely understood in plants. Many of the analyses regarding cultivar-dependent and culture-independent host resistance have been performed in *Arabidopsis*, which does not establish mutualistic associations with nitrogen-fixing rhizobia or with phosphate-acquiring mycorrhizal fungi. It is assumed that the legumes exhibit the same patterns of host resistance as *Arabidopsis* does, and some PRRs have been identified (see references in Zipfel 2008). In addition to cultivar-dependent and culture-independent host resistance, plants deal with pathogens by stopping them at their borders, employing such physical barriers as waxy, extracellular layers and rigid cell walls. Recently, root cap border cells have been evoked as a first line of defense against pathogenic organisms. Martha Hawes and her colleagues have unequivocally demonstrated that the pea root tip is protected from infection, and that this resistance is closely associated with the presence of border cells (Gunawardena & Hawes 2002). By attaching to border cells, fungi and other potential pathogens are discharged from the root surface leaving the root tip free of infection as the border cells are sloughed off. If the integrity of the root cap and border cells is breached, the root tip becomes infected. Experiments suggest that pea seed lectin, which is one of ca. 100 proteins detected in the root cap secretome, is responsible for agglutinating the pathogenic microbes and triggering the innate immunity pathway components, which are released into the secretome (Wen *et al*. 2007).

Interestingly, lectin has long been known as a recognition molecule in the *Rhizobium*-legume symbiosis (see references in Hirsch 1999). The original lectin recognition hypothesis was based on the strong correlation between the ability of legume host-produced lectin to bind to rhizobia that nodulated that host. Lectins are found in the right place, in roots and especially in root hairs, the sites where rhizobial bind. The recognition hypothesis was bolstered by the findings that transgenic legumes carrying an introduced lectin gene from another host were nodulated by the other host's rhizobia, but only if there was a match in Nod factors (Díaz *et al*. 1989, van Rhijn *et al*. 1998, 2001). Rhizobia are able to overcome the plant's basic lines of defense and can penetrate root hair cell walls and enter into root cells via infection threads initiated by endocytosis. Does the interaction between rhizobia and host-produced lectin somehow lead to the masking of determinants that would normally signal the presence of a foe? Or does lectin agglutination of rhizobia lead to a critical mass of bacterial cells that produce sufficient Nod

factor to overcome the host's recalcitrance to infection? The answers are so far unknown.

Final comments

Although there are numerous parallels between how rhizobia cells associate with their legume host versus how a plant pathogenic bacterial species interacts with its host, the outcomes are significantly different (Table I). It may be useful to focus on the parallels between virulent plant pathogens and benign plant symbionts, but it is extremely important to remember that incorporating information from such highly derived plant-microbe interactions into an evolutionary context can produce misleading conclusions. Cultivar-dependent host resistance is a tightly intertwined evolution of both host and pathogen; one changes to overcome the continual challenge by the other (Friedman & Baker 2007, Jones & Dangl 2006). Similarly, most of the *Rhizobium*-legume symbioses that have been investigated in detail are the narrow-host range ones between rhizobia and legume plants that are highly selected for agronomic performance. Many are highly specific with only one or two legume species nodulated by a particular rhizobial strain. The only broad host range rhizobial species that has been thoroughly investigated is *Rhizobium* NGR234, which nodulates ca. 50% of all legumes (Pueppke & Broughton 1999). Interestingly, some rhizobia including NGR234 (Marie *et al*. 2001, Viprey *et al*. 1998) use a Type 3 secretion system (T3SS), a protein injection system that is a hallmark for pathogenic bacteria, both of plants (Gürlebeck *et al*. 2006) and animals (Trosky *et al*. 2008). Proteins secreted through the T3SS by NGR234 make possible this rhizobial species' broad host range (Marie *et al*. 2001). More emphasis needs to be placed on symbioses that occur in nature, particularly those within the Caesalpinioideae, the most primitive group subfamily of the Fabaceae. For some of these legumes, no true nodules are formed although infection threads develop and nitrogen is fixed (de Faria *et al*. 2000).

It is also important to consider other models of prokaryotic-eukaryotic interaction in addition to plant pathogens. Pathogens that exhibit the stealth mode of assault into their mammalian hosts may be particularly good models for the rhizobia-legume symbiosis. Studies of *bacA* in *S. meliloti* and *B. abortus* discussed here have already shown a connection in how the infection is established. Like rhizobia, *Vibrio cholerae* occupies many ecological niches including multiple hosts, one to which it attaches and the other which it invades (Lipp *et al*. 2002). The difference between a competent pathogen or symbiont and an incompetent one is the expression of a set of virulence or symbiotic genes in association with the host.

Merrell & Falkow (2004) have argued that commensalism is the ground state for most human-associated bacteria, which acquire their pathogenic habit via horizontal gene transfer (HGT) and genetic recombination. Similarly, *Rhizobium* species can acquire by HGT genes that allow them to nodulate various host legumes and fix nitrogen within the nodule cells (Sullivan *et al*. 1995, Sullivan & Ronson 1998). However, sometimes the rhizobia are poorly effective or even ineffective after lateral transfer of a symbiotic island (Nandasena *et al*. 2006, 2007); these bacteria are cheaters. Because most studies evaluate symbiotic competence on the basis of whether nitrogen is fixed, strains defective in a single gene critical for nitrogen fixation could be considered symbiotically incompetent. Nevertheless, sanctions may not be imposed upon them if the plant is not starved for nitrogen.

Suffice it to say, as we learn more about the diversity of rhizobia-legume symbiosis, we will learn more about the altered states in which these organisms live. For example, additional similarities between mammalian pathogens and legume symbionts may become evident as studies proceed. The exploration of such convergences should provide a better understanding as to how this agriculturally and ecologically important mutualism between plants and bacteria evolved.

Evolution of virulence in attaching and effacing E. coli

Escherichia coli is a multi-faceted organism. It is an important member of the mammalian gastrointestinal microflora, and an essential tool for biochemical and genetic research. Although most strains are harmless commensals, pathogenic isolates exist, and cause a variety of diseases in human and animal hosts. Virulent *E. coli* strains can be divided into at least eight pathotypes based on clinical features, the epidemiology of infection, and virulence factors produced (Donnenberg & Whittam 2001). These include uropathogenic *E. coli*, an important cause of urinary tract infections, meningitis-associated *E. coli*, a cause of neonatal meningitis, and the diarrheagenic *E. coli* pathotypes which cause a diverse spectrum of gastrointestinal diseases (Nataro & Kaper 1998). Two diarrheal pathotypes, enterohemorrhagic *E. coli* (EHEC O157:H7) and enteropathogenic *E. coli* (EPEC) are members of a family of pathogens that share a common virulence mechanism, the formation of attaching and effacing (A/E) lesions. Recent studies have examined the similarities and differences between A/E family members, and have addressed the acquisition of virulence by these organisms.

EHEC and EPEC virulence determinants

The hallmark of infection by EHEC and EPEC is the formation of attaching and effacing lesions. Bacterial adhere intimately to intestinal epithelial cells, causing a dramatic rearrangement of the cytoskeleton, resulting in degeneration of the microvilli and the formation of actin-rich pedestal structures beneath the adhering bacteria (Knutton *et al*. 1989, Moon *et al*. 1983). Virulence is

mediated by a combination of common and pathotype-specific virulence factors. The major common virulence determinant is the chromosomal LEE (locus of enterocyte effacement) pathogenicity island. The G+C content is significantly lower than the region of the genome flanking the LEE, suggesting that it was acquired horizontally (McDaniel *et al*. 1995, Perna *et al*. 1998). The LEE contains genes required for A/E lesion formation, including a type III secretion system (TTSS), type III secreted effectors (Esps), and the bacterial ligand (intimin) and receptor (tir). EPEC and EHEC have evolved a remarkable mechanism for adherence and A/E lesion formation: They insert their own receptor, the bacterial protein Tir, into the plasma membrane using the TTSS. Tir then binds to the outer membrane protein intimin, resulting in A/E lesion formation (Deibel *et al*. 1998, DeVinney *et al*. 1999, Kenny *et al*., 1997). This mechanism allows EHEC and EPEC to adhere tightly to the host in the absence of a specific host cell receptor. In EPEC but not EHEC, the LEE is sufficient to confer *in vitro* A/E lesion formation activity to non-pathogenic *E. coli* K-12 isolates (Elliott *et al*. 1999, McDaniel & Kaper 1997). Whether the LEE region alone is sufficient to convert K-12 from an avirulent strain to an EPEC-like pathogen is unknown, but highly unlikely due to the requirement for pathotype-specific virulence determinants.

In addition to the LEE, both EPEC and EHEC express pathotype-specific virulence determinants. EPEC contains a large plasmid (pEAF) that contains genes encoding a type IV bundle-forming pilus (BFP) (Sohel *et al*. 1996). The BPF is involved in both the formation of bacterial microcolonies and non-intimate adherence to the intestinal epithelia, and is essential for full EPEC virulence (Bieber *et al*. 1998). EHEC isolates are defined by the production of shiga toxins and the absence of the BFP (Nataro & Kaper 1998). The two shiga toxin subtypes (STX1 and STX2) are encoded on lysogenic phages, and inhibit host cell protein synthesis, resulting in severe gastroenterits and systemic disease. EHEC and EPEC are also distinguished by their host specificity and particular niche within the gastrointestinal tract. EPEC is predominantly a pediatric pathogen, and colonizes the small intestine (Nataro & Kaper 1998). In contrast, EHEC colonizes the large intestine of humans of all ages and ruminants. In humans, EHEC is a pathogen, whereas it can asymptomatically colonize the lower GI tract. Whether this interaction is truly mutualistic is controversial. Although colonization does not result in disease, the interaction is not benign, as EHEC can cause A/E lesions in adult cattle and animals and mount an immune response to STX.

Acquisition of virulence determinants by EPEC and EHEC

An intriguing question is how did virulence arise in *E. coli*? Although numerous possibilities present themselves, experimental data suggests two different mechanisms. Work from Reid and colleagues suggest a stepwise acquisition of virulence, based on multi-locus sequence analysis and the distribution of virulence determinants within 21 different isolates (Reid *et al*. 2000). On the basis of a rate of d_s of $4.7x10^9$/site/year, the authors suggest the radiation of clones began 9 million years ago, and that EHEC O157:H7 and *E. coli* K12 separated from a common ancestor 4.5 million years ago. The authors suggest a stepwise and additive acquisition of virulence factors, with the repeated gain and loss of genes over time. This process began with the insertion of the LEE pathogenicity island in the EPEC and EHEC chromosomes. Acquisition of the LEE is thought to have occurred several times in parallel, with insertion occurring in different chromosomal locations (predominantly pheU and selC tRNA genes) generating distinct clonal lineages. Comparison of the genes encoded by the EHEC O157: H7 and EPEC LEE identified regions with both high sequence similarity and variability (Perna *et al*. 1998). The genes encoding structural components of the TTSS showed low rates of both d_S and d_N substitution, whereas genes encoding Tir, intimin and the type III secreted effectors were highly variable, with more gaps and an increase in both d_S and d_N. This is consistent with observations that genes encoding the secretory apparatus are well conserved across the various bacterial species that express TTSS, but that the secreted effectors are highly variable (Hueck 1998). This also suggests that recombination and mutation occurred within the Tir/intimin and effector genes, (allowing fine tuning for differences in host/lifestyle). Both the EPEC and EHEC lineages subsequently acquired pathotype specific virulence factors, including the genes encoding Shiga toxins and the pO157 plasmid by EHEC, and the plasmid encoding the BFP by EPEC. These factors most likely contribute to differences in host specificity, and the enhanced virulence observed with STX-producing EHEC strains.

A second model suggests that the LEE may have been acquired in multiple steps, rather than by the horizontal transfer of one large region of DNA (Sandner *et al*. 2001). The authors examined the prevalence of LEE-encoded genes for Tir and its chaperone CesT, the type III secreted protein EspB, and the operons encoding the structural subunits of the TTSS, in *E. coli* strains isolated from a variety of species of wild mammals. Markers for the LEE were found in 40% of the strains tested, but surprisingly were not always found together in a given strain. These data suggest that the LEE is a dynamic region, and that the LEE-encoded genes may have other functions when expressed individually in nonpathogenic bacteria. Pathogenic *E. coli* may have acquired LEE-encoded genes by horizontal gene transfer, which were then assembled into a pathogenicity island. A second explanation for these data suggests a loss of virulence by commensal *E. coli*. The LEE may have been initially acquired in its entirety,

and some LEE-encoded genes lost over time through rearrangement and deletion. A compelling question is whether the isolated LEE genes found in wild *E. coli* strains are still functional, and if so, do they play a role in a more mutualistic lifestyle? These questions might be further addressed through additional comparisons of genomes from a compliment of both mutualistic and pathogenic *E. coli* strains. Future research comparing a wider array of *E. coli* genomes may provide further insight into how virulence and pathogenicity islands radiated throughout *E. coli* phylotypes.

Conjugation in Bacteroides *species: A paradigm of efficiency*

Bacteria have evolved sophisticated mechanisms to survive in their environment, and the ability to acquire DNA from the environment confers a selective advantage to many bacteria (Moore & Holdeman 1974, Wang *et al*. 2003). The most efficient method of DNA acquisition is by conjugation, where DNA is transferred from a donor to a recipient bacterium that are in close physical contact (horizontal gene transfer). A large size range of DNA can be transferred by conjugation, including small genes, plasmids and transposons, and even pathogenicity islands. Inter-generic transfer is common, and conjugation-proficient bacteria can acquire genetic material from diverse and frequently, unrelated donors. Conjugation is thus considered a fast and efficient pathway for bacterial evolution.

Members of the genus *Bacteroides* are part of normal human gut flora, and are one of the major anaerobic genera in the colon. *Bacteroides* organisms can grow to high cell density, and are often present at 10^{10}-10^{12} colony forming units per gram fecal matter (approximately 30% by weight of fecal matter). *Bacteroides* are important human symbionts. Among their many functions, one important contribution is aiding in host digestion by degrading plant polysaccharides and other compounds. Polysaccharide breakdown products are further used as energy sources by the bacteria. The extent and importance of this symbiosis is only now being fully appreciated–the recently released genome sequence of *Bacteroides thetaiotaomicron* reveals the presence of an astonishing number of genes whose predicted products are involved in polysaccharide metabolism (Comstock & Coyne 2003). Although *Bacteroides* are normal human commensal flora, events that lead to their spillage from the colon can have serious clinical consequences, including abscess formation and life-threatening infections (Brook 1989, Elliott *et al*. 2000, Hecht *et al*. 1999). The clinical picture may be further compounded by the fact that many *Bacteroides* organisms are resistant to one or multiple antibiotics (Hecht *et al*. 1999). Resistance is mediated by a plethora of genetic elements, many of which are mobile, and can be efficiently transferred within and from the genus. For this reason, *Bacteroides* have been referred to as "reservoirs" of antibiotic resistance (Salyers 1999, Salyers & Amabile-Cuevas 1997, Shoemaker *et al*. 2001).

Mobile genetic elements are responsible for gene transfer

Mobile genetic elements harbored by *Bacteroides* may be either plasmids or transposons (Salyers *et al*. 1995a, b, Smith *et al*. 1998). For efficient DNA transfer, two independent sets of biochemical processes are required-(a) processing of the DNA molecule destined for transfer, and (b) assembly of a mating bridge or conjugation pore across the donor and recipient cell envelopes to allow passage of the transferred DNA. Mobile elements that encode both sets of functions are referred to as conjugative transfer factors, whereas those that encode only the processing functions are referred to as mobilizable transfer factors. Mobilizable elements physically transfer from donor to recipient bacteria only when they are co-resident in the donor cell with a conjugative transfer factor, whose mating bridge they can utilize. In *E. coli* and other aerobic bacteria, mating bridge functions are encoded by large conjugative plasmids (drug-resistance or "R" factors), whereas in *Bacteroides*, conjugation functions appear to be encoded primarily by conjugative transposons. Since the latter are chromosomally localized, stably inherited, and mobile, there is efficient and high-frequency dissemination of antibiotic-resistance and other DNA within and from the *Bacteroides*. More than 80% of clinical *Bacteroides* isolates are now resistant to tetracycline, due to the dissemination of resistance genes mediated by conjugative transposons (Shoemaker *et al*. 2001).

In order to understand how DNA is transferred with high efficiency during conjugation within and from the *Bacteroides*, research has primarily focused on analyses of the initial processing events that result in transfer-ready DNA molecules (Sitailo *et al*. 1998). In the case of both plasmids and transposons, these processing events occur within a specific region of the transferred DNA called the origin of transfer (*oriT*), and are catalyzed by proteins called mobilization (Mob) proteins. A major difference between the Mob proteins encoded by transfer factors from different bacterial genera is exemplified by anaerobic mobilizable transfer factors, many of which require *fewer* Mob proteins to complete initial DNA processing reactions. Multiple small *Bacteroides* and *Clostridium* mobilizable plasmids and transposons encode only one or two Mob proteins (Bass & Hecht 2002, Crellin & Rood 1998, Li *et al*. 1995, Smith & Parker 1998, Vedantam *et al*. 1999) whereas those harbored by *E. coli* and other aerobic bacteria encode 4, 5 or more Mob proteins for DNA processing (Howard *et al*. 1995, Pansegrau *et al*. 1988). Thus, one can argue that Mob proteins derived from anaerobic DNA transfer factors have evolved to be more *efficient* in the DNA processing reactions. In addition, it has been observed that Mob proteins encoded by mobile

elements of anaerobic bacteria retain activity in *aerobic* bacteria as well, where mobilization levels similar to, or higher than those observed in the anaerobic background can be obtained. (Novicki & Hecht 1995, Vedantam *et al*. 1999). It is not completely understood how these proteins are expressed and function in diverse backgrounds. Preliminary analyses reveal that the Mob genes of Gram-negative *anaerobic* bacteria have a % G+C content midway been those of *aerobic* Gram-positive and Gram-negative origin. Such data may provide clues to why anaerobic Mob proteins are functional in diverse backgrounds (Vedantam & Hecht unpublished), and hence, might have evolved from different organisms.

Evolution of mobile cassettes

The plethora of *Bacteroides* transfer factors, their ability to be disseminated within and from the genus, and their stability and further transfer from unrelated genera all raise important questions about the evolution of mobile DNA cassettes. A close analysis of Mob genes from *Bacteroides* reveals that there may be a shuffling of important functional modules that has the potential to generate great diversity of mobile elements. This concept of functional module permutation and combination is now being recognized as an important means of generating gene diversity (Burrus *et al*. 2002, Roberts *et al*. 2001, Rowe-Magnus *et al*. 2002, 2003).

Irrespective of the type of mobile element being transferred in *Bacteroides*, it is likely that the same type of conjugation apparatus will be required to physically transport the DNA. Since the apparatus itself is non-discriminatory in terms of the type of DNA transferred, it then becomes all the more important to gain a deeper appreciation of its nature and structure, so that effective blocking agents can be developed to counter the widespread dissemination of DNA carrying antibiotic-resistance, and pathogenesis-related genes.

In summary, *Bacteroides* harbor a wide variety of mobile DNA transfer elements, many of which carry antibiotic resistance genes, and are capable of inter-genus transfer, survival and expression. In many systems studied to date, the transfer of these elements within the *Bacteroides* is sensitive to, and up-regulated by, sub-inhibitory concentrations of a common, widely used antibiotic, tetracycline. Further, in many cases, transfer factors appear to be composed of functional modules that can mutate, mix and match to generate a great diversity of mobile elements. Thus these normal human commensal organisms have evolved to become "reservoirs of resistance" (Salyers 1999), and infections involving such bacteria can have serious clinical consequences. Since they are part of the normal colonic flora, future research will need to incorporate ingenious approaches to eliminate only antibiotic-resistant variants of these organisms.

Microbial toxins promote biodiversity in a real-life game of rock-paper-scissors

There has been increased interest in the exploration of the role of spatial scale in explaining the maintenance of biological diversity (Chesson 2000, Tilman & Pacala 1993). Ecological theory suggests that local interactions and dispersal promote diversity (Durrett & Levin 1994). Further, multiple species can co-exist when they have non-hierarchical, non-transitive relationships (Durrett & Levin 1997). The children's game of rock-paper-scissors illustrates the concept of non-transitive relationships, with rock crushing scissors, scissors cutting paper and paper covering rock. In this game, with all three phenotypes present, no one wins, the game cycles and thus diversity is maintained.

Evolution of colicin gene clusters

Recent work has focused on developing both *in vitro* and *in vivo* models with which to test the impact of non-hierarchical relationships on microbial diversity (Durrett & Levin 1997, Kerr *et al*. 2002). Some of the more successful of these models are based upon one member (the colicins) of the diverse and abundant family of bacterial toxins known as bacteriocins (Chesson 2000, Kerr *et al*. 2002). The bacteriocin family includes a diversity of proteins in terms of size, microbial targets, modes of action and immunity mechanisms. The most extensively studied, the colicins produced by *Escherichia coli*, share certain key characteristics (James *et al*. 1996). Colicin gene clusters are encoded on plasmids and are comprised of a colicin gene, which encodes the toxin, an immunity gene, which encodes a protein conferring specific immunity to the producer cell, and a lysis gene, which encodes a protein involved in colicin release from the cell. Colicin production is mediated by the SOS regulon and is therefore principally produced under times of stress. Toxin production is lethal for the producing cell and any neighboring cells recognized by that colicin. A receptor domain in the colicin protein that binds a specific cell surface receptor determines target recognition. This mode of targeting results in the relatively narrow phylogenetic killing range often cited for bacteriocins. The killing functions range from pore formation in the cell membrane to nuclease activity against DNA, rRNA, and tRNA targets.

Diversity among colicin phenotypes in natural populations

Various mathematical and experimental models have been used to explore the relationship between the three colicin-related phenotypes found in all natural populations *of E. coli*; sensitive cells (which can be killed by colicin toxins), resistant cells (which have altered receptor and translocation systems and are thus resistant to

Strain below	Wins against	Loses against
Killer	Sensitive	Resistant
Sensitive	Resistant	Killer
Resistant	Killer	Sensitive

Table II. - Chemical warfare among microbes as a non-transitive, three-way game similar to the "rock-scissors-paper" game.

colicins) and producer cells (which carry colicin plasmids and produce toxin when induced) (Durrett & Levin 1994, 1997, Kerr *et al*. 2002). Pair-wise interactions among the strains have the non-transitive structure of the childhood game of rock-scissors-paper (Table II). The colicin producer strain beats the sensitive strain, owing to the toxin's effects on the latter. The sensitive strain beats the resistant strain, because only the latter suffers the cost of resistance. And the resistant strain wins against the producer, because the latter bears the higher cost of toxin production and release, while the former pays only the cost of resistance.

Kerr and coworkers (Kerr *et al*. 2002) employed *in vitro* experimental methods and mathematical modeling to illustrate that the maintenance of diversity in this system requires spatial structure; in the well-mixed environment of liquid nutrients in a flask, diversity is rapidly lost - with producer cells killing sensitive cells and then producer cells replaced with more rapidly growing resistant cells. When spatial structure is introduced, in this case by plating cells onto an agar plate, the three different cell types "chase" each other across the plate - with producer cells chasing, and killing, sensitive cells, sensitive cells chasing resistance cells (as they outgrow them) and resistant cells chasing producer cells (as they outgrow them).

Kirkup & Riley (pers comm) have recently shown that a similar cyclical pattern is observed when the same three cell lines are introduced into a mouse colon model. This *in vivo* model employs streptomycin to rid the mouse colon of its Gram-negative bacterial flora. Streptomycin resistant strains can then be introduced into the system, through the mouse's water source, and the establishment and interaction between strains observed. Kirkup and Riley have shown that, just as was observed when spatial structure was introduced in the *in vitro* model, all three strains (sensitive, producer and resistant) cycle through the mouse colon with the same relationships as predicted by theory, i.e. the sensitive cells are only transiently retained when present with producer cells and resistant cells outgrow producer strains. If migration into this close system of caged mice is allowed, then the predicted cycling between strains ensues.

Understanding the interactions between these three microbial phenotypes does more than simply aid in our understanding of how microbial diversity is maintained in natural communities. Such information can directly inform applied studies as well. For example, one can envision a similar sort of dynamic involved in the evolution of antibiotic resistance. When antibiotics are applied in an environment, it is analogous to introducing a toxin producing strain. The antibiotic rapidly kills sensitive cells and inadvertently selects for resistance. Resistance then dominates until the current antibiotic is no longer useful. Once the selection pressure is removed (antibiotics are no longer applied), then the sensitive population will reinvade the population or community simply because it grows faster than the resistant strains. Viewing the evolution of antibiotic resistance from this perspective allows us to incorporate evolution and ecological theory into drug design. Resistance is usually considered to be an undesirable, but unavoidable consequence of microbial evolution. However, armed with evolutionary theory we can make rational decisions about how to design drugs that are more difficult to resist (thus slowing the transition from sensitive to resistant) or more costly to resist (thus speeding up the transition from resistant to sensitive).

HIV evolution and virulence

A practical implication of the evolution of infection and virulence is adaptive microbial evolution to therapeutic interventions. One good example is the evolution of drug resistance by human immunodeficiency virus type 1 (HIV-1). Drug therapy to HIV infection is typically done with combination therapy has dramatically reduced the rate of HIV-1 and AIDS-related morbidity and mortality. The lack of compliance to drug administration may result in suboptimal therapy, which can lead to drug resistance. Drug resistance limits the clinical benefit of drug treatment and can select for new variant viruses with altered virulence and tropism.

The HIV-1 mutation rate is high (i.e., 4×10^{-5} mutations per target bp per replication cycle, which correlates to about one mutation in every 3 new genomes produced) and likely aids in the rapid development of drug resistance during suboptimal therapy (Mansky & Temin 1995). Transmission of HIV-1 with reduced susceptibility to antiretroviral drugs may compromise the efficacy of drug therapy (Garcia-Lerma *et al*. 2001).

Drugs, drug resistance, increased HIV mutation rates

The impact of drugs and drug-resistant virus on HIV-1 mutation rates was done (Mansky & Bernard 2000) in light of earlier observations made with other retroviruses (Julias *et al*. 1997, Julias & Pathak 1998, Pathak & Temin 1992). Both drugs and drug resistant virus were capable of increasing the virus mutation rate (Mansky & Bernard, 2000). Recent studies with other drugs indicate that drug treatment may generally lead to increased virus mutant frequencies during HIV-1 replication (R Chen & LM Mansky unpublished observations) (Mansky 2003, Mansky *et al*. 2003). These observations suggest that when virus replication occurs in the presence of suboptimal concentrations of drug, drug-resistant virus is selected for and that replication of drug-resistant virus in the presence of drug could further increase the virus mutation rate. This has been shown to be the case (Mansky *et al*. 2002). Interestingly, different drugs used in conjunction with a drug-resistant virus can cause the same affect (Mansky *et al*. 2002). This indicates that when new drugs are added in drug therapy regimens they could also act with the drug-resistant virus to further increase virus mutant frequencies even though the drug-resistance phenotype is associated with another drug.

Although perhaps counterintuitive, an intentional increase in mutation rate has been speculated as a rational approach for antiviral treatment of RNA virus infections (Drake & Holland 1999). RNA viruses have high mutation rates and are particularly vulnerable to increases in mutation rate that could extinguish virus replication, by error catastrophe. The inhibition of RNA virus replication with ribavirin, a ribonucleoside analog, has been associated with error catastrophe for some RNA viruses (Contreras *et al*. 2002, Crotty *et al*. 2000, Severson *et al*. 2003), but not others (LCMV) (Ruiz-Jarabo *et al*. 2003). Promutagenic nucleoside analogs, which are incorporated into the viral genome during nucleic acid replication and result in a progressive accumulation of mutations that would ultimately lead to a drastic reduction in virus replication and fitness, have also been used to extinguish HIV-1 replication (Loeb *et al*. 1999). Given that the majority of mutations are deleterious, selection against such variants would reduce virus yield within a single cycle of replication and allow the maintenance of some significant level of virus fitness within the population. The success of eliminating HIV-1 replication by error catastrophe (also called lethal mutagenesis), has yet to be tested outside of cell culture systems.

Vertebrate host cells have evolved powerful strategies to eliminate retroviral infections by lethal mutagenesis. The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC 3) proteins are cytosine deaminases that provide intrinsic antiviral immunity to HIV-1 infection (Harris & Liddament 2004). In the case of HIV-1, the APOBEC 3G and APOBEC 3F proteins have been shown in particular to attack and destroy infectious virus by C-to-U hypermutation of the viral genome during minus-strand DNA synthesis (Hache *et al*. 2005, Harris *et al*. 2003, Liddament *et al*. 2004, Sheehy *et al*. 2002). These APOBEC 3 proteins attack the viral nucleic acid after being recruited into virus particles. HIV-1 normally evades such attacks by deflecting the APOBEC 3 proteins from particles with the HIV-1 Vif protein, which targets the Vif-APOBEC 3 protein complexes for degradation by the proteasome (Sheehy *et al*. 2003). The development of small molecule inhibitors of the Vif-APOBEC 3 protein interaction will be important for the application of lethal mutagenesis of HIV-1 by the APOBEC 3 proteins.

Antimicrobial drug resistance and increased pathogen mutation rates

There is a growing body of literature indicating that mutator alleles are selected for in microbial populations, particularly in response to environmental stress (Bjedov *et al*. 2003, Sniegowski *et al*. 1997). For instance, the emergence of antimicrobial resistance during drug therapy can increase the likelihood of selection for mutator alleles, as well as increase the probability of failure of subsequent drug therapies (Martinez & Baquero 2000, ONeill & Chopra 2001) (Table III). The generation of drug resistance depends on the rate of emergence of resistant mutants which is defined by the mutation rate. In bacteria, there are many examples indicating that antibiotic treatment not only selects for antibiotic-resistant bacteria, but also selects for mutator alleles which confer a higher mutation rate (Giraud *et al*. 2001, 2002, Kohler *et al*. 1997, Mamber *et al*. 1993, Negri *et al*. 2002, Oliver *et al*. 2000, Ren *et al*. 1999, Tenaillon *et al*. 2001). Correlations between mutation rate and the efficacy of antimicrobial drug treatment have recently been observed (Gerrish & Garcia-Lerma 2003). Error-prone polymerases and mutations of the mismatch repair system, along with mutations of enzymes that protect DNA from DNA damaging agents and enzymes that degrade modified nucleotides, have been implicated as the ultimate mechanisms

Table III. - Examples of antimicrobial resistance associated with increased pathogen mutation rates

Example	Reference
E.coli/rifampin resistance	(2)
E. coli/streptomycin resistance	(5)
S. aureus/vancomycin resistance	(6)
S. pneumoniae/cefotaxime resistance	(3)
P.aeruginosa/rifampicin resistance	(4)
H. pylori/ rifampicin resistance	(1)

responsible for these mutator phenotypes (Table III) (Boshoff *et al*. 2003, Denamur *et al*. 2000, LeClerc *et al*. 1996, Oliver *et al*. 2000, Radman 1999).

In summary, increased HIV-1 mutation rates can be associated with the evolution of drug resistance, and this observation correlates with observations made in bacterial systems with antimicrobial drug resistance. The transmission of drug-resistant HIV-1 along with the development of drug-resistant virus raises concerns about the efficacy of drug regimens due to the presence of mutator phenotypes. Future studies should be directed at determining the risk of these mutator phenotypes in HIV-1 drug resistance. In addition, the unintentional increase in HIV mutagenesis by drugs could be used for improving the efficacy of drug therapy by the rational selection drug combinations that either minimize the potential for HIV mutagenesis or intentionally increase HIV mutagenesis to induce (perhaps along with a mutagen) error catastrophe. Drug resistance may select for new variant viruses with altered virulence.

CONCLUSIONS

The direct impact of "evolved" virulence is subject to many interactions at a variety of levels. Previous thought on the evolution of both pathogenic and mutualistic symbioses considered different roles for interactions between hosts and their microbial partners. Given the variety of examples in this article, and the mechanisms that determine whether they are benign or virulent, raises many questions on the evolution of similar processes of infection and colonization. Understanding, monitoring, and predicting the evolution and spread of infectious disease may depend upon our knowledge and capabilities of anticipating the behavior of symbiotic systems such as those discussed in this paper. The ability to modify or interfere with these infection processes can be approached by first understanding the conditions under which interventions fail. Subsequently, designing protocols to prevent these failures requires the application of evolutionary theory and deciphering complex molecular interactions (i.e. antibiotic resistance). Although there has been a growing interest in understanding the evolution of infectious diseases and anticipating their emergence, the integration of evolutionary biology with the study of pathogen interactions will significantly contribute to the development of predicting disease resistance in such systems. Considering that similar mechanisms exist between mutualistic and pathogenic associations are remarkable yet can be misleading. For example, many model systems have been adapted for use in laboratory experimentation, but do not represent naturally occurring populations in the wild. Comparing both laboratory and field based experiments will help further our understanding of the nature of symbiotic relationships, and their overall evolution as novel "species".

ACKNOWLEDGEMENTS. - MKN is supported by NSF (DEB-0316516 and IOS-0744498) and NIH (S06-GM08136-26 and NIAID 1SC1AI081659-01). AMH's research is supported by NSF (EF-0626896 and IOS-0747516), US-Mexus (200708551) and the Shanbrom Family Foundation. RD is supported by Canadian Institutes for Health Research, the National Sciences and Engineering Council of Canada and the Canadian Bacterial Diseases Network, and is an Alberta Heritage Foundation for Medical Research Scholar. MAR is supported by NIH (GM58433). LMM is supported by the American 797 Cancer Society (RPG0027801), the Campbell Foundation, and the NIH (GM56615 and 798 AI053155).

This mini-review was the outcome of a symposium held at the Society for the Study of Evolution in Chico, CA in June 2003 entitled "Understanding the evolutionary patterns and processes of virulence". SSE partially supported the speakers for travel and registration to attend the symposium.

REFERENCES

Andre JB, Ferdy JB, Godelle B 2003. Within-host parasite dynamics, emerging trade-off, and evolution of virulence with immune system. *Evolution* 57: 1489-1497.

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gómez-Gómez L, Boller T, Ausubel FA, Sheen J 2002. MAP kinase signaling cascade in *Arabidopsis* innate immunity. *Nature* 415: 977-983.

Bass KA, Hecht DW 2002. Isolation and characterization of cLV25, a *Bacteroides fragilis* chromosomal transfer factor resembling multiple *Bacteroides* sp. mobilizable transposons. *J Bacteriol* 184: 1895-1904.

Bassler BL 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr Op Mol Biol* 2: 582-587.

Bates A 2000. The intertidal distribution of two algal symbionts hosted by *Anthopleura xanthogrammica* (Brandt 1835). *J Exp Mar Biol Ecol* 249: 249-262.

Ben-Haim Y, Zicherman-Keren M, Rosenberg E 2003. Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. *Appl Enviro Microbiol* 69: 4236-4242.

Berlocher SH 1998. Can sympatric speciation via host or habitat shift be proven from phylogenetic and biogeographic evidence? *In* Endless Forms: Species and speciation, D J Howard & SH Berlocher eds, Oxford University Press, New York: 99-113.

Bieber, D, Ramer SW, Wu CY, Murray WJ, Tobe T, Fernandez R, Schoolnik GK 1998. Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. *Science* 280: 2114-2118.

Bjedov I, Tenaillon O, Gerard B, Souza V, Denamur E, Radman M, Taddei F, Matic I 2003. Stress-induced mutagenesis in bacteria. *Science* 300: 1404-1409.

Boshoff HI, Reed MB, Barry CE, Mizrahi V 2003. DnaE2 Polymerase Contributes to *In Vivo* Survival and the Emergence of Drug Resistance in *Mycobacterium tuberculosis*. *Cell* 113: 183-193.

Boucher Y, Stokes HW 2006. The roles of lateral gene transfer and vertical descent in *Vibrio* evolution. *In* The Biology of the Vibrios, F L Thompson, B Austin & J Swings eds, ASM Press, Washington, D.C., USA: 84-94.

Braschler T R, Merion S, Tomás JM, Graf J 2003. Complement resistance is essential for colonization of the digestive tract of *Hirudo medicinalis* by *Aeromonas* strains. *Appl Environ Microbiol* 69: 4268-4271.

Brook I 1989. Pathogenicity of the *Bacteroides fragilis* group. *Annu Clin Lab Sci* 19: 360-376.

Browne-Silva J, Nishiguchi MK 2008. Gene sequence of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int J Syst Evol MIcrobiol* 58: 1292-1299.

Burrus V, Pavlovic G, Decaris B, Guedon G 2002. The ICESt1 element of *Streptococcus thermophilus* belongs to a large family of integrative and conjugative elements that exchange modules and change their specificity of integration. *Plasmid* 48: 77-97.

Chesson P 2000a. Mechanisms of maintenance of species diversity. *Annu Rev Ecol Syst* 31: 343-366.

Chun CK, Scheetz TE, Bonaldo Mde F, Brown B, Clemens A, Crookes-Goodson WJ, Crouch K, DeMartini T, Eyestone M, Goodson MS, Janssens B, Kimbell JL, Koropatnick TA, Kucaba T, Smith C, Stewart JJ, Tong D, Troll JV, Webster S, Winhall-Rice J, Yap C, Casavant TL, McFall-Ngai MJ, Soares MB 2006. An annotated cDNA library of juvenile Euprymna scolopes with and without colonization by the symbiont Vibrio fischeri. *BMC Genomics* 7: 154.

Colwell RR 1984. *Vibrios in the environment*. John Wiley and Sons, New York.

Comstock LE, Coyne MJ 2003. *Bacteroides thetaiotaomicron*: a dynamic, niche-adapted human symbiont. *Bioessays* 25: 926-929.

Contreras AM, Hiasa Y, He W, Terella A, Schmidt EV, Chung RT 2002. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. *J Virol* 76: 8505-8517.

Cooper VS, Reiskind MH, Miller JA, Shelton KA, Walther BA, Elkinton JS, Weald PW 2002. Timing of transmission and the evolution of virulence of an insect virus. *Proc Roy Soc Lond B* 269: 1161-1165.

Crellin PK, Rood JI 1998. Tn4451 from *Clostridium perfringens* is a mobilizable transposon that encodes the functional Mob protein, TnpZ. *Mol Microbiol* 27: 631-642.

Crookes WJ, Ding LL, Huang QL, Kimbell JR, Horwitz J, McFall-Ngai MJ 2004. Reflectins: The unusual proteins of squid reflective tissues. *Science* 303: 235-238.

Crotty S, Maag D, Arnold JJ, Zhong W, Lau JY, Hong Z, Andino R, Cameron CE 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. *Nat Med* 6: 1375-1379.

Darnell CL, Hussa EA, Visick KL 2008. The putative hybrid sensor kinase SypF coordinates biofilm formation in *Vibrio fischeri* by actigng upstream of two response regulators, SypG and VpsR. *J Bacteriol* 190: 4941-4950.

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L, McFall-Ngai MJ 2004. NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol* 6: 1139-1151.

de Faria SM, de Lima HC, Olivares FL, Melo RB, Xavier RB 2000. Nodulaçao em especies florestais, especifidade hospedeira e implicaçoes na sistemática de leguminosae. *In* Soil fertility, soil biology, and plant nutrition interrelationships. JO Siquerira, FM Moreira, S Lopes, AS Guilherme, LRG Faquin, V Furtini, AE Neto, JG Carvalho eds, ociedade Brasileira de Ciencias do Solo-Universidade Federal de Lavras Dept, Solos: 67-686

Deibel C, Kramer S, Chakraborty T, Ebel F 1998. EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein. *Mol Microbiol* 28: 463-474.

DeLoney-Marino CR, Wolfe AJ, Visick KL 2003. Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and N-Acetylneuraminic acid, a componenet of squid light-organ mucus. *Appl Environ Microbiol* 69: 7527-7530.

Denamur E, Lecointre G, Darlu P, Tenaillon O, Acquaviva C, Sayada C, Sunjevaric I, Rothstein R, Elion J, Taddei F, Radman M, Matic I 2000. Evolutionary implications of the frequent horizontal transfer of mismatch repair genes. *Cell* 103: 711-721.

Denison RF, Kiers ET 2004. Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. *FEMS Microbiol Lett* 237: 193-198.

DeVinney R, Stein M, Reinscheid D, Abe A, Ruschkowski S, Finlay BB 1999. Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. *Infect Immun* 67: 2389-2398.

Díaz CL, Melchers LS, Hooykaas PJJ, Lugtenberg EJJ, Kijne JW 1989. Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. *Nature* 338: 579-581.

Djordjevic MA, Gabriel DW, Rolfe BG 1987. *Rhizobium*-the refined parasite of legumes. *Annu Rev Phytopathol* 25: 145-168.

Doino Lemus J, McFall-Ngai MJ 2000. Alterations in the proteome of the *Euprymna scolopes* light organ in response to symbiotic *Vibrio fischeri*. *Appl Environ Microbiol* 66: 4091-4097.

Donnenberg MS, Whittam TS 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. *J Clin Invest* 107: 539-548.

Downie JA, Walker SA 1999. Plant responses to nodulation factors. *Curr Opin Plant Biol* 2: 483-489.

Drake JW, Holland JJ 1999. Mutation rates among RNA viruses. *Proc Natl Acad Sci USA* 96: 13910-13913.

Dunlap PV, Ast JC, Kimura S, Fukui A, Yoshino T, Endo H 2007. Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics* 23: 507-532.

Durrett R, Levin S 1997. Allelopathy in spatially distributed populations. *J Theor Biol* 185: 65-171.

Durrett R, Levin SA 1994. Stochastic spatial models: a user's guide to ecological applications. *Phil Trans Roy Soc Lond B* 343: 329-350.

Elliott D, Kufera JA, Myers RA 2000. The microbiology of necrotizing soft tissue infections. *Am J Surg* 179: 361-366.

Elliott SJ, Hutcheson SW, Dubois MS, Mellies JL, Wainwright LA, Batchelor M, Frankel G, Knutton S, Kaper JB 1999. Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. *Mol Microbiol* 33: 1176-1189.

Endre G, Kereszt A, Kevel Z, Mihaceae S, Kaló P, Kiss GB 2002. A receptor kinase regulating symbiotic nodule development. *Nature* 417: 962-966.

Felix G, Duran JD, Voko S, Boller T 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J* 18: 265-276.

Ferguson GP, Datta A, Baumgartner J, Rood II RM, Carlson RW, Walker GC 2004. Similarity to peroxisomal-membrane protein family reveals that Sinorhizobium and Brucella BacA affect lipid-A fatty acids. *Proc Nat Acad Sci USA* 101: 5012-5017.

Finlay BB, Falkow S 1997. Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev* 61: 136-169.

Foster JS, Apicella MA, McFall-Ngai MJ 2000. *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. *Dev Biol* 226: 242-254.

Foster RC, Rovira AD, Cock TW 1983. Ultrastructure of the root-soil interface. *Am. Phytopath Soc*: 1-8.

Frank SA 1996. Host-symbiont conflict over the mixing of symbiotic lineages. *Proc Roy Soc Lond B* 263: 339-344.

Friedman AR, Baker BJ 2007. The evolution of resistance genes in multi-protein plant resistance systems. *Curr Opin Genet Develop* 17: 493-499.

Garcia-Lerma JG, Nidtha S, Blumoff K, Weinstock H, Heneine W 2001. Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naive persons. *Proc Natl Acad Sci USA* 98: 13907-13912.

Gerrish PJ, Garcia-Lerma JG 2003. Mutation rate and the efficacy of antimicrobial drug treatment. *Lancet Infect Dis* 3: 28-32.

Geszvain K, Visick KL 2008. The hybrid sensor kinase RscS integrates positive and negative signals to modulate biofilm formation in *Vibrio fischeri*. *J Bacteriol* 190: 4437-4446.

Gilson L, Kuo A, Dunlap PV 1995. AinS and a new family of autoinducer synthesis proteins. *J Bacteriol* 177: 6946-6951.

Girardin SE, Boneca IG, Carneiro LAM, Antignac A, Jéhanno M, Viala J, Tedin K, Taha MK, Labigne A, Zähringer U, Coyle AJ, DiStefano PS, Bertin J, Sansonetti PJ, Philpott DJ 2003. Nod1 detects a unique muropeptide from gram-negative bacteiral peptidoglycan. *Science* 300: 1584-1587.

Giraud A, Matic I, Radman M, Fons M, Taddei F 2002. Mutator bacteria as a risk factor in treatment of infectious diseases. *Antimicrob Agents Chemother* 46: 863-865.

Giraud A, Radman M, Matic I, Taddei F 2001. The rise and fall of mutator bacteria. *Curr Opin Microbiol* 4: 582-585.

Gómez-Gómez L, Boller T 2000. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol Cell* 5: 1003-1011.

Goodner B, Hinkle G, Gattung S, a o authors 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* 294: 2323-2328.

Graf J, Dunlap PV, Ruby EG 1994. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. *J Bacteriol* 176: 6986-6991.

Graf J, Ruby EG 1998. Host-derived amino acids support the proliferation of symbiotic bacteria. *Proc Nat Acad Sci USA* 95: 1818-1822.

Graf J, Ruby EG 2000. Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. *Mol Microbiol* 7: 168-179.

Guerrero RC, Nishiguchi MK 2007. Identification of light organ symbionts from the genera *Uroteuthis*, *Loliolus*, and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* 23: 1-10.

Gunawardena U, Hawes MC 2002. Tissue specific localization of root infection of fungal pathogens: role of root border cells. *Mol Plant-Microbe Inter* 15: 1128-1136.

Gürlebeck D, Thieme F, Bonas U 2006. Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interacton with the host plant. *J Plant Physiol* 163: 233-255.

Hache G, Liddament MT, Harris RS 2005. The retroviral hypermutation specificity of APOBEC3F and APOBEC3G is governed by the C-terminal DNA cytosine deaminase domain. *J Biol Chem* 280: 10920-10924.

Handfield M LDE, Sanschagrin F, Mahan MJ, Woods DE, Levesque RC 2000. *In vivo*-induced genes in *Psudomonas aeruginosa*. *Infect Immun* 68: 2359-2362.

Harris RS, Bishop KN, Sheehy AM, Craig HM, Petersen-Mahrt SK, Watt IN, Neuberger MS, Malim MH 2003. DNA deamination mediates innate immunity to retroviral infection. *Cell* 113: 803-809.

Harris RS, Liddament MT 2004. Retroviral restriction by APOBEC proteins. *Nat Rev Immunol* 4: 868-877.

Hecht DW, Vedantam G, Osmolski JR 1999. Antibiotic resistance among anaerobes: What does it mean? *Anaerobe* 5: 421-429.

Hensey S, McFall-Ngai MJ 1992. A surface peptide of the bacterium *Vibrio fischeri* plays a key role in specificity and recognition in the symbiosis with the squid *Euprymna scolopes*. *Am Zool* 32: 37A.

Hentschel U, Felbeck H 1993. Nitrate respiration in the hydrothermal vent tubeworm *Riftia pachyptila*. *Nature* 366: 338-340.

Hentschel U, Steinert M, Hacker J 2000. Common molecular mechanisms of symbiosis and pathogenesis. *Trends Microbiol* 8: 226-231.

Hirsch AM, Lum MR, Downie JA 2001. What makes the rhizobia-legume symbiosis so special? *Plant Physiol* 127: 1484-1492.

Hirsch AM 1999. Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Curr Opin Plant Biol* 2: 320-326.

Hirsch AM 2004. Plant-microbe symbioses: A continuum from commensalism to parasitism. *Symbiosis* 37: 345-363.

Hirsch AM, Bauer WD, Bird DM, Cullimore J, Tyler B, Yoder JI 2003. Molecular signals and receptors: Controlling Rhizosphere interations between plants and other organisms. *Ecology* 84: 858-868.

Hirsch AM, McFall-Ngai MJ 2000. Fundamental concepts in symbiotic interactions: light and dark, day and night, squid and legume. *J Plant Growth Regul* 19: 113-130.

Hirsch AM, McKhann HI, Löbler M 1992. Bacterial-induced changes in plant form and function. *Int J Plant Sci* 153: S171-S181.

Howard MT, Nelson WC, Matson SW 1995. Stepwise assembly of a relaxosome at the F plasmid origin of transfer. *J Biol Chem* 270: 28381-28386.

Hueck CJ 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62: 379-433.

Hussa EA, O'Shea TM, Darnell CL, Ruby EG, Visick KL 2007. Two-component response regulators of *Vibrio fischeri*: identification, mutagenesis, and characterization. *J Bacteriol* 189: 5825-5838.

James R, Kleanthous C, Moore GR 1996. The biology of E colicins: paradigms and paradoxes. *Microbiology* 142: 1569-1580.

Janeway CAJ 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54: 1-13.

Jones BW, Lopez JL, Huttenberg J, Nishiguchi MK 2006. Population structure between environmentally transmitted Vibrios and bobtail squids using nested clade analysis. *Mol Ecol* 15: 4317-4329.

Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK 2007. Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. *Microbiol Ecol* 54: 314-323.

Jones BW, Nishiguchi MK 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* 144: 1151-1155.

Jones JD, Dangl JL 2006. The plant immune system. *Nature* 444: 323-329.

Julias JG, Kim T, Arnold G, Pathak VK 1997. The antiretrovirus drug 3'-azido-3'-deoxythymidine increases the retrovirus mutation rate. *J Virol* 71: 4254-4263.

Julias JG, Pathak VK 1998. Deoxyribonucleoside triphosphate pool imbalances *in vivo* are associated with an increased retroviral mutation rate. *J Virol* 72: 7941-7949.

Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB 1997. Enteropathogenic *E coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91: 511-520.

Kerr B, Riley M, Feldman M, Bohannan B 2002. Local dispersal and interaction promote coexistence in a real life game of rock-paper-scissors. *Nature* 418: 171-174.

Kiers ET, Rousseau RA, West SA, Denison RF 2003. Host sanctions and the legume-rhizobium mutualism. *Nature* 425: 78-81.

Kimbell JR, Koropatnick TA, McFall-Ngai MJ 2006. Evidence for the participation of the proteasome in symbiont-induced tissue morphogenesis. *Biol Bull* 211: 1-6.

Kimbell JR, McFall-Ngai MJ, Roderick GK 2002. Two genetically distinct populations of botail squid, *Euprymna scolpes*, exist on the island of O'ahu. *Pac Sci* 56: 347-355.

Knutton S, Baldwin T, Williams PH, McNeish AS 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* 57: 1290-1298.

Kohler T, Michea-Hamzehpour M, Plesiat P, Kahr AL, Pechere JC 1997. Differential selection of multidrug efflux systems by quinolones in *Psudomonas aeruginosa*. *Antimicrob Agents Chemother* 41: 2540-2543.

Koropatnick TA, Engle JT, Apicella MA, Stabb EV, Goldman WE, McFall-Ngai MJ 2004. Microbial factor-mediated development in a host-bacterial mutualism. *Science* 306: 1186-1188.

Koropatnick TA, Kimbell JR, McFall-Ngai MJ 2007. Responses of host hemocytes during the initiation of the squid-*Vibrio* symbiosis. *Biol Bull* 212: 29-39.

LeClerc JE, Li B, Payne WL, Cebula TA 1996. High mutation frequencies among *Escherichia coli* and *Salmonella pathogens*. *Science* 274: 1208-1211.

Lee KH, Ruby EG 1992. Detection of the light organ symbiont, *Vibrio fischeri* in Hawaiian seawater using *lux* gene probes. *Appl Environ Microbiol* 58: 942-947.

Lee KH, Ruby EG 1994a. Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. *J Bacteriol* 176: 1985-1991.

Lee KH, Ruby EG 1994b. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl Environ Microbiol* 60: 1565-1571.

Lee SH, Camilli A 2000. Novel approaches to monitor bacterial gene expression in infected tissue and host. *Curr Op Microbiol* 3: 97-101.

Lee SH, Hava DL, Waldor MK, Camilli A 1999. Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. *Cell* 99: 625-634.

LeVier K, Phillips RW, Grippe VK, Roop II RM, Walker GC 2000. Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival. *Science* 287: 2492-2493.

Li LY, Shoemaker NB, Wang GR, Cole SP, Hashimoto MK, Wang J, Salyers AA 1995. The mobilization regions of two integrated *Bacteroides* elements, NBU1 and NBU2, have only a single mobilization protein and may be on a cassette. *J Bacteriol* 177: 3940-3945.

Liddament MT, Brown WL, Schumacher AJ, Harris RS 2004. APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 *in vivo*. *Curr Biol* 14: 1385-1391.

Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R 2003. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302: 602-633.

Lipp EK, Huq A, Colwell RR 2002. Effects of global climate on infectious disease: the cholera model. *Clin Microbiol Rev* 15: 757-770.

Loeb LA, Essigmann JM, Kazazi F, Zhang J, Rose KD, Mullins JI 1999. Lethal mutagenesis of HIV with mutagenic nucleoside analogs. *Proc Natl Acad Sci USA* 96: 1492-1497.

Lum MR, Hirsch AM 2003. Roots and their symbiotic microbes: strategies to obtain nitrogen and phosphorous in a nutrient-limiting environment. *J Plant Growth Regul* 21: 368-382.

Lupp C, Urbanowski M, Greenberg EP, Ruby EG 2003. The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. *Mol Microbiol* 50: 319-331.

Lynch JD 1989. The gauge of speciation: on the frequencies of modes of speciation. *In* Speciation and its Consequences, D Otte & Endler JA eds, Sinauer, Sunderland, MA: 527-533.

Madsen EB, Madsen LH, Radutoiu S, Olbry M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J 2003. A receptor kinase of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425: 637-640.

Mamber SW, Kolek B, Brookshire KW, Bonner DP, Fung-Tomc J 1993. Activity of quinolones in the Ames *Salmonella* TA102 mutagenicity test and other bacterial genotoxicity assays. *Antimicrob. Agents Chemother.* 37: 213-217.

Mansky LM 2003. Mutagenic outcome of combined antiviral drug treatment during human immunodeficiency virus type 1 replication. *Virology* 307: 116-121.

Mansky LM, Bernard LC 2000. 3'-azido-3'-deoxythymidine (AZT) and AZT-resistant reverse transcriptase can increase the *in vivo* mutation rate of human immunodeficiency type 1. *J Virol* 74: 9532-9539.

Mansky LM, Le Rouzic E, Benichou S, Gajary LC 2003. Influence of reverse transcriptase variants, drugs, and Vpr on human immunodeficiency virus type 1 mutant frequencies. *J Virol* 77: 2071-2080.

Mansky LM, Pearl DK, Gajary LC 2002. Combination of drugs and drug-resistant reverse transcriptase results in a multiplicative increase of human immunodeficiency virus type 1 mutant frequencies. *J Virol* 76: 9253-9259.

Mansky LM, Temin HM 1995. Lower *in vivo* mutation rate of human immunodeficiency virus type 1 than predicted from the fidelity of purified reverse transcriptase. *J Virol* 69: 5087-5094.

Margulis L 1989. Symbiogenesis and Symbionticism. *In* Symbiosis as a source of evolutionary innovation, L Margulis & Fester R eds, The MIT Press, Cambridge, MA: 1-14.

Marie C, Broughton WJ, Deakin WJ 2001. *Rhizobium* type III secretion systems: legume charmers or alarmers? *Curr Opin Plant Biol* 4: 336-342.

Martinez JL, Baquero F 2000. Mutation frequencies and antibiotic resistance. *Antimicrob Agents Chemother* 44: 1771-1777.

McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Nat Acad Sci USA* 92: 1664-1668.

McDaniel TK, Kaper JB 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Mol Microbiol* 23: 399-407.

McFadden CS, Grosberg RK, Cameron BB, Karlton DP, Secord D 1997. Genetic relationships within and between clonal and solitary forms of the sea anemone *Anthopleura eleganitissma* revisited: evidence for the existence of two species. *Mar Biol* 128: 127-139.

McFall-Ngai MJ 1999. Consequences of evolving with bacterial symbionts: Lessons from the squid-vibrio association. *Annu Rev Ecol Syst* 30: 235-256.

McFall-Ngai MJ 2002. Unseen Forces: The influence of bacteria on animal development. *Dev Biol* 242: 1-14.

McKhann HI, Hirsch AM 1994. Does *Rhizobium* avoid the host response? *Curr Top Microbiol Immun* 192: 139-162.

Mekalanos JJ 1985. Cholera toxin: genetic analysis, regulation, and the role in pathogenesis. *Curr Top Microbiol Immun* 118: 97-118.

Merrell DS, Falkow S 2004. Frontal and stealth attack strategies in microbial pathogenesis. *Nature* 430: 250-256.

Millikan DS, Ruby EG 2002. Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Appl Environ Microbiol* 68: 2519-2528.

Millikan DS, Ruby EG 2003. FlrA, a σ^{54}-dependent transcriptional activator in *Vibrio fischeri*, is required for mobility and symbiotic light organ colonization. *J Bacteriol* 185: 3547-3557.

Mithöfer A 2002. Suppression of plant defence in rhizobia-legume symbiosis. *Trends Plant Sci* 7: 440-444.

Montgomery MK, McFall-Ngai MJ 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Eupyrmna scolopes*. *Development* 120: 1719-1729.

Montgomery MK, McFall-Ngai MJ 1995. The inductive role of bacterial symbionts in the morphogenesis of a squid light organ. *Am Zool* 35: 372-380.

Moon HW, Whipp SC, Argenzio RA, Levine MM, Giannella RA 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect Immun* 41: 1340-1351.

Moore WE, Holdeman LV 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl Microbiol* 27: 961-979.

Nandasena KG, O'Hara GW, Tiwari RP, Howieson J 2006. Symbiotically diverse root-nodule bacteria able to nodulate *Biserrula pelecinus* L. emerge six years after introduction of this legume to Australia. *Appl Environ Microbiol* 72: 7365-7367.

Nandasena KG, O'Hara GW, Tiwari RP, Howieson J 2007. *In situ* lateral transfer of symbiosis islands results in rapid evolution of diverse competitive strains of mesorhizobia suboptimal in symbiotic nitrogen fixation on the pasture legume *Biserrula pelecinus* L. *Environ Microbiol* 9: 2496-2511.

Nataro JP, Kaper JB 1998. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11: 142-201.

Nealson K, Cohn D, Leisman G, Tebo B 1981. Co-evolution of luminous bacteria and their eukaryotic hosts. *Annu NY Acad Sci*: 76-91.

Nealson KH, Hastings JW 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev* 43: 496-518.

Nealson KH, Platt T, Hastings JW 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol* 104: 313-322.

Negri MF, Morosini MI, Baquero MR, del Campo R, Blazquez J, Baquero F 2002. Very low cefotaxime concentrations select for hypermutable *Streptococcus pneumoniae* populations. *Antimicrob Agents Chemother* 46: 528-530.

Nishibuchi M, Kaper JB 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. *Infect Immun* 63: 2093-2099.

Nishiguchi MK 2000. Temperature affects species distribution in symbiotic populations of *Vibrio*. *Appl Environ Microbiol* 66: 3550-3555.

Nishiguchi MK 2001. Co-evolution of symbionts and hosts: The sepiolid-*Vibrio* model. *In* Symbiosis: Mechanisms and Model Systems, J Seckbach ed, Cole-Kluwer Academic, Dordrecht, The Netherlands: 757-774.

Nishiguchi MK 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microbiol Ecol* 44: 10-18.

Nishiguchi MK, Jones BW 2004. Microbial biodiversity within the Vibrionaceae. *In* Origins, evolution, and the biodiversity of microbial life, J Seckback ed, Cole-Kluwer Academic Publishers, Dordrecht, The Netherlands: 531-548

Nishiguchi MK, Lopez JE, Boletzky Sv 2004. Enlightenment of old ideas from new investigations: The evolution of bacteriogenic light organs in squids. *Evol Dev* 6: 41-49.

Nishiguchi MK, Nair VA 2003. Evolution of pathogenicity and symbioses in Vibrionaceae: An approach using molecules and physiology. *Int J Syst Evol Bacteriol* 53: 2019-2026.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-*Vibrio* symbioses. *Appl Environ Microbiol* 64: 3209-3213.

Novicki TJ, Hecht DW 1995. Characterization and DNA sequence of the mobilization region of pLV22a from *Bacteroides fragilis*. *J Bacteriol* 177: 4466-4473.

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA, McFall-Ngai MJ 2002. Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* 68: 5113-5122.

Nyholm SV, McFall-Ngai MJ 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl Environ Microbiol* 69: 3932-3937.

Nyholm SV, McFall-Ngai MJ 2004. The winnowing: establishing the squid-vibrio symbiosis. *Nat Rev Microbiol* 2: 632-642.

Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ 2000. Establishment of an animal-bacterial association: Recruiting symbiotic vibrios from the environment. *Proc Nat Acad Sci USA* 97: 10231-10235.

Olafsen JA, Mikkelsen HV, Giaever HM, Hansen GH 1993. Indigenous bacteria in hemolymph and tissues of marine bivalves at low temperatures. *Appl Environ Microbiol* 59: 1848-1854.

Oliver A, Canton R, Campo P, Baquero F, Blazquez J 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288: 1251-1254.

ONeill AJ, Chopra I 2001. Use of mutator strains for characterization of novel antimicrobial agents. *Antimicrob Agents Chemother* 45: 1599-1600.

Pak JW, Jeon KW 1997. A symbiont-produced protein and bacterial symbiosis in *Amoeba proteus*. *Eukary Microbiol* 44: 614-619.

Pansegrau W, Ziegelin G, Lanka E 1988. The origin of conjugative IncP plasmid transfer: interaction with plasmid-encoded products and the nucleotide sequence at the relaxation site. *Biochim Biophys Acta* 951: 365-374.

Pathak VK, Temin HM 1992. 5-azacytidine and RNA secondary structure increase the retrovirus mutation rate. *J Virol* 66: 3093-3100.

Perna NT, Mayhew GF, Posfai G, Elliott S, Donnenberg MS, Kaper JB, Blattner FR 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* 66: 3810-3817.

Pueppke SG, Broughton WJ 1999. *Rhizobium* sp strain NGR234 and R. fredii USDA257 share exceptionally broad, nested host ranges. *Mol Plant Microbe Interact* 12: 293-318.

Radman M 1999. Enzymes of evolutionary change. *Nature* 401: 866-887.

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grønlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425: 585-592.

Reich KA, Biegel T, Schoolnik GK 1997. The light organ symbiont *Vibrio fischeri* possesses two distinct secreted ADP-Ribosyltransferases. *J Bacteriol* 179: 1591-1597.

Reich KA, Schoolnik GK 1994. The light organ symbiont *Vibrio fischeri* possess a homolog of the *Vibrio cholerae* transmembrane transcriptional activator *ToxR J Bacteriol* 176: 3085-3088.

Reich KA, Schoolnik GK 1996. Halovibrin, secreted from the light organ symbiont *Vibrio fishceri,* is a member of a new class of ADP-Ribosyltransferases. *J Bacteriol* 178: 209-215.

Reid SD, Herbelin CJ, Bumbaugh AC, Selander RK, Whittam TS 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* 406: 64-67.

Ren L, Rahman MS, Humayun MZ 1999. *Escherichia coli* cells exposed to streptomycin display a mutator phenotype. *J Bacteriol* 181: 1043-1044.

Roberts AP, Johanesen PA, Lyras D, Mullany P, Rood JI 2001. Comparison of Tn5397 from *Clostridium difficile*, Tn916 from *Enterococcus faecalis* and the CW459tet(M) element from *Clostridium perfringens* shows that they have similar conjugation regions but different insertion and excision modules. *Microbiol* 147: 1243-1251.

Rowe-Magnus DA, Guerout AM, Biskri L, Bouige P, Mazel D 2003. Comparative analysis of superintegrons: engineering extensive genetic diversity in the Vibrionaceae. *Plasmid* 48: 77-97.

Rowe-Magnus DA, Guerout AM, Mazel D 2002. Bacterial resistance evolution by recruitment of super-integron gene cassettes. *Mol Microbiol* 43: 1657-1669.

Ruby EG, McFall-Ngai MJ 1999. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. *Trends Microbiol* 7: 414-420.

Ruimy R, Breittmayer V, Elbaze P, Lafay B, Boussemart O, Gauthier M, Christen R 1994. Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int J Syst Bacteriol* 44: 416-426.

Ruiz-Jarabo CM, Ly C, Domingo E, Torre JC 2003. Lethal mutagenesis of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV). *Virology* 308: 37-47.

Salyers AA 1999. Beware the commensals: Their role in antibiotic resistance spread may be greater then we think. in *ASM News*.

Salyers AA, Amabile-Cuevas CF 1997. Why are antibiotic resistance genes so resistant to elimination? *Antimicrob Agents Chemother* 41: 2321-2325.

Salyers AA, Shoemaker NB, Li LY 1995a. In the driver's seat: the *Bacteroides* conjugative transposons and the elements they mobilize. *J Bacteriol* 177: 5727-5731.

Salyers AA, Shoemaker NB, Stevens AM, Li LY 1995b. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiol Rev* 59: 579-590.

Sandner L, Eguiarte LE, Navarro A, Cravioto A, Souza V 2001. The elements of the locus of enterocyte effacement in human and wild mammal isolates of *Escherichia coli*: evolution by assemblage or disruption? *Microbiol* 147: 3149-3158.

Sandstrom J, Telang A, Moran NA 2000. Nutritional enhancement of host plants by aphids- a comparison of three aphids species on grasses. *J Insect Physiol* 46: 33-40.

Sapp J 1989. Living together: symbiosis and cytoplasmic inheritance. *In* Symbiosis as a source of evolutionary innovation, L Margulis ed, The MIT Press, Cambridge, MA: 15-25.

Schwieger F, Tebbe CC 2000. Effect of field inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*)-Linking of 16S rRNA gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria. *Appl Environ Microbiol* 66: 3556-3565.

Sechi LA, Dupre I, Deriu A, Fadda G, Zanetti S 2000. Distribution of *Vibrio cholerae* virulence genes among different *Vibrio* species isolated in Sardinia, Italy. *J Appl Microbiol* 88: 475-481.

Secord D 2001. Symbioses and their consequences for community and applied ecology. *In* Symbiosis: Mechanisms and model systems, J Seckback ed, Kluwer Academic Publishers, Dordrecht, Netherlands: 47-61

Severson WE, Schmaljohn CS, Javadian A, Jonsson CB 2003. Ribavirin causes error catastrophe during Hantaan virus replication. *J Virol* 77: 481-488.

Sheehy AM, Gaddis NC, Choi JD, Malim MH 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418: 646-650.

Sheehy AM, Gaddis NC, Malim MH 2003. The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med* 9: 1404-1407.

Shoemaker NB, Vlamakis H, Hayes K, Salyers AA 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp and among *Bacteroides* and other genera in the human colon. *Appl Environ Microbiol* 67: 561-568.

Silver AC, Kikuchi Y, Fadl AA, Sha J, Chopra AK, Graf J 2007a. Interaction between innate immune cells and a bacterial type III secretion system in mutalistic and pathogenic associations. *Proc Natl Acad Sci USA* 104: 9481-9586.

Silver AC, Rabinowitz NM, Kuffer S, Graf. J 2007b. Identification of *Aeromonas veronii* genes required for colonization of the medicinal leech, *Hirudo verbana*. *J Bacteriol* 189: 6763-6772.

Sitailo LA, Zagariya AM, Arnold PJ, Vedantam G, Hecht. DW 1998. The *Bacteroides fragilis* BtgA mobilization protein binds to the oriT region of pBFTM10. *J Bacteriol* 180: 4922-4928.

Smith CJ, Parker AC 1998. The transfer origin for *Bacteroides* mobilizable transposon Tn4555 is related to a plasmid family from gram-positive bacteria. *J Bacteriol* 180: 435-439.

Smith CJ, Tribble GD, Bayley DP 1998. Genetic elements of Bacteroides species: a moving story. *Plasmid* 40: 12-29.

Smith H 1998. What happens to bacterial pathogens *in vivo*? *Trends Microbiol* 6: 239-243.

Sniegowski PD, Gerrish PJ, Lenski RE 1997. Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* 387: 703-705.

Sohel I, Puente JL, Ramer SW, Bieber D, Wu CY, Schoolnik GK 1996. Enteropathogenic *Escherichia coli*: identification of a gene cluster coding for bundle-forming pilus morphogenesis. *J Bacteriol* 178: 2613-2628.

Soto W, Gutierrez J, Remmenga MR, Nishiguchi MK 2008a. Synergistic affects of temperature and salinity in competing strains of symbiotic *Vibrio fischeri*. *Microbiol Ecol* DOI 10.1007/s00248-008-9412-9.

Soto W, Lostroh CP, Nishiguchi MK 2008. Physiological responses to stress in the Vibrionaceae: Aquatic microorganisms frequently affiliated with hosts. *In* Cooperation and stress in Biology, J Seckback, Grube M eds, Springer, New York, NY.

Soto W, Nishiguchi MK 2008. Synergistic affects of temperature and salinity in competing strains of symbiotic *Vibrio fischeri*. *Microbial Ecol*. DOI 10.1007/s00248-008-9412-9.

Stabb EV, Reich KA, Ruby EG 2001. *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD^+-glycohydrolases. *J Bacteriol* 183: 309-317.

Stabb EV, Ruby EG 2003. Contribution of *pilA* to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. *Appl Environ Microbiol* 69: 820-826.

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417: 959-962.

Sullivan JT, Patrick HN, Lowther WL, Scott DB, Ronson CW 1995. Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proc Nat Acad Sci USA* 92: 8985-8989.

Sullivan JT, Ronson CW 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-rRNA gene. *Proc Nat Acad Sci USA* 95: 5145-5149.

Tenaillon O, Taddei F, Radman M, Matic I 2001. Second-order selection in bacterial evolution: selection acting on mutation and recombination rates in the course of adaptation. *Res Microbiol* 152: 11-16.

Thomas NA, Finlay BB 2003. Establishing order for type III secretion substrates- a hierarchical process. *Trends Microbiol* 11: 398-403.

Thordal-Christensen H 2003. Fresh insights into processes of nonhost resistance. *Curr Opin Plant Biol* 6: 351-357.

Tilman D, Pacala S 1993. The maintenance of species richness in plant communities.*In* Species diversity in ecological communites, RE Ricklefs & SD eds, Chicago Press, Chicago.

Timmers ACJ, Soupène E, Auriac MC, de Billy F, Vasse J, Boistard P, Truchet G 2000. Saprophytic intracellular rhizobia in alfalfa nodules. *Mol Plant Microbe Interact* 13: 1204-1213.

Trosky JE, Liverman ADB, Orth K 2008. Yersinia outer proteins: Yops. *Cell Microbiol* 10: 557-565.

van Rhijn P, Fujishige NA, Lim PO, Hirsch AM 2001. Sugar-binding activity of pea (*Pisum sativum*) lectin is essential for heterologous infection of transgenic alfalfa (*Medicago sativa L.*) plants by *Rhizobium leguminosarum biovar viciae*. *Plant Physiol* 125: 133-144.

van Rhijn P, Goldberg RB, Hirsch AM 1998. *Lotus corniculatus* nodulation specificity is changed by the presence of a soybean lectin gene. *Plant Cell* 10: 1233-1250.

Vance CP 1983. *Rhizobium* infection and nodulation: a beneficial plant disease? *Annu Rev Microbiol* 37: 399-424.

Vedantam G, Novicki TJ, Hecht DW 1999. *Bacteroides fragilis* transfer factor Tn5520: the smallest bacterial mobilizable transposon containing single integrase and mobilization genes that function in *Escherichia coli*. *J Bacteriol* 181: 2564-2571.

Viprey V, Del Greco A, Golinowski W, Broughton WJ, Perret X 1998. Symbiotic interactions of type III protein secretion machinery in *Rhizobium*. *Mol Microbiol* 28: 1381-1389.

Visick KL, Foster JS, Doino JA, McFall-Ngai MJ, Ruby EG 2000. *Vibrio fischeri lux* genes play and important role in colonization and development of the host light organ. *J Bacteriol* 182: 4578-4586.

Visick KL, McFall-Ngai MJ 2000. An exclusive contract: Specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. *J Bacteriol* 182: 1779-1787.

Visick KL, Skoufos LM 2001. Two-component sensor required for normal symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. *J Bacteriol* 183: 835-842.

Wang X, Heazlewood SP, Krause DO, Florin TH 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. *J Appl Microbiol* 95: 508-520.

Wen F, VanEtten HD, Tsaprailis G, Hawes MC 2007. Extracellular proteins in pea root tip and border cell exudates. *Plant Physiol* 143: 773-783.

Wilkinson HH, Parker MA 1996. Symbiotic specialization and the potential for genotypic coexistence in a plant-bacterial mutualism. *Oecologia* 108: 361-367.

Wilkinson HH, Spoerke JM, Parker MA 1996. Divergence in symbiotic compatibility in a legume-*Bradyrhizobium* mutualism. *Evolution* 50: 1470-1477.

Wood DW, Setubal JC, Kaul R, and a o authors 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* 294: 2317-2323.

Young GM, Schmiel DH, Miller VL 1999. A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc Nat Acad Sci US* 96: 6456-6461.

Zipfel C 2008. Pattern-recognition receptors in plant innate immunity. *Curr Opin Immunol* 20.

Received August 1, 2007
Accepted May 28, 2008
Associate Editor: S v Boletzky

VIE ET MILIEU - LIFE AND ENVIRONMENT, 2008, 58 (2) : 175-184

THE EVOLUTIONARY ECOLOGY OF A SEPIOLID SQUID-VIBRIO ASSOCIATION: FROM CELL TO ENVIRONMENT

S.V. NYHOLM [1], M.K. NISHIGUCHI [2]*

[1] University of Connecticut, Department of Molecular and Cell Biology, BSP 405
91 North Eagleville Rd., Unit 3125, Storrs, CT 06269-3125
[2] New Mexico State University, Department of Biology, Box 30001, MSC 3AF
Las Cruces, NM 88003-8001
** Corresponding author: nish@nmsu.edu*

VIBRIO
MUTUALISM
SEPIOLID
EVOLUTION

ABSTRACT. – Mutualistic relationships between bacteria and their eukaryotic hosts have existed for millions of years, and such associations can be used to understand the evolution of these beneficial partnerships. The symbiosis between sepiolid squids (Cephalopoda: Sepiolidae), and their *Vibrio* bacteria (gamma Proteobacteria: Vibrionaceae), has been a model system for over 20 years, giving insight as to the specificity of the association, and whether the interactions themselves give rise to such finely tuned dialog. Since the association is environmentally transmitted, selection for specificity can evolve from a number of factors; abiotic (temperature, salinity), as well as biotic (host species, receptors, cell/cell interactions). Here, we examine the transition between these forces effecting the symbiosis, and pose possible explanations as to why this association offers many attributes for understanding the role of symbiotic competence.

INTRODUCTION

All metazoans form life-long beneficial partnerships with microbial symbionts. Most of these host-microbe interactions are highly specific and environmentally transferred, meaning that symbiont(s) must colonize the hosts each generation.

Bacterial associations that arise with these metazoan hosts have undergone substantial selective pressures that assure maintenance of specificity, as well as the ability of the microbial partner(s) to remain in the association without being detected by the host's immune capabilities (McFall-Ngai 2005). In order to establish and maintain such highly intimate associations, well-developed mechanisms must be in place to ensure and maintain successful colonization. These mechanisms often involve complex and well-regulated molecular signaling events that act as a type of "conversation" between the partners. Although the host and symbiont usually dictate a certain degree of selection through this molecular dialogue, particularly when mutualistic associations require that all partners participate in the relationship, it is the ecology that often determines whether symbionts that are environmentally transmitted possess a certain degree of flexibility each time they infect a new host (Colwell 1984). These life-history trade-offs between environment and host selection are equivalent in the manner in which they shape the evolution of these associations, as well as the interactions that are so important for maintaining stability within those populations (Anderson & May 1979).

A large number of mutualistic, marine associations have been vastly studied to examine the chasm between the ecology and cellular interactions that drive environmentally transmitted symbioses (Haddad *et al*. 1995, Haygood & Distel 1993, Hoegh-Guldberg *et al*. 2007, Newton *et al*. 2007). One type of symbiosis that has recently been developed as a model system includes the associations between sepiolid squids (Cephalopoda: Sepiolidae), and luminous bacteria (from the genera *Vibrio* and *Photobacterium*; McFall-Ngai & Ruby, 1991). Light organ symbioses occurring with luminous bacteria of the genera *Vibrio* and *Photobacterium* are found in two families of squids: Loliginidae and Sepiolidae (Fig. 1; Herring 1977, Mangold & Boletzky 1988). In most species of sepiolids, squids are bioluminescent, owing to the presence of bacterial symbionts contained in a complex, bilobed, light-emitting organ (Fig. 2; Montgomery & McFall-Ngai 1998, Nishiguchi *et al*. 2004). The light organ itself is used in a behavior termed counter-illumination or silhouette reduction (Jones & Nishiguchi 2004). Light produced by bacteria is used to match down-welling

Fig. 1 – The bobtail squid, *Euprymna scolopes*. Mantle length ~ 4 cm.

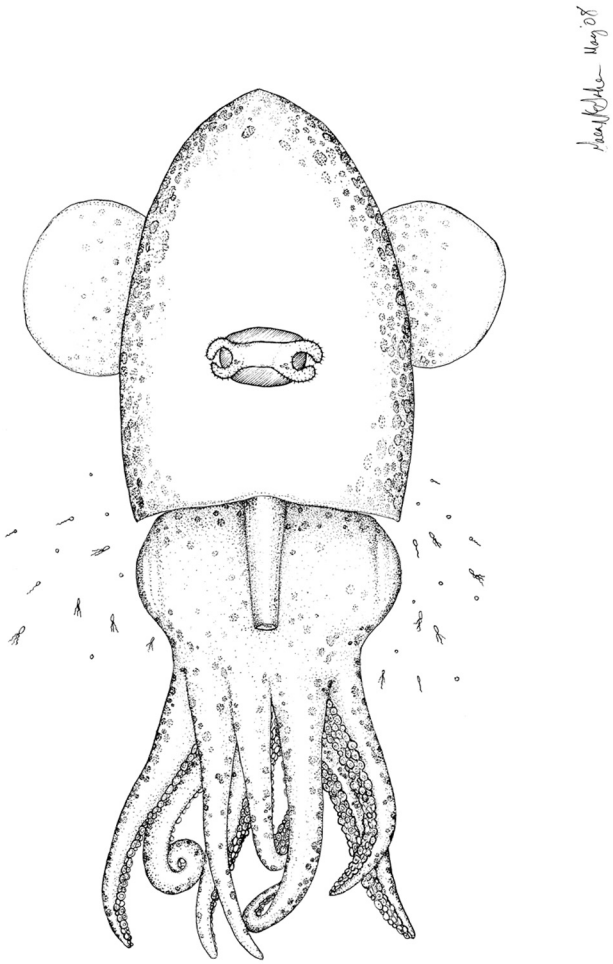


Fig. 2 – Cartoon diagram of *Euprymna*, with the placement of the light organ within the mantle cavity. During respiration/ventilation, the squid uptakes water laden with approximately 10^6 bacteria/mL. *Vibrio fischeri* bacteria comprise approximately < 1% of the total number of bacteria that are found in seawater surrounding host squids. Specificity may be influenced by changes in temperature, salinity, or competition between bacteria in the seawater prior to infection. Size of bacteria in reference to host squid is not to scale.

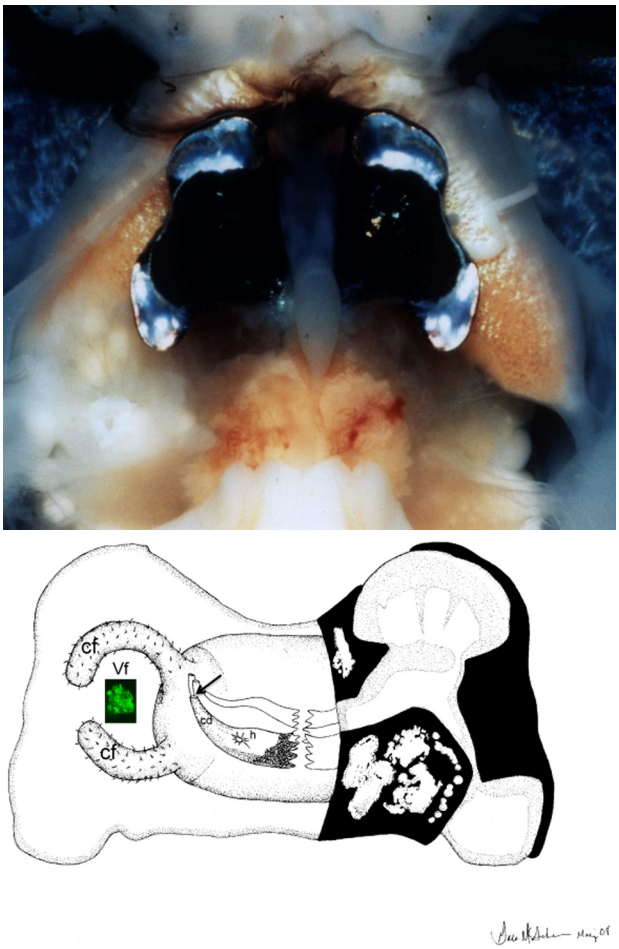


Fig. 3. – **A**, Central dissection of *E. scolopes*, exposing the light organ and ink sac surrounding the light organ complex. **B**, Schematic representation of the light organ, with one side exposed. After hatching, the ciliated fields (cf) create currents and secrete mucus that aggregate Gram-negative bacteria including cells of *Vibrio fischeri* (Vf, green) that out-compete non-symbiotic bacteria for space in these structures. Aggregated *V. fischeri* migrate through one of three pores (arrow) on either side of the light organ where they colonize the light organ after navigating past ciliated ducts (cd) and host hemocytes (h).

moonlight, so that squids avoid detection by predators or prey from below. The morphology of the light organ is such that all light is directed ventrally, which then diffuses through the mantle cavity and hides any shadow being produced (McFall-Ngai & Montgomery 1990). Emission of the bacterial luminescence is controlled in two ways: (i) by a host-modulated, diel (day/night fluctuations) restriction on the luminescent output per bacterial cell and, (ii) by a series of accessory tissues, which are functionally analogous to the tissues that modulate light quality in the eye (Boettcher *et al*. 1996).

One of these associations, that between the Hawaiian bobtail squid *Euprymna scolopes* and the bacterium *Vibrio fischeri*, has been extensively studied for the past 20 years, and has led to a number of rich discoveries for understanding the underlying mechanisms that regulate the interactions between bacteria and eukaryotic tissues (McFall-Ngai 2002). *E. scolopes* obtains bacteria from the environment each generation as a juvenile. The association is highly specific; *i.e*., only *V. fischeri* can colonize the tissues of a newly-hatched juvenile *E. scolopes* squid (McFall-Ngai & Ruby, 1991). The host squid houses its extracellular bioluminescent symbionts in a bilobed light organ that is part of the ink sac complex contained in the center of the squid's mantle cavity (Fig. 2, 3A). The light organ at this developmental stage is composed of a complex ciliated field with two sets of appendages that entrain seawater towards a set of six pores, three on each side of the light organ (Fig. 3B). Upon hatching, the host ventilats seawater containing a mixture of bacterial species through the mantle cavity. Free-living *V. fischeri* must then pass through these pores and migrate through ciliated ducts that terminate in three separate epithelia-lined crypt spaces on each side of the light organ (Fig. 4). It is in these

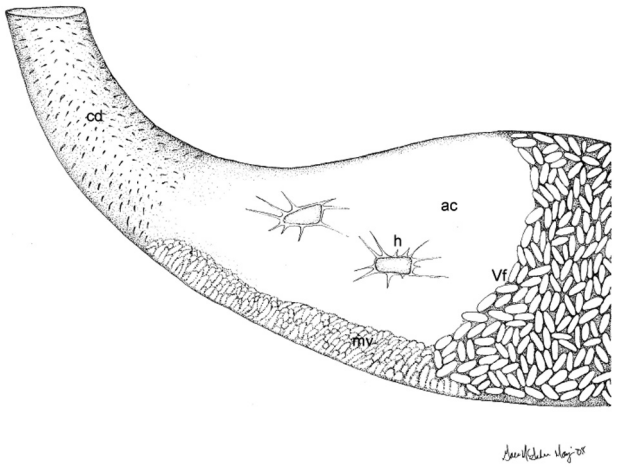


Fig. 4. – Diagram of one of the antechamber (ac) regions leading to the epithelial lined crypt spaces of the light organ. *V. fischeri* (Vf) must overcome a number of host-derived hurdles to successful colonization. Motility is required to traverse the ciliated duct (cd) where these cells also encounter potentially lethal host-derived reactive oxygen species. Successful *V. fischeri* cells swim through the ac and colonize microvilli (mv) of the epithelial cell surfaces of the light organ crypts. A number of cell-signaling events are involved with coordinating the molecular conversation needed for successful colonization (see text).

crypt spaces that colonization finally is established.

Over the past twenty years a number of studies have gone onto characterize a number of developmental effects that *V. fischeri* induces in the squid host, including programmed cell death or apoptosis, epithelial cell swelling, hemocyte trafficking, mucus shedding, and changes in host gene and protein expression (Chun *et al*. 2006, Doino Lemus & McFall-Ngai 2000, Foster & McFall-Ngai 1998, Kimbell & McFall-Ngai 2004, Koropatnick *et al*. 2004, Koropatnick *et al*. 2007, Lamarcq & McFall-Ngai 1998, McFall-Ngai & Ruby, 1991, Montgomery & McFall-Ngai 1994, Nyholm *et al*. 2002). It is the complexity of how *Vibrio* bacteria are first able to locate specific squid hosts in the aquatic environment, to sufficiently colonize this complex organ in a highly specific manner that pervades all levels of symbiosis: physiology, molecular specificity, immunology, and eventually speciation amongst different populations of *Vibrio* bacteria.

ABIOTIC FACTORS AND THE ENVIRONMENT

Vibrio bacteria are cosmopolitan species; they are commonly found in both fresh and oceanic waters including rivers and lakes, as well as a variety of marine habitats (coastal, pelagic deep sea) (Feldman & Buck 1984, Ramesh *et al*. 1989, Urakawa & Rivera 2006). They inhabit a number of ecological niches, such as within the natural bacterioplankton community (Colwell 1984), as saprophytes on dead or decaying matter (Andrews *et al*. 1984), as pathogens to both humans and other metazoans (Owens & Busico-Salcedo 2006, Wong & Wang 2004), and as commensals or mutualists with many marine invertebrates (Dunlap *et al*. 2007, Nyholm & McFall-Ngai 2004, Sawabe 2006). With such great breadth in their natural ability to adapt to such a wide diversity of habitats, it is of no surprise that variation exists among strains that are isolated from differing environments.

Abiotic factors have been previously shown to have substantial effects on the selection of highly adaptable Vibrio strains (Nealson & Hastings 1979). These include temperature, salinity, nutrient concentration, and UV irradiation (Czyz *et al*. 2000, Rosenberg *et al*. 2007, Ruby & Nealson 1978, Soto *et al*. 2008a, Soto *et al*. 2008b). For instance, temperature has significant effects on the shaping of ecological associations between *Vibrio* bacteria and their host organisms (Hilton *et al*. 2006, Larsen *et al*. 2004). Many virulence factors that influence colonization and infection are strongly regulated by temperature, and can have dramatic effects upon host populations, often killing large numbers of individuals (Rosenberg *et al*. 2007). For mutualistic Vibrios that colonize the light organs of sepiolid squids, there are multiple abiotic factors that may influence the ability of colonization to be effective, as well as which types of *Vibrio* species may be more prevalent in the light organ. Initial studies measuring the abundance and types of *V. fischeri* bacteria that were found in habitats containing *Euprymna scolopes* indicated that not only were symbiotically competent Vibrios found in Hawaiian waters, but many of those strains were not accountable by direct plating methods (Lee & Ruby 1995). Since sepiolid squid vent out 90-95% of their bacterial light organ composition each day with the onset of dawn (Boettcher *et al*. 1996), the bacteria released are viable and have a profound effect on the abundance of *V. fischeri* in habitats where host squids are abundant (Jones *et al*. 2007, Lee & Ruby 1994). This has been shown not only in Hawaiian *E. scolopes* populations (Lee & Ruby 1992), but in *E. tasmanica* populations throughout Australia (Jones *et al*. 2007). In addition, both morning and evening measurements infer that the diurnal venting behavior of *E. tasmanica* has an effect on the number of detectable bacteria in the water column during those times, particularly in Botany Bay, New South Wales, which is a semi- enclosed body of water with a constant population of *E. tasmanica*. The data suggests that interactions between *Vibrio* bacteria in the water column and resident host populations are very similar between the two *Euprymna* species.

In contrast, symbiotic *Vibrio* bacteria in Banyuls-sur-Mer, France, are more affected by temperature than by the behavior or specificity of resident host squids. *Sepiola*, which is the genus of sepiolids found in the Mediterranean, harbor two species of *Vibrio* bacteria, *V. fischeri* and *V. logei* (Fidopiastis *et al*. 1998). These two species of *Vibrio* are similar both genetically and in their phylogenetic placement among other Vibrios (Browne-Silva &

Nishiguchi 2008, Nishiguchi & Jones 2004, Nishiguchi & Nair 2003), but have subtle differences in their ecological niches. *V. logei* is a psychrophile, and is commonly found in colder temperatures compared to *V. fischeri*. Thus, when examining the population structure of *V. fischeri* and *V. logei* according to depth, the expected outcome was to observe differences in abundance between the two species. However, there was no difference in abundance of either species, despite previous results examining species of *Sepiola* collected at greater depths, which harbored more *V. logei* then *V. fischeri* (Nishiguchi 2000). What was evident from this study were differences between summer and winter collection periods of both *V. fischeri* and *V. logei*. Both species were significantly higher in their density in the winter sampling compared to the summer season, as well as the overall concentrations of bacteria in the Vibrionaceae. This may in part be due to the availability of nutrients during the winter when the thermocline disappears, as well as less competition for resources from other bacteria. Since both species are represented in higher concentrations during the winter than the summer months, this may also influence the amount of bacteria present in squids during those times of the year as well. Earlier studies suggest that no host specificity exists in either *V. fischeri* and *V. logei*, but rather temperature drives which *Vibrio* species is more prevalent in *Sepiola* (Nishiguchi 2000). Infection between these two species of *Vibrio* may be equally parsimonious, but may be temperature limited under high nutrient conditions (i.e., in squid light organs). Further studies examining the distribution of symbiotic *Vibrio* bacteria in areas where multiple species exist may help determine how strongly environment selects for specific features in competent bacteria, and whether factors such as temperature determines which symbiont is more capable of adapting and eventually evolving to a new ecological niche.

Salinity has also been a major abiotic factor for determining how well *Vibrio* bacteria are capable of infecting host tissues. All extant species of *Euprymna* are allopatric and found within the Indo-West Pacific, whereas species of *Sepiola* are found sympatrically in the Mediterranean Sea. Thus, *Vibrio* bacteria infecting *Euprymna* hosts are specialists, since they have a hierarchical degree to which they infect different *Euprymna* species (Nishiguchi *et al*. 1998). In contrast, *Sepiola* hosts share the same two species of *Vibrio*, and do not exhibit any competitive dominance during colonization (Nishiguchi 2000). Since there is a major difference as to how these two genera of sepiolid squids obtain their bacteria from the environment, there remains a number of mechanisms that influence how *Vibrio* bacteria respond to changes in salinity and temperature, both separately and synergistically. Previous evidence has already demonstrated how changes in osmolarity affect bioluminescence in symbiotic strains of *V. fischeri* ES114 (Stabb *et al*. 2004). Similarly, comparison of different *V. fischeri* strains from a number of *Euprymna* and *Sepiola* host species shows that *V. fischeri* strains from habitats with greater variation (*V. fischeri* ET101, from Melbourne, Australia), appear more sensitive to changes in both salinity and temperature than strains from more homogeneous environments (*V. fischeri* ES114, Kaneohe Bay, Hawaii; Soto *et al*. 2008a). The same is true for *V. fischeri* strains isolated from two closely related sympatric host species (*E. morsei* and *E. berryi*), but isolated from different habitats (Northern *vs*. Southern Japan). Both strains respond differently to salinity and temperature changes, and may be due to physiological differences resulting from evolution within their respective thermal niches. Interestingly, synergistic effects were also observed between strains grown under colder temperatures and lower salinities (12°C/24 ppt), where microbial allelopathy may also have an important role in determining competitive dominance (Soto *et al*. 2008a, Soto *et al*. 2008b). Therefore, competitive dominance in genera such as *Euprymna* may not be the sole result of native *Vibrio* symbionts having faster generation times than non-native ones upon colonization. Rather, *V. fischeri* strains may be competing prior to their infection while residing in the free-living bacterioplankton community, where the environment is much like minimal media relative to the nutrient rich light organ habitat.

ENVIRONMENTAL TRANSMISSION AND THE POPULATION ECOLOGY BETWEEN SEPIOLID SQUIDS AND *VIBRIO* BACTERIA

Environmentally transmitted symbionts are often subjected to broad and changing environmental regimes, where various factors select for adaptations that are suited for (1) host colonization and persistence, (2) the free-living pre-infective state, or (3) both ecological niches. Yet, broadly distributed host-symbiont populations, like those found in the sepiolid squid-*Vibrio* mutualism, may be subjected to different selective conditions that may result in different population structures due to adaptations occurring in the symbiont. Interestingly, while much work has been completed to identify cospeciating host/symbiont assemblages through studies of parallel cladogenesis (Nishiguchi 2002, Nishiguchi *et al*. 1998), few studies have examined how hosts or their environment may dictate symbiont genotypes, and whether symbionts are capable of host switching across large geographical distances through ecological adaptation.

With the exception of two genera, all sepiolid squids possess a light organ containing luminescent bacteria (Nesis 1982). Studies identifying symbiotic bacteria from different host squids indicate that the composition from each species of sepiolid is comprised of only one to three species of *Vibrio* (*V. fischeri*, *V. logei*, and *V. harveyi*) or *Photobacterium* (*P. leiognathi*), and that no other species of bacteria are found present inside the adult light organ

(Fidopiastis *et al*. 1998, Guerrero & Nishiguchi 2007, Nishiguchi *et al*. 2004). Since light organ pores are continually open to the surrounding seawater providing access for any type of bacterium into the light organ crypts (Fig. 2), the presence of only these species of *Vibrio* or *Photobacterium* in the light organ illustrates the specificity that prevents other types of bacteria from entering and colonizing the light organ (Visick & Ruby 2006).

Vibrio specificity is also hierarchical; all symbiotically competent vibrios are capable of colonizing sepiolid light organs, but native strain vibrios are better suited to colonize the crypts of their own squid host light organ when compared to non-native *Vibrio* competitors (Nishiguchi 2002, Nishiguchi *et al*. 1998). Because viable competent bacteria are "vented" every morning at dawn, this behavior selects for bacteria that have evolved specificity to a particular host, yet must still manage to survive in the surrounding environment once exuded from the light organ (Jones & Nishiguchi 2006, Nyholm & McFall-Ngai 2004).

Population studies examining both sepiolid squids and *Vibrio* bacteria have provided a roadmap for understanding the large-scale dynamics of how symbiotic bacteria are environmentally transmitted within and between host populations. Using haplotype networks in combination with nested clade analysis, variation among three species of *Euprymna* from the Indo-West Pacific (*E. scolopes*, Hawaii; *E. tasmanica*, Australia; and *E. hyllebergi*, Thailand) were examined to determine whether cospeciation was prevalent among all three species pairs (Jones *et al*. 2006). *Euprymna* species were genetically distinct from each other, with little or no migration over large geographical distances. In contrast, *Vibrio* populations contained a much more diverse number of haplotypes, suggesting that both host specificity as well as abiotic factors facilitating long-distance migration determines the population structure of the symbionts. This was especially prevalent between populations of *E. tasmanica* that were separated by temperature gradients (Maugean zone), with specific haplotypes affiliated with either colder (Melbourne and Tasmania) or warmer (Sydney, Great Barrier Reef) water populations.

Preliminary results from genetic studies of sympatric Mediterranean squid-*Vibrio* populations demonstrate separation among different host species as compared to their symbiont populations. Squid haplotypes were geographically localized, with little genetic variability among individuals of the same species from different populations (*i.e*., *S. affinis*). No shared haplotypes were found among different species of *Sepiola*. Conversely, *V. fischeri* and *V. logei* populations were found to be homogenzied in the same area, regardless of the squid host they were isolated from. Similar *Vibrio* haplotypes were found in hosts collected from both Banyuls-sur-Mer (S. France) and Bari, Italy (Adriatic Sea). In other words, a higher degree of genetic variation was found across the Western Mediterranean in terms of the symbiont population. This seems to point at the physical mobility and environmental range of the symbionts and not host specificity. These observations are in concordance with *Vibrio* populaion data from the Indo-west Pacific, where temperature, salinity, and currents either keep particular strains restricted (Maugean zone), or, if conditions are similar, provide inter-clonal exchange between large geographical distances (NE Australia, Hawaii, and Thailand with secondary recolonization events occurring in Hawaii (Jones *et al*. 2006)). *Vibrio* bacteria that are vented daily may be under direct selection by abiotic factors such as water movement, salinity, and temperature, which influences their distribution beyond host movement (Soto *et al*. 2008a, Soto *et al*. 2008b). Interestingly, temperature and salinity gradients between *Vibrio* populations such as those in the Mediterranean and the Adriatic Seas are relatively similar, which may provide a selective advantage for non-native vibrios invading environments where different host species reside at similar conditions. Therefore, if temperature, salinity, and currents keep particular strains restricted, can adaptation occur rapidly enough for *V. fischeri* symbionts to invade new host populations? If so, can temperature and salinity adaptation effect squid-host colonization in habitats where there may be mixing of multiple strains adapted to the same environmental regime? Are the genetic mechanisms for colonization similar enough to "leap frog" from one population to another to infiltrate new host populations, or does adaptation to warmer temperatures select for strains with a higher fitness at increased temperatures?

OVERCOMING CHALLENGES OF ENVIRONMENTAL TRANSMISSION

Horizontal transmission of symbionts often pose a problem for both partners in that both the host and symbiont must locate each other and successfully colonize and maintain the association each generation. In the *E. scolopes*/*V. fischeri* association the host and symbiont have evolved mechanisms for ensuring successful colonization very early in the relationship. Why are these mechanisms necessary in this association? Every half-second the juvenile squid ventilates about 1.3 ml of ambient seawater through its mantle cavity. Comprising less than 0.1% of the total ambient bacteria, *V. fischeri* occurs at fewer than 500 cells/ml in nature. Thus, on average no more than a single *V. fischeri* cell, occupying about one-millionth the volume of the mantle cavity, will be present during each ventilation. Without mechanisms to harvest them, the symbionts would have to find one of the six 15-μm pores on the light organ surface in less than one second before being expelled out of the mantle cavity and back into the environment. How do the partners overcome

these physical and environmental challenges?

In response to peptidoglycan (a major cell wall component of bacteria) the squid host begins to secrete mucus from the ciliated fields (Nyholm & McFall-Ngai 2004, Nyholm *et al*. 2000). *V. fischeri* along with a variety of other Gram-negative bacteria are able to aggregate in this host-derived mucus. Mucus secretions are extremely common in nature and a number of marine organisms use these secretions to harvest small particles from the water column, usually in feeding behaviors. However, in this association, the collection of bacteria from the environment is not one-sided nor is it a passive process. By some, as yet undescribed mechanism, *V. fischeri* is able to outcompete other environmental bacteria in the mucus biofilm and excludes these other cells in the aggregations before successfully colonizing the light organ (Nyholm & McFall-Ngai 2003). The mechanisms underlying this competitive advantage are not known, but *V. fischeri* is able to display positive chemotaxis towards sialic acid, a common component of the host mucus (DeLoney-Marino *et al*. 2003). Bacterial motility is also important during these initial stages of aggregation. Although non-motile mutants of *V. fischeri* can aggregate, they cannot go on to colonize the light organ (see below). Hyper-motile mutants are deficient in both their ability to form aggregations in the host mucus biofilms and thus do not colonize the light organ (Millikan & Ruby 2004).

RUNNING THE GAUNTLET

Once the symbionts aggregate in the host mucus they must then migrate through this mucus to the pores on the surface of the juvenile light organ (Figs. 3 & 4). When they breach this border, they face an assault from a number of host factors that help to ensure that only *V. fischeri* will colonize the light organ crypt spaces. First, these cells must traverse a long ciliated duct with very active host-derived currents that direct seawater out of the pores and into the mantle cavity. Once again, symbiont motility is critical in navigating this physically stressful environment. Non-motile mutants of *V. fischeri*, although capable of forming aggregations, the first step of the colonization process, never make it through the ciliated ducts and are thus excluded from the light organ.

Besides these physical barriers to colonization, the host, armed with an arsenal of reactive oxygen species (ROS), including a squid halide peroxidase and nitric oxide (NO), also presents a challenging chemical milieu to any entering bacterial cell (Davidson *et al*. 2004, Weis *et al*. 1996). In response to these assaults, the symbiont has evolved countermeasures to these host barriers. *V. fischeri* gets around this first challenge by possessing a periplasmic catalase that scavanges hydrogen peroxide, the critical substrate necessary for the production of hypohalous acid, the microbiocidal end product of the reaction catalyzed by the squid halide peroxidase (Visick & Ruby 1998). Nitric oxide and nitric oxide synthase (NOS) are both ROS species that can be associated with host innate immune responses and have been found in the ciliated fields, ducts, and crypt spaces of aposymbiotic (uncolonized) hatchling squid. After colonization both NO and NOS expression are reduced in the squid host, presumably in response to some as yet undescribed symbiont signal (Davidson *et al*. 2004, Visick & Ruby 1998).

Once *V. fischeri* crosses through the ducts they swim through a large epithelial-lined space referred to as the "antechamber" and reach the narrow epithelial crypt spaces where colonization and subsequent growth finally takes place (Sycuro *et al*. 2006). One additional hurdle that these potential symbionts encounter is a population of host macrophage-like hemocytes that appear to act as sentinels in this microenvironment (Nyholm & McFall-Ngai 1998).

Phagocytic hemocytes or macrophages are ubiquitous throughout the animal kingdom where they play an important role in innate defense against pathogens. Among invertebrates, which lack the acquired immune response associated with antibody production, phagocytic hemocytes often play a critical role in host defense (Kurata *et al*. 2006, Stuart & Ezekowitz 2008). In mollusks, phagocytic hemocytes are reported to be involved in defense against pathogens in many tissues and their associated lumina, and the ability of these cells to engulf potentially pathogenic bacteria has been reported (Bayne *et al*. 2001, Canesi *et al*. 2002). However, the role these cells may play in interacting with beneficial bacteria is poorly understood. *E. scolopes* hemocytes traverse the epithelium into the crypt spaces where the symbionts reside and appear to 'sample' these spaces, not unlike the way that mammalian blood cells sample enteric microbiota (Fig. 4). Within the crypts of newly colonized juvenile *E. scolopes*, hemocytes have been observed with internalized bacterial cells (Nyholm & McFall-Ngai 1998). However, within the crypts of adult squid, hemocytes have never been observed to have engulfed bacteria although they are entirely surrounded by *V. fischeri* cells. These preliminary observations suggest that the squid's hemocytes change in response to the persistent presence of the symbionts, perhaps as part of the complex developmental program induced in the host by *V. fischeri*. The role that these hemocytes play in the light organ crypt spaces is at present unknown. They may be there as part of the regulatory function of the host used to maintain the symbiont population, or perhaps they prevent non-symbiotic interlopers from taking hold and out-competing the native symbiont population. Current studies in this system are focusing on how these immune cells interact with symbiotic and non-symbiotic environmental bacteria common to the host's natural environment and how these responses may change during development.

MOLECULAR BASIS OF THE CONVERSATION

Specificity, at least in the *E. scolopes*/*V. fischeri* symbiosis, appears to be established by a number of events, each of which appears to be well regulated and mediated through a fine-tuned molecular conversation that occurs between the symbiont and host. A number of genomics tools have recently become available to researchers that study this association, including a completed and annotated genome for *V. fischeri* and a cDNA expression library for colonized and uncolonized host light organs at various time points during the first three days of the symbiosis (Chun *et al*. 2006, Ruby *et al*. 2005). These new tools will serve as a roadmap for teasing apart and interpreting the host and symbiont genes involved with regulating this conversation. A number of experimental studies have also added insight into how some aspects of this dialogue work. For example, many of the symbiont-induced developmental phenotypes that have been observed in the host appear to be caused by microbial-associated molecular patterns (MAMPs) associated with the symbiont. *V. fischeri* lipopolysaccharide (LPS), peptidoglycan, and a derivative of peptidoglycan called tracheal cytotoxin (TCT) have been shown to induce host apoptosis, mucus secretion and cell regression respectively in *E. scolopes* (Foster *et al*. 2000, Koropatnick *et al*. 2004, Nyholm *et al*. 2002).

The host, in turn must have receptors and well-regulated signaling cascades to recognize and interpret these symbiont cues. MAMPs, especially those constituents that contribute to the cell walls and outer membranes of bacteria are known to interact with a variety of these animal and plant cell receptors (Bittel & Robatzek 2007, Nurnberger *et al*. 2004). A family of peptidoglycan recognition proteins (PGRPs) is known to exist in diverse animal phyla from flies to humans (Chaput & Boneca 2007). Recently, four homologues of the PGRP family have been described in *E. scolopes* from sequencing of an expressed sequence tag (EST) gene expression library (Chun *et al*. 2006, Goodson *et al*. 2005). It is presently unknown what role these receptors may play in the association, but ongoing research is being conducted to determine when and where these PGRPs are expressed during development and if their expression changes in response to the symbiont (M McFall-Ngai, pers comm). MAMPs are also known to stimulate host signaling and gene expression via the Toll/NFkB signaling pathway, another evolutionarily conserved signaling pathway for which *E. scolopes* has several homologues, although like the PGRPs, the function has yet to be determined (Goodson *et al*. 2005).

Many of these signaling pathways are directly tied to effector mechanisms of immune responses. Increasing evidence suggests that in both invertebrates and vertebrates, the host innate immune system plays a critical role in mediating communication between hosts and microbes and these "communications" maybe far more complex than previously thought (Dethlefsen *et al*. 2007). The "simplicity" of invertebrate models has proven valuable in our understanding of the function of these highly conserved molecular interactions. However, the squid/bioluminescent bacterial associations offer the broader research community an opportunity to go a step further and explore how specificity between animal hosts and symbionts can evolve and become fine-tuned to distinguish between a wide range of closely-related hosts and symbionts. Previous work has demonstrated a hierarchy to colonization between different strains of *V. fischeri* and their squid hosts (Nishiguchi *et al*. 1998). Such highly evolved specificity likely involves, not only adaptation to the physical environmental niche of each association but, also selection for highly specific receptor/ligand interactions.

SUMMARY

Interactions between hosts, symbionts, and the environment are becoming more complex as we progress to a better understanding of the molecular dialog between the partners. Environmentally transmitted symbiosis in particular, are set in a natural tug-of-war between balancing selective pressures from both the environment and the host that houses the symbiosis. Sepiolid squids and their *Vibrio* bacteria in general have a number of strategies that ensure that the association is successful (specificity), as well as allowing enough flexibility to allow adaptation to occur between various populations of host squids. This alternative strategy of accommodating a number of ecologically adaptable *Vibrio* strains not only allows for squid hosts to select for the best-fit symbionts, but also permits the bacteria to rapidly change in response to both the host and the environment in which the association is found (Gillespie & Turelli 1989, Leroi *et al*. 1994). Understanding how bacteria are capable of adapting to fluctuating environments has many implications that can be extrapolated to how organisms evolve both during and after transitions between habitats. Indeed, factors such as phenotypic plasticity, genetic polymorphisms, and a wide ecological breadth will influence how an organism responds to a new environment, and whether the phenotypic or genotypic response to the environment or host improves the fitness of the individual after those changes have occurred (Parmesan *et al*. 2005, Schlichting & Pigliucci 1993). Clearly, there is a need to understand how quickly organisms can adapt to different environments; with the noticeable change in global climate, it is to our benefit to determine if these changes will increase or decrease biodiversity on a much larger scale. This process is one of the major factors that will determine whether an organism will succeed or eventually go extinct in our world today.

ACKNOWLEDGEMENTS. - SVN's research is supported in part by the University of Connecticut start up funding. MKN's research is supported in part by NIH-NIAID 1SC1AI081659-01 and NSF IOS-0744498.

REFERENCES

Anderson RM, May RM 1979. Population biology of infectious diseases: part 1. *Nature* 280: 361-367.

Andrews CC, Karl DM, Small LF, Fowler SW 1984. Metabolic activity and bioluminescence of oceanic faecal pellets and sediment trap particles. *Nature* 307: 539-541.

Bayne CJ, Hahn UK, Bender RC 2001. Mechanisms of molluscan host resistance and of parasite strategies for survival. *Parasitology* 123 Suppl: S159-167.

Bittel P, Robatzek S 2007. Microbe-associated molecular patterns (MAMPs) probe plant immunity. *Curr Opin Plant Biol* 10: 335-341.

Boettcher KJ, Ruby EG, McFall-Ngai MJ 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J Comp Physiol B* 179: 65-73.

Browne-Silva J, Nishiguchi MK 2008. Gene sequence of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int J Syst Evol MIcrobiol* 58: 1292-1299.

Canesi L, Gallo G, Gavioli M, Pruzzo C 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. *Microsc Res Tech* 57: 469-476.

Chaput C, Boneca IG 2007. Peptidoglycan detection by mammals and flies. *Microbes Infect* 9: 637-647.

Chun CK, Scheetz TE, Bonaldo Mde F, Brown B, Clemens A, Crookes-Goodson W J, Crouch K, DeMartini T, Eyestone M, Goodson MS, Janssens B, Kimbell JL, Koropatnick TA, Kucaba T, Smith C, Stewart JJ, Tong D, Troll JV, Webster S, Winhall-Rice J, Yap C, Casavant TL, McFall-Ngai MJ, Soares MB 2006. An annotated cDNA library of juvenile *Euprymna scolopes* with and without colonization by the symbiont *Vibrio fischeri*. *BMC Genomics* 7: 154.

Colwell RR 1984. Vibrios in the environment. John Wiley and Sons, New York.

Czyz A, Wrobel B, Wegrzyn G 2000. *Vibrio harveyi* bioluminescence plays a role in stimulation of DNA repair. *Microbiol* 146: 283-288.

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L, McFall-Ngai MJ 2004. NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol* 6: 1139-1151.

DeLoney-Marino CR, Wolfe AJ, Visick KL 2003. Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and N-actylneuraminic acid, a component of squid light-organ mucus. *Appl Environ Microbiol* 69: 7527-7530.

Dethlefsen L, McFall-Ngai M, Relman DA 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449: 811-818.

Doino Lemus J, McFall-Ngai MJ 2000. Alterations in the proteome of the *Euprymna scolopes* light organ in response to symbiotic *Vibrio fischeri*. *Appl Environ Microbiol* 66: 4091-4097.

Dunlap PV, Ast JC, Kimura S, Fukui A, Yoshino T, Endo H 2007. Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics* 23: 507-532.

Feldman KA, Buck JD 1984. Distribution and characterization of luminescent bacteria in a temperate estuary. *Estuaries* 7: 93-97.

Fidopiastis P M, Boletzky Sv, Ruby EG 1998. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. *J Bacteriol* 180: 59-64.

Foster JS, Apicella MA, McFall-Ngai MJ 2000. *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. *Dev Biol* 226: 242-254.

Foster JS, McFall-Ngai MJ 1998. Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Dev Genes Evol* 208: 295-303.

Gillespie JH, Turelli M 1989. Genotype-environment interaction and the maintenance of polygenic variation. *Genetics* 121: 129-138.

Goodson MS, Kojadinovic M, Troll JV, Scheetz TE, Casavant TL, Soares MB, McFall-Ngai MJ 2005. Identifying components of the NF-kappaB pathway in the beneficial *Euprymna scolopes-Vibrio fischeri* light organ symbiosis. *Appl Environ Microbiol* 71: 6934-6946.

Guerrero RC, Nishiguchi MK 2007. Identification of light organ symbionts from the genera *Uroteuthis*, *Loliolus*, and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* 23: 1-10.

Haddad A, Camacho F, Durand P, Cary SC 1995. Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete *Alvinella pompejana*. *Appl Environ Microbiol* 61: 1679-1687.

Haygood MG, Distel DL 1993. Bioluminescent symbionts of flashlight fishes and deep-sea anglerfishes from unique lineages related to *Vibrios*. *Nature* 363: 154-156.

Herring PJ 1977. Luminescence in Cephalopods and Fish. *Symp Zool Soc Lond* 38: 127-159.

Hilton T, Rosche T, Froelich B, Smith B, Oliver J 2006. Capsular polysaccharide phase variation in *Vibrio vulnificus*. *Appl Environ Microbiol* 72: 6986-6993.

Hoegh-Guldberg O, Muller-Parker G, Cook CB, Gates RD, Gladfelter E, Trench RK, Weis VM 2007. Len Muscatine (1932-2007) and his contributions to the understanding of algal-invertebrate endosymbiosis. *Coral Reefs* 26: 731-739.

Jones BW, Lopez JL, Huttenberg J, Nishiguchi MK 2006. Population structure between environmentally transmitted Vibrios and bobtail squids using nested clade analysis. *Mol Ecol* 15: 4317-4329.

Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK 2007. Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. *Microbiol Ecol* 54: 314-323.

Jones BW, Nishiguchi MK 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Mar Biol* 144: 1151-1155.

Jones BW, Nishiguchi MK 2006. *Vibrio fischeri* transcripts reveal adaptations in an environmentally transmitted symbiosis. *Can J Microbiol* 52: 1218-1227.

Kimbell JR, McFall-Ngai MJ 2004. Symbiont-induced changes in host actin during the onset of a beneficial animal-bacterial association. *Appl Environ Microbiol* 70: 1434-1441.

Koropatnick TA., Engle JT, Apicella MA, Stabb EV, Goldman WE, McFall-Ngai MJ 2004. Microbial factor-mediated development in a host-bacterial mutualism. *Science* 306: 1186-1188.

Koropatnick TA, Kimbell JR, McFall-Ngai MJ 2007. Responses of host hemocytes during the initiation of the squid-*Vibrio* symbiosis. *Biol Bull* 212: 29-39.

Kurata S, Ariki S, Kawabata S 2006. Recognition of pathogens and activation of immune responses in *Drosophila* and horseshoe crab innate immunity. *Immunobiology* 211: 237-249.

Lamarcq LH, McFall-Ngai MJ 1998. Induction of a gradual, reversible morphogenesis of its host's epithelial brush border by *Vibrio fischeri*. *Infect Immun* 66: 777-785.

Larsen MH, Blackburn N, Larsen JL, Olsen JE 2004. Influences of temperature, salinity and starvation on the motility and chemotactic response of *Vibrio anguillarum*. *Microbiology* 150: 1283-1290.

Lee KH, Ruby EG 1992. Detection of the light organ symbiont, *Vibrio fischeri* in Hawaiian seawater using *lux* gene probes. *Appl Environ Microbiol* 58: 942-947.

Lee KH, Ruby EG 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl Environ Microbiol* 60: 1565-1571.

Lee KH, Ruby EG 1995. Symbiotic role of the viable but nonculturable state of *Vibrio fischeri* in Hawaiian coastal seawater. *Appl Environ Microbiol* 61: 278-283.

Leroi AM, Lenski RE, Bennett AF 1994. Evolutionary adaptation to temperature. III. Adaptation of *Escherichia coli* to a temporally varying environment. *Evolution* 48: 1222-1229.

Mangold K, Boletzky Sv 1988. Mediterranean cephalopod fauna. I*n* The Mollusca, MR Clarke & Trueman ER eds. Academic Press, San Diego, Calif: 315-330

McFall-Ngai MJ 2002. Unseen forces: the influence of bacteria on animal development. *Dev Biol* 242: 1-14.

McFall-Ngai MJ 2005. The interface of microbiology and immunology: A comparative analysis of the animal kingdom. *In* The influence of cooperative bacteria on animal host biology, MJ McFall-Ngai, B Henderson & EG Ruby eds Cambridge University Press, New York, NY: 35-56

McFall-Ngai MJ, Montgomery MK 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). *Biol Bull* 179: 332-339.

McFall-Ngai MJ, Ruby EG 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* 254: 1491-1494.

Millikan DS, Ruby EG 2004. *Vibrio fischeri* flagellin A is essential for normal motility and for symbiotic competence during initial squid light organ colonization. *J Bacteriol* 186: 4315-4325.

Montgomery MK, McFall-Ngai M 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. *Development* 120: 1719-1729.

Montgomery MK, McFall-Ngai MJ 1998. Late postembryonic development of the symbiotic light organ of *Euprymna scolopes* (Cephalopoda: Sepiolidae). *Biol Bull* 195: 326-336.

Nealson KH, Hastings JW 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev* 43: 496-518.

Nesis KN 1982. Cephalopods of the world. TFH Publications, Neptune City, NJ.

Newton ILG, Woyke T, Auchtung TA, Dilly GF, Dutton RJ, Fisher MC, Fontanez KM, Lau E, Stewart FJ, Richardson PM, Barry KW, Saunders E, Detter JC, Wu D, Eisen JA, Cavanaugh CM 2007. The *Calyptogena magnifica* chemoautotrophic symbiont genome. *Science* 315: 998-1000.

Nishiguchi MK 2000. Temperature affects species distribution in symbiotic populations of *Vibrio*. *Appl*. *Environ Microbiol* 66: 3550-3555.

Nishiguchi MK 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microbiol Ecol* 44: 10-18.

Nishiguchi MK, Jones BW 2004. Microbial biodiversity within the Vibrionaceae. *In* Origins, evolution, and the biodiversity of microbial life, J Seckback ed Cole-Kluwer Academic Publishers, Dordrecht, The Netherlands: 531-548.

Nishiguchi MK, Lopez JE, Boletzky Sv 2004. Enlightenment of old ideas from new investigations: The evolution of bacteriogenic light organs in squids. *Evol Dev* 6: 41-49.

Nishiguchi MK, Nair VA 2003. Evolution of pathogenicity and symbioses in Vibrionaceae: An combined approach using molecules and physiology. *Int J Syst Bacteriol* 53: 2019-2026.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in Sepiolid squid-vibrio symbioses. *Appl Environ Microbiol* 64: 3209-3213.

Nurnberger T, Brunner F, Kemmerling B, Piater L 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev* 198: 249-266.

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA, McFall-Ngai MJ 2002. Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* 68: 5113-5122.

Nyholm SV, McFall-Ngai MJ 1998. Sampling the light-organ microenvironment of *Euprymna scolopes*: description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri*. *Biol Bull* 195: 89-97.

Nyholm SV, McFall-Ngai MJ 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl Environ Microbiol* 69: 3932-3937.

Nyholm SV, McFall-Ngai MJ 2004. The winnowing: establishing the squid-vibrio symbiosis. *Nat Rev Microbiol* 2: 632-642.

Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ 2000. Establishment of an animal-bacterial association: recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci USA* 97: 10231-10235.

Owens L, Busico-Salcedo N 2006. *Vibrio harveyi*: pretty problems in paradise. *In* The Biology of the Vibrios, BAJSFL Thompson, ed ASM Press, Washington, DC: 266-280

Parmesan C, Gaines S, Gonzalez L, Kaufman DM, Kingsolver J, Townsend Peterson A, Sagarin R 2005. Empirical perspectives on species borders: from traditional biogeography to global climate change. *Oikos* 108: 58-75.

Ramesh A, Loganathan BG, Venugopalan VK 1989. Seasonal distribution of luminous bacteria in the sediments of a tropical estuary. *J Gen Appl MIcrobiol* 35: 363-368.

Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I 2007. The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology* 5: 355-362.

Ruby EG, Nealson KH 1978. Seasonal changes in the species composition of luminous bacteria in nearshore seawater. *Limnol Oceangr* 23: 530-533.

Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, Lostroh P, Lupp C, McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Whistler K Visick C, Greenberg EP 2005. Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc Natl Acad Sci USA* 102: 3004-3009.

Sawabe T 2006. The mutual partnership between *Vibrio halioticoli* and abalones. *In* The Biology of the Vibrios, FL Thompson, B Austin & J Swings eds ASM Press, Washington, DC: 219-230.

Schlichting CD., Pigliucci M 1993. Control of phenotypic plasticity via regulatory genes. *Am Nat* 142:366-370.

Soto W, Gutierrez J, Remmenga MD, Nishiguchi MK 2008a. Synergistic effects of temperature and salinity on *Vibrio* symbionts from the Indo-west Pacific. *Microbiol Ecol* DOI 10.1007/s00248-008-9412-9.

Soto W, Lostroh CP, Nishiguchi MK 2008b. Physiological responses to stress in the Vibrionaceae: Aquatic microorganisms frequently affiliated with hosts. *In* Cooperation and stress in biology, J Seckback & Grube M eds Springer, New York, NY: in press.

Stabb EV, Butler MS, Adin DM 2004. Correlation between osmolarity and luminescence of symbiotic *Vibrio fischeri* strain ES114. *J Bacteriol* 186: 2906-2908.

Stuart LM, Ezekowitz RA 2008. Phagocytosis and comparative innate immunity: learning on the fly. *Nat Rev Immunol* 8: 131-141.

Sycuro LK, Ruby EG, McFall-Ngai M 2006. Confocal microscopy of the light organ crypts in juvenile *Euprymna scolopes* reveals their morphological complexity and dynamic function in symbiosis. *J Morphol* 267: 555-568.

Urakawa H, Rivera ING 2006. Aquatic environment. *In* The Biology of the Vibrios FL Thompson, B Austin, J Swings eds ASM Press, Washington, DC: 175-189.

Visick KL, Ruby EG 1998. The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. *J Bacteriol* 180: 2087-2092.

Visick KL, Ruby EG 2006. *Vibrio fischeri* and its host: it takes two to tango. *Curr Opin Microbiol* 9: 632-638.

Weis VM, Small AL, McFall-Ngai MJ 1996. A peroxidase related to the mammalian antimicrobial protein myeloperoxidase in the *Euprymna-Vibrio* mutualism. *Proc Natl Acad Sci USA* 93: 13683-13688.

Wong HC, Wang P 2004. Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *J Applied Microbiol* 96: 359-366.

Received May 16, 2008
Accepted June 2, 2008
Associate Editor: S v Boletzky

Nishiguchi, M.K. and R. Mapes. 2008. Cephalopoda. In: W. Ponder and D. Lindberg (eds), Phylogeny and Evolution of the Mollusca. University of California Press, Berkeley, CA, pp. 163-199.

8

Cephalopoda

Michele K. Nishiguchi and Royal H. Mapes

Cephalopoda is one of the most intriguing and diverse classes of molluscs. Modern forms comprise the octopuses, squids, cuttlefish, and pearly nautilus (Figure 8.1). Cephalopods differ greatly from other molluscs—they are more active, fast-moving, intelligent carnivores, with highly advanced visual and nervous systems that allow them to be competitive and efficient predators. Their ability to sense their surrounding environment and adapt rapidly using camouflage or complex behavioral patterns, which have been observed during courtship, reproduction, and mating, demonstrates how complex these animals have become.

All fossil and modern taxa are marine, with a few found in estuarine habitats (as low as 15 ppt salinity). Fossil forms include the ammonoids, which became extinct 65 million years ago; the nautiloids (both orthoconic and coiled), of which *Nautilus* and *Allonautilus* are the only living descendants; and the Coleoidea, the order that accommodates all other living cephalopods.

Modern cephalopods have gained notoriety through being the subject of myths or science fiction (e.g., Verne 1896) and as an important food source. Importantly, they are used as model systems for a large variety of research studies (Hodgkin and Huyley 1952; Makman and Stefano 1984), including areas such as neurobiology, behavior, physiology, development, symbiosis, and growth (Arnold 1962; Hanlon *et al.* 1990; Gilly and Lucero 1992; Boletzky 2002; Forsythe *et al.* 2002; Boletzky 2003; Lee *et al.* 2003). There are close to 1,000 species of living cephalopods (Nesis 1987) found in all oceans, from polar seas to the tropics, with more in the Indo-West Pacific than elsewhere (Norman 2000). They inhabit a variety of marine ecosystems, including estuarine, benthic, pelagic, and the deep (>1,000 m) ocean. Because of their abundance and availability, they are economically important in many of the large fishing industries of Europe, Asia, Australia, New Zealand, and the Americas. It has been estimated that in the western United States alone, 2.7–3.6 million metric tons of squid, worth US $7 billion, are harvested annually (California Department of Fish and Game 2003). They represent a large percentage of the biomass in the ocean and are important in marine food webs, where they play significant roles as predators (mainly of crustaceans, fishes, and other molluscs) as

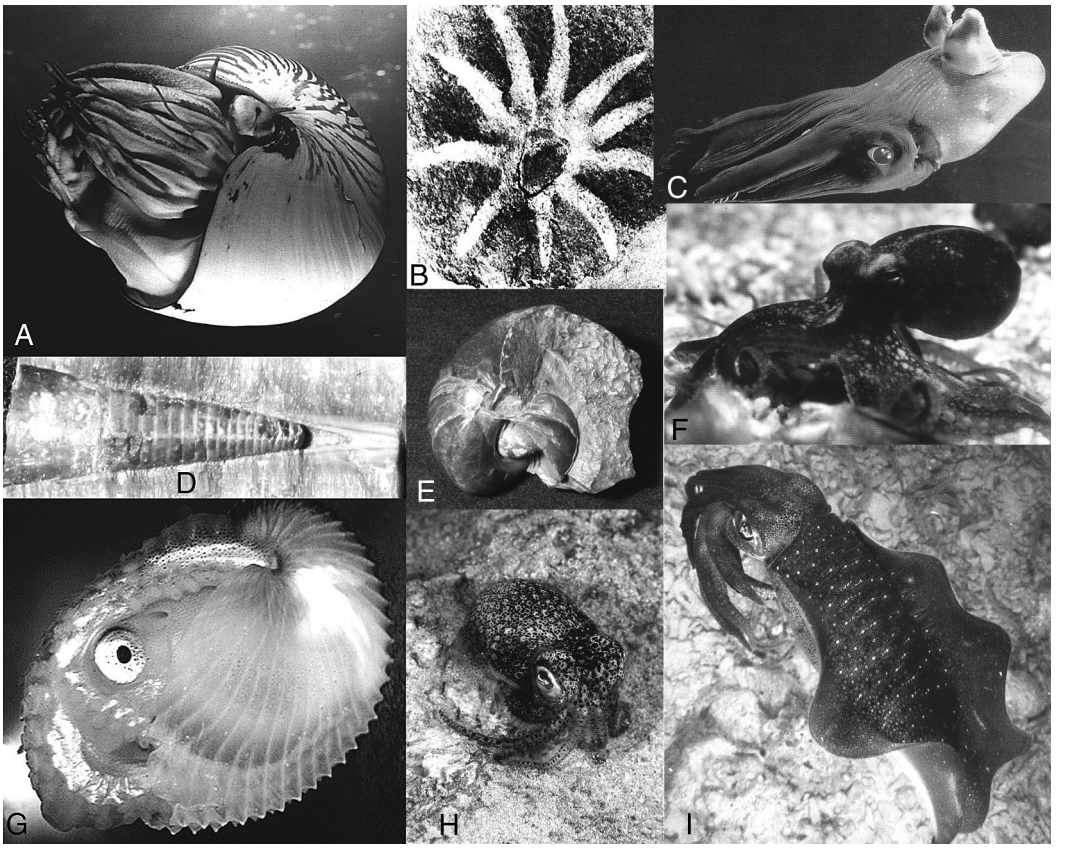


FIGURE 8.1. Cephalopod diversity. (A) *Nautilus pompilius* (M. Norman). (B) *Jeletzkya* (R. Johnson and E. Richardson, Jr.). (C) *Vampyroteuthis infernalis* (K. Reisenbichler). (D) *Pachyteuthis* sp. (D. Lindberg). (E) *Eutrephoceras* sp. (D. Lindberg). (F) *Octopus aegina* (M. Norman). (G) *Argonauta nodosa* (M. Nishiguchi). (H) *Euprymna tasmanica* (M. Norman). (I) *Sepioteuthis lessoniana* (M. Norman).

well as prey for other squids, fishes including sharks, seabirds, and marine mammals (Boyle and Boletzky 1996; Clarke 1996; Croxall and Prince 1996; Norbert and Klages 1996).

The cephalopod fossil record spans more than 450 million years, although this record is patchy because organic remains decrease in quality of preservation and information content with increasing time. Additionally, most fossils are only the remains of the hard, more durable skeletal material, which, in cephalopods as in most other molluscs, is calcium carbonate. Occasional cephalopod-bearing Lagerstätten (i.e., fossil bearing rock units with organisms having exceptional preservation, often including tissues and whole organs; Botter *et al.* 2002) have provided information not seen in typical fossils. In addition to the exceptionally well-preserved cephalopod body fossils from the Konservat Lagerstätten of the Middle Jurassic (Callovian) of England and the Late Jurassic (Tithonian) lithographic limestone of Solenhofen, Germany, fossil cephalopods and their shells are also known from the Lower Carboniferous Bear Gulch Limestone (Hagadorn 2002) in Montana and the Upper Carboniferous Buckhorn Asphalt (Crick and Ottensman 1983; Squires 1973) in southern Oklahoma (both in the United States).

FOSSIL CEPHALOPODS

GROWTH

Like modern *Nautilus* and the coleoids, fossil cephalopods are believed to have determinate

growth (i.e., the termination of growth and shell secretion presumably coincided with sexual maturity). Some fossil cephalopods reached large sizes; the largest nautiloid fossil recorded was four meters in length, whereas the largest ammonite was two meters in diameter (Lehmann 1981; Stevens 1988; Nixon and Young 2003). The smallest adult ammonoid shell we are aware of is *Maximites* from the Upper Carboniferous, with maturity attained at about 10 mm diameter (Frest *et al*. 1981).

SHELL MORPHOLOGY AND TERMINOLOGY

Almost always it is only the mineralized shell that is available for study in fossil cephalopods. The terminology for the hard parts of the shell is extensive; a good set of definitions is provided by Teichert (1964b). In general the shell is divided into a chambered phragmocone, used for buoyancy control, and the body chamber, which contained most of the bulk of the animal tissues and organs. In coleoids, the phragmocone and body chamber, if present, are partly or entirely covered by a rostrum or guard if a hard skeleton is present. Variations of these and other features provide the basis of the identification of different taxa. These morphological characters used in fossil cephalopods include the degree of coiling; chamber spacing; siphuncle position; septal neck shape; presence or absence of carbonate deposits within the chambers and, where present, the shape and placement of those deposits; protoconch shape and size; cross-sectional shape of the shell; rate of shell taper; suture pattern formed by the septa on the inside of the shell; mature shell modification of the body chamber that develops at presumed sexual maturity; shell ultrastructure (discussed next), rostrum composition and ultrastructure; guard shape; and numerous other features.

SHELL ULTRASTRUCTURE

The cephalopod shell is composed of aragonite deposited in two ultrastructural forms: nacreous plates (nacre) and prismatic needles (Bandel and Spaeth 1988). The external shell of living nautiloids, like that of most conchiferans, has an outer organic layer, the periostracum, which covers the outer prismatic needle layer and was presumably present in ammonoids and fossil nautiloids. The inner surface of the shell is composed of an aragonitic layer of nacre, as are structures such as septa and septal necks. Other shell layers can be present, depending on the ontogeny of the shell and the position of the outer whorls if the specimen is coiled. In some coleoids, because the mantle tissue covers the exterior of the shell, a layer of prismatic material (sometimes with organic material) is deposited on top of the original prismatic shell layer (the nacreous layer is missing) around the phragmocone or on the dorsal surface of the phragmocone and body chamber, forming the rostrum or guard seen in many fossil coleoids. The coleoid gladius, or proostracum, is composed of organic material or a combination of organic material and aragonite. In the Belemnoidea the rostrum can be composed of calcite, aragonite, and organic material (Bandel and Spaeth 1988).

Aragonite, the dominant building material of cephalopod shells, is an unstable mineral and can easily alter to calcite. The oldest unaltered cephalopod shell is from the Lower Carboniferous of Scotland (Hallam and O'Hara 1962). In the United States, the Upper Carboniferous Buckhorn Asphalt (Squires 1973) contains much unaltered cephalopod shell material (e.g., Kulicki *et al*. 2002). Other unaltered cephalopod shell occurrences are rare in the Paleozoic, but they are more common in younger units in the Mesozoic and Cenozoic.

BUOYANCY AND EQUILIBRIUM

The evolution of the morphology of the Cephalopoda was controlled, in large part, by the problems of shell equilibrium and the maintenance of neutral buoyancy while swimming, as described in the preceding section. Buoyancy problems persist despite most modern cephalopods lacking an external or internal shell (Figure 8.2).

Teichert (1988: table 1) listed 14 different mechanisms that modern and fossil cephalopods

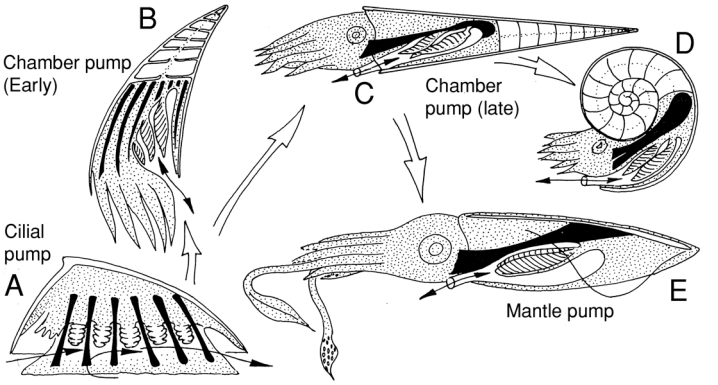


FIGURE 8.2. Diagram showing possible evolutionary directions in the methods of achieving more efficient respiratory currents in the history of the Cephalopoda from a hypothetical ancestor (A), through nautiloids (B–D) to modern coleoids (E) (from House 1988). The "chamber pump" alludes to the presence of a muscular hyponome or funnel.

have used to regulate buoyancy and equilibrium. Some of the most important are truncation of the posterior part of the phragmocone; endosiphuncular deposits in the phragmocone; cameral deposits in the phragmocone; replacing liquid-filling of the chambers with gas; shifting the gas-filled chambers to a position over the body chamber by overlap; shifting the liquid- and gas-filled chambers over the body chamber by coiling; using lighter-density chemicals in special tissues to reduce density; changing the shape or reducing the size and complexity of the phragmocone; thinning of the shell, septa, ornament, or other parts to reduce overall shell weight; construction of a rostrum on the phragmocone; and changing the configuration of the chamber by coiling the shell (Figure 8.2).

SEXUAL DIMORPHISM AND MATURITY

Many fossil cephalopods exhibit changes in shell morphology during growth, especially as the animal approaches and attains full size at maturity. Sexual dimorphism can be expressed by differences in shell size or modifications (Davis *et al.* 1996). Most research on maturity and sexual dimorphism in fossil cephalopods has been done on the Ammonoidea (see Davis *et al.* 1996 for references). Maturity and sexual dimorphism in fossil nautiloids, despite the numerous observations on living nautiloids (Collins and Ward 1987; Ward 1987), have received only modest study. This is also the case for the fossil coleoids.

From the Devonian (416–359 Mya) through the Cretaceous there are numerous cases in which mature ammonoid shells with nearly identical morphologies except for size are found in the same deposits; such cases are usually interpreted as different sexes rather than different species. Other shell morphology changes that have also been used as identifying maturity and sexual dimorphic characteristics include modification of the opening of aperture; septal approximation (i.e., septa becoming closer together); changes in the rate of coiling, ornament or in the cross-sectional shape of the body chamber; simplification of the suture pattern formed by the septa on the inside of the shell; and development of muscle scars (see Davis *et al.* 1996 for a more complete discussion). Additionally, there is approximation of transverse color bands and different patterns on the shells of some otherwise morphologically identical taxa that were presumably mature Triassic ammonoids (Mapes 1987a), which suggests that these specimens are different sexes.

INK

Buckland first described fossil ink from a Jurassic coleoid in 1836. Since then only modest numbers of coleoids from the Mesozoic have been recorded that contain ink (Doguzhaeva *et al.* 2004a) because the preservation of such material requires exceptional geologic conditions. The location and color of black or brown masses in the body chambers of fossil cephalopods

is suggestive that the material could be fossil ink; however, proving that the material is ink has, until recently, been impossible without destroying the specimen, because identification of the melanin-based ink was previously dependent on destructive chemical analysis. The discovery of a new method of identifying fossil ink was made by Doguzhaeva *et al.* (2004a). Doguzhaeva independently discovered that ink from a Jurassic coleoid had a globular ultrastructure when viewed under the scanning electron microscope (SEM) at high magnification (×10,000 to ×20,000) like that of modern coleoid ink from squid, octopus, and cuttlefish. Similar results were obtained on ink from Carboniferous coleoids (Doguzhaeva *et al.* 2004a; Doguzhaeva *et al.,* in press, a). To date the oldest confirmed coleoid ink is from the Upper Carboniferous in the United States (Oklahoma and Illinois), indicating that the use of ink as a method of predator avoidance has been long utilized (Doguzhaeva *et al.* 2004a; Doguzhaeva *et al.,* in press, a).

Although only some fossil coleoids are known to have ink, this is not surprising given the rarity of suitable preservation. It is possessed by most, but not all, modern coleoids, so some extinct coleoid taxa may also have not possessed it. No nautiloids are known to have ink, but there are unconfirmed reports that some Ammonoidea did possess it (Lehmann 1967; Doguzhaeva *et al.* 2004a). However, Lehmann (1988) reinterpreted his original material and concluded that his 1967 report was in error. Later Doguzhaeva *et al.* (2004b; in press, b) analyzed a Jurassic ammonoid using SEM techniques and identified preserved mantle tissue and the possible presence of ink. Thus, the debate as to whether some ammonoids had ink remains unresolved.

BEAK AND RADULAE

In general details, the beak (or mandibles, a modified jaw) and radulae of fossil cephalopods are known for most geological time periods, with many reports back to the Carboniferous (Mapes 1987). There are few reports of Devonian beaks and none of radulae, and no reports of either structure prior to the Devonian. Reported occurrences of beaks are much more common than those of radulae; most are from ammonoids (Kennedy *et al.* 2002), with only a few nautiloid jaws having been recovered (Mapes 1987; Müller 1974). Jaws, in general, appear to be moderately conservative structures in regards to evolutionary innovations with all known Upper Paleozoic examples being chitinous. In the Mesozoic, some ammonoids replaced the chitinous lower jaw with two massive calcareous plates. The function of these plates is debated, with suggestions that either they functioned only as lower jaws or they had a dual function by acting as a lower jaw and as a protective operculum, equivalent to the hood on modern *Nautilus* (Lehmann 1981; Morton 1981; Seilacher 1993; Kennedy *et al.* 2002; Tanabe and Landman 2002). Radulae are known from several species of ammonoids (e.g., Saunders and Richardson 1979; Nixon 1996; and their citations) and coleoids (e.g., Saunders and Richardson 1979). These structures are similar to those found in extant coleoids. In general it is possible to separate nautiloid, ammonoid, and coleoid mandibles and radulae by their morphologies; however, it is seldom possible to separate them on a generic or specific level.

TISSUES AND ORGANS

Only a few reports have been published on the organs and soft tissues of ammonoids and nautiloids, with most being from fossil coleoids. Impressions of tentacle-bearing bodies assigned to octopods have been described from the Cretaceous and Jurassic of the Middle East and Europe (Kluessendorf and Doyle 2000; Haas 2003; Fuchs *et al.* 2003). The oldest impression of soft body tissues we are aware of is from the Mazon Creek Lagerstätte (Upper Carboniferous) in Illinois. This deposit has yielded several important coleoids. One is *Jeletzkya,* the famous ten-armed impression of a coleoid with arm hooks but without a well-preserved phragmocone (Figure 8.1). Another is an octopus-like

form described as *Pohlsepia,* which has eight arms, two modified arms, a poorly defined head (including eyes, funnel, and beaks with a radula preserved between them) and fins; there is no evidence of any internal or external shell associated with the fossil. Other coleoids from this Lagerstätte are also known (Saunders and Richardson 1979; Allison 1987; Doguzhaeva *et al.*, in press, a). However, there are numerous reports of Mesozoic coleoids with preserved soft parts including arms with arm hooks, mantle tissue, gills, beaks, and radulae (e.g., Naef 1922; Doguzhaeva *et al.* 2002a; and citations therein). Gills are known only from Mesozoic coleoids (Bandel and Leich 1986; Mehl 1990).

Internal organs such as the stomach, crop, intestines, and circulatory system have been rarely reported in fossil cephalopods. In all reported cases involving the digestive system, the actual organs are not preserved. Instead, the undigested calcareous, chitinous, or phosphatic skeletal remains of meals are found clustered in specific areas of the body and are interpreted to mark the positions of the crop, stomach, or the intestine. In ammonoids, Lehman (1981) reported crinoid fragments, aptychi (lower beak/operculum) from smaller ammonoids, foraminiferans, and ostracods (Lehmann 1981; Nixon 1988). In orthoconic nautiloids, Quinn (1977) and Mapes and Dalton (2002) reported that the ammonoid clusters surrounding the body of large (1–3 meters in length) actinoceratid nautiloids from the Lower Carboniferous of Arkansas were the stomach contents of the nautiloid (Mapes and Dalton 2002). The oldest crop/stomach contents were discovered in an "orthoconic nautiloid" from the Lower Carboniferous Bear Gulch Lagerstätte (Landman and Davis 1988); these contents appear to be mostly composed of macerated fish scales. Subsequent study has shown that this specimen is a new coleoid (Mapes *et al.* 2007).

The circulatory system in fossil cephalopods is known only from impressions or grooves on the internal parts of mineralized structures such as the shell, cameral deposits, or rostrum and the siphuncle in fossil nautiloids and ammonoids, which contained arteries and veins. In Permian ammonoids from Nevada, segments of the siphuncular tissues replaced by phosphate have been discovered and described (Tanabe *et al.* 2000).

COLOR PATTERNS

It is unknown whether fossil cephalopods had chromatophores like those seen in the mantle tissue of many modern coleoids. *Nautilus* and *Allonautilus* have reddish-colored transverse bands across the shell, and the tissue of the hood has reddish markings, which are not capable of changing in life (in contrast to modern coleoids). While it is unknown whether the head-foot region of ammonites and extinct nautiloids had similar coloration to modern nautiloids, the shells of different fossil cephalopod taxa exhibited a variety of different color patterns.

The actual color of the patterns on the fossil ammonoid and nautiloid shells is unknown because fossilization has destroyed the pigments, leaving patterns of gray in different shades (Teichert 1964a; Mapes and Evans 1995; Mapes and Davis 1996; Gardner and Mapes 2001); such patterns have not been discovered on fossil coleoid shells. Patterns include zigzag lines, transverse bands, longitudinal bands, and uniformly colored shells. Interestingly, while modern *Nautilus* and *Allonautilus* have transverse bands that do not follow the growth lines, all transverse bands on ammonoid forms do. Additionally, at maturity, in *Nautilus* and *Allonautilus* the transverse bands are confined to the umbilical sides of the shell, leaving the lateral and ventral sides of the shell uniform creamy white. In mature shells of ammonoids with transverse color bands, the color pattern extended entirely across the shell and covered the body chamber to the edge of the aperture at maturity.

In addition to the color patterns on nautiloids and ammonoids, a different kind of coloration was present in some fossils. This is not the product of a pigment but instead the result of scattering and differential absorption of light by the ultrastructure of the outer layers of the shell. Unaltered ammonoid shells from Poland,

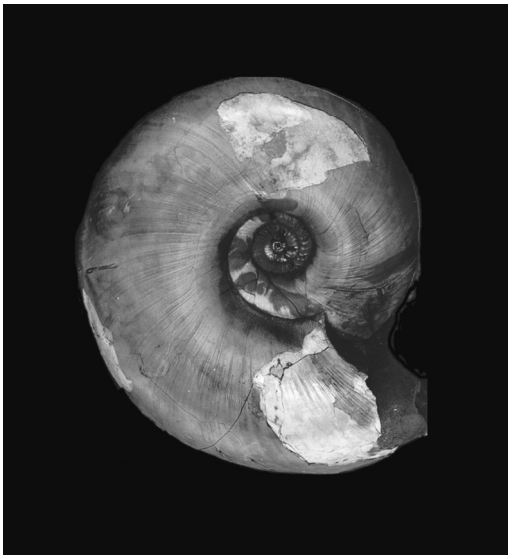


FIGURE 8.3. Longitudinal color pattern on *Cadoceras* sp., an ammonoid from Jurassic sediments near Luków, Poland. Except in the places where the shell is broken (white areas), the conch also exhibits an overall dark (reddish) iridescence; a longitudinal band of lighter iridescence can be seen on the darker (red) background around the umbilical region.

Russia, and Madagascar (all Mesozoic; precise ultrastructural and stratigraphic details are under study; Mapes, personal observation) with narrow longitudinal bands of red to reddish green on a darker red background have been observed (Figure 8.3). Such external colors would appear dark brown or dark gray at water depths below 10 meters. Interior shell layers appear to exhibit only deeper blue to purple colors; however, these colors would not be exposed during normal living conditions. The use of different refractions and reflections at different wavelengths of light to produce color patterns is not known in modern cephalopods, and indeed, we are not aware of any cases in modern taxa belonging to the phylum.

BIOLOGY OF LIVING CEPHALOPODS

Living cephalopods are divided into two major groups usually treated as subclasses: the Nautiloidea, containing *Nautilus* and *Allonautilus*, and the Coleoidea with all remaining taxa. Characteristics that distinguish these two subclasses are the presence of an external, many-chambered shell and multiple (60–90) suckerless tentacles in nautiloids, whereas coleoids have reduced (*Spirula*), internalized, or no shell, bearing eight to ten prehensile suckered appendages (arms and tentacles). Coleoids have a modified seven-element radula and a chitinous beak (similar to many ammonoids, see previous discussion), whereas nautiloids have a 13-element radula with a chitin and calcium carbonate beak. The nautiloid funnel has two separate folds, and they have two pairs of ctenidia (tetrabranchiate) and nephridia. Coleoids have a closed single tube for a funnel and one pair of ctenidia (dibranchiate) and nephridia. Coleoid eyes are more complex than the pinhole-camera eyes of nautiloids, and some even contain a cornea and lens (much like vertebrate eyes, an excellent example of convergent evolution). Modern nautiloids have no ink sac or chromatophores, whereas most coleoids have both. *Nautilus* has a simple type of statocyst, an oval cavity completely lined with hair cells (Young 1965). Coleoids have two types of statocysts: the "octobrachian" statocyst, which contains a spherelike sac with one gravity receptor system and an angular acceleration receptor system subdivided into nine segments (Young 1960; Budelmann *et al.* 1997), or the "decabrachian" statocyst, which is irregularly shaped with three gravity receptor systems and an angular acceleration receptor system subdivided into four segments (Budelmann *et al.* 1997). Both octobrachian and decabrachian statocysts are species specific in size and levels of organization and have been used in phylogenetic comparisons (Young 1984).

EGG LAYING

Laying of fertile eggs by modern *Nautilus* has been observed only in aquariums (Arnold 1987; Uchiyama and Tanabe 1999). Under these conditions single large (about 25 mm in diameter), yolky eggs are attached to a hard substrate. Embryo maturation takes 269 to 362 days, and the hatchling is a miniature of a full-sized adult.

Egg laying for modern coleoids is usually in two basic modes: those whose life histories are restricted to coastal and shelf habitats and those that are pelagic (Boyle and Rodhouse 2005). Cephalopods such as cuttlefish, most octopuses, and loliginid squids lay eggs that are attached to some type of substrate such as coral or kelp. Most of these squids ensheath their eggs with material from the nidamental glands and usually attach the eggs in areas where they are inconspicuous. Octopus eggs are more individually attached by a short stalk into strings or festoons, which are then attached to the substrate (Boyle and Rodhouse 2005). All octopod species brood their eggs, no matter whether or not they are attaching them to a specific substratum, and this characteristic has been used to delineate species complexes (i.e., *Octopus*; Boletzky, personal communication). For pelagic species, some ommastrephid squids extrude their eggs in a gelatinous mass, and some, such as the gonatid squids, are known to carry their eggs (Seibel *et al.* 2000). Young hatch directly from this gelatinous mass and are then fledged into the water column as paralarvae (see below). Other pelagic species, such as the Enoploteuthidae, release eggs singly into the water column (Young 1985), and the juvenile squids are then hatched in a pelagic state.

EARLY DEVELOPMENT

All cephalopods are direct developers; there is no larval stage (although juvenile cephalopods are referred to as paralarvae) or metamorphosis. This direct development is linked with the advent of swimming using jet propulsion (Boletzky 2003). Because coleoids lack an external shell, the development into a free-living, jet-propelled swimmer may have selected for direct development, rather than keeping the shell that would hinder this type of behavior. Cephalopod eggs are laden with yolk and vary in developmental time from a few days (O'Dor and Dawe 1998) to more than one year (Voight and Grehan 2000). The development time is dependent not only on species but on temperature (Boletzky 2003). Juvenile development is also variable with respect to arm crown morphologies among species. Hatchlings that develop from species with large eggs generally have fully developed arms, whereas species that produce smaller eggs have juveniles with short arms and tentacles with fewer suckers that cannot be immediately used like adult tentacles to capture prey (Boletzky 2003). Therefore, these juveniles use their arms to capture prey until the tentacles and suckers fully develop.

In *Nautilus*, the hatchlings have a shell diameter of about 25 mm. At maturity, shell diameters can exceed 210 mm diameter in the largest species, with estimates ranging from 2.5 to 15 years for *Nautilus* to achieve maturity depending on the species and the method of study (Landman and Cochran 1987). In *Nautilus* the sexes are separate and laying the single eggs continues for the life span of the female, which can be years or perhaps decades. In modern coleoids sexes are also separate and, while most species spawn their eggs only once, there are species that have several spawning events throughout their adult life span.

GROWTH

Modern cephalopods are the largest known invertebrates, reaching total lengths (mantle plus tentacles) ranging from 15 mm in pygmy squids (*Idiosepius*) to 20 m (*Architeuthis*). Some cephalopods grow rapidly, depending on temperature and age to senescence (Pecl and Moltschaniwskyj 1997; Semmens and Moltschaniwskyj 2000; Jackson and Moltschaniwskyj 2001; Moltschaniwskyj 2004). Often, in one season, there are two separate breeding cohorts that reach sexual maturity within a few months of one another (Moltschaniwskyj 2004), demonstrating that modern cephalopods can accelerate their growth rates depending on both environmental and physiological constraints.

MOVEMENT

Early cephalopods had a ventral apertural sinus that enabled jet propulsion for movement (House 1988). In all living cephalopods, the funnel enables movement through jet propulsion.

In squids and cuttlefishes, the funnel tube and collar close the entire length of the mantle. In some oegopsid squids (mainly Ommastrephidae), the funnel tube sits in a depression (funnel groove) mainly located in the lower head region. Certain aspects of the funnel groove (folds or ridges within the groove) can be used to distinguish subfamilies from one another. In myopsids, the groove is more like a depression and not as well defined as in Oegopsida, whereas in some octopuses the funnel tube is partially or fully embedded in the head tissues and is free only at the head end. In many of the fast-swimming squids, the anterior part of the funnel groove is bordered in front of the funnel aperture by a cuticular ridge. A pair of anterior adductor muscles are attached to the funnel anteriorly, and the posterior-lateral sides of the funnel are connected to the sac of the gladius or to the dorsal side of the mantle cavity by much stronger retractor muscles. For example, in the Jumbo Flying Squid, *Dosidicus,* the swimming velocity can increase from 4 to 14 knots (2–7.2 m/s) between becoming airborne and re-entering the sea as a result of the rapid expulsion of water from the mantle cavity (Packard 1985; House 1988). In most squids, cuttlefishes, and vampyromorphs, there is a funnel valve that supposedly strengthens or supports the funnel wall when the squid is swimming. There is no valve in octopuses or in some oceanic squids (Cranchiidae). The valve is also used to change the shape of the ink cloud when it is ejected through the funnel. Along with this, a funnel organ (Verrill's organ) is located on the dorsal side of the funnel just behind the valve. Its function is unknown, but it has been hypothesized that the mucus it secretes helps in maintaining the structure of the funnel or decreasing turbulence while swimming. The shape of this organ is species specific, and thus it is another useful character for taxonomy and systematics.

Fins are also used for locomotion in squids, even those that are not good swimmers. There are mainly five types of fin shapes, which can be used as a good taxonomic character for delineating families. These include marginal or fringing, rhomboidal or heart-shaped (cordate), kidney-shaped (reniform), round, and tonguelike fins (Nesis 1980). Smaller fins are usually less adapted to speed than larger ones but are better at maneuverability. It was previously thought that fins could be a good synapomorphy for Coleoidea (Young *et al.* 1998), and this may be true for modern forms, but they are preserved in very few fossils, so their distribution in fossil coleoids is uncertain.

FEEDING MODES

The mouth (buccal aperture) is highly adapted for predatory behavior. In the Devonian and Carboniferous and in most later fossil forms the mandibles (jaws) are a horny beak divided into an upper (shorter) and lower (longer) halves. In modern cephalopods, both mandibles contain pharyngeal plates and a frontal plate covering the lateral walls. These plates fuse, forming the rostrum and the cutting edges of the mandibles. Relative size and structure of the mandibles, as well as indentations along the cutting edge, differ greatly among cephalopods and are useful characters for the study of cephalopod evolution. In dibranchiate cephalopods, the radula consists of seven longitudinal rows of teeth, with a median (= central or rhachidian) tooth and the first, second, and third lateral teeth. Marginal plates may be present on either side but are not present in all species (they are absent in sepiolids and most oegopsids but are more developed in octopuses). Radulae are highly reduced in *Spirula* and finned octopuses. In other squids, such as *Gonatus,* the radula consists of five rows of teeth and the first lateral tooth is absent. Radulae have also been useful in determining differences among closely related species (Lindgren *et al.* 2005). The radula in most coleoids is used for gripping pieces of food torn by the beak and transferring it to the pharynx.

MODIFICATION OF ARMS AND TENTACLES

Cephalopods have the most modified foot among molluscs. The large muscular appendage has been divided into several appendages that can

be used to manipulate and capture prey or be used for mating. The main characteristic that separates the Decabrachia from the Octobrachia is the number of arms, with the squids and cuttlefishes having ten arms, of which two are modified as retractable tentacles, and the octopuses having eight. There is debate on whether vampyromorphs are sister to the decabrachians (with their two reduced arms considered to be "squid-like") or whether they are more like octopuses. Arms and tentacles are attached to the outer lip surrounding the mouth by the buccal membrane. There are initially eight lappets or small triangular flaps that support the buccal membrane during development, at which point the first and sometimes fourth arms may merge together. Where the buccal lappet supports are attached is of great taxonomic significance for decabrachians, since in eight families of oegopsids, the supports are attached to the fourth arms dorsally, while in the remaining families of Oegopsida and Myopsida, they are attached ventrally (Nesis 1980).

Tentacles are always positioned between the third and fourth arms and not connected to the buccal funnel. In cuttlefishes, the tentacles are very elastic and can be retracted into special pockets, whereas in other squids the tentacles can be retracted slightly, but not entirely. Although all squids have tentacles (synapomorphic character), they may lose this feature as adults (as in the Octopoteuthidae and some Gonatidae), or obtain them later during juvenile development (Idiosepiidae). Arms have also been modified for functionality; not only are they used for capturing prey, but in the octopod *Argonauta* they are used for building a pseudo-shell (the "paper nautilus") to brood eggs.

The presence of arms or tentacles around the mouth is a synapomorphic character of the Cephalopoda. The oldest tentacle preservation is *Pohlsepia* from the Carboniferous period (Mazon Creek) (Klussendorf and Doyle 2000) and *Jeletzkya* (Johnson and Richardson 1968). No ammonoid or nautiloid tentacles or impressions made by tentacles are currently known. Suckers on the arms of squids and cuttlefishes are stalked and are hemispherical in shape, and the stalks can either be long or short, thin or thick, with outgrowths in the middle forming a cup (Nesis 1980). They are often arranged in two rows, but can also be found in four rows, particularly in the Sepiidae, Sepiolidae, and Gonatidae. The suckers can also increase in number toward the base of the arm and enlarged suckers may be found in both or one of the sexes. Suckers can also be absent or rearranged into one row. Squids with hectocotylzed arms (used for passing spermatophores to the female) have modifications of the suckers, with the larger ones found in the middle or near the tips and growing smaller towards the ends of the arms. Suckers also differ on the tentacular club and can vary in the number of rows (four and up to fifty, in some Sepiolidae and Mastigoteuthidae). Larger suckers are found in the center of the club (manus) and are smaller at the ends of the club (dactylus). Most suckers of squids and cuttlefishes are armed with horny rings but are smooth in Crahnchiidae or armed with teeth in some Loliginidae. Suckers are also modified into hooks (some Gonatidae and all Enoploteuthidae) and are either on median parts of the arms or only on a few arms. Hooks always develop from suckers by uneven elongation, bending, and longitudinal folding of the distal edge of the ring or by elongation of one or two teeth during the later stages of ontogenesis. Arms of octopuses and vampyromorphs have only suckers with neither stalks nor horny rings. Finned octopods and vampyromorphs have suckers flanked by a small row of cirri, which alternate between the suckers.

NERVOUS SYSTEM

Cephalopods have a more developed nervous system than any other invertebrate, with a highly developed brain and optic lobes (Budelmann *et al.* 1997). It has been previously noted that certain octopuses are capable of learning by observation (Fiorito and Scotto 1992) as well as by testing (Wells and Wells 1977). Of importance to neural biology was the discovery and subsequent use of the giant axon in *Loligo*

vulgaris (J.Z. Young 1936, 1977). In addition, a highly advanced visual system, particularly in the coleoids, gives the capacity to recognize surroundings. *Nautilus* eyes are of the pinhole type, in which the lumen is filled with seawater. Both House (1988) and Lehmann (1985) speculated that this was probably the same for the ancestral Ammonoidea. Coleoids have a lens and a cornea, which improve their vision and is coupled with the ability to camouflage and signal using their chromatophores. This feature probably evolved within the endocochleate (having an internalized shell) Coleoidea, in which the development of chromatophores led to a change in defensive and offensive strategies for seeking or hiding from prey and predators (House 1988).

Not only is the visual system used to match surrounding habitats, but other adaptive features such as light organs, used for counterillumination and signaling, exist in a number of squids and a few octopuses (Young and Roper 1976; R.E. Young 1977; Young *et al.* 1979b; Jones and Nishiguchi 2004; Nishiguchi *et al.* 2004). Cephalopods have either autogenic bioluminescence (luminescence produced by themselves with a eukaryotic luciferase) or bacteriogenic light organs (luminescence produced by symbiotic bacteria with prokaryotic luciferase). Squids are capable of visually detecting changes in light attenuation and can thereby mask their shadow so that predators or prey below cannot easily see them. This is especially important at night when many cephalopods are active and the only potential illumination is moonlight (Young *et al.* 1980; Jones and Nishiguchi 2004).

BEHAVIOR

Behavior is also presently used as a way to distinguish not only species, but populations as well (Hanlon 1988; Packard 1988; Hanlon and Messenger 1996). There is a multitude of behaviors that have been documented by cephalopod researchers and are related to the complexity of the nervous system and the brain. Some of these include camouflage and body patterning, defense, communication, reproduction, and interspecies interactions (Hanlon and Messenger 1996). These intricate behaviors are not found in any of the other molluscan classes and have been key features that render the Cephalopoda unique within the phylum. Most of their behavioral features are ecologically similar to those of modern fishes, and probably the evolution of modern teleosts was largely responsible for the decline of cephalopods since the Mesozoic (Aronson 1991). Supposedly, many behavioral features were selected when fishes and reptiles living in coastal waters forced ectocochleate (having an external shell) cephalopods into deeper habitats (Packard 1972), but this is now refuted for modern cephalopods, with the exception of *Nautilus* (Aronson 1991).

Given such selective pressures, cephalopods have evolved a variety of behavioral traits linked to many of the senses (mechano-, chemo-, and photoreceptors) that allow the effectors (such as muscle, chromatophores, reflectors, photophores and the ink sac) to work in response to external stimuli. The brain, which has been mapped in a few key species (*Nautilus*, *Octopus*, and *Sepia*), forms a much more developed central nervous system and concentrated ganglia than in any other mollusc (Budelmann *et al.* 1997; Young 1988a), enabling the animal to efficiently organize the information received from all the sensory structures. This leads to the ability of matching habitat complexity, detecting and capturing prey, defense against predators (crypsis, flight, aggression), communication, learning, as well as complex mating rituals (which may be species specific) and subsequent brooding of eggs prior to hatching (Hanlon and Messenger 1996). Many of these behaviors can also be specific to species or even populations of individuals, which may then lead to subsequent sympatry and genetic differentiation between populations.

SHELL REDUCTION AND LOSS

Shell reduction (i.e., the change from having an external shell to having a reduced internal shell or no internal shell or supporting structure) was

accomplished by the Carboniferous (*Pohlsepia*), and, based on the fossil record, the reduction did not appear to have a major impact on cephalopod evolution (Kluessendorf and Doyle 2000). Because of *Pohlsepia* (Kluessendorf and Doyle 2000) from the Upper Carboniferous, it is probable that shell reduction in the Coleoidea may have occurred at different times within different coleoid lineages. For example, a single evolutionary event giving rise to the Decabrachia as presented by Hass (2003) does not appear to be resolvable using a single lineage and the known fossil record.

In the *Pohlsepia* lineage, which is not well understood at this time, the complete loss of the internal chambered phragmocone, rostrum, and any kind of supporting structure (such as a pen), occurred much earlier in the overall evolution of the Coleoidea than had been expected given knowledge of the fossil record prior to 2000. With recognition of the early geologic age of this evolutionary internal shell reduction condition, it would appear that loss of buoyancy and equilibrium control by gas-filled chambers in the phragmocone or even a simple mineralized supporting structure did not provide a major evolutionary advantage to the coleoids. However, the coleoids did manage to survive three major extinction events (the Permian-Triassic, the Triassic-Jurassic, and the Cretaceous-Cenozoic), whereas the externally shelled ammonoids, which are the most abundant cephalopod group from the late Paleozoic to the end of the Mesozoic, survived only two of these extinctions. Interestingly, the ancestors of modern *Nautilus* and *Allonautilus* with their external shells managed to survive all three extinction events. The observation that the ammonoids were more abundant than the coleoids and that they were the dominant cephalopod group in the Late Paleozoic and Mesozoic is supported by the numerous marine Lagerstätten and other marine deposits around the world that preserve fossil cephalopods. Based on a nonquantifiable impression of collecting fossil cephalopods for decades and the understanding that externally shelled cephalopods were more easily fossilized, coleoid fossils are considered to be very rare through the Late Paleozoic and Mesozoic; whereas, ammonoids are relatively common and are the dominant (in terms of abundance, diversity, and geographic distribution) fossil cephalopod group.

EVOLUTION AND PHYLOGENY OF CEPHALOPODS

SISTER GROUP RELATIONSHIPS

The two main competing hypotheses for cephalopod relationships with other molluscan classes have considered the placement of Cephalopoda with Gastropoda (as the Cyrtosoma) within the Conchifera (Haszprunar 1996; Salvini-Plawen and Steiner 1996; Haszprunar 2000) or whether the Scaphopoda are sister to the gastropod + cephalopod clade (Lindberg and Ponder 1996; Haszprunar 2000). Most recently, cephalopods have been widely accepted as sister to both Scaphopoda and Gastropoda based on molecular and morphological data (Waller 1998; Giribet and Wheeler 2002; Steiner and Dreyer 2003; Giribet *et al.* 2006).

FOSSIL GROUPS

In fossil cephalopods, the general higher-level details of the overall evolution and phylogeny of many of the nautiloid taxa (variably treated as orders, superorders, or even subclasses; Figure 8.4) appear to be moderately stable, though little research has been done since the 1970s (Orlov 1962; Teichert 1964a, 1967). Although most of the main details of cephalopod evolutionary trends appear to be well established, presumed ancestral links between many higher groups remain uncertain. Ammonoid evolution is known to be very complex because these animals have durable shells that quickly evolved complex features. These animals also had a worldwide distribution over a long span of geologic time, and they have received much more attention over the past two centuries because they are very useful in determining the age of different rock units (Moore 1957, 1964;

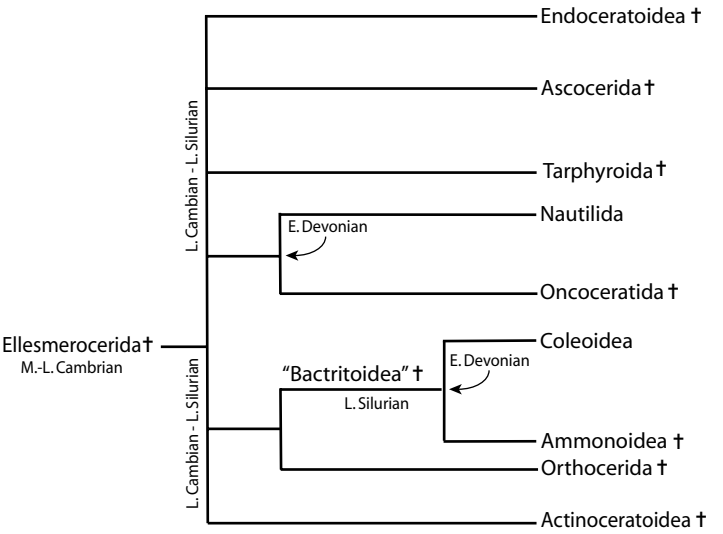


FIGURE 8.4. Generalized cephalopod phylogeny through time showing the general evolutionary trends from an Ellesmerocerida ancestor in Middle to Late Cambrian time to the extant Nautilida and Coleoidea in today's oceans. The ancestral molluscan that gave rise to the Ellesmerocerida was probably a monoplacophoran. The Ellesmerocerida lineage developed into a number of distinct subclasses, superorders, and orders, including the Orthocerida, between Late Cambrian and Late Silurian time. Only two genera with external shells (*Allonautilus* and *Nautilus*), which belong to the Nautilida, survive today. The Bactritoidea arose out of the Orthocerida in the Early Devonian. Slightly later in the Early Devonian, the Bactritoidea gave rise to the Ammonoidea, which became extinct at the end of the Cretaceous. Additionally, the Bactritoidea gave rise to the Coleoidea, which is the only other cephalopod group to survive today. The timing of the evolutionary origin of the Coleoidea from the Bactritoidea is presently unknown, with the majority of authorities suggesting a Devonian or Early Carboniferous timing for this significant evolutionary event. In order to clearly show the extant nautiloid and coleoid placements on the tree, some major groups are omitted from the diagram; all major cephalopod groups and their known geologic ranges are shown in Figure 8.7. Extinct taxa are designated with a dagger (†). E. = Early; L. = Late.

Orlov 1962; House 1981; Becker and Kullmann 1996; Page 1996; Wiedmann and Kullmann 1996; Kullmann 2002) (Figure 8.5). Even though the Nautiloidea have similar characteristics, the evolution of this group has received only modest attention because they evolved more slowly (Woodruff *et al.* 1987; Wray *et al.* 1995; Ward and Saunders 1997). To our knowledge only segments of the Ammonoidea phylogeny have been evaluated using cladistic analysis.

In contrast to the research pattern seen in the Nautiloidea, new coleoid material has been described during the past ten years that has significantly altered parts of the classifications proposed by pre-1995 coleoid researchers (e.g., Naef 1922; Jeletzky 1966; Donovan 1977). New classifications, including some using cladistic methodologies, have been proposed (Engeser 1996; Pignatti and Mariotti 1996; Mariotti and Pignatti 1999; Haas 2002) (Figure 8.6). Despite this, the early phylogeny of the Coleoidea remains poorly understood.

The oldest cephalopod fossils are from the lower and middle part of the Yenchou Member of the Fengshan Formation (late Middle Cambrian) of northeast China (Chen and Teichert 1983). Only the genus *Plectronoceras* has been recovered from the lower part of this unit. This genus represents what is probably the best cephalopod archetype and presumably arose from ancient monoplacophorans with longiconic or breviconic shells similar to the

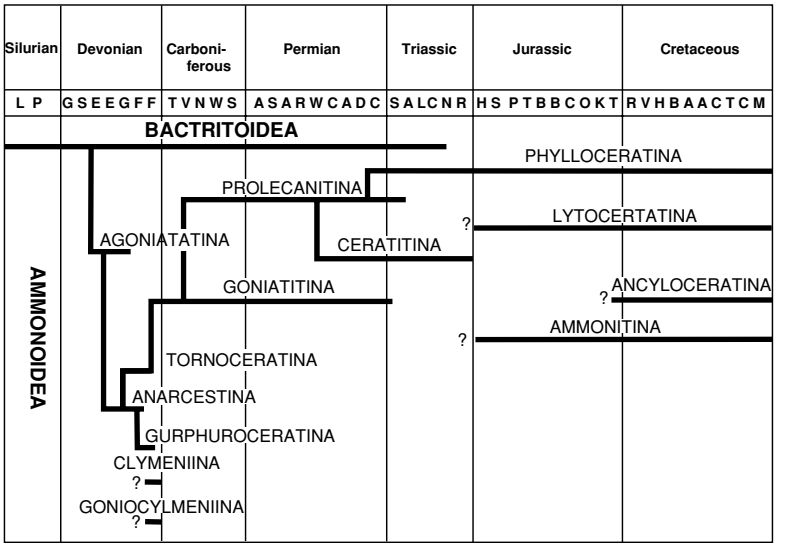


FIGURE 8.5. The evolutionary phylogeny of the Ammonoidea at the ordinal and subordinal level as derived from the Bactritoidea based on published data by Gordon 1966; Donovan *et al*. 1981; Glenister and Furnish 1981; Kullmann 1981; Tozer 1981; Wright 1981; House 1981, 1993; Wiedmann and Kullmann 1988, 1996; Becker and Kullmann 1996; Page 1996; and Kröger and Mapes 2007. Based on these works, there has been general agreement on most the phylogenetic relationships among most authorities, but some differences are unresolved. The overall relationships between the ammonoid suborders look deceptively simple; however, note that five of the 13 ammonoid suborders begin their origination with a question mark, indicating that the ancestral origins are presently unknown. There are also many unresolved ancestor and descendant relationships at the superfamily and family levels. A total number of ammonoid species is not yet available; however, Wiedmann and Kullmann (1996) indicated there are over 3,700 described ammonoid species in the Devonian, Carboniferous, and Permian. Given this impressive diversity and the fact that this cephalopod order has been studied for more than 250 years, it is surprising that subordinal ancestral-descendant relationships within the order are still unknown. This problem is even more complicated and extensive when one attempts to determine the phylogenies of the Bactritoidea, Nautiloidea, Coleoidea, and the other cephalopod orders.

Upper Cambrian *Hypseloconus* (Teichert 1988). During late Yenchou time, almost all nautiloid genera became extinct. This extinction was followed by a large evolutionary radiation seen in the overlying Wanwankou Member of the Fengshan Formation (Teichert 1988). By the Middle Ordovician, the nautiloids had diversified to the greatest morphological diversity recorded in the fossil record for ectocochleate cephalopods (Teichert and Matsumoto 1987). Almost all of the established major clades arose from Ellesmerocerida ancestors. These are the Intejocerida, Endocerida, Actinocerida, Discosorida, Ascocerida, Orthocerida, Barrandeocerida, Nautilida, Tarphycerida, and Oncoceratida (for details of these nautiloid groups see Moore 1964: fig. 7). Two were the ancestors to all the living cephalopods: the Nautilida, which gave rise to modern nautiloids (*Nautilus* and *Allonautilus*), the two living externally shelled cephalopod genera, and the Bactritoidea, which gave rise to the ammonoids and the coleoids (Chen and Teichert 1983) (Figures 8.5, 8.7).

The coleoids are generally thought to consist of eleven ordinal-level taxa, of which six are extinct (House 1988). The extinct groups with internal phragmocones are the Donovaniconida (Doguzhaeva *et al*., in press, a), Aulacocerida, Phragmoteuthida, Belemnitida, Hematitida and Belemnoteuthida (Jeletzky 1966; Engeser and Reitner 1981; Teichert 1988; Figure 8.6, Table 8.1). The five living orders are the Octopoda, Sepiida,

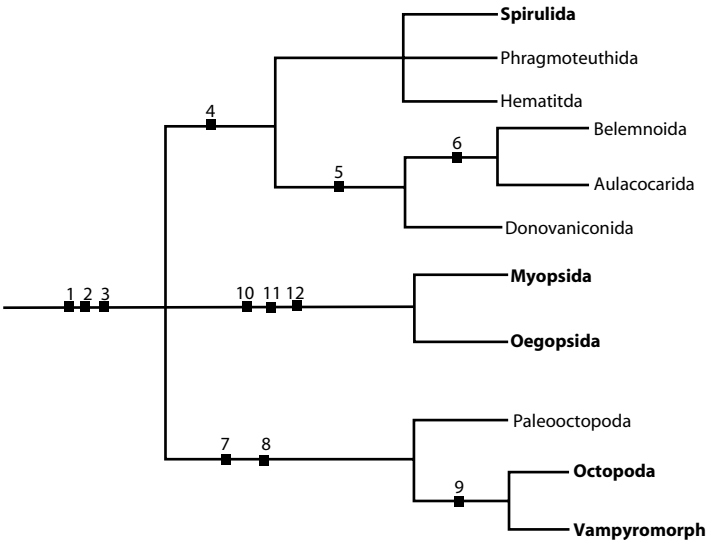


FIGURE 8.6. Proposed evolution of extinct and modern coleoids. Apomorphic characters include: (1) external shell; (2) 10 undifferentiated arms; (3) no suckers; (4) interior phragmocone; (5) arm hooks; (6) closing membrane in first chamber (suppression of prosiphon and cecum); (7) no internal shell; (8) modified arms; (9) presence of suckers; (10) arm 2 modified; (11) suppression of phragmocone; and (12) arm 4 modified. The ancestral bactritoid form probably had apomorphic characters (1), (2), and (3). This analysis evaluates most of the lineages of the Coleoidea. However, many other characters have not yet been evaluated, and we recommend that additional study of these evolutionary pathways is necessary to delineate the phylogeny of the Coleoidea more completely and accurately. Extant taxa are indicated in bold. See also Table 8.1.

Spirulida, Teuthida, and Vampyromorpha. Of these, the best documented in the fossil record is the Sepiida, since they retain all the morphological elements of the belemnitid shell (Jeletzky 1966, 1969). There are no known transitional taxa showing clear intermediate features between any of the higher coleoid groups.

Most characters used for classification of fossil coleoids include the phragmocone, rostrum or guard, and so forth. However, in general the Teuthida lack shelly hard parts but do have a modified proostracum (pen or gladius) made of organic material or a combination of organic and some carbonate material (Teichert 1988). These gladii typically do not fossilize well, and other hard parts such as phragmocones, although calcareous, are also rare as fossils. The Carboniferous coleoid taxon *Pohlsepia*, assigned to Palaeoctopoda, (Kluessendorf and Doyle 2000), lacks hard parts, and reveals the existence of coleoids without shells at that time. However, from the Middle Carboniferous through the last of the late Paleozoic and through the early Mesozoic there has been no other recovery of coleoid material assignable to the Octopoda. Significantly, below the Middle Carboniferous there are no transitional forms from the primitive bactritoid stock that presumably gave rise to this important coleoid order.

New techniques to identify fossils as coleoids include the SEM analysis of fossil ink (see the section on ink above) and the identification of unique coleoid shell ultrastructures using SEM. For example, a Carboniferous "*Bactrites*" from Texas (Miller 1930) was reinterpreted as a coleoid belonging to the Spirulida (Doguzhaeva *et al.* 1999), extending the range of Spirulida from the Cretaceous (the oldest previously known spirulid) to the Carboniferous. Such discoveries, however, are rare (especially in the Paleozoic and early Mesozoic), given the generally poor fossilization potential of most coleoids.

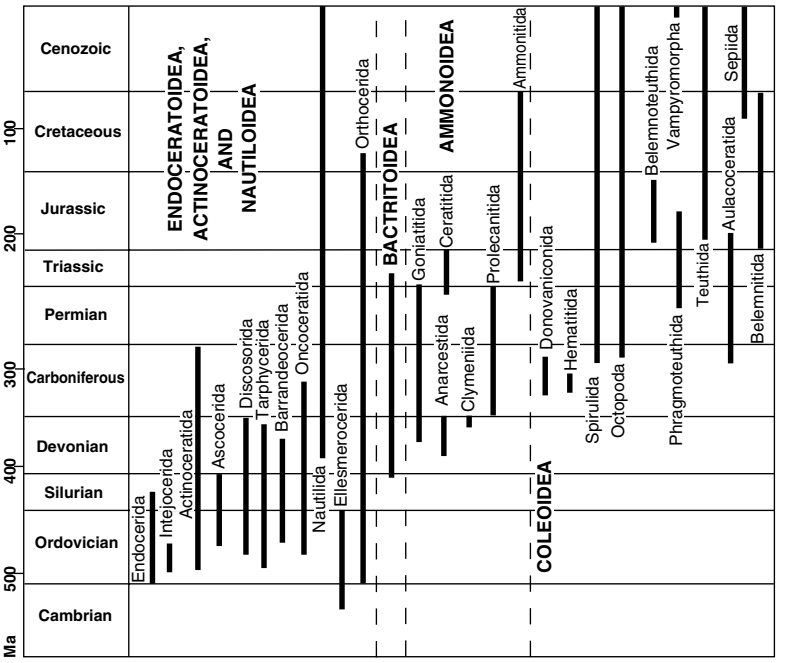


FIGURE 8.7. Geologic ranges of the major extinct and extant subclasses, superorders, and orders of the Cephalopoda (modified from House 1988; note that the terminology as to rank varies considerably in the literature). Some of the ancestor-descendant relationships within the Endoceratoidea, Actinoceratoidea, Nautiloidea, Bactritoidea, and Ammonoidea are known. However, the recent discovery of numerous new fossil coleoids from the Carboniferous has put to question some of the geologic ranges and the proposed origins of some of the fossil coleoid orders. Also, the fact that the origin of the earliest coleoids is unknown remains an unresolved evolutionary problem that complicates the development of a unified phylogeny for the Coleoidea.

The origin of the Coleoidea (Figure 8.4) is presently accepted as being from the Bactritoidea in the Paleozoic (Devonian and/or Carboniferous) (Figure 8.7). The Bactritoidea (Silurian to Triassic) have an egg- or ball-shaped protoconch in which the typical phragmocone of the genus *Bactrites* has a ventral marginal siphuncle with simple disk-shaped septa and an orthoconic (i.e., straight) shell (e.g., Mapes 1979). Primitive coleoids have phragmocones with these same characteristics. *Lobobactrites,* another bactritoidean, is similar in gross morphology to *Bactrites* except that the subspherical protoconch is twice as large in diameter and length as that of *Bactrites*. *Lobobactrites* is considered the probable ancestor of Ammonoidea because the earliest known ammonoids also have large, egg-shaped protoconchs (Erben 1964; Teichert 1988). The main difference between the Bactritoidea and the Ammonoidea is that all bactritoids have orthoconic shells, whereas those of the most primitive ammonoids are cyrtoconic (slightly curved) shells. By the end of the early Devonian, the ammonoid protoconch had generally begun to reduce in size and the shell became more tightly coiled. By the end of the Devonian, all ammonoids had a small, more or less spherical protoconch, and the shell was tightly coiled.

The ammonoid groups underwent several major extinctions and re-radiations from the early Devonian to the end of the Cretaceous (e.g., House 1988). The first extinction was near the end of the Devonian, the second at the Permo-Cenozoic boundary, and the third at the Triassic-Jurassic boundary. After surviving these three extinctions, ammonites became extinct at the Cretaceous-Cenozoic boundary (Figure 8.5). At

TABLE 8.1
Classification of Paleozoic and Modern Coleoids

Subclass Coleoidea Bather, 1888

Superorder Belemnoida Gray, 1849 (Carboniferous–Cretaceous)

Order Hematitida Doguzhaeva, Mapes, and Mutvei, 2002[a] (Carboniferous)

Family Hematitidae Gustomesov, 1976

Hematites (Flower and Gordon 1959): Upper Mississippian, Lower Eumorphoceras Zone (= Serpukhovan), Utah, Arkansas, United States

Bactritimimus (Flower and Gordon 1959): Upper Mississippian, Lower Eumorphoceras Zone (= Serpukhovan), Arkansas, United States

Paleoconus (Flower and Gordon 1959): Upper Mississippian, Lower Eumorphoceras Zone (= Serpukhovan), Arkansas, United States

Order Phragmoteuthida Jeletzky in Sweet, 1964 (Upper Permian–Jurassic)

Family Phragmoteuthididae Mojsisovics, 1882

Permoteuthis groenlandica Rosenkrantz, 1946: Upper Permian, Foldvik Creek Formation, Clavering Island, East Greenland

Order Donovaniconida Doguzhaeva, Mapes, and Mutvei[b] (Carboniferous)

Family Donovaniconidae Doguzhaeva, Mapes and Mutvei, 2002[c]

Donovaniconus (Doguzhaeva *et al*. 2002a): Upper Carboniferous, Desmoinesian, Oklahoma, United States

Saundersities (Doguzhaeva *et al*., in press, b): Upper Carboniferous, Desmoinesian, Illinois, United States

Family Rhiphaeoteuthidae Doguzhaeva, 2002

Rhiphaeteuthis Doguzhaeva, 2002: Upper Carboniferous, Orenburgian, Southern Urals, Kazakhstan Republic (former USSR).

Family Uncertain

New genus: Lower Carboniferous, Bear Gulch Limestone, Montana, United States (Mapes *et al*. 2007)

Order Aulacoceratida Stolley, 1919 (Carboniferous–Jurassic)

Family Mutveiconitidae Doguzhaeva, 2002

Mutveiconites Doguzhaeva, 2002: Upper Carboniferous, Orenburgian, Southern Urals, Kazakhstan Republic (former Soviet Union).

Superorder Decembrachiata Winckworth, 1932 (Carboniferous–Holocene)

Order Spirulida Haeckel, 1896 (Carboniferous–Holocene)

Family Shimanskyidae Doguzhaeva, Mapes, and Mutvei, 1999

Shimanskya (Doguzhaeva *et al*. 1999): Upper Pennsylvanian, Virginian (= Stephanian); Texas, United States

Family Spirulidae Owen, 1836

Order Oegopsida Leach, 1917

Family Architeuthidae Pfeffer, 1900

Family Brachioteuthidae Pfeffer, 1908

Family Batoteuthidae Young and Roper, 1968

Family Chiroteuthidae Gray, 1849

Family Joubiniteuthidae Naef, 1922

TABLE 8.1
(continued)

- Family Magnapinnidae Vecchione and Young, 1998
- Family Mastigoteuthidae Verrill, 1881
- Family Promachoteuthidae Naef, 1912
- Family Cranchiidae Prosch, 1847
- Family Cycloteuthidae Naef, 1923
- Family Ancistrocheiridae Pfeffer, 1912
- Family Enoploteuthidae Pfeffer, 1900
- Family Lycoteuthidae Pfeffer, 1908
- Family Pyroteuthidae Pfeffer, 1912
- Family Gonatidae Hoyle, 1886
- Family Histioteuthidae Verrill, 1881
- Family Psychroteuthidae Thiele, 1920
- Family Lepidoteuthidae Naef, 1912
- Family Octopoteuthidae Berry, 1912
- Family Pholidoteuthidae Voss, 1956
- Family Neoteuthidae Naef, 1921
- Family Ommastrephidae Steenstruup, 1857
- Family Onychoteuthidae Gray, 1847
- Family Thysanoteuthidae Keferstein, 1866

Order Myopsida Naef, 1916

- Family Australiteuthidae Lu, 2005
- Family Loliginidae Lesueur, 1821

Order Sepioidea Naef, 1916

Suborder Sepiida Keferstein, 1866

- Family Sepiidae Keferstein, 1866

Suborder Sepiolida Naef, 1916

- Family Sepiadariidae Fischer, 1882
- Family Sepiolidae Leach, 1817

Superorder Vampyropoda Boletzky, 1992 (Carboniferous–Holocene)

Order Octopoda Leach, 1817 (Carboniferous–Holocene)

- Family Palaeoctopodidae Dollo, 1912
  - *Pohlsepia mazonensis* (Kluessendorf and Doyle 2000): Middle Carboniferous, Desmoinesian, Francis Creek Formation, Illinois, United States

Suborder Cirrata Grimpe, 1916

- Family Cirroteuthidae Keferstein, 1866
- Family Stauroteuthidae Grimpe, 1916
- Family Opisthoteuthidae Verrill, 1896

Suborder Incirrata Grimpe, 1916

- Family Amphitretidae Hoyle, 1886
- Family Bolitaenidae Chun, 1911
- Family Octopodidae Orbigny, 1840, in Ferussac and Orbigny, 1834–1848

TABLE 8.1
(continued)

Family Vitreledonellidae Robson, 1932
Superfamily Argonautoida Naef, 1912
Family Alloposidae Verrill, 1881
Family Argonautidae Cantraine, 1841
Family Ocythoidae Gray, 1849
Family Tremoctopodidae Tryon, 1879
Order and family uncertain
"*Bactrites" woodi* (Mapes 1979: pl. 18: figs. 8, 12): Upper Carboniferous, Missourian, Kansas, United States
Undescribed Stark coleoids (see Doguzhaeva *et al.* 2002c): Upper Pennsylvanian, Missourian (= Kasimovian), Nebraska, United States
Superfamily Bathyteuthoida V
Family Bathyteuthidae Pfeffer, 1900
Family Chtenopterygidae Grimpe, 1922
Family Idiosepiidae Fischer, 1882
Problematic specimens
Boletzkya longa (Bandel *et al.* 1983): Devonian (Emsian), Hunsrückschiefer, Kaisergrube, Hunsrück, Germany
Naefiteuthis breviphragmoconus (Bandel *et al.* 1983): Devonian (Emsian), Hunsrückschiefer, Kaisergrube, Hunsrück, Germany
Protoaulacoceras longirostris (Bandel *et al.* 1983): Devonian, Hunsrückschiefer, Kaisergrube, Hunsrück, Germany
Eoteuthis sp. (Termier and Termier 1971): Devonian of Morocco, North Africa
Aulacoceras? sp. (de Konick 1843): Locality and age (?Devonian/Carboniferous)
Eobelemnites caneyensis (Flower 1945): Unknown locality and age
Jeletzkya douglassae (Johnson and Richardson 1968): Upper Carboniferous, Desmoinesian, Francis Creek Formation, Illinois, United States
Unnamed coleoid from Czech Republic (Kostak *et al.* 2002): Early Carboniferous, Moravica Formation, Northern Moravia, Czech Republic
Unnamed coleoid by Allison (1987): Upper Carboniferous, Desmoinesian, Francis Creek Formation, Illinois, United States
Palaeobelemnopsis sinensis Chin, 1982:Upper Permian from China

NOTE: From Doyle 1993, Doyle *et al.* 1994, Pignatti and Mariotti 1995, Young *et al.* 1998, Doguzhaeva *et al.* 1999, 2002a,b, 2003, in press a, Haas 2002, and the web site maintained by T. Engeser (http://userpage.fu-berlin.de/~palaeont/fossilcoleoidea/hhierarchicalclassification.html) and by R. Young (http://tolweb.org/tree?group=Cephalopoda&contgroup=Mollusca).
[a]Doguzhaeva *et al.* 2002b.
[b]Doguzhaeva *et al.*, in press, b.
[c]Doguzhaeva *et al.* 2002a.
Taxa without stratigraphic ranges are Recent.

each extinction event, only one or a few genera survived, and it was those survivors that rapidly diversified into the empty ecological niches in the world's oceans. Thus, the Ammonoidea have an important place in invertebrate paleontology and stratigraphy, not only because of their diversity but through their pelagic development, which facilitated the attainment of worldwide distributions.

There are literature reports of putative coleoids from the Devonian (de Konick 1843),

Eoteuthis from Morocco (Termier and Termier 1971) and *Protoaulacoceras, Boletzkya,* and *Naefiteuthis* from Germany (Bandel and Boletzky 1988, Bandel *et al.* 1983). Unfortunately, none of these have been confirmed as being coleoids. De Konick's (1843) specimens were inadequately illustrated and are apparently lost, and the specimen identified by Termier and Termier (1971) has been determined to be a bactritoid (Doyle *et al.* 1994). The identification of specimens reported by Bandel *et al.* (1983) and reanalyzed by Bandel and Boletzky (1988) are questionable (Doyle *et al.* 1994), making the coleoid evolution of the Devonian period difficult to assess.

Of the known fossil coleoids, the oldest are *Hematites, Paleoconus,* and *Bactritimimus* (Flower and Gordon 1959; Gordon 1964) from the lower Carboniferous of Arkansas and Utah, United States, with only *Hematites* represented by numerous, well-preserved specimens. Doguzhaeva *et al.* (2002b) determined that *Hematites* and probably the other two genera were unique in many ways and established a new order, Hematitida. Some unique characteristics included the thick, blunt rostrum, which was partly calcified and organic. During early ontogeny, the animal initially grew the breviconic phragmocone with chambers containing internal cameral deposits and a short body chamber. The rostrum was secreted only as the animal neared maturity. Of over 100 *Hematites* specimens examined with rostrums, only one retained a visible protoconch,[1] described as relatively large and spherical in shape. A spherical protoconch and the characteristics of the phragmocone support the interpretation that the bactritoids were the ancestral stock of the coleoids, which probably originated in the earliest Carboniferous or perhaps the Devonian.

The Carboniferous is now known to contain several additional different coleoid genera. Many of these taxa have been described in the past ten years and have lead to the establishment of a number of new families (Table 8.1) complicating previous hypotheses of the early coleoid phylogeny (e.g., Teichert and Moore 1964: K101, fig. 70; Teichert 1967: 198–199, fig. 20; Engeser and Bandel 1988; Pignatti and Mariotti 1996) (Figure 8.6). There has been general agreement that the origination of coleoids occurred in either the Early Carboniferous or the Devonian and that they were derived from a bactritoidean, with the implication that the group is monophyletic, arising from a paraphyletic Nautiloidea (Figure 8.4). Little consideration has yet been given to the possibility that several different taxa belonging to the Bactritoidea may have been involved in the origin of the Coleoidea at different times in the Devonian and Carboniferous. A polyphyletic origin of Coleoidea might help to explain the great diversity of different morphologies seen in the Carboniferous coleoids and why no closely related coleoid taxa have been conclusively documented from the Devonian.

Currently, there is little agreement among coleoid researchers as to how the higher groups are related to each other and how and when their originations occurred. In part, this lack of agreement is due to the lack of suitable fossils. Additionally, the understanding of the phylogeny of fossil Coleoidea may also have been confused by the premature application of cladistic analysis using data sets subject to major changes with the discovery of new fossils. This is not to suggest that cladistic analysis should not be applied to the coleoid data set, but rather that the results of such preliminary analyses should be treated with caution.

LIVING GROUPS

The first attempt at understanding cephalopod relationships among extant species began in the early 1800s, with the establishment of the subclasses Tetrabranchiata and Dibranchiata based on the number of ctenidia present (Owen 1836). Both Decapoda (Decabrachia, *sensu* Boletzky 2003) and Octopoda (Octobrachia, *sensu* Boletzky 2003) have been acknowledged since the works of d'Orbigny (d'Orbigny 1845) and are distinguished by a number of characters including the number of arms, presence or

1. Specimen now misplaced.

absence of chitinous sucker rings and sucker stalks, presence or absence of a buccal crown and lappets, a wide canal between the afferent and efferent vessels of the gill, reduced internal shell (common in both groups), broad neck fusion (found in both groups), and a mediodorsal sac of the mantle cavity (Clarke 1988). These "superorders" were further subdivided into several groups usually treated as orders (Spirulida, Sepiida, Sepiolida, and Teuthida in the Decabrachia; Octopodida and Vampyromorphida in the Octobrachia) (e.g., Sweeney and Roper 1998). The majority of the earliest classifications were initiated by Naef (1921–1923, 1928), who used a large amount of detailed morphological, embryological, and paleontological data in his monographs. Although many of his groupings are still currently recognized today, some of his classification schemes have since been challenged. He did not use many of the adult morphological characters that are commonly used today, but nonetheless his observations provided a solid foundation and are some of the most influential and important works on cephalopod evolution.

Numerous characters have provided information in delineating family- to species-level differences (Clarke 1988), such as the gladii (Donovan and Toll 1988), mouth parts such as the buccal mass and beaks (Clarke and Maddock 1988a; Nixon 1988), statoliths (Clarke and Maddock 1988b; Young 1988b), ontogenetic development (Boletzky 1997, 2002, 2003), brain morphology (Budelmann *et al.* 1997; Nixon and Young 2003; Young 1988a) and photophores or the presence of a light organ (Young *et al.* 1979a; Young and Bennett 1988; Nishiguchi *et al.* 1998, 2004; Nishiguchi 2002). Since many of these characteristics are shared among sister taxa (e.g., bacteriogenic light organs—those that produce light by symbiotic luminous bacteria—are found only in two families of squids, the Loliginidae and the Sepiolidae), they provide additional information regarding the evolution of such structures and whether those features are derived, are synapomorphic, or have evolved independently several times.

EVOLUTION AND PHYLOGENETIC ANALYSIS OF RECENT CEPHALOPODS

CURRENT CLASSIFICATIONS

Overall, there has been strong support for the monophyly of Cephalopoda (Berthold and Engeser 1987; Salvini-Plawen and Steiner 1996; Lindgren *et al.* 2004; Passamaneck *et al.* 2004; Giribet *et al.* 2006). Most research in the past century has focused solely on describing groups, from levels of orders to species. In living cephalopods, Nautiloidea has one family (Nautilidae) and Coleoidea has two major groups (Octopodiformes and Decapodiformes). Several ordinal-group taxa have been recognized: Vampyromorpha (vampire squids) and Octopoda (shallow-water benthic and deep-water and pelagic octopus) within the Octopodiformes, and Oegopsida, Myopsida, Sepioidea (cuttlefish and bobtail squids) and Spirulida (ram's horn squid) (Young *et al.*, 1998). There are many variations on this classification that differ in detail (number of orders, suborders, superfamilies recognized, and their rank). For example, the orders Cirroctopodida and Octopodida have also been classified as suborders Cirrata and Incirrata, using Octopoda as the order designation and Octopodiformes as the superorder designation (Young and Vecchione 1999; Norman 2000). An additional level of controversy exists when fossil taxa are incorporated in classifications (see also previous section) and different classifications result (Berthold and Engeser 1987) (Figure 8.6).

MORPHOLOGICAL DATA

The most extensive morphological phylogenetic analysis of coleoids is that of Young and Vecchione (1996), who examined 50 morphological characters of 24 species from 17 families to determine whether Cirrata, Incirrata, and Decabrachia were monophyletic and whether vampyromorphs were included within the Octobrachia or Decabrachia. Because of problems with character independence, lack of apomorphic characters, and possible presence of homoplasy among several key features, many characters were disregarded prior to analysis. Their analysis resulted

in a better understanding of the relationships between incirrate and cirrate octopods, as well as the placement of Vampyromorpha as sister to the Octobrachia. Taxa within the Decabrachia were unresolved, although that group was not the primary focus of this analysis. In the ten years since their study, numerous Paleozoic coleoids have been described (see previous section and Table 8.1), which will undoubtedly have an impact on our understanding of early coleoid evolution. Other morphological studies focused on one or a few key characters for higher-level relationships (Roper 1969; Toll 1982; Boletzky 1987; Hess 1987; Nesis 1987; Voight 1997; Young *et al*. 1998; Vecchione *et al*. 1999; Haas 2002), but none were able to provide robust phylogenies for family-level relationships (e.g., within the Decabrachia).

MOLECULAR PHYLOGENETICS

SINGLE-GENE TREE PHYLOGENIES

The first attempt at using molecular data to determine family-level relationships of coleoids was by Bonnaud and co-workers (Bonnaud *et al*. 1994) using a 450–580 bp sequence of the 16S rDNA locus from 27 species of decabrachian squids, representing eight families. This study supported many of the higher order-level relationships resolved in some of the previous morphological studies (e.g., Young and Vecchione 1996; Young *et al*. 1998), yet it unsuccessfully delineated many of the family-level relationships. Following this, three more extensive molecular phylogenetic analyses were completed using loci from the mitochondrial genome; the cytochrome *c* oxidase subunit I (COI) and combined cytochrome *c* oxidase subunits II and III for 48 and 17 taxa respectively (Bonnaud 1995; Bonnaud *et al*. 1997; Carlini and Graves 1999). These studies demonstrated the monophyly of Coleoidea, Octobrachia, Vampyromorpha, and Decabrachia; that Vampyromorpha is sister to the Octobrachia; the polyphyly of Sepioidea; and the lack of resolution of lower-level taxa, particularly within the Decabrachia. *Spirula,* which was previously included within the Sepioidea (e.g., Voss 1977), was separated in both studies and grouped with the teuthoids. There were also some discrepancies between the analyses, with placement of Idiosepiidae either with Sepiadariidae (Carlini and Graves 1999), or within the Oegopsida (Bonnaud *et al*. 1997). A separate study used the actin gene family to determine coleoid phylogeny from 44 representative taxa (Carlini *et al*. 2000). The results recognized that multigene families of actin existed with the Cephalopoda (therefore producing gene trees more than taxon-specific trees), and the information had little resolution of decabrachian relationships, particularly within the orders of Teuthoidea, Sepioidea, and the teuthid suborder Oegopsida, but gave support to the Myopsida.

MULTILOCUS AND COMBINED PHYLOGENETIC ANALYSES

Concurrently, there has been a recognition that additional genes, or a combination of genes and morphology, was needed to provide more resolution, not only at the higher levels within coleoid cephalopods but also within "orders" of the Decabrachia. The first analysis using both molecular and morphological data sets (Carlini *et al*. 2001) reevaluated previous COI data (Carlini and Graves 1999) for octopod phylogeny in light of previous morphological evidence (Young and Vecchione 1996). Although a number of congruencies were supported using both data sets (monophyly of the Octopoda and of Cirrata), other discrepancies have not yet been resolved (monophyly of Incirrata). Studies using additional mitochondrial loci (Takumiya *et al*. 2005) or entire mitochondrial genomes (Yokobori *et al*. 2004; Akasaki *et al*. 2006) supported higher-level coleoid relationships but were still not able to resolve family-level hierarchies, particularly in the Decabrachia.

Because of conflict between molecular and morphological data sets, more recent analyses have used a combination of molecular data (using multiple genes) and morphology with a variety of analyses to determine both higher- and lower-level relationships within cephalopods (Lindgren *et al*. 2004; Strugnell *et al*. 2005). Morphology has been successfully used to define higher-level classification among the Octobrachia and Decabrachia, but

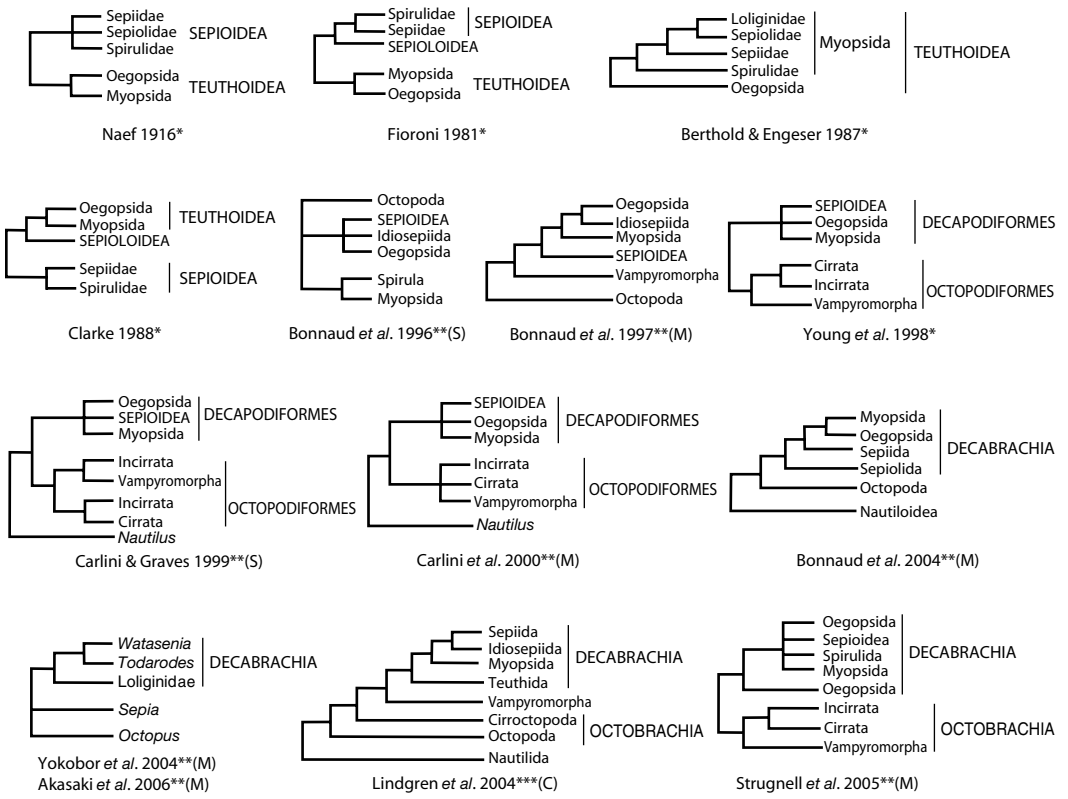


FIGURE 8.8. Summaries of phylogenetic hypotheses of several models of cephalopod evolution based on morphological (*), molecular (**), and combined data (***). Note the differences between many of the morphologically based phylogenies, as well as those using various molecular (single [S] or multiple [M] genes) and combined (molecular and morphology, [C]) data sets.

the lower-level relationships have been better resolved using molecular systematics. This has also been true for other metazoan groups where the diversity among classes is quite high (Giribet *et al.* 2001; Giribet and Edgecombe 2006). Incongruence between morphological and molecular data is not uncommon in other phylogenetic studies (Giribet 2003), and therefore, further investigation of the resolution between individual genes, synonymous and non-synonymous substitutions, and the use of coding genes (Strugnell *et al.* 2005) may help increase support for unresolved relationships. Inclusion of fossil data to "fill in" information regarding rapid radiations or extinctions may also help support nodes that contain problematic taxa (e.g., Vampyromorpha).

Comparisons of individual trees derived from a variety of analyses of single genes and morphological data have provided information regarding which loci/characters have more or less resolution. This can be of use, since genes that evolve faster will have higher resolution at the family/species/population level, whereas slower-evolving or more conserved genes (such as the ribosomal genes) will have more resolution at the basal nodes of larger groups. For example, more conserved loci, such as 18S and 28S rDNA, provide information regarding the monophyly for Cephalopoda and Coleoidea but have little resolution among the lower-level relationships among orders and families (Lindgren *et al.* 2004, table 1). Combined analyses (Lindgren *et al.* 2004), along with several other studies (Bonnaud *et al.* 1994, 1997; Carlini and Graves 1999; Nishiguchi *et al.* 2004; Strugnell *et al.* 2005) have demonstrated that relationships among Sepiolida, Sepiida, Idiosepiida, and the Loliginidae are well supported by the addition of molecular data, while the position

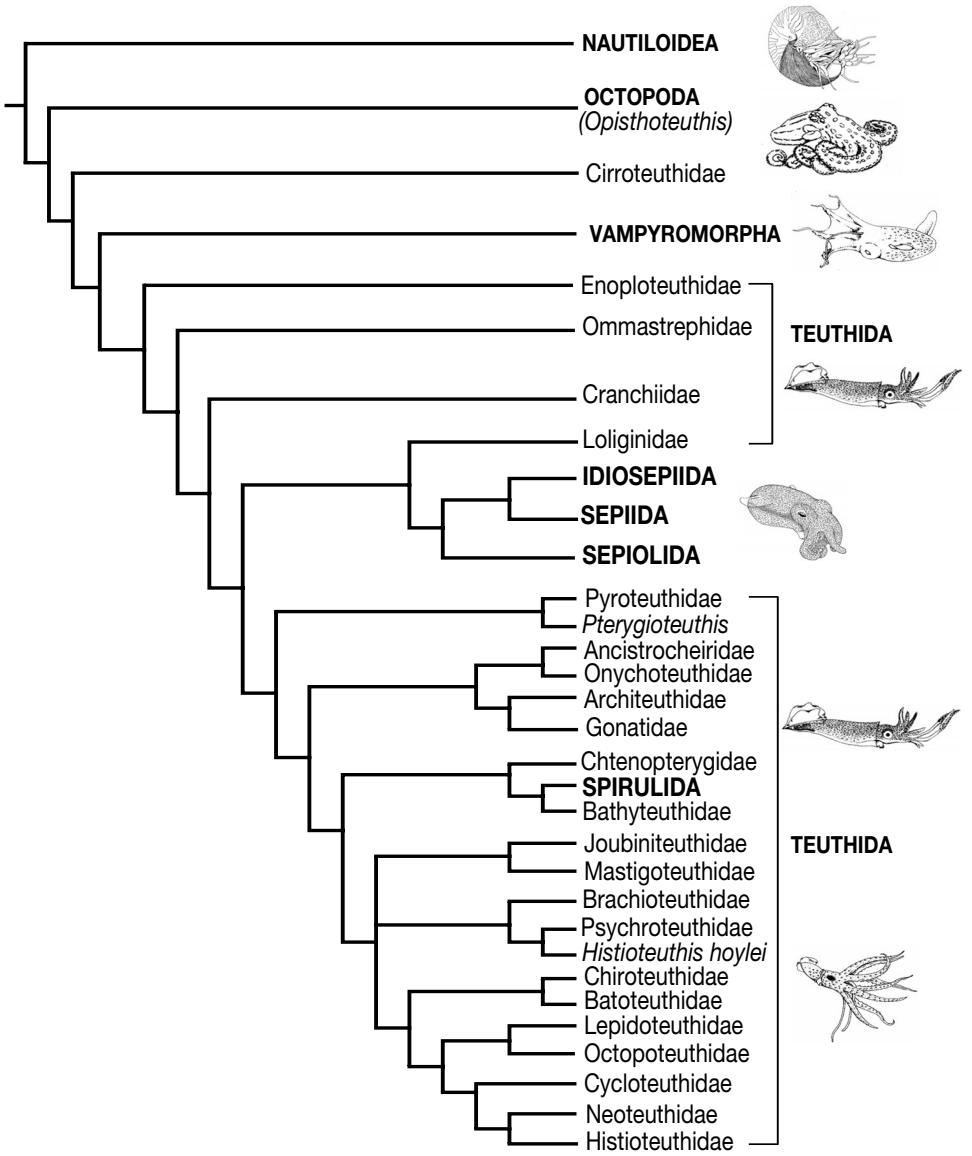


FIGURE 8.9. Schematic representation of modern cephalopod relationships based on the optimal parameter set using direct optimization (via parsimony) for the combined analysis of 101 morphological characters and molecular data from four loci (mitochondrial cytochrome *c* oxidase subunit I, nuclear 18S rDNA, the D3 expansion fragment of 28S rDNA, and histone H3). Taxa in capitals represent orders of cephalopods that appeared monophyletic in the analysis (from Lindgren *et al.* 2004). Drawings by G. Williams.

of *Spirula* within the sepioid groups remains to be resolved (Figures 8.8, 8.9). Although the molecular data has added increased resolution between the orders, it still does not resolve relationships between large family groups, such as the Oegopsida. This is probably because this order radiated rather quickly and, because sampling some of the deeper water families is difficult, they are under-represented in most molecular studies to date.

One of the most debatable relationships is the placement of Vampyromorpha within the Decabrachia (Figures 8.8, 8.9). Earlier studies have placed Vampyromorpha with the Octobrachia based on embryological and developmental data (Naef 1928, Young and Vecchione 1996;

Boletzky 2003) as well as several morphological, including ultrastructural, characters (Toll 1982, 1998; Healy 1989, Lindgren *et al*. 2004). However, molecular studies (Bonnaud *et al*. 1997; Carlini and Graves 1999; Lindgren *et al*. 2004) have shown different results depending on which molecular loci were tested and which outgroup taxa and parameter variations were used. The main conflict for this work was between the morphological and the molecular/combined data. Morphological data suggests a sister relationship between Vampyromorpha and the Octobrachia, whereas the molecular and combined data suggest a closer relationship between Vampyromorpha and the Decabrachia. Additional studies are needed to resolve these and other internal relationships, and the inclusion of more taxa (particularly from those groups that have been poorly sampled and therefore under-represented in previous results) will help provide a more detailed phylogeny of cephalopod evolution.

Additional molecular studies have recently been used to investigate decabrachian evolution with respect to the placement of *Spirula* using 18S rDNA (Warnke *et al*. 2003); lower-level relationships within families (16S rDNA for cirrate octopod relationships [Piertney *et al*. 2003] and microsatellite and 16S rDNA for loliginid relationships [Shaw *et al*. 1999; Anderson 2000; Reichow and Smith 2001]); identification of juvenile and adult gonatid squids (Seibel *et al*. 2000; Lindgren *et al*. 2005); as well as investigating the evolution of species complexes among octopods (Söller *et al*. 2000; Strugnell *et al*. 2004; Allcock *et al*. 1997; Guzik *et al*. 2005) and sepiolids (Nishiguchi *et al*. 2004), to name a few. Population genetic studies have also helped provide information regarding the migration of specific haplotypes and the phylogeography of sister species (Anderson 2000; Herke and Foltz 2002; Jones *et al*. 2006).

DEVELOPMENT

More recently, sophisticated techniques have improved our understanding of the development and neontology of cephalopods. Naef provided much of the foundation for the embryology of many cephalopod species, which is elegantly presented in his monograph (Naef 1928). This work provided cephalopod researchers with a large number of characters that were useful for morphological comparisons and, subsequently, cephalopod systematics and evolution. Recent techniques using *in situ* hybridization have allowed the expansion of a new field of research, namely evolutionary developmental biology, to help understand how specific genes are expressed among related taxa. This information can provide insight as to whether the same gene in a variety of organisms controls key developmental traits, or whether these genes have been co-opted to function for other developmental programs. For instance, master control genes such as *Hox* and *Pax6* have demonstrated the relationship of closely related molluscs and the conservative nature of these sequences (Halder *et al*. 1995, see also Wanninger *et al*., Chapter 16). Although the genetic information may not give the resolution needed to solve internal cephalopod nodes, the observation of protein expression can provide insights into the differentiation of cephalopod morphology and the co-option of genes for numerous functions (Tomarev *et al*. 1997; Callaerts *et al*. 2002; Hartmann *et al*. 2003; Lee *et al*. 2003).

Following developmental programs and combining them with phylogenetic information may also give insights as to the evolution of specific morphological features and provide evidence for relationships that are linked only by these features, such as bacteriogenic light organs in both sepiolids and loliginid squids (Foster *et al*. 2002; Nishiguchi *et al*. 1998, 2004) and symbiosis between squids and bacteria in accessory nidamental glands (Grigioni *et al*. 2000; Pichon *et al*. 2005). Elegant work has recently provided detailed maps of the neurodevelopment of myopsid, oegopsid, and idiosepiid squids (Shigeno *et al*. 2001a, b; Shigeno and Yamamoto 2002), exhibiting major differences among these squids. Protein regulation and gene expression has recently been examined in squids for use in diagnosing induction

of tissue morphogenesis and apoptosis (Crookes *et al.* 2004; Doino and McFall-Ngai 1995; Foster and McFall-Ngai 1998; Montgomery and McFall-Ngai 1992; Small and McFall-Ngai 1998; Zinovieva *et al.* 1993). Finally, advanced tracer techniques, biochemical analyses, and direct *in situ* measurements of growth and other physiological attributes are providing information regarding the growth, life history strategies, and behavior of squids, both from the wild and in the laboratory (Forsythe *et al.* 2001, 2002; Hanlon *et al.* 1997; Huffard *et al.* 2005; Jones and Nishiguchi 2004; Kasugai 2000; Landman *et al.* 2004; Moltschaniwskyj 1994; Pecl and Moltschaniwskyj 1997; Shea 2005; Steer *et al.* 2004).

All these comparative studies have proven to be useful in providing additional information not only for delineating function and life history characteristics but also to delve into the possible evolutionary trajectories that may explain similar characteristics shared among distantly related cephalopods. Combining this information with molecular studies may help evolutionary biologists test homology hypotheses of morphological features or shared developmental patterns in gene expression, which may be evolutionarily derived. These can then be used as key innovations and included in cladistic analyses.

ADAPTIVE RADIATIONS

In fossil cephalopods, the focus of the few studies on adaptive radiation have been on the progressive changes of the external shell through geologic time as predators became more efficient (for an extended discussion of the theory of evolution and escalation see Vermeij 1987; also see Mapes and Chaffin 2003). Ward (1981) determined that the ornament on the shells of ammonoids became rougher through time (Devonian to Cretaceous). Signor and Brett (1984) analyzed the tarphycerids, barrandeoceriids, and nautilids in part of the Paleozoic and determined that the origin of the durophagous (shell-crushing) predators in the Silurian and Devonian coincided with a gradually increased robustness of the ornament in these nautiloid orders. As an additional observation on fossil cephalopod evolutionary radiations, Vermeij (1987) observed that the external shells of ancient nautiloids have not proven to be an effective counter to predation in the long run. The rationale behind this observation is that, given the evolutionary success of durophagous predators through time, and given that modern cephalopods without external shells (i.e., the Coleoidea) are so much more abundant, diverse, and worldwide in their distribution today as compared to modern *Nautilus* and *Allonautilus* with their external shells and limited geographic distribution, the externally shelled cephalopods were not competitive in the long run. This conclusion seems obvious given the then-known fossil record of ectocochleate cephalopods versus the abundance of endocochleate cephalopods living today. However, the externally shelled cephalopods (e.g., the Nautiloidea and Ammonoidea) had a long and diverse history (see Figure 8.5, for example, showing the complexity of ammonoid phylogeny) and that these lineages survived many extinction events and reradiated to refill and dominate the world's oceans. Notably, we now know that the evolution of shell-less coleoids occurred prior to the Upper Carboniferous and that, while these shell-less coleoids must have also survived the same extinction events in the Late Paleozoic and Mesozoic as the externally shelled cephalopods, they did not overwhelm them and become the dominant cephalopod group after those extinction events. It was only after almost all the externally shelled forms had become extinct (all the Ammonoidea and virtually all of the Nautiloidea) at the end of the Mesozoic that the coleoids radiated to become the dominant cephalopod group in the world's oceans.

Cephalopods, with their sensory/nervous/visual systems, are excellent predators, being entirely carnivorous on a large variety of prey species. Not only does their advanced nervous system allow for a great ability for capturing prey, but it also increased their ability to invade

a multitude of niches. Based on observations of modern coleoids, the evolution of a complex visual system and chromatophores was probably related to the development of a well developed nervous system (Budelmann *et al*. 1997; Hanlon and Messenger 1996), which, through integration with a highly developed system of dermal chromatophores, enables most coleoid cephalopods to change color rapidly. Such color changes are used for camouflage; to display specific patterns toward predators, prey, other cephalopod species; or for intraspecific behavioral interactions, such as sexual displays (Hanlon and Messenger 1996), and presumably played a large part in the success of the group. The static presumed cryptic coloration of the shell and animal of living *Nautilus* is in marked contrast. The evolution from a shelled to a nonshelled coleoid probably began in the Devonian and/or early Carboniferous (see above) because more efficient and active predators evolved at that time. By shell reduction and the evolutionary selection of other advanced traits (i.e., complex brain driving other characters), the coleoids evolved elaborate sensory systems fulfilling tasks achieved by effectors (i.e., camouflage) to counter the evolutionary pressures created by efficient and more active predators (Nixon and Young 2003).

Along with major changes in behavior due to a highly advanced nervous system, cephalopods have evolved various mechanisms for controlling buoyancy and propulsion. As previously mentioned, buoyancy in cephalopods is controlled by the presence of gas spaces (as in taxa such as *Nautilus, Spirula,* and *Sepia,* which have a shell or cuttlebone). Cephalopods with no gas-filled compartments must rely on using jet propulsion to move continually through the water column to maintain buoyancy, or they achieve it by an increase in solutes such as ammonium chloride within the coelomic space or in vacuoles within their tissues (Boyle and Rodhouse 2005). The evolution of fins also helped to orient and maneuver the body during swimming, depending on the lifestyle of the squid (epipelagic fast swimmers versus slower, coastal species). Finally, the evolution of arms and tentacles enabled cephalopods to possess the ability to handle prey efficiently, allowing them to feed on a large variety of prey items. The arm crown, which is derived from the mouth of cephalopods (Packard 1972), is much more versatile for capturing and handling prey (Boyle and Rodhouse 2005). Along with arms and tentacles that possess suckers for holding and attaching onto prey, these adaptive characteristics contributed to the success of cephalopods as predators compared to other molluscan groups.

FUTURE STUDIES

The largest unresolved problems in the fossil Coleoidea are the problematic origins of the order and the relationships between the different families and genera that are assigned to the orders. To refine our understanding of the evolutionary events that gave rise to the Coleoidea will require new material, especially from Devonian-aged rock units. Efforts should focus on Lagerstätten where tissues can be preserved and there is excellent shell preservation. Paleoenvironments that had well-oxygenated water columns and anoxic conditions at the water/sediment interface are likely candidates for yielding well-preserved fossil coleoids.

There are also significant problems with obtaining living taxa. Many species do not survive commonly used collection techniques (such as deep-water trawls), suffering extensive damage and frustrating morphological studies. Similarly, there are difficulties in obtaining eggs or juveniles to study development with pelagic taxa and in obtaining rare or deep-water taxa for molecular studies.

The resolution of the problem of whether the coleoids have a monophyletic or paraphyletic origin will require additional research on existing specimens and the collection and analysis of new living and fossil material. Increased taxon sampling (both extinct and extant species) as well as the addition of larger molecular data sets (complete mitochondrial genomes, additional nuclear genes) will provide resolution

both at the higher level relationships and those that have been particularly problematic (e.g., Decabrachia). Thus, we need to obtain and combine more fossil, morphological, developmental, and molecular data in order to increase our understanding of the interrelationships of these amazingly diverse and ecologically important molluscs.

ACKNOWLEDGMENTS

The authors would like to acknowledge the invaluable advice from S. v. Boletzky, whose expertise in many aspects of cephalopod evolution were greatly appreciated, as well as the three reviewers who provided additional insights for this chapter. The work is partially supported by NIH SO6 GM008136-31 and NSF DEB-0316516 to M. K. N. We would like to thank S. v. Boletzky, D. Lindberg, M. Norman, Conch Books, and *Science* magazine for reproduction of photographs used in this chapter.

REFERENCES

Akasaki, T., Nikaido, M., Tsuchiya, K., Segawa, S., Hasegawa, M., and Okada, N. 2006. Extensive mitochondrial gene rearrangements in coleoid Cephalopoda and their phylogenetic implications. *Molecular Phylogenetics and Evolution* 38: 648–658.

Allcock, A. L., Brierley, A. S., Thorpe, J. P., and Rodhouse, P. G. K. 1997. Restricted gene flow and evolutionary divergence between geographically separated populations of the Antarctic octopus *Pareledone turqueti*. *Marine Biology* 129: 97–102.

Allison, P. A. 1987. A new cephalopod with soft parts from the Upper Carboniferous Francis Creek Shale of Illinois, USA. *Lethaia* 20: 117–121.

Anderson, F. E. 2000. Phylogeny and historical biogeography of the loliginid squids (Mollusca: Cephalopoda) based on mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution* 15: 191–214.

Arnold, J. M. 1962. Mating behavior and social structure in *Loligo pealeii*. *Biological Bulletin* 123: 53–57.

———. 1987. Reproduction and embryology of *Nautilus*. In *Nautilus: The Biology and Paleobiology of a Living Fossil*. Edited by W. B. Saunders and N. H. Landman. New York: Plenum Press, pp. 13–16.

Aronson, R. B. 1991. Ecology, paleobiology and evolutionary constraint in the octopus. *Bulletin of Marine Science* 49: 245–255.

Bandel, K., and Boletzky, S. v. 1988. Features of development and functional morphology in the reconstruction of early coleoid cephalopods. In *Cephalopods Present and Past*. Edited by J. Wiedmann and J. E. Kullmann. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung, pp. 229–246.

Bandel, K., and Leich, H. 1986. Jurassic Vampyromorpha (dibranchiate cephalopods). *Neues Jahrbuch für Geologie und Palaeontologie, Abhandlungen* 3: 129–148.

Bandel, K., Reitner, J., and Stürmer, W. 1983. Coleoids from the Lower Devonian Black Slate ("Hunsrück-Schiefer") of the Hunsrück (West Germany). *Neues Jahrbuch für Geologie und Palaeontologie, Abhandlungen* 165: 397–417.

Bandel, K., and Spaeth, C. 1988. Structural differences in the ontogeny of some belemnite rostra. In *Cephalopods Present and Past*. Edited by J. Wiedmann and J. E. Kullmann. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung, pp. 247–261.

Becker, T. R., and Kullmann, J. 1996. Paleozoic ammonoids in time and space. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 711–753.

Berthold, T., and Engeser, T. 1987. Phylogenetic analysis and systematization of the Cephalopoda. *Verhandlungen des Naturwissenschaftlichen Vereins Hamburg* 29:187–220.

Boletzky, S. v. 1987. Ontogenetic and phylogenetic aspects of the cephalopod circulatory system. *Experientia* 43: 478–483.

———. 1997. Developmental constraints and heterochrony: a new look at offspring size in cephalopod molluscs. *Geobios Memoire Special* 21: 267–275.

———. 2002. Yolk sac morphology in cephalopod embryos. In *Cephalopods Present and Past*. Edited by H. Summesberger and H. E. A. Daurer. Vienna: Abhandlungen der Geologischen Bundesanstalt, pp. 57–68.

———. 2003. Biology of early life stages in cephalopod molluscs. *Advances in Marine Biology* 44: 143–203.

Bonnaud, L. 1995. *Phylogenie des céphalopodes decapodes*. Paris: Museum National d'Histoire Naturelle.

Bonnaud, L., Boucher-Rodoni, R., and Monnerot, M. 1994. Phylogeny of decapod cephalopods based on partial 16S rDNA nucleotide sequences. *Comptes Rendus de l'Académie des Sciences de Paris* 317: 581–588.

———. 1997. Phylogeny of cephalopods inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 7: 44–54.

Botter, D., Etter, W., Hagadorn, J. W., and Tang, C. M. 2002. *Exceptional Fossil Preservation: a Unique View on the Evolution of Marine Life*. New York: Columbia University Press.

Boyle, P. R., and Boletzky, S. v. 1996. Cephalopod populations: definitions and dynamics. *Philosophical Transactions of the Royal Society Series B: Biological Sciences* 351: 985–1002.

Boyle, P. R., and Rodhouse, P. 2005. *Cephalopods. Ecology and Fisheries*. Oxford: Blackwell.

Budelmann, B. U., Schipp, R., and Boletzky, S. v. 1997. Cephalopoda. In *Microscopic Anatomy of Invertebrates*. Edited by F. W. Harrison and A. J. Kohn. New York: Wiley-Liss, pp. 119–414.

California Department of Fish and Game. 2003. *Final Market Squid Fishery Management Plan*. California Department of Fish and Game. http://www.dfg.ca.gov/mrd/msfmp/index.html.

Callaerts, P., Lee, P. N., Hartmann, B., Farfan, C., Choy, D. W. Y., Zkeo, K., Fischbach, K.-F., Gehring, W. J., and Couet, H. G. d. 2002. *Hox* genes in the sepiolid squid *Euprymna scolopes*: Implications for the evolution of complex body plans. *Proceedings of the National Academy of Sciences of the United States of America* 99: 2088–2093.

Carlini, D. C., and Graves, J. E. 1999. Phylogenetic analysis of cytochrome *c* oxidase I sequences to determine higher-level relationships within the coleoid cephalopods. *Bulletin of Marine Science* 64: 57–76.

Carlini, D. C., Reece, K. S., and Graves, J. E. 2000. Actin gene family evolution and the phylogeny of coleoid cephalopods (Mollusca: Cephalopoda). *Molecular Biology and Evolution* 17: 1353–1370.

Carlini, D. C., Young, R. E., and Vecchione, M. 2001. A molecular phylogeny of the Octopoda (Mollusca: Cephalopoda) evaluated in light of morphological evidence. *Molecular Phylogenetics and Evolution* 21: 388–397.

Chen, J.-Y., and Teichert, C. 1983. Cambrian cephalopods. *Geology* 11: 647–650.

Clarke, M. R. 1988. Evolution of Recent cephalopods—a brief review. In *The Mollusca, Paleontology and Neontology of Cephalopods*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 331–341.

———. 1996. Cephalopods as prey. III. Cetaceans. *Philosophical Transactions of the Royal Society Series B: Biological Sciences* 351: 1053–1065.

Clarke, M. R., and Maddock, L. 1988a. Beaks of living cephalopods. In *The Mollusca, Paleontology and Neontology of Cephalopods*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 123–131.

———. 1988b. Statoliths from living species of cephalopods and evolution. In *The Mollusca, Paleontology and Neontology of Cephalopods*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 169–184.

Collins, D., and Ward, P. D. 1987. Adolescent growth and maturity in *Nautilus*. In *Nautilus: The Biology and Paleobiology of a Living Fossil*. Edited by W. B. Saunders and N. H. Landman. New York: Plenum Press, pp. 421–432.

Crick, R. E., and Ottensman, V. M. 1983. Sr, Mg, Ca, and Mn, chemistry of skeletal components of a Pennsylvanian and Recent nautiloid. *Chemical Geology*: 147–163.

Crookes, W. J., Ding, L.-L., Huang, Q. L., Kimbell, J. R., Horwitz, J., and McFall-Ngai, M. J. 2004. Reflectins: The unusual proteins of squid reflective tissues. *Science* 303: 235–238.

Croxall, J. P., and Prince, P. A. 1996. Cephalopods as prey. I. Seabirds. *Philosophical Transactions of the Royal Society Series B: Biological Sciences* 351: 1023–1043.

d'Orbigny, A. 1845. *Mollusques vivants et fossiles, ou description de toutes les espèces de coquilles et de mollusques*. Paris: Adolphe Delahays.

Davis, R. A., Landman, N. H., Dommergues, J.-L., Marchand, D., and Bucher, H. 1996. Mature modifications and dimorphism in ammonoid cephalopods. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 464–539.

de Konick, L. G. 1843. Notice sur une coquille fossile de terrains anciens de Belgique. *Bulletin de l'Académie Royale des Sciences, des Lettres et des Beaux-Arts de Belgique* 10: 207–208.

Doguzhaeva, L. A., Mapes, R. H., and Mutvei, H. 1999. A Late Carboniferous spirulid coleoid from the Southern Mid-continent (USA). In *Advancing Research on Living and Fossil Cephalopods*. Edited by F. Olóriz and Rodríguez-Tovar, New York/Boston: Kluwer Academic/Plenum Publishers, pp. 47–57.

———. 2002a. The coleoid with an ink sac and a body chamber from the Upper Pennsylvanian of Oklahoma, USA. *Berliner Paläobiologische Abhandlungen* 1: 34–38.

———. 2002b. Early Carboniferous coleoid *Hematites* Flower and Gordon, 1959 (Hematitida ord. nov.) from Midcontinent (USA). In *Cephalopods Present and Past*. Edited by H. Summesberger, K. Histon, and A. Daurer. Vienna: Abhandlungen Geologische Bundesanstalt. pp. 299–320.

———. 2004a. Occurrence of ink in Paleozoic and Mesozoic coleoids (Cephalopoda). *Mitteilungen des Geologisch-Paläontologischen Institutes der Universität Hamburg* 88: 145–156.

Doguzhaeva, L. A., Mutvei, H., Summesberger, H., and Dunca, E. 2004b. Bituminous soft body

tissues in the body chamber of the Late Triassic ceratitid *Austrotrachyceras* from the Austrian Alps. *Mitteilungen des Geologisch-Paläontologischen Institutes der Universität Hamburg* 88: 37–50.

Doguzhaeva, L. A., Mapes, R. H., Mutvei, H., and Pabian, R. K. 2002c. The late Carboniferous phragmocone-bearing orthoconic coleoids with ink sacs: their environment and mode of life. In *Geological Society of Australia Abstracts*, number 68. Edited by G. A. Brock and J. A. Talent, First International Palaeontological Congress, July 2002, Macquarie University, NSW Australia: p. 200.

Doguzhaeva, L. A., Mapes, R., Summesberger, H., and Mutvei, H. In press, a. A late Carboniferous coleoid from the Mazon Creek (USA) with a radula, arm hooks, mantle tissues, and ink. In *Cephalopods Present and Past*. Edited by N. H. Landman, R. A. Davis, R. H. Mapes, and W. L. Manger. Berlin: Springer-Verlag.

———. In press, b. The preservation of soft tissues, shell, and mandibles in the ceratitid ammonoid *Austrotrachyceras* (Late Triassic), Austria. In *Cephalopods Present and Past*. Edited by N. H. Landman, R. A. Davis, R. H. Mapes, and W. L. Manger. Berlin: Springer-Verlag.

Doino, J. A., and McFall-Ngai, M. J. 1995. A transient exposure to symbiosis-competent bacteria induces light organ morphogenesis in the host squid. *Biological Bulletin* 189: 347–355.

Donovan, D. T. 1977. Evolution of the dibranchiate Cephalopoda. *Symposia of the Zoological Society of London* 38: 15–48.

Donovan, D. T., and Toll, R. B. 1988. The gladius in coleoid (Cephalopoda) evolution. In *The Mollusca, Paleontology and Neontology of Cephalopods*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 89–101.

Donovan, D. T., Callomon, J. H., and Howarth, M. K. 1981. Classification of the Jurassic Ammonitina. In *The Ammonoidea*. Edited by M. R. House and J. R. Senior. New York: Academic Press, pp. 101–155.

Doyle, P., Donovan, D. T., and Nixon, M. 1994. Phylogeny and systematics of the Coleoidea. *University of Kansas Paleontology Contributions, New Series* 5:1–15.

Engeser, T. 1996. The position of the Ammonoidea within the Cephalopoda. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 3–19.

Engeser, T., and Bandel, K. 1988. Phylogenetic classification of coleoid cephalopods. In *Cephalopods: Present and Past*. Edited by J. Wiedmann and J. Kullmann. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung, pp. 105–115.

Engeser, T., and Reitner, J. 1981. Beiträge zur Systematik von phragmokontragenden Coleoiden aus dem Untertithonium (Malm zeta, Solnhofener Plattenkalk) von Solnhofen und Eichstätt (Bayern). *Neues Jahrbuch für Geologie und Paläontologie, Monatshefte* 1981: 527–545.

Erben, H. K. 1964. Die Evolution der ältesten Ammonoidea (Lieferung I). *Neues Jahrbuch für Geologie und Paläontologie, Monatshefte* 120: 107–212.

Fiorito, G., and Scotto, P. 1992. Observational learning in *Octopus vulgaris*. *Science* 256: 545–547.

Flower, R. H. 1945. A belemnite from a Mississippian boulder of the Caney shale. *Journal of Paleontology* 19:490–503.

Flower, R. H., and Gordon, M., Jr. 1959. More Mississippian belemnites. *Journal of Paleontology* 33: 809–842.

Forsythe, J., Lee, P., Walsh, L., and Clark, T. 2002. The effects of crowding on the growth of the European cuttlefish, *Sepia officinalis* Linnaeus, 1758 reared at two temperatures. *Journal of Experimental Marine Biology and Ecology* 269: 173–185.

Forsythe, J. W., Walsh, L. S., Turk, P. E., and Lee, P. G. 2001. Impact of temperature on juvenile growth and age at first egg-laying of the Pacific reef squid *Sepioteuthis lessoniana* reared in captivity. *Marine Biology* 138:103–112.

Foster, J. S., Boletzky, S. v., and McFall-Ngai, M. J. 2002. A comparison of the light organ development of *Sepiola robusta* Naef and *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). *Bulletin of Marine Science* 70: 141–153.

Foster, J. S., and McFall-Ngai, M. J. 1998. Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelia tissues. *Development, Genes, and Evolution* 208: 295–303.

Frest, T. J., Glenister, B. F., and Frunish, W. M. 1981. Pennsylvanian-Permian cheiloceratacean ammonoid families Maximitidae and Pseudohaloritidae. *Journal of Paleontology Memoir 11* 55: 1–46.

Fuchs, D., Keupp, H., and Engeser, T. 2003. New records of soft parts of *Muensterella scutellaris* Muenster, 1842 (Coleoidea) from the Late Jurassic plattenkalks of Eichstätt and their significance for octobrachian relationships. *Berliner Paläobiologische Abhandlungen* 3: 101–111.

Gardner, G., and Mapes, R. 2001. Relationships of color patterns and habitat for Lower Triassic ammonoids from Crittenden Springs, Elko County, Nevada. *Revue de Paléobiologie Genève* 8: 109–122.

Gilly, W. F., and Lucero, M. T. 1992. Behavioral responses to chemical stimulation of the olfactory organ in the squid *Loligo opalescens*. *Journal of Experimental Biology* 162: 209–229.

Giribet, G. 2003. Stability in phylogenetic formulations and its relationship to nodal support. *Systematic Biology* 52: 554–564.

Giribet, G., and Edgecombe, G. D. 2006. Conflict between data sets and phylogeny of centipedes: an analysis based on seven genes and morphology. *Proceedings of the Royal Society of London Series B: Biological Sciences* 273:531–538.

Giribet, G., Edgecombe, G. D., and Wheeler, W. C. 2001. Arthropod phylogeny based on eight molecular loci and morphology. *Nature* 413: 157–161.

Giribet, G., Okusu, A., Lindgren, A. R., Huff, S. W., Schrodl, M., and Nishiguchi, M. K. 2006. Evidence for a clade composed of molluscs with serially repeated structures: Monoplacophorans are related to chitons. *Proceedings of the National Academy of Sciences of the United States of America* 103: 7723–7728.

Giribet, G., and Wheeler, W. C. 2002. On bivalve phylogeny: a high-level analysis of the Bivalvia (Mollusca) based on combined morphology and DNA sequence data. *Invertebrate Zoology* 121: 271–324.

Glenister, B. F., and Furnish, W. M. 1981. Permian Ammonoids. In *The Ammonoidea*. Edited by M. R. House and J. R. Senior. New York: Academic Press, pp. 49–64.

Gordon, M., Jr. 1964. Carboniferous cephalopods of Arkansas. *Geological Survey Professional Paper* 460.

Gordon, M., Jr. 1966. Classification of Mississippian coleoid cephalopods. *Journal of Paleontology* 40: 449–452.

Grigioni, S., Boucher-Rodoni, R., Demarta, A., Tonolla, M., and Peduzzi, R. 2000. Phylogenetic characterization of bacterial symbionts in the accessory nidamental glands of the sepioid *S. officinalis* (Cephalopoda: Decapoda). *Marine Biology* 136: 217–222.

Guzik, M. T., Norman, M. D., and Crozier, R. H. 2005. Molecular phylogeny of the benthic shallow-water octopuses (Cephalopoda: Octopodinae). *Molecular Phylogenetics and Evolution* 37: 235–248.

Haas, W. 2003. The evolutionary history of the eight-armed Coleoidea. *Abhandlungen der Geologischen Bundesanstalt, Wien* 57: 341–351.

Hagadorn, J. W. 2002. Bear Gulch: an exceptional Upper Carboniferous plattenkalk. In *Exceptional Fossil Preservation: a Unique View on the Evolution of Marine Life*. Edited by D. Botter, W. Etter, and C. M. Tang, New York: Columbia University Press, pp. 167–183.

Halder, G., Callaerts, P., and Gehring, W. J. 1995. Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* 267: 1788–1792.

Hallam, A., and O'Hara, M. J. 1962. Aragonite fossils in the Lower Carboniferous of Scotland. *Nature* 195: 273–274.

Hanlon, R. T. 1988. Behavioral and body patterning characters useful in taxonomy and field identification of cephalopods. *Malacologia* 29:247–264.

Hanlon, R. T., Cooper, K. M., Budelmann, B. U., and Pappas, T. C. 1990. Physiological color change in squid iridophores. I. Behavior, morphology and pharmacology in *Lolliguncula brevis*. *Cell Tissue Research* 259: 3–14.

Hanlon, R. T., Claes, M. F., Ashcraft, S. E., and Dunlap, P. V. 1997. Laboratory culture of the sepiolid squid *Euprymna scolopes*: A model system for bacteria-animal symbiosis. *Biological Bulletin* 192: 364–374.

Hanlon, R. T., and Messenger, J. B. 1996. *Cephalopod Behaviour*. Cambridge, UK: Cambridge University Press.

Hartmann, B., Lee, P. N., Kang, Y. Y., Tomarev, S. I., de Couet, H. G., and Calherts, P. 2003. *Pax*6 in the sepiolid squid *Euprymna scolopes*: evidence for a role in eye, sensory organ and brain development. *Proceedings of the National Academy of Sciences of the United States of America* 93: 13683–13688.

Haszprunar, G. 1996. The Mollusca: Coelomate turbellarians or mesenchymate annelids? In *Origin and Evolutionary Radiation of the Mollusca*. Edited by J. Taylor. Oxford: Oxford University Press, pp. 1–28.

———. 2000. Is the Aplacophoran monophyletic? A cladistic point of view. *American Malacology Bulletin* 15: 115–130.

Healy, J. M. 1989. Spermatozoa of the deep-sea cephalopod *Vampyroteuthis infernalis* Chun: ultrastructure and possible phylogenetic significance. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* 323: 589–600.

Herke, S. W., and Foltz, D. W. 2002. Phylogeography of two squid (*Loligo pealei* and *L. plei*) in the Gulf of Mexico and northwestern Atlantic Ocean. *Marine Biology* 140: 103–115.

Hess, H. C. 1987. Comparative morphology, variability, and systematic applications of cephalopod spermatophores (Teuthoidea and Vampyromorpha). Ph.D. thesis, The University of Miami, Coral Gables, FL.

Hodgkin, A., and Huyley, A. 1952. Currents carried by sodium and potassium ions through the membrane of the giant squid axon of *Loligo*. *Journal of Physiology* 116: 449–472.

House, M. R. 1981. On the origin, classification and evolution of the early Ammonoidea. In *The Ammonoidea*. Edited by M. R. House and J. R. Senior. New York: Academic Press, pp. 3–36.

———. 1988. Major features of cephalopod evolution. In *Cephalopods Present and Past*. Edited by J. Wiedmann

and J. Kullmann. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung, pp. 1–16.

House, M. R. 1993. Fluctuations in ammonoid evolution and possible environmental controls. In *The Ammonoidea: Environment, Ecology, and Evolutionary Change*. Edited by M. R. House. Oxford, England: Clarendon Press, pp. 13–34.

Huffard, C. L., Boneka, F., and Full, R. J. 2005. Underwater bipedal locomotion by octopuses in disguise. *Science* 307: 1927.

Jackson, G. D., and Moltschaniwskyj, N. A. 2001. Temporal variation in growth rates and reproductive parameters in the small near-shore tropical squid *Loliolus noctiluca*; is cooler better? *Marine Ecology Progress Series* 218: 167–177.

Jeletzky, J. A. 1966. Comparative morphology, phylogeny, and classification of fossil Coleoidea. *University of Kansas Paleontology Contributions and Articles* 30: 1–162.

———. 1969. New or poorly understood Tertiary sepiids from southeastern United States and Mexico. *University of Kansas Paleontology Contributions and Articles* 41: 1–39.

Johnson, R. G., and Richardson Jr., E. S. 1968. Ten-armed fossil cephalopod from the Pennsylvanian of Illinois. *Science* 159: 526–528.

Jones, B. W., Lopez, J. E., Huttenberg, J., and Nishiguchi, M. K. 2006. Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Molecular Ecology* 15: 4317–4329.

Jones, B. W., and Nishiguchi, M. K. 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Marine Biology* 144: 1151–1155.

Kasugai, T. 2000. Reproductive behavior of the pygmy cuttlefish *Idiosepius paradoxus* in an aquarium. *Venus, the Japanese Journal of Malacology* 59: 37–44.

Kennedy, W., Landman, N. H., Cobbin, A., and Larson, N. L. 2002. Jaws and radulae in *Rhaeboceras*, a late Cretaceous ammonite. In *Cephalopods Present and Past*. Edited by H. Summesberger, K. Histon, and A. Daurer. Vienna: Abhandlungen Geologische Bundesanstalt, pp. 113–132.

Kluessendorf, J., and Doyle, P. 2000. *Pohlsepia mazonensis*, an early "octopus" from the Carboniferous of Pennsylvanian of Illinois. *Paleontology* 43: 919–926.

Kostak, M., Marek, J., Neumann, P., and Pavel, M. 2002. An early Carboniferous coleoid (Cephalopoda Dibranchiata) fossil from the Kulm of Northern Moravia (Czech Republic). In *Berliner Paläobiologisch Abhandlungen*. Edited by K. Warnke. International Symposium "Coleoids Cephalopods Through Time." Program and Abstracts 1: 58–60.

Kröger, B., and Mapes, R. H. 2007. On the origin of bactritoids (Cephalopoda). In *Seventh International Symposium Cephalopods—Present and Past,* Hokkaido University, Sapporo, Japan, Abstracts Volume, pp. 9–10.

Kulicki, C., Landman, N. H., Heaney, M. J., Mapes, R. H., and Tanabe, K. 2002. Morphology of the early whorls of goniatites from the Carboniferous Buckhorn Asphalt (Oklahoma) with aragonite preservation. In *Cephalopods Present and Past*. Edited by H. Summesberger, K. Histon, and A. Daurer. Vienna: Abhandlungen Geologische Bundesanstalt, pp. 205–224.

Kullmann, J. 1981. Carboniferous goniatites. In *The Ammonoidea*. Edited by M. R. House and J. R. Senior. New York: Academic Press, pp. 37–48.

Kullmann, J. 2002. Ammonoid evolution during critical intervals before and after the Devonian-Carboniferous boundary and the mid-Carboniferous boundary. In *Cephalopods Present and Past*. Edited by H. Summesberger, K. Histon, and A. Daurer. Vienna: Abhandlungen Geologische Bundesanstalt, pp. 371–377.

Landman, N. H., Cochran, J. K., Cerrato, R., Mak, J., Roper, C. F. E., and Lu, C. C. 2004. Habitat and age of the giant squid (*Architeuthis sanctipauli*) inferred from isotopic analyses. *Marine Biology* 144: 685–691.

Landman, N. H., and Davis, R. A. 1988. Jaw and crop preserved in an orthoconic nautiloid cephalopod from the Bear Gulch Limestone (Mississippian, Montana). *New Mexico Bureau of Mines and Mineral Resources, Mem* 44: 103–107.

Landman, N. H., and Cochran, J. K. 1987. Growth and longevity of *Nautilus*. In *Nautilus: The Biology and Paleobiology of a Living Fossil*. Edited by W. B. Saunders and N. H. Landman. New York: Plenum Press, pp. 401–420.

Lee, P. N., Callaerts, P., de Couet, H. G., and Martindale, M. Q. 2003. Cephalopod *Hox* genes and the origin of morphological novelties. *Nature* 424: 1061–1065.

Lehmann, U. 1967. Ammoniten mit Tintenbeutel. *Paläontologische Zeitschrift* 41: 132–136.

———. 1981. *The Ammonites: Their Life and Their World*. New York: Cambridge University Press.

———. 1985. Zur Anatomie der Ammoniten: Teintenbeutel, Kieman, Augen. *Palaeontologische Zeitschrift* 59:99–108.

———. 1988. On the dietary habits and locomotion of fossil cephalopods. In *Cephalopods Present and Past*. Edited by J. Wiedmann, and J. Kullmann. Stuttgart: E. Schweizerbart'sche Verlagsbuchhandlung, pp. 633–640.

Lindberg, D. R., and Ponder, W. F. 1996. An evolutionary tree for the Mollusca: Branches or roots? In *Origin and Evolutionary Radiation of the Mollusca*. Edited by J. Taylor. Oxford: Oxford University Press, pp. 135–154.

Lindgren, A. R., Giribet, G., and Nishiguchi, M. K. 2004. A combined approach to the phylogeny of Cephalopoda (Mollusca). *Cladistics* 20: 454–486.

Lindgren, A. R., Katugin, O. N., Amezquita, E., and Nishiguchi, M. K. 2005. Evolutionary relationships among squids of the family Gonatidae (Mollusca: Cephalopoda) inferred from three mitochondrial loci. *Molecular Phylogenetics and Evolution* 36: 101–111.

Makman, M. H., and Stefano, G. B. 1984. Marine mussels and cephalopods as models for study of neuronal aging. In *Invertebrate Models in Aging Research*. Edited by D. H. Mitchell and T. E. Johnson. Boca Raton, FL: CRC Press, pp. 165–189.

Mapes, R. H. 1979. Carboniferous and Permian Bactritoidea in North America. *University of Kansas Paleontological Contributions* 64:1–75.

———. 1987. Upper Paleozoic cephalopod mandibles: frequency of occurrence, modes of preservation, and paleoecological implications. *Journal of Paleontology* 61: 521–538.

Mapes, R. H., and Chaffin, D. T. 2003. Predation on cephalopods: a general overview with a case study from the upper Carboniferous of Texas. In *Predator-Prey Interactions in the Fossil Record*. Edited by P. H. Kelley, M. Kowalewski, and T. A. Hansen. New York: Kluwer Academic/Plenum Publishers, pp. 177–213.

Mapes, R., and Dalton, R. B. 2002. Scavenging or predation? Mississippian ammonoid accumulations in carbonate concretion halos around *Rayonnoceras* (Actinoceratoidea—Nautiloidea) body chambers from Arkansas. In *Cephalopods Present and Past*. Edited by H. Summesberger, K. Histon, and A. Daurer. Vienna: Abhandlungen Geologische Bundesanstalt, pp. 407–422.

Mapes, R. H., and Davis, R. A. 1996. Color patterns in ammonoids. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 104–127.

Mapes, R., and Evans, T. S. 1995. The color pattern on a Cretaceous nautilid from South Dakota. *Journal of Paleontology* 69:785–786.

Mapes, R. H., Weller, E. A., and Doguzhaeva, L. A. 2007. An early Carboniferous coleoid cephalopod showing a tentacle with arm hooks and ink sac (Montana, USA). In *Seventh International Symposium Cephalopods – Present and Past*, Hokkaido University, Sapporo, Japan, Abstracts, pp. 123–124.

Mariotti, N., and Pignatti, J. S. 1999. The Xiphoteuthididae Bather, 1892 (Aulacocerida, Coleoidea). In *Advancing Research on Living and Fossil Cephalopods*. Edited by F. Olóriz and F. J. Rodríguez-Tovar. New York: Kluwer Academic/Plenum Publishers, pp. 161–170.

Mehl, J. 1990. Fossilerhaltung von Kiemen bei *Plesioteuthis prisca* (Rüppel 1829) aus untertithonen Plattenkalken der Altmühlalb. *Archaeopterix* 8: 77–91.

Miller, A. K. 1930. A new ammonoid fauna of Late Paleozoic age from western Texas. *Journal of Paleontology* 4: 383–412.

Moltschaniwskyj, N. A. 1994. Muscle tissue growth and muscle fibre dynamics in the tropical Loliginid squid *Photololigo* sp. (Cephalopoda: Loligindae). *Canadian Journal of Fisheries and Aquatic Sciences* 51: 830–835.

———. 2004. Understanding the process of growth in cephalopods. *Marine and Freshwater Research* 55: 379–386.

Montgomery, M. K., and McFall-Ngai, M. J. 1992. The muscle-derived lens of a squid bioluminescent organ is biochemically convergent with the ocular lens. *Journal of Biological Chemistry* 267: 20999–21003.

Moore, R. C. 1957. *Treatise on Invertebrate Paleontology*. Lawrence, KS: Geological Society of America and University of Kansas Press.

———. 1964. *Treatise on Invertebrate Paleontology*. Lawrence, KS: Geological Society of America and University of Kansas Press.

Morton, N. 1981. Aptychi: the myth of the ammonite operculum. *Lethaia* 14: 57–61.

Müller, A. H. 1974. Über den Kieferapparat fossiler und rezenter Nautiliden (Cephalopoda) mit Bemerkungen zur Ökologie, Funktionsweise und Phylogenie. *Freiberger Forschungshefte* C 298: 7–17.

Naef, A. 1916. Ueber neue Sepioloiden aus dem Golf von Neapel. *Pubblicasioni della Stazione Zoologica di Napoli* 1: 1–10.

———. 1921–1923. *Cephalopoden. I. Teil. 1. Band: Systematik*. Fauna i Flora Golfo Napoli, Monograph 35-1 (English translation available from Smithsonian Institution Libraries, Washington, DC, United States).

———. 1922. *Die fossilen Tintenfische—eine paläozoologische Monographie* [English translation, Berliner Paläobiologische Abhandlungen, Band 5 (2004)].

———. 1928. *Die Cephalopoden (Embryologie)*. Fauna i Flora Golfo Napoli, 35(I-2) [English translation (2000) available from the Smithsonian Institution Libraries, Washington, DC, United States].

Nesis, K. N. 1980. Sepiids and loliginids: A comparative review of distribution and evolution of neritic

cephalopods. *Zooogicheskii Zhurnal, Moscow* 59: 677–688.

———. 1987. *Cephalopods of the World*. Neptune City, NJ: T.F.H. Publications.

Nishiguchi, M. K. 2002. The use of physiological data to corroborate cospeciation events in symbiosis. In *Molecular Systematics and Evolution: Theory and Practice*. Edited by R. DeSalle, W. Wheeler, and G. Giribet. Basel: Birkhäuser, pp. 237–246.

Nishiguchi, M. K., Lopez, J. E., and Boletzky, S. v. 2004. Enlightenment of old ideas from new investigations: The evolution of bacteriogenic light organs in squids. *Evolution and Development* 6: 41–49.

Nishiguchi, M. K., Ruby, E. G., and McFall-Ngai, M. J. 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-*Vibrio* symbioses. *Applied and Environmental Microbiology* 64: 3209–3213.

Nixon, M. 1988. The feeding mechanisms and diets of cephalopods—living and fossil. In *Cephalopods Present and Past*. Edited by J. Wiedmann and J. Kullmann. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung, pp. 641–952.

———. 1996. Morphology of the jaws and radula in ammonoids. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 23–42.

Nixon, M., and Young, J. Z. 2003. *The Brains and Lives of Cephalopods*. New York: Oxford University Press.

Norbert, T., and Klages, W. 1996. Cephalopods as prey. II. Seals. *Philosophical Transactions of the Royal Society Series B: Biological Sciences* 351: 1045–1052.

Norman, M. 2000. *Cephalopods: a World Guide*. Hackenheim, Germany: Conch Books.

O'Dor, R. K., and Dawe, E. G. 1998. *Illex illecebrosus*. In *Squid Recruitment Dynamics—The Genus* Illex *as a Model, the Commercial* Illex *Species and Influences on Variability*. Edited by P. G. Rodhouse, E. G. Dawe, and R. K. O'Dor. FAO Fisheries Technical Paper 376. Rome: Food and Agriculture Organization of the United Nations, pp. 77–104.

Orlov, Y. A. 1962. Fundamentals of paleontology. *Mollusca–Cephalopoda* 5: 887.

Owen, R. 1836. Description of some new and rare Cephalopoda. *Proceedings of the Zoological Society of London* 4: 19–24.

Packard, A. 1972. Cephalopods and fish: the limits of convergence. *Biological Reviews* 47: 241–307.

———. 1985. A tale from the deep. *The Times* (London), October 26, 1985, No. 62,279: 11.

———. 1988. Visual tactics and evolutionary strategies. In *Cephalopods Present and Past*. Edited by J. Wiedmann and J. Kullmann. Stuttgart: Schweizebart'sche Verlagsbuchhandlung, pp. 89–103.

Page, K. 1996. Mesozoic ammonoids in time and space. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 755–794.

Passamaneck, Y. J., Schander, C., and Halanych, K. M. 2004. Investigation of molluscan phylogeny using large-subunit and small-subunit nuclear rRNA sequences. *Molecular Phylogenetics and Evolution* 32: 25–38.

Pecl, G. T., and Moltschaniwskyj, N. A. 1997. Changes in muscle structure associated with somatic growth in *Idiosepius pygmaeus*, a small tropical cephalopod. *Journal of Zoology London* 242: 751–764.

Pichon, D., Gaia, V., Norman, M. D., and Boucher-Rodoni, R. 2005. Phylogenetic diversity of epibiotic bacteria in the accessory nidamental glands of squids (Cephalopoda: Loliginidae and Idiosepiidae). *Marine Biology* 147: 1323–1332.

Piertney, S. B., Hudelot, C., Hochberg, F. G., and Collins, M. A. 2003. Phylogenetic relationships among cirrate octopods (Mollusca: Cephalopoda) resolved using mitochondrial 16S ribosomal DNA sequences. *Molecular Phylogenetics and Evolution* 27: 348–353.

Pignatti, J. S., and Mariotti, N. 1996. Systematics and phylogeny of the Coleoidea (Cephalopoda): a comment upon recent works and their bearing on the classification of the Aulacocerida. *Palaeopelagos* 5: 33–44.

Quinn, J. H. 1977. Sedimentary processes in *Rayonnoceras* burial. *Fieldiana Geology* 33: 511–519.

Reichow, D., and Smith, M. J. 2001. Microsatellites reveal high levels of gene flow among populations of the California squid *Loligo opalescens*. *Molecular Ecology* 10: 1101–1109.

Roper, C. F. E. 1969. Systematics and zoogeography of the worldwide bathypelagic squid *Bathyteuthis* (Cephalopoda: Oegopsida). *Bulletin of the United States National Museum* 291: 1–210.

Salvini-Plawen, L. v., and Steiner, G. 1996. Synapomorphies and plesiomorphies in higher classification of Mollusca. In *Origin and Evolutionary Radiation of the Mollusca*. Edited by J. Taylor. Oxford: Oxford University Press, pp. 29–51.

Saunders, W. B., and E. S. Richardson. 1979. Middle Pennsylvanian (Desmoinesean) (*sic*) Cephalopoda of the Mazon Creek fauna, northeastern Illinois. In *Mazon Creek Fossils*. Edited by M. H. Nitecki. New York: Academic Press, pp. 333–359.

Seibel, B. A., Hochberg, F. G., and Carlini, D. C. 2000. Life history of *Gonatus onyx* (Cephalopoda:

Teuthoidea): deep-sea spawning and post-spawning egg care. *Marine Biology* 137: 519–526.

Seilacher, A. 1993. Ammonite aptychi: how to transform a jaw into an operculum? *American Journal of Science* 293A: 20–32.

Semmens, J. M., and Moltschaniwskyj, N. A. 2000. An examination of variable growth in the loliginid squid *Sepioteuthis lessoniana*: a whole animal and reductionist approach. *Marine Ecology Progress Series* 193: 135–141.

Shaw, P. W., Pierce, G. J., and Boyle, P. R. 1999. Subtle population structuring within a highly vagile marine invertebrate, the veined squid *Loligo forbesi*, demonstrated with microsatellite DNA markers. *Molecular Ecology* 8: 407–417.

Shea, E. K. 2005. Ontogeny of the fused tentacles in three species of ommastrephid squids (Cephalopoda, Ommastrephidae). *Invertebrate Zoology* 124: 25–38.

Shigeno, S., Kidokoro, H., Tsuchiya, K., Segawa, S., and Yamamoto, M. 2001a. Development of the brain in the Oegopsid squid, *Todarodes pacificus*: An atlas up to the hatchling stage. *Zoological Science* 18: 527–541.

Shigeno, S., Tsuchiya, K., and Segawa, S. 2001b. Embryonic and paralarval development of the central nervous system of the loliginid squid *Sepioteuthis lessoniana*. *Journal of Comparative Neurology* 437: 449–475.

Shigeno, S., and Yamamoto, M. 2002. Organization of the nervous system in the pygmy cuttlefish, *Idiosepius paradoxus* Ortmann (Idiosepiidae, Cephalopoda). *Journal of Morphology* 254: 65–80.

Signor, P. W., and Brett, C. E. 1984. The mid-Paleozoic precursor to the Mesozoic marine revolution. *Paleobiology* 10: 229–245.

Small, A. L., and McFall-Ngai, M. J. 1998. A halide peroxidase in tissues interacting with bacteria in the squids *Euprymna scolopes*. *Journal of Cellular Biochemistry* 72: 445–457.

Söller, R., Warnke, K., Saint-Paul, U., and Blohm, D. 2000. Sequence divergence of mitochondrial DNA indicates cryptic biodiversity in *Octopus vulgaris* and supports the taxonomic distinctiveness of *Octopus mimus* (Cephalopoda: Octopodidae). *Marine Biology* 136: 29–35.

Squires, R. L. 1973. Burial environment, diagenesis, mineralogy, and Mg and Sr contents of skeletal carbonates in the Buckhorn Asphalt of Middle Pennsylvanian age, Arbuckle Mountains, Oklahoma. PhD. thesis, Department of Paleontology, California Institute of Technology, Pasadena.

Steer, M. A., Moltschaniwskyj, N. A., Nichols, D. S., and Miller, M. 2004. The role of temperature and maternal ration in embryo survival: using the dumpling squid *Euprymna tasmanica* as a model. *Journal of Experimental Marine Biology and Ecology* 307: 73–89.

Steiner, G., and Dreyer, H. 2003. Molecular phylogeny of Scaphopoda (Mollusca) inferred from 18S rDNA sequences: support for a scaphopod-cephalopod clade. *Zoologica Scripta* 32: 343–356.

Stevens, G. R. 1988. Giant ammonites: a review. In *Cephalopods Present and Past*. Edited by J. Wiedmann and J. Kullmann. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung, pp. 140–166.

Strugnell, J., Norman, M. D., Drummond, A. J., and Cooper, A. 2004. The octopuses that never came back to earth: neotenous origins for pelagic octopuses. *Current Biology* 18: R300–R301.

Strugnell, J., Norman, M., Jackson, J., Drummond, A. J., and Cooper, A. 2005. Molecular phylogeny of coleoid cephalopods (Mollusca: Cephalopoda) using a multigene approach; the effect of data partitioning on resolving phylogenies in a Bayesian framework. *Molecular Phylogenetics and Evolution* 37: 426–441.

Sweeney, M. J., and Roper, C. F. E. 1998. Classification, type localities, and type repositories of Recent Cephalopoda. In *Systematics and Biogeography of Cephalopods*. Edited by N. A. Voss, M. Vecchione, R. B. Toll, and M. J. Sweeney. Smithsonian Contributions to Zoology 586. Washington, DC: Smithsonian Institution, pp. 561–599.

Takumiya, M., Kobayashi, M., Tsuneki, K., and Furuya, H. 2005. Phylogenetic relationships among major species of Japanese coleoid cephalopods (Mollusca: Cephalopoda) using three mitochondrial DNA sequences. *Zoological Science* 22: 147–155.

Tanabe, K., and Landman, N. H. 2002. Morphological diversity of the jaws of Cretaceous Ammonoidea. In *Cephalopods Past and Present*. Edited by H. Summesberger, K. Histon, and A. Daurer. Vienna: Abhandlungen Geologische Bundesanstalt, pp. 157–165.

Tanabe, K., Mapes, R., Sasaki, T., and Landman, N. H. 2000. Soft-part anatomy of the siphuncle in Permian ammonoids. *Lethaia* 33: 83–91.

Teichert, C. 1964a. Glossary of morphological terms used for nautiloids. In *Treatise on Invertebrate Paleontology*. Edited by R. C. Moore. Lawrence, KS: Geological Society of America and University of Kansas Press, pp. K54–K59.

———. 1964b. Morphology of hard parts. In *Treatise on Invertebrate Paleontology*. Edited by R. C. Moore. Lawrence, KS: Geological Society of America and University of Kansas Press, pp. K13–K53.

———. 1967. Major features of cephalopod evolution. In *Essays of Paleontology and Stratigraphy*. Edited

by C. Teichert and E. L. Yochelson. Lawrence: University of Kansas Press, pp. 162–210.

———. 1988. Main features of cephalopod evolution. In *The Mollusca*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 11–79.

Teichert, C., and Matsumoto, T. 1987. The ancestry of the genus *Nautilus*. In *Nautilus: The Biology and Paleobiology of a Living Fossil*. Edited by W. B. Saunders and N. H. Landman. New York: Plenum Press, pp. 25–32.

Teichert, C., and Moore, R. C. 1964. Classification and stratigraphic distribution. In *Treatise on Invertebrate Paleontology*. Edited by R. C. Moore, Lawrence, KS: Geological Society of America and University of Kansas Press, pp. K94–K106.

Termier, H., and Termier, G. 1971. Les Prebelemnitida: un nouvel ordre des céphalopodes. *Annales de la Sociétéé Géologique du Nord* 90: 109–112.

Toll, R. B. 1982. The comparative morphology of the gladius in the order Teuthoidea (Mollusca: Cephalopoda) in relation to systematics and phylogeny. Ph.D. thesis, University of Miami, Coral Gables, FL.

———. 1998. The gladius in cephalopod systematics. In *Systematics and Biogeography of Cephalopods*. Edited by N. A. Voss, M. Vecchione, R. B. Toll, and M. J. Sweeney. Smithsonian Contributions to Zoology 586. Washington, DC: Smithsonian Institution, pp. 55–68.

Tomarev, S. I., Callaerts, P., Kos, L., Zinovieva, R., Halder, G., Gehring, W., and Piatigorsky, J. 1997. Squid *Pax*-6 and eye development. *Proceedings of the National Academy of Sciences of the United States of America* 94: 2421–2426.

Tozer, E. T. 1981. Triassic Ammonoidea: classification, evolution and relationship with the Permian and Jurassic forms. In *The Ammonoidea*. Edited by M. R. House and J. R. Senior. New York: Academic Press, pp. 49–100.

Uchiyama, K., and Tanabe, K. 1999. Hatching of *Nautilus macromphalus* in the Toba Aquarium, Japan. In *Advancing Research on Living and Fossil Cephalopods*. Edited by F. Olóriz and F. J. Rodríguez-Tovar. New York: Kluwer Academic/Plenum Publishers. pp. 13–16.

Vecchione, M., Young, R. E., Donovan, D. T., and Rodhouse, P. G. 1999. Reevaluation of coleoid cephalopod relationships based on modified arms in the Jurassic coleoid Mastigophora. *Lethaia* 32: 113–118.

Vermeij, G. 1987. *Evolution and Escalation. An Ecological History of Life*. Princeton, NJ: Princeton University Press.

Verne, J. 1896. *Twenty Thousand Leagues under the Sea*. New York: Grosset and Dunlap.

Voight, J. R. 1997. Cladistic analysis of the octopods based on anatomical characters. *Journal of Molluscan Studies* 63: 311–325.

Voight, J. R., and Grehan, A. J. 2000. Egg brooding by deep-sea octopuses in the North Pacific Ocean. *Biological Bulletin* 198: 94–100.

Voss, G. L. 1977. Appendix II: Classification of recent cephalopods. *Symposium Zoological Society of London* 38: 575–579.

Waller, T. R. 1998. Origin of Molluscan class Bivalvia and a phylogeny of major groups. In *Bivalves: An Eon of Evolution*. Edited by P. A. Johnston, and J. W. Haggart. Calgary, Alberta, Canada: University of Calgary Press, pp. 1–45.

Ward, P. D. 1981. Shell sculpture as a defensive adaptation in ammonoids. *Paleobiology* 7: 96–100.

———. 1987. *The Natural History of* Nautilus. Boston: Allen and Unwin.

Ward, P. D., and Saunders, W. B. 1997. *Allonautilus*: A new genus of living nautiloid cephalopod and its bearing on phylogeny of the Nautilida. *Journal of Paleontology* 71: 1054–1064.

Warnke, K., Plötner, J., Santana, J. I., Rueda, M. J., and Llinas, O. 2003. Reflections on the phylogenetic position of *Spirula* (Cephalopoda): preliminary evidence from the 18S ribosomal RNA gene. In *Coleoid cephalopods through time*. Edited by K. Warnke, H. Keupp, and S. v. Boletzky. Berlin: Berliner Paläobiologische Abhandlungen, pp. 253–260.

Wells, M. J., and Wells, J. 1977. Cephalopoda: Octopoda. In *Reproduction of Marine Invertebrates,* Vol. 4: *Molluscs: Gastropods and Cephalopods*. New York: Academic Press, pp. 291–336.

Wiedmann, J. and Kullmann, J. 1988. Crises in ammonoid evolution. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 795–813.

Wiedmann, J., and J. Kullmann. 1996. Crises in ammonoid evolution. In *Ammonoid Paleobiology*. Edited by N. H. Landman, K. Tanabe, and R. A. Davis. New York: Plenum Press, pp. 795–813.

Woodruff, D. S., Carpenter, M. P., Saunders, W. B., and Ward, P. D. 1987. Genetic variation and phylogeny in *Nautilus*. In *Nautilus: The Biology and Paleobiology of a Living Fossil*. Edited by W. B. Saunders, and N. H. Landman. New York: Plenum Press, pp. 65–83.

Wray, C. G., Landman, N. H., Saunders, W. B., and Bonacum, J. 1995. Genetic diversity and geographic diversification in *Nautilus*. *Paleobiology* 21: 220–228.

Wright, C. W. 1981. Cretaceous Ammonoidea. In *The Ammonoidea*. Edited by M. R. House and J. R. Senior. New York: Academic Press, pp. 157–174.

Yokobori, S.-I., Fukuda, N., Nakamura, M., Aoyama, T., and Oshima, T. 2004. Long-term conservation

of six duplicated structural genes in Cephalopod mitochondrial genomes. *Molecular Biology and Evolution* 21: 2034–2046.

Young, J. Z. 1936. The giant nerve fibers and epistellar body of cephalopods. *Quarterly Journal of Microscopy Sciences* 78: 367–386.

———. 1960. The statocysts of *Octopus vulgaris*. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* 152: 3–29.

———. 1965. The central nervous system of *Nautilus*. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* 249: 1–25.

———. 1977. Brain, behavior, and evolution of cephalopods. *Symposium of the Zoological Society of London* 38: 377–434.

———. 1984. The statocysts of cranchid squids (Cephalopoda) *Journal of Zoology London* 203: 1–21.

———. 1988a. Evolution of the cephalopod brain. In *The Mollusca, Paleontology and Neontology of Cephalopods*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 215–228.

———. 1988b. Evolution of the cephalopod statocyst. In *The Mollusca, Paleontology and Neontology of Cephalopods*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 229–239.

Young, R. E. 1977. Ventral bioluminescence countershading in mid-water animals: evidence from living squid. *Science* 191: 1046–1048.

———. 1985. The common occurrence of oegopsid squid eggs in near-surface oceanic waters. *Pacific Science* 39: 359–366.

Young, R. E., and Bennett, T. M. 1988. Photophore structure and evolution within the Enoploteuthinae (Cephalopoda). In *The Mollusca, Paleontology and Neontology of Cephalopods*. Edited by M. R. Clarke and E. R. Trueman. San Diego: Academic Press, pp. 241–251.

Young, R. E., Kampa, E. M., Maynard, S. D., Mencher, F. M., and Roper, C. F. E. 1980. Counterillumination and the upper depth limits of midwater animals. *Deep-Sea Research* 27A: 671–691.

Young, R. E., and Roper, C. F. E. 1976. Intensity regulation of bioluminescence during countershading in living midwater animals. *Fishery Bulletin* 75: 239–252.

Young, R. E., Roper, C. F. E., Mangold, K., Leisman, G., and Hochberg, F. G. 1979a. Luminescence from non-bioluminescent tissues in oceanic Cephalopods. *Marine Biology* 53: 69–77.

Young, R. E., Roper, C. F. E., and Walters, J. F. 1979b. Eyes and extraocular photoreceptors in midwater cephalopods and fishes: their roles in detecting down-welling light for counterillumination. *Marine Biology* 51: 371–380.

Young, R. E., and Vecchione, M. 1996. Analysis of morphology to determine primary sister-taxon relationships within coleoid cephalopods. *American Malacological Bulletin* 12: 91–112.

———. 1999. *Octopodiformes*. Tree of Life web site. Tucson: The University of Arizona College of Agriculture and Life Sciences. http://tolweb.org/Octopodiformes.

Young, R. E., Vecchione, M., and Donovan, D. T. 1998. The evolution of coleoid cephalopods and their present biodiversity and ecology. In *Cephalopod Biodiversity, Ecology, and Evolution*. Edited by A. I. Payne, M. R. Lipinski, M. R. Clarke, and M. A. C. Roeleveld. *South African Journal of Marine Science* 20: 393–420.

Zinovieva, R. D., Tomarev, S. I., and Piatigorsky, J. 1993. Aldehyde dehydrogenase-derived omega-crystallins of squid and octopus. Specialization for lens expression. *Journal of Biological Chemistry* 268: 11449–11455.

Microb Ecol (2009) 57:140–150
DOI 10.1007/s00248-008-9412-9

MICROBIOLOGY OF AQUATIC SYSTEMS

Salinity and Temperature Effects on Physiological Responses of *Vibrio fischeri* from Diverse Ecological Niches

W. Soto · J. Gutierrez · M. D. Remmenga · M. K. Nishiguchi

Received: 12 September 2007 / Accepted: 22 May 2008 / Published online: 28 June 2008

Abstract *Vibrio fischeri* is a bioluminescent bacterial symbiont of sepiolid squids (Cephalopoda: Sepiolidae) and monocentrid fishes (Actinopterygii: Monocentridae). *V. fischeri* exhibit competitive dominance within the allopatrically distributed squid genus *Euprymna*, which have led to the evolution of *V. fischeri* host specialists. In contrast, the host genus *Sepiola* contains sympatric species that is thought to have given rise to *V. fischeri* that have evolved as host generalists. Given that these ecological lifestyles may have a direct effect upon the growth spectrum and survival limits in contrasting environments, optimal growth ranges were obtained for numerous *V. fischeri* isolates from both free-living and host environments. Upper and lower limits of growth were observed in sodium chloride concentrations ranging from 0.0% to 9.0%. *Sepiola* symbiotic isolates possessed the least variation in growth throughout the entire salinity gradient, whereas isolates from *Euprymna* were the least uniform at <2.0% NaCl. *V. fischeri* fish symbionts (CG101 and MJ101) and all free-living strains were the most dissimilar at >5.0% NaCl. Growth kinetics of symbiotic *V. fischeri* strains were also measured under a range of salinity and temperature combinations. Symbiotic *V. fischeri* ES114 and ET101 exhibited a synergistic effect for salinity and temperature, where significant differences in growth rates due to salinity existed only at low temperatures. Thus, abiotic factors such as temperature and salinity have differential effects between free-living and symbiotic strains of *V. fischeri*, which may alter colonization efficiency prior to infection.

W. Soto · J. Gutierrez · M. K. Nishiguchi (✉)
Department of Biology, MSC 3AF, New Mexico State University, Box 30001, Las Cruces, NM 88003-8001, USA
e-mail: nish@nmsu.edu

M. D. Remmenga
Economics/International Business and University Statistics Center, Box 30001, Las Cruces, NM 88003-8001, USA

Introduction

Years of research with various members of *Vibrionaceae* have shown temperature and salinity to be integral agents in governing *Vibrio* population dynamics [1, 2], physiological stress responses [3], and evolution [2, 4]. For example, brackish, coastal, and pelagic waters are each uniquely inhabited by distinct *Vibrio* populations [2]. Since temperature and salinity gradients are known to change over these environments, their variability can determine the fitness of each unique *Vibrio* population. Furthermore, *Vibrio* species found in freshwater are prominent since they possess a low Na^+ requirement for growth and starvation survival (e.g., *Vibrio cholerae* and *Vibrio mimicus* [2]). In addition, members of the Vibrionaceae occur naturally in the digestive tract and on the skin surface of marine animals [1]. In general, the genus *Vibrio*—along with their close relatives such as *Photobacterium*—are thought to be especially adapted to engaging in pathogenic and benign host–microbe interactions, with these symbiosis traits probably having a deep and ancient common ancestry, arising independently numerous times during the evolution of Vibrionaceae [5].

The association between sepiolid squids (Mollusca: Cephalopoda) and *Vibrio fischeri* has become a model system for studying the physiological and molecular signaling between hosts and their bacterial symbionts. The association is mutualistic, since the bacterially produced bioluminescence provides camouflage for the

squid hosts in a cryptic behavior termed counterillumination [6]. Interestingly, *V. fischeri* is also a bioluminescent symbiont of monocentrid fishes, including the genera *Monocentris* and *Cleidopus* [7]. Moreover, some strains of *V. fischeri* that are free-living are unable to develop a light organ association with squid or fish hosts, making them symbiotically incompetent [8]. *V. fischeri* isolated from monocentrid fishes are only capable of colonizing sepiolid squids in laboratory experiments at a reduced efficiency and possess a lower carrying capacity within cephalopod hosts [9]. Prior data has also demonstrated that symbiotically incompetent *V. fischeri* and those colonizing *Euprymna*, *Sepiola*, and monocentrid fishes are genetically distinct from each other [5, 10–13].

All extant species of *Euprymna* (Cephalopoda: Sepiolidae) are largely allopatric and distributed in the Indo-West Pacific [14]. Previous research has shown that *V. fischeri* strains native to one *Euprymna* species will out-compete conspecific symbionts that are non-native [9, 15]. These and other data suggest that host specialization and competitive dominance may be the result of symbionts locally adapting to *Euprymna* species in their environment. However, observations of the stratigraphical distributions of *V. fischeri* and *V. logei*, two symbiotic species found in Mediterranean sepiolids, determined that temperature and not host squids established *Vibrio* distribution in the Mediterranean Sea [16, 17]. Numerous *Sepiola* species exist sympatrically in the Mediterranean, and most of these host species simultaneously co-occur with both *V. fischeri* and *V. logei* [16, 18]. Thus, contrary to the competitive dominance observed in *Euprymna*, *Vibrio* symbionts in *Sepiola* are host generalists [16]. Other studies with bivalve and vertebrate hosts have also demonstrated that salinity and temperature influence host colonization [19–22], abundance and distribution [23–25], physiological state and survival [26–28], and the adhesive capabilities to host epithelia [29]. Hence, previous research has made it apparent that salinity and temperature influence all life cycle stages of *Vibrio* species, including the biogeography of free-living cells, host attachment with subsequent proliferation during symbiosis, and the alternative evolutionary trajectories available to different host ranges.

Especially important is the question as to how evolutionary lifestyle as a host generalist, host specialist, fish versus squid symbiont, and free-living cell influences growth limits of *V. fischeri* to abiotic factors such as salinity and temperature. Studying the microbial growth of marine symbionts constantly experiencing shifts between marine free-living and host phases is critical since the osmotic pressure can change dramatically between these two environments [30, 31]. This subject definitely needs to be addressed with the remarkable illumination in recent years that virulence and osmoregulation possess links within pathogenic *Vibrio* species [32]. Therefore, we studied the physiological performances of various *V. fischeri* strains isolated from different host and free-living environments over a gradient of NaCl concentrations ranging from 0.0–9.0% to observe any correlations between *V. fischeri* lifestyle and osmotic effects on microbial growth. We also measured synergistic effects of salinity and temperature on *V. fischeri* growth to determine if these factors had any influence on bacterial fitness during competition with one another.

Methods

Bacterial Strains, Media, and Culture Maintenance

Table 1 lists collection sites, squid hosts, and isolated strains of *V. fischeri* used in this study. Once isolated from squid light organs [9], strains were stored at −80°C in cryo-vial tubes containing a final concentration of 20% glycerol in either seawater tryptone (SWT: 70% 32 ppt Instant Ocean artificial seawater, 0.5% Bacto tryptone, 0.3% Bacto yeast extract, 0.3% glycerol, pH 7.5–8.0; [33]) or Luria–Bertani high salt liquid media (LBS: 1.0% Bacto tryptone, 0.5% Bacto yeast extract, 2% NaCl, 0.3% glycerol, 50 mM Tris–HCl, pH 7.5; [34]). The day before each experiment, strains from the −80°C freezer stock were streaked for use onto SWT agar plates (1.5%) to isolate single colonies.

Bacterial Growth over Salinity Gradients

To study *V. fischeri*'s ability to grow over a wide salinity range, isolates were acquired from diverse niches (Table 1), namely as obligately free-living (i.e., symbiotically incompetent and unable to colonize a fish or squid host), fish symbionts (procured from *Monocentris* and *Cleidopus*), squid-host generalist symbionts (isolated from *Sepiola* squid), and squid-host specialist symbionts (isolated from *Euprymna* squid). Individual colonies of each strain from SWT plates were used to inoculate 18×150 mm test tubes containing 5 mL SWT. These test tubes served as starter cultures for the experiment. Tubes were incubated at 28°C while shaking at 225 rpm for 16 h. Thereafter, 10 μL of each overnight starter culture was used to inoculate test tubes containing 5 mL of fresh SWT liquid media. The subsequent cultures were incubated at 28°C and shaken at 225 rpm for 3 h. After 3 h of growth, a Uvikon XL spectrophotometer was used to measure optical density (OD_{600}) of all cultures. Cultures were then inoculated into test tubes containing 5 ml LBS with salinities spanning 0.0–9.0% NaCl. All cultures began at the same initial cell density of 5×10^5 colony forming units (CFU)/mL. NaCl concentrations were increased by 1.0% NaCl, except

Table 1 Strains of *Vibrio fischeri* used in this study

Strain	Host	Location
WH1	Free-living	USA (Massachusetts, Woods Hole)
MDR7	Free-living	USA (California, Marina del Rey)
CB37	Free-living	Australia (Coogee Bay, New South Wales)
ATCC 7744	Free-living	American Type Culture Collection
MJ101	*Monocentris japonicus*	Japan (Tokyo Bay)
CG101	*Cleidopus gloriamaris*	Australia (Townsville, Queensland)
SR5	*Sepiola robusta*	France (Banyuls sur Mer)
SL518	*Sepiola ligulata*	France (Banyuls sur Mer)
SA1G	*Sepiola affinis*	France (Banyuls sur Mer)
SI66	*Sepiola intermedia*	Italy (Bari)
EM17	*Euprymna morsei*	Japan (Tokyo Bay)
ET101	*Euprymna tasmanica*	Australia (Melbourne, Victoria)
ET401	*Euprymna tasmanica*	Australia (Townsville, Queensland)
EB12	*Euprymna berryi*	Japan (Tosa Bay)
ES114	*Euprymna scolopes*	USA (Kaneohe Bay, Hawaii)

between 0.0–1.0% and between 6.0–7.0%. Increments between these concentrations were at 0.1%, since these were the minimum and maximum limits of growth for all cultures. Test tubes were placed in a shaker for 24 h at 28°C and 225 rpm. Optical density (OD_{600}) readings of each culture were measured at each concentration ($n=5$).

Monoculture Growth Studies on *V. fischeri* from *Euprymna* Hosts

Strains of *V. fischeri* isolated from various *Euprymna* species (Table 1) were grown at different temperatures (12°C, 28°C, and 32°C) and salinities (24, 32, and 38 ppt) to observe how these parameters affect generation times in SWT. Salinity was measured using a refractometer (ATAGO® Co., LTD, Japan). These particular temperatures and salinities were chosen since they are representative of the environments *V. fischeri* encounters in nature outside the host. Three-hour cultures of each strain were grown in the same manner as the optical density–salinity gradient studies.

After 3 h of growth, a Uvikon XL spectrophotometer was used to take optical density measurements (OD_{600}) of each culture. Cultures were inoculated in triplicate into 125-mL flasks containing 50 mL of SWT to bring the initial cell density to 5×10^5 CFU/mL. Salinity and temperature were measured at the following settings: 24 ppt/12°C, 24 ppt/28°C, 24 ppt/32°C, 32 ppt/12°C, 32 ppt/28°C, 32 ppt/32°C, 38 ppt/12°C, 38 ppt/28°C, and 38 ppt/32°C. We also examined the effect of nutrient-limiting media on growth rates using minimal ribose media [35] with two symbiotic *V. fischeri* strains, ES114 and EM17. Flasks (125 mL) were aerated at 225 rpm and maintained at the appropriate temperatures for three hours prior to inoculation to guarantee the media was at the correct temperature. OD_{600} measurements were measured from each of the flask cultures every 30 min for 8 h to obtain growth curves for each strain. OD_{600} measurements were natural log transformed to calculate each strain's generation time. Since the experiment was designed with a two-way factorial (or two crossed factors) in a completely randomized design, our analysis used a two-way ANOVA with interaction and when the interaction was present, means of the factor combinations were separated by pair-wise *t* tests. When interaction was not present, the means at the three temperatures and salinities were separated by pair-wise *t* tests ($\alpha=0.05$).

V. fischeri ES114 and *V. fischeri* EM17 Competition Growth Studies

To search for the possibility of antagonism or allelopathy between strains, competition growth experiments were completed with *V. fischeri* ES114 and EM17, since both had similar growth rates across the entire range of salinities and temperatures examined. Triplicate 125-mL flasks with 50 mL SWT were co-inoculated with equal numbers (50:50 ratio) of both strains. The initial cell densities of *V. fischeri* ES114 and EM17 were each half of the monoculture inoculations (2.5×10^5). This was to achieve the same starting total cell population as in the monoculture growth studies. Salinity of the SWT media and temperature at which they were incubated were as follows: 24 ppt/12°C, 32 ppt/28°C, and 38 ppt/32°C, which represented low, intermediate, and high conditions. Flasks were aerated at 225 rpm and maintained at the experimental temperatures for 3 h prior to inoculation to guarantee the media was at the correct temperatures. Cell enumeration of each strain was ascertained through plate counts by sampling from each of the replicate flasks once every hour. Bacterial ratios were obtained by counting the number of visibly luminous colonies in the dark (EM17), and subsequently the total

number of colonies in the light (EM17+ES114). The difference between the two counts yields the total number of *V. fischeri* ES114 colonies. Since *V. fischeri* ES114 is not visibly luminous, this allows quantification of both strains when grown together [36]. The competition growth rate data was then subjected to *t* tests to detect any significant differences using the software package SAS. The usage of either optical density (OD_{600}) or cell density (CFU/mL) yielded similar growth rates for identical strains; therefore, we used the cell density as an approximation of each strain.

Results

Effects of Salinity Gradients on *V. fischeri* from Different Ecological Niches

V. fischeri native to *Euprymna* species exhibited variable growth throughout the salinity gradient compared to *Sepiola* strains (Figs. 1 and 2). This trend was especially important at lower salinities (<2.0 NaCl, Fig. 1), even when comparing those data to non-squid strains (Fig. 3). In contrast, *V. fischeri* isolated from *Sepiola* squids exhibited the most uniform growth throughout the entire salinity gradient (Fig. 2). *V. fischeri* ET401 and EB12 were the least able to grow at low salinity (<1.0% NaCl) of all the strains isolated from host animals. Conversely, non-squid *V. fischeri* exhibited more variability at high salinities (>5.0% NaCl, Fig. 3). No bacterial growth was observed for any of the strains at either the low or high ranges measured in this study (0.0, 7.0, 8.0, or 9.0% NaCl; Figs. 1, 2, and 3).

Of all the strains, *V. fischeri* ES114 demonstrated the best overall growth at low salinities (<3.0% NaCl) and *V. fischeri* ET401 at higher salinities (>4.0% NaCl). The one exception was free-living *V. fischeri* CB37, which exhibited the best overall growth over most of the salinity range (Fig. 3). Of all the strains examined, *V. fischeri* ATCC 7744 (a free-living isolate) had the most constrained growth across the entire salinity gradient. Therefore, host generalists *V. fischeri* (from *Sepiola* species) were the least different from each other in their ability to grow from 0.0–9.0% NaCl when comparing them to *V. fischeri* from the host genus *Euprymna* (host specialists) and non-squid niches (fish symbionts and obligately free-living bacterioplankton).

Temperature and Salinity Growth Studies of *V. fischeri* from *Euprymna*

Growth rates of the Australian and Japanese *V. fischeri* were tested to assess if any correlations existed between generation times for symbionts as result of being isolated from the same host species (Fig. 4) or the same geographical location (Fig. 5). Mean generation times for strains at each salinity–temperature combination examined (α=0.05) are shown in Table 2. Generation times with dissimilar letters (a, b, c, or d) within the column of each strain were significantly different from each other, whereas growth rates possessing the same letter are statistically equivalent. For example, all values with the letter "a" are equal to one another, and all values with the letter "b" are the same. However, all generation times with the letter "a" are statistically different from those with the letter "b". Temperature significantly affected all five *V. fischeri* strains, whereas a significant salinity result was detected only in *V. fischeri* ES114 and ET101. A significant synergistic interaction between temperature and salinity was also observed within these same two strains (Figs. 4 and 5). At 12°C, growth rates for all strains were significantly lower than those at 28°C and 32°C, while growth rates between these two later temperatures were similar (Figs. 4 and 5). Significant salinity effects for *V. fischeri* ES114 and ET101 were observed only at 12°C. Increasing the salinity at this temperature led to more rapid generation times for *V. fischeri* ET101 (Table 2). Mean generation times for the

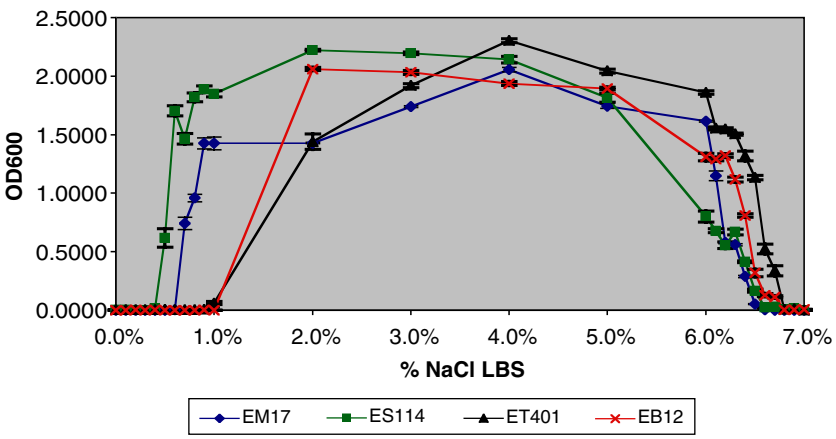


Figure 1 Salinity effects on growth (±standard error) of host specialist *V. fischeri* isolated from *Euprymna*. *Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain designations

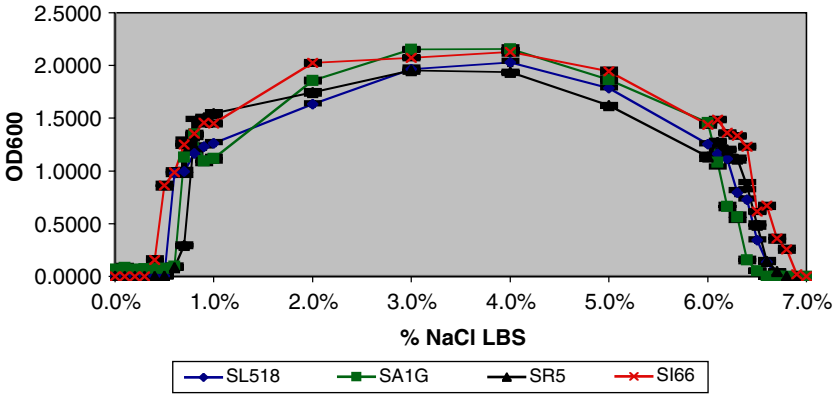


Figure 2 Salinity effects on growth (±standard error) of host generalist *V. fischeri* isolated from *Sepiola* squid. *Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain designations

competition growth studies of *V. fischeri* ES114 and EM17 in nutrient-rich SWT were not significantly different from monoculture generation times of these two strains in the same media at higher salinity and temperature conditions (Table 3). However, a significant difference was observed between the competition and monoculture generation times at 24 ppt/12°C (Fig. 6). Monoculture generation times of *V. fischeri* ES114 and EM17 in minimal ribose media were significantly slower than those in SWT for the same temperature (Table 3). Generation times (as noted with different letters, Table 3) were also significantly different from each other.

V. fischeri EM17 and ET401 generation times behave most similarly to each other than to any other *Vibrio* symbionts across different temperature and salinity conditions (Table 2), as neither displayed a significant salinity–temperature interaction. Salinity and temperature had a significant interaction on the growth rates of *V. fischeri* ET101 and ES114. Similar to *V. fischeri* EM17 and ET401, these two strains have similar generation times at 28°C and 32°C. However, salinity had a dissimilar significant effect on *V. fischeri* ET101 and ES114 growth rates at 12°C. Although *V. fischeri* ET401, ET101, and EM17 are the only strains to annually experience temperatures as low as 12°C (Table 4), they seem to lack growth rates that are uniquely adapted for those temperatures. No significant difference in generation times among *Vibrio* symbionts was observed at 12°C.

Comparably, *V. fischeri* ES114 is from Hawaii, where temperature is nearly constant: the difference between surface temperature and that of 100 m below sea level is only a few degrees centigrade (www.nodc.noaa.gov), yet *V. fischeri* ES114 generation time at 28°C is not significantly faster than the other symbionts (Table 3). In this regard, *V. fischeri* EM17 and ET401 are derived from environments extensively variable in temperature throughout the year. *V. fischeri* ES114 experienced a significant salinity effect and salinity–temperature interaction on its growth rate where *V. fischeri* EM17 and ET401 did not, implying the microbial physiology of *V. fischeri* ES114 is more sensitive to variable environments. However, this does not necessarily allude to the conclusion that *V. fischeri* EM17 and ET401 are better adapted to variable environments.

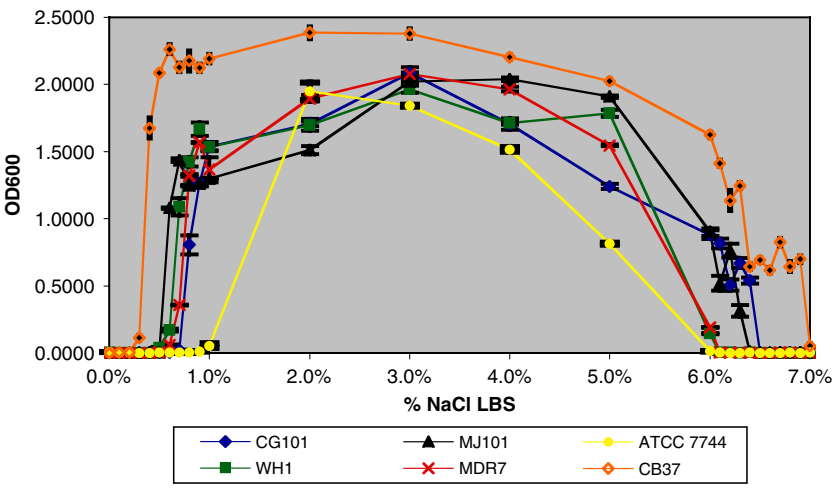


Figure 3 Salinity effects on growth (±standard error) of fish symbionts and free-living *V. fischeri*. *Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain designations

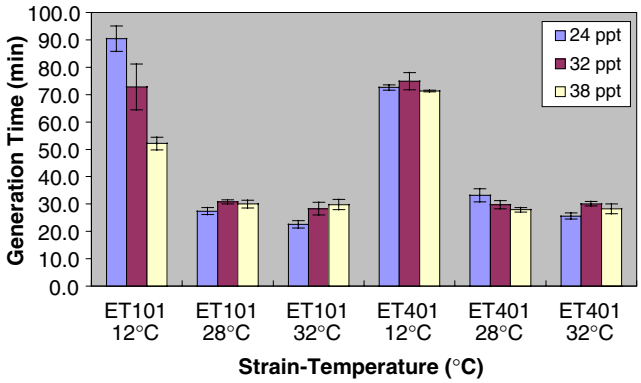


Figure 4 Mean generation times (±standard error) of *V. fischeri* ET101 (Melbourne, VIC) and ET401 (Townsville, QLD) isolated from *E. tasmanica* measured at three different temperature and salinity conditions. *Standard error bars* were calculated using the unbiased estimator for the mean

Discussion

V. fischeri is a cosmopolitan microbe with a ubiquitous distribution in oceans, estuaries, brackish waters, and marine sediments throughout the world [37], as either part of free-living bacterioplankton or as a mutualistic symbiont [7, 38]. Although most *V. fischeri* strains are "facultative" symbionts with cyclical free-living and mutualistic lifestyles, *V. fischeri* strains exist that persist strictly as members of the bacterioplankton and are symbiotically incompetent, essentially becoming obligately free-living [8]. Clearly, *V. fischeri* is establishing its worldwide dissemination through oceanic water currents as host animals are known to be limited in their dispersal ability [10, 11]. Ocean temperatures can range between −1.0°C and 30°C with salinities ranging between 5 to 38 ppt (www.nodc.noaa.gov) [39]. Previous studies investigating the microbial ecology of luminous bacteria suggests that

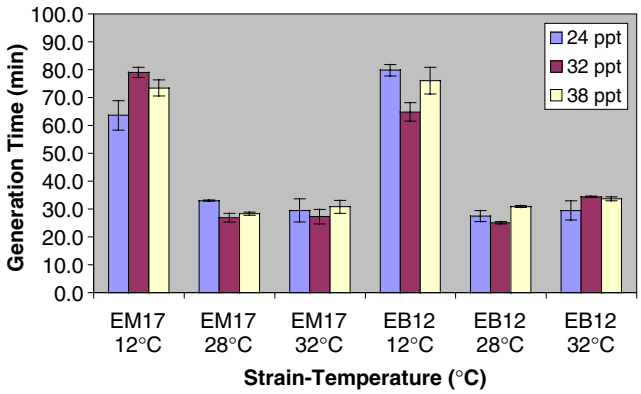


Figure 5 Mean generation times (±standard error) of *V. fischeri* EM17 and EB12 obtained from *Euprymna morsei* (EM17) or *Euprymna berryi* (EB12) measured at three different temperature and salinity conditions. *Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain and host designation

Table 2 Mean generation times of *Vibrio fischeri* strains grown in monoculture at each salinity and temperature condition (n=3)

Salinity–temp (ppt °C)	EB12[a] (min)	EM17[a] (min)	ES114[a, b, c, d] (min)	ET101[a, c, e] (min)	ET401[a] (min)
24–12	77.8a	57.6c	73.0a	90.4e	63.5c
24–28	25.4b	33.0b	29.3b	27.4b	31.8b
24–32	29.5b	29.5b	25.9b	22.6b	25.6b
32–12	68.2c	72.2a	79.1a	72.8a	74.9a
32–28	25.1b	26.9b	29.2b	25.8b	29.8b
32–32	34.4b	27.3b	26.8b	28.3b	30.1b
38–12	76.0a	72.0a	63.1c	52.1d	71.3a
38–28	30.9b	27.4b	28.3b	30.0b	27.9b
38–32	33.7b	30.8b	27.4b	29.8b	28.2b

Generation times with different letters denote values significantly different from each other. For example, all values with the letter "a" are equal to one another, and all values with the letter "b" are also equivalent. However, all generation times with the letter "a" are statistically different from those with the letter "b"

[a] Significant temperature effect (p<0.0001)

[b] Significant salinity effect (p<0.01)

[c] Significant salinity effect (p<0.05)

[d] Significant salinity–temperature interaction (p<0.01)

[e] Significant salinity–temperature interaction (p<0.0001)

species composition of a particular environment was largely determined by patterns of temperature, salinity, nutrient concentration, solar radiation, and other abiotic factors [7, 40–43].

Although this idea may continue to hold for microfloral planktonic communities, more recent research has demonstrated that selective pressures in marine bioluminescent bacteria for specificity toward their host fishes and cephalopods with light organs can preside over normal evolutionary physiological requirements. For instance, *Photobacterium leiognathi* typically is more abundant as a free-living microbe in warmer waters; however, this species can be found as a symbiont in both temperate-water and tropical leiognathid fishes [7]. *V. fischeri* itself is usually a temperate-water species but can be found in hosts inhabiting both tropical and temperate waters [38]. This provides evidence that the distribution, ecology, and evolution of luminescent bacterial species in marine environments can be partially driven by symbiosis as opposed to abiotic factors.

Previous work has demonstrated luminous *Vibrio* species colonizing light organs of the Mediterranean genus *Sepiola* was determined by temperature and not squid-host specificity [16, 18]. Alternatively, *V. fischeri* symbionts colonizing the squid genus *Euprymna* from the Indo-west Pacific were primarily determined by host specificity [9, 15]. Such outcomes governed by abiotic or host specificity may be dependent on the number of hosts available, utilization of different host animals, and whether hosts are allopatric or sympatric. Additionally, the host animal can directly

Table 3 Mean generation times (min) for *Vibrio fischeri* ES114 and *V. fischeri* EM17 from competition and minimal media growth studies (monoculture generation times in SWT are included in parentheses for comparison)

Strain	24 ppt 12°C	32 ppt 28°C	38 ppt 32°C	Minimal ribose 28°C
EM17	80.3d (63.6c)	30.4b (26.9b)	26.4b (30.8b)	89.8d (30.0b[a])
ES114	82.7d (73.0a)	28.4b (30.8b)	29.9b (27.4b)	114.5e (28.9b[a])

Generation times with different letters and colors denote values significantly different from each other. For example, all values with the letter "a" are equal to one another, and all values with the letter "b" are also equivalent. However, all generation times with the letter "a" are statistically different from those with the letter "b"

[a] Mean generation times obtained from averaging different growth studies completed at 28°C

influence symbiont abundance and distribution via seeding the oceanic water column with bacteria through daily venting cycles [44].

Due to the complexity of host interactions and abiotic factors in directing the community structure of marine luminescent microorganisms such as *V. fischeri*, roles of both ecological determinants need further investigation to better understand how this microbe resides in the diverse niches it occupies [7, 17]. Our study measured the effects of salinity and temperature on growth rates (i.e., generation times) of *V. fischeri* from several *Euprymna* species in nutrient-rich media (Table 2). Nutrient-rich media have previously been used to simulate a host environment when studying effects of salinity and temperature on the microbial physiology of *Vibrio* species [26]. Since growth rates of microorganisms have characteristics that represent underlying physiological processes of single cells (e.g., biosynthesis of macromolecules), understanding how abiotic factors influence *Vibrio* generation times will facilitate the illumination of the cellular events responsible for changes in microbial populations during symbiosis [45].

V. fischeri ET101 encounters considerable variation in temperature (Table 4), and growth appears more sensitive to changes in salinity and temperature than *V. fischeri* ES114. Correlations between significant effects on generation times by abiotic factors and constant/variable environments are absent. Finally, *V. fischeri* ET101 and ET401, two *E. tasmanica* symbionts isolated from squid from two distinct locations (Table 1) has demonstrated no detectable competitive dominance [9]. However, both *V. fischeri* isolates are genetically distinguishable [11]. *V. fischeri* ET101 was isolated from *E. tasmanica* inhabiting Melbourne, Victoria and *V. fischeri* ET401 was isolated from *E. tasmanica* living in Townsville, Queensland (Table 1). Waters near Victoria typically range in temperature from about 12–17°C, whereas Queensland is much warmer (24–26°C). *V. fischeri* ET101 and ET401 possess generation times that were uniquely affected by salinity and temperature (Fig. 4). For instance, *V. fischeri* ET401 possess the fastest logarithmic growth at low salinity and low temperature (24 ppt/12°C), yet *V. fischeri* ET101 has the most rapid generation time at high salinity and low temperature (38 ppt/12°C; Table 2, Fig. 4). Salinity only has this growth-altering effect at 12°C and not at the higher temperatures. This outcome may be the result of underlying differences in regional physiological adaptations.

V. fischeri EB12 and EM17 are isolates from two Japanese hosts, *E. morsei* and *E. berryi*. *E. morsei* tends to occur in cooler temperate waters of northern Japan, while *E. berryi* is more frequently found in southern Japan's temperate warm waters, including along the coast of China [46]. Distributions of *E. morsei* and *E. berryi* do overlap partially. Nevertheless, these hosts are believed to be sibling species that are either reproductively isolated or hybridization is rare [47]. Similar to *V. fischeri* ET101 and ET401, generation times of Japanese strains may respond differently to salinity and temperature due to physiological differences resulting from evolution within their respective thermal niches. At low salinity and low temperature (24 ppt/12°C), *V. fischeri* EM17 had a shorter generation time than *V. fischeri* EB12. As the salinity increased at low temperature

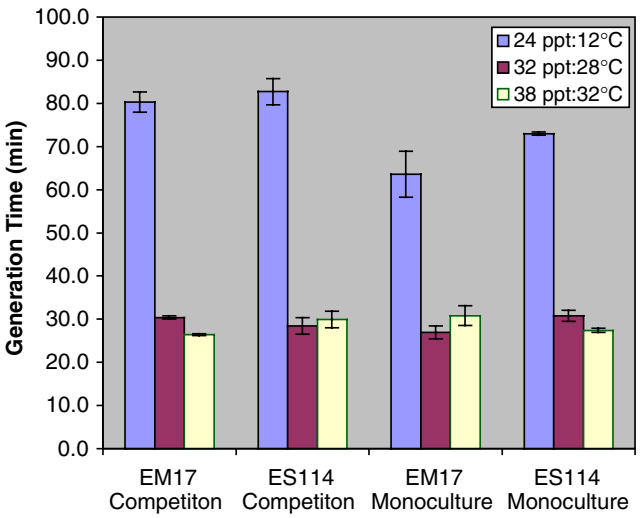


Figure 6 Mean generation times (±standard error) of *V. fischeri* ES114 and EM17 grown together at different temperature and salinity combinations (competition). Monoculture mean generation times are included in parentheses for comparison. Refer to Table 1 for location of hosts and isolates. *Standard error bars* were calculated using the unbiased estimator for the mean

Table 4 Annual mean temperatures and mean salinities for *Euprymna* species (www.nodc.noaa.gov, www.cephbase.utmb.edu)

Host squid	Distribution	0–100 m usual temperature range	0–100 m usual salinity range
E. tasmanica	Australia/Tasmania	12–25°C	20.0–35.8 ppt
E. morsei	Japan (cool temperate waters)	2–17°C	32.2–34.0 ppt
E. berryi	Japan (warm temperate waters)	17–25°C	34.2–34.8 ppt
E. scolopes	Hawaiian Islands	22–26°C	34.6–35.2 ppt
E. hyllebergi	Thailand	21–28°C	31.4–34.4 ppt
E. hoylei	Philippines/North Western Australia/Marshall Islands	21–29°C	34.0–35.0 ppt

Biogeographic data was obtained from the National Oceanographic and Atmospheric Administration and published literature [46, 47, 55]

(12°C), the generation times between these two strains became more similar (Table 2, Fig. 5). Although *V. fischeri* EB12 infects *E. berryi*, which is restricted to sub-temperate/warm waters (17–25°C), this strain may also experience temperatures as low as 2°C during its free-living planktonic phase in northern Japanese waters.

V. fischeri ES114 and EM17 both have generation times of approximately 30 min at 32 ppt/28°C in SWT media, but *V. fischeri* ES114 still out-competes EM17 in *E. scolopes* under similar environmental conditions [15]. Interestingly, a "competition" effect was observed at 24 ppt/12°C; both strains grew significantly slower in the presence of the other. Additionally, *V. fischeri* EM17 generation time was more negatively affected by the presence of *V. fischeri* ES114 than ES114 was by EM17 at 24 ppt/12°C (Fig. 6). Hence, microbial allelopathy may at least be partially responsible for competitive dominance in *Euprymna*, especially at lower temperatures (e.g., 12°C). *Vibrio* symbionts have quite similar generation times over the temperatures and salinities examined in nutrient-rich media, yet native strains still out-compete non-native ones under laboratory conditions that approximately simulate natural habitats. If these results can be extrapolated to the nutrient-rich environment of the host, competitive dominance in *Euprymna* would not be solely the result of native *Vibrio* symbionts possessing faster generation times or growth rate constants (growth parameters g and k) than non-native ones while in the host. Rather, *V. fischeri* strains may be more actively competing against one another via faster generation times when they are part of the free-living bacterioplankton, where the oceanic water column serves as a semi-starving environment relative to the host. Growth rates of *V. fischeri* ES114 and EM17 in minimal media demonstrate that symbionts can have differential growth rates when nutrients may be more limiting. The possibility remains that competitive dominance may be a combination of faster growth rate parameters and microbial amensalism, as these two phenomena are not mutually exclusive.

Despite the presence of competitive dominance of native *V. fischeri* during *Euprymna* colonization [15], population genetic surveys of host squid and *V. fischeri* symbionts suggest secondary colonizations occur [11], whereby previously allochthonous strains become established in a novel animal host. Particular attributes of the *Vibrio*-sepiolid squid symbiosis engender native symbionts of *Euprymna* spp. susceptible to at least partial displacement by non-native invaders. Attachment and proliferation of *V. fischeri* within axenic squid hatchlings emerging from eggs can be initiated and completed with as little as ten cells [8], creating severe symbiont founder effects and genetic bottlenecks during the colonization of the hosts each generation. Symbiont populations may undergo considerable genetic drift upon acquiring new hosts, exposing residential symbionts to potential deleterious effects of Muller's ratchet [48, 49]. An upper bound then crystallizes and confines the magnitude of adaptation that native symbionts achieve to their hosts, diminishing the likelihood of a permanent evolutionarily enduring advantage gained by native vibrios over non-native ones in host colonization. Specific morphological changes are triggered within squid hatchlings upon colonization by *Vibrio* cells, and these immense transformations continue to occur throughout the early stages of the symbiosis [50], which make continuous and serial re-colonization from free-living symbionts less probable after maturity of the association.

These properties could permit invading non-native *V. fischeri* to retain occupancy of foreign *Euprymna*, despite the prevailing presence of competitive dominant strains in an area, providing the non-native symbionts arrive and settle into host animals first. Non-native *V. fischeri* initially outnumbering native strains is key to this scenario to offset competitive dominance. Low temperature environments (i.e., winters, cold temperate climates) appear to foster conditions and alternative salinities where allochthonous vibrios could accomplish this inside hosts during early stages of symbiosis by exploiting their faster generation times (Table 2), if optimal conditions were sustained. Perhaps localities combining low temperature, low salinity, and semi-starvation with free-living bacterioplankton in estuaries and deltas during cold periods provide the best opportunities to function as reservoirs for non-native *Vibrio* symbionts. Although marine environments normally range in salinity from 3.3–3.7% [40], investigating the effects of salinity on *V. fischeri* growth is important considering this

species ability to invade and thrive within novel hosts and environments is related to its capacity to manage stress [51].

The genus *Vibrio* includes some of the most common culturable marine bacteria, but the general ecology of *Vibrio* in the oceans still remains poorly characterized [52]. Furthermore, the physical, chemical, and biological variation that serves as the impetus for the adaptation and diversification of *Vibrio* species is yet to be well described. This study demonstrates different *V. fischeri* strains have various ranges of salinity that they are able to tolerate and grow (Figs. 1, 2, 3). Surprisingly, slight changes in salinity (e.g., Δ0.1% NaCl$\approx\Delta$1 ppt) led to dramatic changes in growth. As a result, the most prevalent strain within a given geographical region can be quite dynamic, depending on the level of salinity fluxes that occur as a consequence of local water currents and seasonal changes. In some instances, sudden and sharp demarcations existed where a particular strain grew significantly and where it did not grow at all, indicating gradual zones of decreasing growth are not always present with subtle changes in salinity. Hence, minute salinity changes in the marine environment could dramatically influence host squid and *V. fischeri* symbiotic relationships (Figs. 1, 2, 3). Some overlap existed in the physiological response among different strains isolated from *Euprymna*, *Sepiola*, and non-squid (i.e., obligately free-living and fish symbionts). These overlapping salinities may represent where different strains can coexist simultaneously in the open ocean. Obligately free-living strains failed to grow above 6.0% NaCl. Therefore, undertaking a symbiotic lifestyle may select for *V. fischeri* more adapted to higher salinities, although this needs to be further investigated. *V. fischeri* CB37 was the only symbiotically incompetent strain able to grow above 6.0% NaCl (Fig. 3).

Understanding how temperature and salinity modulate the biogeographical distribution of *Vibrio* populations can allow us to predict whether these populations are greatly influenced by abiotic pressures. For example, *V. fischeri* ET101 and ET401 both occur in Australian waters. The former grows faster at 24 ppt/12°C and the later at 38 ppt/12°C. The two strains have similar generation times at all other combinations of salinity and temperature. The salinity 38 ppt is not a common salinity in their habitat (Table 4). Stabilizing selection of growth rates at high and low salinities at 12°C is not a suitable explanation of their co-occurrence within *E. tasmanica*. Instead, a more plausible hypothesis is *V. fischeri* ET401 maintains its population by growing more quickly within this host at low salinity and is more prone to expulsion from the light organ through ventilation cycles, while *V. fischeri* ET101 maintains its presence by colonizing the squid more efficiently throughout the host's temperature and salinity ranges. Therefore, *V. fischeri* ET101 would be less likely to be expulsed into the water column. This is analogous to an *r*-selected (ET401) versus *K*-selected (ET101) strategy of survival. Further studies investigating intra-strain variation throughout large environmental gradients are planned in future studies.

Although microbial growth was principally sensitive at high and low NaCl concentrations, other parameters such as oxygen concentration, trace metals, radiation, hydrostatic pressure, and stress responses affect marine microbial populations and need to be considered [40]. For instance, stress is known to affect the quality of organic carbon produced by vibrios living in simple, microbial loop foodwebs. This phenomenon influences the quality of carbon available to other trophic levels [53]. Illumination in recent years that the multitude of microbial physiological responses to stress (e.g., heat shock, starvation) are coupled and cross-talk elevates the complexity and provides fertile ground for intriguing research [54]. Future work will examine the extent to which *V. fischeri* symbionts are capable of adapting to different environmental niches (extreme temperatures, low/high salinities, and feast/famine nutrient conditions) and whether the evolutionary potential to adapt to these environments are correlated with those *Vibrio* symbionts (e.g., strains, species) most abundant in the habitats, leading to a greater understanding of microbial diversity, speciation, and evolution.

Acknowledgments This work was supported in part by grants from the National Science Foundation Population Biology program (DEB-0316516) and the National Institutes of Health (SO6 GM008136-32S2-1) to M.K.N. W. Soto and J. Gutierrez were both supported by the NIH-MBRS RISE program (GM-61222-01) and the Howard Hughes Medical Institute (52005881). The authors would also like to thank A. Burris and J.G. Easterling (MBRS-RISE) for additional help with this study.

References

1. Farmer J III, Janda JM (2001) Family Vibrionaceae. In: Brenner D, Krieg NR, Staley JT (eds) Bergy's manual of systematic bacteriology, vol 2. Springer, New York, NY, pp 491–555
2. Urakawa H, Rivera ING (2006) Aquatic Environment. In: Thompson FL, Austin B, Swings J (eds) The biology of Vibrios. ASM, Washington, D.C., pp 175–189
3. McDougald D, Kjelleberg S (2006) Adaptive responses to Vibrios. In: Thompson FL, Austin B, Swings J (eds) The biology of Vibrios. ASM, Washington, D.C., pp 133–155
4. Bartlett DH (2006) Extremophilic Vibrionaceae. In: Thompson FL, Austin B, Swings J (eds) The biology of Vibrios. ASM, Washington, D.C., pp 156–171
5. Nishiguchi MK, Nair VA (2003) Evolution of pathogenicity and symbioses in Vibrionaceae: a combined approach using molecules and physiology. Int J Syst Bacteriol 53:2019–2026
6. Jones BW, Nishiguchi MK (2004) Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Mar Biol 144:1151–1155

7. Ruby EG, Lee K-H (1998) The *Vibrio fischeri–Euprymna scolopes* light organ association: current ecological paradigms. Appl Environ Microbiol 64:805–812
8. Ruby EG (1996) Lessons from a cooperative, bacterial–animal association: the *Vibrio fischeri–Euprymna scolopes* light organ symbiosis. Annu Rev Microbiol 50:591–624
9. Nishiguchi MK (2002) Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. Microbiol Ecol 44:10–18
10. Kimbell JR, McFall-Ngai MJ, Roderick GK (2002) Two genetically distinct populations of bobtail squid, *Euprymna scolopes*, exist on the island of O'ahu. Pac Sci 56:347–355
11. Jones BW, Lopez JL, Huttenberg J, Nishiguchi MK (2006) Population structure between environmentally transmitted Vibrios and bobtail squids using nested clade analysis. Mol Ecol 15:4317–4329
12. Boettcher KJ, Ruby EG (1994) Occurrence of plasmid DNA in the sepiolid squid symbiont *Vibrio fischeri*. Current Microbiology 29:279–286
13. Lee K-H, Ruby EG (1994) Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. J Bacteriol 176:1985–1991
14. Nesis KN (1982) Cephalopods of the world. T.F.H., Neptune City, N.J
15. Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid–Vibrio symbioses. Appl Environ Microbiol 64:3209–3213
16. Nishiguchi MK (2000) Temperature affects species distribution in symbiotic populations of Vibrio. Appl Environ Microbiol 66:3550–3555
17. Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK (2007) Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. Microbiol Ecol 54:314–323
18. Fidopiastis PM, Boletzky Sv, Ruby EG (1998) A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. J Bacteriol 180:59–64
19. Kaspar CW, Tamplin ML (1993) Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl Environ Microbiol 59:2425–2429
20. Marco-Noales E, Biosca EG, Amaro C (1999) Effects of salinity and temperature on long-term survival of the eel pathogen *Vibrio vulnificus* biotype 2 (serovar E). Appl Environ Microbiol 65:1117–1126
21. Amaro C, Biosca EG, Fouz B, Alcaide E, Esteve C (1995) Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. Appl Environ Microbiol 61:1133–1137
22. Motes ML, DePaola A, Cook DW, Veazey JE, Hunsucker JC, Garthright WE, Blodgett RJ, Chirtel SJ (1998) Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Appl Environ Microbiol 64:1459–1465
23. Jiang SC (2001) *Vibrio cholerae* in recreational beach waters and tributaries of Southern California. Hydrobiol 460:157–164
24. Jiang SC, Fu W (2001) Seasonal abundance and distribution of *Vibrio cholerae* in coastal waters quantified by a 16S–23S intergenic spacer probe. Microbiol Ecol 42:540–548
25. Louis VR, Russek-Cohen E, Choopun N, Rivera ING, Gangle B, Jiang SC, Rubin A, Patz JA, Huq A, Colwell RR (2003) Predictability of *Vibrio cholerae* in Chesapeake Bay. Appl Environ Microbiol 69:2773–2785
26. Hijarrubia MJ, Lazaro B, Sunen E, Fernandez-Astorga A (1996) Survival of *Vibrio vulnificus* under pH, salinity and temperature combined stress. Food Microbiol 13:193–199
27. Hoff KA (1989) Survival of *Vibrio anguillarum* and *Vibrio salmonicida* at different salinities. Appl Environ Microbiol 55:1775–1786
28. Wolf PW, Oliver JD (1992) Temperature effects on the viable but non-culturable state of *Vibrio vulnificus*. FEMS Microbiol Ecol 10:33–39
29. Bordas MA, Balebona MC, Zorrilla I, Borrego JJ, Morinigo MA (1996) Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of Gilt-Head Sea Bream (*Sparus aurata* L.). Appl Environ Microbiol 62:3650–3654
30. Xu C, Ren H, Wang S, Peng X (2004) Proteomic analysis of salt-sensitive outer membrane proteins of *Vibrio parahaemolyticus*. Res Microbiol 155:835–842
31. Xu C, Wang S, Ren H, Lin X, Wu L, Peng X (2005) Proteomic analysis on the expression of outer membrane proteins of *Vibrio alginolyticus* at different sodium concentrations. Proteomics 5:3142–3152
32. Sleator RD, Hill C (2001) Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol Rev 26:49–71
33. Nealson KH (1978) Isolation, identification, and manipulation of luminous bacteria. Methods Enzymol 57:153–165
34. Dunlap PV (1989) Regulation of luminescence by cyclic AMP in cya-like and crp-like mutants *Vibrio fischeri*. J Bacteriol 171:1199–1202
35. Lupp C, Hancock REW, Ruby EG (2002) The *Vibrio fischeri* sapABCDF locus is required for normal growth, both in culture and in symbiosis. Arch Microbiol 179:57–65
36. Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1997) Phenotypic bioluminescence as an indicator of competitive dominance in the *Euprymna–Vibrio* symbiosis. In: Hastings JW, Kricka JW, Stanley PE (eds) Bioluminescence and chemiluminescence: molecular reporting with photons. Wiley, New York, NY, pp 123–126
37. Nishiguchi MK, Jones BW (2004) Microbial biodiversity within the Vibrionaceae. In: Seckback J (ed) Origins, evolution, and the biodiversity of microbial life. Kluwer, Dordrecht, The Netherlands, pp 531–548
38. Nishiguchi MK, Lopez JE, von Boletzky S (2004) Enlightenment of old ideas from new investigations: the evolution of bacteriogenic light organs in squids. Evol Dev 6:41–49
39. Atlas RM, Bartha R (1998) Microbial ecology: fundamentals and applications. Cummings, Menlo Park, CA
40. Yetinson T, Shilo M (1979) Seasonal and geographic distribution of luminous bacteria in the eastern Mediterranean Sea and the Gulf of Elat. Appl Environ Microbiol 37:1230–1238
41. O'Brien CH, Sizemore RK (1979) Distribution of the luminous bacterium *Beneckea harveyi* in a semitropical estuarine environment. Appl Environ Microbiol 38:928–933
42. Ruby EG, Nealson KH (1978) Seasonal changes in the species composition of luminous bacteria in nearshore seawater. Limnol Oceangr 23:530–533
43. Shilo M, Yetinson T (1979) Physiological characteristics underlying the distribution patterns of luminous bacteria in the Mediterranean Sea and the Gulf of Elat. Appl Environ Microbiol 38:577–584
44. Lee K-H, Ruby EG (1994) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl Environ Microbiol 60:1565–1571
45. White D (2000) The physiology and biochemistry of the Prokaryotes. Oxford University Press, Oxford, UK
46. Norman MD, Lu CC (1997) Redescription of the southern dumpling squid *Euprymna tasmanica* and a revision of the genus *Euprymna* (Cephalopoda: Sepiolidae). J Mar Biol Assoc UK 77:1109–1137

47. Nateewathana A (1997) The Sepiolidae (Cephalopoda) of the Andaman Sea, Thailand, with description of *Euprymna hyllebergi* sp. nov. Phuket Mar Biol Cent Spec Pub 17:465–481
48. Rispe C, Moran NA (2000) Accumulation of deleterious mutations in endosymbionts: Muller's ratchet with two levels of selection. Am Nat 156:425–441
49. Moran NA (1996) Accelerated evolution and Muller's ratchet in endosymbiotic bacterial. Proc Nat Acad Sci USA 93:2873–2878
50. McFall-Ngai MJ (1999) Consequences of evolving with bacterial symbionts: insights from the squid–*Vibrio* association. Annu Rev Ecol Syst 30:235–256
51. Bennett AF, Lenski RE (1999) Experimental evolution and its role in evolutionary physiology. Am Zool 39:346–362
52. Giovannoni SJ, Sting U (2005) Molecular diversity and ecology of microbial plankton. Nature Rev Genet 437:343–348
53. Odic D, Turk V, Stopar S (2007) Environmental stress determines the quality of bacterial lysate and its utilization efficiency in a simple microbial loop. Microbiol Ecol 53:639–649
54. Vattanaviboon P, Mongkolsuk S (2001) Unusual adaptive, cross-protection response and growth phase resistance against peroxide killing in a bacterial shrimp pathogen, *Vibrio harveyi*. FEMS Microbiol Lett 47:111–116
55. Okutani T, Horita E (1987) Identity of *Euprymna berryi* Sasaki, 1929 (Cephalopoda: Sepiolidae). Venus 46:91–107

RESEARCH LETTER

Characterization of two host-specific genes, mannose-sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri–Euprymna tasmanica* mutualism

Don Sanjiv Ariyakumar & Michele K. Nishiguchi

Department of Biology, New Mexico State University, NM, USA

Correspondence: Michele K. Nishiguchi, Department of Biology, New Mexico State University, MSC 3AF, PO Box 30001, Las Cruces, NM 88003-8001, USA. Tel.: +1 575 646 3721; fax: +1 575 646 5665; e-mail: nish@nmsu.edu

Present address: Don Sanjiv Ariyakumar, Veterinary Diagnostic Labs, University of Minnesota, E220 VetDL, 6184, 1333 Gortner Ave., St Paul, MN 55108, USA

Received 19 May 2009; accepted 13 July 2009.

DOI:10.1111/j.1574-6968.2009.01732.x

Editor: David Clarke

Keywords
symbiosis; *Vibrio*; colonization; adhesion; squid; biofilm; mutualism; pathogenesis.

Abstract

While much has been known about the mutualistic associations between the sepiolid squid *Euprymna tasmanica* and the luminescent bacterium, *Vibrio fischeri*, less is known about the connectivity between the microscopic and molecular basis of initial attachment and persistence in the light organ. Here, we examine the possible effects of two symbiotic genes on specificity and biofilm formation of *V. fischeri* in squid light organs. Uridine diphosphate glucose-6-dehydrogenase (UDPDH) and mannose-sensitive hemagglutinin (*mshA*) mutants were generated in *V. fischeri* to determine whether each gene has an effect on host colonization, specificity, and biofilm formation. Both squid light organ colonization assays and transmission electron microscopy confirmed differences in host colonization between wild-type and mutant strains, and also demonstrated the importance of both UDPDH and *mshA* gene expression for successful light organ colonization. This furthers our understanding of the genetic factors playing important roles in this environmentally transmitted symbiosis.

Introduction

Mutualistic associations that occur in the marine environment have provided numerous examples of beneficial capabilities that aid in the success of both host and symbiont (Boucher *et al.*, 1982). One such relationship that has been studied over the past 20 years is the mutualistic association between sepiolid squids (*Cephalopoda*: *Sepiolidae*) and their *Vibrio* bacteria (*Gammaproteobacteria*: *Vibrionaceae*) (Nishiguchi & Jones, 2004). During the onset of the symbiosis, free-living *Vibrio* bacteria are environmentally acquired by aposymbiotic juvenile squids through initial invasion and colonization steps, and are eventually housed in a specialized bilobed light organ (Montgomery & McFall-Ngai, 1993). Once *Vibrio* bacteria successfully colonize the naïve squid light organ, those bacteria are able to bioluminescence via quorum sensing, and eventually produce diffuse, downward focused light, which is used by the squid in a behavior known as counterillumination (Jones & Nishiguchi, 2004). In return, the host provides a haven for the bacteria to reproduce at a faster rate, and eventually aid in the daily release of bacteria into the environment through a venting behavior that occurs with the onset of dawn (Lee & Ruby, 1994).

During the initial infection and colonization process *Vibrio* bacteria have developed sophisticated mechanisms to invade the host light organ and eventually persist in this complex (Nyholm & McFall-Ngai, 2004; Nyholm & Nishiguchi, 2008). During such infections they are involved in inducing a variety of extracellular factors such as adhesions, synthesis of polysaccharides, and toxin regulators (Montgomery & Kirchman, 1993). Specifically, adhesion and biofilm formation are two mechanisms that are considered to be crucial for initial colonization (O'Toole *et al.*, 2000; Darnell *et al.*, 2008; Geszvain & Visick, 2008; Hussa *et al.*,

2008). Genes that encode for proteins used in adhesion and biofilm production are oftentimes unique, and can be easily distinguished by their differential gene expression during symbiosis (prior or during colonization; Jones & Nishiguchi, 2006). Two such examples of genes that are differentially expressed by *Vibrio fischeri* are uridine diphosphate glucose-6-dehydrogenase (UDPDH), and mannose-sensitive hemagglutinin (*mshA*). UDPDH is considered to be a main factor for biofilm formation (Nesper *et al.*, 2001), while *mshA* is believed to be essential for initial adhesion and colonization (Bomchil *et al.*, 2003). *mshA* has also been demonstrated as a necessary component in *V. cholerae* for initial attachment and biofilm formation to abiotic substrates using the type IV pili (Watnick & Kolter, 1999). Earlier studies using a technique termed selective capture of transcribed sequences (SCOTS) have shown the expression of UDPDH solely in the light organ, whereas *mshA* is exclusively expressed under natural environmental conditions (Jones & Nishiguchi, 2006). Therefore, the goals of this study were to determine whether both UDPDH and *mshA* were necessary for successful colonization by *V. fischeri* in the sepiolid squid *Euprymna tasmanica*. Mutations were initiated to disrupt gene function for both genes and to determine whether colonization efficiency was decreased when squids were infected with either UDPDH or *mshA* mutants. We also examined whether these mutations had a decreased ability to attach to the brush border epithelia of *E. tasmanica* light organs. Finally, complementation of both mutant strains was used to regain loss of function, both *in vitro* and in colonization of naïve juvenile squids.

Materials and methods

Strains, plasmids, and growth conditions

All strains used in this study are listed in Table 1. Wild-type *V. fischeri* strain ETJB1H was isolated from the light organs of *E. tasmanica* from Jervis Bay, Australia, as described previously (Jones *et al.*, 2006). All *V. fischeri* and *E. coli* strains used in this study were grown in seawater tryptone (SWT) and Luria–Bertani (LB) broth at 28 and 37 °C, and supplemented with appropriate antibiotics for selection of mutant strains.

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant genotype or description
V. fischeri strains	
ETJB1H	Wild type
UDPDH$^-$	UDP-glucose-6-dehydrogenase mutant
mshA$^-$	Mannose sensitive hemagglutinin mutant
UDPDH$^+$	UDP-glucose-6-dehydrogenase complement
mshA$^+$	Mannose sensitive hemagglutinin complement
Plasmids	
pEVS122	R6K, Erm^R
pVSV105	pES213 replicon, Cm^R

Erm^R, erythromycin resistance; Cm^R, chloramphenicol resistance.

PCR

All loci in this study were amplified using 50-μL volume PCR reactions as described previously (Jones *et al.*, 2006; Table 2).

Mutant construction

Mutants were constructed using plasmid insertion where, an internal (partial) fragment of each targeted gene was PCR amplified and cloned directly into pEVS 122 (Dunn *et al.*, 2005; Dunn & Stabb, 2008). This plasmid was introduced into *V. fischeri* by triparental mating (Stabb & Ruby, 2002). The *V. fischeri* strains that had undergone homologous recombination between the genome and the internal gene fragment were selected on SWT-erythromycin plates and were verified via Southern blots.

Biofilm assays

All bacterial strains (wild type, mutant, and complement) were grown and biofilm assays were performed as described previously (Nair, 2006). Biofilm assays for every strain were completed in triplicate and statistically analyzed by Student's *t*-test.

Motility assays

All bacterial strains were grown overnight at 28 °C and 250 r.p.m. The following day, 20 μL of bacterial culture was inoculated and strains were regrown in fresh 32 p.p.t. SWT media to an $OD_{600\,nm}$ of 0.1. Ten microliters of each culture was spotted onto an SWT plate containing 0.5% agar. Motility was determined by measuring the diameter of the spot after 24 h. Each motility assay for every strain was completed in triplicate and statistically analyzed by Student's *t*-test.

Colonization assay

To determine the colonization efficiency between the mutant and the wild-type bacterial strains, colonization assays of different *V. fischeri* strains were completed as described previously (Nishiguchi, 2002). Briefly, both wild-type and mutant strains were grown in 5 mL SWT overnight in a shaking incubator (250 r.p.m.) at 28 °C. Strains were transferred the next morning and regrown in 5 mL of fresh SWT media to an $OD_{600\,nm}$ of 0.3–0.5. Cultures were diluted to approximately 1×10^3 CFU mL^{-1} and used to inoculate 5 mL of sterile seawater with axenic juvenile squids. After inoculation, seawater in all vials was changed every 12 h and bioluminescence was measured using a luminometer (Turner

Table 2. UDPDH$^-$, *mshA*$^-$, UDPDH$^+$, and *mshA*$^+$ primer sequences used for constructing mutant strains

Primers	Primer sequence 5′–3′	PCR product size (bp)
mshA-forward	AGC AGA TCT TTT ATG GTA AAG CCG CGA TT	~180
mshA-reverse	AGC AGA TCT GCT GCA GTT GGG TTA TCT GA	~180
UDP-forward	AGC AGA TCT AA AAT CGC CTA TTT CAG ATG T	~160
UDP-reverse	AGC AGA TCT GCT TCA ACA GAG CCC GTA TT	~160
mshA-Comp-F	CCC GGG GAT CAG TGA GAA TGG CCG TA	~450
mshA-Comp-R	CCC GGG CGA TTG TTG ATA CGC CAG AA	~450
UDP-Comp-F	CCC GGG ATT CAG GTC GCA GGT TTC AG	~700
UDP-Comp-R	CCC GGG TTC AGA TTG CTC ACC CAC AA	~700

F, forward primer sequence; R, reverse primer sequence; bp, base pairs.

Designs, Sunnyvale, CA) over a period of 48 h. During incubation, infected animals were kept in 12-h/12-h light/dark cycle. Infected juvenile squids were sacrificed at 48 h and plated onto SWT agar. The number of CFUs on each plate represented the colonization efficiency of each strain in a squid light organ. Results were analyzed using Student's *t*-test.

Electron microscopy

Juvenile squids infected for 48 h with either strain were fixed and processed for transmission electron microscopy (TEM) as described previously (Nair, 2006).

All bacterial strains examined using scanning electron microscope (SEM) were grown overnight at 250 r.p.m. and 28 °C in 5 mL SWT. The following day, 20 μL of each bacterial culture was used to inoculate a new test tube with 3 mL sterile SWT media and regrown at 250 r.p.m. and 28 °C, until each culture reached an OD_{600nm} of 0.1. One milliliter of each culture was transferred into 50-mL falcon tubes with a sterile cover slip half immersed in the media and incubated 24 h without shaking at 28 °C. Cover slips were then washed with 32 p.p.t. seawater to remove excess particles other than the biofilm formed by each bacterial culture. Excess seawater was blotted dry, and all cover slips were incubated in 1 mL of a 0.2% aqueous solution of crystal violet at room temperature for 1 h. Cover slips from each strain were viewed with the Hitachi S3400 SEM (Hitachi, Schaumburg, IL).

Results and discussion

Growth curves and bioluminescence

Vibrio fischeri ETJB1H and the mutant strains generated in this study are listed in Table 1.

Growth analyses were completed for both UDPDH$^-$ and *mshA*$^-$ mutants and compared with *V. fischeri* ETJB1H. ETJB1H exhibited a doubling time of 25.7 min compared with UDPDH$^-$ and *mshA*$^-$, which had doubling times of 76.2 and 56.4 min. Simultaneously, luminescence of both mutant strains and the wild-type ETJB1H was measured. Comparisons between all three strains demonstrated that mutants had lower levels of bioluminescence/OD values at 13.7 relative light units (RLU) for *mshA*$^-$ and 181 RLU for UDPDH$^-$ compared with values of > 7205 RLU for ETJB1H.

In addition, growth analyses were also completed for complemented UDPDH$^+$ and *mshA*$^+$ strains and compared with their respective mutant strains. UDPDH$^+$ exhibited a doubling time of 29.6 min, which was similar to the rate of the wild-type parental strain. *mshA*$^+$ had a doubling time of approximately 33.3 min. Luminescence of UDPDH$^+$ and *mshA*$^+$ strains was also measured simultaneously. Interestingly, luminescence/OD of complemented strains exhibited a lower amount of bioluminescence/OD compared with the parental strain, with values of 18 RLU for UDPDH$^+$ and 3.4 RLU for *mshA*$^+$.

In vitro biofilm assays

Both *V. fischeri* UDPDH$^-$ and *mshA*$^-$ strains exhibited a deficiency in biofilm formation with values of 0.1 at $OD_{595\,nm}$ when eluted with the alcohol : acetone mixture (Fig. 1a). These values were compared with the parental wild type, which had values of 0.35 at $OD_{595\,nm}$ (Fig. 1a). Test analysis between *V. fischeri* ETJB1H UDPDH$^-$ and *mshA*$^-$ compared with parental wild-type showed that average mean values were significantly different. UDPDH$^+$ and *mshA*$^+$ complemented strains also had an increase in biofilm formation with mean values of 0.3 at $OD_{595\,nm}$. *mshA*$^+$ (Fig. 1b) had a greater value than parental wild-type *V. fischeri* ETJB1H. Statistical analysis of biofilm production gave significant differences between UDPDH$^-$ and UDPDH$^+$ strains, with *P*-values of 0.0001 at $\alpha = 0.05$. Additionally, the *mshA*$^-$ and the *mshA*$^+$ strains exhibited significant differences, with *P*-values of 0.002 ($\alpha = 0.05$).

Biofilm morphology

Biofilm produced from *V. fischeri* ETJB1H parental and mutant strains were visualized using SEM. Significant morphological differences were observed between parental *V. fischeri* ETJB1H (Fig. 1c) and mutants UDPDH$^-$ (Fig. 1d) and *mshA*$^-$ (Fig. 1e). *Vibrio fischeri* ETJB1H exhibited

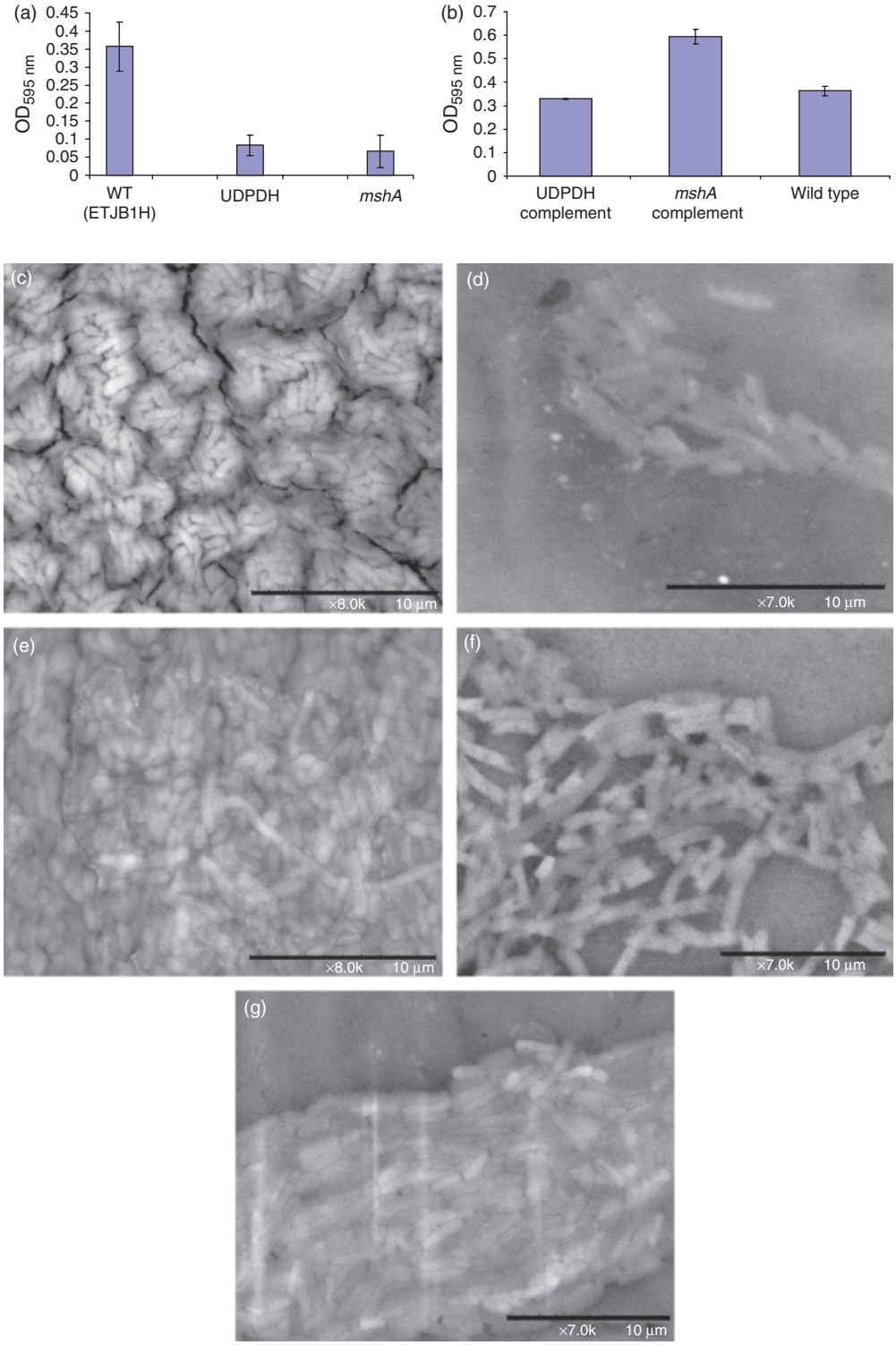


Fig. 1. (a) Biofilm produced by *Vibrio fischeri* ETJB1H wild-type and *V. fischeri* ETJB1H UDPDH$^-$ had a *P*-value of 0.0034 at $\alpha = 0.05$ and *mshA*$^-$ mutant strains had a *P*-value of 0.005 ($\alpha = 0.05$) Bars represent ± 1 SE. (b) Biofilm produced by *V. fischeri* ETJB1H parental wild type and both *V. fischeri* ETJB1H UDPDH$^+$ and *mshA*$^+$ complemented strains (bars represent ± 1 SE). (c) Scanning electron micrograph (SEM) of biofilm formed by the wild-type strain *V. fischeri* ETJB1H. Scale bar = 10 μm. (d) SEM of biofilm formed by *V. fischeri* ETJB1H UDPDH$^-$ strain. Scale bar = 10 μm. (e) SEM of biofilm formed by *V. fischeri* ETJB1H *mshA*$^-$ strain. Scale bar = 10 μm. (f) SEM of biofilm formed by *V. fischeri* ETJB1H UDPDH$^+$ complement strain. Scale bar = 10 μm. (g) SEM of biofilm formation by *V. fischeri* ETJB1H *mshA*$^+$ complement strain. Scale bar = 10 μm.

compact bacterial cells that were attached to one another in a three-dimensional structure. This was in contrast to both mutant strains, which were thinner and less dense than the wild type. Additionally, bacterial biofilms from the complemented UDPDH$^+$ (Fig. 1f) and *mshA*$^+$ strains (Fig. 1g) regained the ability to form well-organized biofilms, and appeared to form complexes similar to their wild-type parent (Fig. 1c).

Motility assay

Wild-type ETJB1H exhibited the highest degree of motility compared with both mutant strains (Fig. 2). Both UDPDH$^-$ and the wild-type strains exhibited significant differences (Student's *t*-test). Motility of complemented strains was also measured and exhibited greater motility compared with their respective mutant strains.

Colonization assay

Axenic juvenile *E. tasmanica* squids were infected with both mutants as well as wild-type *V. fischeri* ETJB1H. Seven animals were used in this study for each strain. Single strain infections were measured by homogenizing juvenile squid after 48 h of infection and calculating the number of CFUs per milliliter (Nishiguchi, 2002). *Vibrio fischeri* ETJB1H parental wild type had a mean colonization efficiency of 1×10^5 CFU mL^{-1} compared with UDPDH$^-$ and *mshA*$^-$, which had values of 1×10^3 CFU mL^{-1} and 1×10^4 CFU mL^{-1}, respectively (Fig. 3).

TEM

Examination of the crypt regions of juvenile *E. tasmanica* that were infected with either parental wild-type *V. fischeri* ETJB1H, *V. fischeri* ETJB1H UDPDH$^-$ or *V. fischeri* ETJB1H *mshA*$^-$ was completed using TEM. Parental wild-type ETJB1H (Fig. 4a) had complete colonization of the crypt region, whereas *V. fischeri* ETJB1H UDPDH$^-$ (Fig. 4b) exhibited little or no change in microvillar density to the brush border epithelia. *Vibrio fischeri* ETJB1H *mshA*$^-$ mutant (Fig. 4c) showed deficiency in colonization of the crypt region, where few bacteria were found along the brush border epithelial lining.

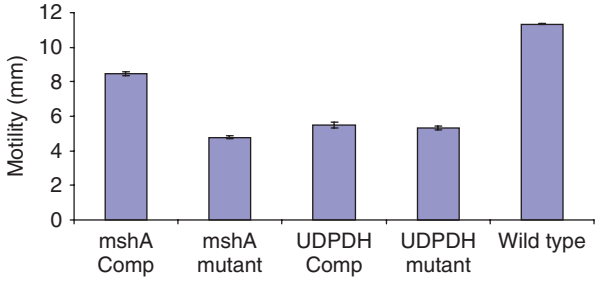


Fig. 2. Mean average motility between *Vibrio fischeri* ETJB1H *mshA*$^-$ and *mshA*$^+$ strains, *V. fischeri* ETJB1H UDPDH$^-$, and UDPDH$^+$ strains and the wild-type *V. fischeri* ETJB1H strains. Each sample was run in triplicate. Both UDPDH$^-$ and *mshA*$^-$ strains exhibited *P*-values of 0.001 and 0.0007 accordingly at $\alpha = 0.05$ compared with the wild-type strains. The UDPDH$^-$ and UDPDH$^+$ did not show significant difference, whereas the *mshA*$^-$ and *mshA*$^+$ had significant *P*-values of 0.001 at $\alpha = 0.05$. Bars represent ± 1 SE.

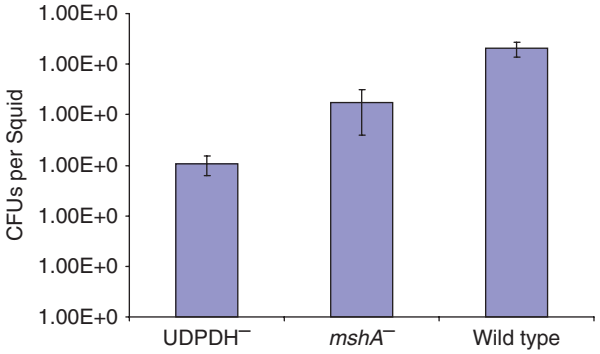


Fig. 3. Colonization assay 48 h postinfection of juvenile *Euprymna tasmanica* by the parental wild-type *V. fischeri* ETJB1H and both *V. fischeri* ETJB1H mutant strains (UDPDH$^-$ and *mshA*$^-$). Both UDPDH$^-$ and *mshA*$^-$ compared with the wild-type strains exhibited significant differences with *P*-values of 1.4E-10 and 7.8E-5 at $\alpha = 0.05$. Bars within each graph represent the SE within ± 1 of the mean.

In this study we examined how mutations in either UDPDH and *mshA* genes in *V. fischeri* affect infection and colonization in juvenile *E. tasmanica* light organs. As an environmentally transmitted bacterium, symbiotic *V. fischeri* must be capable of adapting to the selective environment of the squid light organ upon initial infection. Following successful colonization, squids will vent 90–95% of the bacteria from their crypt spaces every morning at dawn (McFall-Ngai, 2000), which may allow the streamlining of better adapted *V. fischeri* strains to be maintained in the colonized light organ. Earlier studies have provided evidence that bacterial biofilms are present in squid light organs (Nair, 2006). Therefore, the question of whether biofilm formation is important for colonization and persistence within the squid light organ may be of interest for determining specificity.

UDPDH

Biofilm formation has been shown to be one factor responsible for persistence of the bacterium *Pseudomonas aeruginosa* within infected lungs of human patients (Lam *et al.*, 1980). In addition, studies using *E. coli* and *P. aeruginosa* indicate the importance of colanic acid biosynthesis for capsular formation, which increases the ability of those bacteria to adhere to substrates. Colonic acid production is also believed to enhance the ability of bacteria to form

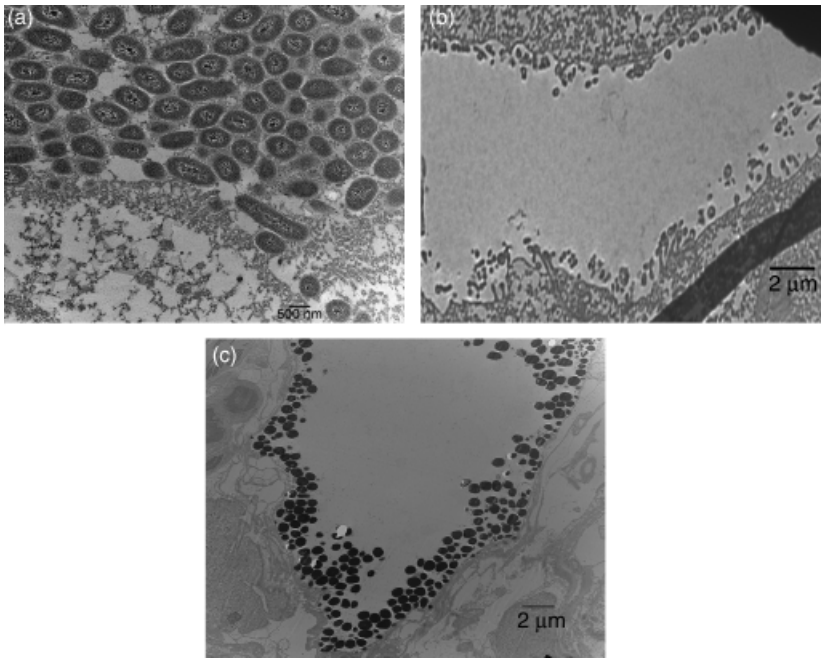


Fig. 4. Transmission electron micrographs of the squid light organ infected by (a) *Vibrio fischeri* ETJB1H wild-type strain (scale bar = 500 nm), (b) *V. fischeri* ETJB1H UDPDH$^-$ mutant (scale bar = 2 μm), and (c) *V. fischeri* ETJB1H *mshA*$^-$ mutant strains (scale bar = 2 μm).

biofilm production upon initial infection of epithelial cells in the lungs of cystic fibrosis patients (Davies *et al.*, 1993). Biosynthesis of this acid first begins with the pathways of four different nucleotide sugars, UDP-galactose, UDP-glucose, UDP-D-glucuronate, and guanine-diphosphate-L-fucose, where UDPDH catalyzes UDP-glucose to UDP-D-glucuronic acid (Stevenson *et al.*, 1996). Interestingly, this gene was only expressed in the symbiotic state in both *E. tasmanica* and *Euprymna scolopes* light organs (Jones & Nishiguchi, 2006). Because biofilm production is also linked to increased infectivity in a number of pathogenic bacteria (Austin & Zhang, 2006; Allegrucci *et al.*, 2006), it may also be involved in colonization by mutualistic bacteria such as *V. fischeri*. Additional studies have demonstrated that biofilms in *V. fischeri* are highly regulated and important for symbiosis to occur in the *E. scolopes* light organs (Yip *et al.*, 2005, 2006; Darnell *et al.*, 2008; Geszvain & Visick, 2008; Hussa *et al.*, 2008). Thus, biofilms are an integral part of the infection, colonization, and persistence processes of mutualistic vibrios in this dynamic symbiosis.

Previous studies in *V. cholerae* also indicate the importance of UDP glucose dehydrogenase for the synthesis of lipopolysaccharide in addition to colanic acid biosynthesis. UDP glucose dehydrogenase mutant strains also exhibit a deficiency in capsular formation in pathogenic strains of *V. cholerae* (Nesper *et al.*, 2001). Similar results were observed during colonization assays with *V. fischeri* ETJB1H UDPDH$^-$ mutant strains, which exhibited a 10^{-2} decrease in colonization compared with wild-type parental strains (Fig. 3). This may provide initial evidence that UDPDH has an additional role in the biosynthetic pathway producing lipopolysaccharide via glucuronic acid synthesis in *V. fischeri*. Previous studies have shown the role between lipopolysaccharide and peptidoglycan that together trigger morphogenesis in juvenile squid light organs (Foster *et al.*, 2000; Koropatnick *et al.*, 2004). Peptidoglycan is also known to be essential for mucin synthesis (Nyholm *et al.*, 2000, 2002; Nyholm & McFall-Ngai, 2003). Induction of mucus secretion in *E. scolopes* demonstrates the presence of gram-negative bacteria such as *V. fischeri*. Together the morphogenesis and mucus production enhances the capability of specific *V. fischeri* to recognize and infect their squid hosts. This is one of the mechanisms that is responsible for selecting symbiotically competent bacteria from all others in the surrounding seawater.

TEM observations in this study also support the hypothesis that UDPDH is responsible for colanic acid biosynthesis thereby affecting capsular and biofilm formation in symbiotic *V. fischeri*. UDPDH$^-$ mutant *V. fischeri* were not found in any part of the crypt region of the light organs upon examination (Fig. 4b). In addition, the ability to produce biofilm *in vitro* was measured to verify whether biofilm production was reduced in the UDPDH mutant strains. *In vitro* biofilm assays exhibited a threefold reduction in biofilm production by *V. fischeri* ETJB1H UDPDH$^-$ mutants compared with parental wild-type *V. fischeri* ETJB1H (Fig. 1). Similar deformations in biofilm production were observed in UDP glucose mutants in *V. cholerae* where mutant strains are not capable of inducing biofilm production (Nesper *et al.*, 2001).

Additionally, motility assays were completed to verify whether the UDPDH gene is linked to motility, because

motility is associated with a decrease in colonization by *V. fischeri* (Millikan & Ruby, 2002). *Vibrio fischeri* UDPDH$^-$ mutants did exhibit significant differences in motility compared with the wild-type parental strain. This result indicated that mutating the UDPDH locus did effect the motility of the bacterium, providing evidence that colonization deficiencies at this locus were linked not only to the decreases in biofilm production but also motility.

mshA

Studies examining *V. cholerae* have provided information regarding the role of *mshA* during initial infection before biofilm formation (Watnick *et al.*, 1999). This gene is responsible for the formation of type IV bundle-forming pili, which are crucial for the initial attachment to abiotic substrates. In addition, type IV bundle pili are vital for twitching motility in *P. aeruginosa*, which is considered to be an essential trait for the spread of infection following initial attachment and biofilm production (O'Toole & Kolter, 1998; Skerker & Berg, 2001). Earlier studies using SCOTS in symbiotic *V. fischeri* detected two genes (*pilM* and *mshA*) that were responsible for pili formation and attachment, and are solely expressed in seawater cultures (Jones & Nishiguchi, 2006). Expression of both *pilM* and *mshA* before colonization (in the free-living stage) suggests that both have important roles in initiating symbiosis with *E. tasmanica*. For example, another *pil* locus (*pilA*) has decreased colonization when mutant (*pilA*$^-$) *V. fischeri* were infected in *E. scolopes* juveniles (Stabb & Ruby, 2003), as well as establishing specificity during the early stages of colonization in enteropathogenic *E. coli* (Hicks *et al.*, 1998). It has also been demonstrated that a high degree of variability exists at this operon when comparing symbiotic and free-living strains of *V. fischeri* (Browne-Silva & Nishiguchi, 2008). This is yet another example of how subtle differences at a particular symbiotic locus can determine specificity in this association, and may be responsible for the differences observed in infection (commencement of the interaction) and colonization (growth and persistence of the association) between a number of symbiotically competent *V. fischeri* isolated (Nishiguchi *et al.*, 1998; Nishiguchi, 2002).

Studies have also shown that juvenile *E. scolopes* contain mannose along the crypt region of the light organs, enhancing colonization by *V. fischeri* (Visick & McFall-Ngai, 2000). Symbiotic *V. fischeri* were also able to agglutinate to guinea pig red blood cells and that exogenous mannose blocked colonization, indicating the presence of mannose-recognizing adhesions (McFall-Ngai *et al.*, 1998). In addition, further studies have provided evidence for mannose residues on the contact surface of host epithelial lining, implicating a receptor–ligand interaction between symbionts and the brush border epithelia of squid light organs (Visick & McFall-Ngai, 2000). Interactions such as those between symbiotic bacteria and mannose or other sugar-containing residues are commonly found in environmentally transmitted associations (Nishiguchi *et al.*, 2008; Nyholm & Nishiguchi, 2008), providing yet another mechanism for recognizing specific partners. This is particularly important in associations where a few bacteria are responsible for initiating the colonization of a new host amidst a number of abiotic and biotic forces that may delay or deter colonization from ever occurring (Soto *et al.*, 2009a, b).

In this study, *mshA* mutants were generated to examine whether this gene was involved in colonization processes. Results indicate that *V. fischeri* ETJB1H *mshA*$^-$ mutants had deficiencies in their colonization ability compared with the parental wild type, with a reduction of mutants observed inside the crypt region after infection (Fig. 4c). *Vibrio fischeri* ETJB1H *mshA*$^-$ mutants also exhibited severe deficiencies in biofilm production when compared with the wild-type parent. These results were similar to *V. cholerae* *mshA*$^-$ mutants where the *mshA*$^-$ mutants lack biofilm production and are severely deficient in motility (Watnick *et al.*, 1999). Motility is believed to be essential for the colonization of the squid light organ, because nonmotile *V. fischeri* are incapable of successful colonization (Millikan & Ruby, 2002). The lack of motility in *V. fischeri* *mshA*$^-$ mutants is additional support that movement to the light organ pores as well as through the duct region is required to colonize the crypts of the light organ after initial infection (Millikan & Ruby, 2002, 2003).

Complementation of mutant strains

Both *V. fischeri* UDPDH$^+$ and *mshA*$^+$ were able to regain the loss of function significantly for biofilm production, growth rate, and motility. The only phenotype that was not regained by complementation was bioluminescence with either UDPDH$^+$ or *mshA*$^+$. This suggests that both UDPDH and *mshA* genes may be linked to luminescence-related behavior (quorum sensing), and disruption of these genes interferes not only with bioluminescence production, but the ability for bacteria to quorum sense. Quorum sensing is activated through a cascade of genes in the *lux* operon, so that disruption of UDPDH may have possibly interfered with other potential downstream genes, regulated through bioluminescence production (Callahan & Dunlap, 2000). Although *lux* genes were not found to be downstream from either UDPDH or *mshA* in *V. fischeri* ES114 (Ruby *et al.*, 2005), it may differ in the *V. fischeri* ETJB1H strain that was used in this study. Further studies examining whether UDPDH is transcriptionally or translationally regulated via the *lux* operon would be required to verify the presence of either *cis*- or *trans*-acting regulatory elements linked to quorum sensing.

Conclusions

This study has examined whether UDPDH and *mshA* genes were involved in the early events of colonization of squid light organs. Results from our experiments indicate that both genes are involved in the establishment of the mutualism between *V. fischeri* and *E. tamanica*, where early attachment, colonization, and biofilm production are important for a successful environmentally transmitted symbiosis to occur. Experimental comparisons of environmentally expressed genes to those that are expressed solely in symbiosis can help us better understand whether trade-offs evolve to benefit either situation, such as the expression of *mshA* in the free-living state and UDPDH in the squid light organ, and how both genes are beneficial during different steps in establishing mutualistic relationships. Additionally, our understanding of how different pathways are co-opted from either a benign or pathogenic association can provide clues as to the evolution of virulence, and whether those genes involved in the symbiosis have been selected for among a wide variety of genotypes (Nishiguchi *et al*., 2008). The selective pressures that bacteria are exposed to (abiotic and biotic) have an incredible amount of influence that determines the evolutionary trajectory of a specific genotype; balancing those forces is one feat that symbiotic bacteria have overcome to be successful in nearly all ecological niches, including those that invade eukaryotic host tissues (Soto *et al*., 2009a, b). Continued research in the genetic basis behind *V. fischeri*'s adaptations to biotic and abiotic factors will continue to illuminate the underpinnings of symbiosis in the years to come.

Acknowledgements

The authors would like to thank A. Dunn and E. Stabb for their gift of the plasmids used in this study. This work was supported by NIH SO6 GM008136-32S2-1, NIH-NIAID 1SC1AI081659-01, and NSF IOS-0744498 to M.K.N.

References

Allegrucci M, Hu FZ, Shen K, Hayes J, Ehrlich GD, Post JC & Sauer K (2006) Phenotypic characterization of *Streptococcus pneumoniae* biofilm development. *J Bacteriol* **188**: 2325–2335.

Austin B & Zhang XH (2006) *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Lett Appl Microbiol* **43**: 119–124.

Bomchil N, Watnick P & Kolter R (2003) Identification and characterization of a *Vibrio cholerae* gene, *mbaA*, involved in maintenance of biofilm architecture. *J Bacteriol* **185**: 1384–1390.

Boucher DH, James S & Keeler KH (1982) The ecology of mutualism. *Annu Rev Ecol Syst* **13**: 315–347.

Browne-Silva J & Nishiguchi MK (2008) Gene sequences of the pil operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int J Syst Evol Micr* **58**: 1292–1299.

Callahan SM & Dunlap PV (2000) LuxR- and acyl-homoserine-lactone-controlled non-*lux* genes define a quorum-sensing regulon in *Vibrio fischeri*. *J Bacteriol* **182**: 2811–2822.

Darnell CL, Hussa EA & Visick KL (2008) The putative hybrid sensor kinase SypF coordinates biofilm formation in *Vibrio fischeri* by acting upstream of two response regulators, *sypG* and *vpsR*. *J Bacteriol* **190**: 4941–4950.

Davies DG, Chakrabarty AM & Geesey GG (1993) Exopolysaccharide production in biofilms; substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl Environ Microb* **59**: 1181–1186.

Dunn AK & Stabb EV (2008) The twin arginine translocation system contributes to symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. *FEMS Microbiol Lett* **279**: 251–258.

Dunn AK, Martin MO & Stabb EV (2005) Characterization of pES213, a small mobilizable plasmid from *Vibrio fischeri*. *Plasmid* **54**: 114–134.

Foster JS, Apicella MA & McFall-Ngai MJ (2000) *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. *Dev Biol* **226**: 242–254.

Geszvain K & Visick KL (2008) The hybrid sensor kinase RscS integrates positive and negative signals to modulate biofilm formation in *Vibrio fischeri*. *J Bacteriol* **190**: 4437–4446.

Hicks S, Frankel G, Kaper JB, Dougan G & Phillips AD (1998) Role of intimin and bundle-forming pili in enteropathogenic *Escherichia coli* adhesion to pediatric intestinal tissue *in vitro*. *Infect Immun* **66**: 1570–1578.

Hussa EA, Darnell CL & Visick KL (2008) RscS functions upstream of SypG to control the *syp* locus and biofilm formation in *Vibrio fischeri*. *J Bacteriol* **190**: 4576–4583.

Jones BW & Nishiguchi MK (2004) Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Mar Biol* **144**: 1151–1155.

Jones BW & Nishiguchi MK (2006) Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. *Can J Microbiol* **52**: 1218–1227.

Jones BW, Lopez JE, Huttenburg J & Nishiguchi MK (2006) Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol Ecol* **15**: 4317–4329.

Koropatnick TA, Engle JT, Apicella MA, Stabb EV, Goldman WE & McFall-Ngai MJ (2004) Microbial factor-mediated development in a host–bacterial mutualism. *Science* **306**: 1186–1188.

Lam J, Chan R, Lam K & Costerton JW (1980) Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect Immun* **28**: 546–556.

Lee K-H & Ruby EG (1994) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl Environ Microb* **60**: 1565–1571.

McFall-Ngai MJ (2000) Negotiations between animals and bacteria: the 'diplomacy' of the squid-*Vibrio* symbiosis. *Comp Biochem Physiol A* **126**: 471–480.

McFall-Ngai MJ, Brennan C, Weis V & Lamarcq LH (1998) Mannose adhesin–glycan interactions in the *Euprymna scolopes–Vibrio fischeri* symbiosis. *New Developments in Marine Biotechnology* (Le Gal Y & Halvorson HO, eds), pp. 273–276. Plenum Press, NY.

Millikan DS & Ruby EG (2002) Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Appl Environ Microb* **68**: 2519–2528.

Millikan DS & Ruby EG (2003) FlrA, an o^{54}-dependent transcriptional activator in *Vibrio fischeri*, is required for mobility and symbiotic light organ colonization. *J Bacteriol* **185**: 3547–3557.

Montgomery MK & McFall-Ngai MJ (1993) Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. *Biol Bull* **184**: 296–308.

Montgomery MT & Kirchman DL (1993) Role of chitin-binding proteins in the specific attachment of the marine bacterium *Vibrio harveyi* to chitin. *Appl Environ Microb* **59**: 373–379.

Nair VS (2006), The evolution of symbiosis in Vibrionaceae and factors influencing host colonization in squid-*Vibrio* mutualism. PhD Dissertation, New Mexico State University, Las Cruces, NM.

Nesper J, Lauriano CM, Klose KE, Kapfhammer D, Krai A & Reidl J (2001) Characterization of *Vibrio cholerae* O1 El Tor *galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infect Immun* **69**: 435–445.

Nishiguchi MK (2002) Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microbiol Ecol* **44**: 10–18.

Nishiguchi MK & Jones BW (2004) Microbial biodiversity within the Vibrionaceae. *Origins* (Seckbach J, ed), pp. 531–548. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Nishiguchi MK, Ruby EG & McFall-Ngai MJ (1998) Competitive dominance during colonization is an indicator of coevolution in an animal–bacterial symbiosis. *Appl Environ Microb* **64**: 3209–3213.

Nishiguchi MK, DeVinney R, Hirsch AM, Riley M, Mansky L & Vendatum G (2008) Perspective: evolution of virulence: deciphering the mechanisms between pathogenic and benign symbioses. *Vie Milieu* **58**: 87–106.

Nyholm SV & McFall-Ngai MJ (2003) Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl Environ Microb* **69**: 3932–3937.

Nyholm SV & McFall-Ngai MJ (2004) The winnowing: establishing the squid–*Vibrio* symbiosis. *Nat Rev Microbiol* **2**: 632–642.

Nyholm SV & Nishiguchi MK (2008) The evolutionary ecology of a sepiolid squid–*Vibrio* association: from cell to environment. *Vie Milieu* **58**: 175–184.

Nyholm SV, Stabb EV, Ruby EG & McFall-Ngai MJ (2000) Establishment of an animal–bacterial association: recruiting symbiotic vibrios from the environment. *P Nat Acad Sci USA* **97**: 10231–10235.

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA & McFall-Ngai MJ (2002) Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microb* **68**: 5113–5122.

O'Toole G & Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**: 295–304.

O'Toole G, Kaplan HB & Kolter R (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* **54**: 49–79.

Ruby EG, Urbanowski M, Campbell J *et al.* (2005) Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *P Natl Acad Sci USA* **102**: 3004–3009.

Skerker JM & Berg HC (2001) Direct observation of extension and retraction of type IV pili. *P Nat Acad Sci USA* **98**: 6901–6904.

Soto W, Gutierrez J, Remmenga MR & Nishiguchi MK (2009a) Synergistic affects of temperature and salinity in competing strains of symbiotic *Vibrio fischeri*. *Microbiol Ecol* **57**: 140–150.

Soto W, Lostroh CP & Nishiguchi MK (2009b) Physiological responses to stress in the Vibrionaceae: aquatic microorganisms frequently affiliated with hosts. *Cooperation and Stress in Biology* (Seckbach J & Grube M, eds), Springer, New York, NY.

Stabb EV & Ruby EG (2002) RP-4 Based plasmids for conjugation between *Escherichia coli* and members of the *Vibrionaceae*. *Methods Enzymol* **358**: 413–426.

Stabb EV & Ruby EG (2003) Contribution of *pilA* to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. *Appl Environ Microb* **69**: 820–826.

Stevenson G, Andrianopoulos K, Hobbs M & Reeves PR (1996) Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J Bacteriol* **178**: 4885–4893.

Visick KL & McFall-Ngai MJ (2000) An exclusive contract: specificity in the *Vibrio fischeri–Euprymna scolopes* partnership. *J Bacteriol* **182**: 1779–1787.

Watnick PI & Kolter R (1999) Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* **34**: 586–595.

Watnick PI, Fullner KJ & Kolter R (1999) A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *J Bacteriol* **181**: 3606–3609.

VIE ET MILIEU - LIFE AND ENVIRONMENT, 2009, 59 (3/4): 277-285

BIOLOGICAL PROPERTIES (*IN VITRO*) EXHIBITED BY FREE-LIVING AND SYMBIOTIC *VIBRIO* ISOLATES

V. NAIR [+], *M. K. NISHIGUCHI* [*]

Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA
** Corresponding author: nish@nmsu.edu*
[+] Present address Research Technologies Section, RTB, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, Montana 59840, USA

VIBRIONACEAE
SYMBIOSIS
SEPIOLID SQUID
PILI
BIOFILM

ABSTRACT. – Adhesion and biofilm forming ability of symbiotic bacteria play a crucial role in host colonization and tissue infection. Bacteria benefit by adhering to their host in a manner that allows them to successfully maintain contact for the exchange of nutrients, hormones, or other necessary products. This study examined pili morphology, motility, and biofilm formation exhibited by *Vibrio fischeri* strains (free-living and symbiotic). Since these symbiotic factors contribute in some fashion to the interaction between *V. fischeri* and their squid host, variation between strains may be a contributing factor that leads to specificity among different hosts. *V. fischeri* strains examined in this study demonstrated considerable variation in their biological properties when observed *in vitro*. In addition to differences observed between strains isolated from several different host species, we observed variation between strains isolated from the same host species from diverse geographical locations. This study suggests that subtle differences in the biological properties of closely related *V. fischeri* strains may influence the nature of the interaction among *V. fischeri* and their sepiolid hosts.

INTRODUCTION

Bacteria are oftentimes found associated with animals, plants, fungi, and protists, as well as other bacteria. They have been successful in colonizing both extra-cellular and intracellular tissues of their host, enabling some specific function or capability. Likewise, metazoans such as sponges, corals, nematodes, molluscs, and humans can be hosts to symbiotic bacteria that possess different ecological paradigms (mutualistic, pathogenic, or commensal). These hosts obtain their symbionts by a number of mechanisms that deliver the symbionts horizontally (host directly to a new host), vertically (parent to offspring, oftentimes maternally), or environmentally (from the surrounding environment to the host), and can occur prior or subsequent to embryogenesis (Nishiguchi 2001, Stabb & Ruby 2002, Nyholm & Nishiguchi 2008). In particular, environmental transmission has a number of obstacles prior to infection as well as during colonization and persistence that bacteria are constantly subjected to (Stabb & Ruby 2002). Oftentimes, host species develop mechanisms that increase their probability of being colonized by beneficial microorganisms, while discouraging nonspecific ones (Nyholm & McFall-Ngai 2004). This is usually achieved by creating conditions that only their specific symbionts are capable of accommodating (Nyholm *et al*. 2000). To counter these obstacles, traits exhibited by bacteria can vary depending upon the specificity of each association. Symbiotic bacteria develop complex traits such as surface adhesions, chemoattraction to specific polysaccharides, motility towards recognizable receptors, and resistance to specific immune response proteins (*i.e*., catalase), to improve their chances of pioneering host colonization. Most colonizing bacteria are capable of expressing adhesions, and/or pili, and have the ability to form biofilms, making these components key factors in bacterial colonization and symbiosis (Klemm & Schembri 2000, Lee & Schneewind 2001).

Microbial communities have been known to use pili to form biofilms by attaching to both abiotic and biotic surfaces. These pili help form three-dimensional structures through which nutrients diffuse through and waste products diffuse out (Watnick *et al*. 1999). Biofilms formed by gram-negative bacteria are also known to be influenced by environmental conditions (O'Toole *et al*. 2000), and have been known to differentially express extracellular proteins used in biofilm production in response to specific environmental cues (Branda *et al*. 2005).

Pili production has also been affiliated with biofilm production in a number of gram-negative bacteria that respond to both environmental and host cues. *Escherichia coli* are capable of expressing conjugative pili that result in non-specific cell-to-cell and cell-to-surface associations, thereby accelerating initial adhesion and biofilm development (Ghigo 2001, Branda *et al*. 2005). *Pseudomonas aeruginosa* uses type IV pili differentially, based on the external environment it resides in. Type IV pili in these bacteria have been shown to mediate surface attachment and twitching motility. Depending on which carbon source is available, they can differentially use their type IV pili for biofilm formation (Branda *et al*. 2005). In *Vibrio cholerae* El Tor strain, the MshA pili mediated by a

type IV pilus are responsible for attachment to abiotic surfaces in the environment (Watnick & Kolter 1999). Once inside the host intestine, they recruit the toxin-coregulated pilus (TCP), a type IV bundle forming pilus to adhere to the intestinal cells, thereby switching between a benign, environmental niche to a pathogenic one.

Motility and chemotaxis also play key roles in the formation of biofilms (Pratt & Kolter 1998). Mechanisms by which bacteria respond to their symbiotic environment have been found to be similar to those that coordinate regulation of motility and colonization genes in pathogenic associations (Millikan & Ruby 2002). Therefore, bacterial adhesion, biofilm formation, and motility all might be important mechanisms used by symbiotic bacteria to colonize their hosts.

The sepiolid squid – *Vibrio* mutualism represents an environmentally transmitted symbiosis where host embryogenesis is completed in absence of the bacterial symbiont (Ruby & McFall-Ngai 1992, McFall-Ngai & Ruby 1998, Nyholm & McFall-Ngai 2003). Newly hatched juvenile squids obtain their symbiotic bacteria from the surrounding seawater within a few hours, and are then colonized for the rest of their lives (Nyholm & Nishiguchi 2008). These nocturnal squids use bioluminescent *V. fischeri* in a behavior referred to as counterillumination, which eliminates the squid's silhouette in order to evade predators or be inconspicuous to their prey while hunting (Jones & Nishiguchi 2004). At sunrise, squids will bury in the sand, and vent approximately 90-95 % of their bacterial symbiont population. The remaining 5 % of bacteria repopulate the light organ crypts for use the next evening.

Previous work has demonstrated that species in the genus *Euprymna* prefer native *Vibrio fischeri* over non-native *V. fischeri*, even though both types of bacteria colonize juvenile squids equally well by themselves (Nishiguchi *et al*. 1998, Nishiguchi 2002). More recently, different genetic isolates have been found within populations of *Euprymna*, providing additional evidence that subtle differences exist among all competent strains of symbiotic *Vibrio* (Jones *et al*. 2006, Guerrero-Ferreira & Nishiguchi 2007, Mandel *et al*. 2009, Wollenberg & Ruby 2009). Although this general specificity is very common among Indo-West Pacific sepiolids (Nishiguchi *et al*. 1998, Nishiguchi 2002), very little is known about which bacterial traits provide native strains a competitive edge over non-native strains as well as environmental isolates of *V. fischeri* that cannot colonize sepiolids. Previous studies investigating the distribution of native and non-native *V. fischeri* strains in *E. tasmanica* light organs revealed symbiotic bacterial cells expressing at least two different morphological types of pili within the light organ crypts (Nair *et al*. in review). From this observation, we deduced that pili formation and adhesion during light organ colonization is linked to strain differentiation and specificity. Since earlier studies in other gram-negative bacteria suggest the importance of pili in adhesion, biofilm formation, and motility, we decided to investigate and compare these biological properties of both free-living and symbiotic vibrios.

METHODS

Biofilm assay: Biofilm assays were performed to determine whether bacteria could form biofilms *in vitro* (O'Toole & Kolter 1998). All bacteria strains (Table I) used in this study were previously isolated and described in earlier studies (Nishiguchi & Nair 2003). Bacterial strains were grown overnight at 28°C with aeration (250 rpm). The following day strains were re-grown in fresh 32 parts/thousand (ppt) Sea Water Tryptone (SWT) medium to an optical density of 0.1 at 600 nm. OD_{600} was adjusted by using fresh SWT media to ensure that the bacterial strains were in logarithmic growth phase. One mL of each strain was then transferred to fresh sterile test tubes and incubated without shak-

Table I. – *Vibrio fischeri* strains used in this study

Strain designation	Source (host)	Location
Vibrio fischeri ETBB1B	*Euprymna tasmanica*	Australia (Botany Bay)
Vibrio fischeri ET401	*Euprymna tasmanicaI*	Australia (Townsville)
Vibrio fischeri ET301	*Euprymna tasmanica*	Australia (Sydney)
Vibrio fischeri ET101	*Euprymna tasmanica*	Australia (Melbourne)
Vibrio fischeri SR5	*Sepiola robusta*	France (Banyuls-sur-Mer)
Vibrio fischeri SL518	*Sepiola ligulata*	France (Banyuls-sur-Mer)
Vibrio fischeri SA1G	*Sepiola affinis*	France (Banyuls-sur-Mer)
Vibrio fischeri SI1D	*Sepiola intermedia*	France (Banyuls-sur-Mer)
Vibrio fischeri ESP915	*Euprymna scolopes*	Hawaii (Paiko)
Vibrio fischeri ES114	*Euprymna scolopes*	Hawaii (Kaneohe Bay)
Vibrio fischeri EB12	*Euprymna berryi*	Japan (Tokyo Bay)
Vibrio fischeri EM17	*Euprymna morsei*	Japan (Tokyo Bay)
Vibrio fischeri CG101	*Cleidopus gloriamaris*	Australia (Townsville)
Vibrio fischeri MG101	*Monocentris japonicus*	Japan
Vibrio fischeri MDR7	Free-living	USA (Marina del Rey, CA)
Vibrio fischeri WH1	Free-living	USA (Woods Hole, MA)

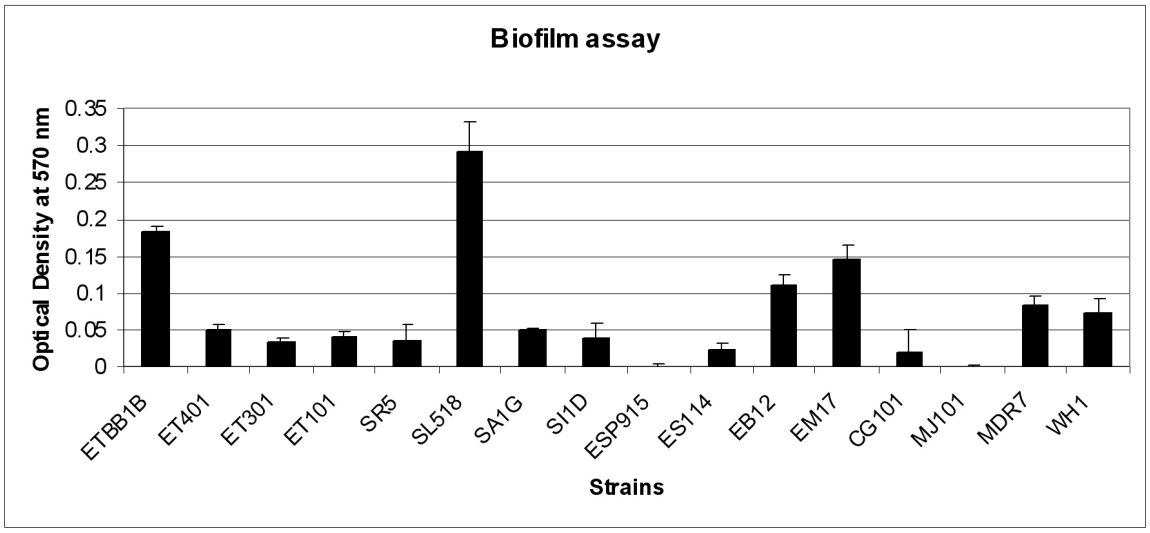


Fig. 1. – Average biofilm produced by closely related *Vibrio fischeri* strains. All measurements were made in triplicate and the average was calculated for each strain (Error bars represent standard error). For strain designation, see Table I.

ing at 28°C for 24 hours. Three replicate samples were measured for each strain. SWT media with no inoculum were used as a negative control for this experiment. After incubation, tubes were washed three times with 32 ppt seawater. Tubes were left upside down for 5 minutes to drain excess seawater. Subsequently, one mL of a 0.2 % aqueous crystal violet solution was added to each tube and incubated at room temperature for one hour. The crystal violet was poured out, and tubes were rinsed three times with 32 ppt seawater. During the last two washes, test tubes were vortexed briefly to ensure complete removal of crystal violet that remained on the sides of each test tube and away from the region of biofilm formation. Tubes were left inverted for 10-15 minutes to drain excess water. Crystal violet bound to the biofilm was then eluted by adding one mL of 80:20 (vol/vol) mixtures of ethyl alcohol and acetone. The tubes were vortexed till all the crystal violet attached to the glass was eluted. Using a UVIKON XL Spectrophotometer (Research instruments Inc.) the amount of biofilm formed was then quantified based on the OD reading at 570 nm, which is directly proportional to the amount of crystal violet binding to the bacterial biofilm produced in each test tube. Standard error was calculated for each of the strains tested.

Motility assay: Bacterial strains were grown overnight at 28°C with rapid shaking (250 rpm). The following day strains were re-grown in fresh 32 ppt SWT medium to 0.1 OD_{600}. Strains that exceeded 0.1 OD_{600} were adjusted by using fresh SWT media. 10 µL of each bacterial culture was plated as spots on a 32 ppt SWT plate using 0.5 % motility agar. Motility was determined by measuring the diameter of each spot after 24 hours incubation at 28°C. Motility assays for every strain were completed in triplicate and photographs of each plate were taken using BioRad phosphorimager (BioRad, Hercules, CA). Distance measurements for each strain were computed and an average of each strain (from the three samples) was calculated along with the standard error.

Transmission electron microscopy of pili: Bacteria were grown overnight at 28°C without shaking. A 200 mesh formvar coated nickel grid was floated on 50 µL of each bacterial culture for 5 minutes. Grids were rinsed for 30 seconds in distilled water and stained with 2 % uranyl acetate for two minutes. Excess uranyl acetate was blotted from the grid, and grids were then rinsed for 30 seconds and air dried. Samples were observed using a Hitachi H-7000 Transmission electron microscope (TEM) at an accelerating voltage of 75kv. Photographs were taken on 3.25 x 4 Kodak EM film and scanned on an Epson flatbed scanner.

RESULTS

Biofilm data

Biofilm formation was measured based on the optical density (570 nm) of the crystal violet solution eluted from the dissolved biofilm formed on each glass test tube. Biofilm concentrations were calculated by subtracting the OD_{570} measured from each of the control tubes (sterile SWT media) from the OD_{570} measurement of the eluted crystal violet/biofilm solution. This value was averaged from the three replicates and then used to determine whether differences existed among the 16 *Vibrio* strains isolated from different host species and habitats (Fig. 1).

Both free-living *V. fischeri* MDR7 and WH1 strains had similar concentrations of biofilm formed, with OD_{570} of approximately 0.07-0.08 (Fig. 1). For symbiotic strains, *V. fischeri* ETBB1B (*E. tasmanica*, Australia), SL518 (*S. ligulata*, France) EM 17 (*Euprymna morsei*, Japan) and EB12 (*E. berryi*, Japan) isolates had the highest levels of biofilm formation, ranging from 0.11 to nearly 0.3 OD_{570}. All *E. tasmanica* (ET) *V. fischeri* strains, with the exception of ETBB1B, had similar biofilm production,

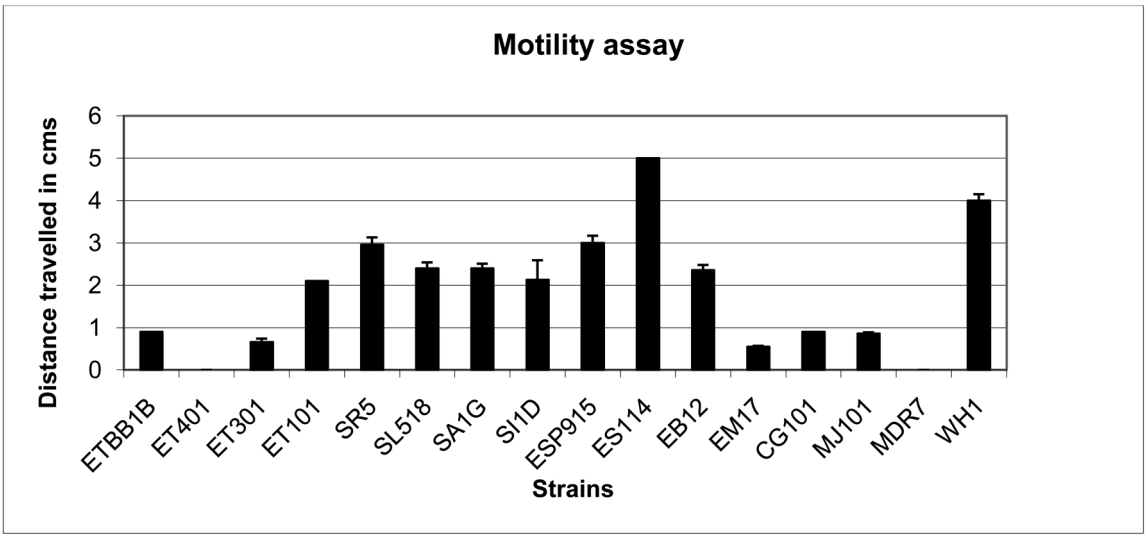


Fig. 2. – Motility measurements (in centimeters) for all 16 *Vibrio fischeri* strains. 10 µL of culture (0.1 OD_{600}) was placed in the center of each agar Petri dish and allowed to grow for 24 hours at 28°C. Measurements were made in triplicate, and the average was made for each strain. Error bars represent standard error. For strain designation, see Table I.

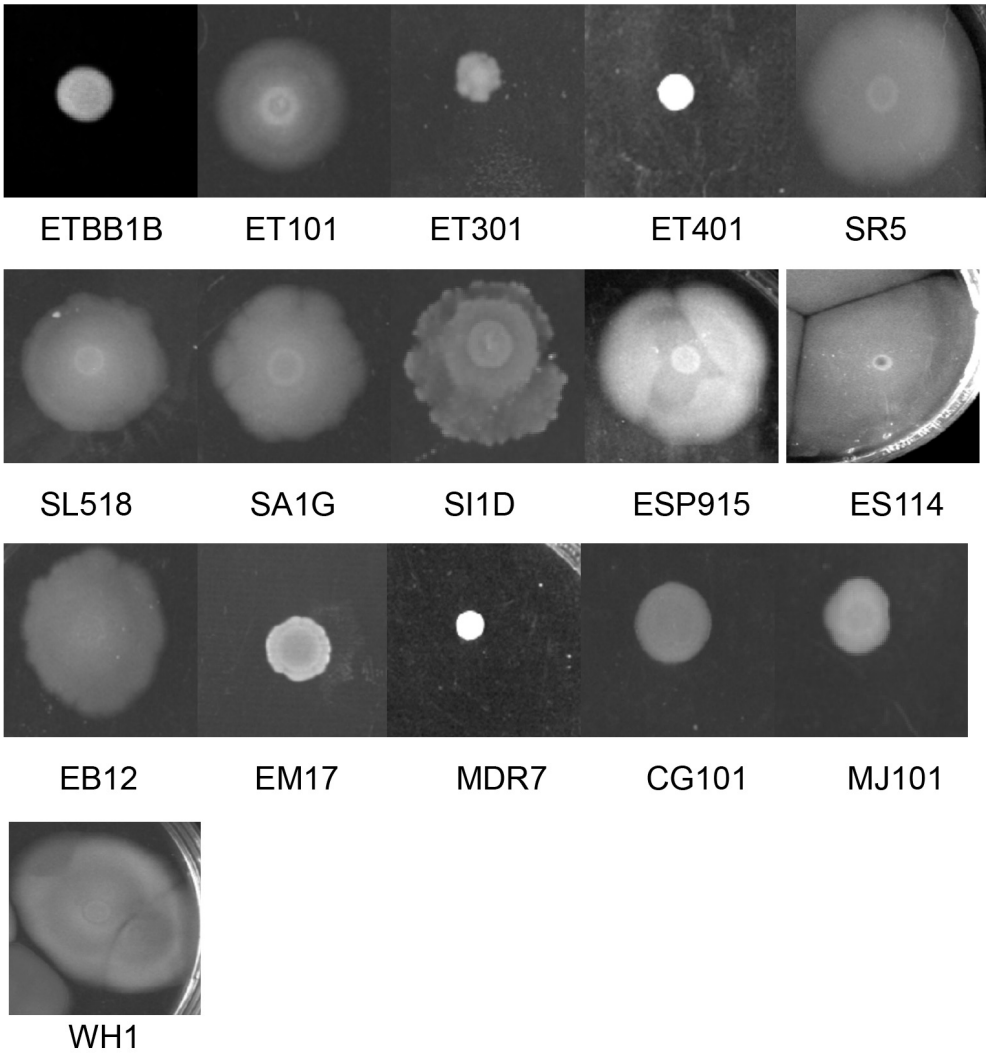


Fig. 3. – 24 hr motility time point on 0.5 % SWT agar plates for free-living and symbiotic *Vibrio fischeri* strains. For strain designation, see Table I.

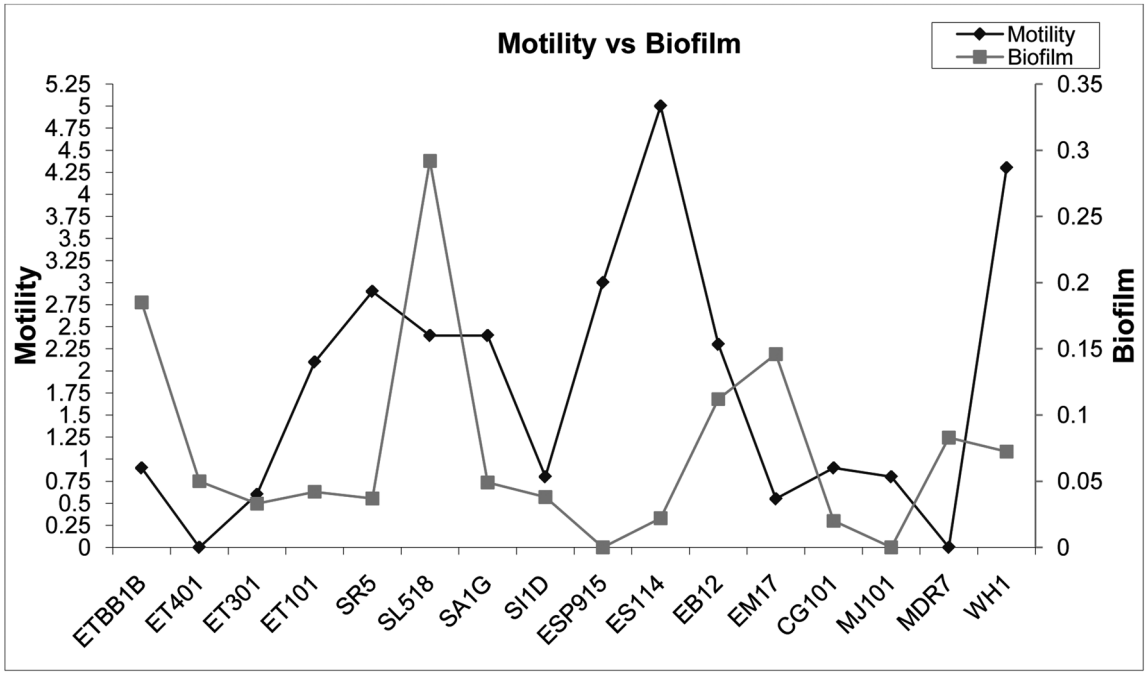


Fig. 4. – Motility vs biofilm formation for all *Vibrio fischeri* strains examined in this study. For strain designation, see Table I.

with an average of 0.04 OD_{570}. Interestingly, *V. fischeri* ETBB1B exhibited four-times more biofilm production when compared to other ET strains isolated from *E. tasmanica* squid hosts from different populations (Fig. 1, Table I). In contrast, both *V. fischeri* ES114 and ESP915 (*E. scolopes*, Hawaii) exhibited minimal biofilm formation, even though both strains were isolated from two different squid populations. Only one fish symbiont (*V. fischeri* CG101) examined was capable of producing biofilm, but was comparably less than other strains tested (0.01 OD_{570}). *V. fischeri* MJ101, a fish symbiont of *Monocentrus japonicus*, did not produce any detectable biofilm.

Motility assays

Motility for all *V. fischeri* strains was measured by calculating the distance traveled by each bacterial strain on low agar (0.5 %) concentration SWT plates (Figs. 2, 3). Symbiotic *V. fischeri* ES114 (from *E. scolopes*) exhibited the highest motility (5 cm) followed by the free-living strain *V. fischeri* WH1 (3.9 cm). Among all the *E. tasmanica* strains studied, *V. fischeri* ET101 was observed to exhibit the highest motility (1.7 cm). Both fish symbionts *V. fischeri* CG101 and *V. fischeri* MJ101 demonstrated similar motility capabilities, between 0.1 and 0.2 cm. Unlike the other symbiotic strains tested for motility *V. fischeri* ET301 (*E. tasmanica*, Australia) and *V. fischeri* SI1D (*S. intermedia*, France) demonstrated a more globular/non-uniform edge for each spot after incubation (Fig. 3). Interestingly, all ET strains from Australia differed in their motility rates (slow to fast) during *in vitro* growth (Figs. 3, 4), despite the fact that they infect the same species of *Euprymna*. Figure 4 demonstrates the relative biofilm formation and motility of each of the strains tested.

Pili morphology

Results from the transmission electron microscopical (TEM) observations made evident that closely related *V. fischeri* strains exhibited a number of different pili morphotypes (Fig. 5). *V. fischeri* ES114 was observed to have the smallest pili, and individual cells exhibited bifurcation among those pili (Fig. 5A). This was morphologically different from pili expressed by another Hawaiian symbiotic *V. fischeri* strain, ESP915, found in the same host species (Fig. 5B). Both *V. fischeri* strains colonize *E. scolopes*, but are from different populations (ES114 is from Kaneohe Bay, and ESP915 from Paiko, see Table I). Aggregated bacterial strains were observed to produce more pili than solitary bacteria, oftentimes with the pili interacting between each cell (Fig. 5D). Strains such as *V. fischeri* SL518, which was one of the strongest biofilm producers, also produced dense pili (Fig. 5D).

Both *V. fischeri* ETB1B and *V. fischeri* EM17 (*E. tasmanica* and *E. morsei* hosts) exhibited longer pili (Figs. 5C, 5G), and were noted to be less dense than *V. fischeri* SR5 and *V. fischeri* SL518, to both of which were strains from Mediterranean squid hosts (*S. robusta* and *S. ligulata*). It was also noted that *V. fischeri* EB12 (*E. berryi*, Japan), had long, continuous pili forming between two

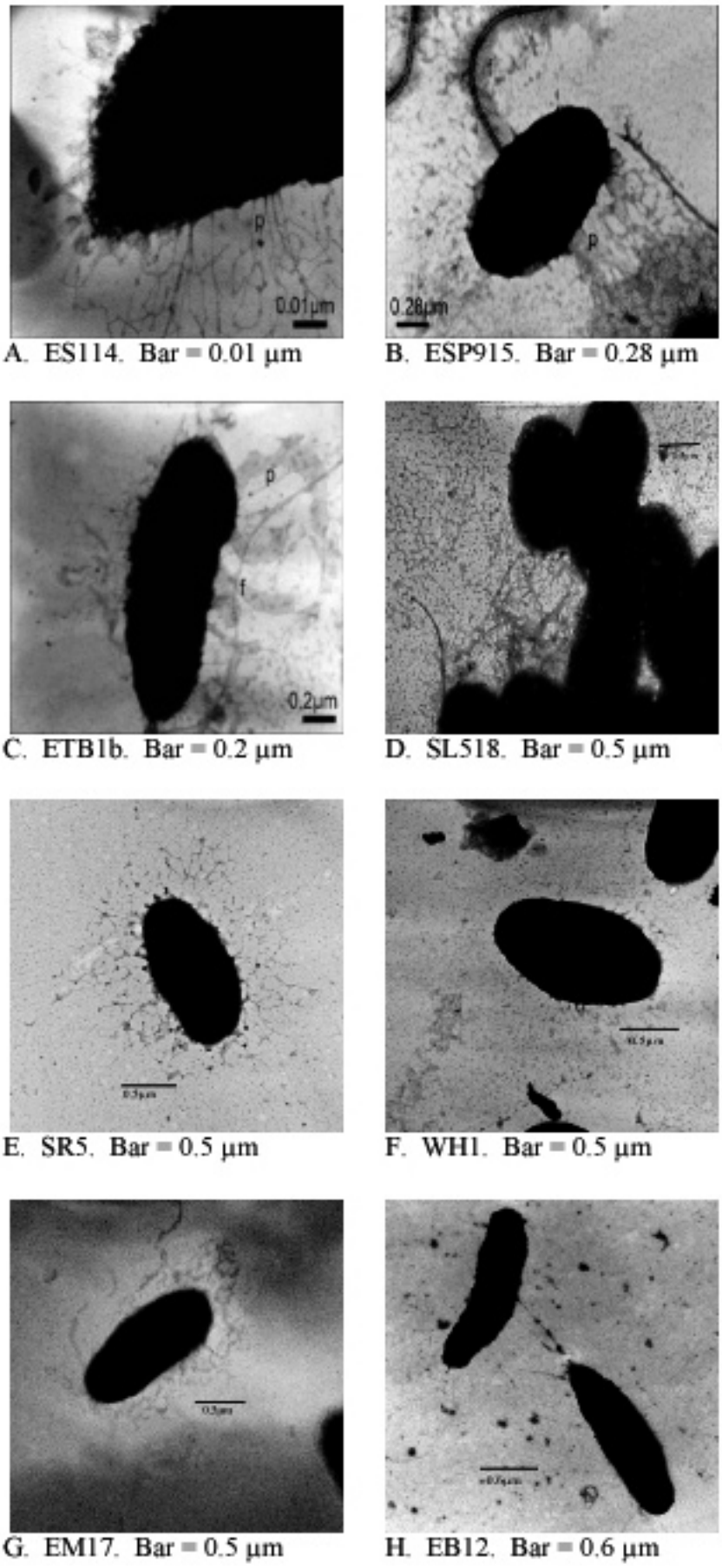


Fig. 5. – Negatively stained *Vibrio fischeri* cells exhibiting different pili morphologies in culture. For strain designation, see Table I.

cells (Fig. 5H). Finally, *V. fischeri* WH1, a free-living isolate, had short, stubby pili along the cell membrane (Fig. 5F). Other strains of *V. fischeri* screened did not reveal any pili (data not included).

DISCUSSION

The goal of this study was to determine whether symbiotic and free-living, *V. fischeri* strains differed in their motility, ability to form biofilm, and produce pili *in vitro*. Our interests were to examine possible differences between wild type strains that may affect infection and colonization capabilities before and after invading squid host light organs. Results from this study revealed that (1) both free-living and symbiotic *V. fischeri* strains have different biofilm forming capabilities; (2) most *V. fischeri* strains demonstrated their capacity to twitch and swim during *in vitro* motility assays; (3) there was heterogeneity in pili morphology among closely related *V. fischeri*.

With the exception of symbiotic *V. fischeri* CG101 and *V. fischeri* MJ101 fish strains, the majority of *V. fischeri* strains studied were isolated from squid light organs. *V. fischeri* MDR7 and *V. fischeri* WH1 were the only two free-living vibrios that were closely related to the other symbiotic *V. fischeri* (Nishiguchi & Nair 2003). Since all squid host species obtain their bacterial symbionts from the surrounding seawater within a few hours after hatching (McFall-Ngai & Ruby 1991, Montgomery 1993), it would be of particular interest if differences in symbiont behavior affect this event. Environmental persistence and host colonization pose very different challenges for symbiotic bacteria (Nishiguchi 2001, Hsiao *et al*. 2006, Jones *et al*. 2006), since vibrios have developed the ability to not only survive outside the host as free-living organisms, but within the host as a symbiotic mutualist. Each niche necessitates *V. fischeri* to develop, express, or modify specific structures like flagella and/or pili, allowing the accommodation of both habitats when the symbiont encounters a different situation. Previous work has demonstrated that genes specific for motility are particularly necessary for the initial infection of *V. fischeri* into the *E. scolopes* light organ; without this structure, they are incapable of efficiently colonizing its squid host (Millikan & Ruby 2002, Millikan & Ruby 2003). Additionally, specific genes that are only expressed during the free-living stage prior to infection demonstrate that chemotaxis (*che* loci), pili assembly (*pilM*), and precursors to biofilm formation (*mshA*) are present during this portion of the life history of *V. fischeri*, and not when inside the light organ of its squid host (Ariyakumar & Nishiguchi 2009, Jones & Nishiguchi 2006). This exemplifies the breadth that *V. fischeri* exhibits and is capable of achieving between periods of symbiosis and host venting. Whether subtle changes exhibited by each strain determines host specificity, or is driven by environmental constraints that may be dictated by each particular habitat, such factors may be correlated to the differences observed in closely related *V. fischeri* strains in this study.

Environmental signals and cellular structures have also been shown to be required for adhesion to the intestinal epithelium, and are controlled under various regulatory pathways leading to biofilm formation (Watnick *et al*. 1999). For example, nutrient dependence is not the only factor that induces formation of biofilms, and that numerous bacterial species can form biofilms even in nutrient deficient environments (Pratt & Kolter 1998). In addition, quorum sensing has also been linked to the formation of biofilm, which may play an important role in regulating inter-microbial communication within and between species in the biofilm matrix (Hooper & Gordon 2001). For example, *V. cholerae*, acyl homoserine lactone CAI-1 (cholera autoinducer 1) plays a significant role in biofilm formation, and has also been shown to control LuxO activity (Hsiao *et al*. 2006). Similarly, *V. fischeri* ES114 has a conserved cluster of genes (*syp* operon) that promotes symbiotic colonization as well as biofilm formation (Yip *et al*. 2005, Yip *et al*. 2006). Both examples suggest the possibility that autoinducers and regulatory elements may have an additional role in biofilm formation as well as colonization and luminescence within squid hosts.

Prior work with the *V. fischeri* genome validates that 10 separate pilus gene clusters are contained within the genome, including mannose sensitive hemagglutinin (*mshA*) as one of the eight type IV pilus loci (Ariyakumar & Nishiguchi 2009, Ruby *et al*. 2004). In addition, both *V. fischeri* ESP915 (another ES strain) and *V. fischeri* ETJB (an ET strain) express these genes either in their free-living or symbiotic state (Jones & Nishiguchi 2006). Vibrio bacteria have been shown to be capable of recruiting different types of pili, depending on whether they are in association with their host (Watnick & Kolter 1999). Employing different pili morphotypes as needed or at different times may increase chances of survival in seawater as well as increasing successful initiation of infection and colonization of a new host. Based on this, it was expected that symbiotic *V. fischeri* strains would exhibit different biofilm formation when compared to free-living *V. fischeri* strains that are not able to colonize animal hosts. The hypothesis that differences in biofilm production exist between strains of *V. fischeri* isolated from distinct host species was confirmed by our studies (Fig. 1). Furthermore, strains isolated from the same squid host but different populations (*i.e*., *E. tasmanica*) displayed different levels of biofilm formation, even though all strains can equally colonize *E. tasmanica* hosts (Nishiguchi 2002). Previous work has demonstrated that *V. fischeri* strains are commonly shared across a network of *Euprymna* hosts, and occupy niches that may contain huge biogeographical distances (Jones *et al*. 2006). Therefore, squid specificity is not what is driving morphological differences such as pili and flagellar formation, but the combina-

tion of environmental survival and host infection prior to colonization.

This study suggests that *V. fischeri* symbionts contain a number of mechanisms that are phenotypically plastic, and have the ability to contribute to host specificity. Recently, we have begun to investigate the role of symbiotic genes in the formation of biofilm during symbiosis. Particularly, genes such as UDP-glucose dehydrogenase, which is a precursor for biofilm formation, is solely expressed by bacteria during symbiosis (Allegrucci *et al*. 2006, Jones & Nishiguchi 2006). The fact that this gene is solely expressed in *V. fischeri* present in the squid light organ strongly suggests bacterial biofilm is being formed in the light organ crypts. Preliminary evidence has demonstrated that bacteria deficient at this locus are unable to adhere to the epithelial lined crypts of the squid light organ, which does not allow them to successfully colonize the light organ to the level of the parental wild type strain (Ariyakumar & Nishiguchi 2009).

Given that most chronic infections are initiated through biofilms (Stewart & Costerton 2001) and that pili play a crucial role in biofilm formation (Watnick & Kolter 1999), it is also likely that biofilm formation in squid light organs is pili driven and not just *mshA* or UDP-glucose dehydrogenase dependent. Based on this assumption, it was expected that pili morphotypes expressed by the bacteria as well as their biofilm forming ability would assist colonization at different stages of this mutualism. The initial step would be entering the light organ of the squids. Our results from motility assays demonstrate a huge amount of variation that *Vibrio* bacteria have in their motility rates *in vitro*. Since colonization of newly hatched *Euprymna* requires the bacteria to locate the external pores (Nyholm *et al* 2000, Wolfe *et al*. 2004), higher motility rates may confer a selective advantage by allowing them to out-compete other symbiotic vibrios prior to entering the squid light organ. Once the bacteria are inside the light organ, the ability to adhere (pili) and form matrices (biofilm) with other bacteria or squid epithelia may provide yet another selective advantage for vibrio bacteria to be better at infection and colonization. Our observations strongly suggest that vibrios are highly plastic in their abilities to accommodate their immediate surroundings, which supports the hypothesis that squid host specificity may not be the only factor that drives specificity in this closely-knit mutualism.

The goal of this study was to illustrate whether symbiotic and free-living *V. fischeri* strains varied in qualities that are important for colonization. Biofilm formation, motility and twitching, and pili morphotypes were all found to be different among both symbiotic and free-living strains, with no correlation to geographical location or squid host species. Such implications suggest that specificity among *Vibrio* bacteria is not a simple, straightforward matter; and that the evolution of such environmentally transmitted associations are molded by both abiotic factors surrounding the free-living stage, as well as the importance of the light organ (biotic) during the symbiosis stage. Future mutational studies on biofilm forming and pili synthesis genes, followed by the ability of these mutated strains to colonize their host would provide more insight on the roles each of these factors have in host colonization.

ACKNOWLEDGEMENTS. –The authors would like to acknowledge W Soto and BRIDGES undergraduates J Clyde and M Weaver who assisted with biofilm and motility assays. This work was supported by NSF DEB-0316516, NSF IOS-0744498 and NIH-S06 GM08136-32S2 and NIH-NIAID 1SC1AI081659 to MKN. Undergraduates J Clyde and M Weaver were supported by the BRIDGES to Native American Indians (NIH-0GM48998).

REFERENCES

Allegrucci M, Hu FZ, Shen K, Hayes J, Ehrlich GD, Post JC, Sauer K 2006. Phenotypic characterization of *Streptococcus pneumoniae* biofilm development. *J Bacteriol* 188: 2325-2335.

Ariyakumar DS, Nishiguchi MK 2009. Characterization of two host specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. *FEMS Microbiol Lett* 299: 65-73.

Branda SS, Vik S, Friedman L, Kolter R 2005. Biofilms: The matrix revisited. *Trends Microbiol* 13: 20-26.

Ghigo JM 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412: 442-445

Guerrero-Ferreira RC, Nishiguchi MK 2007. Identification of light organ symbionts from the genera *Uroteuthis, Loliolus*, and *Euprymna*. *Cladistics* 23: 1-10.

Hooper LV, Gordon JI 2001. Commensal host-bacterial relationships in the gut. *Science* 292: 1115-1118.

Hsiao A, Liu Z, Joelsson A, Zhu J 2006. *Vibrio cholerae* virulence regulator-coordinated evasion of host immunity. *Proc Natl Acad Sci USA* 103: 14542-14547.

Jones BW, Huttenberg J, Lopez JE, Nishiguchi MK 2006. Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol Ecol* 15: 4317-4329.

Jones BW, Nishiguchi MK 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* 144: 1151-1155.

Jones BW, Nishiguchi MK 2006. Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. *Can J Microbiol* 52: 1218-1227.

Klemm P, Schembri MA 2000. Bacterial adhesins: Function and structure. *Int J Med Microbiol* 290: 27-35.

Lee VT, Schneewind O 2001. Protein secretion and the pathogenesis of bacterial infections. *Genes Dev* 15: 1725-1752.

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG 2009. A single regulatory gene is sufficient to alter bacterial host range. *Nature* 458: 215-220.

McFall-Ngai MJ, Ruby EG 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial symbiosis. *Science* 254: 1491-1494.

McFall-Ngai MJ, Ruby EG 1998. Sepiolids and Vibrios: When first they meet. *Bioscience* 48: 257-265.

Millikan DS, Ruby EG 2002. Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Appl Environ Microbiol* 68: 2519-2528.

Millikan DS, Ruby EG 2003. FlrA, a sigma^{54}-dependent transcriptional activator in *Vibrio fischeri*, is required for mobility and symbiotic light organ colonization. *J Bacteriol* 185: 3547-3557.

Montgomery MK, M. J. McFall-Ngai 1993. Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. *Biol Bull* 184: 296-308.

Nair VS, Ghoshroy S, Nishiguchi MK 2009. Distribution and localization of native and non-native vibrios in the bobtail squid, *Euprymna tasmanica* (Mollusca: Cephalopoda). *Microbiol Ecol* in review.

Nishiguchi MK 2001. Co-evolution of symbionts and hosts: The sepiolid-*Vibrio* model. *In* Seckbach J ed, Symbiosis: Mechanisms and Model Systems, Cole-Kluwer Academic, Dordrecht, The Netherlands: 757-774.

Nishiguchi MK 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microbiol Ecol* 44: 10-18.

Nishiguchi MK, Nair VA 2003. Evolution of pathogenicity and symbioses in Vibrionaceae: An combined approach using molecules and physiology. *Int J Syst Evol Bacteriol* 53: 2019-2026.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-*Vibrio* symbioses. *Appl Environ Microbiol* 64: 3209-3213.

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA, McFall-Ngai MJ 2002. Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* 68: 5113-5122.

Nyholm SV, McFall-Ngai MJ 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl Environ Microbiol* 69: 3932-3937.

Nyholm SV, McFall-Ngai MJ 2004. The winnowing: Establishing the squid-*Vibrio* symbiosis. *Nat Rev Microbiol* 2: 632-642.

Nyholm SV, Nishiguchi MK 2008. The evolutionary ecology of a sepiolid squid-*Vibrio* association: From cell to environment. *Vie Milieu* 58: 175-184.

Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ 2000. Establishment of an animal-bacterial association: Recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci USA* 97: 10231-10235.

O'Toole G, Kolter R 1998. Flagellar and twitching motility are necessary for *Psuedomonas aeruginosa* biofilm development. *Mol Microbiol* 30: 295-304

O'Toole G, Kaplan HB, Kolter R 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* 54: 49-79.

Pratt LA, Kolter R 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30: 285-293.

Ruby E, Henderson B, McFall-Ngai M 2004. Microbiology. We get by with a little help from our (little) friends. *Science* 303: 1305-1307.

Ruby EG, McFall-Ngai MJ 1992. A squid that glows in the night: Development of an animal-bacterial mutualism. *J Bacteriol* 174: 4865-4870.

Stabb EV, Ruby EG 2002. RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. *Methods Enzymol* 358: 413-426.

Stewart PS, Costerton WJ 2001. Antibiotic resistance of biofim. *The Lancet* 358: 135-138.

Watnick PI, Fullner KJ, Kolter R 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *J Bacteriol* 181: 3606-3609.

Watnick PI, Kolter R 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* 34: 586-595.

Wolfe AJ, Millikan DS, Campbell JM, Visick KL 2004. *Vibrio fischeri* s^{54} controls motility, biofilm formation, luminescence, and colonization. *Appl Environ Microbiol* 70: 2520-2524.

Wollenberg MS, Ruby EG 2009. Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two Oahu populations. *Appl Environ Microbiol* 75: 193-202.

Yip ES, Geszvain K, DeLoney-Marino CR, Visick KL 2006. The symbiosis regulator RscS controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. *Mol Microbiol* 62: 1586-1600.

Yip ES, Grublesky BT, Hussa EA, Visick KL 2005. A novel, conserved cluster of genes promotes symbiotic colonization and s54-dependent biofilm formation by *Vibrio fischeri*. *Mol Microbiol* 57: 1485-1498.

Received March 30, 2009
Accepted June 4, 2009
Associate Editor: Sv Boletzky

VIE ET MILIEU - LIFE AND ENVIRONMENT, 2009, 59 (3/4): 307-313

ULTRASTRUCTURE OF LIGHT ORGANS OF LOLIGINID SQUIDS AND THEIR BACTERIAL SYMBIONTS: A NOVEL MODEL SYSTEM FOR THE STUDY OF MARINE SYMBIOSES

R. C. GUERRERO-FERREIRA[1†], *M. K. NISHIGUCHI*[1*]

[1] *Department of Biology, New Mexico State University, Las Cruces, NM 88003-8001, USA*
[†] *Present address: Department of Pediatrics, Division of Pediatric Infectious Diseases, Emory University School of Medicine, Atlanta, GA 30322, USA*
* *Corresponding author: nish@nmsu.edu*

CEPHALOPODA
LIGHT ORGAN
VIBRIO
SYMBIOSIS
UROTEUTHIS
LOLIGINIDAE

ABSTRACT. – The class Cephalopoda (Phylum Mollusca), encompassing squids and octopuses, contains multiple species that are characterized by the presence of specialized organs known to emit light. These complex organs have a variety of morphological characteristics ranging from groups of simple, light-producing cells, to highly specialized organs (light organs) with cells surrounded by reflectors, lenses, light guides, color filters, and muscles. Bacteriogenic light organs have been well characterized in sepiolid squids, but a number of species in the family Loliginidae are also known to contain bacteriogenic light organs. Interest in loliginid light organ structure has recently arisen because of their potential as ecological niches for *Vibrio harveyi*, a pathogenic marine bacterium. This also implies the importance of loliginid light organs as reservoirs for *V. harveyi* persistence in the ocean. The present study utilized transmission and scanning electron microscopy to characterize the morphology of loliginid light organs and determined the location of bacterial symbiont cells within the tissue. It was determined that the rod-shaped loliginid symbionts lack flagella, as similarly observed in other light organ-associated bacteria. Also, the interaction of individual cells to light organ tissue is not as defined as reported for other squid-*Vibrio* systems. In addition, SEM observations show the presence of two pores leading to the bacterial chamber. Data presented here offer support for the hypothesis of environmental transfer of bacterial symbionts in loliginid squids.

INTRODUCTION

Several families within the class Cephalopoda (Mollusca) are characterized by the presence of light-producing organs, also known as photophores (Young 1972). Cephalopod photophores have a wide range of morphological features ranging from simple groups of photogenic cells, to organs with photogenic cells surrounded by reflectors, lenses, light guides, color filters, and muscles. These luminescent organs vary from very small, simple photophores (less than 0.2 mm in diameter) such as those of the mesopelagic squid *Abralia trigonura* (Young & Arnold 1982), to larger, more complex organs from squid of the family Sepiolidae (McFall-Ngai & Montgomery 1990). Complex photophores, also referred to as light organs, are found in the widely studied sepiolid squid *Euprymna scolopes* (McFall-Ngai & Montgomery 1990, Nishiguchi *et al*. 2004), as well as a number of other species in the families Sepiolidae and Loliginidae (Naef 1912a). Light organs found in sepiolids are oftentimes comparable in complexity to the compound eyes of many animals, including cephalopods themselves.

Light organs are able to readily adjust color, intensity, and angular distribution of light produced from within (Ferguson & Messenger 1991). In oceanic cephalopods, photophores emit intrinsic luminescence (autogenic) with light emanating from their own photocytes (Pringgenies & Jorgensen 1994). These cells are also able to emit different spectra of light (Herring *et al*. 1992). In contrast, photophores of most neritic cephalopods have extrinsic luminescence (bacteriogenic), with light produced by bacteria housed in a specialized light organ complex within the mantle cavity of their host (Naef 1912b, McFall-Ngai & Montgomery 1990, McFall-Ngai & Ruby 1991). Studies observing *E. scolopes* demonstrate that down-welling light intensity can be matched by the squids luminescence in a behavior termed counterillumination (Jones & Nishiguchi 2004). This confirms the hypothesis proposed by Young & Roper (1977) that production of light ventral to the squid mantle cavity is used for counter-shading down-welling light from the moon. Light organs of similar morphology are also found in oceanic teuthid squids (epipelagic and mesopelagic), such as *Chiroteuthis* spp., *Chiropsis* spp. and *Taningia* spp. However, according to Herring (1977), these anal light organs never house luminous bacteria.

Within the family Loliginidae, a number of species are known to posses bacteriogenic light organs (Alexeyev 1992). Loliginids are neritic, muscular squids, ranging from 3 to 100 cm in total body length. In contrast to sepiolids, loliginids have a gladius that extends along the mantle. They are also characterized by the existence of a

branchial canal in the gill (Vecchione 2008). Light organs in these squids are bilobed and situated on the ventral side of the ink sac near the anus. They are known to harbor luminous bacteria from the family Vibrionaceae, which have been characterized both by molecular methods (Guerrero-Ferreira & Nishiguchi 2009, 2007) and biochemical and morphological assays (Guerrero-Ferreira *et al*. in review). These studies have provided evidence confirming that the pathogenic marine bacterium *V. harveyi* colonizes light organs in loliginid squid, and posed questions regarding the role of loliginid light organs as a reservoir for *V. harveyi* persistence in the ocean. However, the morphology of loliginid light organs has not been well described in the scientific literature, most likely because their potential as *V. harveyi* reservoir was not entirely understood. Thus, the objective of this study was to describe the anatomy and ultrastructure of loliginid light organs using transmission electron microscopy and scanning electron microscopy techniques. In addition, the presence of symbiotic bacteria in the light organ would clarify whether loliginid light organs resembled those of sepiolid squids. The results of this study describe in detail the morphology of light organs in loliginid squids from the genus *Uroteuthis,* as well as the location of symbiotic bacteria existing inside the light organ complex.

MATERIALS AND METHODS

Sample collection and fixation: Specimens (~10 of each species) of the loliginid squids *Uroteuthis chinensis* and *Uroteuthis etheridge* were collected off the coasts of Cairns and Townsville (Australia) respectively, by trawl netting at daytime. Light organs were dissected from the mantle cavity and immediately fixed in 4 % paraformaldehyde and 0.5 % glutaraldehyde (Electron Microscopy Sciences, Hartfield, PA, USA) in 0.1 M marine phosphate buffer (MPB, 0.66 M NaCl, 0.2 M phosphate buffer, pH 7.2). After fixation, light organs were kept at 4°C until processed for light microscopy and transmission electron microscopy. Tissue samples were simultaneously collected for confirmation of squid species identity by cytochrome c oxidase gene (COI) sequencing using universal primers (Folmer 1994) under the following amplification conditions: initial denaturation at 94°C followed by 25 cycles of 94°C for 50 s, 50°C for 75 s, and 72°C for 90 s. Sequences were compared with the National Center for Biotechnology Information (NCBI) database using BLAST 2.2.11 (Basic Local Alignment Search Tool, NCBI, NLM, NIH, Bethesda, MD) to confirm species identity.

Sample preparation for scanning and transmission electron and light microscopy: Scanning electron micrographs were directly taken using a Hitachi TM-1000 tabletop microscope, without further sample preparation. For preparation of light organs for transmission electron microscopy and light microscopy, samples were rinsed with 0.1 M MPB and post-fixed with 0.5 % osmium tetroxide in 0.1 M MPB for 1 hour, stained with 1 % tannic acid (in dH_20_2) for 1.5 hours, rinsed with water and subsequently dehydrated using a 50-100 % ethyl alcohol gradient. Light organs were incubated for 1 hour in each ethanol solution and finally incubated in 100 % ethyl alcohol overnight. Samples were then infiltrated with 3:1 ethanol: Spurr's resin for 1 hour (Electron Microscopy Sciences, Hartfield, PA, USA), and then transferred to a 1:1 ethanol: Spurr's solution overnight. A 1:3 ethanol: Spurr's media was used to infiltrate the samples on the second day overnight, and then finally transferred to 100 % Spurr's resin overnight for the last infiltration. All infiltration steps were completed on a rotating table at room temperature (~22°C). Tissue was embedded in Spurr's resin in plastic molds. Resin was polymerized at 68°C overnight.

Thick Sectioning and imaging: Polymerized blocks were trimmed and sectioned using a Leica UC6 Ultramicrotome (Leica, Bannockburn, IL, USA). Thick sections (2 μm) were collected onto droplets of double distilled water on ethanol-washed glass slides. Slides were dried on a heat block for approximately five minutes to guarantee complete attachment

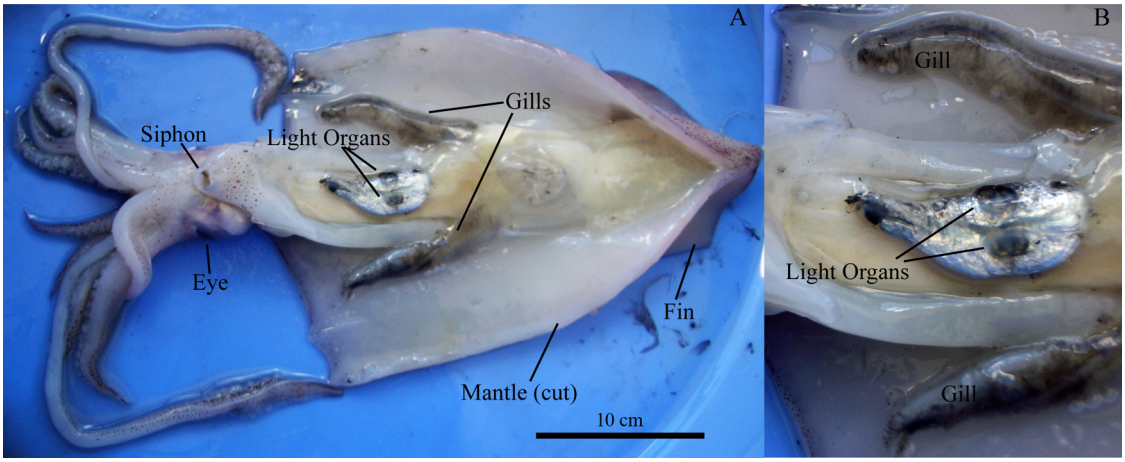


Fig. 1. – Ventral view of the mantle cavity of an adult *Uroteuthis etheridge*. A: Whole specimen B: closeup of the light organs. Dorsal mantle length: 30 cm.

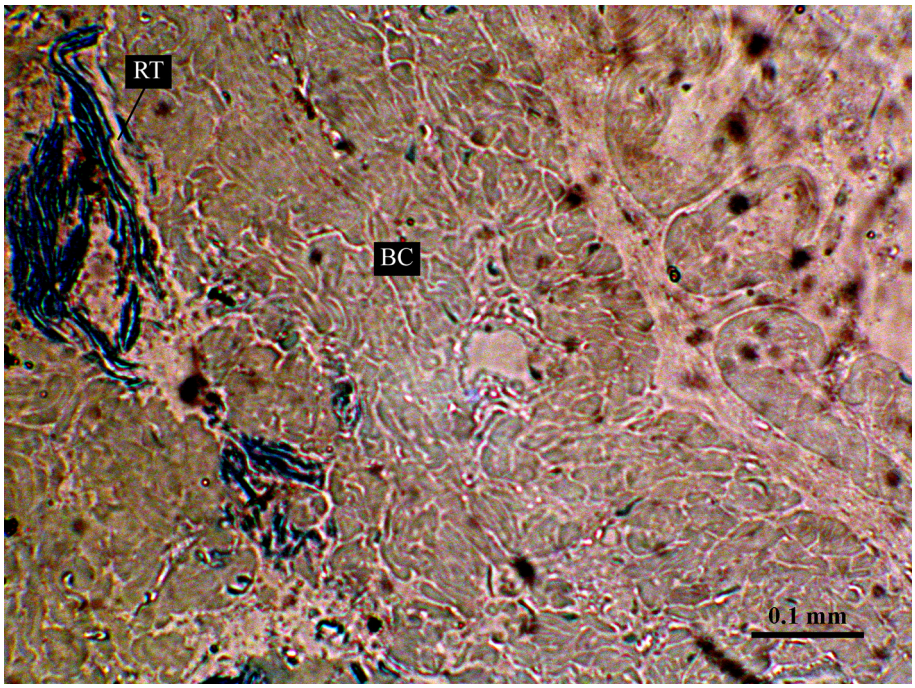


Fig. 2. – Light micrograph of a section showing the bacterial chamber (BC) of the loliginid squid *Uroteuthis etheridge*. Highly electron dense material corresponds to the reflector tissue (RT). Scale bar: 0.1 mm

of the sections to the slide. Subsequently, sections were broadly covered with 1 % toluidine blue epoxy stain (EMS, Hatfield, PA) for 1 minute. Sections were then extensively washed with double distilled water to ensure that all excess stain was removed from the slides. Samples were then viewed with a Nikon E800 upright epifluorescence microscope (Nikon Inc., Melville, NY).

Thin sectioning and imaging: Thin sections were collected onto a 300 mesh (squared) copper grid (Electron Microscopy Sciences, Hatfield, PA, USA) previously washed with a mild solution of hydrochloric acid. Subsequently, sections were positively stained with 2 % uranyl acetate (EMS, Hatfield, PA) for 10 min. and then with 1 % lead citrate (Polysciences Inc., Warrington, PA) for 10 min. Sections were washed with distilled water for one minute between stains. Stained samples were viewed through a Hitachi H7650 Transmission Electron Microscope (Hitachi, Schaumburg, IL, USA) at an accelerating voltage of 80kV.

RESULTS

Specimens were identified through sequencing of the cytochrome *c* oxidase subunit one gene (COI) and when compared with the NCBI database using BLAST 2.2.11, were confirmed as two species of the family Loliginidae, *Uroteuthis chinensis* and *Uroteuthis etheridge*.

Light organs in these species of squid are located on the ventral face of the ink sac. With respect to the size of *U. chinensis* and *U. etheridge* specimens (approximately 30 cm of dorsal mantle length), the light organs are considerably smaller when compared to the homologous structures in members of the family Sepiolidae, such as the Hawaiian bobtail squid *Euprymna scolopes* (McFall-Ngai & Montgomery 1990). Ventral dissection of the squid *U. etheridge* reveals the relatively small, bilobed light organ situated within the mantle cavity (Fig. 1).

A histological cross-section of one lobe of the light organ reveals the existence of labyrinths of crypts where bacteria are housed (Fig. 2). Light microscopy also illustrates the location of the reflector tissue (RT) with respect to the light organ crypts within the bacterial chamber (BC). These observations confirmed results made by Pringgenies & Jorgensen (1994), which illustrated a layer of tissue representing a reflector that was immediately in contact with the bacterial chamber (Fig. 2). Their results were also similar to earlier descriptions of light organ morphology in sepiolid squids (summarized in Nyholm & Nishiguchi 2008), where bacterial crypts are also surrounded by reflector tissue (dorsal) as well as a lens (ventral).

By completing transmission electron microscopy of the reflector tissue area, it was possible to confirm the presence of flat, structural platelets or lamellae organized in alternate layers (Fig. 3). Secreted ink granules from the ink sac are also clearly visible in the micrograph. The lamellae have a constant width of approximately 100 nm, but their length varies considerably. These structures have been studied in *E. scolopes* and have been found to contain proteins called reflectins (Crookes *et al*. 2004) which makes them an exception to most aquatic animals that contain reflectors made of crystals of purinic nucleotides, specially guanine and hypoxanthine (Denton & Land 1971).

Uroteuthis etheridge light organs (Fig. 1) are known to house luminescent bacteria of the family Vibrionaceae

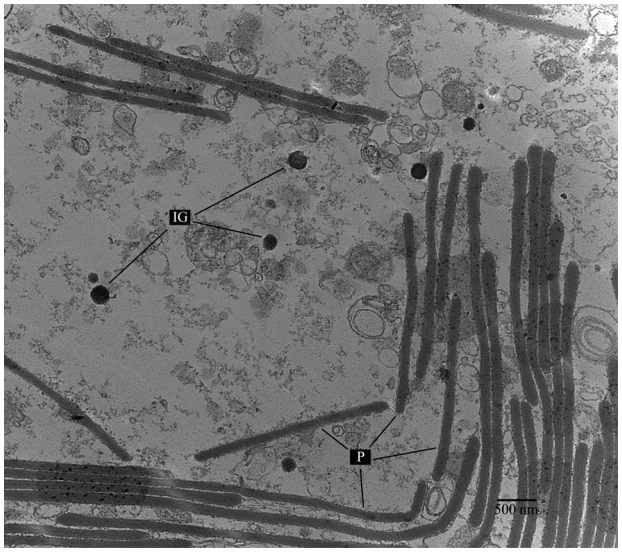


Fig. 3. – TEM image of a region of *U. etheridge* light organs. Ink granules (IG) and platelets (P) are shown in the field. Scale bar: 500 nm.

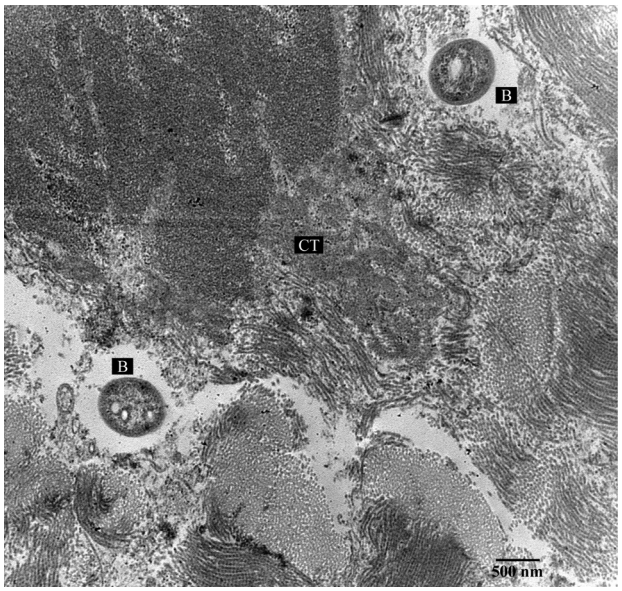


Fig. 4 – *Vibrio* bacteria (B) associated to *Uroteuthis chinensis* squid tissue (CT for connective tissue). Cells are located inside crypt spaces. Scale bar: 500 nm.

(Guerrero-Ferreira & Nishiguchi 2007). Because recent studies in our lab have demonstrated that at least one member of the bacterial symbiotic population within these organs is the marine pathogen *Vibrio harveyi*, it was of great interest to structurally describe the state of the interaction between the two partners. A particular goal of this study was to determine where in the light organ crypts these bacterial symbionts reside, as well as to gather information regarding the symbiotic morphology of the bacteria, particularly the presence of bacterial cell appendages which might facilitate adhesion to squid tissues.

Figure 4 portrays the location of the bacterial cells

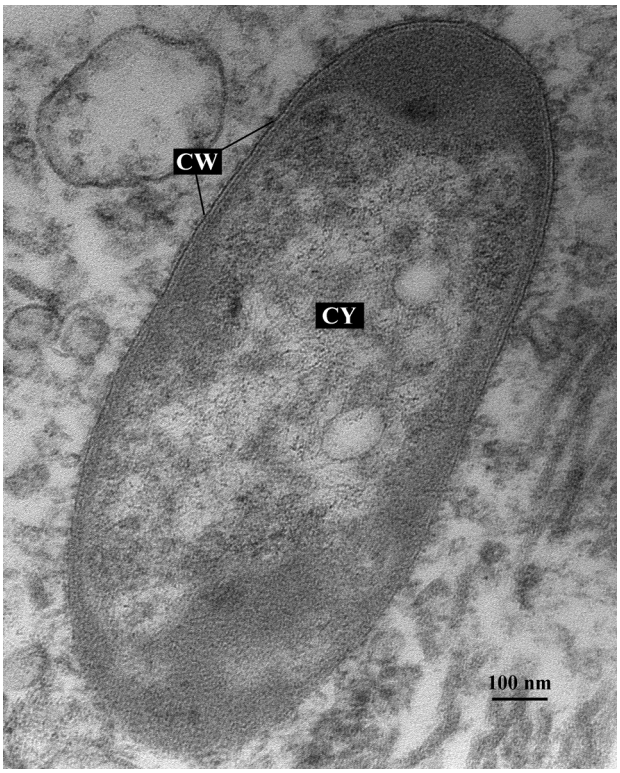


Fig. 5. – Bacterial symbiont within the crypt region of *Uroteuthis etheridge* light organ. CW: Cell wall. CY: Cytoplasm. Scale bar: 100 nm.

when associated with *U. chinensis* squid tissue. The most noticeable feature of the symbionts is the absence of flagella. Even though bacteria from the family Vibrionaceae are known to possess either monotrichous or peritrichous flagella (Baumann 1984), bacterial cells in *U. chinensis* light organ lack flagella. Previous research studying the association between *E. scolopes* and *V. fischeri* has also shown that loss of flagella is a characteristic process that occurs upon establishment of the association (Ruby & Asato 1993).

The morphology of the bacteria colonizing light organs was the characteristic rod shape of members in the family Vibrionaceae. Figure 5 illustrates one of these symbionts and clearly defines the cell wall (CW) and cytoplasm (CY) of the bacterium. In general, transmission electron microscopy of the bacteria reveals they have the ovoid or oblong shape, characteristic of vibrios. Their sizes vary, with smaller bacteria measuring approximately 0.5 μm in length, whereas the largest having dimensions of up to 3.0 μm in length. Symbiotic bacteria residing in the light organ exhibit a dense cytoplasm, with the presence of vacuoles approximately 100 nm in diameter (Fig. 6). In addition, cell appendage pili are present that might allow for bacterial attachment to squid tissue, as it is known to occur in *V. fischeri* bacteria colonizing *E. scolopes* (Nair and Nishiguchi 2009, McFall-Ngai & Ruby 1991).

Scanning electron microscopy was also used to determine the presence of pores that are necessary for initial

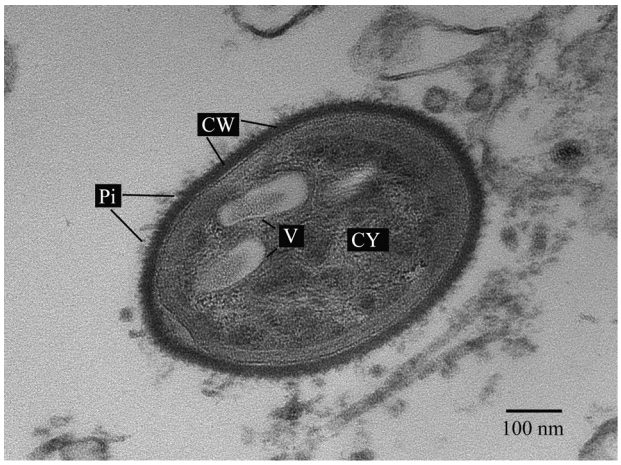


Fig. 6. – Bacterial cell associated to the squid *Uroteuthis etheridge* with pili (P) and vacuoles (V) present. Scale bar: 100 nm.

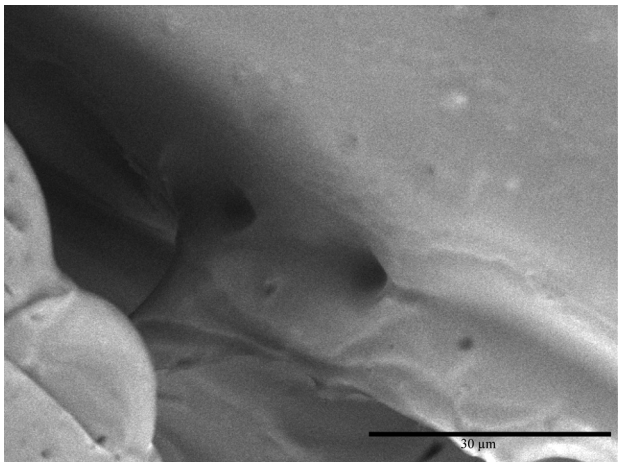


Fig. 7. – Scanning electron micrograph of *Uroteuthis chinensis* light organ showing two pores leading into the bacterial chamber. Scale bar: 30 μm.

colonization by symbionts infecting the crypt region of the light organ. In the *E. scolopes* light organ complex, three pores exist in the juvenile squid after hatching. Upon successful colonization by their symbiotic partner *V. fischeri*, developmental changes occur leading to the formation of a single pore that allows for the dial cycle of bacterial venting that occurs throughout the life of the mollusc (Foster *et al*. 2002). Interestingly, SEM illustrates that *U. chinensis* has a light organ with two pores leading into the bacterial chamber in the adult squid (Fig. 7). The size of these pores is approximately 5 μm, which is similar to the size reported by Montgomery and McFall-Ngai in sepiolid squids (1993). Previous results have shown that pore number varies among sepiolid squids; the genus *Sepiola* has 4 pores on each light organ lobe prior to infection by their *Vibrio* bacteria (Foster *et al*. 2002). The functional advantage for the occurrence of two pores in adult *U. chinensis* is still unclear, but their presence may be required for similar venting behaviors to those found occurring in adult sepiolids.

DISCUSSION

This study provides evidence that physical interactions between bacterial cells and loliginid squid light organs is somewhat sporadic. Electron micrographs illustrate an infrequent distribution of bacterial cells with respect to each other *(i.e.*, no aggregation) and with the squid host light organ tissue. This is in agreement to the description given by Pringgenies and Jorgensen (1994). According to their study, scarcity of bacterial cells was due to the crypts in the light organ being used exclusively for expulsion of excretion products from both symbiont and host. However, it is currently understood that the symbiont community in sepiolid light organs has a diurnal fluctuation (McFall-Ngai & Ruby 1991, Ruby & McFall-Ngai 1992), which may account for the low density of bacteria observed in the micrographs presented here. In addition, absence of a strong physical connection between host cells and symbionts may account for the lack of species-specificity found in loliginid light organ symbiosis and may explain the diversity of bacterial genotypes found in their crypts. Recent studies have established that more than one species of Vibrionaceae bacteria colonize light organs of squids in the family Loliginidae (Guerrero-Ferreira & Nishiguchi 2007). Further studies would need to address whether light organ venting occurs with the onset of dawn in loliginid squids, and if the lack of pili corresponds to a less specific interaction on the cellular level.

This is also true in the case of light organs in the leiognathid fish *Nuchequula nuchalis,* where *Vibrio harveyi* was recently found as a member of the bacterial community (Dunlap *et al*. 2008). In this species, more than two distinct ecological variants of *Photobacterium leiognathi* were also found colonizing the light organ tissue. Transmission electron micrographs of *N. nuchalis* show a similar scattered distribution of symbionts within the light organ tubules, which supports the aforementioned idea of a relationship between lack of specificity and limited physical attachment to host tissue.

In addition, vacuole-like, refractive granules were observed within the symbionts in quantities varying from one to five. These granules have been previously proposed to be poly-β-hydroxybutyrate (PHB) (Dunlap *et al*. 2008), a compound that is produced in *V. harveyi* cells at high density in the presence of endogenous *lux* autoinducer, *N*-(3-D-hydroxybutanoyl) homoserine lactone (Sun *et al*. 1994). This restricts the production of PHB to *V. harveyi* strains living under confined conditions (such

as the light organ of loliginid squids) which might account for the classification of *V. harveyi* cells as PHB negative (Baumann 1981). Because of this, it is possible that PHB containing bacteria living in conditions such as those of fish and squid light organs were misclassified as species different than *V. harveyi*, as observed by Miyamoto *et al*. (1998).

Tissue organization in loliginid light organs observed through TEM resembles the structure of sepiolid light organs with reflector and lens tissue surrounding the chambers where the bacterial symbionts are located (McFall-Ngai & Montgomery 1990). In sepiolid squids, the arrangement of these various tissue types allows the animal to utilize light produced by the bacteria in a behavior known as counterillumination (Jones & Nishiguchi 2004). Our results present morphological evidence suggesting that *Uroteuthis* spp. use their association with Vibrionaceae bacteria for the same purpose. Further behavioral studies investigating light production under controlled down welling light intensity will contribute to our understanding of the evolutionary significance of this association for loliginids. Additionally, temporal studies with light organs from squids collected at several times of the day would help determine whether low bacterial densities within light organ crypts are due to diurnal variation in bacterial populations in loliginid squids.

Moreover, we can argue that association of loliginid squids with their bacterial symbionts is currently in an ancestral state. Nishiguchi *et al*. (2004) presented a hypothesis for the origin of bacteriogenic light organs from the accessory nidamental gland (ANG) complex of sepiolid squids. This organ complex has more than 20 strains of bacteria present that are acquired from the surrounding seawater environment (Kaufman *et al*. 1998). If ANG in loliginid squids contributes to the symbiont population in loliginid light organs, this may explain the diversity of bacteria found in these complexes and the lack of a strong physical association between partners (Guerrero-Ferreira & Nishiguchi 2007). Strong selection by the host for particular strains or species of *Vibrio* bacteria will need to occur in order to accommodate strain specificity and cospeciation (Nishiguchi *et al*. 1998, Nishiguchi 2002, Nyholm & Nishiguchi 2008). Likewise, these environmentally transmitted symbionts that colonize loliginid light organs will need to be successful in their free-living stages in order to out-compete other bacteria for their niche in this mutualism. Trade-offs between these two life history strategies appear to be the ultimate driving forces for the evolution of this dynamic and evolutionary significant symbiosis; whether multiple or single strains can coexist needs to be taken into careful consideration when contemplating the origin of bacteriogenic light organs (Nishiguchi *et al*. 2008, Nyholm & Nishiguchi 2008).

ACKNOWLEDGEMENTS. - The authors thank R Al-Katib from the NMSU Electron Microscopy Lab for his assistance with this project. Field work for RGF was in part funded by a travel grant from the Associated Students of New Mexico State University. This work was supported by NSF-IOS 0744498 and NIH-NIAID 1SC1AI081659-01 to MKN.

REFERENCES

Alexeyev DO 1992. The systematic position of bioluminescent squids of the family Loliginidae (Cephalopoda: Myopsida). *Zool Zhurnal* 71: 12-23.

Baumann P, Baumann L 1981. The marine Gram-negative eubacteria. *In* Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG eds. The Prokaryotes, a Handbook on Habitats, Isolation, and Identification of Bacteria. Springer Verlag, New York: 1352-1394.

Baumann P, Schubert RHW 1984. Family II. Vibrionaceae. *In* Krieg RN, Holt JG eds. Bergy's Manual of Systematic Bacteriology, Vol 1, The Williams and Wilkins Co, Baltimore: 516-517.

Crookes WJ, Ding LL, Huang QL, Kimbell JR, Horwitz J, McFall-Ngai M J2004. Reflectins: The unusual proteins of squid reflective tissues. *Science* 303: 235-238.

Denton EJ, Land MF 1971. Mechanism of reflexion in silvery layers of fish and cephalopods. *Proc R Soc Lond B Biol Sci* 178(50): 43-61.

Dunlap PV, Davis KM, Tomiyama S, Fujino M, Fukui A 2008. Developmental and microbiological analysis of the inception of bioluminescent symbiosis in the marine fish *Nuchequula nuchalis* (Perciformes: Leiognathidae). *Appl Environ Microbiol* 74(24): 7471-7481.

Ferguson GP, Messenger JB 1991. A countershading reflex in cephalopods. *Proc R Soc B* 243(1306): 63-67.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3(5): 294-299.

Foster JS, Boletzky Sv, McFall-Ngai MJ 2002. A comparison of the light organ development of *Sepiola robusta* Naef and *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). *Bull Mar Sci* 70(1): 141-153.

Guerrero-Ferreira RC, Gorman C, Willie S, Nishiguchi MK. Identification of *Vibrio harveyi* bacterial isolates from light organs of loliginid squids (Mollusca: Cephalopoda) from Thailand. *Microb Ecol* (in review).

Guerrero-Ferreira RC, Nishiguchi MK 2009. Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. *Environ Microbiol Rep* DOI:10.1111/j.1758-2229.2009.00077.

Guerrero-Ferreira RC, Nishiguchi MK 2007. Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis*, *Loliolus* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* 23(5): 497-506.

Herring PJ 1977. Luminescence in cephalopods and fish. *Symp Zool Soc Lond* 38: 127-159.

Herring PJ, Widder EA, Haddock SHD 1992. Correlation of bioluminescence emissions with ventral photophores in the mesopelagic squid *Abralia veranyi* (Cephalopoda: Enoploteuthidae). *Mar Biol* 112: 293-298.

Jones BW, Nishiguchi MK 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* 144: 1151-1155.

Kaufman MR, Ikeda Y, Patton C, van Dykhuizen G, Epel D 1998. Bacterial symbionts colonize the accessory nidamental gland of the squid *Loligo opalescens* via horizontal transmission. *Biol Bull* 194: 36-43.

McFall-Ngai MJ, Montgomery MK 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). *Biol Bull* 179: 332-339.

McFall-Ngai MJ, Ruby EG 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial symbiosis. *Science* 254: 1491-1494.

Miyamoto CM, Sun W, Meighen EA 1998. The LuxR regulator protein controls synthesis of polyhydroxybutyrate in *Vibrio harveyi*. *Biochim Biophys Acta (BBA) - Protein Struct Mol Enzym* 1384(2): 356-364.

Montgomery MK, McFall-Ngai MJ 1993. Embryonic development of the light organ of the Sepiolid squid *Euprymna scolopes* Berry. *Biol Bull* 184: 296-308.

Naef A 1912a. Teuthologische Notizen. 2. Gattungen der Sepioliden. *Zool Anz* 39: 244-248.

Naef A 1912b. Teuthologische notizen. 3. Die Arten der Gattungen *Sepiola* und *Sepietta*. *Zool Anz* 39: 262-271.

Nair VS, Nishiguchi MK 2009. Biological properties (*in vitro*) exhibited by free-living and symbiotic *Vibrio* isolates. *Vie Milieu* 59(3/4): 277-285.

Nishiguchi MK, DeVinney R, Hirsch AM, Riley M, Mansky L, Vendatum G 2008. Perspective: Evolution of virulence: Deciphering the mechanisms between pathogenic and benign symbioses. *Vie Milieu* 58: 87-106.

Nishiguchi MK 2002. The use of physiological data to corroborate cospeciation events in symbiosis. *In* DeSalle R, Wheeler W, Giribet G eds. Molecular Systematics and Evolution: Theory and Practice. Birkhäuser, Basel, Germany: 237-246.

Nishiguchi MK, Lopez JE, Boletzky Sv 2004. Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. *Evol Dev* 6(1): 41-49.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-*Vibrio* symbioses. *Appl Environ Microbiol* 64: 3209-3213.

Nyholm SV, Nishiguchi MK 2008. The evolutionary ecology of a sepiolid squid-*Vibrio* association: From cell to environment. *Vie Milieu* 58: 175-184.

Pringgenies D, Jorgensen JM 1994. Morphology of the luminous organ of the squid *Loligo duvauceli* d'Orbigny, 1839. *Acta Zool-Stockholm* 75(4): 305-309.

Ruby EG, Asato LM 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Arch Microbiol* 159: 160-167.

Ruby EG, McFall-Ngai MJ 1992. A squid that glows in the night: Development of an animal-bacterial mutualism. *J Bacteriol* 174(15): 4865-4870.

Sun W, Cao JG, Teng K, Meighen EA 1994. Biosynthesis of poly-3-hydroxybutyrate in the luminescent bacterium, *Vibrio harveyi*, and regulation by the lux autoinducer, N-(3-hydroxybutanoyl)homoserine lactone. *J Biol Chem* 269(32): 20785-20790.

Vecchione M 2008. Loliginidae Lesueur, 1821. Version 04 March 2008. *In* The Tree of Life Web Project (http://tolweb.org).

Young RE 1972. Function of extra-ocular photoreceptors in bathypelagic cephalopods. *Deep-Sea Res* 19: 651-660.

Young RE, Arnold JM 1982. The functional morphology of a ventral photophore from the mesopelagic squid, *Abralia trigonura*. *Malacologia* 23: 135-183.

Young RE, Roper CFE 1977. Intensity regulation of bioluminescence during countershading in living midwater animals. *Fish Bull* 75: 239-252.

Received May 11, 2009
Accepted June 4, 2009
Associate Editor: S v Boletzky

In: J. Seckbach and M. Grube (eds), *Symbiosis and stress; Joint ventures in biology*. Springer, New York, NY., pp. 409-426 (2010).

PHYSIOLOGICAL RESPONSES TO STRESS IN THE VIBRIONACEAE

WILLIAM SOTO[1], C. PHOEBE LOSTROH[2], AND MICHELE K. NISHIGUCHI[1]

[1]*New Mexico State University, Department of Biology,Box 30001, MSC 3AF, Las Cruces, NM, 88003-8001 USA;* [2]*Colorado College, Department of Biology 14 E Cache La Poudre Avenue, Colorado Springs, CO, 80903 USA*

1. The Vibrionaceae

1.1. A GENERAL DESCRIPTION

The family Vibrionaceae (Domain Bacteria, Phylum Proteobacteria, Class Gammaproteobacteria) is comprised of mostly motile gram-negative chemoorganotrophs, possessing at least one polar flagellum (Farmer III and Janda, 2005; Thompson and Swings, 2006). Vibrios are facultative anaerobes, having both respiratory and fermentative metabolisms, and the mol% G+C of the DNA is 38-51% (Farmer III and Janda, 2005). Cells are usually 1 μm in width and 2-3 μm in length, and most are oxidase positive. The vast majority of vibrios require Na^+ for growth and survival, usually 0.5-3% NaCl for optimum growth. Additionally, most species are susceptible to the vibriostatic agent 0/129 (Thompson and Swings, 2006). In recent years, a two-chromosome configuration, one large and the other small (both circular), has been discovered to be a universal feature for all members of the Vibrionaceae (Iida and Kurokawa, 2006). The Vibrionaceae are ubiquitously distributed throughout aquatic habitats, freshwater and marine waters (Madigan and Martinko, 2006), including rivers, estuaries, lakes, coastal and pelagic oceanic waters, the deep sea, and saltern ponds (Urakawa and Rivera, 2006). Although as many as eight genera have been assigned to the *Vibrionaceae*, the two most specious are *Vibrio* and *Photobacterium* (Thompson and Swings, 2006). A third genus, *Salinivibrio* is worthy of mention due to its unusual ability to grow in a wide range of salinity (0-20% NaCl; Ventosa, 2005) and temperature (5-50°C; Bartlett, 2006) (refer to Table 1.).

Numerous species are pathogenic and cause disease in aquatic animals and humans (Farmer III *et al*., 2005), *Vibrio cholerae* being the most notorious example as the causative agent of cholera (Colwell, 2006). *V. vulnificus* and *V. parahaemolyticus* can also cause severe illness in humans as a result of consuming contaminated seafood (Hulsmann *et al*., 2003; Wong and Wang, 2004). Furthermore, every year *V. harveyi* (Owens and Busico-Salcedo, 2006), *V. anguillarum* (Miyamoto and Eguchi, 1997; Crosa *et al*., 2006), and *V. parahaemolyticus* (Austin, 2006) cause substantial economic losses to the aquaculture industry worldwide.

Study of the Vibrionaceae also has applications in ecoysystem health and conservation biology, especially in light of increasing contemporary concerns about human-induced global climate change. It is already clear that temperature is an abiotic factor that is critical for numerous vibrio symbioses (as discussed below), and it is possible that anthropogenic increases in the prevailing ocean temperature could have profound effects on ecosystems mediated partly through alterations in these symbioses. For example, *V. shiloi* is a pathogen of corals that causes coral bleaching at warmer ocean temperatures such as those expected to prevail in the future (Banin *et al*., 2003). For these reasons, the Vibrionaceae has galvanized tremendous basic and applied research. Increasing interest in recent years in the utilization of the genes responsible for light production from the bioluminescent bacteria *V. fischeri* for developing bioreporter monitoring and biosensor technologies illustrates this (Ripp *et al*., 2006).

1.2. SYMBIOSES WITHIN VIBRIONACEAE

Vibrio species not only occur as free-living members of the bacterioplankton but also regularly form symbioses—relationships between two or more organisms that encompass parasitisms, mutualisms, and commensalisms—with other aquatic organisms, including fish, invertebrates, algae, and other microorganisms (Nishiguchi and Nair, 2003; Meibom *et al.*, 2005). Within marine animals, *Vibrio* species are commonly found in the digestive tract and on their surfaces, including skin and chitinous exoskeletons (Urakawa and Rivera, 2006). Host-associated vibrios are provided with a microenvironment rich in nutrients and organic molecules compared to the surrounding seawater (Urakawa and Rivera, 2006). Hence, the vibrio population within or on the host is often several orders of magnitude higher than in the oceanic water column (10^2 cells/ml). *V. cholerae* reach a population level as high as 10^4-10^6 cells/copepod, while *V. halioticoli* can reach a population size at 10^6-10^9 cells/g of fresh gut in abalones (*Haliotis discus hannai*; Sawabe *et al*., 1995). Although some vibrios are pathogenic towards their hosts, numerous *Vibrio* species are part of the normal microflora of animals living in the ocean, such as oysters (Olafsen *et al*., 1993), blue crabs (Davis and Sizemore, 1982), sharks (Grimes *et al*., 1985), and hydroids (Stabili *et al*., 2006). The metabolic, physiological, and genetic traits permitting the Vibrionaceae to attach, colonize, proliferate in, and circumvent the defense mechanisms of their hosts to cause disease are undoubtedly homologous to those responsible for the establishment of mutualisms. These traits have a common and ancient evolutionary origin, giving rise to many different independent lineages (Nishiguchi and Nair, 2003).

In some instances, vibrios are intimate symbionts providing such an essential role that their hosts would be unable to survive in nature without them (Douglas, 2002). These roles include protection from pathogens, enhanced metabolic function, elevated environmental tolerance, or nutrient acquisition. The symbiosis between *V. halioticoli* and abalones (*Haliotis*) is one such example. In this case, the bacterial partner serves in alginate degradation, a brown algal polysaccharide the abalone consumes while grazing, and provides the gastropod host with an important energy source (Sawabe, 2006). Another example is *V. fischeri*, which is a bioluminescent symbiont of sepiolid squids and monocentrid fishes, and benefits these animals through a behavior termed counterillumination, allowing the hosts to conceal themselves from potential predators or prey (Jones and Nishiguchi, 2004). Considering host interactions partaken by vibrio

bacteria encompass the entire symbiosis continuum, from pathogen to indispensable microbial mutualist (Nishiguchi, 2001; Nishiguchi and Jones, 2004), a paradigm shift is emerging where some *Vibrio* species are considered beneficial and may have potential in the development of probiotics for commercially important aquaculture animals (Verschuere *et al*., 2000).

2. Stress regulation

2.1. GENERAL DESCRIPTION

Despite the fact that biologists uniformly recognize some environments as stressful, attempts to unequivocally define or quantify stress are difficult (Lenski and Bennett, 1993). The *Oxford Dictionary of Ecology* defines stress as, "A physiological condition produced by excessive pressures that are detrimental to an organism" (Allaby 2005), while the *Dictionary of Ecology, Evolution, and Systematics* states a stress is "…Any environmental factor that restricts growth and reproduction of an organism or population or causes a potentially adverse change in an organism or biological system; any factor acting to disturb the equilibrium of a system" (Lincoln *et al*., 1998). For many evolutionary biologists and ecologists, a more satisfying definition is one treating stress as any environmental factor (biotic or abiotic) reducing fitness (Lenski and Bennett, 1993).

"Stress," broadly considered, must also include any biotic or abiotic factors that fluctuate, and thus require organisms to adapt to them physiologically in order to survive. Most bacteria encounter such stressful changes in the environment, including the Vibrionaceae. They grow and survive in a multitude of habitats while possessing various lifestyles: aquatic sediments, fresh and brackish waters, oceans, symbionts of host organisms, saprophytes on detritus, and as free-living cells (Nishiguchi and Jones, 2004; Urakawa and Rivera, 2006; Dunlap *et al*., 2007). These different environments and lifestyles should not be viewed as static and permanent but rather as transient and cyclical (Urakawa and Rivera, 2006; Dunlap *et al*., 2007), where microbes migrate between each habitat while encountering stressful conditions (McDougald and Kjelleberg, 2006). These different habitats vary in a myriad of abiotic and biotic factors; consequently, the Vibrionaceae have evolved diverse physiological responses to stress and variable environments.

Previous research has shown fluctuating environments and stressors (e.g., oxygen and reactive forms, extreme salinities/temperatures) have important influences in symbiosis (Xu *et al*., 2004). For instance, a temperature downshift from 26°C to 18°C caused dramatic changes in the microbiota of the gastrointestinal tract in red hybrid tilapia, with tremendous proliferation of *Vibrio* spp. and a concomitant decrease in *Flavobacterium* (LeaMaster *et al*., 1997). Vibrio bacteria have also been shown to be distributed differentially both within host species located in different habitats, as well as in various seasons throughout the water column (Jones *et al*, 2007; Jones *et al*., 2006). The effect of fluctuating environments on the growth of non-host associated vibrios has been investigated less, but there are still some intriguing recent findings. For example, saline stress has been shown to affect the quality of organic carbon produced by vibrios

living in simple, microbial loop foodwebs. This phenomenon affects the quality of carbon available to other trophic levels (Odic *et al*., 2007).

The purpose of this review is to discuss the physiological responses of non-cholera vibrios to stress, especially to stressors likely encountered during symbiosis or during transitions from one host or lifestyle to another. We will also draw connections, wherever possible, among work that addresses vibrios from evolutionary, ecological, and molecular physiological points of view. We refer readers interested in *V. cholerae* to another recent review (Prouty and Klose, 2006).

2.2. TEMPERATURE

Vibrios encounter a broad range of temperatures, from those prevailing in marine habitats, to the higher temperatures tolerated by vibrios that can infect humans. Temperature is a significant determinant in shaping ecological associations of vibrios with countless host organisms, including eels (Amaro *et al*., 1995; Marco-Noales *et al*., 1999), squid (Jones *et al*., 2006; Nishiguchi, 2000), sea bream (Bordas *et al*., 1996), oysters (Kaspar and Tamplin, 1993), and coral (Rosenberg *et al*., 2007). For example, *V. shiloi* and *V. coralliilyticus*, both pathogens of coral, produce virulence factors implicated in bleaching and killing their hosts. In both cases, the production of these virulence factors is strongly regulated by temperature. At winter temperatures (16-20°C), virulence factors are not produced, while summer temperatures (25-30°C) induce virulence factor production (Rosenberg *et al*., 2007).

Temperature is a critical abiotic factor affecting other pathogenic symbioses, too. For example, chemotaxis is important for virulence of the fish pathogen *V. anguillarum*, and it is strongly affected by temperature. *V. anguillarum* is most robustly chemotactic at 25°C, and the chemotactic response diminishes in both cooler (5°C, 15°C) and warmer (37°C) conditions (Larsen *et al*., 2004). The stationary-phase associated sigma factor encoded by *rpoS* is required for *V. vulnificus* to survive heat shock (Hulsmann *et al*., 2003). An important virulence factor in *V. vulnificus* is capsular polysaccharide (CPS); CPS production appears to be controlled by a phase variation mechanism that can be detected by examining colony phenotype. Encapsulated cells make opaque colonies, while *cps*- cells make translucent colonies. Conversion from CPS+ to *cps*- (from opaque to translucent) is affected by temperature, as increasing the temperature from 23°C to 37°C increased switching for several different isolates (Hilton *et al*., 2006).

Since Vibrionaceae are aquatic microorganisms residing mostly within oceans, which are the largest cold environment on earth (Urakawa and Rivera, 2006) making up 71% of the earth's surface (Atlas and Bartha, 1998), some members of this group have been extensively selected to thrive in cold temperatures (Bartlett, 2006). Thus, although clinical and human pathogenic Vibrionaceae are mesophilic and capable of growth at ≥37°C, some members of this bacterial family's ancient lineage have adapted to low temperatures. Examples include *Photobacterium profundum*, *V. logei*, *V. wodanis*, and *V. salmoncida*. *Photobacterium* spp. have been more frequently observed to be the more prevalent member of the Vibrionaceae in the cold deep-sea, whereas the genus *Vibrio* is more common in cold ocean surfaces. These species are capable of growth at ≤5°C. Vibrios such as *V. diabolicus*, isolated from a deep-sea hydrothermal vent annelid

Alvinella pompejana, are heat tolerant, but no evidence exists that any member of the Vibrionaceae are thermophilic (Urakawa and Rivera, 2006).

Cold shock responses have been studied in *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* (McGovern and Oliver, 1995; Bryan *et al*., 1999; Datta and Bhadra, 2003; Huels *et al*., 2003; Lin *et al*., 2004;). Within the later two species, cold shock response increases survival at lower temperatures by a translation-dependent process. Research in this area is particularly important when considering applications of low temperature usage for food storage of shellfish (Bryan *et al*., 1999). As is generally true of stress responses in microorganisms, cold shock response involves changes in gene expression. Expression of cold shock proteins (CSPs) reach maximal levels during acclimation and includes the up-regulation of several proteins, including small homologous peptides 65-70 residues long in the CspA family (Ermolenko and Makhatadze, 2002). Most studies of the CspA family have been studied in greater detail in bacteria such as *E. coli* and *B. subtilis*. Proteins in this family have five antiparallel β strands that form a β barrel, creating a characteristic cold-shock protein domain well conserved throughout all three domains of life. CSPs often bind single-stranded mRNA and DNA, and are believed to assist bacteria in coping with unstable secondary structures at lower temperatures during ribosomal translation, mRNA degradation, termination of transcription, and perhaps nucleoid condensation, thereupon giving CSPs the function of nucleic acid chaperones. Additionally, there may also be a suppression of protein synthesis to prevent miscoding of polypeptides until the cold shock response is initiated (Ermolenko and Makhatadze, 2002).

To maintain functional membrane fluidity with decreasing temperature, vibrios are known to increase the unsaturation of fatty acids comprising their cell membranes. Adaptation to a fully psychrophilic lifestyle regularly, but not always, involves a decrease in enthalpy-driven interactions for the catalytic activity of enzymes, increasing the number of functional conformations permitted for enzyme-substrate complexes (Bartlett, 2006). For instance, the amino acid residues of psychrophilic enzymes within the cytosol display additional hydrophilic associations with the solvent, while simultaneously lessening internal hydrophobic interactions. This yields enzymes that are less condensed relative to mesophilic counterparts, which can result by increasing the α-helix and decreasing the β-sheet character of the secondary structure.

2.3. pH STRESS

pH stress is ecologically and evolutionarily significant because symbiotic vibrios include some gastrointestinal pathogens that must somehow survive the acidic challenge encountered in the digestive environment. Recently, molecular mechanisms of survival in the face of pH stress have been studied most intensively in the species *V. vulnificus*. This microorganism is an opportunistic pathogen of humans, acquired by ingesting contaminated seafood. There appears to be multiple overlapping signal transduction networks that together sense and respond to acid challenge. For example, the alternative sigma factor encoded by the *rpoS* gene is required for *V. vulnificus* to survive acid stress (pH <5) in both stationary and exponential phases of microbial growth (Hulsmann *et al*., 2003; Park *et al*., 2004). Other regulatory proteins such as CadC (accessory protein for the Cd^{2+} efflux ATPase CadA), SoxR (superoxide response regulator), and Fur (ferric

uptake regulator protein) are also needed for survival of acid stress. The CadC regulator in *V. vulnificus* induces *cadAB* expression, leading to the production of CadA (lysine decarboxylase) and CadB (lysine-cadaverine antiporter). Lysine decarboxylation is one step toward the production of cadaverine, which accumulates in the extracellular space during an acid stress response. The AphB transcription factor also enhances expression of *cadAB* under stressful acidic conditions, by directly activating a promoter that drives *cadC* production (Rhee *et al*., 2002; 2006).

The physiological response that leads to survival of acid stress is connected to the response that protects *V. vulnificus* from superoxide stress. The SoxR regulator, already known to regulate genes important for surviving oxidative damaging agents, also induces the *cadAB* operon (Kim *et al*., 2006). This induction does not require the CadC regulator, and cells lacking CadA are more sensitive to oxidizing agents than wild-type cells. Furthermore, simply increasing the amount of *cadAB* expression by supplying these genes on a multicopy plasmid is sufficient to reduce the induction of a superoxide dismutase that the cells would normally produce upon challenge with the oxidizing agent methyl viologen. Together, these results suggest that extracellular cadaverine not only neutralizes the local environment surrounding cells in acidic medium but also scavenges superoxide radicals (Kim *et al*., 2006).

Yet another connection between survival of acid and oxidative stresses in *V. vulnificus* was revealed with the discovery that cells grown to exponential phase and then subsequently exposed to acidic (pH=5.0) conditions induce the expression of *sodA*, a locus encoding a manganese-containing superoxide dismutase (MnSOD; Kim *et al*., 2005). MnSOD is positively regulated by SoxR and negatively regulated by Fur, but is not transcriptionally regulated by *rpoS*. Regulation by SoxR is likely indirect, while regulation by Fur is direct via binding to the *sodA* promoter. An explanation for the induction of MnSOD upon low pH is that shocked cells accumulate superoxides. In fact, use of a scavenger to prevent intracellular superoxide accumulation eliminated pH-dependent induction of MnSOD. Thus, it appears that MnSOD is not induced directly by acidic conditions per se, but rather by oxidizing agents that are themselves produced in response to acid shock. Deletion of any SOD in *V. vulnificus* (FeSOD, encoded by *sodB*; CuZnSOD, encoded by *sodC*, or MnSOD) led to decreased survival of exponential cells exposed to acid stress (pH=5.0). Therefore, acid resistance in *V. vulnificus* involves not only stress responses to acidity itself but also counteractions to superoxides that accumulate intracellularly upon acid stress (Kim *et al*., 2005). Additional investigations of pH stress in vibrios other than *V. vulnificus* are beginning to expand this area of research (Wong and Wang, 2004).

2.4. NUTRITIONAL STRESS

Ninety-five percent of the open ocean is oligotrophic, averaging a scant 50 g of carbon fixed per square meter per year by primary productivity (Atlas and Bartha, 1998). Host organisms, however, are nutrient rich. As vibrios experience transient free-living and host-associated life cycles, these microbes thus encounter feast or famine conditions in which they are either host-associated (feast) or living in the water column or sand (famine). They must therefore undergo long intervals with little or no growth and metabolic dormancy in their free-living state, followed by brief periods of rapid growth during symbiosis (McDougald and Kjelleberg, 2006). Given this natural history, it is no

surprise that many *Vibrio* species possess extraordinarily quick generation times during periods of high nutrient availability, enabling them to out-compete and outgrow other microbial species (Eilers *et al*., 2000; Giovannoni and Rappe, 2000).

They also appear to have been selected for effective starvation response mechanisms, and the molecular basis of these responses has been studied in some detail (Urakawa and Rivera, 2006). Researchers found that incubation of *V. vulnificus* in chambers suspended in natural estuarine waters led to *in situ* expression of both *rpoS* and *katG* (catalase peroxidase), regardless of different prevailing temperature and salinity conditions found in the summer and winter (Smith and Oliver, 2006). Perhaps these genes were expressed specifically to adapt to nutritional stress during the *in situ* incubation; regardless of the time of year, the dissolved organic carbon was only 2.83 mg/L. This *in situ* work is consistent with previous findings, which demonstrated that *rpoS* mutants were less able to survive starvation conditions initially, compared with wild-type, but after 14 days exhibited survival identical to wild-type (Hulsmann *et al*., 2003).

A study of *V. anguillarum*, which are fish pathogens, demonstrated a linkage between nutritional stress and virulence. Chemotaxis is an essential activity during infection, and starving (through incubation in phosphate-buffered saline) and *V. anguillarum* cells remained virulent as exponential-phase cells after 2 days, and were still chemotactic post 8 days starvation using LD_{50} (Larsen *et al*., 2004).

2.5. DNA DAMAGE

Like all cells, *Vibrios* must have mechanisms for repairing DNA damage caused by common environmental assaults such as exposure to UV irradiation. In *V. vulnificus*, *rpoS* mutants are much more sensitive to UV irradiation than their wild-type counterparts in exponential phase (Park *et al*., 2004). In *V. harveyi*, the small GTP-binding protein CgtA is required for survival upon exposure to ultraviolet light. Its role in the repair of damaged DNA is likely indirect, as CgtA stimulates *recA* gene expression (Zielke *et al*., 2003). The coevolution of DNA-interacting proteins and genome dialects, intergenome differences as a result of horizontal gene transfer, has recently been attributed to stress (Paz *et al*., 2005). Evolution of bioluminescence as a mechanism to aid DNA repair via the activation of light-dependent photolyase has also been proposed (Czyz *et al.*, 2003).

2.6. OXIDATIVE STRESS

Vibrio species encounter oxidative stress during colonization of animal hosts, even during mutualistic associations such as the symbiosis between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* (Ruby and McFall-Ngai, 1999). Thus, the question of the mechanisms by which certain *Vibrio* species survive oxidative stress has been under intense investigation. Several groups have investigated the role of the *V. vulnificus* sigma factor encoded by *rpoS* in survival following a challenge with the oxidizing agent H_2O_2. In one circumstance (strain C7184o), an *rpoS* mutant was much more sensitive to H_2O_2 than its wild-type counterpart during stationary phase (Hulsmann *et al*., 2003). Another case using a different pathogenic isolate of *V. vulnificus* (ATCC 29307), the *rpoS* mutant was more sensitive than wild-type to H_2O_2 challenge during exponential phase, but not during stationary phase (Park *et al*., 2004). However, the

ATCC 29307) *rpoS* mutant had reduced catalase activity in both exponential and stationary phases, despite the fact that differential survival upon challenge with H_2O_2 was only observed in exponential phase. Therefore, different roles of *rpoS* during oxidative challenge between these two *V. vulnificus* isolates might indicate that adaptation to oxidative stress has taken different pathways (i.e., convergent evolution) in distinct *V. vulnificus* isolates.

The SoxRS regulon and superoxide dismutases (SODs) have also been implicated in physiological responses needed to survive oxidative stress. In many cases, the production of SODs is linked to survival of multiple stressors. For example, in *V. vulnificus*, extracellular acid stress provokes the accumulation of intracellular superoxides and so further provokes a superoxide response (Kim *et al*., 2005; 2006). An extracellular superoxide dismutase is an important virulence factor in the coral pathogen *V. shiloi*; its production is induced by high temperature, indicating a connection between survival of oxidative stress and temperature stress (Banin *et al*., 2003). In *V. harveyi*, a pathogen of the farmed black tiger prawn, exposure to the superoxide generating drug menadione induces expression of both the OxyR and SoxRS regulons. *V. harveyi* also seem to exhibit physiological adaptation to oxidative stress, as exposure to sub-lethal doses of menadione protect *V. harveyi* cells from subsequent exposure to otherwise lethal concentrations of H_2O_2. Growing *V. harveyi* cells in high-salinity medium prior to exposure to menadione also led to increased protection to this oxidizing agent, suggesting a coupling between osmotic stress physiology and oxidative stress responses in this organism (Vattanaviboon and Mongkolsuk, 2001).

V. harveyi, like most vibrios, are bioluminescent. The enzyme luciferase directly catalyzes the production of photons and is encoded by the *luxAB* genes. The LuxD protein, encoded in the same operon, is an acetyltransferase that produces fatty acid substrates for the luminescence reaction. Mutants with null mutations in *luxA* or *luxB*, but not mutants with a null mutation in *luxD*, are hypersensitive to several oxidative stressors such as H_2O_2, cumene hydroperoxide, t-butyl hydroperoxide, and ferrous ions. Curiously, this hypersensitivity was found over a narrow range of concentrations of these agents, occurring neither above nor below this range. Nevertheless, *luxA* and *luxB* mutants were rescued by supplied antioxidants in the growth medium. This suggests that bioluminescence may have evolved as a response to oxidative stress (Barros and Bechara, 1998; Szpilewska *et al*., 2003).

As in *V. harveyi*, bioluminescence in *V. fischeri* consumes reducing power. The physiology suggests a possible relationship between redox homeostasis, responses to oxidative stress, and bioluminescence. Recent evidence demonstrates that the ArcAB system in *V. fischeri* represses expression of the *luxICDABEG* operon. Possible inactivation of the ArcA repressor by oxidative stress, experienced during the early stages of host colonization, likely derepresses *luxICDABEG* expression when *V. fischeri* colonize *E. scolopes*. This hypothesis, may explain why some strains of symbiotically competent *V. fischeri* such as ES114 are not visibly luminescent *in vitro* yet are visibly luminous during symbiosis (Bose *et al*., 2007).

Additional connections between oxidative stress physiology and host colonization may be present due to the need to survive stressors produced by the host. For example, in halophilic *V. fluvialis*, an opportunistic pathogen that causes gastroenteritis in humans, requires the *hupO* gene for surviving exposure to H_2O_2 during exponential phase (Ahn *et al*., 2005). HupO is a virulence factor that binds to hemin and likely affects intracellular

accumulation of hemin-associated iron during infection. The mechanism of *hupO*-associated H_2O_2 resistance is independent of catalase activity; therefore, the molecular details of how iron deficiency is connected to oxidative stress through HupO remain to be determined.

A relatively unexplored but related topic is the question of survival in the face of nitrosative stress, which symbionts also encounter when colonizing a host. It is increasingly clear that *V. fischeri* encounter nitric oxide during host colonization, as the host tissues lining the spaces where *V. fischeri* must traverse to colonize juvenile squids contain cells that produce NO and nitric oxide synthase (NOS; Davidson *et al*., 2004). NO production is normally considered to be a defensive strategy to prevent harmful bacterial infections, so it is striking that, in this case NO seems to function as part of the normal process of host-symbiont colonization that leads to a highly specialized and mutually beneficial symbiosis.

2.7. OSMOTIC STRESS

Vibrios live in environments that vary in salinity, and therefore experience high (hyperosmolar) and low (hypoosmolar) osmotic stress. During hypoosmolarity, the obstacles to cellular homeostasis are maintaining appropriate cytoplasmic concentrations of metabolites and ions, preventing cell lysis, and preserving ionic strength and pH (Bartlett, 2006). During hypoosmotic shock, some vibrios may increase putrescine content to compensate for decreased K^+ that are necessary to stabilize the phosphate backbones of nucleic acids. Hyperosmolarity, however, promotes dehydration and shriveling of cells. Microorganisms must be able to import or synthesize counterbalancing solutes that are compatible with metabolic and physiological functions. K^+ uptake is frequently stimulated to compensate for the increased external osmolarity. However, negative counter-ions (e.g., glutamate) must also be concurrently imported into the cell or synthesized de novo to sustain the same intracellular net charge (Sleator and Hill, 2001). Alternatively, cells can forgo K^+ uptake and import or synthesize neutral compatible solutes, as they carry no charge. Ectoine is such an example and its biosynthesis may be unique to the genus *Vibrio* (Bartlett, 2006). *V. fischeri* is also known to possess the ability to synthesize the disaccharide trehalose, which is also a neutral compatible solute for high osmolar stress. Incorporating polyunsaturated fatty acids in the cell membrane may also alleviate vibrios of excess toxic Na^+ by allowing their departure through the more fluid membrane (Valentine and Valentine, 2004).

As mentioned previously, *V. vulnificus* is an opportunistic human pathogen that can survive a range of osmolar conditions, from high-salt (or sugar) environments used to curb colonization of shellfish intended for human consumption, to those typical in marine environments and lower osmolarites as encountered in some compartments of the human body. For example, *rpoS* mutant *V. vulnificus* (C7184o) in stationary phase were much more sensitive to hyperosmolarity stress than their wild-type counterparts (Hulsmann *et al*., 2003). In contrast, *rpoS* mutant *V. vulnificus* (ATCC 29307) was no more sensitive to hyperosmolarity stress than its wild-type counterpart (Park *et al*., 2004). Perhaps these isolate-specific observations indicate that the *rpoS* regulon is not identical across all *V. vulnificus* isolates, evidence that a strain-specific genomic context is present for gene expression. Also in *V. vulnificus*, loss-of-function mutations in the *putAP* operon cause hypersensitivity to high osmolarity (Kim *et al*., 2006). The operon

encodes a proline dehydrogenase and a proline permease; proline dehydrogenase is part of a pathway that converts proline into glutamate, a well-known osmoprotectant. Two promoters, separated by 6 base pairs, control production of two transcripts from this operon. One is monocistronic and encodes only *putA*, while the second encodes both *putA* and *putP*. Expression of mRNA that hybridizes to a *putA* probe declines in stationary phase, while both *putA* and *putAP* transcripts are induced by proline and negatively regulated by glutamate (Kim *et al*., 2006). In contrast, high osmolarity induced higher *putA* mRNA levels but did not affect levels of bicistronic *putAP* mRNA, suggesting that only one of the two promoters is responsive to osmotic conditions. Additionally, both *putA* and *putAB* transcripts were dramatically reduced in a *crp*-mutant, suggesting a possible connection between survival of acid stress and nutritional status (as surveyed by intracellular cAMP levels; Lee and Choi, 2006). *Crp* mutants in other bacteria, such as *E. coli*, can have pleiotropic effects, so this relationship between nutritional status and survival of saline stress remains tentative in vibrio bacteria.

Osmotic stress has also been observed to have effects in other vibrios. For example, in the fish pathogen *V. anguillarum*, chemotactic responses to serine are decreased by high osmolarity (≥1.8% NaCl) relative to optimal osmolarity conditions (0.8% NaCl; Larsen *et al*., 2004). Proteomic analyses have been completed of *V. alginolyticus* and *V. parahaemolyticus* at different NaCl concentrations to examine resultant changes in gene expression through these physiological shifts (Xu *et al*., 2004; Xu *et al*., 2005). Since marine pathogens constantly face changes in osmolarity as they shift between marine waters and their native hosts, proteins such as outer membranes are selected to accommodate such changes. Outer membrane proteins OmpW, OmpV, and OmpTolC were discovered to be responsive osmotic stress proteins in *V. alginolyticus* (Xu *et al*., 2005). OmpV was expressed at low NaCl concentrations, but not at higher concentrations. Conversely, OmpW and OmpTolC displayed reverse changes, being expressed at high NaCl concentrations and down-regulated at low NaCl levels. Interestingly, differential expression of outer membrane proteins has been suggested by several researchers to play significant roles in symbiosis, including immunogenicity and virulence (Jones and Nishiguchi 2006; Xu *et al*., 2005). Not only were OmpW and OmpV identified in *V. parahaemolyticus* osmoregulation, but elongation factor TU and polar flagellin were implicated as well (Xu *et al*., 2004). Elongation factor TU and polar flagellin were respectively downregulated and upregulated at higher salinities, while OmpW and OmpV showed analogous patterns of expression, as in *V. alginolyticus*.

3. Experimental evolution and the viable but non-culturable state

3.1. EXPERIMENTAL EVOLUTION WITH VIBRIOS

In recent years, experimental evolution with microorganisms has emerged as an exciting new sub-discipline of evolutionary biology addressing diverse issues (Lenski *et al*., 1991; Bennet, 2002; Lenski, 2002), including microbial adaptation to variable environments and stress (Bennett and Lenski, 1999; Bennett and Lenski, 1997; Lenski and Bennett, 1993). The elegance of this scientific approach is the ability investigators have to control the selective regimen of the experimental conditions, to observe evolution and adaptation on a human time scale due to short generation times of

microorganisms, and the ability to compare evolving lineages from different evolutionary time points directly to a specifically known ancestor through the usage of a -80°C "frozen fossil record." This cryogenically preserved "fossil record" allows identification of changes in gene expression responsible for adaptation and loci subject to selection through subsequent genetic analysis (Riehle, *et al*., 2003). Although experimental evolution studies in the past were largely initiated with *Escherichia coli* as the major study microorganism, the list of other microbial species used in parallel studies has expanded in recent years. To date, the usage of Vibrionaceae in experimental evolution has principally been absent; however, such work is currently underway in our laboratory, as we are in the process of conducting serial passage experiments with *V. fischeri* derived from the sepiolid squid *Euprymna scolopes* (Hawaiian species) and evolving them through the novel host congener *E. tasmanica* (Australian species; Nishiguchi *et al*., 1998; Nishiguchi, 2002). Moreover, we are expanding such experimental evolution projects to address the ability of *V. fischeri* to adapt to abiotic factors at extreme limits of permissible growth based on previous studies that have shown introgression of various *V. fischeri* haplotypes to different habitats (Jones *et al*., 2006), as well as seasonal changes that cause changes in the viable *V. fischeri* baceterioplankton community (Jones *et al*., 2007).

3.2. THE VIABLE BUT NON-CULTURABLE STATE

A substantial literature now exists and continues to develop surrounding the viable but nonculturable (VBNC) state, whereby microorganisms normally culturable do not grow in liquid or agar media because of their entry into a dormancy where cells are still metabolically active and presumed to enhance resistance and survival to stress, a phenomenon first widely reported in the Vibrionaceae but is now believed to exist in other prokaryotes (Roszak and Colwell, 1987; Colwell, 2000). Nevertheless, the existence of VBNC cells has been contested and continues to be challenged (Bogosian and Bourneuf, 2001; Wong and Wang, 2004; McDougald and Kjelleberg, 2006). Past research on cells ostensibly in the VBNC state have included the identification of molecules and mechanisms (e.g., temperature upshift) that apparently resuscitate VBNC cells, enabling them to re-grow in microbiological culture media once again. Nonetheless, skepticism persists because of the possibility that any observed re-growth is the result of injured cells having recovered their healthy state, and not the result of resuscitating cells from a genuine VBNC condition (Bogosian and Bourneuf, 2001). Skeptics point out, as of yet, that no genes have been identified through null mutations or knockouts that may be responsible for vibrios entering a developmental program or pathway leading to a physiologically differentiated VBNC state. Convincing evidence would perhaps require loss-of-function experiments followed by complementation or over-expression gain-of-function studies (Bogosian and Bourneuf, 2001; McDougald and Kjelleberg, 2006). Proponents of the VBNC state remain convinced of its validity, perhaps not least because of the state's power to explain some ecological observations related to isolation of vibrio colony-forming units during different times of the year. Continued work into this area will surely lead to intriguing research, along with lively debate, for years to come.

4. Conclusions

Finally, extended examinations into the genetic traits and physiological responses characteristic of Vibrionaceae—quorum sensing, biofilm formation, two-chromosome architecture, induction of recombination machinery in the utilization of integrons and horizontal gene transfer—is essential to more completely understand their roles in regulating cellular homeostasis against stress (Boucher and Stokes, 2006; Iida and Kurokawa, 2006; Rowe-Magnus *et al*., 2006). There has not been much recent work specifically on stress and its effects on any of these phenomena, providing fertile ground for additional physiological investigations. Future work in areas such as experimental evolution, community ecology and population structure of vibrios in the environment, as well as specific trade-offs between symbiotic and free-living lifestyles should provide key insights into the adaptive radiation and speciation of this extensive group of bacteria.

5. Acknowledgements

The authors would like to thank members of the Nishiguchi lab for their insightful discussions on the topic of vibrio stress. This work was supported in part by grants from the National Science Foundation Population Biology program (DEB-0316516) and the National Institutes of Health (SO6 GM008136-32S2-1) to M.K.N. W.S. was supported by the NIH-MBRS RISE program at New Mexico State University (GM-61222-01).

6. References

Ahn,S.-H.; J.-H. Han; J.-H. Lee; K.-J. Park and I.-S. Kong. (2005) Identification of an iron-regulated hemin-binding outer membrane protein, hupo, in *Vibrio fluvialis*: effects on hemolytic activity and the oxidative stress response. *Infection and Immunity* **73:**722-729.

Allaby, M. (2005) *Oxford Dictionary of Ecology*. Oxford University Press. Oxford, New York.

Amaro, C.; E.G. Biosca; B. Fouz; E. Alcaide and C. Esteve. (1995) Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. *Applied and Environmental Microbiology* **61:**1133-1137.

Atlas, R.M. and R. Bartha. (1998) Ch. 9. Microorganisms in their natural habitats: air, water, and soil microbiology. pg. 322-384. *Microbial Ecology: Fundamentals and Applications*. 4th Ed. Benjamin/Cummings Science Publishing. Menlo Park, California.

Austin, B. (2006) Ch. 22. Miscellaneous animal pathogens In: F.L. Thompson, B. Austin and J. Swings (eds) *The Biology of the Vibrios* ASM Press. Washington, D.C. . pg. 297-308.

Banin, E.; D. Vassilakos; E. Orr; R.J. Martinez and E. Rosenberg. (2003) Superoxide dismutase is a virulence factor produced by the coral bleaching pathogen *Vibrio shiloi*. *Current Microbiology* **46:**418-422.

Barros, M.P. and E.J. Bechara. (1998) Bioluminescence as a possible auxiliary oxygen detoxifying mechanism in elaterid larvae. *Free Radical Biology and Medicine* **24:**767-777.

Bartlett, D.H. (2006) Ch. 11. Extremophilic vibrionaceae. pg. In: F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios*. ASM Press. Washington, D.C. 156-171.

Bennett, A.F. (2002) Experimental Evolution: an overview pg. 339-342. *Encyclopedia of Evolution*. *Vol. 1*. M. Pagel (ed). Oxford University Press. New York, New York.

Bennett, A.F. and R.E. Lenski. (1997) Phenotypic and evolutionary adaptation of a model bacterial system to stressful thermal environments. In: R. Bijlsma and V. Loeschcke (eds). *Environmental Stress, Adaptation, and Evolution* Birkhauser. Basel, Switzerland. pg. 135-154.

Bennett, A.F. and R.E. Lenski. (1999) Experimental evolution and its role in evolutionary physiology. *American Zoologist* **39:**346-362.

Bogosian, G. and E.V. Bourneuf. (2001) A matter of bacterial life and death. *EMBO Reports* **2:**770-774.

Bordas, M.A.; M.C. Balebona; I. Zorrilla; J.J. Borrego and M.A. Morinigo. (1996) Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains of skin mucus of gilt-head sea bream (*Sparus aurata* L.). *Applied and Environmental Microbiology* **62:**3650-3654.

Bose, J.L.; U. Kim; W. Bartkowski; R.P. Gunsalus; A.M. Overley; N.L. Lyell; K.L. Visick and E.V. Stabb. (2007) Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA. *Molecular Microbiology* **65:**538-552.

Boucher, Y. and H.W. Stokes. (2006) Ch. 7. The roles of lateral gene transfer and vertical descent in vibrio evolution. In: F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios* ASM Press. Washington, D.C. . pg. 84-94.

Bryan, P.J.; R.J. Steffan; A. DePaola; J.W. Foster and A.K. Bej. (1999) Adaptive response to cold temperatures in *Vibrio vulnificus*. *Current Microbiology* **38:**168-175.

Colwell, R.R. (2000) Viable but nonculturable bacteria: a survival strategy. *Journal of Infection and Chemotherapy* **6:**121-125.

Colwell, R.R. (2006) Ch. 1. A global and historical perspective of the genus *Vibrio*. In: F.L. Thompson, B. Austin and J. Swings (eds) *The Biology of the Vibrios*. ASM Press. Washington, D.C. pg. 3-11.

Crosa, J.H.; L.A. Actis and M.E. Tolmasky. (2006) Ch. 18. The biology and pathogenicity of *Vibrio anguillarum* and *Vibrio ordalii*. In: F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios*. ASM Press. Washington, D.C. pg. 251-265.

Czyz, A.; K. Plata and G. Wegrzyn. (2003) Stimulation of DNA repair =as an evolutionary drive for bacterial luminescence. *Luminescence* **18:**140-144.

Datta, P.P. and R.K. Bhadra. (2003) Cold shock response and major cold proteins of *Vibrio cholerae*. *Applied and Environmental Microbiology* **69:** 6361-6369.

Davidson, S.K.; T.A. Koropatnick; R. Kossmehl; L. Sycuro and M.J. McFall-Ngai. (2004) NO means 'yes' in the squid-*Vibrio* symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cellular Microbiology* **6:**1139-1151.

Davis, J.W. and R.K. Sizemore. (1982) Incidence of *Vibrio* species associated with blue crabs (*Callinectes sapidus*) collected from Galveston Bay, Texas. *Applied and Environmental Microbiology* **43:**1092-1097.

Douglas, A.E. (2002) Symbiosis. pg. 1093-1099. *Encyclopedia of Evolution*. *Vol. 2*. M. Pagel (ed). Oxford University Press. New York, New York.

Dunlap, P.V.; J.C. Ast; S. Kimura; A. Fukui; T. Yoshino and H. Endo. (2007) Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics* **23:**507-532.

Eilers, H.; J. Pernthaler and R. Amann. (2000) Succession of pelagic marine bacteria during enrichment: a close look at cultivation-induced shifts. *Applied and Environmental Microbiology* **66:**4634-4640.

Ermolenko, D.N. and G.I. Makhatadze. (2002) Bacterial cold-shock proteins. *Cellular and Molecular Life Sciences* **59:**1902-1913.

Farmer III, J.J. and J.M. Janda. (2005) Vibrionaceae. In: D.J. Brenner; N.R. Krieg and J.R. Staley (eds). *Bergey's Manual Systematic Bacteriology: The Proteobacteria, Vol. 2*. 2nd Ed Springer-Verlag. New York. pg. 491-494.

Farmer III, J.J.; J.M. Janda; F.W. Brenner; D.N. Cameron and K.M. Birkhead. (2005) *Vibrio*. In: *Bergey's Manual Systematic Bacteriology: The Proteobacteria, Vol. 2*. 2nd Ed. D.J. Brenner; N.R. Krieg and J.R. Staley (eds). Springer-Verlag. New York. pg. 494-546.

Giovannoni, S. and M. Rappe. (2000) Ch. 3. Evolution, diversity, and molecular ecology of marine prokaryotes. In: *Microbial Ecology of the Oceans*. Wiley-Liss, Inc. New York, New York. pg. 47-84.

Grimes, D.J.; P. Brayton; R.R. Colwell and S.H. Gruber. (1985) Vibrios as autochthonous flora of neritic sharks. *Systematic and Applied Microbiology* **43:**221-226.

Hilton, T; T. Rosche; B. Froelich; B. Smith and J. Oliver. (2006) Capsular polysaccharide phase variation in *Vibrio vulnificus*. *Applied and Environmental Microbiology* **72:** 6986-6993.

Huels, K.L.; Y.J. Brady; M.A. Delaney and J.A. Bader. (2003) Evidence of a cold shock response in *Vibrio vulnificus*, a human pathogen transmitted by via raw eastern oysters, *Crassotrea virginica*, from the Gulf of Mexico. *Journal of Shellfish Research* **22:**336.

Hulsmann, A.; T.M. Roschle; I.-S. Kong; H.M. Hassan; D.M. Beam and J.D. Oliver. (2003) RpoS-dependent stress response and exoenzyme production in *Vibrio vulnificus*. *Applied and Environmental Microbiology* **69:**6114-6120.

Jones, B.W., A. Maruyama, C.C. Ouverney, and M.K. Nishiguchi. 2007. Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. *Microbial Ecol*ogy **54**:314-323.

Jones, B.W., J.E. Lopez, J. Huttenberg, and M.K. Nishiguchi. 2006. Population structure between environmentally transmitted *Vibrios* and bobtail squids using nested clade analysis. *Molecular Ecol*ogy **15**:4317-4329.

Jones, B.W. and M.K. Nishiguchi. 2006. *Vibrio fischeri* transcripts reveal adaptations in an environmentally transmitted symbiosis. *Canadian Journal of Microbiol*ogy **52**:1218-1227.

Jones, B.W. and M.K. Nishiguchi. (2004). Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Marine Biology* **144**:1151-1155.

Iida, T. and K. Kurokawa. (2006) Ch. 5. Comparative genomics: genome configuration and the driving forces in the evolution of vibrios. In:. F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios* ASM Press. Washington, D.C. pg. 67-75.

Kaspar, C.W. and M.L. Tamplin. (1993) Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Applied and Environmental Microbiology* **59:**2425-2429.

Kim, J.-S.; S.H. Choi and J.K. Lee. (2005) Lysine decarboxylase expression by *Vibrio vulnificus* is induced by SoxR in response to superoxide stress. *Journal of Bacteriology* **188:** 8586-8592.

Kim, H.J.; J.H. Lee; J.E. Rhee; H.S. Jeong; H.K. Choi, H.J. Chung; S. Ryu and S.H. Choi. (2006) Identification and functional analysis of the putAP genes encoding *Vibrio vulnificus* proline dehydrogenase and proline permease. *Journal of Microbiology and Biotechnology* **12:**318-326.

Larsen, M.H.; N. Blackburn; J.L. Larsen and J.E. Olsen. (2004) Influences of temperature, salinity and starvation on the motility and chemotactic response of *Vibrio anguillarum*. *Microbiology* **150:**1283-1290.

LeaMaster, B.R.; W.A. Walsh; J.A. Brock and R.S. Fujioka. (1997) Cold stress-induced changes in the aerobic heterotrophic gastrointestinal tract bacterial flora of red hybrid tilapia. *Journal of Fish Biology* **50:**770-780.

Lee, J.H. and S.H. Choi. (2006) Coactivation of *Vibrio vulnificus* putAP operon by cAMP receptor protein and PutR through cooperative binding to overlapping sites. *Molecular Microbiology* **60:**513-524.

Lenski, R.E. (2002) Experimental Evolution: a long-term study with *E. coli*. pg. 342-344. *Encyclopedia of Evolution*. *Vol. 1*. M. Pagel (ed). Oxford University Press. New York, New York.

Lenski, R.E. and A.F. Bennett. (1993) Evolutionary response of *Escherichia coli* to thermal stress. *Evolution* **142:**S47-S64.

Lenski, R.E.; M.R. Rose; S.C. Simpson and S.C. Tadler. (1991) Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *American Naturalist* **128:**1315-1341.

Lin, C.; R.-C. Yu and C.-C. Chou. (2004) Susceptibility of *Vibrio parahaemolyticus* to various environmental stresses after cold shock treatment. *International Journal of Food Microbiology* **92:**207-215.

Lincoln, R.; G. Boxshall and P. Clark. (1998) *A Dictionary of Ecology, Evolution, and Systematics*. 2nd Ed. Cambridge University Press. Cambridge, United Kingdom.

Madigan, M.T. and J.M. Martinko. (2006) Ch. 12. Prokaryotic diversity: the Bacteria. pg. 355-357. *Brock Biology of Microorganisms*. 11th Ed. Pearson Prentice Hall. Upper Saddle River, NJ.

Marco-Noales, E.; E.G. Biosca and C. Amaro. (1999) Effects of salinity and temperature on long-term survival of the eel pathogen *Vibrio vulnificus* biotype 2 (serovar E). *Applied and Environmental Microbiology* **65:**1117-1126.

McDougald, D. and S. Kjelleberg. (2006) Ch. 10. Adaptive responses of vibrios. In: F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios*.ASM Press. Washington, D.C. pg. 133-155.

McGovern, V.P. and J.D. Oliver. (1995) Induction of cold-responsive proteins in *Vibrio vulnificus*. *Journal of Bacteriology* **177:**4131-4133.

Meibom, K.L.; M. Blokesch; N.A. Dolganov; C.-Y. Wu and G.K. Schoolnik. (2005) Chitin induces natural competence in *Vibrio cholerae*. *Science* **310:**1824-1827.

Miyamoto, N. and M. Eguchi. (1997) Response to low osmotic stress in a fish pathogen, *Vibrio anguillarum*. *FEMS Microbiology Letters* **22:**225-231.

Nishiguchi, M.K. (2000) Temperature affects species distribution in symbiotic populations of *Vibrio* spp. *Applied and Environmental Microbiology* **66:**3550-3555.

Nishiguchi, M.K. (2001) Co-evolution of symbionts and hosts: The sepiolid-Vibrio model.. In: J. Seckbach (ed). *Symbiosis: Mechanisms and Model Systems*. Cole-Kluwer Academic Publishers. Dordrecht, The Netherlands. pg. 757-774.

Nishiguchi, M.K. (2002) Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microbial Ecology* **44:**10-18.

Nishiguchi, M.K. and B.W. Jones. (2004) Microbial biodiversity of within the *Vibrionaceae*.. In: *Origins, evolution, and biodiversity of microbial life*. J. Seckback (ed). Cole-Kluwer Academic Publishers. Dordrecht, The Netherlands. pg. 531-548.

Nishiguchi, M.K. and V.S. Nair. (2003) Evolution of symbiosis in the *Vibrionaceae*: a combined approach using molecules and physiology. *International Journal of Systematic and Evolutionary Microbiology* **53:**2019-2026.

Nishiguchi, M.K.; E.G. Ruby and M.J. McFall-Ngai. (1998) Competitive dominance during colonization is an indicator of coevolution in an animal-bacterial symbiosis. *Applied and Environmental Microbiology* **64:**3209-3213.

Odic, D.; V. Turk and S. Stopar. (2007) Environmental stress determines the quality of bacterial lysate and its utilization efficiency in a simple microbial loop. *Microbial Ecology* **53:**639-649.

Olafsen, J.A.; H.V. Mikkelsen; H.M. Glever and G.H. Hansen. (1993) Indigenous bacteria in hemolymph and tissues of marine bivalves at low temperatures. *Applied and Environmental Microbiology* **59:**1848-1854.

Owens, L. and N. Busico-Salcedo. (2006) Ch. 19. *Vibrio harveyi*: pretty problems in paradise. In: F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios*. ASM Press. Washington, D.C. pg. 266-280.

Park, K.-J. M.-J. Kang; S.H. Kim; H.-J. Lee; J.-K. Lim; S.H. Choi; S.-J. Park and K.-H. Lee. (2004) Isolation and characterization of rpoS from a pathogenic bacterium, *Vibrio vulnificus*: role of sigma (S) in survival of exponential-phase cells under oxidative stress. *Journal of Bacteriology* **186:**3304-3312.

Paz, A.; V. Kirzhner; E. Nevo and A. Korol. (2005) Coevolution of DNA-interacting proteins and genome "dialect". *Molecular Biology and Evolution* **23:**56-64.

Prouty, M.G. and K.E. Klose. (2006) *The Biology of the Vibrios*. Ch. 23. *Vibrio cholerae* the genetics of pathogenesis and environmental persistence. In: F.L. Thompson, B. Austin and J. Swings (eds). ASM Press. Washington, D.C. pg. 311-339.

Rhee, J.E.; H.-M. Ju; U. Park; B.C. Park and S.H. Choi. (2006) Identification of the *Vibrio vulnificus* cadC and evaluation of its role in acid tolerance. *Journal of Microbiology and Biotechnology* **14:**1093-1098.

Rhee, J.E.; J.H. Rhee; P.Y. Ryu and S.H. Choi. (2002) Identification of the cadBA operon from Vibrio vulnificus and its influence on survival to acid stress. *FEMS Microbiology Letters* **208:**254-251.

Riehle, M.R.; A.F. Bennett; R.E. Lenski and A.D. Long. (2003) Evolutionary changes in heat-inducible gene expression in lines of *Escherichia coli* adapted to high temperature. *Physiological Genomics* **14:**47-58.

Ripp, S.; P. Jegier; M. Birmele; C.M. Johnson; K.A. Daumer; J.L. Garland and G.S. Sayler. (2006) Linking bacteriophage infection to quorum sensing signaling and bioluminescent bioreporter monitoring for direct detection of bacterial agents. *Journal of Applied Microbiology* **100:**488-499.

Rosenberg, E.; O. Koren; L. Reshef; R. Efrony and I. Zilber-Rosenberg. (2007) The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology* **5:**355-362.

Roszak, D.B. and R.R. Colwell. (1987) Survival strategies of bacteria in the natural environment. *Microbiological Reviews* **51:**365-379.

Rowe-Magnus, D.A.; M. Zouine and D. Mazel. (2006) Ch. 8. The adaptive genetic arsenal of pathogenic *Vibrio* species: the role of integrons. In: F.L. Thompson, B. Austin and J. Swings (eds) *The Biology of the Vibrios*. ASM Press. Washington, D.C. . pg. 95-111.

Ruby, E.G. (1996) Lessons from a cooperative animal-bacterial association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. *Annual Review of Microbiology* **50:**591-624.

Ruby, E.G. and M.J. McFall-Ngai. (1999) Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. *Trends in Microbiology* **7:**414-420.

Sawabe, T. (2006) Ch. 15. The mutual partnership between *Vibrio halioticoli* and abalonesIn: F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios*. ASM Press. Washington, D.C. . pg. 219-230.

Sawabe, T.; Y. Oda; Y. Shiomi and Y. Ezura. (1995) Alginate degradation by bacteria isolated from the gut of sea urchins and abalones. *Microbial Ecology* **30:**192-202.

Sleator, R.D. and C. Hill. (2001) Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS Microbiology Reviews* **26:**49-71.

Smith, B. and J.D. Oliver. (2006) In situ gene expression by *Vibrio vulnificus*. *Applied and Environmental Microbiology* **72:**2244-2246.

Stabili, L.; C. Gravili; S. Piraino; F. Boero and P. Alifano. (2006) *Vibrio harveyi* associated with *Aglaophenia octodonta* (Hydrozoa, Cnidaria). *Microbial Ecology* **52:**603-608.

Szpilewska, H.; A. Czyz and G. Wegrzyn. (2003) Experimental evidence for the physiological role of bacterial luciferase in the protection of cells against oxidative stress. *Current Microbiology* **47:**379-382.

Thompson, F.L. and J. Swings. (2006) Ch. 3. Taxonomy of the vibrios. In: F.L. Thompson, B. Austin and J. Swings (eds) *The Biology of the Vibrios*.. ASM Press. Washington, D.C. pg. 29-43.

Urakawa, H. and I.N.G. Rivera. (2006) Ch. 12. Aquatic environment. In: F.L. Thompson, B. Austin and J. Swings (eds). *The Biology of the Vibrios*. ASM Press. Washington, D.C. pg. 175-189.

Valentine, R.C. and D.L. Valentine. (2004) Omega-3 fatty acids in cellular membranes: a unified concept. *Progress in Lipid Research* **43:**383-402.

Vattanaviboon P. and S. Mongkolsuk. (2001) Unusual adaptive, cross-protection response and growth phase resistance against peroxide killing in a bacterial shrimp pathogen, *Vibrio harveyi*. *FEMS Microbiology Letters* **47:**111-116.

Ventosa, A. (2005) *Salinivibrio*. In: D.J. Brenner; N.R. Krieg and J.R. Staley (eds). *Bergey's Manual Systematic Bacteriology: The Proteobacteria, Vol. 2*. 2nd Ed Springer-Verlag. New York. pg. 552-555.

Verschuere, L.; G. Rombaut; P. Sorgeloos and W. Verstraete. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64:**655-671.

Wong, H.C. and P. Wang. (2004) Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *Journal of Applied Microbiology* **96:**359-366.

Xu, C.; H. Ren; S. Wang and X. Peng. (2004) Proteomic analysis of salt-sensitive outer membrane proteins of *Vibrio parahaemolyticus*. *Research in Microbiology* **155:**835-842.

Xu, C.; S. Wang; H. Ren; X. Lin; L. Wu and X. Peng. (2005) Proteomic analysis on the expression of outer membrane proteins of *V. alginolyticus* at different sodium concentrations. *Proteomics* **5:**3142-3152.

Zielke, R.; A. Sikora; R. Dutkiewicz; G. Wegrzyn and A. Czyz. (2003) Involvement of the cgtA gene function in stimulation of DNA repair in *Escherichia coli* and *Vibrio harveyi*. *Microbiology* **49:**1763-1770.

environmental microbiology reports

Environmental Microbiology Reports (2010) **2**(4), 514–523 doi:10.1111/j.1758-2229.2009.00077.x

Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches

Ricardo C. Guerrero-Ferreira[†] and Michele K. Nishiguchi*

Department of Biology, New Mexico State University, Las Cruces, NM 88003-8001, USA.

Summary

Luminescent bacteria (γ-*Proteobacteria*: *Vibrionaceae*) are found in complex bilobed light organs of both sepiolid and loliginid squids (Mollusca: Cephalopoda). Despite the existence of multiple strain colonization between *Vibrio* bacteria and loliginid squids, specificity at the genus level still exists and may influence interactions between symbiotic and free-living stages of the symbiont. The environmentally transmitted behaviour of *Vibrio* symbionts bestows a certain degree of recognition that exists prior and subsequent to the colonization process. Therefore, we identified bacterial genes required for successful colonization of loliginid light organs by examining transcripts solely expressed in either the light organ or free-living stages. Selective capture of transcribed sequences (SCOTS) was used to differentiate genes expressed by the same bacterium when thriving in two different environments (i.e. loliginid light organs and seawater). Genes specific for squid light organs included vulnibactin synthetase, outer membrane protein W and dihydroxy dehydratase, which have been associated with the maintenance of bacterial host associations in other systems. In contrast, genes that were solely expressed in the free-living condition consisted of transcripts recognized as important factors for bacterial survival in the environment. These transcripts included genes for methyl accepting chemotaxis proteins, arginine decarboxylase and chitinase. These results provide valuable information regarding mechanisms determining specificity, establishment, and maintenance of bacteria–squid associations.

Received 7 May, 2009; accepted 11 August, 2009. *For correspondence. E-mail nish@nmsu.edu; Tel. (+1) 575 646 3721; Fax (+1) 575 646 5665. [†]Present address: Department of Pediatrics, Division of Pediatric Infectious Diseases, Emory University School of Medicine, Atlanta, GA 30322, USA.

Introduction

Bacterial–host interaction during mutualistic or pathogenic symbioses can be a dynamic association where microorganisms use survival and reproduction strategies to fight the normal defence mechanisms of the host (Mekalanos, 1985; McFall-Ngai and Ruby, 1998). Because expression of virulence determinants in bacteria is regulated by both environmental and host factors (Heithoff *et al*., 1997; Jones and Nishiguchi, 2006; Soto *et al*., 2009), many novel genes that are not expressed during *in vitro* growth are known to be regulated by *in vivo* factors within the host (Heithoff *et al*., 1997) at the onset of symbiosis. For instance, Graham and Clark-Curtiss (1999) demonstrated differential gene expression of *Mycobacterium tuberculosis* upon interaction with cultured primary human microphages and during their free-living state. Likewise, studies on *Salmonella typhimurium* showed variation in expression profiles following colonization of mouse tissue when compared with growth outside of the host (Slauch *et al*., 1994). Both studies elucidated the importance of genes coding for membrane, stress and regulatory functions in the establishment and maintenance of these associations. Interactions between bacteria in the family *Vibrionaceae* and squid light organs is not an exception to this rule, with *Vibrio fischeri* undergoing differential gene expression upon colonization of the light organs of bobtail squids in the genus *Euprymna* (Mollusca: Cephalopoda) (Jones and Nishiguchi, 2006).

Associations between bobtail squids (Cephalopoda: Sepiolidae) and *Vibrionaceae* bacteria (γ-proteobacteria: *Vibrionaceae*) have been previously studied to understand the evolution and regulation of specificity of environmentally transmitted symbioses (Nishiguchi *et al*., 1998; Nishiguchi, 2002; Jones *et al*., 2006). Ruby and Asato (1993) demonstrated that luminous bacteria in squid light organs benefit from this association and exhibit higher growth rates than their free-living counterpart. Likewise, there is a benefit for the squid host, which uses the light produced by its bacterial partner for counterillumination (Jones and Nishiguchi, 2004).

Similarly, squid species in the family Loliginidae (Mollusca: Cephalopoda) are known to possess bacteriogenic light organs (Alexeyev, 1992; Anderson, 2000). The

economic importance of loliginid squid fisheries (Chotiyaputta *et al*., 2002; Nootmorn and Chotiyaputta, 2002) has furthered scientific interest in the characterization of bacterial populations colonizing these specialized tissue complexes (Guerrero-Ferreira and Nishiguchi, 2007). Previous studies have provided evidence of an association between the marine pathogen *Vibrio harveyi* with light organs of loliginid squids. These findings have raised questions regarding the potential of this symbiosis as a temporary reservoir for pathogenic *Vibrio* species such as *V. harveyi* (Guerrero-Ferreira and Nishiguchi, 2007; Dunlap *et al*., 2008). Considering the dual life history of *V. harveyi*, it is important to understand the genetic factors involved in its transition from pathogenic to mutualistic lifestyles to obtain valuable clues on how these unique associations have arisen.

Research approaches to identify genes selectively expressed by bacteria during their symbiotic states within host animals or cells have revealed a great deal about how virulence factors are regulated (Slauch *et al*., 1994; Graham and Clark-Curtiss, 1999; Daigle *et al*., 2001; Somboonwiwat *et al*., 2006), as well as identified factors required for successful colonization and persistence (Camilli and Mekalanos, 1995; Faruque *et al*., 2004; Jones and Nishiguchi, 2006). SCOTS (Selective Capture Of Transcribed Sequences) has been successfully used to compare gene expression of the same bacterium existing in two different environments (Graham and Clark-Curtiss, 1999; Daigle *et al*., 2001; Hou *et al*., 2002; Jones and Nishiguchi, 2006). This technique has been recognized as a useful tool to understand selective pressures associated with persistence of bacteria in the environment as well as host colonization. Therefore, we examined differences in gene expression of environmental (seawater) and symbiotic (light organ associated) bacterial isolates from the loliginid squid *Uroteuthis chinensis* using SCOTS to determine bacterium responsiveness to either host or environment.

Results and discussion

SCOTS has been proven to be a successful method for identification of genes expressed either during symbiosis (associated with a host) or in its free-living state (Graham and Clark-Curtiss, 1999; Graham *et al*., 2002; Dozois *et al*., 2003; Faucher *et al*., 2006). Successful use of SCOTS in mutualistic relationships such as the *Euprymna–V. fischeri* association has contributed to the knowledge of factors responsible for colonization and persistence of the symbiont within the light organ of the squid as well as prior to infection in the surrounding environment (Jones and Nishiguchi, 2006). We examined the utility of this method by comparing light organ-expressed genes with those solely expressed in seawater. A total of 47 genes were found, with 27 transcripts identified from light organ isolated bacteria and 20 transcripts from those isolates grown in seawater (Tables 1 and 2). Contamination by ribosomal RNA (rRNA) after capture hybridiza-

Table 1. Genes expressed by vibrio isolates in the light organs of loliginid squids.

Class	Clone name	Homology	Gene/protein coded
Cellular processes	Xba086,135	VVA1310	Vulnibactin synthetase, amide synthase subunit
Cellular processes	Xba171	VIBHAR_06625	Sensory histidine kinase CreC
Cellular processes	Xba103,014	VIBHAR_04770	Cell wall-associated hydrolase
Cellular processes	Xba064	VF-2110	Putative transporter YaaJ
Cellular processes	Xba065	VIBHAR_06639	*omp*W gene for outer membrane protein W
Cellular processes	Xba175	*Vibrio harveyi* HY01	Cell wall-associated hydrolase
Metabolism	Xba010	VIBHAR_00348	Gamma glutamyltransferase
Metabolism	Xba112	pVHA1-VHW-1	Quaternary ammonium compound resistance protein
Metabolism	Xba126	VIBHAR_00512	Partial *ilv*D gene
Metabolism	Xba106	VIBHAR_03235	Uridylate kinase (UMP kinase) PyrH
Metabolism	Xba008	VIBHAR_02101	Non-ribosomal peptide synthetase modules
Information storage and processing	Xba111	VIBHAR_00057	30S ribosomal protein S12
Information storage and processing	Xba006	VIBHAR_06651	*lys*R family transcriptional regulator
Information storage and processing	Xba005	VIBHAR_06734	Ribosomal protein S6 modification protein
Information storage and processing	Xba169	*Vibrio harveyi* HY01-A1Q_5079	Pseudouridine synthase, Rsu
Information storage and processing	Xba134	VIBHAR_0565	Integrase IntI
Poorly characterized	Xba172	VV1_1061	Orf122-like protein
Poorly characterized	Xba168	VIBHAR_00255	RNA-binding protein
Hypothetical	Xba108	VIBHAR_00327	Hypothetical protein
Hypothetical	Xba170	VV1_0932	Hypothetical protein
Hypothetical	Xba174	V12B01_06372	Hypothetical protein
Hypothetical	Xba176	A55_B0062	Hypothetical protein
Hypothetical	Xba177	A55_B0062	Hypothetical protein
Hypothetical	Xba178	VIBHAR_01012	Hypothetical protein
Hypothetical	Xba179	VC274080_B0002	Conserved hypothetical protein
Unknown	Xba173	VV20845	CMCP6 locus tag, product unknown

Table 2. Genes expressed by vibrio isolates in seawater.

Class	Clone name	Homology	Gene/protein coded
Cellular processes	Sal105,141	VIBHAR_01497	Arsenate reductase (*ars*C)
Cellular processes	Sal003	VIBHAR_05872	ATP-dependent chaperone *clp*B
Cellular processes	Sal198	VIBHAR_05104	Putative arsenate reductase
Cellular processes	Sal077	VIBHAR_04747	Methyl-accepting chemotaxis protein (MTA/SAH nucleosidase)
Cellular processes	Sal205	VIBHAR_05559	Heat shock protein
Cellular processes	Sal209	VIBHAR_02921	Tyrosine-phosphatase
Cellular processes	Sal005	VIBHAR_04747	Methyl accepting chemotaxis protein
Metabolism	Sal203	VIBHAR_05421	phenylalanine monooxygenase
Metabolism	Sal127	VIBHAR_06354	D-serine deaminase
Metabolism	Sal142	VIBHAR_00053	Nitrite reductase (*nir*B)
Metabolism	Sal143,180	VIBHAR_06737	Arginine decarboxylase (*spe*A)
Metabolism	Sal197,204	VIBHAR_03711	D-aspartate kinase
Metabolism	Sal103,163	VIBHAR_00698	Aminoglycoside acetyltranferase
Metabolism	Sal104	VIBHAR_05656	Chitinase
Metabolism	Sal107	VIBHAR_03431	Cellobiose phosphorylase
Metabolism	Sal 202	VIBHAR_01806	Adenosylmethionine-8amino-7-oxononanoate transaminase
Metabolism	Sal217	VIBHAR_00151	Manganese superoxide dismutase
Poorly characterized	Sal207	VIBHAR_03535	GTP-binding protein
Poorly characterized	Sal019	VIBHAR_07101	RNA binding protein
Hypothetical	Sal 090	VIBHAR_00469	Hypothetical protein
Hypothetical	Sal102	VIBHAR_00194	Hypothetical protein

tions, which is one of the concerns during SCOTS, was ruled out by completing southern blot hybridizations during primary verification. Tables 1 and 2 also show distribution of transcripts by gene types indicating that seawater-expressed genes are most associated with cellular processes (seven transcripts) and metabolism (10 transcripts). Light organ transcripts detected by SCOTS showed a more uniform distribution among different gene categories including genes for cellular processes (six transcripts), metabolism (five transcripts), and information storage and processing (five transcripts) (Fig. 1). Lack of detection of seawater transcripts under the functional category of information storage and processing (translation, ribosomal structure and biogenesis, transcription, DNA replication, recombination and repair) after SCOTS may indicate that these genes are almost equally expressed under both conditions, therefore being blocked during enrichment of each SCOTS library. However, specific genes (shown in Table 1) are selectively expressed during the bacteria symbiotic lifestyle, which highlights their importance for symbiosis.

Gene expression of vibrio isolates in the light organ of loliginid squids

Graf and Ruby (1998) argued that the crypt epithelium within the light organ provides amino acids and potentially other nutrients to the symbiotic bacteria housed therein. This might indicate that colonization of the nutrient-rich, light organ environment provides a selective advantage to the symbiont over free-living ecological variants. However, the same experiments demonstrated that auxotrophic mutants of squid symbionts were able to grow and establish themselves within the light organ environment at lower densities than wild-type isolates. Similarly, this study demonstrated that some amino acids required for bacterial growth are found in low levels within the light organ environment. Because of this, genes required for

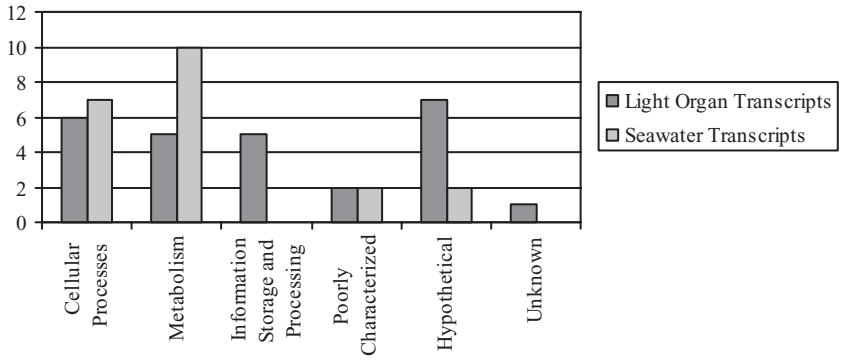


Fig. 1. Distribution of gene categories of transcripts expressed by free-living and light organ-associated bacteria captured through SCOTS.

amino acid synthesis are expected to be expressed in the light organ. In our study, we confirmed that genes required for the production of specific amino acids are being expressed during symbiosis, with *ilv*D (dihydroxyacid dehydratase) being solely expressed in the light organ. *ilv*D (Tarleton and Ely, 1991) catalyses the fourth step in the metabolic pathway leading to production of isoleucine (from pyruvate) and valine (from alpha-oxobutyrate). The product of the reaction catalysed by dihydroxyacid dehydratase is also the starting point in the alternative pathway producing leucine (Gottschalk, 1986). Production of leucine and valine is necessary within the light organ matrix where levels of these two amino acids are low, reaching only 0.07 and 0.09 mM respectively in *Euprymna scolopes* (Graf and Ruby, 1998). Conversely, concentrations of the same amino acids measured within symbiotic cells (including free and peptide forms) are among the highest, with 1.58 mM of leucine and 1.26 mM of valine, indicating that these molecules are being synthesized within the squid light organ. This suggests a major role for this amino acid in the proliferation of the association. Production of *ilv*D mutants of *Vibrio* bacteria and further colonization studies on squids would give insights on the role of *ilv*D on the persistence of the association.

An underlying role of *ilv*D is evident in *Vibrio cholerae*, the causative agent of cholera. In a human *in vivo* expression technology study (IVET), Lombardo and colleagues (2007) identified *ilv*D as one of the *V. cholerae* genes selectively expressed during human infection. In a different study, a large-scale signature-tagged mutagenesis screen (Merrell *et al*., 2002) also demonstrated the requirement of *ilv*D for human colonization by *V. cholerae*. This study showed that mutations affecting the entire *ilv* operon were associated with decreased virulence in the mouse model. Contribution of *ilv*D to the capability of *Vibrio* bacteria to colonize loliginid squid is yet to be characterized. However, since *V. cholerae* requires this gene for colonization of epithelial tissues, it may also indicate a connection between *ilv*D expression and colonization of eukaryotic tissue (such as squids) by *Vibrio* bacteria in general.

Our study also demonstrates selective expression of the gene for the amide synthetase subunit of vulnibactin synthetase, the enzyme responsible for production of vulnibactin, a siderophore first isolated from low-iron cultures of *Vibrio vulnificus* (Noriyuki *et al*., 1994). Acquisition of iron from the host is a requirement for colonization by host tissue-associated microbes. It is a common feature in pathogenic systems such as *Vibrio anguillarum* (Actis *et al*., 1986; Wolf and Crosa, 1986), *V. cholerae* and *V. harveyi* (Henderson and Payne, 1994; Owens *et al*., 1996). In mutualistic associations, studies with *V. fischeri* mutants with depleted siderophore production revealed that the ability to respond to limited iron levels may be connected to symbiosis efficiency (Graf and Ruby, 2000). Owens and colleagues (1996) also established a correlation between siderophore production and the type of bacterial isolates, and determined that free-living strains of *V. harveyi* produce higher amounts of siderophore compared with isolates associated with invertebrates. This difference in siderophore production was in contrast with our findings that light organ-associated vibrios selectively expressed the vulnibactin synthetase gene, necessary for bacterial siderophore production within the light organ. However, because Owens and colleagues (1996) focused on measurements of *V. harveyi* siderophore activity on agar plates prepared, their results do not reflect the expression of genes related with siderophore-producing pathways *in vivo*. Few studies have focused on iron sequestration and regulation of sidephore production of symbiotic bacteria during mutualistic associations with animal tissues (Bagg and Neilands, 1987; Aznar and Alcaide, 1992; Naidu and Yadav, 1997; Moeck and Coulton, 1998). Since bacteria harboured in the light organ are found in high concentrations (~10^8–10^{10} in an adult *Euprymna*), the necessity of having siderophores to capture iron resources may be fuelled by the competition among this community (i.e. limited resources in the light organ). Further examination of siderophore activity and regulation of gene expression associated with iron assimilation is necessary to fully comprehend the role of iron sequestration in colonization of loliginid light organs by *Vibrionaceae* bacteria.

Comparable to most siderophores, vibriobactin is a small cyclic peptide with the ability to bind environmental iron. This siderophore–iron complex is recognized by bacterial surface receptors such as *omp*W and transported into the cytoplasm (Moeck and Coulton, 1998). Expression of the *omp*W gene for outer membrane protein W was also detected in our SCOTS analysis. The importance of iron sequestration in symbiosis, specifically with regards to growth and replication of cytochrome systems of bacterial symbionts residing in eukaryotic hosts, has been previously observed (Bagg and Neilands, 1987; Henderson and Payne, 1994; Dozois *et al*., 2003). It is also known that production of siderophore is frequently accompanied by the synthesis of outer membrane proteins acting as receptors for the iron–siderophore complex (Bagg and Neilands, 1987; Dai *et al*., 1992).

In addition, outer membrane proteins have been shown to enhance adhesion and colonization when tested in animal models. However, their exact role in virulence is not completely understood (Faruque *et al*., 2004). In mutualistic associations, Jones and Nishiguchi (2006) reported *V. fischeri* expression of an integral membrane protein during colonization of *E. scolopes* light organs. Moreover, Aeckersberg and colleagues (2001) presented

evidence connecting the presence of a specific bacteria outer membrane protein (i.e. OmpU) for the establishment of successful mutualistic associations with sepiolid squids. Results from our SCOTS screening indicate that expression of genes for membrane proteins, such as *omp*W in symbiotic bacteria, may be necessary for successful colonization of the loliginid light organ, because of their direct relevance to *in vivo* recognition of iron–siderophore complexes.

Equally important for maintenance of colonization efficiency is the expression of the *pyr*H gene. This gene codes for the protein uridine monophosphate kinase (UMP kinase) that participates in pyrimidine metabolism catalysing the conversion of UMP into UDP (Voet and Voet, 2004). Kim and colleagues (2003) identified *pyr*H as one of the genes in *V. vulnificus* that is expressed preferentially *in vivo*. *Vibrio vulnificus* mutants unable to produce UMP kinase exhibited retarded growth on media and a reduction in the ability to infect HeLa cells. Therefore, *pyr*H expression has relevance in host colonization by *Vibrio* bacteria by directly reducing bacterial growth and decreasing infectivity.

Gene expression of vibrio isolates in seawater

Analysis of gene expression profiles of seawater-grown bacteria yielded a set of genes dominated by those responsible for bacterial metabolism. Among transcripts identified by SCOTS, a chitinase gene was hypothesized to be present, since *Vibrio* species use chitin as a carbon source (Yu *et al*., 1991; Svitil *et al*., 1997). Vibrios play a critical role in transforming chitin, a highly insoluble polysaccharide, into a form usable to other organisms (Bassler *et al*., 1991). Moreover, it has been reported that some species in the family *Vibrionaceae* are able to monitor surrounding environmental conditions by recognizing and migrating towards low concentrations of chitin oligosaccharides (Yu *et al*., 1991). Similarly, in most cooperative interactions studied in nature, hosts provide nutrient-rich environments for bacterial growth, whereas the bacteria contribute by providing the host with products of specific bacterial processes. It might be expected that free-living vibrios would use chitin metabolic machinery to monitor host tissue until successfully detecting chitin contained in the squid light organ (McFall-Ngai, 1998). This type of mechanism would potentially be part of the interactions that account for the first encounter between bacteria and host in the squid–*Vibrio* symbiosis. Upon first contact, other processes such as mucus and nitric oxide production occur (Nyholm *et al*., 2000; Davidson *et al*., 2004) that allow for the successful and permanent establishment of the symbionts within the crypts of the light organ (McFall-Ngai and Ruby, 1998). Our results not only support the importance of host-produced chitin on the initiation of squid-vibrio symbiosis but also imply a more active role of the symbiont in the establishment of this association.

In addition, our screening exhibited arginine decarboxylase (ADC) expression by *V. harveyi* loliginid light organ isolates grown in seawater. ADC degrades arginine to produce agmatine, and increases pH by removing acidic carboxyl groups and releasing CO_2 from their substrates (Stim and Bennett, 1993). A potential role of ADC in the environment is the formation of biofilm. ADC, similar to other amino acid decarboxylases, is involved in the production of polyamines (Stim and Bennett, 1993) that modulate bacterial biofilms within *Vibrionaceae* species (Kierek-Pearson and Karatan, 2005; Patel *et al*., 2006). Studies have shown that factors that modulate biofilm formation (such as ADC and uridine diphosphate dehydrogenase, UDPDH) by *Vibrionaceae* bacteria are important in light organ symbiosis. For example, analysis of *V. fischeri* UDPDH mutants demonstrated that their biofilm-forming ability is reduced *in vitro*. In addition, these mutants were not detected in any part of the crypt region during colonization assays (Ariyakumar, 2007; Ariyakumar and Nishiguchi, 2009).

Also important for bacterial colonization of eukaryotic hosts is the expression of genetic factors responsible for DNA methylation (Mahan *et al*., 2000). This study provides evidence for expression in seawater of the 5′-methylthioadenosine/*S*-adenosylhomocysteine (MTA/SAH) nucleosidase gene. Methylation reactions depend on the appropriate methyl donor to be available when necessary. While *S*-adenosylmethionine (SAM) is the main donor of methyl groups in the cell, the end-product of the reaction is *S*-adenosylhomocysteine (SAH) that negatively regulates SAM-dependent methyltransferases. Therefore, SAH needs to be readily metabolized by MTA/SAH nucleosidase to adenine and *S*-ribosylhomocysteine. The activity of MTA/SAH nucleosidase is indispensable for the maintenance of methylation reactions in bacteria (Stepkowski *et al*., 2005). It has been demonstrated that severe defects in colonization of eukaryotic tissue occur when methylation processes are affected in methylation-defective *S. typhimurium* mutants (Heithoff *et al*., 1999). Such increases in methylation events and efficient regulation of methylation reactions may be accomplished by preferential expression of genes coding for MTA/SAH nucleosidases. Also, with methylation reactions being linked to successful eukaryotic colonization by bacteria, selective expression of methylation genes may be important for improving the likelihood of successful colonization of squid tissue by *Vibrio* bacteria.

Also important in metabolic processes in bacteria is the expression of chaperone molecules such as ClpB. The gene coding for this chaperone molecule was exclusively expressed during seawater growth of symbiotic bacteria.

ClpB is important for the refolding of luciferase (Zavilgelsky *et al*., 2004), the molecule responsible for light emission and important for quorum sensing in bacteria (Bassler, 1999). Zavilgelsky and colleagues (2004) demonstrated that presence of ClpB accelerated the refolding of luciferase and increased its efficiency approximately 10-fold. Considering that luciferases of *Vibrionaceae* bacteria are sensitive to thermoinactivation (Zavilgelsky *et al*., 2004), activity of ClpB would be considerably important in locations where water temperatures can change between winter and summer months (Jones *et al*., 2006; Soto *et al*., 2009), or where temperatures can exceed 28°C (Soto *et al*., 2009). Since bioluminescence is an important aspect of light organ symbioses, this phenomenon is extremely important for the fitness of light-producing bacteria.

Physiological adaptation is essential to maintain homeostasis. Metabolic processes responsible for such adaptations are regulated by the activity of sensory and regulatory proteins that control gene expression and enzymatic activity. Some of these mechanisms are conserved across bacterial species, while others are the result of microbial adaptation to specific environmental niches. Our research presents evidence of gene expression profiles that may explain the ability of a bacterium to transition between a free-living to mutualistic lifestyle, given its mechanisms of transmission (environmental). This study also provides support for differential gene expression relative to the ecology of *V. harveyi* bacteria and their ability to occupy multiple niches when grown in seawater and in squid hosts. Examining the processes that select for the evolution of symbiosis in the ocean can be better understood by defining important genetic factors that contribute to successful initiation and persistence of symbiotic associations. Future studies will include detailed investigations of these genes and the metabolic pathways related to synthesis or degradation of their products, and will address questions concerning host specificity and the array of mechanisms necessary for beneficial associations.

Experimental procedures

Bacterial growth conditions and extraction of total RNA

Specimens of *U. chinensis* were obtained from squid trawls off the coast of Cairns, Australia during the 2008 summer season. Light organs were dissected from the mantle cavity and a portion of it homogenized in sterile seawater. The rest of the light organs were kept in RNA Later (Applied Biosystems/Ambion, Austin, TX) for further extraction of mRNA. Serial homogenate dilutions were incubated on seawater tryptone agar (70% seawater v/v, 0.5% tryptone w/v, 0.3% yeast extract w/v, 0.3% glycerol v/v and 1.5% technical grade agar) at room temperature for 16 h. Individual colonies of luminous bacteria were isolated and used to inoculate 5 ml of SWT media and shaker incubated (250 r.p.m.) overnight. An aliquot (900 μl) of bacterial suspension was used for glycerol stocks. To identify transcripts selectively expressed by the *U. chinensis* isolates in seawater, bacteria were grown in sterile seawater (non-artificial) in a 28°C shaker incubator (250 r.p.m.) for 48 h. At this time an optical density (OD_{600}) of about 0.5 was reached. Seawater was obtained from Santa Catalina Island, CA. Bacteria were concentrated by centrifugation and RNA extracted following the protocol described below.

For light organ-expressed transcripts, three (partial) *U. chinensis* light organs were homogenized in RNA Later (Applied Biosystems/Ambion, Austin, TX) and centrifuged at 12 000 *g* for 2 min to pellet eukaryotic tissue, separating bacterial cells from the rest of the squid light organ. RNA extraction was completed using a protocol modified from Mangan and colleagues (1997). This protocol was incorporated in the RiboPure™ kit (Applied Biosystems/Ambion, Austin, TX), and manufacturer instructions were followed. Following RNA isolation, removal of rRNA from total RNA samples was performed with MICROB*Express* (Applied Biosystems/Ambion, Austin, TX). This additional step removes considerable amounts of rRNA from bacterial total RNA samples, reducing the chance of rRNA-derived complementary DNAs (cDNAs) being generated during SCOTS.

cDNA library construction

Complementary DNA libraries were constructed using the method proposed by Graham and Clark-Curtiss (1999; 2000). The first strand of cDNA was synthesized from total RNA using a Superscript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA), following manufacturer's instructions. Oligonucleotides with a random 9-mer at the 3′ end were used for this first step of cDNA library construction (Table 3). Second-strand synthesis was performed with the Klenow fragment of DNA polymerase (New England Biolabs, Beverly, MA) following manufacturer's instructions. Double-stranded cDNA was purified from remaining salts, enzymes and unincorporated nucleotides using the PCR purification kit (Qiagen, Valencia, CA).

Isolation of rRNA operon

In order to block cDNA derived from rRNA during SCOTS, the full ribosomal operon was PCR-amplified from total DNA using primers 16S Forward (Edwards *et al*., 1989) and 23S Reverse (Jones and Nishiguchi, 2006) (Table 3). PCR reactions yielded a product of approximately 5 kb in length, which was excised from a 1% agarose gel in 1× TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0) using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified ribosomal operon was subsequently inserted into the pCR® 2.1-TOPO® cloning vector (3.9 kb) from the TOPO® TA Cloning kit (Invitrogen Corporation, Carlsbad, CA).

Selective Capture Of Transcribed Sequences (SCOTS)

Normalization of cDNA libraries was conducted by three rounds of capture hybridization following a protocol from

Table 3. Primers for SCOTS protocol and verification.

Name	Sequence (5′–3′)	Application
Xba random	TGCTCTAGACGTCCTGATGGTT9(N)	cDNA library
Xba	TGCTCTAGACGTCCTGATGGTT	cDNA amplification
Sal random	ATATGTCGACTGAATTCCGTAGG9(N)	cDNA library
Sal	ATATGTCGACTGAATTCCGTAGG	cDNA amplification
16S forward	AGAGTTTGATCMTGGCTCAG	rRNA cloning
23S reverse	ATGGTTAAGCCTCACGGGCA	rRNA cloning
*spe*A sense	GTTAAACCACGTCTTGGCTTGCGT	Secondary verification
*spe*A antisense	ACCACCACCCACATCGAGGTATTT	Secondary verification
Chitinase sense	TCAGTCGGTGGATGGACGCT	Secondary verification
Chitinase antisense	GACGCTGATAATGGCGACAT	Secondary verification
*ilv*D sense	TCCGTCGAATACATGGTCAA	Secondary verification
*ilv*D antisense	TTTCAAACGCTGCTTTGTTG	Secondary verification
*omp*W sense	CCACCTACCTTTATGGTCC	Secondary verification
*omp*W antisense	GGTTTGTCGAATTAGCTTCACC	Secondary verification
*vib*H sense	TTGATGGCTACAGCTTGCAC	Secondary verification
*vib*H antisense	ATTGATCCACAGCGGTAAGG	Secondary verification

Jones and Nishiguchi (2006), modified from Graham and Clark-Curtiss (1999). Briefly, 30 μg of sonicated, biotinylated, genomic DNA (gDNA) and 50 μg of sonicated, cloned, ribosomal operon (rRNA) were precipitated and re-suspended in 40 μl of 10 mM 3-[4-(2-Hydroxyethyl)-1-piperazinyl] propanesulfonic acid (EPPS) (Sigma-Aldrich, St. Louis, MO). Simultaneously, 125 μl of amplified cDNAs from each condition were equally precipitated and re-suspended in 40 μl of EPPS. Then, hybrids and cDNAs were denatured (2 min at 98°C), normalized (30 min at 55°C) and mixed with 10 μl of 1 M sodium chloride. After an additional incubation at 55°C for 1 h, a single gDNA–rRNA hybridization reaction was added to each cDNA library and incubated at 55°C overnight.

Following overnight hybridizations, gDNA–rRNA hybrids were recovered using Dynal streptavidin-coated magnetic beads (Dynal/Invitrogen Corporation, Carlsbad, CA) with a magnetic stand (Applied Biosystems/Ambion, Austin, TX). Biotinylated gDNA–cDNA hybrids were washed and cDNA eluted from gDNA using 100 μl of 0.5 M NaOH and 0.1 M NaCl.

Eluted cDNA was PCR-amplified with a non-random primer (Table 3) specific for each growth condition, then purified and normalized through four additional rounds of capture hybridizations. Conversely, all reactions were completed using 1/10 of the volumes used in the first round.

Enrichment of cDNA was conducted in a way similar to capture hybridizations. However, biotinylated gDNA, blocked with rRNA, was additionally blocked with 25 μl of cDNA that was amplified after the last round of capture hybridization (seawater-derived cDNA to examine genes expressed in the light organ and vice versa). Hybrids were collected using Dynal streptavidin-coated magnetic beads. cDNA was eluted, PCR-amplified and purified using the aforementioned protocols. PCR products (Xba-amplified libraries for light organ-expressed transcripts and Sal libraries for seawater-expressed transcripts) were cloned using the TOPO® TA Cloning kit (Invitrogen Corporation, Carlsbad, CA).

Southern hybridizations for primary verification

Southern hybridizations were carried out to eliminate false-positive sequences that escaped the subtraction process. Both Xba (light organ-expressed) and Sal (seawater-expressed) libraries were denatured with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min and treated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.4) for 5 min and 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) for 5 min. Samples were then transferred onto a positively charged nylon membrane and cross-linked utilizing a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was then completed against DIG-labelled probes created from the fifth round captured sequences used to block the cross-linked sequences during the enrichment reactions.

Membranes were pre-hybridized in 15 ml of hybridization solution at 40°C and then hybridized at 40°C overnight. Hybridization was performed with 20 ng ml^{-1} of probe in DIG EasyHyb hybridization solution that was added to the membrane. After hybridization, two 2 min washes were completed using a low-stringency buffer (2× SSC, 0.1% SDS), followed by a 20 min washes at 65°C (with rotation) in high-stringency buffer (0.1× SSC, 0.1% SDS). A final wash with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) was executed for 5 min at room temperature.

Detection was completed by exposing the membrane to 0.5 ml of anti-DIG antibody/alkaline phosphatase enzyme conjugate for 20 min and washing twice with 4 ml of maleic acid buffer for 10 min. Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$) was then used for 5 min to start visualization with the enzyme substrate solution [4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4chloro-3-indolyl phosphate]. No signal indicated that the clone was exclusively expressed in either the light organ or the seawater condition.

Sequence analysis

Positive clones were sequenced using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA), and edited using Sequencher v 4.6 (Gene Codes Corporation, Ann Arbor, MI). Sequences were then compared with the National Center for Biotechnology Information (NCBI) database using BLAST 2.2.11 (Basic Local Alignment Search Tool, NCBI, NLM, NIH, Bethesda, MD) for initial confirmation of sequence identity. In addition, the presence and location of transcripts in

the *V. harveyi* genome was determined (Tables 1 and 2) by comparisons with the *V. harveyi* genome (Bassler *et al*., 2007).

Secondary verification using reverse transcriptase PCR

Four clones were picked for secondary verification using reverse transcription with the SuperScript™ One-Step RT-PCR system (Table 3). Primers were developed for each gene based on their corresponding sequence to the *V. harveyi* genome (Bassler *et al*., 2007). PCR reactions were executed as described previously by Jones and Nishiguchi (2006).

Acknowledgements

The authors thank B.W. Jones for his valuable advice on the SCOTS protocol and S. Edmands for collecting seawater from the USC Catalina Marine Station. C. Gorman assisted with sequencing of seawater and light organ transcripts. Funding was provided by NSF-IOS 0744498 and NIH-NIAID 1SC1AI081659 to M.K.N.

References

Actis, L.A., Fish, W., Crosa, J.H., Kellerman, K., Ellenberger, S.R., Hauser, F.M., and Sanders-Loehr, J. (1986) Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775 (pJM1). *J Bacteriol* **167:** 57–65.

Aeckersberg, F., Lupp, C., Feliciano, B., and Ruby, E.G. (2001) *Vibrio fischeri* outer membrane protein ompU plays a role in normal symbiotic colonization. *J Bacteriol* **183:** 6590–6597.

Alexeyev, D.O. (1992) The systematic position of bioluminescent squids of the family Loliginidae (Cephalopoda: Myopsida). *Zool Zhurnal* **71:** 12–23.

Anderson, F.E. (2000) Phylogenetic relationships among loliginid squids (Cephalopoda: Myopsida) based on analyses of mulitiple data sets. *Zool J Linnean Soc* **130:** 603–633.

Ariyakumar, D.S. (2007) Genetic factors regulating the symbiosis between sepiolid squids (Cephalopoda: Sepiolidae) and their *Vibrio* symbionts. Masters Thesis. New Mexico State University.

Ariyakumar, D.S., and Nishiguchi, M.K. (2009) Characterization of two host specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri–Euprymna tasmanica* mutualism. *FEMS Microbiol Lett* **299:** 65–73.

Aznar, R., and Alcaide, E. (1992) Siderophores and related outer membrane proteins produced by pseudomonads isolated from eels and freshwater. *FEMS Microbiol Lett* **98:** 269–275.

Bagg, A., and Neilands, J.B. (1987) Molecular mechanisms of regulation of siderophore-mediated iron assimilation. *Microbiol Rev* **51:** 509–518.

Bassler, B., Clifton, S.W., Fulton, L., Delehaunty, K., Fronick, C., Harrison, M., *et al.* (2007) *The* Vibrio harveyi *Genome Sequencing Project*. Bethesda, MD, USA: Genetics, Genome Sequencing Center, National Center for Biotechnology Information, NIH.

Bassler, B.L. (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr Opin Microbiol* **2:** 582–587.

Bassler, B.L., Gibbons, P.J., Yu, C., and Roseman, S. (1991) Chitin utilization by marine bacteria. Chemotaxis to chitin oligosaccharides by *Vibrio furnissii*. *J Biol Chem* **266:** 24268–24275.

Camilli, A., and Mekalanos, J.J. (1995) Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol Microbiol* **18:** 671–683.

Chotiyaputta, C., Nootmorn, P., and Jirapunpipat, K. (2002) Review of cephalopod fishery production and long term changes in fish communities in the Gulf of Thailand. *Bull Mar Sci* **71:** 223–238.

Dai, J.-H., Lee, Y-S., and Wong, H.-D. (1992) Effects of iron limitation of production of a siderophore, outer membrane proteins, and hemolysin and on hydrophobicity, cell adherance, and lethality for mice of *Vibrio parahaemolyticus*. *Infect Immun* **60:** 2952–2956.

Daigle, F., Graham, J.E., and Curtiss R., III (2001) Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). *Mol Microbiol* **41:** 1211–1222.

Davidson, S.K., Koropatnick, T.A., Kossmehl, R., Sycuro, L., and McFall-Ngai, M.J. (2004) NO means 'yes' in the squid–vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol* **6:** 1139–1151.

Dozois, C.M., Daigle, F., and Curtiss, R., 3rd (2003) Identification of pathogen-specific and conserved genes expressed *in vivo* by an avian pathogenic *Escherichia coli* strain. *Proc Natl Acad Sci USA* **100:** 247–252.

Dunlap, P.V., Davis, K.M., Tomiyama, S., Fujino, M., and Fukui, A. (2008) Developmental and microbiological analysis of the inception of bioluminescent symbiosis in the marine fish *Nuchequula nuchalis* (Perciformes: Leiognathidae). *Appl Environ Microbiol* **74:** 7471–7481.

Edwards, U., Rogall, T., Blocker, H., Emde, M., and Bottger, E.C. (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17:** 7843–7853.

Faruque, S.M., Nair, G.B., and Mekalanos, J.J. (2004) Genetics of stress adaptation and virulence in toxigenic *Vibrio cholerae*. *DNA Cell Biol* **23:** 723–741.

Faucher, S.P., Porwollik, S., Dozois, C.M., McClelland, M., and Daigle, F. (2006) Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. *Proc Natl Acad Sci USA* **103:** 1906–1911.

Gottschalk, G. (1986) *Bacterial Metabolism*. New York, NY, USA: Springer,

Graf, J., and Ruby, E.G. (1998) Host-derived amino acids support the proliferation of symbiotic bacteria. *Proc Natl Acad Sci USA* **95:** 1818–1822.

Graf, J., and Ruby, E.G. (2000) Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. *Mol Microbiol* **37:** 168–179.

Graham, J.E., and Clark-Curtiss, J.E. (1999) Identification of *Mycobacterium tuberculosis* RNA's synthesized in response to phagocytosis by human macrophages by

selective capture of transcribed sequences (SCOTS). *Proc Natl Acad Sci USA* **96:** 11554–11559.

Graham, J.E., Peek, R.M.J., Krishna, U., and Cover, T.L. (2002) Global analysis of *Helicobacter pylori* gene expression in human gastric mucosa. *Gastroenterology* **123:** 1637–1648.

Graham, J.E., and Clark-Curtiss, J.E. (2000) Identifying *Mycobacterium tuberculosis* virulence determinants – new technologies for a difficult problem: response. *Trends Microbiol* **8:** 100.

Guerrero-Ferreira, R.C., and Nishiguchi, M.K. (2007) Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis, Loliolus* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* **23:** 497–506.

Heithoff, D.M., Conner, C.P., Hanna, P.C., Julio, S.M., Hentschel, U., and Mahan, M.J. (1997) Bacterial infection as assessed by *in vivo* gene expression. *Proc Natl Acad Sci USA* **94:** 934–939.

Heithoff, D.M., Sinsheimer, R.L., Low, D.A., and Mahan, M.J. (1999) An essential role for DNA adenine methylation in bacterial virulence. *Science* **284:** 967–970.

Henderson, D.P., and Payne, S.M. (1994) *Vibrio cholerae* iron transport systems: roles of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. *Infect Immun* **62:** 5120–5125.

Hou, J.Y., Graham, J.E., and Clark-Curtiss, J.E. (2002) *Mycobacterium avium* genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). *Infect Immun* **70:** 3714–3726.

Jones, B.W., and Nishiguchi, M.K. (2004) Counterillumination in the bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* **144:** 1151–1155.

Jones, B.W., and Nishiguchi, M.K. (2006) Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. *Can J Microbiol* **52:** 1218–1227.

Jones, B.W., Lopez, J.E., Huttenburg, J., and Nishiguchi, M.K. (2006) Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol Ecol* **15:** 4317–4329.

Kierek-Pearson, K., and Karatan, E. (2005) Biofilm development in bacteria. *Adv Appl Microbiol* **57:** 79–111.

Kim, Y.R., Lee, S.E., Kim, C.M., Kim, S.Y., Shin, E.K., Shin, D.H., *et al.* (2003) Characterization and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. *Infect Immun* **71:** 5461–5471.

Lombardo, M.-J., Michalski, J., Martinez-Wilson, H., Morin, C., Hilton, T., Osorio, C.G., *et al.* (2007) An *in vivo* expression technology screen for *Vibrio cholerae* genes expressed in human volunteers. *Proc Natl Acad Sci USA* **104:** 18229–18234.

McFall-Ngai, M.J. (1998) The development of cooperative associations between animals and bacteria: establishing détente among domains. *Am Zool* **38:** 593–608.

McFall-Ngai, M.J., and Ruby, E.G. (1998) Sepiolids and vibrios: when first they meet. *Bioscience* **48:** 257–265.

Mahan, M.J., Heithoff, D.M., Sinsheimer, R.L., and Low, D.A. (2000) Assessment of bacterial pathogenesis by analysis of gene expression in the host. *Annu Rev Genet* **34:** 139–164.

Mangan, J.A., Sole, K.M., Mitchison, D.A., and Butcher, P.D. (1997) An effective method of RNA extraction from bacteria refractory to disruption, including mycobacteria. *Nucleic Acids Res* **25:** 675–676.

Mekalanos, J.J. (1985) Cholera toxin: genetic analysis, regulation, and the role in pathogenesis. *Curr Top Microbiol Immun* **118:** 97–118.

Merrell, D.S., Hava, D.L., and Camilli, A. (2002) Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. *Mol Microbiol* **43:** 1471–1491.

Moeck, G.S., and Coulton, J.W. (1998) TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. *Mol Microbiol* **28:** 675–681.

Naidu, A.J., and Yadav, M. (1997) Influence of iron, growth temperature and plasmids on siderophore production in *Aeromonas hydrophila*. *J Med Microbiol* **46:** 833–838.

Nishiguchi, M.K. (2002) Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microbiol Ecol* **44:** 10–18.

Nishiguchi, M.K., Ruby, E.G., and McFall-Ngai, M.J. (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid–*Vibrio* symbioses. *Appl Environ Microbiol* **64:** 3209–3213.

Nootmorn, P., and Chotiyaputta, C. (2002) Species diversity, biomass and community structure of cephalopods off Adang-Rawi Archipelago, Thailand. *Bull Mar Sci* **71:** 591–600.

Noriyuki, O., Saito, M., Yamamoto, S., Yoshida, T., Miyoshi, S., and Shinoda, S. (1994) Structure of vulnibactin, a new polyamine-containing siderophore from *Vibrio vulnificus*. *Biometals* **7:** 109–116.

Nyholm, S.V., Stabb, E.V., Ruby, E.G., and McFall-Ngai, M.J. (2000) Establishment of an animal–bacterial association: recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci USA* **97:** 10231–10235.

Owens, L., Austin, D.A., and Austin, B. (1996) Effect of strain origin on siderophore production in *Vibrio harveyi* isolates. *Dis Aquat Organ* **27:** 157–160.

Patel, C.N., Wortham, B.W., Lines, J.L., Fetherston, J.D., Perry, R.D., and Oliveira, M.A. (2006) Polyamines are essential for the formation of plague biofilm. *J Bacteriol* **188:** 2355–2363.

Ruby, E.G., and Asato, L.M. (1993) Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Arch Microbiol* **159:** 160–167.

Slauch, J.M., Mahan, M.J., and Mekalanos, J.J. (1994) *In vivo* expression technology for selection of bacterial genes specifically induced in host tissues. *Methods Enzymol* **235:** 481–492.

Somboonwiwat, K., Supungul, P., Rimphanitchayakit, V., Aoki, T., Hirono, I., and Tassanakajon, A. (2006) Differentially expressed genes in hemocytes of *Vibrio harveyi*-challenged shrimp *Penaeus monodon*. *J Biochem Mol Biol* **39:** 26–36.

Soto, W., Gutierrez, J., Remmenga, M.D., and Nishiguchi, M.K. (2009) Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. *Microb Ecol* **57:** 140–150.

Stepkowski, T., Brzezinski, K., Legocki, A.B., Jaskólski, M., and Béna, G. (2005) Bayesian phylogenetic analysis

reveals two-domain topology of *S*-adenosylhomocysteine hydrolase protein sequences. *Mol Phylogenet Evol* **34:** 15.

Stim, K.P., and Bennett, G.N. (1993) Nucleotide sequence of the *adi* gene, which encodes the biodegradative acid-induced arginine decarboxylase of *Escherichia coli*. *J Bacteriol* **175:** 1221–1234.

Svitil, A.L., Chadhain, S., Moore, J.A., and Kirchman, D.L. (1997) Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. *Appl Environ Microbiol* **63:** 408–413.

Tarleton, J.C., and Ely, B. (1991) Isolation and characterization of *ilvA*, *ilvBN*, and *ilvD* mutants of *Caulobacter crescentus*. *J Bacteriol* **173:** 1259–1267.

Voet, D., and Voet, J.G. (2004) *Biochemistry*. Hoboken, NJ, USA: John Wiley & Sons.

Wolf, M.K., and Crosa, J.H. (1986) Evidence for the role of a siderophore in promoting *Vibrio anguillarum* infections. *J Gen Microbiol* **132:** 2944–2952.

Yu, C., Lee, A.M., Bassler, B.L., and Roseman, S. (1991) Chitin utilization by marine bacteria. A physiological function for bacterial adhesion to immobilized carbohydrates. *J Biol Chem* **266:** 24260–24267.

Zavilgelsky, G.B., Kotova, V.Y., Mazhul, M.M., and Manukhov, I.V. (2004) The effect of Clp proteins on DnaK-dependent refolding of bacterial luciferases. *Mol Biol (Moscow)* **38:** 427–433.

Guerrero-Ferreira, R.C. and M.K. Nishiguchi. 2011. Bacterial biodiversity in natural environments. In: J. López-Pujol (ed), Biodiversity/ Book 3. In Tech, Open access publisher. http://www.intechopen.com/articles/show/title/bacterial-biodiversity-in-natural-environments.

Bacterial Biodiversity in Natural Environments

Ricardo C. Guerrero-Ferreira and Michele K. Nishiguchi
Emory University. School of Medicine. Department of Pediatrics. Division of Pediatric Infectious Diseases. Atlanta, GA
New Mexico State University. Department of Biology. Las Cruces, NM
United States of America

1. Introduction

Increased accessibility to the technologies for high-throughput sequencing has revealed the diversity and dynamism of bacterial genomes. It is now known that variation in gene content between bacterial strains may encompass 30–35% of the genes in the genome. Because this genetic diversity and genome variability triggers the emergence of pathogens as well as novel metabolic capabilities in the newly originated strains, there are implicit consequences to human health and the economy. Equally, genomic flexibility is understandably an impacting factor for bacterial populations because of the important role in their evolution and speciation. Conversely, in natural environments, bacteria species are constantly exposed to chemical, physical, and trophic gradients, as well as intra- and inter-specific interactions that may play an additional role in shaping bacterial biodiversity.

More specifically in interactions between bacteria and hosts, it is well accepted that the bacterial counterpart are highly susceptible to genetic changes. They usually have increased generation times when compared to eukaryotic organisms, and are genetically more diverse (Steinert et al., 2000). These aspects, in addition to the production of extremely large populations, allow bacterial species to be efficient at acquiring novel metabolic traits that facilitate their success in colonizing new environments.

Highly controlled processes of genetic regulation and genetic diversity are responsible for the ability of bacteria to live and survive under environmental conditions that are continuously changing. Processes that give rise to the genetic variability in bacteria are ultimately responsible for bacterial adaptation. Such processes are represented by point mutations, homologous recombination, and horizontal gene transfer events. Genetic and phenotypic variation is more frequently observed among bacteria since they are haploid organisms and are more susceptible to such changes that are not masked by recombination.

2. Horizontal/lateral gene transfer and biodiversity

Horizontal or lateral gene transfer (HGT or LGT) is one factor, if not the most important mechanism, influencing genomic variability and diversity in bacteria. New research efforts have recognized the importance of this process and aim to understand the rates of genetic exchange in bacterial species in natural settings. Whole genome analysis has corroborated that bacterial evolution may occur by horizontal gene flow between a range of species and genera. The current section briefly describes the role of gene transfer processes between

various bacterial species, and whether this influences microbial biodiversity in a variety of ecological niches in natural environments.

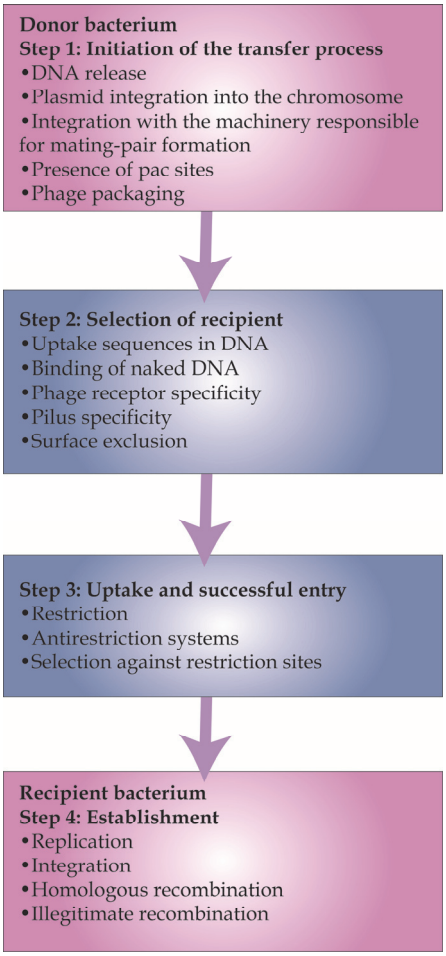


Fig. 1. Modified from Thomas & Nielsen (2005). This figure outlines the steps that take place during transfer of DNA from a donor to a recipient bacterium. The process starts with the availability of DNA from the donor cell and ends with DNA being acquired permanently by the recipient bacterium.

HGT has been observed in a wide variety of species, both in the Archaea and Bacteria domains (Smets & Barkay 2005). A number of mechanisms have accounted for the amount of transfer in specific groups, namely gene acquisition, homologous recombination, and orthologous replacement (Boucher & Stokes 2006). These processes are particularly important for changing the ability of an organism that is "clonal" and never changing, to one that has newly acquired traits that allow adaptation, speciation, and evolution to a new ecological niche. Numerous studies have documented the similarity between species of

bacteria based on phylogenetic analysis of specific genes. For example, the HMG-coenzyme A reductase gene (*mvaA*), responsible for lipid metabolism, is found in a number of *Vibrio* species and was likely transferred from an archaeal donor, since mevalonate biosynthesis/degradation is an archaeal trait (Boucher & Doolittle 2000). Likewise, studies analyzing metabolic networks in *Escherichia coli* have demonstrated that particular changes are due to HGT, with very little contribution from gene duplication events (Pal et al., 2005). These changes can be linked to bacterial response to the environment, particularly when the change requires some specific metabolic capability allowing the organism to adapt more quickly to the selection imposed by the surrounding habitat. Such HGT events are usually driven by newly acquired genes that are coupled by their enzymatic pathways (*i.e.*, operons), which allow processes such as transport and degradation of external nutrients, or accommodation of an abiotic pressure (temperature, salinity). Interestingly, most HGT loci that are environment-specific are not expressed under normal laboratory conditions, demonstrating that selection of HGT loci is in part driven by adaptation to novel environments (Pal et al., 2005). This supports that HGT is a mechanism that is probably more common in natural environments than previously thought; indeed, when analyzing genes that are physiologically coupled, their functions are specific for certain environmental conditions (*i.e.*, arabinose or mannitol uptake; (Pal et al., 2005; Thomas & Nielsen 2005).

HGT has also been examined via phylogenetic reconstruction, where similar suites of genes that group together do not have a common ancestor (Gogarten & Townsend 2005). Unexpected phylogenetic distributions can therefore be explained as either HGT or an ancient gene duplication followed by differential gene loss. Oftentimes, deep-branching lineages with commonly used loci (rRNA) may also contain artifacts that are exhibited during phylogenetic reconstruction, and may provide discordance when compared to less conserved (faster adapting) molecules. This can be observed in genes that experience little or no purifying selection and are oftentimes saturated with substitutions, resulting in little phylogenetic information (Gogarten & Townsend 2005). Interestingly, examining the ratio of non-synonymous to synonymous substitutions (K_a/K_s) between *E. coli* and *Salmonella enterica* demonstrated that most horizontally acquired genes were under purifying selection, despite the K_a/K_s ratio being higher than other *E. coli* genes (0.19 vs. 0.05; (Daubin & Ochman 2004). Another example of this "neutral" selection is found in *Vibrio splendidus* (Thompson et al., 2005), where diversity at the genome level is huge compared to the sequence divergence at the 16S rRNA locus. Genome size differed between 4.5 and 5.6 Mb, eluding that there are multiple subpopulations that have unique ecological niches, despite that most of the HGT events are neutral to the recipient. If HGT events are rare, they have the probability of becoming fixed (due to selective sweeps), and are not detected under modern molecular analysis (Gogarten & Townsend 2005). Thus, in contrast to network modeling predictions, HGT may be selectively filtered against in order to deter any novel deleterious functions that may override adaptive advantages to a novel environment.

Clearly, the acquisition of genes through HGT is a much quicker and effective way for an organism to adapt to changing environments rather than their evolution via natural selection (Smets & Barkay 2005). This can be supported by observations of beneficial gene acquisition, such as antibiotic resistance, degradation pathways for xenobiotics, and bioremediation. But such observations may not be driven by environmental change alone; specific gene cassettes or mobile genetic elements may be augmented due to the increased

presence of substrates that are useable by such organisms. Recently, there have been *in vitro* experiments on microbial communities to determine whether HGT events are induced by changes in environmental conditions through plasmid transfer (Sorensen et al., 2005). Such studies have allowed the detection of environmental hotspots that influence the rate of transfer via conjugation. Combining this experimental information with mathematical models (Sorensen et al., 2005) that utilize variables such as the rate of transfer, formation of new conjugants, density of donors and recipients, cell growth, and plasmid loss in homogeneous and mixed communities will be helpful in determining whether HGT is an important mechanism for driving ecological adaptations. This is particularly important in epidemics where pathogenic bacteria are more increasingly virulent. Since HGT events basically drive the evolution of bacterial "chimeras", categorizing whether a particular strain or species is genetically similar is becoming more and more difficult with modern technology (Gevers et al., 2005). The combination of both genetic background and ecological specificity will undoubtedly be the future criteria used for understanding how HGT drives microbial evolution in natural populations.

3. The role of bacterial viruses in bacterial biodiversity

In addition to the inter-specific relations that occurs within bacterial populations in nature, the association between bacteria and their viruses (bacteriophages or phages) is, quantitatively speaking, the dominant host-pathogen relationship in nature (Calendar 2006). Interactions between bacteria and phages are also expected to be particularly important, owing to the considerably fast rates of evolution of the two counterparts, the essential role bacterial communities play in ecosystem dynamics, and the emerging interest on phages as an alternative to antibiotics in the control and treatment of bacterial infections in agricultural and clinical settings (Levin & Bull 2004). More importantly, recent studies on soil bacteria and their phages have demonstrated that ecological interactions alone are not sufficient to explain the structure, population dynamics, and function of microbial communities in nature, but that rapid coevolution of bacteria and bacteriophages is also indispensable (Gómez & Buckling 2011).

Bacteriophages (also known as phages) are viruses that infect bacteria. They are widespread, with many known groups existing and found in abundance in open and coastal waters, sediments, soils, and animal tissues (Ackermann 2003). Their general life cycle (Fig. 2) varies between phage families, but generally involves adsorption, infection, and release from the host (Calendar 2006). During this cycle of phage production, the cell's metabolic machinery is reprogrammed to continually produce new phage particles with the components of the biosynthetic apparatus rerouted from basal tasks necessary for bacterial growth (Campbell 2003).

Among bacteriophage groups, infection by temperate bacteriophages often results in modification of existing properties or the acquisition of new capabilities in the bacterial host (Waldor 1998). Bacteriophages are able to integrate within the host genome during infection (a process known as lysogenic conversion), making them accountable for bacterial adaptation to new niches (Canchaya et al., 2003) and known contributors to host virulence (Rajadhyaksha & Rao 1965; Takeda & Murphy 1978; Waldor & Mekalanos 1996; Lee et al., 1999; Oakey & Owens 2000). In actuality, the process of lysogenic conversion is a key player in the evolution of Gram-positive and Gram-negative pathogens.

By definition, lysogeny is the process by which bacteriophage genome is stored in a quiescent state within the genome of a host bacterium (lysogen) (Canchaya et al., 2003). During this harboring period, transcription of the phage (temperate) genome does not take place, allowing the bacterial host to remain functional. Activation of phage transcription at this time would result in cell death (Campbell 2001). Exchange of genetic material from the virus to the bacteria can be so all-encompassing that bacteriophages have become recognized as considerable, if not the most important drivers of bacterial evolution (Krisch 2003). Temperate phages are thought of as important players in bacterial evolution because of their ability to establish long-term genetic symbioses with their host bacterium (Abedon & Lejeune 2005).

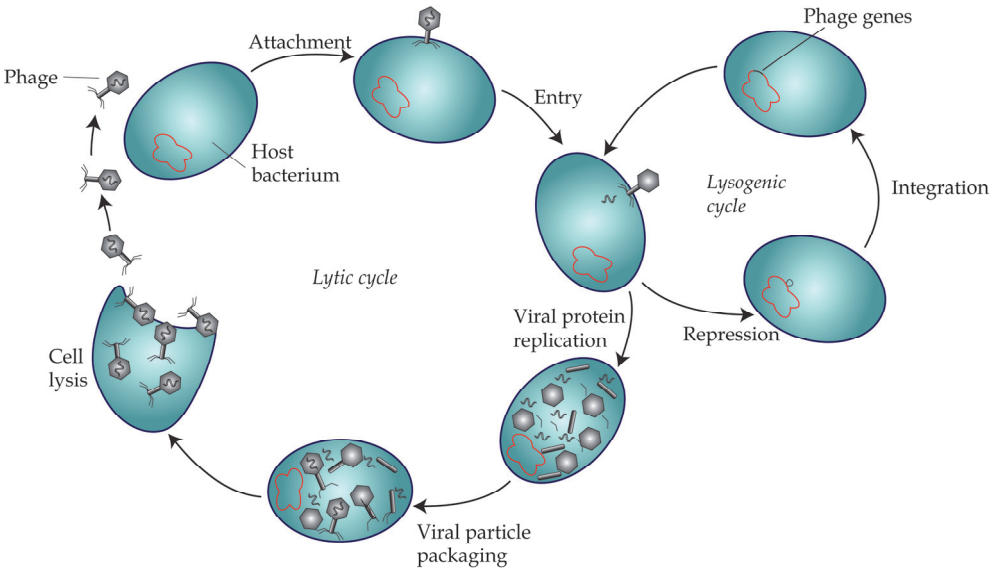


Fig. 2. Basic Phage life cycle, modified from Campbell (2003). Adsorption includes extracellular search (diffusion-mediated), random encounter between phage and host bacterium, attachment of phage to bacterium via a specific receptor, and injection of nucleic acids into the bacterial cytoplasm. This figure represents infection by a temperate phage. Phage development is temporarily repressed and phage DNA integrates into the bacterial chromosome (lysogenic cycle). Virulent phages, as well as temperate phages during their lytic cycles assemble by means of the bacterial metabolic machinery. Lastly, the cell lyses for phage progeny release.

These types of genetic associations have severe consequences in human populations owing to the variety of bacterial virulence factors that are known to be of prophage origin (Brussow et al., 2004). Among others, human diseases such as botulism, diphtheria, cholera, and *E. coli* associated conditions are virus mediated. For a more comprehensive review of prophage associated diseases please refer to (Boyd et al., 2001; Boyd & Brüssow 2002; Brussow et al., 2004).

In a recent work by Canchaya et al. (2004) it was determined that prophages are particularly abundant in the genomes of bacterial pathogens. As expected, the authors confirmed that

the presence of these prophages was in most cases responsible for encoding virulence genes and that the phenotypic characteristics that allow a strain its "uniqueness" within a bacterial consortia were contributed by the viral genome. However, this observation is not unique to pathogenic bacteria since other types of symbioses may require the bacterium to acquire particular functions to successfully colonize a host. For instance, in the gut commensal *Lactobacillus johnsonii*, it has been demonstrated that prophage derived genetic material contributes to approximately 50% of strain-specific DNA (Ventura et al., 2003).

Mechanistically, it would not be beneficial for a bacterium to fix an entire prophage genome. On the other hand, phage-derived functions that have been co-opted by the host bacterium would very likely be subjected to fixation (Casjens 2003). This makes sense considering that new ecological niches can be exploited by a bacterial species more rapidly with the acquirement of genetic material in the form of mobile DNA of phage origin. Genes of viral origin that are of no intrinsic evolutionary value to the bacterium are consequently expected to be deleted (Casjens 2003; Brussow et al., 2004). Considering that a very small amount of prophage DNA is found in the bacterial chromosome, this raises the question of why phages do not accumulate in large numbers in most cases. Campbell (2001) suggested that some genes may remain phage-borne instead of being incorporated into the bacterial genome when the host does not benefit constantly, but rather intermittently, from the product of these genes.

Prophages from bacterial pathogens that encode virulence factors have two situations that are observed (Brussow et al., 2004). Firstly, a phage-encoded toxin could be directly responsible for causing the specific disease. This is the case of *Vibrio cholerae*, Shiga toxin-producing *Escherichia coli*, *Corynebacterium diphtheriae*, and *Clostridium botulinum* (Abedon & Lejeune 2005). Conversely, the bacterial host may carry more than just the prophage material, and each phage-encoded factor contributes incrementally to the fitness of the host (either by direct contribution to fitness or by causing disease).

4. The role of biofilms in bacterial biodiversity

It is widely understood that most bacteria found in natural environments, as well as clinical and industrial settings, exist in biofilms. These are complex communities of microorganisms attached to surfaces or to the tissues of specific hosts, or any substrate with the adequate supply of nutrients and water (Costerton et al., 1987). These surface-associated communities are often composed of more than one species that interact with one another and their environment, and are distinct from bacteria growing in a free-living, planktonic state (Stewart & Franklin 2008).

Biofilm formation has evolved as a strategy of bacteria to establish themselves as a substrate-associated community in the environment or to become more persistent and less invasive to a host, while simultaneously taking advantage of the availability of nutrients found in those settings. The biofilm state is considered the stable period in a biological cycle that is comprised of several steps, namely initiation, maturation, maintenance, and dissolution (Fig. 3). Cells initially attach to a surface, which in most cases requires swimming motion generated by rotating flagella, and is initiated in response to specific environmental stimuli, such as nutrient availability. In most cases, the organisms undertake a series of physiological and morphological changes, transitioning from free-living, planktonic cells to non-motile, surface-attached cells. Biofilms continue to persist and grow for as long as the nutrient requirements are met. Once they are nutrient deprived, the cells separate from the surface and initiate to a free-living state (O'Toole et al., 2000).

Due to the variations in environmental conditions within the biofilm, represented by both chemical and biological heterogeneity, members of a biofilm community are subject to different selective pressures according to their location within the biofilm matrix. Therefore, bacterial cells not only express phenotypic traits that allow adaptation for growth in these surface-associated communities (as opposed to planktonic growth), but they also display phenotypic variability that allows them to thrive within a chemically heterogeneous environment.

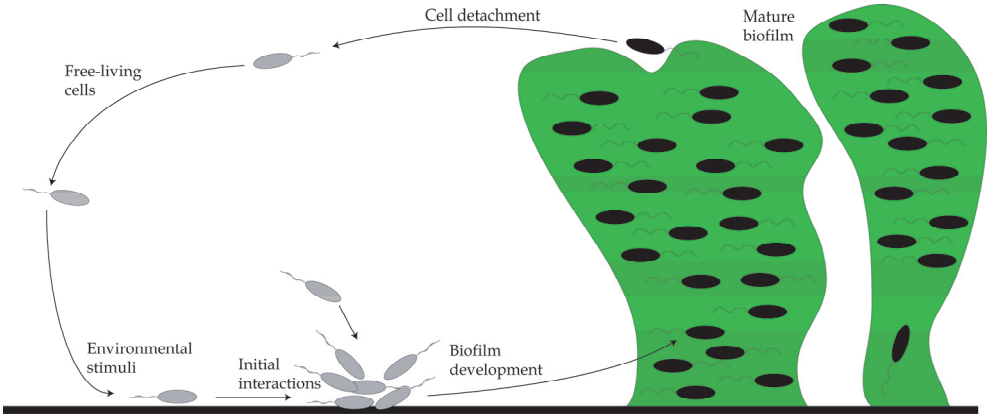


Fig. 3. Model of biofilm development. Modified from O'Toole et al. (2000). Free-living cells establish contact with other cells or with surfaces, which results in the formation of microcolonies and further maturation of the biofilm matrix. Cells from a mature biofilm can go back to a planktonic lifestyle to complete the cycle of biofilm formation.

It is expected that the chemical variability within a biofilm matrix would lead to considerable variability in the physiology of the cells that occupy the various areas within the community (de Beer et al., 1994; Xu et al., 1998). As observed in liquid cultures *in vitro,* where varying growth conditions such as temperature, aeration, and nutrient availability may impair the ability of the bacteria to grow, it is not surprising that limiting conditions within specific regions of a mature biofilm may slow or even completely stop bacterial growth and activity (Chavez-Dozal & Nishiguchi 2011). Also, metabolic waste accumulation would have an effect on the physiological state of the bacteria, mostly by changes in pH within the matrix (Stewart & Franklin 2008).

One important aspect affecting the success of a multi-species biofilm community is the ability of each member of the consortium to adapt to the presence of a second species. In a two-species community (*Acinetobacter* sp. (strain C6) and *Pseudomonas putida* (strain KT2440)), Hansen et al. (2007) demonstrated that selection in an environment such as a biofilm leads to the evolution of unequal interactions. Specific mutations in the genome of one species lead to adaptation to the presence of the other. The resulting community proved to be more successful in stability and productivity, than the ancestral community. This indicates that simple mutations due to the interactions in the biofilm generated a more intimate and specialized association.

Biofilms are ideal for the exchange of genetic material of various origins (bacterial or viral). Several studies have also demonstrated that bacterial conjugation (horizontal transfer of

genetic material between two cells by physical contact) occurs within biofilms (Christensen et al., 1998; Hausner & Wuertz 1999) and this process is known to have a high impact in the evolution of bacterial lineages (Ochman et al., 2000). In a study of *E. coli* K12 biofilms, Ghigo (2001) studied how conjugative plasmids directly contribute to the ability of a bacterial cell to establish a biofilm. In this study, the author demonstrated that natural conjugative plasmids expressed factors that promote the transition of the bacteria to a biofilm forming state from a free-living state, and argued that this process supports the infectious transfer of the plasmid. Considering that antibiotic resistance is carried by bacteria through conjugative plasmids, the use of antibiotics and biocides in clinical and agricultural settings may have promoted the selection for resistant strains (bearing specific plasmids) that are more likely to form a biofilm.

It is clear that in order to be successful in the environment, a bacterial community needs to be efficient in growth and reproduction. However, it is equally important to be able to avoid, tolerate, and defend themselves against natural predators. Most studies on bacterial predation have looked at the strategies they use to increase their survival under grazing pressure by protozoans (Matz & Kjelleberg 2005). Among these adaptive traits, cell surface properties (Wildschutte et al., 2004), motility (Matz & Jurgens 2005), microcolony establishment, and quorum sensing (Matz et al., 2004) are the most studied and their results suggest that grazing by protozoans is an important contributor to bacterial diversification and to the selection of specific adaptations to defend themselves against predators. Biofilm formation has therefore emerged as an adaptive response to flagellate predation. Previous results (Matz et al., 2004) have demonstrated that *Pseudomonas aeruginosa* cells transition into a microcolony forming state upon encounter with a predator. These cell conglomerates reach a size that is beyond the prey size of the protozoan. In addition, mature biofilms build up acute toxicity to the flagellate predator via quorum sensing-mediated up-regulation of lethal compounds.

Bacterial-host interactions during mutualistic symbiosis are another, well studied example of associations in which bacteria utilize adaptive strategies of survival and reproduction in order to fight the normal defense mechanisms of the host (McFall-Ngai 1994; McFall-Ngai 1998). Similar to virulence determinants in bacteria which are regulated in their expression by both environmental and host factors (Heithoff et al., 1997; Soto et al., 2009), many novel genes are selectively expressed during the establishment and persistence of a mutualistic association (Jones & Nishiguchi 2006; Guerrero-Ferreira & Nishiguchi 2010). An example of this type of association is the mutualistic interaction between *Vibrio fischeri* and the bobtail squid *Euprymna scolopes.* It is understood that the bacterial symbionts are able to establish themselves within the host tissue by forming biofilm in the epithelium-lined crypts of the squid light organ. This was demonstrated by Ariyakumar & Nishiguchi (2009), where *V. fischeri* mutants with a reduced ability to form biofilm in vitro were unable to successfully colonize squid light organs and were not detected in any section of the crypt region.

Biofilms are the leading cause of contamination of medical devices and in industrial and agricultural settings. The initial adhesion and further colonization of bacteria onto solid surfaces is essential for biofilm formation, and therefore is the cause of infections of material of biological or medical use (Shemesh et al., 2010). Formation of microcolonies within a biofilm facilitates genetic exchange, favors genetic diversity, and promotes phenotypic variability within bacterial communities. Further understanding of these phenomena is necessary to understand the mechanisms bacterial communities utilize to infect and persist

in humans and other organisms and surfaces. Deciphering the factors that control bacterial diversity will not only permit a more vigorous model of bacterial evolution and speciation but also a more comprehensive analysis of the likelihood of emergence of new biofilm-forming infectious agents.

5. Conclusion

Bacterial diversity in natural populations is continually being revitalized and revisited due to the availability of whole genomes, *in situ* measurements of HGT, and manipulation of regulatory genes that are influenced by changes in the natural environment. It is especially important to consider the diversity of bacteria, and what selection pressures have driven the evolution of species or strains that can accommodate such a wide ecological breadth. Combining phylogenetics, metabolic networks, models of HGT, and phenotypic characterization of ecotypes, will help provide meaningful explanations of how bacteria can adapt so quickly to specific abiotic and biotic factors, and what forces are important to create the diversity of microbes we observe today.

6. Acknowledgements

The authors would like to thank the editors of this book for including this interesting topic in bacterial diversity. R.C.G-F. is supported by grants from the National Institutes of Health. M.K.N. is supported by grants from the National Science Foundation and the National Institutes of Health.

7. References

Abedon, S. T., & Lejeune, J. T. (2005). Why bacteriophage encode exotoxins and other virulence factors. *Evolutionary Bioinformatics Online*, Vol. 1, No. 1, pp. 97-110.

Ackermann, H. W. (2003). Bacteriophage observations and evolution. *Research in Microbiology*, Vol. 154, No. 4, pp. 245-251.

Ariyakumar, D. S., & Nishiguchi, M. K. (2009). Characterization of two host-specific genes, mannose-sensitive hemagglutinin (mshA) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri–Euprymna tasmanica* mutualism. *FEMS Microbiology Letters*, Vol. 299, No. 1, pp. 65-73.

Boucher, Y., & Doolittle, W. F. (2000). The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Molecular Microbiology*, Vol. 37, No. 4, pp. 703-716.

Boucher, Y., & Stokes, H. W. (2006). The roles of lateral gene transfer and vertical descent in Vibrio evolution, In: *The Biology of Vibrios*. F. L. Thompson, B. Austin & J. Swings, pp. 84-94. ASM Press, Washington, D.C.

Boyd, E. F., & Brüssow, H. (2002). Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *Trends in Microbiology*, Vol. 10, No. 11, pp. 521-529.

Boyd, E. F., Davis, B. M., & Hochhut, B. (2001). Bacteriophage-bacteriophage interactions in the evolution of pathogenic bacteria. *Trends in Microbiology*, Vol. 9, No. 3, pp. 137-144.

Brussow, H., Canchaya, C., & Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews*, Vol. 68, No. 3, pp. 560-602.

Calendar, R. (2006). *The Bacteriophages*, Oxford University Press, Inc., New York, NY.

Campbell, A. (2003). The future of bacteriophage biology. *Nature Reviews Microbiology*, Vol. 4, No. 6, pp. 471-477.

Campbell, A. M. (2001). *Lysogeny*, John Wiley & Sons, Ltd.

Canchaya, C., Fournous, G., & Brüssow, H. (2004). The impact of prophages on bacterial chromosomes. *Molecular Microbiology*, Vol. 53, No. 1, pp. 9-18.

Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M. L., & Brussow, H. (2003). Phage as agents of lateral gene transfer. *Current Opinion in Microbiology*, Vol. 6, No. 4, pp. 417-424.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A., & Brussow, H. (2003). Prophage genomics. *Microbiology and Molecular Biology Reviews*, Vol. 67, No. 2, pp. 238-276.

Casjens, S. (2003). Prophages and bacterial genomics: what have we learned so far? *Molecular Microbiology*, Vol. 49, No. 2, pp. 277-300.

Chavez-Dozal, A., & Nishiguchi, M. K. (2011). Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri*. *Journal of Basic Microbiology*, Vol. 51, pp. 1-7.

Christensen, B. B., Sternberg, C., Andersen, J. B., Eberl, L., Moller, S., Givskov, M., & Molin, S. (1998). Establishment of new genetic traits in a microbial biofilm community. *Applied and Environmental Microbiology*, Vol. 64, No. 6, pp. 2247-2255.

Costerton, J. W., Cheng, K.-J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. *Annual Review of Microbiology*, Vol. 41, No. 1, pp. 435-464.

Daubin, V., & Ochman, H. (2004). Bacterial genomes as new gene homes: the genealogy of ORFans in E. coli. *Genome Research*, Vol. 14, No. 6, pp. 1036-1042.

de Beer, D., Stoodley, P., Roe, F., & Lewandowski, Z. (1994). Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnology and Bioengineering*, Vol. 43, No. 11, pp. 1131-1138.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F. L., & Swings, J. (2005). Opinion: Re-evaluating prokaryotic species. *Nature Reviews Microbiology*, Vol. 3, No. 9, pp. 733-739.

Ghigo, J. M. (2001). Natural conjugative plasmids induce bacterial biofilm development. *Nature*, Vol. 412, No. 6845, pp. 442-445.

Gogarten, J. P., & Townsend, J. P. (2005). Horizontal gene transfer, genome innovation and evolution. *Nature Reviews Microbiology*, Vol. 3, No. 9, pp. 679-687.

Gómez, P., & Buckling, A. (2011). Bacteria-Phage Antagonistic Coevolution in Soil. *Science*, Vol. 332, No. 6025, pp. 106-109.

Guerrero-Ferreira, R. C., & Nishiguchi, M. K. (2010). Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. *Environmental Microbiology Reports*, Vol. 2, No. 4, pp. 514-523.

Hansen, S. K., Rainey, P. B., Haagensen, J. A., & Molin, S. (2007). Evolution of species interactions in a biofilm community. *Nature*, Vol. 445, No. 7127, pp. 533-536.

Hausner, M., & Wuertz, S. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied and Environmental Microbiology*, Vol. 65, No. 8, pp. 3710-3713.

Heithoff, D. M., Conner, C. P., Hanna, P. C., Julio, S. M., Hentschel, U., & Mahan, M. J. (1997). Bacterial infection as assessed by in vivo gene expression. *Proceedings of the National Academy of Sciences*, Vol. 94, No. 3, pp. 934-939.

Jones, B. W., & Nishiguchi, M. K. (2006). Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. *Canadian Journal of Microbiology*, Vol. 52, No. 12, pp. 1218-1227.

Krisch, H. M. (2003). The view from Les Treilles on the origins, evolution and diversity of viruses. *Research in Microbiology*, Vol. 154, No. 4, pp. 227-229.

Lee, S. H., Hava, D. L., Waldor, M. K., & Camilli, A. (1999). Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. *Cell*, Vol. 99, No. 6, pp. 625-634.

Levin, B. R., & Bull, J. J. (2004). Population and evolutionary dynamics of phage therapy. *Nature Reviews Microbiology*, Vol. 2, No. 2, pp. 166.

Matz, C., Bergfeld, T., Rice, S. A., & Kjelleberg, S. (2004). Microcolonies, quorum sensing and cytotoxicity determine the survival of Pseudomonas aeruginosa biofilms exposed to protozoan grazing. *Environmental Microbiology*, Vol. 6, No. 3, pp. 218-226.

Matz, C., & Jurgens, K. (2005). High motility reduces grazing mortality of planktonic bacteria. *Applied and Environmental Microbiology*, Vol. 71, No. 2, pp. 921-929.

Matz, C., & Kjelleberg, S. (2005). Off the hook--how bacteria survive protozoan grazing. *Trends in Microbiology*, Vol. 13, No. 7, pp. 302-307.

McFall-Ngai, M. J. (1994). Animal-Bacterial Interactions in the early life history of marine invertebrates: The *Euprymna scolopes/Vibrio fischeri* symbiosis. *American Zoologist*, Vol. 34, No. 4, pp. 554-561.

McFall-Ngai, M. J. (1998). The development of cooperative associations between animals and bacteria: establishing detente among domains. *American Zoologist*, Vol. 38, No. 4, pp. 593-608.

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm formation as microbial development. *Annual Review of Microbiology*, Vol. 54, No. 1, pp. 49-79.

Oakey, H. J., & Owens, L. (2000). A new bacteriophage, VHML, isolated from a toxin-producing strain of *Vibrio harveyi* in tropical Australia. *Journal of Applied Microbiology*, Vol. 89, No. 4, pp. 702-709.

Ochman, H., Lawrence, J. G., & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature*, Vol. 405, No. 1, pp. 299-304.

Pal, C., Papp, B., & Lercher, M. J. (2005). Adaptive evolution of bacterial metabolic networks by horizontal gene transfer. *Nature Genetics*, Vol. 37, No. 12, pp. 1372-1375.

Rajadhyaksha, A. B., & Rao, S. S. (1965). The role of phage in the transduction of the toxinogenic factor in *Corynebacterium diphtheriae*. *Journal of General Microbiology*, Vol. 40, No. 3, pp. 421-429.

Shemesh, M., Tam, A., Aharoni, R., & Steinberg, D. (2010). Genetic adaptation of Streptococcus mutans during biofilm formation on different types of surfaces. *BMC Microbiology*, Vol. 10, No. 1, pp. 51.

Smets, B. F., & Barkay, T. (2005). Horizontal gene transfer: perspectives at a crossroads of scientific disciplines. *Nature Reviews Microbiology*, Vol. 3, No. 9, pp. 675-678.

Sorensen, S. J., Bailey, M., Hansen, L. H., Kroer, N., & Wuertz, S. (2005). Studying plasmid horizontal transfer in situ: a critical review. *Nature Reviews Microbiology*, Vol. 3, No. 9, pp. 700-710.

Soto, W., Gutierrez, J., Remmenga, M. D., & Nishiguchi, M. K. (2009). Salinity and temperature effects on physiological responses of Vibrio fischeri from diverse ecological niches. *Microbial Ecology*, Vol. 57, No. 1, pp. 140-150.

Steinert, M., Hentschel, U., & Hacker, J. (2000). Symbiosis and pathogenesis: evolution of the microbe-host interaction. *Naturwissenschaften*, Vol. 87, No. 1, pp. 1-11.

Stewart, P. S., & Franklin, M. J. (2008). Physiological heterogeneity in biofilms. *Nature Reviews Microbiology*, Vol. 6, No. 3, pp. 199-210.

Takeda, Y., & Murphy, J. R. (1978). Bacteriophage conversion of heat-labile enterotoxin in *Escherichia coli*. *Journal of Bacteriology*, Vol. 133, No. 1, pp. 172-177.

Thomas, C. M., & Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology*, Vol. 3, No. 9, pp. 711-721.

Thompson, J. R., Pacocha, S., Pharino, C., Klepac-Ceraj, V., Hunt, D. E., Benoit, J., Sarma-Rupavtarm, R., Distel, D. L., & Polz, M. F. (2005). Genotypic diversity within a natural coastal bacterioplankton population. *Science*, Vol. 307, No. 5713, pp. 1311-1313.

Ventura, M., Canchaya, C., Pridmore, D., Berger, B., & Brussow, H. (2003). Integration and distribution of Lactobacillus johnsonii prophages. *Journal of Bacteriology*, Vol. 185, No. 15, pp. 4603-4608.

Waldor, M. K. (1998). Bacteriophage biology and bacterial virulence. *Trends in Microbiology*, Vol. 6, No. 8, pp. 295-297.

Waldor, M. K., & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science*, Vol. 272, No. 5270, pp. 1910-1914.

Wildschutte, H., Wolfe, D. M., Tamewitz, A., & Lawrence, J. G. (2004). Protozoan predation, diversifying selection, and the evolution of antigenic diversity in Salmonella. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 101, No. 29, pp. 10644-10649.

Xu, K. D., Stewart, P. S., Xia, F., Huang, C. T., & McFeters, G. A. (1998). Spatial physiological heterogeneity in Pseudomonas aeruginosa biofilm is determined by oxygen availability. *Applied and Environmental Microbiology*, Vol. 64, No. 10, pp. 4035-4039.

Applied and Environmental Microbiology, Jan. 2011, p. 642–649 Vol. 77, No. 2
0099-2240/11/$12.00 doi:10.1128/AEM.02105-10

Phylogeographical Patterns among Mediterranean Sepiolid Squids and Their *Vibrio* Symbionts: Environment Drives Specificity among Sympatric Species[▽]†

D. J. Zamborsky and M. K. Nishiguchi*

Department of Biology, MSC 3AF, New Mexico State University, P.O. Box 30001, Las Cruces, New Mexico 88003-8001

Received 6 September 2010/Accepted 2 November 2010

Bobtail squid from the genera *Sepiola* and *Rondeletiola* (Cephalopoda: Sepiolidae) form mutualistic associations with luminous Gram-negative bacteria (*Gammaproteobacteria*: *Vibrionaceae*) from the genera *Vibrio* and *Photobacterium*. Symbiotic bacteria proliferate inside a bilobed light organ until they are actively expelled by the host into the surrounding environment on a diel basis. This event results in a dynamic symbiont population with the potential to establish the symbiosis with newly hatched sterile (axenic) juvenile sepiolids. In this study, we examined the genetic diversity found in populations of sympatric sepiolid squid species and their symbionts by the use of nested clade analysis with multiple gene analyses. Variation found in the distribution of different species of symbiotic bacteria suggests a strong influence of abiotic factors in the local environment, affecting bacterial distribution among sympatric populations of hosts. These abiotic factors include temperature differences incurred by a shallow thermocline, as well as a lack of strong coastal water movement accompanied by seasonal temperature changes in overlapping niches. Host populations are stable and do not appear to have a significant role in the formation of symbiont populations relative to their distribution across the Mediterranean Sea. Additionally, all squid species examined (*Sepiola affinis*, *S. robusta*, *S. ligulata*, *S. intermedia*, and *Rondeletiola minor*) are genetically distinct from one another regardless of location and demonstrate very little intraspecific variation within species. These findings suggest that physical boundaries and distance in relation to population size, and not host specificity, are important factors in limiting or defining gene flow within sympatric marine squids and their associated bacterial symbionts in the Mediterranean Sea.

Population structure can be determined in nature by a number of intrinsic elements in the environment, including selective pressures resulting from abiotic variables. Many of these factors are the result of physical change in the environment over long periods of time (change in landscape, habitat fragmentation), while some, such as temperature, currents, and salinity, are constantly in flux (3, 7, 21). Small-scale changes in salinity and temperature can have significant effects and are exacerbated in marine environments, where osmotic balance and physical gradients are naturally established. Specifically, temperature may affect the distribution, viability, and fitness of organisms that have not readily adapted to such changing conditions (38, 57). This is pertinent to organisms living in symbiosis with each other, where one partner is dependent upon the other for a number of capabilities, such as physiological functions, nutritional requirements, and predator/prey avoidance (16, 20, 35). Interestingly, very few marine symbioses span across broad geographic ranges, since an enormous spectrum of abiotic and biotic conditions may prevent the ability of the symbiont to move between host populations. Studies of population structure within marine symbioses have increasingly pointed toward the underlying importance of understanding the nonliving elements of the environment as they apply to the distribution of symbiotic partners (either host or symbiont) across broadly distributed landscapes (42).

Population structure between host and symbionts in many symbioses, particularly mutualistic associations, often produce evidence of cospeciation between established partner lineages in both terrestrial and marine environments (1, 34, 37). The evidence for cospeciation or parallel cladogenesis is often attained through comparative phylogenetic analysis, which may neglect to unveil or account for various environmental factors. Abiotic characteristics of individual habitats may drive ecological or physiological processes, which in turn influence population distribution and interspecies interactions for both host and symbiont, particularly those that are environmentally transmitted. This may also influence either benefits or costs that arise due to the adaptability of each partner to accommodate not only environmental changes but also changes occurring with respect to each other. Considering the potential influencing power of the environment, it is prudent to explore the relative impact of nonliving habitat characteristics on symbiont-host distributions as it applies to the phylogeography and population history of both host and symbiont. This is particularly true for the mutualistic symbiosis between sepiolid squids (Mollusca: Cephalopoda) and bioluminescent bacteria in the genus *Vibrio* (*Gammaproteobacteria*: *Vibrionaceae*).

Symbiosis between sepiolid squids (Cephalopoda: Sepiolidae) and *Vibrio* bacteria has become an ideal model to study environmentally transmitted symbioses between cooperative microbes and their eukaryotic hosts (19, 44, 67). The best-known association within this family of squids is between members of the genus *Euprymna* and its symbiont *Vibrio fischeri* (21,

* Corresponding author. Mailing address: New Mexico State University, Department of Biology, MSC 3AF, Box 30001, Las Cruces, NM 88003-8001. Phone: (575) 646-3721. Fax: (575) 646-5665. E-mail: nish@nmsu.edu.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 12 November 2010.

TABLE 1. Collection sites of sepiolid squid species and their bacterial symbionts

Location and date specimen collected	Host species	Symbiont species	Depth/temp (m/°C)
Banyuls-sur-Mer, France (42°35′29′′N, 3°2′22′′E), 2003–2008	*Sepiola affinis*	*Vibrio fischeri*, *V. logei*	3–10/20–22
	S. robusta	*V. fischeri*, *V. logei*	20/13
	S. intermedia	*V. fischeri*, *V. logei*	20–30/13
	Rondeletiola minor	*Photobacterium leiognathi*	70/13
Bari, Italy (42°35′29′′N, 3°2′22′′E), 2005	*S. affinis*	*V. fischeri*, *V. logei*	3–10/21–24
	S. robusta	*V. fischeri*, *V. logei*	10–20/14–18
	S. intermedia	*V. fischeri*, *V. logei*	10–20/14–18
Bay of Biscay, France (43°35′43′′N, 01°48′08′′E), 2007	*R. minor*	*P. leiognathi*	80–120/12–16

29). The symbiosis is established when environmentally transmitted, symbiosis-competent vibrios infect the light organs of newly hatched juvenile squid (30, 53). Upon entrance into the light organ, the bacteria flourish in a relatively nutrient-rich, protected environment and in turn achieve high densities within the organ (59). When a sufficiently high number of symbiont cells is achieved in the light organ, the bacteria become luminescent based on a communication mechanism known as quorum sensing (9, 12, 28, 31). *Vibrio* bacteria then induce a program of drastic morphological changes in the squid host, which helps to establish the symbiont population and completes development of the naïve light organ (33). Development and timing of this *Vibrio* population coincides with the nocturnal hunting behavior of the squid, in which the bacteriogenic luminescence is used in a predator/prey avoidance behavior known as counterillumination (20).

At dawn, the squid bury themselves in the sand and expel 90 to 95% of the symbiotic bacteria from the light organ (25, 54). This expulsion results in a "seeded" environment in which newly hatched, axenic juvenile squids can acquire symbiotically competent *Vibrio* bacteria. The result of this local acquisition of symbionts previously inhabiting hosts from the same population may lead to high degrees of specificity between symbiotic partners (37, 39). In the association between bobtail squids of the genus *Euprymna* (*E. scolopes* and *E. tasmanica* in the Indo-West Pacific Ocean) and *Vibrio fischeri*, this high degree of mutual exclusivity has been observed with *V. fischeri* (43, 67). Although *V. fischeri* is the only symbiont present in most *Euprymna* species, strain variation has been observed within and between populations, indicating that vibrios can migrate among host species complexes, changing the geographic mosaic of these symbionts within their host squids (21, 68). This distribution is affected mainly by other factors, such as temperature, which has been observed to influence the location of other marine populations in similar habitats (21, 22).

In comparison, mutualistic symbioses between Mediterranean squid from the genera *Sepiola* and *Rondeletiola* and Gram-negative bacteria from the genera *Vibrio* and *Photobacterium* (11, 18) demonstrate that species specificity is not always common among all sepiolid genera (17, 38). Furthermore, temperature has been observed to be a major factor determining symbiont distribution both within hosts as well as in their free-living state (22, 38, 57). Therefore, abiotic factors have a much larger influence in the establishment of environmentally transmitted symbiosis than just recognition and specificity to a particular squid host. The Mediterranean Sea harbors 10 species of squid from the genus *Sepiola* and one species from the genus *Rondeletiola* (*R. minor*), all of which possess a bacteriogenic light organ (6, 38, 41, 55). Sepiolid squid species in the Mediterranean exist in sympatry, with overlapping population boundaries along the northern coast of the Mediterranean (55). Also of interest pertaining to the Mediterranean sepiolid fauna is the fact that each squid can harbor two species of symbionts in their light organs (17, 38). Therefore, the aim of this study was to understand whether the population structure of different sympatric host species had a direct effect on symbiont distribution or if the combined influence of abiotic factors (temperature, water movement, and salinity) and multiple symbiont species influences the association among sympatric populations. This is in contrast to previous studies of allopatric populations, in which *Vibrio* bacteria were specialists with their specific host species (21). We hypothesized that a lack of specificity occurs between host squid and the *Vibrio* species found in Mediterranean sepiolids (*V. fischeri*, *V. logei*, and *Photobacterium leiognathi*) and that temperature was a major force in determining these host-symbiont assemblages. Using a phylogeographical approach, we tested whether any type of genetic structure existed among species of sepiolids in the Mediterranean Sea and if their *Vibrio* symbionts' distribution was influenced by host specificity or environment.

MATERIALS AND METHODS

Collection of specimens. Specimens used in this study were collected from sites in southern France (Banyuls-sur-Mer), eastern Italy (Bari, Italy), and the North Atlantic (Bay of Biscay) (Table 1). Banyuls-sur-Mer is located in the south of France near the Spanish border at the foot of the Pyrenees Mountains. Specimens were collected by trawling (*R. minor*, *S. robusta*, and *S. intermedia*) or by hand during SCUBA dives at night (*S. affinis*). Italian sepiolid squid were collected by trawl net off the coast of Bari, Italy, while samples of *R. minor* were collected via mid-water trawl on several cruises in the Bay of Biscay. Other sequence data were incorporated for individual squid (*S. robusta* and *S. affinis*) from previously published sources as well (27, 40, 41). To increase the sample population size, other hosts and associated symbiotic strains from previous work were incorporated into the overall analysis (27, 40, 41). These samples were included in order to equalize the number of strains and host species for each collection site.

DNA isolation and sequencing. All specimens of squid were anesthetized by placing the animal on ice and subsequently removing the light organ through ventral dissection. The light organ from each individual was then homogenized in a 2-ml tube to isolate the symbiotic bacteria inside. Each homogenate was placed through a series of dilutions in sterile filtered seawater before plating the bacteria. These homogenates were spread onto seawater tryptone agar plates (SWT; 0.5% tryptone, 0.3% yeast extract, 70% seawater, 0.3% glycerol, and 15%

agar). After growth on SWT (12 to 24 h, 20 to 28°C), 10 to 15 individual colonies from each plate (representing one squid light organ) were selected, and each colony was then stab inoculated into an SWT agar vial for transport back to New Mexico State University. This sampling regime is applicable to any strain variation that may be observed in an individual squid light organ (68).

DNA was extracted from each individual isolate using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA). Approximately 5 to 10 ng of the extracted genomic DNA was subsequently used in 4 separate PCRs to amplify the 16S rRNA locus of the symbiotic bacteria using *Vibrio*-specific primer sets (see Table S1 in the supplemental material). A total of 5 to 10 ng of the template DNA was also used to amplify a 650-base-pair region of the glyceraldehyde phosphate dehydrogenase locus (*gapA*) using both previously published *Vibrio*-specific primers (40) and primers newly designed specifically for *V. logei* (see Table S1). The *gapA* locus was used to analyze the bacterial symbiont populations in this study, as it has been shown to be amenable to phylogeographic and phylogenetic analyses for *Vibrio* populations (21, 40). This is due to the fact that the *gapA* locus is less conserved than other metabolic, constitutively expressed genes in the *Vibrio* genus and is capable of differentiating between strains of the same species of bacteria. The *V. logei* primers were designed and utilized due to broad problems associated with amplifying the *gapA* locus in a number of light organ isolates determined to be vibrios. DNA from host squid tissue was extracted by homogenizing a 20- to 25-mg piece of tissue dissected from the anterior mantle. The homogenate was processed using the same DNeasy blood and tissue kit (Qiagen, Valencia, CA). After DNA extraction, approximately 5 ng of host DNA was used in a PCR to amplify the cytochrome *c* oxidase subunit I (COI) and 12S and 16S rRNA loci (21, 27, 41) (see Table S1 in the supplemental material).

PCRs to amplify the above-specified loci from both host squid and *Vibrio* bacteria were performed as 25-μl reaction mixtures containing 5 to 10 ng of extract DNA, 0.2 μM both forward and reverse primers, 0.2 U of *Taq* polymerase (GoTaq Flexi DNA polymerase; Promega, San Luis Obispo, CA), 2.5 mM magnesium chloride ($MgCl_2$), a final concentration of 200 μM dGTP, dATP, dCTP, and dCTP (50 μM each), and 1× buffer (Tris-HCl, KCl, and 0.1% Triton X-100). PCRs were completed in an MJ Research Dyad Disciple thermal cycler (Waltham, MA). Following PCRs, amplified fragments were purified using either a QIAquick PCR purification kit (Qiagen, Valencia, CA) or the QIAEX II gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were used for presequencing reactions in an MJ Dyad Disciple thermal cycler (Waltham, MA) using BigDye Terminator 3.1 (Applied Biosystems, Foster City, CA). Reactions were cleaned of excess dideoxynucleotides and fluorescent dyes by centrifuging the samples through 400 μl of dehydrated Sephadex (Sigma, St. Louis, MO) and subsequently collected in a 96-well sequencing plate. All samples were sequenced using the Applied Biosystems 3100 automated capillary sequencer (Applied Biosystems, Foster City, CA) at New Mexico State University. Sequences were aligned and edited using Sequencher, version 4.6 (Gene Codes Corporation, Ann Arbor, MI).

Analysis of variance. The baseline variance of initial sample populations of both host squid and symbiotic bacteria was measured using an analysis of molecular variance (AMOVA) using the program ARLEQUIN, version 3.0 (15). Variance calculated in this program was indicative of variation within populations at the different collection sites (North Atlantic, France, and Italy) and between each of the populations sampled. Variation observed within a population at each site was calculated with a base-pair-by-base-pair polymorphic measure, theta.

Nested clade analysis. Analysis of the sequence data for building haplotype networks was completed in TCS version 1.12 (10). TCS v. 1.12 utilizes a statistical parsimony-based procedure based on Templeton's rules (61). The resulting symbiont and host networks were nested, and potential ambiguities and complementary problems were addressed and resolved using the nesting rules according to Templeton et al. (60, 63). The final nested clade information was then analyzed by the program GEODIS (50). GEODIS analyzes the haplotype network and interprets the nesting to describe intrinsic variation between clades, assuming the specific levels of nesting according to the associated geographical locations.

Phylogenetic analysis. Corroboration of the hierarchical nesting scheme that was revealed through the formation of the haplotype network and the nesting of unique clades was generated using the parsimony-based phylogenetics program POY, version 4.1.2 (66). Equal weights for transitions-transversions were assigned for all trees, for both host squid populations and bacterial symbionts, by the use of the respective loci (combined analyses using all molecular loci).

TABLE 2. Symbiont species present at each collection site[a]

Location	Symbiont species (no. of isolates)[b]	% of total symbiont population
Banyuls-sur-Mer, France	*V. fischeri* (74)	78
	V. logei (16)	16
	P. leiognathi (12)	6
Bari, Italy	*V. fischeri* (58)	85
	V. logei (10)	15
Bay of Biscay, Atlantic	*P. leiognathi* (8)	100*

[a] Species identification is based on the 16S rRNA sequence for all symbiotic bacteria isolated from squid light organs. Percentages are based on the total population within one particular site. *, all symbionts isolated from the *R. minor* host were *Photobacterium leiognathi*. No *Vibrio* species were isolated from these hosts.

[b] $n = 178$.

RESULTS

Genetic variation and haplotype prevalence. Variation in the sequence loci from both host squid and *Vibrio*/*Photobacterium* symbionts was measured from a subsampling of 178 symbiont strains. *V. fischeri* and *V. logei* strains were isolated from all *Sepiola* species, and *P. leiognathi* only was isolated from *R. minor* host populations (for a total of 56 individual squids sampled). *Vibrio*/*Photobacterium* identification was determined using the 1,600-base-pair 16S rRNA locus. After each symbiont strain was identified as either *V. fischeri*, *V. logei*, or *P. leiognathi*, the 650-base-pair *gapA* locus was used in the nested clade analysis for the bacterial symbionts. Numerical distribution of species for each geographical location reflects the relative prevalence of each symbiont species in the populations sampled in this study (Table 2). A high occurrence of *V. fischeri* symbionts was found in both French and Italian populations (Fig. 1).

Both *Vibrio* and *Photobacterium* populations used in this study represented a total of 31 haplotypes. There were, respectively, 23, 6, and 2 species-specific haplotypes found in the analysis belonging to *V. fischeri*, *V. logei*, and *P. leiognathi*. One haplotype (haplotype 24) was shared among *V. logei* symbionts from both French and Italian localities (Fig. 1). In total, 24 strains identified in the initial 16S rRNA species identification were not used in the formation of haplotype networks due to incomplete *gapA* sequence information.

The distribution of haplotypes of symbiotic *V. fischeri* demonstrates grouping in geographical "clusters," which was also reflected in the final nesting. In the Mediterranean Sea, French and Italian clusters were clearly recognizable in the nesting scheme (Fig. 1). The genetic distance between populations of *V. fischeri* from the two Mediterranean sites was comparatively small; the closest association between a French *V. fischeri* haplotype and an Italian *V. fischeri* haplotype was a 3-base-pair difference or 3 unsampled haplotypes (Fig. 1). This sampling discrepancy occurs between the second most numerous French haplotype and the largest Italian haplotype (haplotype 15) (Fig. 1). Final nesting was the most parsimonious arrangement, which resulted in no confirmable false-positive inferences regarding colonization events or other population-level indices (64). Only one closed-loop structure arose in the construction

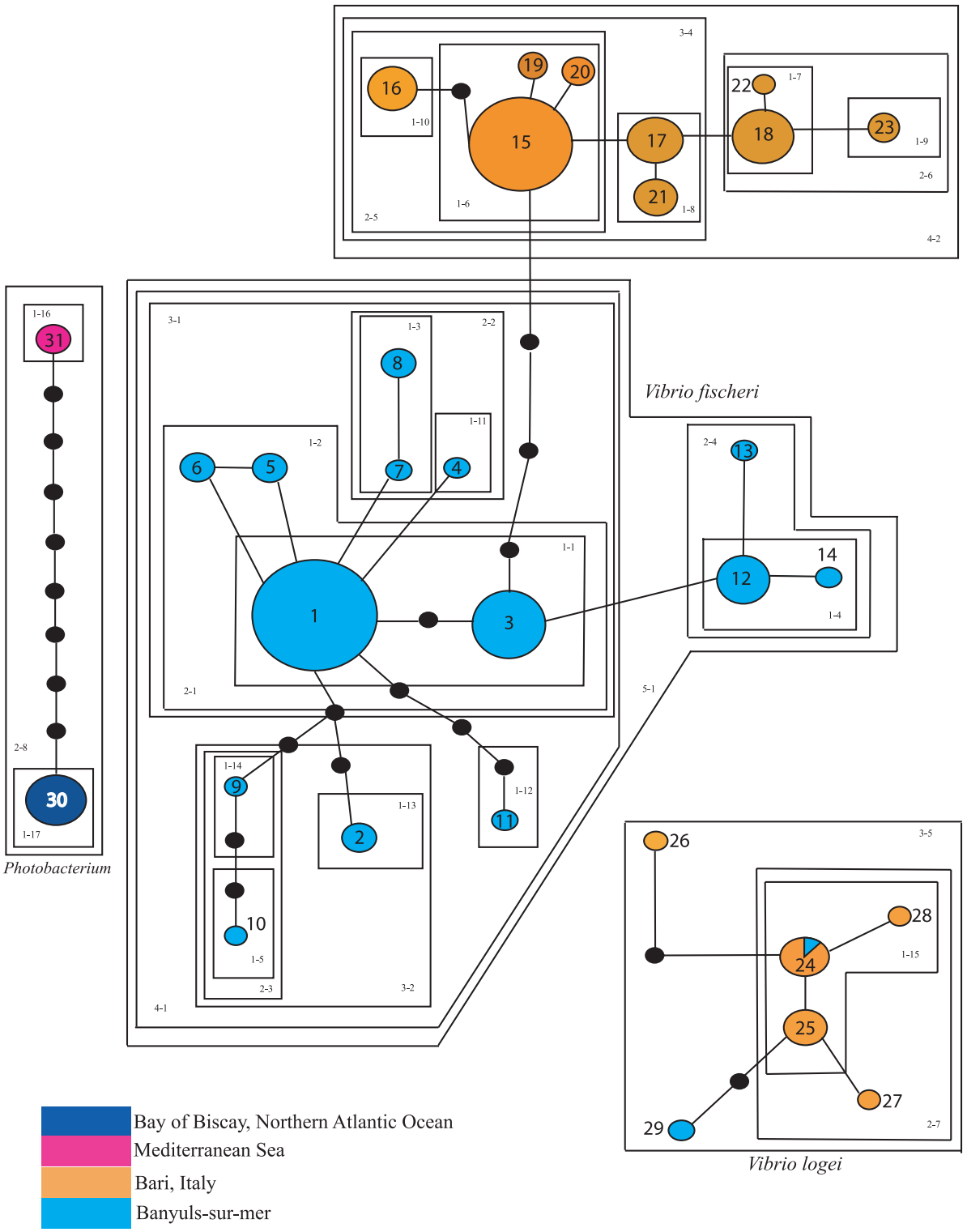


FIG. 1. TCS-generated haplotype network and nested clades for Mediterranean vibrios and *Photobacterium leiognathi* symbionts. Each line represents one base pair difference, with black circles representing an unsampled intermediate haplotype. Each numbered circle represents a distinct haplotype, with color denoting collection site. The size of each circle is proportional to the number of individuals representing each given haplotype. Nested clades are marked by two-number identifiers within each box, signifying the representative nested clade.

of the symbiont haplotype network, which was resolved according to Templeton's rules (62) (Fig. 1).

The *V. logei* network was comparably small, reflecting the total proportion of *V. logei* bacteria within the total symbiont sample population. This network consists of 6 distinct haplotypes (24–29) (Fig. 1), with one haplotype containing both French and Italian strains (haplotype 24). The nesting of these haplotypes followed an interior-to-exterior scheme, with relat-

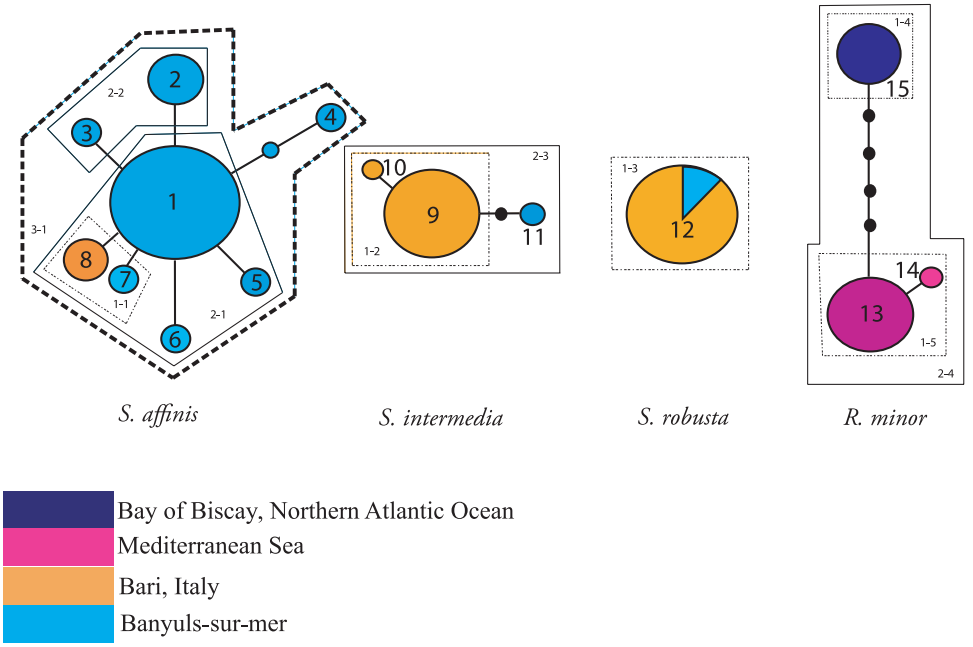


FIG. 2. Haplotype network and corresponding nesting schematic of individual species haplotype networks for both Mediterranean and Northern Atlantic (Bay of Biscay) squids. All species were genetically distinct at the COI, 12S, and 16S loci by a 10 bp-difference, precluding any connection between separate species.

edness, not geography, being the most parsimonious nesting result.

Complete cladograms for symbiotic bacteria from the sampled populations infer a lack of evidence for restriction of gene flow between populations (Fig. 1). Clades 1-3, 1-4, and 2-3 show potential restriction in gene flow and possible habitat fragmentation, which may be a reflection of the terminal, exterior location of these clades within the total cladogram. Overall, the nesting diagrams for the symbiont populations demonstrate geographical structure. Nesting at the fourth level (clades 4-1 and 4-2) (Fig. 1) is indicative of an association between genetic relatedness and local environment.

All *Vibrio* symbionts formed one clade in the phylogenetic analysis based on location (see Fig. S1 in the supplemental material). This confirms the geographical bias of the haplotype network and subsequent nesting diagrams (Fig. 1). Interestingly, *Vibrio* isolates (both *V. fischeri* and *V. logei*) were contained with the *Photobacterium* clade, with Italian *V. logei* sister to Italian *V. fischeri*. All French *V. fischeri* isolates analyzed were sister to the Italian *V. fischeri*/*V. logei* clade.

For host squids, a total of 15 haplotypes were found in all sample populations (Fig. 2). Initial networks that were calculated using a single-gene model (COI) revealed a relatively restricted network, supporting strong species delineation. To diversify the network, two additional genes (12S and 16S rRNA) were incorporated into the analysis to uncover unique variation inherent in other genes within species and among host populations. *S. affinis* had the largest number of COI haplotypes from all host species sampled, with a total of 9 distinct haplotypes, resulting in the most diverse and possibly least parsimonious set of haplotypes of all genes examined. Both 12S and 16S rRNA data had a more limited set of haplotypes for *S. affinis* (6 and 7 haplotypes, respectively), compared to 8 when using all 3 loci (Fig. 2). *S. intermedia* and *R. minor* each showed a relatively low number of representative haplotypes. These particular species demonstrate a smaller, more inclusive set of haplotypes, with both *S. intermedia* and *R. minor* having only 3 haplotypes. *R. minor* haplotypes are split between two distant geographic locales, Banyuls-sur-Mer and the Bay of Biscay (Fig. 2). Interestingly, all *S. robusta* hosts (8 total) sampled in this study possessed the same haplotype for all three loci investigated (haplotype 12). Haplotypes were all species specific, with no introgression of any individual haplotype between squid species sampled for all loci. Additionally, combined phylogenetic analysis of all loci confirmed the structure and orientation of the nested haplotypes as they occur within the populations sampled, with *S. affinis* and *S. intermedia* sister to *S. robusta* and with all *R. minor* individuals being sister to the *Sepiola* clade (see Fig. S2 in the supplemental material).

Host squid networks and nested diagrams demonstrated no introgression between sympatric species (Fig. 2). No connectable distances were found between species, regardless of distance or geographical location. The largest number of base pair differences among any one species was two in the population of *S. affinis*, nesting at the highest level of 3 (clade 3-1) (Fig. 2). Evidence for range expansion or fragmentation was unsubstantiated in this analysis due to a lack of direct evidence for the geographical separation of any of the host species (recall that all species are sympatric).

Variation within total populations of both hosts and symbionts was calculated using the measurement theta (θ). Symbiont theta values indicate higher per-base-pair variation, ranging from 0.00 to 0.0331. All symbiont populations, except the relatively small sample size of *P. leiognathi*, demonstrated some degree of genetic variation within each population (Table 3).

TABLE 3. Within-population variance (theta, θ) for each host species at each respective collection site[a]

Host species	Collection site	No. of hosts	Host θ	Symbiont θ
S. affinis	Banyuls-sur-Mer	18	0.0292	0.0331
	Bari	9	0.0220	0.0297
S. intermedia	Banyuls-sur-Mer	4	0.0026	0.0055
	Bari	7	0.0000	0.0082
S. robusta	Banyuls-sur-Mer	4	0.0000	0.0071
	Bari	6	0.0000	0.0025
R. minor	Banyuls-sur-Mer	4	0.0184	0.0000*
	Northern Atlantic	4	0.0000	0.0000*

[a] Values were calculated only for hosts from which at least two *Vibrio* or *Photobacterium* strains were isolated. *, all symbionts are *P. leiognathi*. Variation among this species was observed only between populations in the Mediterranean versus the North Atlantic (Bay of Biscay).

The largest amount of within-population variation (θ = 0.0331) was observed with *V. fischeri* sampled from Banyuls-sur-Mer, France, from *S. affinis* (Table 3). Conversely, no genetic variation was found in symbiotic *P. leiognathi* isolated from the Bay of Biscay (Table 3). Additionally, no significant variation within populations of *P. leiognathi* was observed over the time period sampled, as only 3 derivative novel haplotypes arose chronologically in the course of this study (Fig. 1).

Theta values for host squids ranged from 0.00 to 0.0292 (range indicative of all 3 loci sampled for the entire host population) (Table 3). *S. robusta* hosts from both French and Italian sample sites demonstrated no genetic variation among individuals and therefore have a θ value of 0. Relatively low theta values for host variation across all loci indicate little or no divergence within populations of species, regardless of the location of populations.

DISCUSSION

Symbiont population structure. Symbiotic bacteria that were sampled from collected host squids exhibited much more dynamic patterns across geographical locations than their respective host squids. Only one closed-loop structure was observed with the initial haplotype network construction within the *V. fischeri* population from Banyuls-sur-Mer (France). This ambiguity occurred between the most highly represented haplotype (Fig. 1, haplotype 1) and less-represented satellite haplotypes (haplotypes 5 and 6). These structures in future haplotype networks need to be carefully considered due to the potential for false-positive results (23, 48).

The most obvious trend apparent in the nesting of individual haplotypes is a strong correlation between relatedness and geography, with different amounts of variation depending on the species sampled. In *V. fischeri* populations, geographical grouping is evident (Fig. 1; see also Fig. S1 in the supplemental material). Within this species, no haplotypes were shared between Banyuls-sur-Mer and Bari. This suggests that genetic differences are due to geographical boundaries for symbiotic bacteria. In *V. logei* populations, a shared haplotype between sample sites was found, indicating that a small amount of introgression has occurred between geographical locations and that not all populations are in complete isolation from each other. *V. logei* is a known psychrophile and has been shown to prefer colder habitats (17, 38, 65), which may reduce the amount of movement during warmer seasons. Differences also exist between *V. fischeri* and *V. logei* in their concentration throughout different times of the year (22). Thus, introgression of certain haplotypes may occur when abiotic factors may be conducive for bacteria to have higher residence times in the water column when habitats share similar features. Since seasonal temperature fluctuations seem to be driving population dynamics within other symbiotic *Vibrio* communities (21, 58), it is likely that introgression may readily occur in Mediterranean vibrios when habitats are similar.

Niche-specific differences driven by abiotic factors like temperature may also play a major role in defining specific genetic differences which have ultimately led to a clear distinction between the two *Vibrio* species in the northern Mediterranean (57, 58). This has direct implications for the population structure of the squid-*Vibrio* symbiosis. Deeper-dwelling, colder-water sepiolid species (i.e., *S. robusta* and *S. intermedia*) may be discriminately exposed to a higher proportion of *V. logei*, thereby favoring colonization by one *Vibrio* species over another (38, 42, 58). Although this *in situ* evidence is only suggestive, additional studies of such populations could add evidence as to how symbiont structure is formed in these particular sepiolids over both time and habitat preference.

P. leiognathi isolates from Mediterranean *R. minor* hosts all shared the same haplotype (Fig. 1, haplotype 29) compared to those from the North Atlantic (Fig. 1, haplotypes 29 and 30). This genetic distinction may be due to differences in abiotic factors that shape their ecology, as well as a large geographical distance between sample sites (Atlantic versus Mediterranean seas). The locational disparity may also be exacerbated by the very narrow Strait of Gibraltar, which symbiotic bacteria would need to pass through to access either host site while adjusting to differential conditions between the Atlantic Ocean and the Mediterranean Sea (Table 1) (45, 46). Additionally, water movement into the Mediterranean Sea from the Atlantic Ocean would most likely prevent significant physical movement and gene flow of most organisms out of the Mediterranean (2, 5, 26, 32, 57).

Host population structure. The population structure of host squid species used in this study suggests strong species delineations within sympatric populations (Fig. 2; see also Fig. S2). Individual squids within each species shared similar haplotypes regardless of collection site (Fig. 2). There was also no significant temporal or geographical change in host populations based on the genetic information, considering squid were sampled within a period of 6 years (Table 1). Lack of significant change over time (between 3 to 7 years) suggests a slow mutation rate in the COI and rRNA-coding loci or active gene flow between populations such that all species populations are homogeneous (8).

Genetically similar populations occurring in geographically distinct areas suggest a number of possible evolutionary scenarios. One is a recent dispersal or radiation event which separated genetically similar populations into various geographical locations. Since the Mediterranean Sea has been shown to have surface water currents driven by strong seasonal changes, this may provide a mechanism for juvenile squids to

be moved to different locations at specific times of the year (56). Another possible consideration is that certain loci do not provide sufficient phylogenetic resolution to expose fine differences between geographically different populations of host squid (4). This second scenario is less likely, given that previous studies of Indo-West Pacific sepiolids and other Mediterranean fauna using mitochondrial loci provide strong support for determining population structure (13, 14, 21, 47). Additional mitochondrial genes (12S and 16S rRNA) reinforce this species exclusivity through parallel networks resulting in only two additional haplotypes when combined with the COI sequence data (haplotypes 9 and 13) (Fig. 2).

Influence of abiotic factors. The Mediterranean Sea is a relatively shallow, contained body of ocean, which is accessed by other bodies of water at only a small number of places: the Strait of Gibraltar, the Dardanelles, and the Bosporus. This results in the lack of water movement between the Mediterranean and any other significantly large body of water. The northern Mediterranean is also absent of ocean-like currents, which could facilitate movement around the coastal margins of the Mediterranean (49, 51). Previous work evaluating the population genetic structure of sepiolid squid and their symbionts in the Indo-West Pacific demonstrated some specificity between allopatric *Euprymna* species and their *Vibrio* symbionts, with observed movement of *V. fischeri* strains between host populations that had similar environmental regimes (21). This supports the idea that although *Euprymna* hosts can share *V. fischeri* from other *Euprymna* hosts (host specificity) throughout the Indo-West Pacific, *Vibrio* bacteria have the ability to move around and infect new populations if environmental factors facilitate their transport, survival, and fitness (57, 58).

Thus, one major difference between the Indo-West Pacific and the Mediterranean that may lead to a lack of specificity between host squids and their vibrio symbionts is temperature. The Mediterranean Sea is subject to shallow thermoclines, especially during summer months, which can drastically change temperatures within the water column (Table 1) (52). These seasonal temperature differences may create population differences due to temperature adaptation that separates the habitat ranges of prospective symbionts (22, 38, 57). Thus, particular temperature conditions of host habitats may directly influence which species/strain of symbiont predominates in their light organs (38, 39, 57, 58). Newly hatched juveniles of species which are found at relatively deeper depths, such as *S. ligulata*, *S. intermedia*, and *S. robusta*, are more likely to be inoculated by *V. logei*, which has a selective advantage at colder temperatures (38). Since sepiolid squids are found continuously yearround, various generations of *Sepiola* may have been colonized by different *Vibrio* bacteria, depending on when and where they were infected (6, 36, 38). In our study, the ratio of *V. fischeri* to *V. logei* found in *S. affinis* (found in shallower, warmer waters) (Table 1) was significantly higher, suggesting that the warm-water-preferring *V. fischeri* has an advantage in terms of its potential to form symbioses with sepiolid squids in those areas. This has also been observed in laboratory experiments, in which temperature rather than host specificity is a strong force in forming these associations (38).

The influence of abiotic factors on the establishment and persistence of this symbiosis in sepiolid squid has only recently emerged as a major force in determining the natural structure of the symbiosis (57, 58). This study has demonstrated a lack of specificity between sympatric species of light organ-bearing sepiolid squids and their *Vibrio* symbionts in the Mediterranean. Adaptation to multiple squid populations in the Mediterranean seems to be driven mainly by environmental determinants, but host specificity is still important in the establishment of the population genetic structure, since *Vibrio* or *Photobacterium* species are the sole symbionts of sepiolid squids in both the Indo-West Pacific and Mediterranean seas (41). The combined influence of host specificity and abiotic factors contribute to the complexity of the population genetic structure of environmentally transmitted host-symbiont associations. Understanding the behavior of symbiotic bacteria among eukaryotic hosts and the phenomena that determines their phylogeographical matrix may help aid in our understanding of the population dynamics of other related *Vibrio* species in nature (including pathogens such as *V. cholerae* and *V. parahaemolyticus*) and what factors are important for their survival in both their free-living and symbiotic states.

ACKNOWLEDGMENTS

We thank B. W. Jones and J. E. Lopez for their help with population genetic software and analysis. We also thank N. Moltschaniwskyj for her assistance with the molecular analysis of the sepiolid squid and S. V. Boletzky for helpful discussions concerning Mediterranean cephalopod fauna. A very special thanks to B. Hesse, C. LaBrune, and J.-L. Martinez (Laboratoire Arago, Banyuls-sur-Mer, France), G. Cricket (University of Perpignan, France), and G. Bello (Instituto Arion, Bari, Italy) for helping collect squid specimens used in this study.

Funding for this study was provided by NIH- NIAID 1SC1AI081659, NIH-NIAID 3SC1AI81659-02S1, and NSF-IOS 0744498 to M.K.N.

REFERENCES

1. **Ashen, J. B., and L. J. Goff.** 2000. Molecular and ecological evidence for species specificity and coevolution in a group of marine algal-bacterial symbioses. Appl. Environ. Microbiol. **66:**3024–3030.
2. **Astraldi, M., S. Balopoulos, J. Candela, J. Font, M. Gacic, G. P. Gasparini, B. Manca, A. Theocharis, and J. Tintore.** 1999. The role of straits and channels in understanding the characteristics of Mediterranean circulation. Prog. Oceanogr. **44:**65–108.
3. **Aurelle, D., T. Cuillemaud, P. Alfonso, T. Morato, P. Wirtz, R. S. Santos, and M. L. Cancela.** 2003. Genetic study of *Coris julis* (Osteichtyes, Perciformes, Labridae) evolutionary history and dispersal abilities. C. R. Biol. **326:**771–785.
4. **Ballard, J. W. O.** 2000. When one is not enough: introgression of mitochondrial DNA in *Drosophila*. Mol. Biol. Evol. **17:**1126–1130.
5. **Bargelloni, L., J. A. Alarcon, M. C. Alvarez, E. Penzo, A. Magoulas, C. Reis, and T. Patarnello.** 2003. Discord in the family Sparidae (Teleostei): divergent phylogeographical patterns across the Atlantic-Mediterranean divide. J. Evol. Biol. **16:**1149–1158.
6. **Bello, G.** 1995. A key for the identification of the Mediterranean sepiolids (Mollusca: Cephalopoda). Bull. Inst. Oceanogr. (Monaco) **16:**41–56.
7. **Bollmann, A., and H. J. Laanbroek.** 2002. Influence of oxygen partial pressure and salinity on the community composition of ammonia-oxidizing bacteria in the Schelde estuary. Aquat. Microb. Ecol. **28:**239–247.
8. **Brierley, A. S., P. G. Rodhouse, J. P. Thorpe, and M. R. Clarke.** 1993. Genetic evidence of population heterogeneity and cryptic speciation in the ommastrephid squid *Martialia hyadesi* from the Patagonian Shelf and Antarctic Polar Frontal Zone. Mar. Biol. **116:**593–602.
9. **Camilli, A., and B. L. Bassler.** 2006. Bacterial small-molecule signaling pathways. Science **311:**1113–1116.
10. **Clement, M., D. Posada, and K. Crandall.** 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. **9:**1657–1660.
11. **Dunlap, P. V., and M. J. McFall-Ngai.** 1987. Initiation and control of the bioluminescent symbiosis between *Photobacterium leiognathi* and leiognathid fish. Ann. N. Y. Acad. of Sci. **503:**269–283.
12. **Dunlap, P. V.** 1999. Quorum regulation of luminescence in *Vibrio fischeri*. J. Mol. Microbiol. Biotech. **1:**5–12.
13. **Duran, S., G. Giribet, and X. Turon.** 2004. Phylogeographical history of the sponge *Crambe crambe* (Porifera, Poecilosclerida): range expansion and recent invasion of the Macronesian islands from the Mediterranean Sea. Mol. Ecol. **13:**109–122.

14. **Duran, S., C. Palacín, M. A. Becerro, X. Turon, and G. Giribet.** 2004. Genetic diversity and population structure of the commercially harvested sea urchin *Paracentrotus lividus* (Echinodermata, Echinoidea). Mol. Ecol. **13:** 3317–3328.
15. **Excoffier, L. G., and S. Schneider.** 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinform. Online **1:**47–50.
16. **Felbeck, H., J. J. Childress, and G. N. Somero.** 1981. Calvin-Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. Nature **293:**291–293.
17. **Fidopiastis, P. M., S. von Boletzky, and E. G. Ruby.** 1998. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. J. Bacteriol. **180:**59–64.
18. **Guerrero-Ferreira, R. C., and M. K. Nishiguchi.** 2007. Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis*, *Loliolus*, and *Euprymna* (Mollusca: Cephalopoda). Cladistics **23:**497–506.
19. **Hanlon, R. T., M. F. Claes, S. E. Ashcraft, and P. V. Dunlap.** 1997. Laboratory culture of the sepiolid squid *Euprymna scolopes*: a model system for bacteria-animal symbiosis. Biol. Bull. **192:**364–374.
20. **Jones, B. W., and M. K. Nishiguchi.** 2004. Counterillumination in the bobtail squid, Euprymna scolopes (Molluca: Cephalopoda). Mar. Biol. **144:**1151–1155.
21. **Jones, B. W., J. E. Lopez, J. Huttenberg, and M. K. Nishiguchi.** 2006. Population structure between environmentally transmitted *Vibrio*s and bobtail squids using nested clade analysis. Mol. Ecol. **15:**4317–4329.
22. **Jones, B. W., A. Maruyama, C. C. Ouverney, and M. K. Nishiguchi.** 2007. Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. Microb. Ecol. **54:**314–323.
23. **Knowles, L. L., and W. P. Maddison.** 2002. Statistical phylogeography. Mol. Ecol. **11:**2623–2635.
24. **Kotoulas, G., A. Magoulas, N. Tsimenides, and E. Zouros.** 1995. Marked mitochondrial DNA differences between Mediterranean and Atlantic populations of the swordfish, *Xiphias gladius*. Mol. Ecol. **4:**473–482.
25. **Lee, K., and E. G. Ruby.** 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. **60:**1565–1571.
26. **Lefkaditou, E., and P. Kaspiris.** 2005. Distribution and abundance of sepiolids (Mollusca: Cephalopoda) off the Northeastern Greek coasts. Belg. J. Zool. **135:**199–204.
27. **Lopez, J.** 2003. Symbiosis among sepiolid squid: origins of the symbiotic light organ and examination of the host population structure at the intraspecies level. M.S. thesis. New Mexico State University, Las Cruces, NM.
28. **Lupp, C., and E. G. Ruby.** 2005. *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. J. Bacteriol. **187:**3620–3629.
29. **McFall-Ngai, M. J.** 1994. Animal-bacterial interactions in the early life history of marine invertebrates: the *Euprymna scolopes*/*Vibrio fischeri* symbiosis. Am. Zool. **34:**554–561.
30. **McFall-Ngai, M. J., and E. G. Ruby.** 1998. Sepiolids and vibrios: when first they meet. Bioscience **48:**257–265.
31. **Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55:**165–199.
32. **Millot, C.** 1999. Circulation in the western Mediterranean Sea. J. Mar. Sys. **20:**423–442.
33. **Montgomery, M. K., and M. J. McFall-Ngai.** 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development in the squid *Euprymna scolopes*. Development **120:**1719–1729.
34. **Munkasci, A. B., J. J. Pan, P. Villesen, U. G. Mueller, M. Blackwell, and D. J. McLaughlin.** 2004. Convergent coevolution in the domestication of coral mushrooms by fungus-growing ants. Proc. R. Soc. Lond. B **21:**1777–1782.
35. **Muscatine, L., and J. W. Porter.** 1977. Mutualistic symbioses adapted to nutrient-poor environments. Bioscience **27:**454–460.
36. **Nesis, K. N.** 1982. Cephalopods of the world. TFH Publications, Neptune City, NJ.
37. **Nishiguchi, M. K., E. G. Ruby, and M. J. McFall-Ngai.** 1998. Competitive dominance during colonization is an indication of coevolution in an animal-bacterial symbiosis. Appl. Environ. Microbiol. **64:**3209–3213.
38. **Nishiguchi, M. K.** 2000. Temperature affects species distribution in symbiotic populations of *Vibrio*. Appl. Environ. Microbiol. **66:**3550–3555.
39. **Nishiguchi, M. K.** 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. Microb. Ecol. **44:**10–18.
40. **Nishiguchi, M. K., and V. S. Nair.** 2003. Evolution of symbiosis in Vibrionaceae: a combined approach using molecules and physiology. Int. J. Syst. Evol. Microbiol. **53:**2019–2026.
41. **Nishiguchi, M. K., J. L. Lopez, and S. van Boletzky.** 2004. Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. Evol. Dev. **6:**41–49.
42. **Nyholm, S. V., and M. K. Nishiguchi.** 2008. The evolutionary ecology of a sepiolid squid-Vibrio association: from cell to environment. Vie Milieu Paris **58:**175–184.
43. **Nyholm, S., and M. J. McFall-Ngai.** 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. Appl. Environ. Microbiol. **69:**3932–3937.
44. **Nyholm, S., and M. J. McFall-Ngai.** 2004. The winnowing: establishing the squid-*Vibrio* symbiosis. Nat. Rev. Microbiol. **2:**632–642.
45. **Patarnello, T., F. A. M. J. Volckaert, and R. Castilho.** 2007. Pillars of Hercules: is the Atlantic-Mediterranean transition a phylogeographical break? Mol. Ecol. **16:**4426–4444.
46. **Pérez-Losada, M., A. Guerra, G. R. Carvalho, A. Sanjuan, and P. W. Shaw.** 2002. Extensive population subdivision of the cuttlefish *Sepia officinalis* (Mollusca: Cephalopoda) around the Iberian Peninsula indicated by microsatellite DNA variation. Heredity **89:**417–424.
47. **Pérez-Losada, M., M. J. Nolte, K. A. Crandall, and P. W. Shaw.** 2007. Testing hypotheses of population structuring in the Northeast Atlantic Ocean and Mediterranean Sea using the common cuttlefish *Sepia officinalis*. Mol. Ecol. **16:**2667–2679.
48. **Petit, R. J.** 2008. The coup de grace for the nested clade phylogeographic analysis? Mol. Ecol. **17:**516–518.
49. **Pinardi, N., and E. Masetti.** 2000. Variability of the large scale general circulation of the Mediterranean from observations and modeling: a review. Palaeogeogr. Palaeoclimatol. Palaeoecol. **158:**153–173.
50. **Posada, D., K. A. Crandall, and A. R. Templeton.** 2000. GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. Mol. Ecol. **9:**487–488.
51. **Robinson, A. R., W. G. Leslie, A. Theocharis, and A. Lascaratos.** 2001. Mediterranean Sea circulation, p. 283–298. *In* J. H. Steele, K. K. Turekian, and S.A. Thorpe (ed.), Encyclopedia of ocean sciences. Academic Press, London, United Kingdom.
52. **Roussanov, V., E. Stanev, V. Artale, and N. Pinardi.** 1995. A seasonal model of the Mediterranean Sea general circulation. J. Geophys. Res. **100:**13.515–13.538.
53. **Ruby, E. G., and M. J. McFall-Ngai.** 1992. A squid that glows in the night: development of an animal-bacterial mutualism. J. Bacteriol. **174:**4865–4870.
54. **Ruby, E. G., and K. Lee.** 1998. The *Vibrio fischeri-Euprymna scolopes* light organ association: current ecological paradigms. Appl. Environ. Microbiol. **64:**805–812.
55. **Salman, A., T. Katagan, and H. Avni Benli.** 2002. Cephalopod fauna of the Eastern Mediterranean. Turk. J. Zool. **26:**47–52.
56. **Send, U., J. Font, G. Krahmann, C. Millot, M. Rhein, and J. Tintore.** 1999. Recent advances in observing the physical oceanography of the western Mediterranean Sea. Prog. Oceanogr. **44:**37–64.
57. **Soto, W., J. Gutierrez, M. R. Remmenga, and M. K. Nishiguchi.** 2009. Synergistic affects of temperature and salinity in competing strains of symbiotic *Vibrio fischeri*. Microb. Ecol. **57:**140–150.
58. **Soto, W., C. P. Lostroh, and M. K. Nishiguchi.** 2009. Physiological responses to stress in the Vibrionaceae: aquatic microorganisms frequently affiliated with hosts, p. 409–426. *In* J. Seckbach and M. Grube (ed.), Cooperation and stress in biology. Springer, New York, NY.
59. **Stabb, E. V.** 2006. The *Vibrio fischeri-Euprymna scolopes* light organ symbiosis, p. 204–218. *In* F. L. Thompson, B. Austin, and J. Swings (ed.), The biology of vibrios. ASM Press, Washington, DC.
60. **Templeton, A. R., E. Beorwinkle, and C. F. Sing.** 1987. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and analysis of alcohol dehydrogenase activity in *Drosophila*. Genetics **117:**343–351.
61. **Templeton, A. R., K. A. Crandall, and C. F. Sing.** 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. III. Cladogram estimation. Genetics **132:**619–633.
62. **Templeton, A. R., and C. F. Sing.** 1993. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. Genetics **134:**659–669.
63. **Templeton, A. R., E. Routman, and C. A. Phillips.** 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Abystoma tigrunum*. Genetics **140:**767–782.
64. **Templeton, A. R.** 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. Mol. Ecol. **7:**381–397.
65. **Thompson, J. R., M. A. Randa, L. A. Marcelino, A. Tomita-Mitchell, E. Lim, and M. F. Polz.** 2004. Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Appl. Environ. Microbiol. **70:**4103–4110.
66. **Varón, A., L. S. Vinh, and W. C. Wheeler.** 2010. POY version 4: phylogenetic analysis using dynamic homologies. Cladistics **26:**72–85.
67. **Visick, K. L., and M. J. McFall-Ngai.** 2000. An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. J. Bacteriol. **182:**1779–1787.
68. **Wollenberg, M. S., and E. G. Ruby.** 2009. Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two Oahu populations. Appl. Environ. Microbiol. **75:**193–202.

Research Paper

Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri*

Alba Chavez-Dozal and Michele K. Nishiguchi

Department of Biology, New Mexico State University, Las Cruces, New Mexico, USA

Persistence and survival under various environmental stresses has been attributed to the capacity of most bacteria to form biofilms. In aquatic environments, the symbiotic bacterium *Vibrio fischeri* survives variable abiotic conditions during its free-living stage that dictates its ability to colonize the squid host. In the present study, the influence of different abiotic factors such as salt concentration, temperature, static/dynamic conditions, and carbon source availability were tested to determine whether biofilm formation occurred in 26 symbiotic and free-living *V. fischeri* strains. Statistical analysis indicate that most strains examined were strong biofilm producers under salinity concentrations that ranged between 1–5%, mesophilic temperatures (25–30 °C) and static conditions. Moreover, free-living strains are generally better biofilm formers than the symbiotically competent ones. Geographical location (strain origin) also correlated with biofilm formation. These findings provide evidence that abiotic growth conditions are important for determining whether mutualistic *V. fischeri* have the capacity to produce complex biofilms, allowing for increased competency and specificity during symbiosis.

Keywords: Biofilm / Environment / *Vibrio fischeri*

Received: October 25, 2010; accepted: February 01, 2011

DOI 10.1002/jobm.201000426

Introduction

Complex communities known as biofilms are increasingly recognized as the predominant form of microbial biomass in the environment [1]. The formation of these dynamic bacterial populations involves attachment and synthesis of a matrix composed primarily of polysaccharides [2] and is one important avenue for host colonization that eventually leads to persistence, antibiotic resistance, and pathogenesis [3]. Various model systems studying bacterial communities have been used to investigate biofilm properties, such as those formed by *Pseudomonas aeuruginosa* [4] and *Staphylococcus* spp. [5]. Numerous traits, such as quorum sensing, motility, and exopolysaccharide synthesis have been shown to be essential for correct assembly and maintenance of the biofilm architecture [6–8]. Although many of these characteristics occur in pathogenic *Vibrio* bacteria, little is known about benign or mutualistic species that have associations with eukaryotic hosts.

Vibrio fischeri is a bioluminescent bacterium that infects the light organs of sepiolid squids and monocentrid fishes establishing mutualistic associations. Free-living *Vibrio* bacteria colonize aposymbiotic juvenile squids within the first few hours after hatching, and upon colonization form a biofilm in the crypts of the light organ complex [9]. These bacteria produce bioluminescence that is used by the squid to avoid predation in a behavior known as counterillumination [9]. The mutualism is established when the host provides an appropriate niche for the bacteria to reproduce at much higher rates than in their free-living state [10]. Once colonized, symbiotic squids vent 90–95% of their bacteria from their light organ into the environment every morning to repopulate the surrounding bacterioplankton community [11]. During this cyclical period between symbiotic and free-living states, *V. fischeri*

Correspondence: Michele K. Nishiguchi, New Mexico State University Box 30001, MSC 3AF, Las Cruces, New Mexico 88003-8001, USA
E-mail: nish@nmsu.edu
Phone: 575-646-3721
Fax: 575-646-5665

forms a biolfilm-like aggregate outside the sites of infection prior to colonization [9, 11]. The squid host secretes mucus which is suspended above the sites of colonization [12]. Gram negative bacteria aggregate in this mucus and, by an unknown mechanism, *V. fischeri* is able to outcompete other bacteria in order to establish the specific mutualism [12]. Although *V. fischeri* has not been considered a typical model for studying biofilm formation until recently, intercellular signaling or quorum sensing molecules linked to the sociomicrobiology aspect of this particular bacterium make it an attractive organism to examine biofilm regulation. Additionally, recent discovery of hybrid sensor kinases and specific genes such as *rpoN* (encoding for the σ^{54}) [8], mannose-sensitive hemagglutinin (*mshA),* uridyl phosphate dehydrogenase (*UDPH*) [13], and the symbiosis polyssacharide cluster (*syp*) [14], have demonstrated the importance of biofilm formation with respect to the life history strategy of this marine bacterium prior and during symbiosis. *V. fischeri* cells that form biofilms exhibit a variety of physiological and molecular characteristics when compared to those in their planktonic state [2]. As a result, the significance of biofilm production has emerged to accommodate a wide variety of sophisticated regulatory procedures allowing survival and adaptation to extreme environmental changes (that is, between a squid light organ and the surrounding seawater).

Symbiotic *V. fischeri* thrive in many different aquatic environments depending upon which squid host it colonizes; for instance, the genus *Eurymna* is distributed in the Indo-west Pacific, whereas species in the genus *Sepiola* are mostly found in Mediterranean waters and along the western margin of the Pacific Ocean [15]. Moreover, some free-living species are symbiotically incompetent or unable to colonize the light organ, even though they are found in the same waters as light organ containing sepiolids [16].

Previous evidence has demonstrated that environmental or abiotic factors influence both growth and physiology of *Vibrio* bacteria prior to their colonization in a squid light organ [11]. Additionally, significant genetic differences exist among *V. fischeri* strains from different geographical locations [10]. Therefore, in the present study, we determined whether biofilm formation between free-living and symbiotic *V. fischeri* strains differed under various environmental conditions (salinity and temperature changes, static-dynamic conditions, and atmosphere) in an effort to describe part of the strategy for *V. fischeri* survival in aquatic ecosystems.

Materials and methods

Bacterial strains used in the study

V. fischeri strains were isolated from seawater or live squid (*Euprymna* and *Sepiola* species; Nishiguchi, 2002) and are listed in Table 1. Isolated strains were stored as glycerol stocks (−80 °C) and re-grown for 24 h in Luria Bertani high salt (LBS; 10 g tryptone, 5 g yeast extract, 20 g sodium chloride, 50 ml 1 M Tris pH 7.5, 3.75 ml 80% glycerol and 950 ml distilled water) medium at 28 °C with moderate shaking (224 rpm). All strains were subsequently sub-cultured again under the same conditions for use in each of the assays. Biofilm quantification under the various conditions of interest was completed as previously described [17], this quantification is achieved by using crystal violet to stain the biofilm and subsequently solubilizing it with 70%

Table 1. *Vibrio fischeri* strains used in this study.

Strain	Host	Location
WH1	Free-living	USA (Woods Hole, Massachusetts)
MDR7	Free-living	USA (Marina del Rey, California)
CB37	Free-living	Australia (Coogee Bay, Sydney, NSW)
ATCC7744	Free-living	American Type Culture Collection
BSM40	Free-living	France (Banyuls sur mer)
BSM46	Free-living	France (Banyuls sur mer)
BSM50	Free-living	France (Banyuls sur mer)
PP3	Free-living	USA (Kaneohe Bay, O'ahu, Hawaii)
PP42	Free-living	USA (Kaneohe Bay, O'ahu, Hawaii)
VLS2	Free-living	USA (Kaneohe Bay, O'ahu, Hawaii)
SR5	*Sepiola robusta*	France (Banyuls sur mer)
SL518	*Sepiola ligulata*	France (Banyuls sur mer)
SA1G	*Sepiola affinis*	France (Banyuls sur mer)
SI66	*Sepiola intermedia*	Italy (Bari)
SI1D	*Sepiola intermedia*	France (Banyuls sur mer)
EM17	*Euprymna morsei*	Japan (Tokyo Bay)
ET101	*Euprymna tasmanica*	Australia (Townsville, QLD)
ETWW	*Euprymna tasmanica*	Australia (Woy Woy, NSW)
ETBB20	*Euprymna tasmanica*	Australia (Botany Bay, Sydney, NSW)
ETSB1	*Euprymna tasmanica*	Australia (Shark Bay, WA)
EB12	*Euprymna berryi*	Japan (Tosa Bay)
ES114	*Euprymna scolopes*	USA (Kaneohe Bay, O'ahu, Hawaii)
ESP915	*Euprymna scolopes*	USA (Paiko, O'ahu, Hawaii)
ESL5	*Euprymna scolopes*	USA (Kaneohe Bay, O'ahu, Hawaii)
ESC9	*Euprymna scolopes*	USA (Kaneohe Bay, O'ahu, Hawaii)

ethanol and measuring optical density, which is proportional to the amount of biofilm formed.

Biofilm formation assay

Overnight cultures were washed twice with fresh LBS medium and diluted to a concentration of 1×10^8 Colony Forming Units (CFU)/ml. Aliquots of each *V. fischeri* isolate were diluted to the same concentration (OD_{600}) and were added to individual wells (5 wells/strain) located on a sterile, flat-bottom, polystyrene 96-well microtitre plate (Corning 96 well plates, Sigma-Aldrich, CLS3628). Three wells were filled with uninoculated sterile LBS as a negative control. Microtiter plates were filled with 100 μl of the medium, covered loosely, and incubated for 24 h at 28 °C. Planktonic (those not forming biofilm) bacteria were removed from each microtiter well by briskly shaking the microtitre plate. The emptied wells were then washed three times with 200 μl of sterile LBS medium, and 125 μl solution of crystal violet (0.2% diluted in sterile water) was then added to each well and incubated for 30 minutes at room temperature (~22 °C) to stain cells adhered to each well. After incubation, crystal violet was removed by shaking the microtiter dish vigorously over a waste tray and washing the plate five times with distilled water and subsequently air-dried. In order to quantify the amount of biofilm, 200 μl of 95% ethanol was added to each stained well (including controls), and incubated for 20 min at room temperature in order to solubilize the dye. The contents of each well were mixed briefly and 125 μl of crystal violet/ethanol solution was transferred to a separate well in an optically clear flat-bottom 96-well plate. Optical density was measured at 562 nm using a plate reader (Bio-tek FL 800). All samples were repeated in triplicate (3 plates) for a total of 15 replicates per isolate.

Salinity, temperature, carbon source and static/dynamic conditions

The effects of salinity, temperature, carbon source, and aerobic conditions were tested by growing cells under various sodium chloride concentrations, temperatures, carbon sources, or degrees of shaking. *Vibrio* isolates were grown overnight and sub-cultured the next morning as indicated previously. Washed cells were diluted to equal concentrations (1×10^8 CFU/ml) in modified LBS medium. For salinity measurements, we used sodium chloride concentrations ranging from 0% to 9%, where 1% corresponds to 10 parts per thousand (ppt) [11]. For temperature experiments, cultures were grown at 12, 15, 20, 25, 28, 30 and 32 °C in LBS medium at 32 ppt sodium chloride (or 3.2% sodium chloride). Minimal Ribose Medium (MRM) was also used as a growth medium for the above conditions, since it mimics the nutrient-poor composition of the seawater (composition per liter: NaCl 17.53 g, $MgSO_4$ 6.02 g, $CaCl_2$ 1.47 g, K_2HPO_4 0.0575 g, KCl 0.7455 g, NH_4Cl 0.5349 g, Ribose 3.0 g, $FeSO_4 \cdot 7\ H_2O$ 0.00278 g, Tris-HCl 7.88 g) [11]. Isolates were examined by growing cells in different dilutions of MRM (1:2, 1:4, 1:8 and 1:16), using as a diluent a solution with all the components of the MRM except the carbon source (Ribose). Salinity measurements of the different dilutions were calculated using a refractometer.

For static/dynamic conditions, microtiter plates were inoculated and measured after incubation of plates grown at different levels of agitation (0, 60, 120, 180 and 240 rpm) at 28 °C for 24 h on microtitre plates prior to final measurement of biofilm formation.

Data analysis and interpretation

Two different analyses were used to examine the variation of biofilm production from all environmental conditions. Strains were classified according to the amount of biofilm production as described in a previous study [18]: weak [$Ac \leq A \leq (2 \times Ac)$], moderate [$(2 \times Ac) \leq A \leq (4 \times Ac)$] and strong biofilm producers [$A > (4 \times Ac)$]. *Ac* represents the minimum absorbance for low biofilm production, which is calculated as three standard deviations above *A* of the control. A two-way ANOVA statistical design was chosen for the analysis of difference between the average mean of free-living and symbiotic *Euprymna* and *Sepiola* strains. A randomized complete block design was executed by using the Statistical Analysis Software (SAS^R) version 5.1.

Results

V. fischeri is a luminous symbiotic bacterium that can also persist solely in the free-living form, flourishes in a wide variety of coastal estuarine and marine waters, and is found as part of microbial biofilms in the environment [11, 13, 19]. Previous studies have focused on growth dynamics and effects of abiotic factors on these bacterial suspensions [4, 5, 11]; however, it is clear that biofilms constitute a distinct and predominantly structurally complex growth phase that is crucial for shaping the majority of aquatic bacterial communities. These biofilm communities are different from their planktonic state due to the physiological cooperation that dictates a high level of organization [20]. The present study attempts to elucidate the role of such environmental conditions on biofilm development of dif-

ferent *V. fischeri* isolates with various life history strategies.

Table 2 lists an overall view of the capacity to which all *Vibrio* strains examined aggregate and form biofilms. Most isolates were classified as weak to moderate biofilm formers at temperatures ranging from 12, 15, 20, 25 and 30 °C, whereas strong biofilm production was observed at 25 and 28 °C, corresponding to the optimal temperatures found in waters from the Indo-West Pacific Ocean (where the majority of *Euprymna* hosts are located) [15]. Strong biofilms were observed at low salinity concentrations (1–5%); conversely higher salinities (6–9%) resulted in significantly less biofilm for all isolates examined.

In this study, *Sepiola* strains were able to form biofilms at higher salinity ranges when compared with strains obtained from *Euprymna* (Fig. 1A). Previous work also reported the trends for planktonic strains under similar conditions [11]. An interesting finding was observed with free-living *V. fischeri* strains (WH1, MDR7, CB37) that are symbiotically incompetent (cannot colonize squids). Our results indicate that strains exhibit differences in biofilm formation when the salinity is modified, and free-living strains are generally better biofilm formers than symbiotic isolates at higher salinities ($p < 0.05$). This provides partial evidence that stress adaptation (including high osmolarity) are dictating bacterial survival and aggregation, and these responses are partially driven by abiotic factors.

The effect of temperature on biofilm production (Fig. 1B) was not significantly different ($p > 0.05$) among symbiotic and free-living strains. Biofilm formation was observed in temperatures ranging between 12 °C to 32 °C; temperatures outside of this range reduced bacterial viability by 95% (results not shown). Some *V. fischeri* strains have been able to adapt and grow at higher (42 °C) and lower (8–10 °C) temperatures (unpublished data); however, biofilms were not observed for these extreme ranges.

Another survival challenge for many bacteria, including *V. fischeri,* is the degree of dynamism/movement in the environment. These conditions can be related to the effect of water currents on community formation, since it is well known that ocean hydrodynamics differ among habitats. Table 2 indicates that the majority of the strains examined form strong biofilms under static conditions (0 Revolutions Per Minute or RPM) and moderate shaking (60 RPM). Under different levels of dy-

Table 2. Summary of biofilm formation of *Vibrio fischeri* under different environmental conditions.

Condition		Biofilm formation ($n = 26$)					
		Weak No. Strains	OD ± SD	Moderate No. Strains	OD ± SD	Strong No. Strains	OD ± SD
Temperature	12	19*	0.39 ± 0.21	7	0.63 ± 0.09	–	–
(°C)	15	14	0.51 ± 0.15	12	0.79 ± 0.12	–	–
	20	–	–	26	0.83 ± 0.25	–	–
	25	–	–	5	1.10 ± 0.19	21*	1.69 ± 0.24
	28	–	–	–	–	26	1.97 ± 0.29
	30	–	–	26	0.78 ± 0.20	–	–
	32	26	0.19 ± 0.08	–	–	–	–
[a]NaCl	1	–	–	–	–	26	1.42 ± 0.44
(%)	2	–	–	–	–	26	1.57 ± 0.48
	3	–	–	–	–	26	1.60 ± 0.53
	4	–	–	–	–	26	1.61 ± 0.60
	5	–	–	20*	1.13 ± 0.09	6	1.03 ± 0.38
	6	4	0.42 ± 0.07	19*	0.95 ± 0.40	3	1.23 ± 0.06
	7	26	0.41 ± 0.38	–	–	–	–
	8	26	0.03 ± 0.01	–	–	–	–
	9	26	0.01 ± 0.001	–	–	–	–
Agitation (rpm)	0	–	–	–	–	26	1.85 ± 0.39
	60	–	–	9	1.17 ± 0.12	17*	1.77 ± 0.46
	130	8	0.47 ± 0.29	10	1.20 ± 0.18	8	1.75 ± 0.32
	240	19	0.32 ± 0.11	7	0.73 ± 0.15	–	–
MRM	Undiluted	3	0.37 ± 0.08	21*	0.84 ± 0.14	2	1.08 ± 0.07
(Dilution factor)	1:2	–	–	17*	1.08 ± 0.11	9	1.73 ± 0.10
	1:4	26	0.34 ± 0.28	–	–	–	–
	1:8	26	0.15 ± 0.06	–	–	–	–
	1:16	26	0.03 ± 0.01	–	–	–	–

[a] 1% = 10 parts per thousand

* Significant difference within strains ($p < 0.05$) calculated using the Welsch step-up procedure.

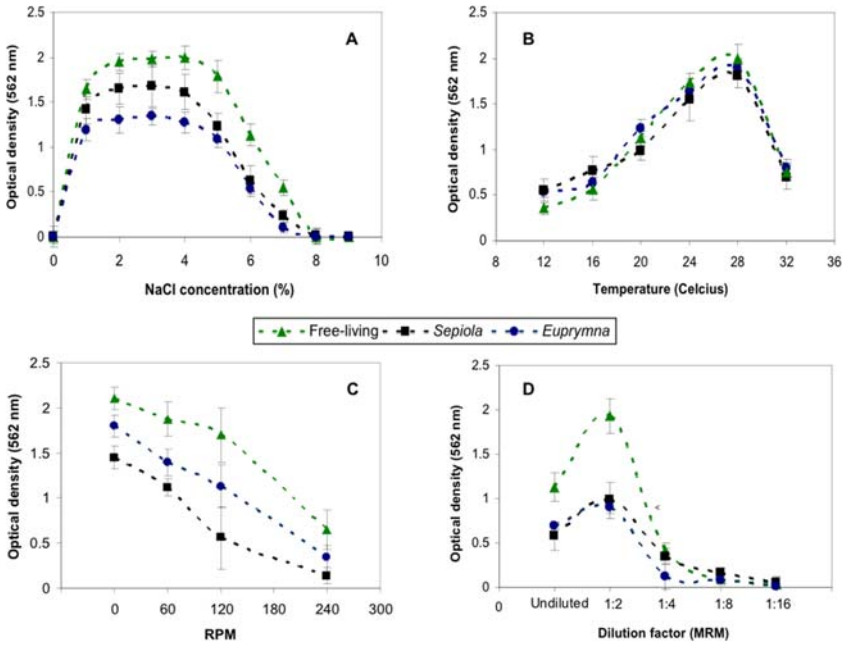


Figure 1. Influence of salinity (A: NaCl concentration, %, where 1% = 10 ppt), temperature (B; °C), carbon source availability (C; Minimal Ribose Media with various concentrations) and dynamic conditions (D; rpm) on biofilm formation when *Vibrio fischeri* isolates are divided between free-living and symbiotic strains. Data shown represents the mean optical density ± standard deviation ($N = 3$).

namic conditions (up to 240 RPM, Fig. 1C), free-living isolates are the best biofilm formers. In a similar manner, *Euprymna* isolates appear to form greater amounts of biofilm at the same dynamic conditions when compared with *Sepiola* isolates (Fig. 1C). Finally, aquatic ecosystems differ in their carbon source availability, depending on whether it is in particulate or dissolved carbon form [30]. Carbon is limited in the ocean and is more abundant inside the host's light organ; however, it is still unclear what preferred metabolic pathway vibrios utilize between their free-living and symbiotic stages [20]. Our study only examined biofilm formation using a nutrient marine medium with compounds that mimic nutrient poor conditions (Minimal Ribose Medium or MRM) [11]. Results demonstrate a significant increase in biofilm formation when the carbon source (Ribose) in the MRM is diluted 1:2 (Fig. 1D). As observed in previous conditions, free-living isolates seem to be the best biofilm formers under oligotrophic environments.

Discussion

It has been shown that abiotic factors such as salinity and temperature are variable among the world's oceans [www.nodc.noaa.gov] and different luminescent bacteria, including *V. fischeri,* are able to survive, colonize different host squids, and adapt to such changing conditions [11]. Moreover, microbial dispersal ability and changing water currents can influence bacterial biogeography, radiation, and evolution [21]. For example, the Mediterranean Sea, which has numerous species of *Sepiola*, has a narrower, yet higher range of salinity (37–40 ppt, or 3.7–4% sodium chloride) when compared with the Indo-West Pacific, where *Euprymna* hosts are found in lower salinity ranges (30–35 ppt, or 3–3.5% sodium chloride). *V. fischeri* found in either location are classified as either host generalists (Mediterranean) or host specialists (Indo-West Pacific) due to their association with either sympatric or allopatric host species [11].

It can be observed in Fig. 1A that the maximum quantity of biofilm is formed at the same salinities (2–4% sodium chloride or 20–40 ppt) for every strain; however, our two-way analysis also indicates that there are differences in the quantity of biofilm that is formed under this range of salinity. These differences are related to life history of the strain examined. For example, free-living strains produce more biofilm than either of the symbiotic strains. Additionally, symbiotic strains adapted to high salinity (from *Sepiola* hosts) produce more biofilm than strains adapted to lower salinity (*Euprymna* hosts).

Due to the capacity of biofilm formation under different salinity concentrations, mechanisms of osmo-

adaptation (described in previous reports for free-living *Vibrio parahaemolyticus*) [22] may be regulated during the biofilm state for *V. fischeri*. The possibility remains that biofilm regulation is also driven by the squid host, since some conditions inside the squid's light organ remain undescribed.

Temperature can be a major factor that affects the ability to produce extracellular polymeric substances [23], which are known to enhance adherence capability and biofilm formation in bacteria [24]. Greater quantities of biofilm were observed at 24 and 28 °C, which are optimal growth temperatures for *Vibrio* and close to the oceanic surface temperatures reported for both Mediterranean (*Sepiola*) and Indo-West Pacific Oceans (*Euprymna*) [9]. Since squid hosts are described as "poikilothermic" invertebrates (body temperature is equal to the temperature of its surroundings) [25], strain adaptation to temperature can be attributed solely to environment, and observations of no significant difference in quantity of biofilm between free-living and symbiotic strains supports this preface (according to our two way analysis). Alternatively, earlier studies have reported temperature effects for generation time of planktonic *V. fischeri,* indicating that the range of temperatures can affect the amount of biofilm produced based on growth of a particular strain [11]. In addition, our findings corroborate earlier data suggesting that temperature is an important factor that dictates specificity of two *Vibrio* species (*V. fischeri* and *V. logei*) within the same squid host [26]. Further experiments need to compare biofilm formation within colonized juvenile squids, to determine if indeed this is true after colonization.

The effect of ocean hydrodynamics can be related to the differences observed in the agitation assays. The strains tested can form biofilms on static conditions and under high degree of dynamism (Fig. 1C), with the general tendency of free-living strains being the best biofilm formers. In some cases, dynamism can be related to oxygen tension or oxygen availability; however, this study is not sufficient to describe the effect of oxygen tension on biofilm formation. This is an important factor that needs to be addressed primarily because the amount of oxygen that is available in the squid light organ is variable [27], and oxygen is an important factor for controlling bioluminescence through expression of the *lux* operon [28]. In addition, this operon is involved in quorum sensing behavior and colony aggregation or biofilm formation [29]. Future studies are needed to investigate subtle differences attributed to aerobic, microaerophilic, and anaerobic conditions in both free-living and symbiotic states.

Differences observed in various dilutions of MRM may be attributed to bacterial responses associated with coordinated alterations in patterns of gene expression of different metabolic networks that are necessary for efficient carbon utilization [31]. For example, the stringent response has been described as a transcriptional program that mediates prokaryotic adaptation to starvation conditions [32]. Previous work demonstrated that the stringent response activates quorum sensing in *Pseudomonas aeruginosa* [33], a process that has been proven to control virulence and biofilm formation in *Vibrio cholerae* [28] and might be an underlying genetic switch that allows *V. fischeri* to survive and associate only when the medium is diluted 1:2 (Fig. 1D).

Results obtained from the present study show that environmental switches exert an important influence in biofilm formation, and although there is no difference in the patterns observed in Fig. 1, there is a distinction of biofilm quantity between free-living and symbiotic strains. It is unclear at this point if these different responses are a result of a specific genetic change that allows adaptation, or as a simple shift in bacterial physiology related to development of a biofilm.

Microbial biofilms are the predominant form for survival of many species of bacteria in the environment, and are important for determining specificity and persistence in both pathogenic and mutualistic associations. The data presented here represent a quantitative analysis that provides a foundation for determining whether abiotic factors are important for biofilm formation at various life history stages of the bacterium. Additionally, this study examined how biofilm quantity differs significantly depending upon the nature of the strain and the conditions during growth *in vitro*. These assays can then be extrapolated to variations that occur between environmental or ecological niches; however, additional studies are needed in order to address the importance of conditions inside the squid's crypts (branched epithelia found inside the squid's light organ). By examining different environmental parameters, we can better understand bacterial survival and aggregation between free-living and symbiotic *V. fischeri* isolates to determine what drives specificity and environmental transmission in this mutualistic association.

Acknowledgements

The authors thank S. Soto for advice in the statistical analysis and D. Hogan for help with biofilm studies. The work was in part supported by NSF IOS-0744498, and NIH-NIAID SC1AI081659 to M.K.N. A. Chavez-Dozal was supported by the NASA-New Mexico Space Grant Consortium and NIH-RISE NIGMS R25GM061222 at NMSU.

References

[1] Kokare, C.R., Chakraborty, S., Khopade, A.N., Mahadik, K.R., 2007. Biofilm: importance and applications. Indian J. Biotechnol., **8**, 159–168.

[2] Yildiz, F.H., Visick, K.L., 2009. *Vibrio* biofilms: so much the same yet so different. Trends in Microbiol., **17**, 109–118.

[3] Lewis, K., 2001. Riddle of biofilm resistance. Antimicrobial Agents and Chemotherapy, **45**, 999–1007.

[4] Whiteley, M., Bangera, M.G., Bumgarner, R.E., Parsek, M.R. *et al*., 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature, **413**, 860–864.

[5] Beenken, K.B., Dunman, P.M., McAleese, F., Macapagal, D. *et al*., 2004. Global gene expression in *Staphylococcus aureus* biofilms. J. Bacteriol., **186**, 4665–4684.

[6] Nadeli, C.D., Xavier, J.B., Levin, S.A., Foster, K.R., 2008. The evolution of quorum sensing in bacterial biofilms. PLoS Biology, **6**, 171–179.

[7] Darnell, C.L., Hussa, E.A., Visick, K.L., 2008. The putative sensor kinase SypF coordinates biofilm formation in *Vibrio fischeri* by acting upstream of two response regulators, SypG and VpsR. J. Bacteriol., **190**, 4941–4950.

[8] Wolfe, A.J., Milikan, D.S., Campbell, J.M., Visick, K.L., 2004. *Vibrio fischeri* σ^{54} controls motility, biofilm formation, luminescence and colonization. Appl. Environ. Microbiol., **70**, 2520–2524.

[9] Jones, B.W., Nishiguchi, M.K., 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Marine Biol., **144**, 1151–1155.

[10] Jones, B.W., Maruyama, A., Ouverney, C.C., Nishiguchi, M.K., 2007. Spatial and temporal distribution on the Vibrionaceae in coastal waters of Hawaii, Australia and France. Microbial Ecol., **54**, 314–323.

[11] Soto, W., Gutierrez, J., Remmega, M.R., Nishiguchi, M.K., 2009. Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. Microbial Ecol., **57**, 140–150.

[12] Nyholm, S.V., Deplancke, B., Gaskins, H.R., Apicella, M.A., McFall-Ngai, M.J., 2002. Role of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbionts colonization of the *Euprymna scolopes* light organ. Appl. Environ. Microbiol., **70**, 2520–2524.

[13] Ariyakumar, D.S., Nishiguchi, M.K., 2009. Characterization of two specific host genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (*UDPH*) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol., **299**, 65–73.

[14] Yip, E.S., Grublesky, B.T., Hussa, E.A., Visick, K.L., 2005. A novel, conserved cluster of genes promotes symbiotic colonization and σ^{54}- dependent biofilm formation by *Vibrio fischeri*. Mol. Microbiol., **57**, 1485–1498.

[15] Nesis, K.N., 2003. Distribution of recent Cephalopoda and implications for plio-pleistoscene events. Berliner Palaeobiologische Abhandlungen, **3**, 199–224.

[16] Lee, K.H., Ruby, E.G., 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol., **60**, 1565–1571.

[17] Merrit, J.H., Kadouri, D.E., O'Toole, G.A., 2005. Growing and analyzing static biofilms. Curr. Protocols Microbiol., Chapter 1: Unit 1B.1.

[18] Di Bonaventura, G., Stepanovic, S., Picciani, C., Pompilio, A., Piccolomini, R., 2007. Effect of environmental factors on biofilm formation by clinical *Streptomonas maltophilia* isolates. Folia Microbiologica, **52**, 86–90.

[19] Stanley, N.R., Lazazzera, B.A., 2004. Environmental signals and regulatory pathways that influence biofilm formation. Mol. Microbiol., **52**, 917–924.

[20] Davey, M.E., O'Toole, G.A., 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev., **64**, 847–867.

[21] Nyholm, S.V., Nishiguchi, M.K., 2008. The evolutionary ecology of a sepiloid squid-*Vibrio* association: from cell to environment. Vie et Milleu, **58**, 175–184.

[22] Naughton, L.M., Blumerman, S.L., Carlberg, M., Boyd, E.F., 2009. Osmoadaptation among *Vibrio* species and unique genomic features and physiological responses of *Vibrio parahaemolyticus*. Appl. Environ. Microbiol., **75**, 2802–2810.

[23] Gottesman, S., Stout, V., 2006. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. Mol. Microbiol., **5**, 1599–1606.

[24] Sutherland, I.W., 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology, **147**, 3–9.

[25] Zuur, A.F., Pierce, G.J., 2004. Common trends in northeast Atlantic squid time series. J. Sea Res., **52**, 57–72.

[26] Nishiguchi, M.K., 2000. Temperature affects species distribution in symbiotic populations of *Vibrio*. Appl. Environ. Microbiol., **66**, 3550–3555.

[27] Ruby, E.G., McFall-Ngai, M.J., 1999. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends in Microbiol., **7**, 414–420.

[28] Lupp, C., Urbanowski, M., Greenberg, E.P., Ruby, E.G., 2003. The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol. Microbiol., **50**, 319–331.

[29] Hammer, B.K., Bassler, B.L., 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. Mol. Microbiol., **50**, 101–104.

[30] Hader, D.P., Kumar, H.D., Smith, R.C., Worrest, R.C., 1998. Effects on aquatic ecosystems. J. Photochem. Photobiol. B: Biology, **46**, 53–68.

[31] Bruckner, R., Titgemeyer, F., 2006. Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiol., **209**, 141–148.

[32] Jain, V., Kumar, M., Chatterji, D., 2005. ppGpp: Stringent response and survival. J. Microbiol., **44**, 1–10.

[33] Van Delden, C., Comte, R., Bally, A.M., 2001. Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. J. Bacteriol., **183**, 5376–5384.

doi:10.1111/j.1558-5646.2011.01547.x

EVOLUTIONARY PERSPECTIVES IN A MUTUALISM OF SEPIOLID SQUID AND BIOLUMINESCENT BACTERIA: COMBINED USAGE OF MICROBIAL EXPERIMENTAL EVOLUTION AND TEMPORAL POPULATION GENETICS

W. Soto,[1] E. B. Punke,[1] and M. K. Nishiguchi[1,2]

[1]*Department of Biology, New Mexico State University, Las Cruces, NM 88003–8001*

[2]*E-mail: nish@nmsu.edu*

Received April 19, 2011
Accepted October 25, 2011

The symbiosis between marine bioluminescent *Vibrio* bacteria and the sepiolid squid *Euprymna* is a model for studying animal–bacterial Interactions. *Vibrio* symbionts native to particular *Euprymna* species are competitively dominant, capable of outcompeting foreign *Vibrio* strains from other *Euprymna* host species. Despite competitive dominance, secondary colonization events by invading nonnative *Vibrio fischeri* have occurred. Competitive dominance can be offset through superior nonnative numbers and advantage of early start host colonization by nonnatives, granting nonnative vibrios an opportunity to establish beachheads in foreign *Euprymna* hosts. Here, we show that nonnative *V. fischeri* are capable of rapid adaptation to novel sepiolid squid hosts by serially passaging *V. fischeri* JRM200 (native to Hawaiian *Euprymna scolopes*) lines through the novel Australian squid host *E. tasmanica* for 500 generations. These experiments were complemented by a temporal population genetics survey of *V. fischeri*, collected from *E. tasmanica* over a decade, which provided a perspective from the natural history of *V. fischeri* evolution over 15,000–20,000 generations in *E. tasmanica*. No symbiont anagenic evolution within squids was observed, as competitive dominance does not purge *V. fischeri* genetic diversity through time. Instead, abiotic factors affecting abundance of *V. fischeri* variants in the planktonic phase sustain temporal symbiont diversity, a property itself of ecological constraints imposed by *V. fischeri* host adaptation.

KEY WORDS: **Adaptation, experimental evolution, fitness, mutualism, sepiolid squid, *Vibrio*.**

The symbiosis between marine bioluminescent *Vibrio fischeri* and their sepiolid squid hosts (Cephalopoda: Sepiolidae) has revolutionized the study of animal–bacteria interactions, serving as a model for the last two decades, because both bacteria and squids can be maintained independently of each other in the laboratory (Nishiguchi 2000). Symbiotic bacteria can be grown in pure culture and the hosts raised axenically. Squid hatchlings emerge from their eggs with gnotobiotic light organs and subsequently are colonized within hours by free-living planktonic *V. fischeri* from seawater (McFall-Ngai and Ruby 1991; Nyholm and Nishiguchi 2008). Adult animals seed the water column via venting 90–95% of the bacteria at dawn, when the squid bury in sand. The remaining 5–10% of the symbionts grow throughout the day and reestablish a full light organ population during this time (Ruby

1996). *Vibrio fischeri* inhabit the mantle cavity of the squid in a specialized, complex, morphological structure termed the light organ (McFall-Ngai and Ruby 1991; Nyholm and Nishiguchi 2008). Research suggests squid utilize the bacterial bioluminescence for counter-illumination concealment during its nocturnal activity (Jones and Nishiguchi 2004), and squid aid the bacteria by providing a microhabitat rich in nutrients (Soto et al. 2009).

The sepiolid squid genera *Euprymna* and *Sepiola* reside in the Indo-West Pacific Ocean and Mediterranean Sea, respectively (Soto et al. 2009). *Euprymna* species distributions are allopatric, whereas those of *Sepiola* co-occur (Nesis 1982). Hybridization among sepiolid squid species is exceedingly rare (Zamborsky and Nishiguchi 2011). *Vibrio* symbionts indigenous to *Euprymna* species are host specialists and exhibit competitive dominance, outcompeting allochthonous isolates. This implies acquired trade-offs during symbiont adaptation to regional *Euprymna* hosts, and these fitness costs manifest themselves when colonizing other sepiolid squids (Nishiguchi 2002; Elena and Lenski 2003). *Vibrio* symbionts in *Sepiola* are host generalists and display no competitive dominance. Despite this competitive dominance of symbionts within *Euprymna* species, previous research suggests secondary colonization events have occurred, whereby nonnative *Vibrio* genotypes have invaded new geographical areas to ultimately become established and simultaneously displace native symbionts from their former *Euprymna* hosts (Jones et al. 2006).

Interestingly, *V. fischeri* also forms light organ mutualisms with monocentrid fishes, and these fish isolates do not colonize squid well (Nishiguchi 2002). Recent work demonstrated that change in a single regulatory gene (*rscS*) was sufficient to dramatically improve colonization capability (Mandel et al. 2009). Host switching is common in the natural history of Vibrionaceae symbioses, regardless of whether the interactions were mutualisms, host–pathogen relationships, or commensalisms. Additionally, obligately free-living *V. fischeri* strains exist as bacterioplankton community members, unable to colonize monocentrid fish or sepiolid squid to initiate light organ symbioses. This abandonment and reacquisition of the symbiont lifestyle from an obligately free-living one has occurred numerous times based on phylogenetic analysis of closely related *Vibrio* species (Nishiguchi and Nair 2003). *Vibrio fischeri* is capable of persisting as a native part of marine sediment and sand biofilm microflora, skin and gut commensals of marine animals, and attached to floating debris, particles, zooplankton, phytoplankton, and carrion (Nishiguchi and Jones 2004; Soto et al. 2010).

Previous work has suggested abiotic factors, microbial allelopathy, and social cooperative behavior can significantly impact growth and abundance of *V. fischeri* symbiont populations existing as part of the free-living bacterioplankton (Soto et al. 2009). For instance, *V. fischeri* symbionts from *Euprymna* differ more from each other than those of *Sepiola* in how they grow over abiotic factor gradients (e.g., salinity) spanning their fundamental niche breadth, including lower and upper growth limits. Hence, abiotic factors provide a mechanism that may allow nonnative *V. fischeri* from foreign geographical areas to invade a particular *Euprymna* host range and extirpate native symbionts through selective amplification of nonnative *V. fischeri* numbers in the oceanic water column while in the free-living phase. Additionally, abiotic factors, allelopathy, and microbial sociality may facilitate the intrusion of nonnative symbionts into a region to the detriment of autochthonous ones by providing the advantage of an early start during squid host colonization (i.e., "running start" or "headstart" colonization; Soto et al. 2009). Moreover, evolution of host specificity by *V. fischeri* may be drastically influenced by founder effects (Soto et al. 2009; Wollenberg and Ruby 2009).

Consequently, competitively dominant *V. fischeri,* locally adapted and specialized to a definitive *Euprymna* squid species, could be outflanked by foreign, more maladapted and less fit isolates during host colonization. Phenomena such as environmental stress, fluctuating abiotic factors, microbial allelopathy, quorum-sensing systems, and tight genetic bottlenecks may grant nonnative *V. fischeri* an opportunity to establish footholds in novel *Euprymna* hosts, despite the continued presence of competitively dominant isolates (Nyholm and Nishiguchi 2008). From these host beachheads, exotic *V. fischeri* could potentially adapt to new and alien *Euprymna* light organ habitats (Soto et al. 2009). Therefore, to test the hypothesis that nonnative strains make headway against competitive dominance, host colonization experiments were implemented in *Euprymna scolopes* and *E. tasmanica* where a nonnative *V. fischeri* strain was given a numerical or running start advantage over an endemic one. In addition, we tested the hypothesis that foreign *V. fischeri* were evolutionarily amenable to expeditious host shifts. *V. fischeri* ES114 from the Hawaiian bobtail squid (*E. scolopes*) was serially passaged through the novel host *E. tasmanica*, the Australian dumpling squid. The goal was to use experimental evolution to adapt nonnative symbiont *V. fischeri* ES114 from Hawaiian *E. scolopes* to Australian host *E. tasmanica* for 500 generations to increase the competitive ability of a derived nonnative symbiont relative to the ancestral clone (Soto et al. 2010). This approach gave the nonnative symbiont a chance to become adapted to the novel host.

Previous research suggests approximately 500–750 generations is sufficient time for bacteria to respond to selection in a novel environment (Lenski et al. 1991). In the following experiments, relative fitness is defined as the proportion of symbionts that have successfully colonized a squid light organ (Nishiguchi et al. 1998; Nishiguchi 2002). Animal colonization experiments with the nonnative lines, evolved in *E. tasmanica,* were conducted in the ancestral Hawaiian squid host *E. scolopes* to detect possible trade-offs. Also, the accompanying effect of host adaptation to *E. tasmanica* on *V. fischeri* microbial physiology was

examined to determine the relationship between symbiosis evolution and bacterial growth response to an abiotic factor ecologically important to *V. fischeri* during the free-living phase (e.g., salinity), including under environmentally stressful conditions (Nyholm and Nishiguchi 2008; Soto et al. 2009, 2010). Thus, the growth of the derived nonnative lines along a sodium chloride (NaCl) gradient, spanning the entire saline niche breadth, was compared to the ancestral state.

To complement the study of *V. fischeri* in squid *E. tasmanica* for 500 generations using microbial experimental evolution, symbiotic *V. fischeri* were isolated over a time span close to a decade from *E. tasmanica* animals (a duration representing 15,000–20,000 generations of *V. fischeri* evolution in this squid host) from wild ocean populations inhabiting a single locality in Australia (Botany Bay, New South Wales). Spatial population genetics studies with *V. fischeri* in allopatric and sympatric squid host populations have revealed high levels of symbiont biodiversity (Boettcher and Ruby 1994; Kimbell et al. 2002; Jones et al. 2006; Zamborsky and Nishiguchi 2011). Because elevated levels of genetic diversity exist in the geographical landscape of *V. fischeri* symbionts, we examined whether this diversity was maintained over temporal scales. Haplotype network analysis was used to examine any fluxes that may be occurring in *V. fischeri* genetic diversity over the sampled time period. Published work in temporal population genetics of natural bacterial populations is scant relative to literature existing on spatial genetic structure (Ramette and Tiedje 2007). Some of these examples include *Escherichia coli* from human hosts over an 11-month period (Caugant et al. 1981) and a *Burkholderia cepacia* stream study over 32 days (Wise et al. 1996). This article is the first to simultaneously integrate both microbial experimental evolution and temporal population genetics, uniquely providing microevolution and macroevolution viewpoints for a marine bioluminescent bacteria-squid symbiosis.

Methods and Materials

IN VIVO ANIMAL EXPERIMENTS

Animal experiments were completed in a 12:12 h dark–light cycle at 25°C in 10-mL scintillation vials containing 5-mL artificial seawater (34 parts per thousand [ppt]). Fresh seawater changes were made every 12 h and accompanied with bioluminescence measurements on a TD-20/20 luminometer (Turner Scientific, Sunnyvale, CA). Axenic squid hatchlings were inoculated with 1×10^3 *V. fischeri* colony forming units (CFUs)/mL for monoculture and competitive colonization experiments. This cell density is sufficient to ensure squid hatchling inoculation with *V. fischeri*. After 3 h of inoculation, animals were rinsed with sterile seawater, allowing colonization to be synchronized within this window (McCann et al. 2003). When the animals were sacrificed, squid light organs were homogenized, serially diluted, and plated onto 70% seawater tryptone (SWT) agar plates for enumeration (Nishiguchi et al. 1998). Symbionts from squid light organ homogenates have nearly 100% plating efficiency (Ruby 1996).

"SUPERIOR NUMBERS" AND "HEADSTART" COLONIZATION

For "superior numbers" and "headstart" colonization experiments, *V. fischeri* strains ES114 (native to Hawaiian *E. scolopes*) and ET401 (native to *E. tasmanica*) were used (Nishiguchi et al. 1997). The two strains are genetically distinct (Nishiguchi and Nair 2003). *Vibrio fischeri* ES114 was the nonnative against *V. fischeri* ET401 in *E. tasmanica*, and *V. fischeri* ET401 was the nonnative to *V. fischeri* ES114 for the cross-experiments performed in *E. scolopes*. Numerical (2:1, 5:1, and 10:1, $n = 20$) and running start (12 h, $n = 20$) advantages were given to the foreign strain. A running start time of 12 h, along with the ratios 2:1, 5:1, and 10:1, were chosen because these nonnative advantages seemed ecologically realistic and form a reasonable simulation of what may happen in nature. Additionally, venting 95% of nonnative symbionts inhabiting the squid light organ after the 12-h headstart provided the most optimal condition for the native strain to infiltrate, since this was when nonnatives were most susceptible and their numbers decimated. Initiation of headstart colonization began once animals hatched in the dark. Sterile (axenic) animals ($n = 20$) served as negative controls. Pure culture inoculations of squid with only native or nonnative symbionts were positive controls ($n = 20$). Animals inoculated 1:1 with native to nonnative *V. fischeri* were competitive dominance controls. For headstart colonization experiments, a 12-h running start advantage was also given to the native strain as an additional control. These experiments were conducted through 48 h, at which point animals were sacrificed (see section "In vivo animal experiments" for further details). *Vibrio fischeri* ET401 is visibly luminescent whereas ES114 is not, and their relative numbers from platings of light organ homogenates containing both can be determined by counting the number of luminescent or bright colonies in the dark ("ET401" colonies; Nishiguchi et al. 1997). Subtracting this previous number from the total colonies on the plate counted (ET401 + ES114) yields the number of "ES114" colonies. This method of strain discrimination and enumeration during competitive co-inoculation experiments of sepiolid squid hosts with symbionts has been published previously (Nishiguchi et al. 1997, 1998). No unusual interactions occur between *V. fischeri* ET401 and ES114 with regard to visible bioluminescence on seawater high nutrient agar plates, and this phenotypic difference is 100% stable between these two specific strains, exhibiting no phase variation since penetrance is complete (Nishiguchi et al. 1997, 1998). Additionally, there is virtually no mutation from one phenotype

to another relating to visible luminescence in *V. fischeri* ET401 and ES114.

IN VIVO EXPERIMENTAL EVOLUTION

Experimental evolution was conducted by serially passaging *V. fischeri* JRM200 (isogenic to *V. fischeri* ES114, see below) through axenic *E. tasmanica* hatchlings ($n = 24$) for 96 h, when animals were sacrificed. Axenic animals remained uninoculated, serving as negative controls ($n = 24$). Light organ homogenate fractions were used to continuously serial passage *V. fischeri* JRM200 through hatchlings for additional 96-h rounds. Homogenates were frozen (20% glycerol final concentration) every five serial passages (25 total) and stored at –80°C to create a "frozen fossil record." After every fifth serial passage, competitive colonization experiments were completed with derived *V. fischeri* JRM200 and ancestral ES114 in *E. tasmanica* ($n = 24$). This assay determines the degree of competitiveness between different strains of symbiotic bacteria (Nishiguchi 2002). The "more fit" competitor will be the symbiont strain that comprises a significantly higher proportion of the final light organ population at 96 h. *Vibrio fischeri* JRM200 is a chloramphenicol-resistant isogenic derivative of *V. fischeri* ES114 (chloramphenicol sensitive; McCann et al. 2003). The spontaneous mutation rate of chloramphenicol resistance was negligible in *V. fischeri* ES114 ($\sim 1 \times 10^{-10}$ CFUs/mL) and no reversion of the marker phenotype to wild-type was observed in *V. fischeri* JRM200 (Soto 2009). Axenic animals ($n = 24$) served as negative controls. Positive control animals ($n = 24$) were exclusively infected with only one of two competitors. Animals were sacrificed, light organs homogenized at 96 h and plated on SWT agar (Ruby 1996). The proportion of light organ colonization by each competitor was then quantified by screening 100 random colonies for chloramphenicol resistance (McCann et al. 2003). Neutral marker status of chloramphenicol resistance in *E. tasmanica* was assessed similarly in two different experiments ($n = 13$, $n = 20$). Chloramphenical resistance has already been shown to be a neutral marker in *E. scolopes* (McCann et al. 2003). Competitive colonization experiments between *V. fischeri* JRM200 and ES114 were also executed in *E. scolopes* (Hawaiian host) over 48 h using the same methodology conducted in *E. tasmanica* (Australian host) for all 24 of the 500-generation derived lines ($n = 24$). Animal experiments with *E. scolopes* were not completed through 96 h because this squid species is more fragile and short lived than *E. tasmanica*.

EFFECTS OF SQUID HOST EVOLUTION ON *V. FISCHERI* GROWTH ALONG A SALINITY GRADIENT

Correlated responses of *V. fischeri* adaptation to novel host *E. tasmanica* on this marine microbe's ability to grow along a wide salinity range were studied using an assay previously published (Soto et al. 2009). Individual colonies from SWT plates for the 24 derived lines, ancestor *V. fischeri* ES114, and unevolved *V. fischeri* JRM200 were used to inoculate 18 × 150 mm test tubes containing 5-mL SWT. These test tubes served as starter cultures for the experiment. Tubes were incubated at 28°C while shaking at 225 rpm for 16 h. Thereafter, 10 μl of each overnight starter culture was used to inoculate test tubes containing 5 mL of fresh SWT liquid media. The subsequent cultures were incubated at 28°C and shaken at 225 rpm for 3 h. After 3 h of growth, a Uvikon XL spectrophotometer (Bio Tek Instruments, Winooski, VT) was used to measure optical density (OD_{600}) of all cultures. Cultures were then inoculated into test tubes containing 5-mL LBS with salinities spanning 0.0–7.0% NaCl. *Vibrio fischeri* does not grow above 7.0% NaCl LBS (Soto et al. 2009). All cultures began at the same initial cell density of 5×10^5 CFU/mL. NaCl concentrations were increased by 0.1% increments between 0.0% and 7.0%. Test tubes were placed in a shaker for 24 h at 28°C and 225 rpm. Optical density (OD_{600}) readings of each culture were measured at each NaCl concentration ($n = 5$).

COLLECTION, ISOLATION, AND CULTURE METHODS FOR HAPLOTYPE NETWORK ANALYSIS

Details of specimen collection and sampling strategy of bacterial symbionts from squid hosts have been described previously (Jones et al. 2006). With the exception of 2001 and 2002, *E. tasmanica* specimens were collected along the eastern Australian (Botany Bay, New South Wales) coast between 2000 and 2009. Squid light organs from adult animals were dissected, rinsed with sterile 34 ppt artificial seawater, homogenized aseptically with a pestle, and serially diluted in sterile 34 ppt seawater, and plated (100 μl) onto SWT agar plates (Nealson 1978; Nishiguchi et al. 1998). Plates were incubated for 24–48 h at 28°C. Ten to 20 colonies per animal for each year were identified using 10 μg Diagnostic Discs with the vibriostatic agent 0129 (Oxoid, Basingstoke, Hampshire, England) on SWT agar plates following manufacturer's instructions, tested for bioluminescence using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), and sequenced at the 16S rRNA locus for verification. Positive isolates were then streaked for isolation onto SWT agar plates and incubated for 24–48 h at 28°C. Single colonies from these plates were used to inoculate 5 mL of SWT liquid media in test tubes for a period of 12–18 h at 28°C at 200 rpm. These pure cultures were then frozen as stocks in a final concentration of 20% glycerol and kept at –80°C until needed.

DNA EXTRACTION, POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION, AND SEQUENCING OF BACTERIAL ISOLATES

Frozen stocks of *V. fischeri* were recovered by streaking onto SWT agar plates and incubating for 24–48 h at 28°C. Single colonies were picked from these cultures and inoculated into 5 mL of

SWT liquid media in test tubes, which were then incubated at 28°C while shaking at 200 rpm for 12–18 h. Bacterial cells were then harvested by centrifugation at 7500 rpm for 10 min. The resulting pellet was resuspended in 180 μl of Buffer ATL (Qiagen DNeasy Blood and Tissue extraction kit, Qiagen Sciences, MD). The bacterial cells were lysed by addition of 20 μl Proteinase K and heated at 56°C for 1–3 h while shaking at 80 rpm. Once lysed, the cells were washed and DNA was extracted according to total DNA bacterial isolation protocols using the Qiagen DNeasy Blood and Tissue extraction kit (Qiagen Sciences) with manufacturer's recommendations for Gram-negative bacteria. The DNA was eluted and stored in 200 μl Buffer AE. The quality and concentration of the extracted DNA was determined using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA).

A total of 5–10 ng of purified DNA was used as a template in the PCR amplification of a ∼350-bp fragment of the glyceraldehyde phosphate dehydrogenase subunit A locus (*gapA*) using the primers: gapAfischeriVFF (5′- TCGAATTGTTTCTAATGGGTCA-3′) and gapAVFR (5′-AGCGGCGCTTCAGTATAGTC-3′; Jones et al. 2006). PCR reactions to amplify the *gapA* locus from *V. fischeri* were performed as 25 μl reactions containing: 5–10 ng of extract DNA, 0.2 μM of both forward and reverse primers, 0.2 U of *Taq* polymerase (GoTaq® Flexi DNA polymerase, Promega, San Luis Obispo, CA), 2.5 mM of magnesium chloride ($MgCl_2$), a final concentration of 200 μM of dGTP, dATP, dCTP, and dTTP (50 μM each), and 1 × buffer (Tris-HCl, KCl, and 0.1% Triton X-100). PCR reactions were completed in a MJ Research Dyad Disciple thermal cycler (Waltham, MA). Samples were amplified using the following PCR cycle: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec for 32 cycles with one final termination step at 72°C for 7 min. PCR products were cleaned using the Qiagen PCR purification kit (Qiagen Sciences, Valencia, CA).

The *gapA* locus was sequenced according to BigDye Terminator version 3.1 Sequencing kit protocol (Applied Biosystems, Foster City, CA). PCR products were combined in a presequencing reaction mixture containing 3–10 ng clean PCR product, forward or reverse primer (10 mM), Big Dye combined with SeqSaver (Sigma, St. Louis, MO) in a 1:1 dilution for a 20% final volume, sequencing buffer (1× final concentration), and PCR H_2O for a final volume of 10 μl. Presequencing reactions were run in an MJ Research Dyad Disciple thermal cycler. The presequenced *gapA* product was spin column purified (Edge Biosystems, Gaithersburg, PA), and mixed with 3–5 μl Hi-Di Formamide prior to sequencing to bring the final reaction volume over the minimum of 10 μl. Samples were sequenced on a 3100 ABI capillary sequencer (Applied Biosystems). Independent forward and reverse sequences were subsequently combined and edited using Sequencher v 4.6 (Gene Codes™, Ann Arbor, MI), and compared against the NCBI database through the Basic Local Alignment Search Tool (BLAST) to confirm gene identity with *V. fischeri* ES114. Sequences were exported in FASTA format and aligned with Clustal W2 (Thompson et al. 1994). Analysis of the sequencedata for building haplotype networks was completed in Arlequin v3.1 (Excoffier et al., 2005) and then used in TCS 1.21 (Clement et al. 2000) to determine genetic variance between the haplotypes and to create a haplotype network, respectively.

DNASP AND TCS ANALYSES

To measure variation within and among populations, genotype data were imported in PHYLIP sequential format into the computer program DnaSP version 4.10.9 (Rozas and Rozas 1999). For each individual population (now defined as comprising all the bacterial isolates from a single year), Arlequin generated measurements of DNA divergence by computing haplotype diversity (Nei 1987). To quantify genetic variation among populations (e.g., 2000 vs. 2007), DnaSP computed Nei's coefficient of gene variation (G_{st}), which varies between 0 and 1 (Nei 1973, 1975). G_{st} approaching 0 infers the majority of variation is found within populations, whereas G_{st} approaching 1 infers the majority of variation is found between populations. The program TCS v. 1.21 (Clement et al. 2000) was utilized to generate a haplotype network depicting symbiont genotype distribution through time for *V. fischeri* isolated from *E. tasmanica* inhabiting Botany Bay, Australia. TCS created the haplotype network from symbiont genotype data by telescoping sequences into haplotypes and calculating the frequency of the haplotypes using a statistical parsimony algorithm (Templeton et al. 1992). Under this method, the probability of parsimony is calculated until it exceeds 0.95. The maximum number of steps connecting parsimoniously two haplotypes is the number of mutational differences just before this 95% cutoff (Clement et al. 2000). The program was run using default settings with gaps treated as a fifth state. A haplotype network was created and displayed as a graph with separate circles representing separate haplotypes, the size of each circle representing the number of isolates in each haplotype, and dissimilar colors signifying different years.

Results and Discussion

"SUPERIOR NUMBERS" AND "HEADSTART" COLONIZATION

Earlier work has addressed conceivable mechanisms of how competitive dominance can be countered (Soto et al. 2009). For example, severe genetic bottlenecks and founder effects inherent during symbiont colonization may place an upper asymptotic limit on the advantage that indigenous *V. fischeri* can gain over nonnatives during host adaptation due to extensive genetic drift (Novella et al.

Table 1. **(A) Colonization experiments with superior numbers of nonnative *Vibrio fischeri* ET401 (2:1, 5:1, and 10:1) over native *V. fischeri* ES114 in *Euprymna scolopes*. (B) Headstart advantage (12 h) of nonnative *V. fischeri* ET401 over native *V. fischeri* ES114. Total inoculum of both strains in artificial seawater is 1 × 10^3 CFUs/mL. Squid hatchlings were sacrificed at 48 h for enumeration of *V. fischeri*. Letters indicate significant differences using fixed-treatment analysis of variance ($P < 0.05$, $\alpha = 0.05$).**

Treatment (n=20)	Percent *Vibrio fischeri* ET401 (nonnative)
(A) Nonnative superior numbers in *Euprymna scolopes*.	
Axenic squid	0
ES114 positive control	0
ET401 positive control	100
1:1 ET401:ES114	2.7^{A}
2:1 ET401:ES114	11.9^{B}
5:1 ET401:ES114	42.6^{C}
10:1 ET401:ES114	53.8^{D}
(B) Nonnative headstart in *E. scolopes*.	
Axenic squid	0
ES114 positive control	0
ET401 positive control	100
1:1 ET401:ES114	6.3^{A}
ES114+12 h ET401	0
ET401+12 h ES114	80.3^{B}

Table 2. **(A) Colonization experiments with superior numbers of nonnative *Vibrio fischeri* ES114 (2:1, 5:1, 10:1) over native *V. fischeri* ET401 in *Euprymna tasmanica*. (B) Headstart advantage (12 h) of nonnative *V. fischeri* ES114 over native *V. fischeri* ET401. Total inoculum of both strains in artificial seawater is 1 × 10^3 CFUs/mL. Squid hatchlings were sacrificed at 48 h for enumeration of *V. fischeri*. Letters indicate significant differences using fixed-treatment analysis of variance ($P < 0.05$, $\alpha = 0.05$).**

Treatment (n=20)	Percent *Vibrio fischeri* ES114 (nonnative)
(A) Nonnative superior numbers in *Euprymna tasmanica*.	
Axenic squid	0
ES114 positive control	100
ET401 positive control	0
1:1 ES114:ET401	5.4^{A}
2:1 ES114:ET401	15.2^{B}
5:1 ES114:ET401	65.7^{C}
10:1 ES114:ET401	98.2^{D}
(B) Nonnative headstart in *E. tasmanica*.	
Axenic squid	0
ES114 positive control	100
ET401 positive control	0
1:1 ES114:ET401	7.6^{A}
ET401+12 h ES114	0
ES114+12 h ET401	100^{B}

1995; Wollenberg and Ruby 2009). Gaining superior numbers or a headstart (Tables 1A, B, 2A, B) in host squid colonization by nonnative *V. fischeri* compared to native strains may also offset and outflank competitive dominance. The possibility remains that any native *V. fischeri* remaining in the light organ, after these initial advantages to nonnatives dissipate, will eventually outcompete inhabiting nonnatives and retake the squid host, especially if natives continue preferential host occupancy during repeated venting events. However, these data (Tables 1A, B, 2A, B) clearly demonstrate that nonnatives can make some headway against competitive dominance with superior numbers and the advantage of "running start" host colonization. Additionally, distinct morphological changes arise in *Euprymna* hatchlings during initial phases of symbiosis consequent to incipient colonization by *Vibrio* symbionts, and these dramatic physical changes make perpetual and successive serial recolonizations from free-living *Vibrio* cells less probable upon maturation of the association. This transformation enables invading nonnative *Vibrio* symbionts to retain inhabitance of foreign *Euprymna,* despite continued existence of competitive dominant strains in seawater. Salinity and temperature have also demonstrated striking and sudden effects on *Vibrio* symbionts isolated from *Euprymna*, which may lead to countervailing of competitive dominance (Soto et al. 2009). Remarkably, slight alterations in salinity (e.g., Δ0.1% NaCl$\approx\Delta$1 ppt) can lead to abrupt changes in microbial growth. For this reason, the strain predominating within a given sphere at a particular time can be kaleidoscopic, depending on the irregularity of salinity occurring in a regional area due to local water currents and seasonal changes. In some instances, precipitous saline demarcations exist, where a specific strain thrives and languishes, suggesting that gradual zones of decreased microbial growth are not always evident (Soto et al. 2009). This is especially true as the boundaries of saline tolerance are approached for *V. fischeri*. A single fluctuation in 0.1% NaCl or 1 ppt at the extreme ends of saline niche breath (lower and upper salinity limits of growth) can determine whether a certain *V. fischeri* isolate will grow or not. Hence, minute salinity deviations in the marine environment can profoundly affect host squid and *V. fischeri* symbiotic relationships by enabling nonnative *V. fischeri*, a superior numbers advantage (or a headstart) over native isolates during host squid colonization, even at moderate oceanic salinities typical of most marine waters (Soto et al. 2009). Incorporating temperature into this sodium chloride scheme only enriches the ecological complexity and the evolutionary dynamics for the sepiolid squid–*V. fischeri* mutualism.

MICROBIAL EXPERIMENTAL EVOLUTION

If advantage of superior numbers or headstart colonization allow nonnative *V. fischeri* to successfully establish an initial beachhead

Table 3. Chloramphenicol resistance in *Vibrio fischeri* strain JRM200 during *Euprymna tasmanica* colonization (two-tailed *t*-test $\alpha = 0.05$).

Sample size	(E)ES114:JRM200	(O)ES114:JRM200
13	61:39	57:43 ns[1]
20	40:60	39:61 ns[1]

[1]ns, not significantly different ($P > 0.05$).

in novel animal hosts, the question is raised whether this new invasive population can adapt to these new hosts, setting the stage for a study with microbial experimental evolution. These experiments also address the extent to which niche-invasion mutations and host range expansion are possible within *V. fischeri* (Cohan 2002). For instance, do evolutionary transitions or mutations exist that would permit a nonnative symbiont to invade a novel host and displace a native symbiont population that is already established? We estimated that *V. fischeri* populations undergo ~10 generations during the first 12 h of growth and colonization inside juvenile squid light organs. *Vibrio* strains reach a carrying capacity of approximately 10^5–10^6 CFUs per *E. scolopes* hatchling (Ruby 1996) and up to 10^7 CFUs per juvenile in larger *E. tasmanica*. Thereafter, the symbiont light organ population passes through ~5 generations per day, as 5% of the population remains after expulsion (Ruby 1996). Over a four-day period (96 h), *V. fischeri* experiences 20–25 generations in the squid host. Independent empirical and theoretical work from other laboratories support these estimates (Schuster et al. 2010). To differentiate *V. fischeri* serially transferred through *E. tasmanica* from antecedent *V. fischeri* ES114, *V. fischeri* JRM200 (isogenic to *V. fischeri* ES114 and chloramphenicol resistant) was utilized for all experiments. Chloramphenicol resistance was a neutral marker and did not affect symbiosis competency in *E. tasmanica* (Table 3) nor in *E. scolopes* (McCann et al. 2003). OD_{600} versus logarithmic cell density curves were used to achieve ratios of *V. fischeri* ES114 and JRM200 at 50:50 in seawater for all colonization experiments. Results were confirmed with agar plate (viable cell) enumeration and live cell counts using a cytometer under light microscopy. The proportion of *V. fischeri* ES114 and JRM200 observed in *E. tasmanica* after 96 h was not significantly different from the inoculation ratios in artificial seawater.

Vibrio fischeri JRM200 was serially passaged (1×10^3 CFUs/mL artificial seawater inoculum) through *E. tasmanica* 25 times and demonstrated an ever increasing proportion of the symbiont light organ population relative to the ancestor at consecutive evolutionary time points in *E. tasmanica*. At 400 and 500 generations, this value was significantly different compared to the ancestor (Table 4). This increase reinforces previous population genetic surveys suggesting allochthonous *V. fischeri* invasions

Table 4. Competitive colonization experiments between *Vibrio fischeri* strains ES114 (ancestor) and JRM200 (derived) at different evolutionary time points in the novel squid host *Euprymna tasmanica.*

Evolutionary time point (Generations)	Expected ES114:JRM200 (Percentage ancestor: percentage evolved)	Observed ES114:JRM200 (Percentage ancestor: percentage evolved)
0 (n=33)	50:50	46:54
100 (n=24)	50:50	47:53
200 (n=24)	50:50	41:59
300 (n=24)	50:50	41:59
400 (n=24)	50:50	35:65[1]
500 (n=24)	50:50	36:64[1]

[1]Significantly different two-tailed *t*-test and sign test ($P < 0.05$, $\alpha = 0.05$).

transpire and can become established, with concurrent displacement of native ones in different *Euprymna* species distributions (Jones et al. 2006). Therefore, *V. fischeri* certainly are evolutionarily fluid and capable of swift adaptation to new hosts. Host adaptation by *V. fischeri* JRM200 to *E. tasmanica* is reminiscent to the step model evolution that has been previously reported in *E. coli* experimental evolution studies (Table 4; Lenski et al. 1991). Relative to ancestral *V. fischeri* ES114, no significant differences in bioluminescence, growth rate in squid light organs, or symbiont population carrying capacity occurred in the novel squid host by *V. fischeri* JRM200 as a result of adapting to *E. tasmanica*.

Subsequently, we investigated possible correlated responses in the ancestral host *E. scolopes* as a result of 400 ($n = 6$) and 500 generations ($n = 24$) of symbiont evolution in *E. tasmanica*. No significant differences were observed in light organ carrying capacity between *V. fischeri* JRM200 and ES114 at 48 h in ancestral *E. scolopes,* either in competition or monoculture experiments. Derived *V. fischeri* JRM200 was consistently less bioluminescent than progenitor *V. fischeri* ES114 over 48 h of colonization in the ancestral host environment, and at 12, 24, and 36 h this disparity was significantly different at 400 generations (Table 5) and over the entire 48 h at 500 generations (Table 6). These observations are consistent with previous findings (Schuster et al. 2010) and are congruent with competitive dominance resulting from host specialization and local adaptation of symbionts to *Euprymna* species in the environment, with the simultaneous acquisition of antagonistic pleiotropy or mutation accumulation. These factors may subsequently lead to trade-offs in other host squid species (Elena and Lenski 2003). For example, a general tendency exists of *V. fischeri* isolated from Australian *E. tasmanica* to be visibly luminescent on seawater nutrient agar plates, whereas those from Hawaiian *E. scolopes* are nonvisibly luminescent (Nishiguchi et al. 1997). Previous results presume nonvisibly luminescent

Table 5. ***Vibrio fischeri*** **JRM200 (six randomly selected clones of 24) reduced bioluminescence in ancestral host** ***Euprymna scolopes*** **after 400 generations of evolution in novel host** ***E. tasmanica*** **(±SE). Standard error (SE) bars were calculated using the unbiased estimator for the mean.**

Hours postinoculation	Mean axenic squid bioluminescence relative light units (n=5)	Mean ES114 bioluminescence relative light units (n=6)	Mean JRM200 bioluminescence relative light units (n=6)
0	0.041 (±0.006)	0.033 (±0.005)	0.031 (±0.007)
12	0.042 (±0.005)	2.809[1] (±0.736)	0.395[1] (±0.161)
24	0.034 (±0.007)	13.94[1] (±3.667)	6.511[1] (±2.940)
36	0.032 (±0.010)	11.46[1] (±2.867)	2.966[1] (±0.990)
48	0.037 (±0.004)	4.212 (±1.275)	3.703 (±1.188)

[1]**Significantly different analysis of variance (**$P < 0.05$, $\alpha = 0.05$**).**

V. fischeri become brighter as a result of host evolution in *E. tasmanica*, yet this study advocates that increased dimness (e.g., secondary loss of luminosity) in *E. scolopes* is perhaps a more likely scenario.

CORRELATED RESPONSES ALONG SALINITY GRADIENTS AFTER 500 GENERATIONS IN *E. TASMANICA*

The ancestral and 24 *V. fischeri* lines serially passaged through *E. tasmanica* for 500 generations were grown along a salinity gradient to note correlated responses in this environment as a result of evolution in a novel squid host. Chloramphenicol resistance has no effect on *V. fischeri* growth along a salinity gradient, as *V. fischeri* ES114 and unevolved JRM200 were indistinguishable (Fig. 1). The ancestral lower and upper limits of microbial growth are 0.4% (SE $= \pm 6.0 \times 10^{-4}$) and 6.9% (SE $= \pm 9.9 \times 10^{-4}$) NaCl, respectively. Ten derived lines serially passaged in *E. tasmanica* for 500 generations exhibited a shift to the right in percent NaCl (increase), where microbial growth first occurred (minimum 0.01 OD_{600}) along a salinity gradient relative to ancestral *V. fischeri* ES114 and unevolved JRM200 (Figs. 2, 3). Another 10 derived lines showed no shift where microbial growth first occurred but demonstrated a shift in percent NaCl where microbial growth last occurred (minimum 0.01 OD_{600}) relative to the ancestor (Figs. 4, 5). The correlated responses of these 24 lines, as a result of undergoing novel host evolution in *E. tasmanica*, were the most common patterns observed. No single evolved line ever simultaneously expanded its lower and upper limit of growth relative to the ancestral osmolar niche as a result of host evolution. Apparently this was an evolutionary genetic or physiological constraint. Perhaps a trade-off exists in *V. fischeri* osmoregulation in its ability to grow at extremely low and high salinities. Two derived lines displayed no shift in percent NaCl where microbial growth initially or finally occurred relative to the ancestor (Fig. 6). However, these two lines still exhibited changes in growth abundance and biomass (i.e., amplitude) along the gradient—characteristics observed in all the derived lines. An additional two lines showed a shift to the left in percent NaCl (decrease) where microbial

Table 6. ***Vibrio fischeri*** **JRM200 reduced bioluminescence in ancestral host** ***Euprymna scolopes*** **after 500 generations of evolution in novel host** ***E. tasmanica*** **(±SE). SE bars were calculated using the unbiased estimator for the mean. "Unevolved" refers to** ***V. fischeri*** **JRM200 before experiencing any serial transfers through** ***E. tasmanica*** **(0 generations), whereas "Evolved" signifies** ***V. fischeri*** **JRM200 after undergoing serial passage through** ***E. tasmanica*** **for 500 generations.**

Hours postinoculation	Mean axenic squid (Negative control) bioluminescence relative light units (n=24)	Mean ES114 (Ancestor) bioluminescence relative light units (n=24)	Mean JRM200 (Unevolved) bioluminescence relative light units (n=24)	Mean JRM200 (Evolved) bioluminescence relative light units (n=24)
0	0.021 (±0.004)	0.026 (±0.006)	0.025 (±0.005)	0.033 (±0.004)
12	0.031 (±0.006)	10.16[1] (±1.043)	10.56[1] (±0.915)	0.689[1] (±0.079)
24	0.021 (±0.005)	38.81[1] (±3.070)	36.81[1] (±3.450)	15.44[1] (±0.668)
36	0.027 (±0.006)	11.56[1] (±1.343)	11.58[1] (±1.286)	6.958[1] (±0.812)
48	0.034 (±0.005)	39.68[1] (±2.044)	39.47[1] (±3.845)	29.78[1] (±2.313)

[1]**Significantly different analysis of variance (**$P < 0.05$, $\alpha = 0.05$**).**

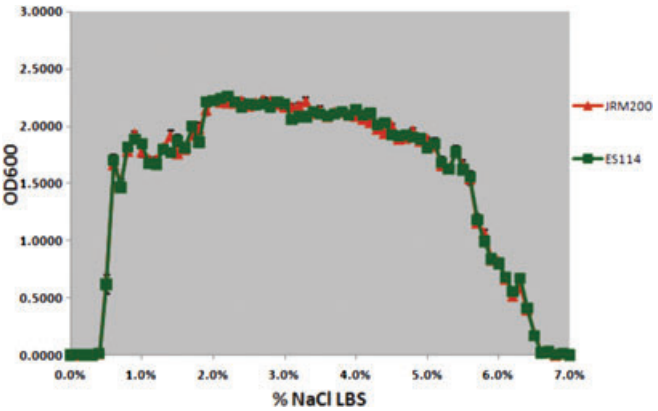


Figure 1. Growth curves of ancestral *Vibrio fischeri* ES114 and unevolved *V. fischeri* JRM200 along an increasing salinity gradient. No differences were observed between ancestral and unevolved strains used in the study.

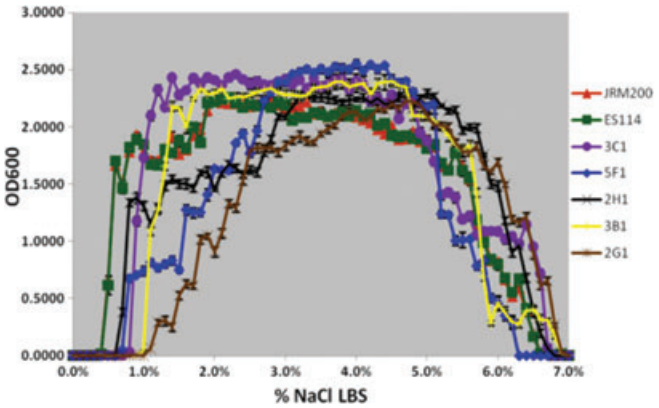


Figure 3. Five derived lines (3C1, 5F1, 2H1, 3B1, 2G1) serially passaged in *Euprymna tasmanica* for 500 generations exhibiting a shift to the right in percent NaCl where microbial growth first occurs (minimum 0.01 OD_{600}) along a salinity gradient relative to ancestral *Vibrio fischeri* ES114 and unevolved JRM200.

growth first occurred (0.2% NaCl, minimum 0.01 OD_{600}) relative to the ancestral osmolar niche (Fig. 7); this was the rarest and most unique (8.3%) correlated response recorded. Presumably, acquiring the capacity to grow at ever lower salinities has physiological limitations (*V. fischeri* is not capable of growth in 0% NaCl culture media or distilled water).

Vibrio fischeri osmoregulation and osmolar niche breadth were clearly affected by evolution through a novel squid host. Although the ancestral osmolar niche breadth was considerably altered as a byproduct of adaptation to *E. tasmancia*, no obvious trends in the correlated responses of the derived lines were noted. Seemingly, the derived *V. fischeri* osmolar niche breadth has been "randomized" from the ancestral state, due to 500 generations of serial transfer in the Australian dumpling squid. Because the crypt spaces in the squid light organ are continuous with the mantle cavity, which itself is extracellular space that merges and is

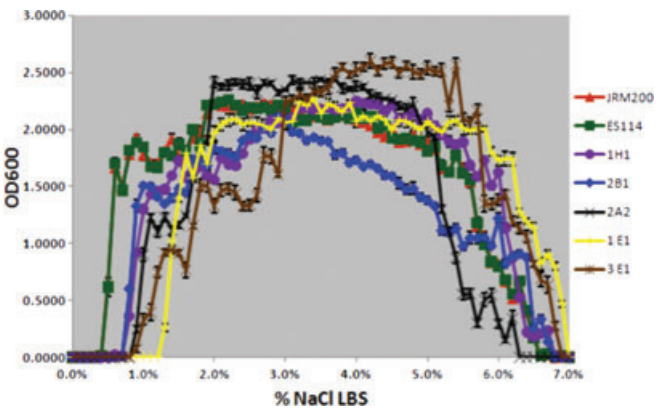


Figure 2. Five derived lines (1H1, 2B1, 2A2, 1E1, 3E1) serially passaged in *Euprymna tasmanica* for 500 generations and showing a shift to the right in percent NaCl where microbial growth first occurs (minimum 0.01 OD_{600}) along a salinity gradient relative to ancestral *Vibrio fischeri* ES114 and unevolved JRM200.

perpetually bathed with the ocean water environment through the siphon during squid ventilation, salinity within the chamber fluids of the squid light organ can only logically be considered as equal to that of the surrounding marine water. Possible complications include exudates or secretions by mucosal cells lining the crypts, and the existence of crypt microenvironments where limited or no mixing occurs. Adequate turbulent and laminar mixing are genuine concerns in batch cultures and bioreactors (Kresta and Brodkey 2004; Szalai et al. 2004), yet the roles of fluid blending and hydraulic principles play in shaping microcosm (squid light organ) salinities remain obscure and merit closer scrutiny. Undoubtedly, *V. fischeri* growth responses and physiological ranges of tolerances (i.e., niche breadths) to other abiotic factors have also been impacted. These results imply that bacterial stress responses are pleiotropically and epistatically influenced by host evolution. Previous studies in microbial experimental evolution investigating stress physiology has been substantial in demonstrating such effects in *E. coli* (Bennett and Lenski 1999; Bennett and Hughes 2009). Additionally, earlier work strongly suggested variable environments and symbiosis were correlated in their effects on *V. fischeri* microbial growth (Soto et al. 2009), providing evidence that stress on microbial physiology induced by abiotic factors and host colonization (e.g., immune defenses) may be coupled (Soto et al. 2010). How bacterial stress responses of host-associated prokaryotes react to challenges imposed by abiotic factors versus host immunity is poorly studied. For instance, do bacterial stress responses compartmentalize and specialize in how they maintain homeostasis against these two origins of stress, or is there cross-reactivity and pathway generalization? Future work is aimed at further phenotypic characterization and genome sequencing of the ancestral and derived *V. fischeri* lines to identify traits and loci underpinning adaptation to *E. tasmanica*.

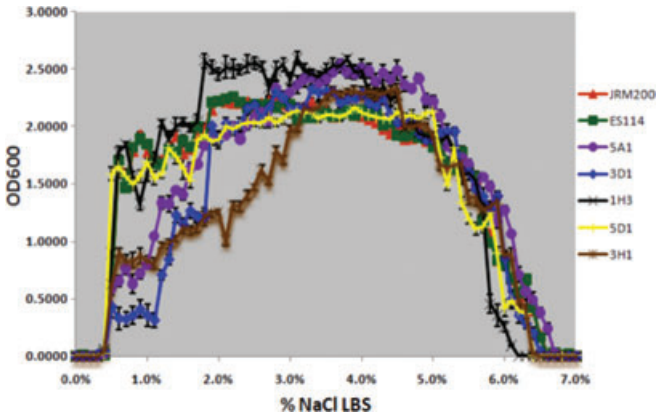


Figure 4. Five derived lines (5A1, 3D1, 1H3, 5D1, 3H1) serially passaged in *Euprymna tasmanica* for 500 generations and exhibiting a shift in percent NaCl where microbial growth last occurs (minimum 0.01 OD_{600}) along a salinity gradient relative to ancestral *Vibrio fischeri* ES114 and unevolved JRM200.

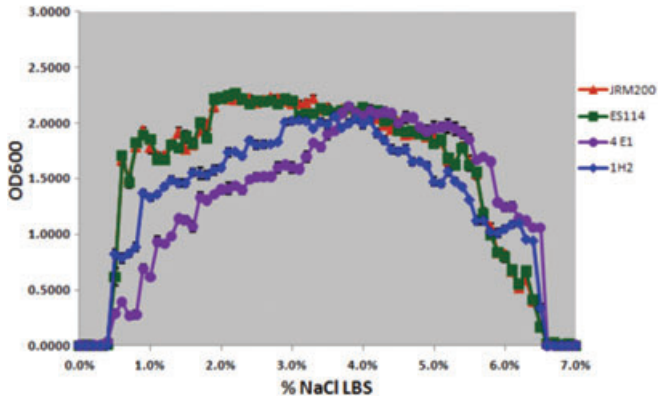


Figure 6. Two derived lines (4E1, 1H2) serially passaged in *Euprymna tasmanica* for 500 generations and exhibiting no shift in percent NaCl where microbial growth initially or finally occurs (minimum 0.01 OD_{600}) along a salinity gradient relative to ancestral *Vibrio fischeri* ES114 and unevolved JRM200. However, both 4E1 and 1H2 still exhibited changes in growth abundance and biomass (i.e., amplitude) along the gradient.

TEMPORAL HAPLOTYPE NETWORK

Variation produced by the *in vivo* experimental evolution study (artificial selection) among 24 lines was compared to wild *V. fischeri* isolates (natural history) procured from field-caught *E. tasmanica* specimens. The biodiversity of natural *V. fischeri* from *Euprymna* squid light organ populations was examined through a period of 10 years, a period of 15,000–20,000 generations in *Vibrio* symbiont evolution (Ruby and Asato 1993; Ruby 1996). The *V. fischeri* experimental evolution study performed inside a *Euprymna* squid host for 500 generations investigated microevolutionary processes (e.g., point mutations). Temporal analysis of diversity among symbiotic *V. fischeri* from *E. tasmanica* supplements experimental evolution studies by observing similar and other processes (e.g., horizontal gene transfer) at a macroevolutionary timescale, showing a haplotype network with extensive genetic diversity through time at the *gapA* locus (Fig. 8). Different colors in the haplotype network represent different years in which *E. tasmanica* were sampled, and the size of the circles signify number of isolates in each haplotype. A total of 24 different *gapA* haplotypes (alleles) were sampled through the years. Genetic distances between dissimilar haplotypes are represented by missing transitional forms, which are illustrated by black dots. For instance, Haplotypes 3 and 5 differ by six base changes, whereas Haplotypes 17 and 19 vary by one.

Vibrio fischeri haplotypes colonizing *E. tasmanica* between the years 2000–2009 (except 2001 and 2002) can be considered as a rising and ebbing tide of biodiversity comprised by multiple

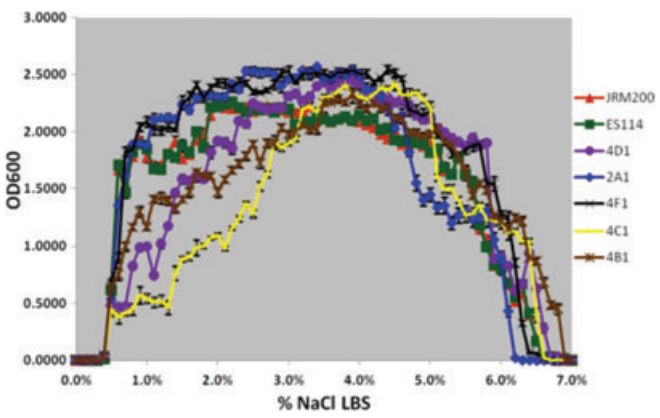


Figure 5. Five derived lines (4D1, 2A1, 4F1, 4C1, 4B1) serially passaged in *Euprymna tasmanica* for 500 generations and exhibiting a shift in percent NaCl where microbial growth last occurs (minimum 0.01 OD_{600}) along a salinity gradient relative to ancestral *Vibrio fischeri* ES114 and unevolved JRM200.

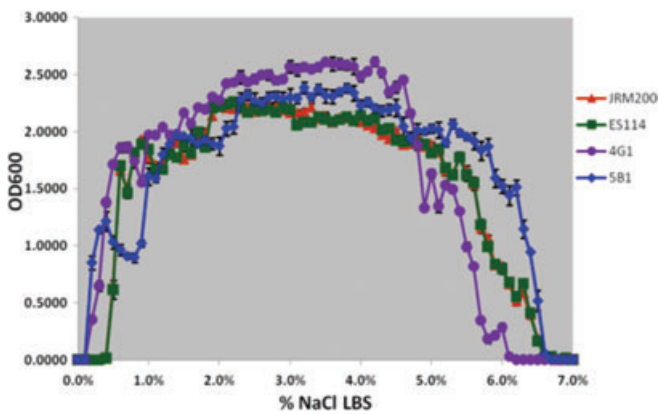


Figure 7. Two derived lines (4G1, 5B1) serially passaged in *Euprymna tasmanica* for 500 generations and exhibiting a shift to the left in percent NaCl where microbial growth first occurs (minimum 0.01 OD_{600}) along a salinity gradient relative to ancestral *Vibrio fischeri* ES114 and unevolved JRM200.

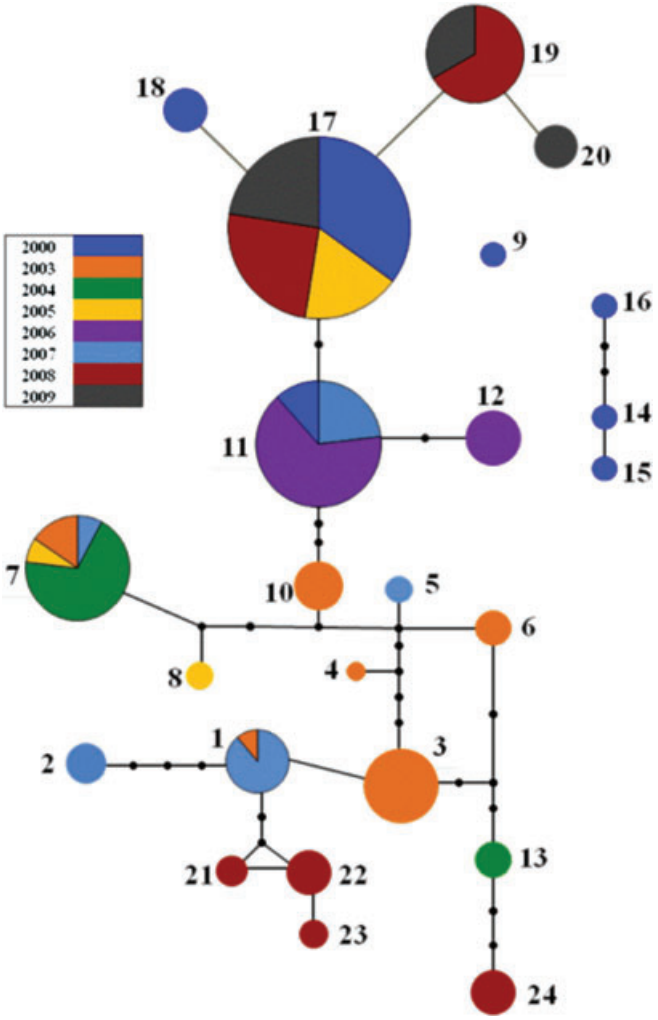


Figure 8. Temporal haplotype network of *Vibrio fischeri* strains from the same *Euprymna tasmanica* population (Botany Bay NSW, Australia) over 10 years (2000–2009) using the *gapA* locus. Two major haplotypes have dominated during this time: haplotype 17, which has the largest sample size (represented by the size of the circle) over the entire span (11 years) and haplotype 11 (second largest representative haplotype). Other related haplotypes (i.e., 2, 8, 10, 20) were found only in one year, and were never recovered during the course of this study. Haplotypes that genetically were not connected to the rest of the network (9, 14, 15, 16) were all from the initial collection time point (2000), and were never recovered in subsequent years (Soto 2009).

variants that populate the squid hosts through successive years (Table 7). Annual diversity values ($\Box_k$) have been corrected for uneven number of isolates and are directly comparable. Distinct haplotypes independently arose several times on separate occasions over a decade, signifying secondary colonization events are occurring. For example, Haplotype 17 was observed in 2000, 2005, 2008, and 2009, and Haplotype 11 is present in 2000, 2006, and 2007 (Fig. 8). Patterns of reemergence in *E. tasmanica* were not consistent for each haplotype; some haplotypes arose in successive years whereas others only recurred years later. Likewise, the prevalence of specific haplotypes at any given year varied greatly. No year existed where only a single haplotype was detected in *E. tasmanica* from the Botany Bay population. Peak diversity was recorded in 2003, whereas the minimum was in 2006 (Table 7). Closed loop *V. fischeri* Haplotypes 1, 21, and 22 represent unresolved ambiguities. Haplotypes 9, 14, 15, and 16 are 2000 *V. fischeri* haplotypes that were never resampled in subsequent years and are therefore disconnected to the main network. These haplotypes may represent locally extirpated, if not extinct, haplotypes or lineages lying in reservoirs that have yet to resurface. Populations of *V. fischeri* are vented daily from *E. tasmanica,* and these vented populations are potential inoculums for subsequent squid hatchlings. Therefore, it could be interpreted that the above patterns are indicative of expelled isolates thriving as opportunistic free-living bacteria colonizing squid hosts when environmental conditions favor their emergence. The *gapA* haplotypes most represented throughout the years (Haplotypes 7, 11, and 17) could represent environmental generalists. Haplotype 17 has also been discovered in a spatial population genetic survey of *V. fischeri* inhabiting light organs of *E. scolopes*, the Hawaiian bobtail squid (Jones et al. 2006), and may represent a *V. fischeri* generalist or biotype more able to shuttle reversibly between Hawaiian and Australian squid host distributions than other haplotypes (Soto 2009). The geographical range of *E. scolopes* relative to *E. tasmanica* is quite restricted in terms of inhabited area and range of environmental factors tolerated (Soto et al. 2009). For instance, *E. scolopes* is restricted to the coast of the Hawaiian archipelago, whereas *E. tasmanica* lives circum-continental to Australia. As a result, *V. fischeri* colonizing *E. scolopes* experience more constant habitats. Average annual temperature and salinity ranges for the marine realms of *E. scolopes* and *E. tasmanica* suggest that such spatial and

Table 7. *Vibrio fischeri* haplotype diversity ($\Box_k$ value) sampled from *Euprymna tasmanica* cyclically increases and decreases through the years. $\Box_k$ values have been corrected for the different number of symbiont isolates per year.

Year	$\Box_k$ value	No. of Haplotypes	No. of isolates
2000	1.6635	7	23
2003	3.2005	6	15
2004	0.4297	2	10
2005	0.6898	3	22
2006	0.3190	2	20
2007	2.0074	5	17
2008	1.1521	6	22
2009	0.8909	3	13
Combined	4.8331	24	142

temporal changes in salinity exist for both host habitats (Soto et al. 2009).

This work examining a decade of symbiont evolution associated with *E. tasmanica* is consistent with *V. fischeri* population genetics through space spanning diverse squid host species and oceans from across the globe (Kimbell et al. 2002; Jones et al. 2006; Zamborsky and Nishiguchi 2011), demonstrating similarity in *V. fischeri* population dynamics and diversity on both spatial and temporal scales. Whether oceanic currents or distributions of animal host metapopulations are chiefly responsible for *V. fischeri* dissemination in the South Pacific is unclear, as unknown *Euprymna* populations scattered amid Polynesian islands could be serving as stepping stone or corridor hosts. Sepiolid squids may be host habitat islands whose environmental complexity and heterogeneity (Travisano and Rainey 2000) cultivate and maintain *Vibrio* symbiont diversity. The *V. fischeri* fish host *Cleidopus gloriamaris* also co-occurs in Australian waters (Nishiguchi and Nair 2003), which may provide an additional source of symbiotic *V. fischeri*; however, prior work demonstrated that fish *V. fischeri* symbionts are quite different in their colonization capabilities than those found in sepiolid squid (Mandel et al. 2009). *Vibrio fischeri* haplotypes colonizing *E. tasmanica* are not continuously supplanted by *V. fischeri* haplotypes appearing in subsequent years. That is, there is an overall absence of anagenic symbiont evolution in sepiolid squid hosts and no direct evidence that *V. fischeri* fitness continuously improves in colonizing sepiolid squid hosts over time. Therefore, competitive dominance (Nishiguchi et al. 1998; Nishiguchi 2002) does not appear to sustain the same genetically distinct *V. fischeri* in *Euprymna* squid hosts over long evolutionary time periods, suggesting other determinants such as abiotic factors, ecological interactions between symbionts (competition, allelopathy, and social cooperative behavior), and founder effects have a strong influence in the sepiolid squid–*Vibrio* symbiosis (Nyholm and Nishiguchi 2008; Wollenberg and Ruby 2009). Host adaptation demonstrated by microbial experimental evolution does not transcend or upscale to long time intervals, however, experimental evolution does explain competitive dominance. Apparently, the same ecological processes fostering competitive dominance also nurture diversifying selection in *V. fischeri* populations in free-living and host-associated phases. Accordingly, competitive dominance does not purge *V. fischeri* haplotype diversity through time in the sepiolid squid–*Vibrio* symbiosis.

As Darwin did in his contrast between artificially selected pigeons and wild populations of rock doves to understand natural history and phenotypic variation of birds (Darwin 1859), contemplating genetic variation of experimentally evolved *V. fischeri* through a novel squid host to that of wild isolates procured from natural squid host populations can provide illumination to the evolution of these bioluminescent marine bacteria. To this end, the correlated responses documented in the salinity gradient by the derived lines from the experimental evolution study may enlighten why competitive dominance of native *V. fischeri* over nonnative isolates in *Euprymna* squid is an illusory barrier to secondary colonization. *Vibrio fischeri* do indeed adapt and specialize to the regional *Euprymna* species, yet this local host adaptation leads to "randomization" of the symbiont osmolar niche breadth (along with those of other abiotic factors). Thus, symbiotic *V. fischeri* vented back into the ocean by squid hosts every dawn are never able to optimize or trek their physiology to environmental conditions of the free-living phase, an especially important detriment to *V. fischeri* when environmental conditions themselves change. This phenomenon could account why no single *V. fischeri* haplotype predominates and persists through time in *E. tasmanica*. Periodic selection and selective sweeps may never be permitted in symbiotic *V. fischeri*, as specific symbiont haplotypes adapted to squid hosts of one generation may fail to consistently colonize hosts of the next generation due to the inability to thrive in the free-living phase under fluctuating environments. Instead alternate *V. fischeri* haplotypes (e.g., nonnatives) more numerous in the ocean under the particular circumstances are the genotypes to colonize the animal hosts. What *V. fischeri* haplotypes colonize *Euprymna* squid from one generation to next from the ocean may be a fortuitous or stochastic process. The existence of obligately free-living *V. fischeri* as bacterioplankton may be a stable evolutionary strategy that evades the physiological constraints imposed by squid host evolution, permitting local adaptation to abiotic factors of the free-living phase. Further mathematical elaboration of "symbiotic" versus "oceanic" *V. fischeri* ecotypes with game theory modeling is a reasonable next step.

Future work in spatial and temporal population genetics surveys of free-living bacterioplanktonic *V. fischeri* in the open ocean should be explored to determine if biodiversity differences exist between the free-living fraction and those colonizing light organ hosts. An important next step is plasmid population genetics and plasmid ecology, unaddressed since Boettcher and Ruby (1994). Examining symbiont plasmid diversity, possible coevolution with chromosomal loci, and how these attributes vary from bacterioplanktonic versus host-associated *V. fischeri* remain relatively unexplored topics in the sepiolid squid–*Vibrio* symbiosis. For microorganisms that alternate between free-living and host-affiliated phases (e.g., *Rhizobium*, *V. fischeri*), identifying sources of host shifts in associations between microbial symbionts and eukaryotic hosts, how these processes translate into patterns through space and time, and which mechanisms predominate at various scales are integral for more fully understanding interactions between hosts and their symbiotic microorganisms. Discerning the consequences of abiotic (salinity, temperature, environmental stress) versus host (immunity) factors on microbial ecology, biogeography, and diversity is necessary for coordinating all of these

processes into a manageable scenario for microbial evolution. These perspectives of mutualisms have implications for medical microbiology and infectious disease, as many microbial pathogens of eukaryotic hosts (including humans) cycle between free-living and symbiotic phases.

ACKNOWLEDGMENTS

Funding was provided by NSF-IOS0744498 and NIH-NIAID 1SC1A1081659–01 to MKN. W. Soto was supported by the NMSU RISE for the doctorate (NIH NIGMS R25GM061222). The authors would also like to thank M. McGrouther, P. Steinberg, N. Wilson, and G. Woolcott for help with squid collecting in Australia.

LITERATURE CITED

Bennett, A. F., and B. S. Hughes. 2009. Microbial experimental evolution. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297:17–25.

Bennett, A. F., and R. E. Lenski. 1999. Experimental evolution and its role in evolutionary physiology. Am. Zool. 39:346–362.

Boettcher, K. J., and E. G. Ruby. 1994. Occurrence of plasmid DNA in the sepiolid squid symbiont *Vibrio fischeri*. Curr. Microbiol. 29:279–286.

Caugant, D. A., B. R. Levin, and R. K. Selander. 1981. Genetic diversity and temporal variation in the *E. coli* population of a human host. Genetics 98:467–490.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9:1657–1659.

Cohan, F. M. 2002. What are bacterial species? Annu. Rev. Microbiol. 56:457–487.

Elena, S. F., and R. E. Lenski. 2003. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat. Rev. Genet. 4:457–469.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin version 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47–50.

Jones, B. W., and M. K. Nishiguchi. 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Mar. Biol. 144:1151–1155.

Jones, B. W., J. E. Lopez, J. Huttenburg, and M. K. Nishiguchi. 2006. Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. Mol. Ecol. 15:4317–4329.

Kimbell, J. R., M. J. McFall-Ngai, and G. K. Roderick. 2002. Two genetically distinct populations of bobtail squid, *Euprymna scolopes*, exist on the island of O'ahu. Pac. Sci. 56:347–355.

Kresta, S. M., and R. S. Brodkey. 2004. Turbulence in mixing applications. Pp. 19–88 in E. L. Paul, V. A. Atiemo-Obeng, and S. M. Kresta, eds. Handbook of industrial mixing: science and practice. John Wiley & Sons, Inc, Hoboken, NJ.

Lenski, R. E., M. R. Rose, S. C. Simpson, and S. C. Tadler. 1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. Am. Nat. 138:1315–1341.

Mandel, M. J., M. S. Wollenberg, E. V. Stabb, K. L. Visick, and E. G. Ruby. 2009. A single regulatory gene is sufficient to alter bacterial host range. Nature 458:215–218.

McCann, J., E. V. Stabb, D. S. Millikan, and E. G. Ruby. 2003. Population dynamics of *Vibrio fischeri* during infection of *Euprymna scolopes*. Appl. Environ. Microbiol. 69:5928–5934.

McFall-Ngai, M. J., and E. G. Ruby. 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science 254:1491–1494.

Nealson, K. H. 1978. Isolation, identification, and manipulation of luminous bacteria. Meth. Enzymol. 57:153–165.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. USA 70:3321–3323.

———. 1975. Molecular population genetics and evolution. North Holland, Oxford, U.K.

———. 1987. Molecular evolutionary genetics. Columbia Univ. Press, New York.

Nesis, K. N. 1982. Cephalopods of the world. T.F.H., Neptune City, NJ.

Nishiguchi, M. K. 2000. Temperature affects species distribution in symbiotic populations of *Vibrio* spp. Appl. Environ. Microbiol. 66:3550–3555.

———. 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. Microb. Ecol. 44:10–18.

Nishiguchi, M. K., and B. W. Jones. 2004. Microbial diversity within the Vibrionaceae. Pp. 531–548 *in* J. Seckbach, ed. Origins, evolution, and biodiversity of microbial life. Cole-Kluwer Academic Publishers, Dordrecht, The Netherlands.

Nishiguchi, M. K., and V. S. Nair. 2003. Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. Int. J. Syst. Evol. Microbiol. 53:2019–2026.

Nishiguchi, M. K., E. G. Ruby, and M. J. McFall-Ngai. 1997. Phenotypic bioluminescence as an indicator of competitive dominance in the *Euprymna-Vibrio* symbiosis. Pp. 123–126 *in* J. W. Hastings, L. J. Kricka, and P. E. Stanley, eds. Bioluminescence and chemiluminescence. John Wiley and Sons, New York.

———. 1998. Competitive dominance during colonization is an indicator of coevolution in an animal-bacterial symbiosis. Appl. Environ. Microbiol. 64:3209–3213.

Novella, I., S. Elena, A. Moya, E. Domingo, and J. Holland. 1995. Size of genetic bottlenecks leading to virus fitness loss is determined by mean initial population fitness. J. Virol. 69:2869–2872.

Nyholm, S. V., and M. K. Nishiguchi. 2008. The evolutionary ecology of a sepiolid squid-*Vibrio* association: from cell to environment. Vie et Milieu 58:175–184.

Ramette, A., and J. M. Tiedje. 2007. Biogeography: an emerging cornerstone for understanding prokaryotic diversity, ecology, and evolution. Microb. Ecol. 53:197–207.

Rozas, J., and R. Rozas. 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15:174–175.

Ruby, E. G. 1996. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri–Euprymna scolopes* light organ symbiosis. Annu. Rev. Microbiol. 50:591–624.

Ruby, E. G., and L. M. Asato. 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch. Microbiol. 159:160–167.

Schuster, B. M., L. A. Perry, V. S. Cooper, and C. A. Whistler. 2010. Breaking the language barrier: experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host. Symbiosis 51:85–96.

Soto, W. 2009. Evolutionary ecology of symbionts within free-living and Host environments in the sepiolid squid-*Vibrio* symbiosis. Pp. 208. Biology Department, New Mexico State University, Las Cruces.

Soto, W., J. Gutierrez, M. D. Remmenga, and M. K. Nishiguchi. 2009. Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. Microb. Ecol. 57:140–150.

Soto, W., C. P. Lostroh, and M. K. Nishiguchi. 2010. Physiological responses to stress in the Vibrionaceae. Pp. 1–16 *in* J. Seckback, and M. Grube, eds. Cooperation and stress in biology. Springer, New York, NY.

Szalai, E. S., M. M. Alvarez, and F. J. Muzzio. 2004. Laminar mixing: a dynamical systems approach. Pp. 89–144 *in* E. L. Paul, V. A. Atiemo-Obeng, and S. M. Kresta, eds. Handbook of industrial mixing: science and practice. John Wiley & Sons, Inc., Hoboken, NJ.

Templeton, A. R., K. A. Crandall, and C. F. Sing. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. III. Cladogram estimation. Genetics 132:619–633.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.

Travisano, M., and P. B. Rainey. 2000. Studies of adaptive radiation using model microbial systems. Am. Nat. 156:S35–S44.

Wise, M. G., J. V. McArthur, C. Wheat, and L. J. Shimkets. 1996. Temporal variation in genetic diversity and structure of a lotic population of *Burkholderia* (*Pseudomonas*) *cepacia*. Appl. Environ. Microbiol. 62:1558–1562.

Wollenberg, M. S., and E. G. Ruby. 2009. Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two Oahu populations. Appl. Environ. Microbiol. 75:193–202.

Zamborsky, D. J., and M. K. Nishiguchi. 2011. Phylogeographical patterns among sympatric populations of sepiolid squids and their *Vibrio* symbionts in the Mediterranean Sea. Appl. Environ. Microbiol. 77:642–649.

Associate Editor: P. Turner

Cell Tissue Res (2012) 348:439–452
DOI 10.1007/s00441-012-1378-8

REGULAR ARTICLE

The VD_1/RPD_2 α1-neuropeptide is highly expressed in the brain of cephalopod mollusks

Tim Wollesen · Michele K. Nishiguchi · Pedro Seixas · Bernard M. Degnan · Andreas Wanninger

Received: 8 November 2011 / Accepted: 13 February 2012 / Published online: 20 March 2012

Abstract In certain gastropod mollusks, the central neurons VD_1 and RPD_2 express a distinct peptide, the so-called VD_1/RPD_2 α1-neuropeptide. In order to test whether this peptide is also present in the complex cephalopod central nervous system (CNS), we investigated several octopod and squid species. In the adult decapod squid *Idiosepius notoides* the α1-neuropeptide is expressed throughout the CNS, with the exception of the vertical lobe and the superior and inferior frontal lobes, by very few immunoreactive elements. Immunoreactive cell somata are particularly abundant in brain lobes and associated organs unique to cephalopods such as the subvertical, optic, peduncle, and olfactory lobes. The posterior basal lobes house another large group of immunoreactive cell somata. In the decapod *Idiosepius notoides*, the α1-neuropeptide is first expressed in the olfactory organ, while in the octopod *Octopus vulgaris* it is first detected in the olfactory lobe. In prehatchlings of the sepiolid *Euprymna scolopes* as well as the squids *Sepioteuthis australis* and *Loligo vulgaris*, the α1-neuropeptide is expressed in the periesophageal and posterior subesophageal mass. Prehatchlings of *L. vulgaris* express the α1-neuropeptide in wide parts of the CNS, including the vertical lobe. α1-neuropeptide expression in the developing CNS does not appear to be evolutionarily conserved across various cephalopod taxa investigated. Strong expression in different brain lobes of the adult squid *I. notoides* and prehatching *L. vulgaris* suggests a putative role as a neurotransmitter or neuromodulator in these species; however, electrophysiological evidence is still missing.

Keywords *Idiosepius* · *Octopus* · Ontogeny · Evolution · Central nervous system

T. Wollesen · A. Wanninger (✉)
Department of Integrative Zoology, Faculty of Life Sciences, University of Vienna,
1090 Vienna, Austria
e-mail: andreas.wanninger@univie.ac.at

M. K. Nishiguchi
Department of Biology, New Mexico State University,
Box 30001, MSC 3AF Las Cruces, NM, USA

P. Seixas
Departamento de Microbiología y Parasitología,
Facultad de Farmacia, Universidad de Santiago de Compostela,
15782 Santiago de Compostela, Spain

P. Seixas
Departamento de Bioquímica y Biología Molecular,
Facultad de Biología, Universidad de Santiago de Compostela,
15782 Santiago de Compostela, A Curuña, Spain

B. M. Degnan
School of Biological Sciences, The University of Queensland,
Brisbane, QLD 4072, Australia

Introduction

Bioactive peptides are important neurotransmitters, neuromodulators and hormones that are involved in crucial physiological and behavioral processes such as feeding, energy flow, growth and water–ion balance in a number of animals (Bogerd et al. 1991; Kastin 2006). Since mollusks represent the second largest group among bilaterian metazoans, they are attractive models to study these

biological processes (Di Cosmo and Di Cristo 2006; Ponder and Lindberg 2008).

The VD_1/RPD_2 system with its endogenous α-neuropeptides was first discovered in the two giant neurons VD_1 and RPD_2 in the visceral and the right parietal ganglion of the adult pond snail *Lymnaea stagnalis* (Fig. 1; Bogerd et al. 1991, 1993, 1994; Kerkhoven et al. 1992, 1993). In this species, both neurons innervate other central neurons but also distinct skin areas, heart auricle and the opening of the respiratory cavity (Kerkhoven et al. 1993). Interestingly, they express a gene that has sequence similarities to the *R15* gene, which is expressed by the R_{15} neuron of the sea slug *Aplysia californica* (Buck et al. 1987; Kerkhoven et al. 1991). Moreover, homology has been claimed for the respective preprohormone of *L. stagnalis* and two R_{15} preprohormones of *A. californica*, which are generated by alternative splicing (Bogerd et al. 1991). In *L. stagnalis*, the ε, δ, α_1 and β neuropeptides as well as a single aspartate can be cleavage products of the VD_1/RPD_2 prohormone (Bogert et al. 1991). VD_1, RPD_2 and R_{15} — and probably their endogenous neuropeptides — are involved in the regulation of cardiorespiratory phenomena (Kerkhoven et al. 1991). The α1-peptide is expressed within the CNS of a number of adult gastropods as well as in two bivalve species (Table 2; Kerkhoven et al. 1993). In *L. stagnalis*, it has been shown that the α1-peptide is expressed in a rather restricted manner in the VD_1 and RPD_2 neurons of the visceral and parietal ganglia but also in other cell somata and nerve fibers of the cerebral, pedal and abdominal ganglia, as well as in a few peripheral nerves (Fig. 1b).

Gastropods, scaphopods (tusk shells), bivalves and monoplacophorans possess a rather simple CNS that consists of a few pairs ganglia (Bullock and Horridge 1965). Thus, it appears surprising that a recent phylogenetic study proposes that the cephalopods, with their highly centralized central nervous system, constitute the basal offshoot of a conchiferan clade comprising Cephalopoda, Scaphopoda, Gastropoda and Bivalvia (Kocot et al. 2011).

Homologies of ganglia and brain lobes between molluscan clades are poorly established and thus specific neuronal markers are sought after in order to shed light on the origin of the complex cephalopod CNS from a simple, gastropod-like CNS. Therefore, we characterized the VD_1/ RPD_2 system in selected cephalopods by applying an antibody directed against the α1-neuropeptide in developmental stages and adults of the following coleoid cephalopods: the decapods *Idiosepius notoides*, *Loligo vulgaris*, *Euprymna scolopes* and *Sepioteuthis australis* and the octopod *Octopus vulgaris*. Intraspecific similarities and differences in the developing and adult cephalopod CNS are discussed and compared to both gastropod and bivalve conditions.

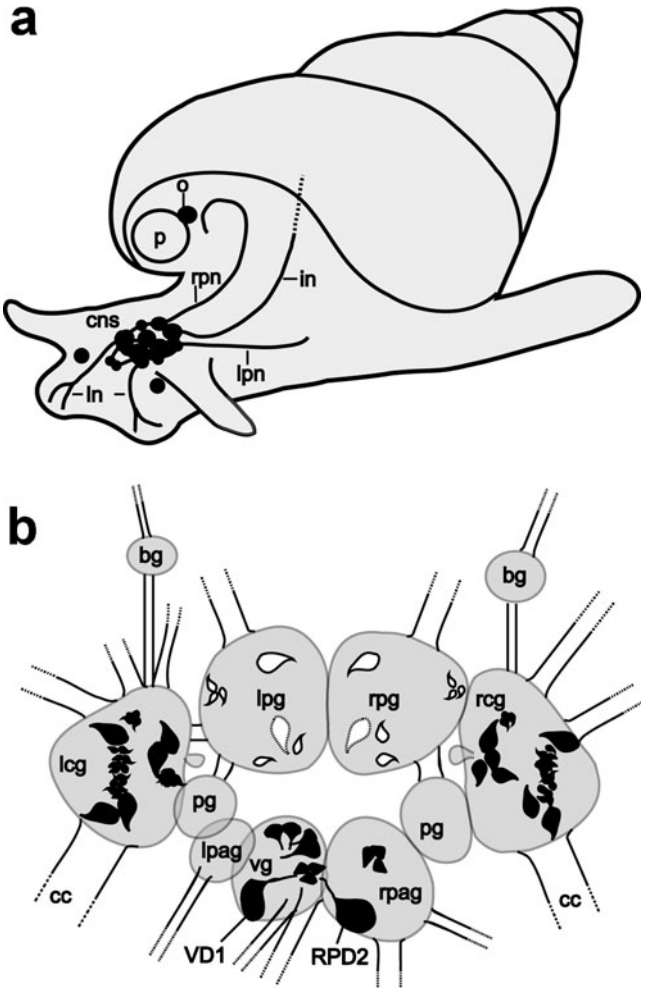


Fig. 1 VRα-ir cell somata within the adult central nervous system of the gastropod *Lymnaea stagnalis*. **a** Schematic outline of the relative position of the CNS. Modified from Kerkhoven et al. (1991). **b** Schematic outline of the relative position of VRα-ir cell somata, including the neurons VD_1 and RPD_2. Dorsal view on the flat-mounted CNS with the cerebral commissure cut and the left and right cerebral ganglia bent aside. Dorsal cell somata are indicated by *solid lines* and ventral cell somata are labeled with *dashed lines*. See Table 1 for abbreviations. Modified from Kerkhoven et al. (1992)

Material and methods

Animal culture and staging of embryos

Idiosepius notoides

Adult specimens of *Idiosepius notoides* were dip-netted in shallow water of seagrass beds at Moreton Bay, Queensland, Australia. Individuals of *I. notoides* were reared in artificial seawater at 25 °C in tanks in a semi-natural environment. Females attached egg clutches to leaves of seagrass or glass plates. These egg clutches were carefully transferred to six-well plates. During embryonic development, water was changed twice daily and completion took place approximately

9–10 days after laying at 25 °C. All individuals were staged according to Yamamoto (1988).

Euprymna scolopes

Egg clutches were shipped from a rearing facility at the Department of Medical Microbiology & Immunology of the University of Wisconsin (Madison, WI, USA) and were transferred to a recirculating aquarium with artificial sea water (24 °C, 32 ppt) at New Mexico State University, where the developmental stages of *E. scolopes* were reared until hatching (approximately 25 days).

Sepioteuthis australis

Egg clutches were collected from shallow water of seagrass beds at Moreton Bay, Queensland, Australia. Embryos were reared in artificial seawater at 25 °C in tanks in a semi-natural environment until hatching.

Loligo vulgaris

Eggs were harvested from the cages of crab-fishermen in Roscoff, France, in spring 2007. They were transferred to an aquarium with an ambient flow-through-seawater system and were reared until hatching.

Octopus vulgaris

Adults of *Octopus vulgaris* were captured off the coast of Galicia, Spain, in the area Cies–Toralla–Cabo Silleiro, in March 2009. Individuals were transferred to suspended cages in the Ria of Vigo. The specimens were individually weighed and placed in a galvanized cage (3 × 1.5 × 1.5 m) that was suspended from a raft used for mussel production (25 × 25 m). The submerged cages were accessible from the top. Adult octopuses (120 individuals with an initial weight of 880± 150 g) were fed mainly fish such as Atlantic horse mackerels (*Trachurus trachurus*), blue whitings (*Micromesistius poutassou*) and occasionally Mediterranean mussels (*Mytilus galloprovincialis*) until they weighed approximately 2.5 kg. Female octopuses were isolated when they laid eggs to allow undisturbed embryonic development. One single female was maintained for up to approximately 1.5 months in order to sample different developmental stages until shortly after hatching. Individual developmental stages were staged according to Naef (1928). For *O. vulgaris*, stages XI to hatchlings (i.e., older than stage XX) were investigated.

Immunocytochemistry

Developmental stages 18 to 30 (cf. Yamamoto 1988) and adults of *Idiosepius notoides*, pre-hatchlings of *Loligo vulgaris*, hatchlings of *Sepioteuthis australis* and various developmental stages of *Euprymna scolopes* [stage XVI to XIX; cf. Naef 1928; i.e., 14 to 17 days post laying (dpl)], as well as *Octopus vulgaris* (stages X-XIII, XV, XVIII to XX; cf. Naef 1928) were slowly chilled and anesthetized with 7.14 % $MgCl_2$ in seawater and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at room temperature (RT) for 2 h. Fixed specimens were stored in PBS with 0.1 % NaN_3 at 4 °C. Alternatively, developmental stages and adults of *I. notoides* were fixed in 4 % paraformaldehyde in 0.1 M 3-(N-morpholino) propane sulfonic acid (MOPS), pH 7.5 and rinsed thrice in 70 % EtOH for 15 min. Fixed specimens were stored in PBS with 0.1 % NaN_3 at 4 °C or in 70 % EtOH at −20 °C respectively. Prior to further processing, the fixed animals were rehydrated into PBS.

Developmental stages of *O. vulgaris*, *L. vulgaris*, *S. australis*, *E. scolopes* and the CNS of adult *I. notoides* were embedded in a gelatin–albumine solution. The embedded samples were stored in PBS with 10 % formaldehyde for 12 h at 4 °C and sectioned into 50–150 μm-thick sections using a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany). Developmental stages of *I. notoides* were processed as whole-mount preparations. The latter and the vibratome sections were rinsed thrice for 15 min each in PBS and another three times for 15 min each in PBS with 2 % Triton X-100 (PBT) to increase tissue permeability. Samples were blocked for 4 to 15 h in PBT with 5 % normal swine serum (NSS; Jackson ImmunoResearch, West Grove, PA, USA) at RT. A primary polyclonal antibody against the VD_1/RPD_2 α_1-peptide (published amino acid sequence: CDMYEGLAGRCQHHPRNCPGFN; see Bogerd et al. 1991), raised in rabbit (CASLO Laboratory, Lyngby, Denmark), was applied in a 1:300 to 1:800 dilution. In addition, a primary monoclonal antibody against acetylated α-tubulin raised in mouse (Sigma, Brøndby, Denmark) was diluted 1:800 in blocking medium. Samples were incubated for 48 h at RT in a cocktail containing both primary antibodies. Specimens were rinsed six times for 20 min each in PBS at RT. Subsequently, both secondary fluorochrome-coupled antibodies were diluted in a blocking solution with 1% NSS and applied in a 1:400 to 1:800 dilution. For VD_1/RPD_2 α_1-peptide visualization, Alexa Fluor 594 (anti-rabbit; Invitrogen, Taastrup, Denmark) and for acetylated α-tubulin visualization, Alexa Fluor 488 (anti-mouse; Invitrogen) were chosen as secondary antibodies. After 15–20 h in the dark at RT, 5 % 4′, 6-diamidin-2-phenylindol (DAPI, Invitrogen) was added for 2 h to label the cell nuclei in the developing CNS (see Wollesen et al. 2009). Subsequently, samples were rinsed in PBT three times for 20 min each and another three times for 1 h in PBS. Finally, whole-mount preparations and vibratome sections were mounted on glass slides, either in Fluoromount G (Southern Biotech, Birmingham, Alabama, USA) or in Elvanol (Rodriguez and Deinhard 1960) according to Wollesen et al. (2009). Samples were stored in the dark at 4 °C for at least 3 days

prior to examination. Previously described neurons reactive against the VD_1/RPD_2 α1-peptide in the adult CNS of *Lymnaea stagnalis* served as positive control (Wollesen, unpublished observation; Kerkhoven et al. 1992).

Negative controls (same procedure but omitting either the primary or the secondary antibody) were performed to assess specificity of the labeling experiments and yielded no signal. Preabsorption experiments employing the α1-neuropeptide and the antibody directed against the latter rendered no signal in any of the species investigated.

Confocal laser scanning microscopy and data analysis

All preparations were examined with a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit (Leica Microsystems, Wetzlar, Germany). Optical sections with a Z-step size of 0.5 μm to 1 μm were generated and digitally merged to yield maximum projection images. These projection images were further processed with Photoshop 9.0.2 software (Photoshop 9.0.2 software (Adobe Systems, Inc., San Jose, CA, USA) to adjust contrast and brightness.

Results

Terminology

The terminology of the CNS applied in this paper is in accordance with Young (1971; 1979), Shigeno and Yamamoto (2002), Yamamoto et al. (2003) and Wollesen et al. (2010a) (Table 1). For consistency with previous neuroanatomical literature, all developmental stages are described with respect to their adult swimming posture. Accordingly, the head is considered anterior, the mantle apex posterior, the funnel ventral and its opposite side dorsal. Elements immunoreactive against the VD_1/RPD_2 α1-peptide are referred to as VRα-ir elements or VRα-immunoreactivity-expressing elements respectively.

Table 1 List of abbreviations used in figures

abl	anterior basal lobe	og	optic gland
acl	anterior chromatophore lobe	ol	optic lobe
alpl	anterior lateral pedal lobe	oo	olfactory organ
asm	anterior subesophageal mass	p	pneumostome
apl	anterior pedal lobe	pcl	posterior chromatophore lobe
bg	buccal ganglion	pdl	peduncle lobe
bm	buccal mass	pg	pleural ganglion
cc	cerebral commissure	plpl	posterior lateral pedal lobe
cns	central nervous system	pml	posterior magnocellular lobe
dbl	dorsal basal lobe	pn	pallial nerve
dll	dorso-lateral lobe	ppl	posterior pedal lobe
dml	dorsal magnocellular lobe	prl	precommissural lobe
e	esophagus	psm	posterior subesophageal mass
ey	eye	pvl	palliovisceral lobe
fl	fin lobe	rcg	right cerebral ganglion
ibl	inferior buccal lobe	rpag	right parietal ganglion
ibrl	intrabrachial lobe	rpg	right pedal ganglion
ifl	inferior frontal lobe	rpn	right parietal nerve
in	intestinal nerve	sbl	superior buccal lobe
itl	interbasal lobe	sfl	superior frontal lobe
iy	internal yolk	sg	stellate ganglion
lbl	lateral basal lobe	spl	subpedunculate lobe
lcg	left cerebral ganglion	spm	supraesphageal mass
ln	labial nerve	stc	statocyst
lpag	left parietal ganglion	svl	subvertical lobe
lpg	left pedal ganglion	vg	visceral ganglion
lpn	left parietal nerve	vml	ventral magnocellular lobe
mbl	median basal lobe	vsn	visceral nerve
msm	middle subesophageal mass	VRα-ir	immunoreactive against the VD_1/RPD_2 α1-peptide
o	osphradium	vsl	visceral lobe
ofl	olfactory lobe	vtl	vertical lobe

Gross anatomy of the adult and the developing cephalopod CNS

The CNS of adult coleoid cephalopods is composed of a brain with two laterally positioned optic lobes (Fig. 2). The brain is penetrated by the esophagus and is composed of a dorsal supraesophageal mass that connects laterally to the ventral subesophageal mass. The periesophageal mass (all magnocellular lobes) constitutes the postero-lateral-most part of the brain close to the statocyst (Fig. 2). For detailed descriptions of the development of the cephalopod CNS, see Yamamoto et al. (2003) and Wollesen et al. (2010a) for *Idiosepius*, Shigeno et al. (2001) for *Sepioteuthis*, Meister (1972) for *Loligo* and Marquis (1989) for *Octopus*.

In general, the neurogenic placodes of the cerebral, intrabrachial, optic, palliovisceral, pedal and stellate ganglia are formed by ingression, migration and accumulation of neuroblasts, when the majority of the yolk syncytium of the embryo is covered by the ecto- and mesentoderm. Subsequently, neuropil develops within the core regions of all ganglia. The palliovisceral ganglia develop into the posterior subesophageal mass and the pedal ganglia give rise to the anterior and the middle subesophageal mass. The cerebral ganglia are the precursors of the supraesophageal mass, while the optic ganglia are termed optic lobes after stage 23. Further development is characterized by increased neuropil growth.

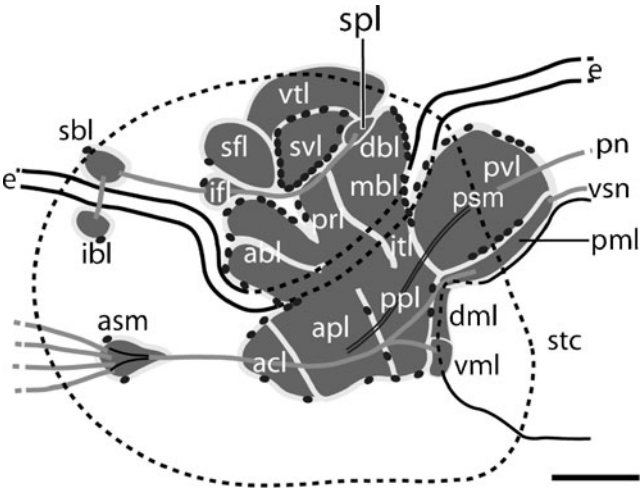
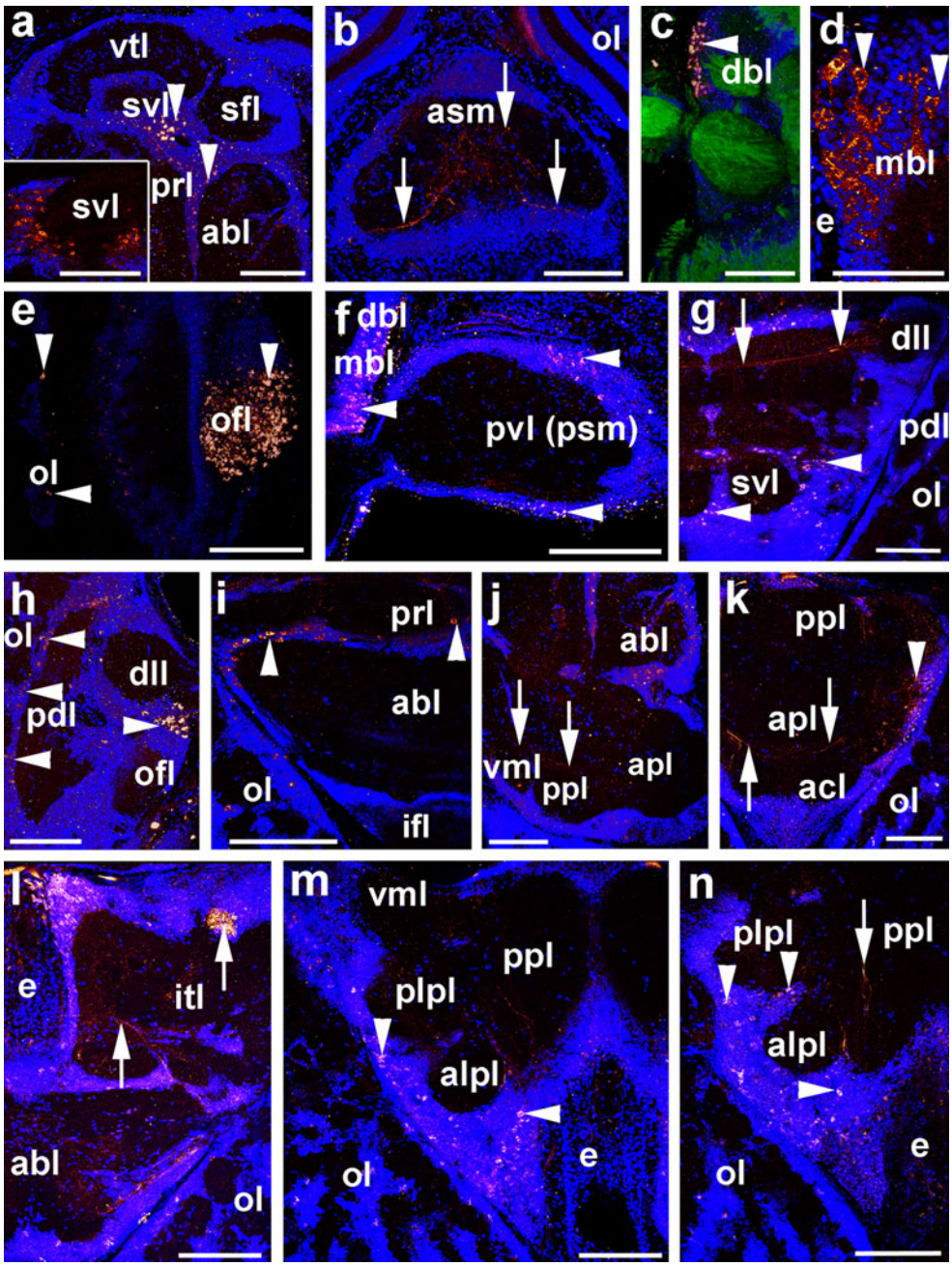


Fig. 2 Schematic outline of the distribution of VRα-ir elements in the CNS of the adult pygmy squid *Idiosepius notoides*. Sagittal section through the middle of the brain with anterior to the left. One optic lobe (*dashed circle*) attaches on each side to the central brain. The brain is divided by the esophagus into a supraesophageal and subesophageal mass. The boundaries of the various brain lobes are often continuous and therefore arbitrary. VRα-ir cell somata (*black dots*) and neurites (*black lines*) are plotted on the CNS with respect to their relative abundance and distribution. In general, faintly fluorescent VRα-ir neurites are distributed throughout the various lobes. Here, only strongly fluorescent VRα-ir neurites are marked. The stellate ganglia, the visceral lobes, the posterior chromatophore lobes, the posterior and anterior lateral pedal lobes, the dorso-lateral lobes, the lateral basal lobes, the fin lobes, the optic tract region and most connectives and commissures are omitted for clarity. The periesophageal mass comprises the ventral magnocellular lobes, the dorsal magnocellular lobes and the posterior magnocellular lobes. All of them are located close to the statocyst (outlined by a *solid black line*). See Table 1 for abbreviations. *Scale bar*=300 μm

The VD_1/RPD_2 system in the adult CNS of *Idiosepius notoides*

VRα-ir elements were found in various lobes of the CNS (Figs. 2, 3 and 4; Table 3). In general, VRα-ir cell somata of the various lobes were intensely immunolabeled (Fig. 3a), in contrast to neurites, which were rather faintly stained (Fig. 3b,g). Labeled neurites do not necessarily connect to labeled cell somata but few immunopositive somata send immunostained neurites into the neuropils (Fig. 3d). Larger populations of VRα-ir somata are present in the subvertical lobes (Figs. 2; 3a,g; 4a), the posterior basal lobes (Figs. 2; 3c,d; 4b,c), the optic lobes (Figs. 3e, 4b), and the olfactory lobes (Figs. 3e; 4b). In the vertical lobe system of the supraesophageal mass, the subvertical lobe, but not the vertical lobe itself, exhibits VRα-ir cell somata (Figs. 2; 3a,g; 4a). Few VRα-ir perikarya are also present in the precommissural lobes (Figs. 2; 3a; 4b). The inferior and superior frontal lobes exhibit only very few VRα-ir cell somata and are devoid of neurites (Figs. 2; 3a,i; 4a,b). Further VRα-ir cell somata are present in the posterior basal lobes such as the dorsal basal (Figs. 2; 3c,f; 4a), the median basal (Figs. 2; 3d,f; 4b), the dorso-lateral (Figs. 3h, 4a) and the lateral basal lobes (Fig. 4c; Table 3). No VRα-ir cell somata or neurites were observed in the subpedunculate lobes (Figs. 2; 4a). The interbasal lobes exhibit no VRα-ir cell somata but VRα-ir neurites (Figs. 2; 3l; 4c). Few VRα-ir neurites are visible in the neuropils of the posterior basal lobes and in those of the anterior basal lobes (Figs. 3c,d,f,h,i,j; 4a–c). The latter possess another population of VRα-ir cell somata (Figs. 2; 3i; 4c). The optic tract region connects both optic lobes to the central brain (Fig. 4b). The peduncle and olfactory lobes comprise VRα-ir cell somata (Figs. 3e,h; 4b; Table 3). In the periesophageal mass, few VRα-ir cell somata are found in the dorsal magnocellular lobe (Figs. 2; 4c; Table 3). In the posterior and ventral magnocellular lobes, no VRα-ir cell somata were observed (Figs. 2; 3m; 4c,d). Individual VRα-ir cell somata are distributed in the posterior subesophageal mass, e.g., the palliovisceral lobe (Figs. 2; 3f; 4b). VRα-ir fibers are part of a nerve strand interconnecting the posterior subesophageal and the middle subesophageal mass (Figs. 2; 3j; 4b,d). The anterior and posterior pedal lobes as well as the anterior and posterior lateral pedal and the chromatophore lobes possess VRα-ir cell somata in their perikaryal layers and neurites in their neuropils (Figs. 2; 3j,k,m,n; 4d; Table 3). VRα-ir neurites are abundant in the neuropil of the anterior subesophageal mass, however,



only very few cell somata are present (Figs. 2; 3b; 4d; Table 3). Few VRα-ir cell somata are present in the buccal ganglia (Fig. 2; Table 3).

The VD_1/RPD_2 system during cephalopod CNS development

In the following, the spatio-temporal distribution of VRα-ir elements in the CNS of coleoid cephalopods is described.

Idiosepius notoides

The earliest VRα-ir elements are cell somata located in the olfactory organ, which is ventrally attached to the optic lobes of stage 27 individuals (Fig. 5c,d; Table 3). Few individuals exhibit VRα-ir cell somata in the labial region and the intrabrachial lobes (Fig. 5e). In stage 29 individuals VRα-ir cell somata are located in the anterior subesophageal mass (not shown), the middle subesophageal mass and the anterior and

Fig. 3 VRα-ir elements (*red*) within the CNS of the adult pygmy squid *Idiosepius notoides*. Neuropil is stained *green* (acetylated α-tubulin) and cell somata are labeled *blue* (DAPI). Sagittal vibratome sections with anterior facing to the right if not stated otherwise. **a** VRα-ir cell somata (*arrowheads*) within the subvertical and precommissural lobes of the vertical lobe system. Note the absence of VRα-ir elements within the vertical lobe. *Inset*: Horizontal section through the right portion of the subvertical lobe (anterior faces down). **b** Horizontal section with anterior facing down, showing rather faintly stained VRα-ir neurites (*arrowheads*) in the anterior subesophageal mass. **c** VRα-ir cell somata (*arrowhead*) within the dorsal basal lobes. **d** Horizontal section through the left medial basal lobe close to the esophagus with VRα-ir cell somata (*arrowheads*) (anterior faces down). **e** The optic lobes and the olfactory lobe house VRα-ir cell somata (*arrowheads*). **f** The posterior basal lobes and the posterior subesophageal mass exhibit VRα-ir cell somata (*arrowheads*) (anterior faces to the left). **g** The dorso-lateral lobes are connected via VRα-ir neurites with each other (*arrows*). VRα-ir cell somata are located in the subvertical lobes (*arrowheads*). **h** The left peduncle lobe, the olfactory lobe and the dorso-lateral lobes exhibit VRα-ir cell somata (*arrowheads*). **i** The anterior basal lobe and the precommissural lobes house many VRα-ir cell somata (*arrowheads*), in contrast to the inferior frontal lobes with only very few somata (not shown) (horizontal section with anterior facing down). **j** The middle subesophageal mass comprises the anterior pedal and posterior pedal lobes, as well as the anterior chromatophore lobes (not shown). Faintly stained VRα-ir neurites (*arrows*) project from the middle subesophageal mass into the posterior subesophageal mass. **k** Horizontal section of the left portion of the middle subesophageal mass, exhibiting VRα-ir neurites (*arrows*). **l** Horizontal section of the left portion of the middle subesophageal mass showing VRα-ir neurites (*arrows*) and an accumulation of VRα-ir neurites in the posterior interbasal lobes. **m** Horizontal section of the right portion of the middle subesophageal mass with the anterior lateral pedal lobes, posterior lateral pedal lobes and VRα-ir cell somata (*arrowheads*). **n** Horizontal section of the right portion of the middle subesophageal mass with VRα-ir neurites (*arrow*) and cell somata (*arrowheads*). See Table 1 for abbreviations. *Scale bars* 150 μm (except **d**: 75 μm)

posterior basal lobes (Fig. 5g). Stage 30 individuals (hatchlings) possess VRα-ir neurites in the anterior and posterior basal lobes and in the posterior middle subesophageal mass (Fig. 5f).

Euprymna scolopes

In stage XVI and XVII individuals, VRα-ir cell somata are present in the periesophageal, posterior, middle and anterior subesophageal masses (Fig. 5c,h; Table 3). In the periesophageal mass, cell somata are located in the perikaryal layers of the ventral magnocellular lobe (Fig. 5c). Further VRα-ir cell somata were found in the stellate ganglia (Fig. 5c). In stage XVIII and XIX individuals, VRα-ir cell somata are also situated in the olfactory organ (Fig. 5c,i) and in the posterior basal lobes, most likely in the perikaryal layers of the dorsal basal lobes (Fig. 5a,j; Table 3). VRα-ir cell somata were also observed in the dorsal magnocellular lobe of the periesophageal mass (Fig. 5c). Further VRα-ir cell somata are situated in the dorsal perikaryal layer of the inferior or superior frontal lobe (Fig. 5a; arrow that points to supraesophageal mass in Fig. 5j).

Octopus vulgaris

It is not until stage XIX and XX that the pre-hatching paralarvae of *O. vulgaris* exhibit VRα-ir cell somata. These are restricted to the posterior olfactory lobule of the optic tract region (Fig. 5b,k; Table 3).

Sepioteuthis australis

In hatchlings of *S. australis*, VRα-ir cell somata are located in the dorsal perikaryal layer of the posterior subesophageal mass and in the dorsal magnocellular lobe (Fig. 5c,l; Table 3). Further VRα-ir cell somata are present in the optic tract region, most likely in the optic gland and in the postero-lateral portion of the subpedunculate lobes (Fig. 5b,m; Table 3).

Loligo vulgaris

For *L. vulgaris*, only the immunohistochemical expression patterns of paralarvae close to hatching were investigated. Within the vertical lobe complex, the vertical lobe houses the largest subset of VRα-ir cell somata and neurites (Figs. 5a; 6a,b; Table 3). The inferior frontal lobes possess some VRα-ir cell somata and neurites, while only a few VRα-ir neurites and no cell somata were observed in the precommissural lobes (Figs. 5a,b; 6b,d; Table 3). The same is true for the subvertical and superior frontal lobes (Figs. 5a; 6a,c; Table 3). The lobes of the posterior basal lobe system do not comprise many VRα-ir elements (Figs. 5a,b; 6c,d; Table 3). The majority of VRα-ir cell somata are associated with the subpedunculate lobes, which exhibit strong VRα-immunoreactivity (Figs. 5a; 6a,c; Table 3). Dorsal basal and dorso-lateral lobes exhibit numerous VRα-ir neurites but no cell somata were observed (Figs. 5a; 6e; Table 3). Some VRα-ir cell somata were observed in the posterior perikaryal layer of the median basal lobes (Figs. 5b; 6d; Table 3). Very few VRα-ir neurites and no cell somata were observed in the lateral basal and interbasal lobes (Fig. 6f; Table 3). The anterior portion of the anterior basal lobes exhibits only few VRα-ir cell somata or neurites compared to the posterior portion of the anterior basal lobes with many VRα-ir cell somata and neurites (Figs. 5b; 6d; Table 3). Among the lobes of the optic tract region, the peduncle lobes possess only few VRα-ir neurites; no VRα-ir cell somata were observed (Figs. 5b; 6d; Table 3). The olfactory lobes house many VRα-ir cell somata and exhibit strong VRα-immunoreactivity in their neuropils (Figs. 5b; 6d; Table 3).

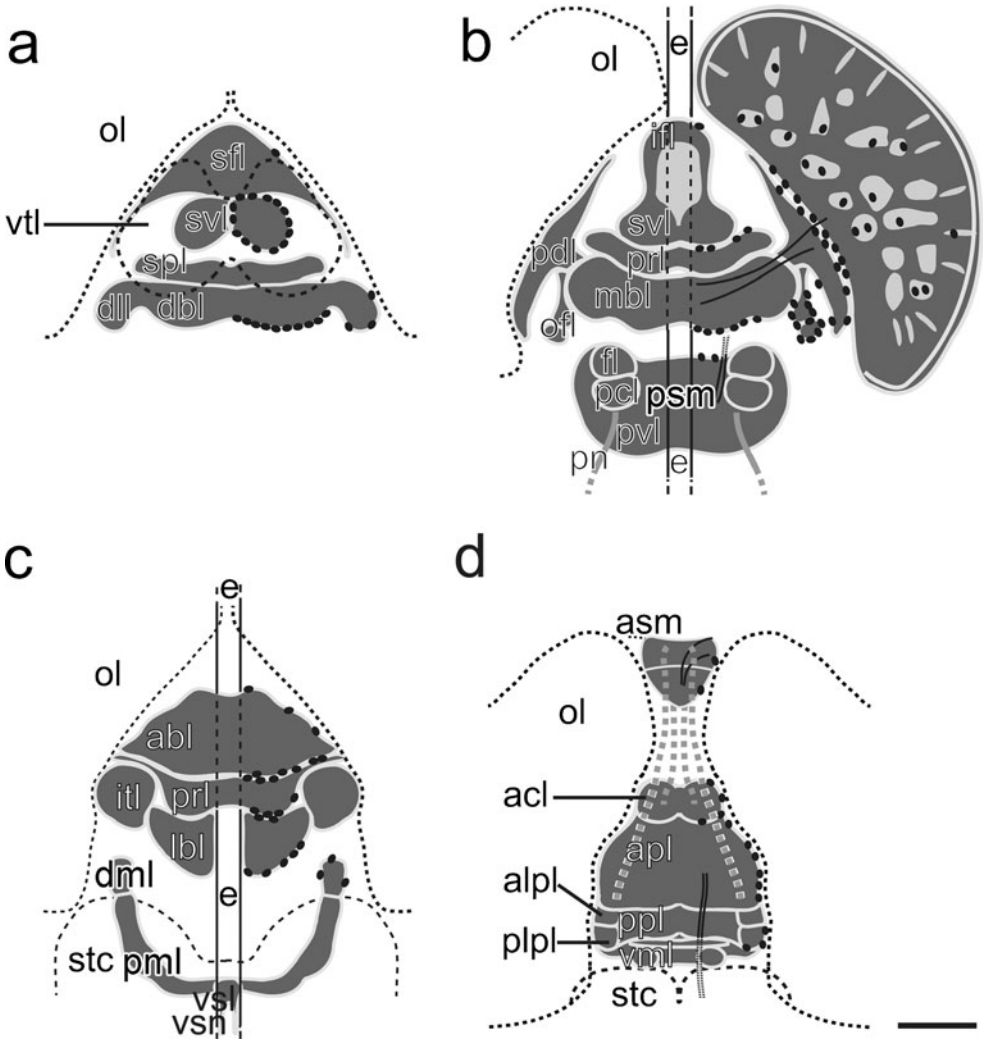


Fig. 4 Schematic outline of the distribution of VRα-ir elements within the CNS of the adult *I. notoides*. VRα-ir cell somata (*black dots*) and neurites (*black lines*) are plotted onto the right hemisphere of the CNS with respect to their relative abundance and topographical distribution. The boundaries of the various brain lobes are often continuous and therefore arbitrarily chosen. In general, faintly fluorescent VRα-ir neurites are distributed throughout the various lobes. Here, only strongly fluorescent VRα-ir neurites are marked. The buccal lobes, stellate ganglia, most connectives and commissures are omitted. **a** The most dorsal portion of the supraesophageal mass. The shape of the vertical lobe is outlined by a *dashed line*. Optic lobes are omitted. **b** Lower portion of the supraesophageal mass and the posterior subesophageal mass. **c** Parts of the supraesophageal mass surrounding the esophagus. The periesophageal mass comprises the posterior, dorsal and the ventral magnocellular lobe (shown in **d**). **d** The anterior subesophageal mass is located anterior to the middle subesophageal mass, which comprises the anterior chromatophore lobe, anterior pedal lobe, posterior pedal lobe, anterior lateral pedal lobe and posterior lateral pedal lobe. See Table 1 for abbreviations. *Scale bar* 500 μm

Within the subesophageal and the periesophageal mass, the largest populations of VRα-ir cell somata are present within the magnocellular lobes and palliovisceral lobe of the posterior subesophageal mass (Figs. 5c; 6f; Table 3). VRα-ir neurites are not very abundant in the middle subesophageal mass (Figs. 5c; 6g–j; Table 3). The majority of VRα-ir neurites are located in the posterior pedal lobes (Figs. 5c; 6g,i; Table 3). VRα-ir neurites are abundant in the neuropil of the anterior subesophageal mass; no VRα-ir cell somata were observed (Figs. 5c; 6j; Table 3).

The superior and inferior buccal lobes exhibit VRα-ir neurites within their neuropils; however, VRα-ir cell somata only appear to be associated with the superior buccal lobes (Figs. 5a; 6a and j; Table 3). The optic lobes contain no or only very few VRα-ir elements (Fig. 6a,d,g,h,j; Table 3).

Discussion

Expression of the VD_1/RPD_2 α1-peptide within the adult cephalopod CNS

VRα-ir cell somata and neurites are distributed throughout the entire CNS of the adult decapod squid *Idiosepius*

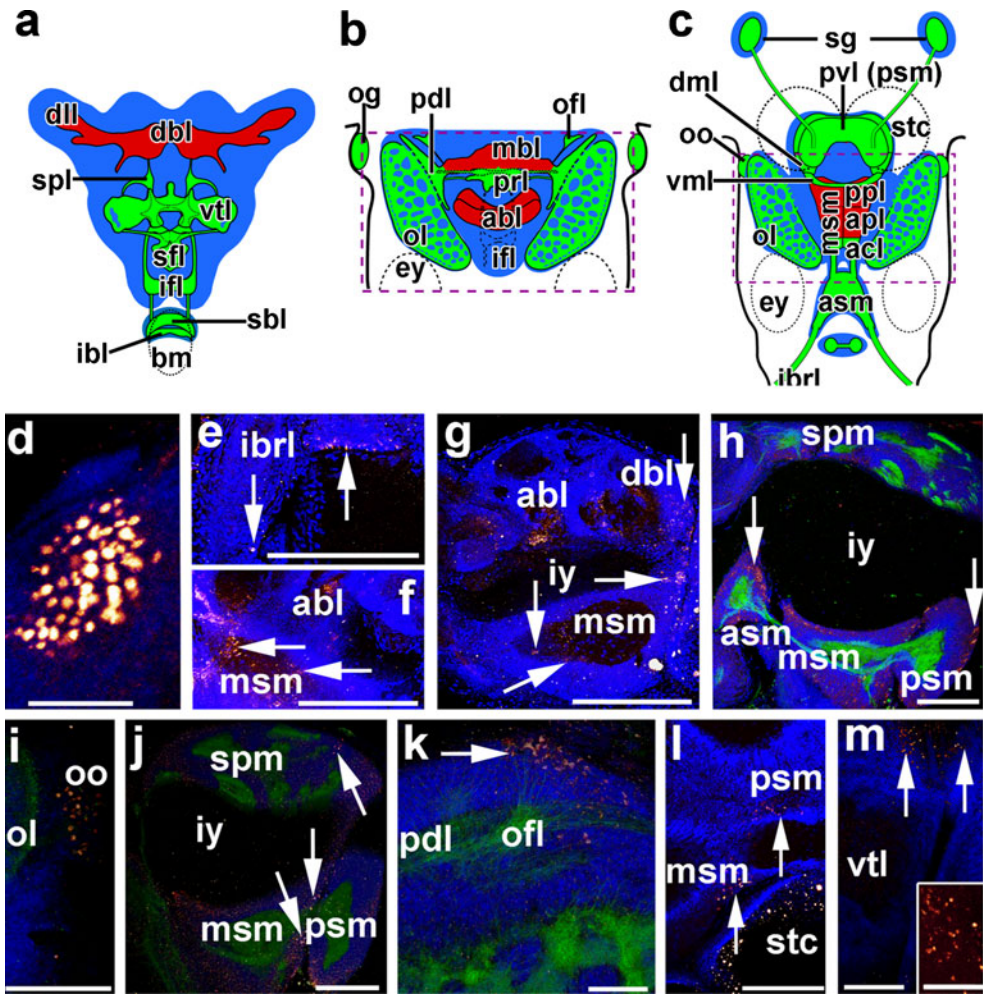


Fig. 5 VRα-ir elements within the developing CNS of the decapods *Idiosepius notoides*, *Sepioteuthis australis*, *Euprymna scolopes* and the octopod *Octopus vulgaris*. **a**–**c** Schematic drawings of horizontal optical sections through the cephalopod CNS, exemplified by a stage 30 individual (hatchling) of *I. notoides* (anterior faces down; not to scale). Brain lobes that exhibit VRα-ir elements are stained *red*, the neuropil is labeled *green* and perikaryal layers are *blue*. The *dashed pink box* in **b** corresponds to the one in **c**. Modified from Wollesen et al. (2010a). Immunochemistry was performed on whole-mount preparations (**d**–**g**) or vibratome sections (**h**–**m**). **a** The dorsal portion of the supraesophageal mass (dorsal third of the brain) includes the vertical and the superior frontal lobes. The inferior frontal lobes are also indicated in **b** (*dashed outline*). The posterior basal lobes comprise the subpedunculate, the dorso-lateral and the dorsal basal lobes. **b** The median portion of the supraesophageal mass comprises, among others, the anterior basal lobes. The peduncle lobe, the olfactory lobe and the optic gland belong to the optic tract region that connects to the optic lobe. **c** The subesophageal mass (ventral third of the brain) is composed of an anterior, middle and a posterior portion. The dorsal and ventral magnocellular lobes belong to the periesophageal mass. The palliovisceral lobe is the largest portion of the posterior subesophageal mass. The olfactory organ is ventrally connected to the optic lobe. **d**–**m** Confocal micrographs showing VRα-ir elements (*red*), neuropil (*green*; acetylated α-tubulin) and cell somata (*blue*; DAPI). All images are sagittal sections with anterior to the left, if not stated otherwise. **d** The earliest VRα-ir elements within the CNS of *I. notoides* are cell somata in the olfactory organ of stage 27 individuals (cf **c**; horizontal section and anterior faces down). **e** In a few stage 27 individuals of *I. notoides* VRα-ir cell somata are present in the labial region and in the intrabrachial lobes (horizontal section and anterior faces down). **f** Stage 30 individuals (hatchlings) of *I. notoides* exhibit VRα-ir elements in the anterior basal lobes, the posterior basal lobes and the posterior middle subesophageal mass (sagittal section with anterior facing to the right). **g** Stage 29 individuals of *I. notoides* exhibit VRα-ir elements in the middle subesophageal mass, the anterior basal lobes and the posterior basal lobes (sagittal section with anterior facing to the left). **h** In stage XVI and XVII individuals of *E. scolopes*, the first VRα-ir cell somata (*arrows*) are present in the posterior, middle and the anterior subesophageal masses (cf **c**) but not in the supraesophageal mass. **i** VRα-ir cell somata within the olfactory organ in a stage XIX individual of *E. scolopes* (cf **c**; horizontal section and anterior faces down). **j** VRα-ir cell somata (*arrows*) within the posterior basal lobes and the middle and posterior subesophageal masses of a stage XIX individual of *E. scolopes* (cf **a** and **c**). **k** VRα-ir cell somata (*arrow*) within the posterior olfactory lobule of a stage XIX *O. vulgaris* (cf **b**; horizontal section and anterior faces to the left). **l** In the CNS of *S. australis* hatchlings, VRα-ir cell somata (*arrows*) are present in the dorsal perikaryal layer of the posterior subesophageal mass and in the dorsal magnocellular lobe (cf **c**). **m** Left hemisphere of the supraesophageal mass of a *S. australis* hatchling (horizontal section with anterior facing down). VRα-ir cell somata are located within the optic tract region, most likely in the optic gland (*right arrow*; cf **b**). Further VRα-ir cell somata are present in the postero-lateral subpedunculate lobes (*left arrow*; (cf **a**). *Inset*: Magnified group of cell somata labeled by *left arrow*. See Table 1 for abbreviations. *Scale bars* 150 μm (except **a**,**c**: 50 μm)

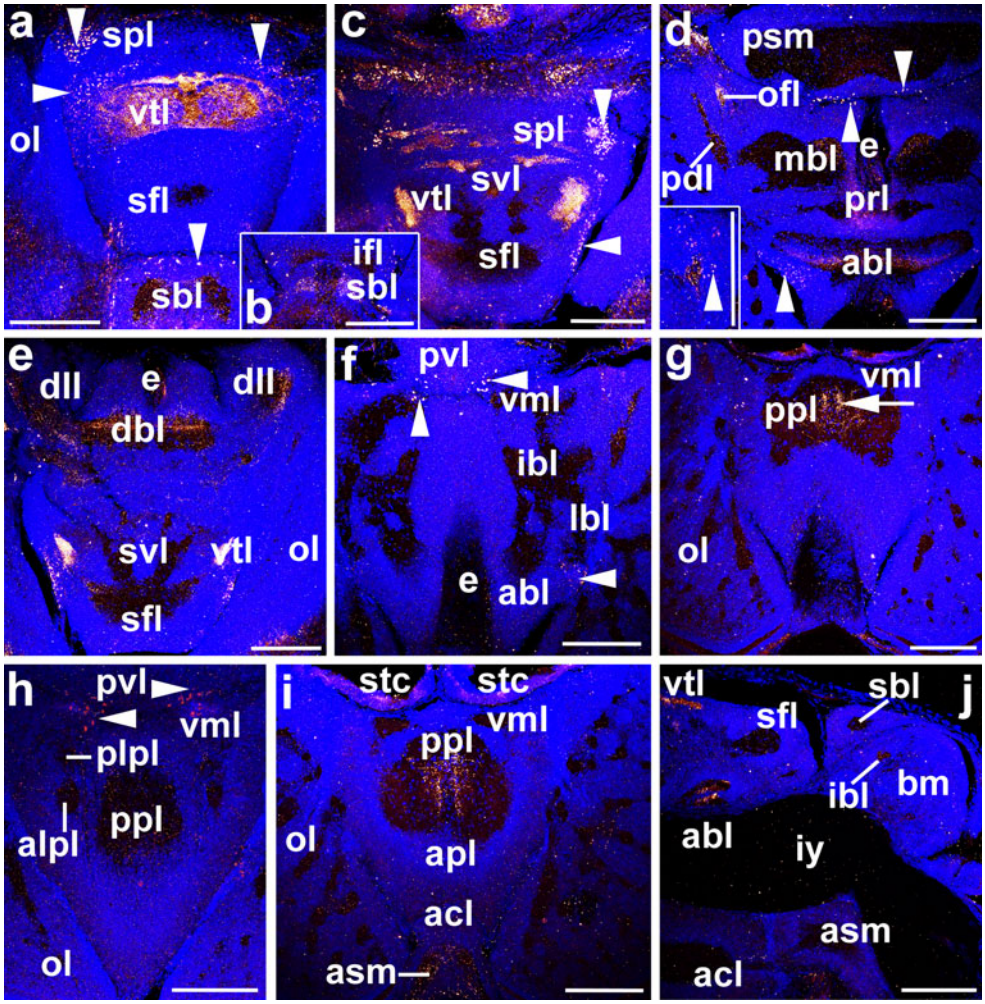


Fig. 6 VRα-ir elements within the CNS of prehatchlings of the decapod *Loligo vulgaris*. Brain lobes that exhibit VRα-ir elements are stained *red* and perikaryal layers are stained *blue*. Horizontal vibratome sections and anterior faces downwards if not stated otherwise. VRα-ir cell somata are labeled with *arrowheads* and neurites with *arrows*. **a** The vertical lobe comprises the largest subset of VRα-ir cell somata and neurites. Other large subsets are associated with the subpedunculate lobes and the superior buccal lobes. No VRα-ir elements are located in the superior frontal lobe. **b** The inferior frontal lobes and the superior buccal ganglia comprise VRα-ir cell somata and neurites. **c** No VRα-ir cell somata and only very few neurites are located in the superior frontal lobe and the subvertical lobes. The subpedunculate lobes also possess many VRα-ir cell somata and neurites. **d** The precommissural lobes contain no or only few VRα-ir elements. Note the subset of VRα-ir cell somata in the anterior perikaryal layer of the posterior subesophageal mass. The olfactory lobes contain numerous VRα-ir cell somata (*inset*). **e** The dorsal basal and dorso-lateral lobes exhibit VRα-ir fibers but lack cell somata. **f** VRα-ir cell somata in the ventral magnocellular lobes, the palliovisceral lobe and the latero-ventral anterior basal lobes (*arrowheads*). No VRα-ir elements were observed in the lateral basal and interbasal lobes. **g** A few neurites exhibit strong VRα-immunoreactivity in the posterior pedal lobes of the middle subesophageal mass. **h** VRα-ir cell somata in the ventral magnocellular lobes and the palliovisceral lobes (*arrowheads*). No VRα-ir elements were observed in the anterior and posterior lateral pedal lobes. **i** VRα-ir neurites in the neuropils of the posterior pedal lobes and the anterior subesophageal mass. **j** This sagittal brain section shows VRα-ir neurites in the vertical lobe, the anterior basal lobes, the superior lobes and the inferior buccal lobes. See Table 1 for abbreviations. *Scale bars* 150 μm

notoides (Figs. 2 and 4; Table 3). Cell somata show intense VD_1/RPD_2 α1-peptide expression, while neurites are less abundant and stain rather weakly. The vast majority of VRα-ir cell somata are located in the perikaryal layers of the various lobes, except for very few cell somata, which are situated in the inner neuropils of the lobes. VRα-ir cell somata are particularly abundant in the subvertical, posterior basal and optic lobes. In *Loligo vulgaris*, the posterior basal lobes, which house a large number of VRα-ir cell somata in adults of *I. notoides*, act as control centers for steering and jet movements (dorsal and median basal lobes) and for reproduction (dorso-lateral lobes) (Young 1977). They also innervate the chromatophores (lateral basal lobes) (Young 1977), although the presence of VRα-ir neurites innervating the chromatophores of *I. notoides* remains to be proven. Nevertheless, these data suggest the involvement of this neuropeptide in important and complex behavioral traits of cephalopod squids.

The subvertical lobes possess one of the largest populations of VRα-ir cell somata and connect the vertical lobe to the remaining CNS. The vertical lobe is the highest integration center and is involved in learning and memory (Young 1979). Further VRα-ir cell somata are present in the optic tract region, including the peduncle lobe and the adjacent optic lobes. The peduncle lobe receives visual input from the optic lobe and a "labyrinthine" input from the statocyst (Messenger 1979).

Expression of the VD_1/RPD_2 α1-peptide during cephalopod CNS development

In our study, VRα-ir elements were found in different but also corresponding regions of the developing CNS. In the decapod *Idiosepius notoides* and the octopod *Octopus vulgaris*, neuropeptide expression commences when the neuropils of the various lobes are already differentiated to a high degree. In both species, the neuropeptide is first expressed in late paralarvae, i.e., in individuals that are close to hatching. Shared expression domains are lobes or organs associated with the optic tract region, such as the olfactory lobe or the olfactory organ. This is also the case for prehatchlings of the decapods *Sepioteuthis australis*, *Euprymna scolopes*, *Loligo vulgaris* and, as already mentioned, for the adult CNS of *I. notoides*. The olfactory organ, which possesses numerous VRα-ir cell somata in two cephalopod species examined, has been suggested to be of chemoreceptive function (Wildenburg 1997). This may be an important aspect for prey location, mate identification and reception and avoidance from predators, all key behaviors commonly observed in adult cephalopods.

In *Loligo vulgaris*, *E. scolopes* and *S. australis*, prehatchlings possess VRα-ir cell somata in the periesophageal mass. Additional cell somata are present in the perikaryal layers of the posterior subesophageal mass of *S. australis*, *L. vulgaris* and *E. scolopes*. Compared to other neuropeptides or neurotransmitters such as FMRFamide-related peptides or serotonin (5-HT), the α1-neuropeptide is expressed relatively late during ontogenesis and not stringently first in the subesophageal mass of the CNS (Wollesen et al., 2010a, 2010b).

Table 2 Expression of the VD_1/RPD_2 α1-peptide within the CNS of the molluscan groups investigated so far, compiled from several sources (Bogerd et al. 1991, 1993, 1994; Kerkhoven et al. 1992, 1993). VRα-ir elements or entire neural structure absent (–)

Ganglion *Species* Gastropoda *Pulmonata*	Caudal regions of visceral (vsg) and right parietal ganglia (rpg)	Cerebral ganglia	Pedal ganglia	Abdominal ganglion	Peripheral nervous system
Bulinus truncates, Lymnaea stagnalis ("Basommatophora")	single giant cell soma & 2–5 medium-sized cell somata	3–5 medium-sized cell somata & 1 cluster of small cell somata	2–4 small cell somata	-	neurites, connectives, peripheral nerves
All Stylommatophora species investigated	2–4 giant cell somata in partly or fully fused vsg and rpg	-	Medium and/or small sized cell somata	-	-
Achatina fulica (Stylommatophora)	1 giant cell soma in caudal margin of rpg near the vsg. 1 giant cell soma in ganglion located at the caudal margin of the vsg. 10–15 medium-sized cell somata in vsg & rpg.	-	-	-	-
Limax maximus (Stylommatophora)	3 giant & 5–8 medium-sized cell somata in rpg & vsg.	-	2–4 medium sized cell somata	-	-
Cepea sp. (Stylommatophora)	2 pairs of adjacent giant cell somata (1 in vsg & 1 in rpg)	-	-	-	small & medium-sized cell somata in different ganglia
Eobania vermiculata (Stylommatophora)	2 giant cell somata in fused vsg/ rpg. Medium-sized cell somata close to them & 1 giant cell somata in rpg	-	-	-	innervation of pedal ganglia
Helix aspersa, H. pomatia (Stylommatophora)	2 giant & several small cell somata in subesophageal ganglion	-	-	-	innervation of pedal ganglia
"Opisthobranchia"					
Aplysia brasiliana, *A. californica*	-	-	-	1 giant cell soma on caudal margin of right hemiganglion, 10–15 medium-sized cell somata in medial part of fused ganglion	-
Caenogastropoda					
Viviparus viviparous, Nassarius reticulata	-	small cell somata & neurites	-	-	other ganglia not studied
Bivalves					
Mytilus edulis, Anodonta sp.	40–50 small cell somata & neurites	several cell somata	-	-	-

VRα-ir elements or entire neural structure absent (-)

Table 3 Expression of the VD_1/RPD_2 α1-peptide within cell somata of the adult and the developing CNS of the cephalopod species investigated in this study. In the literature, the anatomy and terminology of the octopod and decapod CNS differ in parts from each other. Since expression of the VD_1/RPD_2 α1-peptide in octopods is restricted to CNS regions for which the same terms are used as in their decapod counterparts, this common terminology is followed herein

Species, developmental stage			*Idiosepius notoides* adult	*Idiosepius notoides* stage 30 hatchlings	*Euprymna scolopes* stages XVI–XIX	*Sepioteuthis australis* hatchlings	*Loligo vulgaris* almost hatching	*Octopus vulgaris* stages XIX–XX almost hatching
CNS region								
Subesophageal mass	Anterior		+	-	+++ only in stages XVI and XVII	-	-	-
	Middle	Anterior chromatophore lobes	++	-	+++ only in stages XVI and XVII	-	-	-
		Anterior pedal lobes	++	+		-	-	-
		Posterior pedal lobes	+	+		-	++	-
	Posterior	Palliovisceral lobe	+	-	+++ only in stages XVI and XVII	++	++	-
		Posterior chromatophore lobes	+	-	+++ only in stages XVI and XVII		-	-
		Visceral lobes	+	-	+++ only in stages XVI and XVII		-	-
		Fin lobe	+	-	+++ only in stages XVI and XVII		-	-
Periesophageal mass		Ventral magnocellular lobes	-	-	-	-	++	-
		Dorsal magnocellular lobes	+	-	++ only in stages XVIII and XIX	++	-	-
		Posterior magnocellular lobes	-	-	-	-	-	-
Supraesophageal mass	Vertical lobe complex	Vertical lobe	-	-	-	-	+++	-
		Subvertical lobes	+++	-	-	-	-	-
		Superior frontal lobes	+	-	++ (?) only in stages XVIII and XIX	-	-	-
		Inferior frontal lobes	+	-	++ (?) only in stages XVIII and XIX	-	++	-
		Precommissur-al lobes	++	-	-	-	-/+	-
	Posterior basal lobes	Dorsal basal lobes	+++	++	++ (?) only in stages XVIII and XIX	-	-	-
		Dorso-lateral lobes	++	++	-	-	-	-
		Median basal lobes	+++	++	-	-	+	-
		Lateral basal lobes	++	++	-	-	-	-
		Interbasal lobes	-	++	-	-	-	-
		Subpedunculate lobes	-	++	-	+++ (?)	+++	-
	Anterior basal lobes		++	++	-	-	+++	-
	Optic tract region	Peduncle lobes	++	-	-	-	-	-
		Olfactory lobes	++	-	-	-	+++	+++
Optic lobes			+++	-	-	-	+/-	-
Peripheral lobes, ganglia, and tissues	Superior buccal lobes		+	-	-	-	++	-
	Inferior buccal lobes		+	-	-	-	-	-
	Stellate ganglia		n.i.	-	++ only in stages XVI and XVII	n.i.	n.i.	n.i.
	Optic gland		n.i.	-	-	+++ (?)	-	-
	Olfactory organ		n.i.	-	+++ only in stages XVIII and XIX	-	-	-

High (+++), medium (++), and low density (+) of VRα-ir cell somata. No VRα-ir cell somata present (-). Not investigated (n.i.). Identity of brain lobe or tissue ambiguous (?).

Paralarvae of *L. vulgaris* close to hatching exhibit the largest population of VRα-elements (Fig. 6). The largest subset of VRα-cell somata is located in the vertical lobe, a region that does not exhibit VRα-immunoreactivity in any of the other cephalopod species investigated so far (Figs. 2, 4, 5 and 6 in present study). In adults of the cuttlefish *Sepia officinalis* and *I. notoides*, as well as in developmental stages of *I. notoides* and *O. vulgaris*, none or only very few cell somata in the vertical lobe express serotonin (5-hydroxytryptamine) (Boyer et al. 2007; Wollesen et al. 2010b; Wollesen et al. 2012).

In *L. vulgaris*, VRα-ir elements are present in the majority of brain lobes but appear to be entirely missing in the superior frontal lobe (Fig. 6a,b). In developmental stages of all other cephalopod species observed, the superior frontal lobe does not appear to house VRα-ir elements (present study). The same is true for the neurotransmitter serotonin, which is expressed in none or only very few cell somata and neurites in the superior frontal lobe in developmental stages of *I. notoides* and *O. vulgaris*, as well as in adults of *I. notoides* and the cuttlefish *Sepia officinalis* (Boyer et al. 2007; Wollesen et al. 2010b; Wollesen et al. 2012).

Prior to hatching, cephalopods may undergo crucial neurophysiological modifications in order to cope with different environmental regimes as juveniles. In addition, interspecific neuroanatomical and physiological differences exist due to the adaptation to different habitats, i.e., benthic vs planktonic life history strategies. Both facts may account for differences in timing and location of VRα-expression in the specimens investigated.

Comparison of the expression of the VD_1/RPD_2 α1-peptide in the gastropod, bivalve and cephalopod CNS

Table 2 summarizes the different expression domains of the VD_1/RPD_2 α1-peptide in gastropods and bivalves, i.e., all molluscan taxa investigated so far. In all cephalopods investigated, except for developmental stages of *I. notoides* and *O. vulgaris*, VRα-ir cell somata are present in different regions of the supraesophageal mass, which is considered a counterpart of the cerebral ganglia of other mollusks (Table 3; Bullock and Horridge 1965). Homology of the various brain regions of cephalopods and other mollusks, however, is difficult to assess due to the unique degree of complexity of the cephalopod CNS. Only in "prosobranch" and "basommatophoran" gastropods as well as in bivalves, VRα-ir cell somata are present in the cerebral ganglia (Table 2). Except for the "prosobranchs", the largest groups of VRα-ir cell somata are usually present in the partially or fully fused parietal and visceral ganglia of gastropods (Table 2; Fig. 1b).

The posterior subesophageal mass of cephalopods and possibly the visceral ganglia of bivalves, are thought to correspond to the visceral/ pleurovisceral ganglia of gastropods (Bullock and Horridge 1965). In bivalves as well as cephalopods, with the exception of the developmental stages of *I. notoides* and *O. vulgaris*, these ganglia house many VRα-ir cell somata (Tables 1 and 2). Kerkhoven et al. (1993) report VRα-ir cell somata in the visceral ganglia of *Mytilus edulis* and *Anodonta spec*. Since such ganglia are, however, absent in bivalves, these somata may belong to the visceral ganglia of these species instead (cf Bullock and Horridge 1965; Ellis and Kempf 2011). The middle subesophageal mass of adult *I. notoides* and stage XVI and XVII individuals of *E. scolopes* contains another subset of VRα-ir cell somata (Table 3). This portion of the cephalopod CNS is assumed to correspond to the pedal ganglia of other mollusks (Bullock and Horridge 1965). Previous studies have only described VRα-ir cell somata in the pedal ganglia of "basommatophoran" and "stylommatophoran" gastropods (Table 2).

Interestingly, in cephalopods, VRα-ir cell somata are particularly abundant in many brain lobes and organs that are considered evolutionary innovations of this clade, such as the stellate ganglia, optic lobes, lobes of the adjacent optic tract region, vertical lobes, subvertical lobes and olfactory organs and optic glands (Table 3). This demonstrates the high potential of the VRα-ir system for recruitment into novel neural structures. It thus calls for further comparative investigations in other mollusks and the Lophotrochozoa to better understand how neuropeptides are recruited for cognitive processes and behavior in developmental stages and adults.

In summary, VRα-ir elements are abundant and widely distributed throughout the CNS of the adult pygmy squid *Idiosepius notoides* and in *Loligo vulgaris* specimens close to hatching. Future electrophysiological experiments may contribute to assessing whether or not the α1-neuropeptide acts as a neurotransmitter and/or a neuromodulator. Moreover, VRα-ir cell somata are present in high densities in brain lobes that are unique for coleoid cephalopods and thus do not appear to have a counterpart in other mollusks.

The species-specific distribution of VRα-ir cell somata in developmental stages of all cephalopods investigated suggests a specific recruitment of the α1-peptide into the neural control of certain behaviors ascribed to these specific brain regions. The overall spatio-temporal plasticity of α1-peptide expression during cephalopod ontogeny indicates that the expression domains are evolutionary and ontogenetically labile among the various cephalopod species (cf Table 3). Since only adults of a single cephalopod species were investigated, any conclusions concerning the evolutionary conservation of the α1-peptide expression domains in adult cephalopods would be premature. This study and previous investigations on cephalopods, gastropods and bivalves, demonstrate that VRα-ir cell somata are numerous in the diverse ganglia and brain lobes that are assumed to be

homologs of each other (Bullock and Horridge 1965). Although data on scaphopods and monoplacophorans are still missing, it is probable that VRα-ir elements are part of the conchiferan ground pattern. Future work on the VD_1/RPD_2 system in the relatively simple CNS of basal molluscan groups such as polyplacophorans and the worm-shaped neomeniomorph and caudofoveate aplacophorans will allow comparisons with gastropod and cephalopod conditions. These future studies should clarify whether the distribution of the VD_1/RPD_2 α1-peptide was part of the ancestral molluscan neural bodyplan, or whether this system evolved at a later stage in more derived mollusks.

References

Bogerd J, Geraerts WPM, Van Heerikhuizen H, Kerkhoven RM, Joosse J (1991) Characterization and evolutionary aspects of a transcript encoding a neuropeptide precursor of *Lymnaea* neurons, VD1 and RPD2. Mol Brain Res 11:47–54

Bogerd J, Van Kesteren RE, Van Heerikhuizen H, Geraerts WPM, Veenstra J, Smit AB, Joosse J (1993) Alternative splicing generates diversity of VD1/RPD2 α peptides in the central nervous system of *lymnaea stagnalis*. Cell Mol Neurobiol 13:123–136

Bogerd J, Li KW, Jiménez CR, van der Schors RC, Ebberink RHM, Geraerts WPM (1994) Processing, axonal transport and cardioregulatory functions of peptides derived from two related prohormones generated by alternative splicing of a single gene in identified neurons VD1 and RPD2 of *Lymnaea*. Mol Brain Res 23:66–72

Boyer C, Maubert E, Charnay Y, Chichery R (2007) Distribution of neurokinin A-like and serotonin immunoreactivities within the vertical lobe complex in *Sepia officinalis*. Brain Res 1133:53–66

Buck LB, Bigelow JM, Axel R (1987) Alternative splicing in individual *Aplysia* neurons generates neuropeptide diversity. Cell 51:127–133

Bullock TH, Horridge GA (1965) Structure and function in the nervous systems of invertebrates. Freeman, San Francisco

Di Cosmo A, Di Cristo C (2006) Molluscan bioactive peptides. In: Kastin AJ (ed) Handbook of biologically active peptides. Academic, Amsterdam, pp 235–240

Ellis I, Kempf SC (2011) Characterization of the central nervous system and various peripheral innervations during larval development of the oyster *Crassostrea virginica*. Invert Biol 130:251–263

Kastin AJ (2006) Handbook of biologically active peptides. Academic, Amsterdam

Kerkhoven RM, Croll RP, Van Minnen J, Bogerd J, Ramkema MD, Lodder H, Boer HH (1991) Axonal mapping of the giant peptidergic neurons VD_1 and RPD_2 located in the CNS of the pond snail *Lymnaea stagnalis*, with particular reference to the innervation of the auricle of the heart. Brain Res 565:6–16

Kerkhoven RM, Croll RP, Ramkema MD, Van Minnen J, Bogerd J, Boer HH (1992) The VD_1/RPD_2 neuronal system in the central nervous system of the pond snail *Lymnaea stagnalis* studied by in situ hybridization and immunocytochemistry. Cell Tiss Res 267:551–559

Kerkhoven RM, Ramkema MD, Van Minnen J, Croll RP, Pin T, Boer HH (1993) Neurons in a variety of molluscs react to antibodies raised against the VD_1/RPD_2 α-neuropeptide of the pond snail *Lymnaea stagnalis*. Cell Tiss Res 273:371–379

Kocot KM, Cannon JT, Todt C, Citarella MR, Kohn AB, Meyer A, Santos SR, Schander C, Moroz LL, Lieb B, Halanych KM (2011) Phylogenomics reveals deep molluscan relationships. Nature 477:452–456

Marquis F (1989) Die Embryonalentwicklung des Nervensystems von *Octopus vulgaris* lam. (Cephalopoda, Octopoda), eine histologische Analyse. Verh Naturforsch Ges Basel 99:23–76

Meister G (1972) Organogenese von *Loligo vulgaris* LAM. (Mollusca, Cephalopoda, Teuthoidea, Myopsida, Loliginidae). Inaugural dissertation, Universität Basel

Messenger JB (1979) The nervous system of *Loligo*: IV. The peduncle and olfactory lobes. Phil Trans R Soc Lond B 285:275–309

Naef A (1928) Cephalopoda embryology. Fauna and flora of the Bay of Naples. Part I, Vol. II. Translated from German. Smithsonian Institution Libraries, Washington, DC

Ponder WF, Lindberg DR (2008) Phylogeny and evolution of the Mollusca. University of California Press, Berkeley

Rodriguez J, Deinhard F (1960) Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology 12:316–317

Shigeno S, Tsuchiya K, Segawa S (2001) Embryonic and paralarval development of the central nervous system of the loliginid squid *Sepioteuthis lessoniana*. J Comp Neurol 437:449–475

Shigeno S, Yamamoto M (2002) Organization of the nervous system in the pygmy cuttlefish, *Idiosepius paradoxus* Ortmann (Idiosepiidae, Cephalopoda). J Morphol 254:65–80

Wildenburg G (1997) Structure of the so-called olfactory organ of octopods after hatching: Evidence for its chemoreceptive function. Vie Milieu 47:137–142

Wollesen T, Loesel R, Wanninger A (2009) Pygmy squids and giant brains: mapping the complex cephalopod CNS by phalloidin staining of vibratome sections and whole-mount preparations. J Neurosci Meth 178:63–67

Wollesen T, Cummins SF, Degnan BM, Wanninger A (2010a) FMRFamide gene and peptide expression during central nervous system development of the cephalopod mollusk, *Idiosepius notoides*. Evol Dev 12:113–130

Wollesen T, Degnan BM, Wanninger A (2010b) Expression of serotonin (5-HT) during CNS development of the cephalopod mollusk, *Idiosepius notoides*. Cell Tiss Res 342:161–178

Wollesen T, Sukhsangchan C, Seixas P, Nabhitabhata J, Wanninger A (2012) Analysis of neurotransmitter distribution in brain development of benthic and pelagic. J Morphol, in press

Yamamoto M (1988) Normal embryonic stages of the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann. Zool Sci 5:989–998

Yamamoto M, Shimazaki Y, Shigeno S (2003) Atlas of the embryonic brain in the pygmy squid, *Idiosepius paradoxus*. Zool Sci 20:163–179

Young JZ (1971) The anatomy of the nervous system of *Octopus vulgaris*. Clarendon, Oxford

Young JZ (1977) The nervous system of *Loligo*: III. Higher motor centres: The basal supraesophageal lobes. Phil Trans R Soc Lond B 276:351–398

Young JZ (1979) The nervous system of *Loligo*: V. The vertical lobe complex. Phil Trans R Soc Lond B 285:311–354

RESEARCH ARTICLE

Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization

Alba Chavez-Dozal, David Hogan, Clayton Gorman, Alvaro Quintanal-Villalonga & Michele K. Nishiguchi

Department of Biology, New Mexico State University, Las Cruces, NM, USA

Correspondence: Michele K. Nishiguchi, Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA. Tel.: +1 575 646 3721; fax: +1 575 646 5665; e-mail: nish@nmsu.edu

Received 3 November 2011; revised 30 March 2012; accepted 2 April 2012.
Final version published online 21 May 2012.

DOI: 10.1111/j.1574-6941.2012.01386.x

Editor: Julian Marchesi

Keywords
biofilm; mutualism; *Vibrio fischeri*; sepiolid squid; beneficial.

Abstract

Biofilms are increasingly recognized as being the predominant form for survival for most bacteria in the environment. The successful colonization of *Vibrio fischeri* in its squid host *Euprymna tasmanica* involves complex microbe–host interactions mediated by specific genes that are essential for biofilm formation and colonization. Here, structural and regulatory genes were selected to study their role in biofilm formation and host colonization. We have mutated several genes (*pilT*, *pilU*, *flgF*, *motY*, *ibpA* and *mifB*) by an insertional inactivation strategy. The results demonstrate that structural genes responsible for synthesis of type IV pili and flagella are crucial for biofilm formation and host infection. Moreover, regulatory genes affect colony aggregation by various mechanisms, including alteration of synthesis of transcriptional factors and regulation of extracellular polysaccharide production. These results reflect the significance of how genetic alterations influence communal behavior, which is important in understanding symbiotic relationships.

Introduction

In most environments, bacteria form sessile communities attached to a surface known as biofilms, which form a major portion of the microbial biomass present in nature (Yoshida & Kuramitsu, 2002; Moorthy & Watnick, 2004; Kievit, 2009). Biofilm formation is a common strategy utilized for establishment of symbiotic associations, such as mutualisms (Ariyakumar & Nishiguchi, 2009; Morris & Visick, 2010) and pathogenic interactions (Hoyle & Costerton, 1991).

Vibrio fischeri is a marine bacterium that infects the light organs of sepiolid squids and monocentrid fishes (Nishiguchi *et al.*, 2004), establishing an exclusive partnership that is beneficial to both host and symbiont (Nyholm & McFall-Ngai, 2004). Its association in the Hawaiian bobtail squid (*Euprymna scolopes*) has been used as a model system for more than 20 years. At the onset of the mutualism, free-living bacteria infect juvenile aposymbiotic squids within the first few hours after hatching. Host-derived mucus provides a surface that allows bacteria to aggregate (and form a biofilm) prior to colonization (Nyholm *et al.*, 2002), which eventually forms an additional biofilm in the crypts of the squid's light organ complex (Visick & Ruby, 2006). The host provides an appropriate niche for the bacteria to reproduce and form this internal biofilm in the host light organ, providing an environment where the bacteria produce bioluminescence that is used by the squid to avoid predation in a behavior known as counterillumination (Jones & Nishiguchi, 2004). At dawn after the first day of colonization, squids release (or vent) over 90% of the bacteria to the environment to re-populate the bacterioplankton community and infect newly hatched juveniles (Ruby, 1999; Nyholm & McFall-Ngai, 2004). The symbiosis is highly specific and similar to pathogenesis in the dynamics of colonization (Visick & Ruby, 2006).

Successful colonization depends on the activation of numerous genes that lead to the formation of a biofilm. As a result, multiple genes are differentially expressed in biofilms when compared with those in their planktonic counterparts (Eko Niba *et al.*, 2007; Ariyakumar & Nishiguchi, 2009; Chavez-Dozal & Nishiguchi, 2011).

A number of studies have described the genetic basis of biofilm formation of mutualistic vibrios. Some examples

FEMS MICROBIOLOGY ECOLOGY

include the discovery of hybrid sensor kinases such as *rpoN* (encoding for the σ^{54}; Wolfe *et al.*, 2003) and symbiosis polyssacharide cluster (*syp*; Yip *et al.*, 2005), which is transcriptionally regulated either by the RscSypG two-component regulatory system (Morris *et al.*, 2011) or by two proteins, SypA and SypE (Morris & Visick, 2010; Morris *et al.*, 2011). Alternatively, the protein RscS has been reported to play an important role in biofilm formation by inducing expression of the Syp polysaccharide (Mandel *et al.*, 2009). Additional studies also emphasize the importance of mannose-sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (*UDPH*) in *Vibrio* biofilm formation (Ariyakumar & Nishiguchi, 2009).

Remarkably, we know far less about the genetic basis of biofilm formation in mutualistic associations compared with pathogenic associations. Additionally, biofilms formed by *V. fischeri* and the roles that these play in the *Vibrio*–squid symbiosis are still not fully characterized.

Previous studies in other organisms have identified several genes associated with function and formation of bacterial structures that are important for biofilm formation. In *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* two structural genes, *pilT* and *pilU*, have been described to be important in adhesion and biofilm formation by the production of a hexameric ATPase that is required for the retraction of type IV pilus and 'twitching' motility (Withchurch & Mattick, 1994). Genes such as *flgF* are responsible for flagellum synthesis and have been implicated in biofilm formation, particularly related to the synthesis of a protein that is located between the hook–filament junction and proximal rod (Liu & Ochman, 2007). Flagella synthesis depends upon approximately 50 genes (Aldridge & Hiughes, 2002) and FlgF is considered one of the most important highly soluble proteins for flagellar assembly due to its location in flagellar organization (Saijo-Amano *et al.*, 2004). Another example is *motY*, which encodes one component of the sodium-type flagellar motor pump of certain vibrios (Hossain & Tsuyumu, 2006).

Genes important for metabolic processes have also been linked to biofilm formation. Heat shock proteins such as *ibpA* are overexpressed during the biofilm state of *Escherichia coli* (Beenken *et al.*, 2004), but their function in biofilm development has not been established. Another example is *mifB*, which is one of the loci responsible for synthesizing bis-(3′-5′)-cyclic-di-guanosine monophosphate (c-di-GMP). Among the multiple genes responsible for c-di-GMP synthesis, *mifB* (magnesium-dependent induction) has recently been identified as it promotes the synthesis of a DGC (di-guanilate cyclase) that directly controls synthesis of c-di-GMP and (at different concentrations of magnesium) regulates flagellar gene transcription (O'Shea *et al.*, 2006; Wolfe & Visick, 2008). c-di-GMP is a unique novel second messenger that induces extracellular polysaccharide production (Nahamchik *et al.*, 2008), and regulates flagellar biosynthesis, twitching motility and related processes, which also include biofilm formation (Wolfe & Visick, 2008).

Formation of biofilms is a complex and dynamic mechanism that relies both on the presence and concentration of bacteria, targeted gene regulation for bacterial aggregation and colony formation, and on the expression of proteinaceous materials that will eventually become the matrix. Most genetic determinants previously described have been reported to be important for a least one aspect of biofilm formation in other Gram-negative bacteria. Therefore, we have chosen to analyse the role of several structural and regulatory genes (*piU*, *pilT*, *flgF*, *motY*, *ibpA* and *mifB*) that are thought to have an important role in *V. fischeri* biofilm formation and host colonization. This study is also important because previous studies have focused on the effects in colonization of the Hawaiian squid host (*Euprymna scolopes*). Here, we focus on the effects of colonization in a different host (*Euprymna tasmanica*) that has been reported to show similar colonization mechanisms (Nishiguchi, 2002; Nair & Nishiguchi, 2009). Finally, we decided to select and study this set of genes because they have been found to be overexpressed in the biofilm state of *V. fischeri* (RT-PCR studies, our unpublished data). Our hypothesis predicts that they play a crucial role in forming biofilms and are essential in promoting symbiotic colonization by *V. fischeri* over its *E. tasmanica* host.

Materials and methods

Strains, plasmids and growth conditions

All strains used are described in Table 1. *Vibrio fischeri* ETJB1H was isolated from the light organ of *Euprymna tasmanica* from Jervis Bay, Australia (Jones *et al.*, 2006). Strains were grown in either Luria–Bertani (LB; L^{-1}: 10 g tryptone, 5 g yeast extract and 10 g NaCl) or Luria–Bertani high Salt (LBS; L^{-1}: 10 g tryptone, 5 g yeast extract, 20 g NaCl, 50 mL 1 M Tris, pH 7.5, 3.75 mL 80% glycerol and 950 mL dH_2O) media at 37 and 28 °C. For selection of specific mutant strains, erythromycin (25 μg mL^{-1}) was added to the media.

Mutant construction and complementation

Mutants were constructed by insertion of plasmid pEVS122 as described previously (Ariyakumar & Nishiguchi, 2009). All genes were partially amplified with specific primers designed from the sequenced strain ES114 (NCBI

Table 1. Strains and plasmids used in this study

Strain or plasmids	Description
Plasmids	
pEVS122	R6K Erm^R
pVSV105	pES213 replicon, Cm^R
V. fischeri strains	
ETJB1H	Wild-type from Jervis Bay, New South Wales, Australia
pilT$^-$	ETJB1H (*pilT*::pEVS122). PilT insertion mutant
pilU$^-$	ETJB1H (*pilU*::pEVS122). PilU insertion mutant
motY$^-$	ETJB1H (*motY*::pEVS122). MotY insertion mutant
flgF$^-$	ETJB1H (*flgF*::pEVS122). FlgF insertion mutant
ibpA$^-$	ETJB1H (*ibpA*::pEVS122). IbpA insertion mutant
mifB$^-$	ETJB1H (*mifB*::pEVS122). MifB insertion mutant
pilT$^+$	ETJB1H (*pilT*::pEVS122) complemented with pVSV105::*pilT*
pilU$^+$	ETJB1H (*pilU*::pEVS122) complemented with pVSV105::*pilU*
motY$^+$	ETJB1H (*motY*::pEVS122) complemented with pVSV105::*motY*
flgF$^+$	ETJB1H (*flgF*::pEVS122) complemented with pVSV105::*flgF*
ibpA$^+$	ETJB1H (*ibpA*::pEVS122) complemented with pVSV105::*ibpA*
mifB$^+$	ETJB1H (*mifB*::pEVS122) complemented with pVSV105::*mifB*

Erm^R, erythromycin resistance; Cm^R, chloramphenicol resistance.

accession: NC_006840.2; Table 2). PCR products were purified and cloned into suicide vector pEVS122, and wild-type *V. fischeri* strains were transformed by tri-parental mating via conjugation through a helper strain (Stabb & Ruby, 2002). Strains that had undergone single homologous recombination events with the native gene were selected on LBS plates enriched with erythromycin (25 μg mL^{-1}). For complement construction, complete copies of all loci were amplified with specific primers for the entire locus (Table 2), purified and cloned into vector pVSV105. This plasmid was introduced into the particular mutant by tri-parental mating. Strains that had been successfully transformed were selected on LBS plates enriched with erythromycin (25 μg mL^{-1}) for maintenance of the pEVS122 plasmid and chloramphenicol (10 μg mL^{-1}) for maintenance of pVSV105. Mutants and complemented mutants were verified by Southern blotting.

Biofilm assays

All bacterial strains (wild-type, mutants and complemented strains) were grown in LBS media and biofilm assays were performed as previously described (Nair & Nishiguchi, 2009). Strains were inoculated in 96-well microplates and incubated for 18 h at 28 °C. After incubation, planktonic cells were removed and the remaining biofilm was stained with crystal violet, which was immediately solubilized with 70% ethanol. Optical density was measured (562 nm), which directly reflects the amount of biofilm formed (Ariyakumar & Nishiguchi, 2009). Assays were completed from overnight cultures, using five wells in each plate per strain (technical replicate) and three plates (biological replicate) for a total of 15 replicates. Results were analysed statistically via Tukey's *post hoc* test.

Scanning electron microscopy (SEM)

Overnight cultures of all strains were re-inoculated in 5 mL LBS with an immersed sterile cover slip. Strains were incubated for 18 h without shaking (Ariyakumar & Nishiguchi, 2009). Coverslips were washed with sterile seawater (32 p.p.t.) and gold-coated for SEM with a Hitachi S34000-SEM (Schaumburg, IL) as previously described (Greiner *et al.*, 2005). Observations were repeated in triplicate from different overnight cultures.

In vitro chemostat system and confocal-laser scanning microscopy (CLSM)

Biofilm formation was observed under a dynamic environment (continuous-flow) using a modified Kaduri drip-fed chemostat that was assembled and re-designed in our laboratory (Merritt *et al.*, 2005). Overnight cultures were injected with a needle into glass chambers that had been prepared with an inflow (connected to the media reservoir) and outflow (connected to a waste container) siphon. A peristaltic pump supplied fresh LBS media simultaneously to four identical chambers and removed waste at a ratio of 5 mL min^{-1} for 18 h. Chambers were washed with fresh seawater, stained with 1 mL of live/dead stain (SYTO9/propidium iodide, Invitrogen Molecular Probes L3224) for 15 min. Biofilms were subsequently examined by CLSM at the NMSU Fluorescent Imaging Facility (TCS SP5; Leica Microsystems). Samples were measured in triplicate (chemostat was run three different times using different overnight cultures).

Motility assays

Swimming

To test for swarming and motility, *V. fischeri* ETJB1H wild-type, *flgF*$^-$ and *motY*$^-$ strains were examined for any phenotypic changes, such as cell spreading, that leads to colony pattern differentiation. The media consisted of LBS with 0.5% (w/v) Difco bacto-agar, to which glucose

Table 2. Primers used for mutant construction and complementation

Primers	Sequence 5′–3′	PCR product size (bp)
pilT^{-} Forward	GGATCCCTCGTGGCTGTGCTGCTGTGTT	316
pilT^{-} Reverse	TCTAGACGCAAAGCTGAGCGAAGTGCT	316
pilU^{-} Forward	GGATCCGGCTGCGATGACGGGGTATCG	450
pilU^{-} Reverse	TCTAGAGTCTGCAACGCGTGGCGTGT	450
motY^{-} Forward	GGATCCGCCCAATGGGTGAAACTCGTGC	172
motY^{-} Reverse	TCTAGACTGCACCATCACCCGGCATCC	172
flgF^{-} Forward	GGATCCAGCCATGAGTGGCGCAAAGC	456
flgF^{-} Reverse	TCTAGAGCCATCGCTTCAGCTGGTGC	456
ibpA^{-} Forward	GGATCCATGGCGGTAGCTGGCTTTGCT	187
ibpA^{-} Reverse	TCTAGACCATCGTTGCGCCCACCACTT	187
mifB^{-} Forward	GGATCCTGGCTATGGGGATTACCCGTTGGA	920
mifB^{-} Reverse	TCTAGACAACTGAAGGCACTCACCTTGCGT	920
pilT^{+} Forward	ATATCTAGATACTAGTCATAGAAACGATTACCGAGGAAA	1038
pilT^{+} Reverse	TTACCCGGGTGATCATTTGTTTCAGTATTTGATCC	1038
pilU^{+} Forward	ATATCTAGAACTGTGTCATTCAGCCTGACACAAAGGAGTT	1101
pilU^{+} Reverse	TTACCCGGGAATGCCCACCAAACAAATCGCAA	1101
motY^{+} Forward	ATATCTAGAATCGTCAACGGCATGACCCATAGATTTAGG	879
motY^{+} Reverse	TTACCCGGGCGTCCTAGAGAAATGACAACACGACGG	879
flgF^{+} Forward	ATATCTAGATATTATAACTTCAGATAGATTATTGGAGTTC	750
flgF^{+} Reverse	TTACCCGGGACCCATAATGCTGGATTCATT	750
ibpA^{+} Forward	ATATCTAGATACTCACTATTGCTTAAATTAAAGGATAGT	440
ibpA^{+} Reverse	TTACCCGGGACAGCGCCTTTATGTTCAAT	440
mifB^{+} Forward	ATATCTAGAATGGTGATTACTCCCCCTAATGCCCAGGAGC	1971
mifB^{+} Reverse	TTACCCGGGTGCCGGGCCGATATGTGGCT	1971

(5 g L^{-1}) was added as previously described (Rashid & Kornberg, 2000). Swarm plates were inoculated from an overnight culture in LBS agar (1.5% w/v) using a sterile toothpick. Plates were then incubated at 28 °C for 24 h.

Twitching

To examine twitching motility, *V. fischeri* ETJB1H wild-type, *pilU*$^{-}$ and *pilT*$^{-}$ strains were chosen for this portion of the study. Media consisted of LBS with 1% (w/v) Difco bacto-agar. Plates were briefly dried and stab inoculated using a sterile toothpick. Strains were placed at the bottom of the each Petri dish from an overnight culture in LBS agar (1.5% w/v), and incubated at 28 °C for 24 h (Rashid & Kornberg, 2000).

Indole assays

Extracellular indole detection

Differences in production of extracellular indole were measured according to Kuczynska-Wisnik *et al.* (2010). Overnight cultures of *V. fischeri* ETJB1H wild-type and *ibpA*$^{-}$ were subcultured and incubated at 28 °C. Indole was measured at different time points of growth by adding 2 mL Kovac's reagent (10 g *p*-dimethylaminobenzaldehyde, 50 mL 1 M HCl and 150 mL amyl alcohol) to 5 mL of media (after sedimentation of bacteria). This mixture was diluted 1 : 10 in HCl/amyl alcohol solution and the optical density (540 nm) was measured. Assays were performed in triplicate.

Effect of indole addition

The effect of indole on biofilm formation was investigated as described by Lee *et al.* (2008). Overnight strains of *V. fischeri* ETJB1H and *ibpA*$^{-}$ were subcultured in 96-well microplates with LBS media (with 0.25, 0.5 and 1.0 mM indole) and incubated at 28 °C for 18 h. The crystal violet assay was used for quantification of biofilm. All assays were performed in triplicate.

Colonization assays

To determine colonization efficiency, infection assays were performed as previously described (Nishiguchi, 2002). Briefly, overnight cultures of wild-type and mutant strains were regrown in 5 mL fresh LBS media until they reached an $OD_{600\ nm}$ of 0.3. Cultures were then diluted to approximately 1×10^3 CFU mL^{-1} in 5 mL of sterile seawater and added to glass scintillation vials where newly hatched juvenile squids were placed (one individual per vial). Seawater was changed with fresh uninoculated seawater every 12 h over a period of 48 h. Animals were

maintained on a light/dark cycle of 12/12 h. After 48 h, animals were sacrificed and homogenized, and the diluted homogenate was plated onto LBS agar plates. Bacteria (CFUs) were counted the next day to determine the colonization efficiency of each strain. Ten animals per strain were used. Results were analysed using a Tukey *post hoc* test.

Results and discussion

Mutational analysis to determine the importance of multiple genes in biofilm formation and host colonization

In this study we examined how mutations in different structural and regulatory genes affect the organization of *V. fischeri* biofilms both *in vitro* and in juvenile *E. tasmanica*. *Vibrio fischeri* forms biofilms in diverse habitats, including the environment and the squid host, which correspond to different ecological lifestyles. In the environment, there are multiple fluctuations of salinity and temperature that have a direct effect upon colonization and persistence (Soto *et al.*, 2009), but our knowledge of the genes that are important for *in vitro* biofilm formation and hence host colonization is limited.

As biofilms have been shown to be necessary for successful colonization of sepiolid squids, this study was aimed to understand whether specific structural and regulatory genes were essential for biofilm formation in both abiotic and symbiotic environments. Based on previously reported data, we organized the suite of genes into two categories: (1) those responsible for structural components such as flagella and pili, and (2) transcriptional regulators of bacterial metabolism that influence synthesis of the components for the formation of the biofilm matrix and backbone.

To compare the identity of the genes selected with those of known function, a bioinformatics approach comparing protein sequences with high similarity was used. The BLASTP and MATGAT v2 programs were used to compare sequences with those reported for *Vibrio cholerae* O1 biovar El Tor str N16961 (ID 243277). According to this analysis, sequences with known function were: (1) PilU (*V. fischeri* accession number YP_203815.1) with an identity of 77–78% to twitching motility protein; (b) PilT (*V. fischeri* accession number YP_203814.1) with an identity of 80–82% to twitching motility protein; (c) FlgF (*V. fischeri* accession number YP_205256.1) with an identity of 78–80% to flagellar body basal rod protein; (d) MotY (*V. fischeri* accession number YP_204309.1) with an identity of 65–68% to sodium-type flagellar motor protein; (e) IbpA (*V. fischeri* accession number YP_203396.1) having an identity of 79–81% with a 16-kDa heat-shock protein; and (f) MifB (*V. fischeri* accession number YP_206917.1) with an identity of 40–42% to a diguanilate cyclase with a GGDEF domain. Protein function is conserved for sequence identities equal or > 40% (Brenner, 1999). Therefore, the genes (or proteins) described in this study are most likely to share the described functions from other sequenced vibrios.

For this study, we selected an insertional mutagenesis strategy (Ariyakumar & Nishiguchi, 2009) in which the exogenous vector pEVS122 serves as a mutagen and as a molecular tag for identification (Dunn *et al.*, 2005). Complementation was achieved by inserting a complete copy of the gene contained in vector pVSV105. Constructs made utilizing these vectors are stable and do not revert (Dunn *et al.*, 2005). This method has been used successfully in our study, enables rapid construction and has facilitated the screen of defects in the mutated strains, including phenotypic differences (motility, biofilm architecture) and colonization deficiencies. Remarkably, all mutants constructed do not show growth defects when compared with the wild-type (results not shown) and when grown on standard media (LBS).

Biofilms formed by the wild-type strain exhibited a flocculent three-dimensional structure (Fig. 1), and mutated strains showed deficiencies in biofilm formation in at least one of the assays tested, with all mutants deficient in host colonization. The following results detail differences among the mutants examined depending on the function/nature of the genes.

FlgF and MotY

Flagellar motility has been demonstrated to be an important factor in bacterial biofilm formation (Houry *et al.*, 2010). To elucidate the role of the flagella in biofilm formation by *V. fischeri*, we used non-flagellated (*flgF*$^{-}$) and non-motile (*motY*$^{-}$) mutants. It is important to recognize that *flgF* forms part of the flagellar operon (composed of flgBCDEFGHIJKL) in vibrios (Merino *et al.*, 2006), and a mutation at this locus can cause polar effects on downstream genes, thereby creating an aflagellate mutant that is the product of a non-functional operon. Results indicate that both non-functional flagella (*motY*$^{-}$) and an aflagellate phenotype (*flgF*$^{-}$) severely impaired biofilm formation (Figs 2 and 3c, c1, d and d1). Swimming motility was reduced in both mutants (Fig. 4a–c). Observations from our study demonstrate unequal production of flagella, suggesting that a nonfunctional flagellum can still partially form biofilms due to its ability to act as an adhesin in a manner that is independent of motility. This behavior has also been observed for enteropathogenic *E. coli* (Giron *et al.*, 2002). Nevertheless, both mutants were equally deficient in colonizing juvenile squids

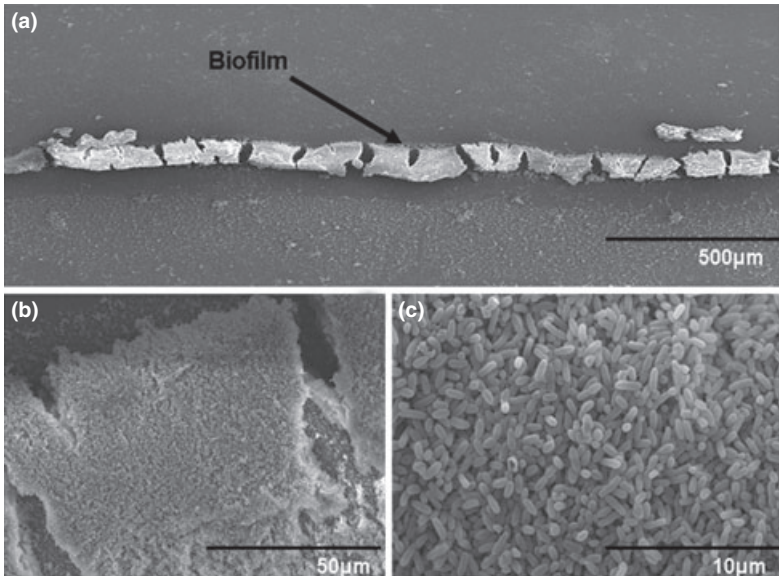


Fig. 1. Scanning electron micrographs of biofilm formed by the wild-type strain (*Vibrio fischeri* ETJB1H) on the liquid/air phase of a coverslip: (a) scale bar = 500 μm, 10× magnification; (b) scale bar = 50 μm, 50× magnification; (c) scale bar = 10 μm, 3000× magnification.

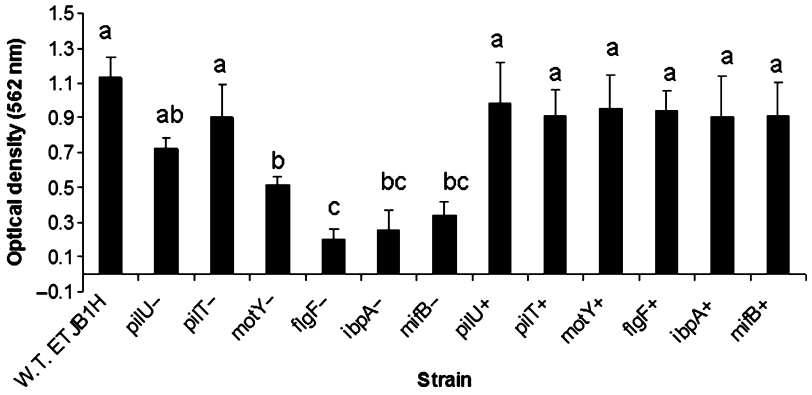


Fig. 2. *In vitro* biofilm formation for the various strains of *Vibrio fischeri* ETJB1H. Data are plotted as the mean $OD_{562\ nm}$ ± SD, with multiple comparisons calculated between groups. Different lower case letters on the abscissa indicate significant differences ($P < 0.05$) between groups, according to the Tukey *post hoc* comparison. According to these data, mutants *pilU*$^-$ and *pilT*$^-$ do not significantly differ from the wild-type (labeled 'a'), *pilU*$^-$ is labeled 'ab', i.e. is not different from *motY*$^-$, mutants *motY*$^-$, *flgF*$^-$, *ibpA*$^-$ and *mifB*$^-$ are significantly different from the wild-type (labeled 'b', 'c' or 'bc'), and *ibpA*$^-$ and *mifB*$^-$ (labeled 'bc') are not different from *flgF*$^-$.

(Fig. 5). Based on these and earlier results (Millikan & Ruby, 2002, 2003, 2004), it is reasonable to propose that flagella are essential for motility and interactions with host cells. How these symbiotic loci are regulated and synchronized during infection still remains an important issue to address in future studies.

PilU and PilT

One of the most renowned roles of pili proteins in biofilm formation is bacterial adherence to surfaces during the initial phases of adhesion (Yildiz & Visick, 2009). *Vibrio fischeri* pili are formed by a number of proteins encoded by genes from the *pil* operon, including *pilA–pilD* (Stabb & Ruby, 2003; Browne-Silva & Nishiguchi, 2008). The *pilU* and *pilT* genes are not part of this particular operon, and their function is related to elongation and retraction, which is important for 'twitching' or 'gliding' motility (Zolfaghar *et al.*, 2003). These genes produce a hexameric ATPase that has previously been described to be an important virulence factor in *P. aeruginosa* (Zolfaghar *et al.*, 2003) and *N. gonorrhoeae* (Firoved & Deretic,

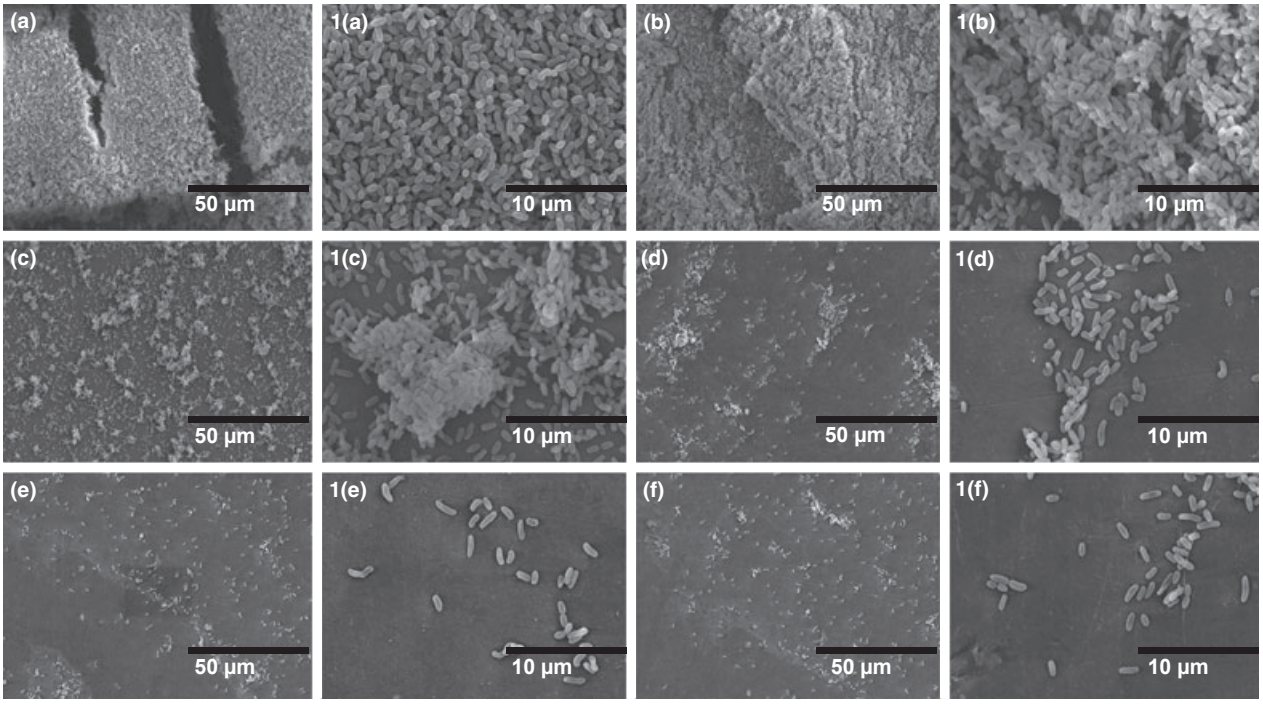


Fig. 3. Scanning electron micrographs of biofilms formed by mutant strains *pilU*$^{-}$ (a, a1), *pilT*$^{-}$ (b, b1), *motY*$^{-}$ (c, c1), *flgF*$^{-}$ (d, d1), *ibpA*$^{-}$ (e, e1), *mifB*$^{-}$ (f, f1); scale bars = 50 μm at 50× magnification and 10 μm at 3000× magnification.

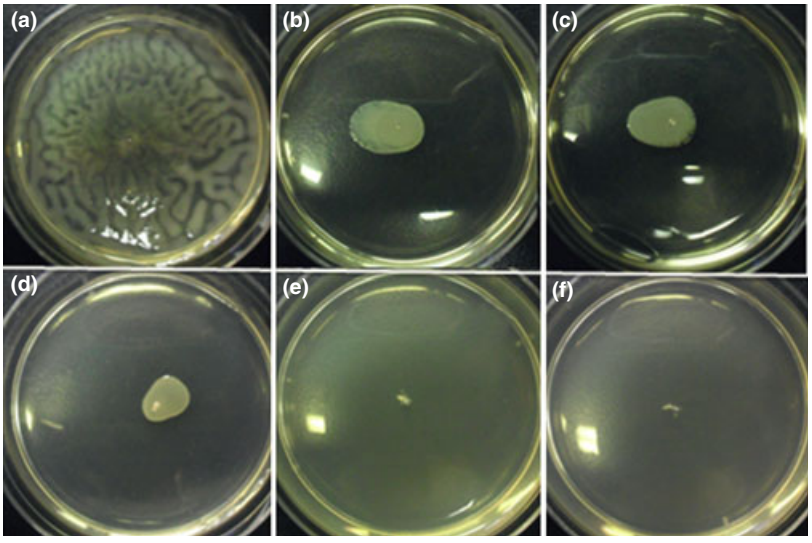


Fig. 4. Motility assays. Swimming for (a) wild-type ETJB1H, (b) *flgF*$^{-}$and (c) *motY*$^{-}$ strains. Twitching for (a) wild-type ETJB1H, (e) *pilT*$^{-}$and (f) *pilU*$^{-}$ strain. Plates were photographed after 24 h incubation.

2003), and are important for biofilm formation in these bacteria.

Visual examination of the parental strain, or wild-type *V. fischeri* ETJB1H (Fig. 1a), revealed a compact flocculent homogeneous organization. At higher magnifications, cells were distinguished by their rod-shaped organization in a mature three-dimensional structure (Fig. 1b, c). A dramatic difference in biofilm architecture was observed in all the mutants. For mutants with disruption of their structural pili genes (*pilU*, *pilT*), biofilm organization remained similar to those observed for the wild-type *V. fischeri* ETJB1H (Fig. 3a, a1, b, b1). These results were

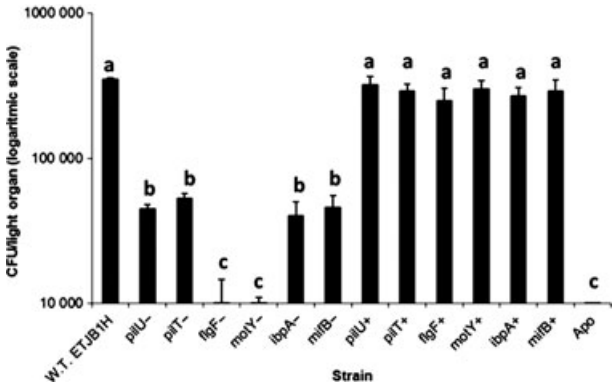


Fig. 5. Colonization assay 48 h post-infection of juvenile *Euprymna tasmanica* by wild-type and mutant strains of *Vibrio fischeri* ETJB1H. Mutant strains exhibited significant differences when compared with wild-type and complemented strains. Apo, aposymbiotic or noninfected juvenile squids. Data are plotted as the mean $OD_{562\ nm} \pm SD$. Multiple comparisons were calculated between groups using the Tukey *post hoc* comparison. Different letters indicate significant differences ($P < 0.05$) between groups. According to these data, *pilU*$^-$, *pilT*$^-$, *ibpA*$^-$ and *mifB*$^-$ are labeled 'b', which indicates that are significantly different from the wild-type (labeled 'a'); *flgF*$^-$ and *motY*$^-$ (labeled 'c') are also different from the wild-type ('a') and from those labeled 'b'. Ten squid were tested per strain.

consistent with those observed in the microtiter plate biofilm assay.

Pil mutants were significantly impaired in colonizing axenic juvenile squids (Fig. 5). These mutants are defined as accommodation mutants, which do not colonize juvenile squid hosts to the same levels as their wild-type congener (Nyholm & McFall-Ngai, 2004). In addition, a microchemostat system was assembled to assess the capacity of various wild-type and mutant strains to form biofilms under a dynamic environment. The tested strains were supplied with a constant carbon source in order to examine growth of the biofilm in real time over 18 h of incubation. Analysis of the samples by confocal microscopy revealed that biofilms from wild-type *V. fischeri* ETJB1H consisted of dense layers of aggregates of cells (Fig. 6a), whereas biofilms formed from all mutants consisted in non-dense and isolated aggregates (Fig. 6b–f). Differences in community formation were observed in mutants for the *pil* locus when compared with SEM observations; *pil* mutants did not form biofilms in the chemostat system (Fig. 6b, c). All other mutants had similar biofilm formation that was consistent with SEM observations. These contrasting observations suggest that biofilm formation is sensitive to hydrodynamic environments, and twitching motility may be important to overcome the multiple barriers that the bacterium encounters before reaching the host light organ (i.e. ducts and ciliated appendages) in order to form a bacterial community. Twitching motility was assayed and defects were noted in both mutants when compared with the wild-type. In addition, previous observations by transmission electron microscopy indicate that these mutants are hyperpilated, similar to earlier results in *P. aeruginosa* (Bertrand *et al.*, 2010; results not shown). This phenotype was attributed to a defect in depolymerization of the pilin proteins during pilus retraction. The hyperpiliated phenotype in mutated *V. fischeri* may have enhanced bacterial adhesion, leading to an increase in community formation observed in our microtiter plate assay. However, it appears that pilus retraction is essential for colonization and biofilm formation under dynamic conditions.

IbpA

IbpA is a heat shock protein that is synthesized from an operon controlled by the σ^{54} subunit of the RNA polymerase, and induced under heat and other stress conditions (Kuczynska-Wisnik *et al.*, 2010). *IbpA* has been detected among stress-response genes that are overexpressed in biofilm populations; however, little is known about its function during biofilm formation. Recent results in *E. coli* demonstrate that lack of IbpAB proteins inhibit formation of biofilm at the air–liquid interface. In the absence of these proteins, cells experience oxidative stress and overproduce extracellular indole (Kuczynska-Wisnik *et al.*, 2010), which is known to be a transcriptional regulator of many genes, including those involved in polysaccharide production (the biofilm matrix) and the quorum sensing cascade (bacterial communication, implicated in community formation). To test differences in indole production, we performed an assay that measures production of indole over time and its effect on biofilm formation. The mutant *ibpA*$^-$ produced significantly more indole when compared with wild-type and complemented strains after 5 h of incubation (Fig. 7a); however, indole significantly reduced biofilm formation (Fig. 7b). IbpA is classified as a small heat shock protein (Kuczynska-Wisnik *et al.*, 2010) and few studies have elucidated the importance of small heat shock proteins (sHsps) in host infection. Deficiencies in biofilm production were more apparent in regulatory mutants (*ibpA*$^-$, *mifB*$^-$), which appeared less complex and dense (Figs 2 and 3e, e1, f, f1). Our findings demonstrate that the *ibpA*$^-$ mutant does not infect the host squid as efficiently as the wild-type (Fig. 5), suggesting that IbpA may also be necessary for colonization of host squid tissues; however, we cannot determine the specific role of this particular protein in colonization. Future studies will examine the specific role

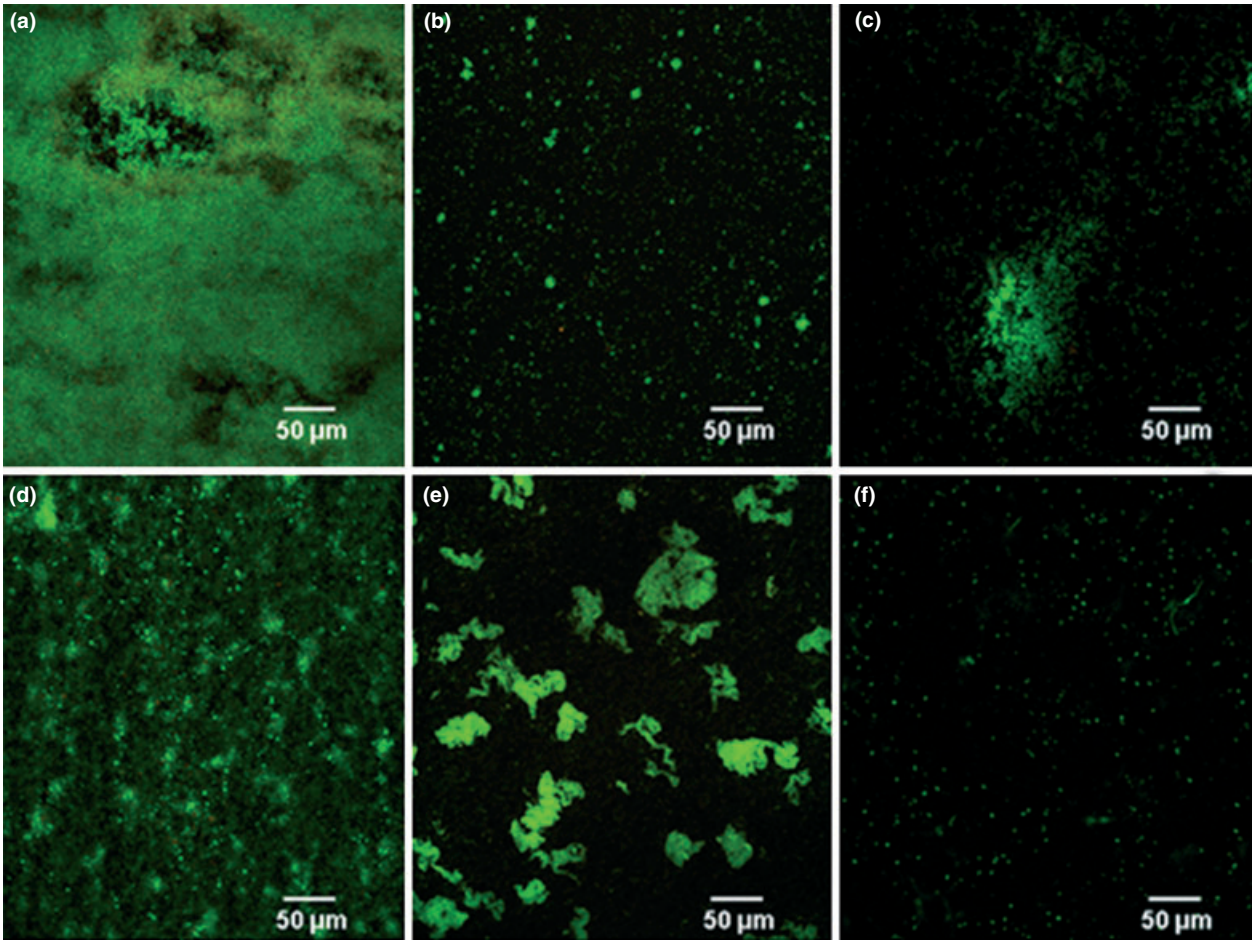


Fig. 6. Confocal scanning laser microscopyof different *Vibrio fischeri* strains. (a) Wild-type ETJB1H (thickness 19.35 ± 3.30 μm), (b) *pilU*$^{-}$ (thickness 3.45 ± 0.18 μm), (c) *pilT*$^{-}$ (thickness 3.66 ± 0.12 μm), (d) *motY*$^{-}$ (thickness 4.84 ± 0.59 μm), (e) *flgF*$^{-}$ (thickness 2.14 ± 0.67 μm), (f) *ibpA*$^{-}$ (thickness 0.93 ± 0.27 μm). Strain *mifB*$^{-}$ is not shown as it looks the same as *ibpA*$^{-}$ (thickness 0.88 ± 0.42 μm). Scale bar = 50 μm at 10× magnification. Mean thickness was calculated from five different image stacks (or 'z' stack).

of IbpA in adhesion and colonization of the squid light organ.

MifB

The second regulatory gene analysed in this study was *mifB*, which is responsible for synthesis of c-di-GMP (Wolfe & Visick, 2008). MifB is a small molecule that acts as a second messenger and regulates many distinct processes in bacteria, including synthesis of virulence factors and cellulose production (Cotter & Stibitz, 2007). Our findings indicate that *mifB*$^{-}$ mutants are neither able to produce biofilms nor impaired in infecting juvenile squid hosts (Figs 2 and 5), which corroborates earlier studies examining *mifB* function related to bioluminescence, motility and colonization (O'Shea *et al.*, 2005, 2006; Visick *et al.*, 2007). The lack of biofilm formation in these mutants may be due to the absence of exopolysaccharide production in this mutant. A recent study determined that c-di-GMP in *Vibrio vulnificus* regulates extracellular polysaccharide production, which is an important component of the biofilm matrix (Nahamchik *et al.*, 2008). Similar to other symbiotic associations, c-di-GMP is proposed to have an important role in regulating changes in gene expression in *V. cholerae* during host infection, specifically regulating transcription of numerous virulence genes (Tamayo *et al.*, 2008). A proposed model suggests that *mifB* catalyses the production of the c-di-GMP pathway to inhibit flagellar synthesis (Visick *et al.*, 2007). Mutation of this gene affects migration, which is directly related to disruption of biofilm formation. Additionally, Visick *et al.* (2007) reported that mutants lacking the *mif* genes are able to synthesize flagella in the presence of abundant Mg^{2+} (present in seawater). This phenomenon may be related to dependent induction of biofilms, and future studies will address the effect of seawater components (such as Mg^{2+} concentration) on biofilm formation.

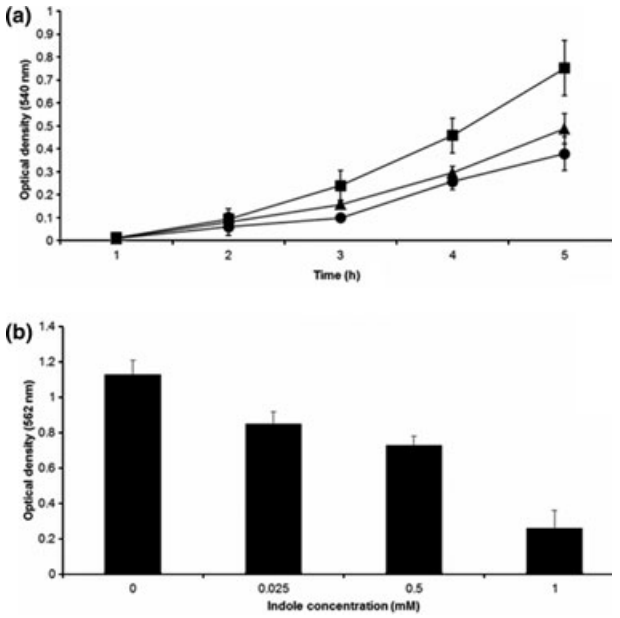


Fig. 7. Indole assays. (a) Indole production test; squares represent the mean for *ibpA*$^-$, triangles represent the mean for the wild-type, and circles represent the mean for the complement (*ibpA*$^+$) strain. Indole production was calculated in triplicate (three different clones from the same strain). Production of indole from the *ibpA*$^-$ strain was significantly different from the other two strains. (b) Effect of indole on formation of biofilms formed from the ETJB1H wild-type strain. Different concentrations of indole were added and biofilm mass was reduced significantly when concentration increased twofold or higher.

Biofilm formation appears to be under regulation of multiple genes. This study complements previous investigations that describe the roles of numerous genes in community biofilm formation in the *Vibrio–Euprymna* association. Other studies have focused on the *syp* operon and its regulation (Yip *et al.*, 2005; Morris & Visick, 2010; Morris *et al.*, 2011) and the role of isolated genes (not part of an operon) such as *mif* (O'Shea *et al.*, 2006), *mshA* and UDPH (Ariyakumar & Nishiguchi, 2009). Elucidation of the genetic mechanisms studied here provides another avenue for understanding the control of biofilm formation and consequently host colonization.

Conclusions

This study focused on deciphering the importance of several structural and regulatory genes in biofilm formation and host colonization. Results from our experiments indicate that all genes in our study are involved in the formation of mature biofilms, which is also important for the successful establishment and persistence of the mutualism between *V. fischeri* and *E. tasmanica*. Furthermore, we demonstrated that there is a difference in biofilm formation in static cultures and hydrodynamic environments when some of the structural genes are mutated (*pilU*, *pilT*), suggesting that there are special requirements for initial attachment prior to biofilm formation (twitching motility). Further research will focus on the regulatory mechanisms of these genes and other various pathways that control biofilm formation and host colonization in order to interpret the mechanisms of symbiotic associations.

Acknowledgements

We thank Drs P. Cooke and E. Stabb for their advice in the experimental design. A.C.-D. was supported by RISE (NIH NIGMS R25GM061222) and NASA (NMSGC). M. K.N. was supported by NSF IOS-0744498, NIH NIAID 1SC1AI081659-01 and NIH ARRA-3SC1AI081659–02S1. The authors have no conflicts of interests to declare.

References

Aldridge P & Hughes KT (2002) Regulation of flagellar assembly. *Curr Opin Microbiol* **5**: 160–165.

Ariyakumar DS & Nishiguchi MK (2009) Characterization of two host-specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPH) that are involved in the *Vibrio fischeri–Euprymna tasmanica* mutualism. *FEMS Microbiol Lett* **299**: 65–73.

Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan S, Blevins JS & Smeltzer MS (2004) Global gene expression in *Staphylococcus aureus* biofilms. *J Bacteriol* **186**: 4665–4684.

Bertrand JJ, West JT & Engel JN (2010) Genetic analysis of the regulation of type IV pilus function by the *chp* chemosensory system of *Pseudomonas aeruginosa*. *J Bacteriol* **192**: 994–1010.

Brenner SE (1999) Errors in genome annotation. *Trends Genet* **15**: 132–133. http://www.serialssolutions.com/images/AL_Button_big.gif.

Browne-Silva J & Nishiguchi MK (2008) Gene sequences of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int J Syst Evol Microbiol* **58**: 1292–1299.

Chavez-Dozal A & Nishiguchi MK (2011) Effect of environmental factors on biofilm formation by *Vibrio fischeri* isolates. *J Basic Microbiol* **5**: 452–458.

Cotter PA & Stibitz S (2007) c-di-GMP-mediated regulation of virulence and biofilm formation. *Curr Opin Microbiol* **10**: 17–23.

Dunn AK, Martin MO & Stabb EV (2005) Characterization of pES213, a small mobilizable plasmid from *Vibrio fischeri*. *Plasmid* **54**: 114–134.

Eko Niba ET, Naka Y, Nagase M, Mori H & Kitakawa M (2007) A genome-wide approach to identify the genes involved in biofilm formation in *E. coli*. *DNA Res* **14**: 237–246.

Firoved AM & Deretic V (2003) Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* **185**: 1071–1081.

Giron JA, Torres AG, Freer E & Kaper J (2002) The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol Microbiol* **44**: 361–379.

Greiner LL, Edwards JL, Shao J, Rabinak C, Entz D & Apicella MA (2005) Biofilm formation by *Neisseria gonorrhoeae*. *Appl Environ Microbiol* **73**: 1964–1970.

Hossain MM & Tsuyumu S (2006) Flagella-mediated motility is required for biofilm formation by *Erwinia carotovora subsp carotovora*. *J Gen Plant Pathol* **72**: 34–39.

Houry A, Briandet R, Ayerich S & Gohar M (2010) Involvement of motility and flagella in *Bacillus cereus* biofilm formation. *Microbiology* **156**: 1009–1018.

Hoyle BD & Costerton WJ (1991) Bacterial resistance to antibiotics: the role of biofilms. *Prog Drug Res* **37**: 91–105.

Jones BW & Nishiguchi MK (2004) Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Mar Biol* **144**: 1151–1155.

Jones BW, Lopez JE, Huttenberg J & Nishiguchi MK (2006) Population structure between environmentally transmitted *Vibrios* and bobtail squids using nested clade analysis. *Mol Ecol* **15**: 4317–4329.

Kievit TR (2009) Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* **11**: 279–288.

Kuczynska-Wisnik D, Matuszewska E & Lakowska E (2010) *Escherichia coli* proteins IbpA and IbpB affect biofilm formation by influencing the level of extracellular indole. *Microbiology* **156**: 148–157.

Lee J, Zhang XS, Hegde M, Bentley WE, Jayaraman A & Wood TK (2008) Indole cell signaling occurs primarily at low temperatures in *Escherichia coli*. *ISME J* **2**: 1007–1023.

Liu R & Ochman H (2007) Origins of flagellar gene operons and secondary flagellar systems. *J Bacteriol* **189**: 798–70104.

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL & Ruby EG (2009) A single regulatory gene is sufficient to alter bacterial host range. *Nature* **458**: 215–218.

Merino S, Shaw JG & Tomas JM (2006) Bacterial flagella: an inducible flagella system. *FEMS Microbiol Lett* **263**: 127–135.

Merritt JH, Kadouri DE & O'Toole GA (2005) Growing and analyzing static biofilms. *Curr Protoc Microbiol* 1B.1.1-1.1.17.

Millikan DS & Ruby EG (2002) Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Appl Environ Microbiol* **68**: 2519–2528.

Millikan DS & Ruby EG (2003) FlrA, a σ^{54}-dependent transcriptional activator in *Vibrio fischeri*, is required for motility and symbiotic light-organ colonization. *J Microbiol* **185**: 3547–3557.

Millikan DS & Ruby EG (2004) Flagellin A is essential for normal motility and for symbiotic competence during initial squid light organ colonization. *J Microbiol* **186**: 4315–4325.

Moorthy S & Watnick PI (2004) Genetic evidence that the *Vibrio cholerae* monolayer is a distinct stage in biofilm development. *Mol Microbiol* **52**: 573–587.

Morris AR & Visick KL (2010) Control of biofilm formation and colonization in *Vibrio fischeri:* a role for partner switching? *Environ Microbiol* **12**: 2051–2059.

Morris AR, Darnell CL & Visick KL (2011) Inactivation of a novel response regulator is necessary for biofilm formation and host colonization by *Vibrio fischeri*. *Mol Microbiol* **82**: 114–130.

Nahamchik A, Wild C & Rowe-Magnus DA (2008) Cyclic-di-GMP regulates extracellular polysaccharide production, biofilm formation, and rugose colony development by *Vibrio vulnificus*. *Appl Enivron Microbiol* **74**: 4199–4209.

Nair VN & Nishiguchi MK (2009) Biological properties (*in vitro*) exhibited by free-living and symbiotic *Vibrio* isolates. *Vie Milieu Paris* **59**: 277–285.

Nishiguchi MK (2002) Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. *Microb Ecol* **44**: 10–18.

Nishiguchi MK, Lopez JE & Boletzky SV (2004) Enlightenment of old ideas from new investigations: the evolution of bacteriogenic light organs in squids. *Evol Dev* **6**: 41–49.

Nyholm SV & McFall-Ngai MJ (2004) The winnowing: establishing the squid-*Vibrio* symbiosis. *Nature* **2**: 632–642.

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA & McFall-Ngai MJ (2002) Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* **68**: 5113–5122.

O'Shea TM, DeLoney-Marion CR, Shibata S, Aizawa S-I, Wolfe AJ & Visick KL (2005) Magnesium promotes flagellation of *Vibrio fischeri*. *J Bacteriol* **187**: 2058–2065.

O'Shea TM, Klein AH, Geszvain K, Wolfe AJ & Visick KL (2006) Diguanylate cyclases control magnesium-dependent motility of *Vibrio fischeri*. *J Bacteriol* **188**: 8196–8205.

Rashid MH & Kornberg A (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *P Nat Acad Sci USA* **97**: 4885–4890.

Ruby EG (1999) The *Euprymna scolopes–Vibrio fischeri* symbiosis: a biomedical model for the study of bacterial colonization of animal tissue. *J Mol Microbiol Biotechnol* **1**: 13–21.

Saijo-Hamano Y, Minamino T, Macnab RM & Namba K (2004) Structural and functional analysis of the C-terminal cytoplasmic domain of FlhA, an integral membrane component of the type III flagellar protein export apparatus in *Salmonella*. *Mol Biol* **343**: 457–466.

Soto W, Gutierrez J, Remmenga MR & Nishiguchi MK (2009) Salinity and temperature effects on the physiological responses of *Vibrio fischeri* from diverse ecological niches. *Microb Ecol* **57**: 140–150.

Stabb EV & Ruby EG (2002) New RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. *Methods Enzymol* **358**: 413–426.

Stabb EV & Ruby EG (2003) Contribution of *pilA* to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. *Appl Environ Microbiol* **69**: 820–826.

Tamayo R, Schild S, Pratt JT & Camilli A (2008) Role of cyclic di-GMP during El Tor biotype *Vibrio cholerae* infection. Characterization of the *in vivo*-induced cyclic di-GMP phosphodiesterase CdpA. *Infect Immun* **76**: 1617–1627.

Visick KL & Ruby EG (2006) *Vibrio fischeri* and its host: it takes two to tango. *Curr Opin Microbiol* **9**: 1–7.

Visick KL, O'Shea TM, Klein AH, Geszvain K & Wolfe AJ (2007) The sugar phosphotransferase system of *Vibrio fischeri* inhibits both motility and bioluminescence. *J Bacteriol* **189**: 2571–2574.

Withchurch CB & Mattick JS (1994) Characterization of a gene, *pilU*, required for twitching motility but not phage sensibility in *Pseudomonas aeruginosa*. *Mol Microbiol* **13**: 1079–1091.

Wolfe AJ & Visick KL (2008) Get the message out: cyclic-Di-GMP regulates multiple levels of flagellum-based motility. *J Bacteriol* **190**: 463–475.

Wolfe AJ, Chang DE, Walker JD, Seitz-Partridge JE, Vidaurri MD, Lange CF, Prüss BM, Henk MC, Larkin JC & Conway T (2003) Evidence that acetyl-phosphate functions as a global signal during biofilm development. *Mol Microbiol* **48**: 977–988.

Yildiz FH & Visick KL (2009) *Vibrio* biofilms: so much the same yet so different. *Trends Microbiol* **17**: 109–118.

Yip ES, Grublesky BT, Hussa EA & Visick KL (2005) A novel, conserved cluster of genes promotes symbiotic colonization and σ^{54}-dependent biofilm formation by *Vibrio fischeri*. *Mol Microbiol* **57**: 1485–1498.

Yoshida A & Kuramitsu K (2002) Multiple *Streptococcus mutans* genes are involved in biofilm formation. *Appl Environ Microbiol* **68**: 6283–6291.

Zolfaghar I, Evans DJ & Fleiszing SMJ (2003) Twitching motility contributes to the role of pili in corneal infection caused by *Pseudomonas aeruginosa*. *Infect Immun* **71**: 5389–5393.

Microb Ecol (2013) 65:214–226
DOI 10.1007/s00248-012-0099-6

HOST MICROBE INTERACTIONS

Characterization of the Bacterial Diversity in Indo-West Pacific Loliginid and Sepiolid Squid Light Organs

Ricardo Guerrero-Ferreira · Clayton Gorman · Alba A. Chavez · Shantell Willie · Michele K. Nishiguchi

Received: 7 February 2012 / Accepted: 7 April 2012 / Published online: 12 August 2012

Abstract Loliginid and sepiolid squid light organs are known to host a variety of bacterial species from the family Vibrionaceae, yet little is known about the species diversity and characteristics among different host squids. Here we present a broad-ranging molecular and physiological analysis of the bacteria colonizing light organs in loliginid and sepiolid squids from various field locations of the Indo-West Pacific (Australia and Thailand). Our PCR-RFLP analysis, physiological characterization, carbon utilization profiling, and electron microscopy data indicate that loliginid squid in the Indo-West Pacific carry a consortium of bacterial species from the families Vibrionaceae and Photobacteriaceae. This research also confirms our previous report of the presence of *Vibrio harveyi* as a member of the bacterial population colonizing light organs in loliginid squid. *pyrH* sequence data were used to confirm isolate identity, and indicates that *Vibrio* and *Photobacterium* comprise most of the light organ colonizers of squids from Australia, confirming previous reports for Australian loliginid and sepiolid squids. In addition, combined phylogenetic analysis of PCR-RFLP and 16S rDNA data from Australian and Thai isolates associated both *Photobacterium* and *Vibrio* clades with both loliginid and sepiolid strains, providing support that geographical origin does not correlate with their relatedness. These results indicate that both loliginid and sepiolid squids demonstrate symbiont specificity (Vibrionaceae), but their distribution is more likely due to environmental factors that are present during the infection process. This study adds significantly to the growing evidence for complex and dynamic associations in nature and highlights the importance of exploring symbiotic relationships in which non-virulent strains of pathogenic *Vibrio* species could establish associations with marine invertebrates.

C. Gorman · A. A. Chavez · S. Willie · M. K. Nishiguchi (✉)
Department of Biology, New Mexico State University,
Las Cruces, NM 88003-8001, USA
e-mail: nish@nmsu.edu

R. Guerrero-Ferreira
Department of Pediatrics, Division of Pediatric Infectious Diseases, Emory University School of Medicine,
Atlanta, GA 30322, USA

Introduction

The family Vibrionaceae (gamma-proteobacteria) is a highly diverse group containing both symbiotic and free-living species [1]. Vibrionaceae is comprised of seven main genera, including *Vibrio*, *Listonella*, *Photobacterium*, *Enterovibrio*, *Aliivibrio*, *Grimontia*, and *Salinivibrio* [2], although recent debates question the overall systematic classification [3]. Vibrios are highly abundant in aquatic environments, where they actively participate in the re-cycling of nutrients and detritus [4]. In addition, a number of luminescent symbionts play a key role in antipredatory behaviors documented in a number of marine organisms [5–7].

Members of the family Vibrionaceae have been frequently detected and isolated from freshwater, estuarine, and marine habitats [8, 9]. Several species such as *Vibrio fischeri* [10, 11], *Vibrio logei* [7], *Vibrio harveyi* [12, 13], and *Photobacterium leiognathi* [14] play important ecological roles because of their life history strategies, including both mutualistic associations with marine organisms and free-living planktonic lifestyles. Moreover, the genus *Vibrio* encompasses several pathogens of humans (e.g., *Vibrio cholerae* [15, 16], *Vibrio parahaemolyticus* [17–22], and *Vibrio vulnificus* [23, 24]) as well as other eukaryotic organisms. Some of these pathogens are known to attach to surfaces of live marine animals without causing disease to their invertebrate host. Examples of such associations include *V. cholerae* and its copepod host, which constitutes an important factor in the epidemiology of cholera disease [16], as well as *V. harveyi*, which causes disease in marine animals, producing mass mortalities in shrimp farms around the

world (luminous vibriosis [25]) and also infecting pearl oysters, fish, seahorses, and lobsters [26]. However, *V. harveyi* is also found mutualistically with the hydrozoan *Aglaophenia octodonta* [13] and fish light organs [27]. Similarly to *V. cholerae*, these non-pathogenic associations are likely to play a role in the epidemiology of vibriosis by *V. harveyi*.

Understanding bacterial diversity in natural environments is of pivotal importance because this information provides a phylogenetic framework to clarify the degree of variation among species in a particular environment. Similarly, characterizing microbial populations is also essential to helping define the structure and diversity of a particular community of microorganisms [28, 29]. Because of the large fraction of non-culturable microbes in nature, establishing these parameters via conventional culture-dependent, physiology-based methods has serious limitations [30]. However, complementing culture-based with molecular methods is an excellent approach to elucidate the nature of bacterial communities, without acquiring the large costs affiliated with wide-scale, genomic-based approaches.

In the specific case of squid symbionts, light organ homogenate, spread-plate cultures yield a great number of luminescent colonies very similar to each other in shape, color, size, and texture [12], which makes them difficult to identify without a combination of microbiological and genetic approaches. Recent studies by our group that explore the diversity of bacterial isolates colonizing light organs of loliginid squids in Thailand have provided evidence that colonization is achieved by multiple species of *Vibrio* [12, 31], including *V. harveyi*, constituting the first report of a marine pathogen in a molluscan mutualism. However, further studies were not implemented to determine the physiological characteristics of these isolates. Here, we report the physiological characterization of Thailand isolates and the results of comparative studies of 16S ribosomal RNA genes using polymerase chain reaction (PCR) in combination with restriction fragment length polymorphism (RFLP). To better characterize variation among loliginid symbionts, we used PCR-RFLP of the 16S rRNA locus to type and identify marine Vibrios associated with light organs of squids in the family Loliginidae (Mollusca: Cephalopoda) from Australian and Thai locations. This method has proven to be time and cost efficient, and is increasingly used as a standard technique to address questions regarding the ecology, distribution, and biodiversity of natural isolates of bacteria [32–38]. Additionally, a battery of microbiological assays were completed in parallel to type and identify isolates through culture based tests including Gram stain, light production (luminescence), growth on thiosulfate/citrate/bile salts (TCBS) agar, and growth on seawater tryptone (SWT) agar at various temperatures in addition to phenotypes of each isolate through electron microscopy.

Materials and Methods

Bacterial Strains, Growth Conditions, and DNA Extraction

Bacterial strains used in this study are listed in Table 1. To isolate bacteria from squid light organs, ten specimens from each location were captured by trawl netting for dissection and their light organs removed and homogenized in sterile seawater [39]. Collection sites in the Indo-West Pacific (a zoogeographical region including the Indian and Pacific oceans) are indicated in Table 1. Serial tenfold dilutions (1/10,000) of the homogenate were plated on seawater tryptone agar (SWT; 70 % seawater *v*/*v*, 0.5 % tryptone *w*/*v*, 0.3 % yeast extract *w*/*v*, 0.3 % glycerol *v*/*v* and 15 % technical grade agar) and grown at 28 °C for 16 h. Individual colonies of luminous bacteria were isolated and used to inoculate 5 mL of SWT broth and incubated for 18 h at 250 revolutions per minute (rpm). An aliquot (900 μL) of the resulting culture was combined with the same volume of 40 % glycerol to be stored at −80 °C for further studies.

Total 16S rRNA Gene Amplification and Sequencing from Bacterial Isolates

Isolates were recovered from glycerol stocks by growing them overnight on SWT agar at 28 ºC. An individual colony was recovered from each plate and inoculated in 5 mL of SWT broth and incubated overnight on a shaking incubator (250 rpm) at 28 ºC. Genomic DNA was isolated from these liquid cultures using the DNAeasy Isolation Kit (Qiagen®, Valencia, CA). Concentration and purity of genomic DNA was estimated with a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). DNA integrity was validated by 1 % agarose gel electrophoresis in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0).

16S rRNA amplification and sequencing was completed using universal primers 16SF (5′-GCAAGCCTGATG CAGCCATG-3′) and 16SR (5′-ATCGTTTACGGCGTG GACTA-3′) at a 0.2 mM concentration per reaction. PCR and sequencing reactions were completed in a DNA peltier thermal cycler (MJ Research, Inc., Watertown, MA). Amplification reactions were executed using 0.05 U/μL of Amplitaq Gold (Applied Biosystems, Foster City, CA) and consisted of an initial hot start at 94 °C for 2 min followed by 29 cycles of: 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. After cycling, the process was terminated at 72 °C for 7 min. Each PCR reaction mix also contained 2.5 mM of $MgCl_2$, 0.5 mM dNTPs (25 μM each, Promega, Madison, WI) and 0.05 U/μL of Taq DNA Polymerase (Promega, Madison, WI), and 10× reaction buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, and 0.1 % Triton X-100). PCR reactions yielded a gene product of about 1,500 bp when analyzed through gel electrophoresis.

Table 1 Environmental and laboratory isolates used in this study

Strain Name	Squid host or source	Location
Group A	*Uroteuthis chinensis*	Cairns, QLD, Australia
Group B	*Uroteuthis etheriogei*	Townsville, QLD,Australia
Group C	*Photololigo noctiluca*	Sydney, NSW,Australia
Vibrio fischeri CG101	*Cleidopus gloriamaris*	Townsville, QLD,Australia
Vibrio fischeri ET101	*Euprymna tasmanica*	Crib Point, VIC,Australia
Vibrio fischeri ETJB	*Euprymna tasmanica*	Jervis Bay, NSW, Australia
Vibrio fischeri ES915	*Euprymna scolopes*	Paiko, O'ahu, Hawaii, USA
Vibrio fischeri MJ101	*Monocentris japonica*	Tokyo, Japan
Vibrio fischeri SL518	*Sepiola ligulata*	Banyuls-sur-mer, France
Vibrio fischeri SR5	*Sepiola robusta*	Banyuls-sur-mer, France
Vibrio fischeri WH1	Free-living	Woods Hole, MA
Vibrio fischeri VLS2	*Euprymna scolopes*	Kaneohe Bay, O'ahu, Hawaii, USA
Vibrio fischeri ES191	*Euprymna scolopes*	Paiko, O'ahu, Hawaii, USA
Photobacterium phosphoreum	Laboratory strain	ATCC 11004
Photobacterium leiognathi	Laboratory strain	ATCC 25521
Photobacterium leiognathi RM1	*Rondeletiola minor*	Banyuls-sur-mer, France
Photobacterium leiognathi LN101	*Uroteuthis noctiluca*	Sydney, NSW, Australia
Vibrio fischeri PP3	Free-living	Kaneohe Bay, O'ahu, Hawaii, USA
Vibrio fischeri PP42	Free-living	Kaneohe Bay, O'ahu, Hawaii, USA
Vibrio anguillarum	Laboratory strain	ATCC 19264
EHP group	*Euprymna hyllebergi*	Phuket, Thailand
UCP group	*Uroteuthis chinensis*	Phuket, Thailand
UCR group	*Uroteuthis chinensis*	Rayong, Thailand
UDP group	*Uroteuthis duvauceli*	Phuket, Thailand

QLD Queensland, *NSW* New South Wales, *VIC* Victoria

16S rDNA amplicons were purified from primers and unincorporated nucleotides using the GeneClean® II DNA purification kit (Bio 101, Carlsbad, CA) and used for subsequent applications. Sequencing reactions were executed by the dideoxy chain termination method using the Big Dye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were obtained through the ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA) and edited using Sequencher v4.6 (Gene Codes Corporation, Ann Arbor, MI). DNA sequences were then compared with the National Center for Biotechnology Information (NCBI) database using BLAST 2.2.11 (Basic Local Alignment Search Tool, NCBI, NLM, NIH, Bethesda, MD) for initial identification of bacterial isolates.

PCR Amplification and Sequencing of Partial 16S rRNA Gene and Uridilate Kinase Gene (*Pyr*h) for Species Identification

Amplification of partial 16S rRNA gene was completed using primers 16S2F (5′-GCAAGCCTGATGCAGCCATG-3′) and 16S3R (5′-ATCGTTTACGGCGTGGACTA-3′) in a DNA thermal cycler (MJ Research, Inc., Watertown, MA). PCR conditions were the following for both genes: hot start at 94 °C for 2 min, followed by 25 cycles of: 94 °C for 2 min, 45 °C for 1.5 min and 72 °C for 2 min. A final termination step at 72 °C for 8 min completed the process. PCR component concentrations were the same as previously stated.

16S rRNA and *pyr*H amplicons were purified and sequenced as mentioned above. DNA sequences were then compared with the NCBI database using BLAST 2.2.11 for initial confirmation of sequence identity. Upon confirmation, partial 16S rRNA gene sequences were incorporated in the combined phylogenetic analysis described below.

Restriction Fragment Length Polymorphism (RFLP) Analysis

RFLP analysis was completed as described by Urakawa et al. [33, 35] using three restriction endonucleases: *Rsa*I (5′ GTAC3′), *Hha*I (5′GCGC3′) and *Dde*I (5′CTNAG3′; Promega Corporation, Madison, WI). Fragments were separated through gel electrophoresis at 2 V/cm in a 1.5 % agarose gel in 0.5× TAE buffer (20 mM Tris acetate, and 0.5 mM EDTA). The BenchTop 1 Kb Ladder was used as a molecular DNA marker (Promega Corporation, Madison, WI). Image analysis and estimation of fragment size were completed with the Kodak Molecular Imaging software v5.0 (Carestream Health Inc., Rochester,

NY). Informative electrophoresis bands derived from restriction endonucleases digestion were scored for presence or absence and entered into a migration distance matrix to determine specific banding patterns for each enzyme. PCR-RFLP restriction pattern, presence–absence matrix, and 16S rRNA gene sequence data were analyzed using the direct optimization method described by Wheeler [40, 41] and implemented in the computer program POY [42, 43]. Previously sequenced 16S rRNA gene sequences were retrieved from GenBank.

Phenotypic Characterization of Bacterial Isolates

Bacterial squid isolates were phenotypically identified following the schemes of Alsina and Blanch [44] and Farmer et al. [2]. Physiological and morphological tests were completed and are listed in Table 2.

Observation of Bacterial Cellular Morphology

Isolates from individual bacterial colonies were used to inoculate 5 mL of SWT media and grown overnight at 28 °C in a shaking incubator (250 rpm). Bacterial samples were prepared using a technique modified from Allen and Baumann [45] for examination of cell appendages by transmission electron microscopy. Briefly, 5 μL of culture was added onto a 200-mesh Formvar coated nickel grid (Electron Microscopy Sciences, Hartfield, PA) and allowed to sit for 10 s. Excess culture media was blotted dry with filter paper. This was followed by the addition and removal of 5 μL of distilled water, which provided the initial wash. Staining was completed with a 1 % aqueous solution of uranyl acetate for 10 s. Excess stain was removed and the grid was allowed to air dry. Conversely, cells grown in solid media were harvested by addition of 5 μL of sterile seawater directly on solid agar media, and immediately homogenized by slow pipetting. Five microliters of the homogenate was collected and processed as mentioned previously. Micrographs were obtained using a Hitachi H-7650 (Hitachi High Technologies America, Pleasanton, CA) transmission electron microscope (TEM) at an accelerating voltage of 80 kV.

Accession Numbers

The 16S rRNA and *pyr*H gene sequences determined in this study were deposited in GenBank and are listed in Tables 3 and 4.

Results and Discussion

Phenotypic Characterization of Thailand Squid Bacterial Isolates

A number of isolates from Thailand loliginid squids had been previously identified as members of the genus *Vibrio* on the basis of their 16S rRNA gene sequence [12] (Table 3). However, these isolates exhibited sequence similarities of 98 % or higher to the 16S rRNA gene sequence of *Vibrio alginolyticus* [46], *V. harveyi* [46], and *Vibrio charchariae* (synonym of *V. harveyi*) [46]. This high percent of sequence similarity did not allow for the specific identification of these isolates solely using their 16S rRNA gene sequence.

We carried out additional tests for physiological, morphological, and biochemical characterization of Thai isolates (Table 1) which provided a more precise species identification. Results from these assays are shown in Table 2. The isolates surveyed belonged to a single species on the basis of their biochemical, physiological, and morphological analysis and were definitively identified as *V. harveyi.* Previous research by Dunlap et al. [27] provided similar evidence of the presence of *V. harveyi* in the light organ of the marine fish *Nuchequula nuchalis* (Perciformes: Leiognathidae) identified by *luxA* sequences. However, our research constitutes the first report confirming the presence of *V. harveyi* as a member of the bacterial population colonizing light organs in loliginid squid.

All Thailand isolates were found to be Gram-negative, luminescent rods, sensitive to the vibriostatic agent 0/129 (at both 10 and 150 μg). These isolates were unable to grow in liquid media without sodium chloride, and exhibited no growth at 4 °C in SWT. As shown in Table 2, the results are consistent with the characteristics of a laboratory strain incorporated in this analysis, as well as other *V. harveyi* laboratory [2] and natural [13] isolates. Similar results were also attained with the oxidase, catalase, Voges–Proskauer, indole, and gelatinase tests. Uniformity between isolates was also achieved in the output of carbon utilization profiles. When compared with other isolates of *V. harveyi*, Thailand strains were equally capable of utilizing L-arabinose, mannose, cellobiose, glucose, trehalose, melibiose, lactose, mannitol, sorbitol, and inositol as unique carbon sources (Table 2).

Interestingly, some differences were evident regarding the ability of individual isolates to produce the enzymes lysine and arginine decarboxylase when compared to a laboratory strain of *V. harveyi*, which is pathogenic in marine environments. Similar results have been reported for lysine decarboxylase in *V. harveyi*, where contradictory results were obtained in tests from the Centers for Disease Control and Prevention *Vibrio* reference lab using standardized enteric media supplemented with marine cations [2]. This may be due to the particular environmental niche each isolate has adapted to, despite being from the same species.

The presence of arginine decarboxylase (ADC) in strains isolated from loliginid squid light organs (Table 2) may be indirectly related with the formation of bacterial biofilms within squid tissues of the light organ, an important factor for successful colonization. ADC is responsible for catalytic

Table 2 Results of physiological and morphological assays of *Vibrio harveyi* squid isolates

Characteristic assay	Thailand bacterial isolates	*Vibrio harveyi*[a]	Results from *Stabili* et al. [13]	Results from Farmer III et al. [2]				
				Vibrio harveyi	*Vibrio fischeri*	*Vibrio alginolyticus*	*Vibrio campbelli*	*Vibrio damsela*
Gram reaction	–	–	–	–	–	–	–	–
Cell morphology	r	r	r	r	r	r	r	r
Luminescence	+	+	+	d	+	–	–	–
0/129 sensitivity								
10 μg	+	+	+	d	+	d	nd	+
150 μg	+	+	+	+	+	d	nd	+
Growth in 0 % NaCl	–	–	–	–	–	–	–	–
Growth in 3 % NaCl	+	+	+	+	+	+	+	+
Growth in 8 % NaCl	+	+	+	+	–	+	+	–
Growth at 4 °C	–	–	–	–	–	–	–	nd
Growth at 30 °C	+	+	+	+	+	+	+	+
Growth at 35 °C	+	+	+	+	d	+	+	+
Oxidation/ fermentation	F	F	F	F	nd	nd	nd	nd
Decarboxylase (NaCl)[b]								
Arginine	+	–	–	–	–	–	–	+
Lysine	–	–	+	+	+	+	+	+
Ornithine	+	+	+	+	–	–	–	–
Acid from Inositol	–	–	nd	nd	nd	nd	nd	nd
Acid from Arabinose	–	–	nd	nd	nd	nd	nd	nd
Acid from Sucrose	–	–	nd	nd	nd	nd	nd	nd
Oxidase	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+[c]	nd	nd	nd
Voges–Proskauer	–	–	–	–	–	+	–	+
Indole	+	+	+	+	–	+	+	–
Gelatinase	+	+	+	+	–	+	+	–
Lipase	–	–	+	+	+	+	+	–
Citrate	–	–	–	+	d	+	d	–
Carbon sources								
L-Arabinose	–	–	–	d	–	–	–	–
Mannose	+	+	+	+	+	d	d	+
Cellobiose	+	+	+	+	+	–	d	–
Glucose	+	+	+	nd	nd	nd	nd	nd
Galactose	d	+	–	d	+	d	–	nd
Trehalose	+	+	+	+	d	+	+	–
Melibiose	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	–
Mannitol	+	+	+	+	+	+	d	–
Sorbitol	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–
Sucrose	–	+	–	d	–	+	–	–

Table 2 (continued)

Characteristic assay	Thailand bacterial isolates	*Vibrio harveyi*[a]	Results from *Stabili* et al. [13]	Results from Farmer III et al. [2]				
				Vibrio harveyi	*Vibrio fischeri*	*Vibrio alginolyticus*	*Vibrio campbelli*	*Vibrio damsela*
Identification	*Vibrio harveyi*	*Vibrio harveyi*	*Vibrio harveyi*					

d diverse, *nd* no data, + positive reaction, – negative reaction, *F* fermentative, *r* rod shape

[a] *Vibrio harveyi* ATCC 14126

[b] (NaCl) indicates that NaCl was added to the standard media to enhance growth

[c] Visick and Ruby [63]

reactions occurring in alternative pathways for the synthesis of putrescine, a precursor of many polyamines [47]. In bacteria, they play a significant role in the formation of biofilms. Patel et al. [48] demonstrated that polyamines are responsible for the formation of biofilms by *Yersinia pestis*. Similarly, polyamines are also responsible for the modulation of bacterial biofilms within Vibrionaceae species. For example, Karatan et al. [49] reported that formation of biofilms by *V. cholerae* is activated by an increase in the environmental concentration of norspermidine, a polyamine. Most importantly, the gene for ADC was previously found to be expressed solely by symbiotic *V. fischeri* ETJB1A in the light organ of the sepiolid squid *Euprymna tasmanica* [50, 51]. This indicates that ADC expression is highly specific during growth and persistence of *V. fischeri* in the light organ, suggesting that this gene has an important role in establishing and maintaining the symbiosis. For instance, some *Vibrio* species also use ADC to regulate pH, which may be linked to the shift between aerobic and fermentative states while colonizing the sepiolid light organ [50]. The source of the *V. fischeri* strain in Farmer et al. [2] is not indicated in their study. However, the negative ADC result reported may indicate that it was a seawater isolate and not a symbiotic one. Furthermore, Guerrero-Ferreira and Nishiguchi [31] reported the expression of ADC gene by symbiotic *V. harveyi*, hypothesizing that environmental production of ADC to degrade ArgA (a molecule associated to *V. cholerae* pathogenesis [52]) may play a pivotal role in the transition of *V. harveyi* from a pathogenic to mutualistic state [53].

Phenotypic variation is not an isolated occurrence in *V. harveyi* strains. In a study by Vidgen et al. [26], differences were evident in phenotypic profiles of five *V. harveyi* strains (four seawater isolates and a pathogenic strain from a diseased prawn *Penaeus monodon*). Those differences were associated with the presence of a specific mobile genetic element, named *V. harveyi* myovirus-like bacteriophage (VHML), which caused the bacterium to elicit variable responses to several phenotypic tests [26]. Another example of this phenomenon is found in the bacterium *Aeromonas veronii*, a digestive tract symbiont of the medicinal leech *Hirudo medicinalis* [54, 55]. When tested for the presence of the enzyme arginine dehydrolase, isolates of *A. veronii* obtained from clinical sources (i.e., respiratory secretions, infected wounds, and stools) were negative [56]. Conversely, strains isolated in their symbiotic state (i.e., in the

Table 3 16S rRNA sequence information from Thailand *Vibrio harveyi* isolates from loliginid squid light organs

Species name	Squid host	Isolate name	Location	Accession number
Vibrio harveyi	*Uroteuthis chinensis*	UCP6	Phuket, Thailand	AY332404
Vibrio harveyi	*Uroteuthis chinensis*	UCP8	Phuket, Thailand	FJ227109
Vibrio harveyi	*Uroteuthis chinensis*	UCP9	Phuket, Thailand	FJ227110
Vibrio harveyi	*Uroteuthis chinensis*	UCP10	Phuket, Thailand	FJ227111
Vibrio harveyi	*Euprymna hyllebergi*	EHP6	Phuket, Thailand	FJ227112
Vibrio harveyi	*Euprymna hyllebergi*	EHP7	Phuket, Thailand	FJ227113
Vibrio harveyi	*Euprymna hyllebergi*	EHP8	Phuket, Thailand	FJ227114
Vibrio harveyi	*Euprymna hyllebergi*	EHP9	Phuket, Thailand	FJ227115
Vibrio harveyi	*Euprymna hyllebergi*	EHP10	Phuket, Thailand	FJ227116
Vibrio harveyi	*Euprymna hyllebergi*	EHP11	Phuket, Thailand	FJ227117
Vibrio harveyi	*Euprymna hyllebergi*	EHP12	Phuket, Thailand	FJ227118
Vibrio harveyi	*Euprymna hyllebergi*	EHP13	Phuket, Thailand	FJ227119

UCP6 16S rRNA sequence from Guerrero-Ferreira and Nishiguchi [12]

Table 4 Species identification of Australian isolates based on protein BLAST of *pyr*H gene sequences. Species names correspond to the highest score of significant alignment using BLAST

Isolate name	Squid host	Symbiont species identification	Accession number
A1-1	*Uroteuthis chinensis*	*Vibrio harveyi*	HQ226045
A1-5	*Uroteuthis chinensis*	*Photobacterium angustum*/*P. leioghnati* [64]	HQ226046
A1-6	*Uroteuthis chinensis*	*P. angustum*/*P. leioghnati*	HQ226047
A2-1	*Uroteuthis chinensis*	*V. harveyi*	HQ226048
B1-1	*Uroteuthis etheriogei*	*V. cyclitrophicus*	HQ226049
C1-1	*Photololigo noctiluca*	*P. angustum*/*P. leioghnati*	HQ226050
C2-7	*Photololigo noctiluca*	*V. cyclitrophicus*/*V. fischeri*	HQ226051
C3-5	*Photololigo noctiluca*	*P. angustum*/*P. leioghnati*	HQ226052
C4-23	*Photololigo noctiluca*	*V. harveyi*	HQ226053
C5-10	*Photololigo noctiluca*	*V. cyclitrophicus*	HQ226054

digestive tract of their leech host) were positive for arginine dehydrolase. Therefore, existing data suggests that within-species variation in arginine metabolism is common in members of the family Vibrionaceae. More interestingly, occurrence of this variation in other bacterial species may be niche related.

Morphology and flagellation patterns of each *Vibrio* symbiont were completed by negative staining TEM (representative micrographs in Fig. 1a, b). After extensive screening, it is evident that isolates appeared rod shaped and displayed a single polar flagellum when grown in liquid media (Fig. 1a). This type of flagellum is commonly observed in species of the genus *Vibrio* grown under these conditions, with the exception of *V. fischeri*, which exhibits lophotrichous flagella (two to eight polar flagella; [57]). Although flagellation pattern was not considered diagnostic for species identification, our microscopic survey confirms that the isolates are not *V. fischeri*. When grown on solid medium, peritrichous (lateral) flagella were observed in addition to the polar flagellum (Fig. 1b). Production of both polar and peritrichous flagella has previously been reported to occur in several species of *Vibrio* (e.g., *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus* [57]).

Amplification and Sequencing of Uridilate Kinase Gene (*pyrH*) for Characterization of Bacterial Consortia in Loliginid Squids

With the purpose of confirming the multi-specific nature of the population of bacteria colonizing loliginid light organs, ten isolates of luminescent strains representing several geographical areas off the coast of Australia were selected for sequencing the uridilate kinase gene (*pyr*H; Table 4). The use of this genetic marker for species identification within the Vibrionaceae family extends from bacterial pathogenesis studies to ecological analysis of both marine and freshwater environments [58–60]. Our results confirm that loliginid light organs are colonized by a luminescent bacterial consortium. This condition is at least common for the selected squid hosts examined in this study, including representatives

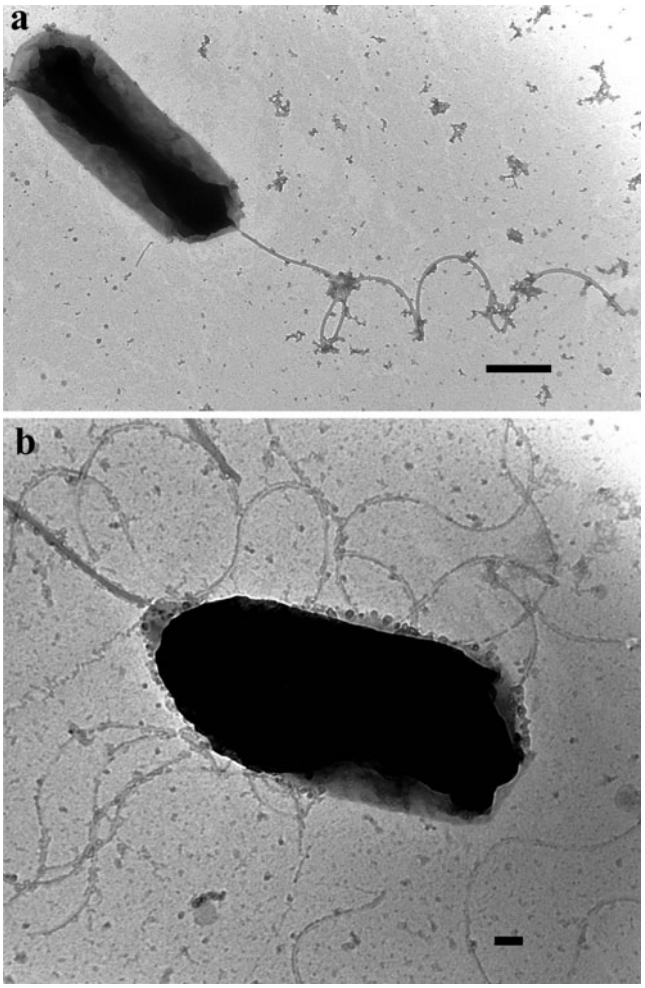


Figure 1 Transmission electron micrographs of *Vibrio harveyi* squid isolates grown in (**a**) seawater tryptone liquid media or (**b**) seawater tryptone agar. *Scale bar*=500 (**a**) and 100 nm (**b**)

of the genera *Uroteuthis* and *Photololigo*. Of the selected isolates, three were identified as *V. harveyi*, further indicating that this species exists in a mutualistic association with loliginid squids in Australia (Table 4).

Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (PCR/RFLP) Confirms Multi-Species Symbiosis in Squids from Thailand and Australia

In a complementary approach to further explore the diversity of bacterial species colonizing light organs in Thai and Australian squids, we analyzed 16S rRNA-PCR/RFLPs of 92 strains including natural isolates and laboratory strains. Amplification of the 16S rRNA gene resulted in a gene product of ~1,400 bp corresponding to the predicted size for this gene amplified under the conditions presented. After digestion with three restriction enzymes, a series of fragment patterns were obtained and are schematically summarized in Fig. 2. The number of restriction banding patterns obtained for each enzyme treatment was: 25 for *Dde*I, 36 for *Hha*I, and 38 for *Rsa*I. Fingerprints constructed with these restriction enzymes exhibited considerable variation when compared among environmental and laboratory isolates. These differences were confirmed by dendograms constructed using restriction patterns (band presence or absence) as input for the phylogenetic analysis (Fig. 3).

Analysis of strains isolated from groups A, B, and C (from Australia) indicates that bacteria colonizing loliginid light organs are represented by more than one species. Interestingly, RFLP patterns consistently grouped strains from *Uroteuthis etheriogei* (Group B) in clusters different than those of *V. fischeri* and *Photobacterium* species. *Hha*I and *Dde*I RFLP analyses resulted in grouping of *Photobacterium* isolates into their own clade (Fig. 3a, b). However, RFLP data from only *Hha*I restriction (independent of *Rsa*I and *Dde*I) generated a phylogeny in which the genus *Photobacterium* grouped independently from the other *Vibrio* isolates (Fig. 3b), specifically *V. fischeri*. These results are in accordance with a study by Urakawa et al. [34], where only *Hha*I RFLP analysis resulted in the separation of *Photobacterium* from *Vibrio* genera. Other enzymes tested in the aforementioned study did not produce RFLP data that separated these two genera into different operational taxonomic units (OTUs). In our study, the cladogram obtained from *Rsa*I restriction profiles neither engendered an apparent *Photobacterium* clade, nor put all *V. fischeri* strains as

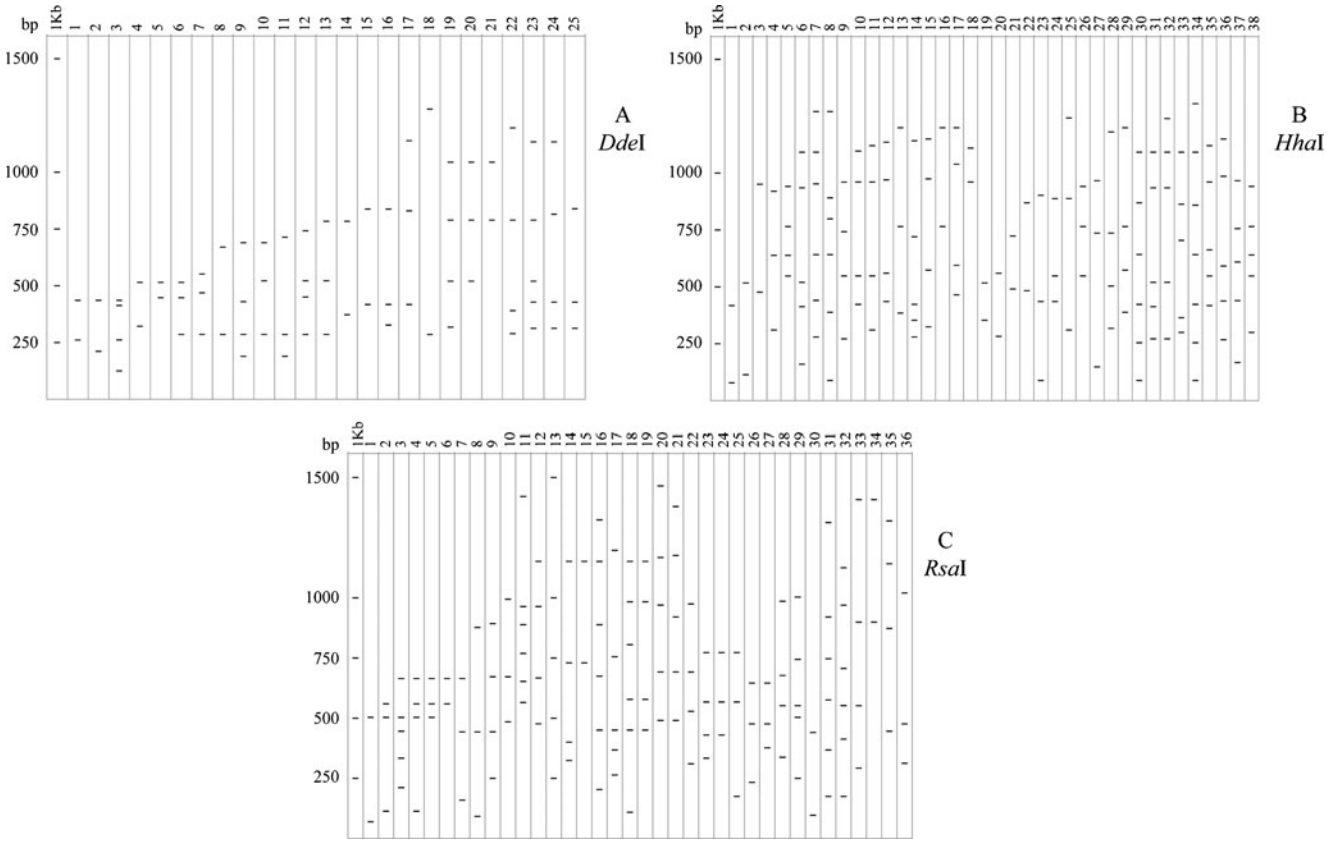


Figure 2 Diagrams representing restriction patterns of 16S rRNA gene digested with *Dde*I (**a**), *Hha*I (**b**) or *Rsa*I (**c**). First column in each diagram corresponds to the banding pattern for the 1 Kb ladder

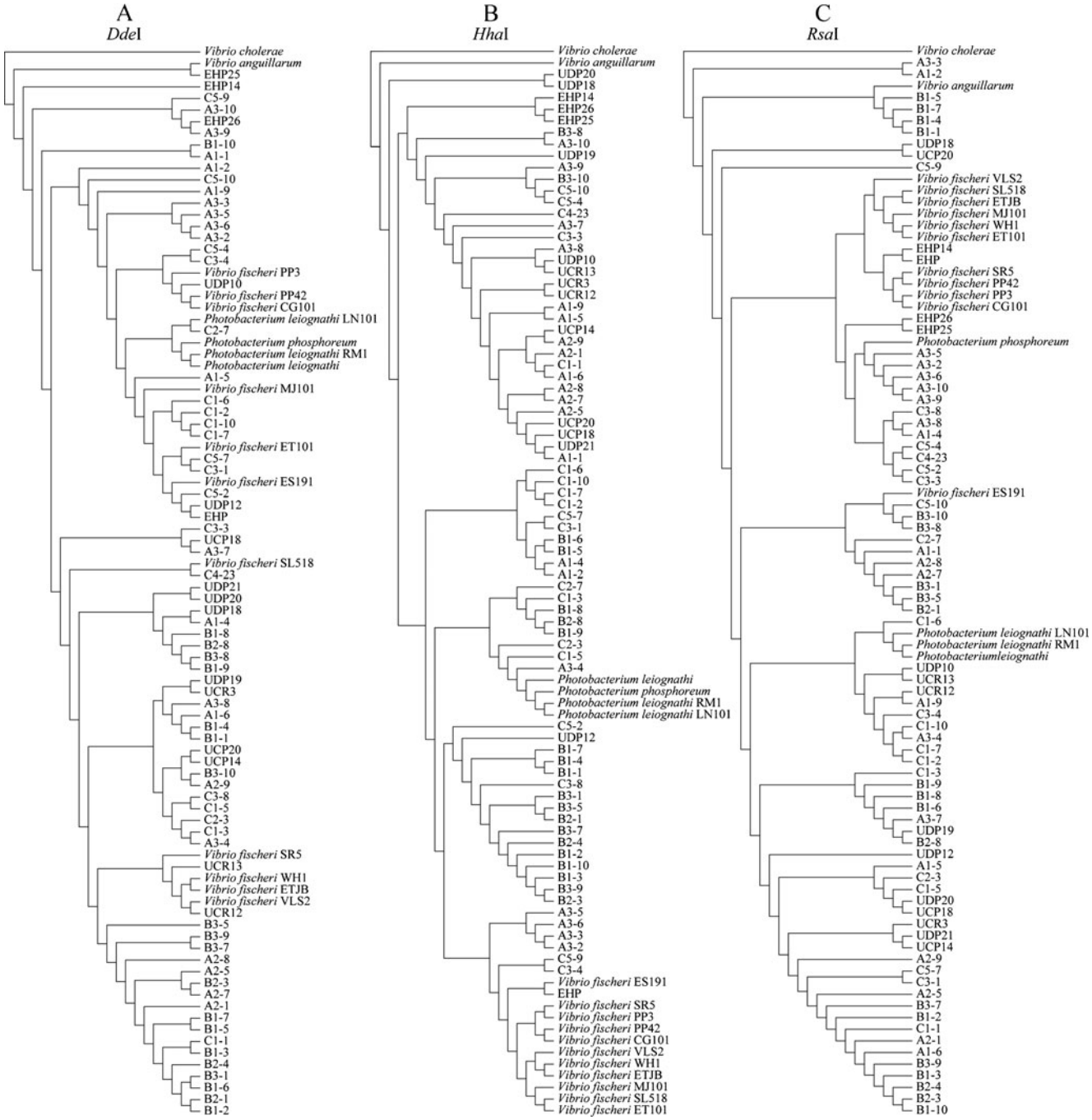


Figure 3 Dendograms built from restriction profiles using parsimony implemented in POY 4.0. Refer to Table 1 for isolates names

sister taxa (Fig. 3c). Not all mutualistic *Vibrio* isolates appear in this group, with free-living *V. fischeri* WH1 grouping separately with Thailand strains.

The distribution of isolates within and among OTUs was neither determined by geographical origin of each isolate nor by its animal host. This is an indication that host biogeography does not play a pivotal role on the phylogenetic history of bacterial populations associated with these species of squids. Australian isolates from groups A, B, and C (three different collection sites in Australia; Table 1) appear scattered throughout the dendograms, indicating no biogeographical partitioning. This lack of pattern is visible in all RFLP derived dendograms, where isolates from Thailand, Australia, Hawaii, and the Mediterranean Sea appear to group together in single clades, despite their geographical origin. This is in contrast to previous studies using more sensitive methods (sequence data) where clear delineation was apparent among *V. fischeri* strains that were allopatric

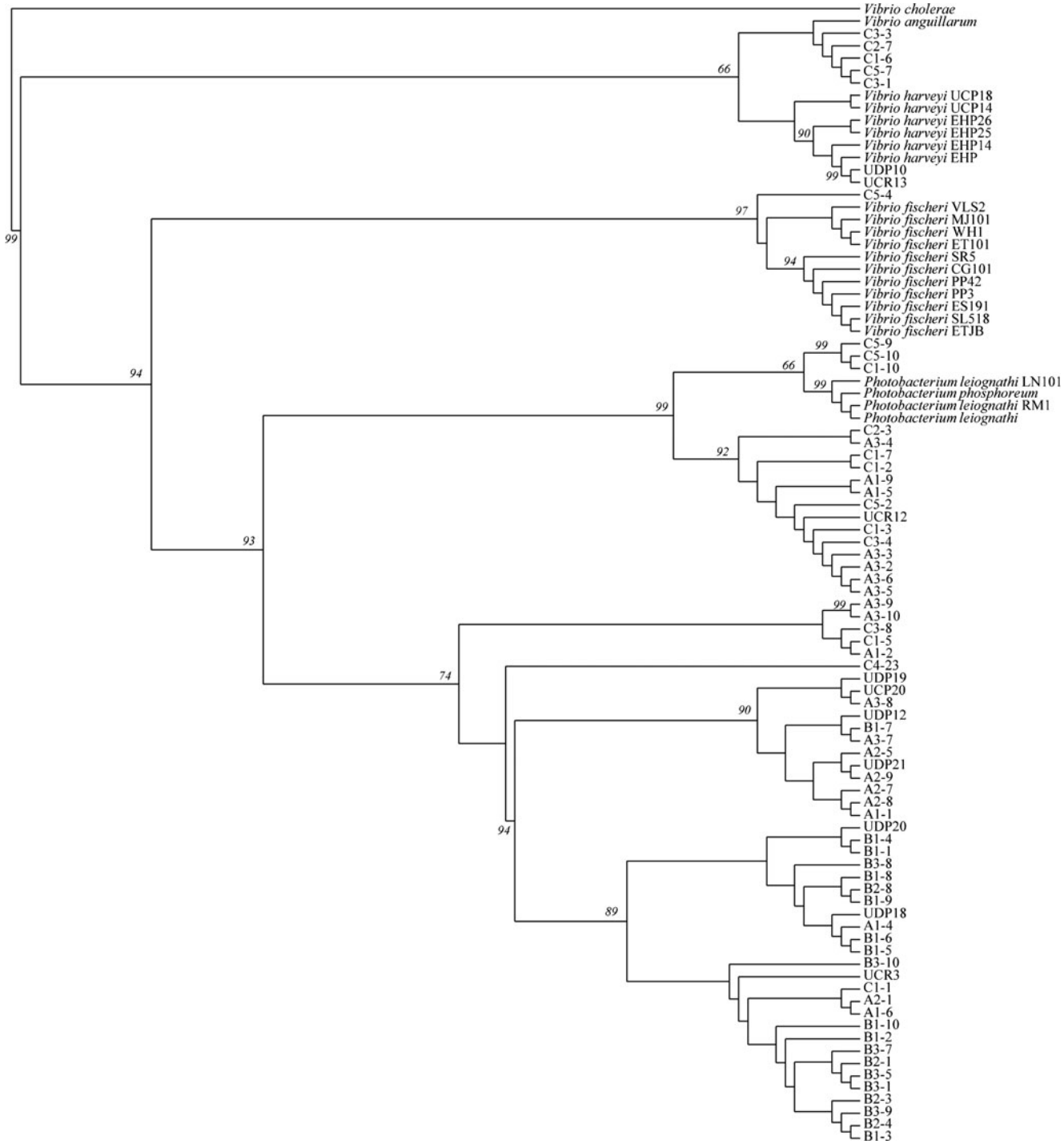


Figure 4 Phylogenetic analysis combining PCR/RFLP data with 16S rRNA gene sequences using parsimony. Jackknife values of more than 50 % are shown as numbers on nodes. Trees were searched by TBR (tree bisection and reconnection) branch-swapping on the best of 100 replicates. One round of tree-fusing was also implemented [65]. At the same time, the command TreeView 0.4.1 was used for visualization of binary trees and PAUP 4.0.10 for consensus tree calculation

and exhibited introgression between closely related populations [61]. Interestingly, sepiolid squids are benthic and do not move between areas as much as loliginid squids, thereby producing more fragmented populations of *Vibrio* bacteria.

Combined Phylogenetic Analysis Using PCR/RFLP *Hha*I Profiles and 16S rRNA Sequence Data

Considering the recognized efficiency of using *Hha*I restriction profiling to distinguish between species of the genera

Photobacterium and *Vibrio* [34, 62], and recognizing the relevance of 16S rRNA gene sequences for the construction of Vibrionaceae phylogenies and the study of the evolution of symbiotic bacteria [1, 12], our study also incorporated a combined phylogenetic approach using both sequence and *Hha*I restriction profile data. Figure 4 depicts the phylogenetic tree resulting from this combined approach. A combination of both data types in a single analysis yielded a distribution of taxa that restricts both the *V. fischeri* group (97 % jackknife support) and the *Photobacterium* genus (99 % jackknife support) into their individual clades. In addition, a number of loliginid squid isolates that the microbiological assays identified as *V. harveyi* were placed within a sole clade, adding strength to our initial conclusions. These results also provide some additional support to previous cladistic analysis, where *Vibrio* and *Photobacterium* were split into separate clades [3].

Conclusion

The use of RFLP of PCR amplified 16S rRNA genes proved to be effective for preliminary screening, evaluation, and characterization of Vibrionaceae populations of bacteria colonizing light organs in loliginid squids. 16S rRNA analysis has been used for the rapid identification of unknown bacterial isolates in samples of fisheries or aquaculture stocks, as well as natural harvests of marine organisms. A systematic development of this technique for *Vibrio* specific groups would contribute to the quick diagnostics of field-collected samples, with the goal of determining whether microbial pathogens (in particular *Vibrio* species) exist as contaminants. In addition, this research further supports that PCR/RFLP analysis is a rapid and economical tool to distinguish the genus *Vibrio* from other members of the family Vibrionaceae, particularly when the number of samples makes phenotypic characterization an expensive and tedious task. Finally, the combination of molecular and biochemical assays has provided additional information regarding species dynamics in *Vibrio*-loliginid squid symbiosis.

Our study also presents additional evidence of a newly recognized association between *V. harveyi* and squids of the family Loliginidae. Our findings contribute to the understanding of bacterial populations in the ocean as it demonstrates that pathogenic bacteria such as *V. harveyi* can also exist as partners in mutualistic associations with loliginid squids. Considering this, there may be some implications regarding the epidemiology of vibriosis in Thailand and Australian coastal areas. Species of sepiolid and loliginid squids are distributed broadly in the Andaman Sea, the Gulf of Thailand, and off the coasts of Australia, and these hosts may represent an ecological niche for pathogens of other marine organisms (including those exploited in aquaculture). *V. harveyi* may utilize these squids as a subtle reservoir for the maintenance of its populations during periods of quiescence. Understanding these survival strategies would better our approaches for assessment of water quality and also clarify the mechanisms of transmission of *Vibrio*-borne diseases and the transition between mutualistic and pathogenic life history strategies. Future studies to examine the distribution of *V. harveyi* throughout the Indo-west Pacific, and the possible existence of specific strains from other locations, may help provide evidence for plausible precursors of vibriosis in the marine environment.

Acknowledgements The authors would like to thank J. Nabhitabhata for help collecting specimens in Rayong and Phuket, Thailand and Rami Al-Khatib from the Electron Microscopy facility at NMSU for help with the Electron Microscopy work. E. Shipp collaborated with DNA amplification and sequencing. Phylogenetic analyses were analyzed on the NetBSD biology computer system at NMSU. This work was supported in part by NIH- SO6 GM008136-32 S2, NIH-NIAID 1SC1AI081659, NSF IOS-0744498, and NSF-DBI-0520956 to M.K.N. Support for C. Gorman and A. Chavez was provided by the NIH-MBRS RISE (NIH-GM-61222-01) and C. Gorman was also supported by the NMSU MARC program (NIH-T34GMO7667-34). S. Willie and E. Shipp were supported by the BRIDGES to Native American Students program (NIH-R25 GM48998) at New Mexico State University.

References

1. Nishiguchi MK, Nair VS (2003) Evolution of symbioses in the Vibrionaceae: a combined approach using molecules and physiology. Int J Syst Evol Micr 53:2019–2026
2. Farmer JJ III, Janda JM, Brenner FW, Cameron DN, Birkhead KM (2005) Genus I. *Vibrio* Pacini 1854, 411^{AL}. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM (eds) Bergey's manual of systematic bacteriology, vol 2. Springer, New York, pp 494–546
3. Dikow RB (2010) Systematic relationships within the Vibrionaceae (Bacteria: Gammaproteobacteria): steps toward a phylogenetic taxonomy. Cladistics 27:9–28
4. Thompson FL, Hoste B, Vandemeulebroecke K, Swings J (2001) Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. Syst Appl Microbiol 24:520–538
5. Jones BW, Nishiguchi MK (2004) Counterillumination in the bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). Mar Biol 144:1151–1155
6. Dunlap PV, McFall-Ngai MJ (1987) Initiation and control of the bioluminescent symbiosis between *Photobacterium leiognathi* and the leiognathid fishes. In: Lee, JJ, Frederick, JF (eds.) *Proceedings of the New York Academy of Sciences Symposium on Endosymbiosis*, pp. 269–283
7. Fidopiastis PM, Boletzky Sv, Ruby EG (1998) A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. J Bacteriol 180:59–64
8. Colwell RR, Huq A (1994) *Vibrios* in the environment: viable but nonculturable *Vibrio cholerae*. In: Wachsmuth IK, Blake PA, Olsvik O (eds) *Vibrio cholerae* and cholera: molecular and global perspectives. American Society of Microbiology Press, Washington, D.C., pp 117–133
9. Yumoto II, Iwata H, Sawabe T, Ueno K, Ichise N, Matsuyama H, Okuyama H, Kawasaki K (1999) Characterization of a facultatively

psychrophilic bacterium, *Vibrio rumoiensis* sp. nov., that exhibits high catalase activity. Appl Environ Microbiol 65:67–72
10. Ruby EG, Morin JG (1979) Luminous enteric bacteria of marine fishes: a study of their distribution, densities, and dispersion. Appl Environ Microbiol 38:406–411
11. Ruby EG, Nealson KH (1976) Symbiotic associations of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*: a model of symbiosis based on bacterial studies. Biol Bull 151:574–586
12. Guerrero-Ferreira RC, Nishiguchi MK (2007) Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis*, *Loliolus* and *Euprymna* (Mollusca: Cephalopoda). Cladistics 23:497–506
13. Stabili L, Gravili C, Piraino S, Boero F, Alifano P (2006) *Vibrio harveyi* associated with *Aglaophenia octodonta* (Hydrozoa, Cnidaria). Microb Ecol 52:603–608
14. Foster JS, Boletzky Sv, McFall-Ngai MJ (2002) A comparison of the light organ development of *Sepiola robusta* Naef and *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). Bull Mar Sci 70:141–153
15. Dalsgaard A, Serichantalergs O, Forslund A, Lin W, Mekalanos J, Mintz E, Shimida T, Wells AJG (2001) Clinical and environmental isolates of *Vibrio cholerae* serogroup O141 carry the CTX phage and the genes encoding the toxin-coregulated pili. J Clin Microbiol 39:4086–4092
16. Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR (1983) Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl Environ Microbiol 45:275–283
17. DePaola A, Ulaszek J, Kaysner CA, Tenge BJ, Nordstrom JL, Wells J, Puhr N, Gendel SM (2003) Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. Appl Environ Microbiol 69:3999–4005
18. Alcaide E, Amaro C, Todoli R, Oltra R (1999) Isolation and characterization of *Vibrio parahaemolyticus* causing infection in Iberian toothcarp *Aphanius iberus*. Dis Aquat Organ 35:77–80
19. Alcaide E, Gil-Sanz C, Sanjuan E, Esteve D, Amaro C, Silveira L (2001) *Vibrio harveyi* causes disease in seahorse, *Hippocampus* sp. J Fish Dis 24:311–313
20. Alcaide E, Sanjuan E, del Ganaa F, Garcia-Gomez A (2000) Susceptibility of Amberjack (*Seriola dumerili*) to bacterial fish pathogens. Bull Eur Ass Fish Pathol 20:153–156
21. Banin E, Israely T, Kushmaro A, Loya Y, Orr E, Rosenberg E (2000) Penetration of the coral-bleaching bacterium *Vibrio shiloi* into *Oculina patagonica*. Appl Environ Microbiol 66:3031–3036
22. Ben-Haim Y, Zicherman-Keren M, Rosenberg E (2003) Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. Appl Environ Microbiol 69:4236–4242
23. Stelma GN Jr, Reyes AL, Peeler JT, Johnson CH, Spaulding PL (1992) Virulence characteristics of clinical and environmental isolates of *Vibrio vulnificus*. Appl Envrion Microbiol 52:2776–2782
24. Aznar R, Ludwig W, Amann RI, Schleifer KH (1994) Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. Int J Syst Bacteriol 44:330–337
25. Owens L, Busico-Salcedo N (2006) *Vibrio harveyi*: pretty problems in paradise. In: Thompson FL (ed) The biology of vibrios. ASM Press, Washington, D.C., pp 266–280
26. Vidgen M, Carson J, Higgins M, Owens L (2006) Changes to the phenotypic profile of *Vibrio harveyi* when infected with the *Vibrio harveyi* myovirus-like (VHML) bacteriophage. J Appl Microbiol 100:481–487
27. Dunlap PV, Davis KM, Tomiyama S, Fujino M, Fukui A (2008) Developmental and microbiological analysis of the inception of bioluminescent symbiosis in the marine fish *Nuchequula nuchalis* (Perciformes: Leiognathidae). Appl Environ Microbiol 74:7471–7481
28. Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522
29. Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol 6:776–788
30. Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345:63
31. Guerrero-Ferreira RC, Nishiguchi MK (2009) Ultrastructure of light organs of loliginid squids and their bacterial symbionts: a novel system for the study of marine symbioses. Vie et Milieu 59:307–313
32. Jamann S, Fernandez MP, Normand P (1993) Typing method for N_2-fixing bacteria based on PCR-RFLP-application to the characterization of *Frankia* strains. Mol Ecol 2:17–26
33. Urakawa H, Kita-Tsukamoto K, Ohwada K (1997) 16S rDNA genotyping using PCR/RFLP (restriction fragment length polymorphism) analysis among the family Vibrionaceae. FEMS Microbiol Lett 152:125–132
34. Urakawa H, Kita-Tsukamoto K, Ohwada K (1998) A new approach to separate the genus *Photobacterium* from *Vibrio* with RFLP patterns by *Hha*I digestion of PCR-amplified 16S rDNA. Curr Microbiol 36:171–174
35. Urakawa H, Kita-Tsukamoto K, Ohwada K (1999) 16S rDNA restriction fragment length polymorphism analysis of psychrotrophic vibrios from Japanese coastal water. Can J Microbiol 45:1001–1007
36. Urakawa H, Kita-Tsukamoto K, Ohwada K (1999) Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. Microbiology 145(Pt 11):3305–3315
37. Yoshimura S, Yoshimura A, Saito A, Kishmoto N, Kawase M, Yano M, Nakagahra M, Ogawa T, Iwata N (1992) RFLP analysis of introgressed chromosomal segments in three near-isogenic lines of rice for bacterial blight resistance genes, *Xa-1*, *Xa-3* and *Xa-4*. Jpn J Genet 67:29–37
38. Marhual NP, Das BK (2009) Use of random amplified polymorphic DNA and restriction fragment length polymorphism for typing of *Vibrio alginolyticus* isolated from black tiger shrimp *Penaeus monodon*. J Pure Appl Microbiol 3:75–82
39. Nishiguchi MK, Lopez JE, Boletzky Sv (2004) Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. Evol Dev 6:41–49
40. Wheeler WC (1996) Optimization alignment: the end of multiple sequence alignment in phylogenetics? Cladistics 12:1–9
41. Wheeler WC (1998) Alignment characters, dynamic programming and heuristic solutions. In: DeSalle R, Schierwater B (eds) Molecular approaches to ecology and evolution, vol 1. Birkhauser Verlag, Basel, pp 243–251
42. Wheeler WC, Gladstein D, DeLaet J (2002) POY v. 3.0
43. Wheeler WC (1995) Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. Syst Biol 44:321–331
44. Alsina M, Blanch AR (1994) Improvement and update of a set of keys for biochemical identification of *Vibrio* species. J Appl Bacteriol 77:719–721
45. Allen RD, Baumann P (1971) Structure and arrangement of flagella in species of the genus *Beneckea* and *Photobacterium fischeri*. J Bacteriol 107:295–302
46. Baumann P, Baumann L, Bang SS, Woolkalis MJ (1980) Reevaluation of the taxonomy of *Vibrio*, *Beneckea*, and *Photobacterium*: abolition of the genus *Beneckea*. Curr Microbiol 4:127–132
47. Kidron H, Repo S, Johnson MS, Salminen TA (2007) Functional classification of amino acid decarboxylases from the alanine

racemase structural family by phylogenetic studies. Mol Biol Evol 24:79–89
48. Patel CN, Wortham BW, Lines JL, Fetherston JD, Perry RD, Oliveira MA (2006) Polyamines are essential for the formation of plague biofilm. J Bacteriol 188:2355–2363
49. Karatan E, Duncan TR, Watnick PI (2005) NspS, a predicted polyamine sensor, mediates activation of *Vibrio cholera* biofilm formation by norspermidine. J Bacteriol 187:7434–7443
50. Jones BW, Nishiguchi MK (2006) Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. Can J Microbiol 52:1218–1227
51. Ariyakumar DS, Nishiguchi MK (2009) Characterization of two host-specific genes, mannose-sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri–Euprymna tasmanica* mutualism. FEMS Microbiol Lett 299:65–73
52. Merrell DS, Hava DL, Camilli A (2002) Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. Mol Microbiol 43:1471–1491
53. Guerrero-Ferreira RC, Nishiguchi MK (2010) Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. Environ Microbiol Rep 2:514–523
54. Graf J (1999) Symbiosis of *Aeromonas veronii* Biovar *sobria* and *Hirudo medicinalis*, the medicinal leech: a novel model for digestive tract associations. Infect Immun 67:1–7
55. Siddall ME, Worthen PL, Johnson M, Graf J (2007) Novel role for *Aeromonas jandaei* as a digestive tract symbiont of the North American medicinal leech. Appl Environ Microbiol 73:655–658
56. Hickman-Brenner FW, MacDonald KL, Steigerwalt AG, Fanning GR, Brenner DJ, Farmer JJ III (1987) *Aeromonas veronii*, a new ornithine decarboxylase-positive species that may cause diarrhea. J Clin Microbiol 25:900–906
57. McCarter LL (2006) Motility and chemotaxis. In: Thompson FL, Austin B, Swings J (eds) The biology of vibrios. ASM Press, Washington, D.C., p 423
58. Pollock FJ, Wilson B, Johnson WR, Morris PJ, Willis BL, Bourne DG (2010) Phylogeny of the coral pathogen *Vibrio coralliilyticus*. Environ Microbiol Rep 2:172–178
59. Bleicher A, Neuhaus K, Scherer S (2010) *Vibrio casei sp. nov.*, isolated from the surface of two French red smear soft cheeses. Int J Syst Evol Microbiol 60:1745–1749
60. Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J (2005) Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Appl Environ Microbiol 71:5107–5115
61. Jones BW, Lopez JE, Huttenburg J, Nishiguchi MK (2006) Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. Mol Ecol 15:4317–4329
62. Kita-Tsukamoto K, Wada M, Yao K, Kamiya A, Yoshizawa S, Uchiyama N, Kogure K (2006) Rapid identification of marine bioluminescent bacteria by amplified 16S ribosomal RNA gene restriction analysis. FEMS Microbiol Lett 256:298–303
63. Visick KL, Ruby EG (1998) The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. J Bacteriol 180:2087–2092
64. Ast JC, Cleenwerck I, Engelbeen K, Urbanczyk H, Thompson FL, De Vos P, Dunlap PV (2007) *Photobacterium kishitanii sp. nov.*, a luminous marine bacterium symbiotic with deep-sea fishes. Int J Syst Evol Microbiol 57:2073–2078
65. Goloboff PA (1999) Analyzing large data sets in reasonable times: solutions for composite optima. Cladistics 15:415–428

Predation Response of Vibrio fischeri Biofilms to Bacterivorus Protists

Alba Chavez-Dozal, Clayton Gorman, Martina Erken, Peter D. Steinberg, Diane McDougald and Michele K. Nishiguchi
Appl. Environ. Microbiol. **2013, 79(2):553. DOI: 10.1128/AEM.02710-12.**
Published Ahead of Print 9 November 2012.

AEM
Journals.ASM.org

Predation Response of *Vibrio fischeri* Biofilms to Bacterivorus Protists

Alba Chavez-Dozal,[a] Clayton Gorman,[a] Martina Erken,[b] Peter D. Steinberg,[c,d] Diane McDougald,[b,e] Michele K. Nishiguchi[a]

Department of Biology, New Mexico State University, Las Cruces, New Mexico, USA[a]; Centre for Marine Biofouling and Bio-Innovation and School of Biotechnology and Biomolecular Sciences, University of South Wales, Sydney, New South Wales, Australia[b]; Centre for Marine Biofouling and Bio-Innovation and School of Biological, Earth and Environmental Science, University of South Wales, Sydney, New South Wales, Australia[c]; Sydney Institute of Marine Science, Mosman, NSW, Australia[d]; Advanced Environmental Biotechnology Centre, Nanyang Technological University, Singapore[e]

***Vibrio fischeri* proliferates in a sessile, stable community known as a biofilm, which is one alternative survival strategy of its life cycle. Although this survival strategy provides adequate protection from abiotic factors, marine biofilms are still susceptible to grazing by bacteria-consuming protozoa. Subsequently, grazing pressure can be controlled by certain defense mechanisms that confer higher biofilm antipredator fitness. In the present work, we hypothesized that *V. fischeri* exhibits an antipredator fitness behavior while forming biofilms. Different predators representing commonly found species in aquatic populations were examined, including the flagellates *Rhynchomonas nasuta* and *Neobodo designis* (early biofilm feeders) and the ciliate *Tetrahymena pyriformis* (late biofilm grazer). *V. fischeri* biofilms included isolates from both seawater and squid hosts (*Euprymna* and *Sepiola* species). Our results demonstrate inhibition of predation by biofilms, specifically, isolates from seawater. Additionally, antiprotozoan behavior was observed to be higher in late biofilms, particularly toward the ciliate *T. pyriformis*; however, inhibitory effects were found to be widespread among all isolates tested. These results provide an alternative explanation for the adaptive advantage and persistence of *V. fischeri* biofilms and provide an important contribution to the understanding of defensive mechanisms that exist in the out-of-host environment.**

Biofilms form at almost every surface that is in contact with water, and they are a natural part of aquatic ecosystems (1, 2). Moreover, biofilm communities offer a refuge toward diverse stresses, such as antibiotics (3–5), dehydration and osmotic stress (5, 6), UV light exposure (6, 7), and starvation (3). The pronounced stress resistance of biofilms has been observed to be prevalent in marine communities, particularly ones comprised of environmental *Vibrio* biofilms (2, 8, 9).

Biofilms formed by *Vibrio* species are ubiquitous in aquatic ecosystems, although no study has specified the prevalence of *Vibrio fischeri* biofilms. *V. fischeri* (and other marine bacteria) in its planktonic state are found over broad geographical ranges, and their biofilms are subjected to multiple physiological stresses that lead to alterations in bacterial physiology (promoting bacterial fitness and bacterial speciation) (10). Thus, the survival of planktonic cells and biofilms in the environment is not only defined by the capacity to overcome abiotic pressures but also by the ability to serve as a protective niche against natural protozoan consumers (11, 12). Thus, an alternative function for the prevalence and relative fitness of *V. fischeri* biofilms is that they serve as refuges to combat a range of predators, including protozoan grazers. Grazing is one of the most common mortality factors of bacterial populations (13–15) and causes rapid changes in the morphology and species composition of microbial communities (13, 16–18). Interactions between bacteria and protozoa within biofilm communities remain largely unexplored; however, recent studies have revealed the impact of grazing on the dynamics of natural biofilm communities. Quorum sensing is an important factor for antipredatory activity in many bacterial species (19–22), and observations have suggested that bacterial genetic diversity enhances grazing resistance (23). Grazing resistance was observed in biofilms of *Pseudomonas aeruginosa* when early and late biofilm communities exhibited antipredatory behavior against two flagellates (*Bodo saltans* and *Rhynchomonas nasuta*) and the ciliate *Tetrahymena* sp. (18). Similar results were also observed when communities of *Vibrio cholerae* prevented predation through an antiprotozoal factor regulated by the response regulator HapR (14, 20). Moreover, *V. cholerae* biofilms exhibit widespread grazing resistance among toxigenic and nontoxigenic isolates, which has an impact on strain distribution and cholera epidemics (20). Previous studies also reported that some bacterial communities synthesize chemical compounds (e.g., violacein) that inhibit protozoan feeding by inducing cell lysis. These specific chemical defenses are prevalent in the tropical aquatic and soil bacterium *Chromobacterium violaceum* and other marine bacteria, such as *Janthinobacterium lividum* and *Pseudoalteromonas luteoviolacea* (24).

The protozoan community in marine ecosystems is cosmopolitan and follows a succession pattern depending on the nature of the grazer. For example, early biofilm colonizers (or generalists, including flagellates and ciliates) are highly motile, allowing fast surface feeding, while intermediate late colonizers (some ciliates and amoebas) are classified as specialists and are abundant in mature biofilms (25, 26). Therefore, the aim of our study was to investigate whether *V. fischeri* biofilms are resistant to protozoan grazing, and if any differences exist in predator avoidance between various free-living and symbiotic strains. We tested symbiotic strains isolated from the light organ of two different squid genera, *Euprymna* (Indo-West Pacific) and *Sepiola* (Mediterranean), along with free-living strains isolated directly from seawater. Three different protozoan predators among the 20 most commonly reported species of predators were chosen for these studies (20, 25) and included two early-feeding flagellates, *Neobodo designis* and

Received 4 September 2012 **Accepted** 26 October 2012
Published ahead of print 9 November 2012
Address correspondence to Michele K. Nishiguchi, nish@nmsu.edu.

doi:10.1128/AEM.02710-12

TABLE 1 *Vibrio fischeri* strains used in this study

Strain	Host	Location
WH1	Free living	USA (Woods Hole, MA)
MDR7	Free living	USA (Marina del Rey, CA)
CB37	Free living	Australia (Coogee Bay, Sydney, NSW)
CB21	Free living	Australia (Coogee Bay, Sydney, NSW)
CHB8	Free living	Australia (Chowder Bay, Sydney, NSW)
CHB12	Free living	Australia (Chowder Bay, Sydney, NSW)
CHB30	Free living	Australia (Chowder Bay, Sydney, NSW)
BSM40	Free living	France (Banyuls sur mer)
BSM46	Free living	France (Banyuls sur mer)
BSM50	Free living	France (Banyuls sur mer)
PP3	Free living	USA (Kaneohe Bay, O'ahu, HI)
PP42	Free living	USA (Kaneohe Bay, O'ahu, HI)
VLS2	Free living	USA (Kaneohe Bay, O'ahu, HI)
SR5	*Sepiola robusta*	France (Banyuls sur mer)
SL518	*Sepiola lingulata*	France (Banyuls sur mer)
SA1G	*Sepiola affinis*	France (Banyuls sur mer)
SA18	*Sepiola affinis*	France (Banyuls sur mer)
SA25	*Sepiola affinis*	France (Banyuls sur mer)
SA70	*Sepiola affinis*	France (Banyuls sur mer)
SI66	*Sepiola intermedia*	Italy (Bari)
SI1D	*Sepiola intermedia*	France (Banyuls sur mer)
EM17	*Euprymna morsei*	Japan (Tokyo Bay)
ET101	*Euprymna tasmanica*	Australia (Townsville, QLD)
ETWW	*Euprymna tasmanica*	Australia (Woy Woy, NSW)
ETBB20	*Euprymna tasmanica*	Australia (Botany Bay, Sydney, NSW)
ETBB45	*Euprymna tasmanica*	Australia (Botany Bay, Sydney, NSW)
ETBB67	*Euprymna tasmanica*	Australia (Botany Bay, Sydney, NSW)
ETSB1	*Euprymna tasmanica*	Australia (Shark Bay, WA)
EB12	*Euprymna berryi*	Japan (Tosa Bay)
ES114	*Euprymna scolopes*	USA (Kaneohe Bay, O'ahu, HI)
ESP915	*Euprymna scolopes*	USA (Paiko, O'ahu, HI)
ESL5	*Euprymna scolopes*	USA (Kaneohe Bay, O'ahu, HI)
ESC9	*Euprymna scolopes*	USA (Kaneohe Bay, O'ahu, HI)

Rhynchomonas nasuta, and the late ciliate colonizer *Tetrahymena pyriformis*. This experimental setting allowed us to identify biofilm survival depending on (i) strain type, (ii) age of biofilm, (iii) protozoan colonizer, and (iv) protozoan feeding type, and these factors have implications for understanding how *V. fischeri* survives, persists, and diversifies in the presence of grazers.

MATERIALS AND METHODS

Bacterial and protozoan strains used in this study. Bacterial strains used in this study are listed in Table 1. *V. fischeri* strains were grown on Luria-Bertani high-salt agar (LBS; with a per liter composition of 10 g tryptone, 5 g yeast extract, 20 g sodium chloride, 50 ml 1 M Tris [pH 7.5], 3.75 ml 80% glycerol, 15 g agar, and 950 ml distilled water) and incubated for 24 h at 28°C. *V. fischeri* strains were isolated from different squid light organs captured live (*Euprymna* or *Sepiola*) or directly from seawater (27). All strains were subsequently subcultured in LBS liquid medium (no agar) and incubated with moderate shaking (200 rpm) for 18 h. The benthic flagellate grazers *R. nasuta* and *N. designis* were isolated from Chowder Bay at the Sydney Institute for Marine Science (SIMS), New South Wales, Australia, treated with an antibiotic cocktail (kanamycin, gentamicin, streptomycin, ampicillin, and trobamycin at 150 μg/ml each), and diluted through many generations (~15) to remove the natural bacterial community (14). *R. nasuta* and *N. designis* were maintained axenically in 0.5× nine salts solution medium (NSS; with a per liter composition of 8.8 g NaCl, 0.735 g Na_2SO_4, 0.04 g $NaHCO_3$, 0.125 g KCl, 0.02 g KBr, 0.935 g $MgCl_2 \cdot 6H_2O$, 0.205 g $CaCl_2 \cdot 2H_2O$, 0.004 g $SrCl_2 \cdot 6H_2O$, 0.004 g H_3BO_3). Additional cultures were supplemented with heat-killed *P. aeruginosa*, which served as prey. The ciliate *T. pyriformis* (CCAP 1630/1W; Culture Collection of Algae and Protozoa, Windmere, United Kingdom) was maintained in PPY medium (with a per liter composition of 20 g proteose peptone and 2.5 g yeast extract). Cultures were incubated at room temperature (20 to 23°C) for 2 weeks.

Grazing assays. Grazing experiments were completed in 24-well microtiter plates as previously described (20) for both early and late biofilms. For the early setup, overnight cultures of all bacterial strains were incubated at a dilution of 10^6 cells per ml in a total of 1 ml of fresh Väätänen-NSS medium (VNSS; with a composition of 1 g peptone, 0.5 g yeast extract, 0.5 g dextrose, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g Na_2HPO_4, mixed with 1 liter of NSS medium) (14), which allowed growth of both bacteria and protozoa. As a negative control, sterile uninoculated VNSS medium was used. Strains were grown for 6 h (to let the early biofilm to become established) at room temperature (28°C) in the 24-well microtiter plates, and subsequently the planktonic population was removed and replaced with 1 ml of fresh VNSS medium that contained overnight cultures of either *R. nasuta* or *N. designis* (early grazers) at an abundance of 10^2 cells per ml. In the late biofilm setup, overnight cultures of all bacterial strains were inoculated as described for the early biofilm setup, but with the bacterial biofilms forming for 24 h prior to addition of the late biofilm grazer *T. pyriformis*. After formation of mature (late) biofilms, the planktonic suspension was removed and *Tetrahymena* was added at a concentration of 10^2 cells per ml. Plates were incubated for 24 h at 20°C with shaking at 50 rpm. Additionally, as a positive control, bacterial cultures were inoculated in parallel (10^6 cells per ml in 1 ml of VNSS medium) and incubated without protozoan grazers for 6 h (early biofilms) or 24 h (late biofilms) with shaking (50 rpm) at 20°C. For each experiment, all samples were inoculated in triplicate (3 wells), and assays were repeated three times (3 independent studies), for a total of 9 wells per bacterial strain.

Enumeration of protozoans and quantification of biofilm formation. Numbers of grazers and grazer growth rates were calculated from direct cell counts using light microscopy. The number of grazers was calculated when 5 μl of supernatant was fixed with acid Lugol's solution (5%, final concentration) and enumerated using direct microscopy. Bacterial biofilms were measured using a colorimetric assay, where the supernatant from each plate was removed and wells were washed three times with 1 ml of sterile VNSS medium. One milliliter of 0.2% crystal violet solution was added to each well, and the mixture was incubated for 30 min at room temperature. After incubation, the crystal violet solution was removed and plates were washed five times with distilled water and dried. One milliliter of ethanol (95%) was added, and plates were incubated for 30 min to allow the dye to solubilize. The contents of each well (including negative controls) were transferred to a new plate, and the optical density was measured at 562 nm using a plate reader (Bio-Tek FLX 800; MTX Lab systems Inc., VA). The optical density observed is directly proportional to the amount of biofilm formed, and values were corrected by blank readings (uninoculated VNSS wells). For biofilm biomass quantifications, pairwise comparisons were performed using two-factor analysis of variance and Tukey's *post hoc* comparisons in order to test for significant differences between treatments (grazing versus nongrazing). Three plates were used for evaluation of statistical significance (3 independent studies).

Scanning electron microscopy. To observe toxic effects or morphological changes in the protozoan cultures, light microscopy and scanning electron microscopy (SEM) were performed. Overnight cultures of all strains were reinoculated in triplicate in glass test tubes containing 5 ml of VNSS with an immersed sterile coverslip. Early (2 sets of tubes) or late (1 set of tubes) biofilms were allowed to form. After 6 or 24 h of incubation, grazers were added (either *Rhynchomonas* or *Neobodo* for the early biofilms and *Tetrahymena* for the late biofilms) and incubated at room temperature for 24 h. After incubation, coverslips were washed with sterile VNSS, fixed with a 0.5% solution of glutaraldehyde, and gold coated for SEM with a Hitachi S34000 SEM apparatus (Hitachi, Schaumburg, IL) as previously described (19).

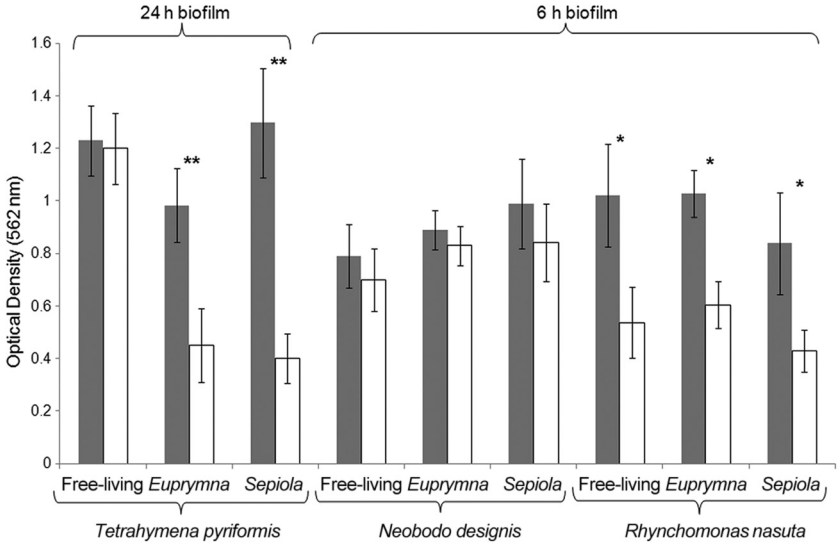


FIG 1 Quantification of biofilm biomass of free-living and symbiotic (*Euprymna* and *Sepiola*) *V. fischeri* strains before grazing (gray bars) and after grazing (white bars) on early (*R. nauta* and *N. designis*) and late (*T. pyriformis*) biofilms. The optical densities were measured after solubilization of crystal violet, with the abundance of biofilm considered directly proportional to the optical density reading. Error bars represent the standard deviations. *, $P < 0.05$ for the difference between grazed and nongrazed bacteria; **, $P < 0.01$. The composite data are from 13 free-living strains, 8 *Sepiola* strains, and 12 *Euprymna* strains (see Table 1).

RESULTS

Resistance to grazing of early and late biofilms. To determine potential inhibitory effects of biofilms (early and late) against three different protozoans with contrasting feeding modes, we compared the quantity of bacterial biofilm that was formed with and without exposure to each predator (Fig. 1). For early symbiotic strains, a significant reduction ($P < 0.001$) in biofilm biomass was observed after addition of the ciliated predator *T. pyriformis*, whereas biofilms formed by free-living strains (Table 1; Fig. 1) did not show any significant differences from nongrazed biofilms. In late biofilms, the raptorial feeder *R. nasuta* had no effect on the biomass of any of the strains examined; however, the flagellate *N. designis* was able to reduce the biofilm biomass of all strains by more than 30% ($P < 0.05$).

Biofilms cause a decrease in predator number. In this study, we hypothesized that a reduction in the number of grazers would occur if the biofilm biomass did not decrease compared to cells that were consumed. Our experiments demonstrated that antiprotozoal effects occurred in both early and late biofilms and that variations in toxicity exist between the two early biofilm flagellates, consistent with other protists that have similar feeding modes (28, 29).

Protist abundance decreased in every case that the biofilms persisted (Fig. 2). Growth rates of *T. pyriformis* (ciliate, late grazer) were significantly reduced when exposed to biofilms formed by free-living strains (isolated from France, Australia, and the United States) (Table 1; Fig. 1), whereas high growth rates were observed when symbiotic biofilms were grazed. The same pattern was observed with *N. designis* (flagellate, early grazer), where numbers increased after grazing of early biofilms formed by both symbiotic and free-living strains. Interestingly, *R. nasuta* (flagellate, early grazer) numbers decreased when grazing on both symbiotic and free-living early biofilms.

Microscopy studies. Biofilms that were preformed on a glass coverslip were exposed to all grazers for 24 h and then examined via SEM in order to visualize possible toxic effects after grazing. *Vibrio* strains that were successfully grazed by the free-swimming filter feeder *T. pyriformis* (including most of the symbiotic strains) were found on glass coverslips with *T. pyriformis* (Fig. 3). The small amount of glass-associated biofilm was distinguishable from the larger ciliated *T. pyriformis* (late grazer). Additionally, evidence of grazing activity was present and included signs of active feeding by a large number of ciliated protozoa (Fig. 3A and B). Discernible changes in grazer morphology were observed in *T. pyriformis* after grazing on free-living strains (Fig. 4). Two different phenotypes in protozoan structure were observed, including grazing morphology (Fig. 4A and B) and cell lysis (Fig. 4C and D). Late biofilms (24 h) were formed at the air-liquid interface of

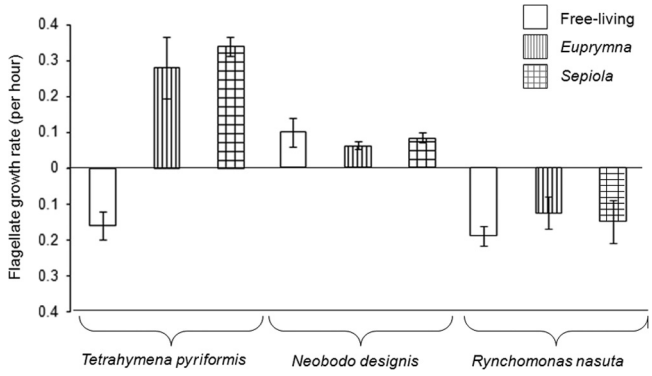


FIG 2 Protozoan growth rates after grazing on early and late biofilms. Negative numbers represent mortality, and positive numbers represent growth. Different bar patterns represent different groups of *V. fischeri* isolates (free-living or symbiotic with *Euprymna* or *Sepiola*). Errors bars are the standard deviations for each treatment. Each protozoan group was significantly different from the others ($P < 0.05$), and within the *Tetrahymena* group there was a significant difference in growth rate when free-living strains were grazed ($P < 0.05$).

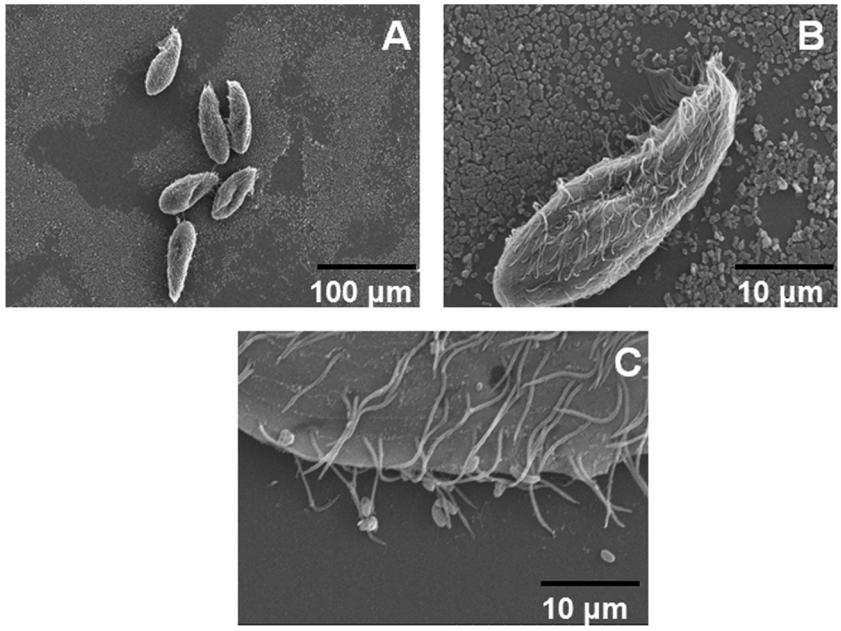


FIG 3 SEM images of *T. pyriformis* grazing dynamics. (A) *T. pyriformis* cells on a mature *V. fischeri* biofilm. (B) Magnification of *T. pyriformis* and the biofilm. (C) *T. pyriformis* grazing through a filter feeding strategy.

coverslips. Immediately after biofilm formation, *Tetrahymena* was added to the cultures, and after 24 h coverslips were analyzed using SEM. We observed two different coverslips per strain (for the protozoa *Tetrahymena*), and 60% of them exhibited a phenotype change for *Tetrahymena*, with protozoan lysis being the most common phenotype (after grazing biofilms formed by free-living strains).

DISCUSSION

Vibrios are a ubiquitous diverse group of heterotrophic bacteria that are found in oceans, estuaries, and marine sediments worldwide. The diversity and dynamics of cooccurring populations has frequently been linked to environmental changes, including fluctuations in temperature, salinity, ocean hydrodynamics, and nutrient composition (10, 30). Therefore, environmental fluctuations lead to considerable bacterial microdiversity and evolution, including evolution of a wide variety of pathogenic and facultative mutualistic strains. The latter include the bioluminescent bacterium *V. fischeri*, which exists in a free-living stage (seawater) or as a mutualist of sepiolid squids and monocentrid fishes (31–33).

Understanding the influences of abiotic factors on *V. fischeri* biofilm populations has become a central theme of our research (10, 27); however, success in the environment is also dictated by the ability of the biofilm to tolerate natural protozoan consumers.

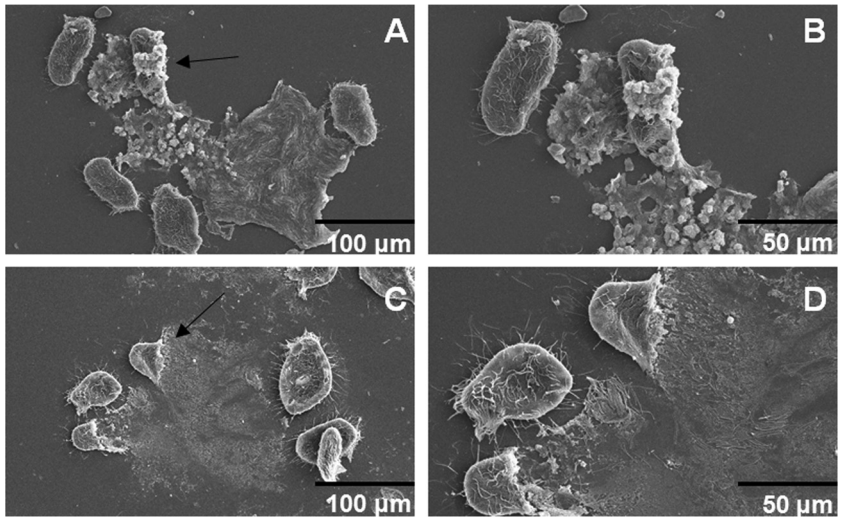


FIG 4 SEM images of the antiprotozoan effects on *T. pyriformis*. (A and B) Morphological changes in the grazer and the bacterial growth around the predator. (C and D) Cell lysis of the grazer. Arrows in panels A and C point to the corresponding grazers that are shown in panels B and D.

Recent studies have emphasized the effects of protozoan grazers (17, 23, 34, 35), particularly in *Vibrio* communities, such as *V. cholerae* (12, 14), but not in communities of other species, including mutualists, such as *V. fischeri* and *V. logei.* In this study, we investigated the effects of common bacteriovores *T. pyriformis* (ciliate, late grazer), *N. designis* (flagellate, early grazer), and *R. nasuta* (flagellate, early grazer) on 33 *V. fischeri* isolates from different origins, including seawater and two squid genera: *Euprymna*, found in Indo-West Pacific waters, and *Sepiola*, found in the Mediterranean Sea. Symbiotic strains were chosen based on their similar growth and infection capabilities (all symbiotic strains could infect juvenile sepiolid squids) despite being from different host squids and geographical locations, whereas free-living strains were chosen based on their inability to infect juvenile squids yet their being found ubiquitously in the ocean.

Our results indicated that biofilms may protect *V. fischeri* from predation. For example, when the generalist ciliate *T. pyriformis* (late grazer) was added to late biofilms, a selective resistance to grazing was exhibited by free-living strains only and not symbiotic strains. Earlier work showed that free-living strains of *V. fischeri* are able to grow under a wider variety of conditions than their symbiotic relatives (10). This demonstrated that free-living strains have better adaptive mechanisms, which may be the result of genetic changes responsible for shifts in bacterial phenotypes (formation of biofilms) (2, 3).

It has been reported that biofilms of different species of environmental isolates secrete antiprotozoan factors, such as violacein (24) and others of an unknown nature (15, 20). These components may be present in *V. fischeri* biofilms, but they have not been identified thus far. *T. pyriformis* is a ciliated protozoa that grazes mature biofilms, similar to the ones found in the environment. Conversely, biofilm-like structures formed in the squid's light organ are expelled daily due to the diurnal cycle of the squid. This cycle allows exponential growth of *V. fischeri* followed by an expulsion of 99% of the symbionts into seawater every day at dawn (33, 36, 37). Thus, diel expulsion of a large number (10^{14}) of bacteria possibly disrupts the preformed biofilm and therefore does not select for production of antiprotozoan compounds. Moreover, these strains may be exposed less frequently to predation and therefore are less capable of responding to predators that are only found in the aquatic environment, as in the case of the protozoan grazers used in this study.

Early biofilms were exposed to the flagellates *N. designis* and *R. nasuta*, which have different feeding modes and are considerably smaller in size than *T. pyriformis* (200 times bigger than *V. fischeri*). Our results revealed that early biofilms exposed to *N. designis* were consumed, resulting in a pronounced increase in flagellate numbers, which was opposite to observations for the flagellate *R. nasuta.* These contrasting results indicate that flagellate size is not necessarily correlated to grazing dynamics. For example, *N. designis* is a direct interception feeder, creating currents that carry suspended or loosely attached bacteria toward the mouth (28). *R. nasuta* is a raptorial feeder that grasps its prey with a proboscis-like structure (26). Another major difference between these two flagellates is that *R. nasuta* has a lower grazing (ingestion) success in sparsely populated biofilms due to its slower moving speed, and it is more successful in waters with higher prey densities (38). These different feeding modes may be crucial to the grazing success regarding *V. fischeri* biofilms. Symbiotic *V. fischeri* strains also exhibit resistance to *R. nasuta* predation (Fig. 1), indicating that some defensive mechanism still exists within biofilms formed by mutualistic strains. These defenses may be coopted to avoid the squid host innate immune response, which is mostly comprised of hemocytes and macrophages (39, 40). Future studies will focus on whether receptor-mediated phagocytosis in protozoa affects immune response evasion through biofilm formation.

In this study, protozoan abundance was reduced after grazing, and antiprotozoan effects on *T. pyriformis* (ciliate, late grazer) were observed (Fig. 2). Morphological changes of *T. pyriformis* occurred with bacteria possibly colonizing the protozoan predator, resulting in subsequent lysis of *T. pyriformis* cells. These two effects may be the result of inactivation of the predator or synthesis of lytic compounds that are released before or after ingestion of *V. fischeri* cells.

Quorum sensing controls biofilm formation (41) and has been described as one possible mechanism for antiprotozoal activity (20). In the case of *V. fischeri*, quorum sensing is under the control of the transcriptional regulator LuxR-LuxI (8). In addition to genes involved in light production, the LuxR regulon activates another number of genes involved in synthesis of efflux proteins, transporters, permeases, and proteases (8). The role of these quorum-sensing proteins in antipredator activity is unknown; however, they may contribute to the competitive fitness of biofilms under grazing pressure. Future studies will help determine the genetic factors responsible for protozoan death and may provide important clues of how survival of *Vibrio* biofilms is linked to quorum-sensing mechanisms.

The nature of the protozoan predator and its feeding characteristics may influence the impact of grazing on *V. fischeri* biofilms. Late/early and generalist/specialist protists have distinct grazing preferences, which are related to the type of *V. fischeri* strain examined (free living or symbiotic). Here we showed that *V. fischeri* biofilms have the ability to quickly adapt to grazing pressure, possibly by releasing antigrazing compounds and products that negatively influence protist survival. The observation that free-living strains are considerably more resistant to grazing pressure than symbiotic strains suggests that host selection may compromise the fitness of *V. fischeri* strains that are more amenable to a stable (and predatorless) environment than the external environment. Future research will address various adaptive mechanisms of bacteria-protist interactions that share fundamental processes with host immune responses.

ACKNOWLEDGMENTS

We thank P. Cooke for helping with the SEM images and two anonymous reviewers who provided helpful suggestions, ideas, and future studies.

This project was partially supported by The Centre for Marine BioInnovation and the Australian Research Council, grant DPDP1096481, and NSF IOS-0744498, NIH NIAID 1SC1AI081659-01, and NIH ARRA-3SC1AI081659-02S1 to M.K.N. A.C.-D. was supported by RISE (NIH NIGMS R25GM061222) and NASA (NMSGC).

We have no conflicts of interests to declare.

REFERENCES

1. **Watnick P, Kotler R.** 2000. Biofilm, city of microbes. J. Bacteriol. **182**: 2675–2679.
2. **Yildiz FH, Visick KL.** 2008. *Vibrio* biofilms: so much the same yet so different. Trends Microbiol. **17**:109–118.
3. **Chavez-Dozal A, Nishiguchi MK.** 2011. Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri.* J. Basic Microbiol. **51**:452S–458S.

4. **Costerton JW, Stewart PS, Greenberg EP.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284**:1318–1322.
5. **Mah TF, O'Toole GA.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. **9**:34–39.
6. **Espeland EM, Wetzel RG.** 2001. Complexation, stabilization, and UV photolysis of extracellular and surface-bound glucosidase and alkaline phosphatase: implications for biofilms microbiota. Microb. Ecol. **42**:572–585.
7. **Dalesman S, Karnik V, Lukowiak K.** 2011. Sensory meadiation of memory blocking stressors in the pond snail *Lymnaea stagnalis.* J. Exp. Biol. **214**:2528–2533.
8. **Antunes LCM, Schaefer AL, Ferreira RBR, Qin N, Stevens AM, Ruby EG, Greenberg EP.** 2007. Transcriptome analysis of the *Vibrio fischeri* LuxR-LuxI regulon. J. Bacteriol. **189**:8387–8391.
9. **Ariyakumar DS, Nishiguchi MK.** 2009. Characterization of two host specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol. Lett. **299**:65–73.
10. **Soto W, Gutierrez J, Remmega MD, Nishiguchi MK.** 2009. Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. Microb. Ecol. **57**:140–150.
11. **Kadouri D, O'Toole GA.** 2005. Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. Appl. Environ. Microbiol. **71**:4044–4051.
12. **Matz C, Kjelleberg S.** 2005. Off the hook: how bacteria survive protozoan grazing. Trends Microbiol. **13**:302–307.
13. **Adiba S, Nizak C, Van Baalen M, Denamur E, Depaulis F.** 2010. From grazing resistance to pathogenesis: the coincidental evolution of virulence factors. PLoS One **5**:e11882. doi:10.1371/journal.pone.0011882.
14. **Erken M, Weitere Kjelleberg M, McDougald SD.** 2011. In situ grazing resistance of Vibrio cholerae in the marine environment. FEMS Microbiol. Ecol. **76**:504–512.
15. **Matz C, Jurgens K.** 2001. Effects of hydrophobic and electrostatic cell surface properties of bacteria on feeding of heterotrophic nanoflagellates. Appl. Environ. Microbiol. **67**:814–820.
16. **Hazlett BA.** 2003. Predator recognition and learned irrelevance in the crayfish *Orconectes virilis.* Ethology **109**:765–780.
17. **Miki T, Jacquet S.** 2008. Complex interactions in the microbial world: underexplored key links between viruses, bacteria and protozoan grazers in aquatic environments. Aquat. Microb. Ecol. **51**:195–208.
18. **Weitere M, Bergfeld T, Rice SA, Matz C, Kjelleberg S.** 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environ. Microbiol. **7**:1593–1601.
19. **Greiner LL, Edwards JL, Shao J, Rabinak C, Entz D, Apicella MA.** 2005. Biofilm formation by Neisseria gonorrhoeae. Infect. Immun. **73**:1964–1970.
20. **Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S.** 2005. Biofilm formation and phenotype variation enhance predation-driven persistence of *Vibrio cholerae.* Proc. Natl. Acad. Sci. U. S. A. **102**:16819–16824.
21. **Queck SY, Weitere M, Moreno AM, Rice SA, Kjelleberg S.** 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environ. Microbiol. **8**:1017–1025.
22. **Turovskiy Y, Rosenberg L, Chikindas M.** 2007. Autoinducer-2-mediated quorum sensing is not involved in *Listeria monocytogenes* adaptive responses to the food preservatives lactic acid and nisin. J. Food Saf. **27**:386–399.
23. **Koh KS, Matz C, Tan CH, Le HL, Rice SA, Marshall DJ, Steinberg PD, Kjelleberg S.** 2012. Minimal increase in genetic diversity enhances predation resistance. Mol. Ecol. **21**:1741–1749.
24. **Matz C, Webb JS, Schupp PJ, Phang SY, Penesyan A, Egan S, Steinberg P, Kjelleberg S.** 2008. Marine biofilm bacteria evade eukaryotic predation by targeted chemical defense. PLoS One **3**:e2744. doi:10.1371/journal.pone.0002744.
25. **Arndt H, Schmidt-Denter K, Auer B, Weitere M.** 2003. Protozoans and biofilms, p 173–189. *In* Krumbein WE, Paterson DM, Zavarzin GA (ed), Fossil and recent biofilms. Kluwer Academic Publishers, Dordrecht, The Netherlands.
26. **Bohme A, Risse-Buhl U, Kusel K.** 2009. Protists with different feeding modes change biofilm morphology. FEMS Microbiol. Ecol. **69**:158–169.
27. **Zamborsky DJ, Nishiguchi MK.** 2011. Phylogeographical patterns among sympatric populations of sepiloid squids and their *Vibrio* symbionts in the Mediterranean Sea. Appl. Environ. Microbiol. **77**:642–649.
28. **Artolozaga I, Ayo B, Latatu A, Azua I, Unanue M, Irriberri J.** 2000. Spatial distribution of protists in the presence of macroaggregates in a marine system. FEMS Microbiol. Ecol. **33**:191–196.
29. **Boenigk J, Arndt H.** 2002. Bacterivory by heterotrophic flagellates: community structure and feeding strategies. Antonie Van Leeuwenhoek **81**:465–480.
30. **Thompson JR, Randa MA, Marcelino LA, Tomita-Mitchell A, Lim E, Polz MF.** 2004. Diversity and dynamics of a north atlantic coastal *Vibrio* community. Appl. Environ. Microbiol. **70**:4103–4110.
31. **Nishiguchi MK, Lopez JE, Boletzky SV.** 2004. Enlightenment of old ideas from new investigations: the evolution of bacteriogenic light organs in squids. Evol. Dev. **6**:41–49.
32. **Nyholm SV, Nishiguchi MK.** 2008. The evolutionary ecology of a sepiolid squid-*Vibrio* association: from cell to environment. Vie Milieu Paris **58**:175–184.
33. **Ruby EG, McFall-Ngai MJ.** 1999. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri.* Trends Microbiol. **7**:414–420.
34. **Jurgens K, Matz C.** 2002. Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. Antonie Van Leeuweenhoek **81**:413–434.
35. **Kicklighter CE, Hay ME.** 2006. Integrating prey defensive traits: contrasts of marine worm from temperate and tropical habitats. Ecol. Monogr. **76**:195–215.
36. **Ruby EG.** 1996. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Annu. Rev. Microbiol. **50**:591–624.
37. **Ruby EG, Lee K.** 1998. The *Vibrio fischeri-Euprymna scolopes* light organ association: current ecological paradigms. Appl. Environ. Microbiol. **64**:805–812.
38. **Erken M, Farrenschon N, Speckmann S, Arndt H, Weitere M.** 2012. Quantification of individual flagellate-bacteria interactions within semi-natural biofilms. Protist **163**:632–642.
39. **Castillo MG, Goodson MS, McFall-Ngai M.** 2009. Identification and molecular characterization of a complement C3 molecule in lophotrochozoan, the Hawaiian bobtail squid *Euprymna scolopes.* Dev. Comp. Immunol. **33**:69–76.
40. **McFall-Ngai MJ, Nyholm S, Castillo M.** 2010. The role of the immune system in the initiation and persistence of the *Euprymna scolopes-Vibrio fischeri* symbiosis. Semin. Immunol. **22**:48–53.
41. **Hammer BK, Bassler BL.** 2003. Quorum sensing controls biofilm formation in Vibrio cholerae. Mol. Microbiol. **50**:101–104.

analytical chemistry

Article

pubs.acs.org/ac

Investigating Catalase Activity Through Hydrogen Peroxide Decomposition by Bacteria Biofilms in Real Time Using Scanning Electrochemical Microscopy

Erwin Abucayon,[†] Neng Ke,[†] Renaud Cornut,[‡] Anthony Patelunas,[§] Douglas Miller,[§] Michele K. Nishiguchi,[§] and Cynthia G. Zoski*,[†]

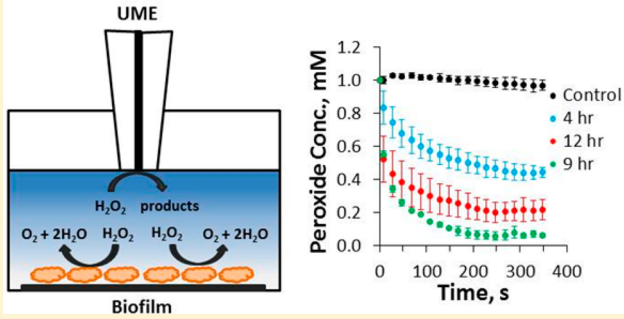
[†]Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, New Mexico 88003, United States

[‡]Laboratoire de Chimie des Surfaces et Interfaces, Gif-sur-Yvette, France

[§]Department of Biology, New Mexico State University, Las Cruces, New Mexico 88003, United States

Supporting Information

ABSTRACT: Catalase activity through hydrogen peroxide decomposition in a 1 mM bulk solution above *Vibrio fischeri* (*γ-Protebacteria-Vibrionaceae*) bacterial biofilms of either symbiotic or free-living strains was studied in real time by scanning electrochemical microscopy (SECM). The catalase activity, in units of micromoles hydrogen peroxide decomposed per minute over a period of 348 s, was found to vary with incubation time of each biofilm in correlation with the corresponding growth curve of bacteria in liquid culture. Average catalase activity for the same incubation times ranging from 1 to 12 h was found to be 0.28 ± 0.07 μmol H_2O_2/min for the symbiotic biofilms and 0.31 ± 0.07 μmol H_2O_2/min for the free-living biofilms, suggesting similar catalase activity. Calculations based on Comsol Multiphysics simulations in fitting experimental biofilm data indicated that approximately (3 ± 1) × 10^6 molecules of hydrogen peroxide were decomposed by a single bacterium per second, signifying the presence of a highly active catalase. A 2-fold enhancement in catalase activity was found for both free-living and symbiotic biofilms in response to external hydrogen peroxide concentrations as low as 1 nM in the growth media, implying a similar mechanism in responding to oxidative stress.



We report the use of scanning electrochemical microscopy (SECM) in investigating catalase activity, a defense mechanism of many bacteria, at the surface of biofilms of the bioluminescent marine bacterium *Vibrio fischeri* (*V. fischeri*). Understanding the processes by which beneficial (i.e., mutualistic) and pathogenic bacteria successfully colonize host tissues is a major goal in biology and biomedicine.[1−8] *V. fischeri* is a beneficial bacterium found within the symbiotic light-emitting organs of sepiolid squids and monocentrid fishes (Mollusca: Cephalopoda). This unique system is used as an experimental model because the natural occurrence between *Vibrio* bacteria and specific host tissues can be used to explore the stages by which such associations develop as well as the mechanisms underlying specificity and host signaling without confounding effects typically observed in the presence of multiple bacterial species.[9] Sepiolid squids are nocturnal predators that hide during the day but emerge each night to forage in the water column for prey. The luminous, gram-negative *V. fischeri* colonize the squid's internal light-emitting organ and generate bioluminescence, which provides a camouflage strategy termed counterillumination that enables the squid to match downwelling moonlight by silhouette reduction.[10] In return, *V. fischeri* bacteria receive nutrients from the squid host.[11] This mutualistic relationship is the basis for a continuing and long-term symbiosis.

All host animals instinctively protect themselves against colonization by inappropriate or pathogenic microorganisms. A central theme in bacteria−host interactions is that the symbiont either avoids damage by host immune defenses and/or communicates with host cells to modulate or regulate them.[12−20] Both mechanisms may contribute to symbiont specificity and are used in the *V. fisheri*-squid symbiosis. One of these multiple selective mechanisms is the imposition of oxidative stress, where oxygen is partially reduced by a squid-activated-membrane oxidase complex to produce hydrogen peroxide.[21] The hydrogen peroxide reacts with squid-produced halide peroxidase to generate hypochlorous acid, a known bactericide. *V. fischeri* bacteria counteract this oxidative stress through the presence of a highly active catalase in their periplasm. The catalase rapidly reduces the effective concentration of hydrogen peroxide (i.e., through decomposition to water and oxygen) near bacteria cell surfaces.[16−18,22] Other

Received: August 5, 2013
Accepted: December 13, 2013
Published: December 13, 2013

symbiotic associations make use of catalase to eliminate host generated hydrogen peroxide during colonization, such as microbes in nasal cavities,[23] plant roots,[24] and in soil where fungi produce peroxide to compete against other organisms.[25] Some bacteria have developed high catalase activity in order to adapt to extreme hydrogen peroxide environments such as encountered in processing plants where hydrogen peroxide is used as a bleaching agent.[26−28] Catalase activity has also been reported for pathogenic bacteria which coexist with hydrogen peroxide producing beneficial bacteria in oral polymicrobial systems.[29] Therefore, current understanding of damage and defense due to oxidative stress continues to be a topic of great interest and has largely been pioneered through studies in *E. coli*.[30]

Catalase is also responsible for the decomposition of hydrogen peroxide produced as a byproduct of oxygen metabolism, and its activity has been found to vary during the bacteria growth cycle.[22,31] For example, catalase activity was enhanced more than 3-fold in planktonic *V. fischeri* cells approaching stationary phase.[22] Addition of micromolar hydrogen peroxide during early logarithmic growth resulted in a 4-fold increase in activity.[22] *V. fischeri* catalase expression has been reported to be controlled by a single catalase gene, *katA*. Kat A is the predominantly expressed catalase enzyme whether hydrogen peroxide is produced internally through metabolic processes or externally from a host.[22] *katA* is also common in many bacteria and is responsible for their catalase expression.[5] Other bacteria, such as *E. coli*, induce a catalase as a result of oxidative stress that is distinct from a separate catalase induced at stationary phase.[30]

Catalase activity is most commonly determined quantitatively using spectroscopy[22,31−33] or Clark-type electrodes[34] to measure hydrogen peroxide decomposition in planktonic cell extracts. It is also measured qualitatively by judging the degree of bubbling due to the addition of hydrogen peroxide to bacteria cells.[22,35] These methods lack the ability to quantify local hydrogen peroxide concentration at the surface of a biofilm. Here we report the use of SECM[36,37] to investigate catalase activity through the change in hydrogen peroxide concentration as a function of time due to its decomposition at the surface of *V. fischeri* biofilms. In SECM, a sensing tip (i.e., an ultramicroelectrode (UME) of size 10−25 μm diameter) is positioned above a substrate through a feedback approach curve (i.e., tip current, i_T, vs distance, d, where d is scanned in the z direction to a fixed position above a substrate such as a biofilm) and measures the local transient hydrogen peroxide concentration over the biofilm in response to its catalase activity.[38] This allows the effective concentration of hydrogen peroxide above a biofilm to be measured *in situ*. We also investigate catalase activity as a function of incubation time of the biofilm and relate this activity to the growth curve of planktonic bacteria. Furthermore, we demonstrate that catalase activity increases in biofilms exposed to hydrogen peroxide concentrations ranging from nM to μM early in the logarithmic phase of growth. Both symbiotic and free-living strains (i.e., cannot infect sepiolid squids but do co-occur) of *V. fischeri* bacteria were studied in order to determine if differences exist in their catalase activity. Electrochemical measurements of hydrogen peroxide concentration have been reported in several studies,[39−45] but none have dealt with hydrogen peroxide decomposition and its relationship to catalase activity as a function of biofilm incubation and increased activity during biofilm growth and on external exposure to hydrogen peroxide during early logarithmic growth. SECM is increasingly being applied to biological systems to measure local concentration over soft biological samples and for imaging.[46−49] For example, SECM was recently used in real-time mapping of local hydrogen peroxide production across a biofilm of oral bacteria and in observing its consumption in a polymicrobial biofilm through a mechanism involving one of the bacteria during spatial scanning,[50] in the discovery of a biofilm electrocline (i.e., of oral bacteria) using real-time 3D metabolite analysis,[51] and in assessing multidrug resistance on cell coculture patterns.[52]

EXPERIMENTAL SECTION

Chemicals. Artificial seawater (Instant Ocean), tryptone (Difco), yeast extract (Difco), glycerol (Sigma), agar (Difco), $MgSO_4{\cdot}7H_2O$ (Fisher Scientific), $CaCl_2{\cdot}2H_2O$ (Fisher Scientific), NaCl (Fisher Scientific), KCl (Fisher Scientific), K_2HPO_4 (Fisher Scientific), NH_4Cl (Fisher Scientific), Tris-HCl (Fisher Scientific), H_2O_2 (Fisher Scientific, 30%).

Media. Bacteria strains were grown in SWT (Seawater-tryptone) media, containing 70% artificial seawater, 0.5% tryptone, 0.3% yeast extract, and 0.3% glycerol. DMM (Defined minimal media, pH 8.0) contained 12.3 g/L $MgSO_4{\cdot}7H_2O$, 1.47 g/L $CaCl_2{\cdot}2H_2O$, 17.5 g/L NaCl, 0.745 g/L KCl, 58.1 g/L K_2HPO_4, 1.0 g/L NH_4Cl, and 3 mL/L glycerol in 50 mM Tris-HCl (pH 7.9). SWT agar plates contained 1.5% agar.

Bacteria Growth Curves. Two wild type *V. fischeri* strains, collected by M.K.N., were used: CB37 (free-living, collected in Australia (Coogee Bay, New South Wales)); ETBB10-1 (symbiotic from *Euprymna tasmanica* squid, collected in New South Wales, Australia (Botany Bay)). *V. fischeri* were cultured overnight in 50 mL of SWT media from cultured freezer stocks stored at −80 °C. The overnight cell suspension (in triplicate) was diluted to an absorbance of 0.3 with SWT. Subcultures contained 1 mL of each diluted overnight suspension inoculated into 200 mL of fresh SWT media. A control flask of 1 mL of SWT in 200 mL of fresh SWT was also prepared. All incubations were performed in a New Brunswick Scientific Excella E25 Incubatore/Shaker programmed at 28 °C, 250 rpm. Volumes of 1 mL were taken from each flask, and absorbance was measured on a ThermoScientific BioMate 3 spectrophotometer at 600 nm at selected times (12 h for *V. fischeri* CB37 and 24 h for *V. fischeri* ETBB10-1).

Biofilm Formation. Overnight cultures (in triplicate) of *V. fischeri* were grown in 50 mL of SWT media from cultured freezer stocks stored at −80 °C. Subcultures contained 1 mL from each overnight culture and placed in 50 mL SWT; bacteria subcultures were incubated to an absorbance (600 nm) of 0.3. All incubations were performed in a New Brunswick Scientific Excella E25 Incubatore/Shaker programmed at 28 °C, 250 rpm; absorbance was measured on a ThermoScientific BioMate 3 spectrophotometer at 600 nm. Cells were pelleted at 2500 rpm for 20 min by centrifugation (Sorvall Legend RT Centrifuge) and resuspended in SWT media to an absorbance (600 nm) of 1.5. The cell suspension (100 μL) was spread on a glass coverslip (Thomas Scientific, #1.5, 18 mm diameter) laid on a freshly prepared SWT agar plate using a bent glass rod. Each coverslip was incubated for 10 min at 28 °C to allow bacteria attachment and then transferred to a fresh Petri dish (VWR Polystyrene, 35 mm × 10 mm, Falcon Easy Grip) containing 500 μL of SWT and incubated at 28 °C under static conditions for specific incubation times ranging from 1 to 24 h. Four coverslips were prepared from each cell suspension for each incubation time. For incubations greater than 6 h, 500 μL

dx.doi.org/10.1021/ac402475m | *Anal. Chem.* 2014, 86, 498−505

of fresh SWT was added as follows: for 9 and 12 h incubations, at 6 h into the incubations; for 24 h incubations, at 6 h, at 12 and 18 h (500 μL of media removed and 500 μL of fresh SWT added). After incubation, each biofilm coverslip was rinsed with SWT by careful dipping three times. The back of each coverslip was dried with Kim Wipes and secured in a fresh Petri dish (VWR Polystyrene, 35 mm × 10 mm, Falcon Easy Grip) with double sided tape (3M) and used in the SECM experiments described below. Each series of incubations for each bacterial strain were repeated with three separate overnight cultures on three separate days.

Electrodes and Electrochemical Cell. Pt wire (10 μm diameter, 99.99% hard, Goodfellow) was used in fabricating ultramicroelectrode (UME) SECM tips, as described elsewhere.[53,54] The SECM tips used in these experiments were shaped to RG = 3, where RG is the ratio between the SECM tip diameter and the diameter of the active UME surface. Prior to use, all SECM tips were polished with 0.05 μm alumina on microcloth pads (Buehler, Lake Bluff, IL) and cycled in 0.1 M H_2SO_4 in the potential range from −0.55 to 0.6 V vs Ag/AgCl, at 0.1 V/s for 80 cycles to a constant cyclic voltammogram. Pt wire (0.5 mm diameter, 99.99%, as drawn, Goodfellow) was used as the counter electrode, and the reference electrode was Ag/AgCl (satd).

The electrochemical cell consisted of a Petri dish (VWR Polystyrene, 35 mm × 10 mm, Falcon Easy Grip) with the biofilm coverslip secured at the bottom center with double sided tape (3M). A solution of 2.5 mL of DMM spiked with H_2O_2 to a concentration of 1 mM was used.

Instrumentation. A CHI 900 SECM (CH Instruments, Texas) coupled with a home-built Faraday cage on a vibration table (VH3030W-OPT, Newport) was used in all SECM experiments. UMEs were characterized and cleaned electrochemically using a CHI 760 D potentiostat (CH Instruments, Texas).

SECM Experiments. The SECM tip was positioned using previously reported procedures.[37] An approach curve (tip current, i_T vs distance, d) was recorded above the biofilm immersed in 2.5 mL of DMM using oxygen as a mediator by holding the tip potential E_T at −0.47 V vs Ag/AgCl (satd.). The distance between tip and biofilm was then fixed at 100 μm above the biofilm in order to avoid effects from the biofilm topography, while still being able to record the current vs time transient for the hydrogen peroxide decomposition.

A background chronoamperometric curve was recorded in the absence of hydrogen peroxide at the same height by pulsing the tip at 0.65 V for 10 s and reversing to 0.15 V for 10 s as shown in Figure S1 in the Supporting Information; the duration of the potential pulse sequence was 360 s, with the last measurement time at 348 s at E_T = 0.65 V. The DMM was then spiked with hydrogen peroxide while stirring toward the end of the 5 s quiet time in order to ensure a uniform final concentration of 1 mM. The current due to hydrogen peroxide oxidation was measured as a function of time during each tip potential pulse at E_T = 0.65 V. This potential pulse was followed by another at E_T = 0.15 V for 10 s in order to electrochemically clean the UME surface. The current at 8 s during each 10 s pulse at 0.65 V was background subtracted and converted to H_2O_2 concentration using calibration curves similar to that shown in Figure S2 in the Supporting Information. Calibration curves were recorded over bare coverslips at peroxide concentrations ranging from 0.2 to 1.2 mM and under identical conditions as the biofilm experiments. The current vs time curves and the concentration vs time curves corresponding to each incubation time were averaged for each of the three separate overnight subcultures. Control experiments in the presence and absence of hydrogen peroxide were performed over a bare, glass coverslip for comparison.

RESULTS AND DISCUSSION

Figure 1 shows the schematic of the experimental setup in which a SECM tip positioned 100 μm above a biofilm monitors

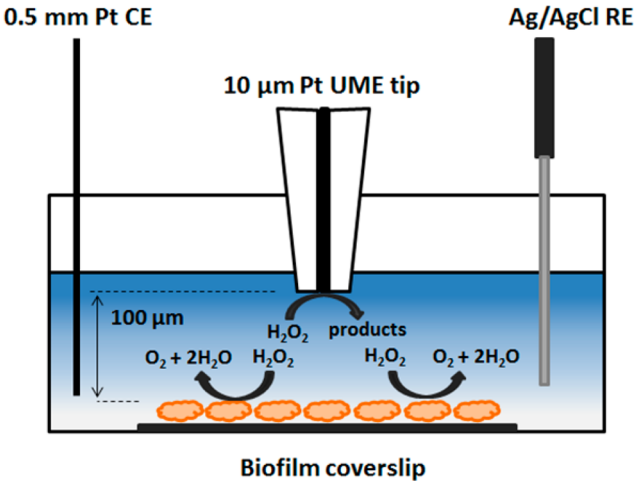


Figure 1. Schematic of the electrochemical experimental setup for SECM real-time measurement of hydrogen peroxide decomposition by a bacterial biofilm.

the hydrogen peroxide concentration gradient that develops with time in the electrochemical cell as a bulk solution of 1 mM hydrogen peroxide is decomposed at a biofilm surface. The decomposition of hydrogen peroxide by *V. fischeri* CB37 (free-living) and *V. fischeri* ETBB10-1 (symbiotic) biofilms as a function of incubation time (e.g., CB37, 1−12 h; ETBB10-1, 1−24 h) are shown in Figures 2a and 3a, respectively. Each concentration point in the graphs corresponds to the chronoamperometric current due to hydrogen peroxide oxidation at a particular time (e.g., t = 8 s during a series of 10 s pulses over a period of 348 s) as shown in the corresponding current vs time plots in Figures S3 and S4 in the Supporting Information and calculated using calibration curves similar to that shown in Figure S2 in the Supporting Information. The error bars in Figures 2a and 3a and Figures S3 and S4 in the Supporting Information were calculated based on three independent bacteria overnight and subcultures and at least three independent measurements within each subculture for each *V. fischeri* strain. Control experiments over bare glass coverslips were recorded in the presence of 1 mM hydrogen peroxide and showed little decrease in peroxide concentration over the duration of the experiment compared to those in the presence of a biofilm as shown in the figures.

For the *V. fischeri* CB37 and ETBB10-1 biofilms, the hydrogen peroxide concentration initially decreased from a bulk concentration of 1 mM to quasi-steady-state concentrations (i.e., corresponding to increasingly less negative currents in Figures S3 and S4 in the Supporting Information). The initial decay and the final quasi-steady-state hydrogen peroxide concentrations were found to be related to the incubation time of the individual biofilms and to the *V. fischeri* strains. Decreasing hydrogen peroxide concentration detected at the

dx.doi.org/10.1021/ac402475m | *Anal. Chem.* 2014, 86, 498−505

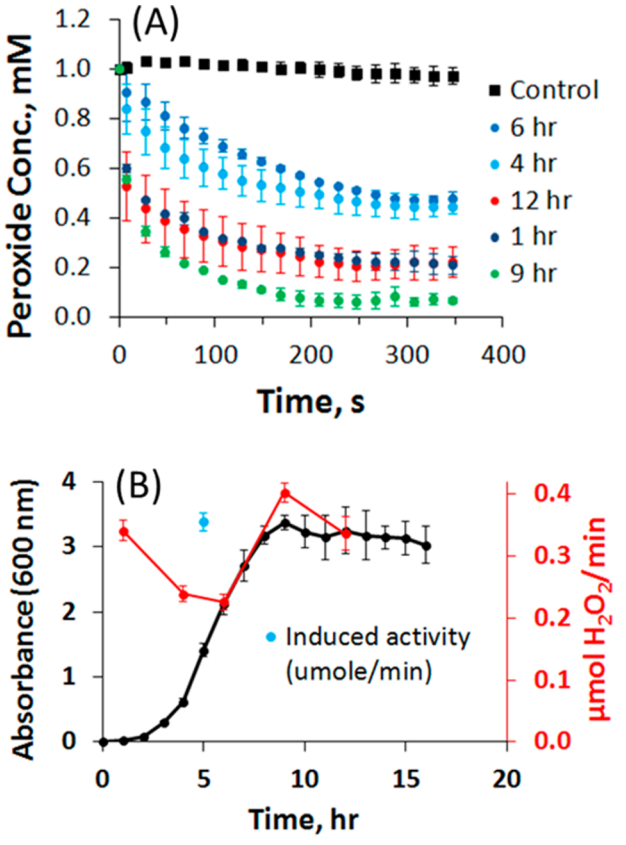


Figure 2. Hydrogen peroxide decomposition activity on *V. fischeri* CB37 biofilms at different incubation times. (A) Hydrogen peroxide concentration vs time curves generated from the corresponding current vs time curves (Figure S3 in the Supporting Information). (B) Comparison of growth curve (left axis, black line and solid dots) for planktonic *V. fischeri* CB37 bacteria and hydrogen peroxide decomposition activity (right axis, red line, and solid dots) on *V. fischeri* CB37 biofilms at designated incubation times. Decomposition activity represents the difference between initial and quasi-steady H_2O_2 moles divided by the analysis time of 348 s. Error bars represent standard deviation based on three independent measurements of each of three overnight cultures. The solid blue dot corresponds to incubation with 10 μM hydrogen peroxide at 4 h and measurement of induced activity at 5 h.

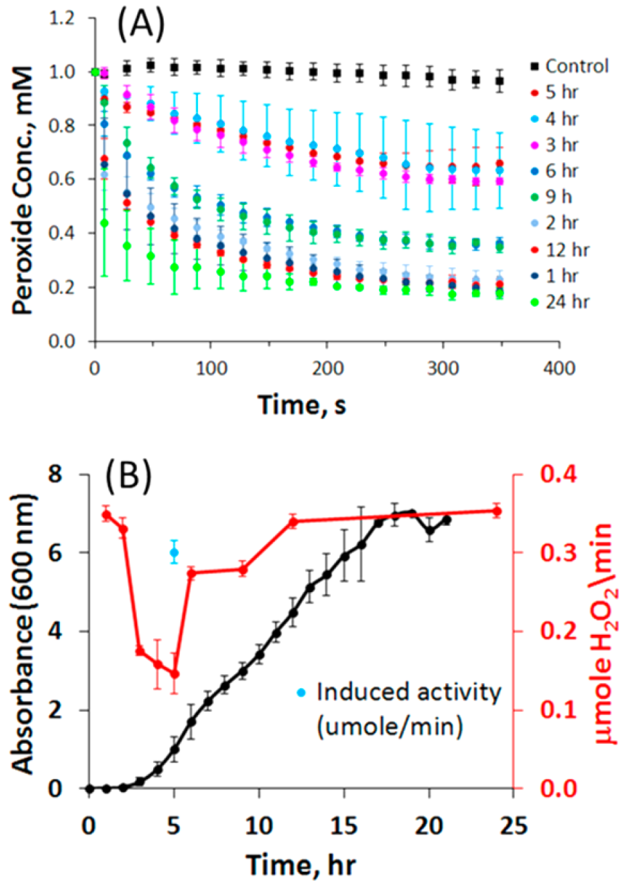


Figure 3. Hydrogen peroxide decomposition activity on *V. fischeri* ETBB biofilms at different incubation times. (A) Hydrogen peroxide concentration vs time curves generated from the corresponding current vs time curves (Figure S4 in the Supporting Information). (B) Comparison of growth curve (left axis, black line and solid dots) for planktonic *V. fischeri* ETBB10-1 bacteria and hydrogen peroxide decomposition activity (right axis, red line and solid dots) at *V. fischeri* ETBB10-1 biofilms at designated incubation times. Decomposition activity represents the difference between initial and quasi-steady H_2O_2 moles divided by the analysis time of 348 s. Error bars represent standard deviation based on three independent measurements of each of three overnight cultures. The solid blue dot corresponds to incubation with 10 μM, 10 nM, and 1 nM hydrogen peroxide at 4 h and measurement of induced activity at 5 h. Error bars represent standard deviation based on three independent measurements of each of three overnight cultures for each peroxide concentration.

SECM tip indicated increased hydrogen peroxide decomposition activity at the surface of the biofilm. We attribute this activity to the presence of catalase in the bacteria of the biofilm.

Correlation of Biofilm Peroxide Decomposition With Planktonic Bacteria Growth Curves. Figure 2A shows the change in the hydrogen peroxide concentration vs time in the presence of *V. fischeri* CB37 biofilms at different stages of incubation. In comparison, the control curve in the absence of *V. fischeri* CB37 biofilm demonstrates that the hydrogen peroxide concentration did not change significantly from the 1 mM bulk concentration over the measurement time. In the presence of the biofilms at different stages of incubation, there was a decrease in hydrogen peroxide concentration in the vicinity of the tip which was found to vary in three general incubation groups (i.e., 6, 4 h, and 12, 1 h, and 9 h) according to the growth stages of the bacteria. Figure 2B shows the growth curve of planktonic *V. fischeri* CB37, recorded as absorbance (600 nm) vs time, at hourly intervals over 16 h. This growth curve features a lag time in growth up to approximately 2 h, a log phase growth between 3 and 9 h and stationary growth after 10 h. Figure 2B also exhibits variation in hydrogen peroxide decomposition activity, expressed in units of micromoles hydrogen peroxide decomposed per minute (i.e., the difference between the initial and quasi-steady H_2O_2 moles divided by the analysis time of 348 s), which was calculated from the hydrogen peroxide concentration vs time curves (Figure 2A). Similar units have been used in previous discussions of catalase activity in planktonic *V. fischeri* bacteria where one U of catalase activity corresponds to 1 μmol of hydrogen peroxide decomposed per minute or, more generally, as U/mg catalase protein or U/cell.[22,55] Here, we similarly correlate hydrogen peroxide decomposition with catalase activity in *V. fischeri* biofilms. Early decrease in catalase activity from 1 h (lag phase) to 4 h incubation (log phase) can be attributed to dilution of catalase as the number of bacteria cells increases without a corresponding increase in catalase synthesis.

dx.doi.org/10.1021/ac402475m | *Anal. Chem.* 2014, 86, 498−505

During this time, existing catalase is divided between two daughter cells during each division.[31] The *V. fischeri* CB37 biofilm formed during 1 h incubation (i.e., lag phase) was found to decompose hydrogen peroxide more efficiently than biofilm formed from 4 h incubations (i.e., log phase, Figure 2A). Similarly, catalase activity of *V. fischeri* CB37 biofilm formed during 6 h incubation (log phase) was only slightly lower than that formed during the 4 h incubation. There was an increase in the catalase activity as the incubation time for the *V. fischeri* CB37 biofilms approached the stationary phase of the *V. fischeri* CB37 growth curve, reaching maximum activity at 9 h incubation (i.e., end of log phase). This decrease in catalase activity during the log phase and increase on approach to the stationary phase has been reported for other planktonic *V. fischeri* strains, where catalase activity was determined in cell extracts of bacteria measured at different times during its growth in rich media with shaking.[22,26−28,31]

Figure 3A shows the change in the hydrogen peroxide concentration vs time in the presence of *V. fischeri* ETBB10-1 biofilms at different incubation times over 24 h. Similar to *V. fischeri* CB37, the presence of *V. fischeri* ETBB10-1 biofilms produced a decrease in hydrogen peroxide concentration in the vicinity of the tip, which varied according to growth stages of the planktonic bacteria. Figure 3B shows the growth curve for planktonic *V. fischeri* ETBB bacteria, recorded as absorbance (600 nm) vs time over 21 h. This growth curve shows that planktonic *V. fischeri* ETBB10-1 develop through two log phases and two semistationary phases before reaching a stable stationary phase at approximately 16 h incubation. In general, the growth curve features a lag time in growth up to about 2 h, log phase growth between 3 and 7 h, semistationary growth between 7 and 10 h, a second log phase growth between 10 and 13 h, and a second semistationary growth starting at 13 h, followed by stationary growth at about 17 h. The two separate log phase periods feature a similar slope that is steeper than the two semistationary slopes, which are also similar to each other. Figure 3B also shows the variation in catalase activity (i.e., expressed as micromoles of hydrogen peroxide decomposed per minute) calculated from hydrogen peroxide concentration vs time curves in Figure 3A at different incubation times and its correlation with the growth curve. There is a dramatic decrease in catalase activity from one and two hour incubation (lag phase) to three hour incubation (log phase), followed by a steady decrease in activity from 3 to 5 h incubation (log phase). At 6 h incubation (log phase) corresponding to the entry into the first semistationary phase, the *V. fischeri* ETBB10-1 biofilm shows a dramatic increase in catalase activity, followed by a slight decrease in activity at 9 h incubation corresponding to the second log phase. The *V. fischeri* ETBB10-1 biofilm shows increased activity at 12 h incubation corresponding to the entry into the second semistationary phase, where the activity is approximately maintained through 24 h incubation (i.e., stationary phase).

V. fischeri ETBB10-1 biofilms show catalase activity comparable to those of *V. fischeri* CB37 up to 5 h incubation. At 6 h incubation, *V. fischeri* ETBB10-1 biofilms show slightly higher catalase activity due to entry into the first semistationary growth phase in comparison to *V. fischeri* CB37 biofilms which are still in the log phase of growth. At 9 h incubation, *V. fischeri* ETBB10-1 biofilms begin a second log phase growth with a corresponding decrease in catalase activity compared to *V. fischeri* CB37 biofilms, which are entering the stationary growth phase. *V. fischeri* ETBB10-1 biofilms enter a second semistationary growth phase at 12 h incubation with catalase activity comparable to that of *V. fischeri* CB37 now in the stationary phase. Overall, the average catalase activity for the common incubation times of 1, 4, 6, 9, and 12 h was found to be 0.28 ± 0.07 μmol H_2O_2/min for the symbiont (i.e., *V. fischeri* ETBB10-1) compared to 0.31 ± 0.07 μmol H_2O_2/min for the free living (i.e., *V. fischeri* CB37) *V. fischeri* biofilms suggesting that catalase activity is similar for both the *V. fischeri* CB37 and ETBB10-1 biofilms, despite differences in the planktonic growth curve behavior as well as life history strategy (i.e., free-living versus symbiotic).

Catalase activity of the *V. fischeri* ETBB10-1 and CB37 biofilms measured using SECM can be compared to those reported for various planktonic *Vibrio* species from different hosts and geographic locations, where the activities were measured using spectroscopy and reported in units of μmol H_2O_2 decomposed/min/cell.[55] Such comparisons are of interest from an evolutionary standpoint since different *Vibrio* bacteria encompass a variety of life history strategies (e.g., free-living, beneficial, pathogenic, commensal, saprophytic) and may have specific uses for catalase besides fending off immune response from a particular host. Examples include *V. fischeri* (host: fish, shrimp; Black Sea) with catalase activities ranging from 1 to 2 × 10^{-7}, *Vibrio harveyi* (host: mussel; Black Sea) with activities ranging from 4 to 7 × 10^{-8}, and *Vibrio sp.* (host: mollasc, fish, shrimp, free living; Black and Azov Seas) with activities ranging from 4 to 8 × 10^{-8}. In comparison, catalase activities for *V. fischeri* ETBB10-1 (host: squid; Botany Bay, Australia) and *V. fischeri* CB37 (free living; Coogie Bay, Australia) biofilms from Figures 2 and 3, respectively, in this work ranged from 2 to 5 × 10^{-9} μmol H_2O_2 decomposed/min/cell (i.e., based on bacteria cell density = 3.5 × 10^7/cm^2 and coverslip area of 2.54 cm^2), which are within 2 orders of magnitude of the planktonic values. Differences in catalase activity can be attributed to several factors including geographic location, type of host, cell density, and planktonic (i.e., the reported activities[55]) vs biofilm (i.e., our work) catalase activity, in addition to methodology (i.e., spectroscopy vs SECM).

Figure 4 shows fitted simulated (solid lines) curves which demonstrate that hydrogen peroxide concentration gradients that developed between the biofilm surface and the SECM tip could be fit to the experimental response (solid dots) by the biofilms upon exposure to 1 mM hydrogen peroxide. Although

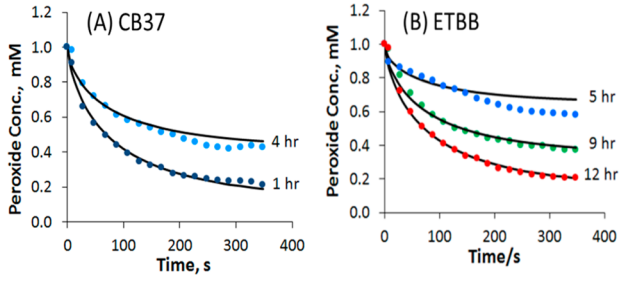


Figure 4. Comparison of simulated (solid black lines) and experimental (solid colored dots) of hydrogen peroxide concentration vs time curves at *V. fischeri* biofilms of CB37 (A) and ETBB10-1 (B) bacteria with an SECM tip located 100 μm above the biofilm surface. The diameter of the SECM tip was 10 μm (RG = 3). Simulated curves were generated with fluxes of (A) CB37: 1.6 × 10^{-10} mol/cm^2 s (4 h) and 2.5 × 10^{-10} mol/cm^2 s (1 h); (B) ETBB10-1: 0.9 × 10^{-10} mol/cm^2 s (5 h), 1.8 × 10^{-10} mol/cm^2 s (9 h), and 2.4 × 10^{-10} mol/cm^2 s (12 h) biofilms.

dx.doi.org/10.1021/ac402475m | *Anal. Chem.* 2014, 86, 498−505

SECM is a very useful method for measuring the local concentration of hydrogen peroxide above the biofilm or 100 μm from the biofilm surface, as previously discussed, it does not give the concentration or flux at the biofilm surface. Digital simulations can be used to determine the exact hydrogen peroxide flux at the biofilm surface in SECM experiments when the tip−substrate distance is known. Key parameters of the simulation model are given in the Supporting Information. The simulation assumes only diffusional mass transfer and a constant hydrogen peroxide flux to the biofilm surface. Competition for hydrogen peroxide by the 10 μm diameter SECM tip at a distance of 100 μm away from the much larger biofilm surface is negligible.[56,57] Fitted hydrogen peroxide fluxes at the *V. fischeri* CB37 biofilm surfaces were found to be 2.5×10^{-10} mol/cm^2 s for 1 h incubation (lag phase) and 1.6×10^{-10} mol/cm^{2}s for the 4 h incubation (log phase). On the basis of a colony forming unit (CFU) count of 9.0×10^8 bacteria/mL, a 0.100 mL bacteria suspension aliquot, and a coverslip area of 2.54 cm^2, the calculated bacteria density was 3.5×10^7 bacteria/cm^2. Thus, approximately 7.1×10^{-18} mol (1 h incubation) and 4.5×10^{-18} mol (4 h incubation) of hydrogen peroxide were decomposed by a single *V. fischeri* CB37 bacterium at the biofilm surface per second. This further corresponds to ~4×10^6 and 3×10^6 molecules of hydrogen peroxide decomposed by a single *V. fischeri* CB37 bacterium at the biofilm surface per second, respectively, in keeping with the fact that catalase is known to be a perfect catalyst with a turnover number of 4×10^7 s^{-1} when the enzyme is saturated with a substrate (e.g., hydrogen peroxide).[58] Similarly, fitted hydrogen peroxide fluxes at the *V. fischeri* ETBB10-1 biofilm surfaces were found to be 0.9×10^{-10} mol/cm^2 s for 5 h incubation (first log phase), 1.8×10^{-10} mol/cm^{2}s for the 9 h incubation (second log phase), and 2.4×10^{-10} mol/cm^{2}s for the 12 h incubation (second semistationary phase). Thus, approximately 2.5×10^{-18} (5 h incubation), 5.2×10^{-18} (9 h incubation), and 6.9×10^{-18} (12 h incubation) mol (i.e., ~2×10^6, 3×10^6, 4×10^6 molecules, respectively) of hydrogen peroxide were decomposed by a single *V. fischeri* ETBB10-1 bacterium at the biofilm surface per second. These calculations provide an important estimate of the amount of hydrogen peroxide decomposed at the bacterial surface, which may be useful in understanding the defense mechanism of a particular bacterial species or interactions with a host in terms of metabolite or external hydrogen peroxide concentration.

Enhanced Catalase Activity in Response to Oxidative Stress. A number of planktonic bacteria (e.g., *H. influenza*,[59] *E. coli*,[30] *V. fischeri*,[22] *V. rumoiensis* S-1T,[26−28] *Aggregatibacter actinomycetemcomitans*[60]) have been shown to respond to the presence of hydrogen peroxide in their external environment by inducing catalase gene expression with a resulting increase in catalase activity. We therefore also used SECM to explore whether the free-living *V. fischeri* CB37 and symbiotic ETBB10-1 biofilms show increased catalase activity in response to external hydrogen peroxide in the culture media. Biofilms were exposed to low concentrations of hydrogen peroxide during early log phase growth (i.e., at 4 h incubation) and catalase activity was measured 1 h after introducing hydrogen peroxide into the growth media (i.e., at 5 h incubation). Hydrogen peroxide concentrations of 1 nM, 10 nM, and 10 μM were used in order to explore the lowest concentration needed to externally enhance catalase activity. These catalase activity results, in units of μmole hydrogen peroxide decomposed per minute, are shown by the solid blue dots in Figures 2B and 3B for *V. fischeri* CB37 and ETBB10-1 biofilms, respectively. Corresponding current vs time and peroxide concentration vs time plots are shown as Figure S7A,B in the Supporting Information for *V. fischeri* ETBB10-1 and Figure S8A,B in the Supporting Information for *V. fischeri* CB37. For the *V. fischeri* ETBB10-1 biofilms, catalase activity was enhanced at 5 h incubation from 0.15 ± 0.03 μmol H_2O_2/min (i.e., no hydrogen peroxide added during incubation) to 0.28 ± 0.01 (10 μM H_2O_2), 0.30 ± 0.01 (10 nM H_2O_2), and 0.32 ± 0.03 (1 nM H_2O_2) μmol H_2O_2/min, suggesting a similar level of catalase gene expression at the three concentrations. The average activity (0.30 ± 0.02 μmol H_2O_2/min) due to external induction at these hydrogen peroxide concentrations as shown in Figure 3B represents an approximately 2-fold increase in catalase activity compared to the 5 h incubation in the absence of hydrogen peroxide. Figure S7A,B in the Supporting Information shows that the activity of the externally induced *V. fischeri* ETBB10-1 biofilm approaches that of the biofilm incubated for 12 h. For the *V. fischeri* CB37 biofilms induced with 10 μM hydrogen peroxide, catalase activity was also enhanced at 5 h incubation to 0.35 ± 0.01 μmol H_2O_2/min (10 μM H_2O_2) compared to 0.24 ± 0.01 μmol H_2O_2/min at 4 h incubation and 0.23 ± 0.01 μmol H_2O_2/min at 6 h incubation. Figure S8A,B in the Supporting Information shows that the activity of the externally induced *V. fischeri* CB37 biofilm approaches that of the biofilm incubated for 9 h. These results indicate that catalase activity is enhanced in free-living *V. fischeri* CB37 and symbiotic *V. fischeri* ETBB10-1 biofilms in response to the presence of hydrogen peroxide in their external environment. On the basis of reported studies of gene regulation and protein expression in planktonic *V. fischeri* as discussed previously,[22] it is likely that the catalase activity enhancement measured in the biofilms reported here is a result of catalase gene expression. The results also suggest that the catalase activity enhancement in response to oxidative stress occurs by a similar mechanism in both the *V. fischeri* free-living CB37 and symbiotic ETBB10-1 biofilms. This suggests that *V. fischeri* bacteria, regardless of whether they are capable of colonizing squid light organs or never see such an environment, rely on high catalase activity and production when in a biofilm. Since biofilms are usually under selective pressures to maintain their integrity due to biotic forces such as grazing by predators, catalase activity may provide one avenue to prevent breakdown and deter bacteriovores from breaking down the complex biofilm community.[61] Such preventative measures are costly but are important for preventing a breach in the biofilm structure.

■ CONCLUSIONS

Using SECM, we monitored the decomposition of 1 mM hydrogen peroxide over the surface of *V. fischeri* biofilms in real time, related catalase activity to micromoles of hydrogen peroxide decomposed over 348 s, and showed that catalase activity depends on the incubation time of the biofilms in comparison with the respective planktonic bacteria growth curves. Examination of biofilms of both free-living and symbiotic *V. fischeri* strains revealed that the average catalase activity of these biofilms was similar for the same incubation times and growth conditions but differing planktonic growth curve behavior. Calculations based on Comsol Multiphysics simulations demonstrated that approximately $(3 \pm 1) \times 10^6$ molecules of hydrogen peroxide were decomposed by a single bacterium at a *V. fischeri* biofilm surface per second, suggesting efficient catalase activity when compared with reported

dx.doi.org/10.1021/ac402475m | *Anal. Chem.* 2014, 86, 498−505

turnover numbers on the order of 10^7 s^{-1} for isolated catalases in general when saturated with a substrate such as hydrogen peroxide. We also used SECM to explore catalase activity in response to external hydrogen peroxide in the growth media and showed up to a 2-fold enhancement in catalase activity at hydrogen peroxide induction concentrations as low as 1 nM and similar catalase activity enhancement in the free-living and symbiotic biofilms. Collectively, these quantitative, real time, and *in situ* catalase activity investigations on *V. fischeri* biofilms by SECM provide hydrogen peroxide spatial, temporal, and decomposition rate data that were not possible from earlier studies of bacteria in liquid culture or bacteria cell extracts. These studies also provide insight into the efficiency of the catalase activity of *V. fischeri* biofilms which is important in combatting the imposition of oxidative stress either through control of oxygen or imposition of hydrogen peroxide by the squid during colonization. The similar catalase activity found for biofilms of symbiotic and free-living *V. fischeri* bacteria suggests that a high level of catalase activity and production may be important in maintaining the integrity of these biofilms, regardless of whether the bacteria are capable of colonizing squid light organs. Further quantitative investigation of the mechanisms that control metabolic and external hydrogen peroxide concentrations with other strains of symbiotic and free living *V. fischeri* will lead to a better understanding of the role of catalase activity in symbiotic bacteria during colonization.

■ ASSOCIATED CONTENT

Supporting Information

Electrochemistry and hydrogen peroxide concentration data; Comsol Multiphysics simulation model and description. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Fax: 575-646-2649. Phone: 575-646-5292. E-mail: czoski@nmsu.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The financial support of the National Science Foundation (Grant CHE-0809966 to C.G.Z) and a New Mexico State University Interdisciplinary Research Grant (C.G.Z., PI; M.K.N., Co-PI) are gratefully acknowledged. C.G.Z. thanks Prof. S. Lusetti and her group members Dr. L. Uranga and I. Menikpurage for valuable discussion and assistance with microbiology methodology and biochemical principles. CFU counts provided by N. Nourabadi and preliminary experiments by W. Castle and A. Devasurendra are acknowledged.

■ REFERENCES

(1) McFall-Ngai, M.; Heath-Heckman, E. A. C.; Gillette, A. A.; Peyer, S. M.; Harvie, E. A. *Sem. Immunol.* **2012**, *24*, 3−8.
(2) Rader, B. A.; Nyholm, S. V. *Biol. Bull.* **2012**, *223*, 103−111.
(3) Bosch, T. C. G.; McFall-Ngai, M. J. *Zoology* **2011**, *114*, 185−190.
(4) Visick, K. L.; Ruby, E. G. *Curr. Opin. Microbiol.* **2006**, *9*, 1−7.
(5) Ruby, E. G. *Nature* **2008**, *6*, 752−762.
(6) Ruby, E. G. *Annu. Rev. Microbiol.* **1996**, *50*, 591−624.
(7) Nishiguchi, M. K.; DeVinney, R.; Hirsch, A. M.; Riley, M.; Mansky, L.; Vendatum, G. *Vie Milieu* **2008**, *58*, 87−106.
(8) Chavez-Dozal, A. A.; Hogan, D.; Gorman, C.; Quintanal-Villalonga, A.; Nishiguchi, M. K. *FEMS Microbiol. Ecol.* **2012**, *81*, 562−573.
(9) Nyholm, S. V.; Nishiguchi, M. K. *Vie Milieu* **2008**, *58*, 175−184.
(10) Jones, B. W.; Nishiguchi, M. K. *Mar. Biol.* **2004**, *144*, 1151−1155.
(11) Graf, J.; Ruby, E. G. *Abst. Annu. Meeting Am. Soc. Microbiol.* **1994**, *94*, 76.
(12) Pennisi, E. *Science* **2013**, *340*, 1159−1160.
(13) Archetti, M.; Ubeda, F.; Fudenberg, D.; Green, J.; Pierce, N. E.; Yu, D. W. *Am. Nat.* **2011**, *177*, 75−85.
(14) Archetti, M. *J. Theor. Biol.* **2011**, *269*, 201−207.
(15) Schleicher, T. R.; Nyholm, S. V. *PLoS One* **2011**, *6*, e25649−e25649.
(16) Visick, K. L.; McFall-Ngai, M. *J. Bacteriol.* **2000**, *182*, 1779−1787.
(17) Ruby, E. G.; McFall-Ngai, M. J. *Trends Microbiol.* **1999**, *7*, 414−420.
(18) Small, A. L.; McFall-Ngai, M. J. *J. Cell. Biochem.* **1999**, *72*, 445−457.
(19) Nishiguchi, M. K. *Microbiol. Ecol.* **2002**, *44*, 10−18.
(20) Castillo, M. G.; Goodson, M. S.; McFall-Ngai, M. J. *Dev. Comp. Immunol.* **2009**, *33*, 69−76.
(21) Small, A. L.; McFall-Ngai, M. J. *J. Cell Biochem.* **1999**, *72*, 445−457.
(22) Visick, K. L.; Ruby, E. G. *J. Bacteriol.* **1998**, *180*, 2087−2092.
(23) Cosgrove, K.; Coutts, G.; Jonsson, I.-M.; Tarkowski, A.; Kokai-Kun, J. F.; Mond, J. J.; Foster, S. J. *J. Bacteriol.* **2007**, *189*, 1025−1035.
(24) Katsuwon, J.; Anderson, J. *Can. J. Microbiol.* **1992**, *38*, 1026−1032.
(25) Kim, K. K.; Fravel, D. R.; Papavizas, G. C. *Phytopathology* **1978**, *78*, 488−492.
(26) Ichise, N.; Hirota, K.; Ichihashi, D..; Nodasaka, Y.; Morita, N.; Okuyama, H.; Yumoto, I. *J. Biosci. Bioeng.* **2008**, *106*, 39−45.
(27) Yumoto, I.; Ichihashi, D.; Iwata, H.; Istokovics, A.; Ichise, N.; Matsuyama, H.; Okuyama, H.; Kawasaki, K. *J. Bacteriol.* **2000**, *182*, 1903−1909.
(28) Ichise, N.; Morita, N..; Hoshino, T.; Kawasaki, K.; Yumoto, I.; Okuyama, H. *Appl. Environ. Microbiol.* **1999**, *65*, 73−79.
(29) Ramsey, M. M.; Whiteley, M. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 1578−1583.
(30) Imlay, J. A. *Nature* **2013**, *11*, 443−454.
(31) McCarthy, B. J.; Hinshelwood, S. C. *Proc. R. Soc. London, Ser. B: Biol. Sci.* **1958**, *150*, 13−23.
(32) Li, Y.; Schellhorn, H. E. *J. Biomol. Tech.* **2007**, *18*, 185−187.
(33) Beers, R. F., Jr.; Sizer, I. W. *J. Biol. Chem.* **1952**, *195*, 133−140.
(34) Rorth, M.; Jensen, P. K. *Biochim. Biophys. Acta* **1967**, *139*, 171−173.
(35) Katsev, A. M.; Wegrzyn, G.; Szpilewska, H. *J. Basic Microbiol.* **2004**, *44*, 178−184.
(36) Bard, A. J.; Mirkin, M. V., Eds. *Scanning Electrochemical Microscopy*, 2nd ed.; CRC Press: New York, 2012.
(37) Fan, F. R. F.; Fernandez, J. L.; Liu, B.; Mauzeroll, J. In *Handbook of Electrochemistry*; Zoski, C. G., Ed. Elsevier: Amsterdam, The Netherlands, 2007; Chapter 12, pp 471−540.
(38) Bard, A. J. In *Scanning Electrochemical Microscopy*, 2nd ed.; Bard, A. J., Mirkin, M. V., Eds.; CRC Press: New York, 2012; Chapter 1, pp 1−14.
(39) Wang, X.; Yang, T.; Feng, Y.; Jiao, K.; Li, G. *Electroanalysis* **2009**, *21*, 819−825.
(40) Mao, L.; Osborne, P. G.; Yamamoto, K.; Kato, T. *Anal. Chem.* **2002**, *74*, 3684−3689.
(41) Liu, X.; Zweier, L. *Free Radical Biol. Med.* **2001**, *31*, 894−901.
(42) Horrocks, B. R.; Schmidtke, D.; Heller, A.; Bard, A. J. *Anal. Chem.* **1993**, *65*, 3605−3614.
(43) Wittstock, G.; Schuhmann, W. *Anal. Chem.* **1997**, *69*, 5059−5066.
(44) Wilhelm, T.; Wittstock, G. *Angew. Chem., Int. Ed.* **2003**, *24*, 2248−2250.

(45) Mezour, M. A.; Cornut, R.; Husssien, E. M.; Morin, M.; Mauzeroll, J. *Langmuir* **2010**, *26*, 13000−13006.
(46) Fan, F. R. F. In *Scanning Electrochemical Microscopy*, 2nd ed.; Bard, A. J., Mirkin, M. V., Eds.; CRC Press: New York, 2012; Chapter 4, pp 53−74.
(47) Mauzeroll, J.; Schougaard, S. B. In *Scanning Electrochemical Microscopy*, 2nd ed.; Bard, A. J., Mirkin, M. V., Eds.; CRC Press: New York, 2012; Chapter 12, pp 379−415.
(48) Beaulieu, I.; Juss, S.; Mauzeroll, J.; Geissler, M. *Anal. Chem.* **2011**, *83*, 1485−1492.
(49) Armemiya, S.; Guo, J.; Xiong, H.; Gross, D. A. *Anal. Bioanal. Chem.* **2006**, *386*, 458−471.
(50) Liu, X.; Ramsey, M. M.; Chen, X.; Koley, D.; Whiteley, M.; Bard, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 2668−2673.
(51) Koley, D.; Ramsey, M. M.; Bard, A. J.; Whiteley, M. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 19996−20001.
(52) Kuss, S.; Polcari, D.; Geissler, M.; Brassard, D.; Mauzeroll, J. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 9249−9254.
(53) Fan, F.-R. F; Demaille, C. In *Scanning Electrochemical Microscopy*, 2nd ed.; Bard, A. J., Mirkin, M. V., Eds.; CRC Press: New York, 2012; Chapter 3, pp 25−51.
(54) Fan, F. R. F.; Fernandez, J. L.; Liu, B.; Mauzeroll, J.; Zoski, C. G. In *Handbook of Electrochemistry*; Zoski, C. G., Ed.; Elsevier: Amsterdam, The Netherlands, 2007; Chapter 6, pp 189−199.
(55) Katsev, A. M.; Wegrzyn, G.; Szpilewska, H. *J. Basic Microbiol.* **2004**, *44*, 178−184.
(56) Zoski, C. G.; Aguilar, J. C.; Bard, A. J. *Anal. Chem.* **2003**, *75*, 2959−2966.
(57) Zoski, C. G.; Luman, C. R.; Fernandez, J. L.; Bard, A. J. *Anal. Chem.* **2007**, *79*, 4957−4966.
(58) Nelson, D. L.; Cox, M. M. *Principles of Biochemistry*, 5th ed.; W. H. Freeman and Co.: New York, 2008; Chapter 6, pp 183−233.
(59) Bishai, W. R.; Smith, H. O.; Barcak, G. J. *J. Bacteriol.* **1994**, *176*, 2914−2921.
(60) Brown, S. A.; Whiteley, M. *J. Bacteriol.* **2007**, *189*, 6407−6414.
(61) Chavez-Dozal, A. A.; Gorman, C.; Erken, M.; Steinberg, P. D.; McDougald, D.; Nishiguchi, M. K. *Appl. Environ. Microbiol.* **2013**, *79*, 553−558.

Ecological Diversification of Vibrio fischeri *Serially Passaged for 500 Generations in Novel Squid Host* Euprymna tasmanica

William Soto, Ferdinand M. Rivera & Michele K. Nishiguchi

Microbial Ecology

ISSN 0095-3628

Microb Ecol
DOI 10.1007/s00248-013-0356-3

Springer

Microb Ecol
DOI 10.1007/s00248-013-0356-3

HOST MICROBE INTERACTIONS

Ecological Diversification of *Vibrio fischeri* Serially Passaged for 500 Generations in Novel Squid Host *Euprymna tasmanica*

William Soto · Ferdinand M. Rivera · Michele K. Nishiguchi

Received: 4 June 2013 / Accepted: 16 December 2013

Abstract *Vibrio fischeri* isolated from *Euprymna scolopes* (Cephalopoda: Sepiolidae) was used to create 24 lines that were serially passaged through the non-native host *Euprymna tasmanica* for 500 generations. These derived lines were characterized for biofilm formation, swarming motility, carbon source utilization, and in vitro bioluminescence. Phenotypic assays were compared between "ES" (*E. scolopes*) and "ET" (*E. tasmanica*) *V. fischeri* wild isolates to determine if convergent evolution was apparent between *E. tasmanica* evolved lines and ET *V. fischeri*. Ecological diversification was observed in utilization of most carbon sources examined. Convergent evolution was evident in motility, biofilm formation, and select carbon sources displaying hyperpolymorphic usage in *V. fischeri*. Convergence in bioluminescence (a 2.5-fold increase in brightness) was collectively evident in the derived lines relative to the ancestor. However, dramatic changes in other properties—time points and cell densities of first light emission and maximal light output and emergence of a lag phase in growth curves of derived lines—suggest that increased light intensity per se was not the only important factor. Convergent evolution implies that gnotobiotic squid light organs subject colonizing *V. fischeri* to similar selection pressures. Adaptation to novel hosts appears to involve flexible microbial metabolism, establishment of biofilm and swarmer *V. fischeri* ecotypes, and complex changes in bioluminescence. Our data demonstrate that numerous alternate fitness optima or peaks are available to *V. fischeri* in host adaptive landscapes, where novel host squids serve as habitat islands. Thus, *V. fischeri* founder flushes occur during the initiation of light organ colonization that ultimately trigger founder effect diversification.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-013-0356-3) contains supplementary material, which is available to authorized users.

W. Soto
Department of Ecology, Evolution, & Behavior, University of Minnesota-Twin Cities, 100 Ecology Building, 1987 Upper Buford Circle, Saint Paul, MN 55108, USA
e-mail: wsoto@umn.edu

F. M. Rivera · M. K. Nishiguchi (✉)
Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003, USA
e-mail: nish@nmsu.edu

Introduction

The Sepiolid Squid–*Vibrio* Mutualism

Sepiolid squids in the genera *Sepiola* and *Euprymna* form light organ mutualisms with marine bioluminescent bacteria from the genera *Vibrio* and *Photobacterium* from the family Vibrionaneae [1]. Sepiolid squids use light produced by their bacterial symbionts for a cryptic behavior termed counterillumination [2], and the light organ bacteria are in turn exposed to a nutrient-rich microcosm in the host [3]. Particularly, the mutualism between *Vibrio fischeri* and *Euprymna* has become a model for studying associations between eukaryotic hosts and bacteria, since both partners can be maintained independently of each other in the laboratory [4, 5]. Axenic juvenile *Euprymna* squids hatch from their eggs with sterile light organs and are quickly colonized by symbiotically competent *V. fischeri* present in bacterioplankton, reaching a light organ carrying capacity (10^4–10^7 colony forming units (CFUs)/light organ) within 12–24 h [6, 7]. Sepiolid squids are nocturnal and, seed the surrounding water with symbiotic competent *V. fischeri* at dawn by venting 90–95 % of the symbiont light organ population. By the next evening, the remaining *V. fischeri* inside the animal grow to repopulate the light organ to full capacity [5]. These vented symbionts in the oceanic water column serve as a source population for colonizing the next generation of squid hatchlings.

Published online: 09 January 2014

Not all strains of *V. fischeri* are capable of colonizing *Euprymna* and *Sepiola* hosts, as some isolates are restricted to planktonic and commensal lifestyles (non-light organ associations with animals) [6]. *V. fischeri* is also able to initiate light organ mutualisms with monocentrid fishes. These mutualisms are each formed with ecologically and genetically distinct *V. fischeri* [8], and strains indigenous to monocentrid fish hosts do not colonize sepiolid squid to the same population levels (i.e., CFUs/light organ) as "squid" isolates [9]. Additionally, *Euprymna* species are distributed allopatrically throughout the Indo-West Pacific Ocean, and *V. fischeri* colonizing this genus are host specialists, exhibiting competitive dominance, whereas strains forming mutualisms with several sympatric *Sepiola* species from the Mediterranean Sea [1, 9–11] are host generalists and display no competitive dominance. Prior evidence from experimentally evolved lines of *V. fischeri* ES114 (native to Hawaiian *Euprymna scolopes*) selected through a non-native host (Australian *Euprymna tasmanica*) for 500 generations exhibited a significant increase in mean fitness relative to the ancestral strain in colonizing *E. tasmanica* [7], yet the traits responsible for this host specificity change remain unknown. Therefore, to determine which phenotypes are subject to host selection, both ancestor and evolved clones were characterized for biofilm formation, motility, carbon source utilization, and in vitro bioluminescence. Wild *V. fischeri* isolates from field-caught *E. tasmanica* ("ET" isolates) and *E. scolopes* ("ES" isolates) were also compared to experimentally evolved strains to determine if the derived lines exhibited any convergent evolution relative to indigenous ET *V. fischeri*.

Methods and Materials

Motility Assay

A substantial literature exists on Vibrionaceae motility, and evidence for swarming in *Vibrio* is prolific and well documented [12–19]. The genus *Vibrio* possesses a dual flagellar system [18, 20, 21]. Polar flagella are produced by cells continuously for facile locomotion such as swimming in liquid, while inducible lateral or peritrichous flagella are manufactured for more arduous navigation via swarming on solid surfaces or through viscous milieus, including 0.5 % agar [18]. Polar and lateral flagella in *Vibrio* spp. are encoded and regulated by distinct gene sets [19]. When performing motility assays on 0.5 % agar with *Vibrio*, swarming can be quantified by measuring the diameter of an expanding and growing bacterial mass. Since twitching motility occurs much more slowly than swarming, the diameter of the expanding bacterial mass does not represent the former. Swimming was not measured in these assays, since the locomotion we observed occurred on the surface of 0.5 % agar rather than within. This was confirmed by examining the motile bacteria under a dissecting microscope. Motility assays were modified from a previously published protocol [22] to monitor swarming. All bacterial strains were grown in 18×150 mm glass test tubes containing 5 mL 70 % 32 ppt seawater tryptone (SWT: 5 g tryptone, 3 g yeast extract, 3.75 mL glycerol, 300 mL double distilled water, and 700 mL 32 ppt artificial seawater made with Instant Ocean®) [23–25] liquid media at 28ºC and 225 rpm for 16 h. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of overnight starter cultures was used to inoculate 18×150 mm glass test tubes with 10 mL 70 % 32 ppt SWT and incubated at 28ºC at 225 rpm until subcultures reached 0.10 OD_{600} (~1.0×10^7 CFUs/mL). Bacterial cells were pelleted by centrifugation (10,000 rpm) at 4 °C for 10 min in 15-mL conical tubes, discarding the supernatant afterward. Bacterial pellets were resuspended with 10 mL washes of either filter-sterilized 34 ppt artificial seawater (Instant Ocean®), minimal ribose media, or 34 ppt SWT to remove residual nutrient media from the overnight culture [26]. This centrifugation and wash procedure was performed three times. After the third centrifugation step and resuspension wash, the cell suspension was once again centrifuged for 10 min and pelleted. The bacterial pellet was then resuspended either with filter-sterilized 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT. For each isolate or strain, 10 μL of the resuspended pellet (~10^5 total cells) was placed directly center onto (0.5 %) swarm motility agar plates [26, 27] containing either 34 ppt artificial seawater, minimal ribose [25], or 34 ppt SWT, representing different nutrient conditions to observe motility (n=3). This was also repeated for negative control (5.0 %) agar plates, where the agar concentration is sufficiently high to prevent swarming. The negative control plates also provided a baseline for comparing motility on each of the different media or nutrient conditions. All agar plates utilized in the motility assays received only a single 10 μL resuspension per isolate. Agar volume placed into each Petri dish for motility assays was measured and kept constant (20 mL). Immediately after the swarm agar plates (including negative controls) solidified, they were allowed to air dry at 25ºC for 24 h before use. The plates were incubated for 24 h at 28ºC, when diameters of swarming bacteria were measured [22, 26]. Fisher least significant differences (LSDs), Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate α= 0.05) [28], were calculated separately for the wild isolates and each evolved time point (100, 200, 300, 400, and 500 generations). Chloramphenicol resistance has no effect on motility, as *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were not significantly different from each other under all conditions. Chloramphenicol resistance is thus a neutral marker with respect to motility between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200.

Microtiter Plate Biofilm Assay

Microtiter plate biofilm assay was adapted from a published methodology initially used for staphylococci [29]. Sixteen-hour *V. fischeri* cultures were grown in 18×150 mm glass test tubes containing 5 mL 70 % SWT in a 28ºC air shaker at 225 rpm. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of overnight starter cultures were each separately inoculated into 18×150 mm glass test tubes with 10 mL of fresh 70 % 32 ppt SWT and incubated in a 28ºC air shaker at 225 rpm. These subcultures were grown to 0.10 OD_{600} (~1.0 X 10^7 CFUs/mL). All 0.10 OD_{600} subcultures were centrifuged (10,000 rpm) at 4 °C, pelleted, resuspended, and washed three times as previously described with either liquid filter-sterilized 34 ppt artificial seawater, minimal ribose, or 34 ppt SWT to ensure all residual 70 % 32 ppt SWT were removed. After the third centrifugation step and resuspension wash, the cell suspension was once again centrifuged for 10 min and pelleted. The bacterial pellet was then resuspended either with filter-sterilized 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT. Cell suspensions (2 μL) were subsequently inoculated into polystyrene 96-well microtiter plates containing 200 μL of either filter-sterilized 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT (n=12). Three liquid media types were used to examine *V. fischeri* biofilm formation in different environments representing varying degrees of nutrient availability. Uninoculated wells remained on each of the three different liquid media microtiter plates to serve as negative controls. Plates were incubated for 18 h at 28 °C without shaking. Media in all wells were then gently removed and washed three times appropriately with either 200 μL filter-sterilized 34 ppt artificial seawater, minimal ribose, or 34 ppt SWT, removing wash liquid each time. Microtiter plates were then incubated at room temperature for 15 min. Two hundred microliters of 0.2 % crystal violet dye was then added to each well and incubated for 30 min at room temperature. After staining, crystal violet was gently removed with a pipet and the wells washed three times with either 200 μL 34 ppt artificial seawater, minimal ribose, or 34 ppt SWT, removing wash liquid after each time. Microtiter plates were allowed to incubate at room temperature for 15 min. Two hundred microliters of 70 % ethanol was added to all the wells in the microtiter plates and incubated at room temperature for 20 min. Absorbance readings were measured for all microtiter plates at 562 nm using a Bio-Tek ELx800™ microplate reader (Winooski, VT). Fisher LSDs, Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate α=0.05) [28], were calculated separately for the wild isolates and each evolved time point (100, 200, 300, 400, and 500 generations). Chloramphenicol resistance has no effect on biofilm formation, as *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were not significantly different from each other under all conditions. Chloramphenicol resistance is thus a neutral marker with respect to biofilms between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200.

Carbon Source Utilization for 95 Substrates

Biolog GN2 microplates (Hayward, CA) for gram-negative bacteria were used, where substrates in each of 95 wells represent different carbon sources, along with one negative control well that contains only distilled water [30, 31]. Utilization of a substrate was recorded by measuring increases in well absorbance relative to the negative control at 590 nm. Manufacturer's instructions (Biolog GN2 MicroPlate™) and protocols were used with modification. All bacterial strains were grown in 18×150 mm glass test tubes containing 5 mL 70 % 32 ppt SWT at 28ºC and 225 rpm for 16 h. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of overnight starter cultures was used to inoculate 125-mL triple-baffled culture flasks with 50 mL of fresh 70 % 32 ppt SWT, which were incubated at 28ºC and shaken at 225 rpm until the flask subculture reached 0.220 OD_{590} (~2.0×10^7 CFUs/mL). Bacterial cells from each 0.220 OD_{590} subculture were pelleted by 4 °C centrifugation (10,000 rpm) for 10 min and resuspended with Biolog GN/GP inoculation fluid (product no. 72101) to remove residual nutrient media from overnight cultures. To meet *V. fischeri* osmolar requirements, the salinity of the Biolog inoculation fluid was adjusted to 3.4 % NaCl final concentration. The centrifugation and wash procedure was completed three times. After the third centrifugation step and resuspension wash, cells were once again centrifuged for 10 min and pelleted. The bacterial pellet was then resuspended with Biolog inoculation fluid. Working *V. fischeri* cell suspensions for each strain were dispensed into Biolog GN2 microplates. All wells in Biolog GN2 microplates were inoculated with 150 μL of washed *V. fischeri* cells at 2.0×10^7 CFUs/mL suspended in Biolog GN/GP inoculation fluid. Uninoculated Biolog GN2 microplate wells containing only Biolog GN/GP inoculation fluid (adjusted to 3.4 % NaCl final concentration) and no bacteria served as negative controls. All negative controls and *V. fischeri* strains were measured in triplicate (n=3). Microplates were incubated at 28ºC for 24 h. Afterwards, optical densities (590 nm) of the wells were measured by placing GN2 plates in a Bio-Tek ELx800™ microplate reader (Winooski, VT). Fisher LSDs, Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate α=0.05) [28], were calculated separately for wild isolates and the 500-generation evolved time point. Chloramphenicol resistance has no effect on carbon source utilization, as *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were not significantly different from each other on any carbon source. Chloramphenicol

resistance is thus a neutral marker with respect to carbon source utilization between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200.

Bioluminescence

Sixteen-hour *V. fischeri* cultures were grown in 18×150 mm glass test tubes containing 5 mL 70 % SWT liquid media in a 28ºC air shaker at 225 rpm. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of the overnight cultures was each separately inoculated into 18×150 mm glass test tubes with 5 mL of fresh 70 % 32 ppt SWT and incubated in a 28ºC air shaker at 225 rpm to obtain exponential growth subcultures at 0.5 OD_{600}. The 0.5 OD_{600} subcultures were used to inoculate 125-mL triple-baffled culture flasks with fresh 50 mL 34 ppt SWT to an initial 5×10^5 CFUs/mL cell density. Culture flasks were incubated at 28ºC and shaken at 225 rpm for 8 h with bioluminescence readings and plate count enumeration taken every 30 min (n=6). Bioluminescence readings were recorded with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Since total bacterial luminescence is dependent on cell density and total volume, measurements were reported as relative light units (RLUs) per $\log_{10}$ (CFUs/mL) per milliliter of liquid culture (RLUs [$\log_{10}$ (CFUs/mL)]$^{-1}$mL^{-1}) over time. Cell density determinations were completed on 34 SWT 1.5 % agar plates, which were incubated at 28 °C for 24 h. During these time interval experiments, *V. fischeri* broth cultures have nearly 100 % plating efficiency [6]. Bioluminescence readings of *Escherichia coli* K12 MG1655 were also monitored as a negative control under the same experimental conditions with the exception that Luria Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1 L double distilled water) and LB 1.5 % agar plates were used. There was no difference in bioluminescence between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 through all time points, showing that chloramphenicol is a neutral marker with this trait. Bioluminescence data were assessed using repeated measures analysis. No assumption was made with sphericity. The quite conservative Extreme Greenhouse-Geisser and Lower Bound corrections were applied separately to statistical main effects [32]. Fisher LSDs, Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate α=0.05) [28], were calculated separately for wild isolates and the 500-generation evolved time point.

Results

Motility

Ten ET (wild isolates from *E. tasmanica*) and ten ES (wild isolates from *E. scolopes*) *V. fischeri* strains were previously shown to be genetically distinct from one another [7, 8, 33]. These field isolates were randomly sampled from their *Euprymna* squid hosts and used as a comparison to the evolved clones for motility (Fig. 1a). ES wild strains were more motile than ET on minimal ribose and 34 ppt SWT. *V. fischeri* ES114 is chloramphenicol sensitive, while *V. fischeri* JRM200 is a *V. fischeri* ES114 derivative which is chloramphenicol resistant [24]. *V. fischeri* JRM200 was used to create 24 lines that were serially transferred through novel squid host *E. tasmanica* for 500 generations [7] and used in this study. All 24 evolved lines improved in colonization of *E. tasmanica* relative to ancestor, as no lineage remained the same or became worse. *V. fischeri* lines serially passaged through *E. tasmanica* for 500 generations became less motile relative to ancestor *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 (zero generations through *E. tasmanica*) on minimal ribose and 34 ppt SWT (Fig. 1b–d for 100, 200, and 500 generations; supplementary Figs. S1A and S1B for 300 and 400 generations). Unlike many bacterial species, *V. fischeri* is capable of motility on minimal media without nutritional supplements such as casamino acids [27]. No growth was observed on 34 ppt artificial seawater 0.5 % agar plates as well as on the motility negative control 5.0 % agar plates. Significant decreases in motility were first observed for all lines by 100 generations (Fig. 1b) on motility SWT plates and continued to decline throughout the remainder of the selection experiment. However, by 500 generations, most derived lines still remained motile on SWT plates relative to SWT motility negative controls (Fig. 1d). Although significant decreases in motility on minimal ribose is evident by 100 generations (Fig. 1b), a marked drop was noted by 200 generations (Fig. 1c), as most of the derived lines are no longer significantly more motile than on minimal ribose motility negative control plates. Motility on minimal ribose plates was completely lost by 300 generations (Fig. S1A). Thus, the derived lines converged on ET motility on minimal ribose and 34 ppt SWT, yet significant differences (polymorphisms) were seen among the various lineages in motility as a result of adaptation to novel host *E. tasmanica.*

Biofilm Formation

No bacterial contamination was observed in uninoculated negative control wells for 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT. Biofilm formation is higher for the ET wild isolates than the ES ones in artificial seawater, minimal ribose, and 34 ppt SWT (Fig. 2a). *V. fischeri* passaged through *E. tasmanica* increased their biofilm formation capacity (Fig. 2b–d for 100, 400, and 500 generations; supplementary Figs. S2A and S2B for 200 and 300 generations). Relative to the loss of motility, the evolution of elevated biofilm formation was delayed, not appearing in great levels until 400 generations (Fig. 2c), when it simultaneously appeared

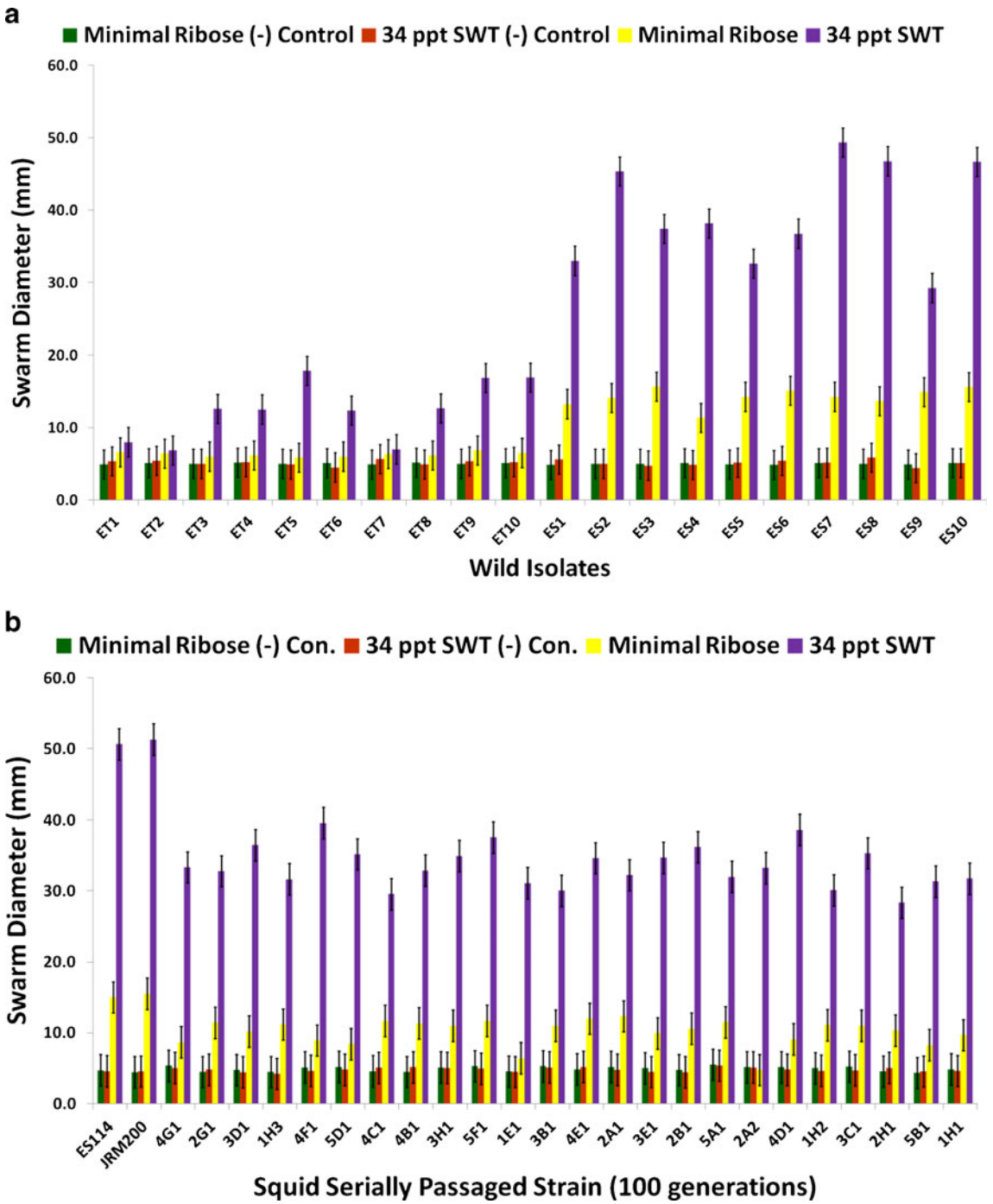


Fig. 1 **a** Motility assays for natural *V. fischeri* isolates extracted from *E. tasmanica* ("ET") and *E. scolopes* ("ES") squid hosts (n=3). Motility assays for *V. fischeri* JRM200 lines serially passaged for **b** 100, **c** 200, and **d** 500 generations through *E. tasmanica* (n=3). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate α=0.05). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

dramatically in minimal ribose and SWT. Heightened biofilm formation in artificial seawater arose by 400 generations (Fig. 2c), as some derived lines were significantly higher than the ancestor in this medium. Interestingly, *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were initially incapable of biofilm growth in artificial seawater. Significant dissimilarity from the ancestral state in biofilm construction did not manifest in the evolved lines in 34 ppt SWT and minimal ribose until 200 and 300 generations, respectively (Figs. S2A and S2B). Thus, like motility, the relative evolutionary change in biofilm formation is nutrient dependent and convergent to ET wild variation, as "domesticated" variation created by artificial

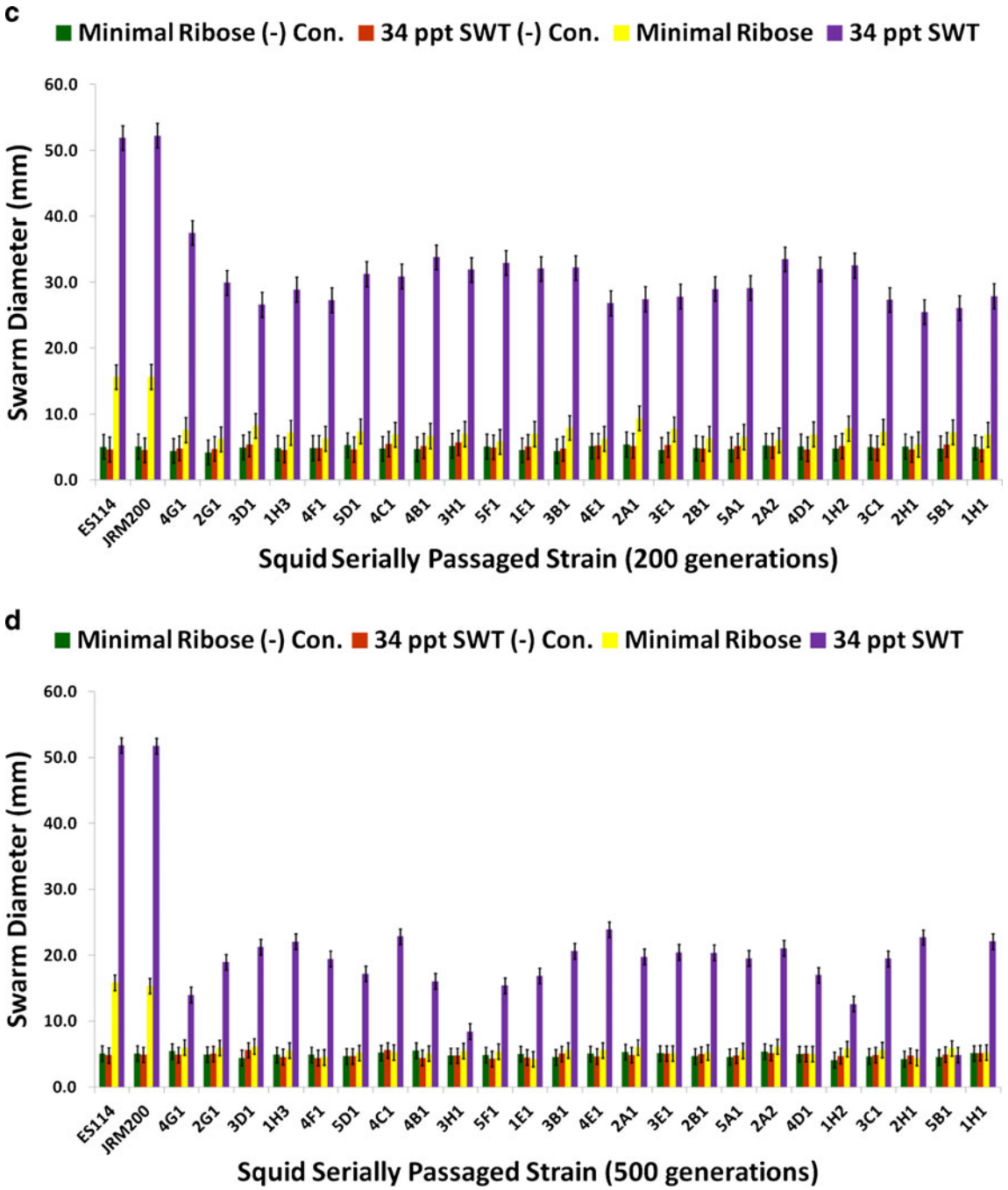


Fig. 1 (continued)

selection through novel host *E. tasmanica* showed a parallel biofilm response in all media types. The evolved lines have also diverged from one another in biofilm formation.

Carbon Source Utilization for 95 Substrates

The goal of measuring carbon source utilization in this study was to identify metabolic or physiological changes in the 24 *V. fischeri* lines as results of adapting to a novel squid host environment in the light organ. Some of these changes may have been directly responsible for adaptation to the new host animal; others may have been the result of tradeoffs, epistasis, pleiotropy, or mutation accumulation [34]. No bacterial contamination was observed on the Biolog GN2 negative control plates. The Biolog GN2 microplate data for *V. fischeri* ES114 and *V. fischeri* unevolved JRM200 (supplementary Table S1) is congruent with what has been previously reported as accessible carbon sources for *V. fischeri*, supporting the validity of

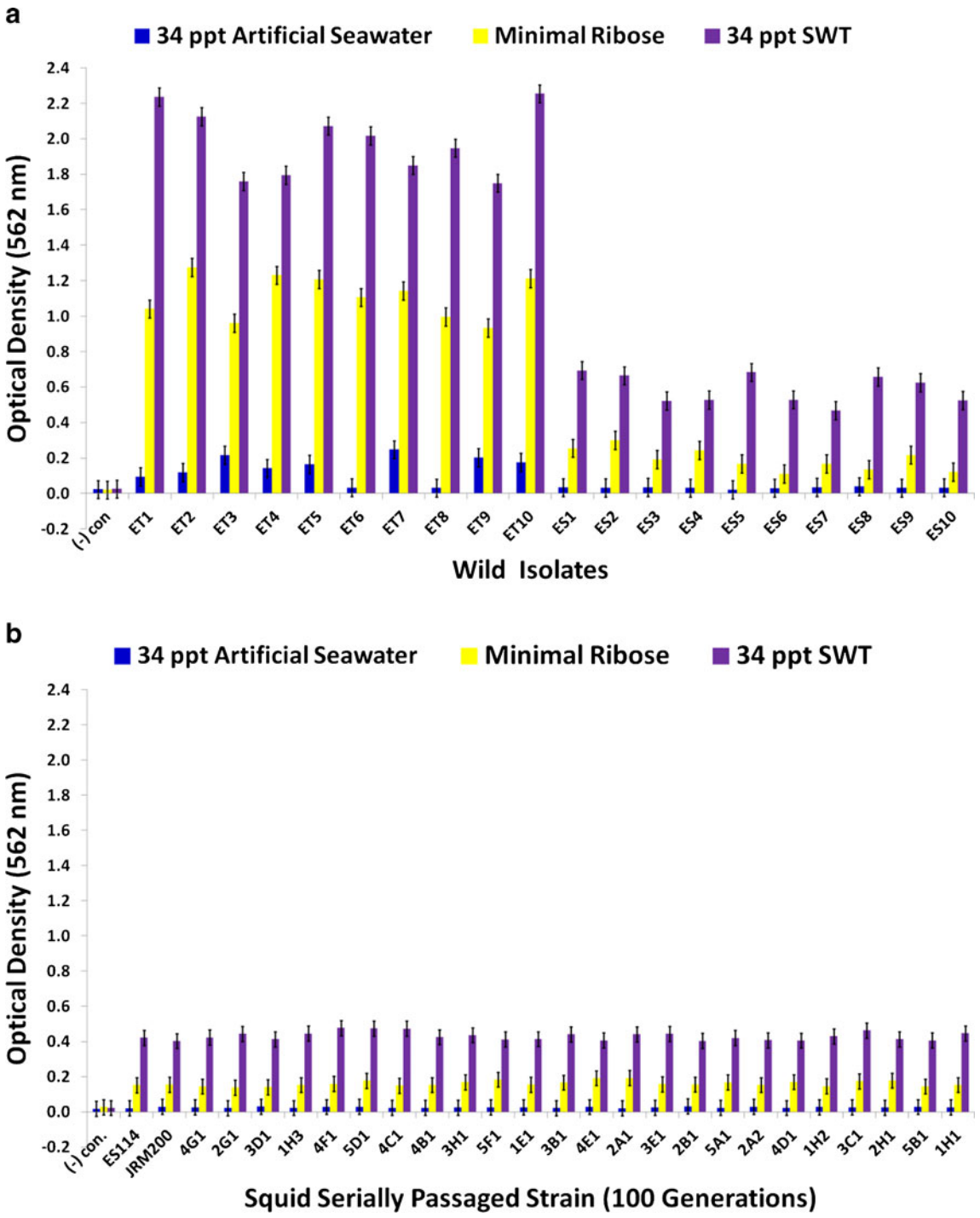


Fig. 2 **a** Biofilm assays for wild *V. fischeri* strains from *E. tasmanica* ("ET") and *E. scolopes* ("ES") specimens collected from the field (n=12). Biofilm assays for *V. fischeri* JRM200 lines serially passaged for **b** 100, **c** 400, and **d** 500 generations through *E. tasmanica* (n=12). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate α=0.05). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

the results [35–40]. ET and ES wild isolates were heterogeneous for carbon substrate utilization (Figs. 3a-4b). Relative to ancestral metabolism, *V. fischeri* lines serially passaged through *E. tasmanica* diversified in their utilization in every substrate in the Biolog microplate initially physiologically procurable (Figs. 5a-6b). As is wild ET variation (Fig. 3a–c), "domesticated" variation (Fig. 5a–c) in carbon utilization from experimentally evolved *V. fischeri* is quite polymorphic. The precise manner of change between the derived lines and the ancestral state was carbon source dependent. For example, the various lineages remained the same or decreased on *N*-acetyl-D-glucosamine, while the evolutionary

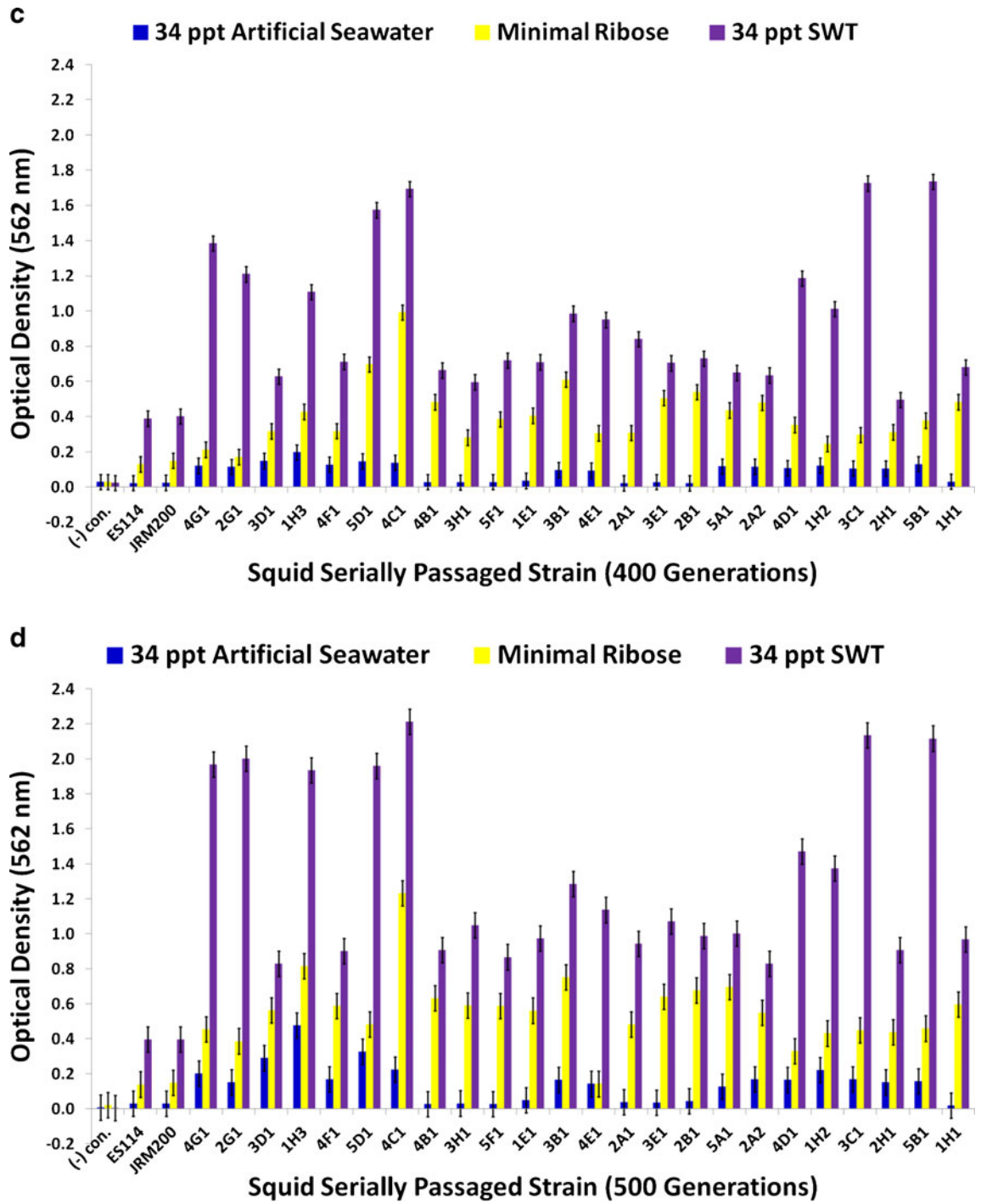


Fig. 2 (continued)

response with α-cyclodextrin was to increase or stay the same (Fig. 5a). On D-psicose (a rare sugar in nature [41]), the evolved lines either gained, lost, or were unaltered (Fig. 5b). However, no compelling convergent evolution was evident relative to wild isolates in most carbon substrates (representative examples shown in Figs. 3a–c and 5a–c).

Genetic evidence exists that these carbon sources play an important role for *V. fischeri* metabolism in its association with sepiolid squid hosts. [3, 42] L-asparaginase, chitinases (liberate *N*-acetyl-D-glucosamine monomers), and dextrinases are produced in the symbiont while in symbiosis with the host squid. Protein importers and permeases for L-serine, D-galactose, and D-fructose have also been found in symbiotic *V. fischeri* [42]. L-Threonine is supplied by *Euprymna* squid to *V. fischeri* within the light organ in nutrient limiting amounts relative to other amino acids [3]. Moreover, a

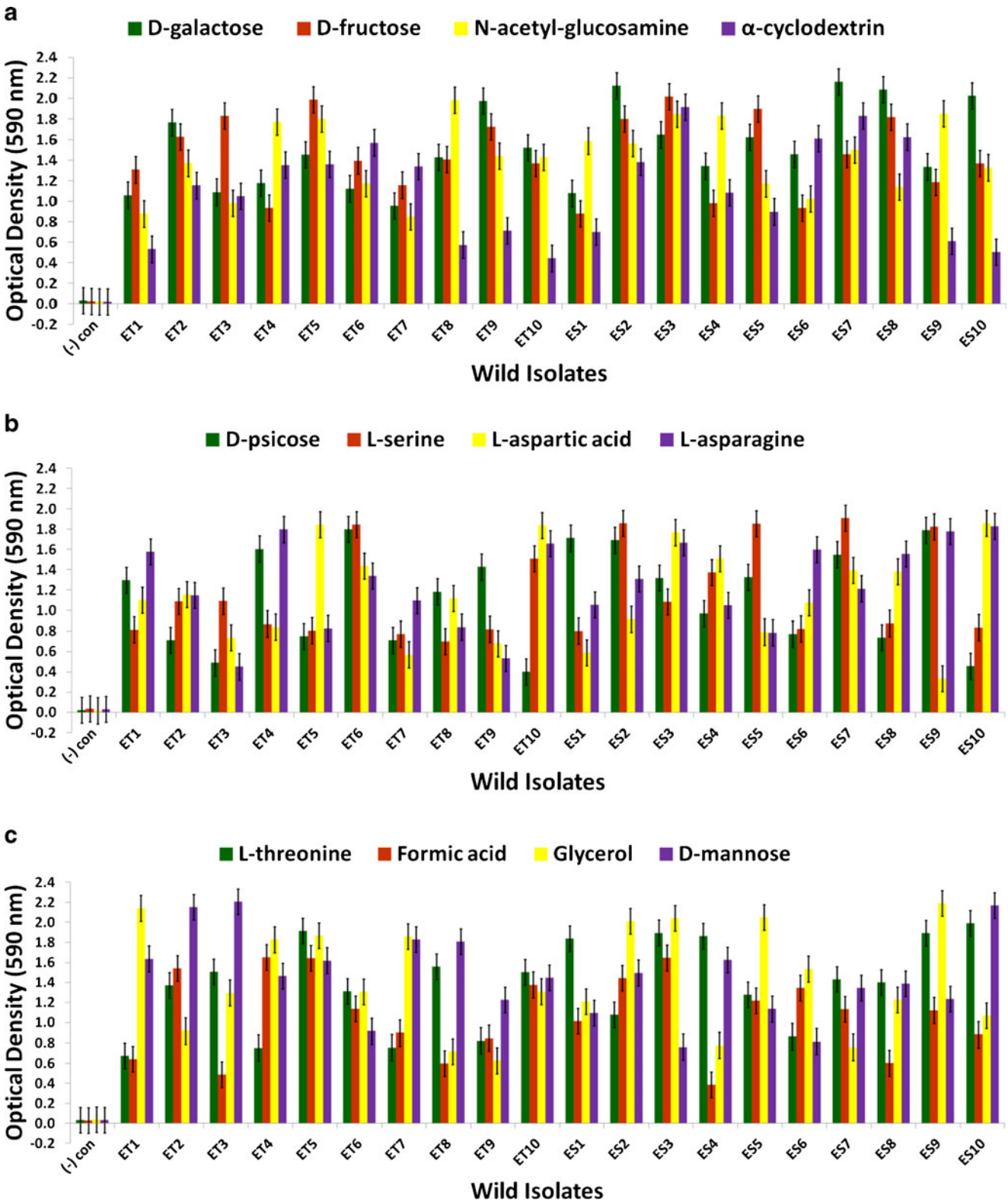


Fig. 3 Optical density (590 nm) measurements in **a** D-galactose, D-fructose, *N*-acetyl-D-glucosamine, and α-cyclodextrin; **b** D-psicose, L-serine, L-aspartic acid, and L-asparagine; and **c** L-threonine, formic acid, glycerol, and D-mannose of wild *V. fischeri* isolates from *E. tasmanica* ("ET") and *E. scolopes* ("ES") specimens collected from the field (n=3). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate α=0.05). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

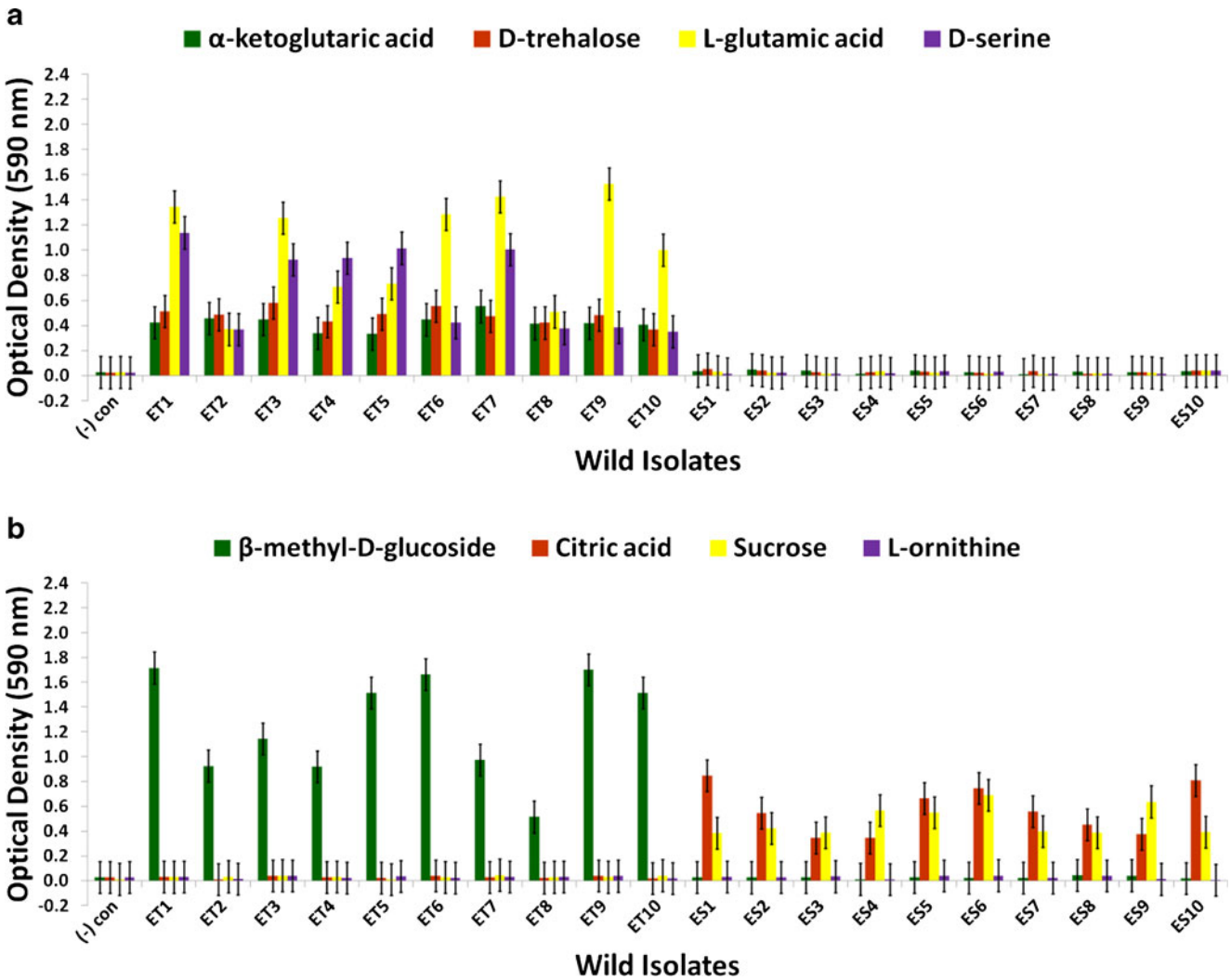
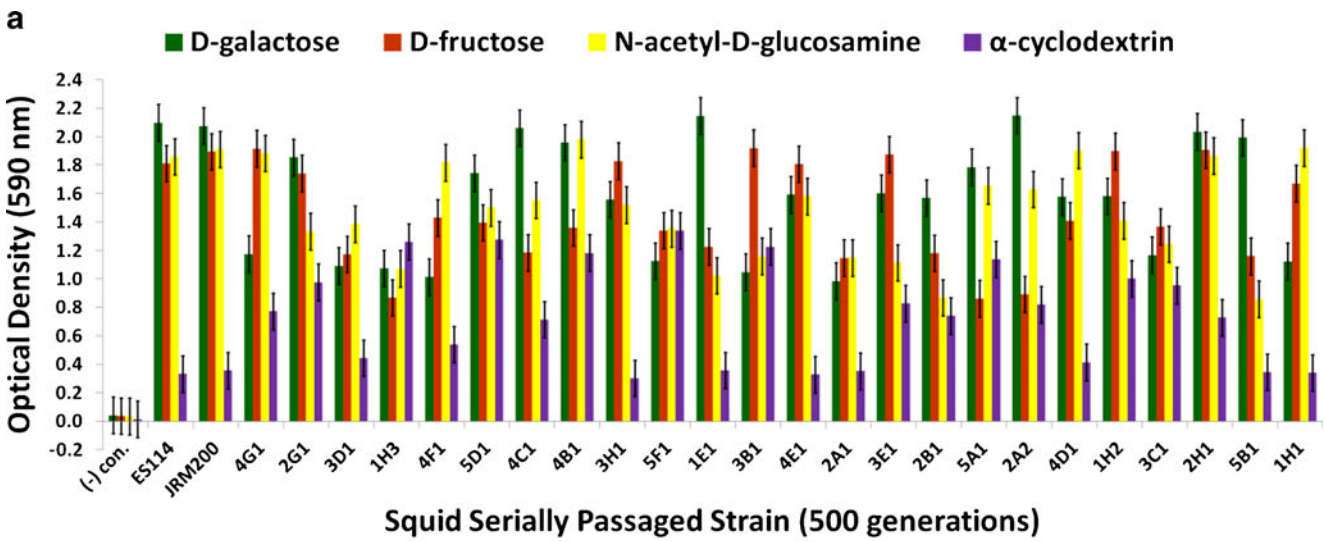


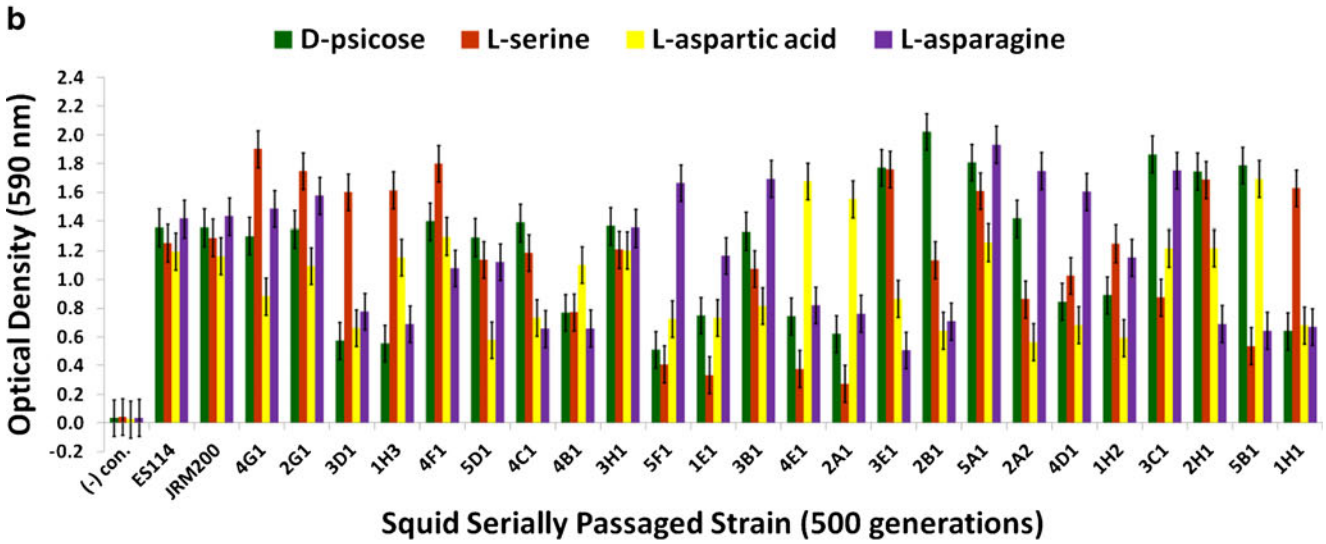
Fig. 4 Optical density (590 nm) measurements in **a** α-ketoglutaric acid, D-trehalose, L-glutamic acid, and D-serine, and **b** β-methyl-D-glucoside, citric acid, sucrose, and L-ornithine of wild *V. fischeri* isolates from Australian *E. tasmanica* ("ET") and Hawaiian *E. scolopes* ("ES") specimens collected from the field (n=3). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate α=0.05). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

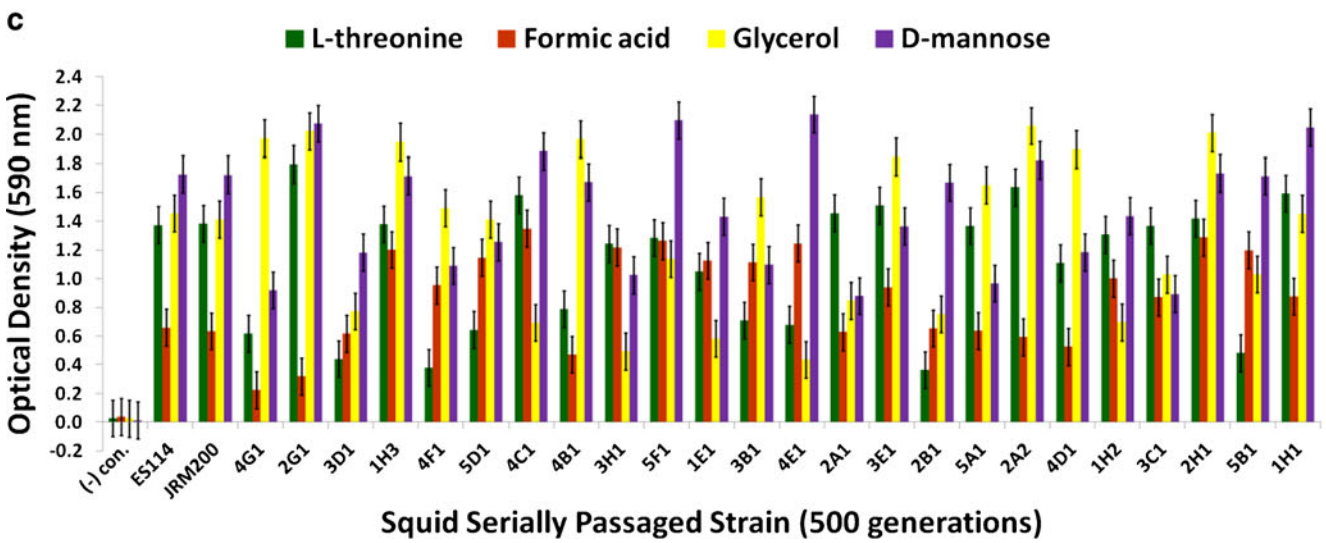
multitude of genes are dedicated to the *V. fischeri* catabolism of glycerol, formic acid, chitin, and fumaric acid while in the light organ. Fumaric acid can be made by the deamination of L-aspartic acid, a reaction carried out by aspartate ammonia-lyase, an enzyme expressed by symbiotic *V. fischeri* [42]. D-Mannose residues coat the epithelial linings of light organs within sepiolid squid hatchlings and play a fundamental role in *V. fischeri* attachment to squid host eukaryotic cells through mannose-recognizing adhesins on the bacterial surface [22, 43], which potentially serve as a source for "mannose" grazing. Prolific variation in catabolism of D-mannose among the derived lines relative to precursor state was evident (Fig. 5c).

For *V. fischeri*, some carbon sources in the Biolog GN2 microplate exhibit the most substantial metabolic diversity (hyperpolymorphic) known for any particular substrate. They include *N*-acetyl-D-galactosamine, L-fucose, α-ketoglutaric acid, D-trehalose, L-glutamic acid, D-serine, β-methyl-D-glucoside, citric acid, sucrose, and L-ornithine [37–40, 44, 45]. The Biolog data for the last eight of these hyperpolymoprhic carbon sources are shown for the wild isolates (Fig. 4a, b).

Fig. 5 Optical density (590 nm) measurements in **a** D-galactose, D-fructose, *N*-acetyl-D-glucosamine, and α-cyclodextrin; **b** D-psicose, L-serine, L-aspartic acid, and L-asparagine; and **c** L-threonine, formic acid, glycerol, and D-mannose of ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations (n=3). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate α=0.05). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]







All ET and no ES strains utilize α-ketoglutaric acid, D-trehalose, L-glutamic acid, and D-serine, while no ET and all ES isolates metabolize citric acid and sucrose. The ability to utilize seven new carbon sources was gained in some *V. fischeri* lines serially passaged through *E. tasmanica* for 500 generations—*N*-acetyl-D-galactosamine, L-fucose, α-ketoglutaric acid, D-trehalose, L-glutamic acid, D-serine, and β-methyl-D-glucoside, relative to forerunners *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 (last five shown in Fig. 6a, b). Conversely, substrates such as citric acid, sucrose, and L-ornithine were lost in some lines (Fig. 6b). *N*-acetyl-D-galactosamine has been found to participate in cell–cell attachment through lectins, carbohydrate-binding proteins on extracellular surfaces, including bioluminescent bacteria [46]. Lectins may be involved in biofilm formation and bacterial microcolony aggregations. Previous research has hypothesized that lectins with *N*-acetyl-D-galactosamine specificity govern symbiosis initiation in bioluminescent bacteria with marine animals, *V. fischeri* included [47]. Furthermore, D-serine, citric acid, α-ketoglutaric acid, β-methyl-D-glucoside, and *N*-acetyl-D-galactosamine have all been listed as carbon substrates dispensable for *Vibrio cholerae*, which are evolutionarily discarded and reacquired as necessary for certain niche environments [48]. Metabolic pathways can evolve modularly in function, some being especially malleable, to accommodate the niche breadth ecologically necessary for

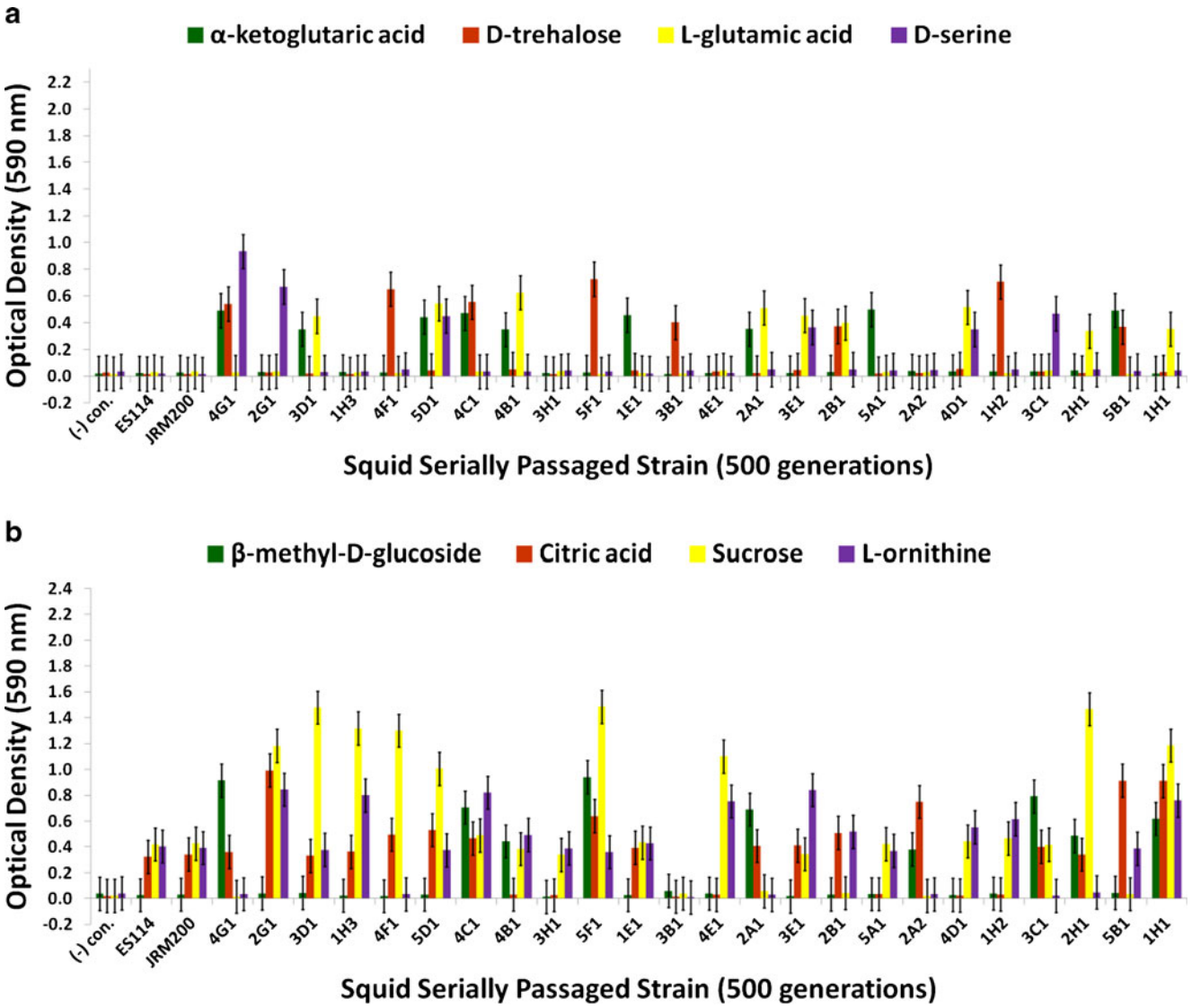


Fig. 6 Optical density (590 nm) measurements in **a** α-ketoglutaric acid, D-trehalose, L-glutamic acid, and D-serine, and **b** β-methyl-D-glucoside, citric acid, sucrose, and L-ornithine of ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations (n=3). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate α=0.05). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

Vibrio populations under specific environmental parameters (e.g., free-living versus host-associated lifestyles). Curiously, no wild isolates from *E. scolopes* and *E. tasmanica* grew on *N*-acetyl-D-galactosamine, L-fucose, and L-ornithine. L-Fucose residues may also serve as attachment sites for bacteria to other cells, such as fucose-sensitive hemagglutinin A (*fshA*) in *V. cholerae* [49]. In summary, ET convergence was observed with α-ketoglutaric acid, D-trehalose, L-glutamic acid, D-serine, β-methyl-D-glucoside, citric acid, and sucrose (Figs. 4a, b and 6a, b). As with motility and biofilm, evolutionary distinction is emerging among the lines in carbon source metabolism.

Bioluminescence

Bioluminescence in *V. fischeri* is necessary for sepiolid squid colonization, as *lux* null mutants display defects in colonization ability [6, 50, 51]. Negative control *E. coli* K12 MG1655 was never luminous. Mean and maximum mean bioluminescence (RLUs $[Log_{10}\ (CFUs/mL)]^{-1}mL^{-1}$) (Fig. 7a, b) and notable characteristics of the "growth-light" curves for the wild isolates and experimentally evolved lines were determined (Tables 1 and 2). All ET strains are brighter than all ES ones (Fig. 7a). ET wild strains are 10.4-fold and 15.6-fold brighter (higher power) than ES strains in mean and maximum mean bioluminescence, respectively. Furthermore, ET *V. fischeri* produce light before ES strains (ET: 195 min versus ES: 225 min) at lower cell densities (ET: 4.80×10^7 CFUs/mL versus ES: 1.00×10^8 CFUs/mL), ET symbionts achieving total photon emission maxima higher (ET: 4,294.2 RLUs versus ES: 264.5 RLUs) and later (ET: 327 min versus ES: 294 min) at higher cell densities (ET: 1.06×10^9 CFUs/mL versus ES: 4.67×10^8 CFUs/mL) than ES isolates in the process (Table 1). A trend in ET growth curves is the appearance of a lag phase, while this growth phase is completely lacking in ES symbiont reproduction. ET symbionts appear to be evolving a quorum-sensing machinery to maximize light production throughout bacterial growth, including producing light earlier at lower populations, prolonging length of time cells are luminous, surging light intensity or wattage, and increasing cell density at which maximum light emission occurs to amplify total amount of light output at an instant.

On average, mean and maximum mean bioluminescence increased 2.5-fold in the derived lines relative to the ancestor. All derived lines increased in mean and maximum mean bioluminescence as a result of adapting to novel host *E. tasmanica* (Fig. 7b); however, modifications of other traits suggest light intensity (i.e., power or wattage, photons emitted per cell per second) was not the only important factor influencing the evolution of bioluminescence. Population growth and quorum sensing apparatus may also have been affected. Some lineages challenged with a novel ET host developed a lag phase, a feature reminiscent of ET symbiont convergence (Tables 1 and 2). Perhaps the ET lag phase is a cost or tradeoff associated with elevated photon production. Moreover, the time point that the evolved lines first ignited occurred earlier and at lower cell densities relative to ancestral *V. fischeri* ES114 and unevolved *V. fischeri* JRM200, while maximal light output occurred later at higher cell densities. Evidently, polymorphisms exist for traits affecting population growth, bioluminescence, and quorum sensing (Fig. 7b; Table 2), as the various lineages are different from each other as well as from the ancestral state, perhaps due to differences in autoinducer sensitivity. As with motility, biofilm formation, and carbon sources demonstrating hyperpolymorphic utilization, bioluminescence displays convergent evolution to ET wild isolates yet simultaneously exhibits ecological diversification among the various lineages.

Discussion

Convergent Evolution

Convergent evolution implies that axenic squid light organs subject colonizing *V. fischeri* to similar selection pressures, and multifarious genetic changes exist to produce the same phenotypic solutions necessary for the symbionts to successfully respond to the evolutionary challenges imposed by a host [52]. Motility plays an integral role in the colonization of sepiolid squid by *V. fischeri* [53] and allows host-associated bacteria to reach the destination and surface desired for further colonization or attachment. Motility results suggest that adaptation to novel host *E. tasmanica* by *V. fischeri* JRM200 mandated first a decrease in motility and then a subsequent boost in biofilm formation. The latter may not have been possible until the first occurred due genetic constraints or evolutionary contingency (i.e., biofilm potential does not increase until mutations decreasing motility happens first) [54], or decreased motility may have been the more immediately important and necessary evolutionary change to adjust to a new host, with a surge in biofilm capacity coming later as a reinforcing adaptation. Interestingly enough is the realization that *V. fischeri* did not show statistically meaningful host adaptation to *E. tasmanica* until 400 generations, yet motility decreased by a significantly different amount by 100 generations [7]. Like motility, biofilm formation is also indispensable in squid colonization for *V. fischeri* [55, 56]. Motility and biofilms from wild symbiont populations in *Euprymna* squid hosts suggest convergent evolution between experimentally evolved lines in *E. tasmanica* and natural ET *V. fischeri* (Figs. 1a–d and 2a–d). ET *V. fischeri* form more prolific biofilms and swarm less than ES *V. fischeri*, while ES *V. fischeri* produce more restricted biofilms and move faster (Figs. 1a and 2a). Perhaps, symbionts in *E. tasmanica* are selected for better host tissue attachment and elevated stress tolerance (more sticky and hardy), while those in *E. scolopes*

are evolutionarily honed for chemotaxis (more nimble locomotion and elevated nutrient sensitivity). Biofilm formation is positively correlated in many host-associated vibrios with colonization potential, immunity avoidance, and eukaryotic cell attachment in hosts [57–59]. Biofilms are known to increase bacterial survival against environmental stress [58–61].

The artificial variation generated from the experimentally evolved lines is congruent with ET natural variation, as *V. fischeri* "domesticated" in *E. tasmanica* appear more phenotypically similar to field ET *V. fischeri* in motility and biofilm production and less like their original ES *V. fischeri*. Figure 2c suggests a threshold event or critical period was

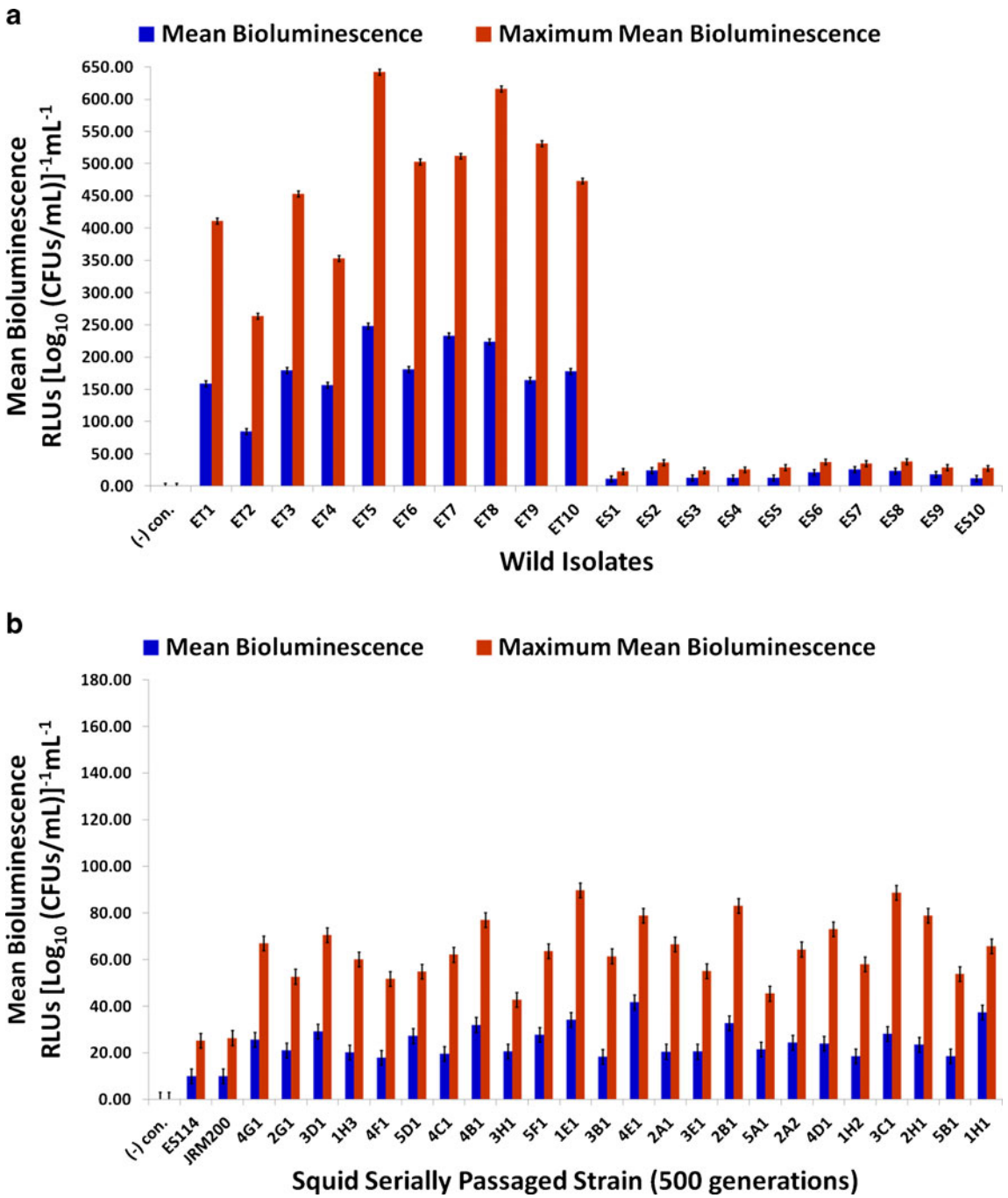


Fig. 7 Mean and maximum mean bioluminescence for **a** wild *V. fischeri* strains from *E. tasmanica* ("ET") and *E. scolopes* ("ES") specimens collected from the field and **b** ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations (n=6). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate α=0.05). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]. *E. coli* K12 MG1655 was used as the negative control

Table 1 Notable characteristics of the "growth-light" curves from wild *V. fischeri* strains from *E. tasmanica* ("ET") and *E. scolopes* ("ES") specimens collected from the field (n=6)

Strain	Time light emission began (min)	Log10 [CFUs/mL] where light was first emitted (±SE)	Log10 [CFUs/mL] where light was first emitted (±SE)	Maximal light output (RLUs) (±SE)	Log10 [CFUs/mL] where maximal light output occurred (±SE)	Lag phase (length)
ET1	210	7.797 (±0.033)	300	3,597.9 (±29.3)	8.754 (±0.046)	No
ET2	240	8.111 (±0.116)	360	2,464.5 (±20.9)	9.347 (±0.127)	No
ET3	180	7.510 (±0.057)	270	3,835.1 (±23.5)	8.464 (±0.140)	No
ET4	150	7.231 (±0.056)	300	3,072.5 (±33.4)	8.696 (±0.053)	No
ET5	180	7.492 (±0.134)	300	5,609.8 (±38.1)	8.737 (±0.077)	Yes (1.0 h)
ET6	210	7.786 (±0.082)	330	4,570.8 (±25.7)	9.088 (±0.060)	No
ET7	210	7.835 (±0.051)	360	4,809.9 (±19.9)	9.398 (±0.146)	No
ET8	210	7.815 (±0.086)	330	5,606.6 (±39.5)	9.096 (±0.048)	Yes (1.0 h)
ET9	180	7.418 (±0.087)	360	4,963.8 (±29.7)	9.338 (±0.122)	Yes (1.0 h)
ET10	210	7.818 (±0.054)	360	4,411.4 (±25.4)	9.317 (±0.092)	No
Mean	195 (±5.8)	7.681 (±0.056)	327 (±5.4)	4,294.2 (±162.6)	9.024 (±0.072)	–
ES1	210	7.793 (±0.109)	270	191.5 (±0.4)	8.375 (±0.048)	No
ES2	240	8.152 (±0.094)	300	316.1 (±5.6)	8.716 (±0.077)	No
ES3	240	8.168 (±0.078)	360	226.8 (±3.6)	9.275 (±0.058)	No
ES4	270	8.407 (±0.093)	330	229.9 (±4.1)	9.045 (±0.093)	No
ES5	240	8.207 (±0.084)	300	250.4 (±2.4)	8.752 (±0.035)	No
ES6	180	7.542 (±0.121)	240	306.9 (±2.5)	8.170 (±0.068)	No
ES7	240	8.111 (±0.052)	300	305.9 (±1.1)	8.696 (±0.033)	No
ES8	240	8.216 (±0.059)	330	345.6 (±0.9)	9.033 (±0.071)	No
ES9	210	7.780 (±0.055)	270	242.8 (±1.5)	8.369 (±0.047)	No
ES10	180	7.628 (±0.117)	240	228.6 (±2.6)	8.254 (±0.073)	No
Mean	225 (±4.8)	8.000 (±0.032)	294 (±6.5)	264.5 (±8.5)	8.669 (±0.099)	–

Negative control *E. coli* K12 MG1655 is omitted from the tables, since it produced no light

SE standard error

reached by 400 generations, since a sharp and sudden expanse in biofilm growth precipitously arises across many of the lineages in different media in a manner suggestive of second messenger signaling, signal transduction cascades, and quorum-sensing autoinducers [62]. The decrease in motility is more progressive and continued throughout the 500 generation time period. Not only was convergent evolution observed in biofilm development and motility between the derived lines and wild isolates but an inverse relationship was also noted. The inverse relationship between biofilm development and motility is known to have a biochemical basis within the genus *Vibrio*, specifically the second messenger cyclic diguanylate (c-di-GMP) [56, 59] and is a topic for future research. Generally, high intracellular concentrations of second messenger c-di-GMP correlate with increased biofilm formation, while lower quantities are associated with elevated motility (e.g., *V. cholerae*) [63]. Inverse relationships between motility and sessility in microbial lifestyles along a "c-di-GMP" continuum within hosts is an intriguing topic to partially explain competitive dominance and resulting tradeoffs observed in *Vibrio* symbionts colonizing *Euprymna* [9, 10]. Possibly, c-di-GMP intracellular pool levels as a "host" point of selection may be dissipated by sympatry of several *Sepiola* host species [1], not permitting for "c-di-GMP" ecological specialization for swarmer or biofilm ecotypes due to more homogenization of host environments in a geographical area. A scattering of few mutations affecting biofilm gene expression and c-di-GMP regulation drives convergent evolution and adaptive radiation in *Pseudomonas fluorescens* in structured microcosms, while simultaneously provoking astounding diversity [64, 65].

As with motility and biofilm formation, some convergent evolution appears to be occurring with carbon metabolism for substrates characteristic of evolutionary fluid allocation and redeployment in the *V. fischeri* physiological repertoire, when weighted against wild symbiont light organ populations from field-caught *E. tasmanica* and *E. scolopes* specimens (Figs. 4a, b, and 6a, b) [37–40, 44, 45]. Analyzing wild *V. fischeri* isolates from *Euprymna* squid hosts provides compelling evidence that citric acid and sucrose metabolic capabilities are abandoned in favor of L-glutamic acid, β-methyl-D-glucoside, D-trehalose, α-ketoglutaric acid, and D-serine for an

Table 2 Notable characteristics of the "growth-light" curves from ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations (n=6)

Strain	Time light emission began (min)	Log_{10} [CFUs/mL] where light was first emitted (±SE)	Log_{10} [CFUs/mL] where light was first emitted (±SE)	Maximal light output (RLUs) (±SE)	Log_{10} [CFUs/mL] where maximal light output occurred (±SE)	Lag phase (length)
ES114	210	8.152 (±0.041)	270	224.8 (±4.5)	8.886 (±0.014)	No
JRM200	210	8.165 (±0.014)	270	234.1 (±3.3)	8.876 (±0.004)	No
Mean	210	8.159 (±0.004)	270	229.5 (±2.4)	8.881 (±0.003)	–
4G1	150	6.624 (±0.006)	270	514.0 (±20.8)	7.769 (±0.045)	Yes (1.5 h)
2G1	180	7.551 (±0.005)	360	481.5 (±8.4)	9.280 (±0.008)	No
3D1	150	6.869 (±0.004)	390	644.5 (±8.6)	9.246 (±0.113)	Yes (1.0 h)
1H3	180	7.595 (±0.016)	390	566.3 (±8.3)	9.539 (±0.110)	No
4 F1	180	7.496 (±0.041)	330	461.9 (±0.1)	9.068 (±0.053)	No
5D1	210	7.817 (±0.051)	450	568.0 (±8.4)	9.770 (±0.053)	No
4C1	150	6.924 (±0.009)	330	536.3 (±0.7)	8.746 (±0.025)	Yes (1.0 h)
4B1	180	7.514 (±0.046)	300	663.6 (±3.1)	8.705 (±0.044)	No
3H1	210	7.801 (±0.030)	300	366.0 (±9.0)	8.705 (±0.004)	No
5F1	150	7.192 (±0.004)	360	583.7 (±37.2)	9.295 (±0.084)	No
1E1	150	6.897 (±0.033)	360	806.3 (±13.1)	9.058 (±0.079)	Yes (1.0 h)
3B1	180	7.702 (±0.007)	300	540.8 (±0.1)	8.916 (±0.004)	No
4E1	150	7.272 (±0.012)	390	749.7 (±19.1)	9.592 (±0.064)	No
2A1	180	7.491 (±0.093)	390	631.9 (±1.8)	9.597 (±0.044)	No
3E1	180	7.459 (±0.049)	330	490.8 (±2.7)	9.038 (±0.020)	No
2B1	150	6.896 (±0.034)	360	741.6 (±19.2)	9.008 (±0.056)	Yes (1.0 h)
5A1	180	7.544 (±0.077)	270	372.2 (±11.1)	8.344 (±0.070)	No
2A2	180	7.487 (±0.068)	330	575.0 (±12.0)	9.045 (±0.045)	No
4D1	180	7.501 (±0.051)	330	651.5 (±16.5)	9.022 (±0.062)	No
1H2	180	7.615 (±0.007)	330	518.3 (±0.7)	9.061 (±0.015)	No
3C1	210	7.807 (±0.041)	300	767.5 (±11.0)	8.732 (±0.044)	No
2H1	180	7.535 (±0.014)	300	684.0 (±4.7)	8.756 (±0.001)	No
5B1	180	7.578 (±0.012)	300	461.0 (±0.6)	8.702 (±0.001)	No
1H1	180	7.420 (±0.005)	390	622.6 (±10.4)	9.579 (±0.007)	Yes (0.5 h)
Mean	175 (±4.0)	7.430 (±0.038)	340 (±9.3)	590.4 (±24.5)	9.033 (±0.093)	–

Negative control *E. coli* K12 MG1655 is omitted from the tables, since it produced no light

SE standard error

ET lifestyle. This is a trend observed in the 24 lines originally retrieved from *E. scolopes* and experimentally adapted to novel host *E. tasmanica.* The functions of these carbon sources in bacteria, the genus *Vibrio*, and in sepiolid squid–*V. fischeri* mutualisms have not been fully elucidated. D-Amino acids are largely restricted to peptidoglycan and teichoic acid in the bacterial cell wall [66], but D-serine could serve as an electron donor with appropriate isomerase function (e.g., mutase, racemase, or epimerase activity), as has been identified in *V. cholerae* [67]. β-Methyl-D-glucoside may serve as an alternative nutrient for bacteria when rapidly metabolizable and more energy efficient catabolites are unavailable [68], as is true for all less preferred carbon sources—but still utilized by *V. fischeri* on the Biolog GN2 microplate. β-Methyl-D-glucoside utilization has been implicated in chemotaxis, motility, phosphorylation by the phosphotransferase system, and *rpoB* mutations affecting RNA polymerase, regulatory proteins, regulatory RNA three-dimensional structures, and translation initiation/termination complexes [69]. β-Methyl-D-glucoside may also be employed in membrane-derived oligosaccharide synthesis and metabolism of cellulose [69, 70], a polymer known to be a component of *V. fischeri* biofilms [56]. D-Trehalose may also function in protection against osmotic stresses [71].

L-Glutamic acid and citric acid increase *V. cholerae* host colonization potential in humans through the phosphotransacetylase–acetate kinase pathway (i.e., acetate switch) [72, 73], which drives ATP generation, recycles acetyl-

CoA when respiration and central metabolism are backlogged, and post-translational regulation of proteins, illustrating the effect carbon sources can have especially on animal host colonization when the substrates themselves are main metabolites easily shuttled or shunted into alternate biochemical pathways (The acetate switch is present in *V. fischeri* [74], but acetic acid did exhibit ET convergence.) [72]. L-Glutamic acid has also been reported to aid bacterial growth during iron limitation [75], which is noteworthy considering the squid light organ is low in available iron [76]. Furthermore, α-ketoglutaric acid is known to function as a substrate for the production of bacterial siderophores to assist iron acquisition in *Vibrio* [77]. α-Ketoglutaric acid is also believed to possess value against oxidative stress [78], and the squid light organ is known to initiate a respiratory burst via innate host immunity that produces toxic oxygen species [79]. Perhaps, the convergent evolution depicted in biofilm formation and these carbon sources are related, either to exploit new resources or increase resistance against unaccustomed stressors present in a novel host. Enhancing colonization and persistence in an unfamiliar host are other possibilities. How alternate carbon sources may be used by *V. fischeri* to manage against various host stresses (e.g., tenacious immune defenses) is a stimulating prospect for future study. For instance, amino acids may be more useful than monosaccharides for some cells against osmotic stress, while the former might be more beneficial to regulate high pH stress if nitrogen is not limiting (otherwise amino sugars such *N*-acetyl-D-glucosamine could be used if available) [80]. Although not relevant to the squid light organ, carbon source is known to affect bacterial susceptibility to heavy metal toxicity, including *V. fischeri* [81]. Convergent evolution coupling metabolism and stress together has been described in mutualisms between invertebrate hosts and microbial symbionts [82, 83].

For bioluminescence, the derived lines displayed convergence to ET wild isolates (Fig. 7a, b; Tables 1 and 2), implying that light emission may be associated with biofilm formation, motility, and the convergent carbon sources. *luxU* links bioluminescence to biofilm formation in *V. fischeri* [84], and *rpoN* controls motility, biofilm, luminescence, and squid colonization ability [85]. Bioluminescence may be tied to biofilm formation, motility, and central metabolism through integrated circuitries involving quorum sensing and c-di-GMP second messenger signaling [86], which is consistent with the simultaneous increase in luminosity, biofilm formation, and lower motility. Relative to the ancestor, light emission occurred earlier and at a lower cell density for the derived lines, while maximal light output ensued later and at a higher cell density (Table 2). Early quorum sensing at low cell densities and late quorum sensing at high cell densities implicate the AinS and Lux signal transduction relays in *V. fischeri*, respectively [87]. AinS quorum sensing has also been associated with the acetate switch [74]. Thus, evidence exists adaptation to *E. tasmanica* and conceivably to novel hosts in general involves selection at multiple layers of cell signaling (e.g., GGDEF, EAL, and PilZ proteins), which could account for appearance of a lag phase [14, 88]. Mutations affecting AinS quorum sensing is known to affect growth curves [89]. Previous efforts failed to find bioluminescence differences among the evolved lines within *E. tasmanica*, and several replicate animals were not examined within a single evolved line (e.g., using n=3 squid hosts with 3B1 instead of one animal each with 3B1, 5B1, and 4F1) [7]. At the time of study, there were insufficient *E. tasmanica* hatchlings available for these experiments. However, derived lines that are significantly brighter at 500 generations relative to ancestral *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 have recently been observed in *E. tasmanica* (unpublished data). Prior investigation showed the derived lines were more dim in the ancestral host *E. scolopes*, giving off less photons per $\log_{10}$ (CFUs/mL) mL^{-1} [7]. *E. scolopes* hatchlings and adults are smaller (with concomitantly smaller light organs as well) than *E. tasmanica* throughout their entire life cycles. Therefore, *E. scolopes* individual hosts carry one to two orders of magnitude less symbionts than *E. tasmanica*, regardless of the ontogenetic stage. Hence, tradeoffs may now exist in the ancestral host *E. scolopes* where *V. fischeri* cell densities and quorum sensing are mistimed for motility, biofilm formation, and bioluminescence due to adaptation of the derived lines to Australian *E. tasmanica*, since symbionts can no longer reach the proper population levels in the Hawaiian squid at the appropriate times of host development, as the symbiont and host life histories are no longer accurately synchronized.

Diversification

Diversifying evolution or radiation predominates over convergent evolution in morphospace when profuse phenotypes prevail to be evolutionarily fit or successful to a particular selection pressure. A multitude of mutations to attain each of these prosperous possibilities in a complex, heterogeneous environment with vacant niches provide ecological opportunity, an attribute characteristic of island evolution, including Galapagos finches, Hawaiian silversword alliance, Hawaiian honeycreepers, *Anolis* lizards, and cichlids in their lake habitat islands [90–92]. Aside from the carbon sources displaying convergent evolution, the remaining carbon sources metabolizable by *V. fischeri* wild isolates depicted a general polymorphism, a tendency also reflected in the derived lines (representative examples in Figs. 3a–c and 5a–c). These polymorphic carbon sources are being used distinctly and variedly, both individually and as an assortment. These results are congruent with other microbial experimental evolution studies [52, 93], including with *E. coli* experiments with less than 800 generations of evolution in homogenous and unstructured environments [94]. This increased "domesticated" variation may be reflective of diversifying selection in the squid light

organ with incipient resource partitioning and changes in nutrient specificities toward carbon resources, perhaps even as part of cross-feeding or syntrophy between other *V. fischeri* ecotypes within the light organ of the same squid host individual [91, 94]. The implication of these results in nature are profound. *Euprymna* squid can live up to a year, which amounts to 1,500–2,000 *V. fischeri* generations [7]. Squid light organs are microcosms full of convoluted chasms with tremendous physical and biochemical complexity and heterogeneity [95], and a single *V. fischeri* clone has immense opportunity to evolve cross-feeding with either other *V. fischeri* subtypes or host cells within a host lifetime. The squid light organ microenvironment varies in spatial and temporal carbon source composition available to *V. fischeri* cells (e.g., *N*-acetyl-D-glucosamine at night and glycerol in the morning) [42]. Moreover, within the light organ of a single individual squid host, different *V. fischeri* subtypes will specialize in where they will settle and reside, with little mixing of individual *V. fischeri* variants within the light organ symbiont population despite the disturbance imposed by daily venting [42, 96]. In turn, this dissimilarity in the spatial localization and structured regional occupancy of the light organ by *V. fischeri* cells leads to differential gene expression in the symbionts, which can be a primer for the evolution of ecological differentiation.

An imperative realization is biofilm formation, motility, convergent carbon sources, and bioluminescence are also exemplifying ecological diversification. Despite the manifestation in convergence to wild ET isolates in these traits, the evolved lines are still becoming divergent from one another. Other laboratories have also proved convergent evolution facilitates ecological diversification in separate lineages, including on other carbon sources [52, 93]. Quorum sensing and biofilms beget diversity, including the modification of population growth (e.g., lag phase changes) [97, 98]. Research with *V. cholerae* verifies alternate carbon sources can be incorporated or substituted into the composition of a biofilm to access new ecological niches [99, 100]. Intriguingly, bioluminescence (i.e., quorum sensing) and *trans*-genetic regulation of the *lux* operon could function as a generator for *V. fischeri* adaptive radiation inside this symbiont's animal hosts, as cAMP receptor protein (a transcriptional regulator) may have a continuum of graded possible effects on *lux* operon gene expression due to the presence of different carbon sources in various light organ microenvironments within sepiolid squids and monocentrid fishes [62]. A captivating possibility is the utilization of the sepiolid squid-*Vibrio* symbiosis to investigate evolvability and versatility in *V. fischeri*. *V. fischeri* possesses three biofilm gene clusters, *syp*, *vpsII* like, and the cellulose operon, yielding over 30 genes that can be independently modified to stimulate biofilm niche differentiation to exploit squid light organ microenvironments in novel ways [101]. This evolutionary process is quite analogous to the customization of the myriad bony elements in the head region of cichlids [102]. Additionally, modularity (as seen in convergent carbon sources, Figs. 4a, b, and 6a, b) increases the evolutionary diversification potential of a lineage, including for adaptive radiation [103, 104].

Squids as Host Habitat Islands

The use of biogeography theory to characterize microbial diversity has been previously applied, including mutualisms, pathogen–host interactions, and microcosms [105–110]. Axenic sepiolid squid hatchlings, first emanating from their eggs, essentially serve as sterile and mobile volcanic islands (i.e., host habitat islands) that subject their bioluminescent symbionts to severe genetic bottlenecks during colonization [7, 106, 108, 111, 112]. Only 6 to 12 *V. fischeri* cells of a 10^0–10^3 CFUs/mL seawater inoculum initiate symbiosis in an animal that ultimately reach an adult light organ carrying capacity of 10^8–10^{11} cells per host [113], providing pioneer *V. fischeri* colonizers with ecological release from the semi-starvation frequently encountered during the oceanic free-living phase. Such episodes of genetic bottlenecks with ensuing abundant proliferation and population recovery are called "founder flushes" [114], which provide opportunities for population movements across valleys via genetic drift to alternative adaptive peaks [90] and facilitate genetic revolutions, unique adaptations, founder effect evolution not normally possible, and novel independent evolutionary trajectories into vacant niches [e.g., island syndromes such as gigantism in the genera *Geochelone* and *Aepyornis*, nanism in *Mammuthus exilis*, and evolution of woody lifestyles by herbaceous plants] [90, 101, 115–118]. Founder flushes have been documented for bacteria, including *V. cholerae* and *Helicobacter pylori*, and are of significance in epidemiology [119]. Within the squid host, founder flushes lead to expanding *V. fischeri* niche breadth and ecological diversification (e.g., alternate metabolic utilization) from initial tight symbiont bottlenecks containing low genetic diversity, as has been observed in heterogeneous and complex microcosm environments with *P. fluorescens* [91, 120]. Moreover, the sepiolid squid light organ is an immensely specialized and complex structure [95], where *V. fischeri* populations can undergo ecological differentiation over the lifetime of the cephalopod host [7, 90, 101]. These conclusions are consistent with population genetics conducted with ES, ET, and *Sepiola* wild isolates of *V. fischeri* from sepiolid squid hosts through vast scales of space encompassing the world's oceans and time spanning 20,000 generations of symbiont evolution [7, 33, 121, 122]. The presence of phenotypic convergent evolution and ecological diversification of *V. fischeri* serially passaged through *E. tasmanica* in motility, biofilm formation, metabolism, and bioluminescence when compared to wild isolates from this animal indicate host adaptive landscapes that have both smooth and rugged, multi-peaked topographies [123, 124],

providing this bacterium with tremendous ecological opportunities to exploit a vast N-dimensional niche hypervolume space within animal hosts [125]. Innumerable evolutionary trajectories and adaptive peaks are available in the fitness landscape of a novel host. Simply put, there are many ways *V. fischeri* can successfully colonize a squid, permitting extensive specialization, resource partitioning, and genetically distinct ecotype subpopulations to crystallize within an animal host—in essence adaptive radiation [120, 126, 127].

Acknowledgments The authors would like to thank the staff at Kewalo Marine Laboratory and the Sydney Institute of Marine Sciences for the help with collecting and maintaining squids prior to shipment. F. Rivera was supported by the NMSU Minority for Access Careers program (NIH GM07667-35). W. Soto was supported by the NIH RISE program at NMSU (NIH NIGMS R25GM061222). This work was supported by NIH NIAID 1SC1AI081659, NIH NIAIDS 3SC1AI081659-02S1, and NSF IOS 074498 to M.K.N.

References

1. Nishiguchi MK (2000) Temperature affects species distribution in symbiotic populations of *Vibrio* spp. Appl Environ Microbiol 66: 3550–3555
2. Jones BW, Nishiguchi MK (2004) Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Mar Biol 144:1151–1155
3. Graf J, Ruby EG (1998) Host-derived amino acids support the proliferation of symbiotic bacteria. Proc Natl Acad Sci 95:1818–1822
4. McFall-Ngai MJ, Ruby EG (1991) Symbiont recognition and subsequent morphogenesis as early events in an animal–bacterial mutualism. Science 254:1491–1494
5. Nyholm SV, Nishiguchi MK (2008) The evolutionary ecology of a sepiolid squid–*Vibrio* association: from cell to environment. Vie Et Milieu Life Environ 58:175–184
6. Ruby EG (1996) Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri–Euprymna scolopes* light organ symbiosis. Annu Rev Microbiol 50:591–624
7. Soto W, Punke EB, Nishiguchi MK (2012) Evolutionary perspectives in a mutualism of sepiolid squid and bioluminescent bacteria: combined usage of microbial experimental evolution and temporal population genetics. Evolution 66:1308–1321
8. Nishiguchi MK, Nair VS (2003) Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. Int J Syst Evol Microbiol 53:2019–2026
9. Nishiguchi MK (2002) Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. Microb Ecol 44:10–18
10. Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1998) Competitive dominance during colonization is an indicator of coevolution in an animal–bacterial symbiosis. Appl Environ Microbiol 64:3209–3213
11. Bello G (1995) A key for the identification of the Mediterranean sepiolids (Mollusca: Cephalopoda). In: Boletzky S (ed) Mediterranean Sepiolidae, vol 16, Bulletin de L'Institute oceanographiique. Monaco, Monaco, pp 41–55
12. O'Shea TM, Klein AH, Geszvain K, Wolfe AJ, Visick KL (2006) Diguanylate cyclases control magnesium-dependent motility of *Vibrio fischeri*. J Bacteriol 188:8196–8205
13. McCarter ML (2010) Bacterial acrobatics on a surface: swirling packs, collisions, and reversals during swarming. J Bacteriol 192: 3246–3248
14. Wolfe AJ, Visick KL (2008) Get the message out: cyclic-di-GMP regulates multiple levels of flagellum-based motility. J Bacteriol 190:463–475
15. Daniels R, Vanderleyden J, Michiels J (2004) Quorum sensing and swarming migration in bacteria. FEMS Microbiol Rev 28:261–289
16. Kim M-Y, Park R-Y, Choi M-H, Sun H-Y, Kim C-M, Kim S-Y, Rhee J-H, Shin S-H (2006) Swarming differentiation of *Vibrio vulnificus* downregulates the expression of the vvhBA hemolysin gene via the LuxS quorum-sensing system. J Microbiol 44:226–232
17. Trimble MJ, McCarter LL (2011) Bis-(3′–5′)-cyclic dimeric GMP-linked quorum sensing controls swarming in *Vibrio parahaemolyticus*. Proc Natl Acad Sci 108:18079–18084
18. McCarter ML (2004) Dual flagellar systems enable motility under different circumstances. J Mol Microbiol Biotechnol 7:18–29
19. McCarter LL (2001) Polar flagellar motility of the Vibrionaceae. Microbiol Mol Biol Rev 65:445–462
20. Merino S, Shaw JG, Tomás JM (2006) Bacterial lateral flagella: an inducible flagella system. FEMS Microbiol Lett 263:127–135
21. Merino S, Tomás JM (2009) Lateral flagella systems. In: Jarrell KF (ed) Pili and Flagella: current research and future trends. Caister Academic Press, Norfolk, UK
22. Ariyakumar DS, Nishiguchi MK (2009) Characterization of two host-specific genes, mannose-sensitive hemagglutinin (mshA) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol Lett 299:65–73
23. Nealson KH (1978) Isolation, identification, and manipulation of luminous bacteria. Methods Enzymol 57:153–165
24. McCann J, Stabb EV, Millikan DS, Ruby EG (2003) Population dynamics of *Vibrio fischeri* during infection of *Euprymna scolopes*. Appl Environ Microbiol 69:5928–5934
25. Soto W, Gutierrez J, Remmenga MD, Nishiguchi MK (2009) Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. Microb Ecol 57:140–150
26. Nair VS, Nishiguchi MK (2009) *Vibrio fischeri* strains exhibit differential biological properties both in their free-living and symbiotic niches. Vie Et Milieu Life Environ 58:175–184
27. Harshey RM (2003) Bacterial motility on a surface: many ways to a common goal. Annu Rev Microbiol 57:249–273
28. Sokal RR, Rohlf FJ (1995) Biometry. W.H. Freeman & Company, New York City
29. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 22:996–1006
30. Bochner BR (2009) Global phenotypic characterization of bacteria. FEMS Microbiol Lett 33:191–205
31. Bochner BR (1989) Sleuthing out bacterial identities. Nature 339: 157–158
32. Abdi H (2010) The greenhouse–Geisser correction. In: Salkind N (ed) Encyclopedia of research design. SAGE Publications, Thousand Oaks, CA, USA
33. Jones BW, Lopez JE, Huttenburg J, Nishiguchi MK (2006) Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. Mol Ecol 15:4317–4329
34. Elena SF, Lenski RE (2003) Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat Rev Genet 4:457–469
35. Hendrie MS, Hodgkiss W, Shewan JM (1971) Proposal that *Vibrio marinus* (Russell 1891) Ford 1927 be amalgamated with *Vibrio fischeri* (Beijerinck 1889) Lehmann and Neumann 1896. Int J Syst Bacteriol 21:217–221
36. Ruby EG, Nealson KH (1976) Symbiotic association of *Photobacterium fischeri* with the marine luminous fish

Monocentris japonica: a model of symbiosis based on bacterial studies. Biol Bull 151:574–586
37. Bryant TN, Lee JV, West PA, Colwell RR (1986) Numerical classification of *Vibrio* and related genera. J Appl Bacteriol 61:437–467
38. Sawabe T, Sugimura I, Ohtsuka M, Nakano K, Tajima K, Ezura Y, Christen R (1998) *Vibrio halioticoli* sp. nov., a non-motile alginolytic marine bacterium isolated from the gut of the abalone *Haliotis discus hannai*. Int J Syst Bacteriol 48:573–580
39. Alcaide E (2003) Numerical taxonomy of Vibrionaceae isolated from cultured amberjack (*Seriola dumerili*) and surrounding water. Curr Microbiol 46:184–189
40. Farmer JJ III, Janda JM, Brenner DJ, Krieg NR, Staley JR (2005) *Vibrio*. In: Brenner DJ, Krieg NR, Staley JR (eds) Bergey's manual systematic bacteriology. Springer-Verlag, New York, pp 494–546
41. Kim H-J, Hyun E-K, Kim Y-S, Lee Y-J, Oh D-K (2006) Characterization of an *Agrobacterium tumefaciens* d-psicose, 3-epimerase that converts, d-fructose to d-psicose. Appl Environ Microbiol 72:981–985
42. Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiassé RP, Schaefer AL, Koroleva I, Splinter-BonDurant S, Brown B, Manzella L, Einat Snir E, Almabrazi H, Scheetz TE, de Fatima BM, Casavant TL, Soares MB, Cronan JE, Reed JL, Ruby EG, McFall-Ngai MJ (2010) Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. Proc Natl Acad Sci 107:2259–2264
43. Visick KL, McFall-Ngai MJ (2000) An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. J Bacteriol 182:1779–1787
44. Noguerola I, Blanch AR (2008) Identification of *Vibrio* spp. with a set of dichotomous keys. J Appl Microbiol 105:175–185
45. Thompson FL, Iida T, Swings J (2004) Biodiversity of *Vibrios*. Microbiol Mol Biol Rev 68:403–431
46. Vydryakova GA, Kirpichenko TV, Lifant'eva AA (2007) Formation of aggregated structures by luminescent bacteria in the presence of carbohydrates. Microbiology 2:282–284
47. Vydryakova GA (2006) Carbohydrate specificity of lectins from luminous bacteria. Appl Biochem Microbiol 42:364–368
48. Keymer DP, Miller MC, Schoolnik GK, Boehm AB (2007) Genomic and phenotypic diversity of coastal *Vibrio cholerae* strains is linked to environmental factors. Appl Environ Microbiol 73:3705–3714
49. Albert MJ, Bhuiyan NA, Talukder KA, Kaisar A, Faruque ASG, Nahar S, Faruque SM, Ansaruzzaman M, Rahman M (1997) Phenotypic and genotypic changes in *Vibrio cholerae* O139 Bengal. J Clin Microbiol 35:2588–2592
50. Soto W, Lostroh CP, Nishiguchi MK (2010) Physiological responses to stress in the Vibrionaceae. In: Seckback J, Grube M (eds) Cooperation and stress in biology: joint ventures in biology, vol 17, Springer. New York, NY, USA, pp 407–426
51. Visick KL, Foster J, Doino J, McFall-Ngai MJ, Ruby EG (2000) *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J Bacteriol 182:4578–4586
52. MacLean RC, Bell G (2003) Divergent evolution during an experimental adaptive radiation. Proc R Soc B Biol Sci 270:1645–1650
53. Millikan DS, Ruby EG (2002) Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. Appl Environ Microbiol 68: 2519–2528
54. Travisano M, Mongold JA, Bennett AF, Lenski RE (1995) Experimental tests of the roles of adaptation, chance, and history in evolution. Science 267:87–90
55. Hussa EA, Darnell CL, Visick KL (2008) RscS functions upstream of SypG to control the *syp* locus and biofilm formation in *Vibrio fischeri*. J Bacteriol 190:4576–4583
56. Yildiz FH, Visick KL (2009) *Vibrio* biofilms: so much the same yet so different. Trends Microbiol 17:109–118
57. McDougald D, Kjelleberg S (2006) Adaptive responses of vibrios. In: Thompson FL, Austin B, Swings J (eds) The biology of vibrios. ASM, Washington, D.C, pp 133–155
58. Chavez-Dozal AA, Nishiguchi MK (2011) Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri*. J Basic Microbiol 51:452–458
59. Chavez-Dozal AA, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK (2012) Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. FEMS Microbiol Lett 81:562–573
60. Landini P (2009) Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. Res Microbiol 160:259–266
61. Chavez-Dozal AA, Gorman C, Erken M, Steinberg PD, McDougald D, Nishiguchi MK (2013) Predation response of *Vibrio fischeri* biofilms to bacterivorus protists/phagotrophic protozoa. Appl Environ Microbiol 79:553–558
62. Miyashiro T, Ruby EG (2012) Shedding light on bioluminescence regulation in *Vibrio fischeri*. Mol Microbiol 84:795–806
63. Jude BA, Taylor RK (2008) Genetics of *Vibrio cholerae* colonization and motility. In: Faruque SM, Nair GB (eds) *Vibrio cholerae* genomics and molecular biology. Caister Academic, Norfolk, pp 67–79
64. McDonald MJ, Gehrig SM, Meintjes PL, Zhang X-X, Rainey PB (2009) Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. IV. Genetic constraints guide evolutionary trajectories in a parallel adaptive radiation. Genetics 183:1041–1053
65. MacLean RC (2005) Adaptive radiation in microbial microcosms. J Evol Biol 18:1376–1386
66. Madigan MT, Martinko JM (2006) Brock biology of microorganisms. Pearson Prentice Hall, Upper Saddle River
67. Lam H, Oh D-C, Cava F, Takacs C-N, Clardy J, de Pedro MA, Matthew K, Waldor MK (2009) D-Amino acids govern stationary phase cell wall remodeling in bacteria. Science 323:1552–1155
68. Tobisch S, Stulke J, Hecker M (1999) Regulation of the *lic* operon of *Bacillus subtilis* and characterization of potential phosphorylation sites of the *licR* regulator protein by site-directed mutagenesis. J Bacteriol 181:4995–5003
69. Perkins AE, Nicholson WL (2008) Uncovering new metabolic capabilities of *Bacillus subtilis* using phenotype profiling of rifampin-resistant *rpoB* mutants. J Bacteriol 190:807–814
70. Kennedy EP (1987) Membrane-derived oligosaccharides. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Shaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella typhimurium*, vol 1. ASM Press, Washington, D.C, pp 672–679
71. DeLoney CR, Bartley TM, Visick KL (2002) Role for phosphoglucomutase in *Vibrio fischeri–Euprymna scolopes* symbiosis. J Bacteriol 184:5121–5129
72. Minato Y, Fassio SR, Wolfe AJ, Hase CC (2013) Central metabolism controls transcription of a virulence gene regulator in *Vibrio cholerae*. Microbiology 159:792–802
73. Wolfe AJ (2005) The acetate switch. Microbiol Mol Biol Rev 69: 12–50
74. Studer SV, Mandel MJ, Ruby EG (2008) AinS quorum sensing regulates the *Vibrio fischeri* acetate switch. J Bacteriol 190:5915–5923
75. Filiatrault MJ, Stodghill PV, Bronstein PA, Moll S, Lindeberg M, Grills G, Schweitzer P, Wang W, Schroth GP, Luo S, Khrebtukova I, Yang Y, Thannhauser T, Butcher BG, Cartinhour S, Schneider DJ (2010) Transcriptome analysis of *Pseudomonas syringae* identifies new genes, noncoding RNAs, and antisense activity. J Bacteriol 192:2359–2372
76. Graf J, Ruby EG (2000) Novel effects of a transposon insertion in the *Vibrio fischeri* glnD gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. Mol Microbiol 37: 168–179

77. Kadi N, Challis GL (2009) Siderophore biosynthesis: a substrate specificity assay for nonribosomal peptide synthetase-independent siderophore synthetases involving trapping of acyl-adenylate intermediates with hydroxylamine. Methods Enzymol 458:432–457
78. Mizunoe Y, Wai SN, Takade A, Yoshida S (1999) Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using H_2O_2-degrading compounds. Arch Microbiol 172:63–67
79. Ruby EG, McFall-Ngai MJ (1999) Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends Microbiol 7:414–420
80. Jaenicke R (1981) Enzymes under extremes of physical conditions. Annu Rev Biophys Bioeng 1:1–67
81. Tekerlekopoulou AG, Tsiamis G, Dermou E, Siozios S, Bourtzis K, Vayenas DV (2010) The effect of carbon source on microbial community structure and Cr(VI) reduction rate. Biotechnol Bioeng 107:478–487
82. Chaston J, Goodrich-Blair H (2010) Common trends in mutualism revealed by model associations between invertebrates and bacteria. FEMS Microbiol Rev 34:41–58
83. Fan L, Reynolds D, Liu M, Stark M, Kjelleberg S, Webster NS, Thomas T (2012) Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. Proc Natl Acad Sci 109:E1878–E1887
84. Ray VA, Visick KL (2012) LuxU connects quorum sensing to biofilm formation in *Vibrio fischeri*. Mol Microbiol 86:954–970
85. Wolfe AJ, Millikan DS, Campbell JM, Visick KL (2004) *Vibrio fischeri* σ54 controls motility, biofilm formation, luminescence, and colonization. Appl Environ Microbiol 70:2520–2524
86. Srivastava D, Waters CM (2012) A tangled web: regulatory connections between quorum sensing and cyclic di-gmp. J Bacteriol 194:4485–4493
87. Lupp C, Ruby EG (2004) *Vibrio fischeri* LuxS and AinS: comparative study of two signal synthases. J Bacteriol 186:3873–3881
88. Visick KL (2005) Layers of signaling in a bacterium–host association. J Bacteriol 187:3603–3606
89. Lupp C, Ruby EG (2005) *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. J Bacteriol 187:3620–3629
90. Whittaker RJ, Fernandez-Palacios JM (2007) Island biogeography: ecology, evolution, and conservation. Oxford University Press, Oxford
91. Travisano M, Rainey PB (2000) Studies of adaptive radiation using model microbial systems. Am Nat 156:S35–S44
92. Rainey PB, Travisano M (1998) Adaptive radiation in a heterogeneous environment. Nature 394:69–72
93. Ostrowski EA, Woods RJ, Lenski RE (2008) The genetic basis of parallel and divergent phenotypic responses in evolving populations of *Escherichia coli*. Proc R Soc B Biol Sci 275:277–284
94. Rosenzweig RF, Sharp RR, Treves DS, Adams J (1994) Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli*. Genetics 137:903–917
95. Sycuro L, Ruby E, McFall-Ngai M (2006) Confocal microscopy of the light organ crypts in juvenile *Euprymna scolopes* reveals their morphological complexity and dynamic function in symbiosis. J Morphol 267:555–568
96. Dunn AK, Millikan DS, Adin DM, Bose JL, Stabb EV (2006) New rfp- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and lux expression in situ. Appl Environ Microbiol 72:802–810
97. Ponciano JM, La H-J, Joyce P, Forney LJ (2009) Evolution of diversity in spatially structured *Escherichia coli* populations. J Bacteriol 75:6047–6054
98. Milton DL (2006) Quorum sensing in vibrios: complexity for diversification. Int J Med Microbiol 296:61–71
99. Chatterjee SN, Chaudhuri K (2003) Lipopolysaccharides of *Vibrio cholerae*. I. Physical and chemical characterization. Biochim Biophys Acta 1639:65–79
100. Sozhamannan S, Yildiz FH (2011) Diversity and genetic basis of polysaccharide biosynthesis in *Vibrio cholerae*. In: Bhattacharya SK, Ramamurthy T (eds) Epidemiological and molecular aspects on cholera. Springer, New York
101. Schluter D (2000) Ecology of adaptive radiation. Oxford University Press, Oxford
102. Galis F, Metz JAJ (1998) Why are there so many cichlid species? Trends Ecol Evol 13:1–2
103. Yang AS (2001) Modularity, evolvability, and adaptive radiations: a comparison of the hemi- and holometabolous insects. Evol Dev 3: 59–72
104. Rainey PB, Cooper TF (2004) Evolution of bacterial diversity and the origins of modularity. Res Microbiol 155:370–375
105. Dickerson JE, Robinson JR, Robinson JV (1985) Microcosms as islands: a test of the MacArthur-Wilson equilibrium theory. Ecology 66:966–980
106. Holt RD (2000) A biogeographical and landscape perspective on within-host infection dynamics. In: Bell CR, Brylinsky M, Johnson-Green P (eds) Proceedings of the 8th international symposium of microbial ecology. Atlantic Canada Society for Microbial Ecology, Halifax, Canada, pp 583–588
107. Papke RT, Ward DM (2004) The importance of physical isolation to microbial diversification. FEMS Microbiol Lett 48:293–303
108. Taylor MW, Schupp PJ, De Nys R, Kjelleberg S, Steinberg PD (2005) Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. Environ Microbiol 7:419–433
109. Green J, Bohannan B (2006) Spatial scaling of microbial biodiversity. Trends Ecol Evol 21:501–507
110. Ramette A, Tiedje JM (2007) Biogeography: an emerging cornerstone for understanding prokaryotic diversity, ecology, and evolution. Microb Ecol 53:197–207
111. Kuris AM, Blaustein AR, Alio JJ (1980) Hosts as islands. Am Nat 116:570–586
112. Van der Gast CJ (2008) Islands shaping thought in microbial ecology. Adv Appl Microbiol 64:167–182
113. Wollenberg MS, Ruby EG (2009) Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two O'ahu populations. Appl Environ Microbiol 75:193–202
114. Slatkin M (1996) In defense of founder-flush theories of speciation. Am Nat 147:493–505
115. Agenbroad LD (1998) New pygmy mammoth (*Mammuthus exilis*) localities and radiocarbon dates from San Miguel, Santa Rosa, and Santa Cruz Islands, California. In: Weigand PW (ed) Contributions to the geology of the northern channel islands southern California. American Association of Petroleum Geologists, Pacific Section, Bakersfield, pp 169–175
116. Yoshida A, Fumihito A, Yamagishi S, Tanida K (2005) Underground temperature change throughout a year measured at a coastal dune bearing sub-fossil egg shell fragments of the elephant bird (*Aepyornis*) in faux-cap, southern Madagascar. J Yamashina Inst Ornithol 36:136–140
117. Templeton AR (2008) The reality and importance of founder speciation in evolution. Bioessays 30:470–479
118. Barton NH, Charlesworth B (1984) Genetic revolutions, founder effects, and speciation. Annu Rev Ecol Evol Syst 15:133–164
119. Garg P, Aydanian A, Smith D, Morris J, Glenn J, Nair GB, Stine OC (2003) Molecular epidemiology of 0139 *Vibrio cholerae*: mutation, lateral gene transfer, and founder flush. Emerg Infect Dis 9:810–814
120. MacLean RC, Bell G (2002) Experimental adaptive radiation in *Pseudomonas*. Am Nat 160:569–581
121. Zamborsky DJ, Nishiguchi MK (2011) Phylogeographical patterns among sympatric populations of sepiolid squids and their *Vibrio*

symbionts in the Mediterranean Sea. Appl Environ Microbiol 77: 642–649
122. Soto W (2009) Evolutionary ecology of symbionts within free-living and host environments in the sepiolid squid-*Vibrio* symbiosis. New Mexico State University, Las Cruces
123. Wright S (1982) Character change, speciation, and the higher taxa. Evolution 36:427–443
124. Colegrave N, Buckling A (2005) Microbial experiments on adaptive landscapes. Bioessays 27:1167–1173
125. Hutchinson GE (1957) Concluding remarks. Cold Spring Harb Symp Quant Biol 22:415–427
126. Habets MGJL, Rozen DE, Hoekstra RF, de Visser JGM (2006) The effect of population structure on the adaptive radiation of microbial populations evolving in spatially structured environments. Ecol Lett 9:1041–1048
127. Hunt DE, David LA, Gevers D, Preheim SP, Alm EJ, Polz MF (2008) Resource partitioning and sympatric differentiation among closely related bacterioplankton. Science 320:1081–1085

VIE ET MILIEU - LIFE AND ENVIRONMENT, 2014, 64: 23-34

A REVIEW OF THE PYGMY SQUID *IDIOSEPIUS*: PERSPECTIVES EMERGING FROM AN "INCONSPICUOUS" CEPHALOPOD

M. K. NISHIGUCHI[1], J. NABHITABHATA[2], N. A. MOLTSCHANIWSKYJ[3], S. V. BOLETZKY[4]*

[1] New Mexico State University, Department of Biology, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA
[2] Centre for Biodiversity of Peninsular Thailand, Faculty of Science, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand
[3] The University of Newcastle, School of Environmental and Life Sciences, Ourimbah NSW, 2258, Australia
[4] CNRS, Observatoire Océanologique de Banyuls, Av du Fontaulé, 66650 Banyuls-sur-Mer, France
** Corresponding author: nish@nmsu.edu*

IDIOSEPIUS
CEPHALOPOD
LIFE HISTORY
EVOLUTION
SQUID
MOLLUSC

ABSTRACT. – The monogeneric family Idiosepiidae (Mollusca: Cephalopoda: Idiosepiida) contains the smallest living representatives of squid. Sexual dimorphism, presence of an adhesive organ on the dorsal mantle integument, lack of fully developed tentacles on hatching, and ability to produce large quantities of eggs relative to their body size during reproduction provide unique characteristics not commonly found in other cephalopods. These "mini-maximalists" have a life history strategy of rapid growth and high fecundity, and species of *Idiosepius* have been used as a model to examine embryonic and post-embryonic development, neurobiology, phylogeny, physiology, and life history strategies. Their small size, rapid generation time, solitary nature, and ease of producing eggs/hatchlings in captivity has provided a solid foundation for better understanding the evolution of an organism that has pushed the boundaries of a multitude of life history characteristics not observed in other metazoans.

INTRODUCTION

Species of the genus *Idiosepius* (Mollusca: Cephalopoda) are very small temperate and tropical squids commonly found in littoral waters in the Indo-Pacific region from South Africa to Japan and southern Australia (Lu & Dunning 1998). They are an ideal model system for laboratory studies due to their short life-span (approximately 80-90 days) and continuous growth throughout their life (Jackson 1989, Tracey *et al*. 2003). It is one of the smallest decabrachians among cephalopod groups (15 mm mantle length; Fig. 1); only few octobrachian relatives are as small in size as an adult *Idiosepius* (Kasugai 2000; Boletzky 2003). The small adult size (10-18 mm total length), the attachment behavior of adults of *Idiosepius*, and direct development raise questions about the ecology of the species. In particular capacity for dispersal during the lifetime, mobility during the post-hatching planktonic phase, patterns of distribution and the related consequences of biological and ecological constraints, and reproductive strategies allowing continuous growth and enhanced reproductive output. Thus, the Idiosepiidae have a diversity of traits that are interesting from a variety of evolutionary perspectives. While there has been a long history of the use of cephalopod systems being used as models to inform function and evolution of vertebrate systems *e.g.*,

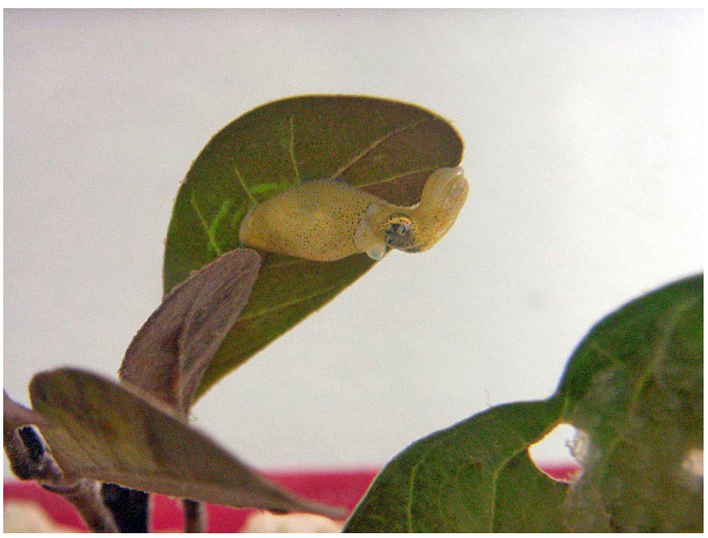


Fig. 1. – *Idiosepius biserialis* from the Andaman Sea, Thailand. The animal sticks to the leaf of an aquarium plant using its dorsal attachment organ; the peculiar position of the arms is exactly as described by Sasaki (1921). Photo courtesy of K. Warnke.

giant axon research and eyes, more recently, *Idiosepius* is being used to explore the evolution of signaling molecules in closed circulatory systems (Yoshida *et al*. 2010a).

There are seven described species of *Idiosepius; I. pygmaeus* Steenstrup, 1881, *I. paradoxus* Ortmann, 1888, *I. picteti* Joubin, 1894, *I. notoides* Berry, 1921, *I. biserialis* Voss, 1962, *I. macrocheir* Voss, 1962, *I. thailandicus* Chotiyaputta, Okutani & Chaitiamvong, 1991, and one undescribed temperate species on the east coast Australia (personal comment Dr A Reid, Australian Museum). Renewed interest in pygmy squid arose half a century ago when Voss (Voss 1962) described two new species of *Idiosepius* (*I. biserialis*, *I. macrocheir*), four decades after the description of *I. notoides* by Berry (Berry 1921). Voss' work brought the number of recognized species to six, three decades before *I. thailandicus* was described (Chotiyaputta *et al*. 1991). The position of *I. minimus* (*c.f.* Jereb & Roper 2005) with the genus was indeed unverified due to the disputed position within the genus. This work, along with studies by Hylleberg and Nateewathana (Hylleberg & Nateewathana 1991b, a) confirmed the presence of a gladius (pen) in *Idiosepius* species, a fact that had been previously disputed. That this family of squids has only one genus with seven described species has motivated malacologists to question the radiation of the family compared to other "cuttlefish-like" squids, with respect to differences in their life history characteristics.

Comparisons between the Idiosepiidae and these related families show many similarities; Sepiidae and Sepiolidae are holobenthic/benthic and mostly found in shallow coastal waters, usually living among sandy bottoms or within seagrass beds, similar to the Idiosepiidae. They have a sedentary lifestyle (except Heteroteuthinae), either resting or burying in or on top of some type of substratum (sand or reef; Sepiidae and Sepiolidae) or as in the case of pygmy squids, adhering to blades of eelgrass and macroalgae with a specialized adhesive organ (which may also reduce energy expenditure; Fig. 2). This characteristic is somewhat similar to that of sepiolid squids, which adhere sand to their mantle. The cryptic behavior of *Idiosepius* spp. decreases the risk of predation since most species are small and are easy prey items (Moynihan 1983a). Most individuals are solitary and rarely seen in large schools like teuthid squids (Moynihan 1983a, b). While all three families are sequential spawners, the small size of the Idiosepiidae represents the physiological edge that most benthic cephalopods can tolerate.

Although the pygmy squids are among the smallest in the entire class of cephalopods, they provide a great deal of information for understanding how a tiny "maximalist" squid can provide detailed information not only to malacologists, but to a wide field of disciplines, ranging from behavior to evolution. This review is a compilation of ideas and research (both published and unpublished) that were brought to our knowledge from a workshop convened at the Cephalopod International Advisory Council meeting held in Phuket, Thailand in February 2003 (Boletzky *et al*. 2005). We have included most of the published works related to *Idiosepius*, as well as "grey" and unpublished literature. The small size, short life span, solitary life, and amenability to laboratory experiments have made it an attractive species for student projects which are lodged in B.Sc. Honors theses, produced at University of Sydney, James Cook University, and the University of Newcastle (Australia) in the last 20 years. Although some studies are limited in time and scope, we felt that they were just as important as the major published research articles, so we have included them in this review. Our goals were to bring together knowledge about all aspects of the pygmy squid (Family Idiosepiidae), as it is becoming a reference cephalopod to study many facets of growth, reproduction, physiology, behavior, and systematics. This review includes topics related to species descriptions, morphology, systematics, distribution,

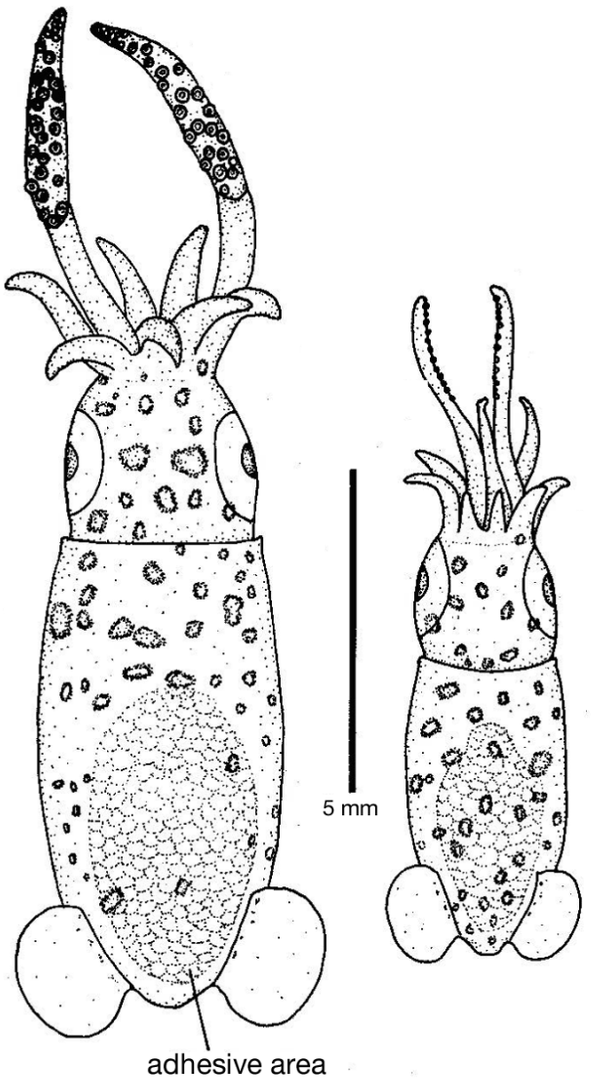


Fig. 2. – Area of adhesive organ on dorsum of *Idiosepius biserialis* (from Hylleberg & Nateewathana 1991a).

behavior, reproduction and embryonic development, and growth for the Idiosepiidae.

FAMILY, GENUS, AND SPECIES DESCRIPTIONS

The first species of *Idiosepius* were described in the late 1800s to early 1900s by Steenstrup [*I. pygmaeus* from the south China Sea (Steenstrup 1881)], Ortmann [*I. paradoxus* from Japan (Ortmann 1888)], Joubin [*I. picteti* from Amboina, Indonesia (Joubin 1984)], and Berry [*I. notoides* from South Australia (Berry 1921)]. These species have many characteristics that are common within the family Idiosepiidae; including absence of an inner arm circle (*i.e.* no buccal lappets), tentacles not present at hatchling stage, anterior edge of mantle not fused with head, nuchal cartilage absent, oval attachment organ on dorsal side of body in the posterior portion of the mantle, cornea locked, short arms, with a small web between the 3rd and 4th arm, arm suckers in two rows, small size, both ventral arms hectocotylized and devoid of suckers along the length, smooth gladius, suckers of all arms enlarged in males, outer oviducts in females developed, with only one (left) functional, and the occurrence of benthic eggs (Steenstrup 1881, Nesis 1987). Only a few characters initially distinguished the earlier described species from one another. These included the number of pairs of suckers and length of ventral arms (in males), and the width of the tentacular club. For instance, in *I. pygmaeus*, 1-3 suckers exist at the base of the ventral arms in males, whereas in *I. paradoxus* there are 4-7 suckers. *Idiosepius picteti* has a much shorter right ventral arm than the left, with only one sucker at the base of each arm. Finally, *I. notoides* has 7-11 pairs of suckers along the entire length of the ventral arm (Nesis 1987).

Grimpe (Grimpe 1931) then subdivided *I. pygmaeus* into three subspecies with *I. pygmaeus hebereri* (S. Pacific Ocean), *I. pygmaeus pygmaeus* (Central Indian Ocean), and *I. pygmaeus paradoxus* (N. Pacific Ocean). Although the species distribution is wide-spread across these geographic areas, modern cephalopod researchers have not accepted these subspecies. Thirty years after this "subdivision", two new sympatric species of *Idiosepius* were described by Voss (Voss 1962) from South Africa. These included *I. biserialis*, and *I. macrocheir*, of which *I. biserialis* has longer and narrower tentacular clubs with 2 rows of suckers, with 4 suckers in the basal part of each ventral arm, whereas *I. macrocheir* has a wider tentacular club with 4 rows of suckers, and the mid-section being wider than the base. Finally, the last newly recorded species was *I. thailandicus* in 1991 (Chotiyaputta *et al*. 1991), which is characterized by only two rows of tentacle suckers and 3-4 suckers on the hectocotylized arm. *Idiosepius thailandicus* and *I. biserialis* are similar in morphology, as well as *I. pygmaeus* and *I. paradoxus*, but size differences (*I. pygmaeus* males are twice as large as *I. thailandicus* males), and number of rows of club suckers are enough to distinguish the species from one another (Chotiyaputta *et al*. 1991).

There has only been one species redescription thus far on the original type species, *I. pygmaeus* (Hylleberg & Nateewathana 1991b). Several additional characters were described, including a thin gladius (Fig. 3), small narrow muscular scar located mid-dorsally above a corresponding oval depression, ventral muscle connecting the mantle to the body, and no presence of a "sinew rod" to the inner ring around the margins of the spongy substratum (loculomenta).

MORPHOLOGY AND ANATOMY

The small size, sedentary nature and relative abundance of the Idiosepiidae make them easy to collect and study. Distinct morphological differences between Idiosepiidae and other cephalopod taxa have been relatively easy to describe due to the small size and the ease of collecting animals. The first published paper on morphological characters specific to *Idiosepius* was by Adam (Adam 1986) on the buccal apparatus who compared radular and beak morphology of *I. pygmaeus* and *I. paradoxus*, and described the denticulation that is unique to this genus. Specifically, the radular structure is reminiscent to that seen in Octopods, where seriation patterns of the rachidian teeth is used for species level identification. Additionally, beaks of both species are denticulated on both upper and lower mandibles, a characteristic that is unique to the Idiosepiidae and not seen in other Decabrachian families. This characteristic is common only among octopus juveniles, establishing the Idiosepiidae as an unusual example of convergent evolution in buccal mass morphology compared to other Decabrachian families.

Morphological observations have also been "renewed" in other species that had been previously described, but not yet recorded in new geographical locations. *Idiosepius biserialis*, a species first described from South Africa (Voss 1962), was also collected from seagrass beds in southern Thailand (Hylleberg & Nateewathana 1991a). Voss had not previously described the internal anatomy of this species of *Idiosepius*, and therefore a more detailed description of the species from Thailand (Andaman Sea) was completed. Using *I. pygmaeus* as a comparison, there were key features unique to both male and female *I. biserialis* specimens that distinguished them from their sympatric species, *I. pygmaeus* (Fig. 3). Hectocotylized arms in males are much longer than arms I-III, and carry 3-7 suckers in various combinations at the base of the arm. A secondary type of cornea, which is thin, smooth, and transparent, distinguishes this species from that of *I. pygmaeus*, which has a much more opaque cornea. As in all other Idiosepiidae, there is a gladius present, which covers the posterior 2/3 of the dorsum. These main features

distinguish *I. biserialis* and *I. pygmaeus* from each other in overlapping habitats. Histology of the dorsal "adhesive organ" of the mantle was described in *I. biserialis*, *I. paradoxus* and *I. pygmaeus* (Yamamoto 1949, Byern & Klepal 2006, Cyran *et al*. 2011).

One important morphological feature in the Idiosepiidae is that their relative small size allows closer investigation of microstructural anatomy of major organ systems. One system in particular is that of the neuronal and brain arrangement in these small squids. Since the Idiosepiidae have been hypothesized to be close relatives of Teuthid squids (Carlini & Graves 1999, Bonnaud 2003, Lindgren *et al*. 2004), the importance of understanding the evolution and development of the nervous system is a key point for determining how such advanced nervous systems have evolved in molluscs. Both nervous system organization and an overall atlas of the brain in the species *I. paradoxus* have been described in detail (Shigeno & Yamamoto 2002, Yamamoto *et al*. 2003, Wollesen *et al*. 2010a, b). Comparisons to other dechabrachians such as *Loligo* and *Sepia* indicate that *I. paradoxus* specimens have a more highly developed optic tract region, with a stellate commissure not observed in *Sepia* or *Sepiola* species. This information can also be compared to recent molecular studies, where transcriptomic and developmental data have supported the mechanisms of cephalopod camera eye evolution (Yoshida & Ogura 2011). Brain structures are very similar to other Decabrachian and Octobrachian species, indicating that developmentally the brains in most coleoid cephalopods are highly conserved (Yamamoto *et al*. 2003). Many of the finer, detailed features of the nervous system differ; apparently reflecting the comparatively inactive nekto-benthic life-history strategy of *Idiosepius* (Shigeno & Yamamoto 2002). Thus, many of the behavioral adaptations of this squid may be reflected in the patterns of organization of the anatomy of the complete nervous system.

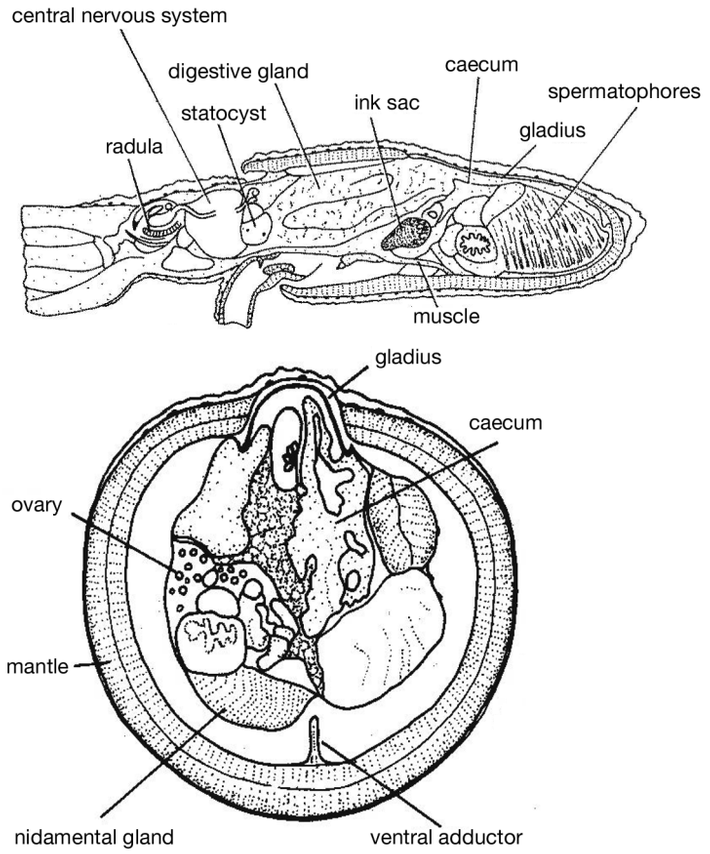


Fig. 3. – Longitudinal section of male *I. pygmaeus* (above) and cross section of posterior female body of *I. beserialis* (from Hylleberg & Nateewathana 1991a, b).

MOLECULAR PHYLOGENY/ SYSTEMATICS

There has been much speculation on the placement of the Idiosepiidae within the Dechabrachian clade of Cephalopods. Early systematic analysis had Idiosepiidae placed sister to Teuthida (Hylleberg & Nateewathana 1991a), but more recent molecular phylogenetic analysis (both total molecules and combined analysis) have suggested that they are sister to Sepiidae and Sepiolidae (Bonnaud *et al*. 1996, Lindgren *et al*. 2004, Nishiguchi *et al*. 2004, Strugnell *et al*. 2006, Lindgren & Daly 2007, Strugnell & Nishiguchi 2007, Nishiguchi & Mapes 2008, Yoshida *et al*. 2010b, Lindgren *et al*. 2012). This relates to the fact that the behavior and life history strategy of the Idiosepiidae align more with other nekto-benthic squids, such as cuttlefishes and bobtail squids. Recent molecular evidence from seven species of *Idiosepius* supports the hypothesis that *I. macrocheir* and *I. thailandicus* are nested within the *I. biserialis* group, and are likely junior synonyms of *I. biserialis* (Byern *et al*. 2012). Given the genetic variability of *I. biserialis* between African and Indo-West Pacific populations and the position of *I. macrocheir* and *I. thailandicus*, the probability of introgression among *I. macrocheir* and *I. thailandicus* populations/species may explain the heterogeneity of these species groups. Additional analysis of the phylogenetic relationships have suggested that climatic conditions, rather than habitat preference or geographical isolation has led to the split between both two and four-rowed species (Byern *et al*. 2012).

Recently there has been a surge to identify the seven species of *Idiosepius* (Jereb & Roper 2005) using molecular analysis as well as morphology. Synapomorphic characters that are well defined in the family are the adhesive organ (or gland) that is restricted to

the posterior part of the dorsal mantle side and fin region (Steenstrup 1881, Sasaki 1921, 1929, Byern *et al*. 2008) (Fig. 1). Suckers tend to be arranged in either two or four rows on the tentacular club and the number of suckers on each hectocotylized ventral arm varies between species. Morphological differences between *I. macrocheir* and *I. thailandicus* did not determine any definitive species differences; both were similar to *I. biserialis* based on the 2-rowed suckers on the tentacle club (Byern & Klepal 2010, Byern *et al*. 2010). Additionally, *I. biserialis* and *I. thailandicus* do not differ in mantle length, number of suckers on the arms, or arrangement of pegs on suckers (Byern *et al*. 2010). The only molecular analysis of the genus analyzed all seven of the species of *Idiosepius*, with a number of species represented from various populations (Byern *et al*. 2010). Phylogenetic analysis using several methods recovered three major clades within the family; all individuals used at the population level were grouped together based on the analysis. Interestingly, the analysis suggests to reduce the number of species to five: *I. biserialis* (= *I. thailandicus* and *I. macrocheir*), *I. notoides*, *I. paradoxus*, *I. pygmaeus*, and *I. picteti* (Byern *et al*. 2010). Unfortunately, due to the condition of the *I. picteti* sample (formalin fixed), this sample did not provide any data for the analysis. The study did provide interesting patterns with respect to the phylogeographic arrangement of the genus. *Idiosepius biserialis* co-occurs both in an African and Indo-West Pacific clade, providing evidence of limited gene flow (both populations are morphologically indistinct). *Idiosepius biserialis* from Japan are morphologically distinct from *I. paradoxus* from the same area, yet molecular data had them grouped within the same clade (Byern *et al*. 2010). Future work should focus on the distribution, behavior, and ecology of the sympatric species, to determine if there is evidence of any gene flow or hybridization occurring between the populations.

DISTRIBUTION

Very little information regarding the location, habitat, and distribution of the Idiosepiidae has been published, except for the initial species descriptions as well as some specific records for species that have been found in different localities as previously noted. Challenges in determining the range of species distributions are associated with their small size, cryptic habit, and that when found in scientific collections are identified to genus level only. Seasonal differences in life history characteristics of *Idiosepius pygmaeus*, has been investigated in one type locality [North Queensland, Australia; (Jackson 1992)]. Unusual findings of *Idiosepius* outside of what has been considered their normal range have been recorded for the temperate *I. paradoxus* in the Russian and Japan Seas (Nesis *et al*. 2002, Sato & Munehara 2013), temperate/tropical *I. biserialis* located in Japan to Indonesia and Mozambique (Byern *et al*. 2005, Byern *et al*. 2010), and new localities for *I. pygmaeus* in Thailand and Indonesia (Suwanmala *et al*. 2005). No population genetic studies, surveys, or records have been found for the other species of *Idiosepius* (Byern & Klepal 2007). An unusual capacity for re-introduction into habitats that do not allow for survival during cold winter water temperatures has been observed, with evidence that large scale passive dispersal is possible by adults (Sato *et al*. 2009). Adherence to drift material that can then be dispersed elsewhere might be an alternative explanation for a benthic dweller to be transported across greater geographical distances.

BEHAVIOR

Idiosepius is a prime candidate for the study of behavior given their ease at observing them in captivity, as well as studying a range of age-specific behaviors from feeding to reproduction. Earliest studies examined the adhering habit of *I. pygmaeus*, since this behavior is unknown among teuthid squids (Sasaki 1921). The morphology of the adhesive organ was described, noting that mostly columnar cells comprise the lining of the organ, in tightly packed bundles with thick cytoplasm laden with fine granules. Sasaki hypothesized that *Idiosepius* uses this organ to adhere itself to the substrate for two functions; during egg deposition and to avoid predation. Subsequently, this adhering behavior was more thoroughly investigated using *I. pygmaeus*, as well as other behaviors observed both in the wild and in captivity (Moynihan 1983a). These included diel rhythm and activity during daylight hours, solitary habits (non-gregarious), habitat preference (sea-grass and large frond macroalgae), various swimming postures (including anchoring), feeding, with notes on tentacular use, ritualized patterns (including light coloration, dark and striped patterning (camoflauge), bar shapes, and spots (dynamics), and inking.

Feeding behavior has been examined in *I. paradoxus* (Kasugai 2001, Kasugai *et al*. 2004) and *I. pygmaeus* (Roberts 1997). *Idiosepius paradoxus* has two stages of feeding upon small prey items, namely attacking and then eating (Table I). The attacking stage can be divided into three phases: attention, positioning, and then seizure as previously described in cuttlefish (Messenger 1968). Once a prey item is captured, the animal inserts the buccal mass into the exoskeleton of the prey item allowing the flesh to be ingested (Kasugai 2001, Kasugai *et al*. 2004). This behavior has also been noted in juvenile teuthid squids (loliginids, E. Vidal pers. obs.). The importance of vision in capture of prey by *I. pygmaeus* was evident when it was demonstrated that better light quality allowed greater success rate at capturing prey and reduced the time spent in the predatory sequence (Roberts 1997). Feeding behavior does not differ among individuals of various sizes or change when different sized prey items are presented.

However, *I. pygmaeus* does selectively choose particular prey items and in this study they actively selected *Acetes sibogae australis* regardless of the relative size of the squid or prey item.

Idiosepius thailandicus from Thailand was used in a comparative study with *I. pygmaeus* to identify elements of similarity as well as to identify any synapomorphic characters. In the laboratory, *I. thailandicus* displays differential sex-specific postures when adhering to eel grass (Nabhitabhata 1998). The two species also differed in their color pattern formation, prey seizure, and the larger size of females. Four types of mating behaviors were observed, including (1) males hovering above an adhering female; (2) both males and females adhering to the same substratum; (3) both males and females hovering above the substratum; and (4) hovering females above an adhering male (Nabhitabhata 1998). Males will try to mate with smaller males, but this was due to false recognition, and ceased once the larger male recognized the other as a male. Both *I. thailandicus* and *I. pygmaeus* show similarities in spawning and developmental patterns and both species fail to display gregarious or schooling behaviors.

Reproductive behavior by *I. paradoxus* has been studied in detail more than the other species of *Idiosepius* (Natsukari 1970, Kasugai 2000, Sato *et al*. 2013a). Laboratory observations of wild-caught animals have determined that there are distinct patterns of mating in *Idiosepius* (also see previous discussion under behavior). Males grasp females at the base of the arm crown and insert the right hectocotylized arm into the female's arm crown, while the left hectocotylized arm takes the spermatangia (evaginated spermatophores) from the funnel and attaches them to the female's body via the tip of the left hectocotylized arm (Kasugai 2000, Sato *et al*. 2013b). Females will mate with either one or many males in-between deposition of batches of eggs, while there is no antagonistic behavior among competing males, there is evidence of cryptic selection of males by females (Sato *et al*. 2014). Female *I. paradoxus* can remove spermatangia after copulation and will remove spermatangia of larger males and male that took longer to deposit spermatophores, appearing to actively select for smaller fast mating males (Sato *et al*. 2013a, 2014). During egg deposition, females adhere to the substrate using the adhesive organ (eel grass, macroalgae, or the side of an aquarium), with the body upside down (Kasugai 2000). Eggs are ejected one at a time, and attached to the substratum within an egg capsule (Natsukari 1970; Table I).

Cross-mating between species has been observed in *Idiosepius biserialis* and *I. thailandicus*, due to the lack of differences between the two species in their reproductive behaviors. These behaviors include both hovering and adhering positions of each sex during copulation (Nabhitabhata & Suwanamala 2008) for crossed pairs and within each species. Interestingly, both species are allopatric, with *I. biserialis* found only in seagrass beds along the Andaman Sea coastline and *I. thailandicus* in mangroves of the eastern Gulf of Thailand. Thus, geographical isolation within the genus does not necessarily constitute major differences in reproductive behavior.

There is evidence that *Idiosepius* can display consistent inter-individual variability in their behaviors and that correlations among behaviors has allowed identification of personality traits (Horsman 2012). This study held adult *Idiosepius* sp. (undescribed eastern Australian species) in aquarium conditions and exposed them to three contexts; a threat, a feeding, and a novel environment. Fourteen behaviors, similar to those described by Sinn & Moltschaniwskyj (2005), were recorded and quantified. Correlations were evident among these behaviors and Horsman (2012) identified three personality traits; "boldness" and "responsiveness" in a threat context, and "activity" in a feeding context. In the "bold" personality trait bold individuals did not move away from the threat quickly or display color changes, while the "responsive" trait was characterized by unresponsive animals stretching the arms out towards the threat and not jetting away from the threat. The "activity" behavioral trait in the feeding context was evident by more active animals undertaking more hunting attempts and spending more time fin swimming. The significance of these behavioral traits in the success of populations is unclear as there is no correlation with body size or sex (Horsman 2012), but the correlation between physiological features and behavior was undertaken by another Honours student on the same species (Felton 2013). However, *Idiosepius* sp. demonstrated no evidence of correlations between mass specific metabolic rate and level of boldness or activity in either the threat or the feeding contexts (Felton 2013).

REPRODUCTION

Most of the research on *Idiosepius* has been on the reproductive life history strategy, since this squid has unusually high fecundity for such a small and short-lived species. Early research described the spawning mode and reproductive output of *I. pygmaeus* (Lewis & Choat 1993). A noticeable coordination between reproductive output and adult growth occurs in both wild-caught and aquarium raised *I. pygmaeus*, with females incorporating at least five times their body weight into eggs and egg coats. In addition, senescence and death is not related to exhaustion of the reproductive potential. Growth in relation to reproductive output was also investigated in *I. pygmaeus* with respect to temperature and food availability (van Camp 1997), where both factors contributed synergistically. There is no evidence that egg number, size, or batch condition decreases with successful clutches of eggs produced by a female, and no trade-offs between the size and number of eggs deposited. The capacity for simultaneous allocation of energy to growth and repro-

Table I. – Comparison of life history and behaviour of pygmy squid species. References: a, (Lewis & Choat 1993); b, (Jackson 1989, Jackson 1993); c, (Nabhitabhata *et al*. 2004); d, (Hylleberg & Nateewathana 1991a); e, (Voss 1963); f, (Jivaluk *et al*. 2003); g, (Sasaki 1929), (Kasugai 2000, 2001), (Kasugai & Ikeda 2003), (Kasugai & Segawa 2005); (Sato *et al*. 2008, Sato *et al*. 2009); h, (Natsukari 1970); i, (Nabhitabhata 1994, Nabhitabhata 1998); j, (Nabhitabhata & Suwanamala 2008); k, (Hylleberg & Nateewathana 1991b); l, (Tracey *et al*. 2003). F, female; M, male; ML, mantle length.

aspects	*Idiosepius pygmaeus*[a]	*Idiosepius paradoxus*[g]	*Idiosepius thailandicus*[i]	*Idiosepius biserialis*[j]	*Idiosepius notoides*[l]
Habitat	littoral, estaury, mangrove	littoral, seagrass bed	littoral, estaury, mangrove	seagrass bed	Seaweed, seagrass bed
Food organisms (in captivity)	glass and mysid shrimps (*Acetes sibogae australis*[b], *Mesopodopsis orientalis*[c])	amphipods (*Ampithoe* sp.), mysid shrimps (*Neomysis japonica*)	mysid shrimps (*Mesopodopsis orientalis*)	mysid shrimps (*Mesopodopsis orientalis*)	
Sex ratio	F > M		F > M	F > M	
Size dimorphism :	F > M	F > M	F > M	F > M	F > M
- female (average ML : mm)	17.5[d]	15, 7.8[h]	10.4	6.4[k]	20
- male	11.7[d]	12	5.9	3.6[k]	16
Mating behaviour:					
- pattern	adhering and hovering[c]	adhering and hovering	adhering and hovering	adhering and hovering	
- motion of male	no dart[c]	dart	no dart	no dart	
- position	head-to-head[c]	head-to-head	head-to-head	head-to-head	
- copulation period (s)			0.5-1	3-7	
- organ used	hectocotylus[c]	hectocotylus	tentacles	tentacles	
- region of spermatophores attachment	arms, buccal, head, mantle[e]	arms, buccal, head, neck, mantle	buccal	buccal	
Spawning behaviour:					
- pattern	adhering[c]	adhering[h]	adhering and hovering	adhering	
- substratum	rock and shell[a] aquarium wall[c]	aquarium wall and bottom[h], eelgrass blade[g]	aquarium wall, glass slide, seaweed fronds	aquarium wall, eelgrass blade	
- organ used	tentacles[a], arms[c]	tentacles[h], arms[g]	arms	arms	
- capsule attachment period (s)	30	30[h], 70-80[g]	5-10	30-50	
- batch size (no)	58.2	17-64	22.2	46.5	
- total eggs (no) / female	640[a], 100-200[c]	51.6	159.5	174.5	
- egg capsule diameter (mm)	2	1.2-1.4	1.8	1.7	
- coating layers		8-10	16-22	14-20	
- embryonic period (d)	7-14[c]	15-17	10-13	6-10	
- temperature(°C)	23-27.5	18.5-22.6	23-28	28	
Hatchling:					
- mode of living	planktonic	planktonic	planktonic	planktonic	
- size (average ML : mm)	1[b], 0.85-0.9[f]	1.16-1.22	1	1	
Life span (statolith ring count)	67-79[b]	140-150			91-115

(citations follow superscripts with taxa at table head, except those with details of the table text)

duction is evident as females continued to grow even during their spawning period. Although, in contrast to studies by van Camp (1997), measurements of *I. pygmaeus* statoliths to determine reproductive investment during winter versus summer months provided evidence that individuals partition a greater amount of energy in gonadal tissues over longer periods of time during winter months which slowed, but did not suspend somatic growth (Jackson 1993). Females also have greater protein concentrations, even at warmer temperatures (contrasting an earlier hypothesis that greater temperatures reduce protein concentrations in metabolically active individuals). Growth rates will slow in cooler temperatures, but accelerate during breeding (English 1981), with larger gonads in the winter season compared to the autumn counterparts (Jackson 1993, Sato *et al*. 2009).

Most research on reproductive biology has focused on the females, with limited descriptions about the *Idiosepius* male reproductive system, like most cephalopod species the males have a seminal receptacle in which the sperm are stored near the bottom of each sac. Females can then store sperm after numerous copulations, and spermatozoa can subsequently move to the seminal receptacle without being depleted after one single spawning event (Sato *et al*. 2010; Table I).

Idiosepius pygmaeus females held in captivity in 28 L glass aquaria and fed *ad libitum* on live *Acetes sibogae australis*, a sergestid shrimp, with one or two males present intermittently through the period of captivity were capable of multiple spawning events, with as many as eight egg batches produced over 22 days, and the total number of eggs produced by any one female in captivity ranged from 53-922. The number of eggs produced in a single batch ranged from 16-287, with the average number of eggs per batch 80.4 eggs (SE 8.9). Fertilization rates ranged from 77 % to 80 % and in all cases the female died within several days of the last spawning episode.

EMBRYONIC DEVELOPMENT

Developmental time of embryos is approximately 15-27 days after oviposition, depending on species (English 1981, Yamamoto 1988), in temperatures between 18-22 °C. The elliptical eggs are encapsulated by a gelatinous coat, with 8-10 spiral layers of a translucent membrane (Kasugai & Ikeda 2003). Eggs are usually 0.87-0.91 mm in length and 0.67-0.72 in width during early stages, again depending on the species (Natsukari 1970; Table I). During embryonic development, approximately 30 stages are observed (Yamamoto 1988). In *I. paradoxus*, the arm formation of the embryo follows the formula $2 > 1 > 3 > 4$, with no tentacles present on hatching (Natsukari 1970). This may be related to the fact that tentacles are solely used in sexual reproduction, *i.e*., copulation, particularly in *I. thailandicus* and *I. biseralis* (Nabhitabhata 1998, Nabhitabhata & Suwanamala 2008). This is not true for all other species of *Idiosepius* (see Table I).

Water temperature has an important role in all aspects of development and hatching of the tropical *I. pygmaeus*. *Idiosepius pygmaeus* fertilized eggs held in constant temperatures (20 °C, 25 °C and 30 °C) and at 12 h light/dark cycles differed in their survival; no mortalities of developing embryos occurred at 30 °C, 5 % mortality at 25 °C and at 20 °C 63-83 % of the embryos died. Embryo death at 20 °C characteristically occurred during the final stages of organogenesis. Gestation rates were also temperature dependent and were fastest at 30 °C; only 9-10 days. The slower embryonic development at 20 °C resulted in an extended gestation period of between 31-35 days. Juveniles that hatched from the surviving eggs also differed as a function of temperature; despite rapid rates of development at 30 °C, juveniles suffered greater mortality during and immediately after hatching, compared with embryos at 25 °C. Temperature also influences the number of days over which hatching occurred which was greatest at the cooler temperatures, with a single batch of eggs held at 20 °C from taking up to 17 days to hatch. Hatching time was reduced at 30 °C, with a maximum of seven days for all embryos to hatch from a single batch. As these water temperatures are typical of tropical waters, the prolonged hatching of *I. pygmaeus* at lower temperatures suggests that as development becomes more protracted the difference in developmental rates of individual eggs becomes greater. Thus, over the winter it would appear that development takes longer and hatching would occur over a longer period than during the summer.

GROWTH AND LIFESPAN

Most publications on growth and lifespan have primarily focused on tropical *I. pygmaeus*. Since *Idiosepius* are short-lived cephalopods, the ability to determine age-class and time to reproductive maturity are important features for interpreting life history changes. This is important since individuals caught in each season will be a different generation and have experienced very different environmental conditions from the generation before. Information about the tropical *I. pygmaeus* and temperate *I. notoides* (both species from Australia) allow comparisons between the two allopatric species, since they are located in different habitats. *Idiosepius pygmaeus* primarily is found in tropical waters, from southeast Asia to northern Australia; *I. notoides* in temperate waters ranging from Tasmania to the south-east coast of Australia. Statolith microstructure analysis in *I. pygmaeus* provides estimates of age at maturity (45-60 days) and growth rates in relation to maturity (Jackson 1989). Females have a much larger variation in size with respect to maturity, and estimates of growth rates and longevity of this species suggest that there are multiple generations within one year (Kasugai & Segawa

2005, Sato *et al*. 2008). In comparison, *I. notoides* statolith microstructure indicates that this species matures between 68 (for males) and 88 (for females) days, with similar sizes for the onset of maturity for both males and females (Tracey *et al*. 2003). Since this species is primarily found in colder waters, this comparative study supports observation from other marine species that temperate species live longer and grow to larger sizes in cooler temperatures, an important fact when considering the short lifespan of this family of squids (Table I).

The structure and function of organ and muscle systems has also been a targeted feature to study in *Idiosepius*, allowing the processes underpinning the rapid and continuous growth to be described. Using *I. pygmaeus*, evidence based on the ultrastructure and proximal composition of the digestive gland demonstrated a strong correlation between numbers of secretory spheres, which are esterase rich, and the time of day that the cells are being produced (Semmens *et al*. 1995). The enzymatic activity of these cells increases during times of feeding, indicating that enzyme production is very responsive to feeding, reducing the time that food is present in the digestive system, and is probably linked to the metabolic demands and physical constraints of this fast growing cephalopod. It is generally thought that cephalopod metabolism primarily uses protein or carbohydrate as an energy store; with no evidence that the digestive gland (analogous to the liver) is not used for lipid storage, but predominantly excretion in *I. pygmaeus* (Semmens *et al*. 1995). Contrary to this, ultrastructural evidence in *I. notoides* provides evidence of lipid storage in the form of large droplets of lipid-like spheres, which are contained in the caecum and digestive gland of wild-caught and captive-fed *Idiosepius* (Eyster & Van Camp 2003). These lipid stores disappeared from the digestive gland when animals were starved in the laboratory, and reappeared several days later in the caecum. Although the use of lipids is not definitive, it is suggested that *Idiosepius* uses these lipid droplets for either energy storage and/or for buoyancy control. As of this time, no further studies have been completed to determine if this is related to growth and feeding/starvation, but it is possible that the capacity for lipid storage in *Idiosepius* is species specific. Lipid storage is problematic for small individuals as internal space is limited, but *Idiosepius'* capacity to attach to the substrate reduces some of the buoyancy issues associated with lipid stores.

Along with digestion, muscle structure and function in mantle tissues has been investigated in *I. pygmaeus*, with respect to changes associated with growth, nutrition, reproduction, senescence, and captivity (Pecl & Moltschaniwskyj 1997). Using a variety of conditions, animals are plastic in respect to their growth in muscle fiber (number and size) and muscle block size. The process of growth rates are altered during captivity as a function of the relative contributions of hyperplasia and hypertrophy (Pecl & Moltschaniwskyj 1999). This reflects the fact that cephalopods in general are malleable in their allocation of energy to growth and reproduction, and are highly responsive to environmental variables. Additionally, *I. paradoxus* was used as a model to determine developmental patterns of vascular endothelial growth factors (VEGF's), which are important for inducing development and regulation of permeability of blood vessels in invertebrates (Yoshida *et al*. 2008). Interestingly, this growth factor is expressed in the blood vessels of arms and regions adjacent to the optic lobes, probably due to the formation of blood islands, a feature that is commonly found in open blood-vascular systems of other molluscs (Yoshida *et al*. 2010a).

CONCLUSIONS

The Idiosepiidae are small, yet dynamic cephalopods that represent a unique and specialized group of molluscs. Research undertaken to date provides knowledge about their distribution, behavior and reproduction, life history strategies, growth, and past evolution of the family. Yet, there are questions on the monophyly of some species pairs or groups within the genus, as well as whether all seven species are indeed "good taxa". Developmentally, the evolution of the adhesive gland is intriguing, largely because no other cephalopod species has such an apparatus for attachment and may be evolutionary related to Hoyle's organ for hatching (Cyran *et al*. 2013). The blocked tentacle development at the embryonic bud stage is unique in the decabrachia, but in fact it is a matter of heterochronic displacement. If possible, future work should focus on the rearing of the *Idiosepius* embryos to allow standardization of post-embryonic stages in life history. Difficulties in rearing newly hatched individuals past a week is because we know nothing has led to the disparate knowledge about the habitat and diet of these very small individuals. Given that these squids comprise the far-end extreme of metabolic scaling (size vs. growth) and short life cycle indeed makes them the true "mini-maximalist" of all cephalopod species.

ACKNOWLEDGEMENTS. – The authors would like to thank all participants in the 2003 Cephalopod International Advisory Committee workshop for their ideas and discussions on "*Idiosepius* ecology, biology, and biogeography of a mini-maximalist", in Phuket, Thailand (Boletzky *et al*. 2005). M.K.N. is supported by the National Science Foundation and the National Institutes of Health. Figure 1 photograph courtesy of K Warnke.

REFERENCES

Adam W 1986. La radule et les mandibules de quelques espèces d'*Idiosepius* Steenstrup, 1881 (Molluska, Cephalopoda, Decapoda). *Bull Inst Rech Sci Nat Belgique: Biol* 56: 149-154.

Berry S S 1921. A review of the cephalopod genera *Sepioloidea, Sepiadarium*, and *Idiosepius*. *Rec S Aust Mus* 1: 347-364.

Boletzky S v 2003. A lower limit to adult size in coleoid cephalopods: Elements of a discussion. *In* Warnke K, Keupp H & Boletzky Sv (eds), Coleoid Cephalopods through Time. Freie Universität Berlin, Germany: 19-28.

Boletzky S v, Nishiguchi M K, Nabhitabhata J, Nugranad J 2005. *Idiosepius*: Ecology, biology, and biogeography of a mini-maximalist. *Phuket Mar Biol Cent Res Bull* 66: 11-22.

Bonnaud L, Boucher-Rodoni R, Monnerot M 1996. Relationships of some coleoid cephalopods established by 3' end of the 16S rDNA and cytochrome oxiidase III gene sequence comparison. *Am Malacol Bull* 12: 87-90.

Bonnaud L, Pichon D, Boucher-Rodoni R 2003. Molecular approach of cephalopod phylogeny: the case of *Idiosepius*. *In* CIAC 2003: Biology, Recruitment, and Culture of Cephalopods, Phuket, Thailand: 9 p.

Byern J V, Klepal W 2006. Adhesive mechanism in cephalopods: a review. *Biofouling* 22: 329-228.

Byern J V, Klepal W 2007. Occurrence of *Idiosepius pygmaeus* (Cephalopoda: Idiosepiidae) in Indonesian waters. *Ann Naturhist Mus Wien,* 108B: 137-144.

Byern J V, Klepal W 2010. Re-evaluation of taxonomic characters of *Idiosepius* (Cephalopoda, Mollusca). *Malacologia* 52(1): 43-65.

Byern J V, Nurnberger S, Shuichi S 2005. Distribution pattern of a minimalist-new records for *Idiosepius biseralis* (Idiosepiidae, Cephalopoda). *In* 2nd International Symposium - Coleoid Cephalopods through Time, Prague: 38-43.

Byern J V, Rudoll L, Cyran N, Klepal W 2008. Histochemical characterization of the adhesive organ of three *Idiosepius* spp. species. *Biotechnol Histochem* 83: 29-46.

Byern J V, Söller R, Steiner G 2010. Molecular phylogenetic relationships of the genus *Idiosepius* (Cephalopoda: Idiosepiidae). *Ferrantia* 59: 184-190.

Byern J V, Söller R, Steiner G 2012. Phylogenetic characterization of the genus *Idiosepius* (Cephalopoda; Idiosepiidae). *Aquat Biol* 17: 19-27.

Carlini D B, Graves J E 1999. Phylogenetic analysis of cytochrome *C* oxidase I sequences to determine higher-level relationships within the coleoid cephalopods. *Bull Mar Sci* 64: 57-76.

Chotiyaputta C, Okutani T, Chaitiamvong S 1991. A new pygmy cuttlefish from the Gulf of Thailand *Idiosepius thailandicus* n. sp. *Venus Jpn J Malacol* 50: 165-174.

Cyran N, Staedler Y, Schönenberger J, Klepal W, von Byern J 2013. Hatching glands in cephalopods- A comparative study. *Zool Anz* 253: 66-82.

Cyran N, Klepal W, von Byern J 2011. Ultrastructural characterization of the adhesive organ of *Idiosepius biserialis* and *Idiosepius pygmaeus* (Mollusca: Cephalopoda). *J Mar Biol Ass UK* 91(07): 1499-1510.

English S 1981. The Biology of two species of estuarine cephalopods from the Sydney region. Undergraduate Honors thesis, Department of Biology. University of Sydney, Sydney, NSW: 125 p.

Eyster L S, Van Camp L M 2003. Extracellular lipid droplets in *Idiosepius notoides*, the southern pygmy squid. *Biol Bull* 205: 47-53.

Felton M 2013. Relationship between metabolic rate and personality in two species of cephalopods (*Euprymna tasmanica* and *Idiosepius* sp.). Undergraduate Honors thesis, School of Environmental & Life Sciences. University of Newcastle, Newcastle, NSW: 78 p.

Grimpe G 1931. Teuthologische Mitteilungen XIII. Über die Cephalpoden der Sunda-Expedition Rensch. *Zool Anz* 95: 149-174.

Horsman M 2012. Individuality: Personality traits in the temperate pygmy squid *Idiosepius* sp. Undergraduate Honors thesis, School of Environmental & Life Sciences. University of Newcastle, Newcastle, NSW: 55 p.

Hylleberg J, Nateewathana A 1991a. Morphology, internal anatomy, and biometrics of the cephalopod *Idiosepius biserialis* Voss, 1962. A new record for the Andaman Sea. *Phuket Mar Biol Cent Res Bull* 56: 1-9.

Hylleberg J, Nateewathana A 1991b. Redescription of *Idiosepius pygmaeus* Steenstrup, 1881 (Cephalopoda: Idiosepidae), with mention of additional morphological characters. *Phuket Mar Biol Cent Res Bull* 55: 33-42.

Jackson G D 1989. The use of statolith microstructures to analyze life history events in the small tropical cephalopod *Idiosepius pygmaeus*. *Fish Bull US* 87: 265-272.

Jackson G D 1992. Seasonal abundance of the small tropical sepioid *Idiosepius pygmaeus* (Cephalopoda: Idiosepiidae) at two localities off Townsville, North Queensland, Australia. *Veliger* 35: 396-397.

Jackson G D 1993. Seasonal variation in reproductive investment in the tropical loliginid squid *Loligo chinensis* and the small tropical sepioid *Idiosepius pygmaeus*. *Fish Bull* 91: 260-270.

Jereb P, Roper C F E 2005. Cephalopods of the World. An annotated and illustrated catalogue of cephalopod species known to date. FAO Species Catalogue for Fishery Purposes.

Jivaluk J, Nabhitabhata J, Nateewathana A 2003. Description of hatchling of Thai pygmy squid, *Idiosepius thailandicus*, Chotiyaputta, Okutani and Chitiamvong, 1991. *In* CIAC 2003: Biology, Recruitment, and Culture of Cephalopods, Phuket, Thailand: 40 p.

Joubin L 1884. Céphalopodes d'Amboine. *Rev Suisse Zool* 2: 23-64.

Kasugai T 2000. Reproductive behavior of the pygmy cuttlefish *Idiosepius paradoxus* in an aquarium. *Venus Jpn J Malacol* 59: 37-44.

Kasugai T 2001. Feeding behavior of the Japanese pygmy cuttlefish *Idiosepius paradoxus* (Cephalopoda: Idiosepidae) in captivity: evidence for external digestion? *J Mar Biol Ass UK* 81: 979-981.

Kasugai T, Ikeda Y 2003. Description of the egg mass of the pygmy cuttlefish, *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) with special reference to its multiple gelatinous layers. *Veliger* 46: 105-110.

Kasugai T, Segawa S 2005. Life cycle of the Japanese pygmy squid *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) in the *Zostera* beds of the temperate coast of central Honshu, Japan. *Phuket Mar Biol Cent Res Bull* 66: 249-258.

Kasugai T, Shigeno S, Ikeda Y 2004. Feeding and external digestion in the Japanese pygmy squid *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae). *J Molluscan Stud* 70: 231-236.

Lewis A R, Choat J H 1993. Spawning mode and reproductive output of the tropical cephalopod *Idiosepius pygmaeus*. *Can J Fish Aquat Sci* 50: 20-28.

Lindgren A R, Pankey M S, Hochberg F G, Oakley T H 2012. A multi-gene phylogeny of Cephalopoda supports convergent morphological evolution in association with multiple habitat shifts in the marine environment. *BMC Evol Biol* 12: 129.

Lindgren A R, Daly M 2007. The impact of length-variable data and alignment criterion on the phylogeny of Decapodiformes (Mollusca: Cephalopoda). *Cladistics* 23: 464-476.

Lindgren A R, Giribet G, Nishiguchi M K 2004. A combined approach to the phylogeny of the Cephalopoda (Mollusca). *Cladistics* 20:1-34.

Lu C C, Dunning M C 1998. Subclass Coleoidea. *In* Beesley P L, Ross G J B, & Wells A (eds), Mollusca: The southern synthesis Fauna of Australia. CSIRO Publishing, Melbourne, VIC: 499-563.

Messenger J B 1968. The visual attack of the cuttlefish, *Sepia officinalis*. *Anim Behav* 16: 342-357.

Moynihan M 1983a. Notes on the behavior of *Idiosepius pygmaeus* (Cephalopda: Idiosepiidae). *Behavior* 85: 42-57.

Moynihan M 1983b. Notes on the behavior of *Euprymna scolopes* (Cephalopoda: Sepiolidae). *Behavior* 85: 25-41.

Nabhitabhata J 1994. Rearing of Thai pygmy cuttlefish, *Idiosepius thailandicus* Chotiyaputta, Okutani, and Chaitiamvong. II: Mating and spawning behavior. *Rayong Coastal Aquacult Stn*19. Rayong, Thailand.

Nabhitabhata J 1998. Distinctive behavior of Thai pygmy squid, *Idiosepius thailandicu*s Chotiyaputta, Okutani, & Chaitiamvong, 1991. *Phuket Mar Biol Cent Spec Publ* 18: 25-40.

Nabhitabhata J, Suwabanaka J 2008. Reproductive behaviour and cross-mating of two closely related pygmy squids *Idiosepius biserialis* and *Idiosepius thailandicus* (Cephalopoda: Idiosepiidae). *J Mar Biol Ass UK* 88: 987-993.

Nabhitabhata J, Nilaphat P, Reureng A, Promboon P. 2004. Culture, growth, and behaviour of sharp-tail pygmy squid, *Idiosepius pygmaeus* Steenstrup, 1881. *Rayong Coastal Fish Res Dev Cent* 51.

Natsukari Y 1970. Egg-laying behavior, embryonic development and hatched larva of the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann. *Bull Faculty Fish Nagasaki Univ* 30: 15-29.

Nesis K N 1987. Cephalopods of the World. TFH Publications, Neptune City, NJ.

Nesis K N, Katugin O N, Ratnikov A V 2002. Pygmy cuttlefish *Idiosepius paradoxus* (Ortmann, 1888) (Cephalapoda) - first record of Idiosepiidae in Russian seas. *Ruthenica* 12: 81-84.

Nishiguchi M K, Lopez J E, Boletzky S v 2004. Enlightenment of old ideas from new investigations: The evolution of bacteriogenic light organs in squids. *Evol Dev* 6**:** 41-49.

Nishiguchi M K, Mapes R 2008. Cephalopoda. *In* Ponder W & Lindberg D R (eds), Phylogeny and Evolution of the Mollusca. University of California Press, Berkeley, CA: 163-199.

Ortmann A S 1888. Japanische Cephalopoden. *Zool Jahrb Abt Syst* 3: 639-670.

Pecl G T, Moltschaniwskyj N A 1997. Changes in muscle structure associated with somatic growth in *Idiosepius pygmaeus*, a small tropical cephalopod. *J Zool Lond* 242: 751-764.

Pecl G T, Moltschaniwskyj N A 1999. Somatic growth processes: How are they altered in captivity? *Proc R Soc B: Biol Sci* 266: 1133-1139.

Roberts D A 1997. Factors affecting the predatory behaviour of *Idiosepius pygmaeus*. Undergraduate honors thesis, Marine Biology. James Cook University, Townsville: 76 p.

Sasaki M 1921. On an adhering habit of pygmy cuttlefish, *Idiosepius pygmaeus* Steenstrup. *Annot Zool Jpn* 10: 209-213.

Sasaki M 1929. A monograph of the Dibranchiate cephalopods of the Japanese and adjacent waters. *J. Coll Agric, Hokkaido Imp Univ* 20: 1357.

Sato N, Munehara H 2013. The possibility of overwintering by *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) at the northern limits of its distribution. *Am Malacol Bull* 31: 101-104.

Sato N, Kasugai T, Munehara H 2008. Estimated life span of the Japanese pygmy squid, *Idiosepius paradoxus* from statolith growth increments. *J Mar Biol Ass UK* 88: 391-394.

Sato N, Awata X, Munehara H 2009. Seasonal occurrence and sexual maturation of Japanese Pygmy Squid *(Idiosepius paradoxus*) at the northern limits of their distribution. *ICES J Mar Sci 66*: 811-815.

Sato N, Kasugai T, Ikeda Y, Munehara H 2010. Structure of the seminal receptacle and sperm storage in the Japanese pygmy squid. *J Zool* 282: 151-156.

Sato N, Kasugai T, Munehara H 2013a. Sperm transfer or spermatangia removal: postcopulatory behaviour of picking up spermatangium by female Japanese pygmy squid. *Mar Biol* 160: 553-561.

Sato N, Yoshido MA, Fujiwara E, Kasugai T 2013b. High-speed camera observations of copulatory behaviour in *Idiosepius paradoxus*: function of the dimorphic hectocotyli. *J Molluscan Stud* 79: 183-186.

Sato N, Kasugai T, Munehara H 2014. Female pygmy squid cryptically favour small males and fast copulation as observed by removal of spermatangia. *Evol Biol* 41: 221-228.

Semmens J M, Moltschaniwskyj N, Alexander C G 1995. Effect of feeding on the structure of the digestive gland of the tropical sepioid squid *Idiosepius pygmaeus*. *J Mar Biol Ass UK* 75: 885-897.

Sinn D L, Moltschaniwskyj N A 2005. Personality traits in dumpling squid (*Euprymna tasmanica*): Context-specific traits and their correlation with biological characteristics. *J Comp Psychol* 119: 99-110.

Shigeno S, Yamamoto M 2002. Organization of the nervous system in the pygmy cuttlefish, *Idiosepius paradoxus* Ortmann (Idiosepiidae, Cephalopoda). *J Morphol* 254: 65-80.

Streenstrup J 1881. *Sepiadarium* og *Idiosepius* to nye Slaegter af Sepiernes Familie. Med Bemaekninger om de to beslaegtede Former *Sepioloidea* D'Orb. og *Spirula* Lamarck. *Videnskabernas Selskab Skrifter* 3: 235-242.

Strugnell J, Jackson J, Drummond A J, Cooper A 2006. Divergence time estimates for major cephalopod groups: evidence from multiple genes. *Cladistics* 22: 89-96.

Strugnell J, Nishiguchi M K 2007. Molecular phylogeny of coleoid cephalopods (Mollusca: Cephalopoda) inferred from three mitochondrial and six nuclear loci: a comparison of alignment and analysis methods. *J Molluscan Stud* 73: 393-398.

Suwanmala J, Byern J V, Nabhitabhata J 2005. Observation of *Idiosepius pygmaeus* (Cephalopoda, Idiosepiidae) at Klong Bangrong, Phuket Island, Thailand. *In* 2nd International Symposium-Coleoid Cephalopods through Time, Prague.

Tracey S R, Steer M A, Pecl G T 2003. Life history traits of the temperate mini-maximalist *Idiosepius notoides* (Cephalopoda: Sepioidea). *J Mar Biol Ass UK* 83: 1297-1300.

Van Camp L M 1997. The effects of temperature and food availability on growth and reproductive output: using a cephalopod model. Undergraduate honors thesis, Marine Biology. James Cook University, Townsville: 81 p.

Voss G L 1962. South African cephalopods. *Trans R Soc S Afr* 36: 245-272.

Wollesen T, Cummins S F, Degnan B M, Wanninger A 2010a. FMRFamide gene and peptide expression during central nervous system development of the cephalopod mollusk, *Idiosepius notoides*. *Evol Dev* 12(2): 113-130.

Wollesen T, Degnan B M, Wanninger A 2010b. Expression of serotonin (5-HT) during CNS development of the cephalopod mollusk, *Idiosepius notoides*. *Cell Tissue Res* 342(2): 161.

Yamamoto M 1988. Normal embryonic stages of the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann. *Zool Sci* 5: 989-998.

Yamamoto M, Shimazaki Y, Shigeno S 2003. Atlas of the embryonic brain in the pygmy squid, *Idiosepius paradoxus*. *Zool Sci* 20: 163-179.

Yamamoto T 1949. Sexual dimorphism of *Loligo bleekeri* and I*diosepius paradoxus* on their mantle length. *Venus* 15: 94-96.

Yoshida MA, Tsuneki, Furuya H 2008. Function of vascular endothelial growth factor receptor of cephalopod, *Idiosepius paradoxus*. *Dev Biol Abstr*: 319

Yoshida MA, Ogura A 2011. Genetic mechanisms involved in the evolution of the cephalopod camera eye revealed by transcriptomic and developmental studies. *BMC Evol Biol*11: 180.

Yoshida MA, Tsuneki K, Furuya H 2010a.Venous branching asymmetry in the pygmy squid *Idiosepius* (Cephalopoda: Idiosepiida) with reference to its phylogenetic position and functional significance. *J Nat Hist* 44: 2031-2039.

Yoshida MA, Shigeno S, Tsuneki, Furuya H 2010b. Squid vascular endothelial growth factor receptor: a shared molecular signature in the convergent evolution of closed circulatory systems. *Evol Dev* 12: 25-33.

Received on February 6, 2014
Accepted on June 3, 2014
Associate Editor: R Villaneuva

OPEN ACCESS Freely available online

PLOS ONE

Gene-Swapping Mediates Host Specificity among Symbiotic Bacteria in a Beneficial Symbiosis

Alba A. Chavez-Dozal[1], Clayton Gorman[1], C. Phoebe Lostroh[2], Michele K. Nishiguchi[1]*

1 New Mexico State University, Department of Biology, Las Cruces, New Mexico, United States of America, **2** Colorado College, Department of Biology, Colorado Springs, Colorado, United States of America

Abstract

Environmentally acquired beneficial associations are comprised of a wide variety of symbiotic species that vary both genetically and phenotypically, and therefore have differential colonization abilities, even when symbionts are of the same species. Strain variation is common among conspecific hosts, where subtle differences can lead to competitive exclusion between closely related strains. One example where symbiont specificity is observed is in the sepiolid squid-*Vibrio* mutualism, where competitive dominance exists among *V. fischeri* isolates due to subtle genetic differences between strains. Although key symbiotic loci are responsible for the establishment of this association, the genetic mechanisms that dictate strain specificity are not fully understood. We examined several symbiotic loci (*lux*-bioluminescence, *pil* = pili, and *msh*-mannose sensitive hemagglutinin) from mutualistic *V. fischeri* strains isolated from two geographically distinct squid host species (*Euprymna tasmanica*-Australia and *E. scolopes*-Hawaii) to determine whether slight genetic differences regulated host specificity. Through colonization studies performed in naïve squid hatchlings from both hosts, we found that all loci examined are important for specificity and host recognition. Complementation of null mutations in non-native *V. fischeri* with loci from the native *V. fischeri* caused a gain in fitness, resulting in competitive dominance in the non-native host. The competitive ability of these symbiotic loci depended upon the locus tested and the specific squid species in which colonization was measured. Our results demonstrate that multiple bacterial genetic elements can determine *V. fischeri* strain specificity between two closely related squid hosts, indicating how important genetic variation is for regulating conspecific beneficial interactions that are acquired from the environment.

Citation: Chavez-Dozal AA, Gorman C, Lostroh CP, Nishiguchi MK (2014) Gene-Swapping Mediates Host Specificity among Symbiotic Bacteria in a Beneficial Symbiosis. PLoS ONE 9(7): e101691. doi:10.1371/journal.pone.0101691

Editor: Christophe Beloin, Institut Pasteur, France

Received November 19, 2013; **Accepted** June 10, 2014; **Published** July 11, 2014

Funding: A.A.C-D. was supported by NMSU National Institutes of Health (NIH) RISE to the doctorate (NIH NIGMS R25GM061222) and NASA (NMSGC) fellowships. C.G. was supported by the NMSU NIH MARC program (NIH-GM0007667-34). Support for the research was through National Science Foundation (NSF) and NIH awards (NSF IOS-0744498, NIH NIAID1SC1AI081659-01 and NIH ARRA-3SC1AI081659–02S1) to M.K.N. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: nish@nmsu.edu

Introduction

Environmentally transmitted symbioses occur through the acquisition of bacteria from the environment into a naïve, uncolonized juvenile host [1]. This type of transmission strategy can be complex, since bacteria are obtained anew for each generation of hosts, and is dependent upon the population and type of symbionts present when transmission occurs [2,3]. Both host and environment have strong influences upon symbiont fitness, and it is the interplay between these two forces that determine whether specific symbiotic strains are able to colonize and persist generation after generation [4,5]. One example where both host and environment exert notable selection pressures upon symbiotic bacteria is in the sepiolid squid-*Vibrio* mutualism [5].

Complex molecular dialogs (including genetic interdependence) exist between sepiolid squid hosts and their *Vibrio* bacteria, leading to a highly specific association and subsequent cospeciation [5–8]. The complex processes by which hosts and symbionts find each other (among the tremendous marine bacterial community) in order to initiate a successful mutualism include a myriad of well-defined molecular signaling events that dictate a certain "conversation" between partners [9]. Additionally, it has been reported that both bacterial specificity (where Vibrios preferentially colonize particular species of hosts as well as environment [6,10,11] dictate which symbionts are successful in squid light organ symbioses. Studies competing native strains with non-native strains in both allopatric Australian *Euprymna tasmanica* and Hawaiian *Euprymna scolopes* indicate the existence of competitive dominance and intraspecific recognition of environmentally transferred symbionts [6,8,11]. Along with host specificity, environmental temperature is also an important factor for colonization and dominance of specific *Vibrio* strains when colonizing different squid host species living in sympatry [4]. Thus, bacterial specificity is dictated both by host mechanisms of selection (particular *Vibrio* spp. are host specialists) or the environment (vibrio bacteria as a group are host generalists), but the exact means of this specificity have not been determined [5].

Recent studies have been devoted to defining bacterial mechanisms (for example gene activation, horizontally transmitted elements, mutations, duplications, *etc.*) for host specificity. Recenty, a study demonstrated that, under laboratory conditions, host specificity between sepiolid squids and one species of monocentrid fish was determined by the presence of a single gene in *V. fischeri* (*rscS*), which regulates luminescence and synthesis of the symbiosis

polysaccharide locus (*syp*) that is important for host colonization and biofilm formation [7]. What subtle genetic factors are responsible for the dramatic competitive fitness differences between various isolates of *V. fischeri* among all sepiolid squids, rather than between squids and a completely different vertebrate host, is the focus of this study.

Two closely related *V. fischeri* isolates (ETJB1H from the Australian host *Euprymna tasmanica*, and ES114 from the Hawaiian host *E. scolopes*) were examined in order to determine whether differences in symbiotic loci were important for strain specificity and host recognition. These two *V. fischeri* strains were selected because they can each colonize aposymbiotic hatchlings from both species of squid equally well in 48 hour colonization assays so long as they are the only strain of *V. fischeri* present; and they nevertheless also demonstrate competitive dominance when they are presented together to their native host. That is to say Australian *E. tasmanica* hosts are preferentially colonized by *V. fischeri* ETJBH1 when *V. fischeri* ETJBH1 and *V. fischeri* ES114 bacteria are both present, and *E. scolopes* are preferentially colonized by *V. fischeri* ES114 when *V. fischeri* ETJBH1 bacteria are also present [6,8,11]. It is likely that subtle differences in specific symbiotic loci are responsible for this complex phenotype. The two particular bacterial strains were also selected because they have full or partial sequenced genomes, allowing easy genetic comparisons between both strains [10].

Materials and Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Biosafety Committee of New Mexico State University (Permit Number: 1306NMD20103) and under the guidelines of the NMSU's Institutional Animal Care and Use Committee (85-R-009 and OLAW A4022-01 and IACUC license 2013-029). Animals were appropriately handled with care and under appropriate conditions to minimize any suffering [5]. Adult *Euprymna tasmanica* were collected from Botany Bay, New South Wales, Australia with permits from the Australian Government, Department of Sustainability, Environment, Water, Population, and Communities (Export permit WT2013-10343), the New South Wales Government, Industry and Investment (Collection permit P04/0014-6.0), and the Australian Government Department of Agriculture, Fisheries, and Forestry Biosecurity (AQIS invoice ELS0016507329). *Euprymna scolopes* (Kane'ohe Bay, Honolulu, O'ahu, GPS coordinates- N 21°26′ W 157°47′) were not required to have any collection permits at this site. Both species of *Euprymna* are not endangered or are protected in either location.

Bacterial strains and growth conditions

Two *V. fischeri* strains were chosen for this study: *V. fischeri* ETJB1H isolated from the light organ of *Euprymna tasmanica* from Jervis Bay, Australia and *V. fischeri* ES114 isolated from the light organ of *Euprymna scolopes* from Kane'ohe Bay, Hawaii. Both strains were grown in Luria Bertani high Salt (LBS; per liter composition: 10 g tryptone, 5 g yeast extract, 20 g NaCl, 50 mL 1 M Tris pH 7.5, 3.75 mL 80% glycerol and 950 mL dH_2O) media and shaken at 225 rpm at 28°C overnight.

Mutant construction

Campbell mutations. Luciferase (*lux*) mutants of both strains (*V. fischeri* ETJB1H and ES114) were constructed by insertion of the plasmid pEVS122 as described previously (11) and constructs are listed in Table S2. Briefly, the *luxA* gene was partially amplified with specific primers designed from the sequenced strain *V. fischeri* ES114 (NCBI accession: NC_006840.2). PCR products were purified and cloned (after double digestion of PCR products and plasmid with *Xba*I and *Xma*I, with posterior ligation in a 1:3 plasmid-insert ratio) into the suicide vector pEVS122, and wild type *V. fischeri* strains were transformed by tri-parental mating via conjugation through a helper strain [12]. Strains that had undergone single homologous recombination events with the native gene were selected on LBS plates enriched with erythromycin (25 μg/mL). Strains constructed were defined as ES114::pACH101 (for the *lux* mutant of the Hawaiian *V. fischeri* strain ES114) and ETJB1H::pACH102 (for the *lux* mutant of the Australian *V. fischeri* strain ETJB1H). Constructs were verified by Southern blotting.

Allelic exchange. *msh* and *pil* mutants (*mshINQ* and *pilABCD*) were constructed by allelic replacement of the chromosomal loci as described previously [13]. 500 bp of neighbor genes were amplified and cloned (the first insert was cloned after digestion of plasmid and PCR product with *Sma*I and *Bam*H1, with posterior ligation in a 1:5 plasmid-insert ratio; the second insert was cloned after digestion of plasmid and PCR product with *Spe*I and *Xba*I, with posterior ligation in a 1:10 plasmid-insert ratio) into the suicide vector pSW7848 containing the P_{BAD} promoter and a chloramphenicol resistant cassette (Tables S1, S2). After transformation of ultracompetent cells (NEB 10-beta competent *E. coli*, New England BioLabs, MA, USA), selection was achieved through antibiotic enrichment (5 μg/mL choloramphenicol). Recipient cells (*V. fischeri*) were transformed by tri-parental mating as described above. Transformed strains (with respective deletions) were selected through colony patching after inoculation in LBS media enriched with 2% arabinose to allow dismissal of inserted constructs through expression of the toxic gene *ccdB* by activation of the P_{BAD} promoter after incubation in LBS media with arabinose. To easily discriminate transformants, colonies from the original tri-parental mating that were initially resistant to chloramphenicol and eventually insensitive to CcdB toxicity were selected. Constructs were named by their respective deletion (Table S2) and were verified by PCR.

Complement construction

GAPture or TAR cloning. Complementation of the *lux* operon with the opposite strain's loci (*lux* in ETJB1H for ES114::pACH101 and *lux* in ES114 for ETJB1H::pACH102) was achieved through operon mobilization ("TAR cloning" or "GAPture") as previously described [14]. TAR cloning technique was achieved by using the yeast homologous recombination pathway. 700 bp of neighboring genes (upstream and downstream) from the *luxCDABEG* operon (with 40 nucleotides of the 5′ end that were homologus with vectors pCRG13 and pCRG23) were PCR amplified and purified. Vector pCRG23 was digested with *Srf*I and pCRG13 was digested with *Eco*RV. In *Saccharomyces cerevisiae* transformation, the yeast strain CRY1-2 (containing the genotype ura^-, leu^-, $cyh2^R$, that confers sensitivity to cycloheximide and cannot grow in media without uracil) was co-transformed with the two digested plasmids and the amplified upstream and downstream genes using lithium acetate transformation. After transformation, yeast colonies were plated on synthetic URA medium (per liter composition: adenine hemisulfate 0.18 g, arginine HCl 0.12 g, glutamic acid 0.6 g, histidine HCl 0.12 g, myo-inositol 0.2 g, isoleucine 0.18 g, leucine 0.18 g, lysine HCl 0.18 g, methionine 0.12 g, p-aminobenzoic acid 0.02 g, phenylalanine 0.3 g, homoserine 0.6 g, tryptophan 0.24 g, tyrosine 0.18 g, valine 0.9 g, Difco yeast nitrogen base

without aminoacids 6.67 g, glucose 20 g) and incubated at 30°C for 4 days. After incubation, colonies were suspended *en masse* with 10 mL of TE and transferred into a 15 mL falcon tube, spun, and the construct (two plasmids + upstream/downstream genes) was extracted with glass beads and 200 µL of glass beading solution (per liter composition: 5 mL 20% sodium dodecil sulfate, 10 mL 1 M NaCl, 1 mL 1 M Tris-HCl pH 8.0, 1 mL 0.1 M EDTA, 2 mL Triton X100) and purified with Phenol/Chloroform. Plasmid DNA was transformed with ultracompetent *E. coli* cells (NEB 10-beta competent *E. coli*, New England BioLabs, MA, USA) and incubated for 24 hours at 37°C. Transformed cells (~ 10 colonies) were re-inoculated and plasmid DNA was extracted using the Qiagen plasmid Maxi kit (QIAGEN Inc., CA, USA). For *lux* operon cloning step, CRY 1–2 yeast cells were transformed ($CaCl_2$ spheroplast transformation procedure) with 1 µg of the plasmid extract and 5 µg of genomic DNA (from either *V. fischeri* ES114 or ETJB1H). Yeast cells were plated onto TYC1/Cycloheximide plates (per liter composition: D-sorbitol 182.2 g, Difco yeast nitrogen base without aminoacids 6.75 g, dextrose 0.98 g, adenine 0.2 g, arginine 0.2 g, aspartic acid 1 g, histidine 0.2 g, leucine 0.59 g, lysine 0.53 g, methionine 0.2 g, phenylalanine 0.4 g, threonine 2 g, tryptophan 0.2 g, tyrosine 0.3 g and 3 mg/mL cycloheximide) and incubated at 30°C for 7 days. Plasmids (containing the *lux* operon) were extracted from yeast spheroplasts using the Stratagene strataprep plasmid miniprep kit (Fisher Scientific, PA, USA). Constructs were verified by PCR and Southern blotting. *E. coli* ultracompetent cells were transformed and triparental mating was achieved as described previously. *V. fischeri* ES114::pACH101 was complemented with the *lux* operon of *V. fischeri* ETJB1H and strain *V. fischeri* ETJB1H::pACH102 was complemented with the *lux* operon of *V. fischeri* ES114.

Cloning using the conjugal vector pVSV105. Complete copies of the loci for *msh* and *pil* operons were amplified with specific primers for the entire locus (Table S1). PCR products and the vector pVSV105 [15] were double digested with *Xba*I and *Xma*I digests were ligated in a 1:3 plasmid-insert ratio and transformed into ultracompetent *E. coli* cells (NEB 10-beta competent *E. coli*, New England BioLabs, MA, USA). Cells were selected with chloramphenicol enrichment (25 µg/mL) and *V. fischeri* recipient cells were transformed by tri-parental mating and selected after chloramphenicol enrichment (5 µg/mL). Lastly, we constructed complements that contain each strain's native loci by cloning the respective locus into vector pVSV105. Transformation was then performed as described previously (see Table S2 for complete list of complements). Complemented strains were verified by Southern blot.

Colonization assays

Colonization assays were performed as described previously [16]. Overnight cultures of *V. fischeri* wild-type strains (ES114 and ETJB1H), mutants and complements were regrown in 5 mL of fresh LBS media until they reached an OD_{600} of 0.3. For single and competition infection experiments, cultures were then diluted to approximately 1×10^3 CFU/mL in 5 mL of sterile artificial seawater and added to glass scintillation vials where newly hatched juvenile squids were placed (one individual/vial). Seawater was changed with fresh uninoculated artificial seawater every 12 hours over a period of 48 hours. Animals were maintained on a light/dark cycle of 12/12. After 48 hours, animals were sacrificed and homogenized, and the diluted homogenate was plated onto LBS agar plates for the wild-type *V. fischeri*, LBS with erythromycin (25 µg/mL) for the *V. fischeri* mutants, and LBS with chloramphenicol (5 µg/mL) for the *V. fischeri* complements. A second set of animals were selected for competition studies where juvenile squids were co-infected with the native strain and a respective complement (Table S2), sacrificed after 48 hours, homogenized, and plated onto the various media as reported above. Colony forming units (CFUs) were counted the next day to determine colonization efficiency of each strain. A total of 8 animals/strain were used for each competition assay, and 10 non-infected (aposymbiotic) juveniles were used as negative controls.

Statistical analysis

To compare bacterial populations (wild-type, mutant, and complement constructs), one way ANOVA followed by the Tukey comparison was performed on calculated CFU numbers. Three technical replicates and 10 biological replicates (representing 3 treatments with 10 animals/strain and one set of 10 non-infected or aposymbiotic animals for the negative control).

Results and Discussion

We disrupted loci from three operons that were previously reported to be important for host colonization and persistence: *lux* (light production), *msh* (biofilm formation) and *pil* (attachment to host). First, gene disruption was achieved via single recombinational events and allelic exchange. Secondly, complementation in *trans* of mutants with copies from the other strain was achieved by the *Saccharomyces cerevisiae*-based molecular tool (GAPture) for operon manipulation and mobilization (*lux*) and extrachromsomal maintenance (*pil* and *msh*). For detailed information of plasmids and strains constructed and used in this study, see supplementary data (Tables S1, S2). Finally, animal colonization experiments were performed using host-specific (or native) strains and complemented mutants in both squid host species (*E. tasmanica* or *E. scolopes*) to determine whether these loci were involved in strain recognition with the purpose of describing how competitive hierarchy is linked to the manipulated symbiotic operons. Using single and competitive colonization experiments for all strains constructed (Table S1), colonization studies were performed in naïve hatchlings of both *E. scolopes* and *E. tasmanica* animals (Figures S1–S3). Competition assays between the two wild type *V. fischeri* strains (ETJB1H and ES114) exhibit the expected host preference for the native strain, where native *V. fischeri* significantly out-competed non-native strains during colonization, supporting earlier work [5,6].

Infection studies in Hawaiian juvenile *E. scolopes* competed native Hawaiian *V. fischeri* ES114 against non-native Australian *lux- V. fischeri* ETJB1H strain complemented with either the native ES114 *lux* or the ETJB1H *lux* genes (Figs. 1, S1). The *lux*- non-native *V. fischeri* ETJB1H had equal competitive ability against native *V. fischeri* ES114 exclusively when its *lux* mutation had been complemented with native ES114 *lux* (Fig. 1). That is, *lux*- non-native ETJB1H behaved like ES114 exclusively when the *lux*- mutation was complemented with the ES114 *lux* operon. Furthermore, when the *lux*- native Hawaiian strain (ES114) was complemented with the non-native *lux* operon from the Australian strain (ETJB1H) and competed against native *V. fischeri* ES114, the wild type dominated the complemented strain (Fig. 1). That is to say the *lux*- ES114 strain complemented with the *lux* operon from non-native ETJB1H behaved like the non-cognate ETJB1H strain.

Results of similar experiments completed in *E. tasmanica* juveniles (where *V. fischeri* ETJB1H is native, and *V. fischeri* ES114 is non-native) produced the expected reciprocal results. For example, when *lux* from non-native Hawaiian *V. fischeri* (ES114) was replaced with native *lux* from Australian *V. fischeri* ETJB1H (strain –*lux*ES114::*lux*ETJB1H), there was an increase in colonization efficiency of the competitor as if using the native wild type

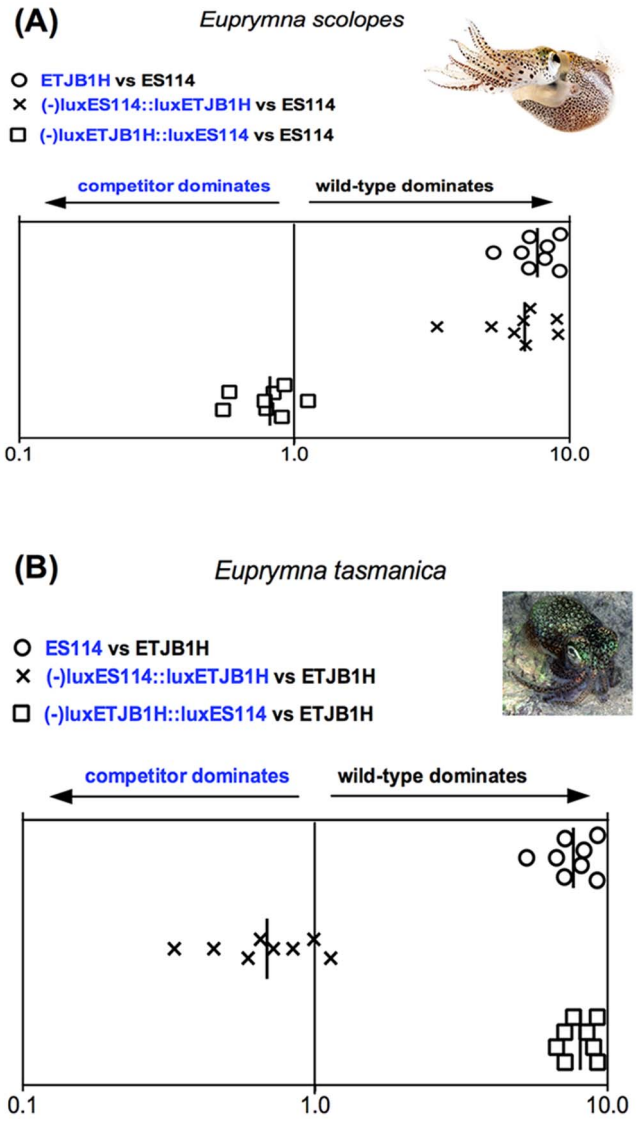


Figure 1. *lux* operon data. Colonization assays 48-hour post-infection of juvenile (A) *Euprymna scolopes* and (B) *Euprymna tasmanica* by their respective wild-type (ES114 or ETJB1H), mutant, and complement strains of the *lux* operon for *Vibrio fischeri*. Infection efficiency data is plotted as the log values of the relative competitiveness index (RCIs), calculated by dividing the ratio of mutant to wild-type by the starting ratio [28]. If the RCI is <1 the mutant strain was outcompeted by the wild-type, the wild-type strain was outcompeted by the mutant if the value is >1, and a RCI equal to 1 indicates no competitive difference. Data points represent individual animals and the position of the figures on the y axis is merely for spacing. Vertical line represents the median value of each data plot.
doi:10.1371/journal.pone.0101691.g001

Australian ETJB1H strain. Additionally when native Australian ETJB1H strain was mutated (-*lux*) and complemented with non-native Hawaiian ES114 *lux* (strain –*lux*ETJB1H::*lux*ES114), colonization efficiency was as if the native wild type Australian ETJB1H strain had been competed against itself (Figs. 1, S1). These results indicate that complementary *lux* genes are equally proficient at determining host preference in both *E. tasmanica* and *E. scolopes* squid hosts, indicating that phenotypic plasticity at one locus can give a subtle advantage to a non-native symbiont, even though it may not be the only gene responsible for symbiont recognition and specificity.

The *lux* operon is responsible for biosynthesis of luciferase, which has a crucial role in *V. fischeri* bioluminescence and fitness. Light production is used by the squid to avoid predation via silhouette reduction in a behavior known as counterillumination [16,17]. The *lux* operon is present in *V. fischeri* as a conserved, contiguous, and coordinately expressed set of genes that have thought to have been acquired through horizontal gene transfer (HGT) among closely related bacterial clones and through vertical inheritance between bacterial families (*e.g.*, *Vibrionaceae* and *Enterobacteriaceae*). Results from this part of our study indicate that although both *lux* operons produce bioluminescence and their structural proteins are similar in primary sequence, host specificity can be obtained through artificial HGT of the *lux* operon alone [18]. Our cloning method intentionally included 5′ and 3′ noncoding sequences flanking the operons; perhaps noncoding sequences contribute to the observed host preference. The *lux* operon might therefore drive evolutionary strain speciation through non-reproductive transmission of *lux* genes, when *lux* DNA is available in the environment and there are no other constraints on integration of operons into the recipient cell (e.g. the action of restriction endonucleases). Additionally, natural competence has been previously observed in *V. fischeri* after expression of the transcriptional regulators *tfoX* and *tfoY* (chitin-sensing regulators); this earlier study highlights a conserved mechanism of genetic exchange in the presence of chitin [19].

Multiple genes comprise the entire *msh* operon (including *mshABCDGIJLMNOPQ*), which is responsible for the synthesis of type IV pseudopili, important for biofilm formation [20] and attachment (or adherence) to abiotic surfaces [21]. The *msh* operon has also been reported to be crucial for attachment to certain host tissues, which is an important step for successful colonization and persistence [11]. We specifically targeted *mshI*, *mshQ* and *mshN*, since these proteins exhibit high variability in their primary sequence among multiple strains of *V. fischeri*, including Hawaiian ES114 and Australian ETJB1H (C. Lostroh, unpublished data). Loci from the *msh* operon were mutated by means of insertional inactivation and complemented by extrachromosomal maintenance [13,15,22]. Similar to the *lux* operon experiments, ES114 mutant strains were complemented with the ETJB1H *msh* gene, and vice versa. Colonization tests using all mutant strains were then completed in both *E. tasmanica* and *E. scolopes* juvenile squids. Results of *mshI*, *mshN* and *mshQ* loci after colonization are illustrated in Figure 2. Due to the difficulty of obtaining a large number of animals from one clutch to complete all infection experiments with *msh* strains, we used three different clutches from *E. scolopes* and two from *E. tasmanica*. Inter-clutch colonization variability was observed between groups, and reflected in low numbers in competition experiments; however, animals from the same clutch were used to replicate the same competition experiment to avoid variation in colonization efficiency.

Mutation of the *msh* genes in Hawaiian *V. fischeri* ES114 caused a significant reduction in colonization efficiency in *E. scolopes* (data not shown), demonstrating that the *msh* operon is important for symbiotic competence. When Hawaiian *E. scolopes* were infected with non-native ETJB1H complemented with native *mshI* (-*mshI*ETJB1H::*mshI*ES114), colonization efficiency was equal to the native wild-type (ES114). Results were similar for *mshL* and *mshQ*. These observations indicate that *mshI*, *mshL*, or *mshQ* are all important in conferring host specificity between *E. scolopes* and *E. tasmanica* squids. Interestingly, colonization in *E. tasmanica* juveniles did not mirror these results as they did for the *lux* operon. Overall levels of colonization in this case were very low (Fig. S1). The *mshI*

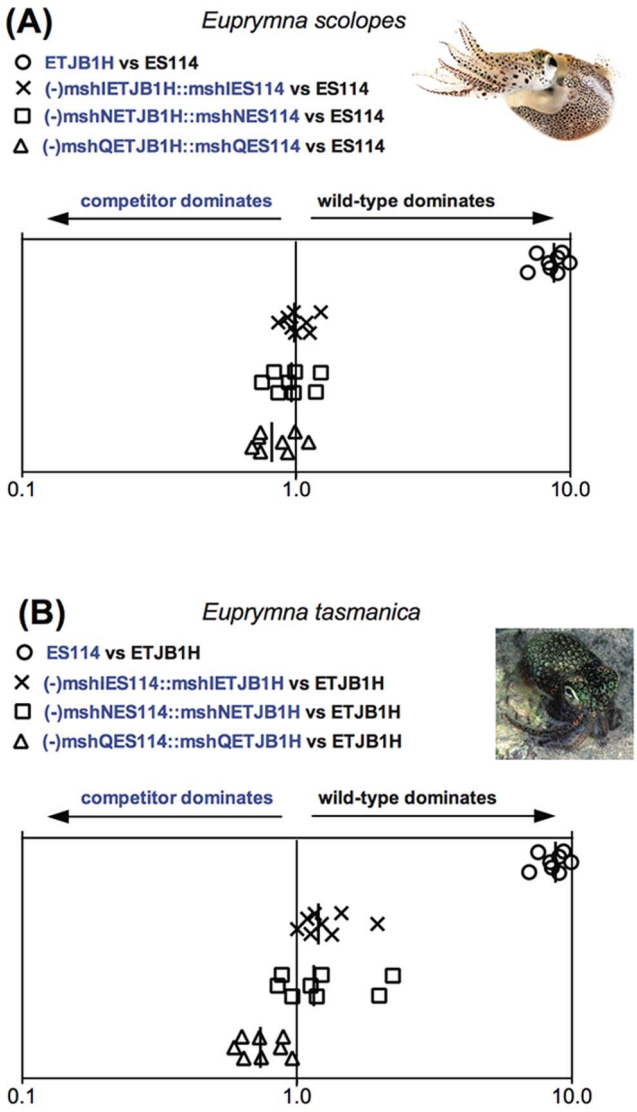


Figure 2. *msh* operon data. Colonization assays 48-hour post-infection of juvenile (A) *Euprymna scolopes* and (B) *Euprymna tasmanica* by their respective wild-type (ES114 or ETJB1H), mutant, and complement strains of *msh* genes for *Vibrio fischeri*. Infection efficiency data is plotted as the log values of the relative competitiveness index (RCIs), calculated by dividing the ratio of mutant to wild-type by the starting ratio [28]. If the RCI is <1 the mutant strain was outcompeted by the wild-type, the wild-type strain was outcompeted by the mutant if the value is >1, and a RCI equal to 1 indicates no competitive difference. Data points represent individual animals and the position of the figures on the y axis is merely for spacing. Vertical line represents the median value of each data plot.
doi:10.1371/journal.pone.0101691.g002

and *mshN* ES114 strains complemented with their ETJBH1 *msh* counterparts were out-competed by wild type ETJBH1, while the *mshQ* ES114 strain complemented with its ETJBH1 *msh* counterpart competed slightly better for colonization than wild type ETJBH1. Alternatively, ES114 complemented with native ETJB1H *mshQ* locus (-*mshQ*ES114::*mshQ*ETJB1H) outcompeted the wild-type strain. Thus, results for *mshQ* closely resemble those for *lux*, whereas *mshI* and *mshN* favored one *V. fischeri* strain (ETJB1H) but not the other (ES114). In our mixed competitions using *mshI* or *mshN* mutants, *E. tasmanica* hosts select against all complemented bacteria, keeping total levels of each symbiont low (Figs. 2, S2). In addition to clutch variability, *E. tasmanica* hosts may exert stronger sanctions against non-native *V. fischeri* more than its congener *E. scolopes* due to the presence of a genetically diverse group of *V. fischeri* symbionts available for colonization in the *E. tasmanica* habitat, whereas *V. fischeri* symbionts from *E. scolopes* are more homogeneous and host squids sample from only a small set of *V. fischeri* genotypes [5]. Having the ability to discern amongst a large, genetically diverse pool of *V. fischeri* may give squids an advantage to also differentiate cheaters to allow for a more successful beneficial symbiosis [23]. Recent work has demonstrated changes in particular symbiotic traits (luminescence, biofilm production, motility, carbon source utilization, growth) of Hawaiian *V. fischeri* strain ES114 when evolved in *E. tasmanica* hosts [24]. These traits differ quite dramatically, with the evolved strain gaining traits similar to the native strain over time. Thus, our results indicate that the *msh* operon is not only important for successful colonization of sepiolid squids, but also determines host range and accommodation from a large pool of available *Vibrio* symbionts.

We also created null *pil* mutants, and complemented them *in trans* to examine host selection. Colonization experiments in Hawaiian *E. scolopes* hatchlings indicate that *pilA, pilB* and *pilD* have important roles in host specificity (Figs. 3, S3). When constructs containing the native complemented gene were competed with either native or non-native wild-type strains, colonization efficiency of the constructed strains was equal or greater than the wild-type strain (Fig. 3). Similar results were observed in the case of *E. tasmanica* infection studies; however, *pilD* was the only locus that demonstrated host specificity in *E. tasmanica* (and not *pilA* or *pilB*). Genes from the *pil* operon (*pil ABCD*) encode for assembly of type IV pili, and are essential for bacterial attachment to both abiotic surfaces and to host cells [16,19]. In *V. fischeri*, pilus subunits are synthesized by the *pilABCD* operon, where *pilA* contributes to colonization effectiveness and encodes a protein similar to type IV-A pilins (where *mshA* is a close relative [25,26]. Phylogenetic and molecular differences have also been observed in *pilB* and *pilD* loci among multiple *V. fischeri* strains isolated from different squid hosts [27]. Our study demonstrates that *V. fischeri* ETJB1C *pilC* complemented with non-native Hawaiian *V. fischeri* ES114 *pilC* (-*pilC* ETJB1H::*pilC* ES114) is dominant in *E. scolopes*, but loses in *E. tasmanica*, since there is a competitive dominance for the native *pilC* locus (Fig. S3). Similar results are observed with *V. fischeri* ES114 *pilC* complemented with non-native Australian *V. fischeri* ETJB1H from *E. tasmanica*. This may be due to PilC being a phase variable protein (with minor differences in 3–5 aminoacids [25], which besides being implicated in type IV pilus biogenesis, mediates cell adherence [26]. Also, the heterogeneity of pili morphology means that multiple minor proteins composed of PilC subunits have evolved to be variable in order to compete for pilus receptors in host cells [25]. The intriguing question of how Pil-dependent binding is modulated and controlled between closely related host species may explain how host-switching can be accomplished through slight variations at this locus.

Additionally, we constructed complements containing the *native genes* and performed single colonization experiments as well as competition studies. Single colonization assays indicated that the complement was able to regain the colonization efficiency observed in the wild-type strain (data not shown), and the competition experiments (where the wild-type strain is used to co-infect the host with the native complement) indicated equivalent colonization efficiency between the two strains (Fig. S4).

To determine whether deletion of the various genetic elements had polar effects, we performed additional experiments to observe

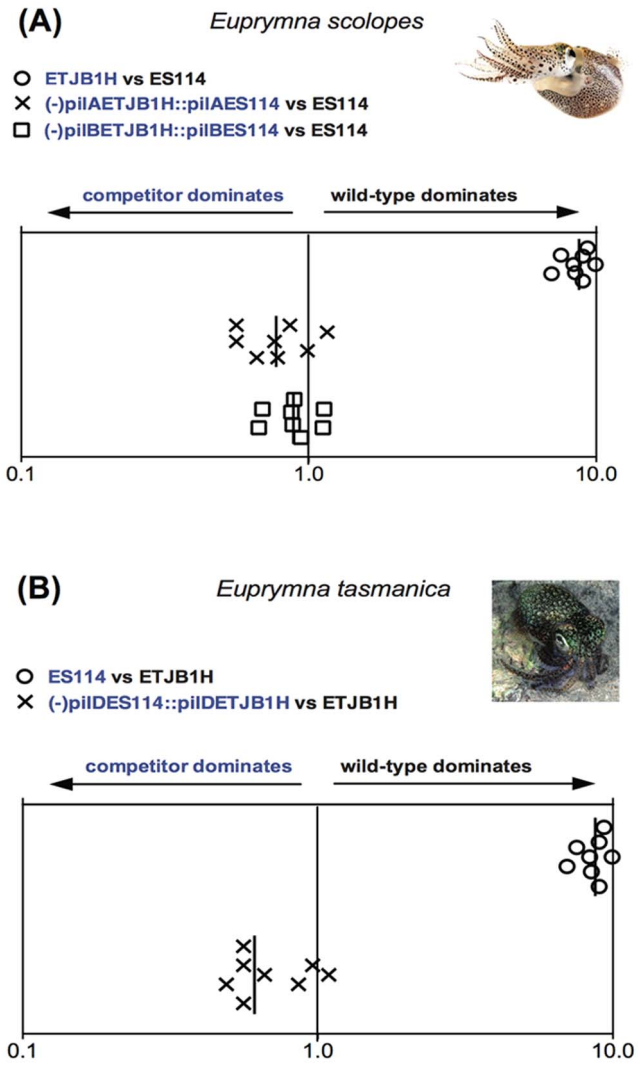


Figure 3. *pil* operon data. Colonization assays 48-hour post-infection of juvenile (A) *Euprymna scolopes* and (B) *Euprymna tasmanica* by their respective wild-type (ES114 or ETJB1H), mutant, and complement strains of *pil* genes for *Vibrio fischeri*. Infection efficiency data is plotted as the log values of the relative competitiveness index (RCIs), calculated by dividing the ratio of mutant to wild-type by the starting ratio [28]. If the RCI is <1 the mutant strain was outcompeted by the wild-type, the wild-type strain was outcompeted by the mutant if the value is >1, and a RCI equal to 1 indicates no competitive difference. Data points represent individual animals and the position of the figures on the y axis is merely for spacing. Vertical line represents the median value of each data plot.
doi:10.1371/journal.pone.0101691.g003

whether our mutants can affect phenotypes related to the function of downstream genes in the operon. For example, *msh* and *pil* influence adhesion and biofilm formation [21], and *lux* is responsible for light production [9]. We quantified biofilm and light production in both mutants and mutants complemented with the native gene, or in the case of *lux*, genes. Biofilm production decreased in mutant *msh* and *pil* strains, and light production was also impaired in *lux* mutants. Each phenotype was recovered in the respective native complements (data not shown), indicating that polarity effects may not be present; however, to be certain of this assumption additional studies are planned to determine if there is an influence in additional phenotypes. Future studies include transcriptional profiling and genetic analyses of metabolic pathways that might be affected in the various mutant strains.

Previous work examining experimental evolution of *V. fischeri* demonstrated polymorphic changes in phenotype (e.g., bioluminescence, biofilm, motility, growth) when strains are evolved in a novel host, allowing greater colonization efficiency of evolved strains when competed against their non-evolved ancestor [5,24]. These results are consistent with our directionally mutated strains reported here, where non-native strains complemented with native loci (in both Hawaiian *E. scolopes* and Australian *E. tasmanica*) outcompeted non-native strains and competed favorably over native parental strains during colonization in both host species examined. Our results indicate that the operons examined here are critical host-specificity factors and sufficient to dictate host recognition among closely related strains of *V. fischeri* from different geographical origins. Thus, strain specificity between two closely related *V. fischeri* symbionts from similar hosts is not mediated by a single or few loci, but rather multiple bacterial genetic elements that determine host range in allopatric Indo-west Pacific *Euprymna-Vibrio* associations.

Colonization of the squid host is multifactorial, and different studies from our laboratory have demonstrated that genes that are responsible for phenotypes associated to colonization are important for successful infection; additionally, experimental evolution of closely related strains does lead to competitive dominance of non-native strains [5,24]. Although these studies suggest that these loci are important for host preference, there is the possibility that compensatory mechanisms could overtake the effect of a mutation and regulatory mechanisms (along with genetic factors) are responsible for colonization efficiency.

This study provides additional support of how bacterial diversity can be maintained through host selection, and key symbiotic loci are just one factor in determining host specificity. Determining whether these loci are acting in concert with one another to further push the selective advantage of beneficial vibrios is crucial for our understanding the evolution of symbiotic associations. How these subtle differences arise in wild populations, and whether they confer a greater selective advantage in bacterial fitness, will give insight into the processes of ecological adaptation in *Vibrio* bacteria.

Supporting Information

Figure S1 **A**) Colonization assays 48-hour post-infection of juvenile *Euprymna scolopes* by wild-type, mutant, and complement strains of the *lux* operon for *Vibrio fischeri*. Single strain infection experiments are represented when only a single bar is shown (□). Competition experiments are represented with two bars (□ is the first strain and ■ is the second strain), and each strain used is indicated below each competition. Wild-type ES114 significantly colonized the host better than non-native ETJB1H. Apo = aposymbiotic or non-infected juvenile squids. Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the Tukey PostHoc comparison. Different letters indicate significant differences ($p<0.05$) between groups or infection sets. See Table S1 for a complete description of strains and Table S2 for a complete description of colonization experiments. **B**) Colonization assays 48-hour post-infection of juvenile *Euprymna tasmanica* by wild-type, mutant, and complement strains of the *lux* operon for *Vibrio fischeri*. Single strain infection experiments are represented with a single bar (□). Competition experiments are represented with two bars (□ is the first strain and ■ is the second strain), and each strain used is indicated below each competition. Wild-type

Australian *V. fischeri* ETJB1H significantly colonized the host better than the non-native Hawaiian *V. fischeri* ES114. Apo = aposymbiotic or non-infected juvenile squids. Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the Tukey PostHoc comparison. Different letters indicate significant differences ($p<0.05$) between groups or infection sets.
(TIFF)

Figure S2 **A**) Colonization assays 48-hour post-infection of juvenile *Euprymna scolopes* by wild-type, mutant, and complement strains of *msh* genes for *Vibrio fischeri*. Single strain infection experiments are represented when only a single bar is shown (□). Competition experiments are represented with two bars (□ is the first strain and ■ is the second strain), and each strain used is indicated below each competition. Wild-type ES114 significantly colonized its native host better than non-native ETJB1H. Apo = aposymbiotic or non-infected juvenile squids. Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the Tukey PostHoc comparison. Different letters indicate significant differences ($p<0.05$) between groups or infection sets. **B**) Colonization assays 48-hour post-infection of juvenile *Euprymna tasmanica* by wild-type, mutant, and complement strains of the *msh* genes for *Vibrio fischeri*. Single strain infection experiments are represented when only a single bar is shown (□). Competition experiments are represented with two bars (□ is the first strain and ■ is the second strain), and each strain used is indicated below each competition. Wild-type ETJB1H significantly colonized the host better than the non-native ES114. Apo = aposymbiotic or non-infected juvenile squids. Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the Tukey PostHoc comparison. Different letters indicate significant differences ($p<0.05$) between groups or infection sets.
(TIFF)

Figure S3 **A**) Colonization assays 48-hour post-infection of juvenile *Euprymna scolopes* by wild-type, mutant, and complement strains of the *pil* genes for *Vibrio fischeri*. Single strain infection experiments are represented when only a single bar is shown (□). Competition experiments are represented with two bars (□ is the first strain and ■ is the second strain), and each strain used is indicated below each competititon. Wild-type ES114 significantly colonized the host better than the non-native ETJB1H. Apo = aposymbiotic or non-infected juvenile squids. Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the Tukey PostHoc comparison. Different letters indicate significant differences ($p<0.05$) between groups or infection sets. **B**) Colonization assays 48-hour post-infection of juvenile *Euprymna tasmanica* by wild-type, mutant, and complement strains of *pil* genes for *Vibrio fischeri*. Single strain infection experiments are represented when only a single bar is shown (□). Competition experiments are represented with two bars (□ is the first strain and ■ is the second strain), and each strain used is indicated below each competition. Wild-type ETJB1H significantly colonized the host better than the non-native ES114. Apo = aposymbiotic or non-infected juvenile squids. Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the Tukey PostHoc comparison. Different letters indicate significant differences ($p<0.05$) between groups or infection sets.
(TIFF)

Figure S4 **A**) Colonization assays 48-hour post-infection of juvenile *Euprymna scolopes* by wild-type and complement strains of the native genes for *Vibrio fischeri*. Single strain infection experiments are represented when only a single bar is shown (■). Competition experiments are represented with two bars (■ is the first strain and □ is the second strain). Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the one-way ANOVA test and Tukey PostHoc comparison. There was no significant difference between strains (as indicated by the *P* value of each column factor). **B**) Colonization assays 48-hour post-infection of juvenile *Euprymna tasmanica* by wild-type and complement strains of native genes for *Vibrio fischeri*. Single strain infection experiments are represented when only a single bar is shown (■). Competition experiments are represented with two bars (■ is the first strain and □ is the second strain). Data are plotted as the mean of Colony Forming Units (CFUs) counted for each strain. Multiple comparisons were calculated between groups using the one-way ANOVA test and Tukey PostHoc comparison. There was no significant difference between strains.
(TIF)

Table S1 Plasmids used and constructed in this study.
(DOCX)

Table S2 Strains used and constructed in this study.
(DOCX)

Acknowledgments

Photo credits in Figures 1–3 are Kahi Kai (E. scolopes) and Mark Norman (E. tasmanica).

Author Contributions

Conceived and designed the experiments: AC CPL MKN. Performed the experiments: AC CG. Analyzed the data: AC CG CPL MKN. Contributed reagents/materials/analysis tools: MKN. Wrote the paper: AC CPL MKN.

References

1. Bright M, Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. Nat Rev Microbiol 8(3):218–30.
2. Stats M, Morris E, Gates RD (2008) Functional diversity in coral-dinoflagellate symbiosis. Proc Nat Acad Sci USA 105(27):9256–61.
3. Sachs JL, Skophammer RG, Regus JU (2011) Evolutionary transitions in bacterial symbiosis. Proc Nat Acad Sci USA 108(2):10800–07.
4. Nyholm SV, Nishiguchi MK (2008) The evolutionary ecology of a sepiloid squid-*Vibrio* association: From cell to environment. Vie et Milleu 58:175–184.
5. Soto W, Punke E, Nishiguchi MK (2012) Evolutionary perspectives in a mutualism of sepiolid squid and bioluminescent bacteria: Combined usage of microbial experimental evolution and temporal population genetics. Evolution 66:1308–1321.
6. Nishiguchi MK (2002) Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. Micro Ecol 44 (1): 10–18.
7. Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG (2009) A single regulatory gene is sufficient to alter bacterial host range. Nature 458 (7235):215–218.
8. Naughton LM, Blumerman SL, Carlberg M, Boyd EF (2009) Osmoadaptation among *Vibrio* species and unique genomic features and physiological responses of *Vibrio parahaemolyticus*. Appl Environ Microbiol 75:2802–2810.
9. Nyholm SV, McFall-Ngai MJ (2004) The winnowing: establishing the squid-*Vibrio* symbiosis. Nature 2:632–642.
10. Ruby E, Henderson B, McFall-Ngai M (2004) We get by with a little help from our (little) friends. Science 303:1305–1307.
11. Ariyakumar DS, Nishiguchi MK (2009) Characterization of two host-specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol Lett 299:65–73.

12. Stabb EV, Ruby EG (2002) New RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. Meth Enzymol 358:413–426.
13. Le Roux F, Binesse J, Saulnier D, Mazel D (2007) Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. Appl Environ Microbiol 73:777–784.
14. Gaida A, Becker MM, Schmid CD, Buhlmann T, Louis EJ, et al. (2011) Cloning of the repertoire of individual *Plasmodium falciparum var* genes using transformation associated recombination (TAR). PLoS One 6(3):e17782.
15. Dunn AK, Millikan DS, Adin DM, Bose JL, Stabb EV (2006) New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression in situ. Appl Environ Microbiol 72(1):802–810.
16. Jones BW, Nishiguchi MK (2004) Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Mar Biol 144:1151–1155.
17. Ruby EG, McFall-Ngai MJ (1999) Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends Microbiol 7:414–420.
18. Urbanczyk H, Ast JC, Kaeding AJ, Oliver JD, Dunlap PV (2008) Phylogenetic analysis of the incidence of *lux* gene horizontal transfer in *Vibrionaceae*. J Bacteriol 190(10):3494–3504.
19. Pollack-Berti A, Wollenberg MS, Ruby EG (2010) Natural transformation of *Vibrio fischeri* requires *tfoX* and *tfoY*. Environ Microbiol 12(8):2302–2311.
20. Watnick P, Kotler R (2000) Biofilm, city of microbes. J Bacteriol 182(10): 2675–2679.
21. Yildiz FH, Visick KL (2008) *Vibrio* biofilms: so much the same yet so different. Trends Microbiol 17 (3):109–118.
22. Dunn AK, Millikan DS, Adin DM, Bose JL, Stabb EV (2006) New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression in situ. Appl Environ Microbiol 72(1):802–810.
23. Castle W (2013) Conflict and cooperation in the squid-*Vibrio* symbiosis. New Mexico State University, Ph.D. dissertation, 99 pgs.
24. Soto W, Rivera FM, Nishiguchi MK (2014) Ecological diversification of *Vibrio fischeri* serially passaged for 500 generations in novel squid host *Euprymna tasmanica*. *Microb Ecol*. 67(3):700–721.
25. Rudel T, Boxberger HJ, Meyer TF (1995) Pilus biogenesis and epithelial cell adherence of *Neisseria gonorrhoeae pilC* double knock-out mutants. Mol Microbiol 17:1057–1071.
26. Scheuerpflug I, Rudel T, Ryll R, Pandit J, Meyer TF (1999) Roles of PilC and PilE proteins in pilus-mediated adherence of *Neisseria gonorrhoeae* and *Neisseria meningitidis* to human erythrocytes and endothelial and epithelial cells. Infect Immun 67(2):834–843.
27. Browne-Silva J, Nishiguchi MK (2008) Gene sequences of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*. J Syst Evol Microbiol 58:1292–1299.
28. Hussa EA, O'Shea TM, Darnell CL, Ruby EG, Visick KL (2007) Two-component response regulators of *Vibrio fischeri*: Identification, mutagenesis and characterization. J Bacteriol 189(16):5825–5838.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 09 December 2014
doi: 10.3389/fmicb.2014.00593

Microbial experimental evolution as a novel research approach in the Vibrionaceae and squid-*Vibrio* symbiosis

***William Soto*[1] *and Michele K. Nishiguchi*[2] ***

[1] *BEACON Center for the Study of Evolution in Action, Michigan State University, East Lansing, MI, USA*
[2] *Department of Biology, New Mexico State University, Las Cruces, NM, USA*

Edited by:
Monica Medina, Pennsylvania State University, USA

Reviewed by:
Natacha Kremer, Université Claude Bernard Lyon 1, France
Catherine Masson-Boivin, Institut National de la Recherche Agronomique, France

***Correspondence:**
Michele K. Nishiguchi, Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Foster Hall- Horseshoe and Sweet Streets, Las Cruces, NM 88003-8001, USA
e-mail: nish@nmsu.edu

The Vibrionaceae are a genetically and metabolically diverse family living in aquatic habitats with a great propensity toward developing interactions with eukaryotic microbial and multicellular hosts (as either commensals, pathogens, and mutualists). The Vibrionaceae frequently possess a life history cycle where bacteria are attached to a host in one phase and then another where they are free from their host as either part of the bacterioplankton or adhered to solid substrates such as marine sediment, riverbeds, lakebeds, or floating particulate debris. These two stages in their life history exert quite distinct and separate selection pressures. When bound to solid substrates or to host cells, the Vibrionaceae can also exist as complex biofilms. The association between bioluminescent *Vibrio* spp. and sepiolid squids (Cephalopoda: Sepiolidae) is an experimentally tractable model to study bacteria and animal host interactions, since the symbionts and squid hosts can be maintained in the laboratory independently of one another. The bacteria can be grown in pure culture and the squid hosts raised gnotobiotically with sterile light organs. The partnership between free-living *Vibrio* symbionts and axenic squid hatchlings emerging from eggs must be renewed every generation of the cephalopod host. Thus, symbiotic bacteria and animal host can each be studied alone and together in union. Despite virtues provided by the Vibrionaceae and sepiolid squid-*Vibrio* symbiosis, these assets to evolutionary biology have yet to be fully utilized for microbial experimental evolution. Experimental evolution studies already completed are reviewed, along with exploratory topics for future study.

Keywords: *Vibrio*, sepiolid squid, cospeciation, experimental evolution, environmental transmission

THE VIBRIONACEAE

The family Vibrionaceae (Domain Bacteria, Phylum Proteobacteria, Class Gammaproteobacteria) encompass gram-negative chemoorganotrophs that are mostly motile and possess at least one polar flagellum (Farmer III and Janda, 2005; Thompson and Swings, 2006); although, the gut symbiont *Vibrio halioticoli* to the abalone *Haliotis discus hannai* has been described as non-motile (Sawabe et al., 1998). Vibrionaceae are facultative anaerobes, having both respiratory (aerobic and anaerobic) and fermentative metabolisms. Nitrogen fixation and phototrophy have both been reported (Criminger et al., 2007; Wang et al., 2012). Agarases and alginases have been noted from *Vibrio* (Fu and Kim, 2010; Dalia et al., 2014). Most cells are oxidase positive with a dimension 1 μm in width and 2–3 μm in length. Sodium cations are a requirement for growth and survival, but *Vibrio cholerae* and *V. mimicus* are unusually tolerant to low sodium waters. Most species are susceptible to the vibriostatic agent 0/129 (Thompson and Swings, 2006). Vibrionaceae are ubiquitously distributed throughout aquatic habitats, including freshwater, brackish, and marine waters (Madigan and Martinko, 2006). Vibrionaceae have been isolated from rivers, estuaries, lakes, coastal and pelagic oceanic waters, the deep sea, and saltern ponds (Urakawa and Rivera, 2006). Vibrionaceae can also be microbial residents of aquatic animals as either commensals, pathogens, and mutualists (Soto et al., 2010). Bacteria may exist as planktonic free-living cells or as biofilms attached to solid subtrates present in sediments of aquatic habitats or alternatively adhered to floating particulate matter or debris. Vibrionaceae may also form biofilms on the surfaces of animal, algal/phytoplanktonic, protoctistal, or fungal hosts the cells colonize, as this prokaryotic family is quite able to initiate and establish vigorous biofilms on eukaryotic cells and chitin surfaces (e.g., invertebrate exoskeletons and fungal cell walls; Polz et al., 2006, Pruzzo et al., 2008; Soto et al., 2014). Vibrionaceae have also been found to be intracellular inhabitants of eukaryotic microorganisms (Abd et al., 2007). Although as many as eight genera have been assigned to the Vibrionaceae, the two most specious are *Vibrio* and *Photobacterium* (Thompson and Swings, 2006). *Salinivibrio* possesses an unusual ability to grow in a wide range of salinity (0–20% NaCl) and temperature (5–50°C; Ventosa, 2005; Bartlett, 2006). Numerous species in the Vibrionaceae are pathogenic and cause disease in aquatic animals and humans (Farmer III et al., 2005), *V. cholerae* being the most notorious example as the causative agent of cholera (Colwell, 2006). *V. vulnificus* and *V. parahaemolyticus* can also cause severe illnesses in humans as a result of consuming contaminated seafood (Hulsmann et al., 2003; Wong and Wang, 2004). Furthermore, every year *V. harveyi* (Owens and Busico-Salcedo, 2006), *V. anguillarum*

(Miyamoto and Eguchi, 1997; Crosa et al., 2006), and *V. parahaemolyticus* (Austin, 2006) cause substantial economic losses to the aquaculture industry worldwide. The genera *Vibrio* and *Photobacterium* include opportunistic pathogens capable of infecting marine animals and humans, and are able to enter preexisting wounds or body openings of especially susceptible hosts that are already ill, stressed, fatigued, or immunocompromised (Urbanczyk et al., 2011). Given the heightened ability of Vibrionaceae to cement themselves to eukaryotic cells through peptide and polysaccharide modification of their exopolysaccharide, lipopolysaccharide, and capsules (Sozhamannan and Yildiz, 2011), the lack of additional human pathogens is curious. Perhaps the reason is foreign extracellular protein and polysaccharide are the same materials the mammalian immune system specifically targets, neutralizes, and removes as non-self antigens with exquisite capacity (Owen et al., 2013). Vibrionaceae have also been recently investigated for the development of probiotics, antimicrobials, and pharmaceutical drugs with potential clinical and economic value for veterinary medicine, animal husbandry, aquaculture, and human health—molecules antagonistic toward cancer cells, fungi, algae, protoctists (a term frequently preferred over protist or protozoan), bacteria, and viruses. Metabolites produced by the Vibrionaceae have also been found to have quorum sensing-disrupting properties against other bacteria, which may open an entire horizon for the advancement of "quorum sensing" antibiotics (i.e., quorum quenching) (Gatesoupe, 1999; Mansson et al., 2011).

MICROBIAL EXPERIMENTAL EVOLUTION

Conventional evolutionary studies seeking to understand adaptation and speciation implement the comparative or historical approach (e.g., phylogenetics). This approach compares organisms from different environments and attempts to understand the evolutionary processes that may have produced the current distributions and adaptations of descendent populations from ancestral ones (Bennett, 2002). Since this methodology generates informed explanations based on extant organisms retrospectively and with hindsight, it naturally must make numerous assumptions on the evolutionary relationships of the organisms under study and their likely mode of evolution, even when the use of fossil data is available. Experimental evolution, however, allows one to begin with an ancestral population and empirically observe the adaptation and radiation that result in the descendent lineages under different selective regimens. Experimental evolution studies can be implemented under controlled and reproducible conditions to study evolution, usually in the laboratory and on model organisms. Less assumptions in environmental conditions, the selection pressures involved, or in the ancestral and evolving populations are necessary, since there is more control by the investigator (Bennett, 2002). Experimental evolution permits tractability for the study of evolutionary biology by allowing experiments to be manipulated and repeated with replication (Lenski, 1995; Bennett, 2002). Bacteria, including *Vibrio*, are ideal organisms for such studies. For instance, these organisms have short generation times which allow evolution and adaptation to be observable on a human time scale (Lenski, 1995). Microorganisms also usually possess the advantage of achieving large population sizes ($>1 \times 10^9$ cells/mL in liquid culture) in the environments for which experimental evolution studies are executed, providing ample opportunity for rare beneficial mutations to arise and reach fixation by natural selection. Deleterious mutations are likely to become extinct via purifying selection, since evolution by genetic drift is negligible in huge gene pools. Moreover, a "frozen fossil record" can be generated with bacteria by storing evolving lineages at different evolutionary time points in a −80°C freezer. Hence, one can later compare relative fitness of the ancestral clone with a derived one in novel or ancestral environments (Bennett, 2002; Lenski, 2002). As a result, evolutionary tradeoffs can be measured during the course of adaptation in the novel environment. The −80°C fossil record also permits the determination of the evolutionary episode that a novel adaptive trait first evolved. Likewise, evolution may be "replayed" from various time points to see if subsequent outcomes are contingent on prior genetic changes or previously modified traits (Kawecki et al., 2012; Barrick and Lenski, 2013). Finally, the ancestral and derived bacteria can subsequently be analyzed to observe the exact genetic changes that have occurred and which specific ones are responsible for novel adaptive traits (Lenski, 1995; Bennett, 2002). Experimental evolution is the only direct method for studying adaptation and the genetic changes responsible, which complements genetic, physiological, biochemical, and phylogenetic approaches.

ATTENUATION AND VACCINE DEVELOPMENT WITH VIBRIOS: INSIGHTS FOR MICROBIAL EXPERIMENTAL EVOLUTION

Microbial experimental evolution is a thrilling sub-discipline of evolutionary biology which has risen in the last twenty to thirty years to address diverse issues (Soto et al., 2010; Conrad et al., 2011). Although initial work largely began with *Escherichia coli* (Lenski et al., 1991), the inclusion of other microbial species has continued to grow. However, despite a few exceptions (Schuster et al., 2010; Soto et al., 2012, 2014), surprisingly little work has been completed to date with members of the Vibrionaceae. Considering the Vibrionaceae possess colossal metabolic, biochemical, ecological, and genetic diversity, the general absence of this bacterial family as an established model in microbial experimental evolution has been heedless. Nonetheless, classical efforts to attenuate pathogenic bacteria for human vaccine development were endeavors analogous to experimental evolution (Kawecki et al., 2012). Virulent bacterial isolates would be repeatedly subcultured under laboratory conditions on growth medium, in tissue/cell culture, or in animal models to introduce random deleterious mutations in the microorganism under study. Alternatively, the microbe would be continuously subjected to chemical or physical mutagens (e.g., ultraviolet light). The exact mutations that occurred and the loci undergoing genetic changes were frequently unknown initially, and attempts to their identification only coming later with additional research (Frey, 2007). For *V. cholerae*, nitrosoguanidine frequently served as a chemical mutagen to induce several attenuating mutations, including auxotrophy (Bhaskaran and Sinha, 1967; Baselski et al., 1978). Although attenuation by random mutagenesis yielded some products that demonstrated promising results in animal models and humans, this approach is less common today (Frey,

2007). The construction of attenuated vibrios containing large targeted deletions of loci known to contribute to virulence is currently more desirable, since microbial reversion to pathogenicity is deemed less probable through this practice. Side effects are also a concern (Honda and Finkelstein, 1979; Cameron and Mekalanos, 2011). *V. cholerae* attenuation by the continual introduction of random mutations, resulting in numerous deleterious genetic lesions across many loci, frequently fails to sufficiently incapacitate virulence (Cameron and Mekalanos, 2011), as the microbe finds alternative ways to thrive and persist in the human host. An evolutionary conclusion coming from vaccine work with *V. cholerae* is that numerous ways of making a successful living exist in a potential host for the genus *Vibrio*; many potential niches exist, as evidenced by the continued ability of *V. cholerae* to initiate successful and alternative symptomatic infections (e.g., reactogenicity) despite the introduction of several deleterious mutations into its genome. An implication of this observation is that vibrios are evolutionarily versatile for host colonization and proliferation. For instance, medical reports exist of *V. cholerae*'s ability to initiate bacteremia, malaise, fever, chills, and skin lesions in humans, even in the absence of a gastrointestinal infection (Ninin et al., 2000). Such symptoms are more typically characteristic of *V. vulnificus* infections and raise the interesting question of whether there may be common virulence factors in *V. cholerae* and other pathogenic vibrios which are overshadowed by the exuberant effect of choleragen. More broadly, determinants and mechanisms responsible for the colonization of host animals (or attachment to eukaryotic cells) by vibrios may possess overlap across diverse interactions (e.g., commensalism, pathogenesis, and mutualism; Hentschel et al., 2000). Hence, microbial selection experiments with vibrios have potential to provide novel insights into evolution of the varied interactions the genus *Vibrio* possesses with its hosts, and vibrio vaccine research is a great repository of information and useful starting point to ask scientific questions, construct hypotheses, and to find focus topics for real world applications and practical value.

SEPIOLID SQUID-*VIBRIO* SYMBIOSIS: A CASE STUDY FOR MICROBIAL EXPERIMENTAL EVOLUTION WITH THE VIBRIONACEAE

As mentioned previously, many members of the Vibrionaceae are able to form associations with eukaryotic hosts, including phytoplankton, protoctists, algae, aquatic fungi, invertebrates, fishes, and aquatic mammals, which may range from harmful, neutral, and beneficial to the host (Soto et al., 2010; Urbanczyk et al., 2011). One particular mutualistic interaction is the partnership between marine bioluminescent *Vibrio* and sepiolid squid. The sepiolid squid-*Vibrio* symbiosis has been a model system for studying developmental biology, immunology, physiology, and molecular biology underpinning interactions between bacteria and animals for over two decades (McFall-Ngai and Ruby, 1991), since both partners can easily be maintained in the laboratory independently of each other. Sepiolid and loliginid squids (**Figure 1A**) are colonized by bioluminescent *Vibrio* (Fidopiastis et al., 1998; Guerrero-Ferreira and Nishiguchi, 2007). The bioluminescent bacteria inhabit a morphological structure called the light organ (**Figure 1B**) within the squid mantle cavity and benefit from their association with the cephalopod host by inhabiting a microenvironment rich in nutrients relative to the oceanic water column. The squid hosts prosper from the presence of bioluminescent bacteria by utilizing the light produced for a cryptic behavior called counterillumination (Jones and Nishiguchi, 2004; **Figure 1C**). Female squid fertilized by males lay their eggs on solid substrates such as rocks, where the embryos develop. Since female sepiolid squids are sequential egg layers, they can produce several clutches over 4–5 months after sexual maturity, with each clutch being 50–500 eggs each (Moltschaniwskyj, 2004). Axenic squid hatchlings emerge from their eggs (usually at twilight or at night) with sterile light organs, which are colonized within a few hours by specific free-living bioluminescent *Vibrio* present in the ocean (Soto et al., 2012). The colonizing bacteria quickly reproduce to fill and occupy the light organ crypt spaces (i.e., lumina, **Figure 1D**). Daily at dawn 90–95% of the light organ symbionts are vented exteriorly to the ocean by the squid host prior to burying in the sand. The remaining bacterial fraction in the squid host re-grows throughout the day to reinstate a complete light organ population by sunset (Soto et al., 2012). At dusk the squid emerge from the sand to engage in their nocturnal activity, including foraging and mating. [More detailed and comprehensive information can be found in recent reviews (Dunn, 2012; Stabb and Visick, 2013)]. Since bioluminescent symbionts can be grown in pure culture, cryopreserved with possible subsequent resuscitation, genetically manipulated and analyzed, and used to inoculate recently hatched gnotobiotic squid juveniles, the sepiolid squid-*Vibrio* mutualism is a promising prospect for experimental evolution studies aiming to understand symbioses. The juvenile squids are born without their *Vibrio* symbionts, lending the ability to infect the juveniles with any strain of *Vibrio* bacteria to examine colonization rates, ability to colonize, and persistence. Additionally, these bacteria can be used in competition experiments, which allows one to test different wild type strains against one another, mutant strains against their original wild type strain or versus other mutants, and experimentally evolved strains against the original ancestor (Nishiguchi et al., 1998; Nishiguchi, 2000, 2002; Soto et al., 2012).

Nevertheless, this mutualism has only recently been tapped as a resource for microbial experimental evolution studies in recent years (Schuster et al., 2010; Soto et al., 2012, 2014). Early work has shown *V. fischeri* are able to adapt to a novel squid host within 400 generations (**Table 1**), and such evolution may create tradeoffs in the ancestral animal host environment or in the free-living phase as a physiological correlated response to an important abiotic factor (Soto et al., 2012). Two sepiolid squid genera, *Euprymna* and *Sepiola*, are in the same taxonomic family. Several different *Euprymna* species are distributed allopatrically throughout the Indo-West Pacific Ocean, while numerous *Sepiola* species simultaneously co-occur sympatrically in the Mediterranean Sea (Nishiguchi et al., 1998; Nishiguchi, 2000, 2002; Soto et al., 2009). *Vibrio* symbionts colonizing *Euprymna* are host specialists and outcompete allochthonous isolates, a phenomenon termed competitive dominance, while those colonizing *Sepiola* are host generalists. *Vibrio* symbionts display no competitive dominance within *Sepiola* (Nishiguchi et al., 1998; Nishiguchi, 2000, 2002; Wollenberg

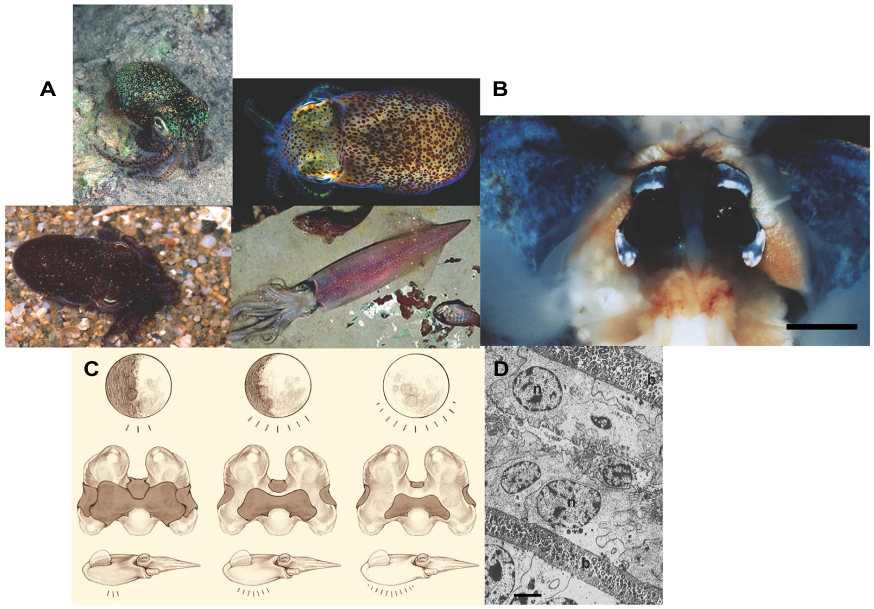


FIGURE 1 | The sepiolid squid-*Vibrio* symbiosis. (A) Representative species from the families Sepiolidae and Loliginidae (clockwise from upper left): *Euprymna tasmanica* (Sepiolidae), *E. scolopes* (Sepiolidae), *Photololigo noctiluca* (Loliginidae), and *Sepiola affinis* (Sepiolidae). **(B)** Ventral dissection of *E. scolopes*, showing the bilobed light organ surrounded by the ink sac. Bar = 0.5 cm. **(C)** Diagram how the light organ operates under different phases of the moon. The progressive decrease in shading from left to right symbolizes increased illumination by the light organ. **(D)** A transmission electron micrograph of an area of the epithelium-lined crypts containing symbiotic bacteria: (n) = nucleus of squid cell, (b) = bacteria in crypts (bar = 10 μm). Photo credits: Mark Norman, Mattias Oremstedt (Kahikai), M. K. Nishiguchi, R. Young, S. Nyholm, R. Long, M. Montgomery. Light organ illustration by Robert Long-Nearsight graphics.

Table 1 | Competitive colonization experiments between *Vibrio fischeri* strains ES114 (ancestor) and JRM200 (derived) at different evolutionary time points in the novel squid host *Euprymna tasmanica*.

Evolutionary time point (Generations)	Expected ES114:JRM200 (Percentage ancestor: percentage evolved)	Observed ES114:JRM200 (Percentage ancestor: percentage evolved)
0 (n = 33)	50:50	46:54
100 (n = 24)	50:50	47:53
200 (n = 24)	50:50	41:59
300 (n = 24)	50:50	41:59
400 (n = 24)	50:50	35:65[1]
500 (n = 24)	50:50	36:64[1]

[1] *Significantly different two-tailed t-test and sign test ($P < 0.05$, $\alpha = 0.05$).*

and Ruby, 2012). Despite the presence of competitive dominance, data from population genetics and phylogenetics suggested secondary colonization events have occurred (Nishiguchi and Nair, 2003; Jones et al., 2006; Urbanczyk et al., 2011), creating a puzzling conundrum for years. Population genetics surveys fueled this enigma by consistently observing high levels of genetic diversity within the squid light organ (Jones et al., 2006), indicating light organ populations are not dominated by single or few genotypes through space and evolutionary time, an observation not consistent with competitive dominance. Competitive dominance results from squid host specialization by the symbionts, which should presumably purge genetic diversity of *V. fischeri* populations inside light organs. Microbial experimental evolution shed light on these mysteries and helped resolve these paradoxes with a complementing temporal population genetics survey spanning a decade—about 20,000 *V. fischeri* generations of evolution within the squid host—revealed the same evolutionary forces begetting competitive dominance were also responsible for driving *V. fischeri* genetic and phenotypic diversity within the squid light organ (Soto et al., 2012, 2014). *V. fischeri* indigenous to the Hawaiian bobtail squid (*E. scolopes*) was serially transferred for 500 generations through the Australian dumpling squid (*E. tasmanica*), a novel host (Soto et al., 2012; **Figure 2**).

Results demonstrated as *V. fischeri* adapted to *E. tasmanica*, the ability of the derived lines to grow along a salinity gradient significantly changed relative to the ancestor. Moreover, no obvious pattern to the growth changes was evident across the salinity continuum, suggesting *V. fischeri* microbial physiology had been "randomized." Salinity is known to impact *Vibrio* population levels and distributions worldwide (Soto et al., 2009). *V. fischeri* subjected to novel host evolution created polymorphic reaction norms for salinity, an abiotic factor integral to shaping symbiont ecology during the free-living phase. Furthermore, experiments indicated a "superior numbers" or a "running start" advantage to foreign strains over native ones in animal host colonization that could outflank competitive dominance. Thus, *V. fischeri* strains most abundant (perhaps due to salinity) during the free-living phase where squid hosts resided were the ones most likely to colonize

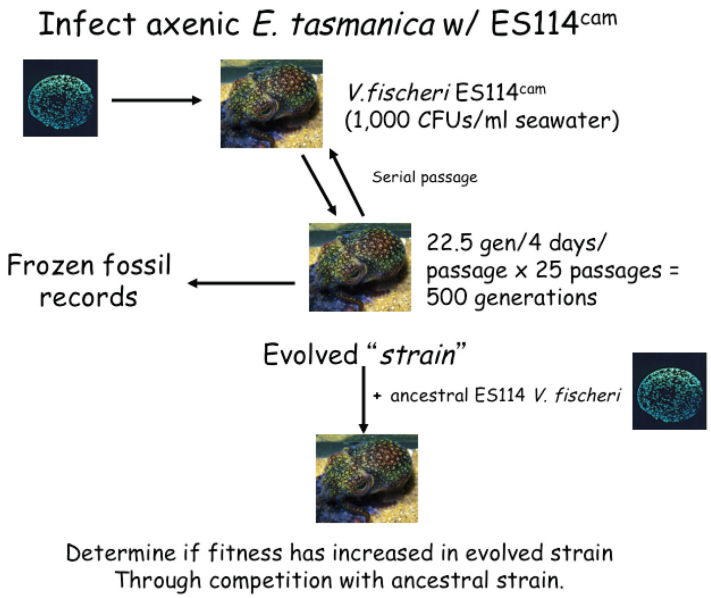


FIGURE 2 | Flow chart of experimental evolution studies in the Southern dumpling squid, *E. tasmanica*. *Vibrio fischeri* ES114^{CAM} is a isogenic strain of *V. fischeri* ES114 (native to the Hawaiian bobtail squid, *E. scolopes*), with a neutral chloramphenicol-resistance marker (CAM). This strain was passaged through a non-native host for 500 generations to determine whether fitness would improve over each generation. Throughout the study, frozen fossil records can be made to eventually compare ancestor to evolved clones. Similar experiments can be completed in *E. scolopes*, including control animals (Soto et al., 2012).

the cephalopod, not strains best adapted to the squid (Soto et al., 2012). A similar process may occur with *Photobacterium* in fish hosts due to temperature (Urbanczyk et al., 2011). Additionally, the *V. fischeri* lines serially passaged through *E. tasmanica* surged in biofilm formation and bioluminescence but lessened in motility (Soto et al., 2014). Increases and decreases in the utilization of select carbon sources also transpired. Interestingly, evolutionary differentiation occurred in the derived lines relative to the ancestor and to each other for biofilm formation, motility, bioluminescence, and carbon source metabolism, results consistent when compared to *V. fischeri* wild isolates obtained from light organs of *E. scolopes* and *E. tasmanica* specimens collected in the field (Soto et al., 2014). Squid host specialization by the symbionts promotes competitive dominance and diversifying selection. Perhaps clonal interference prevents selective sweeps in the squid light organ. The lineages serially transferred through *E. tasmanica* also exhibited decreased levels of bioluminescence in the ancestral host *E. scolopes* (Soto et al., 2012). In an independent study, *V. fischeri* strains previously incapable of establishing a persistent association (chronic infection) with sepiolid squids were shown to be capable of doing so after serial passage in *E. scolopes* (Schuster et al., 2010). Since *V. fischeri* possesses a life history where bacteria are cyclically associated with an animal host (sepiolid squids and monocentrid fishes) and then outside the host as free-living bacteria in the ocean, researchers can use microbial selection experiments with *V. fischeri* to simultaneously study symbiosis evolution and microbial evolution in the natural environment where microbes are not partnered to a host. [Some recent work suggests *V. fischeri* may also be a bioluminescent symbiont in the light organs of fishes belonging to the taxonomical families Moridae and Macrouridae (Urbanczyk et al., 2011).] Additionally, *V. fischeri* strains exist which are completely unable to colonize the light organs of sepiolid squids and monocentrid fishes, permitting evolutionary biologists to study a continuum of interactions between a microbe and animal host when studying the squid-*Vibrio* mutualism. Given the Sepiolidae is a diverse family of squids that include allopatric and sympatric species distributions, testing whether host speciation affects selection for host specialist versus host generalist evolutionary strategies within *Vibrio* symbionts is possible.

TYPE STRAIN MENTALITY AND OTHER BIOLUMINESCENT SYMBIONTS FOR SEPIOLID SQUIDS

Early work characterizing the molecular biology of *V. fischeri* colonizing *Euprymna* squid focused on the strain *V. fischeri* ES114 and the host *E. scolopes* (with occasional studies in *Sepiola*), since only the Hawaiian squid host was routinely available (McFall-Ngai and Ruby, 1991; Fidopiastis et al., 1998). Furthermore, reductionism was desired to understand the fundamentals of the symbiosis. Nonetheless, caution is warranted to avoid development of a "type strain" or "type host" mentality. Recent work has expanded to regularly include other strains of *V. fischeri* and *Euprymna* species (Ariyakumar and Nishiguchi, 2009; Chavez-Dozal et al., 2012; Soto et al., 2012). This will aid in identifying more general results from those that are specific to a particular symbiont strain or host species. In addition, initial characterization of the sepiolid squid-*Vibrio* symbiosis described *V. fischeri* as the only bioluminescent symbiont present in the squid light organ (McFall-Ngai and Ruby, 1991). Subsequently, *V. logei* was discovered as a symbiont in the genus *Sepiola* (Fidopiastis et al., 1998;

Nishiguchi, 2000). More recently, *V. harveyi* and *Photobacterium leiognathi* have been included as symbionts of *E. hyllebergi* and *E. albatrossae* from Thailand and the Philippines, respectively (Guerrero-Ferreira and Nishiguchi, 2007; Guerrero-Ferreira et al., 2013). An important prospect to consider is that *V. fischeri* and *V. logei* may have evolved fundamentally distinct and different traits for colonizing sepiolid squids, even when considering the same host species. Clearly, new and thrilling perspectives are surfacing around the sepiolid squid-*Vibrio* mutualism. Several species in the Vibrionaceae are bioluminescent. An interesting remaining question is why only a few of these form light organ symbioses with sepiolid squid hosts. For example, why is bioluminescent *V. orientalis* never found in squid light organs (Dunlap, 2009)? Are researchers simply not looking thoroughly enough?

BIOGEOGRAPHY OF *VIBRIO* BACTERIA AND EXPERIMENTAL EVOLUTION IN THE FIELD

Experimental evolution in the lab with *Vibrio* bacteria has only been completed in one species of *Vibrio* (*V. fischeri*), and strains used in those studies were either from the squid host *E. scolopes* (Hawaii) or pinecone fish *Monocentris japonicas* (Schuster et al., 2010; Soto et al., 2012). Given that a number of symbiotic *V. fischeri* from squid can colonize and survive in nearly all allopatric *Euprymna* hosts of the Indo-West Pacific, it provides a road map whether other *V. fischeri* strains can adapt to additional potential host species closely related to *Euprymna* (e.g., *Rondeletiola minor*) or even ones from a different phylum (Nishiguchi et al., 2004). Naturally occurring strains may be subjected to movement between hosts that are along a specific environmental gradient (Soto et al., 2010). Obviously, similar cues must be used for these bacteria to recognize a comparable, yet novel host, and then colonize and establish a persistent association in the outré animal for the symbionts to secure their distribution in the new host population (Wollenberg and Ruby, 2009). Only 6–12 *V. fischeri* cells are required to initiate a squid light organ infection. Once these bacteria colonize a squid host, they can reproduce much faster than in seawater. New *V. fischeri* clones encountering a squid host species for the first time will then be expelled every 24 h, increasing the cell numbers of *V. fischeri* new arrivals that can infect even more juvenile squid of the exotic host species (Lee and Ruby, 1994b, 1995). Whether these symbiont founder flushes truly occur in nature is not known, but observations in the laboratory have shown that alien *V. fischeri* genotypes can invade and take root where a preexisting genetic variety was already entrenched (Lee and Ruby, 1994a; Soto et al., 2012). Whether this commonly leads to a dominant symbiont genotype in a host population in a given geographical area over the long term must be investigated more closely.

TWO-CHROMOSOME GENOMIC ARCHITECTURE IN VIBRIONACEAE, EVOLVABILITY, AND VERSATILITY

Research has shown an absence of parallel coevolution between *V. fischeri* symbionts and their light organ animal hosts, which implies significant host switching has occurred (Nishiguchi and Nair, 2003). Host switching has been a common evolutionary phenomenon for *Vibrio* and *Photobacterium* species involved in symbioses, regardless of whether the interaction was commensalism, pathogenesis, or mutualism (Urbanczyk et al., 2011). Extensive host switching could suggest this microbe, along with *Vibrio* species in general, are evolutionarily plastic and malleable organisms. Vibrionaceae possess two circular chromosomes, one large (Chromosome I) and one small (Chromosome II; Tagomori et al., 2002). With this complex genome arrangement, *V. fischeri*'s ability to exploit numerous lifestyles is easy to understand, as the *Vibrio* genome structure is dynamically unstable (Kolsto, 1999). The modular two-chromosome architectural structure of Vibrionaceae genomes has been hypothesized to be the inception for the versatility and ubiquity of this cosmopolitan bacterial family, with ecological specialization being the essence of the smaller and more genetically diverse Chromosome II with its superintegron island gene-capture system and genes encoding for solute transport and chemotaxis (Heidelberg et al., 2000; Ruby et al., 2005; Grimes et al., 2009). Intrachromosomal and interchromosomal recombination is clearly present, along with inversions, indels, and rearrangements (Kolsto, 1999; Heidelberg et al., 2000; Tagomori et al., 2002). Such genomic architecture permits the evolutionary potential for functional genetic specialization to occur among the two chromosomes (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000), promoting ecological opportunity in adapting and radiating into numerous niches (Soto et al., 2014). For example, *V. cholerae* and *V. parahaemolyticus* genomic studies have discovered that house-keeping genes (DNA replication, transcription, translation, cell division, and cell wall synthesis) and pathogenicity are mainly restricted to the large chromosome (Heidelberg et al., 2000).

Chromosome II appears to be a genetic module for DNA and a source for innovation, perhaps evolutionarily functioning analogous to plasmids, possessing significantly more foreign loci that appear to have been acquired horizontally from other microbial taxa (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000). The presence of a gene capture system (i.e., integron island) and genes usually found on plasmids support this claim (Heidelberg et al., 2000). Furthermore, loci involved in substrate transport, energy metabolism, two-component signal transduction, and DNA repair are prominently carried on Chromosome II (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000). The loci involved in substrate transport consist of a large repertoire of proteins with diverse substrate specificity. Genes that subdivide cellular functions and that are intermediaries of metabolic pathways also are found on Chromosome II. These genetic auxiliaries potentially serve as the raw material for adaptation and specialization (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000). The structure and size of the large chromosome appears relatively constant throughout the Vibrionaceae, whereas Chromosome II is more acquiescent and flexible to genetic reorganization, rearrangement, recombination, and large indel events (Okada et al., 2005). Genes encoding function for starvation survival and quorum sensing are located on both chromosomes. Thus, interchromosomal functional regulation is present in Vibrionaceae. As a result, specific and novel mechanisms involved in the regulation, replication,

and segregation of both chromosomes are thought to have evolved in this bacterial family (Waldor and RayChaudhuri, 2000; Egan et al., 2005).

Interestingly, *V. cholerae* colonization factors (e.g., genes responsible for pili formation) primarily reside on Chromosome I. Consequently, different *V. fischeri* ecotypes could be the result of evolution at loci involved in metabolism as opposed to those involved in tissue colonization (Browne-Silva and Nishiguchi, 2007; Soto et al., 2014). Experimental evolution studies with *E. coli* have demonstrated that resource partitioning and alternative substrate specialization is sufficient for ecological polymorphisms to arise in prokaryotes (Rosenzweig et al., 1994). In summary, the two-chromosome architecture provides *V. fischeri* with enormous evolutionary fluidity. Particularly, Chromosome II may possess ecological or symbiosis islands which could account for this microorganism's broad ecological range (Tagomori et al., 2002). For example, differences in the pathogenicity islands present on Chromosome II appear to determine whether or not *V. parahaemolyticus* strains are pathogenic. Similarly, the pliant nature of *V. fischeri* could explain why there is extensive host switching. Chromosome II may well be a gene repository outfitted to respond to environmental change, habitat heterogeneity through space and time, and stress (Dryselius et al., 2007; Soto et al., 2012). Future studies will be thrilling and exciting, as modern bioinformatics and genomics offer high hopes and allow unprecedented visions. Recent advances in high throughput sequencing technologies and genome editing techniques (e.g., MuGENT) will greatly increase the potential of experimental evolution to understand adaptation (MacLean et al., 2009; Dalia et al., 2014).

TOPICS FOR FUTURE STUDY

BIOFILM FORMATION AND MOTILITY

Motility and biofilms are modes by which *V. fischeri* strains can niche specialize in their *Euprymna* squid hosts (Yildiz and Visick, 2009; Soto et al., 2014). Biofilms are aggregates of microorganisms attached to a surface that are frequently enmeshed within a matrix of exopolysaccharide and can be comprised of a pure culture population or a community (Davey and O'toole, 2000; Stoodley et al., 2002). This community is much more resistant to antimicrobials, ultraviolet light, pH shifts, osmotic shock, desiccation, and other environmental stresses (Gilbert et al., 1997; Davey and O'toole, 2000). The role of biofilms in disease and host colonization is well documented, where bacterial pathogens establishing biofilms in animals may be more recalcitrant to phagocytosis by host macrophages, resistant to respiratory bursts by immune cells, and insensitive to antimicrobials produced by host defenses (Davey and O'toole, 2000). In addition, biofilm development has a major role in *V. fischeri* colonization of sepiolid squid hosts (Chavez-Dozal and Nishiguchi, 2011; Chavez-Dozal et al., 2012; **Figure 3**). When movement on surfaces is necessary, swarming with flagella is the motility mechanism for Vibrionaceae (McCarter, 2001). Swarming is specialized mobilization or locomotion on a surface as opposed to the swimming and tumbling done by individual cells. As *V. fischeri* swarms with concurrent cell division (e.g., growth), cells differentiate from a vegetative state to a swarmer one. Swarmer cells are hyperflagellated and longer than vegetative counterparts (Harshey, 2003), and provide a steady state supply of nutrients until motility ceases. Motility plays an integral role in the colonization of sepiolid squid by *V. fischeri* and allows host-associated bacteria to reach the destination and surface desired for further colonization or attachment (Millikan and Ruby, 2004). Since swarming is an energetically expensive process, chemotaxis has a role mediating how a bacterial cell should physiologically respond. Through

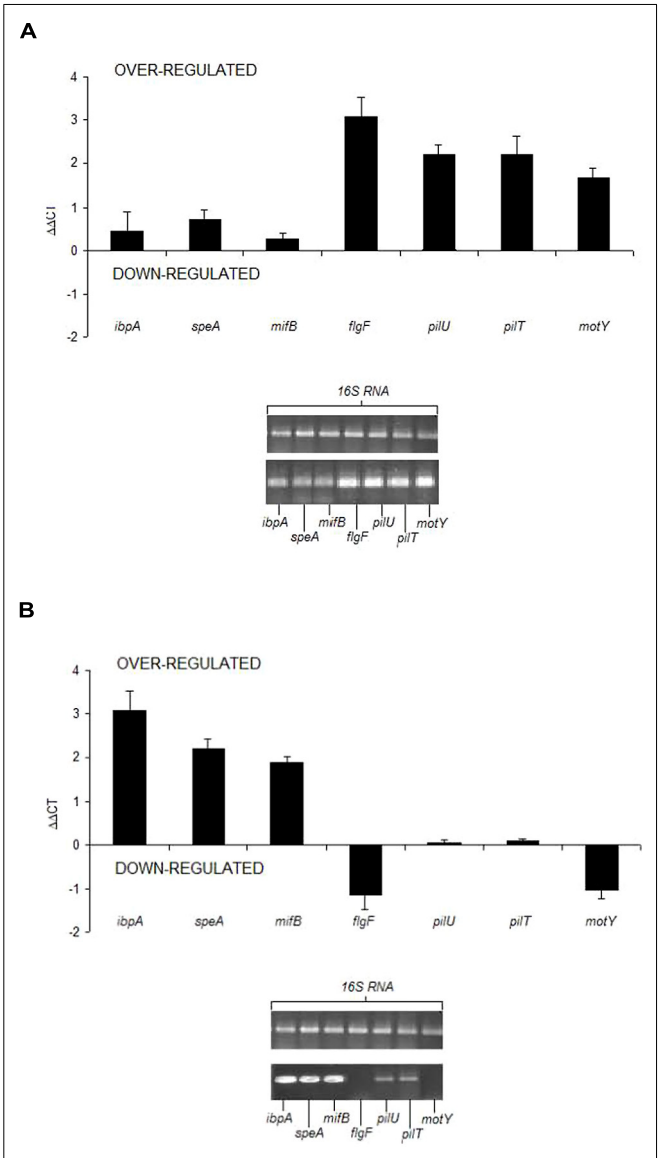


FIGURE 3 | Early and late gene expression (mRNA) of various biofilm related loci in *V. fischeri* ETJB1H. (A) Early (4 h) gene expression of flagellum biosynthesis (*flgF*), type IV pili formation and adhesion (*pilU, pilT*), and the sodium-type flagellar motor pump for motility (*motY*) loci. **(B)** Late gene expression of genes important for mature biofilm production (24 h) include expression of heat shock protein (*ibpA*), magnesium-dependent induction for c-di-GMP synthesis (*mifB*), and arginine decarboxylase (*speA*). Modified from Chavez-Dozal et al. (2012). dCCT is the change in relative expression of each gene compared to the standard control (in this case 16S rRNA). The gel represents the amount of 16S rRNA (top) and the amount of mRNA expressed in each gene examined. Error bars represent SD of three replicates.

years of studying diverse bacteria as motility model systems, research has shown many regulatory pathways controlling motility also affect biofilm formation (Harshey, 2003; Verstraeten et al., 2008). Bacterial populations must resolve whether to institute motile machinery for expedient colonization of surfaces or engage biofilm systems when an appropriate location for initial contact and attachment has been found, a critical choice affecting survival between competitors. Experiments are underway where *V. fischeri* lines are being selected for increased biofilm formation and motility. Accompanying these experiments are ones where *V. fischeri* lines are being alternately or cyclically selected for biofilm and motility lifestyles (oscillatory selection). The relative abilities of these lines to colonize squid hosts will be assessed.

PARASITISM, PREDATION, AND GRAZING ON *VIBRIO* BACTERIA

Substantial work exists on how protoctistan predators are effective grazers on *Vibrio* or other bacteria, particularly when they form biofilms (Matz and Kjelleberg, 2005; Matz et al., 2008). Previous research has demonstrated that certain species of *Vibrio* (e.g., *V. cholerae* and *V. fischeri*) are better able to ward off microbial eukaryotic predators when in their biofilm state compared to their planktonic counterparts (Erken et al., 2011). Earlier work provides strong evidence that when *Vibrio* biofilms are grazed by protoctistans, the bacteria release toxic compounds capable of killing the predators, the dead grazers themselves then become a meal and carbon source for the *Vibrio* (Chavez-Dozal et al., 2013). Depending on the species, and even strain type, *Vibrio* biofilms make an excellent model to determine if grazing can affect biofilm growth, structure, and production of chemicals to inhibit grazers (Barker and Brown, 1994). Current microbial selection studies are ongoing to examine adaptive responses of *V. fischeri* to various grazers and how these evolutionary outcomes impact sepiolid squid colonization. Bacteriophage, predatory bacteria (e.g., *Bdellovibrio*, *Bacteriovorax*, *Micavibrio*, and "wolfpack" feeders such as myxobacteria), and aquatic fungi also prey on the Vibrionaceae (Atlas and Bartha, 1998; Richards et al., 2012). How these natural enemies affect *V. fischeri* evolution and the sepiolid squid-*Vibrio* symbiosis are worthy of future investigations. For instance, *Vibrio* chitinases attacking fungal cell walls may be a means to avoid grazing by marine yeast. Chitinases are known to be utilized by *V. fischeri* symbionts when interacting with the squid host (Wier et al., 2010).

EVOLUTION DURING THE FREE-LIVING PHASE, ABIOTIC FACTORS, AND BACTERIAL STRESS RESPONSES

Prior work has shown that *V. fischeri* host adaptation to sepiolid squids and monocentrid fishes affects this species' ability to grow within a gradient of an abiotic factor (e.g., tolerance limits to environmental stress) while in the free-living or planktonic phase (Soto et al., 2009, 2012), implicating that natural selection could be acting on the bacterial stress responses to better accommodate the symbiont against the unprecedented stressful environments presented by a new animal host (e.g., novel immune defenses; Soto et al., 2010). The coupling of different bacterial stress responses to one another and their correlation to successful symbiosis initiation, host immunity evasion, pathogenesis, and virulence mechanisms is becoming necessary for understanding bacterial evolution (Nishiguchi et al., 2008). Future experimental evolution work will focus on the adaptability of *V. fischeri* to abiotic factor stresses, such as high and low tolerance limits of salinity, temperature, and pH while in the free-living or planktonic phase. In turn, correlated responses of *V. fischeri* adapting to these environmental stresses will be investigated in sepiolid squid hosts (Abucayon et al., 2014). Understanding how *V. fischeri* stress evolution affects its relationship with sepiolid squids will lead to new insights in the dynamic evolutionary forces that shape associations between hosts and symbionts. Because both free-living and host environments impose dramatically different selection pressures to microorganisms (e.g., evasion of immune host defenses), these perspectives have implications into infectious disease and virulence mechanisms, as genetic and physiological components responsible for mutualisms and pathogenesis are frequently identical or homologous (Ruby et al., 2005; Nishiguchi et al., 2008; Buckling et al., 2009). Stress evolution and stress-induced mutagenesis are known to be capable of creating cryptic genetic variation through varying gene-by-gene and gene-by-environment interactions which can be invisible to natural selection during the original circumstances in which they materialize but either beneficial or detrimental to bacterial fitness when conditions change (Tenaillon et al., 2004; McGuigan and Sgro, 2009; Paaby and Rockman, 2014). The evolutionary significance of cryptic genetic variation in patterning interactions between animal hosts and bacteria is unclear. *V. fischeri* adapting to a novel squid host was found to increase this symbiont's ability to form biofilms in artificial seawater containing no organic carbon while in the free-living phase. This result suggests symbiosis evolution can affect *V. fischeri*'s ability to tolerate starvation or oligotrophic conditions when subsequently outside the host (Soto et al., 2014). *V. fischeri* adapting to selective pressures imposed by abiotic factors or environmental stressors during the free-living phase may either reinforce or decouple coevolution between *Vibrio* symbionts and their animal hosts (Soto et al., 2009, 2012). A static microcosm or standing culture of *Pseudomonas fluorescens* where wrinkly spreader, fuzzy spreader, and smooth morph colonies arise over several days has become a model system for studying microbial adaptive radiation, a process known to be affected by oxygen depletion and nutrient availability (Rainey and Travisano, 1998; Travisano and Rainey, 2000). Alterations in *Vibrio* colony morphology is known to affect animal host colonization (Mandel et al., 2009). *V. fischeri* adaptive radiation during the free-living phase and the subsequent consequences on symbiosis are poorly understood. The use of microbial experimental evolution with heterogeneous environments will provide insight into how *V. fischeri* biodiversity (e.g., Shannon-Wiener Index) in the free-living phase affects symbiont population variation within the squid light organ across gradients of various abiotic factors.

METABOLISM

Biolog plates were developed for global phenotype analysis of microorganisms that allows a comprehensive survey of microbial physiological traits (Bochner, 1989; Bochner et al., 2001). The aim is to identify unique characteristics of individual microbes

and common metabolism to particular taxa or ecological populations. These plates also provide functional data to complement genetic analyses and gene expression studies of microbes. For instance, mutants can be screened efficiently to compare phenotypic consequences relative to wild type. This is especially important for examining metabolic polymorphisms, physiological heterogeneity, and distinguishing between different ecotypes within the same bacterial species, since different substrates can be shunted into alternate biochemical pathways (Rosenzweig et al., 1994; White, 2007). Additionally, how metabolism of the same substrate (i.e., D-glucose) is disproportionately distributed among numerous biochemical pathways (glycolysis versus pentose phosphate pathway) may also vary among different individual cells of the same bacterial species (Rosenzweig et al., 1994), as hypothesized by the nano-niche model of bacterial evolution (Wiedenbeck and Cohan, 2011). For example, most members of an *E. coli* population may move the carbon flow from the breakdown of D-glucose via glycolysis, but a small proportion of the remaining population may shuttle more intermediates of D-glucose degradation through Entner-Doudoroff pathway for an alternate way of making a living (e.g., physiological tradeoffs, resource partitioning, and ecological nutrient specialization by differentiation in usage of metabolic pathways; Rosenzweig et al., 1994; Cooper and Lenski, 2000; Travisano and Rainey, 2000; MacLean et al., 2004; White, 2007). Within the lifetime of just one adult squid host, a single *V. fischeri* clone has ample time to evolve cross-feeding with either other *V. fischeri* cells or host cells, since this has been documented in *E. coli* in less than 800 generations in a homogenous and unstructured environment (Rosenzweig et al., 1994). *V. fischeri* adapting to novel animal hosts undergo ecological diversification in carbon source utilization within 500 generations (Soto et al., 2014). With the use of Biolog plates, microbial experimental evolution can provide keen insight in the role of metabolism in *V. fischeri* ecological diversification and sepiolid squid colonization (MacLean and Bell, 2002).

CHEMOTAXIS

Support exists biofilms, motility, carbon metabolism, and bioluminescence are entwined or interlaced with one another. Possible crossroads for their roles in *V. fischeri* include chemotaxis, intracellular second messengers (c-di-GMP), and bacterial stress responses. Methyl-accepting chemotaxis proteins (MCPs) are central for chemotaxis, as these proteins are chemoreceptors that monitor the chemical composition of the environment and transmit this information interiorly to the cell (Bren and Eisenbach, 2000; Brennan et al., 2013). MCPs are versatile receptors to chemical stimuli, adept at mediating taxis to diverse signals (Hsing and Canale-Parola, 1996). A single MCP is incredibly sensitive. It is able to discern differences in stereochemistry between isomers, sense relative asymmetries in chemical concentrations of a substance along a gradient, and integrate diverse information of multiple chemical stimuli present in the environment simultaneously (Hsing and Canale-Parola, 1996; Bren and Eisenbach, 2000). An MCP is capable of a graded, measured, and progressive selective response to chemical stimuli. MCP function is further elaborated by being present on bacterial cell membranes as a mass complex of several interacting MCPs bundled together into a chemo-antenna cluster network, amplifying the synergistic interactions possible in chemotaxis and signal transduction (Bren and Eisenbach, 2000). Additionally, single amino acid substitutions can have colossal effects in sensitivity, affinity, specificity, and function of an MCP (Derr et al., 2006). Hence, MCPs and redistributable metabolism may allow *V. fischeri* populations to better colonize novel hosts by resculpting its N-dimensional niche hypervolume space quickly (Hutchinson, 1957). In a study using comparative genomics and a network biology-based approach to understand how genes select for multigenic phenotypes such as virulence in *V. cholerae*, loci encoding MCPs and others associated with chemotaxis were among those identified as most responsible (Gu et al., 2009). MCPs couple chemotaxis to diverse metabolites and their gradients, supplying one potential route a symbiont can adapt to unaccustomed host physiology. Experimental evolution with microorganisms to analyze chemotaxis can be completed by placing small volumes of bacteria onto the centers of motility agar plates with different chemoattractants at the periphery. Over an incubation time at an appropriate temperature, cells from the leading edge closest to the chemoattractant are serially transferred onto the centers of new motility plates (DeLoney-Marino et al., 2003). Derivations of this method can be used to select for bacteria with increased aversion to chemorepellents. Another avenue is to use a rendition of the glass capillary tube chemotaxis assay that involves continuous subculturing (Adler, 1973).

QUORUM SENSING, BIOLUMINESCENCE, SOCIAL EVOLUTION, AND ECOLOGICAL INTERACTIONS

Quorum sensing was first described in *V. fischeri* in 1970 in connection with bioluminescence (Nealson et al., 1970). Since then, quorum sensing is now known to govern many more traits other than bioluminescence, including but not limited to exoenzyme secretion, siderophore production, antibiotic synthesis, cell division, DNA replication, cell surface anabolism (cell wall, cell envelope, and capsule), biofilm development, and motility (Miller and Bassler, 2001). Bioluminescence is frequently used as a proxy quorum sensing measurement. Regulation of the *lux* operon involves input from the quorum sensing apparatus that couples to other microbial physiological pathways and cascades (Miyashiro and Ruby, 2012). Clever designs can permit microbial selection experiments that investigate quorum sensing and bioluminescence. In a plate selection scheme, ImageJ (image processing freeware produced by National Institutes of Health) may be used to single out brighter and dimmer colonies on agar plates for serial transfers that have been digitally imaged in lit and dark rooms ("digital replica plating" or "replica imaging"). The Vibrionaceae possess a hierarchical and sophisticated quorum sensing machinery comprised of "low cell density" (LCD) and "high cell density" (HCD) gene expressions (Camara et al., 2002). Microbial selection experiments with *V. fischeri* mutants locked or defaulted into LCD and HCD gene expressions will permit studies into group selection, kin selection, social evolution, and greenbeard genes (Travisano and Velicer, 2004). LCD and HCD gene expressions can each secrete a different and distinct subset of public goods not produced by the other (e.g., extracellular nuclease and metalloprotease for LCD and HCD, respectively; Blokesch and Schoolnik, 2008; Natrah et al., 2011; Bruger and Waters, 2014).

Experimentally evolved lines possessing constitutive HCD and LCD gene expressions would be compared to the quorum sensing wild type strain (ancestral or derived) for a particular selection regimen. LCD lines could serve as "cheaters" or "defectors" for a public good produced by HCD or wild type lines at high cell density (e.g., extracellular metalloproteases). An investigator could ask if an LCD cheater line initially at low frequency could invade an HCD line (at low or high cell density) or a quorum sensing wild type line at high cell density. HCD lines could analogously serve as cheaters for extracellular nuclease. The ability to control microbial growth and dilution rates with chemostats using select media might also be another way. The use of quorum sensing enhancers and quorum quenching molecules or drugs are additional avenues for future experiments (Rasmussen and Givskov, 2006; Defoirdt et al., 2008). Serial transfers of liquid cultures performed at particular cell densities (specific transmission bottlenecks) or with spent (conditioned) media may permit inquiries into quorum sensing.

Other possible ecological interactions between microbes include competition (interference and exploitation) and microbial allelopathy (e.g., chemical warfare; Atlas and Bartha, 1998). Additionally, one must recognize that one microbe may be more fit than another because of increased efficiency in resource utilization or better able to convert assimilatory carbon and reducing power into more offspring (i.e., a shorter generation time growing on D-glucose). Yet an interesting facilitation is cross-feeding. Cross-feeding can also occur between cells of different strains or species, where one cell type secretes a waste product that is utilized by another as a nutrient or useful resource. Understanding the diversity of social dynamics is valuable. Within the social evolution context, when a participant (the actor) benefits from harming another (recipient), the interaction is termed selfishness (West et al., 2006). When the actor suffers a negative effect by harming the recipient, the interaction is called spite. Altruism occurs when the recipient benefits and the actor is harmed, but mutualism takes place with both partners benefiting. Commensalism occurs if the actor benefits and the recipient experiences no effect. In amensalism, the actor is unaffected but the recipient is disserviced (Atlas and Bartha, 1998). (Predation was addressed previously.) As alluded to earlier, an initial effort to characterize the assortment of social interactions between bacteria can be done by placing washed cells in the filter-sterilized spent media of a competitor. NMR and mass spectroscopy can possibly be used to identify any interesting molecular components that can be isolated or purified. Excellent questions linger. What are the roles of cooperation, cheating, competition for limiting resources, microbial allelopathy, and other ecological interactions in shaping the squid-*Vibrio* symbiosis? At what stages do each of these processes most predominate (e.g., free-living versus host associated)? Is cheating among symbionts suppressed by the squid host when *V. fischeri* are in the light organ? Are bacteriocins produced by *V. fischeri* strains (i.e., vibriocins) against other conspecific subtypes in the squid light organ?

VIABLE BUT NON-CULTURABLE STATE

The viable but non-culturable (VBNC) state is a phenomenon frequently observed in the Vibrionaceae and other prokaryotes, including *V. fischeri* (Lee and Ruby, 1995). Bacteria normally culturable no longer grow in liquid culture or on agar media, because the cells enter a dormancy where still metabolically active and presumed to have elevated tolerance or resistance to environmental stressors (extreme conditions of an abiotic factor such as temperature or salinity), harmful compounds or noxious chemicals, starvation, and heavy metal toxicity (Ordax et al., 2006; Nowakowska and Oliver, 2013). Escape from digestion after phagocytosis or endocytosis by ameba and macrophages has also been hypothesized to be another function of the VBNC condition, permitting these eukaryotic cells to serve as reservoirs for survival and dispersal (Rahman et al., 2008). Published research has reported molecules and mechanisms (e.g., temperature upshift) that appear to restore culturability to VBNC cells upon their return to liquid media or agar plates. This putative revival of VBNC dormancy has been termed "resuscitation." However, many researchers doubt the existence of a VBNC state and its resuscitation, claiming the supporting evidence is lacking or marginal at best (Bogosian and Bourneuf, 2001). Skepticism arises because resuscitation is thought to be re-growth of injured cells that have regained their health. Disbelievers point out genes responsible for a pathway or developmental program leading to a physiologically differentiated VBNC state have been slow to identify through the use of null mutations and knockout studies (Soto et al., 2010). Nothing analogous to endospore formation has surfaced. Definitive evidence of VBNC cells will require loss-of-function experiments with subsequent complementation or overexpression gain-of-function studies to describe a "VBNC" regulon or modulon (Bogosian and Bourneuf, 2001). Microbial experimental evolution is a remarkable approach to addressing the validity of VBNC cells. After 24–48 h of growth in nutrient rich media (28°C, 200–225 rpm), most of a *V. fischeri* liquid culture is non-culturable, if not entirely dead, as the plating efficiency rapidly decreases. (Static liquid cultures do not experience this phenomenon and can remain culturable for weeks). The exact result is strain dependent, as some strains are more susceptible than others in their failure to re-grow upon subculturing to fresh media or transfer to agar plates. By serially transferring what few *V. fischeri* cells continue to grow from shaking and aging liquid cultures undergoing a decay in culturability, a population can be increasingly selected for resistance to non-culturability.

CONCLUSION

Bioinformatics will provide additional insight into experimental evolution with the Vibrionaceae, including genomics, transcriptomics, proteomics, and metabolomics. For microorganisms such as *V. fischeri*, which cycle between host-associated and free-living phases, consideration of the operating selection pressures unique to each environment, relative magnitudes, and respective contributions in driving microbial evolution merits consideration (Nyholm and Nishiguchi, 2008). Since prokaryotes possess tremendous genetic and metabolic diversity, understanding the factors that shape bacterial biogeography and ecology will provide insights into bacterial adaptation and natural history.

ACKNOWLEDGMENTS

The authors would like to thank helpful discussions with members of the Nishiguchi lab and the *Euprymna–Vibrio* community

of researchers. This work was supported by NIH NIAID 1SC1AI081659, NIH NIAIDS 3SC1AI081659-02S1, and NSF IOS 074498 to Michele K. Nishiguchi. We would also like to thank Michigan State University – BEACON Center for the Study of Evolution in Action for support.

REFERENCES

Abd, H., Saeed, A., Weintraub, A., Nair, G. B., and Sandstrom, G. (2007). Vibrio cholerae O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. *FEMS Microbiol. Ecol.* 60, 33–39. doi: 10.1111/j.1574-6941.2006.00254.x

Abucayon, E., Ke, N., Cornut, R., Patelunas, A., Miller, D., Nishiguch, M. K., et al. (2014). Investigating catalase activity through hydrogen peroxide decomposition by bacterial biofilms in real time using scanning electrochemical microscopy. *Anal. Chem.* 86, 498–505. doi: 10.1021/ac402475m

Adler, J. (1973). A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *J. Gen. Microbiol.* 74, 77–91. doi: 10.1099/00221287-74-1-77

Ariyakumar, D. S., and Nishiguchi, M. K. (2009). Characterization of two host-specific genes, mannose-sensitive hemagglutinin (mshA) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. *FEMS Microbiol. Lett.* 299, 65–73. doi: 10.1111/j.1574-6968.2009.01732.x

Atlas, R. M., and Bartha, R. (1998). *Microbial Ecology: Fundamentals and Applications*. New York: Benjamin Cummings USA.

Austin, B. (2006). "Miscellaneous animal pathogens," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Barker, J., and Brown, M. R. W. (1994). Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. *Microbiology*140, 1253–1159. doi: 10.1099/00221287-140-6-1253

Barrick, J. E., and Lenski, R. E. (2013). Genome dynamics during experimental evolution. *Nat. Rev. Genet.* 14, 827–839. doi: 10.1038/nrg3564

Bartlett, D. H. (2006). "Extremophilic vibrionaceae," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press), 156–171. doi: 10.1128/9781555815714.ch11

Baselski, V. S., Upchurch, S., and Parker, C. D. (1978). Isolation and phenotypic characterization of virulence-deficient mutants of *Vibrio cholerae*. *Infect. Immun.* 22, 181–188.

Bennett, A. F. (2002). "Experimental evolution: an overview," in *Encyclopedia of Evolution*, ed. M. Pagel (New York: Oxford University Press), 339–342.

Bhaskaran, K., and Sinha, V. B. (1967). Attenuation of virluence in *V. cholerae*. *J. Hyg.* 65, 135–148. doi: 10.1017/S0022172400045642

Blokesch, M., and Schoolnik, G. K. (2008). The extracellular nuclease dns and its role in natural transformation of *Vibrio cholerae*. *J. Bacteriol.* 190, 7232–7240. doi: 10.1128/JB.00959-08

Bochner, B. R. (1989). Sleuthing out bacterial identities. *Nature* 339, 157–158. doi: 10.1038/339157a0

Bochner, B. R., Gadzinski, P., and Panomitros, E. (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res.* 11, 1246–1255. doi: 10.1101/gr.186501

Bogosian, G., and Bourneuf, E. V. (2001). A matter of bacterial life and death. *Eur. Mol. Biol. Organ. Rep.* 2, 770–774. doi: 10.1093/embo-reports/kve182

Bren, A., and Eisenbach, M. (2000). How signals are heard during bacterial chemotaxis: protein-protein interactions in sensory signal propagation. *J. Bacteriol.* 182, 6865–6873. doi: 10.1128/JB.182.24.6865-6873.2000

Brennan, C. A., DeLoney-Marino, C. R., and Mandel, M. J. (2013). Chemoreceptor VfcA mediates amino acid chemotaxis in *Vibrio fischeri*. *Appl. Environ. Microbiol.* 79, 1889–1896. doi: 10.1128/AEM.03794-12

Browne-Silva, J., and Nishiguchi, M. K. (2007). Gene sequence of the pil operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int. J. Syst. Evol. Microbiol.* 58, 1292–1299. doi: 10.1099/ijs.0.65370-0

Bruger, E., and Waters, C. M. (2014). *Quorum Sensing Inhibits the Breakdown of Cooperative Behavior by Social Cheaters in Vibrio bacteria. Evolution (N. Y.) 2014.* Raleigh: Society for the Study of Evolution.

Buckling, A., Maclean, R. C., Brockhurst, M. A., and Colegrave, N. (2009). The beagle in a bottle. *Nature* 457, 824–829. doi: 10.1038/nature07892

Camara, M., Hardman, A., Williams, P., and Milton, D. (2002). Quorum sensing in *Vibrio cholerae*. *Nat. Genet.* 32, 217–218. doi: 10.1038/ng1002-217

Cameron, D. E., and Mekalanos, J. J. (2011). "Live attenuated cholera vaccines: flagella and reactogenicity," in *Replicating Vaccines: A New Generation*, eds P. R. Dormitzer, C. W. Mandl, and R. Rappuoli (New York, NY: Springer).

Chavez-Dozal, A. A., Gorman, C., Erken, M., Steinberg, P. D., McDougald, D., and Nishiguchi, M. K. (2013). Predation response of *Vibrio fischeri* biofilms to bacterivorus protists/phagotrophic protozoa. *Appl. Environ. Microbiol.* 79, 553–558. doi: 10.1128/AEM.02710-12

Chavez-Dozal, A. A., Hogan, D., Gorman, C., Quintanal-Villalonga, A., and Nishiguchi, M. K. (2012). Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. *FEMS Microbiol. Lett.* 81, 562–573. doi: 10.1111/j.1574-6941.2012.01386.x

Chavez-Dozal, A. A., and Nishiguchi, M. K. (2011). Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri*. *J. Basic Microbiol.* 51, 452–458. doi: 10.1002/jobm.201000426

Colwell, R. R. (2006). "A global and historical perspective of the genus *Vibrio*," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Conrad, T. M., Lewis, N. E., and Palsson, B. O. (2011). Microbial laboratory evolution in the era of genome-scale science. *Mol. Syst. Biol.* 7, 509–519. doi: 10.1038/msb.2011.42

Cooper, V. S., and Lenski, R. E. (2000). The population genetics of ecological specialization in evolving *Escherichia coli* populations. *Nature* 407, 736–739. doi: 10.1038/35037572

Criminger, J. D., Hazen, T. H., Sobecky, P. A., and Lovell, C. R. (2007). Nitrogen fixation by *Vibrio parahaemolyticus* and its implications for a new ecological niche. *Appl. Environ. Microbiol.* 73, 5959–5961. doi: 10.1128/AEM.00981-07

Crosa, J. H., Actis, L. A., and Tolmasky, M. E. (2006). "The biology and pathogenicity of *Vibrio anguillarum* and *Vibrio ordalii*," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Dalia, A. B., McDonough, E., and Camilli, A. (2014). Multiplex genome editing by natural transformation. *Proc. Natl. Acad. Sci. U.S.A.* 111, 8937–8942. doi: 10.1073/pnas.1406478111

Davey, M. E., and O'toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* 64, 847–867. doi: 10.1128/MMBR.64.4.847-867.2000

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., and Bossier, P. (2008). Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from in vivo work. *Int. Soc. Microbial Ecol. J.* 2, 19–26. doi: 10.1038/ismej.2007.92

DeLoney-Marino, C. R., Wolfe, A. J., and Visick, K. L. (2003). Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and N-acetylneuraminic acid, a component of squid light-organ mucus. *Appl. Environ. Microbiol.* 69, 7527–7530. doi: 10.1128/AEM.69.12.7527-7530.2003

Derr, P., Boder, E., and Goulian, M. (2006). Changing the specificity of a bacterial chemoreceptor. *J. Mol. Biol.* 355, 923–932. doi: 10.1016/j.jmb.2005.11.025

Dryselius, R., Kurokawa, K., and Iida, T. (2007). Vibrionaceae, a versatile bacterial family with evolutionarily conserved variability. *Res. Microbiol.* 158, 479–486. doi: 10.1016/j.resmic.2007.04.007

Dunlap, P. V. (2009). "Microbial bioluminescence," in *Encyclopedia of Microbiology*, ed. M. Schaechter (Amsterdam: Elsevier), 45–61. doi: 10.1016/B978-012373944-5.00066-3

Dunn, A. K. (2012). "*Vibrio fischeri* metabolism: symbiosis and beyond," in *Advances in Microbial Physiology: Bacterial Respiratory Physiology*, ed. R. K. Poole (London: Academic Press).

Egan, E. S., Fogel, M. A., and Waldor, M. K. (2005). Divided genomes: negotiating the cell cycle in prokaryotes with multiple chromosomes. *Mol. Microbiol.* 56, 1129–1138. doi: 10.1111/j.1365-2958.2005.04622.x

Erken, M., Weitere, M., Kjelleberg, S., and McDougald, D. (2011). *In situ* grazing resistance of *Vibrio chloerae* in the marine environment. *FEMS Microbiol. Ecol.* 76, 504–512. doi: 10.1111/j.1574-6941.2011.01067.x

Farmer III, J. J., and Janda, J. M. (2005). "Vibrionaceae," in *Bergey's Manual Systematic Bacteriology*, eds D. J. Brenner, N. R. Krieg, and J. R. Staley (New York: Springer-Verlag).

Farmer III, J. J., Janda, J. M., Brenner, D. J., Krieg, N. R., and Staley, J. R. (2005). "*Vibrio*," in *Bergey's Manual Systematic Bacteriology*, eds D. J. Brenner, N. R. Krieg, and J. R. Staley (New York: Springer-Verlag), 494–546.

Fidopiastis, P. M., von Boletzky, S., and Ruby, E. G. (1998). A new niche for Vibrio logei, the predominant light organ symbiont of squids in the genus *Sepiola*. *J. Bacteriol.* 180, 59–64.

Frey, J. (2007). Biological safety concepts of genetically modified live bacterial vaccines. *Vaccine* 25, 5598–5605. doi: 10.1016/j.vaccine.2006.11.058

Fu, X. T., and Kim, S. M. (2010). Agarase: review of major sources, categories, purification method, enzyme characteristics and applications. *Mar. Drugs* 8, 200–218. doi: 10.3390/md8010200

Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. *Aquaculture* 180, 147–165. doi: 10.1016/S0044-8486(99)00187-8

Gilbert, P., Das, J., and Foley, I. (1997). Biofilms susceptibility to antimicrobials. *Adv. Dent. Res.* 11, 160–167. doi: 10.1177/08959374970110010701

Grimes, D. J., Johnson, C. N., Dillon, K. S., Flowers, A. R., Noriea , N. F. III and Berutti, T. (2009). What genomic sequence information has revealed about *Vibrio* ecology in the ocean-a review. *Microb. Ecol.* 58, 447–460. doi: 10.1007/s00248-009-9578-9

Gu, J., Wang, Y., and Lilburn, T. (2009). A comparative genomics, network-based approach to understanding virulence in *Vibrio cholerae*. *J. Bacteriol.* 191, 6262–6272. doi: 10.1128/JB.00475-09

Guerrero-Ferreira, R., Clayton Gorman, C., Chavez, A. A., Willie, S., and Nishiguchi, M. K. (2013). Characterization of the bacterial diversity in Indo-West Pacific loliginid and sepiolid squid light organs. *Microb. Ecol.* 65, 214–226. doi: 10.1007/s00248-012-0099-6

Guerrero-Ferreira, R. C., and Nishiguchi, M. K. (2007). Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis, Loliolus,* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* 23, 497–506. doi: 10.1111/j.1096-0031.2007.00155.x

Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annu. Rev. Microbiol.* 57, 249–273. doi: 10.1146/annurev.micro.57.030502.091014

Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., et al. (2000). DNA sequence of both chromosomes of the cholerae pathogen *Vibrio cholerae*. *Nature* 406, 477–483. doi: 10.1038/35020000

Hentschel, U., Steinert, M., and Hacker, J. (2000). Common molecular mechanisms of symbiosis and pathogenesis. *Trends Microbiol.* 8, 226–231. doi: 10.1016/S0966-842X(00)01758-3

Honda, T., and Finkelstein, R. A. (1979). Selection and characteristics of a *Vibrio cholerae* mutant lacking the A (ADP-ribosylating) portion of the cholera enterotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 76, 2052–2056. doi: 10.1073/pnas.76.4.2052

Hsing, W., and Canale-Parola, E. (1996). Methyl-accepting protein involved in multiple-sugar chemotaxis by *Cellulomonas gelida*. *J. Bacteriol.* 178, 5153–5158.

Hulsmann, A., Roschle, T. M., Kong, I.-S., Hassan, H. M., Beam, D. M., and Oliver, J. D. (2003). RpoS-dependent stress response and exoenzyme production in *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 69, 6114–6120. doi: 10.1128/AEM.69.10.6114-6120.2003

Hutchinson, G. E. (1957). Concluding remarks. *Cold Spring Harb. Symp. Quant. Biol.* 22, 415–427. doi: 10.1101/SQB.1957.022.01.039

Jones, B. W., Lopez, J. E., Huttenburg, J., and Nishiguchi, M. K. (2006). Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol. Ecol.* 15, 4317–4329. doi: 10.1111/j.1365-294X.2006.03073.x

Jones, B. W., and Nishiguchi, M. K. (2004). Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Mar. Biol.* 144, 1151–1155. doi: 10.1007/s00227-003-1285-3

Kawecki, T. J., Lenski, R. E., Ebert, D., Hollis, B., Olivieri, I., and Whitlock, M. C. (2012). Experimental evolution. *Trends Ecol. Evol.* 27, 547–560. doi: 10.1016/j.tree.2012.06.001

Kolsto, A.-B. (1999). Time for a fresh look at the bacterial chromosome. *Trends Microbiol.* 7, 223–226. doi: 10.1016/S0966-842X(99)01519-X

Lee, K.-H., and Ruby, E. G. (1994a). Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. *J. Bacteriol.* 176, 1985–1991.

Lee, K.-H., and Ruby, E. G. (1994b). Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl. Environ. Microbiol.* 60, 1565–1571.

Lee, K.-H., and Ruby, E. G. (1995). Symbiotic role of the viable but nonculturable state of *Vibrio fischeri* in Hawaiian coastal seawater. *Appl. Environ. Microbiol.* 61, 278–283.

Lenski, R. E. (1995). "Evolution in experimental populations of bacteria," in *Population Genetics of Bacteria*, eds S. Baumberg, J. P. W. Young, E. M. H. Wellington, and J. R. Saunders (Cambridge: Cambridge University Press), 193–215.

Lenski, R. E. (2002). "Experimental evolution: a long-term study with *E. coli*." in *Encyclopedia of Evolution*, ed. M. Pagel (New York: Oxford University Press), 342–344.

Lenski, R. E., Rose, M. R., Simpson, S. C., and Tadler, S. C. (1991). Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* 138, 1315–1341. doi: 10.1086/285289

MacLean, D., Jones, J. D. G., and Studholme, D. J. (2009). Application of 'next-generation' sequencing technologies to microbial genetics. *Nat. Rev. Microbiol.* 7, 287–296. doi:10.1038/nrmicro2088

MacLean, R. C., and Bell, G. (2002). Experimental adaptive radiation in *Pseudomonas*. *Am. Nat.* 160, 569–581. doi: 10.1086/342816

MacLean, R. C., Bell, G., and Rainey, P. B. (2004). The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8072–8077. doi: 10.1073/pnas.0307195101

Madigan, M. T., and Martinko, J. M. (2006). *Brock Biology of Microorganisms*. Upper Saddle River, NJ: Pearson Pratice Hall.

Mandel, M. J., Wollenberg, M. S., Stabb, E. V., Visick, K. L., and Ruby, E. G. (2009). A single regulatory gene is sufficient to alter bacterial host range. *Nature* 458, 215–218. doi: 10.1038/nature07660

Mansson, M., Gram, L., and Larsen, T. O. (2011). Production of bioactive secondary metabolites by marine vibrionaceae. *Mar. Drugs* 9, 1440–1468. doi: 10.3390/md9091440

Matz, C., and Kjelleberg, S. (2005). Off the hook- how bacteria survive protozoan grazing. *Trends Microbiol.* 13, 302–307. doi: 10.1016/j.tim.2005.05.009

Matz, C., Moreno, A. M., Alhede, M., Manefield, M., Hauser, A. R., Givskov, M., et al.(2008). *Psuedomonas aeruginosa* uses type III secretion system to kill biofilm-associated amoebae. *Int. J. Syst. Evol. Microbiol.* 2, 843–852.

McCarter, L. L. (2001). Polar flagellar motility of the *Vibrionaceae*. *Microbiol. Mol. Biol. Rev.* 65, 445–462. doi: 10.1128/MMBR.65.3.445-462.2001

McFall-Ngai, M. J., and Ruby, E. G. (1991). Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* 254, 1491–1494. doi: 10.1126/science.1962208

McGuigan, K., and Sgro, C. M. (2009). Evolutionary consequences of cryptic genetic variation. *Trends Ecol. Evol.* 24, 305–311. doi: 10.1016/j.tree.2009.02.001

Miller, M. B., and Bassler, B. L. (2001). Quorum sensing in bacteria. *Ann. Rev. Microbiol.* 55, 165–199. doi: 10.1146/annurev.micro.55.1.165

Millikan, D., and Ruby, E. G. (2004). *Vibrio fischeri* flagellin a is essential for normal motility and for symbiotic competence during initial squid light organ colonization. *J. Bacteriol.* 185, 3547–3557. doi: 10.1128/JB.185.12.3547-3557.2003

Miyamoto, N., and Eguchi, M. (1997). Response to low osmotic stress in a fish pathogen, *Vibrio anguillarum*. *FEMS Microbiol. Lett.* 22, 225–231. doi: 10.1111/j.1574-6941.1997.tb00374.x

Miyashiro, T., and Ruby, E. G. (2012). Shedding light on bioluminescence regulation in *Vibrio fischeri*. *Mol. Microbiol.* 84, 795–806. doi: 10.1111/j.1365-2958.2012.08065.x

Moltschaniwskyj, N. A. (2004). Understanding the process of growth in cephalopods. *Mar. Fresh. Res.* 55, 379–386. doi: 10.1071/MF03147

Natrah, F. M. I., Ruwandeepika, H. A. D., Pawar, S., Karunasagar, I., Sorgeloos, P., Bossier, P., et al. (2011). Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. *Vet. Microbiol.* 154, 124–129. doi: 10.1016/j.vetmic.2011.06.024

Nealson, K. H., Platt, T., and Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial bioluminescent system. *J. Bacteriol.* 104, 313–322.

Ninin, E., Caroff, N., Kouri, E., Espaze, E., Richet, H. H., Quilici, M. L., et al. (2000). Nontoxigenic *Vibrio cholerae* O1 bacteremia: case report and review. *Eur. J. Clin. Microbiol. Infect. Dis.* 19, 488–491. doi: 10.1007/s100960000296

Nishiguchi, M. K. (2000). Temperature affects species distribution in symbiotic populations of *Vibrio* spp. *Appl. Environ. Microbiol.* 66, 3550–3555. doi: 10.1128/AEM.66.8.3550-3555.2000

Nishiguchi, M. K. (2002). Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microb. Ecol.* 44, 10–18. doi: 10.1007/BF03036870

Nishiguchi, M. K., DeVinney, R., Hirsch, A. M., Riley, M., Mansky, L., and Vendatum, G. (2008). Evolution of virulence: deciphering the mechanisms between pathogenic and benign symbioses. *Vie Et Milieu-LifeEnviron.* 58, 175–184.

Nishiguchi, M. K., Lopez, J. E., and Boletzky, S. V. (2004). Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. *Evol. Dev.* 6, 41–49. doi: 10.1111/j.1525-142X.2004.04009.x

Nishiguchi, M. K., and Nair, V. S. (2003). Evolution of symbiosis in the *Vibrionaceae*: a combined approach using molecules and physiology. *Int. J. Syst. Evol. Microbiol.* 53, 2019–2026. doi: 10.1099/ijs.0.02792-0

Nishiguchi, M. K., Ruby, E. G., and McFall-Ngai, M. J. (1998). Competitive dominance during colonization is an indicator of coevolution in an animal-bacterial symbiosis. *Appl. Environ. Microbiol.* 64, 3209–3213.

Nowakowska, J., and Oliver, J. D. (2013). Resistance to environmental stresses by *Vibrio* vulnificus in the viable but nonculturable state. *FEMS Microbiol. Ecol.* 84, 213–222. doi: 10.1111/1574-6941.12052

Nyholm, S. V., and Nishiguchi, M. K. (2008). The evolutionary ecology of a sepiolid squid-*Vibrio* association: from cell to environment. *Vie Et Milieu-Life Environ.* 58,175–184.

Okada, K., Tetsuya Iida, T., Kita-Tsukamoto, K., and Takeshi Honda, T. (2005). *Vibrios* commonly possess two chromosomes. *J. Bacteriol.* 187, 752–757. doi: 10.1128/JB.187.2.752-757.2005

Ordax, M., Marco-Noales, E., Lopez, M. M., and Biosca, E. G. (2006). Survival strategy of *Erwinia amylovora* against copper: induction of the viable-but-nonculturable state. *Appl. Environ. Microbiol.* 72, 3482–3488. doi: 10.1128/AEM.72.5.3482-3488.2006

Owen, J., Punt, J., and Stranford, S. (2013). *Kuby Immunology*, 7th Edn. New York, NY: W. H. Freeman and Company.

Owens, L., and Busico-Salcedo, N. (2006). "*Vibrio harveyi*: pretty problems in paradise," in *The Biology of Vibrios,* eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Paaby, A. B., and Rockman, M. V. (2014). Cryptic genetic variation: evolution's hidden substrate *Nat. Rev. Genet.* 15, 247–258. doi: 10.1038/nrg3688

Polz, M. F., Hunt, D. E., Preheim, S. P., and Weinreich, D. M. (2006). Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. *Philos. Trans. R. Soc. B* 361, 2009–2021. doi: 10.1098/rstb.2006.1928

Pruzzo, C., Vezzulli, L., and Colwell, R. R. (2008). Global impact of *Vibrio cholerae* interactions with chitin. *Environ. Microbiol.* 10, 1400–1410 doi: 10.1111/j.1462-2920.2007.01559.x

Rahman, M., Abd, H., Romling, U., Sandstrom, G., and Mollby, R. (2008). *Aeromonas–Acanthamoeba* interaction and early sh ift to a viable but nonculturable state of Aeromonas by *Acanthamoeba. J. Appl. Microbiol.* 104, 1449–1357. doi: 10.1111/j.1365-2672.2007.03687.x

Rainey, P. B., and Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature* 394, 69–72. doi: 10.1038/27900

Rasmussen, T. B., and Givskov, M. (2006). Quorum-sensing inhibitors as anti-pathogenic drugs. *Int. J. Med. Microbiol.* 296, 149–161. doi: 10.1016/j.ijmm.2006.02.005

Richards, G. P., Fay, J. P., Dickens, K. A., Parent, M. A., Soroka, D. S., Boyd, E. F., et al. (2012). Predatory bacteria as natural modulators of *Vibrio* parahaemolyticus and *Vibrio vulnificus* in seawater and oysters. *Appl. Environ. Microbiol.* 78, 7455–7466. doi: 10.1128/AEM.01594-12

Rosenzweig, R. F., Sharp, R. R., Treves, D. S., and Adams, J. (1994). Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli. Genetics* 137, 903–917.

Ruby, E., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., et al. (2005). Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3004–3009. doi: 10.1073/pnas.0409900102

Sawabe, T., Sugimura, I., Ohtsuka, M., Nakano, K., Tajima, K., Ezura, Y., et al. (1998). *Vibrio halioticoli* sp. nov., a non-motile alginolytic marine bacterium isolated from the gut of the abalone Haliotis discus hannai. *Int. J. Syst. Bacteriol.* 48, 573–580. doi: 10.1099/00207713-48-2-573

Schuster, B. M., Perry, L. A., Cooper, V. S., and Whistler, C. A. (2010). Breaking the language barrier: experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host. *Symbiosis* 51, 85–96. doi: 10.1007/s13199-010-0074-2

Soto, W., Gutierrez, J., Remmenga, M. D., and Nishiguchi, M. K. (2009). Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. *Microb. Ecol.* 57, 140–150. doi: 10.1007/s00248-008-9412-9

Soto, W., Lostroh, C. P., and Nishiguchi, M. K. (2010). "Physiological responses to stress in the *Vibrionaceae*," in *Cooperation and Stress in Biology: Joint Ventures in Biology*, eds J. Seckback and M. Grube (New York: Springer), 407–426.

Soto, W., Punke, E. B., and Nishiguchi, M. K. (2012). Evolutionary perspectives in a mutualism of sepiolid squid and bioluminescent bacteria: combined usage of microbial experimental evolution and temporal population genetics. *Evolution* 66, 1308–1321. doi: 10.1111/j.1558-5646.2011.01547.x

Soto, W., Rivera, F. M., and Nishiguchi, M. K. (2014). Ecological diversification of *Vibrio fischeri* serially passaged for 500 generations in novel squid host *Euprymna tasmanica. Microb. Ecol.* 67, 700–721. doi: 10.1007/s00248-013-0356-3

Sozhamannan, S., and Yildiz, F. H. (2011). "Diversity and genetic basis of polysaccharide biosynthesis in *Vibrio cholerae*,"in *Epidemiological and Molecular Aspects On Cholera*, eds S. K. Bhattacharya and T. Ramamurthy (London: Springer).

Stabb, E. V., and Visick, K. L. (2013). "*Vibrio fischeri*: squid symbiosis," in *The Prokaryotes,* eds E. Rosenberg, E. F. Delong, E. Stackebrand, S. Lory, and F. Thompson (Berlin: Springer).

Stoodley, P., Sauer, D., Davies, G., and Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Ann. Rev. Microbiol.* 56, 187–209. doi: 10.1146/annurev.micro.56.012302.160705

Tagomori, K., Iida, T., and Honda, T. (2002). Comparison of genome structures of vibrios, bacteria possessing two chromosomes. *J. Bacteriol.* 184, 4351–4358. doi: 10.1128/JB.184.16.4351-4358.2002

Tenaillon, O., Denamur, E., and Matic, I. (2004). Evolutionary significance of stress-induced mutagenesis in bacteria. *Trends Microbiol.* 12, 264–270. doi: 10.1016/j.tim.2004.04.002

Thompson, F. L., and Swings, J. (2006). "Taxonomy of the vibrios," in *Biology of the Vibrios,* eds B. A. F. L Thompson and J. Swings (Washington, DC: ASM Press), 29–43. doi: 10.1128/9781555815714.ch3

Travisano, M., and Rainey, P. B. (2000). Studies of adaptive radiation using model microbial systems. *Am. Nat.* 156, S35–S44. doi: 10.1086/303414

Travisano, M., and Velicer, G. J. (2004). Strategies of microbial cheater control. *Trends Microbiol.* 12, 72–78. doi: 10.1016/j.tim.2003.12.009

Urakawa, H., and Rivera, N. G. N. G. (2006). "Aquatic environment," in *The Biology of Vibrios,* eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Urbanczyk, H., Ast, J. C., and Dunlap, P. V. (2011). Phylogeny, genomics, and symbiosis of *Photobacterium. FEMS Microbiol. Rev.* 35, 324–342. doi: 10.1111/j.1574-6976.2010.00250.x

Ventosa, A. (2005). "Salinovibrioin," in *Bergey's Manual Systematic Bacteriology: The Proteobacteria. Springer-Verlag,* eds D. J. Brenner, N. R. Krieg, and J. R. Staley (New York: Springer-Verlag), 552–555.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., et al. (2008). Living on a surface: swarming and biofilm formation. *Trends Microbiol.* 16, 496–506. doi: 10.1016/j.tim.2008.07.004

Waldor, M. K., and RayChaudhuri, D. (2000). Treasure trove for cholera research. *Nature* 406, 469–470. doi: 10.1038/35020178

Wang, Z., O'Shaughnessy, T. J., Soto, C. M., Rahbar, A. M., Robertson, K. L., Nikolai Lebedev, N., et al. (2012). Function and regulation of *Vibrio campbellii* proteorhodopsin: acquired phototrophy in a classical organoheterotroph. *PLoS ONE* 7:e38749. doi: 10.1371/journal.pone.0038749.g001

West, S. A., Griffin, A. S., Gardner, A., and Diggle, S. P. (2006). Social evolution theory for microorganisms. *Nat. Rev. Microbiol.* 4, 597–607. doi: 10.1038/nrmicro1461

White, D. (2007). *The Physiology and Biochemistry of the Prokaryotes.* Oxford: Oxford University Press.

Wiedenbeck, J., and Cohan, F. M. (2011). Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiol. Rev.* 35, 957–976. doi: 10.1111/j.1574-6976.2011.00292.x

Wier, A. M., Nyholm, S. V., Mandel, M. J., Massengo-Tiassé, R. P., Schaefer, A. L., Koroleva, I., et al. (2010). Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2259–2264. doi: 10.1073/pnas.0909712107

Wollenberg, M. S., and Ruby, E. G. (2009). Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two Oahu populations. *Appl. Environ. Microbiol.* 75, 193–202. doi: 10.1128/AEM.01792-08

Wollenberg, M. S., and Ruby, E. G. (2012). Phylogeny and fitness of *Vibrio fischeri* from the light organs of *Euprymna scolopes* in two Oahu, Hawaii populations. *Int. Soc. Microbial Ecol. J.* 6, 352–362.

Wong, H. C., and Wang, P. (2004). Induction of viable but nonculturable state in *Vibrio* parahaemolyticus and its susceptibility to environmental stresses. *J. Appl. Microbiol.* 96, 359–366. doi: 10.1046/j.1365-2672.2004.02166.x

Yildiz, F. H., and Visick, K. L. (2009). Vibrio biofilms: so much the same yet so different. *Trends Microbiol.* 17, 109–118. doi: 10.1016/j.tim.2008.12.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 May 2014; accepted: 20 October 2014; published online: 09 December 2014.

*Citation: Soto W and Nishiguchi MK (2014) Microbial experimental evolution as a novel research approach in the Vibrionaceae and squid-Vibrio symbiosis. Front. Microbiol. **5**:593. doi: 10.3389/fmicb.2014.00593*

This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology.

Chapter 15
Euprymna hyllebergi and *Euprymna tasmanica*

Jaruwat Nabhitabhata and Michelle K. Nishiguchi

Abstract Bobtail squids of the genus *Euprymna* are small in size with a benthic habit. Such small size results in their insignificance in fisheries and aquaculture focused for human consumption. The unique ability of the voluntary adhesion system and symbiotic bacteria used for bioluminescence is now a primary research focus with potential industrial and biomedical applications. Their small size is well suited for the home aquarium with small volume. Culture of this cephalopod group can therefore serve both research and recreational purposes. Aquaculture in the laboratory provides valuable information for culture methodology that is utilized throughout the entire life cycle of several consecutive generations. This small size and benthic habit of *Euprymna* are advantageous for small-scale closed or open seawater culture systems. Major trends for culturing *Euprymna* are similar to other cephalopod groups, particularly benthic octopus that also produce planktonic hatchlings. Reduction of the cost of production is necessary for future large-scale production, with novel protocols for live feed requirements of planktonic young in the nursing phase.

Keywords *Euprymna* · Small size · Benthic habit · Small-scale culture · Closed and open seawater systems · Research and recreational purposes

15.1 Importance of the Species

The Thai bobtail squid, *Euprymna hyllebergi* Nateewathana 1997, is a common species occurring in the Andaman Sea of Thailand (Indian Ocean) and the Gulf of Thailand (Pacific Ocean; Nateewathana 1997; Nateewathana et al. 2001; Aungtonya et al. 2011). This species is small (20–40-mm mantle length, ML),

J. Nabhitabhata (✉)
Excellence Centre for Biodiversity of Peninsular Thailand, Faculty of Science,
Prince of Songkla University, Hatyai, Songkhla 90112, Thailand
e-mail: jaruwat.n5@gmail.com; e-mail: jaruwat.n@psu.ac.th

M. K. Nishiguchi
Department of Biology – MSC 3AF, New Mexico State University,
PO Box 30001, Las Cruces, NM 88003-8001, USA
e-mail: nish@nmsu.edu

J. Iglesias et al. (eds.), *Cephalopod Culture,* DOI 10.1007/978-94-017-8648-5_15,

Fig. 15.1 Partial sand-coated *Euprymna hyllebergi* shortly after emerging from its burrowing site. (Photograph of J Nabhitabhata)

neritic and strictly nektobenthic, inhabiting coastal waters in a similar manner to its congeners that occur in the Indo-west Pacific region (Summers 1985; Norman and Lu 1997; Reid and Jereb 2005). *E. tasmanica* (Pfeffer 1884), otherwise known as the "southern dumpling squid", is a species that resides around the continent of Australia and is found in similar habitats as *E. hyllebergi* (Reid and Jereb 2005). *E. tasmanica* is somewhat larger in size than *E. hyllebergi,* with an approximate ML of 30–40 mm (Norman and Lu 1997). In Thailand, *E. hyllebergi* and congeners are captured as by-catch of commercial fishing, particularly push netting and trawling (Nateewathana 1997). The yields are discarded as trash fishes due to their small size and low economic value. Because of this, fishing statistics of both species are not available (Nateewathana et al. 2001; Reid and Norman 1998; Reid and Jereb 2005).

Although the economic value of *Euprymna* as human food is low, there is a growing importance of the animals as scientific experimental models. The unique behaviour of *Euprymna* is its capability to retain a "coat" of sand or other debris on its dorsum (Fig. 15.1) when it emerges from its daily buried state to hunt prey at night (Anderson et al. 2002). The function of the sand coat is presumably for camouflage, making the squid difficult to be detected visually by its predators and prey (Anderson and Mather 1996; Shears 1988). The stickiness of the sand coat depends upon secretions of the ectodermal epithelium (Moynihan 1982). Choice between being sticky and nonsticky is voluntary and variable (Moynihan 1982). This indicates that the ability to use a sand coat might have evolved from the initial use of the behaviour for sand consolidation when the squid buries (Shears 1988). The ability to begin sand coating starts 5–7 days after hatching, simultaneous to their burrowing ability (Nabhitabhata et al. 2005). Additionally, bobtail squids are presently used as a model organism to identify a new generation of biomimetic adhesives and marine antifouling compounds with potential industrial value (Byern and Grunwald 2010).

Symbiotic associations between *Euprymna* and the bioluminescent bacterium *Vibrio fischeri* has been a recent focus as a model for investigating the process of bacterial colonization of host tissues and its effect on host development (Ruby 1999, Ruby and Lee 1998). *V. fischeri* and other luminous bacteria form a variety of pathogenic and cooperative associations with marine animals; more recently, they are being increasingly recognized as causes of invertebrate diseases (Guerrero-Ferreira

and Nishiguchi 2011; Guerrero-Ferreira et al. 2012). Since the process of bacterial colonization of the squid light organ begins immediately after hatching (Ruby and McFall-Ngai 1992), independent aquaculture of the squids and their luminous bacterial partners could yield valuable results for biotechnological and biomedical sciences (McFall-Ngai et al. 2012; Nyholm and Nishiguchi 2008). Because of these newly developed models for basic research, the advancement of culturing techniques for species of *Euprymna* has been especially important for monitoring fitness between generations, effects of inbreeding and, more importantly, diet and stress under laboratory conditions (Nabhitabhata et al. 2005; Sinn 2005; Sinn et al. 2008; Moltschaniwskyj and Carter 2010). Additionally, more information on their development, growth and time to reproduction can indicate whether all species have similar life-history strategies, and if this is dependent upon habitat or other abiotic factors.

15.2 State of the Art

Both *E. hyllebergi* and *E. tasmanica* are small in size and found living within benthic habitats. This is an advantage to provide culture conditions on a smaller scale with lower cost and less requirement of facilities compared to those used for pelagic and large-sized species. *Euprymna* can be cultured through several consecutive generations ensuring a supply of broodstocks. Broodstocks collected from the wild can spawn in captivity and are maintained throughout the life history of the animal. *E. hyllebergi* hatchlings are fed with wild-collected live feed for approximately 30 days, but later can be trained to accept dead feed. *E. tasmanica* hatchlings are fed small mysid shrimp two times a day for approximately 6 weeks, and then moved to a diet of ghost shrimp for the duration of their lives while in captivity. Interestingly, *E. tasmanica* adults were not trained to feed on dead material, and prefer not to eat food items that do not move. These same facilities for raising juvenile squids can be used for culture throughout the squid's entire life cycle. The daily growth rate for *E. hyllebergi* is 2.4% in length and 7.5% in weight through the culture period of 100 days. Growth rates for *E. tasmanica* were approximately 3.5% in length and 10% in weight for approximately 60 days. Growing demands for these squid for use as both biotechnological and biomimetic experimental models as well as ornamental animals for home aquaria and teaching laboratories are beneficial for aquaculture and biomedicine, e.g. Nabhitabhata et al. (2005), Moltschaniwskyj et al. (2007), Sinn and Moltschaniwskyj (2005) and Sinn et al. (2008).

15.3 Broodstocks Maintenance

Broodstocks of the Thai bobtail squid, *E. hyllebergi,* are collected live from otter board trawlers and beam trawlers, operating along the eastern part of the Gulf of Thailand, South China Sea and Pacific Ocean. Onboard, the squids are maintained in cylindrical fibreglass tanks of 50-L capacity containing 30 L of fresh seawater

with aeration and then, upon landing, are transported to the cephalopod hatchery. The broodstocks are maintained in an open system of cylindrical concrete tanks of 2 m^3 with flow-through filtered seawater (for the seawater supply system in this chapter, see also Chap. 7 "Aquaculture to Restocking"). Artificial substrates, made from pieces of longitudinal-cut polyvinyl chloride (PVC) pipe (50-mm diameter, 150-mm length), are previously placed on the tank bottom as shelters or "dens".

Broodstocks of southern dumpling squids, *E. tasmanica,* are collected by seine net in shallow waters of Botany Bay, New South Wales, Australia. Adult animals are transported to running open seawater facilities located at the Sydney Institute of Marine Sciences (SIMS) at Chowder Bay, NSW. Adults are acclimated to the conditions in the tanks (34 psu, 18 °C) and transported to New Mexico State University within 2–3 days. Transport of the squids takes approximately 36 h tank to tank in aquaria bags with less than 1 L of seawater. Animals are then acclimated to the contained recirculating artificial seawater tanks (100 L) at New Mexico State University under the same culturing conditions. Each tank is divided into eight cubic sectionals (each 0.3 m^2), which holds three to four adult individuals. Sexes are continuously kept separate, since the presence of males can stress female behaviour. The only time males are placed with females is when a planned mating is scheduled. In this manner, we can document which particular male has mated with which female (and therefore, track fecundity of each female). Males are placed in the female cubical (usually at a 1:1 or 1:2 male to female ratio) and are removed anytime between 4 and 10 h. PVC pipe cut longitudinally is placed in the female cubical and used as artificial substrates for the females to lay their eggs after they have been mated.

Squids will mate and spawn in the tanks. Mating occurs without prior pair formation for *E. hyllebergi,* and controlled conditions (noting which pairs are mated, and how many times) are maintained for *E. tasmanica*. Spawning behavioural pattern is similar for both species. The male responds to the presence of a swimming female by initially approaching and then grasping her from below in a male to female neck position. The female is pulled down to the bottom where copulation takes place. Copulation takes 7–10 min and then the pair separate. Spawning is observed at dawn, 2–3 days after mating. Prior to spawning, the female investigates substrates for attaching her eggs by swimming around, and touching the substrata with the tip of her arm cone. In the tanks, the female attaches her eggs in clusters to the inner surface of the artificial substrates (Fig. 15.2). The time period for attaching is 40–60 s for one egg. Intervals between each egg attachment lengthens as the number of eggs increases, up to 2–3 min. Spawning is intermittent and irregular and may be extended over several weeks. The total number of eggs per female is about 100–470 with an average of 200 eggs. Females can spawn up to three to four clutches in her lifetime, with the number of eggs decreasing as the female becomes older (Steer et al. 2004; Nabhitabhata et al. 2005). For *E. tasmanica,* adults reared in captivity (F1 generation) live longer (2–3 months) and produce larger and more clutches per female. Wild-caught adult *E. tasmanica* at maturity produce approximately 3 clutches while in captivity, ranging from 25 to 100 eggs per clutch (with one exceptional female, which laid approximately 500 eggs in one clutch). F1

Fig. 15.2 Egg capsules of *Euprymna hyllebergi* attached to the inner side of the artificial substratum, a piece of cut polyvinyl chloride (*PVC*) pipe. (Photograph of J Nabhitabhata)

generation *E. tasmanica* females lay up to 5 clutches/lifetime, with sizes ranging from 100 to 250 eggs per clutch. Hatching rate from the F2 clutches is approximately 99% for the first clutch, with a decrease leading up to approximately 20% (~80% hatching rate) for later clutches. F1 adults are larger and thus far have lived for 1 year in captivity (Nishiguchi, unpublished). F2 adults have similar longevities, but hatching rates for the F3 generation was somewhat lower (70–80%)

15.4 Nursing of Eggs

15.4.1 Egg Characteristics

Eggs are single, stalkless and opaque white, having a droplet shape and calcareous leather-like coating capsule (Fig. 15.3). The size of each egg is about 4 mm along its major axis, 3 mm in its minor axis and weighs about 0.02 g. About 2 h after being laid, the outer coat (or capsule) turns brown, leathery and rigid in *E. hyllebergi* (Nabhitabhata et al. 2005), whereas in *E. tasmanica* the egg is orange from wild-caught specimens. In F1 and subsequent generations of *E. tasmanica,* egg capsules are white to opaque and remain so during development. This solid protection allows the developing embryo to become a "sessile organism" during the extended period of development (Boletzky 1998). Eggs are telolecithal. Asymmetric eight-cell cleavage occurs 10 h after fertilization. Clockwise rotation of the embryo occurs from days 3 to 8, at 28 °C. Organogenesis occurs from day 4. The unique bilobed character of the external yolk sac appears after day 5 when the capsule becomes more transparent and the embryo is now visible. Chromatophores develop from day 6, and four diverticula of the internal yolk sac from day 8. The first hatching occurs at day 12 (Fig. 15.4) for *E. hyllebergi,* and day 32 for *E. tasmanica*. The embryonic phase is about 12–18 days, after approximately 14 days at 28 °C for *E. hyllebergi,* and 21–28 days at 18 °C for *E. tasmanica*. The hatching period of eggs in the same clutch takes 5 days from first to the last eggs and primarily occurs on the third day. Average hatching rate is about 94% (82–100%) for both species.

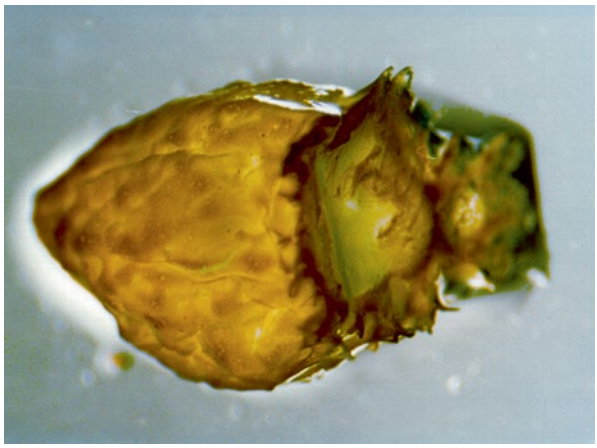


Fig. 15.3 Egg capsule of *Euprymna hyllebergi*; surface is colored brown by attached diatoms (40x). (Photograph of J Nabhitabhata)

Fig. 15.4 Hatchling of *Euprymna hyllebergi* (dorsum, 17x). (Photograph of J Nabhitabhata)

15.4.2 System Requirements and Management

Artificial substrates with egg clusters are transferred to hatch in fibreglass tanks of 50-L capacity containing 40-L filtered seawater. Two pieces of longitudinal-cut PVC pipe (25-mm diameter, 400-mm length) equipped with aeration devices are placed in each tank, facing in the same direction, to generate and direct an artificial current (Fig. 15.5). Tanks are cleaned by siphoning out the old water and replaced by a volume of 50 %. Temperature change is minimised by means of outside running water around the tank base (Nabhitabhata et al. 2005). The average temperature

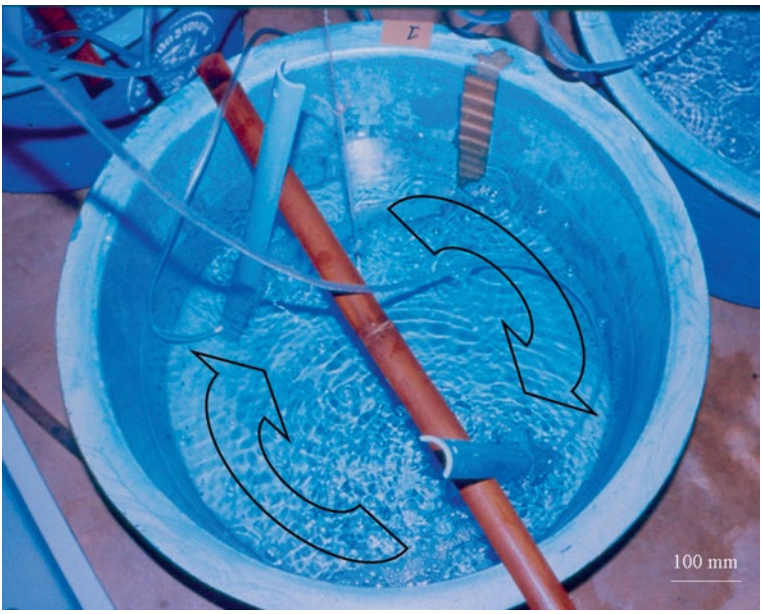


Fig. 15.5 A culture tank of *Euprymna hyllebergi* equipped with current generator devices (*arrows* indicate current direction). (Photograph of J Nabhitabhata)

can be maintained at approximately 28.2 °C, pH 8.0 and salinity at 32.5 psu. For *E. tasmanica,* clutches are placed in a 100-L polycarbonate tank with ultraviolet (UV)-filtered artificial seawater. Each individual clutch is placed in a cage made out of 2 pieces of PVC tubing, cut longitudinally and then glued back together with mesh netting in between. The clutches are placed in these cages so as not to have hatchlings mix with other clutches, as well as receiving enough aeration from below during development. Each cage is aerated with water from an individual spout that provides oxygenated seawater (Fig. 15.6). Water is kept at constant temperature (20 °C) with pH 8.0 and salinity 34.0 psu. Water changes are completed every other day to maintain salinity due to evaporation.

15.5 Nursing of Young

15.5.1 Hatchling Characteristics

The living mode of the hatchling includes a planktonic phase lasting from 6 to 8 h before the hatchling gradually adopts a benthic habit. The settling stage is approximately 5 days. Juvenile squids still enter the water column on a regular basis, alternatively planktonic and benthic, until 25–30 days after hatching. The internal yolk sac is still visible through the transparent mantle from hatching until the third day (Fig. 15.4).

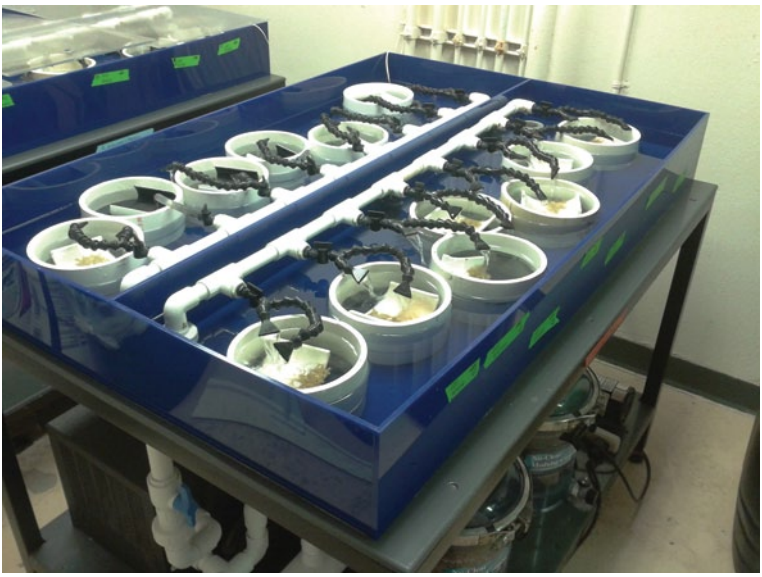


Fig. 15.6 Culture facilities for clutches/hatchlings for *Euprymna tasmanica*. (Photograph of MK Nishiguchi)

15.5.2 System Requirements and Management

Nursing of young is performed using the same system as for nursing of eggs for both species.

15.5.3 Feeding

The general task is to feed planktonic food to juvenile squids before the settling stage, at which time the squids are column feeders. Subsequently, benthic food is provided after the settling stage, when the juvenile squids settle to the bottom (Hanlon 1990; Hanlon et al. 1997; Nabhitabhata et al. 2005). *E. hyllebergi* hatchlings are fed with live, hatchery-produced penaeid shrimp larvae (*Penaeus merguiensis, P. monodon*) of the protozoea and mysis stages for 5 days after hatching (Fig. 15.7). Postlarvae of penaeid shrimps of the same species as well as wild mysids (*Mesopodopsis orientalis*) are also fed to the squids from hatching to 40 days. The planktonic young seize and eat its prey in the water column while hovering. After 25 days, the juvenile gradually changes to a benthic feeder, seizing its prey in the water column and then consuming it on the bottom substrate.

After 30 days, supplementary prey organisms for *E. hyllebergi* are palaemonid shrimps (*Palaemon styliferus*) and wild mysids (*Acetes* spp.). Training the squids to feed on dead fish meat (*Caranx leptolepis*) begins during this

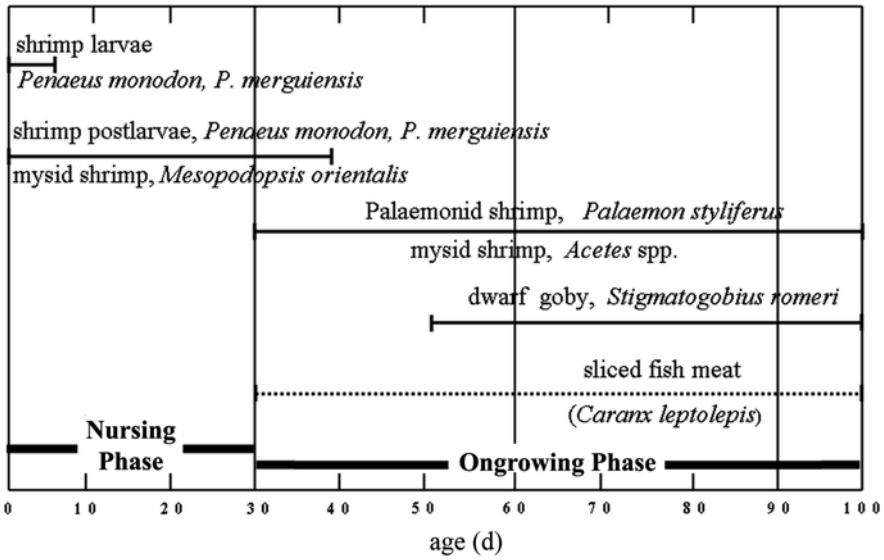


Fig. 15.7 Diagram of feeding of cultured *Euprymna hyllebergi* in the nursing phase (0–30 d) and ongrowing phase (after 30 d settlement) with live feed (*full line*) and dead feed (*dotted line*). (After Nabhitabhata et al. 2005)

period. Size grading is also initiated and continued every 10 days. The density is reduced from the initial 2–6 individuals L^{-1} by at least 25% after each grading (Nabhitabhata et al. 2005). *E. tasmanica* juveniles are fed on live, mysid shrimp for the first month (~30 days), i.e. *Tasmanomysis oculata, Paramesopodopsis refa,* and then are moved to smaller, post-larval panaeid shrimp (*Penaeus* sp.). Since *E. tasmanica* are larger when hatched, they are capable of obtaining bigger prey items earlier in their development than *E. hyllebergi*. Enriched brine shrimps (*Artemia parthenogenetica* and *A. franciscana*) can be used as substituted food when mysids are unavailable (Sinn 2005; Sinn and Moltschaniwskyj 2005; Sinn et al. 2008) although they are generally less preferred. *E. tasmanica* does not take dead prey, although there is no attempt to train juveniles to feed on this type of material.

15.5.4 Euprymna hyllebergi Growth

Hatchlings of Thai bobtail squid grow from about 2-mm ML and 0.004-g weight to 7-mm ML and 0.26 g in the first month (Fig. 15.8a, b; Nabhitabhata et al. 2005). The daily or instantaneous relative growth rate (IGR) is the highest between 10 and 20 days after hatching, about 5% in length and 17% in weight (Fig. 15.8b). The survival in the nursing phase from hatching to settling stage (0–30 days) is approximately 30%.

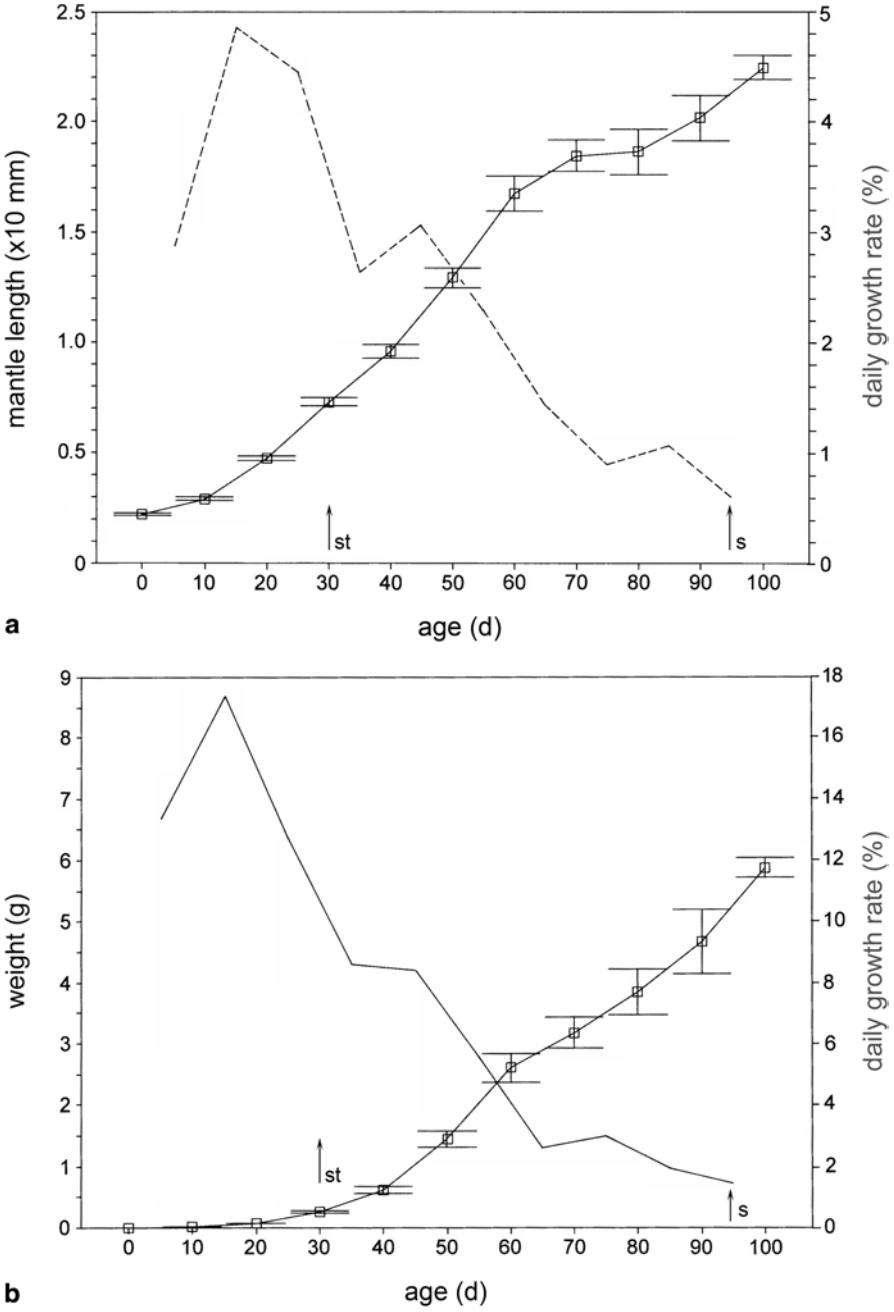


Fig. 15.8 Growth of *Euprymna hyllebergi* in terms of (*above*) mantle length (x10 mm), instantaneous relative growth rate (*IGR*: %) and age (*d*) after hatching and (*below*) weight (*g*), *IGR* (%) and age (*d*) after hatching. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

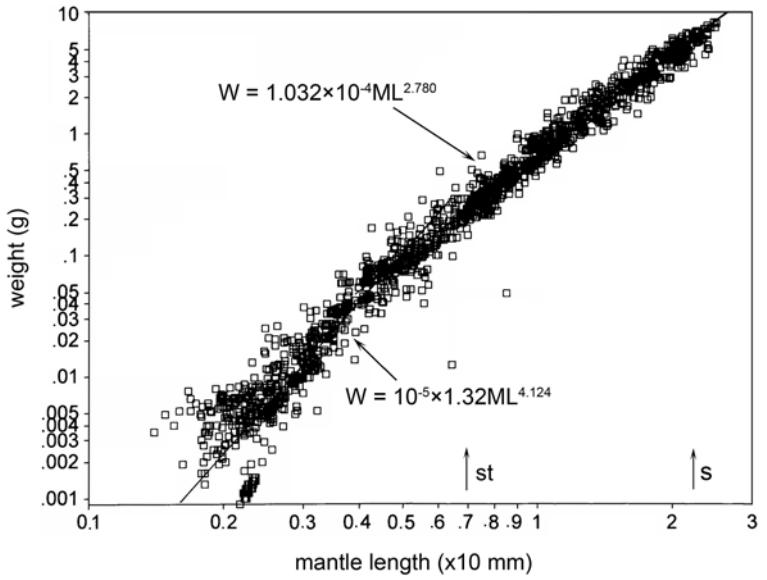


Fig. 15.9 Relationships between mantle length (x10 mm) and weight (*g*) of *Euprymna hyllebergi*; intercept of the two regressions at 5.5 mm mantle length. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

Growth models demonstrate two phases of growth. The early phase was from hatching to 30 days where the relationships between the ML (mm) and weight (g) can be expressed as a power regression model (Fig. 15.9; Nabhitabhata et al. 2005):

$$W = 1.230 \times 10^{-4}\ ML^{4.124}. \tag{15.1}$$

The relationships between ML and age (d: days after hatching, Fig. 15.10; Nabhitabhata et al. 2005) and between weight (g) and age (d, Fig. 15.11; Nabhitabhata et al. 2005) can be expressed as the exponential models:

$$ML = 1.988e^{4.205\times10^{*}(-2)A} \tag{15.2}$$

$$W = 2.750 \times 10^{-3} e^{0.153A}. \tag{15.3}$$

15.6 Ongrowing

15.6.1 System Requirements and Management

For *E. hyllebergi,* ongrowing phase starts after the benthic young are able to accept dead fish meat. Tanks for ongrowing are the same tank used for the nursing phase and with similar management for both species. The density of *E. hyllebergi*

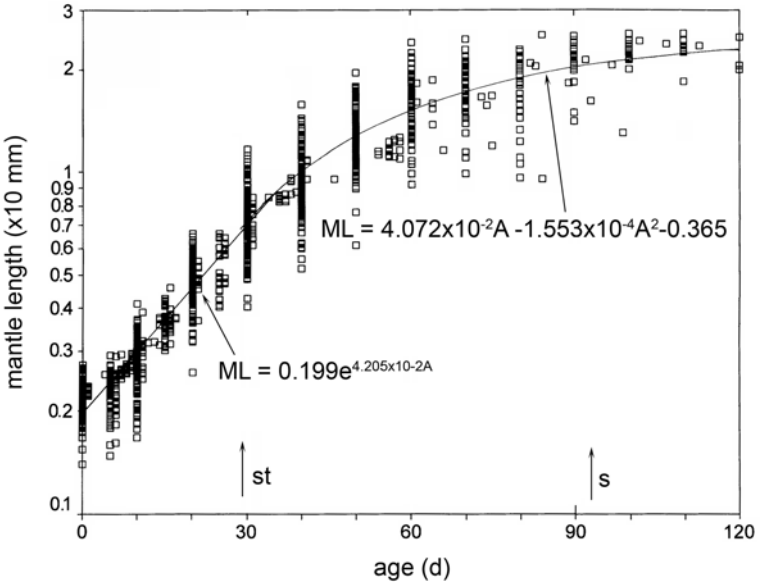


Fig. 15.10 Relationships between mantle length (x10 mm) and age (*d*) of *Euprymna hyllebergi*. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

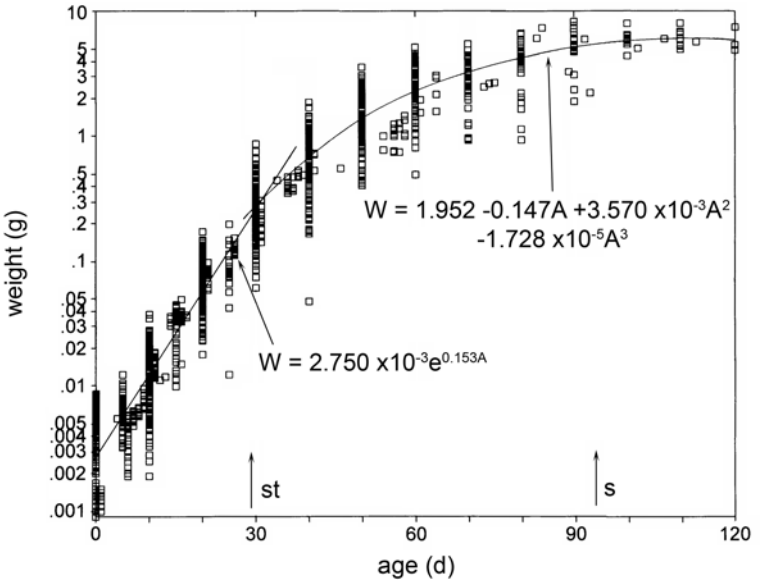


Fig. 15.11 Relationships between weight (*g*) and age (*d*) of *Euprymna hyllebergi*. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

is changed from a water volume oriented to an (bottom) area oriented as 4–5 individuals m^{-2}. *E. tasmanica* juveniles are initially raised in round glass bowls (approximately 2 L) with sand at the bottom so the juveniles can settle. Unlike *E. hyllebergi* juveniles, *E. tasmanica* immediately settle on the bottom once they are hatched. Water is changed daily since the volume is small and there is greater evaporative loss from this volume. At approximately 2–3 weeks, juvenile squids are transferred to 40-L aquaria with sand on the bottom and raised until sexually mature (2 months). Generally, 20 squids are kept in an aquarium this size due to space limitations, but this number does not seem to affect their behaviour with any visible signs of stress. Since *E. tasmanica* F1 and F2 generations have higher growth rates than those caught in the wild, these individuals are moved earlier to the adult cubicals.

15.6.2 Euprymna hyllebergi *Growth*

The growth rate from hatching to 100 days of age for *E. hyllebergi* is approximately 2.4 % in length and 7.5 % in weight. At 60 days after hatching, the squid had grown to 17-mm length and 2.6-g weight and 22 mm and 6 g at 100 days. Food consumption of about 0.2 g d^{-1} or 37 % body weight d^{-1} enables calculation of the food conversion efficiency of about 37 % (range 14–99) from hatching to 100 days. This rate increases from 30 to 40 % after hatching to 60–70 % during 40–60 days with a peak of about 64 % between 50 and 60 days (Fig. 15.12). These values potentially relate to the storage of energy for the consequent reproductive period (Nabhitabhata et al. 2005). At 90 days after hatching, the survival from hatching is approximately 10 % and from settlement is 70 %.

Transition in growth phases is reflected in the nature of the growth models. The stage where the models shifted to a higher elevation is at about 30 days after hatching, and this corresponds to the observed settlement stage (Figs. 15.8–15.11). The second growth phase is from 30 to 122 days. The relationships between ML (mm) and weight (W, g) can also be expressed as a power regression model (Nabhitabhata et al. 2005) as happened in the early phase (Fig. 15.9):

$$W = 1.032 \times 10^{-3}\ ML^{2.780}. \tag{15.4}$$

The relationships between ML and age (d, days after hatching) and between weight (g) and age can be expressed as the quadratic equation (Fig. 15.10; Nabhitabhata et al. 2005) and a cubic regression model (Fig. 15.11; Nabhitabhata et al. 2005):

$$ML = 0.407A - 1.553 \times 10^{-3}\ A^2 - 3.648 \tag{15.5}$$

$$W = 1.952 - 0.147A + 3.570 \times 10^{-3} A^2 - 1.728 \times 10^{-5} A^3. \tag{15.6}$$

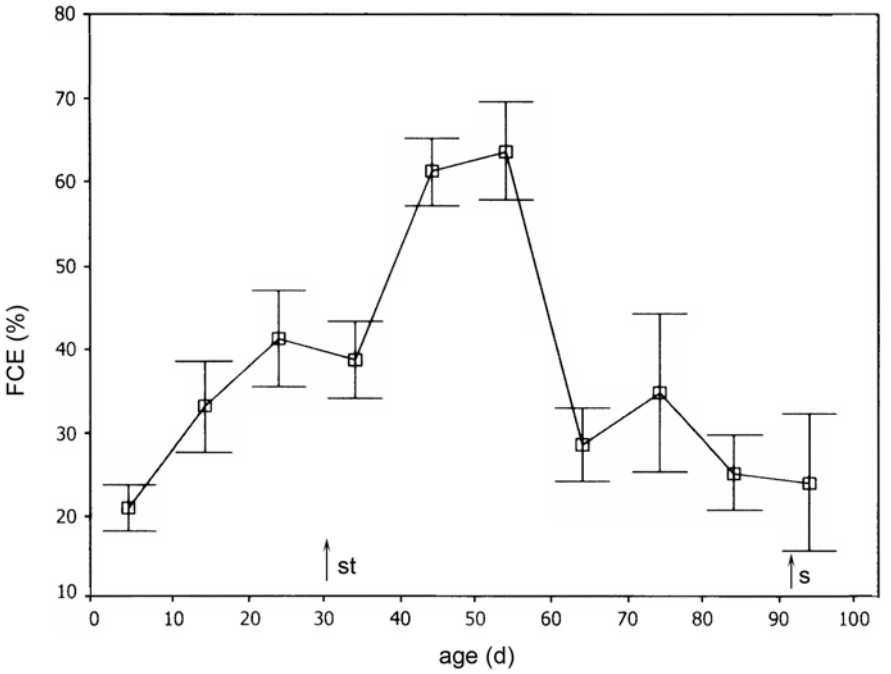


Fig. 15.12 Food conversion efficiency (*FCE*: %) of cultured *Euprymna hyllebergi* during growth (age: d). *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

15.6.3 Euprymna tasmanica *Growth*

Detailed studies on growth of *E. tasmanica* are scarce. Growth rate of *E. tasmanica* is rapid, with hatchlings reaching adult size in 2 months at 18 °C. *E. tasmanica* can grow from a ML of approximately 1.7 mm and a weight of 0.012 g at hatching (Steer et al. 2004) to 0.06 g at 21 days and to 6.8 g at 112 days (Sinn et al. 2008). The daily growth rate from day 21 to 63 is 7–9 %, from 63 to 84 days decreases to 2–4 % and from 84–112 days 1–2 % at 18 °C (Sinn 2005).

The relationships between weight and age are exponential from 7 to 44 days after hatching and linear from 58 to 140 days (Moltschaniwskyj and Carter 2010) which can be expressed as:

$$\mathrm{InW} = 0.069\mathrm{A} - 4.06 \tag{15.7}$$

$$\mathrm{W} = 0.07\mathrm{A} - 3.28. \tag{15.8}$$

15.7 Trends in Research and Industry

The main purpose of culture *Euprymna* is obviously not for human consumption. Present research in the fields of marine pharmacology, biotechnology and mimetic engineering requires small and “easy to culture” squids. Rapidly growing demand

in the ornamental aquaculture trade also requires organisms of similar characters, specifically size and ease of care. The small adult body size, benthic habit and good adaptability to culture conditions of *Euprymna* are prominent character suites that are well adapted to the aforementioned purposes. Based on such qualities, bobtail squids should be cultured on a small scale in order to reduce the cost of production. Additionally, a small-scale culture has advantages of the reduced size and benthic habits of the squids. The variety of flow-through open or closed seawater systems can yield different results and should be further studied to better quantify which systems are best for maximising production and those appropriate for each species.

Culture of *Euprymna* similarly encounters a bottleneck during the nursing phase similar to other cephalopods, since young innately feed on live feed. Future research should focus on developing feeds, both live and artificial. However, small-scale culture of live food organisms is more appropriate for small-scale culture of *Euprymna* in view of low operating costs at present. Development of artificial feed is necessary to reduce costs, but it could be postponed on a small scale. Artificial feed for cephalopods has not been commercially developed anywhere, but many studies are being completed, focusing on species that are aimed to be cultured as human food. Investigating various types of feed may give insight as to whether bobtail squids can also use artificial feed in such a manner.

E. hyllebergi and *E. tasmanica* can be cultured through multiple consecutive generations (3 generations for both *E. hyllebergi* and *E. tasmanica*) with similar growth rates (under similar conditions) without apparent effects of inbreeding on decreased growth (Nabhitabhata et al. 2005). Similar growth among generations enables a reliable supply of broodstocks for aquaculture and provides an alternative to continued fishing for wild-caught specimens, which can be time consuming and costly. However, the feasibility of inbreeding effects on decreasing of growth and fertility must be considered when producing future generations from the same broodstock. Broodstocks cannot rely solely on cultured batches, and wild broodstocks should be added intermittently to provide both genetic variation and possibly the induction of beneficial microbes that are necessary to keep squid healthy during their lifetime. Growth in captivity and culture methodology of both *E. hyllebergi* and *E. tasmanica* as well as their congeners should be further studied in views of maximising the aquaculture production and increasing our ability to provide a useful resource for a variety of research studies as well as the development of model aquaculture cephalopods.

15.8 Conclusions

The ability to maintain and grow small benthic squids such as *Euprymna* has opened up a new avenue for instigating the use of these animals as model systems in both bioengineering (adhesion) and biomedical (beneficial bacteria) research. The requirements for housing, maintaining and raising sepiolids is minimal and not as costly as other, more gregarious squid species, and this allows laboratories to set up facilities that may not necessarily be close to the ocean (such as NMSU). Presently, there are 14 laboratories in the USA alone that have culture facilities for raising

Euprymna; such facilities would not be feasible unless these animals were easy to transport long distances and maintained continually and without a nearby marine station or source of seawater. Additional research must be considered for the effects of inbreeding (when maintaining a constant broodstock) as well as comparing species for different traits that can be used for certain research foci. The inception of using cephalopods as research and not "feed" organisms is a new and exciting avenue that multiple areas of research can benefit from for furthering our knowledge in aquaculture, bioengineering, medicine, ecology and evolutionary biology.

References

Anderson RC, Mather JA (1996) Escape responses of *Euprymna scolopes* Berry, 1911 (Cephalopoda: Sepiolidae). J Mollusc Stud 62:543–545

Anderson RC, Mather JA, Steele CW (2002) The burying behavior of *Euprymna scolopes* Berry, 1913 (Cephalopoda: Sepiolidaae). West Soc Malacol Annu Rep 33:1–7

Aungtonya C, Nateewathana A, Tendal OS, Nabhitabhata J (2011) New records of the Bobtail Squid, *Euprymna hyllebergi* Nateewathana, 1997, with designation of a neotype. Phuket Mar Biol Cent Res Bull 70:15–27

Boletzky Sv (1998) Cephalopod eggs and egg masses. Oceanogr Mar Biol Ann Rev 36:341–371

Byern Jv, Grunwald I (eds) (2010) Biological adhesive systems from nature to technical and medical application. Springer-Verlag, Austria

Guerrero-Ferreira RC, Nishiguchi MK (2011) Bacterial biodiversity in natural environments. In: López-Pujol J (ed) Biodiversity/book 3, In Tech, Open access publisher. http://www.intechopen.com/articles/show/title/bacterial-biodiversity-in-natural-environments

Guerrero-Ferreira RC, Gorman C, Chavez AA, Willie S, Nishiguchi MK (2012) Characterization of the bacterial diversity in Indo-west Pacific loliginid and sepiolid squid light organs. Microb Ecol. doi: 10.1007/s00248–012-0099–6

Hanlon R (1990) Maintenance, rearing, and culture of teuthid and sepiolid squids. In: Gilbert DL, Adelman WJ, Arnold JM (eds) Squids as experimental animals. Plenum Press, New York

Hanlon RT, Claes MF, Ashcraft SE, Dunlap PV (1997) Laboratory culture of the sepiolid squid *Euprymna scolopes*: a model system for bacterial-animals symbiosis. Biol Bull 192:364–374

McFall-Ngai MJ, Heath-Heckman EAC, Gillette AA, Peyer SM, Harvie EA (2012) The secret languages of coevolved symbiosis: insights from the *Euprymna scolopes-Vibrio fischeri* symbiosis. Sem Immunol 24:3–8

Moltschaniwskyj NA, Carter CG (2010) Protein synthesis, degradation, and retention: mechanisms of indeterminate growth in cephalopods. Physiol Biochem Zool 83:997–1008

Moltschaniwskyj NA, Hall K, Lipinski MR, Marian JEAR, Nishiguchi MK, Sakai M, Shulman DJ, Sinclair B, Sinn DL, Staudinger M, Van Gelderen R, Villanueva R, Warnke K (2007) Ethical and welfare considerations when using cephalopods as experimental animals. Rev Fish Biol Fish 17:455–476

Moynihan M (1982) Notes on the behavior of *Euprymna scolopes* (Cephalopoda: Sepiolidae). Behav 85:25–41

Nabhitabhata J, Nilaphat P, Promboon P, Jaroongpattananon C (2005) Life cycle of cultured bobtail squid, *Euprymna hyllebergi* Nateewathana, 1997. Phuket Mar Biol Cent Res Bull 66:351–365

Nateewathana A (1997) The sepiolidae (Cephalopoda) of the Andaman Sea, Thailand, with description of *Euprymna hyllebergi* sp. nov. Phuket Mar Biol Cent Spec Publ 17:465–481

Nateewathana A, Nabhitabhata J, Nilphat P (2001) A new record of a bobtail squid, *Euprymna hyllebergi* Nateewathana, 1997, in the Gulf of Thailand. Phuket Mar Biol Cent Spec Publ 25:501–506

Norman MD, Lu CC (1997) Redescription of the southern dumpling squid *Euprymna tasmanica* and a revision of the genus *Euprymna* (Cephalopoda: Sepiolidae). J Mar Biol Assoc UK 77:1109–1137

Nyholm SV, Nishiguchi MK (2008) The evolutionary ecology of a sepiolid squid-vibrio association: from cell to environment. Vie Milieu 58:175–184

Reid AL, Jereb P (2005) Family Sepiolidae. In: Jereb P, Roper CFE (eds) Cephalopods of the world an annotated and illustrated catalogue of cephalopod species known to date vol. 1 chambered nautiluses and sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae and Spirulidae) FAO Species catalogue for fishery purposes no. 4, vol. 1. FAO, Rome

Reid AL, Norman MD (1998) Sepiolidae bobtail squids. In: Carpenter KE, Niem VH (eds) The living marine resources of the western central Pacific vol 2 cephalopods, crustaceans, holothurians and sharks. FAO, Rome

Ruby EG (1999) The *Euprymna scolopes*-*Vibrio fischeri* symbiosis: a biomedical model for the study of bacterial colonization of animal tissue. J Molec Microbiol Biotech 1:13–21

Ruby EG, Lee K (1998) The *Vibrio fischeri*-*Euprymna scolopes* light organ association: current ecological paradigms. Appl Env Microbiol 64:805–812

Ruby EG, McFall-Ngai MJ (1992) A squid that glows in the night: development of an animal-bacterial mutualism. J Bacteriol 174:4865–4870

Shears J (1988) The use of a sand-coat in relation to feeding and diel activity in the sepiolid squid *Euprymna scolopes*. Malacologia 29:121–133

Sinn DL (2005) From individuals to populations: personality traits in southern dumpling squid (*Euprymna tasmanica* Pfeffer, 1884) and their life history correlates. Doctor of Philosophy Thesis, University of Tasmania

Sinn DL, Moltschaniwskyj NA (2005) Personality traits in dumpling squid (*Euprymna tasmanica*): context-specific traits and their correlation with biological characteristics. J Comp Psychol 119:99–110

Sinn DL, Gosling SD, Moltschaniwskyj NA (2008) Development of shy/bold behaviour in squid: context-specific phenotypes associated with developmental plasticity. Anim Behav 75:433–442

Steer MA, Moltschaniwskyj NA, Nichols DS, Miller M (2004) The role of temperature and maternal ration in embryo survival: using the dumpling squid *Euprymna tasmanica* as a model. J Exp Mar Biol Ecol 307:73–89

Summers WC (1985) Comparative life history adaptations of some myopsid and sepiolid squids. NAFO Sci Stud 9:139–142

Chavez-Dozal et al. BMC Microbiology (2015) 15:226
DOI 10.1186/s12866-015-0560-z

BMC Microbiology

RESEARCH ARTICLE Open Access

Proteomic and metabolomic profiles demonstrate variation among free-living and symbiotic *vibrio fischeri* biofilms

Alba Chavez-Dozal, Clayton Gorman and Michele K. Nishiguchi*

Abstract

Background: A number of bacterial species are capable of growing in various life history modes that enable their survival and persistence in both planktonic free-living stages as well as in biofilm communities. Mechanisms contributing to either planktonic cell or biofilm persistence and survival can be carefully delineated using multiple differential techniques (*e.g.*, genomics and transcriptomics). In this study, we present both proteomic and metabolomic analyses of *Vibrio fischeri* biofilms, demonstrating the potential for combined differential studies for elucidating life-history switches important for establishing the mutualism through biofilm formation and host colonization.

Methods: The study used a metabolomics/proteomics or "meta-proteomics" approach, referring to the combined protein and metabolic data analysis that bridges the gap between phenotypic changes (planktonic cell to biofilm formation) with genotypic changes (reflected in protein/metabolic profiles). Our methods used protein shotgun construction, followed by liquid chromatography coupled with mass spectrometry (LC-MS) detection and quantification for both free-living and biofilm forming *V. fischeri*.

Results: We present a time-resolved picture of approximately 100 proteins (2D-PAGE and shotgun proteomics) and 200 metabolites that are present during the transition from planktonic growth to community biofilm formation. Proteins involved in stress response, DNA repair damage, and transport appeared to be highly expressed during the biofilm state. In addition, metabolites detected in biofilms correspond to components of the exopolysaccharide (EPS) matrix (sugars and glycerol-derived). Alterations in metabolic enzymes were paralleled by more pronounced changes in concentration of intermediates from the glycolysis pathway as well as several amino acids.

Conclusions: This combined analysis of both types of information (proteins, metabolites) has provided a more complete picture of the biochemical processes of biofilm formation and what determines the switch between the two life history strategies. The reported findings have broad implications for *Vibrio* biofilm ecology, and mechanisms for successful survival in the host and environment.

Keywords: *V. fischeri*, Symbiosis, Biofilms, Planktonic, Mass spectrometry, Liquid chromatography, Metabolomics, Proteomics

* Correspondence: nish@nmsu.edu
Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA

Background

Among biofilm communities, there are multiple biochemical interactions that shape the dynamic community that contrasts from free-living planktonic cells. Specifically, the notable stress resistance of biofilms has been associated with physiological changes that bacteria undergo during the transition to the biofilm state [1]. A vast amount of research in the last decade has focused on characterizing unique aspects of microbial biofilms, which include genomics and post-genomic functional approaches. These techniques have allowed a comparative molecular characterization of bacterial communities during various life history stages [2–4]. Numerous techniques include 16S rRNA sequencing for community composition analysis [5], mutational analysis of particular genes, RNA profiling [6], genomics [7], transcriptomics for capturing global views of genetic diversity and expression, and isotope-probing to link phylogeny with community function and processes [8]. These pioneering studies were helpful for their initial differential analysis of biofilm life history strategies, and have opened the way to genetic characterization of biofilm-single cell transitions in phenotype.

New approaches have become available that allow a complete differential profile including proteomic and metabolomic analysis [9]. Proteomic profiles help dissect the complexity of microbial communities by analyzing protein expression, function, modification, and interactions over temporal scales. Specific separation techniques coupled with mass spectrometry (MS) analysis are also essential for proteomic profiling of these extensive and diverse populations. One classic approach is the combination of two-dimensional (2D) protein gel electrophoresis followed by spot identification via isoelectric focusing [10]. In addition, high-throughput approaches are available for protein profiling such as shotgun proteomics, where proteins are digested and the generated peptides are identified by capillary liquid chromatography in tandem with mass spectrometry [10].

Metabolomics refers to the analytical approach used to study different cell products ("chemical fingerprints") that help to understand the physiological state of microorganisms [11]. This analysis is achieved with the use of liquid chromatography coupled with mass spectrometry (LC-MS), followed by detection, and quantification. Subsequent identification of metabolites is then accomplished by cluster analysis and mapping [11]. Although each type of analysis produces an extensive amount of information, combining complementary techniques could significantly contribute to our understanding of biofilm developmental processes. A more recent approach termed "meta-proteomics" aims to identify and combine protein and metabolic data to bridge the gap between phenotypic changes (planktonic cell to biofilm formation) with genotypic changes (reflected in protein/metabolic profiles) [12]. Earlier studies have integrated various system biology analyses (including proteomics, metabolomics and transcriptomics) for biofilms formed by *Bordetella pertussis* [13], *Leptospirillum sp.* [14], and *Pseudomonas fluorescens* [15]. However, there are no studies that integrate data collected via proteomic/metabolomic (meta-proteomics) data for *Vibrio* biofilms.

Vibrio fischeri is a mutualistic bioluminescent bacterium that infects the light organs of sepiolid squids and monocentrid fishes. *V. fischeri* produces bioluminescence that is used by the squid to avoid predation in a behavior known as counterillumination [16]. The mutualism is established when the host provides an appropriate niche for the bacteria to reproduce at much higher rates than in their free-living state [17]. *V. fischeri* is capable of forming biofilms both in seawater during its free-living stage and inside its host squid's light organ while in symbiosis [18]. Environmental and mutualistic biofilms differ in the sense of bacterial diversity, where multispecies biofilms dominate the seawater environment [1] and only one or a few *Vibrio* bacteria colonize and form biofilm inside the squid's light organ [19–22]. The ability of *V. fischeri* to form a biofilm community within its squid host plays a central role in the establishment and maintenance of the mutualism, as well as the degree to what functional molecules are produced and overexpressed for biofilm formation. Therefore, this study aims to describe the metabolic and proteomic profiles of a monospecies biofilm that is crucial for understanding what bacterial molecular components are important for establishing this mutualistic association. Proteomic and metabolomic analyses for both free-living (planktonic) and monospecies biofilm were completed for *V. fischeri* strain ETJB1H to provide the first partial *Vibrio* meta-proteome profile for single cell-to-biofilm physiology. Metabolomic analysis indicates several molecular changes that are the result of different biosynthetic pathways associated with exopolyssacharide (EPS) production and biofilm formation, as well as proteins that are important for persistence in the seawater environment.

Methods

Microorganism and biofilm formation experiments

Vibrio fischeri ETJB1H was isolated from the light organ of *Euprymna tasmanica* from Jervis Bay, Australia [23] and was used throughout this study. *V. fischeri* ETJB1H was routinely cultured on Luria Broth High Salt (LBS, 10 g tryptone, 5 g yeast extract, 20 g sodium chloride, 50 mL 1 M Tris pH 7.5, 3.75 mL 80 % glycerol and 950 mL distilled water) agar (15 %) and sub-cultured on LBS liquid media at 28 °C. Biofilm formation was grown as previously described [24, 25]. Briefly, three flasks of

250 mL with 100 mL of LBS media were inoculated with a 1:100 dilution from an overnight culture (of a 1.0 McFarland standard) and incubated for 24 h at 28 °C under static conditions. After incubation, planktonic cells were removed and flasks were briefly washed with LBS to remove any excess planktonic cells. Biofilm-forming bacteria (the ones that were tightly attached to the glass of the flask) were then removed by placing 100 mL of LBS and sonicating for 10 s three times under low intensity (40 %) power using the Bransonic 220 sonicator (Branson. Ultrasonic, Danbury, CT, USA). Biofilm cells were then concentrated and washed three times by spinning down the cultures at 10,000 xg for 15 min and removing any excess supernatant media. Each of the samples were divided in equal amounts for their proteomic 2-D page and shotgun analysis respectively (3 combined samples for the 2D page analysis and 3 combined samples for the shotgun analysis).

Protein preparation and 2-D PAGE electrophoresis

For protein extraction, we used the EasyLyse™ bacterial protein extraction solution (Epicentre technologies, Madison, WI) following manufacturer's instructions. In brief, a lysis solution was prepared as follows: 0.5 mL of D.I. water, 2 μl of 1 M $MgCl_2$, and 0.5 mL of Lysis Buffer and 1 μl of enzyme were added. A cell pellet consisting of aproximatelly 10^9 cells was added to 200 μL of the above solution. After incubation at room tempareture for 5 min, the samples were centrifugated and the supernatant (cell paste) was transferred to a clean tube. Fifty micrograms of each cell paste was prepared for first dimensional isoelectric focusing by adding four parts of lysis solution (7 M urea, 2 M thiourea, 1 % dithiothreitol, 2 % Pharmalyte 3–10, 0.5 % Triton X-100, 0.14 % phenylmethylsulfonyl fluoride) to one part of protein sample (volume per volume) as described previously [26].

The proteins are initially separated in the first dimension based on their isoelectric points; the focused proteins of the first dimension are subsequentelly separated in a second dimension based on their molecular masses. First, dimensional separation was completed using 17 cm IPG strips, pH 3–10. One microgram of the protein sample was loaded and isoelectric focusing was performed following the manufacturer's protocol (Bio-Rad, Richmond, CA). PROTEAN© IEF cell was used for the first separation at settings of 150 kVh and 23 °C. Strips were then equilibrated for 15 min in a buffer containing 2 % SDS, 6 M urea, 0.05 M Tris–HCl, pH 8.5, and 20 % glycerol with 2 % DTT (Dithiothreitol) and equilibrated again in the same buffer with 2.5 % iodoacetamide. The equilibrated strips were transferred to a PROTEAN II© version xi cell tank for second dimension run (30 mA per gel) in 10 % polyacrylamide gel, and then visualized after staining with Coomasie brilliant blue R250. Stained cells were covered with cellophane and air-dried overnight at room temperature. Gels were analyzed pairwise by eye for differences in their protein patterns [26]. In addition, differential analysis by Guild BioSciences proteome analysis service and a computer densitometric analysis of spots were completed using the Image Master Platinum 5.0 software (GE Healthcare, PA). A threshold of 2-fold change was used to determine significance between biofilm and planktonic groups. Gels were analyzed pairwise by eye for differences in their protein patterns by overlaying the gels on a light table, gels were then scanned into a computer graphics program (Adobe Photoshop 5.0) and one replicated is used as a reference.

Protein shotgun analysis

A fraction of the whole protein extractions (approximately 200 μL, which corresponds to 1 mg of total protein) were trypsin-digested. For digestion, the sample was reduced by adding 5 μL of DDT (Dithiothreitol, 200 mM: 1 mL of 100 M NH_4HCO_3 and 30.86 mg of DTT) and boiled for 10 min and then incubated for 1 h. Alkylation was achieved by adding 4 μL of iodoacetamide (1 M: 200 μL of 100 mM NH_4HCO_3 and 37 mg of iodoacetamide) and incubated for 1 h. Neutralization of the remaining iodoacetamide was achieved by adding 20 μL of DTT and incubating for 1 h. Trypsin was added to the mixture (1 mg for every 50 mg of protein) and complete digestion was accomplished after incubating for 18 h at 37 °C. Protein digests (approximately 100 μM) were analyzed by tandem mass spectrometry through cation exchange-reversed phase chromatography, utilizing a hybrid linear ion trap FT-ICR mass spectrometer with ultra performance liquid chromatograph (UPLC/MS, Agilent Technologies 110 Series, CA) with a capillary system attached to a quadruple ion time (Thermo LQT, Thermo Fisher Scientific, CA). Three technical replicates were analyzed for each combined sample. Peptide libraries were collected in a database (as a single .mgf file for each sample) searched against a marged database composed of reviewed entries of Uniprot database and analyzed with Mascot search engine (www.matrixscience.com). Mascot parameters include proteolysis by trypsin/chymotrypsin with size tolerances of 0.5 Da for peptide fragments, with a 95 % probability that the protein identified is not a random match. The false discovery rate (FDR) was calculated using the automated decoy database tool in MASCOT where decoy statistics were automatically calculated for all matches. Alternatively, FASTA sequences of target peptides previously identified were run in the program peptide cutter (web.expasy.org/peptide_cutter) and resulting fragments were compared to those identified in our analysis. A score of 35 % matching peptides (or higher) indicates a protein match [27].

Metabolomic analysis

To prepare samples for metabolite extraction, strains were inoculated in triplicate in 120 mL of Luria Bertani high salt media (LBS; per litre composition: 10 g tryptone, 5 g yeast extract, 20 g NaCl, 50 ml 1 M tris pH 7.5, 3.75 ml 80 % glycerol and 950 mL dH_2O). When cultures reached an OD_{580} of 1.0, they were pelleted at 10,000 xg for 10 min at 4 °C. Pellets were re-suspended in 10 mL of ice-cold phosphate buffered saline (PBS, pH 7.4) and cells were pelleted again under the same conditions. The supernatant was discarded and pellets were snap-frozen with liquid nitrogen. Bacterial cells were lyophilized for 24 h (Labconco model 7740020) and were analyzed by the Biotechnology Center at the University of Illinois at Urbana-Champaign (Metabolomics Center, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign). The approach used was a two-step LC/MS (Applied Biosystems 5500 QTrap with Agilent 1200 LC, Agilent Technologies, CA and Applied Biosystems CA) followed by targeted identification of differentially expressed metabolites using quadrupole time of flight (Q-TOF) MS/MS. Three technical replicates were used for this analysis.

Results

V. fischeri cells were grown in parallel as the planktonic (free-swimming) culture and as biofilms on the glass surface of the flask to identify and compare differences in protein profiles from the two physiological states. Cell viability was not affected after sonication for the biofilm cells, and there was no statistical difference between the number of Colony Forming Units of planktonic and biofilm samples collected after incubation time (24 h at 28 °C) for an OD_{600} = 1.0 (data not shown). The profiles presented correspond to a mature biofilm (structure achieved between 18 and 24 h of incubation) and free-living cells that did not form any biofilm. Two proteomic analysis approaches were used including i) Complete protein profile obtained by spot analysis, followed by differential two-dimensional gel electrophoresis, and ii) trypsin protein fractionation followed by shotgun identification (UPLC/MS) and peptide analysis (Mascot, peptide cutter). Metabolomic analysis was achieved through liquid chromatography coupled with mass spectrometry (LC/MS).

Identification of biofilm protein fractions by two-dimensional gel electrophoresis

Patterns of protein expression in biofilm communities were complex with an average of approximately 300 spots per gel. Using the planktonic protein gel as the reference, protein spots detected in the biofilm profile were matched against the reference. The number of matched spots was 140 with a total of 75 % gel coverage. Of these, 21 spots were up-regulated in a magnitude of 2 fold or more (Fig. 1 and Additional file 1: Figure S1). Table 1 lists the assay parameters used (isoelectric point and molecular weight of proteins) that correspond to spots numbered in Fig. 1. In addition, there were 59 spots that were unique to the biofilm state (not present in the planktonic stage) that are indicated in Fig. 2 and Table 2. All the proteins listed from this analysis could be detected reproducibly in the range of 15 to 130 KDa.

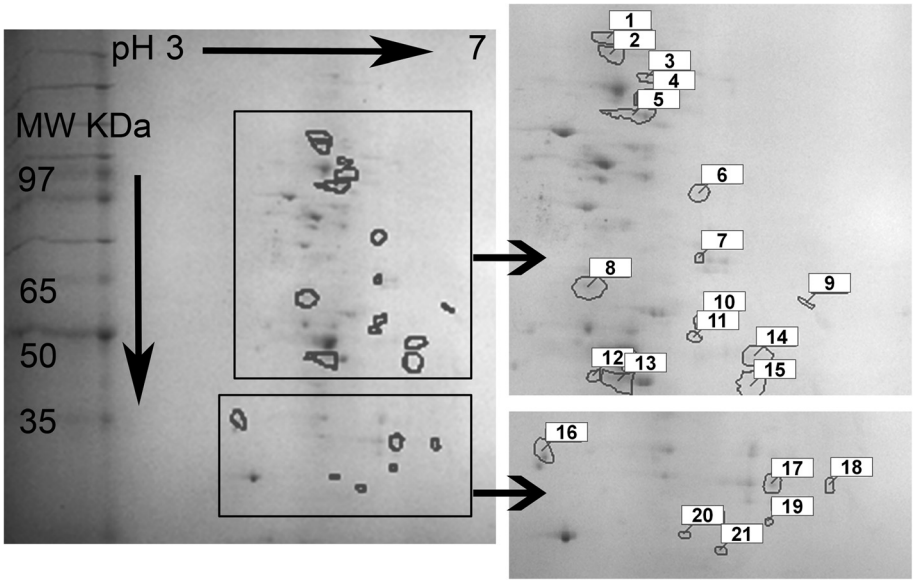


Fig. 1 2D-PAGE gel of the up-regulated *Vibrio fischeri* ETJB1H biofilm protein exudate. Colored squares correspond to magnification of fractions of the gel indicating a better resolution of spots. Numbers identify up-regulated proteins described in Table 1

Table 1 Biochemical properties of proteins identified to be up-regulated in the biofilm state of *Vibrio fischeri* ETJB1H

Spot	Isolectric point	Molecular weight (KDa)
1	3.97	111.51
2	3.98	105.53
3	4.20	96.30
4	4.22	89.63
5	4.12	84.35
6	4.52	70.82
7	4.52	59.62
8	3.85	55.37
9	5.17	53.52
10	4.53	51.82
11	4.49	50.35
12	3.88	47.64
13	4.03	47.27
14	4.86	49.00
15	4.83	46.98
16	3.22	39.60
17	4.68	35.06
18	5.05	34.74
19	4.65	29.76
20	4.12	28.51
21	4.35	30.66

Spot numbers correspond to proteins labeled in Fig. 2

Tandem mass spectrometry of peptides

The first scale proteomic analysis for *V. fischeri* using two-dimensional electrophoresis provides us with differentially expressed protein profiles that include both isoelectric points and molecular weight. In order to identify proteins that were present in the biofilm state of *V. fischeri*, a shot-gun approach was used to detect differentially expressed proteins by matching peptide mass data to available proteome sequence databases (www.ncbi.nlm.nih.gov and www.uniprot.org) using the keyword *"Vibrio fischeri* ES114". Additional analyses utilized the Mascot database. In order for a protein to be identified and considered present, tryptic peptides were required to be the primary identified hit in the database, and digests had to match at least 35 % of the complete protein compared with NCBI and Uniprot databases. In addition, theoretical isolectric points for the protein matches were calculated using algorithms from AnTheProt (antheprot-pbil.ibcp.fr) and Scansite3 (scansite3.mit.edu/#home). The criteria applied for identification resulted in a list of peptides that correlate with the molecular weight and isoelectric point detected for some protein spots observed to be either unique or up-regulated during biofilm production (Table 3).

Proteins related to multiple cellular processes were identified and listed in Table 3. The highest match corresponds to the catalytic enzyme UDP-N acetylglucosomine 1 carboxyvinyltransferase (UDP-GlcNAc) with a calculated MW/IP match to spot 33 (Table 2). Another enzyme detected corresponds to the adenosyltransferase cob(I)yrinic acid a,c-diamide transferase (MW/IP match

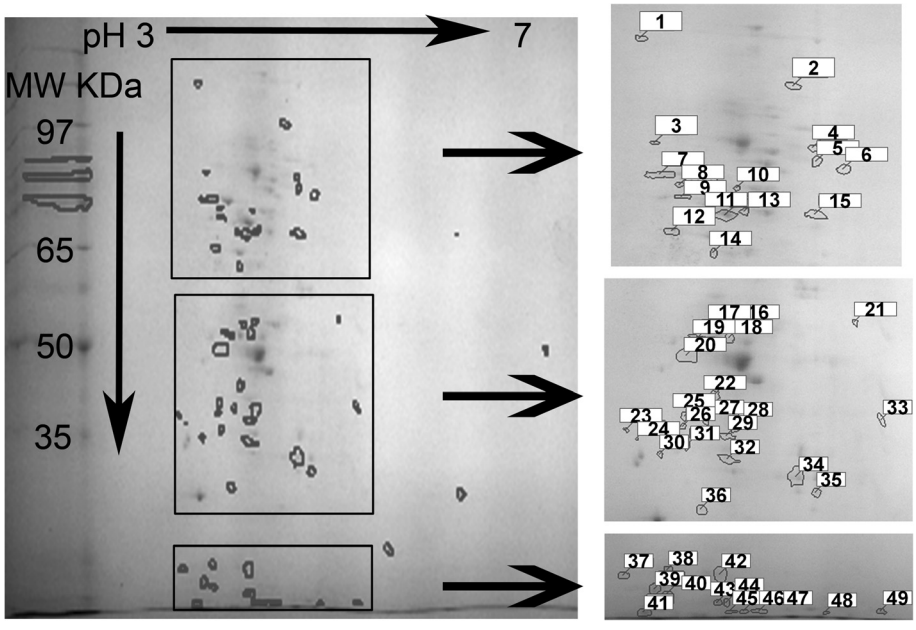


Fig. 2 2D-PAGE gel of unique *Vibrio fischeri* ETJB1H biofilm protein exudates. Colored squares correspond to magnification of fractions of the gel indicating an increased resolution of spots. Numbers identify unique biofilm proteins described on Table 2

Table 2 Biochemical properties of proteins identified to be unique in the biofilm state of *Vibrio fischeri* ETJB1H

Spot	Isolectric point	Molecular weight (KDa)
1	3.33	164.33
2	4.37	121.17
3	3.43	92.69
4	4.51	90.38
5	4.53	85.01
6	4.72	82.48
7	3.47	80.78
8	3.60	78.15
9	3.62	75.72
10	3.99	77.34
11	3.91	72.59
12	3.55	70.00
13	4.04	73.30
14	3.83	65.34
15	4.52	72.74
16	4.01	53.04
17	3.87	52.69
18	3.96	51.41
19	3.7	51.41
20	3.61	49.57
21	5.00	53.39
22	3.84	46.03
23	3.10	42.68
24	3.19	41.60
25	3.59	44.19
26	3.58	43.08
27	3.76	43.74
28	4.02	43.37
29	3.94	41.94
30	3.38	39.32
31	3.61	40.52
32	3.95	38.46
33	5.22	43.83
34	4.52	35.37
35	4.68	32.56
36	3.73	29.77
37	3.16	33.45
38	3.52	32.81
39	3.40	31.40
40	3.52	28.54
41	3.32	15.39
42	3.93	33.38
43	3.91	20.75
44	3.98	20.75
45	4.02	15.77
46	4.13	15.77
47	4.25	15.77
48	4.78	15.39
49	5.22	15.77

in #20 of Table 1). Porins and membrane transporters were detected in *V. fischeri* ETJB1H biofilms, including outer membrane protein U (OmpU; #37 in Table 2), membrane transporter ABC (#11 in Table 2), and multi-drug efflux pump (#2 in Table 2).

Interestingly, multiple stress-related proteins were detected in our study. These include the heat-stress response-related ATPase Clp protease (#6, Table 2), the carbon starvation protein A (#9, Table 1), the specific helicase RuvA related to Holiday junction formation (# 19, Table 1), the DNA double-strand repair protein RecA (#19, Table 1), the chaperone DnaK (#6, Table 1) and the transcriptional activator sigma 54 (#9, Table 1). Another set of proteins include flagellin (#13, Table 1), bioluminescence regulator (Lux R; #5, Table 1), oxidoreductase (#33, Table 1) and phosphate binding protein (#19, Table 1).

Metabolomic analysis of *Vibrio fischeri* ETJB1H biofilms

A mass spectrometry-based profiling method was used for constructing the metabolome of *V. fischeri* ETJB1H planktonic and biofilm stages. This comparison is the first metabolomic study to determine the chemical fingerprint of *V. fischeri* biofilms, as well as the important biochemical pathways involved in formation from planktonic state to the mature biofilm of *V. fischeri*.

Nominated altered chemicals (or biomolecules) were identified in both the planktonic and biofilm samples and are listed in Fig. 3 in a form of a heat map with a subset of color-coded metabolites (around 200) indicating critical fold changes between the two states. A concordance analysis of the two metabolic signatures (corresponding to the average of three analysis per condition, biofilm versus planktonic) indicates significant differences in metabolite profiles. Results for biofilm signatures indicate, up-regulated differences (2 fold) detected for multiple organic acids including carboxylic, phosphoric, aspartic, docosanoic, malonic, hydrobenzoic and keto-gluconic, as well as important sugars such as fructose, mannose, and maltose. Glycerol-derived components were also detected (hexadecanoglycerol, dodecanoglycerol, heptadecanoglycerol, and tetradecanoglycerol), and alcohols included mannitol and tetradecanol. Components that were observed to be significantly down-regulated (2–3 fold) in biofilms and

Table 3 Summary of *Vibrio fischeri* ETJB1H biofilm proteins identified by UPLC/MS

Reference[a]	Protein identified	Theoretical MW(KDa)/IP[b]	No. peptides matched	Coverage (%)
GI:59711008	UDP-N acetylglucosomine 1 carboxyvinyltransferase	44.72/5.28	20	60
GI:59479183	Outer membrane protein U porin OmpU	33.18/3.92	12	55
GI:59712357	Cob(I)yrinic acid a,c-diamide adenosyltransferase	28.31/4.08	10	53
GI:59710825	ABC transporter ATP-binding protein	72.70/4.50	22	48
GI:197317623	carbon starvation protein A	53.51/5.14	13	44
GI:59712372	ATP-dependent Clp protease ATP-binding subunit	82.58/4.8	31	40
GI:31414756	sigma 54 transcriptional activator	53.89/5.15	12	37
GI:59482580	multidrug efflux system protein	122.42/4.87	34	37
GI:59480318	phosphate-binding protein	29.45/5.00	8	36
GI:59710693	Oxidoreductase	43.69/5.25	10	36
GI:121308572	Bioluminescence regulatory protein	84.37/4.20	24	35
GI:172087731	Flagellin	48.09/4.05	9	35
GI:59711558	putative Holiday junction DNA helicase RuvA	29.78/4.71	9	35
GI:148536406	RecA protein	28.54/4.32	8	35
GI:59480175	chaperone, DnaK-like protein	69.88/4.52	10	35

[a]NCBI reference sequence
[b]Calculated using AnTheProt (http://antheprot-pbil.ibcp.fr/) and Scansite3 (http://scansite3.mit.edu/#home)

up-regulated in the planktonic state include threoic acid, hydroxypyrimidine, tyramine, and cellobiose (Fig. 3).

Discussion

In this study, we selected a meta-proteomics approach in order to resolve functional differences between the two life-history stages for *V. fischeri* (biofilms versus planktonic cells), and to describe factors that are important for successful colonization of the host, since the biofilm studied here resembles the community found in sepiolid squid light organs (for example, monospecificity, enriched environment, and availability of carbon sources), *Vibrio fischeri* biofilms and planktonic cells have been observed previously under Scanning Electron Microscopy [28–31]. There are distinct morphological differences; for example, planktonic cells are observed with flagellum and different shapes of fimbriae [28], whereas biofilms are observed as a flocculent bacterial mass encapsulated in a polysaccharide matrix [29, 31] structure that is similar to the one observed in biofilms formed in the squid's light organ [31].

Results indicate differences in protein expression levels, including the number of unique proteins detected, as well as those with enhanced levels of expression (Table 3). The two-dimensional approach used was limited to the inability to identify all the spots through mass spectrometry as previously reported [32]. Alternatively, identification of

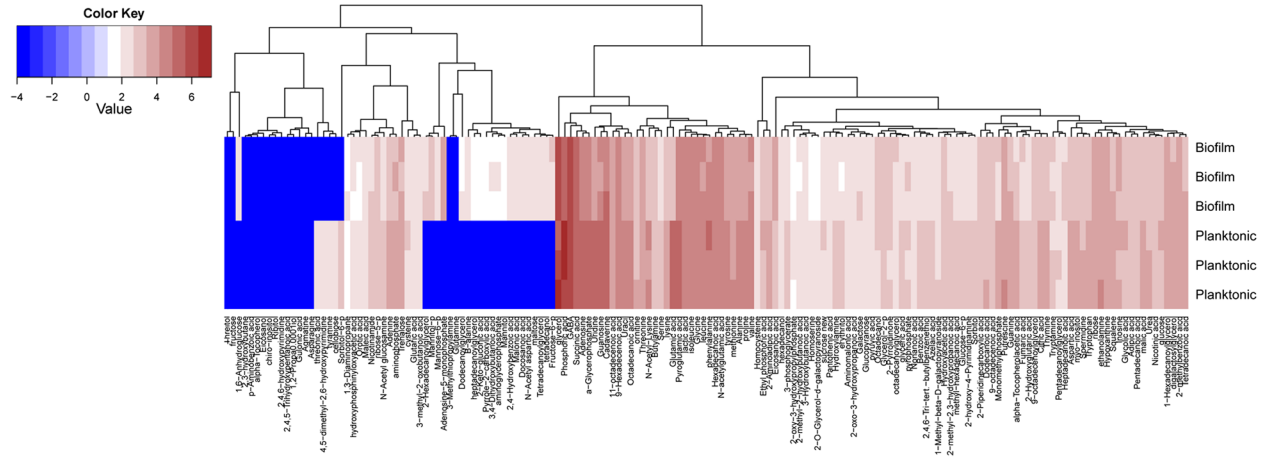


Fig. 3 Heat map indicating the metabolomic profile of biofilm and planktonic ETJB1H *Vibrio fischeri* strains. Data are shown in triplicate as a colored map reflecting the logarithms that relate to the metabolite changes (red areas indicate an increase in metabolite abundancy, blue areas depict a decrease). Log values are color coded as indicated in the scale

some proteins were achieved through shotgun proteomics (a combination of ultra-performance liquid chromatography and mass spectrometry or UPLC/MS), and allowed the comparison of both theoretical molecular weight and isoelectric points to experimental values from proteins identified in the two dimensional gel, which validated the combinational methodology. Proteins identified in this manner were then classified depending on their functions including: a) stress-response regulators, b) catalytic enzymes, c) transporters, d) metabolic enzymes, and e) structural proteins. Proteins that were previously reported overexpressed include those involved in energy generation (*e.g.*, succinyl-CoA synthase) and in biosynthesis (*e.g.*, ribosomal factors) [33–43]. Future studies will focus on more specific differences between these two community phenotypes (environmental versus host biofilms), since it has been observed that environmental versus symbiotic strains respond differently to stress, including fluctuations in temperature and salinity [25].

Upregulated proteins are important for maintenance and integrity of *V. fischeri* biofilms

In *Vibrio fischeri,* flagellin is expressed constitutively, and is esseantial for host colonization [39–41]. *V. fischeri* contains between one and five flagellar filaments that form a tuft of polar sheathed flagella [39]. *V. fischeri* flagellins are more similar to each other than to flagellins of other *Vibrio* species [38, 39], and therefore was not possible to differentiate the type of flagellin detected in this study. However, two proteins detected might be the closest match: (i) the flagellin FlgA that has been reported to be important for initial stages of host colonization [39] and (ii) FlgF, found to be important for host colonization and biofilm formation [39, 41].

Stress response proteins were also identified in this study. Elevated expression of the chaperone Clp was detected here. Clp proteins are known to regulate virulence in pathogenic bacteria such as *Porphyromonas gingivalis* [42] and *Vibrio cholerae* [43], and increased concentrations observed in *V. fischeri* biofilms might be related to increased success in host colonization, since it is believed that strong biofilm formers are also excellent host colonizers [1]. More interestingly, as reported for *V. cholerae*, Clp chaperone may be linked to the control of oxidative stress within the biofilm matrix (this would also include oxidoreductase, which was detected in this study). Oxidative stress is thought to be a result of a combination of slow growth in conjunction with a shift in oxygen at different depths of the biofilm [44]. Additional stress-related proteins were overexpressed in biofilms, including DnaK (molecular chaperone important for protein protection from denaturalization) [45] and the carbon starvation protein A (promotes peptide utilization during carbon starvation) [46]. It has been suggested that different micro-niches within the biofilm community are continuously exposed to various environmental stresses, inducing an increase of stress resistance mechanisms [47, 48].

Stress-inducible biofilm formation also produces DNA damage, which can trigger the bacterial SOS response initiated by the sensor protein RecA [49, 50], which was overexpressed in the biofilm samples. In addition, these methods detected the protein RuvA (responsible for Holiday junction formation as well as initiation of the SOS response). RuvA along with RuvB in the presence of ATP release the cruciform structure formed during strand exchange during homologous recombination [51], which might also occur in *Vibrio* biofilm communities. Since expression of proteins related to DNA repair can be synthesized up to 10 times more in biofilms [52, 53], this may result in undetectable traces in planktonic bacteria.

Another important component detected in *V. fischeri* biofilms is sigma factor 54 (σ^{54}) that has been reported to be an important regulator of a wide range of bacterial processes, including nitrogen metabolism in *Escherichia coli* [54], biogenesis of flagella in *Vibrio parahaemolyticus* and *Vibrio cholerae* [55, 56], and bioluminescence in *Vibrio harveyi* [57]. More interestingly, σ^{54} in *V. fischeri* is encoded by the *rpoN* gene [58] that is overexpressed during the biofilm state, controlling flagellar biosynthesis (motility), nitrogen assimilation, luminescence, and biofilm formation [58].

Biofilms cells expressed a presumptive ABC transporter, which corresponds to a major class of translocation machinery in multiple bacterial species [59, 60]. ABC transporters have been previously identified to be differentially expressed during biofilm formation in *Pseudomonas aeruginosa* and *E. coli* [61, 62] and they may be linked to the transport of small molecules and solutes during the formation and maintenance of the mature biofilm compared to the cells in their planktonic state. In addition, the detected ABC transporter might influence cytoplasmic pH homeostasis by increasing transmembrane fluctuation of ions (for example K+) to allow compensation after pH stress (or osmoprotection). It is known that the light organ of sepiolid squids undergoes anaerobic stress based on fermentation genes expressed solely in the light organ environment [16], and possible drops in pH may be due to the acid by-products accumulating during this time.

The outer-membrane protein detected in this study (OmpU) has been previously identified in symbiotic *V. fischeri*, and has an important role in the initiation of colonization of the squid light organ [37]. In addition, disruption of the *ompU* gene results in increased sensitivity to membrane-disrupting chemical agents such as chlorine and organic acids [37]. These observations indicate that OmpU might have an important role in maintaining membrane integrity during *V. fischeri* biofilm development by providing defense mechanisms that are essential for resistance to the acidic environment within the biofilm matrix.

Biofilm development is guided by several regulatory systems. One of the important processes is formation of the exopolyssacharide (EPS) matrix, a hallmark of bacterial biofilms. UDP-GlcNAc (UDP-N acetylglucosomine 1 carboxyvinyltransferase) was detected in this study, and has an important role in the synthesis of EPS by acting as a transcriptional regulator [63].

Phosphatase-binding proteins have been described to increase production of the second messenger cyclic diguanylic acid (c-di-GMP) in *Pseudomonas aeruginosa* [64] and this protein was prevalent in our study. C-di-GMP is a central regulator of the prokaryote biofilm lifestyle [65], including *V. fischeri* biofilms.

There are multiple proteins that regulate bioluminescence. In particular, biofilm formation and bioluminescence are linked through proteins that regulate bacterial communication or quorum sensing [66]. One protein detected in this study (bioluminescence regulatory protein) may up-regulate the quorum sensing cascade, which, among other functions, has been reported to increase production of EPS [66]. This finding is particularly important for host-related biofilms and host survival since bacterial bioluminescence (increased in high bacterial density, as in the case of biofilms and not planktonic cells) is the main process that sepiolid squids use for the counterillumination (silhouette reduction from the moonlight at night). Therefore, in *V. fischeri*, regulation of bioluminescence is activated when bacterial concentration significantly increases in number and proximity (in the case of biofilms) [66–68]. Provided that the bioluminescence protein was detected in a biofilm that was formed under laboratory conditions, a similar bacterial community (and protein expression) is present in the squid host.

Metabolomic profile revelas an increased number of components of the biofilm matrix

Most *Vibrio* biofilm matrices are composed of polysaccharides, such as the VPS (*Vibrio* polysaccharide), present in *V. cholerae* biofilms [1]. This metabolomic study detected carbohydrates that were present during the biofilm state and absent in planktonic cells. Proteins present consisted of mannose, maltose, fructose, and other monomeric sugars (galactose and glucose). In addition, smaller amounts of N-acetylglucosamine and N-acetyl glutamic acid were detected and have been described to be part of the VPS [69]. The presence of multiple glycerol-derived metabolites suggests that biofilm cells may use phospholipids released from neighboring cells, which possibly serve as a carbon source for amino acid biosynthesis. This metabolomic study revealed the presence of highly phosphorylated (and non-phosphorylated) glucans, which have been identified to be associated with the matrix of strains of *P. aeruginosa* [70]. Metabolites detected are important for synthesis of EPS components, or are related to regulatory processes involving second messengers (such as c-di-GMP). These components are strictly unique of community formation, but further research is required in order to determine if these metabolites are also important for mutualistic associations and what could be the metabolic differences between environmental and mutualistic biofilms. Future studies are needed in order to test whether glycerol and phosphate-derived components (detected in this study) are dominant in the squid's light organ and how these components may contribute to host specificity and maintenance of symbiosis integrity.

Conclusions

The objective of this study was to examine conserved proteomic and metabolomic signatures of both planktonic/free-living *V. fischeri* and their biofilm communities. Our results establish the methodology to utilize meta-proteomic analysis, enabling a more detailed perspective for understanding the biochemistry and metabolism of growth between the free-living/planktonic and community biofilm stages in a mutualistic bacterium. This meta-proteomic approach also improves the understanding of biofilms at a molecular level that is different from a transcriptomic or genomic comparisons (at the "functionality" level, which includes endpoint products, proteins, and metabolites). Results indicate a clear divergence associated with the restructuring of regulatory networks that allow community formation. Unique proteins and metabolites (mostly related to stress-responses, formation of the biofilm matrix and phosphorylated components) were significantly overexpressed in the biofilm state when compare to the free-living planktonic cells. Future work will entail combination of more differential studies (transcriptomics) to link the role of candidate genes to biochemical pathways and protein functionality.

Additional file

Additional file 1: Figure S1. 2D-PAGE gel of unique spots presents in protein exudates from A) *Vibrio fischeri* ETJB1H planktonic cells and B) *Vibrio fischeri* ETJB1H biofilm cells. Circles indicate the spots that are unique for each protein extraction. Spot detection revealed 271 spots for the planktonic cells and 199 spots for biofilm cells. Using planktonic cells as the reference profile, there were a total of 21 spots upregulated and 52 downregulated for the biofilm cells. (DOCX 656 kb)

Abbreviations

V. fischeri: *Vibrio fischeri*; ES114: *Euprymna scolopes* st. 114; ETJB1H: *Euprymna tasmanica* st. Jervis Bay 1H; 2D-PAGE: Two-dimensional PolyAcrylamide Gel Electrophoresis; EPS: Exopolysaccharide; MS: Mass Spectrometry; LC: Liquid Chromatography; LBS: Luria Broth high Salt; IPG: Immobilized pH Gradient; mA: Milliampere; DTT: Dithiothreitol; SDS: Sodium Dodecyl Sulfate; FT-ICR: Fourier Transform-Ion Cyclotron Resonance; Da: Dalton; KDa: Kilodalton; FDR: False Discovery Rate; Q-TOF: Quadruple-Time of Flight; UPLC: UltraPerformance Liquid Chromatography; MW: Molecular Weight; IP: Isoelectric Point; c-di-GMP: Cyclic diguanylate; VPS: *Vibrio* Polysaccharide.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Conceived and designed the experiments: M.K.N., A.C.-D. Performed the experiments: A.C.-D., C.G. Analyzed the data: A.C.-D. Contributed materials/analysis tools: M.K.N. Wrote the paper: M.K.N., A.C.-D. Final approval of manuscript: M.K.N., A.C.-D., C.G. All authors read and approved the final manuscript.

Authors' information
Not applicable.

Acknowledgements
We thank Dr. Augustine A. DiNovo from Guil Biosciences Company for his assistance with the 2D Page electrophoresis proteomic analysis, and Dr. Lucas Li from the Roy J. Carver Biotechnology Center (CBC) at the University of Illinois for the Metabolomic analysis.
A.C.-D. was supported by RISE (NIH NIGMS R25GM061222), NASA (NMSGC) and the IDIP T32 fellowship AI007538 at the University of New Mexico. C.G. was supported by the NMSU NIH MARC program (NIH-GM0007667-34). This project was partially supported by NSF IOS-0744498, NIH NIAID 1SC1AI081659-01, and NIH ARRA-3SC1AI081659-02S1 to M.K.N.

Received: 9 May 2015 Accepted: 8 October 2015
Published online: 23 October 2015

References

1. Yildiz F, Visick K. *Vibrio* biofilms: so much the same yet so different. Trends in Microbiol. 2008;17(3):109–18.
2. Dopheide A, Lear G, Stott R, Lewis G. Relative diversity and community structure of ciliates in stream biofilms according to molecular and microscopy methods. Appl Environ Microb. 2009;75:5261–72.
3. Kim T, Sohn S, Kim H, Lee S. Strategies for systems-level metabolic engineering. J Biotechnol. 2008;3:612–23.
4. Kim S, Wei C. Molecular characterization of biofilm formation and attachment of *Salmonella enterica* serovar *typhimurium* DT104 on food contact surfaces. J Food Prot. 2009;72(9):1841–7.
5. Price L, Liu C, Melendez J, Frankel Y, Engelthaler D, Aziz M, et al. Community Analysis of Chronic Wound Bacteria Using 16S rRNA Gene-Based Pyrosequencing: Impact of Diabetes and Antibiotics on Chronic Wound Microbiota. PLoS ONE. 2009;4(7):1.
6. Resch A, Leicht S, Saric M, Pasztor L, Jakob A, Gotz F, et al. Comparative proteome analysis of *Staphylococcus aureus* biofilm and planktonic cells and correlation with transcriptome profiling. Proteomics. 2006;6(6):1867–77.
7. Jones D, Albrecht D, Dawson K, Schaperdoth I, Freeman K, Pi Y, et al. Community genomic analysis of an extremely acidophilic sulfur-oxidizing biofilm. ISME. 2012;6(1):158–70.
8. Neufeld JD, Wagner M, Murrel JC. Who eats what, where and when? Isotope-labeling experiments are coming of age. ISME. 2007;1(2):103–10.
9. Steinberg C. The actual response: ecological proteomics and metabolomics. Stress Ecology pp. 2012;241–278.
10. Patrauchan M, Sarkisova S, Franklin M. Strain-specific proteome responses of *Pseudomonas aeruginosa* to biofilm-associated growth and to calcium. Microbiology. 2007;153(11):3838–51.
11. Booth SC, Workentine ML, Wen J, Shaykhutdinov R, Vogel HJ, Ceri H, et al. Differences in metabolism between the biofilm and planktonic response to metal stress. J Proteome Res. 2011;10(7):3190–9.
12. Kleiner M, Wentrup C, Lott C, Teeling H, Wetzel S, Young J, et al. Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy source. Proc Natl Acad Sci USA. 2012;109(19):E1173–82.
13. Serra DO, Lucking G, Weiland F, Schulz S, Gorg A, Yantorno OM, et al. Proteome approaches combined with Fourier transform infrared spectroscopy revealed a distinctive biofilm physiology in *Bordetella pertussis*. Proteomics. 2008;8(23–24):4995–5010.
14. Wilmes P, Remis JP, Hwang M, Auer M, Thelen MP, Banfield JF. Natural acidophilic communities reflect distinct organismal and functional organization. The ISME J. 2009;3:266–70.
15. Booth S, George IAS, Zannoni D, Cappelletti M, Duggan GE, Ceri H, Turner RJ (2013) Effect of aluminum and copper on biofilm development of *Pseudomonas pseudoalcaligenes* KF707 and *P. fluorescens* as a function of different media compositions. Metallomics. doi:10.1039/C3MT20240B
16. Jones BW, Nishiguchi MK. Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Marine Biol. 2004;144:1151–5.
17. Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK. Spatial and temporal distribution on the Vibrionaceae in costal waters of Hawaii, Australia and France. Microbial Ecol. 2007;54:314–23.
18. Nyholm SV, McFall-Ngai MJ. The winnowing: establishing the squid-*Vibrio* symbiosis. Nature. 2004;2:632–42.
19. Fidopiastis P, Miyamato C, Jobling M, Meighen E, Ruby E. LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization. Mol Microbiol. 2002;45:131–43.
20. Fidopiastis P, Von B, Ruby E. A new niche for *Vibrio logei*, the predominant light organ symbiont of the Mediterranean sepiolid squids *Sepiola affinis* and *S. robusta*. J Bacteriol. 1998;180:59–64.
21. Nishiguchi MK. Temperature affects species distribution in symbiotic populations of *Vibrio*. Appl Environ Microbiol. 2000;66:3550–5.
22. Guerrero-Ferreira RC, Gorman C, Chavez-Dozal A, Wille S, Nishiguchi MK. Characterization of the bacterial diversity of Indo-West Pacific loliginid and sepiolid squid light organs. Microb Ecol. 2012;65:214–22.
23. Jones BW, Lopez JE, Huttenberg J, Nishiguchi MK. Population structure between environmentally transmitted *Vibrios* and bobtail squids using nested clade analysis. Mol Ecol. 2006;15:4317–29.
24. Merritt JH, Kadouri DE, O'Toole GA (2005) Growing and analyzing static biofilms. Curr Protocols Biol. 1B.1.1-1.1.17. doi:10.1002/9780471729259.mc01b01s00.
25. Chavez-Dozal A, Nishiguchi MK. Effect of environmental factors on biofilm formation by *Vibrio fischeri* isolates. J Basic Microbiol. 2011;5:452–8.
26. Doino Lemus J, McFall-Ngai M. Alterations in the proteome of the *Euprymna scolopes* light organ in response to symbiotic *Vibrio fischeri*. Appl Environ Microbiol. 2000;66(9):4091–7.
27. Kayser JP, Vallet JL, Cerny RL. Defining parameters for homology-tolerant database searching. J Biomol Tech. 2004;15(4):285–95.
28. Nair VN, Nishiguchi MK. Biological properties (in vitro) exhibited by free-living and symbiotic *Vibrio* isolates. Vie et Milieu. 2009;59:277–85.
29. Ariyakumar DS, Nishiguchi MK. Characterization of two host-specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol Lett. 2009;299:65–73.
30. Chavez-Dozal A, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK. Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. FEMS Microbial Ecol. 2012;81(3):562–73.
31. Nyholm SV, Nishiguchi MK. The evolutionary ecology of a sepiolid squid-*Vibrio* association: From cell to environment. Vie et Milieu. 2008;58:175–84.
32. Gauci VJ, Wright EP, Coorssen JR. Quantitative proteomics: assessing the spectrum of in-gel protein detection methods. J Chem Biol. 2011;4(1):4–29.
33. Kalmokoff M, Lanthier P, Tremblay TL, Foss M, Lau PC, Sanders G, et al. Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. J Bacteriol. 2006;188(12):4312–20.
34. Sanchez CJ, Kumar N, Lizcano A, Shivshankar P, Hotopp JCD, Jorgensen JH, et al. *Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. PLoS ONE. 2011;6(12):e28738.
35. Williamson KS, Richards LA, Perez-Osorio AC, Pitts B, McInnerney K, Stewart PS, Franklin MJ (2012) Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. J Bacteriol 194(9): doi:10.1128/JB.00022-12
36. Guerrero-Ferreira R, Nishiguchi MK. Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. Environ Microbiol Rep. 2010;2(4):514–23.
37. Aeckersberg F, Lupp C, Feliciano B, Ruby EG. *Vibrio fischeri* outer membrane protein OmpU plays a role in normal symbiotic colonization. J Bacteriol. 2001;183(22):6590–7.
38. Klose KE, Mekalanos JJ. Differential regulation of multiple flagellins in *Vibrio cholerae*. J Bacteriol. 1998;180(2):303–16.
39. Millikan DS, Ruby EG. Flagellin A is essential for normal motility and for symbiotic competence during initial squid light organ colonization. J Microbiol. 2004;186:4315–25.

40. Millikan DS, Ruby EG. Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. Appl Environ Microbiol. 2002;68:2519–28.
41. Chavez-Dozal A, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK. Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. FEMS Microbial Ecol. 2012;81(3):562–73.
42. Capestany CA, Tribble GD, Maeda K, Demuth DR, Lamont RJ. Role of the Clp system in stress tolerance, biofilm formation, and intracellular invasion in *Porphyromonas gingivalis*. J Bacteriol. 2008;190(4):1436–46.
43. Nag S, Das S, Chaudhury K. *In vivo* induced clpB1 gene of *Vibrio cholerae* is involved in different stress responses and affects *in vivo* cholera toxin production. Biochem Biophys Res Commun. 2005;331(4):1365–73.
44. Boles BR, Singh PK. Endogenous oxidatives stress produces diversity and adaptability in biofilm communities. Proc Natl Acad Sci USA. 2008;105(34):12503–8.
45. Gotoh H, Zhang Y, Dallo SF, Hong S, Kasaraneni N, Weitao T. *Pseudomonas aeruginosa*, under DNA replication inhibition, tends to form biofilms via Arr. Res Microbiol. 2008;159(4):294–302.
46. Schultz JE, Matin A. Molecular and functional characterization of a carbon starvation gene of *Escherichia coli*. J Mol Biol. 1991;218(1):129–40.
47. Bernier SP, Lebeaux D, DeFrancesco AS, Valomon A, Soubigou G, Coppee JV, et al. Starvation, togheter with the SOS response, mediates high biofilm specific tolerance to the fluoroquinolone ofloxacin. PLoS Genet. 2013;9(1):e10033144.
48. Costerton JW, Lewadowski DE, Caldwell DR, Korber DR, Lappin-Scott HM. Microbial biofilms. Annual Rev Micro boil. 1995;49:711–45. ISSN 0066–4227.
49. Gotoh H, Zhang Y, Dallo SF, Hong S, Kasaraneni N, Weitao T. *Pseudomonas aeruginosa*, under DNA replication inhibition, tends to form biofilms via Arr. Res Microbiol. 2008;159(4):294–302.
50. Uranga LA, Balise VD, Benally CV, Grey A, Lusetti SL. The *Escherichia coli* DinD protein modulates RecA activity by inhibiting postsynaptic RecA filaments. J Biol Chem. 2011;286(34):29480–91.
51. Van Gool AJ, Hajibagheri NMA, Stasiak A, West SC. Assembly of the *Escherichia coli* RuvABC resolvasome directs the orientation of holliday junction resolution. Gen Develop. 1999;13:1861–70.
52. Marks LR, Reddinger RM, Hakansson AP. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. mBio. 2012;3(5):e00200–12.
53. Pysz MA, Conners SB, Montero CI, Shocley KR, Johnson MR, Ward DE, et al. Transcriptional analysis of biofilm formation processes in the anaerobic hyperthermophilic bacterium *Thermotoga maritima*. Appl Environ Microbiol. 2004;70(10):6098–112.
54. Reitzer L, Schneider BL. metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. Microbiol Mol Biol Rev. 2001;65(3):422–44.
55. Stewart BJ, McCarter LL. *Vibrio parahaemolyticus* FlaJ, a homologue of FliS, is required for production of a flagellin. Mol Microbiol. 1996;20:137–49.
56. Prouty MG, Correa NE, Klose KE. The novel sigma54 and sigma 28-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. Mol Microbiol. 2001;39(6):1595–609.
57. Lilley BN, Bassler BL. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol Microbiol. 2000;36(4):940–54.
58. Wolfe AJ, Millikan DS, Campbell JM, Visick KL. *Vibrio fischeri* sigma-54 controls motility, biofilm formation, luminescence, and colonization. Appl Environ Microbiol. 2004;70(4):2520–4.
59. Tomii K, Kanehisa K. A comparative analysis of ABC transporters in complete microbial genomes. Genome Res. 1998;8:1048–59.
60. Cuthbertson L, Kos V, Whitfield C. ABC transporters involved in export of cell surface glycoconjugates. Micorbiol Mol Biol Rev. 2010;74(3):341.
61. Hentzer M, Eberl L, Givskov M. Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. Biofilms. 2005;2:37–61.
62. Whitfield C. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu Rev Biochem. 2006;75:39–68.
63. Steiner S, Lori C, Boehm A, Jenal U. Allosteric activation of exopolysaccharide synthesis through cyclic di-GMP-stimulated protein-protein interaction. The EMBO J. 2013;32:354–68.
64. Ueda A, Wood TK. Connecting quorum sensing, ci-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TbpA (PA3885). PLoS Pathog. 2009;5, e1000483.
65. Cotter PA, Stibitz S. c-di-GMP mediated regulation of virulence and biofilm formation. Curr Opin Microbiol. 2007;10(1):17–23.
66. Hammer BK, Bassler BL. Quorum sensing controls biofilm formation in *Vibrio cholerae*. Mol Microbiol. 2003;50(1):101–4.
67. Peat SM, Adams BJ. Natural selection on the *luxA* gene of bioluminescent bacteria. Symbiosis. 2008;46:101–8.
68. Kierek K, Watnick PI. Environmental determinants of *Vibrio cholerae* biofilm development. Appl Environ Microbiol. 2003;69(9):5079–88.
69. Fong JCN, Syed KA, Klose KE, Yildiz FH. Role of *Vibrio* polysaccharide (*vps*) genes in VPS production, biofilm formation and *Vibrio cholerae* pathogenesis. Microbiol. 2010;156(9):2757–69.
70. Coulon C, Vinogradov E, Filloux A, Sodovskaya I. Chemical analysis of cellular and extracellular carbohydrates of a biofilm-forming strain *Pseudomonas aeruginosa* PA14. PLoS ONE. 2010;5(12), e14220.

VIE ET MILIEU - LIFE AND ENVIRONMENT, 2015, 65 (4): 187-200

GENOMICS OF AN ENVIRONMENTALLY-TRANSMITTED SYMBIOSIS: NEWLY SEQUENCED *VIBRIO FISCHERI* GENOMES FROM DIFFERENT HOST SQUIDS AND GEOGRAPHIC LOCATIONS

J. H. HOWARD[1], B. L. WILLIAMS[2], M. BREEN[3], M. K. NISHIGUCHI[*4]*

[1] Pennsylvania State University-Abington, Department of Biology, 1600 Woodland Road, Abington, Pennsylvania 19001-3990, USA
[2] Utah State University Uintah Basin, Department of Biology, 320 N 2000 W (Aggie Blvd), Vernal, Utah 84078-4228, USA
[3] Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, University Rd, Southampton SO17 1BJ, United Kingdom
[4] New Mexico State University, Department of Biology, BOX 30001, MSC 3AF, Las Cruces, New Mexico 88003-8001, USA
** Corresponding author: nish@nmsu.edu*

VIBRIO FISCHERI
GENOME
SYMBIOSIS
BOBTAIL SQUID
EUPRYMNA
SEPIOLA

ABSTRACT. – Environmentally-transmitted symbiotic bacteria must balance selective pressures of host specificity and the abiotic environment. Here, we investigate genomes of three strains of *Vibrio fischeri* isolated from three different squid species living in various temperature and salinity environments. These sequences were compared to others from symbiotic and free-living vibrios for gene orthology, functional subsystem, selection, and phylogenetic analyses. A *V. cf. campbellii* bacterium isolated from seawater also was sequenced and provided an outgroup for the selection and phylogenetic analyses. This investigatory study provides the basis for further directed studies that may elucidate the genetic architecture underlying adaptation to both the free-living environment and host squids in *V. fischeri*. Symbiotic vibrios in this study had relatively conserved genomes with evidence of strong purifying selection and few duplication events. Horizontal gene transfer may provide a mechanism for the acquisition of host and environment-specific genes. Despite spending a majority of time in the free-living stage and a low probability of host-colonization for any one bacterium, symbiotic *V. fischeri* appear to have evolutionary histories molded by their squid hosts.

INTRODUCTION

Environmentally-transmitted symbiotic bacteria must be able to survive both within and external to their specific host. In opposition to vertically transmitted symbioses, hosts are axenic at birth and acquire symbionts from the environment. How symbionts actually balance these two environments has broad implications for pathogen transmission, coevolutionary theory, cooperation, and stability of symbiosis dynamics (Nishiguchi *et al*. 2008, Nyholm & Nishiguchi 2008, Sachs & Simms 2006, Sachs *et al*. 2011). Attempts to map the genetic basis of adaptation to either free-living or host environment have greatly increased with the ability to rapidly sequence whole genomes (Hudson 2008, Nadeau & Jiggins 2010, Stapley *et al*. 2010). For example, genetic signatures for pathogenicity in a *Streptococcus* strain have been identified (Suzuki *et al*. 2011), as have differences in metabolic capabilities in rumen symbionts *Prevotella* (Purushe *et al*. 2010), differences in cold tolerance in *Neurospora* (Ellison *et al*. 2011), and phosphorus acquisition in phosphorus-limited environments in *Pelagibacter* and *Prochlorococcus* (Coleman & Chisholm 2010). Not only can influential point mutations be identified, but whole genome patterns, such as duplication events, expansion or reduction of the core genome, composition of the accessory genome, and rearrangement of regulatory elements can be identified and investigated for their role in adaptation (Gilad *et al*. 2009, Levasseur & Pontarotti 2011, Medina & Sachs 2010).

We investigated an environmentally-transmitted symbiosis between bobtail squids and their *Vibrio* symbionts because of the breadth of host species and environmental conditions, as well as the wealth of prior knowledge available about this symbiotic complex. We examined three strains of *V. fischeri* isolated from different host species of squid from different geographic locations to catalog the genetic diversity of this complex system as a launching point for more in-depth comparative studies that might pinpoint the functional significance of genetic elements involved in adaptation to either host or free-living environment.

*The sepiolid squid-*Vibrio *model system*

Bobtail squids (Cephalopoda: Sepiolidae) harbor bioluminescent bacterial symbionts (γ-Proteobacteria: Vibrionaceae) in a specialized organ that directs light downwards to mask the squids' silhouette from benthic predators (Jones & Nishiguchi 2004). Axenic squid hatchlings are colonized through a "sieve" of host defenses that selects for only coevolved mutualism-competent strains (Nyholm & McFall-Ngai 2004). The squid and their colonists then enter a diel cycle (Boettcher *et al*. 1996), whereby the vibrios first grow and populate the light organ during the day while the squid is quiescent and hidden in the

sand. During the evening the squid emerges and bacterial bioluminescence, which is triggered by quorum sensing that occurs at high bacterial densities (Eberhard 1972, Fuqua *et al*. 1994, Nealson 1977), is directed ventrally by the squid to match downwelling moonlight (Jones & Nishiguchi 2004). Upon sunrise, the squid vents 90 % of the bacteria (Graf & Ruby 1998). The venting process seeds the surrounding seawater with a high number of potential vibrio symbionts capable of colonizing newly-hatched squids (Lee & Ruby 1994, Ruby & Lee 1998). Thus, symbiotic *Vibrio* must navigate both the host and free-living environments.

Coevolution of the symbionts and hosts has influenced colonization success. Some bacterial strains can efficiently colonize multiple squid species, as observed in the Mediterranean Sea where sympatric squid species live (Nishiguchi 2000, Nishiguchi *et al*. 2008, Nyholm & McFall-Ngai 2004, Nyholm & Nishiguchi 2008, Ruby 2008), while others are more host-specific, as demonstrated in the Indo-West Pacific where there is no geographical overlap among squid species (Nishiguchi 2000, Jones *et al*. 2006, Jones *et al*. 2007, Soto *et al*. 2009a, Zamborsky & Nishiguchi 2011). Fine sequence variation in genes required for the mutualism affect host specificity (Chavez-Dozal *et al*. 2014). In competition experiments using *Vibrio* isolates from different squid species, squids show preference for their native bacterial strains, and a hierarchy of competitive dominance exists (Chavez-Dozal *et al*. 2014, Jones *et al*. 2006, Nishiguchi *et al*. 1998, Nishiguchi 2002, Soto *et al*. 2014). Such dominance can be conferred by genes of major effect, which can result in rapid fixation or alter the evolutionary trajectory of a lineage (Nadeau & Jiggins 2010, Orr 2005). For example, introduction of a single regulatory gene (*rscS*) to a strain of *V. fischeri* symbiotic with a fish conferred the ability to colonize a novel host species (bobtail squid); this gene may have played a role in host-switching in this mutualism complex (Mandel *et al*. 2009). Experimentally evolving a strain native to *E. scolopes* in another squid species (*E. tasmanica*) resulted in an increase in colonization efficiency and competitive ability against native *E. tasmanica* strains in under 500 generations (Soto *et al*. 2012). Additionally, experimentally evolving both a free-living strain and a fish symbiotic strain in a squid species leads to a decrease in bioluminescense similar to what native strains exhibit (Schuster *et al*. 2010). In summary, strains appear to be uniquely adapted to their native squid hosts.

Mutualistic strains also illustrate adaptation to their free-living environment. Temperature and salinity tolerance varies among symbiotic strains of vibrios and matches native environmental conditions, which differ geographically (Soto *et al*. 2009a, 2009b). Adaptation to environmental variables can upset host-symbiont dynamics, e.g., native symbionts that are better adapted to colonize their host squid in laboratory settings may not be the most likely to colonize squids in nature, depending on the environmental conditions (Soto *et al*. 2012). In other words, strains that persist in high abundance because of adaptation to the free-living environment can colonize host squids even if other strains illustrated competitive dominance in a laboratory setting (Nishiguchi *et al*. 1998, Nishiguchi 2002, Jones *et al*. 2006, Nyholm & Nishiguchi 2008, Wollenberg & Ruby 2009, Soto *et al*. 2012). Similarly, abiotic factors and geography affect patterns of genetic diversity and colonization competency of *V. cholerae* in human hosts (Keymer *et al*. 2007). For symbionts, arriving at the host early can outweigh weaker colonization ability. In contrast, pleiotropic or epistatic responses in salinity tolerance occurred during experimental evolution in a novel host squid that may or may not be adaptive depending on the environment (Soto *et al*. 2012). Thus, the selective regime of free-living environment clearly produces adaptive responses in symbionts, but confounding variables may maintain the diversity of symbiotic strains that colonize any given host squid and thus also maintain diversity within the population of symbionts.

Study aims

The sepiolid squid-*Vibrio* symbiosis is well suited to explore the genetic scaffolding that enables adaptation to either the host or free-living environment. This well-studied model system has already revealed genetic mechanisms underlying adaptive phenotypes, such as several genes and regulatory elements involved in biofilm formation, which is necessary for host colonization (Yildiz & Visick 2009, Morris & Visick 2010, Chavez-Dozal & Nishiguchi 2011, Chavez-Dozal *et al*. 2012, Chavez-Dozal *et al*. 2014). Some genes and promoters involved in response to nitric oxide (NO) have been found using this model system. NO is a host-derived signal that may function as a population control strategy against the bacterial symbionts (Davidson *et al*. 2004, Wang *et al*. 2010). Other genes involved in circumventing host defenses or facilitating persistence in the free-living environment have been revealed by several transcriptional studies (Chun *et al*. 2008, Guerrero-Ferreira & Nishiguchi 2010, Jones & Nishiguchi 2006, Wier *et al*. 2010). These genet-

Table I. – Strain designations and ecological data for *Vibrio* species whose genomes were sequenced in this study.

Strain	Host	Locality	Temp	Temp °C	Salinity	Salinity ppt
CB37	None	SE Australia	Med	12–25	Med	20.0-35.5
ETJB5C	*Euprymna tasmanica*	SE Australia	Med	12–25	Med	20.0-35.5
EM17	*Euprymna morsei*	Japan	Low	2–17	Low	32.2-34.0
SA1G	*Sepiola affinis*	Mediterranean	Med	12–24	High	37.0-38.0

ic mechanisms appear to play parallel roles in other symbioses as well. Therefore, we used the natural diversity in squid hosts and environments to compare three mutualistic strains from three host squid species, from two temperature ranges, and three salinity levels (Table I). This allowed us to identify unique genes from each genome, differences in functional subsystems, genes currently under selection, point mutations in homologs, and investigate phylogenetic relationships among symbionts. Our findings will enable further studies targeting these points of interest with increased sample sizes and comparative methods focused on one variable (e.g., host, temperature, or salinity).

MATERIALS AND METHODS

Four bacterial genomes were sequenced via 454-pyrosequencing (Supplementary Table I). We chose this platform because longer reads and coverage depth are advantageous for assembly of small genomes like those of bacteria (Hudson 2008, Mardis 2008, Metzker 2010). Three strains of *V. fischeri* were isolated from different geographically located squid hosts (EM17 from *Euprymna morsei* in Tokyo Bay, Japan; ETJB5C from *E. tasmanica* in Jervis Bay, Australia; and SA1G from *Sepiola affinis* in Banyuls-sur-Mer, France; Table I). For comparison, we also sequenced a free-living, non-symbiotically competent *Vibrio* strain, *V. cf. campbellii* (CB37 isolated from Coogee Bay, Australia; Table I) and used it as our outgroup for phylogenetic and selection analyses. High-quality reads were obtained; sequence reads smaller than 40 nucleotides were discarded from analyses (Supplementary Tables I, II).

Pairwise and site-wise comparisons of homologs were completed and core and accessory genomes were identified (orthology analysis; Supplementary Table III). Analysis of these data enabled us to generate a list of genes potentially involved in adaptation to either the symbiotic or free-living state. Comparisons were made with other published genomes for orthology (symbiotic *V. fischeri* strains only) and selection analyses: ES114, a symbiont of *E. scolopes* (Ruby *et al*. 2005); MJ11, a symbiont of the fish *Monocentris japonica* (Mandel *et al*. 2009); SR5, a symbiont of a Mediterranean squid *Sepiola robusta* (Gyllborg *et al*. 2012); *V. campbellii* ATCC BAA-1116, a *Vibrio* strain isolated from a green barrel tunicate (Bassler *et al*. 2007, Lin *et al*. 2010); and two other vibrios, *Photobacterium profundum* 3TCK (Bartlett *et al*. 2006) and *Vibrio* sp. EJY3 (Roh *et al*. 2012). For phylogenetic analyses, we included our four sequenced genomes, and *V. fischeri* SR5 and MJ11.

Orthology analysis of the amino acid sequences from each *Vibrio* strain was performed using OrthoMCL database (Chen *et al*. 2006). Putative proteins encoded by the sequences were categorized as orthologs, paralogs, or orphans. Proteins failing to fall into clusters were analyzed with less stringent parameters (e-value of $1e^{-5}$ and minimum coverage of 50 %) using NCBI's BlastClust package and applying a reciprocal pBlast approach. Clusters were aligned with MUSCLE (Edgar 2004) using default parameters. We identified core clusters, defined as one orthologous sequence per strain, to calculate nucleotide diversity (π) and construct a phylogeny. The parameter Π, measured as the number of nucleotide differences between strains divided by the total length of a nucleotide sequence, was calculated for each set of orthologs in pairwise comparisons. We then averaged the values for all sets of pairwise comparisons to calculate the degree of polymorphism between strains. To visualize results identifying core and accessory genomes, we implemented PERL scripts within the publicly available Venn Diagram tool from VIB / UGent (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Annotated genetic elements were assigned to functional groups such as "Virulence, Disease, and Defense," "Cell Wall and Capsule," and "Stress Response" using Rapid Annotation Using Subsystem Technology (RAST) (Overbeek *et al*. 2014). The number of genes in each subsystem were summarized to highlight differences in allocation to subsystems among our symbionts, a model organism (ES114; (Ruby *et al*. 2005)), and the free-living *Vibrio* strain we sequenced. Annotated genes are provided in Supplementary Table IV.

The proportions of GC content in each open reading frame (ORF) were calculated, including the minimum, maximum, and SD of GC content per genome. Any ORF with GC proportions outside of ± 2 SDs of the mean were noted (Supplementary Table V). Normality of the distribution of GC proportions in the ORFs per genome was examined with Shapiro-Wilk tests.

The Codeml program was implemented from the PAML 4 package (Yang 2007) in a batch mode process on all clusters containing two or more strains to calculate selection ratios, both pairwise and sitewise, on all orthologs. Proteins and sites with Ka/Ks ratios exceeding 1.0 were classified as being under positive selection.

Maximum likelihood phylogenetic analysis was performed on multiple sequence alignments constructed at the protein level then concatenated to form orthologous alignments for the maximum likelihood program PhyML 3.0 (Guindon *et al*. 2010). We adopted the LG amino acid replacement matrix model (Le & Gascuel 2008) and utilized the SPR option, which provides the slowest but most accurate tree-topology search. A bootstrap analysis using 100 replicates also was performed.

RESULTS

Quality of sequence reads

We performed Roche 454 sequencing on the genomes of three *Vibrio fischeri* squid light organ isolates and on a free-living *Vibrio cf. campbellii* strain to increase our understanding of genetic differences that underlie symbionts of different squid species and different marine environments. Genomic DNA was sequenced in NMSU's sequencing facility as described in the Methods. Sequence read lengths of less than 40 nt were discarded. After standard filtering programs were implemented, we obtained good quality reads ranging from 47.9 % (EM17)

Table II. – Strain designations and information for *Vibrio* spp. and a *Photobacterium* sp. whose genomes were obtained from GenBank and used in orthology, positive selection, and sequence comparison analyses.

Species	Strain	Host	Geography	Accession No(s).	Reference
V. fischeri	ES114	*Euprymna scolopes*	Hawaii	NC_006840.2 NC_006841.2 NC_006842.1	(Ruby *et al.* 2005)
V. fischeri	MJ11	*Monocentris japonica*	Sea of Japan	NC_011184.1 NC_011185.1 NC_011186.1	(Mandel *et al.* 2009)
V. fischeri	SR5	*Sepiola robusta*	Mediterranean	CM001400.1 CM001401.1	(Gyllborg *et al.* 2012)
V. harveyi	ATCC BAA-1116	Free-living	Unknown	NC_009783.1 NC_009784.1	(Bassler *et al.* 2007)
Vibrio sp.	EJY3	*Grapsidae* (crab)	South Korea	NC_016613.1 NC_016614.1	(Roh *et al.* 2012)
P. profundum	3TCK	Free-living	San Diego Bay	NZ_AAPH00000000.1	(Bartlett *et al.* 2006)

to 78.4 % (ETJB5C), and averaging 59.0 %, of raw data, with an average read length of 480 nt (Supplementary Table II).

Comparison of Roche 454 sequencing reads to reference genomes

Reference genomes are provided in Table II. The reference genomes used to map sequences from the three *Vibrio fischeri* strains included in this genome-sequencing project were those of *V. fischeri* ES114 (Ruby *et al*. 2005) and *V. fischeri* MJ11 (Mandel *et al*. 2009). Genomic data from *V. campbellii* ATCC BAA-1116 (Lin *et al*. 2010), which originally was identified in GenBank data as a *V. harveyi,* was used as a reference for genome sequencing of the out-group used in analyses, *V. cf. cambellii* CB37. Our three symbiotic strains of *V. fischeri* have an average of 3898 genes, demonstrating slightly reduced genomes compared to an average genome size of 5173 genes found in the other non-*V. fischeri* vibrios used in our analysis (Table II). Overall, genes located on Chromosome I of the symbiotic strains were more conserved than genes located on Chromosome II. On average, the symbiotic strains shared 94.55 % sequence identity with *V. fischeri*'s Chromosome I, while Chromosome II averaged 89.89 %. The plasmid content carried in the symbiotic genomes is extremely variable. Only 20.06 % of the genes in ES114's plasmid are found in the plasmid of the Australian isolate, ETJB5C; roughly half as many are found in the Mediterranean isolate SA16, and none are present in the plasmid of the Japanese Sea isolate, EM17. Instead, the latter two strains share approximately 90 % of their plasmid content with the fish symbiont (MJ11) plasmid, while a very small percentage (less than 2 %) of ETJB5C's plasmid is the same as in MJ11 (Supplementary Table I).

Table III. – Nucleotide diversity (π) of our three symbiotic *V. fischeri* strains and free-living strain CB37, including comparisons to previously-sequenced *V. fischeri* strains ES114, MJ11, and SR5.

	SR5	ETJB5C	MJ11	SA1G	EM17	ES114	CB37
SR5	–	0.0296	0.0286	0.0291	0.0277	0.0301	0.2882
ETJB5C		–	0.0344	0.0364	0.0191	0.0185	0.2883
MJ11			–	0.0176	0.0332	0.0354	0.2883
SA1G				–	0.0359	0.0382	0.2883
EM17					–	0.0195	0.2884
ES114						–	0.2885
CB37							–

Orthology analysis

We identified the genes shared among all of the six genomes, thereby identifying the core *V. fischeri* genome. Including our three newly sequenced genomes, as well as the three reference genomes (ES114 (Ruby *et al*. 2005), SR5 (Gyllborg *et al*. 2012), and MJ11 (Mandel *et al*. 2009)), there are 3018 genes common, listed in Supplemental Table III. This represents 78.4 % of the average genome size. Eliminating the MJ11 genome from analysis increases the core genome for squid symbionts to 3091, whereas eliminating SR5 but leaving MJ11 in increases the core genome size to 3138. Our three newly sequenced genomes alone share 3186 orthologous genes. The overlap in genomic content among different groups of *V. fischeri* strains, along with *V. cf. campbellii* CB37, is depicted in Venn diagrams (Fig. 1). Accessory genomes of all six symbiotic strains, which include strain-specific genes as well as orthologs shared by some but not all of the symbiotic strains, contain on average 833 genes, with each strain possessing an average of 226 unique genes. Orthologs found in more than two but less than six genomes also include varying numbers of paralogous genes, ranging from 20 genes that have undergone at least one duplication

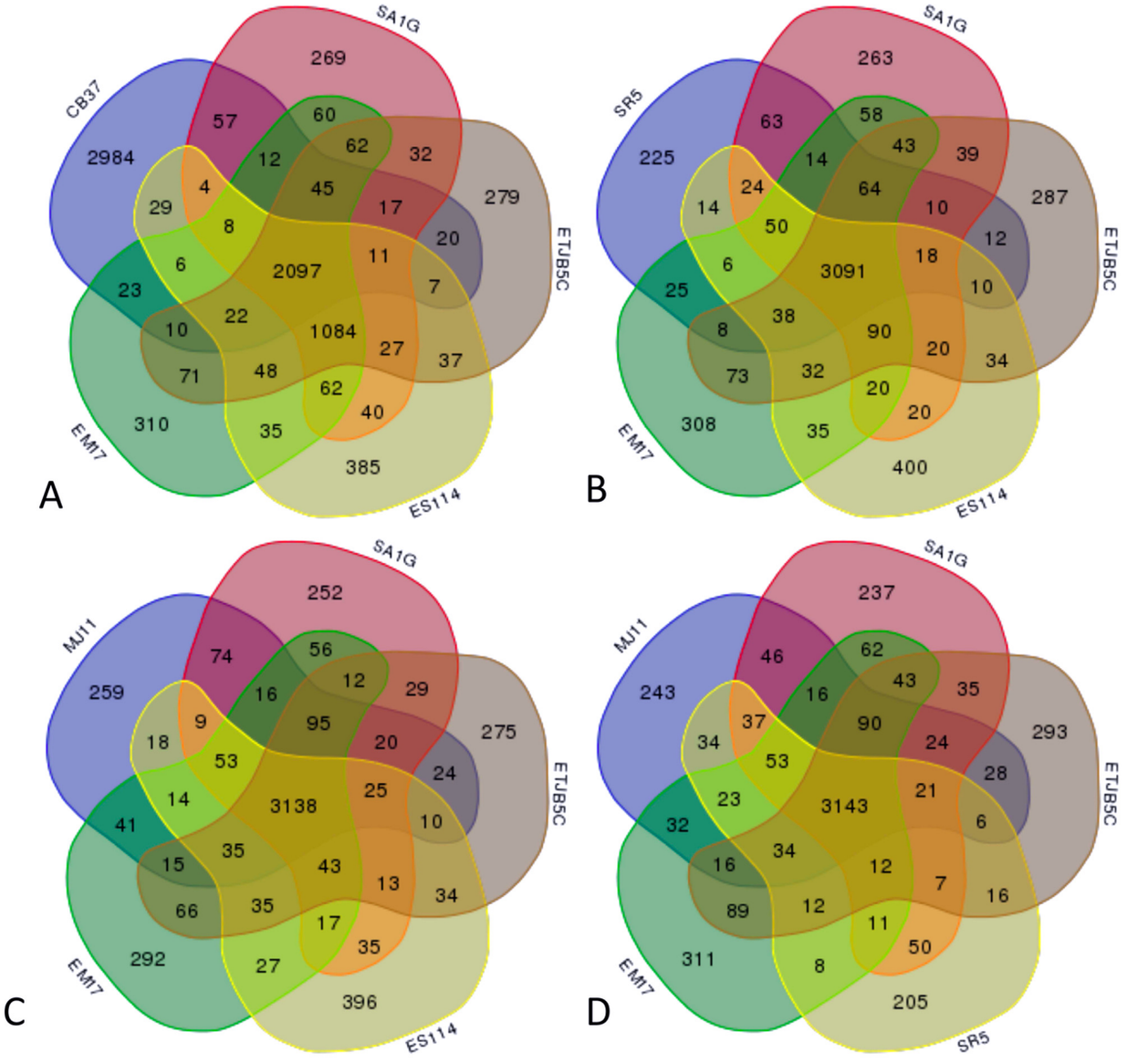


Fig. 1. – Venn diagrams from the Bioinformatics and Evolutionary Genomics Tool depict shared gene content graphically for up to 5 genomes. A. Venn diagram depicting our three sequenced strains of symbiotically competent *V. fischeri* (SA1G, EM17, ETJB5C), a model organism (ES114), and the free-living *V. cf. campbellii* CB37. Here, we compare our free-living strain, CB37, to our symbiotic strains. B. Venn diagram depicting symbiotically competent *V. fischeri* that colonize squid hosts, including our three strains (SA1G, EM17, ETJB5C), a model organism (ES114), and another previously published squid symbiont (SR5). Here, we compare our symbiotic strains to other squid symbiotic strains. C. Venn diagram depicting four strains of symbiotically competent *V. fischeri* with squid hosts (SA1G, EM17, ETJB5C, ES114) and a fish host (MJ11). D. Venn diagram depicting four strains of symbiotically competent *V. fischeri* with squid hosts (SA1G, EM17, ETJB5C, SR5) and a fish host (MJ11). Note that the core genomes when including the fish symbionts (panels C, D) are actually larger (3138 vs. 3143) than the core genome for just the squid symbionts (panel B; 3091), indicating that the fish symbiont is not the most divergent of the symbiotic strains.

event in SR5 to 51 found in EM17 (Supplemental Table VI).

In pairwise comparisons of genetic diversity among the symbiotic *V. fischeri* strains, the greatest difference was seen between SA1G and ES114, with their orthologous genes differing at approximately 3.8 % of nucleotide sites (Table III). The most similar pairing was between SA1G and MJ11, where approximately 1.8 % of their orthologs differed in nucleotide sequence (Table III).

Functional groups analysis

The number of genes assigned to different functional groups varies between symbiotic genomes and the free-living CB37 (RAST analysis; Table IV). Annotations of all specific genes recovered from this RAST analysis are given in Supplementary Table IV.

GC analysis

Distributions of GC proportions by gene for all four genomes sequenced in this study were not normal but were skewed towards ORFs with lower GC content than the genome mean (ETJB5C, $D = 0.065$, $p < 0.01$; SA1G, $D = 0.062$, $p < 0.01$; EM17, $D = 0.060$, $p < 0.01$; CB37, $D = 0.088$, $p < 0.01$; Table V). A high frequency of genes at the edges of the distribution of GC proportions (more than 2 SDs from the mean) for each genome were spatially clustered; that is, they were proximate in the genome (Table V, Supplementary Table V).

Positive selection analysis

A total of 38 genes show evidence in at least one pairwise-comparison of positive selection with ratios greater than 1.0: twenty two in EM17, ten in ES114, four in ETJB5C, and one each in MJ11 and SA1G (Table VI).

Phylogenetic analysis

The maximum likelihood approach of PhyML was used to create a phylogeny of our *V. fischeri* strains. We collected 1,870 orthologs from our three symbiotically competent *V. fischeri* strains (EM17, ETJB5C, SA1G), one free-living strain used as an outgroup (CB37), and three other symbiotic *V. fischeri* strains (ES114 (Ruby *et al*. 2005), MJ11 (Mandel *et al*. 2009), SR5 (Gyllborg *et al*. 2012). These orthologs were aligned and concatenated at the protein level to generate multiple sequence alignments. The best-supported tree identified two clades, one displaying EM17 as sister to ETJB5C and ES114, and the other with SR5 as sister to SA1G and MJ11 (Fig. 2), with *V. cf. campbellii* CB37 as the outgroup. High bootstrap values were obtained for all tree nodes.

Table IV. – Variance in genes comprising functional groups of newly sequenced genomes of *Vibrio* species and one model organism *V. fischeri* ES114.

Subsystem feature counts	CB37	ETJB5C	EM17	SA1G	ES114
Amino Acids and Derivatives	451	324	328	329	329
Carbohydrates	576	375	371	414	414
Cell Division and Cell Cycle	33	31	35	33	33
Cell Wall and Capsule	183	205	176	191	191
Cofactors, vitamins, prosthetic groups, pigments	254	202	202	205	205
DNA metabolism	149	134	151	132	132
Dormancy and Sporulation	7	7	4	4	4
Fatty Acids, Lipids, and Isoprenoids	131	118	107	124	124
Iron Acquisition and Metabolism	83	50	55	72	72
Membrane transport	227	194	200	199	199
Metabolism of aromatic compounds	14	4	4	4	4
Miscellaneous	193	175	170	184	184
Motility and chemotaxis	164	107	108	101	101
Nitrogen metabolism	50	38	38	39	39
Nucleosides and nucleotides	110	95	99	94	94
Phages, prophages, transposable elements, plasmids	9	7	19	9	9
Phosphorus metabolism	58	37	38	36	36
Potassium metabolism	58	53	51	52	52
Protein metabolism	270	232	238	241	241
Regulation and cell signaling	119	99	92	93	93
Respiration	185	131	129	130	130
RNA metabolism	220	141	142	141	141
Secondary metabolism	0	0	0	0	0
Stress response	228	185	184	188	188
Sulfur metabolism	30	32	28	29	29
Virulence, disease, and defense	106	76	75	73	73
Total open reading frames	3908	3052	3044	3117	3117

Table V. – Mean GC content within genomes of four *Vibrio* strains sequenced in this study. Distributions were skewed toward more open reading frames (ORFs) with low GC content (those with GC proportions farther than 2 standard deviations (SDs) from the mean). Clusters, defined as more than 3 ORFs in proximity, with high or low GC content are provided in Supplementary Table V.

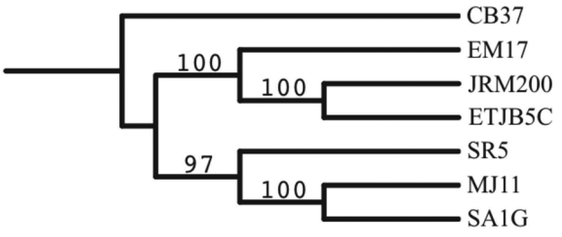
Strain	Mean ORF length (bp)	Mean GC proportion	Total ORFs	MAX GC proportion	MIN GC proportion	SD GC proportion	ORFs GC content above mean	ORFs GC content below mean	Number of ORFs in clusters	Proportion of ORFs in clusters
ETJB5C	952	0.3872	3864	0.5014	0.1795	0.0349	40	156	77	0.3929
SA1G	968	0.3873	3885	0.4987	0.2027	0.0352	42	140	31	0.1703
EM17	953	0.3883	3946	0.5014	0.2068	0.0353	48	146	32	0.1649
CB37	934	0.4535	5340	0.5464	0.2650	0.0318	17	250	78	0.2921

Table VI. – Positively-selected genes identified through pairwise comparative analyses of symbiotic strains.

Strain ID	Compared to:	Gene Annotation	Gene ID	Known or putative function	Reference
ES114	ETJB5C	Hypothetical protein	VF_A0138	Unknown	–
	MJ11	FhuC ferric hydroxamate uptake	VF_A0158	Iron transport	–
	ETJB5C	Hypothetical protein	VF_A0755	Unknown	–
	SA1G	RplP 50S ribosomal subunit protein L16	VF_0243	Translation	–
	SA1G	Crp/Fnr family transcriptional regulator	VF_0318	Environmental stress response	(Soto & Nishiguchi 2014)
	EM17	ProW glycine betaine/proline ABC transporter permease	VF_0786	Osmoprotection	(Chavez-Dozal *et al.* 2014)
	SA1G	Hypothetical protein	VF_1745	Unknown	–
	ETJB5C	Hypothetical protein	VF_1916	Unknown	–
	ETJB5C	AtpI ATP synthase subunit I	VF_2571	Respiration	–
	SA1G	PanB pantothenate hydroxymethyltransferase	VF_2169	Pantothenate biosynthesis (FA, TCA metabolism)	(Jones *et al.* 1993)
ETJB5C	MJ11	ProtC transcriptional regulatory protein	277	Possible antibiotic resistance	(Wietzorrek & Bibb 1997)
	EM17	PriA primosome assembly protein	665	Transcription	–
	ES114	Hypothetical protein	2268	Unknown	–
	EM17; ES114	Phage shock protein	3285	Possible extracytoplasmic stress response	(Maxson & Darwin 2004)
EM17	MJ11	Glutathione S-transferase	190	Detoxification	(Vuilleumier 1997)
	ES114	RpoZ DNA-directed RNA polymerase subunit omega	691	Transcription	–
	ES114	ProQ solute/DNA competence effector	933	Osmoprotectant regulation	(Browne-Silva & Nishiguchi 2008)
	ES114	Hypothetical protein	1289	Unknown	–
	SA1G	Hypothetical protein	1433	Unknown	–
	ETJB5C	Hypothetical protein	1936	Unknown	–
	ETJB5C	Surface protein	1992	Unknown	–
	ES114	Hypothetical protein/possible peptidase	2476	Unknown	–
	ES114; ETJB5C	RpsU 30S ribosomal protein S21	2576	Translation	–
	ETJB5C	PilP pili assembly protein	2620	Conjugation/twitching motility	–
	ES114; ETJB5C	tRNA dihydrouridine synthase B	2709	Translation	–
	ETJB5C	RplU 50S ribosomal protein L21	2794	Translation	–
	ES114	RaiA stationary phase translation inhibitor and ribosome stability factor	3022	Translation	–
	ETJB5C	Type VI secretion system lysozyme-related protein	3088	Possible competition role	(Soto *et al.* 2014)
	ETJB5C	Putative penicillin-binding protein 1C	3124	Antibiotic resistance	–
	ES114	RpsF 30S ribosomal protein S6	3179	Translation	–
	ETJB5C	Hypothetical protein	3250	Unknown	–
	ETJB5C	Hypothetical protein	3804	Unknown	–
	SA1G	RhlE RNA helicase	1433	Transcription	–

Table VI. – Continued.

Strain ID	Compared to:	Gene Annotation	Gene ID	Known or putative function	Reference
	MJ11	Putative lipoprotein	1644	Membrane function/ environmental sensing	–
	ETJB5C	DksA RNA polymerase binding protein	2388	Transcription	–
	ETJB5C	MshO mannose-sensitive hemagglutinin type IV pilus assembly	2722	Host colonization	–
SA1G	MJ11	PilZ Type IV pilus assembly protein	3870	Motility/biofilm formation	(Mattick 2002)
MJ11	ETJB5C	Beta-lactamase	4686	Antibiotic resistance	(Ambler 1980)



Key: CB37 is a free-living *Vibrio harveyi* collected in Australia; EM17 is a *Vibrio fischeri* isolated from *Euprymna morsei* from Japan; JRM200 is a *V. fischeri* isolated from *E. scolopes*, Hawaii; ETJB5C is a *V. fischeri* isolated from *E. tasmanica*, Australia; SR5 is a *V. fischeri* isolated from *Sepiola robusta* from the Mediterranean Sea; MJ11 is a *V. fisheri* fish symbiont isolated from *Monocentris japonica*, Japan; and, SA1G is a *V. fischeri* isolated from *S. affinis* from the Mediterranean Sea.

Fig. 2. – Maximum-likelihood bootstrap consensus tree constructed using PhyML 3.0 and showing relationships among *V. fischeri* strains sequenced in this study. The tree was generated using concatenated amino acid sequences from 1870 orthologous genes shared by all strains. We adopted the LG amino acid replacement matrix model (Le & Gascuel 2008) and utilized the SPR option, which provides the slowest but most accurate tree-topology search. Bootstrap values for nodes were generated using 100 replications.

DISCUSSION

Overview

The plethora of genes divergent among our three symbiotic strains potentially reflect different environmentally-selective regimes and different hosts as well as several divergent between all symbiotic and obligately free-living vibrios (Table III). Rapid Annotation using Subsystem Technology (RAST) uses its own algorithms to discover open reading frames and bin them into functional groups/subsystems (Overbeek *et al*. 2014); different strains have different numbers of genes in each subsystem, which may be indicative of varying environments. Genes under positive selection were also detected and may encompass both host- and free-living effects (Table VI). Within the three symbiotic strains examined, genomes are relatively conserved with evidence of strong purifying selection and few duplication events. Interestingly, a reduced genome size, compared to that of other vibrios we used as reference (mean 3898 vs. 5173 genes), may be indicative of tradeoffs to allow rapid reproduction within host light organs, despite the fact that *V. fischeri* must cycle through a free-living stage. This is common in vertically-transmitted, obligate mutualisms, where bacterial symbionts are streamlined to the specific capabilities that are encompassed during symbiosis (Moran & Mira 2001, Moran 2002, Toh *et al*. 2006, McCutcheon & Moran 2012).

Symbiotic strains demonstrate substantial variation in their accessory genomes presumably due to the necessity of adapting to both the specific host species and unique abiotic environment faced by each *V. fischeri* strain (Fig. 1). Some of these may be acquired via horizontal gene transfer (HGT) as suggested by GC signatures that deviate significantly from mean genome proportions (Table V). The proximity or clustering of ORFs with similar GC frequencies that deviate > 2 SDs from the mean of the genome illustrates that genes with lower than average GC content are not randomly distributed throughout the genome. These blocks of ORFs may be of interest to assess HGT, which may be indicated by either abnormally high or low GC content (Garcia-Vallvé *et al*. 2000, Marcus *et al*. 2000), and which often occurs in the form of pathogenicity islands where several ORFs of an operon are transferred in one event. However, other factors may influence GC content (Hildebrand *et al*. 2010, Hayek 2013), and a further analysis of these blocks is warranted and should be addressed in a separate, more in-depth analysis. Other forces beyond vertical inheritance are also indicated by the phylogenetic analysis, which lacks a biogeographic signature (Fig. 2). Each symbiont's genome contains unique elements that may be shaped by selection from both host and environment; details are summarized in the sections below.

Genome level summary

Our three symbiotic strains of *V. fischeri* exhibited 3,186 orthologous genes (approx. 81.7 %), which comprise the core genome. Our strains combined with two other published squid symbionts had a core genome of 3,091 orthologs; when fish symbiont MJ11 is added to the analysis, there are 3018 (approx. 78 %) shared orthologs (Fig. 1). In comparison, four strains of *V. vulnificus* shared 3,459 genes (Gulig *et al*. 2010), possibly suggesting that pathogenicity in vibrio strains requires a slightly larger suite of conserved genes than does mutualism or

that adaptation to different host squids caused more divergence in our mutualistic genomes.

The accessory genomes uncovered in this study, which are by definition everything exclusive of the core genome, contain an average 833 genes, with each strain possessing an average of 226 unique genes; the remainder were paralogs. The strain-specific genes have no close homologs (> 60 % similarity over 50 % of length) in any of the other five *V. fischeri* strains we examined. Accessory genomes have been hypothesized to be reflective of environmental and host heterogeneity and to equip each strain with the suite of genes most adaptive to its unique ecological niche (Read & Ussery 2006, Mira *et al*. 2010). Strain-specific genes are postulated to arise either from gene duplication, leading to divergence and evolution of new or altered functions, or from lateral gene transfer, in which novel genes are appropriated from other organisms (Zhang 2003, Gevers *et al*. 2004, Treangen *et al*. 2009). However, a more recent analysis found that gene duplication may have a greater effect on gene dosage and less effect on gene neofunctionalization for shaping bacterial genomes (Treangen & Rocha 2011). The strain-specific genes identified through this study are keys to deciphering the relative significance of environmental and host-driven parameters in future studies of *V. fischeri* evolution.

In contrast to findings of significant gene duplication and lineage-specific expansion of protein families within Vibrionaceae (Gu *et al*. 2009), we saw little evidence of widespread gene duplication within symbiotic *V. fischeri* strains. *Vibrio fischeri* EM17 had the most paralogs contained in both its core and accessory genomes (260), while *V. fischeri* SR5 had the fewest (171). The average number of paralogs was 215, representing 5.6 % of the average genome size. Some duplicated genes within each symbiotic genome may have adaptive significance distributed between host and environmental effects, e.g., quadruplets of anaerobic glycerol subunits in SA1G shown to be upregulated during symbiosis (Wier *et al*. 2010) and duplicates of cold shock proteins in MJ11 possibly retained because of environmental effects (Supplementary Table VI).

The proportion of open reading frames associated with different subsystems as assigned during RAST analysis further demonstrates the evolutionary divergence between free-living and symbiotic strains. For example, the free-living *V. cf. campbellii* CB37 has a much larger genome, including many more coding regions associated with nutrient and energy acquisition (Table IV). The symbiotic genomes, in contrast, are streamlined and fairly conserved among each other. In addition, there are fewer elements associated with virulence, disease, and defense in the symbiotic genomes. This analysis showed conservation among the symbiotic genomes in broad functional categories, though some differences are apparent. "Iron acquisition and metabolism" may be limiting in SA1G and ES114 in comparison to ETJB5C and EM17 (Table IV). For example, the former two strains have genes for paraquat-inducible protein A (heme and hemin uptake), while the latter strains lack this gene (Supplementary Table IV). Both SA1G and ES114 have 17 and 22 more genes for "iron acquisition and metabolism" respectively, than EM17, and 22 and 27 more than ETJB5C (Supplementary Table IV). In contrast, the free-living *Vibrio* CB 37 had a total of 83 genes in that subcategory, suggesting that strain experiences the highest selection pressure to sequester a rare resource.

To highlight current levels of natural selection operating on symbiotic *V. fischeri* strains, we calculated ratios of non-synonymous to synonymous (K_a/K_s) substitutions among all possible pairwise comparisons of orthologous proteins. Positive selection was indicated for twenty-two genes in EM17, ten in ES114, four in ETJB5C, and one each in MJ11 and SA1G (Table VI). However, out of a total 19,762 pairwise comparisons, the vast majority of these exhibit very low K_a/K_s ratios, suggesting strong purifying selection occurring on most of the orthologous proteins. Many of the genes exhibiting evidence of positive selection are hypothetical proteins, with no known function. However, the positively-selected genes for which annotations are available include some that are likely influential in adaptation to both host and environment, including mannose-sensitive hemagglutinin *mshO* in *V. fischeri* EM17, expressed during host infection and colonization, and, in *V. fischeri* SA1G, type IV pilus assembly gene *pilZ*, important for motility and biofilm formation (Mattick 2002); see Table VI).

Comparisons with other *Vibrio* species

By comparing our sequence database with genomic sequences available on GenBank of *Vibrio fischeri* and six other vibrios (Table II), we were able to identify genes that are strongly conserved in the symbiotic strains but highly divergent or missing in free-living or pathogenic strains. These genes potentially represent core "symbiotic" genes that are indispensable for host colonization and are inviting candidates for mutational analyses to decipher specific functions that enable *V. fischeri*'s beneficial associations with eukaryotic hosts. Some of these conserved genes include sequences from MJ11, the fish symbiont, while others only include the squid symbionts and may be specific to this host type.

Among the set of conserved orthologs that include MJ11 sequences is *asc1B*, which encodes arylsulfatase regulator and has been shown to be important in other bacterial colonization events (Cheng *et al*. 1992, Morgan *et al*. 2004). Genes in which the MJ11 sequence varies by just one codon include *ntrC*, a gene encoding nitrogen regulatory response regulator/sigma 54 interaction protein that in *V. vulnificus* is involved in membrane saccharide synthesis, biofilm formation, and possibly carbohydrate metabolism (Kim *et al*. 2007) and in *V. fischeri*

is somehow involved in colonization (Hussa *et al*. 2007); and *mshJ*, part of the mannose-sensitive hemagglutinen type IV pilus operon. These genes have functions in the colonization and eventual formation of biofilm, which is crucial for the vibrio community that is established inside the squid light organ (Yip *et al*. 2005, Visick *et al*. 2007, Browne-Silva & Nishiguchi 2008, Geszvain & Visick 2008, Ariyakumar & Nishiguchi 2009, Yildiz & Visick 2009, Chavez-Dozal *et al*. 2012).

Finally, genes conserved within squid symbionts but more divergent in MJ11 include *aepA* encoding an exoenzyme regulatory protein with a putative hydrolase function (Murata *et al*. 1994) and a NADH-dependent flavin oxireductase gene involved in producing the substrate for luciferase (Duane & Hastings 1975). In the phytopathogen *Erwinia carotovora*, *aepA* is necessary for host infection and is up-regulated in response to quorum-sensing signals. Flavin oxireductase, which is instrumental for bioluminescence, has significant sequence divergence from the other *Vibrio* symbionts and is particularly divergent in SA1G, with otherwise close sequence similarity to MJ11. Presumably there are functional attributes of bioluminescence shared between SA1G and MJ11 but distinct from the other symbiotic strains. Whether these attributes relate to an ecological component common between SA1G and MJ11 or is phylogenetic convergence is not presently known.

Phylogenetic analysis

Phylogenetic analysis of the six symbiotic *V. fischeri* strains, using CB37 as the outgroup, revealed a pattern inconsistent with geography, host, or environmental parameters alone and suggests a complex evolutionary history within *V. fischeri* (Fig. 2). One clade consisted of EM17 as sister to ETJB5C and ES114, while a second clade contained SR5 as sister to SA1G and MJ11. These relationships do not have strictly biogeographical signatures; for example, both EM17 and MJ11 are Sea of Japan isolates, yet fall into separate clades. Similarly, the Mediterranean squid symbiotic strain SA1G is more closely related to MJ11 than it is to the other Mediterranean strain SR5. Presumably, different ecological constraints have imposed isolating barriers between *V. fischeri* populations geographically close to one another, despite their hosts sharing multiple species of *Vibrio* bacteria (Fidopiastis *et al*. 1998, Nishiguchi 2000, Zamborsky & Nishiguchi 2011).

Certainly, more extensive taxon sampling might help elucidate evolutionary relationships among symbiotic *V. fischeri* strains, but what our analysis suggests is that divergence of orthologous proteins follows neither clear biogeographical routes nor obvious environmental factors. Instead, evolution of this group of bacteria may operate in a fragmented manner, similar to what has been found in other groups of closely related bacteria, where evolutionary independence of orthologous genes contributes to patchy retention of genes acquired through homologous recombination with proximal populations of *V. fischeri* (Retchless & Lawrence 2010).

CONCLUSION

A selective balance presumably exists between environmental and biogeographical factors that shape the evolution of *V. fischeri* symbiotic strains, likely creating a selection mosaic dependent upon specific interactions between the bacteria, their hosts, and heterogenous environments (Fierer & Jackson 2006, Horner-Devine *et al*. 2004, Yannarell & Triplett 2005). Other researchers have demonstrated similar findings when examining microbial distributions and evolutionary relationships. For example, bacterial communities are driven primarily by environmental heterogeneity rather than geographic distance (Horner-Devine *et al*. 2004); diversity in soil bacterial communities is primarily controlled by edaphic factors (Fierer & Jackson 2006); and, bacterial community composition in shallow lake systems is strongly influenced by local environmental factors (Yannarell & Triplett 2005). The identification of genes that are unique to each strain used in our study, as well as those that exhibit positive selection signatures, is the first step toward disentangling the respective roles that the divergent environments within and outside the host have had on *V. fischeri* evolution.

However, the process of teasing apart environmental and host effects is complicated by the fact that many genes have multiple functions or are upregulated under numerous scenarios. For example, EM17 3088, a Type VI secretion system lysozyme-related protein, could potentially be used against competitors during host colonization (Brooks *et al*. 2013) or against predators in the water column (Pernthaler 2005). In the legume-rhizobia symbiosis, fully one-third of known symbiosis genes are involved in multiple pathways connected to cell metabolism, transcription, signal transduction, and protein modification and regulation (Tian *et al*. 2012). A similar cross-functionalization of symbiotic genes is likely to be valid in the squid-*Vibrio* mutualism.

In summary, *Vibrio fischeri* evolution is likely a patchwork of host- and abiotic environment-driven adaptation. Future work needs to employ comparative methods sampling more strains that differ in only one variable (host, temperature, salinity) but targeting one or a few of the genes/genetic elements we have identified here. While genomic and transcriptional studies have been extremely informative for this symbiosis complex thus far, the proposed strategy will maximize sequencing efficiency and provide more comparative power than previously available.

ACKNOWLEDGEMENTS. – Data are archived in GenBank as BioProject IDs PRJNA212805 (CB37); PRJNA212806 (EM17);

PRJNA212810 (SA1G); and PRJNA212811 (ETJB5C)). We would like to thank NMSU's 454 sequencing staff for help with this project, in particular Dr A Tchourbanov for his help with genome assemblies and Dr A Chavez-Dozal for the DNA preparations. We thank Dr D Houstan for comments on an earlier draft. This research was funded by grants to MKN from the National Institutes of Health NIAID 1SC1AI081659-01 and NIAID ARRA 3SC1AI081659-02S1, and National Science Foundation IOS-074449.

REFERENCES

Ambler RP 1980. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci* 289(1036): 321-331.

Ariyakumar DS, Nishiguchi MK 2009. Characterization of two host-specific genes, mannose-sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. *Fems Microbiol Lett* 299(1): 65-73.

Bartlett DH, Valle G, Lauro FM, Vezzi A, Simonato F, Eloe E, Vitulo N, Stratton TK, D'angelo M, Ferriera S, Johnson J, Kravitz S, Beeson K, Sutton G, Rogers Y, Friedman R, Frazier M, Venter JC 2006. *Photobacterium profundum* 3TCK, whole genome shotgun sequencing project. J Craig Venter Institute, Rockville, MD 20850, USA.

Bassler B, Clifton SW, Fulton L, Delehaunty K, Fronick C, Harrison M, Markivic C, Fulton R, Tin-Wollam AM, Shah N, Pepin K, Nash W, Thiruvilangam P, Bhonagiri V, Waters C, Tu KC Irgon J, Wilson RK 2007. The *Vibrio harveyi* Genome Sequencing Project. Genetics, Genome Sequencing Center, 4444 Forest Park Parkway, St. Louis, MO 63108, USA.

Boettcher KJ, Ruby EG, McFall-Ngai MJ 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J Comp Physiol A Sens Neural Behav Physiol* 179(1): 65-73.

Brooks TM, Unterweger D, Bachmann V, Kostiuk B, Pukatzki S 2013. Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *J Biol Chem* 288(11): 7618-7625.

Browne-Silva J, Nishiguchi M 2008. Gene sequences of the pil operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int J Syst Evol Microbiol* 58(6): 1292-1299.

Chavez-Dozal A, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK 2012. Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. *FEMS Microbiol Ecol* 81(3): 562-573.

Chavez-Dozal AA, Gorman C, Lostroh CP, Nishiguchi MK 2014. Gene-swapping mediates host specificity among symbiotic bacteria in a beneficial symbiosis. *PLoS ONE* 9(7): e101691.

Chavez-Dozal A, Nishiguchi MK 2011. Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri*. *J Basic Microbiol* 51(5): 452-458.

Chen F, Mackey AJ, Stoeckert CJ, Jr Roos DS 2006. OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups. *Nucleic Acids Res* 34(Database issue): D363-368.

Cheng Q, Hwa V, Salyers AA 1992. A locus that contributes to colonization of the intestinal tract by *Bacteroides thetaiotaomicron* contains a single regulatory gene (*chuR*) that links 2 polysaccharide utilization pathways. *J Bacteriol* 174(22): 7185-7193.

Chun CK, Troll JV, Koroleva I, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo MD, Casavant TL, Soares MB, Ruby EG, McFall-Ngai MJ 2008. Effects of colonization, luminescence, and autoinducer on host transcription during development of the squid-*Vibrio* association. *Proc Nat Acad Sci USA* 105(32): 11323-11328.

Coleman ML, Chisholm SW 2010. Ecosystem-specific selection pressures revealed through comparative population genomics. *Proc Nat Acad Sci USA* 107(43): 18634-18639.

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L, McFall-Ngai MJ 2004. NO means 'yes' in the squid-*Vibrio* symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol* 6(12): 1139-1151.

Dong TG, Ho BT, Yoder-Himes DR, Mekalanos JJ 2013. Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *PNAS* 110(7): 2623-2628.

Duane W, Hastings JW 1975. Flavin mononucleotide reductase of luminous bacteria. *Mol Cell Biochem* 6(1): 53-64.

Eberhard A 1972. Inhibition and activation of bacterial luciferase synthesis. *J Bacteriol* 109(3): 1101-1105.

Edgar RC 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acid Res* 32(5): 1792-1797.

Ellison CE, Hall C, Kowbel D, Welch J, Brem RB, Glass NL, Taylor JW 2011. Population genomics and local adaptation in wild isolates of a model microbial eukaryote. *PNAS USA* 108(7): 2831-2836.

Fidopiastis PM, von Boletzky S, Ruby EG 1998. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. *J Bacteriol* 180(1): 59-64.

Fierer N, Jackson RB 2006. The diversity and biogeography of soil bacterial communities. *PNAS USA* 103(3): 626-631.

Fuqua WC, Winans SC, Greenberg EP 1994. Quorum sensing in bacteria - the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* 176(2): 269-275.

Garcia-Vallvé S, Romeu A, Palau J 2000. Horizontal gene transfer in bacterial and archaeal complete genomes. *Genome Res* 10(11): 1719-1725.

Geszvain K, Visick KL 2008. The hybrid sensor kinase RscS integrates positive and negative signals to modulate biofilm formation in *Vibrio fischeri*. *J Bacteriol* 190(13): 4437-4446.

Gevers D Vandepoele K Simillion C Van de Peer Y 2004. Gene duplication and biased functional retention of paralogs in bacterial genomes. *Trends Microbiol* 12(4): 148-154.

Gilad Y, Pritchard JK, Thornton K 2009. Characterizing natural variation using next-generation sequencing technologies. *Trends Genet* 25(10): 463-471.

Graf J, Ruby EG 1998. Host-derived amino acids support the proliferation of symbiotic bacteria. *PNAS USA* 95(4): 1818-1822.

Gu JY, Neary J, Cai H, Moshfeghian A, Rodriguez SA, Lilburn TG, Wang YF 2009. Genomic and systems evolution in Vibrionaceae species. *BMC Genomics* 10(Suppl 1): S11-24.

Guerrero-Ferreira RC, Nishiguchi MK 2010. Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. *Environ Microbiol Rep* 2(4): 514-523.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59(3): 307-321.

Gulig PA, de Crecy-Lagard V, Wright AC, Walts B, Telonis-Scott M, McIntyre LM 2010. SOLiD sequencing of four *Vibrio vulnificus* genomes enables comparative genomic analysis and identification of candidate clade-specific virulence genes. *BMC Genomics* 11: 512.

Gyllborg MC, Sahl JW, Cronin DC 3rd, Rasko, DA Mandel MJ 2012. Draft genome sequence of *Vibrio fischeri* SR5, a strain isolated from the light organ of the Mediterranean squid *Sepiola robusta*. *J Bacteriol* 194(6): 1639.

Haardt M, Bremer E 1996. Use of phoA and lacZ fusions to study the membrane topology of ProW, a component of the osmoregulated ProU transport system of *Escherichia coli*. *J Bacteriol* 178(18): 5370-5381.

Hayek N 2013. Lateral transfer and GC content of bacterial resistance genes. *Front Microbiol* 4: 41.

Hildebrand F, Meyer A, Eyre-Walker A 2010. Evidence of selection upon genomic GC-content in bacteria. *PLoS Genet* 6(9): e1001107.

Horner-Devine MC, Lage M, Hughes JB, Bohannan BJ 2004. A taxa-area relationship for bacteria. *Nature* 432(7018): 750-753.

Hudson ME 2008. Sequencing breakthroughs for genomic ecology and evolutionary biology. *Mol Ecol Resour* 8(1): 3-17.

Hussa EA, O'Shea, TM Darnell, CL Ruby, EG Visick KL 2007. Two-component response regulators of *Vibrio fischeri*: identification, mutagenesis, and characterization. *J Bacteriol* 189(16): 5825-5838.

Jones BW, Nishiguchi MK 2004. Counterillumination in the hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* 144(6): 1151-1155.

Jones BW, Lopez JE, Huttenburg J, Nishiguchi MK 2006. Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol Ecol* 15(14): 4317-4329.

Jones BW, Nishiguchi MK 2006. Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. *Can J Microbiol* 52(12): 1218-1227.

Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK 2007. Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. *Microb Ecol* 54(2): 314-323.

Jones CE, Brook JM, Buck D, Abell C, Smith AG 1993. Cloning and sequencing of the *Escherichia coli* panB gene, which encodes ketopantoate hydroxymethyltransferase, and overexpression of the enzyme. *J Bacteriol* 175(7): 2125-2130.

Keymer DP, Miller MC, Schoolnik GK, Boehm AB 2007. Genomic and phenotypic diversity of coastal *Vibrio cholerae* strains is linked to environmental factors. *Appl Environ Microbiol* 73(11): 3705-3714.

Kim HS, Lee MA, Chun SJ, Park SJ, Lee KH 2007. Role of *ntrC* in biofilm formation via controlling expression of the gene encoding an ADP-glycero-manno-heptose-6-epimerase in the pathogenic bacterium, *Vibrio vulnificus*. *Mol Microbiol* 63(2): 559-574.

Le SQ Gascuel O 2008. An improved general amino acid replacement matrix. *Mol Biol Evol* 25(7): 1307-1320.

Lee KH, Ruby EG 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl Environ Microbiol* 60(5): 1565-1571.

Levasseur A, Pontarotti P 2011. The role of duplications in the evolution of genomes highlights the need for evolutionary-based approaches in comparative genomics. *Biol Direct* 6: 11.

Lin B, Wang Z, Malanoski AP, O'Grady EA, Wimpee CF, Vuddhakul V, Alves N Jr, Thompson FL, Gomez-Gil B Vora GJ 2010. Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii*. *Environ Microbiol Rep* 2(1): 81-89.

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG 2009. A single regulatory gene is sufficient to alter bacterial host range. *Nature* 458(7235): 215-218.

Marcus SL, Brumell JH, Pfeifer CG, Finlay BB 2000. *Salmonella* pathogenicity islands: big virulence in small packages. *Microbes Infect* 2(2): 145-156.

Mardis ER 2008. The impact of next-generation sequencing technology on genetics. *Trends Genet* 24(3): 133-141.

Mattick JS 2002. Type IV pili and twitching motility. *Annu Rev Microbiol* 56: 289-314.

Maxson ME, Darwin AJ 2004. Identification of inducers of the *Yersinia enterocolitica* phage shock protein system and comparison to the regulation of the RpoE and Cpx extracytoplasmic stress responses. *J Bacteriol* 186(13): 4199-4208.

McCutcheon JP, Moran NA 2012. Extreme genome reduction in symbiotic bacteria. *Nat Rev Microbiol* 10(1): 13-26.

Medina M, Sachs JL 2010. Symbiont genomics, our new tangled bank. *Genomics* 95(3): 129-137.

Metzker ML 2010. Sequencing technologies - the next generation. *Nat Rev Genet* 11(1): 31-46.

Mira A, Martin-Cuadrado AB, D'Auria G, Rodriguez-Valera F 2010. The bacterial pan-genome: a new paradigm in microbiology. *Int Microbiol* 13(2): 45-57.

Moran NA, Mira A 2001. The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola*. *Genome Biol* 2(12): RESEARCH0054.

Moran NA 2002. Microbial minimalism: genome reduction in bacterial pathogens. *Cell* 108(5): 583-586.

Morgan E, Campbell JD, Rowe SC, Bispham J, Stevens MP, Bowen AJ, Barrow PA, Maskell DJ, Wallis TS 2004. Identification of host-specific colonization factors of *Salmonella enterica* serovar *Typhimurium*. *Mol Microbiol* 54(4): 994-1010.

Morris AR, Visick KL 2010. Control of biofilm formation and colonization in *Vibrio fischeri*: a role for partner switching? *Environ Microbiol* 12(8): 2051-2059.

Murata H, Chatterjee A, Liu Y, Chatterjee AK 1994. Regulation of the production of extracellular pectinase, cellulase, and protease in the soft rot bacterium *Erwinia carotovora* subsp. *carotovora*: evidence that *aepH* of *E. carotovora* subsp. *carotovora* 71 activates gene expression in *E. carotovora* subsp. *carotovora, E. carotovora* subsp. *atroseptica*, and *Escherichia coli*. *Appl Environ Microbiol* 60(9): 3150-3159.

Nadeau NJ, Jiggins CD 2010. A golden age for evolutionary genetics? Genomic studies of adaptation in natural populations. *Trends Genet* 26(11): 484-492.

Nealson KH 1977. Autoinduction of bacterial luciferase. Occurrence, mechanism and significance. *Arch Microbiol* 112(1): 73-79.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-vibrio symbioses. *Appl Environ Microbiol* 64(9): 3209-3213.

Nishiguchi MK 2000. Temperature affects species distribution in symbiotic populations of Vibrio spp. *Appl Environ Microbiol* 66(8): 3550-3555.

Nishiguchi MK 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microb Ecol* 44(1): 10-18.

Nishiguchi MK, DeVinney R, Hirsch AM, Riley M, Mansky L, Vendatum G 2008. Perspective: Evolution of Virulence: Deciphering the mechanisms between pathogenic and benign symbioses. *Vie Milieu* 58(2): 87-106.

Nyholm SV, McFall-Ngai MJ 2004. The winnowing: Establishing the squid-*Vibrio* symbiosis. *Nat Rev Microbiol* 2(8): 632-642.

Nyholm SV, Nishiguchi MK 2008. The evolutionary ecology of a sepiolid squid-*Vibrio* association: From cell to environment. *Vie Milieu* 58(2): 175-184.

Orr HA 2005. The genetic theory of adaptation: A brief history. *Nat Rev Genet* 6(2): 119-127.

Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S Parrello B, Shukla M 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 42(D1): D206-D214.

Pernthaler J 2005. Predation on prokaryotes in the water column and its ecological implications. *Nat Rev Microbiol* 3(7): 537-546.

Purushe J, Fouts DE, Morrison M, White BA, Mackie RI, Coutinho PM, Henrissat B, Nelson KE 2010. Comparative genome analysis of *Prevotella ruminicola* and *Prevotella bryantii*: insights into their environmental niche. *Microb Ecol* 60(4): 721-729.

Read TD, Ussery DW 2006. Opening the pan-genomics box. *Curr Opin Microbiol* 9(5): 496-498.

Retchless AC, Lawrence JG 2010. Phylogenetic incongruence arising from fragmented speciation in enteric bacteria. *PNAS USA* 107(25): 11453-11458.

Roh H, Yun EJ, Lee S, Ko HJ, Kim S, Kim BY, Song H, Lim KI, Kim KH, Choi IG 2012. Genome sequence of *Vibrio* sp. strain EJY3, an agarolytic marine bacterium metabolizing 3,6-anhydro-L-galactose as a sole carbon source. *J Bacteriol* 194(10): 2773-2774.

Ruby EG, Lee KH 1998. The *Vibrio fischeri-Euprymna scolopes* light organ association: Current ecological paradigms. *Appl Environ Microbiol* 64(3): 805-812.

Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, Lostroh P, Lupp C McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Visick K, Whistler C, Greenberg EP 2005. Complete genome sequence of *Vibrio fischeri*: A symbiotic bacterium with pathogenic congeners. *PNAS USA* 102(8): 3004-3009.

Ruby EG 2008. Symbiotic conversations are revealed under genetic interrogation. *Nat Rev Microbiol* 6(10): 752-762.

Sachs JL, Simms EL 2006. Pathways to mutualism breakdown. *Trends Ecol Evol* 21(10): 585-592.

Sachs JL, Russell JE, Hollowell AC 2011. Evolutionary instability of symbiotic function in *Bradyrhizobium japonicum*. *PLoS ONE* 6(11): e26370.

Schuster BM, Perry LA, Cooper VS, Whistler CA 2010. Breaking the language barrier: experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host. *Symbiosis* 51(1): 85-96.

Smith MN, Crane RA, Keates RA Wood JM 2004. Overexpression, purification, and characterization of ProQ, a posttranslational regulator for osmoregulatory transporter ProP of *Escherichia coli*. *Biochemistry* 43(41): 12979-12989.

Soto W, Gutierrez J, Remmenga MR, Nishiguchi MK 2009a. Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. *Microb Ecol* 57(1): 140-150.

Soto W, Lostroh CP, Nishiguchi MK 2009b. Physiological responses to stress in the Vibrionaceae: aquatic microorganisms frequently affiliated with hosts. Springer, New York.

Soto W, Punke EB, Nishiguchi MK 2012. Evolutionary perspectives in a mutualism of sepiolid squid and bioluminescent bacteria: combined usage of microbial experimental evolution and temporal population genetics. *Evolution* 66(5): 1308-1321.

Soto W, Rivera FM, Nishiguchi MK 2014. Ecological diversification of *Vibrio fischeri* serially passaged for 500 generations in novel squid host *Euprymna tasmanica*. *Microb Ecol* 67(3): 700-721.

Stapley J, Reger J, Feulner PG, Smadja C, Galindo J, Ekblom R, Bennison C, Ball AD, Beckerman AP, Slate J 2010. Adaptation genomics: the next generation. *Trends Ecol Evol* 25(12): 705-712.

Suzuki H, Lefebure T, Hubisz MJ, Bitar PP, Lang P, Siepel A, Stanhope MJ 2011. Comparative genomic analysis of the *Streptococcus dysgalactiae* species group: gene content, molecular adaptation, and promoter evolution. *Genome Biol Evol* 3(168-185.

Tian CF, Zhou YJ, Zhang YM, Li QQ, Zhang YZ, Li DF, Wang S, Wang J, Gilbert LB, Li YR 2012. Comparative genomics of rhizobia nodulating soybean suggests extensive recruitment of lineage-specific genes in adaptations. *PNAS* 109(22): 8629-8634.

Toh H, Weiss BL, Perkin SA, Yamashita A, Oshima K, Hattori M, Aksoy S 2006. Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Res* 16(2): 149-156.

Treangen TJ, Abraham AL, Touchon M, Rocha EPC 2009. Genesis, effects and fates of repeats in prokaryotic genomes. *FEMS Microbiol Rev* 33(3): 539-571.

Treangen TJ, Rocha EP 2011. Horizontal transfer, not duplication, drives the expansion of protein families in prokaryotes. *PLoS Genet* 7(1): e1001284.

Visick KL, O'Shea TM, Klein AH, Geszvain K, Wolfe AJ 2007. The sugar phosphotransferase system of *Vibrio fischeri* inhibits both motility and bioluminescence. *J Bacteriol* 189(6): 2571-2574.

Vuilleumier S 1997. Bacterial glutathione S-transferases: What are they good for? *J Bacteriol* 179(5): 1431-1441.

Wang YL, Dunn AK, Wilneff J, McFall-Ngai MJ, Spiro S Ruby EG 2010. *Vibrio fischeri* flavohaemoglobin protects against nitric oxide during initiation of the squid-*Vibrio* symbiosis. *Mol Microbiol* 78(4): 903-915.

Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiasse RP, Schaefer AL, Koroleva I, Splinter-BonDurant S, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo MD, Casavant TL, Soares MB, Cronan JE, Reed JL, Ruby EG, McFall-Ngai MJ 2010. Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *PNAS USA* 107(5): 2259-2264.

Wietzorrek A, Bibb M 1997. A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an *ompR*-like DNA-binding fold. *Mol Microbiol* 25(6): 1181-1184.

Wollenberg MS, Ruby EG 2009. Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* squid from two Oahu (Hawaii) populations. *Appl Environ Microbiol* 75(1): 193-202.

Yang Z 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol Biol Evol* 24(8): 1586-1591.

Yannarell AC, Triplett EW 2005. Geographic and environmental sources of variation in lake bacterial community composition. *Appl Environ Microbiol* 71(227-239.

Yildiz FH, Visick KL 2009. *Vibrio* biofilms: so much the same yet so different. *Trends Microbiol* 17(3): 109-118.

Yip ES, Grublesky BT, Hussa EA, Visick KL 2005. A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. *Mol Microbiol* 57(5): 1485-1498.

Zamborsky DJ, Nishiguchi MK 2011. Phylogeographical patterns among Mediterranean Sepiolid squids and their *Vibrio* symbionts: environment drives specificity among sympatric species. *Appl Environ Microbiol* 77(2): 642-649.

Zhang JZ 2003. Evolution by gene duplication: an update. *Trends Ecol Evol* 18(6): 292-298.

Zhou A, Chen YI, Zane GM, He Z, Hemme CL, Joachimiak MP, Baumohl JK, He Q, Fields MW, Arkin AP 2012. Functional characterization of Crp/Fnr-type global transcriptional regulators in *Desulfovibrio vulgaris* Hildenborough. *Appl Environ Microbiol* 78(4): 1168-1177.

Received on June 9, 2015
Accepted on August 24, 2015
Associate editor: A Chenuil

These supplementary tables are available on our website:

Supplementary Table I. – Genomic data summary and percent coverage of reference strains available on GenBank corresponding with newly sequenced genomes. NE = not examined.

Supplementary Table II. – Data concerning Roche 454 sequencing read quality.

Supplemental Table III. – List of core genes shared by symbiotic strains of *Vibrio fischeri*. The gene numbers are based on those for the V. fischeri MJ11 reference genome.

Supplementary Table IV. – Genetic elements identified in three symbiotic strains of *Vibrio fischeri* and one free-living *Vibrio* sp. sequenced in this study, in addition to one model organism, *V. fischeri* ES114 (Ruby *et al*. 2005). Annotations provided by RAST (Overbeek *et al*. 2014).

Supplementary Table V. – Patterns of GC content in Vibrio genomes sequenced in this study. Clusters of open reading frames (ORFs) that deviate from mean GC content are provided in blocks separated by spaces. Clusters are defined as 3 or more ORFs in proximity. High or low GC content is defined as GC proportions that fall more the 2 standard deviations from the mean for that genome.

Supplementary Table VI. – Gene duplication within symbiotic *Vibrio fischeri* and the non-symbiotic *V. cf. campbellii* strains. From the orthology analysis, amino acid sequences demonstrating closest-match sequence similarity to other genes within the sequence dataset are listed below. Lengths of the sequences are denoted by nucleotide number. Similarities to most closely-matched reference genomes are shown, as well as putative gene functions for these duplicated gene sequences.

Comparative analysis of quantitative methodologies for Vibrionaceae *biofilms*

Alba A. Chavez-Dozal, Neda Nourabadi, Martina Erken, Diane McDougald & Michele K. Nishiguchi

Folia Microbiologica
Official Journal of the Institute of Microbiology, Academy of Sciences of the Czech Republic and Czechoslavak Society for Microbiology

ISSN 0015-5632
Volume 61
Number 6

Folia Microbiol (2016) 61:449-453
DOI 10.1007/s12223-016-0456-9

Springer

Folia Microbiol (2016) 61:449–453
DOI 10.1007/s12223-016-0456-9

Comparative analysis of quantitative methodologies for *Vibrionaceae* biofilms

Alba A. Chavez-Dozal[1,2] · Neda Nourabadi[3] · Martina Erken[4] · Diane McDougald[5,6] · Michele K. Nishiguchi[3]

Received: 8 October 2015 / Accepted: 9 March 2016 / Published online: 24 March 2016

Abstract Multiple symbiotic and free-living *Vibrio* spp. grow as a form of microbial community known as a biofilm. In the laboratory, methods to quantify *Vibrio* biofilm mass include crystal violet staining, direct colony-forming unit (CFU) counting, dry biofilm cell mass measurement, and observation of development of wrinkled colonies. Another approach for bacterial biofilms also involves the use of tetrazolium (XTT) assays (used widely in studies of fungi) that are an appropriate measure of metabolic activity and vitality of cells within the biofilm matrix. This study systematically tested five techniques, among which the XTT assay and wrinkled colony measurement provided the most reproducible, accurate, and efficient methods for the quantitative estimation of *Vibrionaceae* biofilms.

✉ Michele K. Nishiguchi
nish@nmsu.edu

[1] Section of Infectious Diseases, New Mexico Veterans Healthcare System, Albuquerque, NM 87108, USA

[2] Division of Infectious Diseases, University of New Mexico Health Science Center, Albuquerque, NM 87131, USA

[3] Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA

[4] Centre for Marine Biofouling and Bio-Innovation, School of Biotechnology and Biomolecular Sciences, University of South Wales, Sydney, NSW 2052, Australia

[5] ithree Institute, Faculty of Science, University of Technology, Sydney, Broadway, Sydney, NSW 2007, Australia

[6] Singapore Centre on Environmental Life Sciences Engineering, School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore

Introduction

Biofilms are communities of microbes that are composed of cells attached to a surface and encapsulated in an extracellular matrix (composed primarily of polysaccharides, proteins, and DNA; Watnick and Kotler 2000; Yildiz and Visick 2008). Biofilms develop when cells transition from a planktonic (free-living) lifestyle to surface-attached complex multicellular communities (Watnick and Kotler 2000). These microscopic communities can form unique microbiomes that are common in nature, and can range from a healthy consortium of beneficial bacteria to those that can be the primary source of dangerous chronic diseases (Watnick and Kotler 2000; Costerton et al. 1999).

Biofilms formed by symbiotic bacteria in the family *Vibrionaceae* (pathogenic and mutualistic) have been studied for over 20 years, and diverse methodologies for studying *Vibrio* biofilms under laboratory conditions have been proposed by multiple research groups (Yildiz and Visick 2008). However, this area of research is in constant change and is still under development. For example, a recent methodology developed to measure *Vibrio* biofilm mass included examining cell viability and identification of common biofilm phenotypes (such as formation of wrinkled or rugose bacterial colonies; Ray et al. 2011), while another popular semiquantitative method (that has been used extensively for multiple bacterial biofilms) includes the use of crystal violet in a colorimetric assay to stain biofilms attached to a surface (O'Toole 2011). In the case of fungal biofilms (such as those formed by *Candida* and *Cryptococcus*), there is a commonly used colorimetric assay that accurately shows cellular viability within the biofilm through the metabolic use of formazan salts (Kuhn et al. 2003). Interestingly, this method is not routinely used in *Vibrio* (and other bacterial) biofilms for its quantitative capability or detection limits. All of these proposed methods

have been important tools to measure in vitro formation of biofilms. These procedures vary widely as to their time and cost requirements, and in variation reported in assay performance. An important element of these proposed methods is the necessity to accurately and reproducibly quantify viable cells in the biofilms as can be accomplished by a metabolism-based assay such as the XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide} reduction assay. Therefore, the goals of this current study were to test the efficacy of the XTT assay in *Vibrionaceae* biofilms as well as to make a comparative analysis of time, efficiency, and cost of different quantitative assays. Studies included the use of crystal violet staining, dry cell mass measurement, viable colony counting (direct enumeration of bacteria in biofilms), phenotype observation (wrinkled colony development), and the use of the XTT reduction assay.

Methods

Six wild-type *Vibrio* strains (biosafety level (BSL) 1) were selected for this study: *Vibrio rotiferianus* (Chowdhury et al. 2011), *Vibrio corallilyticus* (ATCC BAA450), *Vibrio parahaemolyticus* (ATCC 17802), two *Vibrio fischeri* strains isolated from *Euprymna* squid hosts: ES114 (*Euprymna scolopes* from Kaneohe Bay, O'ahu, HI, USA) and ETJB1H (*Euprymna tasmanica* from Jervis Bay, New South Wales, Australia), and one free-living (seawater) isolate (*V. fischeri* CB31 from Coogee Bay, New South Wales, Australia). We also selected mutant *V. fischeri* strains (from the ETJB1H isolate) that have been reported to be defective in biofilm formation (Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012). Mutant strains had interruptions in genes responsible for (a) twitching motility and pilus assembly (Δ*pilT*, Δ*pilU*, Δ*mshA*; Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012), (b) flagellum assembly and functionality (Δ*flgF*, Δ*motY*; Chavez-Dozal et al. 2012), and (c) stress responses such as heat shock (Δ*ibpA*) and magnesium-dependent induction (Δ*mifB*; Chavez-Dozal et al. 2012).

To evaluate biofilm formation, cultures were grown overnight at 28 °C and 250 rpm in Luria Bertani high-salt media (LBS; 10 g tryptone, 5 g yeast extract, 20 g sodium chloride, 50 mL 1 mol/L Tris pH 7.5, 3.75 mL 80 % glycerol, and 950 mL distilled water). Biofilm quantification was measured by five different methodologies, including crystal violet (CV) staining (O'Toole 2011), XTT assay (Pierce et al. 2010), dry cell mass measurement (Taff et al. 2012), colony counting (Merrit et al. 2005), and wrinkly colony development (Ray et al. 2011).

For the CV and XTT assays, all strains were subcultured and grown to a cell density of 1×10^8 colony-forming units (CFU)/mL. Aliquots of each *Vibrio* isolate (200 μL) were added to individual wells on a flat-bottom, polystyrene 96-well microtiter plate (Corning, Sigma-Aldrich CLS3628, St. Louis, MO) and incubated for 24 h under conditions previously described (Chavez-Dozal et al. 2012). After incubation, planktonic (those not forming biofilms) cells were removed by briskly shaking the plate and attached cells were washed three times with sterile media. For the CV assay, crystal violet (2 % aqueous solution) was added to each well and incubated at room temperature for 30 min. After incubation, CV was removed and the plate was washed five times with sterile media. CV was then quantified by solubilizing with 95 % ethanol, and optical density (A_{562}) readings were recorded at 562 nm for each biofilm in individual wells. For the XTT assay, planktonic cells were removed and plates washed as previously described (O'Toole 2011; Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012). Metabolic activity was measured by the XTT reduction assay (Pierce et al. 2010). In brief, 0.010 mol/L menadione (Sigma-Aldrich, St. Louis, MO) stock solution (diluted in acetone) was mixed with XTT/Ringer's lactate solution (0.5 g of XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide} from Sigma diluted in 1 L of 1× PBS or Ringer's lactate solution) at a final concentration of 1 μmol/L. An aliquot of the XTT/Ringer's/menadione solution was then added to each prewashed well. The plates were covered in aluminum foil and incubated for 2 h at 28 °C. If the XTT is effectively reduced by metabolically active cells, the original clear solution is transformed into an orange solution that can be measured at A_{490}. For CV and XTT assays, experiments were performed three times independently (biological replicates), each in quadruplicate (technical replicates) including inoculated sterile LBS as a negative control.

For dry cell mass determination, biofilms were formed in 96-well microplates and planktonic bacteria were removed after 24 h of incubation (as described previously; Taff et al. 2012). Biofilms were dried for 30 min at room temperature and then were disrupted by scraping with a sterile spatula and diluted into 500 μL of sterile water. The biofilm suspension was filtered through a preweighted filter (0.45 μm) and dried in an incubator at 105 °C for 2 h, after which the filter was weighed again. The dry mass of the biofilm was calculated based on mass differences between the control and samples.

For enumeration of bacteria in biofilms, the biofilm assay plates were inoculated, incubated, and washed as described for the CV and XTT assays. Each individual well was cut with scissors and 100 μL of 1× PBS was added. The well (plus the PBS) was placed into a separate 10-mL tube containing 1.9 mL of 1× PBS. The sample was sonicated for 5 s at 30 % power (higher sonication times compromised cell viability of some strains). The sample was plated in triplicate onto LBS plates and incubated for 24 h at 28 °C. Viable counts of colony-forming units were performed. For each strain, the experiment was performed in triplicate.

We additionally performed a semiquantitative method to measure biofilm formation by observation of wrinkled colony

development as described previously (Merrit et al. 2005) with minor modifications. In brief, an aliquot of overnight cultures was subcultured in 5 mL of fresh LBS at a 1:100 dilution and grown to an A_{600} of 0.2. After incubation, 1 mL of culture was pelleted and washed twice with 1× PBS, and resuspended in 1 mL of 1× PBS. Ten-microliter subsamples were spotted onto a fresh LBS plate (three spots per plate) and incubated for 24 h at 28 °C. Morphology and spot size were observed, and light micrographs of colonies were acquired using an inverted microscope (Micromaster digital inverted microscope with infinity optics, Fisher Scientific, Waltham, MA). The diameter of the colony was recorded digitally using the data acquisition software Micron 2.0.0 (Westover Scientific, Milpitas, CA). This experiment was performed in triplicate for each strain.

Results were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc or Tukey comparison post-test. Differences between groups were considered to be significant at a P value of <0.05. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Findings

In vitro studies of biofilms have been increasing in number over the last decade. *Vibrio* biofilms play an important role in the environment and have been studied in the laboratory for over a decade (Yildiz and Visick 2008). There are multiple assays that have been proposed for quantification of *Vibrio* biofilms; for example, crystal violet is one of the most commonly used methods (Ray et al. 2011; O'Toole 2011; Kuhn et al. 2003; Chowdhury et al. 2011), and consists of a colorimetric assay where crystal violet solution (water or ethanol based) is used to stain cells and their extracellular matrices. The amount of CV absorbed by the biofilm is quantified by optical density readings of dissolved crystal violet, which is directly proportional to the biofilm mass. An alternative method consists of weighing the dried biofilm. This is one of the techniques used to calculate the total amount of biofilm but does not account for cell viability within the biofilm. The colony-forming unit determination assay (CFU counts) is a labor-intensive method that is solely based on cell viability. Moreover, the recently proposed method of observation of wrinkled colonies provides a more reliable method of quantifying biofilm development, which also allows the evaluation of the tri-dimensional structure and patterning of a particular *Vibrio* biofilm former. Some limitations of CFU and wrinkled colony development assays include lengthy assay time and requirements for previous adjustments to ensure reproducibility. In addition, strains with growth defects are usually difficult to analyze and cells in the viable but non-culturable state will not be detected (McDougald et al. 1998). For those types of assays that require removal of adherent biofilms (dry cell measurement and CFU counting), removal of cells may be inconsistent between samples.

An alternative method that has been widely used and has been proven to be especially useful for the study of fungal biofilms (in particular *Candida albicans*) is a colorimetric assay based on cellular viability involving the use of tetrazolium salts (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide, or XTT) and measurement of its orange-colored formazan product (due to activity of succinoxidase and cytochrome P450 enzymes). Since this assay is easy to perform, we included this analysis in quantification of *Vibrio* biofilms to combine measurements of cell viability with biofilm mass.

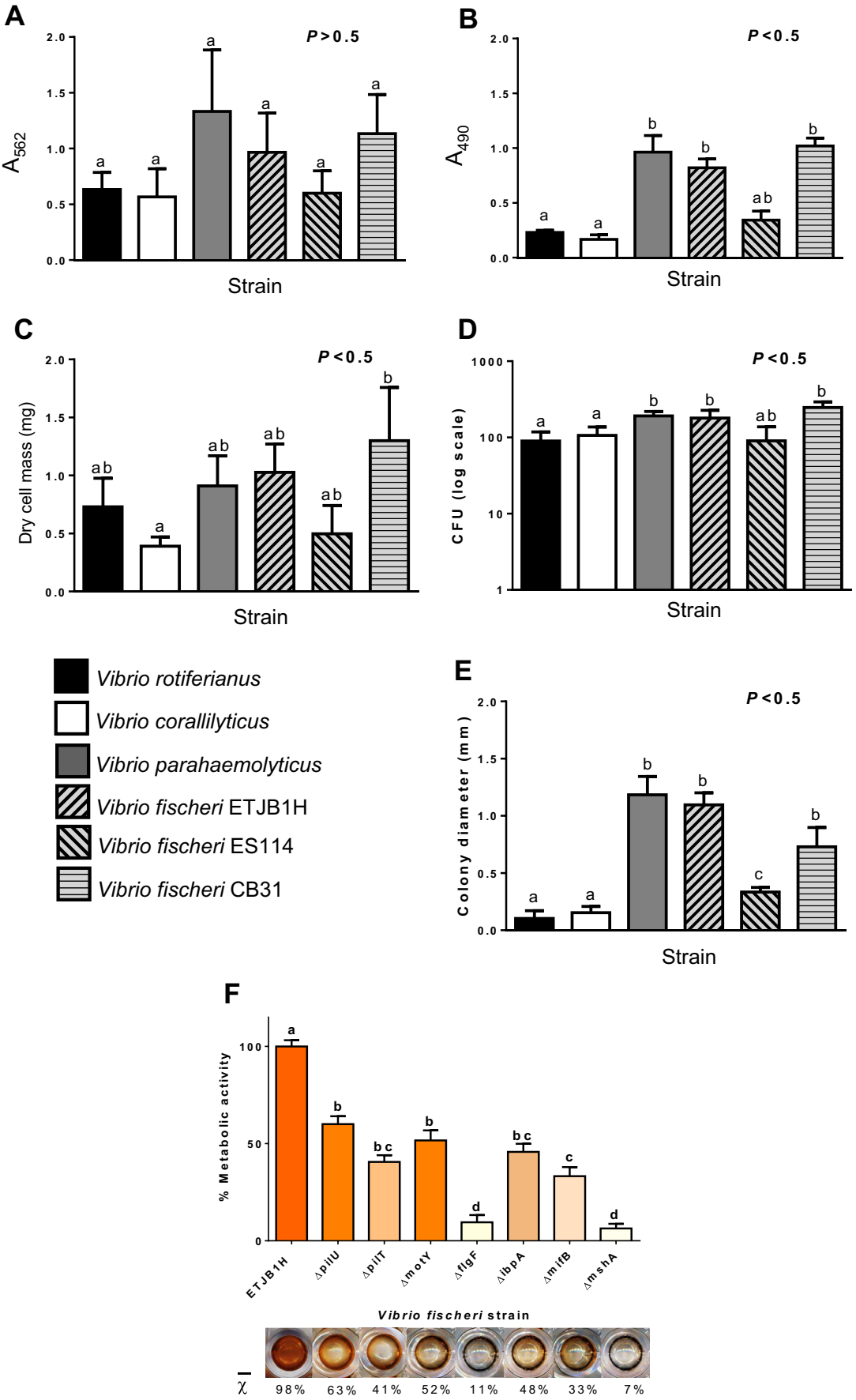
Since it is not advisable to conduct biofilm formation experiments on strains with growth defects, we performed growth curves on all the strains used in this study and none of them exhibited defects in growth. Additionally, for those biofilm formation experiments done in 96-well plates (including crystal violet and XTT), we measured the optical density (A_{600}) of the plate after incubation and prior to addition of either CV or menadione/Ringer's. All strains were between an A_{600} range of 5–6.

Among the assays tested, the most time consuming (but accurate and reproducible) were the CFU counting and the wrinkled colony development, whereas the crystal violet and dry cell mass assays were the most inaccurate and least reproducible (Table 1). Additionally, the XTT assay was the most reliable, the least time consuming, and the least costly. Figure 1 shows a more detailed comparison of the assays tested and their variability according to statistical differences (P values). An additional advantage of the use of metabolism-based assays (XTT assay) was that it allowed comparison of biofilm formation efficacy of mutant cells with the parental strain. This is illustrated in Fig. 1f, where different *V. fischeri* mutants in genes that have been reported to be important for

Table 1 Summary of the different methods used to quantify biofilms

Assay	Accuracy	Reproducibility	Time
XTT assay	++++	++++	+
Crystal violet	+	+	+
Dry cell mass	++	++	++
CFU count	+++	+++	++++
Wrinkled colony development	++++	++++	+++

Accuracy represents whether the data was consistent among technical replicates, as well as whether differences between the strains were significant ($P < 0.05$). Reproducibility was determined by the coefficient of variation (CV) for each set of data between biological replicates; ++++ = CV <0.1, +++ = CV of 0.1–0.15, ++ = CV of 0.15–0.2, and + = CV of >0.2. Time accounts for both the total length of the protocols and the amount of labor required for each (++++ representing the most time-consuming protocols)



◀ **Fig. 1** Comparison of five in vitro biofilm quantification methods for *Vibrionaceae* biofilms. Each graph represents the data of the average (with *error bars* indicating standard deviations) of three independent experiments (biological replicates). *Different letters* on the *abscissa* denote significant differences between groups according to the Tukey post hoc comparison. *P* values indicate significant ($P < 0.5$) or non-significant ($P > 0.5$) overall differences according to the one-way ANOVA test. Absorbancies (A_{562} and A_{490}) of biofilms using **a** crystal violet (CV) or **b** XTT assay, respectively. **c** Biofilm quantification via dry cell mass measurement. **d** Colony-forming unit (CFU) determination of cells in biofilms formed in 96-well microplates. **e** Diameter of wrinkled colonies measured after 24 h of incubation. **f** Metabolic activity of wild-type (ETJB1H) and mutant *Vibrio fischeri* strains. Δ*pilT*, Δ*pilU*, and Δ*mshA* are type IV pilus mutants; Δ*flgF* and Δ*motY* are mutants in flagellum assembly and functionality; Δ*ibpA* is a mutant of a chaperonin responsible for the heat stress response, and Δ*mifB* is a mutant of the magnesium-dependent induction response. Metabolic activity is calculated as percentage in relation to *A* readings (A_{490}) of the wild-type parental strain. *Different letters* indicate significant differences according to the Tukey post hoc comparison test. *Wells* indicate the representative image of the intensity of the orange product as a result of formazan production by each biofilm. $\overline{\chi}$ represents the median value of the metabolic activity (in percentage)

biofilm development (but that are not defective in planktonic growth) were compared based on metabolic activity.

For the *Vibrionaceae* strains tested, we found that the XTT assay is the most reproducible and efficient method for measurement of biofilm biomass. The observation of development of wrinkled colonies could be used as a complementary test as it allows observation of the tri-dimensional structure of the biofilm and complements the colorimetric approach.

Acknowledgments The authors would like to thank Dr. Maurizio Labbate for providing the *Vibrio rotiferianus* strain. A.A.C.D. was supported by the NMSU National Institutes of Health RISE to the doctorate (NIH NIGMS R25GM061222), Institutional Research and Career Development Award, and the Academic Science Education and Research Training Program (IRACDA-ASERT, NIH-K12GM088021).

References

Ariyakumar DS, Nishiguchi MK (2009) Characterization of two host specific genes, mannose sensitive hemagglutinin (mshA) and uridyl phosphate dehydrogenase (UDPH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol Lett 299:65–73

Chavez-Dozal AA, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK (2012) Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. FEMS Microbiol Ecol 81:562–573

Chowdhury PR, Boucher Y, Hassan KA, Paulsen IT, Stokes HW, Labbate M (2011) Genome sequence of *Vibrio rotiferianus* strain DAT722. J Bacteriol 193(13):3381–3382

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA (2003) Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. J Clin Microbiol 41(1):506–508

McDougald D, Rice SA, Weichart D, Kjelleberg S (1998) Nonculturability: adaptation or debilitation? FEMS Microb Ecol 25(1):1–9

Merrit JH, Kadouri DE, O'Toole GA (2005) Growing and analyzing static biofilms. Curr Protocol Microbiol Chapter 1:Unit 1B.1

O'Toole GA (2011) Microtiter dish biofilm formation assay. J Vis Exp 30(47). doi: 10.3791/2437.

Pierce CG, Uppuluri P, Tummala S, Lopez-Ribot JL (2010) A 96 well microtiter plate-based method for monitoring formation and antifungal susceptibility testing of *Candida albicans* biofilms. J Vis Exp 44: 2287

Ray VA, Morris AR, Visick KL (2011) A semi-quantitative approach to assess biofilm formation using wrinkled colony development. J Vis Exp 7(64):e4035. doi:10.3791/4035

Taff HT, Nett JE, Andes DR (2012) Comparative analysis of *Candida* biofilm quantitation assays. Med Mycol 50:214–218

Watnick P, Kotler R (2000) Biofilm, city of microbes. J Bacteriol 182: 2675–2679

Yildiz FH, Visick KL (2008) *Vibrio* biofilms: so much the same yet so different. Trends Microbiol 17:109–118

Chapter 7

Impact of Metabolomics in Symbiosis Research

Alba Chavez-Dozal and Michele K. Nishiguchi

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66631

Abstract

In symbiotic associations, there is a constant molecular complexity that allows establishment and maintenance of the relationship. Metabolomic profiles have enabled researchers to explain symbiotic associations in terms of their underlying molecules and interactions between the symbiotic partners. In this review, we have selected studies on symbioses as examples that have helped to explain the metabolic integration of bacterial symbionts and their hosts in an effort to understand the molecular fingerprint of animal-microbial symbioses.

Keywords: symbiosis, mutualism, metabolomics, co-clustering analysis

1. Introduction

The intimate association between two organisms is a very complex biological phenomenon; nevertheless, it is a very common way of life for every living organism on Earth. Symbiotic associations with one or many phylogenetically different organisms provide a fascinating view into how symbionts adapt and co-evolve. As Chaston and Douglas beautifully described in their comprehensive review [1], the omics revolution has transformed our ability to understand symbiotic associations at the molecular level. Researchers have adopted multiple techniques with great fervor in an effort to decipher the basis and complexity of symbiotic associations. Until recent years, the molecular pathways of symbiotic associations could only be studied in the context of genetic changes (transcriptomic studies) and protein profiles (proteomics); however, it is very likely that the establishment of a mutualistic association involved multiple evolutionary changes in the biochemistry and metabolic network of all the partners involved in the symbiosis [1]. Omics biology brings challenges and opportunities; one of the recent advances is the ability to construct a molecular metabolic catalog of an organism within a symbiotic association.

Metabolomics refers to the analytical approach used to study different cell products ("chemical fingerprints") that help to understand the physiological state of an organism [2].

In this section, we provide a comprehensive description of four experiments where the approach of metabolomics was selected in a particular type of animal-microbial symbiosis, in order to answer specific questions in symbiosis research.

2. Exemplars of metabolomic approaches in symbiosis research

2.1. Inferring metabolic interactions in arbuscular mycorrhizal symbiosis

Our exemplar of metabolomics studies of microbe-plant interactions is a set of observations by Schweiger et al. [3] that describe species-specific leaf metabolic responses to arbuscular mycorrhiza (AM) [4]. Arbuscular mycorrhiza is a unique symbiotic association between root arbuscular mycorrhizal fungi (AMF) and plants [4]. This is an ancient and widespread association where the fungus improves water uptake to the host plant, and in return the fungus receives plant carbohydrates. The fungus is restricted to the roots of the plant; however, the biochemical pathways and the involvement of exchanged substances are reflected on systemic root tissues affecting the chemical composition of plant tissues (defined as "phytometabolome") [4].

Comparative studies conducted on five different plant-AMF associations demonstrate that foliar metabolome is highly plant-species-specific, with low degrees of conservation across species. The experimental design was crucial to the success of this analysis, with the metabolome analysis performed on leaves of five plant species exposed to the worldwide distributed AMF *Rhizophagus irregularis*. Furthermore, the study took into account the implications of metabolite fluctuation at different leaf developmental stages and plant-reproductive status. Additionally, mycorrhizal plants were compared with control plants that received a sterilized inoculum. The results from this study indicate the high specificity of plant metabolome responses to the same AMF colonization; among the most striking findings indicate that metabolomics responses related to phosphate uptake, citric acid cycle, and amino acids were species-specific [3]. **Figure 1** summarizes the most important findings of this interesting study.

2.2. Metabolomic profile of the ryegrass-endophyte symbiosis

Along the lines of microbe-plant interactions, there is an interesting study conducted by Cao et al. [5] that is of particular relevance for symbiosis research. The metabolomics profile of perennial ryegrass (*Lolium perenne*) infected with endophytic fungus (*Neotyphodium lolii*) provided understanding of regulatory biochemical mechanisms for the production of beneficial alkaloids.

N. lolii is a naturally occurring fungus whose complete cycle occurs within perennial ryegrass. The fungus grows between the cells of the host plant drawing nutrients from it, and in return, the endophyte produces chemical compounds that provide resistance to drought, pests, and protection from overgrazing. Therefore, the aim of this study was to gather metabolomics information and combine it with microarray data in order to obtain a better understanding of

http://dx.doi.org/10.5772/66631

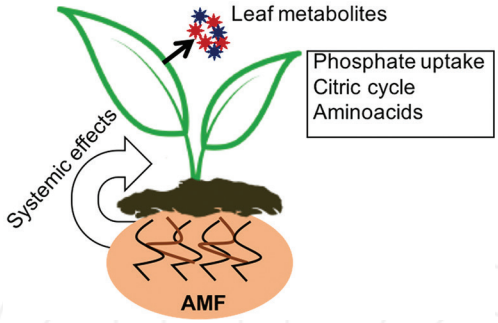


Figure 1. Summary of findings on the leaf phytometabolome when plants are exposed to the same arbuscular mycorrhizal fungi (AMF). Leaf metabolites detected included carbohydrates, organic acids, amino acids and derivatives, cyclic polyols, and sugar alcohols. Metabolites are differentially regulated primordially affecting the phosphate and citric acid cycles.

the biochemical mechanisms involved in the cross talk between partners, with the eventual purpose of achieving genetic manipulation of beneficial metabolite production (in particular manipulation of alkaloids).

Twenty-four perennial ryegrass samples comprising three tissue types (immature leaves, blades, and mature leaves) were examined of both endophyte-infected plants and endophyte free as a control. Targeted metabolomics analysis was used as the quantitative approach that provided identities of 70 metabolites based on the available databases of reference compounds. The use of targeted metabolomics in combination with microarray data provided better identification and classification Accuracy of compounds, as well as greater insights into the dynamics and fluxes of the newly identified metabolites. Results of this comprehensive study included the identification of accumulated alkaloids in the mature tissues of endophyte-infected ryegrass, and the co-clustering analysis of microarray data-identified genes with distinctive expression patterns which coincide with the pattern of alkaloid accumulation [5]. **Figure 2** summarizes the findings of this study. Results of this study indicate that co-clustering analysis is not a straightforward task no matter what kind of algorithm is used, and that the integration of transcriptomics and metabolomics can generate noisy data. However, this study demonstrated that co-cluster analysis could be a comprehensive choice to gain a more complete understanding of a complex biological system involving two entirely different taxa that are intertwined in their metabolic capabilities.

2.3. Metabolomic profile of symbiotic protection against pathogens

It is believed that specific strains from the gut microbiota can influence host immunity and protect from infection by pathogenic bacteria. One example is the early and prevalent gut colonizer *Bifidobacterium*, which is considered part of the healthy normal gut flora. It is believed that different strains of *Bifidobacterium* protect against enteropathogenic *Escherichia coli* O157:H7 infection in mice; however, the potential molecular and cellular mechanisms underpinning this protective effect are still under investigation [6].

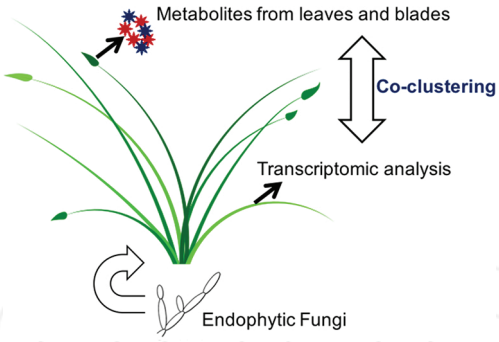


Figure 2. Summary of the study conducted by Cao et al. Co-clustering analysis of microarray and metabolomics data on endophytic-infected ryegrass indicate a set of genes and metabolites that are important for alkaloid production.

One study conducted by Fukuda et al. [7] used a combined "omics" strategy in an effort to gain a better understanding of the protective effect of *Bifidobacterium* over its mice host. Experiments designed comprised mice infected with different species of the symbiotic bacterium *Bifidobacterium* (including *B. longum* and *B. adolescentis*) and the pathogen *E. coli* O157:H7. The life span of co-infected mice was observed and transcriptomic and metabolomic profiles were conducted. **Figure 3** diagrams the experimental design of this study. This sophisticated analysis included a combination of sequencing, the platform used for metabolite detection was HPLC-MS (high-performance liquid chromatography-mass spectrometry) and for the analysis of products, the dataset was subjected to a multivariate analysis method named PLS (partial least squares) projection to latent structures. Typical data-processing flow included detection of signal peaks and normalization of dataset to generate a matrix of the products detected. For their statistical analysis, the method selected was PCA (principal component analysis) and CL (cluster analysis).

Results from this study indicate that mice bearing the strain *B. longum* survived, whereas those infected with *B. adolescentis* died. Metabolomic profiles between the two treatments revealed that the concentration of fatty acids (acetic acid in particular) was significantly elevated in those mice that survived *E. coli* infection. Furthermore, mice that survived showed an increased expression of genes involved in ATP-binding-cassette carbohydrate transporters [7]. Observations from the study suggest that the elevated production of acetic acid improved intestinal defense, thereby enhancing the barrier function of colon epithelial cells inhibiting the transport of *E. coli* toxins.

2.4. Metabolomics of a beneficial marine bacterium

The marine luminescent bacterium *Vibrio fischeri* establishes a symbiotic association with numerous sepiolid squids and monocentric fishes. *V. fischeri* infects a specialized light organ in the mantle (body) cavity of host squids and produces bioluminescence that is used by its host to avoid predation in a behavior known as counterillumination. In return, the squid host provides an enriched habitat for *Vibrio* to reproduce and to form bacterial communities of

monospecies biofilms. The ability of *V. fischeri* to form a biofilm in the light organ of its squid host plays a central role in establishment and maintenance of the symbiotic association. This interesting symbiotic association has been the center of attention of many researchers, and has been investigated for more than 25 years; however, as indicated for other examples of mutualistic associations, the molecular basis of the squid-*Vibrio* symbiosis is still obscure.

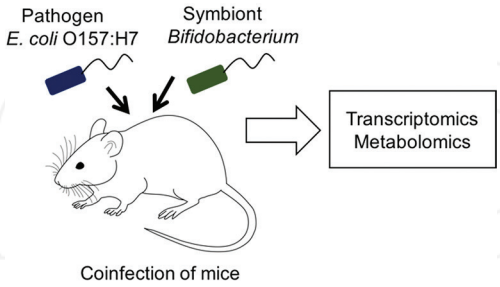


Figure 3. Summary of the experiment conducted by Fukoda et al. Mice were coinfected with beneficial strains of *Bifidobacterium* and the pathogenic strain of *Escherichia coli* O157:H7. Combined transcriptomic and metabolomic profiles revealed an increase in acetate and fructose transporters in those mice that survived lethal infection.

In a recent study conducted by Chavez-Dozal et al. [8], both proteomic and metabolomic profiles were performed in parallel in strains of *V. fischeri* in their biofilm form and compared to profiles of free-living (or planktonic) *V. fischeri* cells of the same strain. The main objective of this study was to obtain a comprehensive profile of the molecular components to provide the first meta-proteome profile of biofilms that are important for establishment of this mutualistic association. A summary of this study is illustrated in **Figure 4**.

Biofilms are a complex microbial community composed of cells encased within a self-produced exopolymeric matrix. Expression profiles of biofilm communities reveal the composition of the matrix, which include a combination of lipids, polysaccharides, proteins, and DNA [9, 10].

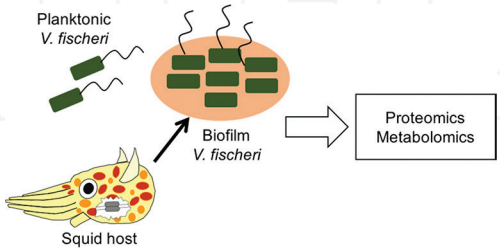


Figure 4. Summary of the experiment conducted by Chavez-Dozal et al. [5]. Proteomic and metabolomic profiles were performed in planktonic cells and biofilm communities of the same strain of *Vibrio fischeri*. Results revealed an upregulation of biofilm matrix components and molecules related to multiple stress responses.

Results of this study revealed a time-resolved picture of approximately 100 proteins and 200 metabolites present in the biofilm state of *V. fischeri*. The most important components found in this study include proteins, sugars, and molecules that form part of the exopolysaccharide matrix of biofilms; surprisingly, an increased concentration of intermediates of the glycolysis pathway was found to be prevalent during the biofilm state [8]. Results from this study suggest that molecules involved in the construction of the biofilm matrix are essential to bacterial community formation, a process that has been known to activate stress responses such as upregulation of alternative anaerobic pathways. The reported findings of this study have broad implications for *V. fischeri* ecology, since many of the symbiosis-regulated genes are not yet described. The combination of proteomics and metabolomics has therefore provided a link between protein regulation and function during different phases of the symbiosis, improving our understanding of the mechanisms that are important for successful host colonization.

3. Concluding remarks

Metabolomic approaches are increasingly selected for multiple purposes of symbiosis research. Although other "omic" approaches are needed to understand molecular function in symbiotic associations, the emerging use of metabolomics provides a new level of biochemical sophistication. The different examples provided in this mini review are only some of the pillar studies that included the use of either metabolomics or a combinational analysis of metabolomics with transcriptomics/proteomics of different mutualistic systems; however, many more studies are in progress using metabolomics profiles to define and characterize molecular and biochemical pathways that are important for establishment and persistence of symbiotic associations. The advancement of technologies that allows higher resolution of minute concentrations of proteins and their modulation will expand the area of metabolomics research and will enable a better perspective of the physiological state of organisms as single entities (otherwise known as the holobiome).

Author details

Alba Chavez-Dozal and Michele K. Nishiguchi*

*Address all correspondence to: nish@nmsu.edu

Department of Biology, New Mexico State University, Las Cruces, NM, USA

References

[1] Chaston J, Douglas AE. Making the most of "omics" for symbiosis research. Biol Bull; 2012;**223**:21–9. doi: 10.1111/j.1365-2133.2012.10859.x.

[2] Tyagi S, Raghvendra, Singh U, Kalra T, Munjal K. Applications of metabolomics, A systematic study of the unique chemical fingerprints: an overview. Int J Pharma Scie Rev and Res; 2010;**3**(1):019.

[3] Schweiger R, Baier MC, Persicke M, Muller C. High specificity in plant leaf metabolic responses to arbuscular mycorrhiza. Nat Comm; 20114;**5**:3886. doi: 10.1094/MPMI-05-14-0126-R.

[4] Schweiger R, Muller C. Leaf metabolome in arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol; 2015;**26**:120–6. doi: 10.1016/j.pbi.2015.06.009.

[5] Cao M, Johnson L, Johnson R, Koulman A, Lane GA, Rasmussen S. Joint analyses of transcriptomic and Metabolomic data to probe ryegrass-endophyte symbiosis. In: Popay A, Thom E (eds). Proceedings of the 6th international symposium on fungal endophytes of grasses. Christchurch, New Zealand, December 2007.

[6] Sugahara H, Odamaki T, Fukuda S, Kato T, Xiao J, Abe F, Kikuchi J, Ohno H. Probiotic *Bifidobacterium longum* alters gut luminal metabolism through modification of the gut microbial community. Sci Rep; 2015;**5**:13548. doi:10.1038/srep13548.

[7] Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T, Taylor TD, Itoh K, Kikuchi J, Morita H, Hattori M, Ohno H. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature; 2011;**469**:543–7. doi: 10.1038/nature09646.

[8] Chavez-Dozal A, Gorman C, Nishiguchi MK. Proteomic and metabolomics profiles demonstrate variation among free-living and symbiotic *Vibrio fischeri* biofilms. BMC Micro; 215;**15**:226. doi: 10.1186/s12866-015-0560-z.

[9] Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. FEMS Microbiol Rev; 2015;**39**(5):649–69. doi: 10.1093/femsre/fuv015.

[10] Jiao J, D'haeseleer P, Dill BD, Shah M, VerBerkmoes NC, Hettich RL, Banfield JF, Thelen MP. Identification of biofilm matrix-associated proteins from an acid mine drainage microbial community. Appl Env Microbiol; 2011;**77**(15):5230–7. doi: 10.1128/AEM.03005-10.

Integrative and Comparative Biology

Integrative and Comparative Biology, volume 57, number 1, pp. 7–17
doi:10.1093/icb/icx004

Society for Integrative and Comparative Biology

INVITED PAPER

Broadening Participation in the Society for Integrative and Comparative Biology

Cheryl A.D. Wilga,[1,*] Michele K. Nishiguchi[†] and Brian Tsukimura[‡]

*Biological Sciences Department, University of Alaska Anchorage, 3101 Science Circle, Anchorage, AK 99508, USA; †Department of Biology, New Mexico State University, PO Box 30001, MSC 3AF, Las Cruces, NM 88003, USA; ‡Department of Biology, California State University, Fresno, 2555 E. San Ramon Avenue, Fresno, CA 93740, USA

[1]E-mail: cwilga@alaska.edu

Synopsis The goal of the Society for Integrative and Comparative Biology's Broadening Participation Committee (SICB BPC) is to increase the number of underrepresented group (URG) members within the society and to expand their capabilities as future researchers and leaders within SICB. Our short-term 5-year goal was to increase the recruitment and retention of URG members in the society by 10%. Our long-term 25-year goal is to increase the membership of URG in the society through recruitment and retention until the membership demographic mirrors that of the US Census. Our plans to accomplish this included establishment of a formal standing committee, establishment of a moderate budget to support BPC activities, hosting professional development workshops, hosting diversity and mentor socials, and obtaining grant funds to supplement our budget. This paper documents broadening participation activities in the society, discusses the effectiveness of these activities, and evaluates BPC goals after 5 years of targeted funded activities. Over the past 5 years, the number of URG members rose by 5.2% to a total of 16.2%, members who report ethnicity and gender increased by 25.2% and 18%, respectively, and the number of members attending BPC activities has increased to 33% by 2016. SICB has made significant advances in broadening participation, not only through increased expenditures, but also with a commitment by its members and leadership to increase diversity. Most members realize that increasing diversity will both improve the Society's ability to develop different approaches to tackling problems within integrative biology, and help solve larger global issues that are evident throughout science and technology fields. In addition, having URG members as part of the executive committee would provide other URG members role models within the society, as well as have a voice in the leadership that represents diversity and inclusion for all scientists.

Introduction

The Society for Integrative and Comparative Biology (SICB) is one of the largest and most prestigious professional associations of its kind. The Society is dedicated to promoting the pursuit and public dissemination of information relating to biological sciences. SICB takes pride in the fact that one of the Society's focal points is to support student members, and that the organization is fundamentally committed to the advancement and development of early career investigators through its programs, meetings, and journal publications (Integrative and Comparative Biology).

The main goal of SICB's Broadening Participation Committee (BPC) is to increase the number of underrepresented group (URG) members within SICB and to expand their capabilities as future researchers within the SICB divisions. SICB has a long-standing mission and commitment to increasing the diversity of URG members, and recognizes the importance of engaging this group of scientists to address future needs within various biological disciplines. Integration among a number of different and diverse disciplines is crucial for our understanding of complex biological questions, and the capability of enabling our future scientists to tackle innovative and interesting ideas regarding organismal variation is in part driven by approaching questions from unique backgrounds and experiences (Schmidt 2010; Kendall 2011). SICB's Broadening Participation activities provide that platform to nurture students,

Advanced Access publication July 5, 2017

postdoctoral researchers, and beginning young investigators, which will create a community that will enhance their participation in the society and beyond (Ely and Thomas 2001). We hope that our activities will equip these young scientists with the toolkit needed to be successful within their careers and eventually become leaders in their fields, utilizing their background and experiences to become our future innovators and teachers (Goode 2004).

The BPC proposed a 5-year Strategic Plan in 2010 to broaden participation within the Society. The proposed objectives were aimed at coalescing and enhancing the experience of participants with new activities that complement those already in place. We established a program that will develop and increase participation of URG (URGs = including minorities, those with disabilities, first generation college attendees, and veterans) by publicizing the benefits of being an active SICB member and encouraging them to participate in leadership roles (Gardner 2013). Our short-term 5-year goal was to increase the recruitment and retention of URG members in the society by 10%. Our long-term 25-year goal is to increase the membership of URG in the society through recruitment and retention until the membership demographic mirrors that of the US Census. Our plans to accomplish this included establishment of a formal standing committee, establishment of a budget to support BPC activities, offering a variety of professional development workshops, hosting diversity socials and obtaining grant funds to supplement our budget. This paper documents broadening participation activities in SICB, discusses the effectiveness of these activities and assesses BPC goals after 5 years of targeted activities.

The importance of diversity in science

There is a deficit of URG members in scientific societies, which is likely due to the deficit of URGs graduating from universities (Smith 1991; Xu and Martin 2011). Diverse learning environments are most effective with all members benefiting from the increased awareness and broader perspectives of its members (Gurin et al. 2004; Peckham et al. 2007; Holly 2013). Thus, groups involving diverse members provide greater critical analyses of problems and solve them in more innovative ways (McLeod et al. 1996). If the trend in the United States showing an increased URG population continues, the majority of children born in the 21st century will belong to URGs, which are underrepresented in STEM fields (NSF 2014). To be responsive to the rapidly changing demographics of the United States, there is a critical need to broaden participation of URGs in STEM fields, such as those represented in SICB. To be effective in promoting the pursuit and dissemination of relevant and timely biological information to the public, membership demographics in SICB must reflect that of society.

Early history of broadening participation in SICB

There are several SICB committees that provide opportunities for students at annual meetings prior to 2002 through the present. The Student/Postdoctoral Affairs Committee hosts a Student First Timer workshop explaining how to get the most out of your SICB meeting, and a topical workshop at each annual meeting. The Student Support Committee is charged with overseeing activities related to student support, including the Charlotte Mangum awards that cover housing or registration, as well as individual research awards (Grants-In-Aid-of-Research). The Education Council arranges for undergraduate students to display their posters near the plenary session at annual meetings. However, until 2002 there were no efforts at increasing membership of URGs. Then-President Marvalee Wake created the BPC in 2002, with the goal of increasing diversity in SICB and fields of Integrative and Comparative Biology. From 2005 until 2009, local faculty and their undergraduate students, along with high school students and their teachers, were recruited to attend and present posters at the annual meeting. Registration and lodging were provided for up to 10 individuals each year. SICB graduate student members were then recruited to mentor the local undergraduate and high school students throughout the meeting. A complimentary breakfast was held on the first day of the meeting for self-identified ethnic minorities to network, and where National Science Foundation program directors spoke about funding opportunities. These were good attempts to introduce science in the SICB to local URG undergraduate and high school students and faculty, but it was not clear how many of these local recruits remained members and attended future annual meetings. The Then-BPC Chair Patricia Hernandez initiated a mechanism for self-identification of ethnicity and gender on annual meeting registration forms for the 2009 annual meeting and the 2010 annual membership renewal. These data continue to be collected on annual (online) membership forms as well as for annual conference attendees.

Formalizing the BPC in SICB

The Executive Committee recognized the need for greater diversity and in 2009 formally recognized

the BPC as a standing committee of the SICB where the Chair was elevated to the SICB Executive Committee, which permits participation in voting on SICB activities, including in budget discussions. This enabled the BPC, which included the authors (Cheryl Wilga, Then-BPC Chair, Brian Tsukimura, Then-Program Officer and Then-BPC member, and Nish Nishiguchi, Then-BPC Member), to develop a regular budget ($10,000 in 2010 that was gradually increased to $15,000 in 2012). SICB students indicated a need for financial support to attend the annual meeting; therefore, the BPC established travel awards to allow more URG members to attend the annual meeting. The BPC started offering two workshops geared toward the needs of URG to provide professional development opportunities. One of these workshops focused on junior members (graduate students) and the other targeted senior members (postdocs and faculty). The BPC supports a meet and greet social on the first day of the annual meeting to allow travel award fellows to meet their cohorts, past cohorts, mentors and the BPC members. The BPC also supports a Diversity Social near the end of the annual meeting where travel award checks are distributed and where travel award fellows can meet and network with other SICB members, including past and current executive committee members, program directors from the National Science Foundation and other invited guests. These activities were initiated at the 2011 annual meeting and continued through the 2017 meeting, and were very successful.

SICB resources

The SICB leadership is very committed to broadening participation in the society and has many resources already in place that enable BPC activities to be implemented effectively. The BPC has retained its annual budget of $15,000. The SICB webmaster, Mr Birenheide, designs and maintains the webpage and online resources for announcements and travel award applications. The SICB has a very effective administrative team (Burke Associates Inc.) that organizes the annual meetings. They are instrumental in allocating rooms for the workshops and socials (along with the Program Officer), organizes food orders for the socials, manage the budget and print award checks. The elevation of BPC to the SICB Executive Committee ensures that all of these activities will be retained in the future. These resources are at the disposal of the BPC who are a dedicated group of volunteer members that have a vision for broadening participation within the Society. The BPC recognizes the importance of creating and sustaining a diverse community of scientists, and is willing to commit time and energy to ensure that our goals are not only embraced but also achieved.

SICB BPC objectives

The main goal of the BPC is to create a culture where members from URGs have access to a number of resources, such as mentors, workshops, funding for travel to annual meetings and a sense of community within the society. By building upon SICB's base of URG members, immediate feedback can be obtained on the needs of those members who are currently at different stages of their careers (graduate students, postdoctoral fellows, junior faculty and senior faculty). The BPC can assess what resources fit the needs for each level of our URG member pool, and reinforce SICB's ability to ensure their success. The BPC census of SICB composition has indicated that the URG numbers show a dramatic decline at the level of postdoctoral and assistant professors (Fig. 1); yet causes for this decline are not apparent from these data. Our objectives include tracking these data to attempt to identify and address as many of the causes as possible, and establish long-term solutions that will enable postdoctoral and assistant professor members to persist and grow within the SICB.

Eight BEST principles were identified as most successful in recruiting and retaining URG in other professional societies and in STEM fields (Wilson and Haynes 2002; Pandya et al. 2007; Payton et al. 2012). The goals of the BPC align closely with the BEST principles: 1) institutional leadership, 2) targeted recruitment, 3) engaged faculty, 4) personal attention, 5) peer support, 6) enriched research experience, 7) bridging to the next level and 8) continuous evaluation. Pandya et al. (2007) identified one pervasive need—financial support—that can easily be addressed by scientific societies. The BPC goals align well with these design principles and have implemented activities that address some of these, which include: 1) provide support for attending our annual meeting in the form of travel awards, which will promote and sustain a URG cohort that in turn will be our future leaders in the society and beyond; 2) increase awareness of broadening participation and building community by promoting events at the annual meeting (socials and workshops); 3) offer workshops that address issues specific for URG members (i.e., career development, leadership, teaching and outreach); 4) recruit new URG members and promote the society to other societies in

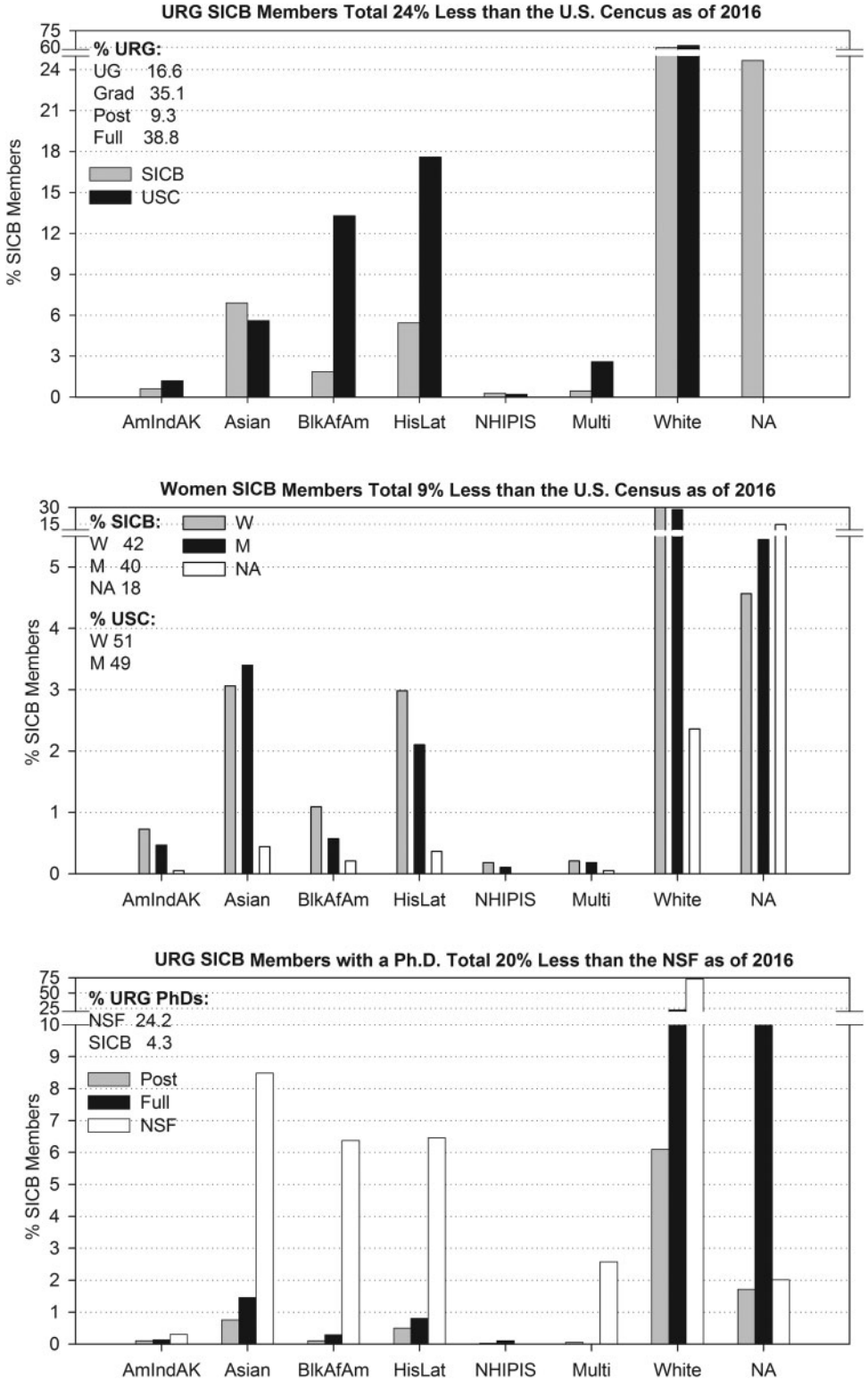


Fig. 1 Disparity in SICB membership relative to the US Census and National Science Foundation (NSF) PhD holders by ethnicity, gender and member level. SICB is comprised of 17% URG members, 4.3% of which hold Doctoral degrees, 42% women members and 8.2% are from URGs. Total 2016 membership is 3855. URG ethnicities: AmInAK, American Indian and Alaska Natives; BlkAfAm, Black and African Americans; HisLat, Hispanic and Latino; Multi, more than one ethnicity; NHIPI, Native Hawaiian and Pacific Islanders. Member level: Full, faculty; Grad, graduate; Post, postdoctorate; UG, undergraduate; Gender: F, female; M, male. NA, not answered. Census: NSF, 2014 NSF census of PhD holders; USC, 2015 US population census.

Table 1 Objectives of the Broadening Participation Committee

Objectives	Outcomes
1 + 2) Initiate a URG cohort and community that will be sustained throughout the career at all levels of membership. Addresses BEST #3–5.	Continue support for attending annual meetings, offer social events that bridge new and loyal URG members.
3) Provide leadership and professional training that sustains beyond their involvement with SICB. Addresses BEST #1, 6–7.	Offer workshops in best research practices such as grant writing, time management, leadership, funding opportunities.
4) Recruit members and promote the society to other organizations serving URG in the biological sciences relevant to the interests of the society. Addresses BEST #2–3.	Individual members who attend meetings such as AISES, SACNAS, MARC, AGEP and McNair can promote the benefits of being an active and diverse SICB member.
5) Determine whether the BPC initiatives are increasing diversity within the society and beyond. Addresses BEST #8.	Continually assess each aspect of the program, revise and redirect (if needed) the goals and objectives of the program.

Notes: AISES, American Indians in Science and Engineering; SACNAS, Society for the Advancement of Chicanos and Native Americans in Science; MARC, Maximizing Access to Research Careers; and AGEP, Alliances for Graduate Education and the Professoriate.

which SICB members are involved and 5) have measurable outcomes that can be used for assessment of BPC goals. Positive outcomes will drive the direction and evolution of future BPC objectives (see Table 1 for current objectives). We will maintain or enhance those activities that we find are successful and modify those that are not working until the percentage of URG members mirrors that of the US population census.

The current BPC goals align with prior key objectives that were identified as most successful in recruiting and retaining URG in other professional societies and in STEM fields (Wilson and Haynes 2002; Pandya et al. 2007; Payton et al. 2012).

SICB BPC activities and assessment

Demography of SICB

SICB URG member gains was measured by implementing a mechanism to collect and assess self-identification of ethnicity, gender and disability (added in 2011) on annual membership (2010–2016) and meeting registration forms (2009–2016). This was a critical step in determining the ethnic, race, gender and disability makeup of our membership compared with 2015 US Census and NSF Doctoral Degree-Holder demographics (NSF 2014; US Census 2015). As of the 2010 annual conference, SICB had 2373 members, of whom 11.0% were from URGs, 49.3% were white (39.7% did not self-identify ethnicity), with 32% women members (30% left gender blank). As of the 2016 annual conference, SICB membership increased to 3855 members, an increase of 62% from 2010 (Fig. 1). URG members increased to 16.2%, with an increase in white members to 59.8% (Fig. 2). Women members increased to 42%, while men members increased by 2% (Fig. 2). Thus, there was a sharp decrease in the percentage of members that failed to self-identify ethnicity (down to 24.7%) and gender (down to 18.1%; Fig. 2). Associated with the 15.0% increase in self-identification is a 10.5% increase in white and a 5.2% increase in URG members (Fig. 2). Even with these remarkable gains, URG membership within the society is only 16.2%, essentially half the 37.9% reported in the 2015 US Census (US Census 2015). SICB members reporting a disability is 1.5% compared with 8.5% of Doctoral Scientists in biological, agricultural and environmental life sciences (ages 16–64 years) of the general population in the United States (NSF 2014; US Census 2015).

Comparing the current membership of SICB to that of the 2015 US Population Census indicates that SICB is doing well attracting some URG groups (Fig. 1). Asians and Native Hawaiian/Pacific Islanders are the only URGs currently in SICB where the percent of members exceeds that of the population census (by 23.2% and 42.7%, respectively) (US Census 2015). Hispanic/Latino and American Indian/Alaskan Natives members together comprise less than or half that of the US population (31.0% and 51.9%, respectively, SICB/US%), with Black/African Americans members totaling only 14.0% of the BlkAfAm US population (SICB/US%). In 2016, SICB added a new category called "multiple ethnicities" to which 0.44% of members selected in lieu of selecting an ethnicity. This made it easier for some members with multiple ethnicities to self-identify (more than one group can be selected); however, this decreases the precision of our assessment. The percentage of white SICB members is slightly less than that of the US Census (97%, SICB/US%). Women members number slightly more than men, 42.3% and 40.1%, respectively, which are proportionately similar to the US Census (50.8% women, 49.2% men). However, unless those 18.1% of

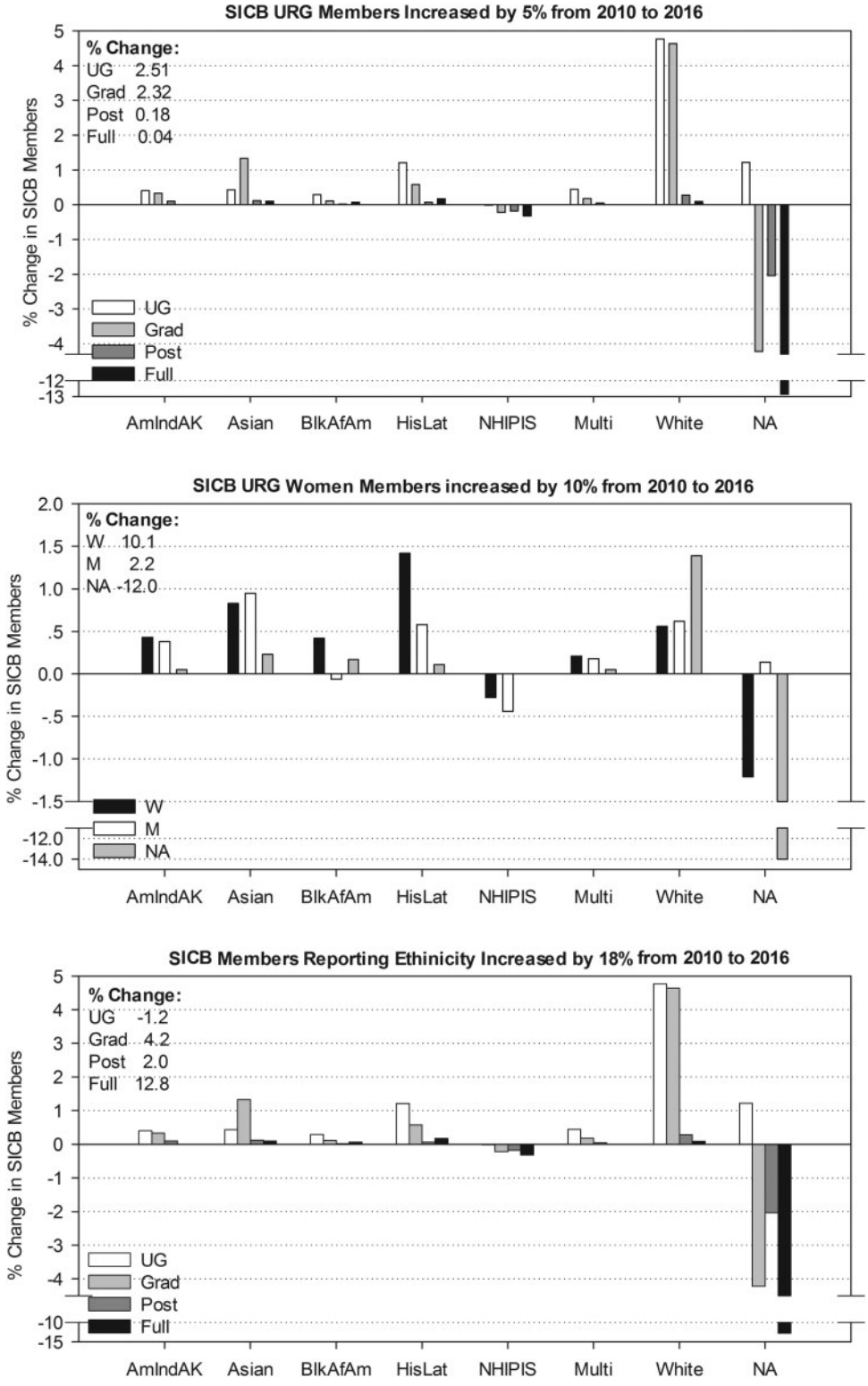


Fig. 2 Effects of BPC activities on SICB membership from 2010 to 2016. URG members increased from 11% to 16%, women members increased from 32% to 42% and member self-identification increased from 60% to 75%. Abbreviations as in Fig. 1.

members that leave gender blank self-identify, it will be unknown whether the gender ratio in SICB is truly equal to that of the US Census (US Census 2015).

SICB Membership consists of doctorate holding members that are Full (35% including 3.6% emeritus) and Postdoctoral (9.3%), with students comprising about half of the members: graduate (35%) and

undergraduate (16.7%) (Fig. 1). Interestingly, most of the emeritus and nearly one-third of full members fail to self-identify ethnicity (79.3% and 28.4%, respectively) compared with postdocs (18.3%), graduate students (16.4%) and high school students (14.3%). On the other hand, undergraduate students (25.4%) fail to self-identify nearly as often as full members. SICB is doing well at attracting graduate students and full members, but is only retaining approximately 26.3% of the graduate student members as postdoctoral members. This may be due to the lack of support for postdoctoral members to attend meetings. Attracting more postdoctoral researchers, who can benefit from professional workshops and networking at the socials could lead to increased SICB members once they obtain faculty positions and bring their postdoctoral researchers and students to the annual meeting. Sharing data about societal composition is a valuable resource for all professional societies to measure their efforts in the recruitment and retention of URG (Freehill and Ivie 2013). However, we were unable to find other scientific societies with similar membership data to compare our data too.

Relating the percentage of SICB members with Doctorates to the NSF Census of Doctoral Holders may be the more appropriate standard with which to compare the demography of a scientific society. Only 4.4% of URG SICB members hold a doctorate degree compared with 21.6% URG Doctoral Scientists reported in the 2014 Census of Doctoral Scientists in biological, agricultural and environmental life sciences in the United States (NSF 2014). SICB Full members are comprised largely of faculty in the academy, thus this may indicate a lack of successful recruitment of URG to the academy, or low URG recruitment to professional societies. URG SICB members with doctorates are fewer than NSF census Doctorate Holders in all ethnic categories: Hispanic/Latino with Doctorates (1.3%) in SICB compared with NSF Census Doctorate holders (6.5%); Asians with PhDs (2.2%) in SICB compared with NSF Census Doctorate holders (8.5%); Black/African Americans with PhDs (0.42%) in SICB compared with NSF Census Doctorate holders (6.4%) (NSF 2014). American Indian/Alaskan Native SICB members with Doctorates (9) are most similar to NSF census Doctoral Holders (0.23% versus 0.30%, respectively) because their populations are relatively low nationally. White SICB members with Doctorates comprise a third that of NSF Doctoral holders (29.3% versus 73.0%, respectively; NSF 2014).

Table 2 Funded travel award demographics from 2011 to 2016 (176 in total)

Ethnicity	Percent	Level and gender	Percent
Hispanic/Latino	55.9	Asst. Professors	7.3
Asian	8.5	Postdoctorates	20.9
Black/African American	13.0	PhD Students	28.8
American/Alaskan Indian	4.0	MS Students	15.8
Native Hawaiian/Pacific Islanders	5.1	UG Students	26.0
White women	13.6	Females	71.8
		Males	32.8

With respect to leadership, SICB has had only nine women and no minority presidents since its inception in 1890, which means that 92% of past presidential terms have been white men (117 terms). In addition, few URG members are holding leadership or divisional positions within SICB. The reason for this lack of leadership may be partly due to the low number of URG SICB members with Doctorates, particularly at the full professor level. Having URG member representation on the executive committee would provide role models within the society, and present a collective societal voice representing diversity and inclusion for all scientists.

The Travel Award Program started with the 2011 annual meeting and has been successful in other scientific societies as well as at SICB (Wilson and Haynes 2002). URG Members from all levels can apply for up to $500 to support travel to the annual meeting. As a mechanism to guide future efforts of the BPC to broaden participation within SICB, applicants are asked to state their career goals, describe two challenges to being a member from an URG in science and suggest workshop topics for the next annual meeting. The BPC was able to fund 86.6% of applicants in 2011, 53.6% of applicants in 2012, 91.6% of applicants in 2013, 92.5% of applicants in 2014, 58% of applicants in 2015 and 33% of applicants in 2016. Hispanic/Latino members received slightly more than half of the Travel Awards, followed by White Women and Black/African American members (Table 2). In 2011, more members were supported at a smaller amount than requested in order to fund most of the applicants. Many of the travel fellows verbally told BPC members that they would not have been able to attend without this funding. In 2012, most members requested the maximum amount stating that they would unlikely attend without full funding. Thus, fewer members were funded but with a higher

Table 3 Professional development workshops from 2011 to 2014

Year	Name, number of attendees	Hosts
2011	Balancing Life and an Academic Career	Greg Florant,[a,b] Nora Espinosa[a,b]
2011	Issues facing new faculty	Denise Dearing,[b] Peggy Biga,[b] Hannah Carey, Michele Nishiguchi,[a,b] Scott McWilliams
2012	Science is a Two-way street: Mentorship and the Mentee	Michele Nishiguchi,[a,b] Billie Swalla (President Elect), Cheryl Wilga[a,b]
2012	Demystifying the Grant Application Process	Cheryl Wilga,[a,b] Michele Elekonich and Bill Zamer (NSF Program Directors)
2013	Effective presentations skills	Manny Azzizi,[a] Patricia Hernandez,[a,b] Andrew Clark[a]
2013	How to negotiate your first job	Gregory Florant,[a,b] Billie Swalla (President)
2014	Recruitment strategies to obtain a diverse and thriving lab and department	Rebecca Calisi-Rodriguez,[a] Michele Nishiguchi,[a,b] Cheryl Wilga[a,b]
2014	Writing grants and manuscripts in a timely manner	Heather Bleakley, Brian Tsukimura[a,b] (Past Program Officer), Michele Nishiguchi[1,2]
2015	The academic juggling trick: how to effectively manage your time during the professoriate	Michele Nishiguchi[a,b]
2015	Don't be such a scientist, part II: How to give dynamic and informative presentations	Jake Socha (SICB Public Affairs Committee)
2016	Integrate diversity awareness into science institutions	Kendra Greenlee,[a,b] Michele Nishiguchi[a,b]

[a]URG member.
[b]BPC member.

level of support. In 2013, the deadline was moved earlier in the year to allow applicants to receive notification of the award before the registration deadline. Applicants requested the earlier deadline so they would know whether they had the funds to attend the meeting before registering for the meeting, however several members missed the earlier deadline. In 2014, BPC members Cheryl Wilga, Michele Nishiguchi and Brian Tsukimura were awarded an NSF Conference Grant for $25,000 to fund two SICB Broadening Participation workshops, URG workshop panelists and URG members at the annual meeting (IOS-1362663). As a result, 64 additional members were funded (92.5% of applicants). In 2014–2016, postdoc and junior faculty URG members were given priority to BPC travel funds in an attempt to increase the low member attendance at those levels. Overall Travel Awards were fairly equally spread out among undergraduate, doctoral and postdoctoral members, with a smaller percentage going to masters student and assistant professor members. Two-thirds of the Travel Award recipients were women.

The BPC offers two Professional Development Workshops at each annual meeting starting with the 2011 meeting. This is a main feature of broadening participation efforts in other scientific societies (Wilson and Haynes 2002). Workshops are chosen from the most requested topics suggested by the Travel Award Fellows. All SICB members are invited, and every workshop thus far has been successful with a mean of 74 members attending (range 30–100) (Table 3). One BPC workshop focuses on professional development for graduate students and the other focuses on faculty and postdocs. Of the 26 workshop hosts from 2011 to 2016: 16 were from URGs; 18 were women; 16 were Full Professors; 2 were Associate Professors; 6 were Assistant Professors; 2 were NSF Program Officers; 6 were current or past Chairs of the BPC; 9 were BPC members and 3 were SICB Executive Committee members (President and Program Officer) (Table 3).

The pre-meeting ''Meet and Greet'' Social is hosted by the current Chair of the BPC on the first day before the plenary lecture and was initiated in 2012. This social brings together travel award fellows and members of the BPC for networking and to establish a cohort that will eventually build community within SICB and increase retention and form lasting post-meeting relationships. The pre-meeting strategy is to create a cohort of members at each meeting that can reconvene throughout the meeting to share thoughts and impressions about the meeting. Appetizers and soft drinks are provided by SICB funds in an informal setting with an attendance of approximately 30–50 members each year.

The Broadening Participation Diversity Social that started with the 2011 meeting has also triumphed in

increasing awareness of the committee and its activities. Here the BPC provides a spread of appetizers, invites guest speakers (BPC members, NSF Program Officers, Past and Current SICB Presidents and SICB Executive Committee Members), recognizes the BPC Travel Fellows, presents the travel awards and provides a comfortable friendly atmosphere for all members to mingle and chat. Attendance started in 2011 at around 100 members, with at least 300 in 2013, and approximately 200 members in 2014–2016. Our hope is that members from URGs feel more comfortable interacting with current and past executive committee and BPC members, NSF program officers, and other members in a smaller, intimate atmosphere and therefore become more involved in the society. This also provides past and future cohorts with a venue for informal networking with leaders in their fields and within the Society.

Impact of BPC activities

Over the past 5 years, the data show an increase in URG members (up by 5.2%), a decrease in members who do not report ethnicity and gender (down by 15% and 12%), and an increase in the number of members attending BPC activities (up to 33% by 2016) (Fig. 3). The striking decrease in the number of members who do not report ethnicity, especially over the last two years, suggests an increased awareness throughout SICB of the benefits of having a diverse membership. We are particularly pleased to see that postdoctoral member attendance is also steadily increasing. BPC efforts have succeeded in increasing diversity within SICB membership as well as those attending annual meetings as indicated by the steady increase in URG members and BPC activity attendance.

Travel awards have increased participation and enhanced attendance of URGs. Assessment of the BPC Travel Award program is very encouraging. Over the past 4 years, the BPC was able to fund 63.6% of the applicants, with 5% withdrawing their applications due to other funding being secured or inability to attend the meeting (Table 4). Funding for travel to annual SICB meetings is critical because 33% of the unfunded applicants did not attend the annual meeting (Table 4). Funding is also critical for continuing membership. Of those applicants that were funded (176 total), 38% remained in SICB as of 2016, with 41% leaving after 1 year of membership and only 14% leaving after 2 years of membership (Table 4). Nearly half of the awardees that leave SICB after at least 1 year are undergraduate students (44%), with graduate students the next largest group (33%). Thus, it appears that most Travel Award applicants decide after two meetings whether SICB fits their needs for a professional scientific society.

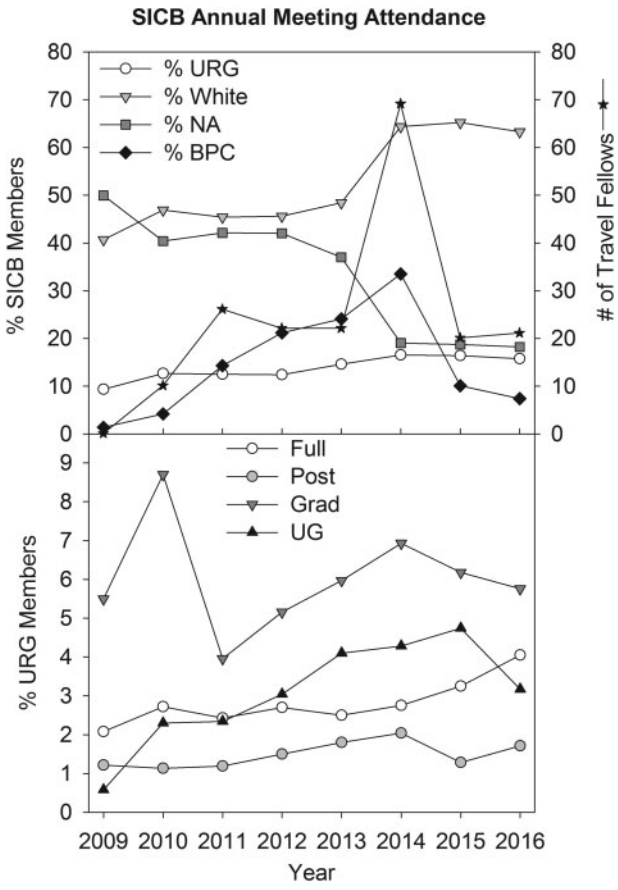


Fig. 3 Effects of BPC activities on SICB member attendance at annual meetings. The top plot shows the percent of all members who attended the annual meeting by ethnicity, failed to self-identify and attended BPC activities. The number of URG members funded by travel awards is shown on the right axis (black asterisk). Note the sharp decline in the percent of members who fail to self-identify ethnicity (NA, gray boxes). Also note the steady increase in URG attendance with increased funding (open circles) and BPC professional development and social activities (black diamonds). The increase in 2014 attendance is due to the increase in BPC URG travel awards funded by a NSF meeting award. The bottom plot shows the demographics of URG members by level who attended the annual meeting. Note the steady increase in the percent of full and postdoctoral members (open and gray circles, respectively).

To maintain or increase retention, it is imperative that a sense of community among the participants be developed (Hassoun and Bana 2001; Peckham et al. 2007; Jones et al. 2008; Koenig 2009; Xu and Martin 2011). Community can be developed through interactional engagement and social activities, such as pre-meeting and intra-meeting socials (Peckham et al. 2007; Jones et al. 2008; Xu and Martin 2011).

Table 4 Assessment of travel award applicants from 2010 to 2016

Travel award applicants = 176 total	%
Unfunded applicants of total	31.0
Unfunded applicants who also did not attend annual meeting	33.3
Withdrawn applicants of total	9.1
Funded applicants of total	63.6
Funded applicant status = 112 total	% funded
Remained SICB members as of 2016	37.9
Left SICB 1 year after funding	40.8
Left SICB 2 years after funding	14.4
Left SICB 3 years after funding	4.0
Left SICB 4 years after funding	2.9
Left SICB 5 years after funding	0.0
Received awards 2 years	10.5
Received awards 3 years	0.7
Received awards 4 years	0.9

URG members with increased interaction with executive committee and divisional leaders can develop a sense of community within SICB, especially if they feel that they are welcomed by the SICB's membership (Informal Professional Networks; Xu and Martin 2011). Mentoring, on informal individual and workshop scales, from peers and SICB leadership, can lower perceived barriers about engaging with senior members (Pandya et al. 2007; Koenig 2009; Tapia 2009; Payton et al. 2012; Wilson et al. 2012). In particular, mentoring early in one's career is critical to enhancing professional success (Jones 2014).

Traditional presentations of the academy, and hence the professional society, are often culturally neutral ignoring participant perception or understanding of basic academy operations (Peckham et al. 2007; Jones et al. 2008). Shared experiences can often bring incongruent backgrounds together and initiate forming a sense of community. Our travel awardees form a natural cohort, which we bring together several times throughout the meeting at BPC socials and workshops so that they might find each other among the crowds, and to find how the meeting itself is a common experience.

We have increased retention through a supportive community formed through the BPC Travel Fellows, workshops and social programs. Continuing and bridging support to the next level of membership can be provided informally by SICB members, but also through BPC workshops. The Travel Fellows Program form a cohort facilitated by the socials and workshops that we hope will stimulate sustained interaction long after the annual meeting. The BPC Pre-meeting and Diversity Socials, open to the entire society, are informal and friendly places where these networks can easily be formed, increasing the potential for URG professional networking and mentoring (Xu and Martin 2011). The very successful and well attended BPC Diversity Social and workshops are already building community within the society and indicates buy in by member attendance.

SICB has already made a significant investment in broadening participation, not only financially, but also with a commitment by its members to increase URG diversity. Members are beginning to realize that increasing diversity will not only impact the Society's ability to successfully facilitate different approaches to tackling problems within integrative biology, but will also help positively impact larger issues that develop throughout science and technology fields. A team composed of diverse members generates a greater breadth of solutions (i.e., "Grand Challenges in Organismal Biology—The need for synthesis" by Padilla et al. 2014). In order to facilitate and build a long-lasting community, SICB has provided the springboard in the BPC to initiate the welcoming of URGs into the society by striving for the goals stated in this paper. The SICB has also committed to a long-lasting and sustainable program that will recapitulate benefits from which all members of the society will gain, especially once SICB demographics match that of the US Census. SICB is a leader among long-lived scientific societies, and by succeeding in the increased awareness and participation of URGs, we will create a community of leaders who can bring their values, ideas and knowledge to improve broader scientific challenges that are of greater importance in today's world.

Acknowledgments

We thank the Executive Committee, Division and Program Officers, Members and Staff in the Society for Integrative and Comparative Biology and Burke Inc. for fully and enthusiastically supporting the BPC's efforts to broaden participation.

Funding

This work was supported by the Society for Integrative and Comparative Biology and by the National Science Foundation [IOS-1362663]. Meeting award to the authors for broadening participation at the annual society meeting.

References

Ely RJ, Thomas DA. 2001. Cultural diversity at work: the effects of diversity perspectives on work group processes and outcomes. Admin Sci Quart 46:229–73.

Freehill LM, Ivie R. 2013. Increasing the visibility on women of color in academic science and engineering: professional society data. New Direct High Educ 163:7–12.

Gardner SK. 2013. The challenges of first-generation doctoral students. New Direct High Educ 163:43–54.

Goode TD. 2004. National center for cultural competence. Georgetown University Center for Child and Human Development, University Center for Excellence in Developmental Disabilities.

Gurin P, Nagda RA, Lopez GE. 2004. The benefits of diversity in education for democratic citizenship. J Soc Issues 60:17–37.

Hassoun S, Bana S. 2001. Practices for recruiting and retaining graduate women students in computer science and engineering, Microelectronic Systems Education, 2001. Proceedings. 2001 International Conference on Microelectric Systems Education, pp. 106–107.

Holly KA. 2013. How diversity influences knowledge, identity, and doctoral education. New Direct High Educ 163:99–105.

Jones KW, Kristof D, Jenkins LC, Ramsey J, Patrick D, Burnham S, Turner IL. 2008. Collaborative technologies: Cognitive apprenticeship, training, and education. Collaborative Technologies and Systems, 2008. International Symposium Microelectric Systems Education, pp. 452–459.

Jones J. 2014. The Chronicle of Higher Education. Clogged Pipelines. March 7, Supplement pp. 45–48.

Kendall FE. 2011. Multicultural education program—evolving community at Cal: equity, inclusion, and diversity, University of California, Berkeley.

Koenig R. 2009. Minority retention rates in science are sore spot for most universities. Science 324:1386–7.

McLeod PL, Lobel S, Cox T. 1996. Ethnic diversity and creativity in small groups. Small Group Res 27:827–47.

National Science Foundation (NSF). 2014. National Center for Science and Engineering Statistics, Survey of Doctorate Recipients, 2013. https://nsf.gov/statistics/2016/nsf16300/digest/.

Padilla DK, Daniel TL, Dickinson P, Grunbaum D, Hayashi C, Manahan DT, Marden JH, Swalla BJ, Tsukimura B. 2014. Addressing grand challenges in organismal biology—the need for synthesis. BioScience 64:1178–87.

Pandya RE, Henderson S, Anthes RA, Johnson RM. 2007. BEST Practices for Broadening Participation in the Geosciences: Strategies from the UCAR Significant Opportunities in Atmospheric Research and Science (SOARS) Program. J Geo Educ 55:500–6.

Payton FC, Suarez-Brown TL, Lamar CS. 2012. Applying IRSS theory: The Clark Atlanta University exemplar. J Innov Educ 10:495–513.

Peckham J, Stephenson PD, Harlow LL, Stuart DA, Silver S, Mederer H. 2007. Broadening participation in computing: issues and challenges 2007. ACM SIGCSE Bulletin 39:9–13.

Schmidt P. 2010. Graduate programs grow less diverse without racial preferences, research suggests. The Chronicle of Higher Education. May 14, 2012 (http://chronicle.com/article/Graduate-Programs-Become-Much/125459/).

Smith DG. 1991. The challenge of diversity: alienation in the academy and its implications for faculty. J Excel College Teach 2:129–37.

Tapia R. 2009. Minority students and research universities: How to overcome the 'mismatch'. The Chronicle of Higher Education 55:A72.

US Census. 2015. Population Estimates Program. Quick Facts. United States. https://www.census.gov/quickfacts/table/PST045216/00.

Wilson DJ, Haynes JK. 2002. ASCB minorities affairs committee goals: ''Strengthening the chain of success''. Cell Biol Educ 1:105–6.

Wilson ZS, Holmes L, deGravelles K, Sylvain MR, Batiste L, Johnson M, McGuire SY, Pang SS, Warner IM. 2012. Hierarchical Mentoring: A transformative strategy for improving diversity and retention in undergraduate STEM disciplines. J Sci Educ Technol 21:148–56.

Xu Y, Martin C. 2011. Gender differences in STEM disciplines: from the aspects of informal professional networking and faculty career development. Gender Issues 28: 134–54.

TITLE: Phylogeographic patterns in the Philippine archipelago influence symbiont diversity in the bobtail squid-*Vibrio* mutualism

AUTHORS: [1]Randy Lane Coryell, [1]Kira Emily Turnham, [2]Evelyn Grace de Jesus Ayson, [2]Celia Lavilla-Pltogo, [3]Angel C. Alcala, [4]Filippina Sotto, [5]Benjamin Gonzales, and [1]Michele Kiyoko Nishiguchi

POSTAL ADDRESSES:

1. New Mexico State University, Department of Biology, MSC 3AF, P.O. Box 30001, Las Cruces NM 88003-8001, USA
2. Southeast Asian Fisheries Development Center (SEAFDEC), Buyu-an, Tigbauan, 5021 Iloilo, Philippines
3. Silliman University, 1 Hibbard Avenue, Dumaguete City, 6200 Negros Oriental, Philippines
4. University of San Carlos, Governor M. Cuenco Ave., Cebu City, 6000 Cebu, Philippines
5. Western Philippine University, San Juan, Aborlan, Palawan, 5302, Philippines

KEYWORDS: *Vibrio*, Squid, Symbiosis, Phylogeography,

CORRESPONDING AUTHOR: Michele K. Nishiguchi, Department of Biology, New Mexico State University, P.O. Box 30001, MSC 3AF, Foster Hall- 1200 S. Horseshoe Dr., Las Cruces, NM 88003-8001, USA

email: nish@nmsu.edu

RUNNING TITLE: Philippine squid-*Vibrio* phylogeography

ABSTRACT

Marine microbes encounter a myriad of biotic and abiotic factors that can impact fitness by limiting their range and capacity to move between habitats. This is especially true for environmentally transmitted bacteria that cycle between their hosts and the surrounding habitat. Since geologic history, biogeography, and other factors such as water temperature, salinity, and physical barriers can inhibit bacterial movement to novel environments, we chose to examine the genetic architecture of *Euprymna albatrossae* (Mollusca: Cephalopoda) and their *Vibrio fischeri* symbionts in the Philippine archipelago using a combined phylogeographic approach. Fourteen separate sites in the Philippine islands were examined using haplotype estimates that were examined via nested clade analysis to determine the relationship between *E. albatrossae* and *V. fischeri* populations and their geographic location. Identical analyses of molecular variance (AMOVA) were used to estimate variation within and between populations for host and symbiont genetic data. Host animals demonstrated a significant amount of variation within island groups, while symbiont variation was found within individual populations. Nested clade phylogenetic analysis revealed that hosts and symbionts may have colonized this area at different times, with a sudden change in habitat. Additionally, host data indicate restricted gene flow, whereas symbionts show range expansion, followed by periodic restriction to genetic flow. These differences between host and symbiont networks indicate that factors “outside the squid” influence distribution of Philippine *V. fischeri.* Our results shed light on how geography and changing environmental factors can impact marine symbiotic associations at both local and global scales.

INTRODUCTION

The dispersal of marine species across suitable habitats can be affected by physical barriers (temperature, distances across oceans, island formations) as well as life history strategies (e.g. dispersal method of larvae, adult motility; (Kool, Paris, Barber, & Cowen, 2011). Biogeographical barriers, as reported by floral and faunal separations, occur worldwide and provide an opportunity to study how physical barriers coupled with other abiotic factors may be affecting species dispersal and ultimately distribution (Lohman et al., 2011; Tonon et al., 2015). Analysis of population structure and physical orientation of the distribution of taxa across these barriers has given us clues to the factors that fragment available habitat (Esselstyn et al., 2010). Although previous work has provided evidence for several causes for speciation among closely related populations in areas where distinct barriers exist, there is less known about species that coexist with one another, and whether rules that govern distribution patterns via allopatric speciation influence such associations (Hellberg, Burton, Neigel, & Palumbi, 2002; S. R. Palumbi, 1994).

One region that has been studied extensively for its unique patterns of biogeography and geologic history is the Indo-Pacific barrier (IPB), which was created by the uprising of the Indonesian archipelago separating the Indian and Pacific oceans (Gaither, Toonen, Robertson, Planes, & Bowen, 2010). Interestingly, dispersal mechanisms and rapid adult motility have allowed certain taxa in the region to cross the IPB due to various dispersal strategies and larval residence time prior to metamorphosis compared to other taxa which are geographically restricted (Horne, 2014; Liu, Chang, Borsa, Chen, & Dai, 2014; Sorenson, Allen, Erdmann, Dai, & Liu, 2014). As part of the

IPB, the Philippine island archipelago is a “hotspot” for species diversity and endism and has warranted investigation of the distribution of taxa across the region (Roberts et al., 2002). In the Philippines, current research has focused on the phylogeographic distribution of some fishes, bent-toed geckoes, as well as bivalves across established biogeographical margins that limit some other terrestrial and marine taxa (Carpenter & Springer, 2005; Esselstyn et al., 2010; Gaither & Rocha, 2013; Huxley, 1868; Lemer et al., 2016; Siler, Oaks, Esselstyn, Diesmos, & Brown, 2010; Wallace, 1860, 1863). Local analysis of the distribution and connectivity of some marine taxa across the Philippines has also been investigated in western populations of the sea star *Linckia laevigata* and the giant clam *Tridacna crocea* near the island of Palawan, as well in the western portion of the Central Visayas (Alcazar & Kochzius, 2016; Juinio-Menez, Magsino, Ravago-Gotanco, & Yu, 2003; Magsino, Ravago, & Juinio-Menez, 2002; Ravago-Gotanco, Magsino, & Juinio-Menez, 2007). Interestingly, very few studies have examined the connectivity of populations across the whole of the Philippine archipelago, and what impact physical factors, life history, and geographical barriers have on the distribution of mutualist partners. This has created a void in the knowledge of how local assemblages of mutualist associations are impacted by geography in this unique area. Therefore, there is a need to better understand whether the physical barriers in this area have shaped the distribution of coexisting marine organisms, and to determine what impact these physical factors have on species interactions, particularly between microbes and their eukaryotic hosts.

Across the globe, sepiolid squids (Cephalopoda: Sepiolidae) form mutualistic associations with bioluminescent bacteria from the genera *Vibrio* and *Photobacterium* (γ-

Proteobacteria: Vibrionaceae (Herring, 1977). *Vibrio* bacteria are housed within a specialized internal organ called the light organ (LO), where the host provides a nutrient rich habitat for the symbiont, and in return *Vibrio* bacteria provide luminescence to the squid to be used in a behavior termed counter-illumination (Jones & Nishiguchi, 2004). Squid hosts use the *Vibrio* produced light to reduce their silhouette during the evening, which enhances their survivability and predation success (McFall-Ngai, Heath-Heckman, Gillette, Peyer, & Harvie, 2012). After each nightly foraging session, approximately 95% of the *Vibrio* bacteria are vented out into the surrounding seawater, seeding the local area with symbiotically viable Vibrios (Boettcher, Ruby, & McFall-Ngai, 1996). Local cycling of symbiotic *V. fischeri* exposes these bacteria to a wide range of abiotic and biotic factors outside the host that can affect their fitness and ability to infect new hosts. This also allows for symbiotically competent free-living bacteria to migrate to new host habitats, where they can invade and colonize different populations of sepiolids (Nyholm, 2004; Nyholm & Nishiguchi, 2008; Nyholm, Stabb, Ruby, & McFall-Ngai, 2000).

Earlier work on sepiolid squids has focused on the influence of geographic distance on symbiont prevalence and genotype in both sympatric and allopatric populations (Jones, Lopez, Huttenburg, & Nishiguchi, 2006; Kimbell, McFall-Ngai, & Roderick, 2002; Zamborsky & Nishiguchi, 2011). Allopatric and sympatric populations for both squids and *Vibrio* bacteria show distinct population breaks that are not necessarily driven by host specificity. Additionally, host mediated factors along with abiotic variables such as water temperature and salinity have been known to shape these mutualist assemblages (McFall-Ngai, 2014; McFall-Ngai et al., 2012; Nishiguchi, 2000; Soto, Gutierrez, Remmenga, & Nishiguchi, 2009). Collectively, either genomic comparison of

115 closely related populations (Bongrand et al., 2016) or haplotype comparisons of
116 allopatric populations of Indo-west Pacific squid and their vibrio symbionts (Jones et al.,
117 2006) do not address the connectivity of populations across physical and
118 biogeographical barriers like those in the Philippines or across the IPB. Therefore, we
119 examined the genetic architecture of *Euprymna albatrossae* (Cephalopoda: Sepiolidae)
120 and their *V. fischeri* symbionts in the Philippine archipelago using a combined
121 phylogeographic approach to determine whether host specificity or geographic location
122 influence the distribution of symbiotic Vibrios in this region. The unique geographic origin
123 of the Philippines, its proximity to deeper and colder water, as well as currents that move
124 through the area allows for investigation of what roles geography and host specificity
125 have in the distribution of mutualistic associations.
126
127 **Methods**
128 ***Specimen collection and bacterial isolation***
129 Squids were collected in the months May, June, July, and August during the
130 years 2010, 2012, 2013, and 2015 at eleven different sites around the Philippine islands
131 (Fig. 1, Table 1). Adult squid, (~5-9 cm in length) were acquired either by dip or seine
132 net. Captured squids were brought back to the laboratory and placed on ice to
133 anesthetize them prior to dissection. Host light organs were subsequently removed via
134 ventral dissection and homogenized to plate on seawater tryptone agar plates (SWT;
135 0.5% tryptone, 0.3% yeast extract, 0.3% glycerol, 1.5% agar, and 70% seawater at 32
136 ppt) to isolate single colonies of *V. fischeri*. Plates used for light organ isolation were
137 made with local seawater from SEAFDEC, while all other plates were made with artificial

seawater containing a mixture of Instant ocean (21 g/L of seawater; Spectrum Brands, VA) and Marine Mix (7 g/L of seawater; Wiegandt GmbH, Germany). Squid tissues were preserved in 95% ethanol for fixation and subsequent DNA extraction for Sanger sequence analysis at New Mexico State University (NMSU).

Light organ homogenates were grown for 12 to 24 hours at 20°-28°C, after which 10-15 individual colonies from each plate were stab inoculated into vials containing SWT agar and sealed for transport back to NMSU. After transport, each sample was re-cultured on SWT agar plates at 28°C for 12-24h. Single colonies were isolated and cultured in liquid SWT at 28°C and shaken at 225 rpm for 12-18 hours in an Innova 43 shaking incubator (New Brunswick Scientific, NJ). Each overnight culture was sub cultured and allowed to reach log phase (2-3 hours at 28°C and 225 rpm), and the log phase cultures were used for DNA extraction and also frozen in 40% glycerol for storage at -80°C.

DNA extraction and amplification

E. albatrossae DNA was extracted by using approximately 25 mg of ethanol preserved tissue that was dissected from the gill or mantle of each squid. Dissected tissues were washed with 100 µL of nuclease free water to remove any residual ethanol. *E. albatrossae* DNA was extracted using the DNeasy© blood and tissue protocol for animal tissues (Qiagen, Valencia, CA). All genomic DNA extractions were visualized on a 1% agarose gel and quantified using a Nanodrop 9600 (Thermo Fischer Scientific, Waltham, MA). Total DNA extracted from each individual squid sample was used to amplify a 658-bp fragment of the cytochrome *c* oxidase subunit I (COI, Table 2; (Folmer,

Black, Hoeh, Lutz, & Vrijenhoek, 1994; Jones et al., 2006; Zamborsky & Nishiguchi, 2011). The cytochrome c oxidase subunit one gene has been shown to be highly conserved, at least at the amino acid level, across invertebrate taxa (Folmer et al., 1994; Jacobs & Grimes, 1986) and has been used extensively to elucidate population structure (Calderon, Garrabou, & Aurelle, 2006; Lessios, Kane, & Evolution, 2003; S. Palumbi, Grabowsky, Duda, Geyer, & Tachino, 1997).

Isolation of DNA from *V. fischeri* light organ isolates was completed using the Qiagen DNeasy© blood and tissue kit (Valencia, CA) gram-negative bacterial protocol. Approximately 2 X 10^9 cells were transferred from each log phase culture to the extraction tube for centrifugation. After, the remaining pellet was used for extraction using the Qiagen protocol. Purified *V. fischeri* DNA was visualized on a 1% agarose gel and quantified using a Nanodrop 9600 (Thermo Fischer Scientific, Waltham, MA). Isolated DNA extracted from each *V. fischeri* isolate was used to amplify a portion of the glyceraldehyde phosphate dehydrogenase (*gapA*) locus (~900 bp) by PCR, using previously described *Vibrio* specific primers (Table 2; (Jones et al., 2006; Nishiguchi & Nair, 2003). The *gapA* locus has been used reliably to estimate deep phylogenetic connections between bacterial families (Nelson, Whittam, & Selander, 1991) within the Vibrionaceae (Thompson, Gomez-Gil, Vasconcelos, & Sawabe, 2007) as well local population structure of mutualist *V. fischeri* (Jones et al., 2006; Nishiguchi & Nair, 2003).

Each PCR amplification reaction (25 µL) contained 2-20 ng of template DNA [0.08-0.8 ng/ µL], GoTaq DNA polymerase [0.05 U/ µL] (Promega, Fitchburg, WI), 5X GoTaq buffer [1x] (Promega, Fitchburg, WI), a 10 mM deoxynucleoside triphosphate mix [0.8 mM] of each nucleotide (Promega, Fitchburg, WI), and both forward and reverse

primers [0.5 μM each] (Table 2). All amplification reactions were run using a MJ Research Dyad Disciple thermocycler (Waltham, MA). Cycle conditions for each reaction are listed in Table 3. Amplicons were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and quantified using a Nanodrop 9600 (Thermo Fischer Scientific, Waltham, MA). Purified amplicons were presequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) and amplified on an MJ Research Dyad Disciple thermocycler (Waltham, MA). Preseqencing samples were cleaned using 96-well Sephadex plates (Edge Biosystems, St. Louis, MO). Samples were sequenced at the NMSU Molecular Biology Sequencing facility using the Applied Biosystems 3130XL sequencer (Applied Biosystems, Foster City, CA). Sequences were assembled and aligned using GENEious (Biomatters Ltd, v7).

Haplotype networks, nested clade analysis, and molecular variance

Haplotype networks for squid and symbiont were generated using TCS v1.12 using statistical parsimony methods outlined by Templeton (Templeton & Sing, 1993). Nested clade analyses were performed using Templeton's nesting algorithm as implemented in GEODIS (Posada, Crandall, & Templeton, 2000). Analysis of molecular variance (AMOVA) was executed using the population genetics software platform ARLEQUIN (Excoffier & Lischer, 2010). Analyses were run for measures of within and among population variation along with a separate analysis assessing variation by island for both host and symbiont data. Concurrently, theta (σ), a base pair by base pair measure of polymorphism was calculated for each mutualist population at each sample site.

Results

Nested clade and molecular variance analysis of E. albatrossae

A total of 81 host COI sequences were used in the nested clade analyses of host genetic data, resulting in 43 distinct squid haplotypes (Genbank; MF379363 - MF379405). Host genetic data yielded three distinct unconnected haplotype networks, with one network containing only samples from the island of Palawan, while the other two networks exhibited introgression from the central island chain but no connection from the Palawan population (Fig. 2). Interestingly, host haplotype networks demonstrate little genetic connection between geographically separated populations. One host network (Clade 4-7, Fig. 2) has most members of this haplotype from the island of Panay with small contributions from populations found near the island of Negros, but no contributions from nearby Cebu populations. Another separate host network (Clade 4-1, Fig. 2) demonstrates genetic connection between Cebu, Negros, and a small contribution from Panay but also no connection to Palawan. The largest haplotype in clade 4-7 (Haplotype 14) is the result of equal contributions of genetic information from SEAFDEC, Santo Niño Sur, and Banate, all from the island of Panay. The dominant haplotype from clade 4-1 (Haplotype 5) has the largest contribution from populations sampled at Cordova and Magellan Bay from the island of Cebu, with a small contribution in this clade from SEAFDEC and Santo Niño Sur populations from the island of Panay. The third host network, clade 4-2, has no connection between Palawan host populations and hosts from the central island chain (Fig. 2).

Final nested clade analysis was performed with no detectable loops according to rules established by Templeton (Templeton & Sing, 1993). Nested clade phylogenetic analysis of host genetic data demonstrates that the null hypothesis of panmixia was rejected in four of the nested clades and for the total cladogram (Fig. 2). Inference from clade 2-3 indicates allopatric fragmentation involving populations from Cebu and Negros (Table 4; Fig.2). Clade 2-11 has restricted gene flow with isolation by distance involving populations from the island of Panay exclusively. Clade 3-1 also demonstrated restricted gene flow with isolation by distance, with clade 2-3 nested within and including genetic contributions from populations from all three central islands sampled (Table 4; Fig. 2). Clade 3-7, which includes subclade 2-11 as an interior clade (Table 4) inferred contiguous range expansion for populations. Total host cladogram inference was inconclusive due to the lack of connection between higher level clades (Table 4).

Analysis of molecular variance of host genetic data revealed that a significant portion of the variance was detected among islands and within populations (66.61%; 29.10%, Table 3). Some of the highest amounts of within-population genetic diversity, reported as theta, were observed at Dumaguete, Sibulan, and Siliman, which are all populations located from the island of Negros (0.1444, 0.0885, 0.0248; Table 1). Additionally, theta measures of populations at Parara, San Juan Barotec, and Santo Niño Sur from the island of Panay demonstrate significant within population diversity at these sites (0.0722, 0.0467, 0.0441; Table 1). Genetic diversity among populations near the island of Palawan was also observed to be a significant source of variation (0.0431, Table 1). The lowest amount of genetic diversity was detected at Cordova on the island

of Cebu, as well as at SEAFDEC and Banate from the island of Panay (0.00135, 0.0055, 0.0067; Table 1).

Nested clade and molecular variance analysis of V. fischeri

Conversely, symbiont genetic architecture in the Philippines displays a different pattern compared to their host squid. Successful initial colonization of juvenile light organs is accomplished by 1-3 strains that persist throughout the life of the animal (Wollenberg & Ruby, 2009). Any identical sequences, isolated from the same light organ were removed. Analysis of 181 symbiont *gapA* sequences yielded one contiguous network of 60 haplotypes (Genbank; MF379406 - MF379465). In contrast to host genetic architecture, symbiont populations are more connected compared to host populations. *Vibrio* genetic data produced a highly connected and diverse network showing genetic continuity between Palawan and the central island chain populations of symbionts (Figs. 3 & 4). Haplotypes 1, 3, 8, 57, and 58 contain representatives from each of the island populations sampled (Fig. 3). Major contributions from the western island of Palawan to haplotypes 8, 57 and 58 occur despite no host genetic introgression (Figs. 2 and 3). The largest haplotype (Haplotype 1) contains a significant number of Cebu haplotypes coupled with populations from Negros and Palawan. Each of the major haplotypes listed require a minimum of one base pair change, with the largest number of changes needed (6) to go from haplotype 1 to haplotype 8.

Contingency analyses of symbiotic *V. fischeri* nesting revealed significant evidence for restricted gene flow with isolation by distance in clades 2-1 and 2-8 (Table 5). Clade 2-1 exhibits restriction within the central island chain and connection between

small haplotypes found near Panay, Negros, and Cebu (Haplotypes 14, 15, and 16. Fig. 4.). Clade 2-8, which includes haplotypes 6, 8, and 9 grouped together with haplotypes 7 and 11 (Fig. 4) shows connection between central island populations and populations from Palawan. Inference from clade 3-5 indicates continuous range expansion of these populations. The grouping of clade 3-5 indicates a genetic connection between Panay and Dumaguete populations specifically from the island of Negros. Clade 4-2 also illustrates continuous range expansion and includes the subclades 2-1 and 2-8, which at the lower nesting level demonstrate restricted gene flow and isolation by distance (Table 5). Clade 4-2 also includes several singleton haplotypes that connect Palawan with the central island populations (Fig. 4). Total cladogram inference indicates, as in some of the lower level clades, restricted gene flow with isolation by distance (Table 5).

An Identical AMOVA analysis of symbiont genetic data revealed that a significant portion of the variance exhibited by these populations exists within and among populations (14.40%, 80.08%; Table 3). Base-pair by base-pair nucleotide diversity of host populations was highest in populations from Atabayan and Parara, Panay (0.0085, 0.0076; Table 1) and from the island of Negros (0.0074, 0.0061, 0.0050; Table 1) which are both in the central island chain. The lowest amount of genetic variation was observed at Magellan Bay, Macatan and Banate, Panay (0.0046, 0.0040; Table 1) this was similar to what was detected in host diversity measures at these sites.

Discussion

Host Genetic Architecture

298 The genetic structure of the *E. albatrossae* sampled for this study indicates that
299 geographical location impacts host distribution. Island effects, as reported by the
300 location and amount of variance during AMOVA analysis were detected in host genetic
301 data, further supporting that geologic origin, physical geography, and possibly
302 environmental factors have shaped the distribution of host squid in the region (among
303 Islands, d.f=3, SS= 1340.934, VC= 23.8035, PV= 66.61%; Table 3). The genetic fixation
304 observed in the host genetic data, reported as F_{ST}, indicate that genetic flow is limited
305 throughout the region and populations are genetically isolated from each other (F_{ST}=
306 0.70897, $p<0.0001$; Table 3). This is reflected in the three distinct host networks
307 detected in the Philippines (Fig. 2), and suggests geography may be influencing host
308 genetic exchange and distribution. This could be due to the benthic lifestyle adult
309 *Euprymna* squid (~5-8 cm) lead as adults rarely migrate, however the semi pelagic
310 nature of newly hatched squid (~3-10 mm) would allow water flow to transport juveniles
311 to novel locations (Kimbell et al., 2002; Villanueva, Vidal, Fernández-Álvarez, &
312 Nabhitabhata, 2016a). Two of the distinct networks occur in the central island chain, and
313 while having similar geological origin, show no genetic connection in habitats that are
314 homogeneous (Allen & Werner, 2002). Clade 4-7 is distinctly made up of mostly squid
315 haplotypes detected around the island of Panay, with small contributions from
316 populations around Negros (Haplotypes 21, 22, 23, 33, Fig. 2). This indicates that
317 populations may have been fragmented due to the result of geologic activity in the
318 region, seasonal changes in currents, or even modern day commercial fishing
319 management- which have all been shown to influence fragmentation of marine habitats
320 in the area (Abesamis, Russ, & Alcala, 2006; Huang, Wu, Zhao, Chen, & Wang, 1997;

Savina & White, 1986; Wyrtki, 1961; Zhou, Ru, & Chen, 1995). Habitat fragmentation was also inferred within clades 2-11 and 3-1 (Table 4), where genetic data indicates restricted gene flow with isolation by distance. Clade 2-11 (Fig. 2), comprised solely of host haplotypes detected around Panay, is closely connected (in some cases only one base pair difference between haplotypes; Fig. 2) to haplotypes with relatively small genetic contributions from the island of Negros. This indicates that the physical geography of the central island chain may be restricting host movement and genetic exchange. Additionally, Clade 3-7 (Fig. 2) inferred contiguous range expansion between populations sampled from the islands of Panay and Negros despite these populations being isolated geographically (Table 4), suggesting that the alternating direction of prevailing currents in the region is one mechanism of dispersal as well as isolation for these squids.

The divergence of the equatorial current (EC) as it approaches the Philippines from the east influences the directional flow of water through the central island chain, particularly north of the island of Panay and south of the islands of Negros and Cebu (Fig. 5; (Huang et al., 1997; Wyrtki, 1961). The amount and speed of water that is funneled around or through the central islands depends on the time of year, and is reflected in the patterns detected in host haplotype networks (Wyrtki, 1961). In late winter/early spring, water flows from east to west from the San Bernardino Strait, south of Masbate and finally around the north of Panay toward the Sulu Sea (Fig. 5A). This flow pattern coupled with the flow of the southern divergence of the EC allows for genetic exchange between geographically isolated populations from Panay, Cebu, and Negros (Fig. 2; Clade 4-1 and 4-7) by allowing squid to be transported to areas they

344 could not reach by themselves. As spring continues, the divergence pattern changes,
345 and only a moderate east to west flow of water north of Panay is produced, while the
346 bulk of the southern divergence is shifted south of the island of Mindanao, temporarily
347 isolating squid populations south of Negros and Cebu from populations to the north of
348 Panay (Fig. 5B). During the summer months (June-August), waters within the central
349 island chain are relatively still, with most of the equatorial current diverted northeast of
350 Luzon and southeast of Mindanao, circling around to the north, into the Sulu Sea (Fig.
351 5C). This isolates the central islands from the western island of Palawan, and further
352 prohibits exchange across the Sulu Sea. The two predominant haplotypes detected from
353 our squid data (Haplotypes 1 and 5; Fig. 2) are separated by only one base pair
354 difference even though the populations that contribute to these haplotypes are
355 separated by physical barriers. This further supports the notion that currents may be
356 influencing the prevalence and direction of gene flow between Panay squid populations
357 and other squid assemblages to the south.
358 While Clade 4-1 had no valid inference (Table 4), clade 3-1, which is fully nested
359 within 4-1 (Fig. 2), had an inference of restricted gene flow with isolation by distance.
360 This indicates that these habitats may have changed over time and influenced the
361 genetic flow between once connected populations of host squid. A third separate
362 network (Clade 4-2, Fig. 2) consisted solely of samples collected around the western
363 island of Palawan provides evidence that the unique geologic origin of Palawan may
364 have fragmented a once continuous population of host animals (Sathiamurthy & Voris,
365 2006; Wallace, 1863; Zhou et al., 1995). Likewise, factors such as the deep water
366 thermocline in the middle of the Sulu Sea, and changes in surface water transport (Fig.

5D) may have isolated these populations from others in the central islands (Chen, Yeh, Chen, & Huang, 2015; Huang et al., 1997; Miao, Thunell, & Anderson, 1994; Stuecker, Timmermann, Jin, McGregor, & Ren, 2013).

Symbiont genetic architecture

Symbiont genetic data indicate that *Vibrio* bacteria seem to be able to mitigate the barriers that restrict host genetic exchange. Analysis (AMOVA) of total symbiont genetic data reveal that most of the genetic variation observed lies within each population in contrast to the partitioning of variation from host genetic data (d.f.= 186, SS= 438.538, VC= 2.6704, PV= 80.08%; Table 3). The level of genetic diversity of the total symbiont population in the region is also indicative of symbiont gene flow between populations of hosts that are isolated from one another (F_{ST}= 0.1991, Table 3). One contiguous haplotype network was detected in the symbiont genetic data (Figs. 3, 4) revealing several genetic connections between symbionts collected from host squid that are genetically and geographically isolated from one another (Fig. 2). The predominant haplotypes found within symbiont genetic data (Haplotypes 1, 3, 8, 57, and 58; Fig. 3) are comprised of samples from all the islands, regardless of geological origin or physical position. In contrast to the pattern detected in host data, symbiont genetic data displays connections between the western island of Palawan and the central Islands to the east (Haplotypes 1, 3, 8, 57, and 58; Fig. 3). Interestingly, *Vibrio* haplotype 8 (Fig. 3) consisting of samples primarily from Palawan, has only 6 base pair changes from the more diverse haplotype 1 (Fig. 3). The composition of these two haplotypes (8 and 1, Fig. 3) is quite different and could be a result of multiple introgressions of these

390 populations by neighboring and distant *V. fischeri* symbionts displaced from their native
391 range. When including intermediate haplotypes from Cebu (Haplotype 40, Fig. 3) and
392 Negros (Haplotype 16, 11, and 12; Fig. 3), there is evidence for connection between
393 symbiont populations where hosts are restricted. While geography appears to have little
394 influence on the population structure of symbiotic *V. fischeri* in this region,
395 oceanographic currents may be influencing the ability of bacteria to cross barriers that
396 restrict hosts.
397
398 **Geography, geologic history, and environmental conditions**
399 Results from this study indicate that geography plays a role in host squid
400 distribution, without demonstrating a significant influence on symbiont distribution. The
401 disparity in these patterns may be a result of differences in dispersal methodology
402 between mutualist partners, *i.e.* host squid have a limited range as adults and rarely
403 travel far from their birthplace due to the limited dispersal ability of direct developing,
404 benthic hatchlings (Kimbell et al., 2002; Villanueva, Vidal, Fernández-Álvarez, &
405 Nabhitabhata, 2016b). Conversely, symbiotically viable *Vibrio* bacteria are cycled out of
406 the host daily exposing them to environmental factors (i.e. currents) that allow for
407 movement into novel areas where they are able to recruit into a new host. While bacteria
408 alone cannot cross great expanses of ocean, the use of rafting has been shown to be an
409 effective dispersal mechanism for marine bacteria like *V. fischeri* (Jones et al., 2006;
410 Theil & Gutow, 2005). The ability for vibrios to cross great expanses of oceans has been
411 previously reported in other marine bacteria and undoubtedly will allow symbiotically

412 viable vibrios to be shuttled to new areas and novel hosts (González-Escalona, Gavilan,
413 Brown, & Martinez-Urtaza, 2015).

414 Prevailing currents in and around the central Philippine island chain vary in
415 direction and magnitude seasonally (Wyrtki, 1961). Since *E. albatrossae* breed all year
416 long, this change in directionality may provide newly hatched squid the opportunity to be
417 carried to new areas, despite their otherwise limited dispersal ability, while being cut off
418 from other available habitats when the prevailing currents change (Hanlon, Claes,
419 Ashcraft, & Dunlap, 1997). The pattern of direction in the symbiont genetic data
420 presented here indicates that introgression across the Sulu Sea, which appears to be a
421 biogeographical margin for host animals, is facilitated by the directional flow of water
422 during the monsoon season (Huang et al., 1997). *Euprymna* hatchlings are known to be
423 “pelagic”, that is, they linger in the water column before settling to their benthic lifestyle
424 (Moltschaniwskyj & Doherty, 1995). This also might heighten the ability of host
425 populations to move to new localities.

426 Geological changes and the physical oceanography of this region may also
427 explain the patterns detected in the genetic data. Glacial maximum sea levels exposed
428 portions of what was host native range within the central island chain, creating a
429 disconnect between populations in the west and central island squid assemblages
430 (Gaither & Rocha, 2013; Gordon, 2005; Zhou et al., 1995). During glacial norms, hosts
431 are restricted by a deep-water thermocline that has persisted since before the Holocene,
432 between Palawan and the central island chain (Miao et al., 1994). Fluctuating sea level
433 during glacial cycles as well as Cenozoic volcanic uprising of the central Visayas may
434 also explain the disjunctive distribution of host animals across this region (Miao et al.,

435 1994; Zhou et al., 1995). Given that many of the more abundant haplotypes examined
436 have prevalence in localities that are geographically distinct, this provides additional
437 evidence that host populations have been established well beyond the geological history
438 of the Philippines (e.g., Palawan).

439 While previous research has shown that symbiont gene flow can be restricted by
440 temperature, symbionts in this region seem to be able to mitigate environmental barriers
441 which hosts cannot, crossing geographical and biological barriers with apparent ease
442 (Nishiguchi, 2000). Symbiont gene flow demonstrates a current dependent directionality
443 of introgression by vibrios from the central islands west to Palawan in the winter, and
444 west to east in the summer months (Huang et al., 1997). The El Niño Southern
445 Oscillation has also been shown to influence not only sea surface temperatures, wind
446 direction, and rainfall in this region, but also the position of this deep water thermocline ,
447 further isolating local populations of host squid while not restricting symbiont distribution
448 (Chen et al., 2015; Stuecker et al., 2013). While other Indo-west Pacific and
449 Mediterranean populations of *Vibrio* demonstrate that some degree of host specificity,
450 geography, or other environmental factors can impact symbiont genetic architecture ,
451 findings from this study indicate that geography alone cannot explain symbiont
452 distribution, and that physical factors (e.g., currents) are important drivers of microbial
453 diversity in the region (Jones et al., 2006; Zamborsky & Nishiguchi, 2011).

454 Beneficial associations like the sepiolid squid-*Vibrio* mutualism will undoubtedly
455 be impacted by reduction of available habitat, highlighting the importance of
456 investigating the influence geography has on symbiont prevalence and distribution.
457 Findings from this study point to a need to better understand the mechanisms that will

458 impact symbiotic associations across a changing landscape, and what factors will
459 influence the fitness of beneficial microbes when they are moved to a novel habitat. Our
460 findings have provided clues as to how established populations of host squids are the
461 foundation for symbiont population structure, yet abiotic factors still influence where
462 vibrios can move and establish new populations.
463
464 **ACKNOWLEDGEMENTS**
465 The authors would like to thank all the collaborators, lab technicians, fisherman, and
466 local merchants without whom this research would not be possible. Without their help,
467 guidance, generosity, hospitality and invaluable local knowledge this project would not
468 be complete. We would also like to thank the administrators, staff, and technicians at the
469 Southeast Asian Fisheries Development Center (SEAFDEC), Siliman University
470 (Negros), the University of San Carlos (Cebu), and Western Philippine University
471 (Palawan) for providing us with lab facilities, transport, and local contacts.
472 We would like to thank M. Sedano and N. Nourabadi for help with collections during the
473 2012 and 2013 field seasons. Funding for this project was supported by New Mexico
474 State University Honors College International fellowship (K.E.T.), Howard Hughes
475 Medical Institute 52008103 (K.E.T.), Department of Biology at NMSU, National Institute
476 of Health Rise to the Postdoctorate-R25GM061222 (R.L.C.), American Association for
477 the Advancement of Science-WIRC/National Science Foundation (M.K.N.), University of
478 New Mexico-ASERT (IRACDA-NIH K12GM088021 (M.K.N.), American Society for
479 Microbiology International Professorship in Asia (M.K.N.), and the NMSU Foundation
480 (M.K.N.).

Data Accessibility:

- Final DNA sequences: Genbank accessions : *Euprymna albatrossae* (COI;MF379363 - MF379405) ; *Vibrio fischeri* (*gapA*; MF379406 - MF379465)

Author Contributions:

1. R.L. Coryell- Extracted DNA, amplified genes of interest, aligned and analyzed sequence data, wrote paper.
2. K.E. Turnham- Collected samples, generated data, analyzed data, co-wrote paper.
3. E.G.J. Ayson- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
4. A. Alacala- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
5. F. Soto- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
6. B. Gonzales- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
7. M.K. Nishiguchi- Coordinated and obtained project funding, initial experimental design, collection and processing of sample animals, co-author of paper, contributing editing and review of manuscript.

REFERENCES

Abesamis, R. A., Russ, G. R., & Alcala, A. C. (2006). Gradients of abundance of fish across no-take marine reserve boundaries: evidence from Philippine coral reefs. *Aquatic Conservation: Marine and Freshwater Ecosystems, 16*(4), 349-371. doi:10.1002/aqc.730

Alcazar, D. S., & Kochzius, M. (2016). Genetic population structure of the blue sea star *Linckia laevigata* in the Visayas (Philippines). *Journal of the Marine Biological Association of the United Kingdom, 96*(3), 707-713. doi:10.1017/S0025315415000971

Allen, G. R., & Werner, T. B. (2002). Coral reef fish assessment in the 'coral triangle'of southeastern Asia. *Environmental Biology of Fishes, 65*(2), 209-214. doi:10.1023/A:1020093012502

Boettcher, K., Ruby, E., & McFall-Ngai, M. (1996). Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *Journal of Comparative Physiology A, 179*(1), 65-73. doi: doi:10.1007/BF00193435

Bongrand, C., Koch, E. J., Moriano-Gutierrez, S., Cordero, O. X., McFall-Ngai, M., Polz, M. F., & Ruby, E. G. (2016). A genomic comparison of 13 symbiotic Vibrio fischeri isolates from the perspective of their host source and colonization behavior. *The ISME Journal, 10*(12), 2907-2917. doi:10.1038/ismej.2016.69

Calderon, I., Garrabou, J., & Aurelle, D. (2006). Evaluation of the utility of COI and ITS markers as tools for population genetic studies of temperate gorgonians. *Journal of Experimental Marine Biology and Ecology, 336*, 184-197. doi:10.1016/j.jembe.2006.05.006

Carpenter, K. E., & Springer, V. G. (2005). The center of the center of marine shore fish biodiversity: the Philippine Islands. *Environmental Biology of Fishes, 72*(4), 467-480. doi:10.1007/s10641-004-3154-4

Chen, C.-T., Yeh, Y.-T., Chen, Y.-C., & Huang, T.-H. (2015). Seasonal and ENSO-related interannual variability of subsurface fronts separating West Philippine Sea waters from South China Sea waters near the Luzon Strait. *Deep Sea Research Part I: Oceanographic Research Papers, 103*, 13-23. doi:10.1016/j.dsr.2015.05.002

Esselstyn, J. A., Oliveros, C. H., Moyle, R. G., Peterson, T. A., McGuire, J. A., & Brown, R. M. (2010). Integrating phylogenetic and taxonomic evidence illuminates complex biogeographic patterns along Huxley's modification of Wallace's Line. *Journal of Biogeography, 37*(11), 2054-2066. doi:10.1111/j.1365-2699.2010.02378.x

Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources, 10*(3), 564-567. doi:10.1111/j.1755-0998.2010.02847.x

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294-299.

Gaither, M. R., & Rocha, L. A. (2013). Origins of species richness in the Indo-Malay-Philippine biodiversity hotspot: evidence for the centre of overlap hypothesis. *Journal of Biogeography, 40*(9), 1638-1648. doi:10.1111/jbi.12126

Gaither, M. R., Toonen, R. J., Robertson, R. D., Planes, S., & Bowen, B. W. (2010). Genetic evaluation of marine biogeographical barriers: perspectives from two widespread Indo-Pacific snappers (*Lutjanus kasmira* and *Lutjanus fulvus*). *Journal of Biogeography, 37*(1), 133-147. doi:10.1111/j.1365-2699.2009.02188.x

González-Escalona, N., Gavilan, R. G., Brown, E. W., & Martinez-Urtaza, J. (2015). Transoceanic Spreading of Pathogenic Strains of Vibrio parahaemolyticus with Distinctive Genetic Signatures in the recA Gene. *PLOS ONE, 10*(2). doi:10.1371/journal.pone.0117485

Gordon, A. L. (2005). Oceanography of the Indonesian Seas. *Oceanography, 18*(4), 14-27. doi:10.5670/oceanog.2005.18

Hanlon, R. T., Claes, M. F., Ashcraft, S. E., & Dunlap, P. V. (1997). Laboratory culture of the sepiolid squid Euprymna scolopes: a model system for bacteria-animal symbiosis. *The Biological Bulletin*.

Hellberg, M. E., Burton, R. S., Neigel, J. E., & Palumbi, S. R. (2002). Genetic assessment of connectivity among marine populations. *Bulletin of marine ….*

Herring, P. J. (1977). Luminescence in cephalopods and fish. *Symposia of the Zoological Society of London, 38*, 127-159.

Horne, J. B. (2014). Thinking outside the barrier: neutral and adaptive divergence in Indo-Pacific coral reef faunas. *Evolutionary Ecology, 28*(6), 991-1002.

Huang, C. Y., Wu, S. F., Zhao, M., Chen, M. T., & Wang, C. H. (1997). Surface ocean and monsoon climate variability in the South China Sea since the last glaciation. *Marine Micropaleontology, 32*(1-2), 71-94 doi:https://doi.org/10.1016/S0377-8398(97)00014-5

Huxley, T. H. (1868). *On the classification and distribution of the Alectoromorphae and Heteromorphae*. London: Royal Institution.

Jacobs, H. T., & Grimes, B. (1986). Complete nucleotide sequences of the nuclear pseudogenes for cytochrome oxidase subunit I and the large mitochondrial ribosomal RNA in the sea urchin Strongylocentrotus purpuratus. *Journal of Molecular Biology, 187*(4), 509 - 527. doi:https://doi.org/10.1016/0022-2836(86)90330-X

Jones, B. W., Lopez, J. E., Huttenburg, J., & Nishiguchi, M. K. (2006). Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Molecular Ecology, 15*(14), 4317-4329. doi:10.1111/j.1365-294X.2006.03073.x

Jones, B. W., & Nishiguchi, M. K. (2004). Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Marine Biology, 144*(6), 1151-1155. doi:10.1007/s00227-003-1285-3

Juinio-Menez, M., Magsino, R., Ravago-Gotanco, R., & Yu, E. (2003). Genetic structure of *Linckia laevigata* and *Tridacna crocea* populations in the Palawan shelf and shoal reefs. *Marine Biology, 142*(4), 717-726.

593 Kimbell, J. R., McFall-Ngai, M. J., & Roderick, G. K. (2002). Two genetically distinct
594 populations of bobtail squid, *Euprymna scolopes*, exist on the island of O'ahu.
595 *Pacific science*. doi:10.1353/psc.2002.0024
596 Kool, J. T., Paris, C. B., Barber, P. H., & Cowen, R. K. (2011). Connectivity and the
597 development of population genetic structure in Indo-West Pacific coral reef
598 communities. *Global Ecology and Biogeography, 20*(5), 695-706.
599 doi:10.1111/j.1466-8238.2010.00637.x
600 Lemer, S., Combosch, D., Sotto, F., Giribet, G., Dumale, D., & Soliman, V. (2016). The
601 family Pinnidae (Bivalvia) in the Philippine archipelago: observations on its
602 distribution and phylogeography. *NAUTILUS*.
603 Lessios, H. A., Kane, J., & Evolution, R.-D. R. (2003). Phylogeography of the pantropical
604 sea urchin Tripneustes: contrasting patterns of population structure between
605 oceans. *Evolution*. doi:10.1554/02-681
606 Liu, S.-Y. V., Chang, F.-T., Borsa, P., Chen, W.-J., & Dai, C.-F. (2014). Phylogeography
607 of the humbug damselfish, *Dascyllus aruanus* (Linnaeus, 1758): evidence of Indo-
608 Pacific vicariance and genetic differentiation of peripheral populations. *Biological*
609 *Journal of the Linnean Society, 113*(4), 931-942. doi:10.1111/bij.12378
610 Lohman, D. J., de Bruyn, M., Page, T., von Rintelen, K., Hall, R., Ng, P., . . . von
611 Rintelen, T. (2011). Biogeography of the Indo-Australian Archipelago. *Annual*
612 *Review of Ecology, Evolution, and Systematics, 42*(1), 205-226.
613 doi:10.1146/annurev-ecolsys-102710-145001
614 Magsino, R. M., Ravago, R. G., & Juinio-Menez, M. A. (2002). *Genetic relationship of*
615 *Linckia laevigata color morphs in the Kalayaan Islands Group, western*
616 *Philippines: preliminary evidence*. Paper presented at the Proceedings of the 9th
617 International Coral Reef Symposium, Bali, Indonesia.
618 http://www.coremap.or.id/downloads/ICRS9th-Magsino.pdf
619 McFall-Ngai, M. (2014). Divining the Essence of Symbiosis: Insights from the Squid-
620 Vibrio Model. *PLoS Biology, 12*(2). doi:10.1371/journal.pbio.1001783
621 McFall-Ngai, M., Heath-Heckman, E., Gillette, A. A., Peyer, S. M., & Harvie, E. A.
622 (2012). The secret languages of coevolved symbioses: Insights from the
623 *Euprymna scolopes–Vibrio fischeri* symbiosis. *Seminars in Immunology, 24*(1), 3-
624 8. doi:10.1016/j.smim.2011.11.006
625 Miao, Q., Thunell, R. C., & Anderson, D. M. (1994). Glacial-Holocene carbonate
626 dissolution and sea surface temperatures in the south China and Sulu seas.
627 *Paleoceanography, 9*(2), 269-290. doi:10.1029/93PA02830
628 Moltschaniwskyj, N. A., & Doherty, P. J. (1995). Cross-shelf distribution patterns of
629 tropical juvenile cephalopods sampled with light-traps. *Marine and Freshwater*
630 *Research, 46*(4), 707-714. doi:10.1071/MF9950707
631 Nelson, K., Whittam, T. S., & Selander, R. K. (1991). Nucleotide polymorphism and
632 evolution in the glyceraldehyde-3-phosphate dehydrogenase gene (gapA) in
633 natural populations of Salmonella and Escherichia coli. *Proceedings of the*
634 *National Academy of Sciences, 88*(15), 6667-6671. doi:10.1073/pnas.88.15.6667
635 Nishiguchi, M. K. (2000). Temperature affects species distribution in symbiotic
636 populations of Vibrio spp. *Applied and environmental microbiology, 66*(8), 3550-
637 3555. doi:http://dx.doi.org/10.1128/AEM.66.8.3550-3555.2000

Nishiguchi, M. K., & Nair, V. S. (2003). Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. *International Journal of Systematic and Evolutionary Microbiology, 53*(6), 2019-2026. doi:10.1099/ijs.0.02792-0

Nyholm, S. V. (2004). The winnowing: establishing the squid–vibrio symbiosis. *Nature reviews. Microbiology, 2*(8), 632-642. doi:10.1038/nrmicro957

Nyholm, S. V., & Nishiguchi, M. K. (2008). The evolutionary ecology of a sepiolid squid-vibrio association: from cell to environment. *Vie et milieu, 58*(2), 175.

Nyholm, S. V., Stabb, E. V., Ruby, E. G., & McFall-Ngai, M. J. (2000). Establishment of an animal–bacterial association: Recruiting symbiotic vibrios from the environment. *Proceedings of the National Academy of Sciences, 97*(18), 10231-10235. doi:10.1073/pnas.97.18.10231

Palumbi, S., Grabowsky, G., Duda, T., Geyer, L., & Tachino, N. (1997). Speciation and Population Genetic Structure in Tropical Pacific Sea Urchins. *Evolution, 51*(5), 1506. doi:10.2307/2411203

Palumbi, S. R. (1994). Genetic divergence, reproductive isolation, and marine speciation. *Annual review of ecology and systematics*. doi:10.1146/annurev.es.25.110194.002555

Posada, D., Crandall, K. A., & Templeton, A. R. (2000). GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology, 9*(4), 487-488. doi:10.1046/j.1365-294x.2000.00887.x

Ravago-Gotanco, R. G., Magsino, R. M., & Juinio-Menez, M. A. (2007). Influence of the North Equatorial Current on the population genetic structure of *Tridacna crocea* (Mollusca: Tridacnidae) along the eastern Philippine seaboard. *Marine Ecology Progress Series, 336*, 161-168.

Roberts, C. M., McClean, C. J., Veron, J. E. N., Hawkins, J. P., Allen, G. R., McAllister, D. E., . . . Werner, T. B. (2002). Marine biodiversity hotspots and conservation priorities for tropical reefs. *Science, 295*(5558), 1280-1284. doi:10.1126/science.1067728

Sathiamurthy, E., & Voris, H. K. (2006). Maps of Holocene sea level transgression and submerged lakes on the Sunda Shelf. *The Natural History Journal of ….*

Savina, G. C., & White, A. T. (1986). A tale of two islands: some lessons for marine resource management. *Environmental Conservation, 13*(2), 107-113. doi:10.1017/S0376892900036699

Siler, C. D., Oaks, J. R., Esselstyn, J. A., Diesmos, A. C., & Brown, R. M. (2010). Phylogeny and biogeography of Philippine bent-toed geckos (Gekkonidae: Cyrtodactylus) contradict a prevailing model of Pleistocene diversification. *Molecular phylogenetics and evolution, 55*(2), 699-710. doi:10.1016/j.ympev.2010.01.027

Sorenson, L., Allen, G. R., Erdmann, M. V., Dai, C.-F., & Liu, S.-Y. (2014). Pleistocene diversification of the *Pomacentrus coelestis* species complex (Pisces: Pomacentridae): historical biogeography and species boundaries. *Marine Biology, 161*(11), 2495-2507. doi:10.1007/s00227-014-2521-8

Soto, W., Gutierrez, J., Remmenga, M., & Nishiguchi, M. (2009). Salinity and Temperature Effects on Physiological Responses of Vibrio fischeri from Diverse Ecological Niches. *Microbial Ecology*. doi:10.1007/s00248-008-9412-9

Stuecker, M. F., Timmermann, A., Jin, F.-F., McGregor, S., & Ren, H.-L. (2013). A combination mode of the annual cycle and the El Nino/Southern Oscillation. *Nature Geoscience, 6*(7), 540-544. doi:10.1038/ngeo1826

Templeton, A. R., & Sing, C. F. (1993). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics, 134*(2), 659-669.

Theil, M., & Gutow, L. (2005). The ecology of rafting in the marine environment. II. The rafting organisms and community. *Oceanography and Marine Biology: An Annual Review, 43*, 279-418.

Thompson, F. L., Gomez-Gil, B., Vasconcelos, A., & Sawabe, T. (2007). Multilocus Sequence Analysis Reveals that Vibrio harveyi and V. campbellii Are Distinct Species. *Applied and Environmental Microbiology, 73*(13), 4279-4285. doi:10.1128/AEM.00020-07

Tonon, L. A., de Silva, B. O., Moreira, A. B., Valle, C., Alves, N., Cavalcanti, G., . . . Thompson, F. L. (2015). Diversity and ecological structure of vibrios in benthic and pelagic habitats along a latitudinal gradient in the Southwest Atlantic Ocean. *PeerJ, 3*. doi:10.7717/peerj.741

Villanueva, R., Vidal, E. A. G., Fernández-Álvarez, F., & Nabhitabhata, J. (2016a). Early mode of life and hatchling size in Cephalopod Molluscs: Influence on the species distributional ranges. *PLOS ONE, 11*(11), e0165334. doi:10.1371/journal.pone.0165334

Villanueva, R., Vidal, E. A. G., Fernández-Álvarez, F., & Nabhitabhata, J. (2016b). Early mode of life and hatchling size in Cephalopod Molluscs: Influence on the species distributional ranges. *PLOS ONE, 11*(11), e0165334. doi:10.1371/journal.pone.0165334

Wallace, A. R. (1860). On the Zoological Geography of the Malay Archipelago. *Journal of the Proceedings of the Linnean Society of London. Zoology, 4*(16), 172-184. doi:10.1111/j.1096-3642.1860.tb00090.x

Wallace, A. R. (1863). On the physical geography of the Malay Archipelago. *The Journal of the Royal Geographical Society of London, 7*(5), 206-213. doi:1798448

Wollenberg, M. S., & Ruby, E. G. (2009). Population structure of Vibrio fischeri within the light organs of Euprymna scolopes squid from Two Oahu (Hawaii) populations. *Applied and environmental microbiology, 75*(1), 193-202. doi:10.1128/aem.01792-08

Wyrtki, K. (1961). Physical oceanography of the Southeast Asian waters. *Scripps Institution of Oceanography*.

Zamborsky, D. J., & Nishiguchi, M. K. (2011). Phylogeographical patterns among Mediterranean sepiolid squids and their Vibrio symbionts: environment drives specificity among sympatric species. *Applied and environmental microbiology, 77*(2), 642-649. doi:10.1128/AEM.02105-10

Zhou, D., Ru, K., & Chen, H. (1995). Kinematics of Cenozoic extension on the South China Sea continental margin and its implications for the tectonic evolution of the region. *Tectonophysics, 251*, 161-177.

Figure Legends

Figure 1. Sampling location where host squid were collected during the months of May, June, July, and August of 2010-2013 and 2015.

Figure 2. TCS nested haplotype network generated from *E. albatrossae* molecular data acquired from animals captured in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent unsampled mutational steps connecting haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two digit indicator with the dotted line enclosures indicating the nesting hierarchy. Each nesting level is labeled with a dashed two-digit label.

Figure 3. TCS haplotype network generated from *Vibrio fischeri* molecular data acquired from isolates harvested from squid light organs in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent un-sampled haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two-digit indicator.

Figure 4. Nested *Vibrio fischeri* haplotype network generated from molecular data acquired in the Philippines during 2010, and 2012 through 2015. Each haplotype is represented by a two-digit identifier (see Fig. 3), with each hierarchical nesting level represented by a 2 to 3 digit dashed identifier and enclosed within dashed and dotted lines. Lines between haplotypes represent the mutational steps required to transition from one genetic station to another, with the small black dots representing un-sampled haplotypes.

Figure 5. Maps of surface currents in the Philippine islands during: A) Winter and early Spring (Dec-Feb), B) Late Spring (Mar-May) C) Early Summer (June-July), D) Late Summer/ Fall (Aug-Nov). Adapted from Wyrtki 1961.

Table 1. Geographic location, sampling years, and sites where *Euprymna albatrossae* and *Vibrio fischeri* were collected in the Philippines during the months of May, June, July, and August of the years listed below. All squids were wild caught adults and approximately 5 cm in mantle length.

Site Name	Abbreviation	Years Collected	Host n	Coordinates (Decimal Degrees)	Host □	*Vibrio* □
Cordova, Mactan	CVCP	2015	9	124.004 10.317	.00135	0.0051
Magellan Bay, Mactan	MGCP	2015	10	124.009 10.528	0.0138	0.0046
Dumaguete, Negros	DBNP	2013	2	123.269 9.186	0.1444	0.0074
Sibulan, Negros	SIBNP	2013	8	123.276 9.396	0.0248	0.0061
Siliman, Negros	SBNP	2013	5	123.310 9.31	0.0885	0.0050
Puerto Bay, Palawan	PBPP	2013, 2015	9	118.733 9.733	0.0431	0.0048
Atabayan, Panay (SEAFDEC)	SEDEC	2010,2012,2013	8	122.400 10.667	0.0055	0.0085
Banate, Panay	BAN	2012	10	122.815 10.983	0.0067	0.0040
San Juan Barotac, Panay	SJBV	2012	7	122.872, 10.027	0.0467	0.0058
Santo Nino Sur, Panay	SNSP	2015	7	122.504 10.679	0.0441	0.0052
Parara, Panay	PARA	2013	6	122.353 10.700	0.0722	0.0076

Table 2. Primer names, sequence, and sequence source used in the amplification of the glyceraldehyde phosphate dehydrogenase (*gapA*) locus from *Vibrio fischeri*, and the cytochrome *c* oxidase subunit 1 (COI) locus from *Euprymna albatrossae* collected in the Philippines from 2010-2015.

Primer Name	Fragment Size	Primer Sequence	Source
gapAF	889 bp	5'-GGATTTGGCCGCATCGGCCG-3'	Jones *et al.* 2006; Zamborski and Nishiguchi 2011
gapAR		5'-GGATTTGGCCGCATCGGCCG-3'	
COI F	658 bp	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Jones *et al.* 2006; Nishiguchi *et al.* 2004
COI R		5'-GGTCAACAAATCATAAAGATATTGG-3'	

Table 3. Results of identical AMOVA analyses performed on host and symbiont genetic data.

Source of *E. albatrossae* Variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among Islands	3	1340.934	23.8035 *	66.61
Within Islands	7	143.175	1.4918	4.29
Within Populations	70	709.212	10.1316 *	29.10
Total	80	2193.321	34.8127	
Overall (F_{ST})		0.70897*		
Source of *V. fischeri* Variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among Islands	3	57.057	0.1799	5.52
Within Islands	7	66.639	0.4693*	14.40
Within Populations	168	438.538	2.6704*	80.08
Total	178	562.235	3.2596	
Overall (F_{ST})		0.1991*		

*, P< 0.001

Table 4. *Euprymna* clades that demonstrated significance during either a permutation contingency analysis or geographic distance analysis. Location of significance is indicated by (D_n), nested clade distance and/or (D_c) the within clade distance. I-T indicates the average distance between a tip clade and an interior clade. S or L indicates the distance measure is significantly small or large at the 5% inference level. Inference steps were performed using the automated inference key in GEODIS, part of the AneCA v1.2 population genetics analysis software platform (Posada, Crandall, & Templeton, 2000).

Clade	Nested Clade	Dist.	Value (S or L)	*P*	Inference Key Steps	Inference
.2-3	1-6(T)	D_n	102.87 S	0.0485	1,19,NO	Allopatric Fragmentation
	1-5(I)	D_c	23.288 S	0.0485		
		D_n	38.528 S	0.0485		
	I-T	D_n	-64.3381 S	0.0485		
2-11	1-21 (T)	D_c	0.0 S	0.0322	1,2,3,4,NO	Restricted gene flow with isolation by distance
3-1	2-1 (T)	D_c	54.307 S	0.0094	1,2,3,4,NO	Restricted gene flow with isolation by distance
3-7	2-9 (T)	D_n	97.411 L	0.048	1,2,11,12, NO	Contiguous range expansion
	2-11 (I)	D_c	31.471 S	0.0406		
		D_n	61.135 S	0.0417		
	I-T	D_c	-57.4872 S	0.0386		
		D_n	-36.2756 S	0.0428		
Total	4-1	D_c	75.7056 S	0.0002	1,2,IO	I-T Status undetermined: Inconclusive outcome
	4-2	D_c	0.000	>0.001		
		D_n	435.1782 L	>0.001		

4-7	D_c	67.0744 S	>0.001
	D_n	82.2657 S	

Table 5. *Vibrio fischeri* clades that demonstrated significance during either a permutation contingency analysis or geographic distance analysis. Location of significance is indicated by (D_n), nested clade distance and/or (D_c) the within clade distance. I-T indicates the average distance between a tip clade and an interior clade. S or L indicates the distance measure is significantly small or large at the 5% inference level. Inference steps were performed using the automated inference key in GEODIS, part of the AneCA v1.2 population genetics analysis software platform(Posada, Crandall, & Templeton, 2000).^{NS}= not significant.

Clade	Nested Clade	Dist.	Value (S or L)	*P*	Inference Key Steps	Inference
2-1	1-1 (T)	D_c	76.77 S	0.0218	1,19,20,2,3,4,NO	Restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species)
		D_n	84.88 S	0.0408		
	1-27 (I)	D_n	117.3675	0.0043		
	I-T	D_c	-76.7722	0.0667^{NS}		
		D_n	32.4858	0.0043		
2-8	1-11(T)	D_c	196.5289 S	0.0183	1,19,20,2,3,4,NO	Restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species)
3-5	2-11 (T)	D_n	99.7825 L	0.0139	1,2,3,4,NO	Contiguous range expansion
	2-12 (I)	D_c	0.833 S	0.0054		
		D_n	73.3769 S	0.0139		
	I-T	D_c	-71.6474 S	0.0054		
		D_n	-26.4056	0.0139		

4-2	3-2 (T)	D_c	240.9458 L	0.0197	1,2,11,12, NO	Contiguous range expansion
		D_n	241.0454 L	0.0045		
	3-3 (I)	D_c	104.4759 S	>0.001		
		D_n	176.6458 S	0.0042		
	I-T	D_c	-136.4699 S	>0.001		
		D_n	-64.3996 S	0.0041		
Total	4-1 (T)	D_c	104.559 S	>0.001	1,2,3,4,NO	Restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species)
		D_n	111.3004 S	>0.001		
	4-3 (T)	D_c	99.2733	0.0508*		
		D_n	97.1363 S	0.0069		
	4-2 (I)	D_c	214.3525 L	>0.001		
		D_n	198.3175 L	>0.001		
	I-T	D_c	110.8023 L	>0.001		
		D_n	89.7202 L	>0.001		

Figure Legends

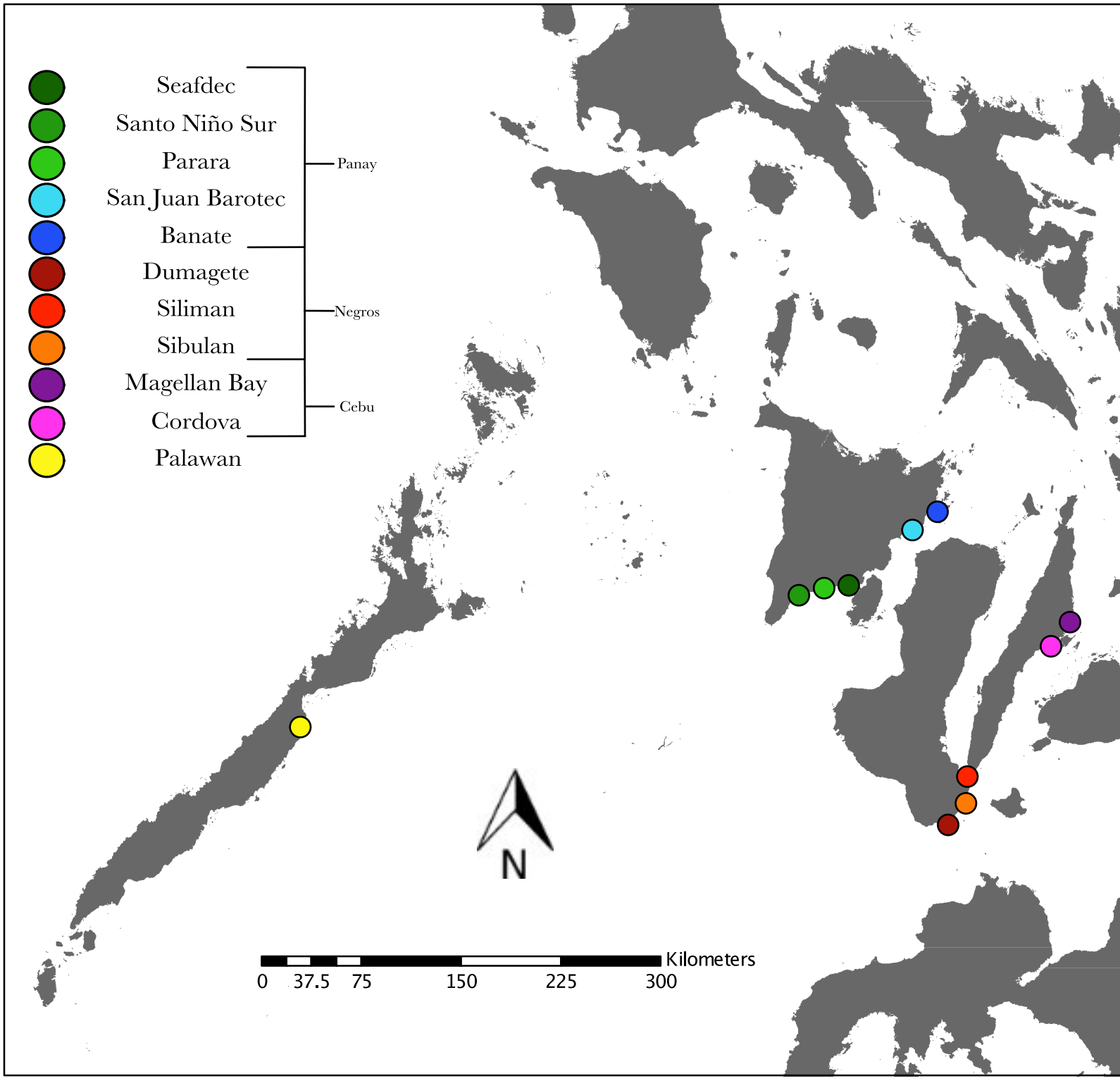
Figure 1. Sampling location where host squid were collected during the months of May, June, July, and August of 2010-2013 and 2015.

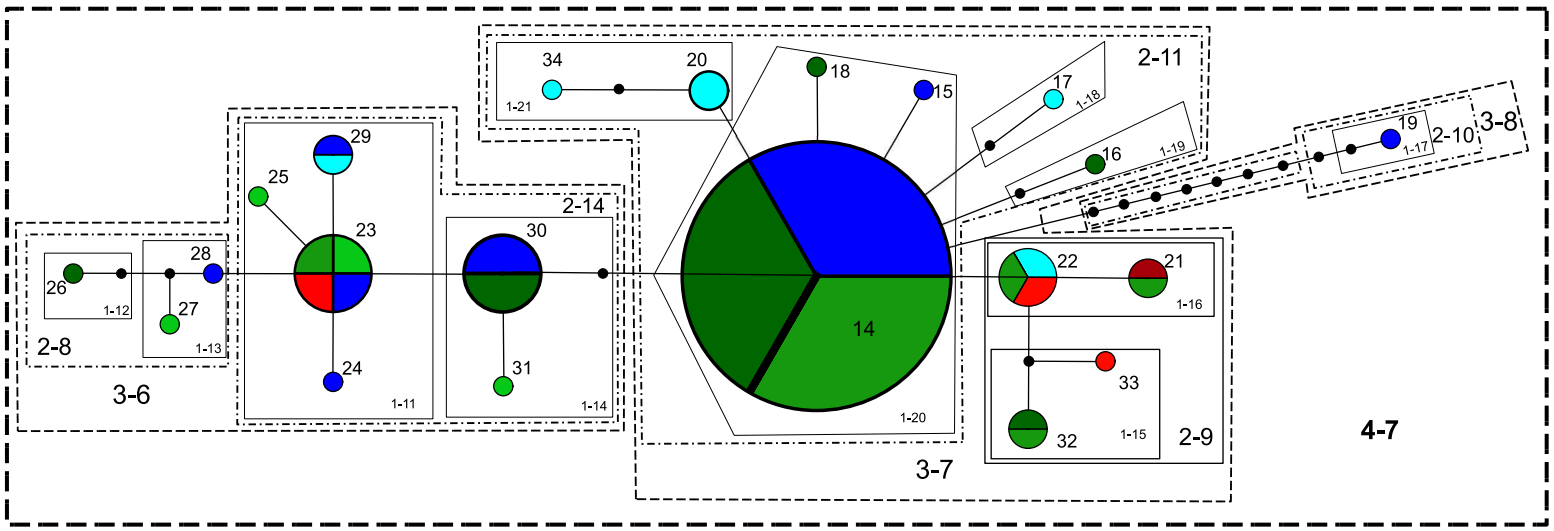
Figure 2. TCS nested haplotype network generated from *E. albatrossae* molecular data acquired from animals captured in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent unsampled mutational steps connecting haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two digit indicator with the dotted line enclosures indicating the nesting hierarchy. Each nesting level is labeled with a dashed two-digit label.

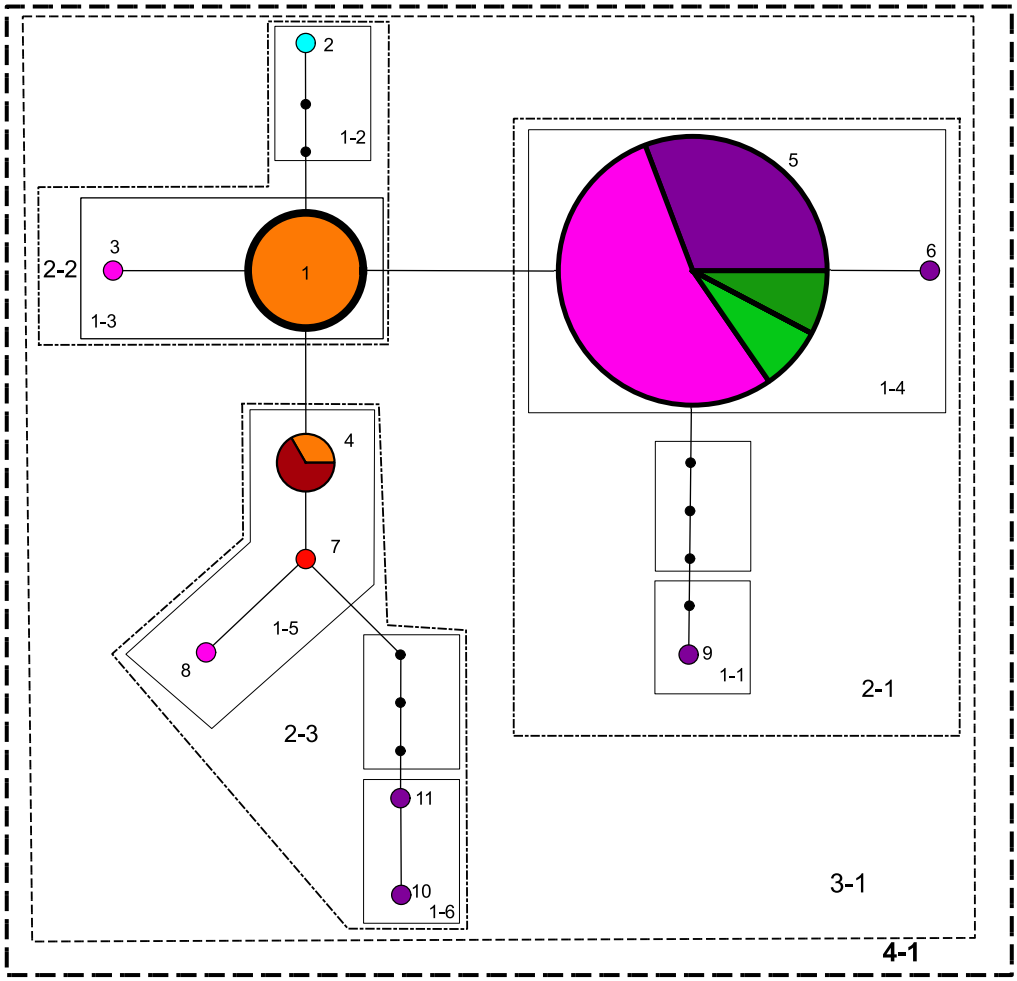
Figure 3. TCS haplotype network generated from *Vibrio fischeri* molecular data acquired from isolates harvested from squid light organs in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent un-sampled haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two-digit indicator.

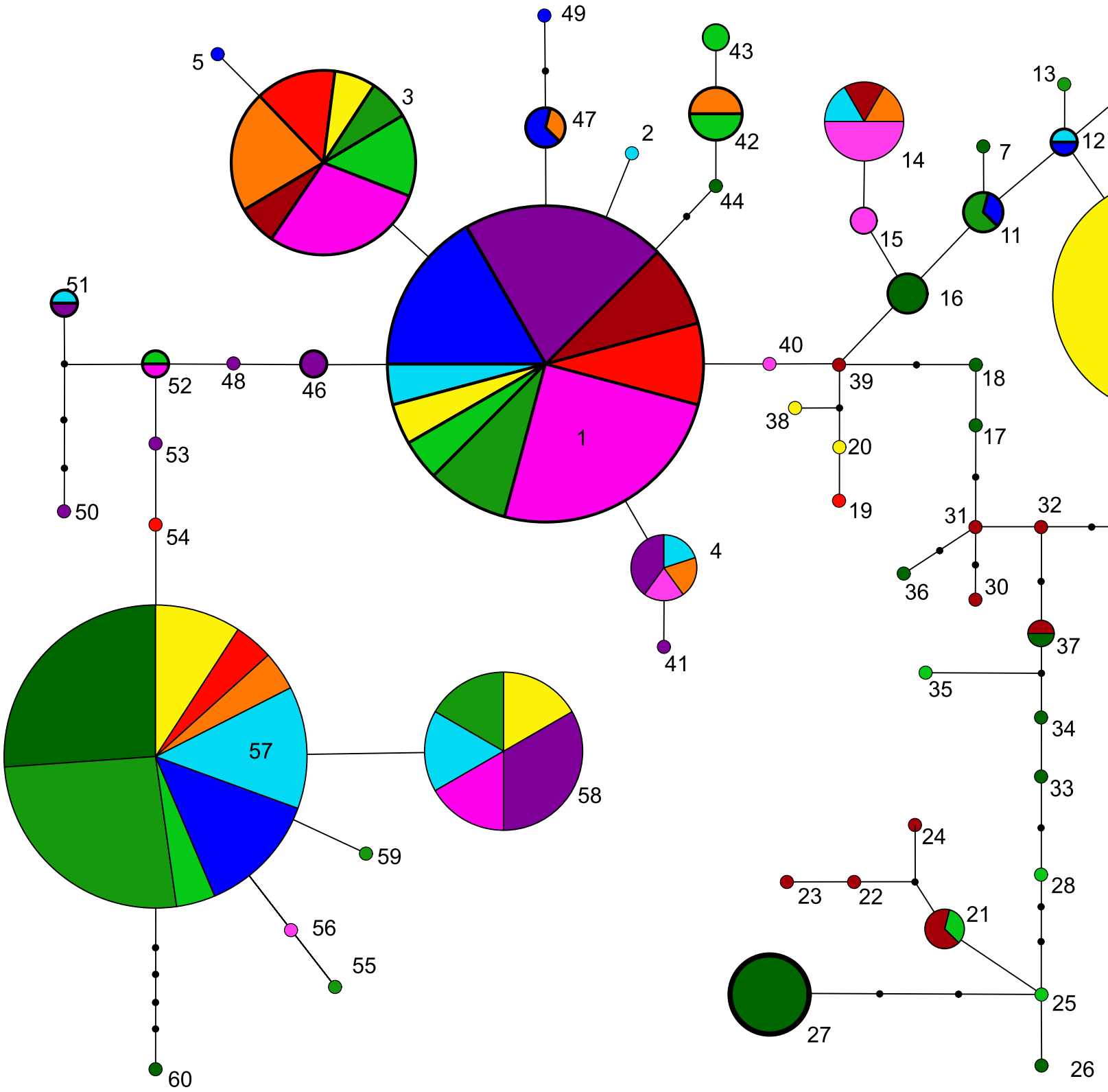
Figure 4. Nested *Vibrio fischeri* haplotype network generated from molecular data acquired in the Philippines during 2010, and 2012 through 2015. Each haplotype is represented by a two-digit identifier (see Fig. 3), with each hierarchical nesting level represented by a 2 to 3 digit dashed identifier and enclosed within dashed and dotted lines. Lines between haplotypes represent the mutational steps required to transition from one genetic station to another, with the small black dots representing un-sampled haplotypes.

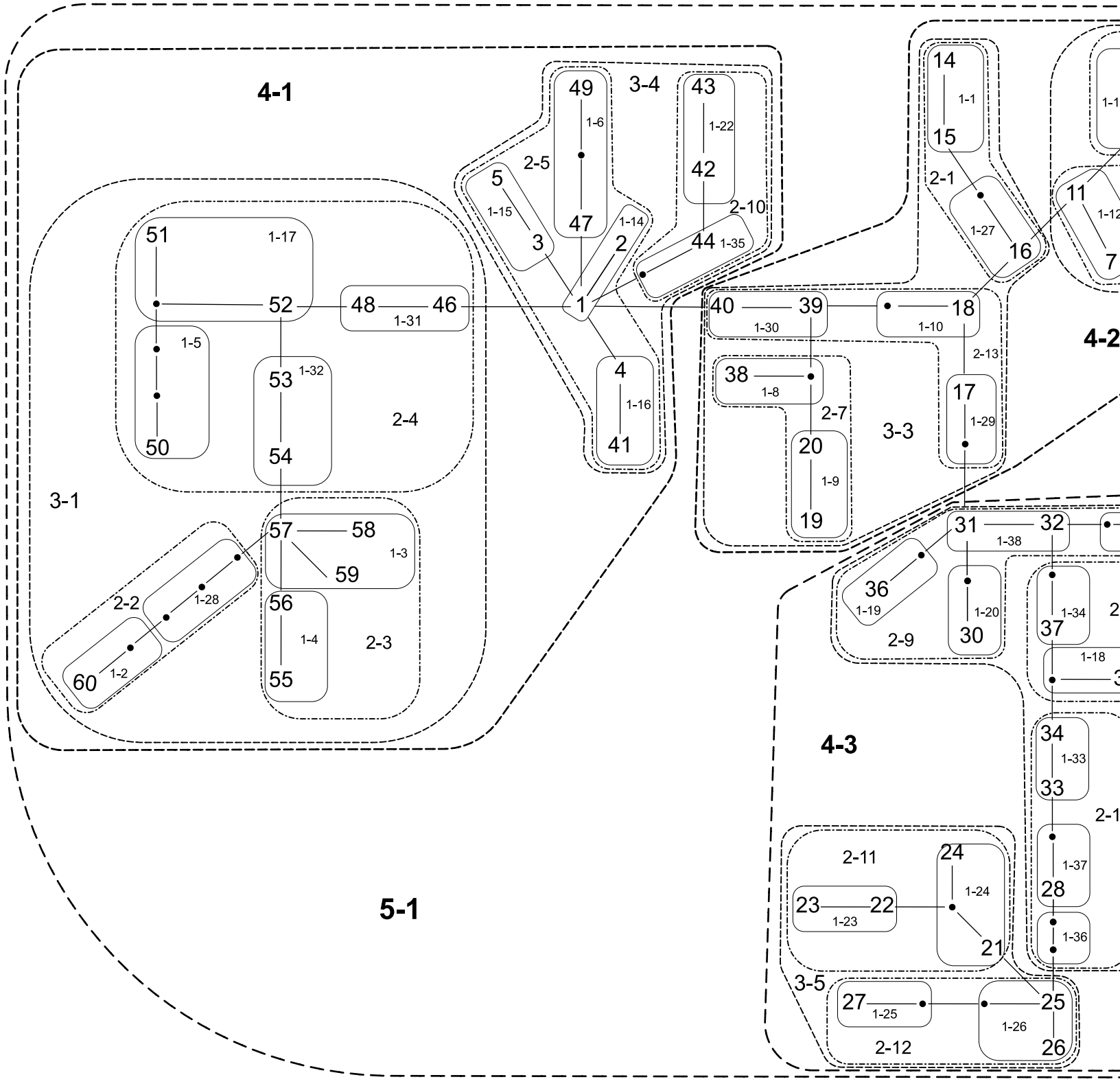
Figure 5. Maps of surface currents in the Philippine islands during: A) Winter and early Spring (Dec-Feb), B) Late Spring (Mar-May) C) Early Summer (June-July), D) Late Summer/ Fall (Aug-Nov). Adapted from Wyrtki 1961.

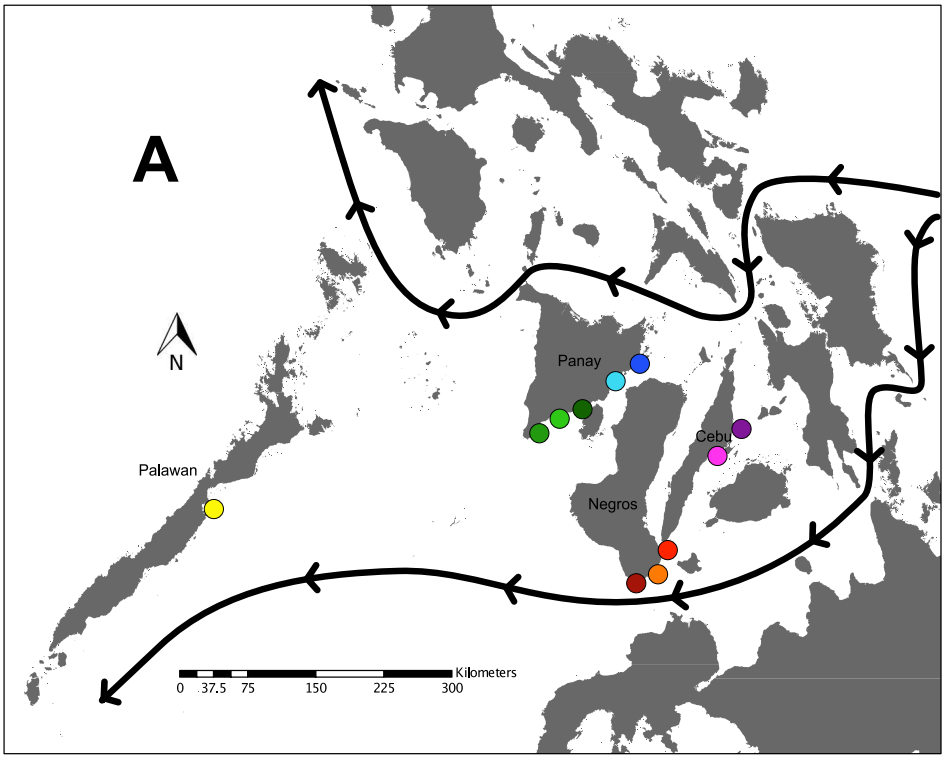

Seafdec
Santo Niño Sur
Parara
San Juan Barotec
Banate
Dumagete
Siliman
Sibulan
Magellan Bay
Cordova
Palawan
Panay
Negros
Cebu
N
0 37.5 75 150 225 300
Kilometers

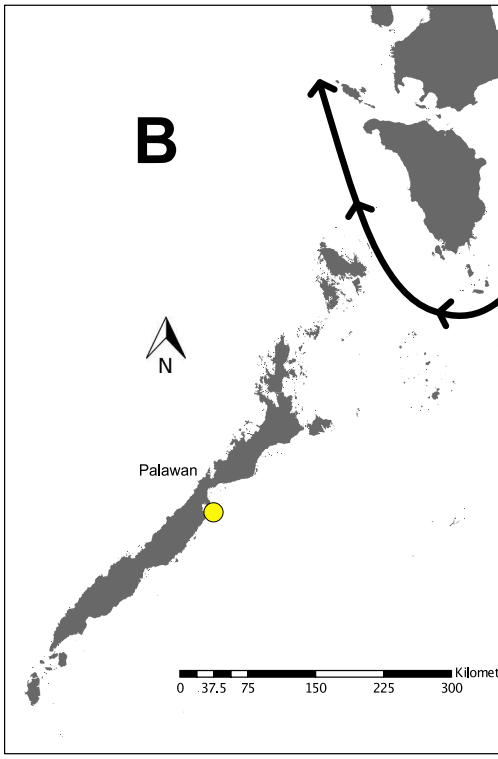

34
20
1-21
18
15
17
1-18
2-11
16
1-19
19
1-17
2-10
3-8
29
25
2-14
23
30
28
26
1-12
27
1-13
2-8
3-6
24
1-11
31
1-14
14
1-20
3-7
22
21
1-16
33
32
1-15
2-9
4-7

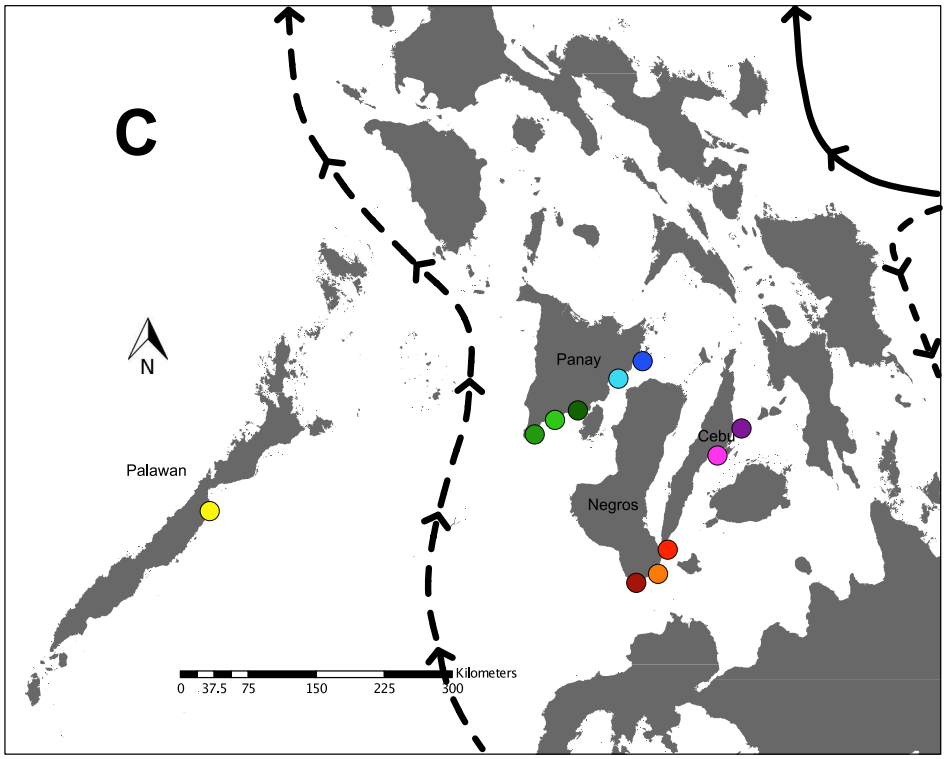

2
1-2
5
6
3
2-2
1
1-3
1-4
4
7
1-5
8
9
1-1
2-1
2-3
11
10
1-6
3-1
4-1

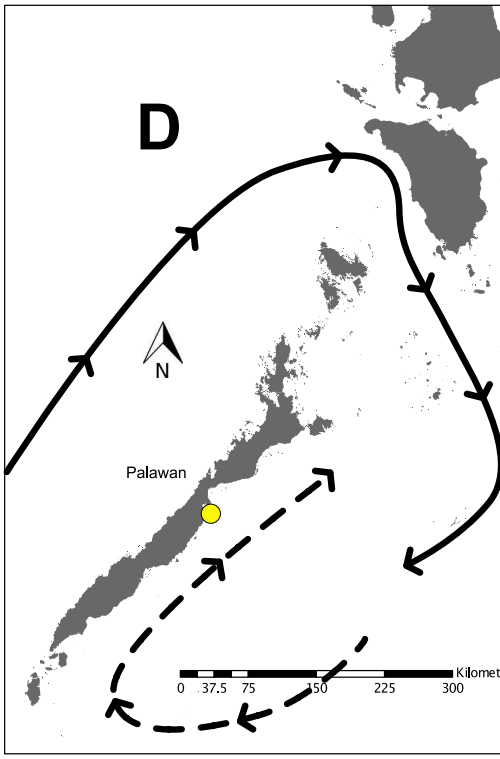

49
5
43
3
47
2
42
44
14
13
7
12
15
11
16
51
40
52
48
46
39
18
38
1
20
17
53
19
50
54
31
32
4
36
30
41
37
35
57
34
58
33
24
59
23
22
28
56
21
55
25
27
60
26

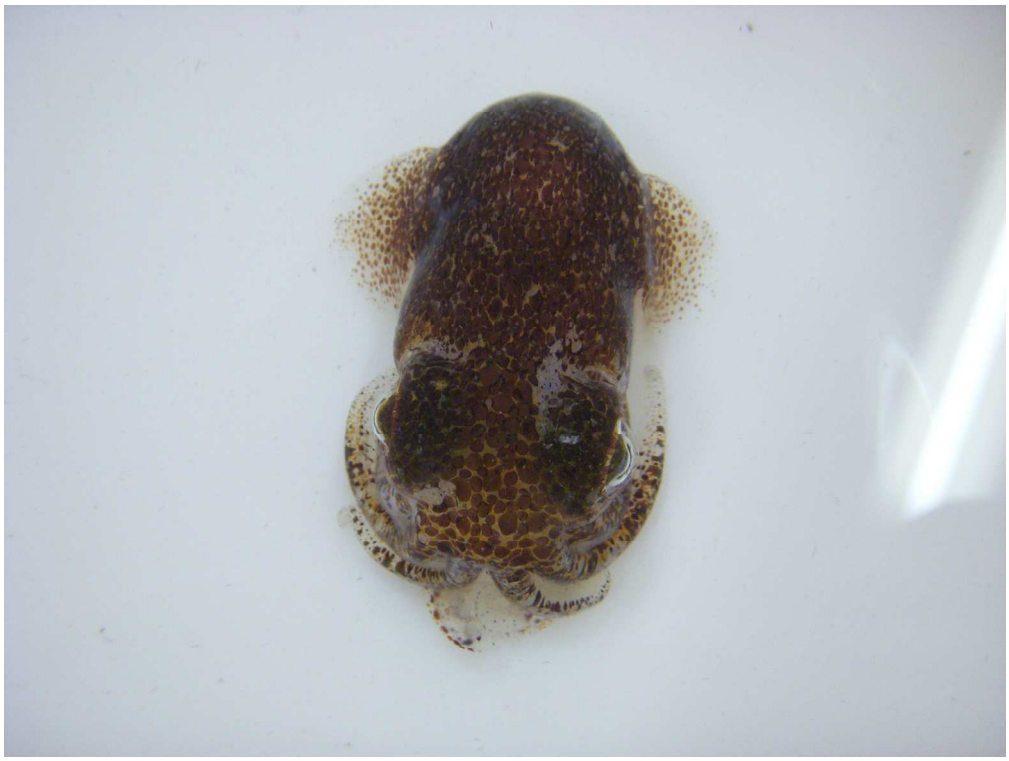

4-1
3-4
49
1-6
43
1-22
2-5
42
5
1-15
47
2-10
1-14
3
2
44
1-35
51
1-17
52
48
46
1-31
1
40
39
1-30
18
1-10
4-2
1-5
53
1-32
4
1-16
38
1-8
2-13
2-4
41
2-7
17
1-29
3-3
50
54
20
1-9
3-1
19
57
58
1-3
59
31
32
1-38
2-2
1-28
56
36
1-19
1-20
1-34
37
2-9
30
1-4
2-3
1-18
60
1-2
55
4-3
34
1-33
33
2-11
24
1-37
1-24
23
22
1-23
28
1-36
21
3-5
27
1-25
25
1-26
2-12
26
5-1


A
N
Panay
Cebu
Palawan
Negros
0 37.5 75 150 225 300
Kilometers


B
N
Palawan
0 37.5 75 150 225 300


C
N
Panay
Cebu
Palawan
Negros
0 37.5 75 150 225 300
Kilometers


D
N
Palawan
0 37.5 75 150 225 300



276x207mm (300 x 300 DPI)

Condensed title: Blood Vessels of the Cephalopod Light Organ

VASCULAR ARCHITECHTURE IN THE BACTERIOGENIC LIGHT ORGAN OF *EUPRYMNA TASMANICA* (CEPHALOPODA: SEPIOLIDAE)

A. J. Patelunas and M. K. Nishiguchi

Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, New Mexico, 88003-8001

Corresponding author: nish@nmsu.edu

Abstract

Symbiosis between Southern dumpling squid, *Euprymna tasmanica* (Cephalopoda: Sepiolidae) and its luminescent symbiont, *Vibrio fischeri*, provides an experimentally tractable system to examine interactions between the eukaryotic host and its bacterial partner. *V. fischeri* luminescence provides light for the squid in a behavior termed "counter-illumination," which allows the squid to mask its shadow amidst downwelling moonlight. Although this association is beneficial, light generated from the bacteria requires large quantities of oxygen to maintain this energy consuming reaction. Therefore, we examined the vascular network within the light organ of juvenile *E. tasmanica* with and without *V. fischeri*. Vessel type, diameter, and location of vessels were measured. Although differences between symbiotic and aposymbiotic squid demonstrated that the presence of *V. fischeri* does not significantly influence the extent of vascular branching at early stages of symbiotic development, these finding do provide an atlas of blood vessel distribution in the organ. Thus, these results provide a framework to understand how beneficial bacteria influence the development of a eukaryotic closed vascular network and provide insight to the evolutionary developmental dynamics that form during mutualistic interactions.

Key words: symbiosis; squid; vasculature; aerobic.

Abbreviations:

Apo= aposymbiotic

Sym= symbiotic

Symbiotic relationships between bacteria and multicellular organisms are very common in nature (Baker 2003; Hirsch and McFall-Ngai 2000; Wang et al. 2011). Beneficial symbioses occur when both members of the association benefit from their interactions with each other. These mutualistic relationships are observed in all major taxa including bacteria, plants, fungi and metazoans (Leigh 2010; Wang et al. 2011; Zamborsky and Nishiguchi 2011). The relationships are highly integrated through their physiologies, metabolic capabilities, and genetic mechanisms that control either the symbiont, or are responsible for host changes to accommodate the symbiont (Bentley et al. 2013; Nadal and Paszkowski 2013; Tschaplinski et al. 2014). Additionally, presence of symbiotic organisms can influence developmental changes in the host, providing evidence of long-term coevolution between the partners (Bergsma and Martinez 2011; Dunlap et al. 2014; Koropatnick et al. 2014). For example, sepiolid squids (Cephalopoda: Sepiolidae) exemplify major morphogenic, biochemical, and physiological changes during infection and colonization by *Vibrio* bacteria from the environment (Foster et al. 2000; Koropatnick et al. 2014; Montgomery and McFall-Ngai 1998). Many of these changes are thought to prevent further colonization by additional environmental symbionts (McFall-Ngai et al. 2010; Nyholm and McFall-Ngai 2004; Nyholm and Nishiguchi 2008; Wier et al. 2010).

Changes in development can be observed and characterized by comparing symbiotic animals to aposymbiotic animals (Claes and Dunlap 2000; Foster et al. 2000; Koropatnick et al.

2004; Sycuro et al. 2006). Extensive vasculature in the light organ of the squid, *Euprymna scolopes,* was observed in earlier studies (McFall-Ngai and Montgomery 1990; Nyholm et al. 2009), but not mapped in detail. Although no hemocytes were tracked in this study, knowledge of how the vasculature develops may provide insight into where hemocytes enter the organ (as previously observed), or how they move to the crypts from the white body where they are produced (Kremer et al. 2014; Nyholm et al. 2009; Schwartzman et al. 2015).

Once infection has occurred by specific *Vibrio* symbionts, the colonized light organ illuminates ventrally to match downwelling moonlight, camouflaging the squid from other benthic organisms during the night. (Jones and Nishiguchi 2004). At dawn, the squid vents 90-95% of the bacteria from the organ into the surrounding seawater, with the remaining 5% re-colonizing the light organ throughout the day. This cycle is repeated daily for the life of the squid. Thus, squids benefit from the bioluminescence of their bacterial symbionts. The host squid in turn provide *Vibrio* bacteria with nutrients, and as a result they grow at least four times as fast in the squid light organ as they do in natural seawater (Lee and Ruby 1994), thereby increasing their overall fitness (Graf and Ruby 2000; Lee and Ruby 1994). While inside the crypts, *V. fischeri* produces light which is regulated by expansion and contraction of the ink sac surrounding the light organ (Jones and Nishiguchi 2004). Production of luminescence via luciferase is aerobically intensive (Goto and Kishi 1968) and may capitalize on oxygen transferred through the squid's circulatory system. Thus, it is plausible that access to oxygen is a limitation to luminescence in the *Euprymna-Vibrio* symbiosis. Since very little is known about whether bacterial colonization induces change in the vasculature of sepiolid squids, the goal of this study was to determine the location and extent of vasculature between symbiotic and aposymbiotic light organs of *E. tasmanica*. Confocal scanning laser microscopy (CSLM) as well

as autofluorescence and Sca*l*e clearing agents (Hama et al. 2011) were used to examine the network of blood vessels in both aposymbiotic and symbiotic squids. The number of branching points (nodes) in each light organ were counted to analyze variation in branching. Diameters of vessels were measured and categorized to determine if there was a clear difference in vasodilation.

Materials and methods

Animal collection and care

Adult *Euprymna tasmanica* were collected from Botany Bay, New South Wales, Australia with permits from the Australian Government, Department of Sustainability, Environment, Water, Population, and Communities (Export permit WT2013-10343), the New South Wales Government, Industry and Investment (Collection permit P04/0014-6.0), and the Australian Government Department of Agriculture, Fisheries, and Forestry Biosecurity (AQIS invoice ELS0016507329). *E. tasmanica* was chosen as the model for this study due to its robustness and ease of rearing in captivity when compared to other species of sepiolids. Animals were transported back to NMSU and kept alive at the NMSU squid facility. Lab raised juvenile *Euprymna tasmanica* were maintained up to 72 hours (3-days) post hatch in 5 mL autoclave sterilized (34 ppt) artificial seawater (Instant Ocean©) in glass scintillation vials (one animal per vial; Nabhitabhata and Nishiguchi, 2014). Symbiotic animals were infected with 5000 colony forming units (CFU) mL^{-1} of *V. fischeri* ETBB 10-1. Animals were maintained with fresh sterilized seawater every 12 hours. Squids were kept on a 12/12 hour light/dark cycle. Colonization of symbiotic animals was confirmed via presence of luminescence in a Berthold Sirius luminometer (Bethold Technologies, Bad Wildbad, Germany).

Animals maintained for longer than 72 hours were raised in 34 ppt autoclave sterilized artificial seawater (Instant Ocean©). One mysid shrimp per squid was placed in a 3 L glass bowl overnight for feeding. Prior to feeding, mysid shrimp were rinsed with autoclaved sterilized DI water, and then maintained in DI water until being placed in the bowl. Any remaining mysid shrimp were removed from the bowl the following morning. Colonization was confirmed via presence of luminescence. For the aposymbiotic tank, 5 μg mL^{-1} of chloramphenicol was added once per week to prevent contamination.

Confocal microscopy

Juvenile squids were appropriately handled with care and under appropriate conditions to minimize any suffering (Nabhitabhata and Nishiguchi 2014). Juveniles were anesthetized on ice for 30 minutes in autoclaved sterilized artificial seawater then fixed with 2.5% glutaraldehyde in 0.1 M imidazole buffer for 48 h. After fixation, animal tissue was initially cleared with Sca*l*eA2 (4 M urea, 10% glycerol, 0.1% Triton X-100) for 2 days, transferred to Sca*l*eB4 (8 M urea, 10% glycerol, 0.1% Triton X-100) for an additional 2 days, and stored in Sca*l*eU2 (4 M urea, 30% glycerol, 0.1% Triton X-100) at room temperature until imaging (Figs. 1A-B; (Hama et al. 2011). Clearing involves making tissue transparent and matching the refractive index throughout the tissue to the refractive index of the immersion media. Mantle cavities were opened from the ventral side and the animal was decapitated directly behind the eyes with a stainless steel razor blade. Whole mantles were opened and placed ventral side down on a 35 mm MatTek glass-bottom dish (MatTek Corporation, Ashland, MA), embedded in 80% glycerol, and covered with a glass coverslip. Dissection, animal orientation, and stereoscopic imaging were completed on a Leica M165FC (Leica Microsystems, Wetzlar, Germany) stereofluorescent microscope.

Autofluorescence was imaged using a laser scanning confocal microscope (Leica TCS SP5 II, Leica Microsystems, Wetzlar, Germany), with a 405 nm UV laser diode, a 488 nm argon-ion laser, and a 561 nm DPSS laser. Autofluorescent spectra for squid tissues were determined by generating λ scans for all available laser lines (Fig. 1C). A 40x 1.25 NA plan-apochromatic oil immersion lens was combined with linear compensation of the photomultiplier tubes (PMT) and acousto-optic tunable filters (AOTF), frame averaging, and stage initiated tiling to generate a high-resolution mosaic of 3 µm optical sections. Blood vessels were identified by comparing region of interest (ROI) emission spectra with known vasculature in the gills (Fig. 2). Different from the surrounding tissue, the ROI spectra inside gill vessels match the ROI spectra inside blood vessels of the light organ. This information was used to infer which pieces of anatomy were blood vessels based on spectra and structural characteristics (tubular, branching, networking structures). Post-processing for confocal images was completed using ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, California, USA).

Morphometric analysis

Diameters were measured manually before and after branch points with the straight-line tool in ImageJ and values were calculated for mean and standard error (SE; (Cartana et al. 2012). Measurements were taken from XY optical sections, and XZ and YZ orthogonal projections. Vessel branches were marked and counted to determine the number of nodes. A minimum of three animals were used for each treatment for biological replicates. All vessels that could be traced were visualized for each animal. Animals were collected and fixed at noon for each of the respective timepoints. A one-way analysis of variance (ANOVA) was used to compare

treatments at the same time point, and the same treatment between time points. *P*-values were corrected with the Holm-Bonferroni test (Holm 1979).

Results

Confocal microscopy

Vessels were divided into four categories based on position relative to the light organ and size: L, the large vessel that runs posterior to anterior; MA, the anterior branch from L going into the lobe; MP, the posterior branch from L going into the lobe; and S, the branches from MA or MP. Lobe refers to one half of the bilobed light organ. Figures 3 and 4 show the position of each of these vessels. L was consistently on the ventral surface of the light organ running proximally posterior to anterior directly under the hindgut on either side. MA and MP form a V branching anterior and posterior, respectively. S vessels were long and branch in no particular pattern. These vessels wrapped around the deep crypts (Figs. 4B, D, F, H, J, L).

Measurements

Comparison by ANOVA of aposymbiotic and symbiotic over three timepoints demonstrated largely insignificant differences, however this analysis provided a descriptive map to the vascular network. The largest trends observed were increases in vessel size at the earliest (1-day) and latest (2-week) timepoints observed. Increases in node number happened between each timepoint as expected during the early stages of development.

In detail: 1-day right lobe L, MA, and MP values are shown in Figure 5 and Table 1 where no differences were observed. Similarly, when 4-day aposymbiotic and symbiotic L, MA, and MP values were evaluated, no differences were found. However, when S measurements were

evaluated for 1-day samples, a significant difference was identified between symbiotic and aposymbiotic animals in the left lobe. Conversely, differences in the number of nodes was observed in the 4-day samples, but not in the 1-day specimens. No major difference was measured in 2-week S vessels, but all other vessels (L, MA, MP) exhibited an increased size in 2-week symbiotic animals.

A comparison of 1-day aposymbiotic and symbiotic left lobes MA and MP were not significantly different (Table 2), but the 1-day L were significantly larger in symbiotic animals. S values of the 1-day specimens were similar to the right lobe. The number of nodes in the left lobe of 1-day samples were not similar.

Four-day samples in the left lobe exhibit no difference between any of the measurements and/or treatments. Comparison of time points within treatments had different results. When the 1-day aposymbiotic animal lobes were compared with the 4-day aposymbiotic animal lobes, MA diameter was not significantly different (P= 0.13 for R and P= 0.95 for L) but the number of nodes were significantly greater in the right lobe. Changes from 1-day symbiotic animal S vessels to 4-day symbiotic animal S vessels were also significant (P= 0.0003 for R and P =0.003 for L) demonstrating fast growth.

When comparing the 2-week specimens, a significant increase in size was observed in the symbiotic L vessels when compared with the aposymbiotic. Most interestingly were the changes from the 4-day samples to the 2-week samples within treatment. Aposymbiotic squid exhibited no significant changes in the number of nodes (P= 0.09 for R and 0.04 for L), but symbiotic animals significantly increased the number of nodes (P= 0.00001 for R and P= 0.003 for L). No other significant differences were consistent between lobes.

Discussion

Oxygen delivery to *V. fischeri* symbionts inhabiting the light organ is crucial for understanding the biochemical reactions that produce luminescence that are necessary for a successful mutualism. Therefore, this study examined the extent of vascularization in the developing bacteriogenic light organ of *E. tasmanica* during early post-hatching development. Previous studies have demonstrated the presence of the blood vessels in adult squids (McFall-Ngai and Montgomery 1990; Nyholm et al. 2009) as well as anaerobic conditions inside the light organ (Ruby and McFall-Ngai 1999; Schwartzman et al. 2015; Wier et al. 2010). These studies provide evidence that the presence of *V. fischeri* in the light organ does not influence angiogenesis. Oxygen is required for the catalysis of luciferase during bioluminescence in *V. fischeri* (Goto 1968), and therefore needs to be present in large quantities to sustain bioluminescence in addition to aerobic metabolism of both *V. fischeri* symbionts and their host squid.

Although the number of branching events does not appear to be significantly different between aposymbiotic and symbiotic animals, there is significance between S vessel diameter in the 1-day sample's left lobe (Fig. 5; Table 1). Within the first 24 hours after inoculation, cell death occurs in the appendages and surface epithelial cells of the light organ (Foster et al. 2000; Koropatnick et al. 2014). Cells directly in contact with *V. fischeri* further differentiate, becoming cuboidal and increasing size (Lamarcq and McFall-Ngai 1998; Montgomery and McFall-Ngai 1994; Sycuro et al. 2006). Since this is a vital time point for symbiont initiation, vasodilation might be initiated by *V. fischeri*. Nitric oxide (NO), which is known to be a vasodilator, is released during the early stages of the symbiosis as a defense against non-symbiotic bacteria accumulation. However, NO is shown to steadily decrease after the initial inoculation of bacteria

(Davidson et al. 2004). In this study, the 4-day time point demonstrated no significant difference between any of the vessel diameters. Blood vessels will return to a resting diameter with a reduction in NO instead of being dilated as observed in 1-day symbiotic samples. This suggests that after symbiont establishment, delivery of substances in the hemolymph (nutrients, oxygen, hemocytes, *etc*.) is reduced to a pre-symbiotic state, similar to that of aposymbiotic animals. Alternatively, this difference could simply be an artifact of low sample size (n=3 for each set). Observations of how these vessels change with concentration of *V. fischeri* during the height of their infection (at night) and pre and post-venting (pre and post dawn), might give additional insight as to how specific vasodialators control size during different stages of the mutualism.

The lack of any difference in the number of nodes in the 1-day samples might be a result of developmental time. Angiogenesis induced by low-oxygen requires continual anaerobic stress (Shweiki et al. 1992). If anaerobic stress is induced by the presence of *V. fischeri*, it would be during the period of highest oxygen demand, or during luminescence. This period only lasts a maximum of ~12 hours during the night when *V. fischeri* are at their highest concentrations. In a 1-day sample, ~12 hours is too short of a period to generate differences in angiogenesis. Longer time points were examined to determine if developmental timing was influential on vascular growth (Fig. 4 E-L). A significant difference was found in the number of nodes for 4-day right lobes, but not for left lobes. This difference between lobes may be due to the low number of samples examined for each time point (n=3). *P* values for 4-day samples were vastly different. However, this is not the only measurement that exhibited such a difference between lobes.

Potential differences between the right and left lobe can be explained by asymmetric development in protostomes. Although it has not been extensively explored, left-right (LR) asymmetry during development has been observed in nematodes and pulmonate snails (Okumura

et al. 2008). Mollusca as a group has evolved and lost symmetry numerous times through evolutionary history. Among cephalopods, the conversion from symmetry to asymmetry is estimated to have changed at least five times with right and left bias evolving simultaneously (Palmer 1996). Light organs of the related congener *Semirossia* also share asymmetry in the ducts leading to the light organ (Boletzky 1970). This similarity may provide indirect evidence for the onset of morphological changes that induced development of the original light organ present in basal sepiolid squids (such as *Semirossia*), and lends the developmental foundation for a haven for bacteria, such as *V. fischeri*, to initiate a beneficial association (Nishiguchi *et al*., 2004; Naef, 1921).

Imaging of juvenile squids shows the posterior aorta branching to feed blood into the light organ. This pathway has been previously observed in *Semirossia tenera* (Boletzky, 1970). Afferent vessels enter the light organ on the ventral side (Fig. 3 and 4A-L) to either side of the hindgut. Our study confirms the vascular anatomical similarities in light organs between distantly related symbiotic squid (Nishiguchi et al. 2004). A decreased node number or lack of change in vessel diameter may compensate oxygen delivery by host squids elevating their heart rate. Future projects might investigate the rate or volume output of the systemic heart, since it may be an alternative way to deliver an increased supply of oxygen and nutrients to the light organ. Additionally, vessel diameter can be examined at varying *in vivo* time points to determine whether the diameter changes over time are due to the daily metabolic activity of the squids.

By examining morphological changes between aposymbiotic and symbiotic sepiolid squids, this study has provided further insights of how beneficial mutualisms are sustained. Additionally, by understanding the location and extent of vascular anatomy in the light organ, the role of hemocyte migration into the crypts can be tracked and analyzed. Other symbiotic systems

have demonstrated nutrient exchange between symbiotic partners (Baker 2003; Denison and Kiers 2011), and only recently has oxygen transfer in the sepiolid squid begun to be explored (Kremer et al. 2014). These results demonstrate that the *E. tasmanica* circulatory system develops the same in both symbiotic and aposymbiotic juveniles, regardless of *V. fischeri* colonization; however, the functional changes in the presence of *V. fischeri* remain to be determined. Live confirmation of vasodilation in the early stages of the symbiosis would provide additional information regarding the developmental ontogeny in a dependent aerobic system located in a dynamic beneficial association. A thorough understanding of how the vascular system adapts under symbiotic stress provides a mechanism for testing the innate immune response on an organismal scale, and an alternative hypothesis for the role of a circulatory system in maintaining beneficial associations such as vertebrate gut microbes.

Acknowledgements

The authors would like to thank P. H. Cook at the NMSU CURRL facility for help with all microscopy. This work was supported by NIH NIAID 1SC1AI081659, NIH NIAIDS 3SC1AI081659-02S1, and NSF IOS 074498 to M.K.N. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Biosafety Committee of New Mexico State University (Permit Number: 1306NMD20103) and under the guidelines of the NMSU's Institutional Animal Care and Use Committee (85-R-009 and OLAW A4022-01 and IACUC license 2013-029).

References

Baker AC. 2003. Flexibility and specificity in coral-algal symbiosis: Diversity, ecology, and biogeography of *Symbiodinium*. Annu. Rev. Ecol. Evol. Syst. 34:661-689.

Bentley K, Jones M & Cruys B. 2013. Predicting the future: Towards symbiotic computational and experimental angiogenesis research. Exp. Cell Res. 319:1240-1246.

Bergsma GS & Martinez CM. 2011. Mutualist-induced morphological changes enhance growth and survival of corals. Mar. Biol. 158:2267-2277.

Boettcher KJ & Ruby EG. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. J. Bacteriol. 177:1053-8.

Boletzky SV. 1970. On Presence of Light Organs in *Semirossia* Steenstrup, 1887 (Mollusca - Cephalopoda). Bull. Mar. Sci. 20:374-&.

Cartana T, Saftoiu A, Gruionu LG, Gheonea DI, Pirici D, Georgescu CV, Ciocalteu A & Gruionu G. 2012. Confocal Laser Endomicroscopy for the Morphometric Evaluation of Microvessels in Human Colorectal Cancer Using Targeted Anti-CS1 Antibodies. PLoS One 7.

Claes MF & Dunlap PV. 2000. Aposymbiotic culture of the sepiolid squid *Euprymna scolopes*: Role of the symbiotic bacterium *Vibrio fischeri* in host animal growth, development, and light organ morphogenesis. J. Exp. Zool. 286:280-296.

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L & McFall-Ngai MJ. 2004. NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. Cell Microbiol. 6:1139-1151.

Denison RF & Kiers ET. 2011. Life Histories of Symbiotic Rhizobia and Mycorrhizal Fungi. Curr. Biol. 21:R775-R785.

Dunlap PV, Takami M, Wakatsuki S, Hendry TA, Sezaki K & Fukui A. 2014. Inception of bioluminescent symbiosis in early developmental stages of the deep-sea fish, *Coelorinchus kishinouyei* (Gadiformes: Macrouridae). Ichthyol. Res. 61:59-67.

Foster JS, Apicella MA & McFall-Ngai MJ. 2000. *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. Dev. Biol. 226:242-254.

Goto T. 1968. Chemistry of Bioluminescence. Pure Appl. Chem 17:421-442.

Goto T & Kishi Y. 1968. Luciferins Bioluminescent Substances. Angew. Chem.-Int. Edit. 7:407-&.

Graf J & Ruby EG. 2000. Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. Mol. Microbiol. 37:168-179.

Hama H, Kurokawa H, Kawano H, Ando R, Shimogori T, Noda H, Fukami K, Sakaue-Sawano A & Miyawaki A. 2011. Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. Nat. Neurosci. 14:1481-U166.

Hirsch AM & McFall-Ngai MJ. 2000. Fundamental concepts in symbiotic interactions: Light and dark, day and night, squid and legume. J. Plant Growth Regul. 19:113-130.

Holm S. 1979. A Simple Sequentially Rejective Multiple Test Procedure. Scand. J. Stat. 6:65-70.

Intaglietta M, Johnson PC & Winslow RM. 1996. Microvascular and tissue oxygen distribution. Cardio. Re. 32:632-643.

Jones BW & Nishiguchi MK. 2004. Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes*; Berry (Mollusca: Cephalopoda). Mar. Biol. 144:1151-1155.

Koropatnick T, Goodson MS, Heath-Heckman EA & McFall-Ngai M. 2014. Identifying the cellular mechanisms of symbiont-induced epithelial morphogenesis in the squid-*Vibrio* association. Biol Bull 226:56-68.

Koropatnick TA, Engle JT, Apicella MA, Stabb EV, Goldman WE & McFall-Ngai MJ. 2004. Microbial factor-mediated development in a host-bacterial mutualism. Science 306:1186-1188.

Kremer N, Schwartzman J, Augustin R, Zhou L, Ruby EG, Hourdez S & McFall-Ngai MJ. 2014. The dual nature of haemocyanin in the establishment and persistence of the squid-vibrio symbiosis. Proc. Biol. Sci. Roy. Soc. 281:20140504.

Lamarcq LH & McFall-Ngai MJ. 1998. Induction of a gradual, reversible morphogenesis of its host's epithelial brush border by *Vibrio fischeri*. Infect. Immun. 66:777-785.

Lee KH & Ruby EG. 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. 60:1565-1571.

Leigh EG. 2010. The evolution of mutualism. J. Evol. Biol. 23:2507-2528.

McFall-Ngai M & Montgomery MK. 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda, Sepiolidae). Biol. Bull. 179:332-339.

McFall-Ngai M, Nyholm SV & Castillo MG. 2010. The role of the immune system in the initiation and persistence of the *Euprymna scolopes-Vibrio fischeri* symbiosis. Sem. Immunol. 22:48-53.

Montgomery MK & McFall-Ngai M. 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Eurpymna scolopes*. Development 120:1719-1729.

Montgomery MK & McFall-Ngai MJ. 1998. Late postembryonic development of the symbiotic light organ of *Euprymna scolopes* (Cephalopoda : Sepiolidae). Biol. Bull. 195:326-336.

Nabhitabhata J & Nishiguchi MK. 2014. *Euprymna hyllebergi* and *Euprymna tasmanica*. In: Cephalopod Culture. Iglesias J, Fuentes L, Villanueva R, eds. pp. 253-269. Springer Netherlands.

Nadal M & Paszkowski U. 2013. Polyphony in the rhizosphere: presymbiotic communication in arbuscular mycorrhizal symbiosis. Curr. Opin. Plant Biol. 16:473-479.

Nishiguchi MK, Lopez JE & von Boletzky S. 2004. Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. Evol. Dev. 6:41-49.

Nyholm SV & McFall-Ngai MJ. 2004. The winnowing: Establishing the squid-*Vibrio* symbiosis. Nat. Rev. Microbiol. 2:632-642.

Nyholm SV & Nishiguchi MK. 2008. The evolutionary ecology of a Sepiolid squid-*Vibrio* association: from cell to environment. Vie Et Milieu-Life Environ. 58:175-184.

Nyholm SV, Stewart JJ, Ruby EG & McFall-Ngai MJ. 2009. Recognition between symbiotic *Vibrio fischeri* and the haemocytes of *Euprymna scolopes*. Environ. Microbiol. 11:483-493.

Okumura T, Utsuno H, Kuroda J, Gittenberger E, Asami T & Matsuno K. 2008. The development and evolution of left-right asymmetry in invertebrates: Lessons from *Drosophila* and snails. Dev. Dyn. 237:3497-3515.

Palmer AR. 1996. From symmetry to asymmetry: Phylogenetic patterns of asymmetry variation in animals and their evolutionary significance. Proc. Natl. Acad. Sci. U. S. A. 93:14279-14286.

Ruby EG & McFall-Ngai MJ. 1999. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends Microbiol. 7:414-420.

Schwartzman JA, Koch E, Heath-Heckman EAC, Zhou L, Kremer N, McFall-Ngai MJ & Ruby EG. 2015. The chemistry of negotiation: Rhythmic, glycan-driven acidification in a symbiotic conversation. Proc. Nat. Acad. Sci. 112:566-571.

Shweiki D, Itin A, Soffer D & Keshet E. 1992. Vascular endothelial growth-factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843-845.

Sycuro LK, Ruby EG & McFall-Ngai M. 2006. Confocal microscopy of the light organ crypts in juvenile *Euprymna scolopes* reveals their morphological complexity and dynamic function in symbiosis. J. Morphol. 267:555-568.

Tschaplinski TJ, Plett JM, Engle NL, Deveau A, Cushman KC, Martin MZ, Doktycz MJ, Tuskan GA, Brun A, Kohler A & Martin F. 2014. *Populus trichocarpa* and *Populus deltoides* exhibit different metabolomic responses to colonization by the symbiotic fungus *Laccaria bicolor*. Mol. plant-microbe interact. 27:546-56.

Wang XR, Pan QA, Chen FX, Yan XL & Liao H. 2011. Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. Mycorrhiza 21:173-181.

Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiasse RP, Schaefer AL, Koroleva I, Splinter-BonDurant S, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo MD, Casavant TL, Soares MB, Cronan JE, Reed JL, Ruby EG & McFall-Ngai MJ. 2010. Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. Proc. Natl. Acad. Sci. U. S. A. 107:2259-2264.

Zamborsky DJ & Nishiguchi MK. 2011. Phylogeographical patterns among Mediterranean sepiolid squids and their *Vibrio* symbionts: Environment drives specificity among sympatric species. Appl. Environ. Microbiol. 77:642-649.

Figure Legends

Figure 1. Bright field imaging of a fixed and fixed then Sca*l*e cleared 24 hour juvenile *E. tasmanica* light organs and emission spectra for Sca*l*e cleared mantle tissue. **A**. Ventral view of an untreated juvenile light organ removed from the squid mantle cavity. Anterior portion of the animal is towards the top of the figure. **B**. Ventral view of a Sca*l*e cleared juvenile light organ removed from the squid mantle cavity. Anterior portion of the animal is towards the top of the figure. **C.** Emission spectra controls for squid muscle tissue exhibiting normalized mean intensity (y-axis) over wavelength in nm (x-axis). The laser line used for the spectrum is in the top right corner of each graph. Scale bar = 500 µm.

Figure 2. Region of interest (ROI) λ scans of blood vessels in the gills (A) and in the light organ (B) of a 12 day old *E. tasmanica*. **A**. XY optical section of the squid gill with the 405 nm laser. The white box indicates the ROI scanned for the emission spectrum. **B**. XY optical section of the central tissue in the light organ with the 405 nm laser. The white box represents the ROI in which the emission spectrum was measured. **C.** Emission spectra for scans in A (black line) and B (grey line). Scale bar = 100 µm.

Figure 3. A diagram proposing the orientation and hierarchy of blood vessels throughout the light organ as described in this paper. Posterior is to the top. SH= systemic heart; MA= mantle; GL= gill; LO= light organ; L= the largest vessel that bifurcates to each lobe of the light organ; MP= the posterior branch of the second tier of vessels; MA= the anterior branch of the second tier of vessels; S= the third tier of vessels that sprawls throughout the organ.

Figure 4. Optical sections of each light organ treatment observed**.** Light organs are oriented from a ventral view with posterior at the top. All images are a merger of laser lines 405 nm (blue), 488 nm (green), and 561 nm (red). Top column represents shallow optical section of the surface of the light organ and large vessel. Deep column represents the deep crypts and associated vessels. **A-D**. 1-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. **E-H**. 4-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. **I-L.** 2-Week aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. ap= anterior appendage; po= pore; cc= crypt Scale bar = 100 µm.

Figure 5. Vessels diameters in µm of light organ lobes comparing aposymbiotic and symbiotic animals in average ± standard error. Columns labeled at the top for the respective lobe. **A and F.** Largest vessel (L) diameters of 1-Day, 4-Day, and 2-week animals. **B and G.** MP diameters of 1-Day, 4-Day, and 2-week animals. **C and H.** MA diameters of 1-Day, 4-Day, and 2-week animals. **D and I.** Smallest vessel (S) diameters of 1-Day, 4-Day, and 2-week animals. **E and J.** Number of branch points (nodes) counted for each of the samples. Significance of $P < 0.5$ is indicated by asterisk (*).

Table 1. Measurements of each lobe for aposymbiotic and symbiotic juvenile squids. L= large vessels, MA= medium anterior vessels; MP=medium posterior vessels; S= small vessels

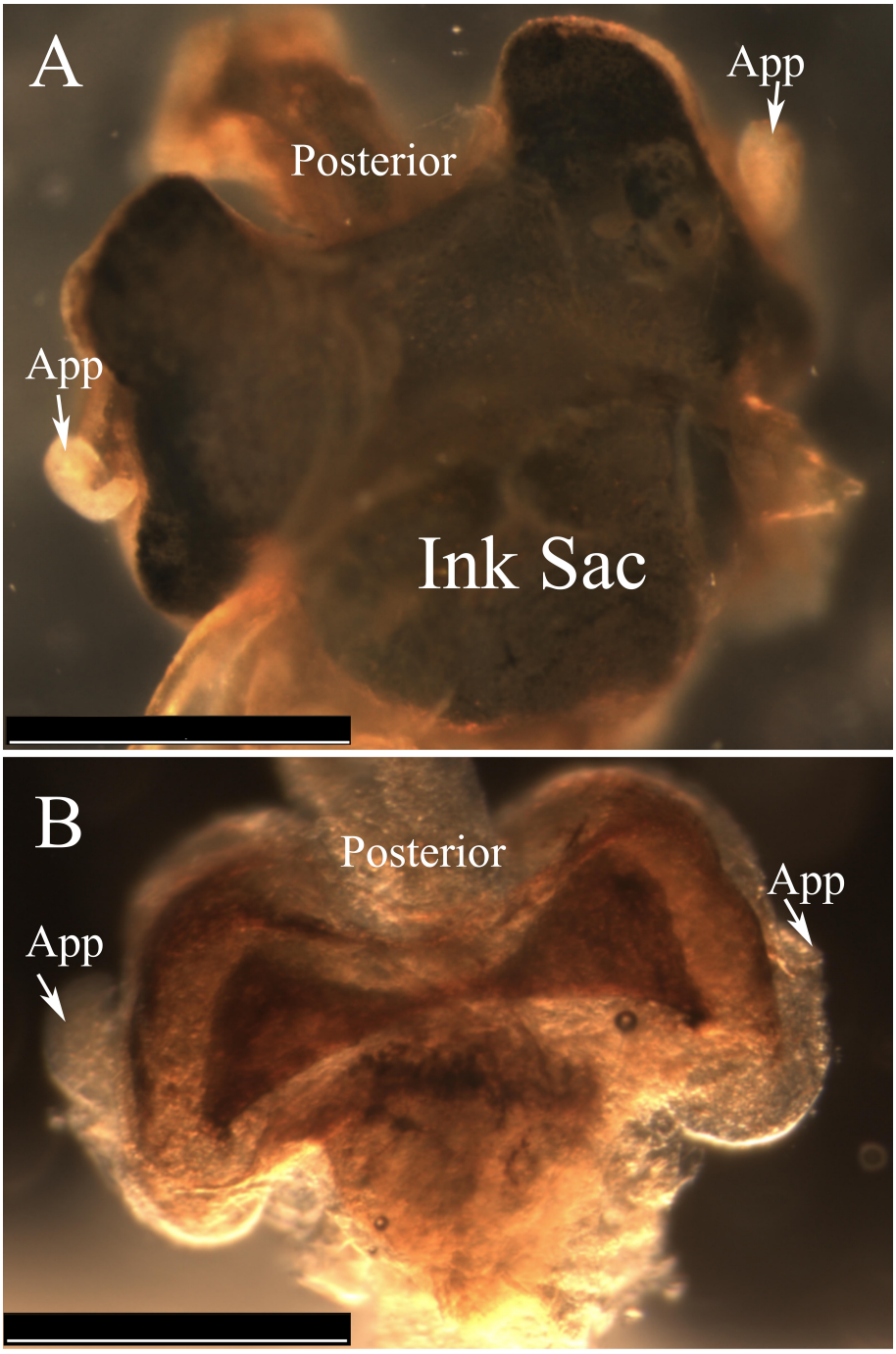
Diameters of blood vessels (µm)						
Right Lobe						
	1 day apo	**1 day sym**	**4 day apo**	**4 day sym**	**2 week apo**	**2 week sym**
N	3	3	3	4	3	3
L	30.02 ± 3.42	31.83 ± 4.32	29.46 ± 0.21	26.71 ± 2.32	20.92 ± 8.08	41.40 ± 2.89
MA	14.23 ± 0.11	11.97 ± 1.60	9.69 ± 3.12	9.71 ± 2.21	11.06 ± 1.91	14.98 ± 0.24
MP	14.05 ± 2.35	12.71 ± 0.87	9.95 ± 1.15	10.45 ± 0.84	11.78 ± 2.39	12.33 ± 2.22
S	5.17 ± 0.66	6.56 ± 0.40	4.75 ± 0.25	4.23 ± 0.23	4.15 ± 0.15	4.40 ± 0.16
Nodes	10.00 ± 0.18	8.33 ± 0.88	12.00 ± 0.58	8.25 ± 0.25	18.67 ± 2.91	17.67 ± 0.21
Left Lobe						
	1 day apo	**1 day sym**	**4 day apo**	**4 day sym**	**2 week apo**	**2 week sym**
N	3	3	3	4	3	3
L	24.28 ± 5.14	36.56 ± 3.85	28.38 ± 2.95	26.93 ± 3.24	30.12 ± 1.31	36.57 ± 5.36
MA	11.23 ± 1.05	12.15 ± 1.04	11.62 ± 1.65	8.82 ± 1.51	12.19 ± 1.56	11.37 ± 1.49
MP	13.12 ± 1.60	14.05 ± 1.82	11.06 ± 1.74	11.15 ± 1.16	10.46 ± 1.82	11.61 ± 2.07
S	4.59 ± 0.26	6.22 ± 0.51	4.02 ± 0.23	4.29 ± 0.25	3.97 ± 0.18	4.13 ± 0.17
Nodes	8.33 ± 0.23	9.33 ± 0.33	10.33 ± 1.20	10.00 ± 0.91	18.67 ± 2.60	18.33 ± 0.31

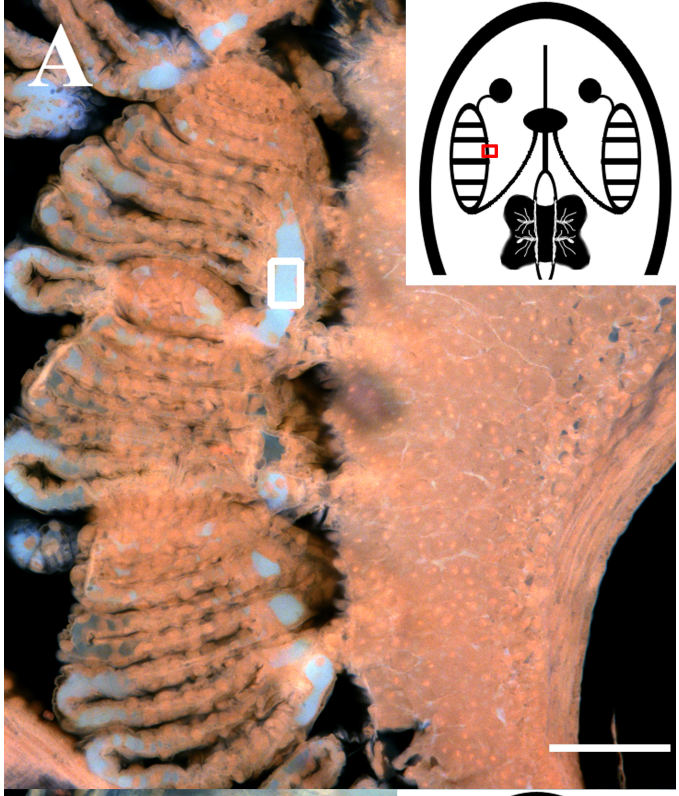
Table 2. *P* values designating comparison between aposymbiotic and symbiotic vessel sizes. Significant values are indicated by bolding and asterisk. L= large vessels, MA= medium anterior vessels; MP=medium posterior vessels; S= small vessels

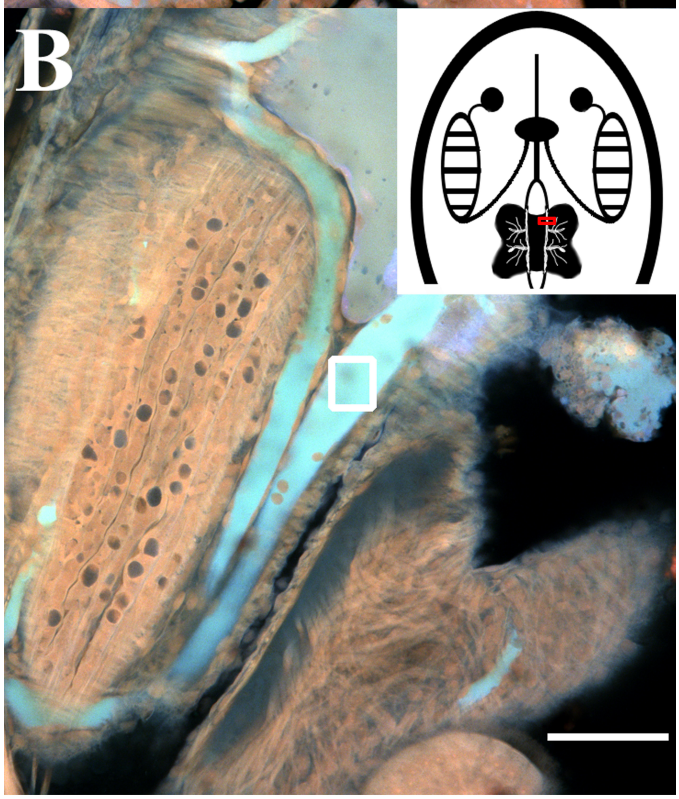
Right Lobe				Left Lobe			
Vessel	**Condition and Time**	**Compared to**	***P***	**Vessel**	**Condition and Time**	**Compared to**	***P***
L	1-Day Sym	4-Day Sym	0.31	L	1-Day Sym	4-Day Sym	0.11
L	1-Day Sym	1-Day Apo	0.76	L	1-Day Sym	1-Day Apo	0.13
L	4-Day Sym	2-Week Sym	**0.01***	L	4-Day Sym	2-Week Sym	0.16
L	4-Day Sym	4-Day Apo	0.36	L	4-Day Sym	4-Day Apo	0.76
L	2-Week Sym	2-Week Apo	**0.01***	L	2-Week Sym	2-Week Apo	0.31
L	1-Day Apo	4-Day Apo	0.88	L	1-Day Apo	4-Day Apo	0.52
L	4-Day Apo	2-Week Apo	0.13	L	4-Day Apo	2-Week Apo	0.62
MA	1-Day Sym	4-Day Sym	0.52	MA	1-Day Sym	4-Day Sym	0.15
MA	1-Day Sym	1-Day Apo	0.23	MA	1-Day Sym	1-Day Apo	0.57
MA	4-Day Sym	2-Week Sym	0.49	MA	4-Day Sym	2-Week Sym	0.81
MA	4-Day Sym	4-Day Apo	0.93	MA	4-Day Sym	4-Day Apo	0.27
MA	2-Week Sym	2-Week Apo	0.69	MA	2-Week Sym	2-Week Apo	0.84
MA	1-Day Apo	4-Day Apo	**0.05***	MA	1-Day Apo	4-Day Apo	0.85
MA	4-Day Apo	2-Week Apo	0.62	MA	4-Day Apo	2-Week Apo	0.31
MP	1-Day Sym	4-Day Sym	0.5	MP	1-Day Sym	4-Day Sym	0.22
MP	1-Day Sym	1-Day Apo	0.62	MP	1-Day Sym	1-Day Apo	0.72
MP	4-Day Sym	2-Week Sym	0.06	MP	4-Day Sym	2-Week Sym	0.82
MP	4-Day Sym	4-Day Apo	0.4	MP	4-Day Sym	4-Day Apo	0.96
MP	2-Week Sym	2-Week Apo	0.25	MP	2-Week Sym	2-Week Apo	0.72
MP	1-Day Apo	4-Day Apo	0.19	MP	1-Day Apo	4-Day Apo	0.43
MP	4-Day Apo	2-Week Apo	0.53	MP	4-Day Apo	2-Week Apo	0.75
S	1-Day Sym	4-Day Sym	**0.0003***	S	1-Day Sym	4-Day Sym	**0.001***
S	1-Day Sym	1-Day Apo	0.06	S	1-Day Sym	1-Day Apo	**0.02***
S	4-Day Sym	2-Week Sym	0.52	S	4-Day Sym	2-Week Sym	0.97
S	4-Day Sym	4-Day Apo	0.13	S	4-Day Sym	4-Day Apo	0.45
S	2-Week Sym	2-Week Apo	0.24	S	2-Week Sym	2-Week Apo	0.62
S	1-Day Apo	4-Day Apo	0.71	S	1-Day Apo	4-Day Apo	0.12
S	4-Day Apo	2-Week Apo	**0.03***	S	4-Day Apo	2-Week Apo	0.56
Nodes	1-Day Sym	4-Day Sym	0.85	Nodes	1-Day Sym	4-Day Sym	0.58
Nodes	1-Day Sym	1-Day Apo	0.19	Nodes	1-Day Sym	1-Day Apo	0.25
Nodes	4-Day Sym	2-Week Sym	**0.00001***	Nodes	4-Day Sym	2-Week Sym	**0.04***
Nodes	4-Day Sym	4-Day Apo	**0.0003***	Nodes	4-Day Sym	4-Day Apo	0.83
Nodes	2-Week Sym	2-Week Apo	0.76	Nodes	2-Week Sym	2-Week Apo	0.91
Nodes	1-Day Apo	4-Day Apo	0.07	Nodes	1-Day Apo	4-Day Apo	0.22
Nodes	4-Day Apo	2-Week Apo	0.09	Nodes	4-Day Apo	2-Week Apo	**0.003***

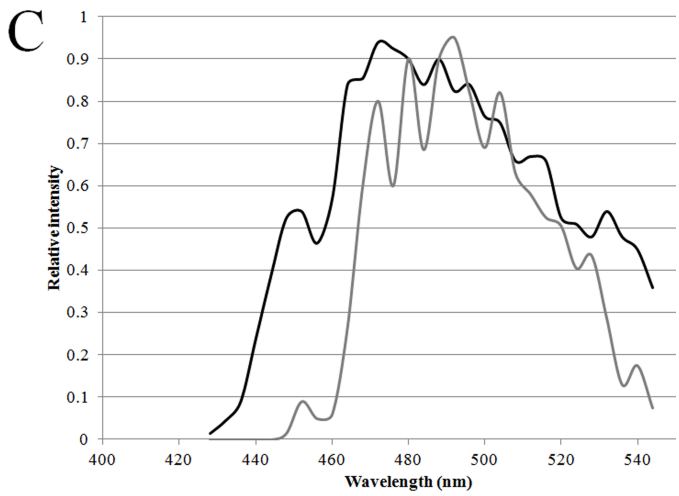
469

470


A
App
Posterior
App
Ink Sac
B
Posterior
App
App
C
1.0
0.8
0.6
0.4
0.2
0
Mean Intensity
400
500
700
800
Wavelength (nm)
405 nm
458 nm
488 nm
514 nm
561 nm
633 nm


A


B


C
Relative intensity
Wavelength (nm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
400
420
440
460
480
500
520
540

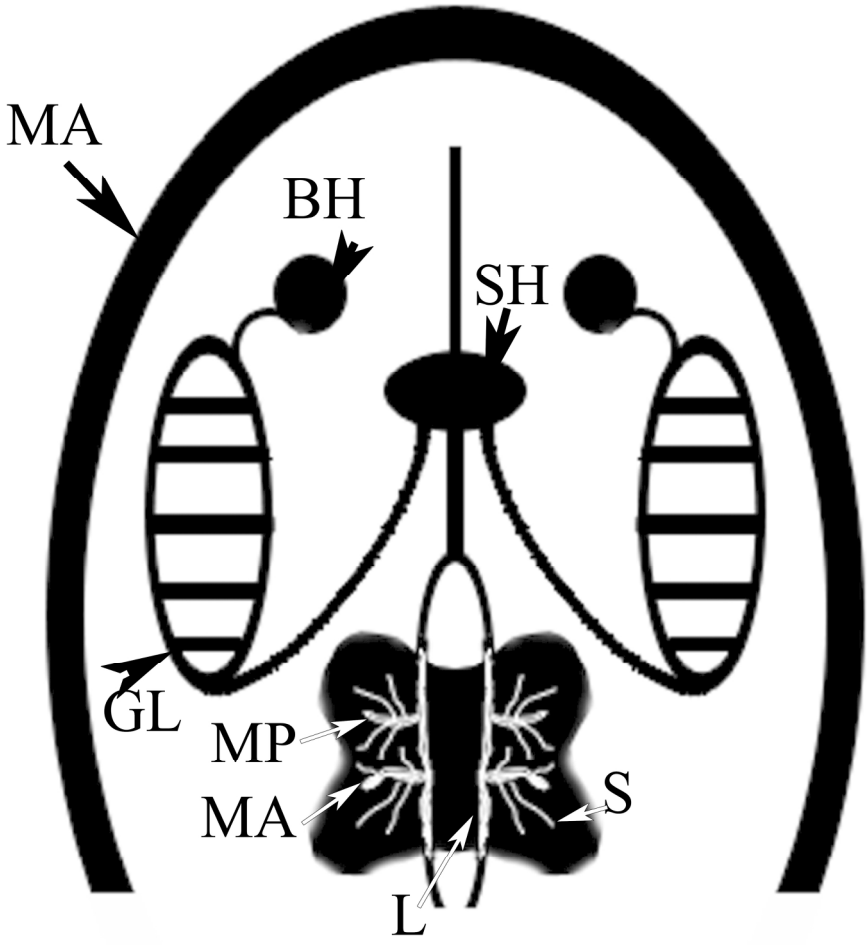


Figure 3. A diagram proposing the orientation and hierarchy of blood vessels throughout the light organ as described in this paper. Posterior is to the top. SH= systemic heart; MA= mantle; GL= gill; LO= light organ; L= the largest vessel that bifurcates to each lobe of the light organ; MP= the posterior branch of the second tier of vessels; MA= the anterior branch of the second tier of vessels; S= the third tier of vessels that sprawls throughout the organ.

96x93mm (600 x 600 DPI)

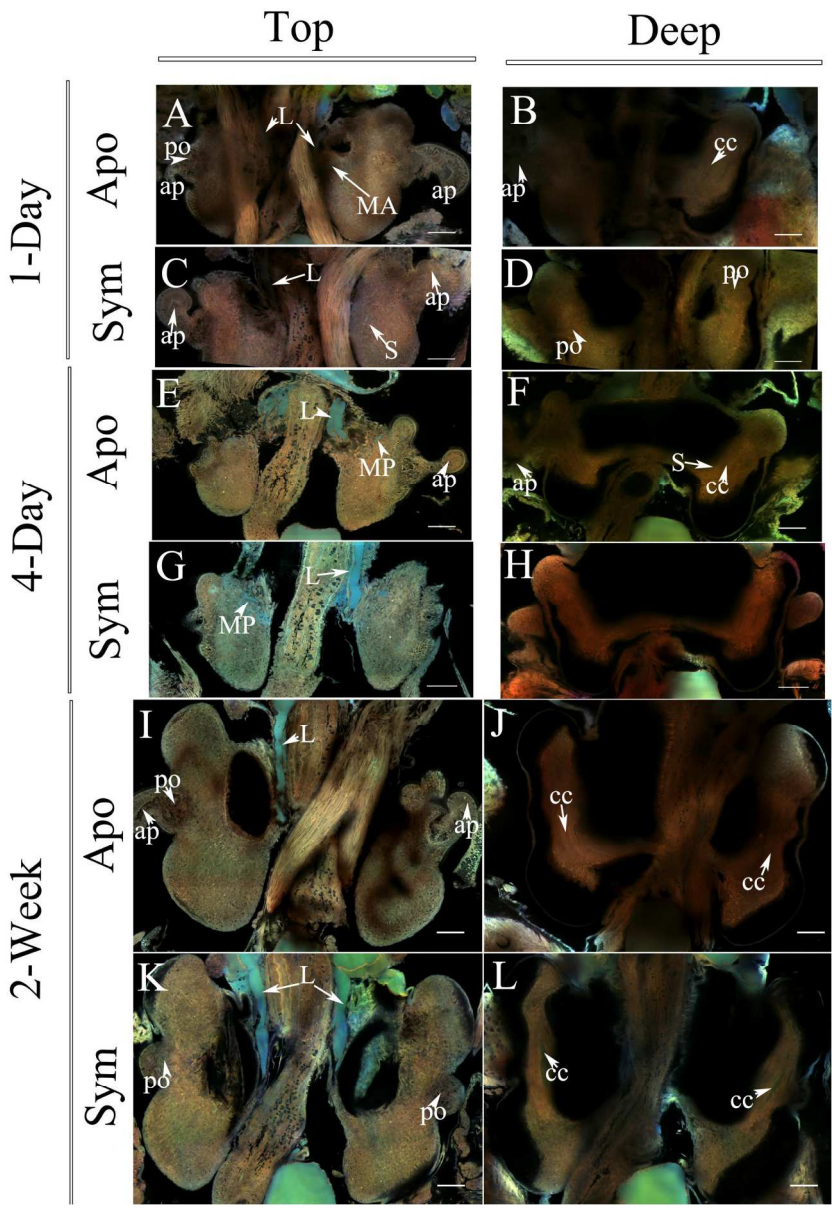


Figure 4. Optical sections of each light organ treatment observed. Light organs are oriented from a ventral view with posterior at the top. All images are a merger of laser lines 405 nm (blue), 488 nm (green), and 561 nm (red). Top column represents shallow optical section of the surface of the light organ and large vessel. Deep column represents the deep crypts and associated vessels. A-D. 1-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. E-H. 4-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. I-L. 2-Week aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. ap= anterior appendage; po= pore; cc= crypt Scale bar = 100 μm.

229x323mm (300 x 300 DPI)

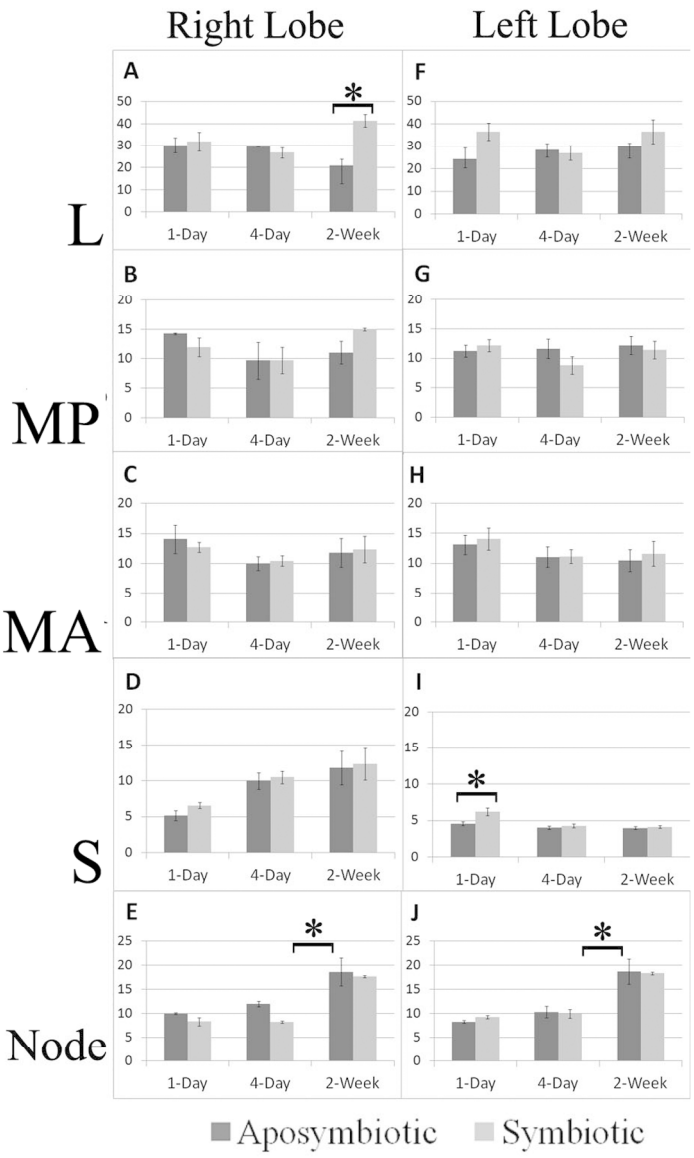


Figure 5. Vessels diameters in µm of light organ lobes comparing aposymbiotic and symbiotic animals in average ± standard error. Columns labeled at the top for the respective lobe. A and F. Largest vessel (L) diameters of 1-Day, 4-Day, and 2-week animals. B and G. MP diameters of 1-Day, 4-Day, and 2-week animals. C and H. MA diameters of 1-Day, 4-Day, and 2-week animals. D and I. Smallest vessel (S) diameters of 1-Day, 4-Day, and 2-week animals. E and J. Number of branch points (nodes) counted for each of the samples. Significance of P< 0.5 is indicated by asterisk (*).

170x291mm (600 x 600 DPI)