LIFE CYCLE EVOLUTION AND SYSTEMATICS OF CAMPANULARIID HYDROZOANS

By

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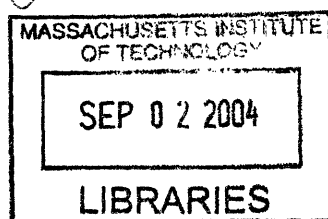
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Abstract

The purpose of this thesis is to study campanulariid life cycle evolution and systematics. The Campanulariidae is a hydrozoan family with many life cycle variations, and provide an excellent model system to study life cycle evolution. Additionally, the unique campanulariid *Obelia* medusae may have been "re-invented" from ancestors without medusae.

Chapter 1 reviews campanulariid life cycles and taxonomy. Chapter 2 presents a phylogeny based on 18S rDNA, calmodulin, 16S rDNA and cytochrome c oxidase I (COI). Ancestral life cycles are reconstructed using parsimony. Medusa loss is common, and *Obelia* may derive from ancestors with typical medusae.

Taxonomic results are discussed in Chapter 3. *Billardia*, a nominal campanulariid, appears phylogenetically distant, while *Bonneviella* spp. (Bonneviellidae), are nested within the Campanulariidae. Campanulariid genera are not monophyletic. *Orthopyxis integra* and *Clytia gracilis* may represent cryptic species, while *Obelia longissima* may be cosmopolitan.

Chapter 4 investigates *Obelia geniculata* phylogeography. Japanese and North Atlantic 16S rDNA and COI sequences are calibrated against the opening of the Bering Strait. Substitution rates are faster than in anthozoans and comparable to non-cnidarian invertebrates. Comparison of Pacific and Atlantic sequences suggests cryptic species exist. Finally, hydroids in New Brunswick, Canada and Iceland may have survived the last glaciation.

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Chapter 1

Introduction

The Hydrozoa

Cnidarians are known for their diversity of life cycles, and their evolution has been a subject of continuing debate for over a century (for example, Brooks 1886; Hyman 1940; Hadzi 1963; Schuchert 1993). Cnidarians are embodied by two basic forms: the polyp and the medusa. The Anthozoa have only the polyp stage, while the Scyphozoa, Cubozoa, and Hydrozoa (collectively termed the Tesserazoa; Salvini-Plawen, 1978 or the Medusozoa; Petersen, 1979) can have both the polyp and medusa stages. Based on a combination of molecular and morphological data, Bridge et al. (1992; 1995) showed that anthozoans are the basal cnidarians. The most parsimonious evolutionary scenario is that the ancestral cnidarian had a polyp-only life cycle, and that there was a single origin of the medusa in the ancestral medusozoan.

The Hydrozoa is a group of cnidarians that may have both the polyp and medusa or only one or the other. Life cycle variation is the hallmark of the Hydrozoa (Boero et al., 1997; Boero et al., 2002). In the typically-presented hydrozoan life cycle, the sexual adult medusa releases either sperm or eggs, which fertilize and form short-lived, lecithotrophic (non-feeding) planulae (Figure 1A). The planulae settle on to a substrate and form larval hydroids (Boero and Bouillon, 1987). The hydroids grow asexually, usually forming colonies. Through a complex process involving the entocodon, or medusary nodule (Boero et al., 1998; Boero et al., 2002), hydroids produce and release medusae, completing the cycle. In many hydrozoans, however, gametes are released from medusoids (with some medusa features but not others, and may or may not be released from the hydroid) or directly from the hydroid ("fixed gonophores" with no medusa features; Figure 1B). The Campanulariidae (Hydrozoa, Leptomedusa) exhibit all of these variations on life cycle, and thus provide an excellent model system for the study of life cycle evolution.

The Campanulariidae

The Campanulariidae (Leptomedusae) are an important, abundant, and widely distributed family of hydrozoans (Cornelius, 1982). Members of the Campanulariidae are common in planktonic and benthic environments around the world. The hydroids grow on a variety of substrates, including rocks, seaweed, bivalve shells, and pilings and floating docks at marinas, and most can be easily found in intertidal and shallow subtidal areas. Campanulariids can be ecologically important; for example, in Georges Bank, pelagic colonies of the hydroid *Clytia gracilis* are important competitors for cod larvae (Madin et al., 1996). Campanulariids have also been used as model organisms to study bioluminescence (e.g., Morin, 1974, Markova et al., 2002) and other aspects of physiology such as growth, aging, and stress responses (e.g., Toth, 1969; Brock, 1970; Brock, 1974; Stebbing, 1981; Stebbing and Santiago-Fandino, 1983; Stebbing, 1985; Crowell, 1991; but see Hughes, 1987).

Campanulariids are particularly interesting because members exhibit a variety of life cycles (Cornelius, 1982; Boero and Sarà, 1987; Boero et al., 1996), providing a model system to study life cycle evolution (Table 1). For example, *Clytia* and *Obelia* produce free medusae, *Orthopyxis* and *Silicularia* produce medusoids, *Gonothyraea* produce meconidia (unique medusoids never released from the hydroid), and *Campanularia* and *Laomedea* form fixed gonophores.

The campanulariid *Obelia*, frequently used as a model hydrozoan in introductory biology classes, is morphologically and developmentally unique among the entire Hydrozoa (Kühn, 1913; Chapman, 1968; Boero et al., 1996). Their unique features include: 1) the presence of hydroid-like chordal (solid), rather than medusa-like hollow, tentacles; 2) a "peduncled" manubrium (flared mouth) that resembles the hydroid hypostome (mouth); 3) the lack of a velum (inner ring of tissue around the bell margin) and true bell cavity resulting in a flattened shape; 4) statocysts positioned at the tentacle bases, rather than at the umbrellar margin; 5) lack of true tentacular bulbs; and 6) gonads that develop from the corners of the manubrium where the radial canals originate, rather

than directly from the radial canals (although they later migrate down the radial canals); and 7) cross-layered, rather than circular, myofibrils which are better suited to a flat, rather than the typical concave and craspedote form (Chapman, 1968). *Obelia* medusa development also differs from all other hydrozoan medusae, in that the entocodon or medusary nodule (a proliferation of ectoderm along the blastostyle that leads to the subumbrellar cavity) seems to disappear early in development (Kühn, 1913; Boero et al., 1996).

Campanulariid gonophore development

Gonophore development is relatively well-studied in the Campanulariidae (Berrill, 1950; 1961). "Gonophore" refers to the structures involved in sexual reproduction in species without medusae (Cornelius, 1995), but here the term is used to refer to the sexually reproducing body, regardless of whether it is released from, or fixed to, the hydroid. Typical medusa (e.g., *Clytia*) development involves the formation of an entocodon, or medusary nodule (Figure 2A). The entocodon is formed along a modified polyp, termed the blastostyle. In *Clytia*, medusae develop along only one side of the blastostyle. There is a thickening of the blastostyle epidermis, forming a nodule (the entocodon) between the apical peripheral epidermis and the adjoining endodermis. The entocodon invaginates to form a cavity which becomes the subumbrellar cavity in the medusa. The adjoining endodermal layer around the entocodon forms the radial canals, which eventually fuse at the distal end to form the circular canal of the medusa. The apical epidermis also gives rise to the tentacle chambers (note medusa tentacles are hollow), and a blastostyle protrusion of the endoderm and adjoining ectoderm into the nascent subumbrellar cavity eventually becomes the manubrium. As the blastostyle grows, the developing medusae are carried to the apical end of the gonotheca, and are liberated. Some unusual developmental variations are found in *Gastroblasta* (a nominal campanulariid genus now recognized as an aberrant *Clytia*), which is similar to *Clytia* except that the medusae have multiple manubria (mouths) and radial canals (Kramp, 1961; Boero, 1980). Another unusual variation is found in *Clytia mccradyi*, where

blastostyle and medusa development takes place on the radial canals of medusae, where the gonads are usually located, rather than in hydroid gonophores; indeed, the existence of a hydroid stage in this species has yet to be confirmed (Carré et al., 1995).

This process is modified in species with medusoids and fixed gonophores (Berrill, 1950; Boero and Bouillon, 1989). In *Orthopyxis*, which produces medusoids, the blastostyle buds off medusoids with a reduced or absent manubrium, which are eventually liberated with the already mature gametes on well-defined radial canals. The medusoids may possess a velum (Hirohito, 1969). Only one or two medusoids are produced from the blastostyle, and the degree of development can differ between the sexes. The medusoid bud has a large entocodon and gonadal mass (Berrill, 1950). Medusoids that are not liberated from the hydroid may be further reduced, remain in gonotheca, and gametes are on the rudimentary radial canals. In *Orthopyxis everta* medusoids are not liberated and females expel their eggs into an acrocyst or external capsule located on top of the gonotheca (Nutting, 1915). *Silicularia* also produces sexually dimorphic medusoids in which the eggs develop along the radial canals and the planulae are brooded in females (Ralph, 1956; Blanco, 1967). The medusoids lack a manubrium and tentacles. There are no records of *Silicularia* medusoids being released, although that possibility has not been ruled out (Vervoort and Watson, 2003).

The process of medusa formation is substantially different in *Obelia* (Figure 2B) (Kühn, 1913; Boero et al., 1996). An entocodon is formed early in development, but subsequently appears to be lost (Figure 2B). Therefore, no velum or true subumbrellar cavity is formed (Figure 3). Development resembles hydranth development (Boero et al., 1996). Thus, the *Obelia* medusa appears to be a chimera of the hydranth and medusa features (Boero et al., 1996). Additionally, medusa development differs from *Clytia* in that medusae can develop along all sides of the blastostyle, and the medusae are smaller and more numerous (Berrill, 1950).

Gonothyraea shares some of *Obelia*'s unusual features but not others. Weismann (1883) and Kühn (1913) describe entocodon formation, but it apparently does not disappear as in *Obelia* (Berrill, 1950). The entocodon is larger than in *Obelia* and

differentiates mainly into gonad (Berrill, 1961). Kühn's (1913) drawings indicate that the meconidia tentacles are solid, endodermal polyp-like tentacles as in *Obelia*. Female meconidia possess more, longer, and more contractile tentacles than male meconidia, which assist in internal fertilization by trapping sperm (Miller, 1973). Female meconidia also have radial canals, which are absent in the males. All meconidia lack a functional manubrium (mouth) (Miller, 1973) and Boero et al. (1996) consider it a spadix, the corresponding structure in fixed gonophores. In females, the hydrocoel currents into the abortive manubrium/spadix are maintained as the planulae develop, perhaps extending the meconidia lifespan (Berrill, 1950). *Gonothyraea* shares some similarities to *Clytia* in that medusae bud off from only one side of the blastostyle, and developing medusae are in between *Clytia* and *Obelia* in terms of number and size of medusa buds (Berrill, 1950).

In fixed gonophore-bearing forms , such as *Laomedea,* the gonophores are further reduced and lack most medusa features. Gametes surround the spadix. In *Laomedea*, gonophore development proceeds through a relatively large entocodon, which differentiates mainly into gonad, as in *Gonothyraea* (Berrill, 1961). The sporosacs rupture and release gametes or planulae while in the gonangium. As in *Obelia* but unlike *Gonothyraea* and *Clytia*, sporosacs bud from all sides of the blastostyle, and the number of buds is similar to *Obelia* although they may be larger. Details of development may vary between species. In *Laomedea flexuosa*, eggs remain in the female sporosac through development, fertilization, and planula development. In contrast *L. calceolifera*, eggs are "ovulated" from the sporosac but remain in the gonangium (Miller 1973). Interestingly, the monotypic *Hartlaubella* also "ovulates", but the eggs are pushed out of the gonangium and fertilization is external (Miller 1973). However, Vervoort and Watson (2003) report gonothecae containing developing planulae in New Zealand *H. gelatinosa*, so this may either be under environmental control or indicative of cryptic speciation. Another gonophore variation is found in *L. inornata* (*Gonothyraea inornata)* and *L. neglecta,* which produce external sacs, or acrocysts, that contain developing planulae (Nutting, 1915; Cornelius 1982; Chapter 3). Note the acrocysts in *L. inornata* are referred to as meconidia in the literature (as *Gonothyraea inornata*; Nutting 1901; Fraser 1946),

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but because they have no medusoid features they are more appropriately viewed as acrocysts (Nutting 1915; Chapter 3). Also only one acrocyst is extruded from the gonangium, in contrast to several meconidia in *Gonothyraea*.

Gonophore development can be, to some extent, influenced by the environment. In some *Orthopyxis integra*, whether or not medusoids are released is under environmental control; however, the factors involved are unclear although season and hydrodynamic conditions are implicated (Cornelius 1982). Another campanulariid example is *Clytia linearis*. In the summer, hydroids produce medusae which grow and reach sexual maturity through series of stages, but in the fall, medusae have mature gonads at birth, live only a few days, and do not attain adult somatic characteristics (Boero and Sarà, 1987). Cornelius (1990) considered reports that *Hartlaubella gelatinosa* could sometimes produce *Obelia*-type medusae instead of fixed gonophores, but the reports were inconclusive because of the possibility of misidentification due to difficulty distinguishing this hydroid from *Obelia bidentata*. Also as noted above, *H. gelatinosa* has been reported both to have internal (Vervoort and Watson, 2003) and external (Miller, 1973) fertilization; however, whether this is due to environmental factors or because they are actually different species remains to be seen. Finally, temperature could potentially affect medusa expression by affecting sex determination. Temperature has been shown to affect sex determination in *Clytia* (Carré and Carré, 2000). If this holds true generally, then temperature may consequently affect gonophore development, as many hydroids have sexually dimorphic gonophores, with the female exhibiting more medusoid features than the male.

Taxonomy

Until recently, rigorous hypothesis testing of life cycle evolution in the Campanulariidae and other hydrozoans has not been possible because of difficulty in classification and species identification. Hydrozoan taxonomy is challenging at both the species level and higher because of difficulties resulting from their life cycles, distinguishing homology, and phenotypic plasticity. Unless an organism has been

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cultured throughout its life cycle, it has not been possible to link the hydroid and medusa stages (although now DNA technology has the potential to do this). Difficulties arose because specialists frequently examined only the hydroid or medusa stage. Consequently, there are many examples where the hydroid and medusa of the same species were given different names (Cornelius, 1977; Cornelius, 1982). For example, among the Campanulariidae, some *Clytia* medusae were originally placed in a different genus, *Phialidium*, than their hydroids, and *Orthopyxis* medusae were originally placed in a different genus, *Agastra*, than their hydroids. Yet, even today, many species, including some campanulariids, remain described only from either the hydroid or medusa stage (Boero and Bouillon, 1993; Bouillon and Boero, 2000).

Gonophore (life cycle) type is used, in part, to define genera in the Campanulariidae and other hydrozoan families (e.g., Cornelius, 1982). However, if life cycle transitions occur often, then this approach could lead to paraphyletic genera (Boero et al., 1996). Recent studies in anthomedusan hydrozoans suggest this may be the case (Petersen, 1990 in the Tubulariidae; Cunningham and Buss, 1993 in the Hydractiniidae).

Another major difficulty challenging hydrozoan taxonomists is determining whether morphological differences are due to environmental or genetic causes (Cornelius, 1990). Early taxonomists described many new species based on small morphological differences. For example, Cornelius (1975) states that 70 species of *Obelia* were described between 1830 and 1948. More recently, authors have attributed many of these differences to environmental causes and combined many of the species. Accordingly, Cornelius (1990) only recognizes 4 species of *Obelia* in the eastern North Atlantic. However, other authors (Stepanjants, 1998; Kubota, 1999; Bouillon and Boero, 2000) recognize other species as valid in other parts of the world. In another example, there were 11 described species of *Silicularia*, distinguished primarily by variations in hydrothecal length. Ralph (1956), however, noted individual *Silicularia* colonies with hydrothecae spanning the range of sizes described for different species, and concluded that these differences were environmental and merged them into 3 species. As a result of

these and other redefinitions, many hydroids now appear to have near-cosmopolitan distributions.

Overall colony form and characteristics of the hydrotheca, or chitinous exoskeleton, are frequently used as taxonomic characters in thecate hydroids such as the Campanulariidae (Cornelius, 1982). However, these features may exhibit considerable phenotypic plasticity (Ralph, 1956), and this presents a major obstacle to their taxonomic utility. Despite this, these characters form the primary basis for species descriptions in the Campanulariidae. Alternatively, morphological characteristics of the nematocysts, or stinging capsules, can be taxonomically useful in many groups of hydroids, including campanulariids (Östman, 1979; Östman 1982; Östman, 1983; Gravier-Bonnet, 1987; Östman, 1987). But again, some nematocyst characters may be plastic (Östman et al., 1987) and they are time consuming and require live material to study.

Molecular genetic data have the potential to clarify both campanulariid taxonomy and evolutionary relationships, because markers relatively independent of life cycle and environment could be used. For example, Östman (1982; 1983) found inter- and intraspecific gel band differences for the enzyme acid phosphatase in Scandinavian campanulariids, and suggested that taken with other characters, isoenzyme banding patterns could be taxonomically useful. There have been no additional enzyme studies, and no studies using DNA sequences, to date on campanulariids. Collection of molecular genetic data, such as DNA sequences, and subsequent phylogenetic analysis has become standard for many taxa, and could be applied to campanulariid hydroids. DNA sequences could be used to both distinguish species by identifying patterns of reciprocal monophyly (Avise, 2000) and investigate higher level evolutionary relationships.

Phylogeography and mitochondrial evolution

Phylogeography examines the role of historical processes in shaping modern species distributions in a phylogenetic framework (Avise, 2000). Mitochondrial DNA is often used in phylogeographic studies of invertebrates, because it is evolving at a faster rate than nuclear DNA and often provides resolution at the intraspecific level (Avise,

2000). However, mitochondrial DNA may evolve exceptionally slowly in cnidarians, although previous studies are based primarily on anthozoans (Romano and Palumbi, 1997; Medina et al., 1999; Van Oppen et al., 1999; Shearer et al., 2002). The only study on a hydrozoan used a relative rate test on COI amino acid sequences, and found that the hydrozoan (Limnomedusa) *Maeotias* sp. was evolving significantly more slowly than echinoderms, molluscs, and arthropods (Shearer et al., 2000).

The North Atlantic is an excellent system for phylogeographic studies because the organisms and recent geological history are relatively well known. Much of the Atlantic fauna and flora is believed to have originated in the Pacific, approximately 3.5 million years ago (mya), with the opening of the Bering Strait (Durham and MacNeil, 1967; Van den Hoek and Breeman, 1990; Vermeij, 1991; Cunningham and Collins, 1998). This migration is referred to as the trans-Arctic interchange, and is useful because it provides an upper bound for divergence estimates (Wares and Cunningham, 2001), especially for taxa like the campanulariids, which are not well represented in the fossil record. Mitochondrial DNA sequences from North Atlantic and North Pacific sister taxa can be analyzed so that substitution rates could be calibrated, the mitochondrial markers can be evaluated for their phylogenetic utility, and the recent history of the species can be elucidated.

Thesis goals

The purpose of this thesis is to study life cycle evolution and systematics of the Campanulariidae. Chapter 2 presents a molecular phylogeny of the Campanulariidae, and tests hypotheses on life cycle evolution, including on the frequency of life cycle transitions and on the origin of the unique *Obelia* medusae. Chapter 3 discusses the taxonomic results of the molecular phylogeny presented in Chapter 2, and revises taxonomic diagnoses accordingly. Chapter 4 examines the mitochondrial evolution and phylogeography of the widely distributed campanulariid, *Obelia geniculata*. The first calibrated hydrozoan substitution rates are presented. Finally, the appendix examines

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species identification from molecular, morphological, and reproductive perspectives in the bivalve-inhabiting hydroid, *Eugymnanthea* (Hydrozoa, Leptomedusa, Eirenidae).

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Table 1. Campanulariid subfamilies and genera and their associated sexual stage. (gonophore type). Following Cornelius (1982), the three subfamily lineages are indicated. *Billardia*, recognized by some (e.g., Vervoort and Watson, 2003), but not others (e.g., Cornelius, 1982) as a campanulariid, is here listed under the Campanulariinae. Following Calder (1991) and Boero et al. (1996), *Tulpa* is placed in the Campanulariinae rather than the Clytiinae, as in Cornelius (1982).

Subfamily	Genus	Gonophore type	Description
Campanulariinae	*Orthopyxis*	Medusoids	With some medusa features (i.e., radial canals) but not others (i.e., tentacles), some with extracapsular development (acrocysts); may or may not be released from the hydroid
	Silicularia	Medusoids	With some medusa features (i.e., radial canals) but not others; not known to be released from the hydroid
	Campanularia	Fixed gonophores	Gonophores with no medusa features; fixed on the hydroid
	Rhizocaulus	Fixed gonophores	"
	Tulpa	Fixed gonophores	"
	Billardia	Fixed gonophores	"
Clytiinae	*Clytia*	Medusae	Typical hydrozoan medusae
Obeliinae	*Obelia*	Medusae	Atypical hydrozoan medusae
	Gonothyraea	Meconidia	With some medusa features (i.e., tentacles), but not others; medusa features similar to *Obelia*, rather than typical, medusae; not released from the hydroid
	Hartlaubella	Fixed gonophores	Gonophores with no medusa features; fixed on the hydroid
	Laomedea	Fixed gonophores	Gonophores with no medusa features; some with extracapsular development (acrocysts); fixed on the hydroid

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Figure 1. Hydrozoan life cycles, adapted from Naumov (1960). **A.** Life cycle with a free medusa stage. The hydroids produce and release medusae, which release gametes that fertilize and form planulae. The planulae settle and form hydroids. **B.** Life cycle where medusoids or fixed gonophores are produced instead of medusae. Medusoids may or may not be released from the hydroid. Fertilization may be internal, and planulae may be brooded within the medusoids or fixed gonophores.

A.
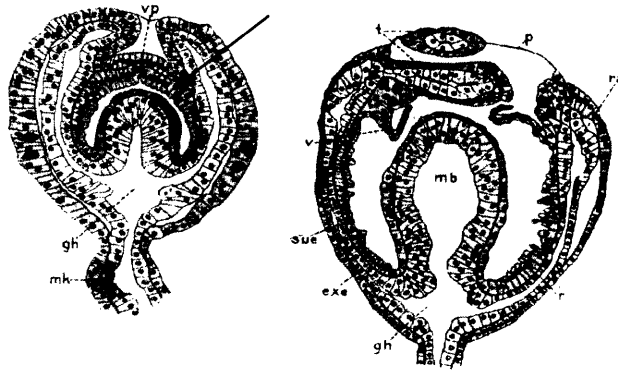
B.
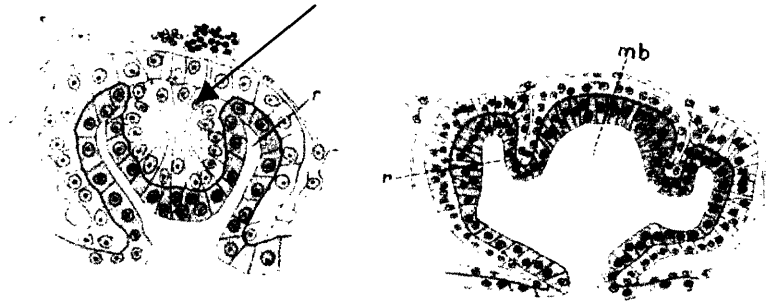
Figure 2. Hydrozoan medusae developmental progression in the typical hydromedusa *Syncoryne sarsii* (**A**) and an *Obelia* medusa (**B**). The entocodon is indicated by arrows. Note the small entocodon and nascent subumbrellar cavity in *Obelia* disappear as development progresses. Images are from Kühn, 1913 and are oriented so that the tentacles are developing towards the top.

A.

Bell cavity

Velum

Hollow tentacles

B.

No bell cavity or velum
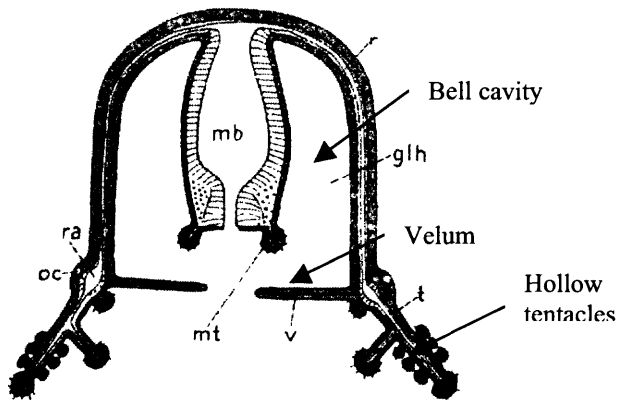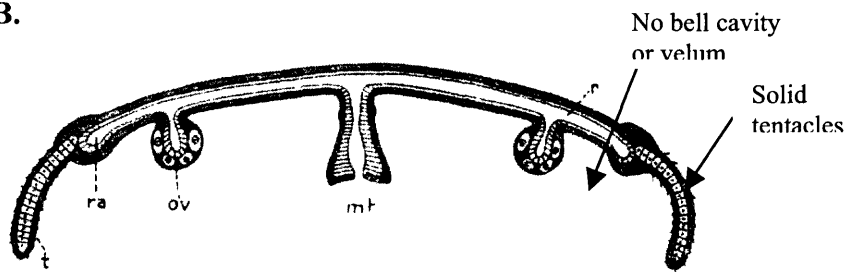
Solid tentacles

Figure 3. Cross section of a typical hydrozoan medusa (**A**) and an *Obelia* medusa (**B**). Note the flat shape, solid tentacles, and lack of a true bell cavity and velum in the *Obelia* medusa. Images are from Kühn, 1913 and are oriented so that the tentacles are pointed down.

Chapter 2

Independent origins of medusa loss in campanulariid hydrozoans*

* manuscript based on this chapter in preparation with Ferdinando Boero and Ken Halanych

Abstract

The goal of this research is to understand the evolution of life cycles in campanulariid hydrozoans (Cnidaria, Leptomedusa). Life cycle diversity is a hallmark of the Hydrozoa, and the Campanulariidae provide an excellent model system in which to study hydrozoan life cycle evolution. Campanulariids, well known by their typically benthic hydroid stage, exhibit an extraordinary array of life cycles, ranging from species with a free medusa stage, to those with a reduced or absent medusa stage. Additionally, based on their unique morphology, the medusae of the campanulariid genus *Obelia* are hypothesized to have been derived from species lacking medusae, thus possibly representing an example of a rare evolutionary reversal. However, inadequate taxonomic resolution has, up to now, prevented testing of this hypothesis. To complement previous morphological studies (which are hampered in a large part by their complex life cycles), a molecular approach was used to resolve campanulariid relationships, using 2 nuclear (18S rDNA and calmodulin) and 2 mitochondrial (16S rDNA and cytochrome c oxidase I [COI]) genes to construct a phylogeny of the Campanulariidae. The results indicate that life cycle transitions have occurred multiple times, and that *Obelia* medusae may be derived from ancestors with typical medusae, rather than medusoids or fixed gonophores, although this conclusion rests on the underlying phylogeny.

Introduction

Marine invertebrates exhibit a diverse array of life cycles, often with multiple morphologically distinct stages separated spatially and temporally. The evolution of such complex life cycles is a subject of considerable study (e.g., Strathmann, 1978; Wray, 1996; McHugh, 1998; Hart, 2000). Many marine invertebrates with complex life cycles,

including echinoderms, bivalves, polychaetes and crustaceans, possess a sessile (or with limited mobility) benthic, sexually-reproducing adult stage that releases gametes. The gametes form free-living planktonic larvae, which after some period of time settle and metamorphose into adults. Frequently the larval stage may be shortened or absent (Giangrande et al., 1994).

Life cycle variation is a hallmark of the Hydrozoa (Cnidaria), and in particular, the Campanulariidae (Leptomedusa) exhibit considerable diversity in their reproductive strategies (Cornelius 1982; Boero and Sarà 1987; Boero et al. 1996), providing a model system to study life cycle evolution. Like other hydrozoans, many campanulariids (e.g., *Clytia, Obelia*) have an asexual benthic polyp, or hydroid, stage that gives rise to a sexual adult planktonic medusa stage. The medusae in turn produce gametes that form short-lived lecithotrophic planulae, which settle and develop into hydroids. In contrast, other campanulariids (e.g., *Laomedea, Campanularia*) have apparently reduced or lost the medusa stage. Campanulariids display several stages of this putative evolutionary transition (Table 1).

The range of complex life cycles, the frequency of medusa loss (e.g., Petersen 1990) and the possibility of "re-invented" medusae (e.g., Boero and Sarà, 1987) have fueled controversy on hydrozoan life cycle evolution and systematics. For example, there are many cases where the hydroid stage is placed in one taxon (species/genus/family etc.) and the medusa stage is placed in another (Boero, 1980). The decoupled rates of morphological evolution occurring in hydroids and medusae (termed "inconsistent evolution" by Naumov [1960] and "mosaic evolution" by Rees [1957] and Morton [1957]) have been a major impediment to hydrozoan classification, and has prevented rigorous testing of phylogenetic hypotheses. Until relatively recently, separate classifications existed for hydroids and medusae. Naumov (1960) was the first to present a unified system. Furthermore, some researchers (Allman 1864; Rees 1957; Millard 1975; Cornelius 1982; Vervoort and Watson, 2003) have either explicitly or implicitly assumed that loss or reduction of the medusa stage is a rare event and have used life cycle type to define genera. Others (Broch 1916; Kramp 1949; Petersen 1979; Petersen 1990;

Cunningham and Buss 1993; Boero et al., 1996) have speculated that medusa loss happens relatively frequently, so that the use of life cycles as a generic character may have resulted in paraphyletic groupings. Finally, it is assumed that the presence of a medusa stage in hydrozoans is ancestral (Bridge et al., 1995) and that medusae have been lost many times; Cornelius (1992a) estimates at least 60 episodes of medusa reduction. However, the many unique features of the campanulariid *Obelia* medusae indicate that this may not always be true, and suggest the possibility that some medusae may have been "re-invented" from forms lacking medusae (Boero and Sarà 1987; Boero et al., 1996).

Obelia medusae

Despite their prominence in textbooks as a model hydrozoan, *Obelia* medusae have morphological and developmental features that are unique among the Hydrozoa, (Kühn, 1913; Chapman, 1968; Boero et al., 1996). These features include: 1) the presence of hydroid-like chordal (solid), rather than medusa-like hollow, tentacles; 2) a "peduncled" manubrium (flared mouth) that resembles the hydroid hypostome (mouth); 3) the lack of a velum (inner ring of tissue around the bell margin) and true bell cavity resulting in a flattened shape; 4) statocysts positioned at the tentacle bases, rather than at the umbrellar margin; 5) lack of true tentacular bulbs; and 6) gonads that develop from the corners of the manubrium where the radial canals originate, rather than directly from the radial canals (although they later migrate down the radial canals); and 7) cross-layered, rather than circular, myofibrils which are better suited to a flat, rather than the typical concave and craspedote form (Chapman, 1968). *Obelia* medusa development also differs from all other hydrozoan medusae, in that the entocodon (a proliferation of ectoderm along the blastostyle that leads to the subumbrellar cavity) seems to disappear early in development (Kühn, 1913; Boero et al., 1996).

Interestingly, another campanulariid, *Gonothyraea*, produces unique medusoids, termed meconidia, whose medusa features are like *Obelia* rather than typical medusae (e.g., solid tentacles) (Boero et al., 1996). Despite the striking features of their medusae

or medusoids, *Obelia* and *Gonothyraea* are placed within the Campanulariidae because of the strong similarity of their hydroids to other campanulariid hydroids. Thus, in addition to contributing to the understanding of medusa loss, the Campanulariidae offer a unique system to investigate the possibility of multiple acquisitions of the medusa form. Understanding this issue would further our knowledge of how bentho-pelagic life cycles evolve relating to the early evolution of major animal lineages by providing insight into the rate of the evolution of development and the likelihood of evolutionary reversals.

Hypotheses of Campanulariid evolution

Three leading hypotheses of campanulariid phylogeny and medusa evolution are shown in Figure 1. Cornelius (1982) divided the Campanulariidae into 3 subfamilies, and hypothesized a progression of medusa loss from medusae to medusoids/meconidia to fixed gonophores (Figure 1A) In this scenario, *Obelia* is the ancestral obeliniid. Östman (1987; Figure 1B) suggested *Gonothyraea* was the ancestral obeliniid, based on the presence of a type of nematocyst also found in other Campanulariids but not *Obelia*. This hypothesis was based on Cornelius (1982) and Boero and Sarà (1987), the latter of whom were the first to suggest that *Obelia* medusae were secondarily derived. Boero et al. (1996; Figure 1C) also modified the hypothesis of Boero and Sarà to include additional morphological features and merged the Clytiinae into the Obeliinae, where it is basal. The ancestral obeliniid in their hypothesis is *Laomedea*.

Goals

Although there has been considerable discussion of hydrozoan morphological and life cycle evolution, evolutionary hypotheses have never been tested in a rigorous manner against an independently derived phylogeny. The goal of this research is to understand life cycle evolution in the Campanulariidae. Towards this end, we have constructed a molecular phylogeny based on 2 nuclear (18S rDNA and calmodulin) and 2 mitochondrial (16S rDNA and cytochrome c oxidase I [COI]) genes to address the following questions: 1) In the Campanulariinae and Obeliinae lineages, did medusoids

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and fixed gonophores evolve only once or many times? 2) Does *Obelia* derive from ancestors with typical medusae, medusoids, or fixed gonophores? Finally, we reviewed the ecological and evolutionary processes that have been implicated in generating hydrozoan life cycle diversity. In order to gain a broader view of campanulariid life cycle evolution, we examined our results in the context of marine invertebrate life history evolution.

Materials and Methods

Sample collection and outgroup selection

The taxa employed in this study and their collection localities are presented in Table 2. A total of 47 putative campanulariid taxa were obtained, including representatives of all recognized genera (except *Hartlaubella* and *Tulpa*). Due to the potential existence of cryptic species in some widely distributed forms, multiple representatives from different locations were included when available. Several thecate hydroids (Leptomedusae) were also collected to be used as outgroups (Table 2). In particular, the Bonneviellidae is thought to be closely related to the Campanulariidae (Bouillon, 1985). Additionally, several 18S rDNA sequences from GenBank were used primarily as outgroups (Table 3).

Data collection

Field-collected hydroids were identified and cut into two portions for preservation: one in 10% formalin for a morphological voucher, and one in 95% ethanol for DNA sequencing. Once in the lab, genomic DNA was extracted using DNEasy extraction kits (Qiagen) following the manufacturer's protocol. The entire 18S rDNA and portions of calmodulin, 16S rDNA, and COI were amplified using procedures and primers described in Halanych et al. (1998) for 18S rDNA, Lindner et al. (in prep) for calmodulin, Cunningham and Buss (1993) for 16S rDNA, and Folmer et al. (1994) for COI. PCR products were visualized on a 1% agarose gel stained with ethidium bromide. In most cases, the entire gene (18S rDNA) or gene fragments (calmodulin, 16S rDNA, COI) were amplified in one reaction; however, in a few taxa, the 18S rDNA gene was

amplified in 3 shorter, overlapping segments. In some cases for calmodulin, the PCR product was re-amplified using 0.5 μl template.

The entire 18S rDNA bands were cut out of the gel and purified using the Qiagen PCR purification kit for gel purification, as occasionally multiple bands were present on the gel. In some cases, the purified PCR product was sequenced directly, while in others, it was cloned using the Promega T-GEM easy kit, and white colonies were screened for the insert by PCR amplification of a portion of the 18S rDNA and visualization on a 1% agarose gel stained with ethidium bromide. Clones containing the insert were grown and purified using the Promega minipreps following manufacturer's protocol. The shorter 18S segments and the calmodulin, 16S rDNA, and COI were purified directly from the PCR product using the Qiagen PCR purification kit according to manufacturer's protocol.

Purified PCR product was cycle-sequenced using either Big Dye 2 or 3 sequencing chemistry (ABI), following the manufacterer's recommendations. For the cloned 18S rDNA, the m13f and m13r primers, as well as 6 internal 18S rDNA primers (Halanych et al., 1998), were used in sequencing reactions. In most cases, unincorporated dideoxynucleotides were removed with a Sephadex G-25 (Sigma, St. Louis, MO) column. The DNA was then sequenced on an ABI 377 or 3700 automated DNA sequencer. DNA sequences were aligned and checked for ambiguities using Autoassembler (ABI) and Sequencher 4.2.2 (Genecodes Corp.).

DNA sequences were aligned using ClustalX (Thompson et al., 1994) and the alignments were confirmed by eye using MacClade (Maddison and Maddison, 2000). Regions that could not be unambiguously aligned from the rDNA were excluded from analyses. No sites were excluded from the calmodulin and COI sequences.

Phylogenetic analysis

Because of uncertainties in hydrozoan systematics, a leptomedusan phylogeny was generated with the 18S rDNA sequences to confirm rooting and delineate the Campanulariidae. The Leptomedusae were rooted with two species of Hydridae (Collins, 2000; 2002) and the closest outgroups to the Campanulariidae were identified. Then, a

new alignment with all 4 markers and including only the Campanulariidae and the most appropriate outgroups was generated for subsequent analyses. The individual markers were analyzed both separately and together.

The best-fit models for maximum likelihood analysis were determined with ModelTest (Posada and Crandall, 1998), and used in PAUP* 4.0b10 (Swofford, 2002). Heuristic searches under maximum parsimony and maximum likelihood were conducted using starting trees obtained by stepwise addition with 10 random addition sequence replicates and TBR branch swapping.

Support for nodes was obtained from parsimony and likelihood nonparametric bootstrap analyses and Bayesian posterior probabilities. 1000 bootstrap replicates were run when the parsimony criterion was used, while 100 bootstrap replicates were run when the likelihood criterion was used. In the parsimony bootstrap analyses, the maximum number of trees saved in each bootstrap replicate was set to 1000 (100 per addition sequence replicate). To minimize computation time, maximum likelihood bootstrap analysis was only run on the combined campanulariid datasets using one addition sequence replicate and limiting the number of rearrangements to 5000. Bayesian posterior probabilities were calculated with MRBAYES 3.0b4 (Ronquist and Huelsenbeck, 2003). Bayesian analyses were run for 1,000,000 generations with 4 chains and a sample frequency of 1000. Burn-in was set at 200, and after analyses were run, a plot of the log likelihoods versus generation was examined to confirm that the likelihood values had leveled off.

Topological hypotheses were tested with the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasagawa, 1999) implemented in PAUP* 4.0b10 (Swofford, 2002) using the RELL test distribution.

Life cycle (gonophore) type was traced on to the parsimony bootstrap consensus tree of the combined dataset, and the ancestral states were reconstructed using equally-weighted parsimony in MacClade 4.0 (Maddison and Maddison, 2000). Life cycle type was designated as typical medusae, medusoids, meconidia, fixed gonophores, or *Obelia* medusae. It was assumed that the ancestral campanulariid possessed a typical medusa

stage. Because losses (e.g., going from medusae to medusoids or fixed gonophores) may be easier than gains (e.g., going from fixed gonophores and medusoids to medusae) and frequent losses may bias the results (Cunningham et al., 1998; Cunningham, 1999), ancestral states were also reconstructed by creating a life cycle transition step matrix where gains were weighted greater than losses. Transition between medusa and medusoid types (typical/*Obelia* and medusoid/meconidia) were considered gains. A sensitivity analysis was conducted to see how sensitive ancestral state reconstruction was by comparing a range of weights applied to gains (e.g., Omland, 1997; Ree and Donoghue, 1998). Gain costs (weights) were tested at 0.1 increments between 1 and 2, and then periodically up to 99.

Because *Gonothyraea* meconidia possess some of the unusual features found in *Obelia* medusae (e.g., solid tentacles and position of the gonads; Boero et al., 1996) while *Orthopyxis* and *Silicularia* medusoids appear more like typical medusae (e.g., they may have a velum; Hirohito, 1969), ancestral state reconstruction was also conducted using only 3 life cycle categories: typical medusae/medusoids, *Obelia* medusae/meconidia, and fixed gonopohores.

Results

18S rDNA and 16S rDNA sequences were obtained from all specimens, and calmodulin and COI were obtained from most (Table 2).

Outgroup rooting and delineation of the Campanulariidae

The leptomedusan phylogeny was based on an alignment including 2057 basepairs (bp) of 18S rDNA. 462 bp were excluded. Of the included sites, 434 bp were variable and 255 bp were parsimony-informative.

The results clearly show that the Campanulariidae are not monophyletic (Figure 2). *Billardia subrufa* falls well outside the primary "campanulariid" clade. *Billardia, Stegella,* and *Melicertissa* form a clade and, along with the sertulariids *Gymnangium* and *Selginopsis*, fall basal to the Campanulariidae. There is a highly supported clade

composed of the campanulariids (except *Billardia*), *Aequorea, Blackfordia, Eugymnanthea, Tiaropsidium, Opercularella*, and *Calycella. Lovenella gracilis* and *Eucheilota bakeri* fall inside the campanulariid clade. The bonneviellids were strongly supported to be deeply nested in the Campanulariidae, in the Campanulariinae. The SH test results were consistent with the phylogenies: inclusion of *Billardia* in the Campanulariidae and exclusion of the bonneviellids were significantly less likely, while the inclusion of *Eucheilota* and *Lovenella* in the Campanulariidae was not significantly less likely (Table 4).

Relationships in the Campanulariidae

Based on Figure 2, *Eugymnanthea, Calycella*, and *Opercularella* were selected as outgroups for the expanded Campanulariid phylogeny. *Lovenella, Eucheilota*, and the bonneviellids were also included because a close relationship with the Campanulariidae was indicated, while *Billardia* was excluded because it appeared phylogenetically distant. A total of 3108 bp could be aligned in the combined dataset (18S: 1612 bp; CAM: 401 bp; 16S: 434 bp; COI: 661 bp). 262 bp of 18S and 176 bp of 16S were excluded. 890 bp were variable (18S: 359; CAM: 125; 16S: 131; COI: 275), and 673 were parsimony-informative (18S: 216; CAM: 107; 16S: 105; COI: 245). In the two protein-coding genes, most of the variation occurred in 3rd codon positions (110/125 variable sites in CAM and 208/275 in COI). The data were analyzed both together (Figure 3) and separately (Figures 4-7), and indicated that most of the phylogenetic signal came from the 18S rDNA sequences, while the others were generally very poorly resolved (by parsimony). Therefore the combined and 18S rDNA results are emphasized below. The combined and 18S analyses yielded similar results, with a few exceptions noted. Maximum likelihood, parsimony, and Bayesian support often agreed, although in some cases the Bayesian posterior probabilities were considerably higher than the likelihood and parsimony bootstrap values. Because Bayesian posterior probabilities may sometimes overestimate support (Erixon et al., 2003; Simmons et al., 2004), only nodes with that also had high bootstrap values were considered well-supported.

The Campanulariidae, inclusive of *Bonneviella* spp. and exclusive of *Eucheilota bakeri* and *Lovenella gracilis*, appears monophyletic but this clade is not well supported (Figure 3, Figure 4). *Eucheilota* and *Lovenella* fall immediately basal to this clade, and these taxa plus the campanulariids and bonneviellids form a very highly supported clade. Within the Campanulariidae, there are 4 major lineages (the Campanulariinae, *Clytia*, and 2 Obeliinae lineages) that are well-supported, with the exceptions of *Clytia hummelincki* and *Obelia bidentata,* which are hard to place. The arrangement of these major lineages with respect to each other is not well resolved.

Monophyly of the Campanulariinae, including the bonneviellids, was very strongly supported (Figure 3, Figure 4). Monophyly of the campanulariniid genera were not well-supported, however. *Orthopyxis* appears to form a monophyletic clade in Figure 3 (but not Figure 4), but this clade does not have either bootstrap or Bayesian support. *Bonneviella* also appears to form a monophyletic clade in Figure 3 (but not Figure 4), which is highly supported by Bayesian, but not bootstrap, analyses. *Campanularia* is not monophyletic in either Figure 3 or 4. In the combined analysis, *Orthopyxis everta* and *Orthopyxis integra* Italy (IT) group together strongly (Figure 3), while in the 18S rDNA analysis, *Orthopyxis everta* and *Campanularia hincksii* group together strongly. The grouping in the combined analysis is probably due to the strong support of that arrangement in the 16S rDNA (Figure 6) and COI (Figure 7).

Clytia appears monophyletic in the combined dataset, although the inclusion of *Clytia hummelincki*, in the basal position, has only moderate support (Figure 3). Without *Clytia hummelincki*, support is higher. In the 18S rDNA, *Clytia hummelincki* falls outside the main *Clytia* clade, although its position there is not supported (Figure 4).

The Obeliinae, although, may be paraphyletic. In the combined data, *Obelia bidentata* plus the *Clytia* clade form a sister group to the rest of the Obeliinae (Figure 3). This position is only minimally supported, however. Both parsimony and Bayesian analyses weakly support the placement of *Obelia bidentata* in the Obeliinae, as the basal member of the clade containing *Laomedea flexuosa, Laomedea inornata*, and *Obelia longissima* (values of 72 and 60, respectively; this arrangement not depicted in Figure 3).

In the Bayesian analysis, when the 4 genes and codon positions for the protein-coding genes are partitioned, the original arrangement in Figure 3 is supported only by a posterior probability of 57. Parsimony analysis of the 18S rDNA data alone highly supports this position (bootstrap value of 95; Figure 4).

With the possible exception of *Obelia bidentata*, the Obeliinae form two well-supported clades (Figure 3; Figure 4). One consists of *Gonothyraea loveni, Obelia dichotoma, Laomedea calceolifera,* and *Obelia geniculata*, and the other consists of *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima*. Thus, the obeliniid genera are not monophyletic.

The SH tests were again consistent with the phylogeny: trees constrained to have monophyletic *Obelia* and *Laomedea* were significantly less likely, while there was no significant difference between the most likely tree and trees constrained to have monophyletic Obeliinae and *Campanularia* (Table 4).

Life cycle evolution and *origin of* Obelia

Obeliniid and possibly campanulariniid genera were not monophyletic (Figure 3; Figure 4; Table 4), indicating that life cycles evolved multiple times. Because both parsimony bootstrap and Bayesian analysis supported (albeit weakly) a basal position of *Obelia bidentata* (which had exceptionally long branches) in the obeliniid *Laomedea flexuosa, Laomedea inornata,* and *Obelia geniculata* clade, in contrast to the likelihood topology (tree) which placed it as the basal *Clytia* (Figure 3), ancestral state reconstruction analysis was conducted on both the likelihood topology (Figure 3) and a modified topology, with *Obelia bidentata* placed in the obeliniid clade (e.g., Figure 13). The 18S rDNA also strongly supported the position of *Obelia bidentata* in the modified topology (Figure 4), and the SH test showed that a monophyletic Obeliinae was not significantly less likely (Table 4). A heuristic maximum likelihood analysis of the combined dataset with *Obelia bidentata* removed yields a topology otherwise identical to Figure 3.

In the likelihood topology, equally-weighted ancestral state reconstruction suggested that the ancestral campanulariid and obeliniid + *Clytia* could not be determined (i.e., equivocal), and that the ancestral campanulariniid and obeliniid had fixed gonophores (Figure 8). A gain cost between 1.1 and 1.4 resulted in the ancestral campanulariid and obeliniid + *Clytia* having typical medusae (Figure 9). Increasing the gain cost to 1.5 and then to 1.6 – 1.9 again resulted in the ancestral campanulariid and obeliniid + *Clytia* being equivocal (Figure 10; Figure 11), although for gain costs 1.6-1.9, the ancestral obeliniid appeared to have an *Obelia*-type medusa (Figure 11). Gain costs of 2.0 and higher gave similar results, except that the ancestral campanulariniid appeared equivocal (Figure 12).

In the modified topology, equally-weighted ancestral state reconstruction indicated that the life cycles of the ancestral campanulariid, *Clytia*, and obeliniid were equivocal, and the ancestral campanulariniid had fixed gonophores (Figure 13). However, when the gain cost was increased to 1.1, the ancestral campanulariid and *Clytia* had typical medusae, the ancestral obeliniid had *Obelia* medusae, and the ancestral campanulariniid had fixed gonophores (Figure 14). Increasing the gain cost had no further effect until it reached 2.0 (Figure 15). In this case, the ancestral campanulariid and *Clytia* had typical medusae, the ancestral obeliniid had *Obelia* medusae, and the ancestral campanulariniid was equivocal. Increasing the gain cost up to 99 had no further effect.

To see how the underlying phylogeny might also affect ancestral state reconstruction in the Campanulariinae, the modified topology was modified further, placing *Campanularia hincksii* in the clade with *Campanularia volubilis*, *Rhizocaulus*, and the bonneviellids (Figure 16). In this case, even when there was no gain cost, the ancestral campanulariniid was equivocal.

Equally-weighted ancestral state reconstruction using 3 life cycle categories (typical medusae/medusoids, *Obelia* medusae/meconidia, and fixed gonophores) on the modified topology (with *Obelia bidentata* placed within the Obeliinae) indicated that the ancestral campanulariid and obeliinid + *Clytia* had typical medusae/medusoids, the ancestral obeliinid had *Obelia* medusae/meconidia, and the ancestral campanulariniid was

equivocal (Figure 17). Some ancestors within the Obeliiniae were equivocal. Gain costs of 1.1 – 99 yielded the same result except that the ancestral campanulariniid had typical medusae/medusoids, and no ancestors within the Obeliinae were equivocal (Figure 18).

Discussion

The results corroborate some aspects of previous classification but refute others. A detailed discussion of the taxonomic results is outside the scope of this chapter, but can be found in Govindarajan and Boero (chapter 3).

Multiple origins of medusa loss and the origin of Obelia

Our results indicate multiple life cycle transitions in the Obeliinae and possibly the Campanulariinae. These results are similar to those in the Tubulariidae (Petersen, 1990) and Hydractiniidae (Cunningham and Buss 1993), suggesting that medusa reduction may be more common than once thought, and adding to the growing list of multiple losses of life history traits in marine invertebrates in general (Hart, 2000). Molecular studies in other hydrozoan lineages will be helpful in revealing the extent of this phenomenon.

In the maximum likelihood topology, when *Obelia bidentata* it is placed in the *Clytia*, under some weighting schemes (gain cost = 1.0 – 1.4) the ancestral obeliniid appears to have fixed gonophores, and *Obelia* medusae appear to have evolved multiple times. Alternatively, at higher gain costs, the relevant ancestors are equivocal. Under these scenarios, either *Obelia* medusae would have to be evolved independently more than once, or *Clytia* medusae would have had to been derived from *Obelia* medusae. Both possibilities indicate that medusa "re-invention" may be easier than previously thought.

However, *Obelia bidentata* has extremely long branches in the maximum likelihood topology, which might have resulted in its erroneous placement. Maximum likelihood bootstrap does not support this position, and both parsimony and bootstrap analyses support, albeit weakly, its position as the basal member of one of the obeliniid

clades. Parsimony analysis of the 18S rDNA alone also strongly supports (95 percent) this position and the SH test indicated that this topology is not significantly less likely than the most likely topology. Thus, caution is warranted in interpreting the ancestral state reconstruction based on this topology as the majority of the support suggests *Obelia bidentata* is misplaced.

Ancestral state reconstruction on the likelihood topology modified to reflect the alternative position of *Obelia bidentata* yielded very different results. Under equally-weighted parsimony, the ancestral forms of the Campanulariidae, Obeliinae, and *Clytia* could not be inferred, but when even a very small gain cost was applied, the ancestral campanulariid and obeliniid + *Clytia* appeared to have typical medusae. This result suggests that *Obelia* medusae derived directly from an ancestor with typical medusae, rather than one with fixed gonophores or meconidia. This scenario is also consistent with a single origin of *Obelia* medusae, which makes sense in light of morphology, as all *Obelia* medusae are nearly identical (Kramp, 1961; Cornelius, 1995). It appears likely that *Obelia* medusae derived directly from typical *Clytia*-like medusae and that the fixed gonophore bearing *Laomedea* evolved multiple times from *Obelia*. This result is also found when life cycle types are placed in 3, rather than 5, categories, and is inconsistent with all hypotheses of campanulariid evolution presented in Figure 1.

One potential caveat, however, is that not all known obeliinids with fixed gonophores, especially *Hartlaubella* (a monotypic genus), could be obtained for this study. Like *Laomedea*, *Hartlaubella* has fixed gonophores; however, hydroid colony morphology differs in some potentially important ways. *Hartlaubella* hydroids are relatively large, polysiphonic, and have toothed hydrothecal margins. These characteristics are generally similar to those found in *Gonothyraea* and some *Obelia* and *Clytia* hydroids but not *Laomedea*. Unless gonophores are present, *Hartlaubella* is easily confused with *Obelia bidentata* (Cornelius 1990). Additional studies are needed to determine if these features indicate a basal position in the Obeliinae. This possibility does not affect the conclusions that the obeliniid genera are not monophyletic and that *Obelia* medusae were likely lost multiple times.

It is not possible to determine whether fixed gonophores evolved once or multiple times within the campanulariniid lineage. Analyses of both campanulariid topologies indicated that at no or low gain costs (1.0 – 1.9), the ancestral campanulariniid had fixed gonophores, but at higher gain costs (≥2.0), the ancestral form was equivocal. If the lower gain costs are correct, it would suggest that the medusoids of *Silicularia* and *Orthopyxis* are an example of an evolutionary reversal. Again, this conclusion rests on the underlying topology: when *Campanularia hincksii*, whose placement is not supported by parsimony bootstrap, is moved into the *Campanularia/Rhizocaulus/Bonneviella* clade, the ancestral campanulariniid appears equivocal even at no gain cost.

Additional caution should be exercised in interpreting the campanulariniid results because they may reflect bias in taxon sampling. All members of the very highly supported campanulariinid/bonneviellid clade were collected in the North Pacific, consistent with a local, geographically restricted radiation there; in particular; all bonneviellids are found only in a few locations in the North Pacific (except *Bonneviella grandis*, which is also found in the North Atlantic [Naumov, 1960]). In contrast, *Campanularia hincksii*, which was the only other *Campanularia* in this study, was collected from the Mediterranean and is a nearly cosmopolitan species (Cornelius, 1995). Other *Campanularia* species exist in other parts of the world (e.g., Millard, 1975) that could not be obtained for this study, but are necessary to determine the frequency of life cycle transitions in this lineage.

Evolutionary mechanisms of medusa loss

The results suggest that if gains are even slightly more likely than losses, medusae may have been reduced multiple times in the Obeliinae (modified topology; Figure 14). Heterochrony, or change in the timing of development, is likely the underlying cause of medusa reduction. Weismann (1883; summarized in Berrill and Liu, 1948) related gonophore type to the location of germ cell differentiation. In free-living medusae, the germ site is on the ectoderm in the medusa. As medusa features are lost, the germ site moves to the entocodon (medusa bud) and gonophore endoderm and eventually to the

hydroid coenosarc, or tissue. The germ cells then migrate to the gonophore ectoderm, where they mature, perhaps blocking medusa development (Boero and Bouillon, 1989). This phenomenon has been interpreted as paedomorphosis, and may occur via neoteny (retardation of somatic development) in large hydroids or progenesis (acceleration of sexual development) in small hydroids (Boero et al. 1997).

The results also indicate that *Obelia* medusae may not be derived from meconidia or fixed gonophore - bearing ancestors, and so are not an example of an evolutionary reversal. Dollo's law contends that complex structures, such as many larval and medusoid features, are more easily lost than gained (Futuyma, 1998). There are few documented exceptions to Dollo's law; these include insect wings (Whiting et al., 2003), mammalian molar teeth (Werdelin 1987), arthropod eyes (Oakley and Cunningham, 2003), and gastropod shell coiling (Collin and Cipriani, 2003). Evolutionary reversals in life history mode include planktotrophy in littorinid gastropods (Reid 1989). Recently Collin (2004) suggested that evolutionary reversals in developmental mode may have possibly occurred multiple times in calyptraeid gastropods, although her results rested on the assumptions of ancestral state reconstruction.

In a survey of marine invertebrate larval feeding structures, Strathmann (1978) found that planktotrophy, once lost, was unlikely to be regained, although it may be more likely in spiralian (molluscs, annelids, echiurans, sipunculans, entoprocts) than oligomeric (lophophorates, hemichordates, echinoderms) taxa. He also pointed out that reacquired planktotrophy would be less likely to have reduced egg size and thus greater fecundity (a benefit usually associated with planktotrophy). From a theoretical viewpoint, Ebenman (1992) argues that a life cycle stage, once lost, would be unlikely to be regained through gradual evolution, because there would be no juvenile-adult selection tradeoff in the derived direct developing phenotype. Finally, from a genetic perspective, when a complex structure (like a life-cycle stage) is lost, the genes involved are not subject to selection and degrade over time unless they are used in other functions (Marshall et al., 1994). Thus re-expression can occur if there is rapid speciation and divergence times are short enough that the genes are not lost. Within a rapidly radiating lineage, a complex

trait can "flicker" on and off. Alternatively, conservation of gene integrity through functioning in different pathways could also allow the re-expression of a lost structure (Marshall et al., 1994). This can happen for meristic traits in the same life cycle stage, or via heterochrony, when the traits are expressed in a different life cycle stage in the ancestor (Collin and Cipriani, 2003), as in hydrozoans.

The re-evolution of medusae is considered unlikely because the medusa is a complex individual, comprising multiple physiological systems working together to perform all life functions necessary for existence – feeding, locomotion, reproduction, etc. In this regard it is not simply the re-emergence of an organ (the gonophore), but of an entity that must function as a whole self-sufficient individual. Our results suggested that *Obelia*, despite its unique features, was probably not a re-expressed medusa, although the possibility that it arose from an ancestor with meconidia or fixed gonophores cannot be completely ruled out. Future studies will need to determine the mechanism of *Obelia*'s unique medusa features. Their similarity in some characteristics to hydranths may indicate that hydranth developmental pathways could be involved. Gene expression studies could be useful in evaluating this hypothesis.

There may be, however, other cases of a secondarily derived planktonic stage in hydrozoans – termed "swimming gonophores", rather than medusae, by Boero and Bouillon (1989), because they lack most medusoid features that typical leptomedusan medusae and medusoids and *Obelia* medusae share, while possessing some features of fixed gonophores. The potential existence of re-expressed hydrozoan medusae or swimming gonophores could be due to any combination of the following mechanisms (adapted from Marshall et al., 1994): 1) the re-expressed medusae diverged relatively recently from fixed gonophore ancestors, and the necessary genes have not been dormant long enough to degrade; 2) the genes involved may have other functions in the hydroid; and 3) changes in developmental timing, such as in gamete maturation, may allow medusa development to complete. Dating species divergences (e.g., Govindarajan et al., submitted), gene expression, and detailed developmental studies will help distinguish between among possibilities.

Ecological correlates and framework for future research

Decades of research have examined the range of developmental modes for marine benthic invertebrates with planktonic larvae (Thorson, 1950; Mileikovsky 1971; Vance, 1973; Strathmann, 1985; Wray and Raff, 1991). Larvae range from being planktotrophic (feeding) and spending several weeks in the plankton, to lecithotrophic (nonfeeding) and spending a short time (minutes to days) in the plankton, to direct development, or brooding within the benthic adult. This continuum is analogous to the range of gonophore types seen in hydrozoans, except that in hydrozoans, it is the adult, not the larval, stage that is planktonic.

As in the Campanulariidae, loss of the planktonic stage appears to be frequent in marine invertebrates with complex life cycles. For example numerous independent losses of planktonic feeding larvae have been hypothesized in echinoids (Emlet, 1990; Wray, 1996); asterinid starfish (Hart et al., 1997); and gastropods (Lieberman et al., 1993; Duda and Palumbi, 1999). The primary difference between hydrozoans and other marine invertebrates is that in hydrozoans it is (usually) the adult rather than the larval stage that is lost; however, the common loss of the planktonic stage suggests that hydrozoans and other marine invertebrates could be responding to common selective pressures.

Dispersal ability is frequently considered in studies of life history evolution of marine invertebrates, although in many cases it appears not to be an important factor. Planktotrophy is generally associated with greater dispersal and larger geographic ranges (e.g., Jablonski 1986; Strathmann, 1986; Scheltema 1989; Emlet 1995) although studies comparing genetic differentiation at different spatial scales give mixed results, and indicate the relationship is not clear-cut (Jackson, 1986; Palumbi, 1995) and hydrodynamic and behavioral factors could limit dispersal (e.g., Hamner et al., 1994). Greater dispersal may be a consequence, rather than a cause, of planktotrophy. Holoplankton, which ostensibly disperse in their adult stage, also can have planktotrophic larvae (Strathmann, 1985). And beyond a certain point, additional time in the plankton may not increase dispersal. For example, Scheltema (1989) found that among gastropods,

planktotrophs had larger geographic ranges, but there was no difference in geographic range between those planktonic for only a few weeks and those planktonic for a few months or more.

Among hydrozoans, species with medusae may also disperse farther than species without them (Boero, 1984). Medusae can live between a few weeks to a few months (Arai,1992), and so have the potential to travel far. On the other hand, hydrodynamic or behavioral processes could limit medusa movements (Arai, 1992; Hamner et al., 1994). If the presence of a medusa stage were important for dispersal, then species with a free-living medusa stage may have greater distributions than those without a medusa. However, Boero and Bouillon (1993) showed that for Mediterranean hydrozoans whose life cycles are known, the percentage of species with a cosmopolitan distribution is significantly higher for species *without* a medusa than those with a medusa.

Within the campanulariid Obeliinae, the lineage that includes members both with and without free medusae, it appears that species with medusae have larger geographic ranges than those without (Table 5). All *Obelia* species have near cosmopolitan distributions, while none of the *Laomedea, Gonothyraea* or *Hartlaubella* do. However, anthropogenic introductions and cryptic speciation may be confounding factors, so this conclusion should be regarded as tentative. It is possible that all *Obelia* "species" represent cryptic species complexes. In a controversial taxonomic revision, Cornelius (1975) synonymized some 120 nominal taxa into 3, and later revised that number to 4 (1990). Others suspect his revisions went too far and recognize additional species (e.g., Kubota, 1999). In this study, *Obelia longissima* from widely separated locations appeared to have very little genetic divergence, indicating it may be truly cosmopolitan (Chapter 3). In contrast, Govindarajan et al. (Chapter 4, submitted) showed that despite a high dispersal potential, there was significant genetic structure and evidence for cryptic speciation in *Obelia geniculata*. Additional genetic studies will be necessary to define species boundaries in other taxa.

A confounding factor in studying the relationship between developmental mode and dispersal is that the planktonic larval/ medusa stage is not the only life cycle stage

that can disperse. Benthic adults (Highsmith 1985; Ingolfsson, 1995; Hobday, 2000) and hydroids (Kramp 1927; Cornelius 1992b; Calder, 1995; Ingolfsson 1995) can grow on seaweeds which could transport them over long distances and thus provide an alternative dispersal mode. Calder (1995) found medusa and fixed gonophore species to be equally likely to be represented on *Sargassum*, but others found that species with fixed gonophores were more likely on algal substrates. In hydrozoans, dispersal may also occur via planulae, which can live anywhere from about a few hours up to a few weeks (Bodo and Bouillon, 1968; Sommer, 1992). Although contrary to what might be expected if a planktonic period were important for dispersal, planulae in medusa-bearing species are longer-lived than in species with fixed gonophores (Sommer, 1992). Hydrozoans also may produce asexual propagules or frustules (Billard 1904; Berrill 1948) that could disperse. Frustules are formed in some *Obelia*, and could provide an alternative dispersal mode when environmental conditions preclude medusa development (Panteleeva, 1999) or perhaps in species without medusae.

Among benthic invertebrates, an extended planktonic period could be deleterious. Pelagic larvae are thought to be exposed to high mortality, due to predation, starvation, and dispersal away from suitable settling habitats, although it is difficult to quantify (Morgan, 1995). In particular, too much dispersal can carry organisms outside of the range of tolerable environmental conditions and away from potential mates, especially for species whose hydroids live in specialized coastal habitats (Edwards 1973) or remote islands (Cornelius 1992b; Calder, 1993). For example, in the "Paradox of Rockall" hypothesis, species with long planktonic periods are unlikely to persist in remote islands (Johannesson, 1988). While they may be able to initially colonize, their larvae may not be retained in sufficient concentration to maintain the population. In contrast, once a species with a reduced or lacking planktonic stage reaches a remote island, perhaps through algal rafting, it is more likely to persist because its larvae are not lost.

Among hydrozoans there are several examples consistent with the paradox of Rockall, suggesting overdispersal is a real possibility. Cornelius (1992a) showed that in the relatively isolated oceanic islands of the Azores, there is a high proportion of

hydroids with no medusa stage, and that these likely arrived to the islands by rafting. He suggested the medusae released from hydroids in remote islands would likely be lost to sea (Cornelius, 1992a). In this case, hydroids with fixed gonophores may reproduce more successfully than those with a medusa stage. Calder (1993) and Kirkendale and Calder (2003) similarly found a relatively higher proportion of fixed gonophore species in Bermuda and Guam, respectively. Similarly, Calder (2000) found the majority of species from 3 seamounts to have fixed gonophores.

Developmental mode is related to adult size, with planktotrophy associated with larger adults and brooding associated with smaller adults (Strathmann and Strathmann, 1982). These authors suggest 3 potential hypotheses to explain the relationship: 1) allometry – brooding space may not be proportional to adult size, and larger adults are less capable of retaining and ventilating their offspring; 2) recruitment variability – larger, longer-lived adults can afford the riskier, but potentially more rewarding, strategy of planktotrophy; and 3) dispersal – larger adults receive greater larval dispersal benefits.

Among thecate hydroids, a relationship between hydroid size and developmental mode is observed (of course, exceptions exist), with relatively larger hydroids associated with brooding, and relatively smaller hydroids associated with medusae (Cornelius, 1990). This trend appears opposite to that in other invertebrates. Cornelius (1990) argued that smaller and presumably shorter-lived or annual hydroids will have only one chance to produce planulae, and it may be more advantageous for them to have a medusoid form so that planulae are spread over a larger area. Larger and presumably longer-lived and perennial hydroids will have many chances to produce planulae that will be able to successfully settle locally. Unlike most other thecates, in the Obeliinae, the relationship may be more like other marine invertebrates, with the larger hydroids more likely to release planktonic medusae (Table 5). *Obelia* are generally larger than *Laomedea*, although *Hartlaubella* and *Gonothyraea* are comparable. More studies on hydroid longevity and recruitment are necessary.

Developmental mode may also be linked to resource allocation (Vance, 1973; Strathmann, 1985). Planktotrophic species produce more, but smaller, eggs than those

with non-feeding development, resulting in higher fecundity. However, planktotrophy is also associated with higher mortality due to the longer pelagic period. Those with lecithotrophic or direct development have a larger parental investment and produce larger, but fewer, eggs that spend less or no time in the plankton. From models, Vance (1973a,b) concluded that the optimal developmental strategy depended on the relative importance of planktonic predation, abundance of planktonic food, and the length of time spent in the plankton, with planktotrophy favored when planktonic predation is low and planktonic food is high.

A general correlation between marine invertebrate developmental mode, latitude, and water depth was first noticed by Thorson (1950), and termed "Thorson's rule" by Mileikovsky (1971). Thorson's rule suggests that pelagic development is prevalent in littoral and sublittoral zones in the tropics and subtropics, while it is considerably less common in high latitudes and deeper water. These trends may be related to the tradeoffs between energy allocation and the hazards of planktonic existence, with lower food supply and temperatures (increasing developmental time) in polar and deep sea regions (Vance, 1973). Many exceptions to Thorson's rule have been documented, however, and phylogenetic and ecological constraints may affect distributional patterns (e.g., Gallardo and Penchaszadeh, 2001).

In hydrozoans, there is also some evidence for a correlation of medusoid production with latitude. Stechow (1924) found that among athecate hydrozoans, species with fixed gonophores were more prevalent in polar regions and deep seas, while those with free medusae were prevalent in warmer areas. The Eutimidae, like the Campanulariidae, is also composed of members with medusae (*Eutima*) and medusoids (*Eugymnanthea*), and while they have overlapping distributions, *Eugymnanthea* is only found in the northernmost of these localities (Kubota 1987). Kubota (1987) suggested that cooler temperatures during the Pleistocene may have driven the evolution of *Eugymnanthea*, because *Eutima* medusae cannot mature at low temperatures. Furthermore, in the Obeliinae, while the cosmopolitan *Obelia* is found in polar waters, it persists there primarily asexually, through frustule production in the hydroid, because the

water is too cold for medusa development (Panteleeva, 1999). *Hartlaubella, Gonothyraea,* and *Laomedea* are usually not found in tropical waters (with the exception of *Laomedea pseudodichotoma*). Additionally in the Campanulariidae, as discussed above, season (which is correlated with temperature) is related to medusa development in *Clytia linearis*, with reduced planktonic medusa development in the fall compared to the summer (Boero and Sarà, 1987).

A comparison of Campanulariid distributions indicates that species without medusae are more likely to be found in higher latitudes or deeper water. Within the Obeliinae, *Laomedea, Gonothyraea,* and *Hartlaubella* are found primarily in temperate and boreal regions, with *Laomedea pseudodichotoma*, found only off the tropical west coast of Africa (Vervoort, 1959), as the sole exception. In contrast, *Obelia* and *Clytia* are represented in warmer waters. In the Campanulariinae, all *Bonneviella* species are found in the North Pacific (*B. grandis* is also in the North Atlantic), especially in deep (100's of meters) water (Nutting 1915; Naumov 1960). *Rhizocaulus* and *Tulpa* are also found exclusively in colder waters (Vervoort, 1972; Cornelius, 1995). *Campanularia* and *Orthopyxis* are widespread (Cornelius, 1995), while *Silicularia* is only found in cooler waters of the southern hemisphere.

While it is clear that the causes and consequences of developmental mode evolution in campanulariids and other hydrozoans are still speculative, comparisons with other invertebrates could provide new insights. While hydrozoans differ in that it is their adult rather than larval stage that is modified, it is important that in both groups the planktonic stage has been reduced multiple times, possibly due to common selective pressures. The diversity of life cycles is likely driven by multiple factors, including allometry, temperature, and resource utilization. Dispersal appears to be less important, but future studies are necessary to sort out the relative contribution of these and potentially other factors. Evaluating ecological shifts in a phylogenetic framework, using the comparative method, will be particularly insightful.

Conclusions

The first molecular phylogeny of the Campanulariidae is presented. Genera are not monophyletic, indicating that life cycle transitions have occurred multiple times within the Campanulariidae. Specifically, within the Obeliinae, medusae may have been reduced to meconidia and fixed gonophores multiple times and the unique *Obelia* medusae may have been derived from an ancestor with typical medusae. These conclusions rest on the underlying phylogeny and the assumption that gains of structure are less likely than losses. Additional research is necessary to corroborate these findings, and to understand the ecological correlates associated with the different life cycle types.

Acknowledgements

Table 1. Campanulariid subfamilies and genera and their associated sexual stage. (gonophore type). * = monotypic genus; ** including *Gastroblasta*.

Subfamily	Genus	Gonophore type	Description
Campanulariinae	*Orthopyxis*	Medusoids	With some medusa features (i.e., radial canals) but not others (i.e., tentacles), some with extracapsular development (acrocysts); may or may not be released from the hydroid
	Silicularia	Medusoids	With some medusa features (i.e., radial canals) but not others; not known to be released from the hydroid
	Campanularia	Fixed gonophores	Gonophores with no medusa features; fixed on the hydroid
	Rhizocaulus*	Fixed gonophores	"
	Tulpa	Fixed gonophores	"
	Billardia	Fixed gonophores	"
Clytiinae	*Clytia***	Medusae	Typical hydrozoan medusae
Obeliinae	*Obelia*	Medusae	Atypical hydrozoan medusae
	Gonothyraea*	Meconidia	With some medusa features (i.e., tentacles), but not others; medusa features similar to *Obelia*, rather than typical, medusae; not released from the hydroid
	Hartlaubella*	Fixed gonophores	Gonophores with no medusa features; fixed on the hydroid
	Laomedea	Fixed gonophores	Gonophores with no medusa features; some with extracapsular development (acrocysts); fixed on the hydroid

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Table 2. Campanulariid taxa and outgroups used in this study, collection locality, and genes sequenced.

Campanulariidae	Taxon	Locality	18S rDNA	calmodulin	16S rDNA	COI
Campanulariinae	*Billardia subrufa*	Antarctic peninsula	x		x	
	Campanularia hincksii	Otranto, Italy	x	x	x	x
	Campanularia volubilis	Monterey, CA, USA	x	x	x	x
	Orthopyxis everta	Torre del Serpe, Italy	x		x	x
	Orthopyxis integra	Italy	x	x	x	x
	Orthopyxis integra	Friday Harbor, WA, USA	x	x	x	
	Orthopyxis integra CA sp. 1	Monterey, CA, USA	x		x	
	Orthopyxis integra CA sp. 2	Monterey, CA, USA	x	x	x	
	Orthopyxis integra	Aleutians, USA	x	x	x	x
	Orthopyxis integra	Sandgerdi, Iceland	x		x	x
	Orthopyxis integra	New Zealand	x	x	x	x
	Orthopyxis sargassicola	Brazil	x	x	x	x
	Rhizocaulus verticllatus	Aleutians, USA	x	x	x	x
	Silicularia rosea	Bay of Islands, New Zealand	x	x	x	
Clytiinae	*Clytia elsaoswaldae*	Brazil	x	x	x	x
	Clytia gracilis	Georges Bank, North Atlantic	x	x	x	x
	Clytia gracilis	Italy	x	x	x	x
	Clytia gracilis	Beaufort, NC, USA	x	x	x	x
	Clytia gracilis	Woods Hole, MA, USA	x	x	x	x
	Clytia hemisphaerica	North Sea	x	x	x	x
	Clytia hummelincki	S. Caterina, Italy	x	x	x	x
	Clytia hummelincki	South Africa	x	x	x	x
	Clytia linearis	Brazil	x	x	x	
	Clytia linearis	Torre Inserraglio, Italy	x	x	x	
	Clytia linearis	Beaufort, NC, USA	x	x	x	x
	Clytia noliformis	Brazil	x	x	x	

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	Clytia paulensis	Otranto, Italy	X	X	X	X
	Clytia sp.	CA, USA	X	X	X	X
Obeliinae	*Gonothyraea loveni*	Dennis, MA, USA	X	X	X	
	Gonothyraea lovenii	Roscoff, France	X	X	X	
	Laomedea calceolifera	Woods Hole, MA, USA	X	X	X	X
	Laomedea flexuosa	Roscoff, France	X	X	X	X
	Laomedea flexuosa	Iceland	X	X	X	X
	Laomedea flexuosa	White Sea	X	X	X	X
	Laomedea inornata	Friday Harbor, WA, USA	X	X	X	X
	Obelia bidentata	Beaufort, NC, USA	X	X	X	X
	Obelia bidentata	North Sea	X	X	X	X
	Obelia dichotoma	Otranto, Italy	X	X	X	X
	Obelia geniculata	Roscoff, France	X	X	X	X
	Obelia geniculata	New Brunswick, Canada	X	X	X	X
	Obelia geniculata	Japan	X	X	X	X
	Obelia geniculata	New Zealand	X	X	X	X
	Obelia longissima	Ryders Cove, MA, USA	X		X	
	Obelia longissima	Antarctic peninsula	X	X	X	X
	Obelia longissima	Dunedin, New Zealand	X	X	X	X
	Obelia longissima	Sandgerdi, Iceland	X	X	X	X
	Obelia longissima	White Sea	X		X	X

Outgroups

Bonneviellidae	*Bonneviella regia*	Aleutians, USA	X	X	X	X
Bonneviellidae	*Bonneviella* sp. 2	Aleutians, USA	X	X	X	X
Bonneviellidae	*Bonneviella* sp. 3	Aleutians, USA	X	X	X	X
Bonneviellidae	*Bonneviella* sp. 4	Aleutians, USA	X	X	X	X
Campanulinidae	*Calycella syringa*	Woods Hole, MA, USA	X	X	X	X
Campanulinidae	*Stegella lobata*	Antarctic peninsula	X		X	
Eirenidae	*Eugymnanthea inquilina*	Taranto, Italy	X	X	X	X
Eucheilotidae	*Eucheilota bakeri*	CA, USA	X	X	X	
Lovenellidae	*Lovenella gracilis*	Wildwood Crest, New Jersey	X		X	
Phiallelidae	*Opercularella pumila*	Woods Hole, MA, USA	X	X	X	

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Table 3. Hydrozoan taxa with 18S rDNA from GenBank and associated accession numbers. *Chlorohydra* and *Hydra* belong to the Hydridae, and the remainder belong to the Leptomedusae.

Family	Species	Accession number
Hydridae	*Chlorohydra viridissima*	AF358081
Hydridae	*Hydra circumcincta*	AF358080
Aequoreidae	*Aequorea aequorea*	AF358076
Aequoreidae	*Aequorea victoria*	AF358077
Aglaopheniidae	*Gymnangium hians*	Z86122
Blackfordiidae	*Blackfordia virginica*	AF358078
Campanulariidae	*Clytia* sp.	AF358074
Laodiceidae	*Melicertissa* sp.	AF358075
Sertulariidae	*Selaginopsis cornigera*	Z92899
Tiaropsidae	*Tiaropsidium kelseyi*	AF358079

Table 4. Results of the Shimodaira - Hasegawa tests for monophyly at the family and generic levels. The best tree (unconstrained) was compared with the best tree under the given constraints.

Constraint	Monophyly tested	-ln L best	-ln L constraint	Diff –ln L	P value
Family 1	Campanulariidae, as defined with *Billardia*, the bonneviellids and *Eucheilota* and *Lovenella*	7442.80525	7547.97789	105.17264	0.000
Family 2	Campanulariidae, as defined without *Billardia*, the bonneviellids, and *Eucheilota* and *Lovenella*	7442.80525	7514.35024	71.54499	0.001
Family 3	Campanulariidae, as defined without *Billardia*, and *Eucheilota* and *Lovenella*, but with the bonneviellids	7442.80525	7446.50419	3.69894	0.255
Family 4	Campanulariidae, as defined with *Eucheilota* and *Lovenella*, and without *Billardia* and the bonneviellids	7442.80525	7485.02453	42.21928	0.009
Obeliinae	*Obelia + Laomedea + Gonothyraea*	20426.69624	20426.85739	0.16110	0.483
Obelia	*Obelia*	20426.69624	20767.61421	340.91797	0.000
Laomedea	*Laomedea*	20426.69624	20746.78747	320.09123	0.000
Campanularia	*Campanularia + Rhizocaulus + Bonneviella* (Campanulariinae with fixed gonophores)	20426.69624	20431.36449	4.66825	0.294

Table 5. Obeliinae ecological features summarized from Cornelius (1982; 1995), Millard (1975), Chaplygina (1993), Nutting (1915), Blanco (1968), Fraser (1946), Vervoort (1959), Vervoort and Watson (2003). * may be the result of an anthropogenic introduction. Colony size represents maximum.

Species	Range	Hydroid colony features
Obelia bidentata	Nearly cosmopolitan	Up to 350 mm, branching, polysiphonic
Obelia dichotoma	Nearly cosmopolitan	Up to 350 mm, branching, mono- or polysiphonic
Obelia geniculata	Nearly cosmopolitan	Up to 40 mm, branching, monosiphonic
Obelia longissima	Nearly cosmopolitan	Up to 350+ mm, branching, usually monosiphonic
Gonothyraea loveni	North Atlantic, North Pacific, Australia, New Zealand, South Africa*	Up to 100 mm, branching, monosiphonic
Hartlaubella gelatinosa	North Atlantic, Mediterranean, New Zealand, Patagonia, NE Pacific	Up to 200 mm, branching, polysiphonic
Laomedea angulata	NE Atlantic, Mediterranean	Up to 15 mm, unbranched, monosiphonic
Laomedea calceolifera	North Atlantic, Mediterranean, Brazil, South Africa*, North Pacific*	Up to 20 mm, unbranched, monosiphonic
Laomedea exigua	NE Atlantic, Mediterranean, Puget Sound*	Up to 4 mm, unbranched, monosiphonic
Laomedea flexuosa	North Atlantic, North Pacific*	Up to 30 mm, sometimes branching, monosiphonic
Laomedea inornata	Puget Sound, China, Argentina	Up to 36 mm, branching, monosiphonic
Laomedea neglecta	North Atlantic	Up to 25 mm, branching, mono- or bisiphonic
Laomedea pseudodichotoma	Tropical West Africa	Up to 50 mm, branching, part polysiphonic

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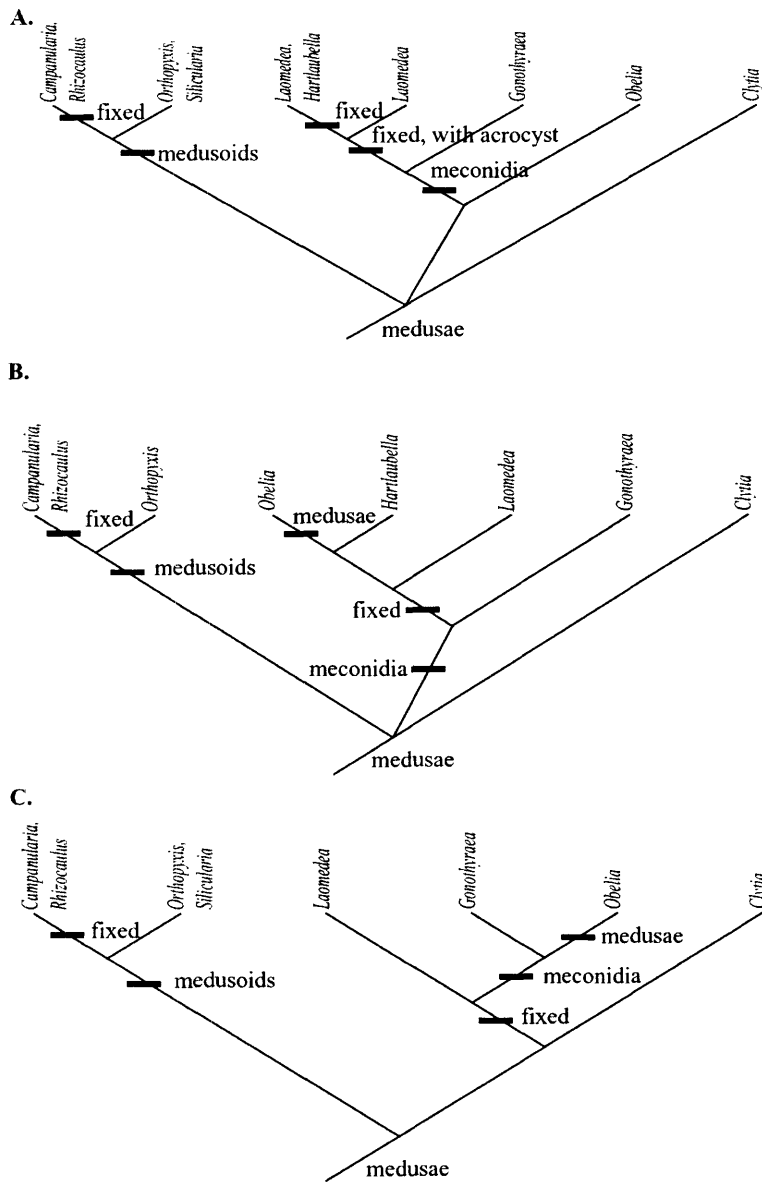
Figure 1. Proposed relationships among the Campanulariidae, reflecting 3 possibilities for the evolution of *Obelia*. **A.** *Obelia* derived from typical medusae (adapted from Cornelius, 1982); **B.** *Obelia* derived from fixed gonophores, which derived from meconidia (adapted from Östman, 1987); **C.** *Obelia* derived from fixed gonophores (adapted from Boero et al., 1996). Cornelius (1982) and Boero et al. (1996) considered *Silicularia* to have fixed gonophores. However, they have medusoids (Ralph, 1956; Blanco, 1967) and their figures are modified here. All hypotheses show a single loss of medusae in the Campanulariinae and Obeliinae lineages. Finally, Boero et al. considers *Clytia* to be part of the Obeliinae rather than a separate lineage as in Cornelius (1982) and Östman (1987).

71
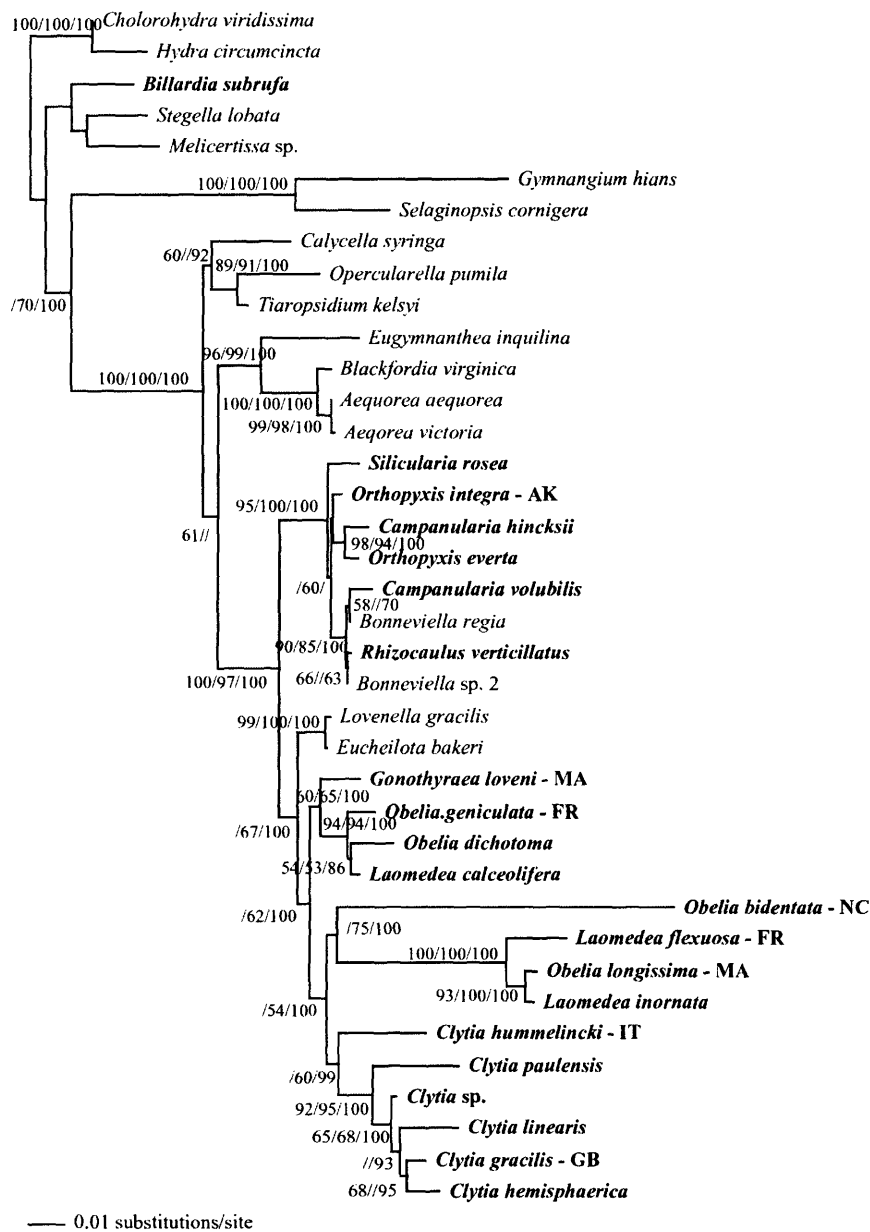
Figure 2. Maximum likelihood phylogeny of the Campanulariidae plus additional Leptomedusae and Hydridae sequences, including those from Genbank. Putative campanulariids are indicated in bold. The phylogeny is from a heuristic search based on 18S rDNA sequences. The best-fit model selected by Modeltest was TrN + I + G. The –ln likelihood for the topology = 7442.80525. First number refers to maximum likelihood bootstrap, second number refers to parsimony bootstrap, and third number refers to Bayesian posterior probability. Location codes: AN=Antarctic peninsula; AS=Aleutians; BR=Brazil; CA=California; FR=France; GB=Georges Bank; IC=Iceland; IT=Italy; JP=Japan; MA=Massachusetts; NB=New Brunswick; NJ=New Jersey; NZ=New Zealand; NC=North Carolina; NS=North Sea; SA=South Africa; WA=Washington.

72

Figure 3. Maximum likelihood phylogeny from a heuristic search based on the combined 18S rDNA, calmodulin, 16S rDNA, and COI sequences. The best-fit model selected by Modeltest was GTR + I + G. The –ln likelihood for the topology = 20426.3370. First number refers to maximum likelihood bootstrap, second number refers to parsimony bootstrap, and third number refers to Bayesian posterior probability. Subfamily lineages are indicated. Location codes are as in Figure 2. Gonophore type indicated in bold after the taxon name. T = typical medusae; D = medusoids; F = fixed gonophores; M = meconidia; O = *Obelia* medusae.

//100   *Eugymnanthea inquilina* **D**

98/78/100   *Calycella syringa* **F**
        Opercularella pumila **F**

100/99/100   *Lovenella gracilis* **T**
        Eucheilota bakeri **T**

100/100/100

99/98/100   *Campanularia hincksii* **F**
    Campanularia volubilis **F**
    Rhizocaulus verticillatus **F**

//94   *Bonneviella* sp. 4 **F**
//99   *Bonneviella* sp. 2 **F**
//98   *Bonneviella regia* **F**
//54   *Bonneviella* sp. 3 **F**

52//96

53//93   *Silicularia rosea* **D**

100/99/100   *Orthopyxis everta* **D**
    Orthopyxis integra IT **D**

65//99   *Orthopyxis integra* CA1 **D**
    Orthopyxis integra NZ **D**

//56   *Orthopyxis sargassicola* **D**

62//97   *Orthopyxis integra* WA **D**
93/89/100   *Orthopyxis integra* CA2 **D**
    Orthopyxis integra AS **D**
72/79/91   *Orthopyxis integra* IC **D**

96/91/

59//88

100/100/100   *Obelia bidentata* NC **O**   Obeliinae
    Obelia bidentata NS **O**

Campanariinae (+ Bonneviellidae)

Obeliinae

100/100/100   *Clytia hummelincki* SA **T**
   Clytia hummelincki IT **T**

/65/89

96/80/100

//80   *Clytia paulensis* **T**
   Clytia gracilis IT **T**

97/55/100   *Clytia gracilis* GB **T**
   Clytia noliformis **T**

84/65/100

100/100/100   *Clytia linearis* BR **T**
100/100/100   *Clytia linearis* IT **T**
  Clytia linearis NC **T**

99/89/100   *Clytia gracilis* MA **T**

91/52/100   *Clytia hemisphaerica* **T**

78/93/98   *Clytia gracilis* NC **T**
66/62/97   *Clytia elsaoswaldae* **T**
99/100/100   *Clytia* sp. **T**

Clytiinae

/53/59

76/81/96

100/100/100   *Gonothyraea loveni* MA **M**
  Gonothyraea loveni FR **M**

100/99/100   *Laomedea calceolifera* **F**

57/99/95   *Obelia dichotoma* **O**
100/100/100   *Obelia geniculata* FR **O**
  Obelia geniculata NB **O**

99/94/100   *Obelia geniculata* JP **O**
85/67/100   *Obelia geniculata* NZ **O**

//61

100/100/100   *Laomedea flexuosa* FR **F**
100/100/100   *Laomedea flexuosa* IC **F**
  Laomedea flexuosa WS **F**

100/100/100   *Laomedea inornata* **F**

99/100/100   *Obelia longissima* AN **O**
  Obelia longissima WS **O**
84/96/89   *Obelia longissima* IC **O**
  Obelia longissima NZ **O**
  Obelia longissima MA **O**

Obeliinae

—— 0.01 substitutions/site

74

Figure 4. Maximum likelihood topology based on 18S rDNA sequences alone. The best-fit model selected by Modeltest was TIM + I + G. The –ln likelihood of the topology = 6469.86106. Numbers before the slash refer to parsimony bootstrap values and numbers after the slash refer to Bayesian posterior probabilities. Subfamily lineages are indicated. Location codes are as in Figure 2.

75
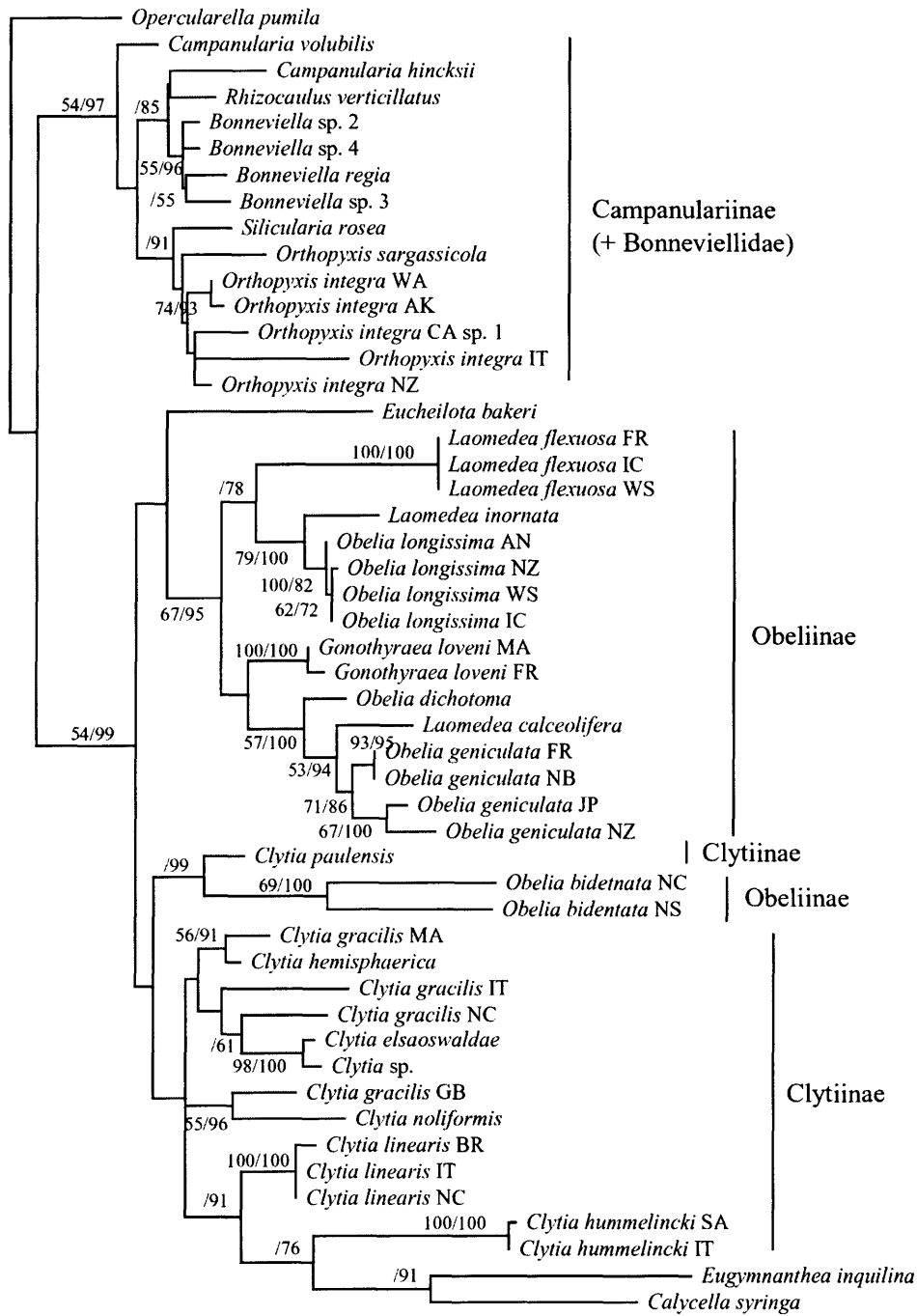
Figure 5. Maximum likelihood topology based on calmodulin sequences alone. The best-fit model selected by Modeltest was TrN + I + G. The –ln likelihood of the topology = 2975.97532. Numbers before the slash refer to parsimony bootstrap values and numbers after the slash refer to Bayesian posterior probabilities. Subfamily lineages are indicated. Location codes are as in Figure 2.

76
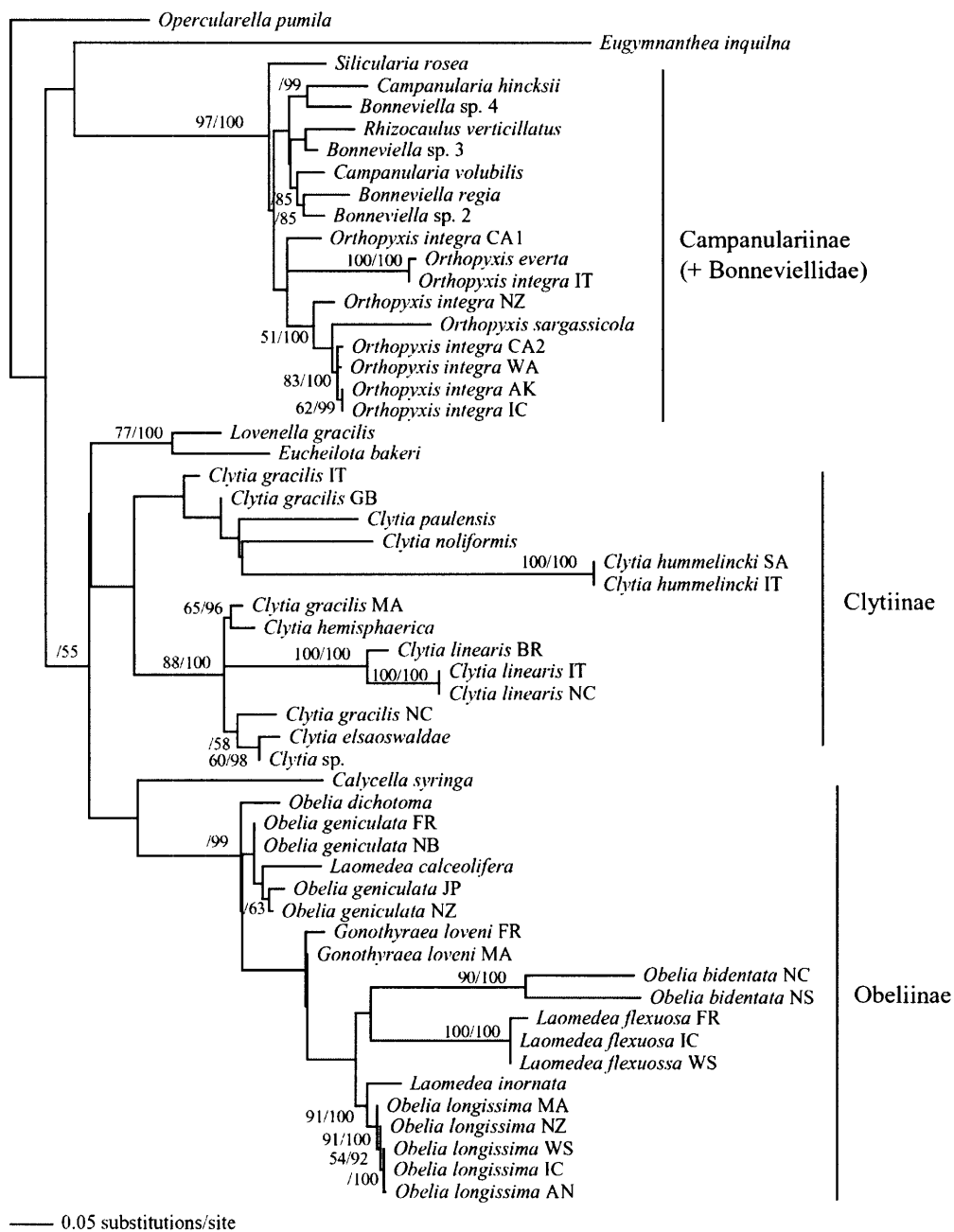
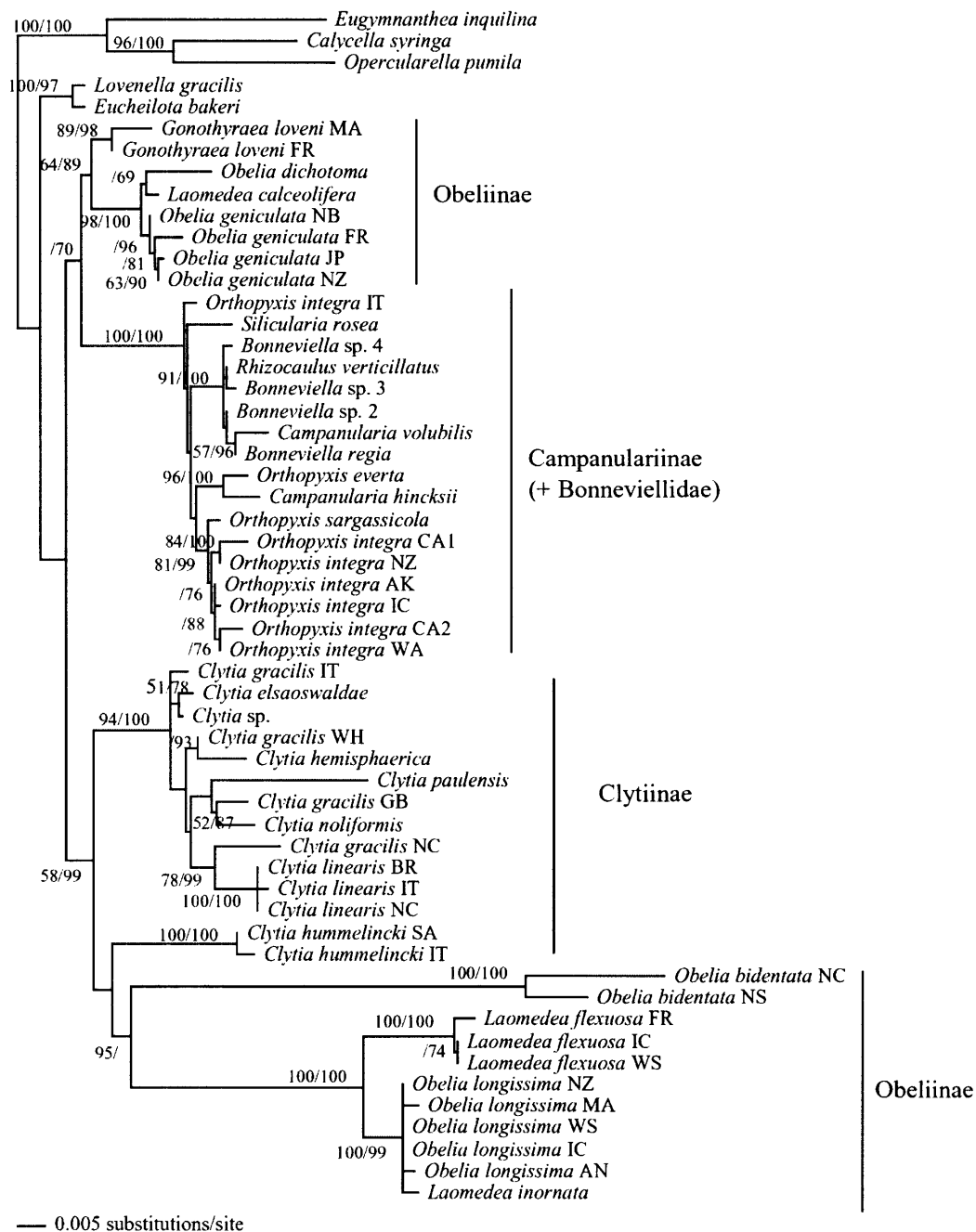**Figure 6.** Maximum likelihood topology based on 16S rDNA sequences alone. The best-fit model selected by Modeltest was GTR + I + G. The –ln likelihood of the topology = 2981.05837. Numbers before the slash refer to parsimony bootstrap values and numbers after the slash refer to Bayesian posterior probabilities. Subfamily lineages are indicated. Location codes are as in Figure 2.

Eugymnanthea inquilina

Calycella syringa

Clytia linearis NC | Clytiinae

Laomedea calceolifera

91/100

Obelia dichotoma

53/96

Obelia geniculata JP

84/100

Obelia geniculata NZ

60/91

Obelia geniculata FR

100/100 — Obelia geniculata NB

Obeliinae

100/100 — Laomedea flexuosa FR

Laomedea flexuosa IC

73/100

100/100 — Laomedea flexuosa WS

Obelia longissima NZ

100/100

Obelia longissima WS

Obelia longissima IC

90/100

Obelia longissima AN

Obelia bidentata NC

Obelia bidentata NS

Clytia gracilis IT

/98

Clytia paulensis

100/100 — Clytia hummelincki SA

Clytia hummelincki IT

Clytiinae

87/97 — Clytia elsaoswaldae

Clytia sp.

87/97

Clytia gracilis NC

Clytia gracilis MA

57/97 — Clytia hemisphaerica

Orthopyxis sargassicola

/96

100/100 — Orthopyxis everta

Orthopyxis integra IT

/100

Orthopyxis integra AK

99/90 — Orthopyxis integra IC

Campanulariinae

Orthopyxis integra NZ

Campanularia hincksii

Clytia gracilis GB | Clytiinae

Rhizocaulus verticillatus

Campanularia volubilis

58/100

Bonneviella sp. 4

Bonneviella sp. 2

/58

Bonneviella regia

Bonneviella sp. 3

Campanulariinae
(+ Bonneviellidae)

—— 0.05 substitutions/site

Figure 7. Maximum likelihood topology based on COI sequences alone. The best-fit model selected by Modeltest was GTR + I + G. The −ln likelihood of the topology = 6877.61370. Numbers before the slash refer to parsimony bootstrap values and numbers after the slash refer to Bayesian posterior probabilities. Subfamily lineages are indicated. Location codes are as in Figure 2.

78

Figure 8. Ancestral state reconstruction on the maximum likelihood topology based on the combined dataset in Figure 3. Gains = losses = 1.0.

Figure 9. Ancestral state reconstruction on the maximum likelihood topology based on the combined dataset in Figure 3. Gains = 1.1 – 1.4. Losses = 1.0.

80

Eugymnanthea inquilina
Calycella syringa
Opercularella pumila
Lovenella gracilis
Eucheilota bakeri
Campanularia hincksii
Campanularia volubilis
Rhizocaulus verticillatus
Bonneviella sp. 4
Bonneviella sp. 2
Bonneviella regia
Bonneviella sp. 3
Silicularia rosea
Orthopyxis everta
Orthopyxis integra IT
Orthopyxis integra CA1
Orthopyxis integra NZ
Orthopyxis sargassicola
Orthopyxis integra WA
Orthopyxis integra CA2
Orthopyxis integra AK
Orthopyxis integra IC
Obelia bidentata NC
Obelia bidentata NS
Clytia hummelincki SA
Clytia hummelincki IT
Clytia paulensis
Clytia gracilis IT
Clytia gracilis GB
Clytia noliformis
Clytia linearis BR
Clytia linearis IT
Clytia linearis NC
Clytia gracilis WH
Clytia hemisphaerica
Clytia gracilis NC
Clytia elsaoswaldae
Clytia sp.
Gonothyraea loveni MA
Gonothyraea loveni FR
Laomedea calceolifera
Obelia dichotoma
Obelia geniculata FR
Obelia geniculata NB
Obelia geniculata JP
Obelia geniculata NZ
Laomedea flexuosa FR
Laomedea flexuosa IC
Laomedea flexuosa WS
Laomedea inornata
Obelia longissima AN
Obelia longissima WS
Obelia longissima NA
Obelia longissima MA

■ Typical medusae
■ Medusoids
■ Meconidia
☐ Fixed gonophores
■ *Obelia* medusae
☐ Equivocal

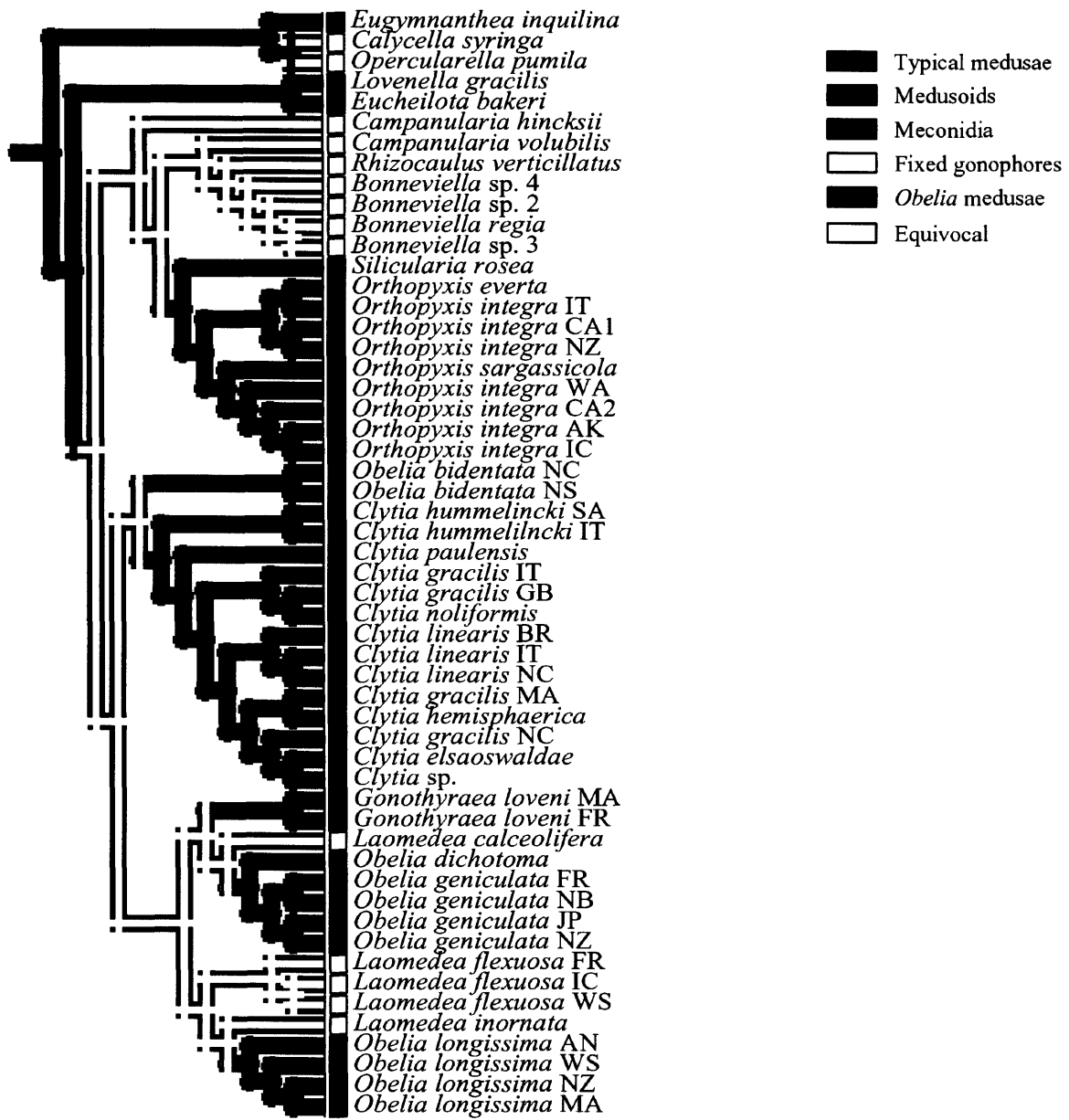**Figure 10.** Ancestral state reconstruction on the maximum likelihood topology based on the combined dataset in Figure 3. Gains = 1.5. Losses = 1.0.
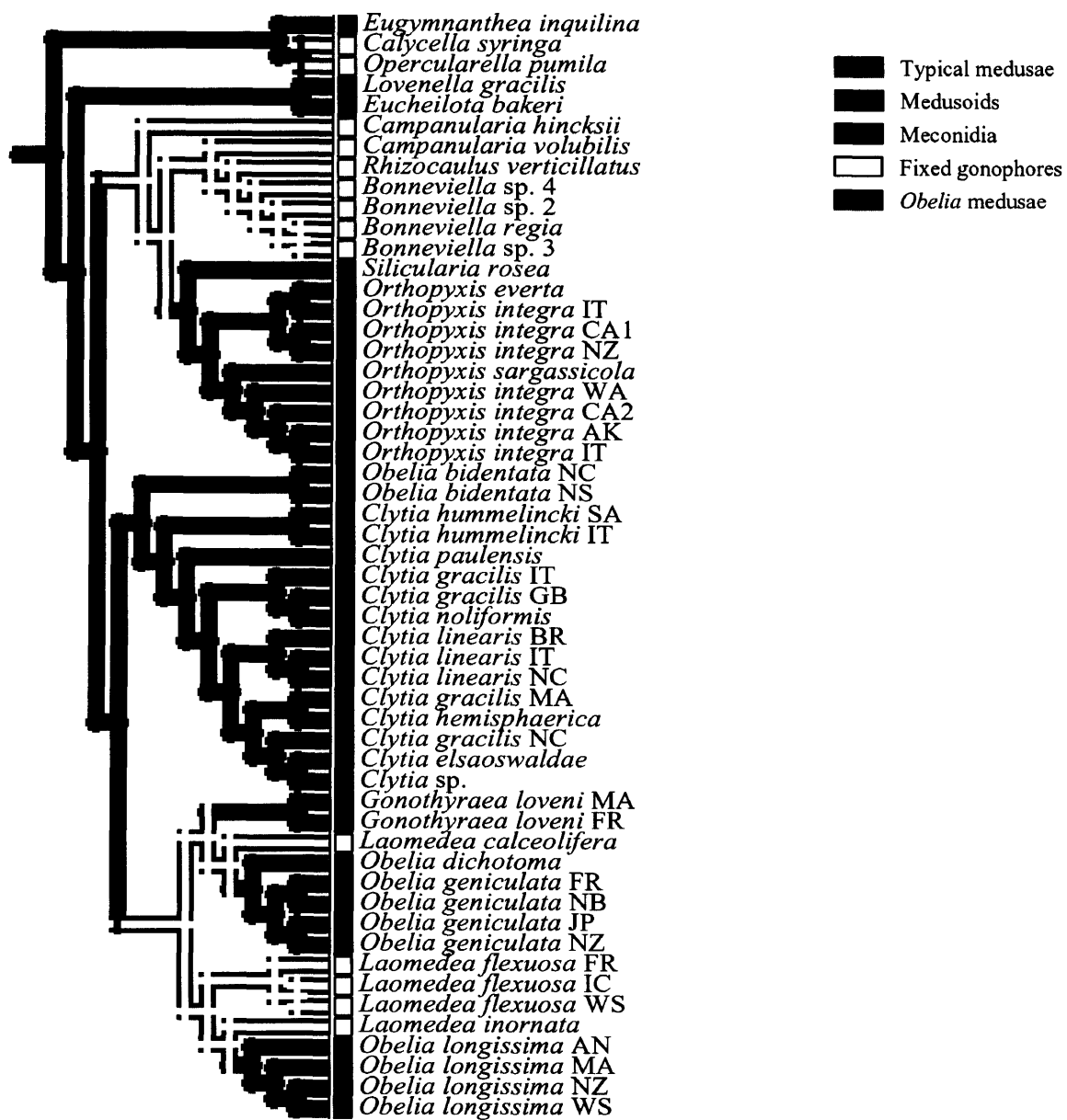
Figure 11. Ancestral state reconstruction on the maximum likelihood topology based on the combined dataset in Figure 3. Gains = 1.6 – 1.9. Losses = 1.0.
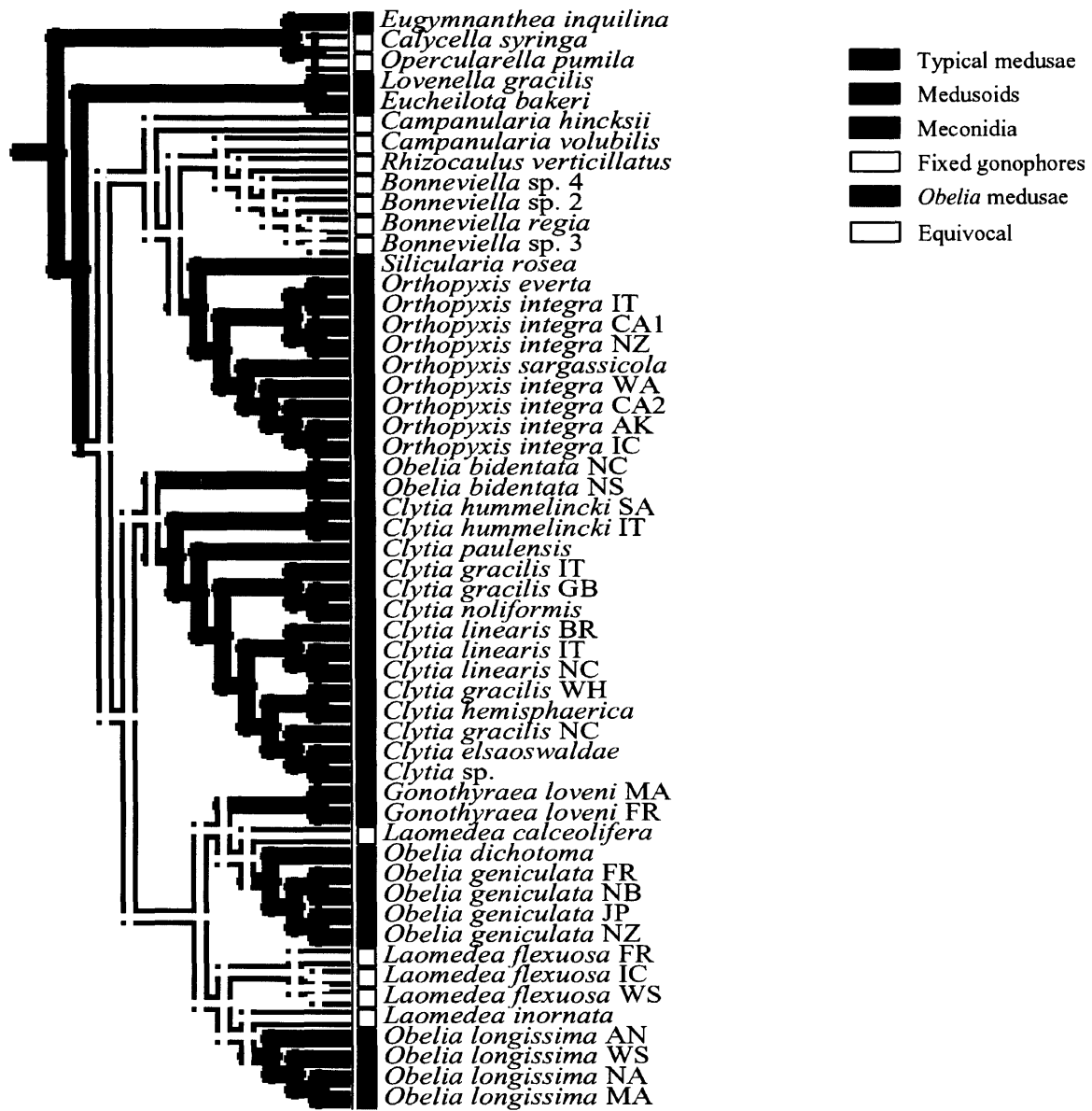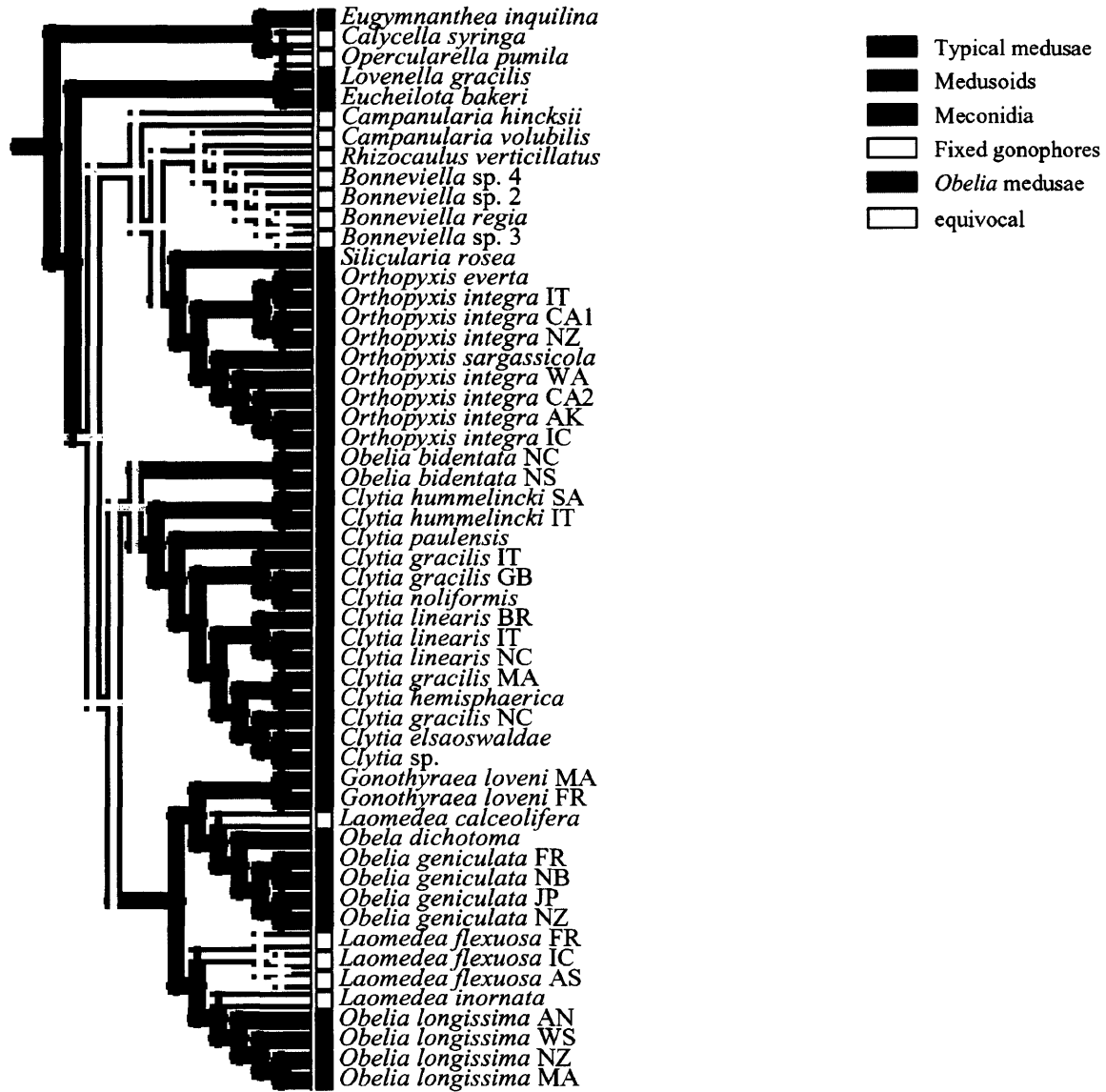
Figure 12. Ancestral state reconstruction on the maximum likelihood topology based on the combined dataset in Figure 3. Gains ≥ 2. Losses = 1.0.

Figure 13. Ancestral state reconstruction on the modified maximum likelihood topology. *Obelia bidentata* is placed as the basal member of the *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima* clade. Gains = losses = 1.0.

Eugymnanthea inquilina
Calycella syringa
Opercularella pumila
Lovenella gracilis
Eucheilota bakeri
Campanularia hincksii
Campanularia volubilis
Rhizocaulus verticillatus
Bonneviella sp. 4
Bonneviella sp. 2
Bonneviella regia
Bonneviella sp. 3
Silicularia rosea
Orthopyxis everta
Orthopyxis integra IT
Orthopyxis integra CA1
Orthopyxis integra NZ
Orthopyxis sargassicola
Orthopyxis integra WA
Orthopyxis integra CA2
Orthopyxis integra AK
Orthopyxis integra IC
Clytia hummelincki SA
Clytia hummelincki IT
Clytia paulensis
Clytia gracilis IT
Clytia gracilis GB
Clytia noliformis
Clytia linearis BR
Clytia linearis IT
Clytia linearis NC
Clytia gracilis MA
Clytia hemisphaerica
Clytia gracilis NC
Clytia elsaoswaldae
Clytia sp.
Gonothyraea loveni MA
Gonothyraea loveni FR
Laomedea calceolifera
Obelia dichotoma
Obelia geniculata FR
Obelia geniculata NB
Obelia geniculata JP
Obelia geniculata NZ
Obelia bidentata NC
Obelia bidentata NS
Laomedea flexuosa WS
Laomedea flexuosa IC
Laomedea flexuosa WS
Laomedea inornata
Obelia longissima AN
Obelia longissima WS
Obelia longissima NZ
Obelia longissima MA

■ Typical medusae
■ Medusoids
■ Meconidia
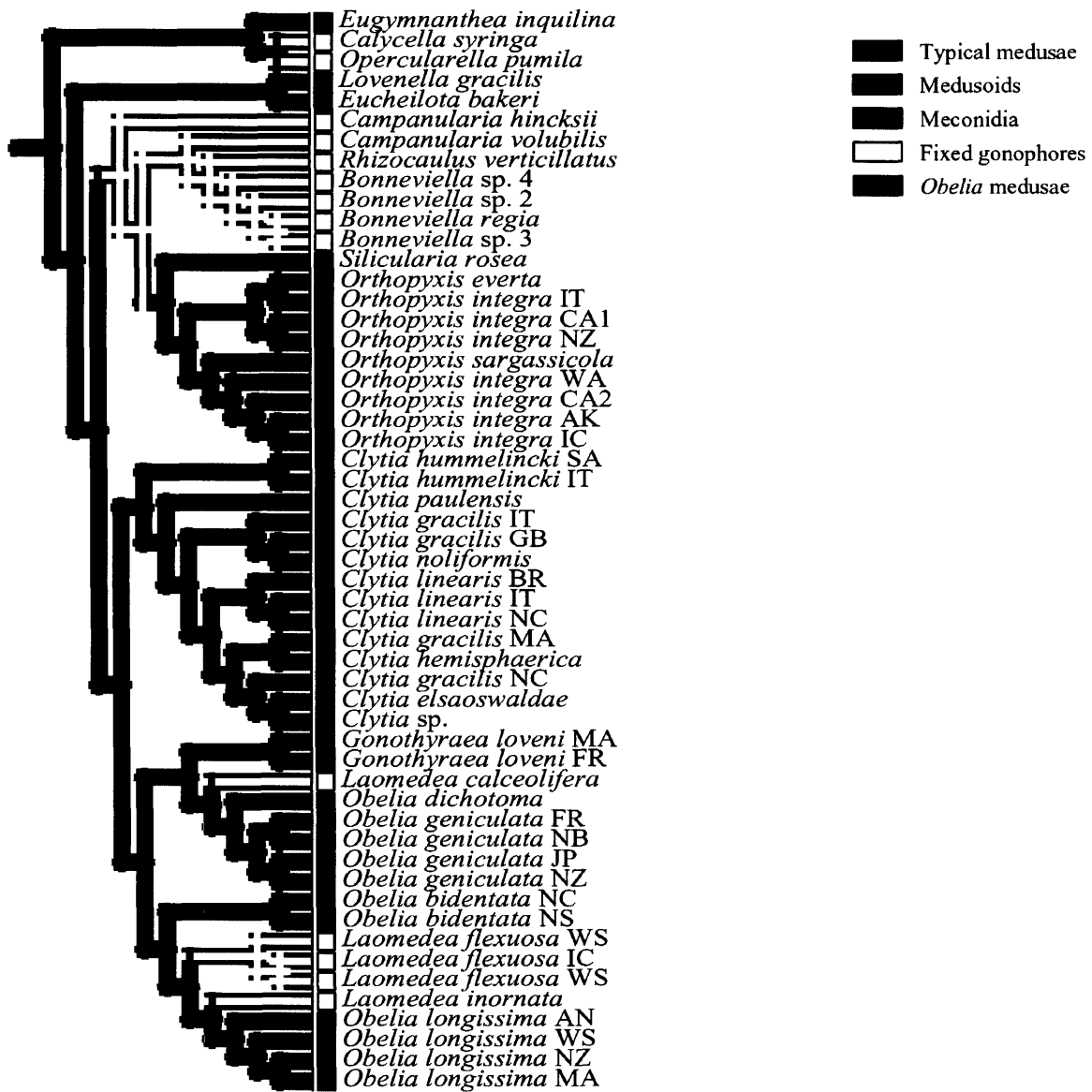☐ Fixed gonophores
■ *Obelia* medusae

Figure 14. Ancestral state reconstruction on the modified maximum likelihood topology. *Obelia bidentata* is placed as the basal member of the *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima* clade. Gains = 1.1 – 1.9. Losses = 1.0.
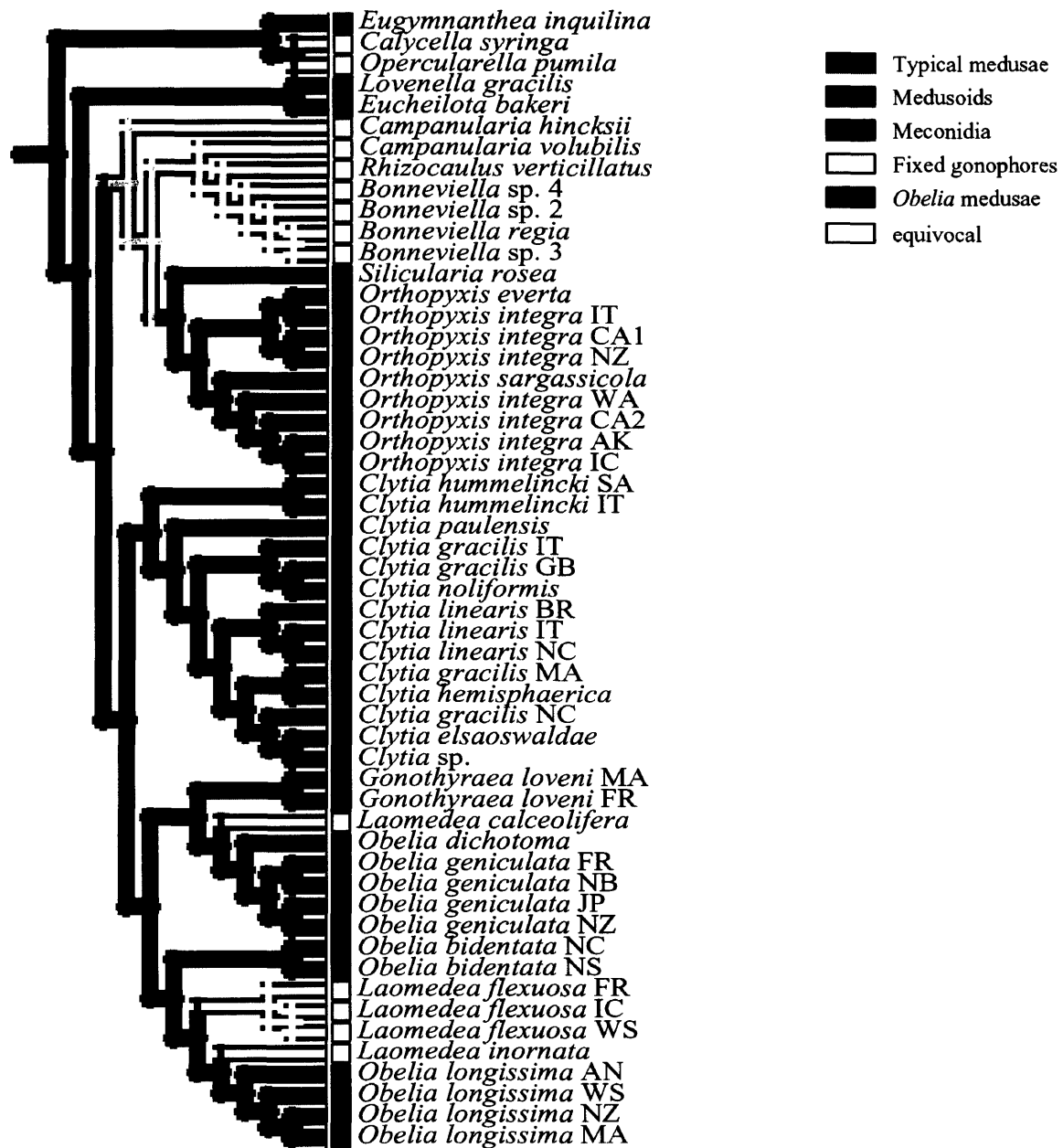
Eugymnanthea inquilina
Calycella syringa
Opercularella pumila
Lovenella gracilis
Eucheilota bakeri
Campanularia hincksii
Campanularia volubilis
Rhizocaulus verticillatus
Bonneviella sp. 4
Bonneviella sp. 2
Bonneviella regia
Bonneviella sp. 3
Silicularia rosea
Orthopyxis everta
Orthopyxis integra IT
Orthopyxis integra CA1
Orthopyxis integra NZ
Orthopyxis sargassicola
Orthopyxis integra WA
Orthopyxis integra CA2
Orthopyxis integra AK
Orthopyxis integra IC
Clytia hummelincki SA
Clytia hummelincki IT
Clytia paulensis
Clytia gracilis IT
Clytia gracilis GB
Clytia noliformis
Clytia linearis BR
Clytia linearis IT
Clytia linearis NC
Clytia gracilis MA
Clytia hemisphaerica
Clytia gracilis NC
Clytia elsaoswaldae
Clytia sp.
Gonothyraea loveni MA
Gonothyraea loveni FR
Laomedea calceolifera
Obelia dichotoma
Obelia geniculata FR
Obelia geniculata NB
Obelia geniculata JP
Obelia geniculata NZ
Obelia bidentata NC
Obelia bidentata NS
Laomedea flexuosa FR
Laomedea flexuosa IC
Laomedea flexuosa WS
Laomedea inornata
Obelia longissima AN
Obelia longissima WS
Obelia longissima NZ
Obelia longissima MA

Typical medusae
Medusoids
Meconidia
Fixed gonophores
Obelia medusae
equivocal

Figure 15. Ancestral state reconstruction on the modified maximum likelihood topology. *Obelia bidentata* is placed as the basal member of the *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima* clade. Gains ≥ 2.0. Losses = 1.0.
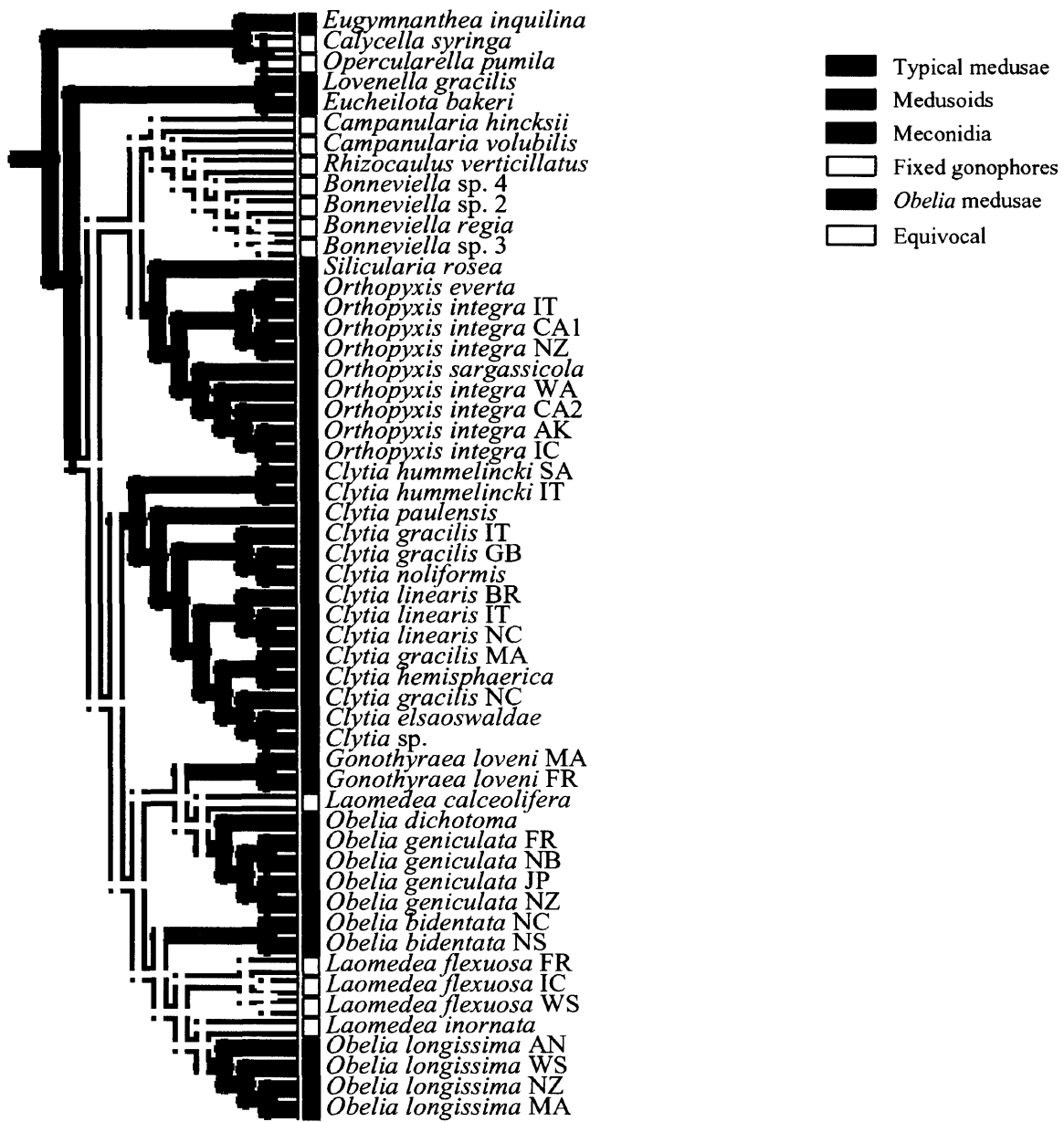
Figure 16. Ancestral state reconstruction on a modified maximum likelihood topology. *Obelia bidentata* is placed as the basal member of the *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima* clade, and *Campanularia hincksii* is placed as the basal member of the *Campanularia, Rhizocaulus,* and *Bonneviella* clade. Gains = losses = 1.0.

Figure 17. Ancestral state reconstruction on the modified maximum likelihood topology. *Obelia bidentata* is placed as the basal member of the *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima* clade. Life cycles are placed in 3 categories. Gains = losses = 1.0.

Figure 18. Ancestral state reconstruction on the modified maximum likelihood topology. *Obelia bidentata* is placed as the basal member of the *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima* clade. Life cycles are placed in 3 categories. Gains $\geq$ 1.1. Losses = 1.0.

Chapter 3

New taxonomic insights on the Campanulariidae (Cnidaria, Hydrozoa)*

*manuscript based on this chapter in preparation with Ferdinando Boero

Abstract

Taxonomy is particularly challenging in the widely distributed hydrozoan family
Campanulariidae (Cnidaria, Leptomedusae) because of their complex life cycles and the
difficulty in attributing morphological differences to genetic or environmental causes.
Traditional morphological characters in the hydroids are based on colony form and theca
characteristics. Nematocyst and hydranth features may be useful, but their utility is
limited because they are time consuming to study and require live material. Life cycle
type is often used to define genera, but frequent life cycle transitions could result in
paraphyletic classifications. DNA sequences have the potential to overcome many of the
shortcomings of traditional hydrozoan taxonomy because they are relatively independent
of environment and life cycle. Here, new taxonomic insights from a molecular phylogeny
based on 2 nuclear (18S rDNA and calmodulin) and 2 mitochondrial (16S rDNA and
cytochrome c I oxidase [COI]) genes are discussed. Some aspects of traditional taxonomy
are corroborated, while others are refuted. In particular, several species of *Bonneviella*,
previously composing the family Bonneviellidae, are shown to be deeply nested in the
Campanulariidae, and within the Obeliinae, multiple life cycle transitions have resulted in
paraphyletic genera. The family and generic definitions are revised accordingly.
Additionally, in some cases, nominal cosmopolitan species collected from multiple
locations are suggested to represent cryptic species, while *Obelia longissima* may be truly
cosmopolitan. Finally, new drawings of the obeliinid *Laomedea (Gonothyraea) inornata*
are presented, showing that what were previously described as meconidia (medusoids)
are really acrocysts (extracapsular development).

Introduction

The Campanulariidae- overview

  The Campanulariidae (Johnston, 1836) is a family of hydrozoans (Cnidaria, Leptomedusae) that are widely distributed throughout the world. It comprises approximately 50+ species, including the well known *Obelia*, which is commonly used as a model hydrozoan in introductory biology courses. Following Cornelius (1982) the Campanulariidae are divided into 3 subfamilies: the Campanulariinae, which includes *Orthopyxis, Silicularia, Campanularia,* and *Rhizocaulus*; the Clytiinae, which includes *Clytia*; and the Obeliinae, which includes *Obelia, Gonothyraea, Hartlaubella,* and *Laomedea*. Within each subfamily, genera are defined in part on the basis of life cycle, or gonophore, type. The purpose of this paper is to review campanulariid taxonomy and incorporate the molecular results of Chapter 2. Revisions of the Campanulariidae and the Obeliinae based on their strongly supported findings are presented.

How many species?

  The number of described campanulariid species is difficult to calculate because some nominal taxa may represent cryptic species complexes, while others may not be valid. The most recent revisions of the Campanulariidae were by Cornelius (1975; 1982; 1990). His revisions created considerable debate among the hydrozoan community because of his extensive synonymizations, particularly for *Obelia*. Among the *Obelia*, he merged 120 nominal species into 3 (Cornelius 1975), although he later increased that number to 4 (Cornelius, 1990). Other authors recognize additional species (e.g., Stepanjants, 1998; Kubota 1999; Bouillon and Boero, 2000).

  Cornelius's (1982) revision of the entire family dealt primarily with those found in the eastern North Atlantic, and the other campanulariid genera have not had a worldwide revision similar to that of *Obelia* (1975). Nominal taxa in these groups may not all represent valid species. In particular, some *Clytia* are known only from the hydroid or medusa stage, and elucidation of their complete life cycles will be important for a future revision (Bouillon and Boero 2000).

Species definitions in the Campanulariidae are primarily based on morphological differences (e.g., Cornelius, 1982), rather than breeding experiments, which would test the biological species concept. Breeding experiments are not feasible for most campanulariids as many, such as *Obelia*, are very difficult to keep in laboratory culture. Also, such experiments obviously require live material. Alternatively, lineage-based species concepts, such as the phylogenetic species concept, identify monophyletic clades (Sites and Crandall, 1997). Monophyletic patterns are often based on, but not limited to, mitochondrial DNA markers. The concordance concept looks for concordant patterns of monophyly using multiple independent markers (Avise, 2000). Lineage-based species concepts have not been applied to the Campanulariidae, however.

Taxonomic characters

Overall colony form and characteristics of the hydrotheca are important taxonomic characters in the Campanulariidae. These characteristics include whether the hydroid colony is reptant (creeping) or erect, the degree of branching, whether the stem is mono- or polysiphonic, hydrothecal dimensions, the type of hydrothecal margin, and the presence/absence of a subhydrothecal spherule (spherical annulation). These characters are useful because they remain intact in preserved specimens. However, they may also exhibit considerable phenotypic plasticity (Ralph, 1956; Cornelius, 1982). The degree to which differences in gross morphology are due to environmental or genetic causes is unknown, and this presents a major obstacle to their taxonomic use.

Morphological characteristics of the nematocysts, or stinging capsules, are taxonomically useful in many groups of hydroids (Gravier-Bonnet, 1987). Östman (1979; Östman 1982; Östman, 1983; Östman et al., 1987; Östman, 1987) has characterized nematocysts of Campanulariid hydroids, and found species-specific differences. However, the degree of phenotypic plasticity in nematocysts is unknown. In their comparison of conspecific Swedish and Italian Campanulariids, Östman et al. (1987) found size differences between nematocysts from northern and southern specimens, suggesting that some nematocyst characters can be plastic.

Hydranth characteristics may also be distinctive; for example, a peduncled (flared or trumpet-shaped), rather than conical, hypostome distinguishes campanulariids from other thecate hydroids. Frustrated with limitations of traditional taxonomic methods, Cornelius (1987a; 1987b) proposed using additional characters from live hydranths. In a preliminary survey of some European hydroids, including some campanulariids, he found that hydranth characters may be less variable than hydrothecal characters. A critical drawback for this approach is that live hydroids must be examined, because, when thecate hydroids are preserved, their hydranths contract into the hydrotheca, rendering them unavailable for study.

Östman (1982; 1983) examined differences in isoenzymes from Scandinavian campanulariids. She found inter- and intraspecific gel band differences for the enzyme acid phosphatase, and suggested that taken with other characters, isoenzyme banding patterns could be taxonomically useful. However, no further work since then has been done with isoenzymes.

Gonophore type as a generic character

An ongoing debate in hydrozoan systematics is whether to use gonophore (sexual stage) development as a generic character. Within families, hydroids bearing liberable medusae, medusoids, or fixed gonophores are frequently placed in separate genera. The use of gonophore type to define genera has been criticized because if gonophore transitions happen frequently, paraphyletic genera result (e.g., in the Tubulariidae [Petersen, 1990] and the Hydractiniidae [Cunningham and Buss, 1993]). Current classifications of the Campanulariidae use gonophore type, in part to define genera, although this approach has been recently questioned by Boero et al. (1996).

Campanulariid phylogeny

Molecular genetic data have the potential to clarify both campanulariid taxonomy and evolutionary relationships, because markers relatively independent of life cycle and environment are available. Govindarajan (Chapter 2) created a molecular phylogeny of

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the Campanulariidae based on 2 nuclear genes (18S rDNA and calmodulin) and 2 mitochondrial genes (16S rDNA and cytochrome c oxidase subunit I [COI]). Their results corroborate some aspects of morphological taxonomy but clearly refute others, indicating that the Campanulariidae, and possibly the entire Leptomedusae, need revision. Their results are summarized in Figures 1 and 2. Figure 1 shows the combined analysis of all 4 genes (corresponding to Figure 3 in Chapter 2), and Figure 2 shows the gonophore type mapped on to a bootstrap consensus tree and the ancestral character states reconstructed using parsimony (corresponding to Figure 14 in Chapter 2). Details of the analyses and figures of the data analyzed individually are in Chapter 2.

Molecular results

Family Campanulariidae

The family Campanulariidae plus *Bonneviella* spp. and exclusive of *Billardia* falls out as a monophyletic group, although it is not well supported by bootstrap analysis (Figure 1). The Campanulariidae appears to be closely related to *Eucheilota bakeri* (Eucheilotidae) and *Lovenella gracilis* (Lovenellidae). Interestingly, the hydroid of *Eucheilota bakeri* was originally placed in the Campanulariidae, as *Clytia bakeri* (Torrey, 1904). However, the medusa displays characters typical of the Eucheilotidae (with lateral cirri), where it was placed by Mayer (1910) and Kramp (1961).

Despite their placement in different families, the hydroids of *Eucheilota bakeri*, found only in southern California and Mexico (Fraser, 1946), and *Lovenella gracilis*, found only in the southeastern USA (Calder, 1971), display many morphological and ecological similarities. Both live on bivalves inhabiting sandy beaches, which is an unusual habitat for hydroids. Morphologically, they are similar, although *Lovenella gracilis* hydroids have an operculum (covering of the hydranth opening), while *Eucheilota bakeri* hydroids, like campanulariid hydroids, do not (Calder, 1971). In addition to their morphological and ecological similarities, the relatively little sequence divergence between the two could suggest they are sister taxa, perhaps separated by the Isthmus of Panama. Further study with additional leptomedusan taxa is necessary to

understand the relationship between *Eucheilota bakeri* and *Lovenella gracilis*, and their relationship with the Campanulariidae.

Family Campanulariidae: the Bonneviellidae

Several species of *Bonneviella* (Bonneviellidae) which were originally designated as outgroups (Chapter 2), fall inside the Campanulariidae (Figure 1). The Bonneviellidae Broch, 1909 is composed of 8 species belonging to the genus *Bonneviella*. All are found in the North Pacific, and one species, *B. grandis*, is also found in the North Atlantic (Nutting, 1915; Naumov, 1960). All possess fixed gonophores rather than medusae or medusoids. The Bonneviellidae is traditionally distinguished from the Campanulariidae by the absence of a peduncled hypostome and the presence of a preoral gastric cavity (Nutting, 1915). Nutting (1915) agreed with Broch (1909) that *Bonneviella* possess a "veloid", or membrane, that divides the gastric cavity. Nutting (1915) further suggested that they have no real proboscis (hypostome), but rather the oral surface is a depression whose lowest point is the mouth. Naumov (1960), however, pointed out that the veloid is actually a typical conical hypostome, and it is actually the tentacle bases projecting into the gastric cavity that divide it. Bouillon (1985), placing the Bonneviellidae in the order Proboscoida Broch, 1909 and superfamily Campanulariidea Bouillon, 1984, suggested affinities with the Campanulariidae and the Lafoeidae.

The molecular phylogeny strongly places 4 *Bonneviella* species deeply nested in the Campanulariidae, in the Campanulariinae (Figure 1). Their inclusion indicates that the peduncled hypostome is not a campanulariid synapomorphy, as long believed (Nutting, 1915). *Bonneviella* appears to be closely related to *Campanularia volubilis* and *Rhizocaulus verticillatus*, which were also collected in the North Pacific.

Family Campanulariidae: Billardia

The genus *Billardia* was first identified and placed in the Campanulariidae by Totton (1930). Currently, it is composed of 3 species found only in the southern hemisphere: *Billardia intermedia* (Blanco, 1967), *B. novaezealandiae* (Totton, 1930), and

B. subrufa (Jäderholm, 1905). Millard (1975) suggested *Billardia* may have affinities with the Lafoeidae, based on their curved bilaterally symmetrical hydrothecae, or the Syntheciidae, based on their occasionally slightly adnate (side partially in contact with stem) hydrothecae. Cornelius (1982) pointed out that if, as Totton (1930) suggested, the blastostyles (gonophores) were produced in place of hydranths, within the hydrothecae, then *Billardia* belongs in the Syntheciidae sensu Millard (1975) rather than the Campanulariidae. The sertulariid genus *Fraseroscyphus* also has gonophores in place of hydrothecae (Boero and Bouillon, 1993). However, *Billardia* has been placed in the Campanulariidae by Ralph (1957), Blanco (1967), Vervoort (1972), and most recently, Vervoort and Watson (2003). The molecular phylogeny, however, (Figure 2) strongly indicates that *Billardia* does not belong in the Campanulariidae, although additional analyses are necessary to examine its placement in the Lafoeidae or Syntheciidae.

The Campanulariinae

The Campanulariinae, including *Bonneviella*, is a strongly supported clade (Figure 1). Within the Campanulariinae clade, many relationships are not that well supported and additional generic markers will probably be necessary to make definitive conclusions.

The *Bonneviella* species form a monophyletic clade, although it is not supported by bootstrap analysis. However, they appear to be closely related to two other species collected in the North Pacific, *Campanularia volubilis* and *Rhizocaulus verticillatus* (Figure 1). Although the position of *Bonneviella* within the Campanulariidae was unexpected, Rees and Thursfield (1967) noted similarities between *Campanularia volubilis and Rhizocaulus verticillatus*. They speculated that *Campanularia volubilis* and *Rhizocaulus verticillatus* might actually be conspecific, although Cornelius (1982) disagreed. While a close relationship between the two is evidenced by the molecular phylogeny, they do not fall out together as would be expected if they were conspecific.

The frequency of medusoid reduction in the Campanulariinae cannot be determined from the molecular phylogeny because of inadequate phylogenetic resolution,

uncertainties in assumptions of ancestral state reconstruction, and possible bias in taxon sampling (Chapter 2). While most fixed gonophore-bearing species fall together in a monophyletic clade (*Bonneviella, Campanularia volubilis, Rhizocaulus verticiillatus*), the apparent monophyly may represent a single, geographically restricted radiation. Members of this clade were all collected in the North Pacific, while *Campanularia hincksii*, the only other fixed gonophore-bearing species included in the phylogeny, comes from the Mediterranean, and falls outside this clade (although its position is not well supported). *Campanularia* species from other parts of the world (e.g., Millard, 1975) could not be obtained for this study and additional sampling on a global scale is necessary.

    Orthopyxis integra is considered to be an exceptionally morphologically variable, cosmopolitan species (Cornelius, 1982). The molecular results suggest that *O. integra* could be a complex of cryptic species. *O. integra* from several locations do not form a monophyletic clade, although specimens from some locations in the North Pacific and North Atlantic appear to fall out together, as do *O. integra* from New Zealand and California (Figure 1). *O. integra* from Italy (formerly *O. asymmetrica*) falls outside this clade and appears allied with another Italian *Orthopyxis, O. everta*. A second form of *O. integra* from Italy, which grows on a variety of substrates as opposed to only on the seagrass *Posidonia* like the *O. asymmetrica* form, was not examined. More samples, especially multiple samples from within locations, are necessary to determine whether the *O. integra* clade represents one or a group of closely related species. Species boundaries should be examined by looking for patterns of reciprocal monophyly with molecular data (e.g., Govindarajan et al., submitted; Chapter 4).

The Obeliinae and Clytiinae

    A close relationship between the Clytiinae and Obeliinae is suggested, although the arrangement of the clades does not have much bootstrap support (Figure 1). This is consistent with the hypothesis of Boero et al. (1996) and in contrast to Cornelius (1982). The implications of this relationship for life cycle evolution are discussed in Chapter 2 and partially summarized in Figure 3. *Clytia* is monophyletic (but note low bootstrap

support for the position of *Clytia hummelincki*), although appears nested within the Obeliinae due to the basal position of *Obelia bidentata* in the maximum likelihood topology (tree) (Figure 1). However, parsimony analysis places *Obelia bidentata* in the basal position of the *Laomedea flexuosa, Laomedea inornata*, and *Obelia longissima* clade (Figure 1; Figure 3). *Obelia bidentata* DNA sequences appear to have very long branch lengths, indicating a lot of change, and making it difficult to ascertain their position in the phylogeny. Additional markers will be necessary to determine the position of *Obelia bidentata* with certainty.

Within the *Clytia* clade, *Clytia hummelincki* is basal (again noting this position has low bootstrap support). *Clytia hummelincki* possesses a typical *Clytia* medusa, although its hydroid has a subhydrothecal spherule (spherical thickening underneath hydranth), typical of most Campanulariinae. The ancestral Campanulariid may have had a form like *Clytia hummelincki*, with one descendent lineage becoming the Campanulariinae, and the other the Clytiinae + Obeliinae. However, the molecular results do not provide much support for the position of *Clytia hummelincki* and the relationship between the *Clytia*, Obeliinae and Campanulariinae lineages, so this hypothesis remains to be tested.

Clytia gracilis collected from different localities do not form a monophyletic clade, indicating that it, like *Orthopyxis integra*, likely represents several cryptic species (Figure 1). Interestingly, Cornelius (1982) synonomized *Clytia gracilis* with the cosmopolitan *Clytia hemisphaerica*, but subsequently *Clytia gracilis* was shown to be distinct on the basis of nematocysts by Östman (summarized in Östman, 1987). In a detailed morphological and life cycle study, Lindner et al. (in prep) showed that a Brazilian "*Clytia gracilis*" was distinct and resurrected the name *Clytia elsaoswaldae* for it. Additional investigations of samples from throughout its global range are necessary to assess its species-level diversity.

There are two main Obeliinae clades. The first includes *Gonothyraea loveni*, *Obelia dichotoma*, *Obelia geniculata*, and *Laomedea calceolifera*. The second clade includes *Obelia longissima, Laomedea flexuosa*, and *Laomedea inornata*. *Obelia*

bidentata has a very long branch length, indicating a lot of change, but may be allied with the second clade (see above; and also based on the 18S data alone [Chapter 2]). There is some morphological and physiological evidence supporting the existence of the two Obeliinae clades. First, the medusae of *Obelia geniculata* and *O. dichotoma* are identical, and specimens identified from the medusa stage as *Obelia lucifera* are thought to consist of both *O. geniculata* and *O. dichotoma* individuals (Cornelius, 1975). *Obelia longissima* medusae may differ slightly from nominal *O. lucifera* medusae (Cornelius, 1990; Kubota 1999). Secondly, Miller (1973) found that gametes from *Gonothyraea loveni* and *Laomedea calceolifera* were attracted to each other, but that gametes from *Laomedea calceolifera* and *Laomedea flexuosa* were not attracted to each other.

The phylogeny suggests that *Obelia* medusae were likely reduced multiple times (Figure 3). The alternative, that medusae were reduced only once, requires multiple independent origins of *Obelia* medusae and so seems less probable if it is assumed that gains in structure are even slightly less likely than losses (Chapter 2). In any case, the obeliinid genera are clearly not monophyletic. Therefore, these results indicate that the taxonomic practice of using gonophore type to define genera is not appropriate in the Obeliinae.

Obelia longissima – *a true cosmopolite?*

Cornelius (1975) synonymized *Obelia longissima*, as well as many other nominal *Obelia* species, into *Obelia dichotoma*. Based on nematocyst differences and electrophoretic patterns, Östman subsequently (1979, 1982a,b, 1983 a,b) showed that *O. longissima* was distinct, and this was recognized by Cornelius (1990; 1995). The molecular results confirm that *Obelia longissima* and *Obelia dichotoma* are distinct; indeed, they fall in different Obeliinae lineages.

O. longissima specimens from the North Atlantic (MA, USA and Iceland), the White Sea, the Antarctic peninsula, and New Zealand were included in the molecular phylogeny (Figure 1). The results indicate a highly supported monophyletic clade with very little sequence divergence in all 4 genes from these widely separated localities,

consistent with what would be expected for a cosmopolitan species. *Obelia longissima* has the potential for long distance dispersal via its hydroids (rafting on algae) and planktonic medusae, although such high dispersal potential may not always be realized (Govindarajan et al., submitted; Chapter 4). An alternative possibility is that the global distribution of *Obelia longissima* might be the result of several anthropogenic introductions, but it should be noted that one of the locations (off of the Antarctic peninsula, Govindarajan et al. in prep) is not near a major port.

In contrast, comparison of mitochondrial genes of *Obelia geniculata* from the North Atlantic, North Pacific (Japan), and South Pacific (New Zealand) suggest that *O. geniculata* is composed of at least 3 cryptic species (Govindarajan et al., submitted; Chapter 4). Surprisingly, of the all the synonomizations in Cornelius (1975), *O. geniculata* generated the least controversy (Cornelius, 1990). Thus, of the 4 species of *Obelia* recognized by Cornelius (1990; 1995), some, like *O. longissima*, may be truly cosmopolitan, while others, like *O. geniculata*, appear to represent multiple cryptic species. In particular, it will be interesting to compare *O. dichotoma* specimens from multiple localities, as this species is the most variable and has the most synonyms.

Laomedea inornata *and acrocysts*

The Obeliinid *Gonothyraea inornata* Nutting 1901 was first discovered in Alaska, and later found in China (Hargitt, 1927), Washington (Fraser, 1937) and Argentina (Blanco, 1968). It was placed in the genus *Gonothyraea* because the gonosome has an acrocyst (external capsule on top of the gonotheca), which was thought to be similar to meconidia, although it possesses no medusoid features (Nutting, 1915). Inspection of fertile colonies obtained from a floating dock in Friday Harbor, WA confirmed that this structure is a simple acrocyst (extracapsular development; Figure 4) and it is referred to as *Laomedea inornata* in Chapter 2 and Figure 1.

This species is similar to *Laomedea neglecta* , which is found in the North Atlantic. *L. neglecta* also posesses an acrocyst, but differs from *L. inornata* in that the hydrothecal margin is toothed, although it sometimes abrades (Cornelius, 1982). Also *L.*

neglecta acrocysts contain relatively few ova (3 reported by Wright, 1863), while *L. inornata* can contain more (6-8 observed; Figure 3).

The acrocyst has been postulated to be an intermediate stage in a series of gonophore reduction, between *Gonothyraea* and other *Laomedea* and *Hartlaubella* (Cornelius, 1982). Molecular analysis indicates that there is neither a straightforward series of gonophore reductions nor any affinities between the acrocyst-bearing *L. inornata* with the meconidia-bearing *Gonothyraea*. Rather, *L. inornata* appears to be the direct result of a medusa reduction of *Obelia longissima*. Additional studies are necessary to determine if *L. inornata* and *L. neglecta* are closely related and the acrocyst evolved only once, or if *L. inornata* and *L. neglecta* are relatively distant and the acrocyst evolved multiple times. Given that medusa reduction apparently occurred multiple times in the Obeliinae, a single origin for the acrocyst should not be assumed.

Campanulariid revision

Here, we present a revision of the Campanulariidae, in light of the molecular phylogeny. The diagnoses are modified from Cornelius (1982) and Bouillon and Boero (2000) to incorporate the new findings. This entailed two major changes which were highly supported: (1) inclusion of the Bonneviellidae, as the genus *Bonneviella* in the Campanulariinae; and (2) merging the Obeliinae genera (*Gonothyraea, Laomedea, Obelia,* and *Hartlaubella*) into a single genus, *Obelia*. Subfamily and generic definitions from Cornelius (1982) and Bouillon and Boero (2000) that were not changed are not presented.

Family Campanulariidae Johnston, 1836

Diagnosis

Medusa: (From Bouillon and Boero, 2000) When present, Leptomedusae with short manubrium; without gastric peduncle; typically with 4 radial canals but sometimes more; with or without velum; with gonads on radial canals, completely surrounding them and separated from the manubrium; hollow or solid tentacles; with or without tenon-like

rudimentary bulbs; without marginal cirri; without excretory papillae or pores; numerous (8-200) closed velar marginal statocysts; no ocelli.

Hydroid: (Modified from Cornelius, 1982) Hydroids forming erect or stolonal colonies; hydrothecae bell-shaped or campanulate, radially or secondarily bilaterally symmetrical; pedicellate, rim cusped or not, lacking operculum, with basal diaphragm or inward annular projection of perisarc; nematophores absent, hydranth when known generally tubular with flared or globose hypostome delimiting a "buccal cavity", with one whorl of filiform tentacles, gastric endoderm of uniform structure; hydrothecal spherules present or not; with free medusae, medusoids, or sporosacs.

Remarks

The medusa diagnosis is not changed from Bouillon and Boero (2000). The hydroid diagnosis is modified to include the Bonneviellidae, which possess conical, rather than peduncled, hypostomes and have a pre-oral gastric cavity. The removal of *Billardia* from the Campanulariidae does not require a change in diagnosis. The molecular phylogeny (Figure 1) also showed a close relationship with *Eucheilota bakeri* and *Lovenella gracilis*. Their potential placement inside the Campanulariidae cannot be ruled out at this time and should be further investigated in the future.

Subfamily Campanulariinae

Diagnosis: Campanulariid hydroids with reptant or branching colonies. Stem usually monosiphonic but polysiphonic in *Rhizocaulus* and some *Bonneviella*. Subhydrothecal spherule usually present. No true hydrothecal diaphragm. Hypostome usually peduncled but conical in *Bonneviella*. Pre-oral gastric cavity in *Bonneviella*. With medusoids that may or may not be released, or fixed gonophores.

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Genus *Bonneviella* Broch, 1909

Diagnosis: Campanulariinid hydroids with conical hypostome and the tentacle bases projecting into the gastric cavity, forming a pre-oral gastric cavity (Naumov, 1960). Subhydrothecal spherule may not be present. With fixed gonophores.

Remarks: If further molecular and morphological investigation indicate that medusoid reduction in the Campanulariinae occurred multiple times, the *Bonneviella* and other campanulariinid genera be combined into a single genus, such as in the Obeliinae (below). At present, however, insufficient taxon sampling and resolution in the molecular phylogeny preclude such a change. With the exception of *Campanularia hincksii*, the molecular phylogeny places the fixed-gonophore bearing Campanulariinae in a single monophyletic clade. However, members of this clade are all of a North Pacific origin, and so may be the result of a single radiation. The position of *Campanularia hincksii*, obtained from Italy, is not well supported. Additional sampling on a global basis and possibly the use of new markers are necessary to determine the frequency of medusoid reduction in the Campanulariinae.

Subfamily Obeliinae

Diagnosis: Campanulariid hydroids with erect hydrocauli forming branched or unbranched, mono- or polysiphonic colonies; true hydrothecal diaphragm; no sub-hyrothecal spherule; stolon not anastomosing; with liberable medusae, medusoids, or fixed gonophores. Campanulariid medusae without velum and with solid tentacles.

Remarks. The diagnosis combines the definitions in Cornelius (1982) and Bouillon and Boero (2000). No new modifications are presented here. The molecular phylogeny suggests a close relationship with *Clytia*, consistent with the scheme presented by Boero et al. (1996), rather than as independent lineages suggested by Cornelius (1982). Indeed, the molecular phylogeny suggests that the Obeliinae and Clytiinae may be paraphyletic

with respect to each other; however, the arrangement of their respective lineages is not well resolved. Therefore, no changes at the subfamily level are undertaken here.

Genus *Obelia* Peron and Lesueur, 1810

Obelia Peron and Lesueur, 1810

Gonothyraea Allman, 1864

Hartlaubella Poche, 1914

Laomedea Lamouroux, 1812

Diagnosis

Hydroid – Colonies erect, branching or unbranching; stolon not anastomosing; hydrocaulus simple or polysiphonic; subhydrothecal spherule always absent; perisarc thickness variable; hydrothecal margin variable (smooth, wavy, or toothed); radially symmetric; hydranth fully retractable into hydrotheca.

Medusae/sexual stage – Medusae, meconidia, or fixed sporosacs, occasionally with acrocysts. Medusae when present with solid tentacles; 16 or more tentacles present upon release; velum absent; flat; one quadrangular manubrium with 4 lips; 4 radial canals; no lateral cirri; and 8 marginal statocysts.

Remarks

Cornelius (1999) justifies the existence of a separate genus for *Hartlaubella* also because of its polysiphonic stem. However, older *Obelia bidentata* colonies have polysiphonic stems as well as occasionally *Obelia dichotoma* and *Obelia longissima*. *Gonothyraea* is separated from *Obelia* solely by its meconidia, while *Hartlaubella* is distinguished by its fixed gonophores, and also by its polysiphonic stem.

Acknowledgements

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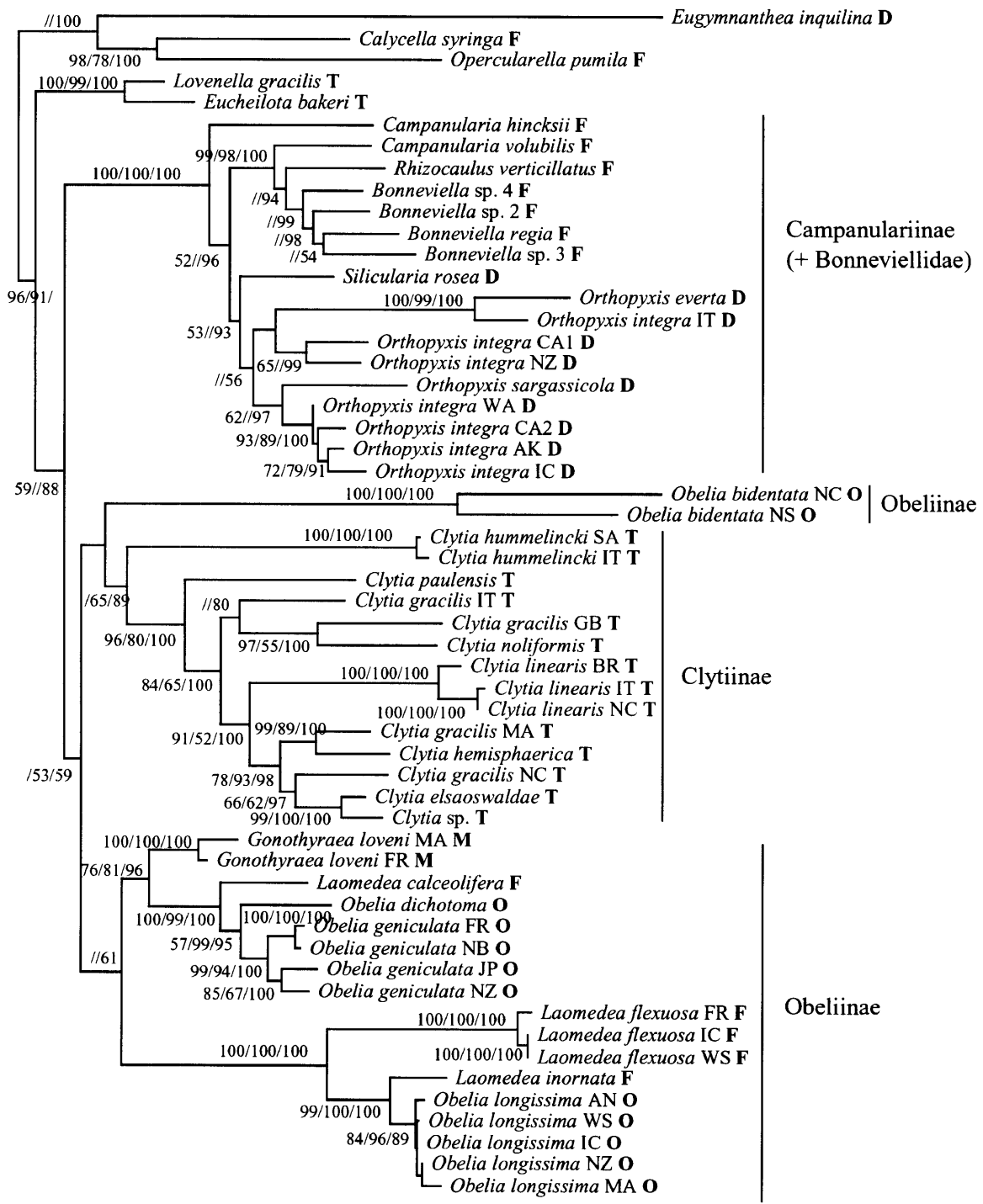
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Figure 1. Maximum likelihood phylogeny from a heuristic search based on the combined 18S rDNA, calmodulin, 16S rDNA, and COI sequences (Figure 3 in Chapter 2). First number refers to maximum likelihood bootstrap, second number refers to parsimony bootstrap, and third number refers to Bayesian posterior probability. In contrast to the likelihood topology shown here, parsimony analyses placed *Obelia bidentata* as the basal member of the clade with *Laomedea flexuosa*, *Laomedea inornata*, and *Obelia longissima* (parsimony bootstrap value = 79). The 18S rDNA sequences alone also strongly support this position for *Obelia bidentata* (Figure 4 in Chapter 2). Subfamily lineages are indicated. Location codes: AN=Antarctic peninsula; AS=Aleutians; BR=Brazil; CA=California; FR=France; GB=Georges Bank; IC=Iceland; IT=Italy; JP=Japan; MA=Massachusetts; NB=New Brunswick; NJ=New Jersey; NZ=New Zealand; NC=North Carolina; NS=North Sea; SA=South Africa; WA=Washington. Gonophore type indicated in bold after the taxon name. T = typical medusae; D = medusoids; F = fixed gonophores; M = meconidia; O = *Obelia* medusae.
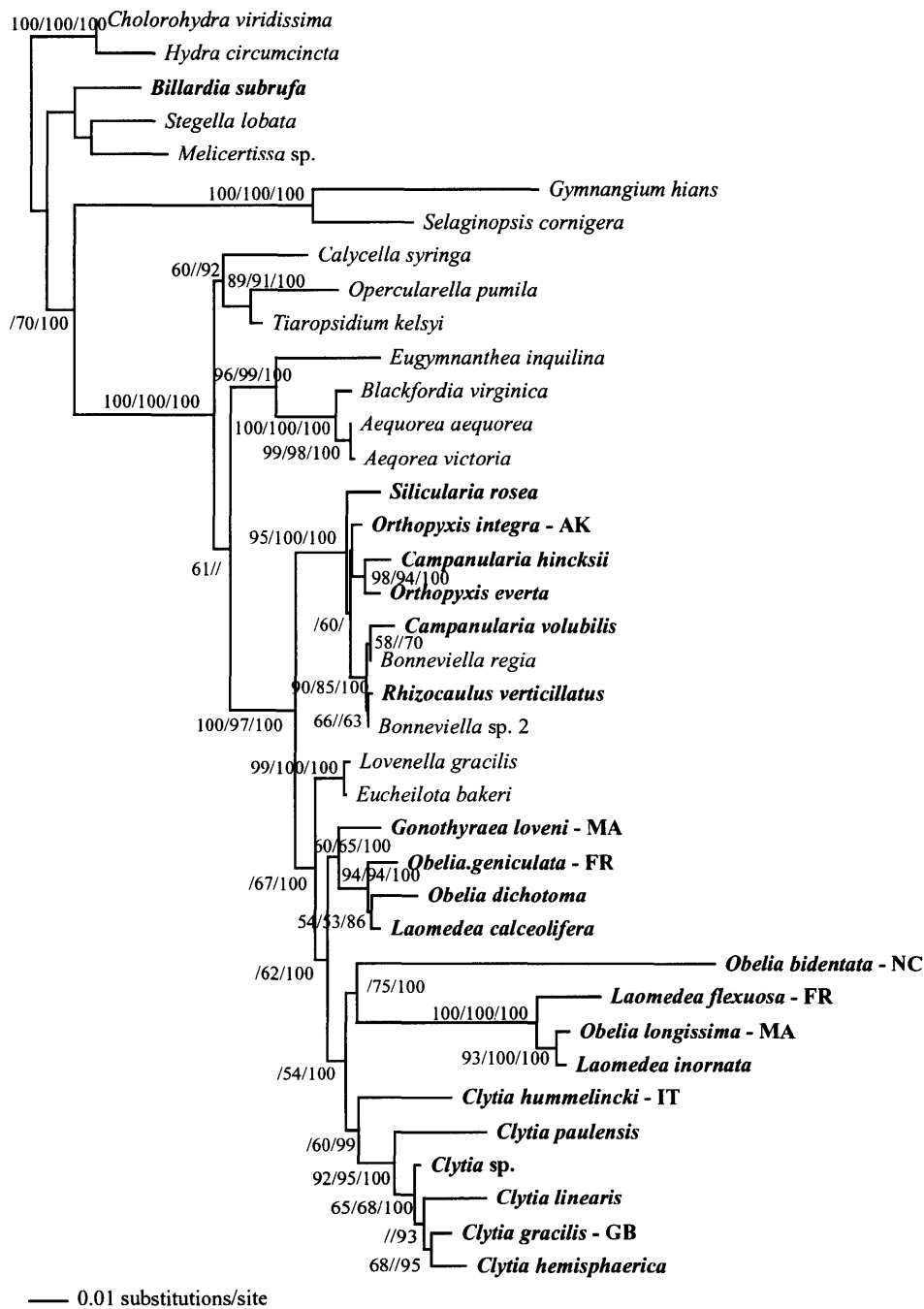
112

//100 — *Eugymnanthea inquilina* **D**

98/78/100 — *Calycella syringa* **F**
— *Opercularella pumila* **F**

100/99/100 — *Lovenella gracilis* **T**
— *Eucheilota bakeri* **T**

100/100/100

99/98/100 — *Campanularia hincksii* **F**
— *Campanularia volubilis* **F**
— *Rhizocaulus verticillatus* **F**

//94 — *Bonneviella* sp. 4 **F**
//99 — *Bonneviella* sp. 2 **F**
//98 — *Bonneviella regia* **F**
//54 — *Bonneviella* sp. 3 **F**

52//96

— *Silicularia rosea* **D**

100/99/100 — *Orthopyxis everta* **D**
— *Orthopyxis integra* IT **D**

53//93 — *Orthopyxis integra* CA1 **D**
65//99 — *Orthopyxis integra* NZ **D**

//56 — *Orthopyxis sargassicola* **D**
— *Orthopyxis integra* WA **D**
62//97 — *Orthopyxis integra* CA2 **D**
93/89/100 — *Orthopyxis integra* AK **D**
72/79/91 — *Orthopyxis integra* IC **D**

96/91/

Campanariinae
(+ Bonneviellidae)

59//88

100/100/100 — *Obelia bidentata* NC **O**
— *Obelia bidentata* NS **O**

Obeliinae

/65/89

100/100/100 — *Clytia hummelincki* SA **T**
— *Clytia hummelincki* IT **T**

96/80/100 — *Clytia paulensis* **T**
//80 — *Clytia gracilis* IT **T**
97/55/100 — *Clytia gracilis* GB **T**
— *Clytia noliformis* **T**

84/65/100

100/100/100 — *Clytia linearis* BR **T**
100/100/100 — *Clytia linearis* IT **T**
— *Clytia linearis* NC **T**

99/89/100 — *Clytia gracilis* MA **T**
91/52/100 — *Clytia hemisphaerica* **T**

78/93/98 — *Clytia gracilis* NC **T**
66/62/97 — *Clytia elsaoswaldae* **T**
99/100/100 — *Clytia* sp. **T**

Clytiinae

/53/59

100/100/100 — *Gonothyraea loveni* MA **M**
— *Gonothyraea loveni* FR **M**

76/81/96

— *Laomedea calceolifera* **F**
— *Obelia dichotoma* **O**
100/99/100 100/100/100 — *Obelia geniculata* FR **O**
57/99/95 — *Obelia geniculata* NB **O**
99/94/100 — *Obelia geniculata* JP **O**
85/67/100 — *Obelia geniculata* NZ **O**

//61

100/100/100 — *Laomedea flexuosa* FR **F**
— *Laomedea flexuosa* IC **F**
100/100/100 — *Laomedea flexuosa* WS **F**

100/100/100

— *Laomedea inornata* **F**
99/100/100 — *Obelia longissima* AN **O**
— *Obelia longissima* WS **O**
84/96/89 — *Obelia longissima* IC **O**
— *Obelia longissima* NZ **O**
— *Obelia longissima* MA **O**

Obeliinae

—— 0.01 substitutions/site

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Figure 2. Maximum likelihood phylogeny of the Campanulariidae rooted with additional Leptomedusae and Hydridae sequences (Figure 2 in Chapter 2). First number refers to maximum likelihood bootstrap, second number refers to parsimony bootstrap, and third number refers to Bayesian posterior probability. Location codes as in Figure 1. Note *Billardia* falls well outside the primary campanulariid clade.
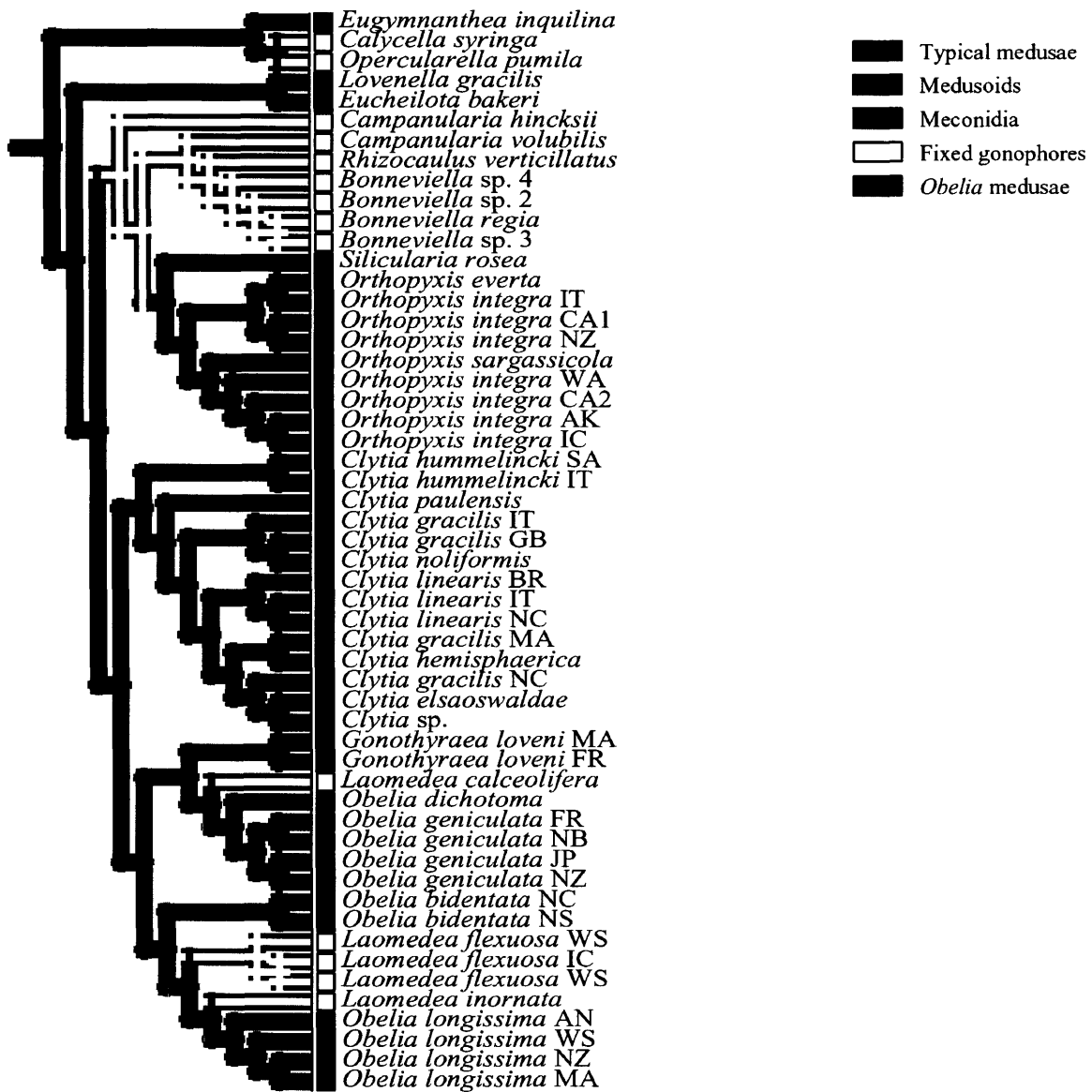
114

Figure 3. Ancestral state reconstruction on the modified maximum likelihood topology (Figure 14 in Chapter 2). *Obelia bidentata* is placed as the basal member of the *Laomedea flexuosa, Laomedea inornata,* and *Obelia longissima* clade. Gains = 1.1 – 1.9. Losses = 1.0.

A.

B.

1mm

0.1mm

acrocyst

C.

0.1mm

1mm

1mm
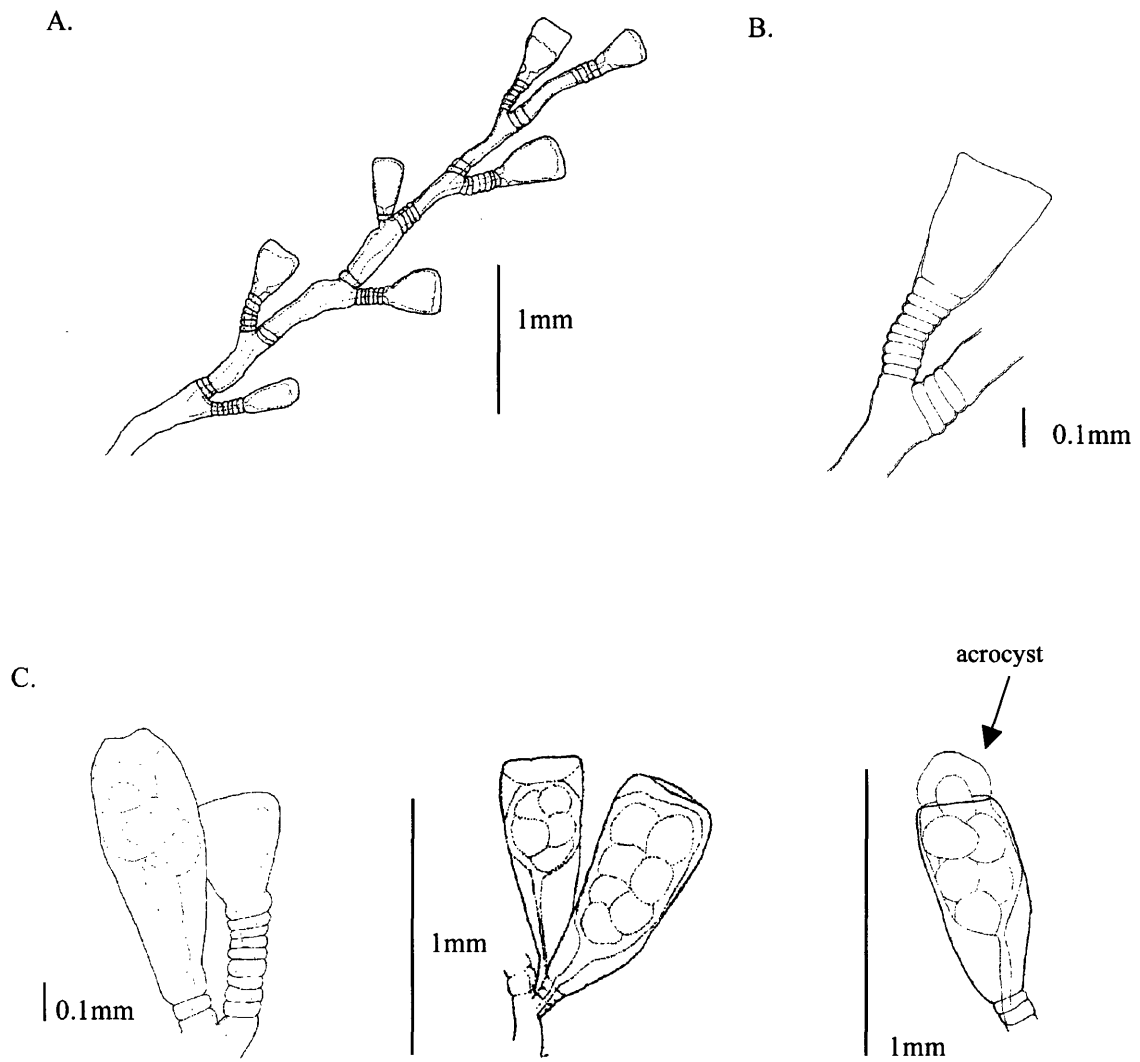
Figure 4. *Laomedea inornata.* **A.** colony branch. **B.** hydranth. **C.** gonophores, showing egg and planulae development and the acrocyst.

Chapter 4

Mitochondrial evolution and phylogeography in the hydrozoan *Obelia geniculata* (Cnidaria)*

Abstract

The distribution and genetic structure of many marine invertebrates in the North Atlantic have been influenced by the Pleistocene glaciation, which caused local extinctions followed by recolonization in warmer periods. Mitochondrial DNA markers are typically used to reconstruct species histories. Here, two mitochondrial markers (16S rDNA and cytochrome c oxidase I [COI]) were used to study the evolution of the widely-distributed hydrozoan *Obelia geniculata* (Linnaeus, 1758) from the North Atlantic and the Pacific, and more specifically in the context of the North Atlantic phylogeography. Samples were collected from six geographic localities between 1998-2002. Hydroids from the North Atlantic, North Pacific (Japan), and South Pacific (New Zealand) are reciprocally-monophyletic and may represent cryptic species. Using portions of the 16S rDNA and COI genes and the date of the last trans-Arctic interchange (3.1 – 4.1 mya), the first calibrated rate of nucleotide substitutions in hydrozoans is presented. Whereas extremely low substitution rates have been reported in other cnidarians, mainly based on anthozoans, substitution rates in *O. geniculata* are comparable to other invertebrates. Despite a life history that ostensibly permits substantial dispersal, there is apparently considerable genetic differentiation in *O. geniculata*. Divergence estimates and the presence of unique haplotypes provide evidence for glacial refugia in Iceland and New Brunswick, Canada. A population in Massachusetts, USA appears to represent a relatively recent colonization event.

Introduction

The rate of mitochondrial nucleotide substitution in Cnidaria, particularly in the Anthozoa, is thought to be considerably lower than in other marine invertebrates (Romano and Palumbi, 1997; Medina et al., 1999; Van Oppen et al., 1999) and may be 10 to 20 times lower than in vertebrates (Shearer et al., 2002). Cnidarian mitochondrial evolution has been best studied in the Anthozoa, because there is a fossil record against which sequence divergence can be calibrated. Generalizations from these studies may not be applicable to other cnidarian clades. Analysis of the scyphozoan *Aurelia aurita* COI suggests that the substitution rate may be higher in this group (Dawson and Jacobs, 2001). The only previous study on a hydrozoan (Shearer et al., 2002) used a relative rate test on COI amino acid sequences, and found that the hydrozoan (Limnomedusa) *Maeotias* sp. was evolving significantly more slowly than echinoderms, molluscs, and arthropods.

A variety of mechanisms, such as selection, a recent bottleneck, introgression, and mismatch repair, could contribute to this unusually slow rate (Shearer et al., 2002). Particularly interesting is the possibility of a mismatch repair system. Mitochondrial gene content is highly conserved among metazoans, but some anthozoans (e.g., octocorals) possess a gene coding for a mismatch repair protein (MSH) that has not been found in any other metazoan mitochondrial genome (Pont-Kingdon et al., 1995; Pont-Kingdon et al., 1998; France and Hoover, 2001). However, it is not known if and how this gene functions in mismatch repair in anthozoan mitochondria (Pont-Kingdon et al., 1998; Shearer et al., 2002).

Biogeographic events can sometimes provide firm upper and lower bounds for divergence when a fossil record is not available (e.g., Cunningham et al., 1991; Knowlton and Weigt, 1998; Wares and Cunningham, 2001). Here, assuming migration through the trans-Arctic interchange (Vermeij, 1991), we provide the first calibrated substitution rates for a hydrozoan, *Obelia geniculata*. We then use our calibrated substitution rates to interpret the timing of phylogeographic events for *O. geniculata* in the North Atlantic.

The hydrozoan *Obelia geniculata* (Leptomedusa, Campanulariidae) is very widely distributed (Cornelius, 1975). It is found on both sides of the north and south Atlantic and Pacific Oceans, but is apparently absent or rare (or undescribed) in the northern Indian Ocean, the tropical western Atlantic, the Great Barrier Reef region, and the Southern Ocean (Cornelius, 1975). The life cycle consists of three stages, in which the adult medusa releases either sperm or eggs forming relatively short-lived, lecithotrophic planula larvae. The planulae settle on to a substrate and metamorphose into hydroids. Hydroids are colonial, and asexually produce medusae, which are released into the plankton, completing the cycle. Additionally, *O. geniculata* hydroids can reproduce asexually by releasing propagules or "capsules" of tissue (Billard, 1904; Berrill, 1948; Panteleeva, 1999). *O. geniculata* hydroids are found from the intertidal down to about 100 m depth, growing on a variety of substrates especially brown algae and occasionally other invertebrates and fish (Cornelius, 1995). This species has the potential for extensive dispersal through planktonic medusae and planulae, or through hydroids rafting on drifting seaweeds (Cornelius, 1992).

The goals of this research were to 1) examine genetic variation and phylogeography in *Obelia geniculata* using portions of the mitochondrial 16S rDNA and COI genes; 2) determine the substitution rates of 16S rDNA and COI by calibrating nucleotide substitutions to the opening of the Bering Strait, and compare these rates with those in other invertebrates; and 3) investigate phylogenetic patterns and divergence relative to the last glacial maximum in the North Atlantic.

Materials and methods

Sample collection and DNA sequencing

Hydroids of *Obelia geniculata* (Linnaeus, 1758) were collected or obtained from colleagues from the North Atlantic and Pacific (Table 1) and preserved in 95% ethanol. Hydroids were identified by A. Govindarajan or P. Schuchert, and vouchers for most samples are available upon request to A. Govindarajan. Genomic DNA was extracted using the DNEasy kit (Qiagen). Portions of the mitochondrial 16S rDNA and cytochrome

c oxidase subunit I (COI) were amplified under standard PCR conditions using the primers of Cunningham and Buss (1993) and Folmer et al., (1994) (LCO1490 and HCO2198), respectively. PCR products were visualized on an agarose gel with ethidium bromide and purified with PCR purification kits (Qiagen). Purified products were cycle-sequenced with either Big Dye 2 or 3 sequencing chemistry (ABI) following the manufacturer's protocol, purified on a Sephadex column, and sequenced in both directions on an ABI 377. Sequences were aligned using Clustal X (Thompson et al., 1994) and confirmed by eye with MacClade (Maddison and Maddison, 2000), although there was virtually no length variation in the sequences (only one indel). ModelTest (Posada and Crandall, 1998) was used to determine the best-fit model for maximum likelihood analyses (described below) conducted with PAUP* 4.0b10 (Swofford 2000). For calculations of substitution rates and divergence estimates, model parameters under the best-fit model were estimated using maximum likelihood in PAUP*.

Phylogeny

Because the mitochondrial genome represents a single locus, the 16S rDNA and COI sequences were combined for each individual sequenced and considered a single mitochondrial haplotype. A maximum likelihood phylogeny was generated from a heuristic search with TBR branch swapping using the ModelTest parameters (corresponding to an HKY85 + I + G model). The starting tree was obtained from the set of most parsimonious trees found from a heuristic search (starting trees obtained via stepwise addition using 10 random addition replicates). Support for the nodes was estimated by conducting a likelihood bootstrap analysis with 300 replicates (identical haplotypes excluded to save computational time).

Substitution rates

Substitution rates were calibrated using PAUP* 4.0b10 (Swofford, 2000) by calculating the average length of the central internal branch of a likelihood phylogeny using the best-fit model between the Pacific and the North Atlantic (New Brunswick,

Icelandic, and French) populations (to get gene, rather than population, divergence, Edwards and Beerli, 2000). A likelihood ratio test (Huelsenbeck and Rannala, 1997) was used to see if the hydroids were evolving in a clock-like fashion. Likelihood scores were calculated in PAUP* using the best-fit model with and without the molecular clock constraint, and were used to generate a χ^2 test statistic with n-2 degrees of freedom (n = number of taxa). Midpoint rooting was used when the molecular clock was enforced.

For comparison, we similarly calculated the substitution rate for 16S rDNA in another hydrozoan, the genus *Hydractinia* (Hydrozoa, Anthomedusa, Hydractiniidae) using previously published sequences (Cunningham and Buss, 1993) and a dated biogeographic event (Cunningham et al., 1991; Young et al., 2002). This calculation had not been done previously.

Phylogeography and North Atlantic population ages

Evidence for geographic subdivision was obtained by conducting an AMOVA between the three groups (North Atlantic, Japan, and New Zealand) and pairwise F_{ST}'s using Arlequin 2.001 (Schneider et al., 2000). The presence of geographically-restricted groups of haplotypes can be used to estimate minimum population ages. This is of interest since several of our populations were in regions thought to have been covered by glaciers during the last glacial maximum.

The age of a clade composed of an ancestral haplotype and its descendants can be estimated using the methods of Saillard et al. (2000). If these form a perfect star phylogeny, the age is easily estimated according to coalescent theory. The more these haplotypes depart from a star phylogeny, the wider the confidence limits.

First, the ancestral haplotype was identified by the method of Castelloe and Templeton (1994) which estimates outgroup weights based on haplotype frequency and connectivity. The haplotype with the highest outgroup weight is most likely the oldest. Following Saillard et al. (2000), the divergence estimate ρ is the average number of links in terms of observed substitutions between the observed haplotypes from the ancestral haplotype:

$$\rho = (n_1l_1 + n_2l_2 + \ldots + n_ml_m)/n)$$

where n = number of individuals with a given haplotype, l = number of steps (links) of a given haplotype to the ancestral haplotype, and m = number of haplotypes. The variance σ is described by:

$$\sigma^2 = (n_1{}^2l_1 + n_2{}^2l_2 + \ldots + n_m{}^2l_m)/n^2$$

The star index $\rho/n\sigma^2$ (Torroni et al., 1998; Saillard et al., 2000), where a value of one equals a perfectly starlike phylogeny, was calculated. As described above, the more starlike, the smaller the confidence intervals. Since ρ is expressed in terms of number of observed substitutions, a per-locus rate of substitution is necessary. This is obtained by multiplying the substitution rate times the number of positions in the combined 16S rDNA and COI data.

Results

Sample collection and DNA sequencing

A total of 51 *Obelia geniculata* hydroids were collected and sequenced from New Zealand, Japan, and four locations in the North Atlantic (Massachusetts, New Brunswick, Iceland, and France). Four hundred forty base pairs of 16S rDNA and 575 base pairs of COI were sequenced for all taxa (GenBank accession numbers AY530328-AY530429). The dataset consisted of 1015 characters, 941 which were constant, 21 which were variable but parsimony-uninformative, and 52 which were parsimony-informative. The alignments were deposited in the EMBL-Align database (accession numbers ALIGN_000710 and ALIGN_000711 for 16S and COI, respectively).

Phylogeny

A total of 31 mitochondrial haplotypes were found. All Japanese individuals, and all but one New Zealand individual, were unique. The likelihood topology indicated three strongly supported (96-100%), reciprocally-monophyletic clades separated by long branches: one for the North Atlantic, one for Japan, and one for New Zealand (Figure 1). The arrangement of the three clades with respect to each other is unresolved.

16S rDNA and COI substitution rates.

Minimum substitution rates were calculated by comparing divergence between North Atlantic and Japanese populations. Three ages for the trans-Arctic interchange were used: the minimum and maximum estimate for the initial opening of the Bering Strait (3.1 and 4.1 mya) (Marincovich and Gladenkov, 2001) and 3.5 mya (Vermeij, 1991). Because these dates represent the initial opening, the resulting substitution rate estimate is the minimum possible rate.

To obtain divergence estimates, a maximum likelihood phylogeny was constructed using the best-fit model. The length of the internal branch between populations estimates the actual age of divergence between populations, and serves to correct for polymorphism in the ancestral population (Edwards and Beerli, 2000). The best-fit models for each data partition (16S rDNA, 16S rDNA + COI, COI, COI 3rd codon positions) and their estimated rates, are presented (Table 2). The molecular clock assumption was tested, but was not rejected (-ln L_{clock} = 1946.40809, -ln $L_{no\,clock}$ = 1913.82546, 2(-ln L_{clock} - -ln $L_{no\,clock}$) = 65.16526, d.f. = 49, critical value at 0.05 significance = 66.339).

Comparison of mitochondrial substitution rates to other invertebrates

The minimum substitution rates for 16S rDNA, COI, and COI 3rd codon positions were higher than those for anthozoans, and within the range of other marine invertebrates (Table 2, Table 3). The 16S rDNA rate of *Obelia geniculata* was comparable to our estimated rate, based on published 16S rDNA sequences, of the hydrozoan *Hydractinia* spp. (Cunningham and Buss, 1993). The maximum date of divergence for Atlantic and Gulf of Mexico populations of the hermit crab host *of*

Hydractinia spp. is $\approx$ 4.1 million years (Cunningham et al., 1991; Young et al., 2002). The divergence between Atlantic and Gulf of Mexico *Hydractinia* spp., using an HKY model is 0.01027, which yields a minimum substitution rate of 1.25 x 10^{-9} for *Hydractinia* 16S rDNA. This is lower than the *Obelia geniculata* rate, but both were considerably higher than published estimates for scleractinian corals (Romano and Palumbi, 1997; Table 3).

Phylogeography

Within the North Atlantic, there appeared to be an ancestral haplotype. As expected (Castelloe and Templeton, 1994), the ancestral haplotype was deeply nested and was shared by Massachusetts (MA), New Brunswick (NB), and Iceland (IC) populations (Figure 2).

The AMOVA indicated that most (92.67%) of the variation corresponded to the three major clades found in the phylogeny (North Atlantic, JP, and NZ). 1.22% of the variation came from between localities within the three major groups (MA, NB, IC, FR, JP, NZ), and 6.11% of the variation came from within locations (Table 4). The pairwise F_{ST} values indicated significant, although in some cases small, genetic differentiation: only one pair (NB-IC) was not statistically different (p > 0.05, Table 5).

Estimates of population age

Both the Icelandic and New Brunswick populations had a number of haplotypes not shared by other populations. In contrast, the Massachusetts sample was entirely composed of haplotypes shared with New Brunswick, consistent with a recent colonization from the north (Hewitt, 2000).

If we consider the ancestral haplotype in the North Atlantic and its descendants that are restricted only to a particular population, we can estimate the minimum age of that population using the method of Saillard et al. (2000). For New Brunswick the estimated age, 143 $\pm$ 47 kya, predates the last glacial maximum (20 kya). Including the

Massachusetts individuals does not change this result (150 ± 82 kya). The Icelandic population appears even older, at 204 ± 68 kya.

To investigate the possibility that the trans-Arctic migration was more recent, thus moving the divergence dates to be more recent than the last glacial maximum, the effects of trans-Arctic migration date on divergence time from the ancestral haplotype and on the corresponding COI 3rd codon position substitution rate were modeled for the New Brunswick samples (Figure 3). The more recent the date of trans-Arctic migration, the more recent the divergence from the ancestral haplotype and the higher the substitution rate. Trans-Arctic migration dates at or after ~750,000 years ago yielded divergence estimates at or after the last glacial maximum. However, the corresponding substitution rates were exceedingly high. For a trans-Arctic migration date of 750,000 years ago, the COI 3rd codon substitution rate was 90.93 x 10^{-9} substitutions site^{-1} year^{-1}, and increased rapidly as trans-Arctic migration dates became more recent.

Discussion

We found three reciprocally-monophyletic clades of *Obelia geniculata* in New Zealand, Japan, and the North Atlantic. The New Zealand and Japanese populations, each collected from a single location, appear to have has much or more haplotype diversity as the four North Atlantic sampling localities combined. This is consistent with a more recent origin for the North Atlantic population, perhaps via an invasion from the Pacific during the trans-Arctic interchange that followed the opening of the Bering Strait 3.1-4.1 mya. However, additional data are necessary to determine the arrangement of the clades with respect to each other.

Our substitution rate calibrations assume a trans-Arctic migration 3.1-4.1 mya, but do not depend on the direction of migration. An earlier opening 5.4 – 5.5 mya has been recently suggested (Marincovich and Gladenkov, 1999; Gladenkov et al., 2002) but, unlike the later opening (Durham and MacNeil, 1967; Briggs, 1970; Vermeij, 1991), it does not appear to have been accompanied by a large faunal migration and so was not considered here. Many species, including molluscs, echinoderms, and algae are thought

to have been introduced to the North Atlantic from the North Pacific when the Bering Strait opened up around 3.5 mya (Durham and MacNeil, 1967; Van den Hoek and Breeman, 1990; Vermeij, 1991; Cunningham and Collins, 1998). Several species of the kelp *Laminaria*, a common substrate of *Obelia geniculata*, were among the invaders from the Pacific (Stam et al., 1988).

We used the opening of the Bering Strait to obtain a minimum estimate of substitution rate for *Obelia geniculata*. For both the COI and 16S rDNA genes, this rate is about an order of magnitude faster than rates estimated from the anthozoans (Table 3). The 16S rDNA rate for *O. geniculata* is also higher than a rate estimated from published sequences for the hydrozoan genus *Hydractinia* (Table 3).

The existence of three well-supported clades separated by long branches suggests that the North Atlantic, Japanese, and New Zealand populations have been separated for a long time. The three clades are reciprocally-monophyletic, suggesting they represent different species according to the phylogenetic species concept (Avise, 2000). Species distinctions in campanulariid hydroids, particularly in the genus *Obelia*, are controversial. Species-level taxonomy is based in a large part on theca (the chitinous covering) morphology, but environmental influences on morphology (e.g., Ralph, 1956) make identification difficult. In a revision of the genus *Obelia*, Cornelius (1975) synonomized about 120 species into 3. He later increased that number to 4 (Cornelius, 1990). However, unlike for other *Obelia* species, his treatment of *O. geniculata* was not controversial (Cornelius, 1990), and *O. geniculata* is considered easily recognizable by the asymmetrical thickening of the perisarc, or exoskeleton (although the degree of thickening is variable). Nevertheless, our results suggest that diversity may be underestimated in *O. geniculata*. Additional genetic studies will be useful in resolving species boundaries in the genus *Obelia*.

This study presents the first calibrated hydrozoan substitution rates. Because our rates are based on the maximum time of divergence, they represent minimum estimates. *Obelia geniculata* COI and 16S rDNA genes are evolving at a rate similar to other

invertebrates, countering the hypothesis that cnidarian mitochondria evolve slowly. Thus mitochondrial sequences should be a useful tool for studying hydrozoan phylogeography.

Anthozoans exhibit very low substitution rates in their mitochondrial sequences, even at COI 3rd codon positions (which are under less selective pressure; Snell et al., 1998; Medina et al., 1999; France and Hoover, 2002). In *Obelia geniculata,* the divergence rate for third codon positions fell within the range for other invertebrates (Wares and Cunningham, 2001).

Noting that sponges appear to have a slow rate of mitochondrial evolution, Shearer et al. (2002) (citing R. Watkins pers. comm.) suggest that mitochondrial evolution was slow in ancestral metazoans and accelerated near the origin of the Bilateria. They point out that if a higher mitochondrial evolutionary rate were found in cnidarian classes other than the Anthozoa, then that accelerated rate likely evolved at the origin of the Medusazoa, as anthozoans are thought to be the basal cnidarians (Bridge et al., 1992; Bridge et al., 1995). Our results are consistent with this interpretation of a second independent origin of rapid mitochondrial evolution.

In addition to the trans-Arctic interchange, the Pleistocene glaciations are thought to have been a major force shaping the history of shallow-water flora and fauna in the North Atlantic by causing local extinctions, especially in the west (Ingolfsson, 1992; Cunningham and Collins, 1998). Our results suggest that it may have been important for hydroids as well.

Although more comprehensive sampling is required, particularly in France, our divergence estimates which predate the last glacial maximum suggest that Icelandic and Canadian populations of *Obelia geniculata* may have survived in northern, glacial refugia. This conclusion is robust to the calibration of the molecular clock. In some cases, trans-Arctic invaders may have come more recently than after the initial opening of the Bering Strait (Palumbi and Kessing, 1991; Van Oppen et al., 1995). If this were the case for *O. geniculata,* the calculated Icelandic and New Brunswick divergence date would be overestimated because the substitution rate estimate would be too low. However, this seems highly unlikely because the minimum substitution rate necessary to push the

divergence dates to the last glacial maximum (requiring a maximum trans-Arctic migration date of ~750 kya) is considerably higher than the substitution rate in all other invertebrates compared except for *Mytilus edulis* (95.1 x 10^{-9} substitutions site^{-1} year^{-1}; Table 3). *M. edulis* is known to have an exceptionally high mitochondrial evolutionary rate, possibly due to its unusual form of mitochondrial inheritance (Hoeh et al., 1996). Thus, it seems most likely that *O. geniculata* had a trans-Arctic migration before 750,000 years ago, supporting our original estimate of pre-glacial ages for *O. geniculata* populations in Iceland and the Canadian Maritimes.

The presence of unique haplotypes in *O. geniculata* from Iceland and New Brunswick also add to the mounting genetic evidence for glacial refugia in these areas (Holder et al., 1999; Dahlgren et al., 2000; Wares et al., 2002; Young et al., 2002). Geological evidence suggests that unglaciated pockets may have existed in northern Norway and Scotland (Dawson, 1992; Siegert, 2001) and in the Canadian Maritimes and Georges Bank (Rogerson, 1983; Pielou, 1991; Holder et al., 1999). These unglaciated pockets may have provided the refugia indicated by the genetic data.

Our results also suggest a recent southward range expansion by two haplotypes from New Brunswick into Massachusetts (the Woods Hole, MA sampling site is located on the southern shore of the biogeographic boundary of Cape Cod [Franz and Merrill, 1980 a; b]). As expected for a recent introduction, genetic diversity was lower than, and a subset of, the putative New Brunswick founding population (Hewitt, 2000). Similarly, Dahlgren et al., (2000) suggested a southern range expansion for the bivalve *Arctica islandica* due to global climate change. This pattern is also seen in the gastropod *Littorina littorea*, a species believed for a long time to have been introduced into the western Atlantic from Europe, but which is now thought to be a recent southward range expansion from Canada (Wares et al., 2002). Such southward range expansions may be common in other invertebrate taxa as well.

Wares and Cunningham (2001) suggest that species capable of long distance dispersal may have persisted through glacial periods because they could take advantage of widely scattered, ephemeral refugia (although the role of dispersal ability in

colonization is complex, Cunningham and Collins, 1998). The presence of a planktonic medusa stage in the life cycle of *Obelia geniculata* could provide a mechanism for substantial dispersal. Additionally, hydrozoans possessing a medusa stage may have relatively longer-lived planulae (Sommer, 1992). In *O. geniculata*, several medusae generations are produced per year, and the medusae may live for several weeks (Kramp, 1927). Planulae of *Obelia* spp. may live from 5 d to 3 wk (Bodo and Bouillon, 1968). Dispersal can also occur via the attached hydroid stage by rafting on algae (Cornelius, 1992; Ingolfsson, 1995). Hydroids living on *Laminaria* spp. live as long as the blades to which they are attached, which may be up to 15 months (Kramp, 1927). Finally, dispersal may occur via asexual propagules (Billard, 1904; Berrill, 1948; Panteleeva, 1999).

Nevertheless, there was significant genetic variation despite the dispersal potential of the planktonic medusae, planulae, propagules, and algal rafting. Not surprisingly, because of the large geographic distances between them, there were large differences between the North Atlantic, Japanese, and New Zealand specimens indicative of cryptic speciation. However, within the North Atlantic, the lack of variation in the Massachusetts samples relative to the New Brunswick samples, and the presence of unique haplotypes in New Brunswick and Iceland, suggest that dispersal may also be restricted at relatively smaller scales. This is counterintuitive given the enormous dispersal potential of *Obelia geniculata*. Boero and Bouillon (1993) suggested that the presence of a medusa stage does not necessarily imply wide dispersal. In fact, they found that among Mediterranean hydrozoans, nominal species with apparently cosmopolitan distributions were less likely to have a medusa stage. Studies of other cnidarian medusae also suggest that a planktonic lifestyle does not lead to genetic homogeneity and that there can be considerable genetic differentiation and even cryptic speciation , as in the scyphozoan *Aurelia aurita* (Dawson and Jacobs, 2001). Oceanographic or behavioral barriers may be responsible for this restricted planktonic dispersal (Hamner et al., 1994). Additional research is necessary to determine the actual, as opposed to potential, dispersal of the several species presently attributed to *Obelia geniculata* and its role in surviving the Pleistocene glaciation.

Acknowledgements

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Table 1. *Obelia geniculata.* Localities sampled for hydroids.

Locality	Sample Code	# Samples	Latitude	Longitude	Sample Date
St. Andrews, New Brunswick, Canada	NB	14	45° 05'	67° 03'	July 2002
Woods Hole, Massachusetts, USA	MA	9	41° 32'	-70° 40'	October 2001
Roscoff, France	FR	4	48° 43'	-3° 59'	April 1998
Garour/Sandgerdi, Iceland	IC	8	64° 04' - 64° 02'	-22° 43' - -22°39'	May 2000
Misaki, Sagami Bay, Japan	JP	8	34° 19'	135° 09'	September 2002
Wellington, New Zealand	NZ	8	-41° 18'	174° 47'	December 2001

Table 2. *Obelia geniculata.* Range of divergence rates based on minimum (3.1mya), maximum (4.1 mya), and commonly used (3.5 mya) estimates for opening of Bering Strait.

Gene	Substitution rate (substitutions site^{-1} yr^{-1}) (x 10^{-9}) 3.1 mya	Substitution rate (substitutions site^{-1} yr^{-1}) (x 10^{-9}) 3.5 mya	Substitution rate (substitutions site^{-1} yr^{-1}) (x 10^{-9}) 4.1 mya	Best-fit model used in rate calculation
16S	2.76	2.44	2.08	HKY
16S + COI	6.13	5.43	4.63	HKY + G
COI	7.38	6.54	5.58	HKY + G
COI 3rd codon	22.00	19.48	16.63	HKY

Table 3. *Obelia geniculata.* Substitution rates of 16S rDNA and COI (all positions) compared with those from other cnidarians (*O. geniculata* in bold). Substitution rate for cytochrome *b* from an anthozoan is included for comparison, although this gene was not sequenced in *O. geniculata.* *O. geniculata* COI 3rd codon position substitution rate is compared with other invertebrates calculated by Wares and Cunningham (2001) with the F84 model. Even if the unlikely earlier, 5.5 mya opening (which was not accompanied by a large faunal migration) is considered, *O. geniculata* rates would be lower, but still higher than anthozoans (16S rDNA: 1.55 x 10^{-9} substitutions site^{-1} yr^{-1}; COI: 4.16 x 10^{-9} substitutions site^{-1} yr^{-1}; COI 3rd codon positions: 12.40 x 10^{-9} substitutions site^{-1} yr^{-1}).

Gene	Phylum	Species	Substitution rate (sub site^{-1} yr^{-1}) (x 10^{-9})	Calibration reference	Source
16S	**Cnidaria (Hydrozoa)**	***O. geniculata***	**2.44**	**Bering Strait (3.5 mya)**	**Present study**
16S	Cnidaria (Hydrozoa)	*Hydractinia*	1.25	Florida (4.1 mya)	Cunningham et al., 1991; Young et al., 2002
16S	Cnidaria (Anthozoa)	Scleractinian corals	0.1-0.6	Fossil record	Romano and Palumbi 1997
COI	**Cnidaria (Hydrozoa)**	***O. geniculata***	**6.54**	**Bering Strait (3.5 mya)**	**Present study**
COI	Cnidaria (Anthozoa)	*Montastraea* spp.	0.5	Fossil record	Medina et al., 1999
Cyt *b*	Cnidaria (Anthozoa)	*Acropora* spp.	0.5-0.9	Fossil record	Van Oppen et al., 1999
COI 3rd codon	**Cnidaria (Hydrozoa)**	***O. geniculata***	**19.5**	**Bering Strait (3.5 mya)**	**Present study**
COI 3rd codon	Arthropoda (Malacostraca)	*Alpheus* spp.	19	Isthmus of Panama	Knowlton and Weigt 1998
COI 3rd codon	Arthropoda (Malacostraca)	*Sesarma* spp.	21	Isthmus of Panama	Schubart et al., 1998
COI 3rd codon	Mollusca (Gastropoda)	*Littorina obtusata*	24	Bering Strait (3.5 mya)	Wares and Cunningham 2001
COI 3rd codon	Arthropoda (Maxillopoda)	*Semibalanus balanoides*	27.6	Bering Strait (3.5 mya)	Wares and Cunningham 2001
COI 3rd codon	Arthropoda (Malacostraca)	*Idotea balthica*	36	Bering Strait (3.5 mya)	Wares and Cunningham 2001
COI 3rd codon	Arthropoda (Maxillopoda)	*Euraphia* spp.	38	Isthmus of Panama	Wares 2001
COI 3rd codon	Mollusca (Gastropoda)	*Nucella lapillus*	44.3	Bering Strait (3.5 mya)	Wares and Cunningham 2001
COI 3rd codon	Echinoderma (Asteroidea)	*Asterias rubens*	48.4	Bering Strait (3.5 mya)	Wares and Cunningham 2001
COI 3rd codon	Mollusca (Bivalvia)	*Mytilus edulis*	95.1	Bering Strait (3.5 mya)	Wares and Cunningham 2001

Table 4. *Obelia geniculata.* AMOVA results. Groups represent the three major clades: North Atlantic, Japan, and New Zealand. Populations represent the 6 populations: Massachusetts (MA), New Brunswick (NB), Iceland (IC), France (FR), Japan (JP), and New Zealand (NZ).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	2	408.972	16.47575	92.67
Among populations within groups	3	8.650	0.21734	1.22
Within populations	45	48.907	1.08682	6.11
Total	50	466.529	17.77990	

Table 5. *Obelia geniculata.* Pairwise F_{ST}'s and associated p-values (in parentheses).

	MA	NB	IC	FR	JP
NB	0.29244				
	(0.00000)				
IC	0.42176	0.03547			
	(0.00000)	(0.13514)			
FR	0.49671	0.19653	0.14118		
	(0.00901)	(0.01802)	(0.03604)		
JP	0.95258	0.94038	0.92409	0.89158	
	(0.00000)	(0.0000)	(0.00000)	(0.00901)	
NZ	0.95557	0.94050	0.92468	0.89326	0.89655
	(0.00000)	(0.0000)	(0.00000)	(0.00000)	(0.00000)
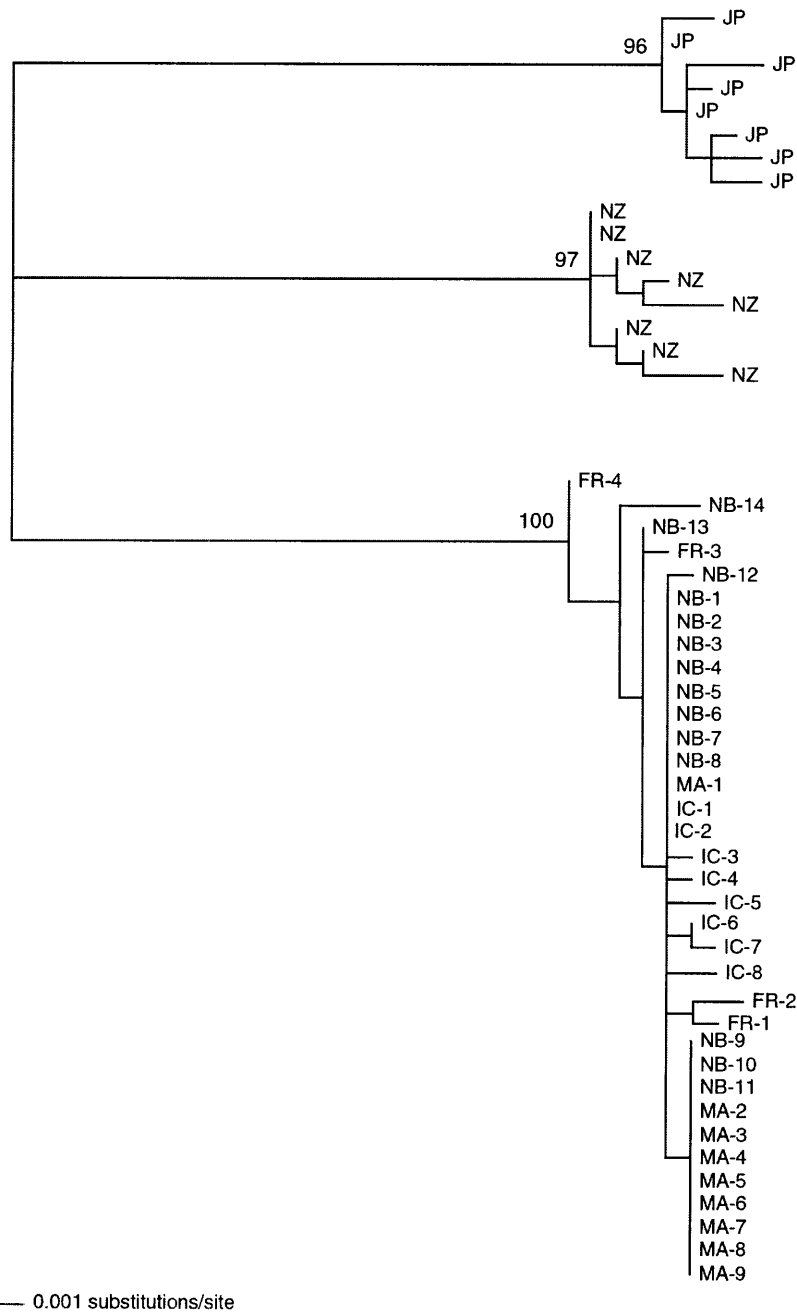
Figure. 1 *Obelia geniculata*. Maximum likelihood topology. Numbers indicate parsimony bootstrap values (300 replicates). Only bootstrap values for the three major clades are shown. Five nodes within these clades had bootstrap values of 50 to 71, and the rest were < 50. North Atlantic sample names correspond to sample names in Figure 2.

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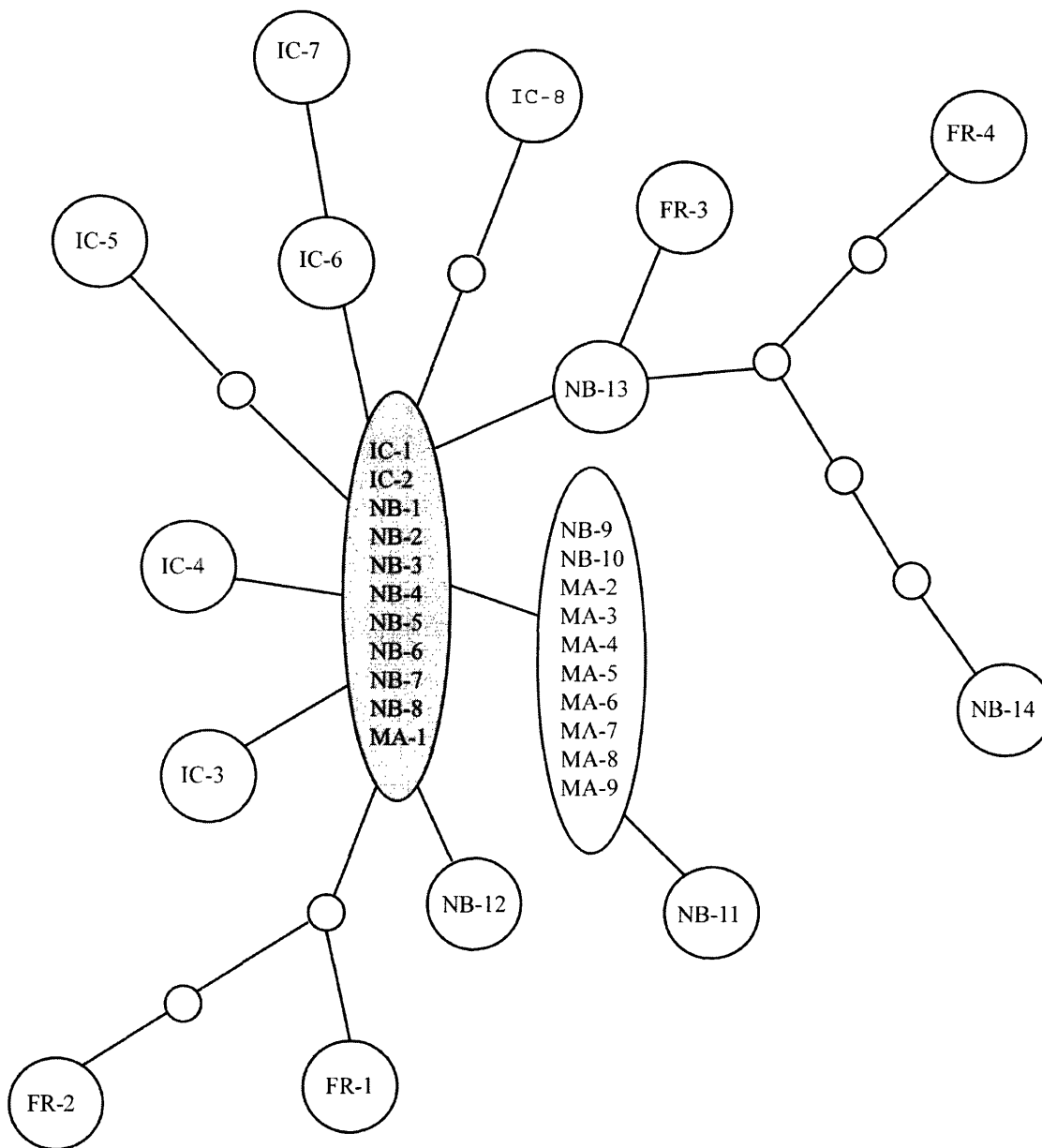
Figure 2. *Obelia geniculata.* Parsimony network for North Atlantic samples. A heuristic search yielded 6 most parsimonious trees, all with 23 steps; the tree identical to the likelihood topology (Figure 1) is represented here. The ancestral haplotype is shaded. Sample names correspond to Figure 1. Sample NB-11 is separated from the NB-MA haplotype by one step; because that change was a deletion, it was coded as missing data in the likelihood analyses and not indicated on Figure1.
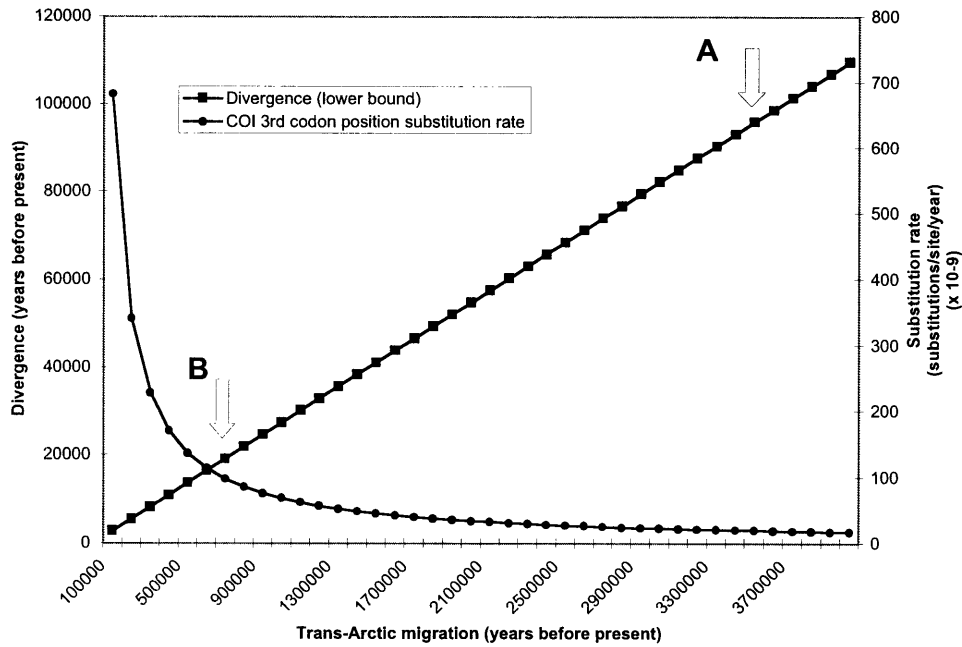
Figure 3. *Obelia geniculata*. Effect of trans-Arctic migration time on divergence of the New Brunswick samples (which diverged more recently than the Icelandic samples) from the ancestral haplotype and on the substitution rate of COI 3rd codon positions (which can be compared to a variety of other invertebrates). To be conservative, the lower bound (mean minus one SD) of the divergence estimate was used. A: assuming migration at the initial opening, 3.5 mya, results in divergence well before the last glacial maximum and a substitution rate as described in the text and Table 3; B: assuming migration at or after 750,000 years ago results in divergence approximately at or after the last glacial maximum (20,552 years ago) and an exceptionally high substitution rate (90.93 x 10^{-9} substitutions site^{-1} year^{-1}).

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Chapter 5

Conclusions

Summary

This thesis has investigated aspects of the evolution, systematics, and phylogeography of the Campanulariidae, which are an important, abundant, and widely distributed group of hydrozoans with a variety of developmental modes and life history strategies. An introduction to the Campanulariidae was provided in Chapter 1, which reviewed their life cycles, development and taxonomy. The important issues in campanulariid biology were outlined, and include: the frequency of life cycle transitions and their ecological and taxonomic consequences, the possibility of an evolutionary reversal and re-invention of the medusa stage, and species-level taxonomy. The specific aims of this thesis were to: 1) investigate the frequency of life cycle transitions and the possibility that *Obelia* medusae are re-invented by constructing a molecular phylogeny of the Campanulariidae and reconstructing ancestral character states; 2) examine the classification of the Campanulariidae in light of the molecular phylogeny at the family, genus, and species levels, and make the appropriate taxonomic revisions; and 3) examine the phylogeography and mitochondrial evolution in the campanulariid *Obelia geniculata*.

In Chapter 2, a molecular phylogeny was constructed using 2 nuclear (18S rDNA and calmodulin) and 2 mitochondrial (16S rDNA and cytochrome c oxidase I [COI]) genes. The genes were analyzed together and separately, and most of the phylogenetic signal came from the 18S rDNA. The calmodulin, 16S rDNA, and COI trees were very poorly resolved by bootstrap methods. The Campanulariinae and *Clytia* lineages appeared monophyletic, and there were at least 2 obeliinid clades. The placement of *Obelia bidentata* was problematic, probably due to its very long branches. The arrangement of the major clades with respect to each other was not resolved. Life cycle types were traced on to the combined phylogeny and the ancestral states reconstructed using parsimony. A sensitivity analysis was conducted to examine the effects of weighting gains greater than losses, and found that even when only a small gain cost was

applied, the fixed gonophore-bearing *Laomedea* likely derived multiple times from an ancestor with *Obelia* medusae, rather than vice versa, and *Obelia* may have derived from an ancestor with typical medusae. The results depended on the underlying phylogeny, however.

The taxonomic results of the combined molecular phylogeny presented in Chapter 2 were discussed in Chapter 3. Some aspects of the traditional taxonomy were supported, while many others were refuted. *Billardia*, referred to the Campanulariidae by some, fell well outside the campanulariid clade. Several species of *Bonneviella* (Bonneviellidae), originally designated as outgroups, were deeply nested within the otherwise monophyletic Campanulariinae lineage. Diagnoses of the Campanulariidae and Campanulariinae were modified accordingly. The taxonomic approach of using life cycle type to define genera was not supported in the Obeliinae, and the obeliinid genera were merged into a single genus, *Obelia*. At the species level, some nominal cosmopolitan species were obtained from several locations, and of these, *Clytia gracilis* appeared to represent multiple cryptic species, while *Obelia longissima* may be truly cosmopolitan. Finally, the putative meconidia (medusoids) of *Gonothyraea* (*Obelia*) *inornata* were shown to be simple acrocysts (external capsules) without any medusoid features.

Chapter 4 examined the phylogeography and mitochondrial evolution of the widely distributed campanulariid *Obelia geniculata*. Phylogeographic studies commonly use mitochondrial DNA markers; however, in cnidarians (mainly anthozoans), the substitution rate is exceptionally low, limiting its utility. Sequence divergence of the mitochondrial 16S rDNA and COI genes in *Obelia geniculata* from the North Atlantic and North Pacific was calibrated against the opening of the Bering Strait, approximately 3.5 million years ago, resulting in the first calibrated substitution rates for a hydrozoan. Substitution rates were considerably faster than in anthozoans, and comparable to other invertebrates. Analysis of *Obelia geniculata* 16S rDNA and COI from New Zealand, Japan, and the North Atlantic revealed that the three localities were reciprocally monophyletic and separated by long branches, consistent with what would be expected for cryptic species. Within the North Atlantic, divergence of Icelandic and New

Brunswick haplotypes from the ancestral haplotype appeared to occur well before the last glacial maximum, suggesting that populations there did not go extinct during the last glaciation, but may have existed in refugia. Hydroids from Woods Hole, MA, USA appeared to be a relatively recent introduction from the north.

The appendix includes a related side project on species identification in the endosymbiotic bivalve-inhabiting hydroids of *Eugymnanthea* (Hydrozoa, Leptomedusa, Eirenidae). These hydroids occupy an unusual habitat, inside bivalves of many species, particularly *Mytilus galloprovincialis*. They are found only in the Mediterranean and Japan, where they have been discovered only relatively recently. Their disjunct distribution, morphological similarity, and the observation that *Mytilus galloprovincialis* was likely introduced to Japan from the Mediterranean have lead to continuing doubt over whether the two localities represent different species, or if the Japanese hydroids were introduced to Japan from the Mediterranean with their hosts. Reciprocal breeding crosses showed that Italian and Japanese *Eugymnanthea* could not interbreed, indicating they are different species according to the biological species concept. Analysis of their 16S rDNA sequences showed that the two forms were reciprocally monophyletic, and diverged by about 12%. A tissue grafting test, previously proposed to identify species, could not distinguish Italian and Japanese specimens. Finally, while hydroids from Italy and Japan were morphologically indistinguishable, there were usually slight differences in the medusoid stage. The results confirm that the two forms are different species, and that the Japanese form may not have resulted from an introduction from Italy.

Broader impacts and future directions

This thesis provided new insight into many aspects of hydrozoan evolution and systematics, and has helped resolve some controversies within the Campanulariidae while suggesting new avenues for future research. Chapter 2 presented the first molecular phylogeny of the Campanulariidae, demonstrating the utility of genetic markers that are relatively independent of the life cycle and environmental biases that plague hydrozoan morphological analyses. Future studies with additional taxa and genetic markers may

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help to determine the arrangement of the major campanulariid lineages which were not resolved here. The phylogeny indicated multiple life cycle transitions within the Obeliinae, suggesting that they are more frequent than sometimes assumed. Studies of other marine invertebrates with complex life cycles suggest several ecological correlates (e.g., dispersal, temperature, latitude, size) to the presence and duration of a planktonic stage, and these should be further examined in the Campanulariidae and other hydrozoans.

It seems likely that the ancestral *Obelia* may have possessed a typical medusa rather than medusoids or fixed gonophores, although alternatives cannot be completely ruled out. More research is necessary to better resolve the underlying phylogeny, and to understand the genetic and developmental changes involved in life cycle transitions. If an ancestor with typical medusae is supported in future studies, it will be interesting to determine the mechanism(s) responsible for effecting so many morphological and developmental changes. For example, similarities between some *Obelia* medusa features to hydranth features indicate that hydranth developmental pathways are involved in *Obelia* medusa development. Gene expression studies may be particularly helpful in understanding the genetic changes and pathways involved, the relative ease of gains versus losses, and the likelihood of evolutionary reversals.

The molecular phylogeny presented in Chapters 2 and 3 indicate the Campanulariidae and indeed, the entire Leptomedusae, may need revision. The exclusion of *Billardia* and especially the inclusion of the Bonneviellidae demonstrated how genetic markers can reveal unexpected relationships. Results also showed that the taxonomic approach of using life cycle type as a generic character can lead to paraphyly and that DNA sequences can be helpful in delineating species boundaries. Traditional morphological taxonomy is exceptionally difficult in the Hydrozoa, largely due to their complex life cycles and phenotypic plasticity. In fact, a unified classification for hydroids and medusae was not even proposed until 1960, and difficulties persist, with several examples of hydroids with features characteristic of one family and their corresponding medusae characteristic of another. Molecular studies, like the one conducted in this

thesis, will be useful in resolving controversies in hydrozoan classification at many levels.

Results from several chapters and the appendix suggest that species-level biodiversity may be underestimated in the Campanulariidae and other hydrozoans, although some true cosmopolites may exist. This is in contrast to the most recent monographs on the Campanulariidae, which synonymized numerous nominal taxa. As in other invertebrates, genetic studies will be crucial in identifying cryptic species. When possible, breeding experiments can corroborate results from DNA studies, and careful morphological examination may identify distinguishing features.

Finally, the first calibrated hydrozoan mitochondrial substitution rates presented in Chapter 4 revealed that hydrozoan mitochondria may be evolving considerably faster than anthozoans, at a rate comparable to other invertebrates. This indicates that mitochondrial markers will be useful in phylogeographic studies of hydrozoans, as they are in many other invertebrates. Analyses of *Obelia geniculata* 16S rDNA and COI revealed likely cryptic speciation in Japan, New Zealand, and North Atlantic. The relationship between these three clades was not resolved, however, so future studies incorporating samples throughout the entire nominal *O. geniculata* range will be helpful in reconstructing the history of this group. Furthermore, the data added to a growing body of evidence that refugia in the North Atlantic existed during the last glaciation, where species that may have went extinct elsewhere could have survived. Similar studies on other hydroids with trans-Atlantic distributions can help determine the extent of this phenomenon.

Appendix

Species identification of bivalve-inhabiting marine hydrozoans of the genus *Eugymnanthea**

Abstract

Species-level identification is difficult in the symbiotic bivalve-inhabiting hydrozoans of the genus *Eugymnanthea* (Cnidaria, Hydrozoa). Morphological differences are detected only in the adult medusoid stage. *Eugymnanthea* is known only from the Mediterranean and the western Pacific, and doubt persists over whether the two localities are inhabited by different species. Because the bivalve host, *Mytilus galloprovincialis*, is thought to be introduced by humans from the Mediterranean to the western Pacific, there has been speculation that the Mediterranean *Eugymnanthea* was also introduced along with its host. Here, we evaluate the species status of the two forms with breeding experiments, morphology, and two recently developed tools for discrimination: a mesoglea cell adhesion and spreading test, and 16S rDNA comparison. Reciprocal crosses of the two forms failed to produce normal offspring, providing evidence that they are indeed different species according to the biological species concept, and suggesting that the Pacific form is not an invasion of the Mediterranean form. The tissue-grafting test failed to distinguish between the two forms, while the morphological and genetic evidence corroborated the breeding results.

Introduction

Many marine hydroids of the family Eirenidae (Hydrozoa, Leptomedusae) occupy an unusual habitat, living in the mantle cavity of some bivalve molluscs, including several species used for human consumption: *Mytilus galloprovincialis* (Cerruti, 1941; Crowell, 1957; Kubota, 1992a), *Crassostrea virginica* (Kubota and Larson, 1990), and *Tivela mactroides* (Narchi and Hebling, 1975). Details of hydroid-bivalve associations

are reviewed in Kubota (1983), Kubota (1987a); and Piraino et al. (1994). In some areas, the frequency of symbiotic hydroids can be quite high; for example, in Taranto, Italy, up to 86% of *M. galloprovincialis* contained hydroids (Kubota, 1989). However, the percentage of infected mussels can vary considerably with location, mussel size, and season (Kubota, 1983; Piraino et al., 1994). *M. galloprovincialis* cultivation is an important industry in Taranto, and the high incidence of infection by the eirinid *Eugymnanthea inquilina* there has led some local fisherman to blame mussel death on the hydroids (although this mortality was ultimately blamed on a dinoflagellate bloom combined with high temperature and oxygen depletion). Despite this, Piraino et al. (1994) found that this hydroid-bivalve relationship is probably mutualistic. The hydroids receive habitat, protection from predators, and a food supply. The mussels may receive some protection against trematode parasites as the hydroids can ingest their sporocysts, and trematode infection is very low in mussels with hydroids (Piraino et al., 1994). Additionally, Tiscar (1992) found no effect of hydroid attachment on mantle tissue.

Members of the eirenid genus *Eugymnanthea* have a complex life cycle: the adult (sexual) stage is a motile medusoid, with some, but not all, characteristics of fully functional hydrozoan medusae (Naumov, 1960). The medusoids can be gonochoric or rarely hermaphroditic (Celiberti et al., 1998; Kubota in press), and fertilization and early developmental stages may still occur within the subumbrellar cavity (Celiberti et al. 1998). The planula larvae settle in a host bivalve, and develop into hydroids, which complete the cycle by budding medusoids (Figure 1). Hydroids can attach to the mantle, labial palp, and foot of the host bivalves. In Japan, hydroids can also attach to the gill, although this is rarely observed in Italy (Kubota, 1989). Instead of a stolon as in other hydroid species, *Eugymnanthea* have a sucker-like, non-penetrating pedal disk that is used for attachment (Kubota, 1983).

Species-level identification of *Eugymnanthea* is problematic unless the mature medusoids can be cultured. In the past, it was thought that there was only a single species; however, Kubota (1991a) raised the medusoids of Japanese and Italian specimens, and based on differences in the manubrium and statoliths, he considered them

two distinct species (*E. japonica* and *E. inquilina*). Kubota (1989) was unable to successfully interbreed the two forms; however, his mutual crossing was only in one direction. In his experiments, he crossed four Mediterranean males with five Japanese females. Only one of these crosses produced offspring, and those degenerated after the third day of the experiment.

To date, *Eugymnanthea* has only been discovered in the Mediterranean Sea and the western Pacific Ocean (Kubota, 2000). *E. japonica* is found in Japan and Taiwan, inhabiting primarily the bivalves *Mytilus galloprovincialis, Crassostrea gigas, Barbatia virescens*, and occasionally other species (Kubota, 1992a; Kubota, 2003). *E. inquilina* is found along the northern coast of the Mediterranean Sea (Italy, France, Spain, Croatia, Greece), and, like *E. japonica,* is found primarily in the mussel *M. galloprovincialis* and occasionally in other species (Piraino et al., 1994; Kubota, 2000; Rayyan et al., 2002; Kubota, in press). The disjunct geographical distribution and the overlap in bivalve host species has resulted in lingering doubt about the species status of the "two forms" and continuing speculation that the Japanese form may have been introduced from the Mediterranean via transfer by the host (Kubota, 1979). It is thought that *M. galloprovincialis* was transported to Japan, initially to the region of Kobe between 1930-1935 (Wilkens et al., 1983; Koehn, 1991; Kubota et al., 1995), possibly from the Mediterranean (Wilkens et al., 1983) and the hydroids could have been introduced along with their hosts.

E. inquilina, first recorded by Palombi (1935), has a relatively long history in the Mediterranean, but *E. japonica* was only discovered in Japan in 1979 (Kubota 1979), despite a relatively long history of research on bivalve-inhabiting hydroids in that country. Furthermore, its range in Japan has been expanding. Originally it was found in only one location (Shimoda, central Japan), but was subsequently discovered in many other locations in southern Japan (Kubota, 1991a; Kubota, 1992a) and Taiwan (Kubota et al., 1999). Explanations for this range shift include: 1) the hydroids are native to the Mediterranean and were introduced to Japan with their host *M. galloprovincialis*, and have subsequently spread from the site(s) of introduction; 2) the hydroids are native to

Japan but were previously rare, and the introduction of *M. galloprovincialis* provided a new host and facilitated their spread; and 3) the hydroids are native to Japan but were previously rare, and other factors, such as habitat modification, have improved environmental conditions and facilitated their spread (Kubota, 1992a).

In order to determine the origin of *Eugymnanthea japonica*, it is necessary to resolve its species-level taxonomy. Various methods have been proposed for species-level identification of cryptic species (Knowlton, 2000), and DNA sequences have proven useful for many taxa. For hydrozoans, the 16S rDNA may be useful to distinguish species (Cunningham and Buss, 1993; Schierwater and Ender, 2000; Govindarajan et al., submitted). Alternatively, Schmid et al. (1992) demonstrated that the mesoglea test, which is based on the adhesion and spread of cells on the mesoglea, may be an effective tool with which to discriminate among hydrozoan species. In this test, tissue fragments from one individual are grafted onto mesoglea from another individual. The tissue fragments should only adhere and spread if the mesoglea is from a closely related (i.e., conspecific) individual.

The goal of this research was to resolve the controversy surrounding *Eugymnanthea*. Morphological and molecular evidence was used 1) to confirm that the two forms are indeed separate species; 2) to assess two methods (the mesoglea test and 16S rDNA sequences) of identifying species accurately and through all life cycle stages; and 3) to deduce whether or not the Japanese form was introduced from the Mediterranean Sea.

Methods

Collection, culture, and morphological observations

E. inquilina was obtained by collecting mussels (*M. galloprovincialis*) from two localities along the southern coast of Italy: Lago Fusaro (40°49'N, 14°03'E; the type locality) and Taranto (40°28'N, 17°14E). The mussels were brought back to the laboratory, where some of them were opened and the hydroids, if present, carefully removed. The hydroids were cultured, with or without their hosts, in small trays or dishes

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filled with natural seawater (salinity = 38--40 $^0/_{00}$). These rearing vessels were kept under constant conditions of 23 °C and 15L:9D photoperiod, and the animals were fed once daily with newly hatched brine shrimp nauplii. The water was changed daily and was continuously aerated. When medusoids were released from the hydroids, all were collected, cultured, and examined daily with a microscope. For all of the medusoids, the primary diagnostic features (the number of statoliths per statocyst and the presence or absence of a manubrium) were recorded. Rarely, when immediate observation was not possible, the newly liberated medusoids were preserved in buffered 10% formalin and examined later. Some hydroids were preserved in 99% ethanol for subsequent DNA analysis.

In Japan, *E. japonica* was collected from the mussel (*M. galloprovincialis*) attached to rafts moored in two localities in Japan facing the Pacific Ocean: Atami, Shizuoka Prefecture (35°05'N, 139°05'E; very close to the type locality in Shimoda) and Shirahama, Wakayama Prefecture (33°42'N, 135°22'E). The hydroids (and subsequent medusoids) were collected, cultured, and analyzed in the same manner as for *E. inquilina*. Additional observations from Kubota (1989; 1991a) were also used for comparison.

Breeding experiments

For each crossing, one pair, i.e. one male and one female, was prepared. Mature medusae of male *E. inquilina* and female *E. japonica*, and female *E. inquilina* and male *E. japonica*, were placed in culture dishes for spawning. Each pair was in a separate culture dish. Pairs of conspecific male and female medusae were also placed together for a control. Three conspecific and 13 interspecific crosses were conducted. Spawning was triggered by light exposure, as male and female medusae of both species release their gametes after about half an hour after the light is turned on (Kubota, unpublished data). Hermaphroditic medusae, easily distinguished by the presence of mature sperm and eggs within the same gonad, were not used, as they may self-fertilize (Celiberti et al., 1998). The presence of late-stage planula larvae (with differentiated endoderm and nematocysts) (Kubota, 1991b) indicated successful mating.

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Mesoglea test

The mesoglea test was performed using live specimens. In this cell adhesion and spreading test on isolated ECM (extracellular matrix, or mesoglea) was carried out following the methods described by Schmid et al. (1992) and Reber-Müller et al. (1994). To summarize, the ECM was excised and the cells were allowed to dissociate from it by incubating in a $Ca^{2+}Mg^{2+}$ --free seawater. Cell dissociation was facilitated by blowing on the ECM with a pipet. The ECM was then washed in distilled water, placed on a coverslip, and allowed to dry. Tissue from the test sample was then excised, washed in filtered seawater, and applied to the ECM. After about an hour, the ECM was examined for cell adhesion. Tissue of *Eugymnanthea* was also grafted on to the ECM of *Podocoryna carnea* (Hydrozoa, Anthomedusae), a phylogenetically distant hydroid species (collected from Naples, Italy and maintained in the Schmid laboratory) as a control.

DNA sequencing

A ~600 bp portion of the 16S rDNA gene was sequenced. DNA was extracted from ethanol-preserved tissue belonging to 3 *E. japonica* and 4 *E. inquilina* with the DNEasy Kit (Qiagen) according to the manufacturer's protocol and amplified with hydrozoan-specific primers (Cunningham and Buss, 1993) following standard protocols (Palumbi, 1996). The PCR product was visualized on a 1% agarose gel with ethidium bromide, and was purified with the PCR purification kit (Qiagen). The purified product was cycle-sequenced using Big Dye 2 sequencing chemistry (ABI) following the manufacturer's protocol. The cycle-sequenced product was purified on a Sephadex column and sequenced on an ABI 377 Sequencer. DNA sequences were aligned using ClustalX 1.8 (Thompson et al., 1997), and the alignment was confirmed by eye. Sequences were analyzed with MacClade 4.0 (Maddison and Maddison, 2000) and PAUP* 4.0b10 (Swofford, 2002) using equally-weighted parsimony (details in figure

legend). Unique sequences were submitted to GenBank (accession numbers AY285162--AY285164).

Results

Morphological observations

The number of statoliths per statocyst was counted in 1555 medusoids of *E. inquilina* from Taranto, Italy and compared with observations on 4597 medusoids of *E. japonica* from Kubota (1991a) (Table 1a). Each medusoid of *E. inquilina* typically had 8 statocysts (2 per interradial sector), but occasionally there were variable numbers of radial canals (3,5, or even 6) and statocysts (5--12) Some statocysts from formalin-preserved medusoids had damaged statoliths, and these were excluded from our analysis. Statolith numbers among statocysts of the same specimen could vary by 1, or rarely 2 or more, statoliths.

A total of 9001 undamaged statoliths were examined in *E. inquilina*. The number of statoliths/statocyst ranged from 0--8, and the mean was 2.4 ±0.83. In this species, fifty – four percent of the statocysts had 1--2 statoliths, which is the statolith number found in 97% of statocysts of *E. japonica* (see tables 1a, 1b). More generally, the number of statoliths per statocyst was significantly different from *E. japonica* (Kolmogorov-Smirnov test, $p < 0.01$), and only 33 medusoids (2.1%) had both 1--2 statoliths per statocyst and a manubrium.

Breeding experiments

All conspecific mating crosses produced healthy, normal planulae, but none of the 13 interspecific crosses did (Table 2). However, some of the interspecific crosses resulted in a few abnormal, short-lived, rotating or swimming larvae without nematocysts and with or without endoderm. These may have been parthenogenetically produced (Kubota, 1987b; Kubota, 1987c; Kubota, 1991b) and all died within a few days.

Mesoglea test

The ectodermal tissue grafted from medusa bud of *E. japonica* adhered to the mesoglea of both other *E. japonica* and *E. inquilina*, but not to the mesoglea of the distantly related *Podocoryna carnea* (Table 3). Ectoderm grafted from *E. inquilina* adhered to the mesoglea of other *E. inquilina*, but not to *P. carnea*. However, *E. inquilina* tissue was not grafted on to mesoglea of *E. japonica*, because, at this point, it was apparent from the previous test (graft of *E. japonica* on to mesoglea of *E. inquilina*) that this method could not distinguish between the two.

DNA sequencing

Five hundred seventy-one base pairs were sequenced and aligned for three individuals of *E. japonica* (two from Atami and one from Shirahama) and four individuals of *E. inquilina* (3 from Taranto and 1 from Lago Fusaro). Of these, 78 characters were variable, including 70 parsimony-informative sites. The three sequences of *E. japonica* were identical, and two (including the Lago Fusaro individual) out of the four sequences of *E. inquilina* were identical. A third differed by one base pair, and the fourth differed from the first two by 14 base pairs, or 2.5%. Sequences of *E. japonica* and *E. inquilina* differed from each other by 68--72 base pairs, or 11.9--12.6 %. *E. japonica* and *E. inquilina* were strongly supported (100% bootstrap) to be reciprocally monophyletic (Figure 2).

Discussion

Our results confirm that *E. inquilina* and *E. japonica* are indeed different species according to the biological species concept, which requires that members of a species should be able to reproduce successfully. In our breeding experiments, none of the crosses produced healthy planulae, while all of the intra-specific crosses did, corroborating the result of Kubota (1989; 1991b). Additionally, the reciprocal monophyly observed in our phylogeny is consistent with the existence of two different species according to the phylogenetic species concept, which is based on descent (Avise, 2000).

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Cryptic species have been found in many marine taxa (Knowlton, 1993).

Knowlton (1993) distinguishes between sibling species and pseudo-sibling species, where sibling species are morphologically indistinguishable and pseudo-sibling species are distinguishable once the appropriate characters are identified. For *Eugymnanthea*, the appropriate characters for identification are only present in two features of the medusoid stage (and even at this stage, there are a few exceptions), and the hydroids are completely indistinguishable.

Ecological and biodiversity studies require accurate species-level discrimination (Maurer, 2000), and molecular methods can provide identification when morphology is inadequate (for example, Bucklin et al., 1995; Hare et al., 2000). Here, we tested two methods: the mesoglea test (Schmid et al., 1992) and 16S rDNA sequence comparison. Our results indicate that the mesoglea test does not have sufficient resolution to determine species-level differences, at least for *Eugymnanthea*. Previously, this test was proven to be effective in species recognition among some, but not all, species of the hydroid *Eudendrium* tested (Schmid et al., 1992) Additional tests in other hydroid families are necessary to prove the extent of applicability of this technique. 16S rDNA sequences, however, clearly distinguish *E. inquilina* and *E. japonica*.

The breeding experiments and DNA sequences also indicate that, unlike the host mussel, the Japanese *Eugymnanthea* is unlikely to be the result of an introduction from the Mediterranean. The three Japanese specimens, unlike those from the Mediterranean, showed no sequence diversity: all 3 sequences are identical. This is probably a result of the small sample size, and is not indicative of a recent invasion, at least not from the Mediterranean population, whose sequences differ from those of the Japanese about 12%. Our sample sizes for the DNA sequences, while small, are sufficient to reject the hypothesis that the two forms belong to the same population (Hey, 1991). Additionally, the observed 12% divergence falls within the range of interspecific divergences found in other Leptomedusae (Govindarajan, unpublished). Govindarajan et al. (submitted) calibrated 16S sequence divergence in another Leptomedusae, *Obelia geniculata,* with the opening of the Bering Strait. Applying their rate of 2.44 x 10^{-9} substitutions/site/year,

which corresponds to 0.49% divergence/million years, suggests *E. inquilina* and *E. japonica* diverged around 24 million years ago. Assuming the 16S substitution rate in *Eugymnanthea* is similar (or slower, as that would increase time since divergence), their divergence predates the closure of the Mediterranean from the Pacific in the late Miocene (Por, 1989) which is what would be expected if, as we argue, *E. japonica* is native to Pacific.

Despite our limited sampling, we feel it is highly unlikely that *E. japonica* is present in the Mediterranean. The hydroid fauna of the Mediterranean is one of the best studied in the world, and it would be surprising if a large population of *Eugymnanthea japonica* (with its unique morphological and ecological features) exists there undetected. Indeed, Kubota (1989, in press) examined thousands of medusoids, obtained from throughout the Italian coasts. Furthermore, it is probable that the small percentage with the aberrant morphologies is simply intraspecific variation. Considerable intraspecific morphological variability is found in other eirenid medusae. For instance, Kubota (1985; Kubota, 1991b; Kubota, 1992a; Kubota, 1992b; Kubota, 1993a; Kubota, 1997) discovered through breeding experiments and observations that the 4 forms of eirenid *Eutima* medusae in Japan belong to the same species (even though one form had features characteristic of a different family!).

The absence of *Eugymnanthea* in other parts of the world, however, may reflect a relict Tethys Sea distribution (Peres, 1985; Boero and Bouillon, 1993) and/or be due to a paucity of studies in those regions. There is a possibility of at least one more species. Salvini-Plawen and Chandrasekhara Rao (1973) described *Anthohydra psammobionta*, a free-living interstitial hydroid, in the meiofauna from the Bay of Bengal, which, due to the presence of a pedal disk, Salvini-Plawen (1987) later referred to as *Eugymnanthea*. On the other hand, Bouillon (1985) placed this species in another family, the Olindiasidae (=Olindiidae) (Limnomedusae). Elucidation of the life cycle and molecular analysis will be necessary to confirm the placement of this enigmatic species.

Kubota (1987a; 2000) discussed the evolutionary origin of *Eugymnanthea*, speculating that the reduced medusae evolved separately in the Mediterranean and Pacific

through independent paedomorphic processes (Boero and Bouillon, 1989). The ancestral form may have had a life cycle like *Eutima*, with a full medusa stage, and the medusa may have been reduced independently in these two lineages. Recent studies have shown that medusa reduction occurs frequently in Hydrozoa, with the resulting taxonomic implication that this feature should not be used as a generic character, as it currently is in *Eugymnanthea* and many others (Petersen, 1990; Cunningham and Buss, 1993; Boero et al. 1997; Govindarajan and Boero, unpublished data [chapter 3]). Thus the presence of the medusoid stage in both *E. inquilina* and *E. japonica* does not necessarily imply a close evolutionary relationship.

It is likely that either *E. japonica* was present in Japan before *M. galloprovincialis*, or that it was introduced from somewhere else in the Pacific. Under the first scenario, *E. japonica* was present in Japan before *M. galloprovincialis* was introduced, although rare and cryptic. Once introduced (from the Mediterranean or elsewhere), *M. galloprovincialis* provided a better host environment than the native bivalves, allowing *E. japonica* to increase. The larger overall population of *E. japonica* may account for the new observations in native bivalve species. Habitat modification may also be causing or contributing to the expansion by providing new habitat for *M. galloprovincialis* and other host species (Kubota, 1992a). If this scenario is correct, then the increase in *E. japonica* is an indirect effect of human activities

In this scenario, is interesting to speculate about why the hydroids were not introduced along with *M. galloprovincialis*. While it is possible that *E. inquilina* was introduced to Japan but remains undetected or is represented by the very small percentage of individuals with the Italian morphology (noted above), given the intense study of Japanese bivalve-inhabiting hydroids by Kubota (1983; 1989; 1991a; 1992a; 2003; in press) it seems unlikely that they simply haven't been discovered. Rather, it seems more likely that *M. galloprovincialis* was introduced without the host via introduction of the larval stage in ballast water (Inoue et al., 1997). As would be expected for a ballast water mediated invasion, the original introduction may have been near the port city of Kobe, and based on a comparison of enzyme polymorphisms with Mediterranean mussels,

Wilkens et al. (1983) concluded that it must have been of a large and continuous nature. Furthermore, based on mitochondrial DNA analysis, Geller et al. (1994) confirmed the presence of *M. galloprovincialis* larvae in cargo ship ballast water.

The second scenario for the origin of *E. japonica*, that it was introduced from somewhere else in the Pacific, is consistent with the invasive species-like history of *E. japonica* in Japan. Its relatively recent discovery and subsequent range expansion, and its comparatively lower variation in 16S rDNA sequences and the number of statoliths/statocysts, could be indicative of a recent anthropogenic introduction. However, for the 16S rDNA, the lower variation may be a consequence of small sample size. Additional genetic samples and studies in other parts of the Pacific will be necessary to fully evaluate this scenario.

This study suggests that DNA sequences will be helpful in identifying cryptic species in hydrozoans. DNA sequence analyses will also be useful in identifying invasions (or confirming endemism) in other hydrozoans, as they have been for other marine invertebrates (Wares et al. 2002). For example, Vainola and Oulasvirta (2001) found used mitochondrial COI sequences to confirm the identity of the introduced limnomedusa, *Maeotias marginata*, in the Baltic Sea. Other possible hydroid introductions have been speculated; for example *Laomedea calceolifera* and *L. flexuosa* in Japan (Chaplygina, 1993), and *Clytia hummelincki* in the Mediterranean (Boero, 2002). These hypotheses could be tested, and the host populations can be identified, using mitochondrial DNA sequences.

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Table 1a. Distributions of statoliths per statocyst from a total of 9001 statocysts observed from medusoids of *E. inquilina* from Taranto, Italy and 12955 statocysts observed from medusoids of *E. japonica* (Kubota, 1991a). The distributions are significantly different (Kolmogorov-Smirnov test, $p \leq 0.01$).

# statoliths/statocyst	# observations *E. inquilina*	# observations *E. japonica*
0	143	149
1	642	11691
2	4221	958
3	3258	144
4	681	11
5	32	1
6	21	1
7	0	0
8	3	0

Table 1b. Morphological overlap in taxonomic characters between *E. inquilina* and *E. japonica*. Values for *E. japonica* are from Kubota (1989; 1991a). Primary diagnostic characters are in bold. For the statoliths per statocyst, the mean, standard deviation, and range (in parentheses) are provided.

Character	*E. inquilina*	*E. japonica*
Hydroids		
Colonial	sometimes	sometimes
Theca	absent	absent
Tentacles	10--30	12--27
Height (mm)	0.2--2.0	0.76--3.5
Medusae		
Manubrium	**absent in 97.9%, present in 2.1%**	**absent in 2.1%, present in 97.9%**
Apical canal	sometimes	sometimes
Gonads	4	4
Radial canals	4	4
Tentacles	0	0
Cirri	0	0
Marginal bulbs	8	8
Statocysts	8	8
Statoliths/statocyst	**2.4 ± 0.83 (0--8)**	**1.08 ± 0.36 (0--6)**
Height (mm)	0.5--1.8	0.59--1.1
Diameter (mm)	0.7--2.6	0.86--1.2

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Table 2. Results of breeding crosses. The fractions indicate the number of successful matings out of the total number of matings attempted. Healthy planulae are significantly more likely to be produced in intraspecific crosses (Fisher 's exact test, p = 0.0018).

	E. japonica male	*E inquilina* male
E. japonica female	1/1	0/9
E. inquilina female	0/4	2/2

Table 3. Results of the mesoglea tests. The fractions indicate the number of successful adhesions and spreading of cells out of the total number of trials. ECM = Extracellular matrix (mesoglea).

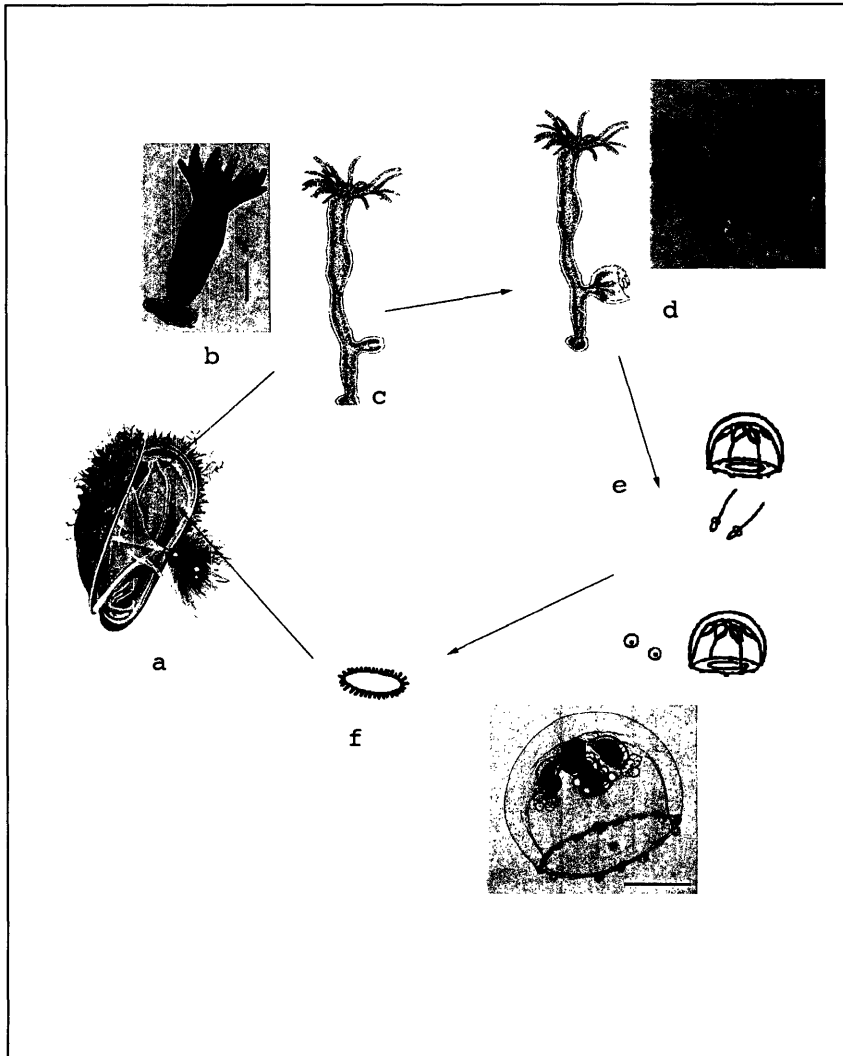|	*E. inquilina* ECM (Taranto, Italy)	*E. japonica* ECM (Shirahama, Japan)	*P. carnea* ECM (Naples, Italy)
E. japonica (Shirahama, Japan)	1/1	1/1	0/2
E. japonica (Atami, Japan)	2/2	Not done	0/2
E. inquilina (Taranto, Italy)	2/2	Not done	Not done

Figure 1. Life cycle of *Eugymnanthea*. Hydroids (a,b) inhabit bivalves, such as *Mytilus galloprovincialis*, and asexually produce medusoids (c,d). The medusoids, which are usually separate sexes, are released from the hydroid and produce gametes (e). The gametes fertilize and form planula larvae (f), which settle in a bivalve host (a) and become hydroids. Scale bars: polyp, 250 μm; medusa, 500 μm.

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Figure. 2. Midpoint-rooted phylogram based on equally-weighted parsimony heuristic search (starting tree through stepwise addition with random addition of taxa (10 replicates), and branch swapping using TBR). Bootstrap numbers are the result of a search with 1000 replicates.