environmental microbiology

Environmental Microbiology (2011) **13**(6), 1467–1476 doi:10.1111/j.1462-2920.2011.02448.x

Composition and dynamics of the gill microbiota of an invasive Indo-Pacific oyster in the eastern Mediterranean Sea

Dror Zurel,[1,2] Yehuda Benayahu,[2] Amitai Or,[2] Amir Kovacs[3] and Uri Gophna[3]*

[1] *The Porter School of Environmental Studies, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel. Department of [2]Zoology and [3]Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel.*

Summary

Gill bacterial communities of *Chama pacifica*, an Indo-Pacific invasive oyster to the eastern Mediterranean Sea, were compared with those of *Chama savignyi*, its northern Red Sea congeneric species. Summer and winter bacterial populations were characterized and compared using 16S rDNA clone libraries, and seasonal population dynamics were monitored by automated ribosomal intergenic spacer analysis (ARISA). Clone libraries revealed a specific clade of bacteria, closely related to marine endosymbionts from the Indo-Pacific, found in both ecosystems, of which one taxon was conserved in oysters from both sites. This taxon was dominant in summer libraries and was weakly present in winter ones, where other members of this group were dominant. ARISA results revealed significant seasonal variation in bacterial populations of Mediterranean Sea oysters, as opposed to Red Sea ones that were stable throughout the year. We suggest that this conserved association between bacteria and oyster reflects either a symbiosis between the oyster host and some of its bacteria, a co-invasion of both parties, or both.

Introduction

Biological invasions in marine habitats represent a recognized worldwide threat to the integrity of native communities, the economy and human health (e.g. Bax *et al*., 2003; Streftaris *et al*., 2005; Galil, 2007; Rilov and Crooks, 2009). Many factors have been found to affect the success of invasion, such as the lack of predators in the invaded ecosystem or physico–chemical conditions that select against or in favour of the invader (reviewed by Vermeij, 1996). One such factor is the presence of microbial pathogens or mutualists that may affect the invader's success to survive and establish a community in its target ecosystem. For example, the microsporidian parasite *Fibrillanosema crangonycis* has been found to promote the invasion of its North American amphipod host *Crangonyx pseudogracilis* to the UK by altering the host's sex ratio towards more females (Galbreath *et al*., 2004). Similarly, the shipworm *Teredo navalis*, hosts cellulose-digesting symbiotic gill bacteria that allowed it to bore into wooden substrates, such as the vessels that have distributed this bivalve worldwide (Norman, 1977).

The eastern Mediterranean Sea (EMS) is susceptible to biological invasions due to its location between the Atlantic, Pontic and Erythrean regions, heavy maritime traffic, and its coastal waters that are teeming with maricultured organisms (Arvanitidis *et al*., 2006). The greatest influx of invaders into the Mediterranean Sea resulted from the opening of the Suez Canal in 1869, which allowed the entry of Indo-Pacific and Erythrean biota (Por *et al*., 1972; Galil, 2007). Several Mediterranean species have been wholly or partially displaced by these invaders (Galil, 2007; Rilov and Crooks, 2009). Such is the case for the Indo-Pacific oyster *Chama pacifica* (class *Bivalvia*, family *Chamidae*), first recorded in the Mediterranean Sea by Tillier and Bavay in 1905, and which has since outnumbered its indigenous congener *C. gryphoides*, which is now hardly encountered due to the new invader that has successfully replaced it (Mienis *et al*., 1993; Galil, 2007).

Chama pacifica has managed to thrive along the Levantine basin (Mienis *et al*., 1993). *Chamidae* species are commonly found attached to sub-littoral hard substrata, in areas with low sedimentation, where they are usually well hidden by overgrowing fouling organisms that extend equally over the natural rocks and shells (Purchon and Purchon, 1981). Previous studies on this species in the Mediterranean Sea focused mainly on the structural effects of *C. pacifica* on the invaded ecosystem, since it

Received 1 December, 2010; accepted 24 January, 2011. *For correspondence. E-mail urigo@tauex.tau.ac.il; Tel. (+972) 3 6409988.

acts as an 'ecosystem engineer' (Streftaris *et al*., 2005). Its heavily calcified shell beds add structural complexity to the Mediterranean rocky habitats, thus providing substrate for settlement and shelter to various invertebrate species (Streftaris and Zenetos, 2006). In contrast, the microbial community of this species, and the role of this community in the invasion of the oyster host, has not been explored.

Bacteria are commonly utilized as food by bivalves and may also inhabit their tissues (reviewed by Harris, 1993). Previous studies on some bivalves dealt with symbiotic bacteria associated with their gill or the gut, which are considered as the primary infection sites (reviewed by Prieur *et al*., 1990). Bacteria may often be symbionts assisting in food digestion, as recorded in the larvae of *Crassostrea gigas* (Prieur *et al*., 1990), and may also supply the bivalve host with growth factors, such as vitamins and amino acids, as revealed in the Pacific vesicomyid clam *Calyptogena magnifica* found at cold seeps (Newton *et al*., 2008). Some symbiotic bacteria have been shown to protect their host from pathogens by either producing antimicrobial agents (Castro *et al*., 2002), or by growing in high densities and thus preventing settlement of other strains (Pujalte *et al*., 1999). We therefore hypothesized that the microbial community (microbiota) composition of invasive *C. pacifica* could be related in composition to closely related Red Sea oysters, due to the similar selective forces operating on both host and microbes. To determine whether such similarity exists, the composition of *C. Pacifica*'s gill microbiota was characterized using 16S rDNA clone libraries. Additionally, the gill microbiota's seasonal dynamics were monitored using automated ribosomal intragenic spacer analysis (ARISA) fingerprinting and compared with those of the surrounding seawater. Oyster gill bacteria have been shown to be more active than those found in digestive glands or gonads (Hernández-Zárate and Olmos-Soto, 2006). As allochthonous bacteria have been found to aggregate in the digestive tract of bivalves (Preiur, 1990), this community is more likely to reflect *C. pacifica's* resident bacteria while minimizing the 'background noise' of bacteria that merely constitute food.

Unlike the dense populations of *C. pacifica* of the eastern Mediterranean, this species is extremely rare in the northern Red Sea (NRS) (H.K. Mienis, pers. comm.). We therefore chose to conduct a concurrent study of *Chama savignyi* Lamy (1921), a widespread Red Sea congener, which is twofold to fivefold smaller, has a longer spawning period, but is highly similar in morphology and exhibits 96% identity of 18S rDNA sequences to those of *C. pacifica* (D. Zurel, Y. Benayahu and U. Gophna, unpubl. data). These comparisons enabled us to identify core bacterial taxa that are shared among these two congeneric species.

Results

Comparison between gill and seawater bacteria

The comparison between the bacterial communities associated with the gills of *C. pacifica* and *C. savignyi* and those of their respective surrounding seawater, using total DNA extraction and ARISA, revealed stark differences in both EMS and NRS oysters in December 2008 (winter) and March 2009 (winter) (Fig. S1). Interestingly, gut microbial communities were more similar to the seawater communities than gill bacteria (Fig. S2). In agreement with the clustering observed in December 2008, gill ARISA profiles from each region (EMS versus NRS) significantly differed from their corresponding seawater profiles (one-way ANOSIM P-values < 0.0014 and < 0.0015 for EMS and NRS, respectively, for both sampling dates).

Phylogenetic conservation in bacterial communities

We obtained partial 16S rRNA gene sequences from 16S clone libraries representing the bacterial communities of *C. pacifica* and *C. savignyi* in both summer (August 2008) and winter (March 2009) (see *Experimental procedures*). Following chimera removal, libraries of *C. pacifica* contained 146 (summer) and 71 (winter) sequences and those of *C. savignyi* 144 (summer) and 172 (winter) (EMBL accession numbers FR666929 to FR667072). The gill communities appeared to be diverse and the libraries were not saturated (Good's coverage ranging from 0.56 to 0.83, also see Fig. S3 for rarefaction curve).

All four libraries showed operational taxonomical units (OTUs) belonging to several bacterial phyla, with a strong dominance of *Proteobacteria*, especially γ-*Proteobacteria* (Figs 1, 2 and S4). One group of phylotypes belonging to this class, of the order *Oceanospirillales*, was dominant in all libraries, representing between 79% and 59% of the observed clones in the *C. pacifica* (Fig. 2A) and 73.8% and 32.12% in the *C. savignyi* August and March libraries (Fig. 2B) respectively. This dominant group, likely to belong to the same family, was termed *Chama*-associated oceanospirillales group (CAOG in short). The closest cultivated relatives to these strains, at a 92–97% 16S sequence identity, were *Endozoicomonas elysicola* and *Spongiobacter nickeltolerans*. The most abundant CAOG clone, termed SAug2 in *C. pacifica* and EAug1 in *C. savignyi*, dominated the August libraries from both species, yet represented only a small fraction of the March libraries (Figs 1, 2 and S4). Several members of γ-*Proteobacteria* and other bacterial Phyla were also observed in both study regions (EMS and NRS). However, only a single phylotype, SAug2/EAug1, was present in all libraries (Figs 1A and B, and S4). In general the phylogenetic structure of the two congeneric oysters 16S rRNA libraries was broadly similar (Figs 1A and B,

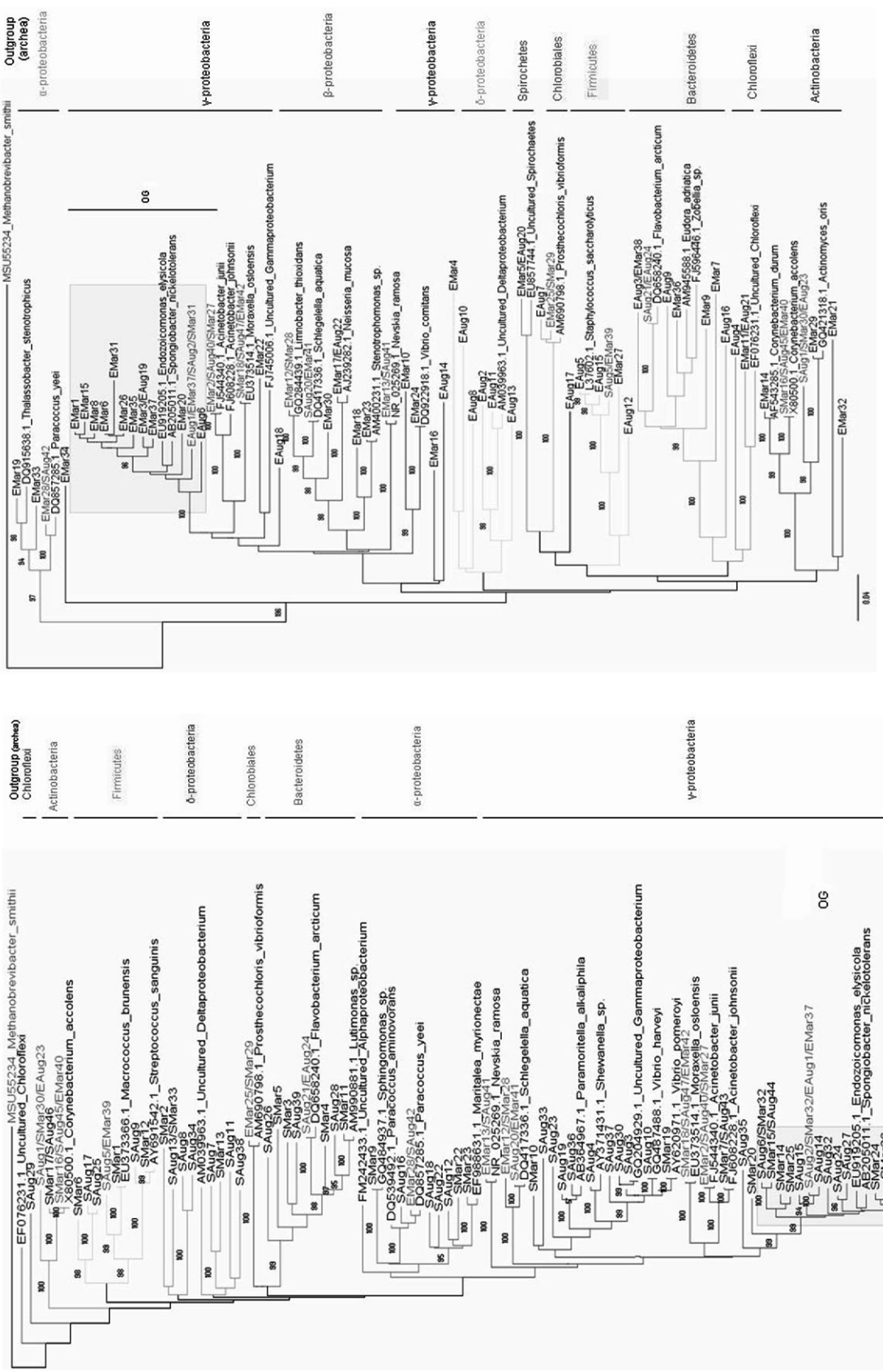

Fig. 1. Phylogenetic analysis of 16S sequences from gill-associated bacteria of *Chama pacifica* (EMS) (A) and *Chama savignyi* (NRS) (B) and their closest GenBank relatives (See Fig. S4 for coloured figure). A bootstrapped neighbour-joining tree corrected for multiple substitutions of all 16S rDNA sequences was constructed from summer (August 2008: S/EAug) and winter (March 2009: S/EMar) sequences, with an archaeal 16S rRNA gene as an outgroup. Numbers represent % bootstrap support based on a 1000 replicate sample sets. Scale bar represents substitutions per site.

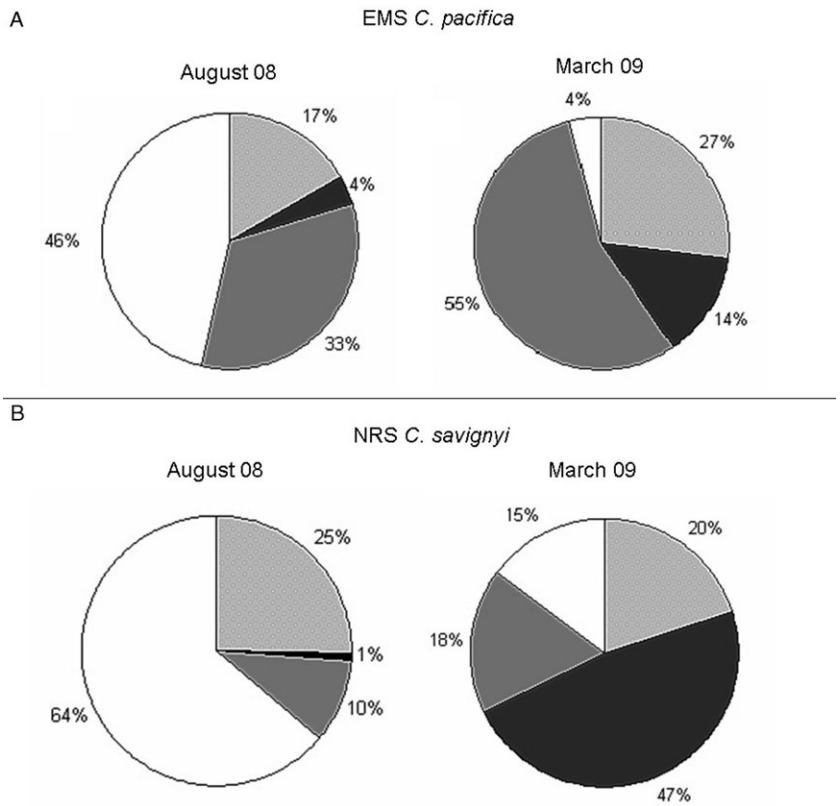

Fig. 2. Pie charts representing percentages of clones belonging to the SAug2/EAug1 clone (blank), other *Oceanospirillales* group 1 (grey), other γ-*Proteobacteria* (black) and other bacterial groups (dotted grey) out of *Chama pacifica* and *C. savignyi* in summer (August 2008: A and C respectively) and winter (March 2009: B and D respectively) clone libraries.

and S4). Accordingly, no statistically significant difference was found between all four clone libraries using either the UniFrac significance test (Lozupone *et al*., 2006) or the P-Test (Martin, 2002), either with or without the use of abundance weights.

Population dynamics of the gill bacterial community

Temporal changes in the gill bacterial communities of *C. pacifica* and *C. savignyi* were examined from August 2008 to August 2009 using ARISA. Notably, a large temporal variation was evident from the ARISA patterns of the EMS *C. pacifica* samples (Fig. 3A and B). Chao1 indices obtained from the clone libraries showed higher species richness in the August libraries relative to the March ones (Table S1). In contrast, the differences between summer and winter were much weaker in the NRS *C. savignyi* (Fig. 3C and D), with close summer and winter Chao1 values (Table S1). To further test these differences, we performed pairwise ANOSIM of Morisita-based similarity values of all sampling months, for each oyster species/location, based on the ARISA data. In agreement with the NMDS (Fig. 3), the most significant temporal differences, reflected in high ANOSIM *R*-values, were evident between August 2008 and March and May 2009 in the EMS *C. pacifica* (Table S2), while in the NRS *C. savignyi* the fluctuations were comparatively low, with ANOSIM *R*-values < 0.5 (Table S3). Notably, when the ARISA profiles of EMS *C. pacifica* and NRS *C. savignyi* were analysed together, they formed two distinct clusters of bacterial communities (Fig. 4).

The *R*-values for one-way ANOSIM between the regions (EMS versus NRS) were all positive, indicating a greater similarity within each group than between groups. *R*-values between these ARISA patterns increased (i.e. differences between communities as opposed to within communities) during December 2008 (winter) and May 2009 (spring), and decreased in August 2009 (summer) (Fig. 5). Thus, there was a greater similarity between the summer bacterial communities of EMS *C. pacifica* and NRS *C. savignyi* compared with the winter ones.

Discussion

In this study we compared the composition and annual dynamics of gill bacteria harboured by two geographically separated oyster communities of the genus *Chama*. The population of *C. pacifica* that has successfully invaded the EMS via the Suez canal was compared with that of *C. savignyi*, indigenous to the NRS. Romero and colleagues (2002) described two kinds of bacteria found within oysters, those who are relatively permanent and associated with the oyster (autochthonous) and those passing through with water and food (allochthonous). Our ARISA

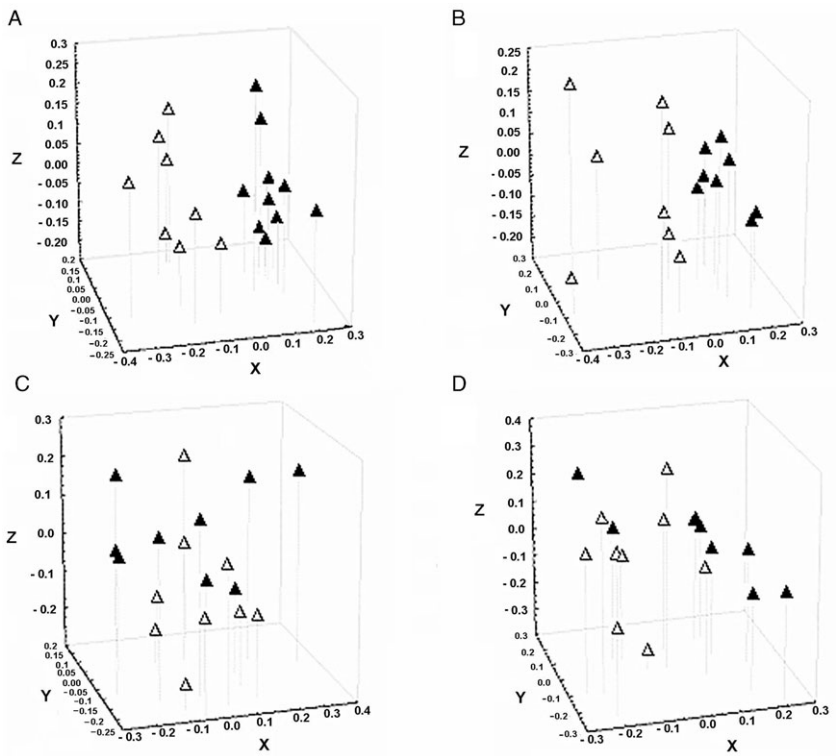

Fig. 3. A comparison of summer gill communities with those of other seasons, using 3D NMDS of ARISA-derived Morisita similarity values. August 2008 samples (empty triangles) of *C. pacifica* (A and B) and *C. savignyi* (C and D) were compared with either March (A and C) or May 2009 (B and D), both represented by black triangles. ANOSIM *R*-values: (A) 0.84, (B) 0.86, (C) 0.27, (D) 0.28; only (A) and (B) had ANOSIM *P*-values < 0.05.

results show a significant difference between the bacterial populations residing in the oysters' gills and those found in its surrounding seawater (Figs S1 and S2), suggesting that the gill microbiota characterized in this study is mainly autochthonous. Our 16S rDNA clone libraries results demonstrate high similarity between the compositions of the gill bacterial populations in both geographic regions (Figs 1, 2 and S4).

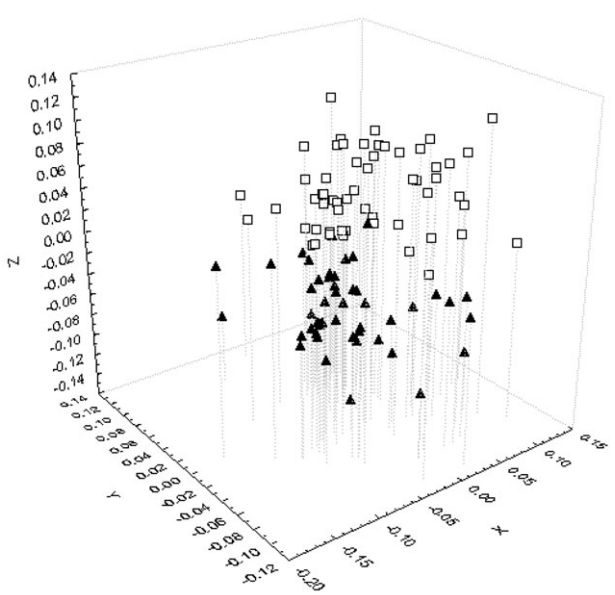

Fig. 4. 3D NMDS of ARISA-derived Morisita similarity values from gill microbial communities of EMS *Chama pacifica* (blank squares) and NRS *C. savignyi* (black triangles) during 2008–2009. ANOSIM *R*-value = 0.43, *P*-value < 0.0001.

Both ARISA and 16S rDNA clone libraries used in our study indicate that the studied *Chama* species harbour a specific and relatively stable gill microbiota, in which CAOG bacteria, closely resembling both *Endozoicomonas elysicola* and *Spongiobacter nickeltolerans*, play a dominant role (Fig. 2). Clones showing > 95.2% 16S rRNA gene sequence identity to *E. elysicola* have been found to be the dominant symbiotic bacteria in sponges in Japan (Kurahashi and Yokota, 2007), several stony corals in the Great Barrier Reef (Bourne and Munn, 2005; Kvennefors *et al*., 2010) and the Caribbean (Cooney *et al*., 2002; Rohwer *et al*., 2002), as well as in a sea anemone in the German North Sea (Schuett *et al*., 2007). This suggests the oyster–bacteria association observed in this study may be a stable symbiosis. Whether this symbiosis is mutualistic remains to be determined, and will require further study, complicated by the fact that we have so far been unable to cultivate CAOG bacteria in the lab.

The vast geographic distribution of *E. elysicola*-related bacteria mentioned above, suggests that CAOG members may have resided in the Eastern Mediterranean prior to the arrival of *C. pacifica*. Indeed, close relatives of *E. elysicola* and *S. nickeltolerans* have also been recorded in the Spanish Mediterranean coast associated

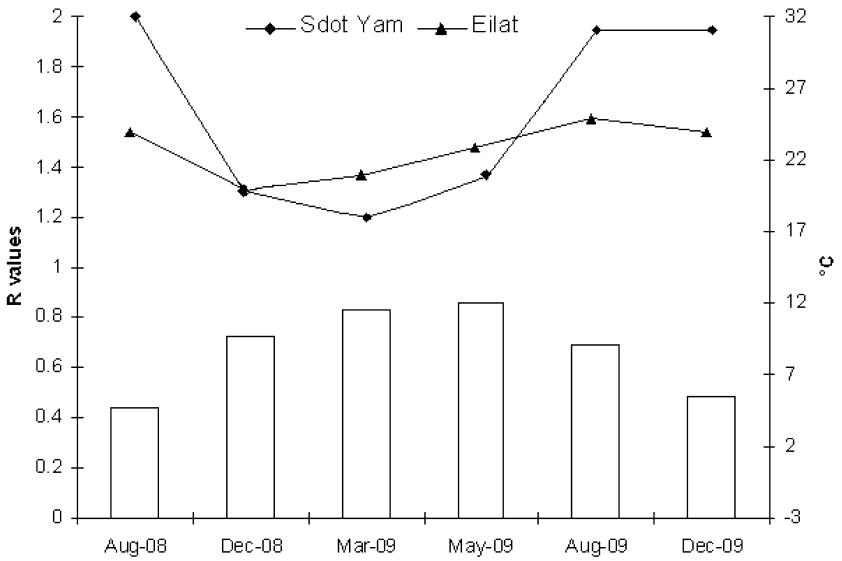

Fig. 5. One-way ANOSIM *R*-values (columns) obtained for Morisita similarities derived from ARISA of EMS *Chama pacifica* and NRS *C. savignyi* during 2008–2009 and the seawater temperatures (lines) measured at each region.

with the colonial ascidian *Cystodytes dellechiajei* (Martinez-Garcia *et al*., 2007) and the Mediterranean sponge *Chondrilla nucula* from Croatia (Thiel *et al*., 2007). Our findings show that one clone, termed SAug2/EAug1, that dominated the EMS August libraries was also the dominant clone in the NRS ones (Fig. 2), and was found in small numbers in the March libraries from both ecosystems as well. While some *C. pacifica* CAOG members may be of Mediterranean origin and could have colonized the oyster post invasion, it is simpler to explain the presence of the dominant bacterium SAug2/EAug1, by a scenario in which it co-invaded the Mediterranean Sea via the Suez Canal along with its host. Alternatively, conditions within the gill may strongly select for particular bacterial species able to persist within them, thus enabling the recruitment of a symbiont from some other marine animal.

While the one-way ANOSIM tests of the NRS ARISA profiles showed a seasonally stable population of the gill microbiota (Table S2), the EMS ones showed significant seasonal changes between summer and winter (Table S3). The temperature fluctuations in the EMS and the relative stability in seawater temperatures in the NRS (Fig. 5) are most likely the reason for the different seasonal population dynamics observed in our current study, although other factors, such as seasonal changes in nutrient levels cannot be altogether dismissed. Seawater temperature has been shown to be the main physico–chemical parameter affecting marine bacterial populations (LeaMaster *et al*., 1997; Gonzalez-Acosta *et al*., 2006), including invertebrate mutualists (Nishiguchi, 2000). Notably, EMS ARISA profiles were more similar to the NRS ones during summer months (August–December), than during winter (March–May), despite the larger temperature differences between sites during summer (Fig. 5). This is in agreement with our 16S rDNA clone libraries results, showing that while August libraries are dominated by one common CAOG member, its relative abundance decreases in the March libraries (Fig. 2), leading to higher Shannon diversity indexes in March Additionally, March Chao1 richness index is significantly lower than the August one in the EMS. The corresponding NRS richness indexes do not differ significantly from one another, suggesting a stable population.

Several Lessepsian co-invasions have been documented, such as that of the Red Sea fish *Siganus rivulatus* and its ectoparasitic monogenean, *Polylabris* cf. *mamaevi* (Pasternak *et al*., 2007). However, not all symbioses may survive the invasion; for example, the symbiosis between the Red Sea ascidian *Herdmania mumus* and the shrimp *Odontonia sibogae* is only observed in the Red Sea, but not in the *H. mumus* population that has invaded the EMS (Shenkar and Loya, 2008). The latter study has suggested that the wide water temperature fluctuations along the EMS may have inhibited the establishment of this symbiotic shrimp. Additionally, elevated seawater temperature, exceeding 31°C, similar to that measured in the EMS during summer months (Fig. 5), caused the Australian sponge *Rhopaloeides odorabile* a loss of symbiotic bacteria followed by establishment of alien microbial populations, including potential pathogenic ones (Webster *et al*., 2008). SAug2/EAug1, the dominant OG member found in this study, appears to be more dominant in the warm temperatures found in the EMS, yet persists even during the colder winter months. Currently, we do not know the nature of this oyster–bacteria relationship, and whether bacterial activity changes with water temperature. Nevertheless, the ability of SAug2/EAug1 to survive large temperature fluctuations may help this relationship persist in the Mediterranean ecosystem.

Experimental procedures

Study sites, sample collection and handling

The study was conducted in two regions: the Israeli EMS and Eilat, NRS. *Chama pacifica* was collected at Sdot Yam (EMS, 29°32′28″N, 53°34′10″E). Seasonal collections were conducted at a depth of 2–6 m by snorkeling and SCUBA during summer (August 2008 and 2009), early winter (December 2008 and 2009), winter (March 2009) and spring (May 2009) ($n = 10$ oysters/collection time). *Chama savignyi* was similarly collected from Eilat reefs (NRS, 30°29′06″N, 55°34′03″E) at a depth of 1–3 m. Seawater temperature was recorded for each date at the sampling depth by a diving computer (Suunto Viper, Vantaa, Finland) (see *Results*).

Oysters were removed from the natural substrate in both regions using hammer and chisel, placed in buckets filled with seawater (collected along with the oysters at the same depth and location) and transferred to Tel Aviv University or the Interuniversity Institute for Marine Sciences at Eilat respectively. Oysters shut tightly when removed from their natural substrates, minimizing the chances for gill contamination. In addition, five 1 l samples of the seawater surrounding the oyster beds were obtained from both regions at each sampling date and kept in sterile bottles. Upon arrival (20–60 min after sampling) the oysters were immediately dissected under sterile conditions and the gills were surgically removed for total (gills and bacteria) DNA extraction using Powersoil DNA extraction kit (MoBio, CA, USA). Each of the sampled seawater bottles was filtered upon arrival at the laboratory, using 0.22 μl filters (Corning). Total seawater bacterial DNA was extracted from the filters using the same kit. DNA concentrations of all samples were determined by a Thermo Scientific NanoDrop 1000 spectrophotometer (Waltham, MA, USA) and DNA samples were then stored at (–20°C), until analysis.

Generation of ARISA profiles

PCR. All reaction templates were normalized to the same DNA concentration of 20 ng μl^{-1} for ARISA. PCR was performed with 1.25 U of Taq DNA polymerase (BIOTAQTM, BIOLINE), 3 mM $MgCl_2$, 2.5 μl 10× PCR buffer, 0.1 mM each dNTP, ultra pure water (Biological Industries) and 10 pmol of primers 1392F and 125R, which was tet-labelled (Hewson and Fuhrman, 2004).

Reactions were prepared in duplicates in a dedicated PCR cabinet with filtered air laminar flow. Negative controls, containing no template, were also prepared to verify lack of contamination. The reaction was performed as follows: 3 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 52°C, 1.5 min at 72°C; and a final elongation step of 6 min at 72°C, using a T-personal PCR thermocycler (BIOMETRA). All PCR products were observed by agarose gel electrophoresis to verify successful amplification and to rule out contamination.

Fragment analysis. The fluorescently labelled PCR products described above were analysed using the ABI PRISM 3100 Genetic Analyzer. The labelled fragments were separated on the capillary sequencer, while an internal size standard, a custom-made marker – CST ROX 250-1150 (Bioventures), was used in each capillary.

Analysis of fingerprinting data

Raw data generated by the ABI PRISM 3100 Genetic Analyzer were initially analysed using GeneMarker (SoftGenetics). After performing accurate size calling and baseline subtraction using the software all data were exported to Microsoft Excel for further analysis. In Microsoft Excel, all OTUs with a relative fluorescence intensity of 40 or lower were excluded. Subsequently, all OTUs were binned as previously described (Brown *et al.*, 2005) and intensities were summed for each bin. Subsequently, the relative intensity for each binned OTU in a certain sample was calculated, and binned OTUs that contributed less than 0.5% to the total intensity of the sample were excluded from analysis. Duplicates were compared, and only OTUs that appeared in both duplicates were used and their new relative intensities were calculated. Finally, the averaged values for each sample were normalized to reflect relative intensity values.

All data were then exported to PAST, a statistical data analysis package (Øyvind Hammer, 2001). Non-metric multidimensional scaling was performed, based on the Morisita similarity index (Morisita, 1959), which has been previously used for DNA fingerprinting methods such as TRFLP (Schmitt-Wagner *et al.*, 2003).

Analysis of similarity (ANOSIM)

ANOSIM is a non-parametric statistical method to compare similarities between and within study groups (Clarke and Warwick, 1994). The ANOSIM statistic R is based on the difference of mean ranks between (r_B) and within (r_W) groups, and is calculated as follows:

$$R = (\text{r\_B} - \text{r\_W})/[N(N-1)/4]$$

ANOSIM R-values range between –1 and 1, with the value 0 indicating completely random grouping.

Clone library construction and analysis

DNA extracted from each oyster was used as a template for amplification of the near-complete 16S rRNA gene using primers 27 forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1391 reverse (5′-GACGGGCGGTGTGTRC-3′), matching the *Escherichia coli* 16S rRNA gene positions 8–27 and 1491–1508, respectively, numbering after Brosius and colleagues (1981). The PCR conditions were 3 mM $MgCl_2$, 2.5 μl 10× PCR buffer, 0.1 mM each dNTP, ultra pure water (Biological Industries) and 1 pmol of each primer, Taq DNA polymerase (BIOTAQTM, BIOLINE), and the standard manufacturer buffer. The PCR reaction conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1.5 min. The final elongation step was 20 min at 72°C. Successful amplification of the 16S rDNA products was verified using electrophoresis in 1% agarose gels and fragments were cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's protocol.

16S rDNA PCR gene clone libraries were constructed from DNA extracted from the gills of 20 oysters, 5 from each region

during two sampling months, August 2008 and March 2009. In total 48 clones were selected randomly from each oyster library for sequencing, and the sequences from individual oyster samples at the same time point/location were then pooled to create four library data sets, each representing one site/season combination; each representative library contained 240 clones. Clones were incubated overnight at 37°C in LB containing Kanamycin, then transferred to LB containing 10% glycerol and frozen in −80°C.

The 960 glycerol stocks (4 libraries*240 clones) were then submitted to the Washington University Genome centre for sequencing. The inserts were sequenced using the 27 forward primer on an ABI 3730 capillary sequencer. Following the removal of low quality sequences and reads that originated from non-16S rRNA gene amplicons (e.g. amplicons of oyster DNA), 600 sequences, 744 bp long on average, were obtained. A distance matrix of unique sequences generated by the PHYLIP software package (Felsenstein, 2005) was imported into DOTUR for phylotype binning (Schloss and Handelsman, 2005), resulting in 160 representative sequences that were run in the Bellerophon server (Huber *et al*., 2004) for chimera identification. Removal of chimeras resulted in 533 sequences. Each of the four libraries was then re-run in the DOTUR software and rarefaction curves were obtained. A bootstrapped neighbour-joining (NJ), tree corrected for multiple substitutions, with *Archea* 16S rDNA as an outgroup, was created using ClustalX (Larkin *et al*., 2007) for each library. A '.txt' file was created with all OTUs named after closest BLAST (Altschul *et al*., 1990) match against the RDP database (Cole *et al*., 2009). Tree and '.txt' file were used for online UniFrac (http://bmf.colorado.edu/unifrac) tests, including the UniFrac significance test, UniFrac Environment Distance Matrix test both with and without use of abundance weights, and the P-Test significance test.

Bootstrapped NJ trees, corrected for multiple substitutions, were created for each sampling site/*Chama* species using ClustalX, and were edited using FigTree (http://tree.bio.ed.ac.uk/software/figtree).

Acknowledgements

We thank Nirit Keren, Adit Naor and Rona Lazary for their help with ARISA data analysis and clone library construction. We thank the staff of the Interuniversity Institute of Eilat and Rami Tzadok of Sdot Yam for their kind hospitality and facilities. Collection of animals complied with a permit issued by the Israel Nature and National Parks Protection Authority. We thank Henk Mienis for assistance in oyster identification. D.Z. was supported by a fellowship from Porter School for Environmental studies. This study was in part supported by the I. Cohen Chair in Environmental Zoology. U.G. is supported by The McDonnel Foundation and the Israeli Ministry of Health.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Arvanitidis, C., Valavanis, V.D., Eleftheriou, A., Costello, M.J., Faulwetter, S., Gotsis, P., *et al.* (2006) MedOBIS: biogeographic information system for the eastern Mediterranean and Black Sea. *Mar Ecol Prog Ser* **316:** 225–230.

Bax, N., Williamson, A., Aguero, M., Gonzalez, E., and Geeves, W. (2003) Marine invasive alien species: a threat to global biodiversity. *Marine Policy* **27:** 313–323.

Bourne, D.G., and Munn, C.B. (2005) Diversity of bacteria associated with the coral *Pocillopora damicornis* from the Great Barrier Reef. *Environ Microbiol* **7:** 1162–1174.

Brosius, J., Dull, T.J., Sleeter, D.D., and Noller, H.F. (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J Mol Biol* **148:** 107–127.

Brown, M.V., Schwalbach, M.S., Hewson, I., and Fuhrman, J.A. (2005) Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environ Microbiol* **7:** 1466–1479.

Castro, D., Pujalte, M.J., Lopez-Cortes, L., Garay, E., and Borrego, J.J. (2002) *Vibrios* isolated from the cultured manila clam (*Ruditapes philippinarum*): numerical taxonomy and antibacterial activities. *J Appl Microbiol* **93:** 438–447.

Clarke, K.R., and Warwick, R.M. (1994) Similarity-based testing for community pattern – the 2-way layout with no replication. *Mar Biol* **118:** 167–176.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., *et al*. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37:** D141–D145.

Cooney, R.P., Pantos, O., Le Tissier, M.D.A., Barer, M.R., O'Donnell, A.G., and Bythell, J.C. (2002) Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques. *Environ Microbiol* **4:** 401–413.

Felsenstein, J. (2005) *PHYLIP (Phylogeny Inference Package)*. Seattle, WA, USA: Department of Genome Sciences, University of Washington, Seattle.

Fishelson, L. (1971) Ecology and distribution of benthic fauna in shallow waters of Red Sea. *Mar Biol* **2:** 113–133.

Galbreath, J.G.M.S., Smith, J.E., Terry, R.S., Becnel, J.J., and Dunn, A.M. (2004) Invasion success of *Fibrillanosema crangonycis*, n.sp., n.g.: a novel vertically transmitted microsporidian parasite from the invasive amphipod host *Crangonyx pseudogracilis*. *Int J Parasitol* **34:** 235–244.

Galil, B.S. (2007) Loss or gain? Invasive aliens and biodiversity in the Mediterranean Sea. *Mar Pollut Bull* **55:** 314–322.

Gonzalez-Acosta, B., Bashan, Y., Hernandez-Saavedra, N.Y., Ascencio, F., and De la Cruz-Aguero, G. (2006) Seasonal seawater temperature as the major determinant for populations of culturable bacteria in the sediments of an intact mangrove in an arid region. *FEMS Microbiol Ecol* **55:** 311–321.

Hammer, Ø., Harper, D.A.T., and Ryan, P.D. (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* **4:** 9pp.

Harris, J.M. (1993) The presence, nature, and role of gut microflora in aquatic invertebrates – a synthesis. *Microb Ecol* **25:** 195–231.

Hernández-Zárate, G., and Olmos-Soto, J. (2006) Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent *in situ* hybridization and polymerase chain reaction. *J Appl Microbiol* **100:** 664–672.

Hewson, I., and Fuhrman, J.A. (2004) Bacterioplankton species richness and diversity along an estuarine gradient in Moreton Bay, Australia. *Appl Environ Microbiol* **70:** 3425–3433.

Huber, T., Faulkner, G., and Hugenholtz, P. (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20:** 2317–2319.

Kurahashi, M., and Yokota, A. (2007) *Endozoicomonas elysicola* gen. nov., sp nov., a gamma-proteobacterium isolated from the sea slug *Elysia ornata*. *Syst Appl Microbiol* **30:** 202–206.

Kvennefors, E.C.E., Sampayo, E., Ridgway, T., Barnes, A.C., and Hoegh-Guldberg, O. (2010) Bacterial communities of two ubiquitous great barrier reef corals reveals both site- and species-specificity of common bacterial associates. *PLoS ONE* **5:** e10401. doi:10.1371/journal.pone.0010401.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., *et al*. (2007) Clustal W and clustal X version 2.0. *Bioinformatics* **23:** 2947–2948.

LeaMaster, B.R., Walsh, W.A., Brock, J.A., and Fujioka, R.S. (1997) Cold stress-induced changes in the aerobic heterotrophic gastrointestinal tract bacterial flora of red hybrid tilapia. *J Fish Biol* **50:** 770–780.

Lozupone, C., Hamady, M., and Knight, R. (2006) UniFrac – an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* **7:** 371.

Martin, A.P. (2002) Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Appl Environ Microbiol* **68:** 3673–3682.

Martinez-Garcia, M., Diaz-Valdes, M., Wanner, G., Ramos-Espla, A., and Anton, J. (2007) Microbial community associated with the colonial ascidian *Cystodytes dellechiajei*. *Environ Microbiol* **9:** 521–534.

Mienis, H.K., Galili, E., and Rapoport, J. (1993)On the presence of the Indo-Pacific bivalve *Chama pacifica* in the Eastern Mediterranean (*Mollusca, Bivalvia, Chamidae*). *Gloria Maris* **32:** 13–18.

Morisita, M. (1959) Measuring of the dispersion of individuals and analysis of the distributional patterns. *Mem Fac Sci Kyushu Univ Ser E* **2:** 214–235.

Newton, I.L.G., Girguis, P.R., and Cavanaugh, C.M. (2008) Comparative genomics of vesicomyid clam (*Bivalvia*: *Mollusca*) chemosynthetic symbionts. *BMC Genomics* **9:** 585.

Nishiguchi, M.K. (2000) Temperature affects species distribution in symbiotic populations of Vibrio spp. *Appl Environ Microbiol* **66:** 3550–3555.

Norman, E. (1977) Geographical distribution and growth of wood-boring Mollusks Teredo-Navalis L, Psiloteredo-Megotara-(Hanley) and Xylophaga-Dorsalis-(Turton) on Swedish West Coast. *Ophelia* **16:** 233–250.

Pasternak, Z., Diamant, A., and Abelson, A. (2007) Co-invasion of a Red Sea fish and its ectoparasitic monogenean, *Polylabris* cf. *mamaevi* into the Mediterranean: observations on oncomiracidium behavior and infection levels in both seas. *Parasitol Res* **100:** 721–727.

Por, F.D., Steinitz, H., Ferber, I., and Aron, W. (1972) Biota of red-sea and eastern mediterranean (1967–1972) a survey of marine life of israel and surroundings. *Isr J Zool* **21:** 459–523.

Prieur, D., Nicolas, J.L., Plusquellec, A., and Vigneulle, M. (1990) Interactions between bivalve mollusks and bacteria in the marine-environment. *Oceanogr Mar Biol* **28:** 277–352.

Prieur, D., Mevel, G., Nicolas, J.-F., Plusquellec, A., and Vigneulle, M. (1990) Interactions between bivalve molluscs and bacteria in the marine environment. *Oceanogr Mar Biol Annu Rev* **28:** 277–352.

Pujalte, M.J., Ortigosa, M., Macián, M.C., and Garay, E. (1999) Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. *Int Microbiol* **2:** 259–266.

Purchon, R.D., and Purchon, D.E.A. (1981) The marine shelled mollusca of West Malaysia and Singapore .1. General introduction and an account of the collecting stations. *J Molluscan Stud* **47:** 290–312.

Rilov, G., and Crooks, J. (2009) *Biological Invasions in Marine Ecosystems: Ecological, Management, and Geographic Perspectives*. Berlin, Germany: Springer.

Rohwer, F., Seguritan, V., Azam, F., and Knowlton, N. (2002) Diversity and distribution of coral-associated bacteria. *Mar Ecol Prog Ser* **243:** 1–10.

Romero, J., Garcia-Varela, M., Laclette, J.P., and Espejo, R.T. (2002) Bacterial 16S rRNA gene analysis revealed that bacteria related to *Arcobacter* spp. constitute an abundant and common component of the oyster microbiota (*Tiostrea chilensis*). *Microb Ecol* **44:** 365–371.

Schloss, P.D., and Handelsman, J. (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71:** 1501–1506.

Schmitt-Wagner, D., Friedrich, M.W., Wagner, B., and Brune, A. (2003) Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). *Appl Environ Microbiol* **69:** 6007–6017.

Schuett, C., Doepke, H., Grathoff, A., and Gedde, M. (2007) Bacterial aggregates in the tentacles of the sea anemone *Metridium senile*. *Helgoland Mar Res* **61:** 211–216.

Shenkar, N., and Loya, Y. (2008) The solitary ascidian *Herdmania momus:* native (Red Sea) versus non-indigenous (Mediterranean) populations. *Biol Invasions* **10:** 1431–1439.

Streftaris, N., and Zenetos, A. (2006) Alien Marine Species in the Mediterranean – the 100 'Worst Invasives' and their Impact. *Mediterranean Marine Science* **7:** 87–118.

Streftaris, N., Zenetos, A., and Papathanassiou, E. (2005) Globalisation in marine ecosystems: the story of non-indigenous marine species across European seas. *Oceanogr Mar Biol – Annu Rev* **43:** 419–453.

Thiel, V., Leininger, S., Schmaljohann, R., Brummer, F., and Imhoff, J.F. (2007) Sponge-specific bacterial associations of the Mediterranean sponge *Chondrilla nucula* (*Demospongiae, Tetractinomorpha*). *Microb Ecol* **54:** 101–111.

Vermeij, G.J. (1996) An agenda for invasion biology. *Biol Conserv* **78:** 3–9.
Webster, N.S., Cobb, R.E., and Negri, A.P. (2008) Temperature thresholds for bacterial symbiosis with a sponge. *ISME J* **2:** 830–842.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. 3D NMDS of ARISA-derived Morisita similarity values from gill's microbial communities of *Chama pacifica* (blank squares) in Sdot Yam (EMS) and of *C. savignyi* in Eilat (NRS) and from microbial communities filtered from their respective surrounding seawater (squares).
Fig. S2. Jaccard similarity index-based cluster analysis of ARISA profiles derived from the gill, gut and surrounding seawater of five *Chama savignyi* oysters from Eilat (NRS), Bootstrap = 1000.
Fig. S3. Rarefaction curves for 16S rDNA clone libraries representing gill bacterial communities from *Chama pacifica* oysters sampled from Sdot Yam (Mediterranean Sea, MS) and *Chama savignyi* from Eilat (Red Sea, RS) during August 2008 and March 2009.
Fig. S4. Phylogenetic analysis of 16S sequences from gill-associated bacteria of *Chama pacifica* (EMS) (a) and *Chama savignyi* (NRS) (b) and their closest GenBank relatives (See SF3 for color figure). A bootstrapped neighbour-joining tree corrected for multiple substitutions of all 16S rDNA sequences was constructed from summer (August 2008: S/EAug) and winter (March 2009: S/EMar) sequences, with an archaeal 16S rRNA gene as an outgroup. Numbers represent % bootstrap support based on a 1000 replicate sample sets. Scale bar represents substitutions per site (colour version).
Table S1. Richness and diversity estimates for bacterial 16S rRNA gene clone libraries from *Chama pacifica* (S – Sdot Yam) and *Chama savignyi* (E – Eilat) gills sampled on August (summer) 2008 and March (winter) 2009, presented by the Shannon diversity index and Chao1 and ACE richness estimators computed using DOTUR.
Table S2. Morisita one-way ANOSIM (*R*-values) between ARISA patterns of *Chama pacifica* gills from the Sdot Yam (SY) during 2008–2009.
Table S3. One-way ANOSIM *R*-values obtained for Morisita similarities derived from ARISA of *Chama savignyi* gills from Eilat (E) 2008–2009.