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The gill microbiota of invasive and indigenous *Spondylus* oysters from the Mediterranean Sea and northern Red Sea

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Summary

The gill tissue of bivalve mollusks hosts rich symbiotic microbial communities that may contribute to the animal's metabolism. *Spondylus spinosus* is an invasive oyster that has become highly abundant along the eastern Mediterranean Sea (EMS) coastline, but is scarce in the northern Red Sea (NRS), its indigenous region. The composition and seasonal dynamics of the gill microbial communities of *S. spinosus* were examined in both regions, using 16S rRNA gene amplicon sequencing. Additionally, two Red Sea *Spondylus* species, *S. avramsingeri* and *S. pickeringae*, were investigated using the same approach. Significant differences were found between microbial communities of the EMS *S. spinosus* and the three NRS species. Bacteria from the family *Hahellaceae* dominated the communities of the EMS *S. spinosus* and the NRS *S. avramsingeri*, oysters that are dominant in their habitat, yet were rare in the NRS *S. spinosus* and *S. pickeringae*, which are only seldom encountered. Bacterial communities of EMS *S. spinosus* were more similar to those of NRS *S. spinosus* than to those of other NRS *Spondylus* species, indicating that either part of the microbiota had co-invaded with their host into the Mediterranean Sea, or that there are species-specific selective constraints on microbial composition.

Introduction

The Suez Canal, which opened in 1869, connecting the Mediterranean and the Red Sea, has caused a massive invasion of marine life into the former, a phenomenon known as the Lessepsian migration (Por, 1971). The spiny oyster *Spondylus spinosus* Schreibers, 1793, is a Lessepsian migrant that was first documented in the Eastern Mediterranean Sea (hereafter EMS) in 1988 (Mienis *et al.*, 1993), and has since expanded its distribution north-west to Turkey (Çeviker and Albayrak, 2006; Katsanevakis *et al.*, 2009; Crocetta *et al.*, 2013; Shabtay *et al.*, 2013). Along the Israeli Mediterranean coast *S. spinosus* forms dense beds together with another invasive oyster, *Chama pacifica*, dwelling on sandstone and artificial structures at a depth of 2–40 m (Mienis *et al.*, 1993; Zurel *et al.*, 2012; Shabtay *et al.*, 2013). Although *S. spinosus* is highly abundant along the Israeli Mediterranean coast, it is extremely scarce in Eilat, Gulf of Aqaba, northern Red Sea (hereafter NRS), which is probably the northernmost edge of its native distribution (Shabtay, 2011; Shabtay *et al.*, 2013).

Several factors, such as climatic conditions, predation pressure and inter-species competition, are known to affect the establishment of an invasive species in its new ecosystem (Vermeij, 1996). An additional factor that cannot be dismissed and is suggested to assist the thriving of a species is the possession of suitable microbiota (Zurel *et al.*, 2011). Microorganisms have been shown to act as inducers of settlement and larval metamorphosis in several bivalve species (Fitt *et al.*, 1990; Tritar *et al.*, 1992; Satuito *et al.*, 1997; Bao *et al.*, 2007). Symbiotic bacteria may also provide their bivalve host with growth factors, such as vitamins and amino acids, assist in food digestion (Waterbury *et al.*, 1983; Prieur *et al.*, 1990) and produce antimicrobial agents that protect their host from pathogen infections (Castro *et al.*, 2002: *Ruditapes philippinarum*). Bacterial communities can be divided into autochthonous, which are stable communities integrated into the host tissues; and allochthonous, which are transients that pass in and out from the surrounding water (Berg, 1996). The microbiota of bivalve gills are considered autochthonous, unlike the microbiota in other organs such as the gonads and digestive system, which are generally allochthonous (Zurel *et al.*, 2011; Wentrup *et al.*, 2013). Bivalve gill tissues are relatively enriched with symbiotic bacteria (Prieur *et al.*, 1990; Zurel *et al.*, 2011) and considered to display higher metabolic activity than in

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Table 1. Number of *Spondylus* oysters collected in the EMS and NRS in each season during 2012–2013.

	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
	2012	2012	2012	2012	2013	2013	2013
EMS *S. spinosus*	8	8	9	4	6	5	5
NRS *S. spinosus*	–	–	–	–	1	3	3
NRS *S. avramsingeri*	–	–	2	2	2	2	2
NRS *S. pickeringae*	–	–	2	2	2	2	2

other organs, such as the digestive glands or gonads (Hernández-Zárate and Olmos-Soto, 2006). Thus, functionality of the gill microbiota is likely to be a critical factor for invasion success. To date, no study has established whether invasive oysters co-invade with their gill microbiota or, rather, recruit a new suite of potential symbionts. In the current study we addressed this issue by examining the gill microbiota of the invasive population of *S. spinosus* in the EMS, and compared it with those of the indigenous *S. spinosus* from Eilat (NRS), as well as to two of its congeners there, *S. pickeringae* Lamprell, 1998, and *S. avramsingeri* Kovalis, 2010. We hypothesized that *S. spinosus* had retained throughout the invasion part of its original (pre-invasion) gill microbiota, which may have contributed to its establishment in the EMS. Specifically, we addressed the questions of whether the bacterial composition of the NRS *S. spinosus*, *S. pickeringae* and *S. avramsingeri* differs from that of the EMS *S. spinosus*, and whether the composition follows a seasonal pattern.

Results and discussion

Here we examined the composition and seasonal dynamics of gill bacterial communities of the oyster *S. spinosus* in the NRS ($n = 7$), its indigenous region, and in the EMS, its invaded region ($n = 45$). We also examined the bacterial composition of two *S. spinosus* NRS congeneric species, *S. avramsingeri* ($n = 10$) and *S. pickeringae* ($n = 10$). Notably, *S. spinosus* and *S. pickeringae* are rare in the NRS, in contrast to *S. avramsingeri*, which is common and is the most abundant *Spondylus* species in that region.

Through 454-pyrosequencing of polymerase chain reaction amplicons, using primers 104F and 530R to target the V2 and V3 regions of the 16S rRNA gene (using reference positions from the *Escherichia coli* 16S rRNA gene), a total of 357 083 high-quality amplicon reads were obtained from 72 samples. The average amplicon sequence length was 407 base pairs after primer and barcode removal. Clustering at an identity level of 97%, a total of 627 operational taxonomic units (OTUs) were obtained. The OTUs were associated to bacterial sequences from 35 known phyla and 3 candidate phyla, which could not be classified as any known phylum, and were therefore named UPYR1, UPYR2 and UPYR3. *Proteobacteria* was the dominant phylum within the bacterial communities of EMS *S. spinosus* oyster gills, with an average relative abundance of 61 ± 22% ($n = 45$) of the total OTUs, and NRS *S. avramsingeri* with 86 ± 12% ($n = 10$), and less abundant but still dominant in NRS *S. spinosus*, with 34 ± 21% ($n = 7$) relative abundance (Fig. S2). NRS *S. pickeringae* was the only species examined whose bacterial communities were dominated by *Spirochaetes* (63 ± 18%, $n = 10$) and where *Proteobacteria* represented only 3 ± 3% of the bacterial communities. Within the phylum *Proteobacteria*, the dominant class was *γ-Proteobacteria*, constituting 84 ± 14% ($n = 45$) of the *Proteobacteria* in EMS *S. spinosus* oysters; 96 ± 7% ($n = 10$) in NRS *S. avramsingeri*; 70 ± 39% ($n = 10$) in NRS *S. pickeringae*; and 70 ± 18% ($n = 7$) in NRS *S. spinosus*. Earlier studies had already shown that *γ-Proteobacteria* are dominant in various marine invertebrates, including bivalves (Sea of Japan: Romanenko *et al.*, 2008; EMS: Zurel *et al.*, 2011), sponges (South China Sea: Li *et al.*, 2006) and corals (Great Barrier Reef: Webster *et al.*, 2004; Gulf of Alaska: Penn *et al.*, 2006).

Seasonality and stability of the gill microbiota in the EMS S. spinosus

Spondylus spinosus oysters were collected by self-contained underwater breathing apparatus (SCUBA) at the Sdot-Yam site (32°29′26.0′N 34°53′09.4′E), the EMS (3–6 m). A total of 45 oysters were collected during the study period (February 2012–July 2013), 4–9 oysters per season: spring (April–May 2012 and 2013), summer (June–August 2012 and 2013), autumn (September–November 2012) and winter (December–March 2012 and 2013) (see Table 1). Unlike the NRS oysters, which are exposed to fairly narrow inter-seasonal temperature fluctuations (21–28°C), the EMS ones encounter a broad seasonal temperature range (16–31°C). Significantly higher alpha-diversity, as measured by the Shannon index, was found in EMS oysters collected when temperatures were relatively high ($T > 30°C$) in comparison to those collected at low temperatures ($T < 18°C$) (Shannon values of 3.1 ± 0.5 and 2.3 ± 0.6, respectively, Mann–Whitney *P*-value = 0.003).

In addition, from the Principal coordinates analysis (PCoA) based on the unweighted UniFrac, it is evident

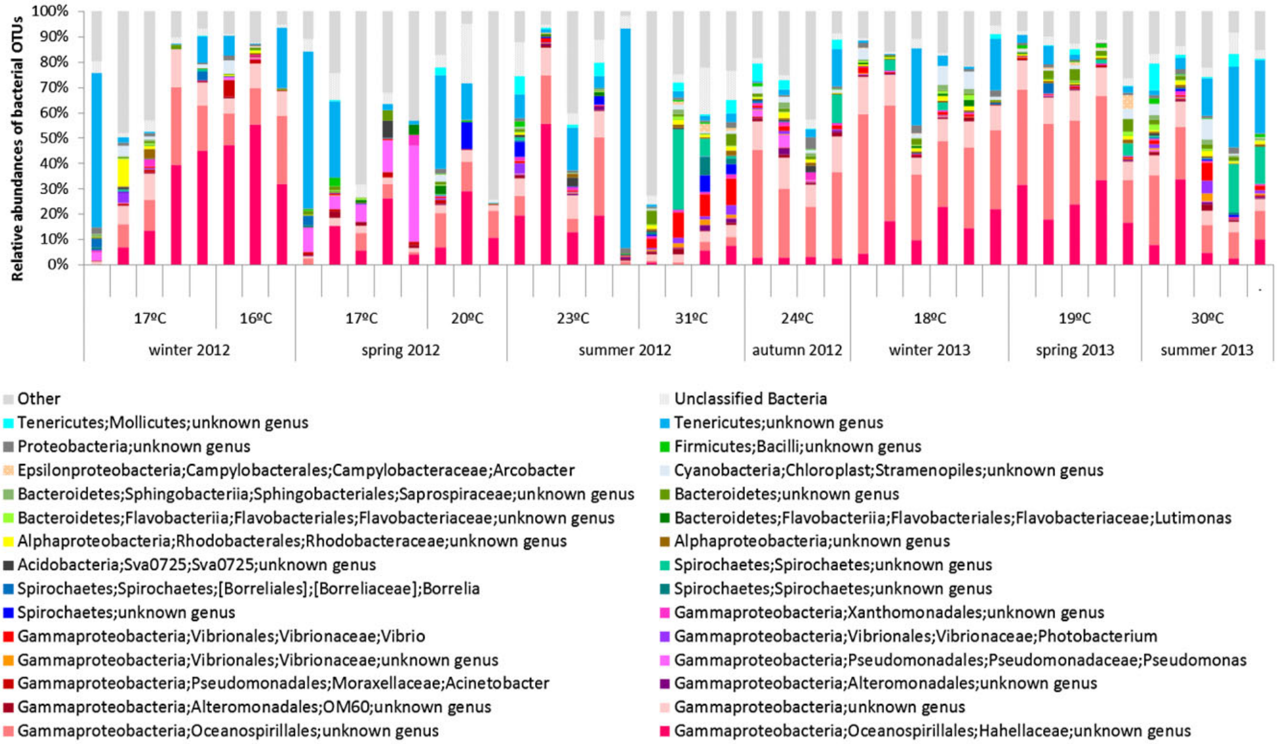

Fig. 1. Relative abundance of bacterial OTUs comprising samples of EMS *Spondylus spinosus* gills in the different seasons. Each colour on the graph represents a distinct genus-level OTU. Only taxa that constituted >2% of an individual sample and were present in at least two samples are presented, and the rest are indicated as ‘others’.

that the summer samples of both 2012 and 2013 are closer to each other in composition than to those of the other seasons. Moreover, when performing pair-wise comparisons of all oysters collected over the different seasons, we observed significant differences between summer communities and those seen in winter and spring (unweighted UniFrac, see Table S1 and Fig. S3).

Within the class *γ-Proteobacteria*, the order *Oceanospirillales*, and within it the family *Hahellaceae*, were the dominant taxa and appeared mostly through autumn to spring (Fig. 1). *Hahellaceae* was present in all EMS *S. spinosus* specimens and comprised on average 16 ± 15% of its total bacteria. The phylum *Spirochaetes* appeared to increase in relative abundance throughout the summer, compared with its yearly mean abundance, in 6 out of 14 individuals (Fig. S4).

Bacterial seasonal dynamics in NRS oysters

The extremely low occurrence of *S. spinosus*, *S. avramsingeri* and *S. pickeringae* in Eilat (29°32′51.9′N 34°57′14.5′E) (Shabtay, 2011), as well as strict conservation constraints, allowed the collection of only 27 oysters there, 10 specimens of both *S. avramsingeri* and *S. pickeringae* and 7 of *S. spinosus*. The collection yielded up to three animals of each species per season in summer (August 2012 and July 2013), autumn (November 2012), winter (February 2012) and spring (April 2013) (Table 1).

The bacterial composition of each NRS oyster species exhibited somewhat different seasonality. Throughout the year, *S. pickeringae* gill microbiota were dominated by *Spirochaetes*, whereas *S. avramsingeri* gills were dominated by *γ-Proteobacteria* (Fig. 2).

Conversely, NRS *S. spinosus* oysters were highly enriched with *γ-Proteobacteria* in winter, which then declined in summer at the same time that *Spirochaetes* increased (Fig. 2). Within the *γ-Proteobacteria* class, an OTU belonging to the candidate genus Candidatus Portiera (family *Halomonadaceae*) and two OTUs from the family *Hahellaceae*, both from the order *Oceanospirillales*, were represented in all of the individual NRS *S. spinosus* oysters (#4248 and #9772, Fig. 3A). Candidatus Portiera were previously identified as primary endosymbionts of the whitefly *Bemisia tabaci* (Baumann *et al.*, 2004; Santos-Garcia *et al.*, 2012).

The gill microbiota of S. spinosus *oysters from the EMS maintain a similarity to the indigenous NRS* S. spinosus *microbiota*

Because *S. spinosus* is a successful invasive species (Shabtay *et al.*, 2013), we investigated whether it had

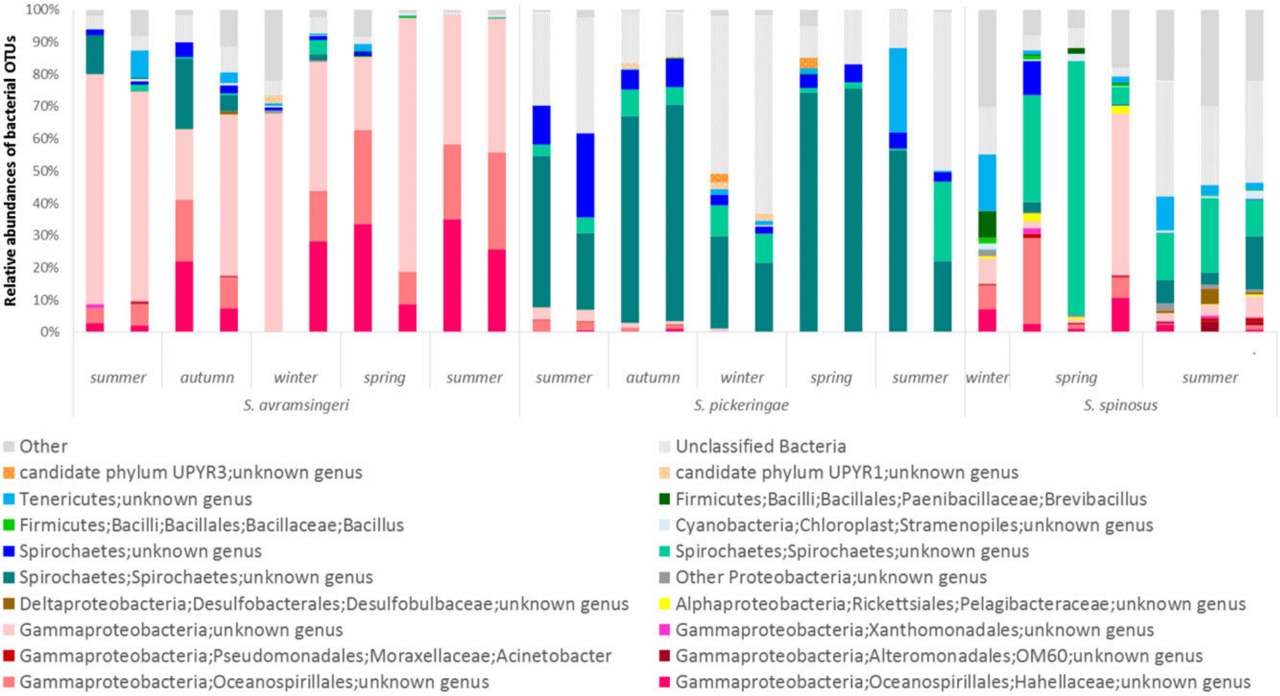

Fig. 2. Relative abundance of bacterial OTUs comprising samples of NRS *Spondylus* species (*Spondylus spinosus*, *S. pickeringae* and *S. avramsingeri*) in the different seasons. Each colour on the graph represents a distinct genus-level OTU. Only taxa that constituted >2% of an individual sample and appeared in at least two samples were presented, and the rest are indicated as 'others'.

retained at least part of its original gill microbiota. Overall, phylum composition (Fig. S2) and unweighted UniFrac analysis (Fig. 4) indicated that bacterial communities of EMS *S. spinosus* were more similar to those of NRS *S. spinosus* oysters than they were to those of the two other *Spondylus* species from the NRS. Nevertheless, the EMS *S. spinosus* were still distinct from the NRS ones in their gill microbiota composition (Analysis of similarity [ANOSIM] R-value = 0.68, *P*-value = 0.0006). Taken together, these results indicate that the invasive *S. spinosus* has probably retained some of its dominant original core microbiota, although the acquisition of new bacteria from the new environment, and the loss of other bacterial members, probably also occurred.

Because temperature is a strong determinant of microbial composition, one would predict a higher similarity between EMS and NRS oyster microbiota composition sampled from water of similar temperature. Although the bacterial communities of NRS *S. spinosus* differed significantly from the EMS ones across all seasons (unweighted UniFrac based ANOSIM $0.7 > R > 0.5$, $P\text{-value} < 0.006$), the NRS *S. spinosus* were most distant in composition from samples of EMS oysters collected in the winters of both 2012 and 2013, and more similar to samples collected in the summers of 2012 and 2013 (unweighted UniFrac-based PCoA Fig. S5).

We used LEfSe (Linear discriminant analysis effect size) (Segata *et al.*, 2011) to identify which of the bacterial taxa differed the most between the NRS and EMS oyster gills, and found that the order *Oceanospirillales*, and particularly the family *Hahellaceae*, were the main determinants for the differences between *S. spinosus* gill communities of the two regions (Fig. S6). While *Oceanospirillales* (median value = 15%, 1%, EMS and NRS respectively) and *Hahellaceae* (median value = 13%, 2%, EMS and NRS respectively) dominated the bacterial communities in EMS oysters, NRS *S. spinosus* oysters were dominated by *Spirochaetes* (median value = 15%, 0%, NRS and EMS respectively). Nonetheless, *Hahellaceae* were present in all *S. spinosus* oysters of both regions, indicating that this family is a core member of the gill microbiota of this oyster species. Moreover, there were 38 OTUs that appeared in over 50% of samples of EMS *S. spinosus*, and all of those OTUs appeared in at least one sample of NRS *S. spinosus*, indicating a possible co-invasion of these OTUs with the oyster host.

NRS Spondylus *species harbour distinct gill microbiota*

Significant differences were observed between the three NRS *Spondylus* species (pair-wise unweighted UniFrac based ANOSIM $R > 0.44$, $P\text{-value} < 0.0072$, see Fig. 4). Notably, alpha diversity (as reflected by the Shannon index) of EMS and NRS *S. spinosus* oysters (2.5 ± 0.7 and 2.4 ± 0.7 respectively) was significantly

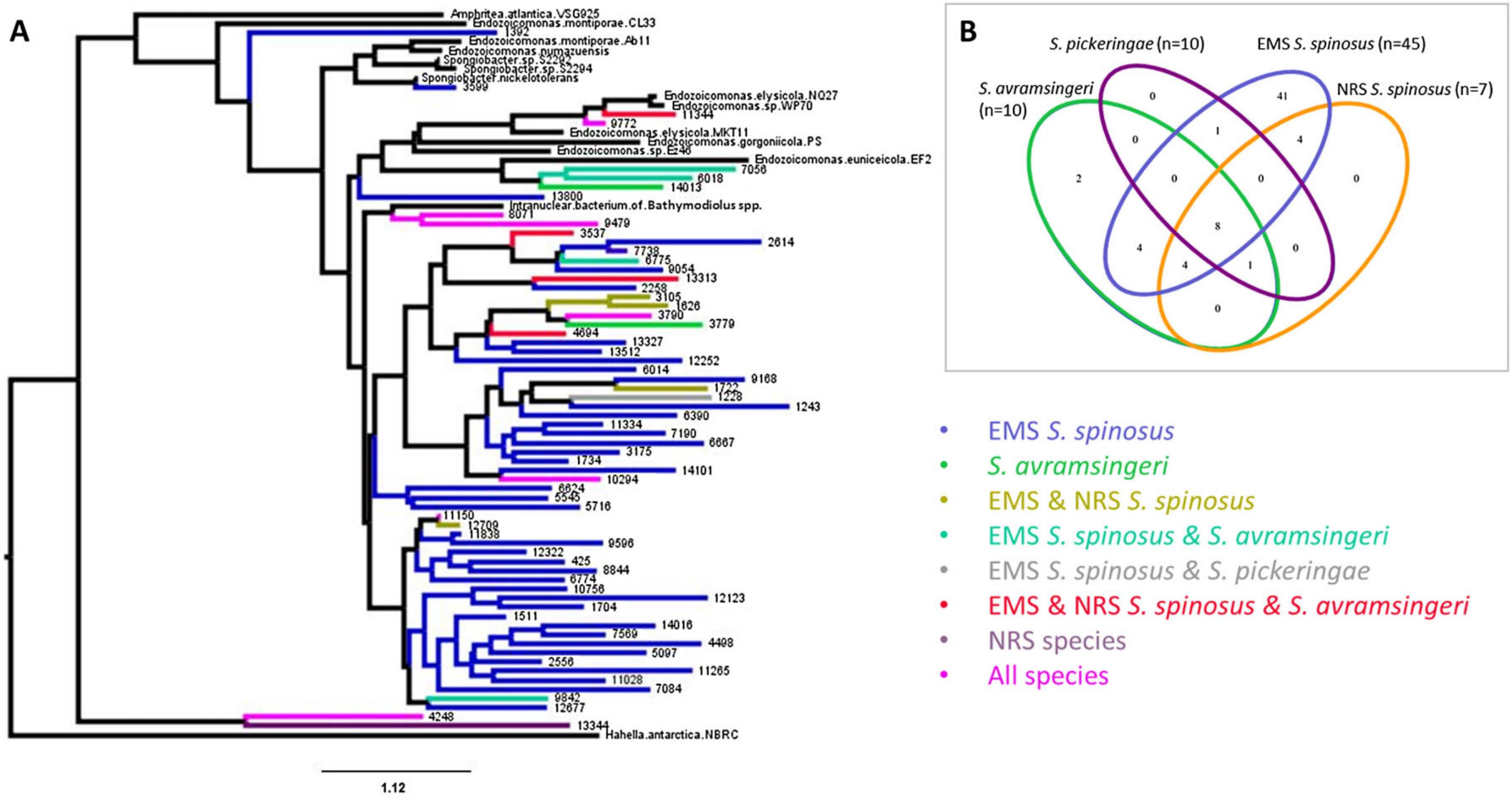

Fig. 3. (A) A neighbour-joining tree of OTUs assigned as *hahellaceae* based on a MAFFT (Katoh *et al.*, 2002) multiple sequence alignment where ambiguous positions were later removed. The tree was visualized using FIGTREE (http://tree.bio.ed.ac.uk/software/figtree/). Branches are colour-coded according to their distribution among oyster species, and reference sequences branches are in black. Bar represents substitutions per nucleotide site. (B) A Venn diagram representing the number of OTUs shared between different species of *spondylus* oysters from different sites.

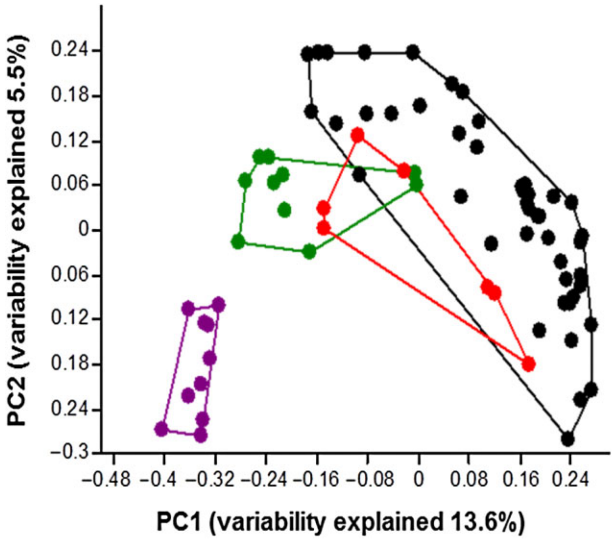

Fig. 4. Principal coordinates analysis (PCoA) of unweighted unifrac matrix showing microbial communities of the EMS *Spondylus spinosus* (black), NRS *S. spinosus* (red), NRS *S. avramsingeri* (green) and NRS *S. pickeringae* (purple). PC, principle coordinate.

higher (Mann–Whitney *P*-value < 0.009) than that of *S. avramsingeri* and *S. pickeringae* (Shannon values of 1.4 ± 0.4 and 1.2 ± 0.2 respectively).

Endozoicomonas*-like bacteria abundance is associated with oyster species abundance*

Interestingly, we revealed a positive association between the relative abundance of *Hahellaceae* in the gills and the abundance of its host species in the NRS and EMS. *Hahellaceae* dominate the EMS *S. spinosus* (Fig. 1) and NRS *S. avramsingeri* (Fig. 2) bacterial communities, both of which are relatively abundant oysters in their respective environments. *Hahellaceae* were present in all *S. avramsingeri* and EMS *S. spinosus* oysters, constituting on average 17 ± 14% and 16 ± 15% of their bacterial assemblages respectively.

In contrast, *Hahellaceae* were only a minor component of the gill microbiota of the NRS *S. spinosus* and NRS *S. pickeringae* oysters (Fig. 2), which are relatively scarce in the examined reefs.

Most of the diverse OTUs assigned as *Hahellaceae* clustered with the cultivated members of the genus *Endozoicomonas* (Fig. 3A). Out of 65 *Hahellaceae* OTUs, 42 were specific to EMS *S. spinosus,* two were exclusive to *S. avramsingeri*, whereas the rest were shared between one or more sample types (different species or samples from different seas, Fig. 3B). The higher diversity of *Endozoicomonas*-like bacteria in EMS *S. spinosus* (*n* = 45) may be at least partially due to higher sampling size compared with the total sample size of NRS oysters (*n* = 27). There are 15 shared OTUs between NRS and EMS *S. spinosus*, and it appears likely that at least some of those strains have co-invaded with their host. These findings are compatible with those of Zurel and colleagues (2011), who suggested that members of the genus *Endozoicomonas*, which dominated the bacterial communities of the Lessepsian migrant oyster *Chama pacifica*, had co-invaded with their host and were tolerant to temperature shifts in the EMS.

The bacterial genus *Endozoicomonas* was first isolated from the sea slug *Elysia ornata* (Kurahashi and Yokota, 2007) and has since then been found to be dominant in a variety of marine invertebrates, such as sponges (Ligurian Sea, Italy and Adriatic Sea, Croatia: Thiel *et al*., 2007; Suruga Bay, Japan: Nishijima *et al*., 2013), mollusks (Izu-Miyake Island, Japan: Kurahashi and Yokota, 2007; EMS: Zurel *et al*., 2011; Zielinski *et al*., 2009; Beinart *et al*., 2014), corals (Kenting, southern Taiwan: Yang *et al*., 2010; Red Sea: Bayer *et al*., 2013; French Mediterranean coast: Bayer *et al*., 2013) and sea anemones (North Sea: Schuett *et al*., 2007). These examples imply a stable symbiotic relationship, although whether this symbiosis is beneficial for the oyster remains to be demonstrated. A parasitic relationship with related taxa was described by Zielinski and colleagues (2009), who found intranuclear *Endozoicomonas*-like bacteria in hydrothermal vent mussels. Although many studies describe different symbiotic and beneficial relationships of microbiota and their host, only a few studies have demonstrated how microbiota may enhance an invasion process and the establishment of their invasive host. One such example is the microsporidian parasite *Fibrillanosema crangonycis*, which has been suggested to enhance the invasion of its North-American amphipod host *Crangonyx pseudogracilis* to the United Kingdom by altering the sex ratio of its host toward more females, thereby increasing the rate of its population growth (Slothouber Galbreath *et al*., 2004).

We therefore speculate that *Endozoicomonas*-related members of *Hahellaceae* may contribute to their *Spondylus* hosts. Unfortunately, despite several efforts and a variety of growth media, we were unable to cultivate any of these oyster-associated *Hahellaceae*. Thus, further studies using metagenomics or single-cell genomics are required in order to elucidate the precise nature of this oyster-bacteria association and the conditions that enable both to flourish in the invaded EMS. Such studies should be combined with a functional approach in which controlled in vivo experiments will determine the degree of dependence of the oyster on its microbiota.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S2. Average relative abundance of bacterial phyla from the EMS *Spondylus spinosus,* NRS *S. spinosus, S. avramsingeri* and *S. pickeringae* gills. Each color on the graph represents a distinct phylum- level OTU. Only taxa that constituted >1% of an individual sample and appeared in at least two samples were presented, and the rest are indicated as "others".

Fig. S3. Unweighted UniFrac- based principal coordinates analysis (PCoA) of microbial communities of the EMS *Spondylus spinosus*. Winter- circle, spring- square, summer- triangle, autumn- diamond. 2012- filled marks, 2013- blank marks.

Fig. S4. Relative abundance of bacterial OTUs from the EMS *Spondylus spinosus* gills in the different seasons. Each color on the graph represents a distinct phylum- level OTU. Only taxa that constituted >1% of an individual sample and appeared in at least two samples were presented, and the rest are indicated as "others".

Fig. S5. Unweighted UniFrac- based principal coordinates analysis (PCoA) of microbial communities of the NRS *Spondylus spinosus* (black) and EMS *S. spinosus* collected during Winter 2012 (dark blue), spring 2012 (dark green), summer 2012 (red), autumn 2012 (brown), Winter 2013 (light blue), spring 2013 (light green) and summer 2013 (pink).

Fig. S6. Taxa differentially distributed between EMS *S. spinosus* (black) and NRS *S. spinosus* (red). LEfSe algorithm was used to identify differential taxa.