Biogeography of sponge-associated bacterial communities and resilience to anthropogenic perturbations

Biogeografía de las comunidades bacterianas asociadas a esponjas y su resiliencia frente a perturbaciones antropogénicas

Lucía Pita Galán

Biogeography of Sponge-Associated Bacterial Communities and Resilience to Anthropogenic Perturbations

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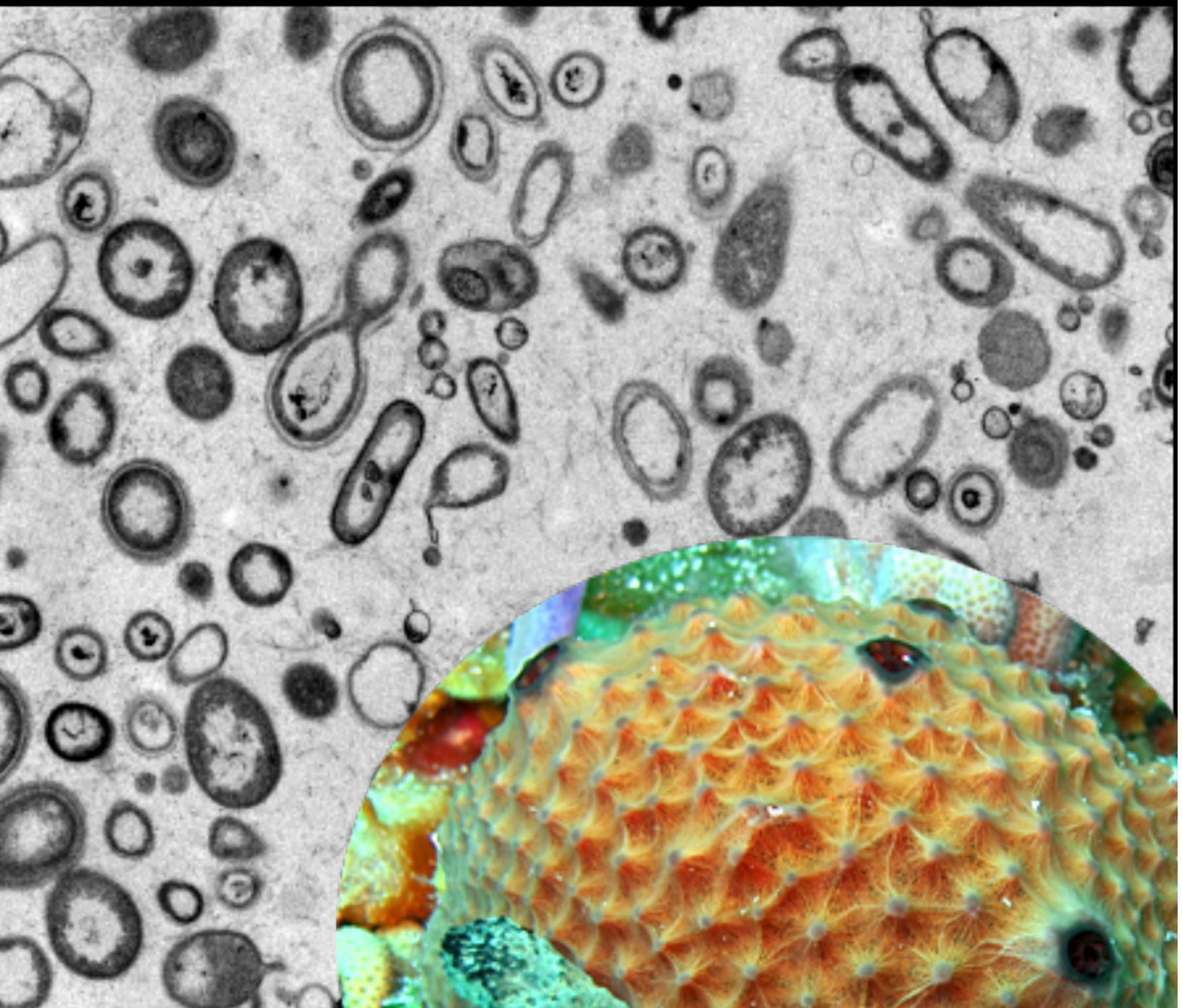

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BIOGEOGRAFÍA DE LAS COMUNIDADES BACTERIANAS ASOCIADAS A ESPONJAS Y SU RESILIENCIA FRENTE A PERTURBACIONES ANTROPOGÉNICAS

Lucía Pita Galán

Doctoral Thesis

2014

Cover

Ircinia felix (Bahamas) and its associated microbial community

Underwater photography: Courtesy of P.M. Erwin

Electron micrograph: L. Pita Galán

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Memoria presentada por

Lucía Pita Galán

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"… Como el comedor de cangrejos,
que para llevar un poco de carne a la boca
tiene que hacer un gran montón de cáscaras."

- Aristón de Chíos

(traducción de A. Cunqueiro en "Fábulas y Leyendas de la Mar")

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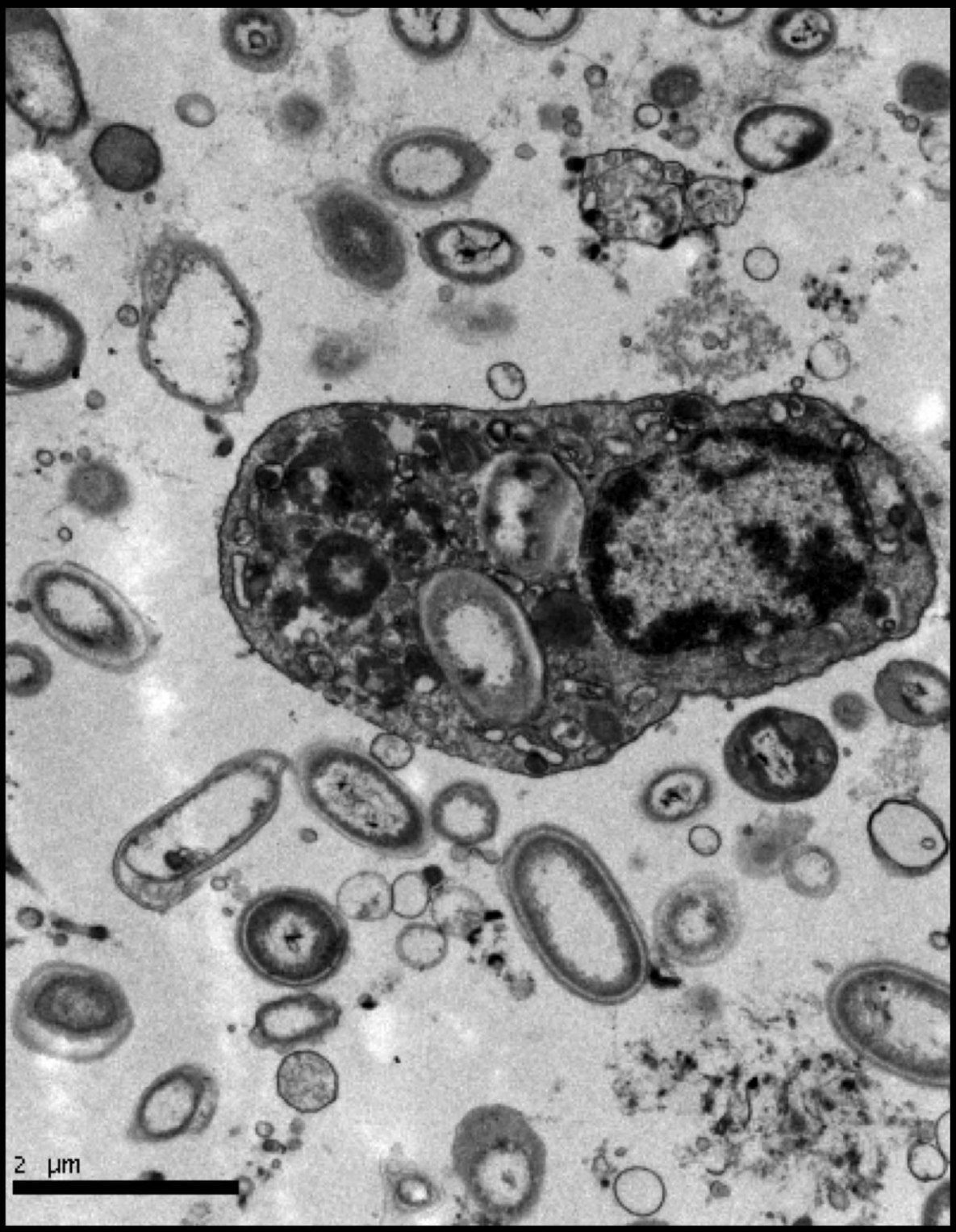
Contents



General Introduction

Electron Micrograph of the mesohyl of *Ircinia strobilina*

L. Pita Galán

A Symbiotic World

Symbiosis is the close association between organisms of different species (De Bary, 1879). Classically, the symbiotic interactions were further classified according to the benefits/costs for each of the partners into three categories: mutualism, commensalism and parasitism (**Fig. 1**). These categories are not closed boxes, but a continuum in which the benefit-cost relation may vary depending on environmental conditions (Palmer *et al.* 2008; LaJeunesse *et al.* 2009; Gsell *et al.* 2013). Thus, symbiotic interactions are dynamic and it is crucial to assess their variability over spatial and temporal scales and their susceptibility to perturbations and artificially-induced changes in their environment (Kiers *et al.* 2010).

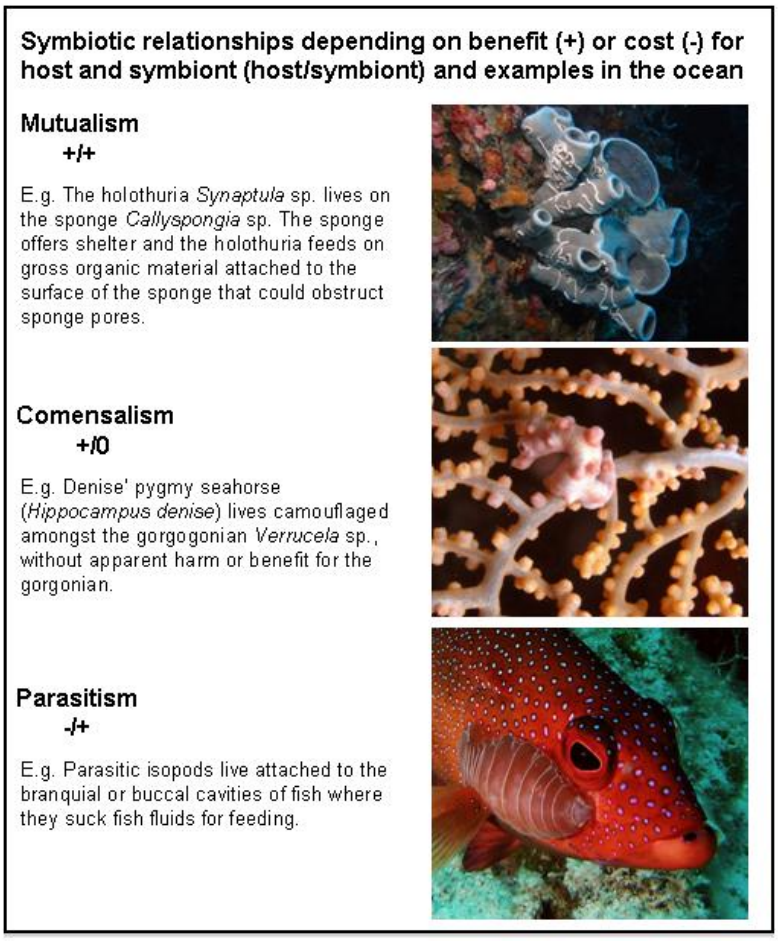


Figure 1. Symbiotic relationships depending on the benefits (+) or costs (-) for each partner and examples in the ocean. Photographies courtesy of Owen Wangensteen and Susanna López-Legentil.

Lynn Margulis (1938-2011), who postulated the symbiotic origin of mitochondria and chloroplasts in eukaryotic cells, was also a pioneer in perceiving that microbial symbionts do not merely cause diseases, but can deeply affect the biology, ecology and evolution of animals (Margulis 1998). Advances in microbial ecology techniques during the last two decades have provided further evidence of the ubiquity and diversity of microbial-animal interactions and further work will probably change the way we define the ecological niches, adaptation and evolution of both animal and microorganisms (McFall-Ngai 2008; McFall-Ngai *et al.* 2013). In fact, the term "holobiont" is now widely used to refer to the host and its metabolically active population of microbial symbionts as the unit of selection and evolution (Mindell 1992; Rohwer *et al.* 2002).

This PhD thesis is focused on the symbiotic relationship between sponges and bacteria, a research field known as sponge microbiology. Research on sponge-associated microbial communities began in the 1970s, when high densities of microbes of different morphologies was observed by microscopy in different sponge species (Sarà 1971; Vacelet & Donadey 1977; Wilkinson 1978). Since then, there has been a growing interest in investigating the "who, how and why" of microbial symbiosis in sponges (Thacker & Freeman 2012). To date, several studies have already demonstrated that sponge-associated microbes are implicated in host metabolism and chemical defense production and thus in the ecology of sponges in marine ecosystems (Taylor *et al.* 2007). However, there is still an open debate about the processes that govern the complex microbial community observed in sponges, their response to biotic and abiotic factors, and their vulnerability to environmental perturbations.

The Sponge Host

Sponges - phylum Porifera, L. *porus* (pores) + *ferre* (bear) - present a porous body plan designed to live attached to the substrate while filtering huge volumes of seawater and feeding on organic particles (**Fig. 2**). The water enters the sponge through pores in their surface (ostia). Their body contains a system of channels and chambers (choanosome) where specialized cells (choanocytes) facilitate the flow of seawater and filter food particles. Seawater exits the sponge by one or more exhalant pores (oscula). Food particles are transferred to the matrix within the sponge (mesohyl) and are mostly digested by ameboid cells (archeocytes). The outer surface of the sponge is called the ectoderm and the inner part, which comprises the choanosome and the mesohyl, is called the endoderm. Microbial symbionts in sponges usually occur extracellularly in the mesohyl, although some species also harbor intracellular symbionts.

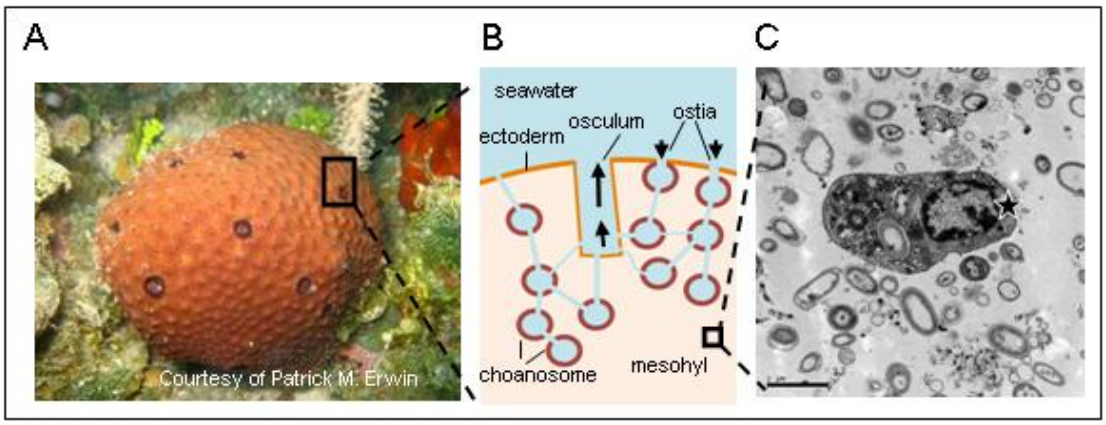


Figure 2. (A) Photography of the marine sponge *Ircinia felix* from the Bahamas **(B)** Schema of the body plan of a typical demosponge. The water enters the sponge through the ostia in the surface (ectoderm) and travels to the choanocyte chambers, where food particles are removed and transferred to the mesohyl to be digested. The filtered water is exhaled through the osculum. **(C)** Electron micrograph of the mesohyl showing a sponge cell (archeocyte) (*star*) surrounded by symbiotic microbial cells.

Sponges are among the most ancient of the Metazoa. Fossils from the Precambrian suggest their appearance around 600 million years ago (Li *et al.* 1998; Love *et al.* 2009). Their ancient origin has prompted many studies using them as a model to identify the events that allowed the emergence of multicellularity and the early evolution of animals (e.g., Srivastava *et al.* 2010; Leys & Riesgo 2012). Marine sponges have colonized all the oceans, from shallow to deep waters. The number of marine sponge taxa exceeds 8,500 described species and this number is still expected to increase (Van Soest *et al.* 2012). Sponges represent a significant component of the ecosystem because of their diversity, abundance and influence on nutrient fluxes (Diaz & Rutzler 2001; De Goeij *et al.* 2013; Fiore *et al.* 2013). For instance, a recent study has evidenced how sponges in coral reefs invest the organic matter they consum in cell regeneration so that dead cells are released into the seawater and taken up by detritivorous, returning the nutrients back into the ecosystem (De Goeij *et al.* 2013). This model has been called “the sponge loop” and could explain at least part of the high productivity of coral reefs in oligotrophic waters (De Goeij *et al.* 2013).

Besides their importance in nutrient fluxes, sponges are also well-known producers of chemical compounds that they use to avoid competition, predation and fouling (Pawlik *et al.* 1995; Pawlik *et al.* 2007; Haber *et al.* 2011). The cytotoxic and antimicrobial activities of some of these compounds make marine sponges the most rich taxon in novel bioactive secondary metabolites with pharmaceutical applications, especially as anti-tumoral drugs (Faulkner 2001; Erwin *et al.* 2010; Paul *et al.* 2011).

However, in most cases, sponges are not solely responsible for the roles described above. In fact, most of these functions are accomplished in association with an abundant and complex symbiotic microbiota (Taylor *et al.* 2007; Thacker & Freeman 2012; Webster & Taylor 2012). The metabolic activity of microbial symbionts expands sponge metabolism by photosynthesis, nitrogen fixation or ammonia oxidation (Weisz *et al.* 2007; Erwin & Thacker 2008b; Fiore *et al.* 2010). In addition, symbionts may actively participate in the chemical defense and the production of bioactive secondary metabolites detected in sponges (Esteves *et al.* 2013; Haber & Ilan 2013). In exchange for their contibution, microbes benefit from living in the protected, nutrient-rich sponge mesohyl (Taylor et al. 2007) and from some metabolic waste released by host cells (e.g., ammonia, López-Legentil *et al.* 2010). Accordingly, this symbiotic association is considered mutualistic in most cases; yet empirical evidence is still scarce (Taylor *et al*. 2007).

Sponge Microbial Symbionts

The striking microbial density and diversity in sponges was first revealed by transmission electron microscopy (Sarà 1971; Vacelet & Donadey 1977). Later, molecular studies confirmed that many sponges harbor a complex microbial community that includes mostly *Bacteria* – 17 described phyla and 12 candidate phyla – and *Archaea*, but also fungi and other eukaryotes (Schmitt *et al.* 2012; Webster & Taylor 2012). Hundreds of bacterial taxa can occur in a single host individual (Webster *et al.* 2010; Lee *et al.* 2011), but the dominant phyla are generally *Proteobacteria* (Class Alpha-, Delta- and Gammaproteobacteria), *Chloroflexi*, *Actinobacteria*, *Acidobacteria* and *Nitrospira* (Webster & Taylor 2012). In addition, many species harbor photoautrotrophic symbionts in the phylum *Cyanobacteria* that reach high densities in the ectoderm of those sponges (Thacker 2005; Erwin & Thacker 2007; Erwin *et al.* 2012b). Archaea in marine sponges belong mostly to the *Thaumarchaeota* phylum, previously known as Marine group I *Crenarchaeota* (Steger *et al.* 2008; Turque *et al.* 2010; Radax *et al.* 2012). Several studies have also shown that the majority of the microbial community is metabolically active in sponge hosts (Mohamed *et al.* 2008a; Kamke *et al.* 2010; Moitinho-Silva *et al.* 2013).

A recent comprehensive analysis repoted a small core bacterial community in sponges, defined as the bacterial phylotypes present in at least 70% of all the analyzed sponges (Schmitt *et al.* 2012). The core bacterial taxa in a particular sponge species are closely related to those found in phylogenetically and geographically distant sponges and absent or rare in other environments (e.g., surrounding seawater,

sediments) (Hentschel *et al.* 2002; Taylor *et al.* 2013). This degree of specificity led to the definition of sponge-specific bacterial clusters (**Box 1**). Notably, some ubiquitous sponge-associated bacteria are also closely related to bacteria in coral microbiota, comprising the sponge-coral clusters (Simister *et al.* 2012a).

Box 1 | **Sponge-specific clusters**

The concept was introduced by Hentschel and collaborators (2002) to define a monophyletic complex (which is composed of, at least, three 16S rRNA gene sequences) representing bacteria that are repeatedly detected in different sponge species or the same species from different geographical locations, but that are distinct from the microorganisms from non-sponge sources. In addition, the cluster must be supported by three independent phylogenetic tree building approaches (neighbor-joining, maximum parsimony and maximum likelihood). A recent comprehensive study including more than 7500 publicly available sponge-associated bacterial 16S rRNA gene sequences confirmed that the concept of sponge-specific microbes is still valid (Simister *et al.* 2012a).

This striking specificity between bacteria and host is probably due to the way symbiont microbial communities are established and maintained in sponges. The general agreement is that a combination of vertical transmission (from parents to progeny) and horizontal acquisition (from surrounding seawater) may interplay in the transmission of sponge-derived symbionts (Taylor *et al.* 2007; Hentschel *et al.* 2012). Vertical transmission was documented by microscopic and molecular studies that confirmed the presence of bacteria in larvae and juveniles of sponges (Ereskovsky & Tokina 2004; Schmitt *et al.* 2007; Lee *et al.* 2009a). However, some "sponge-specific" bacteria have been found in seawater, though at low abundances (Webster *et al.* 2010; Taylor *et al.* 2013), which suggest sponges could potentially uptake at least some of their symbionts from the surrounding seawater; yet the exact mechanism is still unknown.

Sponge hosts have been divided into two groups, high microbial abundance (HMA) and low microbial abundance (LMA) sponges, defined according to different abundances of microorganisms in the mesohyl of each type of sponges (Vacelet & Donadey 1977). However, this division corresponds also to differences in symbiotic structure: microbial communities in HMA sponges are sponge-specific whereas microbial communities in LMA sponges are similar to those in the seawater (Bjork *et al.* 2013; Moitinho-Silva *et al.* 2013). Moreover, the abundance of microorganisms in the mesohyl correlates with different pumping and metabolic activities of each sponge type, suggesting that the presence of the symbiotic community affects the different evolutionary strategies the sponge types have followed (Weisz *et al.* 2007).

An oustanding challenge in sponge microbiology consists of investigating what is not a one host-one symbiont system but a "community affair" (Hentschel *et al.* 2012; Bjork *et al.* 2013), with phylotypes that are diverse yet specific to each host. To date, our knowledge concerning sponge microbiota is still based on a few sponge species collected at a single point in time and space, whereas the potentially dynamism of this interaction is even more understudied. In particular, little is known about the processes shaping the sponge-derived communities at spatiotemporal scales and how do they respond to perturbations in the surrounding environment.

Putative Factors Driving Microbial Symbiont Structure

Microbial biogeography evaluates the patterns in microbial community structure over space and time. Biogeographic patterns emerge primarily from two processes: dispersal limitation and environmental selection (Martiny *et al.* 2006; Fierer 2008; Hanson *et al.* 2012). Dispersal limitation prevents connectivity among distant locations or populations, while environment selection affects microbial community structure as local conditions "pick up" the best-adapted microbes (**Box 2**). In the ocean, microbial dispersal would be passive and restricted by currents and hydrogeographic features (Schauer *et al.* 2000; Galand *et al.* 2009), while local conditions (e.g. salinity, temperature or nutrient levels) would shape marine microbial communities at a given point in time and space (Schauer *et al.* 2003; Flo *et al.* 2011).

Box 2 | **Everything is everywhere *but* the environment selects?**
The principle "everything is everywhere BUT the environment selects" (Baas Becking, 1934) defends that microbes are distributed worldwide but in given environmental conditions only some of them are abundant and active, whereas the others are present only latently and usually in low abundances. Although the assumption "everything is everywhere" is impossible to demonstrate, the development of massive sequencing techniques (e.g., pyrosequencing) has shown that in every environment there is a big pool of "rare" microbes (Pedrós-Alió 2012). Those microorganisms could potentially proliferate and become dominant under particular environmental conditions. Under this scenario, dispersal would seem unlimited in a microbial world. However, isolation-by-distance also appears to affect bacterial communities and be involved in bacterial speciation (reviewed in Hanson *et al.* 2012).

Sponge-associated microbial communities are far from randomly structured; on the contrary, community-level analyses of bacterial symbionts have revealed a remarkable host species-specificity (Taylor *et al.* 2007; Erwin *et al.* 2012a). However,

little is known about the dynamics of these species-specific microbial communities over spatiotemporal scales, with most studies suggesting that these communities are temporally stable and spatially persistent across wide geographic distances (Taylor *et al.* 2003; Webster *et al.* 2004; White *et al.* 2012). A few other studies have detected some degree of differentiation depending on location or seasonality in seawater conditions (Wichels *et al.* 2006; Lee *et al.* 2009b; Anderson *et al.* 2010). The low number of studies and apparently conflicting results make it difficult to draw any conclusions about the biogeography of sponge-derived symbiotic communities. Besides, the stability of sponge-associated microbial communities may vary regarding the species considered, the environmental parameter or the geographic or temporal scale used in the study. In addition, the sampling strategy and comparisons of distantly related host species may also distort the patterns observed and confound the processes involved. Thus, it remains necessary to understand how environmental, geographic and host-related factors interact in shaping sponge-derived communities. This knowledge will also provide a baseline for predicting the persistence and resilience of sponge-symbiont relationship to future perturbations.

Here, we hypothesized that the processes affecting free-living microbial communities (discussed above) are likely to apply to symbiotic communities (**Table 1**). Local conditions may generate variability within the same host species at different locations or homogenize the communities of sympatric sponge species. Host-specific factors, such as internal conditions in the sponge mesohyl, the physiological status of the host or its habitat preference, can also influence the composition and patterns of symbiotic microbial communities over time and space (Thacker 2005; López-Legentil *et al.* 2010).

Table 1. Putative factors affection sponge-derived microbial communities depending on their source (seawater *vs* host) and the nature of the process (selection *vs* dispersal limitation).

	SELECTION	**DISPERSAL LIMITATION**
Seawater	Temperature, light, nutrients, pollutants	Currents, eddies
Host	Sponge mesohyl, physiological status, habitat preference	Host population connectivity

Resilience of Sponge-Microbial Symbiosis

Marine organisms are and will be directly affected by temperature increases, changes in ocean circulation, acidification, and acuteness of seasonal conditions (Harvell *et al.* 2002; Calvo *et al.* 2011; Crisci *et al.* 2011). In addition, seawater warming has been linked to sponge disease outbreaks, a phenomenon affecting sponge populations worldwide (Webster 2007; Coma *et al.* 2009). In the face of increasing human populations and unprecedented environmental extremes resulting from a changing climate, understanding the vulnerability of sponge-microbial symbiosis is critical to preserve the biodiversity and ensure the ecosystem services they provide.

To date, few studies have assessed how sponge-derived communities respond to stressful environmental conditions. In coral-bacteria associations, it has been proposed that symbiotic microbial communities could dynamically respond to changing environmental conditions so that the whole holobiont would adapt rapidly (i.e., days to weeks) to new conditions ("The coral probiotic hypothesis", Reshef *et al.* 2006). In contrast, manipulated experiments mimicking stress (i.e. elevated temperature, presence of pollutants, elevated nutrient levels) suggested that shifts in sponge-derived communities were concomitant with declines in host sponge health (Webster *et al.* 2001; López-Legentil *et al.* 2008; Simister *et al.* 2012c). In temperate regions, sponge-derived bacterial communities changed when exposed to elevated temperatures (Lemoine *et al.* 2007) but remained stable under starvation conditions (Friedrich *et al.* 2001). Further studies are clearly needed to investigate the effect of environmental conditions resulting from human impact on sponge-associated bacterial communities and in order to assess their resilience.

Our Model: Bacterial communities associated with *Ircinia* sponges

The genus *Ircinia* (Dyctioceratida: Irciniidae) exhibits high species richness and occurs widely in tropical and temperate environments. *Ircinia* spp. can reach high densities in the rocky bottoms they inhabit (Parra-Velandia & Zea 2003; Turon *et al.* 2013). In addition, the species of this genus produces a broad spectrum of bioactive compounds that have exhibited antiinflamatory and antifouling activity (Duque *et al.* 2001; Hammami *et al.* 2010) and at least some of them are synthesized by their symbionts (Esteves *et al.* 2013). This PhD thesis is focused on 5 *Ircinia* spp.: *I. felix* and *I. strobilina* from the Bahamas and *I. fasciculata, I. variabilis* and *I. oros* from the Mediterranean Sea (**Fig. 3**).

The *Ircinia* spp. we targeted are HMA sponges that harbor complex and diverse symbiotic communities. Previous studies showed that their bacterial communities are composed by *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Nitrospira*, but also *Chloroflexi, Firmicutes Poribacteria*, *Actinobacteria* and phyla of uncertain affiliation (Schmitt *et al.* 2007; Mohamed *et al.* 2008c; Yang *et al.* 2011; Erwin *et al.* 2012a). Also, the sponges *I. fasciculata*, *I. variabilis* and *I. felix* harbor dense populations of photosymbionts (*Cyanobacteria*) that are absent in *I. oros* and *I. strobilina* sponges. In addition, Schmitt *et al.* (2007) evidenced the vertical transmission (i.e., from adults to larva) of *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes* and *Alpha-, Gamma- and Deltaproteobacteria* in *I. felix*.

Within each region (Bahamas and Mediterranean Sea), these *Ircinia* spp. can often be found living in sympatry. By targeting these sympatric congeneric species, we should be able to discriminate the relative role of host-related *vs* environmental- related factors in shaping the symbiotic communities inhabiting these sponges. Using this approach, Erwin *et al.* (2012a) found that the symbiotic communities in the three Mediterranean *Ircinia* spp. (i.e., *I. fasciculata*, *I. variabilis* and *I. oros*) are comprised primarily of bacteria previously described for coral or other sponge species. However, at the community level, each *Ircinia* species harbored a very specific mix of these bacteria. This pattern of individual ubiquous symbionts and host species-specific structure of entire communities has been defined as a “specific mix of generalists” and suggests that each sponge species contributes to shaping the distinct symbiont mix (Erwin *et al.* 2012a). A recent study showed that *I. strobilina* harbors distinct bacterial communities compared to distantly-related sponges that occurred at the same sampling location (Yang *et al.* 2011). Previous studies on *I. felix* and *I. strobilina* described their bacterial communities using different techniques (Schmitt *et al.* 2007; Mohamed *et al.* 2008c; Yang *et al.* 2011), making it difficult to determine the specificity-level of the symbiosis in *Ircinia* spp. from the Bahamas. Although the bacterial diversity within some of the *Ircinia* species targeted here is well-described, whether those symbiotic communities are maintained over space and time or under stressful conditions remains unknown.

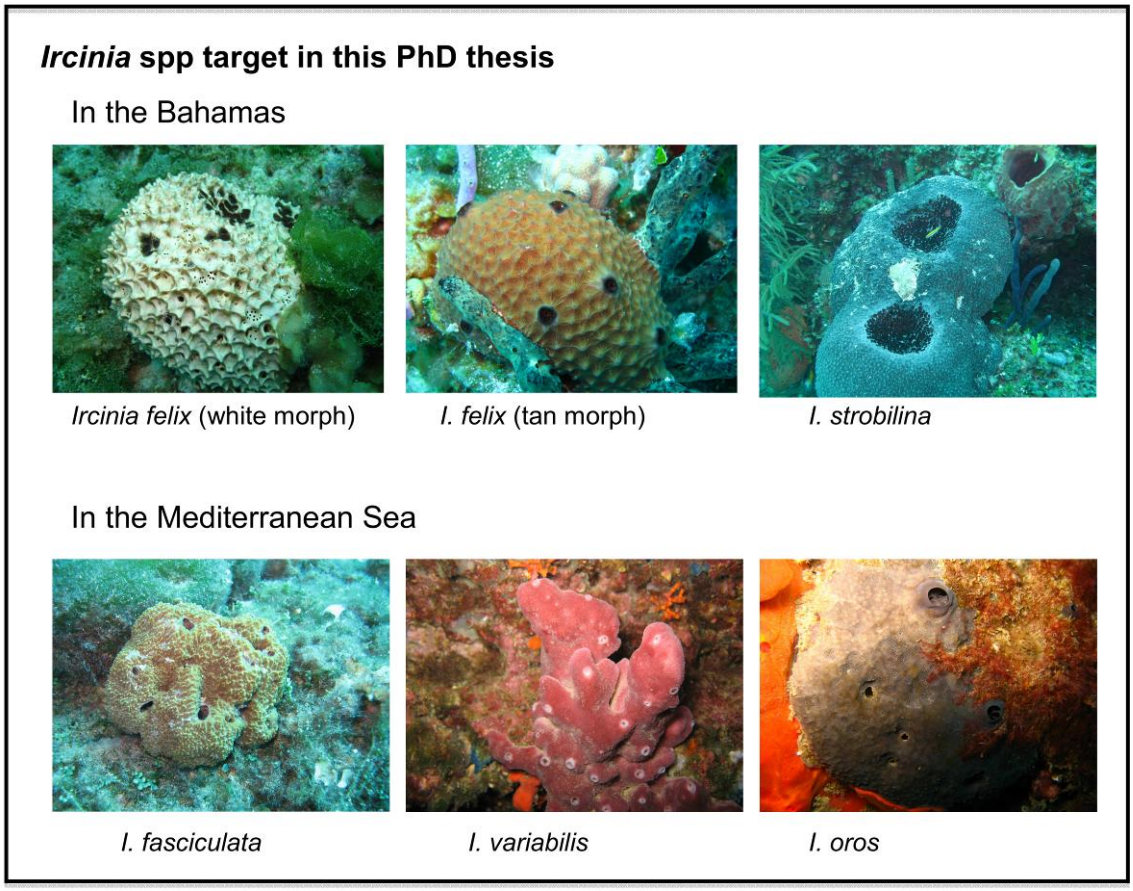


Figure 3. Underwater photographs of the *Ircinia* spp. studied in this PhD thesis. (Photos courtesy of Patrick M. Erwin).

Objectives

The main goal of this PhD thesis is to identify the specificity and persistence of the symbiotic communities associated with HMA sponges and their response to different environmental conditions. Congeneric sympatric sponges of the genera *Ircinia* were used as models to disentangle the relative role of host-specific and environmental factors in shaping sponge symbiotic communities and create a baseline to identify abnormal shifts in symbiotic communities or anticipate conditions where the symbiotic relationship could be compromised, particularly in a global climate change scenario.

In particular, we aimed to test the effects of currents and spatial heterogeneity of water conditions on bacterial symbiotic communities in the *Ircinia* spp. from Bahamas (*Ircinia felix* and *I. strobilina*), at a scale of hundreds of kilometers, and confirm if the same pattern is valid for Mediterranean species (*I. fasciculata*, *I. variabilis* and *I. oros*). As the Mediterranean Sea is characterized by a marked seasonality in seawater conditions (i.e., temperature, irradiance, nutrient levels), we also investigated the temporal dynamic of bacterial communities associated with these *Ircinia* species across seasons. Finally, considering that the recent, episodic mass mortalities of Mediterranean sponges may be related to thermal stresses, we also tested if abnormal environmental conditions may cause symbiont fluctuations and compromise the holobiont health. The characterization and monitoring of bacterial symbiont communities was assessed by electron microscopy and molecular analysis of 16S rRNA gene sequences (clone libraries and DNA fingerprinting). Terminal-restriction fragment length polymorphism (T-RFLP) analysis, a sensitive DNA fingerprinting technique (**annex 1**), allowed the standardized processing of replicates to approach our research questions.

To achieve this goal, the thesis was structured in 4 chapters, each addressing a specific objective. In order to facilitate independent reading, each chapter was written as a standalone unit to allow independent reading and included introduction, material and methods, results and discussion sections. However, all of them are interconnected and may contain cross-references to other chapters.

- **Chapter 1** aims to assess the host specificity and spatial variability (at a scale from 80 to 400s of kilometers) associated with the sympatric sponge species *I. felix* and *I. strobilina* from the Bahamas. For this, we characterized the bacterial assemblages in *I. strobilina* and two color morphs of *I. felix* (tan and white), and surrounding seawater from five different sites at the Bahamas islands, by using terminal-restriction fragment length polymorphism (T-RFLP) and clone library

analysis of bacterial 16S rRNA gene sequences and transmission electronic microscopy (TEM). We also sequenced a fragment of the mitochondrial gene cytochrome oxidase I (COI) to determine the genetic identity and phylogenetic relationships among sponge hosts.

- **Chapter 2** investigates if the spatial patterns observed for the symbiotic communities in the *Ircinia* spp. from the Bahamas are also encountered for the species-specific bacterial communities in Mediterranean *Ircinia* species (*I. fasciculata*, *I. variabilis* and *I. oros*). We used T-RFLP analysis of bacterial 16S rRNA gene sequences to describe the bacterial communities in *Ircinia* spp. from six locations of the shallow waters of coastal Western Mediterranean Sea, including locations affected by different currents and anthropogenic impacts.
- **Chapter 3** seeks to elucidate how sponge-derived bacterial communities respond to natural changes in surrounding seawater conditions such as seasonality of temperature and light irradiance and to identify permanent and transient symbiont taxa in associations with these sponge hosts. To achieve this goal, we monitored the bacterial communities in replicate individuals of each sympatric Mediterranean *Ircinia* host every 3 months for 1.5 years, by T-RFLP and clone libraries analysis of 16S rRNA gene sequences. In addition, we monitored photosynthetic pigments in the tissues of the cyanobacterium-rich sponges *I. fasciculata* and *I. variabilis*, using chlorophyll *a* (chl *a*) quantification.
- **Chapter 4** intends to detect abnormal shifts in the sponge-derived bacterial communities as a result of warmer-than-usual summer seawater conditions (i.e., elevated temperature and stratification of the seawater column). To mimic these conditions, we performed manipulated experiments in aquaria testing 4 different treatments: control, elevated temperature, food shortage and the combination of elevated temperature and food shortage on *I. fasciculata* and *I. oros* symbiotic communities. We studied changes in sponge bacterial communities by T-RFLP analysis of 16S rRNA gene sequences and TEM. Also, in *I. fasciculata*, we quantified chl *a*, as a proxy of photosymbiotic population activity.

Advisors' Report

Box 3 | **Publication status of the chapters**

CHAPTER 1
Biogeography and host-specificity of bacterial communities in *Ircinia* spp. from the Bahamas
Lucía Pita, Susanna López-Legentil, and Patrick M. Erwin
Microbial Ecology (2013), vol. 66: 437-447
Impact factor (2012): 3.277; Q1 Ecology

CHAPTER 2
Host rules: Spatial stability of bacterial communities associated with *Ircinia* spp. in the Western Mediterranean Sea
Lucía Pita, Xavier Turon, Susanna López-Legentil, and Patrick M. Erwin
FEMS Microbiology Ecology (2013), vol. 86: 268-276
Impact factor (2012): 3.563; Q2 Microbiology

CHAPTER 3
Stability of sponge-bacteria symbioses over large seasonal shifts in temperature and irradiance
Patrick M. Erwin, Lucía Pita, Susanna López-Legentil, and Xavier Turon
Applied and Environmental Microbiology (2012), vol. 78(20): 7358-7368
Impact factor (2012): 3.678; Q1 Biotechnology and Applied Microbiology

CHAPTER 4
Till death do us part: Stable sponge-bacteria associations under thermal and food shortage stresses
Lucía Pita, Patrick M. Erwin, Xavier Turon, and Susanna López-Legentil
PLoS ONE (2013), vol. 8(11): e80307
Impact factor (2012): 3.730; Q1 Multidisciplinary Sciences

Dr. **Susanna López-Legentil** and Dr. **Patrick M. Erwin**, co-advisers of the PhD thesis entitled "Biogeography of sponge-associated bacterial communities and resilience to anthropogenic perturbations" certify that the dissertation presented here has been carried out in its totality by **Lucia Pita Galán**, except for Chapter 3 that corresponds to a two-year study initiated by Dr. Patrick M. Erwin and that the PhD candidate completed, earning a second authorship position in the resulting publication. As advisers, we have participated in designing, guiding and correcting earlier drafts of the remaining 3 chapters and manuscripts. Besides the PhD candidate, all the other authors involved in this thesis already have a PhD degree and thus we guarantee that none of the information contained here will be used to elaborate another PhD thesis. Finally, the advisers would like to remark that Lucía was awarded a **3 year fellowship** by the Catalan Government, and that she has conducted the below described work in the allotted time:

Chapter 1: Pita L, López-Legentil S, Erwin PM (2013) Microbial Ecology 66: 437-447. Conceived and designed the study: LP, SL-L, PME. Performed lab analyses: LP. Analyzed the data: LP, SL-L, PME. Contributed reagents/materials/analysis tools: SL-L. Wrote the paper: LP. Revised the paper: SL-L, PME.

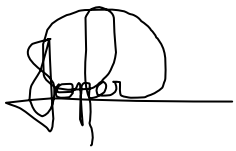
Chapter 2: Pita L, Turon X, López-Legentil S, Erwin PM (2013) FEMS Microbiology Ecology 86: 268-276. Conceived and designed the study: LP, XT, PME. Performed lab analyses: LP. Analyzed the data: LP, XT, SL-L, PME. Contributed reagents/materials/analysis tools: SL-L, XT. Wrote the paper: LP. Revised the paper: XT, SL-L, PME.

Chapter 3: Erwin PM, Pita L, López-Legentil S, Turon X (2012) Applied and Environmental Microbiology 78(20): 7358-7368. Conceived and designed the study: PME, XT. Performed lab analyses: PME, LP. Analyzed the data: PME, LP, XT, SL-L. Contributed reagents/materials/analysis tools: SL-L, XT. Wrote the paper: PME. Revised the paper: LP, SL-L, XT.

Chapter 4: Pita L, Erwin PM, Turon X, López-Legentil S (2013) PLoS ONE 8(11): e80307. Conceived and designed the experiments: LP, PME, XT, SL-L. Performed the experiments: LP, SL-L. Analyzed the data: LP, XT, SL-L, PME. Contributed reagents/materials/analysis tools: SL-L. Wrote the paper: LP. Revised the paper: PME, XT, SL-L.

For all of the above, we consider that the contribution of the PhD candidate grants her the right to defend her thesis in front of a scientific committee.

Barcelona, February 25, 2014



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Chapter 1

Ircinia felix (Bahamas)
Courtesy of P. M. Erwin

Chapter 1

Biogeography and Host Fidelity of Bacterial Communities in *Ircinia* spp. from the Bahamas

Lucía Pita, Susanna López-Legentil & Patrick M. Erwin

Published in: Microbial Ecology (2013) 66:437-447. *(2012-Impact factor: 3.277; Q1 Ecology).*

Abstract

Research on sponge microbial assemblages has revealed different trends in the geographic variability and specificity of bacterial symbionts. Here, we combined replicated terminal-restriction fragment length polymorphism (T-RFLP) and clone libraries analyses of 16S rRNA gene sequences to investigate the biogeographic and host-specific structure of bacterial communities in two congeneric and sympatric sponges: *Ircinia strobilina*, two color morphs of *I. felix* and ambient seawater. Samples were collected from five islands of the Bahamas separated by 80 to 400 km. T-RFLP profiles revealed significant differences in bacterial community structure among sponge hosts and ambient bacterioplankton. Pairwise statistical comparisons of clone libraries confirmed the specificity of the bacterial assemblages to each host species and differentiated symbiont communities between color morphs of *I. felix*. Overall, differences in bacterial communities within each host species and morph were unrelated to location. Our results show a high degree of symbiont fidelity to host sponge across a spatial scale of up to 400 km, suggesting that host-specific rather than biogeographic factors play a primary role in structuring and maintaining sponge-bacteria relationships in *Ircinia* species from the Bahamas.

Biogeografía y fidelidad de las comunidades bacterianas asociadas con esponjas *Ircinia* spp. de Las Bahamas

Resumen

Investigaciones recientes sobre las comunidades microbianas asociadas a esponjas han revelado differentes tendencias en cuanto a la variabilidad geográfica y la especificidad de los simbiontes bacterianos. Aquí hemos estudiado la especificidad y biogeografía de las comunidades bacterianas de dos esponjas congénericas y simpátricas (*Ircinia strobilina* y dos morfotipos de *I. felix*) y el agua circundante a través de análisis de secuencias del gen ARNr 16S bacteriano mediante las técnicas de polimorfismo en la longitud de los fragmentos de restricción terminales (T-RFLP) y librerías de clones. Las muestras de esponjas y agua de mar fueron recogidas en cinco islas de Las Bahamas, separadas un rango de 80-400 km. Los perfiles de T-RFLP revelaron diferencias significativas al comparar la estructura de las comunidades bacterianas de esponjas y el agua circundante. Comparaciones estadísticas de los datos derivados de las librerías de clones confirmaron la especificidad de las comunidades bacterianas a nivel de especie de esponja y cierta diferenciación entre morfotipos en *I. felix*. En general, las diferencias entre comunidades derivadas de individuos de la misma especie fueron independientes de la localidad de muestreo. Estos resultados muestran un alto grado de fidelidad del simbionte hacia su hospedador a lo largo de una escala espacial de hasta 400 km, lo que sugiere que son factores específicos del hospedador y no factores locales los que juegan un papel principal en estructurar y mantener las relaciones esponja-bacteria en especies de *Ircinia* de las Bahamas.

Introduction

Sponges are among the most significant groups in marine benthic communities due to their high abundance and diverse functional roles (Gili & Coma 1998; Diaz & Rutzler 2001; Van Soest *et al.* 2012). However, much of their contribution to benthic ecosystems derives from their association with an abundant and complex microbiota (Taylor *et al.* 2007; Webster & Taylor 2012). The metabolic activity of microbial symbionts within sponges significantly contributes to nutrient fluxes between benthic and pelagic systems and renders sponges critical to healthy ecosystem functioning (Ribes *et al.* 2012). Sponge-microbial relationships have often been considered mutualistic. Sponges may offer a range of nutrient-rich microhabitats and shelter from predators to their microbial symbionts (Sarà 1971; Taylor *et al.* 2007). In exchange, the microbial community can supplement the nutrition of their host via processes like photosynthesis (Erwin & Thacker 2008), nitrogen fixation (Mohamed *et al.* 2008a), or ammonia oxidation (López-Legentil *et al.* 2010). In addition, microbial symbionts can actively participate in the chemical defense of the holobiont by producing secondary metabolites, some of which have interesting biomedical and industrial applications (Newman & Hill 2006; Paul & Ritson-Williams 2008; Erwin *et al.* 2010).

As a result of the biological, ecological and biotechnological importance of the sponge holobiont, studies have begun to focus on understanding the diversity and structuring factors of sponge-associated microbial communities. Similar to free-living microorganisms (Hanson *et al.* 2012), environmental conditions (e.g., distinct bioclimatic zones [Taylor *et al.* 2005] or reefs [Lee *et al.* 2009b; Morrow *et al.* 2012]) and dispersal limitation (i.e., isolation-by-distance) may influence the composition and structure of symbiotic bacterial communities. The relative effect of each process varies depending on the scale of sampling: large scale patterns (tens of thousands of km) appear to be more affected by dispersion limitations and small scale patterns (few km) by environmental conditions, whereas intermediate scale patterns (10-3000 km) are influenced by both processes (Martiny *et al.* 2006). Particular to host-associated microbes, the mode of symbiont transmission may also dictate the specificity and spatial structure of the sponge microbiota.

A recent and comprehensive study (Schmitt *et al.* 2012) reported that the majority of sponge-associated bacteria (55-70%) are present in single host species but form phylogenetic lineages that are shared by numerous sponge hosts, yet absent or rare in the biosphere of bacterioplankton communities. This pattern is explained by a combination of vertical transmission (Usher *et al.* 2001; Ereskovsky *et al.* 2004; Schmitt *et al.* 2007, 2008; Lee *et al.* 2009a) and horizontal acquisition of symbionts (Taylor *et al.*

2007; Schmitt *et al.* 2008; Webster & Taylor 2012). The predominance of vertical transmission would create stable bacterial communities linked to the dispersal and evolutionary trajectory of their host, whereas horizontal acquisition would generate biogeographic patterns related to specific environmental conditions.

Comparisons of the microbiome within the same sponge species across different locations have revealed high similarity of bacterial symbionts in natural host populations within the same latitude (Taylor *et al.* 2003; Webster *et al.* 2004; Taylor *et al.* 2005; Lee *et al.* 2009b), suggesting no biogeographic patterns at intermediate spatial scales. However, Taylor *et al.* (2005) found that the microbiota of *Cymbastela concentrica* hosts inhabiting tropical waters was clearly distinct from those from temperate regions (separated by > 1500 km) and Anderson *et al.* (2010) reported location-specific bacterial communities in *Mycale hentscheli* across a 50 to 1000 km range in New Zealand. The low number of studies and apparently conflicting results highlight the need for additional studies to further pinpoint the factors shaping the structure of sponge-associated bacterial communities over intermediate biogeographic scales.

In this study, we examined bacterial communities in the model sponge species *Ircinia felix* and *I. strobilina*. The genus *Ircinia* (Dictyoceratida: Irciniidae) occurs widely in tropical and temperate environments and produces a broad spectrum of bioactive compounds involved in chemical defense against fouling, infection and competition (Duque *et al.* 2001; Pawlik *et al.* 2002). *I. felix* and *I. strobilina* are high-microbial-abundance (HMA) sponge species commonly found in coral reefs, grass beds, and mangroves throughout the Caribbean Sea (Schmahl 1990; Parra-Velandia & Zea 2003). The ectosome of *I. felix* is rich in *Cyanobacteria* (Maldonado & Young 1998), contrary to *I. strobilina* (Yang *et al.* 2011); and Schmitt *et al.* (2007) demonstrated that diverse bacterial symbionts in *I. felix* were present in adult, larval and juvenile life stages of the host, indicating vertical transmission of at least some of their bacterial symbionts.

The goal of this study was to assess the spatial variability (at a scale from 10s to 100s of km) and host-specificity of the bacteria associated with the sympatric sponge species *Ircinia felix* and *I. strobilina* from the Bahamas. We characterized the bacterial assemblages in *I. strobilina*, two color morphs of *I. felix* (white and tan) and ambient seawater from five islands of the Bahamas, using terminal-restriction fragment length polymorphism (T-RFLP) analysis. We also constructed 16S rRNA gene libraries to assess the composition of sponge-associated bacterial communities and sequenced a fragment of the mitochondrial gene cytochrome oxidase I (COI) to determine the genetic identity and phylogenetic relationships among sponge hosts. We addressed the following hypotheses: (i) bacterial communities will differ significantly among sources (i.e., sponge species and seawater); (ii) bacterial communities will exhibit greater similarity in more

closely related sponge hosts (i.e., greater between *I. felix* color morphs than among *I. felix* morphs and *I. strobilina*); (iii) changes in the bacterial communities within each sponge species will correlate with geographic distances among host populations.

Materials & Methods

Sample collection

The marine sponges *Ircinia strobilina* (Lamarck 1816) and *Ircinia felix* (Duchassaing & Michelotti 1864) and ambient seawater samples were collected from shallow littoral zones (< 20 m depth) of the Bahamas in July 2010 by SCUBA diving (Supplemental Information, **Table S1**). The five sampled populations were separated by 80 to 400 km and were located around islands of different human population densities (http://statistics.bahamas.gov.bs/): San Salvador (24° 03.515N, 074° 32.474 W; < 1,000 inhabitants), Little San Salvador (24° 34.727 N, 075° 57.628 W; < 2,000 inhabitants), Exumas (24° 52.871 N, 076° 47.502 W; < 7,500 inhabitants), Sweeting's Cay, Grand Bahama (26° 33.578 N, 077° 53.036 W; > 45,000 inhabitants) and New Providence (25° 00.771 N, 077° 33.794 W; > 250,000 inhabitants). At each site, ambient seawater (500 mL) was sampled simultaneously and in close proximity (< 1 m) to the sponges. Once on board of the research vessel, sponge samples were immediately preserved in RNAlater (Ambion) and seawater samples were concentrated on 0.2-µm filters prior to preservation. All samples were stored at -20°C.

Transmission electronic microscopy (TEM)

For each sponge species and color morph, a piece of the ectosome was dissected with a sterile scalpel and fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde buffered with filtered seawater and incubated overnight at 4°C. Following incubation, each piece was rinsed at least three times with filtered seawater and stored at 4ºC until processed as described previously (López-Legentil *et al.* 2011). TEM observations were made at the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona on a JEOL JEM-1010 (Tokyo, Japan) coupled with a Bioscan 972 camera (Gatan, Germany). Micrographs were visualized in ImageJ (Abràmoff *et al.* 2004) for bacterial cell counts. The relative abundances of bacteria (bacterial cells/mm^2) were determined as the average (± standard deviation) over 5 TEM micrographs per sample.

DNA extractions

Genomic DNA was extracted from sponge and seawater samples using the DNeasy® Blood & Tissue kit (Qiagen®) according to the manufacturer's instruction. Full-strength and 1:10 diluted DNA extracts were used as templates in PCR amplifications.

Molecular identification of host sponges

A fragment of ca. 1000 bp of the mitochondrial gene cytochrome oxidase I (COI), corresponding to the standard barcoding partition (Folmer *et al.* 1994; Herbert *et al.* 2003) and the I3-M11 partition (Erpenbeck *et al.* 2006) was PCR-amplified using a degenerated version of the universal barcoding forward primer dgLCO1490 (Meyer & Kuever 2008) (5'-GGT CAA CAA ATC ATA AAG AYA TYG G-3') and the reverse primer COX1-R1 (Rot *et al.* 2006) (5'-TGT TGR GGG AAA AAR GTT AAA TT-3'). Amplification was performed in a GeneAmp® PCR machine (Applied Biosystems) as follows: one initial denaturation step for 5 min at 94°C; followed by 30 amplification cycles of 0.5 min at 94°C, 0.5 min of annealing at 42°C, and 1.5 min at 72 °C; and a final elongation step for 7 min at 72°C. Total PCR volume (50 µL) included 10 µM of each primer, 10 nM of each dNTP, 1x Reaction Buffer (Ecogen), 2.5 mM $MgCl_2$, five units of BioTaqTM DNA polymerase (Ecogen) and 5 µL of DNA template. PCR products were cleaned and bi-directionally sequenced at Macrogen, Inc (Seoul, Korea). The consensus sequences obtained in this study for each sponge host and representative sequences from other *Ircinia* species available in GenBank were aligned in Geneious Pro 5.1.6 (Drummond *et al.* 2011). Specifically, the alignment included representative sequences of congeneric species from the Mediterranean Sea (Erwin *et al.* 2012a), the Indo-Pacific (Pöppe *et al.* 2010), and one *I. strobilina* sequence from the Caribbean (Erpenbeck *et al.* 2009). Maximum likelihood (ML) and neighbor joining (NJ) phylogenies were constructed in MEGA v5 (Tamura *et al.* 2011). For ML analyses, we used the GTR+G+I (Tavaré 1986) model and 100 bootstrap replicates (Felsenstein 1985). The NJ tree was built based on the Tamura-Nei model of nucleotide substitution and 1,000 bootstrap replicates. All sequences have been deposited in GenBank (Acc. Nos. JX306085 to JX306089).

T-RFLP analysis

The universal bacterial forward primer Eco8F (Turner *et al.* 1999) (5'-AGA GTT TGA TCC TGG CTC AG-3'), tagged with 6-FAM, and the reverse primer 1509R (Martínez-Murcia *et al.* 1995) (5'-GGT TAC CTT GTT ACG ACT T-3') were used for amplification of ca. 1500 bp fragments of the 16S rRNA gene from all sponge and seawater DNA extracts. PCR was performed in a GeneAmp® PCR machine (Applied Biosystems) as follows: an initial denaturation step for 5 min at 94°C; 35 cycles of 1 min at 94°C, 0.5 min at 50°C, 1.5 min

at 72°C; and a final elongation step for 5 min at 72°C. Total PCR volume (50 µL) included 10 µM of each primer, 10 nM of each dNTP, 1x Reaction Buffer (Ecogen), 2.5 mM $MgCl_2$, 5 units of BioTaqTM DNA polymerase (Ecogen) and 5 µL of DNA template. Products from triplicate PCR reactions were gel-purified and cleaned using the Qiaquick Gel Extraction kit (Qiagen®) and pooled before quantification using the QubitTM fluorometer and Quant-iTTM dsDNA Assay kit (InvitrogenTM). For each sample, 100 ng of purified PCR product were digested with the restriction endonuclease HaeIII and 100 ng with MspI in a total volume of 20 µL, following the manufacturer's protocol (Promega). Restriction reactions were incubated for 4 h at 37°C, followed by ethanol precipitation to remove residual salts. Prior to capillary electrophoresis, samples were fully dried and then eluted in 11.5 µL formamide and 0.5 µL GeneScan 600-LIZ size standard (Applied Biosystems), heated at 94°C for 2 min in a dry bath, and immediately cooled on ice for 2 min. Samples were processed on an automated ABI 3730 Genetic Analyzer (Applied Biosystems) at the Genomics Unit of the Scientific and Technical Services of the University of Barcelona. The lengths of individual terminal-restriction fragments (T-RFs) were determined using the program PeakScanner (Applied Biosystems). T-RFs below 50 fluorescence units (background noise), smaller than 50 bp or larger than 600 bp (beyond the resolution of our internal standard) were excluded from the analysis. T-RFLP peak profiles were uploaded in T-REX (Culman *et al.* 2009) for further filtering, alignment and construction of relative abundance matrices. Data was de-noised applying a cut-off value of 2 standard deviations (Abdo *et al.* 2006) and T-RFs were aligned using a clustering threshold of 1 bp then standardized by relative peak areas.

Statistical analysis of T-RFLP

Bray-Curtis similarity matrices were calculated using square-root transformations of relative T-RF abundances. Non-metric multidimensional scaling (nMDS) plots were constructed for each restriction enzyme to visualize similarities among the bacterial communities recovered from each sample. Permutational multivariate analyses of variance (PERMANOVA) were used for pairwise comparisons of bacterial communities among sources (seawater, sponge species and the two color morphs of *I. felix*) and among locations within each source (nested analysis). PERMDISP was computed for comparing the multivariate dispersions among groups on the basis of Bray-Curtis distance. Calculations were performed in PRIMER v6 (Clarke 1993; Clarke & Gorley 2006) and PERMANOVA+ (Plymouth Marine Laboratory, UK). For all pairwise comparisons, the critical value for significance was corrected using the Benjamini-Yekutieli (B-Y) false discovery rate (Benjamini & Yekutieli 2001). To test for isolation-by-

distance, Mantel tests for each host and enzyme were calculated in R (R Core Team 2012) using the package ade4 (Dray & Dufour 2007).

16S rRNA gene clone library construction.

Clone libraries were constructed for two individuals of each sponge species and color morph collected in Sweeting's Cay and Exumas (ca. 300 km apart). PCR amplification was performed as described for T-RFLP analyses (above), except that no fluorescent tag was attached to the forward primer. PCR products were gel-purified and cleaned using the QIAquick Gel Extraction kit (Qiagen®) and quantified with a Qubit™ fluorometer and Quant-iTTM dsDNA Assay kit (Invitrogen™). Cleaned PCR products were ligated into plasmids using the pGEM®-T Vector System (Promega). In total, 234 positive clones were bi-directionally sequenced using the vector primers T7 and SP6 at Macrogen, Inc. (Seoul, Korea). Raw sequence reads were processed and aligned in Geneious Pro 5.1.6 (Drummond *et al.* 2011) to recover near full-length 16S rRNA gene sequences (range = 1042 to 1563 bp). Low quality sequence reads and sequences identified as chimeric (Schloss *et al.* 2009) were discarded. All sequences were deposited in GenBank (Acc. Nos. JX280152 to JX280385).

Diversity and structure of the bacterial clone libraries

Bacterial 16S rRNA gene sequences were ascribed to 99% operational taxonomic units (OTUs). A 99% sequence identity threshold was used to increase taxonomic resolution and assess fine-scale variability in bacterial communities among hosts. Richness (Observed OTUs, Chao1 estimator) and diversity metrics (Shannon index, Simpson's inverse index) were calculated by source (sponge species or color morph), plotted in rarefaction curves and used to compare the richness, diversity and evenness of recovered bacterial communities. Pairwise differences in bacterial clone libraries of each host species and color morph were determined by LIBSHUFF analyses based on 10,000 randomizations and adjusted using Bonferroni corrections (Sokal & Rohl 1995). All analyses were performed using the mothur software package (Schloss *et al.* 2009). To compare clone library sequences with T-RFs, *in silico* digestions of a representative ribotype of each 99% OTU were generated using the Restriction Analysis option in Geneious Pro 5.1.6 (Drummond *et al.* 2011). A reference database was created consisting of 5'-terminal fragment lengths for each OTU and restriction endonuclease (HaeIII and MspI) and T-RF drift was predicted and corrected as described in Erwin *et al.* (Erwin *et al.* 2012c). This database was then used to match predicted T-RFs based on clone library sequences with empirical T-RFs obtained during T-RFLP analysis using the phylogenetic assignment tool PAT (Kent *et al.* 2003). Default bin sizes and an extra bin

for small T-RFs (2 bp tolerance applied to fragments of 50-100 bp) were applied to PAT analyses.

Phylogenetic analysis of the bacterial clone libraries

Phylogenetic analyses were performed to determine the affiliations between sequences retrieved in this study, top matching sequences from BLASTn searches (Altschul *et al.* 1990) and publicly available *Ircinia*-associated symbionts in the GenBank database (January 2012), including sequences from *I. felix* (Schmitt *et al.* 2007, 2008), *I. strobilina* (Mohamed *et al.* 2008c; Yang *et al.* 2011), Mediterranean *Ircinia* spp. (Muscholl-Silberhorn *et al.* 2008; Erwin *et al.* 2012c) and an Indo-Pacific *Ircinia* sp. (GenBank Acc. No. GQ487629). All sequences were grouped into 99% OTUs and classified using the Ribosomal Database Project II sequence classifier (Cole *et al.* 2003). When bacterial sequences from publicly available database derived from the same sponge species and grouped in the same 99% OTU, only a representative sequence was used for the following analyses to facilitate tree visualization. Finally, sequences were aligned with ClustalX 2.1 (Thompson *et al.* 1997) and a maximum-likelihood (ML) phylogenetic tree was constructed in RAxML (Stamatakis 2006) using the General Time Reversible model with a gamma distribution of variable substitution rates among sites (GTR+G) (Tavaré 1986) and 100 bootstrap replicates (Felsenstein 1985). A binary backbone constraint tree was constructed from long (> 1000 bp) sequences to allow precise placement of shorter sequences as described in Erwin *et al.* (2012a).

Results

Phylogenetic relationship between sponge hosts

Partial COI sequences obtained for each color morph of *I. felix* were more closely related to each other (0.4% divergence) than to *I. strobilina* (> 1% divergence). *I. strobilina* was more closely related to the Mediterranean species *I. fasciculata* and *I. variabilis* (0.5% divergence) than to the sympatric *I. felix*; whereas *I. felix* was more closely related to the Mediterranean species *I. oros* (**Fig. 1**). Caribbean and Mediterranean *Ircinia* species formed a well-supported clade and were a sister group to the Indo-Pacific sponges *I. ramodigitata* and *I. irregularis*.

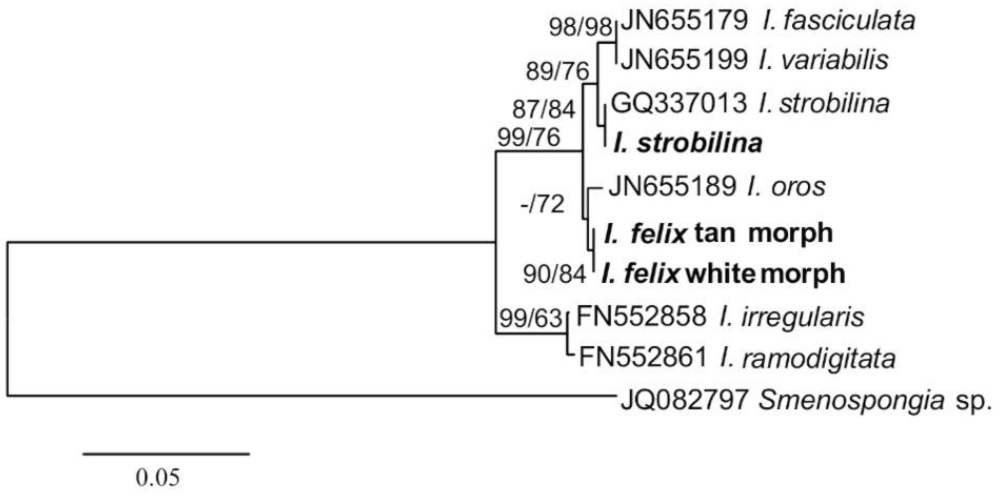

Figure 1. Phylogenetic analysis of host sponges based on a fragment of the mitochondrial gene cytochrome oxidase I. Tree topology was obtained by neighbor joining and numbers on nodes indicate bootstrap values (> 50%) for neighbor joining (left) and maximum likelihood (right) analysis. Terminal node labels show GenBank accession numbers and sponge species. Sequences obtained in this study are highlighted in bold.

Bacterial morphology and ultrastructure

Electron microscopy observations showed that *Ircinia* spp. from the Bahamas harbored diverse microbial communities (**Fig. 2**). Bacteria were mostly distributed extracellularly in the mesohyl of both sponge species (**Fig. 2a, b**) and occurred in high densities (1.197 x 106 ± 0.051 cells/mm^2 in *I. strobilina*, 0.816 x 106 ± 0.142 cells/mm^2 in *I. felix*). Different bacterial morphotypes were distinguishable, including prokaryotic cells with a nucleoid-like structure (**Fig 2a, b**). A cyanobacterium corresponding to the description of *Candidatus Synechococcus spongiarum* (Usher *et al.* 2004) was abundant in the ectosome of *I. felix* (**Fig. 2c**) and was characterized by spiral thylakoids located around the perimeter of the cell. These thylakoids appeared with electron-dense granules in between them. Several cyanobacterial cells were also observed dividing by pinching in the center (**Fig. 2c**). No cyanobacterial symbionts were observed in *I. strobilina*. Sponge cells (archaeocytes) were only observed occasionally in the mesohyl and often contained several phagosomes digesting bacteria (**Fig. 2d**).

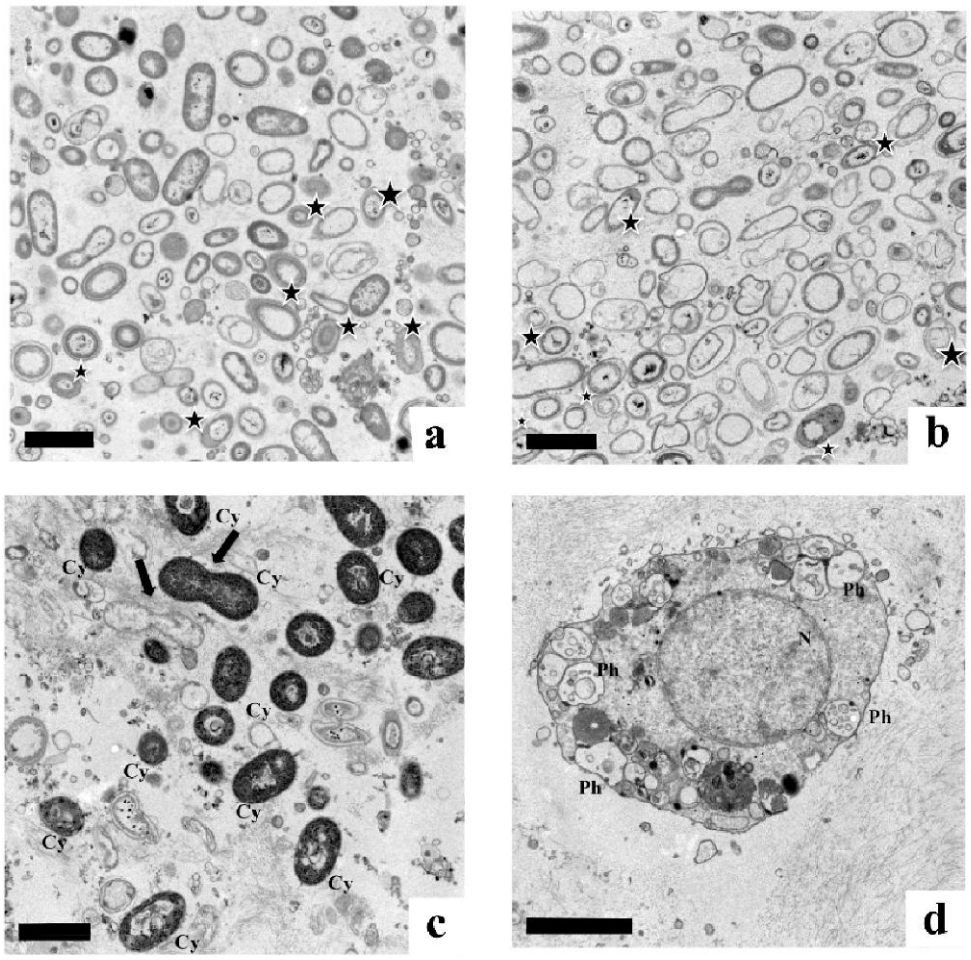

Figure 2. Representative electron micrographs of sponge holobionts. Bacterial diversity in the mesohyl of **(A)** *I. strobilina* and **(B)** *I. felix* tan morph, including morphotypes containing a nucleoid-like structure within the cell (black stars). **(C)** The cyanobacterium *Candidatus Synechococcus spongiarium* (Cy) and active bacterial cell division (black arrows) in the ectosome of the white morph of *I. felix*. **(D)** Sponge cell (archeocyte) in the tan morph of *I. felix* showing the cell nucleus (N), and numerous phagosomes (Ph). Scale bar represents 2 µm.

Host-specificity and biogeography of bacterial communities

A total of 181 unique T-RFs for the restriction enzyme HaeIII (141 in *I. strobilina*, 126 in the white morph of *I. felix*, 109 in the tan morph, and 123 in seawater), and 204 for MspI (158 in *I. strobilina*, 136 in the white morph of *I. felix*, 106 in the tan morph, and 135 in seawater) were recovered. nMDS plots constructed from T-RFLP profiles for both restriction enzymes showed clear differences between seawater and sponge-derived bacteria (**Fig. 3**). Differences were also observed between the bacterial communities of *I. strobilina* and *I. felix* but not between color morphs of *I. felix*. Accordingly, statistical analyses revealed significant differences (PERMANOVA, $P < 0.01$) among all pairwise comparisons of seawater bacteria and sponge-associated bacteria, between *I. strobilina*

and *I. felix*, but not between color morphs of *I. felix* ($P > 0.34$; **Table 1**). PERMDISP results reported significant differences in the homogeneity of dispersion between each sponge host and seawater, but not among sponge sources (**Table 1**). No differences in the bacterial composition of the sponge samples could be attributed solely to location ($P > 0.05$); however, a significant interaction between source and location occurred for the restriction enzyme MspI. Subsequent pairwise comparisons in a nested design and after Benjamini-Yekutieli correction only revealed significant differences between the bacterioplankton communities of Sweeting's Cay and San Salvador (Supplemental Information, **Table S2**). No significant correlations between bacterial community similarity and geographic distance were recovered for any sponge host (Mantel test, $P > 0.233$ for all comparisons).

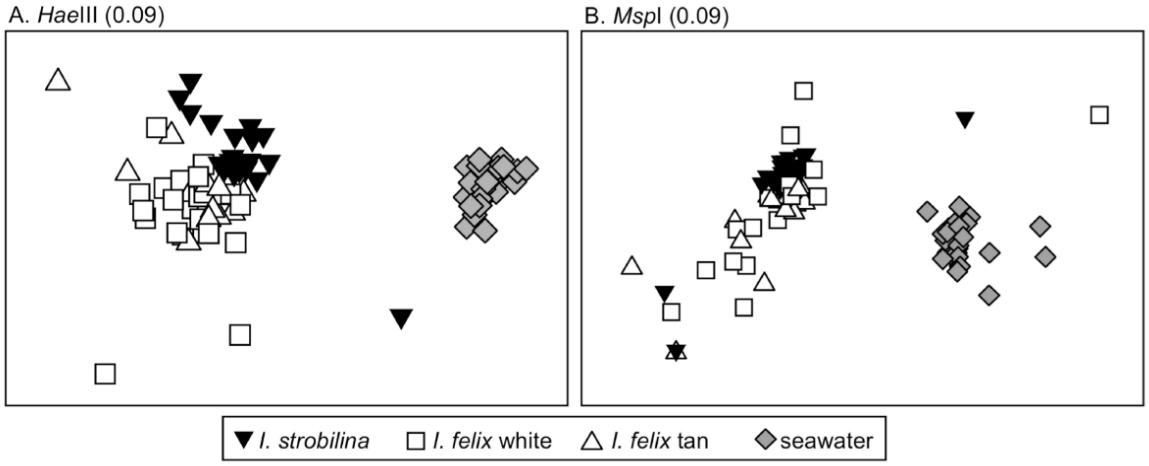

Figure 3. nMDS plots of bacterial community structure in sponge hosts (*I. strobilina* and two color morphs of *I. felix)* and surrounding seawater samples. nMDS ordination based on Bray-Curtis similarity of T-RFLP profiles using the restriction enzymes (A) HaeIII and (B) MspI. Stress values are shown in parenthesis, with values below 0.15 indicating a good representation of similarity matrix distances in the graphical ordination plot.

Diversity and structure of the sponge-associated bacterial communities

16S rRNA gene sequence libraries from *I. strobilina* ($n = 82$), the white morph of *I. felix* ($n = 68$) and the tan morph ($n = 84$) were ascribed to a total of 83 unique OTUs (99% sequence identity). Rarefaction analyses at a similarity level of 99% showed greater OTU saturation for the bacterial communities in both morphs of *I. felix* than for *I. strobilina* (Supplemental Information, **Fig. S1a**). Richness and diversity metrics revealed that *I. strobilina* hosted a more diverse and evenly distributed bacterial community than *I. felix* (**Table 2**). The color morphs of *I. felix* exhibited similar OTU richness values but diversity indices (Shannon and Simpson's inverse index) were much higher for the white morph than for the tan morph (**Table 2**). Rarefaction curves of all estimators (Chao 1, Shannon

and inverse of Simpson's index) approached asymptotes and revealed consistent differences among sponge hosts across sampling effort (Supplemental Information, **Fig. S1b-d**).

Table 1. Permutational statistical analysis of T-RFLP data (HaeIII and MspI enzymes) for bacterial community structure (PERMANOVA) and homogeneity of dispersion (PERMDISP) among sponge hosts and seawater. Main tests of source (sponges and seawater), location (collection site) and an interactive term are shown, along with pairwise comparisons among sources: tan and white morphs of *Ircinia felix* (tan and white *I. felix*, respectively), *I. strobilina* and seawater. Significant comparisons following B-Y correction are indicated with asterisks denoting significance level (*α= 0.05, ** α= 0.01, *** α = 0.005).

	HaeIII		**MspI**	
PERMANOVA Main Test	***F*-ratio**	***P*-value**	***F*-ratio**	***P*-value**
Source	18.167	**0.001*** **	10.779	**0.001*** **
Location	1.707	0.055	1.423	0.128
Source x Location	1.389	0.062	1.573	**0.013***
PERMANOVA Pairwise comparison	***t***	***P*-value**	***t***	***P*-value**
Tan *I. felix* - White *I. felix*	1.039	0.354	0.930	0.508
Tan *I. felix* - *I. strobilina*	2.404	**0.001*** **	1.790	**0.006***
White *I. felix* - *I. strobilina*	2.951	**0.001*** **	1.913	**0.003** **
Tan *I. felix* - Seawater	7.114	**0.001*** **	5.741	**0.001*** **
White *I. felix* - Seawater	7.962	**0.001*** **	5.879	**0.001*** **
I. strobilina - Seawater	7.016	**0.001*** **	6.048	**0.002** **
PERMDISP Pairwise comparison	***t***	***P*-value**	***t***	***P*-value**
Tan *I. felix* - White *I. felix*	0.517	0.648	0.866	0.465
Tan *I. felix* - *I. strobilina*	0.087	0.946	0.590	0.636
White *I. felix* - *I. strobilina*	0.613	0.573	1.435	0.239
Tan *I. felix* - Seawater	3.677	**0.002** **	2.721	0.023
White *I. felix* - Seawater	4.933	**0.001*** **	3.846	**0.001*** **
I. strobilina - Seawater	3.471	**0.001*** **	1.693	0.156

Table 2. Richness (observed OTUs, Chao1) and diversity metrics (Shannon and Simpson's inverse indexes) for the bacterial communities recovered from each sponge host. Confidence intervals at 95% are shown in parentheses.

	I. strobilina	**White *I. felix***	**Tan *I. felix***
Observed OTUs	45	26	30
Expected OTUs (S_{Chao1})	103 (68-194)	39 (30-74)	54 (38-105)
Shannon Index	3.5 (3.2-3.7)	2.9 (2.7-3.1)	2.7 (2.3-2.9)
Simpson's inverse index	28.6 (18.7-60.6)	15.7 (11.2-26.4)	7.1 (4.9-13.3)

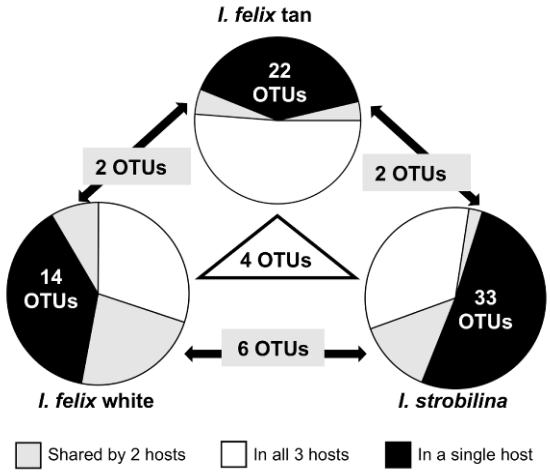

Figure 4. Host specificity of the bacterial communities in *I. strobilina* and two color morphs of *I. felix* based on 16S rRNA gene sequences obtained after clone library construction. Pie charts show the percentage of clones for each symbiont category. Numbers denote the total OTUs (99% sequence identity) in each category

Most bacterial OTUs were unique to one host, with little overlap among the three sponge-associated communities (**Fig. 4**). Only four OTUs (IRCBA01, IRCBA13, IRCBA20 and IRCBA44) were shared among *I. strobilina* and the two color morphs of *I. felix* (hereafter called generalist OTUs). These generalist OTUs were dominant within each bacterial community, in terms of number of sequences retrieved, accounting for 6.0 to 34.5% of all bacterial sequences per host species and morph, except for IRCBA20 (< 2.5% of sequences for all hosts) and IRCBA13 for the white morph of *I. felix* (2.9%). The OTU IRCBA01 represented 8.5% of all the sequences recovered for *I. strobilina*, and 34.5% and 17.5% of the sequences from the tan and white morphs of *I. felix*, respectively. The OTU IRCBA44 accounted for 13.4% of *I. strobilina*-derived sequences and 6.0% of tan *I. felix* and 8.8% of the white *I. felix*-derived sequences. Two additional OTUs were shared between the two color morphs of *I. felix* (IRCBA33 and IRCBA60); these OTUs represented 4.8% and 8.8% of all the sequences retrieved for the tan and white morphs, respectively. Consistent with the little OTU-overlap among host sponges, the symbiotic community associated with each host sponge was significantly different, even among color morphs (LIBSHUFF analysis, **Table 3**). There were no significant differences between the 16S rRNA gene sequences from Sweeting's Cay and Exumas obtained for both color morphs of *I. felix*, while significant differences were detected between populations of *I. strobilina* (**Table 3**).

PAT analysis showed high congruence between bacterial clone libraries and T-RFLP analyses for both restriction enzymes. In fact, 88% of the OTUs obtained with clone libraries were also observed with T-RFLP analysis. Empirical T-RFs obtained with the enzyme HaeIII matched 50.6% of the peaks predicted by in silico digestion, while for MspI, empirical T-RFs matched 55.6% of the predicted peaks.

Table 3. Pairwise statistical comparisons of bacterial community structure (LIBSHUFF analyses) based on 16S rRNA gene sequences obtained from clone libraries of *I. strobilina* and the two color morphs of *I. felix* (tan and white). Comparisons among hosts and between sampling sites (Sweeting's Cay and Exumas) within hosts are shown. Two tests per pairwise comparison (dC_{XY} and dC_{YX}) and corresponding *P*-values (*P*-$value_{XY}$, *P*-$value_{YX}$) were conducted, with significance in either comparison indicating differences in bacterial community structure. Significant comparisons following Bonferroni correction are indicated with asterisks (*α= 0.05, ** α= 0.01, *** α = 0.005).

LIBSHUFF comparisons		dC_{XY} dC_{YX}	*P*-value $_{XY}$ *P*-value $_{YX}$
I. strobilina - Tan *I. felix*		0.0054 0.0038	**0.0016**** **0.0045***
I. strobilina - White *I. felix*		0.0034 0.0089	0.019 **0.001****
Tan *I. felix* - White *I. felix*		0.0048 0.0078	**0.002*** **0.0001*****
Sweeting's – Exumas within	*I. strobilina*	0.0083 0.0026	**0.0161*** 0.2001
	White *I. felix*	0.0027 0.0014	0.2125 0.353
	Tan *I. felix*	0.0040 0.0014	0.0624 0.3014

Phylogenetic analysis of 16S rRNA bacterial sequences

The vast majority of the sequences recovered from each sponge host were closely related with other sponge-associated (73.2% in *I. strobilina*, 94.1% in the white morph of *I. felix*, and 77.4% in the tan morph) and coral-associated bacterial sequences (20.7% in *I. strobilina*, 4.4% in the white morph of *I. felix*, and 20.2% in the tan morph). Some ribotypes matched with seawater-derived sequences (6.1% in *I. strobilina*, 2.4% in the tan morph of *I. felix*, and 0% in the white morph), but mostly at low identity matches (< 97% sequence identity). As in other HMA sponges, the bacterial OTUs recovered herein were distributed into eight known phyla and one unclassified group (Supplemental Information, **Fig. S2-S7**). All three sponge taxa hosted representatives from two classes of *Proteobacteria* (*Delta* and *Gamma*), as well as *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Nitrospira* and *Firmicutes* (**Fig. 5**). *Proteobacteria*, specifically the class *Delta-Proteobacteria* (> 15% total clones in all hosts), was the best-represented phylum in all clone libraries. Sequences related to *Spirochaetes* and *Alpha-Proteobacteria* were only present in *I. strobilina* and the tan morph of *I. felix*. Sequences affiliated to *Cyanobacteria* (*Synechococcus*) were only found in *I. felix* and were more abundant in the white morph than in the tan morph (> 15% and > 2% of total clones, respectively). The generalist OTUs shared by the three sponge hosts corresponded to the class *Delta-Proteobacteria* (IRCBA01), and the phyla *Acidobacteria* (IRCBA13 and IRCBA20) and *Nitrospira* (IRCBA44). These

symbionts formed sponge-specific (IRCBA20 and IRCBA44) and sponge-coral specific (IRCBA01, IRCBA13) clusters (Supplemental Information, **Fig. S2**). The *Delta-Proteobacteria*-affiliated OTU (IRCBA01) was particularly dominant in the bacterial clone libraries (8.5% of the sequences in *I. strobilina*; 17.5% in the white morph of *I. felix*, and 34.5% in the tan morph) and was also common in the Mediterranean species *I. fasciculata*, *I. variabilis* and *I. oros*, as well as in other unrelated sponge species and corals (Suppelemental Information, **Fig. S4**).

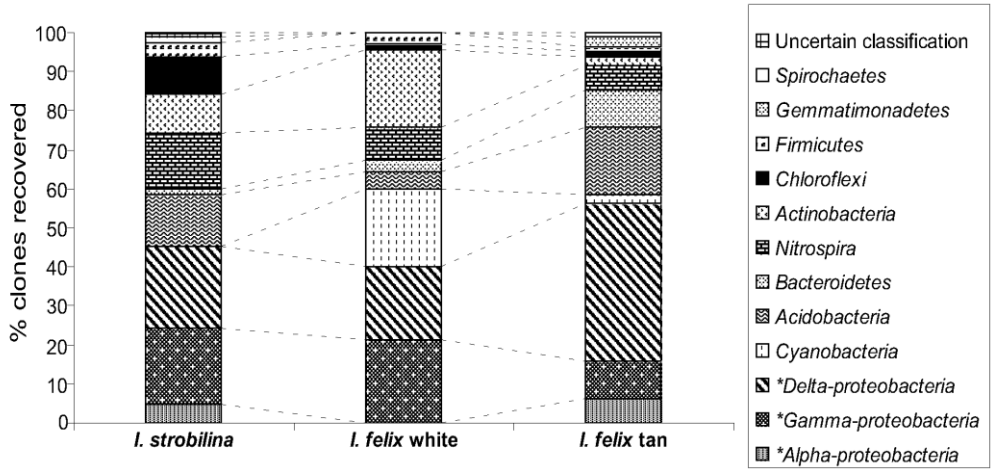

Figure 5. Phylogenetic affiliation of symbiont OTUs (99% sequence similarity) in *I. strobilina* and two color morphs of *I. felix* (tan and white). Bacteria are classified according to phylum or class (*).

Discussion

In this study, we determined whether the bacterial communities associated with the sympatric sponges *I. strobilina* and *I. felix* were stable across islands separated by tens to hundreds of km in the Bahamas. Sequencing of a fragment of the mitochondrial COI gene from host sponges confirmed the taxonomic identification and phylogenetic relationships of *I. strobilina* and two color morphs of *I. felix* (white and tan), allowing for the assessment of the bacterial communities specificity among congeneric and conspecific host individuals. Electron microscopy, T-RFLP analysis and 16S rRNA gene clone libraries confirmed that these sponge taxa harbor host-species specific bacterial communities that are clearly differentiated from the bacterioplankton in the surrounding seawater. T-RFLP profiles further revealed that the bacterial communities in two color morphs of *I. felix* were more similar to each other than to *I. strobilina*.

Within each sponge host, bacterial assemblages were remarkably stable over locations and maintained across host populations and islands.

Our results revealed a major influence of host-related factors in structuring sponge-associated bacterial assemblages. We sampled sponge populations in reefs up to 400 km apart located in islands with distinct human population densities and oceanographic currents (Colin 1995), yielding different environmental conditions, disturbance regimes and dispersal barriers. However, we found high spatial stability of sponge-bacteria symbioses and no isolation-by-distance effect, consistent with previous studies on sponge-derived bacterial communities at geographical scales ranging from tens (Webster *et al.* 2004; Lee *et al.* 2009b; Yang *et al.* 2011) to hundreds of km (Webster & Hill 2001; Taylor *et al.* 2005; Thiel *et al.* 2007a). Other studies suggested that environmental conditions could also influence the structure of symbiont communities (Taylor *et al.* 2005; Yang *et al.* 2011), although these studies involve broader geographic (i.e., inter-ocean) scales and/or genetically distant hosts, thus decoupling the effects of biogeography and host-specificity remained a major obstacle. In contrast, studies that minimize the phylogenetic distance among host species are better suited to distinguish location- and host-related patterns. For instance, Montalvo & Hill (2011) compared the bacteria associated with *Xestospongia muta* and *X. testudinaria* and found that these closely related hosts harbored strikingly similar bacterial communities, despite the fact that they inhabit different oceans (Atlantic and Pacific, respectively).

In addition to spatial stability, our study also assessed host specificity of bacterial communities among congeneric and conspecific sponges. The bacterial sequences derived from 16S rRNA clone libraries for each *Ircinia* host belonged to the same phyla described for other HMA sponges (Webster & Taylor 2012) and were largely consistent with previous studies of *I. strobilina* (Mohamed *et al.* 2008c; Yang *et al.* 2011) and *I. felix* (Schmitt *et al.* 2007, 2008). For example, a sponge-specific cluster of *Bacteroidetes* sequences that was previously detected only in the larvae of *I. felix* (Schmitt *et al.* 2007) was identified herein in both color morphs of adult *I. felix* hosts. TEM micrographs and clone libraries also revealed the absence of *Cyanobacteria* in the microbiota of *I. strobilina*, consistent with a recent molecular-based survey (Yang *et al.* 2011) and the low chlorophyll a content of this sponge host (Erwin & Thacker 2007; Southwell *et al.* 2008). While some *I. strobilina* hosts may harbor nitrogen-fixing cyanobacteria (Mohamed *et al.* 2008a; 2008c), these symbionts are clearly distinct from the dense populations of *Synechococcus spongiarum* consistently reported in *I. felix* (Schmitt *et al.* 2007; Southwell *et al.* 2008). The significance for host metabolism of these divergent bacterial assemblages is still uncertain and further investigation is

necessary to assess whether the net activity of different symbiont microbiota results in overall similar biochemical processes in the holobiont (e.g., in nitrogen flux, [Southwell *et al.* 2008]).

In a broader context, most of the sequences in the bacterial 16S rRNA clone libraries of *Ircinia* spp. from the Bahamas were closely-related to bacterial symbionts in taxonomically distant sponge hosts (e.g., different sponge orders) and from different geographic origins (e.g., Mediterranean and Pacific). Phylogenetic analyses of bacterial clone libraries did not reveal any *Ircinia*-specific or Caribbean *Ircinia*-specific symbiont clusters. The four bacterial OTUs shared by *I. strobilina* and both color morphs of *I. felix* were also described in other sponge (IRCBA20, IRCBA44) and coral (IRCBA01, IRCBA13) hosts from diverse ecosystems. However, at the community level, the bacterial composition in each *Ircinia* host analyzed herein was still host-specific. Similar observations of symbiont structure and specificity were recently described for Mediterranean *Ircinia* spp. and termed a "specific mix of generalists" (Erwin *et al.* 2012a). The outstanding questions are which factors result in the observed distribution of symbiont taxa among hosts and what are the ecological consequences for host-symbiont interactions.

Host-related factors influencing bacterial communities may include particular mesohyl conditions (e.g., different pH and oxygen levels) and the evolutionary history of each sponge species. Although closely related, *I. felix* and *I. strobilina* show striking differences in morphological and physiological traits, such as shape and filter-feeding capacity (Parra-Velandia & Zea 2003; Pile 1997). Pile (1997) demonstrated that *I. strobilina* had higher filtering efficiencies than *I. felix* and suggested that *I. strobilina*, as a tall and massive sponge, contained more aquifer units, retained water inside the sponge body longer, and exhibited more efficient particle uptake than *I. felix*. Such specific features may create distinct conditions in the mesohyl of each host, each supporting particular bacterial consortia. In addition, the evolutionary history of each sponge species may also influence the structure of their bacterial communities. Vertical transmission has been reported in *I. felix* for most of the bacterial taxa (Schmitt *et al.* 2007) and we have confirmed that morphotypes of *I. felix* are more similar to each other than to *I. strobilina*. Thus, while periodic horizontal symbiont transmission is likely to occur and explain the generalist distribution of individual symbiont taxa, continual vertical transmission of specific communities may maintain symbiont structure within host species, and their divergence among host species, over recent evolutionary scales.

In conclusion, the bacterial communities observed in *I. strobilina* and two color morphs of *I. felix* were host-species specific, exhibiting greater similarity within host

species (morphotypes) than between host species (*I. felix* and *I. strobilina*). The bacterial taxa comprising these symbiont communities were also present in other sponge and coral species and thus represent generalist symbionts. As described for Mediterranean *Ircinia* species (Erwin *et al.* 2012a), we conclude that *I. strobilina* and *I. felix* host a specific mix of generalist symbionts and suggest that host-specific factors (mesohyl conditions and host evolutionary history) determine their unique structure in each host. Contrary to our original hypothesis of spatial structure in the bacterial communities associated with *Ircinia* hosts, we found high stability of bacterial communities within each host sponge across different islands and geographic distances up to 400 km, indicating a minimal effect of dispersal limitation and local environmental conditions on symbiont structure. Thus, host-specific rather than biogeographic factors play a primary role in structuring and maintaining sponge-bacteria relationships in *Ircinia* hosts from the Bahamas.

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Supplemental Information

Table S1. Sample collection by site and source (sponge species and seawater).

	Sweeting's Cay	Little San Salvador	San Salvador	Exumas	New Providence
I. strobilina	5	2	2	8	3
I. felix white morph	5	4	3	6	3
I. felix tan morph	5	1	0	6	3
Seawater	19	1	4	3	1

Table S2. Permutational statistical analyses of T-RFLP data (*Msp*I enzyme) comparing bacterial community structure (PERMANOVA) and homogeneity of dispersion (PERMDISP) among locations within each sponge and seawater. For each term, the multivariate version of the *t*-statistic and *P*-values from Monte Carlo's correction are shown. No test = not enough samples available for nested analyses. Critical value of significance was determined following Benjamini-Yekutieli correction (B-Y). Significant P-values after B-Y correction are indicated with an asterisk.

	***I. felix* tan morph**		***I. felix* white morph**		***I. strobilina***		**Seawater**	
PERMANOVA	***t***	***P*-value**	***t***	***P*-value**	***t***	***P*-value**	***t***	***P*-value**
Sweeting's Cay – Exumas	1.555	0.059	0.873	0.549	1.212	0.202	1.533	0.066
Sweeting's Cay – New Providence	1.285	0.185	0.956	0.486	1.089	0.317	1.353	0.107
Sweeting's Cay – Little S. Salvador	0.792	0.656	1.081	0.318	1.284	0.197	0.893	0.534
Sweeting's Cay – San Salvador	No test	No test	1.609	0.068	0.772	0.609	2.175	**0.003***
Exumas – New Providence	1.315	0.171	0.821	0.623	1.156	0.264	0.773	0.59
Exumas – Little S. Salvador	0.985	0.432	1.174	0.238	1.224	0.231	0.405	0.843
Exumas – San Salvador	No test	No test	1.599	0.071	1.098	0.279	1.438	0.161
New Providence – Little S. Salvador	1.124	0.359	0.980	0.41	1.419	0.207	No test	No test
New Providence – San Salvador	No test	No test	1.924	0.041	1.500	0.148	0.856	0.516
Little S. Salvador – San Salvador	No test	No test	1.555	0.087	1.176	0.334	0.813	0.581
PERMDISP	***t***	***P*-value**	***t***	***P*-value**	***t***	***P*-value**	***t***	***P*-value**
Sweeting's Cay – Exumas	2.475	0.054	0.069	0.944	0.580	0.551	1.236	0.378
Sweeting's Cay – New Providence	4.665	0.028	2.228	0.065	1.622	0.383	2.683	0.048
Sweeting's Cay – Little S. Salvador	8.452	0.168	1.015	0.683	0.568	1	2.683	0.048
Sweeting's Cay – San Salvador	No test	No test	0.010	0.986	1.259	0.58	1.165	0.403
Exumas – New Providence	3.674	0.025	1.751	0.228	1.553	0.307	2.200	0.494
Exumas – Little S. Salvador	4.128	0.127	0.967	0.620	1.283	0.661	2.200	0.519
Exumas – San Salvador	No test	No test	0.140	0.936	1.206	0.552	1.521	0.264
New Providence – Little S. Salvador	12.505	0.253	1.832	0.377	23.618	0.118	No test	No test
New Providence – San Salvador	No test	No test	1.822	0.385	0.459	0.799	1.631	0.623
Little S. Salvador – San Salvador	No test	No test	0.830	0.827	No test	No test	1.631	0.626

Figure S1. Rarefaction curves for diversity and dominance metrics of the bacterial 16S rRNA gene sequences obtained from *I. strobilina* (IST) and the tan (IFX-TAN) and white morphs (IFX-WH) of *I. felix* at a similarity level of 99%: Observed OTUs **(A)**, Chao1 estimator **(B)**, Shannon index **(C)** and the inverse of Simpson index **(D)**. For observed OTUs (A), the bounds on the upper (hci) and lower (lci) 95% confidence intervals for each curve are also shown.

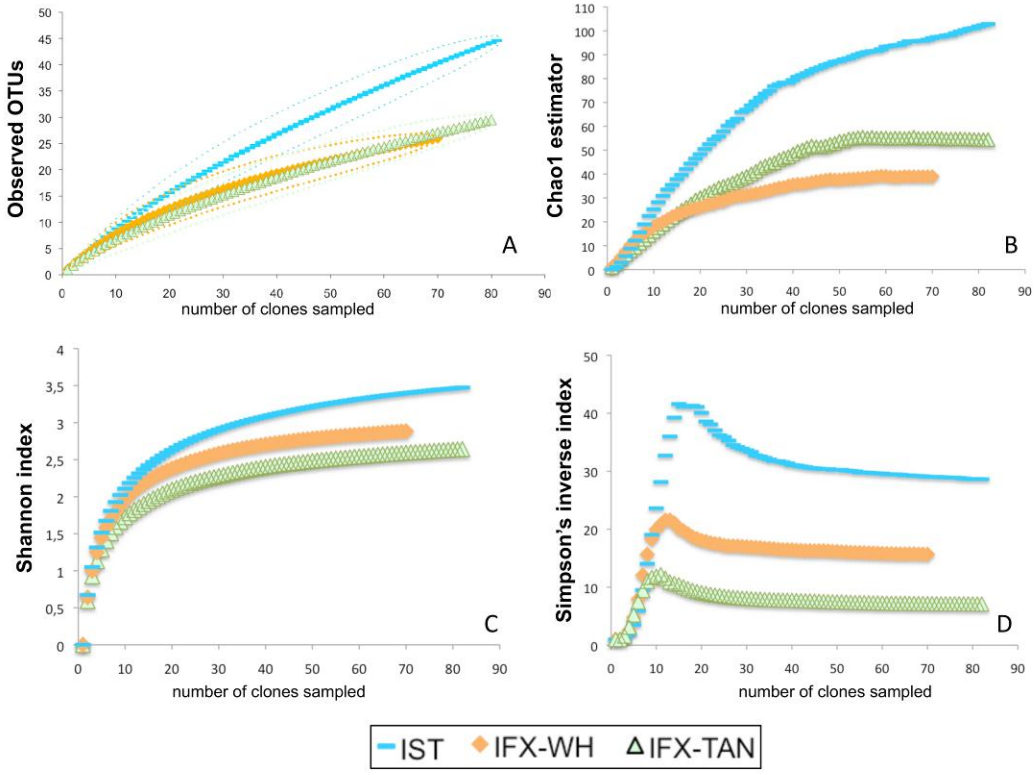


Figure S2. Phylogeny of sponge-associated *Gammaproteobacteria* based on bacterial 16S rRNA gene sequences. The maximum likelihood tree was constructed in RAxML as described in the main text. Bootstrap support values are shown on nodes when >50%. Terminal node labels indicate GenBank accession number and source of each bacterial sequence. Non-sponge sources are specified in parenthesis. Sequences from this study are highlighted in bold and include OTU code and total number of clones obtained (in parenthesis). Reference sequences (ref. seq) obtained from cultured bacteria include GenBank accession number and name of the bacterium. Shaded boxes indicate sponge-specific clades containing sequences from this study.

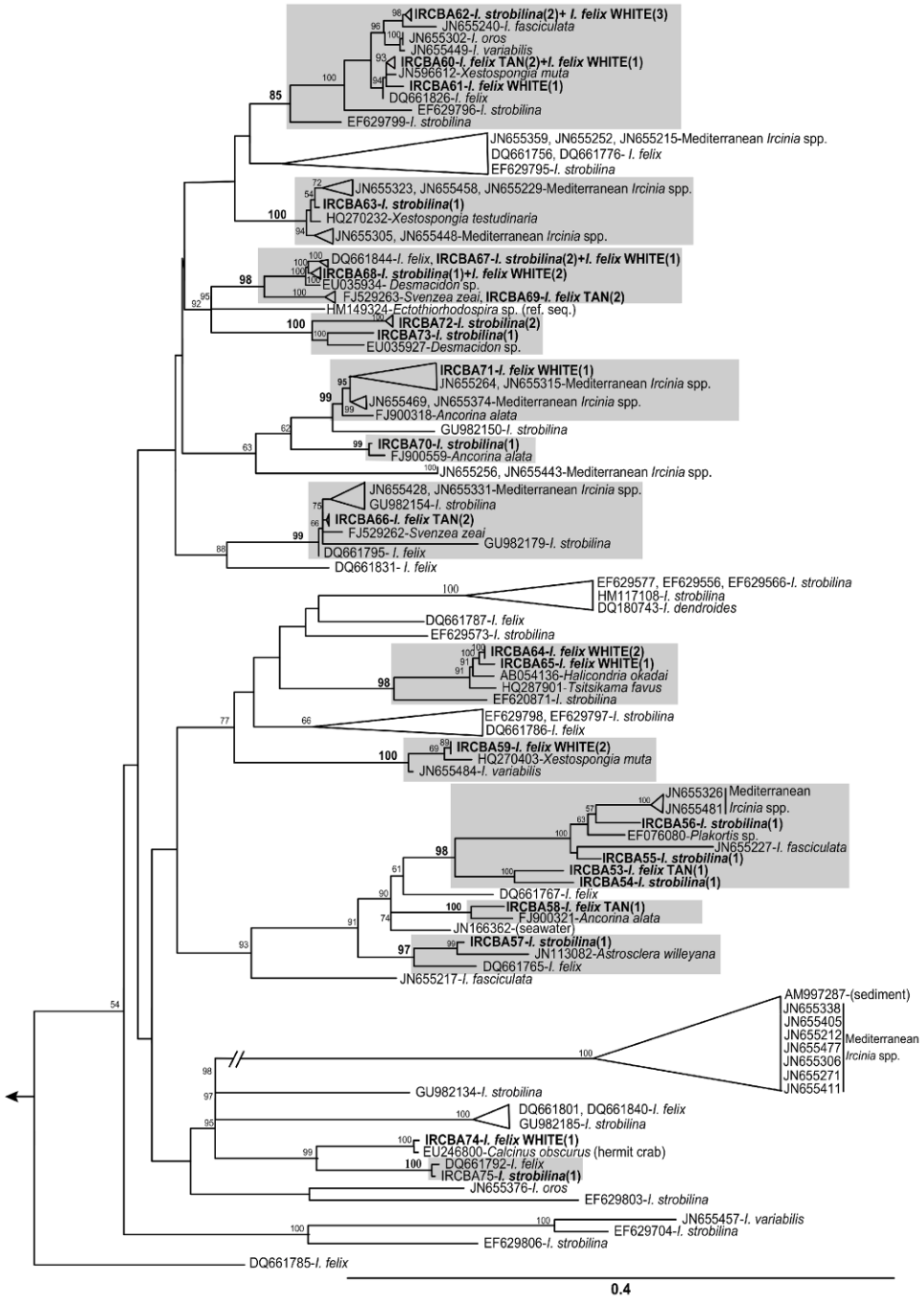


Figure S3. Phylogeny of sponge-associated Alphaproteobacteria based on bacterial 16S rRNA gene sequences. Details are as provided in Fig. S2.

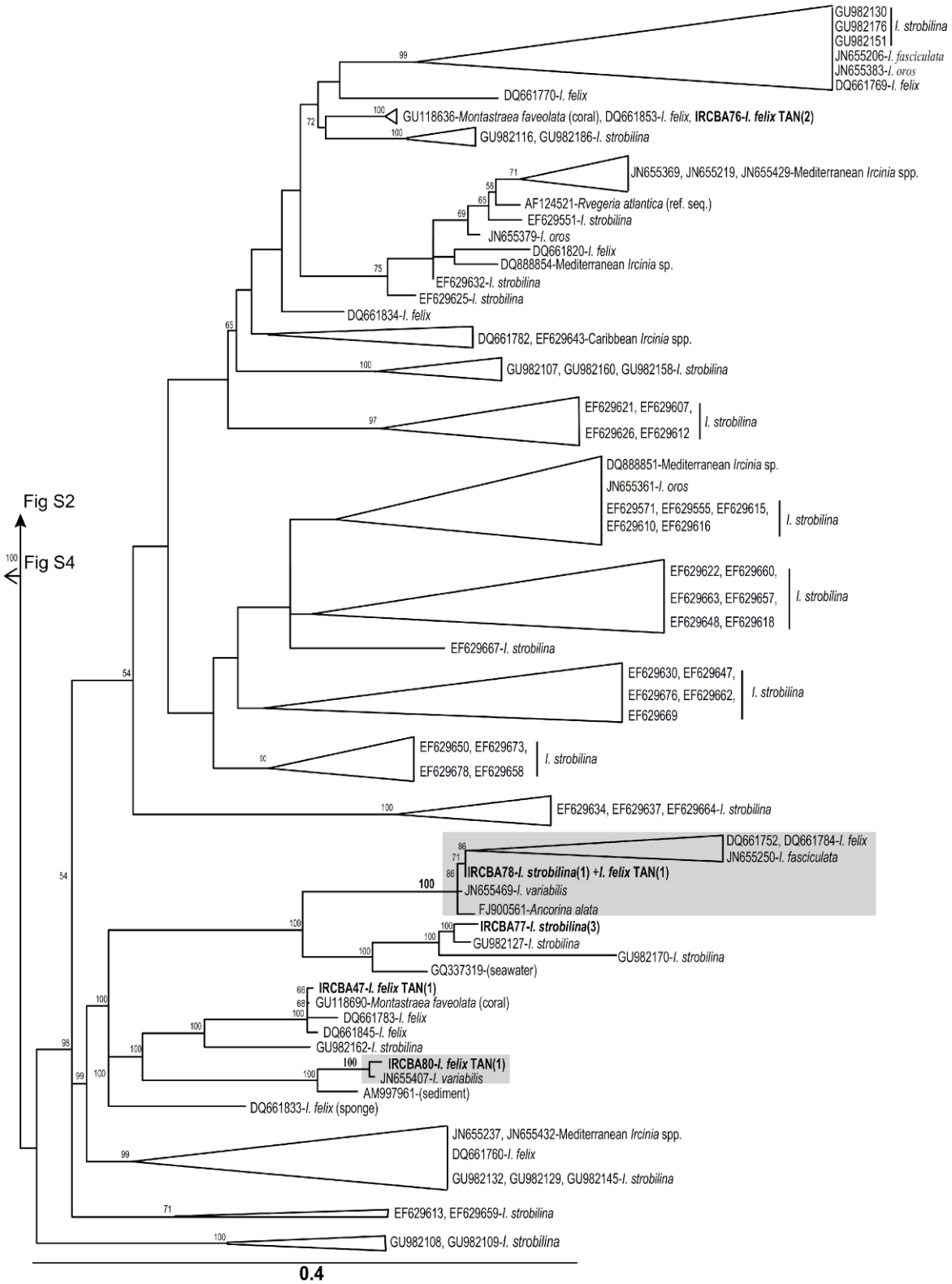


Figure S4. Phylogeny of sponge-associated *Deltaproteobacteria*, *Firmicutes* and *Acidobacteria* based on bacterial 16S rRNA gene sequences. Details are as provided in Fig. S2.

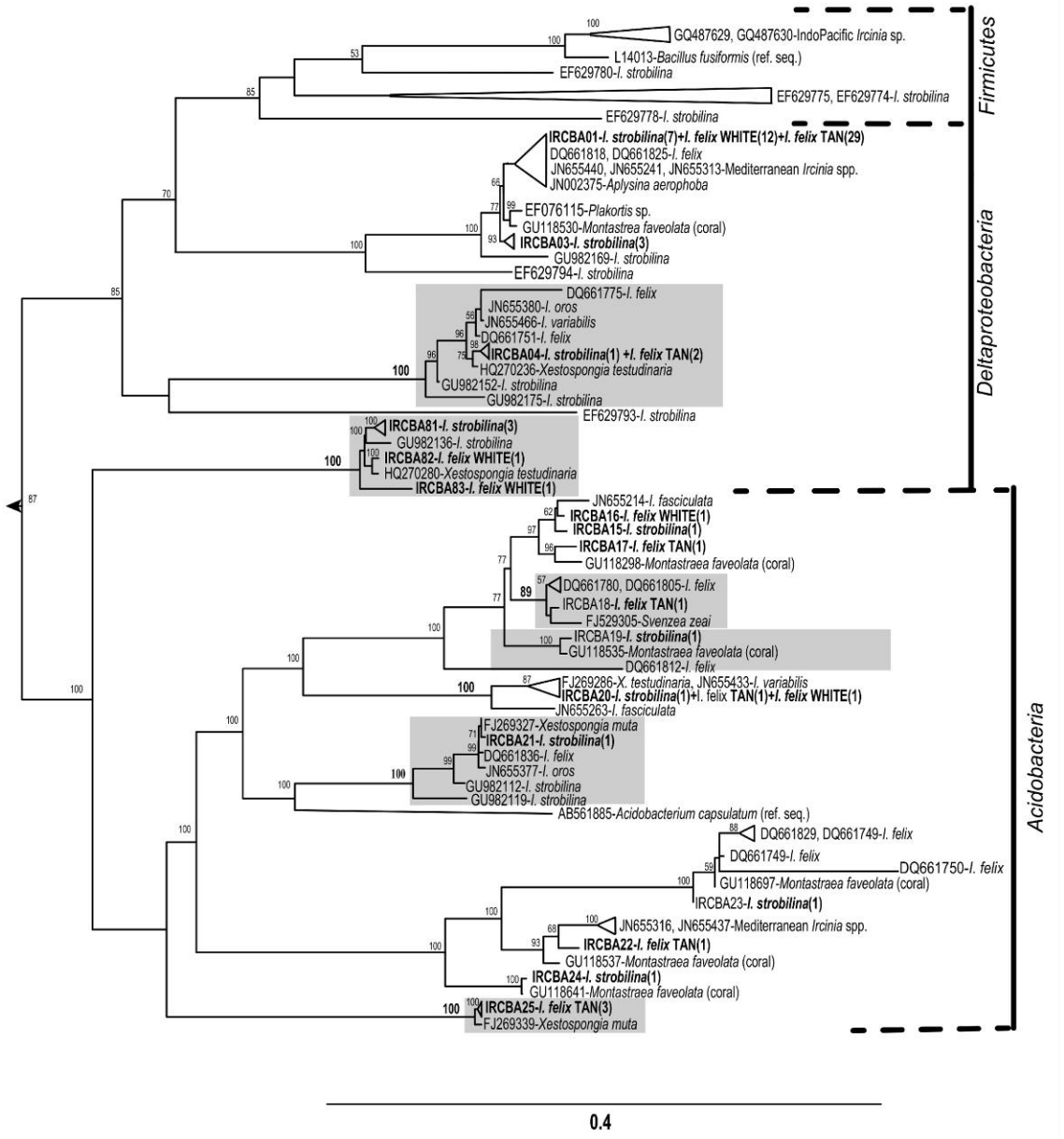


Figure S5. Phylogeny of sponge-associated *Acidobacteria* (SVA0725), *Actinobacteria, Gemmatimonadetes* and *Nitrospira* based on bacterial 16S rRNA gene sequences. Details are as provided in Fig. S2.

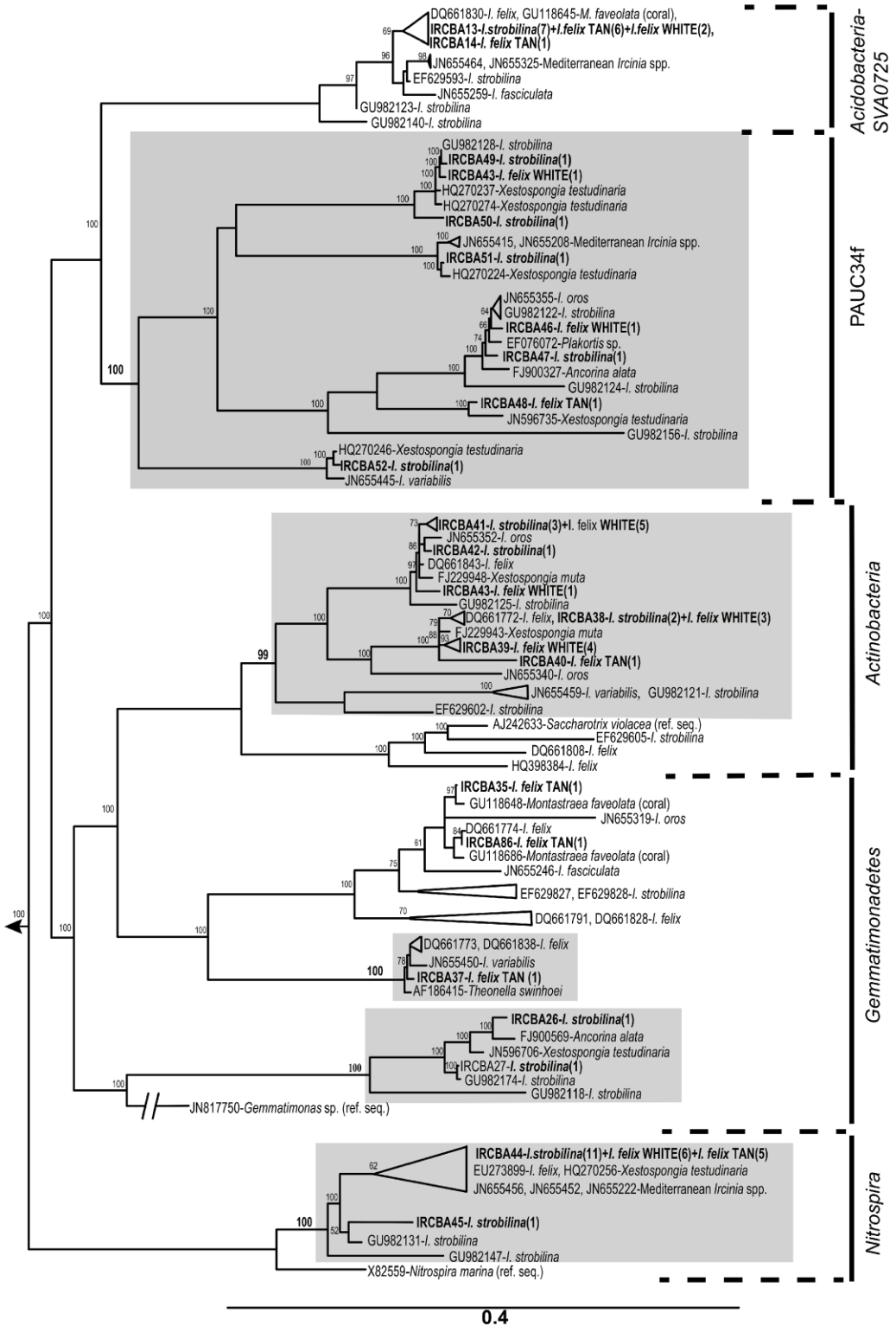


Figure S6. Phylogeny of sponge-associated *Chloroflexi* and the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum based on bacterial 16S rRNA gene sequences. Details are as provided in Fig. S2.

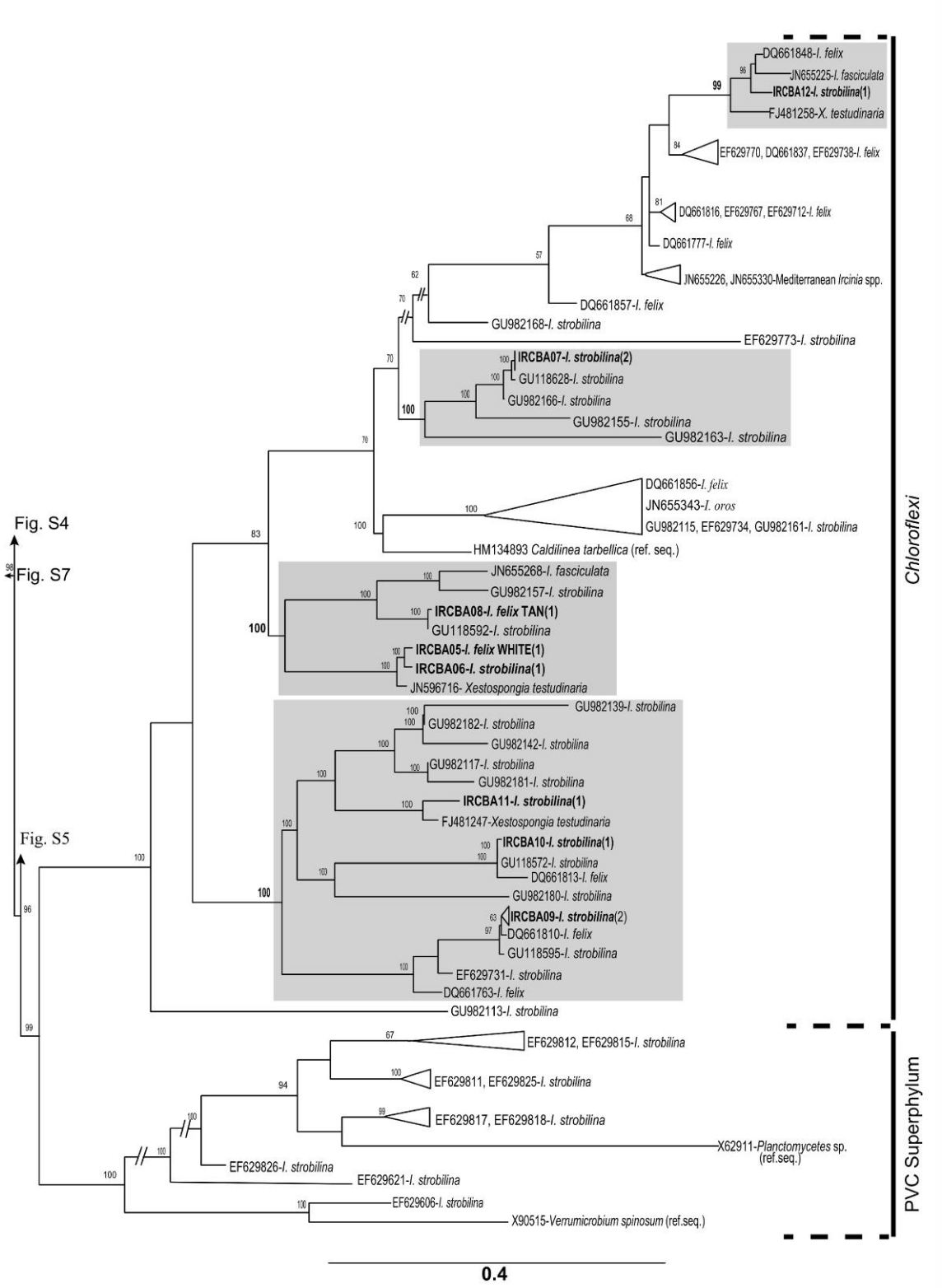
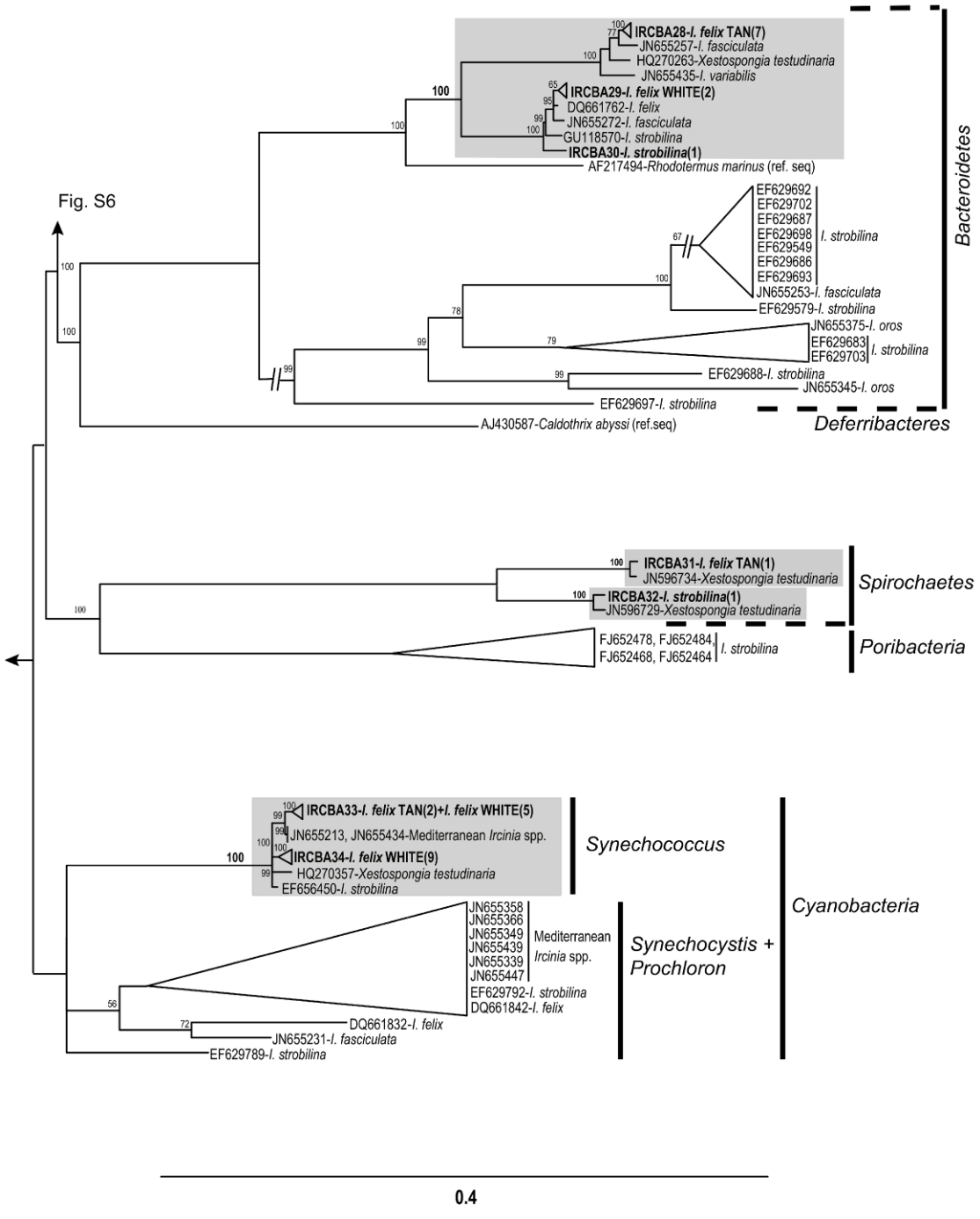


Figure S7. Phylogeny of sponge-associated *Bacteroidetes*, *Deferribacteres*, *Spirochaetes*, *Poribacteria* and *Cyanobacteria* based on bacterial 16S rRNA gene sequences. Details are as provided in Fig. S2.



Chapter 2

Ircinia fasciculata (Tossa de Mar, Spain)

Courtesy of P. M. Erwin

Chapter 2

Host Rules: Spatial Stability of Bacterial Communities Associated with Marine Sponges (*Ircinia* spp.) in the Western Mediterranean Sea

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Abstract

Dispersal limitation and environmental selection are the main processes shaping free-living microbial communities, but host-related factors may also play a major role in structuring symbiotic communities. Here, we aimed to determine the effects of isolation-by-distance and host species on the spatial structure of sponge-associated bacterial communities using as a model the abundant demosponge genus *Ircinia*. We targeted three co-occurring *Ircinia* species and used terminal restriction fragment polymorphism (T-RFLP) analysis of 16S rRNA gene sequences to explore the differentiation of their bacterial communities across a scale of hundreds of kilometers in the Western Mediterranean Sea. Multivariate analysis and non-metric multidimensional scaling plots of T-RFLP profiles showed that bacterial communities in *Ircinia* sponges were structured by host species and remained stable across sampling locations, despite geographic distances (80-800 km) and diverse local conditions. While significant differences among some locations were observed in *Ircinia variabilis*-derived communities, no correlation between geographic distance and community similarity was consistently detected for symbiotic bacteria in any host sponge species. Our results indicate that bacterial communities are mostly shaped by host species-specific factors and suggest that evolutionary processes acting on long term symbiotic relationships have favored spatial stability of sponge-associated bacterial communities.

El hospedador manda: estabilidad espacial en las comunidades bacterianas asociadas con esponjas marinas (*Ircinia* spp.) del Mediterráneo Occidental

Resumen

La limitación de la dispersión y la selección natural son los principales procesos que dan forma a las comunidades microbianas de vida libre, pero factores relacionados con el hospedador pueden desempeñar un papel importante en estructurar las comunidades simbiontes. El objetivo de este trabajo es determinar los efectos del aislamiento por distancia y de la especie de hospedador en la estructura espacial de las comunidades bacterianas asociadas a esponjas, usando como modelo el género *Ircinia*. Nosotros hemos centrado la atención en tres especies simpátricas de *Ircinia* y usamos la técnica de polimorfismo en la longitud de los fragmentos de restricción terminales (T-RFLP) para analizar secuencias del gen ARNr 16S bacteriano con la idea de explorar las comunidades de bacterias a lo largo de una escala espacial de cientos de kilómetros en el Mediterráneo Occidental. Análisis multivariantes y gráficos de escalado multidimensional no métrico de los perfiles de T-RFLP mostraron que las comunidades bacterianas asociadas con esponjas *Ircinia* estaban estructuradas de acuerdo a la especie considerada y se mantenían estables a lo largo de todas las localidades de muestreo, independientemente de la distancia considerada (80-800 km) y diversas condiciones locales. Aunque se detectaron diferencias significativas entre algunas localidades en comunidades bacterianas de *I. variabilis*, no existe correlación entre la distancia geográfica y la similaridad de las comunidades para ninguna especie de esponja. Nuestros resultados indican que las comunidades bacterianas de esponjas se conforman principalmente dependiendo de factores relacionados con la especie de esponja que habitan, lo cual sugiere que procesos evolutivos actuando sobre la relación simbiótica podrían haber favorecido la estabilidad espacial observada.

Introduction

Microbial biogeography studies often evaluate the relationship between community similarity and geographic distance (i.e., isolation-by-distance, also called distance-decay relationships). These patterns respond primarily to two processes: dispersal limitation and environmental selection (e.g., Martiny *et al.* 2006; Fierer 2008). Dispersal limitation prevents connectivity among distant locations or populations, while environmental heterogeneity (e.g. different physico-chemical conditions of seawater in coastal systems) yields variability of the microbial communities among locations as local conditions "pick up" the best-adapted microbes. Disclosing the spatial structure of microbial communities helps to elucidate the relative importance of these two underlying processes (Hanson *et al.* 2012).

Some marine sponges, the so-called high-microbial-abundance sponges (HMA), harbor abundant and diverse bacterial communities (Taylor *et al.* 2007; Hentschel *et al.* 2012). These bacterial communities are far from being randomly structured; rather, their diversity, composition and structure depend on each sponge host (Schmitt *et al.* 2012). Accordingly, each sponge species harbors a specific symbiotic community, resulting from the combination of vertical transmission (from parents to larva) (Usher *et al.* 2001; Ereskovsky *et al.* 2004; Schmitt *et al.* 2007; Lee *et al.* 2009a) and environmental acquisition of bacteria (Schmitt *et al.* 2008; Webster *et al.* 2010; Hentschel *et al.* 2012; Taylor *et al.* 2013).

Recent research on sponge-microbe symbioses has focused on determining whether host specificity of symbiotic communities is maintained across locations. Previous studies have reported high spatial stability of sponge-associated bacteria across geographic distances up to thousands of kilometers (Hentschel *et al.* 2002; Webster *et al.* 2004; Taylor *et al.* 2005; Pita *et al.* 2013a) whereas others have detected differentiation depending on location within the same (Lee *et al.* 2009b) or among different ecosystems (Anderson *et al.* 2010; Yang *et al.* 2011). Thus, it is difficult to draw a general conclusion about the spatial structure of sponge-derived bacterial communities. In addition, sampling strategy and comparison of distantly related host species may confound the processes involved, given the large effect of host sponge species on symbiont community structure.

In this study, we designed a sampling strategy targeting sympatric and congeneric sponges from several western Mediterranean sites. Our goal was to distinguish between the relative contribution of biogeographic (dispersal limitation, environmental selection) and host-related processes (i.e, linked to evolutionary history or biological characteristics) to the spatial structure of bacterial communities associated

with sponges. Herein, we used the term “environment” to refer to the abiotic conditions in ambient seawater external to the host sponges. We investigated the bacterial communities associated with three *Ircinia* species (*I. fasciculata*, *I. variabilis* and *I. oros*) commonly found in the shallow littoral of coastal Mediterranean environments. *Ircinia* bacterial diversity is consistent with other HMA sponges, but each species harbors a unique community composed of generalist sponge symbionts (Erwin *et al.* 2012). The microbial inheritance mode in Mediterranean *Ircinia* species has not yet been studied; although vertical transmission was shown for *I. felix* from the Caribbean (Schmitt *et al.* 2007) and bacterial cells were observed in *I. oros* larva (Ereskovsky & Tokina 2004; Uriz *et al.* 2008). To test whether the host-specific symbiotic communities reported in Mediterranean *Ircinia* spp. were maintained over locations separated by hundreds of kilometers and under different local environmental conditions, we characterized bacterial communities in *Ircinia* spp. from six locations using terminal restriction fragment length polymorphism (T-RFLP) analyses of 16S rRNA gene sequences. We hypothesized that, within each host, a significant distance-decay relationship in bacterial community similarity would be detected as a consequence of (1) dominant currents in the region limiting dispersal of host larvae and bacterioplankton and (2) differences in local conditions generating spatial differentiation of bacterial communities among locations.

Materials & Methods

Sample collection

Tissue samples of *I. fasciculata* (Pallas 1766), *I. variabilis* (Schmidt 1862), and *I. oros* (Schmidt 1864) were collected by scuba diving from shallow littoral zones (depth < 20 m) in September-October 2010 at six different locations from the Western Mediterranean Sea (**Fig. 1**). Seventy-four specimens were sampled (*I. fasciculata*, n = 28; *I. variabilis*, n = 27; *I. oros*, n = 19), including 3-6 replicates per species and site, except for *I. oros* in Caials for which we only had two replicates. All sampled sponges appeared healthy and were collected from sites located 80 to 800 km apart and characterized by different anthropogenic pressures: from marine protected areas (Cabrera National Park, Scandola Nature Reserve in Corsica, Caials-Natural Park of Cap de Creus), to locations near dense human populations (Blanes, Calafat and Alicante). When possible, ambient seawater (500 mL) was simultaneously sampled in close proximity (< 1 m) to the sponges (Caials n = 1; Blanes n = 3; Alicante n = 2). Sponge samples were immediately preserved in absolute ethanol and seawater

samples were concentrated on 0.2-µm filters prior to preservation in ethanol. All samples were stored at -20°C.

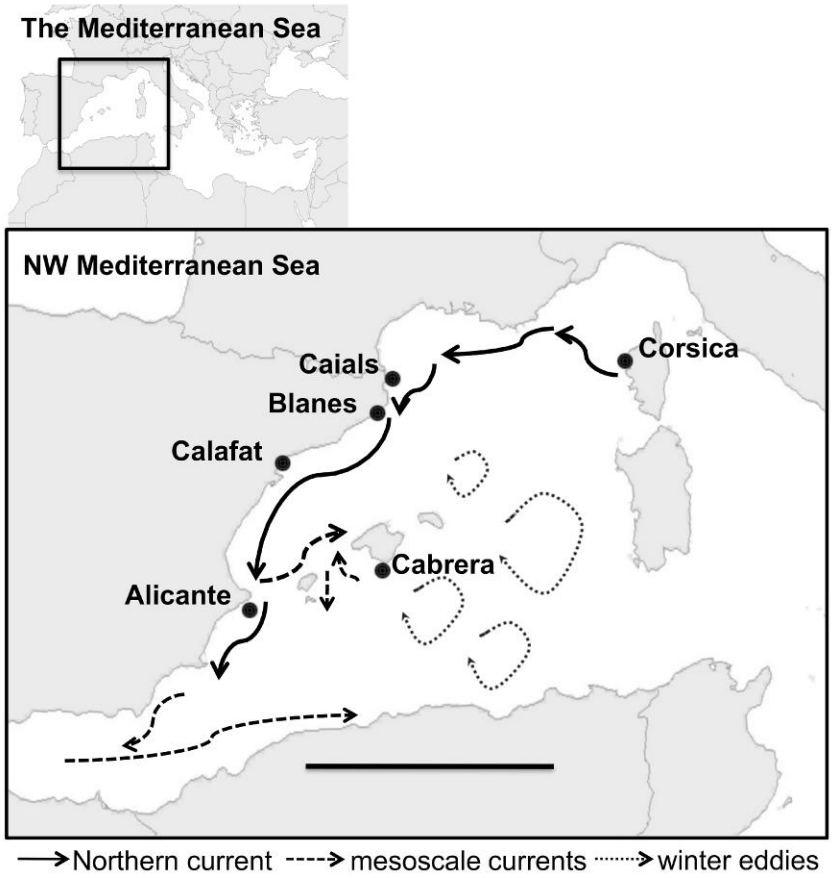

Figure 1. Sampling sites in the Western Mediterranean Sea. Sampling sites and main currents in the region (adapted from Millot 1999) are shown. Scale bar = 422 km.

DNA extractions and T-RFLP analyses

Genomic DNA was extracted from tissue and seawater samples using the DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The universal bacterial forward primer Eco8F (Turner *et al.* 1999), tagged with a 5´-end 6-carboxyfluorescein label (6-FAM), and the reverse primer 1509R (Martínez-Murcia *et al.* 1995) were used for amplification of a *ca*. 1500 bp fragment of the 16S rRNA gene. PCR was performed as follows: one initial denaturation step for 5 min at 94°C; 35 cycles of 1 min at 94°C, 0.5 min at 50°C, 1.5 min at 72°C; and one final elongation step for 5 min at 72°C. Total PCR volume (50 µL) included 10 µM of each primer, 10 nM of each dNTP, 1x Reaction Buffer (Ecogen), 2.5 mM $MgCl_2$, 5 units of BioTaq™ DNA polymerase (Ecogen), and full-strength or 1:10 diluted DNA extracts. Products from triplicate PCR reactions were purified from electrophoresis gels using the Qiaquick Gel

Extraction kit (Qiagen), and quantified using the Qubit™ fluorometer and Quant-iT™ dsDNA Assay kit (Invitrogen, Carlsbad, CA), according to manufacturers' instructions. Separate digestions with the restriction enzymes HaeIII and MspI were performed as described in Pita *et al.* (2013) and analyzed in an automated ABI 3730 Genetic Analyzer (Applied Biosystems) at the Genomics Unit of the Scientific and Technologic Center of the University of Barcelona. The lengths of each terminal-restriction fragment (T-RF) were determined with respect to an internal size standard (LIZ600) using the PeakScanner™ software (Applied Biosystems). T-RFs smaller than 50 bp or larger than 600 bp were discarded because they were beyond the resolution of the size standard. Peak intensities below 50 fluorescence units and relative peak area variation within a cut-off value of two standard deviations (Abdo *et al.* 2006) were discarded as background noise using the T-REX online tool (Culman *et al.* 2009). "True" T-RFs were then aligned in T-REX using a clustering threshold of 1 bp to construct relative T-RF abundance matrices.

Statistical analyses of T-RFLP data

Relative abundance matrices were square root transformed prior to all analyses based on Bray-Curtis distances. For each restriction enzyme, non-metric multidimensional scaling (nMDS) plots were constructed to visualize bacterial community similarity. Permutational multivariate analyses of variance (PERMANOVAs; Anderson 2001; McArdle & Anderson 2001) were used to test for variability across sources (seawater and the three sponge species) and among locations within each sponge host. To compare structure within groups and determine the effect of heterogeneity (dispersion) on significant PERMANOVA outcomes, pairwise comparisons of dispersion (PERMDISP; Anderson 2006) were performed. SIMPER analyses were conducted to identify the individual T-RFs driving the differentiation between groups. Calculations were performed in PRIMER v6 (Clarke 1993; Clarke & Gorley 2006) and PERMANOVA+ (Plymouth Marine Laboratory, UK). Critical values for significance were corrected for multiple pairwise comparisons following the Benjamini & Yekutieli (2001) algorithm (B-Y correction). Mantel tests for each host and restriction enzyme were calculated in R v2.15.2 (The R Core Team 2012) using the package ade4 (Dray & Dufour 2007) to determine whether differences in bacterial community similarity were correlated with geographic distances. We also repeated the Mantel tests excluding the island of Cabrera from the analyses to test if dominant currents in the Western Mediterranean (**Fig. 1**) isolated Cabrera from the peninsular locations, creating a disproportionate differentiation despite short geographic distances and hence distorting the isolation-by-distance effect across the other locations. For each enzyme and

species, we partitioned data matrices into “rare” T-RFs (relative abundance ≤ 1% of each sample) and “abundant” T-RFs (relative abundance > 1%) to determine the influence of rare and abundant T-RFs in the trends observed for the whole community. These threshold values were chosen due to their widespread use in microbial ecology studies (e.g., Pedrós-Alió 2006) and empirical ability to partition the dataset relatively evenly (**Table 1**). Rare and abundant T-RF matrices were analyzed separately with the same procedures described above.

T-RFLP analysis and 16S rRNA gene sequence data

Predicted T-RFs from a reference database were matched with the empirical T-RFs obtained in this study. The reference database consisted of *in silico* digestions by HaeIII and MspI enzymes of *Ircinia*-associated bacterial 16S rRNA gene sequences from a previous study (Erwin *et al.* 2012b). The analysis was performed with the phylogenetic assignment tool PAT (Kent *et al.* 2003), adding an extra bin size for small T-RFs (i.e., 2-bp tolerance applied to fragments of 50-100 bp).

Results

T-RFLP analyses

We identified 183 bacterial T-RFs with the HaeIII enzyme (139 in *I. fasciculata*, 108 in *I. oros*, 140 in *I. variabilis* and 79 in seawater) and 211 using the MspI enzyme (140 in *I. fasciculata*, 145 in *I. oros*, 184 *I. variabilis* and 57 in seawater). The mean and standard error of T-RFs in each category (total, abundant and rare) per source is reported, for HaeIII and MspI enzymes in **Table 1**. Regarding the specificity of the T-RFs, 25.1% (HaeIII) and 20.9% (MspI) were detected in all sources (i.e., present in at least one sample of *I. fasciculata*, *I. variabilis*, *I. oros* and seawater), whereas 19.6% (HaeIII) and 30.3% (MspI) were detected in all sponge species and were absent in the seawater. The proportion of T-RFs that are shared among sources is depicted in Supplemental Information, **Fig. S1**. nMDS plots of all samples (**Fig. 2a**) showed that bacterial communities clustered by source, with sponge-derived samples more similar to each other than to seawater samples. Sponge-derived samples further grouped by host species, but with more discrimination among species for HaeIII than for MspI fingerprints. nMDS graphs for sponge-derived communities (**Fig. 2b**) showed no consistent grouping of sponge-associated bacterial communities based on sampling location. This apparent lack of spatial structure was maintained when nMDS plots were drawn separately for each sponge species (**Fig. 3**). Some *I. variabilis*-derived samples from HaeIII digestions (**Fig. 3b, left**) showed a tendency to cluster according to

sampling location, yet this spatial pattern was not evident for samples from *Msp*I digestions (**Fig. 3b, right**).

Table 1. T-RFs obtained for each sponge species and seawater.

	HaeIII				**MspI**			
	IF	*IO*	*IV*	SW	*IF*	*IO*	*IV*	SW
Total T-RFs	42±3	34±4	41±3	31±6	40±3	42±3	44±3	25±2
Abundant T-RFs	20±1	18±1	20±1	20±2	19±1	21±1	20±1	9±1
Rare T-RFs	22±3	16±3	22±2	12±5	19±3	22±3	25±3	17±1

Shown are the number (average ± standard error) of total, abundant (relative peak area > 1%) and rare (relative peak area ≤1%) T-RFs found per sample within each sponge species and seawater, for each restriction enzyme (HaeIII and MspI). *IF=I. fasciculata*; *IO= I. oros*, *IV= I. variabilis*, SW= seawater.

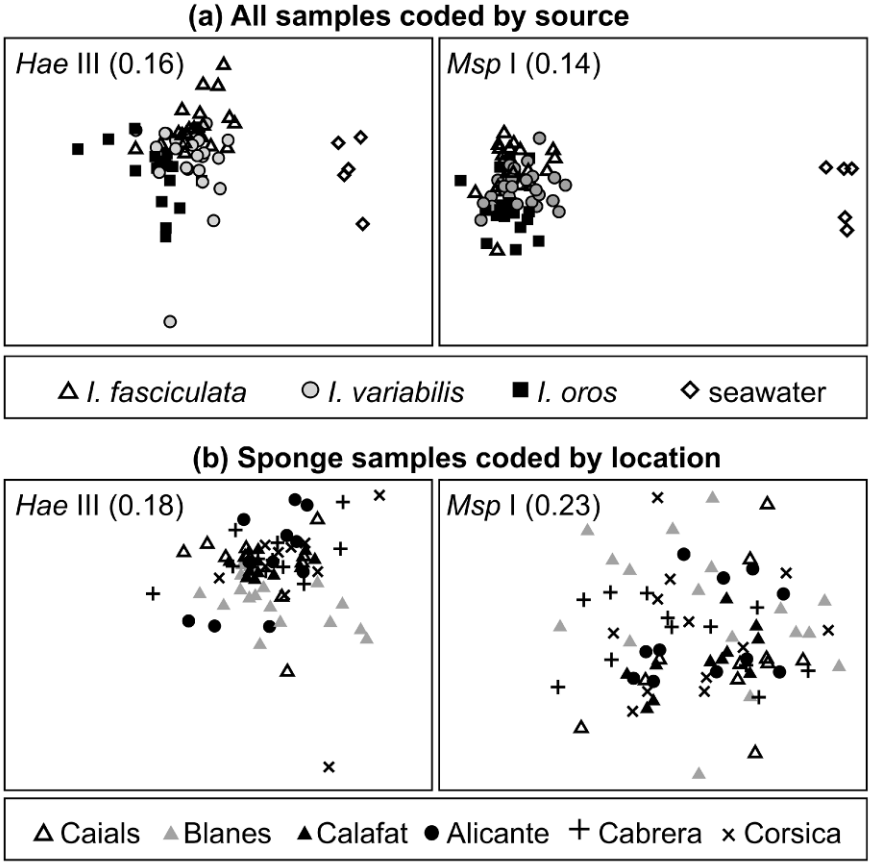

Figure 2. Spatial patterns of bacterial communities in marine sponges and seawater. nMDS plots of bacterial T-RFLP profiles obtained from HaeIII (left) and MspI (right) digestions. **(A)** All samples coded by source; **(B)** sponge samples coded by location. Stress values are shown in parenthesis.

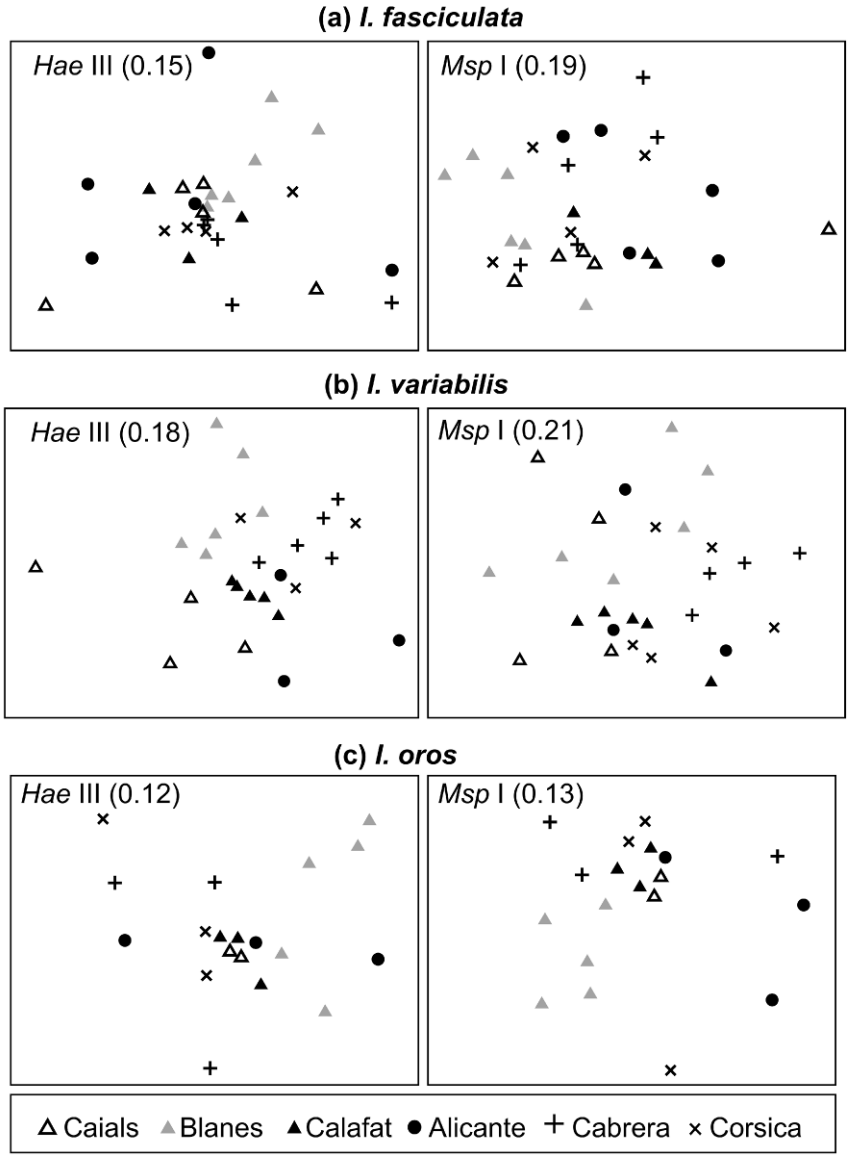

Figure 3. Spatial patterns of bacterial communities in three *Ircinia* sponge species. nMDS plots of bacterial T-RFLP profiles obtained from HaeIII (left) and MspI (right) digestions. (A) *Ircinia fasciculata*-derived samples; (B) *Ircinia variabilis*-derived samples; (C) *Ircinia oros*-derived samples. Stress values are shown in parenthesis.

Comparisons among sources

Pairwise comparisons of T-RFLP profiles among sources (PERMANOVA, **Table 2**) revealed significant differences ($P < 0.05$) among the bacterial communities in each sponge species and seawater for both enzymes and for all comparisons, confirming the patterns visualized in nMDS graphs. The bacterial communities in seawater samples were significantly different from sponge samples, and bacterial communities in sponges were host-species specific. PERMDISP revealed a similar degree of heterogeneity within each source ($P > 0.10$ for all comparisons), and thus the differences between

sources were due to differences in symbiont structure. These results were largely maintained when only rare T-RFs or abundant T-RFs were considered (**Table 2**). The only consistent difference between these data partitions and the entire dataset was that rare *I. variabilis*-derived communities were not different to the rare communities in *I. fasciculata* (for both enzymes), and that the rare communities in *I. fasciculata* and *I. variabilis* did not differ significantly from rare symbionts of *I. oros* for MspI digestions (**Table 2**).

Table 2. Host-specificity of bacterial communities. Multivariate pairwise comparisons of bacterial T-RFLP profiles among sources, for each restriction enzyme (*Hae*III and *Msp*I) applied to the whole community, to the rare partition (relative abundance ≤ 1%) and to the abundant partition (relative abundance > 1%). The multivariate version of *P*-values after 999 permutations from PERMANOVA (above) and PERMDISP (below) tests is reported. Critical values for significance were corrected for multiple comparisons (B-Y correction) and significant values are indicated with asterisks (*α < 0.05, **α < 0.01, ***α < 0.005).

	Whole community		**Rare T-RFs**		**Abundant T-RFs**	
	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI
I. fasciculata-*I. variabilis*	**0.001***** (0.251)	**0.002**** (0.300)	0.063 (0.295)	0.480 (0.530)	**0.001***** (0.152)	**0.005**** **(0.004**)**
I. fasciculata-*I. oros*	**0.001***** (0.150)	**0.001***** (0.574)	**0.001***** (0.359)	0.076 (0.494)	**0.001***** (0.228)	**0.001***** (0.043)
I. variabilis-*I. oros*	**0.001***** (0.706)	**0.001***** (0.767)	**0.006*** (0.810)	0.035 (0.841)	**0.001***** (0.880)	**0.001***** (0.523)
I. fasciculata-Seawater	**0.001***** (0.656)	**0.001***** (0.682)	**0.001***** (0.632)	**0.001***** (0.979)	**0.001***** (0.829)	**0.001***** (0.601)
I. variabilis-Seawater	**0.001***** (0.889)	**0.001***** (0.355)	**0.001***** (0.889)	**0.001***** (0.843)	**0.001***** (0.454)	**0.001***** (0.062)
I. oros-Seawater	**0.001***** (0.606)	**0.001***** (0.496)	**0.001***** (0.915)	**0.001***** (0.719)	**0.001***** (0.408)	**0.001***** (0.136)

Differentiation among locations within sponge hosts

Pairwise comparisons of T-RFLP profiles among locations within each sponge species (nested PERMANOVA, **Table 3**) showed no significant differences in the bacterial communities of *I. fasciculata* and *I. oros* across sampling sites. In *I. variabilis*, Blanes and Cabrera were significantly different in HaeIII-digested T-RFLP profiles and Cabrera-Calafat comparisons were consistently significant for both enzymes. On the whole, PERMDISP analyses (**Table 3**) indicated similar dispersion of the samples within groups, with some exceptions for HaeIII digestions in *I. fasciculata* (Blanes-Alicante) and *I. variabilis* (Blanes-Cabrera, Blanes-Calafat, Calafat-Corsica). For rare

T-RFs, neither PERMANOVA test nor PERMDISP detected significant differences in any pairwise comparison (Supplemental Information, **Table S1**), indicating higher stability and homogeneity of rare sponge symbionts. The analysis of abundant T-RFs revealed additional significant comparisons (i.e., recovered for both enzymes) between Blanes and Calafat for *I. variabilis*, and Blanes and Alicante for *I. fasciculata* (Supplemental Information, **Table S2**).

Table 3. Spatial structure of bacterial communities within sponge hosts. Multivariate pairwise comparisons of bacterial T-RFLP profiles among locations within sponge host and each restriction enzyme (HaeIII and MspI). The multivariate version of *P*-values after 999 permutations from PERMANOVA (above) and PERMDISP (below) tests is reported. Critical values for significance were corrected for multiple comparisons (B-Y correction) and significant values are indicated with asterisks (*α < 0.05). Isolation-by-distance effects were investigated by Mantel tests (*P*-values indicated) for all locations and excluding the island of Cabrera (in italics), for each restriction enzyme.

	I. fasciculata		***I. variabilis***		***I. oros***	
	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI
Multivariate analysis						
Blanes-Alicante	0.291 **(0.003*)**	0.019 (0.344)	0.038 (0.911)	0.192 (0.620)	0.184 (0.842)	0.025 (0.167)
Blanes-Caials	0.122 (0.854)	0.064 (0.935)	0.040 (0.153)	0.080 (0.855)	0.151 (0.048)	0.090 (0.039)
Blanes-Cabrera	0.091 (0.990)	0.049 (0.644)	**0.008*** (**0.004***)	0.029 (0.060)	0.036 (0.492)	0.036 (0.507)
Blanes-Calafat	0.212 (0.400)	0.044 (0.023)	0.017 **(0.005*)**	0.023 (0.125)	0.059 (0.087)	0.029 (0.087)
Blanes-Corsica	0.113 (0.274)	0.116 (0.497)	0.206 (0.319)	0.041 (0.113)	0.046 (0.946)	0.036 (0.329)
Alicante-Caials	0.527 (0.269)	0.193 (0.763)	0.216 (0.547)	0.273 (0.521)	0.468 (0.220)	0.331 (0.213)
Alicante-Cabrera	0.452 (0.174)	0.075 (0.051)	0.061 (0.052)	0.124 (0.222)	0.628 (0.623)	0.251 (0.819)
Alicante-Calafat	0.722 (0.053)	0.205 (0.018)	0.095 (0.057)	0.457 (0.435)	0.317 (0.092)	0.154 (0.093)
Alicante-Corsica	0.450 (0.031)	0.113 (0.198)	0.300 (0.883)	0.310 (0.563)	0.556 (0.892)	0.258 (0.905)
Caials-Cabrera	0.218 (0.958)	0.150 (1)	0.023 **(0.005*)**	0.030 (0.123)	0.400 (0.089)	0.324 (0.381)
Caials-Calafat	0.490 (0.709)	0.104 (0.776)	0.154 (0.017)	0.109 (0.259)	0.365 (0.105)	0.489 (0.114)
Caials-Corsica	0.253 (0.554)	0.175 (0.977)	0.229 (0.861)	0.090 (0.273)	0.403 (0.408)	0.454 (0.314)
Cabrera-Calafat	0.183 (0.884)	0.043 (0.016)	**0.009*** (0.253)	**0.012*** (0.898)	0.250 (0.102)	0.181 (0.103)
Cabrera-Corsica	0.241 (0.568)	0.304 (0.563)	0.225 (0.018)	0.060 (0.161)	0.669 (0.588)	0.450 (1)
Calafat-Corsica	0.301 (0.850)	0.113 (0.092)	0.083 **(0.009*)**	0.098 (0.362)	0.082 (0.099)	0.244 (0.113)
Mantel test (all sites)	0.863	0.931	0.085	0.860	0.950	0.591
Mantel test (no Cabrera)	*0.411*	*0.755*	***0.022****	*0.633*	*0.841*	*0.438*

Isolation-by-distance effect

Mantel tests showed no significant correlation between geographic distances and bacterial community similarity for full datasets (**Table 3**), rare partitions (Supplemental Information, **Table S1**) and abundant partitions (Supplemental Information, **Table S2**); thus, isolation-by-distance effects were not detected in any sponge host or symbiont partition. Results from Mantel tests excluding samples from the island of Cabrera were also not significant with one exception: a significant outcome (P = 0.022) for HaeIII digestions in *I. variabilis* for the full dataset (**Table 3**).

Congruence between T-RFLP analysis and 16S rRNA gene sequence data

PAT analysis showed high congruence between T-RFLP and *in silico* digestions of the reference database containing 16S rRNA gene sequence data from Mediterranean *Ircinia* species (Erwin *et al.* 2012c). The length profiles obtained from the reference database matched 59.1% (HaeIII) and 62.8% (MspI) of the peaks detected empirically in T-RFLP profiles, representing 73.2% (HaeIII) and 79.3% (MspI) of the total peak area. For instance, the T-RF signature of the OTU001, a dominant deltaproteobacterium in all three host species that is closely-related to other sponge- and coral-derived symbionts (Erwin *et al.* 2012a, c), was consistently detected as a conspicuous peak in all sponge species at all locations, with both restriction enzymes. Combining the information from HaeIII and MspI digestions, T-RFLP profiles retrieved 72.5% of the OTUs in the sequence database and included *Deltaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Acidobacteria, Cyanobacteria* (in *I. fasciculata* and *I. variabilis*), *Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Nitrospira, Planctomycetes* and *Verrumicrobia* that were representative of the bacterial communities in Mediterranean *Ircinia* spp. (Erwin *et al.* 2012a, c).

Discussion

The bacterial communities associated with the co-occurring Mediterranean sponges *I. fasciculata*, *I. variabilis* and *I. oros* were structured primarily by host species and remained largely stable across geographic distances of up to 800 km. These results reinforced the key role of host sponge species on the composition of their symbiotic bacterial communities (Montalvo & Hill 2011; Erwin *et al.* 2012a; Hardoim *et al.* 2012) and were consistent with high spatial stability reported in previous studies (Taylor *et al.* 2005; Wichels *et al.* 2006; Thiel *et al.* 2007a; Schöttner *et al.* 2013) including other

Ircinia species (Pita *et al.* 2013a). In addition, we revealed overall similar patterns of spatial stability and host specificity between rare and abundant bacteria, as has been found for free-living microbial communities (Galand *et al.* 2009).

However, rare bacterial symbionts exhibited slightly higher stability over sampled locations than abundant bacterial symbionts, especially for *I. variabilis*. This is contrary to a recent study where we reported the temporal dynamics of microbial communities in these same sponge species (Erwin *et al.* 2012c) and showed remarkable stability in symbiont composition over time with some seasonal variability observed for the rare symbiont taxa. Rare taxa may represent transient bacteria (e.g., from seawater, sediment or fouling) that would be more susceptible to seasonal environmental changes than abundant bacteria (true symbionts), while their spatial stability suggests low selection pressure due to geographic location. Other rare bacterial taxa could be missed in T-RFLP profiles due to technical limitations (Pedrós-Alió 2012). The fewer T-RFs observed for the seawater profiles compared with sponges (an apparent contradiction with previous studies based on cloning and new generation sequencing techniques; e.g. (Webster *et al.* 2010; Erwin *et al.* 2012a) probably result from a lower replication of the seawater samples. Future studies on the spatial structure of bacterioplankton communities in the Western Mediterranean are needed to further reveal the different ecological constraints affecting free-living and sponge-derived communities (Erwin *et al.* 2012c).

At the beginning of this study, we hypothesized that within each host species, bacterial communities derived from sponges in closer locations would exhibit higher similarity (i.e., isolation-by-distance effects) because: (1) vertical symbiont transmission in *Ircinia* spp. (Schmitt *et al.* 2007) may link symbiont dispersal range with that of host larvae, and (2) significant spatial structure and isolation-by-distance patterns were found for other sponge species within the same region studied herein (*Scopalina lophyropoda*, Blanquer & Uriz 2010; *Crambe crambe*, Duran *et al.* 2004). However, we did not observe a significant correlation between bacteria differentiation and geographic distances for any host *Ircinia* species. There are several potential explanations for this lack of differentiation. First, these sponges may disperse further than expected: bacteria in larvae could represent an extra food supply allowing larvae to spend more time in the water column, increasing the probability of successful dispersal, and resulting in high connectivity among *Ircinia* populations (Mariani *et al.* 2006; Uriz *et al.* 2008). Second, host-related factors and symbiotic interactions may exert an intense selective pressure on the bacterial community so that there is no scope for spatial differentiation, even if the connectivity between localities is scarce.

Alternatively, signatures of dispersal limitation may occur yet be masked by the taxonomic resolution of 16S rRNA gene sequences (e.g., Erwin & Thacker 2008a).

In addition to dispersal limitation processes, microbial biogeography patterns may be shaped by environmental selection (Fierer 2008). Local features such as currents, river discharges and human activities generate variability in physico-chemical parameters and spatial differences of bacterioplankton composition among coastal locations in the Western Mediterranean Sea (Schauer *et al.* 2000; Flo *et al.* 2011). While environmental data were not included in our study, it is notable that our sampling sites covered locations near dense human populations (e.g. Blanes, Alicante) and more pristine, protected areas (e.g., Cabrera, Corsica, Caials). However, *Ircinia*-derived bacterial communities persisted across these locations and suggested that the symbiotic community was mostly unaffected by differences in local conditions. A potential exception was observed in bacterial communities associated with two populations of *I. variabilis.* Specifically, differences in symbiont communities occurred between the marine protected area around the island of Cabrera and the populous mainland site of Calafat, which suggests some effect of environmental conditions on the structure of *I. variabilis*-associated communities. Specific features of *I. variabilis* sponges, such as plastic morphology characteristic of this species (Turon *et al.* 2013) or reproductive strategy, could make this species more sensitive to local processes than the other two *Ircinia* spp., which in turn could influence the spatial dynamics of the bacterial community structure (Lee *et al.* 2009b).

Further, a significant isolation-by-distance effect was detected for *I. variabilis* samples after removing Cabrera from the analyses, indicating the inclusion of this site distorts distance-decay trends due to its close geographical proximity yet physical isolation by dominant currents from the remaining sites. Notably, these spatial trends in *I. variabilis* were only detected in T-RFLP profiles with the enzyme HaeIII, which generally exhibited lower resolution than profiles with MspI (e.g., Zhang *et al.* 2008; Erwin *et al.* 2012c; Pita *et al.* 2013a; this study). Since these trends were not detected in both enzymes, they should be interpreted with caution until more data is obtained to confirm these findings.

In this study, we showed that the bacterial communities associated with three co-ocurring *Ircinia* sponges (*I. fasciculata*, *I. variabilis* and *I. oros*) were host-species specific and stable across locations 80 to 800 km apart in the Western Mediterranean Sea. Combined with previous reports of symbiont stability in *Ircinia* spp. over large seasonality in environmental conditions (Erwin *et al.* 2012c), our results support the hypothesis of a unique and stable microenvironment (e.g., mesohyl-specific conditions) within the host sponge body that is largely unaffected by local or seasonal

environmental conditions. Long-term symbiotic interactions shaped by multiple selective pressures (e.g., biotic factors, seasonal and stochastic environmental changes) over time and vertical transmission of key bacteria may have resulted in these persistent bacterial communities. Further studies testing the resilience of these relationships under stressful conditions and investigating how bacterial symbionts metabolically interact with their hosts will provide insights into the vulnerability and resilience of these sponge holobionts in the Mediterranean Sea.

Acknowledgments

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Supplemental Information

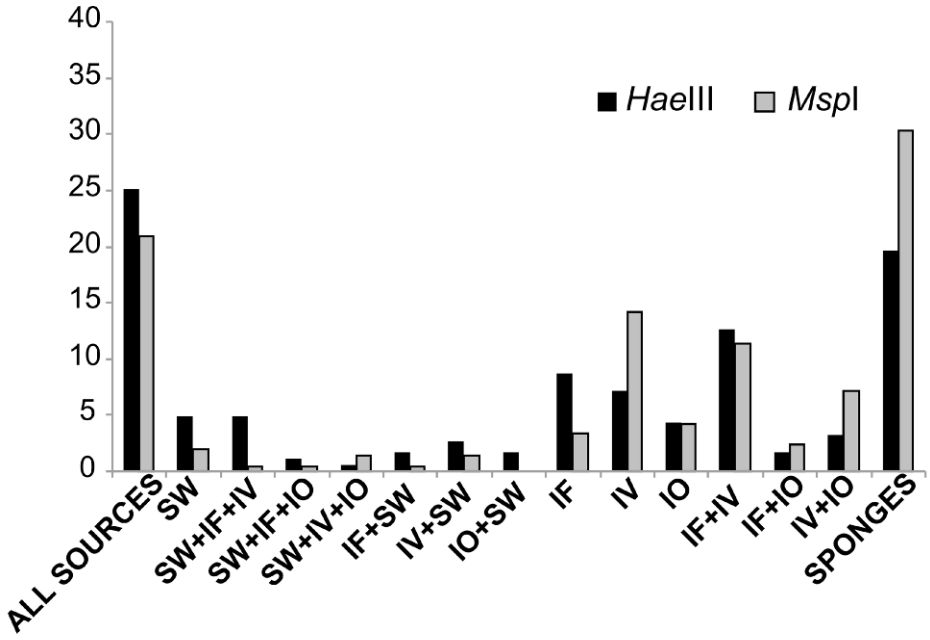
Table S1. Comparisons of rare bacterial communities among locations within sponge host. Multivariate comparisons of rare (relative abundance ≤ 1%) bacterial community structure (PERMANOVA, upper row) and dispersion (PERMDISP, lower row in parentheses), for each restriction enzyme (HaeIII and MspI). Monte Carlo *P*-values after 999 permutations are reported and significance levels were corrected for multiple comparisons (B-Y correction). Isolation-by-distance effect was investigated by Mantel test (*P*-values) for all locations and excluding the island of Cabrera (in italics), for each restriction enzyme.

RARE T-RFs	***I. fasciculata***		***I. variabilis***		***I. oros***	
	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI
Multivariate analysis						
Blanes-Alicante	0.409 (0.886)	0.161 (0.936)	0.161 (1)	0.541 (0.270)	0.621 (0.279)	0.247 (0.320)
Blanes-Caials	0.168 (0.012)	0.119 (0.338)	0.063 (0.872)	0.485 (0.933)	0.346 (0.040)	0.185 (0.039)
Blanes-Cabrera	0.330 (0.467)	0.132 (0.742)	0.049 (0.045)	0.282 (0.334)	0.580 (0.53)	0.140 (0.885)
Blanes-Calafat	0.437 (0.786)	0.139 (0.217)	0.076 (0.703)	0.127 (0.071)	0.191 (0.049)	0.076 (0.123)
Blanes-Corsica	0.266 (0.797)	0.266 (0.037)	0.143 (0.864)	0.101 (0.024)	0.385 (0.053)	0.276 (0.150)
Alicante-Caials	0.249 (0.076)	0.188 (0.275)	0.166 (0.925)	0.540 (0.039)	0.412 (NA)	0.264 (0.318)
Alicante-Cabrera	0.216 (0.406)	0.331 (0.726)	0.104 (0.255)	0.297 (0.285)	0.516 (NA)	0.277 (0.204)
Alicante-Calafat	0.634 (0.705)	0.200 (0.170)	0.144 (0.795)	0.658 (0.682)	0.199 (0.203)	0.151 (0.101)
Alicante-Corsica	0.430 (0.941)	0.339 (0.041)	0.435 (0.903)	0.327 (0.799)	0.329 (NA)	0.369 (0.262)
Caials-Cabrera	0.088 (0.049)	0.147 (0.628)	0.097 (0.306)	0.487 (0.032)	0.301 (NA)	0.353 (0.204)
Caials-Calafat	0.241 (0.102)	0.145 (0.786)	0.289 (0.843)	0.169 (0.105)	0.289 (0.103)	0.466 (0.095)
Caials-Corsica	0.123 (0.075)	0.386 (0.210)	0.178 (1)	0.240 (0.018)	0.387 (NA)	0.337 (NA)
Cabrera-Calafat	0.455 (0.654)	0.148 (0.416)	0.069 (0.658)	0.071 (0.271)	0.157 (0.184)	0.161 (0.098)
Cabrera-Corsica	0.583 (0.676)	0.321 (0.103)	0.240 (0.220)	0.211 (0.025)	0.258 (NA)	0.385 (0.259)
Calafat-Corsica	0.736 (0.893)	0.311 (0.200)	0.251 (0.869)	0.187 (0.381)	0.263 (0.106)	0.412 (0.243)
Mantel test (all sites)	0.781	0.409	0.450	0.483	0.284	0.105
Mantel test (no Cabrera)	*0.562*	*0.391*	*0.393*	*0.391*	*0.346*	*0.198*

Table S2. Comparisons of abundant bacterial communities among locations within sponge host. Multivariate comparisons of abundant (relative abundance > 1%) bacterial community structure (PERMANOVA, upper row) and dispersion (PERMDISP, lower row in parentheses), for each restriction enzyme (HaeIII and MspI). Monte Carlo *P*-values after 999 permutations are reported and significance levels were corrected for multiple comparisons (B-Y correction), significant values are indicated with asterisks (*α < 0.05; **α < 0.01). Isolation-by-distance effect was investigated by Mantel test (*P*-values) for all locations and excluding the island of Cabrera (in italics), for both restriction enzymes.

ABUNDANT T-RFs	***I. fasciculata***		***I. variabilis***		***I. oros***	
	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI
Multivariate analysis						
Blanes-Alicante	0.270 **(0.002**)**	0.016 **(0.003*)**	0.017 (0.492)	0.164 (0.986)	0.142 (0.732)	**0.014*** (0.032)
Blanes-Caials	0.066 (0.560)	0.047 (0.990)	0.058 **(0.013*)**	0.066 (0.641)	0.137 (0.054)	0.034 (0.554)
Blanes-Cabrera	0.107 (0.325)	0.078 (0.322)	**0.004*** (0.038)	0.015 (0.121)	0.033 (0.443)	0.020 (0.161)
Blanes-Calafat	0.216 (0.511)	0.022 (0.181)	**0.009*** (0.041)	**0.012*** (0.411)	0.037 (0.218)	**0.011*** (0.805)
Blanes-Corsica	0.062 (0.096)	0.086 (0.637)	0.171 (0.199)	0.054 (0.849)	0.034 (0.512)	**0.012*** (0.215)
Alicante-Caials	0.354 (0.132)	0.141 (0.474)	0.205 (0.341)	0.308 (0.573)	0.560 (0.205)	0.318 (0.295)
Alicante-Cabrera	0.378 **(0.007*)**	0.082 (0.020)	0.025 (0.038)	0.195 (0.173)	0.642 (0.746)	0.298 (0.525)
Alicante-Calafat	0.650 (0.113)	0.310 (0.020)	0.066 **(0.010*)**	0.469 (0.538)	0.412 (0.104)	0.238 (0.113)
Alicante-Corsica	0.284 **(0.007*)**	0.227 (0.018)	0.328 (0.821)	0.445 (0.760)	0.502 (0.298)	0.299 (0.212)
Caials-Cabrera	0.077 (0.896)	0.126 (0.920)	0.026 **(0.010*)**	0.036 (0.119)	0.400 (0.292)	0.333 (0.576)
Caials-Calafat	0.245 (0.905)	0.055 (1)	0.140 **(0.011*)**	0.097 (0.313)	0.542 (0.101)	0.515 (0.796)
Caials-Corsica	0.115 (0.846)	0.109 (0.992)	0.262 (0.848)	0.148 (0.321)	0.399 (0.390)	0.473 (0.286)
Cabrera-Calafat	0.176 (0.958)	0.052 (0.086)	**0.005*** (0.776)	0.019 (0.656)	0.376 (0.102)	0.247 (0.197)
Cabrera-Corsica	0.172 (0.301)	0.322 (0.279)	0.172 **(0.012*)**	0.085 (0.148)	0.532 (0.217)	0.594 (0.787)
Calafat-Corsica	0.238 (0.574)	0.092 (0.490)	0.075 **(0.006*)**	0.189 (0.433)	0.179 (0.312)	0.422 (0.200)
Mantel test (all sites)	0.700	0.940	0.596	0.910	0.665	0.679
Mantel test (no Cabrera)	*0.487*	*0.816*	*0.503*	*0.926*	*0.646*	*0.682*

Figure S1. Specificity of T-RFs in *I. fasciculata* (IF), *I. variabilis* (IV), *I. oros* (IO), and seawater (SW).



Chapter 3

Ircinia oros (Tossa de Mar)
Courtesy of S. López-Legentil

Chapter 3

Stability of Sponge-Associated Bacteria over Large Seasonal Shifts in Temperature and Irradiance

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Abstract

Complex microbiomes reside in marine sponges and consist of diverse microbial taxa, including functional guilds that may contribute to host metabolism and coastal marine nutrient cycles. Our understanding of these symbiotic systems is based primarily on static accounts of sponge microbiota, while their temporal dynamics across seasonal cycles remain largely unknown. Here, we investigated temporal variation in bacterial symbionts of three sympatric sponges (*Ircinia* spp.) over 1.5 years in the northwestern (NW) Mediterranean Sea, using replicated terminal-restriction fragment length polymorphism (T-RFLP) and clone library analyses of bacterial 16S rRNA gene sequences. Bacterial symbionts in *Ircinia* spp. exhibited host species-specific structure and remarkable stability throughout the monitoring period, despite large fluctuations in temperature and irradiance. In contrast, seawater bacteria exhibited clear seasonal shifts in community structure, indicating that different ecological constraints act on free-living and on symbiotic marine bacteria. Symbiont profiles were dominated by persistent, sponge-specific bacterial taxa, notably affiliated with phylogenetic lineages capable of photosynthesis, nitrite-oxidation and sulfate-reduction. Variability in the sponge microbiota was restricted to rare symbionts and occurred most prominently in warmer seasons, coincident with elevated thermal regimes. Seasonal stability of the sponge microbiota supports the hypothesis of host-specific, stable associations between bacteria and sponges. Further, the core symbiont profiles revealed in this study provide an empirical baseline for diagnosing abnormal shifts in symbiont communities. Considering that these sponges have suffered recent, episodic mass mortalities related to thermal stresses, this study contributes to the development of model sponge-microbe symbioses for assessing the link between symbiont fluctuations and host health.

Estabilidad de las bacterias simbiontes de esponjas a lo largo de cambios estacionales en las condiciones de temperatura e irradiancia

Resumen

Las esponjas de mar albergan complejas comunidades microbianas compuestas por diversos taxones, incluyendo distintos grupos funcionales que pueden contribuir al metabolismo del hospedador y a los ciclos de nutrientes en los sistemas costeros. Nuestra comprensión del sistema simbiótico esponja-microbiota se basa principalmente en descripciones estáticas de la diversidad, mientras que la dinámica temporal a lo largo de ciclos estacionales se desconoce. En este estudio hemos investigado la variación temporal de los simbiontes bacterianos de tres esponjas simpátricas (*Ircinia* spp.) a lo largo de 1.5 años en el noroeste del Mar Mediterráneo, valiéndonos del polimorfismo en la longitud de los fragmentos terminales de restricción (T-RFLP) y librerías de clones de secuencias del gen ARNr 16S bacteriano. Los simbiontes de *Ircinia* spp. mostraron una estructura específica de la especie de hospedador considerada y una notable estabilidad a lo largo de todo el periodo de monitoreo, a pesar de fluctuaciones en las condiciones de temperatura e irradiancia. En cambio, la composición del bacterioplancton mostró una clara estacionalidad, lo que sugiere que los factores ecológicos que afectan a las comunidades de vida libre son distintos de los que afectan a las bacterias simbiontes marinas. Los perfiles de las comunidades simbiontes estuvieron dominados por taxones bacterianos persistentes y específicos de esponja, principalmente incluidos en linages filogenéticos capaces de llevar a cabo la fotosíntesis, la oxidación de nitrito y la reducción de sulfato. La variabilidad en la microbiota de la esponja se limitó a los simbiontes raros y tuvo lugar perceptiblemente en los meses cálidos, coincidiendo con los regímenes de temperatura elevada. La estabilidad estacional de los simbiontes de esponjas apoya la hipótesis de la especificidad respecto a la especie de hospedador y de que las asociaciones entre esponjas y bacterias son estables. Además, la caracterización de los simbiontes persistentes detectados en este estudio proporcionan una base empírica para poder diagnosticar cambios anormales en las comunidades simbiontes. Considerando que estas esponjas han sufrido episodios recientes de mortalidades masivas relacionadas con estrés térmico, este estudio contribuye al desarrollo de un modelo de simbiosis esponja-bacteria que sirve para evaluar el vínculo entre las fluctuaciones en las comunidades simbiontes y la salud del huésped.

Introduction

Sponges are sessile invertebrates that form a species rich phylum at the base of the metazoan tree of life (> 8,500 valid species; [Van Soest *et al.* 2012]). Renowned for their efficient filter-feeding capabilities and bioactive secondary metabolite production, sponges have important ecological and biotechnological relevance as major players in marine nutrient cycles (Diaz & Ward 1997; Diaz & Rutzler 2001; Hoffmann *et al.* 2009) and the most prolific producers of marine natural products (> 6,600 secondary metabolites; [(Erwin *et al.* 2010]). The discovery and characterization of diverse microbial symbionts inhabiting the sponge body have prompted the adoption of the holobiont concept, thereby incorporating microbial symbionts in the study of sponge ecology and evolution (Taylor *et al.* 2011). The resulting field of sponge microbiology has grown rapidly in the past 2 decades (Thacker & Freeman 2012) and revealed a tight ecological link between host health and symbiont composition. Indeed, sponge-associated microbes have been implicated in host metabolism and growth (Wilkinson 1983; Erwin & Thacker 2008b; Freeman & Thacker 2011), chemical defense production (Flatt *et al.* 2005), and susceptibility to biotic (e.g., disease) and abiotic stressors (Lemoine *et al.* 2007; Webster *et al.* 2008a; Webster *et al.* 2008b).

The remarkable diversity of the sponge microbiota has presented a formidable challenge to understanding the structure and function of microbial guilds in sponge hosts (Hentschel *et al.* 2002; Thacker & Freeman 2012; Webster & Taylor 2012). The sponge microbiota includes diverse phylogenetic lineages of *Archaea* and *Bacteria*, as well as fungi and viruses (Taylor *et al.* 2007; Simister *et al.* 2012a). Among bacterial symbionts alone, thousands of taxa have been reported, spanning 17 described phyla and 12 candidate phyla (Schmitt *et al.* 2012), and hundreds of bacterial taxa can occur in a single host individual (Webster *et al.* 2010; Lee *et al.* 2011). Accordingly, considerable effort has focused on describing the vast diversity of the sponge microbiota, while more applied studies of symbiont functioning have targeted specific components (e.g., *Cyanobacteria*; [Thacker & Freeman 2012]) or functional gene pathways (e.g., ammonia oxidation; [López-Legentil *et al.* 2010]) in these communities. As a result, most studies of sponge microbiology have been limited in scope to one or few host species collected at a single time point, and thus, much of our knowledge concerning the sponge microbiota is based on a static representation of these potentially dynamic communities (Thacker & Freeman 2012).

Understanding the complex sponge microbiota requires a basic knowledge of how these communities change over time. The general consensus is that sponge-microbe associations are largely stable over temporal scales (Taylor *et al.* 2007),

including epibionts (Lee *et al.* 2006), cultivatable symbionts (Webster & Hill 2001) and entire bacterial communities (Taylor *et al.* 2003; Thiel *et al.* 2007a; 2007b; White *et al.* 2012). Other studies have reported higher levels of variability across seasons (Wichels *et al.* 2006) and when repeatedly sampling the same individuals over time (Anderson *et al.* 2010), indicating some degree of symbiont fluctuation over time and individual variation among hosts. The prospect of sponge aquaculture for the production of bioactive metabolites has prompted investigations of host-symbiont stability under *ex situ* aquaria conditions, revealing high symbiont stability over short-term time scales (11 days to 12 weeks; [Friedrich *et al.* 2001; Webster *et al.* 2011]), while longer-term maintenance (6 months to 2 years) can result in substantial shifts in symbiont composition (Mohamed *et al.* 2008b; 2008c; Webster *et al.* 2011). Additional studies of temporal variation in sponge-associated bacteria under natural conditions will aid future aquaculture efforts by determining natural variation in the sponge microbiota and its consequences for host-symbiont dynamics. Further, such studies establish the baseline levels of symbiont variability required to define abnormal shifts and ascribe symbiont fluctuations to specific abiotic and biotic factors.

In this study, we investigated temporal variation in the microbiota of three congeneric sponge hosts from the Mediterranean Sea: *Ircinia fasciculata*, *I. variabilis* and *I. oros*. These sponges are common members of coastal benthic communities in the Mediterranean Sea and harbor diverse, host-specific communities of bacterial and cyanobacterial symbionts (Sarà 1971; Usher 2008; Erwin *et al.* 2012a; 2012b). Replicate individuals of each sponge species were tagged *in situ* and sampled quarterly for 1.5 years to monitor their bacterial symbiont communities, using terminal-restriction fragment length polymorphism (T-RFLP) and clone library analyses of bacterial 16S rRNA gene sequences. In addition, photosynthetic pigments were monitored in the tissues of the cyanobacteria-rich sponges *I. fasciculata* and *I. variabilis*, using chlorophyll *a* (chl *a*) quantification. The specific objectives of the study were: 1) to determine the temporal stability of host-symbiont specificity, 2) to identify permanent and transient symbiont taxa in association with sponge hosts, and 3) to document natural variability in symbiont communities over time. Collectively, these objectives contribute to the broader goal of establishing the empirical baselines required to diagnose abnormal symbiont shifts and develop these symbiotic systems as an impact assessment tool in coastal ecosystems.

Material & Methods

Sample collection

The sponge species *Ircinia fasciculata* (Pallas 1766), *I. variabilis* (Schmidt, 1862) and *I. oros* (Schmidt, 1864) were monitored in shallow (< 20 m) littoral zones at two neighboring sites (< 12 km apart) along the Catalan Coast (Spain) in the northwestern (NW) Mediterranean Sea. *I. fasciculata* colonies were studied in Punta de S'Agulla (Blanes; 41° 40' 54.87" N, 2° 49' 00.01" E) and *I. variabilis* and *I. oros* in Mar Menuda (Tossa de Mar; 41° 43' 13.62" N, 2° 56' 26.90" E) from March 2010 to June 2011. Initial sampling of *I. oros* (March 2010) was performed in the nearby Punta Santa Anna (Blanes; 41° 40' 21.48" N, 2° 48' 13.55" E); however, from June 2010 to June 2011, sampling was conducted in Tossa de Mar, due to the onset of heavy construction in the adjacent Blanes Port (< 300 m from the Punta Santa Anna sampling site) in May 2010.

Individual sponges were marked *in situ* and sampled quarterly for genetic analyses and chlorophyll *a* concentrations by scuba diving using a scalpel blade and forceps. At each site, ambient seawater samples (500 ml) were collected simultaneously and in close proximity (< 1 m) to sampled sponges. Sponge and seawater samples were transported in an insulated cooler to the laboratory (ca. 2 h of transit time), where sponge samples for genetic analyses were preserved in 100% ethanol and stored at -20°C and seawater samples were concentrated on 0.2 µm filters and stored at -80°C. Tissue samples for chlorophyll *a* quantification were processed immediately (see below).

Temperature and light measurements

Hourly temperature and light intensity levels were recorded *in situ* at Punta de S'Agulla and Tossa de Mar by Hobo Pendant Temperature/Light Data Loggers (UA-002-64, Onset Computer Corporation) deployed in close proximity (< 2 m) to sampled sponges. Consistent with the distribution of the studied sponge taxa (Erwin *et al.* 2012a), data loggers were deployed at Punta de S'Agulla on horizontal (exposed) substrate, the typical habitat of *I. fasciculata*, and at Tossa de Mar on vertical wall (cryptic) substrate, the typical habitat of *I. variabilis* and *I. oros*. Submarine *in situ* light measurements are complicated by light sensor orientation and the occurrence of sensor encasement fouling. To minimize orientation error, data loggers were attached parallel to the substrate in stable epoxy molds for consistent orientation of light sensors. To minimize fouling error, data loggers were replaced monthly and only the first 7 days of light measurements (70 to 105 data points per month) were used in subsequent analyses. Light measurements were recorded as lux (lumen·m^{-2}), the SI derived unit for luminous

flux density, across a broad spectrum of wavelengths (200 to 1200 nm) and used to compare relative changes in light intensity across sites and seasons. Light duration was calculated as the number of hourly light readings per day greater than 0. Missing data from Tossa de Mar (March 2010 to May 2010) resulted from the loss of data loggers. For comparative analyses, seasons were defined as winter (January, February, March), spring (April, May, June), summer (July, August, September) and fall (October, November, December).

DNA extraction

DNA extracts were prepared from sponge samples containing both ectosome and choanosome for six individuals per host species and time point (*n* = 108) and three replicates of filtered seawater per time point (*n* = 18), using the DNeasy® Blood & Tissue kit (Qiagen®). Dilutions (1:10) of DNA extracts were used as templates in subsequent PCR amplifications.

T-RFLP analysis

PCR amplification of 16S rRNA gene sequences (*ca*. 1500 bp) for T-RFLP analysis was conducted using the universal bacterial forward primer 8F (Reysenbach *et al.* 1994) and reverse primer 1509R (Martínez-Murcia *et al.* 1995), with a 5'-end 6-carboxyfluorescein (6-FAM) label attached to the forward primer. The total PCR volume was 50 µl, and each reaction mix contained 15 pmol of the labeled forward primer, 10 pmol of the reverse primer, 10 nmol of each dNTP, 1x Reaction Buffer (Ecogen) and five units of Biotaq polymerase (Ecogen). Thermocycler reaction conditions were an initial denaturing time of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 0.5 min at 50°C, and 1.5 min at 72°C, and a final extension time of 2 min at 72°C. To minimize PCR amplification biases, a low annealing temperature and low cycle number were used and three separate reactions were conducted for each sample. Triplicate PCR products were gel-purified and cleaned using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) then combined and quantified using a Qubit fluorometer and Quant-i dsDNA assay kit (Invitrogen).

Purified PCR products (*ca*. 100 ng) were digested separately with the restriction endonucleases HaeIII and MspI (Promega) at 37°C for 8 hours and ethanol precipitated to remove residual salts from enzyme buffers. Samples were eluted in 10 µl formamide and 0.5 µl GeneScan 600-LIZ size standard, heated for 2 min at 94°C, cooled on ice and analyzed by capillary electrophoresis on an automated sequencer (ABI 3730 Genetic Analyzer; Applied Biosystems) at the Scientific and Technical Services of the University of Barcelona (Spain). The length of individual terminal-restriction fragments

(T-RF) was determined by comparison with internal size standards using the program GeneScan (PE; Applied Biosystems). T-RFs beyond the resolution of internal size standards (50 to 600 bp) or with peak areas less than 50 fluorescence units were removed, and peak profiles were imported into the program T-REX (Culman *et al.* 2009). Prior to T-RF alignment in T-REX, the objective filtering algorithm of Abdo *et al.* (2006) based on peak area and a cut-off value of 2 standard deviations (SD) was applied to denoise the dataset by eliminating background peaks. Following noise reduction, T-RFs were aligned across samples using a 1-bp clustering threshold, and peak profiles were standardized using relative abundance (percentage total fluorescence).

To compare the similarity of bacterial community profiles, Bray-Curtis similarity matrices were constructed using square root transformations of relative T-RF abundance data and visualized in non-metric multidimensional scaling (nMDS) plots and heatmaps. Permutational multivariate analyses of variance (PERMANOVA) were used to determine significant differences in bacterial community structure across sources (sponge species and seawater) and across seasons within sources (nested analysis). Permutational multivariate analyses of dispersion (PERMDISP) were conducted for all significant PERMANOVA outcomes to test for differences in homogeneity (dispersion) among groups. A significant PERMDISP indicates that differences in community structure detected by PERMANOVA may result from unequal structural variability among groups (i.e., heterogeneity of dispersion) rather than consistent structural shifts. Multiple pairwise comparisons of symbiont structure and dispersion were corrected based on the Benjamini-Yekutieli (B-Y) false discovery rate control (Benjamini & Yekutieli 2001) and an experiment-wise error rate of 0.05. nMDS, PERMANOVA and PERMDISP calculations were performed using PRIMER v6 and PERMANOVA+ (Plymouth Marine Laboratory, UK). Heatmaps were constructed using JColorGrid v1.869 (Joachimiak *et al.* 2006).

Clone library construction and sequence analysis

In a previous study, we provided an initial characterization of bacterial communities in *I. fasciculata*, *I. variabilis* and *I. oros* collected in the winter season (March 2010) by 16S rRNA gene sequence clones libraries (Erwin *et al.* 2012a). In the current study, we resampled the same host individuals in the summer season (September 2010) and constructed clone libraries following the same methodology to: 1) monitor changes in symbiont communities across seasons and 2) identify T-RFLP profile peaks not represented in the winter clone library. In total, 320 clones from the summer clone libraries were bidirectionally sequenced using vector primers at Macrogen, Inc., to

recover near full-length 16S rRNA gene sequences (range = 1,399 to 1,525 bp; average = 1,478 bp). Raw sequence reads were processed in Geneious (Drummond *et al.* 2011) by aligning high-quality forward and reverse reads to yield a final consensus sequence for each clone. Consensus sequences were screened for sequencing anomalies (e.g., chimeras) using Mallard (Ashelford *et al.* 2006) and a reference 16S rRNA gene sequence from *Escherichia coli* (GenBank acc. no. U00096) and confirmed or refuted using Pintail (Ashelford *et al.* 2005) and two related reference sequences.

To determine seasonal overlap and divergence in symbiont communities, sequences were ascribed to operational taxonomic units (OTUs) (99% sequence identity, nearest neighbor algorithm), as implemented in the mothur software package (Schloss *et al.* 2009), and compared to 99% OTUs from the winter clone library (Supplemental Information, **Table S1**). Representative sequences from each 99% OTU were analyzed using the Ribosomal Database Project II (Cole *et al.* 2009) sequence classifier to assess taxonomic affiliations. In addition, OTU-independent statistical tests were conducted to determine seasonal differences in the genetic diversity (homogeneity of molecular variance, HOMOVA), genetic differentiation (analysis of molecular variance, AMOVA) (Stewart & Excoffier 1996) and phylogenetic structure (unweighted UNIFRAC) (Lozupone *et al.* 2007) of bacterial communities within each source. HOMOVA, AMOVA and UNIFRAC analyses were performed as implemented in the mothur software package (Schloss *et al.* 2009).

To match clone library sequences with T-RFLP profile peaks, a reference database (IRC) was created by *in silico* digestions of 16S rRNA gene sequences and consisted of 5'-terminal restriction fragment lengths (reference T-RFs) for each 99% OTU (n = 190) and restriction endonuclease (HaeIII, MspI) combination. Following correction of T-RF drift (see below), the IRC reference database was used to match empirical T-RFs from T-RFLP profiles with known 16S rRNA gene sequences from clone libraries, using the phylogenetic assignment tool (PAT; [Kent *et al.* 2003]) with 1.5-bp bins. Discrepancies between the predicted length of reference T-RFs and actual length of empirical T-RFs can occur due to the phenomenon of T-RF drift (Kaplan & Kitts 2003), where small differences in the molecular weight of fluorescent labels attached to samples (e.g., FAM) and size standards (e.g., LIZ) result in differential capillary migration rates and underestimation of DNA fragment sizes (Pandey *et al.* 2007). To correct for T-RF drift associated with the fluorescent labels used herein, the empirical lengths of T-RFs were determined for eight dominant bacterial OTUs (IRC001, IRC002, IRC003, IRC004, IRC006, IRC007, IRC012, IRC015) using monocultures of each clone as templates for PCR-amplification and T-RFLP analyses, as described above. Regression analysis of the empirical *vs* predicted lengths of T-RFs

from these clones produced a standard curve (R^2 > 0.99, Supplemental Information, **Fig. S1**) used to correct for the discrepancies of T-RF drift and more accurately match DNA sequences with T-RFLP profile peaks.

Chlorophyll *a* Concentrations

Tissue samples for chl *a* quantification were collected from ectosomal regions of *I. fasciculata* (*n* = 48) and *I. variabilis* (*n* = 47) and processed following previously described methods (Erwin, *et al.* 2012b). Due to the absence of photosymbionts in *I. oros* (Erwin *et al.* 2012a), this species was not included in chlorophyll analysis. For *I. fasciculata*, the same eight marked individuals were repeatedly sampled, due to the large size and rapid healing processes of this species. For *I. variabilis*, 3 to 11 non-marked individuals were randomly sampled each month from the same population, as the smaller size and slower healing rate of this species prevented repeated sampling of the same colonies. Accordingly, a one-way repeated-measures analysis of variance (ANOVA) for *I. fasciculata* and a one-way ANOVA for *I. variabilis* were conducted to compare chl *a* concentrations within each species across sampling months. Multiple pairwise comparisons of chl *a* concentrations between species within each month were conducted using Student's *t*-tests with Bonferroni corrections. Statistical analyses were performed using the software Sigmaplot v11.

Nucleotide sequence accession numbers

The sequences determined in this study have been quality checked and are archived in Genbank under accession numbers JX206477 to JX206796.

Results

Seasonal variation in temperature and light intensity

Both monitoring sites exhibited clear seasonal trends in temperature (**Fig. 1**). Annual temperature minima occurred during the winter season, with the lowest average monthly values recorded in March 2010 (12.7°C in S'Agulla, 12.3°C in Tossa) and lowest average daily values in February 2011 (12.4°C in S'Agulla, 12.1°C in Tossa). Annual temperature maxima occurred during the summer season, with the highest average monthly and daily values recorded in August 2010 (23.8°C and 25.3°C in S'Agulla, 22.2°C and 24.8°C in Tossa). Annual temperature fluctuations were accordingly high at both sites (> 12.7°C). Small differences in seawater temperatures between the monitoring sites likely resulted from slightly deeper data logger deployment in Tossa (7 m) compared to S'Agulla (5 m). The summer season was also

characterized by large fluctuations in daily temperatures, averaging 2.2°C (±1.3 SD) in S'Agulla and 1.8°C (±1.2 SD) in Tossa, with > 3°C daily fluctuations recorded on 15 and 12 days in S'Agulla and Tossa, respectively. In contrast, the winter season exhibited minor fluctuations in daily temperatures, averaging 0.4°C (± 0.2 SD) in S'Agulla and 0.3°C (±0.2 SD) in Tossa, and never exceeded 0.8°C at either site. A notable upwelling event occurred in August 2010, causing drastic temperature decreases at both sites and resulting in weekly temperature fluctuations of 7.7°C and 9.4°C and daily fluctuations of 6.9°C and 5.4°C in S'Agulla and Tossa, respectively.

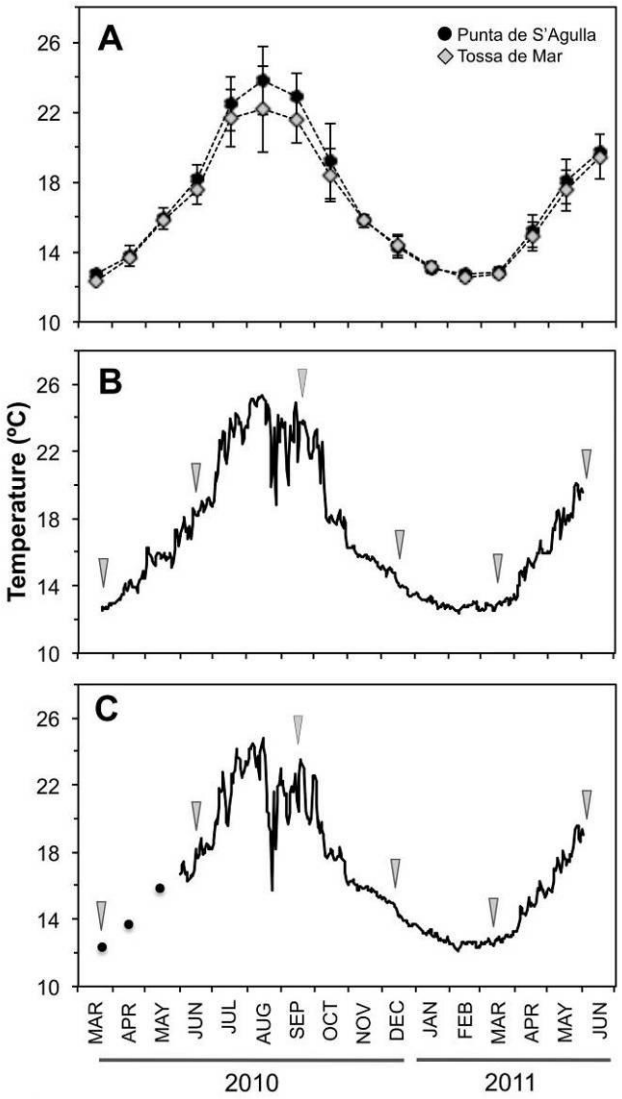

Figure 1. Seasonal variation in seawater temperature from March 2010 to June 2011 at two monitoring sites in the NW Mediterranean Sea. Monthly averages (± SD) for Punta de S'Agulla (*black circles*) and Tossa de Mar (*gray diamonds*) **(A)** and daily averages for Punta de S'Agulla **(B)** and Tossa de Mar **(C)**. Gray triangles highlight sampling times and black dots indicate discrete measurements prior to successful data logger deployment at Tossa de Mar.

Both monitoring sites also exhibited clear trends in irradiance conditions across seasons (**Fig. 2**). Light duration (i.e. day length) was longer in spring and summer than in the fall and winter seasons, which experienced up to 6 h less of light exposure per day. Maximum and average light intensity were higher during the spring and summer seasons than in the fall and winter. Light intensity levels in S'Agulla averaged 1,569 - 10,240 lux per month, with maximum values reaching over 38,000 lux. Lower levels were observed in Tossa, averaging 264 - 1,198 lux per month and maximum values never exceeding 3,700 lux. The large differences in irradiance between sites were consistent with the deployment of data loggers in photophilic (S'Agulla) and semi-sciophilous (Tossa) communities and correspond to the distinct habitats of the host sponge species investigated.

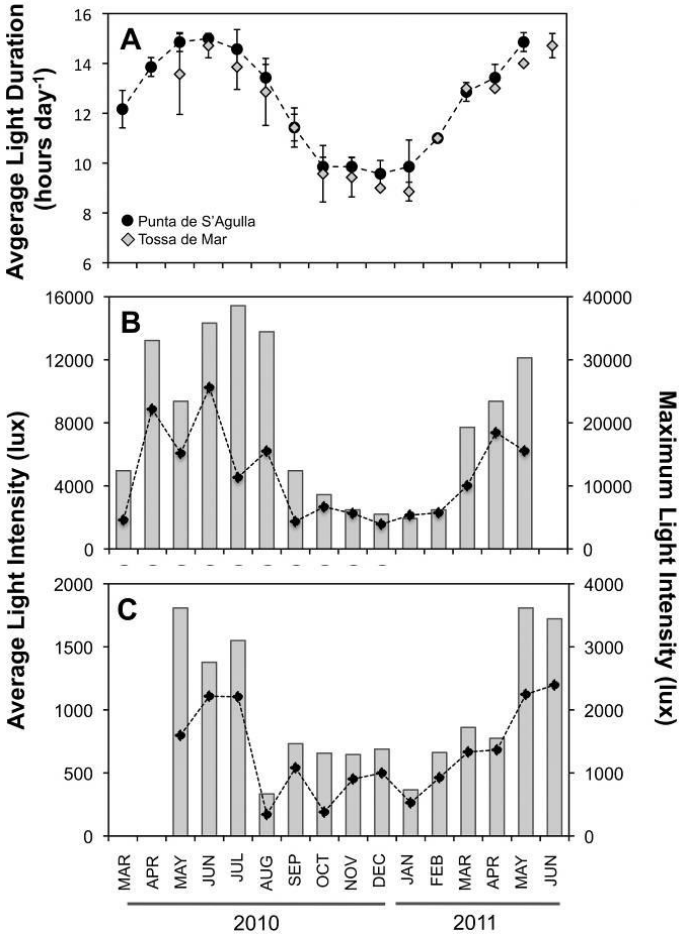

Figure 2. Seasonal variation in light duration (day length) and intensity from March 2010 to June 2011 at two monitoring sites in the NW Mediterranean Sea. Monthly averages (± SD) for day length at Punta de S'Agulla (*black circles*) and Tossa de Mar (*gray diamonds*) **(A)**. Monthly averages (*black diamonds*) and maximum light intensity (*gray bars*) at Punta de S'Agulla **(B)** and Tossa de Mar **(C)**.

Host-specificity of bacterial communities

A total of 213 unique microbial symbiont T-RFs were identified using the restriction enzyme HaeIII (151 in *I. fasciculata*, 149 in *I. variabilis*, 147 in *I. oros*, 144 in seawater) and 237 unique T-RFs with MspI (185 in *I. fasciculata*, 164 in *I. variabilis*, 156 in *I. oros*, 159 in seawater). Binary data analysis of individual T-RFs (presence/absence) revealed highly congruent specificity patterns between the two restriction enzymes used to construct T-RFLP profiles (Supplemental Information, **Fig. S2**). One-third of the unique T-RFs (32.4% for HaeIII and 33.0% for MspI) were sponge-specific, present in one or more host species and absent from seawater, while < 1/10 (8.9% for HaeIII and 5.9% for MspI) were recovered exclusively from seawater (Supplemental Information, **Fig. S2**). The majority of T-RFs were shared among sponges and seawater, present in at least 1 sponge host and seawater (23.0% for HaeIII and 26.6% for MspI) or among all 3 host sponges and seawater (35.7% for HaeIII and 34.6% for MspI) (Supplemental Information, **Fig. S2**). Among the sponge-specific T-RFs, the highest number of unique (host species-specific) T-RFs were detected in *I. fasciculata* (n = 14, HaeIII; n = 11, MspI) and *I. fasciculata* and *I. variabilis* shared more T-RFs than any other pair (n = 12, HaeIII; n = 14, MspI). Similarly, community-level analysis based on the relative abundance of microbial T-RFs revealed clear differentiation of sponge and seawater communities and more similar symbiont communities in *I. fasciculata* and *I. variabilis* than in *I. oros* (**Fig. 3**).

Statistical analyses of community structure (PERMANOVA) revealed significant differences between sponge and seawater microbial fingerprints and among all pairwise comparisons of host sponge species (**Table 1**). Non-metric multidimensional scaling (nMDS) plots exhibited clear spatial segregation of sponge and seawater-derived microbial communities, while among host sponges, symbiont communities consistently clustered by host species across all seasons, with no overlap between *I. fasciculata* and *I. oros* and higher variability in the symbiont profiles of *I. variabilis* (Fig**. 4a** and **c**). Dispersion analysis revealed higher variability within seawater communities compared to sponge-associated bacteria, as pairwise comparisons between sponges and seawater were significant for at least one enzyme while no significant differences in dispersion were found in pairwise comparisons among sponge species (**Table 1**).

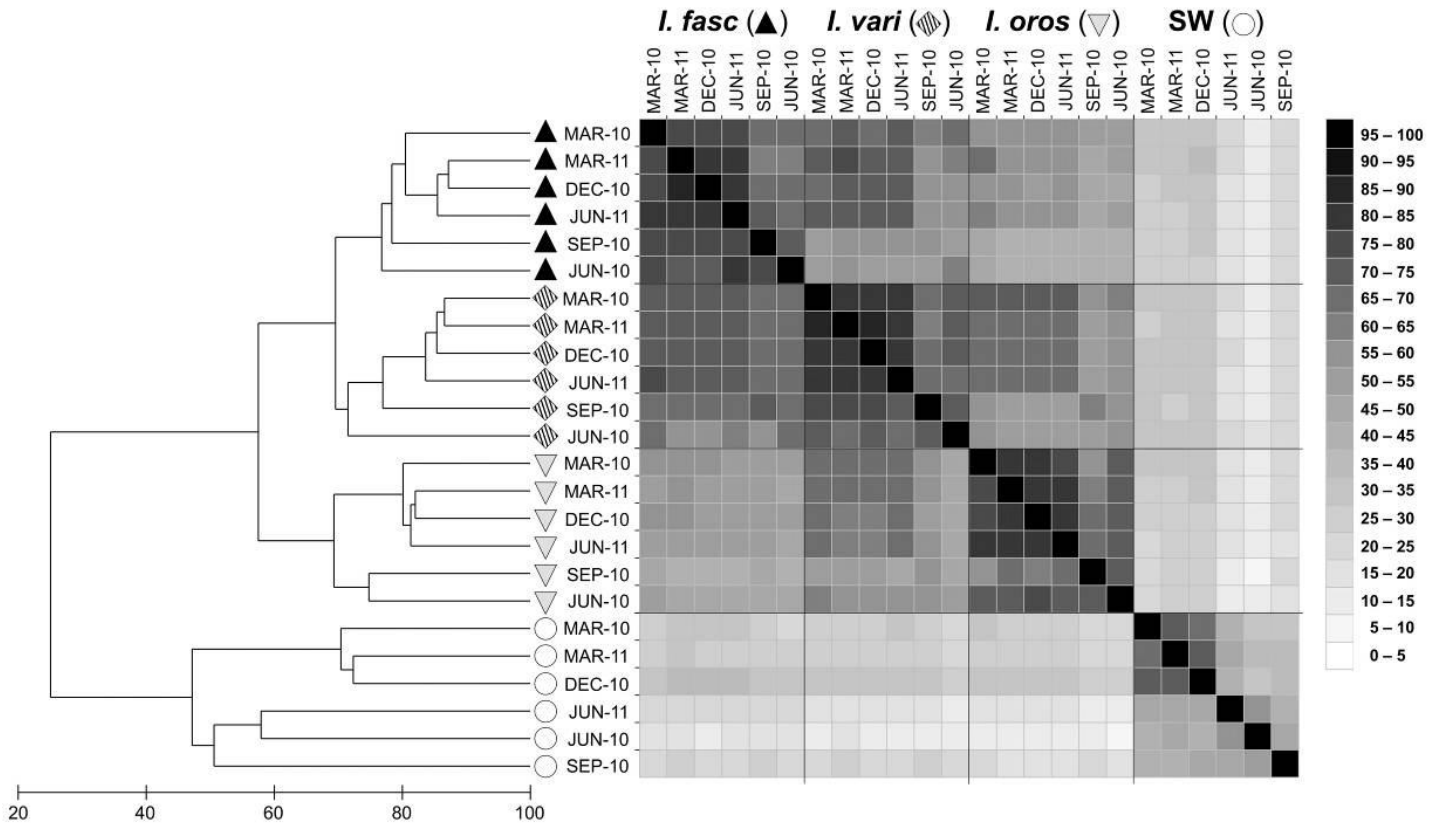

Figure 3. Average similarity of bacterial communities in *I. fasciculata* (*black triangles*), *I. variabilis* (*barred diamonds*), *I. oros* (*gray triangles*) and ambient seawater (*white circles*) over the 1.5-year monitoring period. Dendrogram (*left*) based on Bray-Curtis (BC) similarity values from T-RFLP profiles with HaeIII. Heatmap (*right*) shows all pairwise BC similarity values for both HaeIII (*upper diagonal*) and MspI (*lower diagonal*) datasets.

Table 1. Permutational statistical analyses of T-RFLP data. Analyses included bacterial community structure (PERMANOVA) and dispersion (PERMDSIP) among sponges and seawater.

Analysis	Pairwise Comparison	HaeIII		MspI	
		t	*P (perm)*	*t*	*P (perm)*
PERMANOVA	*I. fasciculata* – *I. variabilis*	3.683	0.001*	3.682	0.001*
	I. variabilis – *I. oros*	5.164	0.001*	4.508	0.001*
	I. oros – *I. fasciculata*	6.988	0.001*	6.637	0.001*
	I. fasciculata – seawater	10.408	0.001*	9.500	0.001*
	I. variabilis – seawater	9.258	0.001*	8.082	0.001*
	I. oros – seawater	9.136	0.001*	10.028	0.001*
PERMDISP	*I. fasciculata* – *I. variabilis*	1.615	0.177	0.848	0.475
	I. variabilis – *I. oros*	0.516	0.639	1.350	0.235
	I. oros – *I. fasciculata*	2.152	0.071	0.456	0.683
	I. fasciculata – seawater	4.016	0.001*	3.575	0.002*
	I. variabilis – seawater	2.451	0.046	2.933	0.015*
	I. oros – seawater	1.997	0.093	4.424	0.002*

*, comparison was found to be significant following B-Y correction (Benjamini & Yekutieli 2001). *P(perm)*, permutation *P* value.

Seasonal variation in bacterial communities

Symbiont communities within each host sponge species exhibited high stability throughout the monitoring period, averaging 69.9% (*I. fasciculata*), 64.0% (*I. variabilis*) and 63.2% (*I. oros*) community similarity in T-RFLP profiles. nMDS plots revealed two tight spatial clusters for *I. oros* and *I. fasciculata* plus *I. variabilis* samples, particularly when considering HaeIII profiles (**Fig. 4b and d**). Each cluster consisted of all samples from 2010-2011 fall and winter and from spring of 2011, as well as some individuals from spring and summer 2010. However, most samples from spring and summer of 2010 were displaced from these central clusters, indicating some change in bacterial profiles during these seasons. In contrast, seawater bacterial communities exhibited clear and consistent seasonal shifts in composition, resulting in spatially segregated clusters in nMDS plots that corresponded to distinct bacterioplankton communities in the fall/winter, spring and summer seasons (**Fig. 4**).

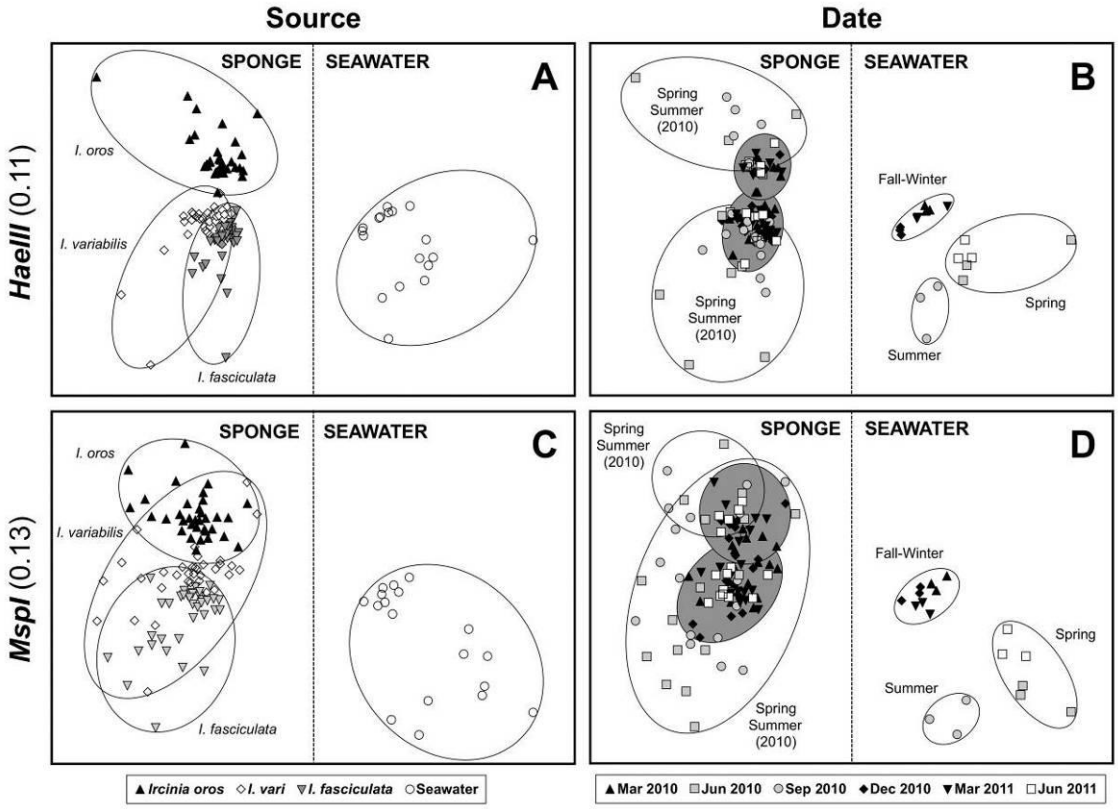

Figure 4. Non-metric multidimensional scaling (nMDS) plots of bacterial community structure from replicate individuals of *I. fasciculata*, *I. variabilis* and *I. oros* and ambient seawater over the 1.5-year monitoring period. nMDS ordination based on Bray-Curtis similarity of T-RFLP profiles for HaeIII **(A, B)** and MspI **(C, D)** datasets. Stress values for two-dimensional ordination are shown in parentheses for each enzyme. Data points are coded by source **(A, C)** with circles encompassing all samples from each source, and by season **(B, D)** with shaded circles denoting core bacterial symbiont profiles and nonshaded circles highlighting deviations from core profiles in spring/summer 2010 **(B, D)**.

Statistical analyses of community structure (PERMANOVA) and dispersion (PERMDISP) revealed significant differences in structure and homogeneity of dispersion among all pairwise comparisons of seawater bacteria (Supplemental Information, **Table S2**), confirming the seasonal shifts in seawater bacteria visualized in nMDS plots. Among host sponges, significant differences in community were observed in the transition from winter to spring and summer to fall of 2010 for at least one enzyme (Supplemental Information, **Table S2**), due to high variability in bacterial community profiles among individuals of each host sponge in spring and summer of 2010. Indeed, PERMDISP analyses revealed significant differences in dispersion during these transitional periods, indicating that heterogeneity was the main driver of structural differences in symbiont communities. Within the fall/winter and

spring/summer seasons, structural differences in sponge-associated bacteria were generally not significant (Supplemental Information, **Table S2**).

Clone library analysis of 16S rRNA gene sequences confirmed the stability of sponge-associated microbial communities over time and the seasonal variability of seawater communities. Comparisons of clone libraries constructed from the same individuals sampled in winter (March) and summer (September) 2010 seasons revealed that a large portion of sponge symbiont communities (57-80% of clones) were stable across seasons, with no significant differences in the genetic differentiation and community structure (**Table 2**; Supplemental Information, **Fig. S3**). Bacterial communities in *I. variabilis* and *I. oros* also exhibited no significant differences in genetic diversity between sampling times, while *I. fasciculata* symbionts showed significantly lower diversity in September 2010, due to increased representation of the dominant cyanobacterium, *Candidatus Synechococcus spongiarum*, in the summer library compared to winter (51% and 26% of clones, respectively). Seawater clone libraries from winter and summer shared few sequences (16-22% of clones) and exhibited significant differences in community structure, genetic diversity and genetic differentiation (**Table 2**; Supplemental Information, **Fig. S3**).

Table 2. Statistical comparisons of genetic diversity and community structure of bacterial communities in sponges (*Ircinia* spp.) and seawater between winter and summer seasons.

	Statistical results for:					
	AMOVA		**HOMOVA**		**UNIFRAC**	
Community	***Fs***	***P***	***B***	***P***	***U***	***P***
I. fasciculata	4.434	0.065	1.834	<0.001	0.670	0.147
I. variabilis	2.241	0.634	0.043	0.248	0.593	0.073
I. oros	3.365	0.397	0.024	0.502	0.584	0.054
Seawater	4.408	<0.001	0.620	0.007	0.734	0.006

Fs, F statistic, *B*, Bartlett's statistic; *U*, unweighted UniFrac value; *P*, p-value

Seasonal variation in bacterial OTUs

Combined analysis of the winter and summer clone libraries revealed 190 bacterial OTUs (99% sequence identity) in sponges and seawater, corresponding to 13 microbial phyla. Within each host sponge species, similar phylogenetic compositions of

bacteria were observed between seasons (**Table 3**), with differences between seasons typically resulting from shifts in rare bacterial OTUs. For example, *I. fasciculata* hosted a single rare OTU (2.6% of clones) affiliated with *Nitrospira* in winter that was absent from summer clone libraries. In contrast, seawater bacteria exhibited large fluctuations in specific lineages and OTUs. For example, cyanobacterial OTUs accounted for only 1.4% of seawater clones in winter and over 1/4 of clones (27.6%) in summer (**Table 3**). Similarly, rank-abundance plots of bacterial OTUs revealed that dominant sponge symbionts were stable across seasons and rare OTUs were more variable, whereas shifts in dominant and rare seawater bacteria were observed between the winter and summer seasons (Supplemental Information; **Fig. S3**).

Clone libraries also revealed the presence of dominant symbiont OTUs in the three sponges species. Overall, eight symbiont OTUs comprised over one-half of all *Ircinia*-associated bacterial clones (51.7%) and were absent from ambient seawater (**Table 4**). Seven of the eight dominant OTUs were recovered from both winter and summer seasons and matched closely (> 98% sequence identity) to other sponge-associated bacteria. The exception was a *Gammaproteobacterium* (IRC012) present only in the winter season and whose closest sequence match was a sediment-derived bacterium (**Table 4**). The most dominant *Ircinia*-associated OTU (IRC002) matched to the sponge-specific cyanobacterium, *Candidatus Synechococcus spongiarum* (Usher *et al.* 2004), and represented the most common symbiont in *I. fasciculata* and *I. variabilis*. The second most dominant OTU (IRC001) matched a member of *Deltaproteobacteria* in the order *Desulfovibrionales* and represented the second most common symbiont in all *Ircinia* hosts. An *Acidobacterium* (IRC003) was the third most dominant OTU and represented the most common symbiont in *I. oros*, while also present in *I. variabilis* yet absent in *I. fasciculata*. The remaining four dominant, sponge-specific OTUs were less abundant (< 5% of clones) and corresponded to symbiont taxa affiliated with *Gammaproteobacteria*, *Nitrospira*, and *Cyanobacteria* (**Table 4**).

Table 3. Composition of bacterial communities in *Ircinia* spp. and ambient seawater sampled in winter (March) and summer (September) seasons.

	% of total clones (no. of 99% OTUs)							
	I. fasciculata		***I. variabilis***		***I. oros***		**Seawater**	
Bacterial phylum	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
Proteobacteria	45.5 (16)	30.0 (11)	61.3 (22)	50.7 (18)	56.1 (17)	47.7 (19)	56.2 (28)	43.7 (21)
*Alphaproteobacteria**	6.5 (4)	2.9 (2)	11.3 (6)	-	12.2 (4)	6.8 (5)	30.1 (15)	32.2 (11)
*Betaproteobacteria**	-	-	1.3 (1)	-	-	-	2.7 (2)	-
*Gammaproteobacteria**	20.8 (10)	12.9 (6)	33.8 (11)	32.0 (13)	28.0 (10)	25.0 (10)	23.3 (11)	11.5 (10)
*Deltaproteobacteria**	18.2 (2)	14.3 (3)	15.0 (4)	18.7 (5)	15.9 (3)	15.9 (4)	-	-
Cyanobacteria	32.5 (2)	61.4 (2)	7.5 (1)	21.3 (1)	-	1.1 (1)	1.4 (1)	27.6 (4)
Acidobacteria	5.2 (3)	1.4 (1)	5.0 (3)	10.7 (1)	18.3 (3)	25.0 (5)	-	1.1 (1)
Bacteroidetes	9.1 (3)	2.9 (1)	3.8 (2)	5.3 (2)	3.7 (2)	-	9.6 (7)	8.0 (4)
Chloroflexi	3.9 (3)	1.4 (1)	-	4.0 (2)	4.9 (2)	14.8 (7)	-	-
Actinobacteria	-	-	3.8 (2)	1.3 (1)	3.7 (2)	1.1 (1)	12.3 (3)	5.7 (3)
Nitrospira	2.6 (1)	-	15.0 (1)	4.0 (1)	2.4 (1)	2.3 (2)	-	-
Bacillariophyta	-	-	2.5 (2)	-	9.8 (5)	-	8.2 (4)	1.1 (1)
Verrucomicrobia	-	-	-	-	-	-	1.4 (1)	12.6 (2)
Firmicutes	-	1.4 (1)	-	-	-	5.7 (2)	1.4 (1)	-
Gemmatimonadetes	1.3 (1)	1.4 (1)	1.3 (1)	1.3 (1)	1.2 (1)	2.3 (2)	-	-
Chlorophyta	-	-	-	-	-	-	4.1 (3)	-
Planctomycetes	-	-	-	1.3 (1)	-	-	1.4 (1)	-
Uncertain	-	-	-	-	-	-	4.1 (2)	-

Table 4. Characteristics of dominant symbiont OTUs in *Ircinia* spp.

	No. (%) of total clones[a]						Taxonomic classification (Bayesian probability)		
OTU	***IF***	***IV***	***IO***	**All *Ircinia* spp.**	**SW**	**Source of closest BLAST match (% sequence identity, accession no.)**	**Taxon[b]**	**Lowest Taxonomic Rank**	**Putative Function**
IRC001	18 (12.2)	16 (10.3)	22 (13.0)	56 (11.9)	0	Sponge-associated (99.2, EU495967)	*Deltaproteobacteria* * (79)	O. *Desulfovibrionales* (70)	Sulfate Reduction
IRC002	56 (38.1)	22 (14.2)	0 (0.0)	78 (16.5)	0	Sponge-associated (98.8, GU981862)	*Cyanobacteria* (100)	G. *Synechococcus* (100)	Carbon Fixation
IRC003	0 (0.0)	10 (6.5)	26 (15.3)	36 (7.6)	0	Sponge-associated (98.7, AJ347029)	*Acidobacteria* (100)	Gp10 (100)	NA[c]
IRC004	2 (1.4)	15 (9.7)	0 (0.0)	17 (3.6)	0	Sponge-associated (99.3, EU183762)	*Nitrospira* (100)	G. *Nitrospira* (100)	Nitrite Oxidation
IRC006	2 (1.4)	11 (7.1)	1 (0.6)	14 (3.0)	0	Sponge-associated (98.8, EU495951)	*Gammaproteobacteria** (100)	*Incertae sedis* (68)	NA
IRC007	0 (0.0)	6 (3.9)	10 (5.9)	16 (3.4)	0	Sponge-associated (98.7, GQ163729)	*Gammaproteobacteria* * (100)	O. *Oceanospirillales* (46)	NA
IRC012	4 (2.7)	6 (3.9)	5 (2.9)	15 (3.2)	0	Sediment bacterium (97.4, GQ143791)	*Proteobacteria* (100)	*Incertae sedis* (84)	NA
IRC015	12 (8.2)	0 (0.0)	0 (0.0)	12 (2.5)	0	Sponge-associated (99.3, JN655231)	*Cyanobacteria* (100)	GpIIa (100)	Carbon Fixation

[a] *IF, I. fasciculata*; *IV, I. variabilis*; *IO, I. oros*; SW, seawater
[b] All taxa are phyla except Deltaproteobacteria and Gammaproteobacteria, which are classes.
[c] NA = not available

Comparison of clone library and T-RFLP data revealed high congruency between these techniques and allowed for the identification of most symbiont taxa in the T-RFLP profiles. *In silico* restriction enzyme digestion of clone libraries predicted 71.6% and 95.8% of all peaks in T-RFLP profiles (HaeIII and MspI data, respectively). Empirical T-RFs of the eight dominant OTUs were well represented in sponge symbiont profiles and accounted for 53.0% (± 2.2% standard error [SE], HaeIII data) and 34.2% (± 1.2%, MspI data) of total profile peak areas, while the same T-RF peaks comprised only a small portion of seawater bacteria profiles (7.5% ± 0.8 and 6.9% ± 1.1, HaeIII and MspI data, respectively). Further, these eight dominant symbionts were present in their respective hosts throughout the seasonal cycle (Supplemental Information, **Table S3**), confirming the stability of these symbionts over annual temporal scales and seasonal environmental conditions.

Seasonal variation in chlorophyll *a* content

The photosymbionts-harboring sponges *I. fasciculata* and *I. variabilis* exhibited different average concentrations and temporal variability in chlorophyll *a* content. Chlorophyll *a* levels were higher in *I. fasciculata* compared to *I. variabilis*, consistent with the habitat preferences of *I. fasciculata* (higher irradiance zones) and *I. variabilis* (lower irradiance zones). Differences between species were significant for all months except June (2010 and 2011; **Fig. 5**), which is, notably, the month with the highest average irradiance levels (**Fig. 2**). For both host sponges, significant variation ($P < 0.001$) in chl *a* content was observed across the monitoring period. In *I. variabilis*, this variation was due to a significant decrease in average chl *a* content in September 2010 (83.3 µg/g), whereas the remaining months exhibited similar average values (131.0 to 162.4 µg/g). Seasonal changes in chl *a* content were more pronounced in *I. fasciculata* and inversely related to daylight hours and light intensity (**Fig. 5**), as lower values occurred during the spring and summer months (149.8 to 210.7 µg/g) and higher values during fall and winter (235.1 to 330.2 µg/g).

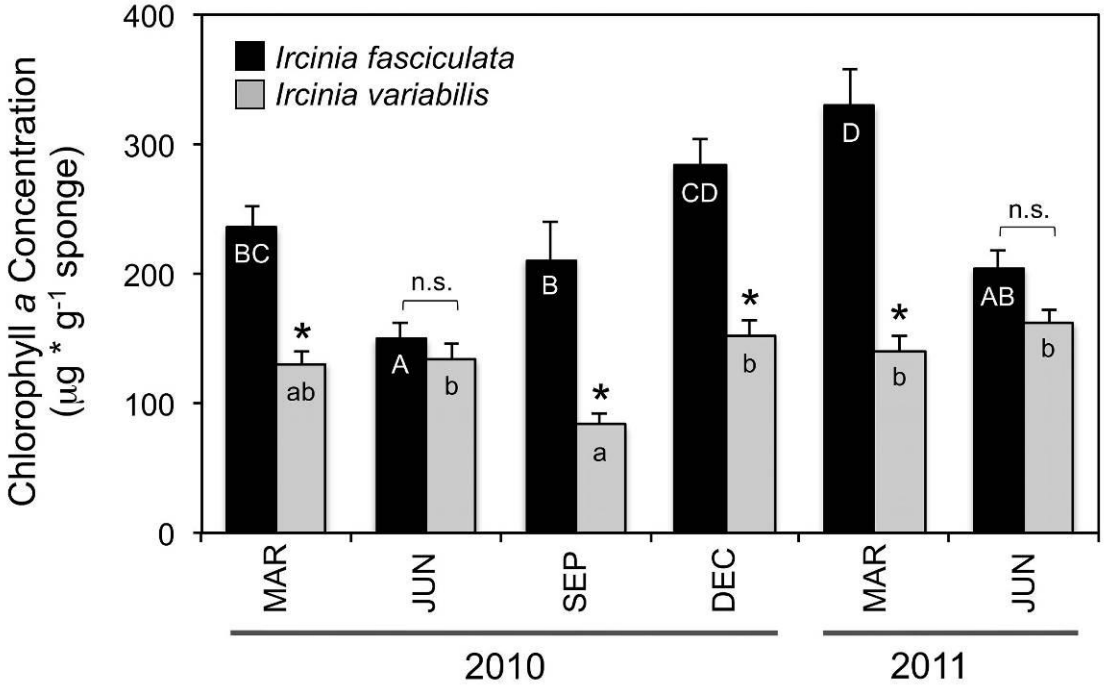

Figure 5. Chlorophyll *a* content of the photosymbiont-bearing sponges *I. fasciculata* (*black bars*) and *I. variabilis* (*gray bars*) over the 1.5-year monitoring period. Asterisks denote significant differences ($P < 0.05$) between host sponge species by month; letters indicate significant differences among months within each host species (uppercase for *I. fasciculata*, lowercase letters for *I. variabilis*). Error bars represent ± 1 SD.

Discussion

Seasonal stability and specificity of sponge microbiota

Temporal monitoring of three *Ircinia* spp. and ambient seawater over 1.5 years revealed remarkable stability and specificity of sponge-associated bacterial symbiont communities, despite large fluctuations in ambient environmental conditions. Across all seasons, each *Ircinia* host maintained a specific bacterial symbiont community, more similar within each host species over time than among hosts. Further, higher symbiont similarity occurred between the microbiota of *I. fasciculata* and *I. variabilis* than with that of *I. oros*, consistent with previous analyses of host-specificity among these species (Erwin *et al.* 2012a). Host-specificity patterns in *Ircinia*-associated bacteria are complex, due to variable levels of symbiont overlap among hosts. Despite the prevalence of generalist symbionts in *Ircinia* microbiotas (i.e., taxa occurring in multiple, unrelated sponge hosts), community level analyses revealed host species-specific symbiont assemblages in each host (Erwin *et al.* 2012a). Here, we show that this phenomenon, termed “a specific mix of generalists”, is maintained over time and

across seasons, with little evidence for symbiont re-structuring or specificity shifts in response to different environmental conditions. The seasonal stability of host-specificity patterns in the *Ircinia* microbiota supports the hypothesis of host-species specific, stable associations between bacteria and marine sponges (Taylor *et al.* 2007; Webster *et al.* 2010; Lee *et al.* 2011; Thacker & Freeman 2012; White *et al.* 2012).

In contrast, seawater bacterial communities exhibited clear temporal shifts in diversity and composition according to a seasonal cycle. Previous studies of surface bacterioplankton in the coastal NW Mediterranean Sea have revealed a similar seasonal succession of seawater bacterial communities (Schauer *et al.* 2000, 2003), including a greater community similarity in the fall and winter seasons as observed here (Anderson *et al.* 2010). Regional stratification of the water column is a seasonal phenomenon in the NW Mediterranean Sea, where restricted upwelling and vertical mixing of nutrient-rich, cold water results in nutrient depletion of surface waters during summer months (Duarte *et al.* 1999). The summer stratification period and its effects on nutrient availability are primary drivers of seasonal microbial dynamics in the Mediterranean Sea (Pinhassi *et al.* 2006). Comparatively, low seasonal dynamics of sponge-associated bacterial community structure suggest that different ecological constraints act on free-living *versus* symbiotic marine bacteria. The effects of nutrient-poor conditions during summer stratification on bacterial communities in the sponge microbiota appear to be limited, supporting the hypothesis of a unique and comparatively stable microbial habitat within the sponge body.

Persistent components of the sponge microbiota

The observed stability of bacterial communities associated with *Ircinia* hosts was driven by the persistent presence of dominant symbiont OTUs. Despite the high diversity of the *Ircinia* microbiota, a small number of symbiont OTUs accounted for the majority of bacteria represented in clone libraries and T-RFLP profiles, similar to what was seen in previous studies of sponge-associated bacteria (Webster *et al.* 2010; Erwin *et al.* 2011). Selective pressures that maintain specific symbiont taxa in the sponge host may result from microbial adaptations to these unique niche microenvironments, as suggested by the presence of unique, vertically transmitted (Schmitt *et al.* 2007) sponge-specific bacterial lineages (Taylor *et al.* 2007; Simister *et al.* 2012a), or the fulfillment of functional roles by particular symbiont guilds that enhance sponge-bacteria holobiont fitness (Thacker 2005; Erwin & Thacker 2008b; Freeman & Thacker 2011). In the latter context, it is noteworthy that several of the dominant symbiont OTUs recovered in *Ircinia* hosts were classified into bacterial lineages with known physiological capabilities, such as photosynthesis (IRC002 and IRC015,

Cyanobacteria), sulfate-reduction (IRC001, *Desulfovibrionales*) and nitrite oxidation (IRC004, *Nitrospira*). The metabolic profile of the sponge microbiome, assessed by both metagenomic (Thomas *et al.* 2010; Liu *et al.* 2012) and nutrient flux (Ribes *et al.* 2012) approaches, has shown diverse and active functional guilds involved in the nutrient cycles of carbon (Thacker & Freeman 2012), nitrogen (Hoffmann *et al.* 2009) and sulfur (Hoffmann *et al.* 2005) that may boost host sponge metabolism and contribute significantly to coastal marine nutrient cycles (Arillo *et al.* 1993; Diaz & Ward 1997; Erwin & Thacker 2007; Jimenez & Ribes 2007). As such, symbiont functionality and its ecological consequences may represent key factors for the selective mechanisms that establish and maintain specific guilds of sponge-associated bacterial symbionts.

Temporal analyses of photosynthetic pigments in *I. fasciculata* and *I. variabilis* provided further insight into symbiont functionality and evidence for seasonal variation in the activity of persistent photosymbiont taxa. *Cyanobacteria* are a key functional guild in the sponge microbiota, capable of photosynthetic carbon assimilation and the transfer of surplus carbon stores to their hosts (Thacker & Freeman 2012). A recent study reported higher photosynthetic activity of cyanobacterial symbionts in *I. fasciculata* than in *I. variabilis*, with differences in symbiont functionality related to ambient irradiance levels in preferred host habitats rather than symbiont composition (Erwin *et al.* 2012b). Here, we show that *I. variabilis* exhibited minimal seasonal fluctuations in chl *a* content, consistent with reduced irradiance levels in the shaded habitats where this species thrives. In contrast, the chl *a* content of photosymbionts in *I. fasciculata* followed a seasonal pattern, with annual minima in summer and peak values in winter, similar to those reported in surface seawater from the NW Mediterranean (Duarte *et al.* 1999; Pinhassi *et al.* 2006; Maldonado *et al.* 2010). Thus, while the factors that determine microbial structure may differ between the sponge niche and open seawater environments (e.g., nutrient levels), some seasonal physiological constraints that dictate microbial function (e.g., irradiance exposure) may be conserved between symbiotic and free-living microbes. Structurally, a single cyanobacterial taxon dominated the symbiotic microbiota in *I. fasciculata* and *I. variabilis* across all seasons; yet functionally, their photosynthetic activity differed among hosts and appears to have a seasonal component in *I. fasciculata*, with potential consequences for host metabolism and growth. The critical ecological link between symbiont structure and function is not well resolved in the sponge microbiota and requires further study, including the potential for seasonal variability in the physiology and functioning of permanent sponge symbionts and its consequence for host metabolism and marine nutrient cycles.

Variable components of the sponge microbiota

Similar to previous studies of temporal variation in the sponge microbiota (Anderson *et al.* 2010), some variability was observed in symbiont communities over time and among individual hosts, though primarily restricted to rare symbiont taxa. Transient components of the sponge microbiota are not unexpected, as microbes recovered from sponge tissue may represent food source bacteria (Pile *et al.* 1996), invasive (Webster *et al.* 2002) or fouling microbes (Lee *et al.* 2006), or simply environmental bacteria present in the sponge filtration system during collection. For example, a common and relatively abundant bacterial OTU (IRC012, 3.2% of sponge clones) was present in the microbiotas of all sponge hosts in winter and absent in the summer. Unlike the majority of sponge-associated bacteria in *Ircinia*, this *Gammaproteobacterium* was not phylogenetically related to other sponge symbionts, but rather matched most closely a sediment-derived sequence. Considering such possible sources of transient microbes in the sponge microbiota, the high degree of bacterial community similarity observed throughout the monitoring period herein is even more extraordinary.

Variability in the composition of bacterial symbionts among conspecific hosts was also detected here by monitoring the same individuals over time, a sampling design rarely utilized to date in the field of sponge microbiology (Anderson *et al.* 2010). Although this variability was minimal compared to differences among host species, some symbionts were consistently recovered from particular individuals and not others. The most notable example is a *Synechocystis*-related cyanobacterium in *Ircinia fasciculata*. A previous report has shown that this cyanobacterium represented a distinct clade of sponge symbiont specific to *I. fasciculata* yet occurred in only one of three *I. fasciculata* individuals studied (Erwin *et al.* 2012b). Here, we report similar findings, with the same *Synechocystis* phylotype recovered in only one of six host individuals, and showed that this association was stable over time, as the cyanobacterium was recovered in winter and summer clones libraries and presented in all symbiont profiles for this particular sponge host. These results show that interindividual variation in the sponge microbiota, often ascribed to the non-specific or transient bacterial associates discussed above, can result from persistent symbionts that occur sporadically among a host population. The implications of interindividual variability in symbiont composition on host ecology and symbiont evolution are unknown for sponge-microbial associations, but have the potential to affect symbiont community function (e.g., photosynthetic activity) and host-symbiont metabolic interactions.

Symbiont fluctuations and thermal thresholds

Recent reports of widespread disease and mass mortality events in *Ircinia* spp. have raised concerns about the future of these sponge populations in the warming Mediterranean Sea. Elevated seawater temperatures are hypothesized to trigger such episodic mortality events, as recurrent disease outbreaks in *I. fasciculata* and *I. variabilis* occurred annually following peak seawater temperatures in summer (Maldonado *et al.* 2010; Stabili *et al.* 2012) and greater disease prevalence has been correlated with the length of exposure to temperatures exceeding threshold values (Cebrian *et al.* 2011). In addition to tissue necrosis, affected sponges also exhibit characteristic changes in their associated microbiota, including the loss of stable symbionts (Cebrian *et al.* 2011) or their replacement by pathogenic microbes (Maldonado *et al.* 2010; Stabili *et al.* 2012). Similar symbiont disruption and proliferation of putatively pathogenic bacteria was reported in a tropical sponge, *Rhopaloeides odorabile*, when exposed to elevated seawater temperatures (Webster *et al.* 2008a), suggesting that symbiont community collapse and host sponge mortality may become widespread as thermal tolerances are exceeded.

A critical question is whether symbiont disruption precedes and precipitates host mortality (e.g., symbiont evacuation followed by colonization of infectious microbes) or simply results from declining host health. In the current study, no sponge mortality events occurred during the monitoring period, consistent with previous surveys of the study area (Cebrian *et al.* 2011), yet deviations from core symbiont communities (i.e., increased heterogeneity) were reported in warmer months, due to fluctuations in rare symbiont taxa within some host individuals. At our monitoring sites, lower temperatures (daily averages > 25°C during only 3 days) were recorded than those that preceded sponge mortality events in other Mediterranean regions (daily averages of 26 to 27°C). Accordingly, no pathogenic lineages (e.g., *Vibrio* spp.) were detected in sponge hosts and the symbiont community shifts observed in our study were minor (i.e., restricted to heterogeneity in rare symbiont's, while dominant symbionts were present throughout) and temporary (i.e., symbiont structure in all sponge hosts reverted to homogeneous core profiles following the 2010 summer season). However, considering the warming trends in the Mediterranean Sea and the proximity of temperature maxima in our study area (25°C) to those preceding sponge mortality events (26 to 27°C), the observed shifts in rare symbiont taxa may represent a precursor to larger symbiont declines and indicate approaching thermal thresholds for Mediterranean sponge-microbe symbioses. Additional monitoring studies and controlled experimentation are required to assess whether elevated seawater temperatures induce shifts in rare symbiont taxa, how these symbiont fluctuations

affect host health, and the utility of symbiont monitoring for predicting sponge mortality events.

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SUPPLEMENTAL INFORMATION

Table S1. Operational taxonomic unit (OTU), isolation source, season and GenBank accession numbers for sponge- and seawater-derived bacterial 16S rRNA gene sequences.

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC001	*Ircinia fasciculata*	Winter	AF10-3-15_C7	JN655221	IRC001	*Ircinia oros*	Summer	TO10-919_C12	JX206619
			AF10-3-7_C11	JN655223				TO10-919_C13	JX206620
			AF10-3-7_C23	JN655236				TO10-919_C16	JX206622
			AF10-3-7_C29	JN655241				TO10-919_C22	JX206627
			AF10-3-7_C8	JN655248				TO10-919_C23	JX206628
			AF10-3-9_C10	JN655251				TO10-919_C4	JX206611
			AF10-3-9_C26	JN655265				TO10-922_C14	JX206650
			AF10-3-9_C27	JN655266				TO10-922_C24	JX206657
			AF10-3-9_C28	JN655267				TO10-922_C8	JX206645
			AF10-3-9_C5	JN655273				TO10-922_C9	JX206646
			AF10-3-9_C7	JN655275				TO10-97_C18	JX206594
			AF10-3-9_C9	JN655276		*Ircinia variabilis*	Winter	TV10-3-12_C14	JN655410
		Summer	AF10-915_C2	JX206520				TV10-3-2_C1	JN655430
			AF10-915_C23	JX206537				TV10-3-2_C2	JN655440
			AF10-915_C26	JX206540				TV10-3-2_C20	JN655441
			AF10-915_C29	JX206543				TV10-3-2_C7	JN655453
			AF10-97_C5	JX206479				TV10-3-7_C17	JN655463
			AF10-99_C17	JX206508				TV10-3-7_C20	JN655467
	Ircinia oros	Winter	PO10-3-1_C27	JN655346			Summer	TV10-912_C13	JX206724
			PO10-3-18_C10	JN655303				TV10-912_C17	JX206728
			PO10-3-18_C11	JN655304				TV10-912_C22	JX206732
			PO10-3-18_C14	JN655307				TV10-912_C27	JX206736
			PO10-3-18_C15	JN655308				TV10-912_C28	JX206737
			PO10-3-18_C2	JN655311				TV10-912_C9	JX206720
			PO10-3-18_C20	JN655312				TV10-92_C22	JX206684
			PO10-3-18_C21	JN655313				TV10-92_C23	JX206685
			PO10-3-7_C14	JN655360				TV10-97_C31	JX206711
			PO10-3-7_C19	JN655365	IRC002	*Ircinia fasciculata*	Winter	AF10-3-15_C10	JN655200
			PO10-3-7_C25	JN655370				AF10-3-15_C11	JN655201

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC002	*Ircinia fasciculata*	Winter	AF10-3-15_C12	JN655202	IRC002	*Ircinia fasciculata*	Summer	AF10-915_C7	JX206525
			AF10-3-15_C13	JN655203				AF10-97_C1	JX206477
			AF10-3-15_C14	JN655204				AF10-97_C11	JX206485
			AF10-3-15_C17	JN655207				AF10-97_C12	JX206486
			AF10-3-15_C2	JN655209				AF10-97_C13	JX206487
			AF10-3-15_C21	JN655211				AF10-97_C15	JX206489
			AF10-3-15_C24	JN655213				AF10-97_C16	JX206490
			AF10-3-15_C3	JN655216				AF10-97_C17	JX206491
			AF10-3-15_C4	JN655218				AF10-97_C18	JX206492
			AF10-3-15_C6	JN655220				AF10-97_C19	JX206493
			AF10-3-7_C20	JN655233				AF10-97_C20	JX206494
			AF10-3-7_C25	JN655238				AF10-97_C23	JX206496
			AF10-3-7_C3	JN655242				AF10-97_C24	JX206497
			AF10-3-7_C30	JN655243				AF10-97_C31	JX206501
			AF10-3-7_C32	JN655244				AF10-97_C4	JX206478
			AF10-3-7_C9	JN655249				AF10-97_C7	JX206481
			AF10-3-9_C16	JN655255				AF10-97_C9	JX206483
			AF10-3-9_C6	JN655274				AF10-99_C1	JX206503
		Summer	AF10-915_C10	JX206527				AF10-99_C19	JX206509
			AF10-915_C12	JX206529				AF10-99_C2	JX206504
			AF10-915_C15	JX206531				AF10-99_C23	JX206512
			AF10-915_C18	JX206533				AF10-99_C28	JX206515
			AF10-915_C19	JX206534				AF10-99_C29	JX206516
			AF10-915_C24	JX206538				AF10-99_C30	JX206517
			AF10-915_C25	JX206539				AF10-99_C32	JX206518
			AF10-915_C3	JX206521		*Ircinia variabilis*	Winter	TV10-3-12_C4	JN655426
			AF10-915_C32	JX206546				TV10-3-2_C13	JN655434
			AF10-915_C4	JX206522				TV10-3-2_C15	JN655436
			AF10-915_C6	JX206524				TV10-3-2_C8	JN655454

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC002	*Ircinia variabilis*	Winter	TV10-3-7_C31	JN655478	IRC003	*Ircinia oros*	Winter	PO10-3-7_C28	JN655373
			TV10-3-7_C8	JN655483			Summer	TO10-919_C27	JX206632
		Summer	TV10-912_C16	JX206727				TO10-919_C30	JX206635
			TV10-92_C14	JX206678				TO10-919_C6	JX206613
			TV10-92_C15	JX206679				TO10-919_C8	JX206615
			TV10-92_C2	JX206667				TO10-922_C13	JX206649
			TV10-92_C24	JX206686				TO10-97_C11	JX206587
			TV10-92_C25	JX206687				TO10-97_C16	JX206592
			TV10-92_C31	JX206692				TO10-97_C17	JX206593
			TV10-92_C32	JX206693				TO10-97_C28	JX206603
			TV10-92_C5	JX206670				TO10-97_C29	JX206604
			TV10-92_C6	JX206671				TO10-97_C3	JX206579
			TV10-92_C8	JX206672				TO10-97_C4	JX206580
			TV10-92_C9	JX206673				TO10-97_C5	JX206581
			TV10-97_C19	JX206701				TO10-97_C8	JX206584
			TV10-97_C26	JX206707		*Ircinia variabilis*	Winter	TV10-3-2_C5	JN655451
			TV10-97_C3	JX206695				TV10-3-7_C18	JN655464
			TV10-97_C30	JX206710			Summer	TV10-912_C23	JX206733
IRC003	*Ircinia oros*	Winter	PO10-3-1_C1	JN655328				TV10-912_C29	JX206738
			PO10-3-1_C13	JN655332				TV10-92_C10	JX206674
			PO10-3-1_C22	JN655341				TV10-92_C17	JX206681
			PO10-3-1_C8	JN655353				TV10-92_C20	JX206682
			PO10-3-1_C9	JN655354				TV10-92_C26	JX206688
			PO10-3-18_C16	JN655309				TV10-92_C30	JX206691
			PO10-3-18_C26	JN655318				TV10-92_C4	JX206669
			PO10-3-18_C3	JN655320	IRC004	*Ircinia fasciculata*	Winter	AF10-3-15_C20	JN655210
			PO10-3-18_C30	JN655321				AF10-3-7_C10	JN655222
			PO10-3-18_C7	JN655325				TV10-3-12_C10	JN655406
			PO10-3-7_C10	JN655356				TV10-3-12_C27	JN655421

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC004			TV10-3-12_C30	JN655424	IRC006	*Ircinia variabilis*	Summer	TV10-912_C5	JX206717
			TV10-3-12_C6	JN655427				TV10-97_C11	JX206698
			TV10-3-2_C10	JN655431				TV10-97_C13	JX206700
			TV10-3-2_C6	JN655452	IRC007	*Ircinia oros*	Winter	PO10-3-18_C18	JN655310
			TV10-3-7_C1	JN655456				PO10-3-18_C32	JN655322
			TV10-3-7_C16	JN655462				PO10-3-18_C8	JN655326
			TV10-3-7_C19	JN655465				PO10-3-7_C22	JN655368
			TV10-3-7_C23	JN655470			Summer	TO10-919_C1	JX206608
			TV10-3-7_C26	JN655472				TO10-919_C29	JX206634
			TV10-3-7_C5	JN655480				TO10-922_C25	JX206658
	Ircinia variabilis	Summer	TV10-912_C4	JX206716				TO10-922_C7	JX206644
			TV10-912_C7	JX206719				TO10-97_C24	JX206599
			TV10-97_C29	JX206709				TO10-97_C7	JX206583
IRC005	*Ircinia oros*	Winter	PO10-3-1_C11	JN655330		*Ircinia variabilis*	Winter	TV10-3-2_C9	JN655455
			PO10-3-1_C15	JN655334				TV10-3-7_C14	JN655461
			PO10-3-7_C16	JN655362				TV10-3-7_C6	JN655481
		Summer	TO10-97_C26	JX206601			Summer	TV10-912_C14	JX206725
			TO10-97_C27	JX206602				TV10-912_C32	JX206740
IRC006	*Ircinia fasciculata*	Winter	AF10-3-15_C22	JN655212				TV10-97_C28	JX206708
		Summer	AF10-99_C21	JX206511	IRC008	Seawater	Winter	PW10-3-I_C11	JN655386
	Ircinia oros	Winter	PO10-3-1_C19	JN655338				PW10-3-I_C15	JN655389
	Ircinia variabilis	Winter	TV10-3-12_C1	JN655405				TW10-3-I_C2	JN655495
			TV10-3-12_C12	JN655408				TW10-3-I_C5	JN655507
			TV10-3-12_C16	JN655412				TW10-3-I_C6	JN655508
			TV10-3-12_C18	JN655413	IRC009	*Ircinia oros*	Winter	PO10-3-1_C16	JN655335
			TV10-3-12_C25	JN655419				PO10-3-1_C23	JN655342
			TV10-3-12_C26	JN655420				PO10-3-1_C25	JN655344
			TV10-3-12_C28	JN655422				PO10-3-18_C9	JN655327
			TV10-3-12_C29	JN655423				PO10-3-7_C17	JN655363

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC009	*Ircinia oros*	Winter	PO10-3-7_C9	JN655383	IRC013	Seawater	Winter	TW10-3-I_C16	JN655492
		Summer	TO10-919_C2	JX206609				TW10-3-I_C22	JN655498
			TO10-919_C31	JX206636				TW10-3-I_C29	JN655502
	Ircinia variabilis	Winter	TV10-3-2_C21	JN655442			Summer	AW10-9I_C28	JX206573
			TV10-3-7_C29	JN655475				AW10-9I_C5	JX206551
IRC010	*Ircinia variabilis*	Winter	TV10-3-2_C18	JN655439				TW10-9I_C10	JX206749
	Seawater	Winter	AW10-3-I_C13	JN655281				TW10-9I_C11	JX206750
			AW10-3-I_C15	JN655283				TW10-9II_C19	JX206784
			TW10-3-I_C19	JN655494				TW10-9II_C20	JX206785
IRC011	*Ircinia fasciculata*	Winter	AF10-3-15_C16	JN655206	IRC014	*Ircinia fasciculata*	Winter	AF10-3-7_C16	JN655228
	Ircinia variabilis	Winter	TV10-3-12_C13	JN655409				AF10-3-9_C31	JN655270
IRC012	*Ircinia fasciculata*	Winter	AF10-3-7_C2	JN655232				AF10-3-9_C4	JN655272
			AF10-3-7_C6	JN655247		*Ircinia fasciculata*	Summer	AF10-915_C27	JX206541
			AF10-3-9_C19	JN655258				AF10-915_C31	JX206545
			AF10-3-9_C32	JN655271		*Ircinia variabilis*	Winter	TV10-3-12_C20	JN655416
	Ircinia oros	Winter	PO10-3-1_C14	JN655333				TV10-3-12_C22	JN655417
			PO10-3-1_C18	JN655337		*Ircinia variabilis*	Summer	TV10-912_C21	JX206731
			PO10-3-1_C3	JN655348				TV10-97_C20	JX206702
			PO10-3-18_C13	JN655306	IRC015	*Ircinia fasciculata*	Winter	AF10-3-7_C18	JN655230
			PO10-3-7_C21	JN655367				AF10-3-7_C19	JN655231
	Ircinia variabilis	Winter	TV10-3-12_C23	JN655418				AF10-3-7_C21	JN655234
			TV10-3-2_C17	JN655438				AF10-3-7_C27	JN655239
			TV10-3-7_C27	JN655473				AF10-3-7_C4	JN655245
			TV10-3-7_C3	JN655476			Summer	AF10-97_C10	JX206484
			TV10-3-7_C30	JN655477				AF10-97_C14	JX206488
			TV10-3-7_C7	JN655482				AF10-97_C21	JX206495
IRC013	Seawater	Winter	AW10-3-I_C19	JN655286				AF10-97_C28	JX206499
			AW10-3-I_C24	JN655292				AF10-97_C30	JX206500
			TW10-3-I_C14	JN655491				AF10-97_C32	JX206502

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC015	*Ircinia fasciculata*	Summer	AF10-97_C8	JX206482	iRC019	*Ircinia variabilis*	Winter	TV10-3-7_C21	JN655468
IRC016	*Ircinia oros*	Winter	PO10-3-18_C25	JN655317			Summer	TV10-912_C11	JX206722
			PO10-3-18_C5	JN655323	IRC020	*Ircinia oros*	Summer	TO10-97_C20	JX206595
	Ircinia oros	Summer	TO10-922_C1	JX206638		*Ircinia variabilis*	Winter	TV10-3-12_C19	JN655414
			TO10-97_C6	JX206582				TV10-3-7_C22	JN655469
	Ircinia variabilis	Winter	TV10-3-7_C11	JN655458				TV10-3-7_C24	JN655471
			TV10-3-7_C28	JN655474	IRC021	*Ircinia oros*	Winter	PO10-3-1_C17	JN655336
IRC017	Seawater	Winter	AW10-3-I_C2	JN655287				PO10-3-1_C4	JN655351
			AW10-3-I_C22	JN655290				PO10-3-7_C2	JN655366
			TW10-3-I_C13	JN655490	IRC022	*Ircinia fasciculata*	Winter	AF10-3-15_C15	JN655205
			TW10-3-I_C7	JN655509				AF10-3-7_C17	JN655229
		Summer	AW10-9I_C7	JX206553	IRC023	*Ircinia oros*	Winter	PO10-3-18_C12	JN655305
			TW10-9II_C17	JX206782				PO10-3-18_C6	JN655324
			TW10-9II_C6	JX206772	IRC024	*Ircinia fasciculata*	Winter	AF10-3-9_C17	JN655256
IRC018	*Ircinia fasciculata*	Winter	AF10-3-15_C19	JN655208		*Ircinia variabilis*	Winter	TV10-3-2_C22	JN655443
			AF10-3-9_C3	JN655269	IRC025	*Ircinia fasciculata*	Winter	AF10-3-9_C13	JN655252
	Ircinia oros	Summer	TO10-919_C10	JX206617				AF10-3-9_C21	JN655261
	Ircinia variabilis	Winter	TV10-3-12_C2	JN655415	IRC026	*Ircinia oros*	Winter	PO10-3-7_C13	JN655359
			TV10-3-2_C28	JN655446				PO10-3-7_C26	JN655371
	Ircinia variabilis	Summer	TV10-912_C19	JX206729				PO10-3-7_C7	JN655381
IRC019	*Ircinia fasciculata*	Winter	AF10-3-9_C25	JN655264	IRC027	*Ircinia oros*	Winter	PO10-3-7_C11	JN655357
	Ircinia oros	Winter	PO10-3-18_C23	JN655315				PO10-3-7_C5	JN655379
			PO10-3-7_C3	JN655374	IRC028	Seawater	Winter	AW10-3-I_C10	JN655278
	Ircinia oros	Summer	TO10-922_C26	JX206659				AW10-3-I_C26	JN655293
			TO10-922_C3	JX206640		Seawater	Summer	TW10-9II_C30	JX206795
			TO10-922_C4	JX206641	IRC029	Seawater	Winter	PW10-3-I_C18	JN655392
			TO10-97_C13	JX206589				PW10-3-I_C9	JN655404
			TO10-97_C14	JX206590	IRC030	*Ircinia fasciculata*	Winter	AF10-3-9_C2	JN655259
			TO10-97_C22	JX206597				AF10-3-9_C22	JN655262

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC030	*Ircinia fasciculata*	Winter	AF10-915_C21	JX206536	IRC038	*Ircinia fasciculata*	Winter	AF10-3-7_C12	JN655224
IRC031	*Ircinia oros*	Winter	PO10-3-18_C1	JN655302				AF10-3-7_C24	JN655237
			PO10-3-18_C22	JN655314			Summer	AF10-915_C9	JX206526
	Ircinia oros	Summer	TO10-919_C11	JX206618		*Ircinia variabilis*	Winter	TV10-3-2_C11	JN655432
			TO10-922_C29	JX206662	IRC039	*Ircinia variabilis*	Winter	TV10-3-12_C9	JN655429
	Ircinia variabilis	Winter	TV10-3-2_C32	JN655449		Seawater	Winter	TW10-3-I_C4	JN655506
		Summer	TV10-912_C20	JX206730			Summer	TW10-9II_C26	JX206791
			TV10-912_C26	JX206735	IRC040	Seawater	Winter	TW10-3-I_C1	JN655486
			TV10-97_C12	JX206699				TW10-3-I_C9	JN655511
IRC032	*Ircinia oros*	Winter	PO10-3-7_C6	JN655380	IRC041	*Ircinia variabilis*	Winter	TV10-3-2_C31	JN655448
	Ircinia variabilis	Winter	TV10-3-2_C24	JN655444				TV10-3-7_C4	JN655479
			TV10-3-7_C2	JN655466			Summer	TV10-92_C16	JX206680
	Ircinia variabilis	Summer	TV10-97_C2	JX206694	IRC042	Seawater	Winter	TW10-3-I_C25	JN655500
			TV10-97_C21	JX206703				TW10-3-I_C26	JN655501
IRC033	*Ircinia fasciculata*	Winter	AF10-3-9_C18	JN655257	IRC043	*Ircinia oros*	Winter	PO10-3-1_C26	JN655345
			AF10-3-9_C20	JN655260				PO10-3-1_C31	JN655350
IRC034	Seawater	Winter	AW10-3-I_C18	JN655285	IRC044	*Ircinia fasciculata*	Winter	AF10-3-15_C27	JN655215
			AW10-3-I_C21	JN655289				AF10-3-7_C22	JN655235
			PW10-3-I_C21	JN655395	IRC044	*Ircinia variabilis*	Summer	TV10-912_C2	JX206714
			PW10-3-I_C25	JN655399				TV10-92_C11	JX206675
IRC035	*Ircinia oros*	Winter	PO10-3-1_C30	JN655349	IRC045	Seawater	Winter	PW10-3-I_C2	JN655394
			PO10-3-7_C18	JN655364				PW10-3-I_C22	JN655396
IRC036	*Ircinia fasciculata*	Winter	AF10-3-9_C14	JN655253	IRC046	*Ircinia oros*	Winter	PO10-3-1_C21	JN655340
			AF10-3-9_C15	JN655254				PO10-3-1_C29	JN655347
IRC037	*Ircinia oros*	Winter	PO10-3-1_C10	JN655329		*Ircinia variabilis*	Summer	TV10-912_C3	JX206715
			PO10-3-7_C32	JN655377	IRC047	*Ircinia variabilis*	Winter	TV10-3-7_C12	JN655459
		Summer	TO10-922_C28	JX206661				TV10-3-7_C13	JN655460
			TO10-97_C30	JX206605	IRC048	*Ircinia oros*	Winter	PO10-3-7_C27	JN655372
			TO10-97_C31	JX206606				PO10-3-7_C4	JN655378

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC048	*Ircinia oros*	Summer	TO10-922_C17	JX206652	IRC075	Seawater	Winter	AW10-3-I_C8	JN655301
IRC049	*Ircinia fasciculata*	Winter	AF10-3-15_C25	JN655214	IRC076	*Ircinia oros*	Winter	PO10-3-1_C12	JN655331
IRC050	*Ircinia fasciculata*	Winter	AF10-3-15_C30	JN655217	IRC077	*Ircinia oros*	Winter	PO10-3-1_C2	JN655339
IRC051	*Ircinia fasciculata*	Winter	AF10-3-15_C5	JN655219	IRC078	*Ircinia oros*	Winter	PO10-3-1_C24	JN655343
IRC052	*Ircinia fasciculata*	Winter	AF10-3-7_C13	JN655225			Summer	TO10-919_C5	JX206612
IRC053	*Ircinia fasciculata*	Winter	AF10-3-7_C14	JN655226				TO10-97_C10	JX206586
IRC054	*Ircinia fasciculata*	Winter	AF10-3-7_C15	JN655227				TO10-97_C32	JX206607
	Ircinia oros	Summer	TO10-922_C21	JX206656	IRC079	*Ircinia oros*	Winter	PO10-3-1_C6	JN655352
IRC055	*Ircinia fasciculata*	Winter	AF10-3-7_C28	JN655240	IRC080	*Ircinia oros*	Winter	PO10-3-18_C24	JN655316
		Summer	AF10-915_C30	JX206544	IRC081	*Ircinia oros*	Winter	PO10-3-18_C27	JN655319
IRC056	*Ircinia fasciculata*	Winter	AF10-3-7_C5	JN655246	IRC082	*Ircinia fasciculata*	Summer	AF10-97_C6	JX206480
		Summer	AF10-915_C11	JX206528				AF10-99_C20	JX206510
IRC057	*Ircinia fasciculata*	Winter	AF10-3-9_C1	JN655250				AF10-99_C27	JX206514
IRC058	*Ircinia fasciculata*	Winter	AF10-3-9_C24	JN655263		*Ircinia oros*	Winter	PO10-3-7_C1	JN655355
IRC059	*Ircinia fasciculata*	Winter	AF10-3-9_C29	JN655268	IRC083	*Ircinia oros*	Winter	PO10-3-7_C12	JN655358
IRC060	Seawater	Winter	AW10-3-I_C1	JN655277	IRC084	*Ircinia oros*	Winter	PO10-3-7_C15	JN655361
		Summer	TW10-9II_C4	JX206770	IRC085	*Ircinia oros*	Winter	PO10-3-7_C23	JN655369
IRC061	Seawater	Winter	AW10-3-I_C11	JN655279	IRC086	*Ircinia oros*	Winter	PO10-3-7_C30	JN655375
IRC062	Seawater	Winter	AW10-3-I_C12	JN655280	IRC087	*Ircinia oros*	Winter	PO10-3-7_C31	JN655376
IRC063	Seawater	Winter	AW10-3-I_C14	JN655282	IRC088	*Ircinia oros*	Winter	PO10-3-7_C8	JN655382
IRC064	Seawater	Winter	AW10-3-I_C17	JN655284	IRC089	Seawater	Winter	PW10-3-I_C1	JN655384
IRC065	Seawater	Winter	AW10-3-I_C20	JN655288	IRC090	Seawater	Winter	PW10-3-I_C10	JN655385
IRC066	Seawater	Winter	AW10-3-I_C23	JN655291	IRC092	Seawater	Winter	PW10-3-I_C13	JN655387
IRC068	Seawater	Winter	AW10-3-I_C30	JN655294	IRC093	Seawater	Winter	PW10-3-I_C14	JN655388
IRC069	Seawater	Winter	AW10-3-I_C31	JN655295	IRC094	Seawater	Winter	PW10-3-I_C16	JN655390
IRC070	Seawater	Winter	AW10-3-I_C32	JN655296	IRC095	Seawater	Winter	PW10-3-I_C17	JN655391
IRC071	Seawater	Winter	AW10-3-I_C4	JN655297	IRC096	Seawater	Winter	PW10-3-I_C19	JN655393
IRC072	Seawater	Winter	AW10-3-I_C5	JN655298	IRC097	Seawater	Winter	PW10-3-I_C23	JN655397
IRC073	Seawater	Winter	AW10-3-I_C6	JN655299	IRC098	Seawater	Winter	PW10-3-I_C24	JN655398
IRC074	Seawater	Winter	AW10-3-I_C7	JN655300	IRC099	Seawater	Winter	PW10-3-I_C27	JN655400

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC100	Seawater	Winter	PW10-3-I_C3	JN655401	IRC118	Seawater	Winter	TW10-3-I_C17	JN655493
IRC101	Seawater	Winter	PW10-3-I_C30	JN655402			Summer	AW10-9I_C29	JX206574
IRC102	Seawater	Winter	PW10-3-I_C31	JN655403	IRC119	Seawater	Winter	TW10-3-I_C20	JN655496
IRC103	*Ircinia variabilis*	Winter	TV10-3-12_C11	JN655407	IRC120	Seawater	Winter	TW10-3-I_C21	JN655497
IRC104	*Ircinia variabilis*	Winter	TV10-3-12_C15	JN655411	IRC121	Seawater	Winter	TW10-3-1_C23	JN655485
		Summer	TV10-92_C29	JX206690			Summer	TW10-9II_C8	JX206774
IRC105	*Ircinia variabilis*	Winter	TV10-3-12_C32	JN655425	IRC122	Seawater	Winter	TW10-3-I_C24	JN655499
IRC106	*Ircinia variabilis*	Winter	TV10-3-12_C8	JN655428	IRC123	Seawater	Winter	TW10-3-I_C3	JN655503
IRC107	*Ircinia variabilis*	Winter	TV10-3-2_C12	JN655433	IRC124	Seawater	Winter	TW10-3-I_C30	JN655504
IRC108	*Ircinia variabilis*	Winter	TV10-3-2_C14	JN655435	IRC125	Seawater	Winter	TW10-3-I_C31	JN655505
IRC109	*Ircinia oros*	Summer	TO10-922_C10	JX206647	IRC126	Seawater	Winter	TW10-3-I_C8	JN655510
			TO10-922_C20	JX206655	IRC127	Seawater	Summer	AW10-9I_C12	JX206558
			TO10-922_C31	JX206664				AW10-9I_C13	JX206559
	Ircinia variabilis	Winter	TV10-3-2_C16	JN655437				AW10-9I_C16	JX206562
IRC110	*Ircinia oros*	Summer	TO10-97_C15	JX206591				AW10-9I_C22	JX206567
	Ircinia variabilis	Winter	TV10-3-2_C26	JN655445				AW10-9I_C6	JX206552
IRC111	*Ircinia variabilis*	Winter	TV10-3-2_C29	JN655447				TW10-9I_C12	JX206751
IRC112	*Ircinia oros*	Summer	TO10-919_C15	JX206621				TW10-9I_C18	JX206756
	Ircinia variabilis	Winter	TV10-3-2_C4	JN655450				TW10-9I_C4	JX206744
		Summer	TV10-912_C31	JX206739				TW10-9II_C16	JX206781
IRC113	*Ircinia variabilis*	Winter	TV10-3-7_C10	JN655457				TW10-9II_C2	JX206769
IRC114	*Ircinia fasciculata*	Summer	AF10-915_C1	JX206519	IRC128	Seawater	Summer	AW10-9I_C27	JX206572
			AF10-915_C28	JX206542				TW10-9I_C1	JX206741
			AF10-915_C5	JX206523				TW10-9I_C21	JX206759
	Ircinia variabilis	Winter	TV10-3-7_C9	JN655484				TW10-9I_C24	JX206761
		Summer	TV10-912_C12	JX206723				TW10-9I_C6	JX206746
IRC115	Seawater	Winter	TW10-3-I_C10	JN655487				TW10-9II_C10	JX206776
IRC116	Seawater	Winter	TW10-3-I_C11	JN655488				TW10-9II_C21	JX206786
IRC117	Seawater	Winter	TW10-3-I_C12	JN655489				TW10-9II_C22	JX206787

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC128	Seawater	Summer	TW10-9II_C28	JX206793	IRC132	*Ircinia variabilis*	Summer	TV10-92_C27	JX206689
			TW10-9II_C9	JX206775	IRC133	*Ircinia oros*	Summer	TO10-919_C24	JX206629
IRC129	*Ircinia oros*	Summer	TO10-97_C23	JX206598				TO10-922_C27	JX206660
	Seawater	Summer	AW10-9I_C18	JX206564				TO10-922_C5	JX206642
			AW10-9I_C25	JX206570				TO10-922_C6	JX206643
			AW10-9I_C26	JX206571	IRC134	Seawater	Summer	AW10-9I_C3	JX206549
			TW10-9I_C13	JX206752				AW10-9I_C8	JX206554
			TW10-9I_C2	JX206742				TW10-9I_C30	JX206765
			TW10-9II_C25	JX206790				TW10-9I_C32	JX206767
			TW10-9II_C29	JX206794	IRC135	*Ircinia variabilis*	Summer	TV10-92_C13	JX206677
IRC130	Seawater	Summer	AW10-9I_C30	JX206575				TV10-92_C21	JX206683
			AW10-9I_C31	JX206576				TV10-92_C3	JX206668
			TW10-9I_C28	JX206764	IRC136	Seawater	Summer	AW10-9I_C15	JX206561
			TW10-9I_C3	JX206743				TW10-9I_C17	JX206755
			TW10-9I_C31	JX206766				TW10-9I_C8	JX206747
			TW10-9I_C5	JX206745	IRC137	*Ircinia oros*	Summer	TO10-919_C18	JX206624
			TW10-9II_C24	JX206789				TO10-919_C25	JX206630
			TW10-9II_C7	JX206773				TO10-97_C21	JX206596
IRC131	Seawater	Summer	AW10-9I_C1	JX206547	IRC138	*Ircinia fasciculata*	Summer	AF10-915_C16	JX206532
			AW10-9I_C14	JX206560				AF10-915_C20	JX206535
			AW10-9I_C24	JX206569	IRC139	Seawater	Summer	TW10-9I_C19	JX206757
			TW10-9I_C15	JX206753				TW10-9I_C20	JX206758
			TW10-9I_C16	JX206754	IRC140	*Ircinia variabilis*	Summer	TV10-912_C6	JX206718
			TW10-9I_C25	JX206762				TV10-97_C4	JX206696
			TW10-9II_C23	JX206788	IRC141	*Ircinia oros*	Summer	TO10-919_C28	JX206633
			TW10-9II_C31	JX206796				TO10-922_C15	JX206651
IRC132	*Ircinia fasciculata*	Summer	AF10-915_C14	JX206530	IRC142	*Ircinia variabilis*	Summer	TV10-97_C25	JX206706
	Ircinia variabilis	Summer	TV10-912_C10	JX206721				TV10-97_C6	JX206697
			TV10-912_C15	JX206726	IRC143	*Ircinia fasciculata*	Summer	AF10-99_C3	JX206505

Table S1 (continued)

OTU	Source	Season	Clone	GenBank Acc	OTU	Source	Season	Clone	GenBank Acc
IRC143	*Ircinia variabilis*	Summer	TV10-97_C22	JX206704	IRC168	*Ircinia oros*	Summer	TO10-922_C19	JX206654
IRC144	*Ircinia fasciculata*	Summer	AF10-97_C25	JX206498	IRC169	*Ircinia oros*	Summer	TO10-922_C2	JX206639
IRC145	*Ircinia fasciculata*	Summer	AF10-99_C15	JX206507	IRC170	*Ircinia oros*	Summer	TO10-922_C30	JX206663
IRC146	*Ircinia fasciculata*	Summer	AF10-99_C25	JX206513	IRC171	*Ircinia oros*	Summer	TO10-922_C32	JX206665
IRC147	*Ircinia fasciculata*	Summer	AF10-99_C8	JX206506	IRC172	*Ircinia oros*	Summer	TO10-97_C1	JX206578
IRC148	Seawater	Summer	AW10-9I_C10	JX206556	IRC173	*Ircinia oros*	Summer	TO10-97_C12	JX206588
IRC149	Seawater	Summer	AW10-9I_C11	JX206557	IRC174	*Ircinia oros*	Summer	TO10-97_C25	JX206600
IRC150	Seawater	Summer	AW10-9I_C17	JX206563	IRC175	*Ircinia oros*	Summer	TO10-97_C9	JX206585
IRC151	Seawater	Summer	AW10-9I_C2	JX206548	IRC176	*Ircinia variabilis*	Summer	TV10-912_C1	JX206713
IRC152	Seawater	Summer	AW10-9I_C20	JX206565	IRC177	*Ircinia variabilis*	Summer	TV10-912_C25	JX206734
IRC153	Seawater	Summer	AW10-9I_C21	JX206566	IRC178	*Ircinia variabilis*	Summer	TV10-92_C1	JX206666
IRC154	Seawater	Summer	AW10-9I_C23	JX206568	IRC179	*Ircinia variabilis*	Summer	TV10-92_C12	JX206676
IRC155	Seawater	Summer	AW10-9I_C32	JX206577	IRC180	*Ircinia variabilis*	Summer	TV10-97_C23	JX206705
IRC156	Seawater	Summer	AW10-9I_C4	JX206550	IRC181	*Ircinia variabilis*	Summer	TV10-97_C32	JX206712
IRC157	Seawater	Summer	AW10-9I_C9	JX206555	IRC182	Seawater	Summer	TW10-9I_C23	JX206760
IRC158	*Ircinia oros*	Summer	TO10-919_C17	JX206623	IRC183	Seawater	Summer	TW10-9I_C26	JX206763
IRC159	*Ircinia oros*	Summer	TO10-919_C19	JX206625	IRC184	Seawater	Summer	TW10-9I_C9	JX206748
IRC160	*Ircinia oros*	Summer	TO10-919_C20	JX206626	IRC185	Seawater	Summer	TW10-9II_C1	JX206768
IRC161	*Ircinia oros*	Summer	TO10-919_C26	JX206631	IRC186	Seawater	Summer	TW10-9II_C12	JX206777
IRC162	*Ircinia oros*	Summer	TO10-919_C3	JX206610	IRC187	Seawater	Summer	TW10-9II_C13	JX206778
IRC163	*Ircinia oros*	Summer	TO10-919_C32	JX206637	IRC188	Seawater	Summer	TW10-9II_C14	JX206779
IRC164	*Ircinia oros*	Summer	TO10-919_C7	JX206614	IRC189	Seawater	Summer	TW10-9II_C15	JX206780
IRC165	*Ircinia oros*	Summer	TO10-919_C9	JX206616	IRC190	Seawater	Summer	TW10-9II_C18	JX206783
IRC166	*Ircinia oros*	Summer	TO10-922_C12	JX206648	IRC191	Seawater	Summer	TW10-9II_C27	JX206792
IRC167	*Ircinia oros*	Summer	TO10-922_C18	JX206653	IRC192	Seawater	Summer	TW10-9II_C5	JX206771

Table S2. Complete results for permutational statistical analyses of bacterial community structure (PERMANOVA, *upper row*) and dispersion (PERMDSIP, *in parenthesis*) within sponge hosts and seawater across the monitoring period (uncorrected *P*-values shown).

	I. fasciculata		***I. variabilis***		***I. oros***		**Seawater**	
Pairwise Comparison	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI
Sequential								
Mar 2010 – Jun 2010	0.026	0.012*	0.061	0.009*	0.003*	0.001*	0.001*	0.001*
	-	(0.011*)	-	(0.002*)	(0.019)	(0.006*)	(0.221)	(0.327)
Jun 2010 – Sep 2010	0.165	0.103	0.102	0.400	0.282	0.086	0.001*	0.001*
	-	-	-	-	-	-	(0.390)	(0.114)
Sep 2010 –Dec 2010	0.083	0.08*	0.053	0.012*	0.021	0.001*	0.001*	0.001*
	-	(0.015*)	-	(0.011*)	-	(0.264)	(0.107)	(0.523)
Dec 2010 – Mar 2011	0.266	0.291	0.268	0.866	0.699	0.409	0.001*	0.001*
	-	-	-	-	-	-	(0.696)	(0.505)
Mar 2011 – Jun 2011	0.163	0.046	0.391	0.604	0.273	0.880	0.001*	0.001*
	-	-	-	-	-	-	(1.00)	(0.105)
Non-Sequential								
Mar 2010 – Sep 2010	0.046	0.015*	0.089	0.007*	0.001*	0.001*	0.001*	0.001*
	-	(0.236)	-	(0.002*)	(0.043)	(0.004*)	(0.099)	(0.090)
Mar 2010 – Dec 2010	0.009*	0.004*	0.360	0.289	0.181	0.001*	0.001*	0.001*
	(0.111)	(0.143)	-	-	-	(0.906)	(0.805)	(1.000)
Mar 2010 – Mar 2011	0.003*	0.004*	0.666	0.331	0.244	0.073	0.001*	0.001*
	(0.085)	(0.009*)	-	-	-	-	(0.813)	(1.000)
Mar 2010 – Jun 2011	0.098	0.042	0.712	0.589	0.049	0.001*	0.001*	0.001*
	-	-	-	-	-	(0.302)	(0.384)	(0.079)
Jun 2010 – Dec 2010	0.055	0.001*	0.048	0.083	0.121	0.002*	0.001*	0.001*
	-	(0.006*)	-	-	-	(0.310)	(0.109)	(1.000)
Jun 2010 – Mar 2011	0.029	0.001*	0.033	0.051	0.09	0.029	0.001*	0.001*
	-	(0.002*)	-	-	-	-	(0.493)	(0.591)
Jun 2010 – Jun 2011	0.158	0.001*	0.100	0.102	0.001*	0.038	0.001*	0.001*
	-	(0.005*)	-	-	(0.003*)	-	(0.496)	(0.102)
Sep 2010 – Mar 2011	0.047	0.001*	0.026	0.001*	0.018	0.001*	0.001*	0.001*
	-	(0.005*)	-	(0.003*)	-	(0.401)	(0.109)	(0.096)
Sep 2010 – Jun 2011	0.115	0.005*	0.073	0.022	0.001*	0.001*	0.001*	0.001*
	-	(0.004*)	-	-	(0.105)	(0.105)	(0.100)	(1.000)
Dec 2010 – Jun 2011	0.337	0.041	0.304	0.829	0.085	0.247	0.001*	0.001*
	-	-	-	-	-	-	(0.277)	(0.201)

*** = Comparison significant following B-Y correction** (Benjamini & Yekutieli 2001)

Table S3. Presence and relative abundance of T-RF signatures from dominant sponge-associated bacteria throughout the monitoring period. Percentage total fluorescence is shown as average values (± SE) per source (sponge and seawater) and sampling time (month).

Source	Time	Enzyme	IRC001	IRC002	IRC003	IRC004	IRC006	IRC007	IRC012	IRC015
Ircinia	Mar	HaeIII	11.22 ±1.07	18.25 ±4.64	4.62 ±0.81	4.53 ±0.60	5.28 ±0.97	n.a.	4.62 ±0.81	1.93 ±1.86
fasciculata	2010	MspI	12.77 ±1.44	9.34 ±3.13	3.02 ±0.90	2.20 ±0.53	1.64 ±1.06	1.01 ±0.38	3.19 ±1.46	2.59 ±2.59
	Jun	HaeIII	4.37 ±0.93	11.98 ±2.92	9.09 ±3.96	3.44 ±0.51	2.57 ±0.89	n.a.	9.09 ±3.96	1.54 ±1.54
	2010	MspI	11.48 ±2.81	7.60 ±3.43	1.00 ±0.36	2.83 ±0.75	2.53 ±1.15	0.28 ±0.19	3.13 ±1.59	2.20 ±2.20
	Sep	HaeIII	8.15 ±1.97	12.92 ±0.89	3.73 ±0.82	1.67 ±0.23	1.71 ±0.37	n.a.	3.73 ±0.82	1.64 ±1.46
	2010	MspI	15.10 ±2.61	15.38 ±3.30	1.95 ±0.32	0.31 ±0.22	1.57 ±0.73	0.32 ±0.21	0.47 ±0.19	n.d.
	Dec	HaeIII	6.98 ±1.02	18.39 ±3.93	2.48 ±0.43	3.53 ±0.65	4.14 ±1.01	n.a.	2.48 ±0.43	0.95 ±0.95
	2010	MspI	7.34 ±0.80	16.80 ±2.56	0.68 ± 0.21	2.55 ±0.58	2.62 ±1.26	0.63 ±0.15	1.29 ±0.51	1.03 ±0.99
	Mar	HaeIII	5.91 ±0.65	12.09 ±1.53	4.02 ±0.44	5.61 ±1.04	4.40 ±0.85	n.a.	4.02 ±0.44	1.86 ±1.82
	2011	MspI	5.85 ±0.99	14.08 ±2.12	0.60 ±0.12	4.03 ±0.87	4.50 ±0.67	0.74 ±0.08	2.65 ±0.73	1.73 ±1.66
	Jun	HaeIII	7.63 ±0.67	14.72 ±3.35	3.06 ±0.37	4.47 ±0.33	2.70 ±0.45	n.a.	3.06 ±0.37	1.68 ±1.68
	2011	MspI	8.12 ±0.72	14.54 ±2.56	1.52 ±0.14	3.05 ±0.58	1.04 ±0.38	0.80 ±0.21	n.d.	n.d.
Ircinia.	Mar	HaeIII	9.80 ±1.76	16.23 ±3.95	10.21 ±1.25	7.79 ±1.36	8.95 ±1.75	n.a.	10.21 ±1.25	n.d.
variabilis	2010	MspI	5.54 ±1.35	13.21 ±2.48	8.93 ±1.37	4.30 ±0.90	6.83 ±2.22	6.47 ±1.65	4.68 ±1.28	0.04 ±0.03
	Jun	HaeIII	3.92 ±0.97	17.17 ±2.48	17.65 ±3.52	4.11 ±1.16	8.70 ±4.01	n.a.	17.65 ±3.52	n.d.
	2010	MspI	4.46 ±1.10	10.70 ±3.67	12.05 ±2.16	1.98 ±0.41	3.34 ±1.45	2.35 ±1.23	4.15 ±1.61	2.34 ±2.14
	Sep	HaeIII	6.40 ±0.96	11.95 ±3.01	23.66 ±6.64	3.17 ±0.65	5.45 ±2.29	n.a.	23.66 ±6.64	n.d.
	2010	MspI	7.49 ±2.44	3.01 ±1.92	16.67 ±3.40	1.66 ±0.44	5.28 ±1.88	2.34 ±1.43	1.20 ±0.76	4.14 ±2.81
	Dec	HaeIII	8.60 ±1.74	10.50 ±3.61	9.71 ±0.91	7.34 ±0.72	11.04 ±2.55	n.a.	9.71 ±7.85	0.02 ±0.02
	2010	MspI	4.62 ±1.16	12.13 ±2.63	9.42 ±0.95	4.16 ±0.46	6.61 ±1.46	3.95 ±1.14	3.02 ±0.90	0.02 ±0.02
	Mar	HaeIII	8.41 ±1.01	17.80 ±2.38	7.85 ±1.32	9.24 ±0.62	10.76 ±1.92	n.a.	7.85 ±1.32	n.d.
	2011	MspI	5.08 ±1.31	17.71 ±2.40	6.28 ±0.75	5.70 ±0.62	4.21 ±2.21	3.84 ±0.89	3.22 ±0.84	0.27 ±0.20
	Jun	HaeIII	11.24 ±0.51	12.31 ±1.59	5.89 ±0.75	6.65 ±0.83	7.26 ±1.57	n.a.	5.89 ±0.75	n.d.
	2011	MspI	7.35 ±1.35	15.89 ±1.23	5.16 ±0.26	3.17 ±0.63	5.02 ±1.86	3.32 ±0.65	1.95 ±0.96	0.02 ±0.02

n.d = not detected, no T-RF fluorescence reading; n.a. =not available, T-RF size larger than sizing standard range

Table S3 (continued)

Source	Time	Enzyme	IRC001	IRC002	IRC003	IRC004	IRC006	IRC007	IRC012	IRC015
Ircinia. oros	Mar 2010	HaeIII	16.20 ±1.34	1.17 ±1.15	10.40 ±2.32	3.66 ±0.72	2.51 ±0.85	n.a.	10.40 ±2.32	n.d.
		MspI	5.13 ±1.05	1.53 ±1.22	6.27 ±1.00	1.94 ±0.38	0.98 ±0.58	7.29 ±1.54	4.75 ±1.08	0.93 ±0.64
	Jun 2010	HaeIII	13.58 ±2.89	n.d.	27.70 ±9.11	3.65 ±2.27	2.66 ±1.62	n.a.	27.70 ±9.11	n.d.
		MspI	2.14 ±0.39	0.10 ±0.09	9.31 ±2.46	1.51 ±0.44	2.20 ±1.08	2.80 ±0.96	8.70 ±2.55	0.28 ±0.26
	Sep 2010	HaeIII	11.37 ±3.25	n.d.	41.36 ±9.45	0.44 ±0.27	0.42 ±0.20	n.a.	41.36 ±9.45	0.21 ±0.19
		MspI	4.66 ±0.97	0.23 ±0.15	16.81 ±2.94	0.35 ±0.16	0.45 ±0.23	2.38 ±0.74	1.26 ±1.15	0.43 ±0.39
	Dec 2010	HaeIII	20.34 ±1.95	0.10 ±0.09	9.48 ±0.92	3.62 ±0.80	2.07 ±0.56	n.a.	9.48 ±0.92	0.12 ±0.11
		MspI	3.24 ±0.80	2.10 ±0.90	5.89 ±1.14	1.76 ±0.50	1.18 ±0.34	6.68 ±0.90	2.20 ±0.56	0.03 ±0.03
	Mar 2011	HaeIII	19.39 ±2.89	n.d.	9.29 ±1.11	6.30 ±0.50	4.07 ±1.10	n.a.	9.29 ±1.11	0.07 ±0.06
		MspI	1.90 ±0.18	1.60 ±0.72	4.59 ±0.54	3.75 ±0.82	1.97 ±1.16	5.97 ±0.69	4.12 ±1.22	1.13 ±0.84
	Jun 2011	HaeIII	16.27 ±3.61	n.d.	9.44 ±1.20	3.44 ±1.14	2.54 ±0.94	n.a.	9.44 ±1.20	0.03 ±0.03
		MspI	2.49 ±0.54	0.28 ±0.28	3.96 ±1.17	3.20 ±0.69	0.20 ±0.13	4.47 ±0.91	3.83 ±1.49	1.11 ±0.81
Seawater	Mar 2010	HaeIII	n.d.	1.18 ±0.25	n.d.	0.34 ±0.04	n.d.	n.a.	n.d.	6.54 ±0.27
		MspI	0.20 ±0.03	8.54 ±1.28	0.34 ±0.03	0.05 ±0.02	n.d.	n.d.	0.70 ±0.07	0.42 ±0.29
	Jun 2010	HaeIII	n.d.	0.84 ±0.33	n.d.	n.d.	n.d.	n.a.	n.d.	1.54 ±0.54
		MspI	n.d.	0.61 ±0.24	n.d.	0.15 ±0.10	n.d.	n.d.	0.61 ±0.21	n.d.
	Sep 2010	HaeIII	n.d.	3.51 ±1.33	3.01 ±0.30	n.d.	n.d.	n.a.	3.01 ±0.30	1.90 ±0.73
		MspI	0.34 ±0.24	1.50 ±0.34	n.d.	n.d.	n.d.	n.d.	0.20 ±0.14	n.d.
	Dec 2010	HaeIII	n.d.	2.38 ±0.39	0.36 ±0.06	1.51 ±0.14	0.11 ±0.08	n.a.	0.36 ± 0.06	5.53 ±0.51
		MspI	0.68 ±0.12	9.83 ±1.42	0.17 ±0.01	0.18 ±0.03	n.d.	0.03 ±0.02	0.14 ±0.10	0.70 ±0.25
	Mar 2011	HaeIII	0.11 ±0.04	1.21 ±0.09	0.05 ±0.03	0.61 ±0.07	0.28 ±0.10	n.a.	0.05 ±0.03	4.19 ±0.47
		MspI	0.98 ±0.11	9.99 ±0.32	0.18 ±0.00	0.05 ±0.02	n.d.	n.d.	0.39 ±0.04	0.12 ±0.08
	Jun 2011	HaeIII	n.d.	0.17 ±0.12	0.06 ±0.04	n.d.	n.d.	n.a.	0.06 ±0.04	6.24 ±0.30
		MspI	0.09 ± 0.03	2.78 ±0.27	0.55 ±0.06	n.d.	n.d.	n.d.	0.91 ±0.35	n.d.

Figure S1. Linear regression analyses showing a negative correlation between T-RF drift and T-RF size (*top*) and positive correlation between empirical T-RF size and predicted T-RF size (*bottom*) used to correct for differential migration rates of labeled primers.

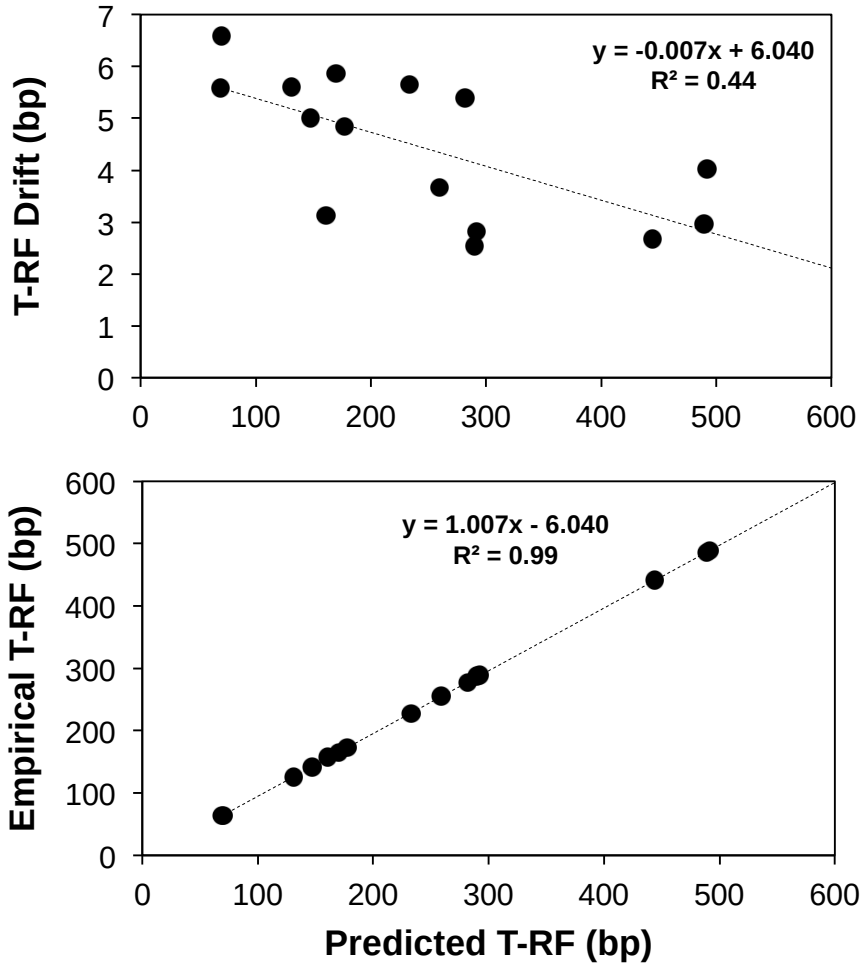


Figure S2. Proportion and specificity of unique bacterial T-RFs in *Ircinia fasciculata* (IF), I. *variabilis* (IV), *I. oros* (IO) and ambient seawater (SW) based on T-RFLP analyses with the restriction enzymes HaeIII (*black bars*) and MspI (*gray bars*).

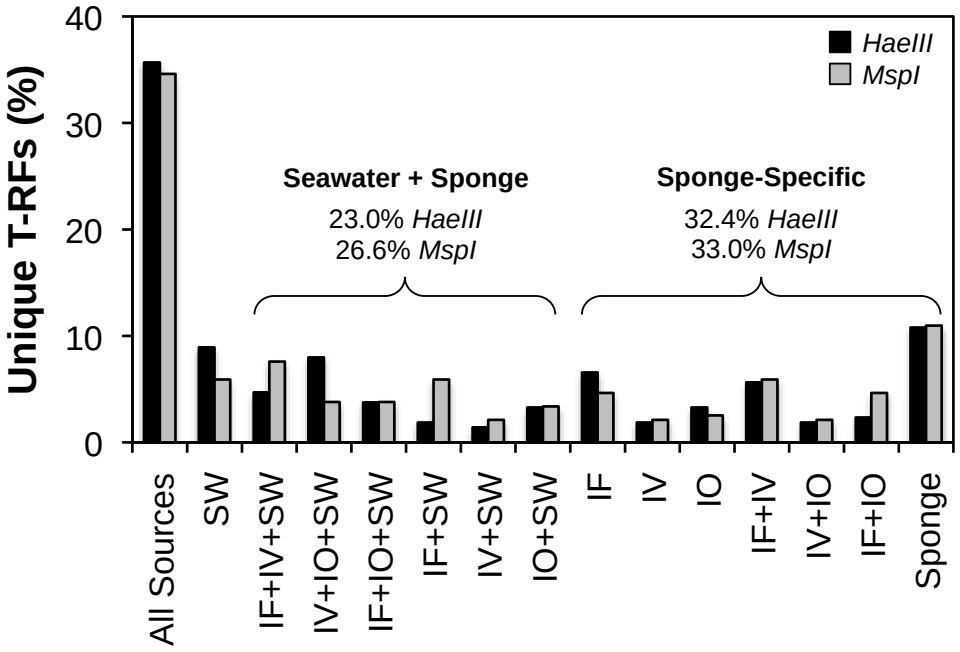
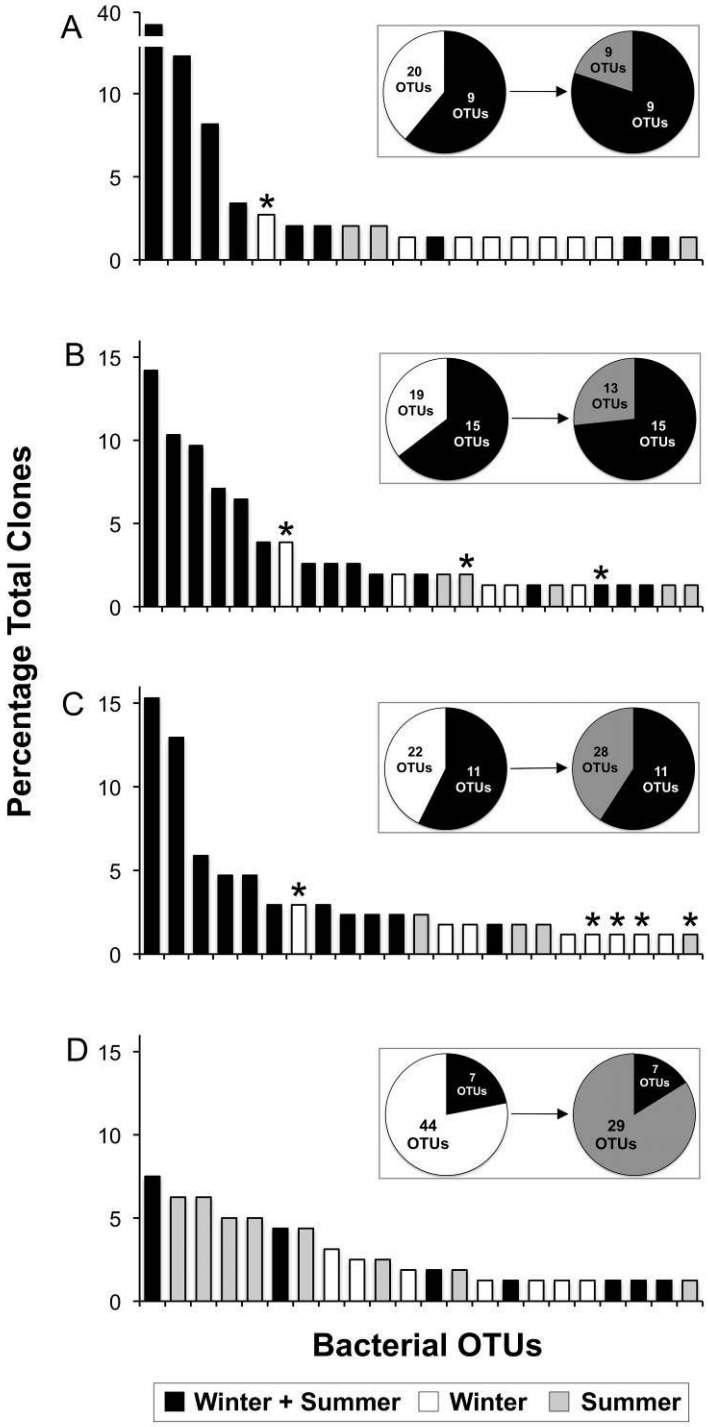


Figure S3. Rank-abundance plots of bacterial OTUs in clone libraries of *Ircinia fasciculata* (**A**)*, I. variabilis* (**B**), *I. oros* (**C**) and ambient seawater (**D**), with singleton OTUs excluded. Bar shading denotes OTUs present during both seasons (*black*), summer only (*gray*) and winter only (*white*); asterisk indicate OTUs matching closest to non-sponge sources. Inset pie charts show entire clone libraries (singleton OTUs included) for winter (*left*) and summer (*right*).



Chapter 4

I. fasciculata in aquaria (Experimental Aquaria Zone, ICM-CSIC)

L. Pita Galán

Chapter 4

Till Death Do Us Part: Stable Sponge-Bacteria Associations under Thermal and Food Shortage Stresses

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Abstract

Sporadic mass mortality events of Mediterranean sponges following periods of anomalously high temperatures or longer than usual stratification of the seawater column (i.e. low food availability) suggest that these animals are sensitive to environmental stresses. The Mediterranean sponges *Ircinia fasciculata* and *I. oros* harbor distinct, species-specific bacterial communities that are highly stable over time and space but little is known about how anomalous environmental conditions affect the structure of the resident bacterial communities. Here, we monitored the bacterial communities in *I. fasciculata* (largely affected by mass mortalities) and *I. oros* (overall unaffected) maintained in aquaria during 3 weeks under 4 treatments that mimicked realistic stress pressures: control conditions (13°C, unfiltered seawater), low food availability (13°C, 0.1 µm-filtered seawater), elevated temperatures (25°C, unfiltered seawater), and a combination of the 2 stressors (25°C, 0.1 µm-filtered seawater). Bacterial community structure was assessed using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene sequences and transmission electron microscopy (TEM). As *I. fasciculata* harbors cyanobacteria, we also measured chlorophyll *a* (chl *a*) levels in this species. Multivariate analysis revealed no significant differences in bacterial T-RFLP profiles among treatments for either host sponge species, indicating no effect of high temperatures and food shortage on symbiont community structure. In *I. fasciculata*, chl *a* content did not significantly differ among treatments although TEM micrographs revealed some cyanobacteria cells undergoing degradation when exposed to both elevated temperature and food shortage conditions.

Arguably, longer-term treatments (months) could have eventually affected bacterial community structure. However, we evidenced no appreciable decay of the symbiotic community in response to medium-term (3 weeks) environmental anomalies purported to cause the recurrent sponge mortality episodes. Thus, changes in symbiont structure are not likely the proximate cause for these reported mortality events.

Hasta que la muerte nos separe: La persistencia de la simbiosis esponja-bacteria frente a estrés térmico y de escasez de alimento

Resumen

Las mortalidades en masa de esponjas registradas en el Mediterráneo tras periodos de temperaturas inusualmente altas y periodos de estratificación más largos de lo normal (i.e., baja disponibilidad de comida) sugieren que estos animales son sensibles a estrés ambiental. Las esponjas mediterráneas *Ircinia fasciculata* e *I. oros* albergan distintas y específicas comunidades de bacterias que son estables a lo largo del tiempo y el espacio pero poco se sabe acerca de cómo condiciones ambientales anómalas afectan la estructura de las comunidades bacterianas simbiontes. Nosotros hemos monitoreado las comunidades de bacterias en *I. fasciculata* (en gran parte afectada por las mortalidades en masa) e *I. oros* (generalmente no afectadas) mantenidas en acuario durante tres semanas bajo cuatro tratamientos que imitan presiones ambientales realistas: condiciones control (13^{o}C, agua sin filtrar), baja disponibilidad de comida (agua filtrada a través de 0.1 µm), temperatura elevada (25^{o}C, agua sin filtrar) y la combinación de sendos estreses (25^{o}C, agua filtrada a través de 0.1 µm). La estructura de las comunidades bacterianas fue determinada a través del polimorphismo de los fragmentos terminales de restricción (T-RFLP) basados en el gen ARNr 16S y microscopía electrónica de tranmisión (TEM). Dado que *I. fasciculata* alberga cianobacterias simbiontes, también medimos los niveles de clorofila *a* (chl *a*) en esta especie. Análisis multivariantes no revelaron ninguna diferencia significativa en los perfiles bacterianos de T-RFLP entre los distintos tratamientos en ninguna de las especies de hospedador estudiadas, indicando que la temperatura elevada y la reducción en la disponibilidad de comida no afectan a la estructura de la comunidad de simbiontes. En *I. fasciculata*, el contenido de chl *a* no varió significativamente entre tratamientos, aunque las micrografías de TEM revelaron algunas células de cianobacterias degradándose cuando los especímenes estuvieron expuestos a la combinación de temperatura elevada y escasez de comida. Se puede decir que tratamientos más largos (meses) podrían finalmente afectar a las comunidades simbiontes. Sin embargo, hemos demostrado que no hay una variación significativa en las comunidades en respuesta a anomalías ambientales a medio término (3 semanas) que podrían ser la causa de los episodios recurrentes de

mortalidad masiva de esponjas. Por tanto, los cambios en la comunidad simbiontes no parecen estar relacionado con las causas de dichas mortalidades.

Introduction

Summer in the Western Mediterranean Sea is getting warmer and longer. Over the past decades, the frequency of seawater temperature anomalies and the period length of stable seawater column (i.e., stratification) have increased (Coma *et al.* 2009; Lejeusne *et al.* 2009; Calvo *et al.* 2011). At the same time and coinciding with years of record temperatures (1-2°C above the mean summer temperature) or prolonged seawater stratification in late summer, mass mortality events were observed for several filter-feeding invertebrates, mainly sponges and cnidarians (Cerrano & Bavestrello 2008; Garrabou *et al.* 2009; Lejeusne *et al.* 2009). A typical summer season in the Mediterranean Sea is characterized by high temperatures (> 18°C) that stratify the seawater column and prevent the upwelling of cooler nutrient-rich water, resulting in nutrient depletion, low turbidity and high irradiance in shallow waters (< 20 m) (Coma *et al.* 2009). Consequently, summer is a energetically-challenging season for filter-feeding invertebrates in the Mediterranean Sea (Coma *et al.* 2000; López-Legentil *et al.* 2013) and together with high temperatures or prolonged stratification, the additional physiological stress that occurs during this season may facilitate the observed episodes of mass mortality (Coma *et al.* 2009).

Marine sponges harbor diverse and host-specific bacterial communities (Taylor *et al.* 2007; Simister *et al.* 2012a) suggesting that the ecology and survival of both the sponge and its bacterial associates are tightly connected; e.g. via nutrient translocation (Weisz *et al.* 2010; Freeman & Thacker 2011). However, despite the potential importance of sponge-bacteria interactions, to date few studies have experimentally assessed the response and stability of these associations under environmental conditions chosen to mimic realistic stress pressures. Most notably, manipulative experiments with the Great Barrier Reef sponge *Rhopaloeides odorabile* showed that the bacterial community associated with this sponge shifted in response to elevated temperatures, high nutrients and pollutants, concomitant with declines in host sponge health (Webster *et al.* 2001; Webster *et al.* 2008a; Simister *et al.* 2012b; Simister *et al.* 2012c). In temperate regions, sponge-derived bacterial communities changed when exposed to elevated temperatures (Lemoine *et al.* 2007) but remained stable under starvation conditions (Friedrich *et al.* 2001). Further studies are needed to investigate the effect of extreme yet realistic environmental conditions on sponge-associated bacterial communities and assess their overall resilience amidst a changing climate.

Sponges in the genus *Ircinia* are ubiquitous in the Western Mediterranean rocky bottoms and harbor a species-specific bacterial community (Erwin *et al.* 2012a) that seems to be adapted to the seasonality of the water column (Erwin *et al.* 2012c).

Recently, *Ircinia* spp. have suffered dramatic episodes of mass mortality linked to extreme summer temperatures (Maldonado *et al.* 2010; Cebrian *et al.* 2011) and the proliferation of an opportunistic *Vibrio*-like bacterium (Maldonado *et al.* 2010; Stabili *et al.* 2012). The factors triggering the proliferation of *Vibrio*-like bacteria in sponge hosts remain unclear, but may be preceded by the disruption of the normal sponge microflora caused by abnormally high seawater temperatures lasting 3 weeks (Cebrian *et al.* 2011). Cebrián *et al.* (2011) observed significant reduction in photosynthetic efficiency in *I. fasciculata* individuals maintained in aquaria at elevated temperatures (27°C for 48 h). Based on these results, the authors suggested that cyanobacteria-harboring sponges such as *I. fasciculata* may be more susceptible to mass mortality events than other sponge species lacking photosymbionts.

In this study, we hypothesized that a high temperature treatment combined with low food availability mimicking an especially hot summer season in the Mediterranean Sea would be accompanied by a shift in the bacterial communities associated with Mediterranean sponges. Based on past studies (Cebrian *et al.* 2011), we expected that sponges harboring photosymbionts would be more susceptible to these shifts than those without them. To test these hypotheses, we performed a series of controlled aquaria experiments for the sympatric sponges *I. fasciculata* (which harbors cyanobacteria and has suffered mass mortality events) and *I. oros* (which does not harbor cyanobacteria, and has remained overall unaffected by mass mortality events). We tested the effect of high seawater temperature (25°C), food shortage (0.1 μm-filtered seawater) and the combination of both treatments on sponge-associated bacterial communities. Bacterial symbiont communities were monitored using terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA gene sequences and transmission electron microscopy (TEM) analyses. We also measured the concentration of chlorophyll *a* (chl *a*) in *I. fasciculata* samples as a proxy for photosymbiont abundance/activity in these hosts.

Material & Methods

Specimen collection

40 individuals of the sponge *Ircinia oros* (Schmidt, 1864) and 40 of *I. fasciculata* (Pallas, 1766) were collected from shallow (< 20 m) rocky reefs in the northwestern Mediterranean Sea (Tossa de Mar, 41°43'13.62" N, 2°56'26.90" E) during January 2011 (*I. oros*) and February 2011 (*I. fasciculata*). Collection during winter months was favored for our experiments because temperatures are more stable during this period (Erwin *et al.* 2012c). Within 2 h, the sponges were transported in insulated coolers from

Tossa de Mar to the Experimental Aquaria Zone (ZAE) located at the Institute of Marine Science (ICM-CSIC) in Barcelona (Spain). *Ircinia* spp. are not endangered or protected by any law and all sampling was conducted outside protected areas following current Spanish regulations (no specific permits were required).

Experimental design

Two experiment sets (one for each sponge species) were conducted in consecutive months, immediately after specimen collection. For each experiment, 40 specimens were placed in separated 2 L aquaria in a flow-through system with direct intake of seawater and an independent supply to each aquarium for a total of 4 weeks. The aquaria were subjected to circadian cycles of 12 h light/12 h dark using artificial light sources. The first week, sponges were maintained at natural (ambient) conditions as an acclimation period. During the following 3 weeks, 4 different treatments were set up (n = 10 individuals per treatment): non-filtered seawater and environmental temperature (control), 0.1 µm-filtered seawater and environmental temperature (FE), non-filtered seawater and hot temperature 25°C (NH), 0.1 µm-filtered seawater and hot temperature (FH). The environmental seawater temperature at the time of the experiments was 13°C. For the heat treatment, the temperature was progressively increased (*ca*. 1.5°C·day^{-1}) during 7 days until reaching 25°C and then maintained at 25°C for the final 2 wk of the experiment. The health status of the sponges was monitored every 2 days by visual inspection for tissue necrosis. Water flux was also controlled every 2 days and readjusted if necessary to obtain a final flux rate through the aquaria of 0.8 L·min^{-1}. Filters were replaced weekly to avoid flux reduction due to particle accumulation.

Experimental sampling

Temperature (°C) and light intensity levels (lx = lumen·m^{-2}) were recorded hourly with Hobo Pendant Temperature/Light Data Loggers (UA-002-64; Onset Computer Corporation). To check for filter efficiency and natural bacterial concentrations in the seawater, 3 samples of water per treatment were collected weekly, before filter replacement. Bacterial concentration was estimated by flow cytometry, based on the method described in Gasol & Giorgio (2000). In short, samples were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde in a phosphate-buffered saline (PBS) solution, incubated in the dark for 10 min, deep frozen in liquid nitrogen and stored at -80°C. For analysis, samples were unfrozen, stained with Syto13 (Molecular Probes) at 5 µM (diluted in dymethil sulfoxyde, DMSO), incubated for 15 min in the dark and run through a GALLIOS flow cytometer with a laser emitting at 480 nm. Bacteria were

detected according to a dot plot of side scatter (SSC, related with cell size) *versus* fluorescent signature (FL1). The number of events (potential bacterial cells) detected by the cytometer was then converted into bacterial cell density (cells·mL^{-1}) by comparing with the events recorded by the machine after injecting a known volume of a solution of 10^6 Syto13-stained beads·mL^{-1}. For each sponge species, the non-parametric Mann-Whitney's U test was used to compare the bacterial cell density in seawater from non-filtered treatments *versus* filtered treatments. Statistical analyses were performed in RStudio (Racine 2012). All cytometry analyses were conducted at the Cytometry Unit of the Scientific and Technological Services of the University of Barcelona.

From all the sponge samples, we randomly selected 3 individuals per treatment that remained healthy throughout the experiment for further analysis (n = 24 per species). Overall, specimens of *I. fasciculata* and *I. oros* remained healthy in all experimental treatments with no tissue necrosis or appreciable biomass loss, except for 1 individual of *I. fasciculata* that died during the acclimation period, and 5 individuals of *I. oros* that died during the first week of experiment (1 from the FE treatment, 3 from the NH, and 1 from the FH). These specimens were not considered in our analysis for several reasons: (i) death was likely due to manipulation rather than to the tested conditions because they all died early during the experiments; (ii) by the end of the experiment, the sponges had been dead for at least two weeks (iii) there were insufficient replicates for robust statistical analysis.

DNA extraction

After the acclimation period (end of week 1) and at the end of the experiments (end of week 4), a tissue sample (*ca.* 2 mm^3) of each selected specimen containing both ectosome and choanosome was preserved in 100% ethanol and stored at -20°C. To characterize the bacterial community in the seawater, 500 mL of water per treatment were filtered through a 0.2 µm filter (Millipore), preserved in 100% ethanol and stored at -20°C. DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen®). Dilutions (1:10) of DNA extracts were used as templates in subsequent PCR amplifications for T-RFLP analysis.

T-RFLP analysis

PCR amplification of 16S rRNA gene sequences was conducted using the universal bacterial forward primer Eco8F (Turner *et al.* 1999), tagged with a 5'-6-carboxyfluorescein (6-FAM) label, and reverse primer 1509R (Martínez-Murcia *et al.* 1995). PCR was performed as follows: one initial denaturation step for 5 min at 94°C;

35 cycles of 1 min at 94°C, 0.5 min at 50°C, 1.5 min at 72°C; and one final elongation step for 5 min at 72°C. Total PCR volume (50 µL) included 10 µM of each primer, 10 nM of each dNTP, 1x Reaction Buffer (Ecogen), 2.5 mM $MgCl_2$ and 5 units of BioTaq™ DNA polymerase (Ecogen). Products from triplicate PCR reactions were pooled and purified from electrophoresis gels using the Qiaquick Gel Extraction kit (Qiagen®), then quantified using the Qubit™ fluorometer and Quant-iT™ dsDNA Assay kit (Invitrogen™) according to manufactures' instructions. Separate enzymatic digestions with HaeIII and MspI were processed as described elsewhere (Pita *et al.* 2013), then analyzed in an automated ABI 3730 Genetic Analyzer (Applied Biosystems) at the Genomics Unit of the Scientific and Technological Services of the University of Barcelona. The lengths of each terminal-restriction fragment (T-RF) were determined against a size standard (600-LIZ) using the PeakScanner™ software (Applied Biosystems). T-RFs smaller than 50 bp or larger than 600 bp were discarded because they were beyond the resolution of the size standard. Background noise was defined by a peak intensity below 50 fluorescence units and by filtering in T-REX (Culman *et al.* 2009) using a cut-off value of 2 standard deviations (Abdo *et al.* 2006). 'True' T-RFs were aligned in T-REX using a clustering threshold of 1 bp and relative T-RF abundance matrices were constructed.

T-RFLP statistical analyses

Samples from each experimental set were analyzed separately to investigate whether the observed response to each treatment depended on sponge species (*I. fasciculata* and *I. oros*). All analyses were based on Bray-Curtis distances calculated from relative abundance matrices, following square root transformation. For each restriction enzyme, non-metric multidimensional scaling (nMDS) plots were constructed to visually compare the bacterial communities. Permutational multivariate analyses of variance (PERMANOVA) (Clarke 1993; Clarke & Gorley 2006) were used to test the effects of source (sponge or seawater) and treatment (control, FE, NH, FH) on bacterial communities. In addition, sponge samples collected after the acclimation period were compared to verify that the specimens harbored similar bacterial communities before experimental treatments were applied. Calculations were performed in PRIMER v6 (Clarke 1993; Clarke & Gorley 2006) and PERMANOVA+ (Plymouth Marine Laboratory, UK). The empirical T-RFs obtained in this study were compared with the available database of *in silico* HaeIII and MspI digestions of 16S rRNA gene sequences derived from the same host sponges in a previous study (Erwin *et al.* 2012c) using the phylogenetic assignment tool PAT (Kent *et al.* 2003).

Transmission electron microscopy (TEM)

At the end of the experiments, a piece of tissue (*ca.* 2 mm^3) from one sponge in each treatment was collected and fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde buffered with filtered seawater and incubated overnight at 4°C. Following fixation, each piece was rinsed at least three times with filtered seawater and stored at 4°C until processed as described previously (López-Legentil *et al.* 2011). TEM observations were made at the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona on a JEOL JEM-1010 (Tokyo, Japan) coupled with a Bioscan 972 camera (Gatan, Germany).

Chlorophyll *a* (chl *a*) concentrations

For chl *a* quantification in *I. fasciculata*, a piece of ectosome was sampled from 5 sponges per treatment at the end of the experiments (n = 20) and processed them using previously described methods (Erwin *et al.* 2012a). *I. oros* was excluded from this analysis because this species lacks photosymbionts (Erwin *et al.* 2012a). One-way ANOVA was performed to test the effect of the factor “treatment” (4 levels; control, FE, NH, FH) on chl *a* concentrations in *I. fasciculata*. The assumptions of the ANOVA were checked by Cramer-von Mises’ normality test and Levene’s homoscedasticity test. Statistical analyses were performed in RStudio (Racine 2012).

Results

Aquaria conditions

Artificial light intensity in the aquaria with *I. fasciculata* samples was 546.7 ± 25.0 lx (mean ± standard error) and in the aquaria with *I. oros* 644.1 ± 8.9 lx. Both light intensity values were in the range of values detected in their natural habitat during winter (Erwin *et al.* 2012c). Environmental water temperature was 13.42 ± 0.01°C and 13.54 ± 0.18°C (mean ± standard error) for the experiment with *I. fasciculata* and with *I. oros*, respectively. For hot temperature treatments, temperature was increased at a rate of 1.49°C·day^{-1} for the aquaria with *I. fasciculata* samples and 1.57°C·day^{-1} for *I. oros* samples during one week, until reaching a final temperature of 25.41 ± 0.01°C and 25.23 ± 0.05°C (mean ± standard error) for the experiment with *I. fasciculata* and with *I. oros*, respectively. The average densities (mean ± standard error) of bacterial cells found in seawater samples from the filtered treatments were (2.4 ± 0.3)·10^4 cells·mL^{-1} in *I. fasciculata* aquaria, and (2.3 ± 0.2)·10^4 cells·mL^{-1} in *I. oros*, while in the unfiltered treatments contained (7.4 ± 1.0)·10^4 cells·mL^{-1} and (6.8 ± 0.5)·10^4 cells·mL^{-1} in aquaria with *I. fasciculata* and *I. oros*, respectively. In spite of the filtering system, bacterial

abundance was only cut by ca. one third. This may relate with decaying filter efficiency with time, in spite of weekly filter changes. Still, the bacterial cell density in seawater samples from non-filtered treatments was statistically higher than in filtered treatments (Mann-Whitney's U, $P < 0.001$) for both *I. fasciculata* and *I. oros* experiments. A one-third reduction in bacterial density is likely a realistic proxy for food shortage conditions in nature.

T-RFLP analysis

A total of 143 unique T-RFs were detected with HaeIII restriction enzyme (101 in *I. fasciculata*, 97 in *I. oros* and 59 in seawater) and 167 with MspI enzyme (117 in *I. fasciculata*, 110 in *I. oros* and 79 in seawater). PERMANOVA analysis of Bray-Curtis similarity matrices from each experiment reported a significant effect of source (sponge *vs* seawater) on the structure of bacterial communities (**Table 1**). No significant differences in bacterial community structure were detected among samples of the same sponge species after the acclimation week ($P > 0.225$, for both enzymes). Likewise, there was not a significant effect of treatment on the bacterial communities of *I. fasciculata* and *I. oros* after 3 weeks (**Table 1**). As the experiment was terminated after 3 weeks, there is no data beyond the duration of the experiments. The lack of structure observed with the nMDS plots further confirmed the similarity of these bacterial communities within host species, despite the different treatments applied (**Fig. 1**). PAT analysis reported that 58.7% (HaeIII) and 71.6% (MspI) of the unique T-RFs obtained in this study for both *I. fasciculata* and *I. oros* matched T-RFs from *in silico* digestions of 16S rRNA sequences from environmental samples of these two species (Erwin *et al.* 2012c).

Table 1. Statistical analysis of T-RFLP profiles to test for an effect of source (seawater *vs* sponge) and treatment on the structure of *Ircinia*-associated bacterial communities.

	I. fasciculata		***I. oros***	
	HaeIII	MspI	HaeIII	MspI
Source (seawater *vs* sponge)	**0.001**	**0.001**	**0.001**	**0.002**
Treatment (control, FE, NH, FH)	0.317	0.328	0.267	0.066

Numbers denote *P*-values from PERMANOVA test after 999 permutations. Significant values at $\alpha = 0.01$ are in bold. Treatments: Control (13^{O}C, unfiltered seawater), FE (13^{O}C, filtered seawater), NH (25^{O}C, non-filtered seawater), FH (25^{O}C, filtered seawater).

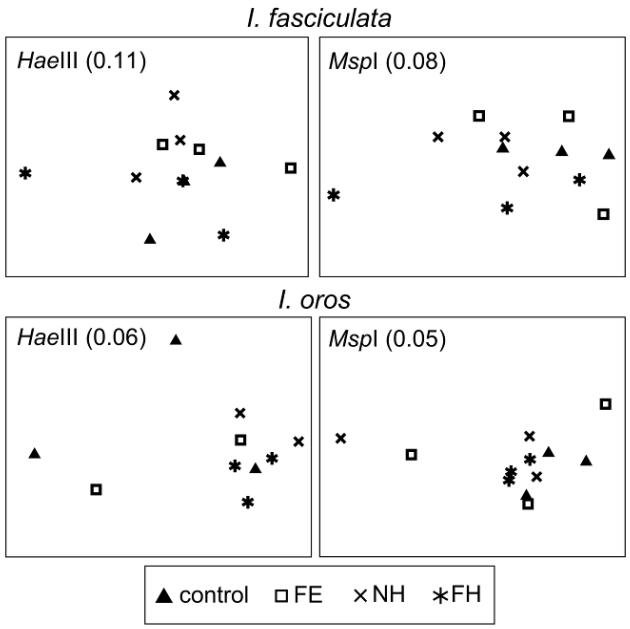

Figure 1. Non-metric multidimensional scaling (nMDS) of sponge-derived bacterial communities at the end of the experiment. Ordination in nMDS plots is based on Bray-Curtis distances between T-RFLP profiles from HaeIII (*left*) and MspI (*right*) digestions of samples of *I. fasciculata* and *I. oros* experiments. Stress values are shown in parenthesis, with values below 0.15 indicating good correlation of similarity matrix distances and ordination in the two-dimension plot. Points are coded by treatment: control (13^{O}C, unfiltered seawater), FE (13^{O}C, filtered seawater), NH (25^{O}C, non-filtered seawater), FH (25^{O}C, filtered seawater).

Transmission electron microscopy

Micrographs of *I. fasciculata* samples from the control treatment showed typical sponge cells with numerous phagosomes and granules of glycogen (**Fig. 2a**). The same sponge cells were observed in all the other treatments. The cyanobacterium *Candidatus 'Synechococcus spongiarum'* dominated the ectosomal tissue of *I. fasciculata* (**Fig. 2b-e**). In the micrographs from the hot temperature (25^{o}C) and filtered seawater treatment (FH), besides healthy cyanobacterial cells, we also observed many cells undergoing degradation (**Fig. 2e-f**). Electron micrographs from *I. oros* samples (**Fig. 3a-d**) showed abundant vacuolated sponge cells surrounded by diverse bacterial morphotypes. No differences in sponge or bacterial cell abundance or morphology were detected for any of the treatments. As expected, no cyanobacterial cells were observed either in this sponge species.

Chlorophyll *a* concentration

Chl *a* levels in *I. fasciculata* at the end of the experiment (3 weeks after acclimation) and for each treatment are depicted in **Fig. 4**. The ANOVA test revealed no significant

differences in chl *a* concentration among treatments (P = 0.4636). The values found here (483.8 ± 20.0 µg·g^{-1} sponge, mean ± standard error) exceeded those observed for this species in the field, where the average concentration reported was 248.1 ± 27.8 µg·g^{-1} sponge (Erwin *et al.* 2012c).

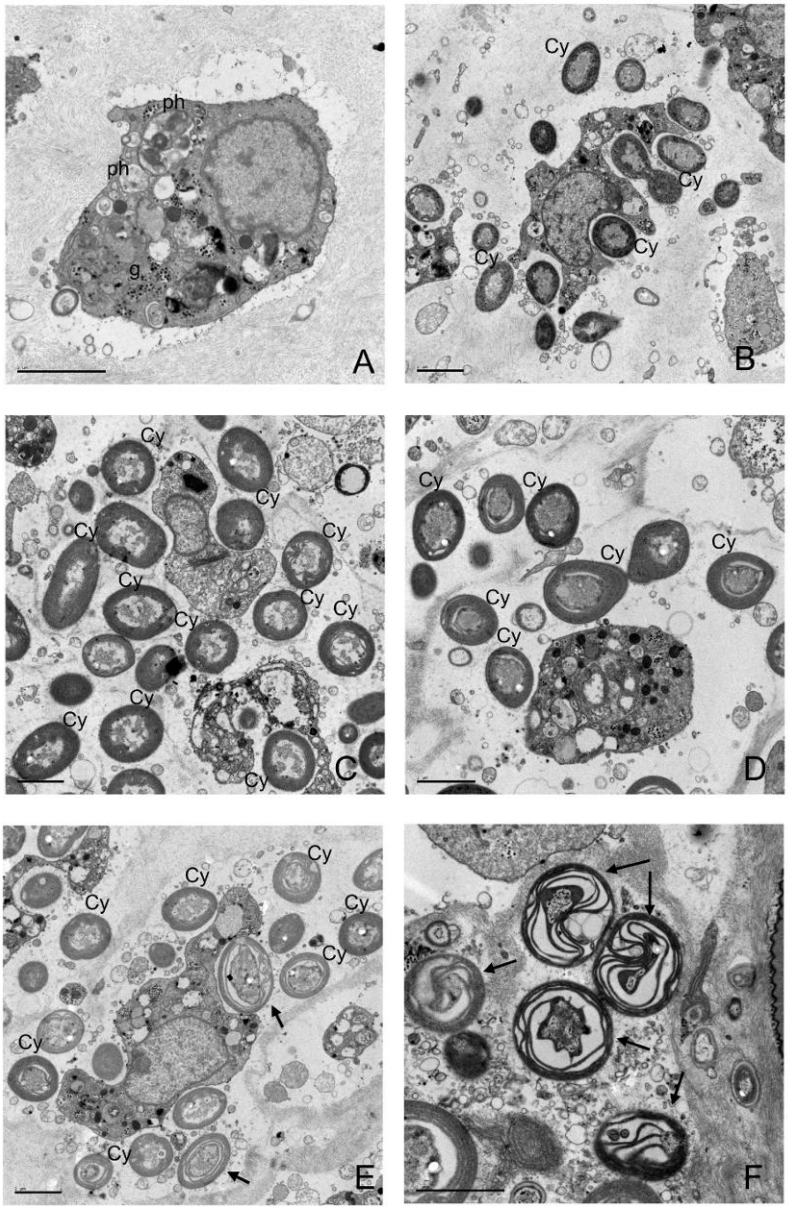

Figure 2. Electron micrographs of *I. fasciculata* bacteria at the end of the experiment. (**A**) Sponge cell in sample from control treatment, containing several phagosomes (ph) and glycogen granules (g). Sponge cells surrounded by multiple *Cyanobacteria* (Cy) and heterotrophic bacteria in the mesohyl of sponges from control treatment (**B**), NH (25°C, non-filtered seawater) treatment (**C**) and FE (13°C, filtered seawater) treatment (**D**). Micrographs of a sponge from FH (25°C, filtered seawater) treatment (**E, F**) showed healthy *Cyanobacteria* (Cy) and *Cyanobacteria* under different stages of degradation (arrows) within the mesohyl. Scale bars represent 2 µm.

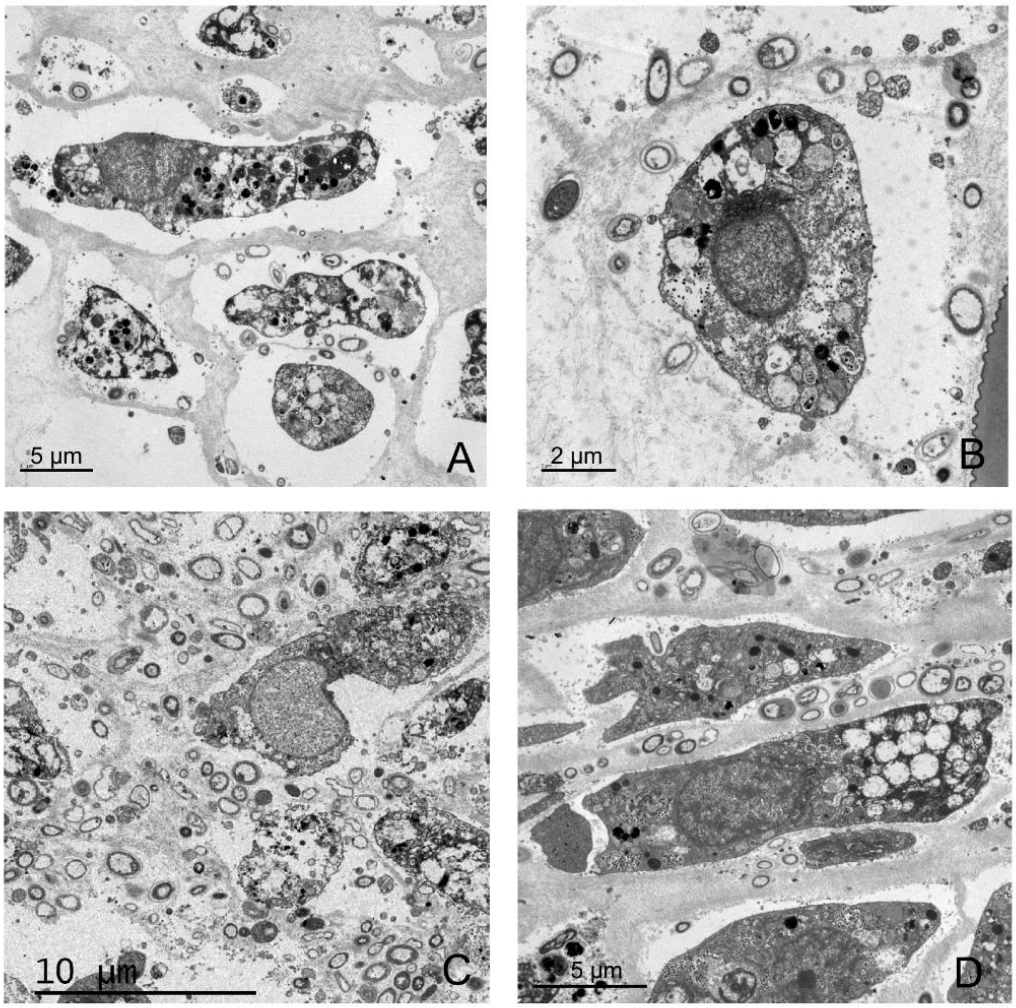

Figure 3. Electron micrographs of *I. oros* bacteria at the end of the experiment. Sponge cells surrounded by numerous bacteria cells of different morphotypes. Samples from control treatment (**A**); 25°C and non-filtered seawater treatment (**B**); 13°C and filtered seawater treatment (**C**); and 25°C and filtered seawater treatment (**D**). Sponge and bacteria cells for all treatments showed no sign of degradation.

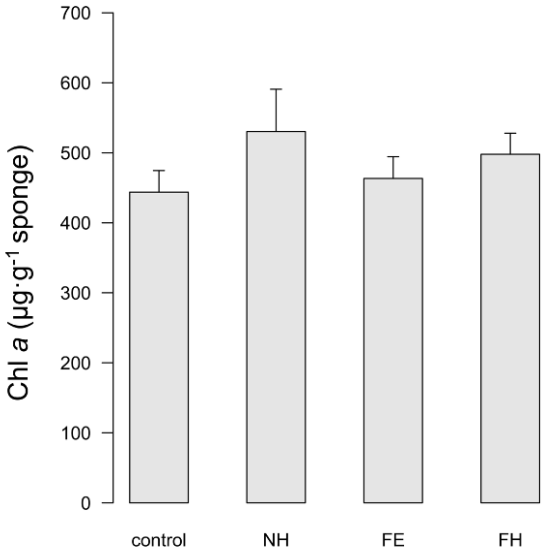

Figure 4. Chlorophyll *a* concentration in *I. fasciculata* for each treatment at the end of the experiment. Control: 13°C and non-filtered seawater; NH: 25°C and non-filtered seawater; FE: 13°C and filtered seawater; FH: 25°C and filtered seawater. Error bars denote standard error.

Discussion

The bacterial communities associated with the Mediterranean sponges *I. fasciculata* and *I. oros* were stable under thermal and food shortage stresses for a period lasting 3 weeks. Comparison of T-RFLP profiles and electron microscopy for each species showed no significant differences among the 4 treatments tested that combined high seawater temperatures (25°C) and low food availability (one-third reduction of the natural bacterial abundance) during three weeks after acclimation. The only noticeable difference consisted of TEM observations of several degraded cyanobacterial cells of *S. spongiarum*, along with healthy looking ones, when *I. fasciculata* specimens were exposed to both thermal and food shortage stresses. However, the presence of degraded cells was not accompanied by a significant decrease in chl *a* concentrations. In fact, chl *a* content was higher in our aquaria samples and for all treatments than what has been observed in the field (Erwin *et al.* 2012c). This increase in chl *a* concentration may be due to a higher density of cyanobacterial cells in the sponge or enhanced photosynthetic activity to compensate for lower ambient irradiance conditions or a poorer diet. Overall, our results indicate that the seawater conditions that characterize anomalously warm summer seasons in the Mediterranean Sea do not affect sponge-associated bacterial communities. Moreover, we did not observe any clear evidence supporting the hypothesis that sponges harboring cyanobacterial symbionts were more vulnerable to the assayed conditions than sponges without them. Other species-specific factors such as habitat-preference or growth dynamics (Turon *et*

al. 2013), alone or in combination, may contribute to the sporadic mass mortality events observed for *I. fasciculata* but not for *I. oros* in the Mediterranean Sea.

One specimen of *I. fasciculata* and 5 of *I. oros* died during the experiments and were excluded from T-RFLP analysis. Necrosis in *I. fasciculata* occurred during the acclimation period and thus was unrelated with the tested treatments. Individual plasticity in resilience to collection and transport or health status at the moment of sampling may have affected the survival of that specimen when moved into aquaria. For *I. oros* sponges, death occurred early during the second week, before the targeted elevated temperature was reached, and sporadically among treatments. Previous studies assaying similar thermal stressors have reported host tissue necrosis and symbiotic cyanobacterial loss in all specimens at elevated seawater temperatures after only 3 to 4 days of treatment (Webster *et al.* 2008a; Simister *et al.* 2012b) . While we cannot be certain of the reason behind the death of these few sponges (i.e. tested treatments or different response to maintenance in aquaria), none of our treatments resulted in mass mortality and the remaining specimens looked healthy through the 3-week experiment.

We cannot disregard that longer-term experiments (months) could result in a significant effect of treatment on bacterial community structure. Stratification of the water column along the Mediterranean coast lasts more than three weeks. Nevertheless, the persistence reported in this study is still remarkable. The high temperature tested here (25°C) represents 3°C more than the summer mean temperature in the study area (Erwin *et al.* 2012c), matched the maximum temperature detected during anomalous summer seasons in years when mass mortality events occurred (Cebrian *et al.* 2011), and represents an increase of > 11°C from ambient conditions at the time of collection. In addition, the time frame of our experiments (3 weeks after acclimation) matched the duration of peaks of temperature in abnormally warm summers (Cebrian *et al.* 2011).

Our results are also in agreement with other studies indicating that sponge-bacteria associations are very stable and able to resist non-lethal stressful conditions. In the Mediterranean sponge *Aplysina aerophoba*, neither food shortage nor antibiotic exposure promoted the consumption of symbionts by the host and the structure of the bacterial community remained unchanged for up to 11 days (Friedrich *et al.* 2001). In the tropical sponge *Rhopaloeides odorabile*, the bacterial community shifted only when sponge tissue necrosis occurred, after exposure to temperatures 2 to 4°C above the mean temperature in the study area (Webster *et al.* 2008a; Simister *et al.* 2012b) . Interestingly, Fan *et al.* (2013) observed that the expression of genes potentially essential for the symbiotic relationship (e.g. proteins involved in cell-cell signaling that

could mediate recognition of symbiont by host) was maintained in partially necrotic sponges although at a lower rate than in healthy ones.

Despite the overall stability of sponge-associated bacteria, cells of dominant cyanobacterium *S. spongiarum* were observed undergoing degradation in *I. fasciculata* sponges exposed to high temperature and food shortage stresses (FH). While not all *S. spongiarum* cells were degrading and chl *a* content did not differ among treatments, the observation of this phenomenon only in the most stressful treatment suggests higher sensitivity of cyanobacteria to these conditions. Previous studies indicated that cyanobacteria-harboring sponges were more vulnerable to elevated temperatures due to photo-oxidative stress (i.e., rising levels of harmful oxygen compounds) derived from temperature-enhanced photosynthesis (Cebrian *et al.* 2011). However, the stability of the symbiotic community and cyanobacterial chl *a* content across treatments observed in this study suggest that the overall photosynthetic activity was not impaired by the degradation of some cyanobacterial cells and that the sponge holobiont is able to resist these conditions for 3 weeks.

The persistence of bacterial symbiont communities despite thermal stress and food shortage conditions lasting 3 weeks is in opposition to one of the predictions of the coral probiotic hypothesis (Reshef *et al.* 2006). According to this hypothesis, the microbial symbionts associated with corals would rapidly shift in response to changing environmental conditions (in days to weeks), thereby conferring an adaptive response to the host. In sponges, it does not seem that rapid changes in bacterial community structures would provide stress tolerance to the host (Simister *et al.* 2012b). Instead, we speculate that, similar to what has been proposed for the human gut microbiome (Bäckhed *et al.* 2005), a persistent symbiotic community in sponges results in constitutive benefits, such as preventing the unexpected proliferation of one or a few bacterial strains within the symbiotic community that yield holobiont death. The empirical demonstration of interactions within the bacterial community and between the bacteria and host that maintain the stability of the symbiotic community under environmental stresses remains a challenge for sponge microbiology.

In conclusion, our experiments for the sympatric sponges *I. fasciculata* and *I. oros* maintained in aquaria mimicking an especially hot summer in the Mediterranean Sea revealed high persistence of sponge-associated bacterial communities. These findings support trends observed in the field showing high symbiont stability across spatial and temporal scales (Erwin *et al.* 2012c; White *et al.* 2012; Turon *et al.* 2013) and also suggest that the disruption of the symbiotic community in response to abnormal thermal and food shortage conditions for a period up to three weeks may not

be the primary cause of the sporadic mass mortality events observed for some *Ircinia* species.

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Discussion & Conclusions

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General Discussion

The aim of this discussion is to integrate all the relevant information from the four different chapters to summarize the diversity patterns we found in sponge bacteria communities, disentangle the underlying processes that shape the symbiotic relationships and debate their response to disturbances. We hypothesized that biogegraphic processes such as dispersal limitation or local selection could generate intraspecific variability on symbiotic bacterial communities over time and space and that perturbations in the surrounding environment would threaten the stability of the sponge-derived bacteria.

This PhD thesis offers a dynamic representation of sponge-bacteria symbiosis from different perspectives (spatial, temporal and in response to stress). We adopted a new approach by investigating the biogeographic patterns and responses to stress in microbial communities associated with closely-related sponges that occurr in sympatry. Our findings contribute to distinguishing environmental *vs* host-related factors that mold HMA sponge-associated microbial communities and provide a baseline to detect abnormal shifts in symbiotic communities, understand the robustness of the symbiosis amidst dramatic changes in their environment, and predict the vulnerability of symbiotic relationships in a rapidly changing world.

Diversity patterns in sponge-associated microbial communities

We assessed the variability of sponge-bacteria associations over spatial and temporal scales. The detection of spatial structure in bacterial symbiotic communities would suggest (1) dispersal limitation of symbionts by dominant currents, or (2) selection of the best adapted community to particular site-specific conditions related with different anthropogenic pressures (i.e., different island size in the Bahamas, protected *vs* unprotected areas in the Mediterranean Sea) or hydrogeographical features (e.g., in the Western Mediterranean Sea, Cabrera presents higher mean temperatures and more oligotrophic waters than coastal and Corsica sampling sites due to dominant currents in the area). As the seawater conditions in the Mediterranean Sea display a marked seasonality in terms of temperature, irradiance and nutrient levels that deeply affect the composition of the free-living bacterioplankton (Pinhassi *et al.* 2006; Alonso-Sáez *et al.* 2007), we also monitored the temporal variability of bacterial communities in Mediterranean *Ircinia* spp.

We found that bacterial communities associated with *Ircinia* sponges are species-specific and persist over space (up to 800 km) and time (despite seasonality in

seawater conditions). We observed that seawater conditions such as temperature, irradiance, currents and anthropogenic pressure provoked significant changes in bacterioplankton communities, but had little effect on sponge-derived communities at temporal and spatial scales. The patterns reported here for *Ircinia* spp. highlight that host species-specific processes mainly govern the composition and structure on HMA sponge symbiotic communities.

Stability of species-specific bacterial communities in sponges

We evidenced the persistence of *Ircinia*-associated communities in two independent studies covering spatial scales ranging 80-400 km in the Bahamas (**chapter 1**) and 80-800 km in the Mediterranean Sea (**chapter 2**). These findings agree with previous studies on sponge-derived bacterial communities at similar geographic scales (Webster *et al.* 2004; Wichels *et al.* 2006; Thiel *et al.* 2007a). Remarkably, we confirmed the pattern observed in Montalvo & Hill (2011), who also minimized the phylogenetic distance among host species (*Xestospongia muta vs X. testudinaria*) and found strikingly similar bacterial communities despite the fact that these sponges inhabit different oceans (Atlantic and Indopacific, respectively). Reveillaud *et al.* (2014) reported the stability of bacterial communities associated with *Hexadella* spp. sponges over bathymetric gradients (shallow and deep waters) and regardless of geographic locations. The overall spatial stability of symbiotic communities in closely-related HMA sponges indicates that site-specific factors (e.g., seawater conditions) and dispersal limitation have little effect on sponge-derived bacterial communities.

Despite the seasonal changes in temperature (differences of 12^{O}C between summer and winter season) and irradiance (*ca.* 5 times higher in summer than in winter; *ca*. 6 h more of daylight in summer), the bacterial communities associated with Mediterranean *Ircinia* spp. were remarkably stable across seasons (**chapter 3**) and constrasted sharply with the significant seasonal shifts in composition and structure of bacterioplankton communities in the surrounding seawater (Schauer *et al.* 2000; 2003; Pinhassi *et al.* 2006; **chapter 3**). The different temporal patterns in sponge-derived *vs* bacterioplankton communities provides strong evidence that different factors structure sponge-associated microbiota compared to free-living bacterial communities.

High dispersal capability of the host's larvae could partially explain the stability of symbiotic community structure, if these symbionts are mostly vertically-transmitted (Ereskovsky *et al.* 2004; Schmitt *et al.* 2007; Lee *et al.* 2009). However, considering the demonstrated lack of correlation found between geographical distance and bacterial community dissimilarity (Schmitt *et al.* 2012; **chapters 1** and **2**) and the possibility of

horizontal acquisition of at least some symbionts (Taylor *et al.* 2007; 2013), it appears that larval dispersal alone is not sufficient to explain the persistence of sponge-bacterial communities over space and time. We propose that host homeostasis and symbiont's metabolism may contribute to foster and maintain stable mesohyl conditions that will in turn act as a buffer against fluctuations in the external environment, thereby preventing marked shifts in sponge-associated bacterial communities.

Our results from 16S rRNA gene sequence clone library analyses confirmed that *Ircinia* spp. from the Bahamas, like those in the Mediterranean Sea (Erwin *et al.* 2012a), harbor a persistent, species-specific bacterial community composed of generalist symbionts closely-related to bacteria found in other sponges and corals (**chapter 1**). Notably, the bacterial communities in the color morphs of *I. felix* were more similar to each other than to those in *I. strobilina*. The phylogenetic affiliation of persistent dominant OTUs in *Ircinia* spp. from the Bahamas and the Mediterranean Sea hints their physiological capabilities, such as photosynthesis (*Cyanobacteria*), nitrite oxidation (*Nitrospira*) or sulfate reduction (*Desulfovibrionales*) (**chapters 1** and **3**). Symbiont functionality and its ecological consequences may also be key for the selective mechanisms that establish and maintain specific guilds of sponge-associated bacterial symbionts (Yang *et al.* 2011).

Intraspecific variability within species-specific bacterial communities in sponges

Species specificity was maintained for both rare and abundant bacterial members of the symbiotic community, yet slight intraspecific variability was occasionally detected (although it was always less than the interspecific variation). In the spatial study done in the Mediterranean Sea (**chapter 2**), we observed certain shifts in the relative abundance of the dominant bacterial members depending on the location, yet the dominance of those members was maintained. In contrast, the seasonal study conducted in the Mediterranean for *Ircinia* spp. (**chapter 3**), revealed interindividual variability due to the occurrence of some bacterial phylotypes in some months and their absence in others; though this fluctuation was always restricted to rare OTUs. Because of the occasional nature of those minor shifts, it is still unkown whether these bacteria are true symbionts or whether they are transient bacteria that represent food, fouling or environmental bacteria (Pile *et al.* 1996; Webster *et al.* 2002; Lee *et al.* 2006).

Interestingly, some bacteria were consistently recovered at each season from some individuals and not others of the same species (**chapter 3**). The persistence of those taxa suggests they are not merely transient associates but true symbionts. This intraspecific variability resulting from persistent symbionts that occur sporadically

among a host population suggests some plasticity in symbiosis establishment. The implications of interindividual variability in symbiont composition on host ecology and symbiont evolution are unknown for sponge-microbial associations, but the activity of those symbionts may bring an advantage in particular new conditions (e.g., Weisz *et al.* 2010).

It is also noteworthy that *I. variabilis* was the species that presented more intraspecific variability in the Mediterranean Sea (**chapters 2**). Similar to what was found by Lee *et al.* (2009a), the different trends observed in *I. variabilis* may be related with specific features of this species, such as a higher degree of morphological plasticity (Turon *et al.* 2013) or a different reproductive strategy. These specific characteristics could influence the establishment of sponge-bacteria associations. Further studies targeting other sympatric closely-related sponge species may help to define which particular species-specific characteristics produce greater variability in bacterial symbiont communities.

The temporal study in Mediterranean species (**chapter 3**) also evidenced the control of host-specific factors, such as habitat preference, on the function of the symbiotic community. In particular, photosymbotic activity (assessed by chlorophyll *a* content) was higher in *I. fasciculata* than in *I. variabilis*. Also, cholorophyll *a* levels in *I. fasciculata* changed seasonally so that the lowest levels were detected in the months of high irradiance and long daylight duration. In contrast, *I. variabilis* generally exhibited annual stability in chl *a* content. Cyanobacterial populations in *I. fasciculata* and *I. variabilis* are composed of *Candidatus Synechococcus spongiarum*, but these populations appeared to respond differently to seasonal changes in seawater conditions. These differences could be related to the different ambient irradiance levels characterizing the habitats favored by each species (i.e., *I. variabilis* thrives in shaded habitats while *I. fasciculata* prefers highly exposed surfaces; Erwin *et al.* 2012a; Turon *et al.* 2013).

Vulnerability of sponge-associated bacterial communities to abnormal perturbations in environmental conditions

In **chapter 4**, we mimicked the stressful conditions that occur in the Western Mediterranean Sea in years of anomalously high temperature and overall longer summer conditions (e.g., longer stratification of the waters). These conditions were previously correlated with episodes of mass mortality of filter-feeding invertebrates (Coma *et al.* 2009; Garrabou *et al.* 2009), including populations of *I. fasciculata* (Cebrian *et al.* 2011). Our results showed that the bacterial communities from the

Mediterranean sponges *I. fasciculata* and *I. oros* were stable despite thermal stress and food shortage conditions in controlled aquaria for a period lasting 3 weeks. Overall, our results indicate that the seawater conditions that characterize anomalously warm summer seasons in the Mediterranean Sea do not significantly affect sponge-associated bacterial communities.

Although we cannot disregard that longer-term experiments (months) could result in a significant effect of treatment on bacterial community structure, the persistence reported in our study is still remarkable. The elevated temperature tested here (25°C) represents 3°C more than the summer mean temperature in the study area (**chapter 3**), matched the maximum temperature detected during anomalous summer seasons (Cebrian *et al.* 2011) and represents an increase of > 11°C from ambient seawater conditions at the time of collection. In addition, the time frame of our experiments (3 weeks after acclimation) matched the duration of peaks of temperature in abnormally warm summers (Cebrian *et al.* 2011).

Studies on sponge-associated community response to stress are scarce but suggest that sponge-bacteria associations are highly persistent until a threshold is surpassed. In the tropical sponge *Rhopaloeides odorabile*, the bacterial community shifted only when sponge tissue necrosis occurred, after exposures to temperatures 2 to 4°C above the mean temperature in the study area (Webster *et al.* 2008a; Simister *et al.* 2012b; 2012c). In the Caribbean, López-Legentil *et al.* (2008) were unable to induce the release of symbiotic cyanobacteria in the giant barrel sponge *Xestospongia muta*, even when sponges were put under temperatures that yielded significant stress to the sponge after a few hours. These studies further confirm that sponge-bacterial associations are extremely stable and persist until death

According to the coral probiotic hypothesis, coral-associated microbes would rapidly shift (days, weeks) as an adaptative response of the holobiont to overcome changing environmental conditions (Reshef *et al.* 2006); but this does not seem to be the case for sponges. The complex microbial community housed in marine sponges, comparable to that found in human gut (Hentschel *et al.* 2012), may confer robustness amidst natural disturbances (e.g., preventing the invasion of opportunist microbes that proliferate in warmer months) but may not be able to help the host survive drastic changes of some environmental parameters such as temperature (Webster *et al.* 2008a; Simister *et al.* 2012b; 2012c). Thus, under a scenario of increasing seawater temperature, acidification and sedimentation (Coma *et al.* 2009; Calvo *et al.* 2011; Crisci *et al.* 2011), the sponge holobiont may be unable to change rapidly enough to surmount the expected drastic and repeated disturbances, and its health may be

ultimately compromised yielding its death (Webster *et al.* 2008a; Simister *et al.* 2012b; Fan *et al.* 2013).

Box 4 | **"Nothing in biology makes sense except in the light of evolution" (Dobzhansky, 1900-1975)**

Although this PhD thesis focused on patterns of sponge-associated bacterial communities over hundreds of km and accross seasons, results can also help shelding some light on the evolution processes that have shaped these symbiotic relationships. First, a less flexible (i.e., species-specific) and highly persistent complex symbiotic community could contribute maintaining host health. For example, it has been proposed for the human gut microbiome that natural selection would have favored symbiotic bacteria that (besides benefitting host fitness or at least being harmless) maintain a metabolic equilibrium and avoid the unexpected proliferation of one or a few bacterial strains that could compromise the health of the holobiont (Bäckhed *et al.* 2005). Second, the intraspecific variability observed for some individuals within a species diversifies the holobiont genetic pool and may bring selective advantages when subjected to new environmental conditions. For instance, some bacteria are known to produce molecules that the host can use for UV protection (Regoli *et al.* 2000), and their presence in some individuals may result in their natural selection under current predictions of higher irradiances.

In conclusion, we investigated the bacterial communities associated with congeneric sponges that occurred in sympatry and showed that host-specific processes, rather than biogeographic factors, are primarily responsible for shaping sponge-derived bacterial communities. Bacterial communities in these sponges were species-specific and persisted over spatial (i.e., hundreds of kilometers) and temporal (i.e., seasonal) scales. Even short-term exposure to elevated seawater temperatures and simulated food limitation conditions did not alter the composition of sponge-associated bacterial communities. Our findings suggest a close and stable HMA sponge-bacterial community association. We speculate that sponge-bacteria symbiosis has probably resulted in a cooperative system where the activity of the community itself as well as the animal's homeostasis contribute to maintain a stable environment in the mesohyl and buffer external disturbances. Under this scenario, the symbioses would have evolved to persist as a unit over different environmental situations (**Box 4**). The threat of habitat degradation and climate change impacts (associated with elevated seawater temperature and extreme seasonality) to sponge diversity entails the loss of their complex symbiotic microbiota and the ecological and biotechnological services they

provide. Our results highlight the importance of considering the holobiont rather than just the sponge when designing future studies on sponge biology, ecology and evolution as well as when deciding on appropriate management strategies to preserve the true biodiversity of these taxa.

Future Perspectives

We have demonstrated that host specific factors mostly determine sponge-derived bacterial communities. In the discussion, we proposed that the sponge mesohyl is a stable habitat that buffers against changes in external seawater conditions. The sponge mesohyl has been defined as an anaerobic, nutrient-rich habitat (Hoffmann *et al.* 2005), but we know little about its physical and chemical conditions. The use of microsensors in experimental studies to record the physical and chemical microenvironmental conditions in the mesohyl would help to define sponge mesohyl microhabitats and their changes under different levels of light or temperature, as published for other marine invertebrates (Kühl *et al.* 2012), or in actively pumping *vs* non-pumping sponges. Also, the application of such techniques to closely-related sponges would allow additional insight into how internal conditions may drive the species-specificity of sponge-bacterial communities.

Although we know more about the factors affecting the *structure* of the bacterial consortia in sponges, studies on the *functional* response of the symbiotic community under different environmental conditions remain limited to few sponge species (e.g., Fan *et al.* 2013) or particular bacterial lineages (e.g., photosymbionts [e.g., this thesis], ammonia oxidizers, [e.g., López-Legentil *et al*. 2010]). Further metagenomic and transcriptomic studies are crucial to understand the role of symbiont metabolism for the holobiont and how similar or different this role is for different sponge species (Liu *et al.* 2011; Thomas *et al.* 2010). In particular, additiona work is needed to assess the response of the holobiont to different environmental conditions (e.g., Fan *et al*. 2013) mimicking current predictions for global climate change and the role of symbionts in holobiont adaptation to these conditions.

Moreover, sponges not only establish symbiotic associations with bacteria. Archaea have also been detected in sponges from different oceans (Steger *et al.* 2008; López-Legentil *et al*. 2010; Turque *et al.* 2010; Radax *et al.* 2012) and are also present in sponge larvae (Schmitt *et al*. 2008), suggesting a tight link between archaeal symbionts and the sponge host. However, the archaeal symbiotic communities in sponges have received less attention than their bacterial counterparts and we know little about their diversity patterns (**Box 5**). In addition, other eukaryotic microorganisms

(e.g., fungi, diatoms and dinoflagellates) are also commonly observed associated with sponges and may also play an important role in holobiont metabolism, yet they are often disregarded in sponge microbiology studies (Hentschel *et al.* 2012). Accordingly, further research in this topic is also needed to obtain a comprehensive view of holobiont complexity and define the role of archaea and microbial eukaryotes in this symbiosis.

Box 5 | **WORK IN PROGRESS: Symbiotic archaea in marine sponges show stability and host specificity in community structure and ammonium oxidation functionality.**

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Abstract. Archaea associated with marine sponges are active and influence the nitrogen metabolism of their hosts. However, we know little about the occurrence, specificity and persistence of this group in marine sponges. We aimed to elucidate the relative importance of host-specific and environmental factors in shaping sponge-associated archaeal communities. We investigated the archaeal communities in sympatric sponges from the Mediterranean Sea (*Ircinia fasciculata* and *I. oros*, sampled in summer and winter) and from the Caribbean (*I. strobilina* and *Mycale laxissima*). Analysis of archaeal 16S rRNA and amoA gene sequences showed that the archaeal community composition and structure was different from those in the seawater and varied between sponge species. The community in *M. laxissima* differed from that in *Ircinia* spp., including the sympatric sponge *I. strobilina*; yet geographical clusters within *Ircinia* spp. were also observed. Whereas archaeal phylotypes in *Ircinia* spp. were persistent and seemed to belong to sponge-specific clusters, archaea in *M. laxissima* were closely related with sequences from diverse habitats (i.e., seawater, sediments). In the four sponge species, the expression of archaeal amoA gene was confirmed. We proposed that host-specific processes, such as host ecological strategy and evolutionary history, rather than surrounding environmental conditions determine sponge-archaeal communities.

This research was mainly conducted during L.P. internship in the group of Russell T. Hill at the Institute of Marine and Environmental Technology from the University of Maryland Center for Environmental Science. This paper has been submitted to FEMS Microbiology Ecology.

Conclusions

CHAPTER 1: Biogeography and host-fidelity of bacterial communities in *Ircinia* spp. from the Bahamas

1. *Ircinia felix* and *I. strobilina* sponges from the Bahamas harbor species-specific bacterial communities, different from those in the surrounding seawater. The symbiont bacterial communities are composed by bacterial phylotypes closely related to bacteria found in other sponge species from different oceans, as reported from T-RFLP and clone libraries of 16S rRNA gene. Bacterial communities in color morphotypes of *I. felix* (white and tan) are more similar to each other than to those in *I. strobilina*
2. Bacterial communities in *Ircinia* spp. in the Bahamas remained stable over locations separated by distances ranging 80-400 km and under different anthropogenic pressure (i.e., islands of different human population densities).
3. Our results show a high degree of symbiont fidelity to host species, suggesting that host-specific rather than biogeographic factors play a primary role in structuring and maintaining sponge-bacteria relationships in *Ircinia* spp. from the Bahamas.

CHAPTER 2: Host rules: spatial stability of bacterial communities associated with marine sponges (*Ircinia* spp.) in the Western Mediterranean Sea

4. Multivariate analysis and nonmetric multidimensional scaling plots of 16S rRNA gene T-RFLP profiles showed that bacterial communities in *Ircinia* sponges were structured by host species and remained stable across sampling locations, despite geographic distances (80-800 km) and different local conditions. Despite the overall stability, slight differentiation among particular locations was observed in *I. variabilis*-derived communities.
5. Host-specicity and spatial stability was observed in both rare and dominant components of T-RFLP profiles within the same sponge species, although the dominant members presented spatial differentiation in terms of relative abundance.
6. No correlation between geographic distance and symbiotic community dissimilarity was consistently detected in any host sponge species.
7. Our results indicate that the structure of bacterial communities and their variability is determined by host species-specific factors.

CHAPTER 3: Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance

8. Bacterial symbionts in *Ircinia* spp. exhibited host species-specific structure and remarkable stability throughout 1.5 year, despite large fluctuations in temperature and irradiance. In contrast, seawater bacteria exhibited clear seasonal shifts in community structure.
9. Variability in the sponge microbiota was restricted to rare symbionts and occurred most prominently in warmer seasons, coincident with elevated thermal regimes.
10. Seasonal stability of the sponge microbiota supports the hypothesis of host-specific, stable associations between bacteria and sponges. Further, the core symbiont profiles revealed in this study provide an empirical baseline for diagnosing abnormal shifts in symbiont communities.

CHAPTER 4: Till death do us part: stable sponge-bacteria associations under thermal and food shortage stresses

11. We detected no significant differences in bacterial T-RFLP profiles within *I. fasciculata* and *I. oros* sponges maintained in aquaria during 3 weeks under 4 treatments that mimicked realistic stress pressures: control conditions (13°C, unfiltered seawater), low food availability (13°C, 0.1 µm-filtered seawater), elevated temperatures (25°C, unfiltered seawater), and a combination of the 2 stressors (25°C, 0.1 µm-filtered seawater).
12. In *I. fasciculata*, chl *a* content did not significantly differ among treatments although TEM micrographs revealed some cyanobacteria cells undergoing degradation when exposed to both elevated temperature and food shortage conditions.
13. Changes in symbiont structure are not likely the proximate cause for recent mortality events reported for *I. fasciculata*, as we evidenced no appreciable decay of the symbiotic community in response to medium-term (3 weeks) environmental anomalies purported to cause the recurrent sponge mortality episodes in filter-feeding invertebrates in the western Mediterranean Sea.

References

Pelecanus conspicillatus (Monkey Mia, Western Australia)
Courtesy of O. Sacristán

References

Abdo Z, Schüette UME, Bent SJ, Williams CJ, Forney LJ *et al.* (2006) Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Environ. Microbiol. 8: 929–938.

Abràmoff MD, Hospitals I, Magalhães PJ & Abràmoff M (2004) Image Processing with ImageJ. Biophotonics Int. 11: 36-42.

Alonso-Sáez L, Balagué V, Sà EL, Sánchez O, González JM *et al.* (2007) Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. FEMS Microbiol. Ecol. 60: 98–112.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment tool. J. Mol. Biol. 215: 403–410.

Anderson M (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26: 32–46.

Anderson M (2006) Distance-based tests for homogeneity of multivariate dispersions. Biometrics 62: 245–253.

Anderson SA, Northcote PT & Page MJ (2010) Spatial and temporal variability of the bacterial community in different chemotypes of the New Zealand marine sponge *Mycale hentscheli*. FEMS Microbiol. Ecol. 72: 328–342.

Arillo A, Bavestrello G, Burlando B & Sarà M (1993) Metabolic integration between symbiotic cyanobacteria and sponges: a possible mechanism. Mar. Biol. 117: 159–162.

Ashelford K, Chuzhanova N, Fry J, Jones A & Weightman A (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl. Environ. Microbiol. 71: 7724–7736.

Ashelford K, Chuzhanova N, Fry J, Jones A & Weightman A (2006) New screening software shows the most recent large 16S rRNA gene clone libraries contain chimeras. Appl. Environ. Microbiol. 72: 5734–5741.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA & Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307: 1915–1920.

Benjamini Y & Yekutieli D (2001) The control of the false discovery rate in multiple testing under dependency. Ann. Stat. 29: 1165–1188.

Bjork JR, Díez-Vives C, Coma R, Ribes M & Montoya JM (2013) Specificity and temporal dynamics of complex bacteria – sponge symbiotic interactions. Ecology 94: 2781–2791.

Blanquer A & Uriz MJ (2010) Population genetics at three spatial scales of a rare sponge living in fragmented habitats. BMC Evol. Biol. 10: 13-21.

Calvo E, Simó R, Coma R, Ribes M, Pascual J *et al.* (2011) Effects of climate change on Mediterranean marine ecosystems : the case of the Catalan Sea. Clim. Res. 50: 1–29.

Cebrian E, Uriz MJ, Garrabou J & Ballesteros E (2011) Sponge mass mortalities in a warming Mediterranean Sea: are cyanobacteria-harboring species worse off? PLoS One 6: e20211.

Cerrano C & Bavestrello G (2008) Medium-term effects of die-off of rocky benthos in the Ligurian Sea. What can we learn from gorgonians? Chem. Ecol. 24: 73–82.

Clarke K (1993) Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18: 117–143.

Clarke K & Gorley R (2006) PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth.

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q *et al.* (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 31: 442–443.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B *et al.* (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37: D141–5.

Colin PL (1995) Surface currents in Exuma Sound, Bahamas, and adjacent areas with reference to potential larval transport. Bull. Mar. Sci. 56: 48–57.

Coma R, Ribes M, Gili J & Zabala M (2000) Seasonality in coastal benthic ecosystems. Trends Ecol. Evol. 15: 448–453.

Coma R, Ribes M, Serrano E, Jiménez E, Salat J *et al.* (2009) Global warming-enhanced stratification and mass mortality events in the Mediterranean. Proc. Natl. Acad. Sci. USA. 106: 6176–6181.

Crisci C, Bensoussan N, Romano J-C & Garrabou J (2011) Temperature anomalies and mortality events in marine communities: insights on factors behind differential mortality impacts in the NW Mediterranean. PLoS One 6: e23814.

Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H & Buckley DH (2009) T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics 10: 171–181.

Diaz M & Ward B (1997) Sponge-mediated nitrification in tropical benthic communities. Mar. Ecol. Prog. Ser. 156: 97–107.

Diaz MC & Rutzler K (2001) Sponges: an essential component of Caribbean coral reefs. Bull. Mar. Sci. 69: 535–546.

Dray S & Dufour A (2007) The ade4 package: implementing the duality diagram for ecologists. J. Stat. Softw. 22: 1–20.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A *et al.* (2011) Geneious v5.4, available from http://www.geneious.com

Duarte CM, Agusti S, Kennedy H & Vaqué D (1999) The Mediterranean climate as a template for Mediterranean marine ecosystems : the example of the northeast Spanish littoral. Prog. Oceanogr. 44: 245–270.

Duque C, Bonilla A, Bautista E & Zea S (2001) Exudation of low molecular weight compounds (thiobismethane, methyl isocyanide, and methyl isothiocyanate) as a possible chemical defense mechanism in the marine sponge *Ircinia felix*. Biochem. Syst. Ecol. 29: 459–467.

Duran S, Pascual M, Estoup A & Turon X (2004) Strong population structure in the marine sponge *Crambe crambe* (Poecilosclerida) as revealed by microsatellite markers. Mol. Ecol. 13: 511–522.

Ereskovsky AV, Gonobobleva E & Vishnyakov A (2004) Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). Mar. Biol. 146: 869–875.

Ereskovsky AV & Tokina DB (2004) Morphology and fine structure of the swimming larvae of *Ircinia oros* (Porifera, Demospongiae, Dictyoceratida). Invertebr. Reprod. Dev. 45: 137–150.

Erpenbeck D, Hooper JNA & Worheide G (2006) CO1 phylogenies in diploblasts and the "Barcoding of Life" - are we sequencing a suboptimal partition? Mol. Ecol. Notes 6: 550–553.

Erpenbeck D, Voigt O, Wörheide G & Lavrov DV (2009) The mitochondrial genomes of sponges provide evidence for multiple invasions by Repetitive Hairpin-forming Elements (RHE). BMC Genomics 10: 591.

Erwin PM & Thacker RW (2007) Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages. J. Mar. Biol. Assoc. UK 87: 1683–1692.

Erwin PM & Thacker RW (2008a) Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts. Mol. Ecol. 17: 2937–2947.

Erwin PM & Thacker RW (2008b) Phototrophic nutrition and symbiont diversity of two Caribbean sponge–cyanobacteria symbioses. Mar. Ecol. Prog. Ser. 362: 139–147.

Erwin PM, López-legentil S & Schuhmann PW (2010) The pharmaceutical value of marine biodiversity for anti-cancer drug discovery. Ecol. Econ. 70: 445–451.

Erwin PM, Olson JB & Thacker RW (2011) Phylogenetic diversity, host-specificity and community profiling of sponge-associated bacteria in the northern Gulf of Mexico. PLoS One 6: e26806.

Erwin PM, López-Legentil S, González-Pech R & Turon X (2012a) A specific mix of generalists: bacterial symbionts in Mediterranean *Ircinia* spp. FEMS Microbiol. Ecol. 79: 619–637.

Erwin PM, López-Legentil S & Turon X (2012b) Ultrastructure, molecular phylogenetics, and chlorophyll a content of novel cyanobacterial symbionts in temperate sponges. Microb. Ecol. 64: 771–783.

Erwin PM, Pita L, López-Legentil S & Turon X (2012c) Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance. Appl. Environ. Microbiol. 78: 7358–7368.

Esteves AIS, Hardoim CCP, Xavier JR, Gonçalves JMS & Costa R (2013) Molecular richness and biotechnological potential of bacteria cultured from Irciniidae sponges in the north-east Atlantic. FEMS Microbiol. Ecol. 85: 519–536.

Fan L, Liu M, Simister R, Webster NS & Thomas T (2013) Marine microbial symbiosis heats up: the phylogenetic and functional response of a sponge holobiont to thermal stress. ISME J. 7: 991–1002.

Faulkner DJ (2001) Marine natural products (1999). Nat. Prod. Rep. 18: 1–49.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39: 783–791.

Fierer N (2008) Microbial biogeography : patterns in microbial diversity across space and time. In: Accessing uncultivated microorganisms: from the environment to organisms and genomes and back. Zengler K (ed). Whashington DC. ASM Press, pp 95-115.

Fiore CL, Baker DM & Lesser MP (2013) Nitrogen biogeochemistry in the Caribbean sponge, *Xestospongia muta*: A source or sink of dissolved inorganic nitrogen? PLoS One 8: e72961.

Fiore CL, Jarett JK, Olson ND & Lesser MP (2010) Nitrogen fixation and nitrogen transformations in marine symbioses. Trends Microbiol. 18: 455–463.

Flatt P, Gautschi J, Thacker RW, Musafija-Girt M, Crews P *et al.* (2005) Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge *Dysidea (Lamellodysidea) herbacea* and symbiotic cyanobacterium *Oscillatoria spongeliae* by CARD-FISH analysis. Mar. Biol. 147: 761–774.

Flo E, Garcés E, Manzanera M & Camp J (2011) Coastal inshore waters in the NW Mediterranean: Physicochemical and biological characterization and management implications. Estuar. Coast. Shelf Sci. 93: 279–289.

Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3: 294–299.

Freeman CJ & Thacker RW (2011) Complex interactions between marine sponges and their symbiotic microbial communities. Limnol. Oceanogr. 56: 1577–1586.

Friedrich AB, Fischer I, Proksch P, Hacker J & Hentschel U (2001) Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. FEMS Microbiol. Ecol. 38: 105–115.

Galand PE, Casamayor EO, Kirchman DL & Lovejoy C (2009) Ecology of the rare microbial biosphere of the Arctic Ocean. Proc. Natl. Acad. Sci. USA. 106: 22427–22432.

Garrabou J, Coma R, Bensoussan N, Bally M, Chevaldonné P *et al.* (2009) Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. Glob. Chang. Biol. 15: 1090–1103.

Gasol JM & Giorgio P (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Sci. Mar. 64: 197–224.

Gili JM & Coma R (1998) Benthic suspension feeders: their paramount role in littoral marine food webs. Trends Ecol. Evol. 13: 316–321.

De Goeij JM, van Oevelen D, Vermeij MJA, Osinga R, Middelburg JJ *et al.* (2013) Surviving in a marine desert: the sponge loop retains resources within coral reefs. Science 342: 108–110.

Gsell AS, Domis LNDS, van Donk E & Ibelings BW (2013) Temperature alters host genotype-specific susceptibility to chytrid infection. PLoS One 8: e71737.

Haber M, Carbone M, Mollo E, Gavagnin M & Ilan M (2011) Chemical defense against predators and bacterial fouling in the Mediterranean sponges *Axinella polypoides* and *A. verrucosa*. Mar. Ecol. Prog. Ser. 422: 113–122.

Haber M & Ilan M (2013) Diversity and antibacterial activity of bacteria cultured from Mediterranean *Axinella* spp. sponges. J. Appl. Microbiol. 116(3): 519-532.

Hammami S, Bergaoui A, Boughalleb N, Romdhane A, Khoja I *et al.* (2010) Antifungal effects of secondary metabolites isolated from marine organisms collected from the Tunisian coast. Comptes Rendus Chim. 13: 1397–1400.

Hanson CA, Fuhrman JA, Horner-Devine MC & Martiny JBH (2012) Beyond biogeographic patterns: processes shaping the microbial landscape. Nat. Rev. Microbiol. 10: 497–506.

Hardoim CCP, Esteves AIS, Pires FR, Gonçalves JMS, Cox CJ *et al.* (2012) Phylogenetically and spatially close marine sponges harbour divergent bacterial communities. PLoS One 7: e53029.

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP *et al.* (2002) Climate warming and disease risks for terrestrial and marine biota. Science 296: 2158–2162.

Hentschel U, Horn M, Friedrich AB, Wagner M & Moore BS (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. Appl. Environ. Microbiol. 68: 4431–4440.

Hentschel U, Piel J, Degnan SM & Taylor MW (2012) Genomic insights into the marine sponge microbiome. Nat. Rev. Microbiol. 10: 641–654.

Herbert P, Cywinska A, Ball S & deWaard JR (2003) Biological identifications through DNA barcodes. Proc. R. Soc. L. B. Biol. Sci. 270: 313–321.

Hoffmann F, Larsen O, Thiel V, Rapp HT, Pape T *et al.* (2005) An anaerobic world in sponges. Geomicrobiol. J. 22: 1–10.

Hoffmann F, Radax R, Woebken D, Holtappels M, Lavik G *et al.* (2009) Complex nitrogen cycling in the sponge *Geodia barretti*. Environ. Microbiol. 11: 2228–2243

Jimenez E & Ribes M (2007) Sponges as a source of dissolved inorganic nitrogen : Nitrification mediated by temperate sponges. Limnol. Oceanogr. 52: 948–958.

Joachimiak M, Weisman J & May B (2006) JColorGrid: software for the visualization of biological measurement. BMC Bioinformatics 7: 225–229.

Kamke J, Taylor MW & Schmitt S (2010) Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA *vs* 16S rRNA gene comparisons. ISME J. 4: 498–508.

Kaplan CW & Kitts CL (2003) Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content. J. Microbiol. Methods 54: 121–125.

Kent AD, Smith DJ, Benson BJ & Triplett EW (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl. Environ. Microbiol. 69: 6768–6776.

Kiers ET, Palmer TM, Ives AR, Bruno JF & Bronstein JL (2010) Mutualisms in a changing world: an evolutionary perspective. Ecol. Lett. 13: 1459–1474.

Kühl M, Behrendt L, Trampe E, Qvortrup K, Schreiber U *et al.* (2012) Microenvironmental ecology of the chlorophyll *b*-containing symbiotic cyanobacterium *Prochloron* in the didemnid ascidian *Lissoclinum patella*. Front. Microbiol. 3: 402.

LaJeunesse TC, Smith RT, Finney J & Oxenford H (2009) Outbreak and persistence of opportunistic symbiotic dinoflagellates during the 2005 Caribbean mass coral “bleaching” event. Proc. Biol. Sci. 276: 4139–4148.

Lee OO, Lau SCK & Qian P (2006) Consistent bacterial community structure associated with the surface of the sponge *Mycale adhaerens* Bowerbank. Microb. Ecol. 52: 693–707.

Lee OO, Chui PY, Wong YH, Pawlik JR & Qian P-Y (2009a) Evidence for vertical transmission of bacterial symbionts from adult to embryo in the Caribbean sponge *Svenzea zeai*. Appl. Environ. Microbiol. 75: 6147–6156.

Lee OO, Wong YH & Qian P-Y (2009b) Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. Appl. Environ. Microbiol. 75: 3513–3521.

Lee OO, Wang Y, Yang J, Lafi FF, Al-Suwailem A *et al.* (2011) Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. ISME J. 5: 650–664.

Lejeusne C, Chevaldonne P, Pergent-Martini C, Boudouresque C & Pérez T (2009) Climate change effects on a miniature ocean : the highly diverse, highly impacted Mediterranean Sea. Trends Ecol. Evol. 25: 250–260.

Lemoine N, Buell N, Hill A & Hill M (2007) Assessing the utility of sponge microbial symbiont communities as models to study global climate change : a case study with *Halichondria bowerbanki*. In: Porifera Research: Biodiversity, Innovatio and Sustainability. Custódio MR, Lôbo-Hajdu G, Hajdu E & Muricy G (eds). Río de Janeiro, Brazil. Série Livros, Museu Nacional, pp. 419–425.

Leys SP & Riesgo A (2012) Epithelia, an evolutionary novelty of metazoans. J. Exp. Zool. B. Mol. Dev. Evol. 318: 438–447.

Li C, Chen J & Hua T (1998) Precambrian sponges with cellular structures. Science 279: 879–882.

Liu M, Fan L, Zhong L, Kjelleberg S & Thomas T (2012) Metaproteogenomic analysis of a community of sponge symbionts. ISME J. 6: 1515–1525.

Liu MY, Kjelleberg S & Thomas T (2011) Functional genomic analysis of an uncultured δ-proteobacterium in the sponge *Cymbastela concentrica*. ISME J. 5: 427–435.

López-Legentil S, Song B, McMurray SE & Pawlik JR (2008) Bleaching and stress in coral reef ecosystems: *hsp70* expression by the giant barrel spone *Xestospongia muta*. Mol. Ecol. 17(7): 1840–1849.

López-Legentil S, Erwin PM, Pawlik JR & Song B (2010) Effects of sponge bleaching on ammonia-oxidizing *Archaea*: distribution and relative expression of ammonia monooxygenase genes associated with the barrel sponge *Xestospongia muta*. Microb. Ecol. 60: 561–571.

López-Legentil S, Song B, Bosch M, Pawlik JR & Turon X (2011) Cyanobacterial diversity and a new acaryochloris-like symbiont from Bahamian sea-squirts. PLoS One 6: e23938.

López-Legentil S, Erwin PM, Velasco M & Turon X (2013) Growing or reproducing in a temperate sea: optimization of resource allocation in a colonial ascidian. Invertebr. Biol. 132: 69–80.

Love GD, Grosjean E, Stalvies C, Fike DA, Grotzinger JP *et al.* (2009) Fossil steroids record the appearance of Demospongiae during the Cryogenian period. Nature 457: 718–721.

Lozupone CA, Hamady M, Kelley ST & Knight R (2007) Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl. Environ. Microbiol. 73: 1576–1585.

Maldonado M, Sánchez-Tocino L & Navarro C (2010) Recurrent disease outbreaks in corneous demosponges of the genus *Ircinia* : epidemic incidence and defense mechanisms. Mar. Biol. 157: 1577–1590.

Maldonado M & Young CM (1998) Limits on the bathymetric distribution of keratose sponges : a field test in deep water. Mar. Ecol. Prog. Ser. 174: 123–139.

Margulis L (1998) Symbiotic planet: a new look at evolution. Basic Books ed. Amherst, Massachusetts.

Mariani S, Uriz M-J, Turon X & Alcoverro T (2006) Dispersal strategies in sponge larvae: integrating the life history of larvae and the hydrologic component. Oecologia 149: 174–184.

Martínez-Murcia A, Acinas S & Rodríguez-Valera F (1995) Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol. Ecol. 17: 247–255.

Martiny JBH, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, *et al.* (2006) Microbial biogeography: putting microorganisms on the map. Nat. Rev. Microbiol. 4: 102–112.

McArdle BH & Anderson MJ (2001) Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecology 82: 290–297.

McFall-Ngai M, Haldfield MG, Bosch TCG, Carey HV, Domazet-Loso T *et al.* (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. USA. 110: 3229–3236.

McFall-Ngai M (2008) Are biologists in “future shock”? Symbiosis integrates biology across domains. Focus symbiosis 6: 789–792.

Meyer B & Kuever J (2008) Phylogenetic diversity and spatial distribution of the microbial community associated with the Caribbean deep-water sponge *Polymastia cf. corticata* by 16S rRNA, *aprA*, and *amoA* gene analysis. Microb. Ecol. 56: 306–321.

Millot C (1999) Circulation in the Western Mediterranean Sea. J. Mar. Syst. 20: 423–442..

Mindell D (1992) Phylogenetic consequences of symbioses: Eukarya and Eubacteria are not monophyletic taxa. Biosystems 27: 53–62.

Mohamed NM, Colman AS, Tal Y & Hill RT (2008a) Diversity and expression of nitrogen fixation genes in bacterial symbionts of marine sponges. Environ. Microbiol. 10: 2910–2921.

Mohamed NM, Enticknap JJ, Lohr JE, McIntosh SM & Hill RT (2008b) Changes in bacterial communities of the marine sponge *Mycale laxissima* on transfer into aquaculture. Appl. Environ. Microbiol. 74: 1209–1222.

Mohamed NM, Rao V, Hamann MT, Kelly M & Hill RT (2008c) Monitoring bacterial diversity of the marine sponge *Ircinia strobilina* upon transfer into aquaculture. Appl. Environ. Microbiol. 74: 4133–4143.

Moitinho-Silva L, Bayer K, Cannistraci CV, Giles EC, Ryu T *et al.* (2013) Specificity and transcriptional activity of microbiota associated with low and high microbial abundance sponges from the Red Sea. Mol. Ecol. (In press). DOI:10.1111/mec.12365.

Montalvo NF & Hill RT (2011) Sponge-associated bacteria are strictly maintained in two closely related but geographically distant sponge hosts. Appl. Environ. Microbiol. 77: 7207–7216.

Morrow KM, Moss AG, Chadwick NE & Liles MR (2012) Bacterial associates of two Caribbean coral species reveal species-specific distribution and geographic variability. Appl. Environ. Microbiol. 78: 6438–6449.

Muscholl-Silberhorn A, Thiel V & Imhoff JF (2008) Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. Microb. Ecol. 55: 94–106.

Newman DJ & Hill RT (2006) New drugs from marine microbes: the tide is turning. J. Ind. Microbiol. Biotechnol. 33: 539–544.

Palmer TM, Stanton ML, Young TP, Goheen JR, Pringle RM *et al.* (2008) Breakdown of an ant-plant mutualism follows the loss of large herbivores from an African savanna. Science 319: 192–195.

Pandey J, Ganesan K & Jain R (2007) Variations in T-RFLP profiles with differing chemistries of fluorescent dyes used for labeling the PCR primers. J. Microbiol. Methods 68: 633–638.

Parra-Velandia FJ & Zea S (2003) Comparación de algunas características de las esponjas del género *Ircinia* (Porifera: Demospongiae) en dos localidades contrastantes del área de Santa Marta, Caribe Colombiano. Bol Invest Mar Cost 32: 75–91.

Paul VJ & Ritson-Williams R (2008) Marine chemical ecology. Nat. Prod. Rep. 25: 662–695.

Paul VJ, Ritson-Williams R & Sharp K (2011) Marine chemical ecology in benthic environments. Nat. Prod. Rep. 28: 345–387.

Pawlik JR, Chanasl B & Robert J (1995) Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. Mar Ecol Prog Ser 127: 183–194.

Pawlik JR, Fall GMC & Zea S (2002) Does the odor from sponges of the genus *Ircinia* protect them from fish predators? J. Chem. Ecol. 28: 1103–1115.

Pawlik JR, Steindler L, Henkel TP, Beer S & Ilan M (2007) Chemical warfare on coral reefs : Sponge metabolites differentially affect coral symbiosis *in situ*. Am. Soc. Limnol. Oceanogr. 52: 907–911.

Pedrós-Alió C (2012) The rare bacterial biosphere. Ann. Rev. Mar. Sci. 4: 449–466.

Pile AJ (1997) Finding Reiswig's missing carbon: quantification of sponge feeding using dual-beam flow cytometry. Proceedings of the 8th International Coral Reef Symposium, 1403–1410.

Pile AJ, Patterson MR & Witman JD (1996) *In situ* grazing on plankton < 10 pm by the boreal sponge *Mycale lingua*. Mar. Ecol. Prog. Ser. 141: 95–102.

Pinhassi J, Gómez-Consarnau L, Alonso-Sáez L, Sala MM, Vidal M *et al.* (2006) Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea. Aquat. Microb. Ecol. 44: 241–252.

Pita L, López-Legentil S & Erwin PM (2013) Biogeography and host fidelity of bacterial communities in *Ircinia* spp. from the Bahamas. Microb. Ecol. 66: 437–447.

Pöppe J, Sutcliffe P, Hooper JNA, Wörheide G & Erpenbeck D (2010) COI barcoding reveals new clades and radiation patterns of Indo-Pacific sponges of the family Irciniidae (Demospongiae: Dictyoceratida). PLoS One 5: e9950.

Racine J (2012) RStudio: A platform-independent IDE for R and Sweave. J. Appl. Econom. 27: 167–172.

Radax R, Hoffmann F, Rapp HT, Leininger S & Schleper C (2012) Ammonia-oxidizing Archaea as main drivers of nitrification in cold-water sponges. Environ. Microbiol. 14: 909–923.

Regoli F, Cerrano C, Chierici E, Bompadre S & Bavestrello G (2000) Susceptibility to oxidative stress of the Mediterranean demosponge *Petrosia ficiformis*: the role of endosymbionts and solar irradiance. Mar. Biol. 137: 453-461.

Reshef L, Koren O, Loya Y, Zilber-Rosenberg I & Rosenberg E (2006) The coral probiotic hypothesis. Environ. Microbiol. 8: 2068–2073.

Reveillaud J, Maignien L, Eren AM, Huber JA, Apprill A *et al.* (2014) Host-specificity among abundant and rare taxa in the sponge microbiome. ISME J. (In press). DOI:10.1038/ismej.2013.227

Reysenbach A, Wickham GS & Pace NR (1994) Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park . Appl. Environ. Microbiol. 60: 2113.

Ribes M, Jiménez E, Yahel G, López-Sendino P, Diez B *et al.* (2012) Functional convergence of microbes associated with temperate marine sponges. Environ. Microbiol. 14: 1224–1239.

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

Rot C, Goldfarb I, Ilan M & Huchon D (2006) Putative cross-kingdom horizontal gene transfer in sponge (Porifera) mitochondria. BMC Evol. Biol. 6: 71.

Sarà M (1971) Ultrastructural aspects of the symbiosis between two species of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variabilis* (Demospongiae). Mar. Biol. 11: 214–221.

Schauer M, Massana R & Pedrós-Alió C (2000) Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. FEMS Microbiol. Ecol. 33: 51–59.

Schauer M, Balagué V, Pedrós-Alió C & Massana R (2003) Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. Aquat. Microb. Ecol. 31: 163–174.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75: 7537–7541.

Schmahl GP (1990) Community structure and ecology of sponges associated with four Southern Florida coral reefs. In: New perspectives in sponge biology. Rützler, K, ed. Washington, DC. Smithsonian Institution Press, pp. 376–383.

Schmitt S, Weisz JB, Lindquist N & Hentschel U (2007) Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. Appl. Environ. Microbiol. 73: 2067–2078.

Schmitt S, Angermeier H, Schiller R, Lindquist N & Hentschel U (2008) Molecular microbial diversity survey of sponge reproductive stages and mechanistic insights into vertical transmission of microbial symbionts. Appl. Environ. Microbiol. 74: 7694–7708.

Schmitt S, Tsai P, Bell J, Fromont J, Ilan M *et al.* (2012) Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. ISME J. 6: 564–576.

Schöttner S, Hoffmann F, Cárdenas P, Rapp HT, Boetius A *et al.* (2013) Relationships between host phylogeny, host type and bacterial community diversity in cold-water coral reef sponges. PLoS One 8: e55505.

Simister R, Deines P, Botté ES, Webster NS & Taylor MW (2012a) Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. Environ. Microbiol. 14: 517–524.

Simister R, Taylor MW, Tsai P, Fan L, Bruxner TJ *et al.* (2012b) Thermal stress responses in the bacterial biosphere of the Great Barrier Reef sponge, *Rhopaloeides odorabile*. Environ. Microbiol. 14: 3232–3246.

Simister R, Taylor MW, Tsai P & Webster N (2012c) Sponge-microbe associations survive high nutrients and temperatures. PLoS One 7: e52220.

Van Soest RWM, Boury-Esnault N, Vacelet J, Dohrmann M, Erpenbeck D *et al.* (2012) Global diversity of sponges (Porifera). PLoS One 7: e35105.

Sokal RR & Rohl FJ (1995) Biometry: the principles and practice of statistics in biological research. Freeman (3ªed), New York, NY.

Southwell MW, Weisz JB, Martens CS & Lindquist N (2008) *In situ* fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. Limnol. Oceanogr. 53: 986–996.

Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier MEA *et al.* (2010) The *Amphimedon queenslandica* genome and the evolution of animal complexity. Nature 466: 720–726.

Stabili L, Cardone F, Alifano P, Tredici SM, Piraino S *et al.* (2012) Epidemic mortality of the sponge *Ircinia variabilis* (Schmidt, 1862) associated to proliferation of a *Vibrio bacterium*. Microb. Ecol. 64: 802–813.

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.

Steger D, Ettinger-Epstein P, Whalan S, Hentschel U, de Nys R *et al.* (2008) Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. Environ. Microbiol. 10: 1087–1094.

Stewart CN & Excoffier L (1996) Assessing population genetic structure and variability with RAPD data: Application to *Vaccinium macrocarpon* (American Cranberry). J. Evol. Biol. 9: 153–171.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M *et al.* (2011) MEGA: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739.

Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. Some mathematical questions in biology-DNA sequence analysis. Miura RM, ed. Providence, RI. American Mathemathics Society, pp. 57–86.

Taylor MW, Schupp PJ, Dahllöf I, Kjelleberg S & Steinberg PD (2003) Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. Environ. Microbiol. 6: 121–130.

Taylor MW, Schupp PJ, de Nys R, Kjelleberg S & Steinberg PD (2005) Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. Environ. Microbiol. 7: 419–433.

Taylor MW, Radax R, Steger D & Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol. Mol. Biol. Rev. 71: 295–347.

Taylor MW, Hill RT & Hentschel U (2011) Meeting report: 1st international symposium on sponge microbiology. Mar. Biotechnol. 13: 1057–1061.

Taylor MW, Tsai P, Simister RL, Deines P, Botte E *et al.* (2013) "Sponge-specific" bacteria are widespread (but rare) in diverse marine environments. ISME J. 7: 438-443.

The R Core Team (2012) R: A language and environment for statistical computing. http://www.r-project.org/.

Thacker RW (2005) Impacts of shading on sponge-cyanobacteria symbioses : A comparison between host-specific and generalist associations. Integr. Comp. Biol. 45: 369–376.

Thacker RW & Freeman CJ (2012) Sponge-microbe symbiosis: recent advances and new directions. Adv Mar Biol 62: 57–111.

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

Thiel V, Neulinger S, Staufenberger T, Schmaljohann R & Imhoff JF (2007b) Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*. FEMS Microbiol. Ecol. 59: 47–63.

Thomas T, Rusch D, DeMaere MZ, Yung PY, Lewis M *et al.* (2010) Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. ISME J. 4: 1557–1567.

Thompson J, Gibson T, Plewniak F, Jeanmougin F & Higgins D (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tools. Nucleic Acids Res. 25: 4876–4882.

Turner S, Pryer KM, Miao VP & Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J. Eukaryot. Microbiol. 46: 327–338.

Turon X, Garriga A & Erwin PM (2013) Lights and shadows: growth patterns in three sympatric and congeneric sponges (*Ircinia* spp.) with contrasting abundances of photosymbionts. Mar. Biol. 160 (10): 2743-2754.

Turque AS, Batista D, Silveira CB, Cardoso AM, Vieira RP *et al.* (2010) Environmental shaping of sponge associated archaeal communities. PLoS One 5: e15774.

Uriz MJ, Turon X & Mariani S (2008) Ultrastructure and dispersal potential of sponge larvae: tufted versus evenly ciliated parenchymellae. Mar. Ecol. 29: 280–297.

Usher KM, Kuo J, Fromont J & Sutton DC (2001) Vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). Hydrobiologia 461: 15–23.

Usher KM, Toze S, Fromont J, Kuo J & Sutton DC (2004) A new species of cyanobacterial symbiont from the marine sponge *Chondrilla nucula*. Symbiosis 36: 183–192.

Usher KM (2008) The ecology and phylogeny of cyanobacterial symbionts in sponges. Mar. Ecol. 29: 178–192.

Vacelet J & Donadey C (1977) Electron microscope study of the association between some sponges and bacteria. J. Exp. Mar. Biol. Ecol. 30: 301–314.

Webster NS & Hill RT (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. Mar. Biol. 138: 843–851.

Webster NS, Webb RI, Ridd MJ, Hill RT & Negri AP (2001) The effects of copper on the microbial community of a coral reef sponge. Environ. Microbiol. 3: 19–31.

Webster NS, Negri AP, Webb RI & Hill RT (2002) A spongin-boring α -proteobacterium is the etiological agent of disease in the Great Barrier Reef sponge *Rhopaloeides odorabile*. Mar. Ecol. Prog. Ser. 232: 305–309.

Webster NS, Negri AP, Munro MMHG & Battershill CN (2004) Diverse microbial communities inhabit Antarctic sponges. Environ. Microbiol. 6: 288–300.

Webster NS (2007) Sponge disease: a global threat? Environ. Microbiol. 9: 1363–1375.

Webster NS, Cobb RE & Negri AP (2008a) Temperature thresholds for bacterial symbiosis with a sponge. ISME J. 2: 830–842.

Webster NS, Xavier JR, Freckelton M, Motti CA & Cobb R (2008b) Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. Environ. Microbiol. 10: 3366–3376.

Webster NS, Taylor MW, Behnam F, Lücker S, Rattei T *et al.* (2010) Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environ. Microbiol. 12: 2070–2082.

Webster NS, Cobb RE, Soo R, Anthony SL, Battershill CN *et al.* (2011) Bacterial community dynamics in the marine sponge *Rhopaloeides odorabile* under *in situ* and *ex situ* cultivation. Mar. Biotechnol 13: 296–304.

Webster NS & Taylor MW (2012) Marine sponges and their microbial symbionts: love and other relationships. Environ. Microbiol. 14: 335–346.

Weisz JB, Hentschel U, Lindquist N & Martens CS (2007) Linking abundance and diversity of sponge-associated microbial communities to metabolic differences in host sponges. Mar. Biol. 152: 475–483.

Weisz JB, Massaro AJ, Ramsby BD & Hill MS (2010) Zooxanthellar symbionts shape host sponge trophic status through translocation of carbon. Biol. Bull. 219: 189–197.

White JR, Patel J, Ottesen A, Arce G, Blackwelder P *et al.* (2012) Pyrosequencing of bacterial symbionts within *Axinella corrugata* sponges: diversity and seasonal variability. PLoS One 7: e38204.

Wichels A, Würtz S, Döpke H, Schütt C & Gerdts G (2006) Bacterial diversity in the breadcrumb sponge *Halichondria panicea* (Pallas). FEMS Microbiol. Ecol. 56: 102–118.

Wilkinson CR (1978) Microbial associations in sponges I. Ecology, physiology and microbial populations of coral reef sponges. Mar. Biol. 49: 161–167.

Wilkinson CR (1983) Net primary productivity in coral reef sponges. Science 219: 410–412.

Yang J, Sun J, Lee O, Wong Y & Qian P-Y (2011) Phylogenetic diversity and community structure of sponge-associated bacteria from mangroves of the Caribbean Sea. Aquat. Microb. Ecol. 62: 231–240.

Zhang R, Thiyagarajan V & Qian P-Y (2008) Evaluation of terminal-restriction fragment length polymorphism analysis in contrasting marine environments. FEMS Microbiol. Ecol. 65: 169–178.

Resumen en Castellano

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INTRODUCCIÓN

Un mundo simbiótico

El botánico alemán Anton de Bary acuñó el término "**simbiosis**" como la asociación estrecha y persistente entre organismos de distintas especies (De Bary, 1879). Tradicionalmente, estas relaciones simbióticas se han clasificado de acuerdo con el beneficio/coste para cada uno de los organismos simbiontes: **mutualismo** si la relación beneficia a ambos socios, **comensalismo** si uno de los organismos resulta beneficiado sin causarle perjuicio al otro, y **parasitismo** si uno de los socios se beneficia a costa de la supervivencia o el éxito reproductivo del otro. Estas categorías no son cajones estancos, sino un continuo en el que el equilibrio beneficio/coste puede variar dependiendo de las condiciones ambientales (Palmer *et al.* 2008; LaJeunesse *et al.* 2009; Gsell *et al.* 2013). Por tanto, las relaciones simbióticas son dinámicas y es esencial evaluar su variabilidad a lo largo de escalas espacio-temporales, así como su susceptibilidad frente a perturbaciones en el ambiente (Kiers *et al.* 2010).

Lynn Margulis (1938-2011), quien postuló el origen simbiótico de las mitocondrias y cloroplastos de las células eucariotas, fue también pionera en percibir que los microorganismos simbiontes no son sólo agentes causantes de enfermedades, sino que pueden influir en la biología, ecología y evolución de los animales y plantas (Margulis 1998). En las últimas décadas, y gracias al desarrollo de nuevas técnicas en ecología microbiana, tenemos evidencias claras de la ubicuidad y la diversidad de las interacciones entre microorganismos y animales. Estos datos prometen revolucionar la forma en la que definimos tanto el nicho ecológico como la adaptación y la evolución de los animales y microorganismos (McFall-Ngai 2008; McFall-Ngai *et al.* 2013). El término “holobionte” es ahora comúnmente utilizado para referirse a la unidad de selección y evolución formada por el animal hospedador y su población metabólicamente activa de organimos simbiontes (Mindell 1992; Rohwer *et al.* 2002).

Esta tesis doctoral está enfocada al estudio de las comunidades microbianas simbiontes en esponjas, un campo que se conoce como “microbiología de esponjas”. El estudio del filo Porifera (esponjas) y su asociación con diversas y específicas comunidades de microorganismos comenzó en la década de los años 70, cuando densas poblaciones de microbios con muy diversas morfologías fueron observadas al microscopio electrónico en varias especies diferentes de esponjas (Sarà 1971; Vacelet & Donadey 1975; Wilkinson 1978). Desde entonces, el interés por investigar el “quién, cómo y por qué” de los microorganismos simbiontes de esponjas ha aumentado de

manera exponencial (Thacker & Freeman 2012). Numerosos estudios han demostrado que esta simbiosis tiene implicaciones directas en el metabolismo de la esponja, su defensa química y, en consecuencia, en la ecología de las esponjas en el ecosistema (Taylor *et al.* 2007). Sin embargo, todavía no están claros los procesos que gobiernan esta simbiosis, su respuesta a factores bióticos y abióticos, y su vulnerabilidad a perturbaciones en el ambiente.

El hospedador: Las esponjas de mar

Las esponjas - filo Porifera, del latín *porus* (poros) + *ferre* (contener) – presentan un cuerpo poroso, diseñado para vivir anclado al sustrato mientras filtran el agua de mar y si alimentan de las partículas orgánicas contenidas en el agua. El agua entra en la esponja a través de unos poros en su superficie (*ostia*). Su cuerpo contiene un sistema de canales y cámaras (coanosoma) donde células especializadas llamadas coanocitos facilitan el flujo y filtran las partículas del agua. El agua abandona la esponja por uno o más poros exhalantes (*oscula*). Las partículas filtradas pasan a la matriz interna de la esponja (mesohilo) y serán principalmente digeridas por unas células ameboides denominadas “arqueocitos”. La capa más superficial de la esponja se denomina pinacodermo, y la parte interna, que incluye el coanosoma y el mesohilo, se denomina endodermo. Los simbiontes microbianos normalmente se encuentran extracelularmente en el mesohilo, aunque algunas especies de esponjas contienen también simbiontes intracelulares.

Las esponjas son uno de los filos más antiguos de los Metazoos. Fósiles del Precámbrico sugieren su aparición hace unos 600 millones de años (Li *et al.* 1998; Love *et al.* 2009). El hecho de que este filo se encuentre en la base del árbol filogenético de los Metazoos lo ha convertido en modelos para identificar los eventos evolutivos que dieron lugar a la multicelularidad y la evolución de los animales (e.g., Srivastava *et al.* 2010; Leys & Riesgo 2012). Las esponjas de mar han colonizado todos los océanos, desde las aguas superficiales hasta las más profundas. El número de taxones descritos supera ya las 8500 especies y se espera que este número se incremente a medida que se vaya resolviendo la filogenia de muchos grupos (Van Soest *et al.* 2012). Las esponjas de mar representan además un componente determinante del ecosistema debido a su diversidad, abundancia e influencia en los ciclos de nutrientes (Diaz & Rutzler 2001; De Goeij *et al.* 2013; Fiore *et al.* 2013).

Además, las esponjas producen compuestos químicos para evitar la competición, la depredación y el asentamiento de epibiontes (Pawlik *et al.* 1995; Pawlik *et al.* 2007; Haber *et al.* 2011). La actividad citotóxica y antimicrobiana de estos

compuestos hacen de las esponjas el taxón más rico en nuevos metabolitos secundarios con potencial commercial para aplicaciones farmacéuticas e industriales, especialmente para el desarrollo de medicamentos antitumorales (Faulkner 2001; Erwin *et al.* 2010; Paul *et al.* 2011).

La mayoría de las características y funciones que hacen relevante a las esponjas derivan de su asociación con una abundante y compleja microbiota (Taylor *et al.* 2007; Thacker & Freeman 2012; Webster & Taylor 2012). La actividad metabólica de los microorganismos simbiontes expande el metabolismo de la esponja gracias a procesos como la fotosíntesis, la fijación de nitrógeno o la oxidación del amonio (Weisz *et al.* 2007; Erwin & Thacker 2008b; Fiore *et al.* 2010). Además, los simbiontes pueden participar activamente en la defensa química de la esponja y, por tanto, en la síntesis de los metabolitos bioactivos detectados en esponjas (Esteves *et al.* 2013; Haber & Ilan 2013). Presumiblemente, los microorganismos se benefician de vivir en un ambiente protegido y rico en nutrientes como es el mesohilo de la esponja y, por tanto, la simbiosis se considera mutualista. Sin embargo, las evidencias empíricas son todavía escasas (Taylor *et al.* 2007). Así, desarrollar nuestro conocimiento sobre la persistencia y resistencia de las asociaciones esponja-microorganismos es crucial desde un punto de vista biológico, ecológico y biotecnológico.

El húesped: Microorganismos simbiontes de esponjas

La sorprendente densidad y diversidad microbiana en esponjas fue revelada por primera vez gracias a la microscopía electrónica de transmisión (Sarà 1971; Vacelet & Donadey 1975). Más adelante, los estudios moleculares confirmaron que la mayoría de las esponjas albergan una compleja comunidad microbiana que incluye principalmente *Bacteria* – 17 filos descritos y 12 filos candidatos – y *Archaea*, pero también hongos y otros eucariotas (Schmitt *et al.* 2012; Webster & Taylor 2012). Es más, en un sólo especimen de esponja se pueden encontrar cientos de taxones bacterianos diferentes (Webster *et al.* 2010; Lee *et al.* 2011). Los filos bacterianos dominantes son: *Proteobacteria* (Clase Alpha-, Delta- y Gammaproteobacteria), *Chloroflexi*, *Actinobacteria*, *Acidobacteria* y *Nitrospira* (Webster & Taylor 2012). Además, muchas especies albergan simbiontes fotoautótrofos del filo *Cyanobacteria* que alcanzan altas densidades en el pinacodermo de esas esponjas (Thacker 2005; Erwin & Thacker 2007; Erwin *et al.* 2012b). Las arqueobacterias (*Archaea*) asociadas a las esponjas marinas pertenecen principalmente al filo *Thaumarchaeota*, antes denominado “Marine group I *Crenarchaeota*” (Steger *et al.* 2008; Turque *et al.* 2010; Radax *et al.* 2012). Además, la mayor parte de la comunidad microbiana es

metabólicamente activa en el interior de la esponja (Mohamed *et al.* 2008a; Kamke *et al.* 2010; Moitinho-Silva *et al.* 2013).

Un amplio estudio publicado recientemente muestra que las esponjas contienen una comunidad basal pequeña, formada por definición por los filotipos bacterianos presentes en al menos el 70% de las esponjas analizadas en el estudio (Schmitt *et al.* 2012). Aún así, los taxones bacterianos presentes en una esponja en particular pueden estar más relacionados con bacterias de esponjas filogenética y geográficamente distantes, y no aparecer o ser raros en otros ambientes (i.e., el agua circundante o los sedimentos) (Hentschel *et al.* 2002; Taylor *et al.* 2012). Este grado de especificidad ha dado lugar a la definición de *clusters* específicos de esponjas. Cabe destacar también que algunas de las más ubicuas bacterias asociadas a esponjas están filogenéticamente relacionadas con bacterias simbiontes de corales y forman los denominados "*clusters* esponja-coral" bacterianos (Simister *et al.* 2012a).

La especificidad mencionada anteriormente podría estar relacionada con la manera en que las comunidades simbiontes se establecen en las esponjas. Está generalmente aceptado que la transmisión vertical (de los padres a su progenie) y adquisición horizontal (desde el agua circundante) se combinan y hacen posible el mantenimiento de la simbiosis (Taylor *et al.* 2007; Hentschel *et al.* 2012). La transmisión vertical ha sido documentada a través de microscopía electrónica de transmisión y estudios moleculares que confirman la presencia de bacterias en las larvas y juveniles de distintas especies de esponjas (Ereskovsky *et al.* 2004; Schmitt *et al.* 2007; Lee *et al.* 2009). Sin embargo, algunas de las bacterias que se consideraban específicas de esponjas han sido encontradas en el agua de mar, aunque en muy baja densidad (Webster *et al.* 2010; Taylor *et al.* 2012), lo que sugiere que las esponjas podrían potencialmente adquirir sus simbiontes del agua circundante, aunque el mecanismo exacto se desconoce todavía.

Tradicionalmente, las esponjas se han divido en dos grupos de acuerdo a la distinta abundancia de microorganismos en el mesohilo: las esponjas con alta densidad de simbiontes (esponjas HMA) y las de baja densidad de simbiontes (esponjas LMA), (Vacelet & Donadey 1977a). Sin embargo, esta división también se corresponde con diferencias en la estructura de la comunidad simbionte: las comunidades de esponjas HMA son específicas del hospedador mientras que las comunidades en esponjas LMA son más similares a las encontradas en el ambiente (Bjork *et al.* 2013; Moitinho-Silva *et al.* 2013). Y es más, la abundancia de microorganimos puede influir la cantidad de agua filtrada por la esponja, por lo que esta clasificación se relaciona también con dos estrategias fisiológicas distintas. Esto

sugiere que la presencia de simbiontes afecta la estrategia evolutiva de las esponjas que los albergan (Weisz *et al.* 2007).

Uno de los principales retos de la investigación en microbiogía de esponjas radica en que no se trata de un sistema formado por un huésped-un hospedador si no que toda una comunidad está involucrada (Hentschel *et al.* 2012), y está formada por filotipos que son diversos pero específicos del hospedador. Hasta ahora, nuestro conocimiento acerca de la microbiota de esponjas proviene principalmente de estudios sobre muestras recogidas en un punto concreto en el tiempo y el espacio, por lo que desconocemos el potencial dinamismo de la interacción. En concreto, sabemos relativamente poco de qué procesos gobiernan las comunidades simbiontes en gradientes espacio-temporales y cómo responden a perturbaciones en su ambiente.

Procesos que pueden afectar la estructura de la comunidad simbionte

La biogeografía microbiana evalúa los patrones en la estructura de las comunidades microbianas en el tiempo y el espacio. Estos patrones pueden emerger principalmente por dos procesos: limitación de la dispersión y selección (Martiny *et al.* 2006; Fierer 2008; Hanson *et al.* 2012). Las barreras a la dispersión limitan la conectividad entre poblaciones o localidades distantes, mientras que las condiciones locales del ambiente seleccionan a los microorganismos mejor adaptados. En el océano, la dispersión de microorganismos se considera pasiva y restringida por las corrientes y estructuras hidrogeográficas (Schauer *et al.* 2000; Galand *et al.* 2009); mientras que las condiciones ambientales que pueden estructurar las comunidades microbianas (por ej., salinidad, temperatura o concentración de nutrientes) pueden también seleccionar la proliferación de ciertos microorganismos y no otros en un momento dado y en una localidad en concreto (Schauer *et al.* 2003; Flo *et al.* 2011).

La estructura que presentan las comunidades microbianas en esponjas de mar no responde puramente al azar; análisis a nivel de comunidad revelan una marcada especificidad respecto a la especie de esponja (Taylor *et al.* 2007; Erwin *et al.* 2012a). Sin embargo, desconocemos la dinámica de estas comunidades específicas a lo largo de escalas espaciotemporales. Diversos estudios sugieren que la comunidad simbionte en esponjas de mar es estable a lo largo de escalas temporales y geográficas (ej., Taylor *et al.* 2003; Webster *et al.* 2004; White *et al.* 2012). Pero también otros trabajos detectaron diferenciación significativa en las comunidades de bacterias dependiendo de la localidad o la época de muestreo (ej., Wichels *et al.* 2006; Lee *et al.* 2009; Anderson *et al.* 2010). Además, la estructura de la comunidad simbionte puede variar de acuerdo con la especie de esponja estudiada, el parámetro

ambiental considerado y/o la escala del studio. También el plan de muestreo o el hecho de comparar esponjas filogenéticamente muy diferentes pueden distorsionar los patrones espacio-temporales y confundir los procesos involucrados. Por todo esto, urge describir cómo los factores ambientales, geográficos y específicos de la esponja interaccionan para conformar la comunidad de simbiontes a lo largo de escalas ecológicas. Conocer la variabilidad natural de esta simbiosis nos servirá también de punto de referencia para detectar la persistencia y resiliencia de la simbiosis frente a futuras perturbaciones.

Nuestra hipótesis es que los procesos que afectan a las comunidades microbianas del plancton son aplicables a las comunidades simbiontes. Condiciones locales pueden generar variabilidad intraespecífica de las comunidades asociadas a esponjas en distintas localidades, o pueden incluso homogeneizar las comunidades bacterianas en esponjas de especies diferentes pero que ocurren en simpatría. Finalmente, los factores relacionados con la especie de hospedador (tales como las condiciones internas en el mesohilo de la esponja, el estado fisiológico de la misma o su hábitat de preferencia) pueden también dirigir la composición y la estructura de las comunidades simbiontes a lo largo de escalas temporales y espaciales.

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Respuesta de la simbiosis esponja-comunidad microbiana frente a perturbaciones ambientales

Los organismos marinos están y estarán directamente afectados por los incrementos de temperatura, los cambios en la circulación oceánica y una cada vez más marcada estacionalidad de las condiciones ambientales (Harvell *et al.* 2002; Calvo *et al.* 2011; Crisci *et al.* 2011). Estas condiciones ligadas al cambio climático se han relacionado con enfermedades y mortalidades de esponjas, un fenómeno cada vez más común en océanos de todo el mundo (Webster 2007; Coma *et al.* 2009). En un escenario de aumento de la densidad de población humana y de las perturbaciones en las condiciones ambientales, cada vez más drásticas y repetidas, surge la necesidad de evaluar la respuesta de la simbiosis esponja-comunidad microbiana frente a estos cambios para una correcta gestión de esta biodiversidad y su preservación como recurso.

Hasta hoy, pocos estudios han descrito cómo las comunidades asociadas a esponjas responden al estrés ambiental. En el caso de las comunidades microbianas de corales, se ha propuesto que éstas pueden responder dinámicamente a variaciones en su ambiente, de manera que el holobionte se adapta rápidamente (en días o

semanas) a las nuevas condiciones (“The coral probiotic hypothesis”, [Reshef *et al.* 2006a]). En cambio, en las comunidades microbianas de esponjas, los resultados derivados de experimentos en acuario con distintas condiciones de estrés (por ej., alta temperatura, presencia de contaminantes, incremento de la concentración de nutrientes) muestran que las alteraciones en la estructura de la comunidad simbionte ocurren simultáneamente al empeoramiento de la salud de la esponja hospedadora (Webster *et al.* 2001; Simister *et al.* 2012c). En regiones templadas, se ha demostrado que la comunidad simbionte puede cambiar en respuesta a altas temperaturas (Lemoine *et al.* 2007), aunque se mantiene estable bajo estrés por reducción de la concentración de partículas en el agua (Friedrich *et al.* 2001). Se necesitan más estudios para investigar el efecto de alteraciones en las condiciones ambientales sobre las bacterias asociadas a esponjas.

Nuestro modelo: las comunidades bacterianas en esponjas del género *Ircinia*

El género *Ircinia* (Dyctioceratida: Irciniidae) exhibe una alta riqueza de especies y se encuentra ampliamente distribuido en zonas troplicales y templadas. Distintas especies de *Ircinia* pueden vivir en simpatría y alcanzar altas densidades en los fondos rocosos en los que habitan (Parra-Velandia & Zea 2003; Turon *et al.* 2013). Además, se ha demostrado que las especies de este género producen un amplio espectro de metabolitos secundarios con actividad citotóxica y antimicrobiana (Duque *et al.* 2001; Hammami *et al.* 2010) que, al menos en algunos casos, se ha demostrado que son sintetizados por sus simbiontes (Esteves *et al.* 2013).

En esta tesis hemos estudiado cinco especies distintas: *I. felix* e *I. strobilina* de Bahamas e *I. fasciculata*, *I. variabilis* e *I. oros* del Mar Mediterráneo. Estas especies son esponjas HMA que albergan complejas comunidades simbióticas, en ellas podemos encontrar bacterias de los filos *Proteobacteria, Acidobacteria, Bacteroidetes, Nitrospira* y también *Chloroflexi, Firmicutes, Poribacteria* y *Actinobacteria* (Schmitt *et al.* 2007; Mohamed *et al.* 2008c; Yang *et al.* 2011; Erwin *et al.* 2012a). Además, las esponjas *I. felix*, *I. fasciculata* e *I. variabilis* albergan densas poblaciones de fotosimbiontes (*Cyanobacteria*) que están ausentes en las comunidades asociadas a *I. oros* e *I. strobilina* (Schmitt et a. 2007; Mohamed *et al.* 2008c, Erwin *et al.* 2012a). Y, como en otras esponjas HMA, los adultos pueden transmitir simbiontes a su descendencia (Schmitt *et al.* 2007; Ereskovsky & Tokina 2004).

Dentro de cada una de las regiones estudiadas en esta tesis (Las Bahamas y Mediterráno Occidental), estas especies de *Ircinia* se pueden encontrar viviendo en simpatría. Por ello, ofrecen la oportunidad de plantear un diseño experimental que permita una mejor discriminación entre la influencia del ambiente y del hospedador sobre las comunidades de bacterias simbiontes, ya que estaremos investigando comunidades simbióticas en esponjas filogenéticamente próximas y que comparten las mismas condiciones ambientales. Utilizando este diseño, Erwin *et al.* (2012a) describieron que las comunidades simbiontes en las tres especies de *Ircinia* del Mediterráneo presentaban bacterias relacionadas con otras que habían sido detectadas también en distintas especies de esponjas o en corales. Sin embargo, a nivel de comunidad, cada especie de esponja alberga una comunidad bacteriana específica, lo que los autores denominaron "cóctel específico de generalistas" ("specific mix of generalists"). En el caso de las especies de Las Bahamas, un estudio reciente demostró que *I. strobilina* alberga comunidades distintas de las de otras esponjas filogenéticamente distantes pero que ocurren en simpatría en ciertas localidades (Yang *et al.* 2011); sin embargo, los estudios en *I. felix* e *I. strobilina* describen sus comunidades de bacterias usando técnicas diferentes (Schmitt *et al.* 2007; Mohamed *et al.* 2008c; Yang *et al.* 2011), lo que dificulta determinar el nivel de especificidad en la simbiosis de las especies de *Ircinia* en Las Bahamas. Aunque la diversidad bacteriana asociada a algunas de las especies de *Ircinia* ha sido descrita anteriormente, se desconoce si el patrón de especificidad que presentan las comunidades asociadas a esponjas del género *Ircinia* se mantiene a lo largo de una escala espacial y temporal o bajo condiciones de estrés.

OBJETIVOS

El principal objetivo de esta tesis es identificar la especificidad y persistencia de las comunidades simbiontes asociadas con esponjas HMA y su respuesta a diferentes condiciones ambientales. Para ello, hemos usado como modelo esponjas simpátricas del género *Ircinia*. Así, podremos distinguir entre el efecto de las condiciones ambientales y de la especie de hospedador considerada sobre la estructura de la comunidad simbiótica. Además, podremos definir una línea de base para identificar alteraciones en la comunidad simbionte o predecir bajo qué condiciones la simbiosis se puede ver comprometida, en especial en un escenario de cambio climático.

En concreto, queremos evaluar el efecto de las corrientes y la heterogeneidad espacial del ambiente sobre las comunidades bacterianas asociadas con esponjas que abundan en Las Bahamas (*Ircinia felix* e *Ircinia strobilina*) a escalas de cientos de

kilómetros, y confirmar si el mismo patrón es también válido para las especies mediterráneas (*Ircinia fasciculata*, *Ircinia variabilis* e *Ircinia oros*). Dado que el Mediterráneo Occidental se caracteriza por una marcada estacionalidad en las condiciones del agua (es decir, temperatura, irradiancia, nivel de nutrientes), también hemos querido investigar la dinámica temporal que potencialmente pueden sufrir sus comunidades bacterianas dependiendo de la estación del año. Finalmente, considerando las recientes mortalidades en masa que han sufrido las esponjas y otros invertebrados filtradores en el Mediterráneo en relación con el estrés térmico, hemos estudiado si cambios anormales en el ambiente (incremento de la temperatura o disminución de la densidad de partículas en el agua) pueden influir en la composición de las comunidades simbiontes y comprometer la supervivencia del holobionte. La caracterización y monitoreo de las comunidades bacterianas simbiontes ha sido evaluada a través de microscopía electrónica de transmisión y estudios moleculares de secuencias del gen ARN ribosomal 16S (librerías de clones y huella molecular). La técnica de huella molecular utilizada ha sido T-RFLP (polimorfismo de la longitud de los fragmentos de restricción terminales) y ha hecho posible el procesamiento estandarizado de muestras replicadas para su análisis estadístico.

Para cumplir con el objetivo principal, esta tesis ha sido estructurada en 4 capítulos que responden a cuatro objetivos específicos. Cada capítulo ha sido escrito de manera independiente con su propia introducción, metodología, resultados y discusión. Pero todos están interconectados y pueden ocasionalmente contener referencias cruzadas a otros capítulos.

El **capítulo 1** evalúa la especificidad del hospedador y la variabilidad espacial (a una escala que va de los 80 a los 400 km) de las comunidades bacterianas asociadas con *Ircinia felix* e *I. strobilina* en Las Bahamas. Para esto, muestreamos individuos de *I. strobilina* y dos morfotipos distintos de *I. felix* (oscuro y claro), así como el agua circundante, en distintas localidades de Bahamas. Las comunidades bacterianas fueron caracterizadas mediante microscopía electrónica y el análisis de T-RFLP y librerías de clones del gen ARNr 16S bacteriano. También secuenciamos un fragmento del gen mitocondrial citocromo oxidasa I (COI) para determinal la identidad y las relaciones filogenéticas entre las especies hospedadoras.

El **capítulo 2** investiga si el patrón de especificidad observado en las comunidades bacterianas asociadas a esponjas *Ircinia* del Mediterráneo (*Ircinia fasciculata*, *I. variabilis* e *I. oros*) se mantiene a una escala espacial de cientos de kilómetros, con un diseño comparable al empleado para estudiar la variabilidad espacial en las especies *Ircinia* de Bahamas. Para ello, usamos el análisis de T-RFLP de del gen ARNr 16S bacteriano para describir las comunidades bacterianas en individuos de *I. fasciculata*,

I. variabilis e *I. oros* muestreados en seis localidades del Mediterráneo Occidental, afectadas por distintas corrientes y bajo distinta presión antropogénica.

El **capítulo 3** busca estudiar cómo las comunidades bacterianas responden a cambios en la estacionalidad de las condiciones del agua (temperatura e irradiancia) para distinguir entre bacterias asociadas permanente o transitoriamente con su esponja hospedadora. Para alcanzar este objetivo, se monitorearon las comunidades bacterianas de individuos de cada especie de *Ircinia* del Mediterráno durante un año y medio, cada tres meses, gracias a análisis de T-RFLP y de las librerías de clones del gen bacteriano ARNr 16S. Además, se midió la concentración de pigmentos (clorofila *a*) en los tejidos de las esponjas ricas en fotosimbiontes (*I. fasciculata* e *I. variabilis*).

En el **capítulo 4** quisimos detectar cambios en las comunidades simbiontes en respuesta a condiciones anormales de temperatura y estratificación de la columna de agua (lo que conlleva una disminución de la concentración de partículas de las que la esponja se alimenta), similares a las observadas en veranos “anormales” que dieron lugar a mortalidades en masa en poblaciones de esponjas. Para imitar estas condiciones, se llevaron acabo experimentos en condiciones controladas de acuario para evalúar el efecto de cuatro tratamientos distintos (control, incremento de temperatura, reducción en la cantidad de partículas en el agua, y combinación de los estrés de temperatura y reducción de alimento) en las comunidades bacterianas asociadas a *I. fasciculata* e *I. oros*. Las comunidades bacterianos fueron caracterizadas a través de análisis de T-RFLP de secuencias del gen bacteriano ARNr 16S y de microscopía electrónica. Además, en *I. fasciculata* calculamos el contenido en clorofila *a* como estima de la abundancia de las poblaciones simbiontes.

RESULTADOS Y DISCUSIÓN

Patrones de diversidad de las comunidades bacterianas en esponjas del género *Ircinia*

En el **capítulo 1** estudiamos la especificidad y biogeografía de las comunidades bacterianas asociadas con *Ircinia strobilina* y dos morfotipos de *I. felix*, que se encuentran a menudo viviendo en simpatría. Muestreamos las esponjas en arrecifes de Las Bahamas, localizados en islas con distinta densidad de población humana y afectadas por distintas corrientes oceanográficas (Colin 1995), en un rango de hasta 400 km de distancia. Los análisis de T-RFLP y las librerías de clones de secuencias del gen ARNr 16S confirmaron que cada una de las especies de *Ircinia* alberga una comunidad bacteriana específica, claramente diferenciada de la comunidad en el

bacterioplancton, y que las comunidades simbiontes de ambos morfotipos de *I. felix* (claro y oscuro) son similares entre sí y distintas de la comunidad en *I. strobilina*. Los análisis filogenéticos de las secuencias bacterianas confirmaron que estas esponjas presentan un patrón similar al observado en las especies del Mediterráneo, con comunidades simbiontes específicas para cada especie pero compuestas por bacterias similares a las encontradas en otras especies de esponjas y en corales ("specific mix of generalists" o "cóctel específico de generalistas"). Además, los perfiles de T-RFLP revelaron que dentro de cada especie de esponja, la comunidad bacteriana se mantenía estable, sin importar la localidad donde fueron muestreadas. Estos resultados muestran un alto grado de fidelidad del simbionte hacia su hospedador a lo largo de una escala espacial de hasta 400 km.

A continuación, quisimos testar si el mismo patrón de estabilidad espacial se cumplía en las comunidades bacterianas asociadas con las esponjas *Ircinia* del Mediterráneo (**capítulo 2**). Los análisis multivariantes de los perfiles de T-RFLP mostraron que las comunidades bacterianas asociadas con esponjas *Ircinia* del Mediterráneo estaban estructuradas de acuerdo a la especie considerada y se mantenían estables a lo largo de todas las localidades de muestreo, independientemente de la distancia considerada (80-800 km). Esta especificidad y estabilidad espacial fue observada tanto en los miembros dominantes de la comunidad simbiontes como en los raros (es decir, aquéllos presentes a baja densidad, picos de T-RF con < 1% abundancia relativa). Aunque se detectaron diferencias significativas entre algunas localidades en comunidades bacterianas de *I. variabilis*, no existe correlación entre la distancia geográfica y la similaridad de las comunidades para ninguna especie de esponja.

Ambos capítulos (**capítulo 1** y **capítulo 2**) demuestran la persistencia de las comunidades asociadas a esponjas del género *Ircinia*, en acuerdo con lo encontrado en otras especies de esponjas a escalas espaciales similares (Webster *et al.* 2004; Wichels *et al.* 2006; Thiel *et al.* 2007a). Además, Montalvo & Hill (2011), que también realizaron un estudio con esponjas congenéricas (*Xestospongia muta vs X. testudinaria*), encontraron que sus comunidades simbiontes eran muy similares, a pesar de que cada especie habita océanos diferentes (Atlántico e Indopacífico, respectivamente). También Reveillaued *et al.* (2014) observaron estabilidad espacial en las bacterias asociadas con esponjas *Hexadella* spp. a lo largo de gradientes batimétricos (aguas someras *vs* aguas profundas) e independientemente de la localidad muestreada. La estabilidad espacial de las comunidades simbiontes en esponjas HMA filogenéticamente cercanas confirma que factores específicos de las

condiciones ambientales locales y la limitación de la dispersión tienen poco efecto sobre las bacterias asociadas a esponjas de mar.

Dado que las condiciones de la columna de agua presentan una marcada estacionalidad en el Mediterráneo en términos de temperatura, irradiancia y disponibilidad de nutrientes, en el **capítulo 3** realizamos un monitoreo de los simbiontes bacterianos en individuos de las tres esponjas simpátricas (*I. fasciculata, I. variabilis* e *I. oros*) a lo largo de 1,5 años en una localidad del noroeste del mar Mediterráneo, muestreando los mismos individuos cada 3 meses. Los simbiontes de *Ircinia* spp. mostraron una notable estabilidad a lo largo de todo el periodo de monitoreo, a pesar de fluctuaciones en las condiciones de temperatura (hasta 12°C más en verano que en invierno) e irradiancia (aproximadamente 5 veces mayor en verano que en invierno, con unas 6 h más de luz en verano). La estabilidad estacional observada en este trabajo concuerda con la encontrada en otros estudios (Thacker & Freeman 2012; Lee *et al.* 2011; Taylor *et al.* 2007), y contrasta con los estacionalidad significativa en la estructura y composición de las comunidades bacterianas en el agua circundante (Schauer *et al.* 2000; 2003; Pinhassi *et al.* 2006; **capítulo 3**). La marcada diferencia en los patrones temporales de las comunidades bacterianas asociadas a esponjas *vs* las comunidades del bacterioplancton pone en evidencia que los factores ecológicos que estructuran las comunidades bacterianas de vida libre son distintos de los que afectan a las que son simbiontes de esponjas.

Una alta dispersión de la esponja hospedadora podría explicar en parte la estabilidad espacial en la estructura de la comunidad simbiótica, si la mayoría de los simbiontes son transmitidos de los adultos a su descendencia (Ereskovsky *et al.* 2004; Schmitt *et al.* 2007; Lee *et al.* 2009). Sin embargo, considerando la demostrada falta de correlación entre distancia geográfica y disimilaridad de las comunidades bacterianas (Schmitt *et al.* 2012; **capítulos 1** y **2**) y la posible transmisión horizonal de los simbiontes (Taylor *et al.* 2007; 2012), parece que no sólo la dispersión del hospedador justifica la alta persitencia de las comunidades bacterianas asociadas a esponjas. En cambio, nosotros proponemos que la homeostasis del animal y la actividad de la comunidad simbionte contribuyen al mantenimiento de unas condiciones estables en el mesohilo de la esponja, tamponando las fluctuaciones en el ambiente externo y manteniendo así la composición y estructura de la comunidad de simbiontes.

La afiliación filogenética de las bacterias dominantes en las *Ircinia* spp. de Bahamas (**capítulo 1**) y del Mediterráneo (**capítulo 3**), derivada de las librerías de clones de secuencias del gen ARNr 16S, nos aporta información acerca de su capacidad metabólica; tales como fotosíntesis (*Cyanobacteria*), oxidación del nitrito

(*Nitrospira*) or reducción del sulfato (*Desulfovibrionales*). Estos procesos metabólicos ocurren exclusivamente en procariotas y se cree que el aprovechamiento por parte del hospedador de los compuestos y nutrientes derivados de estas rutas le confieren una ventaja evolutiva de la cual carecería si la simbiosis no existiera. Se cree que el mantenimiento de esas funciones metabólicas y las consecuencias ecológicas derivadas de ellas podrían también ser claves para los mecanismos de selección que establecen y mantienen gremios específicos en la comunidad bacteriana asociada a las esponjas (Yang *et al.* 2011).

A pesar de la estabilidad general, también detectamos cierta variabilidad intraspecífica en las comunidades bacterianas simbiontes (aunque siempre menor que la variabilidad interespecífica). En el estudio espacial del Mar Mediterráneo (**capítulo 2**), observamos ciertas variaciones significativas en la abundancia relativa de las bacterias dominantes, aunque su dominancia frente a otras bacterias se mantuvo. En cambio, en el estudio temporal (**capítulo 3**), observamos que las comunidades de los meses cálidos eran más variables, con filotipos de bacterias que estaban sólo presentes en esos meses y no eran detectados en las librerías de clones el resto de estaciones del año, pero estas fluctuaciones se limitaron a miembros raros de la comunidad bacteriana (presentes en poca abundancia o sólo en una muestra). No está claro si estos resultados reflejan la adaptación de simbiontes o realmente se trata de bacterias no simbiontes que sirven de comida para la esponja o se encuentran de manera transitoria en el agua de sus canales acuíferos (Pile *et al.* 1996; Webster *et al.* 2002; Lee *et al.* 2006).

Resulta particularmente interesante que algunas bacterias fueron detectadas, a lo largo de todas las estaciones, asociadas a algunos individuos pero no a otros dentro de una misma especie de esponja (**capítulo 3**). La persistencia temporal de estos taxones sugiere que son simbiontes verdaderos. La presencia de simbiontes que ocurren esporádicamente dentro de una especie de esponja sugiere cierta plasticidad en el establecimiento de la simbiosis. Las implicaciones de esta variabilidad en la ecología del hospedador y la evolución de las simbiosis se desconocen, pero potencialmente la actividad de esos simbiontes puede ser ventajosa en determinadas condiciones ambientales.

Cabe destacar que *I. variabilis* fue la especie, de las tres que habitan el Mediterráneo, que presentó más variabilidad intraespecífica en su comunidad de bacterias (**capítulo 2**). Tal y como sugirieron Lee *et al.* (2009a) diferentes patrones en la estructura de la comunidad simbionte podrían emerger en relación con factores específicos de cada especie. En el caso de *I. variabilis*, podrían ser su mayor plasticidad (Turon *et al.* 2013) o su estrategia reproductiva. En todo caso,

características específicas pueden influir en la variabilidad de la asociación esponja-comunidad bacteriana. Sería interesante realizar más estudios en otras especies de esponjas filogenéticamente próximas y que ocurren en simpatría para definir características específicas concretas que pueden potencialmente favorecer una mayor variabilidad en la composición y estructura de la comunidad simbionte.

El **capítulo 3** muestra que factores específicos de la especie de esponjas, como su hábitat de preferencia, también pueden afectar a la función de la comunidad simbionte. En concreto, la actividad fotosimbiótica (evaluada en términos de contenido en clorofila *a*) fue mayor en *I. fasciculata* que en *I. variabilis*. Además, los niveles de clorofila *a* presentaron una marcada estacionalidad en *I. fasciculata*, con valores más bajos en los meses de mayor irradiancia y más horas de luz (Junio y Septiembre). En cambio, *I. variabilis* presentó niveles más uniformes de clorofila *a*, a excepción de un descenso significativo en Septiembre. Las poblaciones de fotosimbiontes en ambas especies de esponjas están formadas por *Candidatus Synechococcus spongiarum*, pero las poblaciones respondieron de manera diferente a la estacionalidad en las condiciones del agua, en concordancia con los distintos niveles de luz del hábitat de preferencia del hospedador, ya que *I. variabilis* se encuentra en hábitats más sombríos y crípticos, mientras que *I. fasciculata* domina en ambientes expuestos y con altos niveles de luz (Erwin *et al.* 2012c; Turon *et al.* 2013). Por tanto, aunque los factores que determinan la comunidad microbiana difieren entre la esponja y el agua de mar o los sedimentos (por ej., la concentración de nutrientes), otras limitaciones fisiológicas como la luz pueden ser compartidas entre las bacterias de vida libre y las simbiontes. Sin embargo, el efecto en la comunidad simbionte va a a estar marcado por la especie de hospedador considerada.

Vulnerabilidad de las comunidades bacterianas simbiontes de esponjas a perturbaciones en su ambiente

En el **capítulo 4**, realizamos experimentos de acuario para testar en las comunidades bacterianas simbiontes el efecto de condiciones de estrés en temperatura y reducción de disponibilidad de alimento semejantes a las que se dieron en el Mediterráneo Occidental en veranos con picos de temperatura anormalmente altos durante días-semanas y que, como consecuencia, aumentaron también la duración de estratificación de la columna de agua. Estos veranos con condiciones atípicas coincidieron con episodios de mortalidades en masa de distintas especies de invertebrados filtradores (Coma *et al.* 2009; Garrabou *et al.* 2009), entre los cuales se encontraba *I. fasciculata* (Cebrian *et al.* 2011).

A pesar de que sometimos a los individuos de *I. fasciculata* e *I. oros* a distintos tratamientos (control, temperatura elevada, agua filtrada y combinación de temperatura elevada y agua filtrada) durante 3 semanas, los perfiles de T-RFLP de secuencias del gen ARNr 16S bacteriano fueron estables, demostrando que no existían diferencias significativas en la comunidad bacteriana simbionte entre los distintos tratamientos testados, para ninguna de las dos especies. Estos resultados nos llevan a concluir que las mortalidades observadas no se relacionan directamente con un declive en la composición de la comunidad simbionte en estas esponjas.

Aunque no podemos descartar que experimentos a más largo plazo (meses) puedan resultar en un efecto significativo, la persistencia encontrada en este estudio es todavía sorprendente. La temperatura testada (25^{o}C) representa 3^{o}C más que la temperatura media en verano del área donde las esponjas del estudio fueron recolectadas (**capítulo 3**), coincidiendo con máximos de temperatura en los años de verano anómalo (Cebrian *et al.* 2011) y representando un incremento de > 11^{o}C respecto a las condiciones ambientales en el momento de recolección. Además, la duración de los experimentos (3 semanas tras la aclimatación) concuerda con la duración de los picos de temperatura de esos años de mortalidades en masa en *I. fasciculata* y otros invertebrados (Cebrian *et al.* 2011).

Los estudios en microbiología de esponjas enfocadas a resolver la respuesta de las comunidades simbiontes al estrés son escasos, pero sugieren que la asociación esponja-comunidad bacteriana es altamente persistente hasta que un determinado umbral es sobrepasado. En la esponja *Rhopaloeides odorabile*, que habita en la Gran Barrera de Coral, la comunidad bacteriana cambia únicamente cuando el tejido de la esponja es necrótico, lo cual ocurrió cuando las esponjas fueron expuestas a temperaturas 2-4^{o}C por encima de la temperatura media del área de estudio (Webster *et al.* 2008a; Simister *et al.* 2012b; 2012c). Estos estudios ejemplifican como, pasada una temperatura umbral, no sólo la comunidad bacteriana cambia sino que todo el holobionte colapsa.

De acuerdo con la hipótesis probiótica en corales ("*coral probiotic hypothesis*"), las comunidades bacterianas asociadas a corales cambian rápidamente (en cuestión de días o semanas) en respuesta a cambios en el ambiente, lo que proporciona una respuesta adaptativa del holobionte para sobrevivir a esas perturbaciones. Pero ése no parece ser el caso en las esponjas. La compleja diversidad de las comunidades bacterianas en esponjas, comparable a la que se encuentra en el intestino humano (Hentschel *et al.* 2012), puede proporcionar robustez frente a las perturbaciones naturales (por ej., la invasión de microbios oportunistas que proliferan en los meses cálidos). Sin embargo, en los estudios de estrés térmico en *R. odorabile*, la comunidad

simbiontes se ve afectada cuando todo el holobionte colapsa (Webster *et al.* 2008a; Simister *et al.* 2012b; 2012c). En un escenario de creciente temperatura del agua, acidificación y aumento de la sedimentación (Coma *et al.* 2009; Calvo *et al.* 2011; Crisci *et al.* 2011), la fiel y estable simbiosis de la esponja y su comunidad bacteriana puede ser incapaz de responder lo suficientemente rápido como para sobrellevar las repetidas perturbaciones y su supervivencia se puede ver comprometida (Webster *et al.* 2008a; Simister *et al.* 2012b; Fan *et al.* 2013).

En conclusión, hemos investigado las comunidades bacterianas asociadas con esponjas del mismo género que ocurren en simpatría y mostramos que procesos específicos de la especie de hospedador, y no factores biogeográficos, son los principales responsables de estructurar las comunidades bacterianas simbiontes de esponjas. Las comunidades bacterianas en las esponjas de *Ircinia* fueron específicas de la especie considerada, y estables en escalas espaciales (cientos de kilómetros) y temporales (a lo largo de distintas estaciones del año). Incluso una exposición a corto plazo a condiciones de temperatura elevada y limitación de las partículas bacterianas que forman parte de la dieta de la esponja, no causaron ningún cambio en la comunidad bacteriana simbionte. Nuestros resultados sugieren una íntima y estable asociación de las esponjas HMA con sus comunidades bacterianas. Además, proponemos que esta simbiosis ha resultado en un sistema cooperativo en el que la actividad de la propia comunidad, junto con la homeostasis del animal hospedador, contribuyen a mantener un microambiente estable en el mesohilo de la esponja y tampona las perturbaciones en el medio externo. Así, la simbiosis habría evolucionado para persistir como unidad bajo distintas condiciones ambientales. La amenaza de la degradación del hábitat y el impacto del cambio climático (asociado al incremento en la temperatura del agua y una extrema estacionalidad), podría conllevar tanto la pérdida de esta compleja simbiosis como de los servicios que proporcionan al ecosistema y como recurso biotecnológico. Por todo ello, es imprescindible abordar las investigaciones en biología de esponjas, ecología y evolución, así como las estrategias de gestión, desde la perspectiva del holobionte (la esponja y sus microorganismos simbiontes).

Perspectivas

Hemos demostrado que los factores específicos de la esponja son los que principalmente determinan las comunidades bacterianas simbiontes. En la discusión, hemos propuesto que el mesohilo de la esponja es un hábitat estable que tampona las

perturbaciones en el ambiente externo. El mesohilo de la esponja ha sido definido como anaeróbico y rico en nutrientes (Hoffmann *et al.* 2005), pero las condiciones fisicoquímicas han sido muy poco estudiadas. El uso de microsensores para describir los parámetros fisicoquímicos (por ej., pH, niveles de O_2 o cantidad de luz, etc.) del mesohilo frente a distintas condiciones externas, tal y como se ha hecho en otros invertebrados marinos (Kühl *et al.* 2012), o comparando esponjas que están bombeando agua activamente frente a las que no, ayudaría a definir los distintos microhábitats y testar su estabilidad. Además, la aplicación de estas técnicas a esponjas filogenéticamente próximas, permitiría arrojar luz a cómo las condiciones internas pueden controlar la especificad de las comunidades bacterianas hacia la especie de esponja.

Aunque ahora conocemos mejor los factores que afectan la *estructura* de las comunidades bacterianas en esponjas, los estudios de la respuesta *funcional* de los simbiontes frente a diferente condiciones ambientales se limita todavía a pocas especies de esponjas (ej., Fan *et al.* 2013) o linajes bacterianos concretos, tales como fotosimbiontes (ej., esta tesis) u organismos oxidadores del amonio (ej., López-Legentil *et al.* 2010). Estudios de metagenómica o transcriptómica son imprescindibles para entender el papel del metabolismo de los simbiontes para el holobionte y para comprobar si este papel es semejante o diferente dependiendo de la especie de esponja considerada (Liu *et al.* 2011; Thomas *et al.* 2010).

Además, las esponjas no sólo mantienen relaciones simbiontes con bacterias. También se han detectado arqueobacterias asociadas a esponjas de distintos océanos (Steger *et al.* 2008; López-Legentil *et al.* 2010; Turque *et al.* 2010; Radax *et al.* 2012) y también están presentes en las larvas de esponjas (Schmitt *et al.* 2008), lo que sugiere un estrecho vínculo entre estas arqueobacterias simbiontes y la esponja hospedadora. Sin embargo, las arqueobacterias asociadas a esponjas han sido menos estudiadas que las bacterias homólogas y conocemos muy poco de sus patrones de diversidad. También existen microorganimos eucariotas (ej, hongos, diatomeas, dinoflagelados) como simbiontes de esponjas y, aunque potencialmente pueden jugar un papel importante en el metabolismo del holobionte, a menudo son ignorados en los estudios de microbiología de esponjas (Hentschel *et al.* 2012). Por tanto, se necesita investigar más en este campo para obtener una visión integradora de la complejidad del holobionte y definir la función de las arqueobacterias y los microorganismos eucariotas en esta simbiosis.

Conclusiones

CAPITULO 1: Biogeografía y fidelidad de las comunidades bacterianas asociadas con esponjas *Ircinia* spp. de Las Bahamas

1. *Ircinia felix* e *I. strobilina*, esponjas abundantes en Las Bahamas, albergan comunidades bacterianas específicas de la especie considerada y distintas de las bacterias en el agua circundante, tal y como indican los resultados de análisis de T-RFLP y librerías de clones de secuencias del gen ARNr 16S. Sus comunidades simbiontes se componen de filotipos bacterianos relacionados con bacterias encontradas en otras especies de esponjas y corales de distintos océanos. Las comunidades bacterianas encontradas en los dos morfotipos de color de *I. felix* (blanco y oscuro) son más similares entre sí que a las comunidades en *I. strobilina*.
2. Las comunidades bacterianas de las especies de *Ircinia* de Las Bahamas mantuvieron su especificidad y permanecieron estables en las distintas localidades muestreadas, separadas por distancias de 80-400 km y bajo distinta presión antropogénica.
3. Nuestros resultados muestran una alta fidelidad de la comunidad simbionte a a la especie de hospedador, lo que sugiere que factores específicos de especie y no factores biogeográficos son los que estructuran y mantienen la simbiosis esponja-bacteria en las *Ircinia* spp. de Las Bahamas.

CAPÍTULO 2: El hospedador manda: estabilidad espacial en las comunidades bacterianas asociadas con esponjas marinas (*Ircinia* spp.) en el Mediterráneo Occidental

4. Los análisis multivariantes y gráficos nMDS de los perfiles T-RFLP derivados de las secuencias del gen ARNr 16S mostraron que las comunidades bacterianas en las esponjas del género *Ircinia* del Mediterráneo Occidental se estructuran de acuerdo con la especie de hospedador y se mantienen estables en distintas localidades separadas por distancias de 80-800 km y distintas condiciones locales. A pesar de la estabilidad espacial general, observamos cierta diferenciación en las comunidades bacterianas de *I. variabilis* en localidades concretas.
5. La especificidad de especie y la estabilidad espacial fueron observadas tanto en las bacterias dominantes como en las raras de los perfiles de T-RFLP derivados de una misma especie de esponja, aunque los miembros

dominantes presentaron cierta diferenciación espacial en términos de abundancia relativa.

6. No detectamos correlación entre la distancia geográfica y la disimilaridad de las comunidades simbiontes en individuos de una misma especie de esponja muestreados en distintas localidades.
7. Nuestros resultados indican que la estructura de las comunidades bacterianas y su variabilidad está determinada por factores específicos de la especie de esponja hospedadora.

CAPÍTULO 3: Estabilidad de las bacterias simbiontes de esponjas a lo largo de cambios estacionales en las condiciones de temperatura e irradiancia

8. Los simbiontes bacterianos de esponjas mediterráneas del género *Ircinia* mostraron una estructura específica y estable a lo largo de un año y medio de muestreo, a pesar de marcadas fluctuaciones en la temperatura del agua y las condiciones de irradiancia. En contraste, las comunidades del bacterioplancton mostraron una clara estacionalidad en su estructura.
9. La variabilidad en las bacterias asociadas a esponjas se limitó a los simbiontes raros y tuvo lugar principalmente en los meses más cálidos y de mayor irradiancia.
10. La estabilidad de las comunidades bacterianas en esponjas a pesar de la estación del año y de los marcados cambios estacionales en las comunidades bacterianas de vida libre del agua, apoyan la hipótesis de la estrecha relación esponja-bacteria. Además, la información derivada de este estudio nos proporciona una base de referencia para detectar posibles cambios anormales en las comunidades simbiontes en condiciones de estrés.

CAPÍTULO 4: Hasta que la muerte nos separe: la persistencia de la asociación esponja-bacteria frente a estrés térmico y de escasez de alimento

11. No se detectaron cambios significativos en los perfiles de T-RFLP, derivados de secuencias del gen ARNr 16S, en individuos de *I. fasciculata* e *I. oros* mantenidos en acuario durante 3 semanas bajo 4 tratamientos distintos: control (13°C, agua sin filtrar), baja disponibilidad de comida (13°C, agua filtrada a través de 0.1 µm), temperatura elevada (25°C, agua sin filtrar), y combinación de temperatura elevada y baja disponibilidad de comida (25°C, agua filtrada a través de 0.1 µm).
12. En *I. fasciculata*, el contenido de chl *a* no varió significativamente entre tratamientos, aunque las micrografías del TEM revelaron células de

cianobacteria en degradación en esponjas expuestas al tratamiento que combinaba elevada temperatura y agua filtrada.

13. No encontramos prueba empírica que apoye la idea de que la disgregación de la comunidad bacteriana simbiontes en condiciones de estrés sea la causa primaria de los episodios recurrentes de mortalidad en esponjas del Mediterráneo Occidental.

Annex

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Annex 1

TERMINAL RESTRICTION FRAGMENT LENGHT POLYMORPHISM (T-RFLP) TECHNIQUE

A great variety of bacteria have been found to be associated with marine sponges. Since cultivation of most symbiotic bacteria is challenging, the study of its diversity is based, in most of the cases, on the use of genetic tools. When aiming to describe patterns in symbiotic communities, DNA fingerprinting techniques provides a standardized method to process a suitable number of samples and replicates for statistical analysis in community patterns in a reasonable amount of time and spending a reasonable amount of money. This offers the possibility of conducting expansive studies to assess spatial and temporal changes in microbial diversity, or to systematically explore the effects of treatments and disturbances on microbial community composition and structure.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) technique uses restriction enzyme digestion of fluorescently tagged PCR-amplicons separated by capillary electrophoresis (Liu *et al.* 1997) (**Fig. 1**). T-RFLP analysis has higher detection sensitivity and reproducibility than another common DNA fingerprinting technique, denaturing gel gradient electrophoresis (DGGE) (Moesenedor *et al.* 1999; Schütte *et al.* 2008; Lee *et al.* 2009). By utilizing automated capillary electrophoresis, T-RFLP is well standardized and allows easy comparison and simultaneous processing of large numbers of samples.

For T-RFLP analysis, DNA is extracted using commercially available extraction kits. Amplification is carried out using a primer set suitable for amplifying a DNA fragment of interest, typically a fragment of the 16S rRNA gene. The PCR protocol needs a hexachlorofluorescein-label added to the forward primer. We performed triplicates of the PCR per each sample for reducing the PCR bias. PCR products from the same samples are pooled together for gel purification and cleaning using commercially available kits. DNA is then quantified to standardize the amount of clean PCR product in the digestion process. After digestion with restriction endonuclease (in our case, two separate digestions per sample, one per restriction enzyme) and then precipitated and dried. Final analyses are conducted on automated sequencers (e.g. ABI Prism 3100) using a size standard. Only the fluorescently labelled terminal restriction fragments are detected, and their sizes are determined by comparison to

those found the internal standard (LIZ600). Results were interpreted PeackScanner (Applied Biosystems).

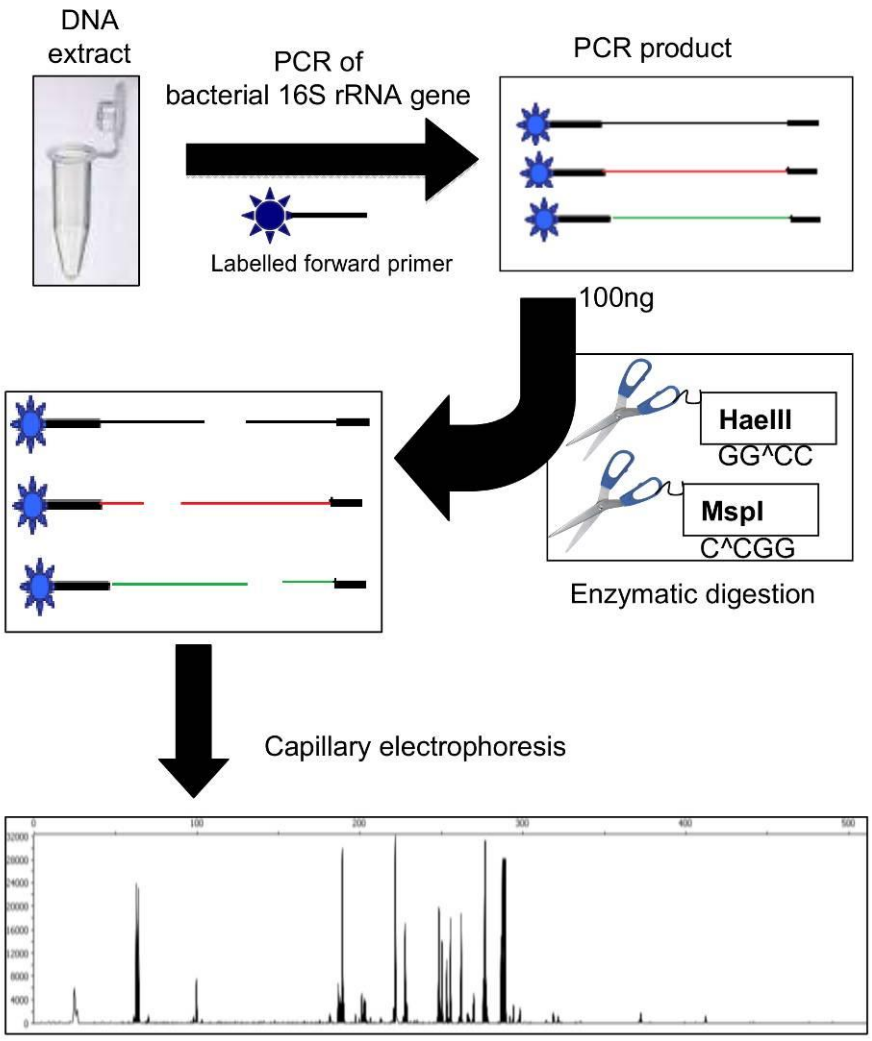

Figure 1. Scheme of T-RFLP technique.

T-REX provides a user-friendly platform to process our raw data in a standardized way and avoid unwanted variability when transforming the data into a format ready for analysis and interpretation (Culman *et al.* 2009). In T-REX we determined a baseline threshold for identification of true peaks over noise. Also, we aligned T-RFs (terminal restriction fragments) in all samples creating bins to overcome any drift between runs of capillary electrophoresis. Finally, we obtain two matrices, one for presence/absence and other for relative abundance of the T-RFs in the samples. Those matrices were used as input for comparing the bacterial communities in the samples based on similarity or distance measures and using multivariate statistics.

References

Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H & Buckley DH (2009) T-REX: Software for the processing and analysis of T-RFLP data. BMC Bioinform 10:171-180.

Doringo U, Volatier L & Humbert J-F (2005) Molecular approaches to the assessment of biodiversity in aquatic microbial communities. Water Res 39: 2207-2218.

Lee OO, Wong YH & Quian PY (2009) Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. Appl. Environ. Microbiol. 75: 3515-3521.

Liu WT, Marsh TL, Cheng H & Forney IJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphism of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63: 4516-4522.

Moesendor MM, Arrieta JM, Muyzer G, Winter C & Herndl GJ (1999) Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gel gradient electrophoresis. Appl. Environ. Microbiol. 65: 3518-3525.

Schütte UME, *et al.* (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. Appl Microbiol Biot 80: 365-380.

Annex 2

THE ONGOING DISCUSSION OF THE SYSTEMATIC POSITION OF *Ircinia* SPECIES

The sponge species targeted in this PhD study possess a skeletal structure of collagenous material but lack a mineral skeleton (i.e., spicules). As a result, the taxonomic status of these taxa and even the broader phylogenetic relationships within the family Irciniidae are uncertain (Cook & Bergquist 2002). This annex aims to provide a summary of the ongoing discussion of the systematic position of these taxa.

Pronzato *et al.* (2004) proposed a solution for the taxonomic status of *Ircinia felix*, *I. fasciculata* and *I. variabilis*, based on morphological characters of available type material and specimens recently collected in the Mediterranean Sea, the Caribbean Sea and the Gulf of Mexico. They supported *I. felix* and *I. variabilis* as valid species, with good descriptions and illustrations in the literature, and proposed *I. variabilis* as the type species of the genus *Ircinia*. However, they conclude that the taxonomic status of *I. fasciculata* and its position within the family Irciniidae remains unclear because of the lack of a fixed holotype and an invalid neotype. They proposed that this species should be included in the genus *Sarcotragus* and the taxon was tentatively transferred to that genus (van Soest *et al.* 2013).

Molecular assessment of the genera *Ircinia*, *Sarcotragus* and *Psammocinia*, the three genera that comprise the family *Irciniidae*, suggests that *Ircinia* is paraphyletic with respect to *Sarcotragus* (Erpenbeck *et al.* 2012). Molecular phylogenetic analyses of mitochondrial and ribosomal markers (Erwin *et al.* 2012) showed that *I. fasciculata* and *I. variabilis* are closely-related but genetically distinct taxa (i.e., species/morphotypes) that clearly belong in the same genus. The phylogenetic analyses presented in this thesis (**chapter 1**) with the taxa from the Mediterranean and the Bahamas confirmed that the sponge species analyzed herein should be considered congeneric species regardless of the higher level taxonomic issues (i.e., paraphyletic genera) in family Irciniidae.

References

Cook SdC & Bergquist PR (2002) Family Irciniidae Gray, 1867. In: Hooper JNA, Soest RWM van, eds. Systema Porifera A guide to the classification of sponges. New York, Boston, Dordrecht, London, Moscow: Kluwer Academic/Plenum Publishers. pp 1022–1027.

Erpenbeck D, *et al.* (2012) Horny sponges and their affairs: On the phylogenetic relationships of keratose sponges. Mol Phylogenet Evol, 63, 809–816.

Erwin PM, Pita L, López-Legentil S, & Turon X (2012) Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance. Appl Environ Microbiol, 78(20), 7358–68.

Pita L, López-Legentil S, & Erwin PM (2013) Biogeography and Host Fidelity of Bacterial Communities in *Ircinia* spp. from the Bahamas. Microbial Ecol, 66(2), 437–47.

Pronzato R, Malva R, & Manconi R (2004) The taxonomic status of *Ircinia fasciculata*, *Ircinia felix* and *Ircinia variabilis* (Dictyoceratida, Irciniidae). Boll Mus Ist Biol Univ Genova, 68, 553–563.

van Soest, RWM (2013) *Hircinia fasciculata* (Pallas, 1766). In: Van Soest R.W.M; Boury-Esnault N.; Hooper J.N.A.; Rützler K.; de Voogd N.J.; Alvarez de Glasby B.; Hajdu E.; Pisera A.B.; Manconi R.; Schoenberg C.; Janussen D.; Tabachnick K.R., Klautau M.; Picton B.; Kelly M.; Vacelet J.; Dohrmann M.; Díaz MC. (2013) World Porifera database.

Annex 3

PUBLICATIONS

Pita L, López-Legentil S & Erwin PM (2013): Biogeography and host fidelity of bacterial communities in *Ircinia* spp from the Bahamas. Microbial Ecology 66:437-447.

Pita L, Turon X, López-Legentil S & Erwin PM (2013): Host rules: spatial stability of bacterial communities associated with marine sponges (*Ircinia* spp) in the Western Mediterranean Sea. FEMS Microbiology Ecology 86:268-276.

Erwin PM, Pita L, López-Legentil S & Turon X (2012): Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance. Applied and Environmental Microbiology 78(20):7358-7368.

Pita L, Erwin PM, Turon X & López-Legentil S (2013): Till death does us apart: Stable sponge-bacteria associations under thermal and food shortage stresses. PLoS One 8:e80307.

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INVERTEBRATE MICROBIOLOGY

Biogeography and Host Fidelity of Bacterial Communities in *Ircinia* spp. from the Bahamas

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Abstract Research on sponge microbial assemblages has revealed different trends in the geographic variability and specificity of bacterial symbionts. Here, we combined replicated terminal-restriction fragment length polymorphism (T-RFLP) and clone library analyses of 16S rRNA gene sequences to investigate the biogeographic and host-specific structure of bacterial communities in two congeneric and sympatric sponges: *Ircinia strobilina*, two color morphs of *Ircinia felix* and ambient seawater. Samples were collected from five islands of the Bahamas separated by 80 to 400 km. T-RFLP profiles revealed significant differences in bacterial community structure among sponge hosts and ambient bacterioplankton. Pairwise statistical comparisons of clone libraries confirmed the specificity of the bacterial assemblages to each host species and differentiated symbiont communities between color morphs of *I. felix*. Overall, differences in bacterial communities within each host species and morph were unrelated to location. Our results show a high degree of symbiont fidelity to host sponge across a spatial scale of up to 400 km, suggesting that host-specific rather than biogeographic factors play a primary role in structuring and maintaining sponge–bacteria relationships in *Ircinia* species from the Bahamas.

Introduction

Sponges are among the most significant groups in marine benthic communities due to their high abundance and diverse functional roles [11, 29, 80]. However, much of their contributions to benthic ecosystems derive from their association with an abundant and complex microbiota [71, 73, 83]. The metabolic activity of microbial symbionts within sponges significantly contributes to nutrient fluxes between benthic and pelagic systems and renders sponges critical to healthy ecosystem functioning [57]. Sponge–microbial relationships have often been considered mutualistic. Sponges may offer a range of nutrient-rich microhabitats and shelter from predators to their microbial symbionts [59, 71]. In exchange, the microbial community can supplement the nutrition of their host via processes like photosynthesis [24], nitrogen fixation [45], or ammonia oxidation [40]. In addition, microbial symbionts can actively participate in the chemical defense of the holobiont by producing secondary metabolites, some of which have interesting biomedical and industrial applications [23, 50, 52].

As a result of the biological, ecological, and biotechnological importance of the sponge holobiont, studies have begun to focus on understanding the diversity and structuring factors of sponge-associated microbial communities [22, 31, 36]. Similar to free-living microorganisms [30], environmental conditions (e.g., distinct bioclimatic zones [70] or reefs [38, 48]) and dispersal limitation (i.e., isolation-by-distance) may influence the composition and structure of symbiotic bacterial communities. The relative effect of each process varies depending on the scale of sampling: large-scale patterns (tens of thousands of kilometers) appear to be more affected by dispersion limitations and small scale patterns (few kilometers) by environmental conditions, whereas intermediate scale patterns (10–3,000 km) are influenced by both processes [43]. Particular to host-associated microbes, the mode of symbiont transmission may also dictate the specificity and spatial structure of the sponge microbiota.

A recent and comprehensive study [62] reported that the majority of sponge-associated bacteria (55–70 %) are present in single host species but form phylogenetic lineages that are shared by numerous sponge hosts, yet absent or rare

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in the biosphere of bacterioplankton communities. This pattern is explained by a combination of vertical transmission [15, 37, 63, 64, 79] and horizontal acquisition of symbionts [63, 71, 83]. The predominance of vertical transmission would create stable bacterial communities linked to the dispersal and evolutionary trajectory of their host [18, 74], whereas horizontal acquisition would generate biogeographic patterns related to specific environmental conditions.

Comparisons of the microbiome within the same sponge species across different locations have revealed high similarity of bacterial symbionts in natural host populations within the same latitude [38, 70, 72, 82], suggesting no biogeographic patterns at intermediate spatial scales. However, Taylor et al. [70] found that the microbiota of *Cymbastela concentrica* hosts inhabiting tropical waters was clearly distinct from those from temperate regions (separated by >1,500 km) and Anderson et al. [4] reported location-specific bacterial communities in *Mycale hentscheli* across a 50- to 1,000-km range in New Zealand. The low number of studies and apparently conflicting results highlight the need for additional studies to further pinpoint the factors shaping the structure of sponge-associated bacterial communities over intermediate biogeographic scales.

In this study, we examined bacterial communities in the model sponge species *Ircinia felix* and *Ircinia strobilina*. The genus *Ircinia* (Dictyoceratida: Irciniidae) occurs widely in tropical and temperate environments and produces a broad spectrum of bioactive compounds involved in chemical defense against fouling, infection, and competition [14, 53]. *I. felix* and *I. strobilina* are high-microbial abundance (HMA) sponges species commonly found in coral reefs, grass beds, and mangroves throughout the Caribbean Sea [51, 61]. The ectosome of *I. felix* is rich in *Cyanobacteria* [41], contrary to *I. strobilina* [86]; and Schmitt et al. [64] demonstrated that diverse bacterial symbionts in *I. felix* were present in adult, larval, and juvenile life stages of the host, indicating vertical transmission of at least some of their bacterial symbionts.

The goal of this study was to assess the spatial variability (at a scale from 10s to 100s of kilometers) and host specificity of the bacteria associated with the sympatric sponge species *I. felix* and *I. strobilina* from the Bahamas. We characterized the bacterial assemblages in *I. strobilina*, two color morphs of *I. felix* (white and tan), and ambient seawater from five islands of the Bahamas using terminal-restriction fragment length polymorphism (T-RFLP) analysis. We also constructed 16S rRNA gene libraries to assess the composition of sponge-associated bacterial communities and sequenced a fragment of the mitochondrial gene cytochrome oxidase I (COI) to determine the genetic identity and phylogenetic relationships among sponge hosts. We addressed the following hypotheses: (1) bacterial communities will differ significantly among sources (i.e., sponge species and seawater); (2) bacterial communities will exhibit greater similarity in more closely related sponge hosts (i.e., greater between *I. felix* color morphs than among *I. felix* morphs and *I. strobilina*); (3) changes in the bacterial communities within each sponge species will correlate with geographic distances among host populations.

Materials and Methods

Sample Collection

The marine sponges *I. strobilina* (Lamarck 1816) and *I. felix* (Duchassaing and Michelotti 1864) and ambient seawater samples were collected from shallow littoral zones (<20-m depth) of the Bahamas in July 2010 by SCUBA diving (Table S1). The five sampled populations were separated by 80 to 400 km and were located around islands of different human population densities (http://statistics.bahamas.gov.bs/): San Salvador (24°03.515 N, 074°32.474 W; <1,000 inhabitants), Little San Salvador (24°34.727 N, 075°57.628 W; <2,000 inhabitants), Exumas (24°52.871 N, 076°47.502 W; <7,500 inhabitants), Sweeting's Cay, Grand Bahama (26°33.578 N, 077°53.036 W; >45,000 inhabitants), and New Providence (25°00.771 N, 077°33.794 W; >250,000 inhabitants). At each site, ambient seawater (500 mL) was sampled simultaneously and in close proximity (<1 m) to the sponges. Once on board the research vessel, sponge samples were immediately preserved in RNAlater (Ambion) and seawater samples were concentrated on 0.2-μm filters prior to preservation. All samples were stored at −20 °C.

Transmission Electronic Microscopy

For each sponge species and color morph, a piece of the ectosome was dissected with a sterile scalpel and fixed in a solution of 2.5 % glutaraldehyde and 2 % paraformaldehyde buffered with filtered seawater and incubated overnight at 4 °C. Following incubation, each piece was rinsed at least three times with filtered seawater and stored at 4 °C until processed as described previously [39]. Transmission electronic microscopy (TEM) observations were made at the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona on a JEOL JEM-1010 (Tokyo, Japan) coupled with a Bioscan 972 camera (Gatan, Germany). Micrographs were visualized in ImageJ [2] for bacterial cell counts. The relative abundances of bacteria (bacterial cells/square millimeter) were determined as the average (±standard deviation) over five TEM micrographs per sample.

DNA Extractions

Genomic DNA was extracted from sponge and seawater samples using the DNeasy® Blood & Tissue kit (Qiagen®) according to the manufacturer's instruction. Full-strength

and 1:10 diluted DNA extracts were used as templates in PCR amplifications.

Molecular Identification of Host Sponges

A fragment of ca. 1,000 bp of the mitochondrial gene cytochrome oxidase I (COI), corresponding to the standard barcoding partition [28, 33] and the I3-M11 partition [17] was PCR-amplified using a degenerated version of the universal barcoding forward primer dgLCO1490 [44] (5′-GGT CAA CAA ATC ATA AAG AYA TYG G-3′) and the reverse primer COX1-R1 [58] (5′-TGT TGR GGG AAA AAR GTT AAA TT-3′). Amplification was performed in a GeneAmp® PCR machine (Applied Biosystems) as follows: one initial denaturation step for 5 min at 94 °C; followed by 30 amplification cycles of 0.5 min at 94 °C, 0.5 min of annealing at 42 °C, and 1.5 min at 72 °C; and a final elongation step for 7 min at 72 °C. Total PCR volume (50 μL) included 10 μM of each primer, 10 nM of each dNTP, 1× Reaction Buffer (Ecogen), 2.5 mM $MgCl_2$, 5 units of BioTaq™DNA polymerase (Ecogen), and 5 μL of DNA template. PCR products were cleaned and bi-directionally sequenced at Macrogen, Inc. (Seoul, Korea). The consensus sequences obtained in this study for each sponge host and representative sequences from other *Ircinia* species available in GenBank were aligned in Geneious Pro 5.1.6 [13]. Specifically, the alignment included representative sequences of congeneric species from the Mediterranean Sea [21], the Indo-Pacific [55], and one *I. strobilina* sequence from the Caribbean [16]. Maximum likelihood (ML) and neighbor joining (NJ) phylogenies were constructed in MEGA v5 [68]. For ML analyses, we used the GTR+G+I [69] model and 100 bootstrap replicates [26]. The NJ tree was built based on the Tamura–Nei model of nucleotide substitution and 1,000 bootstrap replicates. All sequences have been deposited in GenBank (accession nos. JX306085 to JX306089).

T-RFLP Analysis

The universal bacterial forward primer Eco8F [77] (5′-AGA GTT TGA TCC TGG CTC AG-3′), tagged with 6-FAM, and the reverse primer 1509R [42] (5′-GGT TAC CTT GTT ACG ACT T-3′) were used for amplification of ca. 1,500-bp fragments of the 16S rRNA gene from all sponge and seawater DNA extracts. PCR was performed in a GeneAmp® PCR machine (Applied Biosystems) as follows: an initial denaturation step for 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 0.5 min at 50 °C, 1.5 min at 72 °C; and a final elongation step for 5 min at 72 °C. Total PCR volume (50 μL) included 10 μM of each primer, 10 nM of each dNTP, 1× Reaction Buffer (Ecogen), 2.5 mM $MgCl_2$, 5 units of BioTaq™ DNA polymerase (Ecogen), and 5 μL of DNA template. Products from triplicate PCR reactions were gel-purified and cleaned using the Qiaquick Gel Extraction kit (Qiagen®) and pooled before quantification using the Qubit™ fluorometer and Quant-iT™ dsDNA Assay kit (Invitrogen™). For each sample, 100 ng of purified PCR product were digested with the restriction endonuclease *Hae*III and 100 ng with *Msp*I in a total volume of 20 μL, following the manufacturer's protocol (Promega). Restriction reactions were incubated for 4 h at 37 °C, followed by ethanol precipitation to remove residual salts. Prior to capillary electrophoresis, samples were fully dried and then eluted in 11.5 μL formamide and 0.5 μL GeneScan 600-LIZ size standard (Applied Biosystems), heated at 94 °C for 2 min in a dry bath, and immediately cooled on ice for 2 min. Samples were processed on an automated ABI 3730 Genetic Analyzer (Applied Biosystems) at the Genomics Unit of the Scientific and Technical Services of the University of Barcelona.

The lengths of individual terminal-restriction fragments (T-RFs) were determined using the program PeakScanner (Applied Biosystems). T-RFs below 50 fluorescence units (background noise), smaller than 50 bp or larger than 600 bp (beyond the resolution of our internal standard) were excluded from the analysis. T-RFLP peak profiles were uploaded in T-REX [10] for further filtering, alignment, and construction of relative abundance matrices. Data were de-noised applying a cutoff value of 2 standard deviations [1], and T-RFs were aligned using a clustering threshold of 1 bp then standardized by relative peak areas.

Statistical Analysis of T-RFLP

Bray–Curtis similarity matrices were calculated using square-root transformations of relative T-RF abundances. Non-metric multi-dimensional scaling (nMDS) plots were constructed for each restriction enzyme to visualize similarities among the bacterial communities recovered from each sample. Permutational multivariate analyses of variance (PERMANOVA) were used for pairwise comparisons of bacterial communities among sources (seawater, sponge species and the two color morphs of *I. felix*) and among locations within each source (nested analysis). PERMDISP was computed for comparing the multivariate dispersions among groups on the basis of Bray-Curtis distance. Calculations were performed in PRIMER v6 [6, 7] and PERMANOVA+ (Plymouth Marine Laboratory, UK). For all pairwise comparisons, the critical value for significance was corrected using the Benjamini-Yekutieli (B-Y) false discovery rate [5]. To test for isolation-by-distance, Mantel tests for each host and enzyme were calculated in R [56] using the package ade4 [12].

16S rRNA Gene Clone Library Construction

Clone libraries were constructed for two individuals of each sponge species and color morph collected in Sweeting's Cay

and Exumas (ca. 300 km apart). PCR amplification was performed as described for T-RFLP analyses (above), except that no fluorescent tag was attached to the forward primer. PCR products were gel-purified and cleaned using the QIAquick Gel Extraction kit (Qiagen®) and quantified with a Qubit™ fluorometer and Quant-iT™ dsDNA Assay kit (Invitrogen™). Cleaned PCR products were ligated into plasmids using the pGEM®-T Vector System (Promega). In total, 234 positive clones were bi-directionally sequenced using the vector primers T7 and SP6 at Macrogen, Inc. (Seoul, Korea). Raw sequence reads were processed and aligned in Geneious Pro 5.1.6 [13] to recover near full-length 16S rRNA gene sequences (range=1042 to 1563 bp). Low quality sequence reads and sequences identified as chimeric [60] were discarded. All sequences were deposited in GenBank (Acc. Nos. JX280152 to JX280385).

Diversity and Structure of the Bacterial Clone Libraries

Bacterial 16S rRNA gene sequences were ascribed to 99 % operational taxonomic units (OTUs). A 99 % sequence identity threshold was used to increase taxonomic resolution and assess fine-scale variability in bacterial communities among hosts. Richness (Observed OTUs, Chao1 estimator) and diversity metrics (Shannon index, Simpson's inverse index) were calculated by source (sponge species or color morph), plotted in rarefaction curves and used to compare the richness, diversity and evenness of recovered bacterial communities. Pairwise differences in bacterial clone libraries of each host species and color morph were determined by LIBSHUFF analyses based on 10,000 randomizations and adjusted using Bonferroni corrections [65]. All analyses were performed using the mothur software package [60].

To compare clone library sequences with T-RFs, in silico digestions of a representative ribotype of each 99 % OTU were generated using the Restriction Analysis option in Geneious Pro 5.1.6 [13]. A reference database was created consisting of 5'-terminal fragment lengths for each OTU and restriction endonuclease (*Hae*III and *Msp*I) and T-RF drift was predicted and corrected as described in Erwin et al. [19]. This database was then used to match predicted T-RFs based on clone library sequences with empirical T-RFs obtained during T-RFLP analysis using the phylogenetic assignment tool PAT [35]. Default bin sizes and an extra bin for small T-RFs (2 bp tolerance applied to fragments of 50–100 bp) were applied to PAT analyses.

Phylogenetic Analysis of the Bacterial Clone Libraries

Phylogenetic analyses were performed to determine the affiliations between sequences retrieved in this study, top matching sequences from BLASTn searches [3] and publicly available *Ircinia*-associated symbionts in the GenBank database (January 2012), including sequences from *I. felix* [63, 64], *I. strobilina* [46, 86], Mediterranean *Ircinia* spp. [21, 49] and an Indo-Pacific *Ircinia* sp. (GenBank Acc. No. GQ487629). All sequences were grouped into 99 % OTUs and classified using the Ribosomal Database Project II sequence classifier [8]. When bacterial sequences from publicly available database derived from the same sponge species and grouped in the same 99 % OTU, only a representative sequence was used for the following analyses to facilitate tree visualization. Finally, sequences were aligned with ClustalX 2.1 [76] and a maximum-likelihood (ML) phylogenetic tree was constructed in RAxML [67] using the General Time Reversible model with a gamma distribution of variable substitution rates among sites (GTR+G) [69] and 100 bootstrap replicates [26]. A binary backbone constraint tree was constructed from long (>1000 bp) sequences to allow precise placement of shorter sequences as described in Erwin et al. [21].

Results

Phylogenetic Relationship Between Sponge Hosts

Partial COI sequences obtained for each color morph of *I. felix* were more closely related to each other (0.4 % divergence) than to *I. strobilina* (>1 % divergence). *I. strobilina* was more closely related to the Mediterranean species *Ircinia fasciculata* and *Ircinia variabilis* (0.5 % divergence) than to the sympatric *I. felix*; whereas *I. felix* was more closely related to the Mediterranean species *Ircinia oros* (Fig. 1). Caribbean and Mediterranean *Ircinia* species formed a well-supported clade and were a sister group to the Indo-Pacific sponges *Ircinia ramodigitata* and *Ircinia irregularis*.

Bacterial Morphology and Ultrastructure

Electron microscopy observations showed that *Ircinia* spp. from the Bahamas harbored diverse microbial communities (Fig. 2). Bacteria were mostly distributed extracellularly in the mesohyl of both sponge species (Fig. 2a, b) and occurred in high densities ($1.197 \times 10^6 \pm 0.051$ cells/mm^2 in *I. strobilina*, $0.816 \times 10^6 \pm 0.142$ cells/mm^2 in *I. felix*). Different bacterial morphotypes were distinguishable, including prokaryotic cells with a nucleoid-like structure (Fig 2a, b). A cyanobacterium corresponding to the description of *Candidatus Synechococcus spongiarum* [78] was abundant in the ectosome of *I. felix* (Fig. 2c) and was characterized by spiral thylakoids located around the perimeter of the cell. These thylakoids appeared with electron-

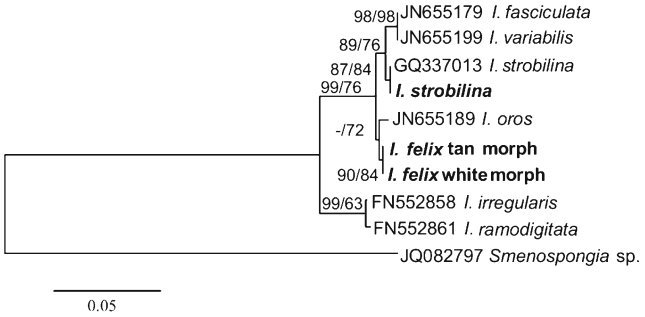

Fig. 1 Phylogenetic analysis of host sponges based on a fragment of the mitochondrial gene cytochrome oxidase I. Tree topology was obtained by neighbor joining and numbers on nodes indicate bootstrap values (>50 %) for neighbor joining (*left*) and maximum likelihood (*right*) analysis. Terminal node labels show GenBank accession numbers and sponge species. Sequences obtained in this study are highlighted in *bold*

dense granules in between them. Several cyanobacterial cells were also observed dividing by pinching in the center (Fig. 2c). No cyanobacterial symbionts were observed in *I. strobilina*. Sponge cells (archaeocytes) were only observed occasionally in the mesohyl and often contained several phagosomes digesting bacteria (Fig. 2d).

Host Specificity and Biogeography of Bacterial Communities

A total of 181 unique T-RFs for the restriction enzyme *Hae*III (141 in *I. strobilina,* 126 in the white morph of *I. felix*, 109 in the tan morph, and 123 in seawater), and 204 for *Msp*I (158 in *I. strobilina,* 136 in the white morph of *I. felix*, 106 in the tan morph, and 135 in seawater) were recovered. nMDS plots constructed from T-RFLP profiles for both restriction enzymes showed clear differences between seawater and sponge-derived bacteria (Fig. 3). Differences were also observed between the bacterial communities of *I. strobilina* and *I. felix* but not between color morphs of *I. felix*. Accordingly, statistical analyses revealed significant differences (PERMANOVA, $P<0.01$) among all pairwise comparisons of seawater bacteria and sponge-associated bacteria, between *I. strobilina* and *I. felix*, but not between color morphs of *I. felix* ($P>0.34$; Table 1). PERMDISP results reported significant differences in the homogeneity of dispersion between each sponge host and seawater, but not among sponge sources (Table 1). No differences in the bacterial composition of

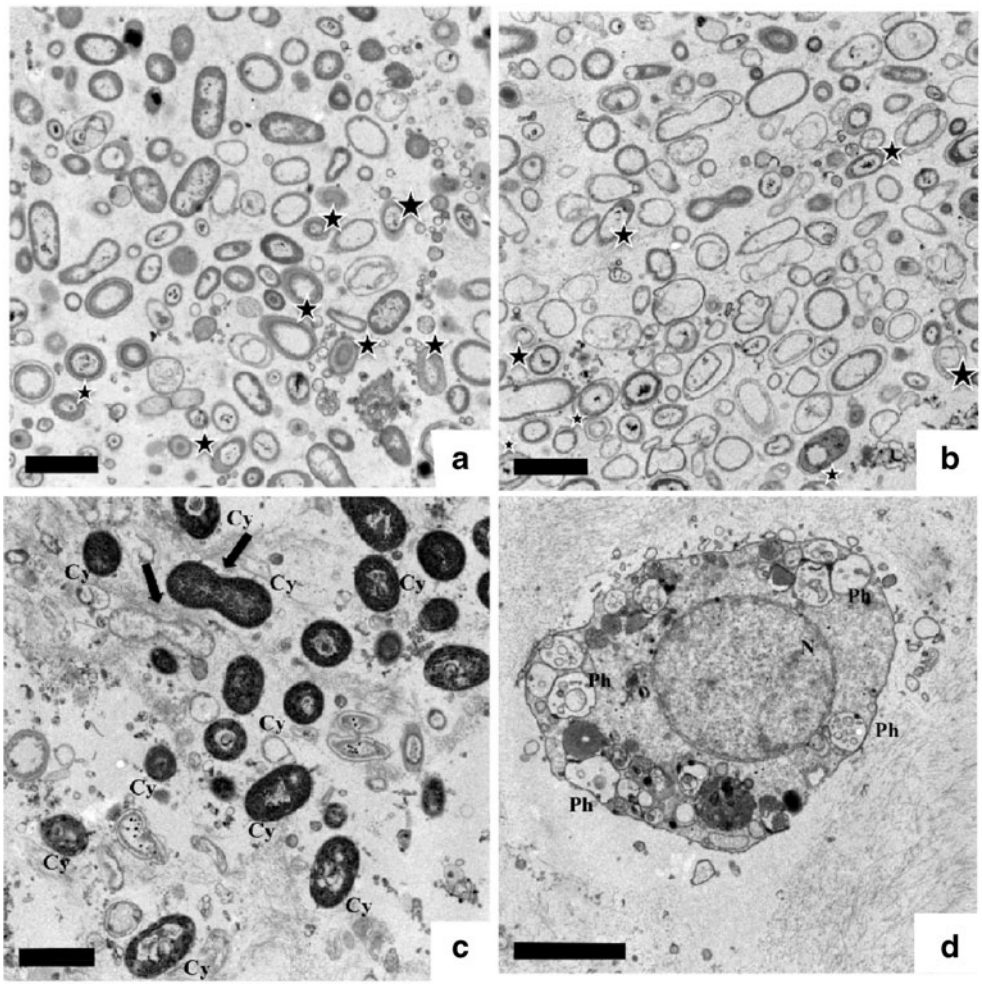

Fig. 2 Representative electron micrographs of sponge holobionts. Bacterial diversity in the mesohyl of **a** *I. strobilina* and **b** *I. felix* tan morph, including morphotypes containing a nucleoid-like structure within the cell (*black stars*). **c** The cyanobacterium *Candidatus Synechococcus spongiarum* (*Cy*) and active bacterial cell division (*black arrows*) in the ectosome of the white morph of *I. felix*. **d** Sponge cell (archaeocyte) in the tan morph of *I. felix* showing the cell nucleus (*N*), and numerous phagosomes (*Ph*). Scale bars represent 2 μm

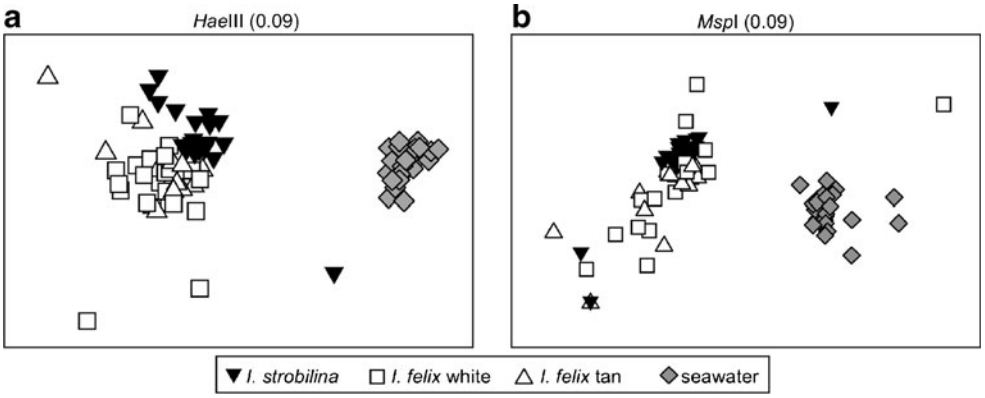

Fig. 3 nMDS plots of bacterial community structure in sponge hosts (*I. strobilina* and two color morphs of *I. felix*) and surrounding seawater samples. nMDS ordination based on Bray–Curtis similarity of T-RFLP profiles using the restriction enzymes **a** *Hae*III and **b** *Msp*I. Stress values are shown in parenthesis, with values below 0.15 indicating a good representation of similarity matrix distances in the graphical ordination plot

the sponge samples could be attributed solely to location ($P>0.05$); however, a significant interaction between source and location occurred for the restriction enzyme *Msp*I. Subsequent pairwise comparisons in a nested design and after Benjamini–Yekutieli correction only revealed significant differences between the bacterioplankton communities of Sweeting's Cay and San Salvador (Table S2). No significant correlations between bacterial community similarity and geographic distance were recovered for any sponge host (Mantel test, $P>0.233$ for all comparisons).

Table 1 Permutational statistical analysis of T-RFLP data (*Hae*III and *Msp*I enzymes) for bacterial community structure (PERMANOVA) and homogeneity of dispersion (PERMDISP) among sponge hosts and seawater

	*Hae*III		*Msp*I	
PERMANOVA Main test	*F* ratio	*P* value	*F* ratio	*P* value
Source	18.167	**0.001*** **	10.779	**0.001*** **
Location	1.707	0.055	1.423	0.128
Source × location	1.389	0.062	1.573	**0.013***
PERMANOVA Pairwise comparison	*t*	*P* value	*t*	*P* value
Tan *I. felix*–white *I. felix*	1.039	0.354	0.930	0.508
Tan *I. felix*–*I. strobilina*	2.404	**0.001*** **	1.790	**0.006***
White *I. felix*–*I. strobilina*	2.951	**0.001*** **	1.913	**0.003****
Tan *I. felix*–seawater	7.114	**0.001*** **	5.741	**0.001*** **
White *I. felix*–seawater	7.962	**0.001*** **	5.879	**0.001*** **
I. strobilina–seawater	7.016	**0.001*** **	6.048	**0.002****
PERMDISP Pairwise comparison	*t*	*P* value	*t*	*P* value
Tan *I. felix*–white *I. felix*	0.517	0.648	0.866	0.465
Tan *I. felix*–*I. strobilina*	0.087	0.946	0.590	0.636
White *I. felix*–*I. strobilina*	0.613	0.573	1.435	0.239
Tan *I. felix*–seawater	3.677	**0.002****	2.721	0.023
White *I. felix*–seawater	4.933	**0.001*** **	3.846	**0.001*** **
I. strobilina–seawater	3.471	**0.001*** **	1.693	0.156

Main tests of source (sponges and seawater), location (collection site), and an interactive term are shown, along with pairwise comparisons among sources: tan and white morphs of *I. felix* (tan and white *I. felix*, respectively), *I. strobilina* and seawater. Significant comparisons following B-Y correction are indicated in bold, with asterisks denoting significance level (*α=0.05, **α=0.01, ***α=0.005)

Diversity and Structure of the Sponge-Associated Bacterial Communities

16S rRNA gene sequence libraries from *I. strobilina* (n=82), the white morph of *I. felix* (n=68) and the tan morph (n=84) were ascribed to a total of 83 unique OTUs (99 % sequence identity). Rarefaction analyses at a similarity level of 99 % showed greater OTU saturation for the bacterial communities in both morphs of *I. felix* than for *I. strobilina* (Fig. S1a). Richness and diversity metrics revealed that *I. strobilina* hosted a more diverse and evenly distributed bacterial community than *I. felix* (Table 2). The color morphs of *I. felix* exhibited similar OTU richness values, but diversity indices (Shannon and Simpson's inverse index) were much higher for the white morph than for the tan morph (Table 2). Rarefaction curves of all estimators (Chao 1, Shannon, and inverse of Simpson's index) approached asymptotes and revealed consistent differences among sponge hosts across sampling effort (Fig. S1b–d).

Most bacterial OTUs were unique to one host, with little overlap among the three sponge-associated communities

Table 2 Richness (observed OTUs, Chao1) and diversity metrics (Shannon and Simpson's inverse indexes) for the bacterial communities recovered from each sponge host

	I. strobilina	White *I. felix*	Tan *I. felix*
Observed OTUs	45	26	30
Expected OTUs (S_{Chao1})	103 (68–194)	39 (30–74)	54 (38–105)
Shannon index	3.5 (3.2–3.7)	2.9 (2.7–3.1)	2.7 (2.3–2.9)
Simpson's inverse index	28.6 (18.7–60.6)	15.7 (11.2–26.4)	7.1 (4.9–13.3)

Confidence intervals at 95 % are shown in parentheses

(Fig. 4). Only four OTUs (IRCBA01, IRCBA13, IRCBA20, and IRCBA44) were shared among *I. strobilina* and the two color morphs of *I. felix* (hereafter called generalist OTUs). These generalist OTUs were dominant within each bacterial community, in terms of number of sequences retrieved, accounting for 6.0 % to 34.5 % of all bacterial sequences per host species and morph, except for IRCBA20 (<2.5 % of sequences for all hosts) and IRCBA13 for the white morph of *I. felix* (2.9 %). The OTU IRCBA01 represented 8.5 % of all the sequences recovered for *I. strobilina*, and 34.5 % and 17.5 % of the sequences from the tan and white morphs of *I. felix,* respectively. The OTU IRCBA44 accounted for 13.4 % of *I. strobilina*-derived sequences and 6.0 % of tan *I. felix* and 8.8 % of the white *I. felix*-derived sequences. Two additional OTUs were shared between the two color morphs of *I. felix* (IRCBA33 and IRCBA60); these OTUs represented 4.8 % and 8.8 % of all the sequences retrieved for the tan and white morphs, respectively. Consistent with the little OTU overlap among host sponges, the symbiotic community associated with each host sponge was significantly different, even among color morphs (LIBSHUFF analysis, Table 3). There were no significant differences between the 16S rRNA gene sequences from Sweeting's Cay and Exumas obtained for both color morphs of *I. felix*, while significant differences were detected between populations of *I. strobilina* (Table 3).

PAT analysis showed high congruence between bacterial clone libraries and T-RFLP analyses for both restriction enzymes. In fact, 88 % of the OTUs obtained with clone libraries were also observed with T-RFLP analysis. Empirical T-RFs obtained with the enzyme *Hae*III matched 50.6 % of the peaks predicted by in silico digestion, while for *Msp*I, empirical T-RFs matched 55.6 % of the predicted peaks.

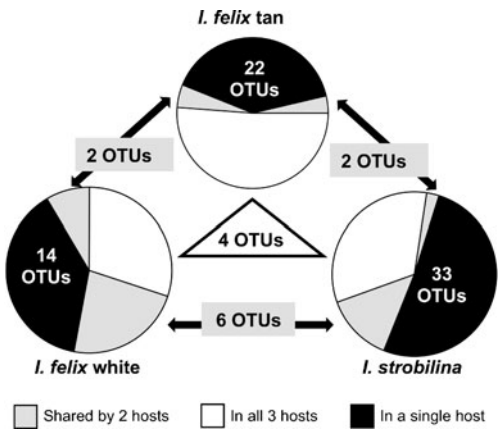

Fig. 4 Host specificity of the bacterial communities in *I. strobilina* and two color morphs of *I. felix* based on 16S rRNA gene sequences obtained after clone library construction. Pie charts show the percentages of clones for each symbiont category. Numbers denote the total OTUs (99 % sequence identity) in each category

Table 3 Pairwise statistical comparisons of bacterial community structure (LIBSHUFF analyses) based on 16S rRNA gene sequences obtained from clone libraries of *I. strobilina* and the two color morphs of *I. felix* (tan and white)

LIBSHUFF comparisons		dC_{XY} dC_{YX}	*P* value $_{XY}$ *P* value $_{YX}$
I. strobilina–tan *I. felix*		0.0054	**0.0016****
		0.0038	**0.0045***
I. strobilina–white *I. felix*		0.0034	0.019
		0.0089	**0.001****
Tan *I. felix*–white *I. felix*		0.0048	**0.002***
		0.0078	**0.0001*****
Sweeting's–Exumas within	*I. strobilina*	0.0083	**0.0161***
		0.0026	0.2001
	White *I. felix*	0.0027	0.2125
		0.0014	0.353
	Tan *I. felix*	0.0040	0.0624
		0.0014	0.3014

Comparisons among hosts and between sampling sites (Sweeting's Cay and Exumas) within hosts are shown. Two tests per pairwise comparison (dC_{XY} and dC_{YX}) and corresponding *P* values (*P* value $_{XY}$, *P* value $_{YX}$) were conducted, with significance in either comparison indicating differences in bacterial community structure. Significant comparisons following Bonferroni correction are indicated in bold, with asterisks denoting significance level (*α=0.05; **α=0.01; ***α=0.005)

Phylogenetic Analysis of 16S rRNA Bacterial Sequences

The vast majority of the sequences recovered from each sponge host were closely related with other sponge-associated (73.2 % in *I. strobilina*, 94.1 % in the white morph of *I. felix*, and 77.4 % in the tan morph) and coral-associated bacterial sequences (20.7 % in *I. strobilina*, 4.4 % in the white morph of *I. felix*, and 20.2 % in the tan morph). Some ribotypes matched with seawater-derived sequences (6.1 % in *I. strobilina*, 2.4 % in the tan morph of *I. felix*, and 0 % in the white morph), but mostly at low identity matches (<97 % sequence identity). As in other HMA sponges, the bacterial OTUs recovered herein were distributed into eight known phyla and one unclassified group (Fig. S2–S7). All three sponge taxa hosted representatives from two classes of *Proteobacteria* (*Delta* and *Gamma*), as well as *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Nitrospira*, and *Firmicutes* (Fig. 5). *Proteobacteria*, specifically the class *Delta-Proteobacteria* (>15 % total clones in all hosts), was the best-represented phylum in all clone libraries. Sequences related to *Spirochaetes* and *Alpha-Proteobacteria* were only present in *I. strobilina* and the tan morph of *I. felix*. Sequences affiliated to *Cyanobacteria* (*Synechococcus*) were only found in *I. felix* and were more abundant in the white morph than in the tan morph (>15 % and >2 % of total clones, respectively). The generalist OTUs shared by the three sponge hosts corresponded to class *Delta-Proteobacteria* (IRCBA01) and

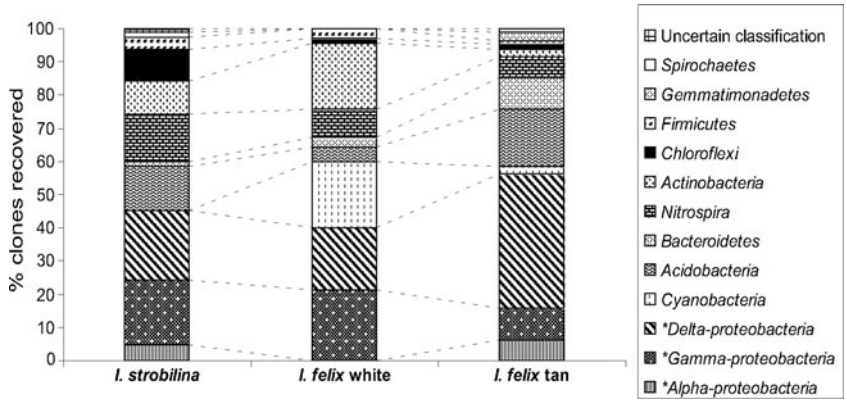

Fig. 5 Phylogenetic affiliation of symbiont OTUs (99 % sequence similarity) in *I. strobilina* and two color morphs of *I. felix* (tan and white). Bacteria are classified according to phylum or class (marked with an *asterisk*)

phyla *Acidobacteria* (IRCBA13 and IRCBA20) and *Nitrospira* (IRCBA44). These symbionts formed sponge-specific (IRCBA20 and IRCBA44) and sponge–coral-specific (IRCBA01, IRCBA13) clusters (Fig. S2). The *Delta-Proteobacteria*-affiliated OTU (IRCBA01) was particularly dominant in the bacterial clone libraries (8.5 % of the sequences in *I. strobilina*; 17.5 % in the white morph of *I. felix* and 34.5 % in the tan morph) and was also common in the Mediterranean species *I. fasciculata*, *I. variabilis* and *I. oros*, as well as in other unrelated sponge species and corals (Fig. S4).

Discussion

In this study, we determined whether the bacterial communities associated with the sympatric sponges *I. strobilina* and *I. felix* were stable across islands separated by tens to hundreds of kilometers in the Bahamas. Sequencing of a fragment of the mitochondrial COI gene from host sponges confirmed the taxonomic identification and phylogenetic relationships of *I. strobilina* and two color morphs of *I. felix* (white and tan), allowing for the assessment of the bacterial communities specificity among congeneric and conspecific host individuals. Electron microscopy, T-RFLP analysis, and 16S rRNA gene clone libraries confirmed that these sponge taxa harbor host–species-specific bacterial communities that are clearly differentiated from the bacterioplankton in the surrounding seawater. T-RFLP profiles further revealed that the bacterial communities in two color morphs of *I. felix* were more similar to each other than to *I. strobilina*. Within each sponge host, bacterial assemblages were remarkably stable over locations and maintained across host populations and islands.

Our results revealed a major influence of host-related factors in structuring sponge-associated bacterial assemblages. We sampled sponge populations in reefs up to 400 km apart located in islands with distinct human population densities and oceanographic currents [9], yielding different environmental conditions, disturbance regimes and dispersal barriers. However, we found high spatial stability of sponge–bacteria symbioses and no isolation-by-distance effect, consistent with previous studies on sponge-derived bacterial communities at geographical scales ranging from tens [38, 82, 85] to hundreds of kilometers [70, 75, 84]. Other studies suggested that environmental conditions could also influence the structure of symbiont communities [70, 86], although these studies involve broader geographic (i.e., inter-ocean) scales and/or genetically distant hosts, thus decoupling the effects of biogeography and host specificity remained a major obstacle. In contrast, studies that minimize the phylogenetic distance among host species are better suited to distinguish location- and host-related patterns. For instance, Montalvo and Hill [47] compared the bacteria associated with *Xestospongia muta* and *Xestospongia testudinaria* and found that these closely related hosts harbored strikingly similar bacterial communities, despite the fact that they inhabit different oceans (Atlantic and Pacific, respectively).

In addition to spatial stability, our study also assessed host specificity of bacterial communities among congeneric and conspecific sponges. The bacterial sequences derived from 16S rRNA clone libraries for each *Ircinia* host belonged to the same phyla described for other HMA sponges [81, 83] and were largely consistent with previous studies of *I. strobilina* [46, 86] and *I. felix* [63, 64]. For example, a sponge-specific cluster of *Bacteroidetes* sequences that was previously detected only in the larvae of *I. felix* [64] was identified herein in both color morphs of adult *I. felix* hosts. TEM micrographs and clone libraries also revealed the absence of *Cyanobacteria* in the microbiota of *I. strobilina*, consistent with a recent molecular-based survey [86] and the low chlorophyll *a* content of this sponge host [25, 66]. While some *I. strobilina* hosts may harbor nitrogen-fixing cyanobacteria [45, 46], these symbionts are clearly distinct from the dense populations of *Synechococcus spongiarum* consistently reported in *I. felix* [64, 66]. The significance for host metabolism of these divergent bacterial assemblages is still uncertain, and further investigation is necessary to assess whether the net activity of different symbiont microbiota results in overall similar biochemical processes in the holobiont [e.g., in nitrogen flux, 66].

In a broader context, most of the sequences in the bacterial 16S rRNA clone libraries of *Ircinia* spp. from the Bahamas were closely related to bacterial symbionts in taxonomically distant sponge hosts (e.g., different sponge orders) and from different geographic origins (e.g., Mediterranean and Pacific), consistent with reports from other HMA sponge hosts [e.g., 27, 32, 34]. Phylogenetic analyses of bacterial clone libraries did not reveal any *Ircinia*-specific or Caribbean *Ircinia*-specific symbiont clusters. The four bacterial OTUs shared by *I. strobilina*, and both color morphs of *I. felix* were also described in other sponge (IRCBA20, IRCBA44) and coral (IRCBA01, IRCBA13) hosts from diverse ecosystems. However, at the community level, the bacterial composition in each *Ircinia* host analyzed herein was still host-specific. Similar observations of symbiont structure and specificity were recently described for Mediterranean *Ircinia* spp. and termed a "specific mix of generalists" [21]. The outstanding questions are which factors result in the observed distribution of symbiont taxa among hosts and what are the ecological consequences for host–symbiont interactions.

Host-related factors influencing bacterial communities may include particular mesohyl conditions (e.g., different pH and oxygen levels) and the evolutionary history of each sponge species. Although closely related, *I. felix* and *I. strobilina* show striking differences in morphological and physiological traits, such as shape and filter-feeding capacity [51, 54]. Pile [54] demonstrated that *I. strobilina* had higher filtering efficiencies than *I. felix* and suggested that *I. strobilina*, as a tall and massive sponge, contained more aquifer units, retained water inside the sponge body longer, and exhibited more efficient particle uptake than *I. felix*. Such specific features may create distinct conditions in the mesohyl of each host, each supporting particular bacterial consortia. In addition, the evolutionary history of each sponge species may also influence the structure of their bacterial communities. Vertical transmission has been reported in *I. felix* for most of the bacterial taxa [64], and we have confirmed that morphotypes of *I. felix* are more similar to each other than to *I. strobilina*. Thus, while periodic horizontal symbiont transmission is likely to occur and explain the generalist distribution of individual symbiont taxa, continual vertical transmission of specific communities may maintain symbiont structure within host species, and their divergence among host species, over recent evolutionary scales.

In conclusion, the bacterial communities observed in *I. strobilina* and two color morphs of *I. felix* were host species specific, exhibiting greater similarity within host species (morphotypes) than between host species (*I. felix* and *I. strobilina*). The bacterial taxa comprising these symbiont communities were also present in other sponge and coral species and thus represent generalist symbionts. As described for Mediterranean *Ircinia* species [21], we conclude that *I. strobilina* and *I. felix* host a specific mix of generalist symbionts and suggest that host-specific factors (mesohyl conditions and host evolutionary history) determine their unique structure in each host. Contrary to our original hypothesis of spatial structure in the bacterial communities associated with *Ircinia* hosts, we found high stability of bacterial communities within each host sponge across different islands and geographic distances up to 400 km, indicating a minimal effect of dispersal limitation and local environmental conditions on symbiont structure. Thus, host-specific rather than biogeographic factors play a primary role in structuring and maintaining sponge–bacteria relationships in *Ircinia* hosts from the Bahamas.

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References

1. Abdo Z, Schuette U, Bent S, Williams C, Forney L, Joyce P (2006) Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Environ Microbiol 8(5):929–938
2. Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophoton Int 11:36–42
3. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment tool. J Mol Biol 215:403–410
4. Anderson SA, Northcote PT, Page MJ (2010) Spatial and temporal variability of the bacterial community in different chemotypes of the New Zealand marine sponge *Mycale hentscheli*. FEMS Microbiol Ecol 72:328–342
5. Benjamini Y, Yekutieli D (2001) The control of the false discovery rate in multiple testing under dependency. Ann Stat 29(4):1165–1188
6. Clarke KR, Gorley RN (2006) PRIMERv6: User manual/Tutorial. PRIMER-E, Plymouth
7. Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18(1):117–143
8. Cole JR, Chai B, Farris FJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM, Bandela AM, Cardenas E, Garrity GM, Tiedje JM (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res 35:D169–D172
9. Colin PL (1995) Surface currents in Exuma Sound, Bahamas and adjacent areas with reference to potential larval transport. Bull Mar Sci 56(1):48–57
10. Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H, Buckley DH (2009) T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinforma 10:171–180
11. Diaz MC, Rützler K (2001) Sponges: an essential component of Caribbean coral reefs. Bull Mar Sci 69(2):535–546
12. Dray S, Dufour AB (2007) The ade4 package: implementing the duality diagram for ecologists. J Stat Softw 22(4):1–20
13. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2011) Geneious v5.4, available from http://www.geneious.com

14. Duque C, Bonilla A, Bautista E, Zea S (2001) Exudation of low molecular weight compounds (thiobismethane, methyl isocyanide, and methyl isothiocyanate) as a possible chemical defense mechanism in the marine sponge *Ircinia felix*. Biochem Syst Ecol 29:459–467
15. Ereskovsky A, Gonobobleva E, Vishnyakov A (2005) Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). Mar Biol 146:869–875
16. Erpenbeck D, Voigt O, Wörheide G, Lavrov DV (2009) The mitochondrial genomes of sponges provide evidence for multiple invasions by Repetitive Hairpin-forming Elements (RHE). BMC Genomics 10:591–604
17. Erpenbeck D, Hooper JNA, Wörheide G (2006) CO1 phylogenies in diploblasts and the "Barcoding of Life"-are we sequencing a suboptimal portion? Mol Ecol Notes 6:550–553
18. Erpenbeck D, Breeuwer JAJ, van der Velde HC, van Soest RWM (2002) Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae, Porifera) using a mitochondrial marker. Mar Biol 141:377–386
19. Erwin PM, Pita L, López-Legentil S, Turon X (2012) Temporal variation in the sponge microbiota: symbiont stability over large seasonal shifts in temperature and irradiance. Appl Environ Microb 78(20):7358–7368
20. Erwin PM, López-Legentil S, Turon X (2012) Ultrastructure, molecular phylogenetics, and chlorophyll *a* content of novel cyanobacterial symbionts in temperate sponges. Microb Ecol 64:771–783
21. Erwin PM, López-Legentil S, González-Pech R, Turon X (2012) A specific mix of generalists: bacterial symbionts in Mediterranean *Ircinia* spp. FEMS Microbiol Ecol 79(3):619–637
22. Erwin PM, Olson JB, Thacker RW (2011) Phylogenetic diversity, host-specificity and community profiling of sponge-associated bacteria in the northern Gulf of Mexico. PLoS One 6(11):e26806
23. Erwin PM, López-Legentil S, Schuhmann PW (2010) The pharmaceutical value of marine biodiversity for anti-cancer drug discovery. Ecol Econ 70(2):445–451
24. Erwin PM, Thacker RW (2008) Phototrophic nutrition and symbiont diversity of two Caribbean sponge-cyanobacteria symbioses. Mar Ecol Prog Ser 362:139–147
25. Erwin PM, Thacker RW (2007) Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages. J Mar Biol Assoc UK 87:1683–1692
26. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
27. Fieseler L, Horn M, Wagner M, Hentschel U (2004) Discovery of the novel candidate phylum "Poribacteria" in marine sponges. Appl Environ Microb 70(6):3724–3732
28. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299
29. Gili J, Coma R (1998) Benthic suspension feeders: their paramount role in littoral marine food webs. Tree 13(8):316–321
30. Hanson CA, Fuhrman JA, Horner-Devine MC, Martiny JBH (2012) Beyond biogeographic patterns: processes shaping the microbial landscape. Nat Rev Microbiol 10(7):497–506
31. Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters. FEMS Microbiol Ecol 55:167–177
32. Hentschel U, Hopke J, Horn M, Friedrich A, Wagner M, Hacker J, Moore B (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. Appl Environ Microb 68(9):4431–4440
33. Herbert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci 270:313–321
34. Hill M, Hill A, Lopez N, Harriott O (2006) Sponge-specific bacterial symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida). Mar Biol 148:1221–1230
35. Kent AD, Smith DJ, Benson BJ, Triplett EW (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl Environ Microb 69(11):6768–6776
36. Lee OO, Wang Y, Yang J, Lafi FF, Al-Suwailem A, Qian P (2010) Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. ISME J 5:650–664
37. Lee OO, Chui P, Wong YH, Pawlik JR, Qian P (2009) Evidence for vertical transmission of bacterial symbionts from adult to embryo in the Caribbean sponge *Svenzea zeai*. Appl Environ Microb 75(19):6147–6156
38. Lee OO, Wong YH, Qian P (2009) Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. Appl Environ Microb 75(11):3513–3521
39. López-Legentil S, Song B, Bosch M, Pawlik JR, Turon X (2011) Cyanobacterial diversity and a new *Acaryochloris*-like symbiont from Bahamian sea-squirts. PLoS One 6(8):e23938
40. López-Legentil S, Erwin PM, Pawlik JR, Song B (2010) Effects of sponge bleaching on ammonia-oxidizing Archaea: distribution and relative expression of ammonia monooxygenase genes associated with the barrel sponge *Xestospongia muta*. Microb Ecol 60(3):561–571
41. Maldonado M, Young CM (1998) Limits on the bathymetric distribution of keratose sponges: a field test in deep water. Mar Ecol Prog Ser 174:123–139
42. Martínez-Murcia AJ, Acinas SG, Rodríguez-Valera F (1995) Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol Ecol 17(4):247–255
43. Martiny JBH, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC, Kane M et al (2006) Microbial biogeography: Putting microorganisms on the map. Nat Rev Microbiol 4(2):102–112
44. Meyer CP, Geller JB, Paulay G (2005) Fine scale endemism on coral reefs: archipelagic differentiation in turbinid gastropods. Evolution 59:113–125
45. Mohamed NM, Coman AS, Tal Y, Hill RT (2008) Diversity and expression of nitrogen fixation genes in bacterial symbionts of marine sponges. Environ Microbiol 10(11):2910–2921
46. Mohamed NVR, Hamann M, Kelly M, Hill RT (2008) Monitoring bacterial diversity of the marine sponge *Ircinia strobilina* upon transfer into aquaculture. Appl Environ Microb 74(13):4133–4143
47. Montalvo N, Hill RT (2011) Sponge-associated bacteria are strictly maintained in two closely related but geographically distant sponge hosts. Appl Environ Microb 77(20):7207–7216
48. Morrow KM, Moss AG, Chadwick NE, Liles MR (2012) Bacterial associates of two Caribbean coral species reveal species-specific distribution and geographic variability. Appl Environ Microb 78(18):6438–6449
49. Muscholl-Silberhorn A, Thiel V, Imhoff JF (2008) Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. Microb Ecol 55(1):94–106
50. Newman D, Hill RT (2006) New drugs from marina microbes: the tide is turning. J Ind Microbiol Biotechnol 33:539–544
51. Parra-Velandia F, Zea S (2003) Comparación de la abundancia y distribución de algunas características de las esponjas del género *Ircinia* (Porifera: Demospongiae) en dos localidades contrastantes del área de Santa Marta, Caribe colombiano. Bol Invest Mar Cost 32:75–91
52. Paul V, Ritson-Williams R (2008) Marine chemical ecology. Nat Prod Rep 25(4):662–695
53. Pawlik JR, McFall G, Zea S (2002) Does the odor from sponges of the genus *Ircinia* protect them from fish predators? J Chem Ecol 28(6):1103–1115

54. Pile A (1997) Finding Reiswig's missing carbon: quantification of sponge feeding using dual-beam flow Cytometry. Proc 8th Int Coral Reef Sym 2:1403–1410
55. Pöppe J, Sutcliffe P, Hooper JNA, Wörheide G, Erpenbeck D (2010) CO1 Barcoding reveals new clades and radiation patterns of Indo-Pacific sponges of the family Irciniidae (Demospongiae: Dictyoceratida). PLoS One 5(4):e9950
56. R Core Team (2012) R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria, available from http://www.R-project.org
57. Ribes M, Jiménez E, Yahel G, López-Sendino P, Diez B, Massana R, Sharp J, Coma R (2012) Functional convergence of microbes associated with temperate marine sponges. Environ Microbiol 14(5):1224–1239
58. Rot C, Goldfarb I, Ilan M, Huchon D (2006) Putative cross-kingdom horizontal gene transfer in sponge (Porifera) mitochondria. BMC Evol Biol 6:71–81
59. Sarà M (1971) Ultrastructural aspects of the symbiosis between two species of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variabilis* (Demospongiae). Mar Biol 11:214–221
60. Schloss P, Westcott S, Ryabin T, Hall J, Hartmann M, Hollister E, Lesniewski R, Oakley B, Parks D, Robinson C, Sahl J, Stres B, Thallinger G, Van Horn D, Weber C (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microb 75(23):7537–7541
61. Schmahl GP (1990) Community structure and ecology of sponges associated with four Southern Florida coral reefs. In: Rützler K (ed) New perspectives in sponge biology. Smithsonian Institution Press, Washington, pp 376–383
62. Schmitt S, Tsai P, Bell J, Fromont J, Ilan M, Lindquist N, Perez T, Rodrigo A, Schupp P, Vacelet J, Webster NS, Hentschel U, Taylor MW (2012) Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. ISME J 6:564–576
63. Schmitt S, Angermeier H, Schiller R, Lindquist N, Hentschel U (2008) Molecular microbial diversity survey of sponge reproductive stages and mechanistic insights into vertical transmission of microbial symbionts. Appl Environ Microb 74(24):7694–7708
64. Schmitt S, Weisz J, Lindquist N, Hentschel U (2007) Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. Appl Environ Microb 73(7):2067–2078
65. Sokal RR, Rohl FJ (1995) Biometry: the principles and practice of statistics in biological research, 3rd edn. Freeman, New York
66. Southwell MW, Weisz JB, Martens CS, Lindquist N (2008) In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. Limnol Oceanogr 53(3):986–996
67. Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22(21):2688–2690
68. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739
69. Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura RM (ed) Some mathematical questions in biology-DNA sequence analysis. Am Math Soc, Providence, pp 57–86
70. Taylor MW, Schupp P, de Nys R, Kjelleberg S, Steinberg P (2005) Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. Environ Microbiol 7(3):419–433
71. Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71(2):295–347
72. Taylor MW, Schupp P, Dahllof I, Kjelleberg S, Steinberg PD (2004) Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. Environ Microbiol 6(2):121–130
73. Thacker RW, Freeman CJ (2012) Sponge-microbe symbioses: recent advances and new directions. Adv Mar Biol 62:57–111
74. Thacker RW, Starnes S (2003) Host specificity of the symbiotic cyanobacterium *Oscillatoria spongeliae* in marine sponges, *Dysidea* spp. Mar Biol 142:643–648
75. Thiel V, Leininger S, Schmaljohann R, Brümmer F, Imhoff J (2007) Sponge-specific bacterial associations of the Mediterranean sponge *Chondrilla nucula* (Demospongiae, Tetractinomorpha). Microb Ecol 54:101–111
76. Thompson J, Gibson T, Plewniak F, Jeanmougin F, Higgins D (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
77. Turner S, Pryer KM, Miao VPW, Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol 46(4):327–338
78. Usher KM, Toze S, Fromont J, Kuo J, Sutton DC (2004) A new species of cyanobacterial symbiont from the marine sponge *Chondrilla nucula*. Symbiosis 36:183–192
79. Usher KM, Kuo J, Fromont J, Sutton DC (2001) Vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). Hydrobiologia 461:15–23
80. Van Soest RWM, Boury-Esnault N, Vacelet J, Dohrmann M, Erpenbeck D, De Voogd NJ, Santodomingo N, Vanhoorne B, Kelly M, Hooper JNA (2012) Global diversity of sponges (Porifera). PLoS One 7(4):e35105
81. Webster NS, Taylor MW, Behnam F, Lucker S, Rattei T, Whalan S (2010) Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environ Microbiol 12(8):2070–2082
82. Webster NS, Negri A, Munro M, Battershill C (2004) Diverse microbial communities inhabit Antarctic sponges. Environ Microbiol 6(3):288–300
83. Webster NS, Taylor MW (2011) Marine sponges and their microbial symbionts: love and other relationships. Environ Microbiol 14(2):335–346
84. Webster NS, Hill RT (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alpha-proteobacterium. Mar Biol 138:843–851
85. Wichels A, Würtz S, Döpke H, Schütt C, Gerdts G (2006) Bacterial diversity in the breadcrumb sponge *Halichondria panicea* (Pallas). FEMS Microbiol Ecol 56:102–118
86. Yang J, Sun J, Lee OO, Wong YH, Qian P (2011) Phylogenetic diversity and community structure of sponge-associated bacteria from mangroves of the Caribbean Sea. Aquat Microb Ecol 62:231–240

RESEARCH ARTICLE

Host rules: spatial stability of bacterial communities associated with marine sponges (*Ircinia* spp.) in the Western Mediterranean Sea

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Abstract

Dispersal limitation and environmental selection are the main processes shaping free-living microbial communities, but host-related factors may also play a major role in structuring symbiotic communities. Here, we aimed to determine the effects of isolation-by-distance and host species on the spatial structure of sponge-associated bacterial communities using as a model the abundant demosponge genus *Ircinia*. We targeted three co-occurring *Ircinia* species and used terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene sequences to explore the differentiation of their bacterial communities across a scale of hundreds of kilometres in the Western Mediterranean Sea. Multivariate analysis and nonmetric multidimensional scaling plots of T-RFLP profiles showed that bacterial communities in *Ircinia* sponges were structured by host species and remained stable across sampling locations, despite geographic distances (80–800 km) and diverse local conditions. While significant differences among some locations were observed in *Ircinia variabilis*-derived communities, no correlation between geographic distance and community similarity was consistently detected for symbiotic bacteria in any host sponge species. Our results indicate that bacterial communities are mostly shaped by host species-specific factors and suggest that evolutionary processes acting on long-term symbiotic relationships have favored spatial stability of sponge-associated bacterial communities.

Introduction

Microbial biogeography studies often evaluate the relationship between community similarity and geographic distance (i.e. isolation-by-distance, also called distance–decay relationships). These patterns respond primarily to two processes: dispersal limitation and environmental selection (Martiny *et al.*, 2006; Fierer, 2008). Dispersal limitation prevents connectivity among distant locations or populations, while environmental heterogeneity (e.g. different physicochemical conditions of seawater in coastal systems) yields variability of the microbial communities among locations as local conditions 'pick up' the best-adapted microbes. Disclosing the spatial structure of microbial communities helps to elucidate the relative importance of these two underlying processes (Hanson *et al.*, 2012).

Some marine sponges, the so-called high-microbial-abundance sponges (HMA), harbor abundant and diverse bacterial communities (Taylor *et al.*, 2007; Hentschel *et al.*, 2012). These bacterial communities are far from being randomly structured; rather, their diversity, composition and structure depend on each sponge host (Schmitt *et al.*, 2012). Accordingly, each sponge species harbors a specific symbiotic community, resulting from the combination of vertical transmission (from parents to larva; Usher *et al.*, 2001; Ereskovsky *et al.*, 2004; Schmitt *et al.*, 2007; Lee *et al.*, 2009b) and environmental acquisition of bacteria (Schmitt *et al.*, 2008; Webster *et al.*, 2010; Hentschel *et al.*, 2012; Taylor *et al.*, 2013).

FEMS MICROBIOLOGY ECOLOGY

Recent research on sponge–microbe symbioses has focused on determining whether host specificity of symbiotic communities is maintained across locations. Previous studies have reported high spatial stability of sponge-associated bacteria across geographic distances up to thousands of kilometres (Hentschel *et al.*, 2002; Webster *et al.*, 2004; Taylor *et al.*, 2005; Pita *et al.*, 2013) whereas others have detected differentiation depending on location within the same (Lee *et al.*, 2009a) or among different ecosystems (Anderson *et al.*, 2010; Yang *et al.*, 2011). Thus, it is difficult to draw a general conclusion about the spatial structure of sponge-derived bacterial communities. In addition, sampling strategy and comparison of distantly related host species may confound the processes involved, given the large effect of host sponge species on symbiont community structure.

In this study, we designed a sampling strategy targeting sympatric and congeneric sponges from several western Mediterranean sites. Our goal was to distinguish between the relative contribution of biogeographic (dispersal limitation, environmental selection) and host-related processes (i.e. linked to evolutionary history or biological characteristics) to the spatial structure of bacterial communities associated with sponges. Herein, we used the term 'environment' to refer to the abiotic conditions in ambient seawater external to the host sponges. We investigated the bacterial communities associated with three *Ircinia* species (*I. fasciculata*, *I. variabilis* and *I. oros*) commonly found in the shallow littoral of coastal Mediterranean environments. *Ircinia* bacterial diversity is consistent with other HMA sponges, but each species harbors a unique community composed of generalist sponge symbionts (Erwin *et al.*, 2012a). The microbial inheritance mode in Mediterranean *Ircinia* species has not yet been studied, although vertical transmission was shown for *Ircinia felix* from the Caribbean (Schmitt *et al.*, 2007) and bacterial cells were observed in *I. oros* larva (Ereskovsky & Tokina, 2004; Uriz *et al.*, 2008). To test whether the host-specific symbiotic communities reported in Mediterranean *Ircinia* spp. were maintained over locations separated by hundreds of kilometers and under different local environmental conditions, we characterized bacterial communities in *Ircinia* spp. from six locations using terminal restriction fragment length polymorphism (T-RFLP) analyses of 16S rRNA gene sequences. We hypothesized that, within each host, a significant distance–decay relationship in bacterial community similarity would be detected as a consequence of (1) dominant currents in the region limiting dispersal of host larvae and bacterioplankton; and (2) differences in local conditions generating spatial differentiation of bacterial communities among locations.

Materials and methods

Sample collection

Tissue samples of *I. fasciculata* (Pallas 1766), *I. variabilis* (Schmidt 1862) and *I. oros* (Schmidt 1864) were collected by scuba diving from shallow littoral zones (depth < 20 m) in September–October 2010 at six different locations from the Western Mediterranean Sea (Fig. 1). Seventy-four specimens were sampled (*I. fasciculata*, $n = 28$; *I. variabilis*, $n = 27$; *I. oros*, $n = 19$), including 3–6 replicates per species and site, except for *I. oros* in Caials for which we only had two replicates. All sampled sponges appeared healthy and were collected from sites located 80–800 km apart and characterized by different anthropogenic pressures: from marine protected areas (Cabrera National Park, Scandola Nature Reserve in Corsica, Caials-Natural Park of Cap de Creus), to locations near dense human populations (Blanes, Calafat and Alicante). When possible, ambient seawater (500 mL) was simultaneously sampled in close proximity (< 1 m) to the sponges (Caials, $n = 1$; Blanes, $n = 3$; Alicante, $n = 2$). Sponge samples were immediately preserved in absolute ethanol and seawater samples were concentrated on 0.2 μm filters prior to preservation in ethanol. All samples were stored at −20 °C.

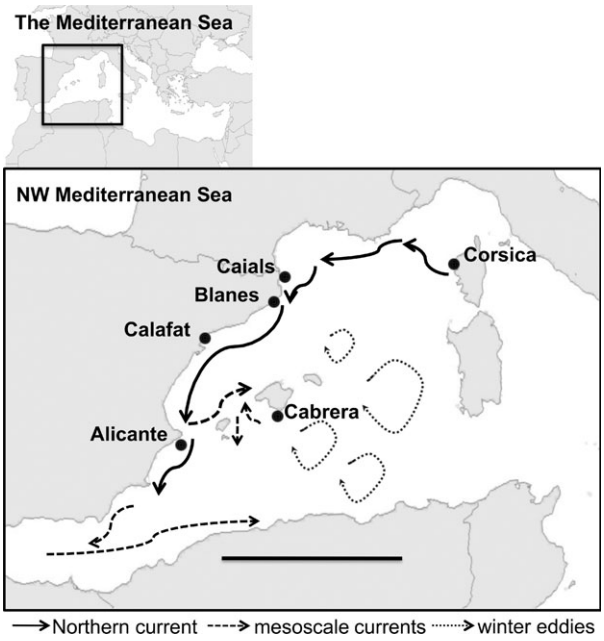

Fig. 1. Sampling sites in the western Mediterranean Sea. Sampling sites and main currents in the region (adapted from Millot, 1999) are shown. Scale bar = 422 km.

DNA extractions and T-RFLP analyses

Genomic DNA was extracted from tissue and seawater samples using the DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The universal bacterial forward primer Eco8F (Turner *et al.*, 1999), tagged with a 5′-end 6-carboxyfluorescein label (6-FAM), and the reverse primer 1509R (Martínez-Murcia *et al.*, 1995) were used for amplification of a *c.* 1500-bp fragment of the 16S rRNA gene. PCR was performed as follows: one initial denaturation step for 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 0.5 min at 50 °C and 1.5 min at 72 °C; and one final elongation step for 5 min at 72 °C. Total PCR volume (50 μL) included 10 μM of each primer, 10 nM of each dNTP, 1 × Reaction Buffer (Ecogen, Barcelona, Spain), 2.5 mM $MgCl_2$, 5 units of BioTaq™ DNA polymerase (Ecogen), and full-strength or 1 : 10 diluted DNA extracts. Products from triplicate PCR reactions were purified from electrophoresis gels using the Qiaquick Gel Extraction kit (Qiagen), and quantified using the Qubit™ fluorometer and Quant-iT™ dsDNA Assay kit (Invitrogen, Carlsbad, CA), according to the manufacturers' instructions. Separate digestions with the restriction enzymes HaeIII and MspI were performed as described by Pita *et al.* (2013) and analyzed in an automated ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Genomics Unit of the Scientific and Technologic Center of the University of Barcelona. The lengths of each terminal-restriction fragment (T-RF) were determined with respect to an internal size standard (LIZ600) using the PeakScanner™ software (Applied Biosystems). T-RFs smaller than 50 bp or larger than 600 bp were discarded because they were beyond the resolution of the size standard. Peak intensities below 50 fluorescence units and relative peak area variation within a cut-off value of two standard deviations (Abdo *et al.*, 2006) were discarded as background noise using the T-REX online tool (Culman *et al.*, 2009). 'True' T-RFs were then aligned in T-REX using a clustering threshold of 1 bp to construct relative T-RF abundance matrices.

Statistical analyses of T-RFLP data

Relative abundance matrices were square root transformed prior to all analyses based on Bray–Curtis distances. For each restriction enzyme, nonmetric multidimensional scaling (nMDS) plots were constructed to visualize bacterial community similarity. Permutational multivariate analyses of variance (PERMANOVAS; Anderson, 2001; McArdle & Anderson, 2001) were used to test for variability across sources (seawater and the three sponge species) and among locations within each sponge host. To compare structure within groups and determine the effect of heterogeneity (dispersion) on significant PERMANOVA outcomes, pairwise comparisons of dispersion (PERMDISP; Anderson, 2006) were performed. SIMPER analyses were conducted to identify the individual T-RFs driving the differentiation between groups. Calculations were performed in PRIMER v6 (Clarke, 1993; Clarke & Gorley, 2006) and PERMANOVA+ (Plymouth Marine Laboratory, UK). Critical values for significance were corrected for multiple pairwise comparisons following the Benjamini & Yekutieli (2001) algorithm (B-Y correction). Mantel tests for each host and restriction enzyme were calculated in R v2.15.2 (The R Core Team, 2012) using the package ADE4 (Dray & Dufour, 2007) to determine whether differences in bacterial community similarity were correlated with geographic distances. We also repeated the Mantel tests excluding the island of Cabrera from the analyses to test if dominant currents in the Western Mediterranean (Fig. 1) isolated Cabrera from the peninsular locations, creating a disproportionate differentiation despite short geographic distances and hence distorting the isolation-by-distance effect across the other locations. For each enzyme and species, we partitioned data matrices into 'rare' T-RFs (relative abundance ≤ 1% of each sample) and 'abundant' T-RFs (relative abundance > 1%) to determine the influence of rare and abundant T-RFs in the trends observed for the whole community. These threshold values were chosen due to their widespread use in microbial ecology studies (Pedrós-Alió, 2006) and empirical ability to partition the dataset relatively evenly (Table 1). Rare and abundant T-RF matrices were analyzed separately with the same procedures described above.

T-RFLP analysis and 16S rRNA gene sequence data

Predicted T-RFs from a reference database were matched with the empirical T-RFs obtained in this study. The reference database consisted of *in silico* digestions by HaeIII and MspI enzymes of *Ircinia*-associated bacterial 16S rRNA gene sequences from a previous study (Erwin *et al.*, 2012b). The analysis was performed with the phylogenetic assignment tool PAT (Kent *et al.*, 2003), adding an extra bin size for small T-RFs (i.e. 2-bp tolerance applied to fragments of 50–100 bp).

Results

T-RFLP analyses

We identified 183 bacterial T-RFs with the HaeIII enzyme (139 in *I. fasciculata*, 108 in *I. oros*, 140 in *I. variabilis*

Table 1. T-RFs obtained for each sponge species and seawater

	HaeIII				MspI			
	IF	IO	IV	SW	IF	IO	IV	SW
Total T-RFs	42 ± 3	34 ± 4	41 ± 3	31 ± 6	40 ± 3	42 ± 3	44 ± 3	25 ± 2
Abundant T-RFs	20 ± 1	18 ± 1	20 ± 1	20 ± 2	19 ± 1	21 ± 1	20 ± 1	9 ± 1
Rare T-RFs	22 ± 3	16 ± 3	22 ± 2	12 ± 5	19 ± 3	22 ± 3	25 ± 3	17 ± 1

Shown are the number (average ± SE) of total, abundant (relative peak area > 1%) and rare (relative peak area ≤ 1%) T-RFs found per sample within each sponge species and seawater, for each restriction enzyme (HaeIII and MspI). IF, *Ircinia fasciculata*; IO, *Ircinia oros*, IV, *Ircinia variabilis*; SW, seawater.

and 79 in seawater) and 211 using the MspI enzyme (140 in *I. fasciculata*, 145 in *I. oros*, 184 *I. variabilis* and 57 in seawater). The mean and standard error of T-RFs in each category (total, abundant and rare) per source is reported for HaeIII and MspI enzymes in Table 1. Regarding the specificity of the T-RFs, 25.1% (HaeIII) and 20.9% (MspI) were detected in all sources (i.e. present in at least one sample of *I. fasciculata*, *I. variabilis*, *I. oros* and seawater), whereas 19.6% (HaeIII) and 30.3% (MspI) were detected in all sponge species and were absent in seawater. The proportion of T-RFs that are shared among sources is depicted in Supporting Information, Fig. S1. nMDS plots of all samples (Fig. 2a) showed that bacterial communities clustered by source, with sponge-derived samples more similar to each other than to seawater samples. Sponge-derived samples further grouped by host species, but with more discrimination among species for HaeIII than for MspI fingerprints. nMDS graphs for sponge-derived communities (Fig. 2b) showed no consistent grouping of sponge-associated bacterial communities based on sampling location. This apparent lack of spatial structure was maintained when nMDS plots were drawn separately for each sponge species (Fig. 3). Some *I. variabilis*-derived samples from HaeIII digestions (Fig. 3b, left) showed a tendency to cluster according to sampling location, yet this spatial pattern was not evident for samples from MspI digestions (Fig. 3b, right).

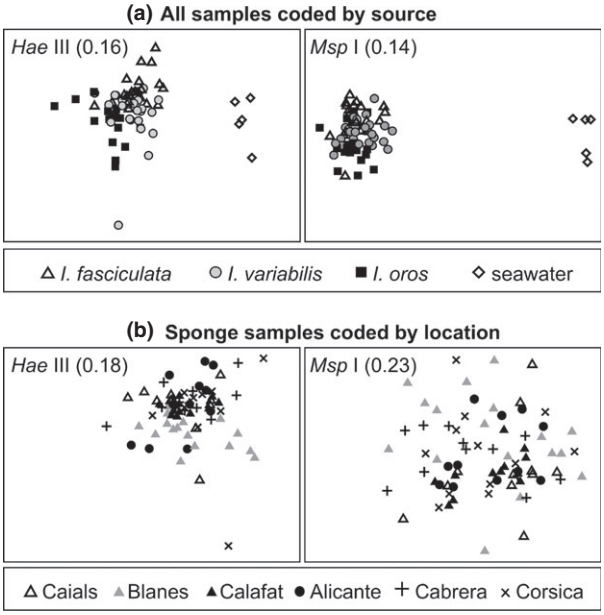

Fig. 2. Spatial patterns of bacterial communities in marine sponges and seawater. nMDS plots of bacterial T-RFLP profiles obtained from HaeIII (left) and MspI (right) digestions. (a) All samples coded by source; (b) sponge samples coded by location. Stress values are shown in parentheses.

Comparisons among sources

Pairwise comparisons of T-RFLP profiles among sources (PERMANOVA, Table 2) revealed significant differences ($P < 0.05$) among the bacterial communities in each sponge species and seawater for both enzymes and for all comparisons, confirming the patterns visualized in nMDS graphs. The bacterial communities in seawater samples were significantly different from sponge samples, and bacterial communities in sponges were host-species specific. PERMDISP revealed a similar degree of heterogeneity within each source ($P > 0.10$ for all comparisons), and thus the differences between sources were due to differences in symbiont structure. These results were largely maintained when only rare T-RFs or abundant T-RFs were considered (Table 2). The only consistent difference between these data partitions and the entire dataset was that rare *I. variabilis*-derived communities were not different from the rare communities in *I. fasciculata* (for both enzymes), and that the rare communities in *I. fasciculata* and *I. variabilis* did not differ significantly from rare symbionts of *I. oros* for MspI digestions (Table 2).

Differentiation among locations within sponge hosts

Pairwise comparisons of T-RFLP profiles among locations within each sponge species (nested PERMANOVA, Table 3)

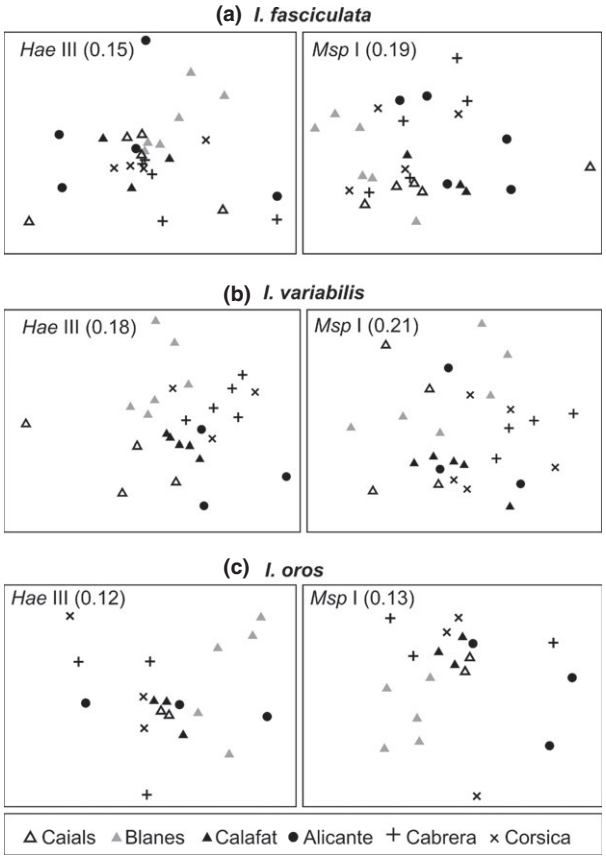

Fig. 3. Spatial patterns of bacterial communities in three *Ircinia* sponge species. nMDS plots of bacterial T-RFLP profiles obtained from HaeIII (left) and MspI (right) digestions. (a) *Ircinia fasciculata*-derived samples; (b) *Ircinia variabilis*-derived samples; (c) *Ircinia oros*-derived samples. Stress values are shown in parentheses.

showed no significant differences in the bacterial communities of *I. fasciculata* and *I. oros* across sampling sites. In *I. variabilis*, Blanes and Cabrera were significantly different in HaeIII-digested T-RFLP profiles and Cabrera–Calafat comparisons were consistently significant for both enzymes. On the whole, PERMDISP analyses (Table 3) indicated similar dispersion of the samples within groups, with some exceptions for HaeIII digestions in *I. fasciculata* (Blanes–Alicante) and *I. variabilis* (Blanes–Cabrera, Blanes–Calafat, Calafat–Corsica). For rare T-RFs, neither PERMANOVA nor PERMDISP detected significant differences in any pairwise comparison (Table S1), indicating higher stability and homogeneity of rare sponge symbionts. The analysis of abundant T-RFs revealed additional significant comparisons (i.e. recovered for both enzymes) between Blanes and Calafat for *I. variabilis*, and Blanes and Alicante for *I. fasciculata* (Table S2).

Isolation-by-distance effect

Mantel tests showed no significant correlation between geographic distances and bacterial community similarity for full datasets (Table 3), rare partitions (Table S1) and abundant partitions (Table S2); thus, isolation-by-distance effects were not detected in any sponge host or symbiont partition. Results from Mantel tests excluding samples from the island of Cabrera were also not significant with one exception: a significant outcome ($P = 0.022$) for HaeIII digestions in *I. variabilis* for the full dataset (Table 3).

Congruence between T-RFLP analysis and 16S rRNA gene sequence data

PAT analysis showed high congruence between T-RFLP and *in silico* digestions of the reference database containing 16S rRNA gene sequence data from Mediterranean *Ircinia* species (Erwin *et al.*, 2012b). The length profiles obtained from the reference database matched 59.1% (HaeIII) and 62.8% (MspI) of the peaks detected empirically in T-RFLP profiles, representing 73.2% (HaeIII) and 79.3% (MspI) of the total peak area. For instance, the T-RF signature of operational taxonomic unit (OTU)001,

Table 2. Host-specificity of bacterial communities

	Whole community		Rare T-RFs		Abundant T-RFs	
	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI
I. fasciculata–*I. variabilis*	0.001*** (0.251)	0.002** (0.300)	0.063 (0.295)	0.480 (0.530)	0.001*** (0.152)	0.005** (0.004**)
I. fasciculata–*I. oros*	0.001*** (0.150)	0.001*** (0.574)	0.001*** (0.359)	0.076 (0.494)	0.001*** (0.228)	0.001*** (0.043)
I. variabilis–*I. oros*	0.001*** (0.706)	0.001*** (0.767)	0.006* (0.810)	0.035 (0.841)	0.001*** (0.880)	0.001*** (0.523)
I. fasciculata–Seawater	0.001*** (0.656)	0.001*** (0.682)	0.001*** (0.632)	0.001*** (0.979)	0.001*** (0.829)	0.001*** (0.601)
I. variabilis–Seawater	0.001*** (0.889)	0.001*** (0.355)	0.001*** (0.889)	0.001*** (0.843)	0.001*** (0.454)	0.001*** (0.062)
I. oros–Seawater	0.001*** (0.606)	0.001*** (0.496)	0.001*** (0.915)	0.001*** (0.719)	0.001*** (0.408)	0.001*** (0.136)

Multivariate pairwise comparisons of bacterial T-RFLP profiles among sources, for each restriction enzyme (HaeIII and MspI) applied to the whole community, to the rare partition (relative abundance ≤ 1%) and to the abundant partition (relative abundance > 1%). The multivariate version of *P*-values after 999 permutations from PERMANOVA and PERMDISP (in parentheses) tests are reported. Critical values for significance were corrected for multiple comparisons (B-Y correction) and significant values are indicated with asterisks (*$\alpha < 0.05$, **$\alpha < 0.01$, ***$\alpha < 0.005$).

Table 3. Spatial structure of bacterial communities within sponge hosts

	I. fasciculata		*I. variabilis*		*I. oros*	
	HaeIII	MspI	HaeIII	MspI	HaeIII	MspI
Multivariate analysis						
Blanes–Alicante	0.291 (0.003*)	0.019 (0.344)	0.038 (0.911)	0.192 (0.620)	0.184 (0.842)	0.025 (0.167)
Blanes–Caials	0.122 (0.854)	0.064 (0.935)	0.040 (0.153)	0.080 (0.855)	0.151 (0.048)	0.090 (0.039)
Blanes–Cabrera	0.091 (0.990)	0.049 (0.644)	0.008* (0.004*)	0.029 (0.060)	0.036 (0.492)	0.036 (0.507)
Blanes–Calafat	0.212 (0.400)	0.044 (0.023)	0.017 (0.005*)	0.023 (0.125)	0.059 (0.087)	0.029 (0.087)
Blanes–Corsica	0.113 (0.274)	0.116 (0.497)	0.206 (0.319)	0.041 (0.113)	0.046 (0.946)	0.036 (0.329)
Alicante–Caials	0.527 (0.269)	0.193 (0.763)	0.216 (0.547)	0.273 (0.521)	0.468 (0.220)	0.331 (0.213)
Alicante–Cabrera	0.452 (0.174)	0.075 (0.051)	0.061 (0.052)	0.124 (0.222)	0.628 (0.623)	0.251 (0.819)
Alicante–Calafat	0.722 (0.053)	0.205 (0.018)	0.095 (0.057)	0.457 (0.435)	0.317 (0.092)	0.154 (0.093)
Alicante–Corsica	0.450 (0.031)	0.113 (0.198)	0.300 (0.883)	0.310 (0.563)	0.556 (0.892)	0.258 (0.905)
Caials–Cabrera	0.218 (0.958)	0.150 (1)	0.023 (0.005*)	0.030 (0.123)	0.400 (0.089)	0.324 (0.381)
Caials–Calafat	0.490 (0.709)	0.104 (0.776)	0.154 (0.017)	0.109 (0.259)	0.365 (0.105)	0.489 (0.114)
Caials–Corsica	0.253 (0.554)	0.175 (0.977)	0.229 (0.861)	0.090 (0.273)	0.403 (0.408)	0.454 (0.314)
Cabrera–Calafat	0.183 (0.884)	0.043 (0.016)	0.009* (0.253)	0.012* (0.898)	0.250 (0.102)	0.181 (0.103)
Cabrera–Corsica	0.241 (0.568)	0.304 (0.563)	0.225 (0.018)	0.060 (0.161)	0.669 (0.588)	0.450 (1)
Calafat–Corsica	0.301 (0.850)	0.113 (0.092)	0.083 (0.009*)	0.098 (0.362)	0.082 (0.099)	0.244 (0.113)
Mantel test (all sites)	0.863	0.931	0.085	0.860	0.950	0.591
Mantel test (no Cabrera)	*0.411*	*0.755*	*0.022**	*0.633*	*0.841*	*0.438*

Multivariate pairwise comparisons of bacterial T-RFLP profiles among locations within sponge host and each restriction enzyme (HaeIII and MspI). The multivariate version of *P*-values after 999 permutations from PERMANOVA and PERMDISP (in parentheses) tests are reported. Critical values for significance were corrected for multiple comparisons (B-Y correction) and significant values are indicated with asterisks (*$\alpha < 0.05$). Isolation-by-distance effects were investigated by Mantel tests (*P*-values indicated) for all locations and excluding the island of Cabrera (in italics), for each restriction enzyme.

a dominant deltaproteobacterium in all three host species that is closely related to other sponge- and coral-derived symbionts (Erwin *et al.*, 2012a, b), was consistently detected as a conspicuous peak in all sponge species at all locations, with both restriction enzymes. Combining the information from HaeIII and MspI digestions, T-RFLP profiles retrieved 72.5% of the OTUs in the sequence database and included *Deltaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Acidobacteria*, *Cyanobacteria* (in *I. fasciculata* and *I. variabilis*), *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes* and *Verrucomicrobia* that were representative of the bacterial communities in Mediterranean *Ircinia* spp. (Erwin *et al.*, 2012a, b).

Discussion

The bacterial communities associated with the co-occurring Mediterranean sponges *I. fasciculata*, *I. variabilis* and *I. oros* were structured primarily by host species and remained largely stable across geographic distances of up to 800 km. These results reinforced the key role of host sponge species on the composition of their symbiotic bacterial communities (Montalvo & Hill, 2011; Erwin *et al.*, 2012a; Hardoim *et al.*, 2012) and were consistent with high spatial stability reported in previous studies (Taylor *et al.*, 2005; Wichels *et al.*, 2006; Thiel *et al.*, 2007; Schöttner *et al.*, 2013), including other *Ircinia* species (Pita *et al.*, 2013). In addition, we revealed overall similar patterns of spatial stability and host specificity between rare and abundant bacteria, as has been found for free-living microbial communities (Galand *et al.*, 2009).

However, rare bacterial symbionts exhibited slightly higher stability over sampled locations than abundant bacterial symbionts, especially for *I. variabilis*. This is contrary to a recent study where we reported the temporal dynamics of microbial communities in these same sponge species (Erwin *et al.*, 2012b) and showed remarkable stability in symbiont composition over time with some seasonal variability observed for the rare symbiont taxa. Rare taxa may represent transient bacteria (e.g. from seawater, sediment or fouling) that would be more susceptible to seasonal environmental changes than abundant bacteria (true symbionts), while their spatial stability suggests low selection pressure due to geographic location. Other rare bacterial taxa could be missed in T-RFLP profiles due to technical limitations (Pedrós-Alió, 2012). The fewer T-RFs observed for the seawater profiles compared with sponges (an apparent contradiction with previous studies based on cloning and next-generation sequencing techniques; e.g. Webster *et al.*, 2010; Erwin *et al.*, 2012a) probably result from a lower replication of the seawater samples. Future studies on the spatial structure of

bacterioplankton communities in the Western Mediterranean are needed to further reveal the different ecological constraints affecting free-living and sponge-derived bacterial communities (Erwin *et al.*, 2012b).

At the beginning of this study, we hypothesized that within each host species, bacterial communities derived from sponges in closer locations would exhibit higher similarity (i.e. isolation-by-distance effects) because: (1) vertical symbiont transmission in *Ircinia* spp. (Schmitt *et al.*, 2007) may link symbiont dispersal range with that of host larvae; and (2) significant spatial structure and isolation-by-distance patterns were found for other sponge species within the same region studied herein (*Scopalina lophyropoda*, Blanquer & Uriz, 2010; *Crambe crambe*, Duran *et al.*, 2004). However, we did not observe a significant correlation between bacteria differentiation and geographic distances for any host *Ircinia* species. There are several potential explanations for this lack of differentiation. First, these sponges may disperse farther than expected: bacteria in larvae could represent an extra food supply allowing larvae to spend more time in the water column, increasing the probability of successful dispersal, and resulting in high connectivity among *Ircinia* populations (Mariani *et al.*, 2005; Uriz *et al.*, 2008). Second, host-related factors and symbiotic interactions may exert an intense selective pressure on the bacterial community so that there is no scope for spatial differentiation, even if the connectivity between localities is scarce. Alternatively, signatures of dispersal limitation may occur yet be masked by the taxonomic resolution of 16S rRNA gene sequences (Erwin & Thacker, 2008).

In addition to dispersal limitation processes, microbial biogeography patterns may be shaped by environmental selection (Fierer, 2008). Local features such as currents, river discharges and human activities generate variability in physicochemical parameters and spatial differences of bacterioplankton composition among coastal locations in the Western Mediterranean Sea (Schauer *et al.*, 2000; Flo *et al.*, 2011). While environmental data were not included in our study, it is notable that our sampling sites covered locations near dense human populations (e.g. Blanes, Alicante) and more pristine, protected areas (e.g. Cabrera, Corsica, Caials). However, *Ircinia*-derived bacterial communities persisted across these locations and suggested that the symbiotic community was mostly unaffected by differences in local conditions. A potential exception was observed in bacterial communities associated with two populations of *I. variabilis*. Specifically, differences in symbiont communities occurred between the marine protected area around the island of Cabrera and the populous mainland site of Calafat, which suggests some effect of environmental conditions on the structure of *I. variabilis*-associated communities. Specific features of *I. variabilis* sponges, such as the plastic morphology characteristic of this species (Turon *et al.*, 2013) or reproductive strategy, could make this species more sensitive to local processes than the other two *Ircinia* spp., which in turn could influence the spatial dynamics of the bacterial community structure (Lee *et al.*, 2009a).

Furthermore, a significant isolation-by-distance effect was detected for *I. variabilis* samples after removing Cabrera from the analyses, indicating that inclusion of this site distorts distance–decay trends due to its close geographical proximity yet physical isolation by dominant currents from the remaining sites. Notably, these spatial trends in *I. variabilis* were only detected in T-RFLP profiles with the enzyme HaeIII, which generally exhibited lower resolution than profiles with MspI (Zhang *et al.*, 2008; Erwin *et al.*, 2012b; Pita *et al.*, 2013; this study). Thus, these trends should be interpreted with caution until more data are obtained to confirm these findings.

In this study, we showed that the bacterial communities associated with three co-ocurring *Ircinia* sponges (*I. fasciculata*, *I. variabilis* and *I. oros*) were host-species specific and stable across locations 80–800 km apart in the Western Mediterranean Sea. Combined with previous reports of symbiont stability in *Ircinia* spp. over large seasonality in environmental conditions (Erwin *et al.*, 2012b), our results support the hypothesis of a unique and stable microenvironment (e.g. mesohyl-specific conditions) within the host sponge body that is largely unaffected by local or seasonal environmental conditions. Long-term symbiotic interactions shaped by multiple selective pressures (e.g. biotic factors, seasonal and stochastic environmental changes) over time and vertical transmission of key bacteria may have resulted in these persistent bacterial communities. Further studies testing the resilience of these relationships under stressful conditions and investigating how bacterial symbionts metabolically interact with their hosts will provide insights into the vulnerability and resilience of these sponge holobionts in the Mediterranean Sea.

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References

Abdo Z, Schüette UME, Bent SJ, Williams CJ, Forney LJ & Joyce P (2006) Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environ Microbiol* **8**: 929–938.

Anderson M (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecol* **26**: 32–46.

Anderson M (2006) Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* **62**: 245–253.

Anderson SA, Northcote PT & Page MJ (2010) Spatial and temporal variability of the bacterial community in different chemotypes of the New Zealand marine sponge *Mycale hentscheli*. *FEMS Microbiol Ecol* **72**: 328–342.

Benjamini Y & Yekutieli D (2001) The control of the false discovery rate in multiple testing under dependency. *Ann Stat* **29**: 1165–1188.

Blanquer A & Uriz MJ (2010) Population genetics at three spatial scales of a rare sponge living in fragmented habitats. *BMC Evol Biol* **10**: 13.

Clarke K (1993) Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol* **18**: 117–143.

Clarke K & Gorley R (2006) *PRIMER v6: User Manual/ Tutorial*. PRIMER-E, Plymouth, MA.

Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H & Buckley DH (2009) T-REX: software for the processing and analysis of T-RFLP data. *BMC Bioinformatics* **10**: 171.

Dray S & Dufour A (2007) The ade4 package: implementing the duality diagram for ecologists. *J Stat Softw* **22**: 1–20.

Duran S, Pascual M, Estoup A & Turon X (2004) Strong population structure in the marine sponge *Crambe crambe* (Poecilosclerida) as revealed by microsatellite markers. *Mol Ecol* **13**: 511–522.

Ereskovsky AV & Tokina DB (2004) Morphology and fine structure of the swimming larvae of *Ircinia oros* (Porifera, Demospongiae, Dictyoceratida). *Invertebr Reprod Dev* **45**: 137–150.

Ereskovsky AV, Gonobobleva E & Vishnyakov A (2004) Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). *Mar Biol* **146**: 869–875.

Erwin PM & Thacker RW (2008) Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts. *Mol Ecol* **17**: 2937–2947.

Erwin PM, López-Legentil S, González-Pech R & Turon X (2012a) A specific mix of generalists: bacterial symbionts in Mediterranean *Ircinia* spp. *FEMS Microbiol Ecol* **79**: 619–637.

Erwin PM, Pita L, López-Legentil S & Turon X (2012b) Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance. *Appl Environ Microbiol* **78**: 7358–7368.

Fierer N (2008) Microbial biogeography: patterns in microbial diversity across space and time. *Accessing Uncultivated Microorganisms: From the Environment to Organisms and Genomes* (Zengler K, ed), pp. 95–115. ASM Press, Washington, DC.

Flo E, Garcés E, Manzanera M & Camp J (2011) Coastal inshore waters in the NW Mediterranean: physicochemical and biological characterization and management implications. *Estuar Coast Shelf Sci* **93**: 279–289.

Galand PE, Casamayor EO, Kirchman DL & Lovejoy C (2009) Ecology of the rare microbial biosphere of the Arctic Ocean. *P Natl Acad Sci USA* **106**: 22427–22432.

Hanson CA, Fuhrman JA, Horner-Devine MC & Martiny JBH (2012) Beyond biogeographic patterns: processes shaping the microbial landscape. *Nat Rev Microbiol* **10**: 497–506.

Hardoim CCP, Esteves AIS, Pires FR, Gonçalves JMS, Cox CJ, Xavier JR & Costa R (2012) Phylogenetically and spatially close marine sponges harbour divergent bacterial communities. *PLoS ONE* **7**: e53029.

Hentschel U, Horn M, Friedrich AB, Wagner M & Moore BS (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol* **68**: 4431–4440.

Hentschel U, Piel J, Degnan SM & Taylor MW (2012) Genomic insights into the marine sponge microbiome. *Nat Rev Microbiol* **10**: 641–654.

Kent AD, Smith DJ, Benson BJ & Triplett EW (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Appl Environ Microbiol* **69**: 6768–6776.

Lee OO, Wong YH & Qian P-Y (2009a) Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. *Appl Environ Microbiol* **75**: 3513–3521.

Lee OO, Chui PY, Wong YH, Pawlik JR & Qian P-Y (2009b) Evidence for vertical transmission of bacterial symbionts from adult to embryo in the Caribbean sponge *Svenzea zeai*. *Appl Environ Microbiol* **75**: 6147–6156.

Mariani S, Uriz MJ & Turon X (2005) The dynamics of sponge larvae assemblages from northwestern Mediterranean nearshore bottoms. *J Plankton Res* **27**: 249–262.

Martínez-Murcia A, Acinas S & Rodríguez-Valera F (1995) Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. *FEMS Microbiol Ecol* **17**: 247–255.

Martiny JBH, Bohannan BJM, Brown JH *et al.* (2006) Microbial biogeography: putting microorganisms on the map. *Nat Rev Microbiol* **4**: 102–112.

McArdle BH & Anderson MJ (2001) Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82**: 290–297.

Millot C (1999) Circulation in the Western Mediterranean Sea. *J Mar Syst* **20**: 423–442.

Montalvo NF & Hill RT (2011) Sponge-associated bacteria are strictly maintained in two closely related but geographically distant sponge hosts. *Appl Environ Microbiol* **77**: 7207–7216.

Pedrós-Alió C (2006) Marine microbial diversity: can it be determined? *Trends Microbiol* **14**: 257–263.

Pedrós-Alió C (2012) The rare bacterial biosphere. *Ann Rev Mar Sci* **4**: 449–466.

Pita L, López-Legentil S & Erwin PM (2013) Biogeography and host fidelity of bacterial communities in *Ircinia* spp from the Bahamas. *Microb Ecol*, in press DOI:10.1007/s00248-013-0215-2

Schauer M, Massana R & Pedrós-Alió C (2000) Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. *FEMS Microbiol Ecol* **33**: 51–59.

Schmitt S, Weisz JB, Lindquist N & Hentschel U (2007) Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. *Appl Environ Microbiol* **73**: 2067–2078.

Schmitt S, Angermeier H, Schiller R, Lindquist N & Hentschel U (2008) Molecular microbial diversity survey of sponge reproductive stages and mechanistic insights into vertical transmission of microbial symbionts. *Appl Environ Microbiol* **74**: 7694–7708.

Schmitt S, Tsai P, Bell J *et al.* (2012) Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J* **6**: 564–576.

Schöttner S, Hoffmann F, Cárdenas P, Rapp HT, Boetius A & Ramette A (2013) Relationships between host phylogeny, host type and bacterial community diversity in cold-water coral reef sponges. *PLoS ONE* **8**: e55505.

Taylor MW, Schupp PJ, De Nys R, Kjelleberg S & Steinberg PD (2005) Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. *Environ Microbiol* **7**: 419–433.

Taylor MW, Radax R, Steger D & Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol Mol Biol Rev* **71**: 295–347.

Taylor MW, Tsai P, Simister RL, Deines P, Botte E, Ericson G, Schmitt S & Webster NS (2013) 'Sponge-specific' bacteria are widespread (but rare) in diverse marine environments. *ISME J* **7**: 438–443.

The R Core Team (2012) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.

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

Turner S, Pryer KM, Miao VP & Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J Eukaryot Microbiol* **46**: 327–338.

Turon X, Garriga A & Erwin PM (2013) Lights and shadows: growth patterns in three sympatric and congeneric sponges (*Ircinia* spp) with contrasting abundances of photosymbionts. *Mar Biol*, in press DOI: 10.1007/s00227-013-2267-8

Uriz MJ, Turon X & Mariani S (2008) Ultrastructure and dispersal potential of sponge larvae: tufted versus evenly ciliated parenchymellae. *Mar Ecol* **29**: 280–297.

Usher KM, Kuo J, Fromont J & Sutton DC (2001) Vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Hydrobiologia* **461**: 15–23.

Webster NS, Negri AP, Munro MMHG & Battershill CN (2004) Diverse microbial communities inhabit Antarctic sponges. *Environ Microbiol* **6**: 288–300.

Webster NS, Taylor MW, Behnam F, Lücker S, Rattei T, Whalan S, Horn M & Wagner M (2010) Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environ Microbiol* **12**: 2070–2082.

Wichels A, Würtz S, Döpke H, Schütt C & Gerdts G (2006) Bacterial diversity in the breadcrumb sponge *Halichondria panicea* (Pallas). *FEMS Microbiol Ecol* **56**: 102–118.

Yang J, Sun J, Lee O, Wong Y & Qian P (2011) Phylogenetic diversity and community structure of sponge-associated bacteria from mangroves of the Caribbean Sea. *Aquat Microb Ecol* **62**: 231–240.

Zhang R, Thiyagarajan V & Qian P-Y (2008) Evaluation of terminal-restriction fragment length polymorphism analysis in contrasting marine environments. *FEMS Microbiol Ecol* **65**: 169–178.

Supporting Information

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

Fig. S1. Specificity of T-RFs in *I. fasciculata* (IF), *I. variabilis* (IV), *I. oros* (IO), and seawater (SW).

Table S1. Comparisons of rare bacterial communities among locations within sponge host.

Table S2. Comparisons of abundant bacterial communities among locations within sponge host.

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Stability of Sponge-Associated Bacteria over Large Seasonal Shifts in Temperature and Irradiance

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Complex microbiomes reside in marine sponges and consist of diverse microbial taxa, including functional guilds that may contribute to host metabolism and coastal marine nutrient cycles. Our understanding of these symbiotic systems is based primarily on static accounts of sponge microbiota, while their temporal dynamics across seasonal cycles remain largely unknown. Here, we investigated temporal variation in bacterial symbionts of three sympatric sponges (*Ircinia* spp.) over 1.5 years in the northwestern (NW) Mediterranean Sea, using replicated terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of bacterial 16S rRNA gene sequences. Bacterial symbionts in *Ircinia* spp. exhibited host species-specific structure and remarkable stability throughout the monitoring period, despite large fluctuations in temperature and irradiance. In contrast, seawater bacteria exhibited clear seasonal shifts in community structure, indicating that different ecological constraints act on free-living and on symbiotic marine bacteria. Symbiont profiles were dominated by persistent, sponge-specific bacterial taxa, notably affiliated with phylogenetic lineages capable of photosynthesis, nitrite oxidation, and sulfate reduction. Variability in the sponge microbiota was restricted to rare symbionts and occurred most prominently in warmer seasons, coincident with elevated thermal regimes. Seasonal stability of the sponge microbiota supports the hypothesis of host-specific, stable associations between bacteria and sponges. Further, the core symbiont profiles revealed in this study provide an empirical baseline for diagnosing abnormal shifts in symbiont communities. Considering that these sponges have suffered recent, episodic mass mortalities related to thermal stresses, this study contributes to the development of model sponge-microbe symbioses for assessing the link between symbiont fluctuations and host health.

Sponges are sessile invertebrates that form a species-rich phylum at the base of the metazoan tree of life (>8,500 valid species [65]). Renowned for their efficient filter-feeding capabilities and bioactive secondary metabolite production, sponges have important ecological and biotechnological relevance as major players in marine nutrient cycles (11, 12, 26) and the most prolific producers of marine natural products (>6,600 secondary metabolites [16]). The discovery and characterization of diverse microbial symbionts inhabiting the sponge body have prompted the adoption of the holobiont concept, thereby incorporating microbial symbionts in the study of sponge ecology and evolution (55). The resulting field of sponge microbiology has grown rapidly in the past 2 decades (59) and revealed a tight ecological link between host health and symbiont composition. Indeed, sponge-associated microbes have been implicated in host metabolism and growth (20, 22, 75), chemical defense production (21), and susceptibility to biotic (e.g., disease) and abiotic (e.g., temperature stress) stressors (33, 66, 72).

The remarkable diversity of the sponge microbiota has presented a formidable challenge to understanding the structure and function of microbial guilds in sponge hosts (24, 59, 70). The sponge microbiota includes diverse phylogenetic lineages of *Archaea* and *Bacteria*, as well as fungi and viruses (52, 56). Among bacterial symbionts alone, thousands of taxa have been reported, spanning 17 described phyla and 12 candidate phyla (50), and hundreds of bacterial taxa can occur in a single host individual (32, 71). Accordingly, considerable effort has focused on describing the vast diversity of the sponge microbiota, while more applied studies of symbiont functioning have targeted specific components (e.g., *Cyanobacteria* [59]) or functional gene pathways (e.g., ammonia oxidation [35]) in these communities. As a result, most studies of sponge microbiology have been limited in scope to one or a few host species collected at a single time point, and thus, much of our knowledge concerning the sponge microbiota is based on a static representation of these potentially dynamic communities (59).

Understanding the complex sponge microbiota requires a basic knowledge of how these communities change over time. The general consensus is that sponge-microbe associations are largely stable over temporal scales (56), including epibionts (31), cultivatable symbionts (68), and entire bacterial communities (57, 60, 61, 73). Other studies have reported higher levels of variability across seasons (74) and when repeatedly sampling the same individuals over time (3), indicating some degree of symbiont fluctuation over time and individual variation among hosts. The prospect of sponge aquaculture for the production of bioactive metabolites has prompted investigations of host-symbiont stability under *ex situ* aquarium conditions, revealing high symbiont stability over short-term time scales (11 days to 12 weeks [23, 67]), while longer-term maintenance (6 months to 2 years) can result in substantial shifts in symbiont composition (39, 40, 67). Additional studies of temporal variation in sponge-associated bacteria under natural conditions will aid future aquaculture efforts by

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determining natural variation in the sponge microbiota and its consequences for host-symbiont dynamics. Further, such studies establish the baseline levels of symbiont variability required to define abnormal shifts and ascribe symbiont fluctuations to specific abiotic and biotic factors.

In this study, we investigated temporal variation in the microbiota of three congeneric sponge hosts from the Mediterranean Sea: *Ircinia fasciculata*, *I. variabilis*, and *I. oros*. These sponges are common members of coastal benthic communities in the Mediterranean Sea and harbor diverse, host-specific communities of bacterial and cyanobacterial symbionts (15, 17, 46, 63). Replicate individuals of each sponge species were tagged *in situ* and sampled quarterly for 1.5 years to monitor their bacterial symbiont communities, using terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of bacterial 16S rRNA gene sequences. In addition, photosynthetic pigments were monitored in the tissues of the cyanobacterium-rich sponges *I. fasciculata* and *I. variabilis*, using chlorophyll *a* (chl *a*) quantification. The specific objectives of the study were (i) to determine the temporal stability of host-symbiont specificity, (ii) to identify permanent and transient symbiont taxa in association with sponge hosts, and (iii) to document natural variability in symbiont communities over time. Collectively, these objectives contribute to the broader goal of establishing the empirical baselines required to diagnose abnormal symbiont shifts and develop these symbiotic systems as an impact assessment tool in coastal ecosystems.

METHODS

Sample collection. The sponge species *Ircinia fasciculata* (Pallas, 1766), *I. variabilis* (Schmidt, 1862), and *I. oros* (Schmidt, 1864) were monitored in shallow (<20 m) littoral zones at two neighboring sites (<12 km apart) along the Catalan Coast (Spain) in the northwestern (NW) Mediterranean Sea. *I. fasciculata* colonies were studied in Punta de S'Agulla (Blanes; 41°40′54.87″N, 2°49′00.01″E) and *I. variabilis* and *I. oros* in Mar Menuda (Tossa de Mar; 41°43′13.62″N, 2°56′26.90″E) from March 2010 to June 2011. Initial sampling of *I. oros* (March 2010) was performed in the nearby Punta Santa Anna (Blanes; 41°40′21.48″N, 2°48′13.55″E); however, from June 2010 to June 2011, sampling was conducted in Tossa de Mar, due to the onset of heavy construction in the adjacent Blanes Port (<300 m from the Punta Santa Anna sampling site) in May 2010.

Individual sponges were marked *in situ* and sampled quarterly for genetic analyses and chlorophyll *a* concentrations by scuba diving, using a scalpel blade and forceps. At each site, ambient seawater samples (500 ml) were collected simultaneously and in close proximity (<1 m) to sampled sponges. Sponge and seawater samples were transported in an insulated cooler to the laboratory (ca. 2 h of transit time), where sponge samples for genetic analyses were preserved in 100% ethanol and stored at −20°C and seawater samples were concentrated on 0.2-μm filters and stored at −80°C. Tissue samples for chlorophyll *a* quantification were processed immediately (see below).

Temperature and light measurements. Hourly temperature and light intensity levels were recorded *in situ* at Punta de S'Agulla and Tossa de Mar by Hobo Pendant Temperature/Light Data Loggers (UA-002-64; Onset Computer Corporation) deployed in close proximity (<2 m) to sampled sponges. Consistent with the distribution of the studied sponge taxa (15), data loggers were deployed at Punta de S'Agulla on the horizontal (exposed) substrate, the typical habitat of *I. fasciculata*, and at Tossa de Mar on the vertical wall (cryptic) substrate, the typical habitat of *I. variabilis* and *I. oros*. Submarine *in situ* light measurements are complicated by light sensor orientation and the occurrence of sensor encasement fouling. To minimize orientation error, data loggers were attached parallel to the substrate in stable epoxy molds for consistent orientation of light sensors. To minimize fouling error, data loggers were replaced monthly and only the first 7 days of light measurements (70 to 105 data points per month) were used in subsequent analyses. Light measurements were recorded as lux (lumen m^{-2}), the SI-derived unit for luminous flux density, across a broad spectrum of wavelengths (200 to 1,200 nm) and used to compare relative changes in light intensity across sites and seasons. Light duration was calculated as the number of hourly light readings per day greater than 0. Missing data from Tossa de Mar (March 2010 to May 2010) resulted from the loss of data loggers. For comparative analyses, seasons were defined as winter (January, February, and March), spring (April, May, and June), summer (July, August, and September), and fall (October, November, and December).

DNA extraction. DNA extracts were prepared from sponge samples containing both ectosome and choanosome for six individuals per host species and time point ($n = 108$) and three replicates of filtered seawater per time point ($n = 18$), using the DNeasy Blood & Tissue kit (Qiagen). Dilutions (1:10) of DNA extracts were used as the templates in subsequent PCR amplifications.

T-RFLP analysis. PCR amplification of 16S rRNA gene sequences (ca. 1,500 bp) for T-RFLP analysis was conducted using the universal bacterial forward primer 8F (44) and reverse primer 1509R (38), with a 5′-end 6-carboxyfluorescein (6-FAM) label attached to the forward primer. The total PCR volume was 50 μl, and each reaction mixture contained 15 pmol of the labeled forward primer, 10 pmol of the reverse primer, 10 nmol of each deoxynucleoside triphosphate (dNTP), 1× reaction buffer (Ecogen), and five units of Biotaq polymerase (Ecogen). Thermocycler reaction conditions were an initial denaturing time of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 0.5 min at 50°C, and 1.5 min at 72°C, and a final extension time of 2 min at 72°C. To minimize PCR amplification biases, a low annealing temperature and low cycle number were used and three separate reactions were conducted for each sample. Triplicate PCR products were gel purified and cleaned using the QIAquick gel extraction kit (Qiagen) and then combined and quantified using a Qubit fluorometer and the Quant-iT dsDNA assay kit (Invitrogen).

Purified PCR products (ca. 100 ng) were digested separately with the restriction endonucleases HaeIII and MspI (Promega) at 37°C for 8 h and ethanol precipitated to remove residual salts from enzyme buffers. Samples were eluted in 10 μl formamide and 0.5 μl GeneScan 600-LIZ size standard, heated for 2 min at 94°C, cooled on ice, and analyzed by capillary electrophoresis on an automated sequencer (ABI 3730 Genetic Analyzer; Applied Biosystems) at the Scientific and Technical Services of the University of Barcelona (Spain). The lengths of individual terminal restriction fragments (T-RF) were determined by comparison with internal size standards using the program GeneScan (PE; Applied Biosystems). T-RFs beyond the resolution of internal size standards (50 to 600 bp) or with peak areas of less than 50 fluorescence units were removed, and peak profiles were imported into the program T-REX (10). Prior to T-RF alignment in T-REX, the objective filtering algorithm of Abdo et al. (1) based on peak area and a cutoff value of 2 standard deviations (SD) was applied to denoise the data set by eliminating background peaks. Following noise reduction, T-RFs were aligned across samples using a 1-bp clustering threshold, and peak profiles were standardized using relative abundance (percentage total fluorescence).

To compare the similarity of bacterial community profiles, Bray-Curtis similarity matrices were constructed using square root transformations of relative T-RF abundance data and visualized in nonmetric multidimensional scaling (nMDS) plots and heat maps. Permutational multivariate analyses of variance (PERMANOVA) were used to determine significant differences in bacterial community structure across sources (sponge species and seawater) and across seasons within sources (nested analysis). Permutational multivariate analyses of dispersion (PERMDISP) were conducted for all significant PERMANOVA outcomes to test for differences in homogeneity (dispersion) among groups. A significant PERMDISP outcome indicates that differences in community structure detected by PERMANOVA may result from unequal structural variability among groups (i.e., heterogeneity of dispersion) rather than consistent structural

shifts. Multiple pairwise comparisons of symbiont structure and dispersion were corrected based on the Benjamini-Yekutieli (B-Y) false discovery rate control (7) and an experiment-wise error rate of 0.05. nMDS, PERMANOVA, and PERMDISP calculations were performed using Primer v6 and Permanova+ (Plymouth Marine Laboratory, United Kingdom). Heat maps were constructed using JColorGrid v1.869 (28).

Clone library construction and sequence analysis. In a previous study, we provided an initial characterization of bacterial communities in *I. fasciculata*, *I. variabilis*, and *I. oros* collected in the winter season (March 2010) by 16S rRNA gene sequence clone libraries (15). In the current study, we resampled the same host individuals in the summer season (September 2010) and constructed clone libraries following the same methodology to (i) monitor changes in symbiont communities across seasons and (ii) identify T-RFLP profile peaks not represented in the winter clone library. In total, 320 clones from the summer clone libraries were bidirectionally sequenced using vector primers at Macrogen, Inc., to recover near-full-length 16S rRNA gene sequences (range, 1,399 to 1,525 bp; average length, 1,478 bp). Raw sequence reads were processed in Geneious (13) by aligning high-quality forward and reverse reads to yield a final consensus sequence for each clone. Consensus sequences were screened for sequencing anomalies (e.g., chimeras) using Mallard (6) and a reference 16S rRNA gene sequence from *Escherichia coli* (GenBank accession no. U00096) and confirmed or refuted using Pintail (5) and two related reference sequences.

To determine seasonal overlap and divergence in symbiont communities, sequences were ascribed to operational taxonomic units (OTUs) (99% sequence identity, nearest-neighbor algorithm), as implemented in the mothur software package (49), and compared to 99% OTUs from the winter clone library (see Table S1 in the supplemental material). Representative sequences from each 99% OTU were analyzed using the Ribosomal Database Project II (9) sequence classifier to assess taxonomic affiliations. In addition, OTU-independent statistical tests were conducted to determine seasonal differences in the genetic diversity (homogeneity of molecular variance [HOMOVA]), genetic differentiation (analysis of molecular variance [AMOVA]) (54) and phylogenetic structure (unweighted UNIFRAC [36]) of bacterial communities within each source. HOMOVA, AMOVA, and UNIFRAC analyses were performed as implemented in the mothur software package (49).

To match clone library sequences with T-RFLP profile peaks, a reference database (IRC) was created by *in silico* digestions of 16S rRNA gene sequences and consisted of 5′-terminal restriction fragment lengths (reference T-RFs) for each 99% OTU ($n = 190$) and restriction endonuclease (HaeIII or MspI) combination. Following correction of T-RF drift (see below), the IRC reference database was used to match empirical T-RFs from T-RFLP profiles with known 16S rRNA gene sequences from clone libraries, using the phylogenetic assignment tool (PAT) (30) with 1.5-bp bins. Discrepancies between the predicted length of reference T-RFs and actual length of empirical T-RFs can occur due to the phenomenon of T-RF drift (29), where small differences in the molecular weight of fluorescent labels attached to samples (e.g., FAM) and size standards (e.g., LIZ) result in differential capillary migration rates and underestimation of DNA fragment sizes (41). To correct for T-RF drift associated with the fluorescent labels used here, the empirical lengths of T-RFs were determined for eight dominant bacterial OTUs (IRC001, IRC002, IRC003, IRC004, IRC006, IRC007, IRC0012, and IRC0015) using monocultures of each clone as the templates for PCR amplification and T-RFLP analyses, as described above. Regression analysis of the empirical versus predicted lengths of T-RFs from these clones produced a standard curve ($R^2 > 0.99$; see Fig. S1 in the supplemental material) used to correct for the discrepancies of T-RF drift and more accurately match DNA sequences with T-RFLP profile peaks.

Chlorophyll *a* concentrations. Tissue samples for chl *a* quantification were collected from ectosomal regions of *I. fasciculata* ($n = 48$) and *I. variabilis* ($n = 47$) and processed following previously described methods (17). Due to the absence of photosymbionts in *I. oros* (15), this species was not included in chlorophyll analysis. For *I. fasciculata*, the same eight marked individuals were repeatedly sampled, due to the large size and rapid healing processes of this species. For *I. variabilis*, three to 11 nonmarked individuals were randomly sampled each month from the same population, as the smaller size and slower healing rate of this species prevented repeated sampling of the same colonies. Accordingly, a one-way repeated-measures analysis of variance (ANOVA) for *I. fasciculata* and a one-way ANOVA for *I. variabilis* were conducted to compare chl *a* concentrations within each species across sampling months. Multiple pairwise comparisons of chl *a* concentrations between species within each month were conducted using Student's *t* tests with Bonferroni corrections. Statistical analyses were performed using the software Sigmaplot v11.

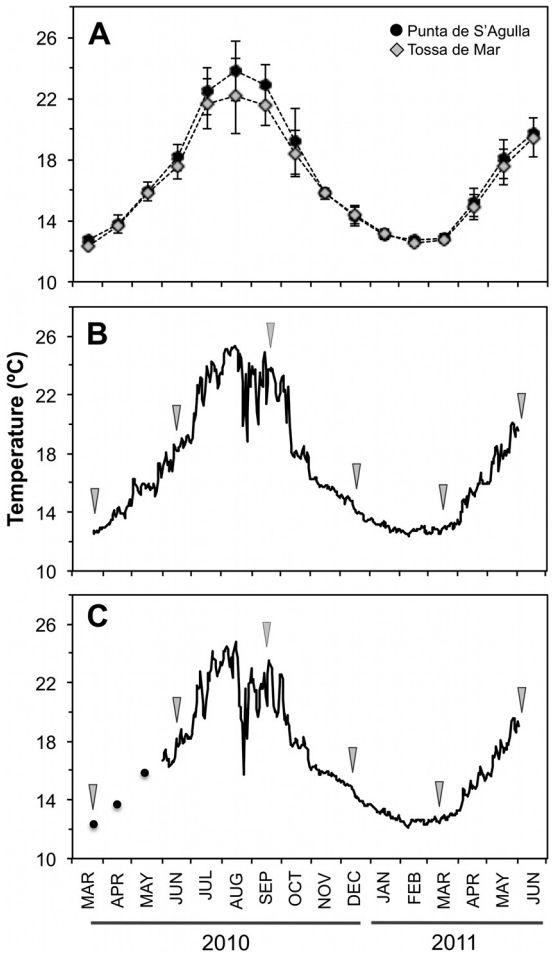

FIG 1 Seasonal variation in seawater temperature from March 2010 to June 2011 at two monitoring sites in the NW Mediterranean Sea. Monthly averages (±SD) for Punta de S'Agulla (black circles) and Tossa de Mar (gray diamonds) (A) and daily averages for Punta de S'Agulla (B) and Tossa de Mar (C). Gray triangles highlight sampling times, and black dots indicate discrete measurements prior to successful data logger deployment at Tossa de Mar.

Nucleotide sequence accession numbers. The sequences determined in this study have been quality checked and are archived in GenBank under accession numbers JX206477 to JX206796.

RESULTS

Seasonal variation in temperature and light intensity. Both monitoring sites exhibited clear seasonal trends in temperature (Fig. 1). Annual temperature minima occurred during the winter season, with the lowest average monthly values recorded in March

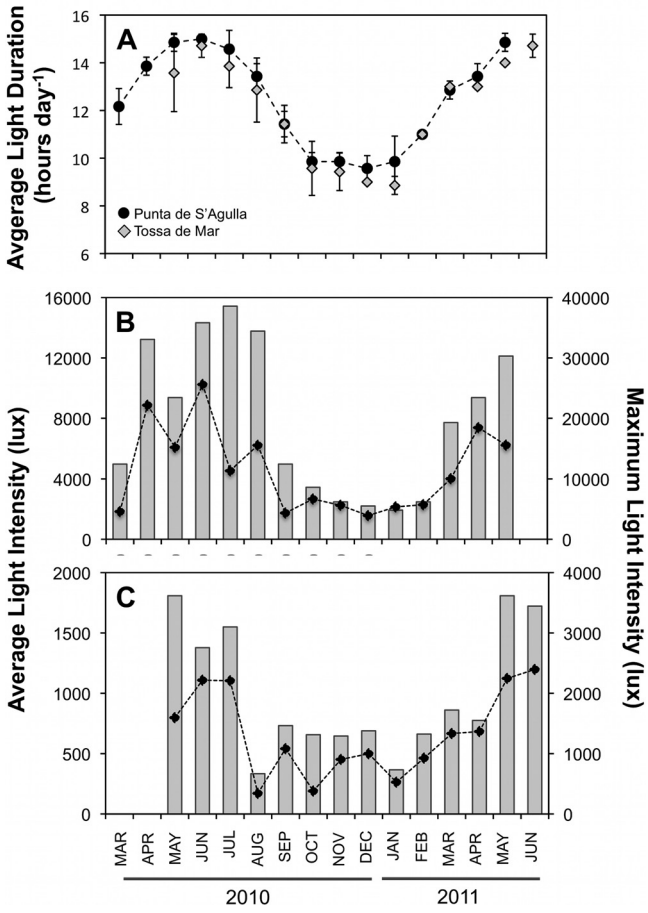

FIG 2 Seasonal variation in light duration (day length) and intensity from March 2010 to June 2011 at two monitoring sites in the NW Mediterranean Sea. Monthly averages (±SD) for day length at Punta de S'Agulla (black circles) and Tossa de Mar (gray diamonds) (A). Monthly averages (black diamonds) and maximum light intensity (gray bars) at Punta de S'Agulla (B) and Tossa de Mar (C).

2010 (12.7°C in S'Agulla and 12.3°C in Tossa) and lowest average daily values in February 2011 (12.4°C in S'Agulla and 12.1°C in Tossa). Annual temperature maxima occurred during the summer season, with the highest average monthly and daily values recorded in August 2010 (23.8°C and 25.3°C in S'Agulla; 22.2°C and 24.8°C in Tossa). Annual temperature fluctuations were accordingly high at both sites (>12.7°C). Small differences in seawater temperatures between the monitoring sites likely resulted from slightly deeper data logger deployment in Tossa (7 m) than in S'Agulla (5 m). The summer season was also characterized by large fluctuations in daily temperatures, averaging 2.2°C (±1.3 SD) in S'Agulla and 1.8°C (±1.2 SD) in Tossa, with >3°C daily fluctuations recorded on 15 and 12 days in S'Agulla and Tossa, respectively. In contrast, the winter season exhibited minor fluctuations in daily temperatures, averaging 0.4°C (±0.2 SD) in S'Agulla and 0.3°C (±0.2 SD) in Tossa and never exceeding 0.8°C at either site. A notable upwelling event occurred in August 2010, causing drastic temperature decreases at both sites and resulting in weekly temperature fluctuations of 7.7°C and 9.4°C and daily fluctuations of 6.9°C and 5.4°C in S'Agulla and Tossa, respectively.

Both monitoring sites also exhibited clear trends in irradiance conditions across seasons (Fig. 2). Light duration (i.e., day length) was longer in spring and summer than in the fall and winter seasons, which experienced up to 6 h less of light exposure per day. Maximum and average light intensity values were higher during the spring and summer seasons than in the fall and winter. Light intensity levels in S'Agulla averaged 1,569 to 10,240 lx per month, with maximum values reaching over 38,000 lx. Lower levels were observed in Tossa, averaging 264 to 1,198 lx per month, and maximum values never exceeded 3,700 lx. The large differences in irradiance between sites were consistent with the deployment of data loggers in photophilic (S'Agulla) and semisciophilous (Tossa) communities and correspond to the distinct habitats of the host sponge species investigated.

Host specificity of bacterial communities. A total of 213 unique microbial symbiont T-RFs were identified using the restriction enzyme HaeIII (151 in *I. fasciculata*, 149 in *I. variabilis*, 147 in *I. oros*, and 144 in seawater) and 237 unique T-RFs with MspI (185 in *I. fasciculata*, 164 in *I. variabilis*, 156 in *I. oros*, and 159 in seawater). Binary data analysis of individual T-RFs (presence/absence) revealed highly congruent specificity patterns between the two restriction enzymes used to construct T-RFLP profiles (see Fig. S2 in the supplemental material). One-third of the unique T-RFs (32.4% for HaeIII and 33.0% for MspI) were sponge specific, present in one or more host species and absent from seawater, while <1/10 (8.9%, HaeIII; 5.9%, MspI) were recovered exclusively from seawater (see Fig. S2 in the supplemental material). The majority of T-RFs were shared among sponges and seawater, present in at least 1 sponge host and seawater (23.0%, HaeIII; 26.6%, MspI) or among all 3 host sponges and seawater (35.7%, HaeIII; 34.6%, MspI) (see Fig. S2). Among the sponge-specific T-RFs, the highest number of unique (host species-specific) T-RFs was detected in *I. fasciculata* ($n = 14$, HaeIII; $n = 11$, MspI), and *I. fasciculata* and *I. variabilis* shared more T-RFs than any other pair ($n = 12$, HaeIII; $n = 14$, MspI). Similarly, community-level analysis based on the relative abundance of microbial T-RFs revealed clear differentiation of sponge and seawater communities and more similar symbiont communities in *I. fasciculata* and *I. variabilis* than in *I. oros* (Fig. 3).

Statistical analyses of community structure (PERMANOVA) revealed significant differences between sponge and seawater microbial fingerprints and among all pairwise comparisons of host sponge species (Table 1). Nonmetric multidimensional scaling (nMDS) plots exhibited clear spatial segregation of sponge and seawater-derived microbial communities, while among host sponges, symbiont communities consistently clustered by host species across all seasons, with no overlap between *I. fasciculata* and *I. oros* and higher variability in the symbiont profiles of *I. variabilis* (Fig. 4A and C). Dispersion analysis revealed higher variability within seawater communities than among sponge-associated bacteria, as pairwise comparisons between sponges and seawater were significant for at least one enzyme while no significant differences in dispersion were found in pairwise comparisons among sponge species (Table 1).

Seasonal variation in bacterial communities. Symbiont communities within each host sponge species exhibited high stability throughout the monitoring period, averaging 69.9% (*I. fasciculata*), 64.0% (*I. variabilis*), and 63.2% (*I. oros*) community similarity in T-RFLP profiles. nMDS plots revealed two tight spatial clusters for *I. oros* and *I. fasciculata* plus *I. variabilis* samples, par-

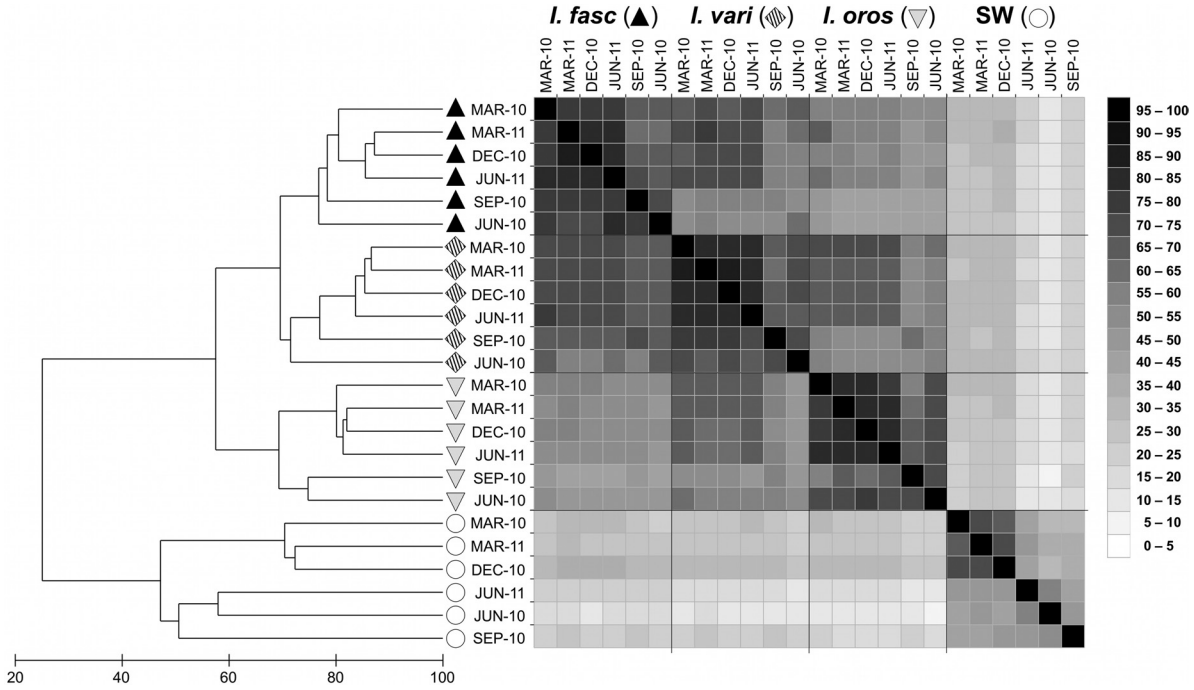

FIG 3 Average similarity of bacterial communities in *I. fasciculata* (black triangles), *I. variabilis* (barred diamonds), *I. oros* (gray triangles), and ambient seawater (white circles) over the 1.5-year monitoring period. Dendrogram (left) based on Bray-Curtis (BC) similarity values from T-RFLP profiles with HaeIII. Heat map (right) shows all pairwise BC similarity values for both HaeIII (upper diagonal) and MspI (lower diagonal) data sets.

ticularly when considering HaeIII profiles (Fig. 4B and D). Each cluster consisted of all samples from the 2010-2011 fall and winter and from spring of 2011, as well as some individuals from spring and summer of 2010. However, most samples from spring and summer of 2010 were displaced from these central clusters, indicating some change in bacterial profiles during these seasons. In contrast, seawater bacterial communities exhibited clear and consistent seasonal shifts in composition, resulting in spatially segregated clusters in nMDS plots that corresponded to distinct bacterioplankton communities in the fall/winter, spring, and summer seasons (Fig. 4).

Statistical analyses of community structure (PERMANOVA) and dispersion (PERMDISP) revealed significant differences in structure and homogeneity of dispersion among all pairwise comparisons of seawater bacteria (see Table S2 in the supplemental material), confirming the seasonal shifts in seawater bacteria visualized in nMDS plots. Among host sponges, significant differences in community structure were observed in the transition from winter to spring and summer to fall of 2010 for at least one enzyme (see Table S2), due to high variability in bacterial community profiles among individuals of each host sponge in spring and summer of 2010. Indeed, PERMDISP analyses revealed significant differences in dispersion during these transitional periods, indicating that heterogeneity was the main driver of structural differences in symbiont communities. Within the fall/winter and spring/summer seasons, structural differences in sponge-associated bacteria were generally not significant (see Table S2).

Clone library analysis of 16S rRNA gene sequences confirmed the stability of sponge-associated microbial communities over time and the seasonal variability of seawater communities. Comparisons of clone libraries constructed from the same individuals sampled in winter (March) and summer (September) 2010 seasons revealed that a large portion of sponge symbiont communities (57 to 80% of clones) were stable across seasons, with no significant differences in the genetic differentiation and community structure (Table 2; see Fig. S3 in the supplemental material). Bacterial communities in *I. variabilis* and *I. oros* also exhibited no significant differences in genetic diversity between sampling times, while *I. fasciculata* symbionts showed significantly lower diversity in September 2010, due to in-

TABLE 1 Permutational statistical analyses of T-RFLP data[a]

Analysis	Pairwise comparison	HaeIII		MspI	
		t	*P* (*perm*)	*t*	*P* (*perm*)
PERMANOVA	*I. fasciculata*/*I. variabilis*	3.683	0.001*	3.682	0.001*
	I. variabilis/*I. oros*	5.164	0.001*	4.508	0.001*
	I. oros/*I. fasciculata*	6.988	0.001*	6.637	0.001*
	I. fasciculata/seawater	10.408	0.001*	9.500	0.001*
	I. variabilis/seawater	9.258	0.001*	8.082	0.001*
	I. oros/seawater	9.136	0.001*	10.028	0.001*
PERMDISP	*I. fasciculata*/*I. variabilis*	1.615	0.177	0.848	0.475
	I. variabilis/*I. oros*	0.516	0.639	1.350	0.235
	I. oros/*I. fasciculata*	2.152	0.071	0.456	0.683
	I. fasciculata/seawater	4.016	0.001*	3.575	0.002*
	I. variabilis/seawater	2.451	0.046	2.933	0.015*
	I. oros/seawater	1.997	0.093	4.424	0.002*

[a] Analyses included bacterial community structure (PERMANOVA) and dispersion (PERMDISP) among sponges and seawater. *, comparison was found to be significant following B-Y correction (7). *P* (*perm*), permutation *P* value.

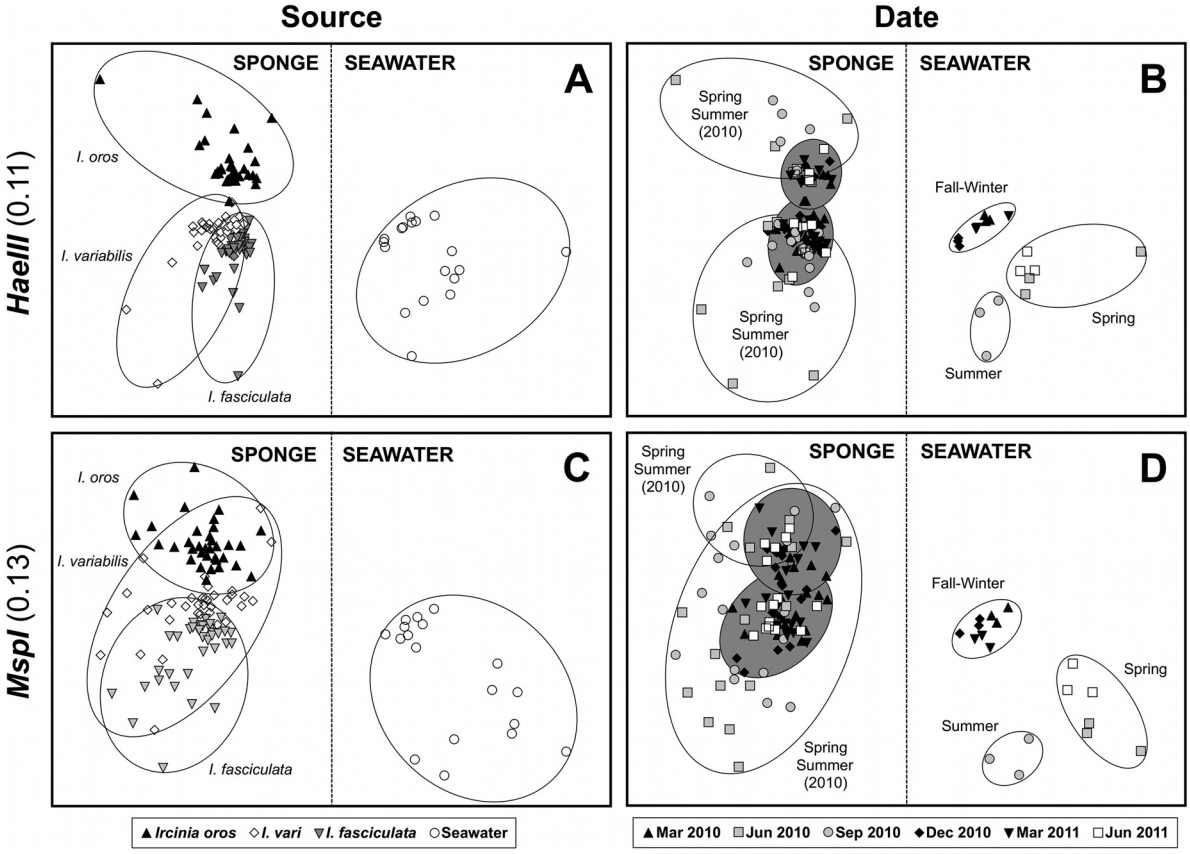

FIG 4 Nonmetric multidimensional scaling (nMDS) plots of bacterial community structure from replicate individuals of *I. fasciculata*, *I. variabilis*, and *I. oros* and ambient seawater over the 1.5-year monitoring period. nMDS ordination based on Bray-Curtis similarity of T-RFLP profiles for HaeIII (A, B) and MspI (C, D) data sets. Stress values for two-dimensional ordination are shown in parentheses for each enzyme. Data points are coded by source (A, C), with circles encompassing all samples from each source, and by season (B, D), with shaded circles denoting core bacterial symbiont profiles and nonshaded circles highlighting deviations from core profiles in spring/summer 2010 (B, D).

creased representation of the dominant cyanobacterium, "*Candidatus* Synechococcus spongiarum," in the summer library compared to the winter one (51% and 26% of clones, respectively). Seawater clone libraries from winter and summer shared few sequences (16 to 22% of clones) and exhibited significant differences in community structure, genetic diversity, and genetic differentiation (Table 2; see Fig. S3).

Seasonal variation in bacterial OTUs. Combined analysis of the winter and summer clone libraries revealed 190 bacterial OTUs (99% sequence identity) in sponges and seawater, corresponding to 13 microbial phyla. Within each host sponge species, similar phylogenetic compositions of bacteria were observed between seasons (Table 3), with differences between seasons typically resulting from shifts in rare bacterial OTUs. For example, *I. fasciculata* hosted a single rare OTU (2.6% of clones) affiliated with *Nitrospira* in winter that was absent from summer clone libraries. In contrast, seawater bacteria exhibited large fluctuations in specific lineages and OTUs. For example, cyanobacterial OTUs accounted for only 1.4% of seawater clones in winter and over one-fourth of clones (27.6%) in summer (Table 3). Similarly, rank-abundance plots of bacterial OTUs revealed that dominant sponge symbionts were stable across seasons and rare OTUs were more variable, whereas shifts in dominant and rare seawater bacteria were observed between the winter and summer seasons (see Fig. S3 in the supplemental material).

Clone libraries also revealed the presence of dominant symbiont OTUs in the three sponge species. Overall, eight symbiont OTUs comprised over one-half of all *Ircinia*-associated bacterial clones (51.7%) and were absent from ambient seawater (Table 4). Seven of the eight dominant OTUs were recovered from both winter and summer seasons and matched closely (>98% sequence identity) other sponge-associated bacteria. The exception was a member of *Gammaproteobacteria* (IRC012) present only in the winter season and whose closest sequence match was a sediment-derived bacterium (Table 4). The most dominant *Ircinia*-associated OTU (IRC002) matched the sponge-specific cyanobacterium

TABLE 2 Statistical comparisons of genetic diversity and community structure of bacterial communities in sponges (*Ircinia* spp.) and seawater between winter and summer seasons[a]

	Statistical result for:					
	AMOVA		HOMOVA		UNIFRAC	
Community	*Fs*	*P*	*B*	*P*	*U*	*P*
I. fasciculata	4.434	0.065	1.834	<0.001	0.670	0.147
I. variabilis	2.241	0.634	0.043	0.248	0.593	0.073
I. oros	3.365	0.397	0.024	0.502	0.584	0.054
Seawater	4.408	<0.001	0.620	0.007	0.734	0.006

[a] *Fs*, F statistic; *B*, Bartlett's statistic; *U*, unweighted UniFrac value.

TABLE 3 Composition of bacterial communities in *Ircinia* spp. and ambient seawater sampled in winter (March) and summer (September) seasons

Bacterial phylum	% of total clones (no. of 99% OTUs)							
	I. fasciculata		*I. variabilis*		*I. oros*		Seawater	
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
Proteobacteria	45.5 (16)	30.0 (11)	61.3 (22)	50.7 (18)	56.1 (17)	47.7 (19)	56.2 (28)	43.7 (21)
Alphaproteobacteria	6.5 (4)	2.9 (2)	11.3 (6)		12.2 (4)	6.8 (5)	30.1 (15)	32.2 (11)
Betaproteobacteria			1.3 (1)				2.7 (2)	
Gammaproteobacteria	20.8 (10)	12.9 (6)	33.8 (11)	32.0 (13)	28.0 (10)	25.0 (10)	23.3 (11)	11.5 (10)
Deltaproteobacteria	18.2 (2)	14.3 (3)	15.0 (4)	18.7 (5)	15.9 (3)	15.9 (4)		
Cyanobacteria	32.5 (2)	61.4 (2)	7.5 (1)	21.3 (1)		1.1 (1)	1.4 (1)	27.6 (4)
Acidobacteria	5.2 (3)	1.4 (1)	5.0 (3)	10.7 (1)	18.3 (3)	25.0 (5)		1.1 (1)
Bacteroidetes	9.1 (3)	2.9 (1)	3.8 (2)	5.3 (2)	3.7 (2)		9.6 (7)	8.0 (4)
Chloroflexi	3.9 (3)	1.4 (1)		4.0 (2)	4.9 (2)	14.8 (7)		
Actinobacteria			3.8 (2)	1.3 (1)	3.7 (2)	1.1 (1)	12.3 (3)	5.7 (3)
Nitrospira	2.6 (1)		15.0 (1)	4.0 (1)	2.4 (1)	2.3 (2)		
Bacillariophyta			2.5 (2)		9.8 (5)		8.2 (4)	1.1 (1)
Verrucomicrobia							1.4 (1)	12.6 (2)
Firmicutes		1.4 (1)				5.7 (2)	1.4 (1)	
Gemmatimonadetes	1.3 (1)	1.4 (1)	1.3 (1)	1.3 (1)	1.2 (1)	2.3 (2)		
Chlorophyta							4.1 (3)	
Planctomycetes				1.3 (1)			1.4 (1)	
Uncertain							4.1 (2)	

"*Candidatus* Synechococcus spongiarum" (64) and represented the most common symbiont in *I. fasciculata* and *I. variabilis*. The second most dominant OTU (IRC001) matched a member of *Deltaproteobacteria* in the order *Desulfovibrionales* and represented the second most common symbiont in all *Ircinia* hosts. An *Acidobacterium* (IRC003) was the third most dominant OTU and represented the most common symbiont in *I. oros*, while also present in *I. variabilis* yet absent in *I. fasciculata*. The remaining four dominant, sponge-specific OTUs were less abundant (<5% of clones) and corresponded to symbiont taxa affiliated with *Gammaproteobacteria*, *Nitrospira*, and *Cyanobacteria* (Table 4).

Comparison of clone library and T-RFLP data revealed high congruency between these techniques and allowed for the identification of most symbiont taxa in the T-RFLP profiles. *In silico* restriction enzyme digestion of clone libraries predicted 71.6% and 95.8% of all peaks in T-RFLP profiles (HaeIII and MspI data, respectively). Empirical T-RFs of the eight dominant OTUs were well represented in sponge symbiont profiles and accounted for

TABLE 4 Characteristics of dominant symbiont OTUs in *Ircinia* spp.

OTU	No. (%) of total clones[a]					Source of closest BLAST match (% sequence identity, accession no.)	Taxonomic classification (Bayesian probability)		Putative function
	IF	*IV*	*IO*	All *Ircinia* spp.	SW		Taxon[b]	Lowest taxonomic rank	
IRC001	18 (12.2)	16 (10.3)	22 (13.0)	56 (11.9)	0	Sponge associated (99.2, EU495967)	*Deltaproteobacteria* (79)	Order *Desulfovibrionales* (70)	Sulfate reduction
IRC002	56 (38.1)	22 (14.2)	0 (0.0)	78 (16.5)	0	Sponge associated (98.8, GU981862)	*Cyanobacteria* (100)	Genus *Synechococcus* (100)	Carbon fixation
IRC003	0 (0.0)	10 (6.5)	26 (15.3)	36 (7.6)	0	Sponge associated (98.7, AJ347029)	*Acidobacteria* (100)	Gp10 (100)	NA[c]
IRC004	2 (1.4)	15 (9.7)	0 (0.0)	17 (3.6)	0	Sponge associated (99.3, EU183762)	*Nitrospira* (100)	Genus *Nitrospira* (100)	Nitrite oxidation
IRC006	2 (1.4)	11 (7.1)	1 (0.6)	14 (3.0)	0	Sponge associated (98.8, EU495951)	*Gammaproteobacteria* (100)	*Incertae sedis* (68)	NA
IRC007	0(0.0)	6(3.9)	10(5.9)	16(3.4)	0	Sponge associated (98.7, GQ163729)	*Gammaproteobacteria* (100)	Order *Oceanospirillales* (46)	NA
IRC012	4 (2.7)	6 (3.9)	5 (2.9)	15 (3.2)	0	Sediment bacterium (97.4, GQ143791)	*Proteobacteria* (100)	*Incertae sedis* (84)	NA
IRC015	12 (8.2)	0 (0.0)	0 (0.0)	12 (2.5)	0	Sponge associated (99.3, JN655231)	*Cyanobacteria* (100)	GpIIa (100)	Carbon fixation

[a] *IF*, *I. fasciculata*; *IV*, *I. variabilis*; *IO*, *I. oros*; SW, seawater.
[b] All taxa are phyla except *Deltaproteobacteria* and *Gammaproteobacteria*, which are classes.
[c] NA, not available.

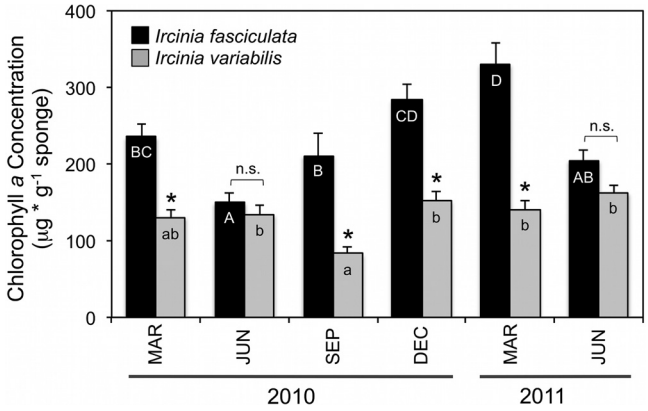

FIG 5 Chlorophyll *a* content of the photosymbiont-bearing sponges *I. fasciculata* (black bars) and *I. variabilis* (gray bars) over the 1.5-year monitoring period. Asterisks denote significant differences ($P < 0.05$) between host sponge species by month; letters indicate significant differences among months within each host species (uppercase letters for *I. fasciculata* and lowercase letters for *I. variabilis*). Error bars represent ±1 SD.

53.0% (±2.2% standard error [SE], HaeIII data) and 34.2% (±1.2%, MspI data) of total profile peak areas, while the same T-RF peaks comprised only a small portion of seawater bacteria profiles (7.5% ± 0.8% and 6.9% ± 1.1%, HaeIII and MspI data, respectively). Further, these eight dominant symbionts were present in their respective hosts throughout the seasonal cycle (see Table S3 in the supplemental material), confirming the stability of these symbionts over annual temporal scales and seasonal environmental conditions.

Seasonal variation in chlorophyll *a* content. The photosymbiont-harboring sponges *I. fasciculata* and *I. variabilis* exhibited different average concentrations and temporal variability in chlorophyll *a* content. Chlorophyll *a* levels were higher in *I. fasciculata* than in *I. variabilis*, consistent with the habitat preferences of *I. fasciculata* (higher irradiance zones) and *I. variabilis* (lower irradiance zones). Differences between species were significant for all months except June (2010 and 2011 [Fig. 5]), which is, notably, the month with the highest average irradiance levels (Fig. 2). For both host sponges, significant variation ($P < 0.001$) in chl *a* content was observed across the monitoring period. In *I. variabilis*, this variation was due to a significant decrease in average chl *a* content in September 2010 (83.3 μg/g), whereas the remaining months exhibited similar average values (131.0 to 162.4 μg/g). Seasonal changes in chl *a* content were more pronounced in *I. fasciculata* and inversely related to daylight hours and light intensity (Fig. 5), as lower values occurred during the spring and summer months (149.8 to 210.7 μg/g) and higher values during fall and winter (235.1 to 330.2 μg/g).

DISCUSSION

Seasonal stability and specificity of sponge microbiota. Temporal monitoring of three *Ircinia* spp. and ambient seawater over 1.5 years revealed remarkable stability and specificity of sponge-associated bacterial symbiont communities, despite large fluctuations in ambient environmental conditions. Across all seasons, each *Ircinia* host maintained a specific bacterial symbiont community, more similar within each host species over time than among hosts. Further, higher symbiont similarity occurred between the microbiotas of *I. fasciculata* and *I. variabilis* than with that of *I. oros*, consistent with previous analyses of host specificity among these species (15). Host specificity patterns in *Ircinia*-associated bacteria are complex, due to variable levels of symbiont overlap among hosts. Despite the prevalence of generalist symbionts in *Ircinia* microbiotas (i.e., taxa occurring in multiple, unrelated sponge hosts), community level analyses revealed host species-specific symbiont assemblages in each host (15). Here, we show that this phenomenon, termed "a specific mix of generalists," is maintained over time and across seasons, with little evidence for symbiont restructuring or specificity shifts in response to different environmental conditions. The seasonal stability of host specificity patterns in the *Ircinia* microbiota supports the hypothesis of host species-specific, stable associations between bacteria and marine sponges (32, 56, 59, 71, 73).

In contrast, seawater bacterial communities exhibited clear temporal shifts in diversity and composition according to a seasonal cycle. Previous studies of surface bacterioplankton in the coastal NW Mediterranean Sea have revealed a similar seasonal succession of seawater bacterial communities (47, 48), including a greater community similarity in the fall and winter seasons as observed here (2). Regional stratification of the water column is a seasonal phenomenon in the NW Mediterranean Sea, where restricted upwelling and vertical mixing of nutrient-rich, cold water results in nutrient depletion of surface waters during the summer months (14). The summer stratification period and its effects on nutrient availability are primary drivers of seasonal microbial dynamics in the Mediterranean Sea (43). Comparatively low seasonal dynamics of sponge-associated bacterial community structure suggest that different ecological constraints act on free-living versus symbiotic marine bacteria. The effects of nutrient-poor conditions during summer stratification on bacterial communities in the sponge microbiota appear to be limited, supporting the hypothesis of a unique and comparatively stable microbial habitat within the sponge body.

Persistent components of the sponge microbiota. The observed stability of bacterial communities associated with *Ircinia* hosts was driven by the persistent presence of dominant symbiont OTUs. Despite the high diversity of the *Ircinia* microbiota, a small number of symbiont OTUs accounted for the majority of bacteria represented in clone libraries and T-RFLP profiles, similar to what was seen in previous studies of sponge-associated bacteria (18, 71). Selective pressures that maintain specific symbiont taxa in the sponge host may result from microbial adaptations to these unique niche microenvironments, as suggested by the presence of unique, vertically transmitted (51) sponge-specific bacterial lineages (52, 56), and/or the fulfillment of functional roles by particular symbiont guilds that enhance sponge-bacteria holobiont fitness (20, 22, 58). In the latter context, it is noteworthy that several of the dominant symbiont OTUs recovered in *Ircinia* hosts were classified into bacterial lineages with known physiological capabilities, such as photosynthesis (IRC002 and IRC015, *Cyanobacteria*), sulfate reduction (IRC001, *Desulfovibrionales*), and nitrite oxidation (IRC004, *Nitrospira*). The metabolic profile of the sponge microbiome, assessed by both metagenomic (34, 62) and nutrient flux (45) approaches, has shown diverse and active functional guilds involved in the nutrient cycles of carbon (59), nitrogen (26), and sulfur (25) that may boost host sponge metabolism and contribute significantly to coastal marine nutrient cycles (4, 12, 19, 27). As such, symbiont functionality and its ecological consequences may represent key factors for the selective mechanisms

that establish and maintain specific guilds of sponge-associated bacterial symbionts.

Temporal analyses of photosynthetic pigments in *I. fasciculata* and *I. variabilis* provided further insight into symbiont functionality and evidence for seasonal variation in the activity of persistent photosymbiont taxa. *Cyanobacteria* are a key functional guild in the sponge microbiota, capable of photosynthetic carbon assimilation and the transfer of surplus carbon stores to their hosts (59). A recent study reported higher photosynthetic activity of cyanobacterial symbionts in *I. fasciculata* than in *I. variabilis*, with differences in symbiont functionality related to ambient irradiance levels in preferred host habitats rather than symbiont composition (17). Here, we show that *I. variabilis* exhibited minimal seasonal fluctuations in chl *a* content, consistent with reduced irradiance levels in the shaded habitats where this species thrives. In contrast, the chl *a* content of photosymbionts in *I. fasciculata* followed a seasonal pattern, with annual minima in summer and peak values in winter, similar to those reported in surface seawater from the NW Mediterranean (14, 43, 47). Thus, while the factors that determine microbial structure may differ between the sponge niche and open seawater environments (e.g., nutrient levels), some seasonal physiological constraints that dictate microbial function (e.g., irradiance exposure) may be conserved between symbiotic and free-living microbes. Structurally, a single cyanobacterial taxon dominated the symbiotic microbiota in *I. fasciculata* and *I. variabilis* across all seasons; yet functionally, their photosynthetic activity differed among hosts and appears to have a seasonal component in *I. fasciculata*, with potential consequences for host metabolism and growth. The critical ecological link between symbiont structure and function is not well resolved in the sponge microbiota and requires further study, including the potential for seasonal variability in the physiology and functioning of permanent sponge symbionts and its consequence for host metabolism and marine nutrient cycles.

Variable components of the sponge microbiota. Similar to previous studies of temporal variation in the sponge microbiota (3), some variability was observed in symbiont communities over time and among individual hosts, though primarily restricted to rare symbiont taxa. Transient components of the sponge microbiota are not unexpected, as microbes recovered from sponge tissue may represent food source bacteria (42), invasive (69) or fouling (31) microbes, or simply environmental bacteria present in the sponge filtration system during collection. For example, a common and relatively abundant bacterial OTU (IRC012, 3.2% of sponge clones) was present in the microbiotas of all sponge hosts in winter and absent in the summer. Unlike the majority of sponge-associated bacteria in *Ircinia*, this *Gammaproteobacterium* was not phylogenetically related to other sponge symbionts but rather matched most closely a sediment-derived sequence. Considering such possible sources of transient microbes in the sponge microbiota, the high degree of bacterial community similarity observed throughout the monitoring period here is even more extraordinary.

Variability in the composition of bacterial symbionts among conspecific hosts was also detected here by monitoring the same individuals over time, a sampling design rarely utilized to date in the field of sponge microbiology (3). Although this variability was minimal compared to differences among host species, some symbionts were consistently recovered from particular individuals and not others. The most notable example is a *Synechocystis*-related cyanobacterium in *Ircinia fasciculata*. A previous report has shown that this cyanobacterium represented a distinct clade of sponge symbionts specific to *I. fasciculata* yet occurred in only one of three *I. fasciculata* individuals studied (17). Here, we report similar findings, with the same *Synechocystis* phylotype recovered in only one of six host individuals, and we showed that this association was stable over time, as the cyanobacterium was recovered in winter and summer clone libraries and present in all symbiont profiles for this particular sponge host. These results show that interindividual variation in the sponge microbiota, often ascribed to the nonspecific or transient bacterial associates discussed above, can result from persistent symbionts that occur sporadically among a host population. The implications of interindividual variability in symbiont composition on host ecology and symbiont evolution are unknown for sponge-microbe associations but have the potential to affect symbiont community function (e.g., photosynthetic activity) and host-symbiont metabolic interactions.

Symbiont fluctuations and thermal thresholds. Recent reports of widespread disease and mass mortality events in *Ircinia* spp. have raised concerns about the future of these sponge populations in the warming Mediterranean Sea. Elevated seawater temperatures are hypothesized to trigger such episodic mortality events, as recurrent disease outbreaks in *I. fasciculata* and *I. variabilis* occurred annually following peak seawater temperatures in summer (37, 53) and greater disease prevalence has been correlated with the length of exposure to temperatures exceeding threshold values (8). In addition to tissue necrosis, affected sponges also exhibit characteristic changes in their associated microbiota, including the loss of stable symbionts (8) and/or their replacement by pathogenic microbes (37, 53). Similar symbiont disruption and proliferation of putatively pathogenic bacteria were reported in a tropical sponge, *Rhopaloeides odorabile*, when exposed to elevated seawater temperatures (66), suggesting that symbiont community collapse and host sponge mortality may become widespread as thermal tolerances are exceeded.

A critical question is whether symbiont disruption precedes and precipitates host mortality (e.g., symbiont evacuation followed by colonization of infectious microbes) or simply results from declining host health. In the current study, no sponge mortality events occurred during the monitoring period, consistent with previous surveys of the study area (8), yet deviations from core symbiont communities (i.e., increased heterogeneity) were reported in warmer months, due to fluctuations in rare symbiont taxa within some host individuals. At our monitoring sites, lower temperatures (daily averages of >25°C during only 3 days) were recorded than those that preceded sponge mortality events in other Mediterranean regions (daily averages of 26 to 27°C). Accordingly, no pathogenic lineages (e.g., *Vibrio* spp.) were detected in sponge hosts, and the symbiont community shifts observed in our study were minor (i.e., restricted to heterogeneity in rare symbionts, while dominant symbionts were present throughout) and temporary (i.e., symbiont structure in all sponge hosts reverted to homogeneous core profiles following the 2010 summer season). However, considering the warming trends in the Mediterranean Sea and the proximity of temperature maxima in our study area (25°C) to those preceding sponge mortality events (26 to 27°C), the observed shifts in rare symbiont taxa may represent a precursor to larger symbiont declines and indicate approaching thermal thresholds for Mediterranean sponge-microbe symbioses. Addi-

tional monitoring studies and controlled experimentation are required to assess whether elevated seawater temperatures induce shifts in rare symbiont taxa, how these symbiont fluctuations affect host health, and the utility of symbiont monitoring for predicting sponge mortality events.

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REFERENCES

1. **Abdo Z, et al.** 2006. Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Environ. Microbiol. **8**:929–938.
2. **Alonso-Sáez L, et al.** 2007. Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. FEMS Microbiol. Ecol. **60**:98–112.
3. **Anderson SA, Northcote PT, Page MJ.** 2010. Spatial and temporal variability of the bacterial community in different chemotypes of the New Zealand marine sponge *Mycale hentscheli*. FEMS Microbiol. Ecol. **72**:328–342.
4. **Arillo A, Bavestrello G, Burlando B, Sará M.** 1993. Metabolic integration between symbiotic cyanobacteria and sponges: a possible mechanism. Mar. Biol. **117**:159–162.
5. **Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ.** 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl. Environ. Microbiol. **71**:7724–7736.
6. **Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ.** 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. Appl. Environ. Microbiol. **72**:5734–5741.
7. **Benjamini Y, Yekutieli D.** 2001. The control of the false discovery rate in multiple testing under dependency. Ann. Stat. **29**:1165–1188.
8. **Cebrian E, Uriz MJ, Garrabou J, Ballesteros E.** 2011. Sponge mass mortalities in a warming Mediterranean Sea: are cyanobacteria-harboring species worse off? PLoS One **6**:e20211. doi:10.1371/journal.pone.0020211.
9. **Cole JR, et al.** 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res. **35**:D169–D172.
10. **Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H, Buckley DH.** 2009. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinform. **10**:171. doi:10.1186/1471-2105-10-171.
11. **Diaz MC, Rützler K.** 2001. Sponges: an essential component of Caribbean coral reefs. Bull. Mar. Sci. **69**:535–546.
12. **Diaz MC, Ward BB.** 1997. Sponge-mediated nitrification in tropical benthic communities. Mar. Ecol. Prog. Ser. **156**:97–107.
13. **Drummond AJ, et al.** 2010. Geneious v5.3. http://www.geneious.com.
14. **Duarte CM, Agustí S, Kennedy H, Vaqué D.** 1999. The Mediterranean climate as a template for Mediterranean marine ecosystems: the example of the northeast Spanish littoral. Prog. Oceanogr. **44**:245–270.
15. **Erwin PM, López-Legentil S, González-Pech R, Turon X.** 2012. A specific mix of generalists: bacterial symbionts in Mediterranean *Ircinia* spp. FEMS Microbiol. Ecol. **79**:619–637.
16. **Erwin PM, López-Legentil S, Schuhmann PW.** 2010. The pharmaceutical value of marine biodiversity for anti-cancer drug discovery. Ecol. Econ. **70**:445–451.
17. **Erwin PM, López-Legentil S, Turon X.** 2012. Ultrastructure, molecular phylogenetics, and chlorophyll *a* content of novel cyanobacterial symbionts in temperate sponges. Microb. Ecol. doi:10.1007/s00248-012-0047-5.
18. **Erwin PM, Olson JB, Thacker RW.** 2011. Phylogenetic diversity, host-specificity and community structure of sponge-associated bacteria in the northern Gulf of Mexico. PLoS One **6**:e26806. doi:10.1371/journal.pone.0026806.
19. **Erwin PM, Thacker RW.** 2007. Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages. J. Mar. Biol. Assoc. U.K. **87**:1683–1692.
20. **Erwin PM, Thacker RW.** 2008. Phototrophic nutrition and symbiont diversity of two Caribbean sponge-cyanobacteria symbioses. Mar. Ecol. Prog. Ser. **362**:139–147.
21. **Flatt P, et al.** 2005. Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge *Dysidea* (*Lamellodysidea*) *herbacea* and symbiotic cyanobacterium *Oscillatoria spongeliae* by CARD-FISH analysis. Mar. Biol. **147**:761–774.
22. **Freeman CJ, Thacker RW.** 2011. Complex interactions between marine sponges and their symbiotic microbial communities. Limnol. Oceanogr. **56**:1577–1586.
23. **Friedrich AB, Fischer I, Proksch P, Hacker J, Hentschel U.** 2001. Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. FEMS Microbiol. Ecol. **38**:105–113.
24. **Hentschel U, et al.** 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. Appl. Environ. Microbiol. **68**:4431–4440.
25. **Hoffmann F, et al.** 2005. An anaerobic world in sponges. Geomicrobiol. J. **22**:1–10.
26. **Hoffmann F, et al.** 2009. Complex nitrogen cycling in the sponge *Geodia barretti*. Environ. Microbiol. **11**:2228–2243.
27. **Jiménez E, Ribes M.** 2007. Sponges as a source of dissolved inorganic nitrogen: nitrification mediated by temperate sponges. Limnol. Oceanogr. **52**:948–958.
28. **Joachimiak MP, Weisman JL, May BCH.** 2006. JColorGrid: software for the visualization of biological measurement. BMC Bioinform. **7**:225–229.
29. **Kaplan CW, Kitts CL.** 2003. Variation between observed and true terminal restriction fragment length is dependent on true TRF length and purine content. J. Microbiol. Methods **54**:121–125.
30. **Kent AD, Smith DJ, Benson BJ, Triplett EW.** 2003. Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl. Environ. Microbiol. **69**:6768–6776.
31. **Lee OO, Lau SCK, Qian PY.** 2006. Consistent bacterial community structure associated with the surface of the sponge *Mycale adhaerens* Bowerbank. Microb. Ecol. **52**:693–707.
32. **Lee OO, et al.** 2011. Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. ISME J. **5**:650–664.
33. **Lemoine N, Buell N, Hill A, Hill M.** 2007. Assessing the utility of sponge microbial symbiont communities as models to study global climate change: a case study with *Halichondria bowerbanki*, p 239–246. *In* Custódio MR, Lôbo-Hajdu G, Hajdu E, Muricy G (ed), Porifera research: biodiversity, innovation, and sustainability. Série livros 28. Museu Nacional, Rio de Janeiro, Brazil.
34. **Liu M, Fan L, Zhong L, Kjelleberg S, Thomas T.** 2012. Metaproteogenomic analysis of a community of sponge symbionts. ISME J. doi:10.1038/ismej.2012.1.
35. **López-Legentil S, Erwin PM, Pawlik JR, Song B.** 2010. Effects of sponge bleaching on ammonia-oxidizing *Archaea*: distribution and relative expression of ammonia monooxygenase genes associated with the barrel sponge *Xestospongia muta*. Microb. Ecol. **60**:561–571.
36. **Lozupone CA, Hamady M, Kelley ST, Knight R.** 2006. Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. Appl. Environ. Microbiol. **73**:1576–1585.
37. **Maldonado M, Sánchez-Tocino L, Navarro C.** 2010. Recurrent disease outbreaks in the corneous demosponges of the genus *Ircinia*: epidemic incidence and defense mechanisms. Mar. Biol. **157**:1577–1590.
38. **Martínez-Murcia AJ, Acinas SG, Rodriguez-Valera F.** 1995. Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol. Ecol. **17**:247–255.
39. **Mohamed NM, Enticknap JJ, Lohr JE, McIntosh SM, Hill RT.** 2008. Changes in bacterial communities of the marine sponge *Mycale laxissima* on transfer into aquaculture. Appl. Environ. Microbiol. **74**:1209–1222.
40. **Mohamed NM, Rao V, Hamann MT, Kelly M, Hill RT.** 2008. Monitoring bacterial diversity of the marine sponge *Ircinia strobilina* upon transfer into aquaculture. Appl. Environ. Microbiol. **74**:4133–4143.
41. **Pandey J, Ganesan K, Jain RK.** 2007. Variations in T-RFLP profiles with differing chemistries of fluorescent dyes used for labeling the PCR primers. J. Microbiol. Methods **68**:633–638.
42. **Pile AJ, Patterson MR, Witman JD.** 1996. *In situ* grazing on plankton

<10 μm by the boreal sponge *Mycale lingua*. Mar. Ecol. Prog. Ser. **141**:95–102.

43. **Pinhassi J, et al.** 2006. Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea. Aquat. Microb. Ecol. **44**:241–252.
44. **Reysenbach AL, Wickham GS, Pace NR.** 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. **60**:2113–2119.
45. **Ribes M, et al.** 2012. Functional convergence of microbes associated with temperate marine sponges. Environ. Microbiol. **14**:1224–1239.
46. **Sarà M.** 1971. Ultrastructural aspects of the symbiosis between two species of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variabilis* (Demospongiae). Mar. Biol. **11**:214–221.
47. **Schauer M, Balagué V, Pedrós-Alió C, Massana R.** 2003. Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. Aquat. Microb. Ecol. **31**:163–174.
48. **Schauer M, Massana R, Pedrós-Alió C.** 2000. Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. FEMS Microb. Ecol. **33**:51–59.
49. **Schloss PD, et al.** 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. **75**:7537–7541.
50. **Schmitt S, et al.** 2012. Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. ISME J. **6**:564–576.
51. **Schmitt S, Weisz JB, Lindquist N, Hentschel U.** 2007. Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. Appl. Environ. Microbiol. **73**:2067–2078.
52. **Simister RL, Deines P, Botté ES, Webster NS, Taylor MW.** 2012. Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. Environ. Microbiol. **14**:517–524.
53. **Stabili L, et al.** 2012. Epidemic mortality of the sponge *Ircinia variabilis* (Schmidt, 1862) associated to proliferation of a *Vibrio* bacterium. Microb. Ecol. doi:10.1007/s00248-012-0068-0.
54. **Stewart CN, Excoffier L.** 1996. Assessing population genetic structure and variability with RAPD data: application to *Vaccinium macrocarpon* (American Cranberry). J. Evol. Biol. **9**:153–171.
55. **Taylor MW, Hill RT, Hentschel U.** 2011. Meeting report: 1st International Symposium on Sponge Microbiology. Mar. Biotechnol. **6**:1057–1061.
56. **Taylor MW, Radax R, Steger D, Wagner M.** 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol. Mol. Biol. Rev. **71**:295–347.
57. **Taylor MW, Schupp PJ, Dahllof I, Kjelleberg S, Steinberg PD.** 2004. Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. Environ. Microbiol. **6**:121–130.
58. **Thacker RW.** 2005. Impacts of shading on sponge-cyanobacteria symbioses: a comparison between host-specific and generalist associations. Integr. Comp. Biol. **45**:369–376.
59. **Thacker RW, Freeman CJ.** 2012. Sponge-microbe symbioses: recent advances and new directions. Adv. Mar. Biol. **62**:57–111. doi:10.1016/B978-0-12-394283-8.00002-3.
60. **Thiel V, Leininger S, Schmaljohann R, Brümmer F, Imhoff JF.** 2007. Sponge-specific bacterial associations of the Mediterranean sponge *Chondrilla nucula* (Demospongiae, Tetractinomorpha). Microb. Ecol. **54**:101–111.
61. **Thiel V, Neulinger SC, Staufenberger T, Schmaljohann R, Imhoff JF.** 2007. Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*. FEMS Microbiol. Ecol. **59**:47–63.
62. **Thomas T, et al.** 2010. Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. ISME J. **4**:1557–1567.
63. **Usher KM.** 2008. The ecology and phylogeny of cyanobacterial symbionts in sponges. Mar. Ecol. **29**:178–192.
64. **Usher KM, Toze S, Fromont J, Kuo J, Sutton DC.** 2004. A new species of cyanobacterial symbiont from the marine sponge *Chondrilla nucula*. Symbiosis **36**:183–192.
65. **Van Soest RWM, et al.** 2012. Global diversity of sponges (Porifera). PLoS One **7**:e35105. doi:10.1371/journal.pone.0035105.
66. **Webster NS, Cobb RE, Negri AP.** 2008. Temperature thresholds for bacterial symbiosis with a sponge. ISME J. **2**:830–842.
67. **Webster NS, et al.** 2011. Bacterial community dynamics in the marine sponge *Rhopaloeides odorabile* under in situ and ex situ cultivation. Mar. Biotechnol. **13**:296–304.
68. **Webster NS, Hill RT.** 2001. The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. Mar. Biol. **138**:843–851.
69. **Webster NS, Negri AP, Webb RI, Hill RT.** 2002. A spongin-boring alpha-proteobacterium is the etiological agent of disease in the Great Barrier Reef sponge *Rhopaloeides odorabile*. Mar. Ecol. Prog. Ser. **232**:305–309.
70. **Webster NS, Taylor MW.** 2011. Marine sponges and their microbial symbionts: love and other relationships. Environ. Microbiol. **14**:335–346.
71. **Webster NS, et al.** 2010. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environ. Microbiol. **12**:2070–2082.
72. **Webster NS, Xavier JR, Freckelton M, Motti CA, Cobb R.** 2008. Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. Environ. Microbiol. **10**:3366–3376.
73. **White JR, et al.** 2012. Pyrosequencing of bacterial symbionts within *Axinella corrugate* sponges: diversity and seasonal variability. PLoS One **7**:e38204. doi:10.1371/journal.pone.0038204.
74. **Wichels A, Würtz S, Döpke H, Schütt C, Gerdts G.** 2006. Bacterial diversity in the breadcrumb sponge *Halichondria panicea*. FEMS Microb. Ecol. **56**:102–118.
75. **Wilkinson CR.** 1983. Net primary productivity in coral reef sponges. Science **219**:410–412.

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Till Death Do Us Part: Stable Sponge-Bacteria Associations under Thermal and Food Shortage Stresses

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Abstract

Sporadic mass mortality events of Mediterranean sponges following periods of anomalously high temperatures or longer than usual stratification of the seawater column (i.e. low food availability) suggest that these animals are sensitive to environmental stresses. The Mediterranean sponges *Ircinia fasciculata* and *I. oros* harbor distinct, species-specific bacterial communities that are highly stable over time and space but little is known about how anomalous environmental conditions affect the structure of the resident bacterial communities. Here, we monitored the bacterial communities in *I. fasciculata* (largely affected by mass mortalities) and *I. oros* (overall unaffected) maintained in aquaria during 3 weeks under 4 treatments that mimicked realistic stress pressures: control conditions (13°C, unfiltered seawater), low food availability (13°C, 0.1 μm-filtered seawater), elevated temperatures (25°C, unfiltered seawater), and a combination of the 2 stressors (25°C, 0.1 μm-filtered seawater). Bacterial community structure was assessed using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene sequences and transmission electron microscopy (TEM). As *I. fasciculata* harbors cyanobacteria, we also measured chlorophyll *a* (chl *a*) levels in this species. Multivariate analysis revealed no significant differences in bacterial T-RFLP profiles among treatments for either host sponge species, indicating no effect of high temperatures and food shortage on symbiont community structure. In *I. fasciculata*, chl *a* content did not significantly differ among treatments although TEM micrographs revealed some cyanobacteria cells undergoing degradation when exposed to both elevated temperature and food shortage conditions. Arguably, longer-term treatments (months) could have eventually affected bacterial community structure. However, we evidenced no appreciable decay of the symbiotic community in response to medium-term (3 weeks) environmental anomalies purported to cause the recurrent sponge mortality episodes. Thus, changes in symbiont structure are not likely the proximate cause for these reported mortality events.

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Introduction

Summer in the Western Mediterranean Sea is getting warmer and longer. Over the past decades, the frequency of seawater temperature anomalies and the period length of stable seawater column (i.e., stratification) have increased [1–3]. At the same time and coinciding with years of record temperatures (1–2°C above the mean summer temperature) or prolonged seawater stratification in late summer, mass mortality events were observed for several filter-feeding invertebrates, mainly sponges and cnidarians [3–5]. A typical summer season in the Mediterranean Sea is characterized by high temperatures (>18°C) that stratify the seawater column and prevent the upwelling of cooler nutrient-rich water, resulting in nutrient depletion, low turbidity and high irradiance in shallow waters (<20 m) [2]. Consequently, summer is a energetically-challenging season for filter-feeding invertebrates in the Mediterranean Sea [6,7] and together with high temperatures or prolonged stratification, the additional physiological stress that occurs during this season may facilitate the observed episodes of mass mortality [2].

Marine sponges harbor diverse and host-specific bacterial communities [8,9] suggesting that the ecology and survival of both the sponge and its bacterial associates are tightly connected; e.g. via nutrient translocation [10,11]. However, despite the potential importance of sponge-bacteria interactions, to date few studies have experimentally assessed the response and stability of these associations under environmental conditions chosen to mimic realistic stress pressures. Most notably, manipulative experiments with the Great Barrier Reef sponge *Rhopaloeides odorabile* showed that the bacterial community associated with this sponge shifted in response to elevated temperatures, high nutrients and pollutants, concomitant with declines in host sponge health [12–15]. In temperate regions, sponge-derived bacterial communities changed when exposed to elevated temperatures [16] but remained stable under starvation conditions [17]. Further studies are needed to investigate the effect of extreme yet realistic

environmental conditions on sponge-associated bacterial communities and assess their overall resilience amidst a changing climate.

Sponges in the genus *Ircinia* are ubiquitous in the Western Mediterranean rocky bottoms and harbor a species-specific bacterial community [18] that seems to be adapted to the seasonality of the water column [19]. Recently, *Ircinia* spp. have suffered dramatic episodes of mass mortality linked to extreme summer temperatures [20,21] and the proliferation of an opportunistic *Vibrio*-like bacterium [21,22]. The factors triggering the proliferation of *Vibrio*-like bacteria in sponge hosts remain unclear, but may be preceded by the disruption of the normal sponge microflora caused by abnormally high seawater temperatures lasting 3 weeks [20]. Cebrián *et al.* [20] observed significant reduction in photosynthetic efficiency in *I. fasciculata* individuals maintained in aquaria at elevated temperatures (27°C for 48 h). Based on these results, the authors suggested that cyanobacteria-harboring sponges such as *I. fasciculata* may be more susceptible to mass mortality events than other sponge species lacking photosymbionts.

In this study, we hypothesized that a high temperature treatment combined with low food availability mimicking an especially hot summer season in the Mediterranean Sea would be accompanied by a shift in the bacterial communities associated with Mediterranean sponges. Based on past studies [20], we expected that sponges harboring photosymbionts would be more susceptible to these shifts than those without them. To test these hypotheses, we performed a series of controlled aquaria experiments for the sympatric sponges *I. fasciculata* (which harbors cyanobacteria and has suffered mass mortality events) and *I. oros* (which does not harbor cyanobacteria, and has remained overall unaffected by mass mortality events). We tested the effect of high seawater temperature (25°C), food shortage (0.1 μm-filtered seawater) and the combination of both treatments on sponge-associated bacterial communities. Bacterial symbiont communities were monitored using terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA gene sequences and transmission electron microscopy (TEM) analyses. We also measured the concentration of chlorophyll *a* (chl *a*) in *I. fasciculata* samples as a proxy for photosymbiont abundance/activity in these hosts.

Materials and Methods

Specimen collection

40 individuals of the sponge *Ircinia oros* (Schmidt, 1864) and 40 of *I. fasciculata* (Pallas, 1766) were collected from shallow (<20 m) rocky reefs in the north-western Mediterranean Sea (Tossa de Mar, 41°43′13.62″ N, 2°56′26.90″ E) during January 2011 (*I. oros*) and February 2011 (*I. fasciculata*). Collection during winter months was favored for our experiments because temperatures are more stable during this period [19]. Within 2 h, the sponges were transported in insulated coolers from Tossa de Mar to the Experimental Aquaria Zone (ZAE) located at the Institute of Marine Science (ICM-CSIC) in Barcelona (Spain). *Ircinia* spp. are not endangered or protected by any law and all sampling was conducted outside protected areas following current Spanish regulations (no specific permits were required).

Experimental design

Two experiment sets (one for each sponge species) were conducted in consecutive months, immediately after specimen collection. For each experiment, 40 specimens were placed in separated 2 L aquaria in a flow-through system with direct intake of seawater and an independent supply to each aquarium for a total of 4 weeks. The aquaria were subjected to circadian cycles of 12 h light/12 h dark using artificial light sources. The first week, sponges were maintained at natural (ambient) conditions as an acclimation period. During the following 3 weeks, 4 different treatments were set up (n = 10 individuals per treatment): non-filtered seawater and environmental temperature (control), 0.1 μm-filtered seawater and environmental temperature (FE), non-filtered seawater and hot temperature 25°C (NH), 0.1 μm-filtered seawater and hot temperature (FH). The environmental seawater temperature at the time of the experiments was 13°C. For the heat treatment, the temperature was progressively increased (ca. $1.5°C \cdot day^{-1}$) during 7 days until reaching 25°C and then maintained at 25°C for the final 2 wk of the experiment. The health status of the sponges was monitored every 2 days by visual inspection for tissue necrosis. Water flux was also controlled every 2 days and readjusted if necessary to obtain a final flux rate through the aquaria of $0.8\ L \cdot min^{-1}$. Filters were replaced weekly to avoid flux reduction due to particle accumulation.

Experimental sampling

Temperature (°C) and light intensity levels (lx = $lumen \cdot m^{-2}$) were recorded hourly with Hobo Pendant Temperature/Light Data Loggers (UA-002-64; Onset Computer Corporation). To check for filter efficiency and natural bacterial concentrations in the seawater, 3 samples of water per treatment were collected weekly, before filter replacement. Bacterial concentration was estimated by flow cytometry, based on the method described in Gasol & Del Giorgio [23]. In short, samples were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde in a phosphate-buffered saline (PBS) solution, incubated in the dark for 10 min, deep frozen in liquid nitrogen and stored at −80°C. For analysis, samples were unfrozen, stained with Syto13 (Molecular Probes) at 5 μM (diluted in dymethil sulfoxyde, DMSO), incubated for 15 min in the dark and run through a GALLIOS flow cytometer with a laser emitting at 480 nm. Bacteria were detected according to a dot plot of side scatter (SSC, related with cell size) *versus* fluorescent signature (FL1). The number of events (potential bacterial cells) detected by the cytometer was then converted into bacterial cell density ($cells \cdot mL^{-1}$) by comparing with the events recorded by the machine after injecting a known volume of a solution of 10^6 Syto13-stained $beads \cdot mL^{-1}$. For each sponge species, the non-parametric Mann-Whitney's U test was used to compare the bacterial cell density in seawater from non-filtered treatments *versus* filtered treatments. Statistical analyses were performed in RStudio [24]. All cytometry analyses were conducted at the Cytometry Unit of the Scientific and Technological Services of the University of Barcelona.

From all the sponge samples, we randomly selected 3 individuals per treatment that remained healthy throughout the experiment for further analysis (n = 24 per species). Overall, specimens of *I. fasciculata* and *I. oros* remained healthy in all experimental treatments with no tissue necrosis or appreciable biomass loss, except for 1 individual of *I. fasciculata* that died during the acclimation period, and 5 individuals of *I. oros* that died during the first week of experiment (1 from the FE treatment, 3 from the NH, and 1 from the FH). These specimens were not considered in our analysis for several reasons: (i) death was likely due to manipulation rather than to the tested conditions because they all died early during the experiments; (ii) by the end of the experiment, the sponges had been dead for at least two weeks (iii) there were insufficient replicates for robust statistical analysis.

DNA extraction

After the acclimation period (end of week 1) and at the end of the experiments (end of week 4), a tissue sample (ca. 2 mm^3) of each selected specimen containing both ectosome and choanosome was preserved in 100% ethanol and stored at −20°C. To characterize the bacterial community in the seawater, 500 mL of water per treatment were filtered through a 0.2 µm filter (Millipore), preserved in 100% ethanol and stored at −20°C. DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen®). Dilutions (1:10) of DNA extracts were used as templates in subsequent PCR amplifications for T-RFLP analysis.

T-RFLP analysis

PCR amplification of 16S rRNA gene sequences was conducted using the universal bacterial forward primer Eco8F [25], tagged with a 5′-6-carboxyfluorescein (6-FAM) label, and reverse primer 1509R [26]. PCR was performed as follows: one initial denaturation step for 5 min at 94°C; 35 cycles of 1 min at 94°C, 0.5 min at 50°C, 1.5 min at 72°C; and one final elongation step for 5 min at 72°C. Total PCR volume (50 µL) included 10 µM of each primer, 10 nM of each dNTP, 1x Reaction Buffer (Ecogen), 2.5 mM $MgCl_2$ and 5 units of BioTaq™ DNA polymerase (Ecogen). Products from triplicate PCR reactions were pooled and purified from electrophoresis gels using the Qiaquick Gel Extraction kit (Qiagen®), then quantified using the Qubit™ fluorometer and Quant-iT™ dsDNA Assay kit (Invitrogen™) according to manufactures' instructions. Separate enzymatic digestions with *Hae*III and *Msp*I were processed as described elsewhere [27], then analyzed in an automated ABI 3730 Genetic Analyzer (Applied Biosystems) at the Genomics Unit of the Scientific and Technological Services of the University of Barcelona. The lengths of each terminal-restriction fragment (T-RF) were determined against a size standard (600-LIZ) using the PeakScanner™ software (Applied Biosystems). T-RFs smaller than 50 bp or larger than 600 bp were discarded because they were beyond the resolution of the size standard. Background noise was defined by a peak intensity below 50 fluorescence units and by filtering in T-REX [28] using a cut-off value of 2 standard deviations [29]. 'True' T-RFs were aligned in T-REX using a clustering threshold of 1 bp and relative T-RF abundance matrices were constructed.

T-RFLP statistical analyses

Samples from each experimental set were analyzed separately to investigate whether the observed response to each treatment depended on sponge species (*I. fasciculata* and *I. oros*). All analyses were based on Bray-Curtis distances calculated from relative abundance matrices, following square root transformation. For each restriction enzyme, non-metric multi-dimensional scaling (nMDS) plots were constructed to visually compare the bacterial communities. Permutational multivariate analyses of variance (PERMANOVA) [30,31] were used to test the effects of source (sponge or seawater) and treatment (control, FE, NH, FH) on bacterial communities. In addition, sponge samples collected after the acclimation period were compared to verify that the specimens harbored similar bacterial communities before experimental treatments were applied. Calculations were performed in PRIMER v6 [32,33] and PERMANOVA+ (Plymouth Marine Laboratory, UK). The empirical T-RFs obtained in this study were compared with the available database of *in silico* *Hae*III and *Msp*I digestions of 16S rRNA gene sequences derived from the same host sponges in a previous study [19] using the phylogenetic assignment tool PAT [34].

Transmission electron microscopy (TEM)

At the end of the experiments, a piece of tissue (ca. 2 mm^3) from one sponge in each treatment was collected and fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde buffered with filtered seawater and incubated overnight at 4°C. Following fixation, each piece was rinsed at least three times with filtered seawater and stored at 4°C until processed as described previously [35]. TEM observations were made at the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona on a JEOL JEM-1010 (Tokyo, Japan) coupled with a Bioscan 972 camera (Gatan, Germany).

Chlorophyll *a* (chl *a*) concentrations

For chl *a* quantification in *I. fasciculata*, a piece of ectosome was sampled from 5 sponges per treatment at the end of the experiments (n = 20) and processed them using previously described methods [18]. *I. oros* was excluded from this analysis because this species lacks photosymbionts [18]. One-way ANOVA was performed to test the effect of the factor "treatment" (4 levels; control, FE, NH, FH) on chl *a* concentrations in *I. fasciculata*. The assumptions of the ANOVA were checked by Cramer-von Mises' normality test and Levene's homoscedasticity test. Statistical analyses were performed in RStudio [22].

Results

Aquaria conditions

Artificial light intensity in the aquaria with *I. fasciculata* samples was 546.7±25.0 lx (mean ± standard error) and in the aquaria with *I. oros* 644.1±8.9 lx. Both light intensity values were in the range of values detected in their natural habitat during winter [19]. Environmental water temperature was 13.42±0.01°C and 13.54±0.18°C (mean ± standard error) for the experiment with *I. fasciculata* and with *I. oros*, respectively. For hot temperature treatments, temperature was increased at a rate of $1.49°C \cdot day^{-1}$ for the aquaria with *I. fasciculata* samples and $1.57°C \cdot day^{-1}$ for *I. oros* samples during one week, until reaching a final temperature of 25.41±0.01°C and 25.23±0.05°C (mean ± standard error) for the experiment with *I. fasciculata* and with *I. oros*, respectively. The average densities (mean ± standard error) of bacterial cells found in seawater samples from the filtered treatments were $(2.4\pm0.3)\cdot10^4$ cells$\cdot mL^{-1}$ in *I. fasciculata* aquaria, and $(2.3\pm0.2)\cdot10^4$ cells$\cdot mL^{-1}$ in *I. oros*, while in the unfiltered treatments contained $(7.4\pm1.0)\cdot10^4$ cells$\cdot mL^{-1}$ and $(6.8\pm0.5)\cdot10^4$ cells$\cdot mL^{-1}$ in aquaria with *I. fasciculata* and *I. oros*, respectively. In spite of the filtering system, bacterial abundance was only cut by ca. one third. This may relate with decaying filter efficiency with time, in spite of weekly filter changes. Still, the bacterial cell density in seawater samples from non-filtered treatments was statistically higher than in filtered treatments (Mann-Whitney's U, $P<0.001$) for both *I. fasciculata* and *I. oros* experiments. A one-third reduction in bacterial density is likely a realistic proxy for food shortage conditions in nature.

T-RFLP analysis

A total of 143 unique T-RFs were detected with *Hae*III restriction enzyme (101 in *I. fasciculata*, 97 in *I. oros* and 59 in seawater) and 167 with *Msp*I enzyme (117 in *I. fasciculata*, 110 in *I. oros* and 79 in seawater). PERMANOVA analysis of Bray-Curtis similarity matrices from each experiment reported a significant effect of source (sponge *vs* seawater) on the structure of bacterial communities (Table 1). No significant differences in bacterial community structure were detected among samples of the same sponge species after the acclimation week ($P>0.225$, for both

Table 1. Statistical analysis of T-RFLP profiles to test for an effect of source (seawater *vs* sponge) and treatment on the structure of *Ircinia*-associated bacterial communities.

	I. fasciculata		*I. oros*	
	*Hae*III	*Msp*I	*Hae*III	*Msp*I
Source (seawater *vs* sponge)	**0.001**	**0.001**	**0.001**	**0.002**
Treatment (control, FE, NH, FH)	0.317	0.328	0.267	0.066

Numbers denote *P*-values from PERMANOVA test after 999 permutations.
Significant values at $\alpha = 0.01$ are in bold.
Treatments: Control (13°C, unfiltered seawater), FE (13°C, filtered seawater), NH (25°C, non-filtered seawater), FH (25°C, filtered seawater).
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enzymes). Likewise, there was not a significant effect of treatment on the bacterial communities of *I. fasciculata* and *I. oros* after 3 weeks (Table 1). As the experiment was terminated after 3 weeks, there is no data beyond the duration of the experiments. The lack of structure observed with the nMDS plots further confirmed the similarity of these bacterial communities within host species, despite the different treatments applied (Fig. 1). PAT analysis reported that 58.7% (*Hae*III) and 71.6% (*Msp*I) of the unique T-RFs obtained in this study for both *I. fasciculata* and *I. oros* matched T-RFs from *in silico* digestions of 16S rRNA sequences from environmental samples of these two species [18].

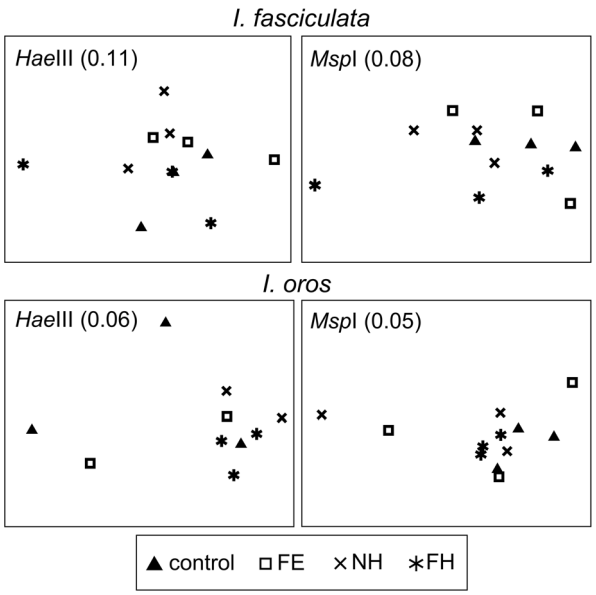

Figure 1. Non-metric multidimensional scaling (nMDS) of sponge-derived bacterial communities at the end of the experiment. Ordination in nMDS plots is based on Bray-Curtis distances between T-RFLP profiles from *Hae*III (left) and *Msp*I (right) digestions of samples of *I. fasciculata* and *I. oros* experiments. Stress values are shown in parenthesis, with values below 0.15 indicating good correlation of similarity matrix distances and ordination in the two-dimension plot. Points are coded by treatment: control (13°C, unfiltered seawater), FE (13°C, filtered seawater), NH (25°C, non-filtered seawater), FH (25°C, filtered seawater).
doi:10.1371/journal.pone.0080307.g001

Transmission electron microscopy

Micrographs of *I. fasciculata* samples from the control treatment showed typical sponge cells with numerous phagosomes and granules of glycogen (Fig. 2a). The same sponge cells were observed in all the other treatments. The cyanobacterium *Candidatus* 'Synechococcus spongiarum' dominated the ectosomal tissue of *I. fasciculata* (Fig. 2b–e). In the micrographs from the hot temperature (25°C) and filtered seawater treatment (FH), besides healthy cyanobacterial cells, we also observed many cells undergoing degradation (Fig. 2e–f). Electron micrographs from *I. oros* samples (Fig. 3a–d) showed abundant vacuolated sponge cells surrounded by diverse bacterial morphotypes. No differences in sponge or bacterial cell abundance or morphology were detected for any of the treatments. As expected, no cyanobacterial cells were observed either in this sponge species.

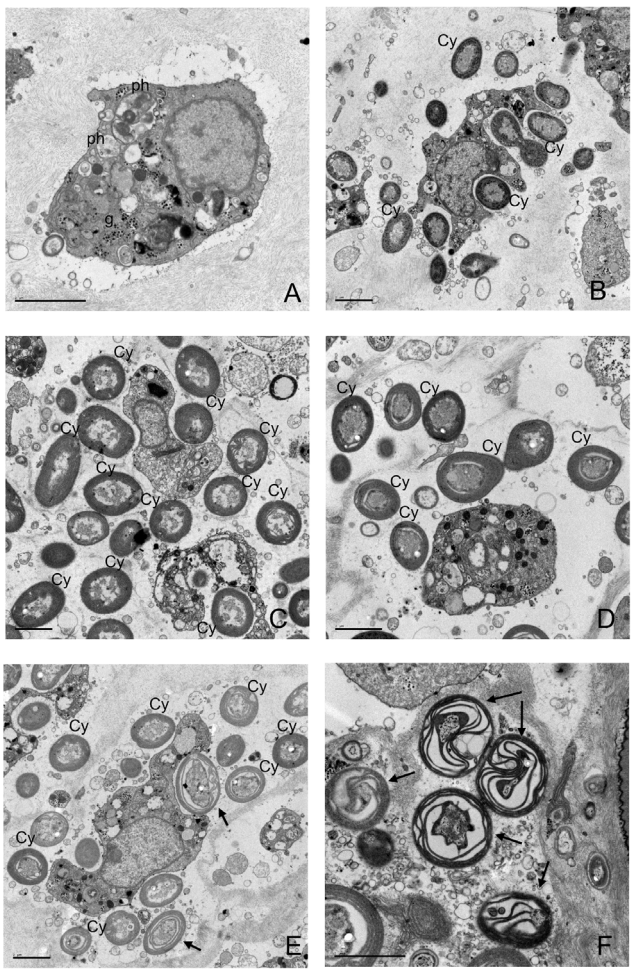

Figure 2. Electron micrographs of *I. fasciculata* bacteria at the end of the experiment. A) Sponge cell in sample from control treatment, containing several phagosomes (ph) and glycogen granules (g). Sponge cells surrounded by multiple *Cyanobacteria* (Cy) and heterotrophic bacteria in the mesohyl of sponges from control treatment (B), NH (25°C, non-filtered seawater) treatment (C) and FE (13°C, filtered seawater) treatment (D). Micrographs of a sponge from FH (25°C, filtered seawater) treatment (E, F) showed healthy *Cyanobacteria* (Cy) and *Cyanobacteria* under different stages of degradation (arrows) within the mesohyl. Scale bars represent 2 μm.
doi:10.1371/journal.pone.0080307.g002

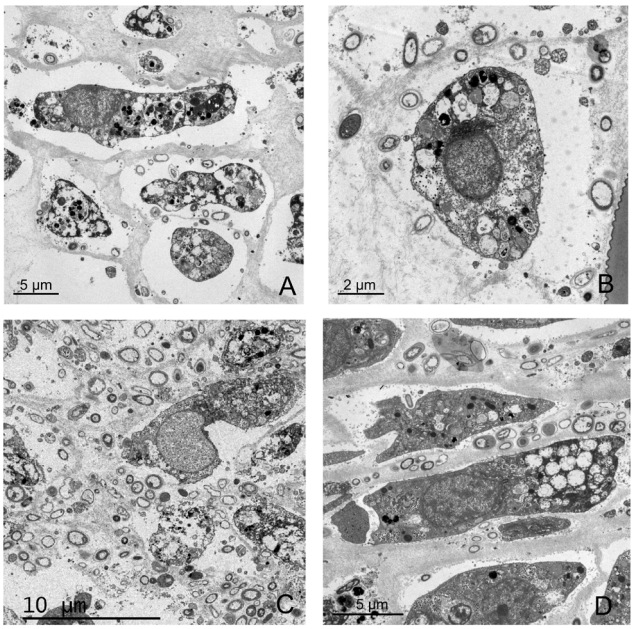

Figure 3. Electron micrographs of *I. oros* bacteria at the end of the experiment. Sponge cells surrounded by numerous bacteria cells of different morphotypes. Samples from control treatment (A); 25°C and non-filtered seawater treatment (B); 13°C and filtered seawater treatment (C); and 25°C and filtered seawater treatment (D). Sponge and bacteria cells for all treatments showed no sign of degradation.
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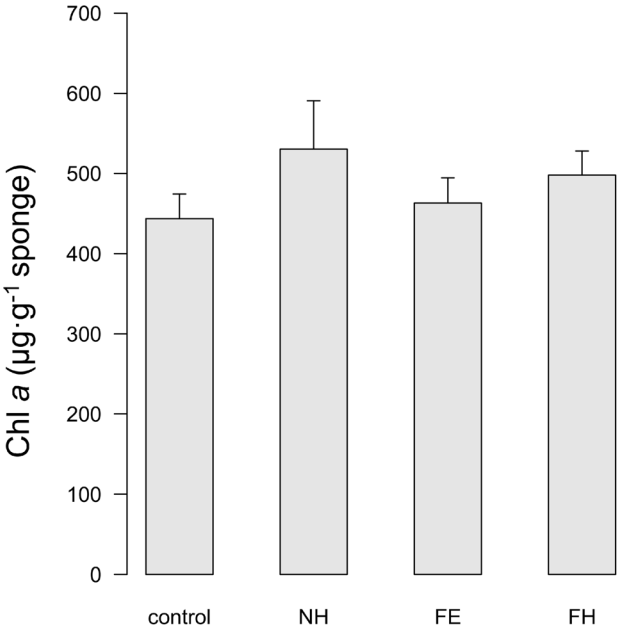

Figure 4. Chlorophyll *a* concentration in *I. fasciculata* for each treatment at the end of the experiment. Control: 13°C and non-filtered seawater; NH: 25°C and non-filtered seawater; FE: 13°C and filtered seawater; FH: 25°C and filtered seawater. Error bars denote standard error.
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Chlorophyll *a* concentration

Chl *a* levels in *I. fasciculata* at the end of the experiment (3 weeks after acclimation) and for each treatment are depicted in Fig. 4. The ANOVA test revealed no significant differences in chl *a* concentration among treatments ($P = 0.4636$). The values found here (483.8 ± 20.0 µg·g^{-1} sponge, mean ± standard error) exceeded those observed for this species in the field, where the average concentration reported was 248.1 ± 27.8 µg·g^{-1} sponge [19].

Discussion

The bacterial communities associated with the Mediterranean sponges *I. fasciculata* and *I. oros* were stable under thermal and food shortage stresses for a period lasting 3 weeks. Comparison of T-RFLP profiles and electron microscopy for each species showed no significant differences among the 4 treatments tested that combined high seawater temperatures (25°C) and low food availability (one-third reduction of the natural bacterial abundance) during three weeks after acclimation. The only noticeable difference consisted of TEM observations of several degraded cyanobacterial cells of *S. spongiarum*, along with healthy looking ones, when *I. fasciculata* specimens were exposed to both thermal and food shortage stresses. However, the presence of degraded cells was not accompanied by a significant decrease in chl *a* concentrations. In fact, chl *a* content was higher in our aquaria samples and for all treatments than what has been observed in the field [19]. This increase in chl *a* concentration may be due to a higher density of cyanobacterial cells in the sponge or enhanced photosynthetic activity to compensate for lower ambient irradiance conditions or a poorer diet. Overall, our results indicate that the seawater conditions that characterize anomalously warm summer seasons in the Mediterranean Sea do not affect sponge-associated bacterial communities. Moreover, we did not observe any clear evidence supporting the hypothesis that sponges harboring cyanobacterial symbionts were more vulnerable to the assayed conditions than sponges without them. Other species-specific factors such as habitat-preference or growth dynamics [34], alone or in combination, may contribute to the sporadic mass mortality events observed for *I. fasciculata* but not for *I. oros* in the Mediterranean Sea.

One specimen of *I. fasciculata* and 5 of *I. oros* died during the experiments and were excluded from T-RFLP analysis. Necrosis in *I. fasciculata* occurred during the acclimation period and thus was unrelated with the tested treatments. Individual plasticity in resilience to collection and transport or health status at the moment of sampling may have affected the survival of that specimen when moved into aquaria. For *I. oros* sponges, death occurred early during the second week, before the targeted elevated temperature was reached, and sporadically among treatments. Previous studies assaying similar thermal stressors have reported host tissue necrosis and symbiotic cyanobacterial loss in all specimens at elevated seawater temperatures after only 3 to 4 days of treatment [13,14]. While we cannot be certain of the reason behind the death of these few sponges (i.e. tested treatments or different response to maintenance in aquaria), none of our treatments resulted in mass mortality and the remaining specimens looked healthy through the 3-week experiment.

We cannot disregard that longer-term experiments (months) could result in a significant effect of treatment on bacterial community structure. Stratification of the water column along the Mediterranean coast lasts more than three weeks. Nevertheless, the persistence reported in this study is still remarkable. The high temperature tested here (25°C) represents 3°C more than the summer mean temperature in the study area [19], matched the maximum temperature detected during anomalous summer seasons in years when mass mortality events occurred [20], and

represents an increase of >11°C from ambient conditions at the time of collection. In addition, the time frame of our experiments (3 weeks after acclimation) matched the duration of peaks of temperature in abnormally warm summers [20].

Our results are also in agreement with other studies indicating that sponge-bacteria associations are very stable and able to resist non-lethal stressful conditions. In the Mediterranean sponge *Aplysina aerophoba*, neither food shortage nor antibiotic exposure promoted the consumption of symbionts by the host and the structure of the bacterial community remained unchanged for up to 11 days [17]. In the tropical sponge *Rhopaloeides odorabile*, the bacterial community shifted only when sponge tissue necrosis occurred, after exposure to temperatures 2 to 4°C above the mean temperature in the study area [13,14]. Interestingly, Fan et al. [35] observed that the expression of genes potentially essential for the symbiotic relationship (e.g. proteins involved in cell-cell signaling that could mediate recognition of symbiont by host) was maintained in partially necrotic sponges although at a lower rate than in healthy ones.

Despite the overall stability of sponge-associated bacteria, cells of dominant cyanobacterium *S. spongiarum* were observed undergoing degradation in *I. fasciculata* sponges exposed to high temperature and food shortage stresses (FH). While not all *S. spongiarum* cells were degrading and chl *a* content did not differ among treatments, the observation of this phenomenon only in the most stressful treatment suggests higher sensitivity of cyanobacteria to these conditions. Previous studies indicated that cyanobacteria-harboring sponges were more vulnerable to elevated temperatures due to photo-oxidative stress (i.e., rising levels of harmful oxygen compounds) derived from temperature-enhanced photosynthesis [20]. However, the stability of the symbiotic community and cyanobacterial chl *a* content across treatments observed in this study suggest that the overall photosynthetic activity was not impaired by the degradation of some cyanobacterial cells and that the sponge holobiont is able to resist these conditions for 3 weeks.

The persistence of bacterial symbiont communities despite thermal stress and food shortage conditions lasting 3 weeks is in opposition to one of the predictions of the coral probiotic hypothesis [36]. According to this hypothesis, the microbial symbionts associated with corals would rapidly shift in response to changing environmental conditions (in days to weeks), thereby conferring an adaptive response to the host. In sponges, it does not seem that rapid changes in bacterial community structures would provide stress tolerance to the host [13]. Instead, we speculate that, similar to what has been proposed for the human gut microbiome [37], a persistent symbiotic community in sponges results in constitutive benefits, such as preventing the unexpected proliferation of one or a few bacterial strains within the symbiotic community that yield holobiont death. The empirical demonstration of interactions within the bacterial community and between the bacteria and host that maintain the stability of the symbiotic community under environmental stresses remains a challenge for sponge microbiology.

In conclusion, our experiments for the sympatric sponges *I. fasciculata* and *I. oros* maintained in aquaria mimicking an especially hot summer in the Mediterranean Sea revealed high persistence of sponge-associated bacterial communities. These findings support trends observed in the field showing high symbiont stability across spatial and temporal scales [19,38,39] and also suggest that the disruption of the symbiotic community in response to abnormal thermal and food shortage conditions for a period up to three weeks may not be the primary cause of the sporadic mass mortality events observed for some *Ircinia* species.

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Author Contributions

Conceived and designed the experiments: LP PME XT SLL. Performed the experiments: LP SLL. Analyzed the data: LP PME XT SLL. Contributed reagents/materials/analysis tools: PME XT SLL. Wrote the paper: LP PME XT SLL.

References

1. Calvo E, Simó R, Coma R, Ribes M, Pascual J, et al. (2011) Effects of climate change on Mediterranean marine ecosystems: the case of the Catalan Sea. Climate Res 50: 1–29.
2. Coma R, Ribes M, Serrano E, Jiménez E, Salat J, et al. (2009) Global warming-enhanced stratification and mass mortality events in the Mediterranean. Proc Natl Acad Sci USA 106: 6176–6181.
3. Lejeusne C, Chevaldonne P, Pergent-martini C, Boudouresque C, Pérez T (2009) Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. Trends Ecol Evol 25: 250–260.
4. Garrabou J, Coma R, Bensoussan N, Bally M, Chevaldonné P, et al. (2009) Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. Glob Chang Biol 15: 1090–1103.
5. Cerrano C, Magnino G, Sarà A (2001) Necrosis in a population of *Petrosia* in relation with environmental stress. Ital J Zool 136: 131–136.
6. Coma R, Ribes M, Gili J, Zabala M (2000) Seasonality in coastal benthic ecosystems. Trends Ecol Evon 15: 448–453.
7. López-Legentil S, Erwin PM, Velasco M, Turon X (2013) Growing or reproducing in a temperate sea: optimization of resource allocation in a colonial ascidian. Invertebr Biol 132: 69–80.
8. Schmitt S, Tsai P, Bell J, Fromont J, Ilan M, et al. (2012) Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. ISME J 6: 564–576.
9. Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol R 71: 295–347.
10. Freeman CJ, Thacker RW (2011) Complex interactions between marine sponges and their symbiotic microbial communities. Limnol Oceanogr 56: 1577–1586.
11. Weisz JB, Massaro AJ, Ramsby BD, Hill MS (2010) Zooxanthellar symbionts shape host sponge trophic status through translocation of carbon. Biol Bull 219: 189–197.
12. Simister R, Taylor MW, Tsai P, Webster N (2012) Sponge-microbe associations survive high nutrients and temperatures. PLoS One 7: e52220.
13. Simister R, Taylor MW, Tsai P, Fan L, Bruxner TJ, et al. (2012) Thermal stress responses in the bacterial biosphere of the Great Barrier Reef sponge, *Rhopaloeides odorabile*. Environ Microbiol 14: 3232–3246.
14. Webster NS, Cobb RE, Negri AP (2008) Temperature thresholds for bacterial symbiosis with a sponge. ISME J 2: 830–842.
15. Webster NS, Webb RI, Ridd MJ, Hill RT, Negri AP (2001) The effects of copper on the microbial community of a coral reef sponge. Environ Microbiol 3: 19–31.
16. Lemoine N, Buell N, Hill A, Hill M (2007) Assessing the utility of sponge microbial symbiont communities as models to study global climate change: a case study with *Halichondria bowerbanki*. In: Custódi MR, Lôbo-Hadju G, Hadju E, Muricy G, (eds). Porifera Research: Biodiversity, Innovation and Sustainability. Série Livros 28. Museu Nacional, Rio de Janeiro. pp. 419–425.
17. Friedrich AB, Fischer I, Proksch P, Hacker J, Hentschel U (2001) Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. FEMS Microbiol Ecol 38: 105–115.
18. Erwin PM, López-Legentil S, González-Pech R, Turon X (2012) A specific mix of generalists: bacterial symbionts in Mediterranean *Ircinia* spp. FEMS Microbiol Ecol 79: 619–637.
19. Erwin PM, Pita L, López-Legentil S, Turon X (2012) Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance. Appl Environ Microbiol 78: 7358–7368.

20. Cebrian E, Uriz MJ, Garrabou J, Ballesteros E (2011) Sponge mass mortalities in a warming Mediterranean Sea: are cyanobacteria-harboring species worse off? PLoS One 6: e20211.
21. Maldonado M, Sánchez-Tocino L, Navarro C (2010) Recurrent disease outbreaks in corneous demosponges of the genus *Ircinia*: epidemic incidence and defense mechanisms. Mar Biol 157: 1577–1590.
22. Stabili L, Cardone F, Alifano P, Tredici SM, Piraino S, et al. (2012) Epidemic mortality of the sponge *Ircinia variabilis* (Schmidt, 1862) associated to proliferation of a *Vibrio* bacterium. Microb Ecol 64: 802–813.
23. Gasol JM, Del Giorgio PA (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Sci Mar 64: 197–224.
24. Racine J (2012) RStudio: A Platform-Independent IDE for R and Sweave. J Appl Econ (Chichester Engl) 27: 167–172.
25. Turner S, Pryer KM, Miao VP, Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol 46: 327–338.
26. Martínez-Murcia A, Acinas S, Rodríguez-Valera F (1995) Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol Ecol 17: 247–255.
27. Pita L, López-Legentil S, Erwin PM (2013) Biogeography and host fidelity of bacterial communities in *Ircinia* spp. from the Bahamas. Microb Ecol 66:437–447.
28. Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H, Buckley DH (2009) T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics 10: 171–181.
29. Abdo Z, Schüette UME, Bent SJ, Williams CJ, Forney LJ, et al. (2006) Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Environ Microbiol 8: 929–938.
30. Clarke K (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18: 117–143.
31. Clarke K, Gorley R (2006) PRIMER v6: User Manual/Tutorial.
32. Kent AD, Smith DJ, Benson BJ, Triplett EW (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl Environ Microbiol 69: 6768–6776.
33. López-Legentil S, Song B, Bosch M, Pawlik JR, Turon X (2011) Cyanobacterial diversity and a new *Acaryochloris*-like symbiont from Bahamian sea-squirts. PLoS One 6: e23938.
34. Turon X, Garriga A, Erwin PM (2013) Lights and shadows: growth patterns in three sympatric and congeneric sponges (*Ircinia* spp.) with contrasting abundances of photosymbionts. Mar Biol 160: 2743-2754.
35. Fan L, Liu M, Simister R, Webster NS, Thomas T (2013) Marine microbial symbiosis heats up: the phylogenetic and functional response of a sponge holobiont to thermal stress. ISME J 7: 991–1002.
36. Reshef L, Koren O, Loya Y, Zilber-Rosenberg I, Rosenberg E (2006) The coral probiotic hypothesis. Environ Microbiol 8: 2068-2073.
37. Bäckhed F, Ley RE, Sonnenburg JL, Peterson D a, Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307: 1915–1920.
38. Pita L, Turon X, López-Legentil S, Erwin PM (2013) Host rules: Spatial stability of bacterial communities associated with marine sponges (*Ircinia* spp.) in the Western Mediterranean Sea. FEMS Microbiol Ecol DOI:10.1111/1574-6941.12159 (In press).
39. White JR, Patel J, Ottesen A, Arce G, Blackwelder P, et al. (2012) Pyrosequencing of bacterial symbionts within *Axinella corrugata* sponges: diversity and seasonal variability. PLoS One 7(6): e38204.