RESEARCH ARTICLE

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

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Received 28 March 2013; revised 26 May 2013; accepted 4 June 2013.
Final version published online 9 July 2013.

DOI: 10.1111/1574-6941.12159

Editor: Gary King

Keywords
Porifera; biogeography; host specificity; spatial variation; symbiosis; isolation-by-distance; distance–decay.

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).

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.

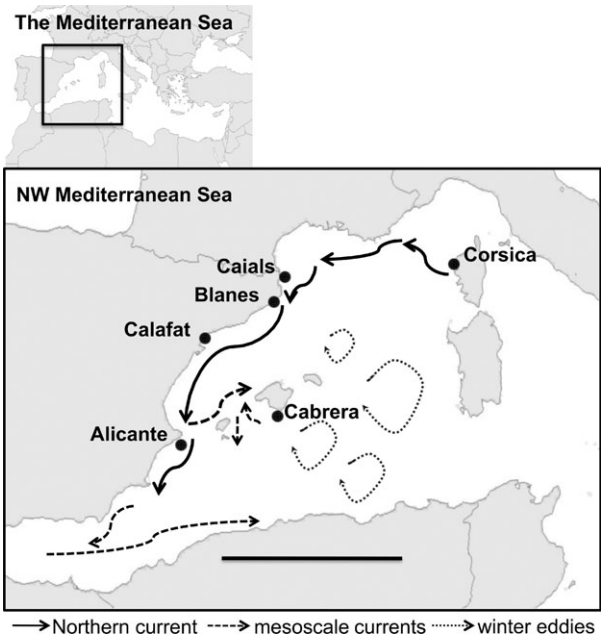

Fig. 1. Sampling sites in the western Mediterranean Sea. Sampling sites and main currents in the region (adapted from Millot, 1999) are shown. Scale bar = 422 km.

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).

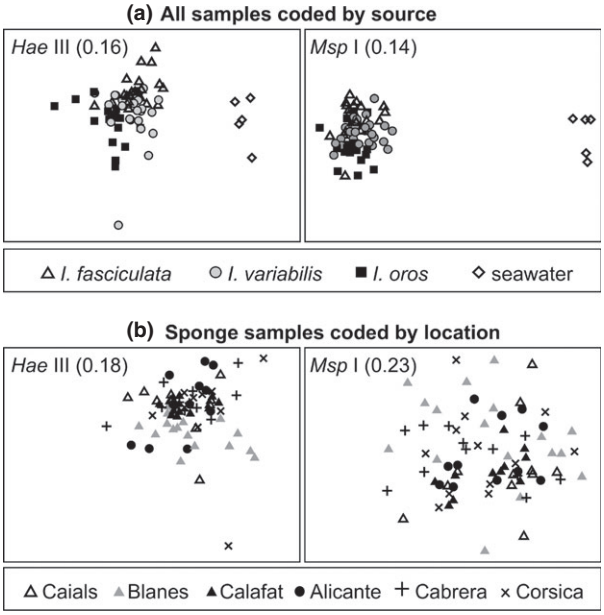

Fig. 2. Spatial patterns of bacterial communities in marine sponges and seawater. nMDS plots of bacterial T-RFLP profiles obtained from HaeIII (left) and MspI (right) digestions. (a) All samples coded by source; (b) sponge samples coded by location. Stress values are shown in parentheses.

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)

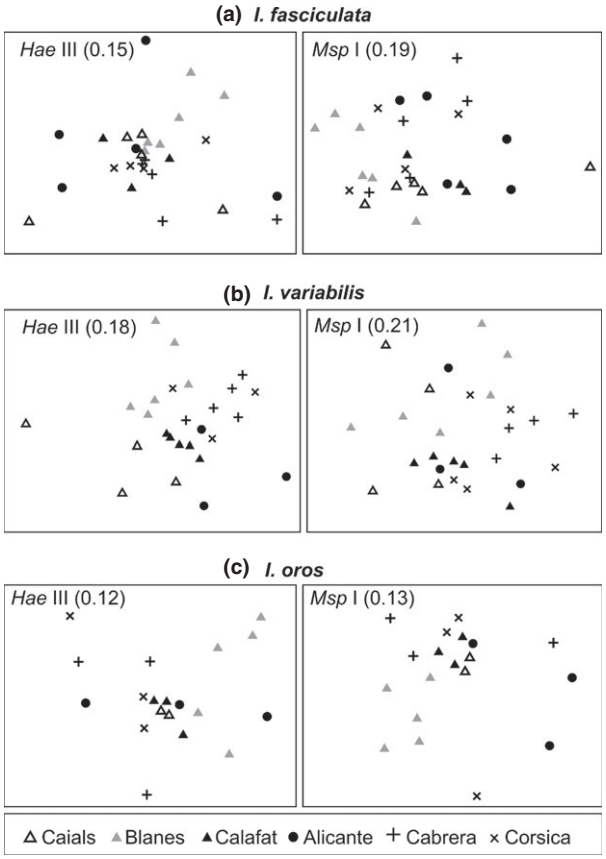

Fig. 3. Spatial patterns of bacterial communities in three *Ircinia* sponge species. nMDS plots of bacterial T-RFLP profiles obtained from HaeIII (left) and MspI (right) digestions. (a) *Ircinia fasciculata*-derived samples; (b) *Ircinia variabilis*-derived samples; (c) *Ircinia oros*-derived samples.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

Acknowledgements

We are grateful to E. Cebrian (CEAB-CSIC) for supplying samples from Cabrera National Park and Scandola Nature Reserve (Corsica). We thank A. Pujals (Universitat Autònoma de Barcelona) for his help with some of the laboratory work. This research was funded by the Spanish Government projects CTM2010-17755 and CTM2010-22218, the Catalan Government grant 2009SGR-484 for Consolidated Research Groups, the US National Science Foundation under grant 0853089, and a FI-DGR fellowship to L.P.

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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.