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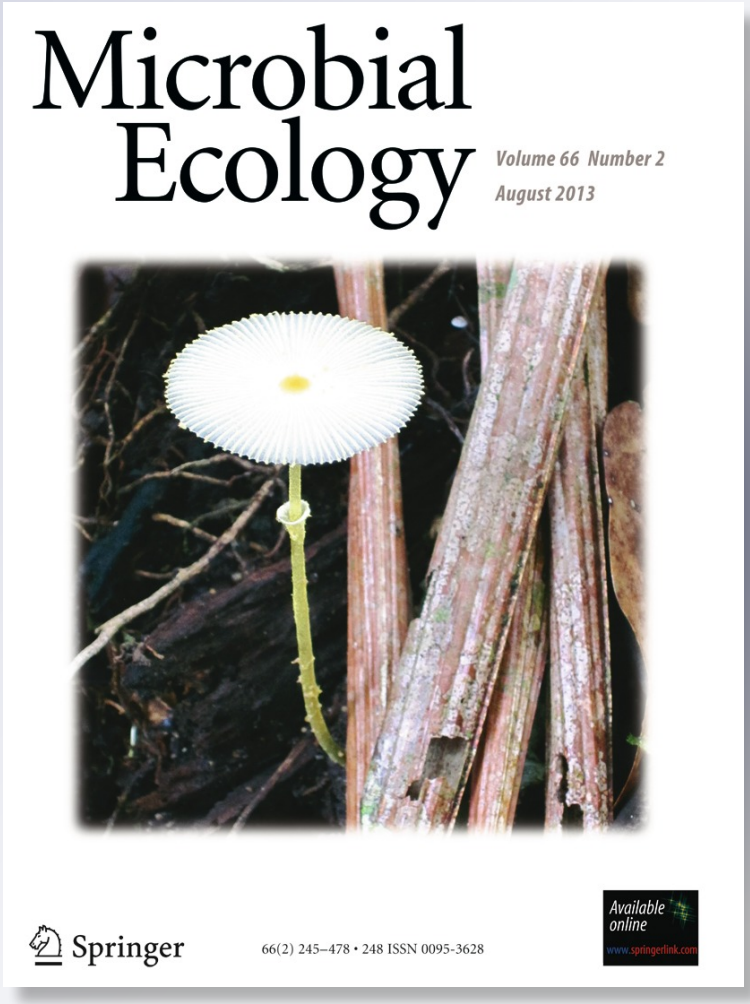

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INVERTEBRATE MICROBIOLOGY

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-

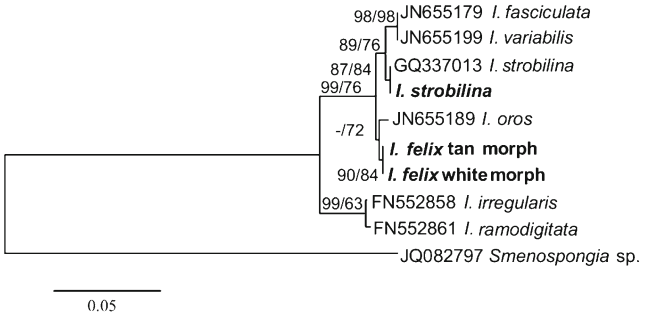

Fig. 1 Phylogenetic analysis of host sponges based on a fragment of the mitochondrial gene cytochrome oxidase I. Tree topology was obtained by neighbor joining and numbers on nodes indicate bootstrap values (>50 %) for neighbor joining (*left*) and maximum likelihood (*right*) analysis. Terminal node 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

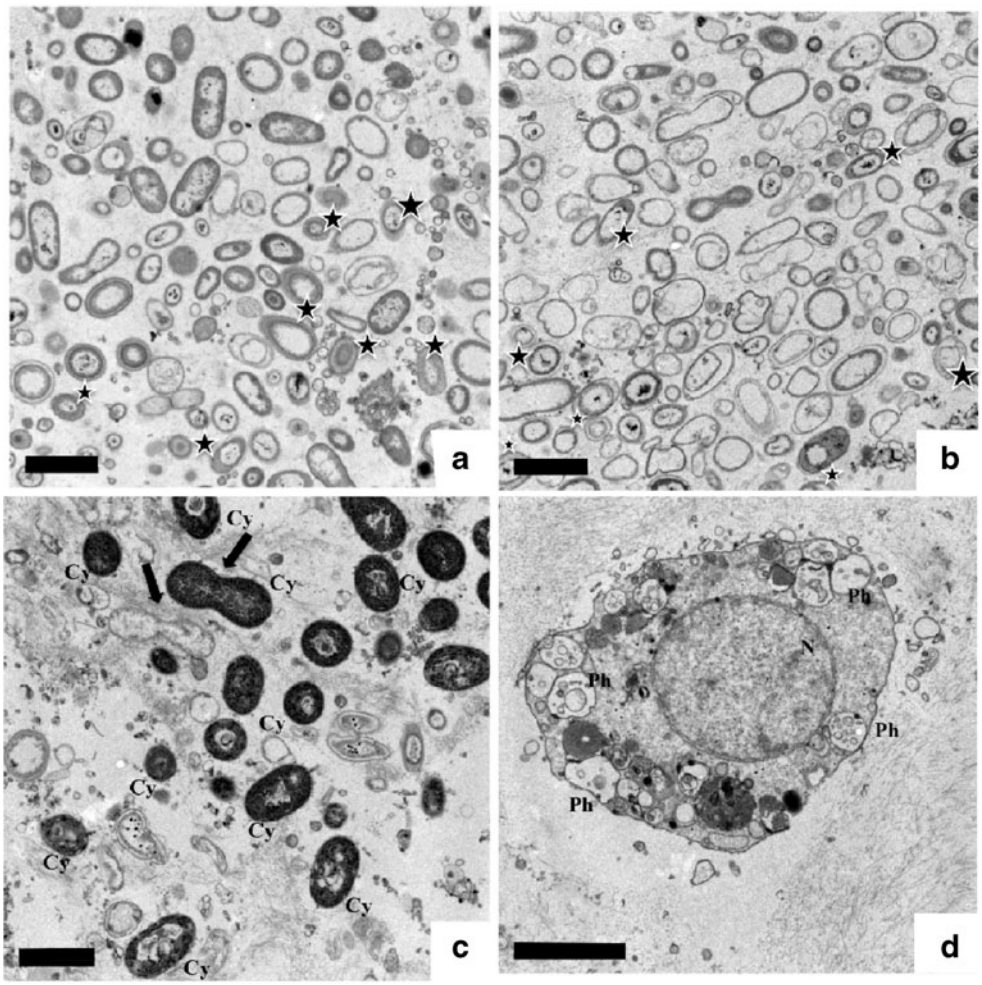

Fig. 2 Representative electron micrographs of sponge holobionts. Bacterial diversity in the mesohyl of **a** *I. strobilina* and **b** *I. felix* tan morph, including morphotypes containing a nucleoid-like structure within the cell (*black stars*). **c** The cyanobacterium *Candidatus Synechococcus spongiarum* (*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

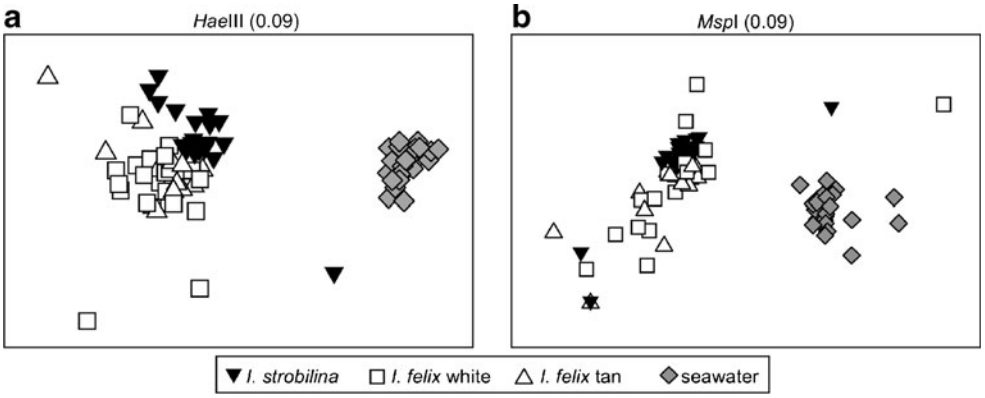

Fig. 3 nMDS plots of bacterial community structure in sponge hosts (*I. strobilina* and two color morphs of *I. felix*) and surrounding seawater samples. nMDS ordination based on Bray–Curtis similarity of T-RFLP profiles using the restriction enzymes **a** *Hae*III and **b** *Msp*I. Stress values are 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.

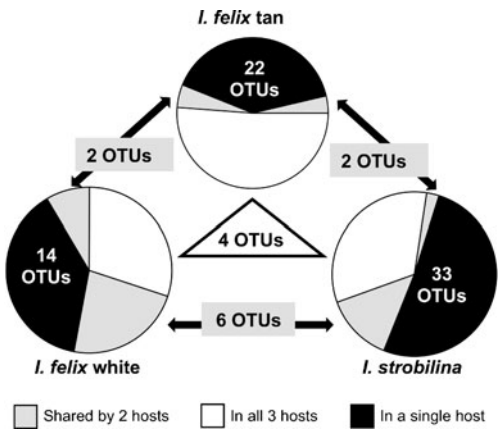

Fig. 4 Host specificity of the bacterial communities in *I. strobilina* and two color morphs of *I. felix* based on 16S rRNA gene sequences obtained after clone library construction. Pie charts show the percentages of clones for each symbiont category. Numbers denote the total OTUs (99 % sequence identity)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

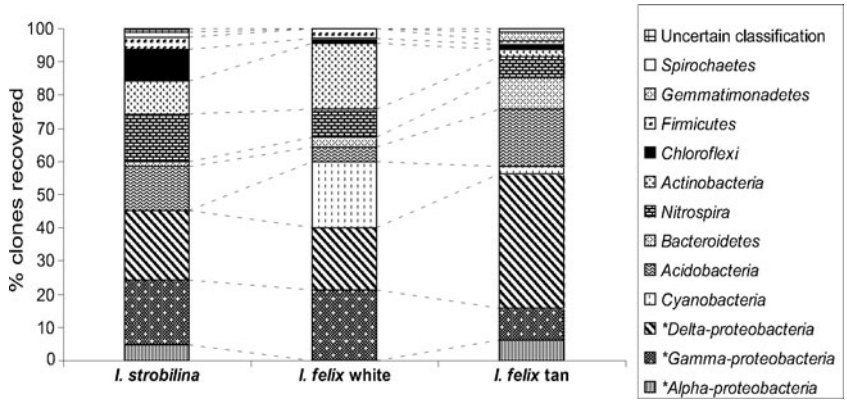

Fig. 5 Phylogenetic affiliation of symbiont OTUs (99 % sequence similarity) in *I. strobilina* and two color morphs of *I. felix* (tan and white). Bacteria are classified according to phylum or class (marked with an *asterisk*)

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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