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Latitudinal variation in the microbiome of the sponge *Ircinia campana* correlates with host haplotype but not anti-predatory chemical defense

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ABSTRACT: Many marine sponges host diverse symbiotic microbial communities that have been implicated in the production of secondary metabolites. These metabolites may defend the host sponge from potential predators. Variability in symbiont communities across the range of the host sponge could alter levels of chemical defense. To investigate the relationship between symbiont composition and chemical defense, the microbiomes and palatability of tissue samples from *Ircinia campana* were characterized from 5 sites along a latitudinal gradient spanning temperate (South Atlantic Bight, SAB) and tropical (Caribbean) regions. Terminal restriction fragment length polymorphism analysis and Illumina sequencing of 16S ribosomal RNA genes revealed that *I. campana* from different locations contained significantly distinct microbiomes and exhibited a consistent relationship of lower symbiont similarity over greater geographic distance (i.e. distance-decay). However, crude organic extracts of all samples of *I. campana* were unpalatable to assay fish *Thalassoma bifasciatum* in laboratory assays, indicating no difference in chemical defense across locations. Distinct haplotypes of *I. campana* were detected in populations from the SAB and Caribbean, correlating with the observed patterns of latitudinal variation in microbial symbiont communities. Our findings indicate that *I. campana* is chemically defended from fish predators across the range of the species and that latitudinal variation occurs in the microbiome of *I. campana*, driven by a combination of host-specific factors and region-specific environmental filtering of symbiont communities.

KEY WORDS: Symbiosis · Microbial community · Chemical defense · Biogeography · Porifera · Intraspecific variation

INTRODUCTION

Many animals and plants host endosymbiotic microbial communities that are distinct from those found in the environment (Gaiero et al. 2013, McFall-Ngai et al. 2013). Although some of these microbes are parasitic, many endosymbionts form beneficial associations that increase host fitness through enhanced nutrient acquisition or decreased susceptibility to pathogens and predators (Moran 2007, Bennett & Moran 2015). In some systems, the benefits that specific endosymbionts provide are well understood, but these hosts usually contain low-diversity microbiomes (McFall-Ngai 2008, Smith & Smith 2011, Shigenobu & Wilson 2011, Davy et al. 2012). It has been more difficult to elucidate the benefits of individual microbial taxa that coexist in symbiotic communities with hundreds or thousands

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of taxa (Hongoh 2011, Robles Alonso & Guarner 2013). Examples of such complex symbiotic systems include the microbiomes of sponges, which can reach densities of 10^8 to 10^{10} microorganisms per gram of sponge tissue and comprise up to 35% of sponge biomass (Hentschel et al. 2006). Recent work has begun to examine sponge microbiomes and the factors that influence their structure and variability (Taylor et al. 2007, Freeman & Thacker 2011, Hentschel et al. 2012, Thacker & Freeman 2012, Webster & Taylor 2012).

Sponge-associated microbial communities are generally distinct from those in adjacent seawater and are largely host species-specific (Thomas et al. 2016), with symbiont communities varying significantly between sponge species but with individuals of the same species harboring similar microbiomes (Lee et al. 2011, Schmitt et al. 2012, Webster et al. 2013, Easson & Thacker 2014, Reveillaud et al. 2014). This high degree of host specificity and symbiont stability is reported across broad longitudinal and bathymetric ranges (Erwin et al. 2012a, Olson & Gao 2013, Pita et al. 2013b,c, Cárdenas et al. 2014, Reveillaud et al. 2014), temporal scales (Thiel et al. 2007, Erwin et al. 2012b, 2015, Simister et al. 2013, Hardoim & Costa 2014) and in response to exposure to sub-lethal temperatures, elevated nutrient levels, and food shortages (Webster et al. 2008, 2011, Simister et al. 2012a,b, Pita et al. 2013a). Notable exceptions to these general trends in sponge microbiology also exist, where significant intraspecific variation in symbiont microbial communities has been detected across different habitats (Cleary et al. 2013, Weigel & Erwin 2016) and latitudes (Taylor et al. 2005, Anderson et al. 2010), thus suggesting that host species is not the only factor that determines microbiome composition. Temperature and other ecosystem attributes vary strongly with latitude and may explain why differences in microbial communities were detected when sampling occurred across large latitudinal gradients and not across large longitudinal ranges.

Marine sponges have long been recognized as prolific producers of bioactive secondary metabolites, many with pharmaceutical applications (Erwin et al. 2010). More recently, the potential and realized contributions of symbiotic microorganisms to host defensive chemistry has suggested a link between the microbial ecology and chemical ecology of marine sponges. Investigations of secondary metabolite production in sponges have found evidence that symbiotic microbes are involved in the production of specific bioactive compounds originally attributed to host sponges, including chemical analyses of host and symbiont cell partitions and the characterization and localization of biosynthetic gene clusters (Bewley & Faulkner 1998, Piel 2009, Hentschel et al. 2012, Flórez et al. 2015, Freeman et al. 2016). Other studies have targeted broader linkages between symbiont community composition and sponge chemistry, for example, documenting co-variation between microbial communities and metabolite profiles of the sponge *Mycale hentscheli* (Anderson et al. 2010). Given the importance of chemical defenses in structuring Caribbean sponge communities (Pawlik 2011, Loh & Pawlik 2014), understanding the relationship between symbiont structure and host palatability has broad ecological importance. Further, sponge-associated microorganisms can contribute to the nutritional ecology of their hosts (Freeman et al. 2013), which may indirectly affect sponge chemical ecology by relaxing energy investment trade-offs between defensive metabolite production, growth and reproduction (Leong & Pawlik 2010).

The objectives of this study were to determine intraspecific variation in the microbial community and chemical defense of *Ircinia campana* across a broad (>2500 km) geographic range spanning temperate and tropical latitudes. *I. campana* was chosen for this study because (1) it exhibits a large latitudinal range (extending from the tropical southern Caribbean to the temperate South Atlantic Bight, SAB); (2) the chemical defenses of this species are well characterized (Pawlik et al. 1995, 2002); (3) the level of chemical defense is previously reported to vary across this range (Ruzicka & Gleason 2009); and (4) members of the genus *Ircinia* are known to host abundant and diverse microbiomes (Erwin et al. 2012a, Pita et al. 2013b,c). Past work found that a mixture of furanosesterterpene tetronic acids protects *I. campana* from predation; however, it is unknown whether these chemicals are produced by the sponge or endosymbiotic microbes (Pawlik et al. 2002, Esteves et al. 2013). Tissue samples from specimens of *I. campana* from 5 locations in the Caribbean and SAB were collected and used to assess differences in chemical defense against fish predators using a standard laboratory palatability assay, and the microbial communities were assessed using terminal restriction fragment length polymorphism (T-RFLP) analyses and Illumina sequencing of partial 16S rRNA gene sequences. The taxonomic identity and haplotype diversity of sponge hosts were determined by sequencing a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (COI).

MATERIALS AND METHODS

Sample collection and DNA extraction

Sampling was conducted between May and September 2014 at 5 locations within the SAB (Wilmington, North Carolina and Gray's Reef, Georgia, USA) and Caribbean (Key Largo, Florida, USA; Carrie Bow Cay, Belize; Bocas del Toro, Panama) (Fig. 1). Tissue samples were non-fatally collected from individual *Ircinia campana* by cutting a notch in the lip of this vase-shaped sponge using a disposable scalpel. At each site, tissue samples from 10 sponges and 3 samples of ambient seawater (500 ml) were collected using SCUBA at depths ranging from 10 to 20 m. Owing to logistical constraints, no seawater was collected in Belize and only 2 seawater samples were collected in Panama. All sampled sponges were free of epibionts and visible signs of disease. For molecular analyses, a 1 ml piece of tissue containing external pinacoderm and mesohyl was collected from each sponge sample, preserved in 100% ethanol and stored at –20°C. For chemical analyses, the remaining sponge tissue was frozen wet and stored at –20°C. Seawater samples were concentrated on 0.2 µm filters, preserved in 100% ethanol and stored at –20°C. Each ethanol-preserved tissue sample and concentrated seawater sample was separately extracted using the DNeasy Blood & Tissue Kit (Qiagen). Full strength and 1:10 diluted DNA extracts were used as templates in subsequent PCR amplifications.

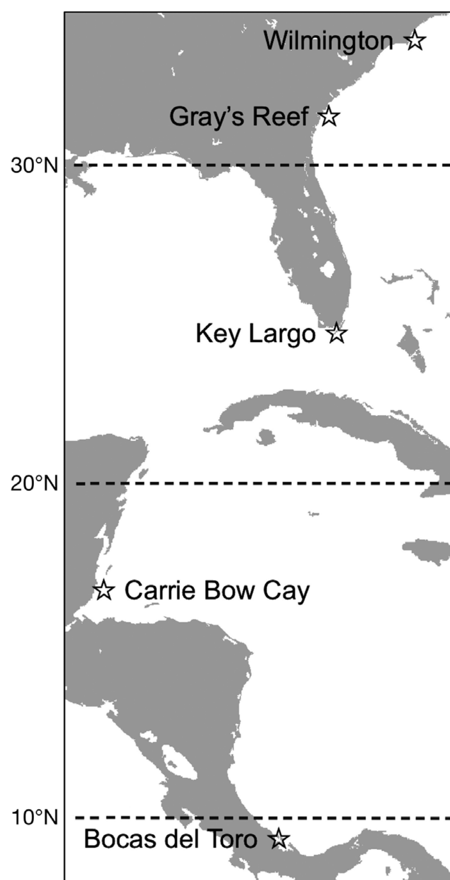

Fig. 1. Collection sites (☆) within the South Atlantic Bight and the Caribbean: Wilmington, North Carolina (34° 7.926′ N, 77° 21.636′ W); Gray's Reef, Georgia (31° 23.790′ N, 80° 53.418′ W); Key Largo, Florida (24° 56.657′ N, 80° 29.768′ W); Carrie Bow Cay, Belize (16° 48.173′ N, 88° 4.928′ W); and Bocas del Toro, Panama (9° 21.639′ N, 82° 16.680′ W)

Molecular identification of host sponges

From each location, 3 sponge samples were haphazardly selected for molecular identification (Belize: C3, C6, C7; Panama: P1, P3, P6; Florida: L2, L4, L5; Georgia: G1, G7, G8; North Carolina: W4, W8, W9). A degenerated version of the universal barcoding forward primer dgLCO1490 (Meyer et al. 2005) (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′) were used for PCR amplification of a fragment (ca. 1000 bp) of the COI gene, corresponding to the standard barcoding ('Folmer') partition (Folmer et al. 1994, Hebert et al. 2003) and the I3-M11 partition (Erpenbeck et al. 2006). Amplification was performed with 1 µl of each primer (10 µM), 12 µl (0.5 units) of MyTaq™ HS Red Mix DNA polymerase (Bioline), 1 to 2 µl of DNA template, and PCR water to a total reaction volume of 25 µl. Thermocycler conditions were an initial denaturating step at 94°C for 5 min, followed by 32 cycles of 94°C for 30 s, 42°C for 30 s and 72°C for 1.5 min, and a final extension at 72°C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and used as templates for sequencing reactions with BigDye terminator v3.1 and the same primer set used during initial amplification step, then analyzed on a 3130xl Genetic Analyzer (Applied Biosystems) at the University of North Carolina Wilmington Center for Marine Science. Consensus sequences were constructed from forward and reverse sequence reads aligned using Geneious v8 (Kearse et al. 2012) and final sequences were deposited in GenBank (accession numbers KR819143 to KR819157). A final alignment including sequences from the congeners *I. fasciculata*, *I. oros* and *I. variabilis* from Spain (Erwin et al. 2012a) and for *I. strobilina* and *I.*

felix from the Bahamas (Pita et al. 2013b) was built using ClustalW (Larkin et al. 2007) with a gap opening penalty of 24 and a gap extension penalty of 4 (Erwin & Thacker 2008). According to the Bayesian information criterion, the best model of nucleotide substitution for maximum likelihood analysis was the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985). A maximum likelihood tree was built using the HKY model, the nearest-neighbor-interchange heuristic method, and 1000 bootstrap replicates (Felsenstein 1985) with Mega v6.06 (Tamura et al. 2013).

Characterization of microbial symbionts by 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-carboxyfluorescein, and the reverse primer 1509R (Martínez-Murcia et al. 1995) (5′-GGT TAC CTT GTT ACG ACT T-3′), were used to amplify a ca. 1500 bp fragment of the 16S rRNA gene from sponge (7 to 10 samples per location) and seawater (2 to 3 samples per location) extracts, using the same reaction mixture detailed above for sponge COI genes. Thermocycler conditions were an initial denaturating step at 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 50°C for 15 s, 72°C for 20 s, and a final extension at 72°C for 5 min. PCR reactions for each sponge and seawater sample were run in triplicate and the products were combined and gel-purified using the QIAquick Gel Extraction Kit (QIAGEN). DNA concentrations were quantified on a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and 100 ng of purified PCR product was digested separately with the restriction enzymes *Hae*III and *Msp*I (FastDigest, Thermo Scientific) and ethanol-precipitated to remove excess salts. Samples were eluted in 10 µl formamide and 0.5 µl GeneScan 500 ROX size standard (Life Technologies), heated at 96°C for 1 min, immediately cooled on ice for 2 min, and analyzed by capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems).

The lengths of terminal restriction fragments (T-RFs) were determined by comparison with internal size standards using the program PeakScanner (Applied Biosystems). T-RFs below 50 relative units of fluorescence (background noise), smaller than 50 bp or larger than 500 bp (beyond size standard resolution) were excluded. Additional filtering and alignment of the T-RFLP peak profiles was carried out in the program T-REX (Culman et al. 2009) and relative abundance matrices were calculated. Data were de-noised by applying a cutoff value of 1.5 SD (Abdo et al. 2006). T-RFs were aligned using a clustering threshold of 1 bp then standardized by relative peak area.

Statistical analyses of T-RFLP profiles were performed in PRIMER v6 (Clarke 1993, Clarke & Gorley 2006) and PERMANOVA+ (Plymouth Marine Laboratory). Square root transformations were applied to relative abundance matrices and a Bray-Curtis similarity matrix was constructed for each restriction enzyme dataset. Non-metric multidimensional scaling (NMDS) plots were generated to visualize bacterial community similarity. Permutational multivariate analyses of variance (PERMANOVAs) (Anderson 2001, McArdle & Anderson 2001) were used to test for variability between sources (seawater and sponges) and among locations (sponges only), with significance determined by permutational p-values or Monte-Carlo asymptotic p-values in comparisons with few permutations. Pairwise comparisons of dispersion (PERMDISPs) were performed to determine the effect of heterogeneity for significant PERMANOVA outcomes. Critical values for significance for all pairwise comparisons were corrected using the Benjamini-Yekutieli (B-Y) false discovery rate (Benjamini & Yekutieli 2001).

Characterization of microbial symbionts by Illumina sequencing

DNA extracts from sponge (3 to 4 replicates per location) and seawater (2 to 3 replicates per location) samples were sent to Molecular Research LP (Shallowater, Texas) for amplification, library construction and multiplexed, paired-end sequencing of partial (V4 region) 16S rRNA gene sequences using the bacterial and archaeal specific primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CVS GGG TAT CTA AT-3′) (Bates et al. 2011). Raw sequences were deposited into the sequence read archive of GenBank (accession number SRP058983).

Raw sequences were processed using mothur v1.33.3 (Schloss et al. 2009). Low-quality sequences were removed using the following parameters: no ambiguous bp, no bp differences from barcode, differences from primer ≤2, a maximum of 8 homopolymers, a minimum length of 200 bp, and a maximum length of 300 bp. Unique sequences were then aligned to a trimmed (V4 region) SILVA v119.4 database (Yilmaz et al. 2014). Poorly aligned sequences were removed and the remaining sequences were trimmed to an overlapping alignment space. Sequences were pre-clustered (differences = 2; Huse et al.

2010) and chimeras were identified and removed using UCHIME (Edgar et al. 2011), as implemented in mothur. Sequences were taxonomically classified using the Greengenes v13.5.99 database (DeSantis et al. 2006) and non-target sequences were removed. Sequences were clustered into operational taxonomic units (OTUs) according to 97% sequence identity (average neighbor algorithm). A shared table was created with the abundance of each OTU in each sponge or seawater sample. The data were subsampled to the lowest read count (n = 9226) to standardize sequencing depth across samples.

Univariate and multivariate statistical analyses were conducted to compare the diversity and similarity of microbial communities. Univariate metrics included observed OTU richness (number of 97% OTUs), expected OTU richness (Chao1 and abundance-based coverage estimator [ACE] indices; Chao 1984, Chao & Lee 1992), and OTU diversity calculations (Simpson's inverse index and Shannon index; Shannon 1948, Simpson 1949). Multivariate statistical analyses were performed in PRIMER v6 and PERMANOVA+, as described above. In addition, similarity percentage (SIMPER) analyses were conducted to identify the individual OTUs driving community-level differentiation between groups.

Testing for isolation-by-distance effects

To determine whether differences in symbiont community similarity were correlated with geographic distances among host sponge populations, Mantel tests were conducted in R v3.1.2 (R Core Team 2014) using the package ade4 (Dray & Dufour 2007). Three separate Mantel tests were run using Bray-Curtis similarity values derived from T-RFLP profiles (*Hae*III and *Msp*I datasets) and Illumina sequence data to test for isolation-by-distance effects (i.e. distance-decay relationships).

Metabolite extraction and laboratory feeding assays

Extraction of secondary metabolites from sponge tissue and subsequent laboratory feeding assays were performed following Marty & Pawlik (2015). Briefly, metabolites were extracted from wet sponge tissue using a 1:1 solvent mixture of dichloromethane and methanol followed by a second tissue extraction with methanol alone, and the solvents were evaporated to provide the crude organic extract. The crude extract was mixed at the same volumetric concentration as it occurred in the sponge tissue with a squid mantle-based food paste containing alginic acid and food coloring to standardize paste color. The paste was extruded from a syringe into a calcium chloride solution, which hardened the paste into a noodle-like strand from which extract-treated pellets could be cut with a scalpel. Control pellets were made in the same way, but without the addition of crude extract.

Feeding deterrence was determined using a well-established laboratory feeding assay with the generalist predatory bluehead wrasse *Thalassoma bifasciatum* (Marty & Pawlik 2015), which produces results consistent with field assays of sponge palatabilty against a natural suite of reef fishes (Chanas et al. 1997), including sponges in the genus *Ircinia* (Pawlik et al. 2002). Control and treated pellets were presented to 10 groups of 3 yellow-phase bluehead wrasses *T. bifasciatum*, with each group held in a separate aquarium cell. Feeding results were evaluated using a modified version of Fisher's exact test, with sponge tissue samples considered deterrent if 6 or fewer pellets were eaten ($p \leq 0.057$; Marty & Pawlik 2015).

RESULTS

Host sponge haplotypes

Partial COI gene sequences (973 bp) were recovered from tissue samples of 15 individuals of *Ircinia campana* and revealed 2 haplotypes of *I. campana* distinguished by a single mutation (pyrimidine transition) in the I3-M11 region. The haplotypes exhibited a biogeographic distribution, with *I. campana* from SAB locations (North Carolina, Georgia) exclusively showing Haplotype 1 and those from Caribbean locations (Belize, Panama) exclusively showing Haplotype 2. Both haplotypes were detected in Florida (1 sample presented Haplotype 1, 2 samples presented Haplotype 2). Phylogenetic analysis grouped all samples of *I. campana* together in a modestly supported monophyletic clade (65% bootstrap support) separated from Mediterranean and Bahamian species of *Ircinia* (Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/m565p053_supp.pdf).

T-RFLP analysis

The *Hae*III digest recovered 114 unique T-RFs across all samples: seawater yielded 76, while *I.*

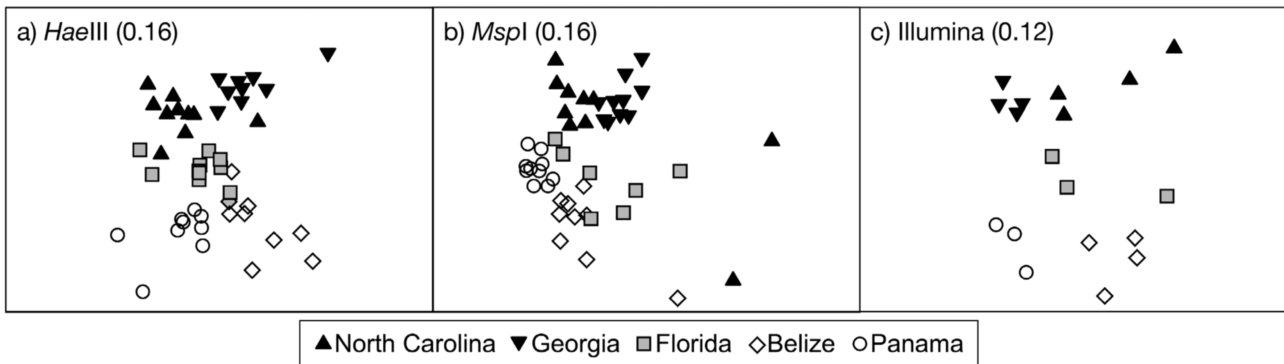

Fig. 2. NMDS plots of microbial community structure in *Ircinia campana* from different collection sites based on Bray-Curtis similarity of (a) T-RFLP profiles using *Hae*III, (b) T-RFLP profiles using *Msp*I and (c) OTU profiles using Illumina sequences. Stress values are shown in parentheses

campana from North Carolina, Georgia, Florida, Belize, and Panama yielded 73, 66, 71, 61, and 65, respectively. The *Msp*I digest yielded 116 unique T-RFs: seawater yielded 72, while *I. campana* from North Carolina, Georgia, Florida, Belize, and Panama yielded 59, 57, 70, 50, and 74, respectively. In NMDS plots for both digests, seawater and sponge-associated bacterial communities were clearly differentiated (Fig. S2 in Supplement 1) with sponge-associated bacterial communities generally clustered by location (Fig. 2). PERMANOVA results revealed significant effects of source (*Hae*III: F = 10.038, p < 0.001; *Msp*I: F = 6.7879, p < 0.002) and location nested within source (*Hae*III: F = 7.3365, p < 0.001; *Msp*I: F = 73.133, p < 0.001) based on microbial community structure. Further, all pairwise comparisons across locations for sponge-associated microbial communities were significant (p < 0.002; Table 1). PERMDISP results reported significant differences in the homogeneity of dispersion between seawater and sponge-associated microbial communities from different locations, except between *I. campana* from North Carolina and seawater in the *Msp*I digest. No significant PERMDISP results were consistently found (i.e. in both enzyme datasets) among sponge-associated microbial communities from different locations, although pairwise comparisons between Georgia and Florida and between Florida and Panama were significant for 1 enzyme dataset (*Msp*I; Table 1).

Table 1. Permutational statistical analysis of T-RFLP data (*Hae*III and *Msp*I digests) for microbial community structure (PERMANOVA) and homogeneity of dispersion (PERMDISP) among sponge tissue samples and seawater samples. Main tests of source (sponge tissue, seawater) and location (collection site) are shown, along with pairwise comparisons among samples of *Ircinia campana* from different locations and seawater samples. **Bold**: significant comparisons following B-Y correction with asterisks denoting significance level (*α = 0.05, **α = 0.01)

Pairwise comparison	PERMANOVA (*Hae*III)		PERMANOVA (*Msp*I)		PERMDISP (*Hae*III)		PERMDISP (*Msp*I)	
	t	p-value	*t*	p-value	*t*	p-value	*t*	p-value
Belize–Georgia	3.8933	**0.001****	4.4639	**0.001****	0.3912	0.757	1.8491	0.091
Belize–Florida	2.8041	**0.001****	2.212	**0.001****	0.9498	0.365	0.9218	0.494
Belize–Panama	2.6786	**0.001****	3.322	**0.001****	0.3562	0.776	2.1272	0.032
Belize–North Carolina	3.6819	**0.001****	2.9231	**0.001****	0.3936	0.718	0.8811	0.508
Georgia–Florida	3.4956	**0.001****	3.4093	**0.002****	0.3919	0.779	3.9254	**0.005***
Georgia–Panama	4.1855	**0.001****	5.2375	**0.001****	0.0148	0.99	0.2897	0.816
Georgia–North Carolina	2.5523	**0.001****	2.5504	**0.001****	0.8014	0.534	2.0779	0.069
Florida–Panama	3.0235	**0.001****	2.8882	**0.001****	0.3868	0.761	4.8230	**0.001****
Florida–North Carolina	2.5612	**0.001****	2.0928	**0.001****	1.6213	0.129	0.2929	0.784
Panama–North Carolina	3.4297	**0.001****	3.1791	**0.001****	0.7381	0.539	2.2020	0.023
Belize–Seawater	5.3204	**0.001****	4.4762	**0.001****	6.7057	**0.001****	4.9844	**0.001****
Georgia–Seawater	5.2698	**0.001****	5.6203	**0.001****	6.5974	**0.001****	8.6570	**0.001****
Florida–Seawater	5.3776	**0.001****	3.7855	**0.001****	8.6670	**0.001****	4.5162	**0.003****
Panama–Seawater	5.4425	**0.001****	4.8767	**0.001****	6.3081	**0.001****	9.4360	**0.001****
North Carolina–Seawater	5.0850	**0.001****	4.3398	**0.001****	7.2846	**0.001****	2.4188	0.048

Table 2. Richness and diversity of microbial communities in tissue samples of *Ircinia campana* and samples of ambient seawater (mean ± SE). Comparisons among all sponge tissue and seawater samples (all) and among samples of *I. campana* from different locations are shown. *Significant ($p < 0.05$) differences between sponge and seawater communities; no significant differences were detected among populations of *I. campana*. S_{obs} = average observed OTU richness; S_{Chao1} = Chao1 expected OTU richness; S_{ACE} = ACE expected OTU richness; H = Shannon diversity index; $1/D$ = inverse Simpson index

Source	S_{obs}	S_{Chao1}	S_{ACE}	H	$1/D$
Seawater (all)	710 ± 26*	2441 ± 127	4583 ± 239	4.15 ± 0.05*	21.5 ± 1.2*
I. campana (all)	615 ± 8*	2326 ± 73	4155 ± 139	4.53 ± 0.02*	44.9 ± 1.5*
I. campana					
–North Carolina	604 ± 21	2197 ± 177	4131 ± 397	4.46 ± 0.06	43.5 ± 3.5
–Georgia	598 ± 14	2367 ± 174	4203 ± 353	4.52 ± 0.04	42.6 ± 1.8
–Florida	608 ± 5	2194 ± 142	4352 ± 402	4.55 ± 0.03	47.6 ± 2.7
–Belize	634 ± 20	2399 ± 170	4228 ± 294	4.50 ± 0.06	42.8 ± 4.7
–Panama	633 ± 19	2481 ± 178	3830 ± 104	4.65 ± 0.03	49.6 ± 3.6

Illumina sequence analysis

A total of 9055 microbial OTUs were recovered, with samples of *I. campana* containing 5480 OTUs from 33 bacterial and archaeal phyla, and seawater samples containing 4363 OTUs from 34 bacterial and archaeal phyla. The seawater microbial community was dominated by *Proteobacteria* (68.2%), which in turn was dominated by *Alphaproteobacteria* and *Gammaproteobacteria* (33.6% and 31% of the total reads, respectively). Other abundant phyla included *Chloroflexi* (6%), *Bacteroidetes* (4.2%), *Acidobacteria* (3%), the bacterial group PAUC34f (3%), *Euryarchaeota* (2.5%), *Cyanobacteria* (2.4%), *Gemmatimonadetes* (1.9%), *Nitrospirae* (1.4%), *Verrucomicrobia* (1.4%), the bacterial group SAR406 (1.4%), and *Poribacteria* (1.1%). Each of the remaining 22 phyla contributed <1% of the total reads for the seawater microbial community. Microbial communities in *I. campana* were dominated by *Proteobacteria* (51.5%), namely the classes *Gammaproteobacteria* and *Alphaproteobacteria* (27.4% and 17.6% of the total reads, respectively). Other abundant taxa included *Chloroflexi* (13.9%), *Actinobacteria* (9.3%), *Bacteroidetes* (5.7%), *Cyanobacteria* (4.6%), the bacterial group SBR1093 (3.5%), *Thaumarcheota* (3%), *Acidobacteria* (2.4%), and the microbial group SAR406 (1.5%). Each of the remaining 26 phyla contributed <1% of the total reads. See Table S1 in Supplement 2 at www.int-res.com/articles/suppl/m565p053_supp.xls for the microbial community composition of each sample.

The microbial communities in *I. campana* exhibited lower richness and greater diversity compared to ambient seawater communities, with *I. campana* from different locations showing similar levels of OTU diversity (Table 2). Observed OTU richness was significantly greater ($p < 0.001$) in seawater compared to *I. campana*, while expected OTU richness (ACE, Chao1) was not significantly different ($p > 0.11$) between seawater and sponge-associated communities. Both diversity indices (Shannon and inverse Simpson) were significantly higher ($p < 0.001$) in *I. campana* compared to seawater communities. Comparisons of microbial community richness and diversity among the different populations of *I. campana* revealed no significant differences ($p > 0.15$), indicating similar levels of symbiont diversity across the investigated biogeographic range of *I. campana*.

A comparison of OTU profiles revealed that few microbial OTUs (8.7%, n = 788) were shared between *I. campana* and seawater samples (Fig. S3 in Supplement 1). Notably, the number of shared OTUs was extremely low (0.01%, n = 11) when rare OTUs were excluded (<0.1% of total reads), indicating that most shared OTUs have low abundances in one or both sources (Fig. S3 in Supplement 1). A similar comparison among samples of *I. campana* from different locations revealed that, although rare OTUs may be location-specific, many abundant microbes were found in *I. campana* from all collection sites (Fig. 3). A total of 291 symbiont OTUs were present in at least 1 sponge sample from all sites (n = 69 with rare OTUs excluded; Fig. 3) and 119 OTUs were present in all samples of *I. campana* from all sites (i.e. 'core' OTUs; Table S1 in Supplement 2), with these core OTUs accounting for the majority of symbiont sequences (79.2 to 87.0%). Accordingly, NMDS plots revealed clear differences between seawater and sponge-associated microbial communities (Fig. S4 in Supplement 1), and sponge-associated microbial communities generally clustered by location (Fig. 2). PERMANOVA results revealed significant effects of source ($F = 18.835$, $p < 0.002$) and location nested within source ($F = 4.256$, $p < 0.001$) on microbial community structure. Pairwise comparisons revealed that microbial communities of *I. campana* from Georgia were significantly different from all other sponge samples except for those from North Carolina, while the microbial communities of *I. campana* from North Carolina were

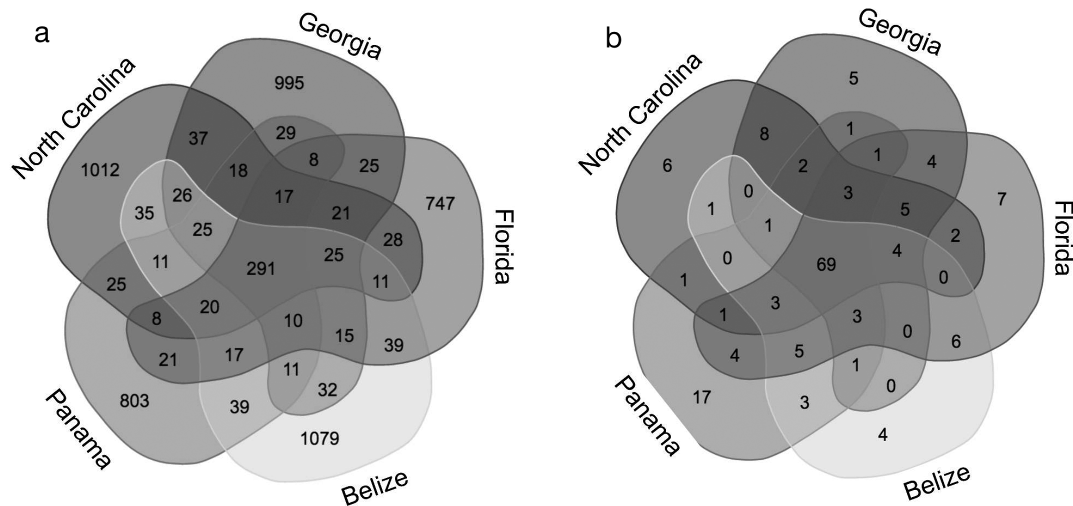

Fig. 3. Venn diagrams comparing the overlap in microbial OTU profiles of *Ircinia campana* from different locations for (a) all symbiont OTUs and (b) abundant symbiont OTUs (≥0.1% of total reads)

significantly different from sponge samples collected in Panama and Belize, but not from samples collected in Florida (Table 3). The microbial communities of *I. campana* from Florida, Belize and Panama were not significantly different from each other (Table 3). PERMDISP results found significant differences in the homogeneity of dispersion between seawater and sponge-associated microbial communities, but not among sponge-associated microbial communities from different locations (Table 3).

SIMPER analyses revealed that differences among microbial communities of *I. campana* samples from different locations were driven by variations in the abundance of multiple symbiont OTUs rather than a single OTU (Table S2 in Supplement 2). A single OTU never contributed more than 6.3% to the total dissimilarity and most contributed less than 3%. Although many of these OTUs maintained relative abundances >1% regardless of collection location, some symbionts were abundant in sponges from one location while existing at very low concentrations in sponges from other sites (Table S2 in Supplement 2). For example, the actinobacterium OTU000015 (family *Acidimicrobiales*) was rare in sponges from Panama (0.16% relative abundance) while common at the remaining sites (1.10 to 4.19%), and the deltaproteobacterium OTU-000053 (family *Syntrophobacterales*) was common in sponges from Belize (2.77%) while rare in sponges from the remaining locations (0.05 to 0.43%; Table S2 in Supplement 2).

Table 3. Permutational statistical analysis of Illumina data for microbial community structure (PERMANOVA) and homogeneity of dispersion (PERMDISP) among sponge tissue samples and seawater samples. Main tests of source (sponge tissue, seawater) and location (collection site) are shown, along with pairwise comparisons among samples of *I. campana* from different locations and seawater samples. **Bold**: significant comparisons following B-Y correction with asterisks denoting significance level (*α = 0.05, **α = 0.01); ^{m}Monte-Carlo asymptotic p-value

Pairwise comparison	PERMANOVA		PERMDISP	
	t	p-value	t	p-value
Belize–Georgia	2.8651	**0.003**^{**m}	5.5179	0.024
Belize–Florida	1.4408	0.097^{m}	0.1241	0.836
Belize–Panama	1.8743	0.035^{m}	3.1695	0.118
Belize–North Carolina	2.0237	**0.009**^{*m}	0.5603	0.554
Georgia–Florida	2.2043	**0.015**^{*m}	7.1253	0.019
Georgia–Panama	3.0662	**0.002**^{**m}	2.1198	0.094
Georgia–North Carolina	1.9746	0.018^{m}	6.8759	0.031
Florida–Panama	1.6217	0.083^{m}	3.9587	0.116
Florida–North Carolina	1.5052	0.092^{m}	0.7740	0.438
Panama-North Carolina	2.2381	0.008^{*m}	4.1331	0.029
Belize–Seawater	4.4062	**0.003****	3.5103	**0.005***
Georgia–Seawater	4.6915	**0.003****	5.6654	**0.004***
Florida–Seawater	3.8975	**0.001**^{**m}	3.1115	**0.015***
Panama–Seawater	3.9866	**0.001****	4.3046	**0.004***
North Carolina–Seawater	4.4309	**0.001****	3.2796	**0.004***

Isolation-by-distance effects

Mantel tests revealed significant correlations between geographic distance and the similarity of sponge-associated microbial communities for all datasets (T-RFLP *Hae*III: r = 0.63, p < 0.001; T-RFLP *Msp*I: r = 0.54, p < 0.001; Illu-

mina: r = 0.68, p < 0.001), indicating a strong distance-decay relationship within the microbiome of *I. campana*.

Laboratory feeding assays

Laboratory feeding assays using the bluehead wrasse *Thalassoma bifasciatum* did not reveal palatability differences between organic extracts of tissue samples of *I. campana* from different locations, and all samples were unpalatable to *T. bifasciatum* (Fig. 4). Mean palatability across locations ranged from 0 to 2 pellets of 10 eaten, well below the threshold considered palatable for this assay (Marty & Pawlik 2015).

DISCUSSION

In this study, variation in the microbial symbiont community and anti-predatory chemical defense of the sponge *Ircinia campana* was investigated along a latitudinal gradient spanning 2500 km from temperate to tropical habitats. Multiple symbiont profiling methods revealed that *I. campana* from different geographic locations hosted significantly different microbial symbiont communities, exhibiting a consistent relationship of lower symbiont similarity across greater geographic distance (i.e. distance-decay). Genetic characterization of host sponges delineated 2 haplotypes whose latitudinal distribution was consistent with variations in the microbiome. No variation in the chemical defense of *I. campana* was detected across the same latitudinal range, with crude extracts of all sponge samples unpalatable to the generalist predator *Thalassoma bifasciatum*. Together, these results revealed latitudinal variation in the microbiome of *I. campana* that correlated with host haplotype while having no effect on host anti-predatory chemical defense.

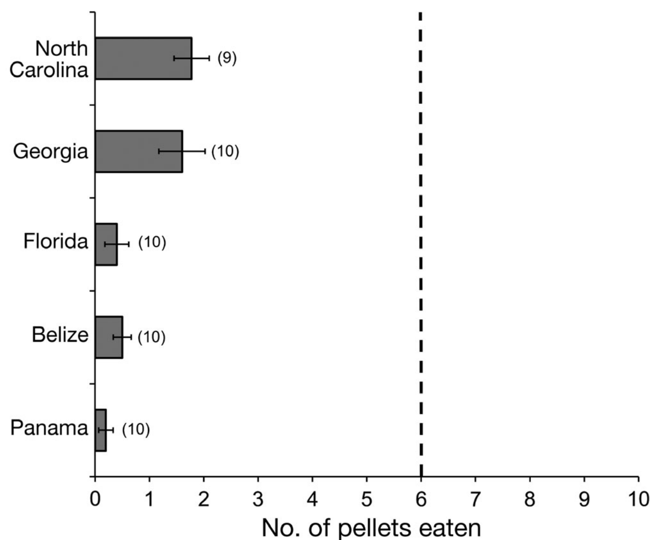

Fig. 4. Consumption by *Thalassoma bifasciatum* of pellets containing volumetric concentrations of crude organic extracts from *Ircinia campana* from different locations. Bars represent the mean number of pellets eaten (±SD) with sample replication shown in parentheses. Extracts were considered deterrent if 6 or fewer pellets (p ≤0.057, Fisher's exact test) were eaten (Marty & Pawlik 2015), indicated by the dashed line

Biogeographic patterns in the microbiomes of *I. campana* were characterized by more similar symbiont communities in temperate SAB regions compared to those from tropical Caribbean sites. Populations of *I. campana* from Florida hosted microbial communities less clearly differentiated from SAB and Caribbean samples, suggesting an influence of structuring factors both from northern and southern regions. The observed variations between microbial communities of *I. campana* were attributed to a complex consortium of symbiont taxa (OTUs). Consistent with previous studies of intraspecific variation in sponge symbionts (Luter et al. 2015, Weigel & Erwin 2016), this consortium included dominant OTUs that were present in all hosts but at different relative abundances, as well as numerous, rare OTUs with regional exclusivity. Notably, most of the microbial taxa that contributed to microbial community variations were absent or extremely rare in free-living seawater communities, indicating that these differences were not strongly influenced by the composition of bacterioplankton in ambient seawater.

Latitudinal variation in sponge-associated microbial communities offers insight into the host-specific and environmental factors that structure host–symbiont relationships. In free-living microbial communities, biogeographic patterns result from 2 key processes: dispersal limitation and environmental selection (Martiny et al. 2006). In host-associated microbial communities, dispersal is dictated by transmission mode: the passage of symbiont taxa via vertical (parent-to-offspring) or horizontal (environmental acquisition) means. Vertical symbiont transmission is documented in numerous sponges (Ereskovsky et al. 2005, Oren et al. 2005, Enticknap et al. 2006, de Caralt et al. 2007, Sharp et al. 2007, Lee et al. 2009, Schöttner et al. 2013), including the genus *Ircinia* (Schmitt et al. 2007), and links the evolutionary trajectory of host and symbiont. Accordingly, reproductive isolation of host populations may restrict

the exchange of vertically transmitted microbes or genes that regulate the microbiome between host populations, resulting in divergent microbial communities over time (Schöttner et al. 2013, Easson & Thacker 2014). The observed geographic distribution of host haplotypes for *I. campana* correlated with symbiont community similarity, suggesting that limited gene flow between SAB and Caribbean populations of *I. campana* may contribute to the observed latitudinal variation in symbiont communities. While larger sample sizes and higher resolution genetic markers are required to corroborate this hypothesis, even low levels of variation in COI sequences are informative, given the slow mutation rate of sponge mitochondrial genes (Huang et al. 2008, López-Legentil & Pawlik 2009), and previous research reports a barrier to gene flow between SAB and Caribbean populations of other marine species (Carlin et al. 2003, Mobley et al. 2010, Betancur-R et al. 2011). Our results highlight the importance of host genetic characterization in discerning the effects of host-specific and environmental factors on microbiome variations across locations, as understanding the genetic structure and gene flow among host populations yields critical information about the dispersal range of symbionts via vertical transmission.

Environmental conditions vary greatly across the broad spatial scale in this study, from temperate SAB regions to tropical Caribbean reefs, and may exert different selective pressures on sponge holobionts that also contribute to the observed variability in microbiomes of *I. campana*. Although larval and adult sponge microbiomes are largely unresponsive to short-term, sub-lethal changes in environmental factors (e.g. temperature and nutrient loads; Webster et al. 2008, 2011, Simister et al. 2012a,b, Pita et al. 2013a), adaptations to local environmental conditions over longer timescales may drive region-specific differences in sponge microbiomes (Luter et al. 2015). Indeed, environmental variation is shown to impact symbiont communities from the same host sponge species, resulting in distinct symbiont communities between open-ocean sites and marine lakes (Cleary et al. 2013), intertidal and subtidal habitats (Weigel & Erwin 2016), and inshore and offshore reefs (Luter et al. 2015). Similar environmental filtering of symbiont communities may contribute to the latitudinal variation detected in the microbial community of *I. campana*, with periodic environment-driven shifts in symbiont communities solidified by recurrent vertical symbiont transmission in isolated host populations, and may also explain the lack of variation in microbiomes of *Ircinia* species previously reported across more environmentally homogenous longitudinal gradients (Pita et al. 2013b,c).

Despite the observed latitudinal variation in symbiont communities, no variation in sponge tissue palatability was detected over the same geographic range using the fish-feeding laboratory assay. All populations of *I. campana* were chemically defended, suggesting that host palatability is decoupled from symbiont structure in *I. campana*. Similar findings are reported for the Caribbean congeneric sponge *I. strobilina*, where shifts in the bacterial symbionts under aquaculture conditions did not alter secondary metabolite profiles (Mohamed et al. 2008). The secondary metabolites responsible for feeding deterrence in *I. campana* were previously identified as a complex mixture of linear furanosesterterpene tetronic acids (Pawlik et al. 2002) and may be produced by shared components of the microbiome (i.e. core symbionts) or by metabolic pathways of the host sponge alone. While shifts in the specific composition of these complex mixtures may underlie the observed unpalatability, such hypothetical changes were not manifested in the ecological function investigated herein (i.e. predator deterrence). Additional ecological roles of secondary metabolites, such antifouling and allelopathic activity, were not investigated in the current study, and warrant investigation in future studies of structure–function relationships in the sponge microbiome.

Crude organic extracts of tissue samples of *I. campana* were consistently unpalatable across locations, with mean assay data ranging from 0 to 2 pellets of 10 eaten. Although mean values were higher at SAB locations, behavioral assay data should not be strictly interpreted to indicate quantitative differences in metabolite concentrations, as small differences in the mean number of pellets eaten more likely reflect variation in assay fish response due to hunger, time or date of assay, minor variations in extraction or assay food preparation among other factors (Pawlik 2012). Similar mean crude extract palatability values are also reported for samples of *I. campana* from Rodriguez Cay in the Florida Keys using the same extraction and assay techniques as the present study (Pawlik et al. 2002). The chemical defense data reported here contradict previous studies that suggest sponges from the SAB (including *I. campana*) are more palatable to fish predators than their conspecifics in the Caribbean (Ruzicka & Gleason 2008, 2009). Importantly, these past studies do not directly compare the palatability of sponge tissue samples collected across a latitudinal gradient with side-by-side feeding assays, but instead compare assay data

for sponges from the SAB alone to assay data from Caribbean sponges published elsewhere and based on different extraction protocols. These methodological differences include lyophilization of tissue and the absence of an aqueous extraction phase (Ruzicka & Gleason 2008, 2009) vs. fresh tissue with inclusion of an aqueous phase (Pawlik et al. 1995), and field-based feeding assays (mixed predators *in situ*; Ruzicka & Gleason 2008, 2009) vs. laboratory feeding assays (single predator *ex situ*; Pawlik et al. 1995). The lower levels of chemical defenses observed in past studies of SAB sponges (Ruzicka & Gleason 2008, 2009) may be attributed to incomplete extraction of more polar secondary metabolites from sponge tissue during sample preparation (Pawlik 2012) and, to a lesser extent, bioassay conditions (given that extracts of *Ircinia* spp. exhibit predator deterrence in both single predator, laboratory assays, and mixed predator, field assays; Pawlik et al. 2002). In contrast, our study employed a standardized methodology (extraction protocols and assay conditions) performed on sponge tissue samples collected across a broad latitudinal gradient, and our results indicate that all tissue samples of *I. campana* were unpalatable to the generalist predator *T. bifasciatum*, regardless of collection site.

In conclusion, we report latitudinal variations and distance-decay relationships in the microbiome of *I. campana* across a 2500 km range extending from temperate SAB regions to tropical Caribbean reefs. Microbiome variations were driven by the different relative abundances of dominant, shared OTUs, as well as the presence/absence of rare, site-specific OTUs. No variation in palatability was detected over the same spatial scale, with chemical extracts from all samples of *I. campana* exhibiting predator deterrence using a standardized fish-feeding bioassay. Notably, distinct sponge haplotypes that correlated with the observed variability of symbiont community structure were detected. Together, these findings revealed latitudinal variation in the microbiome of *I. campana* and suggest that these biogeographic trends are driven by a combination of region-specific environmental filtering of symbiont communities and distinct evolutionary trajectories of host sponge populations.

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