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REPORT

Molecular detection and microbiome differentiation of two cryptic lineages of giant barrel sponges from Conch Reef, Florida Keys

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Abstract The giant barrel sponge, *Xestospongia muta*, represents a dominant member of Caribbean reef communities. Recent microsatellite data have revealed the presence of two genetic clusters of *X. muta* in a monitored population on Conch Reef, Florida Keys, with a reduced abundance of one cluster among the largest individuals. Tracking changes to populations over time and their ecological significance requires rapid identification of each genetic cluster and subsequent studies of biological differences between clusters. Here, we show that single-gene barcoding detected the same intraspecific genetic variation within *X. muta* from Conch Reef as microsatellite data, with mitochondrial gene sequences (cytochrome c oxidase subunit I, I3-M11 partition) from 54 individuals corresponding to 4 known haplotypes within the two genetic clusters. Remarkably, mapping these haplotypes to barrel sponges worldwide revealed positioning on opposite ends of a global network, despite their sympatric occurrence. Further, we investigated whether differences in symbiotic microbial communities could be detected between the two clusters using next-generation (Illumina) sequencing of 16S rRNA gene amplicons. Both clusters exhibited highly diverse microbial communities, with 12,185 total OTUs spanning 38 bacterial and 3 archaeal phyla, but significant differences in microbial community structure (PERMANOVA; $p < 0.001$) and diversity (Shannon diversity index; $p < 0.01$) were detected between the two clusters. As sponges typically exhibit interspecific, but not intraspecific, variability in microbial communities, these findings within a sympatric population provide additional support for ecologically relevant cryptic species of *X. muta*.

Keywords Xestospongia muta · Microbial symbionts · Giant barrel sponge · Cryptic lineages · Sponge ecology · Microbial ecology

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Introduction

Coral reefs represent complex ecological systems, with biodiversity rivaling or surpassing even that of tropical rainforests. Sponges are particularly abundant on Caribbean reefs (Loh and Pawlik 2014), with species richness outnumbering even corals and algae (Diaz and Rützler 2001). As spatial competitors with corals, rapid coral decline has led to concurrent increases in sponge cover on some reefs as sponges colonize newly available space faster than corals (Aronson et al. 2002; McMurray et al. 2010; Bell et al. 2013; Marlow et al. 2019). In the long term, this trend may lead to sponge-dominated reefs replacing coral-dominated reefs (Bell et al. 2013), although this shift will ultimately depend on the causes of coral decline, as sponges may be similarly sensitive to environmental stressors (Powell et al. 2014; reviewed in Pawlik and McMurray 2020).

The increasing abundance of sponges on tropical reefs may amplify the important ecological services these

animals provide. Sponges function as sources of three-dimensional habitat for other marine life (Pawlik and Henkel 2005; Seemann et al. 2018; Chak and Rubenstein 2019), feed spongivorous reef fishes, invertebrates, and sea turtles (Pawlik 1983; Van Dam and Diez 1997; Ruzicka and Gleason 2008), and serve to anchor and reinforce reef structures (Wulff and Buss 1979; Diaz and Rützler 2001), all functional roles that may become increasingly important as reef-building corals continue to decline. Sponges also filter vast quantities of water (Reiswig 1974), removing toxic heavy metals and other pollutants from the water column in the process (Cebrian et al. 2007). Perhaps most importantly, sponges are important in nutrient cycling, including the transformation of nitrogen (Hoffmann et al. 2009) and the recirculation of dissolved organic carbon back into trophic systems, a "sponge-loop" that may support the diverse array of life within reefs that are otherwise nutrient-limited (de Goeij et al. 2013).

The giant barrel sponge *Xestospongia muta* may play an especially important role within Caribbean reef communities. The so-called redwood of the reef can grow to over a meter in height and diameter, and is long-lived, with some individuals estimated to be thousands of years old (McMurray et al. 2008). *Xestospongia muta* is also one of the most abundant sponge species on Caribbean reefs (Loh and Pawlik 2014), and in the Florida Keys, there is evidence that it is experiencing rapid population growth (McMurray et al. 2015). Three species of *Xestospongia* are currently recognized worldwide: *X. muta*, *X. testudinaria*, and *X. bergquistia*; however, Swierts et al. (2017) recently have proposed that these species are included among complexes of cryptic species, based on molecular analyses of mitochondrial and nuclear genes that revealed the presence of 17 distinct genotypes. Using microsatellite markers, Deignan et al. (2018) investigated intraspecific variation within a single population of *X. muta* on Conch Reef, Florida, and detected two distinct, co-occurring genetic clusters (named Cluster 1 and 2). However, the relationship between these sympatric genetic clusters and the genotypes described by Swierts et al. (2017) remains unresolved.

Deignan et al. (2018) further determined that the occurrence of each genetic cluster of *X. muta* was skewed towards different sponge sizes. Cluster 1 sponges dominated the two largest size classes within the population, while Cluster 2 sponges made up about half of the smallest two size classes. As larger sponge individuals should exhibit far greater reproductive capacity (Uriz et al. 1995; Deignan et al. 2018), Cluster 1 sponges would be expected to dominate at all size classes, and to recruit more heavily into the population. However, the recruitment of Cluster 2 sponges was significantly higher than expected, suggesting a population shift may be occurring on Conch Reef (Deignan et al. 2018). A mechanism to quickly and accurately distinguish between these two genetic clusters would greatly facilitate the tasks of tracking these shifts over time, investigating their ecological significance, and determining the selective forces responsible for this genetic structure shift. Here, we used single-gene sequencing (mitochondrial gene cytochrome c oxidase subunit I, I3-M11 partition) to rapidly identify the two genetic clusters of *X. muta* previously recovered using time-intensive microsatellite analyses (Deignan et al. 2018), and related these clusters to the global haplotype network described by Swierts et al. (2017).

Sponges also harbor diverse and highly host-specific microbial communities (Taylor et al. 2007; Erwin et al. 2012a; Fiore et al. 2013; Thomas et al. 2016) that remain relatively conserved between individuals of the same species in the same region, but can vary significantly between different species (Taylor et al. 2004; Montalvo and Hill 2011; Easson and Thacker 2014), including congeneric species (Erwin et al. 2012a). *Xestospongia muta* is a high-microbial abundance (HMA) sponge with a high diversity of microbial symbionts (Montalvo and Hill 2011; Fiore et al. 2013), which have been shown to exhibit regional differences within the Caribbean (Fiore et al. 2013). Recent studies suggest that these differences may be linked to previously undescribed cryptic species in *X. muta* (Swierts et al. 2017, 2018), and indeed intraspecific genetic differences have been shown to affect microbial community composition in some sympatric sponges (Griffiths et al. 2019). Microbial symbionts represent an integral part of the sponge holobiont; not only do they confer important benefits upon their host, such as supplemental sources of nutrition (Erwin and Thacker 2008), but they have also been implicated in many of the important ecological functions sponges perform, such as the cycling of nitrogen (Fiore et al. 2015). Thus, in addition to the objectives outlined above, we also characterized and compared the symbiotic microbial communities of the two genetic clusters of *X. muta* to investigate a potential selective force behind host genetic differentiation and shed light on the ecological consequences of the genetic shift occurring within Florida populations of *X. muta*.

Materials and methods

Sample collection

Prior to this study, SCUBA divers identified the location of every *Xestospongia muta* individual within a single plot (AQS3) on Conch Reef, assigning an *x*, *y* coordinate and a unique 3-digit identifier (McMurray et al. 2010). AQS3 (24°56′59″N; 80°27′13 W) is a permanent circular plot

measuring 16 m in diameter, located at approximately 20 m depth. The site has been surveyed typically once per annum since 2000, with new recruits identified and tagged, and the status (presence/absence) and size classes of all *X. muta* individuals assessed annually for change (McMurray et al. 2008, 2010, 2015). Deignan et al. (2018) sampled every *X. muta* individual in AQS3 for microsatellite analysis. For this study, the same sponge individuals (n = 54) were resampled in June 2017 and classified into genetic Cluster 1 or Cluster 2 based on Deignan et al. (2018). Replicate seawater samples (n = 3; 50 mL) were contemporaneously collected in sterile bottles. Sponge samples were collected with a corer and transferred to separate sterile Whirl–Pak bags at depth. All samples were subsequently stored on ice in an insulated cooler and transported back to the field station (< 16 km away from sampling site). Seawater samples were concentrated onto 0.2-µm membrane filters which were halved and immediately frozen at − 80 °C. Sponge samples were each placed in individual vials and fixed in absolute ethanol before storage at −20 °C. Prior to DNA extraction, a small cube (ca. 2 mm^3) from each sponge sample was isolated using sterile techniques. Each cube contained parts of the endosome and ectosome of the sponge, evident by the color change from white to reddish-brown, respectively, and the outside edges of the sponge were specifically avoided. DNA extractions of sponge cubes and seawater filters were performed using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer protocols.

Haplotype identification of sponge hosts

The I3-M11 partition of the mitochondrial gene cytochrome oxidase I (COI) was sequenced from all sponge samples using the primers C1-Npor2760 and C1-J2165 (Misof et al. 2000; Erpenbeck et al. 2002). PCR amplifications were conducted based on a total volume of 25 µL, consisting of 5 pmol of each primer, 2X MyTaq HS Red Mix (Bioline), and 1 µL (ca. 10 ng) of template DNA on an Eppendorf Mastercycler nexus gradient. The thermocycler program included initial denaturation at 95 °C for 1 min; 35 cycles of 95 °C for 15 s, 45 °C for 15 s, and 72 °C for 10 s; and a final extension at 72 °C for 1 min. Sequencing reactions were then conducted using BigDye Terminator v. 3.1 (Applied Biosystems) and the same primers as above. Sequencing PCR products were purified with BigDye XTerminator (Applied Biosystems) and subsequently sequenced on an AB 3500 genetic analyzer (Applied Biosystems) located at the UNCW Center for Marine Science. Corresponding forward and reverse raw sequences were aligned, cleaned, and trimmed to 534 bp using Geneious version 8.02 (Kearse et al. 2012) to generate consensus sequences. Sequences were then compared to the GenBank database using nucleotide–nucleotide BLAST searches (BLASTn) to confirm host identity based on the highest percent match (Altschul et al. 1990). Final sequences were aligned, and variable sites were identified and compared to known microsatellite clusters (Deignan et al. 2018) and mitochondrial haplotypes of *X. muta* described previously (López-Legentil and Pawlik 2009; Swierts et al. 2017). All consensus sequences were archived in GenBank with the accession numbers MT773455 to MT773508.

Sequencing of microbial symbionts

Microbial communities of *X. muta* and seawater samples were characterized through amplification and sequencing of a ca. 300 bp fragment (V4 region) of the 16S ribosomal RNA (rRNA) gene using the universal bacterial/archaeal forward primer 515f and reverse primer 806r (Caporaso et al. 2011). DNA extracts were amplified in an Eppendorf Mastercycler nexus gradient, using a thermocycler program consisting of an initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 15 s, 50 °C for 15 s, and 72 °C for 20 s; and a final extension at 72 °C for 2 min. All PCR-viable DNA extracts were sent to Molecular Research LP for amplification, library construction, and multiplexed sequencing of partial 16S rRNA gene sequences on an Illumina MiSeq platform, using the same 515f and 806r primers. All raw sequences were submitted to the Sequence Read Archive of NCBI (accession no. PRJNA645299).

Processing of microbial next-generation sequence data

Raw sequence processing was conducted using the mothur software package (v.1.38.0, Schloss et al. 2009), following a slightly modified version of the Illumina MiSeq SOP pipeline (Kozich et al. 2013). In brief, raw sequences were first quality-filtered to remove low-quality sequences and were then aligned to the SILVA reference database (v132; Pruesse et al. 2007). Putative chimeric sequences were culled via self-reference searches with UChime (Edgar et al. 2011). Based on a naïve Bayesian classifier and bootstrap algorithm for confidence scoring (Wang et al. 2007), sequences were classified using the SILVA reference database (v132; Pruesse et al. 2007). Nontarget sequences (chloroplasts, mitochondria, and eukarya) and singletons were removed from the dataset. Using the opticlust clustering algorithm, all remaining sequences were assigned to operational taxonomic units (OTUs) based on 97% sequence similarity, with the taxonomic classification of OTUs established by majority consensus (Schloss and Westcott 2011). In order to standardize sampling depths (i.e., number of sequence reads) across all

samples, each data set (i.e., the microbial community of each sponge individual or seawater sample) was subsampled to the lowest read count (n = 62,700) from the final shared file, and all subsequent data analyses were based on these subsampled data sets. Rarefaction curves (Fig. S1) and coverage calculations (Good's, average = 98.9%, range = 98.5 to 99.3%) confirmed this sampling depth was sufficient to capture the diversity of each sample's microbial community.

Analysis of microbial community diversity

To compare microbial community diversity between the two sponge clusters (Cluster 1 and Cluster 2), alpha diversity metrics for OTU richness and evenness were calculated within mothur, including the OTU richness indices S (total number of observed OTUs) and Chao 1 (expected number of OTUs), and the Simpson evenness index (E1/D), the inverse Simpson index (D), and the Shannon–Weaver diversity index (H′). Diversity indices for the two genetic clusters of *X. muta* were statistically compared using analyses of variance (ANOVA) based on a 95% confidence interval.

Analysis of microbial community structure

To compare host specificity and community structure differences between the two sponge clusters, beta diversity metrics for OTU overlap and similarity of microbial symbiont communities were calculated. Venn diagrams were created within mothur to visualize OTU overlap between the two clusters. Within each cluster, core OTU communities, or those OTUs present in all replicate sponges of a genetic cluster, were identified. To compare the microbial communities of each sponge host, Bray–Curtis similarity (BCS) matrices were constructed in PRIMER (version 7.0.13) based on square root transformations of OTU relative abundances and subsequently visualized using multidimensional scaling plots. Permutational multivariate analyses of variance (PERMANOVA) were performed to compare the structure of microbial communities of the two clusters and sponges compared to seawater. Permutational multivariate analyses of dispersion (PERMDISP) were performed to verify that all significant PERMANOVA results represented actual structural differences, rather than unequal variability of dispersion between the two clusters. Similarity percentage (SIMPER) analyses were performed to identify the OTUs primarily responsible for the majority (> 50%) of observed differences in microbial communities of the two sponge genetic clusters. To correct for the potential error associated with distance-based metric calculations (Warton et al. 2012), a Metastats test was conducted within mothur using 1000 permutations to determine which SIMPER OTUs exhibit significant differences in relative abundance among groups (White et al. 2009). In addition, we determined whether host sponge size influenced the structure of *X. muta* microbial communities by classifying size measurements for each sponge into the 5 size classes (I–V) described in McMurray et al. (2010, 2015). PERMANOVA analyses were conducted to determine whether the structure of microbial communities was affected by host size and genetic cluster.

Rare and abundant symbiont community analysis

To determine whether significant differences in community structure also occurred among rarer OTUs, sequence data were further divided into abundant and rare-OTU data partitions, based on a 0.1% cutoff threshold (Fuhrman 2009). This resulted in a cutoff value of 62 sequences, with OTUs containing > 62 sequences considered "abundant," and OTUs containing ≤ 62 sequences considered "rare". These abundant and rare data partitions were also tested for differences in microbial community structure through the creation of BCS matrices and PERMANOVA and SIMPER analyses, as described above.

Results

Sponge genetic analysis

Sequences of the I3-M11 partition of the COI gene were obtained for all 54 individuals of *X. muta* collected from the AQS3 plot. Nucleotide alignments revealed 5 variable sites which corresponded to 4 known haplotypes (López-Legentil and Pawlik 2009; Swierts et al. 2017) and with the two genetic clusters described by Deignan et al. (2018). The majority of sponges grouped in Cluster 1 (77.8%, n = 42) and represented 100% matches for either haplotypes H1/C2 (38.1%, n = 16) or H2/C9 (61.9%, n = 26). The remaining sponges grouped in Cluster 2 (22.2%, n = 12), and represented 100% matches for either haplotypes H3/C5 (33.3%, n = 4) or H4/C8 (66.7%, n = 8; Table 1). Notably, the haplotypes corresponding to Cluster 1 and Cluster 2 sponges were on opposite ends of the global haplotype network (Fig. S2), thus representing highly divergent haplotypes despite their sympatric occurrence.

Symbiotic microbial community composition

For the microbial community assessment, a total of 3,573,900 sequences, consisting of 13,082 distinct OTUs, were obtained from the 57 samples (54 sponge individuals and 3 seawater samples). Seawater bacterioplankton

Table 1 Genetic cluster and haplotype diversity of *Xestospongia muta* individuals examined in this study

Cluster (Deignan et al. 2018)	Haplotype (López-Legentil and Pawlik 2009)	Haplotype (Swierts et al. 2017)	Locus 11	Locus 22	Locus 28	Locus 133	Locus 347	# of sponges
1	H1	C2	A	T	C	A	G	16
	H2	C9	A	T	T	A	G	26
2	H3	C5	A	A	C	G	G	4
	H4	C8	G	A	C	G	A	8

All 4 known mitochondrial COI haplotypes for the Caribbean *Xestospongia muta* were retrieved. Variable loci with corresponding nucleotide positions are indicated along with the number of individuals assigned to each haplotype. Cluster 1 sponges represented 100% matches for either haplotypes H1/C2 or H2/C9, while Cluster 2 sponges represented 100% matches for haplotypes H3/C5 or H4/C8

communities represented 2,205 OTUs, with 1,308 of these also present in *X. muta* microbial communities (Fig. 1). Seawater microbial communities spanned 38 microbial phyla and were dominated by *Alphaproteobacteria* (66%), *Gammaproteobacteria* (11%), and *Cyanobacteria* (7%; Fig. 2). Sponge symbiont communities represented 12,185 OTUs, with the majority of these (89.3%, $n = 10{,}877$) unique to sponge hosts and not represented within the bacterioplankton community (Fig. 1). Between the two clusters, 6,225 and 1,030 OTUs were unique to Clusters 1 and 2, respectively, with 4,930 OTUs shared by both clusters. Microbial symbionts of *X. muta* spanned 37 bacterial and 5 archaeal phyla (*Asgardaeota*, *Crenarchaeota*, *Euryarchaeota*, *Nanoarchaeota*, and *Thaumarchaeota*), with *Thaumarchaeota* present in all sponge samples, and *Nanoarchaeota* and *Euryarcheaota* present in 98.1% ($n = 53$) and 94.4% ($n = 51$) of all samples for Cluster 1

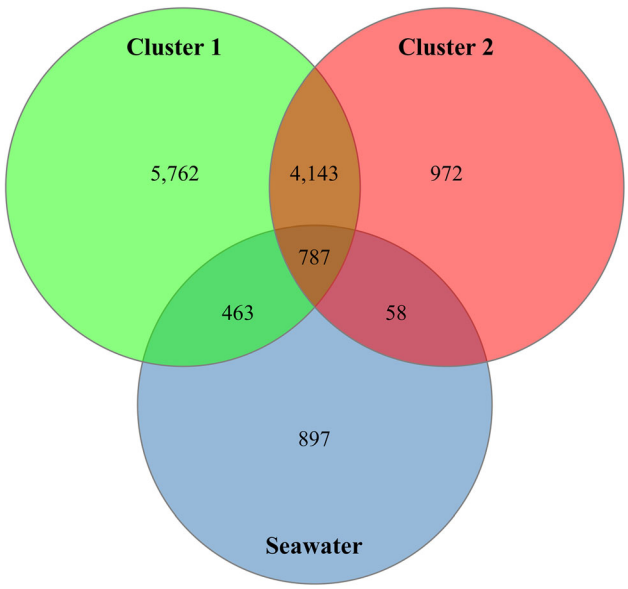

Fig. 1 Venn diagram showing OTU overlap in microbial communities of *Xestospongia muta* genetic clusters 1 (green) and 2 (red), and in seawater (blue). Total richness among all three sources was 13,082 OTUs

and 2, respectively. Microbial communities of Cluster 1 sponges included all 37 bacterial and 5 archaeal phyla (Fig. S3) and were dominated by *Gammaproteobacteria* (16%), *Chloroflexi* (13%), *Thaumarchaeota* (13%), and *Actinobacteria* (10%; Fig. 2). 157 OTUs were core OTUs, present in all Cluster 1 replicates. Of these, only 4 OTUs were core symbionts for Cluster 1 exclusively. Microbial communities of Cluster 2 sponges spanned 31 bacterial phyla and 4 archaeal phyla (Fig. S4) and were dominated by *Gammaproteobacteria* (18%), *Chloroflexi* (15%), *Thaumarchaeota* (11%), and *Acidobacteria* (8%; Fig. 2). 217 OTUs were found to be part of the core community of Cluster 2, and 64 of these core symbionts were exclusive to Cluster 2 (Table 2).

Symbiont diversity

To determine whether differences in microbial community diversity of the two genetic clusters of *X. muta* were statistically significant, alpha diversity metrics were calculated by cluster. Of the alpha diversity metrics investigated, the Shannon diversity index indicated that the genetic cluster of the host sponge had a significant effect on microbial community diversity (ANOVA, $F_{1,52} = 8.407$, $p < 0.01$) and the inverse Simpson evenness index indicated that there were significant differences in the evenness of microbial communities across the two sponge genetic clusters (ANOVA, $F_{1,52} = 6.561$, $p < 0.05$). Other indices detected no significant differences in microbial community alpha diversity between the clusters (ANOVA, $p > 0.05$; Table 3).

Symbiont community structure

Microbial communities clustered significantly in response to source [sponge or seawater; PERMANOVA pseudo-$F = 14.217$, $p(MC) < 0.001$; Fig. S5]. Pairwise comparisons further revealed that when grouped by host-sponge genetic cluster, both clusters exhibited significant differences in microbial community structure compared to

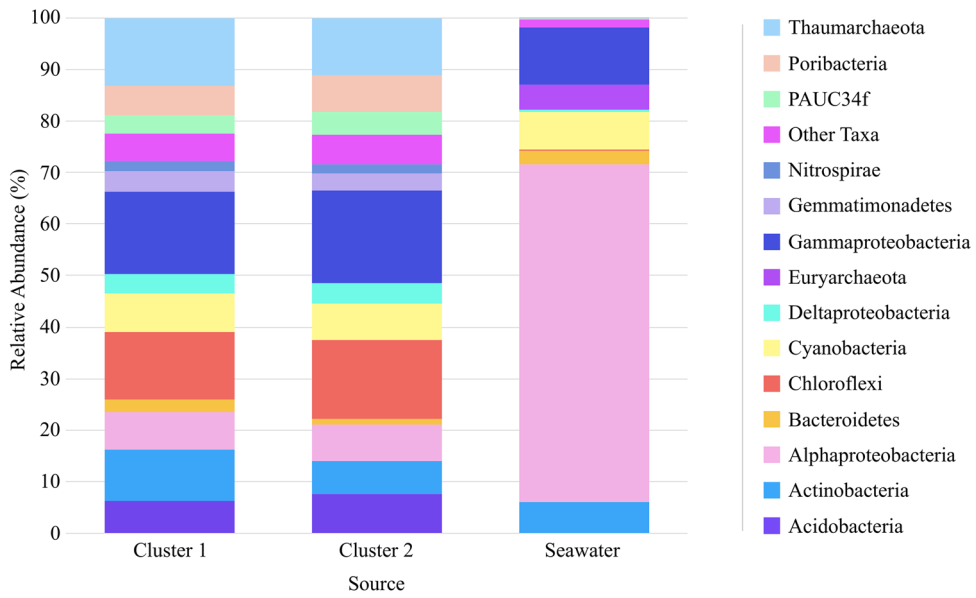

Fig. 2 Microbial community composition averaged for the three sources: *Xestospongia muta* genetic clusters 1 and 2, and seawater. Phylum-level classifications are shown except for *Proteobacteria*, which are divided into major classes (*Alpha-*, *Delta-*, and *Gammaproteobacteria*). "Other taxa" represents less abundant taxa and includes: *AncK6*, unclassified archaea, *Asgardaeota*, unclassified bacteria, *Calditrichaeota*, *Chlamydiae*, *Crenarchaeota*, *Dadabacteria*, *Deinococcus-Thermus*, *Dependentiae*, *Elusimicrobia*, *Entotheonellaeota*, *Epsilonbacteraeota*, *FCPU426*, *Fibrobacteres*, *Firmicutes*, *Fusobacteria*, *Kiritimatiellaeota*, *Latescibacteria*, *LCP-89*, *Lentisphaerae*, *Margulisbacteria*, *Marinimicrobia* (*SAR406_-clade*), *Modulibacteria*, *Nanoarchaeaeota*, *Nitrospinae*, *Omnitrophicaeota*, *Patescibacteria*, *Planctomycetes*, unclassified *Proteobacteria*, *Schekmanbacteria*, *Spirochaetes*, *Tenericutes*, *Verrucomicrobia*, and *Zixibacteria*

seawater [$t = 4.964$ and 4.856 for clusters 1 and 2, respectively, $p(MC) = 0.001$ for both clusters]. Within microbial communities associated with *X. muta*, a significant difference in community structure was also detected between the two sponge clusters [PERMANOVA pseudo-$F = 3.466$, $p = 0.001$; Table 4; Fig. 3], with host sponge cluster alone explaining 26.7% of the observed variation. The structure of microbial communities from sponges within each genetic cluster were on average 60.7% and 60.9% similar to other sponges within the same genetic cluster, for Clusters 1 and 2, respectively. Microbial communities of sponges within different clusters exhibited 58.2% structural similarity on average. However, no significant differences in dispersion were detected between clusters (PERMDISP, $F_{1,52} = 0.532$, $p = 0.589$) for the overall data partition (Table 4). SIMPER analyses revealed that for the overall data partition, a difference in the relative abundance of a proportionally small number of OTUs ($n = 370$; 3.04% of all sponge-associated OTUs) were responsible for the majority (50%) of the observed differences between the microbial communities of the two sponge clusters (Table S1). PERMANOVA analyses revealed no significant effect of sponge size on sponge microbial community structure (PERMANOVA, pseudo-$F = 1.042$, $p = 0.335$), a significant effect of host sponge genetic cluster (PERMANOVA, pseudo-F = 2.718, $p = 0.001$), and no significant interaction effect between sponge size and genetic cluster (PERMANOVA, pseudo-$F = 0.890$, $p = 0.83$).

Rare and abundant symbiont communities

To determine whether differences in OTU relative abundance impacted observed differences in overall microbial community structure, OTUs were sorted into abundant ($> 0.1\%$ relative abundance) and rare ($\leq 0.1\%$) partitions. Partitioning resulted in 660 abundant OTUs, representing 98.4% of all microbial sequences, and 12,422 rare OTUs (1.6% of all microbial sequences). Analyses of both the abundant and rare data partitions indicated significant differences in microbial community structure between the two *X. muta* genetic clusters (PERMANOVA, pseudo-$F = 4.993$, $p = 0.001$ for the abundant data partition, and pseudo-$F = 1.573$, $p = 0.002$ for the rare data partition; Table 4). SIMPER analyses revealed that for the abundant data partition, microbial communities in sponges within Clusters 1 and 2 exhibited 72.7% and 72.9% similarity, respectively. Between the two clusters, sponges were on average 69.9% similar, with the majority of differences between the two clusters stemming from variation in the relative abundance of just 108 OTUs (0.89% of all sponge-associated OTUs). In contrast, for the rare data set, microbial communities within Cluster 1 and 2 sponges were 12.2% and 14.1% similar on average respectively,

Table 2 Top 25 most abundant core OTUs for each *X. muta* genetic cluster, arranged in descending abundance by cluster

Cluster 1				Cluster 2			
OTU	Phylum	Lowest taxonomic classification†	%	OTU	Phylum	Lowest taxonomic classification†	%
1	*Thaumarchaeota*	(F.) *Nitrosopumilaceae*	7.4	1	*Thaumarchaeota*	(F.) *Nitrosopumilaceae*	4.8
2	*Thaumarchaeota*	(F.) *Nitrosopumilaceae*	4.6	3	*Cyanobacteria*	(F.) *Cyanobiaceae*	4.6
3	*Cyanobacteria*	(F.) *Cyanobiaceae*	4.5	2	*Thaumarchaeota*	(F.) *Nitrosopumilaceae*	4.0
4	*Actinobacteria*	(G.) *Microtrichaceae_Sva0996_marine_group*	3.3	6	*Poribacteria*	(P.) *Poribacteria*	3.4
5	*Actinobacteria*	(G.) *Microtrichaceae_Sva0996_marine_group*	3.0	11	*PAUC34f*	(P.) *PAUC34f*	2.6
7	*Cyanobacteria*	(F.) *Cyanobiaceae*	2.7	7	*Cyanobacteria*	(F.) *Cyanobiaceae*	2.4
10	*Actinobacteria*	(G.) *Microtrichaceae_Sva0996_marine_group*	2.6	14	*Proteobacteria*	(G.) *Nitrosococcaceae_AqS1*	2.4
6	*Poribacteria*	(P.) *Poribacteria*	2.6	5	*Actinobacteria*	(G.) *Microtrichaceae_Sva0996_marine_group*	2.3
9	*Proteobacteria*	(G.) *Woeseiaceae_JTB255_marine_benthic_group*	2.2	9	*Proteobacteria*	(G.) *Woeseiaceae_JTB255_marine_benthic_group*	2.2
12	*Proteobacteria*	(F.) *Myxococcales_bacteriap25*	2.3	12	*Proteobacteria*	(F.) *Myxococcales_bacteriap25*	2.0
11	*PAUC34f*	(P.) *PAUC34f*	2.2	13	*Nitrospirae*	(G.) *Nitrospira*	1.8
13	*Nitrospirae*	(G.) *Nitrospira*	2.1	19	*Acidobacteria*	(G.) *PAUC26f*	1.7
16	*Bacteroidetes*	(F.) *Rhodothermaceae*	1.8	10	*Actinobacteria*	(G.) *Microtrichaceae_Sva0996_marine_group*	1.7
15	*Acidobacteria*	(G.) *Thermoanaerobaculaceae_Subgroup_10*	1.6	22	*Poribacteria*	(P.) *Poribacteria*	1.7
14	*Proteobacteria*	(G.) *Nitrosococcaceae_AqS1*	1.5	18	*Proteobacteria*	(O.) *Gammaproteobacteria_UBA10353_marine_group*	1.6
20	*Gemmatimonadetes*	(C.) *Gemmatimonadetes_BD2-11_terrestrial_group*	1.4	17	*Entotheonellaeota*	(F.) *Entotheonellaceae*	1.5
17	*Entotheonellaeota*	(F.) *Entotheonellaceae*	1.3	4	*Actinobacteria*	(G.) *Microtrichaceae_Sva0996_marine_group*	1.4
21	*Chloroflexi*	(C.) *Chloroflexi_TK17*	1.3	15	*Acidobacteria*	(G.) *Thermoanaerobaculaceae_Subgroup_10*	1.4
18	*Proteobacteria*	(O.) *Gammaproteobacteria_UBA10353_marine_group*	1.2	31	*Proteobacteria*	(O.) *Gammaproteobacteria_KI89A_clade*	1.4
19	*Acidobacteria*	(G.) *PAUC26f*	1.2	40	*Chloroflexi*	(O.) *Dehalococcoidia_SAR202_clade*	1.3
30	*Proteobacteria*	(G.) *Albidovulum*	1.2	34	*Chloroflexi*	(O.) *Dehalococcoidia_SAR202_clade*	1.3
27	*Chloroflexi*	(F.) *Caldilineaceae*	1.1	25	*PAUC34f*	(P.) *PAUC34f*	1.2
22	*Poribacteria*	(P.) *Poribacteria*	1.1		*Proteobacteria*	(O.) *Gammaproteobacteria_JTB23*	1.2
28	*Poribacteria*	(P.) *Poribacteria*	1.1		*Chloroflexi*	(F.) *Caldilineaceae*	1.2
25	*PAUC34f*	(P.) *PAUC34f*	1.0		*Proteobacteria*	(O.) *Gammaproteobacteria_KI89A_clade*	1.2

sOTU identity, phylum classification, lowest taxonomic classification, total number of sequences per genetic cluster are shown

†*K* kingdom, *P* phylum, *C* class, *O* order, *F* family, *G* genus, *S* species

Table 3 Diversity metrics for microbial communities in *X. muta* by genetic cluster

Genetic cluster	S	Chao 1	E1/D	D	H′
1	1133.2 ± 208.8^{A}	2871.4 ± 536.0^{A}	0.034 ± 0.010^{A}	38.4 ± 10.9^{B}	4.4 ± 0.222^{B}
2	1187.8 ± 211.2^{A}	2932.5 ± 588.7^{A}	0.041 ± 0.010^{A}	47.6 ± 11.4^{A}	4.6 ± 0.141^{A}

S, observed richness; Chao 1, expected richness; E1/D, Simpson evenness; D, inverse Simpson evenness; H′, Shannon Weaver index. Average values (± 1 SD) are shown, with different superscript letters denoting significantly different means between the two genetic clusters

Table 4 Pairwise statistical comparisons of microbial community structure (PERMANOVA) and dispersion (PERMDISP) in the two genetic clusters of *Xestospongia muta* at overall, abundant, and rare data partition levels

Data partition	PERMANOVA		PERMDISP	
	F	*p*	*F*	*p*
Overall	3.466	0.001	0.532	0.589
Abundant	4.993	0.001	0.180	0.721
Rare	1.573	0.002	38.338	0.003

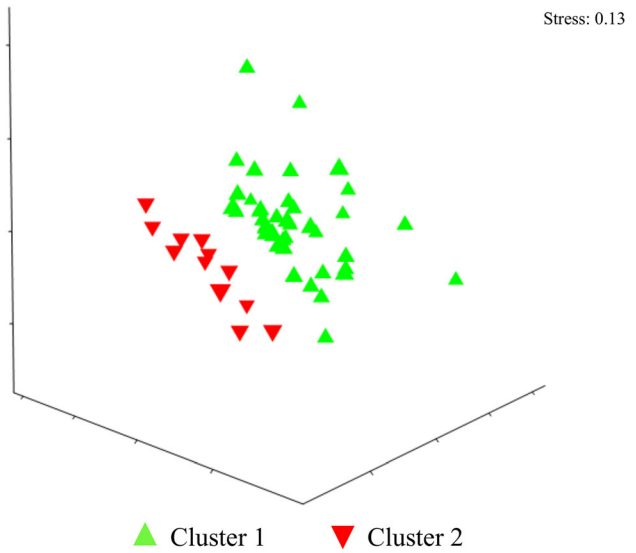

Fig. 3 Non-metric multi-dimensional scaling plot based on Bray–Curtis similarity matrices of microbial communities in *Xestospongia muta* genetic clusters 1 (green triangles) and 2 (red triangles). The spatial grouping of all replicate samples by genetic cluster indicates a high degree of host specificity and distinct structural divisions between Cluster 1 and Cluster 2 microbial communities

with an average similarity between the two clusters of 11.7%, driven primarily (50% contribution) by variation in relative abundance of 1,917 OTUs (15.7% of all sponge-associated OTUs). However, a significant difference in dispersion (PERMDISP, $F_{1,52} = 38.338$, $p = 0.003$) was detected within microbial communities between the two sponge genetic clusters for the rare data partition, but not the abundant data partition (PERMDISP, $F_{1,52} = 0.180$, $p = 0.721$; Table 4).

Discussion

Previous work with microsatellite loci by Deignan et al. (2018) revealed the presence of two genetic clusters of *X. muta* within a single monitored plot on Conch Reef off the Florida Keys. Here, using the same sponge individuals as the 2018 study, we determined that the I3-M11 partition of the mitochondrial COI gene detects the same fine-scale intraspecific genetic variation and exhibits 100% clustering agreement with the microsatellite data (Deignan et al. 2018). In order to investigate potential selective forces that may contribute to this variation and the genetic shift described in Deignan et al. (2018), we examined whether differences existed between the sponge-associated microbial communities of two genetic clusters. We detected significant differences in microbial community structure and diversity between Cluster 1 and Cluster 2 sponges, which given the high host specificity of sponge symbionts, provides additional support for the cryptic speciation of *X. muta* proposed by Swierts et al. (2017) and possibly maintained by multiple reproductive peaks and variability in spawning synchronicity (Neely and Butler 2020). Taxonomic descriptions and species delineations are now required to formalize the molecular and microbiological data for cryptic speciation in *X. muta*.

Determining the causes of the ongoing shift in Conch Reef *X. muta* populations (Deignan et al. 2018) and cascading impacts on coral reef ecosystems requires additional study facilitated by the molecular tool developed herein. The ability of single-gene sequencing to identify Cluster 1 and Cluster 2 sponge individuals will expedite the tracking of population shifts among *X. muta* and the study of physiological and ecological differentiation among lineages. Our results also allow the integration of microsatellite data (Deignan et al. 2018) with global investigations of barrel sponge diversity (Swierts et al. 2017). Importantly, the haplotypes corresponding to Cluster 1 and Cluster 2 sponges were on opposite ends of a global haplotype network despite their sympatric occurrence, when mapped against the mitochondrial network

developed by Swierts et al. (2017) for giant barrel sponges. Thus, the haplotypes within Cluster 1 and Cluster 2 sponges were found to be more similar to haplotypes from the Indian Ocean, Red Sea, and the Indo-Pacific than they were to each other. We hypothesize that this high degree of genetic divergence in sympatric populations of *X. muta* underlies important physiological and ecological differences between lineages.

Assessing such ecological differences will require stratifying individuals of *X. muta* by lineage to determine whether variability in specific traits (e.g., chemical defenses, Loh and Pawlik 2014; reproductive timing, Neely and Butler 2020) relate to cluster status. Gross morphology is currently unable to differentiate lineages of *X. muta*, as sponges within Clusters 1 and 2 at AQS3 exhibited no consistent morphological differences related to surface roughness. Previous work suggested that some surface morphologies may have a genetic basis in *X. muta*, as sponges that possessed smooth surfaces versus those with pronounced digitate extensions were assigned to different haplotypes (H4 and H3, respectively; López-Legentil and Pawlik 2009). These haplotypes correspond to C5 and C8 (Swierts et al. 2017), and both represented Cluster 2 sponges. However, other sponge individuals with the same two haplotypes exhibited the most common morphology (rough surface) shared by Cluster 1 sponges (haplotypes C2 and C9, Swierts et al. 2017). Further, annual time-series photographs of individual sponges on Conch Reef over a decade have revealed changes from smooth to rough surfaces, or the opposite, over time (J Pawlik pers. obs.). Accordingly, no morphological differences in surface features consistently differentiate the Cluster 1 and Cluster 2 genetic lineages. Microbial community characterization can help distinguish between morphologically indistinguishable cryptic sponge lineages (Chambers et al. 2013; Gloeckner et al. 2013; Cuvelier et al. 2014). In addition to possessing host-specific microbial symbionts, sponges form highly stable microbial associations that often remain consistent across broad spatial (Hentschel et al. 2002; Pita et al. 2013b) and temporal scales (Friedrich et al. 2001; Lee et al. 2006; Pita et al. 2013b; Erwin et al. 2015), and in spite of fluctuating or otherwise stressful environmental conditions (Friedrich et al. 2001; López-Legentil et al. 2010; Erwin et al. 2012b; Simister et al. 2012; Pita et al. 2013a). Accordingly, the stability and host specificity of sponge microbial associations suggest their potential usefulness as a tool for delineating between cryptic species, especially in the absence of observable morphological differences.

The detection of distinct microbial communities in each genetic cluster of *X. muta* also provides first insights into biological differences between host lineages. Structurally distinct microbiomes may have ecological consequences for sponge populations if they provide functional contributions to their hosts. The large diversity of symbionts within the microbial communities detected herein, and the varied functional potential described previously for symbionts in *X. muta* (Fiore et al. 2015), suggest that at least some of these microbial symbionts confer benefits upon their sponge hosts. Here, we focus on putative benefits of the most abundant core OTUs, as the sheer abundance and universal distribution of these OTUs among all the sponges sampled suggest that they play critical roles in the overall function of their host. Indeed, increases in symbiont abundance have been linked to enhanced host function in other marine invertebrates (Goffredi et al. 2007). The top 25 most abundant core microbiome OTUs presented here were also present within the seawater samples, though in extremely low abundance. This finding provides further support for *X. muta* sourcing at least some of its symbionts via horizontal acquisition (Schmitt et al. 2008), subsequently amplifying rare members of the environmental microbial "seed bank" (Lynch and Neufeld 2015).

Within the core microbiome, the most common archaeal symbionts detected were all within the family *Nitrosopumilaceae*, known sponge symbionts (Zhang et al. 2014) and ammonia oxidizers (Könneke et al. 2005). Several species of *Nitrosopumilus* have been described as capable of utilizing urea as an ammonia source for energy generation and biomass production (Qin et al. 2017). Another top OTU in the core microbiome of both clusters was classified within the genus *Nitrospira* of the bacterial phylum *Nitrospirae*, representing known bacterial nitrifiers (Van Kessel et al. 2015). Further, OTUs of the family *Woesceiaceae* and specifically of the genus *JTB255* have putative links to denitrification (Mußmann et al. 2017). These three OTUs correspond to microbes directly involved with the nitrogen cycle, providing further evidence of the important role sponges may play in this biogeochemical cycle (Hoffmann et al. 2009; Fiore et al. 2015). Other abundant OTUs included cyanobacterial symbionts that may provide fixed carbon to their sponge hosts through photosynthesis (Erwin and Thacker 2008) and *Poribacteria,* which are well-known sponge symbionts (Fieseler et al. 2004) that have putative links to organic phosphorous consumption (Kamke et al. 2014) and vitamin B_{12} synthesis (Siegl et al. 2011). Finally, *Myxococcales* are known sponge symbionts that produce abundant antibiotically active secondary metabolites (Hoffmann et al. 2013), that may confer anti-fouling or other defense capabilities to the sponge holobiont.

Significant structural differences between microbial communities of each cluster were also evident and may manifest in functional consequences that contribute to the observed genetic shift in host sponge populations. Importantly, these microbiome differences were not a function of

sponge size, a potential confounding variable as Cluster 1 sponges skew towards larger sizes (Deignan et al. 2018). The majority (> 50%) of microbial community dissimilarity between genetic clusters of *X. muta* could be explained by variation in the relative abundance of a proportionally small number of OTUs, including all of the top 25 most abundant universal core OTUs described above. For instance, a top core OTU within Cluster 1 sponges corresponded to a proteobacterial species within the genus *Albidovulum* (OTU00030), a thermophilic lineage most often described in hot spring environmental samples (Travassos et al. 2002; Yin et al. 2012). While this particular OTU was not even within the top 50 OTUs for Cluster 2, Cluster 2 sponges did possess a different *Albidovulum* lineage (OTU00050) within their top 50 most abundant core OTUs, suggesting possible co-divergence of *X. muta* lineages and *Albidovulum* symbionts. Although thermophilic symbionts have been proposed to provide some temperature tolerance capabilities in other marine invertebrate hosts (Lee et al. 2008), such benefits have been suggested to result from physical insulation rather than increased host tolerance (Lee et al. 2008). Another major driver of differences between the two clusters was an OTU within the genus *Cenarchaeum*, a *Thaumarchaeota* lineage (OTU00083). This genus currently has only 3 described species, with the best-studied being the psychrophilic *C. symbiosum*, which is thought to associate exclusively with a single sponge species (Preston et al. 1996), and may benefit the host sponge through B-vitamin synthesis (Hallam et al. 2006; Fan et al. 2012). Genes associated with B-vitamin production have been detected in *X. muta* metagenomes (Fiore et al. 2015), and this particular symbiont exhibited a greater average abundance in Cluster 2 sponges. Variable functional contributions to host physiology may differentially impact fitness between lineages, thereby representing a potential selective force behind the apparent genetic shift occurring on Caribbean reefs that seems to paradoxically favor the recruitment of Cluster 2 sponges. Further, these population shifts may have cascading impacts on community-level processes like nutrient cycling and other important sponge contributions to ecosystem functioning. Additional studies validating functional predictions from taxonomic data (e.g., metatranscriptomic comparisons of the two clusters) and testing hypothesized relationships between symbiont function and host fitness are required to determine the ecological relevance of this apparent population shift.

In summary, we found that single-gene sequencing of the I3-M11 partition of the mitochondrial COI gene easily differentiates between the two genetic clusters defined by Deignan et al. (2018) using microsatellite markers, and thus represents an important molecular tool for future ecological studies of *X. muta* populations (e.g., correlating reproductive timing and host genotype). We mapped these genetic clusters to opposite ends of a global barrel sponge network, revealing high haplotype divergence despite their sympatric occurrence. We also found that the symbiotic microbial communities of *X. muta* exhibit significant differences in community structure and diversity between, but not within, these two genetic clusters. We detected numerous OTUs that represent well-known sponge symbionts and that may potentially provide a multitude of benefits to their host. These lineage-specific differences in the structure and putative functionality of microbial communities provide preliminary evidence for a microbial-mediated driver of genetic shifts in *X. muta* populations currently occurring on Florida reefs.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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