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Microbiota of the major South Atlantic reef building coral *Mussismilia*

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Abstract (256 words)

The Brazilian endemic scleractinian corals, genus *Mussismilia*, are among the main reef builders of the South Atlantic and are threatened by accelerating rates of disease. To better understand how holobiont microbial populations interact with corals during health and disease and to evaluate whether selective pressures in the holobiont or neutral assembly shape microbial composition, we have examined the microbiota structure of *Mussismilia* corals according to coral lineage, environment, and disease/health status. Microbiota of three *Mussismilia* species (*M. harttii*, *M. hispida* and *M. braziliensis*) were compared using 16S rRNA pyrosequencing and clone library analysis of coral fragments. Analysis of biological triplicates per *Mussismilia* species and reef site allowed assessment of variability among *Mussismilia* species and between sites for *M. braziliensis*. From 173,487 V6 sequences 6733 coral- and 1052 water-associated OTUs were observed. *M. braziliensis* microbiota were more similar across reefs than to other *Mussismilia*-species microbiota from the same reef. Highly prevalent OTUs were more significantly structured by coral lineage and were enriched in Alpha and Gamma Proteobacteria. Bacterial OTUs from healthy corals were recovered from a *M. braziliensis* skeleton sample at twice the frequency of recovery from water or a diseased coral suggesting the skeleton is a significant habitat for microbial populations in the holobiont. Diseased corals were enriched with pathogens and opportunists (Vibrios, Bacteroidetes, Thallassomonas, and SRB). Our study examines for the first time intra- and inter-specific variability of microbiota across the genus *Mussismilia*. Changes in microbiota may be useful indicators of coral health and thus be a valuable tool for coral reef management and conservation.
Introduction

Emerging evidence suggests microbes play critical positive roles in the health of reef corals, with beneficial functions such as nutrient acquisition and cycling [1-3] and antimicrobial activities [4-7] increasing the fitness of the coral holobiont (i.e. the association between the coral animal, symbiotic algae and microbes) [8]. Coral diseases are followed by shifts in the composition of coral associated microbial assemblages [9-11], including loss of beneficial microbial activities as well as the proliferation of opportunists and/or pathogens [12-15]. Thus, the composition of the coral-associated microbial assemblage may ultimately serve as an indicator of coral and ecosystem health [16]. The coral reefs of Abrolhos Bank located in eastern Brazil are the largest and richest reefs in the South Atlantic, playing critical ecosystem services and harboring unique marine life. Despite its importance, accelerating rates of coral diseases, particularly white-plague, threaten the Abrolhos reef ecosystem [16].

The coral genus *Mussismilia* is endemic to Brazil and represents more than 70% of the reef structure in several areas of Abrolhos Bank [17]. The fossil record shows that *Mussismilia* is a relict genus that was distributed globally, with origins estimated at 23 Ma to ~400 Ka (reviewed in [18]). At present the genus *Mussismilia* is found only along the Atlantic coast of South America [19]. This genus currently comprises four morphologically distinct species, namely: *M. braziliensis*, *M. harttii*, *M. hispida* and *M. leptophylla*, this latter just recently recognized as belonging to the genus *Mussismilia* [18]. *M. hispida* and *M. braziliensis* appear to harbor distinct mucus-associated microbiotas [20, 21] that can be distinguished from corals with disease symptoms [21].
The purpose of this study was to examine if the microbiota associated with tissue and near-tissue skeleton of the three most abundant *Mussismilia* species (*M. braziliensis*, *M. harttii* and *M. hispida*) were distinct from surrounding seawater and structured by interaction with the coral lineage; to determine whether the coral associated microbiota displays evidence of shared populations (i.e. a “core” microbiota); and to establish a baseline of variability associated with the microbiota of healthy corals relative to two examples of diseased corals. Deep sampling of biological replicates affords the opportunity to examine the interacting roles of natural variability, reef environment, and host lineage on the structure of coral associated microbiota. To this end, we have used 454 pyrosequencing of the 16S rRNA V6 hypervariable region with clone library analysis of near-full length 16S rRNA sequences to characterize the microbiota of twelve presumptively healthy specimens of *Mussismilia* corals collected from two reefs in the Abrolhos Bank, including representatives from the three most abundant *Mussismilia* species. In addition, we have analyzed reef water and two *M. braziliensis* colonies affected by disease including one live coral colony exhibiting symptoms of white plague disease and the bare coral skeleton of a colony recently killed by white plague. This analysis allowed us to determine the baseline microbiota structure in healthy corals to gain insight into how these microbial communities shift during disease and to identify coral lineage-specific microbial associations within the genus *Mussismilia*. Characterizing changes in microbiota during disease may lead to the identification of indicator species of coral health and thus be a valuable tool for coral reef management and conservation.
If coral-associated microbial populations occupy ecological niches that are associated with the holobiont, then community structure may be shaped by selective pressures within the holobiont, and communities associated with a coral host should be most similar among closely related corals and perhaps reveal stably-associated populations i.e. a core microbiota [22-24]. On the other hand, if microbes colonize the holobiont at random (i.e. “neutral assembly”) then coral-associated microbiota would be shaped by the composition of the source community in the surrounding environment and corals from the same reef should harbor more similar microbial assemblages than corals from different reefs. In this case, we would not expect to observe lineage-specific microbiotas in coral-associated microbial assemblages [25]. Herein we present evidence that the *Mussismilia* associated microbiota is both lineage-specific and highly variable suggesting that the holobiont microbial assemblage is shaped by both selection of certain microbial populations within the holobiont and by other unknown and potentially stochastic processes.

**Methods**

*Study Site and Sampling Procedures*

Samples were collected from the Sebastião Gomes (SG) Reef (17°54'42.49"S/39°7'45.94"W) and the Parcel dos Abrolhos (PAB) Reef (17°57'32.7"S/38°30'20.3"W) by SCUBA diving (depth 5 to 8 m) on the 21st and 22nd of January 2009. The SG reef is located in the inner portion of the Abrolhos Bank, in an open access area, whereas the PAB reef is located in the outer portion of the Abrolhos Bank within the Abrolhos National Park, a Marine Protected Area where no fishing is
allowed (i.e. a no-take zone). Fragments were collected from 3 individual colonies of each coral species from genus *Mussismilia*: *M. braziliensis* (PMBR 1-2, PMBR 2-1, PMBR 4-1), *M. hispida* (PMHS 1-3, PMHS 2-4, PMHS 2-3), and *M. harttii* (PMHA 1-5, PMHA 2-5, PMHA 2-6) from the PAB reef and 3 additional fragments of *M. braziliensis* from the SG reef (GMBR 1-8, GMBR 1-7, GMBR 2-7). In addition, fragments of live coral were collected immediately adjacent to coral skeleton that appeared to be recently exposed by White Plague, and from a bare *M. braziliensis* skeleton that was likely a recent (< 1 month) remnant of White Plague due to the minimal degree of visible skeleton colonization by epibionts (R. Moura, pers. Comm.) from the PAB reef. Fragments were processed immediately using a hammer and chisel to remove excess coral skeleton and snap-frozen in liquid nitrogen. Concurrently, 100 ml of seawater from each reef was pre-filtered through a 3.0 µm nucleopore filter, and bacterioplankton were collected on a 0.2 µm STERIVEX™ filter (Millipore, Billerica, MA) and snap frozen in liquid nitrogen. Seawater nutrients, bacterioplankton counts, salinity, and temperature were analyzed by standard oceanographic methods as described previously [26, 27].

*Nucleic Acid Extraction*

Coral fragments of approximately 1 cm² were ground into a powder in liquid nitrogen using a mortar and pestle. Similarly, frozen Sterivex filters containing bacterioplankton were removed from filter cartridges and ground by mortar and pestle. Nucleic acids were extracted from powdered samples using the TRIzol™ reagent according to the manufacturer’s protocol that resulted in isolation of DNA and RNA from each coral specimen. The resulting nucleic acids were further purified using a
phenol:chloroform:isoamylalcohol (25:24:1) extraction to improve quality. Nucleic acids were visualized using agarose gel electrophoresis to evaluate the quality and were quantified using a Nano-drop ND-1000 spectrophotometer.

Bacterial 16S rRNA gene clone libraries

Libraries of nearly full-length 16S rRNA genes were generated for each of the four coral species and reef combinations in the study. Briefly, 50 ng of pooled DNA from each coral species/reef was amplified in triplicate using universal 16S rRNA gene primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) [28] and 1492R (5’-GGTTACCTTGTTACGACTT-3’) [29]. PCRs contained 0.5 Units of Phusion polymerase (Finnzymes Inc., Woburn, MA), 1X buffer, 200 μM dNTPs, and 200 nM of each primer and were subjected to an initial denaturation of 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min; and a final 5 min at 72°C. Gel purified amplicons were cloned using the CloneJET™ PCR Cloning Kit (Fermentas).

Approximately 192 clones from each of the four libraries were screened by PCR and Restriction Fragment Length Polymorphism (RFLP) analysis using HpaII (New England Biolabs). Forty-eight non-redundant RFLP types from each library (192 total) were sequenced uni-directionally using 515F (5’-GTGCCAGCMGCAGCGGTAA-3’) [30] on an ABI 3700. After removal of chimeric sequences identified through Bellerophon [31] sequences were assembled into OTUs at 97% identity using Sequencher 4.010.1 (Gene Codes, Ann Arbor, MI). Representative sequences from each OTU were aligned using Infernal – secondary structure alignment tool [32]. Phylogenetic analysis was performed using maximum likelihood phylogenies within Phyml [33] and was visualized with iTOL.
Nucleotide sequences from 16S rRNA gene clone libraries are deposited at NCBI with accessions JN106583-JN106666.

454 pyrosequencing of the hypervariable V6 region of the bacterial 16S rRNA

16S rRNA gene amplicon libraries of the V6 region were prepared from DNA extracted from 2 water samples and 14 coral samples (Table 1). Multiplex barcodes were used during sequencing to preserve sample identity. PCR primers used contained the Roche 454 sequencing adaptors A- and B- at the 5’ end followed by a 5 nucleotide key and the universal 16S rRNA V6 primer sequence at the 3’ end (A: 5’-gacctccctgcgcctcag-nnnnn-3’, B: 5’-gccttgccagcccgcctcag-nnnnn-3’) [35]. The forward primer was mixture of 5 primers A-967F CNACGCGAAGAACCTTACC, CAACGCGCAGAACCTTACC, ATACGCGARGAACCTTACC, CTAACCGANGAACCTYACC-3’ and the reverse primer was B-1046R CGACRRCCATGCANCACCT-3’ [36]. Triplicate PCRs for each sample were pooled before sequencing to minimize PCR biases. A 20 µl PCR reaction contained 0.5 Units of Phusion DNA polymerase (Finnzymes Inc., Woburn, MA), 1X reaction buffer, 200 µM dNTPs (Invitrogen, Carlsbad, CA), 200 nM of each primer, and 50-100 ng of nucleic acid template or no-template control. The cycling conditions were an initial denaturation of 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension of 72°C for 2 min. Gel purified (Qiagen, Valencia, CA) products were evaluated using ND-1000 Spectrophotometer and on a Bioanalyzer 2100 (Agilent, Palo Alto, CA) using a DNA1000 LabChip and was used for 454 sequencing as described by Sogin et al [35]. Nucleotide sequences from 454
sequencing of the V6 region are available through the Visualization and Analysis of Microbial Population Structures (VAMPS) database (records #LCR0021 to LCR0036).

**Removal of Low-Quality Sequence Tags**

Reads that did not match the primer sequence or nucleotide key, reads <50 bp after primer removal, or contained more than one undetermined nucleotide (N) were removed by the automated VAMPS pipeline [35]. In addition, chimeric sequences were identified using UCHIME [37] and reads that did not align to the secondary structure of the V6 region in the *E. coli* 16S rRNA gene (967-1046 bp) and/or did not form a stable V6 secondary structure were identified using the Infernal secondary structure alignment tool [32] and the CLC RNA Workbench (CLC bio USA, Cambridge, MA), and removed from the dataset.

**Phylogenetic assignment of sequences and estimation of sequence richness**

V6 sequences were processed into operational taxonomic units (OTUs) using the two-step clustering approach outlined in Huse et al [38]. Taxonomic assignment of the V6 OTUs was determined using the Ribosomal Database Project (RDP) classifier tool [39]. A bootstrap threshold of 50% in RDP classifier was used for the V6 sequences to assign sequences to the phylum and sub-phylum levels for comparison across samples [40]. Where individual sequence types are discussed bootstraps >20% are also indicated for tentative classification or designated as “unclassified”. BLASTN was used to compare partial 16S rRNA genes from the four clone libraries to the NCBI nr database and to the V6 OTUs using a threshold of 98% nucleotide identity to determine a match and
sequences identified as derived from chloroplasts were removed from the dataset for subsequent analyses of bacterial community composition.

Statistical analyses

Richness estimators ACE and Chao1 and rarefaction curves were calculated based on the distribution of OTUs in the samples [41]. Bacterial OTU distributions were compared to a model of neutral assembly of single cells using an adaptation of the method by Sloan et al which infers the source community distribution based on averaging local abundances obtained from multiple samples [42]. This method was modified to account for sequencing depth and the robustness of model fit [43]. Principal Coordinate analysis (PCO) of bacterial V6 sequences was determined using resemblance of Bray Curtis similarities constructed from dispersion-weighted standardized abundance data implemented in Primer 6 [44, 45] (Plymouth Marine Laboratory, Plymouth, UK) [46, 47]. For a subset of statistical analyses data was sampled to a common abundance using the downsample function implemented in R (version 2.11.11). Permutational multivariate analysis of variance (Permanova) and SIMPER were implemented in Primer 6 where non-parametric P values for association with coral species or reef site were calculated based on 10,000 iterations using the Monte Carlo method p(MC). SIMilarity PERcentages (SIMPER) was used to identify OTUs contributing to the separation of coral species and were calculated by decomposing average Bray Curtis dissimilarities between all pairs of samples into percentage contributions from each OTU. The abundance of individual OTUs identified by SIMPER or at highest abundance in coral and water samples were visualized using the heat-map tool of Multi Experiment Viewer
(MEV) version 4.7 implementing the Pearson correlation and average linkage method for clustering.

Results

Reef Water Quality

Nutrient and water quality data for the two studied reefs fell within the typical range for continental coastal systems (Table S1) and revealed signatures of elevated nutrients at the near-shore and open-access SG Reef which had 15-fold higher concentration of ammonia (4.5 µM) and ≥2-fold higher concentrations of nitrate (1.15 µM) and silicate (2.10 µM) than the off-shore protected PAB reef. In contrast, higher concentrations of dissolved organic carbon, Chlorophyll-a, and bacterioplankton cell counts were observed at PAB Reef than at the SG reef (Table S1).

Sequencing output and richness

The number of high-quality V6 rRNA sequences obtained from this study was 170,802 with an average of 12,200 sequences per sample (Tables 1, S2, Fig. S1). In addition, a total of 728 clones of nearly full-length 16S rRNA genes were sequenced from the clone libraries (Tables S3, S4, Fig. S2) and used to enhance phylogenetic identification of V6 sequences. The majority of cloned 16S rRNA gene sequences were most similar to populations observed in corals in the Caribbean and Great Barrier Reef (e.g. [11, 48, 49]; Table S4). 27% of the V6 sequence data could be mapped to the 16S rRNA clones (47,033 sequences) (Table S4).
After clustering of V6 sequences at 97% and 95% a total of 5,368 and 5,352 V6-OTUs were identified in the 12 healthy coral samples, respectively with similar OTU distributions per sample (Table 1). Since the clustering method employed has been reported to carry the risk of over-aggregating sequences in complex samples that may result in under estimation of diversity [38] we used the OTUs generated at 97% identity for subsequent microbial community and statistical analysis. Predicted total microbial richness in coral samples ranged from 408 to 5009 bacterial and chloroplast populations (Table 1B) [50-52] and 10,419 (chao1) or 15,296 (ACE) OTUs for the twelve healthy corals combined (Table 1D). In addition, 372 and 2077 OTUs were observed in the diseased sample (WP) and skeleton sample (SK), respectively and 1,052 OTUs in the two water samples (Table 1). No significant differences in the predicted richness of OTUs were observed among the three *Mussismilia* species (ANOVA p=0.859). The rarefaction curves did not reach an asymptote for most samples indicating that richness was under-sampled (Fig. S3).

**Distribution of taxonomic groups in coral and water samples**

The distribution of phyla (and sub-phyla) among the healthy corals revealed a predominance of chloroplasts (4-80%), and sequences that could not be confidently classified (RDP classifier bootstrap >50%) (7-59%) (Fig. 1B, Fig. 2, Table S6). The most abundant sequence type in the healthy coral samples was identified as a Green Algal chloroplast (genus *Ostreobium*) by comparison to the 16S rRNA gene clones (OTU 1) (Tables S4, S6) followed by two unclassified sequence types (OTU 2 and 3) (Fig. 2). Cloned chloroplast sequences had a perfect sequence match to the “bacterial” V6 primers
used in this study. OTUs identified as derived from chloroplasts in the V6 dataset were removed for subsequent analyses focused on the bacterial component of the holobiont. 18 bacterial Phyla were observed in the coral samples while 9 Phyla were observed in the two water samples. Proteobacterial sequences were most abundant in the coral samples (5-67%), dominated by Alpha- (1-35%) and Gamma- (1-22%), followed by the Cyanobacteria (0-23%), Bacteroidetes (1-31%), and Firmicutes (0.25-10%). No significant differences in the distribution of bacterial phyla were observed among the three *Mussismilia* species (ANOVA p = 0.796). The dominant OTUs in coral samples were rare in the water samples and vice-versa. The most frequent sequence types in water samples were Group IIa Cyanobacteria (i.e. *Synechococcus*) (OTUs 9, 37) and Alphaproteobacteria (OTUs 13, 16, 36, 32) (Fig. 2). In contrast, the twelve healthy corals had a unique profile of dominant sequence types that were primarily classified as Bacteroidetes, Firmicutes, Proteobacteria (Alpha-, Beta-, Delta-, and Gamma-), Cyanobacteria, and unclassified Phyla (Fig. 2).

The microbiotas associated with the corals affected by White Plague disease were distinct from the water and deviated from the baseline established from analysis of the 12 healthy corals (Fig. 1C, 3). The diseased sample with live tissue (WP) was dominated by Bacteroidetes (31%), Gammaproteobacteria (21%), and unclassified Proteobacteria (10%) while the most abundant groups in the bare skeleton (SK) were Deltaproteobacteria (15%), Alphaproteobacteria (18%), and Gammaproteobacteria (15%) (Fig. 1). Vibrios represented 2.7% of sequences in the diseased coral compared to <0.025% in the healthy corals (Fig. 3) while Sulfate-Reducing Bacteria-like sequences were enriched in both the diseased coral (12.0%) and the coral skeleton (11.4%) relative
to healthy corals (<0.5%). The most abundant bacterial sequence types corresponded to
Delta-proteobacteria most similar to sulfate reducers (OTUs 8, 14), Alteromonadales
(OTU 17), and Bacteroidetes (OTUs 12, 20). The most abundant bacterial sequence types
in the skeleton included Delta- and Gammaproteobacterial sequences also abundant in the
diseased sample (OTUs 8, 14 and 17) in addition to a Rhodobacterales (OTU 31).

*Shared populations in Mussismilia corals*

Three populations were associated with all 12 healthy *Mussismilia* coral samples:
an Alpha-proteobacteria (OTU19, RDP-65%) most similar to Rhizobiales, and two
unclassified populations most similar to Proteobacteria (OTU6 and OTU26). These three
ubiquitous populations were also observed in the diseased and skeleton samples and were
absent or at much lower frequency in water samples (Fig. 2, S4 A).

Microbiota from the *M. braziliensis* corals collected from two different reefs
(n=6) shared 14 bacterial populations (Figure S4 A) that were also widely distributed
among the other corals. These included: The three ubiquitous populations described
above (OTUs 6, 19 and 26); three Beta-proteobacteria (*Ralstonia* (OTU42, RDP-98%),
*Acidovorax* (OTU249, RDP-88%), *Methyloversatllis* (OTU244, RDP-77%); four
Gammaproteobacteria (OTU24- matching clone MUS012 with 97% identity to
*Pseudoalteromonas issachenkonii*, OTU48, matching clone PHMA012 with 93% identity to
*Vibrio parahaemolyticus*, OTU27 matching clone PMHA018 with 91% identity to
*Methylophaga alcalica*, and an V6 OTU classified as Gammaproteobacterial (OTU242,
RDP-57%); a Firmicute-like population (OTU21, 47%); and three unclassified Bacteria
(OTUs 7, 15, 456) sharing 90-95% nucleotide identity to V6 sequences recovered from other corals [49] and (Table S4).

Prospecting for potential bioindicators of a healthy holobiont

To further explore microbial associations in *M. braziliensis* and to identify potential bioindicators of a healthy holobiont over the sampling period we identified a set of 36 OTUs associated with the majority of healthy *M. braziliensis* samples (4 out of the possible 6 *M. braziliensis* samples, to allow tolerance for undersampling) that were not observed in the skeleton or diseased samples (Fig. S4 B). The majority of the classifiable bacterial populations were Proteobacteria (Alpha and Gamma) with additional populations from the Beta-Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Chlamydiae, Sphingobacteria, and Spirochaetes. Further work is in progress to characterize the distribution of these populations.

Distribution of OTUs in coral and water samples

Principal Coordinate Analysis of bacterial V6 sequences revealed distinction between coral samples, water samples, and the diseased and skeleton samples (Fig. 1 C). To examine whether a robust relationship between coral lineage and microbiota is increasingly evident among more prevalent bacterial populations approximating a core, or stably-associated, microbiota, we sorted the OTUs observed in healthy corals (i.e. HC-OTUs) based on their prevalence in the 12 healthy coral samples ranging from observation in at least 1 coral to detection in all 12 corals. These OTU sets sorted on prevalence were given the categorical labels “In 1” to “In 12”, respectively (Fig. 4). The
majority (76%) of the 5204 HC-OTUs were observed in only a single coral individual (Fig. 4A). The more prevalent an OTU was among the healthy corals, the more likely it was to also be detected in randomized sampling of sequences from the reef water, coral skeleton, or diseased coral sample (Fig. 4B). Proteobacteria (primarily Alpha- and Gamma-) represented an increasing proportion of OTU richness at higher OTU prevalence (Fig. 4C). As expected, the richness of HC-OTUs at all levels of prevalence was highest among the individual healthy corals, however surprisingly HC-OTUs were more likely to be observed in the bare coral skeleton sample than in the diseased coral or water samples, after adjustment for sampling effort (Fig. 4B). While 8.9% of all HC-OTUs (i.e. In 1) were observed in the skeleton, the proportion of skeleton-associated OTUs increased to 34% for HC-OTUs found in at least 3 healthy corals (In 3) and for the OTUs observed in at least 11 healthy corals (In 11) 77% were also found in the skeleton (Fig. 4B).

370 **Lineage-specificity of Mussismilia microbiota**

Permanova of Bray Curtis (BC) similarities followed by Principal Coordinate Analysis (PCO) revealed that Mussismilia microbiota from *M. braziliensis* collected at different sites (i.e. the PAB and SG reefs) were more similar to each other than to microbiota collected from the two other Mussismilia species (i.e. *M. hispida* and *M. harttii* - both from the PAB reef) and that this result was most significant among the 599 OTUs found in at least three corals (In 3) (unbalanced Two-way ANOVA p=0.0069 for coral species) (Figs. 1D, 4D, Table S5) which coincides with the level of biological replication in our study. The species specificity of Mussismilia microbiota structure was
robust and was disrupted by randomization of coral species groupings (p>0.2 for 
randomizations of the “In 3” dataset). Similarity Percentage Analysis (SIMPER) revealed 
that \textit{M. braziliensis} microbiota were enriched in unclassified Bacteria (OTUs 15 and 
518), Proteobacteria (OTUs 27 and 134), an Alphaproteobacteria (OTU19), and an 
Alphaproteobacterium in the genus \textit{Roseovarius} (OTU342). Distinction of the \textit{M. 
hispid}a microbiota was driven by a member of the Betaproteobacterial genus \textit{Shinella} 
(OTU273), while distinction of the \textit{M. hartt}ii microbiota was driven by a population in 
the Alphaproteobacterial Order Rhodospirillales (OTU472) (Fig. 2). While the 
distribution of bacterial OTUs revealed coral species-specificity, this difference was not 
linked to differences in the total bacterial richness or distribution of bacterial phyla, 
which did not appear to vary significantly among coral species or sites.

\textit{Analysis of neutral assembly in the Mussismilia microbiota}

The overall distribution of microbial OTUs in the twelve coral samples did not 
significantly fit a model of neutral assembly of single cells from a common meta-
community (Fig. S5) consistent with structuring of coral-associated communities by non-
random factors such as interactions within the coral holobiont (Fig. S5 A, B). However, 
this does not preclude random assembly of individual populations as single cells or as 
particle-associated biofilms within the holobiont. We noted that the richness of bacterial 
OTU’s in increasing numbers of healthy coral samples showed no saturation, following a 
power-law: \( S = C \times N^z \) where \( S \) is the observed number of OTUs, \( N \) is the number of 
coral individuals and \( C \) and \( z \) are fitting parameters determined by linear regression of the 
log-transformed relationship (\( R^2 > 0.998 \)) where the exponent \( z \) was 0.82-0.85 (Fig. S6).
Such power-law relationships have been previously observed in microbial communities to describe the relationship between species richness and the magnitude of the spatial [53] or temporal [54] interval sampled.

Discussion

Coral-host specificity and a healthy core microbiota

The microbiotas associated with healthy Mussismilia corals were distinct from those in diseased corals or water (Fig. 1B, C, 2 and 3) consistent with previous studies of Mussismilia mucus associated communities [20, 21, 55]. We hypothesized that sets of microbial populations with increasing prevalence in Mussismilia corals would be enriched in microorganisms that were stably-associated with the different coral species and thus would bear signatures of coral lineage-specific selection. Indeed, microbiota from the three Mussismilia species could be distinguished based on their microbiota structure (Table S5), and the effect of coral lineage was most robust when the data was restricted to the 599 OTUs that were observed in three or more coral individuals and are thus likely to be enriched in microbial populations that are stably associated with the coral host. These results suggest coral lineage plays a stronger role than reef site in the assembly of stable microbial associates (Table S5). The microbiotas from M. harttii or M. hispida, which have a broader geographical distribution than M. braziliensis [17], were also structured by coral lineage by clustering analysis and PCO (Fig. 1 C, D). However, whether this clustering is independent of site is an open question that could not be examined in the present study since biological replicates of M. harttii and M. hispida were collected only from a single site.
Similar to the co-dominance of alpha and gamma-proteobacteria among bacterial sequences observed in this study, previous studies of *Mussismilia* mucus microbiota also reveal dominance of Proteobacterial sequences (alpha-, gamma-, and unclassified) [20, 21, 55] where up to 60% of the sequences corresponded to copiotrophic Gammaproteobacteria at a highly degraded coastal reef (Pedra de Leste, Brazil) [55].

Highly represented OTUs in these mucus-microbiota studies were not found to correspond to the most abundant OTUs in the current study (Table 2), possibly due to differences between microbial communities in the coral mucus and the coral tissue/near-tissue skeleton as analyzed in the current study.

**Microbiota of the skeleton and diseased samples**

After adjustment for sample size, the coral skeleton sample harbored a greater number of HC-OTUs than in the diseased sample (WP) or seawater samples (SG and PAB reefs) suggesting that the skeleton may be a habitat for stably associated microbial populations in the *Mussismilia* holobiont (Fig. 4B). Although we cannot rule out that trace levels of free microbial DNA remained on the coral skeleton, several recent studies have pointed to the significance of skeleton-associated (endolithic) microbiota during coral disease [56, 57]. In our study, the most abundant OTU in the skeleton, and many of the healthy coral samples, is a chloroplast sequence from the algae *Ostreobium* that has been described as an endolith among a wide diversity of corals [58, 59] and supporting existence of an active skeleton-associated community of algae and bacteria.

In contrast, the low richness of HC-OTUs in the diseased sample may reflect the decline of stably-associated populations during disease and/or the over-growth of
opportunists that skew the sampling of disease-associated richness. Indeed, rarefaction analysis indicated that the diseased coral was one of two samples where the richness recovered was nearest to saturation indicating that HC-OTUs not sequenced were likely absent or rare in the sample (Fig. S3). Enrichment of bacterial populations within the microbiota of the samples affected by disease (WP and SK) revealed similarities with microbial community composition as reported in previous studies of coral disease, notably enrichment of a *Desulfovibrio* population (OTU 8) in the WP and SK samples [9, 60], enrichment of *Thalassomonas*- and Alteromonadaceae-like sequences in the WP sample [11, 49, 61] and enrichment of *Vibrio* sequences in the WP sample [62-65] (Fig. 4).

*Assembly of coral microbiotas*

The mechanisms and selective pressures that drive the assembly and maintenance of coral microbiotas remain undefined and it is likely that they are structured by a combination of random (neutral) and non-random processes as has been identified in diverse ecosystems including plant communities, soil microbiota, wastewater treatment systems and the human microbiome [66-69]. Increasing evidence suggests that specific microbial populations in the coral holobiont may play important roles in coral physiology, including nutrient acquisition and cycling [1-3] and protection against coral pathogens [4-7]. Whether such beneficial populations are under selection and form part of a “core microbiota” in the coral holobiont is an important open question in coral microbiology.
Core microbiotas have been characterized in an expanding diversity of organisms e.g. [22-24, 70-74] and also have been discounted in others [75, 76]. Microbial genomes defined by their presence in a majority of host individuals have been described as a “core” microbiome [22, 77] and populations possessing these core genes are hypothesized to play a key role in microbiome function [77]. Core microbiotas, i.e. the set of microbial populations defined by their presence in the majority of host individuals, are an imperfect proxy for this core “microbiome” of genome-encoded functions due to functional redundancy among microbial populations. Our study targeted the *Mussismilia* microbiota through 16S rRNA gene sequencing, and revealed 14 microbial populations that were shared among the 6 healthy *M. braziliensis* corals and only 3 microbial populations shared among the 12 *Mussismilia* corals. This level of overlap is not significantly different than what would be expected by chance alone and does not support the hypothesis that *Mussismilia* corals maintain a strictly defined core microbiota that could be detected through our sequencing approach. However, observation that bacterial OTUs with higher prevalence among the corals are more significantly structured by coral lineage than the total set of OTUs supports the idea that this microbial assemblage is indeed structured by interaction with the coral host.

While our finding of lineage-specific trends in microbiota structure indicate that the *Mussismilia* microbiota is structured by interaction with the host or other holobiont members, the vast richness of taxa with low-prevalence among the coral individuals suggests that a subset of the microbiota may be assembled stochastically e.g. by immigration on food particles or from the bacterioplankton. While the overall
Mussismilia-associated bacterial community did not fit a model of neutral assembly (Fig. S5) we have observed that the richness of bacterial OTUs in our samples has a linear relationship on a log-log plot with increasing numbers of coral samples (Fig. 4A and Fig. S6) that is similar to the power-law relationships observed in many different ecological systems between species richness and area [78, 79] or sampling time [54]. Such power law relationships for species richness can be explained by neutral assembly [53, 54] although they do not preclude concurrent niche-based assembly. The prevalence categories, which are represented as log transformed fractions in Figure S6 are correlated to the amount of coral surface area sampled. OTUs in the In 1 category are detected in at least a one of twelve samples, thus the effective area analyzed is at a maximum, corresponding to all 12 samples. On the other hand, OTUs in the In 12 category were detected in every individual coral sample, thus screening of a single coral sample was sufficient to recover the OTU and the effective area sampled is at a minimum. Although this study was not designed to specifically test the existence of a species-area relationship (SAR) or species turn-over rate (STR), variability in the distribution of microbial taxa as a function of space or time may contribute to observed pattern of bacterial richness in our coral samples.

Taken together evidence for lineage-, but not site-, specificity in the microbiota and the high diversity of low-prevalence OTUs in healthy corals suggest that Mussismilia corals may harbor a dynamic assemblage of transitory microbes in addition to a set of microbial populations that are more stably associated and enriched in Alpha- and Gamma-proteobacteria. Observation that several low prevalence/low abundance OTUs in
the healthy corals are enriched in the diseased sample (Fig. 2) further suggests that the dynamic component of the microbiota may include pathogens and opportunistic species. Whether populations with higher prevalence in the microbiota are enriched in functions that are beneficial to the holobiont remains to be determined, however the partitioning of the microbiota with coral lineage and a reduced richness of prevalent OTUs in diseased *M. braziliensis* and the water, suggests some of these populations may interact with the healthy holobiont.

Supplementary information is available online.

The authors declare no conflict of interests.

**Acknowledgments**

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**References**


Table 1. Observed and predicted richness in Abrolhos Bank coral and water samples

Figure 1. Microbial community composition in different species of *Mussismilia* corals. A) Colony and individual polyps of *Mussismilia* species. From top *M. braziliensis*, *M. harttii*, *M. hispida* and *M. braziliensis* with an advancing white plague lesion (images contributed by Pedro Meirelles and JRT). B) Distribution of Phyla and Sub-Phyla in 16S rRNA V6 tag sequences from coral and water samples. C) Principal Coordinate Analysis (PCO) of coral associated bacterial microbiotas performed after chloroplast removal. The microbial community structure in healthy corals are distinctly different from the diseased (White Plague) coral and coral skeleton (both *M. braziliensis*) and from the water. Abbreviations used: PMBR= *M. braziliensis* from Parcel dos Abrolhos reef, GMBR= *M. braziliensis* from Sebastiao Gomes reef, PMHS= *M. hispida* from Parcel dos Abrolhos reef, PMHA= *M. harttii* from Parcel dos Abrolhos reef. PCO axis 1 and 2 together explain 24.3% of the variance for the dataset. D) PCO of 599 OTUs observed in 3 or more coral individuals (i.e. In 3) collected from two reefs *M. braziliensis*, *M. hispida* and *M. harttii* from Parcel de Abrolhos (PAB) and *M. braziliensis* from Sebastian Gomes reef (SG). PCO axis 1 and 2 together explain 37.4% of the variance for the dataset.

Figure 2. Distribution of the most abundant or coral species-associated OTUs in each coral and water samples. OTUs that were in the top 40 most abundant for the
dataset, in the top 5 most abundant for each sample, or were identified by SIMPER analysis as driving the species specific microbiota structure in *Mussismilia* corals.

Heatmap indicates frequency of OTU sequences in the dataset. OTU abundances were standardized to 10,000 sequences per sample, subjected to a log(X+1) transformation followed by addition of 1E-6 to each datapoint to eliminate zeros. Hierarchical clustering of OTUS was carried out in MeV (Multi Experiment Viewer) version 4.6 implementing the Pearson correlation and average linkage method. OTUs indicated by an astrix (*) contributed to the top 10% of the variance between coral species as identified by SIMPER analysis of dispersion weighted data. Taxonomic assignments are based on RDP classifier (bootstraps in brackets) or by match to a cloned 16S rRNA sequence (nucleotide identity given in %). Taxonomic assignments of the V6 sequences represented in the clone library supported use of a 50% RDP classifier bootstrap for phylogenetic assignment of OTUs. For example, with low bootstrap support (RDP 19±11%) 101 Chloroplast V6 OTU’s identified by homology to cloned chloroplast sequences were misidentified by RDP classifier as Bacterial and Archaeal Phyla.

Although for description of individual OTUs we have reported the nearest neighbors for taxonomic assignment any RDP classifier bootstrap below 50% must be interpreted with caution.

![Figure 3. Distribution of Bacterial families harboring characterized coral pathogens and disease associates.](image)

Distribution of sequences with highest identity to the A) family Vibrionaceae, B) Sulfate reducing delta proteobacterial Families C) Family Alteromonadaceae (including *Thalassomonas spp.* and *Alteromonas spp.*) The
classification of each OTU was performed using BLASTN and “RDP Classifier” at a >50% bootstrap. Error bars correspond to standard error of the mean for water and healthy corals while the White Plague (WP) and Skeleton data are from individual samples.

Figure 4. Distribution of the 5,204 bacterial OTUs observed in healthy corals (HC-OTUs) of the genus Mussismilia with increasing prevalence. The prevalence of HC-OTUs in the twelve healthy coral samples is indicated on the independent axis such that “In 1” corresponds to HC-OTUs present in 1 or more coral individuals and represents all OTUs associated with healthy corals while “In 12” corresponds to HC-OTUs present in all 12 healthy coral samples. The shaded area on the plot corresponds to values calculated from 22 or fewer HC-OTUs which are likely to reflect artifacts from small sample size.

(A) The number of OTUs associated with multiple coral samples declines exponentially as the number of samples increase according to $Y = 4581 \times \exp(-0.567X)$; $R^2 = 0.970$ where 5,204 HC-OTUs are in at least one coral (i.e. “In 1”) and the majority of HC-OTUs (76%) are associated with only a single coral sample. (B) The distribution of HC-OTUs in seawater (reefs PAB or SG), While Plague (WP) coral, or Coral Skeleton (SK) reveals that HC-OTUs in each prevalence category were recovered from the bare skeleton sample more frequently than from seawater or the diseased coral. The median and range of HC-OTUs in each healthy sample at each level of prevalence is plotted for reference. (C) Total microbial OTU abundance in healthy corals, seawater, white plague, and skeleton samples were each subsampled to 7,420 sequences with replacement before calculation of percentages. (C) Taxonomic assignment of HC-OTUs at increasing levels of prevalence.
shows an increased proportion of Proteobacteria (RDP classifier bootstrap >50%) (primarily alpha, gamma and unclassified Proteobacteria, data not shown). Error bars are plotted as the standard error of the mean (sem) for each of the 12 coral samples and are not visible outside the symbols. (D) P-values p(MC) for the effect of coral species from two-way PerMANOVA.
Supplementary Materials

Table S1. Water quality parameters for Sebastião Gomes (SG) and Parcel dos Abrolhos (PAB) reefs.

Table S2. V6 tags removed using secondary structure alignment and delta G values.

Table S3. RFLP Screen of 16S rRNA clone libraries prepared from Mussismilia corals.

Table S4. Taxonomic affiliation of cloned 16S rRNA gene sequences and corresponding V6 tag OTUs.

Table S5. Permutational Analysis of Variance of microbiota structure from Mussismilia corals grouped by species and reef site with data filtered by occurrence of OTUs in multiple coral individuals.

Table S6. Excel file with all OTUs, and abundances, with classification from RDP.

Figure S1. Analysis of V6 secondary structure predictions to improve quality control. A) Distribution of ΔG’s for the most stable secondary structure of the 16S rRNA V6 region predicted using the CLC RNA Workbench secondary structure prediction tool (CLC bio USA, Cambridge, MA) with respect to frequency of reads in the current dataset (red) and the V6 reference database at VAMPS (blue). A ΔG value of -4.5 kcal/mol was identified as the minimum ΔG needed to form an acceptable secondary structure. 1.55%
of the reads were removed manually due to misalignment of secondary structure and/or anomalously high delta G values for the V6 stem-loop structure. B) Predicted secondary structures for representative 16S rRNA V6 OTUs.

**Figure S2. Maximum likelihood tree of cloned and sequenced 16S rRNA genes** from each coral species/reef. 16S rRNA from sequenced isolates were included for comparison (*E. coli* region from 515-1300 nt). Sequences were clustered into ribotype groups at 97% identity, and a representative sequence was used for phylogenetic analysis. Type strains are indicated for phylogenetic comparison. Bootstrap values >50% are indicated at branch nodes. Ribotype clusters containing sequences that match 16S V6 tag sequences from this study are highlighted in red and the corresponding V6 tag OTUs are indicated.

**Figure S3. Rarefaction analysis of each sample** based on V6 OTUs generated at 97% identity indicated that diversity was under-sampled despite the deep-sequencing approach. The coral skeleton, and sample PMHS2-3 had notably higher richness than other samples while PMBR2-1 and the White Plague sample appeared to approach saturation. Rarefaction curves were generated using Mothur v.1.9.0. Abbreviations used: PMBR= *M. braziliensis* from Parcel dos Abrolhos reef, GMBR= *M. braziliensis* from Sebastiao Gomes reef, PMHS= *M. hispida* from Parcel dos Abrolhos reef, PMHA= *M. harttii* from Parcel dos Abrolhos reef.

**Figure S4. Identification of populations associated with healthy *M. braziliensis*. A)** Distribution of 15 populations present in n=6 *M. braziliensis*. B) The set of OTUs that
were only observed in association with the healthy *M. braziliensis* samples may be
enriched in populations that serve as bioindicators for a healthy holobiont. 36 microbial
OTUs satisfied the condition of being observed in the majority of healthy *M. braziliensis*
samples (at least 4 of the six) but not observed in the skeleton or diseased sample. These
OTUs may include stable associates of healthy coral tissue that are disrupted during
disease. Heatmaps indicate frequency of OTU sequences in the dataset. OTU abundances
were standardized to 10,000 sequences per sample, subjected to a log(X+1)
transformation followed by addition of 1E-6 to each datapoint to eliminate zeros.
Hierarchical clustering of OTUS was carried out in MeV (Multi Experiment Viewer)
version 4.6 implementing the Pearson correlation and average linkage method.
Taxonomic assignments are based on RDP classifier (bootstraps in brackets) or by match
to a cloned 16S rRNA sequence (nucleotide identity given in %).

**Figure S5. Comparison of the distribution of OTUs in twelve healthy Mussismilia corals to a model of neutral community assembly.** A) The mean relative abundance of
HC-OTUs and the frequency with which the 7525 HC-OTUs appear among the 12
healthy *Mussismilia* corals (i.e. prevalence) is plotted for observed data (points) and
modeled data (line) based on fitting parameter mN as calculated in Sloan et al (2006). B)
Distribution of R^2 values obtained when fitting 50 simulated communities generated
with the fitted parameter mN. The simulated datasets fit the neutral model significantly
better than the observed data (p<0.02), thus a locally-neutral community assembly model
can be rejected for our dataset.
Figure S6. **The average richness of bacterial OTUs (S) in increasing numbers of**

*Mussismilia coral samples* was calculated from i) the total OTU abundance or from OTU abundance in the dataset downsampled without replacement to 583 sequences.

Species accumulation was calculated using the species accumulation function in Primer6 with 1000 permutations considering all coral samples (n=12) (open symbols) or restricted to M. braziliensis corals only (n=6) (crossed symbols). A linear relationship was observed between log-transformed estimates of OTUs observed and number of coral samples under all conditions ($R^2 > 0.998$). Based on this regression the exponent ($z$) was 0.82 to 0.85.
Figure 1
Figure 3
Distribution of OTUs from Healthy Corals

Figure 4
<table>
<thead>
<tr>
<th>Sample</th>
<th>Reads</th>
<th>OTUs (97%)</th>
<th>Chao1 (CI ± 95%)</th>
<th>ACE (CI ± 95%)</th>
<th>OTUs (95%)</th>
<th>Chao1 (CI ± 95%)</th>
<th>ACE (CI ± 95%)</th>
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Sample codes correspond to reef and coral species where
P = Parcel de Abrolhos Reef, G = Sebastião Gomes Reef, MBR = M. braziliensis, MHS = M. hispida, and MHA = M. hartii