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Characterization of Geographically Distinct Bacterial Communities Associated with Coral Mucus Produced by *Acropora* spp. and *Porites* spp.

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Acropora* and *Porites* corals are important reef builders in the Indo-Pacific and Caribbean. Bacteria associated with mucus produced by *Porites* spp. and *Acropora* spp. from Caribbean (Punta Maroma, Mexico) and Indo-Pacific (Hoga and Sampela, Indonesia) reefs were determined. Analysis of pyrosequencing libraries showed that bacterial communities from Caribbean corals were significantly more diverse (H′, 3.18 to 4.25) than their Indonesian counterparts (H′, 2.54 to 3.25). Dominant taxa were *Gammaproteobacteria*, *Alphaproteobacteria*, *Firmicutes*, and *Cyanobacteria*, which varied in relative abundance between coral genera and region. Distinct coral host-specific communities were also found; for example, *Clostridiales* were dominant on *Acropora* spp. (at Hoga and the Mexican Caribbean) compared to *Porites* spp. and seawater. Within the *Gammproteobacteria*, *Halomonas* spp. dominated sequence libraries from *Porites* spp. (49%) and *Acropora* spp. (5.6%) from the Mexican Caribbean, compared to the corresponding Indonesian coral libraries (<2%). Interestingly, with the exception of *Porites* spp. from the Mexican Caribbean, there was also a ubiquity of *Psychrobacter* spp., which dominated *Acropora* and *Porites* libraries from Indonesia and *Acropora* libraries from the Caribbean. In conclusion, there was a dominance of *Halomonas* spp. (associated with *Acropora* and *Porites* [Mexican Caribbean]), *Firmicutes* (associated with *Acropora* [Mexican Caribbean] and with *Acropora* and *Porites* [Hoga]), and *Cyanobacteria* (associated with *Acropora* and *Porites* [Hoga] and *Porites* [Sampela]). This is also the first report describing geographically distinct *Psychrobacter* spp. associated with coral mucus. In addition, the predominance of *Clostridiales* associated with *Acropora* spp. provided additional evidence for coral host-specific microorganisms.

Microorganisms are important to coral reef ecosystems through their roles in carbon/nitrogen cycling (43), coral nutrition (7), response to stress (12), and health and disease (6, 40). Coral bleaching and various coral diseases are increasing due to changes in environmental conditions that result in either an increase in abundance of pathogenic microbes or the expansion of ecological niches occupied by coral pathogens (32, 34, 36). Therefore, understanding the microbial communities associated with corals and how they vary in response to changing environmental conditions is important for understanding the future health of coral reefs.

Previous studies that used both culture-dependent and culture-independent methods demonstrated that coral-associated bacterial communities may be host species specific and differ from those dominating the surrounding reef water (4, 17, 39). Similar bacterial populations have also been found on the same coral species from geographically different locations, and different bacterial communities have been found on different coral species (38, 39). By using clone libraries and sequence analysis, Bourne and Munn (4) found that the majority of clones recovered from the coral tissue of *Pocillopora damicornis* were related to *Gammaproteobacteria*, while *Alphaproteobacteria* were dominant in the coral mucus, thus further supporting the hypothesis that specific bacterium-coral associations exist.

Although many studies have supported the hypothesis that corals harbor unique microbiota, inconsistencies across studies have raised many questions about the specificity and dynamics of associations between corals and microbes. One of the major limitations of these studies is that conventional cloning and sequencing methods do not allow for characterization of the microbial community beyond the most dominant taxa (47). However, current methodologies utilizing pyrosequencing have allowed for detection of rare taxa (45). These rare taxa remain largely unexplored, but they may be extremely important and may become more dominant in response to environmental changes (47).

Currently, little is known about the composition and structure of bacterial communities across reef bioregions and environmental gradients. The genera *Acropora* and *Porites* are dominant corals and important reef builders with representative species in both the Indo-Pacific and Caribbean (48). While acroporids are fast-growing, branching genera, *Porites* are submassive to massive flat corals with a hemispherical shape and slow growth rates. As a result, *Porites* tend to be longer lived, often for hundreds of years, and grow to large sizes (37, 48). The Caribbean *Porites asteroides* may grow up to 1 m but tends to form more numerous, smaller colonies, while *Porites lutea* from Indonesia grows up to a few meters (37). The Caribbean *Acropora palmata* grows up to 2 m, and *Acropora formosa* from Indonesia grows on average up to 1 m. Both genera occur in shallow, tropical reef environments, reef slopes, and in lagoons (48).

This study aimed to apply deep sequencing analysis to compare the bacterial community structures associated with the coral mucus produced by *Porites astreoides* and *Acropora palmata* from

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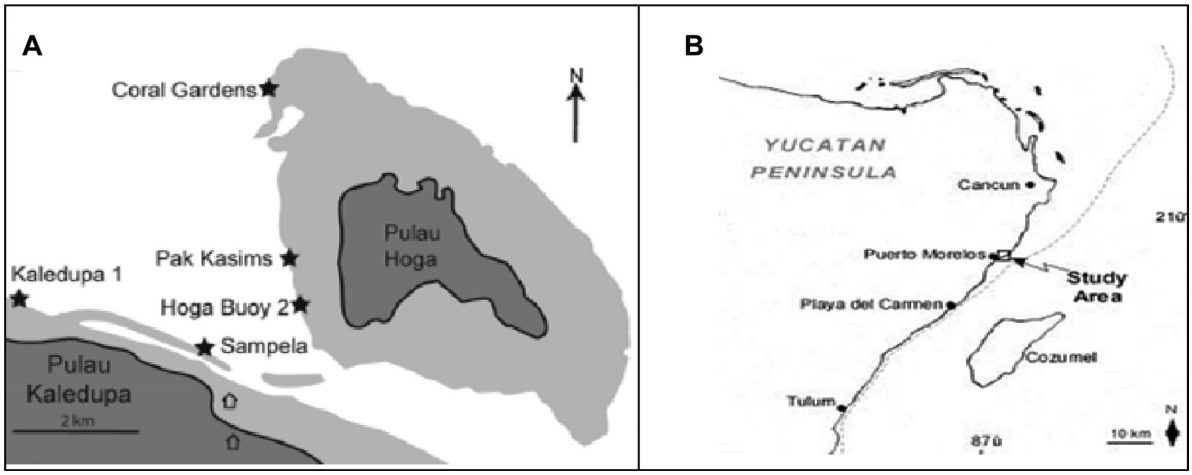

FIG 1 Sampling sites. (A) Map of the Wakatobi National Park, Sulawesi, Indonesia, showing the Sampela and Hoga (Hoga buoy 2) reefs. (Reprinted from reference 19 with permission of the publisher.) (B) Map showing Punta Maroma on the northeastern coast of the Yucatan Peninsula, Mexico.

Mexican Caribbean reefs with those associated with the closely related *Porites lutea* and *Acropora formosa* found in Indonesia (southeast Sulawesi, Hoga and Sampela reefs). Such information will provide a better understanding of the diversity of microorganisms associated with these important coral species and further our understanding of coral physiology, health, and ecosystems.

MATERIALS AND METHODS

Site location and mucus collection. A total of 54 mucus samples (derived from three regions on a colony, from triplicate living colonies) of *Porites astreoides* and *Acropora palmata* at Punta Maroma, Mexican Caribbean (20°43′N, 86°57′W; 18 samples) and the related coral species *Porites lutea* and *Acropora formosa* at Hoga (18 samples) and Sampela (18 samples), Sulawesi, Indonesia (123°46′E, 5°28′S) (Fig. 1) were sampled in July 2007 by using sterile syringes (as described by Ducklow and Mitchell [12]). The mucus samples were filtered using 0.22-μm filters, and the filters were stored at −20°C. Overlying seawater (two 500-ml volumes) adjacent to the coral colonies was also collected at a depth of 10 m, filtered through 0.22-μm filters, and stored at −20°C. The water temperature during collections at both sites was typically 28°C.

DNA extraction, 16S rRNA gene amplification, and DGGE analysis. Total community DNA was extracted from the filtered seawater and mucus samples (from pooled colony regions on each colony replicate) by using a modified beadbeating method (26). Eubacterial 16S rRNA genes were PCR amplified using the primers for positions 341 to 534 in *Escherichia coli* (Table 1) (28). PCRs were performed in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) with the following mixtures (50-μl volumes): 1× buffer (Qiagen), 0.2 mM deoxynucleoside triphosphates (Fermentas), 0.4 μM each primer, 2.5 U *Taq* DNA polymerase (Qiagen), and approximately 25 ng of DNA (26). PCR cycling conditions were as follows: 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and then 72°C for 10 min and holding at 4°C. PCR products were analyzed using 1% (wt/vol) 1× TAE agarose gels (40 mM Tris-acetate, 1.0 mM EDTA; pH 8.0), stained with ethidium bromide (0.5 mg liter^{-1}), and visualized under UV light by using the Gel-Doc system (Bio-Rad). Denaturing gradient gel electrophoresis (DGGE) was performed as described previously (26), except that gels were silver stained (30).

TABLE 1 Summary of PCR primer sequences used in this study

Primer	Sequence (5′–3′)	Reference
pA	AGAGTTTGATCCTGGCTCAG	15
pH′	AAGGAGGTGATCCAGCCGCA	15
341	CCTACGGGAGGCAGCAG	28
534	ATTACCGCGGCTGCTGG	28
GC clamp[a]	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAG	28

[a] Primer 341 contains a 40-nucleotide GC-rich sequence (GC clamp) for DGGE analysis.

Clone libraries. PCR products were obtained using the primers pA/pH′ (Table 1) and cycling conditions previously described (15). The PCR products were ligated into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. Ligations were transformed into high-efficiency JM109 *Escherichia coli* cells (Promega) according to the manufacturer's instructions. Plasmids of transformed *E. coli* were purified using a plasmid purification kit (Qiagen) according to the manufacturer's instructions.

Sequencing. 16S rRNA gene sequencing was performed from selected clones by Geneservice Ltd., Cambridge, United Kingdom. Partial sequences (>500 bp) were obtained from clones using the primer pH′ (Table 1) (15). The closest phylogenetic relatives were compared with those in the GenBank database by using the Basic Local Alignment Search Tool (BLAST) network service (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (1). Sequences were aligned with sequences from GenBank by using the RDP INFERNAL alignment tool (29). Analysis was performed using PHYLIP 3.4 (16) with Jukes-Cantor DNA distance correction and neighbor-joining methods (21, 42). Bootstrap analysis was based on 100 replicates, using SEQBOOT and CONSENSE (PHYLIP 3.4). Tree construction was performed using Treeview (WIN32; version 1.5.2) (31). For pyrosequencing, PCR products were obtained as described above using the primers F341-GC and 534 R (Table 1) (28), except that the forward primer had no GC clamp and a 5′ modification with a 454 amplicon adaptor followed by a unique 10-nucleotide barcode (2, 33). PCR products were quantified with a Nanodrop ND-1000 spectrophotometer, and replicate samples were pooled in equal amounts. Approximately 200 ng of pooled sample was analyzed by pyrosequencing by the NERC Biomolecular Analysis Facility.

Pyrosequence reads were analyzed using the QIIME pipeline and its associated modules (8). All sequences were checked for the presence of correct pyrosequencing adaptors, 10-bp barcodes, and taxon-specific primers, and any sequences containing errors in these primer regions were removed. In addition, sequences of <150 bp or >200 bp in read length, sequences with low quality scores (<20), and sequences containing homopolymer inserts were also removed from further analysis. All pyrose-

quence reads were clustered into operational taxonomic units (OTUs) by using the UClust algorithm (14). Representative sequences from each OTU were identified using the RDP classifier, which assigns taxonomic identities against the RDP database by using a naïve Bayesian classifier (49). Finally, all singletons were removed before further analysis.

Statistical analysis. Similarity between DGGE profiles was calculated using binary data indicating the presence of particular bands (Jaccard's index) and a hierarchal cluster analysis constructed using Primer E software version 6 (9). Compositional changes in bacterial assemblages across coral species and sites were analyzed by nonmetric multidimensional scaling (NMDS) ordination of distance matrices calculated from the OTU pyrosequence-read matrix and using Jaccard's index (24, 25). Differences in composition between sites were assessed using permutation-based multivariate analysis of variance (PERMANOVA), based on the distance matrix calculated using Jaccard's index and using 10,000 randomizations. Geographic location (either the Mexican Caribbean, Sampela, or Hoga) and coral species/water sample (either *Porites*, *Acropora*, or seawater) were used as independent factors, but the interaction between these two factors was not explored.

Species diversity (number and relative abundance of OTUs) was calculated using the Shannon-Wiener index and compared between sites and corals by using a simple randomization test, based on 10,000 randomizations (46). This randomization approach was also used to compare Jaccard index results between coral species. The randomization approach used treats the entire community as a single data set and is an absolute statistical measure that does not require replication to produce probabilities. This approach is commonly employed in community ecology studies where full replication of sampled communities is impossible (25). All analyses were conducted in the R statistical language version 2.7.2 and using the R standard libraries and the community ecology analysis-specific package Vegan (version 2007; R Development Core Team).

Nucleotide sequence accession numbers. Clone sequences were submitted to GenBank and assigned accession numbers HQ456683 to HQ456770.

RESULTS

Community and phylogenetic analyses. Bacterial communities from coral and seawater samples were analyzed by 16S rRNA PCR-DGGE analysis (see Fig. S1 in the supplemental material). There were clear differences in DGGE profiles between corals and their geographical regions (see Fig. S1). Profiles were, however, similar between colony replicates, and so the replicates were pooled for detailed community analysis by pyrosequencing of the 16S rRNA gene. Libraries comprising a total of 9,353 sequences (once sequences of <150 bp or >200 bp in read length, sequences with low quality scores [<20], and sequences containing homopolymer inserts were removed) derived from pooled coral mucus samples produced by *P. astreoides* and *A. palmata* (Mexican Caribbean) and *P. lutea* and *A. formosa* (Hoga and Sampela, Indonesia) were analyzed (Table 2).

NMDS ordination revealed distinct microbial communities associated with samples from geographically distinct regions (Table 2; Fig. 2). PERMANOVA results supported the NMDS ordination and showed that the compositions of the bacterial assemblages were significantly different between geographic sites (PERMANOVA, based on 10,000 randomizations; $F_{1,8} = 2.08$; $P = 0.03$), but not between coral species and seawater samples ($F_{1,8} = 0.58$; $P = 0.88$). The most noticeable result was that bacterial communities from the corals and seawater in the Mexican Caribbean were clearly distinct from those from Indonesia (Fig. 2). Although across all sites there was no significant difference between coral species in the composition of the associated bacterial assemblages (see above), by focusing only on data from the Mexican Caribbean we found significant compositionally distinct assemblages of bacteria from *P. astreoides*, *A. palmata*, and seawater samples (simple pairwise randomization test based on 10,000 randomizations; Jaccard's index, >0.89; $P < 0.01$ in all cases).

Clustering and classification (Table 2) and diversity analysis (Table 2; Fig. 3A and B) of pyrosequencing libraries revealed geographically distinct coral-bacteria associations. In general, bacterial assemblages from the Mexican Caribbean corals were the most diverse (H′, 3.18 to 4.25), followed by samples from Hoga (H′, 3.25), with samples from Sampela containing the least diverse bacterial assemblages (H′, 2.54 to 2.64) (Fig. 3B). However, most samples had similar levels of bacterial diversity, and only in bacterial assemblages from the Mexican Caribbean *Acropora* samples were diversity levels significantly higher than those from other sites or corals (simple pairwise randomization test based on 10,000 randomizations; ΔH′, >0.87; $P < 0.001$ in all cases) (Fig. 3B). Analyses of diversity indices were supported by analysis of rarefied species richness, which provided quantitatively similar results but accounted for differences in sequencing intensities between samples (Fig. 3A).

Overall, *Gammaproteobacteria* dominated pyrosequencing libraries from *Acropora* (69.7%) and *Porites* (59.2%) (at Hoga) and from *Porites* (76.1%) (from the Mexican Caribbean) (Table 2). This was in contrast to *Acropora* and *Porites* libraries from Sampela, which were dominated by *Alphaproteobacteria* (comprising >54.3%). Bacterial communities that differed with coral genera (from either Indonesia or the Caribbean) were also observed. For example, within the *Gammaproteobacteria*, *Halomonas* spp. (49%) and *Alteromonadales* (9.1%) were predominant in *Porites* libraries (from the Mexican Caribbean), compared to *Acropora* at the same location and the corresponding Indonesian corals (<5.6%) (Table 2). Other differences in coral-associated bacteria included *Clostridiales*, which were more predominant from *Acropora* (16.0%) than from *Porites* (5.4%) (at Hoga). Similarly, in the Mexican Caribbean, 7.2% of sequences were related to *Clostridiales* from *Acropora*, compared to 0.2% from *Porites* libraries. In Hoga, cyanobacteria were predominant in *Porites* libraries (28%), compared to *Acropora* (6.4%) and the corresponding corals from Sampela and the Caribbean, where cyanobacteria were much rarer (<5.2%).

As with Indonesia, other distinct sequences were associated with the corals found in the Mexican Caribbean (Table 2). For example, there was a relative dominance of *Firmicutes* (24.1%), primarily bacilli (16.7%), found in *Acropora* libraries, but *Firmicutes* were much rarer (<2.8%) in the *Porites* libraries from the same region. Additionally, within the *Rhodobacteriales*, *Silicibacter* spp. were more dominant in the Mexican Caribbean *Acropora* libraries (6.2%) than *Porites* (2.0%) and the corresponding Indonesian coral libraries (<0.2%). In contrast, *Sulfitobacter* spp. dominated both coral libraries from Sampela (comprising >47.3%) compared to the corresponding coral libraries from Hoga and the Mexican Caribbean (<3.2%).

Similarities were also found with the coral-associated bacterial communities (from either Indonesia or the Caribbean) (Table 2). For example, *Psychrobacter* spp. were ubiquitous in all coral libraries (with the exception of *Porites* from the Mexican Caribbean). *Psychrobacter* spp. were also more dominant in *Acropora* (63.4%) and *Porites* (53.4%) from Hoga (compared to the corresponding corals in Sampela [27.1% and 30.1%, respectively] and *Acropora* [26.7%] from the Mexican Caribbean) (Table 2). Similarly, cya-

TABLE 2 Bacterial assemblages based on 16S rRNA pyrosequencing libraries from coral mucus and seawater

Phylum, class, or order	% of sequences (total *n*) in phylum, class, or order from sample area[a]								
	Indonesia						Mexican Caribbean		
	HA (1,264)	HP (924)	HSW (1,083)	SA (1,110)	SP (1,169)	SSW (820)	MA (1,887)	MP (635)	MSW (461)
Actinobacteria	0	0.1	0	0	0.1	0.2	0.5	0.2	0.6
Bacteroidetes	0	0	**6.2**	**5.8**	2.3	4.1	0.1	0.2	0.4
Firmicutes	**16.5**	**6.4**	0	0.7	1.4	2.1	**24.1**	2.8	2.4
Clostridiales	**16.0**	**5.4**	0	0.2	1.1	0.1	**7.2**	0.2	0.2
Bacilli	0.2	1.0	0	0.5	0.3	2.0	**16.7**	2.7	2.2
Unclassified *Firmicutes*	0.2	0	0	0	0	0	0.2	0	0
Fusobacteria	0.6	1.2	0	0	0.2	0	0	0	0
Proteobacteria	4.4	2.4	**20.0**	**59.0**	**54.3**	**67.7**	**28.3**	**14.0**	**43.2**
Alphaproteobacteria									
Caulobacterales	0.1	0	0	0	0	0	0.3	0	0.7
Rhizobiales	0.2	0	0.1	0.1	0.1	0.4	3.0	1.4	3.3
Rhodobacterales (not including *Silicibacter* spp. or *Sulfitobacter* spp.)	0.7	0.9	1.5	**6.6**	**5.4**	3.9	**11.7**	**7.2**	2.2
Silicibacter spp.	0	0.2	0	0	0	0.4	**6.2**	2.0	1.3
Sulfitobacter spp.	3.2	1.2	**18.2**	**48.3**	**47.3**	**61.8**	1.6	0.3	0.4
Rhodospirillales	0	0	0	0	0	0	0.1	0	0
Sphingomonadales	0.2	0	0.2	4.0	1.4	1.1	1.2	0	**31.9**
Unclassified *Alphaproteobacteria*	0.2	0.1	0	0.3	0.2	0.1	4.5	3.0	3.5
Betaproteobacteria	0	0	0	0	0	0	0	1.7	0
Gammaproteobacteria	**69.7**	**59.2**	**70.1**	**31.2**	**35.2**	**23.6**	**37.0**	**76.1**	**26.7**
Alteromonadales	0	0	0	0	0.4	0	0.3	**9.1**	0.4
Oceanospirillales (not including *Halomonas* spp.)	0.4	0.2	0.6	0.2	0	0	0.8	4.1	0.2
Halomonas spp.	2.0	2.0	3.4	0.6	1.9	3.4	**5.6**	**49.0**	0.4
Pseudomonadales (not including *Acinetobacter* spp., *Psychrobacter* spp.)	2.6	1.8	3.6	3.1	1.6	3.4	1.5	2.1	0.7
Acinetobacter spp.	0	0	0	0	0	0	0.4	**6.9**	**12.8**
Psychrobacter spp.	**63.4**	**53.4**	**63.0**	**27.1**	**30.1**	**18.2**	**26.7**	0.6	**9.1**
Xanthomonadales	0	0	0	0	0	0	0	0	1.1
Vibrionales	0	0.2	0	0	0	0	0	0	0
Unclassified *Gammaproteobacteria*	1.3	1.6	0.3	0.2	0.4	0.6	2.0	3.1	1.1
Verrucomicrobia; *Verrucomicrobiales*	0	0	0	0	0	0	0.1	0	0
Cyanobacteria	**6.4**	**28.0**	1.8	1.8	**5.2**	1.0	3.7	1.7	**23.6**
Planctomycetes	0	0	0	0	0	0	0.2	0	0
Unclassified bacteria	1.8	2.7	0.5	0.1	0.3	0	3.0	1.1	2.4

[a] Samples were from *Acropora formosa* at Hoga (HA), *Porites lutea* at Hoga (HP), seawater at Hoga (HSW), *Acropora formosa* at Sampela (SA), *Porites lutea* at Sampela (SP), seawater at Sampela (SSW), *Acropora palmata* at the Mexican Caribbean (MA), *Porites astreoides* at the Mexican Caribbean (MP), and seawater at the Mexican Caribbean (MSW). Numbers in bold indicate that the group represents >5% of the community.

nobacteria (28%) were also more dominant in *Porites* than in *Acropora* libraries from Hoga (6.4%) and the corresponding coral libraries from Sampela (<5.2%) and the Mexican Caribbean (<3.7%).

Not surprisingly, there were also differences observed in seawater bacterial communities from Indonesia and the Mexican Caribbean (Table 2). For example, *Gammaproteobacteria* (70.1%) were dominant in seawater from Hoga, Indonesia, compared to seawater from Sampela (23.6%) and the Mexican Caribbean (26.7%). Conversely, *Alphaproteobacteria* (67.7%) were more dominant in seawater from Sampela, Indonesia, than in seawater from Hoga (20.0%) and the Mexican Caribbean (42.3%). In addition to *Alpha-* and *Gammaproteobacteria*, seawater from the Mexican Caribbean was dominated by *Sphingomonadales* (31.9%), *Cyanobacteria* (23.6%), and *Acinetobacter* spp. (12.8%).

Clone libraries. In order to obtain almost-full-length 16S rRNA gene sequences for phylogenetic analysis, clone libraries were generated from each coral sample (Fig. 4A and B). Libraries were screened by DGGE, and from a total of 317 clones, 106 partial 16S rRNA gene sequences (of >500 bp) were obtained (distributed relatively evenly across all samples). Generally, there was good agreement between clone and pyrosequencing libraries, with discrete sequence clusters associated with each coral. For example, sequences from *Acropora* and *Porites* (at Hoga) predominantly clustered within the *Gammaproteobacteria* (Fig. 4A), with discrete clusters associated with *Psychrobacter* spp. and *Halomonas* spp.

In addition to *Gammaproteobacteria*, one clone (HP7) from *Porites* (Hoga) grouped with *Synechococcus* spp. within the *Cyanobacteria*, and two clones (HA11 and MA13) from *Acropora* (from Hoga and the Mexican Caribbean, respectively) clustered

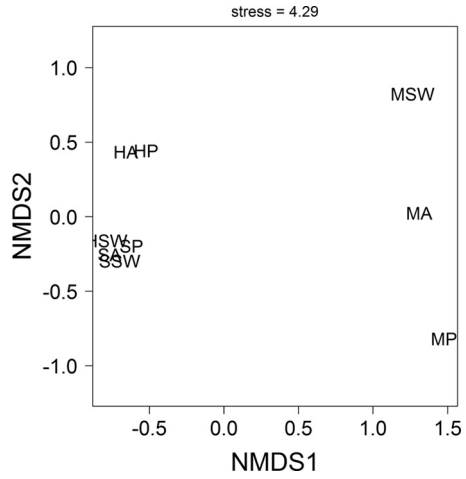

FIG 2 NMDS ordination of distance matrices calculated from the OTU pyrosequence read matrix and using Jaccard's index. Shown are the bacterial communities associated with *Acropora formosa* at Hoga (HA), *Porites lutea* at Hoga (HP), seawater at Hoga (HSW), *Acropora formosa* at Sampela (SA), *Porites lutea* at Sampela (SP), seawater at Sampela (SSW), *Acropora palmata* at the Mexican Caribbean (MA), *Porites astreoides* at the Mexican Caribbean (MP), and seawater at the Mexican Caribbean (MSW) based on the 454 pyrosequencing libraries.

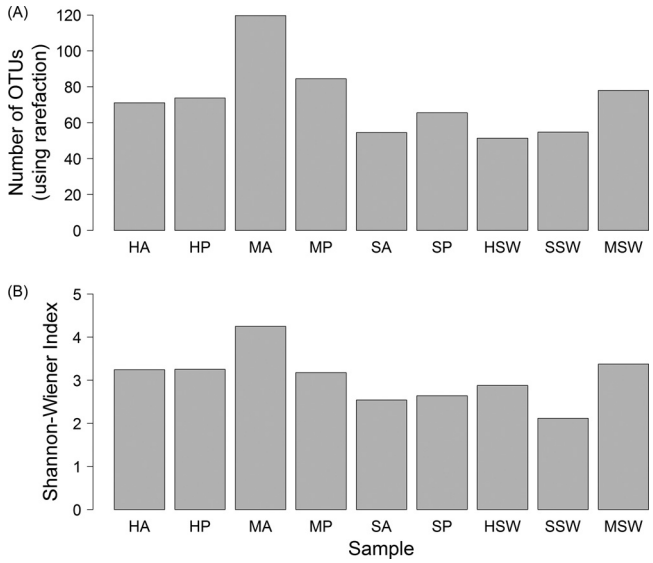

FIG 3 Bacterial community diversity based on the analysis of rarefied species (OTU) richness (A) and the Shannon-Wiener diversity index (B). OTUs were defined using the UClust algorithm with clustering at the 5% level and assigned taxonomic identities using the RDP classifier. Samples are from *Acropora formosa* at Hoga (HA), *Porites lutea* at Hoga (HP), seawater at Hoga (HSW), *Acropora formosa* at Sampela (SA), *Porites lutea* at Sampela (SP), seawater at Sampela (SSW), *Acropora palmata* at the Mexican Caribbean (MA), *Porites astreoides* at the Mexican Caribbean (MP), and seawater at the Mexican Caribbean (MSW).

with an uncultured Gram-positive bacterium with strong bootstrap support (Fig. 4B). Sequences from *Acropora* (Sampela) were dominant within the *Alphaproteobacteria*, followed by *Gammaproteobacteria* (Fig. 4A and B). Dominant sequences from *Acropora* (Sampela) within the *Alphaproteobacteria* were related to *Sulfitobacter* spp., while dominant clone sequences from *Porites* (Sampela) affiliated with *Gammaproteobacteria*, including *Halomonas* spp., *Alteromonas* spp., and *Psychrobacter* spp. In addition to *Gammaproteobacteria* sequences, one clone (SP19) associated with *Porites* from Sampela clustered with *Synechococcus* spp. within the *Cyanobacteria*. Another clone (SP12) associated with *Porites* from Sampela clustered with *Exiguobacterium* spp. within the *Firmicutes*.

In comparison to clones from Indonesian corals, the clone libraries from *Acropora* and *Porites* from the Mexican Caribbean spanned several phyla, including *Gamma-* and *Alphaproteobacteria*, *Firmicutes*, *Altermonadales*, and *Actinobacteria* (Fig. 4A and B). In contrast to the dominance of *Gammaproteobacteria* in samples from Indonesia, only three clones from *Acropora* (Mexican Caribbean) clustered within the *Gammaproteobacteria*. In addition, two clones (MA2 and MA11) were closely related to *Exiguobacterium* spp. within the *Firmicutes* with strong bootstrap support (100%) (Fig. 4B).

Gammaproteobacteria (specifically, *Halomonas* spp. and *Alteromonas* spp.) dominated the *Porites* library (Mexican Caribbean), followed by *Alphaproteobacteria*. We also found that no clone sequences from the *Porites* library (Mexican Caribbean) clustered with *Psychrobacter* spp. In addition, two clones (MA1 and MP1; from *Acropora* and *Porites* libraries, respectively) had 99% sequence identity to *Dietzia* spp. within the *Actinobacteria*, and one clone (MP14) from the *Porites* library grouped with *Paracoccus* spp. with strong bootstrap support (100%).

Although clone libraries generally corroborated information from pyrosequencing libraries, some differences were observed, which may have been due to differences in sequencing efforts. For example, *Alphaproteobacteria* were dominant in the pyrosequencing library from *Porites* for Sampela (54.3%), but only one clone sequence (SP7) was found within the *Alphaproteobacteria* (Fig. 4B). Conversely, *Vibrionales* were rarer in the pyrosequencing library, while three clone sequences (HP6, HP10, and HP13) from *Porites* (Hoga) grouped with *Vibrio* spp. with strong bootstrap support (96%) (Fig. 4A).

DISCUSSION

The coral mucus samples yielded more diverse 16S rRNA pyrosequecing libraries than seawater. This finding, coupled with the fact that particular phylotypes were present in greater abundance, suggests discrete bacterial associations with their coral hosts. Here, we found a high relative abundance of *Gammaproteobacteria* sequences associated with *Porites* from the Mexican Caribbean, followed by *Acropora* from the Mexican Caribbean and *Acropora* and *Porites* from Sampela. Similar findings have been previously reported, whereby there was an association of *Gammaproteobacteria* with *Porites astreoides* from Panama and Bermuda (39). Furthermore, a high relative abundance of *Gammaproteobacteria* in the coral *Montastrea cavernosa* from the Mexican Caribbean has also been found (17). In our study, within the *Gammaproteobacteria* there was a predominance of sequences relating to *Halomonas* spp. associated with *Porites* and *Acropora* from the Mexican Caribbean; in addition, this is the first study to report an abundance of sequences relating to *Psychrobacter* spp. associated with both *Acropora* spp. and *Porites* spp.

In addition to *Gammaproteobacteria*, another study found *Alphaproteobacteria* to be the dominant microbial group within the coral mucus of *Pocillopora damicornis* from the Great Barrier Reef (4). More specifically, 36% of the clones were affiliated with


A
Bacillus subtilis EU382220
SP16
Halomonas sp. AY573034
HA2
HA3
HA4
SP13
SP15
SP2
SP8
MP16
Halomonas meridiana EU441001
Halomonas sp. AJ551131
Halomonas sp. AM403725
Halomonas sp. AJ551139
Halomonas aquamarina EU440965
Halomonas sp. AB022662
Halomonas venusta AB362301
Halomonas axialensis AF212206
Halomonas aquamarina EU430083
Halomonas aquamarina EF143425
Halomonas aquamarina EU684464
HA13
MP22
MA9
SP3
MP7
MP15
MP13
MP11
MP6
Oceanospirillales
SA4
SP10
SP18
Psychrobacter pulmonis EF101551
Psychrobacter marincola EU652050
Psychrobacter salsus AJ539104
Hyphomicrobium sp. AJ551095
Psychrobacter sp. AJ551125
Psychrobacter luti AJ430828
Psychrobacter psychrophilus EU196312
Psychrobacter maritimus AJ609272
Psychrobacter psychrophilus AJ748270
Hyphomicrobium sp. DQ200849
HA1
MA4
Psychrobacter glacincola AY167310
Psychrobacter nivimaris EF101544
MA8
HP5
Psychrobacter meningitidis AY057116
Psychrobacter sp. EU246833
Psychrobacter piscidermidis EU127295
Psychrobacter pacificensis EF179615
Psychrobacter marincola EF179616
HA12
HA6
HA5
HA7
HA10
HA14
HA8
HA9
SA6
SP6
HP9
HP4
HP3
HP14
SA5
Psychrobacter sp. EU781517
Psychrobacter celer AY842259
Psychrobacter celer EF101550
Pseudomonadales
SP5
Pseudoalteromonas sp. AM422562
Pseudoalteromonas ganghwensis DQ011614
Vibrio aestuarianus AJ845016
Vibrio pomeroyi AJ491290
Vibrio splendidus AB038030
Vibrio chagasii AJ490157
Vibrio lentus AJ278881
Vibrio kanaloae AM162657
HP10
HP6
HP13
Vibrionales
Alteromonas alvinellae AF288360
Alteromonas macleodii AM885869
Alteromonas macleodii AM885870
Alteromonas marina AF529061
Alteromonas marina AY881234
Alteromonas sp. AM990853
MP19
MP9
MP21
SP17
SP4
Alteromonas sp. AY138988
SP20
Alteromonadales
Gammaproteobacteria

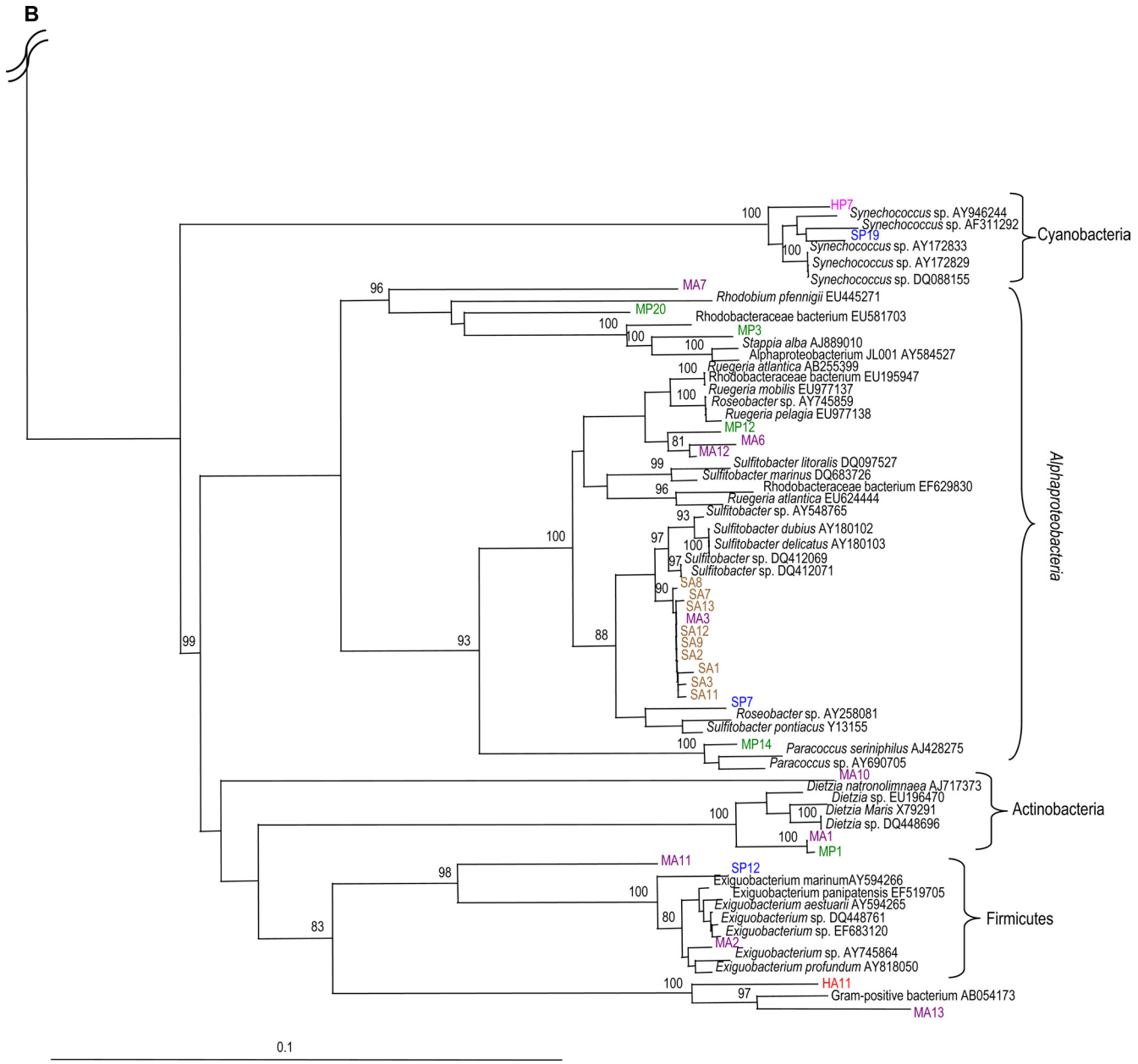

FIG 4 Phylogenetic analysis of the 16S rRNA gene sequences from selected clones. Included are type strains obtained from GenBank. Sequence analysis was performed on common partial sequences (>500 bp) by using Jukes-Cantor DNA distance and neighbor-joining methods. Bootstrap values represent percentages from 100 replicates of the data; percentages of >80% are shown. Bar, 0.1 substitutions per nucleotide base. The 16S rRNA gene sequences from clones clustered within the *Gammaproteobacteria* (A) and within the *Alphaproteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, and uncultured bacteria (B). Clone sequences are color coded as follows: *Acropora formosa* at Hoga (HA; red), *Porites lutea* at Hoga (HP; pink), *Acropora formosa* at Sampela (SA; brown), *Porites lutea* at Sampela (SP; blue), *Acropora palmata* at the Mexican Caribbean (MA; purple), and *Porites astreoides* at the Mexican Caribbean (MP; green). Unique clone identifiers are shown following the species names.

Alphaproteobacteria and only 16% with *Gammaproteobacteria* (4). In our study, *Alphaproteobacteria* also dominated microbial communities associated with *Acropora* and *Porites* from Sampela and *Acropora* and *Porites* from the Mexican Caribbean.

It was previously suggested that mucus of different coral species enriches for different bacterial communities (12, 37). In our study, differences in the library compositions in the coral mucus of *Porites* spp. versus *Acropora* spp. were observed. A metagenomic analysis of the microbial community associated with the coral *P. astreoides* in Panama found that the most prominent bacteria were *Proteobacteria* (68%), followed by *Firmicutes* (10%), *Cyanobacteria* (7%), and *Actinobacteria* (6%) (50), similar to the findings in the present study. Interestingly, in the present study, within the *Firmicutes*, a high abundance of *Clostridiales* sequences was recovered from *Acropora* from Hoga and the Mexican Caribbean (compared to *Porites* and seawater samples from the same location), suggesting that *Clostridiales* are strongly associated with coral mucus from *Acropora* spp. in both regions. It is possible that

the *Clostridia* spp. may play a role in the breakdown of complex carbon compounds present in the mucus produced by *Acropora*, although the available evidence remains inconclusive. Since clostridia are generally obligate anaerobes, it is possible that oxygen becomes depleted during complex carbon degradation, generating anaerobic or low-oxygen microniches within the thick mucus and facilitating their proliferation.

It is known that *Porites* spp. produce more and denser mucus than *Acropora* spp. (12), which may be attributable to its greater tolerance to sedimentation (7, 27). Furthermore, metagenomic studies of microbial communities associated with *P. astreoides* have shown that coral-associated bacteria possess a large number of genes for the uptake and processing of protein and sugars, reflecting the compounds found in coral mucus (50). However, it is also entirely possible that bacteria associated with other corals may possess similar genes.

The Hoga reef in this study has been designated a protected area (10, 11). The Sampela reef is in an enclosed lagoon, buffered from the Hoga-Kaledupa Channel by an outer reef wall and located approximately 400 m away from the Bajau village, which has a population of >1,500 people (3, 44). Due to continued human activities, the Sampela reef has low light availability and high sedimentation rates (10). Specifically, Sampela sedimentation rates are around 2-fold higher (11.5 mg cm^{-2} day^{-1}) than at Hoga (<5 mg cm^{-2} day^{-1}) (20). Consequently, corals at Sampela may be more likely to be stressed and produce more mucus (27).

It has also been suggested that coral bleaching and coral diseases may be more likely to occur in Hoga than Sampela due to high light exposure (5, 18), resulting in either an increase in abundance of pathogens or the expansion of ecological niches occupied by coral pathogens, like *Vibrio* spp. (32, 34, 36). In our study, three clone sequences relating to *Vibrio* spp. (from *Porites* at Hoga) were recovered, suggesting that the corals at Hoga may be more likely to become infected than those from Sampela, where low light penetration may account for the lack of *Vibrio* spp. Furthermore, *Porites* spp. may produce a higher disease prevalence than *Acropora* spp. (19). Sulfite-oxidizing bacteria have also been isolated from black band-diseased corals (12), and in our study, sequences relating to *Sulfitobacter* spp. were found in corals from Sampela.

In the Mexican Caribbean, where the reefs are enclosed in a lagoon (41), a much higher diversity of microorganisms was associated with coral mucus of *Porites* and *Acropora* compared to their Indonesian counterparts. Interestingly, two clone sequences, one from *Acropora* and one from *Porites*, were closely related to *Actinobacteria*. Similar findings have been obtained from the Red Sea coral *Fungia scutaria*, whereas *Actinobacteria* were cultured from the mucus of healthy corals (22, 23). In addition, our results revealed that the bacterial assemblages associated with corals in the Mexican Caribbean had significantly distinct compositions from those from Indonesia, as well significantly distinct communities between *Acropora* spp. and *Porites* spp. within the Mexican Caribbean. These findings highlight that not only are coral host species-specific effects structuring these bacterial assemblages, but also isolation by distance effects due to the relative geographic separation of the main sites contributes. Thus, adding further supporting evidence for the hypothesis that both the dispersal limitation and environmental gradients (biotic coral-host niche) affect the structure microbial communities (13).

It has previously been suggested that the coral host may obtain nutrients, such as nitrogen and phosphorus, from their associated microbial communities, and indeed, nitrogen-fixing bacteria have been identified within the coral holobiont (43). In our study, microorganisms closely affiliated with *Synechococcus* spp. (*Cyanobacteria*) were recovered from *Acropora* and *Porites* in Hoga and to a lesser extent from *Porites* at Sampela, and thus they may provide nitrogen to the coral host.

In conclusion, the microbes associated with mucus from *Porites* and *Acropora* spp. from the Mexican Caribbean and Indonesia (Hoga and Sampela) reefs were determined. The pyrosequence library composition associated with the mucus of *Acropora* spp. and *Porites* spp. was more diverse in the Mexican Caribbean than Indonesia. To our knowledge, this is also the first report describing geographically distinct *Psychrobacter* spp. associated with coral mucus. We found that different coral species harbored different bacterial sequences that were distinct from seawater, and some bacteria-coral relationships appeared to be host specific, such as for *Clostridiales* with *Acropora* spp. Since corals are increasingly faced with changing environmental conditions (35), characterization of coral-associated microbes and their interactions with the coral host is essential in order to better understand the dynamics of coral reef systems and their responses to environmental changes.

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REFERENCES

1. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Alvarez LA, Exton DA, Timmis KN, Suggett DJ, McGenity TJ.** 2009. Characterization of marine isoprene-degrading communities. Environ. Microbiol. **11**:3280–3291.
3. **Bell JJ, Smith DJ.** 2004. Ecology of sponge assemblages (*Porifera*) in the Wakatobi region, southeast Sulawesi, Indonesia: richness and abundance. J. Mar. Biol. Assoc. UK **84**:581–591.
4. **Bourne DG, Munn CB.** 2005. Diversity of bacteria associated with the coral *Pocillopora damicornis* from the Great Barrier Reef. Environ. Microbiol. **7**:1162–1174.
5. **Boyett HV, Bourne DG, Willis BL.** 2007. Elevated temperature and light enhance progression and spread of black band disease on staghorn corals of the Great Barrier Reef. Mar. Biol. **151**:1711–1720.
6. **Breitbart M, Bhagooli R, Griffin S, Johnston I, Rohwer F.** 2005. Microbial communities associated with skeletal tumors on *Porites compressa*. FEMS Microbiol. Lett. **243**:431–436.
7. **Brown BE, Bythell JC.** 2005. Perspectives on mucus secretion in reef corals. Mar. Ecol. Prog. Ser. **296**:291–309.
8. **Caporaso JG, et al.** 2010. QIIME allows analysis of high-throughput community sequence data. Nat. Methods **7**:335–336.
9. **Clarke KR, Gorley RN.** 2006. PRIMER v6: user manual/tutorial. PRIMER-E, Plymouth, MA.
10. **Crabbe MJC, Smith DJ.** 2002. Comparison of two reef sites in the Wakatobi Marine National Park (SE Sulawesi, Indonesia). Using digital image analysis. Coral Reefs **24**:437–441.
11. **Crabbe MJC, Karaviotis S, Smith DJ.** 2004. Preliminary comparison of three coral reef sites in the Wakatobi Marine National Park (S.E. Sulawesi, Indonesia): estimated recruitment dates compared with Discovery Bay, Jamaica. Bull. Mar. Sci. **74**:469–476.
12. **Ducklow HW, Mitchell R.** 1979. Bacterial populations and adaptations in the mucus layers on living corals. Limnol. Oceanogr. **24**:715–725.
13. **Dumbrell AJ, Nelson M, Dytham C, Helgason T, Fitter AH.** 2010. Relative roles of niche and neutral processes in structuring a soil microbial community. ISME J. **4**:337–345.

14. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26**:2460–2461.
15. **Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC.** 1989. Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S-ribosomal RNA. Nucleic Acids Res. **17**: 7843–7853.
16. **Felsenstein J.** 1989. PHYLIP: Phylogeny Inference package (version 3.2). Cladistics **5**:164–166.
17. **Frias-Lopez J, Zerkle AL, Bonheyo GT, Fouke BW.** 2002. Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. Appl. Environ. Microbiol. **68**:2214–2228.
18. **Glynn PW.** 1993. Coral reef bleaching: ecological perspectives. Coral Reefs **12**:1–17.
19. **Haapkylä J, Seymour AS, Trebilco J, Smith DJ.** 2007. Coral disease prevalence and coral health in the Wakatobi Marine Park, southeast Sulawesi, Indonesia. J. Mar. Biol. Assoc. UK **87**:1–12.
20. **Haapkyla et al.** 2009. Spatio-temporal coral disease dynamics in the Wakatobi Marine National Park, South-East Sulawesi. Indones. Dis. Aquat. Organ. **87**:105–115.
21. **Jukes TH, Cantor CR.** 1969. Evolution of protein molecules, p 21–132. *In* Munro HN (ed), Mammalian protein metabolism. Academic Press, New York, NY.
22. **Lampert Y, Kelman D, Dubinsky Z, Nitzan Y, Hill RT.** 2006. Diversity of culturable bacteria in the mucus of the Red Sea coral *Fungia scutaria*. FEMS Microbiol. Ecol. **58**:99–108.
23. **Lampert Y, et al.** 2008. Phylogenetic diversity of bacteria associated with the mucus of Red Sea corals. FEMS Microbiol. Ecol. **64**:187–198.
24. **Lozupone C, Hamady M, Knight R.** 2006. UniFrac: an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics **7**:371.
25. **Maček I, et al.** 2011. Local adaptation to soil hypoxia determines the structure of an arbuscular mycorrhizal fungal community in natural CO_2 springs. Appl. Environ. Microbiol. **77**:4770–4777.
26. **McKew BA, Coulon F, Osborn AM, Timmis KN, McGenity TJ.** 2007. Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary UK. Environ. Microbiol. **9**:165–176.
27. **Meikle P, Richards GN, Yellowlees D.** 1988. Structural investigations on the mucus from six species of coral. Mar. Biol. **99**:187–193.
28. **Muyzer G, de Waal EC, Uitterlinden AG.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695–700.
29. **Nawrocki EP, Eddy SR.** 2007. Query-dependent banding (QDB) for faster RNA similarity searches. PLoS Comput. Biol. **3**:e56. doi:10.1371/journal.pcbi.0030056.
30. **Nicol G, Tscherko D, Embley TM, Prosser JI.** 2005. Primary succession of soil Crenarchaeota across a receding glacier foreland. Environ. Microbiol. **7**:337–347.
31. **Page RDM.** 1996. TreeView: an application to display phylogenetic trees on personal computers. Bioinformatics **12**:357–358.
32. **Pantos O, et al.** 2003. The bacterial ecology of a plague-like disease affecting the Caribbean coral *Montastrea annularis*. Environ. Microbiol. **5**:370–382.
33. **Parameswaran P, et al.** 2007. A pyrosequencing-tailored nucleotide barcode design unveils opportunities for large-scale sample multiplexing. Nucleic Acids Res. **35**:e130.
34. **Remily ER, Richardson LL.** 2006. Ecological physiology of a coral pathogen and the coral reef environment. Microb. Ecol. **51**:345–352.
35. **Riegl B, Bruckner A, Coles SL, Renaud P, Dodge RE.** 2009. Coral reefs: threats and conservation in an era of global change. Ann. N. Y. Acad. Sci. **1162**:136–186.
36. **Ritchie KB.** 2006. Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Mar. Ecol. Progr. Ser. **322**:1–14.
37. **Ritchie KB, Smith GW.** 1997. Physiological comparison of bacterial communities from various species of scleractinian corals, p 521–526. Proc. 8th Int. Coral Reef Symp. Smithsonian Tropical Research Institute, Balboa, Panama.
38. **Rohwer F, Breitbart M, Jara J, Azam F, Knowlton N.** 2001. Diversity of bacteria associated with the Caribbean coral *Montastraea franksi*. Coral Reefs **20**:85–91.
39. **Rohwer F, Seguritan V, Azam F, Knowlton N.** 2002. Diversity and distribution of coral-associated bacteria. Mar. Ecol. Progr. Ser. **243**:1–10.
40. **Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I.** 2007. The role of microorganisms in coral health, disease and evolution. Nat. Rev. Microbiol. **5**:355–362.
41. **Ruìz-Renterìa F, van Tussenbroek BI, Jordán-Dahlgren E.** 1998. Puerto Morelos, Quintana Roo, Mexico, p 57–66. *In* Kjerfve B (ed), CARICOMP: Caribbean coral reef, seagrass and mangrove sites. UNESCO, Paris, France.
42. **Saitou N, Nei M.** 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406–425.
43. **Shashar N, Cohen Y, Loya Y, Sar N.** 1994. Nitrogen-fixation (acetylene reduction) in stony corals: evidence for coral-bacteria interactions. Mar. Ecol. Progr. Ser. **111**:259–264.
44. **Smith DJ, Pilgrim S, Cullen L.** 2007. Coral reefs and people, p 1081–1117. *In* Pretty J, et al (ed), Sage handbook on environment and society. Sage Publications, London, England.
45. **Sogin ML, et al.** 2006. Microbial diversity in the deep sea and the underexplored 'rare biosphere.' Proc. Natl. Acad. Sci. U. S. A. **103**:12115–12120.
46. **Solow AR.** 1993. A simple test for change in community structure. J. Anim. Ecol. **62**:191–193.
47. **Sunagawa S, Woodley CM, Medina M.** 2010. Threatened corals provide underexplored microbial habitats. PLoS One **5**:1–6. doi:10.1371/journal.pone.0009554.
48. **Wallace CC, Chen CA, Fukami H, Muir PR.** 2007. Recognition of separate genera within *Acropora* based on new morphological, reproductive and genetic evidence from *Acropora togianensis*, and elevation of the subgenus *Iso pora* Studer, 1878 to genus (Scleractinia: Astrocoeniidae; Acroporidae). Coral Reefs **26**:231–239.
49. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73**:5261–5267.
50. **Wild C, et al.** 2004. Degradation and mineralization of coral mucus in reef environments. Mar. Ecol. Progr. Ser. **267**:159–171.