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Bacterial Community Associated with Healthy and Diseased Reef Coral *Mussismilia hispida* from Eastern Brazil

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Abstract In order to characterize the bacterial community diversity associated to mucus of the coral *Mussismilia hispida*, four 16S rDNA libraries were constructed and 400 clones from each library were analyzed from two healthy colonies, one diseased colony and the surrounding water. Nine bacterial phyla were identified in healthy *M. hispida*, with a dominance of *Proteobacteria, Actinobacteria*, *Acidobacteria*, *Lentisphaerae*, and *Nitrospira.* The most commonly found species were related to the genera *Azospirillum, Hirschia, Fabibacter, Blastochloris, Stella, Vibrio, Flavobacterium, Ochrobactrum, Terasakiella, Alkalibacter, Staphylococcus, Azospirillum, Propionibacterium, Arcobacter, and Paenibacillus*. In contrast, diseased *M. hispida* had a predominance of one single species of *Bacteroidetes*, corresponding to more than 70% of the sequences. Rarefaction curves using evolutionary distance of 1% showed a greater decrease in bacterial diversity in the diseased *M. hispida*, with a reduction of almost 85% in OTUs in comparison to healthy colonies. ∫-Libshuff analyses show that significant p values obtained were <0.0001, demonstrating that the four libraries are significantly different. Furthermore, the sympatric corals *M. hispida* and *Mussismilia braziliensis* appear to have different bacterial community compositions according to Principal Component Analysis and Lineage-specific Analysis. Moreover, lineages that contribute to those differences were identified as α-Proteobacteria, Bacteroidetes, and Firmicutes. The results obtained in this study suggest host–microbe co-evolution in *Mussismilia*, and it was the first study on the diversity of the microbiota of the endemic and endangered of extinction Brazilian coral *M. hispida* from Abrolhos bank.

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Introduction

The brain coral *Mussismilia* is the most important reef builder of the Abrolhos reef bank, comprising for approximately 70% of the reef structures [1]. This is the largest and most important reef bank of the entire South Atlantic Ocean (Electronic Supplementary Material, Fig. S1) [2].

The genus *Mussismilia* comprises three species (*M. braziliensis*, *M. hispida*, and *M. hartii*) with different morphologies and genotypes [3, 4], while *M. harttii* presents a very distinct morphology, *M. hispida* and *braziliensis* present subtle differences (small colonies may be even difficult to differentiate in the field). *Mussismilia braziliensis* forms hemispheric large colonies (>2 m in diameter), consisting of small polips (10 mm in diameter), whereas *M. hispida* forms smaller colonies (<1 m in diameter) and larger polyps (>15 mm in diameter) [2]. The two species are sympatric in the Abrolhos bank, but the former species occurs only in the Bahia state, while the latter is found from Maranhão to Santa Catarina states (ca. 7,000 km distance). This wide latitudinal distribution reflects a high physiological plasticity of *M. hispida*, particularly related to water quality and temperature tolerance [2]. The *Mussismilia* host harbors a high number of microbes, yet it is not known whether different *Mussismilia* species have different microbiotas [5]. The microbial communities from closely related species of the genus *Acropora* are very similar in corals colonies from the same location, arguing against the classical idea of host–microbe co-evolution in corals [6, 7].

Reef corals are in danger of extinction worldwide [8–10]. Mass mortality of *Mussismilia* has been recently documented for the Abrolhos bank [11]. Global climate changes and infectious diseases appear to be the main causes of mass mortality of *Mussismilia*. There is evidence that diseases are more common in the (late) summer than in the winter and that disease incidence is higher in the more human impacted areas [11]. White plague is one of the most common diseases in the Abrolhos bank, although bleaching is also observed in the Corumbau reef. So far, the microbial aetiological agents of these diseases in the Abrolhos Bank have not been determined.

One possible function of microorganisms found on coral holobiont surfaces may be to provide the corals with protection from pathogens by means of interspecific competition and/or secretion of antibiotic substances [6, 7, 12, 13]. These microorganisms may also supply the coral with nitrogen and phosphorous, which are not provided by their symbiotic zooxanthellae [14–19]. Hence, changes in microbial communities may affect coral health [20]. The role of bacteria on coral health is not yet fully understood [21, 22]. In the first culture-independent study on the bacterial community diversity associated with the Brazilian endemic coral species *M. braziliensis*, the classes *Proteobacteria*, *Cyanobacteria*, and unclassified Bacteria appeared to be dominant in the mucus [23]. Diseased and healthy colonies of *M. braziliensis* had distinct microbiotas, although there was not a clear dominance of a potential aetiological disease agent in the diseased *M. braziliensis*. Because there was no dominance of a particular taxonomic group, it was difficult to pinpoint possible disease agents on that study. Diseased *M. braziliensis* had a highly heterogeneous bacterial microbiota with most of its 16S rDNA sequences appearing as singletons, suggesting that the disruption of holobiont homeostasis resulted in colonization by a diverse group of opportunistic bacteria [23].

The aim of this study was to analyze the bacterial community diversity associated with apparently healthy and diseased (paling and necrosis) colonies of *M. hispida* based on 16S rRNA clone libraries. Statistical analyses allowed the comparison between 16S rRNA libraries obtained from *M. hispida* and *M. braziliensis* in order to disclose possible host specific microbiotas.

Materials and Methods

Site and Sample Collection

Colonies of apparently healthy and diseased (paling and necrosis) *M. hispida* were collected on 03/April/2007 at the AMP3 site, Itacolomis Reef, Corumbau village (16 55′ 02, 3″ S, 39 04′ 39,4″ W; (Electronic Supplementary Material, Fig. S1) by SCUBA diving between depths of 7 and 10 m. Sea water (1 L) of the site was collected in order to measure the water quality physical-chemical parameters [24] (Electronic Supplementary Material, Fig. S3).

Mucus and Water Sampling

The coral mucus was drained from colonies of *M. hispida* using a sterile syringe on board the boat. The mucus samples were placed in sterile flasks. These samples were immediately placed in an ice box and brought to the laboratory for further extraction of total DNA. RNA later (Qiagen; proportion 1:1) was used in order to avoid nucleic acid degradation of mucus bacteria. Sea water of the surrounding environment near the corals was collected in a sterile bottle. One liter of sea water was pre-filtered through an 8.0 μm filter and vacuum filtered through a 0.2 μm membrane (Durapore, Millipore). The membrane was subsequently placed in 4 ml of lysis buffer (40 mM EDTA, 50 mM tris-HCl, 0.75 M sucrose) and kept on ice until DNA extraction was performed.

DNA Extraction

DNA was extracted from mucus of two apparently healthy colonies of *M. hispida* and one diseased colony (paling and necrosis) approximately 5 m apart. The diseased colony was completely infected. The mucus DNA extraction was performed as described by Reis et al. [23]. The water DNA was extracted from the filter using the protocol by Schauer et al. [25] as described in Reis et al. [23].

16S rRNA Amplification, Cloning, and Sequencing

Total purified DNA from the four samples (one from water, two from healthy coral, and one from diseased coral mucus) were used as templates for amplification of the 16S rRNA gene using the universal primer 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) and the domain Bacteria-specific primer 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′). The PCR reactions and the construction of the four 16S rRNA libraries were performed as described in Reis et al. [23].

Sequence Analysis

Sequence quality was checked using the tool available at the site http://www.bioinformatica.ucb.br/electro.html [26] and chimeras were detected using the Chimera-check software at the Ribosomal Database Project (RDP) home page [27]. Trimmed sequences of each library were aligned using Muscle [28] and the alignment was manually edited using Bioedit (http://www.mbio.ncsu.edu/BioEdit/). The hipervariable regions generating unsolvable gaps were clipped out and sequences shorter than 370 bp were removed from the analysis. Jukes–Cantor evolutionary distances were calculated using DNADIST of the PHYLIP 3.63 package (http://evolution.genetics.washington.edu/phylip/getme.html). Aligned sequences were clustered into operational taxonomic units (OTUs) using DOTUR [29]. This software was also used to calculate diversity indices, richness estimators and to construct randomized rarefaction curves. Phylogenetic analyses were performed with the software MEGA [30], using *p* distance model [31].

16S rRNA sequence data of *M. braziliensis* [23] and *M. hispida* and the two corresponding surrounding water samples were compared using UniFrac, a web application available at http://bmf.colorado.edu/unifrac [32]. *M. braziliensis* [23] and *M. hispida* were collected on the same date and site described above. Sequences from both species were aligned using Muscle [28]. Phylogenetic trees based on the p distance model were constructed using MEGA software [30]. Sequence data were also analyzed using Principal Component Analysis (PCA) [33] and Lineage-specific Analysis. The PCA Lineage-specific Analysis with the branch length threshold of 0.15 was obtained from Unifrac software [32].

∫-LIBSHUFF software was used to estimate the significance of differences between the libraries of seawater, *M. hispida* and healthy colonies of *M. braziliensis* [34]. The Classifier tool at the Ribosomal Database Project RDP and BLASTN were used to assign the 16S rRNA sequences from *M. braziliensis* to taxonomic levels [35, 36]. The confidence threshold was 95%. Sequence Match was used to find the closest type of each cluster. Sequence data generated in this study are available at GenBank under the accession numbers GU199606–GU200660.

Results

One thousand and forty-eight high quality 16S rRNA sequences were obtained in this study. The majority of the sequences from water (92% of the total) were related at species level (i.e., >97% sequence similarity) to sequences already deposited in the GenBank. Between 72% and 80% of the sequences of the healthy coral mucus had more than 97% sequence similarity to GenBank sequences, whereas 99% of the sequences of the diseased coral mucus showed 97–100% similarity with the sequences deposited in this database (Electronic Supplementary Material, Fig. S2). Using the Classifier (RDP) with a confidence threshold of 95%, five bacterial phyla were identified in water, eight and nine phyla in each of the healthy colonies and three phyla in the diseased coral (Fig. 1). Unclassified bacteria were

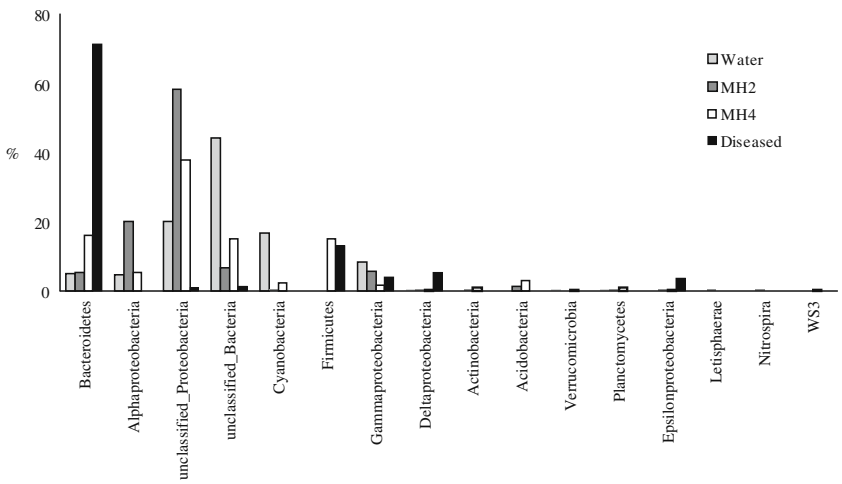

Figure 1 Frequency of bacterial phylum found in the mucus coral and surrounding water according to ribosomal data project with 95% confidence threshold. (▯ water; ▯ healthy MH2; ▯ healthy MH4; ▮ diseased)

Table 1 Dominant bacterial clusters found in the water and coral mucus

Water	Number of sequences	Representative sequence (Accession no.)	Closest neighbor (% similarity)	Accession no. of closest neighbor	Closest cultured neighbor (% similarity)	Accession no. of closest neighbor
W1	18	7H2O_PL1	Uncultured marine bacterium (99)	FJ895254.1	*Prochlorococcus marinus* subsp. *pastoris* (T); (96)	AF180967
W2	7	36H2O_PL1	Uncultured bacterium clone 6C232539 (99)	EU804590.1	*Bartonella alsatica* (T); (84)	AJ002139
W3	42	19H2O_PL1	Uncultured bacterium (99)	EF572981.1	*Rhizobium giardinii* (T); (84)	U86344
W4	11	26H2O_PL1	Uncultured bacterium (99)	EU805158.1	*P. marinus* subsp. *pastoris* (T); (97)	AF180967
W5	4	43H2O_PL1	Uncultured gamma proteobacterium clone D03-W3-C2 (99)	EU315646.1	*Kangiella koreensis* (T); (90)	AY520560
W6	4	32H2O_PL1	Uncultured alpha proteobacterium clone HF10_24P22 (99)	EU361495	*Stella vacuolata* (T); (88)	AJ535711
W7	6	34H2O_PL1	Uncultured bacterium clone W2-D09 (99)	FJ930688.1	*Anabaena cylindrica* (T); (82)	AF091150
W8	8	13H2O_PL1	Uncultured cyanobacterium clone 5kpl2D12 (99)	EF092507.1	*Chlorogloeopsis fritschii* (T); (84)	AB093489
W9	11	37H2O_PL1	Uncultured gamma proteobacterium clone D03-W3-C4 (99)	EU315485.1	*K. koreensis* (T); (87)	AY520560
W10	7	33H2O_PL1	Vibrio sp. NH87-56 (99)	GQ336803.1	*Vibrio neptunius* (T); (99)	AJ316171
W11	5	82H2O_PL2	Uncultured gamma proteobacterium clone SHWN (99)	FJ745243.1	*Thalassolituus oleivorans* (T); (86)	AJ431699
W12	3	36H2O_PL2	Uncultured bacterium clone 4C230061 (99)	EU802699.1	*S. vacuolata* (T); DSM5901; (88)	AJ535711
W13	3	71H2O_PL4	Uncultured gamma proteobacterium clone HF10 (99)	EU361666.1	*Microbulbifer maritimus* (T); (90)	AY377986
W14	22	59H2O_PL2	Uncultured marine bacterium clone SW2-44-8F (99)	FJ895263.1	*Conexibacter woesei* (T); (82)	AJ440237
W15	7	76H2O_PL4	Uncultured bacterium clone MM54 (99)	FJ809096.1	*Ruegeria pomeroyi* (T); (94)	AF098491
W16	3	2H2O_PL2	Uncultured alpha proteobacterium clone WW01G11 (99)	EF630000.1	*Labrenzia aggregata* (T); (88)	D88520
W17	4	91H2O_PL1	Uncultured bacterium clone 6C233081 (99)	EU805095.1	*Sulfurospirillum arcachonense* (T); (91)	Y11561
MH2 Healthy						
H1	51	10MH2_PL2	Uncultured alpha proteobacterium clone CD204D02 (98)	DQ200435.1	*Azospirillum brasilense* (T); (86)	AY150046
H2	126	10MH2_PL4	Uncultured bacterium clone SGUS430 (98)	FJ202840.1	*Hirschia baltica* (T); IFAM 1418 (85)	X52909
H3	11	11MH2_PL2	Uncultured bacterium clone SHFH448 (96)	FJ203413.1	*Fabibacter halotolerans* (T); (87)	DQ080995
H4	3	13MH2_PL4	Uncultured bacterium clone C3D10-2 (96)	FJ930404.1	*Blastochloris sulfoviridis* (T); (91)	D86514
H5	4	14MH2_PL4	Marine magnetic spirillum QH-2 (87)	EU675666.1	*S. vacuolata* (T); (84)	AJ535711
H6	4	28MH2_PL4	Uncultured *Vibrio* sp. (99)	AM183716.1	*Vibrio nereis* (T); (98)	X74716
MH4 Healthy						
M1	28	50MH4_PL4	Uncultured Bacteroidetes bacterium clone CD205C10 (95)	DQ200521.1	*Flavobacterium frigoris* (T); (84)	AJ557887
M2	15	81MH4_PL4	Uncultured alpha proteobacterium clone CL20-G04 (98)	GQ204833.1	*Ochrobactrum tritici* (T); (92)	AJ242584
M3	84	69MH4_PL4	Uncultured alpha	DQ889887.1	*Terasakiella pusilla* (T); (91)	AB006768

Table 1 (continued)

Water	Number of sequences	Representative sequence (Accession no.)	Closest neighbor (% similarity)	Accession no. of closest neighbor	Closest cultured neighbor (% similarity)	Accession no. of closest neighbor
			proteobacterium clone EC154 (98)			
M4	3	93MH4_PL4	Uncultured bacterium clone KspoC6 (95)	EU035944.2	*Alkalibacter saccharofermentans* (T); (82)	AY312403
M5	33	38MH4_PL4	Uncultured bacterium clone nbw292b08c1 (99)	GQ086630.1	*Staphylococcus warneri* (T); (99)	L37603
M6	3	26MH4_PL4	Uncultured alpha proteobacterium clone CD204D02 (99)	DQ200435.1	*A. brasilense* (T); (89)	AY150046
M7	3	61MH4_PL4	Uncultured bacterium clone nbw415g09c1 (99)	GQ092500.1	*Propionibacterium avidum* (T); (94)	AJ003055
M8	7	12MH4_PL2	Uncultured epsilon proteobacterium clone CH-B24 (88)	AY280389.1	*Arcobacter nitrofigilis* (T); (84)	L14627
M9	7	79MH4_PL4	*Paenibacillus sp* (98)	AB366300.1	*Paenibacillus koreensis* (T); (96)	AF130254
M10	5	42MH4_PL1	Uncultured bacterium clone SHFH635 (78)	FJ203563.1	*Candidatus Phytoplasma ulmi* (T); (no significant similarity found)	AY197655
M11	7	24MH4_PL4	Uncultured bacterium clone BB1S16SI-7 (99)	EF433151.1	*Flavobacterium daejeonense* (T); (86)	DQ222427
M12	3	35MH4_PL2	Uncultured bacterium clone EPR3970 (95)	EU491660.1	*Clostridium cylindrosporum* (T); (80)	Y18179
M13	3	14MH4_PL2	Uncultured bacterium (99)	AB294987.1	*Aphanizomenon flos-aquae* (T); (85)	AY038035
MH2 Diseased						
D1	16	86MH2D_PL1	*Desulfovibrio* sp. NA302 (99)	AJ866944.1	*Desulfovibrio acrylicus* (T); W218 (98)	U32578.1
D2	205	38MH2D_PL4	Uncultured Bacteroidetes (99)	AY580708.1	*F. daejeonense* (T); GH1–10 (86)	DQ222427
D3	8	54MH2D_PL3	Uncultured bacterium clone SHFG542 (99)	FJ203140.1	*Arcobacter halophilus* (T); LA31B (92)	AF513455
D4	5	55MH2D_PL1	Uncultured bacterium clone SHFG542 (99)	FJ203140.1	*A. nitrofigilis* (T) (93)	L14627
D5	4	95MH2D_PL2	Uncultured bacterium clone SHFG649 (100)	FJ203237.1	*Pseudoalteromonas aurantia* (T); ATCC 33046T (91)	X82135
D6	4	29MH2D_PL2	Uncultured gamma proteobacterium (99)	FJ654592.1	*Oceanospirillum multiglobuliferum* (T); IFO 13614 (92)	AB006764
D7	3	89MH2D_PL1	Uncultured bacterium clone SGUS484 (99)	FJ202184.1	*Staphylococcus saccharolyticus* (T); (81)	L37602
D8	4	90MH2D_PL4	Uncultured bacterium (99)	FJ202830.1	*Clostridium proteolyticum* (T); DSM 3090 (92)	X73448
D9	9	34MH2D_PL2	Uncultured *Clostridia* (99)	EF629781.1	*Clostridium glycolicum* (T); CIN5 (89)	AY007244
D10	11	19MH2D_PL4	Uncultured bacterium (100)	FJ203165.1	*Peptostreptococcus stomatis* (T); W2278 (86)	DQ160208
D11	13	60MH2D_PL1	Uncultured bacterium (99)	FJ202903.1	*Fusibacter paucivorans* (T); SEBR 4211 (91)	AF050099

predominant in water (44%), followed by Proteobacteria (33%), and Cyanobacteria (17%). Proteobacteria dominated the microbiota of healthy colonies (45-85%) where the great majority of them (40–60% of total bacteria) were allocated to novel Proteobacteria clades. Actinobacteria, Acidobacteria, Lentisphaerae and Nitrospira were found only in the healthy coral samples. On the other hand, close to 70% of the microbiota of the diseased *M. hispida* consisted of Bacteroidetes (Fig. 1). This represented approximately 5-fold more Bacteroidetes in the diseased sample than in the Mh4 healthy sample. A relative increase of Deltaproteobacteria (5-fold) and Epsilonproteobacteria (4-fold) in the diseased sample compared to the Mh4 healthy sample (Fig. 1). Only 1% of the sequences from the

diseased sample were not allocated to any phyla. Shanon diversity indexes (at 0.03 cut-off) were 3.59 for water, 2.06 and 3.16 for healthy Mh2 and Mh4, and 1.82 for diseased colony.

Most sequences of the coral mucus and water formed tight clusters, corresponding to species (Electronic Supplementary Material, Figs. S4, S5, S6, S7 and Table 1). Clusters were defined as at least three sequences having more than 97% similarity [37]. The dominant clusters in water were Cyanobacteria (W1), Alphaproteobacteria (W3) and unclassified bacteria (W14), corresponding to 34% of the sequences. The main clusters from healthy corals were Alphaproteobacteria (clusters H1, H2, M2, and M3), Bacteriodetes (cluster M1) and Firmicutes (cluster M5). The largest cluster in the diseased *M. hispida* (D2) represented 71% of the sequences (N=208) and had 99% sequence similarity towards an unclassified Bacteroidetes sequence (Table 1). The second largest cluster (D1) had 16 sequences of Deltaproteobacteria and three clusters of the Firmicutes (D9, D10, and D11). A large fraction of the sequences from water (32%), healthy (23%) fell into singletons and doubletons, while most of the sequences from diseases *M. hispida* (96%) formed clusters.

Representatives of the Bacteroidetes sequences found in the four libraries were aligned to other Bacteroidetes sequences retrieved from GenBank (i.e., type strains and other environmental bacteroidetes, including those associated to *M. braziliensis*). Using the same parameters already mentioned, a tree was generated (Electronic Supplementary Material, Fig. S8). Bacteroidetes found in the water are heterogeneous and well distributed along the tree. In contrast, Bacteroidetes associated with healthy and diseased *M. hispida* corals formed separated distinct and tight clusters. Bacteroidetes from the diseased coral were grouped in one single cluster. All sequences from the healthy corals (except six), formed a cluster separated from both water and diseased coral sequences.

The rarefaction curves indicated that the number of groups on higher taxonomic levels (i.e., order/class) found in the water, healthy, and diseased corals reached a plateau, between 30 and 50 (Fig. 2). However, the diversity at species level (defined by the threshold of 97% sequence

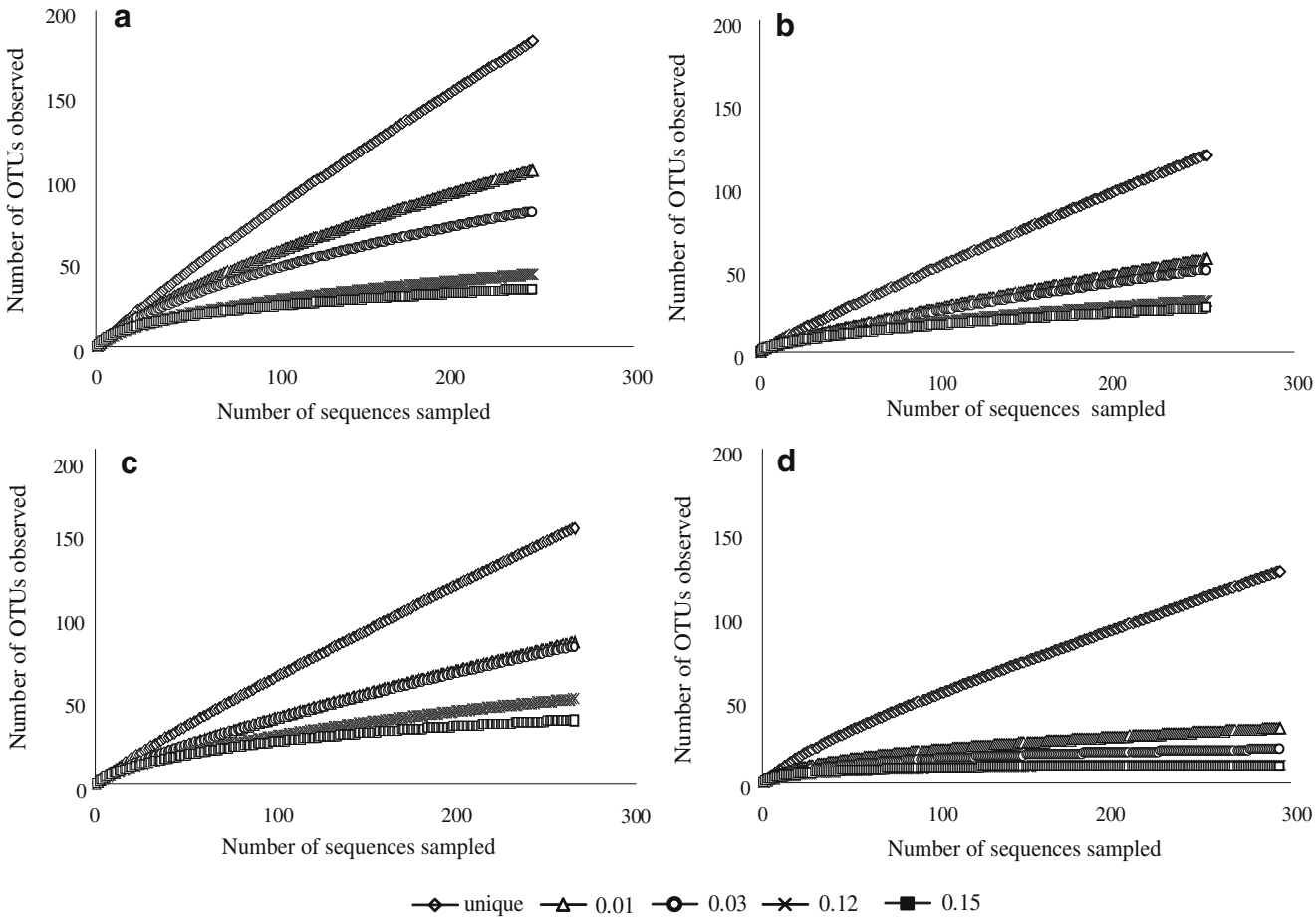

Figure 2 Rarefaction curves from DOTUR analysis of sequences from **a** sea water; **b** healthy MH2; **c** healthy MH4 and **d** diseased coral mucus 16S rRNA gene library for various distance levels. *Error bars* representing a 95% confidence interval were omitted for clarity

similarity) did not reach an asymptote in this study. A total of 80, 50, 80, and 20 species were determined for the water, healthy *M. hispida* Mh2 and Mh4, and diseased *M. hispida*, respectively (Fig. 2). Rarefaction curves using an evolutionary distance of 1% or 3% (both used to delimit species level diversity) showed a greater decrease in diversity in the diseased *M. hispida*, with reduction of almost 85% in the OTUs when compared to the "unique" sequences curve.

The comparison of the bacterial community 16S rRNA sequences of *M. hispida*, *M. braziliensis* and surrounding seawater by means of PCA showed that each coral species has a different microbiota (Fig. 3). ∫-Libshuff analysis with 10,000 randomizations corroborated the Principal Component Analysis (PCA) with a p value <0.0001 (Electronic Supplementary Material, Fig. S9 online).

Discussion

Although 70–99% of bacteria found in *M. hispida* and its surrounding water reaches at least 97% identity to sequences already deposited at GenBank, the majority of these sequences is not recognized to phyla or class levels with confidence (Fig. 1). This is a reflection of recent high throughput sequencing of 16S rRNA using uncultured techniques that enriched the GenBank with "uncultured bacteria". This means that the microbial diversity is being recognized, but it remains not classified at lower taxonomic levels (i.e., order, family, and genus). This is evidenced in Table 1, where, from the 47 clusters found in total, 41 have at least 97% identity to some clone at GenBank. On the other hand, only five clones have more than 97% identity to a type strain, and 26 have less than 90% identity to a type strain.

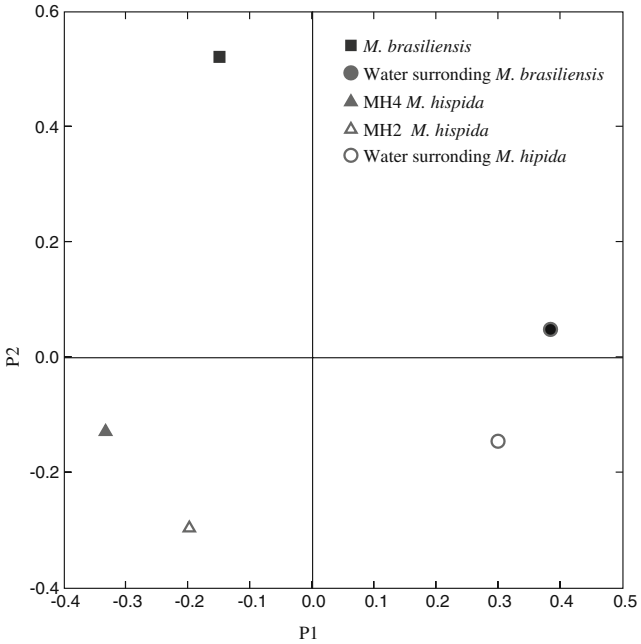

Figure 3 Principal coordinates analysis (PCA) using UniFrac software. 16S rRNA sequences of the *M. hispida*, *M. braziliensis* and water surrounding these coral samples were aligned using Muscle and p distance model in the MEGA software was used to create a single phylogenetic tree containing all the sequences. This tree was used as input to UniFrac to analyze the relationships between theses samples using PCA. *Black square*, *black circle*, *triangles* and *white circle* represent *M. braziliensis*, water surrounding *M. braziliensis*, the two healthy *M. hispida* samples and the water surrounding *M. hispida*, respectively

The bacterial community of diseased *M. hispida* is dominated by a single Bacteroidetes species. The representative Bacteroidetes 16S rRNA sequence (38MH2D_PL4) appears to belong to a widely distributed bacteria, common in the environment, with close matches to sequences from previous studies of bacterioplankton from the Massachusets area [38] and to sequences from experimentally fossilized embryos of the Australian sea urchin *Heliocidaris erythrogramma* [39]. The genetic variability within the Bacteroidetes found in the present study is very low, probably restricted to one species (Figures S7, S8 online supplementary material). Most of the sequences had 98 to 99% similarity to the sequence EF 433144.1 and not to sequences retrieved by [40] (Table 1). The sequence EF 433144.1 originated from Faviid coral from the Northern Red Sea (Eilat) affected by black band disease [41]. Barneah and colleagues found that proportions of Bacteroidetes retrieved from tissue mucus layer adjacent to the black band was 5%, increasing to 16% in the sample from the black band, but this group was not found when samples were retrieved from the mucus layer of the healthy tissue [41].

It is becoming evident that Bacteroidetes plays a role in coral health. Diseased tissues of *Montastrea annularis* have an increased number of Bacteroidetes sequences [42]. Bacteroidetes was also present in black band affected *Siderastrea siderea* [43] and in diseased *Montipora aequitubecurlata* [44]. More recently, a bioassay study using the coral *Porites compressa* and metagenomics of coral microbiota clearly demonstrated an increase in the Bacteroidetes group in stressed corals [40]. *P. compressa* exposed to reduced pH or nutrient enrichment had a 14-fold or 6-fold increase, respectively, in the relative amount of Bacteroidetes DNA sequences. Bioassays have not been performed to demonstrate the Koch's postulates (to establish a causal relationship between a causative microbe and a disease) in this case. Our study shows that Bacteroidetes found in the diseased *M. hispida* are different from Bacteroidetes present in the healthy colonies or in the seawater. This might suggest that bacteroidetes observed in the disease coral, colonized the sample after or during the disease, or, if they were present in the healthy condition, it was in a very low number."

"Bacteroidetes appear to have several properties which allow them to be successful coral pathogens [45]. First, it tolerates high concentrations of salt and low levels of dissolved oxygen. Second, it has cell colonization factors (fimbrils) and produces very potent toxins, most notably zinc-metaloproteases that destroy cell junctions and promote the cleavage of e-cadherin possibly leading to cell death. The genomic plasticity and pathogenicity would be associated with horizontally acquired genetic elements (e.g., transposons). Third, Bacteroidetes are frequently associated to areas of high fecal content and the possible contamination of Corumbau reef by sewage may lead to unbalanced environmental conditions and proliferation of this bacterium in corals."

The microbiota of healthy *M. hispida* seems to be dominated by a diverse group of Proteobacteria, including putative gliding bacteria (cluster H2) related to *Hirschia baltica* IFAM 1418T, diazotrophic bacteria related to *Azospirillum brasilensis* (cluster H1), *Flavobacterium frigoris* (cluster M1) and *Terasakiella pusilla* (cluster M3). Several putative facultative anaerobic bacteria (e.g., *Vibrio nereis*, *Arcobacter nitrofigilis*, and *Staphylococcus warneri*) or micro-aerofilic bacteria (*Propionibacterium avidum, Paenibacillus koreensis* and *Clostridium cylindrosporum*) were also found in association with healthy *M. hispida,* although these sequences have low percent similarity with GenBank sequences suggesting that oxygen may be frequently depleted in the coral holobiont in spite of the zooxanthellae photosynthetic activity. During the night oxygen is depleted in the holobiont by the metabolic activity of the holobiont's heterotrophic community, including Bacteria, Archaea, and Eukaryotes [46]. Diazotrophic bacteria are commonly found in different types of corals. Cyanobacteria and putative diazotrophic were among the dominant groups in healthy *M. braziliensis* [23], possibly indicating a type of mutualistic relationship that is not well understood in this holobiont.

The PCA comparison of the *M. braziliensis* and *M. hispida* bacterial communities, both obtained from the Itacolomis reef, showed a species-specific pattern, forming separated groups. This analysis also indicated that the two bacterial communities of the surrounding seawater appeared together but separated from the corals. Both *M. hispida* samples were closer to each other than to *M. braziliensis*. The ∫-LIBSHUFF test reinforced the differences in bacterial community composition observed in PCA (Electronic Supplementary Material, Fig. S9). ∫-LIBSHUFF is a powerful tool to detect differences between communities, providing the basis for ecological inferences about the association of microbial community composition [34, 47]. These results corroborate the studies on *Montastraea franksi*, *Diploria strigosa*, and *Porites astreoides* [6]. These corals shown marked species-specific patterns when individuals more than 1,000 km apart from one another were analyzed, suggesting host-microbe co-evolution.

In order to identify lineages that are contributing to yield differences between samples that had been shown by the PCA, we performed a "*Lineage-specific Analysis.* Choosing a small distance, which cuts near from the root, we can perform analysis at about the phylum level (see "Materials and Methods"). There are three nodes that showed significant G test p values, indicating that the node had an excess or deficit of sequences in each sample (data not shown). Two important nodes corresponding to α-Proteobacteria and Bacteroidetes sequences were over-represented in M. *braziliensis*. One important node consisting of Firmicutes members had only sequences in healthy *M. hispida*. This analysis suggested that α-Proteobacteria, Bacteroidetes, and Firmicutes distinguishes the microbial communities between M. *braziliensis* and *M. hispida.*

Although there is a recently published paper with *M. hispida* from Buzios, Brazil [48] this is the first culture independent analysis of the bacterial community diversity of the reef builder *M. hispida* from Abrollos bank. This species is sympatric with *M. braziliensis* and yet it has possibly evolved to harbor a specific microbiota. Several recent studies have found a higher richness (greater number of OTUs) in diseased than in healthy corals [21, 23, 49]. Apparently, the loss of homeostasis allows the colonization of the holobionts by a diverse opportunistic bacterial community. Opportunistic microbes may take advantage of the compromised immune system of the coral host and proliferate in diseased colonies. Our study shows the opposite pattern with the dominance of one single bacterial species in the diseased coral. Our study also highlights the need for further bioassays in order to better understand the disease process in this coral species from the early onset of infection to the late disease stages.

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