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Microbial Ecology

ISSN 0095-3628
Volume 65
Number 4

Microb Ecol (2013) 65:1076-1086
DOI 10.1007/s00248-012-0161-4

Springer

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HOST MICROBE INTERACTIONS

Metagenomic Analysis of Healthy and White Plague-Affected *Mussismilia braziliensis* Corals

Gizele D. Garcia · Gustavo B. Gregoracci · Eidy de O. Santos · Pedro M. Meirelles · Genivaldo G. Z. Silva · Rob Edwards · Tomoo Sawabe · Kazuyoshi Gotoh · Shota Nakamura · Tetsuya Iida · Rodrigo L. de Moura · Fabiano L. Thompson

Received: 17 October 2012 / Accepted: 11 December 2012 / Published online: 15 January 2013

Abstract Coral health is under threat throughout the world due to regional and global stressors. White plague disease (WP) is one of the most important threats affecting the major reef builder of the Abrolhos Bank in Brazil, the endemic coral *Mussismilia braziliensis*. We performed a metagenomic analysis of healthy and WP-affected *M. braziliensis* in order to determine the types of microbes associated with this coral species. We also optimized a protocol for DNA extraction from coral tissues. Our taxonomic analysis revealed *Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria*, and *Actinomycetes* as the main groups in all healthy and WP-affected corals. Vibrionales, members of the *Cytophaga–Flavobacterium–Bacteroides* complex, *Rickettsiales*, and *Neisseriales* were more abundant in the WP-affected corals. Diseased corals also had more eukaryotic metagenomic sequences identified as *Alveolata* and *Apicomplexa*. Our results suggest that WP disease in *M. braziliensis* is caused by a polymicrobial consortium.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-012-0161-4) contains supplementary material, which is available to authorized users.

G. D. Garcia · G. B. Gregoracci · E. de O. Santos · P. M. Meirelles · G. G. Z. Silva · R. Edwards · R. L. de Moura · F. L. Thompson
Institute of Biology, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

G. G. Z. Silva · R. Edwards
Computational Science Research Center, San Diego State University, San Diego, CA 92182, USA

T. Sawabe
Laboratory of Microbiology, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan

K. Gotoh · S. Nakamura · T. Iida
Laboratory of Genomic Research on Pathogenic Bacteria, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

F. L. Thompson (✉)
Av. Carlos Chagas Fo. S/N - CCS - IB - BIOMAR - Lab de Microbiologia - BLOCO A (Anexo) A3 - sl 102, Cidade Universitária, Rio de Janeiro CEP 21941-599, Brazil
e-mail: fabiano.thompson@biologia.ufrj.br

Introduction

Coral reefs are the most fascinating biogenic structures of the marine realm. They represent one of the most diverse and complex ecosystems of the Earth, with both economic (i.e., fisheries, tourism, and natural products) and ecological relevance, for instance, as habitat and nursery for a variety of marine life [1–4]. However, corals are facing massive extinction due to local (e.g., overfishing, pollution, predation, storms, and infectious diseases) and global impacts (e.g., warming and acidification) [5, 6]. In the last 30 years, decline of coral coverage has reached 50 and 80 % in the Caribbean and the Indo-Pacific, respectively [7, 8]. The causes of coral extinction vary according to the geographic location. In the Great Barrier Reef in Australia and Okinawa reefs, the major stressors appear to be storms and predation by crown-of-thorns starfish [6, 9]. In the Caribbean region, human sewage and infections appear to play key roles in coral reefs [8], whereas in the Pacific (Line Islands), fishing and microbialization are the major causes of reef degradation [10, 11]. In the last year, connections between coral and human health have become more evident, with the involvement of human pathogens in coral disease [10, 11].

Corals of the Abrolhos Bank—a 45,000-km^2 expansion of the continental shelf south of the Bahia State in Brazil (Fig. 1a)—are not an exception. They have faced extensive decline over the last decade with a remarkable reduction in coral cover. Infections (white plague disease) are among the major stressors, particularly for the *Mussismilia* corals (Fig. 1b) [11]. *Mussismilia braziliensis*, a relic of the Tertiary period, is endemic to the Abrolhos Bank and constitutes approximately 70 % of the reefs (mushroom-like forms or chapeirões) [12]. White plague disease in *Mussismilia* is characterized by paling and progressively irreversible coral tissue loss, as white syndrome and white band [11]. *Vibrio coralliilyticus* is one of the best studied white plague causal agent. *V. coralliilyticus* causes disease in several coral species, *Symbiodinium*, other invertebrates (*Artemia* and *Drosophila*), and even in fish by means of a complex set of metalloproteases and other potent toxins [13–16].

We recently observed that vibrios are less abundant within marine-protected areas than in unprotected areas of the Abrolhos Bank [17]. On the other hand, corals are more abundant and healthier within the protected areas, suggesting a possible connection between *Vibrio* abundance and coral health in this region [17]. In spite of the advances already made in the study of the microbial diversity associated with *Mussismilia* [18–21], we have only a limited understanding about the microbes associated with white plague in *Mussismilia*. Several studies have applied 16S rRNA sequencing in order to study prokaryotic diversity in corals [22–25], but only a few have analyzed the microbial community diversity based on more comprehensive metagenomic approaches, which do not rely on gene amplification and analyze concomitantly the host, symbiotic zooxanthellae, and microbes [26, 27]. This holobiont metagenomic approach would allow us to gain understanding about the complexity of interactions in the coral holobiont disease. Our aim was to investigate the microbial community composition of *M. braziliensis* in order to characterize the metagenomic diversity (both prokaryotic and eukaryotic) of healthy (H) and white plague (WP) of this endemic coral from different locations of the Abrolhos Bank. In addition, we established a modified, fast, and reliable protocol for DNA extraction, to obtain enough quantity of high-quality DNA without the use of GenomiPhi amplification or Percoll gradient centrifugation.

Material and Methods

Sample Collection

Two archipelago reef sites were chosen for coral sampling: (1) Sebastião Gomes (17°54′42.49″/39°7′45.94″), near shore (~20 km), and (2) Parcel dos Abrolhos (17°57′32.7″/38°30′20.3″), located on an off shore area (~70 km), inside the Abrolhos Marine National Park (Fig. 1). Sampling was performed on February 22, 2010 by scuba diving, on both sites, in a 30-m^2 area and 3–8-m depth. Six fragments of the stony coral *M. braziliensis* were collected with a hammer and a chisel: three healthy and three presenting signs of white plague disease, on each site, amounting to 12 coral specimens. Each fragment was immediately stored on polypropylene tubes, identified, and frozen in liquid nitrogen. After transportation to the lab, all samples were preserved, at −80 °C, until processing.

Coral Holobiont DNA Extraction

Several variations of the DNA extraction protocol were tested (schematized in Fig. 2), since there is no consensus protocol in the literature. After a quick removal of calcium carbonate ($CaCO_3$) skeletons with sterile spatulas, about 200 mg of each coral sample was crushed with sterile mortar and pestle, in the presence of liquid nitrogen. The resulting slurry received one of four lysis buffers: (i) 1 ml Lysis G buffer [4 M guanidine hydrochloride (Sigma-Aldrich), 200 mM Tris–HCl (pH8.0), 50 mM EDTA, and 0.5 % (*m*/*v*) sodium *N*-lauryl sarcosine (Sigma-Aldrich)]; (ii) 1 ml cetyl trimethylammonium bromide (CTAB) buffer [2 % (*m*/*v*) CTAB (Sigma-Aldrich), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl (pH8,0), and freshly added 5 μg proteinase

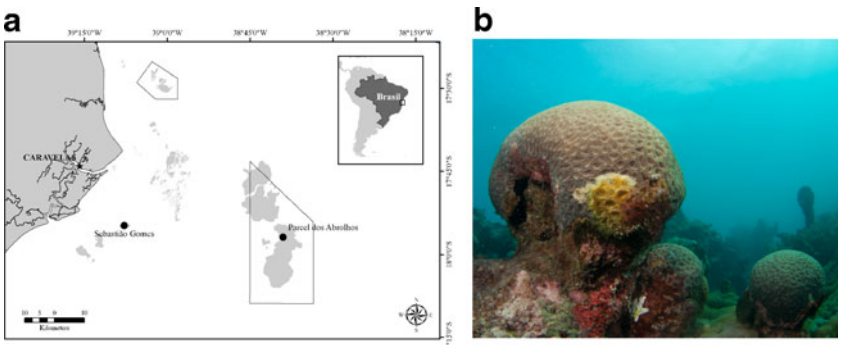

Fig. 1 **a** Study area, the Abrolhos archipelago, with sampling sites (Sebastião Gomes and Parcel dos Abrolhos) highlighted; notice that Sebastião Gomes is outside the protected areas, whereas Parcel dos Abrolhos is in the main protected area. **b** *Mussismilia braziliensis* healthy (*right*) and exhibiting white plague syndrome (*left*) colonies

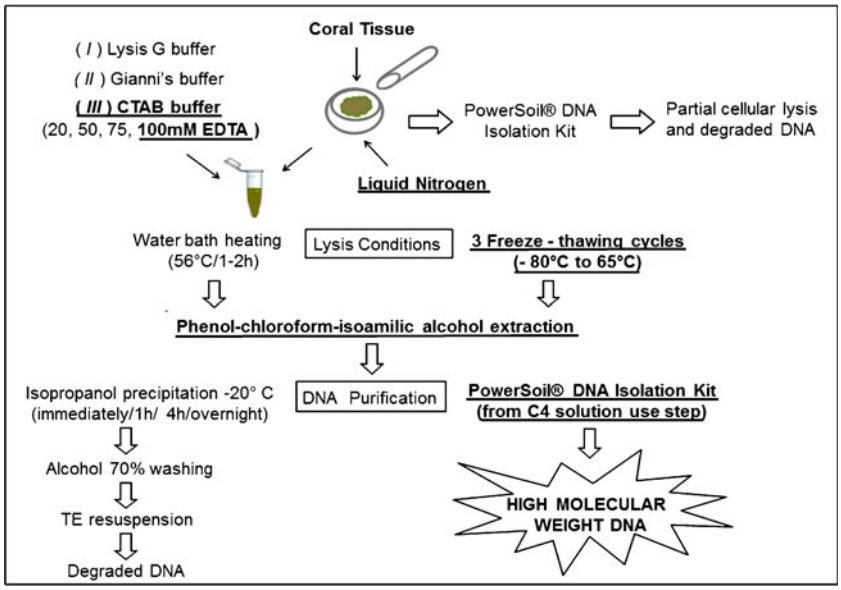

Fig. 2 General workflow of tested protocols to obtain scleractinian coral high molecular weight DNA. Steps in *bold* represent the successful protocol

K and 0.5 (or 1)% (*v*/*v*; Invitrogen) 2-mercaptoethanol (Sigma-Aldrich)]; (iii) Gianni's buffer, 250 µl ice-cold stabilizing solution [20 % (*m*/*v*) sucrose, 50 mM Tris–HCl (pH8,0), 50 mM EDTA] plus 500 µl ice-cold lysis solution [50 mM NaCl, 1 % (*m*/*v*) sodium *N*-lauryl sarcosine (Sigma-Aldrich), and freshly added 25 µg proteinase K]; and (iiii) 1 ml Trizol solution (Invitrogen). Alternative solutions containing increased concentrations of EDTA (50, 75, or 100 mM) were also tested. We compared the usefulness of each of these four buffers. Two lysis conditions were tested with buffers (i), (ii), and (iii). Tubes were alternatively frozen (−80 °C) and heated (water bath 65 °C) in water bath heating (56 °C/1–2 h) and freeze–thawing cycles. Three freeze–thaw cycles were performed, in about 3 min per step, with mixing by inversion in between. For deproteinization, phenol–chloroform–isoamilic alcohol (25:24:1) and chloroform–isoamilic alcohol (24:1) washes were performed (one each) in all cases [28]. DNA purification was obtained by two protocols: (a) isopropanol precipitation, with 3 M ammonium acetate, at −20 °C immediately/for 1 h/for 4 h/overnight, followed by washing/desalinization with 70 % ethanol, air drying, and resuspension in Tris–EDTA solution (10:1) and (b) addition of solution C4 and loading in the purification column of the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Washings with solution C5 and elution with solution C6 followed manufacturer's instructions. The PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) was also fully employed, following manufacturer's instructions. The alternative lysis methods suggested in the manual were tested. The quality and size of the extracted DNAs were evaluated by electrophoresis on 1 % agarose gels stained with GelRed (Uniscience). Further confirmation of the purity of the extraction and quantification was made on a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). PCR amplification of 16S rRNA gene (with bacteria-specific primer 27F and the universal primer 1492R) was performed on holobiont DNA samples, according to [29], in order to assess the quality of the samples for further molecular biology manipulations.

Metagenome Pyrosequencing

Metagenome sequencing of the 12 samples was performed using 454 pyrosequencing technology [30]. Shotgun libraries were individually generated with 0.5 µg of whole DNA and sheared into fragments by nebulization. End-repair and adaptor ligation were performed with the use of GS FLX Titanium kit (Roche), following manufacturer's specifications. Quality control and quantification were done with the use of Agilent 2100 Bioanalyzer (Agilent Technologies) and TBS 380 Fluorometer (Turner Biosystems), respectively. After library construction, 10^6 molecules of each sample were denatured and amplified by emulsion PCR. Sequencing was done using the GS Junior System (Roche).

Dinucleotide Composition Analysis

The dinucleotide composition of the *Mussismilia* metagenomes was compared to other metagenomes obtained from and freely available in the databases, namely *Porites compressa* corals (field and lab samples), *Acropora millepora* corals (healthy and bleached), seawater from the Abrolhos Bank, and soil from Black Soudan mine. Frequency tabulation of the sequence data was performed according to [31], using homemade Perl scripts, and the principal component analysis (PCA) analysis (using covariance) of the

tabulated data was performed in the STATISTICA software (StatSoft®).

Metagenomic Analysis

The taxonomic composition of the coral metagenomes was first evaluated through local nucleotide blast (blastall 2.2.18) against a Coral database (Table S1). Data were organized using the MEGAN4 software [32]. Sequences with no hits against this database (e-value 10^{-5}) were further annotated on the MG-Rast v3 server [33], under taxonomic classification (GenBank database, e-value cutoff 10^{-5}), and both results were pooled together. The functional analysis was performed directly on MG-Rast (SEED database, e-value cutoff 10^{-3}). Databases were last updated in September 2012. Metagenome data are available on the MG-Rast server.

Statistical Analysis

The taxonomic annotation was analyzed using the R statistical package ShotgunFunctionalizeR [34], to evaluate compositional differences between healthy and white plague-affected corals. Gene-centric regression analysis was performed in the data using the Poisson model. Abundance percentage values were normalized based on the number of identified sequences in each domain within each metagenome.

The R statistical package ShotgunFunctionalizeR [34] and the STAMP bioinformatics software [35] were also used for statistical analysis of the functional annotation of MG-RAST [33]. Additionally, the "All annotations" tool of MG-RAST was used to classify *Mussismilia* sequences (both healthy and WP) and also *Porites* [27] and *Acropora* (healthy and bleached) [26] sequences, both available in public databases. Manual curation of this automatic annotation was subsequently performed and resulted in a comparative table of the functions represented in these corals.

Results

Coral Holobiont DNA Extraction

Our DNA extraction protocol using CTAB lysis buffer with 100 mM EDTA and the PowerSoil® purification column resulted in high molecular weight DNA, while all the remaining combinations resulted, invariably, in total or partial DNA degradation. All attempts using this protocol were successful (with an average of 15 μg of DNA per 200 mg wet holobiont material), as well as 16S rRNA gene PCR amplification. The time required for one complete DNA extraction using this protocol was less than 2 h.

General Features of the Metagenomic Libraries

We generated altogether, approx. 344,000 sequences (with an average length of 414 bp), from healthy and diseased *M. braziliensis* by means of pyrosequencing technology (Table 1). Three H and three WP corals from two different locations (Parcel dos Abrolhos and Sebastião Gomes reefs) were analyzed (N=12 coral specimens). We annotated 43,249 (24.0 %) and 25,011 (15.0 %) sequences from H and WP libraries, respectively, based on our Coral Database blast and MG-Rast's GenBank annotation. We obtained the overall taxonomic id of 19.8 %. On the other hand, only 12,953 (3.76 %) sequences were identified on the All annotations option using the SEED database.

Dinucleotide Composition Analysis of the Metagenomic Libraries

We performed a PCA using the calculated dinucleotide frequencies (Karlin's signature) of the different metagenomes. We found a clear distinction of the metagenomes on the basis of this analysis (Fig. 3). All *Mussismilia* metagenomes formed a tight cluster. However, there was no clear distinction among H and WP *Mussismilia* metagenomes. *P. compressa* metagenomes obtained from cultivated corals in the lab were different from their environmental counterparts (Fig. 3).

Taxonomic Assignment of Metagenomic Sequences

Most sequences belonged to Eukaryota, comprising 95.3 % (N=65,085) of the total sequences identified taxonomically (Table 1). Cnidaria were more abundant in healthy corals ($p=7.5\times10^{-3}$). These sequences were related to the Anthozoa genera *Montastraea*, *Acropora*, and *Porites*. *Alveolata* (Dinoflagellata) sequences (n=709) were assigned as *Symbiodinium* (only one sequence identified as clade C) and more represented in WP-affected group ($p=1.2\times10^{-64}$), as well as sequences related to Streptophyta (n=227; $p=4.7\times10^{-21}$), Chlorophyta (n=64; $p=1.2\times10^{-4}$), and *Apicomplexa* (n=45; $p=2.0\times10^{-2}$). Ascomycota (n=108) and Basidiomycota (n=23) were present in both healthy and WP groups without significant difference. A total of 666 (1.02 %) eukaryotic sequences were not identified (Fig. 4). Archaeal sequences were dominated by Euryarchaeota (27) and Thaumarchaeota (15) while only 15 sequences were detected as viral.

Approximately 4.5 % (N=3,060) of total annotated sequences belonged to bacteria, and the bacterial fraction of H and WP *Mussismilia* metagenomes corresponded to 5.8 and 2.3 %, respectively (Table 2). Twenty-five different phyla were identified in H and WP *Mussismilia* metagenomes. *Proteobacteria* was the most abundant phylum,

Table 1 General features of the metagenomic data of *M. braziliensis*

	H1	H2	H3	H4	H5	H6	WP1	WP2	WP3	WP4	WP5	WP6	Total
Post QC size (Mbp)	15,903	4,623	10,306	11,281	10,566	23,289	2,692	6,247	19,467	25,457	6,389	10,833	147,053
Total generated sequences	36,496	10,867	24,120	27,158	27,275	51,737	7,297	17,109	43,531	56,008	15,963	27,214	344,775
Eukaryota	11,085	3,886	8,530	5,093	8,102	3,974	2,064	4,175	2,474	4,198	4,281	7,223	65,085
Bacteria	2,117	98	39	24	212	6	24	104	6	18	215	197	3,060
Archaea	32	1	1	3	7	0	0	0	0	1	1	3	49
Viruses	5	0	2	1	0	0	0	1	0	1	1	10	21
Unclassified sequences	7	2	10	6	4	2	0	0	2	0	5	7	45
Taxonomic identified sequences (%)	13,246 (36.3)	3,987 (36.7)	8,582 (35.6)	5,127 (18.9)	8,325 (30.5)	3,982 (7.7)	2,088 (28.6)	4,280 (25.0)	2,482 (5.7)	4,218 (7.5)	4,503 (28.2)	7,440 (27.3)	68,260 (19.8)
Functional identified sequences (%)	2680 (7.34)	332 (3.05)	761 (3.15)	931 (3.42)	904 (3.31)	1868 (3.61)	223 (3.05)	467 (2.73)	1470 (3.38)	1978 (3.53)	490 (3.07)	872 (3.20)	12976 (3.76)

followed by *Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes*, and *Actinobacteria* (Fig. 5a). Metagenomic sequences assigned to Actinomycetales ($p=1.3\times10^{-8}$), Planctomycetales ($p=3.0\times10^{-4}$), Myxococcales ($p=3.4\times10^{-2}$), Desulfuromonadales ($p=1.3\times10^{-3}$), and Verrucomicrobia ($p=3.2\times10^{-2}$) were more abundant in H *Mussismilia* metagenomes. On the other hand, *Bacteroidetes* ($p=5.0\times10^{-5}$), Cytophagales ($p=2.1\times10^{-17}$), Flavobacteriales ($p=3.0\times10^{-4}$), Vibrionales ($p=2.8\times10^{-6}$), *Rickettsiales* ($p=8.5\times10^{-3}$), and *Neisseriales* ($p=3.8\times10^{-6}$) were more abundant in WP *Mussismilia* metagenomes (Fig. 5b)

Functional Assignment of Metagenomic Sequences

Sequences identified as conserved hypothetical proteins were the most abundant by far (38.3–43.3 % of the metagenomes; 8× the abundance of the second most abundant functional group). Only 4.2 and 3.29 % of the sequences were functionally annotated in H and WP *Mussismilia* metagenomes, respectively. General cellular functions (histone, ribosome, RNA polymerase, and TPR repeat) and energy metabolism enzymes (NADH ubiquinone oxidoreductase and cytochrome C oxidase) were also among the most abundant functional group identified in both H and WP. Retroviral and/or retrotransposon-related sequences were prominent features of *Mussismilia* metagenomes, particularly polyproteins (pol, pol-like, and gag-pol), reverse transcriptase (RT and RT-like), integrase (int, int-like, and int/RNaseH), and retroelements. Healthy *Mussismilia* metagenomes had slightly more sequences in the ABC transporters, transcriptional regulators, RT/RNaseH, transposases, and dynein chains, while WP *Mussismilia* metagenomes had a few more sequences related to NADH dehydrogenases, zinc finger-containing proteins, adaptor protein for phosphatidylinositol 3-kinases, ATP synthases, and translation elongation factors. Multidrug resistance proteins were more abundant in *Mussismilia* metagenomes (0.38–0.51 %) than in *Acropora* and *Porites* corals (0–0.24 %). On the other hand, *Mussismilia* metagenomes had fewer sequences related to photosystem II, cytochrome b, glycogen (starch) synthase, and phage proteins than other coral holobionts.

Discussion

Overall DNA quality (purity and integrity) and quantity are crucial steps for metagenomic analysis of coral holobionts. The protocol established in the present study was methodically tested and represents an efficient and rapid strategy for obtaining DNA from the complex coral matrixes. The resulting amount of DNA from this protocol also avoided the need of GenomiPhi amplification, unlike previous protocols [26, 27]. Even without enrichment strategies, such as

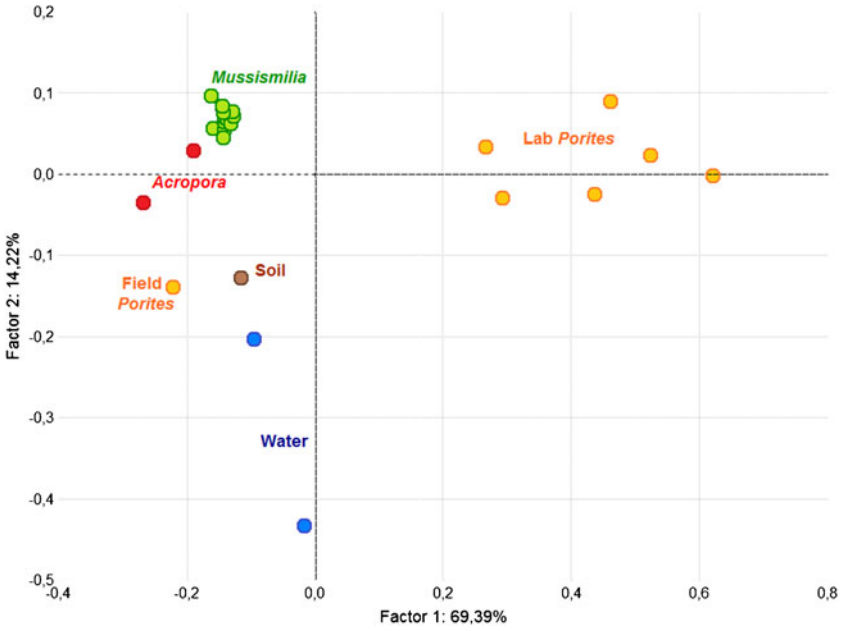

Fig. 3 PCA of dinucleotide signatures of coral, soil, and water metagenomes. Coral samples include healthy and diseased specimens

Percoll gradient, we obtained a similar level of taxonomic identification (19.8 %) as Wegley and co-workers that obtained 21 % [27] and better than Littman and colleagues which obtained 7.1 % [26]. Functional annotation was similar among all corals (3.3 to 4.2 % for *Mussismilia*, 6.6 % for *Porites*, and 2.7 to 3.5 % for *Acropora*), following our strategy. In short, this protocol provided a quick and reliable tool for coral holobiont DNA extraction.

Coral microbial communities demonstrate geographic variation, interspecies variation, and species specificity [36]. Furthermore, differences can be observed among individuals from the same species and place [37]. For such reasons, the analysis of true biological replicates is mandatory and allows the description of patterns, not taking individual variation as real abundance differences [38], and being crucial for the determination of potential roles of microorganisms on coral biology [39]. Previous studies have focused on one sample [27] or pooled samples [26] of holobionts. We opted for the analysis of several samples individually in order to obtain a better understanding of inter-sample variation. Our study illustrates that variation may be observed, for instance, in the different taxonomic groups.

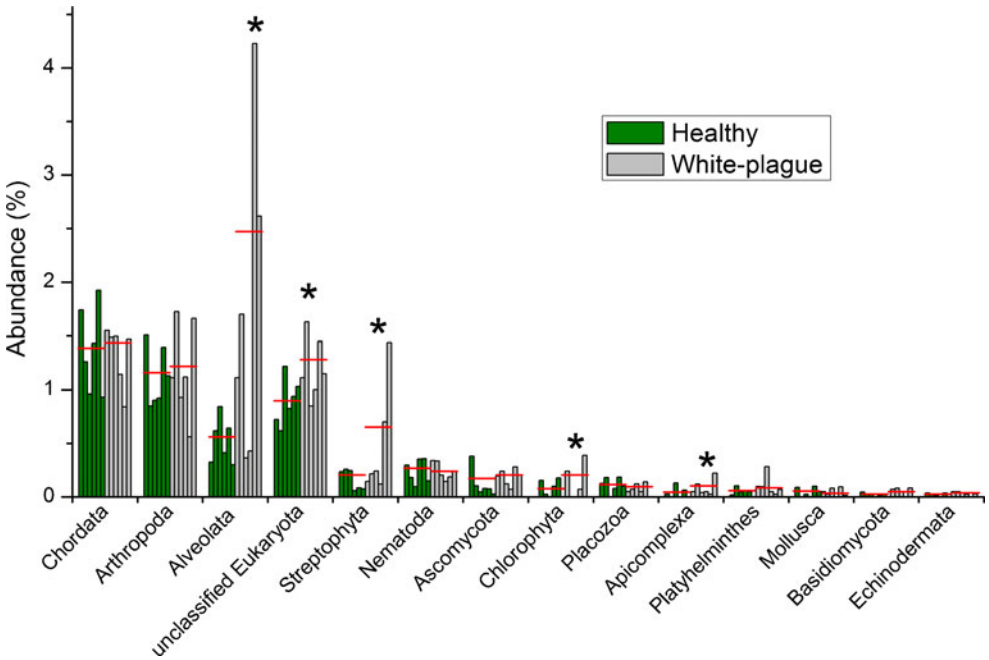

Fig. 4 Eukaryotic phyla distribution in *Mussismilia braziliensis* metagenomes. Cnidarian abundance was omitted to better visualization of other groups. *Red lines* are regression lines using Poisson model and *asterisks* denote statistical significance of the differences ($p<0.05$)

Table 2 Domain distribution of healthy and white plague *M. braziliensis* metagenomic sequences

Domains	Healthy (%)	WP (%)	Total (%)
Eukaryota	40,670 (94.0)	24,415 (97.6)	65,085 (95.3)
Bacteria	2,496 (5.8)	564 (2.3)	3,060 (4.5)
Archaea	44 (0.1)	5 (0.02)	49 (0.1)
Viruses	8 (0.02)	13 (0.1)	21 (0.03)
Unclassified sequences	31 (0.1)	14 (0.1)	45 (0.1)
Total identified sequences	43,249 (100)	25,011 (100)	68,260 (100)

Dinucleotide Signatures Are Useful for Grouping *Mussismilia* Metagenomes

Our PCA of the dinucleotide signature corroborates previous results [31], demonstrating a signature of coral sequences apart from water and soil. Additionally, we also discriminated different coral species, including field and lab samples. The 12 biological replicates of *Mussismilia* analyzed in this study formed a tight group with no evident distinct dinucleotide signature for H and WP *Mussismilia*, suggesting that dinucleotide signatures are useful for grouping these two conspecific groups. The large amount of eukaryotic DNA may have been

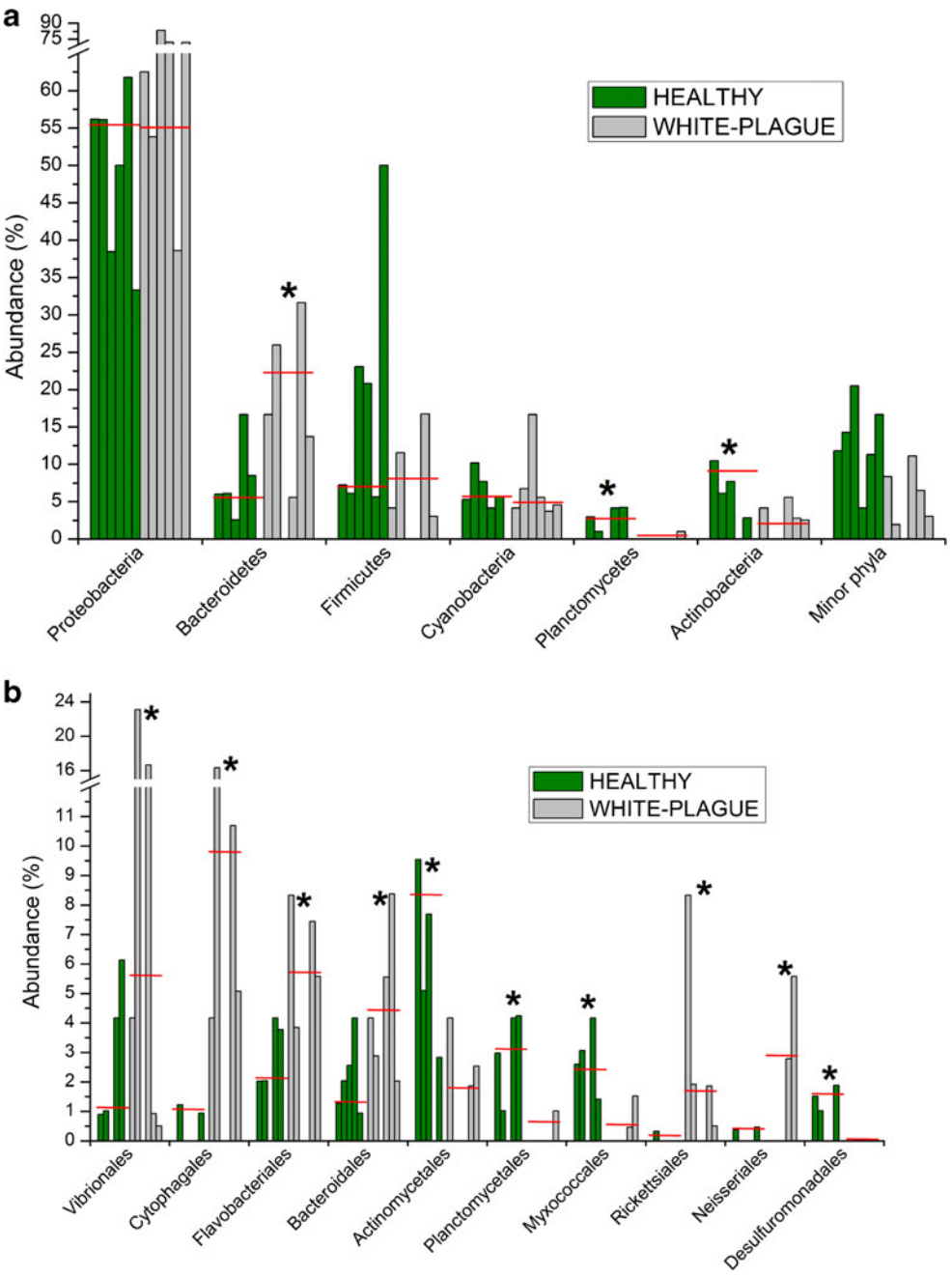

Fig. 5 **a** Bacterial phyla distribution in *Mussismilia braziliensis* metagenomes. **b** Statistically different orders in healthy and white plague *M. braziliensis* metagenomes. *Red lines* are regression lines using Poisson model and *asterisks* denote statistical significance of the differences ($p<0.05$)

the major cause for the dinucleotide similarity among *Mussismilia* metagenomes. For instance, the recent published study of *Acropora digitifera* described a 420-Mbp genome with less than 10 % of gene homology with corals ESTs, suggesting a huge number of coral-specific genes [40]. Additionally, Dinoflagellata have a human-comparable genome size, with 2–4 Gb, which could be larger than its host [41]. Such large genome sizes and the endemic nature of this coral species [42] could explain this particular grouping.

Insights into Eukaryote-Associated *Mussismilia* Corals

Members of *Apicomplexa* phylum, typical animal parasites, were more represented in diseased corals in our study. The presence of coccidians has been previously reported in coral holobiont associations [43] and the analysis of non-photosynthetic plastids coral-associated reveals intermediate lineages between free-living and parasites apicomplexan [44]. Further investigations are needed to clarify their role on coral health and disease. *Alveolata* was more abundant in white plague *M. braziliensis*, as previously observed in bleached *Acropora* [26]. This apparent counterintuitive finding could be explained by the breakdown of symbiosis in diseased corals, supporting Wooldridge's controlled parasitism hypothesis [45]. While healthy corals arrest *Symbiodinium* division [46], keeping the population under control and reaping the benefits of the surplus organic compounds [45], both white-plagued and bleached corals (this study and [26]) are supposedly allowing the faster free-living replication of the dinoflagellate, thus reaching higher relative abundance [45]. The higher abundance of Chlorophyta and Streptophyta in WP corals may indicate secondary colonization by algae. Secondary colonization in WP-affected corals was an evident process in previous studies (see Fig 2d in [47]).

Implications of the Enhancement of *Bacteroidetes, Cytophaga, Flavobacteria, Rickettsiales, Neisseriales*, and *Vibrionales* in WP *Mussismilia* Metagenomes

Altogether, our data suggest that white plague in *Mussismilia* is a polymicrobial disease. Our results relate well with previous studies on corals in the Caribbean and Pacific. Alphaproteobacteria was shown to be increased in the Caribbean WP *Montastraea annularis* and *Montastria faveolata* [23, 48, 49]. We also observed a similar increase of the Alphaproteobacteria *Rickettsiales*, which seems to be involved in the Acroporid white band disease [50, 51]. We have previously observed an increase in *Bacteroidetes* sequences in diseased *Mussismilia hispida* in the Abrolhos [22]. *Bacteroidetes* comprise a metabolic diverse and widespread distributed bacterial group. Bacteroidia is anaerobic and colonizes the gut of mammals [52], suggesting that anaerobic conditions are created in WP *Mussismilia* which allows their colonization and proliferation in this coral. On the other hand, *Cytophaga*, Flavobacteria, and Sphingobacteria are obligate aerobes with widespread distribution in marine and freshwater bacterioplankton [53, 54]. Other studies have considered *Cytophaga* as a primary pathogen in the black band disease affecting the corals *M. annularis*, *Diploria strigosa*, and *Porites lutea* [55, 56]. The abundance of these *Cytophaga–Flavobacterium–Bacteroides* members in the WP *Mussismilia* metagenomes observed in the present study may indicate the possible complexity of holobiont microhabitats, with varying oxygen and nutrient availability.

Our study indicates a possible central role for vibrios in the WP disease in *Mussismilia*. Vibrios respond swiftly to nutrient enrichment and anoxic conditions, duplicating extremely rapidly in numbers in these conditions [57]. They have been the microbial group most strongly associated with coral diseases in a variety of coral groups (e.g., *Acropora*, *Montipora*, and *Pachyseris*) [14, 24, 58]. For instance, *Vibrio shiloi* and *V. coralliilyticus* are proven aetiological agents of bleaching in *Oculina patagonica* and white plague in *Pocillopora damicornis*, respectively [59, 60]. Further studies based on infection of *Mussismilia* with vibrios in the laboratory settings and in the field will enhance our understanding of coral infection, clarifying the role of this microbial group in the pathogenesis process. The altered groups could have bigger than expected roles (based on their relative abundance) in the disruption of the bacterial partners in the *Mussismilia* holobiont. In fact, this would be in accordance with the "keystone pathogens" hypothesis, where one or more low abundance groups (*Bacteroidetes, Vibrionales, Rickettsiales, Neisseriales*, and/or *Apicomplexa*) impact the homeostasis of the whole, leading to a diseased state [61].

A First Glimpse on the Functional Assignment Metagenomic Sequences of *Mussismilia* Holobiont

Our study obtained the first genome sequences of *Mussismilia* holobiont. Previous diversity studies on *Mussismilia* had only focused on the 16S rRNA gene sequence [22, 29], while our approach allowed additional functional information, e.g., the abundance of multidrug resistance proteins in *Mussismilia* holobionts. However, the functional profiles revealed no significant differences between H and WP *Mussismilia* metagenomes, unlike a previous study on bleaching in *Acropora* [26]. Even though a considerable amount of sequences (344,000) was generated, only a small fraction of the functions were recognized in *Mussismilia* samples (3.3–4.2 %), most of which conserved hypothetical or basic metabolic functions, which offer no clue on disease aspects. This implies on a limitation of current databases on coral holobiont functions and a diversity of functions still unexplored in this holobiont.

Manual curation of the functional data allowed an additional serendipitous observation. Unlike the *Porites astreoides* metagenome, which presented abundant phages [27], the *M. braziliensis* and the *A. millepora* metagenomes, both healthy and diseased (this study and [26]), presented abundant sequences related to retrovirus and/or retrotransposons. Retroviruses or retrotransposon particles could perhaps be the unknown VLP particles observed in other coral studies (as reviewed by [62]), given for instance that retrotransposon Ty3 presents icosahedral symmetry with variation in capsid size [63]. Reverse transcriptases, polyproteins, integrases, and their variants were among the most abundant functional categories in these corals and represent genes clearly associated with retrovirus/retrotransposable elements [64]. The absence of *env* genes hints to a retrotransposon, while the presence of *gag*, *pol* (including reverse transcriptase), and *int* sequences points to LTR retrotransposons (which include Ty3-type retrotransposons), although no LTR repeat sequences were found [65]. Although widespread in higher mammals [66–68], invertebrates [69–71], and other eukaryotes [64, 72], there is no previous report to our knowledge recognizing these retroelements in coral (meta)genomes. Besides corroborating the already suspected coexistence of retrovirus and eukaryotes in earlier animals [67], retrotranscribed elements may also be an important component in coral cell biology. There are several examples of retrotransposons as sources of cellular diversity in animals, either generating somatic diversity or altering transcriptomic profiles in differentiating cells [66, 73, 74]; domesticated transposable elements and retroviruses have also been recruited to perform essential functions in the biology of their hosts (reviewed by [65]) and further studies could elucidate this possible role in coral biology.

Conclusions

This study represents the first metagenomic analysis to understand the microbial diversity associated with healthy and white plague-affected *M. braziliensis* corals. We disclosed a microbial consortium (bacteria and protozoa) associated with white plague, possibly reflecting its polymicrobial nature. These microbes, even low abundance ones, may possibly interact synergistically in order to disrupt coral homeostasis and cause the infectious process. Koch's postulates testing for these complex polymicrobial infections may be difficult, but will possibly be highly informative. Our study also shed light, for the first time, on the complex gene repertoire of *Mussismilia* holobiont.

Acknowledgments We acknowledge JST-CNPq, FAPERJ, and CAPES for funding. We also thank the project for the International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, from the Ministry of Education, Science, Sports, Culture, and Technology, Japan.

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