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ORIGINAL ARTICLE

Stable and sporadic symbiotic communities of coral and algal holobionts

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Coral and algal holobionts are assemblages of macroorganisms and microorganisms, including viruses, Bacteria, Archaea, protists and fungi. Despite a decade of research, it remains unclear whether these associations are spatial–temporally stable or species-specific. We hypothesized that conflicting interpretations of the data arise from high noise associated with sporadic microbial symbionts overwhelming signatures of stable holobiont members. To test this hypothesis, the bacterial communities associated with three coral species (*Acropora rosaria*, *Acropora hyacinthus* and *Porites lutea*) and two algal guilds (crustose coralline algae and turf algae) from 131 samples were analyzed using a novel statistical approach termed the Abundance-Ubiquity (AU) test. The AU test determines whether a given bacterial species would be present given additional sampling effort (that is, stable) versus those species that are sporadically associated with a sample. Using the AU test, we show that coral and algal holobionts have a high-diversity group of stable symbionts. Stable symbionts are not exclusive to one species of coral or algae. No single bacterial species was ubiquitously associated with one host, showing that there is not strict heredity of the microbiome. In addition to the stable symbionts, there was a low-diversity community of sporadic symbionts whose abundance varied widely across individual holobionts of the same species. Identification of these two symbiont communities supports the holobiont model and calls into question the hologenome theory of evolution.
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Introduction

The coral holobiont is the assemblage of the coral animal and its symbionts (viruses, *Symbiodinium*, protozoa, endolithic algae, fungi, Archaea and Bacteria, microfauna) and functions as an ecological unit (Rohwer *et al.*, 2002; Knowlton and Rohwer, 2003). Viruses, mostly phage, are the most abundant and diverse members of the holobiont, followed by Bacteria and Archaea (Kellogg, 2004; Wegley *et al.*, 2007; Marhaver *et al.*, 2008; Vega Thurber *et al.*, 2009). Phage are hypothesized to provide the holobiont with a specific immune system (Barr *et al.*, 2013a,b), in addition to a large reservoir of functional genes (Marhaver *et al.*, 2008; Dinsdale *et al.*, 2008; Vega Thurber *et al.*, 2009; Kelly *et al.*, 2014). The Bacteria and Archaea perform a wide array of metabolic functions that influence the physiology of the holobiont, including cycling of carbon, nitrogen, iron and sulfur (Lesser *et al.*, 2004; Wegley *et al.*, 2007; Beman, 2007; Siboni *et al.*, 2008; Raina *et al.*, 2009; Fiore *et al.*, 2010; Kimes *et al.*, 2010). The microbial symbionts may ward off potentially pathogenic bacteria through niche exclusion and production of antibiotics (Ritchie, 2006; Nissimov *et al.*, 2009; Rypien *et al.*, 2010). Similar to corals, other reef organisms such as sponges (Taylor *et al.*, 2004; Schmitt *et al.*, 2012) and various algal guilds (Lachnit *et al.*, 2009; Barott *et al.*, 2011; Lachnit *et al.*, 2011; Nelson *et al.*, 2013) harbor diverse microbial communities that have been shown or hypothesized to provide ecological services to the holobiont (Lachnit *et al.*, 2009; see review of algal–bacterial associations in Egan *et al.*, 2013). The health of the holobiont is linked to the composition of its viral and microbial constituents, which can become disrupted by natural and anthropogenic stressors, usually leading to blooms of pathogen-related viruses and bacteria (Vega Thurber *et al.*, 2008; Vega Thurber *et al.*, 2009), as well as an increased incidence of diseases (Bourne *et al.*, 2008). However, there are documented cases where stressors such as nitrogen (Siboni *et al.*, 2012) and temperature (Berkelmans and Van Oppen, 2006) may increase microbial symbionts that confer

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environmentally important physiologies, as well as cases where the bacterial communities return to their original composition after a stressor is removed (Bourne *et al*., 2008; Sweet *et al*., 2011).

Even though coral reef holobiont research has been very active for over a decade, the stability of these symbioses over time and space remains contentious. Specifically, do viral and microbial symbionts stably associate with their coral or algal hosts? A survey of the literature is summarized in Table 1 and shows that almost half of the reports find species-specific associations, while the remainder demonstrate environment-driven associations between bacteria and corals. This same question arises for algae, as algal-associated bacterial communities show both species specificity (Lachnit *et al*., 2009, 2011) and environmentally driven dynamics (Lachnit *et al*., 2011; Case *et al*., 2011).

To parameterize dynamical models of the holobiont, the field needs robust statistical tests of membership. Here we characterized >350 000 16S rDNA reads from 131 bacterial communities associated with five hosts: three coral species (*Acropora hyacinthus*, *Acropora rosaria* and *Porites lutea*) and two functional groups of benthic algae (Turf and crustose coralline algae (CCA)). A new statistical approach, the Abundance-Ubiquity (AU) test, was developed to objectively identify members of the bacterial community that were stable or sporadic members of these benthic holobionts. Our results support the hypothesis that coral and algal holobionts harbor both a stable microbiome and a locally variable suite of Bacteria.

Materials and methods

Natural history and sampling design

Coral and algal tissue samples were collected from the six most northerly Line Islands. These islands are remote, span a large biogeochemical gradient and include some of the most pristine reefs in the world (DeMartini *et al*., 2008; Dinsdale, 2008; Sandin *et al*., 2008; Barott *et al*., 2010, 2012). Several of the islands are inhabited, leading to significant changes in the microbial, benthic and fish communities (Dinsdale *et al*., 2008; McDole *et al*., 2012; Sandin *et al*., 2008). Coral and algal tissue samples were collected and preserved, and total DNA was extracted in the same way for all samples in this study.

Sample collection

Coral and algae samples were collected from the following six islands of the Line Islands chain of the Central Pacific: Kingman, Palmyra, and Jarvis (USA) and Teraina, Tabuaeran, and Kiritimati (Republic of Kiribati). Tissue samples were taken from apparently healthy tissue of branching *Acropora* spp. (*A. rosaria*, *A. hycinthus*), *P. lutea*, CCA (*Hydrolithon* spp.) and Turf assemblages. A total of 131 samples were collected (Supplementary Table S2). Species-level identification was determined from a combination of field observation and molecular identification using the Internal Transcribed Spacer (ITS) and 18S rDNA. Samples were collected at 5–10 m depth using a hollow punch (0.95 cm diameter) and hammer and placed into individual plastic bags under water. Samples were placed into RNAlater within 1 h of collection and frozen at − 20 °C.

DNA extraction and PCR amplification

Coral tissue was removed from the skeleton by air brushing the samples with phosphate-buffered saline and EDTA (10 μM EDTA). Similarly, algal tissue was removed from substrate with the airbrush and PBS +EDTA solution. In all, 500 μl of tissue slurry was centrifuged at 13 000 *g* for 15 min and resuspended in 500 μl of a lysis buffer (50 mM Tris HCL pH 8.3, Sigma-Aldrich (St Louis, MO, USA); 40 mM EDTA pH 8; 0.75 M sucrose, Sigma-Aldrich). Samples were incubated with lysozyme at a final concentration of 1 mg ml^{-1} for 60 min at 37 °C. Proteinase K (0.5 mg ml^{-1} final concentration, Sigma-Aldrich) and sodium dodecyl sulfate (1% final) were added, and samples were incubated overnight at 55 °C and subsequently 70 °C for 10 min. In all, 60 μl 3 M sodium acetate (Sigma-Aldrich), 1 μl 10 mg ml^{-1} glycogen (Sigma-Aldrich) and 600 μl isopropanol were added, and samples were incubated at − 20 °C for 2 h to precipitate DNA. Precipitated DNA centrifuged at 13 000 *g* at 4 °C, washed with 70% cold Ethanol and resuspended in 567 μl Tris-EDTA.

A CTAB (cetyltrimethylammonium bromide) and subsequent phenol/chloroform extraction method was employed (1% sodium dodecyl sulfate, 0.7 M NaCl and 0.27 mM CTAB (Sigma-Aldrich) solution incubated at 65 °C for 10 min). An equal volume of chloroform was added, mixed vigorously and centrifuged at 13 000 *g* for 2 min. The aqueous layer was then transferred to clean tube. Phenol:chloroform (Sigma-Aldrich) was then added in equal volume, mixed vigorously and centrifuged at 13 000 *g* for 2 min. Another chloroform extraction step was carried out to finalize the phenol/chloroform extraction. A 0.7 volume of isopropanol was added to the final aqueous layer and incubated for 2 h. The extract was centrifuged at 4 °C for 15 min at 13 000 *g* to pellet DNA. DNA was resuspended in 50 μl molecular grade water and stored at − 20 °C until ready for amplification via PCR.

Sample preparation for 454 sequencing using barcoded primers

Sample DNA template was loaded onto 96-well plates and prepped for sequencing. Primers were designed using software tools BARCRAWL and BARTAB included the 454 adaptor sequence and a barcode sequence (454_27F: GCCTTGCCAG CCCGCTCAGTCAGAGTTTGATCCTGGCTCAG and

Table 1 Literature reports of the specificity of coral associated bacterial communities

Study	*Varied*			*Coral species*
	Within species	*Between species*	*By site*	
Tout *et al.* (2014)	NA	Yes	NA	*Acropora aspera*
Sunagawa *et al.* (2010)	NA	Yes	NA	*Acropora cervicornis*
McKew *et al.* (2012)	NA	No	Yes	*Acropora formosa*
Kvennefors *et al.* (2010)	Yes	Yes	Yes	*Acropora hyacinthus*
Littman *et al.* (2009)	Yes	No	Yes	*Acropora millepora*
Li *et al.* (2013)	No	Yes	NA	*Acropora millepora*
Lema *et al.* (2014)	No	NA	No	*Acropora millepora*
Tout *et al.* (2014)	NA	Yes	NA	*Acropora palifera*
Sunagawa *et al.* (2010)	NA	Yes	NA	*Acropora palmata*
McKew *et al.* (2012)	NA	No	Yes	*Acropora palmata*
Littman *et al.* (2009)	Yes	No	Yes	*Acropora tenuis*
Ceh (2010)	Yes	No	NA	*Acropora tenuis*
Littman *et al.* (2009)	Yes	No	Yes	*Acropora valida*
Yang *et al.* (2013)	NA	Yes	NA	*Alcyonium gracillimum*
Lee *et al.* (2012)	No	Yes	Yes	*Astreopora myriophthalma*
Frias-Lopez (2002)	NA	Yes	NA	*Diploria strigosa*
Klaus *et al.* (2005)	Yes	No	Yes	*Diploria strigosa*
Sunagawa *et al.* (2010)	NA	Yes	NA	*Diploria strigosa*
Rohwer *et al.* (2002)	No	Yes	No	*Diploria strigosa*
Ceh (2010)	Yes	No	NA	*Favites abdita*
Tremblay *et al.* (2011)	No	Yes	NA	*Galaxea fascicularis*
Li *et al.* (2013)	No	Yes	NA	*Galaxea fascicularis*
Chen *et al.* (2011)	Yes	NA	NA	*Isopora palifera*
Carlos *et al.* (2013)	No	Yes	No	*Madracis decactis*
Daniels *et al.* (2011)	Yes	NA	NA	*Montastraea annularis*
Frias-Lopez (2002)	NA	Yes	NA	*Montastraea annularis*
Klaus *et al.* (2005)	No	No	No	*Montastraea annularis*
Klaus *et al.* (2007)	Yes	NA	Yes (only shallow)	*Montastraea annularis*
Barott *et al.* (2011)	Yes	NA	Yes	*Montastraea annularis*
Morrow *et al.* (2012)	No	Yes	Yes	*Montastraea annularis*
Frias-Lopez (2002)	NA	Yes	NA	*Montastraea cavernosa*
Sunagawa *et al.* (2010)	NA	Yes	NA	*Montastraea faveolata*
Guppy and Bythell (2006)	Yes	NA	Yes	*Montastraea faveolata*
Kimes *et al.* (2013)	Yes	NA	NA	*Montastraea faveolata*
Sunagawa *et al.* (2010)	NA	Yes	NA	*Montastraea franksi*
Rohwer *et al.* (2002)	Yes	NA	Yes	*Montastraea franksi*
Rohwer *et al.* (2002)	No	Yes	No	*Montastraea franksi*
Carlos *et al.* (2013)	No	Yes	No	*Mpalythoa caribaeorum*
Lins-de-Barros *et al.* (2010)	NA	No	NA	*Mussismilia hispida*
Carlos *et al.* (2013)	No	Yes	No	*Mussismillia hispida*
Tremblay *et al.* (2011)	No	Yes	NA	*Pavona cactus*
Chiu *et al.* (2012)	No	NA	Yes	*Platygyra carnosus*
Bourne and Munn (2005)	Yes	NA	NA	*Pocillopora damicornis*
Ceh (2010)	Yes	No	No	*Pocillopora damicornis*
Lee *et al.* (2012)	No	Yes	Yes	*Pocillopora verrucosa*
Sunagawa *et al.* (2010)	NA	Yes	NA	*Porites astreoides*
Rohwer *et al.* (2002)	No	Yes	No	*Porites astreoides*
Morrow *et al.* (2012)	No	Yes	Yes	*Porites astreoides*
McKew *et al.* (2012)	NA	No	Yes	*Porites astreoides*
Li *et al.* (2013)	No	Yes	NA	*Porites lutea*
McKew *et al.* (2012)	NA	No	Yes	*Porites lutea*
Lee *et al.* (2012)	No	Yes	Yes	*Sarcophyton sp 1*
Lee *et al.* (2012)	No	Yes	Yes	*Sarcophyton sp 2*
Lins-de-Barros *et al.* (2010)	NA	No	NA	*Siderastrea stellata*
Hong *et al.* (2009)	Yes	NA	Yes	*Stylophora pistillata*
Kvennefors *et al.* (2010)	Yes	Yes	Yes	*Stylophora pistillata*
Lee *et al.* (2012)	No	Yes	Yes	*Stylophora pistillata*
Carlos *et al.* (2013)	No	Yes	No	*Tubastraea coccinea*
Yang *et al.* (2013)	NA	Yes	NA	*Tubastraea coccinea*
Tremblay *et al.* (2011)	No	Yes	NA	*Turbinaria reniformis*
Aires *et al.* (2015)	Yes	Yes	NA	*Caulerpa taxifolia*
Aires *et al.* (2015)	Yes	Yes	NA	*C. racemosa var. turbinata*
Aires *et al.* (2015)	Yes	Yes	NA	*C. racemosa var. cylindracea*
Aires *et al.* (2015)	Yes	Yes	NA	*Caulerpa prolifera*

Abbreviation: NA, not available.

454_338R: GCCTCCCTCGCGCCATCAGxxxxxxxxxx xxCATGCTGCCTCCCGTAGGAGT) (Frank 2009). PCR reactions were performed in triplicate. The PCR amplification protocol is as follows: initial start at 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 50 °C 30 s, 72 °C for 1.5 min, and a final extension of 72 °C for 10 min. Samples were held at 4 °C. The three PCR reactions were pooled, and DNA was quantified with PicoGreen (Life Technologies, Carlsbad, CA, USA). Equal amounts of amplicon (normalized to 240 ng) were pooled in a single tube and cleaned using the MoBio Single Tube PCR Cleanup (Carlsbad, CA, USA). This library was then sequenced using the 454 Titanium platform at the San Diego State University.

Sequencing preparation for host identification

18S rDNA fragment was amplified from the total DNA using a custom Illumina-specific primer (Ill_18S_1A: TCGTCGGCAGCGTCAGATGTGTATA AGAGACAAACCTGGTTGATCCTGCCAGT and Ill_ 18S_564R: GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAGGCACCAGACTTGCCCTC). The primer contains the 18S rDNA loci-specific oligo as well as a region for a secondary amplification using Illumina's barcode indices. The PCR amplification protocol for the primary amplification from total DNA was as follows: 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 55 °C 30 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min holding at 4 °C. Ampure beads were used in order to clean PCR reactions for a secondary amplification with eight cycles in order to apply Illumina's index barcodes. A secondary cleaning step using Ampure beads was performed, individual libraries were quantified using the PicoGreen dsDNA Assay Kit (Life Technologies) and pooled in equal quantities. The final library was sequenced on the MiSeq at San Diego State University. In order to identify coral species, a coral-specific primer targeting the ITS region (A18S_F: GATCGAACGGTTTAG TGAGG and ITS4: TCCTCCGCTTATTGATATGC) was used to amplify from total DNA (Takabayashi *et al.*, 1998). The PCR protocol was used as performed by Takabayashi *et al.* (1998) Amplicons were cleaned using Ampure beads and sent for Sanger sequencing at Retrogen (San Diego, CA, USA).

Sequence analysis

Using the Quantitative Insights into Microbial Ecology pipeline (Caporaso *et al.* 2010), reads were denoised, quality filtered with a cutoff quality score of 25, a minimum and maximum length of 200 and 1000 bp, respectively, no ambiguous bases or mismatches were allowed in the primers and checked for chimeras. Barcodes were used to split sequences into their respective libraries. Operational taxonomic units (OTUs) were generated by picking a seed sequence and clustering at a 97% identity threshold using the UCLUST algorithm (Edgar 2010). Representative OTUs were picked from each cluster to be compared against the SILVA database at an 80% confidence (Quast *et al.*, 2013). OTUs that were classified as chloroplasts were filtered from subsequent analysis, and cyanobacteria were subjected to additional comparison against the SILVA rRNA database to filter any additional chloroplast contamination (minimum alignment length of 151 bp, *E*-value $< 10^3$).

In order to identify coral hosts, representative ITS reads were blasted against the NR database. The top hit was used to determine coral species identity. For CCA identification, a representative subsample was taken from each 18S rDNA library and compared against the Silva database as a closed reference using UCLUST. Representative reads were taken from each library, trimmed and aligned using ClustalW, providing phylogenetic distances between reads. Because Turf algae are an assemblage rather than a single species, 18S rDNA libraries for Turf sample identification were clustered into OTUs at a 97% similarity using the SILVA reference database as a closed reference (Quast *et al.*, 2013). OTUs that were assigned to Chlorophyta, Rhodophyceae (excluding CCA) or Phaeophyceae were used as the composition of a given assemblage. In order to determine the range of the composition of turf assemblages, a Bray–Curtis dissimilarity matrix was used to compare differences in assemblage composition. Statistical groupings of turf assemblages were identified using the *simprof* function from the *clustsig* R library. The *mantel* function in R was used to perform mantel tests in order to test for an association between the 18S rDNA dissimlarity of CCA and Turf assemblage composition with respective bacterial community composition. Sequences were submitted to MG-RAST under the study accession number 4663447.3.

All statistical tests were performed in R (version 2.15.3). The *vegdist* function from the *vegan* library was used to create Bray–Curtis dissimilarity matrices. Prior to multivariate analysis, a square root transformation was applied, and the data were normalized to relative abundances in order to satisfy assumptions of homogeneity in variance and to account for libraries of differing sequencing depths. The *isoMDS* function was used to generate nMDS plots of Bray–Curtis dissimilarity matrices. The AU test was implemented as described in Supplementary Appendix S1.

The *betadisper* function from the *vegan* library was used to estimate each host group's associated bacterial community variation using a Bray–Curtis distance matrix. The variation of each community was evaluated by placing the dissimilarity between samples into Euclidean space, making the two-dimensional distances between samples roughly equal the compositional dissimilarity. A group centroid was calculated for each host group, and each sample's distance to their respective group centroid was calculated. These distances to group

centroid were used to test for differences in variability for a given host group.

Results

Characterizing the coral and algal host genotypes

A combination of field observations and molecular marker data was used to identify individual coral and algal genotypes. Coral hosts were identified as *P. lutea*, *A. rosaria* and *A. hyacinthus* using sequences from the ITS region. There was a strong association between the *Acropora* spp. and island ($\chi^2 = 15.51$; $P = 0.003$). All samples from Jarvis were *A. hyacinthus*, while all samples from Tabuaeran and Palmyra were *A. rosaria*; (Supplementary Figure S1; Supplementary Table S2). Both *Acropora* spp. were sampled from Kingman and Teraina; however, neither were sampled on Kiritimati (the most degraded island) owing to inability to locate any *Acropora* colonies. *P. lutea* was collected from every island.

The 18S rDNA locus was used to identify CCA genotypes. All CCA were identified as *Hydrolithon* sp. Genetic distances between aligned 18S rDNA reads were used to create a dissimilarity matrix, and a mantel test was used to test for an association between the genetic distances and island. No association was found between the CCA 18S rDNAs and sampling location (Mantel $R = -0.068$; $P = 0.809$), therefore all CCA samples were considered as a single group.

Turf algae are assemblages of different algal species, containing a mixture of juvenile macroalgae, filamentous algae and cyanobacteria. The composition of each Turf assemblage was characterized based on 18S and 16S rDNA reads assigned to Chlorophyta, Phaeophyceae, Rhodophyta and Cyanobacteria. Turf assemblage composition differences were assessed with the Bray–Curtis dissimilarity, and the corresponding dissimilarity matrix was used to test for an association with island. No association was found between Turf assemblage composition and island (Mantel $R = -0.018$; $P = 0.584$), and therefore, all Turf hosts were considered as a single guild for the subsequent analyses.

In total, we examined five different host groupings: *A. hyacinthus*, *A. rosaria*, *P. lutea*, CCA, and Turf. In total, 8 *A. hyacinthus*, 16 *A. rosaria*, 34 *P. lutea*, 35 CCA and 38 Turf bacterial communities were used in this study. Sample distributions across the islands can be found in Supplementary Table S2. The mean number of post-quality 16S rDNA reads per sample was >1800. The average Shannon diversity ($H' \pm$ s.d.) of associated bacterial communities were 3.2 ± 0.88, 3.3 ± 0.8, 3.6 ± 0.78, 4.4 ± 0.80 and 4.9 ± 1.4 for *A. hyacinthus*, *A. rosaria*, *P. lutea*, CCA and Turf, respectively (Table 2). The mean number of post-quality filtered 18S rDNA reads per CCA and Turf samples were > 57 000. The average Shannon diversity (± s.d.) of the Eukaryotic community associated with CCA and Turf were 1.0 ±0.79 and 1.1 ± 0.74, respectively.

Characterization of the variance in bacterial community composition

Bray–Curtis dissimilarity was used to generate a dissimilarity matrix for the bacterial communities associated with the coral species (*A. hyacinthus*, *A. rosaria* and *P. lutea*) and the two algal guilds (CCA and Turf). From this dissimilarity matrix, the first two principle coordinates were plotted to visualize bacterial community composition in Euclidean space (Figure 1a). A spatial median was then calculated for each host type, and group centroids were generated. For each sample, the distance to the respective group centroid was computed, generating a distribution of distances for each host (Figure 1a).

Table 2 Statistics for stable and sporadic symbionts associated with each host

	A. hyacinthus	A. rosaria	P. lutea	*CCA*	*Turf*
Stable					
OTUs (*n*)	902	2188	3662	8856	18 065
Proportion (% OTUs)	94.9	93.9	91.1	92.5	95.5
Abundance	$6.6 \times 10^{-4} \pm 3.1 \times 10^{-4}$	$6.6 \times 10^{-4} \pm 2.1 \times 10^{-4}$	$5.4 \times 10^{-4} \pm 1.5 \times 10^{-4}$	$2.6 \times 10^{-4} \pm 1.4 \times 10^{-4}$	$2.8 \times 10^{-4} \pm 1.6 \times 10^{-4}$
Diversity (H')	3.7 ± 0.56	2.2 ± 0.68	2.5 ± 0.77	2.7 ± 0.50	2.8 ± 0.79
Sporadic					
OTUs (*n*)	49	143	356	703	861
Proportion (% OTUs)	5.1	6.1	8.9	7.4	4.6
Abundance	$8.2 \times 10^{-3} \pm .006$	$1.0 \times 10^{-3} \pm 0.001$	$1.8 \times 10^{-3} \pm 0.002$	$9.7 \times 10^{-3} \pm 0.001$	$9.9 \times 10^{-4} \pm 0.002$
Diversity (H')	1.6 ± 0.65	1.2 ± 0.59	1.3 ± 0.69	1.1 ± 0.59	0.8 ± 0.55
Overall					
OTUs (*n*)	951	2331	4018	9559	18 926
Diversity (H')	3.2 ± 0.88	3.3 ± 0.80	3.6 ± 0.78	4.4 ± 0.80	4.9 ± 1.40

Abbreviations: CCA, crustose coralline algae; OTU, operational taxonomic unit. Number of OTUs found with each host as assigned sporadic and stable and the percentage of total OTUs that these groups make up of the total number of OTUs in each community. The median relative abundance of OTUs in stable and sporadic groups (± interquartile range) and mean Shannon diversity ($H' \pm$ s.d.) are also included.

These distances represent the variability in the composition of the associated bacterial communities between individuals of each host. A larger median distance-to-centroid indicates higher variation in bacterial communities. The bacterial communities associated with corals were significantly more variable than the algal groups (median distance to centroid ± s.d.: 0.42 ± 0.11 versus 0.36 ± 0.05, respectively; Figure 1b; *P*-values in Supplementary Table S1). Of the coral species, *A. rosaria* and *P. lutea* had the same degree of variation in their bacterial community (0.49 ± 0.1 and 0.53 ± 0.15, respectively) and were both higher than *A. hyacinthus* (0.39 ± 0.09; Figure 1b; Supplementary Table S1).

AU test

To identify the OTUs responsible for the community variability identified above, a novel statistical analysis termed the AU test was developed (described in detail in Supplementary Appendix S1). The purpose of this analysis was to address and account for potential incomplete sampling of the bacterial community. That is, despite thousands to tens-of-thousands of observations per sample, there remains a probability that any particular OTU was present but not sampled. The approach taken here compares the ubiquity of an OTU (that is, the fraction of individual samples in which that OTU was observed for a given host) to its relative abundance within each sample. As shown in Figure 2, the ubiquity of an OTU and its relative abundance within a host were strongly correlated for the majority of OTUs; however, there were also OTUs that strongly deviated from this pattern.

In order to quantify the deviation of an observed AT relationship for an individual OTU and to develop an objective way of identifying the OTUs that exhibit a pattern of high variance in relative abundance, the ubiquity and the pooled relative abundance of OTUs were taken as a pair of test statistics. The distribution of these test statistics was calculated assuming the null hypothesis that an OUT's relative abundance does not vary across all sampled communities. Figure 2 shows the theoretical expectation curve (blue line), as well as a 0.01 significance curve for these distributions (black line). Any OTU above the significance curve has a probability of <0.01 of achieving ubiquity and abundance under the null assumption. The two groups of OTUs identified by the AU test were labeled as (1) stable symbionts, which were identified by their failure to reject the null hypothesis and therefore display a statistically stable relative abundance across many individuals of a given host group, and (2) *sporadic symbionts*, which violated the null hypothesis of the AU test, displaying variability in terms of presence/absence and relative abundance between individuals of the same host (Figure 2).

In general, the stable symbionts were rare, where each OTU made up on average 2.6×10^{-4}–6.6×10^{-4} of the overall community, and the majority were

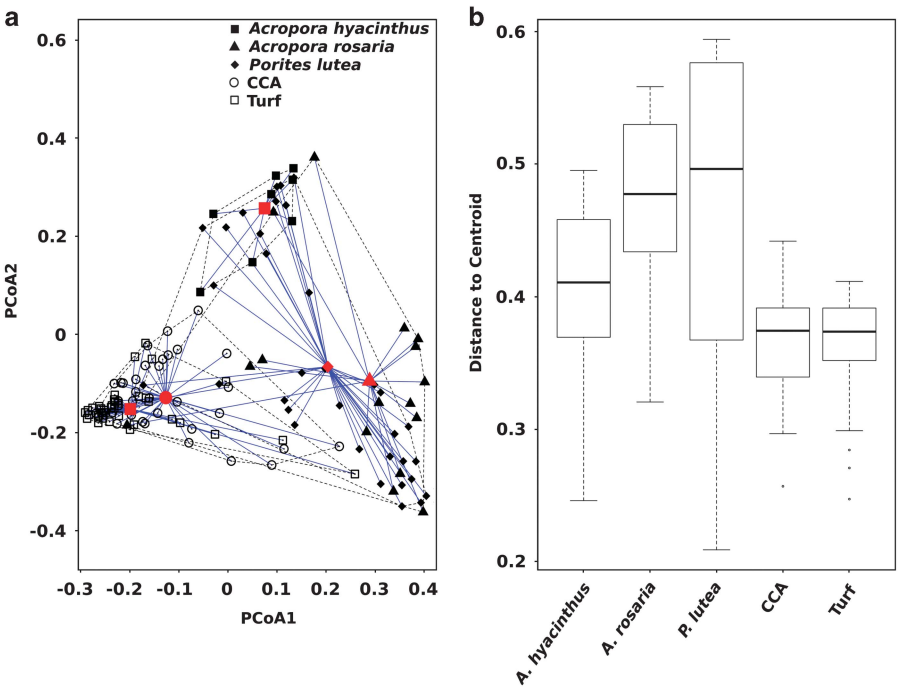

Figure 1 Bacterial communities associated with coral and algal holobionts. Each host's associated community was assessed at the OTU level with the Bray–Curtis dissimilarity and those dissimilarities were reduced to principle coordinates, embedding them in Euclidean space (**a**). The variation of each community was evaluated by measuring the Euclidean distance for each sample associated with a host to the host centroid (**b**). The bar represents the median distance to centroid and the boxes represent upper and lower 25% quartiles. The whiskers represent minimum and maximum values.

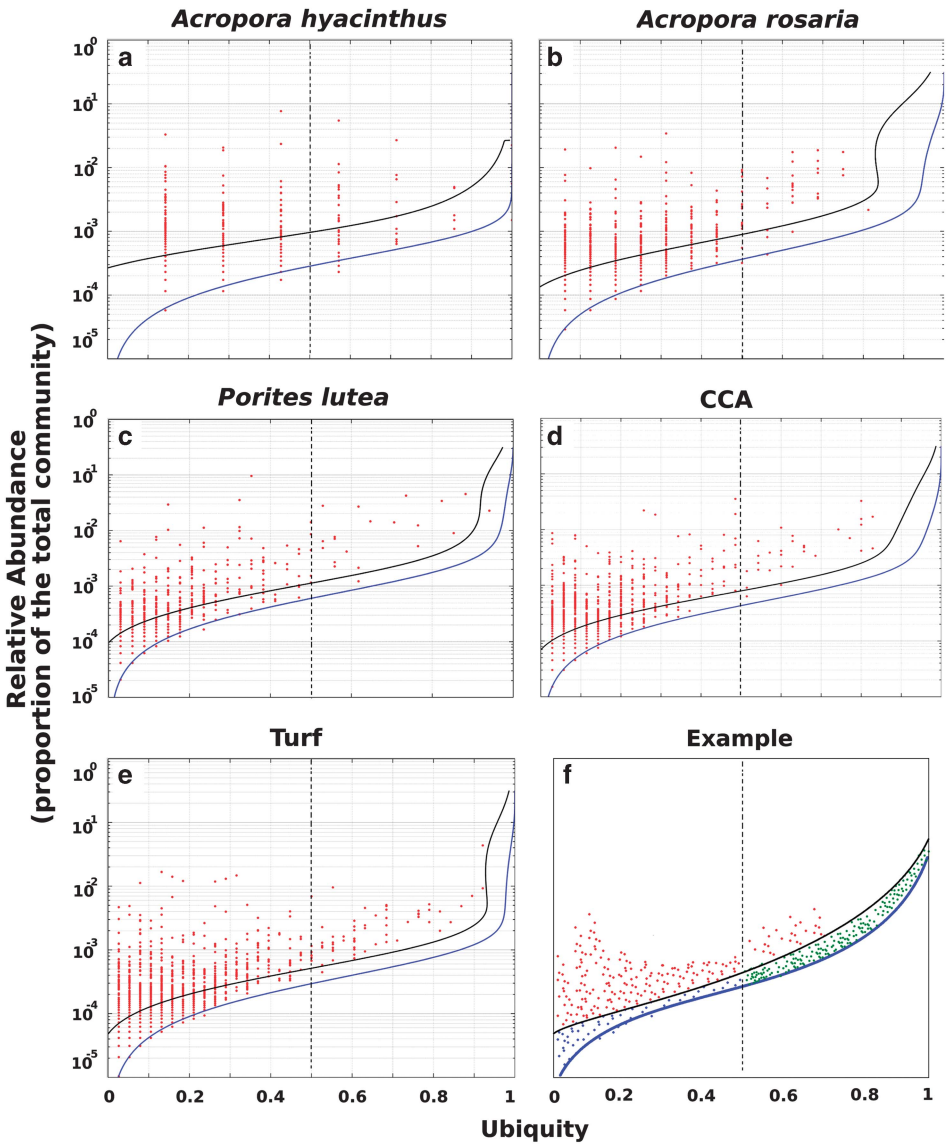

Figure 2 AU method for determining significant deviations in an OTU's distribution across the samples studied. The AU method was utilized to distinguish between OTUs (97% similar) whose community relative abundance did not vary across each community studied and those that did. OTUs that fall above the 0.01 confidence interval (black line) are found in fewer samples than expected given their mean community relative abundance (that is, OTUs whose distribution in the data set is restricted and are therefore classified as sporadic). Panels **a**–**e** represent bacterial communities associated with *Acropora hyacinthus* (**a**), *Acropora rosaria* (**b**), *Porites lutea* (**c**), CCA (**d**) and Turf (**e**). Panel **f** is a mock plot illustrating the different classifications of OTUs based on the AU test. The different color points indicate sporadic (red), stable predicted (blue) and stable observed symbionts (green).

observed in <50% of the samples (that is, low ubiquity). Therefore, we conditionally classify OTUs that failed to reject the AU test's null as stable-predicted symbionts. There were also several of OTUs that had a high ubiquity (>50% of samples) and were therefore classified as stable-observed symbionts (the three groups are illustrated in Figure 2f).

Sporadic symbionts are members of the bacterial community that were observed in fewer samples than expected assuming that bacterial associates were stable across time and space. The total proportion of bacteria that were classified as sporadic in the AU test ranged between 4.5% and 7.4% of total OTUs observed with each host (Figure 2; Table 2). The relative abundance of sporadic OTUs was significantly higher than stable OTUs (9.7×10^{-4}–8.2×10^{-3}; Wilcoxon test; $P<0.001$; Table 2). The communities of sporadic symbionts were significantly less diverse compared with the stable symbionts across all hosts using the Shannon diversity index (sporadic: 0.794–1.632; stable: 2.177–3.715; Wilcoxon test; $P<0.001$; Table 2).

Taxonomic identification of stable and sporadic symbionts

The OTUs were taxonomically identified by using the SILVA database as a closed reference and matching

using UCLUST (Edgar 2010; Quast *et al.*, 2013). Alphaproteobacteria were the most abundant sporadic symbionts associated with all five hosts. Of these, the majority belonged to the Rhizobiales or Rhodobacterales orders. Turf algae were the exception, with the most abundant OTUs assigned as Rickettsiales.

The most abundant stable symbionts associated with Turf and CCA were Alphaproteobacteria of the orders Rhodobacterales, Rhizobiales and Rhodospirales, represented by the genera *Rhodobium* and several uncultured/unclassifiable genera (Supplementary Tables S3–S7). Stable symbionts associated with coral also were mostly Alpha and Gammaproteobacteria represented by genera such as *Rhodobium*, *Pseudomonas* and *Coxiella* (Supplementary Tables S3–S7).

There was a single OTU identified as a *Propionibacterium* sp. that was observed in 100% of the *Acropora* spp. samples and 95% of the *P. lutea* samples. This OTU made up on average 3.3% of the community for *Acropora* spp. and 2.5% on *P. lutea*. The CCA and turf did not have any OTUs that were observed in $>95\%$ of all sampled individuals; however, the most ubiquitously observed OTUs (observed in $>80\%$ of the samples) were all Alphaproteobacteria, mostly belonging to the order Rhodobacterales and Rhizobiales. There was a single Rhodobacterales OTU observed in 92% of turf samples, which on average made 3.1% of the bacterial community. This OTU was also observed in 83% of the CCA samples making up 1.8% of the CCA bacterial communities.

The phylogenetic similarity of stable and sporadic bacteria for each host group was determined using the Bray–Curtis distance at the genus level and a test for significant differences were made using *simprof* (Supplementary Figure S2). There were five significant groupings at the 0.01 confidence level. *A. rosaria* and *P. lutea* stable communities each made up their own grouping while the two algal stable communities made up a significant group on their own (Supplementary Figure S2). The stable community associated with *A. hyacinthus* formed a group with the algal sporadic communities. Finally, the sporadic communities of coral all formed their own significant group.

Discussion

All macroorganisms associate with microbes, forming ecological assemblages called holobionts (Gordon *et al.*, 2013). Reef corals and algae are some of the best-studied holobionts, but it remains unclear how stable these associations are over time and space (Table 1). Here we developed a statistical method called the AU test to identify different associative patterns of bacterial members of the holobiont. The AU test showed that coral and algal holobionts harbor two different groups of bacterial symbionts: (i) sporadic symbionts, whose abundance varies significantly between individual holobionts of the same species or guild, and (ii) stable symbionts, which are usually found associated with the holobionts of a particular grouping. There were no obligate, exclusive symbionts (that is, a single bacteria species always associated with a single host and not found on other hosts).

Sporadic symbionts

Sporadic symbionts were variably associated with an individual host, but as a group sporadic symbionts were abundant within the holobiont community. The sporadic symbionts are likely the product of either stochastic events or in responses to environmental pressures. In support of the latter hypothesis, other studies have shown that biogeochemical regimes (Siboni *et al.*, 2012; Kelly *et al.*, 2014) or position on the reef (for example, internal reef zones versus reef periphery; Schöttner *et al.*, 2012) influence reef- and organism-associated microbes. Kushmaro and colleagues (Siboni *et al.*, 2012) showed that excess nitrogen led to an enrichment of denitrifying Bacteria and Archaea. Changes in the symbiont members can lead to holobionts better adapted for particular conditions, such as the switching of *Symbiodinium* clades with depth (Rowan and Knowlton 1995; Toller *et al.*, 2001). Local adaption of the whole reef microbome to local conditions (for example, nutrient regimes) has also been observed in the Line Islands (Kelly *et al.*, 2014). If horizontal acquisition of microbes is the main mechanism for holobiont responses to changing environmental conditions, then adaption is best described as an ecological dynamic. This means that long-term, evolution trajectories are most probably not selected at the level of the holobiont but rather at the species level (that is, swapping of individual members in and out of the holobiont).

Reassessing the ubiquity of a coral symbiont

The *Porites astreoides* symbiont PA1 (*Endozoicomonas* sp.) is ubiquitously associated with *P. astreoides* from Panama to Bermuda (Rohwer *et al.*, 2002). Here we found a Gammaproteobacteria with 99% sequence similarity to PA1, which was found only on *P. lutea* samples collected from Kingman (10% of all *P. lutea* samples). Kvennefors *et al.* (2010) also reported that bacteria similar to PA1 were common on *A. hyacinthus* and *Stylophora pistillata* from the Great Barrier Reef, and we observed several additional OTUs that were closely related to PA1 on all the five hosts studied here. Additionally, Bayer *et al.* (2013) demonstrated that species from the Endozoicomonas genera closely associated with tissue of the coral *S. pistillata*. Therefore, PA1 and its close relatives appear to associate generally with coral reef macrobes and the abundance of this group is subject to local unknown conditions.

Stable symbionts

Stable bacterial symbionts were relatively rare and highly diverse. These stable symbionts are likely associated with host-constructed niches that are less sensitive to the surrounding environment. For example, *Actinobacter* and *Ralstonia* spp. are closely associated with coral gastrodermal cells and endosymbiotic dinoflagellates, suggesting these bacteria occupy specific niches within the macrobial host (Ainsworth *et al.*, 2015). Despite this niche specificity, stable symbionts were not holobiont specific. That is, any holobiont species has a group of stable symbionts; however, these symbionts may also be found on other hosts. This is similar to what has been found for the human and other mammalian microbiomes (Muegge *et al.*, 2011; Human Microbiome Project Consortium, 2012). Additionally, the diversity of these stable symbionts might have a role in the overall community stability (Yachi and Loreau 1999).

Holobionts or hologenomes?

The Holobiont model posits ecological assemblages of symbionts that may change over the lifetime of the macroorganism. The term holobiont was originally coined by Margulis and Fester (1991) and later refined by Rohwer *et al.* (2002) and Knowlton and Rohwer (2003) to explain the emerging observations about ubiquitous, dynamical symbioses between macrobes and microbes (for example, *Symbiodinium* and bacteria in corals). The main premises of the holobiont model are: (1) all macroorgansisms form symbioses with microbes, (2) the bionts (viruses, microbes, protists, macrobe and so on) assemble, disassemble and reassemble in different combinations based on ecological dynamics (for example, environmental conditions, migration, predation and so on), and (3) each biont member of the holobiont belongs to a metapopulation with their own evolutionary trajectory. The bionts may be evolutionarily selected based on interactions with each other (that is, coevolution), but they are not obligate symbionts (this work) and they migrate between holobionts and/or other reservoirs (for example, seawater, sediment and so on).

The Hologenome Theory of Evolution was based on the holobiont model and incorporates the exact same premises with one important exception: heredity. To quote from Zilber-Rosenberg and Rosenberg (2008), '(1) All animals and plants establish symbiotic relationships with microorganisms. (2) Symbiotic microorganisms are transmitted between generations. (3) The association between host and symbionts affects the fitness of the holobiont within its environment. (4) Variation in the hologenome can be brought about by changes in either the host or the microbiota genomes; under environmental stress, the symbiotic microbial community can change rapidly.' Therefore, the only strong difference between these two models is the transmission between generations (that is, heredity; Figure 3 compares and contrasts these two models). Here we test the hypothesis that symbionts are transmitted by looking across populations of coral and algal holobionts. If bacteria are transmitted between generations, then these bacteria should be ubiquitous and have strong phylosymbiotic signals (Brucker and Bordenstein, 2013). The analysis here shows that this is not true; the stable bacterial symbionts are neither ubiquitous nor specific to one host. Ainsworth *et al.* (2015) came to similar conclusions *sans* an AU-like test. Similar to *Symbiodinium*, the vast majority of bacterial symbionts are moving in and out of the holobiont. This is also similar to what has been observed in the human microbiome (Human Microbiome Project Consortium, 2012), and the AU test described here can be used to assess the generalization of this phenomenon across all holobionts.

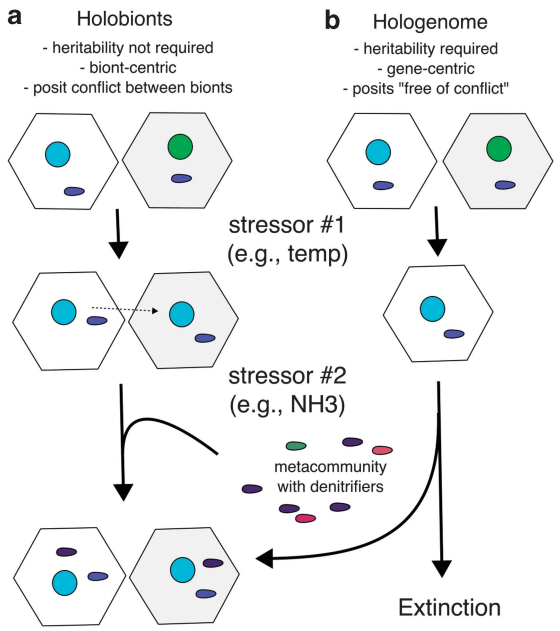

Figure 3 Comparison of the Holobiont model (Rohwer *et al.* 2002; Knowlton and Rohwer, 2003) and Hologenomic Theory of Evolution (Rosenberg *et al.*, 2007). The primary difference between the two hypotheses is the importance of heredity. For the illustration, the coral animal is represented by hexagons, the colored circles represent Symbiodinium and oval shapes represent Bacteria. In actuality, the symbionts also include viruses, Archaea, fungi, protists, other macrobes and so on. In the Holobiont model, (**a**) heritability is not required and the relationships among the bionts are best described by ecological dynamics. Membership in the holobiont may be stable or sporadic as predicted by the AU test (blue ovals in figure) or obligate (for example, plastids or mitochondria). Stable and sporadic symbionts are recruited from other holobionts or from the environment (different color ovals). Changing environmental conditions lead to symbiont switching. Symbionts remain independent entities, may be found outside the holobiont and are sometimes absent from any individual holoboint of the same species (that is, not ubiquitous, similar to PA1). Even in a stable environment, members of the holobiont may be replaced by bionts with similar functions. (**b**) The Hologenome is evolutionary and requires heredity, otherwise it incorporates the same premises laid forth under the Holobiont model and is indistinguishable.

Based on the observations presented here and in Table 1, we propose that the holobiont model best describes what has been observed for macrobe–microbe associations. One place where additional clarity is needed is when two bionts become linked in an obligate manner (for example, Ochman and Moran 2001; Moran *et al.*, 2008; and Brucker and Bordenstein 2013; Bordeinstein and Theis, 2015). Endosymbiotic theory and the subsequent models for understanding this type of symbiosis are established (Margulis and Fester, 1991). To differentiate between obligate symbioses and the more flexible, holobiont-type found in corals, algae and human, we propose that there is a bifurcation called *aboluta iunctio* for 'absolute linkage' where two symbionts become one, indivisible biont (based on some to be determined by probability). The attraction of incorporating *aboluta iunctio* into models of symbioses is that it would be both mathematically tractable and translatable into laboratory and field studies.

What to do with the hologenome?

Despite the eloquent defense of the term by Rosenberg *et al.*, 2007, the term hologenome has become divisive and the older term metagenome should be used to describe the collective genetic material of a particular sample (holobiont or otherwise). As for the Hologenome Theory of Evolution, without clear evolutionary mechanisms, including heredity, this is a misnomer. It might be useful to develop the theory as a null model for multilevel selection, but this will require more quantitative precision than has been forthcoming. Statements that hologenomes are integrated such that they are free from conflict (Rosenberg *et al.*, 2007; Rosenberg and Zilber-Rosenberg, 2013) has eroded any serious consideration by evolutionary theorists. This assertion is clearly not true; symbiont members of the assemblages frequently kill or weaken the holobiont in corals (Kline *et al.*, 2006; Vega Thurber *et al.*, 2008; Vega Thurber *et al.*, 2009). Further, the very name hologenome implies a gene-centric approach reminiscent of the selfish gene. And while it is possible to measure the outcomes of evolutionary processes at the genetic level, this information is only relevant within the context of its environment (for example, in a cell). Bacteria, *Symbiodinium*, corals and the other bionts of the holobiont have remained separate entities despite close association for >200 million years (Timmis *et al.*, 2004; Life and Death of Corals Reefs, chapter 5) and it is the bionts, not just their genetic material, that determines selectable phenotypes (Grafen and Ridley 2007).

Conclusions

The results here support the holobiont model where the microbial and macrobial members have individual evolutionary trajectories. Although there are stable associations between the bacteria and macrobes, these associations are not exclusive and therefore not heritable as a unit. Our emerging view is that coral and algal holobionts assemble and then the environment selects for membership. As the environment changes, there is reassembling and the acquisition of new members. This is similar to the adaptive bleaching model proposed by Buddemeier and Fautin (1993). As with the *Symbiodinium*, our analysis suggests that the stable bacterial symbionts are found on multiple hosts. The fact that the individual elements of the holobiont have remained separate for several hundreds of millions of years also suggests that the unit of selection is at the individual biont level. Until compelling contradictory evidence is presented, the gene-centric proposal of the hologenome is subject to all of the criticisms of the selfish gene hypothesis, as well as the challenges presented by this study (that is, heritability). At this stage, the evidence points toward holobionts. However, to really understand the complex dynamics in these systems, there is a need for strong mathematical models, extensive sampling of naturally occurring holobionts and statistics, such as the AU test.

Conflict of Interest

The authors declare no conflict of interest.

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