

ORIGINAL ARTICLE

The dynamics of microbial partnerships in the coral *Isopora palifera*

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Both bacteria and algal symbionts (genus *Symbiodinium*), the two major microbial partners in the coral holobiont, respond to fluctuations in the environment, according to current reports; however, little evidence yet indicates that both populations have any direct interaction with each other in seasonal fluctuation. In this study, we present field observations of a compositional change in bacteria and *Symbiodinium* in the coral *Isopora palifera* in three separate coral colonies following monthly sampling from February to November in 2008. Using massively parallel pyrosequencing, over 200 000 bacterial V6 sequences were classified to build the bacterial community profile; in addition, the relative composition and quantity of *Symbiodinium* clades C and D were determined by real-time PCR. The results showed that coral-associated bacterial and *Symbiodinium* communities were highly dynamic and dissimilar among the tagged coral colonies, suggesting that the effect of host specificity was insignificant. The coral-associated bacterial community was more diverse (Shannon index up to 6.71) than previous estimates in other corals and showed rapid seasonal changes. The population ratios between clade C and D groups of *Symbiodinium* varied in the tagged coral colonies through the different seasons; clade D dominated in most of the samples. Although significant association between bacteria and symbiont was not detected, this study presents a more detailed picture of changes in these two major microbial associates of the coral at the same time, using the latest molecular approaches.

The ISME Journal (2011) 5, 728–740; doi:10.1038/ismej.2010.151; published online 21 October 2010

Subject Category: microbial ecology and functional diversity of natural habitats

Keywords: coral; bacterial community; *Symbiodinium*; metagenomics; tag pyrosequencing

Introduction

Corals generally associate with a variety of microorganisms, including algae, bacteria, archaea and viruses (Knowlton and Rohwer, 2003; Wegley *et al.*, 2007; Marhaver *et al.*, 2008). Among these associated partners, the most well known are the algal symbionts (genus *Symbiodinium*) and various bacteria. *Symbiodinium* are photosynthetic algal symbionts providing energy for corals (Baker, 2003). Based on their ribosomal DNA sequences, *Symbiodinium* can be categorized into eight distinct phylogenetic clades (A–H), five of which (A–D, F) are associated with corals (Baker, 2003). *Symbiodinium* are geographically specific; the clades C and D are dominant in the corals dwelling in western Pacific Ocean, whereas other types are detected in other oceans, for example, the Caribbean Sea (Baker, 2003). *Symbiodinium* are functionally diverse and, typically, display variations in tolerance against

high temperature and irradiance (Baker, 2003; Stat *et al.*, 2008; Mieog *et al.*, 2009; Takahashi *et al.*, 2009). For instance, *Symbiodinium* clade D is more heat tolerant and is thus found more often in corals subject to conditions of higher temperature or irradiance (Baker, 2001; Rowen, 2004). Because of differences in the physiological characteristics of each type, the population composition of *Symbiodinium* in a coral may change under different environmental conditions, such as season (Chen *et al.*, 2005), latitude (LaJeunesse *et al.*, 2004) and depth (Toller *et al.*, 2001; Frade *et al.*, 2008).

Coral-associated bacteria are highly diverse (Rohwer *et al.*, 2002) and their population compositions are affected by environmental factors such as geographic differences (Hong *et al.*, 2009; Littman *et al.*, 2009), seasonal changes (Koren and Rosenberg, 2006; Hong *et al.*, 2009), coastal pollution (Klaus *et al.*, 2007) and nutrient status (Garren *et al.*, 2009). They potentially have functions relating to coral health, nutrition and disease (Reshef *et al.*, 2006; Rosenberg *et al.*, 2007; Wegley *et al.*, 2007), but their definitive role remains unclear. Many questions also remain about the dynamics of coral-associated bacteria. What factors affect their composition? What are the ecological implications of their compositions and dynamics?

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Received 4 March 2010; revised 29 July 2010; accepted 18 August 2010; published online 21 October 2010

To determine the composition of a coral bacterial population, one of the most common methods has been construction of a 16S rRNA gene clone library (Amann *et al.*, 1995; Rohwer *et al.*, 2002; Lampert *et al.*, 2008; Hong *et al.*, 2009; Reis *et al.*, 2009). Surveying the range of bacterial 16S rRNA gene sequences from such a library provides more detailed taxonomic information of bacterial community than other older methods. This culture-independent method has circumvented the difficulty of unculturable bacteria detection and offers considerable understanding of bacterial biodiversity and ecology (Amann *et al.*, 1995; Rohwer *et al.*, 2002). However, the clone library method has long been limited in the number of colonies picked, mainly because of cost considerations (Rohwer *et al.*, 2002; Lampert *et al.*, 2008; Hong *et al.*, 2009; Reis *et al.*, 2009). As a result, practical application of this method is unfeasible to present the profile of the very diverse bacterial communities found in most environmental samples. To define such a community more precisely, large survey size is crucial. For example, picking 1000 16S rRNA sequences from a clone library would be minimally sufficient to define a coastal bacterial community (Acinas *et al.*, 2004). Similarly, with the current limitations on sampling number, studies of coral-associated bacterial communities with extensive temporal or spatial scales would be impossible.

Massively parallel pyrosequencing (MPP) offers a high-throughput and cost-effective system for survey of bacterial communities by revealing the sequence of a hypervariable region of the 16S rRNA gene (Sogin *et al.*, 2006). Compared with the full-length rRNA gene sequence, the information from the hypervariable region sequence provides nearly equivalent taxonomic data (Huse *et al.*, 2008). This robust method has recently been employed to analyze the biodiversity of complex bacterial communities in different habitats such as the human gut (Huse *et al.*, 2008), hydrothermal vents (Huber *et al.*, 2007) and soil (Roesch *et al.*, 2007). Moreover, modifying the PCR primers with unique sequences in the sequencing reaction makes the method more economical and practical. DNA amplification with different unique-sequence-designed primers for each sample from different sources allows sequencing of all the amplicons from multiple sources in a single reaction run and still offers a sufficient number of sequences for the unbiased analysis of a bacterial community. The sequences in each sample can be simply sorted according to unique sequences of the primers (Binladen *et al.*, 2007; Andersson *et al.*, 2010; Barriuso *et al.*, 2010).

In this study, we adopted the MPP technique and a newly improved quantitative PCR method to study the dynamics of bacterial and symbiotic algal communities in *Isopora palifera* with a monthly sampling survey from February to November in 2008. These rich data sets have provided much detail about the dynamics of these two microbial communities, bacteria and *Symbiodinium*.

Materials and methods

The experimental methods used to analyze the composition of microorganisms in coral *I. palifera* are illustrated in Supplementary Figure S1, which presents a flowchart of the procedure from bench work to bioinformatics analysis.

Study site and sampling

Coral samples and seawater samples were collected from Tan-Tzei Bay (21°57'N, 120°46'E), Ken-Ting National Park, located in Heng-Chun Township at the southern tip of Taiwan. Three colonies of the coral *I. palifera*, at 2 to 3 m depth, were tagged as 351, 352 and 353. *I. palifera* is a plate form in this area (Supplementary Figure S6). Near the tagged coral colonies, we deposited a thermograph to record water temperature.

All tagged colonies were sampled near-monthly for the 7 months of February (winter), April (spring), May (spring), June (summer), July (summer), August (summer) and November (winter) in 2008. Coral fragments (~2 × 2 cm) were collected with hammer and chisel. In addition, 1000 ml sea water was collected from the area near the tagged colonies.

Coral samples were washed twice with sterile synthetic sea water and then sprayed with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) to collect the tissue, using an airbrush. Coral tissue was stored in liquid nitrogen until DNA extraction. Seawater samples were filtered through membrane paper (pore size 0.2 µm; Adventec, Tokyo, Japan) to collect the microbial particles. After filtering, the paper was kept at -20 °C until DNA extraction.

DNA extraction

The coral tissues were recovered from the TE buffer by centrifugation; after discarding the supernatant, tissue pellets were then resuspended with 1 ml TE buffer, and homogenized in a mortar and pestle with liquid nitrogen. The homogenized solution was transferred to a clean tube, and total DNAs were extracted with a PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. To isolate the total DNAs of seawater samples, the filter membrane paper with adsorbed microbial sample was cut into smaller pieces and placed into a clean tube, and the total DNA again extracted using a PowerSoil DNA Isolation Kit (MoBio). Finally, the DNA was resuspended in 150 µl sterile Milli-Q water, aliquoted into PCR microtubes (each tube 10 µl) and stored at -80 °C.

PCR of bacterial V6 hypervariable region of the 16S ribosomal RNA gene and DNA tagging PCR

For amplification of the bacterial V6 hypervariable region of the 16S ribosomal RNA gene, two bacterial universal primers were used: 967F, 5'-CAACGCGA AGAACCTTACC-3' and 1046R, 5'-CGACAGCCATG

CANCACCT-3' (Sogin *et al.*, 2006) with the following PCR conditions: an initial step of 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 10 s and 72 °C for 30 s, followed by a final step of 72 °C for 2 min. The amplification mixture contained 0.5 µl of 5 U *TaKaRa Ex Taq* HS (Takara Bio, Otsu, Japan), 5 µl of 10 × *Ex Taq* buffer, 4 µl of 2.5 mM deoxynucleotide triphosphate mixture, 1 µl of each primer (10 µM) and 5 µl (10–20 ng) template DNA in a volume of 50 µl. The PCR amplicons of the V6 region were checked by 15% native polyacrylamide gel electrophoresis with TAE buffer. The expected DNA band (~100 bp) was cut and DNA extracted by electroelution (Sambrook and Russell, 2001). The quality of the purified product was assayed using a NanoDrop spectrophotometer (Thermo Scientific, Vantaa, Finland).

DNA tagging PCR (DT-PCR) was used to add unique tags to each of the PCR products isolated from different samples (Supplementary Figure S2a). Each tag primer was designed with four overhanging nucleotides at the 5' end of the 967F and 1046R primers. The arrangements of the four nucleotides ensured 256 different kinds of unique tags. In this study, 28 unique tags were used for DT-PCR.

In DT-PCR, the PCR products from individual sample were extended with different unique-tagged primer sets by low-cycle PCR under the following conditions: an initial 94 °C for 3 min, then 5 cycles of 94 °C for 30 s, 57 °C for 10 s and 72 °C for 30 s, followed by 72 °C for 2 min. The DT-PCR mixture contained 0.5 µl of 5 U *TaKaRa Ex Taq* HS, 5 µl of 10 × *Ex Taq* Buffer, 4 µl of 2.5 mM deoxynucleotide triphosphates, 1 µl of each primer (10 µM) and 20 µl (50–100 ng) V6 amplicon in a volume of 50 µl. To confirm the success of the tagging reaction, we checked the PCR products after DT-PCR using non-denatured polyacrylamide gel electrophoresis on 15% gels run at 100 V for 6 h and stained with SYBR-Gold (Invitrogen, Carlsbad, CA, USA). In comparison with the V6 PCR products, the tagged V6 DNA would shift to a higher position in the gel because of their larger molecular mass (Supplementary Figure S2b). Once the tagging reaction was successful, we then purified the tagged amplicon DNAs using PCR Purification Kits (Qiagen, Valencia, CA, USA). The qualities and concentrations of the purified tagged DNA samples were determined using NanoDrop spectrophotometer (Thermo Scientific).

Parallel pyrosequencing

Pooled 200 ng lots of each tagged V6 DNA samples (28 samples in total) were used for parallel pyrosequencing on a Roche 454 Genome Sequencer FLX System at Mission Biotech (Taipei, Taiwan). Ambiguous reads were trimmed off if the sequences had mismatched sequences to the forward and reverse primers or the sequences were shorter than 75 bases. A total of 205 082 sequences were generated and sorted according to unique tags using an in-house sorting tool (<http://tanglab.csie.org/~tanglab/proj1/>).

Determination of Symbiodinium population by PCR length heteroplasmy (PCR-LH) analysis and quantitative PCR

For PCR heteroplasmy analysis, we designed new primers specific for clades C and D of algal symbionts: SymITS2-F (5'-TATGTYTGCTTCAGTGC TTA-3'), Clade-C-ITS2-R (5'-AATTAACAAGCCATT GGC-3'), and Clade-D-ITS2-R (5'-ACTCAGCAAGCC ATGGGC-3') according to the internal transcribed spacer 2 (ITS2) region sequences of *Symbiodinium* from the NCBI (National Center for Biotechnology Information) database (Supplementary Figure S3). The PCR mixture contained 0.5 µl of 5 U *TaKaRa Ex Taq* HS, 5 µl of 10 × *Ex Taq* Buffer, 4 µl of 2.5 mM deoxynucleotide triphosphate mixture, 1 µl of 10 µM SymITS2-F, 0.5 µl of 10 µM Clade-C-ITS2-R, 0.5 µl of 10 µM Clade-D-ITS2-R and 2–5 ng template DNA in a total volume of 50 µl. The amplified condition had the following steps: an initial 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 20 s, and finally 72 °C for 2 min.

After the DNA of ITS2 partial region was amplified, 4 µl of the PCR products of each sample was mixed with 4 µl of 2 × DNA loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA and 10% glycerol), and analyzed by electrophoresis on 15% polyacrylamide gels with 7 M urea. Gels were run at 85 V for 16 h in 0.5 × Tris-borate-EDTA (TBE) buffer (45 mM Tris-borate and 1 mM EDTA) at a constant temperature of 55 °C and stained with SYBR-Gold. The relative intensities of each expected band (clades C and D) were quantified using ImageQuant TL (Amersham Bioscience, GE Healthcare, Waukesha, WI, USA).

For the quantitative PCR analysis (Q-PCR), we designed two pairs of specific primers for the ITS1 partial region (~100 bp) of *Symbiodinium* clades C and D according to the ITS1 region sequences of *Symbiodinium* from the NCBI database. Three primers were designed: QPCR ITS1-F (5'-TGCGGAA GGATCATTCGCAC-3') for both algal symbiont populations, and QPCR-SymITSC-R (5'-CCTCGAGTT CTGCCAGCAGAT-3') specific for clade C and QPCR-SymITSD-R (5'-TGAACGCGCATCTCACCCTG-3') specific for clade D. To avoid pipette error, the total DNA of the coral sample was diluted ninefold before Q-PCR: 10 µl DNA was mixed with 80 µl ddH₂O. The Q-PCR reaction solution contained 9 µl of diluted coral tissue total DNA, 0.2 µM of QPCR ITS1-F (universal primer), 0.2 µM of QPCR-SymITSC-R (for clade C) or QPCR-SymITSD-R (for clade D) and 1 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 µl. Q-PCR analysis was performed in an ABI 7300 Real-Time PCR System (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 10 s and 62 °C for 27 s, followed by 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s for the dissociation step. A standard curve was constructed with a series dilution of template DNA from ITS PCR products of known

concentration. The data were analyzed using ABI 7300 System SDS Software (Applied Biosystems).

Diversity indices and OTU definition

We aligned sequences from individual samples using MUSCLE software (<http://www.drive5.com/muscle/>) and calculated distance matrices based on the Juke-Cantor model. The distance matrices were then used as input to the DOTUR software (Schloss and Handelsman, 2005) to define the operational taxonomic units (OTUs) with a 3% cutoff value and to estimate the Shannon-Weaver diversity index, Chao1 estimator and Simpson index, and to construct rarefaction curves. Calculations of evenness and richness were also based on the number of OTUs.

Taxonomic identification of bacterial V6 sequences

We identified the taxonomic information of the V6 sequences (205 082 reads) using Ribosomal Data Project (RDP) SeqMatch software (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) with 5 KNN match. Those sequences identified as 'unclassified bacteria' by SeqMatch were reconfirmed using an in-house program, Greedy Fetching (GF; C-H Tseng *et al.*, unpublished), based on the BLASTn results to assign taxonomy of V6 reads with a similarity threshold of 0.7 and the approved hits were all at least with 0.91 alignment identity, which is enough to define taxonomy at class level. GF provided a more extensive calculation in sequence similarity searching and enabled us to detect missing information that could not be determined using SeqMatch. The outcome of GF was merged with the results of the RDP SeqMatch analysis.

To analyze the dynamics of the coral-associated bacteria community in the different months, we examined 45 314 unique groups from 205 082 V6 sequences according to sequence similarity; any mismatch or inserted or deleted base between two V6 sequences was regarded as a different unique V6 sequence group. Through plotting and comparing these unique groups in the monthly samples, we were able to survey more detailed information of the compositional changes of the bacterial community. The unique sequence groups were classified taxonomically into different groups at the class level by SeqMatch and GF.

Non-metric multidimensional scaling (nMDS) ordination

The relationships among samples were determined by the statistical method, nMDS ordination (Kenkel and Orłóci, 1986), using each different V6 sequence group as an OTU. To refine OTUs, we aligned these 45 314 unique group sequences from all samples using the program MUSCLE (<http://www.drive5.com/muscle/>) and calculated distance matrices based on the Juke-Cantor model. These distance matrices were input to DOTUR software (Schloss and Handelsman, 2005) to define the OTUs with a

3% cutoff value. This calculation estimated 11 236 OTUs. Then, the relative percentages of each OTU in each sample were incorporated into a matrix to estimate a distance matrix (via City-Block distance) to present the nMDS profile using Primer 6 software (PRIMER-E, Lutton, Ivybridge, UK).

Hierarchical cluster analysis

Hierarchical cluster analysis was used to analyze the relationship between environmental factors (seawater temperature, sunlight intensity and rainfall), bacterial community and symbiotic algal population. We used the software Gene Cluster 3.0 (de Hoon *et al.*, 2004) from <http://www.falw.vu/~huik/cluster.htm> to construct the clusters. The population ratio of each bacterial group was derived from the sequence data sets (classified by SeqMatch and GF at the class level), and the population proportion of the two symbiotic algal groups was determined according to the Q-PCR data sets. Data on sunlight intensity and rainfall were collected from the Central Weather Bureau of Taiwan in Heng-Chun Township, at the southern tip of Taiwan. In the hierarchical cluster analysis, the distance matrix was constructed with normalized data. To normalize the data, we deducted the raw value of each data point from the relevant mean value and then divided by the standard deviation. The cluster constructed was based on the distance matrix with the uncentered correlation distance and complete linkage, two options in Gene Cluster 3.0 (de Hoon *et al.*, 2004). We then visualized the clustering map using Java Treeview software, downloaded from <http://jtreeview.sourceforge.net/>, with a contrast value of 1.5 to enhance readability.

Results

I. palifera-associated bacterial community and its diversity

Coral samples of three tagged *I. palifera* colonies were collected near-monthly in 2008 (from February to November) at Tan-Zi Bay in Ken-Ting National Park, Taiwan. The V6 hypervariable regions of bacterial 16S ribosomal RNA genes were surveyed to determine bacterial community composition of the monthly samples. After V6 DNA amplification by PCR with universal primers and DNA tagging (Supplementary Figure S2), we performed MPP to acquire sequence data.

The number of V6 sequences in each sample varied from 657 to 21 091 (Table 1); however, the rarefaction curves (with a 3% cutoff value) of the samples showed that the sampling size (read number) was generally sufficient (Supplementary Figure S4).

I. palifera-associated bacterial communities were highly diverse (Table 1). Shannon's index ranged from 3.64 to 6.71 in the coral samples and from 3.85 to 6.32 in the sea water. Simpson's index in coral samples ranged from 0.009 to 0.069 and in sea water

Table 1 Sequence information and diversity estimates as represented in V6 tags^a

<i>Index</i>	<i>351-Feb</i>	<i>351-Apr</i>	<i>351-May</i>	<i>351-Jun</i>	<i>351-Jul</i>	<i>351-Aug</i>	<i>351-Nov</i>
<i>S^b</i>	690	304	942	1623	778	863	554
<i>N^c</i>	6724	1751	15 696	19 496	4757	9256	2359
Evenness ^d	0.71	0.80	0.73	0.87	0.76	0.81	0.84
Richness ^e	180.01	93.42	224.27	211.29	217.32	217.32	163.96
Shannon	4.67	4.56	4.98	5.82	5.14	5.49	5.3
Chao 1	959	427	1073	1966	1179	1085	989
Simpson	(887–1057) ^f 0.026	(381–501) ^f 0.029	(1033–1131) ^f 0.031	(1887–2069) ^f 0.017	(1081–1309) ^f 0.028	(1025–1166) ^f 0.009	(872–1149) ^f 0.012
<i>Index</i>	<i>352-Feb</i>	<i>352-Apr</i>	<i>352-May</i>	<i>352-Jun</i>	<i>352-Jul</i>	<i>352-Aug</i>	<i>352-Nov</i>
<i>S^b</i>	392	654	1378	710	460	498	794
<i>N^c</i>	2281	6396	17 256	9523	4592	3296	4300
Evenness ^d	0.85	0.80	0.75	0.76	0.78	0.80	0.74
Richness ^e	116.43	171.58	325.00	178.20	125.34	141.27	218.25
Shannon	5.1	5.20	5.44	5.02	4.79	4.97	4.96
Chao 1	476	811	1701	764	598	643	1071
Simpson	(444–526) ^f 0.014	(763–880) 0.015	(1625–1800) 0.023	(743–800) 0.029	(550–670) 0.023	(597–711) 0.019	(1003–1160) 0.04
<i>Index</i>	<i>353-Feb</i>	<i>353-Apr</i>	<i>353-May</i>	<i>353-Jun</i>	<i>353-Jul</i>	<i>353-Aug</i>	<i>353-Nov</i>
<i>S^b</i>	648	181	588	400	1360	1804	365
<i>N^c</i>	6048	1567	4791	2640	10 867	7785	4545
Evenness ^d	0.74	0.70	0.75	0.84	0.79	0.89	0.79
Richness ^e	171.09	56.34	159.49	116.61	336.71	463.35	99.52
Shannon	4.8	3.64	4.77	5.01	5.67	6.71	4.68
Chao 1	788	262	904	487	2009	2443	643
Simpson	(744–852) 0.051	(226–327) 0.069	(816–1026) 0.032	(454–540) 0.020	(1880–2170) 0.013	(2329–2581) 0.0029	(543–797) 0.018
<i>Index</i>	<i>Sea-Feb</i>	<i>Sea-Apr</i>	<i>Sea-May</i>	<i>Sea-Jun</i>	<i>Sea-Jul</i>	<i>Sea-Aug</i>	<i>Sea-Nov</i>
<i>S^b</i>	606	2753	1373	1085	631	203	215
<i>N^c</i>	10 279	21 091	10 051	12 421	3434	1218	657
Evenness ^d	0.67	0.80	0.78	0.86	0.98	0.72	0.86
Richness ^e	150.80	636.43	342.81	178.18	65.46	65.46	75.95
Shannon	4.28	6.32	5.60	5.57	5.2	3.85	4.63
Chao 1	936	4952	2650	1668	1186	401	469
Simpson	(844–1064) 0.037	(4654–5298) 0.006	(2413–2940) 0.016	(1536–1838) 0.009	(1041–1384) 0.019	(318–544) 0.061	(369–634) 0.021

^aCalculations were based on operational taxonomic units (OTUs) formed at an evolutionary distance of <0.03 (or ~97% similarity).

^b*S* defined as the number of OTUs.

^c*N* defined as the number of sequences.

^dEvenness defined as Shannon/Ln (the number of OTUs).

^eRichness = (number of singleton OTUs-1)/log*N*. The maximum value is (*N*-1)/log*N*.

^fConfidence intervals for the Chao 1 estimator are shown in parenthesis.

ranged from 0.006 to 0.037. Student's *t*-test was used to describe the difference between coral and seawater samples. The *P*-value was 0.96 in Shannon's index and 0.94 in Simpson's index. There was no significant difference in Shannon's and Simpson's indices between the coral and seawater samples. In addition, the seasonal tendency of the diversity indices was irregular. Overall, the bacterial communities in both coral and seawater samples were highly diverse.

Composition of I. palifera-associated bacterial community
The most abundant bacterial groups in the coral or seawater samples were *Alphaproteobacteria* (10–50%) and *Gammaproteobacteria* (12–50%);

however, the specific composition of each bacterial community in the coral tissues clearly differed from the sea water (Figure 1). There were more bacteria of *Betaproteobacteria*, *Actinobacteria*, *Acidobacteria* and *Nitrospira* groups in the coral tissues, whereas seawater samples had more bacteria in the *Flavobacteria* and *Cyanobacteria* groups (Figure 1).

In our study, high proportions (5–50%) of unclassified bacteria were detected in the coral samples using the program SeqMatch from the RDP; in contrast, the seawater samples contained only approximately 5% unclassified bacteria (Figure 1a). To confirm this observation, we assessed the same data sets using a self-developed method, the GF technique (see Materials and methods). In the coral samples, GF reassigned 32 655 unclassi-

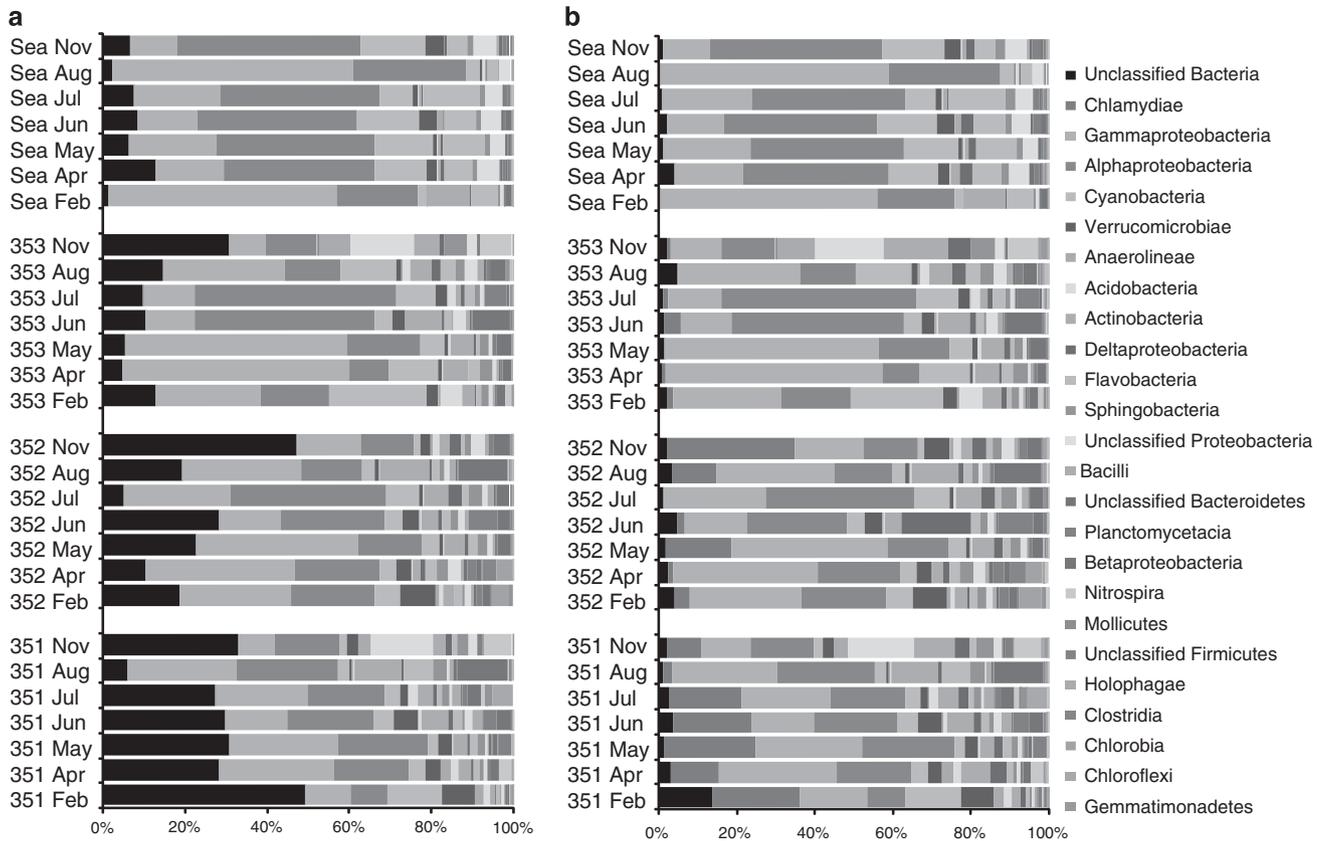


Figure 1 Bacterial composition profile. The bacterial compositions were analyzed using two methods, SeqMatch alone online at RDP (a) and a combination method, SeqMatch-Greedy Fetching (GF) (b). (a) With SeqMatch, high proportions (5–50%) of unclassified bacteria were detected in the samples. (b) With SeqMatch-GF method, the unclassified bacteria were reduced to <5%. In these figures, bacteria composition in *I. palifera* was different from sea water, and the bacterial composition in *I. palifera* varied in different coral colonies and at different months.

fied sequences in the RDP SeqMatch analysis to different taxonomic classes; in all, 28 508 (87%) unclassified sequences were reassigned to known taxa (Figure 1b). In seawater samples, 4790 sequences were assigned to unclassified bacteria by RDP SeqMatch. After GF analysis, these were reduced to 1174 sequences of unclassified bacteria (75% unclassified sequences reassigned to known taxa; Figure 1b).

In addition, by GF refinement, we discovered a group of novel bacteria, which was closely related to *Chlamydia* (Figures 1a and b). The *Chlamydia*-like bacteria (CLB; 88% similarity to *Chlamydia* based on NCBI database) were specifically found only in the coral samples. This bacterial group was the major population (up to 33%) of bacterial communities in the coral samples.

The distribution profile of unique bacterial V6 sequence group

We used the unique V6 sequence group profiles to analyze the bacterial composition below the class level to detect dominant bacteria and their population changes. Overall, most of the bacterial groups in the coral or seawater samples remained

minor components of the population most of the time. It was evident, however, that a few particular bacterial groups in the coral samples, such as CLB, *Rhodobacteraceae*, *Sphingomonadales*, *Escherichia*, *Pseudomonas* and *Chroococcidiopsis*, largely appear at particular times; however, they became dominant for only brief periods of time (Figure 2). In the seawater samples, SAR11 was the constant predominant group (comprising 3–4% of total bacteria in most months).

The variation of the composition of bacterial community within each of the coral samples was mainly because of the temporary predominance of a particular bacteria with a considerable population. For instance, the increase of *Gammaproteobacteria* in April and May (Figure 1 and Supplementary Figure S5) was caused by an increase in *Escherichia* (Figure 2). In colonies 352 and 353, the increase of *Alphaproteobacteria* in June (Supplementary Figure S5) was caused by an increase in *Rhodobacteraceae* (Figure 2). In colony 352, the spike of *Deltaproteobacteria* (20%) in June resulted from increasing presence of an unclassified *Deltaproteobacterial* group. Similarly, the enrichment of *Cyanobacteria* in February (Figure 1 and Supplementary Figure S5) resulted from *Chroococcidiopsis* (Figure 2).

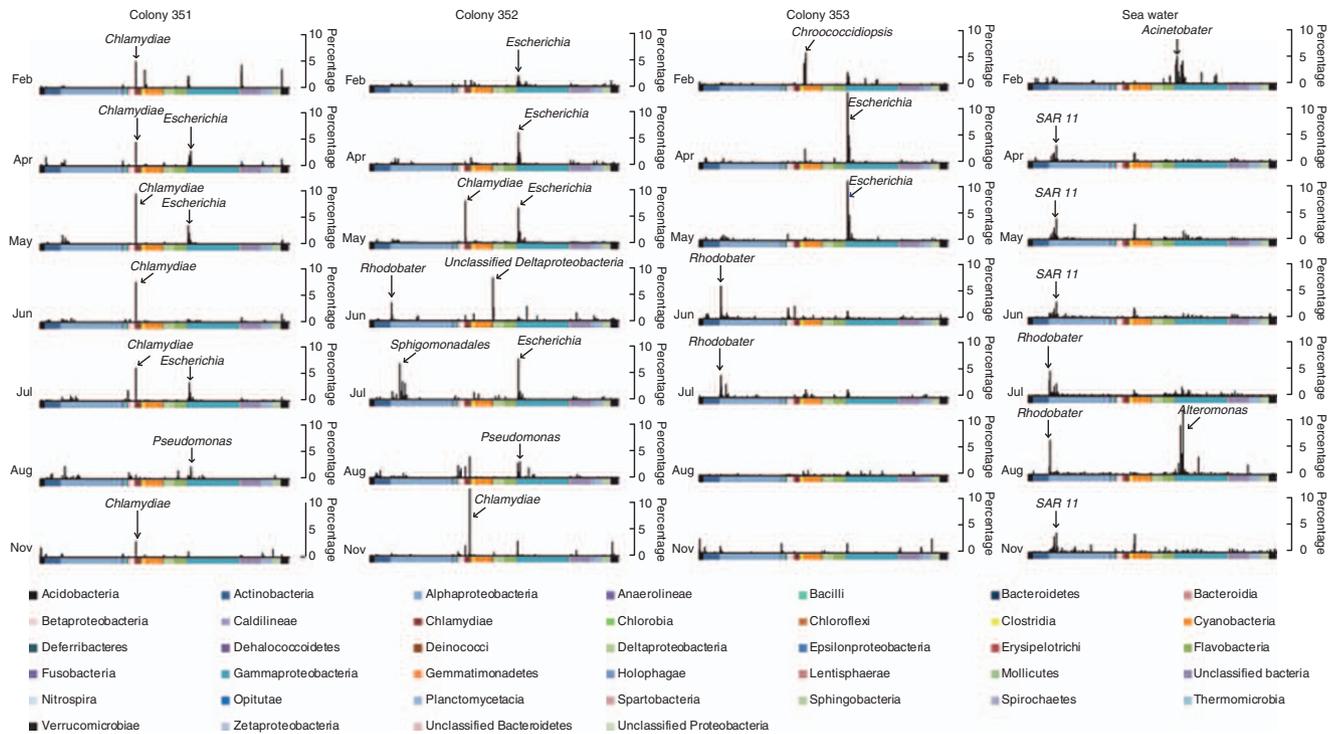


Figure 2 The V6 unique sequence groups in each sample. This profile illustrates dominant bacteria groups and how bacterial communities changed. Variations in the composition of bacterial community within each of the coral samples was rapid and mainly resulted in temporary predominance of bacteria such as *Rhodobacteraceae*, *Sphingomonadales*, *Escherichia* and CLB. The y axis shows the percentage of each unique group in each sample from three colonies and sea water. The color bars on x axis indicate the taxonomy of the bacteria. Each vertical black bar signifies the proportion (%) of a unique bacterial group (labeled). Some dominant groups could not be defined to Family; therefore, these groups were labeled with class name (such as CLB and *Deltaproteobacteria*) or cluster name (such as SAR11).

In addition, the distribution profile of unique bacterial V6 sequence groups provided a more detailed resolution of changes in the bacterial community when compared with an analysis of bacterial groups based on higher taxonomic levels (for example, Class; Figure 1). In the seawater samples, *Gammaproteobacteria* was the major group in February and August; however, the compositions of *Gammaproteobacteria* in these two months were distinct. *Acinetobacter* was the most abundant *Gammaproteobacterial* group in February, but *Alteromonas* was most abundant in August (Figure 2). Similarly, in colony 352, *Gammaproteobacteria* was the major group in June and July; however, the compositions of *Gammaproteobacteria* in these two months were distinct. *Rhodobacter* was predominant in June, whereas *Sphingomonadale* predominated in July. CLB was abundant in May and November in colony 352; however, the predominant group of CLB in May was different to that in November. On the contrary, in colony 351, the predominant groups of CLB remained the same in all months.

Comparison of *I. palifera*-associated bacterial communities

Based on sequence similarity, a total of 11 236 OTUs were classified by DOTUR using the Juke–Cantor

model with a cutoff value of 3%. The OTU percentages in each sample were incorporated into the nMDS matrix and analyzed by city-block distance. The coral-associated bacterial communities were relatively closer to each other and significantly different from the bacterial community of the sea water in the two-dimensional nMDS map, which also supports the result of the analysis of the bacterial taxonomy (Figure 3).

In addition, the bacterial communities of the coral samples collected in the same month clustered together closely (Figure 3). The bacterial compositions of the samples of spring and summer were more similar to each other, whereas the winter samples also congregated together.

Symbiodinium communities

Two methods, PCR-LH and Q-PCR, were adopted to quantify the population of *Symbiodinium* clades C and D (symbiotic algae). Initially, we adopted the PCR-LH technique to detect the population proportions of two *Symbiodinium* clades. Two distinguishable specific PCR products (102 and 96 bp) of ITS2 region of clades C or D were analyzed using urea-polyacrylamide gel electrophoresis (Supplementary Figure S7a). The relative proportions of these two populations in the coral samples were determined by band intensity. The histogram shows the profile

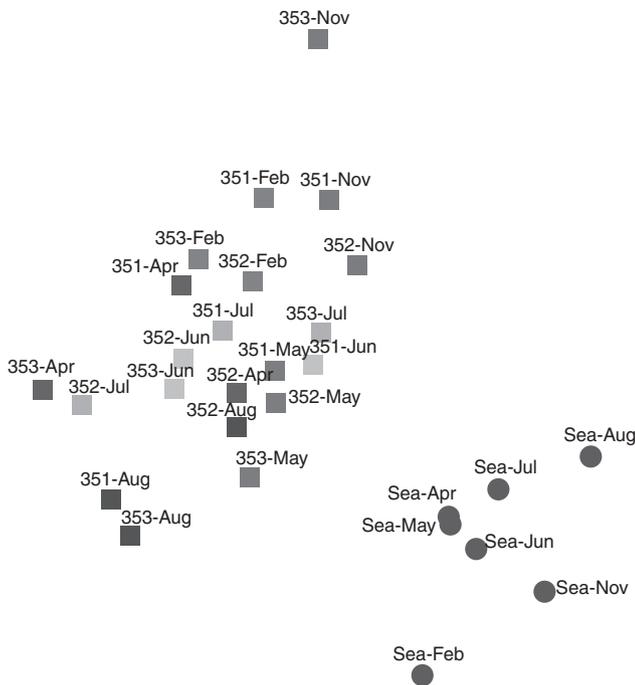


Figure 3 Non-metric multidimensional scaling ordination (nMDS) analysis. We used the relative percentages of each OTU (at 3% cutoff value) in each sample to perform nMDS mapping (see Materials and methods). In this map, each dot signifies the bacterial population of an individual sample. The square boxes show coral samples, and blue circles show seawater samples. In the coral samples, different colors represent different months. The coral-associated bacterial communities were relatively close to each other and significantly different from the bacterial community of the sea water. Moreover, the bacterial compositions of the coral samples collected in the same month tended to cluster together. The color reproduction of this figure is available on the html full text version of the manuscript.

of the C/D ratio in the different coral samples and months (Supplementary Figure S7b). In colony 351, clade D was the constant predominant group in all the samples, and the symbiotic algal communities were more stable than those in colonies 352 and 353.

Using specific primers based on the indel mutation region in ITS1 of clades C and D, we performed three independent Q-PCRs to confirm the result of PCR-LH. The Q-PCR results (Figure 4) were similar to those from PCR-LH (Supplementary Figure S7). However, inaccuracy of the relative ratio of clades C and D occurred when using PCR-LH because that technique failed to represent the correct measured value if the band's color intensity exceeded the saturation threshold, and the proportion of an abundant group was therefore underestimated. For instance, in 351-Feb, the clade D proportion was estimated at ~80% and 99% in PCR-LH and Q-PCR analysis, respectively. On the other hand, in 352-Apr, the clade C proportion was estimated to be 60% in PCR-LH but 80% in Q-PCR.

According to the results of Q-PCR and PCR-LH, all coral colonies contained both clades C and D

(Figure 4 and Supplementary Figure S7). In April, there was a sharp increase in the clade C population (>60% proportion) in colonies 352 and 353. However, the *Symbiodinium* spp. population of colony 351 was dominated by clade D (>98% proportion) throughout our survey (Figure 4). Take together, these results show that *Symbiodinium* composition in *I. palifera* varied at different times and that the extent of this effect varied in different colonies.

Hierarchical clustering analysis

We used hierarchical clustering analysis to examine the relationships between environmental factors (seawater temperature, sunlight intensity and rainfall), bacterial community and symbiotic algal population. The proportion of *Clostridia* reached a maximum in April, whereas proportions of *Chlorobia* and *Chloroflexi* were high in February and April. Rainfall and the population of *Bacilli* had a similar profile (Figure 5), which supported the association of a high *Bacilli* proportion with rainfall (Supplementary Figure S8). There were no associations between seawater temperature or sunlight intensity and the variation of various bacterial groups at the class level (Figure 5). The proportion of *Symbiodinium* clade C increased in April, whereas the clade D proportion decreased at the same time. The changes in *Symbiodinium* proportion were not similar to the variation of bacterial groups at the class level (Figure 5).

Discussion

In this study, we suggest that both bacterial and *Symbiodinium* populations in the coral *I. palifera* vary at different times and to different extents in different colonies; notably, the compositional changes of bacterial community are likely to be associated with seasonal changes. The extensive information provided by the recently developed technique of MPP clearly defines monthly changes of the coral-associated bacterial community that were unavailable in previous surveys using older methods or shorter sampling timescales (Rohwer *et al.*, 2002; Koren and Rosenberg, 2006; Hong *et al.*, 2009).

Factors affecting fluctuation of the *I. palifera*-associated bacterial community

Our results suggest that three factors affected the coral-associated bacterial community, including the environment (rainfall) (Figure 5 and Supplementary Figure S8), seasonal changes (Figure 3) and colony variation (Figures 1 and 2).

Environmental factors were considerable drivers of the structure of the coral-associated bacterial community, as also suggested by other studies (Koren and Rosenberg, 2006; Garren *et al.*, 2009; Hong *et al.*, 2009; Littman *et al.*, 2009). Three factors

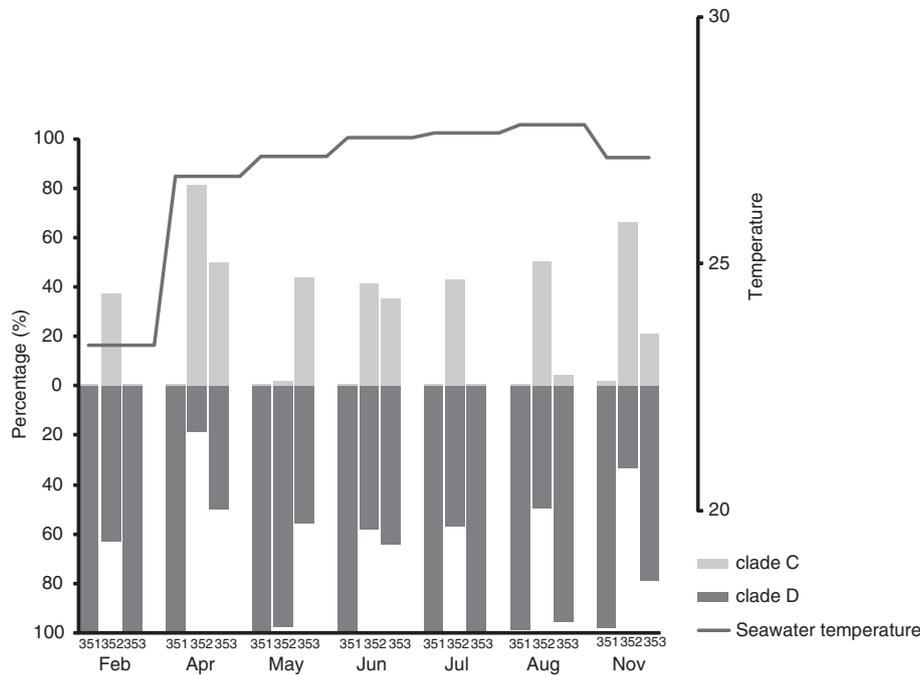


Figure 4 *Symbiodinium* population analysis by real-time quantitative-PCR. Two populations of *Symbiodinium* clades C and D were determined by real-time quantitative PCR of the ITS. All the coral colonies contained both clades C and D but the relative proportion of the *Symbiodinium* members varied at different times and between coral colonies. In April, the temperature shifting point, there was a sharp increase in the clade C population (>60% proportion) in colonies 352 and 353. However, the *Symbiodinium* spp. population of colony 351 was dominated by clade D (>98% proportion) throughout our survey. The green bar is the proportion of clade C and the blue bar is the proportion of clade D. The right y axis is percentage and the left y axis is temperature (°C). The color reproduction of this figure is available on the html full text version of the manuscript.

(seawater temperature, sunlight intensity and rainfall) were available and included in this study in the association analysis with the bacterial groups. Among these environmental factors, rainfall seems to be the factor with greatest effect on the bacterial community of *I. palifera* at Ken-Ting. Changes in the pattern of *Bacilli* bacteria were associated with rainfall (Figure 5 and Supplementary Figure S8). This bacterial group comprised 1% of the total bacterial community in the coral, and many *Bacilli* are soil-borne bacteria. We speculate that these bacteria might come from terrestrial soil because in the summer months (from June to August), abundant rainfall (~500 mm per month; Supplementary Figure S9) flushed out a great amount of soil from the land to the coastal waters around Ken-Ting (Meng *et al.*, 2008). Soil-borne bacteria, such as *Bacilli*, were likely disseminated to the reef where they took up brief residence in coral. A similar phenomenon has also been detected and discussed in another report (Hong *et al.*, 2009), but there has been no other direct evidence.

Based on the cluster analysis, unlike the rainfall effect on coral-associated bacteria (Figure 5), there was no association between seawater temperatures and bacterial group variation (Figure 5). The water temperature in Ken-Ting changed over a small range (24–28 °C) during our study (Supplementary Figure S9). This result suggests that water temperature

might not be a decisive factor triggering the compositional change of bacterial community in *I. palifera* at Ken-Ting. Perhaps, larger temperature changes might have more effect, or other unknown environmental factors that still require further investigation may be involved.

Seasonal variation was another potential factor prompting compositional change of the bacterial communities. In the nMDS analysis, the bacterial composition profiles of the coral samples collected in the same month tended to cluster together (Figure 3). Moreover, the bacterial compositions of the samples of spring (April and May) and summer (June, July and August) were similar to each other, and the samples of winter (February and November) clustered separately (Figure 3), similar to our cluster analysis (data not shown). The composition of the *I. palifera*-associated bacterial community had seasonal-dependent variation. Apart from water temperature, other environmental conditions accompanying seasonal changes may have caused the bacterial composition to alter significantly. To confirm seasonal changes or temporal dynamics on the coral-associated bacterial community, a longer survey and a larger sample size should be able to provide more information.

Alphaproteobacteria and *Gammaproteobacteria* were the two constant predominant groups in *I. palifera*, as seen in previous observations on the

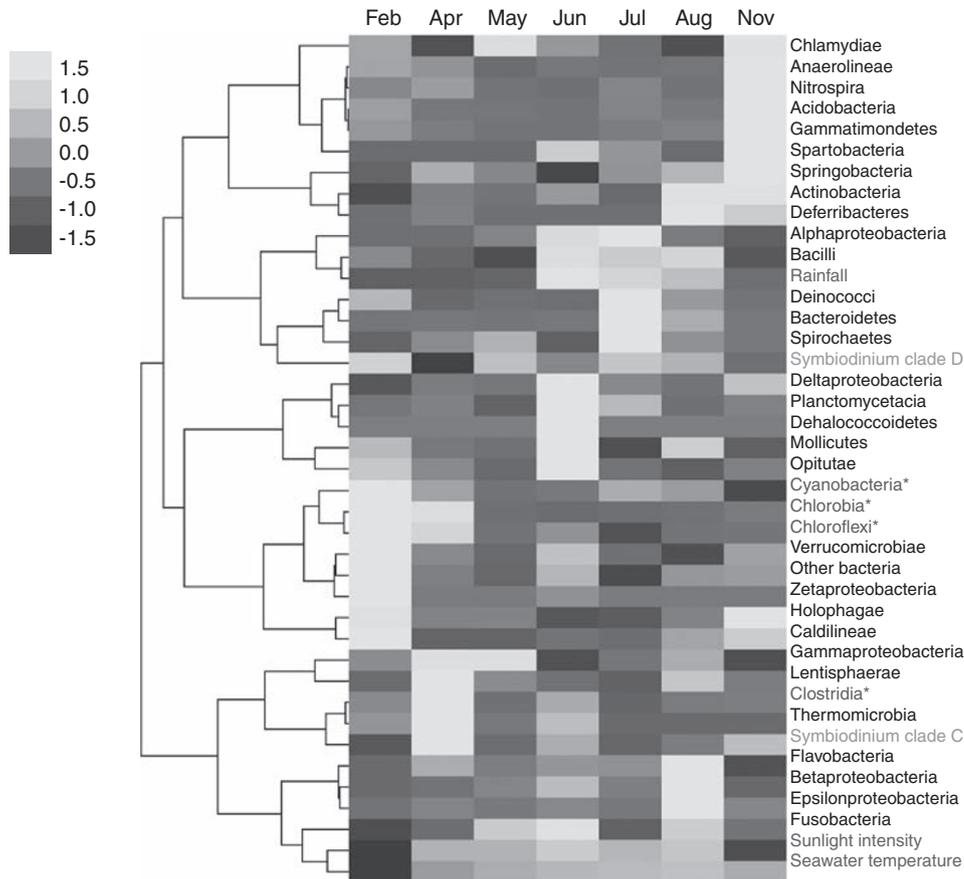


Figure 5 Hierarchical cluster analysis. Single-way clustering method was used to dissect relationships between the abundance of 35 individual bacterial groups at class level, the abundance of *Symbiodinium* (containing both clades C and D) and environmental parameters (seawater temperature, sunlight intensity and rainfall) in the seven months of February, April, May, June, July, August and November. Lighter colors (yellow) mean more members of the bacterial and *Symbiodinium* group detected, or higher values of environmental conditions. Darker colors (dark blue) mean fewer bacteria or *Symbiodinium* or lower values of environmental conditions. In this map, rainfall and population of *Bacilli* were correlated. Potential nitrogen fixation bacteria (*Cyanobacteria*, *Clostridia*, *Chlorobia* and *Chloroflexi*) were enriched in February and April. The proportion of symbiont clade C was increased in April. Heat map intensities were calculated from normalized data (see Materials and methods) with the uncentered correlation distance and complete linkage, the two options in the Gene Cluster 3.0. Blue: potential nitrogen fixation bacteria, labeled by *. Red: environmental factors, seawater temperature, sunlight intensity and rainfall. Orange: the *Symbiodinium* clades. The color reproduction of this figure is available on the html full text version of the manuscript.

coral *Acropora* (a coral genus closely related to *Isopora*) in the Great Barrier Reef (Hong *et al.*, 2009; Littman *et al.*, 2009). Both of these bacterial groups comprised >10% proportions of the bacterial communities in *Acropora* and *I. palifera*. Importantly, however, CLB were abundant (up to 33%) in *I. palifera*, but were not detected in Great Barrier Reef *Acropora* (Littman *et al.*, 2009). This difference might be caused by differences in geography and coral species, or they may have been present but were undetected by previous methods.

Escherichia and *Rhodobacteraceae* were the major groups, respectively, of the *Alphaproteobacteria* and *Gammaproteobacteria* detected in *I. palifera*. *Escherichia*, the dominant group in April and May (Figure 2), appeared consistently in all colonies. In addition, *Escherichia* was assumed to be an animal-associated bacteria (Feng *et al.*, 2002). *Escherichia* presence may be indicative of human and animal

waste, because the coast of Ken-Ting is a popular tourist spot. However, the exact reason for the appearance of *Escherichia* still needs more evidence to prove conclusively. *Rhodobacteraceae*, a group of photosynthetic bacteria (Paoli and Tabita, 1998; Xiong *et al.*, 2000), appeared abundantly in colonies 352 and 353 in June (Figure 2). Potential functions of this particular group in corals have not been described. Notably, June brought together relatively high levels of rainfall, temperature and sunlight, as indicated in the clustering analysis. Is this appearance of *Rhodobacteraceae* associated with these environmental factors? The answer is still uncertain.

The cyanobacteria *Chroococcidiopsis* was abundant in colony 353 in February (Figure 2). *Chroococcidiopsis* has been found on the rock in marine environments (Díez *et al.*, 2007; Goh *et al.*, 2009). However, the reason for its appearance in coral is still unknown.

Diversity of the coral-associated bacteria is very high
In this study with a large survey (>200 000 reads using pyrosequencing), we have shown that the bacterial communities in sea water and coral are highly diverse, with Shannon's index as high as 6.71, much higher than the 4.95 found using the clone library method (reviewed in Reshef *et al.*, 2006). This is the highest index value of diversity ever detected in a coral-associated bacterial community. Despite the difference between the two methods of measuring the index value, our result suggests that the diversity of the coral-associated bacteria is much higher than previously thought. Moreover, our study also highlights that the survey number of a representative gene, such as the 16S ribosomal RNA gene, is critical in revealing the composition of a bacterial community. The rarefaction curves (at a 3% cutoff value) of the samples show that the sampling size (read number) in our survey, with over 1500 sequences for each coral sample (Table 1), was sufficient to reveal the bacterial community in *I. palifera* (Supplementary Figure S4).

Potential nitrogen fixer groups

Corals can take up ammonium and nitrate compounds from surrounding water as a nitrogen source (Grover, 2002, 2003). However, as the reef ecosystem is a nutrient-limited environment (Muscatine and Porter, 1977), bacteria-driven nitrogen fixation can compensate for any deficiency of nitrogen source for coral holobiont (Shashar *et al.*, 1994; Lesser *et al.*, 2004). Nitrogen-fixing bacteria have been detected elsewhere in coral (Shashar *et al.*, 1994; Lesser *et al.*, 2004). In this study, we also detected several potential nitrogen fixers, including *Cyanobacteria* (Meeks, 1998; Berman-Frank *et al.*, 2003), *Clostridia* (Chen *et al.*, 2001), *Chlorobia* (Wahlund and Madigan, 1993) and *Chloroflexi* (Seshadri *et al.*, 2005; Kneip *et al.*, 2007). These potential nitrogen fixer groups in *I. palifera* greatly were enriched in February and April (Figure 5). Although better evidence is still lacking, we speculate that this phenomenon might be caused by a relative nitrogen limitation from February to April (Meng *et al.*, 2008). During this period, the coral might be able to acquire some nitrogen from these potential nitrogen fixers. However, in the summer (June, July and August), the nitrogen-limited condition was released by abundant rainfall flushing a large amount of nutrients, including nitrogen sources, into coastal waters from river outflow as previously observed (Meng *et al.*, 2008), and the potential bacterial nitrogen fixer population proportion also decreased.

The fluctuation of the symbiotic algal composition

The composition of *Symbiodinium* in corals is known to be changeable (Toller *et al.*, 2001; LaJeunesse *et al.*, 2004; Chen *et al.*, 2005; Reimer *et al.*, 2007), varying with environmental conditions

such as season (Chen *et al.*, 2005), latitude (LaJeunesse *et al.*, 2004) and depth (Toller *et al.*, 2001; Frade *et al.*, 2008). We also observed changes in the algal symbiont population in terms of number and composition. According to our PCR heteroplasmy and Q-PCR analyses, the algal symbiont composition in the coral samples continuously fluctuated but both clades C and D were always detectable in all three tagged colonies throughout our survey.

What drives the change of dominance of the algal population? The relevant factors are probably highly complex. One factor may be the difference in physiological properties between the two clades (Rowen, 2004). Most of *Symbiodinium* clade D is heat tolerant and they are commonly found in corals living in shallow and inshore reefs with more radiation and higher temperature fluctuation (Baker, 2003; Rowen, 2004; LaJeunesse *et al.*, 2008). Thus, harboring symbionts of clade D may increase the survival rate of the coral during bleaching events in hot seasons as suggested by Jones *et al.* (2008). However, clade D may not be the perfect symbiotic partner all the time. In juvenile coral stages, corals infected with clade D grew slower than those with clade C (Little *et al.*, 2004), suggesting that members of clade C may provide other growth benefits than clade D algae despite their greater temperature sensitivity (Rowen, 2004). It should also be noted that not all subgroups of clade D confer temperature tolerance in corals (Abrego *et al.*, 2008). Considering the complexity of physiological features in clades C and D, we suggest that a better analysis of each major subgroup of clades C and D may shed more light on their population dynamics during season changes. Although the reasons for the seasonal alterations in clade proportion are still unclear, these changes occurred internally in the coral because we did not detect any of the algal genes in the seawater samples (data not shown).

Issues for use of nucleotide-tag primers in MPP

MPP offers several advantages to surveys of microbial communities, including sufficient data, low cost, low labor requirements and avoidance of bias (Sogin *et al.*, 2006; Binladen *et al.*, 2007; Huse *et al.*, 2008). However, when amplifying the 16S rRNA gene using universal bacterial primers with specific tag sequences, it has been argued that appending different tag sequences to the universal primers would bring extra bias during the amplification by PCR because of the differential hybridization efficiency of different primers. Similarly, we have also observed a similar problem in that universal primers with different code sequences may be a cause of differential amplification efficiency. To avoid such bias from different code sequences, we first amplified the bacterial V6 region with universal bacterial primers without tag sequences, and only then carried out DNA tagging PCR with a very low number of amplification cycles, a process that can specifically

add individual code sequences to different PCR amplicons with universal bacterial primers containing different code sequences. Compared with the previous method, this newly modified technique for DNA coding generates less artificial bias.

We strongly recommend the MPP approach for the study of the population dynamics of microbial patterns in coral because of the highly dynamic nature of such populations in response to temporal or seasonal factors and the evident variation among individual colonies in corals, such as *I. palifera*. The robustness of the technique can provide the capacity to handle a large number of samples with affordable costs and low labor requirements (Sogin *et al.*, 2006; Binladen *et al.*, 2007; Huse *et al.*, 2008).

Acknowledgements

We thank Garmin Hsu and the colleagues of Allen CL Chen for assistance with providing samples and seawater temperature data. This study was supported by thematic project funding of Academia Sinica, Taiwan, and by the Biodiversity Research Center, Academia Sinica, Taiwan.

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