Seasonal Changes in Bacterial Communities Associated with Healthy and Diseased *Porites* Coral in Southern Taiwan

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Seasonal Changes in Bacterial Communities Associated with Healthy and Diseased Porites Coral in Southern Taiwan

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Abstract

We compared the bacterial communities associated with healthy scleractinian coral *Porites* sp. with those associated with coral infected with pink spot syndrome harvested during summer and winter from waters off the coast of southern Taiwan. Members of the bacterial community associated with the coral were characterized by means of denaturing gradient gel electrophoresis (DGGE) of a short region of the 16S rRNA gene and clone library analysis. Of five different areas of the 16S rRNA gene, we demonstrated that the V3 hypervariable region is most suited to represent the coral-associated bacterial community. The DNA sequences of 26 distinct bands extracted from DGGE gels and 269 sequences of the 16S rRNA gene from clone libraries were determined. We found that the communities present in diseased coral were more heterogeneous than the bacterial communities of uninfected coral. In addition, bacterial communities associated with coral harvested in the summer were more diverse than those associated with coral collected in winter, regardless of the health status of the coral. Our study suggested that the compositions of coral-associated bacteria communities are complex, and the population of bacteria varies greatly between seasons and in coral of differing health status.
Keywords: Coral disease; Primer effects; Pink spot syndrome; DGGE.
Introduction

Coral reef is one of the most complex marine ecosystems. Many fish, snails, and lobsters depend on corals for their survival. The global decline in coral populations has endangered this very important ecosystem (Loya et al. 2001; Jones et al. 2004a). Bacteria are ubiquitous in every habitat on earth. The symbiotic relationships between bacteria and corals have been investigated using various techniques (Kellogg 2004; Wegley et al. 2007; Chiou et al. 2010; Kimes et al. 2013; Lema et al. 2014). It is now known that corals harbor many different types of bacteria (Frias-Lopez et al. 2002; Rohwer et al. 2002; Chiou et al. 2010; Ceh et al. 2011; Lema et al. 2014), archaea (Ferris et al. 1996; Kellogg 2004), and fungi (Bentis et al. 2000; Wegley et al. 2007) and their associations are often host species-specific (Rohwer et al. 2001; Rohwer et al. 2002). However, bacteria-coral associations are not always stable: evidence suggests that environmental change could disrupt the microbe-coral association, leading to either a new adaptation or to coral disease (Rosenberg et al. 2007).

During the past 30 years, more than twenty disease signs have been described in corals; however, the causative agents of most coral diseases are still unknown (Rosenberg et al. 2007). Advances in molecular techniques, such as the 16S rRNA...
clone library and terminal restriction fragment length polymorphism (T-RFLP), provide culture-independent methods for comparing the differences in microbial communities between healthy and diseased corals (Ritchie and Smith 1995; Frias-Lopez et al. 2002; Cooney et al. 2002; Frias-Lopez et al. 2004; Chiou et al. 2010). For example, bacteria isolated from healthy elkhorn coral *Acropora palmata* and bacteria from the same coral with type 1 white band disease are significantly different (Pantos and Bythell 2006). Similar surveys have shown that there are numerous toxin-producing heterotrophic bacteria in the mucopolysaccharide layer and bacterial mat near the black band area of *Siderastrea* coral infected with black band disease (BBD) (Sekar et al. 2006). Recently, high-throughput sequencing and microarray technologies have been applied to study microbial communities associated with coral disease, and our knowledge of the abundance, diversity and gene content of microbiota in healthy and diseased coral has increased (Cardenas et al. 2012; Roder et al. 2014; Closek et al. 2014; Ng et al. 2015). These studies not only confirm the changes in bacterial communities of coral affected by disease, but also suggest that dynamic interaction between environmental factors, host immunity, and microbiome plays an important role in coral health, and any
change in these components may have the potential to result in the development of diseases (Cardenas et al. 2012; Roder et al. 2014).

There are several methods currently available for identifying bacteria without prior knowledge of the bacterial community. PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) for DNA amplification and separation techniques coupled with DNA sequencing is one of these methods and has been used successfully to characterize and compare the microbial diversity and community structure in environmental samples (Muyzer et al. 1993). This culture-independent method compares microbial communities by examining how far 16S rDNA fragments of different bacteria move through a polyacrylamide gel. The gel bands are then excised, sequenced and compared with reference 16S rRNA genes in the database. Although this technique is now routinely carried out for the analysis of microbial diversity, selection of the region(s) of the 16S rRNA gene that could provide the most information about the bacterial community is primarily based on experience; selection of the 16S rRNA gene regions, and thus the choice of primer set, can greatly affect the outcome (Yu and Morrison 2004). Even so, few studies have critically analyzed the choice of primers for study of the bacterial communities associated with corals (Yu and Morrison 2004; Sanchez et al. 2007;
Yu et al. 2008). Moreover, only a small number of researchers have analyzed how seasonal changes affect the coral bacteria association (Bourne et al. 2008; Hong et al. 2009; Ceh et al. 2011; Kimes et al. 2013; Lema et al. 2014).

Invasion of trematodes in Porites tissue causes bright pink spots to appear on the surface of the coral. This disease is called pink spot syndrome (PSS) (Aeby 2003). Recently, Benzoni et al. (2010) reported that trematodiasis is only a secondary infection of the already weakened Porites. The bacterial community associated with PSS in coral has never been studied. In the present study, we therefore used PCR-DGGE and DNA sequencing techniques to characterize and compare the dominant members of the bacterial community associated with healthy and PSS Porites. Porites sp. was harvested from Nanwan at Kenting National Park in southern Taiwan. We used five different primer sets to amplify different hypervariable regions of the 16S rRNA and compared the quality of information obtained. We also examined the dynamics of the bacterial communities in coral collected in summer and in winter.
Materials and methods

Sampling

Fragments of separate colonies of *Porites* sp. coral were collected at a depth of approximately 7 meters near Nanwan (21°57'1.78"N, 120°46'13.8"E) at Kenting National Park in southern Taiwan during summer (June, 2007) and winter (November, 2007). The samples (obtained in triplicate) were divided into four groups: healthy colonies collected in summer (SH 1-3), pink spot-diseased colonies collected in summer (SD 1-3), healthy colonies collected in winter (WH 1-3), and the pink spot-diseased colonies collected in winter (WD 1-3). The average seawater temperature in June and November at the times of sampling was 29.0 ± 0.9 and 24.7 ± 0.8 °C, respectively (Hsieh 2009). Coral samples were collected underwater using a hammer and chisel. Each sample was placed in an individual sterile plastic bag with seawater. Samples were delivered to the laboratory in an ice box within 30-60 min, whereupon the seawater within each plastic bag was decanted and the sample stored at -20 °C until analysis.

DNA extraction
Five grams of coral were washed twice with filtered (0.22 \mu m) artificial seawater (24.544 g/L NaCl, 4.676 g/L MgCl$_2$·6H$_2$O, 3.128 g/L MgSO$_4$, 1.323 g/L CaCl$_2$·2H$_2$O, 0.671 g/L KCl, 0.168 g/L NaHCO$_3$, and 2.384 g/L HEPES) and pulverized using a mortar and pestle. The sample was transferred to a 50-mL centrifuge tube, mixed with 10 mL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and shaken at 4 °C for 10 h. Bacterial genomic DNA from the supernatant was extracted using a PowerMax Soil DNA Isolation kit (MO BIO Laboratory, Carlsbad, CA, USA).

**PCR amplification of 16S rRNA fragments**

The 16S rRNA gene sequences from 8 to 1513 bp of *Escherichia coli* were amplified by PCR using a method modified from that used by Muyzer et al. (1993) with the universal bacterial primers 8F (5’-AGAGTTTGATCHTGGCTCAG-3’) and 1492R (5’-ACGGHTACCTGTTACGACTT-3’). The PCR mixture was of a final volume of 50 \mu L and contained 1 \mu L (roughly 10 ng) of coral bacterial genomic DNA, 500 pmoles of each primer, 100 \mu moles of dNTP, 5 \mu L of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl$_2$, and 500 mM KCl), and 2.5 U of Taq
DNA polymerase (Takara Bio, Otsu, Japan). Touchdown PCR was performed using a GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with the following protocol: 1 cycle of 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 65-55 °C (-0.5 °C/cycle), and 90 s at 72 °C; 1 cycle of 10 min at 72 °C. The PCR products were resolved on 2% agarose gels; bands were purified using a QIAquick Gel Extraction kit (Qiagen, Santa Clarita, CA, USA) and sub-cloned into yT&A vector (Yeastern, Taipei, Taiwan) for clone library construction.

Nested PCR was used to amplify five different V regions of the bacterial 16S rRNA following the method of Sanchez et al. (2007), with the above mentioned amplified 16S rRNA gene as the template. The PCR primer sets and annealing conditions used to amplify the specific 16S rRNA sequences for DGGE analysis were as shown in Table 1.

DGGE separation of 16S rRNA fragments

DGGE was performed essentially as described previously (Muyzer and de Waal 1994; Muyzer et al. 1996; Sanchez et al. 2007) using a Bio-Rad D-Code System (Bio-Rad, Hercules, CA, USA). PCR fragments were separated on 6 or
8% (v/v) polyacrylamide (37.5:1 ratio of acrylamide to bisacrylamide) gels. A gradient of 45 to 65% denaturant was used to separate the PCR products; 100% denaturant contained 7 M urea and 40% formamide. Electrophoresis was carried out at 100 V for 14 h in 1.0× TAE buffer at a constant temperature of 60 °C. The detailed DGGE conditions used for different primer sets are listed in Table 1. Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) prepared in 1.0× TAE buffer (1:10000, v/v) for 30 min and finally photographed using a Typhoon Trio scanner (GE Healthcare, Piscataway, NJ, USA).

**Clone library construction and analysis**

Clone libraries were constructed from the amplified 16S rRNA from each sample. Bacterial clones from each library were selected randomly and PCR-amplified with the M13 forward and reverse primers supplied with the cloning kit. The PCR products of the bacterial clones were digested with restriction enzymes HhaI and NlaIII (Fermentas, Hanover, MD, USA) according to the manufacturer’s directions. The resulting restriction fragment length polymorphism (RFLP) patterns were analyzed on a 2% agarose gel stained with
ethidium bromide. Clones were grouped according to RFLP pattern and representative clones were sequenced.

Isolation, PCR, cloning and sequencing of DGGE bands

The DGGE bands were excised from the gel and transferred to a microcentrifuge tube. To elute the DNA from the gel fragment, 30 μL of distilled water were added to the gel fragment and the mixture was ground with a plastic pestle and heated for 10 min at 80 °C. Approximately 1 μL of the mixture was used for PCR amplification. PCR amplification was carried out using a GeneAmp PCR System 2400 with the following temperature profile: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The fragments were cloned using the methods outlined previously. Plasmid purification was performed using a QIAprep Spin Miniprep kit (Qiagen) and DNA sequencing was completed at National Cheng Kung University (Tainan, Taiwan).

DGGE band pattern analysis
DGGE bands were identified using the Quantity One 4.5 free-trial program
(Bio-Rad) with default settings. The differences in the banding pattern between
two gel tracks were converted into binary data, and the distance matrix was
calculated according to the Dice similarity coefficient. The Dice similarity
coefficient \( S_D \) between gel track A and B is defined as
\[
S_D = \frac{2N_{AB}}{N_A + N_B},
\]
where \( N_A \) is the total number of bands in A, \( N_B \) is the total number of bands in B,
and \( N_{AB} \) is the number of bands common to A and B (Eichner et al. 1999). The
unweighted pair group method with arithmetic mean (UPGMA) was used to
create dendrograms. The diversity of the coral-associated bacterial community
was assessed using the Shannon-Wiener diversity index \( (H) \), which is defined as
\[
H = -\sum (P_i \ln P_i),
\]
where \( P_i \) is the importance probability of the bands in a track and
\( \ln \) represents the natural logarithm function. The importance probability is defined
as \( P_i = n_i/N \), where \( n_i \) is the height of a peak and \( N \) is the sum of all peak heights in
the curve (Eichner et al. 1999).

**Sequence analysis**

16S rRNA gene fragments were compared with those in the SILVA SSU Ref
databases release 119 (Pruesse et al. 2007) using the basic local alignment search

tool (BLAST) (Altschul et al. 1990) algorithm to identify known sequences with a high degree of similarity. In addition, we also used the Ribosomal Data Project (RDP) classifier tool (Wang et al. 2007) to classify 16S rRNA gene fragments by bacterial taxonomy. A 70% confidence threshold was used to assign sequences to a taxonomical hierarchy; however, a 50% confidence threshold was applied for assigning sequences at the phylum level (Liu et al. 2008).

To build the phylogenetic tree, all of the sequences were imported into the ARB program (Ludwig et al. 2004). 16S rRNA sequences obtained from the clone libraries were aligned with a data set containing the nearest relative matches using the positional tree server from the ARB program. A neighbor-joining tree was constructed with Jukes-Cantor correction using sequences obtained from the clone libraries. The statistical significance of tree branches was evaluated by bootstrap analysis involving the construction of 1000 trees from randomly resampled data. Short DGGE band sequences were added to the tree using the ARB parsimony insertion tool.

The nucleotide sequences obtained in the present work are available in the GenBank database (KM078980 to KM079077).
Results

DGGE profiles using different primer sets

Five different hypervariable regions of the 16S rRNA sequence from extracts of (A) healthy coral collected in summer (labeled SH 1 to 3), (B) diseased colonies collected in summer (labeled SD 1 to 3), (C) healthy colonies collected in winter (labeled WH 1 to 3), and (D) diseased colonies collected in winter (labeled WD 1 to 3) were amplified using five different primer sets. The amplified DNA fragments of each sample were resolved by DGGE (Fig. 1). Comparing individual samples (A through D), the overall banding patterns of triplicate samples of each group were generally similar. Occasionally, a band was absent in one of the triplicates (as indicated by thin lines) but present in the other two replicates. These slight differences are likely due to local variations of bacteria in different colonies.

When the five gel patterns were compared, it was clear that the banding profile is directly influenced by the primer set used for PCR. It is also clear that regardless of the primer set used, the bacterial community that the corals harbored differed between seasons. The health status of the coral was also observed to be a major factor affecting the bacterial constituents.
As the band patterns of the samples are subject to the specificity of the primer set, it was necessary to evaluate which primer set could provide the most information. We used the Dice similarity coefficient for pairwise comparison of the band profiles for each DGGE gel, then employed the UPGMA clustering method to virtualize their relationships. We found that primer set GC357F-518R (V3 region, Fig. 2C) provided the best cluster in terms of both season and health status, although sample WD1 appeared to be closely related to the SH1, SH2 and SH3 cluster. Primer sets GC63F-518R (V1-V3 region, Fig. 2A) and GC968F-1401R (V6-V8 region, Fig. 2D) clustered the samples according to health status, with the exceptions of WD1 and SD2. Primer set GC357F-907R (V3-V5 region, Fig. 2B) clustered the samples according to season, with the exceptions of SD2 and WD1.

To further evaluate these primer sets, we considered the number of bands and the corresponding Shannon diversity index for each group of coral samples (Fig. 3). The primer set GC357F-518R had both the highest average number of bands (35.9, Fig. 3A) and the highest average Shannon diversity index (3.38, Fig. 3B). The second highest average number of bands and average Shannon diversity index were obtained with GC968F-1401R, being 30.6 and 3.33, respectively.
Two-way ANOVA to compare the number of bands between the different groups of samples showed that the number of bands generated using primer set GC357F-518R was significantly influenced by the season in which the sample was collected (P < 0.001), the health status of the coral (P < 0.001), and the interaction between season and health (P < 0.001). Primer sets GC968F-1401R and GC1055F-1392R also produced significantly different results between seasons (both P < 0.05) and in corals of differing health status (P < 0.05 and P < 0.001, respectively); however, the differences were less significant than those obtained using primer set GC357F-518R. Primer set GC357F-907R resulted in a significant difference only between corals of differing health status (P < 0.05). Primer set GC63F-518R resulted in no differences being observed between corals collected in different seasons and those with differing health status. Interestingly, the same results were obtained using two-way ANOVA to compare the Shannon diversity index between different groups of samples for each primer set.

Based on the band patterns, number of bands, and Shannon diversity indices obtained by analysis of the DGGE banding profiles, we concluded that GC357F-518R was the best primer set for analyzing the bacterial community of the
Primer set GC968F-1401R was the second best, whereas GC357F-907R was least preferable for analysis of the bacterial community of the coral.

DGGE analysis

By examining the numbers of bands and the Shannon diversity indices resulting from the use of the GC357F-518R primer set, as shown in Fig. 3, we found that the number of bands in the DGGE gels varied from 26 to 40, which suggests that the bacterial community associated with the coral is complex. It was also observed that the species richness and species diversity associated with the corals decreased in winter, while these factors increased when the corals were affected by disease. The species richness and diversity of the bacterial community were lowest in healthy coral samples collected in winter.

DGGE band sequence analysis

The 16S rRNA gene fragments of the DGGE bands obtained using primer set GC357F-518R (Fig. 1C) were eluted from the gel and amplified by PCR.
Twenty-six fragments were cloned and their sequences determined. The lengths of these sequences ranged from 164 to 198 bp, with an average length of 183.5 bp. The DNA sequences of these fragments were then compared with those in the SILVA database, and the values for sequence similarity with the closest matched sequences, as well as the phylogenetic affiliations of the 26 distinct clones (B1-B26), are listed in Table S1*. A diverse collection of bacteria, including Gammaproteobacteria (34.43%, 12 clones), Alphaproteobacteria (21.62%, 8), Deltaproteobacteria (18.92%, 7), Actinobacteria (5.41%, 2), Firmicutes (5.41%, 2), Bacteroidetes (2.70%, 1), Chloroflexi (2.70%, 1), Nitrospira (2.70%, 1), Verrucomicrobia (2.70%, 1), and unclassified Proteobacteria (5.41%, 2), was matched with the samples.

Clone library analysis

Clone library analysis was performed in each sample group. A total of 269 clones from the clone libraries were randomly selected for analysis. The numbers of clones in the SH, SD, WH, and WD libraries were 73, 66, 59 and 71, respectively.

* Supplementary Material.
The RFLP patterns of each clone were compared, and 70 different RFLP ribotypes from the clones were identified. The numbers of distinct ribotypes contained in the SH, SD, WH, and WD libraries were 16, 17, 14 and 23, respectively. Clone inserts from the representative ribotypes were partially sequenced and compared with the SILVA database (Table S2"). The average length of distinct ribotypes was 775.96 bp. The taxonomic group percentages of the 269 bacterial clones from corals of differing status are shown in Fig. 4. The bacterial clones inhabiting the corals were predominantly identified as Alphaproteobacteria (46.10%, 124 clones), Gammaproteobacteria (29.38%, 79), Acidobacteria (6.69%, 18), Chloroflexi (4.09%, 11), Betaproteobacteria (4.09%, 11), Deltaproteobacteria (4.09%, 11), Firmicutes (2.23%, 6), Actinobacteria (1.86%, 5), and Gemmatimonadetes (1.49%, 4). In addition, a phylogenetic tree showing the relationships between sequences from four clone libraries and DGGE bands is presented in Fig. 5.

As shown in Fig. 4, identification of several tendencies was possible. (1) Most of the bacteria in the summer coral were Alphaproteobacteria, whereas Alphaproteobacteria and Gammaproteobacteria were found in coral collected in winter. (2) Acidobacteria and Firmicutes were only found in diseased coral. (3) The

* Supplementary Material.
bacteria in healthy coral collected in winter were the least diverse, an observation
that independently supported the DGGE analysis results (obtained using
GC357F-518R, Fig. 3). (4) Actinobacteria species were only found in coral
collected in summer (which was also in good agreement with the DGGE results
shown in Table S1).

Figure S1* shows the taxonomic classifications of the 269 clones as
determined using the RDP classifier tool. Of the 269 clones, approximately half
(50.93%, 137 clones) were related to known genera. Members of the family
Rhodobacteraceae (including genera *Loktanella, Paracoccus, Pseudovibrio,
Roseovarius, Silicibacter, and unclassified Rhodobacteraceae) were the most
abundant clones in the SH (76.71%) and SD (57.58%) samples. Within this family,
members of the genus *Silicibacter represented 46.43% and 39.47% of the clones in
the SH and SD samples, respectively. In contrast, members of the
Rhodobacteraceae family comprised only 0.00% and 7.04% of the clones in the
WH and WD samples, respectively.

The genera *Acinetobacter* (27.12%) and *Pseudoxanthomonas* (22.03%) were
the most abundant clones in the WH sample. The most abundant clones in the WD

* Supplementary Material.
sample were members of the Gammaproteobacteria (25.35%) and Rhizobiales families (14.09%). Most of the clones (77.46%) in the WD sample could not be classified as belonging to any known family.

One ribotype (clone-SD-4) from the SD samples and two ribotypes (clone-WD-7 and clone-WD-9) from the WD samples have previously been identified in BBD-affected Siderastrea siderea colonies (Sekar et al. 2008), and a further ribotype from SD samples (clone-SD-1) and one from WD samples (clone-WD-19) were previously found to be associated with tumors in the coral Platygyra carnosus (Chiu et al. 2012), indicating that unique bacterial communities are involved in disease lesions of different corals. On the other hand, SH samples were found to have one ribotype (clone-SH-9) that was highly similar (100%) to a sequence previously found in association with diseased Platygyra carnosus (Chiu et al. 2012), suggesting that it is likely to be a ubiquitous coral-associated bacteria.
Discussion

Pink spot syndrome is a disease caused by infection of *Porites* coral with trematode *Odocotyloides stenometra*, which leads to swollen pink nodules on the coral colony (Aeby 2003). A previous study reported that the pink spots are due to the mechanical/chemical stress caused by the settling of barnacle larvae on living *Porites* (Benzoni et al. 2010). The phenomenon suggests that although the signs of the disease are similar in *Porites* of different locations, they do not have the same etiology. In addition, parasitic infection might not be the only cause of the disease, and other microorganisms could also play a role in disease formation. Bacterial involvement in other diseases of *Porites* has been demonstrated; for example, bacterial communities have been found to be correlated with white patch syndrome in *Porites* (Sere et al. 2013). The roles of these bacterial species in pink spot syndrome therefore need to be examined.

In this study, we used DGGE, a frequently-used method for the analysis of bacterial communities in various environments, to characterize the bacterial communities associated with pink spot syndrome and analyze the effects of seasonal factors on the diversity of bacteria. DGGE only profiles the dominant microbes within a community (Muyzer and Smalla 1998). In the present study, we
compared the effects of primer sets on bacterial communities inhabiting coral of different sample groups using PCR-DGGE. Our results suggested that the use of primer set GC357F-518R generated a cluster consistent with season and health status. This primer set produced a wealth of information, and was found to be the best primer set for use to examine the bacterial communities of *Porites* coral. Yu and Morrison (Yu et al. 2008) found that use of the GC357F-518R primer set produced DGGE profiles with the highest species richness and Shannon diversity index in a study of the use of eight different bacterial primer sets to analyze bacterial community DNA extracted from the digesta of sheep. In contrast, Sanchez et al. (Sanchez et al. 2007) reported that primer set 357fGC-907RM grouped samples according to season, and was the most suitable of five different primer sets for PCR-DGGE analysis of marine bacterioplankton communities in an oligotrophic coastal marine system. However, it is interesting to note that they predicted that GC357F-518R could match the most abundant marine bacterioplankton sequences in the RDP database using bioinformatic methods (Sanchez et al. 2007), which could further explain our results. Interestingly, they also predicted that 907RM would not be a good primer for marine bacterioplankton analysis using the same method, which was controversial; however, this result
again agreed with our finding that GC357F-907R was the least useful primer set for PCR-DGGE study of coral bacteria in this study. The reason for the difference in selection of the best primer set in different studies is very possibly due to the differences in microbial flora structure in samples of different studies. The present study analyzed and compared the bacterial community structure and composition inhabiting healthy and diseased coral obtained during two different seasons. Both DGGE and clone library data indicated that the bacterial community structures of the coral were diverse and considerably different between seasons as well as between coral of differing health status. Overall, the bacterial assemblages of coral were composed mainly of Alphaproteobacteria, Gammaproteobacteria, Acidobacteria, Chloroflexi, Betaproteobacteria, Deltaproteobacteria, Firmicutes, Actinobacteria, Gemmatimonadetes, Bacteroidetes, Chloroflexi, Nitrospira, Verrucomicrobia, and unclassified Proteobacteria (Tables S1 and S2). This high level of microbial diversity was in agreement with many previous culture-independent surveys of microbial communities of corals (Rohwer et al. 2001; Rohwer et al. 2002; Bourne et al. 2008). From the number of DGGE bands (357F-518R, Fig. 3A) and the Shannon diversity index (357F-518R, Fig. 3B), we concluded that the bacterial communities
inhibiting healthy coral were significantly more diverse in summer than in winter. This result was further confirmed by comparing the diversity of bacterial communities between two seasons from the clone library data at the phylum/class level (Fig. 4). Our observation was in line with the conclusions of Hong et al. (Hong et al. 2009), who reported that the diversity of bacterial communities associated with the coral *Stylophora pistillata* is also higher in the summer. In contrast, *Porites astreoides*-associated bacteria from the Caribbean Sea showed stronger species specificity and little difference between seasons (Wegley et al. 2007). The reason for this difference in temporal influence is not clear, although it has been suggested that temporal influence may be stronger in some regions than others (Littman et al. 2009).

We demonstrated large differences between bacterial communities of the coral in different seasons and in coral of differing health status. More than half of the bacterial clones found in coral collected in summer were members of the Alphaproteobacteria family (Fig. 4). On the other hand, both Gammaproteobacteria and Alphaproteobacteria species were predominant in the samples collected in winter. This shift in bacterial assemblage to Gammaproteobacteria in coral exposed to colder temperatures is parallel with the bacterial communities of the corals
Acropora millepora (Lema et al. 2014) and Stylophora pistillata (Hong et al. 2009).

However, other researchers reported a different succession profile of the bacterial community of coral exposed to warmer temperatures (Koren and Rosenberg 2008; Kimes et al. 2013). For example, Gammaproteobacteria (60.9%) is dominant in summer, and both Alphaproteobacteria (79.5%) and Gammaproteobacteria (21.5%) are dominant in winter in the Mediterranean coral Oculina patagonica (Koren and Rosenberg 2008).

The health status of the coral has a great impact on the bacterial community, as indicated by the increase in the Shannon diversity index (Fig. 3B, primer set 357F-518R) of the bacterial population in diseased coral. Several studies investigating the structure of the bacterial community also observed a distinct difference and a higher diversity in the bacterial communities associated with diseased/bleached corals (Sekar et al. 2006; Pantos and Bythell 2006; Chimetto et al. 2008; Bourne et al. 2008; Sunagawa et al. 2009; Kimes et al. 2013; Closek et al. 2014). Coral holobiont is a dynamic equilibrium between the coral animal and its associated microbes. The high bacterial diversity in diseased coral may be due to a shift in the bacterial community structure from the equilibrium state. Reduction in the immunity of the host may lead to colonization of opportunistic pathogens and
directly or indirectly cause variations in normal microbiota. In addition, several factors, such as disease lesions, symbiont composition, and micro-environmental differences, provide new niches for the growth of specific bacteria, and therefore increase the diversity of coral microbiota (Closek et al. 2014).

Coral physiological states and environmental factors, such as geography, water quality, light exposure and season, have been reported to be important in terms of their effect on the coral-associated bacterial community (Bourne et al. 2008; Koren and Rosenberg 2008; Littman et al. 2009; Hong et al. 2009). In the current study, we examined the influences of two factors, season and health status, on the coral-associated consortia. Clone library data showed that Alphaproteobacteria were dominant in summer, while Gammaproteobacteria and Alphaproteobacteria were dominant in winter in both healthy and diseased corals, which indicate that the seasonal factor may be more important in shaping the coral-associated bacterial community.

BLAST searches of the ribotype clone-SD-14 in GenBank revealed that it was homologous with clone 2D804 (JF411489, 100% identity), an uncultured member of the Alphaproteobacteria family that was previously identified in skeletal tissue growth anomalies by other researchers (Chiu et al. 2012). Similar,
but not identical, clones have been found in BBD (EF123405 and DQ441657) (Sekar et al. 2006), white plague-like disease (FJ203320) (Sunagawa et al. 2009), and Porites white patch syndrome (KF179790) (Sere et al. 2013) coral tissues. This ribotype was also found to correspond with DGGE clone B21 and ribotype clone-SD-7 (Fig. 5), which were present in the bacterial communities associated with the WD and SD samples, respectively. This closely-related group of bacteria, which is consistent in terms of appearance in diseased corals and absence in healthy corals, represents potentially important PSS pathogens, and clearly warrants further study.

We observed that Silicibacter was present in high abundance only in the summer coral samples (35.62% in SH and 22.73% in SD), revealing a shift in bacterial flora in response to changing environmental conditions. To our knowledge, this was the first study to report an abundance of sequences relating to Silicibacter associated with coral in the summer months. A high abundance of Silicibacter clones (greater than 20%) was previously identified in bacterial communities from corals collected in Monterey, California, suggesting the possibility of dominance of Silicibacter in marine environments. Clones of this group were found to be related to microbiota in healthy corals Acropora palmate
(6.2%) and *Porites astreoides* (2.0%) in the Mexican Caribbean (McKew et al. 2012), as well as diseased coral *Montipora aequituberculata* of the Great Barrier Reef (Jones et al. 2004b) and *Siderastrea siderea* of the Bahamas (Sekar et al. 2006).

Deltaproteobacteria, which contain ribotypes related to species of sulfate-reducing bacteria (genus *Desulfarculus*), were detected in the SH (2.74%), SD (3.03%) and WD (9.86%) samples according to clone library data (Fig. 4) and DGGE (Table S1) analysis. Sulfate-reducing bacteria have been proposed as a causative agent for BBD (Sekar et al. 2006); however, it is interesting to note that sulfate-reducing bacteria are also present in healthy corals (Arboleda and Reichardt 2009), suggesting that they are likely to colonize anaerobic niches in coral.

Four actinobacterial clones (B12, clone-SH-10, clone-SD-6 and clone-SD-13) were identified in summer samples of both healthy and diseased coral. Interestingly, BLAST analysis suggested that they are most closely matched to sequences of bacteria associated with the marine sponge. Recently, several studies (Lombo et al. 2006; Lampert et al. 2008; Chen et al. 2012) have shown that corals harbor diverse species of Actinobacteria, some of which can produce antimicrobial
substances. It has been suggested that resident microbial populations compete with invading microbes for nutrients and ecological niches, and might play a major role in coral health (Lampert et al. 2008). Coral has been suggested as an untapped source of microbial diversity of economic importance (Chen et al. 2012). Further studies are needed to confirm the possible production of antimicrobial substances from marine bacteria isolated from *Porites* sp.

In conclusion, we characterized the bacterial communities associated with healthy and diseased *Porites* sp. coral with pink spot syndrome collected off the coast of southern Taiwan. Our results showed that primer set GC357F-518 was most suitable for PCR-DGGE analysis of bacteria associated with healthy and diseased *Porites* sp. coral collected off the coast of southern Taiwan. The bacterial communities of *Porites* sp. were found to be complex, and changed greatly between seasons and in coral of differing health status. The diversity of the bacterial communities associated with the coral increased in coral of a diseased state, as well as in coral collected in the summer. In addition, our findings indicated that the season plays a more important role in shaping the coral-associated bacterial community than health status. Further investigation of interactions between the bacterial
community and coral host under differing environmental conditions will provide
more information to enable elucidation of the roles of bacteria in coral diseases.

Acknowledgement

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of Marine Biology and Aquarium.
References


Hsieh, Y. 2009. Inter-annual variation of lunar periodicity in larval release by reef corals *Pocillopora damicornis* and *Seriatopora hystrix*. Master's Thesis, National Dong Hwa University, Checheng, Pingtung, Taiwan.


Table 1. PCR primers used to amplify specific 16S rRNA sequences for DGGE analysis.

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Primer sequence</th>
<th>Target region</th>
<th>Amplicon size (bp)</th>
<th>Annealing conditions</th>
<th>DGGE conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>63F* 5'-GCCTAACACATGCAAGTC-3'</td>
<td>V1-V3</td>
<td>489</td>
<td>67-57 °C, -1 °C /cycle</td>
<td>6%, 45-65%, 100 V, 14 h</td>
</tr>
<tr>
<td></td>
<td>518R 5'-ATTACCGCGGTGGCTTTCG-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>357F* 5'-CCTACGGGAGGCAGCGAG-3'</td>
<td>V3-V5</td>
<td>586</td>
<td>66-55 °C, -1 °C /cycle</td>
<td>6%, 45-65%, 100 V, 14 h</td>
</tr>
<tr>
<td></td>
<td>907R 5'-CCGTCGTCCTTCCAAGGTT-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>357F* 5'-CCTACGGGAGGCAGCGAG-3'</td>
<td>V3</td>
<td>194</td>
<td>65-55 °C, -1 °C /cycle</td>
<td>8%, 45-65%, 100 V, 14 h</td>
</tr>
<tr>
<td></td>
<td>518R 5'-ATTACCGCGGTGGCTTTCG-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>968F* 5'-AACGCGAGAAGCCTTAC-3'</td>
<td>V6-V8</td>
<td>434</td>
<td>63-53 °C, -1 °C /cycle</td>
<td>6%, 45-65%, 100 V, 14 h</td>
</tr>
<tr>
<td></td>
<td>1401R 5'-CGGTGCTGACAAGACCC-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E</td>
<td>1055F 5'-ATGGCGCTGCGCTGACGC-3'</td>
<td>V8</td>
<td>352</td>
<td>65-55 °C, -1 °C /cycle</td>
<td>8%, 45-65%, 100 V, 14 h</td>
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<tr>
<td></td>
<td>1392R* 5'-ACCGCGGTGTGTCGRC-3'</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Primer with a 40-bp GC clamp at the 5’ or 3’ end.

GC clamp: 5’-CGCCCGCCCGCCGCGCCGGCCGGGGCCGGGGGACGCGGGG-3’
Figure Captions

Fig. 1 DGGE profiles obtained from bacterial community DNA extracted from coral *Porites* sp. using five different 16S rRNA-targeted primer sets: (A) GC63F-518R; (B) GC357F-907R; (C) GC357F-518R; (D) GC968F-1401R; (E) 1055F-1392RGC (SH1-3: healthy summer coral; SD1-3: diseased summer coral; WH1-3: healthy winter coral; WD1-3: diseased winter coral).

Fig. 2 Cluster of bacterial communities associated with coral *Porites* sp. obtained using 16S rRNA-DGGE profiles with five different primer sets: (A) GC63F-518R; (B) GC357F-907R; (C) GC357F-518R; (D) GC968F-1401R; (E) 1055F-1392RGC. Dendrograms were constructed using the unweighted pair group method with arithmetic mean (UPGMA) according to Dice's coefficient (SH1-3: healthy summer coral; SD1-3: diseased summer coral; WH1-3: healthy winter coral; WD1-3: diseased winter coral).

Fig. 3 Distributions of (A) number of bands and (B) Shannon diversity index derived from denaturing gradient gel electrophoresis profiles of the bacterial community associated with *Porites* sp. for each primer set (SH: healthy...
summer coral; SD: diseased summer coral; WH: healthy winter coral; WD: diseased winter coral).

**Fig. 4** Percentage of bacterial phylum/class in each sample group for 269 bacterial clones from the bacterial community associated with *Porites* sp. using clone library analysis. (A) Healthy summer coral; (B) diseased summer coral (C) healthy winter coral; (D) diseased winter coral.

**Fig. 5** Phylogenetic tree of 16S rRNA sequences obtained from clone libraries and DGGE bands of *Porites* sp. samples. The tree was constructed using the ARB program, based on neighbor-joining analysis of a distance matrix using the Jukes-Cantor model. Bootstrap values of greater than 50% are shown at branch points. The 16S rRNA sequences of the *Methanococcus* genus were used as the outgroup. The scale bars represent 0.10 substitutions per nucleotide position. Ribotypes marked with arrows are bacterial sequences mentioned in the Results and Discussion sections.
DGGE profiles obtained from bacterial community DNA extracted from coral *Porites* sp. using five different 16S rRNA-targeted primer sets: (A) GC63F-518R; (B) GC357F-907R; (C) GC357F-518R; (D) GC968F-1401R; (E) 1055F-1392RGC (SH1-3: healthy summer coral; SD1-3: diseased summer coral; WH1-3: healthy winter coral; WD1-3: diseased winter coral).

250x200mm (300 x 300 DPI)
Fig. 2.
Fig. 3.

(A) Numbers of DGGE bands

(B) Shannon diversity index

[Graphs showing data for different primer sets with bars representing different conditions (SH, SD, WH, WD).]
Percentage of bacterial phylum/class in each sample group for 269 bacterial clones from the bacterial community associated with *Porites* sp. using clone library analysis. (A) Healthy summer coral; (B) diseased summer coral (C) healthy winter coral; (D) diseased winter coral.

2474x1748mm (72 x 72 DPI)
Fig. 5.

[Diagram showing various bacterial clones and their relationships]