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

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1 **Seasonal Changes in Bacterial Communities Associated with**
2 **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.

40 Keywords: Coral disease; Primer effects; Pink spot syndrome; DGGE.

41

Draft

42 **Introduction**

43 Coral reef is one of the most complex marine ecosystems. Many fish, snails,
44 and lobsters depend on corals for their survival. The global decline in coral
45 populations has endangered this very important ecosystem (Loya et al. 2001; Jones
46 et al. 2004a). Bacteria are ubiquitous in every habitat on earth. The symbiotic
47 relationships between bacteria and corals have been investigated using various
48 techniques (Kellogg 2004; Wegley et al. 2007; Chiou et al. 2010; Kimes et al.
49 2013; Lema et al. 2014). It is now known that corals harbor many different types of
50 bacteria (Frias-Lopez et al. 2002; Rohwer et al. 2002; Chiou et al. 2010; Ceh et al.
51 2011; Lema et al. 2014), archaea (Ferris et al. 1996; Kellogg 2004), and fungi
52 (Bentis et al. 2000; Wegley et al. 2007) and their associations are often host
53 species-specific (Rohwer et al. 2001; Rohwer et al. 2002). However, bacteria-coral
54 associations are not always stable: evidence suggests that environmental change
55 could disrupt the microbe-coral association, leading to either a new adaptation or to
56 coral disease (Rosenberg et al. 2007).

57 During the past 30 years, more than twenty disease signs have been described
58 in corals; however, the causative agents of most coral diseases are still unknown
59 (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

77 change in these components may have the potential to result in the development
78 of diseases (Cardenas et al. 2012; Roder et al. 2014).

79 There are several methods currently available for identifying bacteria without
80 prior knowledge of the bacterial community. PCR-DGGE (polymerase chain
81 reaction-denaturing gradient gel electrophoresis) for DNA amplification and
82 separation techniques coupled with DNA sequencing is one of these methods and
83 has been used successfully to characterize and compare the microbial diversity and
84 community structure in environmental samples (Muyzer et al. 1993). This
85 culture-independent method compares microbial communities by examining how
86 far 16S rDNA fragments of different bacteria move through a polyacrylamide gel.
87 The gel bands are then excised, sequenced and compared with reference 16S rRNA
88 genes in the database. Although this technique is now routinely carried out for the
89 analysis of microbial diversity, selection of the region(s) of the 16S rRNA gene that
90 could provide the most information about the bacterial community is primarily
91 based on experience; selection of the 16S rRNA gene regions, and thus the choice
92 of primer set, can greatly affect the outcome (Yu and Morrison 2004). Even so, few
93 studies have critically analyzed the choice of primers for study of the bacterial
94 communities associated with corals (Yu and Morrison 2004; Sanchez et al. 2007;

95 Yu et al. 2008). Moreover, only a small number of researchers have analyzed how
96 seasonal changes affect the coral bacteria association (Bourne et al. 2008; Hong et
97 al. 2009; Ceh et al. 2011; Kimes et al. 2013; Lema et al. 2014).

98 Invasion of trematodes in *Porites* tissue causes bright pink spots to appear on
99 the surface of the coral. This disease is called pink spot syndrome (PSS) (Aeby
100 2003). Recently, Benzoni et al. (2010) reported that trematodiasis is only a
101 secondary infection of the already weakened *Porites*. The bacterial community
102 associated with PSS in coral has never been studied. In the present study, we
103 therefore used PCR-DGGE and DNA sequencing techniques to characterize and
104 compare the dominant members of the bacterial community associated with healthy
105 and PSS *Porites*. *Porites* sp. was harvested from Nanwan at Kenting National Park
106 in southern Taiwan. We used five different primer sets to amplify different
107 hypervariable regions of the 16S rRNA and compared the quality of information
108 obtained. We also examined the dynamics of the bacterial communities in coral
109 collected in summer and in winter.

110

111 **Materials and methods**

112 **Sampling**

113 Fragments of separate colonies of *Porites* sp. coral were collected at a depth
114 of approximately 7 meters near Nanwan (21°57'1.78"N, 120°46'13.8"E) at
115 Kenting National Park in southern Taiwan during summer (June, 2007) and winter
116 (November, 2007). The samples (obtained in triplicate) were divided into four
117 groups: healthy colonies collected in summer (SH 1-3), pink spot-diseased
118 colonies collected in summer (SD 1-3), healthy colonies collected in winter (WH
119 1-3), and the pink spot-diseased colonies collected in winter (WD 1-3). The
120 average seawater temperature in June and November at the times of sampling was
121 29.0 ± 0.9 and 24.7 ± 0.8 °C, respectively (Hsieh 2009). Coral samples were
122 collected underwater using a hammer and chisel. Each sample was placed in an
123 individual sterile plastic bag with seawater. Samples were delivered to the
124 laboratory in an ice box within 30-60 min, whereupon the seawater within each
125 plastic bag was decanted and the sample stored at -20 °C until analysis.

126

127 **DNA extraction**

128 Five grams of coral were washed twice with filtered (0.22 µm) artificial
129 seawater (24.544 g/L NaCl, 4.676 g/L MgCl₂·6H₂O, 3.128 g/L MgSO₄, 1.323 g/L
130 CaCl₂·2H₂O, 0.671 g/L KCl, 0.168 g/L NaHCO₃, and 2.384 g/L HEPES) and
131 pulverized using a mortar and pestle. The sample was transferred to a 50-mL
132 centrifuge tube, mixed with 10 mL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM
133 EDTA), and shaken at 4 °C for 10 h. Bacterial genomic DNA from the
134 supernatant was extracted using a PowerMax Soil DNA Isolation kit (MO BIO
135 Laboratory, Carlsbad, CA, USA).

136
137 **PCR amplification of 16S rRNA fragments**

138 The 16S rRNA gene sequences from 8 to 1513 bp of *Escherichia coli* were
139 amplified by PCR using a method modified from that used by Muyzer et al.
140 (1993) with the universal bacterial primers 8F
141 (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R
142 (5'-ACGGHTACCTTGTTACGACTT-3'). The PCR mixture was of a final
143 volume of 50 µL and contained 1 µL (roughly 10 ng) of coral bacterial genomic
144 DNA, 500 pmoles of each primer, 100 µmoles of dNTP, 5 µL of 10X PCR buffer
145 (100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, and 500 mM KCl), and 2.5 U of Taq

146 DNA polymerase (Takara Bio, Otsu, Japan). Touchdown PCR was performed
147 using a GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems, Foster
148 City, CA, USA) with the following protocol: 1 cycle of 5 min at 94 °C; 30 cycles
149 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
150 min at 72 °C. The PCR products were resolved on 2% agarose gels; bands were
151 purified using a QIAquick Gel Extraction kit (Qiagen, Santa Clarita, CA, USA)
152 and sub-cloned into yT&A vector (Yeastern, Taipei, Taiwan) for clone library
153 construction.

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

159

160 **DGGE separation of 16S rRNA fragments**

161 DGGE was performed essentially as described previously (Muyzer and de
162 Waal 1994; Muyzer et al. 1996; Sanchez et al. 2007) using a Bio-Rad D-Code
163 System (Bio-Rad, Hercules, CA, USA). PCR fragments were separated on 6 or

164 8% (v/v) polyacrylamide (37.5:1 ratio of acrylamide to bisacrylamide) gels. A
165 gradient of 45 to 65% denaturant was used to separate the PCR products; 100%
166 denaturant contained 7 M urea and 40% formamide. Electrophoresis was carried
167 out at 100 V for 14 h in 1.0× TAE buffer at a constant temperature of 60 °C. The
168 detailed DGGE conditions used for different primer sets are listed in Table 1. Gels
169 were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) prepared
170 in 1.0× TAE buffer (1:10000, v/v) for 30 min and finally photographed using a
171 Typhoon Trio scanner (GE Healthcare, Piscataway, NJ, USA).

172

173 **Clone library construction and analysis**

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

181 ethidium bromide. Clones were grouped according to RFLP pattern and
182 representative clones were sequenced.

183

184 **Isolation, PCR, cloning and sequencing of DGGE bands**

185 The DGGE bands were excised from the gel and transferred to a
186 microcentrifuge tube. To elute the DNA from the gel fragment, 30 μ L of distilled
187 water were added to the gel fragment and the mixture was ground with a plastic
188 pestle and heated for 10 min at 80 °C. Approximately 1 μ L of the mixture was
189 used for PCR amplification. PCR amplification was carried out using a GeneAmp
190 PCR System 2400 with the following temperature profile: initial denaturation at
191 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s,
192 annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final
193 extension at 72 °C for 10 min. The fragments were cloned using the methods
194 outlined previously. Plasmid purification was performed using a QIAprep Spin
195 Miniprep kit (Qiagen) and DNA sequencing was completed at National Cheng
196 Kung University (Tainan, Taiwan).

197

198 **DGGE band pattern analysis**

199 DGGE bands were identified using the Quantity One 4.5 free-trial program
200 (Bio-Rad) with default settings. The differences in the banding pattern between
201 two gel tracks were converted into binary data, and the distance matrix was
202 calculated according to the Dice similarity coefficient. The Dice similarity
203 coefficient (S_D) between gel track A and B is defined as $S_D = 2N_{AB}/(N_A + N_B)$,
204 where N_A is the total number of bands in A, N_B is the total number of bands in B,
205 and N_{AB} is the number of bands common to A and B (Eichner et al. 1999). The
206 unweighted pair group method with arithmetic mean (UPGMA) was used to
207 create dendrograms. The diversity of the coral-associated bacterial community
208 was assessed using the Shannon-Wiener diversity index (H), which is defined as
209 $H = -\sum(P_i \ln P_i)$, where P_i is the importance probability of the bands in a track and
210 $\ln$ represents the natural logarithm function. The importance probability is defined
211 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
212 the curve (Eichner et al. 1999).

213

214 **Sequence analysis**

215 16S rRNA gene fragments were compared with those in the SILVA SSU Ref
216 databases release 119 (Pruesse et al. 2007) using the basic local alignment search

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

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

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

234

235 **Results**

236 **DGGE profiles using different primer sets**

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

247 When the five gel patterns were compared, it was clear that the banding profile
248 is directly influenced by the primer set used for PCR. It is also clear that regardless
249 of the primer set used, the bacterial community that the corals harbored differed
250 between seasons. The health status of the coral was also observed to be a major
251 factor affecting the bacterial constituents.

252 As the band patterns of the samples are subject to the specificity of the primer
253 set, it was necessary to evaluate which primer set could provide the most
254 information. We used the Dice similarity coefficient for pairwise comparison of the
255 band profiles for each DGGE gel, then employed the UPGMA clustering method to
256 virtualize their relationships. We found that primer set GC357F-518R (V3 region,
257 Fig. 2C) provided the best cluster in terms of both season and health status,
258 although sample WD1 appeared to be closely related to the SH1, SH2 and SH3
259 cluster. Primer sets GC63F-518R (V1-V3 region, Fig. 2A) and GC968F-1401R
260 (V6-V8 region, Fig. 2D) clustered the samples according to health status, with the
261 exceptions of WD1 and SD2. Primer set GC357F-907R (V3-V5 region, Fig. 2B)
262 clustered the samples according to season, with the exceptions of SD2 and WD1.

263 To further evaluate these primer sets, we considered the number of bands and
264 the corresponding Shannon diversity index for each group of coral samples (Fig. 3).
265 The primer set GC357F-518R had both the highest average number of bands (35.9,
266 Fig. 3A) and the highest average Shannon diversity index (3.38, Fig. 3B). The
267 second highest average number of bands and average Shannon diversity index were
268 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

286 coral. Primer set GC968F-1401R was the second best, whereas GC357F-907R was
287 least preferable for analysis of the bacterial community of the coral.

288

289 **DGGE analysis**

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

298

299 **DGGE band sequence analysis**

300 The 16S rRNA gene fragments of the DGGE bands obtained using primer set
301 GC357F-518R (Fig. 1C) were eluted from the gel and amplified by PCR.

302 Twenty-six fragments were cloned and their sequences determined. The lengths of
303 these sequences ranged from 164 to 198 bp, with an average length of 183.5 bp. The
304 DNA sequences of these fragments were then compared with those in the SILVA
305 database, and the values for sequence similarity with the closest matched sequences,
306 as well as the phylogenetic affiliations of the 26 distinct clones (B1-B26), are listed
307 in Table S1^{*}. A diverse collection of bacteria, including Gammaproteobacteria
308 (34.43%, 12 clones), Alphaproteobacteria (21.62%, 8), Deltaproteobacteria
309 (18.92%, 7), Actinobacteria (5.41%, 2), Firmicutes (5.41%, 2), Bacteroidetes
310 (2.70%, 1), Chloroflexi (2.70%, 1), Nitrospira (2.70%, 1), Verrucomicrobia
311 (2.70%, 1), and unclassified Proteobacteria (5.41%, 2), was matched with the
312 samples.

313

314 **Clone library analysis**

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

* Supplementary Material.

318 The RFLP patterns of each clone were compared, and 70 different RFLP ribotypes
319 from the clones were identified. The numbers of distinct ribotypes contained in the
320 SH, SD, WH, and WD libraries were 16, 17, 14 and 23, respectively. Clone inserts
321 from the representative ribotypes were partially sequenced and compared with the
322 SILVA database (Table S2^{*}). The average length of distinct ribotypes was 775.96
323 bp. The taxonomic group percentages of the 269 bacterial clones from corals of
324 differing status are shown in Fig. 4. The bacterial clones inhabiting the corals were
325 predominantly identified as Alphaproteobacteria (46.10%, 124 clones),
326 Gammaproteobacteria (29.38%, 79), Acidobacteria (6.69%, 18), Chloroflexi
327 (4.09%, 11), Betaproteobacteria (4.09%, 11), Deltaproteobacteria (4.09%, 11),
328 Firmicutes (2.23%, 6), Actinobacteria (1.86%, 5), and Gemmatimonadetes (1.49%,
329 4). In addition, a phylogenetic tree showing the relationships between sequences
330 from four clone libraries and DGGE bands is presented in Fig. 5.

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

* Supplementary Material.

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

340 Figure S1^{*} shows the taxonomic classifications of the 269 clones as
341 determined using the RDP classifier tool. Of the 269 clones, approximately half
342 (50.93%, 137 clones) were related to known genera. Members of the family
343 Rhodobacteraceae (including genera *Loktanella*, *Paracoccus*, *Pseudovibrio*,
344 *Roseovarius*, *Silicibacter*, and unclassified Rhodobacteraceae) were the most
345 abundant clones in the SH (76.71%) and SD (57.58%) samples. Within this family,
346 members of the genus *Silicibacter* represented 46.43% and 39.47% of the clones in
347 the SH and SD samples, respectively. In contrast, members of the
348 Rhodobacteraceae family comprised only 0.00% and 7.04% of the clones in the
349 WH and WD samples, respectively.

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

^{*} Supplementary Material.

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

355 One ribotype (clone-SD-4) from the SD samples and two ribotypes
356 (clone-WD-7 and clone-WD-9) from the WD samples have previously been
357 identified in BBD-affected *Siderastrea siderea* colonies (Sekar et al. 2008), and a
358 further ribotype from SD samples (clone-SD-1) and one from WD samples
359 (clone-WD-19) were previously found to be associated with tumors in the coral
360 *Platygyra carnosus* (Chiu et al. 2012), indicating that unique bacterial
361 communities are involved in disease lesions of different corals. On the other hand,
362 SH samples were found to have one ribotype (clone-SH-9) that was highly similar
363 (100%) to a sequence previously found in association with diseased *Platygyra*
364 *carnosus* (Chiu et al. 2012), suggesting that it is likely to be a ubiquitous
365 coral-associated bacteria.

366

367 Discussion

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

380 In this study, we used DGGE, a frequently-used method for the analysis of
381 bacterial communities in various environments, to characterize the bacterial
382 communities associated with pink spot syndrome and analyze the effects of
383 seasonal factors on the diversity of bacteria. DGGE only profiles the dominant
384 microbes within a community (Muyzer and Smalla 1998). In the present study, we

385 compared the effects of primer sets on bacterial communities inhabiting coral of
386 different sample groups using PCR-DGGE. Our results suggested that the use of
387 primer set GC357F-518R generated a cluster consistent with season and health
388 status. This primer set produced a wealth of information, and was found to be the
389 best primer set for use to examine the bacterial communities of *Porites* coral. Yu
390 and Morrison (Yu et al. 2008) found that use of the GC357F-518R primer set
391 produced DGGE profiles with the highest species richness and Shannon diversity
392 index in a study of the use of eight different bacterial primer sets to analyze
393 bacterial community DNA extracted from the digesta of sheep. In contrast, Sanchez
394 et al. (Sanchez et al. 2007) reported that primer set 357fGC-907RM grouped
395 samples according to season, and was the most suitable of five different primer sets
396 for PCR-DGGE analysis of marine bacterioplankton communities in an
397 oligotrophic coastal marine system. However, it is interesting to note that they
398 predicted that GC357F-518R could match the most abundant marine
399 bacterioplankton sequences in the RDP database using bioinformatic methods
400 (Sanchez et al. 2007), which could further explain our results. Interestingly, they
401 also predicted that 907RM would not be a good primer for marine bacterioplankton
402 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

421 inhibiting healthy coral were significantly more diverse in summer than in winter.
422 This result was further confirmed by comparing the diversity of bacterial
423 communities between two seasons from the clone library data at the phylum/class
424 level (Fig. 4). Our observation was in line with the conclusions of Hong et al. (Hong
425 et al. 2009), who reported that the diversity of bacterial communities associated
426 with the coral *Stylophora pistillata* is also higher in the summer. In contrast, *Porites*
427 *astreoides*-associated bacteria from the Caribbean Sea showed stronger species
428 specificity and little difference between seasons (Wegley et al. 2007). The reason
429 for this difference in temporal influence is not clear, although it has been suggested
430 that temporal influence may be stronger in some regions than others (Littman et al.
431 2009).

432 We demonstrated large differences between bacterial communities of the coral
433 in different seasons and in coral of differing health status. More than half of the
434 bacterial clones found in coral collected in summer were members of the
435 Alphaproteobacteria family (Fig. 4). On the other hand, both Gammaproteobacteria
436 and Alphaproteobacteria species were predominant in the samples collected in
437 winter. This shift in bacterial assemblage to Gammaproteobacteria in coral exposed
438 to colder temperatures is parallel with the bacterial communities of the corals

439 *Acropora millepora* (Lema et al. 2014) and *Stylophora pistillata* (Hong et al. 2009).
440 However, other researchers reported a different succession profile of the bacterial
441 community of coral exposed to warmer temperatures (Koren and Rosenberg 2008;
442 Kimes et al. 2013). For example, Gammaproteobacteria (60.9%) is dominant in
443 summer, and both Alphaproteobacteria (79.5%) and Gammaproteobacteria
444 (21.5%) are dominant in winter in the Mediterranean coral *Oculina patagonica*
445 (Koren and Rosenberg 2008).

446 The health status of the coral has a great impact on the bacterial community, as
447 indicated by the increase in the Shannon diversity index (Fig. 3B, primer set
448 357F-518R) of the bacterial population in diseased coral. Several studies
449 investigating the structure of the bacterial community also observed a distinct
450 difference and a higher diversity in the bacterial communities associated with
451 diseased/bleached corals (Sekar et al. 2006; Pantos and Bythell 2006; Chimetto et
452 al. 2008; Bourne et al. 2008; Sunagawa et al. 2009; Kimes et al. 2013; Closek et al.
453 2014). Coral holobiont is a dynamic equilibrium between the coral animal and its
454 associated microbes. The high bacterial diversity in diseased coral may be due to a
455 shift in the bacterial community structure from the equilibrium state. Reduction in
456 the immunity of the host may lead to colonization of opportunistic pathogens and

457 directly or indirectly cause variations in normal microbiota. In addition, several
458 factors, such as disease lesions, symbiont composition, and micro-environmental
459 differences, provide new niches for the growth of specific bacteria, and therefore
460 increase the diversity of coral microbiota (Closek et al. 2014).

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

471 BLAST searches of the ribotype clone-SD-14 in GenBank revealed that it
472 was homologous with clone 2D804 (JF411489, 100% identity), an uncultured
473 member of the Alphaproteobacteria family that was previously identified in
474 skeletal tissue growth anomalies by other researchers (Chiu et al. 2012). Similar,

475 but not identical, clones have been found in BBD (EF123405 and DQ441657)
476 (Sekar et al. 2006), white plague-like disease (FJ203320) (Sunagawa et al. 2009),
477 and *Porites* white patch syndrome (KF179790) (Sere et al. 2013) coral tissues.
478 This ribotype was also found to correspond with DGGE clone B21 and ribotype
479 clone-SD-7 (Fig. 5), which were present in the bacterial communities associated
480 with the WD and SD samples, respectively. This closely-related group of bacteria,
481 which is consistent in terms of appearance in diseased corals and absence in
482 healthy corals, represents potentially important PSS pathogens, and clearly
483 warrants further study.

484 We observed that *Silicibacter* was present in high abundance only in the
485 summer coral samples (35.62% in SH and 22.73% in SD), revealing a shift in
486 bacterial flora in response to changing environmental conditions. To our
487 knowledge, this was the first study to report an abundance of sequences relating to
488 *Silicibacter* associated with coral in the summer months. A high abundance of
489 *Silicibacter* clones (greater than 20%) was previously identified in bacterial
490 communities from corals collected in Monterey, California, suggesting the
491 possibility of dominance of *Silicibacter* in marine environments. Clones of this
492 group were found to be related to microbiota in healthy corals *Acropora palmate*

493 (6.2%) and *Porites astreoides* (2.0%) in the Mexican Caribbean (McKew et al.
494 2012), as well as diseased coral *Montipora aequituberculata* of the Great Barrier
495 Reef (Jones et al. 2004b) and *Siderastrea siderea* of the Bahamas (Sekar et al.
496 2006).

497 Deltaproteobacteria, which contain ribotypes related to species of
498 sulfate-reducing bacteria (genus *Desulfarculus*), were detected in the SH
499 (2.74%), SD (3.03%) and WD (9.86%) samples according to clone library data
500 (Fig. 4) and DGGE (Table S1) analysis. Sulfate-reducing bacteria have been
501 proposed as a causative agent for BBD (Sekar et al. 2006); however, it is
502 interesting to note that sulfate-reducing bacteria are also present in healthy corals
503 (Arboleda and Reichardt 2009), suggesting that they are likely to colonize
504 anaerobic niches in coral.

505 Four actinobacterial clones (B12, clone-SH-10, clone-SD-6 and clone-SD-13)
506 were identified in summer samples of both healthy and diseased coral.
507 Interestingly, BLAST analysis suggested that they are most closely matched to
508 sequences of bacteria associated with the marine sponge. Recently, several studies
509 (Lombo et al. 2006; Lampert et al. 2008; Chen et al. 2012) have shown that corals
510 harbor diverse species of Actinobacteria, some of which can produce antimicrobial

511 substances. It has been suggested that resident microbial populations compete with
512 invading microbes for nutrients and ecological niches, and might play a major role
513 in coral health (Lampert et al. 2008). Coral has been suggested as an untapped
514 source of microbial diversity of economic importance (Chen et al. 2012). Further
515 studies are needed to confirm the possible production of antimicrobial substances
516 from marine bacteria isolated from *Porites* sp.

517 In conclusion, we characterized the bacterial communities associated with
518 healthy and diseased *Porites* sp. coral with pink spot syndrome collected off the
519 coast of southern Taiwan.

520 Our results showed that primer set GC357F-518 was most suitable for
521 PCR-DGGE analysis of bacteria associated with healthy and diseased *Porites* sp.
522 coral collected off the coast of southern Taiwan. The bacterial communities of
523 *Porites* sp. were found to be complex, and changed greatly between seasons and in
524 coral of differing health status. The diversity of the bacterial communities
525 associated with the coral increased in coral of a diseased state, as well as in coral
526 collected in the summer. In addition, our findings indicated that the season plays a
527 more important role in shaping the coral-associated bacterial community than
528 health status. Further investigation of interactions between the bacterial

529 community and coral host under differing environmental conditions will provide
530 more information to enable elucidation of the roles of bacteria in coral diseases.

531

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535

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

Primer pairs		Primer sequence	Target region	Amplicon size (bp)	Annealing conditions	DGGE conditions
A	63F*	5'-GCCTAACACATGCAAGTC-3'	V1-V3	489	67-57 °C, -1 °C /cycle	6%, 45-65%, 100 V, 14 h
	518R	5'-ATTACCGCGGCTGCTGG-3'				
B	357F*	5'-CCTACGGGAGGCAGCAG-3'	V3-V5	586	66-55 °C, -1 °C /cycle	6%, 45-65%, 100 V, 14 h
	907R	5'-CCGTCAATTCMTTGTGAGTTT-3'				
C	357F*	5'-CCTACGGGAGGCAGCAG-3'	V3	194	65-55 °C, -1 °C /cycle	8%, 45-65%, 100 V, 14 h
	518R	5'-ATTACCGCGGCTGCTGG-3'				
D	968F*	5'-AACGCGAAGAACCTTAC-3'	V6-V8	434	63-53 °C, -1 °C /cycle	6%, 45-65%, 100 V, 14 h
	1401R	5'-CGGTGTGTACAAGACCC-3'				
E	1055F	5'-ATGGCTGTCGTCAGCT-3'	V8	352	65-55 °C, -1 °C /cycle	8%, 45-65%, 100 V, 14 h
	1392R*	5'-ACGGGCGGTGTGTRC-3'				

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

GC clamp: 5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG-3'

664 **Figure Captions**

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

670
671 **Fig. 2** Cluster of bacterial communities associated with coral *Porites* sp. obtained
672 using 16S rRNA-DGGE profiles with five different primer sets: (A)
673 GC63F-518R; (B) GC357F-907R; (C) GC357F-518R; (D) GC968F-1401R;
674 (E) 1055F-1392RGC. Dendrograms were constructed using the unweighted
675 pair group method with arithmetic mean (UPGMA) according to Dice's
676 coefficient (SH1-3: healthy summer coral; SD1-3: diseased summer coral;
677 WH1-3: healthy winter coral; WD1-3: diseased winter coral).

678
679 **Fig. 3** Distributions of (A) number of bands and (B) Shannon diversity index derived
680 from denaturing gradient gel electrophoresis profiles of the bacterial
681 community associated with *Porites* sp. for each primer set (SH: healthy

682 summer coral; SD: diseased summer coral; WH: healthy winter coral; WD:
683 diseased winter coral).

684

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

689

690 **Fig. 5** Phylogenetic tree of 16S rRNA sequences obtained from clone libraries and
691 DGGE bands of *Porites* sp. samples. The tree was constructed using the ARB
692 program, based on neighbor-joining analysis of a distance matrix using the
693 Jukes-Cantor model. Bootstrap values of greater than 50% are shown at branch
694 points. The 16S rRNA sequences of the *Methanococcus* genus were used as
695 the outgroup. The scale bars represent 0.10 substitutions per nucleotide
696 position. Ribotypes marked with arrows are bacterial sequences mentioned in
697 the Results and Discussion sections.

698

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.

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.