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Abstract

Dongsha Atoll (also known as Pratas) in Taiwan is the northernmost atoll in the South China Sea and a designated marine national park since 2007. The marine park's scope of protection covers the bio-resources of its waters in addition to uplands, so it is important to have data logging information and analyses of marine flora and fauna, including their physiology, ecology, and genetics. As part of this effort, we investigated *Symbiodinium* associations in scleractinian corals from Dongsha Atoll through surveys carried out at two ~~depths~~ depth ranges (1-5 m and 10–15 m) in 2009 and 2010 and compared them with seawater temperature ~~trends~~profiles. *Symbiodinium* composition was assessed using restriction fragment length polymorphism (RFLP) of nuclear large subunit ribosomal DNA (nlsrDNA). Our results showed that the 807 coral samples from 7 families and 21 genera collected in 2009 and 132 coral samples from 7 families and 12 genera collected in 2010 were mainly associated with *Symbiodinium* clades C and D. The proportion of *Symbiodinium* in corals in 2009 was 71.34%, 19.71%, and 8.95% for *Symbiodinium* C, D, and C+D, respectively. The proportion of *Symbiodinium* D between reef tops and reef bases were 24.48% and 14.88%, respectively. In the 2010, analysis of bleached corals showed presence of *Symbiodinium* C in 84.78% samples and *Symbiodinium* D in 10.87% of samples respectively. However, in those corals that did not bleach, 62.79% were associated with clade D. Denaturing gradient gel electrophoresis (DGGE) of internal transcribed ~~subunit~~ spacer 2 (ITS2) of 175 randomly selected samples showed the presence of 8 *Symbiodinium* C types and 2 *Symbiodinium* D types associated with corals in 2009 and 2010. This study is the first baseline survey on *Symbiodinium* associations in corals of Dongsha Atoll in the South China Sea, and shows the dominance of *Symbiodinium* clade C in the population.

Comment [CL1]: Unclear as to what data

Comment [CL2]: What region? 28S?

Comment [CL3]: Were there also other clades detected?

Comment [CL4]: Misleading or confusing terminology? This sounds like you are reporting the relative abundance within a single coral rather than across multiple species. Consider: The proportion of corals containing Symbiodinium clades C, D, and C+D were…..Or: Clade C, D, and C+D was detected in

Comment [CL5]: Consider referring to them as deep and shallow reefs for more clarity?

Comment [CL6]: Clearly summarize the main points of your findings here rather than fine details which you will explain in Results.

Comment [CL7]: Need to identify this bleaching event before discussing 2010 results. This is a very important factor between the 2 years of your study

Comment [CL8]: Summarize these findings more clearly: Using finer resolution markers, ITS2/DGGE, eight clade C sub-types and two clade D sub-types were detected…….

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Introduction

Coral reefs provide habitat for numerous marine organisms and are considered among the richest ecosystems on earth. In addition to their ecological values, they are also economically important as they contribute toward food, tourism, coastal protection, aesthetics, and cultural significance to people in coastal areas (Moberg and Folke 1999; Hoegh-Guldberg 2004; Wilkinson 2004) and act as ecosystem engineers (Jones *et al*. 1994; Coleman and Williams 2002). As a result of climate change and anthropogenic disturbances, corals and coral reefs have recently suffered an unprecedented decline in terms of species abundance and community degradation (Hoegh-Guldberg 1999; Coles and Brown 2003; Hughes *et al*. 2003; Bellwood *et al*. 2004).

Seawater temperature fluctuation is considered as one of the main causes of this decline. Generally, corals undergo a phenomenon known as bleaching when confronted with 1.0-2.0 ºC above the summer average seawater temperatures. Bleaching results in breakdown of the symbiosis between the coral host and single celled algae *Symbiodinium*, either due to release of *Symbiodinium* cells by the host or escape of the cells from the host. While, majority of corals undergo bleaching, some coral species can resist thermal stress or changes in environmental conditions. This is due either to the ability of the coral host to withstand stress or by it associating with a stress-resistant type of zooxanthellae, or to a combination of the two (Bhagooli *et al.* 2008; Berkelmans and van Oppen 2006; Baird *et al.* 2009). On the other hand, to avoid widespread mortality, an ability to acclimatize and/or adapt might help corals overcome the crisis and effects of global climate change (Hoegh-Guldberg 1999; Berkelmans *et al*. 2004).

Mechanisms of acclimatization include phenotypic re-adjustments to confront environmental stresses (Meesters et al. 2002). Gradual changes in environmental parameters might allow corals to acquire higher tolerance slowly (Coles and Brown 2003). In contrast, adaptation requires genotypic changes that can be subsequently passed on to

Comment [CL9]: Recent decades, 30-40 years

Comment [CL10]: Perhaps some more recent references to add?

Comment [CL11]: Increasing seawater temperatures, increasing frequency of hyper-thermal events not really fluctuations

Comment [CL12]: ??? above mean summer average or above mean summer high? One degree above the maximum monthly mean is called the "bleaching threshold" temperature.

Comment [CL13]: ??? breakdown of symbiosis?? Loss of symbionts results in “bleaching”

Comment [CL14]: ??? different species? All corals?? All reefs??

Comment [CL15]: Introducing a new term here!

Comment [CL16]: Ref chronological order

Comment [CL17]: Maybe good opening sentence for next paragraph??

succeeding generations (Brown 1997). The capacity to shuffle *Symbiodinium* clades/types may be key for their acclimatization and/or adaptation (Baker and Romanski 2007; Mieog *et al*. 2007). The general trend is for thermal-tolerant clades/types to supersede thermal-sensitive clades/types under temperature stress (Jones et al. 2008; Jones and Berkelmans 2010).

Based on ITS2 rDNA (Stat et al. 2009), nuclear partial 5.8S, the ITS2 and D1-D3 regions (Pochon *et al*. 2001), and chloroplast large subunit domain (Pochon *et al*. 2006), *Symbiodinium* has been classified into nine "clades" (A-I) (Pochon and Gates 2010). Among them, depending on the variation at the base-pair level, clades have been classified into "types" based on ITS1 (van Oppen *et al*. 2001; Fabricius *et al*. 2004) or ITS2 DGGE profiles (LaJeunesse and Trench 2000). Different host–symbiont assemblages can ~~confront~~ respond differently to diverse conditions such as temperature, irradiance, and sedimentation disturbance. *Symbiodinium* clades A–D, F, and G are known to be associated with scleractinian corals (Baker 2003; Coffroth and Santos 2005; van Oppen *et al*. 2005b; Pochon et al. 2006). Most *Symbiodinium* clades /types are associated with specific host genera or species (LaJeunesse *et al*. 2004a). In many cases, a *Symbiodinium* clades/types associate with only one or a few host species. However, studies also have shown that one of the two partners is more flexible (Baker 2003) and occupy defined ecological niches and roles within and across coral hosts (LaJeunesse *et al*. 2010; Pochon and Gates 2010; Weber and Medina 2012) based on their physiological responses to various environmental stresses (Iglesias-Prieto *et al*. 2004; Baker 2003a, 2003b; Jones *et al*. 2008; Little *et al*. 2004; Rowan 2004; Sampayo *et al*. 2008; Warner et al. 2006). Recent studies have argued that in addition to the specific *Symbiodinium* clade associated with any given coral species, it is also important to know the *Symbiodinium* distribution at the type level (see Tonk *et al*. 2013, Keshavmurthy *et al*. 2014). For example, *Symbiodinium trenchii* (D1a) is often found in corals present in shallow and turbid locations (LaJeunesse

Comment [CL18]: Rowan & Powers 1991 A molecular genetic classification of zooxanthellae and the evolution of animal-algal symbioses
Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae)

Comment [CL19]: within clade variation detected and classified as sub-types

Comment [CL20]: Specificity, flexibility & shuffling

Comment [CL21]: ???? considerable within-clade diversity?

et al. 2010) or in corals after a bleaching event over a given period of time (Thornhill et al. 2006). In addition to field observations, laboratory experiments have shown differences between *Symbiodinium* types in tolerance to temperature stress (Brading *et al*. 2011; Fisher *et al*. 2012; Wang *et al*. 2012). For example, *Symbiodinium* type C15 is more tolerant than type C3 in terms of photosynthetic efficiency (Fisher *et al*. 2012) and freshly isolated *S. trenchii* showed highest activation energy for inhibiting photosystem II activity when subjected to temperature stress in comparison to *Symbiodinium* types B1, *Symbiodinium goreaui* (C1), C3, and C15 (Wang *et al*. 2012).

The present study was carried out to obtain the baseline information about the *Symbiodinium* composition in scleractinian corals from Dongsha Atoll (also known as Pratas), Taiwan. Dongsha Atoll is located in the northern South China Sea and studies have been conducted on its reef-building corals despite its remote location. Ma (1937) studied growth rates in different coral species. Coral biodiversity (17 genera and 45 species) was first described by Yang et al. (1975), followed by Fang et al. (1990), who reported 28 genera and 63 species. Later, Dai et al. (1995) recorded 34 genera and 101 species of scleractinian corals, 8 genera and 33 species of octocorallians, and 1 genus and 3 species of hydrocorallina. Recently, and especially after 1998, interest has arisen on the effects of mass coral bleaching of the coral reef community of Dongsha Atoll. After the exceptionally high 1997-98 summer temperatures during an *El Niño–Southern Oscillation* (ENSO) bleaching event, different studies reported a decrease in its coral cover and biodiversity (Fang 1998; Li and Fang 2002; Soong *et al*. 2002). Moreover, Li *et al*. (2000) showed a decline in coral species number due to the extensive use of poisons and explosives from illegal fishing. Before 1998, *Porites* and *Acropora* were the most abundant and widespread genera in Dongsha (Fang *et al*. 1990; Dai *et al*. 1995). Although *Porites* has remained a dominant genus from 1998 to the present, it has shared its dominance with other species belonging to the Merulinidae and Fungiidae (Dai 2013).

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Comment [CL22]: Be more clear that the 2009 was the baseline study. Was 2010 sampling actually during the bleaching event?

Comment [CL23]: Move this opening sentence. The rest of the paragraph talks about previous baseline work done on the coral diversity

Although the species diversity of scleractinian corals at Dongsha has been investigated extensively (Fang *et al*. 1990; Li *et al*. 2000; Soong *et al*. 2002; Dai *et al*. 1995; Dai 2005, 2006, 2008, 2013), no studies have been carried out on the Coral - *Symbiodinium* associations. Dongsha Atoll is characterized by well-developed tropical atoll reefs with 281 known scleractinian coral species (Dai 2013). In the present study, restriction fragment length polymorphisms (RFLPs) of nuclear large subunit ribosomal DNA (nlsrDNA) and denaturing gradient gel electrophoresis (DGGE) of internal transcribed spacer 2 (ITS2) were used to examine the *Symbiodinium* association in scleractinian corals collected from the central lagoon of Dongsha Atoll. *Symbiodinium* clades/types was then correlated with seawater temperature trends over time to see how fluctuations in seawater temperature around Dongsha Atoll might influence coral-*Symbiodinium* associations.

Comment [CL24]: Methods

Comment [CL25]: Differences in temperature profiles between 2 years 2009 and 2010? Unbleached vs bleached?

Materials and methods

Study site and sample collection

Dongsha Atoll (20°35'- 47'N and 116°41'- 55'E), also known as Pratas Island, is located 850 km southwest of Taiwan (Fig. 1). This circular atoll is about 25 km in diameter and its central lagoon covers more than 600 km^2. Depths in the lagoon range 10-15 m with a maximum depth of 23.7 m (Dai, 2013). Composed of coral debris, Dongsha Island is small (2,860 m long 865 m wide), 2 m above sea level at most, covers a total surface area of 1.74 km^2, and is located in the western part of the atoll. Anthropogenic disturbances on the atoll are minimal (about 200 military personnel and park rangers) since Dongsha is a military-exclusive area and a designated marine national park. However, there is occasional human disturbance by fishermen from nearby coastal nations, including China, Philippine, and Vietnam (Li 2000). The circular reef flat around the lagoon spreads over 46 km and its maximum width reaches 2 km. The geomorphology of the different patch reefs within the central area of the lagoon is heterogeneous but basically composed of two parts: the reef top (shallow reef, depth range?) and reef base (deep reef, depth range?). Dongsha Atoll's seawater circulation exchange is to the (mainly) northwest and southwest (Chen *et al*. 2008). The Dongsha Atoll Marine National Park was established in 2007 in order to implement the legal jurisdiction for conservation effort.

In June and September 2009 and September 2010 (during the coral bleaching episode), nine sites (except Reef 1) located in the Dongsha Atoll Lagoon (DAL) (Fig. 1) were sampled consistent with a long-term ecological research there. Sampling effort was limited to lagoon due to the military-restricted access to the reef crest and outside the atoll. A total of 939 scleractinian coral samples covering 7 families and 21 genera were collected at two depths: 1-5 m (reef top) and 10-15 m (reef base). Coral samples were preserved in 70% ethanol (v/v) and kept at 4 °C until processing. Scleractinian corals were identified to species when possible. Scleractinian taxonomy followed Veron and Stafford-Smith (2000),

Comment [CL26]: This topic is also in your intro final paragraphs

Comment [CL27]: Perhaps this description of Dongsha should be all together in the final paragraphs of your introduction and then could lead in to why this study was important. This is also where you should bring in the bleaching event in 2010 when you sampled.
For methods descript your sampling strategy (random sampling – what species? Following a transect along a depth gradient? Which species?) shallow vs deep reefs.

Comment [CL28]: Unclear: was there bleaching in both 2009 & 2010?

Comment [CL29]: Reference this?

Comment [CL30]: How were coral sampled? Hammer and chisel?, how much tissue collected?)

Dai and Horng (2009a, b), and Huang *et al*. (2014). *In situ* seawater temperatures at every site and at both depths were recorded with temperature loggers (HOBO, Pendant™, USA) at 30 min intervals (the periods of record were June 17 to September 15, 2009, and May 28 to September 6, 2010).

DNA extraction

Total genomic DNA (coral host + *Symbiodinium*) was extracted by the salting-out method (Ferrara *et al*. 2006). Coral tissue was lysed overnight in a 2 mL Eppendorf tube with 200 µL of lysis buffer [0.25 M Tris, 0.05 M EDTA at pH 8.0, 2% sodium dodecylsulfate (SDS), and 0.1 M NaCl] and 10 µL of 10 mg mL^{-1} proteinase E at 55 °C in a water bath. NaCl (210 µL at 7 M) was added to the lysed tissue in the tube and the sample mixed carefully by inverting the tube. The solution was then transferred to a 2 mL collection tube containing a DNA spin column (Viogene, USA) and centrifuged at 8000 rpm for 1 min. The lysate was washed twice with 500 µL of ethanol (70%) by centrifuging at 8000 rpm for 1 min at each step, with an additional centrifugation step at 8000 rpm for 3 min to dry the spin column. The column was dried further at 37 °C for 15 min and the DNA then eluted by adding 150 µL of distilled water, with a final centrifugation at 14,000 *g* for 3 min. The quality of genomic DNA was checked using a 1% agarose gel and the concentration of eluted DNA was examined using NanoDrop and then stored at -20 °C for further analysis.

Molecular phylotyping of *Symbiodinium*

Restriction fragment length polymorphism (RFLP)

All 939 samples (20 genera in 2009 and 12 genera in 2010) were used to analyze the clade composition. *Symbiodinium* clades were analyzed using the RFLP method modified from Chen *et al*. (2005a, b). The 5' end of nuclear large subunit ribosomal DNA

Comment [CL31]: Onset Computer Corporation Bourne, MA USA

Comment [CL32]: Please explain here how raw temperature data was analyzed – mean hourly? Mean daily?

Comment [CL33]: If this protocol is described fully in your reference, then perhaps a more brief summary here?

Comment [CL34]: First use of this term!!

Comment [CL35]: What gene region? 28S?

Comment [CL36]: Determining Symbiodinium clades

192 (nlsrDNA) was amplified using a *Symbiodinium*-specific primer set, 28Szoox-D1/D2F (5'-
193 CCT CAG TAA TGG CGA ATG AAC A-3') and 28Szoox-D1/D2R (5'-CCT TGG TCC
194 GTG TTT CAA GA-3') (Loh et al. 2001). A 25 µl PCR reaction consisting of 3 µl DNA
195 (10 ng μl^{-1}), 2 µl dNTPs (0.8 mM), 2 µl forward primer (0.16 µM), 2 µl reverse primer
196 (0.16 µM), 3 µl PCR Buffer (1.2X), 0.5 unit *Taq* polymerase (Protech, Taiwan), and 12.5
197 µl distilled water was run on a Px2 thermal cycler (Thermo Scientific, USA). The PCR
198 cycling profile consisted of initial denaturation at 95 °C for 1 min followed by 5 cycles of
199 94 °C for 30 s, 30 cycles of annealing at 55 °C for 1 min, decreasing 1 °C to a final
200 annealing temperature of 50 °C and 2 min at 72 °C. The final extension was at 72 °C for 10
201 min. The PCR product was digested with Rsa I (BioLabs, USA) solution at 37 °C
202 overnight and then run on 3% agarose gel (2% low melting agarose with 1% agarose) at 50
203 V for 3 h. Bands were stained by ethidium bromide (EtBr) and visualized under ultraviolet
204 (UV) radiation.
205
206 *Denaturing gradient gel electrophoresis (DGGE)*

Comment [CL37]: Determining Symbiodinium sub-types might be a better heading

207 For ITS2 *Symbiodinium* type composition, 175 samples were randomly picked from
208 18 genera in 2009 and 12 genera in 2010. The ribosomal internal transcribed spacer 2
209 (ITS2) region of *Symbiodinium* was amplified using the primers ITSintfor2, 5'-GAA TTG
210 CAG AAC TCC GTG-3', and ITS2clamp, 5'-CGC CCG CCG CGC CCC GCG CCC GTC
211 CCG CGG GAT CCA TAT GCT TAA GTT CAGCGG GT-3' (LaJeunesse and Trench
212 2000). Each 50 µl polymerase chain reaction (PCR) comprised 50 ng of genomic DNA,
213 PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM of each primer, and 2 units of Taq
214 polymerase (Invitrogen, USA). The PCR was run on a Px2 thermal cycler (Thermo
215 Scientific, MA, USA) with a touch-down PCR (LaJeunesse 2002) to ensure specificity. An
216 initial denaturation period at 92 °C for 3 min was followed by 20 cycles of 30 s at 92 °C,
217 annealing from 62 °C to a final annealing temperature of 52 °C with decrements of 0.5 °C,

and 30 s at 72 °C. Once the annealing temperature reached 52 °C, a further 20 cycles were performed at that annealing temperature, with a final extension period of 10 min at 72 °C. Each PCR product was loaded onto an acrylamide denaturing gradient gel (45–80%) and electrophoresed at 115 V for 15 h using a CBS Scientific system (Del Mar, CA, USA). Gels were stained with SYBR Green (Molecular Probes, Eugene, OR, USA) for 15 min, and photographed for further analysis. Band patterns were confirmed by sequencing bands cut from the DGGE gel.

Statistical analysis

Reef top and reef base temperature comparisons were analyzed by paired *t*-tests and average temperature values presented as means ± standard deviation (SD). The difference in *Symbiodinium* composition between reef top and reef base was chi-square tested by comparing observed clades relative to theoretical values (Sokal and Rohlf 1995). All graphs were drawn using Aabel (Ver. 3.0, Gigawiz Ltd. Co., USA) or Datagraph (Visual Data Tools, USA) softwares for Macintosh platform.

Comment [CL38]: Exactly what? How can you t-test with stdev if you have not I some way calculated means. Mean hourly? Mean daily? Mean weekly?

Comment [CL39]: I don't understand what theoretical values might be

Results

Seawater temperature

~~Seawater temperature was recorded from June 17 to September 15, 2009, and May 28 to September 6, 2010~~ During the period June to September 2009, daily/hourly/mean(??) temperatures on the shallow reefs were…. (Fig. 2A and B). ~~During the 2009 period~~, temperatures at reef tops (29.55 ± 0.23 °C) were higher than at reef bases (28.82 ± 0.19 °C; Fig. 2; Table 1; paired *t*-test, $p < 0.001$). Similarly, in 2010, temperatures at reef tops (29.94 ± 0.29 °C) were higher than at reef bases (29.72 ± 0.32 °C; Fig. 2; Table 1; paired *t*-test, $p < 0.001$). Overall, ΔT was 0.62 ± 0.3 °C in 2009 and 0.22 ± 0.14 °C in 2010. Daily temperature differences between reef tops and reef bases in 2009 were 0.5-1.0 °C and 0-0.5 °C in 2010.

Symbiodinium clade composition ~~in corals in Dongsha Atoll Lagoon~~ in 2009

A total of 807 samples from 20 genera and 7 families were collected in 2009 (Table 2; Figs. 3 and 4) from all sites (Fig. 1), except Reef 1 and Reef 9 top due to adverse weather conditions. RFLPs band patterns revealed the presence of *Symbiodinium* clade C, D and C + D.

All 20 genera were found to be associated with *Symbiodinium* clade C at both sampling depths (fig. 3). *Leptastrea*, *Fungia*, and *Porites* were found to harbor only *Symbiodinium* clade C at reef tops and reef bases. *Cyphastrea*, *Hydnophora*, *Montipora*, and *Psammocora* were associated only with *Symbiodinium* clade C at reef bases. At reef tops, the proportion of *Symbiodinium* clades was 64.18% (clade C), 24.48% (clade D), and 11.34% (clades C+D). The clade D proportion in comparison to clade C was significantly different at reef tops and reef bases (chi-square test, $p = 0.007$). The clade D proportion was high at reef tops (24.5%) compared to reef bases (14.9%). At both depths, most corals were associated with > 50% of *Symbiodinium* clade C, the exceptions being *Oxypora*,

Comment [CL40]: This needs to be clarified in Methods

Comment [CL41]: Not necessary and redundant? I think figure 3 is perfectly clear and much better representation of the data

Comment [CL42]: I would also display these data in the same bar graph manner as figure 2 for easier comparison

Comment [CL43]: Or make both figure 3 & 4 pie graphs so we can more easily see the differences and similarities

Comment [CL44]: Clade C occurred in all 20 coral genera, at both shallow and deep sites, in 2009…..

Comment [CL45]: How was proportion calculated?

Hydnophora, *Favites*, *Astrea*, and *Pectinia*, which were associated > 50% of the time with clades D or C+D. *Symbiodinium* clade D showed differences among Reefs 2 (top and base) and 3, 10 (top), and 6 (base**)** of 34%, 8.3%, 36.3%, 44.1%, and 7.9%, respectively. At each site, when *Porites* spp. were not included in the analysis, Reefs 5 and 8 (top) also showed significant differences in *Symbiodinium* clade D. The clade C proportion was high at the reef top (78.9%) compared to the reef base (70.2%) at Reef 8. At Reef 6, the clade C proportion was almost the same between the reef top (84.2%) and reef base (86.8%). The other sites had higher proportions of clade C in reef bases than reef tops.

Clade D proportions in *Turbinaria* spp. and *Coelastrea* spp. were significantly different when comparing reef tops to reef bases (chi-square test, $p < 0.05$). The clade D proportion of *Turbinaria* spp. at reef tops (39.5%) was higher than at reef bases (10.8%), and in *Coelastrea* spp. was 46.7% at reef tops and 15% at reef bases.

Symbiodinium clade composition in corals ~~in Dongsha Atoll Lagoon~~ during the 2010 bleaching episode

A total of 132 samples from 12 genera and 7 families were sampled in 2010 from reef tops (Reefs 3, 6, 10; Fig. 1; Table 3) and reef bases (Reefs 3 and 6; Fig. 1; Table 3). *Symbiodinium* clade D showed significant differences (chi-square test, $p < 0.001$) between bleached and non-bleached corals. Clade D proportions were extremely high in non-bleached corals (62.8%) compared to bleached corals (10.9%). Bleached corals were dominated by *Symbiodinium* clade C at each location. *Astreopora* spp., *Psammocora* spp., *Fungia* spp., *Cyphastrea* spp., *Porites* spp., and *Oxypora* spp., all associated with clade C, experienced bleaching in Dongsha Lagoon in the summer of 2010, and *Pavona* spp., *Coelastrea* spp., *Echinopora* spp. still experienced bleaching even though they were associated with clades D or C+D.

Comment [CL46]: First mention of bleaching!!

Comment [CL47]: important

Comment [CL48]: can this be stated and summarized more clearly?? Maybe a simple tble?

Comment [CL49]: ??? doesn't make sense with previous statement

Symbiodinium type composition in corals ~~in Dongsha Atoll Lagoon in~~ 2009 and 2010

Due to random sampling of coral colonies, there was no particular trend in the *Symbiodinium* type composition between two sampling years. However, *Symbiodinium* composition was dominated by different types of *Symbiodinium* C. ITS2 DGGE analysis of DNA samples revealed the presence of nine *Symbiodinium* types (C1, C1b, C3, C15, C21a, C27, C30 and C40 Table 4). C15 was mainly associated with corals *Montipora* and *Porites*. The composition of *Symbiodinium* type D consisted of only 2 types; *Symbiodinium glynii* (D1) and *Symbiodinium trenchii* (D1a), either separately or in combination, depending on the genus. Out of 18 genera ~~analysed~~analyzed in 2009, genus *Montipora, Acropora, Pavona, Osypora, Echinopora Favites, Astrea, Coelastrea, Gonipora, Isopora, Hydnopora and Pachyseries* were associated with *S. glynii* and/or *S. trenchii*. Whereas out of 12 genera analyzed in 2010, genus *Pavona, Turbinaria, Oxypora, Echinopora, Favites, Astrea* and *Gonipora* were associated with *S. glynii* and/or *S. trenchii*.

S. glynii has been, until now, found to be associated mainly with *Pocillopora* (see LaJeunesse *et al*. 2016). This could due to inefficient separation of the bands of *S. glynii* and *S. trenchii* on the ITS2 DGGE gel. Moreover, these two species of *Symbiodinium* are classified based on one base-pair difference. Because of such fine difference, we ran the gels with appropriate markers for *S. glynii* and *S. trenchii* and also sequenced the cut bands from the ITS2 DGGE gels to reveal the presence of *S. glynii* in several genera of corals sampled from DAL (Fig S1).

Comment [CL50]: No. samples from the 2 years should not be lumped together for analysis. Keep them separate

Comment [CL51]: Reorganize table 4

Comment [CL52]: Discussion

Discussion

This is the first study to investigate *Symbiodinium* association in the corals of Dongsha Atoll Lagoon (DAL). The main results of this study are that (1) *Symbiodinium* composition in DAL consists mainly of two clades, C and D; (2) *Symbiodinium* C diversity more *compared* *Symbiodinium* D diversity; and (3) up to 80% of coral species surveyed and associated with *Symbiodinium* C underwent moderate to severe bleaching in the year 2010. Since we have utilized both RFLP and DGGE for analysis, to avoid confusion, throughout the discussion, clades and types will not be used and instead we will just use either *Symbiodinium* C or *Symbiodinium* D. And use species name for those *Symbiodinium* types that have been designated formally.

Comment [CL53]: Did you find any other clades??

Comment [CL54]: ??

Comment [CL55]: I believe referring to these as clade C and clade D has been accepted as standard nomenclature. Using Symbiodinium C & D seems to infer that Symbiodinium C & D are a species while now we think they may actually genera!! Great to use species names for those sub-clades that are known

Symbiodinium association in corals in 2009 and 2010

Out of 939 samples collected from 21 genera of scleractinian corals, 606 hosted *Symbiodinium* C, suggesting its dominance in the South China Sea (see Table 5). Association of corals with *Symbiodinium* C could be due to generally below bleaching threshold seawater temperatures in Dongsha (Fig. 2, Table 1 and 2).

From ITS2 DGGE analysis, DAL corals were found to be associated with 8 *Symbiodinium* C (*Symbiodinium goreaui* (C1), C1b, C3, C15, C21a, C27, C30 and C40) and this number is greater than in the corals at Kenting, southern Taiwan (Keshavmurthy *et al*. 2014). Whereas, only 2 *Symbidinium* D types were observed; *Symbiodinium gylnii* (D1) and *Symbiodinium trenchii* (D1a). The presence of D in corals sampled in 2010 was more obvious, and this we believe might have been due to the influence of seawater temperature (Table 4). Overall, in the corals we sampled, *Symbiodinium* D proportion was high in reef tops (24.5%) compared to reef bases (14.9%). This trend was also reflected in the corals sampled in 2009. The occurrence *of S. glynii* and *S. trenchii* was more in corals sampled form the reef tops. The frequency of temperature fluctuations > 1 °C at reef tops was higher than at reef bases, which might be one of the reasons for the higher proportion of

335 *Symbiodinium* D at reef tops than at reef bases. In addition, our results show that some
336 corals associated with *Symbiodinium* D were dominant at reef tops and not at reef bases,
337 suggesting the possibility of acclimatization of the corals associated with *Symbiodinium* D
338 to warm waters.

339 In the present study, *Astreopora*, *Pavona*, *Montipora*, *Psammocora*, *Porites*, and
340 *Leptastrea* showed stable associations with *Symbiodinium* C at reef tops and reef bases in
341 DAL, suggesting that either host might be physiologically adjusted or acclimated to
342 temperature stress or that the *Symbiodinium* C with which they associate might have
343 greater photosynthetic stability under thermal stress (Berkelmans *et al*. 2004; Thornhill *et*
344 *al*. 2005; van Oppen *et al*. 2005a). *Turbinaria*, *Coelastrea*, and *Favites*, which were
345 dominated by *Symbiodinium* D at reef tops compared to reef bases, suggest that these
346 corals have acclimatized to hot water (reef tops) via *Symbiodinium* D. The coral
347 *Echinopora* had a stable association with *Symbiodinium* D in reef tops and reef bases,
348 which might be due to its ability to adjust to higher temperatures and populate quickly in
349 shallow environments. In the present study, the proportion of *Symbiodinium* D showed a
350 significant difference only in some locations, which might be due to host diversity (habitat
351 diversity) and abundance (habitat availability) and these may be the factors that influence
352 *Symbiodinium* composition. Our results also showed that *S. glynii* was dominant in many
353 coral genera, both in 2009 (12 genera out of 20) and 2010 (7 genera out of 12), indicating
354 that *S. glynii* may not be specific to any particular host species (LaJeunesse 2002; Baker
355 2003). This is the first work to show the presence of *S. glynii* in such large number of coral
356 genera and hence we believe that *S. glynii* may not be specific or rare, as it has been
357 thought to be, in the previous studies.

358 In 2010, scleractinian corals at Dongsha suffered from high seawater temperature
359 stress caused by a La Niña event. In a previous study, the critical coral bleaching
360 temperature threshold at Dongsha was determined to be 29.6 °C (Dai, 2008). During May

Comment [CL56]: Is this threshold mean daily temp?

Comment [CL57]: This might also be included in Introduction? It is an important point This threshold temp should be used to compare your results, not 30C

Comment [CL58]:

28 to September 6, 2010, average temperatures at reef tops (29.9 °C) and reef bases (29.7 °C) within DAL exceeded the critical value and may have induced coral bleaching (Fig. 5 B). The 2010 bleaching event might have been due to prolonged high seawater temperatures in terms of degree heating weeks, irrespective of daily temperature fluctuations (Fig. 5 B).

Comment [CL59]: Most likely!!

From our analysis, we believe that the corals in DAL hosting *Symbiodinium* C and D may not have shuffled them since only those corals associated with *Symbiodinium* D survived after bleaching. *Symbiodinium* D might have enhanced the survival of these corals compared to the ones associated with *Symbiodinium* C (Cunning *et al*. 2015). However, we cannot rule out the absence of shuffling, since corals were not tagged in this study. Another possibility is that the corals associated with *Symbiodinium* C might have bleached and died rather than shuffling to survive, hinting that not all corals shuffle even when associated with different clades and that the percentage of corals that can shuffle is very low.

Conclusions

The present study has shown that *Symbiodinium* C is dominant in Dongsha Atoll in the South China Sea corals and that natural stressors such as seawater temperatures can influence *Symbiodinium* associations in Dongsha Atoll corals. The baseline information provided, on the composition of *Symbiodinium* in corals from Dongsha Atoll, through the present work will help to carry out comprehensive and detailed studies on the diversity of *Symbiodinium* in corals by conducting more detailed survey and sampling over time. By analyzing data from normal and coral-bleaching years, we have shown the composition of *Symbiodinium* in some corals is different between years in remote and undisturbed locations such as Dongsha Atoll. Such, studies are important to understand the future response of corals to climate change in remote atolls. With increasing threats to coral health, mainly from temperature induced bleaching events as was seen in the Great Barrier Reef in 2016, studies documenting the *Symbiodinium* associations in corals of Donghsa Atoll will help in educating and informing the park managers of Dongsha Atoll marine

387 national park about management and conservations measures The focus on the status of coral reefs
388 in the South China Sea, including Dongsha Atolls, has gained attention in the past couple of years,
389 particularly due to recent destruction by mean of land-filling of reefs several Atolls in the South
390 China Sea. Although the reefs on the offshore atolls in the South China Sea are found to be in better
391 condition than near-shore reefs (Hughes *et al*. 2013), they can still undergo bleaching and mortality
392 as a result of climate change (Graham *et al*. 2006) and other stressors (Hughes *et al*. 2013).
393 Transboundary cooperation in the South China Sea region to maintain and conserve the various
394 Atolls and islands has become one of the important cause for conservation efforts in the South
395 China Sea (McManus *et al*. 2010) As part of the conservation program in Dongsha Atoll, it is
396 necessary to combine the influence of natural disturbances and the effects of anthropogenic
397 stressors such as fishing activities in order to understand the alterations of the present and their
398 influences on future coral communities.

399

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