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A community change in the algal endosymbionts of a scleractinian coral following a natural bleaching event: field evidence of acclimatization

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The symbiosis between reef-building corals and their algal endosymbionts (zooxanthellae of the genus *Symbiodinium*) is highly sensitive to temperature stress, which makes coral reefs vulnerable to climate change. Thermal tolerance in corals is known to be substantially linked to the type of zooxanthellae they harbour and, when multiple types are present, the relative abundance of types can be experimentally manipulated to increase the thermal limits of individual corals. Although the potential exists for this to translate into substantial thermal acclimatization of coral communities, to date there is no evidence to show that this takes place under natural conditions. In this study, we show field evidence of a dramatic change in the symbiont community of *Acropora millepora*, a common and widespread Indo-Pacific hard coral species, after a natural bleaching event in early 2006 in the Keppel Islands (Great Barrier Reef). Before bleaching, 93.5% ($n=460$) of the randomly sampled and tagged colonies predominantly harboured the thermally sensitive *Symbiodinium* type C2, while the remainder harboured a tolerant *Symbiodinium* type belonging to clade D or mixtures of C2 and D. After bleaching, 71% of the surviving tagged colonies that were initially C2 predominant changed to D or C1 predominance. Colonies that were originally C2 predominant suffered high mortality (37%) compared with D-predominant colonies (8%). We estimate that just over 18% of the original *A. millepora* population survived unchanged leaving 29% of the population C2 and 71% D or C1 predominant six months after the bleaching event. This change in the symbiont community structure, while it persists, is likely to have substantially increased the thermal tolerance of this coral population. Understanding the processes that underpin the temporal changes in symbiont communities is key to assessing the acclimatization potential of reef corals.

Keywords: *Symbiodinium*; bleaching; clade D; coral; thermal tolerance; acclimatization

1. INTRODUCTION

Coral reefs owe their success to the symbiosis between reef-building corals and intracellular, phototrophic dinoflagellates of the genus *Symbiodinium* (zooxanthellae) that supply up to 95% of the coral host's energy requirements (Muscatine 1990). Under stressful environmental conditions, such as abnormally high water temperatures in combination with high light, this symbiosis can break down and the algae are lost in a process known as 'bleaching'. Such conditions have occurred on reefs globally (Hoegh-Guldberg 1999; Wilkinson 2004) and are predicted to become more frequent as a result of global warming (Donner *et al.* 2005; Hoegh-Guldberg *et al.* 2007). Therefore, coral bleaching is considered one of the biggest threats to coral reefs (Marshall & Schuttenberg 2006).

Nuclear ribosomal and chloroplast DNA markers show that the genus *Symbiodinium* is highly diverse. The genus is currently divided into eight distinct clades (categorized as A–H), each containing multiple subclades, strains or types (Coffroth & Santos 2005; Pochon *et al.* 2006; Stat *et al.* 2006). This level of genetic diversity appears to be matched by appreciable levels of physiological diversity within and between clades. For instance, symbiont types differ in their photosynthetic response to light (Iglesias-Prieto *et al.* 2004) and temperature stress (Robinson & Warner 2006). Reef-building corals can form associations with members of six of the eight *Symbiodinium* clades (A–D, F and G; reviewed by Baker 2003) and some of these associations seem to be more flexible than others (van Oppen *et al.* 2004). *Symbiodinium* C is the most common symbiont type in *Acropora* corals on the Great Barrier Reef (van Oppen *et al.* 2001; LaJeunesse *et al.* 2004; Smith 2004) and certain types within this clade have been shown to be particularly sensitive to heat stress (Berkelmans & van Oppen 2006). *Symbiodinium* clade D is common in *Acropora* corals on shallow and inshore reefs and has been shown to be relatively tolerant to high temperatures (Glynn *et al.* 2001; Baker 2004; Fabricius *et al.* 2004; van Oppen *et al.* 2005*b*; Ulstrup *et al.* 2006).

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Some corals are known to harbour multiple types within a single colony (Rowan & Knowlton 1995; Rowan *et al.* 1997; Ulstrup & van Oppen 2003), which may allow for changes in the relative abundances of each symbiont type under influence of the environment (symbiont 'shuffling'). One way in which this change can occur is by the predominant, thermally sensitive symbiont population being replaced by a population of thermally tolerant symbionts that arise from the presence of less abundant 'background' symbionts (Baker 2003). As a result, the entire coral colony becomes more thermally tolerant. This acclimatization mechanism has been shown to occur in at least one population of *Acropora millepora* on the Great Barrier Reef after transplantation to a different thermal environment (Berkelmans & van Oppen 2006). However, only a few species of coral have been shown to shuffle their symbiont communities (Goulet & Coffroth 2003; Thornhill *et al.* 2003; Goulet 2006, 2007) and the longest symbiont monitoring study to date indicates stability rather than wholesale changes in symbiont communities (Thornhill *et al.* 2006). Symbiont 'switching', i.e. the acquisition of new symbionts from the surrounding environment (Baker 2003), may be another way by which corals can achieve a functional change in their predominant symbiont population, but so far this has not been demonstrated in scleractinian corals. Since bleaching is predicted to become more frequent as a consequence of climate change (Dunbar *et al.* 1994; Hoegh-Guldberg 1999; Hoegh-Guldberg *et al.* 2007), shuffling to more heat-resistant symbiont types may be an important acclimatization mechanism, but it must operate at the scale of populations and communities if reefs are to acclimatize and become more resistant to subsequent events (Buddemeier & Fautin 1993; Buddemeier *et al.* 2004). To date, this has not been shown to occur in a natural setting.

In this study, we characterize the *Symbiodinium* community in an inshore population of *A. millepora* and compare the *Symbiodinium* community in the same tagged colonies before and after a natural bleaching that took place in 2006. This is the first field study that follows changes in *Symbiodinium* genotypes in specific colonies over 3 years that includes a natural bleaching event. We show a dramatic shift in the symbiont community within this host population as a result of the disturbance, which is likely to have increased its thermal tolerance. We argue that if this shift is sustained and is community wide, the reefs in this area are likely to have substantially increased their capacity to withstand the next bleaching event.

2. MATERIAL AND METHODS

(a) *Study site*

Our study site is a reef flat adjacent to Miall Island (23°09′ S 150°54′ E), which is 1 of 15 islands in the Keppel Island group, in the southern inshore Great Barrier Reef. Miall Island, like many of the islands in this group, has an extensive reef flat on its leeward shore with an average coral cover of approximately 50%, dominated by colonies of the corymbose, Indo-Pacific stony coral *A. millepora* (van Woesik & Done 1997). The region suffered moderate to severe mass bleaching (more than 60% corals bleached) in February 2002 (Berkelmans *et al.* 2004) and severe bleaching in January/February 2006 (89% corals bleached; R. Berkelmans & A. M. Jones 2006, unpublished data).

(b) *Coral sampling*

To determine the *Symbiodinium* community composition before the bleaching, 460 colonies were tagged on the reef flat at Miall Island between September 2004 and March 2005. A small (2–3 cm) branch was sampled from the central area of each colony and placed in a labelled bag for subsequent storage in 100% ethanol. Symbiont changes were monitored in a subset of 79 tagged colonies that survived the bleaching three and six months (May and August, respectively) after the bleaching event in January/February 2006. The subset of 79 colonies was chosen haphazardly from surviving colonies and comprised 58 with predominantly C2-type (no background types detected), 15 with predominantly D-type (no background types detected) and 6 with both C2 and D types present. To minimize confounding of temporal trends in symbiont community by intracolony variation in symbiont types, we sampled from the same area within each colony on each sampling occasion and ensured that only the tips of branches were used for DNA extraction. Mortality in the *A. millepora* population was assessed six months after the bleaching event in August 2006 by visually estimating the percentage of live and dead coral tissue on 159 haphazardly chosen tagged colonies using pre-bleaching photos of each colony as a reference.

(c) *Genotyping and sequencing*

DNA was extracted from coral tissue based on the method of Wilson *et al.* (2002). A combination of single-stranded conformation polymorphism (SSCP) analysis, cloning and DNA sequencing was used for symbiont identification. The internal transcribed spacer 1 (ITS1) region was amplified as described by van Oppen *et al.* (2001). SSCP analysis was used to identify the predominant symbiont type in each colony and estimate the relative abundance of *Symbiodinium* types within each sample when more than one type was identified. Relative abundances of less than 5–10% are not detected using SSCP (Fabricius *et al.* 2004). SSCP bands that were faint compared with another more intense band in the same sample were identified as background and predominant types, respectively. The presence of two equally intense bands in the same sample was interpreted as the colony hosting equal amounts of each type. Fabricius *et al.* (2004) found that this was a reliable method for estimating the relative abundance of different *Symbiodinium* types. SSCP profiles were assigned to symbiont type by comparing to reference samples of known identity and by cloning and sequencing in the case of novel SSCP profiles. Phylogenetic analyses are described in detail in the electronic supplementary material.

(d) *Statistical analysis of symbiont community changes*

Counts of colonies of *A. millepora* before the bleaching were analysed using a Pearson's chi-squared contingency table to compare the frequencies of colonies with different combinations of predominant symbiont types C2, C1 and clade D with the null hypothesis that there were no differences in the observed and expected cell frequencies.

In addition, two separate multinomial loglinear regressions were used to test for significant changes in the (i) predominant- and (ii) low-level background symbiont types in the subset of 79 colonies three and six months after bleaching with the null hypotheses that there were no differences in the log ratios of the observed and expected cell frequencies. Predominant

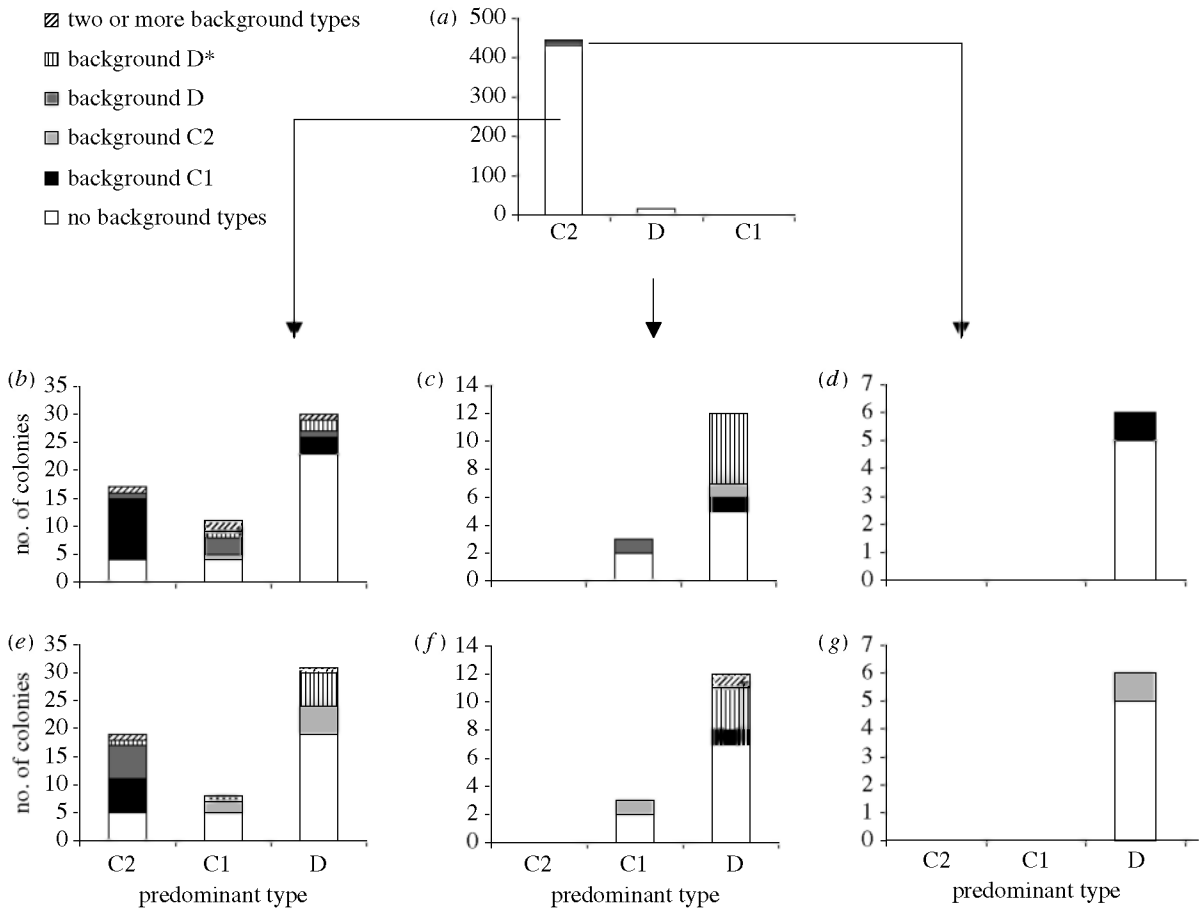

Figure 1. *Acropora millepora* colonies sampled (*a*) prior to bleaching between September 2004 and March 2005 ($n=460$), (*b–d*) three months after bleaching in May 2006 ($n=79$) and (*e–g*) six months after bleaching in August 2006 ($n=79$) at Miall Island reef (Keppel Islands, southern Great Barrier Reef). (*b,e*) Original C2 type, $n=58$, (*c,f*) original D type, $n=16$, (*d,g*) original C2/D type, $n=6$.

symbiont types (C2, D and C1) and background types (C2, D, C1, D*, multiple types and no background types) before bleaching and three and six months after bleaching were fixed factors in the analyses, and cases were weighted by the number of colonies of each type. The parameter estimates derived from the multinomial loglinear regressions were used to show the nature of any significant changes. All statistical analyses were performed with SPSS v. 15.0.

3. RESULTS

(a) *Symbiont diversity at Miall Island before and after bleaching*

Before the bleaching in 2006, *A. millepora* at Miall reef associated predominantly with *Symbiodinium* type C2 (93.5%, *sensu* van Oppen *et al.* 2001) and to a much lesser extent with *Symbiodinium* clade D (3.5%) or mixtures of C2 and D (3.0%; $\chi^2_1=398$, $p<0.001$, $n=460$, figure 1*a*). Cloning and sequencing of five clade D and six clade C ITS1 PCR products (370 bp in length) showed that these differed by 1–6 bp within clades, which we assume represents intragenomic variation. By late February 2006 when bleaching was at its most intense, the relative difference in bleaching susceptibility between corals predominated by C2 and D was clearly evident, with the former bleaching white and the latter normally pigmented (figure 2). Tagged corals harbouring a mix of *Symbiodinium* C2 and D were mostly pale in appearance.

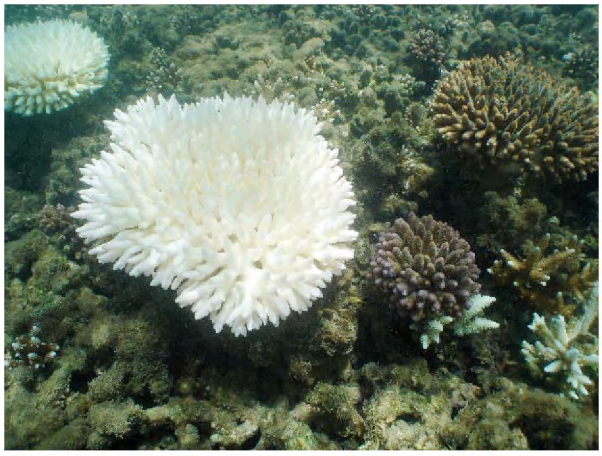

Figure 2. A stark landscape of 100% bleached *A. millepora* colonies predominated by *Symbiodinium* clade C2 and unbleached colonies with clade D symbionts at Miall Island reef in the Keppel region of the southern Great Barrier Reef during the January/February 2006 summer bleaching confirm the differential bleaching susceptibility of corals with these symbiont types.

In May 2006, three months after the bleaching, a major shift to thermally tolerant type D and C1 symbiont communities occurred in the surviving colonies. Of 58 C2-predominant colonies monitored post-bleaching, 30 became predominant in type D symbionts (most without

detectable levels of C2 symbionts; figure 1*b*). Type C1 was not detected in any of the SSCP gels of samples just before the bleaching. By May 2006, however, C1 became the predominant type in 11 out of the 58 colonies and was clearly evident as a background type in 11 out of 17 colonies that remained C2 predominant. C2-predominant colonies without other detectable background symbiont types (lower detection limit 5–10%) made up only 4 out of the 58 colonies three months after bleaching. Of the 15 original D-predominant colonies monitored post-bleaching, 12 retained their D predominance while 3 changed to C1 predominance. All six colonies that initially hosted C2 with background clade D became D predominant by May 2006. A variant of D which we called D* was not apparent in any colonies prior to bleaching but was detectable at low levels in 10% of colonies after bleaching (figure 1*b*,*c*). The appearance of previously undetected C1 and D* led to an increased diversity of symbiont types three months after bleaching.

By August 2006, six months after bleaching, the proportion of predominant symbiont types in each of the three initial groups of colonies (C2 or D predominant and C2 with D) remained stable, but there were substantial changes in the mix of background types. In the group that changed from C2 to D predominance, none had detectable background levels of C2 in May but C2 reappeared in five colonies in August. In addition, the other two groups also showed a slight increase in the background occurrence of C2 in August, possibly suggesting the start of a drift back to pre-bleaching C2 predominance. By contrast, more colonies had C1 in May compared with August while the abundance of D* increased from May to August (figure 1*b*,*e*). The loglinear regressions showed that a significant change occurred in the predominant symbiont types of the colonies at Miall Island. The C2-predominant colonies were more likely to have changed to clade D predominance than to have remained unchanged or changed to C1 predominance in both May and August ($Z=-15.0$, $p<0.001$, d.f.$=10$). Type C2 colonies were more likely to occur with clade D than any other type or combination of types (C1, C2, D or D*) in May ($Z=29.7$, $p<0.001$, d.f.$=70$) and August ($Z=34.4$, $p<0.001$, d.f.$=70$).

While the symbiont community change in surviving colonies was dramatic (71% changed predominance from C2 to D or C1 ($n=79$)), selective mortality also played a substantial role in shifting the symbiont community in the coral population. Of 159 colonies monitored for survival, 147 were initially C2 predominant and of these, 54 colonies suffered 100% mortality and a further 34 suffered more than 50% partial mortality (figure 3). Only 1 of 15 colonies that were initially D predominant died. The difference in mortality between clades was statistically significant ($p=0.043$, $\chi^2_1=4.1$), confirming their differential thermal tolerance.

In terms of relative contribution to symbiont community change, selective mortality accounted for 37% of the change while altered symbiont-type predominance accounted for 42% of the change ($n=159$). Just over 20% of the original C2-predominant population survived and maintained C2 as their predominant symbiont (cf. 93.5% prior to bleaching).

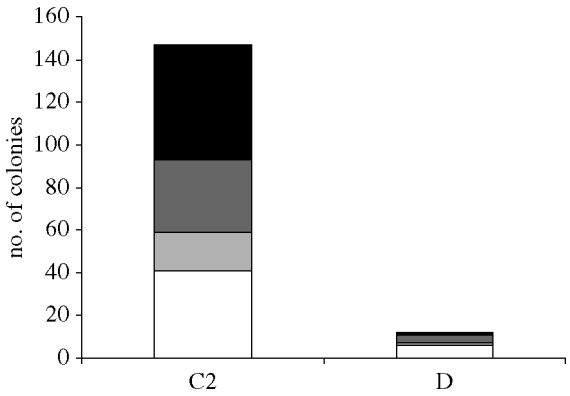

Figure 3. Summary of partial mortality in tagged *A. millepora* colonies at Miall Island ($n=159$). 37% of colonies with type C2 suffered 100% mortality compared with only 8% of clade D colonies ($\chi^2_1=4.1$, $p=0.043$). D, colonies with predominantly clade D; C2, colonies with predominantly type C2; and C2/D, colonies with type C2 and clade D. Within-colony mortality: black, 100%; dark grey, 51–99%; light grey, 11–50%; white, less than 10%.

4. DISCUSSION

We have shown field evidence of a dramatic shift in the symbiont community in a reef-building coral as a result of bleaching. The balance of the symbiosis shifted from a predominant association between *A. millepora* and *Symbiodinium* type C2 to a predominance of type D and to a lesser extent to predominance of type C1. This shift resulted partly from a change of symbionts within coral colonies that survived the bleaching event (42%) and partly from selective mortality of the more bleaching-sensitive C2-predominant colonies (37%). While these numbers are event, population and location specific, they do confirm that several interrelated processes play a role in shaping reef symbiont communities after bleaching episodes (Baker 2003). We propose that symbiont shuffling is a more likely explanation for the observed shift in symbiont communities than switching (i.e. de novo uptake) because (i) all 14 colonies that harboured low levels of D-type symbionts prior to the bleaching event survived and changed from C2 to D predominance, (ii) SSCP analysis is known to lack the sensitivity to detect symbiont types at a relative abundance of less than 5–10% and (iii) cloning and sequencing a subset of samples before bleaching revealed D and C1 below the detection limits of SSCP, the presence of which predicted their appearance after bleaching if shuffling was the mechanism of change. This is supported by the observation of novel symbiont types three and six months after the bleaching. Although de novo uptake cannot be ruled out, mathematical modelling of the recovery of symbiont populations after bleaching suggests that such rapid changes are more easily explained by upward and downward regulations of existing symbiont populations (Jones & Yellowlees 1997).

As a direct result of the shift in symbiont community, the Miall Island *A. millepora* population is likely to have become more thermo-tolerant. We base this conclusion on the experimental evidence of Berkelmans & van Oppen (2006) who found that differences in thermal tolerance in *A. millepora* from the same area is driven by symbiont type rather than the host coral. Furthermore, a shift from bleaching-sensitive type C2 to clade D increased the

thermal tolerance of this species by 1–1.5°C. These findings are supported by our observation of differential bleaching susceptibility between C2- and D-predominant colonies during the 2006 bleaching event (figure 2). We suggest that *A. millepora* colonies that host predominantly C1-type symbionts are also more thermally tolerant than their counterparts with C2. Unbleached colonies of the staghorn coral *A. formosa* sampled in February 2006 at Miall Island harboured predominantly C1 symbionts whereas white-bleached colonies of this species hosted C2. These observations, together with the high occurrence of C1 in acroporid corals (van Oppen *et al.* 2001) at one of the most thermo-tolerant reefs on the Great Barrier Reef (Berkelmans 2002), suggest that C1 may confer thermal tolerance to some species, just like D-type symbionts. Given the direct experimental evidence of increased thermal tolerance of *A. millepora* with D-type symbionts and the circumstantial evidence of similar thermal tolerance in this species with C1-type symbionts, the symbiont community change documented in this study is therefore likely to have resulted in increased thermal resistance for the majority of the *A. millepora* population. If the symbiont community drifts back to C2 predominance, the increased thermal tolerance will be lost. A drift back to pre-bleaching symbiont types was suggested for *Montastraea annularis* in the Florida Keys (Thornhill *et al.* 2006), and there are signs of a similar drift back to pre-bleaching C2 predominance in this study six months after bleaching.

Our results strongly support the reinterpreted adaptive bleaching hypothesis of Buddemeier *et al.* (2004), which postulates that a continuum of changing environmental states stimulates the loss of bleaching-sensitive symbionts in favour of symbionts that make the new holobiont more thermally tolerant. However, such a change may come at a physiological cost such as loss of photosynthetic efficiency (Rowan 2004) leading to lower energy reserves (Hoogenboom *et al.* 2006; Loram *et al.* 2007) and slower growth (Little *et al.* 2004). Our field observations provide the first extensive colony-specific documentation and quantification of temporal symbiont community change in the field in response to temperature stress, suggesting a population-wide acclimatization to increased water temperatures. If this shift is sustained and extends to other species, the reefs in this area are likely to have substantially increased their capacity to withstand the next bleaching event. However, at this stage, it is unknown whether the increased thermal tolerance, even if it persists, will necessarily translate into increased reef resilience, particularly if growth and carbonate accretion are depressed to levels whereby bioerosion outweighs net accretion.

This study highlights the importance of improving our understanding of multi-clade symbiotic partnerships (Baker & Romanski 2007). Our results show an increase in the diversity of symbionts after bleaching together with a considerable change in the make-up of the symbiont community within individual colonies over time scales as short as three months. This increase in the diversity and variation of symbionts has not been previously shown following a bleaching event. Most studies that have followed the *Symbiodinium* community during bleaching (Glynn *et al.* 2001; Guzman & Cortes 2001; Baker 2003; Van Woesik *et al.* 2004) have not used molecular techniques sensitive enough to detect the low-density symbiont genotypes and genetic variations of rDNA types (Apprill & Gates 2007). A recent study has shown that the majority of scleractinian corals are likely to harbour symbiont types at levels that are undetectable using electrophoretic genetic techniques (Mieog *et al.* 2007), suggesting that symbiont flexibility may also be more common than previously thought. Subtle seasonal and spatial shifts in symbiont populations that occur as a result of even minor changes in environmental variables such as temperature and light may underwrite the more permanent, climate-driven shifts following dramatic bleaching events (Thornhill *et al.* 2006). Smith (2005) found that four months before a major bleaching event in early 2002, 20 out of 20 *A. millepora* colonies at Miall Island were predominant in type C2, while van Oppen *et al.* (2005*a*) found that five months after the 2002 bleaching event, 6 out of 19 were predominant in type D. Although the sample sizes in these studies are small, these results suggest that the *A. millepora* symbiont community underwent a similar shift towards clade D predominance as a result of the 2002 bleaching event and then drifted back to C2 predominance 4 years later just prior to the 2006 bleaching event. This poses the question of why some coral population retain thermally tolerant symbionts while others revert back to former sensitive types. Baird *et al.* (2007) hypothesize that symbiont community shuffling to clade D may persist only as a result of enduring changes in environmental conditions, e.g. repeated warm summers. This may be evident at Magnetic Island, where temperatures exceed 30.5°C during most summers (Berkelmans 2002) and *A. millepora* have harboured exclusively clade D symbionts over many years (van Oppen *et al.* 2001; Berkelmans & van Oppen 2006). Conditions similar to those currently occurring at warm reefs such as Magnetic Island have been projected to occur on in the southern Great Barrier Reef by 2020–2030 (Done *et al.* 2003). Understanding the role of these backgrounds symbionts and the process and conditions under which they are up- and downregulated is the key to assessing the acclimatization potential of coral reefs and their ability to withstand future thermal stress events in an era of climate change.

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Supplementary material

Phylogenetic analysis of *Symbiodinium* ITS1

Following amplification of the ITS1 region and SSCP analysis, four different bands were observed (Fig. 1), and the ITS1 region of 8 representative samples were cloned and/or sequenced. Amplification products of about 370 bp length were obtained for all samples. Sequences were compared and aligned to closely matching sequences in Genbank using BioEdit (version 7.0) and CLUSTER W (version 1.7, Thompson *et al.* 1994). Primer sequences were removed from the alignments before analysis. Phylogenetic analyses were conducted using the Maximum Parsimony (MP) method (Saitou & Nei 1987) in PAUP (Version 4.0b10 for 32-bit Microsoft Windows). Either exhaustive or branch-and-bound searches were conducted, with gaps treated as a fifth base. Support for MP clusters was tested by bootstrap analysis with 1000 replicates (heuristic search). Type C and type D ITS1 sequences were analysed separately because this region is virtually un-alignable among clades.

The *Symbiodinium* ITS1 clade C alignment consisted of 253 alignment positions and 13 taxa (Taxa for which the ITS1 sequences were obtained for this study are shown in Table 1). Six positions were variable, two of which were parsimony informative. The *Symbiodinium* ITS1 clade D alignment consisted of 8 taxa and 329 characters, six of which were variable but not parsimony-informative. The clade D sequences obtained from the Keppel island *A. millepora* colonies are identical or very similar to clade D obtained from *A. millepora* at other Great Barrier Reef locations (van Oppen *et al.* 2001, accession number EU024793). Phylogenetic

analysis distinguished the two subclades C1 and C2 within clade C (van Oppen *et al.* 2001, Berkelmans & van Oppen 2006) as well as a few other sequence variants (Fig. 2 a). There was one clade D variant (named type D*) that appeared on SSCP in a number of samples before the bleaching (Fig. 1). Sequence analysis of the clone matching the D* SSCP profile revealed a single base pair change from the most common type D variant (Fig. 2 b).

Cloning of samples that appeared to harbour one symbiont type (according to SSCP) in some samples revealed low-abundance background types that had missed detection (Fig. 3). For instance, nineteen clones of sample 292, harbouring only C1 according to the uncloned sample SSCP gel image, were sequenced and multiple variants of C1 and one C2 clone were detected. Only samples that contained clear, sharp SSCP gel image bands of type C2 or clade D or equal bands of both were chosen for direct sequencing (see below).

Comparison of phylotypes with other studies

Comparisons of the *Symbiodinium* clade C type nomenclature between studies is often hampered by the use of different parts of the nrDNA region. To overcome this, the entire *Symbiodinium* nrDNA-ITS region (comprising ITS1, 5.8S and ITS2) of a small subset of five uncloned type C1 and C2 (from ITS1 genotyping) samples from the tagged colonies was amplified (three sampled before bleaching and two sampled after bleaching) and sequenced as described by Baillie *et al.* (2000). Forward primer, 'zITSf': 5'- CCG GTG AAT TAT TCG GAC TGA CGC AGT GCT-3' (Hunter *et al.* 1997) and reverse primer 'zITSr': 5'- TCC TCC GCT TAT TGA TAT GC-3' (White *et al.* 1990) were used to obtain approximately 650 base

pair PCR products (Table 1). Sequences were compared and aligned to previously sequenced ITS1 samples and closely matching sequences in Genbank using BioEdit (version 7.0) and CLUSTER W (version 1.7, Thompson *et al.* 1994).

The uncloned full-length ITS sequences were assigned to type C1 or C2 based on their match to the ITS1 region of the previously sequenced representative ITS1 region samples (Table 1) and the SSCP gel profile. The sequences were similar but not identical to the LaJeunesse ITS2 sequence regions of *Symbiodinium* type C1e & f (Genbank accession numbers AY258489, AY258490) found in *Fungia scutaria* (Hawaii LaJeunesse *et al.* 2004), C1b (AY239363) and C1c (AY239364) from the Great Barrier Reef (2003) and to the entire ITS sequence region of *Symbiodinium goreaui* type C1 (AF333515 LaJeunesse 2001). The alignment of the full length ITS sequences from this study and the LaJeunesse C1 sequence (AF333515) consisted of six taxa and 542 alignment positions. Nine alignment positions were variable, 7 of which were parsimony informative. Phylogenetic analysis of the six full-length ITS sequences obtained from before and after the bleaching and the *Symbiodinium goreaui* C1 sequence (AF333515) confirmed the assignment to types C1 and C2 (Fig. 2c).

Twenty sequences have been submitted to Genbank under the accession numbers EU189435 - EU189455 (Table 1). All except one cloned and one uncloned ITS1 sequence had at least one or two base pair variations from previous Genbank entries. All sequences used as references, standards and matches from Genbank are shown in Table 2.

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Table 1. Samples from which ITS1 and full ITS DNA sequences were obtained in this study via either direct sequencing of the PCR product ('direct') or cloning of the PCR product followed by sequencing of a number of clones ('cloned'). Phylogenetic analysis of the ITS1 and full length ITS regions of a subset of the samples confirmed the presence of clade D and type C1 and C2 *Symbiodinium*. Sequenced samples were used as references in subsequent SSCP analysis to confirm the predominant symbiont type of the colonies in this study. Note, samples are classified by a prefix that represents the reef name (e.g. M= Miall Island, K=Keppels) and the colony tag number, and suffixed with a dash and a number if cloned.

Inter-transcribed Spacer Region 1 (ITS1)			Full length Inter-transcribed Spacer Region (ITS, comprising ITS1, 5.8S and ITS2)	
Type C		Type D	Type C	
Before bleaching	After bleaching	Before bleaching	Before bleaching	After bleaching
cloned	cloned	cloned	uncloned	uncloned
M20-3 EU189440	M292-1 EU189447	M283-1 EU189451	M365 EU189438	M365 EU189435
M20-8 EU189441	M292-2 EU189448	M283-5 EU189452	M392 EU189436	M296 EU189439
M20-11 EU189442	M296-1 EU189449	M283-11 EU189453	M265 EU189437	
	uncloned	M283-12 EU189454		
	M291 EU189443	M283-13 EU189455		
	M296 EU189444	uncloned		
	M349 EU189445	M071 EU189450		

	M2 EU189446			

Table 2. Additional *Symbiodinium* types whose full ITS and ITS1 sequences were referred to in this study.

Species	Clade	Host	Location	Citation	Accession No.
Symbiodinium sp.	C1	*A. tenuis*	Magnetic Island	(van Oppen *et al.* 2001)	AF380555
Symbiodinium sp.	C2	*A. millepora*	Orpheus Island	(van Oppen *et al.* 2001)	AF380549
Symbiodinium sp.	D	*A. millepora*	Magnetic Island	(van Oppen *et al.* 2001)	EU024793
Symbiodinium sp.	C	*A. millepora*	Keppel Islands	(Berkelmans 2006)	AY643496
Symbiodinium sp.	D	Various spp.	Palau	(Fabricius *et al.* 2004)	AY457965
Symbiodinium goreaui	C1	*Rhodactis lucida*	Carribean, Jamaica	(LaJeunesse 2001)	AF333515
Symbiodinium sp	C1b, c	Various spp.	Heron Island	(LaJeunesse *et al.* 2003)	AY239363/4
Symbiodinium sp	C1e	Various spp.	Hawaii	(LaJeunesse *et al.* 2004)	AY258489
Symbiodinium sp.	C1f	*Fungia scutaria*	Hawaii	(LaJeunesse *et al.* 2004)	AY258490

Fig 1. Several SSCP variants of *Symbiodinium* types C and D were found before and after bleaching in *Acropora millepora* colonies from the Keppels. Cloning and sequencing of one of these (D*) revealed a difference of only one base pair from other type D sequences. Clade C variants that matched C1 and C2 had either none or 3 to 6 base pair differences.

Fig 2 a-c. Phylogenetic relationships derived by Maximum Parsimony analysis of **(a)** the clade C ITS1 and **(**b**)** clade D ITS1 and **(**c**)** clade C full length ITS regions of *Symbiodinium* sequences from *A. millepora* colonies in the Keppel region and matches from GenBank. (Trees are branch-length informative and numbers at nodes represent bootstrap support values based on 1000 replicates). Two major clades were identified using this method, C and D (van Oppen *et al.* 2001). Standards with Genbank accession numbers used in SSCP analysis are shown in boldface. Branch lengths represent phylogenetic distances. Host names are shown in italics followed by the name of the reef. Type C samples taken before the bleaching are marked with ►. Subclades are labelled on the right. Cloned sequences are labelled with a dash following the sample number. M=Miall Island or Magnetic Island, K=Keppel islands.

a. Rooted *Symbiodinium* ITS1 type C (MP) phylogenetic relationships (1000 replicates) showing branch lengths to scale (Mega 3).

b. Rooted *Symbiodinium* ITS1 type D (NJ) phylogenetic relationships (1000 replicates). No type D samples were sequenced after the bleaching in January 2006. Branch lengths indicate the strength of the relationships. *denotes type D* which had a higher SSCP band compared to all other clade D sequences.

c. Rooted *Symbiodinium* full length nrDNA-ITS type C sequence (MP) consensus phylogenetic relationships (1000 replicates).

Figure 3. SSCP gel image showing diversity of symbiont types within a single sample after recovery from bleaching. The wide band of the uncloned sample 292 (far left) suggests multiple type C1 variants. Cloning and sequencing revealed 4 different sequences, unexpectedly including C2. The sample was taken after the colony bleached and then recovered. The SSCP image of the sample taken before bleaching showed a sharp C2 band with no obvious evidence of other background symbiont types. Greater than 90% of the colony (mostly the tips of branches) had mortality in August 2006, six months after bleaching.

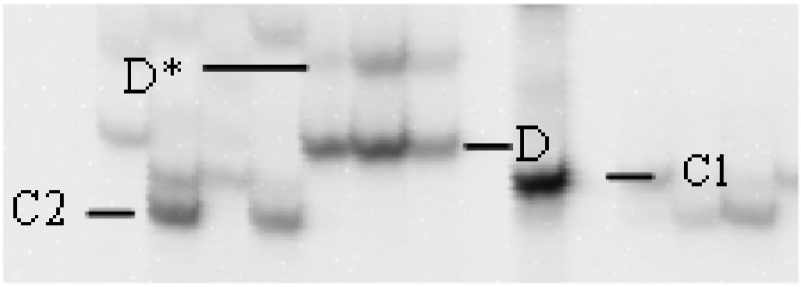

Fig 1

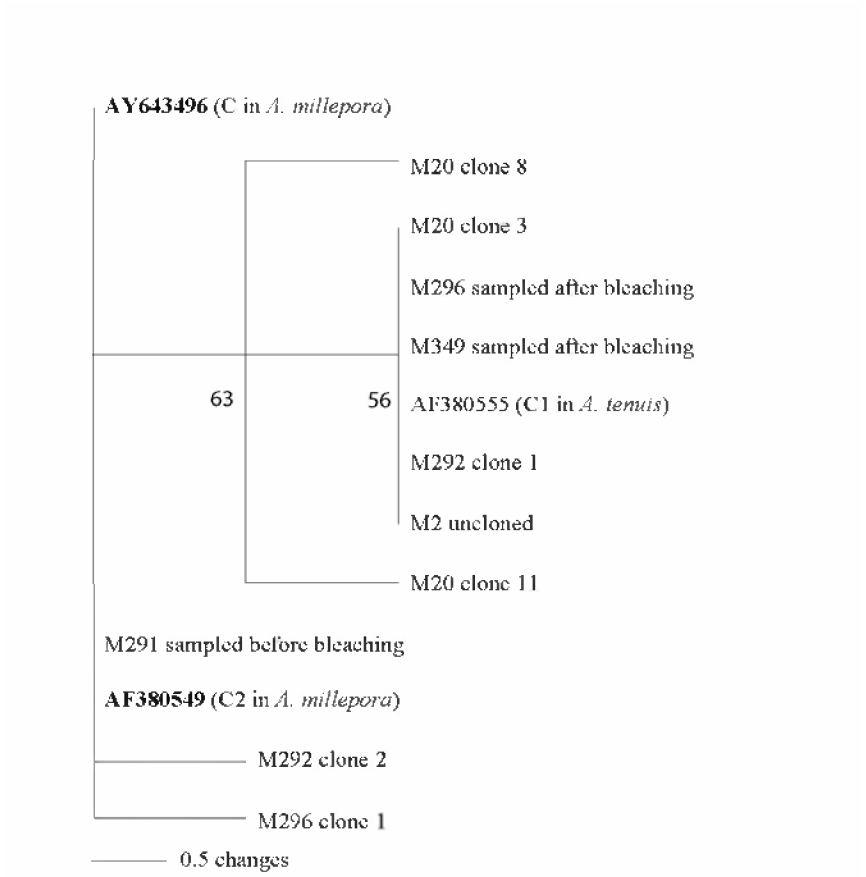
AY643496 (C in A. millepora)
M20 clone 8
M20 clone 3
M296 sampled after bleaching
M349 sampled after bleaching
63
56
AF380555 (C1 in A. tenuis)
M292 clone 1
M2 uncloned
M20 clone 11
M291 sampled before bleaching
AF380549 (C2 in A. millepora)
M292 clone 2
M296 clone 1
0.5 changes

Fig 2 a

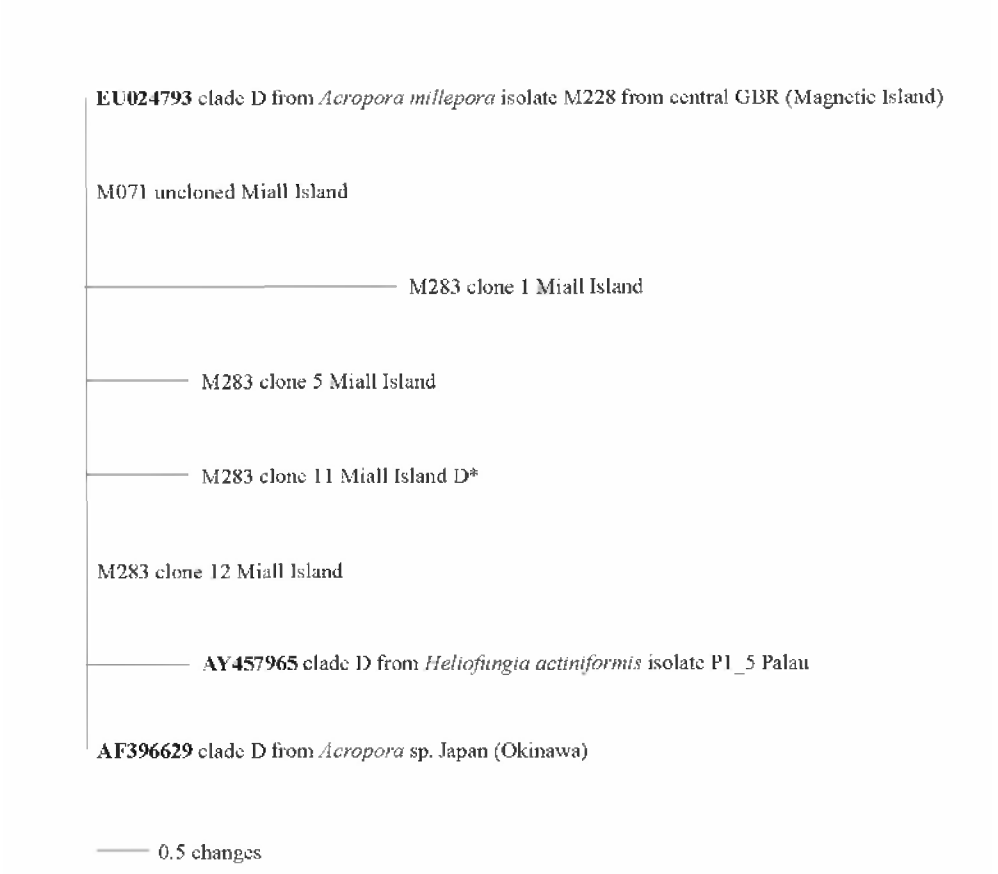
EU024793 clade D from *Acropora millepora* isolate M228 from central GBR (Magnetic Island)
M071 uncloned Miall Island
M283 clone 1 Miall Island
M283 clone 5 Miall Island
M283 clone 11 Miall Island D*
M283 clone 12 Miall Island
AY457965 clade D from *Heliofungia actiniformis* isolate P1_5 Palau
AF396629 clade D from *Acropora* sp. Japan (Okinawa)
0.5 changes

Fig 2 b

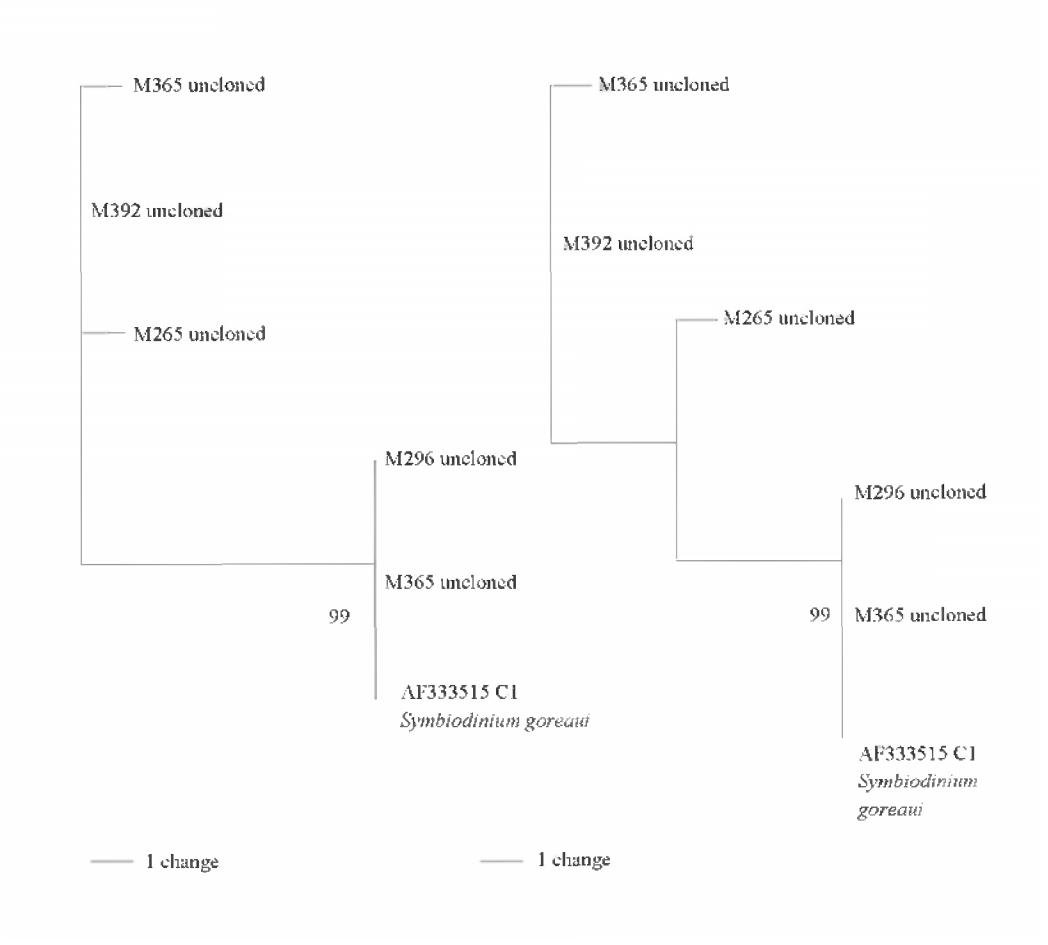
M365 uncloned
M392 uncloned
M265 uncloned
M296 uncloned
M365 uncloned
99
AF333515 C1
Symbiodinium goreaui
1 change
M365 uncloned
M392 uncloned
M265 uncloned
M296 uncloned
99
M365 uncloned
AF333515 C1
Symbiodinium goreaui
1 change

Fig 2 c

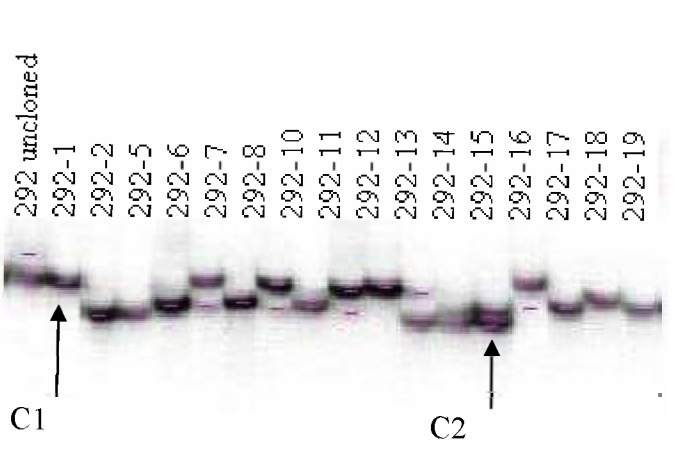
292 uncloned
292-1
292-2
292-5
292-6
292-7
292-8
292-10
292-11
292-12
292-13
292-14
292-15
292-16
292-17
292-18
292-19
C1
C2

Fig 3