Real-time PCR reveals a high incidence of Symbiodinium clade D at low levels in four scleractinian corals across the Great Barrier Reef

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REPORT

Real-time PCR reveals a high incidence of *Symbiodinium* clade D at low levels in four scleractinian corals across the Great Barrier Reef: implications for symbiont shuffling

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Abstract Reef corals form associations with an array of genetically and physiologically distinct endosymbionts from the genus *Symbiodinium*. Some corals harbor different clades of symbionts simultaneously, and over time the relative abundances of these clades may change through a process called symbiont shuffling. It is hypothesized that this process provides a mechanism for corals to respond to environmental threats such as global warming. However, only a minority of coral species have been found to harbor more than one symbiont clade simultaneously and the current view is that the potential for symbiont shuffling is limited. Using a newly developed real-time PCR assay, this paper demonstrates that previous studies have underestimated the presence of background symbionts because of the low sensitivity of the techniques used. The assay used here targets the multi-copy rDNA ITS1 region and is able to detect *Symbiodinium* clades C and D with >100-fold higher sensitivity compared to conventional techniques. Technical considerations relating to intragenomic variation, estimating copy number and non-symbiotic contamination are discussed. Eighty-two colonies from four common scleractinian species (*Acropora millepora*, *Acropora tenuis*, *Stylophora pistillata* and *Turbinaria reniformis*) and 11 locations on the Great Barrier Reef were tested for background *Symbiodinium* clades. Although these colonies had been previously identified as harboring only a single clade based on SSCP analyses, background clades were detected in 78% of the samples, indicating that the potential for symbiont shuffling may be much larger than currently thought.

Keywords Coral · *Symbiodinium* · Zooxanthella · Background clade · Shuffling · Real-time PCR

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Introduction

Many marine invertebrates and protists (e.g., corals, anemones, jelly fish, giant clams, and Foraminifera) form obligate mutualistic symbioses with algae of the genus *Symbiodinium*. The genus is several tens of millions of years old and comprises eight phylogenetic clades (A–H) based on ribosomal and chloroplast DNA (Pochon et al. 2006). Each clade encompasses multiple strains or types (Baker 2003; Coffroth and Santos 2005). The high diversity of *Symbiodinium* is often linked to physiological performance. In vitro studies using genetically distinct *Symbiodinium* cultures have found differences in photoacclimatory responses to light (Iglesias-Prieto and Trench 1997) and in growth (Kinzie et al. 2001; Robison and Warner 2006). Freshly isolated symbionts from different host species have shown distinct responses to heat-stress (Bhagooli and Hidaka 2003). *In hospite*, the genetic identity of the symbionts has been linked to a two to threefold difference in growth rates (Little et al. 2004) and 1–1.5°C difference in heat-tolerance (Rowan 2004; Berkelmans and van Oppen 2006) within a coral species. These results show

that changes in a coral's zooxanthella community may result in changes in the physiology of the symbiosis.

There are two proposed ways by which changes in the symbiont population can occur: symbiont switching and symbiont shuffling (Baker 2003). Symbiont switching is the uptake of a new symbiont type from the environment, whereas in symbiont shuffling, the new symbiont does not come de novo from the environment, but is already present at low abundance in the coral tissues. Uptake of exogenous zooxanthellae by adult individuals has been shown experimentally in anemones (Kinzie et al. 2001) and in a soft coral (Lewis and Coffroth 2004), but has so far not been observed in scleractinian corals. Instead, a coral's temporal window for uptake of zooxanthellae may be narrow and restricted to the juvenile stage (Little et al. 2004). Symbiont shuffling may play an important role in scleractinian corals, as recent studies have shown that resident algal populations consisting of more than one type of alga may change in relative abundance: (1) on a seasonal basis (Chen et al. 2005); (2) while recovering from a natural bleaching event (Thornhill et al. 2006b); and (3) after bleaching following transplantation (Berkelmans and van Oppen 2006).

In a recent review, Goulet (2006) surveyed 442 species of both hard and soft corals from 43 published studies in the literature and concluded that the simultaneous occurrence of multiple symbiont clades is low (23%) and, therefore, the promise of symbiont shuffling as a mechanism to cope with climate change, has been overestimated. While their conclusion is consistent with current data, absence of evidence is not necessarily evidence of absence. This study investigated whether the low value is an artifact caused by the low sensitivity of the most commonly used survey techniques for symbiont detection: denaturing gradient gel electrophoresis (LaJeunesse 2002), single strand conformational polymorphism (Fabricius et al. 2004), restriction fragment length polymorphism (Diekmann et al. 2002), and DNA fingerprinting (Goulet and Coffroth 2003a). None of these methods can detect clades, which are present at levels below 5–10% of the total symbiont population, and direct sequencing of PCR products will also only detect the dominant symbiont.

Here, a highly sensitive, real-time PCR assay is presented that quantifies the nuclear ribosomal DNA (nrDNA) Internal Transcribed Spacer 1 (ITS1) of clade C symbionts versus clade D symbionts within a single sample. The goal is to provide a new tool for a reassessment of the presence of low abundance, background or cryptic clades. The new assay was tested by resurveying a set of samples that were previously identified as having a single symbiont clade based on SSCP analyses and by verifying the nrDNA results with a similar assay based on chloroplast DNA. The implications of the results are discussed in terms of the current view about the importance of symbiont shuffling, and in terms of the prevalence and significance of cryptic symbionts.

Materials and methods

Collection and preparation of the coral samples

A collection of 82 scleractinian coral samples spanning 11 locations and 4 species (Fig. 1, Table 1) was assayed for background clades. Small pieces were taken from 2–5 colonies/species and fixed in absolute ethanol. DNA was extracted using the DNeasy tissue kit (Qiagen) following the manufacturer's protocol for animal tissues and using 150 µl elution buffer. The DNA samples were the same as those used in van Oppen et al. (2005) (*Acropora millepora*, *Acropora tenuis* and *Stylophora pistillata*), and in Ulstrup et al. (2006) (*Turbinaria reniformis*).

The real-time PCR assay

Clade C- and clade D-specific ITS1 primer pairs were obtained from Ulstrup and van Oppen (2003) (Table 2). A 25 µl real-time PCR reaction contained: 1× IQ SYBR Green supermix (BioRAD), 180 nM clade *Symbiodinium*-universal forward primer, 180 nM clade C- or D-specific reverse primer and 2.5 µl coral DNA template (MilliQ H_2O in case of no-template controls). Amplifications were run on the ICycler IQ Real-Time PCR Detection System (BioRAD). After an initial heating step to activate the Taq-polymerase following the manufacturer's instructions, the

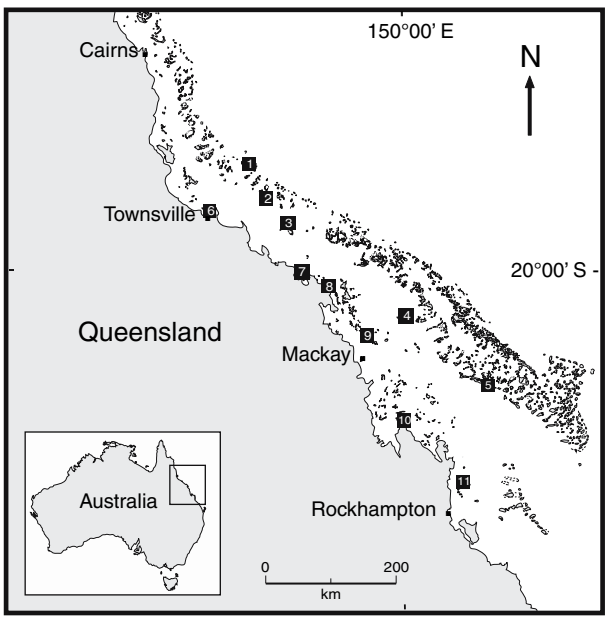

Fig. 1 Sampling locations. See Table 1 for location names, coordinates and sampling numbers

Table 1 Species, locations and symbiont types/clades based on SSCP and real-time PCR

Species	Location name	Lat-long	Fig. 1	Dominant type based on SSCP[a]	Dominant clade, back-ground clade based on real-time PCR
Acropora millepora	Chicken Reef	18.5 S, 147.5 E	1	C2 $(2)^1$	C$(2)^1$, D $(1)^2$
	Darley Reef	19.1 S, 148.1 E	3	C2 (5)	C(5), D (5)
	Stone Island	20.0 S, 148.2 E	7	D (4)	D(4), C2 (4)
				C2 (1)	C(1), D (1)
	Credlin Reef	20.3 S, 150.0 E	4	C2 (5)	C(5), D (5)
	Miall Island	23.1 S, 150.5 E	11	C1, C2 (2)	C(2), D (2)
				D (2)	D(2), C (2)
Acropora tenuis	Chicken Reef	18.5 S, 147.5 E	1	C2 (5)	C(5), D (3)
	Darley Reef	19.1 S, 148.1 E	3	C2 (5)	C(5), D (4)
	Credlin Reef	20.3 S, 150.0 E	4	C1 , C2, C3 (5)	C(5), D (4)
	Miall Island	23.1 S, 150.5 E	11	C1 (3)	C(3), D (2)
Stylophora pistillata	Chicken Reef	18.5 S, 147.5 E	1	C1, Cn (5)	C(5), D (3)
	Darley Reef	19.1 S, 148.1 E	3	C1, Cn (4)	C(4), D (4)
	Credlin Reef	20.3 S, 150.0 E	4	Cn (5)	C(5), D (5)
	Brampton Island	20.5 S, 149.2 E	9	C1 (3)	C(3), D (3)
	Mumford Island	22.0 S, 149.5 E	10	C1, Cn (5)	C(5), D (5)
Turbinaria reniformis	Big Broadhurst Reef	18.5 S, 147.4 E	2	C1 (4)	C(4), D (1)
	Magnetic Island	19.1 S, 147.5 E	6	C1 (5)	C(5), D (3)
	Grassy Island	20.1 S, 148.4 E	8	C1 (4)	C(4), D (2)
	Heralds Reef Prong	21.3 S, 151.2 E	5	C1 (3)	C(3), D (2)
	Miall Island	23.1 S, 150.5 E	11	C1 (5)	C(5), D (3)

$(n)^1$ = number of samples analyzed, $(n)^2$ = number of samples with a background clade

[a] van Oppen et al. (2005) and Ulstrup and van Oppen (2003)

Table 2 Nuclear and chloroplast primers used in real-time PCR

Primer	Sequence
nITS1 universal forward[a]	5′-AAGGAGAAGTCGTAACAAGGTTTCC-3′
nITS1 C-specific reverse[a]	5′-AAGCATCCCTCACAGCCAAA-3′
nITS1 D-specific reverse[a]	5′-CACCGTAGTGGTTCACGTGTAATAG-3′
cp23S C forward	5′-GGGATAAAACTTGGGTAACATTC-3′
cp23S C reverse	5′-CCAATTAAACAGTGGTCTTAGGAG-3′
cp23S D forward	5′-AACCCCCGATTGGCCTAG-3′
cp23S D reverse	5′-CTTGATTGGGCCATTAAGCA-3′

[a] Ulstrup and van Oppen (2003)

profile consisted of: 40 two-step cycles of 15 s at 95°C and 1 min at 60°C. At the end of each run, a melt curve was generated by starting at 60°C and increasing the temperature by 0.5°C each 5 s for 70 cycles. Data collection took place during the 1 min at 60°C in each cycle, and during each temperature step of the melt curve.

The cycle-threshold (C_T) is the PCR cycle at which the fluorescence of a sample exceeds the chosen threshold limit. Setting the fluorescent threshold to a fixed value allows the comparisons of C_T values between runs. Duplicate clade C and D reactions were run for each sample along with negative (no-template) and positive controls in each run. The positive controls showed distinct peaks in the melt curve for clades C and D. Any runs with peaks not within 1°C of these temperatures were discarded as having primer dimer signals (which occurred rarely). In some runs, the no-template controls showed a small signal during the last few cycles of the run, which is common in real-time PCR and caused by the formation of non-specific fluorescence. To avoid the inclusion of false positives, the end of the detection range was set three cycles prior to the C_T of the no-template controls that showed a non-specific fluorescence signal. Samples with C_T's above the cut-off were treated as false positives and scored as no background clade present. Runs were analyzed using the IQ software V3.1 (BioRAD).

D:C cell number ratios were calculated using the formula: D:C = $\left(2^{C_T(C)-C_T(D)}\right)/K^{DC}$, where $C_T(C)$ is the threshold cycle for the clade C specific reaction, $C_T(D)$ the threshold cycle for the clade D specific reaction, and K^{DC} the ratio of average copy number per cell between clades D and C (see next subheading). The average D:C cell number ratio and SE were calculated for each species per location, including the samples in which no backgrounds were detected as zero. A typical run for one coral sample including calculations is shown in Fig. 2.

Estimating average copy number

Ribosomal DNA loci can exhibit large variations in copy numbers between individuals (Zhang et al. 1990; Rogers and Bendich 1987; Govindaraju and Cullis 1992) thus affecting the translation from ITS1 copy number ratios to cell number ratios. To assess the extent of this potential bias, the cell-to-cell copy number variability within a C- and D-dominated coral colony was investigated, and the average copy number differences between colonies was determined from bulk cell analyses.

The cell-to-cell variability of ITS1 copy numbers within a clade was measured in a clade C-only and clade D-only population using single-cell real-time PCR. Clade C and clade D zooxanthellae were isolated from *A. tenuis* and *A. millepora*, respectively, obtained from Magnetic Island. Coral tissue was airbrushed off the skeleton into 1 µm filtered seawater (FSW). The slurry was spun down (400 g, 5 min) and the zooxanthella pellet washed three times with FSW. Cell suspensions were quantified using a haemocytometer and diluted in FSW to one cell per 2 µl. Ten microliter were pipetted onto a microscope slide and viewed under a dissecting microscope (70× magnification). Single cells were then collected in 0.5 µl of FSW using a pipette with a heat-elongated plastic pipette tip (to narrow the diameter of the tip), and were added directly to the real-time PCR reaction mix, which were thoroughly mixed by pipetting.

The 20 µl PCR reactions consisted of: 180 nM universal Forward Primer (uFP), 180 nM C- or D-specific Reverse Primer (RP), 1× SYBR Green PCR master mix (ABI), and 0.5 µl FSW containing one zooxanthella. Runs were performed on a Rotor-Gene RG-3000A (Corbett Research) and analyzed using the Rotor-Gene v6.0 software. The reaction profile was the same as described in the previous section. A standard curve was prepared from purified PCR products of ITS1 with known DNA concentrations. DNA used for the standard curves was diluted in MilliQ with 20% FSW to compensate for the FSW added to the single cell reactions.

To obtain an indication of average copy numbers per clade, zooxanthellae were isolated and quantified from four C-dominated (2× *A. millepora*, 1× *A. tenuis* and 1× *Pocillopora damicornis*) and two D-dominated (2× *A. millepora*) colonies as described above. An aliquot containing 100,000 cells was spun down (5 min, 400 g) and the DNA extracted following an adapted version of a method used for the black tiger shrimp, *Penaeus monodon* (Wilson et al. 2002). DNA isolations were performed in duplicate, and DNA pellets were dissolved in 200 µl 0.01 M Tris buffer pH 9. Two microliter template was used in the real-time PCR reaction mixes, which were prepared and run as described above. Values were calculated against

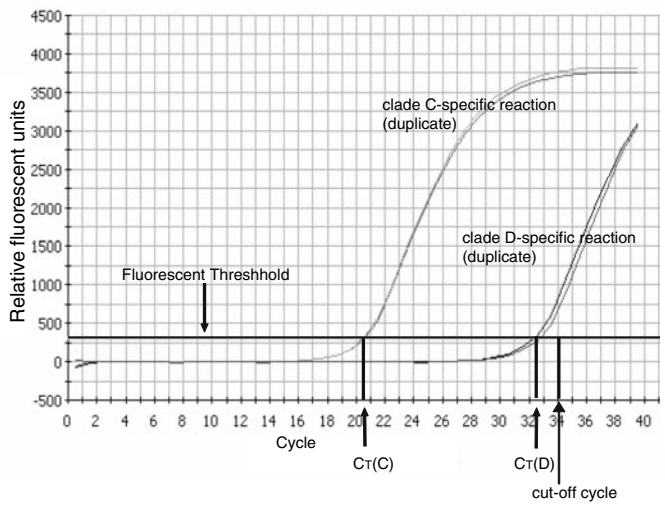

Fig. 2 A typical real-time PCR profile for the detection of a clade D background in a clade C dominated coral sample. The D-specific amplications begin later indicating the low, background abundance of this clade. The D:C cell nr. ratio is calculated as: D:C = $\left(2^{C_T(C)-C_T(D)}\right)/K^{DC} = 2^{(20.5-32.5)}/3 = 8.1 \times 10^{-5}$. The sensitivity for this sample is $2^{(20.5-34)}/3 = 2.9 \times 10^{-5} = 0.003\%$

the standard curve (diluted with 0.01 M Tris buffer pH 9) and divided by 1,000 (only 1/100 of the sample was used in the PCR) to obtain the mean ITS1 copy number per cell per colony.

Intragenomic variation

Ribosomal DNA copies within a genome evolve in concert (Arnheim et al. 1980), usually resulting in rapid sequence homogenization within individuals and populations through gene conversion and unequal crossing over (Dover 1982). However, the homogenization is assumed not to be complete in *Symbiodinium* (Apprill and Gates 2007) and the occurrence of intragenomic variants may affect the real-time PCR assay if they interfere with the correct binding of the clade C- and clade D-specific primers. To ensure that detected background clades were not due to intragenomic variants of ITS1 or real-time PCR artifacts, and to cross-test the accuracy of our ITS1 real-time PCR assay, 12 coral samples were re-analyzed using a second real-time PCR assay.

The 23S region of the chloroplast ribosomal DNA was quantified using two separate primer pairs (Table 2). Invitrogen's Platinum SYBR green 2× PCR mastermix was used as more consistent results were obtained in this assay with this mastermix than with the ABI real-time PCR mastermix. The 20 µl reactions contained: 400 nM C- or D-specific forward primer, 400 nM C- or D-specific reverse primer, 1× Platinum SYBR Green PCR master mix, 2 µl template. Cycle conditions were: 2 min at 50°C, 2 min at 95°C, followed by 15 s at 95°C and 30 s at 60°C for 40 cycles. Calculations were identical to the ITS1 assay except that no K^{DC} was used.

Results

Specificity, efficiency and sensitivity of the ITS1 real-time PCR assay

An initial PCR amplification of cloned clade C and D ITS1 PCR products (i.e., using plasmids as template) verified that the clade C and D reactions were specific to their respective clades. The fluorescent threshold was set at 300 relative fluorescent units in all Icycler runs. Clade C and D reactions with identical plasmid concentrations differed in their C_T values by less then 0.5, which allowed direct comparison of C_T(C) and C_T(D) of one sample.

In order to calculate the relative abundances of the two clades using the differences in C_T values, near-equal PCR efficiencies were required. PCR efficiency is 100% when the product doubles every cycle. Using a dilution series of a clade C and D sample, a C_T range of 18–32 was obtained for each clade. The ΔC_T was plotted against the Log of the relative template concentrations and the slope of the linear regression line was –0.092, which showed that PCR efficiencies were well matched and around 93%.

Sensitivity is the ability to detect very low levels of DNA. The range of background clade detection was between the C_T of the dominant symbiont up to the cut-off cycle (set at 34, Fig. 2), thus the sensitivity varied per sample. On average, background clade copy numbers could be detected down to 0.004% of the total zooxanthella population, representing a ca. 1,000-fold greater sensitivity as compared to conventional assays. However, translating these values into relative abundances of the two clades requires caution because abundance is affected by copy number variability between individual zooxanthella cells (see next subheading). Thus, a more conservative overall estimate of the sensitivity is suggested, at minimally a 100-fold increase over conventional methods.

Copy number variability and intragenomic variants

The limit of detection for single-cell real-time PCR runs was set at 500 copies, as the negative controls showed relatively high non-specific fluorescence. The fluorescent threshold was set at 0.1 normalised fluorescence in all Rotorgene runs. ITS1 copy number per cell ranged from <500 to 22,000 ($N = 20$) and from 2,300 to 12,000 ($N = 13$) for clade C and D, respectively.

Based on bulk analysis of 100,000 isolated cells per colony, cell-average ITS1 copy number for clade C was 984 ± 109 (over four colonies) and 3,181 ± 69 (over two colonies, mean ± SE) for clade D. Therefore, the K^{DC} is estimated at three and reflects the difference in average copy number between clades C and D.

The comparison between D:C ratios calculated from ITS1 and the cp23S (Table 3) showed good agreement within a factor of 10. Therefore, D:C cell nr. ratios should be interpreted as ''order of magnitude'' estimates. In two cases the assays disagreed, where the ITS1 assay detected a background clade but the 23S assay did not.

Re-assessing the incidence of background clades in scleractinian corals

Using the ITS1 assay, background clades were detected in 78% of 82 colonies, which based on previous SSCP analysis, were originally thought to harbor only a single symbiont clade (Table 1). *A. millepora* and *S. pistillata* had the highest frequency of backgrounds (20 out of 21 and 20 out of 22 colonies, respectively). *A. tenuis* (13 out of 18) and *T. reniformis* (11 out of 21) were less successful in acquiring/maintaining backgrounds. For both *A. millepora* and *S. pistillata*, the only colonies without detectable background

Table 3 Accuracy of ITS1 D:C ratio compared with an independent chloroplast marker

Species	Location	Cp 23S background (D:C cell nr. ratio)	ITS1 background (D:C cell nr. ratio)	Relative difference (ITS1/23S)
Acropora millepora	Darley Reef	2.0×10^{-4}	6.8×10^{-5}	0.3
	Darley Reef	5.5×10^{-4}	1.9×10^{-4}	0.3
	Miall Island	4.5×10^{2}	8.9×10^{1}	0.2
	Stone Island	2.5×10^{2}	6.1×10^{1}	0.2
Acropora tenuis	Chicken Reef	0	0	–
	Darley Reef	2.0×10^{-5}	1.5×10^{-5}	0.7
	Credlin Reef	0	8.0×10^{-4}	x
Stylophora pistillata	Chicken Reef	0	0	–
	Darley Reef	3.0×10^{-4}	3.1×10^{-4}	1.0
Turbinaria reniformis	Grassy Island	0	0	–
	Miall Island	1.6×10^{-1}	2.9×10^{-2}	0.2
	Magnetic Island	0	4.8×10^{-4}	x

clades were found at Chicken Reef, whereas *A. tenuis* and *T. reniformis* had colonies without backgrounds at all locations.

Symbiodinium clade C was the dominant endosymbiont at all locations for three out of the four species (*A. tenuis*, *S. pistillata*, *T. reniformis*), with average D:C ratios ranging from $4.3 \times 10^{-6} \pm 1.5 \times 10^{-6}$ (mean ± SE) for *S. pistillata* at Credlin Reef to $5.8 \times 10^{-3} \pm 5.7 \times 10^{-3}$ for *T. reniformis* at Miall Island (Fig. 3b–d). Clade D dominance was found in six *A. millepora* colonies sampled at the inshore locations of Stone and Miall Islands (Fig. 3a). These colonies had a relatively high background level of clade C compared to the clade D backgrounds in the other samples (D:C ratio = $7.4 \times 10^{1} \pm 1.2 \times 10^{1}$). Again, it is cautioned that all D:C cell nr. ratios should be interpreted as ''order of magnitude'' estimates.

Symbiodinium clade D backgrounds were detected in 71% of the colonies. The average clade D background density was 1 D-cell per 150–15,000 C-cells. Scleractinian corals have been estimated to harbor ~1,500,000 zooxanthella cells cm^{-2} of colony surface (Drew 1972), which translates into an average clade D background level of 100–10,000 cells cm^{-2}.

No latitudinal gradient of D:C cell nr. ratio was apparent. Cross-comparison of off-shore and mid-shore reefs showed a tendency of C > D on offshore reefs and D > C on nearshore reefs in *A. millepora*, but not in the other species.

Discussion

Sensitivity, accuracy and caveats of the ITS1 real-time PCR assay

Real-time PCR can detect background or cryptic clades over eight orders of magnitude and is at least 100 times more sensitive than previously used techniques. However, the high copy number variability found between symbiont cells within both clades with the ''single-cell'' analyses means that the conversion of ITS1 copy numbers into an estimate of the number of symbiont cells has limited accuracy. On the other hand, the average ITS1 copy number estimates of clades C and D, obtained from ''bulked cell'' analyses, were remarkably stable for each clade. These results indicate that cell-to-cell variation has only minor impact when enough cells are analyzed, but low-density background clade measurements will be more strongly affected. It should also be considered that the measured cell-to-cell variation might be an over-estimation of the real variation, caused by the difficult nature of single-cell amplifications. The approximate threefold discrepancy in average copy numbers between clades C and D led to the use of the correction factor K^{DC} in an effort to get background abundance estimates as accurate as possible. The relative difference between the ITS1 and 23S assays varied within tenfold, which is still reasonable over the range measured, and indicates that D:C cell nr. ratios are meaningful when interpreted as ''order of magnitude'' estimates.

The high agreement between the ITS1 and 23S assays indicated that the measured background clade signals were not due to intragenomic variation or PCR artifacts. For the two samples where the assays did not agree, it is assumed that the 23S D-specific reactions failed as opposed to the absence of a D background, as it was noted that the 23S assay was generally less robust compared to the ITS1 assay.

As a result of the high sensitivity of the real-time assay, it is possible that non-symbiotic zooxanthellae were detected, present as ingested cells or surface contaminants. Abundances of genuine free-living *Symbiodinium* are unknown, although progress is being made in detecting

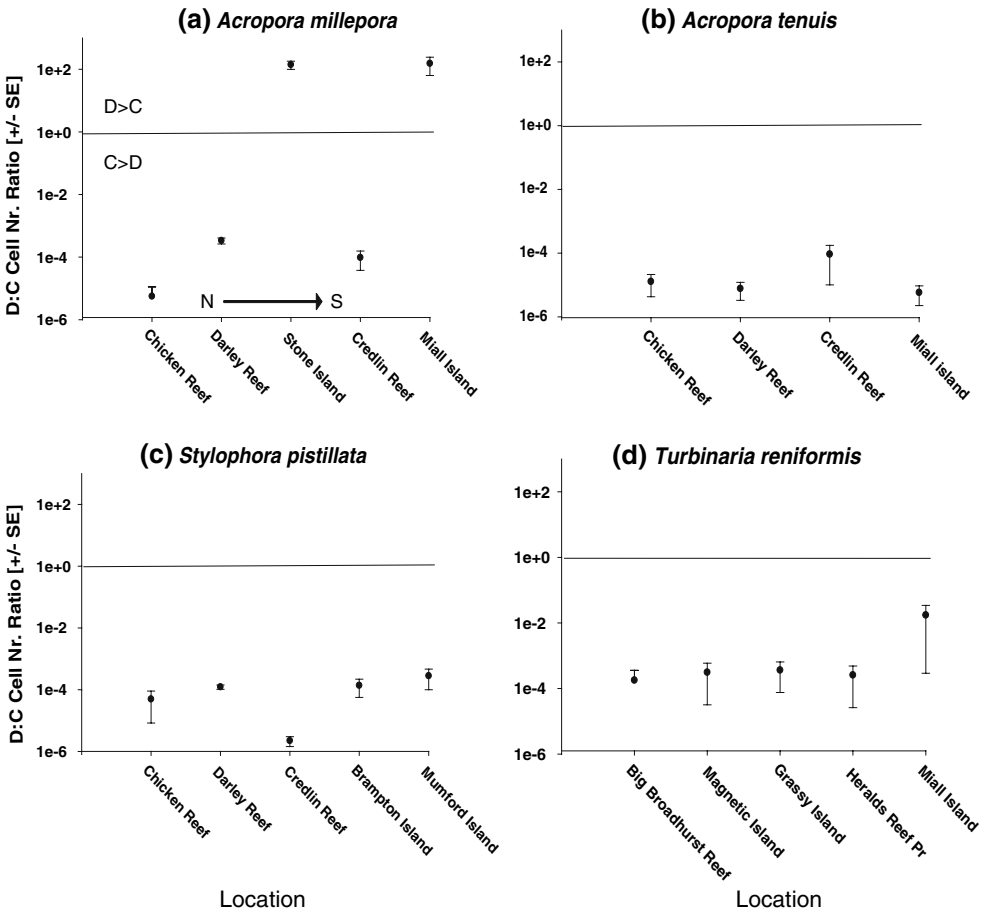

Fig. 3 The mean relative abundance of *Symbiodinium* clade D versus clade C in the four coral species at each of the sampling locations. Values should be regarded as ''order of magnitude'' estimates only

non-symbiotic, environmental populations (Coffroth et al. 2006). However, given the average background abundance detected in this study of 100–10,000 cells per cm^2 of coral surface, it is unlikely, in our opinion, that these zooxanthellae were non-symbiotic.

Significance of background clades

Ninety-three percent of the colonies tested were dominated by clade C and 76% of these had a D background. A number of studies have shown that clade D symbionts are amongst the most thermo-tolerant types know to date, whereas clade C types are often relatively thermo-sensitive (Rowan 2004; Tchernov et al. 2004; Berkelmans and van Oppen 2006; Ulstrup et al. 2006). Clade D is also found on reefs that chronically experience unusually high temperatures (Fabricius et al. 2004) or that have recently been impacted by bleaching events (Baker et al. 2004), suggesting that temperature stress can favor clade D. It is proposed here that the clade D backgrounds detected in this study can potentially act as a safety-parachute, allowing corals to become more thermo-tolerant through symbiont shuffling as seawater temperatures rise due to global warming.

For species that have already been found to be dominated by clade D symbionts at some locations, such as *A. millepora* and *T. reniformis*, shuffling between these two clades seems a likely mechanism. Could shuffling act as a mechanism to acclimatize to increasing sea surface temperatures in more than a few species? At present this question remains open. It is likely that not all coral species will have a shuffling capacity, as certain coral species have a high symbiont stability (Goulet and Coffroth 2003b; Thornhill at al. 2006a, b) which may not allow them to shuffle even when recovering from bleaching (Thornhill et al. 2006a). However, the ratio between shuffling and non-shuffling species is currently unclear and needs further study.

The results of this study indicate that the potential for symbiont shuffling is higher than previously thought. However, symbiont shuffling is likely to represent a trade-off and comes at a cost. First, coral colonies may have to bleach first before the background symbionts can proliferate (Baker 2001; Berkelmans and van Oppen 2006; Buddemeier and Fautin 1993), possibly causing high mortality in the process. Second, newly shuffled corals that have successfully recovered from bleaching are still likely

to be impaired in growth and reproduction (Baird and Marshall 2002). Third, if the stressor disappears for a prolonged period of time, the corals may shuffle back to the original symbiont (Thornhill et al. 2006b), leaving them again vulnerable to subsequent bleaching events. Lastly, the extra heat-resistance that corals may gain by shuffling (1–1.5°C) may be insufficient to help these populations cope with the predicted increases in average tropical sea temperatures over the next 100 years (Berkelmans and van Oppen 2006). Nevertheless, symbiont shuffling is likely to play a role in the way some corals cope with global warming conditions, leading to new competitive hierarchies and, ultimately, help shape the coral community assemblages of the future.

Conclusions and future directions

Real-time PCR can detect background clades over eight orders of magnitude, thus offering an assay that is at least 100 times more sensitive than previously used techniques. Furthermore, previous studies have failed to detect the majority of background clades because of the low sensitivity of the techniques used. This has led to an underestimation of the potential for symbiont shuffling.

The assay presented here should be used as a starting point to optimize the real-time PCR technique for coral-*Symbiodinium* research. First, at present it only distinguishes between clades C and D and not between types within clades, i.e., C_1, C_2, etc. Because *Symbiodinium* physiology is highly diverse and thermally tolerant types are known to exist within a single clade (Tchernov et al. 2004), future efforts will need to be directed towards the development of type-specific real-time PCR assays. Second, while rDNA-ITS continues to be the main marker in use, it has many unfavorable properties associated with its multi-copy nature. The development of novel single-copy markers for *Symbiodinium* should be a research priority and may become available from EST libraries that are currently under development.

The application of this technique in broadscale surveys for background *Symbiodinium* clades/types in many coral species, will allow the re-evaluation of the prevalence of background symbionts. The next challenge will be to decipher how symbiont shuffling is regulated (by the coral host, through competition between symbiont types inside the host tissues, and/or by environmental factors), and to understand how many corals harboring multiple symbiont types are able to undergo temporal changes in their symbiont communities.

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