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Stable *Symbiodinium* Composition in the Sea Fan *Gorgonia ventalina* During Temperature and Disease Stress

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Abstract. Like most Caribbean octocorals, *Gorgonia ventalina,* the common sea fan, harbors endosymbiotic dinoflagellates belonging to the genus *Symbiodinium.* When stressed, the host can lose these algal symbionts, a phenomenon termed "bleaching." Many cnidarians host multiple types of algal symbionts within the genus *Symbiodinium,* and certain types of algae may be more tolerant of stress than others. We examined the effects of temperature, temperature-induced bleaching, and infection by *Aspergillus sydowii,* a fungal pathogen, on *Symbiodinium* types harbored by the sea fan *Gorgonia ventalina* in the Florida Keys. Symbiont type, identified on the basis of variation in small subunit nuclear ribosomal genes or large subunit chloroplast ribosomal genes, did not vary with temperature treatment or infection status. Although allelic variation based on one microsatellite locus was found among samples and reef site, it did not consistently correlate with temperature, treatment, or disease status, suggesting that the symbiont-host relationship is stable. An aberrant PCR product was found in samples collected at one site and could be used to differentiate *Symbiodinium* populations among sites in the Florida Keys.

Introduction

Gorgonian corals, including the common sea fan *Gorgonia ventalina* Linnaeus, 1758, are prevalent on reefs across the Caribbean Sea (Kinzie, 1973; Opresko, 1973; Sánchez *et al.,* 1998; Jordán-Dahlgren, 2002). Like many other cnidarians, octocorals harbor endosymbiotic dinoflagellates of the genus *Symbiodinium.* These endosymbionts, known collectively as zooxanthellae, can be phylogenetically sorted into groups, or clades, based on restriction fragment length polymorphism (RFLP) analysis of the 18S small subunit rDNA (Rowan and Powers, 1991a; Rowan and Powers, 1991b). *Gorgonia ventalina* adults, like most Caribbean octocorals, harbor *Symbiodinium* clade B zooxanthellae (Goulet, 1999; Coffroth *et al.,* 2001; LaJeunesse, 2002; Santos *et al.,* 2003a; Goulet and Coffroth, 2004). However, many cnidarians harbor multiple types of algal symbionts, and the composition can vary in response to environmental factors (Rowan and Knowlton, 1995; Rowan *et al.,* 1997; Baker, 2001; Toller *et al.,* 2001; van Oppen *et al.,* 2001; Knowlton and Rohwer, 2003; Douglas, 2003). If an environmental change is extreme, the host may lose algal endosymbionts, a phenomenon termed "coral bleaching." High and low temperature, light, salinity, and other stresses have been implicated in coral bleaching (Steen and Muscatine, 1987; Glynn and D'Croz, 1990; Buddemeier and Fautin, 1993; Glynn, 1996; Hoegh-Guldberg, 1999; Downs *et al.,* 2002; Douglas, 2003; McClanahan, 2004; Strychar *et al.,* 2004).

Disease and parasitism can also lead to a change or loss of algal symbionts. For example, Toller *et al.* (2001) reported variation in *Symbiodinium* type in diseased *versus* healthy areas of colonies of *Montastraea* spp. affected by yellow blotch disease. Bacteria of the genus *Vibrio* infect corals and induce bleaching (Kushmaro *et al.,* 1996, 2001; Banin *et al.,* 2001; Ben-Haim *et al.,* 2003a, b). These bacteria infect only corals harboring endosymbiotic dinoflagellates, and they cause death and eventual expulsion

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of the dinoflagellates (Banin *et al.,* 2001; Kushmaro *et al.,* 2001; Ben-Haim *et al.,* 2003a, b).

Bleached octocorals (and possibly other cnidarians) can acquire *Symbiodinium* from the water column and from cryptic populations within the colony (Rodriguez-Lanetty *et al.,* 2003; Lewis and Coffroth, 2004). These can be a source for repopulation after a bleaching event, opening the way for changes in symbionts (Buddemeier and Fautin, 1993; Rowan *et al.,* 1997; Rowan, 1998; Kinzie *et al.,* 2001; Toller *et al.,* 2001; Douglas, 2003; Baker, 2004; Buddemeier *et al.,* 2004; Lewis and Coffroth, 2004). Especially under stressful conditions, this may lead to recombination between host and endosymbiont.

Over the past 15 years, *Gorgonia ventalina* and *Gorgonia flabellum* have suffered widespread mortality associated with an infection from *Aspergillus sydowii* (Bainier & Sartory 1913) Thom & Church 1926, a common soil saprobe (Smith *et al.,* 1996; Nagelkerken *et al.,* 1997a, b; Geiser *et al.,* 1998; Harvell *et al.,* 1999; Kim *et al.,* 2000; Alker *et al.,* 2001). The fungus may be introduced to the marine environment through soil runoff (Smith *et al.,* 1996; Nagelkerken *et al.,* 1997a; Jolles *et al.,* 2002) or air-borne dust (Shinn *et al.,* 2000). Once in the water column, it can be spread to other sea fans *via* water currents (Nagelkerken *et al.,* 1997a; Jolles *et al.,* 2002). *Aspergillus* causes lesions and tissue loss in the host (Nagelkerken *et al.,* 1997b; Alker *et al.,* 2001; Petes *et al.,* 2003), which triggers a nonspecific innate immune response that includes production of antifungal and antibacterial compounds (Kim *et al.,* 2000; Petes *et al.,* 2003). Furthermore, severity of *Aspergillus* infections is highest in areas of poor water quality (*i.e.,* high nitrogen concentrations and low water clarity) (Kim and Harvell, 2002) and those with experimentally elevated nutrient levels (Bruno *et al.,* 2003).

Aspergillus sydowii infection, a stressful condition, could lead to the loss of some *Symbiodinium* strains and replacement with others more tolerant to stress. We characterized the symbiont genetic diversity within *Gorgonia ventalina,* examined *Symbiodinium* endosymbionts present in healthy and naturally infected *G. ventalina* sea fans, and experimentally tested the effects of temperature stress and bleaching on symbiont diversity.

Materials and Methods

In situ *comparison of* Symbiodinium *within healthy and diseased hosts*

To compare the symbiont taxa in healthy and diseased sea fans, *Gorgonia ventalina* was sampled at Pickles Reef (24° 59.079′ N, 80° 24.978′ W) and Eastern Sambo Reef (24° 30.329′ N, 81° 39.260′ W) in the Florida Keys. At each site, 2 cm^2 of tissue was cut from healthy and diseased colonies. From each diseased fan, a second 2 cm^2 of tissue was removed from a healthy area about 10 cm from the infection. Forty colonies (20 healthy, 20 diseased) were sampled at Pickles Reef, and 25 healthy and 15 diseased colonies were sampled at Eastern Sambo. Half of each sample (1 cm^2) was preserved in 95% ethanol for molecular analysis and half in 5% formalin for quantification of zooxanthellae densities.

Laboratory experiments

Elevated temperature. Five pieces (each 36 cm^2) were collected from each of 36 *Gorgonia ventalina* colonies on Eastern Sambo Reef and transported to the Mote Tropical Research Laboratory, Summerland Key, Florida. One fragment from each colony was processed immediately as an initial control, and three pieces were blocked across temperature treatments: 27° (control), 30° and 31.5 °C in temperature-controlled aquaria. The fifth fragment served as an *in situ* control ($n = 36$). *In situ* controls were mounted on 5-cm-tall polyvinyl chloride (PVC) racks attached to the substrate with nails and two-part marine epoxy. The racks were situated within 5 m of the collection area. Each fragment was held on the rack with a hose clamp at the fragment base. After 24 h of acclimation, all pieces from a particular colony received either control or fungal inoculations using sterile cotton wicks embedded with agar only (control) or agar and a GFP-modified strain of *Aspergillus sydowii* (treatment). The fragments were sampled 6 days post-inoculation and preserved for *Symbiodinium* density counts (5% formalin) and *Symbiodinium* identification (95% ethanol). *In situ* controls all received control inoculations to prevent spread of the pathogen on the reef.

Temperature duration. The effects of duration of temperature stress were tested with a second experiment with the following temperature treatments: 27 °C for 12 days (no temperature stress), 30.5 °C for 12 days (long-duration temperature stress), 30.5 °C for 6 days followed by 27 °C for 6 days (short-duration temperature stress). All collections and methods were the same as those in the elevated temperature experiment. Treatments were sampled 6 and 12 days post-inoculation. An additional 36-cm^2 fragment was collected from each sea fan colony sampled for the experiment to serve as an *in situ* control ($n = 36$).

Molecular analyses

Molecular determination of Symbiodinium *type. Symbiodinium* clade was determined using restriction fragment length polymorphisms (RFLP) of the 18S small subunit ribosomal DNA (18S ssrDNA). *Symbiodinium* DNA was extracted from the 1-cm^2 samples collected during surveys or from experimentally manipulated fragments. Standard methods were used (Coffroth *et al.,* 1992), with the following modifications. Due to high levels of contaminating compounds, half of the samples were extracted twice with

phenol/chloroform/isoamyl alcohol (25:24:1). Other samples were extracted one time with phenol/chloroform/isoamyl alcohol and then further purified using a polyethylene glycol precipitation (Glenn *et al.,* 1999). Some samples were transferred to a sodium-chloride-saturated, 20% dimethyl sulfoxide solution 2 weeks prior to extraction (Seutin *et al.,* 1991) to aid in the removal of contaminating compounds.

The 18S ssrDNA region was amplified with a universal primer, SS5, and a dinoflagellate-biased primer, SS3Z (Rowan and Powers, 1991a). PCR products were digested with *Taq* I restriction enzyme and compared with standards to identify *Symbiodinium* clade. Some samples did not amplify with SS5 and SS3Z primers, and for these the PCR was repeated using the universal primers SS5-SS3 (Rowan and Powers, 1991a) to verify the presence of amplifiable DNA. If a product was obtained with these primers, then the PCR was repeated using the dinoflagellate-biased primer set SS5Za+b and the universal primer SS3 (Rowan and Powers, 1991a).

To further resolve *Symbiodinium* taxa, the hypervariable region of domain V in the chloroplast 23S large subunit of ribosomal DNA (cp-23S rDNA type) was examined from a subset of the samples. These included all of the *in situ* comparison samples from each of the treatments from Pickles Reef and Eastern Sambo (healthy, diseased, and healthy portions of diseased colonies) and from most of the samples from the elevated temperature study ($n = 114$). Reaction conditions followed those of Santos *et al.* (2003a) using the primer set 23SHYPERUP and 23SHYPERDNM13, and fragments were separated on a 6.5% Long Ranger (FMC Bioproducts, Rockland, ME) gel on a LI-COR Gene ReadIR 4200 automatic DNA sequencer (LI-COR Biotechnology Divison, Lincoln, NE) (Santos *et al.,* 2003a). Base pair length of the fragments was determined using ladders and standards of known size.

A single microsatellite locus (CA 6.38) was examined to increase taxon resolution. Primers were developed using zooxanthellae isolated from the octocoral *Pseudopterogorgia elisabethae* (Santos and Coffroth, 2003). The locus was amplified and analyzed on a 6.5% Long Ranger gel on a LI-COR Gene ReadIR 4200 automatic DNA Sequencer (Santos and Coffroth, 2003). The base pair size of the microsatellite alleles was determined by comparing the samples to a ladder and standards of known size.

Sequencing aberrant 18S ribosomal DNA amplicons. To identify an aberrant *Symbiodinium* PCR product amplified by the dinoflagellate-specific 18S rDNA primer set SS5 and SS3z, four samples from the East Sambo field survey and laboratory experiment were gel-purified and sequenced bi-directionally using IRD-800 M13 primers (LI-COR Biotechnology Division, Lincoln, NE) and the SequiTherm EXCEL II DNA sequencing kit-LC (Epicentre Technologies, Madison, WI) following manufacturer's specifications (Santos *et al.,* 2003b). The sequencing products were visualized on a LI-COR Gene ReadIR 4200 automatic DNA sequencer using a 5.5% Long Ranger gel. The forward and reverse sequences were compared to remove ambiguities using AlignIR version 1.2 (LI-COR Biotechnology Division, Lincoln, NE). Consensus sequences of all four samples were identical, and one was deposited into Genbank under accession number DQ191042.

Symbiodinium *cell counts*

Symbiodinium densities were determined by homogenizing 1 cm^2 of tissue in 1 ml filtered seawater, washing twice, and resuspending in 1 ml of 5% formalin. Four independent aliquots from each sample were stained with Lugol's solution and counted on a hemacytometer at 40×. All data were checked for normality and homogeneity of variances and subsequently analyzed with ANOVA. *Symbiodinium* densities from field survey data were analyzed with a one-way ANOVA. To analyze the change in *Symbiodinium* density from initial controls to experimental pieces used in laboratory experiments a three-way nested ANOVA (fan nested within fungal treatment and temperature) was used (SAS ver. 8.0).

Results

In situ *comparison of* Symbiodinium *within healthy and diseased hosts*

Healthy and diseased sea fans from Pickles and Eastern Sambo harbored only *Symbiodinium* clade B zooxanthellae. Analysis of a hypervariable region within the large subunit of the chloroplast ribosomal gene of algal symbionts isolated from healthy colonies and from both healthy and diseased areas of diseased colonies at both sites yielded a PCR product of 184 bp (Cp-23S-rDNA type, $n = 110$). A single sample contained the 184-bp allele and a 206-bp allele. Therefore, almost all colonies harbor the same type of *Symbiodinium* within clade B, namely B184 (B1) (*sensu* Santos *et al.,* 2003a; LaJeunesse, 2002).

An aberrant band appeared in the PCR product of the 18S small subunit rDNA in many of the Eastern Sambo samples (Fig. 1). The band, 565 bp in length, occurred either alone (Fig. 1a, Lane 3) or with the normal 1800-bp product (Fig. 1a, Lane 2). Samples ($n = 122$) containing only the aberrant band, and missing the normal 1800-bp PCR product, were amplified with the SS5Za+b-SS3 dinoflagellate-biased primers. For most of the samples, this resulted in a normal (1.8-kb) product in addition to the 565-bp band. These samples were identified as *Symbiodinium* clade B when compared to clade standards. Very little DNA was recovered from samples containing just the 565-bp band ($n = 37$),

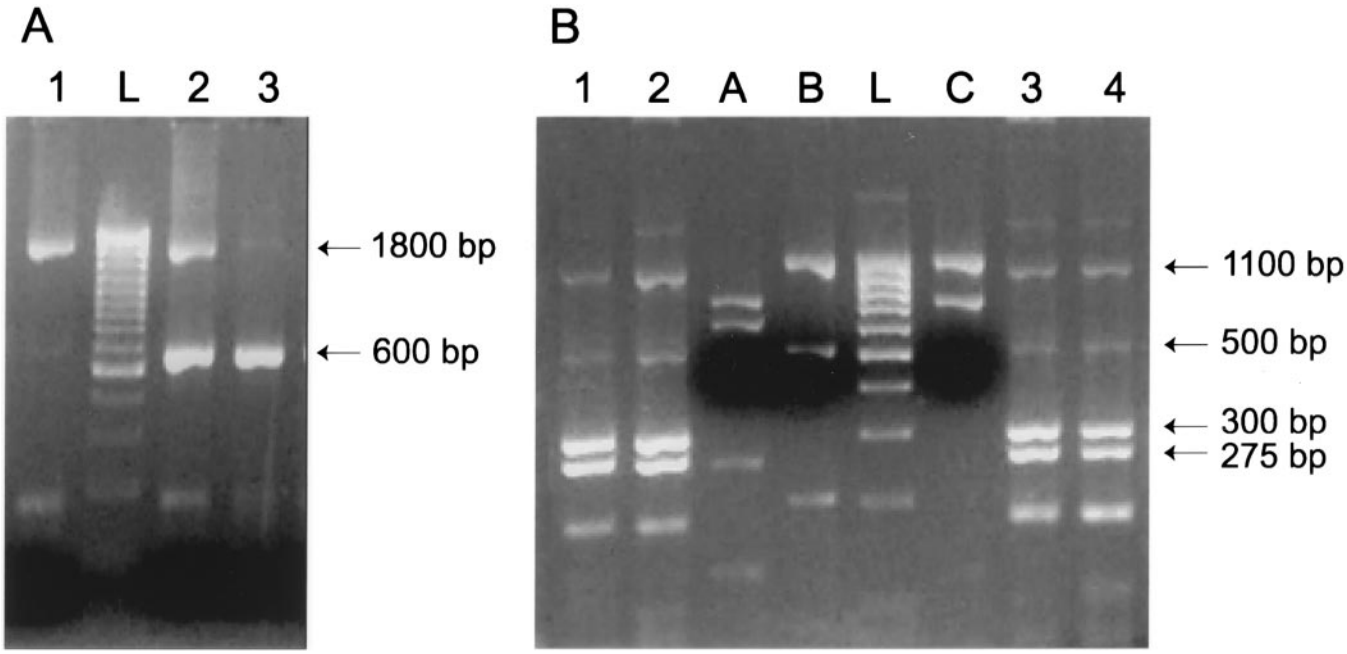

Figure 1. (A) A 2% agarose gel with PCR products amplified using the dinoflagellate-biased 18S rDNA primers SS5–SS3Z. The normal PCR product is 1800 bp long, and the aberrant product is approximately 600-bp long (Lanes 1–3). All samples collected from Eastern Sambo. L is a molecular size ladder. (B) RFLP banding pattern created with *Taq* 1 restriction enzyme of the PCR products in A (Lanes 1–4). Clade standards are labeled A, B, and C. Samples 1–4 contain an abnormal banding pattern. These samples correspond to the samples containing the aberrant PCR product. The bands in the clade standards are homologous to the samples except where noted.

which may explain our inability to amplify the functional copy of the gene. No samples from Pickles Reef contained the abnormal PCR product.

Cp-23S-rDNA type was determined for 54 of the samples from Eastern Sambo. These were samples that had either the 565-bp fragment, the normal 1.8-kb PCR product for the 18S ssrDNA, or both. All of the samples yielded a PCR product of 184 bp, typical of *Symbiodinium* in clade B.

The CA 6.38 microsatellite locus was polymorphic, with four distinct alleles (Table 1). Significant differences in allele frequencies were found between the Pickles and Eastern Sambo sites ($\chi^2 = 25.064$, df = 3, $P < 0.05$). *Symbiodinium* isolated from healthy (H:D) and diseased areas (D:D) of the same colony contained the same allele in all cases ($n = 35$), confirming that the symbiont taxon was ubiquitous throughout the diseased fan. At Eastern Sambo, four alleles were detected among the colonies sampled, and allele frequencies were not significantly different between healthy and diseased colonies ($\chi^2 = 6.975$, df = 3, $P > 0.05$). In contrast, there was a significant difference in the frequency of the alleles in *Symbiodinium* isolated from healthy and diseased colonies at Pickles Reef: diseased colonies were more likely to harbor symbionts with the 108-bp allele ($\chi^2 = 8.179$, df = 1, $P < 0.05$).

Laboratory experiments

In both laboratory experiments (elevated temperature and temperature duration) all *Symbiodinium* yielded an RFLP pattern characteristic of *Symbiodinium* clade B. The cp-23S rDNA type of a subset of these samples was determined and all were B184 ($n = 114$). Fifty-seven percent of the 70

Table 1

Allelic variation at microsatellite locus CA6.38 among Symbiodinium *isolated from* Gorgonia ventalina *collected at Pickles and Eastern Sambo Reefs*

		Allele size (bp)			
Reef	Fan type	104	106	108	110
East Sambo					
	H	0	8	15	0
	D	1	1	12	1
Pickles					
	H	0	18	0	0
	D	0	12	7	0

The number of samples with each allele size for samples from healthy (H) fans (not visibly infected with the *Aspergillus* fungi) and samples from diseased (D) fans (those inoculated with the fungi) are shown.

colonies harbored *Symbiodinium* with the 565-bp fragment, but presence of the extra band was not correlated with treatment. Furthermore, in the laboratory experiment, no differences in allele frequency were detected at the CA 6.38 microsatellite locus in *Symbiodinium* isolated from the initial healthy tissue *versus* tissue blocked across all temperatures and treatments.

Symbiodinium *cell counts*

Symbiodinium densities were quantified for samples from Pickles Reef. Healthy areas of healthy fans (H:H) contained more zooxanthellae than both healthy (H:D) and diseased (D:D) areas of diseased fans (H:H *vs.* H:D—Student's $t = -2.48$, df = 57, $P = 0.0418$; H:H *vs.* D:D—$t = -7.35$, df = 57, $P = < 0.0001$). Healthy areas of diseased fans contained more zooxanthellae than diseased areas of the same fans (H:D *vs.* D:D—$t = -4.87$, df = 57, $P = < 0.0001$) (Table 2). Although the data were not normal (Shipiro-Wilk, $W = 0.987$, $P = 0.0008$; Kolmogorov-Smirnov, $D = 0.047$, $P = 0.0213$) the variances were homoscedastic (Levene's, $P = 0.4193$). A three-way nested ANOVA (fan nested within fungal treatment and temperature) showed a significant difference between *Symbiodinium* density, temperature treatments, and inoculations ($F = 4.29$, $P = 0.0143$).

Discussion

Symbiodinium clade B is the predominant symbiont taxa in adult *Gorgonia ventalina* (LaJeunesse, 2002; Goulet and Coffroth, 2004). All *Symbiodinium* isolated from sea fans sampled at Pickles Reef and Eastern Sambo were within clade B, regardless of sea fan health or sample site. In the laboratory experiments, all the sea fans harbored *Symbiodinium* clade B throughout the experiment, regardless of temperature treatment or length of exposure, indicating that *Symbiodinium* clade does not change when the sea fan host is infected with the *Aspergillus* fungus. All samples except one harbored the B184 type of the hypervariable region of domain V of the chloroplast 23S large subunit rDNA. This is the most common cp-23S-rDNA type found in Caribbean gorgonians (Santos *et al.,* 2003a) and is equivalent to ITS-type B1 (based on the internal transcribed spacer region of the nuclear rDNA), as described in LaJeunesse (2002).

Table 2

*Density of zooxanthellae (*Symbiodinium*) in samples from healthy and diseased (infected with* Aspergillus *fungi) specimens of* Gorgonia ventalina

Sample type	Mean $\times 10^5$ cells cm^{-2} ($\pm$ std. dev.)
H:H	5.27 (1.46)
H:D	4.45 (1.64)
D:D	2.83 (2.42)

Cell counts are given as the average of 20 samples with the standard deviations. (H:H) are healthy fans, (D:D) are infected areas of diseased fans, and (H:D) samples are taken from diseased fans peripheral to the infection site.

In the laboratory studies and at Eastern Sambo, allele frequencies at the microsatellite locus did not correlate with infection status. However, in the samples collected at Pickles Reef, the 108-bp allele was associated with infected *G. ventalina* more frequently than expected. Given that this is a single microsatellite locus, which is a putative neutral marker, it is unclear whether this finding is significant to the etiology of the disease. This allele was found abundantly in healthy fans from East Sambo. Therefore, it seems unlikely that the correlation between algae with this allele and infection status has any biological relevance. Significant differences in allele frequencies were also detected between sites. Larger sample sizes using more loci are currently being analyzed to determine the population structure of the algal symbionts harbored by *G. ventalina* in the Florida Keys. Previous work by Santos *et al.* (2003) also detected population structure among the symbionts of another member of the Gorgoniidae, *Pseudopterogorgia elisabethae,* on Bahamian reefs, with each reef harboring a unique *Symbiodinium* genotype.

Fewer algal symbionts were present in diseased tissue than in healthy tissue, although these tissues always harbored the same symbiont type. The loss of algal symbionts is highly correlated with infection with *Aspergillus sydowii.* Symbiont density is higher in samples from tissue peripheral to the infection site than in samples from the diseased tissue itself, but lower than in tissue sampled from healthy fans. Loss of algal symbionts from diseased fans could be considered bleaching caused by infection with the fungal pathogen. However, it is also possible that reduced zooxanthellar density is due to a reduction in the number of polyps in diseased tissue (Kim and Harvell, 2002).

Among the *Symbiodinium* isolated from Eastern Sambo colonies in the field, 64.4% ($n = 104$) of the samples produced a 565-bp fragment when amplified with the dinoflagellate-biased 18S-rDNA primers. The presence of a smaller amplicon in addition to the expected full-length rDNA following PCR has been reported in a number of free-living and symbiotic dinoflagellates (Santos *et al.,* 2003b). Blast searches of Genbank have found that the 565-bp fragments are most similar to clade B 18S small subunit rDNA (M88509 submitted to Genbank by Rowan and Powers, 1992; and AF238257 submitted to Genbank by Toller *et al.,* 2001). In the case reported here, the truncated copy is created by a deletion of approximately 1200 base pairs, similar to that described in Santos *et al.* (2003b). Some samples, when amplified with the primers SS5-SS3Z, contained only the shortened length of DNA. If a deletion

generated the short segment of DNA (*i.e.,* a pseudogene), a larger, functional copy of the gene must also be present. When another dinoflagellate-biased primer set, SS5Za+b-SS3, was used, the normal-sized DNA fragment was recovered. This indicates that the normal, functional copy of the 18S rDNA gene is present in the zooxanthellae from these samples and, because the samples also amplified with the universal primers SS5-SS3 (Rowan and Powers, 1991a), it suggests that a mutation may be present in the flanking region of the 18S rDNA near the binding site of the SS3Z primer.

As in the case of free-living dinoflagellates and other symbiotic dinoflagellates, the presence or absence of the putative "pseudogene" can be used to differentiate *Symbiodinium* types present in different study sites and experimental treatments (Santos *et al.,* 2003b). Only *G. ventalina* collected from Eastern Sambo harbored *Symbiodinium* containing the aberrant PCR product. No samples from Pickles Reef or samples from *Gorgonia ventalina* in previous studies contained this aberrant copy of the 18S rDNA (Goulet, 1999; Goulet and Coffroth, 2004). The aberrant PCR product occurred—regardless of treatment and temperature—in 57.1% of 70 samples used in the laboratory experiment, and in 64.4% of all 104 colonies collected in Eastern Sambo. The presence or absence of the putative pseudogene can be used to distinguish taxa of *Symbiodinium* among sites, but no correlation occurs between the pseudogene and the infection status of the sea fan.

The *Gorgonia ventalina* symbiosis does not respond to stresses such as increased temperatures or fungal infection through symbiont shuffling or switching (*sensu* Baker, 2003). Although changes in symbiont type are correlated with increased temperature and some coral diseases (*i.e.,* Rowan *et al.,* 1997; Toller *et al.,* 2001), symbiont change is not universal and appears to depend on the host species. Further studies will be necessary to understand the interaction of the host and symbiont under these stresses. This work further supports a growing number of studies that have demonstrated that many symbioses (perhaps the majority, see references in Goulet and Coffroth, 2003b) are extraordinarily stable and do not change seasonally or under different environmental conditions (Billinghurst *et al.,* 1997; Diekmann *et al.,* 2002; Goulet and Coffroth, 2003a, b; Rodriguez-Lanetty *et al.,* 2003; Thornhill *et al.,* in press.). Future work must focus not only on how symbioses might change, but on how less flexible symbioses survive in the face of disease and environmental change.

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