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Genetic diversity of potentially free-living *Symbiodinium* in the Xisha Islands, South China Sea: Implications for the resilience of coral reefs

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Free-living Symbiodinium have been the focus of considerable attention among coral reef researchers because they represent a source of symbionts for both recently recruited corals and adult corals that are subject to environmental stress (e.g. climate change). We evaluated the genetic diversity of free-living Symbiodinium in seawater collected from the Xisha Islands in the South China Sea using sequence analysis of the hypervariable region of the chloroplast 23S. We sequenced a total of 267 cloned gene fragments representing Symbiodinium in clades A, B, C, D, F, and G, of which 34 were distinct types. The diversity of free-living Symbiodinium types was high at all sites. The majority of Symbiodinium sequences belonged to clade C. There was no significant difference in Symbiodinium sequence types among sites or depths. Our results highlight the high diversity of free-living Symbiodinium pools in the coral reef environment and provide a foundation for future studies of the spatiotemporal distribution of Symbiodinium. Such information is critical to understand and predict the influence of symbiotic dinoflagellates on the adaptation of their host to environmental changes.

Keywords: chloroplast 23S hypervariable region, dinoflagellate, zooxanthellae

Introduction

The high productivity and diversity of coral reef ecosystems is largely dependent on endosymbiotic dinoflagellates of the genus *Symbiodinium* (also called zooxanthellae), which provide the host corals with photosynthetic products (Hoegh-Guldberg, 1999). Coral bleaching, the breakdown of the symbiosis between corals and their symbiotic

dinoflagellate *Symbiodinium*, occurs in response to a range of environmental stressors (Buddemeier and Fautin, 1993; Baker, 2003). Bleaching events may cause coral death and reef degradation when environmental stress is severe, repeated, or prolonged (Hoegh-Guldberg, 1999). However, the adaptive bleaching hypothesis suggests that coral bleaching events also represent an opportunity for scleractinian corals to adapt to the changing environmental

conditions by switching (uptake of a new symbiont from the environment) or rearranging (increased growth of a background resident population) their *Symbiodinium* communities (Buddemeier and Fautin, 1993; Baker, 2003; Rowan, 2004).

Corals can initially acquire symbiotic algae in two ways, vertical transmission (coral eggs inherit the symbionts maternally) or horizontal transmission (symbionts are obtained from the environment) (Baker, 2003). Most cnidarian host species acquire *Symbiodinium* from the environment, suggesting that free-living *Symbiodinium* are critical to the proper functioning of coral reef ecosystems. Free-living populations may serve as a reservoir of genetic types that have distinct ecological functions from which corals can select (Coffroth et al., 2006; Manning and Gates, 2008). Aposymbiotic cnidarians are able to acquire multiple types of *Symbiodinium*, often exceeding the symbiotic diversity found in their adult hosts (Thornhill et al., 2006). In addition, recent studies have shown that corals can change the cell density or type of their symbionts to adapt to changes in the natural environment or to experimental conditions (Lewis and Coffroth, 2004; Berkelmans and van Oppen, 2006; Coffroth et al., 2006). Taken together, these observations suggest that free-living *Symbiodinium* cells are abundant in the coral reef environment and play an important role in the adaptation of reef corals to changes in the environment.

The genus *Symbiodinium* consists of 11 named species (Baker, 2003). There has been an extensive phylogenetic analysis of the genetic diversity of symbiotic *Symbiodinium* based on analyses of nuclear ribosomal DNA and chloroplast rDNA (23S) (Rowan, 1991; Santos et al., 2002; LaJeunesse et al., 2004). Currently, nine genetic clades (A through I) have been proposed (Baker, 2003; Pochon and Gates, 2010). Each clade contains a number of closely related “types” that are found in distinctive hosts and geographical niches (LaJeunesse et al., 2004; Pochon et al., 2006). However, our understanding of *Symbiodinium* that occur outside a host (referred to as “free-living”) is relatively poor in comparison with those that occur in symbioses (Pochon et al., 2010). Recently, a number of researchers have investigated the diversity of free-living *Symbiodinium* in the seawater column, benthic sands, macroalgae, and fish feces in the coral reef environment using culture and genetic techniques (Carlos et al., 1999; Gou et al., 2003; Coffroth et al., 2006; Hirose et al., 2008; Littman

et al., 2008; Manning and Gates, 2008; Porto et al., 2008; Pochon et al., 2010; Venera-Ponton et al., 2010).

The coral reefs of the South China Sea (SCS) include the fringing reefs found in southern China’s coastal seawaters and those associated with islands and atolls in the SCS. Prior studies have shown that *Symbiodinium* clades C and D form symbioses with the scleractinian corals in the Xisha Islands and on Zhubi Reef in the Nansha Islands, suggesting that these corals possess higher sensitivity to thermal stress (Huang et al., 2006; Dong et al., 2009). However, little is known about potentially symbiotic *Symbiodinium* species that occur outside of coral hosts (free-living) in this region. To address this, we evaluated the distribution and diversity of free-living *Symbiodinium* in the seawater column at remote coral reef locations within the SCS using culture-independent cloning. Our results provide insight into the role of free-living *Symbiodinium* in the adaptation of corals and the resilience of reef ecosystems.

Materials and Methods

Study site and sampling

North Reef and Xishazhou Island are located north of the Xisha Islands in the SCS, 247.4 km away from Hainan Island (Figure 1). North Reef is an elongated ring shaped reef with a semi-enclosed lagoon. We compared the diversity of free-living *Symbiodinium* at three sites, the inner and outer reefs of North Reef (both near-surface and near-bottom) and the reef flat on Xishazhou Island (control site). All sampling was conducted in May 2009 (Table 1). During each sampling period, we collected seawater samples at the target depth using sterile 5 l plastic bottles. Each seawater sample (2–5 l depending on seawater quality) was filtered through a 5 mm nucleopore track etch membrane filter (Whatman) using a hand-held vacuum pump. The filter retaining the plankton cells was placed in 0.5 ml DNA buffer (0.1 M EDTA, 1% sodium dodecyl sulfate) in a microcentrifuge tube.

DNA extraction, polymerase chain reaction and sequencing

We added proteinase K (final concentration of 200 $\mu\text{g ml}^{-1}$) to each sample prior to DNA extraction. The samples were then centrifuged at

Table 1. Summary of sampling stations, times, depths, and total filtered volume of seawater samples collected in Xisha Islands for the detection of *Symbiodinium* in the water column. NR: North Reef, XSZ: Xishazhou Island, B: near bottom seawater, S: near surface seawater.

Location	Sample	Station	Time (h)	Depth (m)	Filtered volume (l)
North Reef	NR-1S	1	12:00	0.3	3.8
	NR-1B	1	12:00	5	4.3
	NR-2S	2	12:00	0.3	5
	NR-2B	2	12:00	10	5
	NR-3S	3	12:00	0.3	4.95
	NR-3B	3	12:00	15	5
	NR-4S	4	12:00	0.3	4.6
	NR-4B	4	13:00	5	4.65
	NR-5S	5	13:00	0.3	3.9
	NR-5B	5	13:00	10	3.9
	NR-6S	6	13:00	0.3	3.8
	NR-6B	6	13:30	15	4
Xishazhou Island	XSZ-7S	7	12:00	0.3	5
	XSZ-7B	7	12:00	10	5

3,000 × g for 20 min at 4°C and the supernatant was carefully removed. The pellets were resuspended in 100 μl DNA extraction buffer and incubated at 55°C for 16 h. We isolated the DNA by adding 17 μl each of 5 M NaCl and 10% cetyltrimethylammonium bromide in 0.7 M NaCl and incubating at 55°C for 10 min, followed by one chloroform extraction and one phenol-chloroform extraction. Last, the DNA solution was further purified by being eluted twice through a DNA Clean and Concentrator column (Zymo Research, Canada) using 30 μl distilled and deionized water. The purified DNA was stored at –20°C until the samples could be analyzed using polymerase chain reaction (PCR).

We evaluated the diversity of *Symbiodinium* genomic DNA using the hypervariable region of domain V in the large subunit of the chloroplast ribosomal array (cp23S-HVR). The cp23S-HVR region was amplified using the forward primer “23SHYPERUP” (5'-TCAGTACAAA TAATATGCTG -3') (Santos et al., 2002) and the reverse primer “23HYPERDN” (5'-TTATCGCCCCAATTAACAGT -3'). The PCR thermal cycle consisted of an initial denaturation for 2 min at 94°C followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were purified using a DNA cleanup and concentration column and cloned into PMG-T Vectors (Tiangen Biotech, China). Colonies

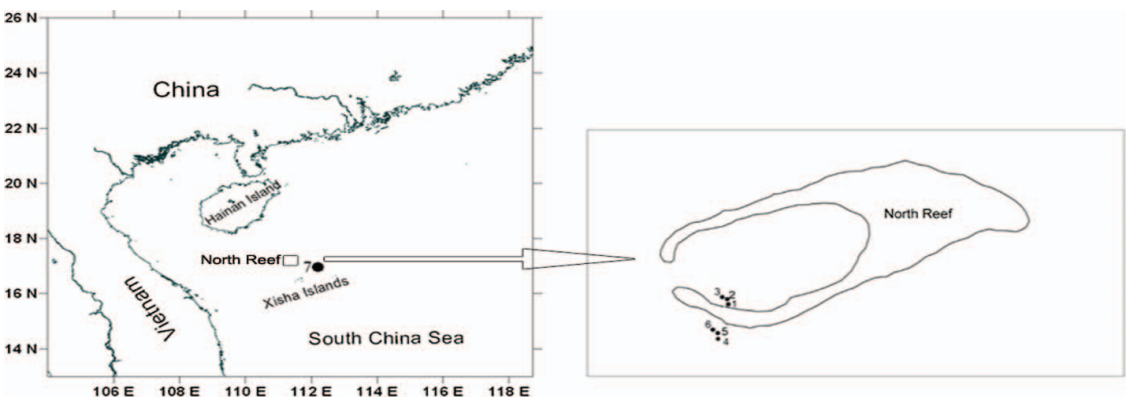


Figure 1. Study sites in the Xisha Islands, South China Sea. The rectangle and dot represent sampling locations and stations, respectively. (Color figure available online.)

carrying a target gene fragment were randomly selected and approximately 20 clones from each seawater sample were sequenced using an ABI 3730 automated DNA sequencer (Applied Biosystems, USA). A total of 267 clones were sequenced.

Phylogenetic analyses

The sequences were aligned using Clustal W (Thompson et al., 1994). Cladal alignments consisted of one representative from each unique sequence type. The use of PCR has been associated with overestimation of sequence diversity. Therefore, we examined the alignments of all *Symbiodinium* sequences and identified those that were recovered from two or more clone libraries (representing independent seawater samples) or that differed from a published sequence by three or more bp substitutions (Pochon et al., 2010). New sequence types that occurred only once and that differed from published sequences by only one or two substitutions were treated as a match to their top BLAST hit. Phylogenetic analyses were conducted in MEGA v4.0 (Tamura et al., 2007) using the maximum-parsimony and neighbor-joining method. We performed bootstrap testing and reconstruction 500 times to confirm the reliability of the phylogenetic tree.

Statistical analyses

We used the Bray–Curtis coefficient of similarity (S) in Primer v6.0 (Clarke and Warwick, 2001) to evaluate the spatial distribution of free-living *Symbiodinium* 23S-HVR types recovered from the 14 seawater samples. The abundance of

Symbiodinium 23S-HVR sequences were grouped by sites and environment (near-surface seawater, near-bottom seawater), and standardized to relative frequencies within each grouping. The data were square root transformed to give more weight to low frequency sequences. We compared *Symbiodinium* 23S-HVR sequences within each grouping using the Bray–Curtis coefficient of similarity. We used a one-way analysis of similarity (ANOSIM) to compare the *Symbiodinium* cp23S-HVR sequence types between sites and depths (Primer v6.0).

Results

We successfully amplified and sequenced the *Symbiodinium* spp. cp23S-HVR region from all 14 seawater samples. A total of 267 cp23S-HVR sequences were obtained from the two regions. Among these sequences, 34 distinct sequence types were detected, which fell into six cladal groups. *Symbiodinium* clade C was the dominant type of free-living *Symbiodinium* (61.4% of 267), the remainder consisting of clades A, B, D, F, and G (Figure 2). Our phylogenetic analyses revealed that all the *Symbiodinium* isolated from the seawater belonged to differing clades (Figure 3). Clade C contained closely related sequences to sister clades B, D, F, and G, which in turn were sister to clade A. However, the overall grouping of clades C and D and the novel sequences, despite forming a monophyly, was not well supported (bootstrap values $\leq 50\%$). Clades A, B, F, and G formed a well-supported (bootstrap values $> 70\%$) monophyly.

We used the Bray–Curtis similarity coefficient and ANOSIM to evaluate the spatial distribution of the *Symbiodinium* 23S-HVR sequence types at the

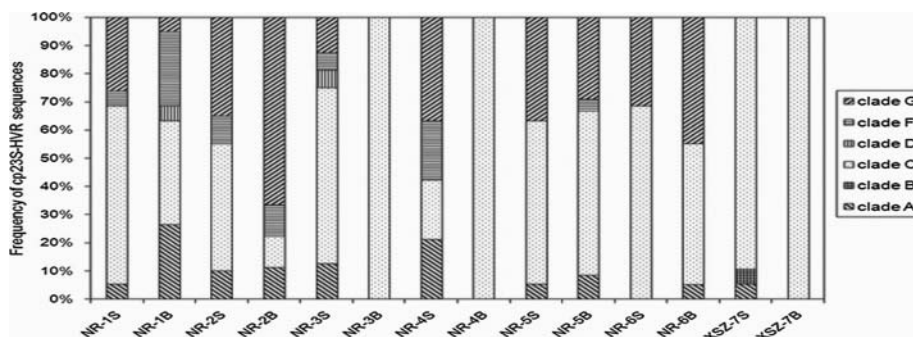


Figure 2. Frequency of *Symbiodinium* sequences at the clade level in samples of seawater collected at North Reef and Xishazhou Island, Xisha Islands. Sample names are given and correspond to locations shown in Table 1.

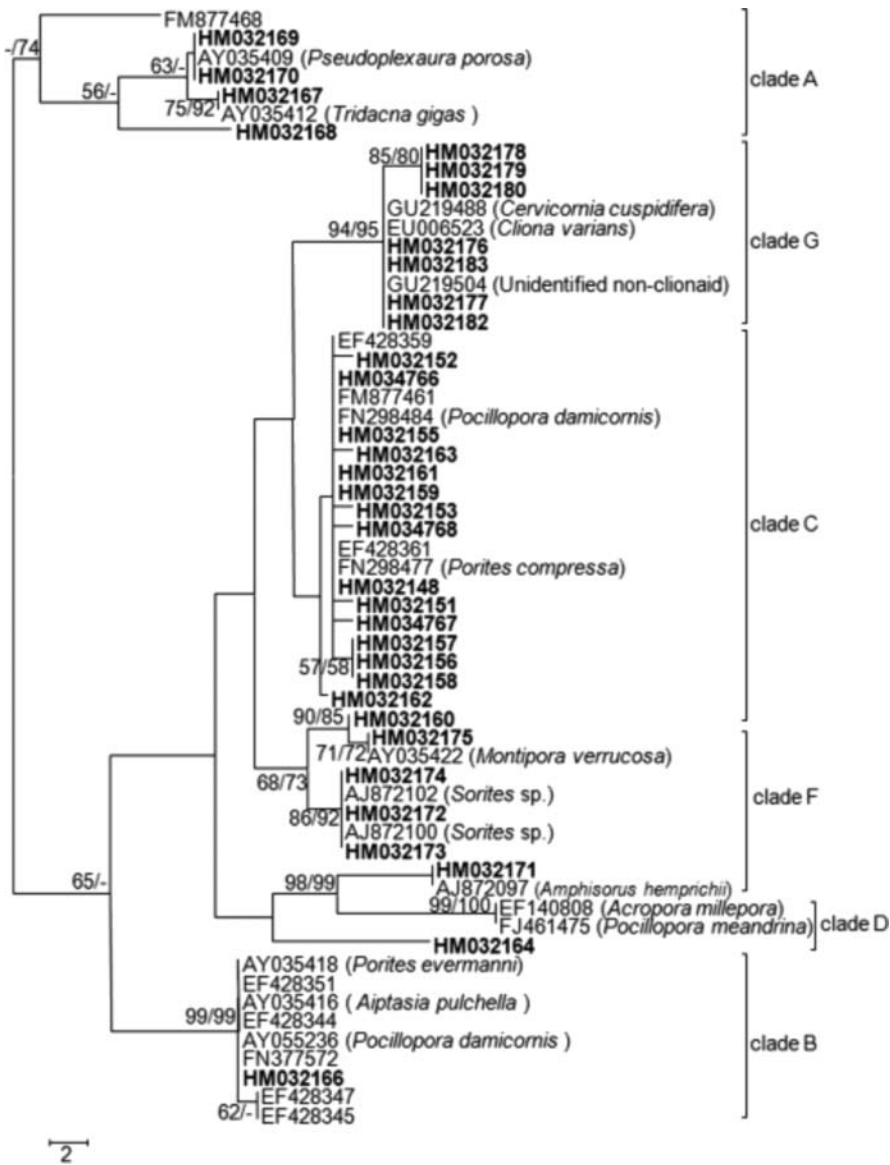


Figure 3. Phylogenetic reconstruction was inferred from *Symbiodinium* cp23S-HVR sequences based on the methods of maximum parsimony (MP) and neighbor-joining (NJ). Sequence types found in this study (highlighted in black) as well as previously published types are indicated by their GenBank accession number. Names in parenthesis indicate their host origin. MP/NJ bootstrap values are indicated for internal nodes with probabilities above 50%.

cladal level (A, B, C, D, F and G). There was no significant difference among *Symbiodinium* types between the samples collected at the surface or the bottom (ANOSIM: $R = 0.01$, $P = 0.318$). Similarly, there was no difference in types among the different sites, including between inner and outer sections of North Reef (Figure 4).

Discussion

Molecular diversity of free-living Symbiodinium in seawater

Our results confirm that the cp23S-HVR primer set is highly specific to free-living *Symbiodinium*

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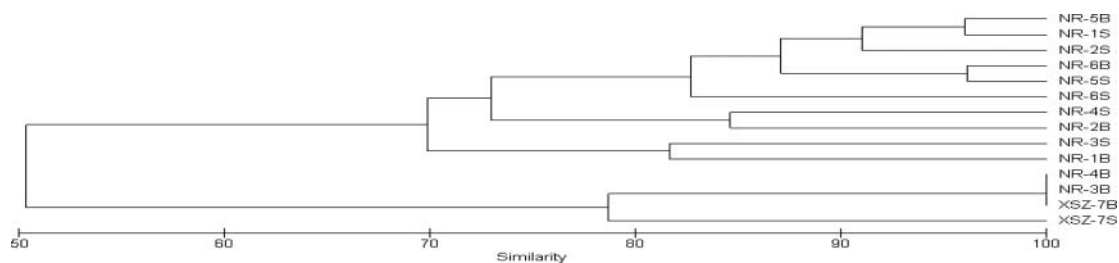


Figure 4. Dendrograms of *Symbiodinium* community similarity using group-average clustering from Bray-Curtis similarities for samples in North Reef and Xishazhou Island, Xisha Islands. Sample names are shown adjacent to the branch tips and correspond to stations shown in Table 1.

(Manning and Gates, 2008; Pochon et al., 2010). We isolated six clades of *Symbiodinium* (clades A–D, F, and G) from seawater in the Xisha Islands. To our knowledge, this is the first time clades A, B, F, and G have been found in the Xisha Islands. Our results suggest that *Symbiodinium* clade C is the dominant free-living *Symbiodinium* type, which is consistent with prior studies showing that clade C is the dominant type in scleractinian corals in the SCS (Huang et al., 2006; Dong et al., 2009). Similarly, Manning and Gates (2008) reported that the dominant types of *Symbiodinium* associated with scleractinian corals and in the water column were identical among both Caribbean and Pacific reefs. The recovery of several distinct sequence variants within *Symbiodinium* clade C was consistent with previous observations of relatively high diversity within this clade, as compared with other clades (LaJeunesse et al., 2004).

Baker (2003) reported that *Symbiodinium* of clades A–D formed a symbiosis with a wide range of invertebrates and were the predominant symbionts of scleractinians. Clade B was particularly common in the Western Atlantic. These clades have been found both in seawater and sand sediment from the Pacific and the Caribbean (Manning and Gates, 2008; Pochon et al., 2010). Clades C and D have been associated with foraminifera, while clades F and G have been found (rarely) in scleractinian corals (Pochon and Pawłowski, 2006), but commonly in foraminifera (Baker, 2003; Coffroth and Santos, 2005; Stat et al., 2006). *Symbiodinium* in clades F and G have rarely been detected in seawater or sand sediment (Hirose et al., 2008; Manning and Gates, 2008; Porto et al., 2008; Pochon et al., 2010). We did not find members of clades E and H, but clade H has been isolated from seawater collected in Kaneohe Bay (Manning and Gates, 2008). *Symbiodinium* clade E has never been

directly detected in the coral reef environment and has only been identified in two cultured *Symbiodinium*-like isolates from seawater collected in two temperate locations (Jiaozhou Bay, P.R. China, and Wellington Harbor, New Zealand), where symbiotic hosts were not prevalent, suggesting this type is entirely free-living (Santos et al. 2002; Gou et al., 2003; Pochon et al., 2006).

We documented a relatively high level of genetic diversity among *Symbiodinium* collected in the current study. Furthermore, we may have isolated additional *Symbiodinium* types by sequencing a greater number of clones per library. Interestingly, Coffroth et al. (2006) demonstrated that not all *Symbiodinium* strains isolated from environmental samples were capable of establishing symbiosis with recently settled polyps of aposymbiotic cnidarians. In addition, corals are able to continuously release healthy zooxanthellae, a post-mitotic control measure for regulating density within the coral tissue (Jones and Yellowlees, 1997). Thus, it is possible that some *Symbiodinium* found in the current study were in hospite, associated with soritid foraminifera or macroscopic ciliates or from symbiotic larvae. It is unclear whether the free-living *Symbiodinium* we detected had the potential to become symbiotic, an interesting topic of future research. Similarly, the diversity of symbiotic algae associated with soft corals and giant clams in this region is unclear, but deserves attention.

Given the typical mobility and diurnal migration of free-living *Symbiodinium*, their distribution is likely influenced by complex interactions between a range of physical and biological factors (e.g. seawater flow, temperature, and light intensity). These patterns are also likely to vary both spatially and temporally (Fitt and Trench, 1983; Yacobovitch et al., 2004). Previous studies have suggested that there is limited dispersal of *Symbiodinium*,

despite the high levels of population genetic structure (Howells et al., 2009; Thornhill et al., 2009). Even given the variability in free-living *Symbiodinium* diversity among sites, we found no evidence of partitioning of types among sites or depths. However, the variability in free-living *Symbiodinium* distribution remains unknown, as we did not measure cell density at each site.

Implications of free-living *Symbiodinium* for coral adaptation and resilience

The majority of spawning juvenile corals assimilate symbiotic algae from the surrounding seawater during their planktonic phase (Baker, 2003). Moreover, coral larvae have the potential to acquire additional symbiotic dinoflagellates, a process that may increase larval survivorship and thus play an important role in larval dispersal (van Oppen et al., 2001). Furthermore, it has been proposed that corals can adapt to changing environment conditions by the switching or rearrangement of less beneficial symbiont types for better adapted symbionts from the exogenous environment under stressful circumstances. (Buddemeier and Fautin, 1993; Baker et al., 2004; Lewis and Coffroth, 2004; Little et al., 2004). Indeed, some corals have recovered from bleaching by repopulation from stress-tolerant in hospite symbionts that remained within the host during bleaching-induced stress (Berkelmans and van Oppen, 2006; Mieog et al., 2007; Jones et al., 2008; LaJeunesse et al., 2009). More recently, Coffroth et al. (2010) found that the coral *Porites divaricata* had the ability to assimilate exogenous symbionts but could not maintain a stable symbiosis during bleaching. Thus, it is reasonable to assume that a coral host will revert back to the original symbionts in the absence of stressful conditions. This is likely due, in part, to the competitive advantage the original symbionts have under normal conditions, as they are likely better adapted to the local environment in which the host is established (Little et al., 2004; LaJeunesse et al., 2009, 2010). Taken together, these observations suggest that free-living *Symbiodinium* may be crucially important to the recovery, adaptation, and resilience of host invertebrates.

Conclusions

In light of climate change and associated bleaching, it is important that we gain a better under-

standing of the distribution and abundance of free-living *Symbiodinium* dinoflagellates. In particular, the mechanisms of host recruitment and their role in coral reef resilience deserve attention. Similarly, it is unclear whether free-living *Symbiodinium* can form a symbiosis with hosts such as giant clams, anemones, sponges, and foraminifera, or with corals that are recovering from bleaching in the present study. The coral reefs in the SCS, as with those in other regions of the world, are facing degradation due to the effects of climate change and more direct human activities. Our results provide insight into the symbionts that are available for host uptake and the formation of new holobionts that can adapt to climate changes. Continuing this line of research may help predict how corals will respond to the changing climate and how to better protect these reefs.

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