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Marine Biology Research

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/smar20>

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To cite this article: Hui Huang, Guowei Zhou, Jianhui Yang, Sheng Liu, Feng You & Xinming Lei (2013): Diversity of free-living and symbiotic Symbiodinium in the coral reefs of Sanya, South China Sea, *Marine Biology Research*, 9:2, 117-128

To link to this article: <http://dx.doi.org/10.1080/17451000.2012.708045>

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ORIGINAL ARTICLE

Diversity of free-living and symbiotic *Symbiodinium* in the coral reefs of Sanya, South China Sea

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Abstract

Unicellular photosynthetic dinoflagellates from the genus *Symbiodinium* are found either free-living or *in hospite* with a wide variety of marine invertebrate hosts including scleractinian corals. The linkages between free-living and endosymbiotic *Symbiodinium* remain largely unstudied and constitute a major gap in knowledge. In this study, the diversity of free-living *Symbiodinium* spp. and those associated with scleractinian corals from coral reefs in Sanya CRNR, South China Sea were determined by *Symbiodinium*-specific primers for the hypervariable region of the chloroplast 23S domain V (cp23S-HVR). The results illustrated that the free-living *Symbiodinium* spp. were highly spatially heterogeneous with high diversity related to clades A, B, C, D, F, G and H. However, the reef corals mainly hosted only one symbiont type of either clades C or D, and few species could harbour both concurrently. Surprisingly, *Symbiodinium* spp. in scleractinian corals were totally different from free-living forms in adjacent waters. These results suggest that the corals did not recruit *Symbiodinium* spp. from adjacent waters and the exchange of *Symbiodinium* spp. between corals and waters is limited in this region.

Key words: Coral, free-living *Symbiodinium*, diversity, resilience

Introduction

It is well known that coral reef ecosystems have extremely high biodiversity and productivity despite their occurrence in oligotrophic waters. Dinoflagellates in the genus *Symbiodinium* (also called zooxanthellae) are found in association with reef-dwellers including corals, molluscs, sponges and protists, and play important functional roles in their hosts for organic matter synthesis, vital nutrient cycling and calcification (Hoegh-Guldberg 1999; Baker 2003). *Symbiodinium* form an obligate symbiosis with most of their hosts, and dissociation of the symbiosis under persistent stress conditions (loss of symbionts from the host tissues leading to a reduction in colour termed bleaching) can lead to partial or complete mortality of the host colony (Glynn 1993; Hughes et al. 2003).

To date, the *Symbiodinium* spp. have been designated to at least nine clades (A–I) based on analyses

of nuclear ribosomal DNA (Rowan & Powers 1991; LaJeunesse 2005; Pochon & Gates 2010) and the chloroplast 23S rDNA (Santos et al. 2002). Each clade contains numerous closely related ‘subclades’ or ‘types’ in distinctive hosts or geographical niches (LaJeunesse et al. 2004; Pochon et al. 2006). Six of them (A–D, F and G) are known to form associations with scleractinian corals (Baker 2003; Pochon et al. 2006). Additionally, endosymbionts showed distinct physiological and ecological functions strongly related to different holobionts (coral host plus algal symbionts) (Baker 2003; Sampayo et al. 2008; Stat et al. 2008). For example, Glynn et al. (2001) reported that the coral *Pocillopora* containing clade D *Symbiodinium* did not bleach, whereas colonies that contained clade C *Symbiodinium* bleached severely in Panama waters during the 1997–98 bleaching event. Most recently, increasing reports documented that *Symbiodinium* spp. could

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Published in collaboration with the University of Bergen and the Institute of Marine Research, Norway, and the Marine Biological Laboratory, University of Copenhagen, Denmark

live outside their hosts (referred to as ‘free-living’) in the water column, benthic sands, macroalgal beds and fish faeces. Such free-living *Symbiodinium* spp. could be reservoirs for cnidarian hosts, and may be of particular importance if symbionts are lost during periods of stress (Carlos et al. 1999; Gou et al. 2003; Coffroth et al. 2006; Hirose et al. 2008; Manning & Gates 2008; Porto et al. 2008; Pochon et al. 2010; Takabayashi et al. 2011).

Generally, corals have two modes to acquire symbionts at the onset of symbiosis during the early life stages: vertical inheritance (from the parental colony to the eggs) and horizontal acquisition (uptake from the environment by aposymbiotic eggs or larvae). Most cnidarians must acquire *Symbiodinium* spp. from adjacent waters in each generation, indicating that free-living *Symbiodinium* spp. should play an important role for coral recruitment and resilience in coral reef ecosystems (Coffroth et al. 2006, 2010; Rodriguez-Lanetty et al. 2006; Adams et al. 2009; Baird et al. 2009; Pochon et al. 2010). Jones et al. (2008) reported that 71% of colonies of the coral *Acropora millepora* (Ehrenberg, 1834) could replace their symbionts for more heat-tolerant types to adapt to increased temperature during a bleaching event. This result indicated that the potential ability of corals to respond to future climate change, and the exchange of symbionts (via switching or shuffling) could result in adaptation of the holobiont to a changed environment as proposed by the theory of Adaptive Bleaching Hypothesis (ABH) (Buddemeier & Fautin 1993; Baker 2003; Berkelmans & van Oppen 2006). Free-living *Symbiodinium* spp. populations are the essential foundation of adaptation/acclimation of symbiosis, and serve as a reservoir of symbiont with distinct physiological and ecological functions (Coffroth et al. 2006; Manning & Gates 2008; Adams et al. 2009).

Although there is great understanding of diversity of *Symbiodinium* spp. associated with their host invertebrates, little attention has been focused on the diversity and distribution of free-living as opposed to symbiotic *Symbiodinium* spp. Sanya National Coral Reef Nature Reserve (CRNR) in the South China Sea (SCS) located near Sanya City, Hainan Island, was established in 1990. The coral reefs in the CRNR are in decline as is the case worldwide due to the effects of global warming, ocean acidification, coral disease, and anthropogenic activities (Hughes et al. 2003; Huang 2005; Hoegh-Guldberg et al. 2007), and the rate of decay is likely to accelerate (IPCC 2007). The present study was conducted using molecular methods to directly investigate the diversity of both free-living and endosymbiotic *Symbiodinium* spp. in water samples and 36 scleractinian coral species in the CRNR. These data are essential to understand the role of free-living *Symbiodinium* spp. in coral reefs and will help us recognize and evaluate the adaptation of corals and resistance/resilience of CRNR in response to future climate change.

Materials and methods

Study site description

Sampling sites were located in Sanya CRNR, southernmost coast of Hainan Island, northern part of SCS (Figure 1). Xiaodonghai Bay (XDH), Luhuitou Bay (LHT), Ximao Island (XD) and Xipai Island (XP) are part of the CRNR. The best developed coral reef communities at all sites have a fringing structure with a clearly defined reef flat and a reef slope that descends from a depth of 1–3 m (back-reef) down to a depth of 12 m (fore-reef) except at LHT, where the distribution of coral is limited to 6 m.

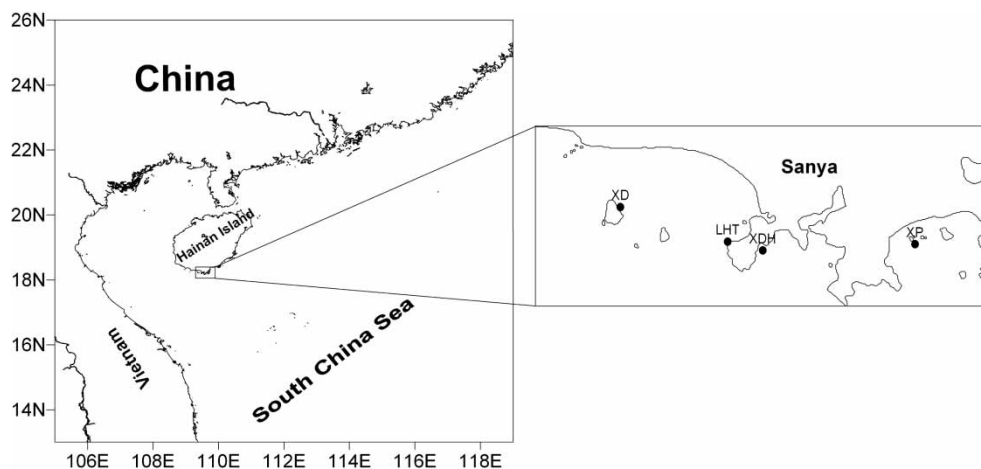


Figure 1. Map of Sanya National Coral Reef Nature Reserve with four marked sampling sites. XDH, Xiaodonghai Bay; LHT, Luhuitou Bay; XD, Ximao Island; XP, Xipai Island.

Reef transects and coral collection

In October 2008, the diversity of cnidarians hosts at the four research sites was surveyed using belt transects 60 m long and 1 m wide at both 3 m depth on the back-reef and at 10 m depth on the fore-reef (in LHT the depth is 3 m and 6 m). For each transect, lines were draped over the substratum parallel to depth contours, digital video taken along the transect and coral cover analyzed using a 0.5 m Point Intercept Transect (PIT) method (English et al. 1997). The common coral colonies were randomly selected and samples around 5 cm² in size were placed in pre-labelled plastic bags and immediately transported back to the laboratory in fresh seawater and then preserved in 100% ethanol for DNA extraction.

Water sampling

Water samples were collected by filling sterile 5-litre plastic bottles from the four sites in July 2009. Near surface water samples (0.5 m) were collected at the back-reef of each site, and both near surface and near bottom water samples were collected at the fore-reef of each site. Each water sample (the final volume is 5 litres) was filtered through a 50- μ m mesh followed by a 5- μ m Nucleopore track-etch membrane filter (Whatman) using a hand-held vacuum pump. Each filter paper was immersed in DNA buffer (0.1 M EDTA, 1% sodium dodecyl sulphate) and stored in a freezer (-20°C) until DNA extraction.

DNA extraction and molecular phylotyping

For filters already in DNA buffer, proteinase K was added to reach a final concentration of 200 $\mu\text{g ml}^{-1}$. The samples were incubated overnight at 55°C . DNA was extracted using a cetyltrimethylammonium bromide protocol. Each resultant DNA solution was further purified using a Zymo DNA cleanup and concentration column (Zymo Research, Orange, CA) to improve PCR efficiency, and eluted in 50 μl of ddH₂O at -20°C . The diversity of *Symbiodinium* spp. in genomic DNA isolated from the seawater samples was evaluated using the hypervariable region of domain V in the large subunit of the chloroplast ribosomal array (cp23S-HVR) with the forward '23SHYPERUP' (5'-TCAGTCAAATAATATGCTG-3'; Santos et al. 2002) and reverse '23SHYPERDN' primers (5'-TTATCGC CCAATTAAACAGT-3'; Manning & Gates 2008), and under the following thermal cycler conditions: initial denaturing period of 2 min at 94°C , 36 cycles consisting of 94°C for 30 s, 50°C for 1 min, 72°C for 1 min, and a final extension period of 10 min at 72°C . PCR products were purified

using a DNA cleanup and concentration column and cloned into PMG-T Vectors (TIANGEN). Colonies carrying a target gene fragment were randomly selected, and approximately 20 clones were sequenced for each water sample by using an ABI 3730 automated DNA sequencer (Applied Biosystems, USA). A total of 218 clones were sequenced.

For *Symbiodinium* spp. associated with coral, the DNA was isolated according to Huang et al. (2006). The cp23S-HVR was also PCR-amplified as described above. The resulting PCR product was purified and directly sequenced.

Sequence analysis

Sequence chromatograms were manually checked with BioEdit (Hall 1999) and identified via the Basic Local Alignment Search Tool (BLAST) in GenBank. *Symbiodinium* sequences belonging to clades C and D were aligned using the Clustal W (Thompson et al. 1994) and analysed phylogenetically using maximum parsimony (MP) and statistical parsimony implemented in the program MEGA (Kumar et al. 1993) and TCS v1.21 (Clement et al. 2000), respectively. The support for nodes within MP tree topologies was determined using bootstrap analyses with 500 replicates. Networks were delineated with 95% certainty, with gaps being treated as a fifth state. Additional published 23S-HVR sequence types previously isolated from water samples were included in the analysis, and referred to here as M1 (FM877475), M2 (EF428359), M3 (EF428360), P1 (FM877461), and P2 (FM877453) (Manning & Gates 2008; Pochon et al. 2010). Sequences within the same TCS-defined 'haplotype' were all scored as the same *Symbiodinium* type; each 'haplotype' took a representative name, for example Cchv1; uppercase letter that indicates the clade, chv indicates the locus (chloroplast hypervariable) and then a sequential number.

Only *Symbiodinium* sequences recovered from two or more clone libraries (representing independent water samples) or differing from a published sequence by three or more bp substitutions (Pochon et al. 2010) were included in the downstream analysis.

Statistical analyses

Similarity among sites, based on the percentage cover of coral species or genera at each site, was discerned with nonmetric multidimensional scaling (MDS) analysis. The Bray-Curtis coefficient of similarity (S) was used to determine if the free-living *Symbiodinium* 23S-HVR types recovered from the 12 water samples exhibited significant patterns in spatial distribution. The abundance of *Symbiodinium*

23S-HVR sequences were grouped by site and depth (near surface water, near bottom water), and standardized to relative frequencies within each grouping. Two-way crossed analysis of similarity (ANOSIM) was calculated with factors sites and depth. All data were square-root transformed prior to the statistical test. All calculations were performed in Primer v6.0 (Clarke & Warwick 2001).

Results

Coral cover and similarities among assemblages

Overall, the average cover (four sites) of live reef-building coral was $14.2 \pm 9.2\%$, ranging from 3% to 27.3% (Table I). Among these sites, *Galaxea fascicularis* (Linnaeus, 1767) was predominant in all sites except for LHT. *Porites* sp. was commonly seen in LHT, XD and XDH. *Acropora* sp. was dominant in LHT, XDH and XP at the back-reef. At all sites, *Montipora* sp., *Diploastrea heliopora* (Lamarck, 1816) were more abundant than other coral species. Sea anemones and sponges were seen at all sites, the soft corals *Simularia* sp. and *Sarcophyton* sp. were especially abundant in XP at the fore-reef. The MDS ordination plots of live coral community data (Figure 2) illustrate the relationships of the assemblages on each transect and the separation of transects and assemblages according to depth and sites. An ANOSIM of the similarity

matrix between sites showed no significant differences in coral assemblages both across sites and with depth ($R = 0.486$, $p = 0.198$).

Molecular diversity of endosymbiotic and free-living Symbiodinium spp.

A total of four *Symbiodinium* cp23S-HVR types were detected from 38 coral species belonging to 19 genera and 9 families. A summary of coral species and *Symbiodinium* types sampled from four sites is given in Table II. The *Symbiodinium* types characterized were Cchv1 (HQ832737), Cchv2 (HQ832738), Cchv3 (HQ832739) and Dchv1 (HQ832740). The results showed that *Symbiodinium* clade C was found in almost all coral species. The dominant cp23S-HVR type Cchv1 was an exact match to previously published cp23S sequence types (e.g. FN298484). The cp23S-HVR used in the present study could not resolve the diversity within clade. Clade D was found in five coral species (13.2%). The only one cp23S-HVR type Dchv1 was an exact match to previously published sequence types (JN558007). The coral *Acropora cerealis* (Dana, 1846), *Acropora microclados* (Ehrenberg, 1834), *Galaxea fascicularis* and *Pocillopora damicornis* (Linnaeus, 1758) can be associated with *Symbiodinium* clades C or D or with both simultaneously.

Of the 12 water samples collected, *Symbiodinium* cp23S-HVR region was successfully amplified and

Table I. Average percent cover of species from different sites in the Sanya CRNR, Hainan Island, South China Sea. (XDH, Xiaodonghai Bay; LHT, Luhuitou Bay; XD, Ximao Island; XP, Xipai Island. Each abbreviation followed by number indicates the depth.)

Name	Average percent cover							
	LHT3	LHT6	XD3	XD10	XDH3	XDH10	XP3	XP10
<i>Acanthastrea echinata</i>					0.333			
<i>Cyphastrea serailia</i>	0.167							
<i>Porites</i> sp.	3.500	2.167	1.333	2.167	2.167	0.667		
<i>Favites</i> sp.				0.167	1.333			
<i>Favia</i> sp.					0.167	0.167		
<i>Goniastrea</i> sp.	1.000		0.167		0.333			
<i>Hydnophora contignatio</i>	0.500		0.167		0.167			
<i>Montipora</i> sp.	0.500				7.833			
<i>Acropora</i> sp.	2.167				5.333		2.667	
<i>Goniopora</i> sp.	0.833			0.333		0.333		0.167
<i>Echinopora gemmacea</i>							0.333	
<i>Podabacia crustacea</i>		0.167						
<i>Galaxea fascicularis</i>			9.667	1.167	8.333	14.667	1.167	20.500
<i>Platygyra</i> sp.			0.167		1.167		2.833	
<i>Pocillopora</i> sp.			0.333				2.000	
<i>Diploastrea heliopora</i>				3.000		6.500		1.833
<i>Psammocora contigua</i>					0.167			
<i>Lobophyllia hemprichii</i>						1.167		
<i>Herpolitha limax</i>								1.000
Milleporidae	0.833	0.667						
Sea anemone	2.000				0.667	1.667		
Sea sponge	2.667			0.333		2.667		
<i>Sarcophyton</i> sp.					0.167		0.500	0.167
<i>Simularia</i> sp.						0.833	1.167	16.333

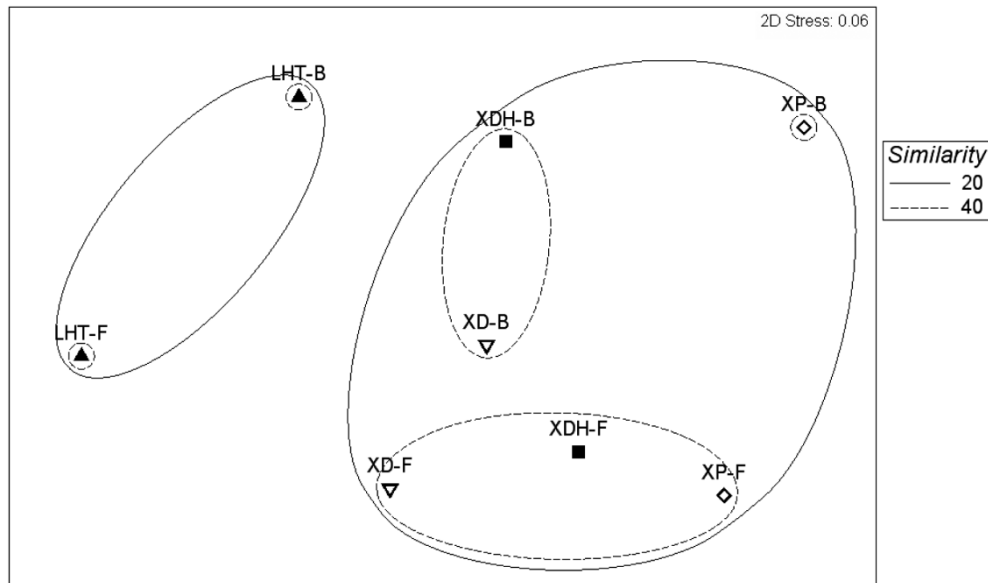


Figure 2. Multidimensional ordinations (MDS) plots showing the relationship between coral assemblages on different transects. Each point represents a single transect, and in the MDS ordination proximity represents increased similarity. Circles represent minimum% similarity between sites. XDH, Xiaodonghai Bay; LHT, Luhuitou Bay; XD, Ximao Island; XP, Xipai Island. For example, XDH-F means the transect line on the fore-reef in Xiaodonghai Bay.

sequenced from all samples. In total, 218 *Symbiodinium* spp. sequences were obtained, representing 24 distinct sequence types. Among these sequences, 2 belonged to clade A (HM032169, HM032170), 1 to clade B (HM032166), 12 to clade C (Cchv4–15: HM032148–HM032150, HM032154, HM032155, HM032157, HM032159, HM032161, HM034766–HM034769), 2 to clade D (Dchv2, Dchv3: HM032164, HM032165), 2 to clade F (HM032172, HM032174), 4 to clade G (HM032177, HM032181–HM032183), and 1 to clade H (HM032184). Most of the distinct sequences were in clade C (Figure 3). Species accumulation curves generated from the cumulative number of *Symbiodinium* types against a measure of the sequencing effort indicated that further sampling effort would be likely to detect additional *Symbiodinium* types (Figure 4).

The diversity of free-living *Symbiodinium* in seawater and their endosymbiotic counterparts combined with published data for *Symbiodinium* clades C and D were analysed phylogenetically (Figure 5). Both methods provided the same topology. The analysis of the cp23S-HVR provides a low level of phylogenetic resolution and reveals no overlap in *Symbiodinium* diversity between host corals and adjacent seawater.

Diversity of free-living Symbiodinium spp. across different sites

The Bray–Curtis similarity coefficient and ANOSIM were employed to statistically test whether the seven

Symbiodinium 23S-HVR sequence types at the clade level (A–D, F–H) recovered varied significantly among the sampling sites and depth. Significant variation in the clade-level *Symbiodinium* spp. types among different sites was observed (ANOSIM: $R=0.551$, $P=0.001$). The samples from the same sites were clustered together with high similarity, except for samples taken from XP, which were placed in separate groups (Figure 6). In addition, the results of ANOSIM test showed insignificant variance for the *Symbiodinium* clade composition irrespective of sampling depth (surface and bottom).

Discussion

In the present study, highly diverse free-living *Symbiodinium* communities did not overlap with symbiotic communities occurring in reef-building corals characterized by complex spatial patterns. This study provides new understanding of *Symbiodinium* and has important implications about the response of corals to climate change.

Coral cover and symbiotic algae diversity

Low live coral cover was observed in all sites in the present study, especially when compared to historical records. For example, it has been reported that the coral cover was > 70% in LHT in the 1960s, reduced to 38% in 1994, and during surveys in 2002 coral cover was 19% (Huang 2005). Further, this study showed that coral cover in LHT was

Table II. Host species and symbiont type of samples from Sanya CRNR, Hainan Island, South China Sea. Numerals in parentheses next to symbiont type indicate the number of individual colonies found to have a particular symbiont. ‘Types’ separated by solidus were identified together in the same sample. v, vertical transmitter; h, horizontal transmitter (Willis et al. 1985; Baird et al. 2009).

	Coral host	Symbiont type			
		LHT	XD	XP	XDH
Acroporidae	<i>Acropora valida</i> (Dana, 1846) (h)	Cchv1(2)			
	<i>Acropora hyacinthus</i> (Dana, 1846) (h)	Cchv1			
	<i>Acropora microclados</i> (Ehrenberg, 1834) (h)	Cchv1, Cchv1/ Dchv1 (2)			
	<i>Acropora cerealis</i> (Dana, 1846) (h)				Cchv1/ Dchv1
	<i>Acropora anthocercis</i> (Brook, 1893) (h)				Cchv1
	<i>Acropora abrotanoides</i> (Lamarck, 1816) (h)	Cchv1 (2)			
	<i>Acropora intermedia</i> (Brook, 1891) (h)			Cchv1	
	<i>Acropora formosa</i> (Dana, 1846) (h)				Cchv1
	<i>Acropora humilis</i> (Dana, 1846) (h)	Cchv1			
	<i>Astreopora myriophthalma</i> (Lamarck, 1816) (h)	Cchv1, Cchv3			
	<i>Montipora mollis</i> (Bernard, 1897) (v)	Cchv1 (2)			
	<i>Montipora grisea</i> (Bernard, 1897) (v)		Dchv1		
	<i>Montipora turgescens</i> (Bernard, 1897) (v)	Cchv2			
	<i>Pavona decussata</i> (Dana, 1846) (h)	Cchv1			
Dendrophylliidae	<i>Turbinaria mesenterina</i> (Lamarck, 1816) (h)			Cchv1	
	Faviidae	<i>Cyphastrea serailia</i> (Forskål, 1775) (h)	Cchv1 (2)		
<i>Diploastrea heliopora</i> (Lamarck, 1816)		Cchv1	Cchv1		Cchv1
<i>Favia speciosa</i> (Dana, 1846) (h)					Cchv1
<i>Favia mathaii</i> (Vaughan, 1918) (h)			Cchv1		
<i>Favia veroni</i> (Moll and Borel-Best, 1984)		Cchv1			
<i>Favites pentagona</i> (Esper, 1794)		Cchv1			Cchv1
<i>Goniastrea aspera</i> (Verrill, 1905) (h/v)		Cchv1			
<i>Leptoria Phrygia</i> (Ellis and Solander, 1786)					Cchv1
<i>Leptastrea purpurea</i> (Dana, 1846) (h)			Cchv1		
<i>Platygyra daedalea</i> (Ellis and Solander, 1786) (h)		Cchv1	Cchv1 (2)	Cchv1	Cchv1
<i>Platygyra sinensis</i> (Milne Edwards and Haime, 1849) (h)			Cchv1		Cchv1
<i>Platygyra lamellina</i> (Ehrenberg, 1834) (h)			Cchv1		
<i>Platygyra crosslandi</i> (Matthai, 1928)			Cchv1		
<i>Platygyra pini</i> (Chevalier, 1975) (h)					Cchv1 (2)
Merulinidae	<i>Hydnophora exesa</i> (Pallas, 1766)	Cchv1	Cchv1		
	<i>Hydnophora contignatio</i> (Forskål, 1775)	Cchv1			
Mussidae	<i>Symphylia radians</i> (Milne Edwards and Haime, 1849) (h)			Cchv1	
Oculinidae	<i>Galaxea fascicularis</i> (Linnaeus, 1767) (h)		Cchv1 (2), Dchv1 (3), Cchv1/Dchv1	Dchv1 (6)	
Pocilloporidae	<i>Pocillopora damicornis</i> (Linnaeus, 1758) (v)	Dchv1 (2)	Cchv1		Dchv1 (2)
	<i>Pocillopora verrucosa</i> (Ellis and Solander, 1786) (v)	Cchv1	Cchv1(2)	Cchv1	Cchv1
Poritidae	<i>Porites lutea</i> (Milne Edwards and Haime, 1851) (v)		Cchv1 (2)	Cchv1	
Siderastreidae	<i>Psammocora contigua</i> (Esper, 1797)		Cchv1	Cchv1	
	<i>Psammocora digitata</i> (Milne Edwards and Haime, 1851)			Cchv1	

only 8.7% and 3% at 3 and 6 m depths, respectively. Similarly, coral cover showed similar patterns of decline at other sites (Huang 2005). Located near Sanya City, the CRNR has been impacted by human activities including destructive fishing, tourist diving and coastal development. Additionally, sedimentation and freshwater incursion had adversely impacted the coral reefs in Sanya. Partial bleaching and severe mortality of *Acropora* sp. were documen-

ted in shallow water of XP during our investigation in 2007, possibly because of the impacts of a typhoon.

The presence of stress-tolerant clade D symbionts may allow resistance to bleaching by some coral species (Baker et al. 2004; Berkelmans & van Oppen 2006; Huang et al. 2011). Only 13.2% of sampled reef-building coral species were associated with *Symbiodinium* clade D, and most of these occurred

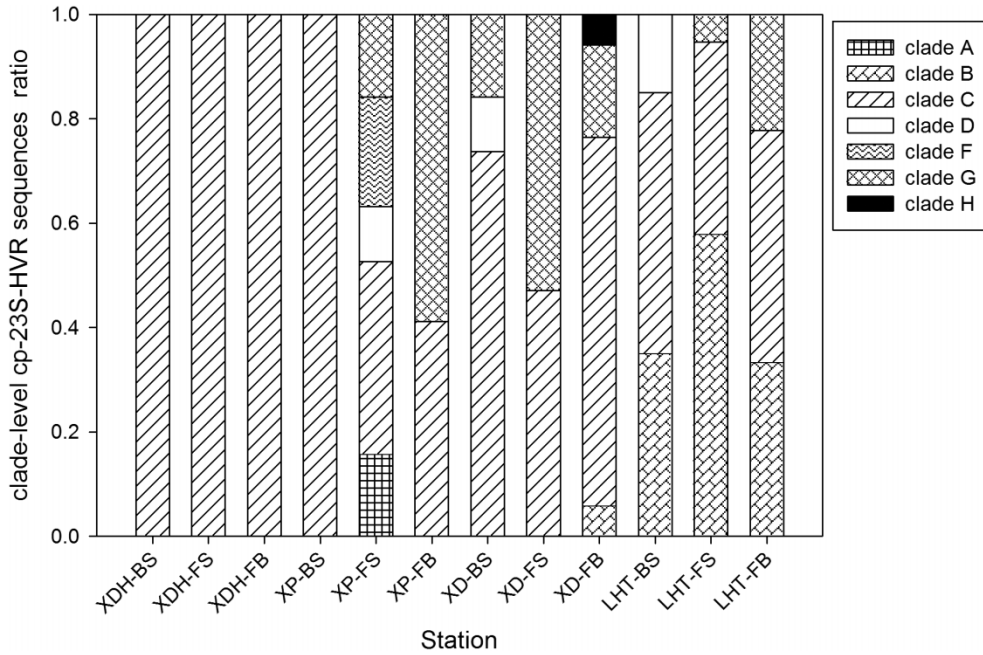


Figure 3. The ratio of *Symbiodinium* clade retrieved from water samples taken at different sites and depths from Sanya CRNR. For site labels see Figure 2 (S, surface; B, bottom). For example, XDH-FS means the surface water sample collected on the fore-reef in Xiaodonghai Bay.

in coral species that are commonly found with D symbionts, such as *Galaxea* and *Pocillopora*, and most corals harboured the stress-sensitive clade C. Importantly, variation in symbiont tolerances to heat stress among within-clade symbiont types (Sampayo et al. 2008; Mieog et al. 2009) indicates that the

subclades or types within clade are complex despite the low-level diversity resolved by the cp23S-HVR marker used in this study. The cp23S-HVR network reduces the complexity of *Symbiodinium* diversity by collapsing most of the sequences into a single type (Figure 6).

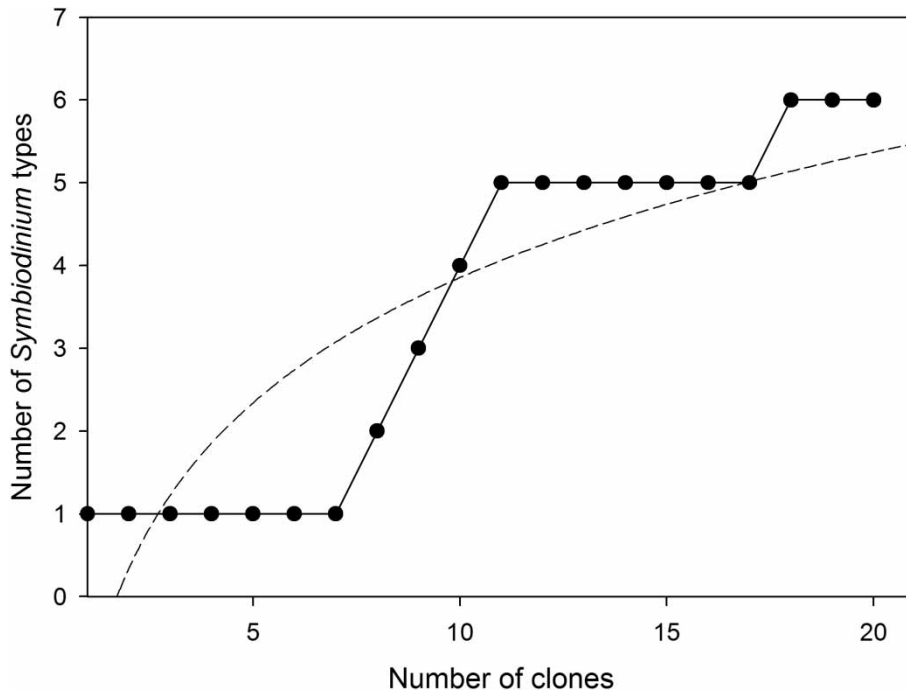


Figure 4. Species accumulation curves derived from cp23S-HVR clone library data for *Symbiodinium* types detected. Dotted lines represent the estimated curve and the solid lines represent species accumulation based on true values.

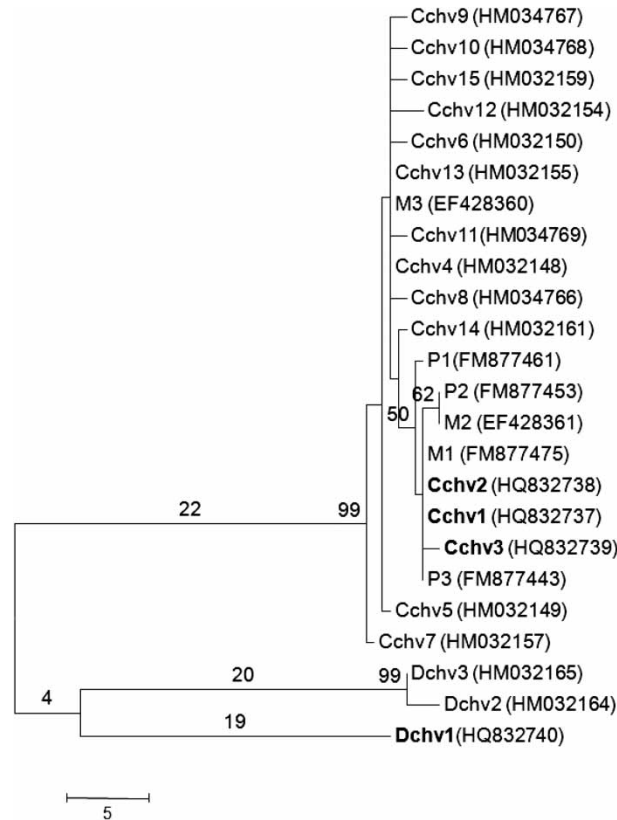


Figure 5. Maximum-parsimony phylogenetic tree constructed from *Symbiodinium* cp23S-HVR sequences. Sequence types found in this study including water and reef corals as well as previously published types are followed by their GenBank accession number. MP bootstrap values are indicated for internal nodes with probabilities above 50%. Symbiotic types are in bold.

Corals associated with more than one symbiotic alga are more flexible and resistant to bleaching events than others (Baker 2003; Little et al. 2004; Baird et al. 2007). However, most scleractinian

species sampled in our study were spawning corals with horizontal symbiont transmission that harboured only one symbiont clade. The proportion of corals harbouring multiple symbionts was lower than

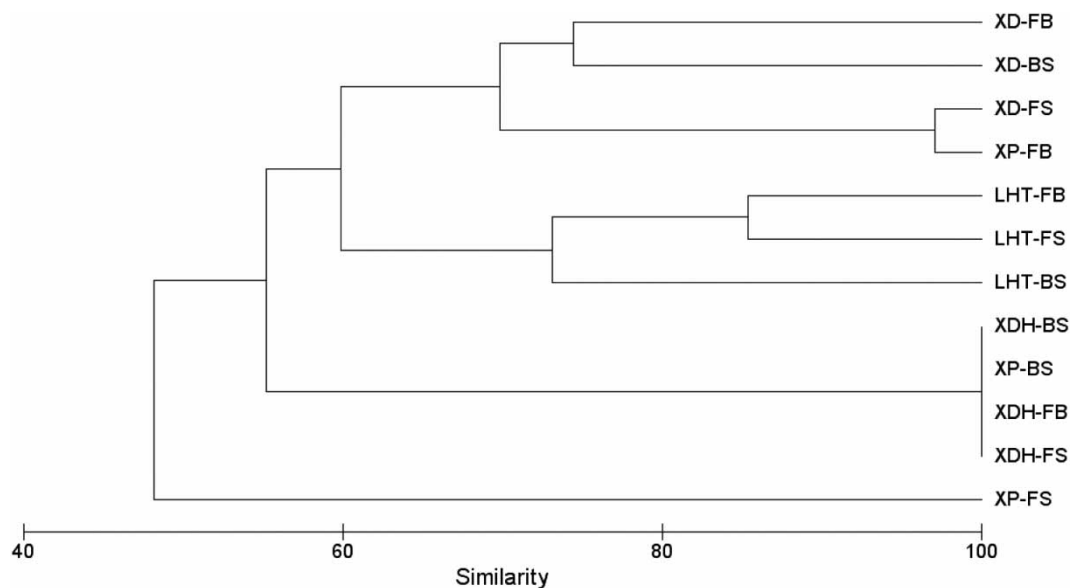


Figure 6. Cluster analyses (Bray-Curtis) of free-living *Symbiodinium* communities from all sampling sites. For site labels see Figure 2; e.g. XDH-FS means the surface water sample collected on the fore-reef in Xiaodonghai Bay.

in other regions of the Indo-Pacific, which may be partly due to the low resolution of the cp23S-HVR marker used in the present study and consequently the *Symbiodinium* spp. diversity may be underestimated (Goulet 2006; Pochon et al. 2010).

Diversity and distribution of free-living Symbiodinium spp.

A total of 24 distinct types representing clades A–D, F–H were retrieved from the water column across the four sites in the CRNR. Except for clades C and D, the clade diversity of free-living *Symbiodinium* is reported for the first time in Sanya CRNR and is greater than previously found in the water column from other regions (Manning & Gates 2008; Pochon et al. 2010). The present study did not detect clade E in water samples, but this has only been identified from two cultured *Symbiodinium* spp.-like isolates from water samples taken from temperate locations (Santos et al. 2002; Gou et al. 2003). The majority of sequences retrieved from Sanya CRNR water samples were clade C, consistent with the overall abundance of these clades in the ecosystem. This indicates that clade C endosymbionts largely dominate reef corals, as has been reported in other regions of the SCS (Huang et al. 2006; Dong et al. 2009; Zhou & Huang 2011) and more broadly, whereas other clades are relatively rare in the Indo-Pacific regions (Baker 2003; LaJeunesse 2005; LaJeunesse et al. 2004, 2010). Additionally, there were more types found within clade C than in other types, in accordance with a previous report showing relatively high diversification within this clade compared to others (LaJeunesse 2005).

The ANOSIM analysis showed that free-living *Symbiodinium* spp. communities have high spatial variability. Typical free-living dinoflagellates are mobile and are capable of diurnal migration (Fitt & Trench 1983; Yacobovitch et al. 2004). Therefore, it can be assumed that free-living *Symbiodinium* spp. are capable of independent dispersal through the water column, resulting in spatiotemporal heterogeneity. The distribution of free-living *Symbiodinium* spp. will be influenced by physical factors such as water flow, light and temperature. In the present study, the distribution of free-living *Symbiodinium* spp. was related to sampling sites but not to depth.

No difference in *Symbiodinium* composition between water column positions was found in the present study, indicating that the distribution of free-living *Symbiodinium* spp. is homogeneous during the day. A previous study reported that mobile organisms such as corallivorous fish have the ability to disperse viable *Symbiodinium* (Parker 1984). Several studies have noted that *Symbiodinium* spp.

is mobile and responds to host chemical attractants, possibly enabling *Symbiodinium* to aggregate near potential hosts (Fitt 1984; Yacobovitch et al. 2004). Additionally, corals can continuously release healthy *Symbiodinium* as a post-mitotic control for regulating density within coral tissue (Jones & Yellowlees 1997). Ultimately, there are numerous biotic and abiotic factors controlling the distributions of free-living *Symbiodinium* spp.

Comparison of endosymbiotic and free-living Symbiodinium and the diversity resolved using cp23S

None of the 218 sequences obtained from our environmental survey of free-living *Symbiodinium* matched those of the endosymbionts in neighbouring reef-building corals. This result is consistent with Pochon et al. (2010), who found that cp23S-HVR sequence types from the free-living *Symbiodinium* in water was distinct from those of dominant symbiotic algae in neighbouring reef corals.

Although multiple symbiont types are initially acquired by corals during early ontogeny, only a subset of those strains is retained subsequently (Little et al. 2004; Adams et al. 2009). There is some evidence that symbiont recognition may occur before infection into host cells in larvae of the coral *Fungia scutaria* (Rodriguez-Lanetty et al. 2006). Furthermore, in most adult corals, the symbiosis is extremely specific and stable over time, although exogenous symbionts may be temporarily acquired under environmental stress (Goulet 2006; Sampayo et al. 2008; Thornhill et al. 2009; Coffroth et al. 2010). Moreover, a large diversity of *Symbiodinium* has only been found in water in the current study, suggesting that not every *Symbiodinium* spp. type does or can exist in a symbiotic relationship (Pochon et al. 2010). The findings presented here are provocative in terms of the ABH (Buddemeier & Fautin 1993) and suggests that the potential for exchange between symbiotic and environmental pools of *Symbiodinium* in Sanya CRNR may be more limited than previously thought. Recently, Coffroth et al. (2010) found that the coral *Porites divaricata* had the ability to take up exogenous symbionts, but could not maintain these in a stable symbiosis. All available evidence indicates that the pools of free-living *Symbiodinium* are entirely different in composition compared to the *in hospite* pool, and this has significant implications in terms of acquisition, success and specificity.

It should be noted that a possible explanation for this discrepancy is given as follows. First, given typical free-living dinoflagellates' mobility and diurnal migration (Fitt & Trench 1983; Yacobovitch et al. 2004), free-living *Symbiodinium* spp.

distribution is likely to vary spatially and temporally (Littman et al. 2008). Because the samplings were not carried out simultaneously in the present study, they could be underestimating the actual diversity of free-living *Symbiodinium*. Second, *Symbiodinium* are also present in sediment with a higher abundance of cells (Littman et al. 2008) and more sequence types (Takabayashi et al. 2011) and may provide an additional source for symbiont acquisition in corals (Adams et al. 2009). Unfortunately, the current study did not investigate this important source. Third, the present study only checked the diversity of symbiotic algae in common reef-building corals, whereas other invertebrate hosts have not been inspected. Lastly, although cp23S-HVR it is the only marker specific to environmental samples (Manning & Gates 2008; Pochon et al. 2010) and may be directly linked to physiological performance (Takabayashi et al. 2011), it provides less taxonomic resolution. As sampling effort increases in frequency and spatial resolution, and is combined with sufficient variable markers (Manning & Gates 2008; Pochon et al. 2010), it is likely that additional *Symbiodinium* spp. diversity will be discovered in Sanya CRNR and it is possible that the endosymbionts of reef invertebrates will also be identified in free-living compartments of the ecosystem.

Ecological implication for resilience of coral reefs

Symbiodinium spp. play a key role in adaptation, resilience and recovery of host invertebrates, as the future of these organisms continues to be critically compromised (Buddemeier & Fautin 1993; Lewis & Coffroth 2004; Coffroth et al. 2006; Baird et al. 2007). Although the increased incidence of mass bleaching events associated with climate change threatens the future of coral reefs, coral communities with diverse symbiotic combinations and abundant symbiont pools may be more resilient than others to climate change (Baker 2003; Berkelmans & van Oppen 2006; Jones et al. 2008).

In conclusion, our study found no overlap between symbiotic and free-living *Symbiodinium* assemblages, and supports the hypothesis that the host corals may acclimatize to environmental changes via shuffling of pre-existing symbiont types (Berkelmans & van Oppen 2006; Coffroth et al. 2010; Pochon et al. 2010). On the other hand, host factors including growth and disease have been demonstrated to be critical to stress susceptibility and cannot be neglected. Much remains to be learnt of the extent of the possible availability and distribution of free-living *Symbiodinium* spp., which

potentially have a pivotal role in the coral reef ecosystem.

Acknowledgements

We thank the administration and staff of the Sanya National Coral Reef Nature Reserve, China for permit and logistical support. Particularly, Yuyang Zhang and Ting Su are acknowledged for their assistance during the sample collection and experiments. We are grateful to Zhao Sun and Jingbin Feng for valuable discussions. This work was supported by grants from the National Natural Science Foundation of China (Nos. 40776085, 40830850) and the Main Direction Program of Knowledge Innovation of Chinese Academy of Sciences (No. KZCX2-YW-227).

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Editorial responsibility: David McKinnon