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Non-seasonal clade-specificity and subclade microvariation in symbiotic dinoflagellates (*Symbiodinium* spp.) in *Zoanthus sansibaricus* (Anthozoa: Hexacorallia) at Kagoshima Bay, Japan

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SUMMARY

While much work has investigated the genetic diversity of symbiotic dinoflagellate genus *Symbiodinium* Freudenthal in cnidarians, investigations into such diversity over temporal scales (seasonal and/or annual) remain scarce. Here, we have sequenced the internal transcribed spacer of ribosomal DNA (ITS-rDNA) of *Symbiodinium* from samples of designated *Zoanthus sansibaricus* Carlgren (Anthozoa: Hexacorallia) colonies collected for 12 months (August 2004–July 2005) at a high latitude non-reefal coral community at Sakurajima, Kagoshima Bay, Japan (31°35′N, 130°35′E). Our results show that despite large ocean temperature changes (15.0–29.0°C) throughout the one-year experimental period, *Z. sansibaricus* colonies contained only clade C *Symbiodinium* from many different subclade C1/C3-related novel types not previously reported. While no temporal changes in clade-level associations were seen, there were consistent and extremely large amounts (145 unique sequences out of 153 total obtained sequences) of genotypic microvariation observed in our obtained sequences. Despite *Z. sansibaricus* acquiring *Symbiodinium* horizontally and the presence of various other *Symbiodinium* clades (A, G) and subclades (e.g. C15 and derived subclades) in the immediate environment, *Z. sansibaricus* at Sakurajima specifically associates with subclade C1/C3-related *Symbiodinium*. While subclades C1/C3 have been found in a variety of different environments and are believed to be ancestral, 'generalist' types of *Symbiodinium,* C1/C3-related clades such as seen here may be more adapted to specialized niches. We theorize that specific and year-round association with many different types of subclade C1/C3-related *Symbiodinium* helps *Z. sansibaricus* to survive in the fluctuating Sakurajima environment.

Key words: internal transcribed spacer of ribosomal DNA, *Symbiodinium*, symbiosis, *Zoanthus*, zooxanthellae.

INTRODUCTION

Many marine organisms (i.e. foraminifers (Pochon *et al*. 2001), cnidarians (Rowan & Powers 1991), and mollusks (Carlos *et al*. 1999)) are in symbioses with symbiotic dinoflagellates (zooxanthellae) of the genus *Symbiodinium* Freudenthal *(*order Suessiales) and in particular, such algae–host symbiotic relationships are numerous in the coral reef ecosystems of the world's shallow subtropical and tropical oceans (Muscatine & Porter 1977). Recently, the phenomenon of bleaching, where *Symbiodinium* are degraded and/or expelled from hosts, resulting in drastic whitening and/or death of hosts, has been observed worldwide.

Much work has been carried out on the genetic diversity of *Symbiodinium*, and it has been established that there are at least eight major 'clades' (of unknown taxonomic level) of this dinoflagellate genus (Pochon *et al*. 2004; Pochon *et al*. 2006). Additionally, changes in *Symbiodinium* populations have been seen temporally within individual hosts, particularly before and after major bleaching events (i.e. Rowan *et al*. 1997 and Toller *et al*. 2001; both in coral *Montastraea* spp.),

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but also on a seasonal basis in some organisms (e.g. in the coral *Acropora palifera* Lamarck (Chen *et al*. 2005)). Such observations have led to the proposal of the Adaptive Bleaching Hypothesis (ABH), which states that the composition (i.e. genetic diversity) of populations of *Symbiodinium* in hosts can change over time to adapt to changing environmental conditions (Buddemeier & Fautin 1993). However, the presence or absence of seasonality in the genetic diversity of symbiotic *Symbiodinium* populations in a wide range of host cnidarian species in different climates remains to be determined.

Previously, investigations into the zoanthid encrusting anemone *Zoanthus sansibaricus* Carlgren (Anthozoa: Hexacorallia) over a latitudinal range in southern Japan have shown a flexibility in their association with *Symbiodinium*: *Z. sansibaricus* at two northern sites (Kokubu = 31°41′N, Sakurajima = 31°35′N) contained *Symbiodinium* closely related to subclade C1, while at a more southern site (Yakushima = 30°16′N) subclades related to C1 and C15 were present and at the most southern site (Amami = 28°27′N) subclades related to A1 and C1 were present (Reimer *et al*. 2006c). Other studies have shown *Zoanthus* spp. can associate with multiple *Symbiodinium* clades even within a single individual colony (LaJeunesse 2002). These data combined with the knowledge that *Z. sansibaricus* acquire their *Symbiodinium* from the environment (horizontal transmission) (Ono *et al*. 2005) make *Zoanthus* spp. a good candidate for investigating seasonal/temporal changes in *Symbiodinium* association. However, no investigation into the characterization of the genetic diversity of *Symbiodinium* over time in any zoanthid has yet been reported.

To investigate *Symbiodinium* population changes in zoanthid hosts over time, we have characterized *Symbiodinium* spp. at the molecular level using internal transcribed spacer of ribosomal DNA (ITS-rDNA) in four marked colonies of *Z. sansibaricus* from samples collected monthly at a high-latitude colonical cnidarian ecosystem (Sakurajima, Japan) over a one-year period in order to answer the following question: Is there any monthly or seasonal change in the genotypic diversity of *Symbiodinium* spp. in *Z. sansibaricus,* and if so at what level does variation occur?

MATERIALS AND METHODS

Sampling

Clonal polyps of *Z. sansibaricus* were collected monthly between August 2004 and July 2005 from four marked colonies at a field site located at Hakamagoshi, Sakurajima, Kagoshima, Japan (31°35′N, 130°35′E), except in November 2004 when samples were not collected. Each of the four colonies was of a different coloration pattern, corresponding to the previously presumed species *Z.* aff. *pacificus* (colony I), *Z. sansibaricus* (colony II), *Z.* aff. *erythrochloros* (colony III), and *Z.* aff. *gnophodes* (colony IV). These previously presumed species (see Uchida 2001) have recently been shown to be conspecific morphotypes (Reimer *et al*. 2004, 2006a) and are grouped in the species *Z. sansibaricus*. All four colonies were at depths of 3 m. During sampling, ocean temperature was recorded at 3 m depth.

Sample polyps were collected from the center of each colony, and immediately placed in 99.5% ethanol and stored at –30°C until further analysis.

DNA extraction and polymerase chain reaction-amplification (PCR)

Small pieces of tissue were cut from the top one-third of each sampled *Zoanthus* polyp. Pieces were seen to contain innumerable symbiont zooxanthellae. Total DNA was extracted from these samples using a spin-column DNeasy Animal DNA Extraction kit (QIAGEN, Tokyo, Japan). PCR amplification was performed using HotStarTaq DNA polymerase (QIAGEN) according to the manufacturer's instructions. ITS-rDNA was amplified using primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al*. 1990) and zooxanthellae-specific zITSf (5′-CCG GTG AAT TAT TCG GAC TGA CGC AGT-3′) (Rowan & Powers 1992; Hunter *et al*. 1997).

Cloning and sequencing

PCR-amplified products from *Z. sansibaricus* colonies were obtained and processed as described in Reimer *et al*. (2006b). PCR-amplified products from October 2004, January 2005, and April 2005 from all four colonies were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). Several clones of ITS-rDNA from each sample were sequenced with an ABI PRISMTM 3700 DNA Analyzer (PE Applied Biosystems, Foster City, CA, USA) using a BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The sequences were analyzed using DNASIS Mac v3.6 (Hitachi Software Engineering Company, Ltd, Tokyo, Japan). PCR-amplified products from colonies I-IV from other months (August 2004–June 2005) were sequenced directly.

Phylogenetic analyses

Previously reported ITS-rDNA sequences of *Symbiodinium* clade C (AF195144, AF195157 (Baillie *et al*. 2000); AJ291514 (Pawlowski *et al*. 2001); AJ311944 (Pochon *et al*. 2001); AY186567 (Rodriguez-Lanetty & Hoegh-Guldberg 2003); AY237296, DQ068036, DQ072720 (Bui *et al*. unpubl.)) and our previous (July 2003) *Symbiodinium* sequence data from the marked

Z. sansibaricus colony I at Sakurajima (AB190274-AB190276, Reimer *et al.* 2006c) and a *Z. sansibaricus* colony at Yakushima (AB190278-279, AB207184, Reimer *et al.* 2006c) were retrieved from the DNA Data Bank of Japan (DDBJ) and were aligned with our present data using Clustal W version 1.8 (Thompson *et al*. 1997). Additionally, a sequence from *Symbiodinium* subclade C91 (AJ291519 (Pawlowski *et al*. 2001)) was included as an outgroup.

The alignment was inspected by eye and manually edited. The alignment dataset is available on request from the corresponding author. The dataset (140 taxa/631 sites) was subjected to analyses with the neighbor-joining (NJ) (Saitou & Nei 1987) method. The distances were calculated using Kimura's two-parameter model (Kimura 1980). Support for NJ branches was tested by bootstrap analysis (Felsenstein 1985) of 1000 replicates. PAUP version 4.0 was used for these phylogenetic analyzes (Swofford 2000).

Using the full ITS-rDNA region dataset, Bayesian trees were reconstructed by using MrBayes 3.0 (Ronquist & Huelsenbeck 2003) with an input tree generated by BIONJ with the general time-reversible model (Rodriguez *et al*. 1990) incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + Γ). The proportion of invariable sites, a discrete gamma distribution, and base frequencies of the model were estimated from the dataset. One cold and three heated Markov chain Monte Carlo (MCMC) chains with default-chain temperatures were run for 1 000 000 generations, sampling log-likelihoods (InLs), and trees at 100-generation intervals (10 000 InLs and trees were saved during MCMC). The likelihood plot for the dataset suggested that MCMC reached the stationary phase after the first 30 000 generations. Thus, the remaining 970 000 trees were used to obtain clade probabilities and branch-length estimates, respectively.

RESULTS

A summary of our obtained full ITS-rDNA region sequences is shown in Table 1 and in Fig. 1. New sequences obtained in the present study were deposited in GenBank (Accession Numbers DQ335255 – DQ335411, AB253787 – AB253789). All PCR-amplified products could be sequenced directly with minimal (<0.5%) ambiguity (i.e. observable double peaks), indicating that samples possessed *Symbiodinium* of very similar genetic identity (i.e. to the subclade level, as in Rodriguez-Lanetty & Hoegh-Guldberg 2003), although the possible presence of very small amounts of other *Symbiodinium* clades cannot be confirmed by direct sequencing (Santos *et al*. 2001). All sequences from the *Zoanthus* colonies were able to be unambiguously aligned only with clade C rather than

Table 1. Summary of *Symbiodinium* spp. found in *Zoanthus sansibaricus* colonies at Sakurajima, July 2003 and August 2004–July 2005 with ocean temperature

Genus	Species	Morphotype†	Depth (m)‡	Acc. #	Colony #	*Symbiodinium* composition (subclade) and number of clones (x) by sampling date (YY/MM)‡									
						03.07§	04.08	04.09	04.10	04.12	05.01	05.02	05.03	05.04	05.05	05.06	05.07
Zoanthus	*sansibaricus*	pacificus	3.0	AB190274-276	I	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3
				DQ335255-294		(3)			(12)		(9)			(8)			
		sansibaricus	3.0	DQ335295-335	II	NA	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3
									(13)		(12)			(7)			
		erythro-chloros	3.0	DQ335336-372	III	NA	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3
									(9)		(10)			(10)			
		gnophodes	3.0	DQ335373-411	IV	NA	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3	C1/C3
									(10)		(10)			(11)			
		Sakurajima ocean temperature (depth = 3.0 m) on sampling date (°C)				27.0	29.0	28.0	24.0	20.1	17.0	15.0	15.2	16.2	20.3	22.0	25.0

†For morphotype information, refer to Reimer *et al*. (2004); ‡Subclade data with number of subclones in parentheses are the product of cloning experiments, data with no numbers in parentheses are the product of direct sequencing; §data for 03.07 from Reimer *et al*. (2006b). All additional cloned sequences from Reimer et al. 2006c) from six other Sakurajima *Z. sansibaricus* colonies at various depths (2.0–10.0 m) were all *Symbiodinium* subclade C1/C3-related.

NA, data not obtained due to no sampling.

Table 2. Matrix summary of site differences of *Symbiodinium* internal transcribed spacer of ribosomal DNA (ITS-rDNA) (total length = 624 sites) between cloned sequences from *Zoanthus sansibaricus* colony I (October 2004)

1	–											
2	3	–										
3	3	5	–									
4	12	14	9	–								
5	14	15	10	1	–							
6	10	11	7	4	5	–						
7	12	13	9	6	7	2	–					
8	10	11	7	4	5	0	2	–				
9	5	6	1	12	13	7	10	7	–			
10	14	15	11	5	6	4	5	4	11	–		
11	15	14	10	8	9	3	7	3	14	8	–	
12	15	15	12	5	6	5	6	5	15	5	9	–
One0410 Clone #	1	2	3	4	5	6	7	8	9	10	11	12

other clades, clearly indicating that they belonged to clade C. Furthermore, based on the phylogenetic analyses of clade C the obtained sequences were closely related to subclades C1 and C3 (both NJ bootstrap support and Bayesian posterior probability = 100%) (*sensu* LaJeunesse 2005). While our obtained sequences match most closely with subclade C1, they do not form a robust C1-specific monophyly in the tree, grouping with both C1 and C3-related sequences. Thus we will refer to these sequences as 'subclade C1/C3-related'. Obtained full ITS-rDNA region sequences were most closely related (98–100% base pair matching, Accession Nos. AB190265-AB190277) to clade C ITS-rDNA sequences from *Z. sansibaricus* samples collected in summer 2003 and summer 2004 at Sakurajima (Reimer *et al*. 2006c). Based on full ITS-rDNA region sequences, no changes in clade composition were seen in any *Z. sansibaricus* colony during the one-year sampling period.

However, large amounts of ITS-rDNA microvariation were present, both between sequences from different colonies and from the same colony from different sampling months, as well as among sequences cloned from single colonies (for an example see Table 2). As explained later in the *Discussion*, we do not feel that this microvariation is the result of a sequencing error. While all analyzed *Z. sansibaricus* full ITS-rDNA region sequences (n = 153, 32 direct, 121 cloned sequences) were unambiguously closely related to *Symbiodinium* subclades C1/C3, almost all sequences were unique (145 unique sequences of 153 total) compared to other obtained sequences. Observed microvariation did not appear to have any seasonal, temporal or colony-by-colony (i.e. *Z. sansibaricus* morphotype) pattern. Our observed microvariation confirms high levels of microvariation previously seen in *Symbiodinium* from *Z. sansibaricus* of the 'sansibaricus' morphotype (Reimer *et al*. 2006c).

We additionally examined only the ITS-2 rDNA region as this region has been used extensively by researchers to identify subclades of *Symbiodinium* (see LaJeunesse *et al*. 2003, 2004a,b; LaJeunesse 2005). The topology of the resulting ITS-2 rDNA tree was similar to the full ITS-rDNA tree with less resolution between subclades (Reimer *et al*. 2006b).

DISCUSSION

Our results here confirm previous results that show *Z. sansibaricus* at Sakurajima harbors *Symbiodinium* related to subclades C1/C3 (Reimer *et al*. 2006c). Despite large annual ocean temperature variations (approximately 15.0–29.0°C) at the Sakurajima site, no observable seasonal change in *Symbiodinium* genetic diversity at the clade level was observed, similar to host-symbiont fidelity observed between the coral *Oulastrea crispata* Lamarck and *Symbiodinium* clade D in Hong Kong and Taiwan (Chen *et al*. 2003) and stable *Symbiodinium* association in the Caribbean octocoral *Gorgonia ventalina* Linnaeus (Kirk *et al*. 2005), and counter to seasonal *Symbiodinium* clade C and D population shifts observed by Chen *et al*. (2005) in *Acropora palifera* in Taiwan.

As *Z. sansibaricus* acquire *Symbiodinium* from the environment with each new generation (Ono *et al*. 2005), *Z. sansibaricus* at Sakurajima has an apparent selective specificity of zooxanthellae to C1/C3-related subclades. Certainly, other more distantly related *Symbiodinium* C subclades (e.g. C15 in *Z.* aff. *vietnamensis,* Accession No. = AB253787) and clades (G in soft coral *Nephthea* spp., AB253788; A1 in the zoanthid *Zoanthus* aff. *vietnamensis*, AB253789) are present at the Sakurajima site. *Z.* aff. *vietnamensis* colonies are often intermingled with *Z. sansibaricus*, and all four of our examined colonies had *Z.* aff. *vietnamensis* colonies

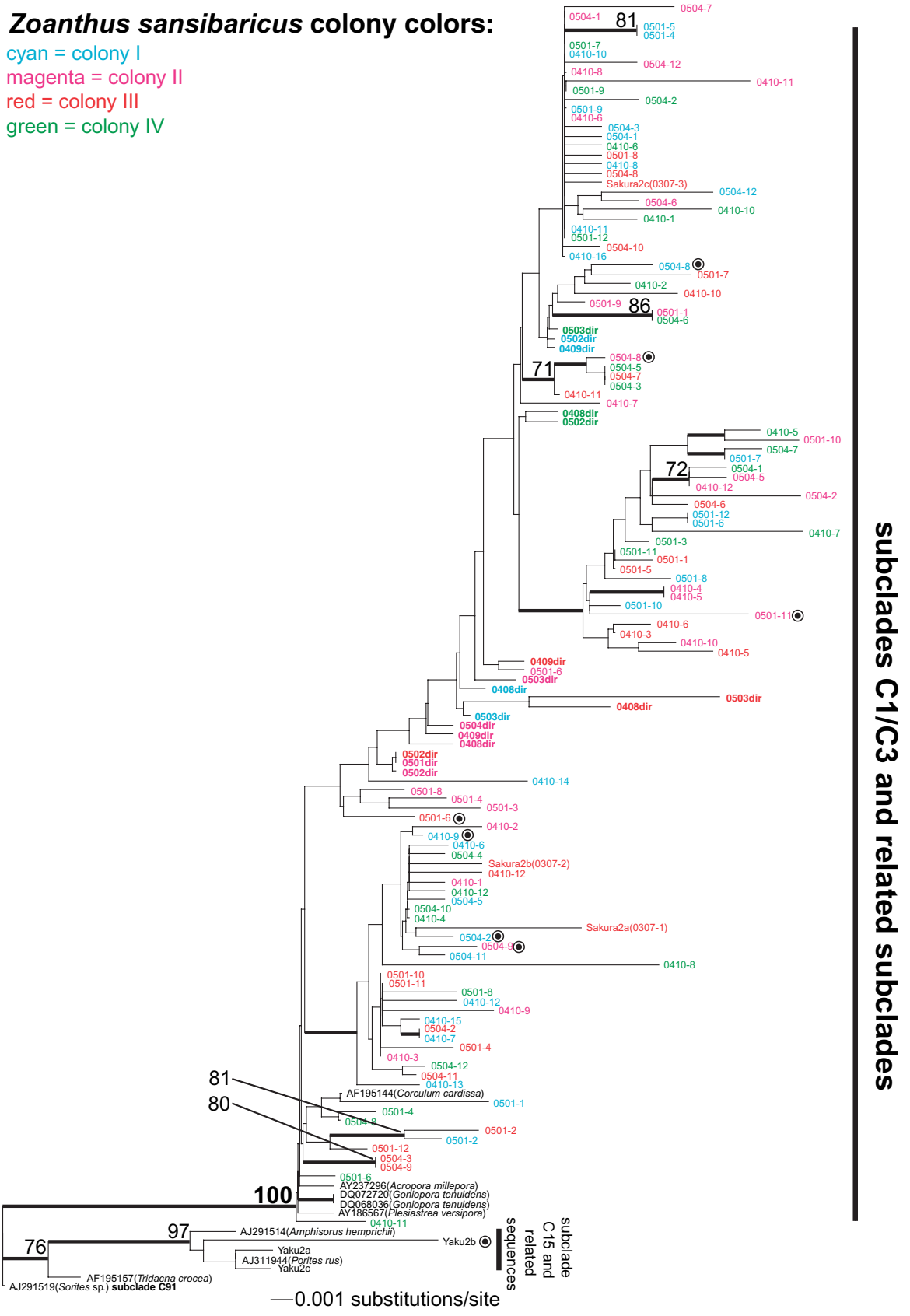
Zoanthus sansibaricus colony colors:
cyan = colony I
magenta = colony II
red = colony III
green = colony IV
0504-7
0504-1
81
0501-5
0501-4
0501-7
0410-10
0504-12
0410-8
0410-11
0501-9
0504-2
0501-9
0410-6
0504-3
0504-1
0410-6
0501-8
0410-8
0504-8
Sakura2c(0307-3)
0504-12
0504-6
0410-10
0410-1
0410-11
0501-12
0504-10
0410-16
0504-8
0501-7
0410-2
0410-10
0501-9
86
0501-1
0504-6
0503dir
0502dir
0409dir
0504-8
71
0504-5
0504-7
0504-3
0410-11
0410-7
0408dir
0502dir
0410-5
0501-10
0504-7
0501-7
0504-1
72
0504-5
0410-12
0504-2
0504-6
0501-12
0501-6
0410-7
0501-3
0501-11
0501-1
0501-5
0501-8
0410-4
0410-5
0501-10
0501-11
0410-6
0410-3
0410-10
0410-5
0409dir
0501-6
0503dir
0408dir
0503dir
0408dir
0503dir
0504dir
0409dir
0408dir
0502dir
0501dir
0502dir
0410-14
0501-8
0501-4
0501-3
0501-6
0410-2
0410-9
0410-6
0504-4
Sakura2b(0307-2)
0410-12
0410-1
0410-12
0504-5
0504-10
0410-4
Sakura2a(0307-1)
0504-2
0504-9
0504-11
0410-8
0501-10
0501-11
0501-8
0410-12
0410-9
0410-15
0504-2
0410-7
0501-4
0410-3
0504-12
0504-11
0410-13
81
80
AF195144(Corculum cardissa)
0501-1
0501-4
0504-8
0501-2
0501-2
0501-12
0504-3
0504-9
0501-6
100
AY237296(Acropora millepora)
DQ072720(Goniopora tenuidens)
DQ068036(Goniopora tenuidens)
AY186567(Plesiastrea versipora)
0410-11
97
AJ291514(Amphisorus hemprichii)
Yaku2b
76
Yaku2a
AJ311944(Porites rus)
Yaku2c
AF195157(Tridacna crocea)
AJ291519(Sorites sp.) subclade C91
subclade C15 and related sequences
0.001 substitutions/site
subclades C1/C3 and related subclades

Fig. 1. Neighbor-joining (NJ) tree of internal transcribed spacer of ribosomal DNA (ITS-rDNA) of *Symbiodinium* clade C from *Zoanthus sansibaricus* colonies at Sakurajima. Values at branches represent NJ bootstrap probability (> 70%), and thick branches Bayesian posterior probability > 95%. Sequences acquired in this study are designated by 'year, month, clone number'; thus '0504-1' is '2005 April clone 1' sequence. Bold sequences with 'dir' endings were obtained by direct sequencing. Sequences from colony I are in cyan, colony II magenta, colony III red, and colony IV green. Sequences acquired in previous studies are designated by Accession Number and host species name, except for six sequences (shown as Sakura 2a–c and Yaku 2a–c, Accession Numbers AB190274-AB190276 and AB190278-279 plus AB207184, respectively) acquired in July 2003 (Reimer et al. 2006c). Filled-in circles after sequence names indicate sequences with deletions (>5 b.p.).

within one meter, but in all 162 sequences examined to date (nine sequences from Reimer *et al*. 2006c) plus 153 analyzed sequences obtained here) only subclade C1/C3-related *Symbiodinium* has been seen in *Z. sansibaricus* at Sakurajima, regardless of colony morphotype or depth of the colony (2–10 m). Similarly, individual *Z. sansibaricus* colonies at the more southern site of Amami have been shown to only contain one clade of *Symbiodinium* (either subclade C1/C3-related or A1-related). Our results suggest that despite the presence of a variety of *Symbiodinium* clades and fluctuating and overlapping temperature ranges at Sakurajima and Amami, individual *Z. sansibaricus* colonies stably associate with subclade C1/C3-related *Symbiodinium*. Ancestral *Symbiodinium* subclade C1/C3 has been theorized to be a host 'generalist' strain of zooxanthellae, widespread over the Eastern Pacific and found in a variety of different environments (LaJeunesse 2005). However, *Symbiodinium* types derived from ancestral C1/C3 have been speculated to be more adapted to specific environmental conditions (see LaJeunesse 2002).

Here, specific and year-round association with our observed different novel subclade C1/C3-related types may allow *Z. sansibaricus* to survive in the fluctuating Sakurajima environment. This is a different (i.e. in number of *Symbiodinium* types in symbiosis with the host) yet similar (lacking seasonal *Symbiodinium* clade variation) mechanism to cold-water inhabiting (7–10°C in winter) northwest Pacific *O. crispata*, which contains 'stress-tolerant' *Symbiodinium* clade D year-round (Chen *et al*. 2003). It remains to be determined if the presence (i.e. *Acropora* and *Symbiodinium* clades C and D in Chen *et al*. 2005) or absence (as seen here with *Z. sansibaricus* and subclade C1, and in *O. crispata* with clade D in Chen *et al*. 2003) of changes in *Symbiodinium* clades are due to the host species, the environment, or a combination of these two factors.

Preliminary experiments conducted during this study on clonal cultures of *Symbiodinium* sp. HA3-5 (clade A) (Acc. No. AF184948, Baillie *et al.* 2000) resulted in no variation in acquired ITS-rDNA sequences. Additionally, an examination of *Symbiodinium* ITS-rDNA sequences from 35 zoanthid *Palythoa* spp. (Anthozoa: Hexacorallia: Zoanthidea: Sphenopidae) samples collected over a 1000+ km latitudinal range in Japan found only subclade C1-related *Symbiodinium* with almost no microvariation (Reimer *et al*. 2006b). However, both studies investigating *Symbiodinium* in *Zoanthus* (Reimer et al. 2006c; this study) have consistently shown large amounts of ITS-rDNA microvariation despite using identical methods. In this study, seven of our obtained sequences have large deletions (>5 b.p., see Fig. 1, Table 3), similar to as seen in Reimer *et al*. (2006b). If microvariation is the result of multiple copies of the ITS-rDNA region within a single genome (as suggested by Santos *et al*. 2003) we would expect all of our experiments and not only *Zoanthus*-based investigations to show *Symbiodinium* ITS-rDNA microvariation. Observed microvariation of ITS-rDNA only from *Symbiodinium* in *Zoanthus (*and no microvariation in HA3-5 or in *Symbiodinium* from *Palythoa*) suggests extreme subclade C1/C3-related microvariation in *Zoanthus* is the result of multiple genotypes present in samples, although there may be inherent differences in the number of copies of the ITS-rDNA region between clades (i.e. C and A) or even between different genotypes within subclades C1/C3.

Table 3. Summary of obtained internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences from *Symbiodinium* in *Zoanthus sansibaricus* with deletions

Sequence name	Region of deletion	Deletion length (b.p.)
Three0501-6	ITS-1	54
One0504-8	5.8S	6
Two0410-9	5.8S	25
One0501-11	ITS-2	13
One0504-9	5.8S – ITS-2	66
Two0504-2†	ITS-2	38
Two0504-8†	ITS-2	38

†deletions identical.

While no seasonal changes in *Symbiodinium* clades were seen in *Z. sansibaricus* during our study, it should be noted that ocean temperatures during this period were not more than ±0.5°C higher or lower than annually expected at Sakurajima. Similarly, no cnidarian bleaching at Sakurajima was seen during weekly dives over the entire experimental period (SO, pers. obs.). However, ABH suggests that shifts in *Symbiodinium*

composition of hosts most often occur during times of stress (i.e. not only 'expected' seasonal temperature changes but also during 'abnormal' (extreme high or low temperature) bleaching events) (Buddemeier & Fautin 1993). Continued long-term monitoring of our marked *Z. sansibaricus* colonies is needed to ascertain if shifts of *Symbiodinium* clade composition predicted in times of extreme conditions do occur.

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