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Latitudinal and intracolony ITS-rDNA sequence variation in the symbiotic dinoflagellate genus *Symbiodinium* (Dinophyceae) in *Zoanthus sansibaricus* (Anthozoa: Hexacorallia)

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SUMMARY

We sequenced the internal transcribed spacer of ribosomal DNA (ITS-rDNA) of *Symbiodinium* spp. (Freudenthal) from conspecific *Zoanthus sansibaricus* (Carlgren) colonies along a latitudinal gradient in Japan. Phylogenetic analysis reveals that *Zoanthus* in the two northern sites of Kokubu and Sakurajima harbor exclusively *Symbiodinium* subclade C1, whereas Yakushima *Zoanthus* harbors *Symbiodinium* subclades C1 and C15, and southernmost Amami *Zoanthus Symbiodinium* subclades A1 and C1, indicating holobiont flexibility. Individual *Zoanthus* colonies associated exclusively with one single subclade, but unexpectedly there was small variation between *Symbiodinium* ITS-rDNA clone sequences obtained from within individual *Zoanthus* colonies. There was also a large deletion in the ITS-2/28S rDNA boundary region in one clone sequence, and another large deletion in the 5.8S rDNA region in another clone. Our intracolony sequence heterogeneity might be a result of the presence of multiple copies of the ITS-rDNA region within individual *Symbiodinium* genomes, or result from the possible presence of closely related *Symbiodinium* genotypes in the host.

Key words: cloning, deletion, holobiont, *Symbiodinium*, zoanthid, *Zoanthus*, zooxanthellae.

INTRODUCTION

The symbiotic dinoflagellate genus *Symbiodinium* (order Suessiales) plays a critical role in tropical-temperate shallow marine ecosystems. *Symbiodinium* is found in a wide variety of marine organisms, from foraminifers (Pochon *et al*. 2001) to cnidarians (Rowan & Powers 1991a,b) to mollusks (Carlos *et al*. 1999; Baillie *et al*. 2000; Belda-Baillie *et al*. 2002), providing their host with photosynthetic products from their chloroplasts (Muscatine & Porter 1977). The existence of coral reefs in a nutrient-poor environment is a result, primarily, of the *Symbiodinium*-coral host relationship (Muscatine & Porter 1977).

Originally it was thought that these dinoflagellates, which exist in hospite primarily in their vegetative non-motile state, consisted of a single species, *Symbiodinium microadriaticum* (Freudenthal 1962; Taylor 1974). However, research conducted in the last 25 years has shown that there is a remarkable biological diversity in *Symbiodinium* spp., with a multitude of undescribed 'species' (i.e. Rowan 1998; LaJeunesse 2001), with varying physiological characteristics (e.g. Tchernov *et al*. 2004).

Original work dealing with *Symbiodinium* phylogenetics was conducted using nuclear 18S ribosomal DNA (Rowan & Powers 1991a,b), whereas recent work has focused on 28S rDNA (Baker 1999), plastid 23S rDNA (Santos *et al*. 2002; Pochon *et al*. 2005), *psbA* (Takishita *et al*. 2003) and the internal transcribed spacer of nuclear rDNA (ITS-rDNA) (Hunter *et al*. 1997; Loh *et al*. 1998; LaJeunesse 2001; LaJeunesse 2002). ITS-rDNA has been used as a marker for *Symbiodinium* at the species level and below because of its high evolutionary rate (Gonzalez *et al*. 1990; Coleman & Mai 1997; Hunter *et al*. 1997). Thus far, the genus *Symbiodinium* has been divided into eight 'clades' (according to obtained nuclear and chloroplastic sequences) (Pochon *et al*. 2004, 2005), with varying numbers of individual strains within each clade. Although some general geographic and physiological trends have been discovered (e.g. Iglesias-Prieto & Trench 1994; Baker 2001), much remains to be studied.

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Research conducted using methods that minimize the observed genetic variation in *Symbiodinium* from one host individual (e.g. direct sequencing (Loh *et al*. 1998; LaJeunesse & Trench 2000; LaJeunesse 2001; Diekmann *et al*. 2002; LaJeunesse 2002; Rodriguez-Lanetty & Hoegh-Guldberg 2003) and/or culturing (LaJeunesse & Trench 2000; LaJeunesse 2001; Goulet & Coffroth 2003; Santos & Coffroth 2003)) report, little, if any, intraindividual host variation in *Symbiodinium*. In contrast, when intraindividual variation in *Symbiodinium* has been looked for (at the clade or subclade level), it has generally been found (i.e. Rowan & Knowlton 1995; Rowan *et al*. 1997; Carlos *et al*. 2000; Toller *et al*. 2001; Ulstrup & van Oppen 2003; van Oppen 2004). Similarly, Santos *et al*. (2001) show that cultured *Symbiodinium* samples do not correspond to original *in situ Symbiodinium* diversity, suggesting the presence of multiple 'types' within a single colony. If a single host can have *Symbiodinium* spp. from different clades or subclades, it is possible that there might also be genotypic variation in *Symbiodinium* at the subclade level within some hosts, especially if hosts are flexible or selecting for a certain genotype of *Symbiodinium* (Belda-Baillie *et al*. 2002). Investigating intraindividual *Symbiodinium* variation in different individual hosts of the same species would help clarify this issue. Sequence variation in cloned *Symbiodinium* ITS-rDNA sequences inside single individual host colonies has not yet been researched to a large degree.

However, much work has been conducted on the presence or absence of latitudinal variation in *Symbiodinium* clades present in the same host species over large areas. Rodriguez-Lanetty *et al*. (2001) found clade C in *Plesiastrea versipora* in low latitudes on the eastern seaboard of Australia, and further south clade B *Symbiodinium* were found. Similarly, LaJeunesse and Trench (2000) observed latitudinal variation in *Symbiodinium* in *Anthopleura elegantissima* along the Pacific coast of the USA. In addition, longitudinal variation has been observed in *Symbiodinium* in an Indian Ocean zoanthid, *Palythoa caesia* (Burnett, 2002). These investigations, along with reports of multiple clades and subclades of *Symbiodinium* in individuals of other host species (Rowan & Knowlton 1995; Rowan *et al*. 1997; Toller *et al*. 2001; van Oppen 2004) clearly show that cnidarian hosts do not always specifically associate with one *Symbiodinium* genotype. Additionally,Rowan and Knowlton (1995) show that irradiance is the most important environmental parameter influencing what *Symbiodinium* is found in which hosts, suggesting that differences in environment lead to latitudinal *Symbiodinium* variation.

The zoanthid genus *Zoanthus* (Hexacorallia), and the species *Zoanthus sansibaricus* was chosen as the host subject for this work for several reasons. Previous work (LaJeunesse 2002; LaJeunesse *et al*. 2003; T. C. LaJeunesse, 2004 pers. comm.) shows that zoanthids might contain *Symbiodinium* from a variety of genotypes and clades within individual colonies. For *Zoanthus* in general (Ryland 1997), and in particular in southern Japan (Ono *et al*. 2005), transmission of *Symbiodinium* spp. is exclusively horizontal, and not vertical/maternal, allowing for the possibility of a variety of *Symbiodinium* to be present in *Zoanthus* within and between different locations.

Here we have performed polymerase chain reaction (PCR)-amplification, cloning and subsequent sequencing of ITS-rDNA in *Symbiodinium* samples obtained from polyps in conspecific *Z. sansibaricus* colonies (according to previously obtained cytochrome oxidase I-mDNA: see Reimer *et al*. (2004)) collected from four sites in southern Japan covering a subtropical-temperate range of marine habitats. Subsequently, we investigated the following questions:

- Is there genetic variation in *Symbiodinium* from *Z. sansibaricus* colonies along a latitudinal gradient?
- Focusing on one sampling location, is there variation in *Symbiodinium* from *Zoanthus* colonies occupying different microhabitats?
- Does microvariation in obtained ITS-rDNA sequences exist in *Symbiodinium* sequences obtained from individual *Zoanthus* colonies?

MATERIALS AND METHODS

Sampling

Single polyps of conspecific *Z. sansibaricus,* according to cytochrome oxidase I sequence data (Reimer *et al*. 2004) and unpublished nuclear gene sequence data (JDR, unpubl.) containing *Symbiodinium* spp., were collected from the center upper-surface (the location most exposed to irradiance) of two or more colonies. We sampled in June–August 2003 and June–August 2004 at four field sites (Kokubu, Sakurajima, Yakushima and Amami) (Tables 1,2,3 and Fig. 1) in Kagoshima Prefecture, Japan. The samples were stored in 100% ethanol at –20°C until further analysis. In each location, two *Zoanthus* colonies were chosen, located directly next to each other (but not touching) so the colonies would experience as much as possible the same ocean temperatures, irradiance levels, wave action, and other environmental parameters. Further *Zoanthus* polyps at three of the sites (Sakurajima, Yakushima and Amami) were collected from *Zoanthus* colonies living in different microenvironments to investigate the full potential range of *Symbiodinium* existing within each site (Tables 2 and 3). To avoid confusion, we have referred to individual polyp samples as 'colony' or 'polyp' samples throughout the text.

Table 1. Summary of field sites

Site name	Latitude	Site description	Average temperature (°C) (2002–2003)†	*Zoanthus* distribution depth (m)
Kokubu	31°41′N	Volcanic rock and rubble, some sulfur vents, moderate current with little wave action	21.3	–2 to –4
Sakurajima	31°35′N	Recent volcanic rock (<100 years old), strong current with little wave action	21.2	–2 to –9
Yakushima	30°16′N	Granite rock with some hard coral, strong current with intense wave action	23.5	+1.0 to –2
Amami	28°27′N	Coral reef front, moderate current with intense wave action	24.3	+1.5 to –1

†Data obtained from field site measurements (July 2002–September 2003) taken at a depth of 3 m (see Materials and Methods for details).

Table 2. List of obtained *Symbiodinium* isolates (the present study) used in phylogenetic analyses, as well as geographic origin, sampling depth, microenvironment details and GenBank accession numbers. All isolates obtained from *Zoanthus sansibaricus*

Site	Colony number	Sample depth (m)	Microenvironment	Clade	Accession number(s)
Kokubu	1	–2.5	Subtidal, non-shaded	C1	AB190265–267
Kokubu	2	–2.5	Subtidal, non-shaded	C1	AB190268–272
Sakurajima	1	–2.0	Subtidal, non-shaded	C1	AB190273
Sakurajima	2	–2.5	Subtidal, non-shaded	C1	AB190274–276
Sakurajima	3	–2.0	Subtidal, non-shaded	C1	AB207185
Sakurajima	4	–2.0	Subtidal, non-shaded	C1	AB207186–187
Sakurajima	5	–5.0	Subtidal, shaded	C1	AB207188
Sakurajima	6	–9.0	Subtidal, non-shaded	C1	AB207189
Yakushima	1	+0.5	Intertidal, non-shaded	C1	AB190277
Yakushima	2	+0.5	Intertidal, non-shaded	C15	AB190278–279 AB207184
Yakushima	3	–2.0	Subtidal, shaded	C1	AB207190–191
Amami	1	+1.0	Intertidal, non-shaded	A1	AB190280–282 AB207197–204
Amami	2	+1.0	Intertidal, non-shaded	A1	AB190283–285
Amami	3	+1.0	Intertidal, shaded	C1	AB207192–195
Amami	4	+1.0	Intertidal, non-shaded	A1	AB207205–207
Amami	5	+0.5	Intertidal, non-shaded	A1	AB207208–209
Amami	6	–1.0	Subtidal, non-shaded	A1	AB207210
Amami	7	–1.0	Subtidal, shaded	C1	AB207196

Environmental data

Zoanthus distribution data at each field site were recorded by scuba diving and snorkeling (Tables 2 and 3). We recorded ocean temperature data (all locations, depth = 3 m) and tide pool temperature data (Yakushima and Amami only) on monthly research trips to each field site from July 2002 to October 2003 using a YSI 600 XLM Multi-parameter sonde and a YSI 650MDS handheld logging and display system (Yellow Springs, OH, USA).

DNA extraction and polymerase chain reaction amplification

Small pieces were removed from the top one-third of each sampled *Zoanthus* polyp. Pieces were seen to contain innumerable symbiont zooxanthellae. Total DNA was extracted from 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 ITS-4 (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

The purified PCR-amplified DNA fragments were cloned into the pCR2.1 vector of the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Several clones of

Table 3. List of obtained isolates (previous studies) used in phylogenetic analyses in the present study, as well as geographic origin, host species and GenBank accession numbers

Symbiodinium Clade C			
CcFIZ	West Pacific, Palau	*Corculum cardissa*	AF195144
Am8	Southwest Pacific Great Barrier Reef	*Acropora millepora*	AY237296
Amakusa I isolate 9	West Pacific, Kyushu	*Plesiastrea versipora*	AY186567
1675a	West Pacific, Guam	*Porites* rus.	AJ311944
1366	Red Sea, Gulf of Elat	*Amphisorus hemprichii*	AJ291514
TcFIZ	West Pacific, Palau	*Tridacna crocea*	AF195157
Symbiodinium clade A			
'clade A'	Caribbean, Jamaica	*Cassiopea xamachana*	AF427466
C20B	Red Sea, Gulf of Elat	*Millepora* sp.	AJ311946
C3B	Red Sea, Gulf of Elat	*Acropora* sp.	AJ311947
'clade A'	Caribbean, Florida Keys	*Aiptasia pallida*	AF427465
PHMSHP 1A	West Pacific, Philippines	*Hippopus porcellanus*	AF195151
PHBOTS 3B	West Pacific, Philippines	*Tridacna squamosa*	AF195149

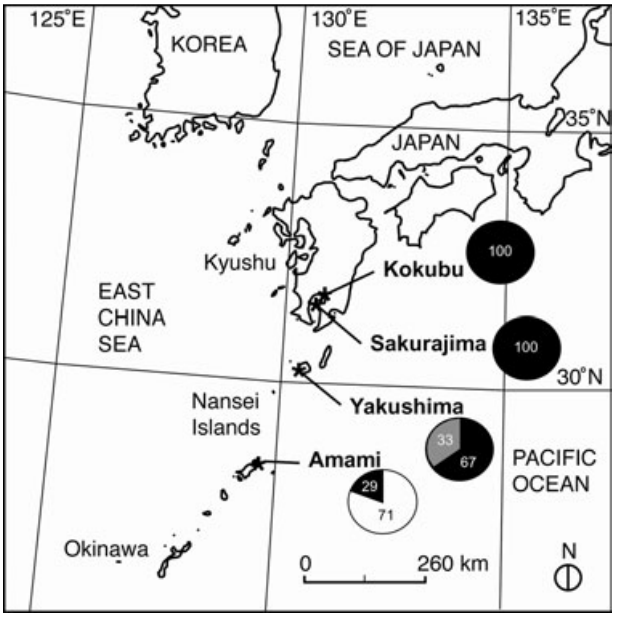

Fig. 1. Map of sampling sites and frequency distribution of *Symbiodinium* subclades in *Zoanthus sansibaricus*. The distance from the northernmost site at Kokubu to the southernmost site at Amami is approximately 400 km, and spans from temperate inland waters in the north to subtropical coral reefs on the open ocean at Amami. Within each pie diagram the frequency of *Symbiodinium* symbionts is shown, with subclade A1 in white, subclade C15 in gray, and subclade C1 in black. Numbers within each pie diagram section refer to percentage of sampled colonies possessing each *Symbiodinium* subclade.

ITS-1 – 5.8S rDNA – ITS-2 from each site 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, Foster City, CA, USA). The sequences were analyzed using DNASIS Mac version 3.6 (Hitachi Software Engineering, Tokyo, Japan).

Phylogenetic analyses

Internal transcribed spacer of ribosomal DNA sequences of *Symbiodinium* clade A and clade C were retrieved from the DNA Data Bank of Japan (Tables 2 and 3). ITS-rDNA alignments of clade A and clade C independently generated by CLUSTAL W version 1.8 (Thompson *et al*. 1997) were inspected by eye and manually edited. The alignment datasets of ITS-rDNA are available on request from the corresponding author. The datasets of clade A (26 taxa/633 sites) and clade C (33 taxa/593 sites) were separately subjected to analyses with maximum-likelihood (ML), neighbor-joining (NJ) (Saitou & Nei 1987) and maximum parsimony (MP) methods. ML analyses were performed using PhyML (Guindon & Gascuel 2003). PhyML was performed using an input tree generated by BIONJ with the general time-reversible model (Rodriguez *et al*. 1990) of nucleotide substitution 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. PhyML bootstrap trees (100 replicates) were constructed using the same parameters as the individual ML trees. The NJ tree was constructed using Kimura's 2-parameter model (Kimura 1980). Support for NJ branches was tested by bootstrap analysis (Felsenstein 1985) of 1000 replicates. The MP tree was based on the tree-bisection-reconnection branch-swapping algorithm with stepwise addition (the closest option) of taxa under the heuristic search method (50% confidence level). We conducted bootstrap analysis of 1000 replicates using the heuristic search method (50% confidence level) to assess the confidence levels of branches

in the MP tree. PAUP version 4 was used for all phylogenetic analyses in the present study (Swofford 2000).

RESULTS

Internal transcribed spacer of ribosomal DNA sequences

Clade C

All sequences from Kokubu and Sakurajima, as well as those from *Zoanthus* colonies Yaku1, Yaku3 and Amami7 matched most similarly (98–99%) with the sequence AF195144 obtained from *Symbiodinium* in the clam *Corculum cardissa* in Palau (Baillie *et al*. 2000). AF195144's ITS-rDNA region rDNA is identical to sequences AF333515 (LaJeunesse 2001), AF380533 and AF380539 (van Oppen *et al*. 2001), which fall into *Symbiodinium* subclade C1 (Rodriguez-Lanetty & Hoegh-Guldberg 2003). All designations of subclades follow the nomenclature used by LaJeunesse (2001, 2005).

All 3 obtained *Zoanthus* sequences from colony Yakushima2 matched most similarly (97–99%) with those of AF195153–AF195157 obtained from *Symbiodinium* in five different giant clam species (Baillie *et al*. 2000) in Palau. AF195153–AF195157 are identical to that of AF333518 (LaJeunesse 2001), which belongs to subclade C15 (LaJeunesse 2005).

All 24 of our aligned C1-matching sequences showed heterogeneity, both between different colonies (intercolony) as well as intracolony (within individual colony) heterogeneity. Aligned subclade C1-matching sequences had 53 variable sites (37 transitions, 12 transversions, 4 indels over 589bp). Additionally, a clone from *Zoanthus* colony Amami3 matching closely (99%) with AF195144 (Amami3a) was not used in our alignment because its short length had a large (47 bp) deletion in the 5.8S rDNA region.

Aligned subclade C15-matching sequences were 720 bp in length with 10 variable base pairs (8 transitions, 1 transversion, 2 1 bp insertions). Clones Yaku2a and Yaku2c had identical sequences and sequence Yaku2b had an 8 bp deletion in the ITS-1 region.

When all clade C-matching sequences were aligned together there were 73 variable sites (52 transitions, 15 transversions, 7 indels, with 1 variable site having both 1 transition and 1 single base pair deletion) and over 593 base pairs, not including the aforementioned Yaku2b 8 bp deletion.

Initially, a clade C tree was constructed using *Symbiodinium* clade F from *Sorites* sp. (AJ311949, Pochon *et al*. 2001) as an outgroup. Clade F is distantly related to clade C and, therefore, the resolution of the resulting tree was low. Thus, here we have presented clade C as an unrooted tree with identical topology as the rooted tree, showing higher resolution within clade C (Fig. 2). In the resulting clade C NJ tree (Fig. 2), all C1-matching sequences clustered with previously reported C1 sequences with 100% bootstrap support (both NJ and MP methods). Subclade C15-matching sequences (2 Yakushima sequences, Yaku2a/2c and Yaku2b) formed another monophyletic group with previously reported subclade C15 sequences with 100% bootstrap support (both NJ and MP methods). From this, we assigned subclade C1 and C15 identifications to these sequences.

Clade A

All sequences from the Amami *Zoanthus* colonies Amami1, Amami2, Amami4 and Amami5 had sequence microvariation and matched most closely with the sequence of AF427466 (98–99%) obtained from the jellyfish *Cassiopea xamachana* in Jamaica (Santos *et al*. 2002), which belongs to subclade A1 (LaJeunesse 2001). Novel ITS-rDNA sequence Amami6a matched closely with AJ311946 (99%) (obtained from *Millepora* sp. coral in the Gulf of Elat) and AF427466 (99%).

We next aligned the Amami clone sequences with several subclade A1, A3 and A4 sequences from previous studies, designating subclade A3 sequences as outgroup (see Tables 2 and 3). Each novel ITS-rDNA subclade A1-matching clone sequence again showed heterogeneity both between and within colonies. For the total 633 bp length there were 31 variable sites (23 transitions, 6 transversions, 4 single base pair deletions, with 1 variable site having both a transition and deletion, and another variable site having a transition and transversion). Additionally, the novel sequence Amami2b had a large deletion of 42 bp, spanning from the end of the ITS-2 region and to the beginning section of 28S-rDNA.

These Amami sequences were clustered with previously reported subclade A1 sequences with high bootstrap values (NJ: 97%, MP: 95%, Fig. 3).

We did not include some sequences mentioned above in our phylogenetic trees as they were more truncated than other sequences available.

DISCUSSION

Variation of *Symbiodinium* internal transcribed spacer of ribosomal DNA sequences within individual *Zoanthus* colonies (intracolony variation)

Our results clearly show that individual *Zoanthus* colonies at our study sites possess multiple genotypes or sequence copies of *Symbiodinium,* even though they are from only one subclade. Our results differ from the genetic heterogeneity results seen on the Yucatan Peninsula in which one individual *Zoanthus sociatus* colony possessed *Symbiodinium* from three clades and four subclades (A3, A4, B1, C1) (LaJeunesse 2002).

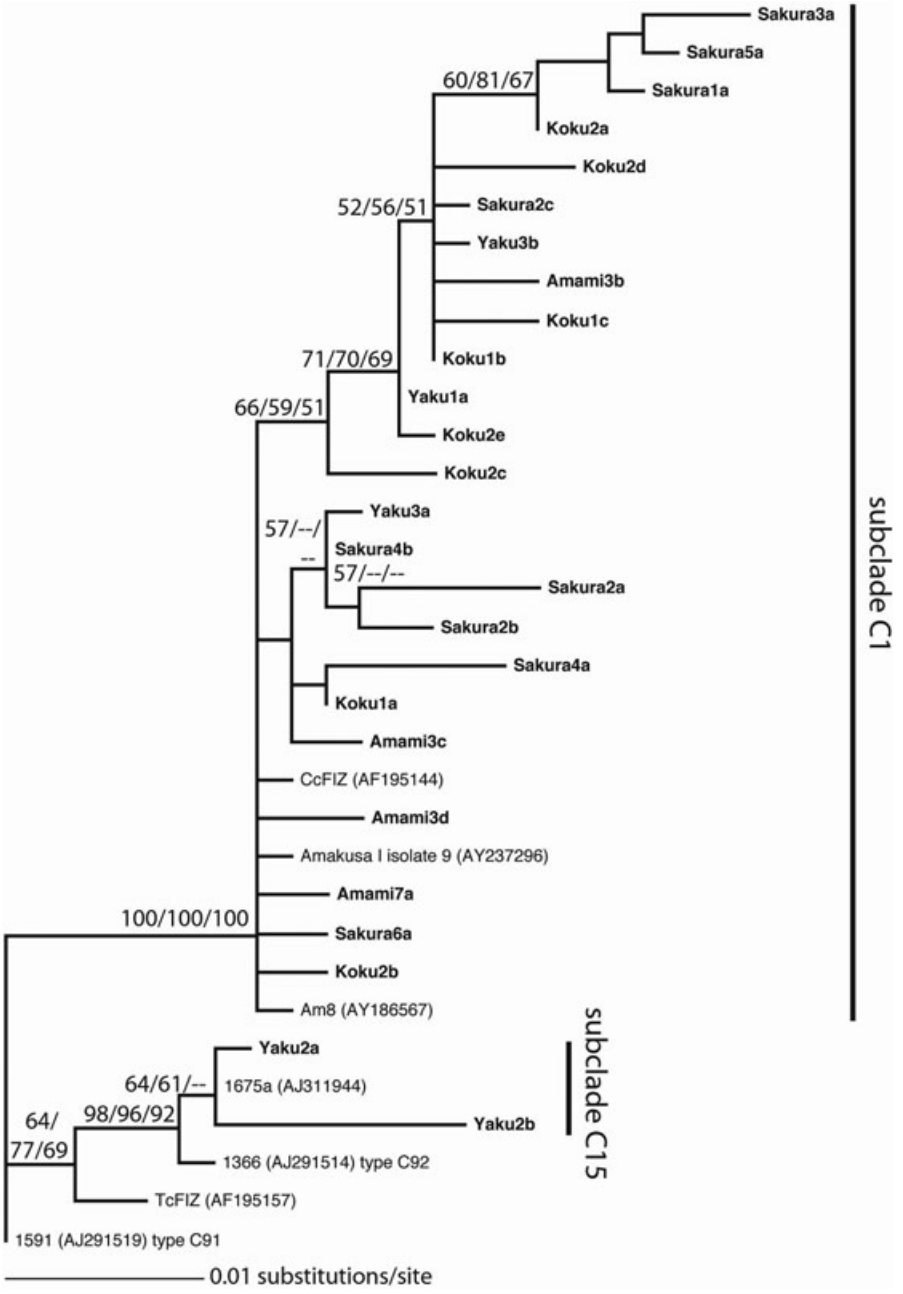

Fig. 2. Maximum-likelihood tree of the full internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences of symbiotic *Symbiodinium* dinoflagellates (clade C) associated with *Zoanthus sansibaricus*. Bootstrap values by the branches show maximum-likelihood, neighbor-joining and maximum parsimony values, respectively. (–) indicates the bootstrap value was less than 50%. Sequences in bold without GenBank accession numbers shown are ITS-rDNA sequences obtained in this study. Koku, Kokubu; Sakura, Sakurajima; Yaku, Yakushima; Amami, Amami Oshima Island.

Although it is conceivable that misincorporation inherent in the PCR process could be responsible for some of the diversity seen in our *Symbiodinium* sequences, this is unlikely as 71% (52 of 73 variable sites) of clade C and 74% (23 of 31 variable sites) of clade A base pair substitutions were transitions and, therefore, sequence variation cannot solely be a result of PCR error. Chimeras, a potential source of error (see Rodriguez-Lanetty & Hoegh-Guldberg 2003), occur in very low frequencies (2%) in samples with only a single original environmental sequence (Speksnijder *et al*. 2001).

In a recent study, microsatellite flanking region diversity in *Symbiodinium* from the Caribbean at the reef scale within a common lineage of clade B has been observed despite subject samples all belonging to a single ITS-rDNA lineage (Santos *et al*. 2004). However, it has been shown that ITS-rDNA evolves much more slowly in clade B (Santos *et al*. 2004), and it follows that ITS-rDNA heterogeneity in *Symbiodinium* other than clade B inside individual hosts might exist, echoing the clade B microsatellite flanking region diversity observed by Santos *et al*. (2004).

Zoanthus sansibaricus at the Sakurajima site has been shown to annually sexually reproduce (Ono *et al*. 2005). *Zoanthus* planulae are azooxanthellate (Ryland 1997), and must acquire their *Symbiodinium* from the environment. Therefore, long-term clone–symbiont

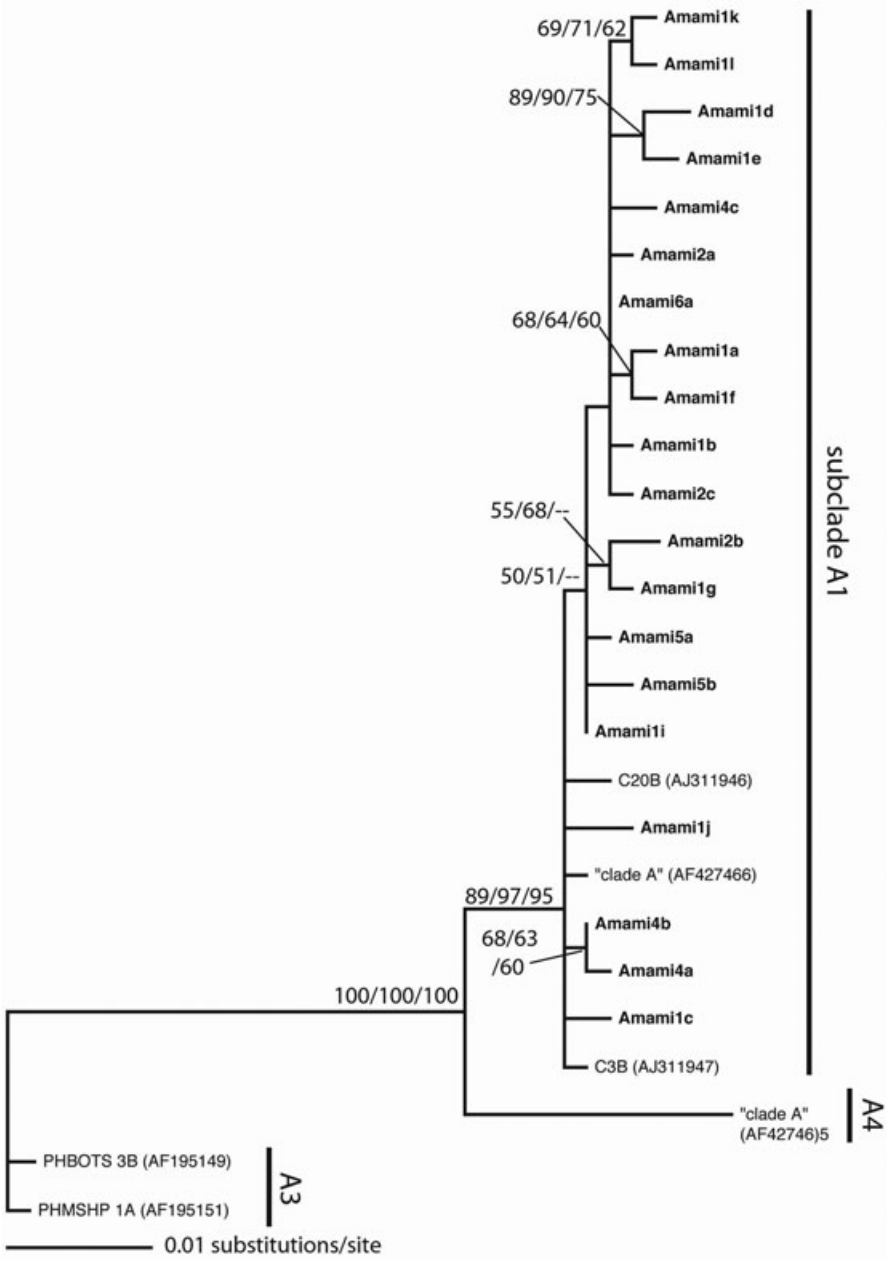

Fig. 3. Maximum-likelihood tree of the full internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences of symbiotic *Symbiodinium* dinoflagellates (clade A) associated with *Zoanthus sansibaricus*. Bootstrap values by the branches show maximum-likelihood, neighbor-joining and maximum parsimony values, respectively. Sequences in bold without GenBank accession numbers shown are ITS-rDNA sequences obtained in this study. For phylogenetic trees showing the relationship between *Symbiodinium* clades A and C (based on 28S and ITS2 rDNA data), refer to Pochon *et al*. (2004). Amami, Amami Oshima Island.

association cannot be considered as a possible explanation for the observed ITS-rDNA sequence heterogeneity. Further negating this theory, there appears to be no relation between mode of zooxanthellae transmission (horizontal or vertical/maternal) and *Symbiodinium* diversity, at least in the case of host acropoid corals (van Oppen 2004).

If *Symbiodinium* has only a single copy of the ITS-rDNA region, as shown in a preliminary study (Baillie *et al*. 2000), then it is reasonable to explain our results as intracolony genotypic variation. However, sample numbers in Baillie *et al*. (2000) are limited, so it is impossible to draw definite conclusions about our observed microvariation. Adding to this uncertainty, 3 of our samples possessed deletions, including a 42 bp deletion seen in Amami2b that spanned the end of the ITS-2 region and the beginning of the 28S rDNA, and a deletion in Amami3a of 47 bp incorporated entirely within the 5.8S rDNA region. In the dinoflagellate genus *Alexandrium* both 18S rDNA (Scholin *et al*. 1993; Scholin & Anderson 1994) and 28S rDNA (Yeung *et al*. 1996) have been shown to have pseudogenes. Also, in *Symbiodinium* the presence of an 18S rDNA pseudogene is suggested by Santos *et al*. (2003). It is highly possible that individual *Symbiodinium* has multiple copies of ITS-rDNA, either included in a single genome (as observed by Yokouchi *et al*. 2004) or as a pseudogene. Alternately, *Zoanthus* might possess many

closely related genotypes from a single *Symbiodinium* subclade, resulting in an adaptive mechanism allowing the holobiont (host + symbiont) greater flexibility to acclimate to different environments, just as it has been suggested for possessing multiple *Symbiodinium* clades in hospite (Buddemeier *et al*. 2003). The answer to this issue is of great importance in understanding the *Symbiodinium*-host relationship, as *Symbiodinium* with single base pair differences in the ITS-rDNA region have been shown to occupy distinct habitats (LaJeunesse *et al*. 2003).

Intercolony and latitudinal variation of *Symbiodinium* internal transcribed spacer of ribosomal DNA sequences

Our *Symbiodinium* ITS-rDNA data show an unambiguous latitudinal distribution of clades and subclades, with subclade C1 in the north (Kokubu and Sakurajima), a mixture of subclades C1 and C15 in the middle (Yakushima), and subclades A1 and C1 in the south (Amami). As stated previously, irradiance is the most important environmental parameter influencing what *Symbiodinium* is found in which hosts (Rowan & Knowlton 1995). According to this observation and based on our data, it would appear that subclade C1 is less adapted and subclade A1 more adapted to higher light and temperature levels, with subclade C15 in between.

At Kokubu and Sakurajima *Zoanthus* colonies are found only subtidally (at depths of 2–10 m) (Tables 2 and 3), and light levels reaching subclade C1-bearing *Zoanthus* at Kokubu and Sakurajima are much lower than light levels experienced by more southern and intertidal Yakushima and Amami *Zoanthus*. All sampled *Zoanthus* colonies across the full range of depth distribution at Sakurajima possessed only subclade C1 *Symbiodinium*. In addition, the waters of Kagoshima Bay at Kokubu and Sakurajima are consistently less clear than waters at Yakushima and Amami (SO, JDR, JT, 2005, unpubl. data), further reducing irradiance reaching *Zoanthus* colonies at the two northern sites. Ocean temperatures at the two northern sites were also consistently lower than at the two southern sites (Table 1). *Zoanthus* at Yakushima experiences higher temperature and irradiation levels than Kokubu or Sakurajima, as unlike at Kokubu and Sakurajima *Zoanthus* is it found intertidally (Tables 2 and 3), and thermally tolerant *Symbiodinium* subclade C15 (see LaJeunesse 2005) was present in one intertidal *Zoanthus* colony.

Zoanthus at the Amami site exist in a much more inconstant environment than at the other three sites in this study. Aside from being primarily intertidal (unlike Sakurajima and Kokubu) (Tables 2 and 3), *Zoanthus* colonies exist on the outer edge of a large coral reef flat (unlike Yakushima), and are directly exposed to waves as well as strong sunlight. In addition, tide pools at the Amami site are have less shade and are much shallower than Yakushima tide pools and, therefore, more susceptible to intertidal temperature change than at Yakushima (Tables 2 and 3). However, within the two *Zoanthus* colonies at Amami investigated in shaded microenvironments (Tables 2 and 3), *Symbiodinium* subclade C1 was exclusively found.

Previous results support our observed *Symbiodinium* patterns. *Symbiodinium* of subclade C1 is an Indo-Pacific generalist, whereas subclade C15 is more thermally tolerant (LaJeunesse 2005). Rowan and Knowlton (1995) and Rowan *et al*. (1997) found *Symbiodinium* clade A in shallow (0–6 m) waters in *Montastrea*, whereas clade C was found at all depths. Similarly, Toller *et al*. (2001) and LaJeunesse (2002) found clade A in shallow waters with high irradiance. Banaszak *et al*. (2000) show that only *Symbiodinium* in clade A produce ultraviolet-absorbing mycosporine-like amino acids. These results suggest that clade A is adapted to highly irradiated environments. Furthermore, different *Symbiodinium* genotypes have different high-end thermal tolerances (for example Tchernov *et al*. 2004), and our observed latitudinal variation of different subclades of *Symbiodinium* might be a result, in part, to differing temperatures between the sites. It appears that *Zoanthus*-clade A1 *Symbiodinium* holobionts on Amami Island are adapted to a highly irradiated and constantly changing intertidal microenvironment with higher temperatures and larger temperature ranges compared to the more northern clade C1-bearing *Zoanthus* colonies that inhabit slightly cooler water (in terms of both lower ambient seawater temperatures (Kokubu, Sakurajima and to a lesser degree Yakushima) and lower 'low tide' intertidal temperatures (Yakushima) and/or experience lower light levels (colonies subtidal at Kokubu and Sakurajima, in deeper tide pools with more shade on Yakushima).

Although we did not detect any *Symbiodinium* subclades other than C1, C15 and A1 in our 49 novel clone sequences, we cannot discount the presence of cryptic (non-dominant) *Symbiodinium* from other clades. However, even if such cryptic populations exist in our samples, they do not affect the fact of our observed latitudinal variation, nor the observed intracolony sequence variation.

Conclusions

In summary, it appears that conspecific host *Z. sansibaricus* colonies have a flexible association with *Symbiodinium* over latitudinal and microenvironmental (microhabitat) gradients (such as temperature and light levels). Additionally, individual *Zoanthus* colonies have fine-scale sequence variation within *Symbiodinium* subclades. Whether this intracolony variation is a result of a variety of symbiont genotypes allowing host flexi-

bility (reminiscent of latitudinal variation) or a result of multiple copies of ITS-rDNA within individual *Symbiodinium* cells (as the majority of previous evidence suggests) remains to be studied. Further studies using common host species investigating ITS-rDNA *Symbiodinium* diversity should be conducted to help ascertain whether the situation observed here in *Zoanthus* is an exception or the norm.

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