ZOOLOGICAL SCIENCE **24**: 346–359 (2007)

Molecular Evidence Suggesting Interspecific Hybridization in *Zoanthus* spp. (Anthozoa: Hexacorallia)

James Davis Reimer[1*†], Kiyotaka Takishita[1], Shusuke Ono[2], Junzo Tsukahara[3] and Tadashi Maruyama[1]

[1]*Research Program for Marine Biology and Ecology, Extremobiosphere Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan*
[2]*Miyakonojo Higashi High School, Mimata, Miyazaki 889-1996, Japan*
[3]*Department of Developmental Biology, Faculty of Science, Kagoshima University, Korimoto 1-21-35, Kagoshima, Kagoshima 890-0065, Japan*

Interspecific hybridization has been proposed as a possible explanation for the incredible diversity seen in reef-dwelling corals, but until now little proof of such hybridization in other reef-dwelling anthozoans has been reported. Without further observation of hybridization, the question of such a phenomenon being widespread in Anthozoa remains. Here we have examined the mitochondrial cytochrome oxidase I gene (COI) and the nuclear internal transcribed spacer of ribosomal DNA (ITS-rDNA) from three species of the mass-spawning, encrusting anemone genus *Zoanthus* (*Z. sansibaricus, Z. kuroshio, Z. gigantus*) to investigate possible hybridization. The three species coexist at two of three sampling locations in southern Japan. *Zoanthus* spp. ITS-rDNA region spacers (ITS-1 and ITS-2) were shown to have very high rates of divergence. At locations where all three species co-existed, several of our sampled *Z. sansibaricus* individuals (with identical "sansi" COI sequences) possessed two very divergent (*i.e.*, species-level difference) ITS-rDNA alleles, the expected "sansi" allele and the divergent "B" allele. Additionally, two *Z. sansibaricus* individuals possessed only "B" alleles despite having "sansi" COI sequences. These results indicate that *Z. sansibaricus* has possibly experienced interspecific hybridization at least once with a *Zoanthus* partner possessing the "B" allele, and that these resulting hybrids may also sexually reproduce, demonstrating potential hybridization occurring in the order Zoantharia (Hexacorallia).

Key words: COI, hybridization, ITS-rDNA, zoanthid, *Zoanthus*

INTRODUCTION

It has been proposed that the bewildering variety and apparently unclear species boundaries in many genera of corals may be at least partially due to interspecific hybridization and subsequent reticulate evolution (Veron, 1995). Similarly to plants, cnidarians of the orders Octocorallia (soft corals) and Hexacorallia (corals, zoanthids, and anemones) often display a wide diversity of morphospecies over geographical (*i.e.*, ocean current) gradients, and many different morphospecies often inhabit the same marine environment (Veron, 1995). Many different species and genera of Hexacorallia have also been shown to reproduce in synchronous phase with the moon in mass spawning events (see Levitan *et al*., 2004; Penland *et al*., 2004; Ono *et al*., 2005). Such conditions appear to be ideal for hybridization events between species (Veron, 1995).

Genetic studies investigating potential hybridization require the use of molecular markers with a high evolutionary rate, and the most widely used marker in cnidarians has been the internal transcribed spacer region of ribosomal DNA (ITS-rDNA). The ITS-rDNA region (used along with mitochondrial DNA sequence data), despite the potential presence of pseudogenes (Alvarez and Wendel, 2003; Marquez *et al*., 2003), has been particularly useful in exploring evolutionary patterns due to its extremely non-conservative nature (for example, see Hunter *et al*., 1997; Odorico and Miller, 1997; Medina *et al*., 1999; van Oppen *et al*., 2000, 2002; Diekmann *et al*., 2001; Marquez *et al*., 2003; Fukami *et al*., 2004).

Several recent studies have strongly suggested the occurrence of interspecific hybridization in clonal cnidarians. Hatta *et al*. (1999) and Marquez *et al*. (2002) have shown that several species in the genus *Acropora* can hybridize in vitro, albeit with varying degrees of reproductive success. McFadden and Hutchinson (2004) have shown that according to ITS-rDNA data, the soft coral species *Alcyonium*

* Corresponding author. Phone: +81-98-895-8542;
Fax : +81-98-895-8576;
E-mail: jreimer@jamstec.go.jp

† Present address: Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan
doi:10.2108/zsj.24.346

hibernicum may be of hybrid origin. Utilizing the ITS-rDNA region, other cnidarian genera shown to hybridize include *Madracis* (Diekmann *et al*., 2001), *Acropora* (Odorico and Miller, 1997, van Oppen *et al*., 2001), and *Montipora* (van Oppen *et al*., 2004), suggesting that hybridization, reticulate evolution, and/or introgression (hybrids backcrossing with parents) may be widespread in Anthozoa.

Here, we investigated three species of *Zoanthus* (Hexacorallia: Zoanthidae) at three sites in southern Japan using the nuclear DNA marker ITS-rDNA to explore the possibilities of hybridization between species, and utilized the mitochondrial DNA marker cytochrome oxidase I gene (COI) to confirm species-level relationships. Previous research has shown that that *Zoanthus sansibaricus* investigated here reproduces in synchrony with the moon phase as a hermaphroditic mass spawner (Ono *et al*., 2005). While nothing is known of the sexual reproduction of the other two *Zoanthus* species investigated here, previous research has shown gamete maturation to occur in summer months for both Pacific (*Z*. aff. *pacificus*; Cooke, 1976) and Atlantic (*Zoanthus* spp.; Karlson, 1981) *Zoanthus* species. Similarly, *Palythoa* (*Protopalythoa*) on the Great Barrier Reef has been also shown to spawn in synchrony with moon phase as a hermaphroditic mass spawner (Babcock and Ryland, 1990; Ryland and Babcock, 1991). At all three sites, large numbers of *Zoanthus* coexist within very small areas in the infra- and sub-littoral zones, increasing the potential of hybridization events occurring in situ. Based on our genetic results, we discuss hybridization and possible evolutionary patterns in *Zoanthus* spp.

MATERIALS AND METHODS

Sampling

Samples of *Zoanthus* spp. were collected from three field sites (Sakurajima, Yakushima, Amami) in Kagoshima Prefecture, Japan, in June-August 2003, June-September 2004, and December 2005, and stored in 80–100% ethanol at –20°C. Samples were collected of all *Zoanthus* morphotypes observed at each site across the full range of depths and environments and are preserved in collections at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC, Yokosuka, Japan) and museums worldwide (see Reimer *et al*., 2006a for details). As samples were collected *in situ*, photographs were taken to assist in identification and for the collection of morphological data (oral disk/polyp diameter, color, tentacle count, polyp form). A sample of *Parazoanthus gracilis* was collected from Jogasaki, Shizuoka Prefecture, Japan in November 2004 to use as an outgroup for phylogenetic analyses.

Sample nomenclature

Samples were assigned names based on species, sampling site, and sampling number (Table 2). For example, sample ZAT1 is sample *Zoanthus sansibaricus* from Amami, Tomori Beach, sample number 1 at this site. Sample names with hyphens and terminal letters or numbers represent ITS-rDNA sequence clones (*i.e*., ZAT1-1). Samples and sequences from Reimer *et al*. (2004) follow the nomenclature used in that article, with an explanation attached in Table 2.

DNA extraction, PCR Amplification, cloning, and sequencing

DNA was extracted from samples weighing 5–20 mg using the spin-column DNeasy Animal Extraction protocol (Qiagen, Santa Clarita, CA, USA) (LaJeunesse and Trench, 2000). PCR amplification using genomic DNA as a template was performed using HotStarTaq DNA polymerase (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. The COI gene was amplified using primer set LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al*., 1994), with the following thermal-cycle conditions: 35 cycles of 1 min at 94°C, 1 min at 40°C, and 90 sec at 72°C, with a final elongation step of 7 min at 72°C. The ITS-rDNA region was amplified with newly designed Zoanthidae-specific primer set Zoan-f (5'-CTT GAT CAT TTA GAG GGA GT-3') and Zoan-r (5'-CGG AGA TTT CAA ATT TGA GCT-3'), except for *Z. kuroshio* samples, which were amplified with Zoan-f and a general primer (pITS-r; 5'-TCC TCC GCT TAT TGA TAT GC-3') (Sugita *et al*., 1999). All ITS-rDNA samples were amplified under the following thermal-cycle conditions: 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a final elongation step of 10 min at 72°C. The amplified products were checked by 1.5% agarose gel electrophoresis. Some PCR-amplified DNA fragments were cloned into the pCR2.1 vector from a TOPO TA Cloning Kit (Invitrogen, Berkeley, CA, USA) and sequenced with an ABI PRISMTM 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). The sequences were analyzed using GENETYX-MAC version 8.0 (Software Development, Tokyo, Japan) and DNASIS Mac v3.6 (Hitachi Software Engineering Company, Ltd., Tokyo, Japan).

Phylogenetic analyses

New sequences obtained in the present study were deposited in DDBJ and GenBank (accession numbers AB214123–AB214178, AB252665–AB252676, and DQ442406–DQ442493). By using CLUSTAL X version 1.8 (Thompson *et al*., 1997), the nucleotide sequences of the COI gene and 5.8S rDNA from *Zoanthus* species were separately aligned with COI and 5.8S rDNA sequences from

Table 1. Summary of *Zoanthus* species sampled.

Name	Habitat/depth	Oral disk diameter (mm)	Colony/polyp form	Oral disk color	Polyp color
Z. sansibaricus	Exposed or shaded rocks & coral, +1.5 to -10.0m	3~12	Connected growth of polyps, polyps extending from coenenchyme	Wide variety (green, purple, red, blue, yellow, brown, orange)	Dark purple
Z. kuroshio[1]	Exposed or shaded rocks & coral, +0.5 to -3.0m	6~10	Connected growth of polyps, polyps embedded in coenenchyme	Little variety, (usually pale pink)	Pale purple
Z. gigantus[1]	Shaded cracks in rocks, +1.0 to -2.0m	6~24	Small colonies, polyps connected to less-developed coenenchyme	Some variety (green, blue, brown)	Dark purple with white markings or stripes near oral disk

[1]New species described in Reimer *et al*. (2006a).

Table 2. List of samples

Species	Sample name[a]	Depth (m)	Oral disk diameter (mm)	Oral disk color(s)	COI clade[b]	ITS clade (# clones)[b]	Designation
Z. sansibaricus	SakZpac1	–2	6~10	bright green	sansi[1]	sansi (4)	pure sansi
	SakZpac2	–2	6~10	bright green	sansi[1]	sansi	pure sansi
	SakZ1	–3	6~10	green-brown	sansi[1]	sansi	pure sansi
	SakZ2	–3	6~10	green-brown	sansi[1]	sansi	pure sansi
	SakZery1	–3	6~10	red, blue, white	sansi[1]	sansi	pure sansi
	SakZery2	–3	6~10	red, blue, white	sansi[1]	sansi	pure sansi
	SakZ3	–3	6~10	yellow	sansi[1]	sansi	pure sansi
	SakZ4	–3	6~10	yellow	sansi[1]	sansi	pure sansi
	SakZ5	–3	6~10	red, white	sansi[1]	sansi	pure sansi
	SakZgnop	–3	6~10	green, blue	sansi[1]	sansi	pure sansi
	SakZ6	–3	6~10	yellow	sansi[1]	sansi	pure sansi
	SakZ7	–3	6~10	white	sansi[1]	sansi	pure sansi
	SakZsansi	–3	6~10	purple	sansi[1]	sansi	pure sansi
	SakZ8	–3	6~10	white	sansi[1]	sansi	pure sansi
	SakZ9	–3	6~10	green	sansi[1]	sansi	pure sansi
	ZSH1	–5	6~8	light green	sansi	sansi	pure sansi
	ZSH2	–5	6~8	light green	sansi	sansi	pure sansi
	ZSH17	–1	6~8	light green	sansi	sansi	pure sansi
	ZSH23	–9	6~8	white	sansi	sansi	pure sansi
	YakZpac1	1	6~10	bright green	sansi[1]	mixed	sansi/B F_1
	YakZpac2	1	6~10	bright green	sansi[1]	sansi	pure sansi
	YakZ1	1.5	6~8	green	sansi[1]	sansi	pure sansi
	YakZ2	–1	8~12	white, green	sansi[1]	B (16)	sansi/B F_{2+}
	YakZ5	–1	8~12	purple, white	sansi[1]	mixed	sansi/B F_1
	AmamiZpac1	0.5	6~10	bright green	sansi[1]	mixed	sansi/B F_1
	AmamiZpac2	0.5	6~10	bright green	sansi[1]	mixed	sansi/B F_1
	AmamiZ1	0	4~6	green	sansi[1]	mixed	sansi/B F_1
	AmamiZ2	0.5	4~8	purple	sansi[1]	sansi	pure sansi
	AmamiZ5	2	4~8	purple	sansi[1]	mixed	sansi/B F_1
	ZAT1	0	6~8	bright green	sansi	sansi	pure sansi
	ZAT2	0	6~8	white	sansi	sansi/B (9/4)	sansi/B F_1
	ZAT3	0	3~6	light purple	sansi	sansi	pure sansi
	ZAT4	0	3~6	light yellow	sansi	sansi	pure sansi
	ZAT5	0.5	3~6	white	sansi	sansi/B (5/3)	sansi/B F_1
	ZAT6	–1	6~8	bright green	sansi	mixed	sansi/B F_1
	ZAT7	–1	6~8	purple	sansi	sansi	pure sansi
	ZAT11	1.5	3~6	white, purple	sansi	B	sansi/B F_{2+}
Z. kuroshio	ZkYS1	–1.5	6~8	white, light pink	kuro	kuro	pure kuro
	ZkAT9	–1	6~8	white, light pink	kuro	kuro (6)	pure kuro
	ZkAT12	–3	6~8	white, light pink	kuro	kuro (11)	pure kuro
	ZkYS21	–0.5	8~12	white, pink	kuro	kuro (6)	pure kuro
	ZkYS22	–0.5	8~12	white, pink	kuro	kuro (8)	pure kuro
	ZkYS23	–0.5	8~12	white, pink	kuro	kuro (11)	pure kuro
Z. gigantus	AmamiZg4	1	6~10	fl. green	giga[1]	giga (6)	pure giga
	ZgYS8	0	8~20	mint green, white	giga	giga (11)	pure giga
	ZgYS9	0	8~20	mint green, white	giga	giga (12)	pure giga
	ZgYS10	0	8~20	mint green, white	giga	giga (9)	pure giga
	ZgYS11	–0.5	10~20	blue, dark green	giga	giga (11)	pure giga
	ZgYS12	–0.5	10~20	blue, dark green	giga	giga (12)	pure giga
	ZgYS13	–0.5	14~20	NA	giga	giga (12)	pure giga
	ZgYS15	0	12~20	blue, dark green	giga	giga (12)	pure giga
	ZgYS16	0	12~20	blue, dark green	giga	giga (12)	pure giga
Parazoanthus gracilis	PgIJ1	–18	1~3	bright yellow	para	para	pure para

[a]Sample name abbreviations: Z=*Zoanthus* (*sansibaricus* species), Zg=*Z. gigantus*, Zk=*Z. kuroshio*, Pg=*Parazoanthus gracilis*, SH=Sakurajima (site), Hakamagoshi (locale), YS=Yakushima, Sangohama, AT=Amami ,Tomori, IJ=Izu Penninsula, Jogasaki. All sample names from this study followed by a sample number. Other sample names (*i.e*. SakZpac1) follow nomenclature used in Reimer *et al*. (2004), with the following abbreviations: Sak=Sakurajima, Yak=Yakushima, Zpac=Z. aff. *pacificus* morphotype, Zery=*Z*. aff. *erythrochloros* morphotype, Zgnop=*Z*. aff. *gnophodes* morphotype, Zsansi=*Z*. aff. *sansibaricus* morphotype, Z=unknown *Zoanthus* sp., and Zg=*Z. gigantus*. [b]Sequence abbreviations: sansi=sansibaricus, B=allele B, kuro=kuroshio, gigan=gigantus, mixed=direct PCR resulted in "mixed" signals, para=parazoanthus.

[1]Sequences previously obtained from Reimer *et al*. (2004); Genbank Accession #s **AB128893**~**AB128898** and **AB194014**~**AB194036** (supplemental data).

Numbers in parentheses represent number of clones (no numbers indicate data collected by direct sequencing). Novel sequences obtained in this study are **AB214123**~**AB214178**, **AB252665**~**AB252676** and **DQ442406**~**DQ442493**.

Parazoanthus gracilis as outgroups (ITS-1 and ITS-2 rDNA regions were unalignable; see Table 3 for ITS-1 and ITS-2 lengths acquired from the samples). The alignments were inspected by eye and manually edited. All ambiguous sites of the alignments were removed from the data set for phylogenetic analyses. In this manner, we generated two aligned datasets: 1) 592 sites of 50 sequences (the COI gene); and 2) 157 sites of 92 sequences (5.8S rDNA). The alignment data are available on request from the corresponding author.

For phylogenetic analyses of the COI gene and 5.8S rDNA sequences, the same methods were independently applied. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and 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 data sets. PhyML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees.

The neighbour-joining (NJ) method (Saitou and Nei, 1987) was performed using PAUP* Version 4.0 (Swofford, 1998) with the Kimura-2 parameter model (Kimura, 1980). NJ bootstrap trees (1,000 replicates) were constructed using the same model.

Bayesian trees were also reconstructed by using the program MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) under GTR+I+Γ. One cold and three heated Markov-chain Monte-Carlo (MCMC) analyses with default-chain temperatures were run for 1,000,000 generations, with log-likelihoods (lnLs) and trees sampled at 100-generation intervals (10,000 lnLs and trees were saved during MCMC). The likelihood plots for the COI and 5.8S rDNA data sets suggested that MCMC reached the stationary phase after the first 40,000 and 50,000 generations, respectively. Thus, the remaining 960,000 and 950,000 trees of COI and 5.8S rDNA, respectively, were used to obtain clade probabilities and branch-length estimates.

Minimum-spanning networks were constructed with the program TCS version 1.2.1 (Clement *et al*., 2000) for the COI and 5.8S rDNA data sets using the variable sites of each data set.

ITS-rDNA utility assessment and intragenomic polymorphism

In order for the ITS-rDNA region to be an accurate species-specific marker, the amount of intra-genomic polymorphism (IGP) must be screened and corroborated with data from other loci (Vollmer and Palumbi, 2004; Worheide *et al*., 2004). Based on our cloning results, the ITS-rDNA region appeared to be an accurate

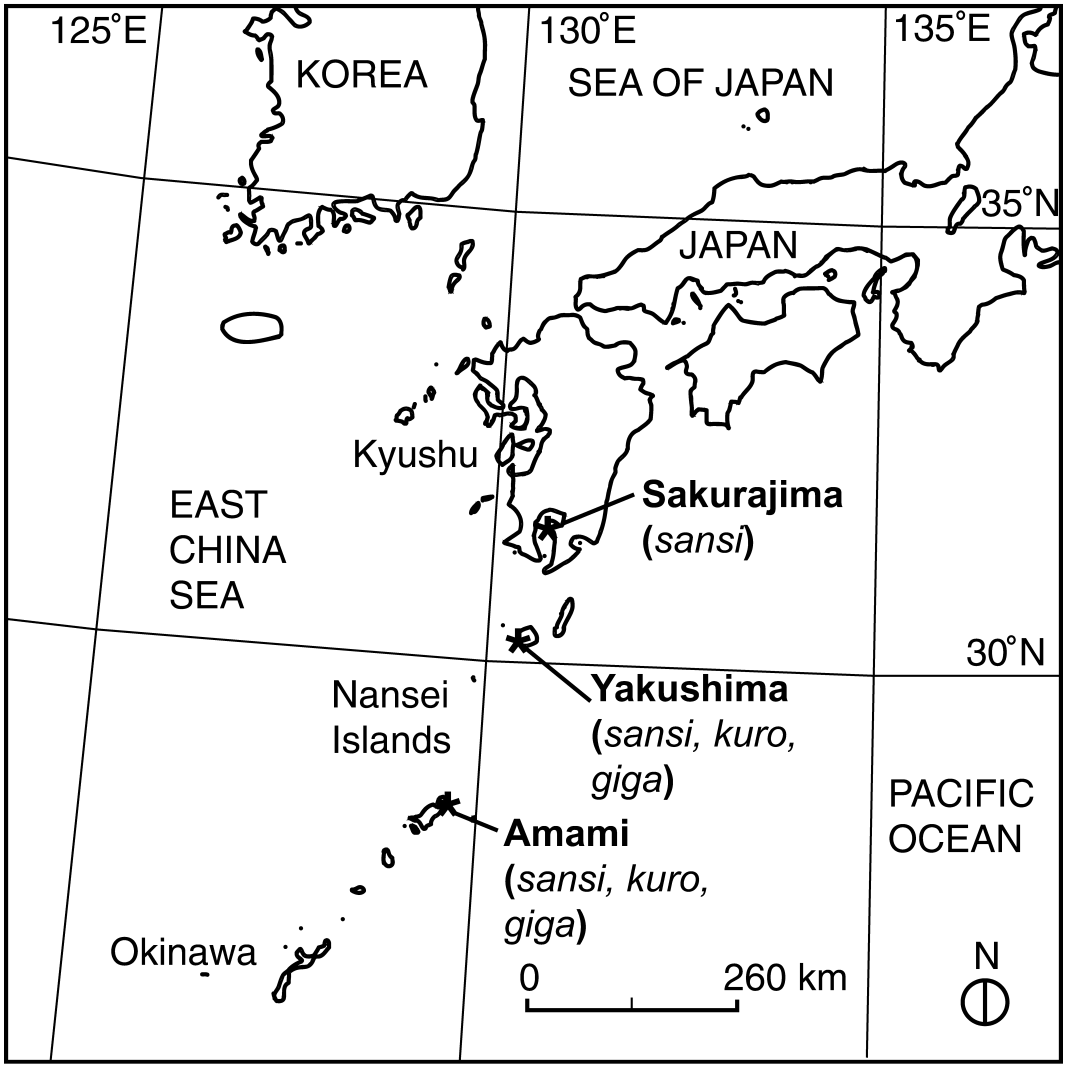

Fig. 1. Map of sampling locations in southern Japan and the *Zoanthus* species found at each location. Abbreviations: *sansi, Z.sansibaricus*; *kuro, Z. kuroshio*; *giga, Z. gigantus*.

marker for investigating *Zoanthus* species. Preliminary experiments showed IGP in samples of *Z. gigantus* and *Z. sansibaricus* to be at acceptable levels (6/911bp=0.7% and 1/782bp=0.1%, 5 clones each), while interspecific ITS-rDNA variation between these two species was very high (see alignment in Fig. 5), particularly in the ITS-1 and ITS-2 regions, which were highly divergent in length (Table 3). Based on these results, we chose the ITS-rDNA region as the nuclear marker we utilized along with COI mtDNA.

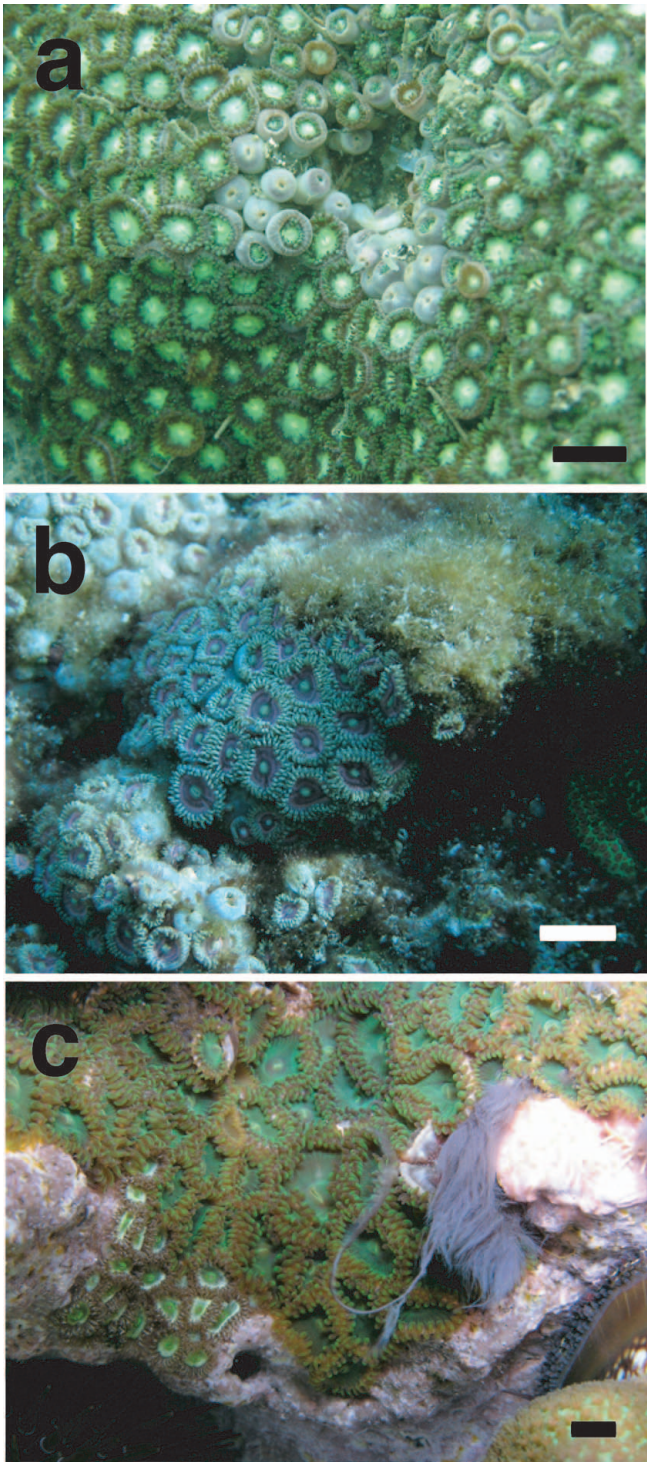

Fig. 2. In situ photographs of **(a)** *Zoanthus sansibaricus* (sample ZSH1, Sakurajima, depth -5 m), **(b)** *Z. kuroshio* (sample ZkYS1, Yakushima, depth -1 m), and **(c)** *Z. gigantus* (sample ZgYS1, Yakushima, depth -1.5 m). All depths relative to extreme low tide line. In (c), note *Z. sansibaricus* colony (white and green) in lower left corner for size comparison. White/black scales=1 cm.

RESULTS

Zoanthus sampling

Based on morphology, our *Zoanthus* samples grouped into three species groups; a) *Zoanthus sansibaricus*, corresponding to a variety of morphotypes previously shown to be conspecific (including *Z*. aff. *pacificus*, *Z*. aff. *erythrochloros*, and *Z*. aff. *gnophodes*; see Reimer *et al*., 2004, 2006a), b) *Zoanthus kuroshio*, generally light pink in oral-disc color and polyps much more embedded in the coenenchyme than *Z. sansibaricus*, and c) *Zoanthus gigantus*, much larger (>2X) both in oral-disc diameter and polyp size than either *Z. sansibaricus* or *Z. kuroshio*, with white or pale pink striping patterns on the upper external surface of the polyp (Tables 1 and 2). Detailed species descriptions of the new species *Z. kuroshio* and *Z. gigantus* have appeared elsewhere (Reimer *et al*., 2006a). Only *Z. sansibaricus* morphotypes were observed at Sakurajima, while all three species were seen at Yakushima and Amami (Figs. 1 and 2). *Zoanthus sansibaricus* was abundant at all three sites investigated, while *Z. kuroshio* was abundant at Yakushima and Amami but not present at Sakurajima. *Zoanthus gigantus* (also found at Yakushima and Amami) colonies were comparatively much less abundant than the other two species. For additional data on the distribution and morphological characteristics of the *Zoanthus* spp. used in this study, refer to Fig. 2 and Table 2.

Sequences and phylogeny of the COI gene

The ML tree based on the COI sequences is shown in Fig. 3. All assumed *Z. sansibaricus* samples formed a monophyletic group, with strong ML bootstrap support (98%). All presumed *Z. kuroshio* formed a separate monophyletic group sister to *Z. sansibaricus*, with lower ML bootstrap support (81%). *Zoanthus gigantus* samples also formed a monophyletic group, with high ML bootstrap support (88%). Bayesian posterior probability values for the monophyletic groups of *Z. kuroshio* and *Z. gigantus* were not so high (less than 80%), but as our alignment showed clear species-specific substitutions, we are confident in the phylogenetic topology reconstructed here. Overall, *Z. kuroshio* COI sequences differed from *Z. sansibaricus* by 0.7% (4/592 bp), and *Z. gigantus* COI sequences differed from *Z. sansibaricus* by 1.3% (8/592 bp). There were no differences in COI sequences within each of the three species groups. Based on our present results regarding the COI phylogeny and past exam-

Table 3. Varying base pair lengths and GC% for obtained ITS-rDNA sequences for different alleles in *Zoanthus* spp.

allele	ITS-1	5.8S	ITS-2	GC content (%) of entire ITS-rDNA
"sansi"	236~237	156	234~241	44.6
"B"	337~343	156	175	52.4
"giga"	346~348	156	246~250	51.6
"kuro"	295~297	156	190~192	58.8

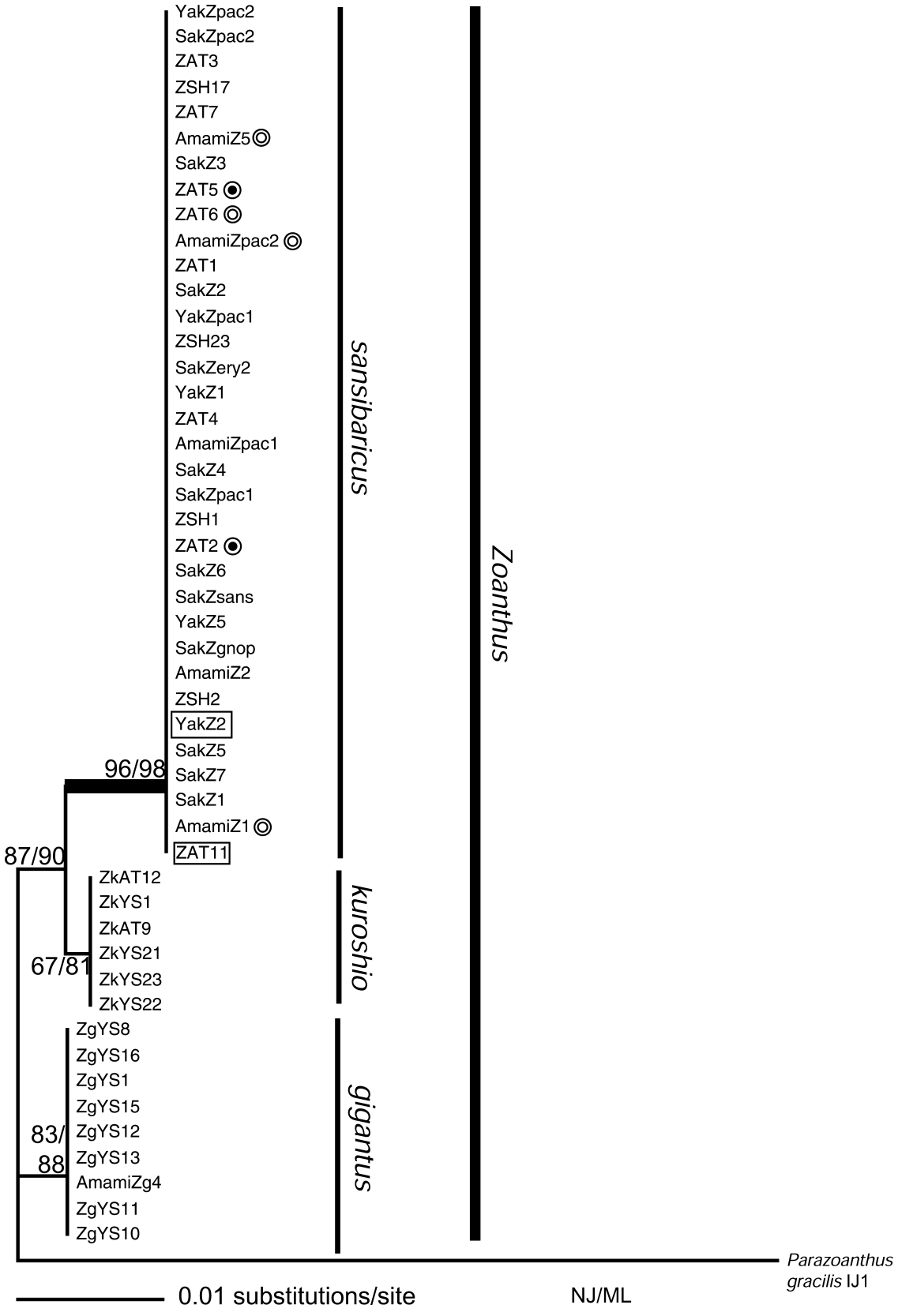

Fig. 3. Maximum-likelihood tree of obtained cytochrome oxidase I gene (COI) sequences. Values at branches represent NJ and ML bootstrap probabilities, respectively (>50%). Bayesian posterior probabilities >95% are represented by thick branches. For sample name abbreviations, see Table 2. Samples in boxes represent "B" only samples, samples with filled-in target symbols represent confirmed sansi/B hybrids, and samples with empty target symbols represent putative sansi/B hybrids.

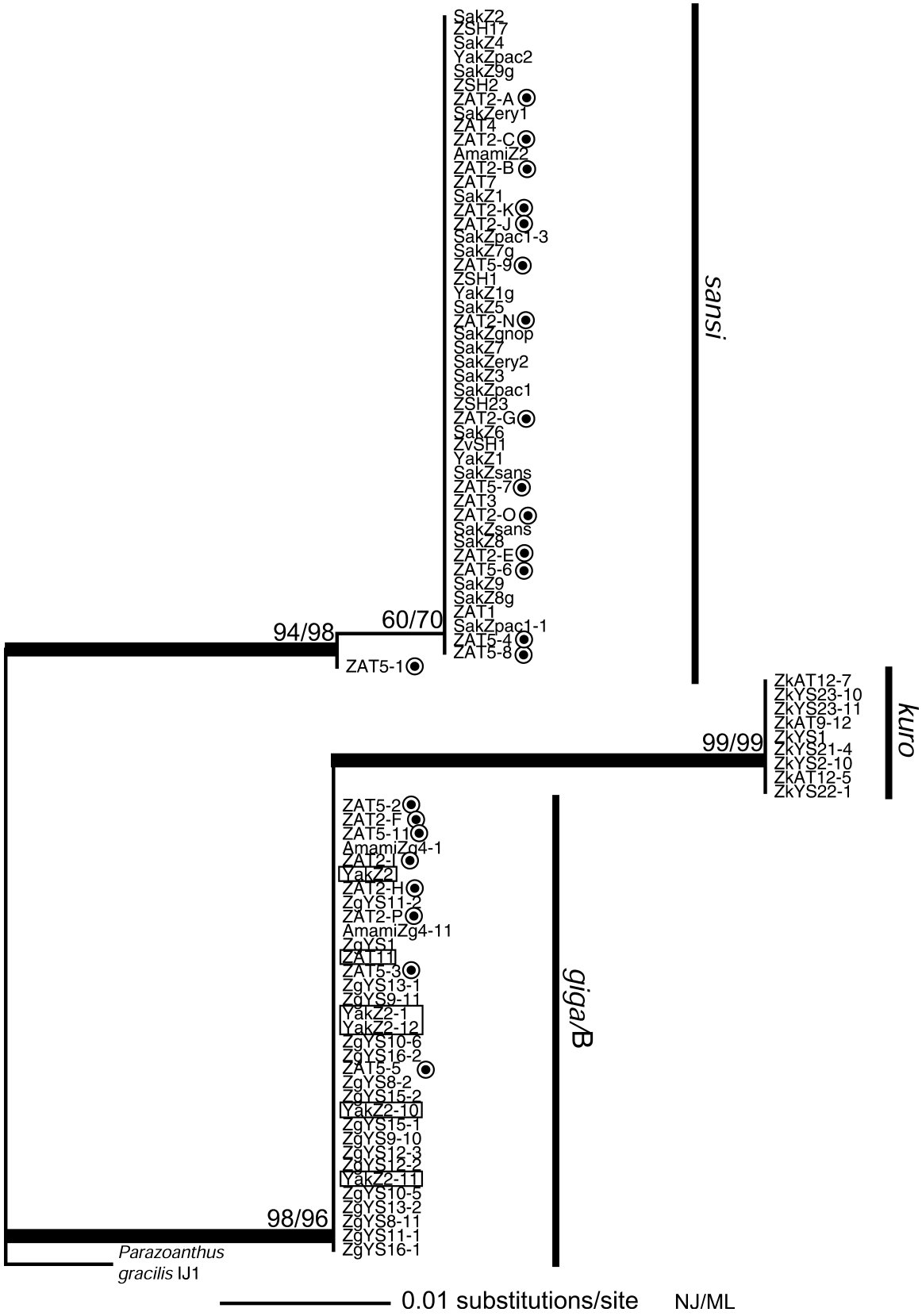

Fig. 4. Maximum-likelihood tree of obtained 5.8S rDNA sequences. Values at branches represent NJ and ML bootstrap probabilities, respectively (>50%). Bayesian posterior probabilities >95% are represented by thick branches. For sample-name abbreviations, see Table 2. Sample names ending in a hyphenated number or letter represent clones. Samples in boxes represent "B" only samples, samples with filled-in target symbols represent confirmed sansi/B hybrids, and samples with empty target symbols represent putative sansi/B hybrids.

inations of COI data in Anthozoa (Medina *et al*., 1999; Reimer *et al*., 2004, 2006a; van Oppen *et al*., 2004), as well as mt 16S rDNA sequence data (Reimer *et al*., 2006a) and morphological characteristics, our divergence results (0.7–1.3%) are consistent with anthozoan species-level differences, and we interpreted our three presumed *Zoanthus* species to be true, separate species.

ITS-rDNA sequences and phylogeny

We designated monophyletic groups of ITS-rDNA with sequence variation of less than 5% to be "alleles" (see Worheide *et al*., 2004). *Zoanthus sansibaricus* samples (n=19) from Sakurajima all possessed almost identical ITS-rDNA sequences we designated as the "sansi" allele (Table 2). *Zoanthus kuroshio* (n=6) and *Z. gigantus* (n=9) samples possessed one ITS-rDNA allele each, designated "kuro" and "giga", respectively (Table 2). Unexpectedly, some *Z. sansibaricus* from Yakushima and Amami possessed both the "sansi" ITS-rDNA allele as well as another highly divergent allele we designated "B". Individual *Z. sansibaricus* from

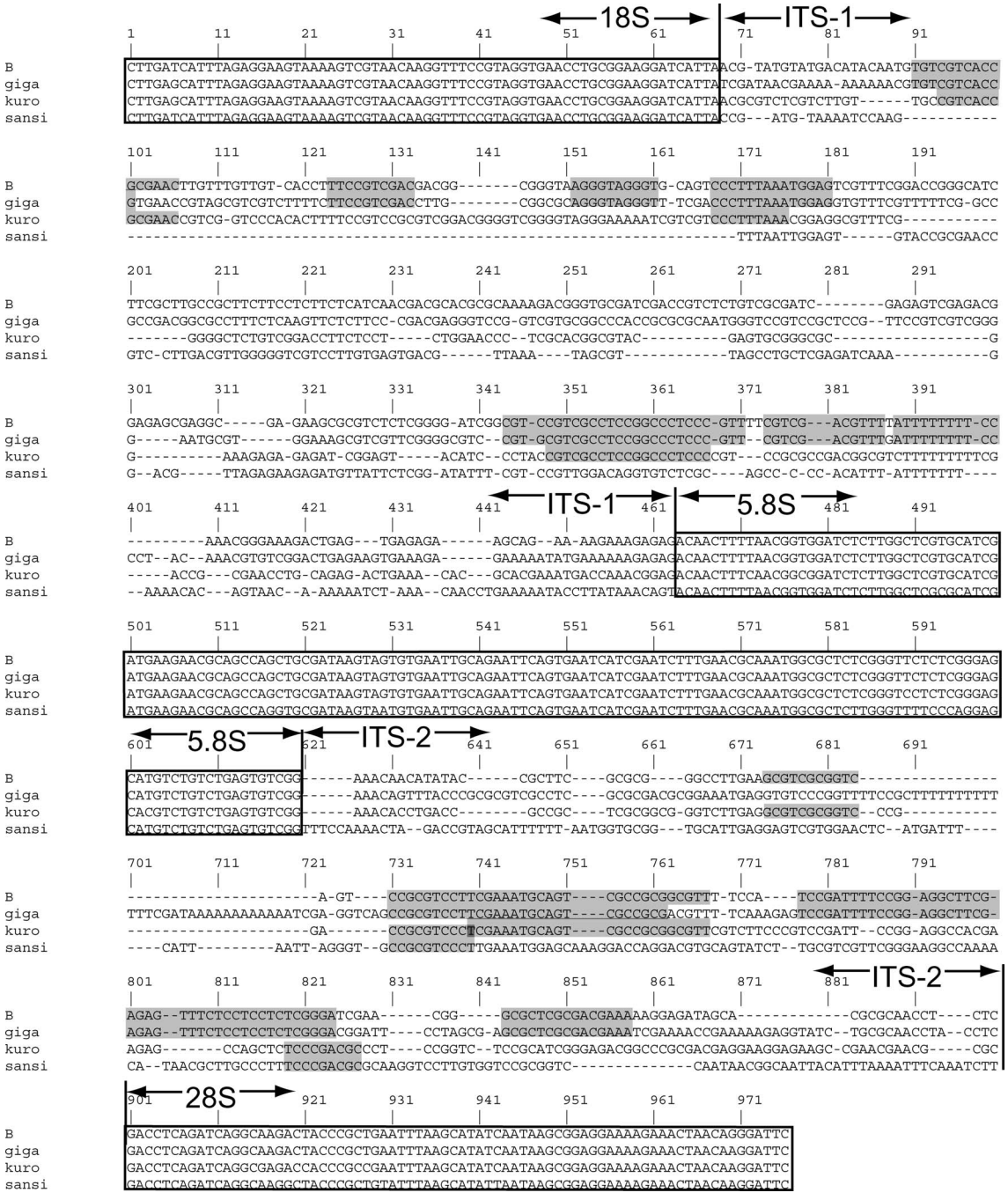

Fig. 5. Alignment of the ITS-rDNA region for four alleles: "sansi", "kuro", "B", and "giga". Areas in black boxes represent conservative regions (18S, 5.8S, 28S-rDNA), and shaded gray areas represent identical regions of 9 or more bp in ITS-1 and ITS-2 shared by two or more alleles. "giga", AmamiZg4-1; "B", ZAT2-F; "sansi", ZAT5-4; "kuro", ZkAT12-7.

Yakushima and Amami were shown to possess "sansi" only (seven samples, confirmed by direct sequencing), both "sansi" and "B" (nine samples total; all samples directly sequenced resulted in "mixed" ITS-rDNA sequences, and presence of "sansi" and "B" was confirmed by cloning in two samples [ZAT2, ZAT5], with 13 and 8 clones respectively), or "B" only (two samples; YakZ2 confirmed by cloning [16 clones] and ZAT11 confirmed by direct sequencing) (Table 2). All samples possessing single alleles confirmed by cloning could be directly sequenced, while no samples possessing two alleles ("sansi" and "B") could be directly sequenced (*i.e*., double peaks in the raw sequence data). In general, while the sequences of 3'-terminal 18S and 5.8S and 5'-terminal 28S-rDNA were easily alignable, the ITS-1 and ITS-2 regions were highly variable in length and base-pair sequence between alleles, and we did not have confidence in the resulting alignments (Table 3). For this reason we did not align the entire ITS-rDNA region for subsequent phylogenetic analyses, but instead aligned only the 5.8S rDNA region. Note that even though we did not use the entire ITS-rDNA region alignment for phylogenetic analyses, we have presented an alignment in Fig. 5 to show both shared ITS-rDNA regions and the high levels of sequence differences between different alleles. Additionally, 5.8S rDNA sequence-difference levels between species were higher than for COI (Table 4).

To reconfirm the levels of IGP within each allele, all sequences obtained of the entire ITS-rDNA region (not only 5.8S rDNA) for each species or allele group were aligned and edited, and the total number of variable sites calculated (Table 5). Additionally, we calculated intra-individual IGP levels between the two most variable (most sequence differences) individuals of each allele (Table 5). Worheide *et al*. (2004) have stated that the ITS-rDNA region is phylogenetically informative when IGP levels do not obscure within- or between-species differences. Our *Zoanthus* ITS-rDNA sequences, while having moderate IGP levels (all IGP below 5%; see Table 5), show very clear between-allele differences, making ITS-rDNA valid as a species marker.

We obtained the following number of ITS-rDNA sequences for each allele: "sansi", n=43; "B", n=24; "giga", n=95; and "kuro", n=43. However, we did not include some sequences in our 5.8S rDNA phylogenetic tree due to redundancy.

The ML tree based on 92 5.8S rDNA sequences is shown in Fig. 4. As with the COI data, the "sansi" allele formed a monophyletic group, with only 70% ML bootstrap probability and 67% Bayesian posterior probability, although these low probabilities are due to the inclusion of sequence ZAT5-1 (see below). The ITS-rDNA ZAT5-1 sequence had an ITS-1 "B" sequence and an ITS-2 "sansi" sequence, with a 5.8S sequence intermediate between "sansi" and "B" (the first 53 bp of 5.8S were identical to "B", while the remaining 104 bp were identical to "sansi"). This sample is located basally in the "sansi" clade with very high bootstrap support (98% ML bootstrap probability and 100% Bayesian posterior probability). Unlike in the COI alignment, the "kuro" allele monophyletic group (99% ML bootstrap probability and 100% Bayesian posterior probability) was derived from a basal "giga/B" monophyletic group (96% ML bootstrap probability and 98% Bayesian posterior probability). "Giga" and "B" also shared ten identical regions 9 bp or greater in length in their ITS-1 and ITS-2 sequences, while "kuro" and "B" shared five such regions, and "kuro" and "giga" shared four regions (Fig. 5). The "sansi" allele shared no regions in ITS-1 and ITS-2 with "B" or "giga", and two ITS-2 regions with "kuro". For ease of understanding, we have referred to all morphotypic *Z. sansibaricus* in the text as *Z. sansibaricus*, regardless of their ITS-rDNA allele makeup. In cases where distinctions between ITS-rDNA allele types are necessary, "sansi/sansi" have been referred to as "only "sansi"", hybrids as "sansi/B", and "B/B" as "only "B"".

Minimum-spanning networks

Minimum-spanning networks (MSN) were created for both the COI and 5.8S rDNA data sets (Fig. 6). A MSN created for the entire ITS-rDNA region resulted in most allele and haplotype groups being unconnected, and is thus not shown here. Both the COI and 5.8S rDNA MSNs are relatively simple, as there were low numbers of haplotypes in both data sets. The COI MSN showed *Z. sansibaricus* and *Z. gigantus* COI sequences to be 8 steps apart, with *Z.*

Table 4. Comparison of cytochrome oxidase I (COI) and 5.8S ribosomal DNA (5.8S rDNA) sequence difference levels in three species of *Zoanthus*.

Comparison species	COI sequence difference (% and number of base-pair differences of total base pairs)	5.8S rDNA sequence difference (% and number of base-pair differences of total base pairs)
Zoanthus sansibaricus — Zoanthus gigantus	1.3%, 8/592	4.4%, 7/158
Zoanthus sansibaricus — Zoanthus kuroshio	0.7%, 4/592	7.0%, 11/158
Zoanthus gigantus — Zoanthus kuroshio	0.8%, 5/592	2.5%, 4/158

Table 5. Intra-genomic polymorphism (IGP) levels for different alleles of the ITS-rDNA region in *Zoanthus* spp.

allele (base pair length used in calculations[1])	Intra-allele (IGP) (total sequences used)	Highest intra-individual IGP observed (sample name, sequences used)
"sansi" (679)	4/679=0.59% (48 sequences)	0/670=0.00% (ZAT2, 9 sequences)
"B" (540)	23/540=4.26% (26 sequences)	16/540=2.96% (YakZ2, 16 sequences)
"giga" (840)	31/840=3.69% (69 sequences)	19/840=2.26% (ZgYS13, 12 sequences)
"kuro" (710)	11/710=1.55% (24 sequences)	4/710=0.56% (ZkYS23, 12 sequences)

[1] note allele base pair length differs from ITS-1+5.8S+ITS-2 total length from Table 3 due to different alignments used to maximize number of sequences investigated for IGP calculations.

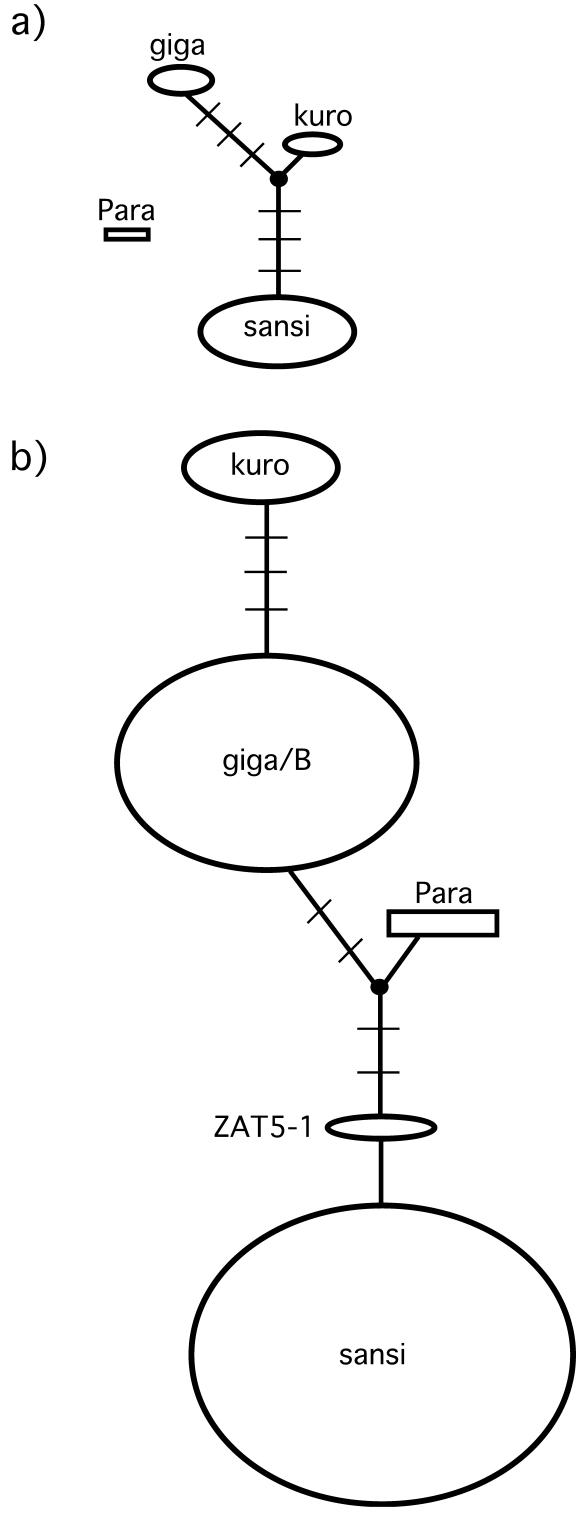

Fig. 6. Minimum-spanning networks for **(a)** *Zoanthus* spp. COI sequences and **(b)** *Zoanthus* spp. 5.8S rDNA sequences. Species/allele abbreviations: giga, *Zoanthus gigantus*; kuro, *Zoanthus kuroshio*; sansi, *Zoanthus sansibaricus*; B, B allele; Para, *Parazoanthus gracilis*; ZAT5-1, sample ZAT5 subclone 1. Note that the *P. gracilis* COI sequence was unconnected to the network in (a). *Zoanthus* haplotypes are displayed as ovals, *Parazoanthus* haplotypes as squares. The size of the oval or square indicates haplotype frequency.

kuroshio in the middle, 5 steps apart from both *Z. sansibaricus* and *Z. gigantus* (Fig. 6a). In the 5.8S rDNA MSN, ZAT5-1 was 1 step apart from the sansi allele, while the giga/B allele was 7 steps from sansi, and kuro an additional 4 steps removed from giga/B (for a total of 11 steps removed from sansi) (Fig. 6b).

DISCUSSION

Utility of COI-mtDNA and ITS-rDNA in *Zoanthus*

Our results confirm previous speculation that COI is a suitable species-level marker for the genus *Zoanthus* (Reimer *et al*., 2004). All samples examined fell into one of three monophyletic species groups, with the resulting phylogenetic trees having relatively high bootstrap support. The three *Zoanthus* species examined here differed by four to eight base pairs from each other.

Additionally, 5.8S rDNA proved to be useful in examining phylogenetic relationships, with a divergence rate slightly higher than seen in COI (Table 4). However, it should be noted that the 5.8S rDNA region utilized here for phylogenetic analyses failed to distinguish between the ITS-rDNA alleles “giga” and “B”. Furthermore, the ITS-1 and ITS-2 regions obtained between species proved to be virtually unalignable, negating the potential use of these regions in phylogenetic analyses. Furthermore, while our 5.8S rDNA data confirms monophylies seen with the COI data, the topologies of the two resulting trees are different. Possible reasons for this will be discussed later in this paper.

The highly variable ITS-1- and ITS-2-region sequences for *Zoanthus* obtained here were highly useful for a) inferring the presence of hybrids, and b) acting as species-level markers based on the length of each ITS region (similarly to Takabayashi *et al*., 1998; Hung *et al*., 2004). Our results also show that *Zoanthus* spp. have a rate of ITS-1 and ITS-2 divergence that is very high compared to mitochondrial DNA divergence and much higher than that reported for ITS-1 and ITS-2 between species in most other genera of Hexacorallia (*i.e*., only 2% variation in the *Montastrea annularis* complex [Medina *et al*., 1999], 4.9% in *Madracis* spp. [Diekmann *et al*., 2001], and 11% in *Porites* spp. [Hunter *et al*., 1997]). We estimate the ITS-rDNA region variation (despite difficulty in alignment) in the genus *Zoanthus* (between “sansi” and “B”; Fig. 5) at approximately 45% (and up to 70% for ITS-1), even higher than the extreme diversity (approximately 20%) reported between *Acropora* spp. (Marquez *et al*., 2003).

We recommend that any future study utilizing the ITS-rDNA region first investigate intragenomic polymorphism levels through cloning (as suggested by Worheide *et al*., 2004) and compare the sequences obtained with data from at least one other, single copy locus (as suggested by Vollmer and Palumbi, 2004). The ITS-rDNA region alone does not appear to be accurate nor informative enough to draw informed phylogenetic conclusions on the evolution of *Zoanthus* species, but when used along with a mitochondrial (as seen here) or another nuclear marker, ITS-rDNA data can be a powerful tool in investigating potential hybridization, as well as concerted evolution.

“B” most closely related to *Z. gigantus*

While the “B” ITS-rDNA sequences are definitely of

Zoanthus origin based on the 5.8S rDNA phylogeny, their source remains undetermined. As shown in Figs. 4 and 5, the "giga" ITS-rDNA sequence shares a completely identical 5.8S rDNA region with the "B" allele, with 7 bp differences (substitutions) from the "sansi" 5.8S rDNA sequence. Additionally, "giga" and "B" share ten other identical regions in the highly non-conservative ITS-1 and ITS-2 regions, much more than shared between any other two alleles (Fig. 5). Sibling species with varying ITS-1 rDNA length yet sharing some conservative ITS-1 regions have been reported in the ladybug subfamily Coccinellinae (von der Schulenberg *et al*., 2001). It is expected, based on "B" and "giga" ITS-rDNA similarities, that the species possessing "B" ITS-rDNA would be a sibling species to *Z. gigantus,* and possibly *Z. kuroshio*. There are, however, several possible scenarios that may explain the "sansi/B" and "only B" samples.

Explanations for the presence of "B" ITS-rDNA

I. Pseudogenes and polymorphism

All *Z. sansibaricus* COI sequences were identical, as were ITS-rDNA sequences (designated "sansi") from the majority of *Z. sansibaricus* colonies. However, at least two *Z. sansibaricus* at Yakushima and Amami possessed both "sansi" and "B" cloned ITS-rDNA sequences in approximately equal numbers (Table 2). Additionally, two colonies (YakZ2 and ZAT11) had only "B" ITS-rDNA sequences despite having "sansi" COI (Table 2) and mt 16S rDNA (data not shown). The presence of both "sansi" only and "B" only *Z. sansibaricus* colonies and the fact that "B" had an identical 5.8S-rDNA sequence as "giga" strongly suggests that both alleles are functional, eliminating the possibility of a retained, ancestral pseudogene. Similarly, ITS-rDNA polymorphism appears to be an unlikely explanation for our observed results, as *Z. sansibaricus* samples from Sakurajima had no ITS-rDNA allele besides "sansi" despite this being the most extensively sampled site (n=19). If ancestral polymorphism were truly present in *Z. sansibaricus*, we would expect at least some Sakurajima samples to also possess the "B" allele.

Furthermore, Feliner *et al*. (2004) state that ancestral polymorphism is unlikely to be the ultimate cause of intragenomic variation in ITS-rDNA due to the presence of concerted evolution, and that the merging of different ITS-rDNA copies (*i.e*., from hybridization) is the more likely explanation. It is interesting to note that one of our acquired sequences (ZAT5-1) is a combination of the "sansi" and "B" alleles, although this may be not due to homogenization/concerted evolution but a chimera.

II. Are "B"-only samples the cryptic species or subspecies parental source of "B" ITS rDNA?

One possible explanation for samples possessing "only B" ITS-rDNA is that these samples represent a cryptic *Zoanthus* species (which we designate as *Z*. sp. B) and are the other parent in the "sansi/B" equation. In other words, samples possessing "sansi/B" ITS-rDNA would be F_1 or further generation hybrid offspring of *Z. sansibaricus* and *Z*. sp. B.

It has been noted that COI appears to have a very slow evolutionary rate in Anthozoa (Shearer *et al*., 2002), both in Hexacorallia (Romano and Palumbi, 1997; Knowlton, 2000) and Octocorallia (France and Hoover, 2002). Indeed, species-level differences between *Z. sansibaricus* and *Z. kuroshio* (3/600 bp=0.5%) are much lower than in the jellyfish genus *Aurelia* (Scyphozoa) (10–20%) (Dawson and Jacobs, 2001). It may be possible that COI fails to resolve differences between *Z. sansibaricus* and *Z*. sp. B., although an examination of mt 16S rDNA data (to be presented elsewhere) showed results identical to COI, with potential *Z*. sp. B. samples having "sansi" mt 16S rDNA sequences. Due to slow anthozoan mtDNA evolution, mtDNA-sequence and morphological differences between *Z. sansibaricus* and *Z*. sp. B may have not yet accumulated. However, *Z. sansibaricus, Z. kuroshio*, and *Z. gigantus* all displayed clear morphological differences from each other, and a monophyletic group of COI and 5.8S rDNA data for each species was observed. Why this would not happen between *Z*. sp. B. and *Z. sansibaricus* remains obscure, especially considering that theoretically *Z*. sp. B would be closely related to *Z. gigantus*, not *Z. sansibaricus*. Thus, we feel COI failing to resolve differences between *Z. sansibaricus* and *Z*. sp. B is an unlikely explanation for our observed data.

Another scenario is that *Z*. sp. B may be a subspecies of *Z. sansibaricus*, but this seems highly unlikely at best, as subspecies possessing such highly variable ITS-rDNA regions have not been reported from any taxa to date, and we feel that the "sansi" and "B" ITS-rDNA sequences are different at least to the species level (see Table 2).

Morphological and distributional data are inconclusive regarding this hypothesis. One "only B" *Zoanthus* colony was found relatively low in the intertidal zone (YakZ2 in Table 2) at Yakushima and morphologically appeared to be a normal *Z. sansibaricus* colony. The other "only B" colony (ZAT11, Table 2) was found very high in the intertidal range of *Z. sansibaricus* at Amami, and was quite small (yet still within the size range of *Z. sansibaricus*) in oral disk diameter. Interestingly, other *Zoanthus* samples collected from Amami high in the intertidal zone possessing the same "smaller" morphology as ZAT11 that have been genetically confirmed (COI, mt 16S rDNA) to be *Z. sansibaricus* have been shown to possess clade A *Symbiodinium* zooxanthellae, as opposed to most *Z. sansibaricus* samples from Amami, Yakushima, and Sakurajima, which possess clade C *Symbiodinium* (Reimer *et al*., 2006b). However, as only two "only B" *Z. sansibaricus* colonies have been found thus far, further "only B" samples are needed to confirm or negate the subspecies hypothesis.

III. Importance of hybridization in Zoanthus

In recent years, evolution has been proposed and shown to not always be a clear-cut case of branching "trees" of species gradually diverging over time. Over the past three decades, another form of evolution has been proposed in which species can continuously rejoin (hybridize) and split with one another, resulting in "repackaging" of the same genetic data over time (Grant, 1981; Veron, 1995). This theory can help explain the growing body of data in which phylogenies of recently diverged species based on nuclear-encoded genes do not agree with mitochondrial or chloroplast gene phylogenies (for example, see Shaw, 2002; Lihova *et al*., 2004), as seen in this study, where the 5.8S rDNA tree topology does not match the COI tree topology. Such "reticulate evolution" has been suggested to arise when a group of species possessing ancestral allopatric lineages moves

into a new environment that provides interspecific hybrids the chance to evolve into niches (often extreme) not available to the parent species (Schliewen and Klee, 2004). Reticulate evolution differs from incomplete lineage sorting in that the parent species involved are diverged species possessing diverged alleles, while incomplete lineage sorting involves diverged or diverging species still possessing ancestral alleles that have not diverged. Genetic evidence has been shown for reticulate evolution in many plant groups, including peonies (*i.e.* Sang *et al*., 1995), the genera *Cardamine* (Lihova *et al*., 2004), *Leucadendron* (Pharmawati *et al*., 2004), and *Armeria* (Aguilar and Feliner, 2003), as well as recently for animals (*e.g*., cichlids; Schliewen and Klee, 2004).

One potential clue to reticulate evolution has been suggested by Odorico and Miller (1997), who predicted that reticulate evolution should result in "species" with multiple, homologous nuclear sequences inherited from different parent species (our "sansi", "sansi/B", and "B" only ITS-rDNA samples), while diverging "evolutionary units" should have interspecifically different yet intraspecifically identical sequences (as seen with "kuro" and "giga" ITS-rDNA).

If reticulate evolution is occurring, then "sansi" only ITS-rDNA colonies are a parent species, "sansi/B" colonies would be F_1 or further generation hybrids, and "B" only colonies may possibly be the result of either backcrossing (as seen by Vollmer and Palumbi, 2002, 2004) or hybrid-hybrid sexual reproduction (F_{2+}), although this explanation fails to take into account recombination within a potentially multicopy ITS-rDNA region. Judging from our results, it appears that only female *Z. sansibaricus* and male "B" may be capable of hybridization, resulting in the conservation of maternal (mitochondrial) genes (COI and mt 16S rDNA).

From the observation that "B" and "giga" share the most sequence similarities in the ITS-rDNA region, it may be possible that the "B" allele entered into to the genome of an ancestor of *Z. sansibaricus* from an ancestor of *Z. gigantus*. Subsequent divergence has resulted in the present "B" allele in *Z. sansibaricus*. The ancestor of *Z. gigantus* also underwent divergence, resulting in the "giga" ITS-rDNA seen today. The "B" donor species may or may not be extant.

Another possibility is that "sansi" has entered the *Z. sansibaricus* genome, and that "B" is the original *Z. sansibaricus* ITS-rDNA sequence. This would explain the close relationship between "B", "kuro", and "giga". While the "sansi" allele is unambiguously of zoanthid origin, in this scenario the possible source of "sansi" remains unknown. In addition, if this scenario is true, the "sansi" allele would apparently confer some advantage over the "B" allele, as it is much more common than "B", especially at Sakurajima (19 *Z. sansibaricus* samples, no "B"). We feel this scenario is less likely than the "B" allele entering the *Z. sansibaricus* genome from outside sources, but it must be considered.

Discerning between reticulate evolution and incomplete lineage sorting is problematic at best, but given the extreme divergence between "sansi" and "B" ITS-rDNA sequences, we feel that incomplete lineage sorting is not occurring in our samples. Similarly to Buckley *et al*. (2006), who also observed no shared alleles between sympatric species of alpine *Maoricicada* cicadas, we conclude that the most likely explanation for our ITS-rDNA sequence data and the incongruence between the ITS-rDNA and COI trees is introgression by the common ancestor of the "giga" and "B" ITS-rDNA alleles into *Z. sansibaricus*.

Regardless of where "B" and/or "sansi" ITS-rDNA originated from, our results suggest that interspecific hybridization occurs in the genus *Zoanthus*, expanding the taxonomic range in which this phenomenon has been potentially observed. Whether our results show only first-generation hybrids (under the *Z*. sp. B scenarios), or also subsequent F_{2+} hybrids (under the reticulate evolution scenario), remains debatable.

ACKNOWLEDGMENTS

We express our gratitude and thanks to Dr. Yoshihiro Fujiwara, Dr. Masashi Tsuchiya, Masaru Kawato, and Hirokazu Kuwahara at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) in Yokosuka, Japan for their helpful comments and assistance throughout the course of this study. Atsushi Iwama, Denny Probizanski, Mika Reimer, and Ryan Cox helped with sample collection in Kagoshima. Dr. Steven Vollmer pointed us in the right direction regarding analyses of potential hybrids. JDR was supported at JAMSTEC by a Foreign Researcher Post-Doctoral Fellowship (#04868) from the Japan Society for the Promotion of Science (JSPS). Three anonymous reviewers' comments greatly improved the quality of this manuscript.

REFERENCES

Alvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. Mol Phylogenet Evol 29: 417–434

Aguilar JF, Feliner GN (2003) Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). Mol Phylogenet Evol 28: 430–447

Babcock RC, Ryland JS (1990) Larval development of a tropical zoanthid (*Protopalythoa* sp.). Invertebr Reprod Dev 17: 229–236

Buckley TR, Cordeiro M, Marshall DC, Simon C (2006) Differentiating between hypotheses of lineage sorting and introgression in New Zealand alpine cicadas (*Maoricicada* Dugdale). Syst Biol 55: 411–425

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657–1660

Cooke WJ (1976) Reproduction, growth, and some tolerances of *Zoanthus pacificus* and *Palythoa vestitus* in Kaneohe Bay, Hawaii. In "Coelenterate Ecology and Behaviour" Ed by GO Mackie, Plenum Press, New York, pp 281–288

Dawson MN, Jacobs DK (2001) Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). Biol Bull 200: 92–96

Diekmann OE, Bak RPM, Stam WT, Olsen JL (2001) Molecular genetic evidence for probably reticulate speciation on the coral genus *Madracis* from a Caribbean fringing reef slope. Mar Biol 139: 221–233

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299

France SC, Hoover LL (2002) DNA sequences of the mitochondrial COI gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). Hydrobiologia 471: 149–155

Fukami H, Budd AF, Levitan DR, Jara J, Kersanach R, Knowlton N (2004) Geographic differences in species boundaries among members of the *Montastraea annularis* complex based on molecular and morphological markers. Evolution 58: 324–327

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol

52: 696–704
Grant V (1981) Plant Speciation. Columbia University Press, New York 2nd Ed
Hatta M, Fukami H, Wang W, Omori M, Shimoike K, Hayashibara T, Ina Y, Sugiyama T (1999) Reproductive and genetic evidence for a reticulate evolutionary history of mass-spawning corals. Mol Biol Evol 16: 1607–1613
Hung YT, Chen AC, Wu WJ, Li CC, Shih CJ (2004) Phylogenetic utility of the ribosomal transcribed spacer 2 in *Strumigenys* spp. (Hymenoptera: Formicidae). Mol Phylogenet Evol 32: 407–415
Hunter CL, Morden CW, Smith CM (1997) The utility of ITS sequences assessing relationships among zooxanthellae and corals. Proc 8th Int Coral Reef Symp 2: 1559–1602
Karlson RH (1981) Reproductive patterns in *Zoanthus* spp. from Discovery Bay, Jamaica. Proc 4th Int Coral Reef Symp 2: 699–704
Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120
Knowlton N (2000) Molecular genetic analyses of species-boundaries in the sea. Hydrobiologia 420: 73–90
LaJeunesse TC, Trench RK (2000) The biogeography of two species of *Symbiodinium* (Freudenthal) inhabiting the intertidal anemone *Anthopleura elegantissima* (Brandt). Biol Bull 199: 126–134
Levitan DR, Fukami H, Jara J, Kline D, McGovern TM, McGhee KE, Swanson CA, Knowlton N (2004) Mechanisms of reproductive isolation among sympatric broadcast-spawning corals of the *Montastraea annularis* species complex. Evolution 58: 308–323
Lihova J, Aguilar JF, Marhold K, Feliner GN (2004) Origin of the disjunct tetraploid *Cardamine amporitana* (Brassicaceae) assessed with nuclear and chloroplast DNA sequence data. Amer J Bot 91: 1231–1242
Marquez LM, van Oppen MJH, Willis B, Miller DJ (2002) Sympatric populations of the highly cross-fertile coral species *Acropora hyacinthus* and *Acropora cytherea* are genetically distinct. Proc R Soc Lond B 269: 1289–1294
Marquez LM, Miller DJ, MacKenzie JB, van Oppen MJH (2003) Pseudogenes contribute to the extreme diversity of nuclear ribosomal DNA in the hard coral *Acropora*. Mol Biol Evol 20: 1077–1086
McFadden CS, Hutchinson MB (2004) Molecular evidence for the hybrid origin of species in the soft coral genus *Alcyonium* (Cnidaria: Anthozoa: Octocorallia). Mol Ecol 13: 1495–1505
Medina M, Weil E, Szmant AM (1999) Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. Mar Biotechnol 1: 89–97
Odorico DM, Miller DJ (1997) Variation among the ribosomal internal transcribed spacers and 5.8S rDNA among five species of *Acropora* (Cnidaria; Scleractinia): patterns of variation consistent with reticulate evolution. Mol Biol Evol 14: 465–473
Ono S, Reimer JD, Tsukahara J (2005) Reproduction of *Zoanthus sansibaricus* in the infra-littoral zone at Taisho Lava Field, Sakurajima, Kagoshima, Japan. Zool Sci 22: 247–255
Penland L, Kloulechad J, Idip D, van Woesik R (2004) Coral spawning in the western Pacific Ocean is related to solar insolation: evidence of multiple spawning events in Palau. Coral Reefs 23: 133–140
Pharmawati M, Yan G, Sedgley R, Finnegan PM (2004) Chloroplast DNA inheritance and variation in *Leucadendron* species (Proteaceae) as revealed by PCR-RFLP. Theor Appl Genet 109: 1694–1701
Reimer JD, Ono S, Fujiwara Y, Takishita K, Tsukahara J (2004) Reconsidering *Zoanthus* spp. diversity: molecular evidence of conspecificity within four previously presumed species. Zool Sci 21: 517–525
Reimer JD, Ono S, Iwama A, Tsukahara J, Takishita K, Maruyama T (2006a) Morphological and molecular revision of *Zoanthus* (Anthozoa: Hexacorallia) from southwestern Japan with description of two new species. Zool Sci 23: 261–275
Reimer JD, Takishita K, Ono S, Maruyama T, Tsukahara J (2006b) Latitudinal and intracolony ITS-rDNA sequence variation in the symbiotic dinoflagellate genus *Symbiodinium* (Dinophyceae) in *Zoanthus sansibaricus* (Anthozoa: Hexacorallia). Phycol Res 54: 122–132
Rodriguez F, Oliver JL, Marin A, Medina JR (1990) The general stochastic model of nucleotide substitution. J Theor Biol 142: 485–501
Romano SL, Palumbi SR (1997) Molecular evolution of a portion of the mitochondrial 16S ribosomal gene region in scleractinian corals. J Mol Evol 45: 397–411
Ronquist F, Huelsenbeck JP (2003) Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574
Ryland JS, Babcock RC (1991) Annual cycle of gametogenesis and spawning in a tropical zoanthid, *Protopalythoa* sp. Hydrobiologia 216–217: 117–123
Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425
Sang T, Crawford DJ, Stuessy TF (1995) Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc Natl Acad Sci USA 92: 6813–6817
Schliewen UK, Klee B (2004) Reticulate sympatric speciation in Cameroonian Crater lack cichlids. Front Zool 1: 5
Shaw KL (2002) Conflict between mitochondrial and nuclear DNA phylogenies of a recent species radiation: what mitochondrial reveals and conceals about modes of speciation in Hawaiian crickets. Proc Natl Acad Sci USA 99: 16122–16127
Shearer TL, van Oppen MJH, Romano SL, Worheide G (2002) Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol 11: 2475–2487
Sugita T, Nishikawa A, Ikeda R, Shinoda T (1999) Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. J Clin Microbiol 37: 1985–1993
Swofford DL (1998) PAUP*. Phylogenetic analysis using parsimony (*and other methods) Vers 4. Sinauer Associates, Sunderland, MA, USA
Takabayashi M, Carter D, Ward S, Hoegh-Guldberg O (1998) Inter- and intra-specific variability in ribosomal DNA sequence in the internal transcribed spacer region of corals. In "Proceedings of the Australian Coral Reef Society 75th Anniversary Conference, Heron Island October 1997" Ed by JG Greenwood, NJ Hall, School of Marine Sciences, The University of Queensland, Brisbane, pp 237–244
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882
van Oppen MJH, Koolmees EM, Veron JEN (2004) Patterns of evolution in the scleractinian coral genus *Montipora* (Acroporidae). Mar Biol 144: 9–18
van Oppen MJH, McDonald BJ, Willis B, Miller DJ (2001) The evolutionary history of the coral genus *Acropora* (Scleractinia, Cnidaria) based on a mitochondrial and a nuclear marker: reticulation, incomplete lineage sorting, or morphological convergence? Mol Biol Evol 18: 1315–1329
van Oppen MJH, Willis BL, Rheede TV, Miller DJ (2002) Spawning times, reproductive compatibilities and genetic structuring in the *Acropora aspera* group: evidence for natural hybridization and semi-permeable species boundaries in corals. Mol Ecol 11: 1363–1376

van Oppen MJH, Willis BL, Van Vugt HWJA, Miller DJ (2000) Examination of species boundaries in the *Acropora cervicornis* group (Scleractinia, Cnidaria) using nuclear DNA sequence analyses. Mol Ecol 9: 1363–1373

Veron JEN (1995) Corals of the World: The Biogeography and Evolution of the Scleractinia. University of New South Wales Press, Sydney

Vollmer SV, Palumbi SR (2002) Hybridization and the evolution of coral reef diversity. Science 296: 2023–2025

Vollmer SV, Palumbi SR (2004) Testing the utility of internally transcribed spacer sequences in coral phylogenetics. Mol Ecol 13: 2763–2772

von der Schulenburg JHG, Hancock JM, Pagnamenta A, Sloggett JJ, Majerus MEN, Hurst GDD (2001) Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Mol Biol Evol 18: 648–660

Worheide G, Nichols SA, Goldberg J (2004) Intragenomic variation of the rRNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies. Mol Phylogenet Evol 33: 816–830

(Received September 7, 2006 / Accepted October 20, 2006)