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High Levels of Morphological Variation Despite Close Genetic Relatedness Between *Zoanthus* aff*. vietnamensis* and *Zoanthus kuroshio* (Anthozoa: Hexacorallia)

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Recent investigations into the encrusting anemone genus *Zoanthus* using molecular and morphological techniques have begun to bring order to this taxonomically neglected group. Previous studies have confirmed the existence of three distinct species present in southern Japan: *Z. sansibaricus, Z. kuroshio*, and *Z. gigantus*. Results from such studies show species of *Zoanthus* to be highly morphologically plastic, often incorporating morphotypes with varying oral disk color and oral disk diameter. Literature lists the species *Z.* aff. *vietnamensis* as occurring in southern Japan and throughout the western Pacific Ocean, but due to the morphological plasticity of *Zoanthus* species, a re-examination of *Z.* aff. *vietnamensis* using molecular techniques was needed. Here, using mitochondrial 16S rDNA and the nuclear internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences, as well as morphological data, we have examined several nominal *Z.* aff. *vietnamensis* samples collected from Kagoshima Bay and Yakushima Island, Japan. Based on polyp length and diameter, oral disk diameter, mesentery and tentacle numbers, and colony form, *Z.* aff. *vietnamensis* is easily distinguishable from *Z. sansibaricus, Z. kuroshio*, and *Z. gigantus.* However, despite these clear morphological differences, our mitochondrial and nuclear sequence-based phylogenies indicate that *Z.* aff. *vietnamensis* and *Z. kuroshio* are very closely related (perhaps conspecific), highlighting the morphological plasticity of this genus and the difficulty of species identification based on morphological data alone.

Key words: ITS-rDNA, mt 16S rDNA, morphology, zoanthid, *Zoanthus*

INTRODUCTION

Taxonomy of species of the genus *Zoanthus* (Anthozoa: Hexacorallia) has been in a state of disarray since its establishment by Lamarck (1801), due to the morphological plasticity of species, lack of research, and no established criteria for species identification (Fossa and Nilsen, 1998). Taxonomic uncertainty has been compounded by confusion surrounding type species and specimens (Burnett *et al*., 1995, 1997; Ryland and Lancaster, 2003). However, recent work using molecular techniques has allowed researchers to begin to clarify the taxonomic status and biodiversity levels of *Zoanthus* species (Burnett *et al*., 1995, 1997; Reimer *et al*., 2004, 2006a). Molecular results have confirmed the morphological plasticity of certain species (*e.g*., intraspecific variation in oral disk color and diameter, tentacle number, and polyp length in *Z. sansibaricus*) (Reimer *et al*., 2004), while allowing the redefinition of previously described species and the identification of new species (Reimer *et al*., 2006a).

Currently, three species have been described from the shallow tropical and sub-tropical waters of Japan: *Z. sansibaricus* (Carlgren, 1900)*, Z. kuroshio* (Reimer and Ono, 2006), and *Z. gigantus* (Reimer and Tsukahara, 2006) (all described in Reimer *et al*., 2006a). However, Uchida (2001) mentions the existence in southern Japan of *Z.* aff. *vietnamensis* (Pax and Muller, 1957), characterized by relatively small colonies, large polyps 2–3 cm in length, and a pale pink oral disk with white mouth, although the species has not yet been properly described (Fautin, 2004). Other reports list *Z. vietnamensis* in its type locality in Vietnam (Pax and

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Muller, 1957), as well as Australia (Burnett *et al.*, 1997). While several colonies of *Zoanthus* fitting Uchida's (2001) description have been observed at Sakurajima and Yakushima Island, both in Kagoshima, Japan, the morphological plasticity of *Zoanthus* species calls the existence of *Z.* aff. *vietnamensis* as a species separate from *Z. sansibaricus, Z. kuroshio*, and *Z. gigantus* into question. Here, we have collected and examined, both morphologically and molecularly, nominal *Z.* aff. *vietnamensis* specimens in an attempt to further understand *Zoanthus* spp. diversity in southern Japan.

MATERIALS AND METHODS

Nominal *Z.* aff. *vietnamensis* specimens (Fig. 1a, Table 1) were collected from Hakamagoshi, Sakurajima, Kagoshima Bay, Japan (31°35'N, 130°35'E) and from Sangohama, Kurio, Yakushima Island, Japan (30°16'N, 130°25'E) at depths of 0 to 5 m. Samples were placed in 99.5% ethanol and stored at –30°C. DNA extraction and mitochondrial 16S rDNA and nuclear internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequencing were conducted following procedures described in Reimer *et al.* (2004, 2006a) and Sinniger *et al.* (2005). Morphological examinations were conducted following procedures described in Reimer *et al.* (2006a). From *in situ* images and cross and longitudinal sections, data were collected on tentacle number, mouth/oral disk/polyp/tentacle color, minimum and maximum oral disk diameter, polyp height, mesogleal thickness, and mesentery number.

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank under accession numbers AB235397-AB235412 and AB255640-AB255645. By using CLUSTAL X version 1.8 (Thompson *et al.*, 1997), the nucleotide sequences of mt 16S rDNA and 5.8S rDNA (the ITS-1 and ITS-2 rDNA regions were unalignable) from *Zoanthus* species were separately aligned with *Palythoa* spp. sequences as outgroups (Table 1). The alignments were inspected by eye and manually edited. All ambiguous sites in the alignments were removed from the data sets for phylogenetic analyses. We generated two aligned data sets: 1) 787 sites for 13 taxa (mt 16S rDNA), and 2) 157 sites for 16 taxa (5.8S rDNA). The aligned sequences are available on request from the corresponding author.

For phylogenetic analyses of mt 16S rDNA and 5.8S rDNA sequences, the same methods were independently applied. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel, 2003). ML analyses were 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 set. ML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees. The neighbor-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 (500 replicates) were constructed using the same model.

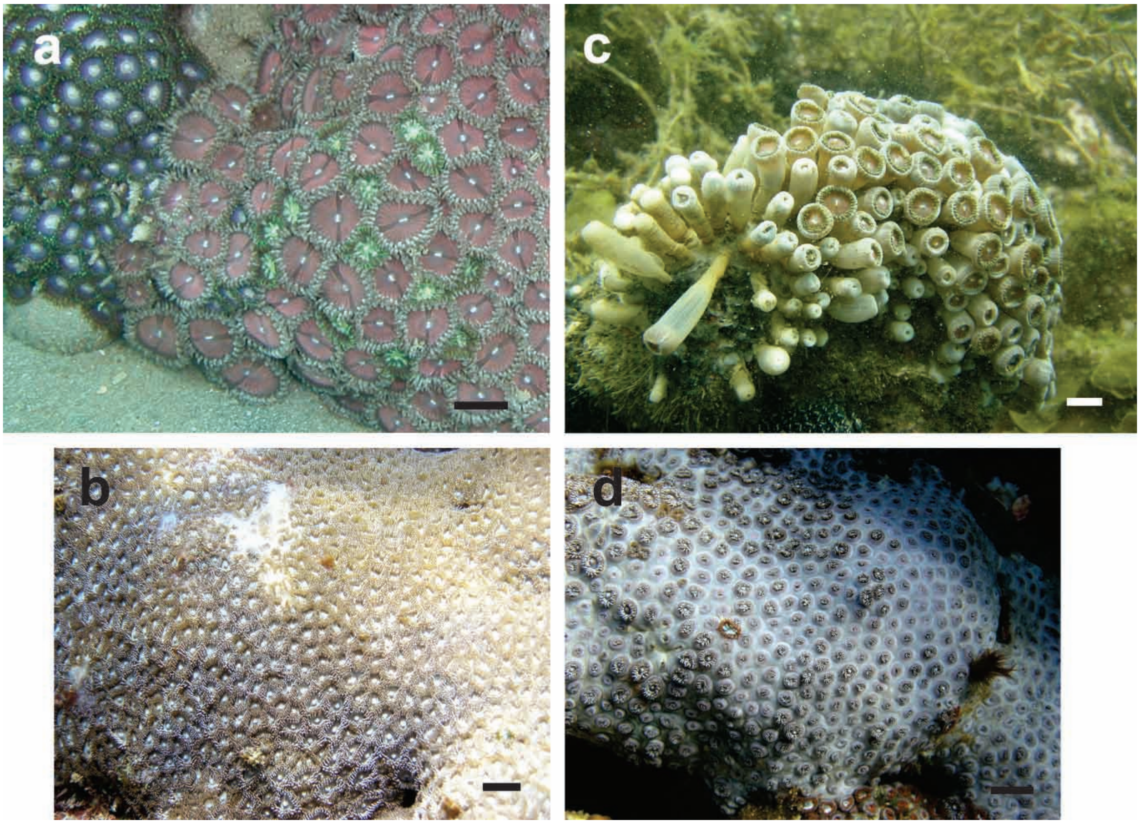


Fig. 1. **a)** *Zoanthus* aff. *vietnamensis* colony ZvSH1 (right) *in situ* at Hakamagoshi, Sakurajima, Kagoshima Bay, Japan. A colony of *Z. sansibaricus* (morphotype *sansibaricus*) is to the left of *Z.* aff. *vietnamensis,* with some smaller *Z. sansibaricus* (morphotype *pacificus*) polyps (green) intermingled with the *Z.* aff. *vietnamensis* colony. Depth=3 m. **b)** *Zoanthus kuroshio* colony NSMT-CO 1445 *in situ* at Sangohama, Kurio, Yakushima Island, Japan. Depth=1.5m **c)** *Z.* aff. *vietnamensis* colony ZvSH1 with oral disks closed, showing 'liberae' polyps. **d)** *Z. kuroshio* colony NSMT-CO 1445 with oral disks closed, showing 'immersae' polyps. All bars=1 cm.

Table 1. *Zoanthus* aff. *vietnamensis* samples examined in this study and corresponding GenBank Accession Numbers plus congener and outgroup samples.

Species/ group	Sample #*	Collected by	Collection date (Year/Month)	Location	Depth (m)	16S rDNA Accession Number	ITS-rDNA Accession Number
Zoanthus aff. *vietnamensis*	ZvSH1	SO	2005.07	Sakurajima	3	NA**	NA**
	ZvSH2	SO	2005.07	Sakurajima	3	AB235407	NA
	ZvSH3	SO	2005.07	Sakurajima	3	AB235408	AB235397
	ZvSH4	SO	2005.07	Sakurajima	3	NA	AB235398
	ZvSH5	SO	2005.07	Sakurajima	3	NA	AB235399
	ZvSH6	SO	2005.07	Sakurajima	3	AB235409	AB235400
	ZvSH7	SO	2005.07	Sakurajima	3	NA	AB235401
	ZvSH8	SO	2005.07	Sakurajima	3	NA	AB235402
	ZvSH9	SO	2005.07	Sakurajima	3	NA	AB235403
	ZvSH11	SO	2005.07	Sakurajima	3	NA	AB235404
	ZvSH12	SO	2005.07	Sakurajima	3	NA	AB235405
	ZvYS1	JDR	2005.12	Yakushima	1	AB255640	AB255643
	ZvYS2	JDR	2005.12	Yakushima	0.5	AB255641	AB255644
	ZvYS3	JDR	2005.12	Yakushima	0.5	AB255642	AB255645
Z. kuroshio	ZkU1	JT	2005.05	Uji Islands	0.5	AB235410	NA
	ZkYS1 (NSMT-CO 1445) [1]	JDR	2004.07	Yakushima	1.5	AB219191[2]	NA
	ZkYS23	JDR	2005.12	Yakushima	0.5	NA	DQ442480[3]
Z. gigantus	ZgKe1	JDR	2005.06	Kerama	7	AB235411	NA
	ZgYS1	JDR	2004.07	Yakushima	1.5	AB219192[2]	NA
	ZgYS8	JDR	2005.12	Yakushima	0	NA	DQ442406[3]
Z. sansibaricus	ZN1	SO	2005.06	Nagashima	2	AB235412	NA
	ZSH23	JDR	2004.07	Sakurajima	9	AB219187[2]	NA
	SakZpac1	JDR	2003.06	Sakurajima	2	NA	AB214123[3]
Palythoa mutuki 1	PpYS1 (YakuPalyBr)	JDR	2003.06	Yakushima	0	NA	AB235406
Palythoa tuberculosa	PtMiI1	JDR	2005.06	Miyakejima	2	AB219199[4]	NA

*All samples conserved in JDR's collection at JAMSTEC unless otherwise noted.

**NA=sequence not obtained or not used in analyses in this study.

[1] NSMT-CO 1445 is the holotype for *Z. kuroshio*, preserved at the National Science Museum in Tokyo.

[2] From Reimer *et al.* (2006a).

[3] From Reimer *et al.* (unpublished).

[4] From Reimer *et al.* (2006b).

RESULTS

Phylogenetic analyses

The resulting ML trees for mt 16S rDNA (Fig. 2a) and 5.8S rDNA (Fig. 2b) sequences show similar topologies. Bootstrap support for the monophyly of *Z.* aff. *vietnamensis* sequences with *Z. kuroshio* sequences was very high for mt 16S rDNA (ML=100%, NJ=99%) and 5.8S rDNA (ML=98%, NJ=99%). In the mt 16S rDNA tree, *Z. kuroshio* sequences formed a separate, moderately supported clade within the *Z.* aff. *vietnamensis* clade (ML=93%, NJ=67%), differing by a single base pair from *Z.* aff. *vietnamensis* sequences. Similarly, some *Z.* aff. *vietnamensis* ITS-rDNA region sequences (ZvSH3, ZvSH5, ZvSH9, ZvSH12) showed only very slight differences (two insertions in ITS-1, one of 4 bp, one of 1 bp) from the remaining *Z.* aff. *vietnamensis* and previously acquired *Z. kuroshio* sequences, which were identical (Table 2). However, overall, ITS-rDNA sequences of putative *Z.* aff. *vietnamensis* and *Z. kuroshio* samples showed no more variation (11/687 bp=1.6%) than intraspecific ITS-rDNA variation seen in other *Zoanthus* species (Reimer *et al.*, unpublished). *Zoanthus* aff. *vietnamensis* ITS-1 and ITS-2 sequences were of different lengths (Table 2) and virtually unalignable with *Z. sansibaricus* and *Z. gigantus* sequences, further confirming the large amounts of interspecific ITS-1 and ITS-2 variation seen in this genus (Reimer *et al.*, unpublished).

Table 2. Varying base pair lengths of different regions of obtained ITS-rDNA sequences for different *Zoanthus* spp.

species/group	ITS-1	5.8S	ITS-2
sansibaricus[1]	236~237	156	234~241
gigantus[1]	346~348	156	246~250
kuroshio[1]	295~297	156	190~192
vietnamensis	295~300	156	190~192

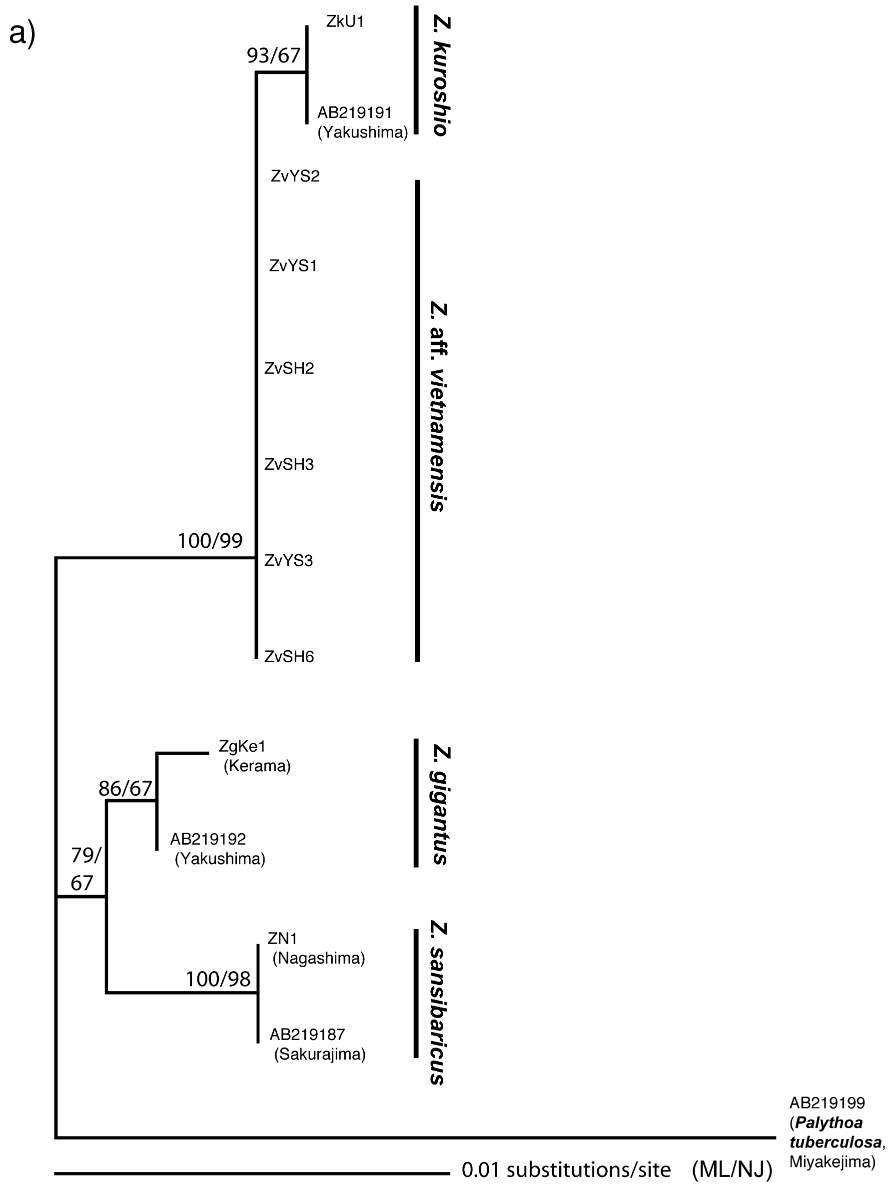
[1] From Reimer *et al.* (unpublished).

Morphology

Morphologically, *Z.* aff. *vietnamensis* showed clear differences from the other three *Zoanthus* species described thus far from southern Japan (Table 3). In cross-sections, *Z.* aff. *vietnamensis* was larger than both *Z. sansibaricus* and *Z. kuroshio*, but smaller than *Z. gigantus* (Table 3). Additionally, polyp heights of the *Z.* aff. *vietnamensis* samples we examined were longer than those of all other Japanese *Zoanthus* species (Table 3). Of particular interest, despite having mt 16S rDNA and ITS-rDNA sequence data virtually identical to that of *Z. kuroshio*, *Z.* aff. *vietnamensis* was morphologically very different from *Z. kuroshio* in polyp form, polyp height, colony form, polyp diameter, tentacle count, mesentery count, and maximum colony size (Fig. 3, Table 3).

DISCUSSION

Our genetic analyses indicate that *Z.* aff. *vietnamensis* and *Z. kuroshio* are very closely related. *Zoanthus* aff. *vietnamensis* mt 16S rDNA sequences were very similar to those of *Z. kuroshio* (sharing 8 of 9 base pair differences compared to *Z. sansibaricus*), but as mitochondrial genes have been shown to be highly conservative in cnidarians (Romano and Palumbi, 1997; Knowlton, 2000; Shearer *et al.*, 2002), these data alone are inconclusive regarding whether or not *Z.* aff. *vietnamensis* is a separate species from *Z. kuroshio*. This is a situation similar to that previously seen with identical mt 16S rDNA sequences occurring in *Palythoa tuberculosa* and *Palythoa mutuki* (Reimer *et al.* 2006b). However, ITS-rDNA sequences from *Z.* aff. *vietnamensis* were virtually identical to those previously obtained from *Z. kuroshio*. Differences in ITS-rDNA between other species of *Zoanthus* are clear (Reimer *et al.*, unpublished), and thus based solely on mt 16S rDNA and ITS-rDNA data, it appears that *Z.* aff. *vietnamensis* and *Z. kuroshio* are con-



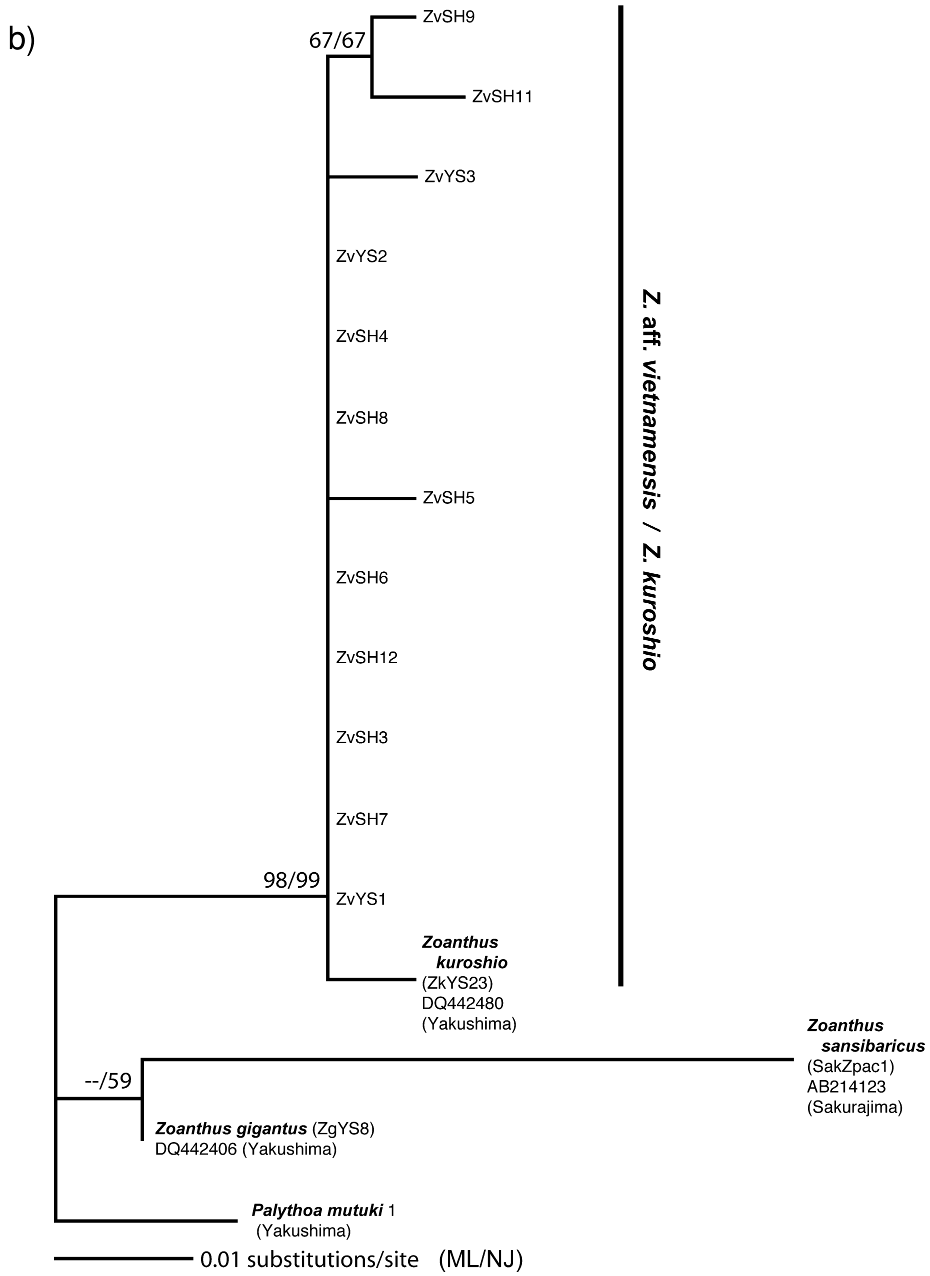


Fig. 2. Maximum likelihood (ML) trees for **a)** mt 16S rDNA and **b)** 5.8S rDNA sequences. Values at branches represent ML and NJ bootstrap probabilities, respectively (>50%). Sequences labeled without accession numbers were obtained in this study.

specific.

The morphological data do not match with the genetic data. *Zoanthus* aff. *vietnamensis* samples have very clear and consistent differences from *Z. kuroshio*, with much longer and wider polyps that are "liberae" in form (Pax, 1910), wider oral disk diameters, and much higher mesentery and tentacle counts (Table 3). There are some similarities between *Z.* aff. *vietnamensis* and *Z. kuroshio*, such as generally pink oral disk coloration and specific association with endosymbiotic *Symbiodinium* dinoflagellates of subclade C15 (*sensu* LaJeunesse, 2005) at Sakurajima and Yakushima (see Reimer *et al.*, 2006c). However, except for color, the differences in form are more striking than conspecific variation seen in the various morphotypes of *Z. sansibaricus* (Reimer *et al.*, 2004, 2006a), and even more striking than the morphological differences often observed between *Zoanthus* species.

Different morphotypes from all other *Zoanthus* species from southern Japan examined show overlap in mesentery and tentacle count, and other *Zoanthus* spp. in southern Japan have a generally consistent colony structure and polyp shape (Reimer *et al.*, 2006a). While our *Z.* aff. *vietnamensis* samples were often much longer in polyp length than the three other *Zoanthus* species found in southern Japan, polyp length may be influenced by currents and/or waves. At the Sangohama (Yakushima Island) site, *Z. kuroshio* (with short polyps embedded in a well-developed coenenchyme) is found only in areas of high wave activity, while

Table 3. Comparison of morphological and ecological characteristics of *Zoanthus* aff. *vietnamensis*, *Z. sansibaricus*, *Z. kuroshio*, and *Z. gigantus*.

Species/group	Oral disk color	Mesogleal thickness (μm)[1]	Polyp diameter (μm)[2]	Polyp height (μm)[3]	Usual polyp form[4]	Mesentery number[2]	Max. colony size[5]	Habitat current/wave activity	Depth (m)
Z. aff. *vietnamensis*	pale - dark pink	10–50 (30)	395–555	750–870	liberae	55–63	small	low - moderate	2.0 (Sakurajima) 0.0 – 5.0 (Yakushima)
Z. kuroshio[6]	white - pale pink	10–100 (20)	280–380	130–210	immersae	42–48	large	high	+0.5 – 3.0 (Yakushima)
Z. gigantus[6]	varied	30–100 (50)	600–750	500	liberae	57–63	small	moderate	0.0 – 5.0 (Yakushima)
Z. sansibaricus[6]	varied	10–75 (40)	240–400	460–580	liberae	48–60	large	low - high	2.0 – 10.0 (Sakurajima) +1.0 – 1.0 (Yakushima)

[1] Minimum and maximum, with average in parentheses.
[2] From cross-sections of polyps at maximum width.
[3] From longitudinal sections. Samples preserved in 99.5% ethanol, samples *in situ* would be much larger.
[4] Based on Pax (1910).
[5] Size definitions: small=<500 polyps/colony, large=1m^2 or larger (*e.g.* thousands of polyps).
[6] From Reimer *et al.* (2006a) supplemented by data collected by A.I.

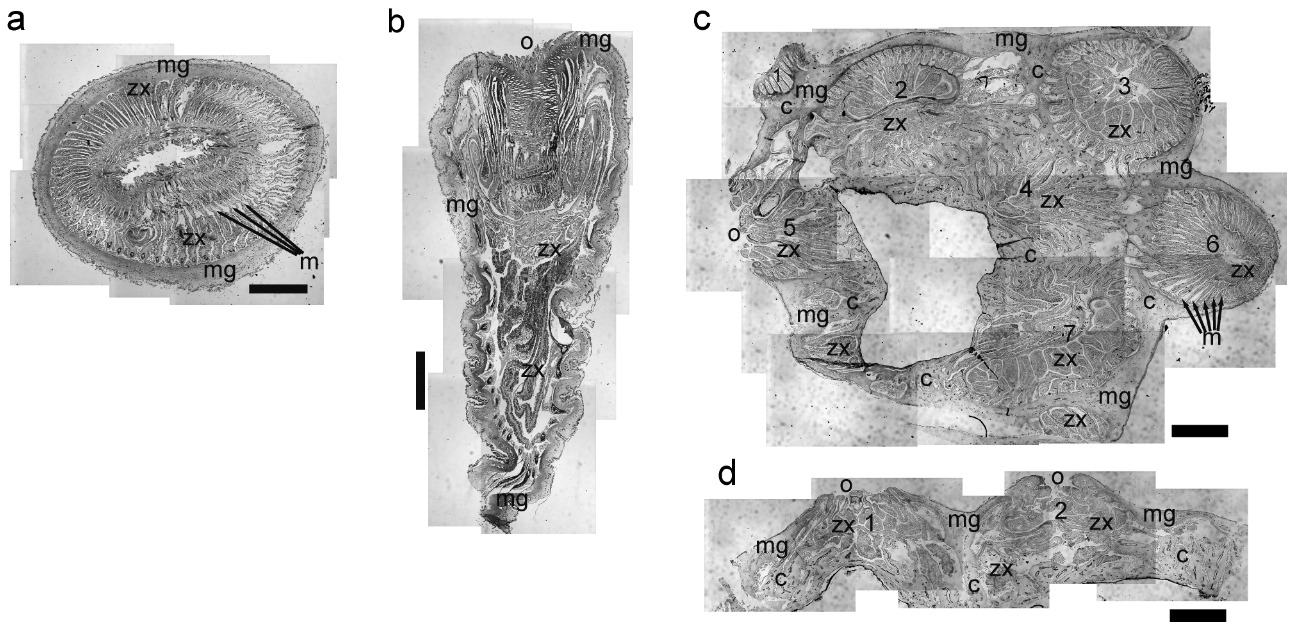


Fig. 3. **a)** *Zoanthus* aff. *vietnamensis* colony ZvSH1 (JDR collection) cross section and **b)** longitudinal section; **c)** *Z. kuroshio* colony NSMT-CO 1445 cross section and **d)** longitudinal section (c and d from Reimer *et al.*, 2006a). Abbreviations: m=mesentery, mg=mesoglea, o=oral opening, zx=zooxanthellae, c=coenenchyme. *Z. kuroshio* numbers refer to individual polyps. All black bars=1000 μm.

Z. aff. *vietnamensis* colonies (with longer polyps) appear in more sheltered areas (Table 3). Similarly, at the Sakurajima site in Kagoshima Bay, wave activity is much more limited than at Yakushima, and no *Z. kuroshio* morphotypes have been found. However, no colonies with features intermediate between these *Z. kuroshio* and *Z.* aff. *vietnamensis* have been seen, despite extensive sampling, contrary to what would be expected if morphology were based solely on environmental factors. Furthermore, how waves and/or currents could cause the internal morphological differences seen here (*e.g.*, mesentery and tentacle number) remains to be demonstrated.

There are two possible explanations to reconcile the genetic and morphological data seen in this study. Although speculative, one explanation is that *Z.* aff. *vietnamensis* and *Z. kuroshio* are conspecific morphospecies, as seen often in many hard coral species. For example, there are morphological differences in growth form of the hard coral *Pocillopora damicornis* (Linnaeus, 1758), with compact and sturdy branches in habitats with strong currents and/or waves, and thin and delicate branches in habitats with less current or wave influence (Veron, 2000). Such morphological differences are virtually identical to the differences observed between 'immersae' *Z. kuroshio* found in areas with high wave activity and ‘liberae’ *Z.* aff. *vietnamensis* found in areas with less wave activity. This hypothesis could also explain the very high level of similarity in mt 16S rDNA and ITS-rDNA sequences, but fails to explain why we did not observe any morphotypes intermediate between *Z.* aff. *vietnamensis* and *Z. kuroshio.*

The other explanation for our results is that *Z.* aff. *vietnamensis* and *Z. kuroshio* are very closely related yet different species. This explanation allows us to easily explain the observed differences in morphology with no observed intermediate forms, as well as the differences in habitat preference. If *Z.* aff. *vietnamensis* and *Z. kuroshio* are truly different species, they would be much more closely related than the other previously observed *Zoanthus* spp. (*Z. sansibaricus, Z. gigantus*) in southern Japan. Investigations into the sexual reproduction of *Z.* aff. *vietnamensis* and *Z. kuroshio* (*i.e.*, whether or not they are reproductively isolated) are necessary to confirm their true taxonomic status.

Regardless of the evolutionary relationship (*i.e.*, conspecific but differing morphotypes, or separate but closely related species) of *Z.* aff. *vietnamensis* and *Z. kuroshio*, our results highlight the morphological plasticity of *Zoanthus* species, and the dangers of basing species identifications solely on morphological data. Morphology alone does not indicate relatedness in this genus. Our results here confirm the contention by Burnett *et al.* (1997) that genetic data appear to be the best way to determine *Zoanthus* species groups and how they relate to one another.

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