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Reconsidering *Zoanthus* spp. Diversity: Molecular Evidence of Conspecifity Within Four Previously Presumed Species

James Davis Reimer[1*], Shusuke Ono[2], Yoshihiro Fujiwara[3], Kiyotaka Takishita[3] and Junzo Tsukahara[1]

[1]*Department of Developmental Biology, Faculty of Science, Kagoshima University, Korimoto 1-21-35, Kagoshima , 890-0065, Japan*
[2]*Miyakonojo Higashi High School, Mimata, Miyazaki, 889-1996, Japan*
[3]*Marine Ecosystems Research Department, Japan Marine Science and Technology Center, 2-15 Natsushima-cho, Yokosuka, Kanagawa, 237-0061, Japan*

ABSTRACT—We have conducted the first phylogenetic study to our knowledge of *Zoanthus* in the northern hemisphere by sequencing and analysing the mitochondrial cytochrome oxidase subunit 1 (COI) gene. Various unidentified *Zoanthus* specimens and samples of what have been assumed to be four discrete species (*Z. pacificus, Z. sansibaricus, Z. gnophodes, Z. erythrochloros*) were collected from four field sites in Kagoshima Prefecture, Japan. Based on our obtained COI gene sequences, all but one of our collected *Zoanthus* samples appear to be conspecific, with nearly 100.00% base pair matching. Genetic results are further backed up by collected polyp diameter, tentacle count, and mesentary count data. These results indicate a need to reconsider and re-analyze current *Zoanthus* classification and identification. Possible reasons for the large morphological variation in the same genotype in *Zoanthus* are also discussed.

Key words: *Zoanthus*, cytochrome oxidase subunit 1, morphotype, conspecific

INTRODUCTION

The zooxanthellate-encrusting anemone genus *Zoanthus* is worldwide in distribution, and is commonly found on rocky and hard substrates in tropical and sub-tropical waters, and especially in coral reef environments. Uchida and Soyama (2001) list 5 species of *Zoanthus* as occurring in Japanese waters; *Zoanthus sansibaricus* (Pax and Mueller, 1957), *Z. gnophodes* (ibid.), *Z. pacificus* (first described in Walsh and Bowers, 1971), *Z. erythrochloros* (Pax and Mueller, 1957), and *Z. vietnamensis* (ibid.). For a summary of *Z. pacificus, Z. sansibaricus, Z. gnophodes,* and *Z. erythrochloros*, refer to the summary of diagnostic characteristics in Table 1 and the color photographs in Fig. 1. Historically, *Zoanthus* diagnostic characters for identification purposes have been oral disk color, polyp diameter, tentacle count, and mesentary count (Pax and Mueller, 1957; Walsh and Bowers, 1971; Uchida and Soyama, 2001). However, the classification and identification of the entire genus is in chaos (Burnett *et al*., 1995; Burnett *et al*., 1997). Very little work has been done on identification to the species level, although some work was done on *Zoanthus* species of the Great Barrier Reef and northern Australia, which found that "species" or discrete groups had considerable morphological variation (Burnett *et al*., 1997). Fautin's (2003) database includes 320 descriptions of Zoanthidea, but only 16 have proper species descriptions. Similarly, there were almost 120 species of *Zoanthus* listed in this database (last updated in July 2003), but how many of these are actually true "species" is unknown (Fautin, 2003).

At Sakurajima, Japan, alone, there exists over 20 different color variations of *Zoanthus* at one inter-tidal site (personal observation), including the five presumed *Zoanthus* species mentioned above. Whether these are separate species or simply color variations of the same species is unknown.

The cytochrome oxidase subunit 1 (COI) gene has been shown in previous literature to be an accurate species-level marker (Dawson and Jacobs, 2001; Erpenbeck *et al*., 2002; Otranto *et al*., 2003). We sequenced the COI gene from collected *Zoanthus* samples to investigate the following question.

Are the various morphological types of *Zoanthus* different species as described in previous literature (for example, Uchida and Soyama, 2001), or are they conspecific?

* Corresponding author: Tel. +81-99-285-8161;
FAX. +81-99-285-8029.
E-mail: zoanthid@hotmail.com

Table 1. Summary of *Zoanthus* species morphological characteristics in previous literature and this study.

***Zoanthus* Species**	**Pax & Mueller (1957)**			**Walsh & Bowers (1971)**			**Uchida & Soyama (2001)**		**this study**		
	oral disk (polyp) diameter (mm)	tentacle count	mesentary count	oral disk (polyp) diameter (mm)	tentacle count	mesentary count	oral disk (polyp) diameter (mm)	tentacle count	oral disk (polyp) diameter (mm)	tentacle count	mesentary count
Z. erythrochloros	7	~60	54	NA	NA	NA	8	48~58	6~10	54	53
Z. gnophodes	6	NA	56	NA	NA	NA	8	~60	6~10	54~56	52~53
Z. pacificus	NA	NA	NA	7	60	60	6	54	6~10	54~58	52
Z. sansibaricus	NA	NA	NA	NA	NA	NA	7	56	6~10	54~56	52

NA = not available

MATERIALS AND METHODS

Sampling and DNA extraction, and PCR amplification

Samples of *Zoanthus* spp. (Table 2, Fig. 1) containing *Symbiodinium* spp. were collected from four field sites (Fig. 2) in Kagoshima Prefecture, Japan, in June~August 2003 and stored in 100% ethanol at –20°C. As samples were collected photographs were also taken to assist in identification and for collection of diagnostic character data (oral disk/polyp diameter and tentacle count) (Table 1, Fig. 1). The number of mesentaries of samples of the four presumed species *Z. pacificus, Z. sansibaricus, Z. gnophodes,* and *Z. erythrochloros* were counted from cross-sections (Table 1). During field sampling, *Palythoa* samples were collected from Amami and Yakushima field sites to provide outgroup sequences for the following phylogenetic analyses.

Usual *Zoanthus* tissue has a large number of ZX, and this makes it difficult to obtain uncontaminated *Zoanthus* DNA (personal observation, and see Maier *et al*., 2001 for a review of such problems). However, polyps contained zooxanthellae (ZX)-free gametes. ZX-free gametes were carefully removed from sample polyps using a dissecting microscope, and immediately processed.

A total of 29 samples were analyzed. ZX-free gamete samples weighed 0.4~1.8 mg. DNA was extracted from the samples using a spin-column DNeasy Animal DNA Extraction protocol (Qiagen, Santa Clarita, CA, USA) (LaJeunesse and Trench, 2000).

The primers used were the universal primers HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' and LCO1490 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al*., 1994).

PCR amplification was performed on the samples under the following conditions: an initial denaturing step at 95.0°C for 1 minute, followed by 35 cycles of 1 minute denature at 95.0°C, 1 minute annealing at 40.0°C, and 90 s extension at 72.0°C, followed by 7 minutes' extension at 72.0°C.

Sequence Analysis:

Cycle sequencing was accomplished in both directions using the forward and reverse primers separately. Reagents and reaction conditions were as specified in the ABI Prism Big Dye Terminator Cycle Sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA). Reaction products were analyzed on an Applied Biosystems 310 genetic analyzer (Division of Perkin Elmer, Foster City, CA, USA). The sequences were analyzed by DNASIS Mac v3.6 (Hitachi Software Engineering Company, Ltd., Tokyo, Japan).

By using CLUSTAL X v 1.8 (Thompson *et al*., 1997), the nucleotide sequences from *Zoanthus* taxa obtained in this study were aligned with those from other related species that were retrieved from GenBank. The alignment data are available on request from the corresponding author. A distance tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with ClustalX. Bootstrap values were obtained for 1000 replicates of dataset to assess relative branch support.

RESULTS

Morphological diagnostic character data

Obtained diagnostic character data for the four presumed species *Z. pacificus, Z. sansibaricus, Z. gnophodes,* and *Z. erythrochloros* are shown in Table 1, along with previous results from other studies. For all four presumed species there was overlap in polyp diameter (6~10 mm), tentacle count (54~58), and mesentary count (52~53), despite showing small variation between individual polyps. *Z. pacificus, Z. sansibaricus, Z. gnophodes,* and *Z. erythrochloros* morphological diagnostic character data overlapped with previous literature's data (Table 1). Wide variation was seen in oral disk color in all 29 sequenced samples (Table 2), while there was less variation in tentacle count (54~58) (data not shown), and oral disk/polyp diameter data (4~12 mm) (Table 2).

All *Zoanthus* samples were from clonal interconnected colonies except for Amami *Zoanthus* 4, which was found in small groups with individual polyps separated (Fig. 1e). It should be noted that polyp diameter (8 mm) and tentacle count (54) of Amami *Zoanthus* 4 are within the range of the other *Zoanthus* samples, however (Table 1, Table 2).

Zoanthus COI sequences

The alignment of our obtained sequences is shown in Fig. 3. The six different sequences were submitted to GenBank (Ascension Numbers AB128893 to AB128898). The phylogenetic tree based on the COI gene sequences is shown in Fig. 4.

All of our *Zoanthus* samples excepting Amami *Zoanthus* 4 (Fig. 1e) were found to be almost identical in their COI sequences (Figs. 3 and 4). In fact, all of the *Zoanthus* sample sequences were 100.00% identical over the 649 base pair length of the COI gene (designated "Zoanthus" in Fig. 3), excepting Sakurajima *Zoanthus* 1 (3 base pairs, or 0.46% different relative to the COI sequence "Zoanthus") and Sakurajima *Zoanthus* 9, which differed by four base

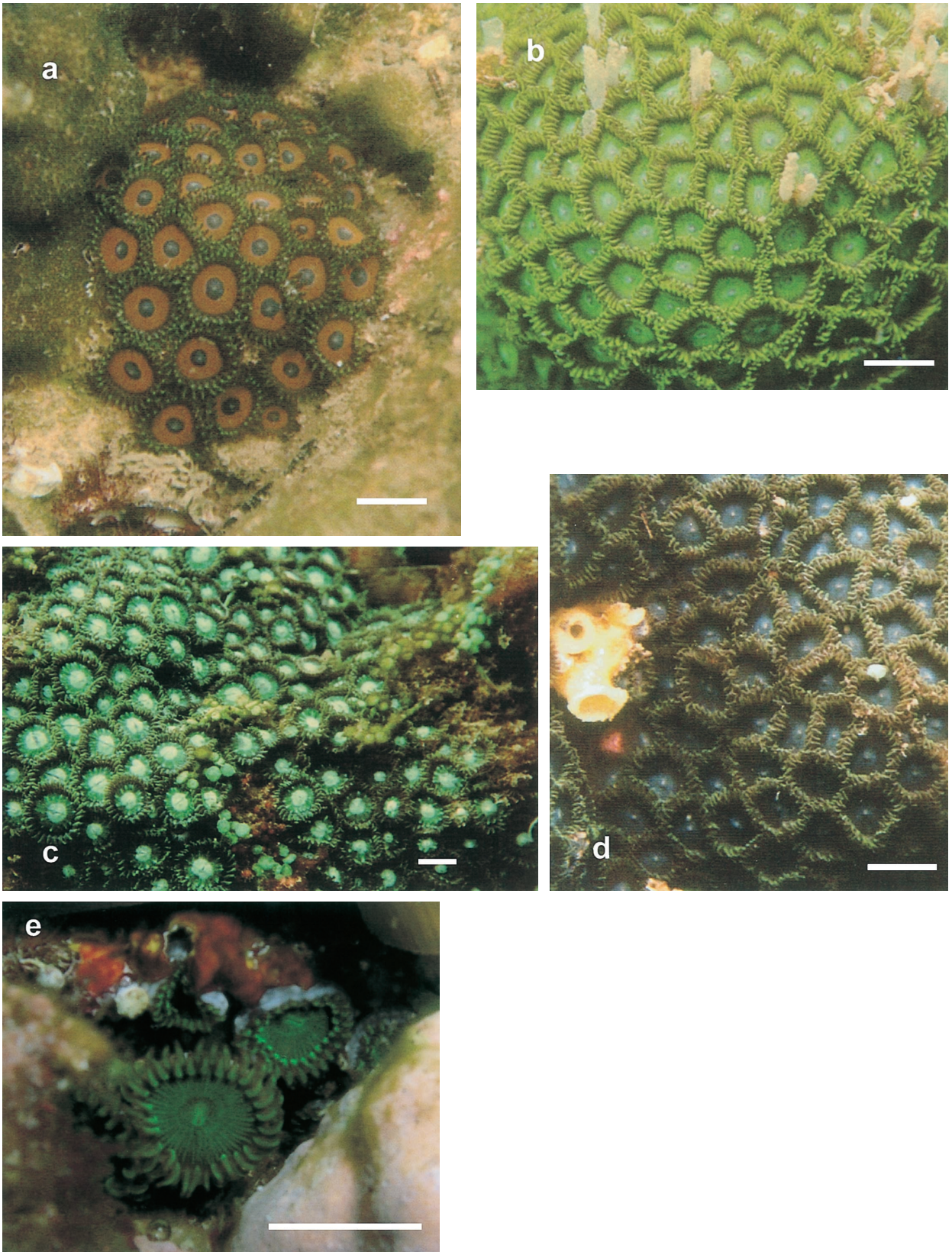

Fig. 1. Photographs of presumed species samples. All samples shown were collected from field sites in summer 2003. a) *Zoanthus erythrochloros*, b) *Z. gnophodes*, c) *Z. pacificus*, d) *Z. sansibaricus*, e) Amami *Zoanthus* 4. Please note that *Z. pacificus* was sampled from all four field sites. Diagnostic characters for identification visible in these photographs are oral disk color, polyp diameter, and tentacle number. White bar scale = 1 cm.

Table 2. List of collected samples with coloration, oral disk diameter, depth, and cytochrome oxidase subunit 1 (COI) sequence type.

Site ♣	Sample #	Disk Color	Tentacle Color	Disk Diameter (mm)	Depth (m)	COI type
K	Kokubu *Z. pacificus* 1 and 2	bright green	bright green	6~ 8	–2	Zoanthus
S	Sak *Z. pacificus*	bright green	bright green, brown	6~10	–2	Zoanthus
Y	Yaku *Z. pacificus* 1 and 2	bright green	bright green	6~10	+1	Zoanthus
A	Amami *Z. pacificus* 1 and 2	bright green	bright green	6~10	+0.5	Zoanthus
S	Sak *Zoanthus* 1	green-brown	green	6~10	–3	SakZoan1
S	Sak *Zoanthus* 2	green-brown	green	6~10	–3	Zoanthus
S	Sak *Z. erythrochloros* 1 and 2	red, blue, w/ white center dot	red-brown	6~10	–3	Zoanthus
S	Sak *Zoanthus* 3	yellow	green	6~10	–3	Zoanthus
S	Sak *Zoanthus* 4	yellow	green	6~10	–3	Zoanthus
S	Sak *Zoanthus* 5	red w/ white center dot	green or purple	6~10	–3	Zoanthus
S	Sak *Z. gnophodes*	green, blue	bright green	6~10	–3	Zoanthus
S	Sak *Zoanthus* 6	yellow	brown	6~10	–3	Zoanthus
S	Sak *Zoanthus* 7	white	purple	6~10	–3	Zoanthus
S	Sak *Z. sansibaricus*	purple w/ lighter center	purple	6~10	–3	Zoanthus
S	Sak *Zoanthus* 8	white	green	6~10	–3	Zoanthus
S	Sak *Zoanthus* 9	green	green	6~10	–3	SakZoan9
Y	Yaku *Zoanthus* 1	green w/ bright green center	purple	6~ 8	+1.5	Zoanthus
Y	Yaku *Zoanthus* 2	white, green w/ bright green dots and center, octogonal	red-brown, feathery	8~12	–1	Zoanthus
Y	Yaku *Palythoa*	brown	brown	10~20	+1.5	YakuPaly
Y	Yaku *Zoanthus* 5	purple w/ white center	blue-gray, feathery	8~12	–1	Zoanthus
A	Amami *Palythoa*	green	brown	10~20	+2	AmamiPaly
A	Amami *Zoanthus* 1	red, blue, w/ white center dot	green	4~ 6	0	Zoanthus
A	Amami *Zoanthus* 2	purple w/ white center dot	purple	4~ 8	+0.5	Zoanthus
A	Amami *Zoanthus* 4	bright green, polyps not clonal	green	6~10	+1	AmamiZoan4
A	Amami *Zoanthus* 5	pink	purple	4~ 8	+2	Zoanthus

♣ - Abbreviations: K=Kokubu, S=Sakurajima, Y=Yakushima, and A=Amami.
Depth is in meters relative to extreme low tide (thus "–" values are lower than the extreme low tide line, and "+" values above the extreme low tide line).

pairs (0.61%) from the "Zoanthus" sequence. Based on these results, it can be concluded that these samples are most likely intraspecific, despite their differing morphotypes. Presumed *Z. pacificus* sample COI sequences obtained from all 4 sites were 100.00% identical.

Amami *Zoanthus* 4's sequence was different by 7 base pairs (1.07%) from the "Zoanthus" sequence, which indicates Amami *Zoanthus* 4 may be of a different species.

Palythoa tuberculosa sequences were shown to be 24 to 25 base pairs (3.69%~3.85%) different from obtained

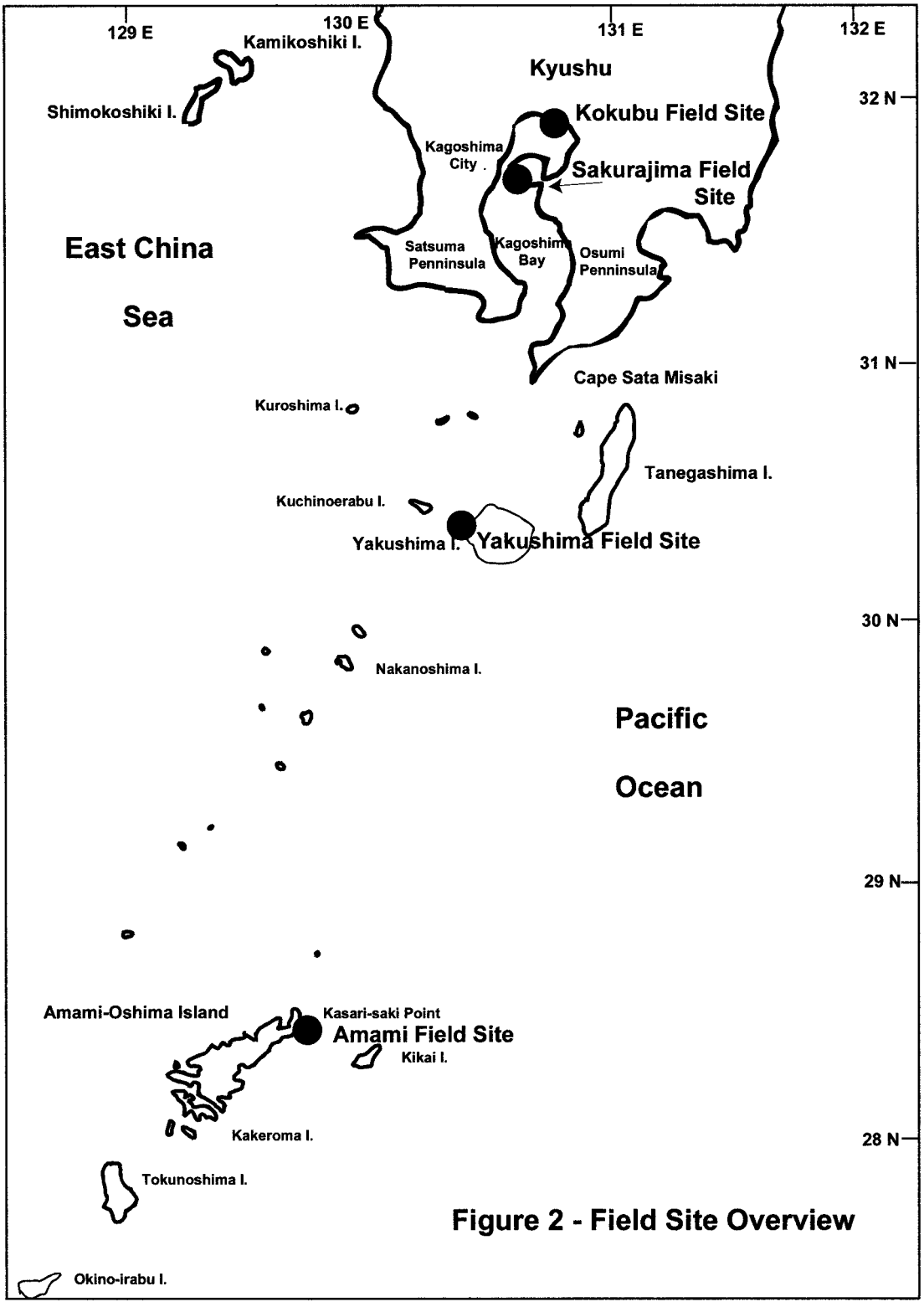

Fig. 2. Map of field sites. The distance from the northernmost site at Kokubu to the southernmost site at Amami is approximately 400 km, and spans from temperate inland waters in the north to sub-tropical coral reefs on the open ocean at Amami.

Zoanthus sequences. The two sampled *Palythoa* (green oral disk from Amami and brown oral disk from Yakushima) differed by two base pairs from each other, indicating a relationship within the species level.

DISCUSSION

DNA sequencing was performed on the mitochondrial cytochrome c oxidase 1 (COI) subunit gene. The COI gene

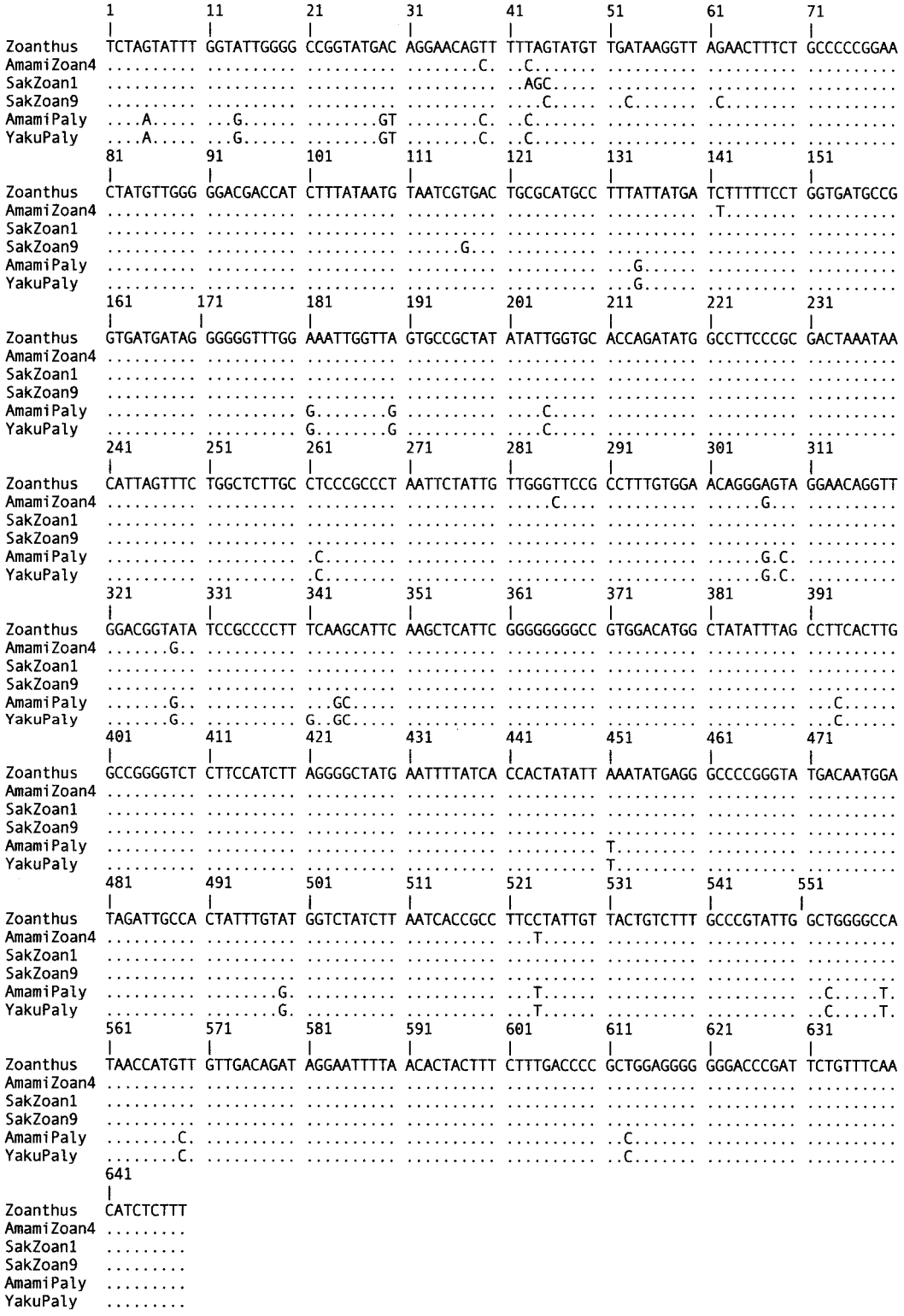

Fig. 3. Mitochondrial cytochrome oxidase subunit 1 gene sequence alignment of *Zoanthus* and *Palythoa* samples. The sequences are 649 base pairs long.

has been shown to be an adequate species-level marker (Dawson and Jacobs, 2001; Erpenbeck *et al.*, 2002; Otranto *et al.*, 2003). While investigating *Aurelia* (Cnidaria, Scyphozoa), Dawson and Jacobs (2001) suggested a 10% to 20% sequence difference as the benchmark of distinct species, based on their results and an examination of previous literature. In warble flies (Oestridae) Otranto *et al.* (2003) found intraspecific pairwise differences between 0.14%~1.59%,

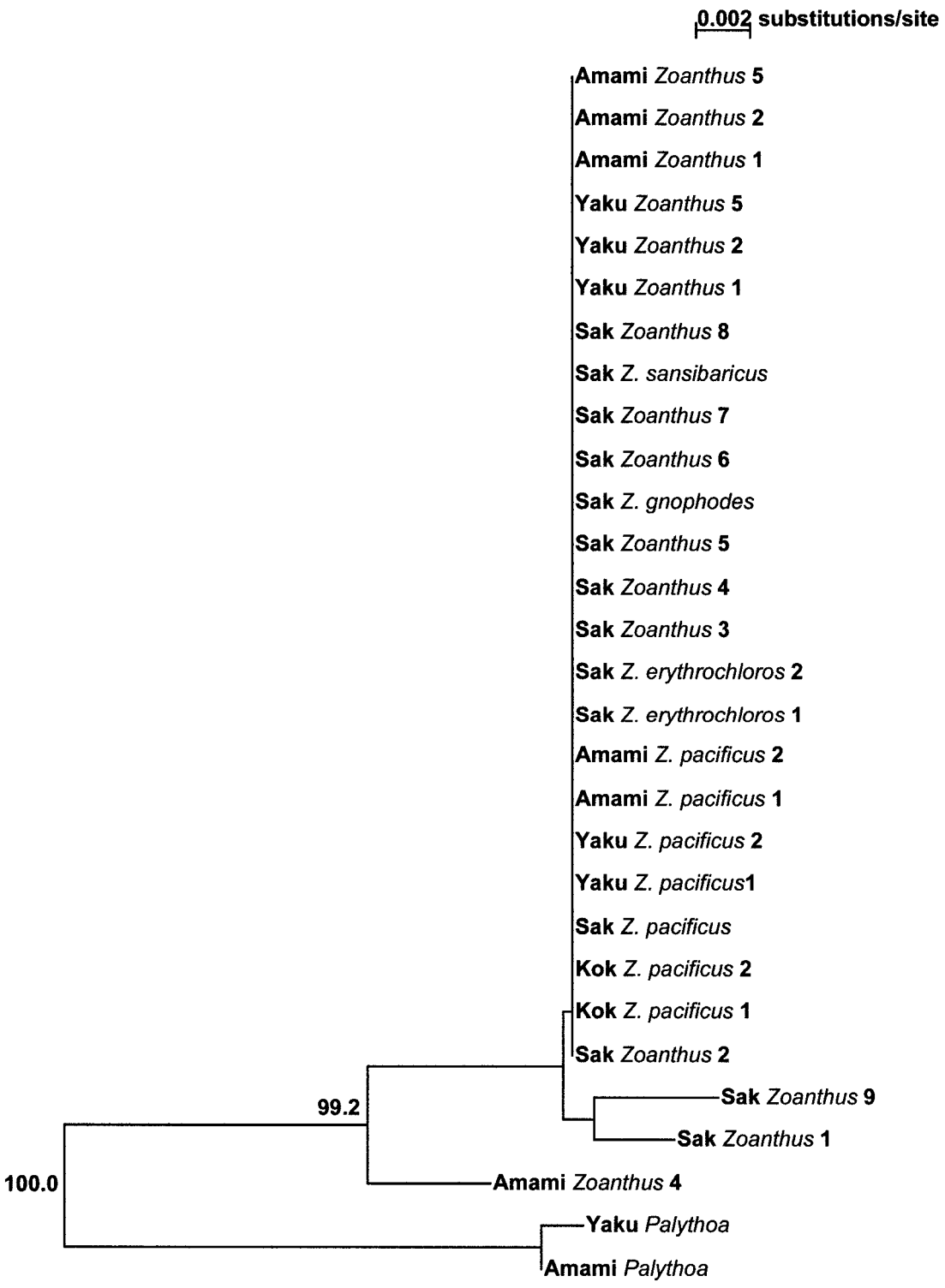

Fig. 4. Phylogenetic analysis of the COI gene sequences from collected samples. *Palythoa* species were used to root the tree. Numbers at the nodes refer to percentage of bootstrap support.

while interspecific differences were 0.7%~27%. While Dawson and Jacobs' (2001) benchmark value is much higher than the value calculated by Otranto *et al*. (2003), both Knowlton (2000) and Romano and Palumbi (1997) do mention that molecular evolution in cnidarians appears to be slower than in most other taxa. Supporting this hypothesis, France and Hoover (2002) found divergence levels in COI sequences among deep-sea octocorals were lower (0.4%~10.3%) than expected.

From our present results, regardless of which COI species benchmark is used it is clear that all four sites harbor the same species of *Zoanthus*, despite morphological varia-

tion in oral disk color between samples. Variation in other diagnostic characters, such as polyp diameter, tentacle count, and mesentary count is much less pronounced and shows overlap in data ranges.

Only whether Amami *Zoanthus* 4 is a distinct and separate species or not is open to speculation, as the difference of 1.07% in its COI sequence lies in the overlapping range between intra- and interspecific values. Morphologically Amami *Zoanthus* 4 showed slight differences as well, as polyps were found to be individual and not clonal unlike all other samples sequenced. Sequencing of different genes and further investigation may help clarify the classification of this sample.

Zoanthus larvae are thought to be able to live for long periods; a minimum of ~3 weeks (Burnett *et al*., 1995) up to several weeks before settling (Ryland, 1997) which could explain how similar genotypes could be distributed over such a wide range. The persistence of clonal genotypes and the amount of energy directed into asexual reproduction (i.e. rapid budding and changes in colony size etc.) as opposed to sexual reproduction may also contribute to similar genotypes being widespread (Ryland, 1997).

Previous literature dealing with classification and identification of *Zoanthus* species has been scarce, but literature up to now has identified 5 species of *Zoanthus* (Z. *vietnamensis, Z. sansibaricus, Z. gnophodes, Z. erythrochloros, Z. pacificus*) living in Japanese waters (Uchida and Soyama, 2001). While we collected *Z. vietnamensis* samples, we were unable to perform successful sequencing of these samples. However, the other four previously identified species samples, as well as other *Zoanthus* morphotypes collected appear to be conspecific genetically and morphologically, excepting oral disk color. Similar results were reported by Burnett *et al*. (1997) in Australia, who found that *Zoanthus* samples of various color morphotypes (excepting *Z. vietnamensis*) were all most likely conspecifiic, based on allozyme electrophoretic analyses of fourteen allozyme loci coding for eleven different enzymes. However, their study did not investigate the COI gene, so it is impossible to speculate on whether our *Zoanthus* and the studied Australian *Zoanthus* are conspecific or not.

What is clear is that current *Zoanthus* classification is in need of serious revision and review. The number of *Zoanthus* species worldwide, estimated at 120 (Fautin, 2003), may be far lower if our results are any indication.

On the other hand, if these various color morphotypes are not separate species, what accounts for their seeming morphological plasticity in oral disk color? Unlike other organisms, it appears that host tissue color is controlled in Anthozoa (including *Zoanthus*) solely by the green fluorescent protein (GFP) gene (Kelmanson and Matz, 2003). Kelmanson and Matz (2003) have shown that phenotypic plasticity in the great star coral *Montastraea cavernosa* exists in color variation. They suggest that several different loci on the (GFP) gene code for three different colors that are expressed in varying degrees, resulting in color morphs (Kelmanson and Matz, 2003). Thus, despite possessing identical GFP genotypes, color morphotypes exist in nature due to differing GFP expression. Such color variation appears to have evolved independently in several different classes of Anthozoans (Labas *et al*., 2002). This may be the case with *Zoanthus*, and investigating GFP genotypes in our samples is the next logical step in investigating the mechanism of oral disk color variation in *Zoanthus*. The underlying reason for such variation in color expression is not yet understood, although this may be related to the environment (depth, lighting, etc.). Further data collection is necessary.

In other work currently being conducted, it appears that *Zoanthus* colonies at the four study sites in this study harbor *Symbiodinium* zooxanthellae of different clades (Reimer *et al*., unpublished data). Thus, the *Zoanthus-Symbiodinium* "holotype" may be genetically and physiologically different from site to site despite the host *Zoanthus* being genetically identical, allowing this symbiosis to adapt to a variety of environments.

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