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

Authors: James Davis Reimer, Shusuke Ono, Yoshihiro Fujiwara, Kiyotaka Takishita, and Junzo Tsukahara
Source: Zoological Science, 21(5) : 517-525
Published By: Zoological Society of Japan
URL: https://doi.org/10.2108/zsj.21.517
Reconsidering Zoanthus spp. Diversity: Molecular Evidence of Conspecificity Within Four Previously Presumed Species

James Davis Reimer¹*, Shusuke Ono², Yoshihiro Fujiwara³, Kiyotaka Takishita³ and Junzo Tsukahara¹

¹Department of Developmental Biology, Faculty of Science, Kagoshima University, Korimoto 1-21-35, Kagoshima, 890-0065, Japan
²Miyakonojo Higashi High School, Mimata, Miyazaki, 889-1996, Japan
³Marine 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 Muller, 1957), Z. gnophodes (ibid.), Z. pacificus (first described in Walsh and Bowers, 1971), Z. erythrochloros (Pax and Muller, 1957), and Z. viettamensis (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?
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/polydiameter and tentacle count) (Table 1, Fig. 1). The number of mesenteries 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 1 minute 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 Cycle Sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA) (LaJeunesse and Trench, 2000).

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.

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

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oral disk (polydiameter (mm))</td>
<td>tentacle count</td>
<td>oral disk (polydiameter (mm))</td>
<td>tentacle count</td>
</tr>
<tr>
<td>Z. erythrochloros</td>
<td>7</td>
<td>~60</td>
<td>54</td>
<td>NA</td>
</tr>
<tr>
<td>Z. gnophodes</td>
<td>6</td>
<td>NA</td>
<td>56</td>
<td>NA</td>
</tr>
<tr>
<td>Z. pacificus</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>7</td>
</tr>
<tr>
<td>Z. sansibaricus</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>7</td>
</tr>
</tbody>
</table>

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 polydiameter (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/polydiameter 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 polydiameter (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 (Accession 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

Downloaded From: https://bioone.org/journals/Zoological-Science on 15 Jun 2019
Terms of Use: https://bioone.org/terms-of-use
Fig. 1. Photographs of presumed species samples. All samples shown were collected from field sites in summer 2003. a) *Zoanthus erythrochlo-ros*, 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.
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

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

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

*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).*
Evidence of Conspecificity in *Zoanthus* spp. 521

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. 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.
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%,
Evidence of Conspecifity in Zoanthus spp. 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-

---

**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.
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. erythrorholoros, 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 conspecific, 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 Anthozoa (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.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude and thanks to Professor Masao Sakai for his comments and software help throughout the course of this study. At the Japan Marine Science and Technology Center (JAMSTEC) in Yokosuka Dr. Tadashi Maruyama graciously provided helpful comments in helping with this study. In the JAMSTEC lab, Masaru Kawato gave invaluable and gracious guidance. Mika Wada and Denny Probizanski assisted with the field studies. This research was in part funded by a Ministry of Education scholarship.

REFERENCES


Evidence of Conspecifity in Zoanthus spp.


(Received December 17, 2003 / Accepted January 16, 2004)