# Molecular Evidence Suggesting Species in the Zoanthid Genera Palythoa and Protopalythoa (Anthozoa: Hexacorallia) Are Congeneric

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# Molecular Evidence Suggesting Species in the Zoanthid Genera *Palythoa* and *Protopalythoa* (Anthozoa: Hexacorallia) Are Congeneric

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**Taxonomic status of the zoanthid genera *Palythoa* and *Protopalythoa* has been in question for almost a century. Separation of the two genera has been based on traditional morphological methods (colony and polyp form, nematocyst size and form, and number of septa), with *Palythoa* polyps embedded in a well developed coenenchyme and *Protopalythoa* polyps standing free and clear of the coenenchyme. Here we sequenced two mitochondrial regions, the cytochrome oxidase I (COI) gene and 16S ribosomal DNA (16S rDNA) genes, from *Palythoa* and *Protopalythoa* samples from various parts of the world and performed phylogenetic analyses of the sequence data. The phylogenetic trees for both COI and 16S rDNA from *Palythoa* and *Protopalythoa* show four monophyletic groups (designated *Palythoa tuberculosa*, *Palythoa heliodiscus*, *Palythoa mutuki* 1, and *Palythoa mutuki* 2), with levels of sequence divergence (COI and 16S rDNA divergence approximately 0.0~1.1%) similar to or lower than that previously found among congeneric species within the closely related genus *Zoanthus*. Surprisingly, sequence differences among *Palythoa tuberculosa*, *Palythoa mutuki* 1, and *Palythoa mutuki* 2 were negligible (0.0~0.2% for both COI and 16S rDNA), potentially indicating relationships below the species level. Our sequences align well with the few *Palythoa* and *Protopalythoa* sequences reported to date. These findings strongly indicate that our samples represent a minimum of two and possibly up to four species (the *Palythoa tuberculosa* - *P. mutuki* 1 - *P. mutuki* 2 group, and *P. heliodiscus*) within the genus *Palythoa*, and that the genus *Protopalythoa* is erroneous nomenclature.**

**Key words:** 16S rDNA, COI, *Palythoa*, *Protopalythoa*, zoanthid

## INTRODUCTION

Zoanthid taxonomy has long been in a state of chaos, due to the morphological plasticity of species as well as a lack of research establishing reliable criteria to identify and delineate species and genera (Fossa and Nilsen, 1998). A striking example of this taxonomic problem is the status of the two genera *Palythoa* and *Protopalythoa*; arguments for and against the separation of these two genera have been ongoing for almost a century (see Verrill, 1900; Pax, 1910; Ryland and Lancaster, 2003). Morphologically, both genera are generally well described (for a review see Ryland and Lancaster, 2003). The major criteria for the delineation of *Palythoa* and *Protopalythoa* have been the structure and shape of polyps and the coenenchyme (Fig. 1), with *Palythoa* having polyps virtually immersed in a well developed coenenchyme (deemed "immersae" in form by Pax [1910]), and *Protopalythoa* having polyps standing free and clear of the coenenchyme, which forms basal lamellae ("intermediae" and "liberae" forms [Pax, 1910]). Other morphological characters cited include septa number, polyp column diameter, and nematocyst characters, as well as reproductive characters (Ryland and Lancaster, 2003; 2004), but all of these are extremely difficult to properly investigate, especially in the field, and may vary according to environment (Ryland and Lancaster, 2003).

A recent genetic study in the zoanthid genus *Zoanthus* has grouped *Z. kuroshio* (embedded polyps, "intermediae") and *Z. gigantus* and *Z. sansibaricus* (free-standing polyps, "liberae") (Reimer *et al*., 2006) into three monophyletic congeneric groups. These *Zoanthus* species differ from each other by 0.7~1.3% in mitochondrial cytochrome oxidase I (COI) gene sequence, while sequence differences between the related genera *Zoanthus* and *Palythoa* (both in the suborder Brachycnemina) are approximately 3–4%. Addition-

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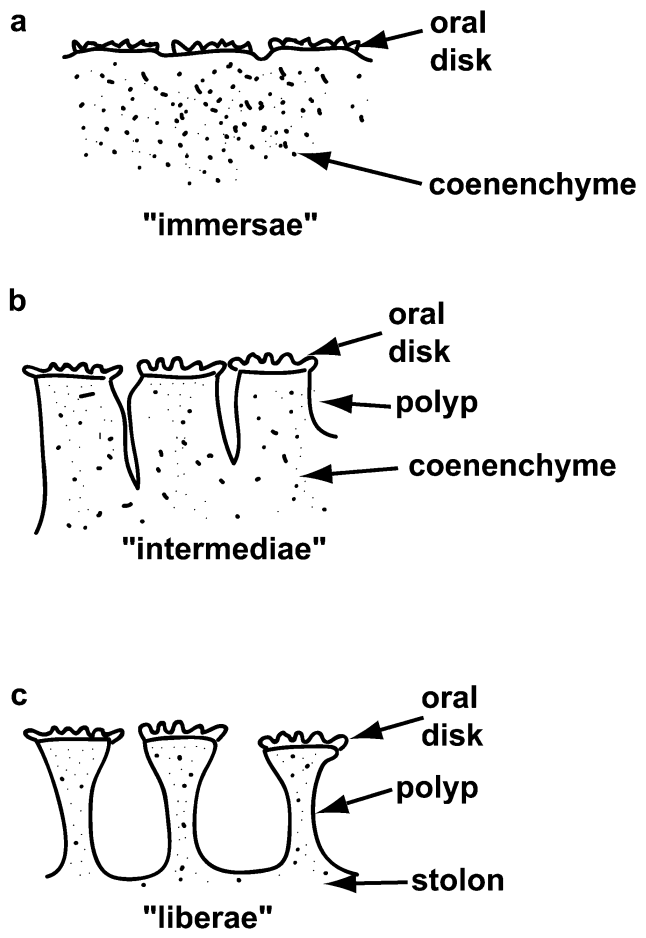


**Fig. 1.** Diagram of colony and polyp structure forms of zoanthids. a) "immersae" form, with polyps deeply embedded in a well-developed coenenchyme; b) "intermediae" form, intermediate in form, usually with well-developed, thick polyps; c) "liberae" form, with free-standing polyps extending well above a thin coenenchyme (stolons), often with space between oral disks. Adapted from Pax (1910) and Fossa and Nilsen (1998).

ally, genetic results show individual species in *Zoanthus* comprising a wide range of morphotypes, with *Z. sansibaricus* encompassing four previously presumed species (Reimer *et al*., 2004). Similarly, 16S and 12S rDNA sequences from a single sample each of *Palythoa* and *Protopalythoa* (Sinniger *et al*., 2005) showed differences below the genus level (<1% for both genes), indicating that *Palythoa* and *Protopalythoa* might indeed be one genus.

Here we use mitochondrial COI and 16S ribosomal DNA (16S rDNA) sequences from samples identified morpholgically as putative *Palythoa* and *Protopalythoa* from southern Japan, Saipan, Madagascar, and the Caribbean to clarify the phylogenetic relationship of these two genera. We also compare our sequences with previously obtained *Palythoa* and *Protopalythoa* sequences.

## MATERIALS AND METHODS

### Sample collection

Samples of *Palythoa* spp. (mostly appearing to be *Palythoa tuberculosa* as described by Uchida and Soyama, 2001) and *Protopalythoa* spp. were collected from various sites in southern Japan and Saipan between August 2003 and April 2005 and stored in 80–100% ethanol at –20°C. As samples were collected *in situ* photographs were taken to assist in identification and for collection of morphological data (colony and polyp form, in situ coloration, *etc*.), and sampling data (depth, environment, date) were recorded. Samples of *Palythoa* cf. *caribaeorum* (Utila, Honduras, collected February 2004), *Protopalythoa* sp. (Madagascar, February 2004), and *Palythoa* sp. (NW Madagascar, January 2004) were kindly supplied by Frederic Sinniger of the University of Geneva. A sample of *Parazoanthus gracilis* (Jogasaki, Izu, Japan) was used to obtain outgroup sequences for phylogenetic analyses.

### DNA extraction, PCR amplification, and sequencing

DNA was extracted from samples (5–25 mg) following procedures outlined in Reimer *et al*. (2004). The COI gene and 16S rDNA were amplified following procedures outlined in Reimer *et al*. (2004) and Sinniger *et al*. (2005), respectively. Amplification products were checked by 1.5% agarose gel electrophoresis and sequenced with an ABI PRISM$^{TM}$ 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDyeTM 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

Our new nucleotide sequences were deposited in GenBank (accession numbers AB219195–AB219225). COI and 16S rDNA sequences were aligned separately, using CLUSTAL X version 1.8 (Thompson *et al*. 1997), with sequences from *Zoanthus* spp. (COI sequences AB214172, AB214177 [J. Reimer, unpublished data] and AB219182 [Reimer *et al*., 2006]; 16S rDNA sequences AB219187, AB219191, AB219192 [Reimer *et al*. 2006]) and from *Parazoanthus gracilis* (COI, AB214178 [J. Reimer, unpublished data]; 16S rDNA, AB219194 [Reimer *et al*., 2006]). Two putative *Palythoa* and *Protopalythoa* spp. COI sequences obtained from a previous study (AB128895, AB128896; Reimer *et al*., 2004) were also included in the COI alignment. 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 comprising 533 sites from 29 taxa for the COI gene, and 870 sites from 12 taxa for 16S rDNA. These alignments are available on request from the corresponding author.

The same methods were independently applied for phylogenetic analyses of the COI and 16S rDNA sequences. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel, 2003). An input tree for PhyML was 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 + $\Gamma$). The proportion of invariable sites, a discrete gamma distribution, and base frequencies were estimated from the data set. 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 (1000 replicates) were constructed using the same model.

Maximum parsimony (MP) analyses were performed by PAUP* Version 4.0 (Swofford, 1998), using heuristic searches with closest stepwise addition of taxa and tree-bisection-reconnection (TBR) branch-swapping. A bootstrap analysis of 1000 replicates was conducted by the heuristic search method to assess the confidence of branches in the MP tree.

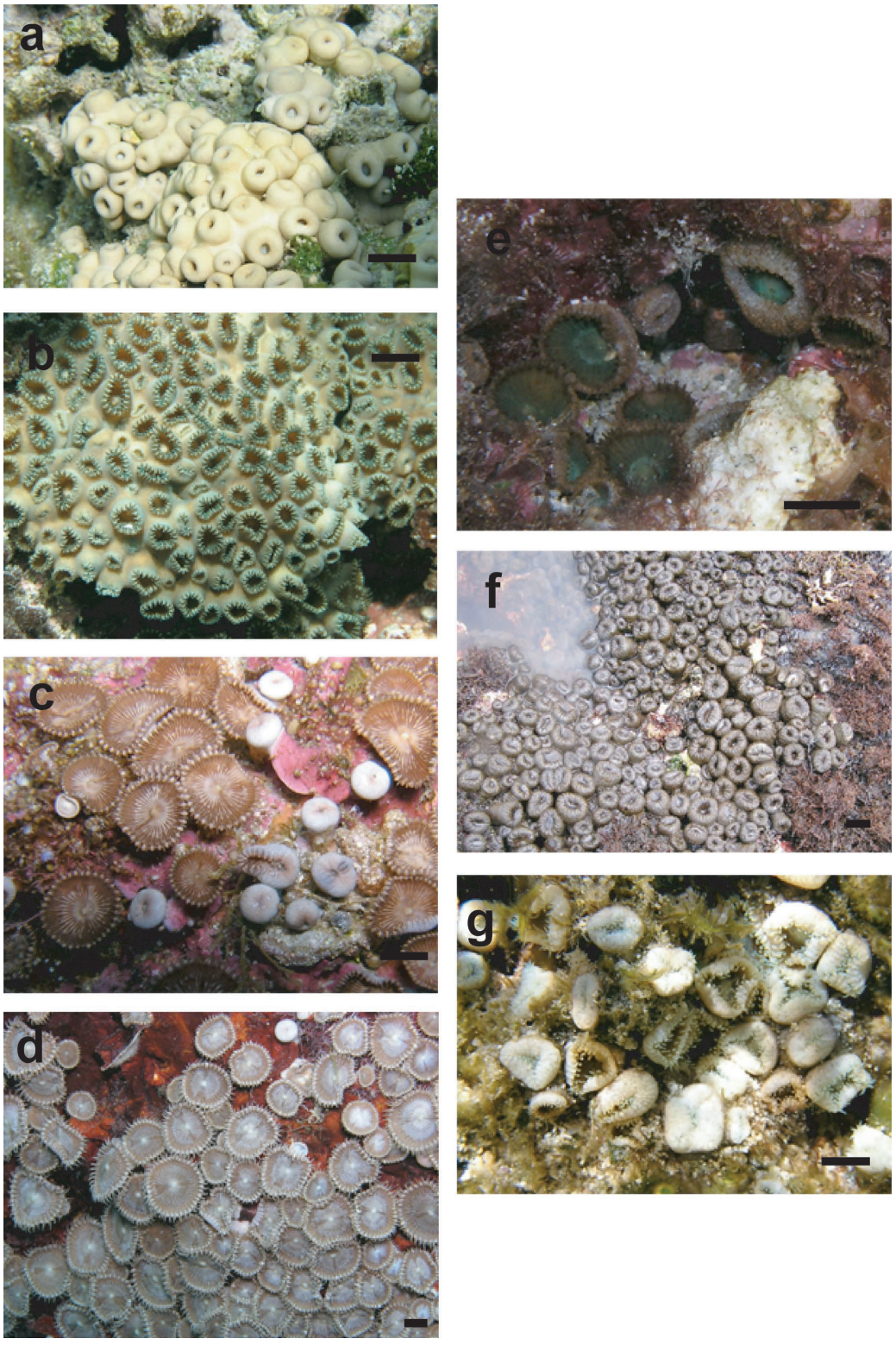


**Fig. 2.** Examples of *Palythoa* samples used in the current study. a) and b) *Palythoa tuberculosa*, "immersae" form, with individual polyps deeply embedded in a well-developed coenenchyme (a, sample PtYoS1, Shin's Reef, Yoron, Kagoshima, Japan, depth=1.0m; b, PtYS1, Sangohama, Yakushima, Kagoshima, Japan, 9.0m); c) and d) *Palythoa heliodiscus*, "liberae" form, individual polyps standing free and clear of a basal coenenchyme, and with polyps spaced more apart than *P. tuberculosa*, (c, PpEK1, Kaito, Erabu, Kagoshima, Japan, 19.0m; d, PpSaiLL1, Lau Lau, Saipan, 3.0m); e) and f) *Palythoa mutuki* 1, generally "intermediae" form, with colony and polyp structure intermediate between *P. tuberculosa* and *P.heliodiscus* (e, PpYS1, Sangohama, Yakushima, Kagoshima, Japan, 0.0m; f, PpMil1, Izushita, Miyakejima, Tokyo, Japan, 0.0m); g) *Palythoa mutuki* 2, morphologically virtually identical to *P. mutuki* 1 (g, PpAT1, Tomori, Amami, Kagoshima, Japan, +0.5 m). Black bars = 1 cm.

## RESULTS

### Sequences and phylogeny of the COI gene

All samples of presumed *Palythoa* spp. and *Protopalythoa* spp. (Fig. 2, Table 1) formed a monophyletic group separate from *Zoanthus* and *Parazoanthus*, with relatively strong ML bootstrap support (80%). *Protopalythoa* spp. samples did not form a separate clade from *Palythoa*. Instead, some *Protopalythoa* samples (PpAT1, PpAT2, AmamiPaly, PpYS1, PpMiI1, PpBA1) formed a cluster with the *Palythoa* clade. Samples of putative *Palythoa* from Madagascar (PM1, PM2) and Honduras (PcH1) were also in the *Palythoa* clade. *Protopalythoa* samples from Madagascar and Saipan (PpSaiLL1, PpM1) constituted another distinct monophyletic group (ML bootstrap support 100%). The resulting ML tree is shown in Fig. 3.

### Sequences and phylogeny of 16S rDNA

All samples of presumed *Palythoa* spp. and *Protopalythoa* spp. (Fig. 2, Table 1) formed a monophyletic group separate from *Zoanthus* and *Parazoanthus*, with strong MP bootstrap support (86%). The *Protopalythoa* spp. samples did not form a separate clade from *Palythoa*. Instead, four *Protopalythoa* samples (PpYS1, PpMiI1, PpAT1, PpAT2) were within the *Palythoa* clade (ML bootstrap support 90%). Sequence AF398920 (Burnett, unpublished data) of *Protopalythoa* sp. from Bali was also in the *Palythoa* clade, but was not included in the 16S rDNA alignment, as the sequence was too short. Sequences AF282931 from *Palythoa caesia* and AF282932 from *Palythoa caribaeorum* (Burnett, unpublished data) similarly belonged to the *Palythoa* clade, but as with *Protopalythoa* from Bali, were not included in the 16S rDNA alignment asthe sequences were too short. *Pro-*

**Table 1.** Zoanthid samples used in this study

| Assumed species | Sample #* | Sampling location | Depth (m) | Phylogenetic conclusion |
|---|---|---|---|---|
| *Palythoa tuberculosa* | YakuPalyBr[1] | Sangohama, Yakushima, Japan | + 1.5 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtAT1 | Tomori, Amami, Japan | – 2.0 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtAT2 | Tomori, Amami, Japan | – 1.5 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtBA1 | Akamizu, Bonotsu, Japan | – 1.0 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtWK1 | Kushimoto, Japan | – 1.0 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtMiI1 | Izushita, Miyakejima, Japan | – 2.0 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtYS1 | Sangohama, Yakushima, Japan | – 9.0 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtSaiLL1 | Lau Lau, Saipan | – 3.0 | *P. tuberculosa* |
| *Palythoa* sp. | PtIsO1 | Onsen, Ishigaki, Japan | – 8.5 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtIsK3 | Kataguwa, Ishigaki, Japan | –10.0 | *P. tuberculosa* |
| *Palythoa* sp. | PtYoS1 | Shin's Reef, Yoron, Japan | – 1.0 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtEO1 | Okidomari, Erabu, Japan | 0.0 | *P. tuberculosa* |
| *Palythoa tuberculosa* | PtKK1 | Kuroshima, Okinawa, Japan | – 6.0 | *P. tuberculosa* |
| *Palythoa anthoplax* | PaIsK2 | Kataguwa, Ishigaki, Japan | – 9.5 | *P. tuberculosa* |
| *Palythoa* cf. *caribaeorum* | PcH1[2] | Utila, Honduras | – 8.0 | *P. tuberculosa* |
| *Palythoa* sp. | PM1[2] | Tanikely, Madagascar | NA** | *P. tuberculosa* |
| *Palythoa* sp. | PM2[2] | NW coast, Madagascar | NA** | *P. tuberculosa* |
| *Protopalythoa* sp. | AmamiPalyGr[1] | Tomori, Amami, Japan | + 2.0 | *P. mutuki 2* |
| *Protopalythoa* sp. | PpAT1 | Tomori, Amami, Japan | + 0.5 | *P. mutuki 2* |
| *Protopalythoa* sp. | PpAT2 | Tomori, Amami, Japan | + 1.0 | *P. mutuki 2* |
| *Protopalythoa* sp. | PpYS1 | Sangohama, Yakushima, Japan | 0.0 | *P. mutuki 1* |
| *Protopalythoa* sp. | PpSaiLL1 | Lau Lau, Saipan | – 3.0 | *P. heliodiscus* |
| *Protopalythoa* sp. | PpEK1 | Kaito, Erabu, Japan | –19.0 | *P. heliodiscus* |
| *Protopalythoa* sp. | PpBA1 | Akamizu, Bonotsu, Japan | 0.0 | *P. mutuki 1* |
| *Protopalythoa* sp. | PpM1[2] | Madagascar | NA** | *P. heliodiscus* |
| *Protopalythoa* sp. | PpMiI1 | Izushita, Miyakejima, Japan | 0.0 | *P. mutuki 1* |

[1]from Reimer *et al*. (2004).

[2]samples collected by Frederic Sinniger. Samples without superscript were collected by JDR. All samples conserved in JDR's collection.

*Sample name abbreviations: Samples are designated by assumed species, sample site, sample locale, and sample number. Thus, sample PtAT1 is Palythoa tuberculosa, Amami, Tomori, sample 1. Abbreviations: Pt=*Palythoa tuberculosa*, P=*Palythoa* sp. Pa=*Palythoa anthoplax*, Pc=*Palythoa* cf. *caribeorum*, Pp=*Protopalythoa* sp. Sample sites and locales are as listed. Samples from Reimer *et al*. (2004) use nomenclature identical to that study.

**NA=not available

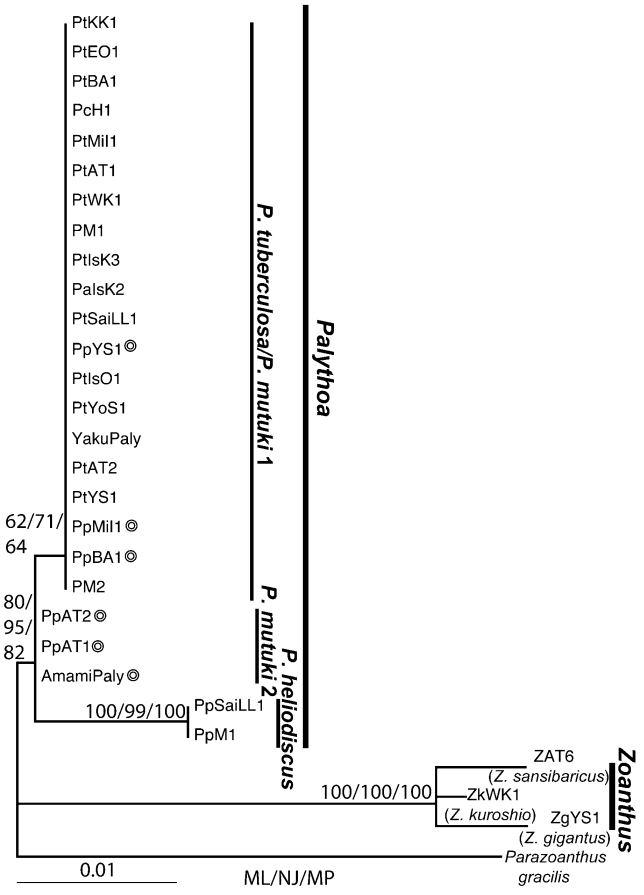


**Fig. 3.** Maximum likelihood tree of cytochrome oxidase I gene (COI) sequences. Values at branches represent ML, NJ, and MP bootstrap probability, respectively (>50%). Refer to Table 1 for sample name abbreviations.

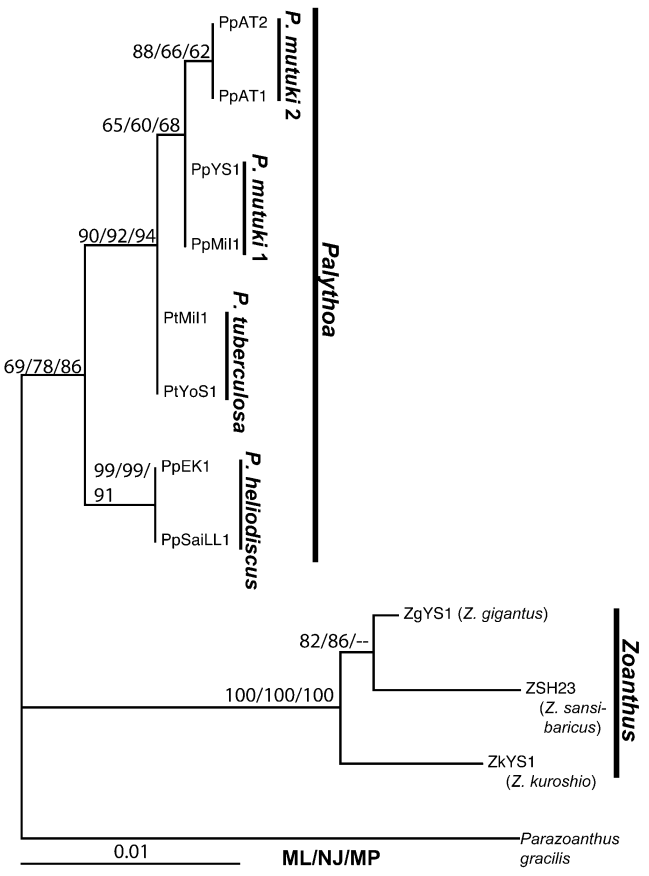


**Fig. 4.** Maximum likelihood tree of 16S ribosomal DNA (16S rDNA) sequences. Values at branches represent ML, NJ, and MP bootstrap probability, respectively (>50%). Refer to Table 1 for sample name abbreviations.

*topalythoa* samples from Erabu, Japan and Saipan (PpEK1, PpM1) constituted another distinct clade (ML bootstrap support 99%). The resulting ML tree is shown in Fig. 4.

## DISCUSSION

### Diversity of *Palythoa/Protopalythoa* species

As shown in Figs. 3 and 4, all of our samples of presumed *Palythoa* (but not presumed *Protopalythoa*), regardless of the species identification and collection area, belonged to the same monophyletic group, with very little divergence among samples (0.0~0.1% for COI and 0.0% for 16S rDNA). Similar results on a smaller scale were obtained by Burnett *et al*. (1994) when they examined *Palythoa caesia* on the Great Barrier Reef and found that this single species encompassed a wide range of morphotypes. Based on our genetic results from worldwide samples, it appears that our samples of presumed *Palythoa* from the Caribbean, Indian Ocean, and Pacific may all be conspecific. Similarly, our samples of putative *Protopalythoa* species only formed three non-monophyletic groups, indicating that many of the 217 currently described species of *Palythoa/Protopalythoa* (Fautin, 2004) in all likelihood constitute varying morphotypes of the same species. This result is similar to that reported for *Zoanthus* spp. (Reimer *et al*., 2004).

Species of the *Palythoa/Protopalythoa* group have often been described simply on the basis of color, or polyp shape and size (*e.g*., Zunan, 1998), but as is shown in Fig. 2 (compare 2a and 2b, or 2e, 2f, and 2g), *Palythoa/Protopalythoa* colonies can vary greatly in polyp form and color with variations in environment (*e.g*., current strength and duration, degree of wave action, sediment color). The most striking examples of this were the samples from Miyakejima, Japan (PpMiI1, shown to be *Palythoa mutuki* 1 [Fig. 2f], and PtMiI1, *P. tuberculosa*, not shown), which were dark gray in polyp color due to volcanic ash incorporated into their tissue, in stark contrast to all other samples from all other sites, which were white, light tan, or brown in color. Additionally, *P. tuberculosa* from Shin's Reef, Yoron, Japan (sample PtYoS1), had a unique colony and polyp form (Fig. 2a), much more "intermediae" than all other *P. tuberculosa* samples, and yet was unambiguously within the *P. tuberculosa* clade for both the COI and 16S rDNA data sets. Similarly, sample PaIsK2, which appeared to be *Palythoa anthoplax* as described by Zunan (1998), with smaller polyps than *Palythoa tuberculosa* samples, was unambiguously within the COI *P. tuberculosa* clade.

**Are *Palythoa* and *Protopalythoa* separate genera?**

The COI and 16S rDNA trees clearly show that the *Palythoa* and *Protopalythoa* species groups are no more divergent than species groups within the genus *Zoanthus* (Table 2, Figs. 3, 4). COI sequences of *Zoanthus* congeners are divergent by 0.8~1.1%, while our assumed *Palythoa* and *Protopalythoa* samples are divergent by 0.0~1.1%. Similarly, 16S rDNA results show *Zoanthus* congeners divergent by 0.7~1.3% and *Palythoa* and *Protopalythoa* sequences divergent by a maximum of 0.9%. These results are congruent with those of Sinniger *et al*. (2005), who saw 0.5% divergence between *Palythoa tuberculata* and *Protopalythoa* sp. utilizing 16S rDNA, and 0.2% utilizing 12S rDNA. On the basis of our COI and 16S rDNA phylogenies and past examinations of COI data in Anthozoa (Medina *et al*., 1999; Reimer *et al*., 2004; van Oppen *et al*., 2004), as well as morphological characteristics described earlier, we interpreted our presumed *Palythoa* spp. and *Protopalythoa* spp. samples to be congeners (Table 2).

It is true that when polyps are expanded, many *Zoanthus* species look strikingly similar, but often when polyps are closed, colony and coenenchyme structure appear different (J. Reimer, personal observation). Many *Palythoa/Protopalythoa* species' polyps are closed during the daytime, making coenenchyme and polyp differences more striking to most observers (in the daytime) than in *Zoanthus* spp. Overall, however, morphological differences between *Palythoa* and *Protopalythoa* are not as pronounced as other generic-level comparisons (*e.g*., *Zoanthus* [no sand incorporated into polyps] and *Palythoa* [sand incorporated into polyps], or *Palythoa* [colonial] and *Sphenopus* [non-colonial], the latter two both members of family Sphenopidae).

**Species examined in the current study**

All samples of presumed *Palythoa/Protopalythoa* species investigated during the course of this study were placed genetically within one of four clades. Examination of *in situ* photographs and collection data (depth, *etc*.) showed that no clear morphological divisions could be made that were congruent with the majority of our genetic data. Morphological variation (colony shape, colony size, polyp shape, polyp size, oral disk and polyp color) existed within three of the following four clades (excepting *Palythoa heliodiscus*).

**1. *Palythoa tuberculosa*** (Fig. 2a, b) as described in Uchida and Soyama (2001). This group includes all presumed *Palythoa* samples (YakuPaly, PtAT1, PtAT2, PtBA1, PtWK1, PtMil1, PtYS1, PtSaiLL1, PtIsO1, PtIsK3, PtYoS1, PtEO1, PtKK1, PaIsK2, PcH1, PM1, PM2), with colonies generally "immersae" in form (Pax 1910), but occasionally "intermediae" (Fig. 2a, b). Samples from this clade were found worldwide (Japan, Saipan, Madagascar, Honduras) and correspond to *Palythoa tuberculosa* from Japan described in Uchida and Soyama (2001). One sample from Kataguwa Reef, Ishigaki Is., Japan, had smaller polyps and more closely resembled *Palythoa anthoplax* (as described in Zunan, 1998), but the COI and 16S rDNA sequences were identical to our *P. tuberculosa* sequences. It should be noted that *P. tuberculosa* (and *P. mutuki* 1) samples were found on the north end of Miyakejima Island, Japan, and we believe that this is the farthest north *Palythoa* has been recorded in the western Pacific.

**2. *Palythoa heliodiscus*** Ryland and Lancaster, 2003 (Fig. 2c, d). Samples PpSaiLL1, PpEK1, and PpM1, which formed a monophyletic group in both our COI and 16S rDNA analyses, correspond to *Protopalythoa heliodiscus* (Ryland and Lancaster, 2003) and *Palythoa* (*Protopalythoa*) *lesueuri* (as described in Uchida and Soyama, 2001). The level of sequence difference of this clade from *Palythoa tuberculosa* (1.1% COI, 0.9% 16S rDNA) and its very high bootstrap support (100% for ML, both COI and 16S rDNA) clearly show that this group is a separate *Palythoa* species from all other observed *Palythoa/Protopalythoa* samples in this study. We have chosen here to use the name *Palythoa heliodiscus,* as the species description in Ryland and Lancaster (2003) is highly detailed. Similarly to observations made by Burnett *et al*. (1997) and Ryland and Lancaster (2003), this species was generally found to be subtidal (Table 1), and to occur in areas of lower light (*i.e*., on rock/coral ledges and shelves under other coral colonies, away from direct light exposure) than the other putative *Palythoa* species described here. *Palythoa heliodiscus* specimens had shorter tentacles than *Palythoa mutuki* 1-2 (see below), as well as a white mouth opening. Polyps were "liberae" (Pax, 1910) in form (Fig. 2c, d). For a morphological description separating *P. heliodiscus* and *P. mutuki* (*Protopalythoa* in the text), refer to Ryland and Lancaster (2003).

**3. *Palythoa mutuki* 1** (Fig. 2e, f) (as described in Ryland and Lancaster, 2003). Samples of this species (PpYS1, PpBA1, PpMil1) were virtually indistinguishable from *Palythoa mutuki* 2 samples (see below), with very similar habitat, polyp size and structure, as well as polyp spacing and coenenchyme appearance. The only noticeable morphological differences observed were that *P. mutuki* 1 had a green oral disk with a pale green center and dark-colored tentacles, whereas *P. mutuki* 2 had a brown oral disk with a white oral opening and pale radii (marking septa), and

**Table 2.** Comparison of cytochrome oxidase I (COI) and mitochondrial 16S ribosomal DNA (16S rDNA) sequence difference levels in zoanthids

| Comparison groups | 16S rDNA sequence difference | COI sequence difference | Taxonomic relation level |
|---|---|---|---|
| *Palythoa heliodiscus – P. tuberculosa* | 0.9% | 1.1% | species |
| *P. mutuki 1 – P. tuberculosa* | 0.1% | 0.0% | species or subspecies |
| *P. mutuki 2 – P. tuberculosa* | 0.2% | 0.2% | species or subspecies |
| *Zoanthus sansibaricus – P. tuberculosa* | 2.1% | 3.4% | genera |
| *Z. sansibaricus – Z. gigantus* | 0.7% | 1.1% | species |
| *Z. sansibaricus – Z. kuroshio* | 1.3% | 0.8% | species |

comparatively lighter tentacles than *P. mutuki* 1. However, differences in color must be interpreted with extreme caution, as shown in the genus *Zoanthus*, where many different color morphotypes have been clearly shown to be conspecific (Reimer *et al*., 2004; 2006). Whether these samples are truly a new cryptic species, or a subspecies or different genotype of either *P. tuberculosa* or *P. mutuki* 2, is open to speculation; the COI sequences were identical to *P. tuberculosa* and only one base pair different from *P. mutuki* 2, and the 16S rDNA sequences differed from both *P. tuberculosa* and *P. mutuki* 2 by only one base pair. In the future, less conservative genetic markers more appropriate for examining interspecific relationships, such as the internal transcribed spacer (ITS) rDNA region, should be utilized to clarify the taxonomic status of the *P. tuberculosa* - *P. mutuki* 1 - *P. mutuki* 2 phylogenetic group.

**4. *Palythoa mutuki* 2** (Fig. 2g) (as described in Ryland and Lancaster, 2003). *Palythoa mutuki* 2 samples (Amami-Paly, PpAT1, PpAT2) were found to be either "liberae" or "intermediae" in form, having much larger and thicker polyps connecting the oral disk to the coenenchyme than *P. heliodiscus* (Fig. 2g), but with a coenenchyme not as well-developed as that of *P. tuberculosa*. All *P. mutuki* 2 samples were found intertidally in areas experiencing strong waves and/or currents, and only at the Amami sampling location. As mentioned above, *P. mutuki* 1 and *P. mutuki* 2 are morphologically indistinguishable, and genetically very closely related (perhaps below the species level) to each other and to *P. tuberculosa*; thus *P. mutuki* 1 and/or 2 may have a subspecies-level relationship with *P. tuberculosa* and/or each other. Despite an apparent close taxonomic relationship to *P. tuberculosa*, *P. mutuki* 1 and 2 samples are morphologically very different from *P. tuberculosa*. Further investigation into the taxonomic relationship between *P. tuberculosa* and *P. mutuki* 1 and 2 is clearly needed.

## Conclusions

Taken as a whole, our molecular genetic evidence, plus an examination of what morphologically constitutes genera in other zoanthid groups, strongly suggest that *Palythoa* and *Protopalythoa* are not truly separate genera, but instead comprise congeneric species and/or subspecies within the genus *Palythoa*. The sequence similarities between putative, "immersae"-form *P. tuberculosa* and the morphologically very similar "intermediae/liberae"-form *P. mutuki* 1 and *P. mutuki* 2 show that zoanthid species assignment based solely on morphology is uncertain speculation at best. Further genetic investigations utilizing other genetic markers and other presumed *Palythoa* and *Protopalythoa* species besides the ones examined here (and in particular the *Palythoa* and *Protopalythoa* type species, *Palythoa mamillosa* and *Protopalythoa variabilis*) will help solidify our conclusions as well as shed further light on the true level of diversity found in this genus.

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## REFERENCES

Burnett WJ, Benzie JAH, Beardmore JA, Ryland JS (1994) High genetic variability and patchiness in a common Great Barrier Reef zoanthid (*Palythoa caesia*). Mar Biol 121: 153–160

Burnett WJ, Benzie JAH, Beardmore JA, Ryland JS (1997) Zoanthids (Anthozoa, Hexacorallia) from the Great Barrier Reef and Torres Strait, Australia: systematics, evolution, and a key to species. Coral Reefs 16: 55–68

Fossa SA, Nilsen AJ (1998) The Modern Coral Reef Aquarium, Vol. 2. Birgit Schmettkamp Verlag, Bornheim

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704

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

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

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

Pax F (1910) Studien an westindischen Actinien. In "Ergebnisse einer Zoologischen Forschungreise nach Westindien von Prof. W. Kukenthal und Dr. R. Hartmeyer im Jahre, 1907" Ed by JW Spengel, Zoologische Jahrbucher Supplement 11: 157–330

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, Takishita K, Tsukahara J, Maruyama T (2006) Morphological and molecular revision of *Zoanthus* (Anthozoa: Hexacorallia) from Southwestern Japan with description of two new species. Zool Sci 23: (in press)

Rodriguez F, Oliver JL, Marin A, Medina JR (1990) The general stochiatic model of nucleotide substitution. J Theor Biol 142: 485–501

Ryland JS (1997) Reproduction in Zoanthidea (Anthozoa: Hexacorallia). Invert Reprod Dev 31: 177–188

Ryland JS, Lancaster JE (2003) Revision of methods of separating species of *Protopalythoa* (Hexacorallia: Zoanthidea) in the tropical West Pacific. Invert Systemat 17: 407–428

Ryland JS, Lancaster JE (2004) A review of zoanthid nematocyst types and their population structure. Hydrobiol 530/531: 179–187

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425

Sinniger F, Montoya-Burgess JI, Chevaldonne P, Pawlowski J (2005) Phylogeny of the order Zoantharia (Anthozoa, Hexacorallia) based on mitochondrial ribosomal genes. Mar Biol 147: 1121–1128

Swofford DL (1998) PAUP*. V Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882

Uchida H, Soyama I (2001) Sea Anemones in Japanese Waters. TBS Britannica, Tokyo (in Japanese)

van Oppen MJH, Koolmees EM, Veron JEN (2004) Patterns of evolution in the scleractinian coral genus *Montipora* (Acroporidae)

Mar Biol 144: 9–18
Verrill AE (1900) Additions to the Anthozoa and Hydrozoa of the Bermudas. Trans Connecticut Acad Arts Sci 10: 551–572
Zunan P (1998) Coelenterata Actiniaria Ceriantharia Zoanthidea. Science Press, Beijing

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