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REPORT

Morphological and molecular characterization of a new genus and new species of parazoanthid (Anthozoa: Hexacorallia: Zoantharia) associated with Japanese Red Coral

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Abstract The Order Zoantharia has long been taxonomically neglected primarily due to difficulty in examining the internal morphology of sand-encrusted zoanthids. However, recent work using molecular markers has shown an unexpectedly high diversity of previously "hidden" taxa (families and genera) within Zoantharia (=Zoanthidea, Zoanthiniaria). In this study, unidentified sediment-encrusting zoanthid specimens ($n = 8$) were collected from living Japanese Red Coral *Paracorallium japonicum* (Family Coralliidae) during precious coral harvesting by Remotely Operated Vehicle (ROV) and manned submersible (February 2004–January 2006) at depths of 194–250 m at six locations between Ishigaki-jima Island and Kikai-jima Island, southern Japan. DNA sequences (mitochondrial 16S ribosomal DNA [mt 16S rDNA], cytochrome oxidase subunit I [COI], nuclear internal transcribed spacer of ribosomal DNA [ITS-rDNA]) unambiguously place these specimens in a previously undescribed, new monophyletic lineage within the family Parazoanthidae. *Corallizoanthus tsukaharai,* gen. n. et sp. n. is the first reported zoanthid species associated with the family Coralliidae and unlike other described gorgonian-associated zoanthids (*Savalia* spp.) does not secrete its own hard axis. Morphologically, *C. tsukaharai* sp. n. is characterized by generally unitary polyps and bright yellow external coloration.

Keywords Zoanthid · *Paracorallium* · mt 16S rDNA · COI · ITS-rDNA

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Introduction

Recent investigations using molecular phylogeny have prompted a reconsideration of species diversity and taxonomy in a wide variety of marine invertebrates (e.g., Knowlton and Weigt 1997). A proper understanding of marine invertebrate diversity levels and how to identify different taxa is a critical first step not only in further research, but also in making subsequent conservation and management decisions. Thus, a molecular re-examination of previously misunderstood or taxonomically "neglected" taxa is important in helping clarify situations where morphological conclusions are ambiguous.

One such taxonomically "neglected" group is the Order Zoantharia (=Zoanthiniaria, =Zoanthidea) (Cnidaria: Hexacorallia). Zoanthids are generally colonial (but sometimes unitary/solitary) benthic hexacorallians having two rows of tentacles and a single ventral siphonoglyph, with most families except one (Zoanthidae) using sand and other

particles to help make their structure. Despite being worldwide in distribution, the true levels of diversity within this group are unknown (see Ryland and Muirhead 1993; Burnett et al. 1997), although recent investigations strongly indicate the number of genera is much higher than previously thought using solely traditional morphological identification characteristics such as septa arrangement, sphincter muscle anatomy, etc. A combination of morphological, ecological, and molecular methods have resulted in the identification of new zoanthid families and genera (i.e., Reimer et al. 2007a), and species (Reimer et al. 2006a), and also in the identification of synonymous taxa (Reimer et al. 2004, 2006b).

One group within Zoantharia in need of reorganization is the family Parazoanthidae and in particular the genus *Parazoanthus*. Formerly a "catch-all" for almost any zoanthid not producing a scleroprotein axis (as opposed to the genus *Savalia*), and in association with sessile marine invertebrates, *Parazoanthus* was shown to be paraphyletic in Sinniger et al. (2005) through the use of mitochondrial 12S ribosomal DNA and mitochondrial 16S ribosomal DNA (mt 16S rDNA) suggesting the genus *Parazoanthus* should be reorganized to include several genera. Each clade can be relatively easily identified by substrate specificity (e.g., one clade primarily associates with hydrozoans, etc.), and all clades form well-supported phylogenetic monophyletic groups based on obtained molecular data (Sinniger et al. 2005).

In this study, specimens ($n = 8$) of an unknown azooxanthellate zoanthid were found living on seven living Japanese Red Coral *Paracorallium japonicum* (Family Coralliidae) colonies at six locations (depths 194–250 m) in southern Japan during commercial precious coral harvesting. As no previously described zoanthids are known to associate with Coralliidae precious coral species, collected specimens were examined using molecular (cytochrome oxidase subunit I [COI], mt 16S rDNA, internal transcribed spacer of ribosomal DNA [ITS-rDNA]), ecological (substrate, depth, water temperature), and morphological (polyp dimensions, mesentery and tentacle count, polyp and colony structure, nematocyst type) data to characterize these enigmatic zoanthids' phylogenetic position within the order Zoantharia.

Materials and methods

Sample collection and identification of precious corals

The zoanthid samples were obtained at depths of 194–250 m from six locations between Kikai-jima and Ishigaki-jima, Japan (Fig. 1) during commercial precious coral harvesting dives using either the Remotely Operated Vehicle (ROV) submersible *Hakuyo 2000* or the manned submersible *Hakuyo* between February 2004 and January 2006 (see Electronic Supplementary Material (ESM) Table S1 for sampling details). Both submersibles were operated by SNK Ocean Co. Ltd. (Tokyo, Japan), and observation of harvesting by Okinawa Churaumi Aquarium (hereafter designated "OCA", Motobu, Japan) staff was undertaken through a collaborative agreement. As part of the observation agreement and as precious coral harvesting is strictly regulated and subject to potential poaching, the exact coordinates of the precious coral sampling locations cannot be divulged.

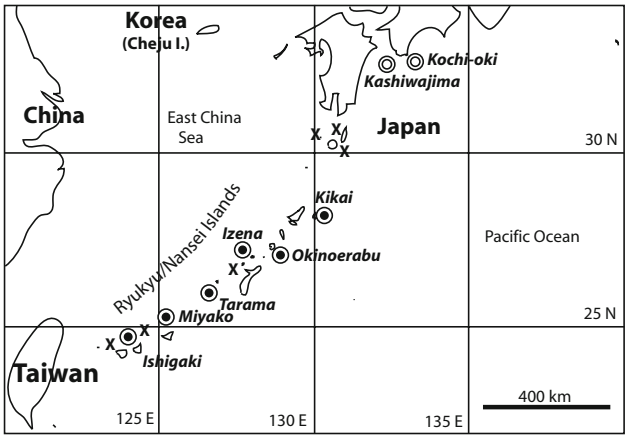

Fig. 1 Map of sampling locations in this study. Closed circles indicate locations where the zoanthid *Corallizoanthus tsukaharai* sp. n. was found on Japanese Red Coral *Paracorallium japonicum* (Family Coralliidae). Crosses indicate locations where *P. japonicum* was found, but with no *C. tsukaharai* present. Open circles indicate locations of records in Japan where potential *Corallizoanthus* specimens have been previously found on Coralliidae precious coral

During commercial precious coral harvesting, 86 living Coralliidae specimens were collected. Specimens were identified according to Kishinouye (1903, 1904), Bayer (1956), and Bayer and Cairns (2003). 50 specimens had prickle-like twigs on the front and sides of branches, dark red axes with nearly white branch tips, and eight-radiate sclerites dominating the coenenchyme, and were identified as *Paracorallium japonicum* (Kishinouye 1903). Seven of these specimens had unknown zoanthids associated with them.

Most zoanthids ($n = 6$) were photographed and immediately preserved in 99.5% ethanol, excepting two specimens (OCACn20040228-001 and OCACn20040228-002; both on the same *P. japonicum* colony; see ESM Table S1), which were brought back to OCA live in an aquarium for further observation following procedures as in Nonaka et al. (2006). As the submersibles used had no high-resolution cameras, no high-resolution in situ images were obtained, and detailed images of living specimens were instead obtained in the aquarium at OCA.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from six of the collected zoanthid samples (Table S1) (5–20 mg) following procedures outlined in Reimer et al. (2004) by using a DNEasy Tissue Kit for animals (QIAGEN, Tokyo, Japan).

The mitochondrial cytochrome oxidase c subunit I (COI) gene was amplified using the zoanthid-specific primers COIZoanF (3′ TGATAAGGTTAGAACTTTCTGCCCCG GAAC 5′) and COIZoanR (3′ AGGCTAAATATAGCCA TGTCCACG 5′) (Reimer et al. 2007a). The following thermal cycle conditions were utilized: 35 cycles of: one minute at 94°C, one minute at 40°C, 1 min 30 s at 72°C, and followed by a seven minute extension at 72°C.

Mitochondrial 16S ribosomal DNA (mt 16S rDNA) was amplified using zoanthid-specific primers described by Sinniger et al. (2005), with the following thermal cycle conditions: 40 cycles of: one minute at 94°C, 1 min at 52°C, 2 min at 72°C, and followed by a seven minute extension at 72°C.

The nuclear internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was amplified using zoanthid-specific primers previously reported in Reimer et al. (2007b). The following thermal cycle conditions were utilized: 35 cycles of: one minute at 94°C, 1 min at 50°C, 2 min at 72°C, and followed by a 10 min extension at 72°C.

The amplified PCR products were checked by 1.5% agarose gel electrophoresis. The PCR-amplified DNA fragments were sequenced with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). The sequences were analyzed using GENETYX-MAC version 8.0 (Software Development, Tokyo, Japan) and DNASIS Mac v3.6 (Hitachi Software Engineering Company, Ltd., Tokyo, Japan).

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank (accession numbers EU035617-EU035634). By using CLUSTAL X version 1.8 (Thompson et al. 1997) on default settings, the nucleotide sequences of the COI gene, mt 16S rDNA, and ITS-rDNA from samples were separately aligned with previously obtained Zoantharia from all known families (Abyssoanthidae, Parazoanthidae, Sphenopidae, and Zoanthidae–Table 1) excepting Epizoanthidae, which has been shown to be highly divergent in both molecular phylogeny (Sinniger et al. 2005) and ecology (i.e., see Muirhead et al. 1986) from the other zoanthid families. The COI and mt 16S rDNA alignments contained sequences from both described genera in Parazoanthidae (*Parazoanthus* and *Savalia*) and included members from all potentially generic-level clades within *Parazoanthus* (see Sinniger et al. 2005). The alignments were inspected by eye and manually edited. All ambiguous sites of the alignments ((i.e., codes that were not G, C, T, or A; $n = <10$ for all alignments) were removed from the dataset or edited based on other zoanthid sequences for phylogenetic analyses. Consequently, three alignment datasets were generated: (1) 784 sites of 23 taxa (mt 16S rDNA), (2) 310 sites of 31 taxa (the COI gene) and (3) 905 sites of 9 taxa (ITS-rDNA). Phylogenetic analyses of ITS-rDNA sequences included only sequences from Parazoanthidae as other families such as Zoanthidae and Abyssoanthidae with ITS-rDNA sequences available to the public from GenBank are extremely divergent and thus very difficult to align with any confidence. The alignments are available as NEX files in the Electronic Supplementary Material.

For the phylogenetic analyses of the mt 16S rDNA, COI gene, and ITS-rDNA the same methods were independently applied. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel 2003). PhyML was performed using an input tree generated by BIONJ with the general time-reversible model (Rodriguez et al. 1990) of nucleotide substitution incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + Γ). The proportion of invariable sites, a discrete gamma distribution, and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees.

Bayesian trees were also reconstructed by using the program MrBayes 3.0 (Ronquist and Huelsenbeck 2003) under GTR + I + Γ. One cold and three heated Markov chains Monte Carlo (MCMC) with default-chain temperatures were run for 1,000,000 generations, sampling log-likelihoods (InLs), and trees at 100-generations intervals (10,000 InLs and trees were saved during MCMC). The likelihood plot for mt 16S rDNA, COI, and ITS-rDNA datasets suggested that MCMC reached the stationary phase after the first 30,000 generations for mt 16S rDNA and COI analyses (potential scale reduction factor [PSRF] = both 1.000; standard deviation of split frequencies = 0.006778 and 0.009022, respectively), and after 50,000 generations for ITS-rDNA (potential scale reduction factor [PSRF] = 1.000; standard deviation of split frequencies = 0.003993). Thus, the remaining 9,700 trees of mt 16S rDNA and COI, and the remaining 9,500 trees of ITS-rDNA were used to obtain clade probabilities and branch-length estimates.

Light microscope analyses

Initial observation of samples and polyp surfaces were made using a dissecting microscope. Due to the presence of detritus in the endoderm of zoanthids (excepting family Zoanthidae) obtaining sections is unusually difficult unless potentially dangerous hydrofluoric acid is used. Rough

Table 1 Sequence data of Order Zoantharia from previous studies used in phylogenetic analyses

Order	Family	Genus and species	Locality	Collected by	Mt 16S rDNA Acc. Number	COI Acc. Number	ITS-rDNA Acc. Number	Reference
Zoantharia	Abyssoanthidae	*Abyssoanthus nankaiensis*	Nankai Trough, Japan	Y. Fujiwara & T. Sato	AB247344-AB247346	AB247362-AN247364	NA	Reimer et al. (2007a)
	Parazoanthidae	*Parazoanthus swiftii*	Utila, Honduras	F. Sinniger	AY995936	AB247350	NA	Sinniger et al. (2005), Reimer et al.(2007a)
		Parazoanthus puertoricense	Utila, Honduras	F. Sinniger	AY995933	AB247351	NA	Sinniger et al. (2005), Reimer et al. (2007a)
		Parazoanthus aff. *puertoricense*[a]	Izu, Japan	J. Reimer	NA	AB247352	NA	Reimer et al. (2007a)
		Parazoanthus parasiticus	Utila, Honduras	F. Sinniger	AY995938	NA	NA	Sinniger et al. (2005)
		Parazoanthus axinellae	Marseille, France	F. Sinniger	NA	AB247355	EU363364	Reimer et al. (2007a), this study
		Cape Verde zoanthid	Sal Island, Cape Verde	P. Wirtz	NA	AB247357	EU363365	Reimer et al. (2007a); this study
		Yellow polyps	Aquarium trade[b]	NA	NA	AB247358	NA	Reimer et al. (2007a)
		Savalia savaglia	Gran Canaria, Canary Islands	P. Wirtz	AY995930	NA		Sinniger et al. (2005)
		Savalia savaglia	Marseille, France	F. Sinniger	AY995925	AB247356	NA	Sinniger et al. (2005), Reimer et al. (2007a)
			Embiez, France	F. Sinniger	NA	NA	EU346888	This study
		Parazoanthus tunicans	Utila, Honduras	F. Sinniger	AY995940	AB247353	NA	Sinniger et al. (2005), Reimer et al. (2007a)
		Parazoanthus gracilis	N. Sulawesi, Indonesia	M. Boyer	AY995942	NA	NA	Sinniger et al. (2005)
			Izu, Japan	J. Reimer	NA	AB214178	AB214161	Reimer et al. (2007b)
	Sphenopidae	*Palythoa* sp.	Aquarium trade[b]	NA	AY995943	NA	NA	Sinniger et al. (2005)
		Palythoa heliodiscus	Erabu, Japan	J. Reimer	AB219224	NA	NA	Reimer et al. (2006b)
			Lau Lau, Saipan	J. Reimer	NA	AB219201	NA	Reimer et al. (2006b)
		Palythoa mutuki 2	Amami, Japan	J. Reimer	AB219221	AB219212	NA	Reimer et al. (2006b)
		Palythoa sp. 289	Sakatia, Madagascar	F. Sinniger	NA	AB247359	NA	Reimer et al. (2007a)
		Palythoa sp. 296	Sakatia, Madagascar	F. Sinniger	NA	AB247360	NA	Reimer et al. (2007a)
	Zoanthidae	*Isaurus* sp.	Aquarium trade[b]	NA	AY995945	NA	NA	Sinniger et al. (2005)
		Isaurus tuberculatus	Otsuki, Japan	F. Iwase	NA	AB247361	NA	Reimer et al. (2007a)
		Acrozoanthus sp.	Aquarium trade[b]	NA	AY995946	NA	NA	Sinniger et al. (2005)
		Zoanthus gigantus	Amami, Japan	J. Reimer	NA	AB128893	NA	Reimer et al. (2004)
		Zoanthus gigantus	Yakushima, Japan	J. Reimer	AB219192	NA	NA	Reimer et al. (2006a)
		Zoanthus gigantus	Amami, Japan	J. Reimer	NA	AB219184	NA	Reimer et al. (2006a)
		Zoanthus sansibaricus	Sakurajima, Japan	J. Reimer	NA	AB194021	NA	Reimer et al. (2004) supp.
		Zoanthus sansibaricus	Sakurajima, Japan	J. Reimer	AB219187	NA	NA	Reimer et al. (2006a)

Table 1 continued

Order	Family	Genus and species	Locality	Collected by	Mt 16S rDNA Acc. Number	COI Acc. Number	ITS-rDNA Acc. Number	Reference
		Zoanthus sansibaricus	Amami, Japan	J. Reimer	NA	AB128897	NA	Reimer et al. (2004)
		Zoanthus kuroshio	Yakushima, Japan	J. Reimer	NA	AB214177	NA	Reimer et al. (2007b)
		Zoanthus kuroshio	Yakushima, Japan	J. Reimer	NA	AB214176	NA	Reimer et al. (2007b)

NA, Not available or not utilized in this study

[a] Corresponds *to Parazoanthus* aff. *puertoricense* as described in Uchida (2001), but our sequence data clearly shows this specimen is not conspecific with *P. puertoricense* from Utila, Honduras. in the Caribbean

[b] As noted in Sinniger et al. (2005), samples were acquired in the aquarium trade but are assumed to be from Indonesia

cross-sections of polyps were made, however, and complete mesentery number obtained.

Additional data collected include tentacle number, which is approximately the same as mesentery number in zoanthids. Also, polyp dimension data (expanded polyp diameter (living samples OCACn20040228-001 and OCACn20040228-002)and height, closed polyps' aboral end maximum diameter) were obtained. As most specimens ($n = 6$, see ESM Table S1) were preserved in ethanol, polyps closed and retracted tentacles to varying degrees, and thus we avoided recording potentially erroneous oral end diameter data. Aboral maximum diameter is not as prone to such size changes upon polyp closure, and thus was recorded for all six preserved specimens ($n = 10$ polyps/specimen). Polyps were randomly chosen and only unitary polyps selected to avoid selecting potentially immature polyps. Average maximum aboral diameter and standard deviation for each colony were calculated, as were average maximum aboral diameter and standard deviation for all data from the six colonies combined ($n = 60$ polyps).

Finally, squashes of polyps ($n = 6$ specimens, with 50 undischarged nematocysts/specimen examined) were made and nematocyst types from mesenterial filament tissue observed, following the classifications described in Ryland and Lancaster (2004).

Electron microscope analyses

In order to examine materials encrusted within the zoanthid specimens' tissue, a scanning electron microscope (SEM) (Keyence VE-8800) was used for high-magnification (200×) observations. Host *P. japonicum* tissue was also examined. Observed sclerites within the zoanthid specimens were compared to *P. japonicum* sclerites. For SEM examinations, sclerites were separated from tissue using sodium hypochlorite solution.

Sources of material

Abbreviations for collections used in the text are as follows.

MHNG Natural History Museum of the City of Geneva, Switzerland
NSMT National Museum of Nature and Science, Tokyo, Japan
USNM National Museum of Natural History, Washington D.C., USA
OCA Okinawa Churaumi Aquarium, Motobu, Japan

New taxa

Order Zoantharia, Gray 1870
Family Parazoanthidae Delage & Hérouard, 1901

Genus *Corallizoanthus*, new genus Reimer
Type species: *Corallizoanthus tsukaharai*

Etymology

Named for type species association with the Japanese Red Coral *Paracorallium japonicum* (family Coralliidae), with ending in relation to other Zoantharia genera.

Diagnosis

Unlike all previously described zoanthid genera except *Savalia*, *Corallizoanthus* gen. n. found on living gorgonians (Order Alcyonacea: Coralliidae), and unlike gorgonian-associated *Savalia*, does not secrete its own scleroproteinous axis. Polyps are primarily but not always unitary (solitary; non-colonial).

Remarks

As only one species for this genus is described herein, the above diagnosis may change if additional species within *Corallizoanthus* are described.

Corallizoanthus tsukaharai, new species Reimer (Figs. 2–4; Tables 2–4, ESM Table S1).

Holotype

JAPAN, Okinawa, Miyako-jima, depth = 239 m, 18 August 2005 (ROV Hakuyo 2000), operated by OCA and SNK staff. NSMT-Co 1511, removed from specimen NSMT-Co 1513.

Paratypes

1, JAPAN, Kagoshima, Okinoerabu-jima, depth = 250 m, 4 June 2005 (submarine Hakuyo), operated by OCA and SNK staff, NSMT-Co 1512, removed from NSMT-Co 1514; 2, JAPAN, Okinawa, Izena-jima, depth = 194 m, 9 January, 2006 (ROV Hakuyo 2000), operated by OCA and SNK staff, MHNG-INVE 60950, removed from MHNG-INVE 60951; 3, JAPAN, Okinawa, Izena-jima, depth = 208 m, 20 December 2005 (ROV Hakuyo 2000), operated by OCA and SNK staff, USNM 1102464.removed from USNM 1110399.

Other material

JAPAN, Okinawa, Tarama-jima, depth = 214 m, 28 November 2005 (ROV Hakuyo 2000), operated by OCA and SNK staff, OCACn20051128-014; JAPAN, Kagoshima, Kikai-jima, depth = 212 m, 13 June 2005 (submarine Hakuyo), operated by OCA and SNK staff, OCACn20050613-014; JAPAN, Okinawa, Ishigaki-jima, depth = 222 m, 28 February 2004 (ROV Hakuyo 2000), operated by OCA and SNK staff, OCACn20040228-001; JAPAN, Okinawa, Ishigaki-jima, depth = 222 m, 28 February 2004 (ROV Hakuyo 2000), operated by OCA and SNK staff, OCACn20040228-002.

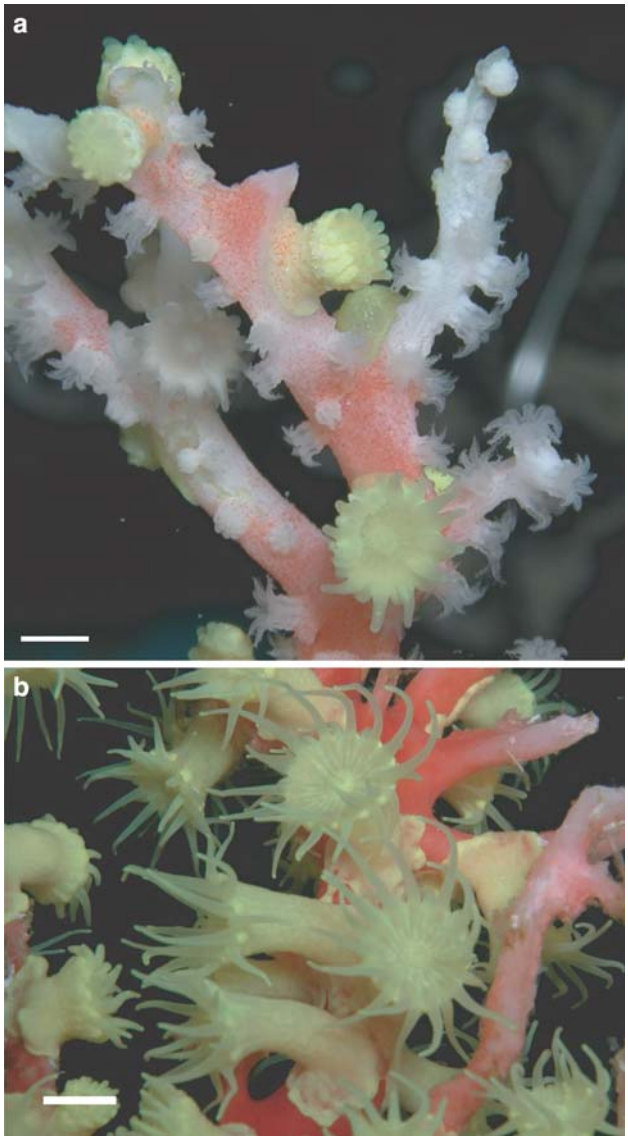

Fig. 2 Living *Corallizoanthus tsukaharai* sp. n. specimen OCACn20040228-001 with expanded polyps in an aquarium at Okinawa Churaumi Aquarium, on Japanese Red Coral *Paracorallium japonicum*. Note that the *P. japonicum* colony, although alive in (**a**), died in captivity before image (**b**) was taken. Specimen OCACn20040228-002 (large white polyp) is visible in center left of (**a**). Also note elongation of tentacles in (**b**) compared to (**a**). Dates: (**a**) 6 March 2004 (just after collection), (**b**) 27 March 2007. Scale = 0.5 cm

Etymology

Species named for Dr. Junzo Tsukahara, long-time Kagoshima University professor and the mentor of J. D. Reimer.

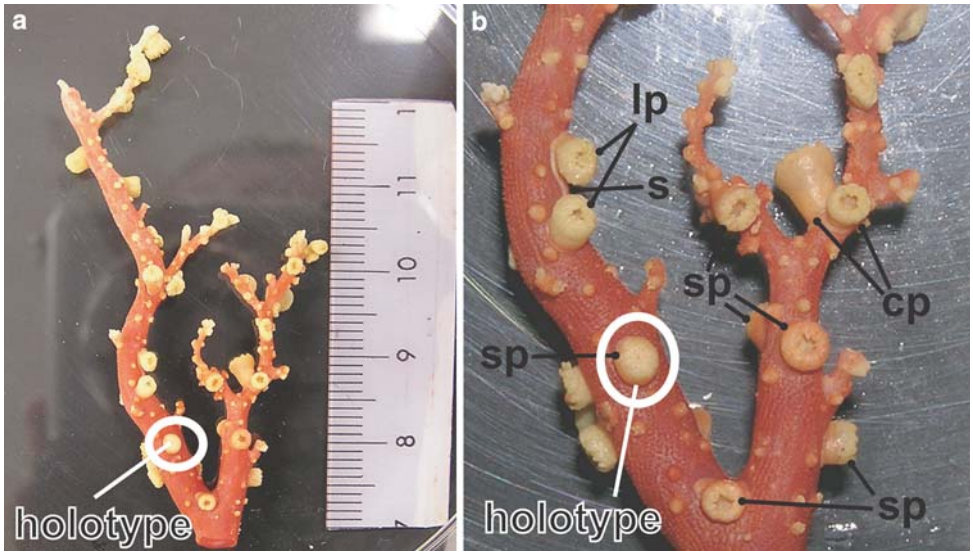

Fig. 3 *Corallizoanthus tsukaharai* sp. n. holotype and associated polyps. (**a**) NSMT-Co 1513 on a branch of Japanese Red Coral *Paracorallium japonicum*. Holotype polyp (specimen NSMT-Co 1511) subsequently removed from the red coral branch is circled. Scale in centimeters. (**b**) close-up of polyps in a. Abbreviations: sp, unitary singular polyps; lp, linked polyps; s, joined by a stolon; cp, conjoined polyps

Species description

External anatomy

Generally unitary (solitary, non-colonial), occasionally colonial zoanthid (see Table 2) known only from the surface of Japanese Red Coral *P. japonicum* (Family Coralliidae) (coral living in all specimens observed). When colonial, polyps connected by thin stolon growing over Japanese Red Coral (Fig. 3b). Polyps generally spaced regularly over axis of *P. japonicum*, often at "bends" or "joint" areas of branches, also located on tips of small branches (see Fig. 3). Outside of polyps, coenenchyme, oral disk, and tentacles generally were all bright yellow (Figs. 2, 3); color was not due to encrusted particles but most likely due to pigments in zoanthid tissue. Outer polyp surface rough with encrusted particles. Aboral maximum polyp diameter was (closed preserved polyps) 1.7–3.9 mm (average 2.5 ± 0.6 mm, $n = 60$) (Table 2). Expanded oral disks were estimated (from in situ images) at approximately 6.5 ± 1.2 mm ($n = 10$) in diameter, expanded polyps were up to approximately 1.2 cm in height (Fig. 2). Polyps were relatively uniform in diameter toward both oral opening and base, slightly wider at oral end of polyp when both closed and expanded (Figs. 2, 3). 18–24 capitular ridges were clearly visible in closed polyps. Tentacle count was 18–22 (Fig. 2), mesentery number likely close to tentacle and capitular ridge numbers were based on observations of number of complete mesenteries (9–11).

Internal anatomy

Zoanthid body wall was encrusted, containing sclerites from *P. japonicum* and foraminifer tests, sponge spicules, other detritus, based on electron microscope observations (Fig. 4). Mesenteries were macrocneminic; fifth mesentery from dorsal directive was complete. There were 9–11 complete mesenteries. Nematocyst types from mesenterial filaments composed almost primarily of small holotrichs types B and C (10–20 µm in length) described in Fig. 1 in Ryland and Lancaster (2004), also some (~15 µm in length) basitrichs.

Description of the holotype

One unitary polyp (circled in Fig. 3a, b) was removed from a *P. japonicum* branch (height 57 mm, width 29 mm) that originally contained 42 polyps, 32 polyps unitary and 10 polyps connected by stolon (Fig. 3).

Holotype polyp had a maximum diameter of 2.74 mm, minimum diameter 2.48 mm. Polyp was bright yellow, surface appears rough due to encrusted sediment on and in mesoglea.

Connected polyps on original *P. japonicum* branch were extremely "liberae" in form (see Pax 1910). Unitary polyp had aboral average maximum diameter 2.0 ± 0.2 mm ($n = 10$) (range 1.7–2.2 mm) (Table 2). Polyps were bright yellow and cylindrical. Sediment visibly encrusted on and in mesoglea surrounding ectoderm, consisting of *P. japonicum* sclerites, sponge spicules, sclerites from other corals, foraminifer tests, and other debris (Fig. 4b). Holotype was fixed in 99.5% ethanol.

Distribution

Known only on Japanese Red Coral *P. japonicum*, at depths of 194–250 m between Ishigaki-jima Island, Okinawa, Japan and Kikai-jima Island, Kagoshima, Japan (Fig. 1, ESM Table S1), with ocean temperatures of 15–20°C (ESM Table S1).

Table 2 Polyp dimensions and other polyp data (connected/unitary %) of *Corallizoanthus tsukaharai*, gen. n. et sp. n

Specimen number/name	Red coral max. height (mm)	Red coral max. width (mm)	# Polyps measured	Max. aboral diameter range	Avg max. aboral diameter ± S.D.	# Unitary polyps	# Connected polyps	Total # polyps	% of Unitary polyps
NSMT-Co 1513[a]	57	29	10	1.7–2.2	2.0 ± 0.2	32	10	42	76
NSMT-Co 1514[b]	62	50	10	1.9–3.7	2.7 ± 0.6	57	4	61	93
MNHG XXXXX[c]	101	50	10	2.9–3.9	3.3 ± 0.3	73	22	95	77
USNM 1110399[d]	63	19	10	1.8–2.8	2.2 ± 0.3	35	8	43	81
OCACn20051128–014	29	19	10	1.7–2.8	2.3 ± 0.4	12	4	16	75
OCACn20050613-014	46	31	10	1.7–2.8	2.4 ± 0.4	23	0	23	100
Average/totals	NA	NA	60	1.7–3.9	2.5 ± 0.6	232	48	280	84 ± 10

NA = not available/not applicable

[a] Specimen from which holotype polyp (NSMT-Co 1511) was removed

[b] Specimen from which paratype 1 polyp (NSMT-Co 1512) was removed

[c] Specimen from which paratype 2 polyp (MHNG-XXX) was removed

[d] Specimen from which paratype 3 polyp (USNM 1102464) was removed

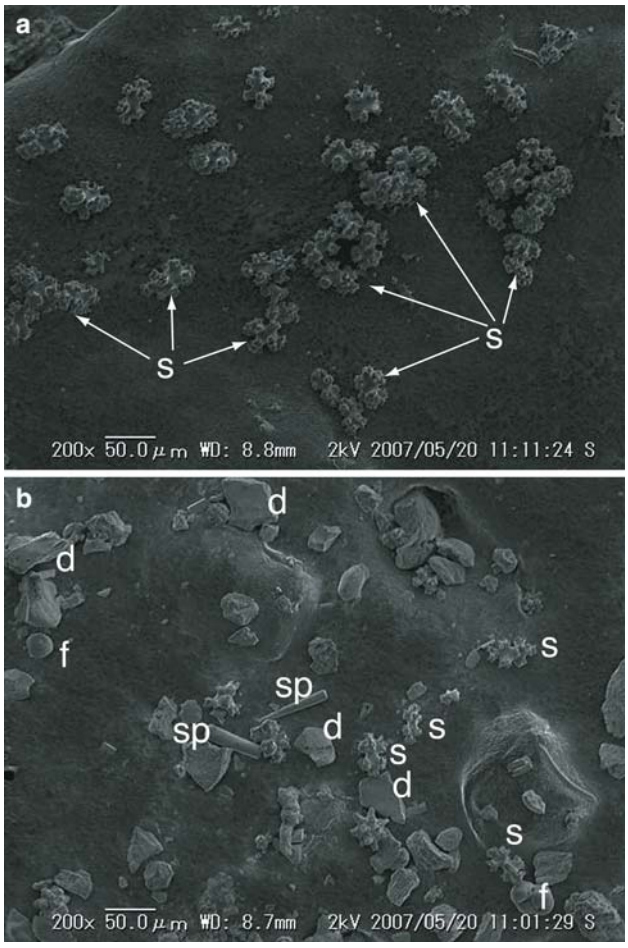

Fig. 4 (**a**) Sclerites of host Japanese red coral *Paracorallium japonicum* (=coral that NSMT-Co 1511 and NSMT-Co 1513 are attached to) as seen at 200× under SEM. (**b**) Various pieces of detritus from *Corallizoanthus tsukaharai* sp. n. NSMT-Co 1513 living on *P. japonicum* at same magnification as b. Abbreviations: s, *P. japonicum* sclerites; sp, sponge spicules; f, foraminifer tests; d, other debris. Scales = 50 μm

Remarks

Paratypes 1–3 and other specimens do not show any significant morphological differences from the holotype aside from differing dimensions of the substrate *P. japonicum* branches, slightly differing maximum aboral polyp diameters, and total polyp number per specimen (for non-type specimens) (Table 2).

While most polyps were unitary (depending on specimen, 75–100% of total polyps unitary), occasionally polyps were connected by thin yellow coenenchyme/stolon growing over the surface of *P. japonicum* (Fig. 3, Table 2). It is speculated unitary polyps were originally connected to other polyps by a stolon that subsequently disappeared (as is seen in *Parazoanthus parasiticus* and other related parazoanthids—i.e., asexual reproduction). Long-term rearing observations will help further confirm how this species asexually reproduces.

All *Corallizoanthus tsukaharai* sp. n. specimens collected in situ were seen to be bright yellow in coloration, but one specimen (OCACn20040228-002) on *P. japonicum* turned very pale yellow after being brought back to OCA, although genetically this sample was identical to all other specimens of *C. tsukaharai* sp. n. (Figs. 5, 6). This may be simply a color variant of this species, or alternately the coloration may be different due to this zoanthid colony having grown considerably in the aquarium, and not at depth in situ. This phenomenon remains to be investigated.

Historically, this species or a similar *Corallizoanthus* sp. may have been first mentioned in Kishinouye (1903, 1904), although this is not clear. There is a brief mention of "an anemone living on top of a (precious) coral (*Paracorallium inutile*; in family Coralliidae the same as *P. japonicum*), with coral sclerites within the anemone's body" from

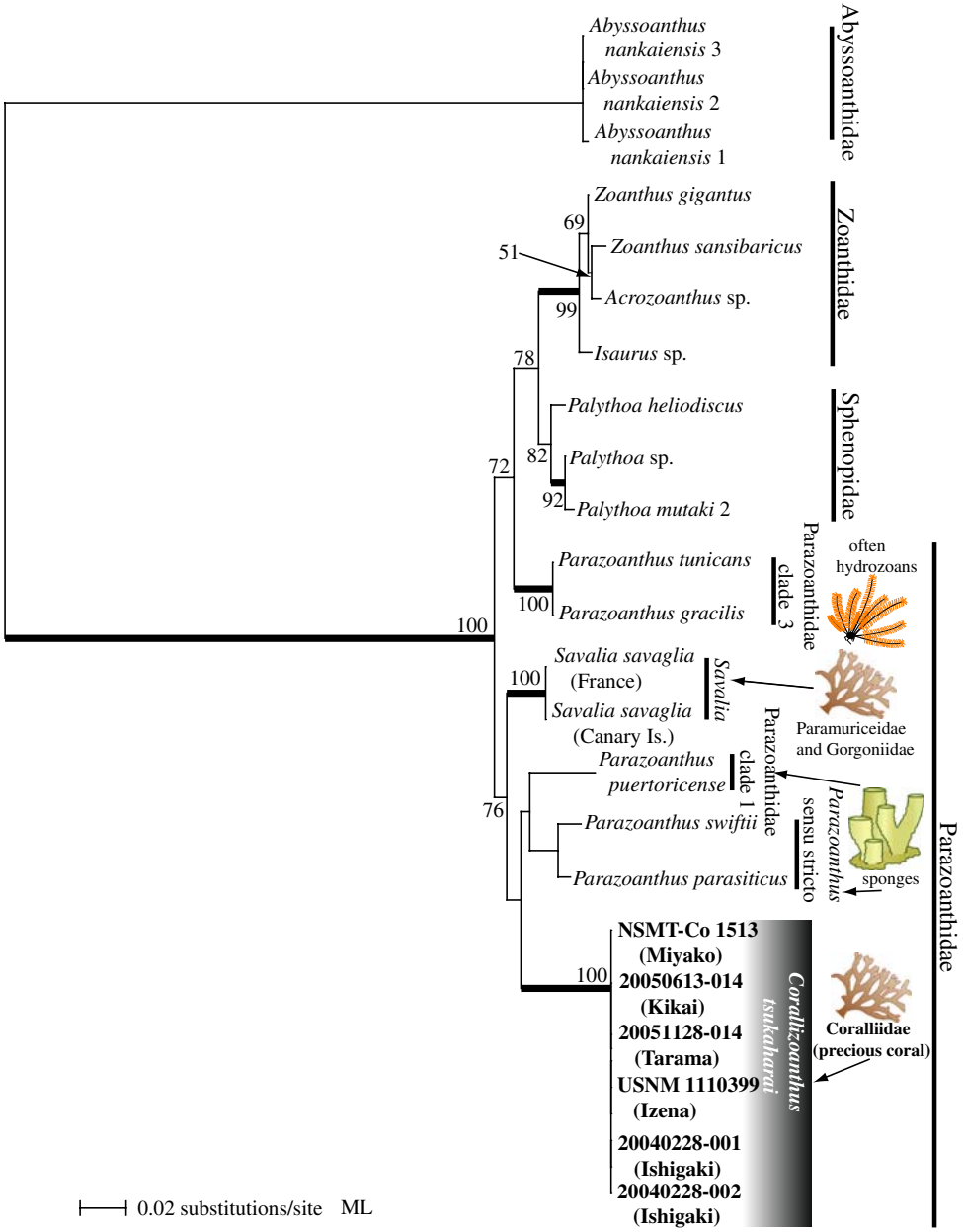

Fig. 5 Maximum likelihood tree of obtained and previous mitochondrial 16S ribosomal DNA (mt 16S rDNA) sequences for the order Zoantharia. Values at branches represent ML bootstrap probabilities (>50%). Bayesian posterior probabilities of >0.95 are represented by thick branches. For specimen information please refer to Tables 1 and S1. Organisms to the left of clades in Parazoanthidae represent specific substrates of each clade. Sequences from this study in bold

Kashiwajima, Tosa (now Kochi), Japan (Kishinouye 1904), which was proposed to be a commensal *Actinea* species (Kishinouye 1903). It was also noted that this "anemone" was not found on any other precious coral species (Kishinouye 1903). While there is no further description of this "anemone", it is likely to have been a zoanthid, as only zoanthids and not anemones have incrustations within their bodies. The largely unitary (non-colonial) nature of *C. tsukaharai* and perhaps potentially related zoanthid species could have led Kishinouye to misidentify this commensal as an *Actinea* species. Unfortunately, the description is very brief, with no figures or other data, and the true identity of this imperfectly characterized "*Actinea*" associated with *P. inutile* remains uncertain.

Bayer (1956, 1993) mentioned that most examined specimens of the precious coral *Corallium salomonense tortuosum* were "infested" with a zoanthid that pitted and distorted the axis, but no more information is given on these unidentified zoanthids. Additionally, "symbiotic" zoanthids have been noted to be present on the base of some *Corallium thrinax* colonies, but again little other information is given (Bayer 1996).

A zoanthid specimen similar to *C. tsukaharai* (informally described by H. Uchida, pers. comm.) was collected off Kochi from Kochi-oki, Japan (see Fig. 1, ESM Table S1) (collected 15 March 1993 by Fumihito Iwase), but the specimen was preserved in 10% SW formalin so no molecular examination was possible. Although some morphological characteristics of

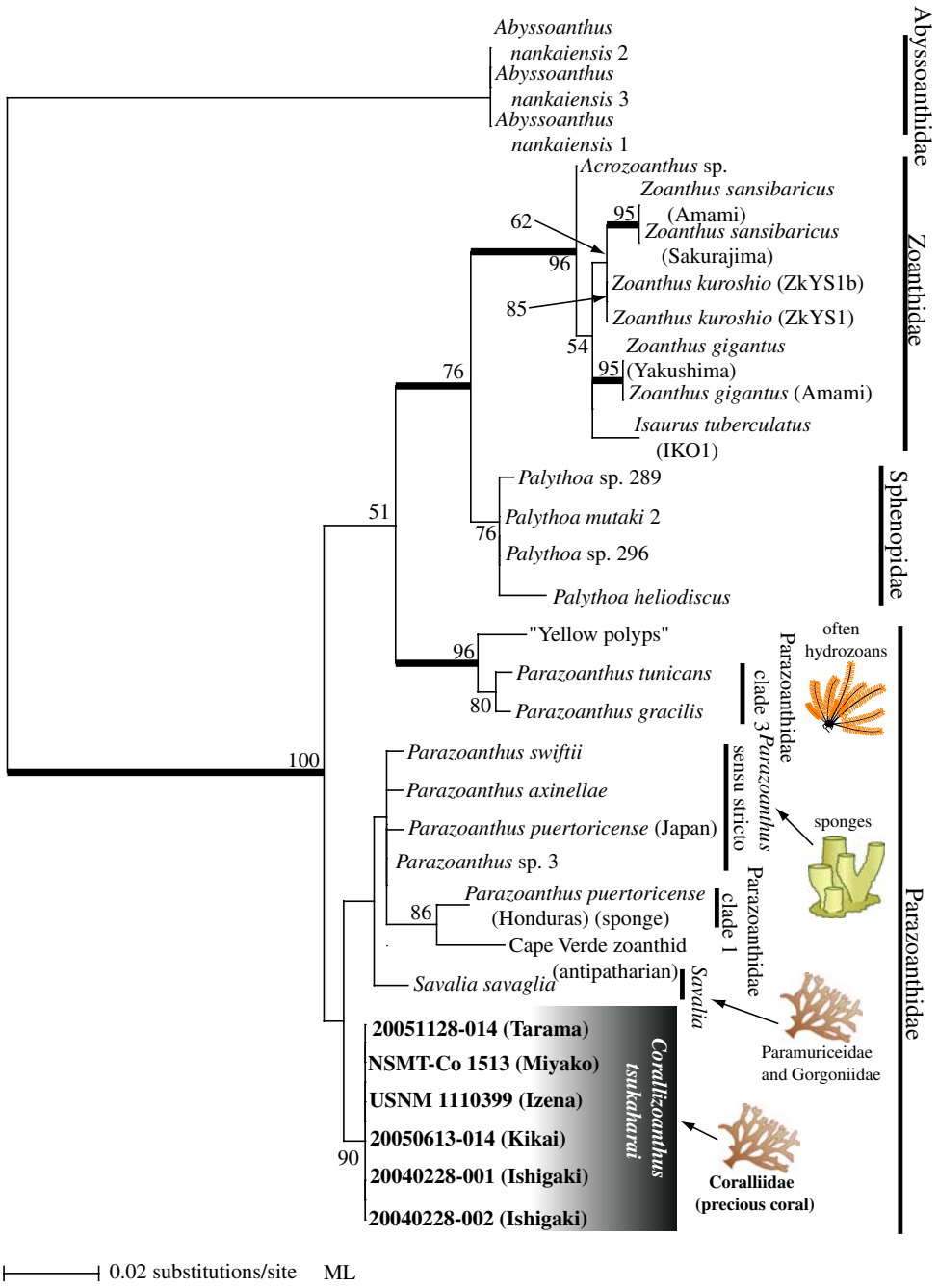

Fig. 6 Maximum likelihood tree of obtained and previous cytochrome oxidase I gene (COI) sequences for the order Zoantharia. Values at branches represent ML bootstrap probabilities (>50%). Bayesian posterior probabilities of >0.95 are represented by thick branches. For specimen information please refer to Tables 1 and S1. Organisms to the left of clades in Parazoanthidae represent specific substrates of each clade. Sequences from this study in bold

this specimen are similar to *C. tsukaharai* (bright yellow, generally unitary, on *P. japonicum*), it has several morphological characters different than *C. tsukaharai*; polyps are much smaller (1.0–1.4 mm in diameter, polyps are much less prominent and form low "bumps" on Red Coral axis) with only 8–10 capitular ridges. Based on the fact that this zoanthid had *P. japonicum* as its substrate, this specimen is assigned to the genus *Corallizoanthus*. It is possible that this specimen is an undescribed *Corallizoanthus* congener, or alternatively a morphological variant of *C. tsukaharai*.

Other bright yellow undescribed parazoanthids are known from shallower waters in Australia and South Africa, but both of these zoanthids are not found on anthozoans, and it is highly unlikely these are *Corallizoanthus* species.

The current generic description for *Corallizoanthus* is for zoanthids associated with gorgonians that do not secrete their own hard axis. Two examined South Australia Museum specimens (F. Sinniger, data not shown) consisting of small zoanthid fragments associated with unidentified gorgonians from 500–900 m in waters of Australia and Antarctica were shown through mt 16S rDNA sequence analyses to be potential but not likely congeners to *C. tsukaharai* (e.g., mt 16S rDNA divergence rates from *C. tsukaharai* of 20/727 base pairs = 2.75%—compare with

Table 3 DNA sequence differences between zoanthid taxa

Comparison	Level of comparison	COI % variation	Mt 16S rDNA % variation	Notes	Reference(s)
Palythoa tuberculosa—Palythoa mutuki	Intrageneric	0.0–0.2	0.1–0.2	Evidence of past reticulate evolution based on ITS-rDNA.	Reimer et al. (2006b)
Zoanthus sansibaricus—Zoanthus gigantus	Intrageneric	0.7	1.1	Evidence of past reticulate evolution based on ITS-rDNA.	Reimer et al. (2006a, 2007b)
Palythoa tuberculosa—Palythoa heliodiscus	Intrageneric	0.9	1.1		Reimer et al. (2006b)
Zoanthus sansibaricus—Zoanthus kuroshio	Intrageneric	1.3	0.8		Reimer et al. (2006a, 2007b)
Zoanthus sansibaricus—Acrozoanthus sp.	Intrafamily	1.3	0.9	Status of *Acrozoanthus* as a valid genus unclear.	Reimer et al. (2008)
Zoanthus sansibaricus—Isaurus tuberculatus	Intrafamily	1.9	1.3		Reimer et al. (2008)
Parazoanthus swiftii—Savalia savaglia	Intrafamily	1.3	2.0*		Reimer et al. (2007a)
Corallizoanthus tsukaharai—Parazoanthus swiftii	Intrafamily	1.9	1.9*		This study
Corallizoanthus tsukaharai—Savalia savaglia	Intrafamily	1.6	3.0*		This study
Zoanthus sansibaricus—Palythoa mutuki	Interfamily	3.2	1.9		Reimer et al. (2006b)
Parazoanthus swiftii—Abyssoanthus nankaiensis	Interfamily or inter-suborder?	11.3	12.6[a]	Abyssoanthidae phylogenetically very distant to other zoanthids.	Reimer et al. (2007a)

[a] Indicates insertion and/or deletion events present in comparative alignment, which were counted as a single difference

Table 3 values). Unfortunately, the specimens are in poor condition due to damage during sampling and little more information can be obtained from them. Furthermore, as shown by previous undetailed reports (Kishinouye 1903, 1904; Bayer 1956, 1993, 1996) of zoanthids found on Coralliidae, it is highly likely many other *Corallizoanthus* spp. also await discovery and description. Clearly, further sampling and research is needed in other areas where Coralliidae and other gorgonian species are found.

As *P. japonicum* is found from Sagami Bay to Okinawa and the Bonin Islands, Japan (roughly 26°N–36°N–see Grigg 1984), it is highly likely that additional populations of *C. tsukaharai* await discovery and description.

Genetic sequences

mitochondrial 16S ribosomal DNA:
EU035623-EU035628
Cytochrome oxidase c subunit I:
EU035629-EU035634
Internal transcribed spacer of ribosomal DNA:
EU035617-EU035622

Sequence results

The resulting ML trees of mt 16S rDNA (Fig. 5), COI (Fig. 6), and ITS-rDNA (Fig. 7) sequences all show the monophyly of *C. tsukaharai.* Bootstrap support for the monophyly of *C. tsukaharai* samples was very high for mt 16S rDNA (Maximum Likelihood [ML] = 100%, Bayesian posterior probability [Bayes p.p.] = 1.00) and high for COI and ITS-rDNA (ML = 90% and 93%, Bayes p.p. = 0.87 and 1.00, respectively). The *C. tsukaharai* monophyly was unambiguously within the order Zoantharia and the family Parazoanthidae for mt 16S rDNA, COI and ITS-rDNA trees, sister to a monophyly consisting of *Parazoanthus* sensu stricto (i.e., the clade/grouping containing the type species *P. axinellae* Schmidt), and another *Parazoanthus* clade (COI tree), to *Savalia* (ITS-rDNA tree) or to a monophyly consisting of *Parazoanthus sensu stricto,* another *Parazoanthus* clade and *Savalia* (mt 16S rDNA tree), although bootstrap support for these sister clades was often low (ML = <50% and Bayes p.p. = <0.5 for mt 16S rDNA and COI markers). *Corallizoanthus tsukaharai* sequences for both COI and mt 16S rDNA were consistently shown to be monophyletic and distinct from all other analyzed zoanthid sequences, which consisted of representatives from both described Parazoanthidae genera (*Parazoanthus* and *Savalia*) and all known clades with the genus *Parazoanthus*. ITS-rDNA results further supported the monophyletic distinctiveness of *C. tsukaharai* within Parazoanthidae.

Discussion

Substrate specificity in the suborder Macrocnemina

The observed monophyly of the *P. japonicum*-associated zoanthid *C. tsukaharai* seen in this study reinforces recent

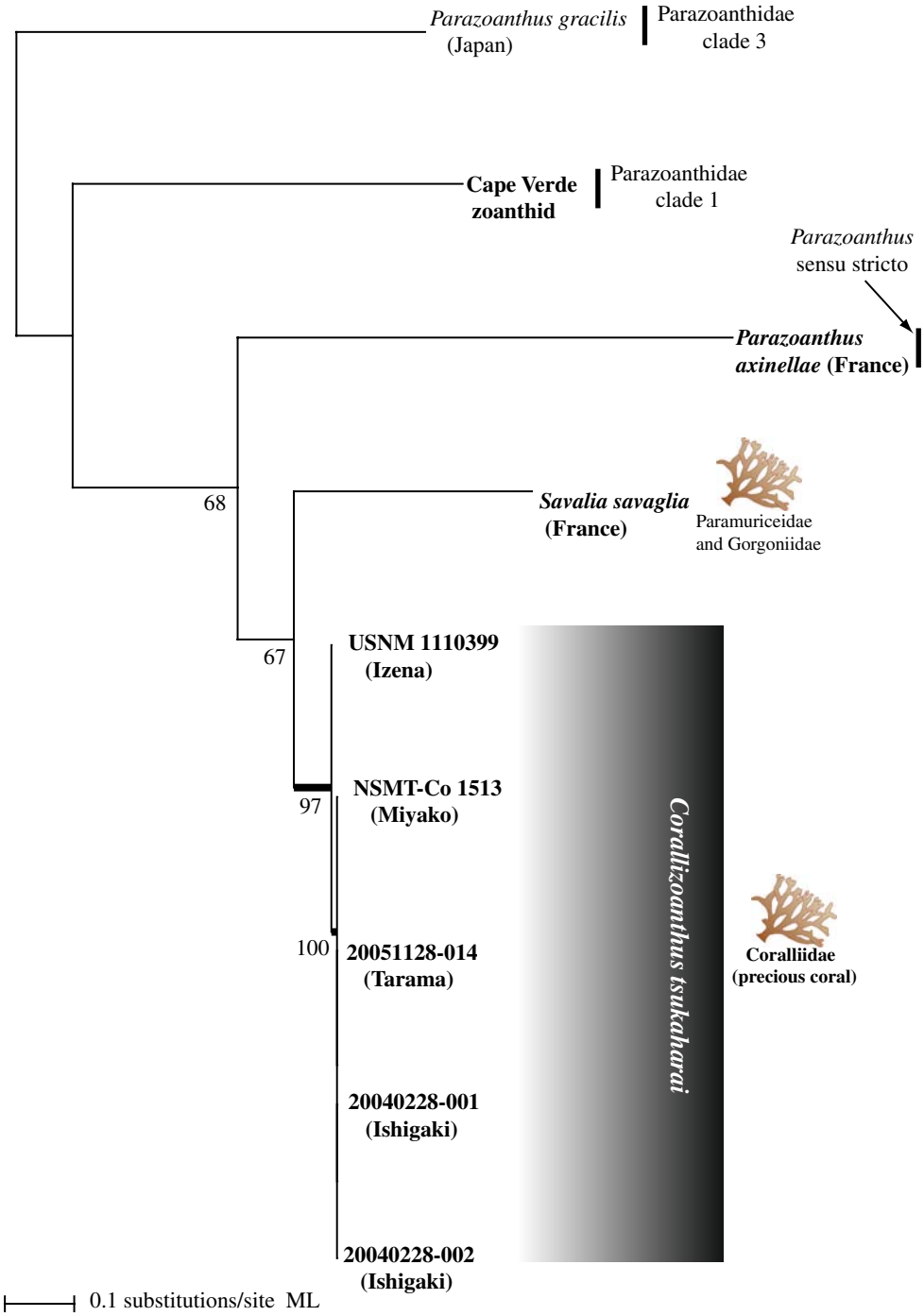

Fig. 7 Maximum likelihood tree of obtained and previous internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences for the family Parazoanthidae. Values at branches represent ML bootstrap probabilities (>50%). Bayesian posterior probabilities of >0.95 are represented by thick branches. For specimen information please refer to Tables 1 and S1. Organisms to the left of clades in Parazoanthidae represent specific substrates of each clade. Sequences from this study in bold

phylogenetic conclusions seen within the Parazoanthidae. The genus *Parazoanthus* as currently described included zoanthids associated with sponges, antipatharians, hydrozoans, etc., but molecular data (COI, mt 12S rDNA, mt 16S rDNA, ITS-rDNA) has conclusively shown *Parazoanthus* can be divided into strongly supported monophyletic groups by major substrate groupings (Sinniger et al. 2005). mt 16S rDNA and COI sequence divergence rates for *C. tsukaharai* when compared with other Parazoanthidae and to other zoanthid intergeneric comparisons clearly validate the creation of a new genus for the specimens examined in this study, with divergence values between *C. tsukaharai* and *P. swiftii* (1.9% for both markers) comparable to divergence values between *Savalia savaglia* and *P. swiftii* (1.3% and 2.0% for COI and mt 16S rDNA, respectively—Table 3). Additionally, *C. tsukaharai* had similar levels of sequence divergence from *S. savaglia* (1.6% and 3.0%, respectively—Table 3).

The only zoanthids other than *Corallizoanthus* formally described thus far as being associated with living anthozoans belong to the genus *Savalia*, which are both different

Table 4 Comparative table of morphological and ecological characteristics of other zoanthid genera in the family Parazoanthidae with *Corallizoanthus* n. gen

Family	Genus/Species	Primary substrate	Obligate?	Hard axis secretion?	Main growth form	Parasitic/ symbiotic
Parazoanthidae	Parazoanthus[a]	Sponges	No	No	Colonial	Symbiotic
	Savalia	Plexauridae, Gorgoniidae	Yes	Yes	Colonial	Parasitic
	Parazoanthidae "clade 3"[b]	Hydrozoans	No	No	Colonial	Parasitic
	Corallizoanthus tsukaharai[c]	Coralliidae	Yes	No	Unitary	Symbiotic?

[a] This refers to *Parazoanthus* sensu stricto—i.e., the phylogenetic grouping/clade that includes the type species of *Parazoanthus* (*P. axinellae*) (see Sinniger et al. 2005)

[b] This clade has been shown to be phylogenetically distinct from other Parazoanthidae, and awaits taxonomic revision (see Sinniger et al. 2005)

[c] As *Corallizoanthus tsukaraharai* is the only species described from *Corallizoanthus* thus far, these data may change when or if additional species are described. Hard axis secretion is not expected to change, as this is a diagnostic character separating *Savalia* from other Parazoanthidae

morphologically (secreting their own hard scleroprotein axis) and molecularly from *C. tsukaharai*. Additionally, Japanese Red Coral, although in the same order (Alcyonacea) as *Savalia*'s gorgonian substrates *Paramuricea* and *Eunicella*, belongs to a different family (family Coralliidae as opposed to families Plexauridae and Gorgoniidae, respectively), and thus it is not unexpected that *C. tsukaharai*, the first zoanthid observed to use Coralliidae as a substrate, forms another new and previously unknown zoanthid monophyly/genus within Parazoanthidae (see Table 4 for a summary of ecological characteristics of genera in Parazoanthidae).

The well-supported monophyletic groups of zoanthid clades on specific substrates within Parazoanthidae (Sinniger et al. 2005) suggest that each monophyletic group has a long evolutionary association with its respective substrate (Figs. 5–7), particularly as two of these phylogenies are based on mitochondrial sequence data, which are known to have very slow rates of divergence in Anthozoans (Shearer et al. 2002).

Phylogenetic placement of *Corallizoanthus*

Although mt 16S rDNA, COI, and ITS-rDNA trees (Figs. 5–7) all had slightly differing topologies, based on both phylogenetic and ecological data it is most likely that *Corallizoanthus* is most closely related to *Savalia*. This relationship is suggested in the mt 16S rDNA tree (but not the COI tree).

The strongest evidence for the *Savalia-Corallizoanthus* relationship is from ITS-rDNA sequences. Bootstrap support for these two genera being sister is not very high (ML = 67%, Bayes p.p = 0.85; Fig. 7), but in the ITS-rDNA alignment both *C. tsukaharai* and *S. savaglia* possess more similar indels when compared to other parazoanthid groups (Fig. 8). Additionally, only these two genera of zoanthids are known to associate with gorgonians, making a sister relationship seem more likely. Further examination of zoanthids known to associate with other gorgonians will help confirm the phylogenetic position of *Corallizoanthus*.

Prevalence of *Corallizoanthus tsukaharai* on *Paracorallium japonicum*

During the commercial harvesting of precious corals, 50 *P. japonicum* colonies were collected (34 by ROV *Hakuyo 2000* and 16 by manned submersible *Hakuyo*). Colonies of *P. japonicum* were observed at depths between 125 and 250 m (water temperature = 15–20°C). Despite the large number of *P. japonicum* colonies collected, only seven colonies had yellow zoanthids on their branches, and all of these zoanthids were shown to be *C. tsukaharai*. Thus, 14% (seven zoanthid-bearing colonies/50 total colonies) of *P. japonicum* over the range investigated had *C. tsukaharai* associated with them.

Although in this study no additional zoanthids were found during precious coral sampling at sites further north in the Nansei Islands (see Fig. 1), it remains to be seen if *C. tsukaharai* is limited in distribution to the southern Nansei Islands. As described above, further sampling of other red and precious coral populations as well as other gorgonians should help in understanding of *Corallizoanthus* abundance and distribution patterns.

Is *Corallizoanthus tsukaharai* parasitic or symbiotic?

One important issue that remains to be investigated in detail is the lifestyle ecology of *C. tsukaharai*, i.e., is it parasitic or symbiotic? At first observation, for all collected samples, both *P. japonicum* and *C. tsukaharai* sp. n. were living, both with extended polyps clearly visible in situ, supporting the case for symbiosis between these two organisms. Additionally, there were no areas of "irritation" or tissue necrosis (as often seen between neighboring and competing anthozoans/cnidarian species, i.e., see Sammarco et al.

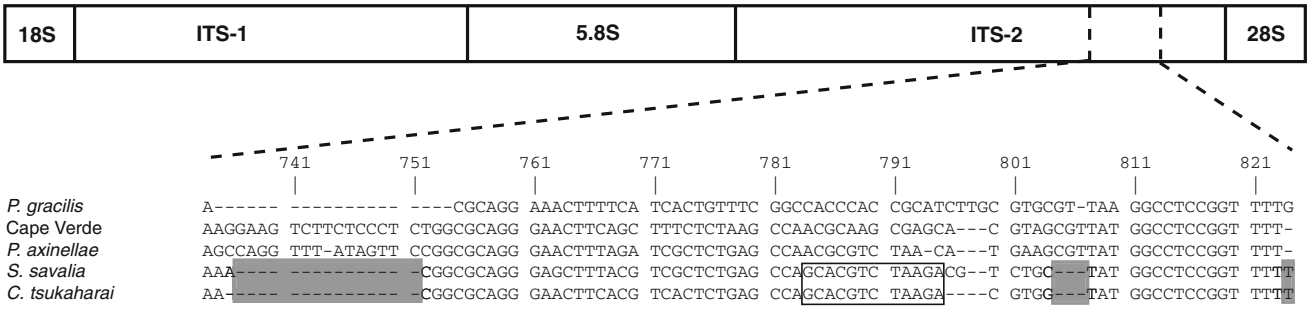

Fig. 8 Alignment of a portion of the internal transcribed spacer region of ribosomal DNA (ITS-rDNA) from ITS-2 showing sequences obtained from representative parazoanthids group specimens. Areas in shaded boxes are indels shared between only *Savalia savaglia* and *Corallizoanthus tsukaharai*, while areas in open boxes are areas of identical sequences shared between only *S. savaglia* and *C. tsukaharai*. Alignment position numbers are identical to numbers in the ITS-rDNA alignment used in analyses

1985) in the areas bordering between the zoanthids and Japanese Red Corals, further lending support to the symbiosis hypothesis. This may be similar to observations made by Kishinouye (1904), who observed that the unknown zoanthid or anemone (see discussion above) living commensally on the precious coral *Paracorallium inutile* from Kashiwajima, Japan (location shown in Fig. 1) adhered to the axis of the coral without damaging the host.

However, although unlikely, it may be possible that *C. tsukaharai* is parasitic upon *P. japonicum*. It is known that most zoanthids incorporate debris obtained from the water column to help make their structure (Isa 1991; Mueller and Haywick 1995). Under electron microscopic observation, examined *C. tsukaharai* specimens were shown to have encrustations containing many sclerites that are most likely from *P. japonicum* along with sponge spicules, foraminifer tests, sclerites from other species, debris, etc. (Fig. 4b). While it is not known if *C. tsukaharai* is actively obtaining sclerites to encrust, or if the encrustation simply reflects the immediate living environment of *C. tsukaharai*, *P. japonicum* does not freely shed large numbers of sclerites, and thus such an opportunistic encrustation of host sclerites could be parasitic in nature. However, Bayer (1956) noted that "infesting" zoanthids on the precious coral *Corallium salomonense tortuosum* pitted and distorted the coral axis, something that was not observed with *C. tsukaharai* on *P. japonicum*, and thus it is highly likely this relationship is symbiotic and not parasitic. Further sampling collection and observation should help clarify the ecological characteristics of *C. tsukaharai*.

This study demonstrates the importance of further investigation of zoanthids in areas of the ocean not accessible by conventional means (i.e., SCUBA). Additionally, further sampling and in-depth in situ observation of *C. tsukaharai* are required to further characterize this intriguing new genus. The combination of morphological and molecular analyses as utilized in this study may help uncover more new zoanthid taxa in the future.

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