Zoologica Scripta

Phylogeny of the highly divergent zoanthid family Microzoanthidae (Anthozoa, Hexacorallia) from the Pacific

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Submitted: 8 February 2011
Accepted: 26 April 2011
doi:10.1111/j.1463-6409.2011.00479.x

Fujii, T. & Reimer, J. D. (2011). Phylogeny of the highly divergent zoanthid family Microzoanthidae (Anthozoa, Hexacorallia) from the Pacific. — *Zoologica Scripta*, *40*, 418–431.
In this study, one new family, one new genus and two new species of zoanthids from rubble zones spanning the temperate, subtropical and tropical Pacific Ocean are described. Two new species are described, *Microzoanthus occultus* sp. n. and *Microzoanthus kagerou* sp. n., both belonging to the new genus *Microzoanthus* and new family Microzoanthidae, and they can be clearly distinguished both morphologically and genetically from each other and other zoanthids by their very small size, reduced or absent stolon, habitat usually on the bottom side of rubble zone rocks, and divergent and distinct DNA (cytochrome oxidase subunit I, mitochondrial 16S ribosomal DNA, internal transcribed spacer region of ribosomal DNA) sequences. The phylogenetic analyses clearly show Microzoanthidae fam. n. to be genetically far different from all other hexacorallians at the order level, but the macrocnemic arrangement of mesenteries and other morphological characters (colonial specimens with narrow stolons, two rows of tentacles sand encrustation) clearly place these specimens within the order Zoantharia. This study demonstrates how it is highly likely the existence of many marine invertebrate taxa remains overlooked, and that widely distributed groups such as Microzoanthidae fam. n. remain to be discovered.
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Introduction

Rubble zones, areas composed mainly of dead coral rubble interspersed with sandy areas, are a ubiquitous part of coral reefs. However, few studies about the biodiversity of marine species in rubble zones have been conducted to date, and such ecosystems tend to be underrepresented in biodiversity surveys, even though unique benthic animals found specifically in this zone have been described (e.g. Obuchi *et al.* 2010).

In recent studies, unidentified zoanthid colonies (order Zoantharia = Zoanthiniaria = Zoanthidea, subclass Hexacorallia, class Anthozoa) were found under stones and rubble from the rubble zones of shallow subtropical seas (intertidal coral reef moat to 30 m depths on reef slopes) around Okinawa Island, Japan and the Galapagos Islands, Ecuador (Reimer *et al.* 2008; Reimer 2010; Reimer & Fujii 2010). These zoanthids were initially believed to belong to a single undescribed species as no zoanthids of such small sizes (expanded oral disc diameter <3 mm) specifically inhabiting rubble zones had previously been described.

Zoanthids belong to the subclass Hexacorallia (Cnidaria, Anthozoa), a group currently comprising six orders Actiniaria, Antipatharia, Ceriantharia, Corallimorpharia, Scleractinia and Zoantharia. Each order in Hexacorallia is considered to be monophyletic, however, the relationships between the orders are still uncertain (see Daly *et al.* 2003; Sinniger *et al.* 2007). One of the most considerable problems for acquiring a clear understanding of the phylogeny within Hexacorallia is the confusion and lack of taxonomic studies at the species or the genus level (Daly *et al.* 2007).

Zoanthids can be characterized by having two rows of tentacles, and are typically colonial, with a single ventral siphonoglyph and detritus encrustation in their mesoglea. Zoanthids are often difficult to identify to species level utilizing only morphological characters due to their variable shape influenced by micro-environment and high levels of

intraspecific variation (Burnett *et al.* 1997; Reimer *et al.* 2004). Moreover, internal morphological structures that are used as diagnostic taxonomic characters to identify to the level of suborder or family are often difficult to observe due to zoanthids' sand encrustation. Recently, molecular analyses have helped overcome such issues and have helped in advancing the taxonomic study of zoanthids (e.g. Burnett *et al.* 1997; Reimer *et al.* 2006; Sinniger & Häusserman). Phylogenetic analyses can often help to distinguish between zoanthid species in cases where diagnostic morphological characters are problematic (Reimer *et al.* 2006) and the order Zoantharia has undergone a revision of its taxonomy in recent years, with several new species, genera and families being described (e.g. Reimer *et al.* 2007a; Swain 2009a; Sinniger & Häusserman 2009; Reimer & Fujii 2010).

In this study, we formally describe one new family, one new genus and two new species of zoanthids from specimens from the rubble zone spanning the temperate, subtropical and tropical Pacific Ocean and examine their phylogeny.

Materials and methods

Sample collection

Specimens from various locations in the Pacific Ocean were collected by snorkeling or scuba from the intertidal zone to depths of 30 m (Fig. 1). As specimens were collected, *in situ* digital images were taken to assist in identification and morphological analyses (oral disc/polyp diameter, colour, polyp form, etc.). Collected samples were fixed and preserved in 99.5% ethanol (n = 41 colonies; Table S1). Some large colonies (colonies of >6 polyps) were subdivided into subsamples. Polyps of subsamples were relaxed with magnesium chloride ($MgCl_2$) and subsequently fixed in 5–10% sea water (SW) formalin to be utilized in making anatomical sections for internal morphological analyses (n = 7 colonies).

Morphological analyses

The length of individual polyps and maximum column diameter of preserved specimens were measured using a dissecting microscope. Gross shape of colonies (e.g. unitary polyps or polyps connected by stolon), colour of the polyps, and number and length of the tentacles were recorded utilizing *in situ* images. Horizontal and vertical sections of polyps were made by hand cutting with razor, or after paraffin embedding following Reimer *et al.* (2010). After decalcification utilizing Bouin's fluid for 24 h, sand and other detritus remaining on the surface of column were removed as much as possible by tweezers under a dissecting microscope. The specimens were dehydrated through an ethanol–xylene series and then embedded in paraffin. Eight micometre thick sections made by embedding were stained with hematoxylin and eosin. Some paraffin embedded polyps (n = 7) were sectioned for only half of their body or cut by hand with a razor (n = 5) and deparaffinized through a xylene–ethanol series. Deparaffinized specimens were immersed in *t*-butanol and freeze-dried, sputter-coated with gold-palladium, and then the cut surfaces were observed by scanning electron micro-

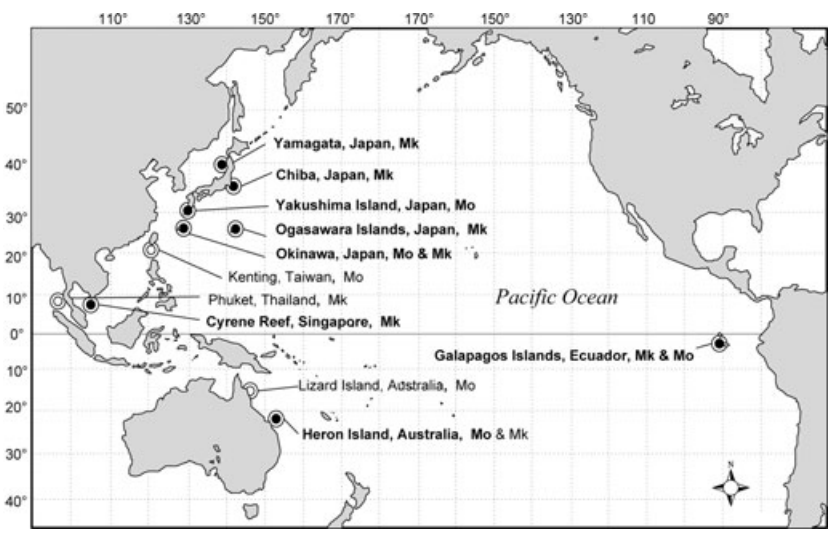

Fig. 1 Map of zoanthid specimen locations in the Pacific Ocean. Locations for specimens collected in this study represented by closed symbols, locations for which only photographic evidence exists represented by open symbols. Locations in bold indicate specimens for which molecular data are available. Species abbreviations after locations: Mo, *Microzoanthus occultus* sp. n.; Mk, *Microzoanthus kagerou* sp. n.

scope (SEM; JSM-5310LV or JSM-6060LV; JEOL, Tokyo, Japan). Acceleration voltage was set at 15 kV.

Cnidae

Undischarged cnidae were measured from tentacles, column, actinopharynx and mesenterial filaments of polyps (specimens examined $n = 3$ polyps/species group). Images of the cnidae were obtained by differential interference contrast microscopy, and measured using the software ImageJ (National Institute of Health, Bethesda, Maryland, USA). Cnidae nomenclature generally followed England (1991) and Ryland & Lancaster (2003). However, both Schmidt (1974) and Hidaka and co-workers (1987, 1992) have suggested basitrichs and microbasic b-mastigophores are the same type of nematocyst, and in this study, these two types were treated as the same type of cnidae.

DNA extraction and PCR amplification

DNA was extracted from ethanol preserved samples by following a guanidine extraction protocol (Sinniger *et al.* 2010). PCR amplifications were performed for mitochondrial cytochrome oxidase subunit I (COI), mitochondrial 16S ribosomal DNA (mt 16S rDNA) and the internal transcribed spacer region of ribosomal DNA (ITS-rDNA) region using the primer pairs HCO (Folmer *et al.* 1994) and COzoanf (Reimer *et al.* 2007a), 16SarmL (modified primer for mt 16S rDNA used in Sinniger *et al.* 2008: 5′ GGC CTC GAC TGT TTA CCA AA 3′) and 16SBmoH (Sinniger *et al.* 2005), and ITSf and ITSr (Swain 2009b), respectively. The following thermal cycle conditions were used: 35 cycles of: 30 s at 94 °C, 1 min at 40 °C, 1 min 30 s at 72 °C and followed by a 7 min extension at 72 °C for COI; 40 cycles of: 1 min at 95 °C, 1 min at 52 °C, 2 min at 72 °C and followed by a 7 min extension at 72 °C for 16S rDNA; 35 cycles of: 1 min at 95 °C, 1 min at 50 °C, 2 min at 72 °C, and followed by a 10 min extension at 72 °C for ITS-rDNA. Amplified PCR products were sequenced either by an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) or by Macrogene Japan (Tokyo, Japan).

Phylogenetic analyses

New sequences obtained in this study were deposited in GenBank (accession numbers HQ912792-HQ912861; Table S1). Obtained DNA sequences were aligned using Bioedit ver. 7.0.5.3. (Hall 1999) and the attached application Clustal W (Thompson *et al.* 1994), and the nucleotide sequences of COI and mt 16S rDNA from samples were separately aligned with previously obtained zoanthid sequences from all known zoanthid families (Epizoanthidae, Parazoanthidae, Sphenopidae, Zoanthidae, Neozoanthidae; for GenBank accession numbers, see Table S2) excepting Abyssoanthidae, which has only relatively short COI sequences available in GenBank. For outgroups, sequences of the phylogenetically highly divergent zoanthid genus *Isozoanthus* and Actiniaria were used for both mt 16S rDNA and COI trees. ITS-rDNA sequences obtained in this study (particularly ITS-1 and ITS-2 spacers) were highly divergent from other obtained zoanthid ITS-rDNA sequences, and thus an ITS-rDNA alignment was made consisting only of the sequences obtained in present research with a single *Parazoanthus* sequence as an outgroup (accession number EU418298; Table S2). Indels were kept unedited in the alignments of 16S rDNA and ITS-rDNA. All phylogenetic alignments are available from the corresponding author, and at the webpage http://web.me.com/miseryukyu/.

For phylogenetic analyses of COI, mt 16S rDNA and ITS-rDNA, the same methods were independently applied. The neighbor-joining (NJ) method was performed using MEGA4 (Tamura *et al.* 2007), with 1000 replicates of bootstrapping. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon & 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 made by Mr. Bayes 3.1.2 (Ronquist & Huelsenbeck 2003) under GTR+I+Γ. One cold and three heated Markov chains Monte Carlo (MCMC) with default-chain temperatures were run for 20 million generations, sampling log-likelihoods (InLs), and trees at 1000-generation intervals (20 000 InLs and trees were saved during MCMC). The likelihood plots for COI, mt 16S rDNA and ITS-rDNA datasets suggested that MCMC reached the stationary phase after the first 2 million generations for COI and mt 16S rDNA (standard deviation of split frequencies = 0.01362 and 0.002737, respectively), and after 3 million generations for the ITS-rDNA analysis (standard deviation of split frequencies = 0.002450). Thus, the remaining 18 000 trees of COI and mt 16S rDNA, and the remaining 17 000 trees of ITS-rDNA were used to obtain clade probabilities and branch-length estimates.

Systematics

Suborder Macrocnemina Haddon and Shackleton, 1891

Family MICROZOANTHIDAE, fam. n.

Type genus. Microzoanthus gen. n.

Etymology. As for the type genus with ending as in other zoanthid families.

Diagnosis. Colonies attached to bottom side (downward facing side) of dead coral rubble, asperous stones, inside narrow cracks, or occasionally on dead coral rubble on muddy seafloor. Azooxanthellate, macrocnemic. Polyps connected by narrow stolon or solitary. Sand particles encrusted in column. Irregularly sized sand particles encrusted into ectoderm. Tentacles two to three times as long as expanded oral disc diameter. Edge of oral disc shaped in regular, repeating zig-zagged pattern.

Remarks. No previously described macrocnemic zoanthid families have encrusting sand particles in their ectoderm but not in mesoglea. Additionally, family Microzoanthidae fam. n. is phylogenetically very divergent from all described zoanthid species at the level of family or order. Fixed specimens of Microzoanthidae fam. n. somewhat resembles the recently described genus *Terrazoanthus* Reimer & Fujii 2010 in being relatively small (column diameter <3 mm) for a zoanthid and found in cracks and under stones, however, numbers of tentacles and mesenteries for Microzoanthidae fam. n. species are fewer than in the genus *Terrazoanthus*. Microzoanthidae fam. n. does not have large holotrichs in their column, unlike *Terrazoanthus* species. Capitular ridges, base of tentacles and associated tissue are raised comparatively high above oral disc, resulting in oral disc forming a concave depression, unlike as in other zoanthid in which oral disc is much flatter.

Genus *Microzoanthus* gen. n.

Type species. *Microzoanthus occultus* sp. n.

Etymology. Named from the latin 'micro' meaning small, as polyp sizes of specimens of this group are generally too small to observe details *in situ* with the naked eye, with ending as in other zoanthid families. Gender is masculine.

Diagnosis. Only one genus of family Microzoanthidae, as for family above.

Microzoanthus occultus sp. n. (Fig. 2)

Holotype. NSMT-Co1536, Sesoko Island, Okinawa, Japan (26°38′52N, 127°51′16E), 0.5 m depth, collected by Takuma Fujii (TF), 6 June 2008, fixed in 99% ethanol (99% EtOH), deposited in National Museum of Nature and Science, Tokyo, Japan (NSMT).

Paratypes. Paratype 1, RUMF-2G-04367, Teniya, Okinawa, Japan, 1 m, collected by TF, 24 May 2008, fixed in 99% EtOH, deposited in Ryukyu University Museum Fujukan, Okinawa, Japan (RUMF); paratype 2, USNM-1150461, Oku, Okinawa, Japan, 23 m, collected by TF, 22 September 2008, fixed in 99% EtOH, deposited in National Museum of Natural History, Smithsonian Institution, Washington, D.C., USA (USNM); paratype 3, MHNG-INVE-77144, Minna Island, Okinawa, Japan, 1 m, collected by TF, 23 October 2008, fixed in 99% EtOH, deposited in Natural History Museum of Geneva, Geneva, Switzerland (MHNG).

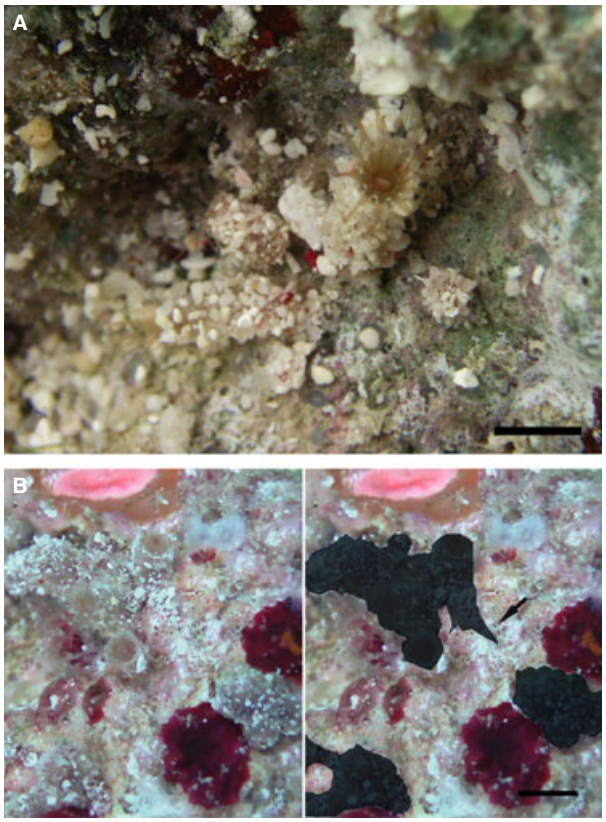

Fig. 2 A–B. *Microzoanthus occultus* sp. n. *in situ* in Okinawa, Japan. —A. Holotype NSMT-Co-1536 with an expanded, solitary polyp. —B. A stolon without polyps on its end, indicating either stolon growth or stolon recession (arrow). The blacked out area indicates location of the polyps. Scale bar: approximately 2 mm.

Other materials. Other specimens in addition to type specimens listed above are deposited in the Molecular Invertebrate Systematics and Ecology (MISE) Laboratory collection at the University of the Ryukyus, Nishihara, Okinawa, Japan (Table S1): MISE-TF-2, 3, 4, 5, 6, Odo, Okinawa, Japan, collected by TF, 3 September 2007, fixed in 99% EtOH (MISE-TF2-4, 1.5 m; MISE-TF-5, 3 m; MISE-TF-6, 1 m); MISE-TF7-a, 8, 11, Sesoko Island, Okinawa, Japan, collected by TF, 6 May 2008, fixed in 99% EtOH (MISE-TF7-a, 1 m; MISE-TF-8, 1.7 m; MISE-TF-11, 0.5 m); MISE-TF7-b, divided from the specimen MISE-TF7-a, fixed in 5–10% saltwater formalin; MISE-TF-15, Cape Hedo, Okinawa, Japan, 0.3 m, collected by TF, 22 May 2008, fixed in 99% EtOH; MISE-TF-22, 24, Teniya, Okinawa, Japan, 1 m, collected by TF, 24 May 2008, fixed in 99% EtOH; MISE-TF-23b, Teniya, Okinawa, Japan, 1 m, collected by TF, 24 May 2008,

fixed in 5–10% sea water formalin; MISE-TF36, Hateruma Island, Okinawa, Japan, 3 m, collected by TF, 8 September 2008, fixed in 99% EtOH; MISE-TF-41, Oku, Okinawa, Japan, 1.5 m, collected by TF, 22 September 2008, fixed in 99% EtOH; MISE-TF69, Yakushima Island, Kagoshima, Japan, 9.7 m, collected by James Davis Reimer (JDR), 17 June 2009, fixed in 99% EtOH; MISE-TF85, Yoron Island, Kagoshima, Japan, in the low intertidal, collected by JDR, 3 March 2010, fixed in 99% EtOH; MISE-JDR460, Espanola, Galapagos Islands, Ecuador, 9.5 m, collected by Angel Chiriboga (AC), 12 March 2007, fixed in 95% EtOH; MISE-HI241, Heron Island, Queensland, Australia, 16 m, collected by JDR, 25 November 2009, fixed in 95% EtOH; MISE-HI242-244, Heron Island, Queensland, Australia, 16 m, collected by JDR, 25 November 2009, fixed in 95% EtOH.

Etymology. Named from the latin 'occultus' meaning 'hidden', as colonies of this species are hard to recognize *in situ* because of their tiny body size, clear colour and cryptic sandy appearance. In particular this species is difficult to find *in situ* colonies consist of only a few polyps are solitary.

Common name. Hagure-tsubu-sunaginchaku (new Japanese name).

Description of holotype. Colony of four adjacent polyps. On 5 × 2 mm rubble fragment substrate. Polyps 0.4 × 0.9 mm, 0.4 × 0.6 mm, 0.5 × 1.4 mm and 0.5 × 1.7 mm maximum diameter and heights, respectively. Polyps and coenenchyme encrusted with various sand particles up to 0.8 mm in diameter. Distal tip of contracted polyps rounded

Diagnosis. Colonial polyps connected by narrow stolon with <1 cm between polyps or occasionally solitary polyps. Polyps cylindrical, diameter approximately up to 3 mm, height up to approximately 10 mm when living. *Microzoanthus occultus* has 22–24 tentacles 2–3 times as long as diameter of oral disc. Capitular ridges half number of tentacles. Capitular ridges, base of tentacles and associated tissue raised above main central portion of oral disc to height of more than 1/4 of diameter of oral disc, resulting in oral disc forming concave depression. Polyps and coenenchyme encrusted with sand in ectoderm, no encrustations in mesoglea. Tentacles, oral disc and column transparent, some colonies with slight reddish tinge and fluorescent green on tentacles or oral disc locally.

Internal anatomy. Mesogleal thickness approximately 90 μm. Mesentery macrocnemic arrangement (Fig. 3). Zooxanthellae absent.

Cnidae. Holotrichs, basitrichs, microbasic p-mastigophores, spirocysts (see Table 1; Fig. 4).

Distribution and habitat. *Microzoanthus occultus* sp. n. occurs on the underside (=bottom side, downward facing side) of rubble on the seafloor in shallow subtropical to tropical waters in the Pacific Ocean. Particularly, preferred habitat appears to be rubble on sandy seafloors in areas with some tidal current and/or water circulation. Specimens found from the low intertidal to a depth of 23 m, from Yakushima Island southwards in the Nansei Islands (Japan), around Heron Island, Queensland, on the Great Barrier Reef (Australia), and in the Galapagos Islands (Ecuador), and therefore it is expected that *M. occultus* sp. n. is distributed widely in the subtropical to tropical Indo-Pacific Ocean.

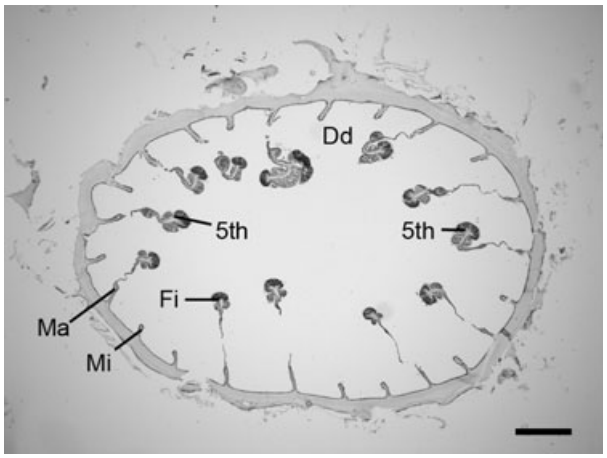

Fig. 3 Cross-section of *Microzoanthus occultus* sp. n. MISE-TF-10 polyp at the region of filaments showing macrocneme (with filament) and microcneme. Dd, dorsal directive; Fi, filament; Ma, macrocneme (complete mesentery); Mi, microcneme (incomplete mesentery); Si, siphonoglyph; 5th, 5th mesentery from the dorsal directive mesentery. Scale: 100 μm.

Remarks. *Microzoanthus occultus* sp. n. is similar to *Microzoanthus kagerou* sp. n., but can be clearly distinguished both morphologically and genetically. *Microzoanthus occultus* sp. n. never has a well-developed stolon and polyps are usually solitary. Additionally, *M. occultus* sp. n. has microbasic p-mastigophores in the actinopharynx (Table 1; Fig. 4), unlike *M. kagerou* sp. n. Both solitary polyps of *M. occultus* sp. n. and colonies (multiple polyps) with stolons <1 cm in length between polyps were observed. Polyps occurring together at close range (within 1 cm) are usually connected by a stolon and therefore form small colonies (observed maximum of seven polyps). The polyps of each colony were composed of both relatively large and small polyps (e.g. holotype). Moreover, some stolons did not have polyps on their ends, indicating either new stolon growth or stolon recession (Fig. 2B). These observations lead us to believe that *M. occultus* sp. n. is 'semi-colonial' and reproduces asexually by budding, and newly grown polyps subsequently become separated by stolon recession.

Table 1 Cnidae types and sizes of different areas of polyps of new zoanthid species in this study

	Microzoanthus occultus		*Microzoanthus kagerou* 'group k1'		*M. kagerou* 'group k2'	
	Length × width (μm)	Frequency[a]	Length × width (μm)	Frequency[a]	Length × width (μm)	Frequency[a]
Tentacles						
Holotrichs large	26–15 × 9–6	Common (3/3)	23–19 × 10–8	Occasional (1/3)	27–11 × 10–5	Occasional (3/3)
Holotrichs small	14–6 × 5–3	Occasional (2/3)	–	–	–	–
Basitrichs	18–9 × 5–2	Numerous (3/3)	13–9 × 4–2	Numerous (3/3)	14–10 × 4–2	Common (2/3)
p-Mastigophores	17–10 × 6–3	Common (2/3)	–	–	–	–
Spirocysts	16–10 × 4–2	Occasional (1/3)	16–9 × 4–2	Numerous (3/3)	15–9 × 5–2	Numerous (3/3)
Column						
Holotrichs large	25–16 × 12–7	Occasional (3/3)	26–12 × 11–4	Occasional (3/3)	32–12 × 10–5	Occasional (3/3)
Holotrichs small	14–8 × 8–3	Occasional (2/3)	–	–	–	–
Basitrichs	29–26 × 6–4	Rare (1/3)	–	–	–	–
Spirocysts	19–11 × 4–2	Rare (1/3)	–	–	–	–
Actinopharynx						
Holotrichs large	25–18 × 9–7	Common (2/2)[b]	30–17 × 10–7	Common (3/3)	28–15 × 4–2	Common (2/3)
Holotrichs small	12–6 × 5–2	Rare (1/2)[b]	10–8 × 4–3	Rare	13–5 × 4–3	Rare (1/3)
Basitrichs	16–9 × 5–2	Common (1/2)[b]	18–11 × 5–1	Numerous (3/3)	19–105 × 8–3	Numerous (3/3)
p-Mastigophores	15–7 × 4–2	Common (1/2)[b]	–	–	–	–
Spirocysts	14–8 × 3–2	Occasional (1/2)[b]	15–8 × 4–2	Rare (2/3)	14–9 × 4–2	Rare (3/3)
Mesenterial filaments						
Holotrichs large	29–22 × 22–7	Occasional (1/3)	28–15 × 10–6	Common (3/3)	25–14 × 10–5	Common (3/3)
Holotrichs small	9–6 × 4–3	Occasional (2/3)	11–7 × 5–3	Common (3/3)	9–6 × 6–2	Occasional (2/3)
Basitrichs	19–9 × 5–2	Numerous (3/3)	25–12 × 4–2	Numerous (3/3)	18–11 × 4–3	Numerous (2/3)
p-Mastigophores	16–11 × 6–4	Occasional (2/3)	18–11 × 5–3	Numerous (2/3)	17–12 × 5–1	Common (2/3)
Spirocysts	17–10 × 4–3	Occasional (2/3)	–	–	15–10 × 5–2	Rare (2/3)

[a]Frequency within samples: frequency in decreasing order; numerous, common, occasional, rare (*N* = number of specimens found in/total specimens examined).
[b]Tissue of actinopharynx could be obtained from only two specimens due to conditions of specimens.

As a result of sand particles encrusted in the column, anatomical sections in good condition could not be obtained except for some cross-sections that were not through the pharynx (Fig. 3). The arrangement of mesenteries was estimated from cross-sections and confirmed by observation with SEM.

Microzoanthus kagerou sp. n. (Fig. 5A–C)

Holotype. NSMT-Co1537. Chatan, Okinawa, Japan (26°19′34N, 127°44′34E), 18 m, collected by TF, 7 July 2008, fixed in 5–10% sea water (SW) formalin, a part of the colony was fixed in 99% EtOH, deposited in National Museum of Nature and Science, Tokyo, Japan.

Paratypes. Paratype 1, RUMF-2G-04369, Yona, Okinawa, Japan, 12 m, collected by TF, 27 September 2009, fixed in 5–10% SW formalin; paratype 2, RUMF-2G-04368, subspecimen of RUMF-2G-04368, fixed in 99% EtOH, deposited in Ryukyu University Museum Fujukan, Okinawa, Japan; paratype 3, USNM-1150462 Oura Bay, Okinawa, Japan, 20 m, collected by Masami Obuchi (MO), 8 November 2009, fixed in 5–10% SW formalin; paratype 4, USNM-1150463, subspecimen of USNM-1150462, fixed in 99% EtOH deposited in National Museum of Natural History, Smithsonian Institution, Washington, D.C.; paratype 5, MHNG-INVE-77145, Oura Bay, Okinawa, Japan, 20 m, collected by MO, 7 October 2010, fixed in 5–10% SW formalin; paratype 6, MHNG-INVE-55146, subspecimen of MHNG-INVE-77145, fixed in 99% EtOH, Natural History Museum of Geneva, Geneva, Switzerland.

Other materials. Other specimens are deposited in the MISE Laboratory collection at the University of the Ryukyus, Nishihara, Okinawa, Japan (Table S1): MISE-TF21-a, Teniya, Okinawa, Japan, 1 m, collected by TF, 24 June 2008, fixed in 99% EtOH; MISE-TF21-b, divided from the colony MISE-TF21-a, fixed in 5–10% SW formalin; MISE-TF55, 56, Mizugama, Okinawa, Japan, collected by TF, 6 April 2009, fixed in 99% EtOH (MISE-TF-55, 7 m, MISE-TF-56, 5 m); MISE-JDR427, Wolf Island, Galapagos Islands, Ecuador, 10.7 m, collected by JDR, 4 March 2007, fixed in 99% EtOH; MISE-TFCh, Katsuura, Chiba, Japan, low intertidal, collected by Kensuke Yanagi (KY), 25 June 2008, fixed in 5–10% SW formalin; MISE-TF16, Cape Hedo, Okinawa, Japan, 5 m, collected by TF, 24 June 2008, fixed in 99% EtOH; MISE-TF25, Teniya, Okinawa, Japan, 1 m, collected by JDR, 25 June 2008, fixed in 99% EtOH; MISE-TF40, Oku, Okinawa, Japan, 1.5 m, collected by TF, 22 September 2008, fixed in 99% EtOH; MISE-TF45, Yona, Oki-

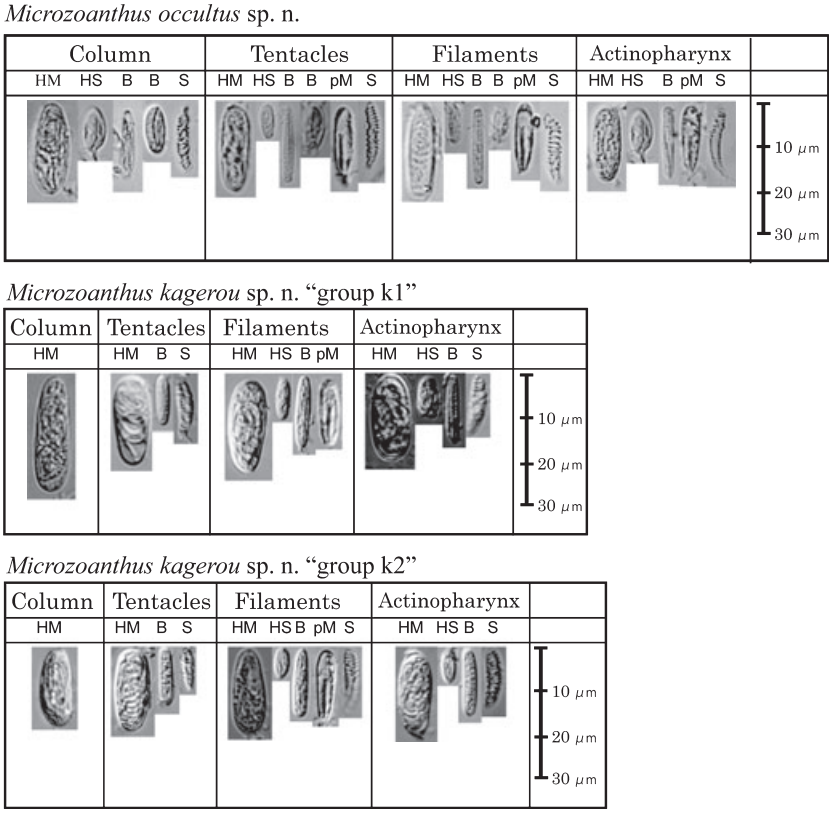

Fig. 4 Cnidae in the tentacles, column, pharynx and filament of *Microzoanthus occultus* sp. n., *Microzoanthus kagerou* sp. n. 'group k1', and *M. kagerou* sp. n. 'group k2', respectively. HM, medium holotrichs; B, basitrichs; pM, microbasic *p*-mastigophores; S, spirocysts.

nawa, Japan, 15 m, collected by TF, 24 September 2008, fixed in 99% EtOH; MISE-TF51, Unten, Okinawa, Japan, 6 m, collected by TF, 28 December 2008, fixed in 99% EtOH; MISE-TF53, Teniya, Okinawa, Japan, 1.7 m, collected by TF, 25 January 2009, fixed in 99% EtOH; MISE-TF58, Chichi Island, Tokyo, Japan, 1 m, collected by JDR, 25 March 2009, fixed in 99% EtOH; MISE-TF74, Kamo, Yamagata, Japan, 5.7 m, collected by TF, 15 September 2009, fixed in 99% EtOH; MISE-628, Cyrene Reef, Singapore, low intertidal, collected by JDR, 8 June 2008, fixed in 99% EtOH.

Etymology. Named from the Japanese 'kagerou' meaning 'heat haze', as their clear colour tentacles looks like heat haze rising up from the polyp.

Common name. Kagerou-tsubu-sunaginchaku (new Japanese name).

Description of holotype. Colony of approximately 15 polyps connected by narrow stolon in small hollow on fragment of dead coral rubble, rubble approximately 8 × 7 × 6 cm. Polyps approximately 1.0–3.0 mm in diameter, and approximately 1.0–4.5 mm in height from coenenchyme. Some stolons more than 1.5 cm in length between polyps. Polyps and coenenchyme encrusted with various sized sand particles. Distal tip of contracted polyps rounded.

Diagnosis. Colonial, polyps connected by narrow stolon. Polyps cylindrical, maximum diameter approximately 3 mm, maximum height approximately 10 mm when living. Stolon often longer than 1 cm between polyps. *Microzoanthus kagerou* has 20–26 tentacles two to three times as long as diameter of the column. Capitular ridges half the number of tentacles. Capitular ridges, base of tentacles and associated tissue raised above oral disc to height of more than 1/4 of diameter of oral disc, resulting in oral disc forming a concave depression. Polyps and coenenchyme encrusted with sand in ectoderm, encrustation not extending to mesoglea. Tentacles, oral disc and column transparent, some colonies with slight reddish tinge, oral disc locally coloured fluorescent green (Fig. 5B,C).

Internal anatomy. Mesogleal thickness approximately 90 μm. Marginal sphincter muscle in mesoglea near ectoderm distally, linked to endoderm proximally. Mesenteries in macrocnemic arrangement (Fig. 6A–D). Zoxanthellae absent.

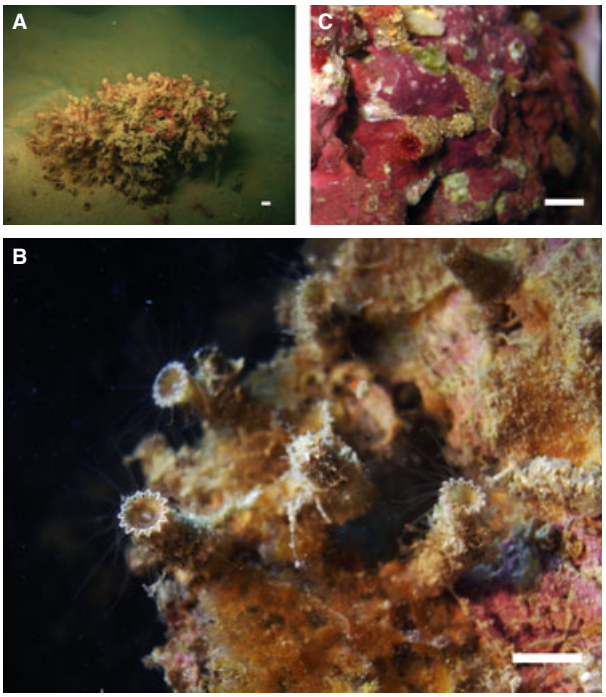

Fig. 5 A–C. *Microzoanthus kagerou* sp. n. *in situ* in Okinawa, Japan. —A. Colony of paratype USNM-1150462. Photograph by Masami Obuchi. —B. Polyps of paratype MHNG-INVE-77145. —C. Colony of holotype NSMT-Co1537 showing well-developed stolon. Scale bar: approximately 2 mm.

Cnidae. Holotrichs, basitrichs, microbasic p-mastigophores, spirocysts (Table 1; Fig. 3).

Distribution and habitat. *Microzoanthus kagerou* sp. n. occurs on rubble on muddy seafloors, on the under-side of rubble on sandy seafloors and in small cracks of rocky walls of shallow tropical to temperate waters in the Pacific Ocean. Found from the intertidal zone to a depth of 20 m, in this study *M. kagerou* sp. n. was found from Yamagata on the Sea of Japan (northern Japan), Chiba (Japan), the Nansei (Ryukyu) Islands (Japan), and in Singapore and the Galapagos Islands (Ecuador). Therefore, *M. kagerou* sp. n. likely has a wide distribution across the Pacific Ocean. *Microzoanthus kagerou* sp. n.'s range may extend further north and south into colder, more temperate water than *M. occultus* sp. n., which was found only in subtropical and tropical areas.

Remarks. Based on morphologic and phylogenetic results, *M. kagerou* sp. n. is closely related to *M. occultus* sp. n., but can be clearly distinguished both morphologically and genetically from its congener. *Microzoanthus kagerou* sp. n. often has a long stolon (>1 cm) connecting polyps (see Fig. 5C). Although *M. kagerou* sp. n. colonies that consist of only a few polyps are hard to distinguish from *M. occultus* sp. n. colonies, they can be distinguished by microbasic p-mastigophores being absent in actinopharynx (Table 1; Fig. 3), unlike as in *M. occultus* sp. n. Phylogenetically, *M. kagerou* sp. n. is unique from both *M. occultus* sp. n. and all other known zoanthid species (Fig. 7; Fig. S1).

Two specimens found on rubble on the muddy seafloor of Oura Bay, Okinawa, were designated as type specimens based on their excellent preservation condition, but it should be noted the muddy environment of Oura Bay does not appear to this *M. kagerou* sp. n.'s typical habitat. Anatomical sections of *M. kagerou* sp. n. were obtained from only one colony [specimen USNM-1150462, see Fig. 5A] due to the presence of encrusted sand. The resulting section also was partially broken when encrusted sand was being removed by hand before embedding for sectioning. However, from the results of SEM observations we were able to conclude that sand particles were encrusted only in the ectoderm and not in the mesoglea.

Phylogenetic results

COI

The phylogenetic tree resulting from ML analyses of the COI sequence alignment is shown in Fig. 6A. In the phylogenetic tree, Microzoanthidae fam. n. formed a completely supported monophyletic group (ML = 100%, Bayes = 1.00). The distance between all currently known zoanthids and Microzoanthidae fam. n. was as great as the distance from all known zoanthids to the Actiniaria (sea anemones) [pairwise distance from closest anemone, *Urticina* 0.234–0.223; from *Epizoanthus* (closest zoanthids) 0.247–0.242]. In other words, Microzoanthidae fam. n. was located almost equidistant between known zoanthids and the sea anemones. Microzoanthidae fam. n. was divided into two large clades (ML = 88%, Bayes = 0.96; ML = 70%, Bayes = 0.53, respectively). One of the clades [consisting of specimens TF6, TF22, TF36, TF41, USNM-1150461, MHNG-INVE-77144, TF85, HI241, HI242, HI243, JDR460] corresponded to specimens with 'less colonial' morphological characters, designated as *M. occultus* sp. n. The other clade, consisted of specimens with 'more colonial' colonies, designated as *M. kagerou* sp. n., was divided into at least two subclades. Within the *M. kagerou* sp. n. clade, one subclade formed a well-supported (ML = 95%, Bayes = 0.87) monophyletic group [=NSMT-Co1537, TF55, TF56, RUMF-2G-04368, USNM-1150463, JDR427] designated 'group k1', and the remaining specimens formed a polyphyletic grouping (=TF25, TF40, TF45, TF51, TF53, TF74), designated 'group k2'.

mt 16S rDNA

The phylogenetic tree resulting from analyses of the mt 16S rDNA sequence alignment is shown in Fig. 6B. The

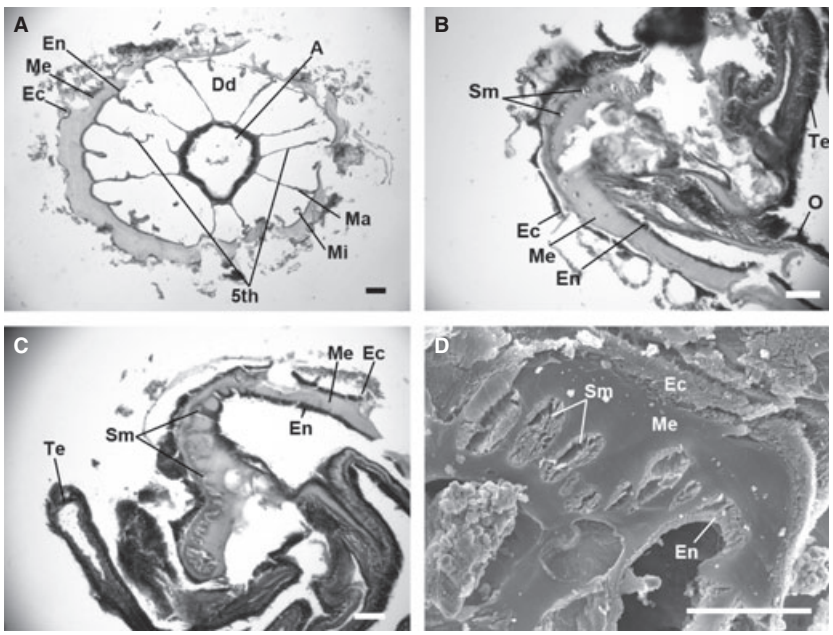

Fig. 6 A–D. Histological sections of *Microzoanthus kagerou* sp. n. paratype USNM-XXXXX (TF81-b) polyp. —A. Cross-section of polyp at the region of pharynx. —B–C. Mesogleal sphincter muscles in longitudinal sections of polyp, muscle showing different shape by sections in a polyp, pictures were taken by optical microscope. —D. Picture of mesogleal sphincter muscle taken by scanning electron microscope (SEM). A, actinopharynx; DD, dorsal directive; Ec, ectoderm; En, endoderm; Ma, macrocneme; Mi, microcneme; Me, mesoglea; O, oral disc; Si, siphonoglyph; SM, sphincter muscle; T, tentacle; 5th, 5th mesentery from the dorsal directive mesentery. Scale: 50 μm.

phylogenetic tree shows Microzoanthidae fam. n. forming a completely supported monophyletic group (ML = 100%, Bayes = 1.00). Inside Microzoanthidae fam. n., there were three clades and the topology of mt 16S rDNA was therefore somewhat different from the COI tree, which had only two clades. Specimens with 'less colonial' colonies formed a well-supported (ML = 98%, but Bayes <0.5) monophyletic clade [=NSMT-Co1536, TF22, RUMF-04367, TF36, TF41, USNM-1150461, MHNG-INVE-77144], corresponding to *M. occultus* sp. n. The other specimens (=*M. kagerou* sp. n.) were divided to two moderately supported (ML = 84%, Bayes = 0.95; ML = 55%, Bayes = 0.96, respectively) sister clades, 'group k1' [=NSMT-Co1537, TF56] and 'group k2' (=TF53, TF25, TF74).

ITS-rDNA

ITS-rDNA phylogenetic results are shown in Fig. 8. Sequences could not obtained from specimens belonging to 'group k2' despite repeated attempts. Obtained ITS-rDNA sequences of Microzoanthidae fam. n. were highly divergent from sequences of all known zoanthid species, and it was therefore somewhat difficult to align these sequences with ITS-rDNA sequences from other zoanthid species, although the 18S and 5.8S rDNA regions provided reliable anchors for successfully making an alignment. The resulting phylogenetic tree showed that specimens were divided into two well-supported clades (ML = 71%, Bayes = 0.94; ML = 99%, Bayes = 1.00, respectively), corresponding to *M. occultus* sp. n. [=TF2, TF3, TF7, TF8, NSMT-Co1536, TF15, TF22, TF24, TF36 and MHNG-INVE-77144] and *M. kagerou* sp. n. [=TF21 and NSMT-Co1537].

Discussion

Classification of Microzoanthidae

Phylogenetic results clearly showed Microzoanthidae fam. n. to be genetically far different from all other hexacorallians at the order level as sequences from specimens were equidistant between zoanthids and sea anemones (Figs 7 and 8; Fig. S1). However, the arrangement of the mesenteries and other morphological characters (colonial specimens with narrow stolons, two rows of tentacles, sand encrustation) clearly place these specimens within the order Zoantharia. The arrangement of mesenteries is considered an important character when considering the evolution of Cnidaria (Daly *et al.* 2003), and this system is currently used to place anthozoans into the various orders. Thus, based on our cross-section results, Microzoanthidae fam. n. must be placed within the order Zoantharia.

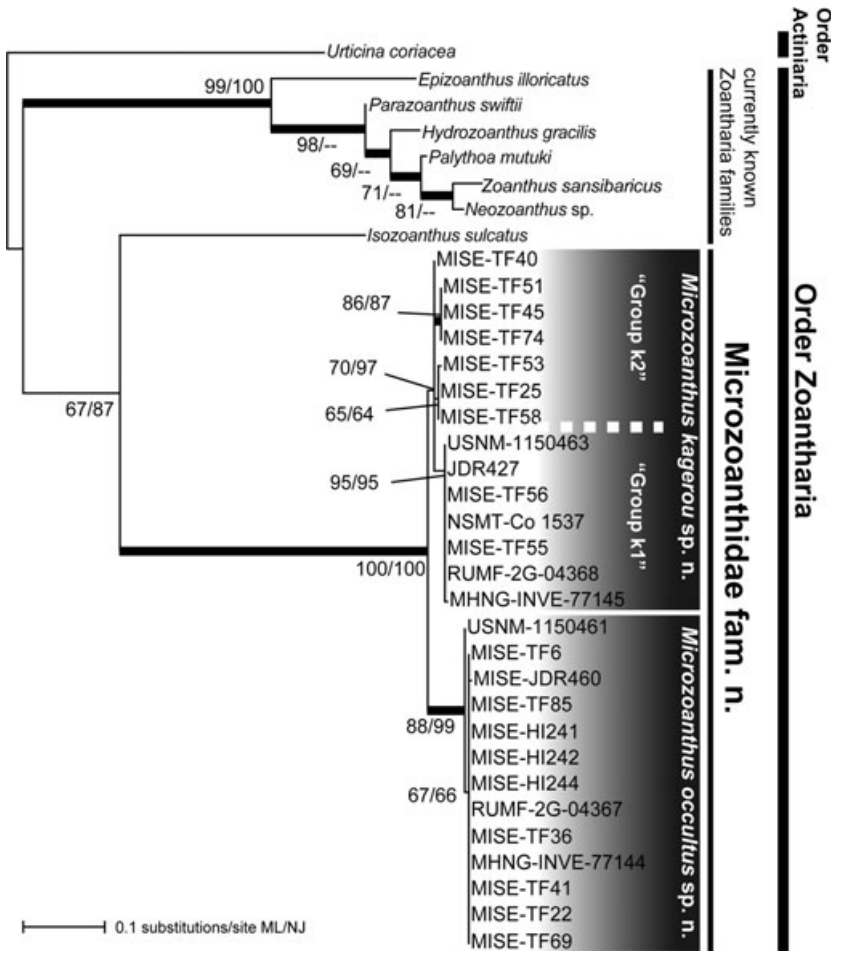

Fig. 7 Maximum likelihood (ML) tree of cytochrome oxidase subunit I (COI), for newly obtained sequences from zoanthid specimens in this study along with previously published GenBank sequences. Values at branches represent ML and neighbor-joining (NJ) probabilities (>60%). Monophylies with Bayesian posterior probabilities >0.95 are shown by thick branches. Sequences for new species in this study in larger font. Sequences/species names from previous studies in regular font. For specimen information, see Table S1.

The cross-sections of Microzoanthidae fam. n. specimens showed their sphincter muscles were within the mesoglea. A mesogleal sphincter muscle is currently a key character of the zoanthid family Epizoanthidae, in contrast to other zoanthid families (including Parazoanthidae) that have an endodermal sphincter muscles. However, some Microzoanthidae fam. n. sphincter muscles were at the outer edge of the endoderm, so we cannot completely exclude the possibility of endodermal sphincter muscle (Fig. 6C,D). Moreover, both Sinniger *et al.* (2009) and Swain (2010) have suggested that the position of the sphincter muscle alone may be inadequate as a useful taxonomical character in zoanthids. On the other hand, when taken in sum, the morphological characters of these new specimens, such as their encrustation of large pieces of sand and the shape of colonies (solitary or stoloniferous colonies), make this group easily distinguishable from all known zoanthid taxa, and the molecular phylogenetic analyses support this. Therefore, specimens in this research have been classified as a new zoanthid family, Microzoanthidae.

The molecular phylogenetic trees (COI, mt 16S rDNA, Fig. 7; Fig. S1) showed Microzoanthidae fam. n. can be separated into at least two putative species-level clades. *Microzoanthus occultus* sp. n. formed a clear monophyly in all examinations, and therefore we consider this grouping to clearly be a single species. The other two clades are described here together as the new species *M. kagerou* as they could not be separated by any discernable morphological differences. Phylogenetic analyses showed that one of the two *M. kagerou* sp. n. subclades consisted of specimens that had identical sequences (see Fig. 7; Fig. S1; 'group k1') while the other subclade formed a complex group contain-

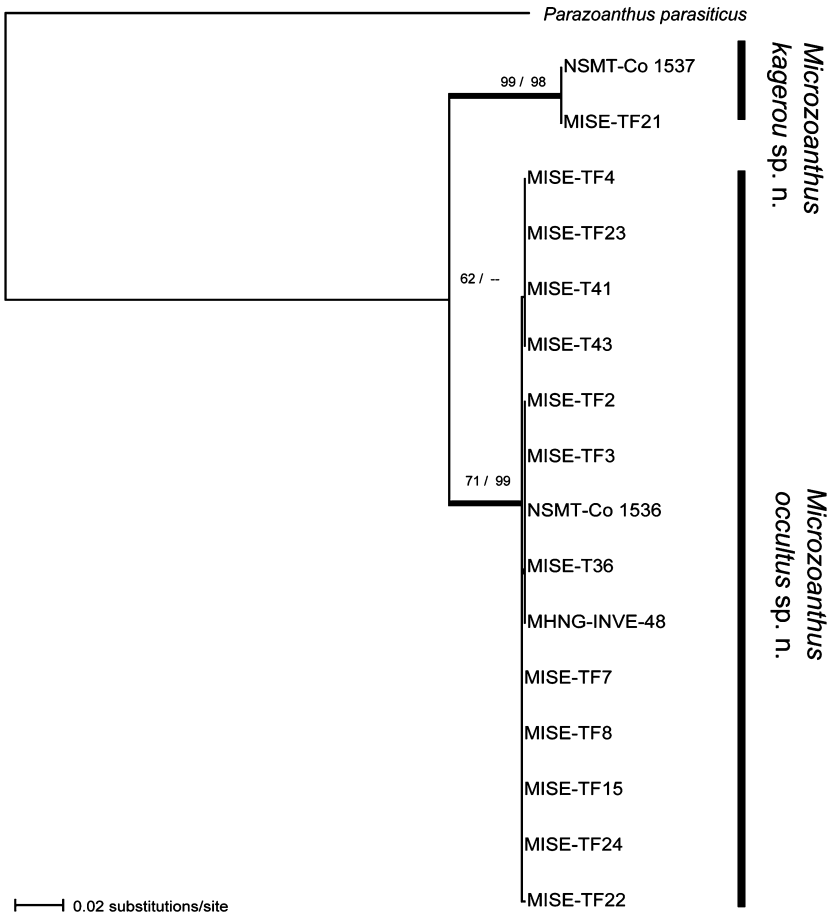

Fig. 8 Maximum likelihood (ML) tree of internal transcribed spacer of ribosomal DNA (ITS-rDNA) for *Microzoanthus* gen. n. specimen sequences. Values at branches represent ML and neighbor-joining (NJ) probabilities (>60%). Monophylies with Bayesian posterior probabilities >0.95 shown by thick branches. For specimen information, see Table S1.

ing some different genotypes ('group k2'; see Fig. 7; Fig. S1). Additionally, ITS-rDNA sequences could not be acquired from 'group k2' despite the use of the same primer set as used for other Microzoanthidae fam. n. However, these two subclades, despite their genetic differences, could not be separated morphologically, and are sister clades based on our results. Thus, based on the sum of our molecular and morphological results, in this study we have described these two subclades as a single species, *M. kagerou* sp. n., and have chosen all type specimens from a single subclade. In the future, specimens from 'group k2' may be split from 'group k1' *M. kagerou* sp. n. into another *Microzoanthus* gen. n. species if diagnostic characters can be found to morphologically distinguish specimens.

Phylogeny of hexacorals

Recently, the molecular phylogeny of hexacorals has been the focus of much research as this group apparently is one of the oldest eumetazoan groups, and genome projects of scleractinian and actiniarian species are currently underway (e.g. Putnam *et al.* 2007; Wang *et al.* 2009). However, phylogenetic relationships within hexacorals are still not well understood. Some recent research has suggested that asides from Ceriantharia, the order Zoantharia may be the ancestral group within Hexacorallia (Medina *et al.* 2006; Brugler & France 2007; Sinniger *et al.* 2007). Additionally, past research has theorized that Ceriantharia may merit separate subclass status outside of Hexacorallia, which would make Zoantharia potentially the ancestral group within Hexacorallia. Recent research has also demonstrated that Actiniaria is apparently the sister group of Zoantharia (Medina *et al.* 2006; Brugler & France 2007; Sinniger *et al.* 2007).

Sequences of both COI and mt 16S rDNA from *Isozoanthus* were highly divergent from all zoanthids including Microzoanthidae fam. n. (see Table S2; Fig. 7, pairwise

distances: 0.213–0.229 for COI, 0.127–0.179 for mt 16S rDNA). Similarly, Sinniger *et al.* (2009) mentioned that *Isozoanthus sulcatus* (family Parazoanthidae), used as an outgroup in this study, is clearly distinct from other *Isozoanthus* species. Additionally, *I. giganteus* sequences, referred to in Swain (2010) and also utilized in this study, are also highly divergent from both known zoanthid sequences and Microzoanthidae fam. n. sequences (Fig. S1). Thus, as well as further examinations of Microzoanthidae fam. n., a taxonomical re-examination of the genus *Isozoanthus* is required to properly estimate the relationship between order Zoantharia and Actiniaria.

From the results of the molecular phylogenetic and morphological analyses in this research, Microzoanthidae fam. n. is clearly different from all known hexacorals. Microzoanthidae fam. n. encrusts particles in their ectoderm but not in their mesoglea. In addition, Microzoanthidae fam. n. has either a primitive colonial form or is solitary. These morphological characters are not typical characters of the majority of zoanthids, which usually have a well-developed coenenchyme (but see *Corallizoanthus* and *Sphenopus*), and encrust sand particles in the body to help strengthen their structure (except for the family Zoanthidae), and *Microzoanthus* fam. n. can be thought of as an intermediate group between Actiniaria and other Zoantharia.

The morphological, ecological and genetic characters of Microzoanthidae fam. n. lead us to theorize that this group is evolutionary ancestral to other zoanthids. In future research, it will be necessary to perform analyses including all representative groups of not only Zoantharia but also of Actiniaria to fully confirm this ancestral phylogenetic placement of Microzoanthidae fam. n. These future studies may demonstrate the importance of Microzoanthidae fam. n. in the evolutional phylogeny of Hexacorallia and help in obtaining a clearer understanding of the ancient evolution of Hexacorallia. However, for now, the monophyly and uniqueness of this new zoanthid family is unequivocal.

Finally, this study demonstrates how it is highly likely the existence of many marine invertebrate taxa remains overlooked, and that widely distributed groups such as Microzoanthidae fam. n. remain to be discovered.

Acknowledgements

The following people and institutions are thanked for their support in sampling and logistics; in the Galapagos; Dr Cleveland Hickman, Jr, Angel Chiriboga, the Darwin Initiative 'Galapagos Coral Conservation' Project, Charles Darwin Research Station, captains and crew of *Tip Top IV* and *Pirata*; in Singapore, Dr Peter Todd, the Raffles Biodiversity Museum, captain and crew of *RV Mudskipper*, Marine Biology Laboratory students (all National University of Singapore); in Australia, Dr Julian Caley, Shawn Smith, the Census of Coral Reef Ecosystems Australia Project (part of the Census of Marine Life); in Japan, Dr Frederic Sinniger, Dr Kensuke Yanagi, Dr Masami Obuchi, Tetsuo Sasaki, Shin Nishihira, Tsuruoka Kamo Aquarium, and Molecular Invertebrate Systematics and Ecology laboratory members (2007–2010). Specimens from Australia were collected under Great Barrier Reef Marine Park Authority permit #G32313.1 and Queensland Fisheries permit #95152. Sampling in the Ogasawara Islands (Japan) was made possible by a permit for collection of aquatic organisms by scuba diving from the Government of Tokyo (#21–12). TF was supported in part by post-COE program at the University of the Ryukyus. JDR was partially supported by the Rising Star Program and the 21st Century Center of Excellence Program at the University of the Ryukyus, a grant from the Japan Society for the Promotion of Science ('Wakate B' #2170088), and the Biological Institute on Kuroshio (Otsuki, Kochi, Japan). The Census of Coral Reef Ecosystems (CReefs) Australia Project was sponsored by BHP Bilton in partnership with the Great Barrier Reef Foundation and the Australian Institute of Marine Science. Comments from two anonymous reviewers and Dr Daphne Fautin comments helped improve this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Maximum likelihood (ML) tree of mitochondrial 16S ribosomal DNA sequences, for newly obtained sequences from zoanthid specimens in this study along with previously published GenBank sequences. Values at branches represent ML and Neighbor-joining (NJ) prob-

abilities (>60%). Monophylies with Bayesian posterior probabilities >0.95 are shown by thick branches. Sequences for new species in this study in larger font. Sequences/species names from previous studies in regular font. For specimen information see Table S1.

Table S1 Examined zoanthid specimens from this study from the Pacific Ocean, and corresponding GenBank accession numbers.

Table S2 GenBank accession numbers of order Zoantharia sequences from previous studies used in phylogenetic analyses in this study.

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