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ORIGINAL PAPER

New records and molecular characterization of *Acrozoanthus* (Cnidaria: Anthozoa: Hexacorallia) and its endosymbionts (*Symbiodinium* spp.) from Taiwan

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Abstract During a recent survey of the zoanthid (Cnidaria: Anthozoa) fauna of Taiwan, specimens resembling *Acrozoanthus australiae* (family Zoanthidae) were found at Kenting and Green Island, Taiwan, attached to eunicid worm tubes growing out from under large *Porites* coral colonies in coral reef environments. As this species had previously been described only from eunicid worm tubes in mud flats in Australia and Indonesia, and no studies had specifically examined its phylogenetic position, molecular and morphological examinations were conducted to determine the identity of the Taiwan specimens, and its phylogenetic relationships with other Zoanthidae genera and species. At the same time, endosymbiotic *Symbiodinium* types within specimens were also investigated. Results from the phylogenetic analyses of sequences of three DNA markers [cytochrome oxidase subunit I, mitochondrial 16S ribosomal DNA, internal transcribed spacer 2 of ribosomal DNA (ITS-2)] strongly suggested that the Taiwan specimens were identical with *A. australiae*. Based on endosymbiont ITS-2 sequences, these colonies were in symbiosis with *Symbiodinium* clade D1a (= *S. trenchii*), theorized to be adapted to both comparatively cold and hot marine environments. Furthermore, phylogenetic analyses from all three zoanthid DNA markers suggest that *Acrozoanthus* may be within the closely related genus *Zoanthus*. This study demonstrates the overall lack of data on zoanthid species' distributions, and it is recommended the diversity of zoanthids within the nearby Coral Triangle be investigated to link Indo-Australian zoanthid data with information from Japan and the northwest Pacific.

Keywords *Acrozoanthus* · *Symbiodinium* · Zoanthid · COI · mt 16S rDNA · ITS-rDNA

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Introduction

Zoanthids are an order (Zoantharia, = Zoanthidea, = Zoanthiniaria) of generally colonial, benthic anthozoans found in many different marine ecosystems. These hexacorallians can be usually distinguished from other closely related groups such as scleractinians and actiniarians by the presence of sand and detritus in their ectoderm, a character unique to this order. However, not all zoanthids take up sand, with one family, Zoanthidae, consisting of three genera with no sand in their body walls. The family Zoanthidae contains three genera, *Zoanthus* Lamarck, 1801, *Isaurus* Gray, 1828, and *Acrozoanthus* Saville-Kent 1893, which are zooxanthellate, containing endosymbiotic *Symbiodinium* (Reimer et al. 2006c, 2007b, 2008b). *Zoanthus* species are found worldwide in tropical and sub-tropical shallow oceans, and are particularly common

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in coral reef environments. This genus is the most speciose of the family, with approximately 150 species described in the literature (Fautin 2009), although the true number of species remains unknown (Burnett et al. 1997; Reimer et al 2004). The species of the genus *Isaurus* are also found in both the Atlantic and Indo-Pacific, and similarly found in shallow, tropical or subtropical environments, although this genus appears to be much less specious, with colonies also occurring at much lower frequencies than *Zoanthus* spp. (Reimer et al. 2008b). *Isaurus* is distinguishable from *Zoanthus* by usually having numerous tubercules (bumps) on the outside of their polyps, and with polyps being recumbent (not standing upright), although there are species in both genera that are somewhat intermediate with regards to these features (Reimer et al. 2008b).

The third genus of Zoanthidae, *Acrozoanthus*, is monotypic, only comprising *Acrozoanthus australiae* Saville-Kent 1893 described from the Great Barrier Reef, Australia. Despite its very similar appearance, *A. australiae* was placed into a genus separate from *Zoanthus* due to the presence of an axial skeleton (Saville-Kent 1893). Later it was shown that this skeleton was in fact a result of habitat preference as *Acrozoanthus* inhabits the outside of eunicid worm tubes, and the genus was subsequently merged back again into the genus *Zoanthus* (Haddon 1895). Subsequent to its original description, this species was not mentioned in literature again until its rediscovery by Ryland (1997), based on examination of a single specimen. Further work by Ryland and co-workers described the nematocysts of *A. australiae* (Ryland et al. 2004) and also an unusual "budding" method of asexual reproduction (Ryland 1997), which was theorized to potentially confirm the placement of this species in its own genus. Three additional specimens have been reported from Ambon, southern Sulawesi (both in Ryland 1997), and northern Sulawesi, Indonesia (Sinniger et al. 2005), and thus, although data are scarce, *A. australiae* has been thought to be distributed in the "Coral Triangle" (Indonesia) region, the Great Barrier Reef and the northern coast region of Australia on worm tubes along somewhat muddy shores (Ryland 1997).

Recent phylogenetic examinations of zoanthids using both mitochondrial and nuclear DNA, combined with ecological and morphological data, have allowed for a re-examination of zoanthid taxonomy. These new investigations have resulted in the description of new species (Reimer et al. 2006a; Sinniger and Häussermann 2009), new genera (Reimer et al. 2008a; Sinniger et al. 2010; Reimer and Fujii 2010), and new families (Reimer et al. 2007; Sinniger et al. 2010), while also suggesting the combination of other genera (Reimer et al. 2006b) and species (Burnett et al. 1997; Reimer et al. 2006a). Such results demonstrate both the morphological simplicity and plasticity of zoanthids, and also show that their evolution and higher-level systematics are much more complex than previously realized. Thus, a critical re-examination of as many taxa as possible is needed to truly understand zoanthid diversity and evolution. In the family Zoanthidae, while much research has focused on *Zoanthus* (Burnett et al. 1995, 1997; Reimer et al. 2004; 2006a, 2006c, 2007c) and some on *Isaurus* (Muirhead and Ryland 1985; Reimer et al. 2008b), the genus *Acrozoanthus* remains largely unexamined with these new techniques (but see Sinniger et al. 2005).

During recent expeditions to southern Taiwan, numerous *Acrozoanthus*-like colonies were discovered living on the outer surfaces of eunicid worm tubes growing on the bottom sides of massive *Porites* coral colonies in coral reef environments. As these colonies were far away from the known distribution region of *A. australiae* and in a different environment, specimens were collected for examination. Here, we report on the results of our molecular examinations, which investigated the following questions:

1. Are these new specimens in fact *Acrozoanthus* species? If so, are they conspecific to *A. australiae*?
2. Does *Acrozoanthus* belong in a genus separate from *Zoanthus*?
3. What types of *Symbiodinium* do Taiwan specimens possess? As different *Symbiodinium* clades, subclades and types have different physiologies and adaptations (e.g. Sampayo et al. 2008), characterization of *Symbiodinium* can allow us to understand the ecology of the holobiont (host + symbiont) (Reimer and Todd 2009).

Materials and methods

Specimen collection and initial identification

Eight zoanthid specimens (in this study specimens= colonies) were collected by SCUBA diving from two locations in Taiwan, Ho-bi-hoo at Kenting (n=6), and Dabaisha at Green Island (n=2) in September and November 2009, respectively (Table 1, Figs. 1, 2). An additional piece of tissue from an *Acrozoanthus australiae* specimen previously utilized (referred to as specimen 199 in Sinniger et al. 2005) from northern Sulawesi was also acquired for comparison, and designated as specimen "MISE 1053" in this study.

Specimens were preliminarily identified as *Acrozoanthus* using morphological characteristics used in past literature (e.g. on the outside of eunicid worm tubes, no encrustation) based on in situ photographs (taken with an Canon Powershot digital camera in an underwater housing) and ex situ physical examination. Specimens were subsequently stored in 99% ethanol at ambient temperature. All samples were finally deposited at the University of the Ryukyus

Table 1 *Acrozoanthus* specimens from Taiwan and Indonesia examined in this study

Specimen number	Collection location	Depth (m)	Collected by	Collection month	COI GenBank acc. no.	16S GenBank acc. no.	ITS rDNA GenBank acc. no.	*Symbiodinium* clade, GenBank acc. no.
MISE 1053	N. Sulawesi, Indonesia	9	MB	September 2003	HM171915, EF672670	HM171921, AY996947	AB517555	NA
MISE K33a	Ho-bi-hoo, Kenting	9	JDR	September 2009	HM171914	HM171919	NA	D, HM171907
MISE K33b	Ho-bi-hoo, Kenting	9	JDR	September 2009	HM171913	HM171920	NA	D, HM171909
MISE K34	Ho-bi-hoo, Kenting	9	JDR	September 2009	NA	HM171918	NA	D, HM171910
MISE K35	Ho-bi-hoo, Kenting	9	JDR	September 2009	HM171912	HM171917	HM171905	D, HM171908
MISE K36	Ho-bi-hoo, Kenting	10	JDR	September 2009	NA	HM171916	HM171906	D, HM171911
MISE K37	Ho-bi-hoo, Kenting	12	JDR	September 2009	NA	HM171922	NA	D, NA
MISE Gr4	Dabaisha, Green Island	10	TF, YI	November 2009	NA	NA	NA	NA
MISE Gr5	Dabaisha, Green Island	10	TF, YI	November 2009	NA	NA	NA	NA

COI cytochrome oxidase subunit I DNA, *16S* mitochondrial 16S ribosomal DNA, *ITS rDNA* internal transcribed spacer 2 of ribosomal DNA; *MB* M Boyer, *JDR* JD Reimer, *TF* T Fujii, *YI* Y Irei; *NA* not acquired

(Nishihara, Okinawa, Japan) in 99.5% ethanol at –20°C. Specimens from Taiwan were originally assigned names based on sampling location and order (Table 1).

DNA extraction, PCR amplification, cloning, and sequencing

DNA was extracted from Kenting specimen portions (tentacles and column) weighing 5-20 mg using a spin-column Dneasy Animal Extraction protocol (Qiagen, Santa Clarita, Calif., USA). PCR amplification using the genomic DNA as a template was performed using HotStarTaq DNA polymerase (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Mitochondrial (mt) 16S rDNA was amplified following procedures outlined in Sinniger et al. (2005). Cytochrome oxidase subunit I (COI) DNA was amplified using the zoanthid-specific primer COIZoanF (Reimer et al. 2007a) and general COI primer HCO2198

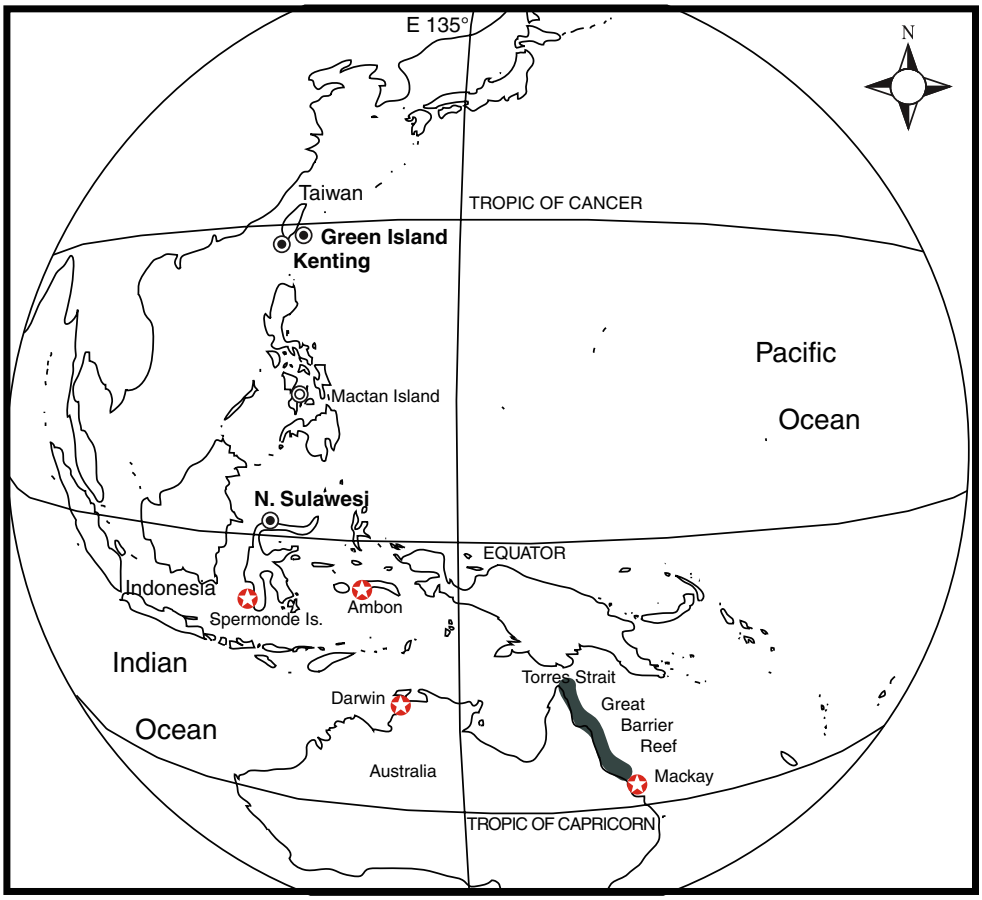

Fig. 1 Locations of historical *Acrozoanthus* records (*red stars*) and specimens examined in this study (*filled target symbols*, *bold location names*). *Empty target symbols* signify locations where distribution has been confirmed but no specimens exist. The original described distribution of *Acrozoanthus australiae* is the *shaded gray area* along the Great Barrier Reef, and around Darwin, Australia

Fig. 2 *Acrozoanthus australiae* in situ at Kenting, Taiwan. Specimen MISE K36, at 10.3 m. *Scale bar* 1 cm

(Folmer et al. 1994). PCR amplification was performed on the samples under the following conditions: an initial denaturing step at 95°C for 15 min, followed by 35 cycles of 1 min denaturing at 94°C, 1 min annealing at 40°C, and 90 s extension at 72°C, followed by 7 min extension at 72°C. The internal transcribed spacer region of the ribosomal DNA (ITS-rDNA) of *Acrozoanthus* was amplified using zoanthid-specific primer ZoanF (Reimer et al. 2007c) and general ITS-rDNA primer pITSr (Sugita et al. 1999). PCR amplification was performed on the samples under the following conditions: an initial denaturing step at 95°C for 15 min, followed by 35 cycles of 1 min denaturing at 94°C, 1 min annealing at 50°C, and 2 min extension at 72°C, followed by 7 min extension at 72°C. The ITS-rDNA region of *Symbiodinium* was amplified following procedures outlined in Reimer et al. (2006c) using primers that amplify all *Symbiodinium* clades. The amplified products were visualized by 1.0% agarose-gel electrophoresis. PCR products were treated with exonuclease I and alkaline phosphatate (Shrimp) (Takara) prior to sequencing reactions using DTCS Quick Start Master Mix (Beckman Coulter). The products were analysed using a CEQ8800 (Beckman Coulter) automated DNA sequencing system.

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank (accession numbers HM171905-HM171922). Nucleotide sequences of mt 16S rDNA, COI and ITS-rDNA from samples were manually aligned with previously published sequences from various Zoanthidae and Neozoanthidae species representing the genera *Zoanthus*, *Acrozoanthus*, *Isaurus*, and *Neozoanthus*, with outgroup *Palythoa* sequences. While mt 16S rDNA and ITS-rDNA (consisting of mainly ITS-2) alignments were easily aligned, some COI sequences for *Isaurus*, *Zoanthus praelongus*, and *Neozoanthus* were shorter than other COI sequences, and consequently, to examine as many different scenarios as possible, three COI alignments of differing taxa and lengths were analyzed. These are designated "COI long" (least taxa, longest alignment), "COI medium" and "COI short" (most taxa, shortest alignment).

For *Symbiodinium* ITS-rDNA sequences, different *Symbiodinium* clade sequences are highly divergent from each other, and thus we only examined clade D sequences with a clade G outgroup (GenBank accession number AJ291537) after initially identifying our new sequences through NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST). This alignment consisted of mainly the second internal ribosomal spacer of ribosomal DNA (ITS-2), which has been shown to have great utility in identifying *Symbiodinium* types (LaJeunesse 2002).

All alignments were inspected by eye and manually edited. All ambiguous sites of the alignments (observed only at the 5' and 3' ends of alignments) were removed from the dataset for phylogenetic analyses. Consequently, six alignment datasets were generated: (1) 655 sites of 16 sequences (mt 16S rDNA); (2) 302 sites of 21 sequences (COI short); (3) 466 sites of 19 sequences (COI medium); (4) 575 sites of nine sequences (COI long); (5) 482 sites of 23 sequences (zoanthid ITS-2); and (6) 345 sites of 15 sequences (*Symbiodinium* ITS-2). The alignment data are available on request from the corresponding author.

For the phylogenetic analyses of the six alignments, the same methods were applied independently. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel 2003) and neighbour-joining (NJ) methods. PhyML was performed using an input tree generated by BIONJ with the general time-reversible model (Rodriguez et al. 1990) of nucleotide substitution incorporating a discrete gamma distribution (eight categories) (GTR + Γ). The discrete gamma distribution, and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (1,000 replicates) were constructed using the same parameters as the individual ML trees. The distances were calculated using Kimura's two-parameter model (Kimura 1980). Support for NJ branches was tested by bootstrap analysis (Felsenstein 1985) of 1,000 replicates. PAUP* Version 4.0 was used for phylogenetic analyses (Swofford 1998). Phylogenetic trees were edited using NJplot v2.3 (Perrière and Gouy 1996).

Bayesian trees were reconstructed by using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under the GTR model for all six alignment data sets. One cold and three heated Markov chain Monte Carlo (MCMC) chains with default-chain temperatures were run for 1 million generations, sampling log-likelihoods (InLs), and trees at 100-generation intervals (20,000 InLs and trees were saved during MCMC). The first 100,000 generations of all runs were discarded as "burn-in" for all datasets except for mt 16S rDNA, in which the first 200,000 generations were discarded. The likelihood plots for datasets also showed

that MCMC reached the stationary phase by these points (COI long, COI medium, COI short, mt 16S rDNA, and *Symbiodinium* ITS-rDNA PSRFs=all 1.002; zoanthid ITS-rDNA PSRF=1.004). Thus, the remaining 9,000 trees (= 900,000 generations) (except 8,000 trees for mt 16S rDNA) were used to obtain posterior probabilities and branch-length estimates, respectively.

Shimodaira-Hasegawa (SH) and approximately unbiased (AU) tests

We assessed the congruity (or incongruity) among the ML trees inferred from COI and mt 16S rDNA alignments. The first alternative tree was generated by modifying the ML tree from COI long alignment along with the ML trees from COI medium and short alignments. As COI long alignment lacks *I. tuberculatus* and *Z. praelongus*, these taxa were excluded from the resultant alternative trees. The analyses of COI medium and short suggest an intimate affinity between *I. tuberculatus* and *Neozoanthus* sp. (Fig. 3b, c). Thus, *Neozoanthus* sp. was treated as the representative of the robust clade of *I. tuberculatus* and *Neozoanthus* sp. in the first alternative tree. Note that the two tree topologies became identical by removing *Z. praelongus* (details shown in Supplementary Materials). We took a similar approach to generate the second alternative tree, which corresponds to the ML tree from mt 16S rDNA alignment. *Z. pulchellus*, a closely relative of *Z. sansibaricus* (Fig. 3d), was excluded from this tree, since the COI long alignment lacks this species. *Neozoanthus* sp. was treated as the replacement of *I. tuberculatus* in the second alternative trees. *P. tuberculosa*, *P. mutuki* and *P. heliodiscus* were tightly grouped together in the analysis of mt 16S rDNA alignment, but the latter two species were absent in COI long alignment. Thus, the second alternative tree considered only *P. tuberculosa* as the representative of the clade of *Palythoa* species.

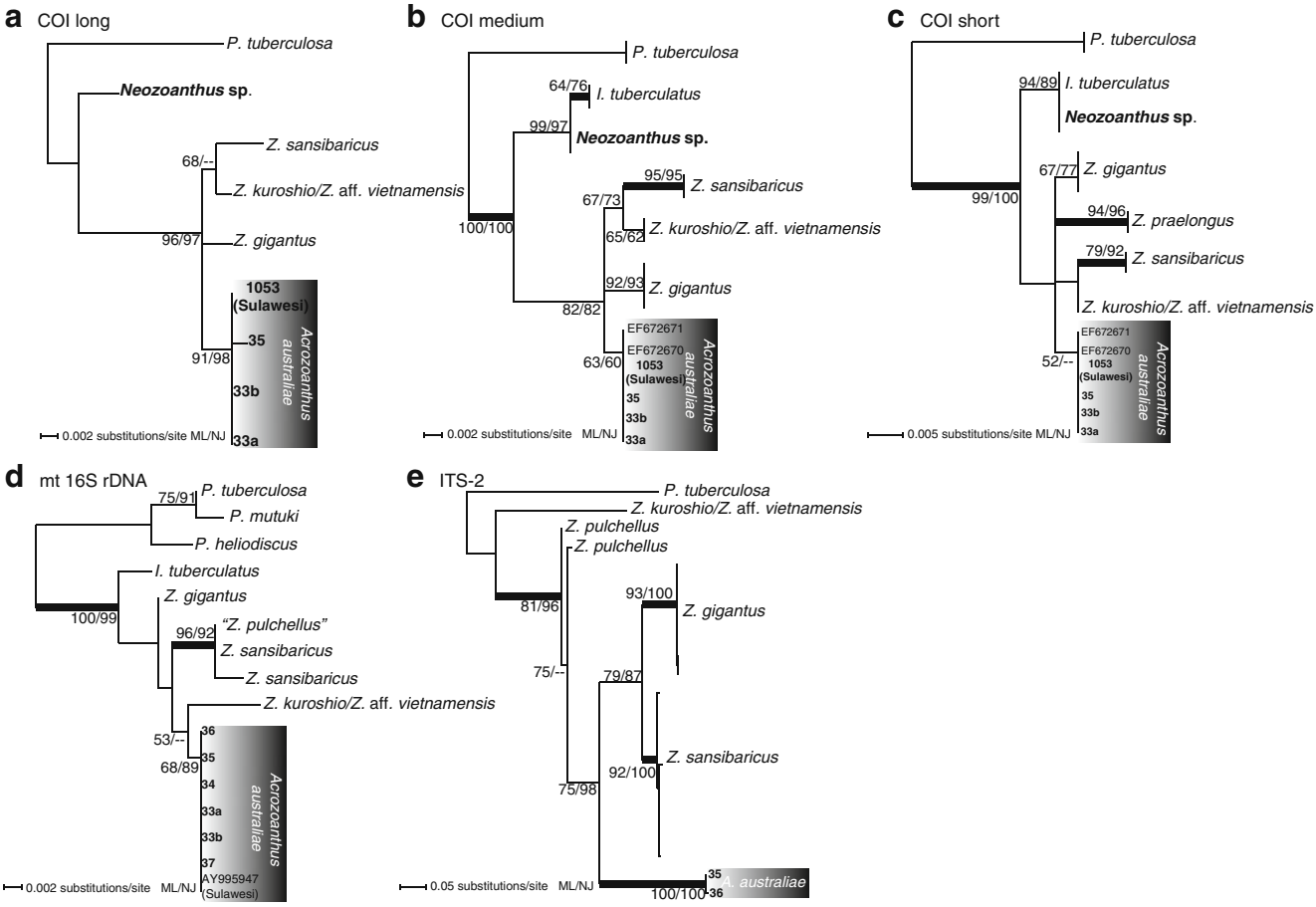

Fig. 3 Maximum likelihood trees of (**a**) "COI long" alignment, (**b**) "COI medium" alignment, **c**) "COI short" alignment, **d**) mt 16S rDNA, and (**e**) ITS-2 sequences for *Acrozoanthus* specimens including specimens from this study. Values at branches represent ML and NJ bootstrap probabilities, respectively. *Thick branches* indicate Bayesian posterior probability values equal or greater than 0.95. New sequences from this study in *bold*

Finally, the branching pattern in the clade of *Acrozoanthus australiae* in the ML tree from COI long alignment and the two alternative trees (described above) were collapsed, since the relationship amongst *A. australiae* isolates was poorly resolved in the ML analysis of COI long alignment (Fig. 3a). These trees were then used for SH and AU tests. For each test tree, site-wise log-likelihoods were calculated by PhyML with the GTR+Γ model based on COI long alignment (model parameters were estimated from the alignment by the ML method). The site-wise log-likelihood data were then subjected to weighted and unweighted SH tests and AU test (Shimodaira and Hasegawa 1999; Shimodaira 2002) implemented in CONSEL v.0.1k (Shimodaira and Hasegawa 2001).

SH and AU tests were repeated by another set of test trees (see below) and mt 16S rDNA alignment. We prepared two alternative trees by modifying the ML tree from the mt 16S rDNA alignment along with those from COI long and COI medium/short alignments. The difference in taxon sampling amongst the alignments used in this study was accommodated as described in the previous paragraph. The details of calculation of site-wise log-likelihoods, and the SH and AU tests were same as described above.

Results

Morphological analyses

From images taken in situ (Fig. 2) and ex situ examinations, Kenting and Green Island specimens were morphologically identified as *Acrozoanthus* using the following four characteristic traits: (1) polyp height 5-10 mm, polyp diameter 2-6 mm, and oral disk diameters of approximately 6 mm, connected by a reduced and stoloniferous coenenchyme, occasionally unitary; (2) approximately 50 tentacles, which were pale yellow-green or pale purple occasionally with fluorescent mint-green markings, black tips, and very long (up to twice oral disk diameter); (3) light purple/brown to white coenenchyme and outer surface of polyps, with oral disks generally pale purple or brown, occasionally with fluorescent mint-green markings; (4) found exclusively on the outside of eunicid worm tubes.

COI

The phylogenetic trees produced from the maximum likelihood analyses of the cytochrome oxidase subunit I (COI) alignments are shown in Fig. 3a-c. As mentioned in the Materials and methods, three different alignments were analysed.

1. "COI long"

The first alignment, "COI long" (Fig. 3a), included three *Zoanthus* spp., *Neozoanthus* sp., and a *Palythoa* outgroup, along with the new putative *Acrozoanthus* sequences. The resulting tree shows *Acrozoanthus* specimens forming a well-supported subclade [maximum likelihood bootstrap support (ML)=91%, neighbour-joining (NJ) = 98%, Bayesian posterior probability (Bayes)=0.94] that was part of a large clade with the three *Zoanthus* species. These four species groups formed a very well supported Zoanthidae sensu stricto clade (ML=96%, NJ=97%, Bayes=1.00), separate from *Neozoanthus* sp (family Neozoanthidae). The log likelihood of the ML phylogenetic tree was -982.395421.

2. "COI medium"

The "COI medium" tree (Fig. 3b) additionally included sequences from *Isaurus tuberculatus*, and previously acquired *Acrozoanthus* sequences from Sinniger et al. (2008). The resulting tree again shows putative *Acrozoanthus* specimens together with previous *Acrozoanthus* sequences as a clade, albeit with lower support (ML= 63%, NJ=60%, Bayes<0.50), which is part of a larger clade containing the three *Zoanthus* species. This large clade is moderately well supported (ML=82%, NJ=82%, Bayes=0.99), and is clearly distinct from a *Neozoanthus*/*Isaurus* clade (ML=99%, NJ=97%, Bayes=0.91). Overall, the two clades form one large, fully supported "Zoanthidae" clade (Zoanthidae sensu stricto+*Neozoanthus*, currently within its own family, Neozoanthidae) (ML=100%, NJ= 100%, Bayes=1.00). The log likelihood of the ML phylogenetic tree was -795.543525.

3. "COI short"

The "COI short" tree (Fig. 3c) further includes sequences from *Zoanthus praelongus* (Reimer et al. 2008b). Once again, *Acrozoanthus* forms a poorly supported clade (ML= 52%, NJ<50%, Bayes<0.50), part of a larger clade including all four *Zoanthus* species (ML<50%, NJ<50%, Bayes<0.50). This clade, together with a well-supported *Neozoanthus*/*Isaurus* clade (ML=94%, NJ=89%, Bayes= 0.95), forms a very well supported "Zoanthidae" clade (ML= 99%, NJ=100%, Bayes=1.00), despite the fact that *Neozoanthus* is currently placed within its own family, Neozoanthidae. The log likelihood of the ML phylogenetic tree was -524.555290.

mt 16S rDNA

The phylogenetic tree resulting from the analyses of mt 16S rDNA sequences is shown in Fig. 3d. Putative *Acrozoanthus* specimens from Taiwan, together with a specimen

from Sulawesi, formed a moderately supported sister clade within the *Zoanthus* group (ML=68%, NJ=89%, Bayes< 0.50) (Fig. 3d). Using this DNA region, the *Acrozoanthus* specimens were placed within a group with *Zoanthus kuroshio/Zoanthus* aff. *vietnamensis*, albeit with low bootstrap support (ML=53%, NJ<50%, Bayes<0.50). This group was contained within a larger *Zoanthus*/*Isaurus* (= Zoanthidae sensu stricto) clade (ML=100%, NJ=99%, Bayes=1.00). Support within this large clade was generally low for most nodes. The log likelihood of the ML phylogenetic tree was -1141.875285.

ITS-2

The ML tree resulting from the phylogenetic analyses of ITS-2 is shown in Fig. 3e. Only two sequences were obtained from putative *Acrozoanthus* specimens from Taiwan, which formed a totally supported sister clade (ML=100%, NJ=100%, Bayes=1.00) to a *Zoanthus gigantus*/*Zoanthus sansibaricus* clade (ML=79%, NJ= 87%, Bayes=0.94). Additionally, a shorter (240 bp) sequence (AB517555; Aguilar and Reimer 2010) from a *Acrozoanthus* from Sulawesi in previous study also matched closely (238/240 bp; = 99% similarity) with these two *Acrozoanthus* sequences, but was not included in the alignment as it was much shorter than other sequences. The *Acrozoanthus*/*Z. gigantus*/*Z. sansibaricus* clade (ML=75%, NJ=98%, Bayes=0.97) was derived from *Zoanthus pulchellus* and *Zoanthus* aff. *vietnamensis* (=closely related to *Z. kuroshio*). The log likelihood of the ML phylogenetic tree was -1770.932599.

Shimodaira-Hasegawa (SH) and approximately unbiased (AU) tests

The results of the topology tests are given in Supplementary Table S1. Overall, two topologies, COI long and COI medium/short, were dismissed in the mt 16S rDNA based AU test (significance level=<0.05), although they were not dismissed in the SH and weighted Shimodaira-Hasegawa (WSH) tests (Table S1). From these results, it can be inferred that the mt 16S rDNA phylogeny showed better resolution than the COI long alignment's ML phylogeny.

Symbiodinium ITS-2

Results of the phylogenetic analyses of ITS-2 sequences of *Symbiodinum* isolated from putative *Acrozoanthus* specimens from Kenting are shown in Fig. S1. Resulting ITS-2 sequence data were clear in both forward and reverse directions with no "double-peaks", and thus no cloning was performed. All putative *Acrozoanthus* specimens were shown to have clade D1a *Symbiodinium trenchii* LaJeunesse et al. (2010), 100% identical to the previously reported sequences AB294667 and AF396631. Together with five other sequences, including EU333714 from *Palythoa mutuki* in Singapore, these sequences formed a very well supported subclade (ML=88%, NJ= 100%, Bayes=0.88)), different from potentially free-living clade D types from Ogasawara (described in Reimer et al. 2010).

Discussion

Are these new specimens in fact *Acrozoanthus* species? If so, are they conspecific to *A. australiae*?

The observed and collected specimens in this study are unequivocally *Acrozoanthus*, and very likely belong to the species *Acrozoanthus australiae*, based on combined morphological, ecological, and phylogenetic (mt 16S rDNA, COI, ITS-2) data. In particular, acquired sequences for all three zoanthid DNA markers were identical to sequences from *Acrozoanthus australiae* specimen 1053 (utilized in Sinniger et al. 2005; 2008) from Indonesia. As *Acrozoanthus* is monospecific, divergence rates of ITS-rDNA for different species within this genus are incalculable. However, it has been shown that ITS-rDNA evolves at a relatively fast rate in *Zoanthus* spp. (Reimer et al. 2007c) and perhaps in the suborder Brachycnemina when compared with Macrocnemina zoanthid species (Sinniger et al. 2010), and thus the ITS-rDNA results in this study show with very high probability that the Taiwan specimens are *A. australiae*. In addition, specimens were very similar in external appearance (coloration, tentacles approximately 50 in number) to *A. australiae* as described in Ryland (1997) and Saville-Kent (1893). Specimens were found exclusively on eunicid worm tubes, as previously noted in the literature (Haddon 1895; Ryland 1997). The only other zoanthid known to exclusively associate with eunicid worm tubes is *Epizoanthus illoricatus*, which is quite different morphologically (encrusted) and phylogenetically, within the family Epizoanthidae.

The discovery of *Acrozoanthus* in Taiwan represents a major and surprising range extension for this species, as the literature previously mentioned this species from only Australia (Saville-Kent 1893; Ryland 1997) and Indonesia (Sinniger et al. 2005). It should be noted, however, that the presence of *Acrozoanthus* (no specimens) has been confirmed from Mactan Island in the Philippines (P. Poppe, pers. comm.), and it may be that this species is much more widely distributed than previously thought. As noted by Ryland (1997), *A. australiae* inhabits somewhat muddy shores not regularly investigated by "coral reef" researchers, and this may contribute to the overall paucity of data on this

Table 2 Genetic differences between *Acrozoanthus* and other brachycnemic zoanthid taxa

Comparison	COI	mt 16S DNA	Relationship	Reference
Acrozoanthus – Zoanthus sansibaricus	1.7% (5/302)	1.1% (7/655)	Intrafamily. intergeneric?	This study
Acrozoanthus – Zoanthus gigantus	1.0% (3/302)	0.6% (4/655)	Intrafamily. intergeneric?	This study
Acrozoanthus – Zoanthus kuroshio	1.0% (3/302)	1.1% (7/655)	Intrafamily. intergeneric?	This study
Acrozoanthus – Zoanthus praelongus	1.3% (4/302)	NA	Intrafamily. intergeneric?	This study
Acrozoanthus – Isaurus tuberculatus	1.7% (5/302)	1.2% (8/655)	Intrafamily. intergeneric?	This study
Zoanthus sansibaricus – Zoanthus gigantus	0.7%	1.1%	Intrageneric	Reimer et al. 2008b
Zoanthus sansibaricus – Zoanthus kuroshio	1.3%	0.8%	Intrageneric	Reimer et al. 2008b
Zoanthus kuroshio – Zoanthus vietnamensis	0.0%	0.1%	Intrageneric	Reimer et al. 2008b
Palythoa heliodiscus – Palythoa tuberculosa	0.9%	1.1%	Intrageneric	Reimer et al. 2008b
Palythoa tuberculosa – Palythoa mutuki	0.0-0.2%	0.1-0.2%	Intrageneric	Reimer et al. 2008b
Acrozoanthus – P. tuberculosa	10/302=3.3%	17/655=2.6%	Interfamily	This study

species. Additionally, based on results of this study to 12 m and the sample in Sinniger et al (2005) from 9 m, it appears that *A. australiae* is not strictly limited to mud flats, but is found in areas of low light and/or heavy sedimentation where eunicid worms are present. Finally, the specimens in this study were discovered inadvertently while examining the reefs of southern Taiwan for other brachycnemic zoanthid species. Future research into this unique species should focus on examining the locations of habitats of eunicid worms.

Does *Acrozoanthus* belong in a genus separate from *Zoanthus*?

The results of our phylogenetic analyses were inconclusive regarding the location of *Acrozoanthus* within Zoanthidae, although the family Zoanthidae was generally very well supported. Thus, while it is very clear that *Acrozoanthus* belongs within Zoanthidae, each DNA marker showed *Acrozoanthus* with a different phylogenetic position within the family (Fig. 3). The three COI trees showed *Acrozoanthus* as a subclade within *Zoanthus*, albeit with generally low bootstrap support (Figs. 3a-c). The other mitochondrial marker, mt 16S rDNA, which has thus far proven to be quite reliable in examining relationships between many zoanthid species (Sinniger et al. 2008), also showed *Acrozoanthus* well within *Zoanthus*, sister group to *Zoanthus kuroshio/Zoanthus* aff. *vietnamensis*, again with low support (Fig. 3d). Finally, nuclear ITS-rDNA placed *Acrozoanthus* as derived from *Zoanthus pulchellus*, and *Zoanthus kuroshio/Zoanthus* aff. *vietnamensis*, and basal to *Zoanthus gigantus* and *Z. sansibaricus* (Fig. 3e). Our topology test results showed only few significant differences between COI and mt 16S rDNA topologies (Table S1) demonstrating the generally superior phylogenetic resolution of the mt 16S rDNA alignment, further supporting the placement of *Acrozoanthus* within *Zoanthus*.

One explanation is that *Zoanthus* is actually composed of multiple genera, which would then make *Acrozoanthus* a potential sister taxon to one of the genera. However, this possibility is deemed unlikely at best given the phylogenetic monophyly of *Zoanthus* and rates of divergence of mitochondrial DNA in zoanthids (see below) combined with the abundance of morphologic and ecologic similarities between species in this group, such as lack of encrustation and presence of endosymbiotic *Symbiodinium*.

From these varied results, the phylogenetic position of *Acrozoanthus* cannot be clearly determined, although it appears that this genus does in fact belong within *Zoanthus*. However, it is obvious that additional data are needed due to the low bootstrap support for relations within Zoanthidae phylogenies. One option would be to find a DNA marker that evolves at a faster rate than anthozoan mitochondrial DNA (which is known to occur at very slow rates (Shearer et al. 2002; Huang et al. 2008)), yet slower than ITS-rDNA. ITS-rDNA is so variable in many brachycnemic zoanthids that often alignment becomes difficult (Reimer et al. 2007c), suggesting the "true" phylogenetic signal may be saturated as seen in other anthozoans (e.g. Aguilar and Sánchez 2007). Generally, the mt 16S rDNA and COI results present a clearer phylogenetic signal at higher than species levels (e.g. genera and higher) than ITS-rDNA in zoanthid phylogeny for the reasons stated above, and thus the results point to *Acrozoanthus* belonging to *Zoanthus*. Furthermore, regardless of the differing observed topologies inside Zoanthidae, all three trees with the highest log likelihoods (COI long, mt 16S rDNA, ITS-rDNA) all placed *Acrozoanthus* inside Zoanthidae.

Additionally, we examined the overall sequence differences between *Acrozoanthus*, *Zoanthus*, and *Isaurus* species to see if these values are comparable with those seen between other zoanthid groups. It has been suggested that congeneric species within Macrocnemina have COI sequence differences of 0.0% to approximately 1.0%, while

species belonging to the same family but different genera have 1.0–4.0% sequence differences, and levels above 4.0% signify species from different families (Sinniger et al. 2010). The differences in COI and mt 16S rDNA sequences between *Acrozoanthus* and *Zoanthus*, *Isaurus*, and *Palythoa* (0.6-1.7%) suggest that the amounts of sequence divergence between *Acrozoanthus* and other taxa are in the range differentiating congeneric species or genera (Table 2). Most *Zoanthus* congeners (0.0-1.3%) and some *Palythoa* species (0.0-1.1%) have similar levels of differences between each other (Table 2 in Reimer et al. 2008b).

For now, despite its ambiguous position within Zoanthidae and potential placement within *Zoanthus*, due to its unique ecology and other characteristics, as mentioned in Ryland (1997), we do not formally merge the genus *Acrozoanthus* into *Zoanthus*, with the caveat that this will likely occur when more molecular data (with more robust bootstrap support) become available. Future research with more molecular data should help ascertain the true evolutionary history and phylogenetic position of *Acrozoanthus*.

What types of *Symbiodinium* do Taiwan specimens possess?

All examined specimens of *Acrozoanthus* from Kenting, Taiwan were shown to possess clade D1a *Symbiodinium* (= *S. trenchii*). It may be that Green Island specimens possess different *Symbiodinium* zooxanthellae, as previously some flexibility has been observed in zooxanthellae associations in *Zoanthus sansibaricus* (Reimer et al. 2006c), *Palythoa caesia* (Burnett 2002), and *P. tuberculosa* (Reimer and Todd 2009), this remains to be investigated. Clade D has previously been hypothesized to be adapted to more "stressful" conditions, such as unusually cold (e.g. <18°C) or hot (>30°C) marine environments (Chen et al. 2003), or warm, turbid locations (LaJeunesse et al. 2010). In zoanthids, clade D has not been previously reported from Zoanthidae species, but is known from *Palythoa* in the Indian Ocean (Fig. S1, also Burnett 2002; Reimer and Todd 2009). The marine environment where *Acrozoanthus* was found in Taiwan is not a particularly "cold" (<18°C) or "hot" (>30°C) location, as it is usually influenced by the warm Kuroshio Current. However, the location of *Acrozoanthus* colonies on eunicid worm tubes on the undersides of large *Porites* colonies is likely to have relatively low light levels, especially as most colonies of *Acrozoanthus* were found to be completely shaded by the massive coral colonies above them. Likewise, a similar strain of clade D has been found in *Palythoa tuberculosa* and *P. mutuki* colonies in turbid locations in Singapore (Reimer and Todd 2009). Thus, our results support that clade D *Symbiodinium* zooxanthellae are adapted to not only hot or cold environments, but to generally low light conditions as well (Ulstrup and Van Oppen 2003; Fabricius et al. 2004).

Future zoanthid research

This research emphasizes how little is known of many zoanthid species' distribution. In particular, the Coral Triangle (Hoeksema 2007) is well known as the area of highest scleractinian (hard coral) diversity, and as a sister order within the subclass Hexacorallia, it may be that zoanthids also have very high diversity in this region. Specifically, zooxanthellate zoanthids of the families Zoanthidae, Sphenopidae, and Neozoanthidae are ecologically similar in many ways to hermatypic corals, and future investigations should not neglect to examine their diversity in the Coral Triangle. As most zoanthids from coral reefs have been described from various locations in the northern or southern hemisphere without much examination of the equatorial Coral Triangle region, there exists the possibility of synonyms as mentioned in previous studies (Burnett et al. 1997; Reimer et al. 2004). Thus, a detailed investigation into zoanthid species diversity in the Coral Triangle may help to clear up taxonomic confusion.

Additionally, as brachycnemic zooxanthellate zoanthids have evolved a wide variety of lifestyles and strategies, they would make excellent subjects from an evolutionary ecology point of view. Clarifying their complex phylogeny will allow this next step in analyses to begin.

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