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PRELIMINARY MOLECULAR EXAMINATION OF ZOOXANTHELLATE ZOANTHIDS (HEXACORALLIA: ZOANTHARIA) AND ASSOCIATED ZOOXANTHELLAE (*SYMBIODINIUM* SPP.) DIVERSITY IN SINGAPORE

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ABSTRACT. **–** Singapore is located at the southern tip of the Malaysian peninsula between the Indian and Pacific Oceans. Despite being in such an important biogeographical location, many groups of marine invertebrates from this area remain poorly understood. One such group is the zoanthids (Order Zoantharia, = Zoanthidea, Zoanthiniaria), hexacorallians known from almost all marine environments but historically taxonomically neglected. In this study we examined the species diversity of zoanthids from the genera *Zoanthus* and *Palythoa*, as well as their associated symbiotic dinoflagellates (*Symbiodinium* spp.), for the first time in Singapore. After specimen collection (n = 44) and preliminary identification using traditional morphological characters (oral disk color and polyp shape, etc.) we used two host DNA markers, mitochondrial 16S ribosomal DNA (mt 16S rDNA) and cytochrome oxidase subunit I (COI), to confirm all collected specimen identities (to species or species group level). Results show the presence of five putative species of zoanthids [*Z. sansibaricus* (n=11), *Z. vietnamensis* (n = 17), *P. tuberculosa* (n = 13), *P. mutuki* or closely related (n = 2), and a potential new species designated *P*. sp. "singapura" (n = 1)] in Singapore. *Symbiodinium* from these zoanthids generally followed patterns previously seen in Japan; *Z. sansibaricus* and *P*. sp. "singapura" possessing C1/C3-derived *Symbiodinium*, *Z. vietnamensis* with C15/C91-derived *Symbiodinium*, and *P. mutuki* with generalist C1/C3 *Symbiodinium*. However, nine of 12 *P. tuberculosa* examined, as well as one *P. mutuki* possessed clade D *Symbiodinium*, were previously hypothesized to be tolerant to extreme hot or cold temperatures. Our results demonstrate the necessity of further worldwide zoanthid sampling in order to properly understand the distribution and diversity of zooxanthellate zoanthids and their *Symbiodinium*.

KEY WORDS. **–** Mitochondrial 16S ribosomal DNA, cytochrome oxidase subunit I, *Zoanthus*, *Palythoa*, Anthozoa.

INTRODUCTION

The order Zoantharia (= Zoanthidea, Zoanthiniaria) is one of the most taxonomically neglected and least examined orders of Cnidaria, despite a worldwide distribution in marine environments. Zooxanthellate zoanthids from the genera *Palythoa* (family Sphenopidae) and *Zoanthus* (Zoanthidae) are particularly common in tropical and subtropical shallow waters. Confusion surrounding the taxonomy and species diversity in these two genera is largely attributable to high morphological variability (i.e. polyp shape and size, oral disk color, etc.) within species (see Burnett et al., 1997; Reimer et al., 2006a), the lack of accurate morphological markers to properly discern species, and the paucity of accurate species descriptions in past literature. Additionally, most zoanthids (asides from species in Zoanthidae) incorporate sand and other detritus from their surrounding environment into their tissues to help enhance their structure, making sectioning and internal morphological examination for taxonomic purposes extremely difficult.

Recent molecular examinations utilizing mitochondrial 16S ribosomal DNA (mt 16S rDNA), mitochondrial cytochrome oxidase subunit I (COI) and the internal transcribed spacer of ribosomal DNA (ITS-rDNA) data from *Zoanthus* and *Palythoa* spp. specimens, combined with the more

traditional morphological methods, have begun to bring taxonomic order to these two genera (Reimer et al., 2004, 2006b, 2006c, 2007c, 2007d). The same molecular markers have also successfully been utilized in examining other zoanthid taxa (Sinniger et al., 2005; Reimer et al., 2007a). While the evolutionary rates between COI and mt 16S rDNA in zoanthids can be slightly different (Reimer et al. 2008), the phylogenetic tree topology resulting from data sets of both markers are generally congruent (e.g. see Reimer et al., 2007a).

Similar to many other coral reef-inhabiting invertebrates, both *Zoanthus* and *Palythoa* spp. possess symbiotic dinoflagellates (order Sussiales) of the genus *Symbiodinium* (zooxanthellae). Despite morphological similarity, various molecular markers (see Rowan & Powers, 1991a, 1991b; Baker, 1999; Santos et al., 2002; Takishita et al., 2003), including the internal transcribed spacer region of ribosomal DNA (ITS-rDNA) (Hunter et al., 1997; Loh et al., 1998; LaJeunesse, 2001, 2002), have identified at least eight major "clades" and numerous "types" of *Symbiodinium* (Pochon et al., 2004, 2006). Additional studies utilizing ITS-rDNA from symbiotic zooxanthellae (*Symbiodinium* spp.) from *Zoanthus* and *Palythoa* in Japan have shown various levels of symbiont specificity and flexibility among zoanthid species (Reimer et al., 2006d, 2006e, 2007b). As different *Symbiodinium* types potentially have different physiologies (Tchernov et al., 2004), the characterization of these types within zoanthids can help us understand the ecology and biogeography of the holobiont (host + associated symbionts).

While zoanthids are commonly known to exist in the waters surrounding Singapore (e.g. Vohra, 1971), and despite a long history of marine biology-related research in the area, only a few published scientific records of any zoanthid exist for Singapore, and these refer to only one *Palythoa* species. This species was identified as *Gemmaria variabilis* Duerden 1898 by Heider (1899) and later redescribed as *Protopalythoa singaporensis* Pax & Muller 1956. Both of these were later synonymized with *Pr. mutuki* Haddon & Shackleton 1891 by Ryland & Lancaster (2003). Molecular research has shown that it is likely that the genus *Protopalythoa* is within *Palythoa* (Burnett et al. 1997; Reimer et al. 2006c, 2007c), and here we use the name *P. mutuki* to indicate this species. In fact, only two other records of *Palythoa* spp., both from southern Sumatra (Pax, 1924) have been noted previously for this area of South East Asia, and there are no records for *Zoanthus*.

We used both molecular and morphological methodologies to examine the species diversity of zooxanthellate zoanthids and their zooxanthellae in Singapore for the first time. Upon collection, zoanthid specimens were assigned tentative species identifications based solely on morphology. Further examinations of mt 16S rDNA, COI, as well as ITS-rDNA from *Symbiodinium*, allowed us to: 1) identify zoanthid specimens to the species or species group level and examine the accuracy of initial morphological identifications, and; 2) compare *Symbiodinium* types in zoanthids from Singapore with types from other locations.

MATERIAL AND METHODS

***Specimen collection.* –** Forty-four zooxanthellate zoanthid specimens (in this study specimens = colonies) representing all observed zoanthid morphotypes were collected by snorkeling or SCUBA from three locations in Singapore (Raffles Lighthouse, Lazarus Island and Kusu Island; Fig. 1) in November and December 2006 (Table 1, Table 2). Specimens were preliminarily identified using morphological characteristics used in past literature e.g. oral disk color, polyp form, etc. (Table 1, Table 2, Fig. 2) based on in situ photographs (taken with an Olympus™ C745 digital camera in an underwater housing), ex situ photographs and ex situ physical examination. Specimens were subsequently stored in 75% ethanol at ambient temperature. All samples were finally deposited at the University of the Ryukyus (Nishihara, Okinawa, Japan) in 99.5% ethanol at −20°C.

***Specimen nomenclature.* –** Specimens were assigned names based on sampling location and an assigned sampling number (Table 2). Thus, sample RL1 is sample 1 from Raffles Lighthouse, K1 sample 1 from Kusu Island, and L1 sample 1 from Lazarus Island.

***DNA extraction, PCR amplification, cloning, and sequencing.* –** DNA was extracted from specimen portions (tentacles and column) weighing 5–20 mg using a spin-column Dneasy Animal Extraction protocol (Qiagen, Santa Clarita, CA, 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). COI was amplified following procedures outlined in Reimer et al. (2004). The ITS-rDNA region of *Symbiodinium* was amplified following procedures outlined in Reimer et al. (2006e). The amplified products were visualized by 1.5 % agarose gel electrophoresis.

***Phylogenetic analyses.* –** New sequences obtained in the present study were deposited in GenBank (accession numbers EU333653-EU333743). By using CLUSTAL X version 1.8 (Thompson et al., 1997), the nucleotide sequences of mt 16S rDNA and COI from samples were aligned with previously published mt 16S rDNA and COI sequences (Table 3) from various zoanthid species representing the genera *Palythoa*, *Zoanthus*, *Isaurus* and *Acrozoanthus*. The outgroup sequences for both mt 16S rDNA and COI trees were from the genus *Parazoanthus*.
For *Symbiodinium* ITS-rDNA sequences, different *Symbiodinium* clade sequences are highly divergent from each other, making interclade alignments of ITS-rDNA very difficult, and therefore two different alignments were created. The first alignment consisted solely of clade C *Symbiodinium*, with newly acquired sequences from this study along with other representative C sequences (Table 4). The second alignment consisted of clade D newly

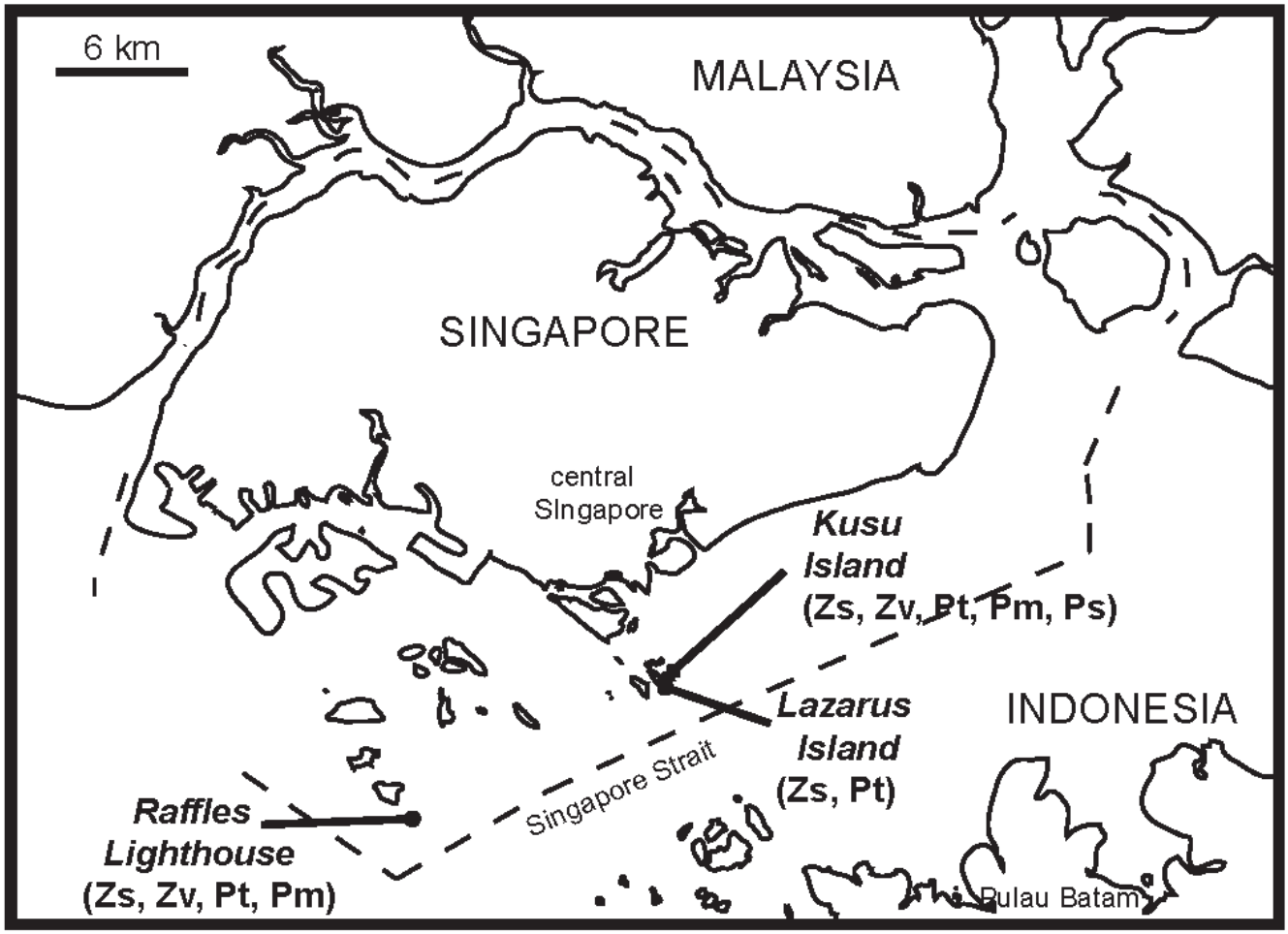

Fig. 1. Singapore and the position of the three sampling locations with zooxanthellate zoanthid species found at each location. Abbreviations: Zs, *Zoanthus sansibaricus*; Zv, *Z. vietnamensis*; Pt, *Palythoa tuberculosa*; Pm, *P. mutuki*-related; Ps, *P*. sp. "singapura".

acquired sequences from this study and other representative D sequences (Table 4).

All alignments were inspected by eye and manually edited. All ambiguous sites of the alignments were removed from the dataset for phylogenetic analyses. Consequently, four alignment datasets were generated: 1) 756 sites of 22 sequences (mt 16S rDNA); 2) 297 sites of 44 sequences (COI); 3) 616 sites of 35 sequences (*Symbiodinium* clade C ITS-rDNA); and 4) 646 sites of 15 sequences (*Symbiodinium* clade D ITS-rDNA). The alignment data are available on request from the corresponding author.

For the phylogenetic analyses of the four alignments, the same methods were applied independently. Alignments were subjected to analyses with the maximum-likelihood (ML) with PhyML (Guindon & 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 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 tree. The distances were calculated using a Kimura's 2-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).

RESULTS

In situ morphological zoanthid identification. – Preliminary identifications of zoanthids were made in situ based on morphological data collated from Japanese zoanthids and other previous literature (see Table 1). While identifications to the generic level (*Zoanthus* or *Palythoa*) could be made with confidence, species level assignments often proved difficult. In particular, *Zoanthus* spp. were problematic due to members of this genus exhibiting high degrees of color and polyp shape variation (see Reimer et al., 2004, 2006a). Nevertheless, preliminary species-level identifications were made for 35 of 44 (= 80%) specimens collected (Table 1), representing four species (*Z. sansibaricus* Carlgren, 1900, *Z. vietnamensis* Pax & Muller, 1956, *P. tuberculosa* Klunzinger, 1877, and *P. mutuki* Haddon & Shackleton, 1891). The remaining nine specimens (all *Zoanthus* spp.)

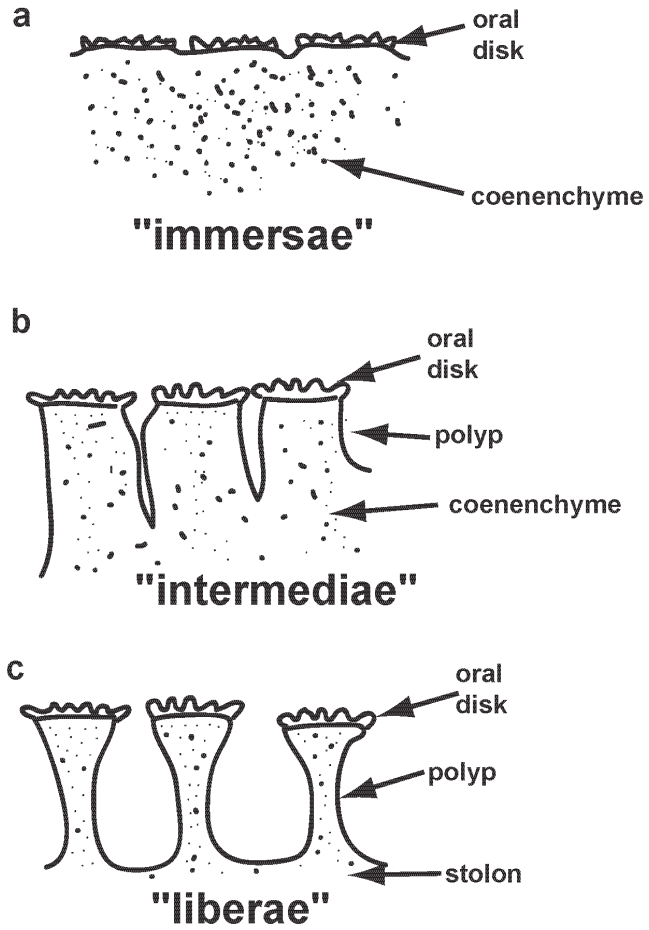

Fig. 2. Diagram of colony and polyp structure forms of zoanthids: a, "immersae" form, with polyps deeply embedded in a well-developed coenenchyme; b, "intermediae" form, intermediate in form, usually with well-developed, thick polyps; c, "liberae" form, with free-standing polyps extending well above a thin coenenchyme (stolons), often with comparatively much space between oral disks and polyps. This figure originally in Reimer et al. (2006c); adapted from Pax (1910) and Fossa & Nilsen (1998).

appeared to be the same species, but were morphologically unlike any *Zoanthus* previously examined by the authors or described in previous literature, with large (~ 10mm diameter) light mint green oral disks and tentacles—similar to *Z. vietnamensis* sensu Burnett et al. (1997)—but with 'liberae' polyps (see Pax, 1910) and no conspicuous markings on the outside of the smooth polyp surface (Tables 1, 2).

***DNA sequence-based zoanthid identification.* –** Unlike the morphological preliminary identifications, DNA sequence results showed that the collected specimens represented five species of zooxanthellate zoanthids; *Zoanthus sansibaricus* (n = 11), *Zoanthus vietnamensis* (n = 17), *Palythoa tuberculosa* (n = 13), *Palythoa mutuki* or closely related (n = 2), and a potentially undescribed *Palythoa* species (n = 1) closely related to *Palythoa heliodiscus* (Ryland & Lancaster, 2003) that had been originally placed with *P. mutuki* specimens (Tables 1, 2). Based on DNA sequences, the nine *Zoanthus* specimens that were not identified morphologically all belonged to the *Z. vietnamensis* clade.

***mt 16S rDNA results.* –** As shown in Fig. 3, based on mt 16S rDNA, all collected zoanthid specimens belonged to a described zoanthid species clade with very high probability (maximum likelihood [ML] = 95–100%, neighbor-joining [NJ] = 98–100%). Although specimens K2, L4, and L1 had small differences (2–4 base pairs) in acquired mt 16S rDNA sequences, all fitted within the *P. tuberculosa/P. mutuki* monophyly. As these specimens also had morphology ("immersae" polyps) consistent with *P. tuberculosa,* they were thus identified as *P. tuberculosa*.

***COI results.* –** Similar to the mt 16S rDNA results, COI sequences and the resulting tree (Fig. 4) showed that putative *Z. sansibaricus*, *Z. vietnamensis*, and *P. heliodiscus*-related specimens clustered with high support (ML = 80–98%, NJ = 83–96%) with known zoanthid species. Putative *P. tuberculosa* specimens all formed a monophyletic clade, although support was low (ML = 57%, NJ = 58%). Although COI sequences for our *P. mutuki*-related specimens (n = 2) did not match exactly with *P. mutuki* (differing by 1 base pair), morphology and habitat closely matched with *P. mutuki*, and we have designated these two specimens as *P. mutuki*-related. Additionally, specimen K12 had a slightly different mt 16S rDNA sequence (by 1 base pair) than *P. heliodiscus,* and also different morphology and habitat and was therefore judged to be a potential new species (designated *P.* sp. "singapura" hereafter).

Symbiodinium ITS-rDNA. – The *Symbiodinium* types associated with the zoanthid specimens based on acquired ITS-rDNA sequences are shown by sampling location in Tables 2 and 5. Each of the zoanthid colonies associated with only a single type of *Symbiodinium*, with the possible exception of two *P. tuberculosa* specimens which had "mixed" unclear ITS-rDNA sequences (e.g. multiple peak signals) suggesting two or more types of *Symbiodinium* may have been present. More than one (n = 2–6) attempt at amplification was tried unsuccessfully on such specimens.

All *Z. sansibaricus* specimens had only C1/C3-derived *Symbiodinium* (n = 10) (Fig. 5). All *Z. vietnamensis* specimens similarly had C15/91-derived *Symbiodinium* (n = 16) (Fig. 5).

The potentially undescribed *Palythoa* specimen contained *Z. sansibaricus*-specific C1/C3-derived *Symbiodinium* (Fig. 5), although the obtained ITS-rDNA sequence was substantially shorter than other acquired sequences and was not included in the final clade C ITS-rDNA alignment. One *P. mutuki*-related colony possessed "generalist" C1/C3 *Symbiodinium* (Fig. 5), while the other *P. mutuki*-related colony possessed clade D *Symbiodinium* (Fig. 6). *Palythoa tuberculosa* colonies possessed either "generalist" C1/C3 *Symbiodinium* (n = 1) (Fig. 5) or *Symbiodinium* from clade D (n = 9) (Fig. 6).

Table 1. Summary of morphological characteristics of collected zooxanthellate zoanthids (*Zoanthus* spp. and *Palythoa* spp.) from Singapore (Nov.–Dec.2006) with preliminary morphological identification and references.

Group (n)	Sand present in mesoglea?	Outer surface of polyp color	Observed oral disk colors and notes	Oral disk diameter (mm)[a]	Polyp form[b]	Tentacle number, color	Preliminary morphological identification with authority	Recent morphological reference(s) with similar descriptions
Ia (8)	No	Light to dark purple	Light to dark pink; septae visible	8–14	Liberae	46–54, light green, gray	*Zoanthus vietnamensis* Pax & Muller, 1956	Uchida 2001; Reimer et al., 2006a
Ib (9)	No	Light to dark purple	Light "mint" green; septae visible	4–14	Liberae	48–52, light green	Unknown *Zoanthus*	Similar in color only to *Z. vietnamensis* sensu Burnett et al., 1997
II (11)	No	Dark purple	Wide variety; red, blue, white, light to dark green, orange, brown, gray; septae not visible	4–8	Liberae	48–60, often brown or dark green	*Zoanthus sansibaricus* Carlgren, 1900 or *Z. coppingeri* Haddon & Shackleton, 1891	Reimer et al. 2006b; Burnett et al., 1997, respectively
III (13)	Yes	Light to dark brown, occasionally fluor. yellow	Brown, tan, cream; septae visible	6–16	Intermediae to immersae	38–52, white, tan, or brown	*Palythoa tuberculosa* Klunzinger, 1877, or *P. caesia* Dana, 1846	Reimer et al., 2006c; Burnett et al., 1997, respectively
IVa (2)	Yes	Brown	Fluorescent light to dark green; septae visible	10–18	Liberae	60–74, white, tan, or brown	*Palythoa mutuki* Haddon & Shackleton, 1891	Ryland & Lancaster, 2003; Reimer et al., 2006c
IVb (1)	Yes	Dark brown	Dark brown, septae barely visible	10–20	Liberae	Approx. 80, gray-blue	*Palythoa mutuki*? Haddon & Shackleton, 1891	Ryland & Lancaster, 2003; Reimer et al., 2006c

[a] = For expanded polyps
[b] = Polyp shape as defined by Pax (1910); see Fig. 2.

Table 2. Zooxanthellate zoanthid specimens (*Zoanthus* spp. and *Palythoa* spp.) collected from Singapore (Nov.–Dec.2006), their preliminary morphological identification, DNA sequences and species identity conclusions.

Sample number	Preliminary morphological identification (see Table 1)	COI sequence identity	Mt 16S rDNA sequence identity	*Symbiodinium* ITS-rDNA sequence identity	Taxonomic Conclusion (species group)
RL1	*P. tuberculosa*	*P. tuberculosa*	NA	D	*P. tuberculosa*
RL3	*P. tuberculosa*	*P. tuberculosa*	NA	D	*P. tuberculosa*
RL4	*P. tuberculosa*	*P. tuberculosa*	NA	D	*P. tuberculosa*
RL6	Unknown *Zoanthus*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RL8	*P. tuberculosa*	*P. tuberculosa*	NA	D	*P. tuberculosa*
RL9	*P. tuberculosa*	*P. tuberculosa*	NA	D	*P. tuberculosa*
RL10	*P. tuberculosa*	*P. tuberculosa*	NA	D	*P. tuberculosa*
RL11	*Z. sansibaricus*	NA	NA	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
RL12	*P. tuberculosa*	*P. tuberculosa*	NA	D	*P. tuberculosa*
RL14	*Z. sansibaricus*	*Z. sansibaricus*	NA	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
RL15	Unknown *Zoanthus*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RL16	*Z. vietnamensis*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RL17	*Z. sansibaricus*	*Z. sansibaricus*	NA	NA	*Z. sansibaricus*
RL18	Unknown *Zoanthus*	*Z. sansibaricus*	NA	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
RL19	Unknown *Zoanthus*	*Z. vietnamensis*	NA	*Zoanthus*-specific C1/C3 derived	*Z. vietnamensis*
RL20	*P. mutuki*	*P. mutuki*-related	NA	General C1/C3	*P. mutuki*
RL21	*Z. vietnamensis*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RLB	Unknown *Zoanthus*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RLD	Unknown *Zoanthus*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RLE	*Z. vietnamensis*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RLA1	*Z. vietnamensis*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RLC1	*Z. sansibaricus*	*Z. sansibaricus*	NA	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
RLE1	*Z. vietnamensis*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RL102	*Z. vietnamensis*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RL103	*Z. vietnamensis*	*Z. vietnamensis*	NA	C15/C91-derived	*Z. vietnamensis*
RL105	*Z. sansibaricus*	NA	NA	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
RL106	*Z. sansibaricus*	*Z. sansibaricus*	NA	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
RLX	*Z. sansibaricus*	NA	NA	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
K1	*Z. vietnamensis*	NA	*Z. vietnamensis*	C15/C91-derived	*Z. vietnamensis*

Table 2 (continued).

Sample number	Preliminary morphological identification (see Table 1)	COI sequence identity	Mt 16S rDNA sequence identity	*Symbiodinium* ITS-rDNA sequence identity	Taxonomic Conclusion (species group)
K2	*P. tuberculosa*	*P. tuberculosa*	*P. tuberculosa*	D	*P. tuberculosa*
K3	Unknown *Zoanthus*	NA	*Z. vietnamensis*	NA	*Z. vietnamensis*
K4	*Z. sansibaricus*	*Z. sansibaricus*	*Z. sansibaricus*	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
K5	*Z. vietnamensis*	*Z. vietnamensis*	*Z. vietnamensis*	C15/C91-derived	*Z. vietnamensis*
K6	Unknown *Zoanthus*	*Z. vietnamensis*	*Z. vietnamensis*	C15/C91-derived	*Z. vietnamensis*
K7	*P. tuberculosa*	*P. tuberculosa*	*P. tuberculosa*	D	*P. tuberculosa*
K8	*Z. sansibaricus*	*Z. sansibaricus*	*Z. sansibaricus*	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
K9	*Z. vietnamensis*	*Z. vietnamensis*	*Z. vietnamensis*	C15/C91-derived	*Z. vietnamensis*
K11	*P. mutuki*	*P. mutuki*- related	NA	D	*P. mutuki*
K12	*P. mutuki*	*P. heliodiscus*-related	*P. heliodiscus*	*Zoanthus*-specific C1/C3 derived	*P.* sp. “singapura”
L1	*P. tuberculosa*	*P. tuberculosa*	*P. tuberculosa*	Mixed	*P. tuberculosa*
L3	*P. tuberculosa*	*P. tuberculosa*	*P. tuberculosa*	NA	*P. tuberculosa*
L4	*P. tuberculosa*	*P. tuberculosa*	*P. tuberculosa*	General C1/C3	*P. tuberculosa*
L5	*Z. sansibaricus*	*Z. sansibaricus*	*Z. sansibaricus*	*Zoanthus*-specific C1/C3 derived	*Z. sansibaricus*
L6	*P. tuberculosa*	*P. tuberculosa*	*P. tuberculosa*	Mixed	*P. tuberculosa*

Notes:

1. NA = not available
2. Sample name abbreviations: RL = Raffles Lighthouse, K = Kusu Island, L = Lazarus Island
3. All specimens collected in 1 to 3m of water. All specimens collected by J. D. Reimer except for K12 collected by Danwei Huang (NUS).

Table 3. Zoanthid specimens with previously published sequences used in this study.

Species	Specimen name[a]	Sampling location[b]	Depth (m)	Date collected	Collector[c]	mitocondrial 16S rDNA Accession No.	COI Accession No.	References
Zoanthus praelongus	WAMZ 40080	Favourite I., Jurien Bay, Western Australia	4.0–7.3	Apr.2005	GC, RB, AS	EF452255	EF452275	Reimer et al., 2008
Zoanthus sansibaricus	ZSH23[e]	Hakamagoshi, Sakurajima	9.0	Jul.2004	JDR	AB219187	AB214166	Reimer et al., 2004; 2006b
Zoanthus kuroshio	ZkYS1[e]	Sangohama, Yakushima	1.5	Jul.2004	JDR	AB219191	AB214175	Reimer et al., 2004; 2006b
Zoanthus gigantus	ZgYS1[e]	Sangohama, Yakushima	1.5	Jul.2004	JDR	AB219192	AB214177	Reimer et al., 2004, 2006b
Acrozaonthus sp.	"Sulawesi"[g]	Northern Sulawesi, Indonesia	9.0	Sep.2003	MB	AY995947	NA	Sinniger et al., 2005
Isaurus tuberculatus	IYS1	Sangohama, Yakushima	+0.5	Dec.2005	JDR	EF452239	EF452258	Reimer et al., 2008
Palythoa mutuki	PmMiI1[d]	Izushita, Miyakejima	0.0	May 2005	JDR	AB219225	AB219217	Reimer et al., 2006c
Palythoa tuberculosa	PtMiI1[d]	Izushita, Miyakejima	2.0	May 2005	JDR	AB219218	AB219199	Reimer et al., 2006c
Palythoa heliodiscus	PhSaiLL1[d]	Lau Lau, Saipan	3.0	Dec.2004	JDR	NA	AB219214	Reimer et al., 2006c
Palythoa heliodiscus	PhEK1	Kaito, Erabu	19.0	May 2005	JDR	AB219224	NA	Reimer et al., 2006c
Parazoanthus gracilis sensu Uchida, 2001	PgChK1	Kamogawa, Chiba	15.0	Nov.2006	JDR & FI	EF452257	NA	Reimer et al., 2008
Parazoanthus gracilis sensu Uchida, 2001	PgIJ1[f]	Jogasaki, Izu	17.0	Nov.2004	JDR	NA	AB214178	Reimer et al., 2004

Notes:
[a]Specimens collected in previous studies retain sample names assigned by the original collector/institution.
[b]All locations in Japan unless otherwise noted.
[c]Name abbreviations: JDR = J. Reimer, FI = F. Iwase, MB = Marcel Boyer, GC = G. Clapin, RB = R. Babcock, AS = A Sampey
*NA = data not acquired, not available, or not used in this study.

Table 4. Previously published *Symbiodinium* sequences used in this study.

Sequence Accession No.	*Symbiodinium* clade, type, or designation	Host species	Location	Reference
Yaku2a	C15/C91 derived	*Zoanthus sansibaricus*	Yakushima, Japan	Reimer et al., 2006e
AJ311944	C (sp. 1675a)	*Porites rus*	Guam	Pochon et al., 2001
AJ291514	C (sp. 1366)	*Amphisorus hemprichii*	Gulf of Elat, Isreal	Pawlowski et al., 2001
AF195157	C (sp. TcFIZ)	***Tridacna crocea***	Palau	Baillie et al., 2000
AJ291519	C (sp. 1591)	*Sorites* sp.	Guam	Pawlowski et al., 2001
Three0501-11	*Zoanthus*-specific C1/C3 derived	*Zoanthus sansibaricus*	Sakurajima, Japan	Reimer et al., 2007b
Two0504-12	*Zoanthus*-specific C1/C3 derived	*Zoanthus sansibaricus*	Sakurajima, Japan	Reimer et al., 2007b
Four0501-9	*Zoanthus*-specific C1/C3 derived	*Zoanthus sansibaricus*	Sakurajima, Japan	Reimer et al., 2007b
AF195144	General C1/C3	*Corculum cardissa*	Palau	Baillie et al., 2000
AY186567	General C1/C3	*Plesiastrea versipora*	Amakusa, Japan	Rodriguez-Lanetty & Hoegh-Guldberg, 2003
AY237296	General C1/C3	*Acropora millepora*	Great Barrier Reef, Australia	Bui et al., unpublished
PtIsK1-6	General C1/C3	***Palythoa tuberculosa***	Ishigaki, Japan	Reimer et al., 2006d
AB294667	D	***Corculum cardissa***	Okinawa, Japan	Kii et al. (unpublished data)
AF396631	D	***Entacmaea quadricolor***	Okinawa, Japan	Santos et al., 2003
AJ311948	D (sp. 1655)	***Acropora* sp.**	Guam	Pochon et al., 2001
EU074900	D1a	NA	NA	Thornhill et al., 2007
EU074906	D1a	NA	NA	Thornhill et al., 2007

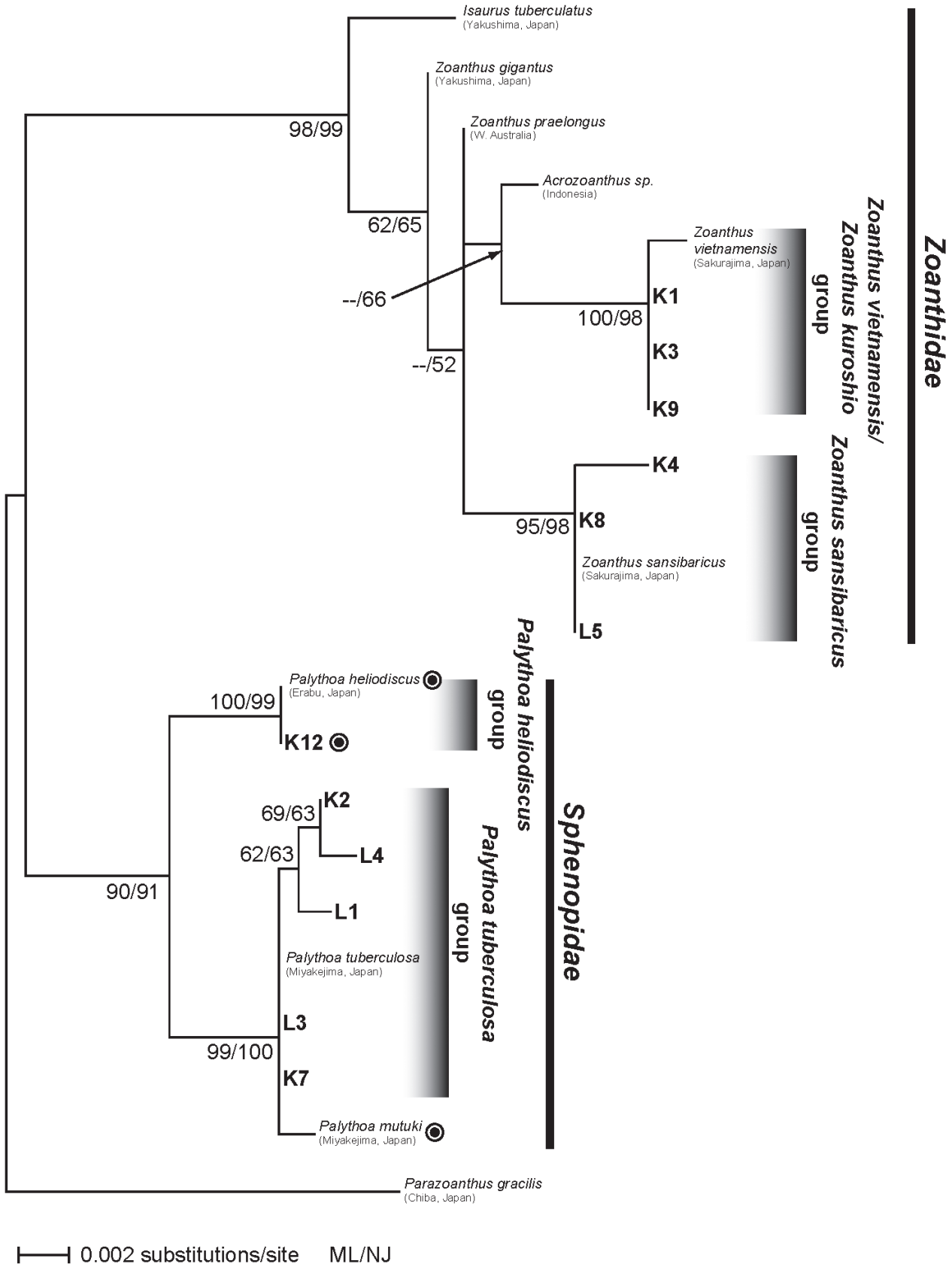

Fig. 3. Maximum likelihood tree of mitochondrial 16S ribosomal DNA (mt 16S rDNA) sequences for zooxanthellate zoanthid specimens. Values at branches represent ML and NJ bootstrap probabilities, respectively (> 50%). New sequences from this study in **bold.** For sample name abbreviations see Table 2. Sample names with Accession Numbers are from previous studies (see Table 3). Closed circles after some *Palythoa* spp. specimens indicate a "liberae" polyp form (polyps clear of the coenenchyme–see Pax, 1910, and Fig. 2), as opposed to other, non-marked *Palythoa* specimens, with "immersae" polyps (polyps embedded in coenenchyme).

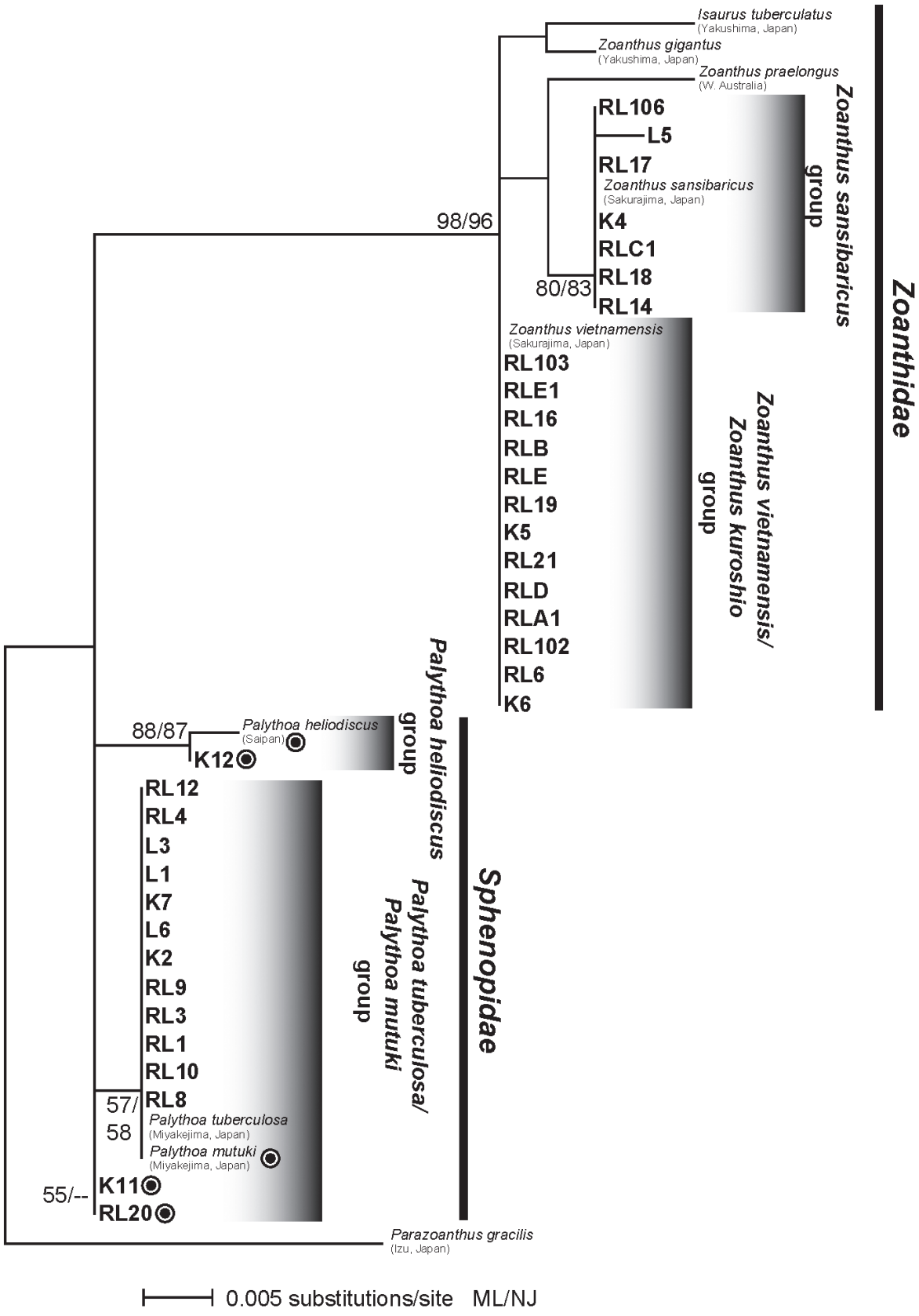

Fig. 4. Maximum likelihood tree of mitochondrial cytochrome oxidase subunit I (mt COI) sequences for zooxanthellate zoanthid specimens. Values at branches represent ML and NJ bootstrap probabilities, respectively (> 50%). New sequences from this study in **bold.** For sample name abbreviations see Table 2. Sample names with Accession Numbers are from previous studies (see Table 3). Closed circles after some *Palythoa* spp. specimens indicate a "liberae" polyp form (polyps clear of the coenenchyme–see Pax, 1910, and Fig. 2), as opposed to other, non-marked *Palythoa* specimens, with "immersae" polyps (polyps embedded in coenenchyme).

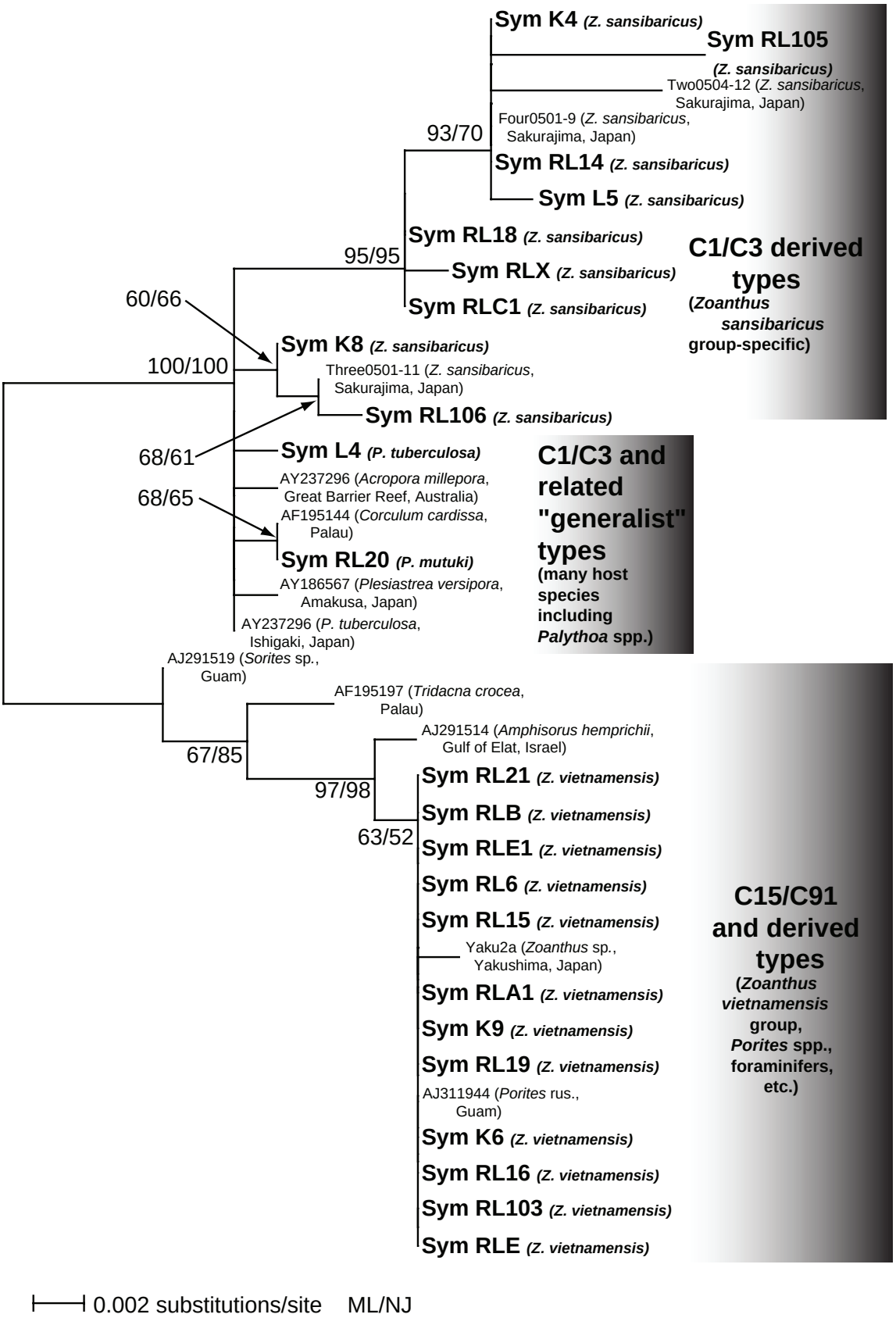

Fig. 5. Maximum likelihood tree of internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences from clade C *Symbiodinium* from zooxanthellate zoanthid specimens. Values at branches represent ML and NJ bootstrap probabilities, respectively (> 50%). For sample name abbreviations see Table 2. New sequences from this study in **bold**, followed by host species in parentheses. Sample names with Accession Numbers are from previous studies (see Table 4).

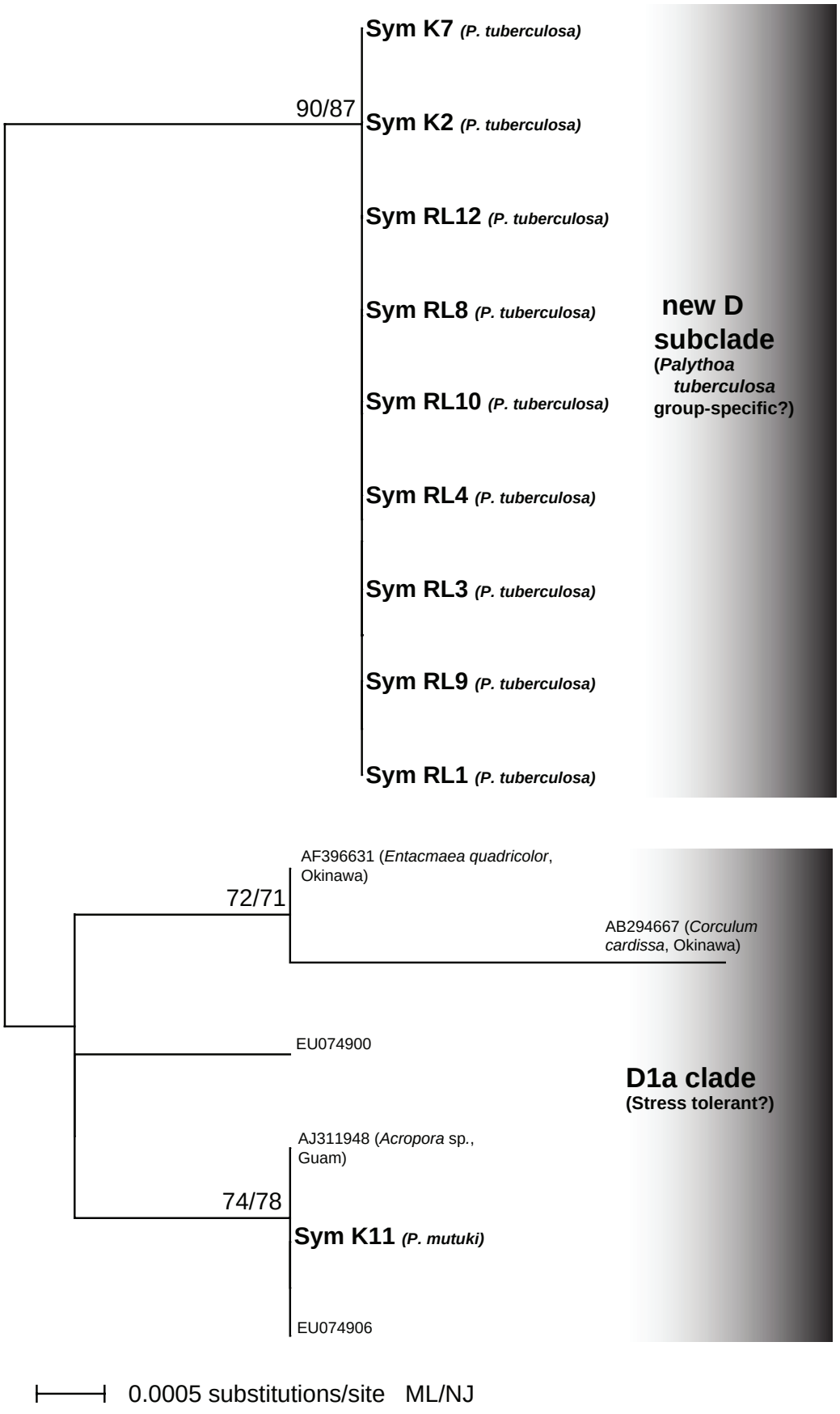

Fig. 6. Maximum likelihood tree of internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences from clade D *Symbiodinium* from zooxanthellate zoanthid specimens. Values at branches represent ML and NJ bootstrap probabilities, respectively (> 50%). For sample name abbreviations see Table 2. New sequences from this study in **bold**, followed by host species in parentheses. Sample names with Accession Numbers are from previous studies (see Table 4).

DISCUSSION

Accuracy of morphological identification. **–** During the preliminary identification process, 80% of the zoanthid specimens (i.e. 35 / 44) were correctly identified to the species level using only morphological criteria. In particular, the various color morphotypes of *Z. sansibaricus* (Fig. 7A and see Reimer et al., 2004), and *P. tuberculosa* (Fig. 7C), *P. mutuki*-related specimens (Fig. 7D), and pink oral disk morphotypes of *Z. vietnamensis*, proved to be readily identifiable in the field using only oral disk coloration and polyp form.

Conversely, the mint green color morphotype of *Z. vietnamensis* (Fig. 7B), not seen in previous studies in Japan and different in polyp shape ("liberae" vs. "intermediae" or embedded in Burnett et al., 1997) to previous literature, proved to be difficult to identify due to a paucity of zoanthid data for most parts of the world—especially the areas between Japan and Singapore. Additionally, *P.* sp. "singapura" (Fig. 7E) was initially misidentified as *P. mutuki* due to its "liberae" polyps, and similar brown oral disk coloration and size, relatively small colony size, and shallow water habitat to *P. mutuki*. Thus, while some zoanthid species were identifiable based on their morphology, encounters with previously unseen morphotypes and/or new species (particularly cryptic species) are often difficult to identify using morphology alone due to the variation seen within *Zoanthus* and *Palythoa* species.

Based on these observations, we suggest that any zoanthid taxonomic sampling in a relatively unexplored area should not only conduct specimen identifications based on morphology, but also use DNA molecular methods (mt 16S rDNA, COI) for identification confirmation, especially for potential new morphotypes and species. The mt 16S rDNA and COI sequences acquired in the present study were able to correctly place 100% of specimens within a species group.

Zooxanthellate zoanthids in Singapore. **–** From the sampling trips undertaken during this study, it became apparent that most Singaporean zooxanthellate zoanthids live in waters in 1–2 m of water with few or none found deeper than 3 m below mean sea level. While there exists little data on depth distribution for *Palythoa* and *Zoanthus* from areas close by, zoanthids in Singapore appear to be limited to much shallower water than in other tropical regions such as Japan and Saipan (pers. obs.). This phenomenon is likely due to the year-round sediment-associated poor light penetration in the waters surrounding Singapore (Todd et al., 2004). Indeed, zooxanthellate scleractinian corals are also restricted to much shallower waters than would be expected for tropical Indo-Pacific reefs (Chou, 1996).

From the three locations surveyed, we found five species of zooxanthellate zoanthids.. Whether this relatively low species diversity is due to the turbid waters of Singapore or the limited number of locations investigated is unknown. *Zoanthus sansibaricus* is found in many Japanese locations near cities, potential pollution and poor visibility (i.e. Sakurajima site, see Reimer et al., 2004).

Palythoa tuberculosa, a species that thrives in a wide range of habitats (Reimer et al., 2006d), was found at all sites. Similar to Singapore, *P. mutuki* exists in mainly shallow waters in Japan. *Zoanthus vietnamensis*, however, was not found on Lazarus Island, and may fare better in the slightly clearer waters at Kusu Island and Raffles Lighthouse. Similarly, in Japan, the very closely related *Z. vietnamensis* and *Z. kuroshio* Reimer & Ono, 2006 (see Reimer et al., 2006b) prefer locations more towards the open sea, with strong currents and good visibility, and are rarely found at more sheltered and/or turbid locations. *Zoanthus gigantus* Reimer & Tsukahara, 2006 (see Reimer et al., 2006b) and *Isaurus tuberculatus* Gray, 1828, (see Reimer et al., 2008) from Japan are associated with even clearer waters, and were not seen at the three Singapore sites. Additionally, *Z. praelongus* Carlgren, 1954 (see Reimer et al., 2008) from Western Australia, and *P. heliodiscus* from slightly deeper waters of Indo-Pacific coral reefs (see Reimer et al., 2006c) were not recorded during this study.

Palythoa sp. "singapura" is only known from one colony and more research is needed to determine whether this is truly an undescribed species. Naturally, it is impossible to speculate on its distribution and ecology until more specimens and data have been collected.

Symbiodinium diversity in zoanthids in Singapore. – Acquired *Symbiodinium* ITS-rDNA sequences reflect previously seen patterns of association in *Zoanthus*, with *Zoanthus sansibaricus* (C1/C3-derived *Symbiodinium*) and *Zoanthus vietnamensis* (C15/C91-derived) both showing identical zooxanthellae types as those found in Japan and other Indo-Pacific locations (Reimer et al., 2006e; 2007b). C1/C3-derived *Symbiodinium* have previously been shown to be a subclade unique to *Z. sansibaricus* derived from the major subclades C1 and C3, and similarly C15/C91-derived *Symbiodinium* may be unique to *Z. vietnamensis* and *Z. kuroshio* (Reimer et al., 2006e).

The majority of *Palythoa tuberculosa* (n = 9) and one *P. mutuki*-related colony were associated with *Symbiodinium* clade D, previously seen in zoanthids (Burnett 2002) and corals (e.g. Chen et al., 2003; Thornhill et al., 2006). Only one specimen each of *P. tuberculosa* and *P. mutuki*-related was associated with "generalist" type C1/C3, the most commonly observed *Symbiodinium* in *Palythoa* in Japan (Reimer et al., 2006d). Clade D has been hypothesized to be relatively tolerant of both high (~ 35°C) and low (~ 12°C) ocean temperatures (e.g. Chen et al., 2003; Pochon et al., 2006; Thornhill et al., 2006). *Palythoa tuberculosa* and *P. mutuki* are flexible in their association with *Symbiodinium*, as also seen in *P. caesia* Dana 1846 with clades C and D in the Indian Ocean (Burnett, 2002). It is unusual that all *P. tuberculosa* from Raffles Lighthouse and Kusu Island associated with clade D only, while on the more turbid Lazarus Island *P. tuberculosa* had clade C1/C3 or a mixed group of *Symbiodinium*. Further investigations of *Symbiodinium* from *P. tuberculosa* found at other turbid

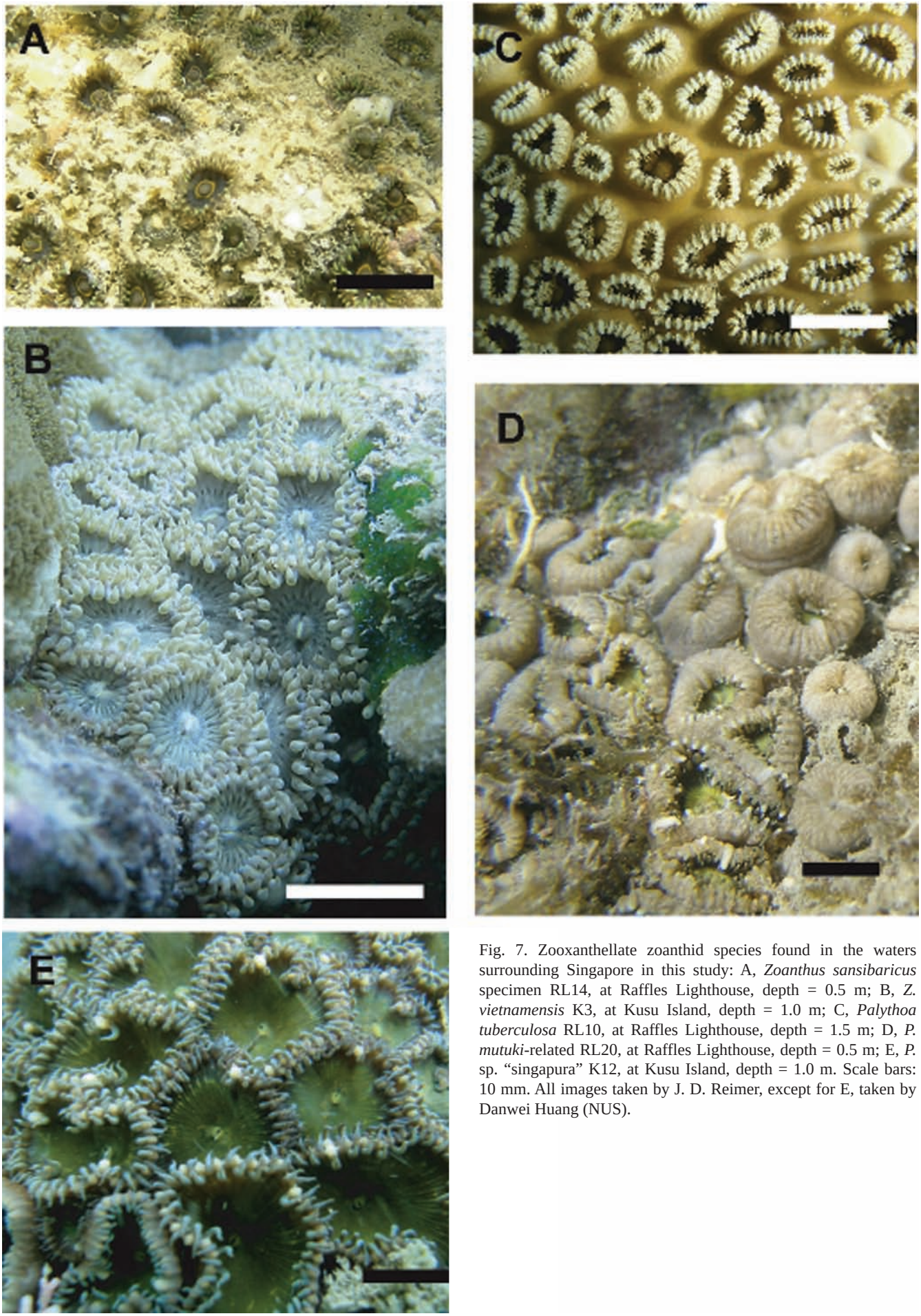

Fig. 7. Zooxanthellate zoanthid species found in the waters surrounding Singapore in this study: A, *Zoanthus sansibaricus* specimen RL14, at Raffles Lighthouse, depth = 0.5 m; B, *Z. vietnamensis* K3, at Kusu Island, depth = 1.0 m; C, *Palythoa tuberculosa* RL10, at Raffles Lighthouse, depth = 1.5 m; D, *P. mutuki*-related RL20, at Raffles Lighthouse, depth = 0.5 m; E, *P.* sp. "singapura" K12, at Kusu Island, depth = 1.0 m. Scale bars: 10 mm. All images taken by J. D. Reimer, except for E, taken by Danwei Huang (NUS).

locations in Singapore may help us determine the reason behind this varying *Symbiodinium* association pattern observed in *P. tuberculosa*.

The lone colony of *Palythoa* sp. "singapura" associated with the same type of *Symbiodinium* (C1/C3-derived) as seen in *Z. sansibaricus* represents the first time this *Symbiodinium* type has been seen outside of *Zoanthus*. This further demonstrates that *P*. sp. "singapura" is a different species than *P. mutuki* despite the close morphological resemblance.

Future taxonomic questions. **–** Based on similar morphological characteristics, it may be that *P. tuberculosa* and *P. caesia* are conspecifics, and this warrants future examination. Similarly, *Zoanthus coppingeri* Haddon & Shackleton 1891 sensu Burnett et al. (1995) from eastern Australia and the Torres Strait may be the same species as *Z. sansibaricus*, and this should also be investigated to help clarify the taxonomy of *Zoanthus*. Additional examinations of zoanthid specimens collected in this study using faster-evolving molecular markers (e.g. ITS-rDNA) may also increase our understanding of within-species group diversity [as seen in Reimer et al., (2007c, 2007d)].

Conclusions. – While this survey was by no means exhaustive, we have documented the presence of zooxanthellate zoanthids in Singapore for the first time. Five species were identified here, and the possibility remains that more species exist at other sites not yet investigated. Most of the individual zoanthid colonies in this study associated with one type of *Symbiodinium*, and all except for *P. tuberculosa* and *P. mutuki* had a specific association with one *Symbiodinium* type. *Palythoa tuberculosa* associated with both "generalist" C1/C3 *Symbiodinium* as noted in most previous studies, and also with clade D *Symbiodinium* as reported by Burnett (2002) in *P. caesia* from the eastern Indian Ocean. Additionally, we made the first discovery of flexibility in the *P. mutuki-Symbiodinium* association.

Most importantly, the results of this study highlight the need for further sampling and examination of zooxanthellate zoanthids from far more regions to help complete the global picture of zoanthid and associated zooxanthellae distribution patterns. By using a combination of molecular and morphological techniques, zoanthid diversity in new locations can be investigated easily, and thus increase our understanding of the taxonomy and diversity of this long neglected order.

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Table 5. Summary of identified zoanthid specimens from Singapore and their *Symbiodinium* type by sampling location.

Site/Species group	n	*Symbiodinium* type (n)
Raffles Lighthouse	**28**	**27**
Zoanthus sansibaricus	8	*Zoanthus*-specific C1/C3 derived (7)
Zoanthus vietnamensis	12	C15/C91 derived (12)
Palythoa tuberculosa	7	D (7)
Palythoa mutuki	1	general C1/C3 (1)
Kusu Island	**11**	**10**
Zoanthus sansibaricus	2	*Zoanthus*-specific C1/C3 derived (2)
Zoanthus vietnamensis	5	C15/C91 derived (4)
Palythoa tuberculosa	2	D (2)
Palythoa mutuki	1	D (1)
Palythoa sp. "singapura"	1	*Zoanthus*-specific C1/C3 derived (1)
Lazarus Island	**5**	**4**
Zoanthus sansibaricus	1	*Zoanthus*-specific C1/C3 derived (1)
Palythoa tuberculosa	4	general C1/C3 (1) mixed (2)
Total	**44**	**41**

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