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Epizoanthus spp. Associations Revealed Using DNA Markers: A Case Study from Kochi, Japan

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Zoanthids (Cnidaria, Hexacorallia) of the genus *Epizoanthus* are often found in association with other marine invertebrates, including gastropods and hermit crabs. However, little information exists on the specificity and nature of these associations due to a lack of investigation into *Epizoanthus* species diversity, and the taxonomy of *Epizoanthus* is therefore confused. In this study, analyses of morphological data (tentacle number, polyp size, etc) and molecular data (mitochondrial cytochrome oxidase subunit 1 = COI, 16S ribosomal DNA = 16S rDNA) were used to examine *Epizoanthus* specimens from Tosa Bay, Kochi, Japan. The *Epizoanthus* specimens were found on both live gastropods (*Gemmula unedo*) and hermit crabs (*Paguristes palythophilus*) inhabiting *G. unedo* and *G. cosmoi* shells. While morphological analyses did not show clear differences between examined specimens, both COI and mt 16S rDNA clearly divided the specimens into two groups, one associated only with hermit crabs (= *Epizoanthus* sp. C), and another associated only with living gastropods (= *Epizoanthus* sp. S). Unexpectedly, DNA sequences from both groups did not match with two previously reported *Epizoanthus* species from Japan (*E. indicus*, *E. ramosus*), indicating they both may be undescribed species. These results highlight the utility of DNA "barcoding" of unknown zoanthids, and will provide a foundation for re-examinations of *Epizoanthus* species diversity and specificity, which will be critical in understanding the evolution of these unique marine invertebrates.

Key words: zoanthid, *Epizoanthus*, COI, mt 16S rDNA, symbiosis, barcoding

INTRODUCTION

Epizoanthus is a genus of generally colonial zoanthids (Cnidaria: Hexacorallia: Zoantharia) commonly found in many marine ecosystems, from shallow waters to the deep sea. This genus is notable for containing both free-living (i.e. attached to the ocean floor directly) and symbiotic (living upon other organisms) species. Symbiotic substrate organisms include, but are not limited to, hermit crabs (Muirhead et al., 1986; Ates, 2003; Williams and McDermott, 2004; Schejter and Mantelatto, in press), live shells (Rees, 1967), eunicid worms (Sinniger et al., 2005), and the stalks of glass sponges (Beaulieu, 2001). Despite their relatively common occurrence, little research has been done on *Epizoanthus* species in recent years, particularly with regards to their taxonomy and symbiotic associations (Ates, 2003). In particular, aside from a few well-known species (*Epizoanthus illoricatus* on eunicid worms), little is known on the specificity of epizoanthid symbioses. Many *Epizoanthus* species originally described as symbiotic with hermit crabs are subsequently mentioned in symbioses with live shells, or vice-versa. Thus, until a detailed examination of *Epizoanthus* species diversity and symbiotic specificity is undertaken, research on the ecological nature of such symbioses will remain at a standstill (Ates, 2003).

Much of this lack of taxonomic knowledge stems from the difficulty in zoanthid species identification. Many species

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of zoanthids (particularly in the suborder Brachycnemina) have high levels of intraspecific morphological variation, particularly with regards to polyp shape and size, color of the outer surface of colonies, polyps, and oral disks (Burnett et al., 1997; Reimer et al., 2004). Correct identification is further confounded by the utilization of sand and detritus in the body wall of most zoanthids, which results in extreme difficulty in internal examination due to difficulty of sectioning (Reimer et al., 2010). *Epizoanthus* species are often particularly difficult to identify due to their relative lack of external colony color (Fig. 1) and the fact that many species live in the deep sea below the depths accessible by SCUBA. Thus, when colonies are brought to the surface for examination, their polyps are closed and valuable data, such as tentacle number and oral disk color, are unobtainable.

A search of the literature reveals three species commonly mentioned in the waters around Japan. *Epizoanthus ramosus* Carlgen 1934 is described living on shells inhabited by the hermit crabs *Paguristes balanophilus* Alcock 1905 (Okada, 1965) and *Paguristes palythophilus* Ortmann 1892 (Komai, 2004). *Epizoanthus paguriphilus* Verrill 1863 is described associated with *Paguristes palythophilus*, as well as *Parapagurus holthuisi* Lemaitre 1989 (= *Parapagurus pilosimanus*) (Okada, 1965). *Epizoanthus indicus* Lwowsky 1913 is described as inhabiting the shell of *Pleurotoma symbiotes* Wood-Mason & Alcock 1891 (Balss, 1924), and also possibly on *Parapagus* spp. crabs (Carlgren, 1934) (associations summarized in Table 1). However, as outlined above, the specificity and true diversity of the *Epizoanthus* species in Japanese waters is much in doubt, as little research has been focused on these subjects.

In this study, we examined *Epizoanthus* specimens from Tosa Bay, Kochi, Japan. This bay is generally sandy-bottomed, and *Epizoanthus* species are frequently caught in the nets of bottom-trawls, found on live *Gemmula unedo* Kiener 1839 gastropods and the hermit crab *Paguristes palythophilus* Ortmann 1892 inhabiting empty shells of *G. unedo* and *G. cosmoi* Sykes 1830. External coloration and other morphological data from epizoanthid specimens did not clearly reveal the number of *Epizoanthus* species present in Tosa Bay, and thus phylogenetic techniques (DNA markers) recently used with success in other zoanthid groups (Reimer et al., 2006; Sinniger and Hausermann, 2009) were applied to reveal the associations between *Epizoanthus* specimens from Tosa Bay with living gastropods and hermit crabs, and further compared with known specimens of *E. ramosus* and *E. indicus*. For this study we chose relatively short and easy-to-use DNA fragments (< 400 base pairs in length) to examine the accuracy of "DNA barcoding" in zoanthids, as proposed in Sinniger et al. (2008).

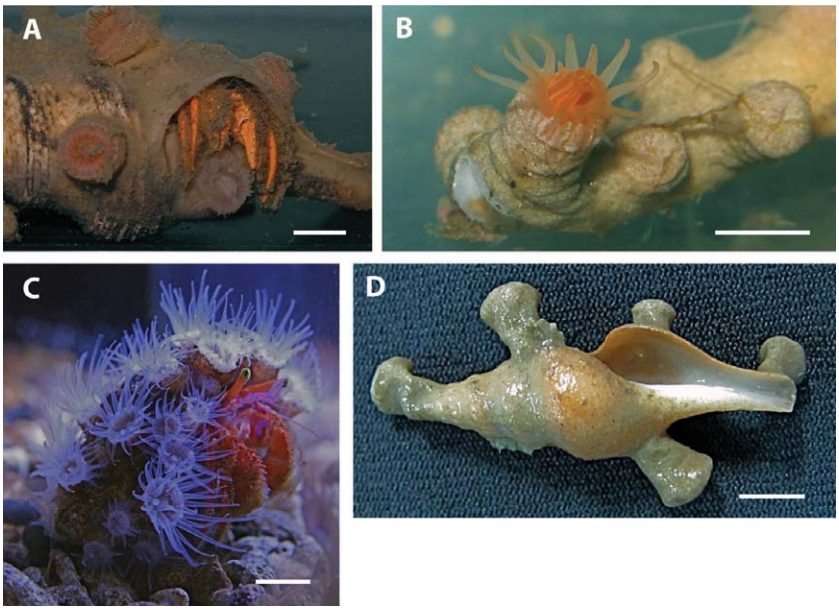

Fig. 1. *Epizoanthus* species examined in this study, including **(A)** *Epizoanthus* sp. C associated with the hermit crab *Paguristes palythophilus*, **(B)** *Epizoanthus* sp. S associated with the gastropod *Gemmula unedo*, **(C)** *Epizoanthus ramosus* associated with the hermit crab *Paguristes balanophilus*, and **(D)** *Epizoanthus indicus* associated with the shell *Pleurotoma symbiotes*. All scale bars = approximately 1 cm.

MATERIAL AND METHODS

Specimen collection

Unidentified *Epizoanthus* specimens (n = 10) associated with gastropods and hermit crabs were collected at depths of 40–60 m in Tosa Bay, Kochi, Japan (33°04′N, 133°08′E) by trawl fishery between April and September 2009 (Table 2). Specimens were immediately preserved in 99% ethanol until further examination. Specimens of *E. ramosus* (n = 3) were obtained from the Natu-

Table 1. Summary of *Epizoanthus* species in this study and their symbiotic associations from this study and past literature. *This hermit crab species was found inhabiting empty shells of the species *Gemmula cosmoi* and *G. unedo*, both of which were encrusted with *Epizoanthus* (Table 2).

Species and authority	Partner	Reference(s) or location
E. ramosus Carlgren 1934	*Paguristes* spp.? (shell)	Carlgren 1934 (original description)
	Paguristes balanophilus (hermit crab)	Natural History Museum, Wakayama
	Paguristes palythophilus (hermit crab)	Komai 2001
	Guildfordia triumphans (shell)	Natural History Museum, Wakayama
E. indicus Lwowsky 1913	*Pleurotoma symbiotes* (shell)	Lwowsky 1913 (original description); Balss 1924
	Parapagurus spp. (hermit crab)	Carlgren 1934; Uchida 2001
E. paguriphilus Verrill 1863	*Paguristes palythophilus* (hermit crab)	Okada 1965
Unknown *Epizoanthus* specimens	*Gemmula unedo* (shell)	This study
	Paguristes palythophilus (hermit crab)*	This study

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Table 2. *Epizoanthus* specimens examined in this study. *These samples were collected from an aquarium at the Natural History Museum (Kainan, Wakayama). The original *Epizoanthus ramosus* colony was found inhabiting the shell of a *Paguristes balanophilus* hermit crab, and subsequently colonized other surfaces in the aquarium, including rocks, and both living gastropods (*Guildfordia triumphans*) and dead shells.

Species	Specimen number	Sampling location	Sampling date	Collector	Depth	Substrate	16S rDNA Accession No.	COI Accession No.
Epizoanthus sp. C	EK01	Tosa Saga, Kochi, Japan	2009.4.16	T. Nishisaka	40–60 m	*Gemmula cosmoi* shell (hermit crab)	HM040882	HM040872
Epizoanthus sp. S	EK02	Tosa Saga, Kochi, Japan	2009.4.16	T. Nishisaka	40–60 m	*Gemmula unedo* shell (live)	HM040880	HM040865
Epizoanthus sp. C	EK03	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula cosmoi* shell (hermit crab)	NA	HM040873
Epizoanthus sp. C	EK04	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula cosmoi* shell (hermit crab)	NA	HM040871
Epizoanthus sp. C	EK05	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula unedo* shell (hermit crab)	HM040883	NA
Epizoanthus sp. C	EK06	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula unedo* shell (hermit crab)	NA	HM040870
Epizoanthus sp. S	EK07	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula unedo* shell (live)	HM040879	HM040867
Epizoanthus sp. S	EK08	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula unedo* shell (live)	NA	HM040866
Epizoanthus sp. S	EK09	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula unedo* shell (live)	NA	HM040868
Epizoanthus sp. S	EK10	Tosa Saga, Kochi, Japan	2009.9.11	T. Nishisaka	40–60 m	*Gemmula unedo* shell (live)	HM040881	HM040869
E. ramosus	ErWS1	Shirahama, Wakayama, Japan	2006.4.15	H. Tanase	80–90 m	Hermit crab and shell	HM040878	HM040874
E. ramosus	ErWS2	Shirahama, Wakayama, Japan	2006.4.15	H. Tanase	80–90 m	Hermit crab and shell	NA	NA
E. ramosus	ErWS3	Shirahama, Wakayama, Japan	2006.4.15	H. Tanase	80–90 m	Hermit crab and shell	NA	NA
E. indicus	EI1	East China Sea	2009.11.20	M. Nonaka	339 m	*Pleurotoma symbiotes* shell (live)	NA	HM040875

ral History Museum of Wakayama Prefecture (Kainan, Wakayama), and a specimen of *E. indicus* (n = 1) was collected by Churaumi Aquarium (Motobu, Okinawa, Japan) in the East China Sea. Specimens were identified by the first author. Collection details are given in Table 2. All samples were finally deposited at the University of the Ryukyus (Nishihara, Okinawa, Japan) in 99.5% ethanol at –20°C.

Morphological analyses

Digital images of specimens in situ or in aquaria were used to count tentacle number and confirm tentacle color where possible, and were supplemented by data from the original descriptions or other literature if available. Measurements of polyp dimensions (diameter, height, and spacing from other polyps) were performed on fixed specimens in the laboratory.

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 using zoanthid-specific primers and following procedures outlined in Sinniger et al. (2005). COI was amplified using zoanthid-specific primers and following procedures outlined in Reimer et al. (2007). The amplified products were visualized by 1.0% agarose gel electrophoresis.

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank (accession numbers HM040865-HM040883). Phylogenetic analyses of all markers were conducted primarily to visually demonstrate the relative degree of relatedness between specimens and known *Epizoanthus* species, and thus figures in this study should not be used to draw phylogenetic conclusions without caution.

Nucleotide sequences of mt 16S rDNA and COI from samples were manually aligned with previously published mt 16S rDNA and COI sequences from various *Epizoanthus* species (downloaded from GenBank). Outgroup sequences for both mt 16S rDNA and COI trees were from the genus *Hydrozoanthus*.

All alignments were inspected by eye and manually edited. All ambiguous alignment sites of were removed from the dataset for phylogenetic analyses. Consequently, two alignment datasets were generated: 1) 361 sites of 17 sequences (mt 16S rDNA); and 2) 270 sites of 18 sequences (COI). While most acquired sequences were longer than the final alignments, some specimens yielded only relatively short fragments, and in order to compare all possible species, these relatively short alignments were utilized. The alignment data are available on request from the corresponding author.

Genetic distances between all sequences for each marker were calculated using both uncorrected and Kimura's (1980) two parameter (K2P) models implemented in Phylip version 3.67 (Felsenstein, 1989). K2P distances have been shown to be most accurate when genetic distances are low (Hebert et al., 2003).

For the phylogenetic analyses of the two alignments, the same methods were applied independently. Alignments were subjected to analyses with maximum-likelihood (ML) method with PhyML (Guindon and Gascuel, 2003) and neighbor-joining (NJ) method with PAUP* version 4.0 (Swofford, 1998). PhyML was performed

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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 1000 replicates.

RESULTS

Morphological analyses

The results of morphological analyses are summarized in Table 3, with representative specimens shown in Fig. 1.

Table 3. Summary of morphological data from *Epizoanthus* spp. examined in this study.

Species	Number of tentacles[1]	Tentacle color	Polyp diameter (cm)[3]	Polyp spacing (cm)[3]	Polyp height (cm)[3]
Epizoanthus sp. C	24	Transparent red	0.4–0.6	1 to 2	Up to 1.0
Epizoanthus sp. S	Approx. 24[2]	Transparent red	0.2–0.5	Usually < 1	Up to 0.6
Epizoanthus ramosus	26 (34–36)	White	< 0.4	< 0.6	Up to 0.3
Epizoanthus indicus	(32–36)	NA	0.4	Approx. 1	Up to 1.0

[1] Numbers in parentheses from Carlgren (1934), numbers with no parentheses from this study.
[2] Uncertain, as only one partially open polyp was observed.
[3] For preserved (70% EtOH) specimens (closed polyps).
NA = not available.

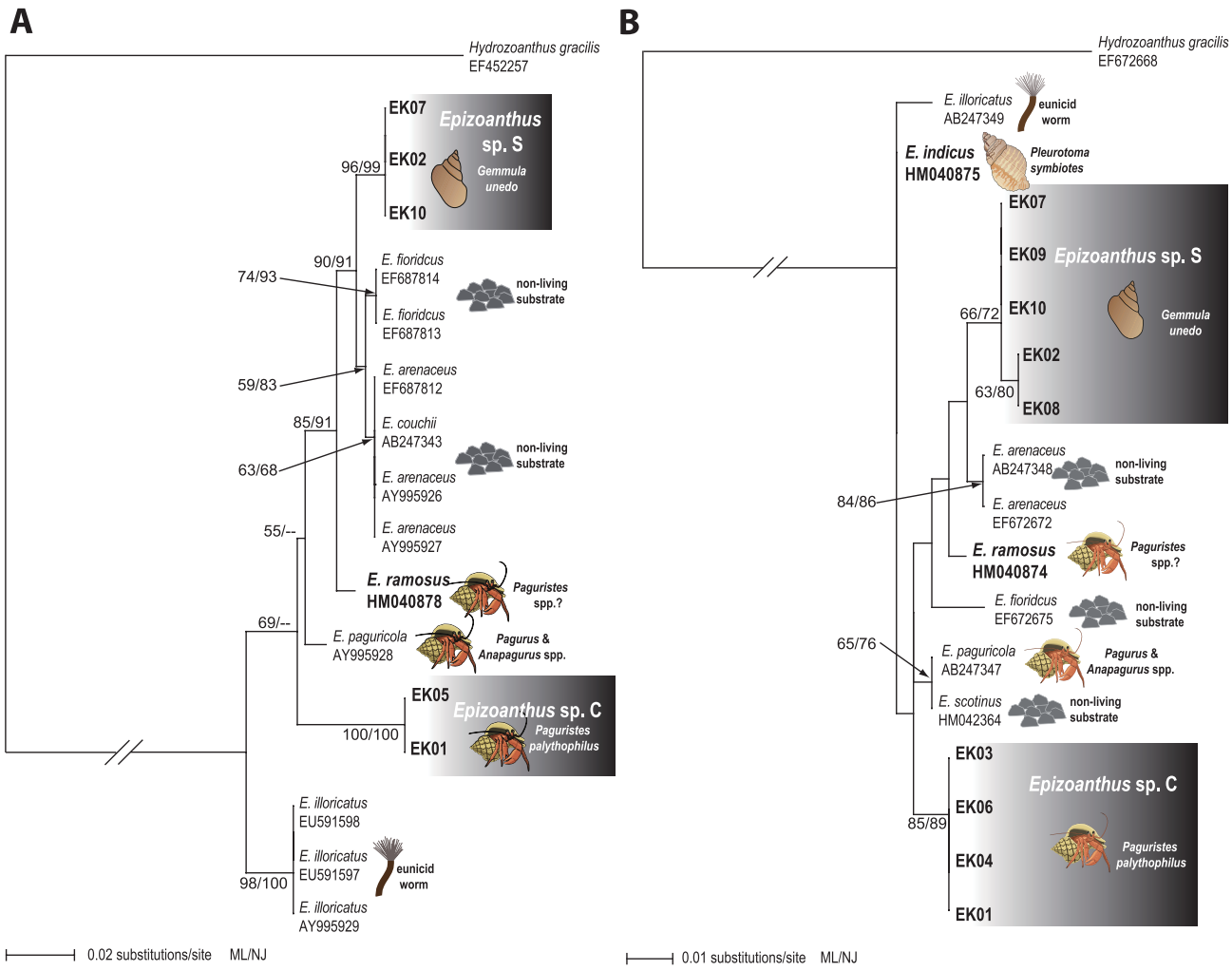

Fig. 2. Maximum likelihood (ML) trees of **(A)** mitochondrial 16S ribosomal DNA, and **(B)** cytochrome oxidase subunit I (COI) sequences for zoanthid specimens. Values at branches represent ML and neighbor-joining (NJ) probabilities (> 50%), respectively. Sequences newly obtained in this study in bold. Sequences/species names from previous studies in regular font with GenBank Accession Numbers. Specimen information in Table 2.

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Generally speaking, while there were some differences in polyp diameter and spacing between specimens associated with crabs (hereafter *Epizoanthus* sp. C) and specimens associated with living shells (hereafter *Epizoanthus* sp. S), the data overlapped to some degree, and it proved difficult to make clear distinctions based solely on these data. Tentacle number and color also proved not to be indicative of any large differences between the two groups, although it was difficult to obtain many images of open polyps. However, the two groups were clearly different morphologically from both *E. ramosus* and *E. indicus* based on tentacle number, polyp spacing, and polyp height (for *E. ramosus*) (Table 3).

Phylogenetic analyses

mt 16S rDNA

Mitochondrial 16S ribosomal DNA (mt 16S rDNA) phylogenetic results are shown in Fig. 2A, and genetic distances in Supplemental Table S1 online. The two groups' sequences differed from each other by twenty base pairs (out of 360 b.p. = 5.6%). The results were similar to the COI results below, with sequences from the two groups of specimens forming two well-supported and distinct monophyletic groups (*Epizoanthus* sp. S ML = 96%, NJ = 99%; *Epizoanthus* sp. C ML = 100%, NJ = 100%) that did not match with other *Epizoanthus* species. *Epizoanthus* sp. S (EK02, EK07, EK10) was sister to a large group of benthic specimens (*E. fiordicus*, *E. couchii*, *E. arenaceus*/*E. vagus*), and this large group was well-supported (ML = 90%, NJ = 91%). *Epizoanthus ramosus* was placed basal to this group, and was clearly distinct. The *Epizoanthus* sp. C group (EK01, EK05) was basal to all other *Epizoanthus* sequences except for those of *E. illoricatus*. Genetic distances between *Epizoanthus* sequences ranged from 0 to 0.059086 (uncorrected) and 0 to 0.059851 (K2P), while *Hydrozoanthus gracilis* sequence distances from *Epizoanthus* species were 0.210303 to 0.234480 (uncorrected) and 0.210708 to 0.237693 (K2P) (Supplemental Table S1 online).

COI

Cytochrome oxidase subunit I (COI) phylogenetic results are shown in Fig. 2B, and genetic distances in Supplemental Table S2 online. Both groups of Tosa Bay *Epizoanthus* (sp. S and sp. C) specimens examined in this study formed monophylies clearly distinct from all other *Epizoanthus* specimens included in the analyses. The two groups' sequences differed from each other by five to six base pairs (out of 270 b.p. = 1.9 to 2.2%). *Epizoanthus* sp. C (specimens EK01, EK03, EK04, EK06) made a strongly supported monophyly (ML = 85%, NJ = 89%) sister to two clades, one containing *E. scotinus* and *E. paguricola* (ML = 65%, NJ = 76%), and one containing all other *Epizoanthus* sequences except for those of *E. illoricatus* and *E. indicus* (ML and NJ < 50%). Together, these three subclades formed one large clade that was not well supported (ML and NJ < 50%). *E. illoricatus* and *E. indicus* were seen to be divergent basal to this large clade. *Epizoanthus* sp. S (EK07, EK09, EK10 + EK02, EK08) formed a moderately supported subclade (ML = 66%, NJ = 72%) within this lineage, with two sequences (= EK02, EK08) grouping separately within this subclade (ML = 63%, NJ = 80%). Genetic distances between *Epizoanthus* sequences ranged from 0 to 0.030231 (uncorrected) and 0 to 0.030329 (K2P), while *Hydrozoanthus gracilis* sequence distances from *Epizoanthus* species were 0.129003 to 0.146808 (uncorrected) and 0.129674 to 0.146813 (K2P) (Supplemental Table S2 online).

DISCUSSION

The results of the molecular analyses using the mitochondrial markers 16S ribosomal DNA and cytochrome oxidase I (COI) clearly show the presence of two different *Epizoanthus* spp.: one found on living *G. unedo* shells and the other found on *Paguristes palythophilus* hermit crabs inhabiting *G. unedo* and *G. cosmoi* shells. The sequences from the two species formed clear well-supported monophylies and were clearly distinct, with differences of 1.9% to 5.6% (also see Supplemental Tables S1 and S2 online), and such values have previously been shown to be in line with or above congeneric, interspecific differences in other zoanthids (Reimer et al., 2006; Sinniger et al., 2008; Sinniger et al., 2010). Furthermore, sequences from both markers from both species unexpectedly did not match with either *E. ramosus* or with *E. indicus*. From these preliminary results we can infer two major lines of investigation for future studies; a) the diversity of *Epizoanthus* species in Japanese waters is higher than previously thought, and b) the specificity of certain *Epizoanthus* species may be much more limited than previously thought (i.e. dwelling only on living shells or only on hermit crabs).

While information on *Epizoanthus* species in Japan is limited, it may be that the both species examined here are new, undescribed species. The hermit crab-associated *Epizoanthus* specimens in this study associated with *Paguristes palythophilus*, as seen with *E. paguriphilus*, the morphology of the colonies (thin coenenchyme covering *Gemmula* spp. shells) is completely different from the well developed and incrusting morphology of *E. paguriphilus* as described in other research (Muirhead et al., 1986; Williams and McDermott, 2004), and it is extremely unlikely that specimens in this study are *E. paguriphilus*. Additionally, the true identities of "*E. ramosus*" (originally described on *Paguristes* spp. in Carlgren, 1934) found on living gastropods and of "*E. indicus*" (originally described on the gastropod *Pleurotoma symbiotes* in Lwowsky, 1913) found on crabs clearly need to be reexamined (also Table 1).

While much research has demonstrated the monophyly of the genus *Epizoanthus* using a variety of DNA markers (Sinniger et al., 2005; Reimer et al., 2007), no research has yet closely examined the phylogenetic relations within the genus. The sequences used in this study are relatively short "barcodes", and further examinations with longer sequences may help unravel the phylogenetic relationships within *Epizoanthus*. Thus, while conclusions on the evolution of substrate specificity of *Epizoanthus* subclades are beyond the scope of this examination, it may be that species within this genus may have lost or acquired "free-living", "crab" and "gastropod" lifestyles more than once. It is worth noting that substrate specificity has been shown to have taxonomic utility within the zoanthid families Parazoanthidae and Hydrozoanthidae (Sinniger et al., 2010). The evolution of substrate specificity in *Epizoanthus* is worth examining in future studies with more species and multiple DNA markers of a longer

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length.

It is clear that DNA barcoding and phylogenetic analyses are powerful tools for zoanthid taxonomic analyses. At the same time, shorter DNA sequences as used in this study are sufficient for the "barcoding" of *Epizoanthus* species, further supporting the recommendation in Sinniger et al. (2008) that both DNA (COI and mt 16S rDNA) and often substrate information can be used to accurately identify zoanthid specimens at least to generic levels, and often to species or species-group levels.

As mentioned in Ates (2003), most research on *Epizoanthus* species and their living substrates to date has focused on the non-zoanthid partner. However, until the diversity of both parties is more clearly understood, ecological research into such associations will remain at a standstill (Ates, 2003), and such questions such as what benefits each partner derives from such relationships, and how specificity occurs will remain enigmatic. It is hoped that this research will be a basis for the start of a new era of epizoanthid-host research utilizing both ecological and molecular techniques.

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