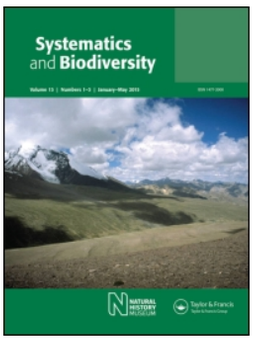

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Research Article

A molecular phylogeny of carcinoecium-forming *Epizoanthus* (Hexacorallia: Zoantharia) from the Western Pacific Ocean with descriptions of three new species

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Many cnidarians have been reported in association with hermit crabs from shallow waters to the deep sea. Some of these actiniarians and zoantharians produce a carcinoecium, a chitin-like pseudo-shell. Many studies have been conducted on hermit crab–actiniarian symbioses, while hermit crab–zoantharian symbioses have received less attention due to the difficulty of specimen collection as they are exclusively found in the deep sea. In this study, 11 carcinoecium-forming specimens associated with hermit crabs of several genera were collected from the western Pacific Ocean. We formally described the collected specimens as *Epizoanthus xenomorphoideus* sp. nov., *E. australis* sp. nov., and *E. gorgonus* sp. nov. based on results of molecular phylogenetic analyses (COI, mt 12S-rDNA, mt 16S-rDNA, 18S-rDNA, ITS-rDNA) and morphological observations. Our phylogenetic results showed that there are two different subclades within a carcinoecium clade, and these subclades have different external morphologies. Further studies with more taxon sampling will enable a more comprehensive evaluation of the evolution and phylogenetic relationships of carcinoecium-forming zoantharian species.

Key words: deep sea, diversity, hermit crab, pseudo-shell, sea anemone, zoantharian

Introduction

The orders Actiniaria Hertwig, 1882 and Zoantharia Rafinesque, 1815 within the subclass Hexacorallia Haeckel, 1896 contain hermit crab-associated species. Actiniarians within the genera *Adamsia* Forbes, 1840, *Calliactis* Verrill, 1869, and *Paracalliactis* Carlgren, 1928 contain species actively positioned on shells inhabited by hermit crabs (Gusmao & Daly, 2010). Additionally, some actiniarian species belonging to genus *Stylobates* produce a carcinoecium, a chitin-like pseudo-shell, that grows as the inhabiting hermit crab also grows, thus eliminating the need for the hermit crab to change shells (Crowther, Fautin, & Wallace, 2011; Dunn, Devaney, & Roth, 1980). Overall, at least 30 associations between different actiniarian and hermit crab species have been noted (Ross, 1974). Much research has been conducted on such symbioses; not only ecological and ethological studies such as examining the symbiotic relationships between species within *Adamsia* and hermit crabs (McLean & Mariscal, 1973; Ross, 1970, 1971), but also several phylogenetic and taxonomic studies (Crowther et al., 2011; Daly, Ardelean, Cha, Campbell, & Fautin, 2004; Dunn et al., 1980; Gusmao & Daly, 2010). However, research on hermit crab-associated zoantharian species lags behind that of Actiniaria due to difficulty of collection as these associated species are almost exclusively found in the deep sea (Ates, 2003; Ryland & Ward, 2016). In particular, there have been only limited studies on the diversity and phylogeny of zoantharians living on shells

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inhabited by hermit crabs (e.g., Ates, 2003; Reimer, Hirose, Nishikawa, Sinniger, & Itani, 2010; Ryland & Ward, 2016). The genus *Epizoanthus* Gray, 1867 within the family Epizoanthidae Delage & Hérouard, 1901 is known to have associations with hermit crabs, and 82 species are valid within the genus *Epizoanthus*. Of these, at least 21 species are associated to shells inhabited by hermit crabs, and of these, at least 13 species are known as carcinoecium-forming species; *E. abyssorum* Verrill, 1885, *E. carcinophilus* Carlgren, 1923, *E. chuni* Carlgren, 1923, *E. frenzeli* Pax, 1937, *E. michaelsarsi* Carlgren, 1923, *E. mortenseni* Carlgren, 1934, *E. paguricola* Roule, 1900, *E. paguriphilus* Verrill, 1883, *E. parasiticus* (Verrill, 1864), *E. papillosus* Johnston, 1842, *E. studeri* Carlgren, 1923, *E. vatovai* Pax & Lochter, 1935, and *E. valdiviae* Carlgren, 1923 (Ates, 2003; Williams & McDermott, 2004). It is known that some of the carcinoecium-producing *Epizoanthus* species listed above dissolve the original mollusc shell while growing in tandem with the hermit crab (Muirhead, Tyler, & Thurston, 1986).

In addition, Muirhead et al. (1986) reported on the reproduction and growth of three carcinoecium-forming *Epizoanthus* species by examining different stages of the development of the relationship. As a result, they suggested that symbiotic relationships between *Epizoanthus* and hermit crabs are a type of mutualism, and these relationships continue during their growth (Muirhead et al., 1986). A subsequent study by Schejter and Mantelatto (2011) also supported the presence of mutualism, although this hypothesis was based on actiniarian–hermit crab associations. Moreover, only a few phylogenetic and diversity studies focusing on carcinoecium-forming species have been performed. Further studies on the ecology, evolution, behaviour, and physiology of *Epizoanthus*–hermit crab associations have not been conducted. Thus, an understanding of the diversity and phylogeny of carcinoecium-forming *Epizoanthus* species is needed to form the basic framework for further studies.

In this study, we collected 11 carcinoecium-forming specimens from the western Pacific Ocean and sequenced five genetic markers (mitochondrial cytochrome oxidase subunit I, mt 12S ribosomal DNA, mt 16S ribosomal DNA, nuclear 18S ribosomal DNA, and internal transcribed spacer region of ribosomal DNA) to investigate the phylogenetic relationships of carcinoecium-forming species within the genus *Epizoanthus*. Based on our results, three new carcinoecium-forming *Epizoanthus* species are formally described herein, as *E. xenomorphoideus* sp. nov., *E. australis* sp. nov., and *E. gorgonus* sp. nov.

Materials and methods

Specimen collection

Epizoanthus specimens were collected by deep water beam trawls in Australia and Japan (Table S1, see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2019.1693439).

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from tissue preserved in 70–99.5% ethanol either by following a guanidine extraction protocol (Sinniger, Reimer, & Pawlowski, 2010) or by using a spin-column DNeasy Blood and Tissue Extraction kit following the manufacturer's instructions (Qiagen, Tokyo, Japan). PCR amplification using Hot Star Taq Plus Master Mix Kit (Qiagen, Tokyo, Japan) was performed for each of COI (mitochondrial cytochrome oxidase subunit I), mt 12S-rDNA (mitochondrial 12S ribosomal DNA), mt 16S-rDNA (mitochondrial 16S ribosomal DNA), 18S-rDNA (nuclear 18S ribosomal DNA), and ITS-rDNA (nuclear internal transcribed spacer region of ribosomal DNA). COI was amplified using the universal primer set LCO1490 and HCO2198 (Folmer et al., 1994) following the protocol by Montenegro, Sinniger, and Reimer (2015). mt 12S-rDNA was amplified using the primer sets ANTMTf and ANTMTr (Chen, Wallace, & Wolstenholme, 2002), and 12S1a and 12S3r (Sinniger, Montoya-Burgos, Chevaldonne, & Pawlowski, 2005), following protocols by Chen et al. (2002) and Sinniger et al. (2005), respectively. mt 16S-rDNA was amplified using the primer set 16SarmL (Fujii & Reimer, 2011) and 16SbmoH (Sinniger et al., 2005), following the protocol by Fujii & Reimer (2011). 18S-rDNA was amplified using the primer set 18SA and 18SB (Medlin, Elwood, Stickel, & Sogin, 1988) and sequenced using 18SL, 18SC, 18SY, and 18SO (Apakupakul, Siddall, & Burreson, 1999), following the protocol by Apakupakul et al. (1999). ITS-rDNA was amplified using the primer set ITSf and ITSr (Swain, 2009), following the protocol by Fujii & Reimer (2011). All PCR products were purified with 1 U of shrimp alkaline phosphatase (SAP) and 5 U of Exonuclease I (Takara Bio Inc., Shiga, Japan) at 37 °C for 40 min followed by 80 °C for 20 min. Cleaned PCR products were sequenced in both directions on an ABI 3730Xl (Fasmac, Kanagawa, Japan). Obtained sequences in this study were deposited in GenBank under accession numbers MN497568–MN508037 (Table S1, see supplemental material online).

Molecular phylogenetic analyses

Sequences were initially aligned in Geneious v10.2.3 (Kearse et al., 2012) using global alignments with free end gaps with the default configuration. Thereafter the sequences were manually trimmed and realigned using MAFFT (Katoh & Standley, 2013) with the algorithm L-INS-i. The above procedure was used for all molecular markers with exception of 18S-rDNA; where the three partial sequences optioned from the primer's pairs 18SA-18SL, 18SC-18SY, and 18SB-18SO were manually concatenated with no overlapping positions. These alignments were combined with GenBank sequences (Table S2) to obtain a dataset of 459 bp for 18 sequences of COI, 700 bp for 17 sequences of mt 12S-rDNA, 611 bp for 23 sequences of mt 16S-rDNA, 1717 bp for 15 sequences of 18S-rDNA, and 579 bp for 24 sequences of ITS-rDNA. These alignments were subsequently concatenated to obtain a final dataset of 4066 bp for 26 OTUs. A minimum of three markers' data was established as the threshold to include or exclude OTUs from the final concatenation (Table S2, see supplemental material online). All aligned datasets are available from the Dryad repository (http://datadryad.org).

Phylogenetic analyses were performed over the concatenated dataset using Maximum likelihood (ML) and Bayesian inference (BI). TOPALi v2.5 (Milne et al., 2009) was used to select the best fitting model for each molecular marker, independently for ML and BI. The best selected models were HKY + G (010010) for COI, HKY (010010) for 18S-rDNA, JC + G (000000) for mt 12S-rDNA, SYM + G (012345) for mt 16S-rDNA, and K80 + G (010010) for ITS-rDNA. Phylogenetic estimations were performed using model partition per each region in RAxML v8.2.11 (Stamatakis, 2014) for ML, and MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003) for BI. RAxML was configured to use the substitution model GTR GAMMA with the '-f a –x 1' algorithm, 1000 bootstrap replicates, and 1 parsimony random seed. MrBayes was configured following the models and parameters as indicated by TOPALi, 4 MCMC heated chains were run for 5,000,000 generations with a temperature for the heated chain of 0.2. Chains were sampled every 200 generations. Burn-in was set to 1,250,000 generations at which point the average standard deviation of split frequency (ASDOSF) was steadily below 0.01. ITS-rDNA has been demonstrated to be the most variable genetic region to delineate species within Zoantharia (e.g., Reimer, Takishita, Ono & Maruyama, 2007). Therefore, we performed phylogenetic analyses focused on ITS-rDNA. The phylogenetic reconstruction of the ITS-rDNA was performed following the best fitting model as indicated by TOPALi for ML and BI. BI phylogenies were generated in the same manner as for the concatenated alignment, but ML trees were generated using the Geneious plug-in of PhyML v3.3.201806221 (Guindon et al., 2010) with the following configuration: 1000 bootstraps, fixed proportion of invariant sites = 0, substitution rate categories = 4, and Gamma distribution = 'Estimated'. *Microzoanthus occultus* was used as the outgroup in ML and BI analyses.

Morphological analyses

Morphological data were collected from preserved specimens, photographs and histological sections; the lengths and diameters of individual polyps, carcinoeciums, polyp dimensions (oral disc diameter, polyp height), tentacle lengths and numbers, colour of polyps, and diameters of oral discs were measured using preserved specimens and photographs. Histological sections of 8–10 µm thickness were made using microtome RV-240 (Yamato Kohki, Asaka, Japan) and stained with haematoxylin and eosin after decalcification with Morse solution for 48 h (1:1 vol; 20% citric acid: 50% formic acid). Classification of marginal muscle shapes followed Swain, Schellinger, Strimaitis, and Reuter (2015). Additionally, marginal muscle position and type, and mesenterial arrangement and number of mesenteries were observed by hand-cutting polyps. Cnidae analyses were conducted using undischarged nematocysts from tentacles, column, actinopharynx, and mesenteries filaments of a single holotype polyp under a Nikon Eclipse80i stereomicroscope (Nikon, Tokyo). Cnidae sizes were measured using ImageJ ver. 1.45 s (Rasband, 2012). Although cnidae classification generally followed England (1991) and Ryland and Lancaster (2004), basitrichs and microbasic b-mastigophores were considered as the same type of nematocyst based on studies by Hidaka (1992), Hidaka, Miyazaki, and Yamazato (1987), and Schmidt (1974), as has been done in other recent zoantharian species descriptions (Kise, Maeda, & Reimer, 2019), and these two types were pooled together.

Abbreviations used

NSMT: National Science Museum, Tsukuba, Ibaraki, Japan; QM: Queensland Museum, Queensland, Australia; MISE: Molecular Invertebrate Systematics and Ecology Laboratory, University of the Ryukyus, Nishihara, Okinawa, Japan.

Results

Systematics

Order Zoantharia Rafinesque, 1815

Suborder Macrocnemina Haddon & Shackleton, 1891
Family Epizoanthidae Delage & Hérouard, 1901
Genus *Epizoanthus* Gray, 1867

Type species: *Dysidea papillosa* Johnston, 1842, by monotypy (see also Opinion 1689, ICZN 1992).

Diagnosis: Macrocnemic zoantharians with simple mesogleal muscle, readily distinguishable from *Palaeozoanthus* by the presence of non-fertile micromesenteries (Sinniger & Häussermann, 2009).

Epizoanthus xenomorphoideus sp. nov.

ZooBank ID (LSID): urn:lsid:zoobank.org:act:D94988E7-5F76-44FB-B632-78BF5FB9C0A6.

Material examined: Holotype. NSMT-Co 1688 (MISE-HK193), Sea of Kumano, Mie, Japan 33°58′30.8″–33°56′79.4″N, 136°21′07″–136°20′51.1″E, 271–351 m, trawl net, coll. T. Moritaki on the fishing trawlers *Jinsho-maru*, 24 January 2014, divided into two pieces, one portion fixed in 5–10% saltwater formalin, and the other in 99.5% ethanol. Deposited in NSMT.

Etymology: The new species is named after a fictional alien xenomorph creature in the famous 1979 movie Alien, as this species resembles the 'face hugger' xenomorph. 'Xenomorph' is combined with the Latin word 'oideus' meaning 'resembling'.

Japanese common name: Yadokari-sunaginchaku

Description: External morphology. Coenenchyme strongly developed, shell inhabited by hermit crab completely dissolved. Carcinoecium-forming. Preserved carcinoecium size 80.1 mm in diameter, 42.7 mm in thickness. Surface of column smooth, and ectoderm with net-like appearance. Cylindrical polyps. Three dorsal polyps and 12 marginal polyps (Fig. 1.1), with one ventral polyp (Fig. 1.3). No polyps attached on the dissolved aperture of shell inhabited by hermit crab. Diameter of capitulum either as large or smaller in comparison to scapus when contracted. Contracted marginal polyps usually well developed and 9.6–22.5 mm in height, 8.4–13.7 mm in diameter ($n = 8$), contracted dorsal polyp 2.9–14.7 mm in length, 7.8–11.1 mm in diameter ($n = 3$), contracted ventral polyp 4.0 mm in length, 5.0 mm in diameter ($n = 1$) when preserved. Capitulary ridges present but not strongly pronounced when contracted. Capitulum of marginal polyps usually located horizontally. Ectoderm and mesoglea of scapus and coenenchyme encrusted with a small amount of sand and silica particles, while ectoderm and mesoglea of capitulum heavily encrusted with numerous sand and silica particles. Tentacles in two rows, 68–76 tentacles in number ($n = 5$), light orange in colouration. Oral disc ~8.0–20.5 mm in diameter, longer than tentacle length. Living colony light orange in colouration, and preserved colony light beige in colouration.

Internal morphology. Zooxanthellae absent. Mesenteries ~66–76, in macrocnemic arrangement. Mesogleal thickness 0.5–2.9 mm and gradually wider in direction from capitulum towards scapus. Mesoglea thicker than ectoderm. Reticulate mesogleal muscle (Fig. 1.5). Marginal muscle completely occupies mesoglea, and lacunae shapes of marginal muscle elliptical. Reticulate marginal muscle smoothly curved into oral disc and usually encrusted by sand and silica particles. Siphonoglyph distinct and U-shaped. Mesenterial filaments present (Fig. 1.6). The basal canals of mesenteries could not be observed as there were numerous sand particles heavily encrusted into ectoderm and mesoglea.

Cnidae. Basitrichs and microbasic *b*-mastigophores, microbasic *p*-mastigophores, holotrichs, and spirocysts (Fig. 2.1, Table S1a, see supplemental material online).

Habitat and distribution: *Epizoanthus xenomorphoideus* sp. nov. was found on muddy bottoms from the Sea of Kumano at a depth of 271–351 m in this study. Additionally, previous records of specimens matching this description also occur in the Gulf of Bengal at a depth of 1483 m (Lwowsky, 1913).

Associated host: *E. xenomorphoideus* sp. nov. has been observed associated with *Sympagurus dofleini* (Balss, 1912).

Remarks: In terms of morphology, *Epizoanthus xenomorphoideus* sp. nov. is identical to carcinoecium-forming specimens collected from the Gulf of Bengal by Lwowsky (1913). Lwowsky (1913) reported these carcinoecium-forming specimens as *E. paguriphilus*. However, Carlgren (1923) examined specimens from Lwowsky (1913) and noted that the specimens from Lwowsky (1913) were morphologically different from *E. paguriphilus*; colonies of *E. paguriphilus* consisted of both ventral and marginal polyps without any dorsal polyps, while the specimens from Lwowsky (1913) consisted of ventral, marginal, and dorsal polyps. Therefore, we conclude that the specimens examined by Lwowsky (1913) are *E. xenomorphoideus* sp. nov., and that this new species can be distinguished from *E. paguriphilus* by colony form and polyp arrangement. Moreover, *Epizoanthus xenomorphoideus* sp. nov. is identical to carcinoecium-forming specimens collected from the Sea of Enshu, Japan by Hertwig (1882). However, Hertwig's (1882) description was lacking a ventral polyp, and there is no illustration for the ventral side of the carcinoecium colony. Hertwig (1882) reported these carcinoecium-forming specimens as *E. parasitiscus* (Verrill, 1864),

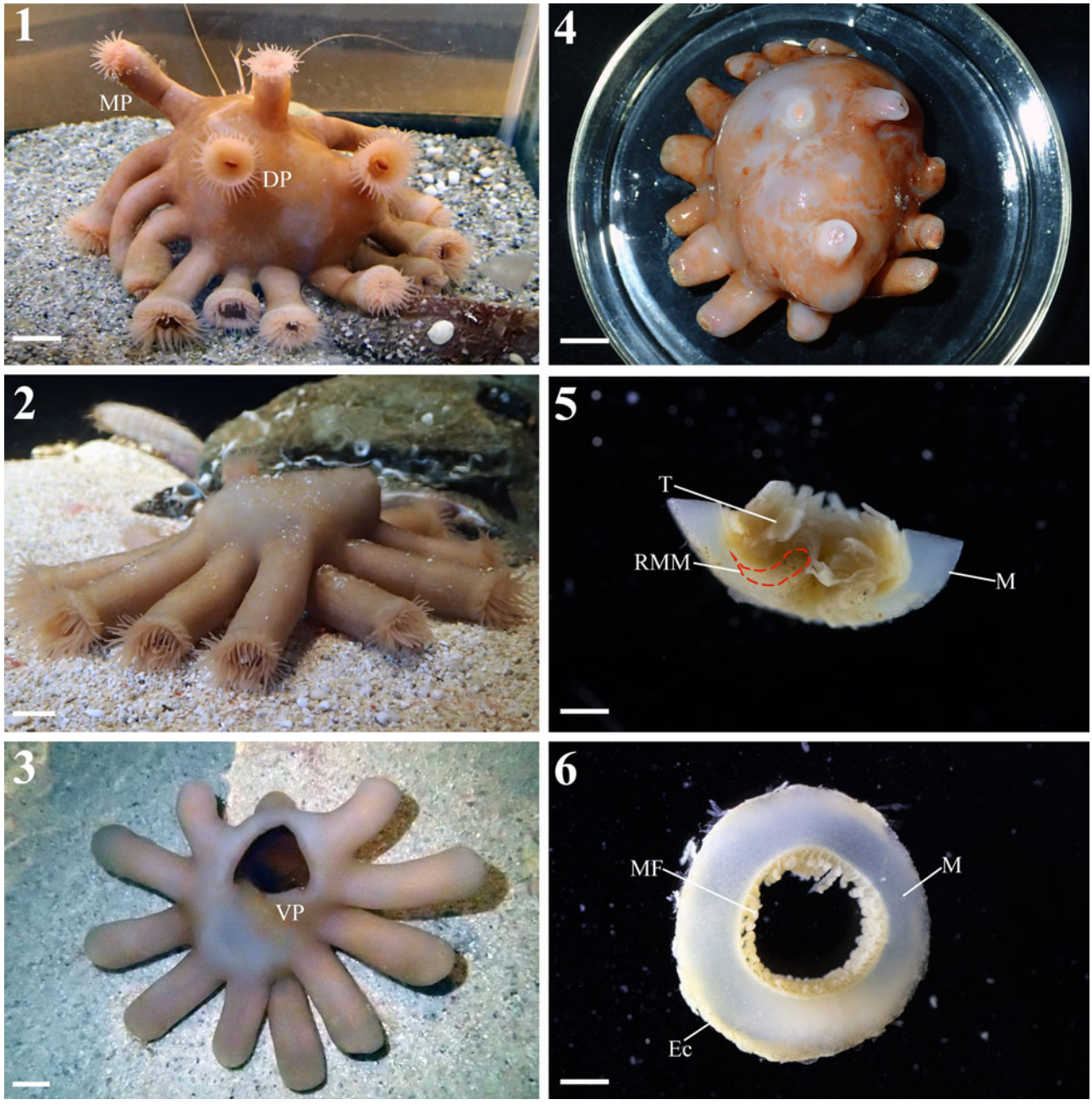

Fig. 1. Images of external and internal morphology of *Epizoanthus xenomorphoideus* sp. nov. (holotype: NSMT-Co 1688). (1) living carcinoecium colony from the dorsal side, (2) living carcinoecium colony from the marginal side, (3) living carcinoecium colony from the ventral side, (4) preserved carcinoecium colony from the dorsal side, (5) longitudinal section of polyp, (6) cross-section of polyp. DP, dorsal polyp; MP, marginal polyp; VP, ventral polyp; T, tentacle; M, mesoglea; RMM, reticulate marginal muscle; MF, mesenterial filament; Ec, Ectoderm. Scale: (1–4) 20 mm, (5–6) 2 mm.

although the polyps of the specimens Hertwig (1882) examined were separated into dorsal and marginal, while the original description of *E. parasiticus* mentioned that the polyps arise in all directions. Therefore, the specimens examined by Hertwig (1882) may correspond to *Epizoanthus xenomorphoideus* sp. nov. However, further investigation with specimens collected from the Sea of Enshu is needed to confirm the taxonomic relationship between *Epizoanthus xenomorphoideus* sp. nov. and the *E. parasitiscus* of Hertwig (1882). In addition, the morphology of *E. valdiviae* from the East African coast is similar to *E.*

xenomorphoideus sp. nov. in having dorsal polyps, but *E. xenomorphoideus* sp. nov. is unique in having not only dorsal polyps but also one ventral polyp.

Epizoanthus australis sp. nov.

ZooBank ID (LSID): urn:lsid:zoobank.org:act:27ECDCD4-E816-4281-A04E-33B1293E2302.

Material examined: Holotype: QM G337292, Central Eastern Commonwealth Marine Reserve (CMR), New South Wales, Australia, 30°05′56.4″–30°07′40.8″S, 153°34′15.6″–153°35′45.6″E, 1194–1257 m, beam trawl, coll. Merrick Ekins on *RV Investigator*, Cruise IN2017_V03, Sample 80-186, 05 June 2017, fixed in 5–10% saltwater formalin. Paratype: QM G337592, off Byron Bay, New South Wales, Australia, 28°03′14.4″–28°05′49.2″S, 154°04′51.6″–154°04′58.8″E, 999–1013 m, beam trawl, coll. Merrick Ekins on *RV Investigator*, Cruise IN2017_V03, Sample 100-193, 09 June 2017, fixed in 99.5% EtOH.

Etymology: 'Australis' is the Latin word 'southern', as this species was first discovered in waters off New South Wales, off south-eastern Australia.

Japanese common name: Kanmuri-yadokari-sunaginchaku

Description: External morphology. Coenenchyme strongly developed, shell originally inhabited by hermit crab completely dissolved. Carcinoecium-forming. Preserved carcinoecium size 18.4–20.0 mm in diameter, 6.7–12.2 mm in thickness. Surface of column rough, ectoderm net-like appearance. Cylindrical polyps. One dorsal polyp and four to six marginal polyps, without ventral polyp (Fig. 3.1, 2). No polyps attached on the aperture of shell inhabited by hermit crab. Capitulum swollen, and diameter of capitulum larger than scapus when contracted. Contracted marginal polyps usually well developed and 6.9–12.6 mm in length, 4.6–8.1 mm in diameter ($n = 5$), and dorsal polyp 1.1–1.9 mm in length, 4.3–5.8 mm in diameter when preserved ($n = 2$). Capitulary ridges clearly present, 25–28 in number. Capitulum of marginal polyps face upwards. Ectoderm and mesoglea of polyps and coenenchyme heavily encrusted with numerous sand and silica particles. Encrusted particles smaller in mesoglea in comparison to those in ectoderm. Tentacles in two rows, totalling 50–56 ($n = 3$) in number. Living colony pink-beige in colouration, and preserved colony light beige in colouration.

Internal morphology. Zooxanthellae absent. Mesenteries ∼50–56, in macrocnemic arrangement. Mesoglea thickness 0.2–0.9 mm and gradually wider in the direction from capitulum towards scapus. Mesoglea thicker than ectoderm. Reticulate mesogleal muscle (Fig. 3.6). Marginal muscle completely occupies mesoglea, and lacunae shapes of marginal muscle elliptical. Reticulate marginal muscle bends at a right angle. Siphonoglyph distinct and V-shaped. Mesenterial filaments present, and actinopharynx short (Fig. 3.4). Basal canals of the mesenteries absent. Cnidae. Basitrichs and microbasic *b*-mastigophores, microbasic *p*-mastigophores, holotrichs, and spirocysts (Fig. 2.2, Table S1a, see supplemental material online).

Habitat and distribution: *Epizoanthus australis* sp. nov. has been found on muddy bottoms in the deep-sea (999–1257 m) off eastern Australia.

Associated host: *Epizoanthus australis* sp. nov. has been observed associated with *Oncopagurus minutus* (Henderson, 1896) and *Parapagurus furici* Lemaitre, 1999.

Remarks: We obtained specimens that were in different stages of development in this study. The initial primary polyp grows around the aperture of the gastropod shell, followed by secondary polyps growing towards the apex. Polyps regularly grow in a row around the gastropod shell. The carcinoeciums of some specimens were still under development, and the body whorl of shell could still be observed. However, dorsal polyps of such specimens were not found, suggesting that the dorsal polyp grows during the final stage of carcinoecium formation. Several characters of the external morphology of *Epizoanthus australis* sp. nov. resemble *E. chuni* from the East African coast (Carlgren, 1923); *E. australis* sp. nov. and *E. chuni* have polyps with swollen capitulum, and marginal polyps in which the capitulum is facing upwards. Additionally, the number of tentacles of these species overlaps (*Epizoanthus australis* sp. nov. 50–56 vs. *E. chuni* 50–60). However, these species are distinguishable based on polyp arrangement and encrustation. *E. chuni* colonies consist of four marginal polyps and a ventral polyp without any dorsal polyp, and encrustation is limited to the swollen capitulum. On the other hand, *E. australis* sp. nov. consists of several marginal polyps and a dorsal polyp without any ventral polyp, and encrustations are observed not only on the swollen capitulum but also on the whole colony.

Epizoanthus gorgonus sp. nov.

ZooBank ID (LSID): urn:lsid:zoobank.org:act:8F11F6F0-B3B8-4705-AD4F-023D347FB77E.

Material examined: Holotype: QM G337296, off Fraser Island, Queensland, Australia, 25°19′31.1″–25°21′04.7″S, 154°04′05.9″–154°04′33.6″E, 2342–2350 m, beam trawl, coll. Merrick Ekins on *RV Investigator*, Cruise IN2017_V03, Sample 115–109, 11 June 2017, fixed in 5–10% saltwater formalin.

Paratypes: QM G337589, Hunter Commonwealth Marine Reserve (CMR), New South Wales, Australia,

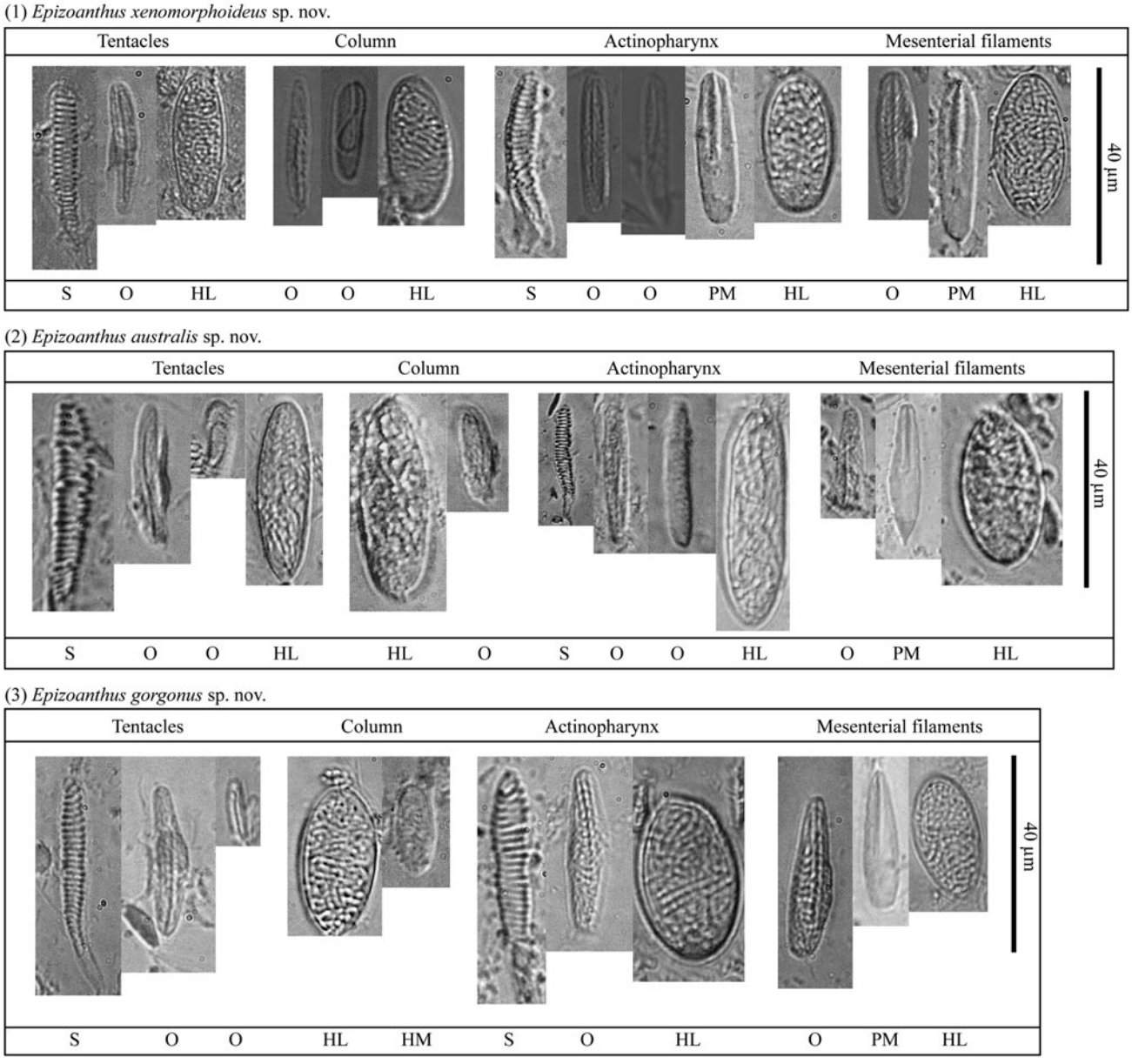

Fig. 2. Cnidae in the tentacles, column, actinopharynx, and mesenterial filaments of holotypes of new species in this study. (1) cnidae of *Epizoanthus xenomorphoideus* sp. nov., (2) cnidae of *E. australis* sp. nov., (3) cnidae of *E. gorgonus* sp. nov. HL, holotrichs large; HM, holotrichs medium; O, basitrichs and microbasic b-mastigophores; PM, microbasic p-mastigophores; S, spirocysts.

32°34′30.0″–32°37′53.9″S, 153°08′31.2″–153°09′42. 1″E, 2474–2595, beam trawl, coll. Merrick Ekins on *RV Investigator*, Cruise IN2017_V03, Sample 70-160, 03 June 2017, fixed in 99.5% EtOH. QM G337581, Bass Strait, Tasmania, Australia, 39°27′43.2″–39°27′54.0″S, 149°14′31.2″–149°16′33.6″E, 2692–2760 m, beam trawl, Coll. Merrick Ekins on *RV Investigator*, Cruise IN2017_V03, Sample 22-167, 22 May 2017, fixed in 99.5% EtOH. QM G337576, Freycinet CMR, Tasmania, Australia, 41°43′49.8″–41°47′28.7″S, 149°07′10.9″ –149°09′20.9″E, 2751–2760 m, beam trawl, coll. Merrick Ekins on *RV Investigator*, Cruise IN2017_V03, Sample 4-163, 18 May 2017, fixed in 99.5% EtOH. NSMT-Co 1689 (MISE-JDR1267), off Boso Peninsula, Chiba, Japan (35°04.17.6″–35°05. 8.6″N, 140°51.32″–140°52.11.8″E), between depths of 959–1011 m, beam trawl, coll. Suguru Ohta on *Tansei-maru*, 18 November 2003, fixed in 99.5% EtOH.

Etymology: From the Greek word 'gorgon', the monster Medusa with multiple snake heads, as the marginal polyps and their colouration are reminiscent of vipers.

Japanese common name: Beni-yadokari-sunaginchaku

Description: External morphology. Coenenchyme strongly developed, original shell inhabited by hermit crab completely dissolved. Carcinoecium-forming. Preserved carcinoecium 28–49 mm in length, 13.8–26.4 mm in thickness. Surface of column smooth, and ectoderm with net-like appearance. Dorsal side of colony dented and wrinkled. Cylindrical polyps. One ventral polyp and five to seven marginal polyps, without any dorsal polyps (Fig. 4.1, 2). No polyps attached on the aperture of shell inhabited by hermit crab. Capitulum swollen, diameter of capitulum either as large or larger in comparison to scapus when contracted. Contracted marginal polyps well developed, 11.2–26.1 mm in length, 5.0–26.6 mm in diameter ($n = 10$), and ventral polyp 6.1 mm in height, 11.1 mm in diameter ($n = 4$) when preserved. Capitulary ridges present but not strongly pronounced when contracted. Capitulum of marginal polyps usually but not always facing upwards. Column of capitulum transparent, marginal muscle and endodermal tissue usually visible. Ectoderm and mesoglea of polyps and coenenchyme encrusted with a small amount of sand particles. Tentacles in two rows, totalling 50 to 60 in number ($n = 5$). Living colony purple and pink in colouration, and preserved colony in alcohol dark violet in colouration.

Internal morphology. Zooxanthellae absent. Mesenteries ~48–60, in macrocnemic arrangement. Mesoglea thickness 0.2–2.6 mm and gradually wider in direction from capitulum towards scapus. Mesoglea thicker than ectoderm. Reticulate mesogleal muscle (Fig. 4.6). Marginal muscle completely occupies mesoglea, and lacunae shapes of marginal muscle elliptical. Reticulate marginal muscle smoothly curved into oral disc. Developed siphonoglyph distinct and U-shaped (Fig. 4.4). Mesenterial filaments present.

Cnidae. Basitrichs and microbasic *b*-mastigophores, microbasic *p*-mastigophores, holotrichs, and spirocysts (Fig. 2.3, Table S1a, see supplemental material online).

Habitat and distribution: *Epizoanthus gorgonus* sp. nov. was found on muddy bottoms of the deep sea (2342–2820 m) off the south-eastern coast of Australia. In addition, this species was also found off Boso Peninsula, Chiba, Japan (959–1011 m).

Associated host: *Epizoanthus gorgonus* sp. nov. has been observed associated with *Parapagurus latimanus* Henderson, 1888, *Parapagurus furici* Lemaitre, 1999, *Parapagurus richeri* Lemaitre, 1999, and Paguroidea sp. Latreille, 1802.

Remarks: *Epizoanthus gorgonus* sp. nov. resembles *E. paguriphilus* from the Atlantic Ocean (Carlgren, 1913; Haddon & Shackleton, 1891; Verrill, 1883) in not having a dorsal polyp and thus having only marginal polyps and a single ventral polyp. Additionally, both species have a posterior polyp which is markedly smaller than the other marginal polyps. However, these two species are distinguishable based on colony size and by the total number of polyps. Colonies of *E. gorgonus* sp. nov. are smaller than *E. paguriphilus* (28–49 mm vs up to 60 mm) and have only five to seven marginal polyps, while marginal polyps of *E. paguriphilus* have six to 14.

We could not observe detailed external and internal morphology for the single specimen from Japanese waters due to its poorly preserved condition. Although sequences of this specimen formed a monophyletic clade with other *E. gorgonus* sp. nov. specimens collected from Australian waters, it is possible that a Japanese specimen is a different species as one base pair insertion/deletion were found in mt 12S-rDNA and ITS-rDNA, respectively. Additionally, the carcinoecium colony of the Japanese specimen is larger than those of the specimens from Australia (28 mm vs 49 mm). However, we have described this clade as single species *E. gorgonus* sp. nov., based on phylogenetic results. Additional specimens from Japanese waters are needed to confirm the taxonomic position of the Japanese specimen examined in this study.

Molecular phylogeny

The phylogenetic tree from the concatenated dataset (COI + mt 12S-rDNA + mt 16S-rDNA + 18S-rDNA + ITS-rDNA) showed that carcinoecium-forming *Epizoanthus* species including *Epizoanthus incrustatus*, *E. paguricola*, *E. xenomorphoideus* sp. nov., *E. australis* sp. nov., and *E. gorgonus* sp. nov. were recovered as a monophyletic clade with moderate support (ML= 65%, BI = 1) (Fig. 5). Within this 'carcinoecium clade', sequences were divided into two groups; one group with polyps arising in all directions as seen in *E. incrustatus* (=*E. papillosus*), with strong support (ML = 98%, BI= 1), and the other group with polyps arranged relatively regularly (e.g., dorsal, marginal, ventral) as seen in the three new species, with strong support (ML = 99%, BI= 1). Additionally, the non-associated species *E. scotinus* Wood, 1957 was placed within the 'all-direction' clade while the free-living species *E. lindahli* Carlgren, 1913 was located in the 'regular arrangement' clade. The phylogenetic tree from ITS-rDNA had almost the same topology with that of the concatenated dataset for both ML and BI with the exception of the

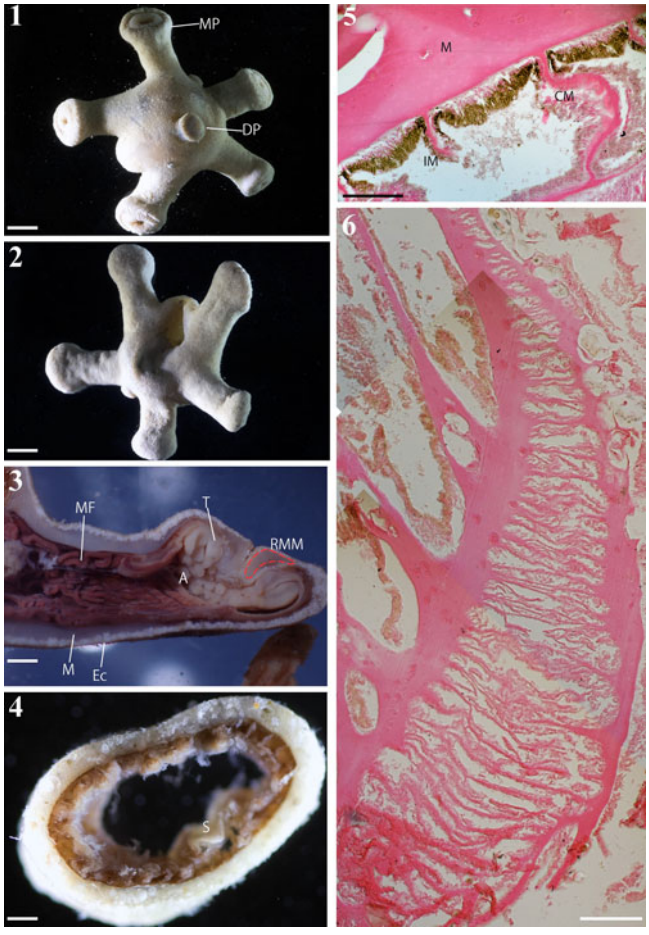

Fig. 3. Images of external and internal morphology of *Epizoanthus australis* sp. nov. (holotype: QM G337292). (1) preserved carcinoecium colony from the dorsal side, (2) preserved carcinoecium colony from the ventral side, (3) longitudinal section of polyp, (4, 5) cross section of polyp, (6) close-up image of reticulate marginal muscle. DP, dorsal polyp; MP, marginal polyp; A, actinopharynx; T, tentacle; M, mesoglea; S, siphonoglyph; CM, complete mesentery; IM, incomplete mesentery; RMM, reticulate marginal muscle; MF, mesenterial filament; Ec, Ectoderm. Scale (1–2) 5 mm, (3–4) 1 mm, (5) 0.5 mm (6) 0.2 mm.

positions of *E. lindahli* and *E. paguricola* (Fig. S1, see supplemental material online).

Discussion

A relatively large number of taxonomic and diversity studies of host hermit crabs have been performed (e.g., Lemaitre, 1996; Lemaitre, Rahayu, & Komai, 2018). From these studies, the taxonomic understanding of host has been improved (Ates, 2003). Taxonomic studies of carcinoecium-forming *Epizoanthus* have placed disproportionate weight on species from the Atlantic Ocean, particularly *E. abyssorum*, *E. papillosus*, and *E. paguriphilus*, which are mainly found from the North Atlantic Ocean and are well-studied (see Ryland & Ward, 2016). However, examinations into the phylogenetic relationships among carcinoecium-forming species have never previously been focused on. In the Indo-Pacific Ocean, several carcinoecium-forming species of *Epizoanthus* have been described in the past by expeditions such as the Challenger Expedition (Hertwig, 1882) and the Deutsche Tiefsee-Expedition (Carlgren, 1923), yet subsequent taxonomic studies of carcinoecium-forming species have not been conducted in the almost 100 years since. Consequently, the specificity of symbiont-host associations still remains unclear (Ates, 2003; Lemaitre et al., 2018). In this study, three new species were found from the Indo-Pacific Ocean, and these species were distinguished from already described carcinoecium-forming species by a combination of morphological data and molecular phylogeny. Our results indicate that polyp arrangement, as previously suggested by Carlgren (1923), may be a key characteristic for delineating carcinoecium-forming species.

Sequences of new carcinoecium-forming species were almost identical in their mitochondrial marker sequences due to the high rates of conservation in anthozoan mitochondrial genes (Shearer, Oppen, Romano, & Wörheide, 2002). However, the current three closely related new species can be distinguished by a combination of mitochondrial and nuclear markers as well as morphological data. Some specimens of *Epizoanthus* sp. having only primary polyps (i.e., QM G337591, QM G337595, and QM G337597) were found in this study, and these specimens were genetically different from the other *Epizoanthus* species we examined. However, we could not obtain complete morphological data as polyps were possibly at an early stage and very small. Therefore, we have kept these specimens as unidentified in this study. Further collections from the Australian deep-sea are needed to confirm the taxonomic position of these unidentified specimens.

Our phylogenetic analyses suggest a single origin for carcinoecium-forming species as they grouped into a monophyletic clade within *Epizoanthus*. Although *E. ramosus*, within another clade, is known as having a symbiotic relationship with hermit crabs, the production of a carcinoecium has not been observed in this species. Carcinoecium-forming *Epizoanthus* species usually have obligate associations with hermit crabs belonging to the family Parapaguridae, which consists of exclusively deep-water species (200–3000 m; Lemaitre, 1996), while other hermit crab-associated *Epizoanthus* species that do not produce a carcinoecium such as *E. ramosus* can be found with relatively shallow-water hermit crab species within the families Diogenidae and Paguridae (Ates

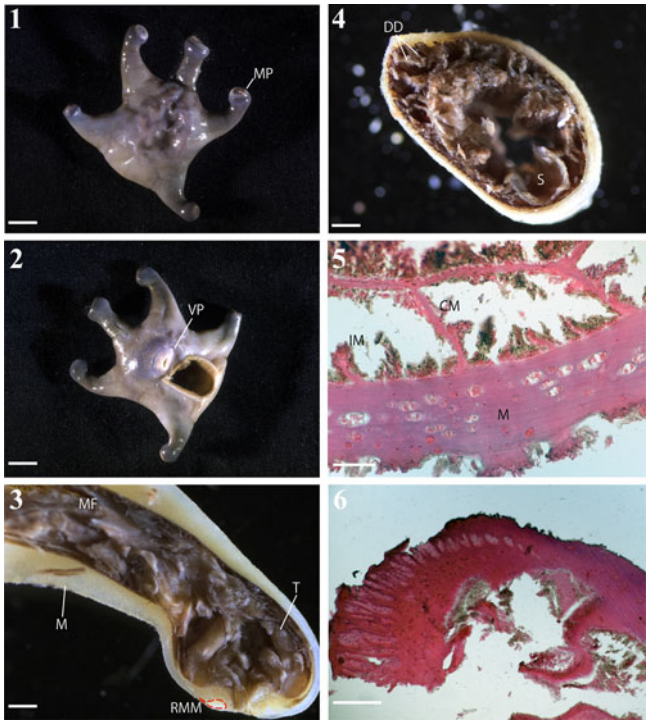

Fig. 4. Images of external and internal morphology of *Epizoanthus gorgonus* sp. nov. (1) preserved carcinoecium colony from the dorsal side (paratype: QM G337589), (2) preserved carcinoecium colony from the ventral side (paratype: QM G337589), (3) longitudinal section of polyp (holotype: QM G337296), (4, 5) cross section of polyp(holotype: QM G337296), (6) close-up image of reticulate marginal muscle (holotype: QM G337296). DP, dorsal polyp; VP, ventral polyp; T, tentacle; M, mesoglea; S, siphonoglyph; DD, dorsal directive; CM, complete mesentery; IM, incomplete mesentery; RMM, reticulate marginal muscle; MF, mesenterial filament. Scale (1–2) 10 mm, (3–4) 1 mm, (5–6) 0.5 mm.

2003). Deep-water benthic environments are usually muddy bottoms that may be uninhabitable for sessile anthozoans (Ryland & Ward, 2016) and where calcium carbonate (e.g., gastropod shell) is rapidly soluble (Correns, 1955), and thus the supply of shells for hermit crabs may be limited (Balss, 1924). However, hermit crabs living in a carcinoecium-forming colony of *Epizoanthus* do not need to change shells as it grows with the crab (Muirhead et al. 1986). Moreover, carcinoecium-forming colonies may not dissolve in deep-water environments as seen in actiniarians (e.g., Dunn et al., 1980). Therefore, the symbiotic relationship between *Epizoanthus* and hermit crabs along with carcinoecium production may have evolved to adapt to such environments.

Two clades ('all-direction' and 'regular arrangement') existed within carcinoecium-forming species based on results from this study. Species within the 'all-direction' clade usually encrust comparatively more sand and silica particles than species within the 'regular arrangement' clade. On the other hand, 'regular arrangement' species usually formed larger carcinoecium colonies than 'all-direction' species. Such morphological differences may occur due to associations with different host hermit crabs or/and original gastropod shells before carcinoecium-forming species settle. In fact, different forms and shapes of carcinoecium have been observed in *E. paguricola* based on the differences in original gastropod shells (Schejter & Mantelatto, 2011). For host hermit crabs, carcinoecium-forming zoantharian species have associations with hermit crabs of seven genera within three different families according to Ates (2003), Williams and McDermott (2004), and this study.

Previous studies have demonstrated that sizes of spirocyst and some nematocyst types are correlated with polyp size (Francis, 2004; Ryland, Brasseur, & Lancaster, 2004). Therefore, we theorize that the sizes of spirocysts and nematocysts in the new carcinoecium-forming species are larger than those in other *Epizoanthus* species as these new carcinoecium-forming species have bigger polyps. Ryland and Ward (2016) showed that carcinoecium-forming *E. papillosus* (= *E. incrustatus* that is subjective synonym of *E. papillosus* in phylogenetic tree (Fig. 5)) within the 'all-direction' clade has no holotrichs in the tentacles. However, the new carcinoecium-forming species colonies in this study have spirocysts and nematocysts including large holotrichs in their tentacles. Such cnidom differences between 'all-direction' and 'regular arrangement' are perhaps due to differences in the interactions with host hermit crabs including feeding and protection. Thus, accurate identifications of both gastropod and hermit crab species as well as descriptions of cnidae are needed to better understand the evolutionary relationships between 'all-direction' and 'regular arrangement' species. In this study, we could not obtain any sequences of previously described species such as *E. paguriphilus*. However, we were successful in examining two carcinoecium-forming species within the 'all-direction' clade. Further taxon sampling will enable the construction of a more comprehensive phylogeny of carcinoecium-forming species.

Some carcinoecium-forming species are known to also have a free-living form. In this study, we found the free-living species *E. lindahli* within the carcinoecium-forming clade, and no sequences of carcinoecium-forming species matched with those of *E. lindahli*. In the original description of *E. lindahli*, Carlgren (1913) mention that *E. lindahli* does not form a carcinoecium.

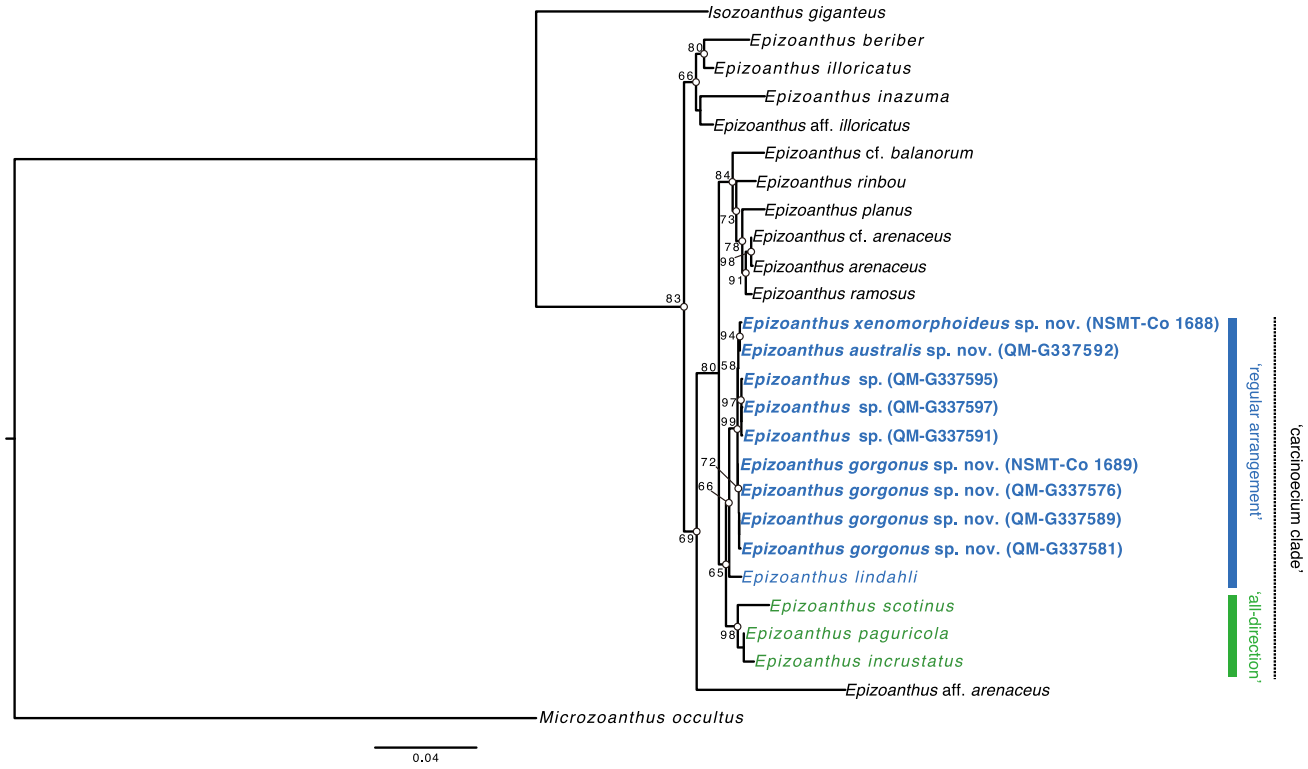

Fig. 5. Maximum likelihood tree based on combined dataset of COI, mt 12S-rDNA, mt 16S-rDNA, 18S-rDNA, and ITS-rDNA. Number at nodes represent ML bootstrap values (>50% are shown). White circles on nodes indicate high support of Bayesian posterior probabilities (>0.95).

Although our phylogenetic results suggest that *E. lindahli* may have both free-living and carcinoecium-forming colonies as this species was located within the carcinoecium-forming clade, no sequences of ITS-rDNA for *E. lindahli* are available. Moreover, the carcinoecium-forming species *E. paguricola* is phylogenetically close to the non-associated species *E. scotinus* (Fig. 5). However, sequences of ITS-rDNA for *E. paguricola* are not available. Therefore, the phylogenetic position of *E. lindahli* and *E. paguricola* may be amended in the future.

It is known that morphological characteristics between carcinoecium-forming and free-living colonies are different. For instance, free-living *E. papillosus* colonies have fewer polyps than carcinoecium-forming colonies of the same species (3–5 polyps vs up to 17 polyps) (Ryland & Ward, 2016). Therefore, it is difficult to match free-living and carcinoecium-forming colonies by basing decisions only on morphological aspects. However, studies focused on phylogenetic comparisons between these forms have never been attempted. Thus, the matching of carcinoecium-forming and free-living colonies through DNA barcodes is needed to avoid misidentification and misunderstanding of the relationships between these forms.

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Supplemental data

Supplemental data for this article can be accessed here: https://doi.org/10.1080/14772000.2019.1693439.

Disclosure statement

No potential conflict of interest was reported by the authors.

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