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ORIGINAL ARTICLE

Epizoanthidae (Hexacorallia: Zoantharia) associated with *Granulifusus* gastropods (Neogastropoda: Fasciolariidae) from the Indo-West Pacific

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Abstract

There have been few detailed studies on the associations between cnidarians and gastropod mollusks. Several hypotheses have been suggested to explain the origins of these symbioses. However, respective benefits for gastropod mollusks and cnidarians have generally not been well examined, as there are many understudied cnidarian taxa associated with gastropod mollusks, and in particular, species of the order Zoantharia in the family Epizoanthidae remain poorly studied. In this study, we examined family Epizoanthidae specimens associated with *Granulifusus* gastropods via morphological observations combined with molecular phylogenetic analyses. Based on our results, we formally describe a new *Epizoanthus* species from the northwest Pacific Ocean. Our phylogenetic analyses recovered at least two independent origins for associations with gastropod in Epizoanthidae. Furthermore, during the course of this work, we reconfirm the existence of the enigmatic species *Paleozoanthus reticulatus*, the only species of the genus *Paleozoanthus*, for the first time since its original description, and we add information on this species' morphological characteristics. This study provides a basis for evolutionary and behavioral studies of symbiotic associations between zoantharians and gastropods. Continued investigations examining the diversity of gastropod-associated zoantharians have the potential to greatly expand our overall comprehension of anthozoan-gastropod symbioses.

Keywords Symbiosis · Evolution · Behavior · Phylogeny · New species

Introduction

Some cnidarians are known to live on the shells of live gastropods. These symbiotic associations between cnidarians and shelled gastropods have been reported in several studies such as between the actiniarian *Allantactis parasitica* Danielssen (1890), and *Buccinum undatum* Linnaeus, 1758 (Mercier & Hamel, 2008), and between the hydrozoan *Cytaeis capitata* (Puce et al., 2004), and *Nassarius globosus* (Quoy & Gaimard, 1833) (Puce et al., 2004).

However, there have been few detailed studies of the associations between cnidarians and live gastropod mollusks with an external shell, particularly in comparison to cnidarian association with gastropod shells occupied by hermit crabs (e.g., Brooks & Gwaltney, 1993; Gusmão et al., 2020; Williams & McDermott, 2004). Several hypotheses have been suggested to explain symbioses between cnidarians and live gastropod mollusks. For instance, the symbiosis between *A. parasitica* and *B. undatum* provides advantages to both partners, including mobility, substrate, and food acquisition to *A. parasitica*, while *B. undatum* may receive

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protection from *A. parasitica* through camouflage as well as from the cnidarian's nematocysts (Mercier & Hamel, 2008). However, overall, respective benefits for gastropod mollusks and cnidarians have generally not been well examined (Puce et al., 2008), and there are many understudied cnidarian taxa associated with gastropod mollusks. Therefore, basic fundamental studies in terms of taxonomy and ecology on such understudied cnidarians are needed.

One such understudied taxon is the order Zoantharia Rafinesque, 1815. Symbiotic associations between several species within the zoantharian family Epizoanthidae Delage & Hérouard, 1901, and gastropods have been documented (e.g., Haddon & Duerden, 1896; Lwowsky, 1913; Carlgren, 1924; Reimer et al., 2010; Kise & Reimer, 2019) at mesophotic and deeper depths (Reimer et al., 2019). Within Epizoanthidae, some species of the genera *Epizoanthus* Gray, 1867, and *Paleozoanthus* Carlgren, 1924, are known to be associated with shelled gastropods, such as *E. egeriae* Haddon & Duerden, 1896, associated with *Murex* spp., *E. indicus* (Lwowsky, 1913) associated with *Borsonia symbiotes* (Wood-Mason & Alcock, 1891) from the Indo-West Pacific (see Kise & Reimer, 2019), *E. mediterraneus* Carlgren, 1935, associated with *Murex* spp. from the Mediterranean Sea, and *Paleozoanthus reticulatus* Carlgren, 1924, associated with *Granulifusus rubrolineatus* (Sowerby II, 1870) from off South Africa. Although many *Epizoanthus* species associated with gastropods have been reported on, the monotypic species *Paleozoanthus reticulatus* has never subsequently been reported since its original description. In summary, our knowledge of Epizoanthidae-mollusk associations is still fragmentary (Reimer et al., 2010).

In this study, to help address this knowledge gap, we examined Epizoanthidae specimens associated with gastropods collected from the northwest Pacific Ocean, as well as a single specimen in the collection of the KwaZulu-Natal Museum in South Africa. Our morphological observations combined with molecular phylogenetic analyses led us to formally describe some Pacific Ocean specimens as a new zoantharian species, *Epizoanthus protoporos* sp. nov. In addition, we examine the symbiosis between *E. protoporos* sp. nov. and its host *G. niponicus*. Furthermore, we report on the existence of *P. reticulatus* for the first time since its original description, and add information on this species' morphological characteristics.

Materials and methods

Specimen collection

Regarding specimens from Japan, three *Epizoanthus* specimens associated with *Granulifusus niponicus* were collected at depths of 250 to 300 m via trawl net on the fishing trawler *Jinsho-maru* from the Sea of Kumano, Mie, Japan (33°54′44.6″N–33°56′0.03.7″N, 136°17′47.8″E–136°19′37.6″E) by Moritaki on December 26, 2016, and March 17, 2019. After observation of living specimens, specimens were initially fixed in 5–10% seawater formalin and were then later preserved in 70% ethanol for morphological observations. Subsamples were preserved in 99.5% ethanol for molecular analyses. Newly collected specimens were deposited in the National Science Museum, Tsukuba, Ibaraki, Japan.

Furthermore, we examined a single specimen associated with *Granulifusus rubrolineatus* in the Mollusca collections at KwaZulu-Natal Museum (NMSA-P1196), collected from off South Africa.

Morphological observation

External morphological characters of the preserved specimen were examined using in situ images and dissecting microscope. Internal morphological characters were examined by histological sections; 10–15 mm thickness of serial section were made with microtome LEICA RM2145 (Leica, Germany) and stained with hematoxylin and eosin after decalcification with Morse solution for 48 h (1:1 vol; 20% citric acid: 50% formic acid) and desilication with 20% hydrofluoric acid for 18–24 h. Classification of marginal muscle shapes followed Swain et al. (2015). Cnidae analyses were conducted using undischarged nematocysts and spirocysts from tentacles, column, actinopharynx, and mesenterial filaments using a Nikon Eclipse80i stereomicroscope (Nikon, Tokyo). Cnidae sizes were measured using ImageJ v1.45 s (Rasband, 2012). The reported frequencies are the relative amounts based on numbers from all slides in the cnidae analyses. Cnidae classification generally followed England (1991) and Ryland and Lancaster (2004) exception for the treatment of basitrichs and microbasic b-mastigophores as mentioned in Kise et al. (2019).

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from tissue by using a spin-column DNeasy Blood and Tissue Extraction kit following the manufacturer's instructions (Qiagen, Hilden, Germany). PCR amplification using Hot Star Taq Plus Master Mix Kit (Qiagen, Hilden, Germany) and TaKaRa Ex Taq™ (Takara Bio Inc., 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 et al. (2015). mt 12S-rDNA was amplified using the primer sets ANTMTf and ANTMTr (Chen et al., 2002), and 12S1a and 12S3r (Sinniger et al., 2005), following protocols by Chen et al. (2002) and Sinniger et al. (2005), respectively. mt 16S-rDNA was amplified using the primer set (Sinniger et al., 2010) and 16SbmoH (Sinniger et al., 2005), following the protocol by Sinniger et al. (2005). 18S-rDNA was amplified using the primer set 18SA and 18SB (Medlin et al., 1988) and sequenced using 18SL, 18SC, 18SY, and 18SO (Apakupakul et al., 1999), following the protocol by Swain (2010). ITS-rDNA was amplified using the primer set ITSf and ITSr (Swain, 2009), following the protocol by Swain (2010). All PCR products were purified with shrimp alkaline phosphatase (SAP) and 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 by Fasmac (Kanagawa, Japan) and Macrogen (Tokyo, Japan). Obtained sequences in this study were deposited in GenBank under accession numbers ON007050 – ON074339 (Table S1).

Molecular phylogenetic analyses

Sequences were initially aligned in Geneious v10.2.3 (Kearse et al., 2012). Thereafter, the sequences were manually trimmed and realigned using MAFFT (Katoh & Standley, 2013) with the auto algorithm under default parameters for all genetic markers. A minimum of data from three markers was established as the threshold to include or exclude OTUs from the final combined dataset (Table S1). All aligned datasets are available from Figshare (https://doi.org/10.6084/m9.figshare.19545556). Phylogenetic analyses were performed on the concatenated dataset using Maximum likelihood (ML) and Bayesian inference (BI). ModelTest-NG v0.1.6 (Darriba et al., 2019) under the Akaike information criterion was used to select the best fitting model for each molecular marker independently for both ML and BI analyses. The best selected models for ML and BI analyses were TPM2uf + I + G (GTR + G) for COI, TPM3uf + G (HKY + G for BI) for mt 12S-rDNA, TIM3 + G (GTR + G) for mt 16S-rDNA, HKY + I for 18S-rDNA, TVM + G (GTR + G for BI) for ITS-rDNA, and TIM1 + I + G (GTR + I + G for BI) for 28S-rDNA. Independent phylogenetic analyses were performed using model partitioning per each region in RAxML-NG v0.9.0 (Kozlov et al., 2019) for ML, and MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003) for BI. RAxML-NG was configured to use 12,345 initial seeds, search for the best tree among 100 preliminary parsimony trees, branch length was scaled and automatically optimized per partition, and model parameters were also optimized. MrBayes was configured following the models and parameters as indicated by ModelTest-NG, 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. Parazoanthidae genera sequences were used as the outgroup in ML and BI analyses (Table S1).

Results

Taxonomic account.

Order Zoantharia Rafinesque, 1815

Suborder Macrocnemina Haddon & Shackleton, 1891

Family Epizoanthidae Delage & Hérouard, 1901.

Genus *Paleozoanthus* Carlgren, 1924

Type species. *Paleozoanthus reticulatus* Carlgren, 1924, by original designation and monotypy.

Paleozoanthus reticulatus — Carlgren (1938: 103), fig. 53.

Diagnosis. Macrocnemic zoantharians with a simple mesogleal muscle, and both complete and incomplete mesenteries fertile.

Paleozoanthus reticulatus Carlgren, 1924

Figure 1a–c

Material examined. NMSA-P1196, collected from off the Mgazi River mouth, South Africa (43°0′09.0″S, 29°30′05.0″E) at a depth of 100 m, collected by National Research Institute for Oceanology of the Council for Scientific and Industrial Research, 15 July 1982, fixed in 75% ethanol, deposited in the KwaZulu-Natal Museum, South Africa.

Description. External morphology. Colonial macrocnemic zoantharian associated with gastropod *Granulifusus rubrolineatus*. The examined specimen consists of six truncated cone-like shaped polyps, 1.7–3.1 mm in height and 3.5–5.5 mm in diameter when contracted (Fig. 1a, b). The six polyps connected by thin mesh-like coenenchyme and regularly arranged on the shell margin. Coenenchyme completely covering the gastropod shell (Fig. 1a). No polyps attached on the aperture of the *G. rubrolineatus* shell (Fig. 1b). Surface of column rough, and ectoderm and mesoglea of scapus and coenenchyme encrusted with numerous sand and silica particles. Tentacles in two rows, number not available, but estimated as 20–24 based on numbers of capitulary ridges. Capitulary ridges present, 10–12 in number, visible in contracted polyps.

Internal morphology. We could not obtain cross-sections or images to observe internal morphology due to the poor condition of the specimen.

Cnidae. Basitrichs and microbasic b-mastigophores, holotrichs, and spirocysts (Fig. 2, Table 1).

Distribution. The type locality of *Paleozoanthus reticulatus* is the Agulhas Bank, South Africa (35°16′00.0″S, 22°26′07.0″E). With the current specimen, we confirmed the presence of this species in the Eastern Cape, South Africa, as the examined specimen was collected from off the Mgazi River mouth, South Africa.

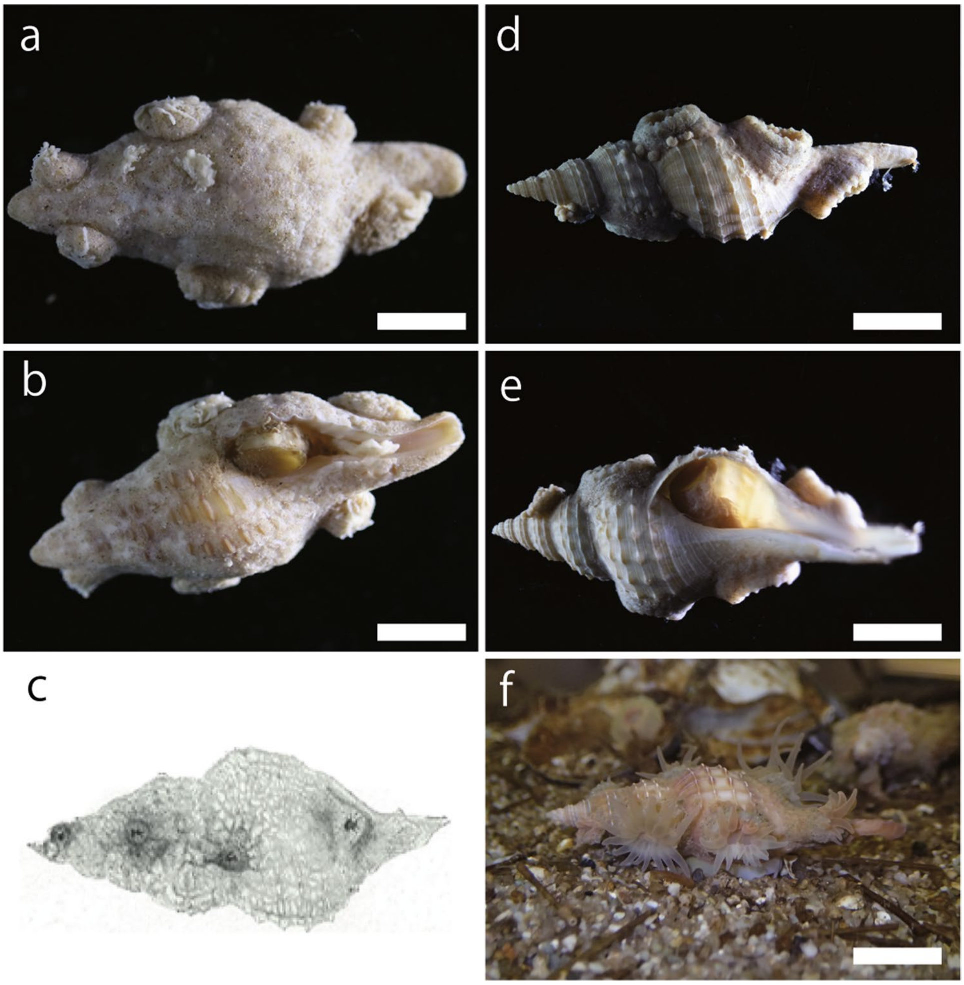

Fig. 1 External morphology of *Palezoanthus reticulatus* and *Epizoanthus protoporos* sp. nov. **a**, **b** Overall view of the preserved specimen of *P. reticulatus* (NMSA-P1196). **c** Original drawing of *P. reticulatus* (Carlgren, 1924). **d**, **e** Overall view of the preserved specimen of *Epizoanthus protoporos* sp. nov. (holotype: NSMT-Co1797). **f** A living colony on *Granulifusus niponicus* in aquarium. Scale bars: 1 cm (**a**, **b**, **d**–**f**)

Associated host. *Paleozoanthus reticulatus* is associated with *Granulifusus rubrolineatus*.

Remarks. *Paleozoanthus reticulatus* was described in brief by Carlgren (1924) based on a single specimen collected from the Agulhas Bank, off South Africa (Fig. 1c). The original description is not very complete, although it includes internal and external morphology, as Carlgren (1924) examined a single specimen that was poorly preserved. In general, gametes of zoantharians develop only on complete mesenteries (Ryland, 1997), while *P. reticulatus* is unique in having gametes develop in both complete and incomplete mesenteries (Carlgren, 1924, 1938). The present study could not confirm the presence of fertilized incomplete mesenteries due to the poor condition of the examined specimen, although in other regards the examined specimen corresponds well to the description of Carlgren (1924). Although the capitulary ridges of examined specimens are 10–12, the original description reported 24 capitulary ridges with 24 tentacles and mesenteries. The numbers of the capitulary ridges are usually half the tentacles and mesenteries (Swain et al., 2016), and therefore, the number of the capitulary ridges determined in this study is likely correct. Although *P. reticulatus* had been treated as a taxon inquirendum due to a lack of information (Reimer & Sinniger, 2020), this study reports on the existence of *P. reticulatus* for the first time since its original description, based on our identification made with the associated gastropod species and the zoantharian's external morphological characters.

Genus *Epizoanthus* Gray, 1867

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

Duseideia papillosa — Johnston (1842: 190–191), fig. 18, *Mammillifera incrustata* — Düben & Koren (1847: 268), *Sidisia barleei* — Gray (1858: 557–560), pl 5, fig. 8, *Zoanthus couchii* — Landsborough (1852: 225), *Zoanthus incrustatus* — Sars (1860), 141, *Epizoanthus americanus* — Verrill (1864: 34, 45), *Epizoanthus incrustatus* — Haddon & Shackleton

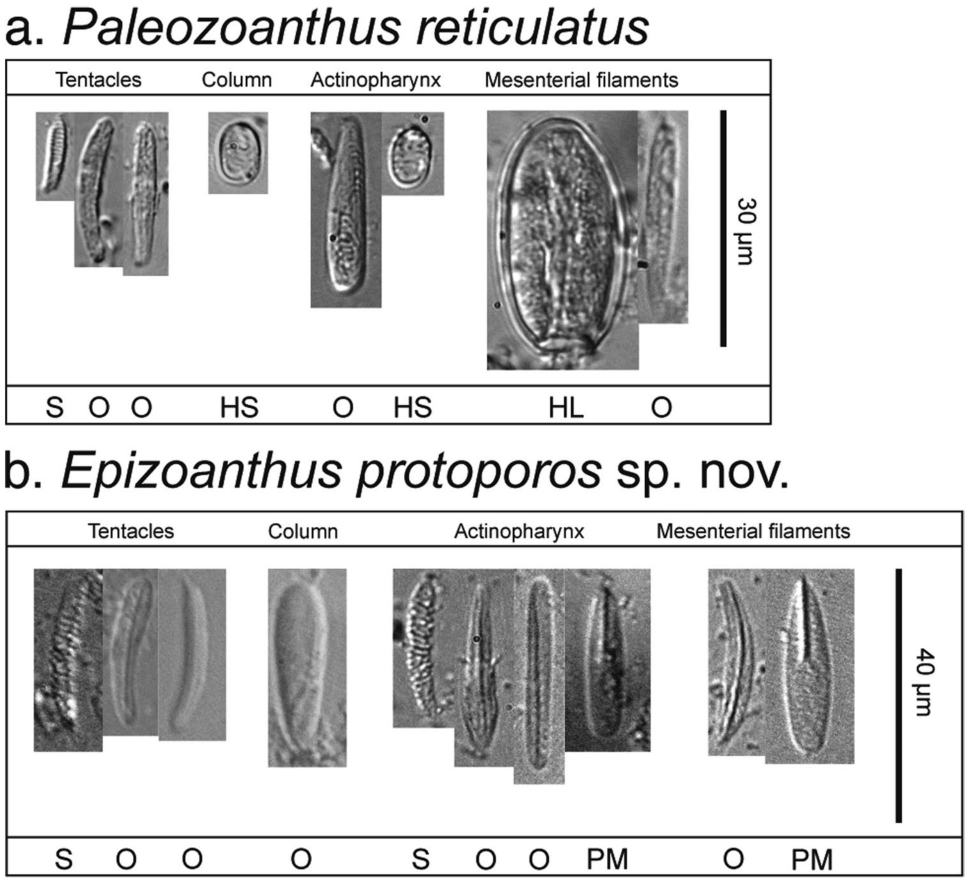

Fig. 2 Cnidae in the tentacles, column, actinopharynx, and mesenterial filaments of *Palezoanthus reticulatus* and *Epizoanthus protoporos* sp. nov. (holotype: NSMT-Co 1797). Abbreviations: HL: holotrich large, HS: holotrich small, O: basitrichs and microbasic *b*-mastigophores, PM: microbasic *p*-mastigophores, S: spriocysts

(1898: 636–616), pl 58, fig. 1- 22, pl 59, fig. 2, pl 60, 1, *Epizoanthus papillosum* — Cutress & Pequegnat (1960: 98).

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

Epizoanthus protoporos sp. nov.

Figure 1d–f, Fig. 4a–f

Material examined. Holotype: Sea of Kumano, Mie, Japan (33°56′03.7″N, 136°19′37.6″E), 300 m depth, December 26, 2016, NSMT-Co 1797. Paratypes: Sea of Kumano, Mie, Japan (33°54′44.6″N, 136°17′47.8″E), 250 m depth, March 17, 2019, NSMT-Co 1798; Sea of Kumano, Mie, Japan (33°54′44.6″N, 136°17′47.8″E), 250 m depth, March 17, 2019, NSMT-Co 1799.

Description. External morphology. Colonial macrocnemic zoantharian associated with gastropod *Granulifusus niponicus* (Smith, 1879). The holotype is a colony consisting of seven polyps on a *G. niponicus* shell (Fig. 1d–f). Polyps of living holotype truncated and cone-like in shape, and 2.0–3.9 mm in height, 9.1–14.5 mm in diameter when expanded (Fig. 1f). Polyps of preserved holotype dark beige in coloration, 2.7–4.7 mm in height from coenenchyme, 5.3–9.1 mm in diameter when contracted. Polyps partially connected by thin coenenchyme and regularly arranged on the shell margin (Fig. 1d, e). No polyps attached on the aperture of the *G. niponicus* shell (Fig. 1e). Ectoderm and mesoglea of scapus and coenenchyme heavily encrusted with numerous sand and silica particles, while ectoderm and mesoglea of capitulum encrusted with a small amount of sand and silica particles. Tentacles in two rows, 28–32 in number, light beige and/or pale red in coloration. Tips of tentacles usually cream in coloration. Tentacles thick and longer than expanded oral disk diameter, 3.5–14.1 mm in length and 1.0–2.6 in diameter. Capitulary ridges present and strongly pronounced when contracted, 14–16 in number. Oral disk small, 5.0–6.4 mm in diameter, light beige and/or pale red in coloration, oval protrusion has a slit-like mouth when expanded. There was no noteworthy variation between holotype and paratypes.

Internal morphology. Zooxanthellae absent. Mesenteries approximately 28–32, in macrocnemic arrangement (fifth mesentery complete). Mesoglea thickness 250–300 µm at the actinopharynx region. Encircling sinus consisting of oval and flattened lacunae present. Large lacunae in mesoglea and ectoderm resulting from dissolution of encrustations consist of sand and

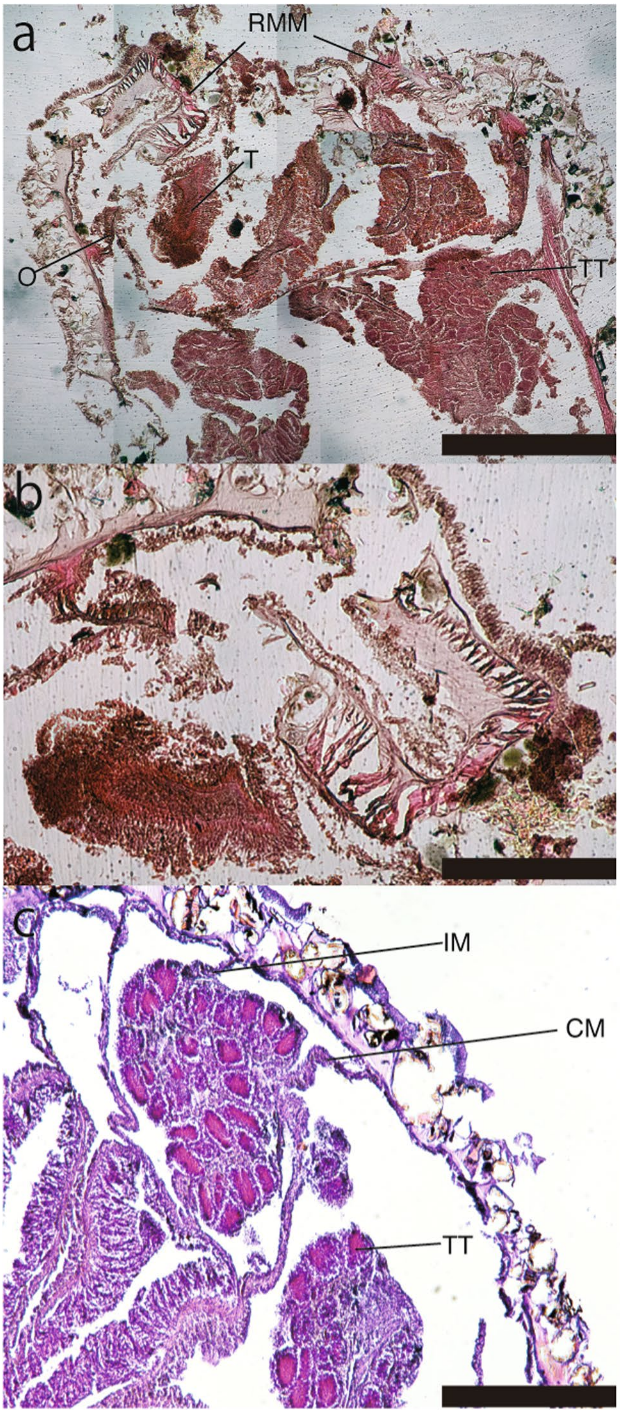

Fig. 3 Internal morphology of *Epizoanthus protoporos* sp. nov. (holotype: NSMT-Co 1797). **a** Longitudinal section of polyp. **b** Closed-up image of reticulate marginal muscle. **c** Cross-section of polyp at level of mesenterial filaments. Abbreviations: CM: complete mesentery, IM: incomplete mesentery, O: oral disk, RMM: reticulate marginal muscle, T: tentacle, TT: testis. Scale bars: 3 mm (**a**), 200 µm (**b**), 500 µm (**c**)

silica particles by hydrofluoric acid. Mesoglea thicker than ectoderm. Reticulate mesogleal muscle. Reticulate marginal muscle bends at a right angle (Fig. 3a, b). Complete mesenteries fertile (Fig. 3c).

Cnidae. Basitrichs and microbasic b-mastigophores, microbasic p-mastigophores, and spirocysts (Fig. 2, Table 1).

Distribution. *Epizoanthus protoporos* sp. nov. has been only found in Japanese waters around the Sea of Kumano, Mie, at depths of 250–300 m.

Notes. Although *Granulifusus niponicus* was not often active in the aquarium, we observed some behaviors. Occasionally, the front end of its foot stretched and stroked the polyps of *Epizoanthus protoporos* sp. nov. (Fig. 4). At this time, the tip of the foot was bifurcated so as to pinch the polyps of *Epizoanthus protoporos* sp. nov., and the foot twisted strongly against the distal polyps to rotate the shell and bring the tip of the foot closer to the polyps.

Molecular phylogeny. The results of the phylogenetic analyses using the concatenated dataset are shown in Fig. 5. Sequences of *Antipathozoanthus remengesaui*, *B. puetoricense*, *Paraozoanthus darwini*, *Savalia savaglia*, and *Umimayanthus chanpuru* were used as outgroup. *Epizoanthus* formed a monophyletic clade with complete support (ML = 100%, BI = 1.00). Within the *Epizoanthus* clade, *Epizoanthus protoporos* sp. nov. was sister to *E. rinbou* Kise and Reimer 2019 with moderate support (ML = 59%, BI = 0.99). Another gastropod-associated species, *Epizoanthus* sp. S02, was located within another clade consisting of *Epizoanthus ramosus*, and is known to have an association with hermit crabs within the families Diogenidae and Paguridae (Ates, 2003). Thus, this study recovered at least two independent origins for symbioses between zoantharians and gastropods.

Remarks. Including the current study, there are two zoantharian species associated with *Granulifusus* gastropods; *Paleozoanthus reticulatus* and *Epizoanthus protoporos* sp. nov. However, there are distinct morphological differences separating these species. The polyp size of *Epizoanthus protoporos* sp. nov. is larger than that of *P. reticulatus* (2.7–4.7 mm in height and 5.3–9.1 mm in diameter vs 1.7–3.1 mm in height and 3.5–5.5 mm in diameter). Additionally, *Epizoanthus protoporos* sp. nov. has 28–32 tentacles, while *P. reticulatus* has 20–24 tentacles. *Epizoanthus protoporos* sp. nov. is also distinguished from other Indo-Pacific gastropod–associated *Epizoanthus* species, *E. thalamophilus* Hertwig, 1888, *E. egeriae*, *E. indicus*, and *E. rinbou*, by combinations of morphological characteristics and molecular differences (Table 2). *Epizoanthus protoporos* sp. nov. can be easily distinguished from both *E. thalamophilus* and *E. indicus* as the coenenchyme of the two latter species are continuous and cover associated gastropod shells completely, while

Table 1 Cnidae types and sizes observed in *Paleozoanthus reticulatus* and *Epizoanthus protoporos* sp. nov. Frequency: relative abundance of cnidae type in decreasing order; numerous, common, occasional, rare (n = number of cnidae)

		Paleozoanthus reticulatus				*Epizoanthus protoporos* sp. nov			
		Length (min–max, average)	Width (min–max, average)	*n*	Frequency	Length (min–max, average)	Width (min–max, average)	*n*	Frequency
Tentacles	Spirocysts	9.0–16.0, 12.2	1.0–3.0, 2.0	47	Numerous	14.0–31.0, 22.6	2.0–6.0, 3.5	180	Numerous
	Bastrichs and microbasic b-mastigophores	12.0–21.0, 17.7	2.0–4.0, 2.8	98	Numerous	18.0–31.0, 23.4	1.0–6.0, 3.5	153	Numerous
Column	Bastrichs and microbasic b-mastigophores	—	—	—	—	13.0–30.0, 27.0	3.0–9.0, 5.8	32	Common
	Holotrich small	7.0–8.0, 7.7	4.0–6.0, 5.0	3	Rare	—	—	—	—
Pharynx	Spirocysts	—	—	—	—	19.0–23.0, 21.6	3.0–4.0, 3.6	7	Rare
	Bastrichs and microbasic b-mastigophores	11.0–24.0, 21.4	3.0–5.0, 4.3	36	Common	20.0–36.0, 29.0	2.0–6.0, 3.5	93	Numerous
	Microbasic p-mastigophores	—	—	—	—	22.0–22.4, 22.8	4.0–6.0, 5.3	4	Rare
	Holotrich small	6.0–10.0, 7.9	2.0–5.0, 3.5	11	Occasional	—	—	—	—
Mesenteries	Bastrichs and microbasic b-mastigophores	18.0–26.0, 22.9	2.0–5.0, 3.4	27	Common	21.0–33.0, 28.0	3.0	3	Rare
	Microbasic p-mastigophores	—	—	—	—	19.0–29.0, 24.9	4.0–9.0, 6.4	114	Numerous
	Holotrich large	32.0	14.0	1	Rare	—	—	—	—

the coenenchyme of *Epizoanthus protoporos* sp. nov. is not continuous and only partially covers associated *Granulifusus* gastropod shells. Additionally, *E. indicus* is generally larger than *Epizoanthus protoporos* sp. nov. in polyp size (*E. indicus* polyps: 4.0 mm in height and 10.0 mm in diameter). Additionally, associated gastropod species are different between *E. thalamophilus* and *Epizoanthus protoporos* sp. nov.; the former species is associated with *Borsonia symbiotes*, while the latter is associated with *Granulifusus niponicus*. *E. rinbou*, and *Epizoanthus* sp. S sensu Reimer et al. (2010) are smaller than *Epizoanthus protoporos* sp. nov. in polyp size. Moreover, holotrichs were not found in any tissues of *Epizoanthus protoporos* sp. nov., while holotrichs are present in the tissues of *E. rinbou*. Finally, the molecular phylogeny clearly showed that *Epizoanthus protoporos* sp. nov., *E. rinbou*, and *Epizoanthus* sp. S are placed in different groups (Fig. 5).

Etymology. The specific name is derived from the Greek word protopóros meaning "explorer", as this species colonizes on rocket-like gastropod shells to gain mobility.

Japanese common name. Naganishi-yadori-sunaginchaku.

Discussion

In hexacorallian groups, actiniarian-gastropod associations have been the subject of some research attention, and results have demonstrated that such associations provide benefits to hosts and associates as both partners receive protection and can increase their survival rates (Pastorino, 1993; Riemann-Zürneck, 1994; Mercier & Hamel, 2008). On the other hand, research on zoantharian-gastropod associations has lagged considerably compared to other cnidarian-gastropod associations due largely to the difficulty of specimen collection, as zoantharian-gastropod associations are exclusively found in mesophotic to deeper waters. Among the few existing zoantharian-gastropod studies, a recent taxonomic study suggested that *Epizoanthus rinbou* has an obligate association with the gastropod *Guildfordia triumphans* based on molecular and morphological datasets (Kise & Reimer, 2019). In addition, in this study, we theorize that an obligate association between *Epizoanthus protoporos* sp. nov. and *Granulifusus niponicus* exists, as *Epizoanthus protoporos*

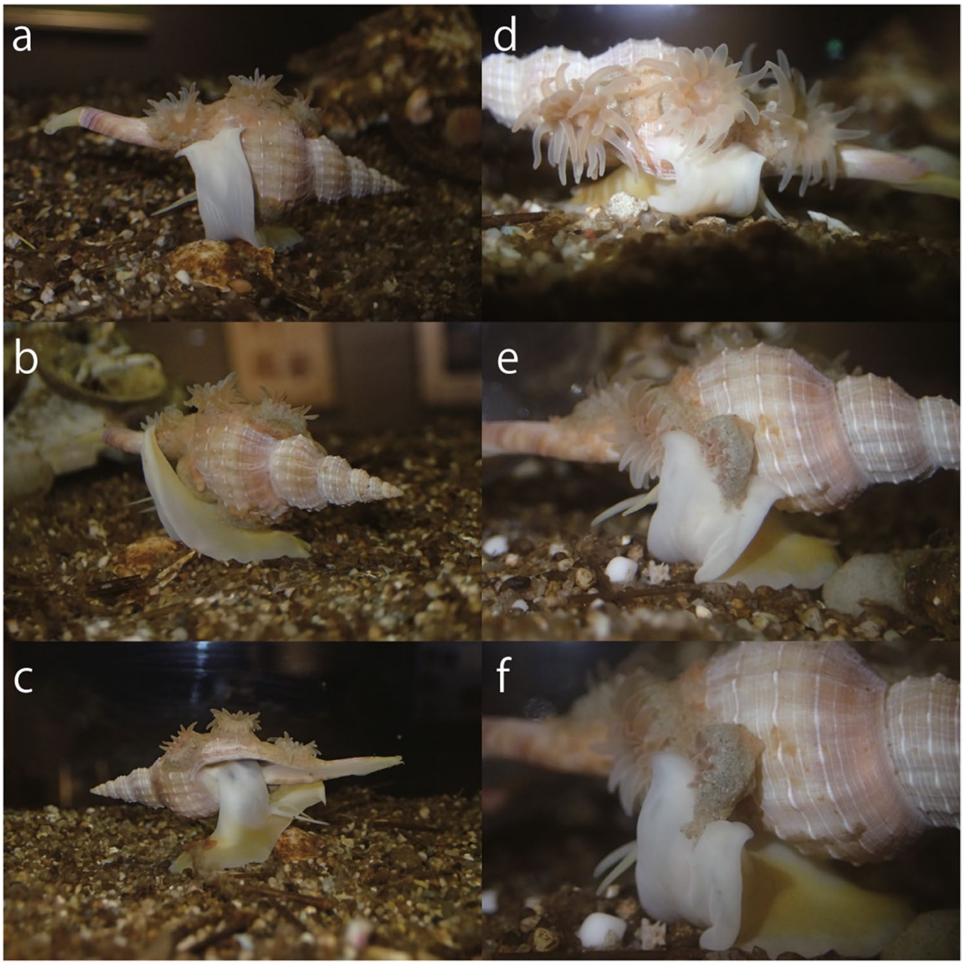

Fig. 4 Images of *Granulifusus niponicus* acting on *Epizoanthus protoporos* sp. nov. **a**–**c** The front, side, backside of image that the front end of *G. niponicus*'s foot stretched and stroked the polyps of *Epizoanthus protoporos* sp. nov. **d**–**f** Closed-up image of the front end of *G. niponicus*'s foot acting on *Epizoanthus protoporos* sp. nov

sp. nov. was not found in situ on other invertebrates or on rocks, and was always found exclusively on shells of living *G. niponicus*.

We observed a unique behavior of *G. niponicus* towards *Epizoanthus protoporos* sp. nov. in aquaria during this study. Polyps of *Epizoanthus protoporos* sp. nov. are regularly arranged on the shell margin around the aperture but are not found on the other side of the aperture, and thus, we theorize that this polyp arrangement may depend on the distance that the foot of *G. niponicus* can reach, with polyps only present out of range of the foot. *Epizoanthus protoporos* sp. nov. may receive several advantages from *G. niponicus* such as mobility, substrate, and relatively easy food acquisition. Furthermore, similar advantages may be considered in the association between *Paleozoanthus reticulatus* and *Granulifusus rubrolineatus*, as polyp arrangement of *P. reticulatus* is identical with that of *Epizoanthus protoporos* sp. nov. However, the advantages to *G. niponicus* from such a symbiosis are still not clear as *Epizoanthus protoporos* sp. nov. lacks holotrichs in all tissues, while other gastropod-associated species such as *E. rinbou* have holotrichs in their tissues (Kise & Reimer, 2019), particularly since holotrichs have been characterized as used in aggression (Rotjan & Dimond, 2010). Therefore, potential protection benefits to *G. niponicus* via the nematocysts of *Epizoanthus protoporos* sp. nov. are uncertain. To better understand this symbiotic association, experiments on the influence of predators under controlled laboratory settings are necessary.

Epizoanthus protoporos sp. nov. and *P. reticulatus* both associate with hosts belong to genus *Granulifusus*, suggesting that *Paleozoanthus* and *Epizoanthus* may be congeneric. This is further supported by the fact that their external morphology resembles each other. However, the presence of fertilized incomplete mesenteries was not confirmed in *Epizoanthus protoporos* sp. nov. or *P. reticulatus* in this study. Thus, further studies are needed to confirm if fertilized incomplete

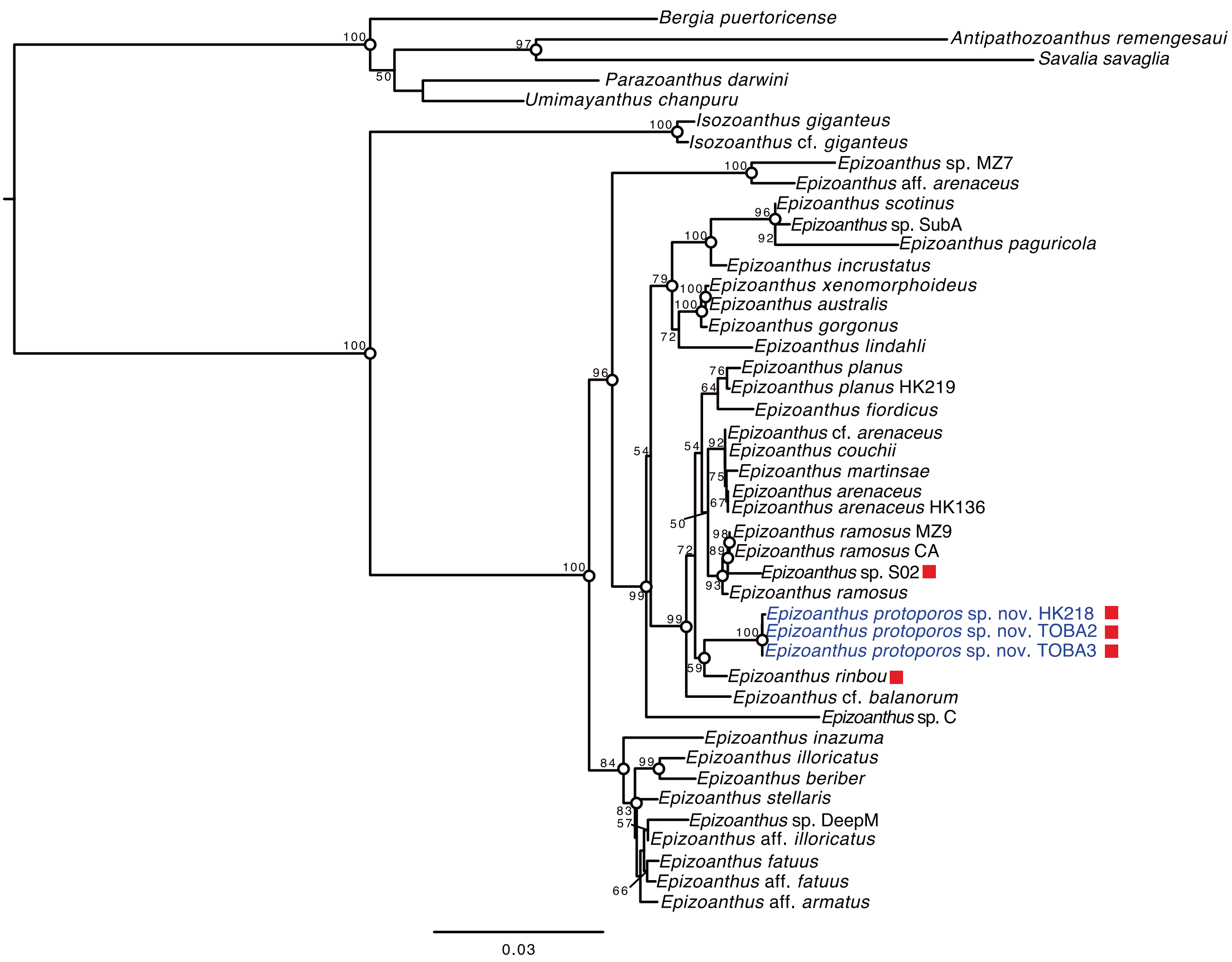

Fig. 5 Maximum likelihood tree based on combined dataset of COI, mt 12S-rDNA, mt 16S-rDNA, 18S-rDNA, ITS-rDNA, and 28S-rDNA sequences. Number at nodes represent ML bootstrap values (> 50% are shown). White circles on nodes indicate high support of Bayesian posterior probabilities (> 0.95). Red squares indicate species associated with gastropod mollusks

mesentery are a diagnostic characteristic to separate the genera *Epizoanthus* and *Paleozoanthus*. Confirmation should be achievable via molecular phylogenetic analyses of *P. reticulatus*. At the same time, it must be noted that based on similarities in sphincter musculature, Low et al. (2016) suggested that *Paleozoanthus* may correspond to genus *Terrazoanthus* in the family Hydrozoanthidae.

Based on molecular phylogenetic analyses, we found at least two independent origins for associations with gastropods in Epizoanthidae. In comparison to *Epizoanthus protoporos* sp. nov. and *E. rinbou*, *Epizoanthus* sp. S is located within a subclade consisting of hermit crab-associated *E. ramosus*. *Epizoanthus* sp. S is known to associate with the gastropod *Unedogemmula unedo* (Kiener, 1839). Furthermore, *Epizoanthus* sp. C sensu Reimer et al. (2010) is found on the empty shells of *U. unedo* inhabited by hermit crabs, although these two *Epizoanthus* species are clearly distinct by morphology and molecular phylogeny (Reimer et al., 2010), suggesting that mechanisms for the establishment of the symbiotic associations with gastropods and hermit crabs are different.

The numbers of symbiotic studies on zoantharian-gastropod associations conducted until now are few compared to those on actiniarian-gastropod associations. Thus, continued investigations examining the diversity of gastropod-associated zoantharians have the potential to greatly expand our overall comprehension of anthozoan-gastropod symbioses.

Table 2 Summary of morphological data from Epizoanthidae species associated with gastropods

Species	Polyp height (mm)	Polyp diameter (mm)	Number of tentacles	Coloration of tentacle	Number of polyps on shell	Number of capitulary ridges	Coenenchyme	Associated gastropod	Depth (m)	References
Paleozoanthus reticulatus	1.7–3.1	3.5–5.5	Approx. 20–24	NA	6–7	10–12	Continuous	*Granulifusus rubrolineatus* (Sowerby II, 1870)	155	(Carlgren, 1924), this study
Epizoanthus indicus	Up to 10.0	10.0	32–44	NA	Approx. 7	16–22	Sparse	*Borsonia symbiotes* (Wood-Manson & Alcock, 1891)	1200–1800	(Lwowsky, 1913; Reimer et al., 2010)
Epizoanthus thalamophilus	2.0–10.3	9.0–10.5 mm	30–40	NA	15–20	Approx. 30–40	Continuous	*Fusus* spp.	3950	(Hertwig, 1888)
Epizoanthus egeriae	1.0–2.0	5.0	Approx. 24–36	NA	NA	12–18	Continuous	*Murex pecten* Lightfoot, 1786	82–86	(Haddon & Duerden, 1896)
Epizoanthus rinbou	0.5–3.0	2.2–5.9	38–42	Transparent	50	19–21	Sparse	*Guildfordia triumphans* (Philippi, 1841)	280–900	(Kise & Reimer, 2019)
Epizoanthus sp. S	Up to 6.0	2.0–5.0	Approx. 24	Transparent red	NA	Approx. 12	Sparse	*Unedogemmula unedo* (Kiener, 1839)	40–60	(Reimer et al., 2010)
Epizoanthus protoporos sp. nov	2.7–4.7	5.3–9.1	28–32	Light beige and/or pale red	7	14–16	Sparse	*Granulifusus niponicus* (Smith, 1879)	250–300	This study

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Author contribution HK wrote the draft manuscript with input from JDR. JDR supervised this study. TM collected the examined specimens and provided research materials, and all authors contributed and approved the final manuscript.

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Data availability All sequences were deposited in GenBank, and all aligned datasets for phylogenetic analyses are available from Figshare.

Declarations

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Consent for publication All authors approved the final version of the manuscript for publication.

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