

Carcinoecium-Forming Sea Anemone *Stylobates calcifer* sp. nov. (Cnidaria, Actiniaria, Actiniidae) from the Japanese Deep-Sea Floor: A Taxonomical Description with Its Ecological Observations

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Abstract. Here we describe *Stylobates calcifer* sp. nov. (Cnidaria, Actiniaria, Actiniidae), a new carcinoecium-forming sea anemone from the deep-sea floor of Japan. *Stylobates* produces a carcinoecium that thinly covers the snail shells inhabited by host hermit crabs *Pagurodoyleinia doederleini*. The new species is distinct from other species by the shape of the marginal sphincter muscle, the distribution of cnida, the direction of the oral disk, and host association. The species' novelty is supported by the data of its mitochondrial genes *12S*, *16S*, and *COIII* and nuclear genes *18S* and *28S*. Also, we conducted behavioral observation of this new species, focusing on the feeding behavior and interaction with the specific host hermit crab. Our observations suggest that this sea anemone potentially feeds on the suspended particulate organic matter from the water column or the food residuals of hermit crabs. When the host's shell changed, intensive

manipulation for transference of *S. calcifer* sp. nov. was recorded. However, although the hermit crab detached and transferred the sea anemone to the new shell after shell change, the sea anemone did not exhibit active or cooperative participation. Our data suggest that the sea anemone may not produce a carcinoecium synchronously to its host's growth, contrary to the anecdotal assumption about carcinoecium-forming sea anemones. Conversely, the host hermit crab's growth may not depend entirely on the carcinoecium produced by the sea anemone. This study is perhaps the first observation of the behavioral interaction of the rarely studied carcinoecium-forming mutualism in the deep sea.

Introduction

Interspecific interactions are key evolutionary forces driving the evolution of species in a biological community (Darwin, 1862; Nilsson, 1988; Thompson, 1994). Symbiosis has been a research focus on co-evolution in evolutionary biology; remarkable morphological, behavioral, and adaptive strategies are involved in symbiotic relationships in terrestrial and marine habitats (Wiebes, 1979; Kato *et al.*, 2003; Izumi *et al.*, 2018). However, the natural histories—for example, species diversity, lifestyle, and symbiotic interaction—of the organisms living on the deep-sea floor remain unclear due to the inaccessibility of the organisms. Studying these natural histories is necessary for understanding these organisms' diversification and co-evolution, which are topics of interest in evolutionary biology.

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Abbreviations: ASDSF, average standard deviation of split frequencies; BI, Bayesian inference; CF, carcinoecium forming; CMNH, Coastal Branch of the Natural History Museum and Institute, Chiba; DDBJ, DNA Data Bank of Japan; EOD, extending oral disk; ML, maximum likelihood; NSMT, National Museum of Nature and Science, Tsukuba; PCR, polymerase chain reaction; SB, shrinking body;

Online enhancements: videos.

The symbiotic association between hermit crabs and sea anemones is an example of mutualism. At least 35 symbiotic sea anemone species belonging to 14 genera have been found on the chelipeds of hermit crabs or the shells they inhabit (Williams and McDermott, 2004; Antoniadou *et al.*, 2013). Carcinoecium-forming mutualism is a remarkable relationship between the sea anemones (*i.e.*, genera *Calliactis* Verrill, 1869, *Paracalliactis* Carlgren, 1928, and *Stylobates* Dall, 1903) and hermit crabs living on the deep-sea floor (Daly *et al.*, 2004; Gusmão, 2010; Crowther *et al.*, 2011; Gusmão *et al.*, 2020). Carcinoecium-forming (CF) sea anemones settle on the gastropod shells inhabited by the hermit crabs and produce a pseudo-snail shell (carcinoecium) covering over the shell (Gusmão and Daly, 2010; Antoniadou *et al.*, 2013; Yoshikawa *et al.*, 2019; Gusmão *et al.*, 2020) that is likely beneficial for both parties; the hermit crab may not have to find new, larger shells, and the sea anemone may not have to change the substrate (Dunn *et al.*, 1980; Antoniadou *et al.*, 2013).

A novel, rare CF anemone, collected at a depth of 100–400 m around the Pacific side (middle of Honshu Island to Kyushu) of Japan (Uchida and Soyama, 2001; Yanagi, 2006; Yoshikawa *et al.*, 2019), was found on the shells inhabited by the hermit crab *Pagurodofoleinia doederleini* (Doflein, 1902). This species, known as “*Isadamsia* sp. J” (Japanese name: *Hime-kin-kara-isoginchaku*) in Japan (Uchida and Soyama, 2001; Yanagi, 2006), has not undergone detailed morphological and molecular investigation. Because *Isadamsia* is a junior synonym of *Stylobates* (Dunn *et al.*, 1980; Daly and Fautin, 2021), here we describe this species as *Stylobates calcifer* sp. nov., based on the 16 specimens collected from 2017 to 2020 exclusively on the shells inhabited by the host hermit crab *P. doederleini*.

The genus *Stylobates* previously comprised four species, all of which exclusively inhabit deep water: *S. aeneus* Dall, 1903 from Guam and Hawaii (402–797 m); *S. birtlesi* Crowther, Fautin & Wallace, 2011 from northeast Australia (590–964 m); *S. cancrisocia* (Carlgren, 1928) from East Africa (818 m); and *S. loisetteae* Fautin, 1987 from northwest Australia (320–508 m). However, little is known about the symbiotic interactions between the organisms and species diversity, because of the difficulties associated with sample collection and specimen management.

Compared with other CF anemone species, for example, *Calliactis* Verrill, 1869 and *Paracalliactis* Carlgren, 1928, the various *Stylobates* species form a carcinoecium that more closely resembles a gastropod shell. *Stylobates* was first established as a gastropod species with a flexible shell by Dall (1903), and *Stylobates aeneus* was assigned as a direct child species. However, Dall (1919) later noted that the flexible shell differed, depending on the host hermit crab, and concluded that it was produced by a sea anemone; at this point, *Stylobates* was assigned as Actinia. Subsequently, Carlgren (1928) described *Isadamsia* as a new genus of shell-making anemone and described a new species from the Pemba Channel off of East Africa, *Isadamsia cancrisocia*, as a type species

of this genus. Dunn *et al.* (1980) then reinvestigated the morphology of *S. aeneus* and *I. cancrisocia* and confirmed that both species were in the same genus.

The behavioral relationship between *Stylobates* species and the host hermit crab is largely unknown. For hermit crab-associated sea anemones in shallow water—for example, the genera *Calliactis* Verrill, 1869, and *Verrillactis* England, 1971—when host hermit crabs change their shells as they grow, they use tactile stimulation to detach the sea anemones from the previous shells and transfer them to the new substrate. These sea anemones respond to this stimulation by actively releasing their pedal disk from the shell (Ross, 1974, 1975; Yoshikawa *et al.*, 2018). Conversely, in a specific CF sea anemone association, *Paracalliactis* spp. inhabits depths of 50–1000 m (Hand, 1975; Ross and Zamponi, 1982) and produces thin cuticles of carcinoecium on the host snail shells; however, unlike shallow-water species, it does not respond to artificial and/or mechanical stimulation (Ross, 1974; Ross and Zamponi, 1982). Therefore, the host hermit crab of *Paracalliactis* spp. shows no active responses toward the associated sea anemone. However, *Paracalliactis mediterranea* is able to respond to the new snail shell and transfer/climb onto it when the host hermit crab is removed or lost. In another CF sea anemone that secretes thin cuticles of carcinoecium, *Calliactis palliata* (Fabricius, 1779), which is abundant at depths of 15–60 m (Gusmão, 2010), the species can relax, extend, or detach without difficulty following tactile stimulation applied around the pedal edge of the anemone-free host hermit crab *Pagurus prideaux* Leach, 1815.

However, all of the previous records and discussions were based on metridioidean species. Indeed, there have been no attempts to observe the phylogenetically different groups of Actinoidea to which *Stylobates* belongs. In *Stylobates*-hermit crab associations, it is anecdotally considered that the host hermit crab does not necessarily seek new, larger, or more suitable shells. As a result, the frequency of shell changing may remain lower, shaping species-specific relationships in the deep sea (Dunn *et al.*, 1980; Fautin, 1987; Crowther *et al.*, 2011; Antoniadou *et al.*, 2013). To further discuss the co-evolutional pattern and convergent evolution of the CF sea anemone association, knowledge on the behavioral relationship between a host hermit crab and *Stylobates* anemone is necessary. Therefore, here we examined the partnership between the newly identified species and its hermit crab host by observing their interaction during the shell change behavior of the host hermit crab.

Materials and Methods

Sample collection and preservation

Stylobates calcifer sp. nov. was found on the snail shells used by hermit crab *Pagurodofoleinia doederleini* (Doflein, 1902). A total of 16 specimens were collected by beam trawl from the Sea of Kumano, Japan, at the depth of 100–400 m during an R/V cruise *Seisui-maru* (cruise 1722) on November 9,

2017, and fishery trawling at Kiinagashima, Kihoku-Cho, Mie, and Heda, Numazu, Shizuoka, in 2018 to 2020 (Fig. 1; Table 1). The locality, collection date, depth, and usage of the type specimens are summarized in Table 1. Of these, 6 specimens of *S. calcifer* sp. nov. (CMNH-ZG09764–CMNH-ZG09769) were fixed in 99% ethanol immediately for DNA extraction. All other specimens were anesthetized with isotonic magnesium chloride ($MgCl_2$) for morphological and/or histological observations, sampled for DNA extraction, fixed in 10%–20% formalin in seawater, washed in running fresh water overnight (about 12 h), and preserved in 70% ethanol. One specimen (CMNH-ZG09774), collected from the Sea of Kumano and exhibited at the Toba Aquarium, was used for behavioral observation and then for external morphological observations. The shells used by the sea anemones and the host hermit crabs were identified following their descriptions by Okutani (2000). Identification of the shell species was further confirmed by referring to their nomenclature in the World Register of Marine Species (WoRMS) database. A holotype specimen (NSMT-Co 1794) and 15 paratypes were deposited at the National Museum of Nature and Science, Tsukuba (NSMT), and the Coastal Branch of the Natural History Museum and Institute, Chiba (CMNH), respectively.

Preparation of histological sections

Specimens (NSMT-Co 1794) were removed from snail shells and dissected to obtain mesenteries, sphincter muscles, and tentacles for morphological observation. Specimen CMNH-ZG09774 was maintained on the petri dish with running seawater before fixation to observe the entire mesenteries. All

of the tissues were dehydrated by ethanol (80% and 90% for 2 h each, 99% for 1 h, and absolute for 30 min twice) and cleared in xylene (EtOH-xylene, xylene I, and II for 20 min each), embedded in paraffin (xylene-paraffin for 30 min, paraffin I for 2 h, and paraffin II for more than 2 h), and sliced into 8- μ m sections, mounted, and stained (Masson, 1928; Presnell and Schreiber, 1997). The taxonomic descriptions followed the morphological terminology of Carlgren (1949), Dunn *et al.* (1980), and Crowther *et al.* (2011).

Cnidae observation

Tentacle, actinopharynx, mesenterial filament, column, and limb tissues were mounted on slide glasses with 50% glycerin in seawater. The cnidae observations were conducted using the two *S. calcifer* sp. nov. samples, that is, the holotype (NSMT-Co 1794) and the paratype (NSMT-Co 1796). The cnidae were imaged using differential interference contrast microscopy (Yanagi *et al.*, 2015), named (Mariscal, 1974), and measured using ImageJ version 1.49 (Abramoff, 2004).

Molecular analysis

Total DNA was extracted from the tissue of specimens CMNH-ZG09764, CMNH-ZG09765, CMNH-ZG09771, and NSMT-Co 1796, using the High Pure polymerase chain reaction (PCR) template kit (Roche, Basel, Switzerland). Polymerase chain reaction was used to amplify about 700 bp of the *COXIII* gene, 500 bp of the mitochondrial *16S* rRNA gene, 800 bp of the *12S* rRNA gene, 1700 bp of the nuclear *18S* rRNA gene, and 3200 bp of the *28S* rRNA gene. The PCRs were performed in 25- μ L reactions, with 2.5 μ L each of the forward and reverse primers, 2.5 μ L of Ex Taq buffer (Takara Bio, Shiga, Japan), 2.0 μ L of deoxyribonucleoside triphosphate (dNTP; Takara Bio), 0.13 μ L of Ex Taq polymerase (Takara Bio), and 14.87 μ L of distilled water. The PCR was run at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, at various annealing temperatures for 90 s, 72 °C for 120 s, and 72 °C for 10 min. The amplified products were verified on 1% agarose gel, purified using a High Pure PCR Product Purification Kit (Roche), and sequenced (Eurofins Genomics, Louisville, KY) using PCR primers for *COXIII*, *12S*, and *16S* or PCR and sequencing primers for *18S* and *28S*. The primers and the procedures for each amplification followed those of Izumi *et al.* (2019). The sequence data were assembled using GeneStudio Professional version 2.2.0.0 and deposited in the DNA Data Bank of Japan (DDBJ) (Table A1).

A total of 174 operational taxonomic units were used for the present phylogenetic analysis. Of these, the four sequences of Anthemoneae acted as an outgroup (DDBJ; Table A1). Metridioidean species were included in the present analysis to verify that the newly identified species was not a Hormathiidae. The data set was aligned by MAFFT version 7.402 (Katoh and Standley, 2013) under default settings. Ambiguously aligned regions were eliminated by Gblocks version 0.91b (Castresana,

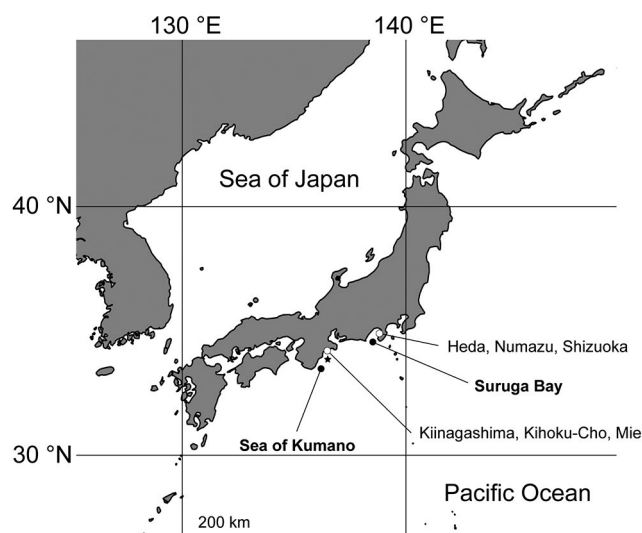


Figure 1. Sampling locations of *Stylobates calcifer* sp. nov. Locations included the areas sampled with the deep-sea trawling net by R/V *Seisui-maru*, Mie University (November 17, 2017) (star); the fishing trawlers; the area where the fisheries were conducted (filled circles); and the location of the fishing ports (open circles).

Table 1

Specimens of *Stylobates calcifer* sp. nov. used in the present study

Registration no.	Host gastropod		Station	Coordinate	Water depth (m)	Methods	Date	Preservation solution (% ethanol)	Usage	No. of the preparation specimen		
	shell	Tentacle								Longitudinal sections	Transverse sections	Nematocyst preparations
CMNH-ZG09764	<i>Trochocerithium shikoensis</i>	—	Kumano-Nada, central Japan	34°06'42'' N, 136°37'48'' E	355–373	Beam trawl on R/V <i>Seisui-maru</i>	Nov. 9, 2017	99	pa	—	—	—
CMNH-ZG09765	<i>Trochocerithium shikoensis</i>	—	Kumano-Nada, central Japan	34°06'42'' N, 136°37'48'' E	355–373	Beam trawl on R/V <i>Seisui-maru</i>	Nov. 9, 2017	99	pa	—	—	—
CMNH-ZG09766	<i>Clavus</i> sp.	—	Kumano-Nada, central Japan	34°06'42'' N, 136°37'48'' E	355–373	Beam trawl on R/V <i>Seisui-maru</i>	Nov. 9, 2017	99	mo	—	—	—
CMNH-ZG09767	<i>Bathybembix argenteonitens</i>	—	Heda, Numazu, Shizuoka, Japan	—	~300	Trawl net on <i>Hinode-maru</i>	Feb. 18, 2018	70	mo	—	—	—
CMNH-ZG09768	<i>Bathybembix argenteonitens</i>	—	Heda, Numazu, Shizuoka, Japan	—	~300	Trawl net on <i>Hinode-maru</i>	Nov. 17, 2018	70	mo	—	—	—
CMNH-ZG09769	Unidentified	—	Heda, Numazu, Shizuoka, Japan	—	~300	Trawl net on <i>Hinode-maru</i>	Feb. 14, 2019	70	mo	—	—	—
CMNH-ZG09770	Naticidae gen. et sp. indet.	—	Heda, Numazu, Shizuoka, Japan	—	~300	Trawl net on <i>Hinode-maru</i>	Feb. 14, 2019	70	mo	—	—	—
CMNH-ZG09771	Naticidae gen. et sp. indet.	—	Heda, Numazu, Shizuoka, Japan	—	~300	Trawl net on <i>Hinode-maru</i>	Feb. 14, 2019	70	pa	—	—	—
CMNH-ZG09772	Unidentified	—	Kiinagashima, Kihoku-Cho, Mie, Japan	—	~300	Trawl net on <i>Jinsho-maru</i>	May 19, 2019	70	mo	—	—	—
CMNH-ZG09773	<i>Bathybembix argenteonitens</i>	—	Kiinagashima, Kihoku-Cho, Mie, Japan	—	~300	Trawl net on <i>Jinsho-maru</i>	May 19, 2019	70	mo	—	—	—
CMNH-ZG09774	<i>Bathybembix argenteonitens</i>	179	Kiinagashima, Kihoku-Cho, Mie, Japan	—	~300	Trawl net on <i>Jinsho-maru</i>	Feb. 15, 2020	70	bo	—	—	—
CMNH-ZG09775	<i>Bathybembix argenteonitens</i>	188	Kiinagashima, Kihoku-Cho, Mie, Japan	34°05'50.2'' N, 136°32'2.81'' E	320–340	Trawl net on <i>Jinsho-maru</i>	June 3, 2020	70	ho	—	187	—
CMNH-ZG09776	<i>Bathybembix argenteonitens</i>	—	Kiinagashima, Kihoku-Cho, Mie, Japan	34°05'50.2'' N, 136°32'2.81'' E	320–340	Trawl net on <i>Jinsho-maru</i>	June 3, 2020	70	mo	72	—	—
NSMT-Co 1794*	<i>Bathybembix argenteonitens</i>	178	Kiinagashima, Kihoku-Cho, Mie, Japan	34°01'10.0'' N, 136°23'10.0'' E	190–300	Trawl net on <i>Jinsho-maru</i>	Oct. 27, 2019	70	ho/co	3	14	6
NSMT-Co 1795	<i>Bathybembix argenteonitens</i>	190	Kiinagashima, Kihoku-Cho, Mie, Japan	34°01'10.0'' N, 136°23'10.0'' E	190–300	Trawl net on <i>Jinsho-maru</i>	Oct. 27, 2019	70	mo	—	—	—
NSMT-Co 1796	<i>Bathybembix argenteonitens</i>	181	Kiinagashima, Kihoku-Cho, Mie, Japan	34°01'10.0'' N, 136°23'10.0'' E	190–300	Trawl net on <i>Jinsho-maru</i>	Oct. 27, 2019	70	pa	—	3	5

For registration numbers, an asterisk indicates the holotype. For usage, pa indicates phylogenetic analysis; mo indicates morphological observation; bo indicates behavioral observation; ho indicates histological observation; co indicates cinidae observation.

2002). The file was then processed by Kakusan 4 (Tanabe, 2011) to test its substitution models for the analyses of RAxML and MrBayes. Maximum-likelihood (ML) analyses were performed by RAxML-VI-HPC (Stamatakis, 2006) with the GTR+ Γ evolutionary model recommended by Kakusan 4 and evaluated by 1000 bootstrap replicates. Bayesian inference (BI) was conducted using MrBayes version 3.2.6 (Ronquist and Huelsenbeck, 2003). The substitution parameters were GTR+ Γ for *COXIII*, *12S*, and *28S*, HKY85+ Γ for

16S, and SYM+ Γ for *18S*. Two independent runs of the Markov chain Monte Carlo were carried out simultaneously for 5,000,000 generations, sampling trees every 100 generations and calculating the average standard deviation of split frequencies (ASDSFs) every 100,000 generations. Because ASDSF was calculated based on the last 75% of the samples, the initial 25% of the sampled trees were discarded as burn-in. Finally, two resultant trees were combined by FigTree version 1.4.4 (Rambaut, 2018); low bootstrap (<50) and posterior probability

(<0.50) values were manually deleted on each node. The evolutionary divergence (interspecific and/or intraspecific variation) of each sequence was also estimated using the Kimura two-parameter (K2P) distance model (Kimura, 1980) in MEGA X (Kumar *et al.*, 2018). Interspecific variation was calculated between the most closely related and/or congeneric species in the phylogenetic tree.

Behavioral observations

A hermit crab and sea anemone pair (CMNH-ZG09774) was used for the behavioral observation. It was brought back to the laboratory, kept in a running seawater aquarium at 10 °C to 15 °C, and observed multiple times, using a Canon EOS Kiss X9i from February 17 to March 1, 2020. The pair's feeding response for mashed krill (observations F1–F5, Video S1, available online), krill (*Euphausia* spp.) (observations F6–F8, Video S2), slices of skipjack tuna (*Katsuwonus* sp.) (observation F9, Video S3), brine shrimps (observation F10, Video S3), and living shrimps (*Palaemon* spp., etc.) (observation F11, Video S3) were recorded.

We also observed the anemone's interaction with the hermit crab continuously while focusing on the host's shell change behavior. We then provided new shells (*Glossaulax reiniana*) (Dunker, 1877) to the hermit crab and observed its subsequent behavior (observation S1 in Video S4, available online; observations S2-1 to S2-4 in Videos S5–S8). The time schedule of video recordings is shown in Figure A1.

Results

Taxonomic description

Order ACTINIARIA

Suborder ENTHEMONAE

Superfamily ACTINIOIDEA Rafinesque, 1815

Family ACTINIIDAE Rafinesque, 1815

Genus *Stylobates* Dall, 1903

Stylobates calcifer sp. nov. Yoshikawa and Izumi

[Japanese name: *Hime-kin-kara-isoginchaku*]

(Figs. 2–6, A2)

Synonyms: *Isadamsia* sp. J: Uchida and Soyama (2001), p. 81; Yanagi (2006), p. 141 (pl. I-I).

Stylobates sp.: Yoshikawa *et al.* (2019), p. 285 (fig. 1A); sea anemone (*Isadamsia* sp.): Jimi *et al.* (2021), p. 6 (fig. 1a, b, c).

ZooBank LSIDs (Life Science Identifiers): urn:lsid:zoobank.org:pub:D947FADF-9E1F-4136-A6A0-38227AAB5A32.

Material examined: Holotype: NSMT-Co 1794, collected from the Sea of Kumano, off of the coast of the Kumano Region of the Kii Peninsula, Honshu, Japan (34°01'10.0'' N,

136°23'10.0'' E), by the bottom trawl net on the fishing vessel *Jinsho-maru*, on October 27, 2019, at a depth of 190–300 m.

Paratypes: CMNH-ZG09764, CMNH-ZG09765, CMNH-ZG09766, CMNH-ZG09772, CMNH-ZG09773, CMNH-ZG09774, CMNH-ZG09775, and CMNH-ZG09776, collected from the type locality at a depth of about 300 m; NSMT-Co 1795 and NSMT-Co 1796, collected in a similar manner to NSMT-Co 1794 (34°01'10.0'' N, 136°23'10.0'' E) at a depth of 190–300 m; CMNH-ZG09774, CMNH-ZG09775, and CMNH-ZG09776, collected from the type locality at a depth of about 300 m; CMNH-ZG09767, CMNH-ZG09768, CMNH-ZG09769, CMNH-ZG09770, and CMNH-ZG09771, collected from Sagami Bay, the coast of Heda, Numazu, Shizuoka, Japan, at a depth of about 300 m. All examined specimens are summarized in Table 1.

Morphology: Pedal disk thin, concave, conforming to the shape of the host shell. Diameter of the pedal disk depended on the shell's length and shape. Limbus thin, smooth with an irregular outline dependent on the shape of the shell, covering the entire shell except for the part under the hermit crab (Fig. 2). Column smooth, thin, flat, usually not cylindrical on the shells, with mesenterial insertions as dark lines (Fig. 2B, F), 13.7–16.7 mm in height in fixed holotype, diameter flexible depending on the host shell's size and the sea anemone's conditions. Mesenterial arrangement biradial symmetry with its plane of symmetry perpendicular to the directive axis and its orthogonal axis. Oral disk flat with an oval mouth, 16.0–20.7 mm in diameter in fixed holotype, with two prominent siphonoglyphs (Figs. 2A, 3E, 4A, B). Mouth weakly swollen in the live animal (Fig. 2C, D), flat in the preserved specimen (Fig. 2E). Tentacles thin and pointed, inner ones 9.25–16.51 mm in length and 1.19–2.62 mm in diameter, outer shorter than inner, 1.46–7.81 mm and 0.37–1.95 mm, respectively (in fixed holotype). The number of tentacles, 178 to 190 (178 in holotype) (Table 1). The ectodermal musculature longitudinal in tentacles, radial in oral disks. Actinopharynx shallowly ribbed, with two symmetrical siphonoglyphs (Figs. 3E, 4A, B). Marginal sphincter muscle well developed, circumscribed, pinnate, with a short, radiate primary axis (Fig. 3A, B).

Mesenterial arrangement in 96 pairs in cycles of 6, 6, 12, 24, and 48 (Fig. 4). Six pairs of the first cycle perfect, two pairs of the first mesentery directives each attached to siphonoglyph (Fig. 4B). The third to fifth cycles with mesenterial filaments (Fig. 4A, D). The fourth and fifth cycles fertile (Figs. 3, 4). Retractor muscles of mesenteries diffuse (Figs. 3E, F, 4B, C). Parieto-basilar muscle poorly developed (Fig. 4D). Sexes separate, holotype male (Fig. 3), paratype (CMNH-ZG09775) female (Fig. 4). Mature spermatid vesicles 0.12–0.41 mm in diameter (holotype collected in November), mature oocytes 0.44–0.85 mm in diameter (paratype CMNH-ZG09775 collected in February). All of the developmental stages of the ova seen in a single mesentery.

Carcinocodium, varying from a thin chitinous coating on the gastropod shell to a coating with a shell extension and

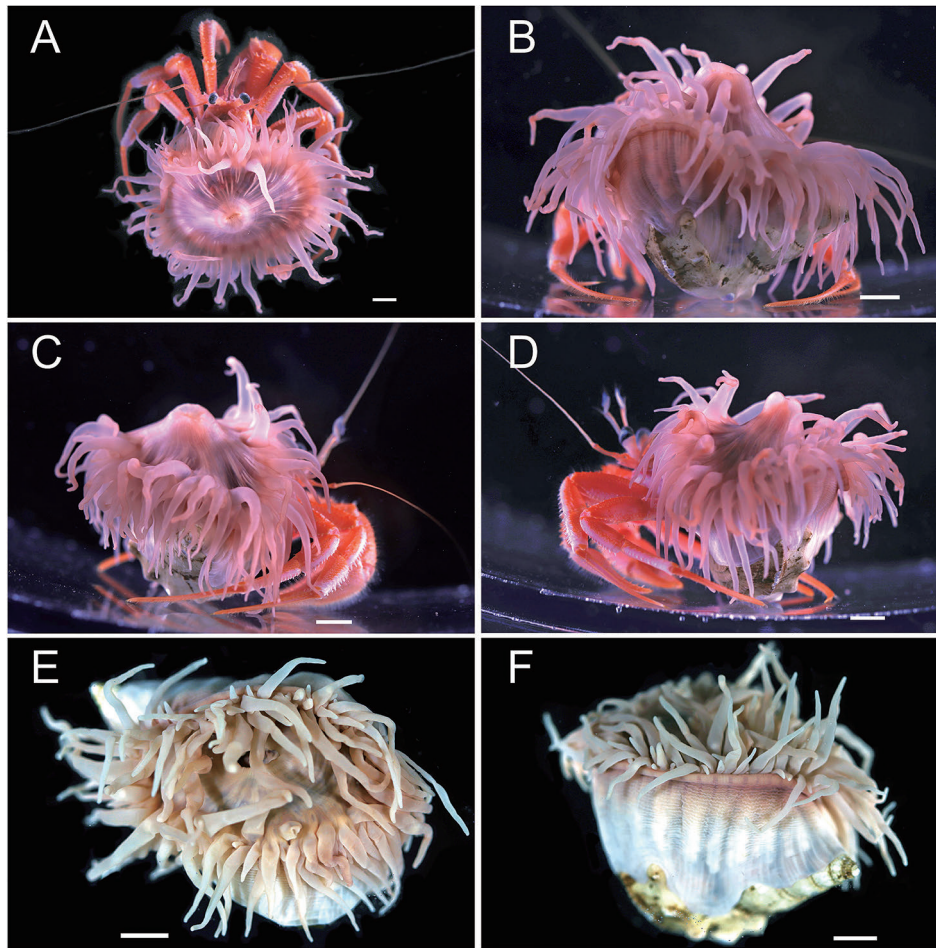


Figure 2. External morphology of *Stylobates calcifer* sp. nov. (holotype: NSMT-Co 1794) with host hermit crab *Pagurodofeinia doederleini*. The upper (A) and posterior (B) views and the marginal views of the right and left sides (C, D, respectively) of the living specimen. The upper and marginal views (E, F, respectively) of the preserved specimen. Scale bars = 5 mm.

only extension of the shell aperture, thinner (1.0–5.0 mm) than the snail shell, dark brown without gloss (Fig. 5). The growth rings recognized on the surface; the layered structures absent, including dark-colored and white particles, fine sand, forams, and diatoms. Outer surface smoother and fewer exposed particles than the inner surface.

Cnidome: Spirocyts in the tentacle, basitrichs in the tentacle, actinopharynx, column, limbus, and microbasic *b*-mastigophores and *p*-mastigophores in the mesenterial filament (Figs. 6, A2, A3; Table 2).

Coloration in life: Base and column light red or pearl pink with dark mesenterial insertions (Fig. 2A, B, C, D). Tentacles, oral disk, lips, actinopharynx, and siphonoglyphs with the same coloring (Fig. 2A, C, D). Right after collection, coloration semitransparent, then turning whitish and somewhat opaque after several weeks in the aquarium.

Distribution and habitat: All of the samples were collected from the Sea of Kumano (Uchida and Soyama, 2001; Yoshi-

kawa *et al.*, 2019) and Suruga Bay. All of the previous records were from the Pacific Ocean facing the middle of Honshu Island to Kyushu (Uchida and Soyama, 2001; Yanagi, 2006). The specimens were distributed at 100–400 m from the fine sand and soft mud (Fig. 1; Table 1).

Ecological note: *Stylobates calcifer* sp. nov. was exclusively found on the shells inhabited by *Pagurodofeinia doederleini*, consistent with previous studies (Uchida and Soyama, 2001; Yanagi, 2006; Yoshikawa *et al.*, 2019). However, *P. doederleini* without *S. calcifer* sp. nov. was sometimes collected in this study. One individual was usually attached to one host hermit crab, consistent with previous studies (Uchida and Soyama, 2001; Yanagi, 2006; Yoshikawa *et al.*, 2019).

Genetic analysis: In total, 6867 bp of *12S* rDNA, *16S* rDNA, *18S* rDNA, *28S* rDNA, and *COIII* genes were obtained from the 4 specimens of the newly identified species (CMNH-ZG09764, CMNH-ZG09765, CMNH-ZG09771, and NSMT-Co 1796). These sequences were compared to those of

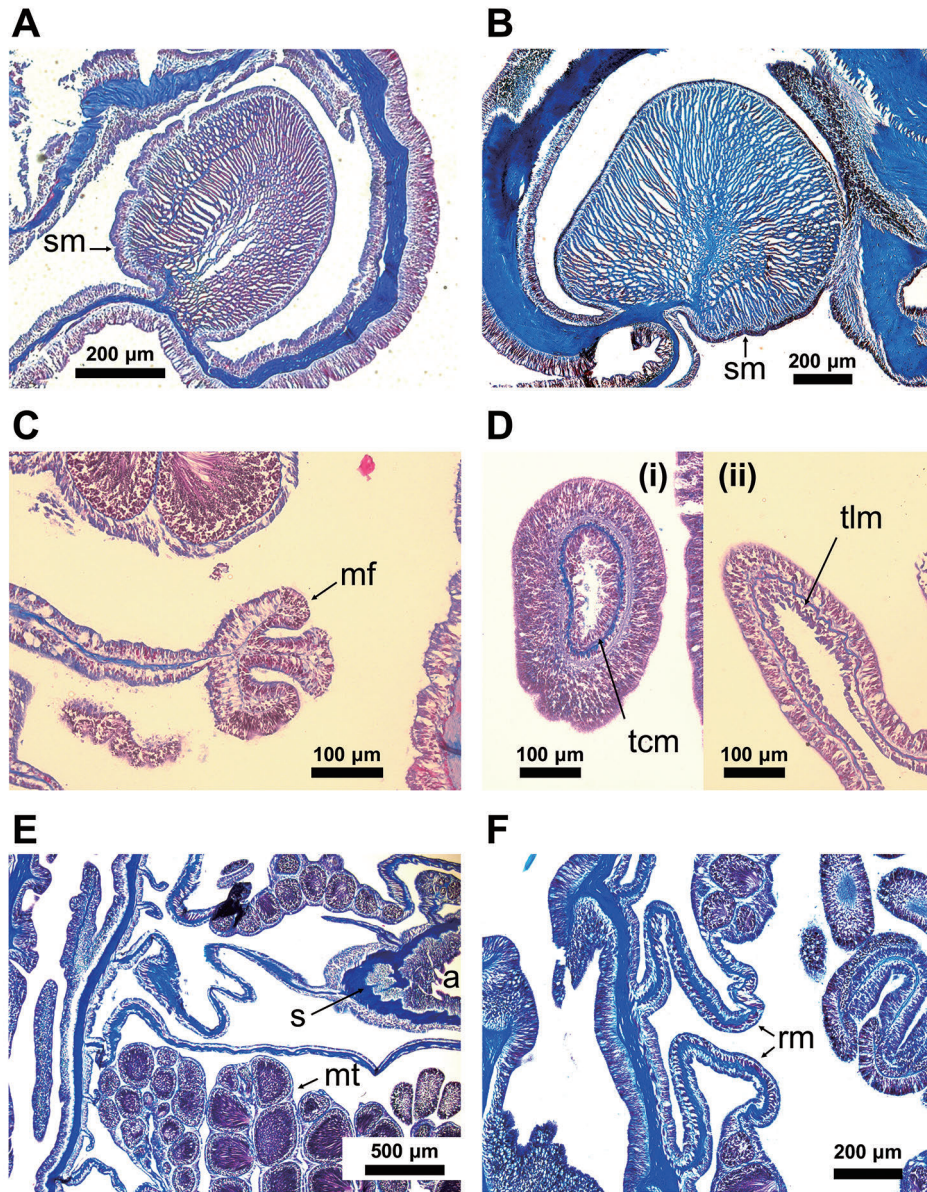


Figure 3. Internal anatomy of *Stylobates calcifer* sp. nov. (holotype: NSMT-Co 1794; paratype: NSMT-Co 1796). (A) The longitudinal section of the marginal sphincter muscle of the holotype (NSMT-Co 1794). (B) The longitudinal section of the marginal sphincter muscle of the paratype (NSMT-Co 1796). (C) The transverse section of a mesenterial filament of the holotype (NSMT-Co 1794). (D) The transverse section of the tentacle (i) and longitudinal section of a tentacle tip (ii) of the holotype (NSMT-Co 1794). The transverse section of a column with a well-developed siphonoglyph (E) and one with diffuse longitudinal muscles on the fifth mesenteries (F) of the holotype (NSMT-Co 1794). a, actinopharynx; mf, mesenterial filament; mt, matured testis cyst; rm, retractor muscle; s, siphonoglyph; sm, sphincter muscle; tcm, tentacular circular muscle; tlm, tentacular longitudinal muscle.

other Actinioidea species (DDBJ; Table A1) to examine the phylogenetic relationships between Actinioidea. In the phylogenetic tree of those three regions (Fig. 7), *Stylobates* belonged to sub-order Enthemonae (node A of Fig. 7) and superfamily Actinioidea (node B of Fig. 7), with the ML bootstrap values/BI posterior probabilities of 100%/1 and 31%/1, respectively. All of the *S. calcifer* sp. nov. sequences formed a monophyletic

clade supported by 99% of the bootstrap values and one posterior probability (node C of Fig. 7). The sister clade of *S. calcifer* sp. nov. was comprised of *Stylobates loisetteae*, with high support rate (node D) with ML bootstrap value/BI posterior probability of 99%/1. The interspecific variation of each sequence divergence was calculated as follows between the 2 congeneric species in the phylogenetic tree: 0.000 in *12S*, 0.002 in *16S*,

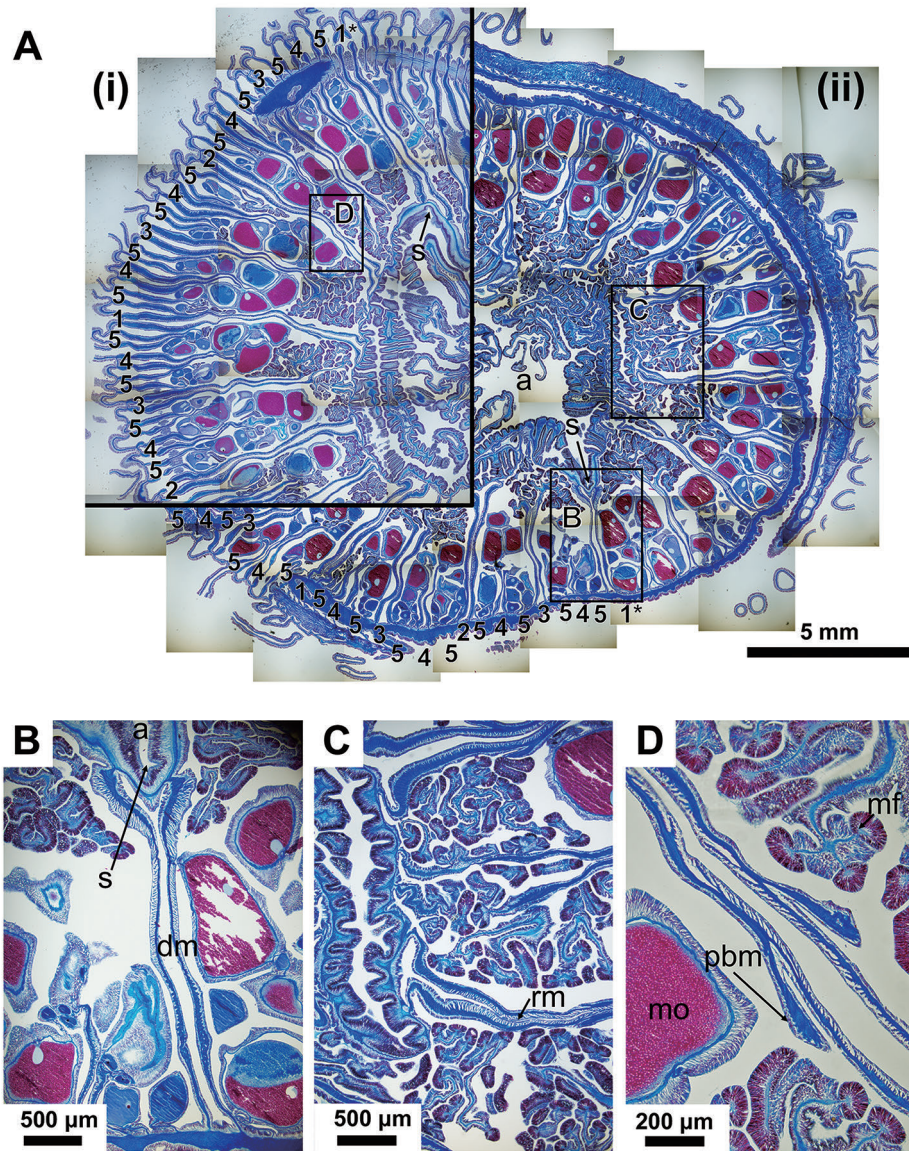


Figure 4. Histological section of a column of *Stylobates calcifer* sp. nov. (paratype: CMNH-ZG09775). (A) The entire transverse serial section of the actinopharynx. The cycle of mesenteries is indicated by numbers (asterisk indicates directive mesentery). (i) The upper transverse section near the tentacles and mouth. (ii) The transverse section of the middle of the column. (B) The full view of the ventral directives (first mesenteries), diffuse longitudinal muscles, and siphonoglyph. (C) The enlarged view of the second and third mesenteries and diffuse longitudinal muscles. (D) The parieto-basilar muscle of the second mesenteries. a, actinopharynx; dm, directive mesentery; mf, mesenterial filament; mo, matured ovary cyst; pbm, parietal basilar muscle; rm, retractor muscle; s, siphonoglyph.

0.094 in *18S*, 0.281 in *28S*, and 0.000 in *COIII*; the intraspecific variation was usually 0.000 or <0.004 (Table A2).

Etymology: The specific name “calcifer” is driven from the name of a resident fire-demon Calcifer, which appeared in *Howl’s Moving Castle*, a fantasy novel by British author Diana Wynne Jones, published in 1986; the story is the original version of the Japanese animated film of the same name, directed by Hayao Miyazaki (animated by Studio Ghibli). The

shell-making ability of the new species in the species-specific relationship appears as if Calcifer was in a magical contract with the Wizard Howl, constructing his Moving Castle.

Behavior: *feeding response of Stylobates calcifer* sp. nov.

Two feeding responses of *S. calcifer* sp. nov. (CMNH-ZG09774) were observed: the shrinking body (SB) type and

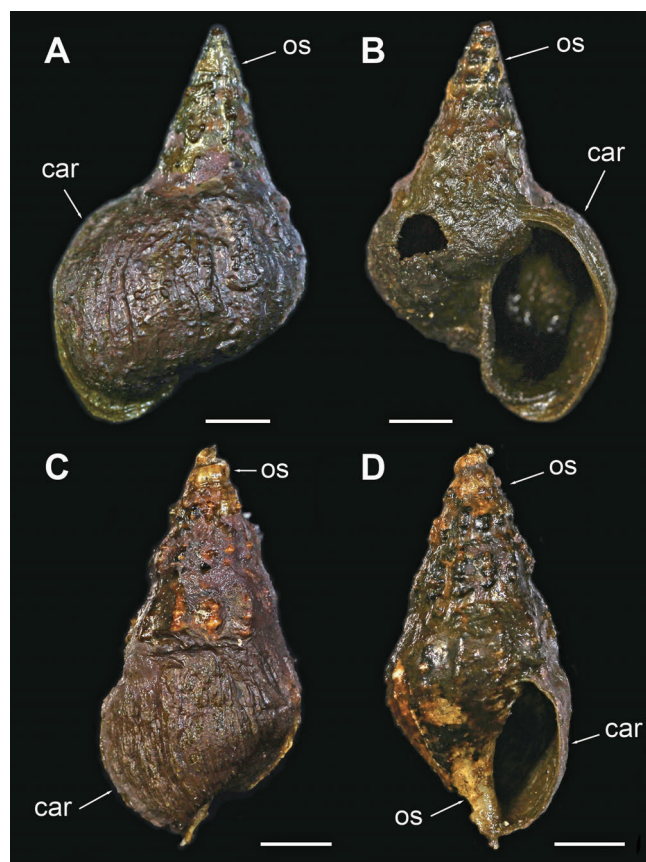


Figure 5. External morphology of the carcinoecium produced by *Stylobates calcifer* sp. nov. (A, B) The dorsal and ventral view of the carcinoecium on the shell of *Trochocerithium shikoensis* (Yokoyama, 1928) (CMNH-ZG09764). (C, D) The dorsal and ventral view of the carcinoecium on the shell of *Clavus* sp. (CMNH-ZG09766). Scale bars = 5 mm. car, carcinoecium; os, original shell.

the extending oral disk (EOD) type. An SB-type response occurred when the sea anemone obtained mashed foods. It began to shrink its sphincter muscles and opened its mouth widely immediately after the mashed foods fell on its mouth or tentacles (Video S1, available online). The SB-type response was recorded five times in this study. The EOD-type response occurred when the sea anemone obtained krill and small shrimps. It extended its mouth in the food's direction and slightly shrank the marginal disk and tentacles to carry the food toward the mouth (Video S2, available online). The EOD-type response was recorded three times. In contrast, the sea anemone had no feeding responses to the skipjack tuna, brine shrimps, and live shrimps (e.g., *Palaemon* spp.) (Video S3, available online).

Behavioral relationship with host hermit crabs

The first host hermit crab's shell change behavior was observed on February 20, 2020 (Video S4, available online). Af-

ter the hermit crab entered the new shell, it walked to the empty shell and started to detach the sea anemone from the shell (observation S1: Video S4). The detaching behavior of the hermit crab consisted of short and quick tapping, pinching of the sea anemone with its walking legs and chelae, and massaging with its walking legs and its shell (Fig. 8A; observation S1: Video S4). The hermit crab often tapped the edge of the pedal disk of the sea anemone and sometimes pinched the sea anemone's tentacles and oral disk, using both chelipeds. Moreover, the hermit crab rode on the sea anemone and shook and tapped it with the shell rhythmically and constantly at about six stimulations per minute. Although the hermit crab had been continually giving the tactile stimulations to the sea anemone, there was no observed cooperative reaction, which was an apparent or quick reaction observed in other symbiotic sea anemones in shallow water (e.g., *Calliactis* spp. and *Verrillactis* sp.) (Ross, 1974, 1975; Yoshikawa *et al.*, 2018). Then, the host hermit crab repeated the above behavioral series continuously; however, failure in transference of the sea anemone and its subsequent return to the previous shells are shown in observation S1 in Figure A1.

The host hermit crab's second shell change behavior was observed on February 28, 2020 (Fig. A1). Although no apparent reaction was observed with this sea anemone, its position was gradually moved and peeled off from the shell (Fig. 8B; observation S2-1: Video S5, available online). During the hermit crab's transference behavior, the column extended to the paniculate shape from the thin and flat shape (original shape) (observation S2-1: Video S5). Finally, rather than being spontaneously detached, the sea anemone was peeled off from the shell by the hermit crab about 12 hours after the crab's shell change (observation S2-2: Video S6, available online).

After detachment, despite the intense effort of the hermit crab to replace the sea anemone by carrying and fitting to the shell, the sea anemone showed no apparent shell-mounting action (observation S2-2: Video S6). However, the host hermit crab continued to carry the sea anemone and fit it onto the shell. When the sea anemone was turned to the upside-down position (facing up the pedal disk) (Fig. 8C), the hermit crab rode on the pedal disk of the sea anemone and attempted to fit the shell to the curved line of the pedal disk (Fig. 8C, D). The host hermit crab kept trying until the sea anemone started shell mounting.

After about 43 h from the host's shell change and 18 h from detachment, the sea anemone began to mount the shell (observation S2-3: Video S7, available online). While the sea anemone was moving to the shell, the hermit crab was still tapping and pinching the sea anemone as in its detaching behavior (Fig. 8E; observation S2-4: Video S8, available online). Finally, the sea anemone settled down on the new shell of the host hermit crab with a usual position facing the upper oral disk (Fig. 8F; observation S2-4: Video S8).

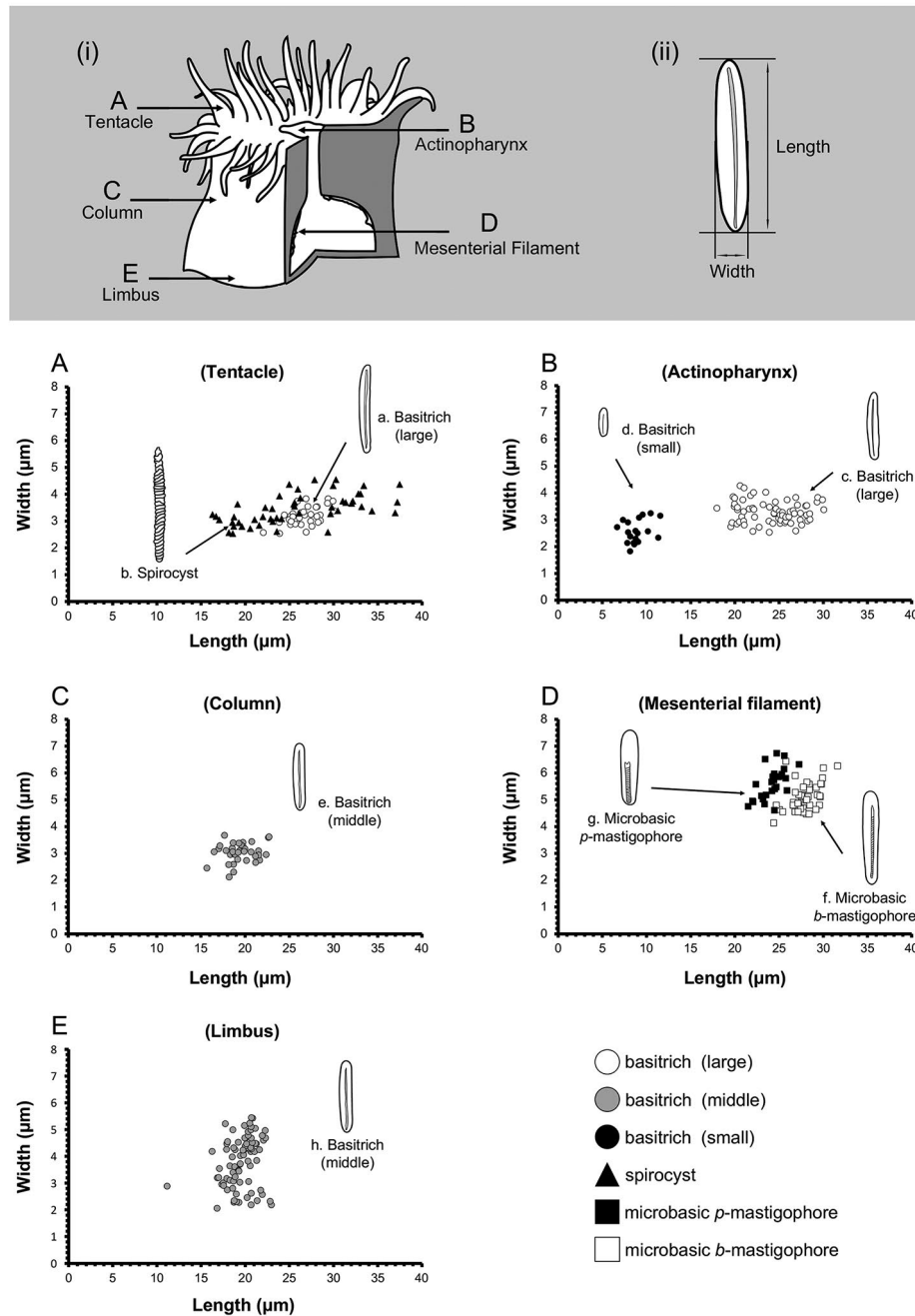


Figure 6. Size distribution of the cnidae of *Stylobates calcifer* sp. nov. (holotype: NSMT-Co 1794). (i) The examined positions of the sea anemone and the corresponding scatter diagrams (A–E). (ii) The measured parameters of the cnidae.

In summary, the transfer of the sea anemone took more than 24 h after the second shell change of the host hermit crab, that is, 12 h for detachment from the previous shell, 12 h for the mounting behavior after detachment, and 4 h to complete the transfer (Fig. A1).

In summary, the host hermit crab's shell change behavior was observed twice on February 20 (Video S4) and Febru-

ary 28, 2020. In the first trial (observation S1), the hermit crab tried to transfer the CF anemone from 22:00 hours on February 20 to 24:00 hours on February 22 but failed, returning to the previous shell on February 22. However, in its second attempt (observations S2-1 to S2-4), it successfully transferred the CF anemone onto the new shell (observation S2-4: Video S8). Despite the persistent manipulation by the host

Table 2

Cnidae size ranges of *Stylobates calcifer* sp. nov. (NSMT-Co 1794 and NSMT-Co 1796).

Type of cnidae		NSMT-Co 1794 (holotype)			NSMT-Co 1796 (paratype)		
Category in this study	Category in Crowther <i>et al.</i> , 2011	Length (μm), mean \pm SD (min.–max.)	Width (μm), mean \pm SD (min.–max.)	<i>n</i>	Length (μm), mean \pm SD (min.–max.)	Width (μm), mean \pm SD (min.–max.)	<i>n</i>
Tentacle							
a.	(a) Basitrich (large)	26.64 \pm 1.79 (22.1–30.1)	3.19 \pm 0.34 (2.54–3.83)	35	28.81 \pm 2.35 (20.08–32.44)	2.96 \pm 0.37 (2.24–3.76)	47
b.	(c) Spirocyst	25.81 \pm 5.89 (16.3–37.48)	3.45 \pm 0.54 (2.54–4.6)	49	27.92 \pm 5.07 (20.06–37.77)	3.37 \pm 0.46 (2.51–4.02)	24
Actinopharynx							
c.	(d) Basitrich (large)	24.2 \pm 3.05 (17.98–30.08)	3.3 \pm 0.39 (2.54–4.27)	69	23.8 \pm 3.37 (19.82–30.53)	3.4 \pm 0.52 (2.51–4.25)	13
d.	(f) Basitrich (small)	8.9 \pm 1.24 (6.7–11.6)	2.58 \pm 0.41 (1.83–3.25)	19	8.63 \pm 1.09 (6.22–10.17)	2.68 \pm 0.28 (2.28–3.06)	9
Column							
e.	Basitrich (middle)	19.45 \pm 1.68 (15.69–22.72)	3.03 \pm 0.35 (2.12–3.67)	36	19.07 \pm 2.85 (12.44–24.89)	2.91 \pm 0.42 (2.4–3.69)	16
Mesenterial filament							
f.	(i) Microbasic <i>b</i> -mastigophore Basitrich (i) in Crowther <i>et al.</i> , 2011	27.88 \pm 1.51 (24.4–31.63)	5.11 \pm 0.52 (4.13–6.43)	42	29.72 \pm 2.03 (24.63–34.02)	4.16 \pm 0.48 (3.17–5.22)	43
g.	(m) Microbasic <i>p</i> -mastigophore	24.24 \pm 1.31 (21.5–27.28)	5.61 \pm 0.57 (4.6–6.73)	27	25.83 \pm 1.44 (23.79–28.33)	5.41 \pm 0.45 (4.57–6.41)	19
Limbus							
h.	Basitrich (middle)	19.61 \pm 1.77 (11.16–22.97)	3.8 \pm 0.93 (2.06–5.44)	81 (two slides)	20.74 \pm 1.34 (18.19–24.5)	3.78 \pm 0.5 (2.79–4.95)	36

hermit crab for more than 12 h, the sea anemone did not show a visible, consistent reaction to the crab.

Discussion

Taxonomic discussion

The species in *Stylobates* genus have a smooth column, pinnate or palmate marginal sphincter muscles, about 200 short tentacles, 5 or 6 cycles of mesenteries with several perfect mesenteries, and diffuse mesenterial retractor muscles (Carl-gren, 1928; Dunn *et al.*, 1980). *Stylobates calcifer* sp. nov. has all of these characteristics.

In addition, the results from the molecular analysis indicated that the new species was located near *Stylobates loisetteae* but differed morphologically with respect to the marginal sphincter muscle (pinnate with short radiate primary axis in *S. calcifer* sp. nov. vs. pinnate with reticulate pattern in *S. loisetteae*), the size range of basitrichs in the actinopharynx (two in *S. calcifer* sp. nov. vs. one in *S. loisetteae*), cnidae distribution of mesenterial filament (microbasic *b*-mastigophore in *S. calcifer* sp. nov. vs. basitrichs in *S. loisetteae*), distribution, and host associations. Thus, *S. calcifer* sp. nov. likely belongs to the genus *Stylobates*.

This study showed that *S. calcifer* sp. nov. could be distinguished morphologically and ecologically from other *Stylobates* species, such as *S. aeneus*, *S. birtlesi*, and *S. cancrisocia*, by the upward-facing oral disk; from *S. birtlesi*, *S. aeneus*, and *S. cancrisocia* by the two size ranges of basitrichs in the actinopharynx; from *S. birtlesi*, *S. aeneus*, *S. cancrisocia*, and *S. loisetteae* by the middle size of basitrichs in the column; from *S. aeneus*, *S. cancrisocia*, and *S. loisetteae* by the absence of basitrichs and existence of the microbasic *b*-mastigophore in mesenterial filament; from *S. birtlesi*, *S. aeneus*, *S. cancrisocia*, and *S. loisetteae* by distribution; from *S. birtlesi*, *S. aeneus*, *S. cancrisocia*, and *S. loisetteae* by host association; and from *S. birtlesi*, *S. aeneus*, and *S. cancrisocia* by the shallower site of collection (Dunn *et al.*, 1980; Fautin, 1987; Crowther *et al.*, 2011).

Moreover, each species established a highly specific association with a host hermit crab. *Stylobates birtlesi* and *S. cancrisocia* are always found on the shells inhabited by *Sympagurus trispinosus* (Balss, 1911), and *S. aeneus* and *S. cancrisocia* are found only on *Sympagurus dofleini* (Balss, 1912) and *Sympagurus brevipes* (de Saint Laurent, 1972), respectively (Dunn *et al.*, 1980; Fautin, 1987; Crowther *et al.*, 2011). Given their styles of association, the differences between

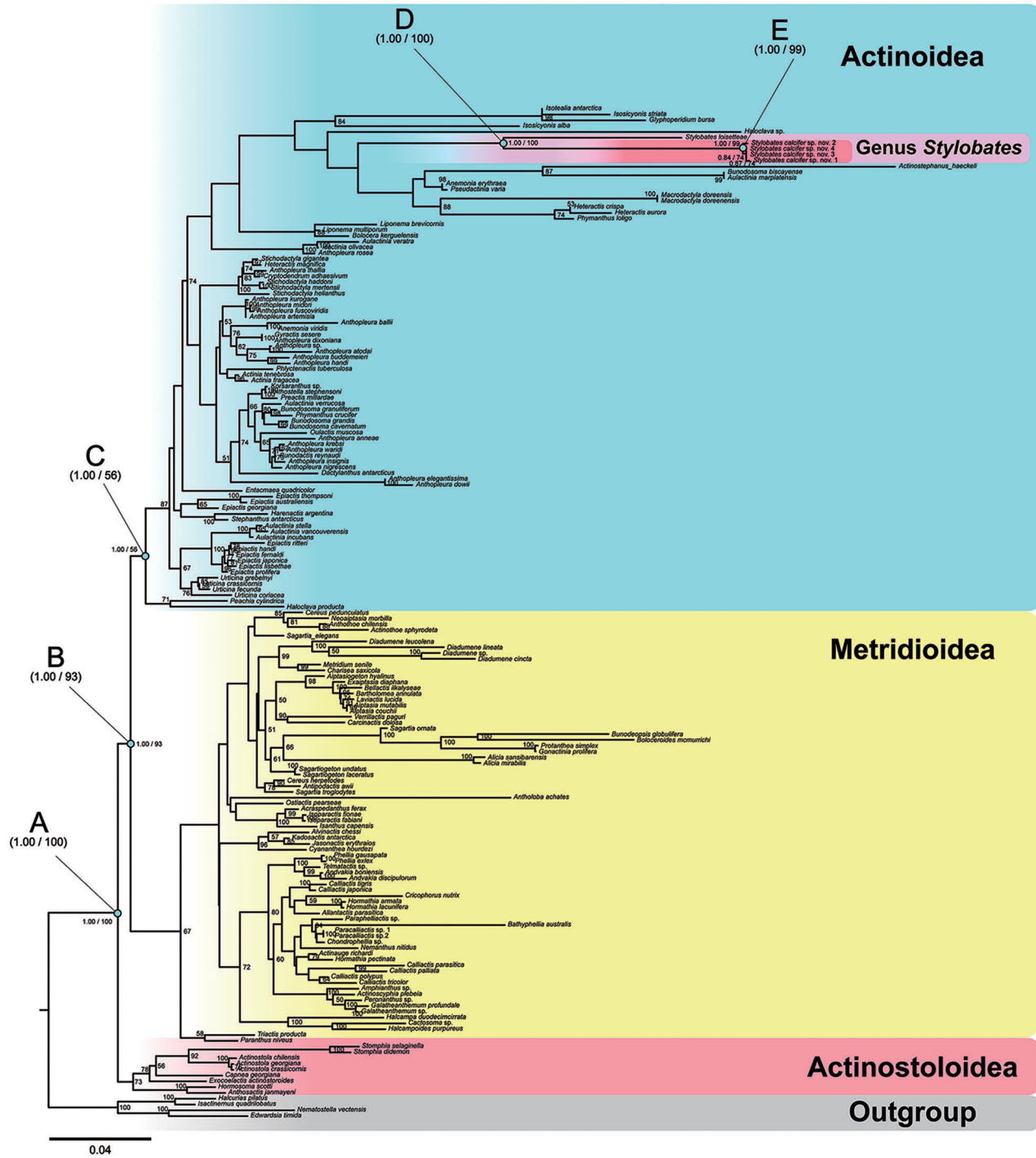


Figure 7. Maximum likelihood (ML) phylogenetic tree of Actiniidae, including *Stylobates calcifer* sp. nov., based on the combined data of the 18S, 28S, 16S rDNA, 12S rDNA, and COXIII genes. The numbers written with slashes above or below the branches indicate the ML bootstrap support values followed by Bayesian inference (BI) posterior probabilities of each node. Those without slashes indicate only the ML bootstrap support values. Green and pink spaces indicate the species of superfamily Actinoidea and the genus of *Stylobates*, respectively. The position of *S. calcifer* sp. nov. is indicated in red. The values less than 50 of bootstrap support or 0.50 of posterior probability are shown by a dash. The numbers after *S. calcifer* sp. nov., 1 (CMNH-ZG09764), 2 (CMNH-ZG09765), 3 (CMNH-ZG09771), and 4 (NSMT-Co 1796), correspond to the registration numbers of the specimens.

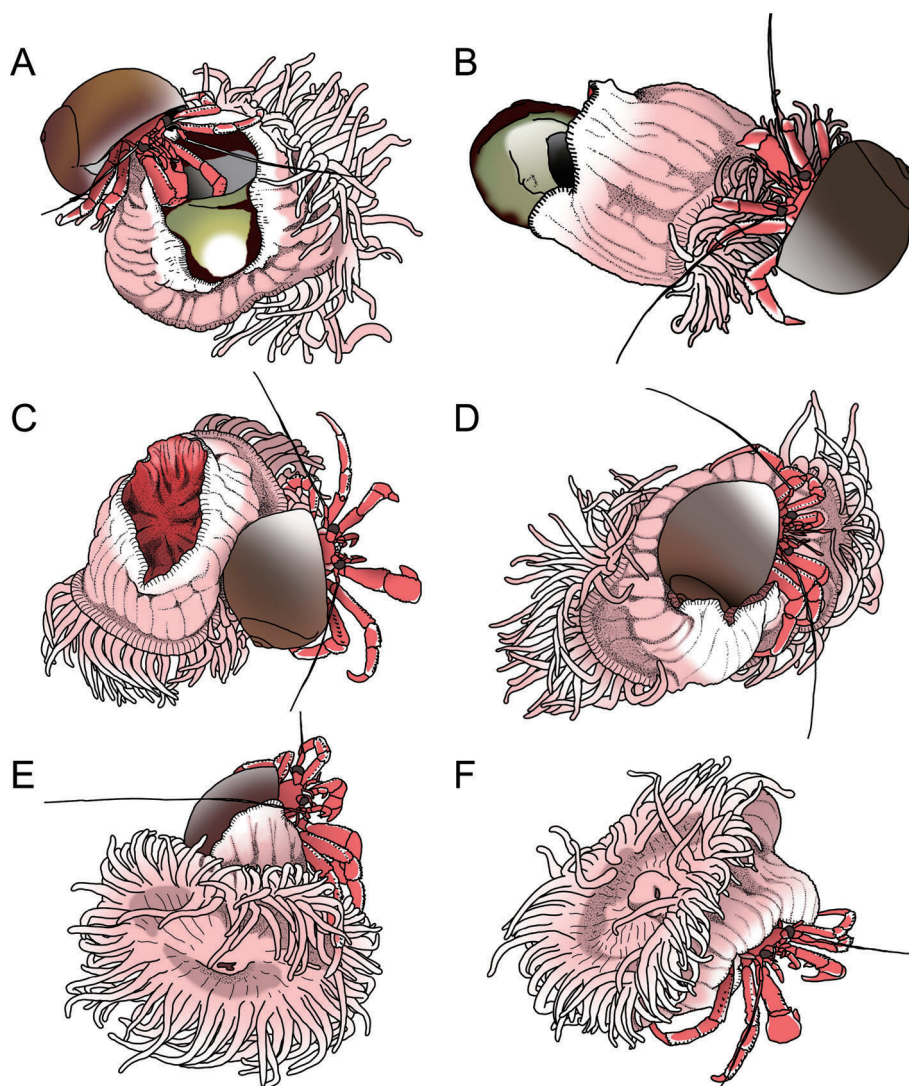


Figure 8. Behavioral sequence of the hermit crab *Pagurodoifleinia doederleini* transferring the sea anemone *Stylobates calcifer* sp. nov. from the original snail *Glossaulax reiniana* shell to a new one. (A) The hermit crab begins tapping the pedal disk and column of the sea anemone with its walking legs and chelipeds. (B) The hermit crab uses one or both of its chelipeds to pinch and remove *S. calcifer* sp. nov. from the *G. reiniana* shell. (C) *Stylobates calcifer* sp. nov. displays no shell-mounting action after being removed from the original shell. (D) The sea anemone is turned to the upside-down position (facing up the pedal disk) by the crab. (E) The hermit crab rides on the pedal disk of the sea anemone and fits the shell to the curved line of the pedal disk. (F) The sea anemone settles down in a new shell of the host hermit crab with a usual position.

the host use patterns are also key ecological characteristics by which the species can be distinguished.

In addition, *S. calcifer* sp. nov. can be differentiated from *S. aeneus* by the short radiate primary axis of the pinnate marginal sphincter muscle, from *S. birtlesi* by the pinnate marginal sphincter muscle and the absence of basitrichs in mesenterial filament, and from *S. loisetteae* by the short radiate primary axis of the pinnate marginal sphincter muscle and two size ranges of basitrichs in the actinopharynx. Therefore, *S. calcifer* sp. nov. is conclusively distinguishable from the other four species and is the first species described based on

scientific specimens from the Japanese deep-sea water of the Pacific Ocean. See Table 3 for the differences of all species of *Stylobates* in detail.

Crowther *et al.* (2011) did not distinguish the basitrichs and microbasic *b*-mastigophores in the mesenterial filament of *S. birtlesi*. Basitrichs, reported as basitrich (*i*) in Crowther *et al.* (2011), should be a microbasic *b*-mastigophore because the shape of the capsule and shaft of (*i*) is noticeably different from the other basitrichs in figure 5 of Crowther *et al.* (2011). Therefore, the basitrich (*i*) in Crowther *et al.* (2011) was incorrectly compared to the basitrich (F) of *S. aeneus* (fig. 4F in

Table 3

Morphology, biogeography, and symbiotic association with a hermit crab of *Stylobates calcifer* sp. nov. compared to other *Stylobates* species

	<i>S. calcifer</i> sp. nov.	<i>S. aeneus</i>	<i>S. birtlesi</i>	<i>S. cancrisocia</i>	<i>S. loisetteae</i>	
Morphology	Cycles of mesenteries	5	Not examined	5	Not examined	5 or 6 (uncertain description)
	Number of perfect mesenteries	First to third	Several cycles (uncertain description)	First to third	Several cycles (uncertain description)	At least first to second (uncertain description)
	Direction of oral disk	Upward-facing position	Facing-toward position	Facing-toward position	Facing-toward position	Upward-facing position
	Marginal sphincter muscle	Pinnate (short radiate primary axis)	Pinnate (strong mesogleal axis)	Palmate	Pinnate (short radiate primary axis)	Pinnate (reticulate pattern)
	Number of tentacles	178–190	Not examined	96 to more than 200	Not examined	160–200
Cnidom	Basitrichs in actinopharynx	Large (17.98–30.08; 2.54–4.27) and small (6.7–11.6; 1.83–3.25)	Large (26.2–37.7; 2.9–4.1) and middle (17.2–21.3; 2.5–3.1)	Large (27.8–37.1; 2.9–4.3), middle (19.9–21.2; 3.3), and small (6.4–11.2; 1.9–2.8)	Large (27.1–31.2; 2.5–3.3)	Large (26.0–37.2; 2.5–3.7)
	Basitrichs in column	Middle (15.69–22.72; 2.12–3.67)	Large (29.5–36.1; 2.9–3.5)	Large (19.9–30.5; 2.6–4.1) and small (7.3–9.2; 1.9–3.6)	Large (23.0–28.7; 2.7–3.3)	Large (21.1–33.5; 2.5–3.7)
	Basitrichs in mesenterial filament	–	○	○	○	○
	Microbasic <i>b</i> -mastigophore in mesenterial filament	○	–	○	–	–
	Distribution and habitat	Sea of Kumano (off the coast of Kumano Region of the Kii Peninsula in central Japan)	Between Oahu and Molokai in the Hawaiian Islands, US	Coral Sea, off the coast of Tully, Queensland, Australia (17°45.99' S, 148°39.09' E)	Pemba Channel off east Africa	Off the northwest coast of Port Hedland, Western Australia (18°41' S, 116°45' E)
Type locality						
Distribution	Pacific side of Japan	Guam and Hawaii	Northeast Australia	East Africa	Northwest Australia	
Depth (m)	100–400	402–797	590–964	818	320–508	
Substrate	Fine sand, mud	Sand, rock	Mud, rock	Not recorded	Mud	
Host association	Host hermit crab	<i>Pagurodoyleinia doederleini</i>	<i>Sympagurus dofleini</i>	<i>Sympagurus trispinosus</i>	<i>Sympagurus trispinosus</i>	<i>Sympagurus brevipes</i>
	Responses for the stimulations from the host hermit crab	No typical responses were observed	Not examined	Not examined	Not examined	Not examined
	Transference of the host hermit crab	Transferred	Not examined	Not examined	Not examined	Not examined

Table 3 (Continued)

	<i>S. calcifer</i> sp. nov.	<i>S. aeneus</i>	<i>S. birtlesi</i>	<i>S. cancrisocia</i>	<i>S. loisetteae</i>
References	Uchida and Soyama, 2001; Yanagi, 2006; Yoshikawa <i>et al.</i> , 2019; this study	Dall, 1903; Dunn <i>et al.</i> , 1980; Crowther <i>et al.</i> , 2011	Crowther <i>et al.</i> , 2011	Carlgren, 1928; Dunn <i>et al.</i> , 1980; Crowther <i>et al.</i> , 2011	Fautin, 1987; Crowther <i>et al.</i> , 2011

The size range (min.–max.) of the cnida's length and width is in μm . The unique cnida characteristics of *S. calcifer* sp. nov. are in bold. Circles and dashes indicate the existence or absence, respectively, of each cnida.

Dunn *et al.*, 1980) and the basitrich (f) of *S. loisetteae* (fig. 6F in Fautin, 1987; Crowther *et al.*, 2011). Thus, we have updated the comparison table of the cnida distribution of *Stylobates* species, including *S. calcifer* sp. nov. (Table A3).

Taxonomic confusion and synonym of *Stylobates calcifer* sp. nov.

Stylobates calcifer sp. nov. was known as “*Isadamsia* sp. J” in Japan because it was first introduced in the Japanese general guidebook of sea anemones (Uchida and Soyama, 2001). However, Uchida and Soyama (2001) showed only one photograph of a living specimen and a short diagnosis without any morphological comparison to other *Stylobates* species. Uchida and Soyama (2001) mentioned that *Isadamsia* sp. J possessed the mesenteries arranged in 96 pairs in 5 cycles and a pinnate marginal sphincter muscle with the habitable depth of 100–400 m, species specific with *Pagurodofleinia doederleini* and the sampling locality near the coast of the Kumano Region of the Kii Peninsula in central Japan. Since these characteristics and sampling locality agree with those of *S. calcifer* sp. nov., we have assigned *Isadamsia* sp. J as a synonym of the new species.

Yanagi (2006) also reported CF anemones on the shells inhabited by *P. doederleini*. Yanagi (2006) examined the CF anemones' external morphology, carcinoecium production, and host association, following Uchida and Soyama's description (2001), and identified these specimens as *Isadamsia* sp. J. Therefore, we have included *Isadamsia* sp. J, reported by Yanagi (2006), in the list of synonyms of *S. calcifer* sp. nov.

The four specimens identified as *Stylobates* sp. in Yoshikawa *et al.* (2019), SMBL-V00001 to SMBL-V00004, were collected by the beam trawl on R/V *Seisui-maru* on the same research cruise of this study (no. 1722), at the same locality, same depth, and same date as CMNH-ZG09764, CMNH-ZG09765, and CMNH-ZG09766. We have, thus, assigned these specimens in the list of synonyms of *S. calcifer* sp. nov.

The sea anemones (*Isadamsia* sp.) photographed in figure 1a, b, and c of Jimi *et al.* (2021) were also collected at a similar location and depth as type specimens of *S. calcifer* sp. nov. Moritaki (one of the authors of the abovementioned and present studies) reported that *Isadamsia* sp. was exclusively found

on the snail shells of *P. doederleini* through their study. Therefore, we conclude that these records are of *S. calcifer* sp. nov.

Behaviors of sea anemone

The videos obtained in this study suggest that *S. calcifer* sp. nov. potentially feeds on suspended particulate organic matter from the water column. Because the species displayed no typical or clear feeding responses for the deposited organisms on the floor in the sequential observations, including the observation of the symbiotic relationship, it might feed on the falling macroscopic aggregates of detritus and inorganic matter from the water column, possibly marine snow, particulate organic matter, and the food residuals of hermit crabs. Because it displayed no aggressive reactions to living foods, it seems not to prey on living animals. Moreover, this species exhibited active mounting behavior at the usual upward-facing position after being transferred to the new shell by the hermit crab (Video S8, available online). Thus, the upward-facing position may be significant for it to catch the accumulating organisms drifting in the water column.

Attached position and direction of the oral disk

In hermit crab-associated sea anemones, the attached position and/or the direction of the oral disk will potentially be a key ecological or behavioral diagnosis. For example, *Verrillactis paguri sensu* England (1971) is always found on the shell aperture of the host snail's shell (England, 1971; Yoshikawa *et al.*, 2018). In *Paracalliactis* spp., the direction of the oral disk differs between species (*e.g.*, *Paracalliactis azorica* is oriented ventrally, whereas *Paracalliactis consors* is oriented upward and/or dorsally) (Doumenc, 1975; Daly *et al.*, 2004; Gusmão, 2010). In *Stylobates* species, differences in the direction of the oral disk have previously been reported as a means of differential diagnosis as follows: “The position of the oral disc of *Stylobates birtlesi* sp. n. is near the aperture of carcinoecium, like in *Stylobates aeneus* and *Stylobates cancrisocia*, whereas that of *Stylobates loisetteae* is on the side of the ultimate whorl of the carcinoecium, away from the aperture” (see “Differential diagnosis” in Crowther *et al.*,

2011, pp. 41–42). Therefore, the direction of the oral disk can also be considered an ecological or behavioral characteristic by which species of *Stylobates* can be distinguished.

The significance of the attaching position of *Stylobates* was previously discussed as an adaptation to avoid injury when being dragged along the seafloor by a host hermit crab (Gusmão *et al.*, 2019). For example, *Sympagurus dofleini* (Balss 1912), which is associated with *S. aeneus*, has long legs and can probably carry the anemone high above the floor. In contrast, *S. loisetteae*, which is associated with *Sympagurus brevipes*, might avoid potential injury from being dragged along the seafloor by attaching itself in an upward-facing position. However, observational evidence to support this claim has yet to be reported.

Our observational data, however, lead to a new hypothesis related to the relationship between attaching position and feeding style. The upward-facing position of *S. calcifer* sp. nov. may be an adaptation for feeding on the suspended particulate organic matter from the water column. The downward-facing position, recognized in *S. aeneus*, *S. birtlesi*, and *S. cancri-socia*, might have significance for feeding on the deposited organisms beneath. Continuous observations of the downward-facing feeding style of these three species and the upward-facing position of *S. loisetteae* are needed to verify the significance of the attaching position as an adaptation to the deep-sea habitat.

Notably, however, the direction of the oral disk may temporarily differ or vary, depending on the transfer process conducted by the host hermit crab. In our behavioral observations, *S. calcifer* sp. nov. was first attached to the ventral side of the shell and then climbed to its usual position (facing upward) (see observations S2-3 to S2-4 in Videos S7 and S8, available online; Fig. 8D–F). Therefore, although we have included the direction of the oral disk as a diagnosis of *S. calcifer*, the reliability of this characteristic should be considered according to the relevant ecological and/or behavioral conditions and the process of transference involving the hermit crab and sea anemone.

Symbiotic relationship with hermit crab

Our observational data are likely the first video records of the behavioral interaction between the conspicuous CF Actinoidean sea anemone *Stylobates* and a hermit crab. The host's shell change observed here was in contrast to the previous prediction on the CF mutualism: "Unless it were displaced by a more aggressive individual, as occurs in typical hermit crabs (Hazlett 1966, 1970), there should be no reason for the crustacean to leave a shell it had not outgrown" (Dunn *et al.*, 1980, p. 386). Otherwise, *S. calcifer* sp. nov. might not produce and expand the carcinoecium at an ideal speed for the growth of the hermit crab, which would lead to the host potentially seeking new, larger snail shells as it grows.

The host hermit crab persistently tried to transfer *S. calcifer* sp. nov., as if its relationship with the CF anemone were essential. However, the sea anemone's active or cooperative participation was not observed (the sea anemone detached

passively). Moreover, the behavioral manner of *S. calcifer* sp. nov. may resemble the shell response of *Paracalliactis mediterranea*, although a conspicuously longer period until the detached sea anemone climbed onto the shell was observed for *S. calcifer* sp. nov. (*S. calcifer* sp. nov.: 22 h [in this study]; *P. mediterranea*: about 60–160 min [Ross and Zanponi, 1982]).

This observation leads to the following hypothesis related to symbiotic style: the sea anemone produces the carcinoecium constantly (not synchronously with the host growth rate) and waits until a well-suited host hermit crab uses it after the last host's shell change. Practically, although *S. calcifer* sp. nov. is exclusively found on snail shells inhabited by *P. doederleini*, the host hermit crab species was collected without *S. calcifer* sp. nov. or any other symbiotic sea anemones throughout the present study (AY, pers. obs.). Given this, the anemone-free hermit crabs may steal the sea anemone in the middle of the transference of other pairs. Additional research on behavioral interactions under conditions involving multiple individuals will be needed to strengthen and/or verify (or refute and extend) our hypothesis.

Because of our limited observations due to difficulties in collecting specimens, we conducted all behavioral observations on a single specimen. Nevertheless, our behavioral observations are suggestive of the remarkable symbiotic relationships in the actiniarian CF mutualisms in the deep sea. To understand how the sea anemone builds and retains its highly specific association with other organisms on the deep-sea floor, further exploration on species recognition systems between other *Stylobates* species is required.

Possible merits of shell change and sea anemone transferring

Hermit crabs are critical physical ecosystem engineers in various marine habitats. At least 550 invertebrate species from 16 phyla, from the intertidal/littoral zone to the deep sea, are associated with the shells inhabited by hermit crabs (Jones *et al.*, 1994, 1997; Williams and McDermott, 2004). Of these, some communal associations of Polynoidae are thought to affect the host's survival because the species inside the shell are known to steal food from the hermit crab's mouth and feed on crab eggs soon after extrusion (Brightwell, 1951; Williams and McDermott, 2004). A communalistic scale-worm species, *Eunoe issunboushi* Jimi, Hookabe, Moritaki, Kimura & Imura, 2021, was recently reported from inside the carcinoecium of *S. calcifer* sp. nov. (Jimi *et al.*, 2021). It may indicate that the long-term use of the same carcinoecium increases the risk of trespassing by the Polynoidae species on *P. doederleini*.

Thus, our observational data provide new insights into host hermit crab behavior; accordingly, we suggest the following hypothesis: the constant shell changes of the host hermit crab might be an adaptation to avoid the risk of trespassing from other communal species into the shell; that is, the behavior is not only for the crab's own growth success. Further research

focusing on the interactions between communal associations (e.g., symbiotic rate and host preference for species; quantification of the disadvantages of hermit crabs and/or sea anemone) will be necessary to verify this proposition. Such research could also uncover the function of CF association as an ecosystem engineer in the biological communities that exist on the soft bottom of the deep sea.

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Data Accessibility

All of the DNA sequences reported in this article have been deposited in GenBank (accession numbers LC642139–LC642149).

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Appendix

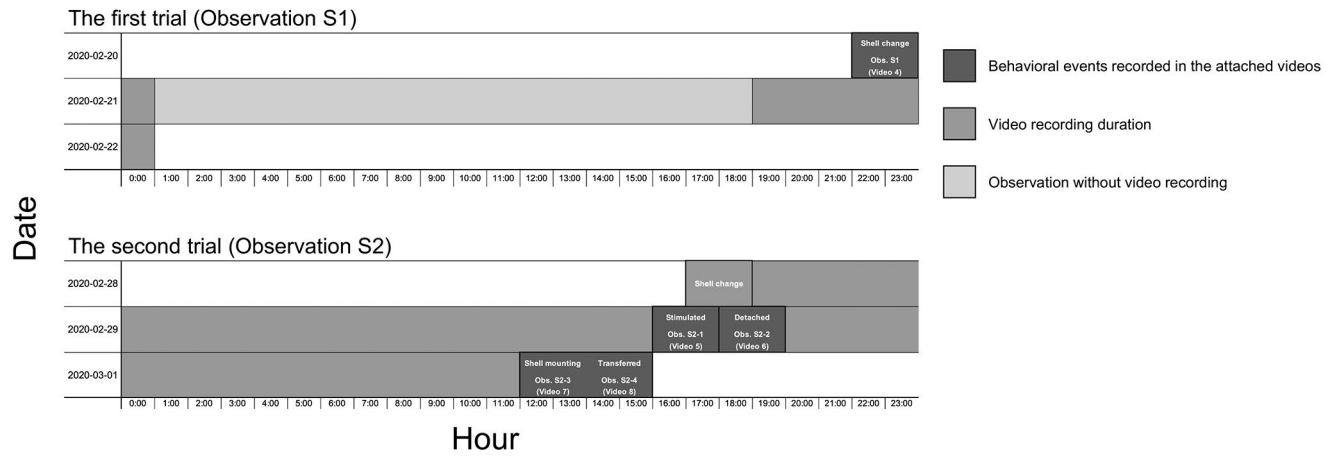


Figure A1. Time sequencing of the behavioral observations (S1 and S2) on the symbiotic relationship between the hermit crab *Pagurodotheimia doederleini* and the sea anemone *Stylobates calcifer* sp. nov.

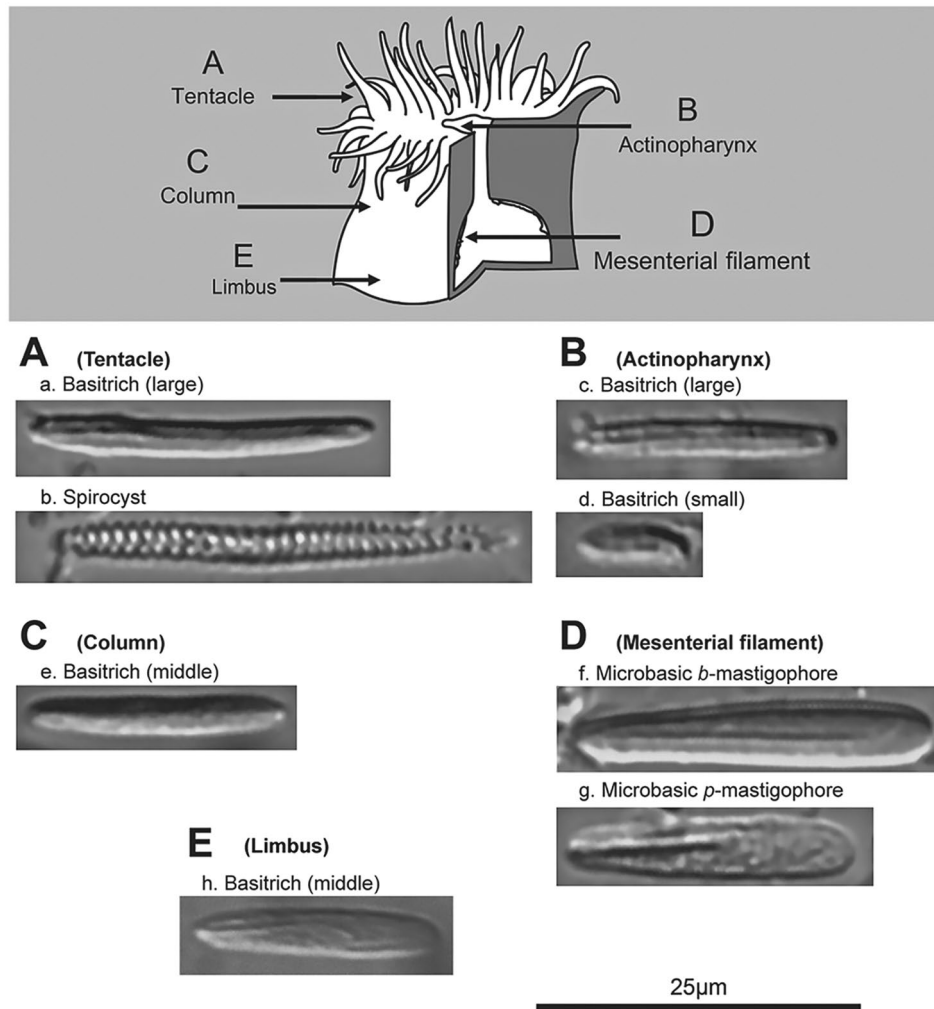


Figure A2. Cnidae of *Stylobates calcifer* sp. nov. (holotype: NSMT-Co 1794). (A–E) The examined position of the sea anemone (the upper diagram), including the large basitrich in tentacles (a), spirocyst in tentacles (b), large (c) and small (d) basitrich in actinopharynx, middle basitrich in the column (e), microbasic *b*-mastigophore (f) and *p*-mastigophore (g) in mesenterial filament, and middle basitrich (h) in limbus.

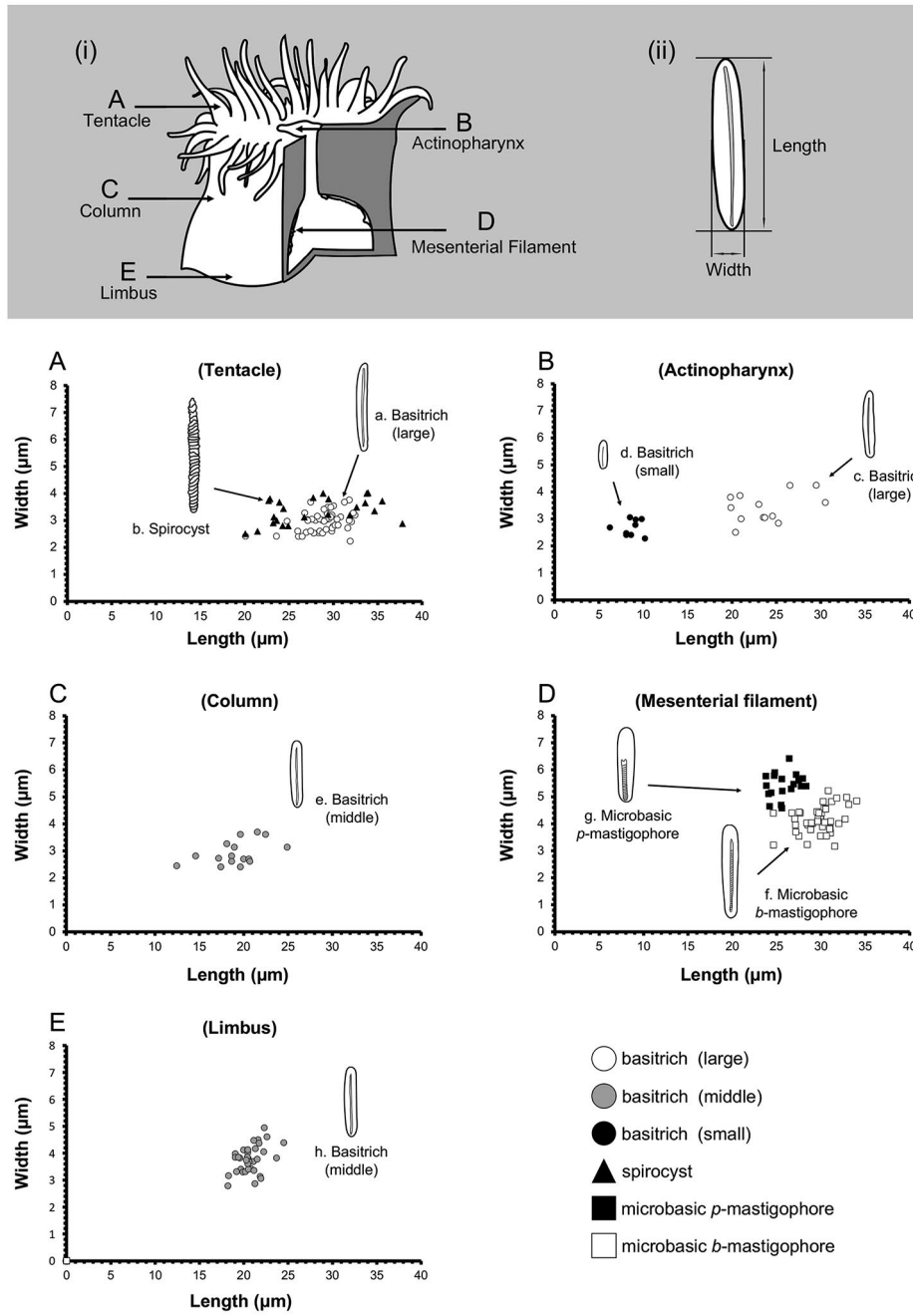


Figure A3. Size distribution of the cnidae of *Stylobates calcifer* sp. nov. (paratype: NSMT-Co 1796). (i) indicates the examined positions of the sea anemone and the corresponding scatter diagrams (A–E). (ii) indicates the measured parameters of the cnidae.

Table A1

Base sequences in the phylogenetic analyses

Higher taxon	Family	Genus	Species	12S	16S	18S	28S	COIII
Actiniaria								
Enthemonae								
Actinoidea	Actiniidae	<i>Actinia</i>	<i>fragacea</i>	EU190714	EU190756	EU190845	KJ483085	GU473334
		<i>Actinia</i>	<i>tenebrosa</i>	KT852045	KT852111	KT852174	—————	KT852330
		<i>Anemonia</i>	<i>erythraea</i>	KY789302	KY789335	—————	—————	KY789271
		<i>Anemonia</i>	<i>viridis</i>	EU190718	EU190760	EU190849	EU190806	GU473335
		<i>Anthopleura</i>	<i>anneae</i>	KY789327	KY789360	—————	KY789392	KY789293
		<i>Anthopleura</i>	<i>artemisias</i>	KT852015	KT852081	KT852148	—————	KT852300
		<i>Anthopleura</i>	<i>atodai</i>	KT851993	KT852055	KT852123	KT852247	KT852275
		<i>Anthopleura</i>	<i>ballii</i>	KY789311	KY789346	—————	KY789376	KY789281
		<i>Anthopleura</i>	<i>buddemeieri</i>	KY789316	KY789351	—————	KY789381	—————
		<i>Anthopleura</i>	<i>dixoniana</i>	KY789307	KY789341	—————	—————	KY789276
		<i>Anthopleura</i>	<i>dowii</i>	KY789318	KY789353	—————	KY789383	KY789286
		<i>Anthopleura</i>	<i>elegantissima</i>	EU190713	EU190755	EU190844	KT852248	GU473333
		<i>Anthopleura</i>	<i>fuscoviridis</i>	KY789303	KY789336	—————	KY789369	KY789272
		<i>Anthopleura</i>	<i>handi</i>	KT852013	KT852079	KT852146	KY789387	KT852298
		<i>Anthopleura</i>	<i>insignis</i>	KY789331	KY789364	—————	KY789395	KY789297
		<i>Anthopleura</i>	<i>krebsi</i>	KY789305	KY789339	—————	KY789372	KY789275
		<i>Anthopleura</i>	<i>kurogane</i>	KY789321	KY789355	—————	KY789385	KY789288
		<i>Anthopleura</i>	<i>midori</i>	KY789324	—————	—————	KY789388	KY789289
		<i>Anthopleura</i>	<i>nigrescens</i>	KY789309	KY789343	—————	KY789373	KY789278
		<i>Anthopleura</i>	<i>rosea</i>	KT852039	KT852104	KT852168	—————	KT852324
		<i>Anthopleura</i>	sp.	KY789304	KY789338	—————	KY789371	KY789274
		<i>Anthopleura</i>	<i>thallia</i>	KY789333	KY789366	—————	KY789397	KY789300
		<i>Anthopleura</i>	<i>waridi</i>	KY789301	KY789334	—————	KY789368	KY789270
		<i>Anthostella</i>	<i>stephensoni</i>	JQ810719	JQ810721	JQ810723	KJ483132	JQ810726
		<i>Aulactinia</i>	<i>incubans</i>	KT852014	KT852080	KT852147	KT852256	KT852299
		<i>Aulactinia</i>	<i>marplatensis</i>	KT851999	KT852061	KT852129	KT852249	KT852281
		<i>Aulactinia</i>	<i>stella</i>	KT852044	KT852110	KT852173	KT852263	KT852329
		<i>Aulactinia</i>	<i>vancouverensis</i>	KT852019	KT852085	KT852151	—————	KT852305
		<i>Aulactinia</i>	<i>veratra</i>	KT852001	KT852063	KT852131	—————	KT852283
		<i>Aulactinia</i>	<i>verrucosa</i>	EU190723	EU190766	EU190854	KT852250	FJ489484
		<i>Bolocera</i>	<i>kerguelensis</i>	KJ482925	KJ482965	KJ483029	KJ483133	KJ482985
		<i>Bunodactis</i>	<i>reynaudi</i>	KT852041	KT852106	KT852170	KT852260	KT852326
		<i>Bunodosoma</i>	<i>biscayense</i>	KY789315	KY789350	—————	KY789380	KY789284
		<i>Bunodosoma</i>	<i>cavernatum</i>	KY789313	KY789348	—————	KY789378	KY789282
		<i>Bunodosoma</i>	<i>grandis</i>	EU190722	EU190765	EU190853	KJ483083	GU473336
		<i>Bunodosoma</i>	<i>granuliferum</i>	KY789314	KY789349	—————	KY789379	KY789283
		<i>Epiactis</i>	<i>australiensis</i>	KT852000	KT852062	KT852130	—————	KT852282
		<i>Epiactis</i>	<i>fernaldi</i>	KT852005	KT852068	KT852136	KT852252	KT852288
		<i>Epiactis</i>	<i>georgiana</i>	KT852007	KT852070	KT852138	KT852254	KT852290
		<i>Epiactis</i>	<i>handi</i>	KT851988	KT852050	KT852118	KT852245	KT852269
		<i>Epiactis</i>	<i>japonica</i>	KT851991	KT852053	KT852121	—————	KT852272
		<i>Epiactis</i>	<i>lisbethae</i>	KT852006	KT852069	KT852137	KT852253	KT852289
		<i>Epiactis</i>	<i>prolifera</i>	KT851989	KT852051	KT852119	KT852246	KT852270
		<i>Epiactis</i>	<i>ritteri</i>	KT851994	KT852056	KT852124	—————	KT852276
		<i>Epiactis</i>	<i>thompsoni</i>	KT852011	KT852074	KT852142	—————	KT852294
		<i>Glyphoperidium</i>	<i>bursa</i>	KJ482923	KJ482961	KJ483033	KJ483136	KJ482982
		<i>Gyractis</i>	<i>sesere</i>	KT852012	KT852078	KT852145	KY789386	KT852297
		<i>Isactinia</i>	<i>olivacea</i>	—————	KT852077	KT852144	—————	KT852296
		<i>Isosicyonis</i>	<i>alba</i>	—————	KJ482959	KJ483030	KJ483134	KJ482981
		<i>Isosicyonis</i>	<i>striata</i>	EU190736	EU190781	EU190864.1	KJ483137	FJ489493
		<i>Isotealia</i>	<i>antarctica</i>	JQ810720	JQ810722	—————	—————	JQ810727
		<i>Korsaranthus</i>	sp.	KJ482920	KJ482958	KJ483017	KJ483117	KJ482987
		<i>Macroductyla</i>	<i>doreenensis</i>	EU190739	EU190785	EU190867	KJ483049	GU473342
		<i>Oulactis</i>	<i>muscosa</i>	KT852033	KT852097	KT852162	KY789391	KT852317
		<i>Phlyctenactis</i>	<i>tuberculosa</i>	KY789326	KY789359	—————	—————	KY789292
		<i>Pseudactinia</i>	<i>varia</i>	KY789328	KY789361	—————	—————	KY789294
		<i>Stylobates</i>	<i>calcifer 1</i>	—————	—————	LC642145	LC642146	—————

Table A1 (Continued)

Higher taxon	Family	Genus	Species	12S	16S	18S	28S	COIII
		<i>Stylobates</i>	<i>calcifer 2</i>	LC642139	LC642142	—————	LC642147	LC642149
		<i>Stylobates</i>	<i>calcifer 3</i>	LC642140	LC642143	—————	LC642148	—————
		<i>Stylobates</i>	<i>calcifer 4</i>	LC642141	LC642144	—————	—————	—————
		<i>Stylobates</i>	<i>loisitteae</i>	MN266876	MN266875	MN266881	MN266882	MN295037
		<i>Urticina</i>	<i>coriacea</i>	GU473282	EU190797	KT852176	KT852266	GU473351
		<i>Urticina</i>	<i>crassicornis</i>	KT851997	KT852059	KT852127	—————	KT852279
		<i>Urticina</i>	<i>fecunda</i>	KT852004	KT852067	KT852135	—————	KT852287
		<i>Urticina</i>	<i>grebelnyli</i>	KT852034	KT852098	KT852163	—————	KT852318
	Actinodendridae	<i>Actinostephanus</i>	<i>haeckeli</i>	KJ482936	EU190762	KJ483034	EU190808	GU473353
	Capneidae	<i>Capnea</i>	<i>georgiana</i>	—————	KJ482951	KJ483022	KJ483050	KJ482990
	Condylanthidae	<i>Charisea</i>	<i>saxicola</i>	KT852020	KT852086	KT852152	—————	KT852306
	Haloclavidae	<i>Haloclava</i>	<i>producta</i>	EU190734	EU190779	AF254379	EU190823	JF833008
		<i>Haloclava</i>	sp.	KJ482924	KJ482963	KJ483031	KJ483138	KJ482989
		<i>Harenactis</i>	<i>argentina</i>	KJ482926	KJ482964	KJ483026	KJ483047	KJ482984
		<i>Peachia</i>	<i>cylindrica</i>	EU190743	EU190789	KJ483015	EU190832.1	—————
		<i>Stephanthus</i>	<i>antarcticus</i>	KJ482927	KJ482960	KJ483019	KJ483092	KJ482983
	Liponematidae	<i>Liponema</i>	<i>brevicornis</i>	EU190738	EU190784	EU190866	KJ483139	KJ483001
		<i>Liponema</i>	<i>multiporum</i>	KJ482922	KJ482962	—————	—————	—————
	Phymanthidae	<i>Phymanthus</i>	<i>crucifer</i>	KJ910343	KJ910345	MH670399	MH670928	KJ910346
		<i>Phymanthus</i>	<i>loligo</i>	EU190745	EU190791	EU190871	—————	GU473345
	Preactiidae	<i>Dactylanthus</i>	<i>antarcticus</i>	GU473272	AY345877	AF052896	KJ483086	GU473358
		<i>Preactis</i>	<i>milliardae</i>	KJ482921	KJ482957	KJ483018	KJ483118	KJ482986
	Stichodactylidae	<i>Entacmea</i>	<i>quadricolor</i>	MK519405	MK519459	MK519568	MK519643	MK522443
		<i>Heteractis</i>	<i>aurora</i>	MK519414	MK519469	—————	MK519647	MK522453
		<i>Heteractis</i>	<i>magnifica</i>	EU190732	EU190777	EU190862	KJ483093	KJ482988
		<i>Heteractis</i>	<i>crispa</i>	MK519423	MK519478	—————	MK519654	MK522462
		<i>Macroactyla</i>	<i>dorensis</i>	EU190739	EU190785	EU190867	KJ483049	GU473342
		<i>Stichodactyla</i>	<i>gigantea</i>	EU190747	EU190793	—————	EU190835	KY789299
		<i>Stichodactyla</i>	<i>haddoni</i>	MK519435	MK519491	MK519583	MK519662	MK522476
		<i>Stichodactyla</i>	<i>helianthus</i>	MK519436	MK519492	MK519563	MK519667	MK522477
		<i>Stichodactyla</i>	<i>mertensii</i>	—————	MK519496	MK519584	MK519663	MK519662
	Thalassianthidae	<i>Cryptodendrum</i>	<i>adhaesivum</i>	KC812142	KC812164	KC812190	KC812214	KC812235
Actinostoloidea	Actinostolidae	<i>Actinostola</i>	<i>chilensis</i>	—————	GU473285	GU473302	KJ483110	GU473357
		<i>Actinostola</i>	<i>crassicornis</i>	—————	EU190753	EU190843	KJ483098	GU473332
		<i>Actinostola</i>	<i>georgiana</i>	KJ482928	KJ482952	KJ483032	KJ483099	KJ482991
		<i>Antholoba</i>	<i>achates</i>	GU473269	GU473284	GU473301	KJ483128	GU473356
		<i>Anthosactis</i>	<i>janmayeni</i>	KJ482938	GU473292	GU473308	KJ483091	GU473363
		<i>Hormosoma</i>	<i>scotti</i>	EU190733	EU190778	EU190863	KJ483090	GU473366
		<i>Paranthus</i>	<i>niveus</i>	GU473277	GU473295	GU473311	KJ483072	GU473344
		<i>Stomphia</i>	<i>didemon</i>	KJ482929	EU190795	EU190875	KJ483127	GU473348
		<i>Stomphia</i>	<i>selaginella</i>	GU473280	GU473298	GU473314	GU473331	GU473349
Metridioidea	Exocoelactinidae	<i>Exocoelactis</i>	<i>actinostoloides</i>	KP793003	KP793004	—————	—————	—————
	Actinoscyphiidae	<i>Actinoscyphia</i>	<i>plebeia</i>	EU190712	EU190754	FJ489437	KJ483067	FJ489476
	Aiptasiidae	<i>Aiptasia</i>	<i>couchii</i>	KP761199	KP761254	KP761301	—————	KP761405
		<i>Aiptasia</i>	<i>mutabilis</i>	JF832963	FJ489418	FJ489438	KJ483115	FJ489505
		<i>Aiptasiogeton</i>	<i>hyalinus</i>	KR704266	KP761257	KR704268	—————	—————
		<i>Bartholomea</i>	<i>annulata</i>	EU190721	EU190763	EU190851	KJ483068	FJ489483
		<i>Bellactis</i>	<i>ilkalyseae</i>	—————	KP761238	KP761316	—————	KP761393
		<i>Exaiptasia</i>	<i>diaphana</i>	KP761188	KP761239	KP761312	—————	KP761386
		<i>Laviactis</i>	<i>lucida</i>	KP761192	KP761243	KP761296	—————	KP761402
		<i>Neoiptasia</i>	<i>morbilla</i>	—————	—————	—————	KJ483075	JF833010
	Aliciidae	<i>Alicia</i>	<i>mirabilis</i>	KP761213	—————	KP761310	KP761329	KP761410
		<i>Alicia</i>	<i>sansibarensis</i>	KJ482933	KJ482953	KJ483016	KJ483116	KJ483000
		<i>Triactis</i>	<i>producta</i>	EU490525	—————	EU190876	KJ483125	GU473350
	Amphianthidae	<i>Amphianthus</i>	sp.	FJ489413	FJ489432	FJ489450	FJ489467	FJ489502
		<i>Peronanthus</i>	sp.	KJ482917	KJ482956	KJ483014	KJ483066	KJ482976
	Andvakiidae	<i>Andvakia</i>	<i>boninensis</i>	EU190717	EU190759	EU190848	KJ483053	FJ489479
		<i>Andvakia</i>	<i>discipulorum</i>	GU473273	GU473287	GU473316	KJ483051	—————
		<i>Telmatactis</i>	sp.	JF832968	JF832979	KJ483013	KJ483135	—————
	Antipodactinidae	<i>Antipodactis</i>	<i>awii</i>	GU473271	GU473286	GU473303	KJ483074	GU473337

Table A1 (Continued)

Higher taxon	Family	Genus	Species	12S	16S	18S	28S	COIII
	Bathypheiliidae	<i>Bathypheilia</i>	<i>australis</i>	FJ489402	FJ489422	EF589063	EF589086	FJ489482
	Boloceroideidae	<i>Bolocerooides</i>	<i>mcmurrici</i>	GU473270	—	EU190852	EU190810	—
		<i>Bunodeopsis</i>	<i>globulifera</i>	KJ482940	KJ482949	KJ483025	KJ483122	KJ482992
	Diadumenidae	<i>Diadumene</i>	<i>cincta</i>	EU190725	EU190769	EU190856	KJ483106	FJ489490
		<i>Diadumene</i>	<i>leucolena</i>	JF832957	JF832977	JF832986	KJ483123	JF833006
		<i>Diadumene</i>	<i>lineata</i>	JF832965	JF832973	JF832987	KJ483107	JF833007
		<i>Diadumene</i>	sp.	JF832960	JF832976	JF832980	KJ483130	JF833005
	Galatheanthemidae	<i>Galatheanthemum</i>	<i>profundale</i>	KJ482919	KJ482954	KJ483011	KJ483119	KJ482978
		<i>Galatheanthemum</i>	sp.	KJ482918	KJ482955	KJ483012	KJ483065	KJ482977
	Gonactiniidae	<i>Gonactinia</i>	<i>prolifera</i>	KJ482935	—	KJ483008	KJ483112	KJ482994
		<i>Protanthea</i>	<i>simplex</i>	KJ482939	KJ482970	KJ483010	KJ483078	KJ482993
	Halcampidae	<i>Cactosoma</i>	sp.	—	GU473297	GU473313	GU473329	GU473346
		<i>Halcampa</i>	<i>duodecimcirrata</i>	JF832966	EU190776	AF254375	EU190820	—
		<i>Halcampoides</i>	<i>purpureus</i>	EU190735	EU190780	AF254380	KJ483100	—
	Hormathiidae	<i>Actinauge</i>	<i>richardi</i>	EU190719	EU190761	EU190850	KJ483055	FJ489480
		<i>Allantactis</i>	<i>parasitica</i>	FJ489399	FJ489420	FJ489439	KJ483056	FJ489478
		<i>Calliactis</i>	<i>japonica</i>	FJ489403	FJ489423	FJ489441	KJ483057	FJ489486
		<i>Calliactis</i>	<i>palliata</i>	FJ489398	FJ489419	FJ489436	KJ483101	FJ489474
		<i>Calliactis</i>	<i>parasitica</i>	EU190711	EU190752	EU190842	KJ483102	FJ489475
		<i>Calliactis</i>	<i>polypus</i>	FJ489407	FJ489427	FJ489445	KJ483058	FJ489485
		<i>Calliactis</i>	<i>tricolor</i>	FJ489405	FJ489425	FJ489443	KJ483059	FJ489488
		<i>Chondrophellia</i>	sp.	FJ489406	FJ489426	FJ489444	KJ483060	FJ489489
		<i>Cricophorus</i>	<i>nutrix</i>	—	KT852066	KT852134	—	KT852286
		<i>Hormathia</i>	<i>armata</i>	EU190731	EU190775	EU190861	KJ483062	FJ489491
		<i>Hormathia</i>	<i>lacunifera</i>	FJ489409	FJ489428	FJ489446	KJ483063	FJ489492
		<i>Hormathia</i>	<i>pectinata</i>	FJ489415	FJ489430	FJ489448	FJ489465	FJ489497
		<i>Paracalliactis</i>	sp. 1	FJ489411	FJ489429	FJ489447	—	FJ489496
		<i>Paracalliactis</i>	sp. 2	MK801513	MK801515	MK801511	MK801517	MK801562
		<i>Paraphelliactis</i>	sp.	FJ489412	FJ489431	FJ489449	FJ489466	FJ489498
	Isanthidae	<i>Isanthus</i>	<i>capensis</i>	JF832967	GU473291	GU473291	KJ483096	GU473362
		<i>Isoparactis</i>	<i>fabiani</i>	JF832964	GU473283	GU473300	KJ483124	GU473355
		<i>Isoparactis</i>	<i>ferax</i>	KC700002	—	KC700005	KC700006	KC700008
		<i>Isoparactis</i>	<i>fionae</i>	KC700001	KC700003	KC700004	—	KC700007
	Kadosactinidae	<i>Alvinactis</i>	<i>chessi</i>	GU473278	GU473296	GU473312	KJ483052	GU473352
		<i>Cyananthea</i>	<i>hourdezi</i>	GU473275	GU473293	GU473309	KJ483081	GU473364
		<i>Jasonactis</i>	<i>erythraios</i>	—	GU473289	GU473305	KJ483079	GU473339
		<i>Kadosactis</i>	<i>antarctica</i>	FJ489410	EU190782	EU190865	KJ483080	FJ489504
	Metridiidae	<i>Metridium</i>	<i>senile</i>	KT852024	EU190786	AF052889	EU190829.1	FJ489494
	Nemanthidae	<i>Nemanthus</i>	<i>nitidus</i>	EU190741	EU190787	EU190868	KJ483064	FJ489495
	Ostiactinidae	<i>Ostiactis</i>	<i>pearseae</i>	EU190751	EU190798	EU190878	KJ483082	GU473365
	Phellidae	<i>Phellia</i>	<i>exlex</i>	JF832958	JF832978	JF832984	KJ483121	JF833004
		<i>Phellia</i>	<i>gausapata</i>	EU190744	EU190790	EU190870	—	FJ489473
	Sagartiidae	<i>Actinothoe</i>	<i>sphyrodeta</i>	FJ489401	FJ489421	FJ489440	KJ483111	FJ489481
		<i>Anthothoe</i>	<i>chilensis</i>	FJ489397	FJ489416	FJ489434	FJ489453	FJ489470
		<i>Carcinactis</i>	<i>dolosa</i>	MN266878	MN266877	MN266880	MN266874	MN295038
		<i>Cereus</i>	<i>herpetodes</i>	JF832956	JF832969	JF832983	JF832992	—
		<i>Cereus</i>	<i>pedunculatus</i>	EU190724	EU190767	EU190855	EU190813	FJ489471
		<i>Sagartia</i>	<i>elegans</i>	—	JF832970	JF832989	JF832994	JF833012
		<i>Sagartia</i>	<i>ornata</i>	JF832959	JF832975	JF832985	JF832997	JF833011
		<i>Sagartia</i>	<i>troglydites</i>	EU190746	KT852107	EU190872	KT852261	FJ489499
		<i>Sagartiogeton</i>	<i>laceratus</i>	EU190748	EU190794	EU190874	KJ483071.1	FJ489500
		<i>Sagartiogeton</i>	<i>undatus</i>	FJ489400	FJ489417	FJ489435	KJ483070	FJ489472
		<i>Verrillactis</i>	<i>paguri</i>	FJ489414	FJ489433	FJ489451	KJ483046.1	FJ489503
Anenthemonae (outgroup)								
Actinernoidea	Actinernidae	<i>Isactinernus</i>	<i>quadrolobatus</i>	KJ482932	KJ482968	KJ483024	KJ483105	KJ482998
	Halcuriidae	<i>Halcurias</i>	<i>pilatus</i>	KJ482931	KJ482967	KJ483020	KJ483109	KJ482997
Edwardsioidea	Edwardsiidae	<i>Edwardsia</i>	<i>timida</i>	GU473281	KT852113	GU473315	KJ483088	KT852332
		<i>Nematostella</i>	<i>vectensis</i>	EU190750	AY169370	AF254382	KJ483089	FJ489501

The obtained sequences (in bold) were deposited at the DNA Data Bank of Japan (DDBJ). The species of Anenthemonae acted as the outgroups.

Table A2

Estimates of evolutionary divergence between sequences of the *Stylobates* species calculated using the Kimura two-parameter (K2P) distance model (Kimura, 1980)

		Registration no.	Accession nos.	1	2	3	4
<i>12S</i> (590 bp)							
1	<i>S. loisetteae</i>	—	MN266876				
2	<i>S. calcifer</i>	CMNH-ZG09765	LC642139	0.000			
3	<i>S. calcifer</i>	CMNH-ZG09771	LC642140	0.000	0.000		
4	<i>S. calcifer</i>	NSMT-Co 1796	LC642141	0.000	0.000	0.000	
<i>16S</i> (435 bp)							
1	<i>S. loisetteae</i>	—	MN266875				
2	<i>S. calcifer</i>	CMNH-ZG09765	LC642142	0.002			
3	<i>S. calcifer</i>	CMNH-ZG09771	LC642143	0.002	0.000		
4	<i>S. calcifer</i>	NSMT-Co 1796	LC642144	0.002	0.000	0.000	
<i>18S</i> (1618 bp)							
1	<i>S. loisetteae</i>	—	MN266881				
2	<i>S. calcifer</i>	CMNH-ZG09764	LC642145	0.094			
<i>28S</i> (851 bp)							
1	<i>S. loisetteae</i>	—	MN266882				
2	<i>S. calcifer</i>	CMNH-ZG09764	LC642146	0.281			
3	<i>S. calcifer</i>	CMNH-ZG09765	LC642147	0.281	0.002		
4	<i>S. calcifer</i>	CMNH-ZG09771	LC642148	0.281	0.001	0.004	
<i>COIII</i> (621 bp)							
1	<i>S. loisetteae</i>	—	MN295037				
2	<i>S. calcifer</i>	CMNH-ZG09765	LC642149	0.000			

Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). CMNH, Coastal Branch of the Natural History Museum and Institute, Chiba; NSMT, National Museum of Nature and Science, Tokyo.

Table A3
 Comparison of the cnidae size range between *Stylobates calceifer* sp. nov. and other *Stylobates* species

Type of cnidae	<i>S. calceifer</i> sp. n.				<i>S. aeneus</i>				<i>S. hirtlesi</i>				<i>S. camarciscia</i>				<i>S. loisiretae</i>			
	Category in this study	Category in Crowther et al., 2011	Length (µm), mean ± SD (min.–max.)	Width (µm), mean ± SD (min.–max.)	n	Length (µm), mean ± SD (min.–max.)	Width (µm), mean ± SD (min.–max.)	n	Length (µm), mean ± SD (min.–max.)	Width (µm), mean ± SD (min.–max.)	n	Length (µm), mean ± SD (min.–max.)	Width (µm), mean ± SD (min.–max.)	n	Length (µm), mean ± SD (min.–max.)	Width (µm), mean ± SD (min.–max.)	n			
Tentacle																				
a.	(a) Basitrich (large)		26.64 ± 1.79 (22.1–30.1)	3.19 ± 0.34 (2.54–3.83)	35	—	—	—	—	—	—	—	—	—	—	—	—	—		
	(b) Basitrich (middle)		—	—	—	(27.9–36.1)	(3.1–3.9)	43	(26.6–38.8)	(2.6–4.6)	63	(27.1–30.3)	(2.5–3.3)	11	(29.8–39.7)	(2.5–3.7)	70	—		
b.	(c) Spirocyst		25.81 ± 5.89 (16.3–37.48)	3.45 ± 0.54 (2.54–4.6)	49	—	—	—	((11.9) 14.2–19.9)	(2.6–3.4)	12	—	—	—	(9.9–16.1)	(1.6–2.5)	11	—		
	Actinopharynx		—	—	—	((25.4) 28.7–44.3)	(2.9–4.1 (4.9))	40	(17.9–39.8 (46.3))	(2.2–4.6)	51	(23.8–39.4)	(2.7–3.3)	10	(21.1–55.8)	(2.5–3.7)	63	—		
c.	(d) Basitrich (large)		24.2 ± 3.05 (17.98–30.08)	3.3 ± 0.39 (2.54–4.27)	69	—	—	—	—	—	—	—	—	—	—	—	—	—		
	(e) Basitrich (middle)		—	—	—	(26.2) 29.5–37.7)	(2.9–4.1)	38	(27.8–37.1)	(2.9–4.3)	61	(27.1–31.2)	(2.5–3.3)	10	(26.0–37.2)	(2.5–3.7)	59	—		
d.	(f) Basitrich (small)		8.9 ± 1.24 (6.7–11.6)	2.58 ± 0.41 (1.83–3.25)	19	—	—	—	(17.2–21.3)	(2.5–3.1)	9	—	—	—	—	—	—	—		
	Column		—	—	—	(6.4–11.2)	(1.9–2.8)	20	—	—	—	—	—	—	—	—	—	—		
e.	(g) Basitrich (large)		—	—	—	(29.5–36.1)	(2.9–3.5)	24	(19.9–30.5)	(2.6–4.1)	65	(23.0–28.7)	(2.7–3.3)	12	(21.1–33.5)	(2.5–3.7)	46	—		
	(h) Basitrich (small)		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	Mesenterial filament		—	—	—	—	—	—	(7.3–9.2)	(1.9–3.6)	10	—	—	—	—	—	—	—		
	Basitrich (large)		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	(j) Basitrich (large)		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	(k) Basitrich (middle)		—	—	—	(29.5–36.1)	(2.5–3.9)	21	(28.5–33.2)	(2.8–3.8)	18	—	—	—	(28.5–37.2)	(2.5–3.5)	11	—		
	(l) Basitrich (small)		—	—	—	(15.6–23.0)	(2.1–3.3)	58	(15.9–21.4)	(2.1–3.1)	51	(14.8–18.0)	(2.1–2.5)	8	(12.4–16.1)	(1.9–3.1)	10	—		
f.	(i) Microbasic <i>b</i> -mastigophore		27.88 ± 1.51 (24.4–31.63)	5.11 ± 0.52 (4.13–6.43)	42	—	—	—	(7.9–11.9)	(1.9–2.9)	21	—	—	—	—	—	—	—		
	Crowther et al., 2011		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	(m) Microbasic <i>p</i> -mastigophore		24.24 ± 1.31 (21.5–27.28)	5.61 ± 0.57 (4.6–6.73)	27	—	—	—	(27.5–37.1)	(4.4–6.7)	25	—	—	—	—	—	—	—		
g.	Basitrich (middle)		19.61 ± 1.77 (11.16–22.97)	3.8 ± 0.93 (2.06–5.44)	15	Not examined	—	—	(21.2–30.0)	(4.4–7.9)	45	(18.9–23.0)	(4.1–5.7)	12	(23.6–32.2)	(3.5–6.2)	50	—		
	References		This study	Dunn et al., 1980	Dunn et al., 1981	Crowther et al., 2011	Dunn et al., 1987	Fautin, 1987	Not examined	Not examined	Not examined	Not examined	Not examined	Not examined	Not examined	Not examined	Not examined	Not examined		

The unique cnidae characteristics of *S. calceifer* sp. nov. are shown in bold