Systematic and Evolutionary Studies of Ascidians (Chordata: Tunicata)

（海鞘類（脊索動物門・被嚢動物亜門）の体系学・進化学的研究）

A Ph.D. dissertation

submitted

to

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Disclaimer

According to Article 8.3 of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature, 1999), the new name proposed in this work*, Clavelina ossipandae,* is to be disclaimed for nomenclatural purposes and thus is not become available by this work.

Abstract

The classification system of ascidians below the family level remains to be organized; a non-negligible number of species and genera still require taxonomic scrutiny. Faunal investigations in Japanese waters are insufficient in the Ryukyu Islands and the deep sea, from which discoveries of species that are either new to the geographic area or even science can be expected. Meanwhile, the size of organisms has consistently intrigued researchers across various disciplines in biology. However, the evolutionary process of zooid miniaturization in colonial animals, including ascidians, persists as an enigmatic topic. To augment our knowledge of the systematics and evolutionary biology of ascidians, especially about the ones occurring in waters around Japan, the following four studies are reported in this dissertation: *i*) a systematic study of a styelid in Hokkaido, the taxonomic identity of which was questionable, *ii*) a systematic study of a deep-sea ascidiid, which was previously unknown from Japan, *iii*) a systematic study of an undescribed clavelinid from Okinawa, and *iv*) an evolutionary study of coloniality, especially the miniaturization of zooids in colonies, in Stolidobranchia.

This dissertation consists of five chapters. Following a general introduction to ascidians in Chapter 1, Chapter 2 deals with a styelid, *Syncarpa composita* (Tokioka, 1951), which has been suspected as a junior synonym of *S. oviformis* Redikorzev, 1913. To assess the taxonomic identity of *S. composita*, the syntypes and freshly collected topotypes were morphologically compared with one of the syntypes *S. oviformis*. As a result, differences were found in the number of oral tentacles, the number of size classes of transverse vessels, and the number of anal lobes between the two. In addition, a phylogenetic position of *Syncarpa* was inferred within Styelidae based on the 18S rRNA and cytochrome *c* oxidase subunit I (COI) gene sequences. In the resulting phylogenetic tree, *Syncarpa* formed a well-supported clade together with *Dendrodoa* MacLeay, 1824. In *Syncarpa* and *Dendrodoa*, a single gonad is situated on the right side of the body, which is unique among Styelidae, and thus can be a synapomorphy for this clade.

Chapter 3 gives a morphological redescription of *Fimbrora calsubia* Monniot and Monniot, 1991, an ascidiid previously recorded from the South Pacific at depths of 1000–1860 m. The taxonomic status of *Fimbrora* remained ambiguous because characteristics in its branchial papillae and neural-gland opening were incompletely known in previous studies. At the same time, these traits are essential for distinguishing other ascidiid genera. Before the present study, no nucleotide sequence representing *F.*

calsubia had been available. In this study, a single specimen of *F. calsubia* was collected using the manned submersible *Shinkai 6500* at a depth of 2027 m, about 400 km off the Pacific coast of Honshu, Japan. This was the deepest record, as well as the first report from the North Pacific, for the species. A morphological examination indicated that *Fimbrora* is similar to another ascidiid genus, *Psammascidia* Monniot, 1962, by having only secondary branchial papillae in the pharynx. A phylogenetic analysis based on the 18S rRNA and COI genes indicated that acquisitions of carnivorousness by deep-sea members happened independently at least three times in the evolutionary history of the entire Ascidiacea.

In Chapter 4, a colonial ascidian called *gaikotsu-panda-hoya* in Japanese, literally meaning 'skeleton panda ascidian', is described as a new species. Its strange appearance was introduced on the Internet by a diving shop in Kumejima Island, Japan several years ago. Its taxonomic identity, however, was unidentified. To confirm the taxonomic status of this species, fresh samples were collected from a diving point off the coast of Kumejima Island. The new species is morphologically distinguished from 44 congeners in the genus *Clavelina* Savigny, 1816. Partial sequences (810 bp) of the COI gene from the holotype and one of the paratypes differed at 10 sites from each other (1.23% p-distance) but were the same when translated into amino acids. A phylogenetic tree supported that this species is included in the genus *Clavelina*.

In Chapter 5, a directionality of zooid miniaturization through an evolutionary process of coloniality was indicated based on a phylogenomic analysis and ancestral-state reconstruction in stolidobranch ascidians. A phylogenomic relationship mainly within Stolidobranchia was inferred using transcriptomes of a total of 42 ascidians; from 17 species sampled in Israel and Japan and transcriptome data from 25 species sourced from a previous study and a database. Through ancestral-state reconstruction, results indicated a clear directional change: following the acquisition of coloniality, zooids tended to become progressively smaller. This miniaturization was likely an adaptive response, enabling organisms to swiftly colonize limited marine substrate. A mathematical model was formulated suggesting that zooid miniaturization, due to living space constraints, would result in shortening the period for daughter zooids to start budding and accelerated expansion in a colony. The phylogenomic results also suggested that coloniality evolved independently three times within Stolidobranchia. Moreover, once colonial traits are established, they appear to be consistently preserved, underscoring their biological importance in the colonial lineage.

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Contents

Chapter 1

General introduction

Systematics of ascidians

Historically, ascidians had been looked upon as members of Mollusca for a long time. Aristotle regarded solitary ascidians (termed τήθυα, tethya) as being related to molluscs in Περὶ τὰ ζῷα ἱστορίαι (History of Animals) around 330 B.C. (Cresswell 1883); colonial ascidians were initially recorded and called *uva marina* ("marine grapes" in Latin) by Rondeletius (1555). Linnaeus (1767) included solitary ascidians among molluscs, whereas colonial ascidians among zoophytes in the 12th edition of Systema Naturae; ascidians were not contained in any of the earlier editions. Savigny (1816) classified solitary and colonial ascidians, along with pyrosomes and salps, into the family Ascidiae. For this taxon, Lamarck (1816) created the name Tunicata. Milne Edwards (1843) added the Bryozoa to the Tunicata in the class Molluscoidea; Hancock (1850) added the Brachiopoda to this group. Huxley (1851a, b) first suggested that tunicates are different from the Brachiopoda, Bryozoa, and Mollusca, and that appendicularians should be united with the Tunicata. Haeckel (1874) regarded tunicates not as members of the phylum Mollusca but as the phylum Vermes, which also contained acorn worms; he coined Chordonia (now Chordata) for a hypothetical common ancestor of lancelets, tunicates, and vertebrates; earlier, Kowalevsky (1866) discovered the notochord in ascidian tadpole larvae. The name Urochordata had not been used until Balfour (1881) created it to emphasize that tunicates are relative to chordates. Haeckel (1894) redefined Chordonia, including Tunicata as one of its members. Currently, the majority of ascidian researchers accept that the Tunicata is one of the three subphyla in the phylum Chordata, although Satoh et al. (2014) proposed that Cephalochordata, Tunicata, and Vertebrata should be ranked at the phylum level under the superphylum Chordata.

The subphylum Tunicata (or Urochordata) consists of three classes, Appendicularia, Ascidiacea, and Thaliacea. All members of Tunicata share the following five characteristics (Nishikawa 1986; Ballarin and Burighel 2002): *i*) the body is fully covered with a tunic that contains cellulose-like tunicin fibers (Robinson et al. 1983); the English word "ascidian" derives from ασκιδιον (askidion) meaning 'small wine skin' in Greek because the appearance of some ascidians covered with leathery

tunics evokes an image of leathery bags for making wine (Rehder 2008), *ii*) the pharynx is equipped with stigmata in the peripharyngeal cavity, *iii*) the central nervous system is situated in the dorsal side, *iv*) the heart is in the ventral side, and *v*) the endostyle is on the internal surface of the ventral side of the pharynx.

The class Ascidiacea is a group of sea squirts, differentiated from the other two classes, Appendicularia and Thaliacea, in that the tadpole-like larvae metamorphose into sessile adults, instead of planktonic (e.g., Nishikawa 1986, 2017; Holland 2016; Kocot et al. 2018; Brauna et al. 2020). Multiple classification systems have been proposed for this class. In this study, I follow the most widely accepted classification system reviewed by Shenkar and Swalla (2011). In it, Ascidiacea is divided into three orders according to the structure of the adult pharynx, a classification first proposed by Lahille (1886): Aplousobranchia (simple pharynx, having no transverse vessels in the pharynx), Phlebobranchia (having transverse vessels but not folds in the pharynx), and Stolidobranchia (having both transverse vessels and folds in the pharynx). To date, 25 families, 171 genera, and approximately 3000 species of ascidians have been described (Shenkar et al. 2023), since Schlosser and Ellis (1756) initially made a clear description of a colonial ascidian, currently known as *Botryllus schlosseri* (Pallas, 1766). The ratio of solitary and colonial forms is 4:6 (Shenkar and Swalla 2011). The species discovery rate has been accelerated since the 1950s when the great taxonomists (P. Kott, C. and F. Monniot, C.P. Sluiter, R.H. Millar, and W.A. Herdman) were actively describing ascidians (Shenkar and Swalla 2011). Ascidians are distributed throughout the world's oceans, from tropical to polar regions, in shallow to deep waters. While some ascidians occur in brackish waters, no freshwater form has ever been known; for survival, ascidians generally require salinity above approximately 25‰ (Lambert 2007). Many ascidians attach their ventral side of tunic or root structures to natural or artificial substrates including corals, rocks, sea plants, seaweeds, shells, concrete blocks, glass, metals, and plastics. A few are known to bury themselves in soft substrates such as sand and mud.

Molecular phylogeny of tunicates

Traditionally, Tunicata was regarded as a member of Protochordata along with cephalochordates (or lancelets) (Barrington 1965). However, recent molecular phylogenetic analyses suggest that Tunicata is a sister group of vertebrates and thus Protochordata is a paraphyletic group (e.g., Bourlat et al. 2006; Singh et al. 2009; Edgecombe et al. 2011; Rubinstein et al. 2013; Voskoboynik et al. 2013; Pisani et al. 2015). Although Monniot et al. (1975) elevated the carnivorous family Hexacrobylidae

to a class Sorberacea, independent of Ascidiacea, a phylogenetic analysis based on partial 18s RNA gene sequences supported the inclusion of Sorberacea/Hexacrobylidae within Molgulidae (Tatián et al. 2011).

Subsequent phylogenetic studies have elucidated relationships within Tunicata. Analyses by Yokobori et al. (2006) and Tsagkogeorga et al. (2009) identified Aplousobranchia and Phlebobranchia as sister taxa to Thaliacea, and Stolidobranchia as closely related to Appendicularia. Delsuc et al. (2018) refined these results, proposing Aplousobranchia + Phlebobranchia as a sister group to Thaliacea and positioning Appendiculara alongside Ascidiacea and Thaliacea.

Within ascidians, phylogenetic/phylogenomic analyses were conducted for some specific groups: Octacnemidae based on 18S rRNA (18S) gene (Kurabayashi et al. 2003), Stolidobranchia (18S) (Zeng et al. 2006), Clavelinidae based on the mitochondrial cytochrome oxidase *c* subunit I (COI) gene (Pérez-Portela and Turon 2008), Polyzoinae and Styelidae (18S and COI) (Pérez-Portela et al. 2009), Ascidiidae (COI) (Nishikawa et al. 2014), Dideminidae (Oliveira et al. 2017), Styelidae (1306 genes) (Alié et al. 2018), and Botryllinae (200 genes) (Nydam et al. 2021). As Ascidiacea includes 25 families (Shenkar and Swalla 2011), these studies represent analyses of roughly a quarter of all families.

Evolutionary studies of zooid size

The body size of organisms has been a matter of research topic among biologists, with its related factors so far considered including latitudinal variations, physiological implications, and life history strategies (Grischenko et al. 2002). Yet, much of our understanding of the evolutionary shifts in animal body size is derived primarily from solitary metazoans rather than colonial ones (Liow and Taylor 2019).

Colonies of sessile aquatic animals consist of modular individuals, termed polyps and zooids (Beklemishev 1969). The modules within a single colony are clones, proliferating through asexual reproduction (Ryland and Warner 1986). Coloniality has independently evolved across various animal higher taxa (Hiebert et al. 2021), including Bryozoa (Schwaha et al. 2020), Cnidaria (Barbeitos et al. 2010; Kayal et al. 2018), Hemichordata (Cannon et al. 2013), Kamptozoa (Fuchs et al. 2010), and Tunicata (Alié et al. 2021). Within each taxon, the modules of colonial organisms are typically smaller than solitary individuals (Ryland and Warner 1986; Mukai 2001). Although zooid miniaturization has been noticed in various taxa for approximately 40 years (e.g., Kott 1985; Ryland and Warner 1986; Davidson et al. 2004; Sogot et al. 2014; Kocot 2016; Braun and Stach 2018; Nanglu et al. 2023), its evolutionary process still remains a topic

of inquiry.

Taxonomy of ascidians in Japan

In Japan, sea squirt is called *hoya*. This word has been used since the late 7th century A.D. to refer to the solitary ascidian *Halocynthia roretzi*, known as *maboya* and documented on ancient wooden tablets as a fermented delicacy in imperial cuisine (Nishikawa 2017; cf. Nara National Research Institute for Cultural Properties 2023). The word *hoya* is regarded to have originated from an ancient Japanese name for mistletoe, likely due to the resemblance between the mistletoe roots and the ascidian's tunic attachment structures (Nishikawa 2017).

Prior to the late Edo period, in his work "*Hoya-ko*" (On ascidians) written around the 8th year of the Bunsei era (1825) (National Diet Library 2023a), H. Ansho, a medical practitioner in the Tokushima clan, lamented the lack of detailed descriptions of ascidians in previous literature; he documented his observations and consumption of what was believed to be *H. roretzi* (cf. Nishikawa 2021). Subsequently, *H. roretzi* was clearly illustrated in color in the scholarly essay "*Inteizatsuroku*" (National Diet Library 2023b) by the Japanese classical scholar N. Kitamura in 1843 and the shellfish compendium "*Mokuhachifu*" (National Diet Library 2023c) by S. Musashi, an herbalist, in 1845 (cf. Nishikawa 2021).

The modern taxonomy of ascidians in Japan initially unfolded through the work of foreign taxonomists like Herdman (1882, 1886), who based their studies on specimens collected during the HMS Challenger expedition in 1875, along with others like Drasche (1884), Traustedt (1885), and Hartmeyer (1906). Since the first Japanese taxonomist of ascidians Prof. A. Oka of the Tokyo Higher Normal School (a predecessor of University of Tsukuba) established *Diplosoma mitsukurii* Oka, 1892 and *Didemnum misakiense* (Oka and Wiley, 1892) (cf. Oka 1892; Oka and Wiley 1892), 106 species had been recorded in his studies (Nishikawa 2017). Prof. T. Tokioka of the Seto Marine Biological Laboratory, Kyoto University reviewed Japanese ascidian fauna (approximately 300 species) (Tokioka 1963). Prof. T. Nishikawa of Nagoya University and Toho University provided a series of taxonomic revisions on ascidians in the Sea of Japan, with descriptions of 158 species based on examinations of newly collected samples, type specimens, and other museum specimens (Nishikawa 1990, 1991, 1992). Japanese ascidian developmental biologists, Profs. Y. Saito and H. Watanabe of the Shimoda Marine Research Center, University of Tsukuba, and their collaborators reported new colonial ascidians through life-history observations and allorecognition experiments (e.g., Watanabe and Tokioka 1972, 1973; Saito et al. 1981a, b; Saito and

Watanabe 1985; Okuyama and Saito 2001, 2002; Saito and Nagasawa 2003; Saito and Okuyama 2003; Atsumi and Saito 2011). Understanding the ascidian fauna, however, still requires further taxonomic revisions, which should incorporate both molecular analyses and morphological observations (Nishikawa 2017). There are some regions where ascidian fauna has been less studied like Ryukyu Islands and the deep-sea areas (cf. Tokioka 1963; Nishikawa 1990, 1991).

Aims of this dissertation

The ultimate goal of my study is to enhance our knowledge on the systematics and evolutionary biology of ascidians, mainly the ones occurring in Japanese waters. To approach this goal, the following four studies were conducted: *i*) a taxonomic study of the styelid *Syncarpa composita* (Tokioka, 1951), which was suspected as a junior synonym of another congener, *S. oviformis* Redikorzev, 1913 (Chapter 2); *ii*) morphological and phylogenetic studies on the deep-sea ascidiid *Fimbrora calsubia* Monniot and Monniot, 1991, which is herein reported from Japan for the first time (Chapter 3); *iii*) taxonomic description of a new species in Clavelinidae from Okinawa (Chapter 4); and *iv*) an evolutionary study of coloniality, especially zooid miniaturization, based on a phylogenomic analysis and an ancestral-state reconstruction in Stolidobranchia (Chapter 5).

Chapter 2

A redescription of *Syncarpa composita* (Ascidiacea, Stolidobranchia) with an inference of its phylogenetic position within Styelidae

Introduction

Syncarpa Redikorzev, 1913 is a member of the ascidian family Styelidae and consists of two species, *Syncarpa composita* (Tokioka, 1951) and *S. oviformis* Redikorzev, 1913. The two nominal species *S. corticiformis* Beniaminson, 1975 and *S. longicaudata* Skalkin, 1957, all from the Northwest Pacific, have been synonymized with *S. oviformis* by Sanamyan (2000). This genus is defined by the following four characteristics: *i*) colonial, with zooids reproducing asexually, *ii*) a single, well-developed fold is present on each side of the pharynx, *iii*) a single gonad is situated on the right side of the body, and *iv*) the gonad has several branches. *Syncarpa composita* is only known by the original description based on material from Akkeshi, Japan (Tokioka 1951). It was originally placed in a new monotypic genus *Syndendrodoa* Tokioka, 1951, which has been synonymized with *Syncarpa* by Nishikawa (1995).

The phylogeny of ascidians including styelids has been investigated by Zeng et al. (2006), Pérez-Portela et al. (2009), Tsagkogeorga et al. (2009), Alié et al. (2018), and Delsuc et al. (2018). Among these, Alié et al.'s (2018) analysis was based on 4908 genes and included 16 OTUs from Styelidae. It recovered Styelidae as monophyletic with maximum branch-support values, which turned out to be sister to part of paraphyletic Pyuridae. Alié et al.'s (2018) phylogeny showed three major clades for Styelidae: *i*) Polyzoinae + Botryllinae, *ii*) *Dendrodoa* + *Polycarpa* + *Polyandrocarpa zorritensis* (Van Name, 1931), and *iii*) *Asterocarpa* + *Styela*. However, no member of *Syncarpa* has ever been placed in a phylogenetic context in any of the previous studies.

The aims of this study are to assess the taxonomic identity of *S. composita* based on type specimens and freshly collected topotypes and to infer the phylogenetic position in Styelidae. In this chapter, I redescribe the species and present the results of multi-gene molecular analysis.

Methods

Eleven topotype colonies of *S. composita* were freshly collected by dredging, snorkeling, and SCUBA diving in the type locality, Akkeshi Bay, at depths of 3–5 m in

June, August, and September 2017, and July 2018 (Table 1). One of the colonies was photographed underwater and in the laboratory with a Nikon COOLPIX AW130 digital camera. The live colonies were anesthetized with menthol; then a part of a zooid was cut off along with the tunic from each colony and preserved in 99% EtOH for DNA extraction. The colonies were preserved in 10% formalin-seawater for morphological observation; zooids were removed from the colonies and then dissected for morphological examination. Larvae for histological observation were dehydrated in an ethanol series, cleared in xylene, embedded in paraffin wax, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. After sections were mounted on glass slides in Entellan New (Merck), they were observed under an Olympus BX51 compound microscope and photographed with a Nikon D5200 digital camera. These voucher specimens have been deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan. For comparison, specimens deposited in the Seto Marine Biological Laboratory (SMBL), Shirahama, Japan, and the Zoological Institute of the Russian Academy of Sciences (ZIRAS), St. Petersburg, Russia, were also examined.

Total genomic DNA was extracted from a piece of the body wall tissue for eight specimens of *S. composita* as well as one specimen each of *Botrylloides violaceus* Oka, 1927, *Pyura mirabilis* (Drasche, 1884), *Styela clava* Herdman, 1881, and *Styela plicata* (Lesueur, 1823) (Table 1). The tissue was placed in a 1.5 mL tube after being air-dried, then mixed with 180 μL of ATL buffer (Qiagen) and 20 μL of proteinase K (> 700 U/mL, Kanto Chemical, Tokyo, Japan), and incubated at 55°C for ca. 10 h. To the lysis solution, 200 μL of AL buffer (Qiagen) was added and incubated at 70°C for 10 min; then 210 μL of 99% EtOH was added. The rest of the DNA extraction was carried out following Boom et al.'s (1990) silica method.

Two gene markers were amplified from the genomic DNA by PCR. A partial sequence of 18S was amplified with the primer pair 1F/9R (Giribet et al. 1996). COI sequence was amplified with the primers Sty_COI_F2 (5′-TTTGCCTTTAATAGTAAGAAGTCC-3′) and Sty_COI_R1 (5′-CATCAAAACAGATGCTGATA-3′) for *S. composita* and with the primer pair LCO1490/HCO2198 (Folmer et al. 1994) for the other ascidians. PCRs were performed in a 10-μL total reaction volume with 3 μL of each primer pair (10 μM), 0.5 μL of TaKaRa Ex Taq (TaKaRa), 10 μL of 10 × Ex Taq Buffer (TaKaRa), 8 μL of dNTP mixture (TaKaRa), 1 μL of extracted DNA, and 68.5 μL of deionized water. Thermal cycling condition was 94°C for 2 min; 35 cycles of 94°C for 45 sec, 52°C for 90 sec (for 18S) or 55°C for 50 sec (for COI), and 72°C for 55 sec; then 72°C for 5 min.

Amplification was verified by electrophoresis in 1% agarose gel. The PCR products were purified through enzymatic reaction with 24 mU/μL of Exonuclease I (TaKaRa) and 4.9 mU/μL of Shrimp Alkaline Phosphatase (TaKaRa). The purified PCR products were sequenced directly with a BigDye Terminator ver. 3.1 Cycle Sequence Kit (Thermo Fisher Scientific) and 3730 Genetic Analyzer (Thermo Fisher Scientific), using the same primer pairs for amplification, as well as the following internal primers for 18S: 3F, 5R (Giribet et al. 1996); and 2, bi (Whiting et al. 1997). Base calling was performed with GeneStudio Professional Edition ver. 2.2.0.0 (GeneStudio).

To infer the phylogenetic position of *S. composita*, 18S and COI sequences of 24 species of Styelidae were obtained from GenBank (Table 2). For 18S, alignment was carried out by MAFFT ver. 7 using the *E-INS-i* strategy (Katoh and Standley 2013); ambiguous sites were removed by using Gblocks ver. 0.91b (Castresana 2002). For COI, nucleotide sequences were manually edited by MEGA ver. 5.2.2 (Tamura et al. 2011) so that translated amino acid sequences were aligned straightforwardly without indels. 18S and COI sequences were concatenated by using MEGA ver. 5.2.2 (Tamura et al. 2011).

Bayesian inference (BI) was performed using MrBayes ver. 3.2.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The best-fit substitution models selected by PartitionFinder ver. 2.1.1 (Lanfear et al. 2016) for BI were GTR + I + G for 18S and GTR + G for all the three codon positions of COI. Each Markov chain was initiated from a random tree and run for 5×10^6 generations; trees were sampled every 100 generations from the chain. A burn-in fraction was set to be 0.25. A consensus of sampled trees was computed using the "sumt" command, and the posterior probability (PP) for each interior branch was obtained to assess the robustness of the inferred relationships. Values of run convergence indicated that sufficient amounts of trees and parameters were sampled (average standard deviation of split frequencies = 0.009823; average estimated sample size of tree lengths = 205.35; potential scale reduction factor of tree lengths = 1.005). Run convergence was also assessed with Tracer ver. 1.6 (Rambaut et al. 2014) to see if the effective sample size of each parameter exceeded 200. Maximum Likelihood (ML) analysis was performed by RAxML ver. 8.2.3 (Stamatakis 2014). One thousand fast-bootstrap replicates were conducted to evaluate branch support by using the same partition as the BI analysis.

Result and discussion

Taxonomy

Family Styelidae Sluiter, 1895

Genus *Syncarpa* Redikorzev, 1913

***Syncarpa composita* (Tokioka, 1951)**

(Figs 1–4)

Syndendrodoa composita Tokioka, 1951: 14–16, fig. 11.

?*Syncarpa longicaudata* Skalkin, 1957: 297–298, figs a, b.

Material examined. Thirteen specimens: SMBL 104 (syntypes, two colonies); ICHUM 5815–5825 (non-types, each represented by a single colony).

Comparative material examined. ZIRAS 508-911, one of the syntypes of *Syncarpa oviformis* Redikorzev, 1913.

Description. Colonies ca. 30–50 mm (40 mm and 50 mm in syntypes) in thickness and ca. 40–130 mm (45 mm and 100 mm in syntypes) in diameter. Tunic grayish violet to black or red in life, tough and leathery; zooids more or less protruded and thus externally discernible from each other (Fig. 1A–C). Zooids 12–50 mm long (21 mm and 22 mm in syntypes) and ca. 8 mm wide (Fig. 1D). Posterior extension of zooids varying in length within the colony and among different colonies; while main zooid length (L_a) varied from 9 mm to 20 mm, posterior extension length (L_b) varied from 3 mm to 22 mm among 20 zooids from 11 colonies, with L_b/L_a ratio being 0.33–1.83 (Fig. 1E, Table 3). Siphons four-lobed, reddish in life, close together. Approximately 30 oral tentacles present (Fig. 2A), comprised of larger and smaller ones alternating almost regularly. Approximately 30 atrial tentacles present and ca. 0.3 mm long. Ciliated aperture of the dorsal tubercle C-shaped, with its interval directing leftward (Fig. 2B). Prepharyngeal band consisting of a single lamina running close to the ring of oral tentacles; prepharyngeal band V shaped around the dorsal tubercle. Neural ganglion close to dorsal tubercle. Dorsal lamina smoothly margined. One pharyngeal fold and one reduced pharyngeal fold present on each side of pharynx with formula:

L D. 0 (7–8) 2 (2) 3 V.

R D. 0 (7) 2 (3) 3 V.

Thirteen–twenty stigmata per mesh between endostyle and first longitudinal vessel from endostyle. Transverse vessels comprised of larger and smaller ones almost regularly alternating antero-posteriorly (Fig. 2C); when running across each pharyngeal fold (as well as reduced pharyngeal fold) on outer surface of pharynx, larger ones always taking ‘shortcut’ and bridging over fold valley, while smaller ones ‘detour’ and go along valley

(Fig. 2D, E). Parastigmatic vessels present. Stigmata straight. Gut located on left side (Fig. 3A). Alimentary system occupying approx. half of the left side of body; intestinal loop J-shaped. Esophagus short and slightly curved; its length being one-third of stomach (Fig. 3A). Stomach spindle-shaped, shorter than one-third of body length and has no plication or striation on its outer surface; stomach lying almost parallel to longitudinal axis of body (Fig. 3A), with its internal wall having at least 22 well-defined, regularly arranged, parallel, longitudinal folds (Fig. 3B). Intestine gently curving from pyloric part. Anus lying almost beneath atrial aperture. Diameter of intestine almost uniform from pylorus to anus. Anus without lobes. Gonad with 2–5 branches, situated only on right side of body (Fig. 3A). Ovaries spherical, occupying medial side of gonad; oviduct slightly bending at its end to peripharyngeal cavity before opening on right side of body at almost same level as pylorus. Male follicles located laterally within gonad, surrounding ovaries. Many endocarps present on inner surface of body wall (Fig. 3A).

Hatched tadpole larvae found in peripharyngeal cavity of ICHUM 5824 and 5825; trunk spindle-shaped, ca. 1 mm in length (Fig. 3C). Three adhesive papillae arranged in triangle. Approximately 35 elongated ampullae discerned on anterior half of trunk surface. Photolith present in cerebral vesicle but invisible from the outside (Fig. 4). Tail twice as long as trunk.

Remarks. *Syncarpa composita* and *S. oviformis* are different in terms of the number of oral tentacles, the number of size-classes of transverse vessels, and the number of anal lobes (Table 4). In addition, the transverse vessels in *S. composita* alternate ‘shortcut’ and ‘detour’ when crossing the valley of pharyngeal folds, while all the transverse vessels in *S. oviformis* make a shortcut and bridge over the valley of pharyngeal folds (Fig. 5A, B). Based on the consistent, discontinuous differences discovered in the present study, I conclude to leave *S. composita* as a valid species as opposed to *S. oviformis*, until molecular data settle the issue of conspecificity.

Syncarpa composita and *S. longicaudata* were supposed to be differentiated by the ratio of the lengths of the zooid’s main body (L_a) to its posterior extension (L_b), expressed as L_b/L_a (Fig. 1E). The values of this character for *S. composita* and *S. longicaudata*, based on the original figures (Tokioka 1951, figs 11.2, 11.3; Skalkin 1957, fig. a), are 0.40 and 1.00, respectively. In this study, however, I discovered that the L_b/L_a values could vary from 0.33 to 1.83 even intra-colonially in *S. composita* (Table 3), completely encompassing the character state of *S. longicaudata*. Although *S. longcaudata* has been considered a junior synonym of *S. oviformis*, I think that it is more similar to *S. composita* (Table 4). Extensive population genetic studies on

potentially different populations of these species from the Northwest Pacific would help to improve my understanding of the taxonomy of this genus.

Phylogeny

In the phylogenetic tree, *Syncarpa* formed a well-supported clade together with *Dendrodoa* (Fig. 6). These two genera have a single gonad positioned on the right side of the body. This feature is likely to represent a synapomorphy for this clade. The only difference between *Syncarpa* and *Dendrodoa* is that the former is colonial while the latter is solitary. The latter currently consists of eight species (Shenkar et al. 2023). Future studies should ascertain the possible reciprocal monophyly of the two genera by analyses with expanded taxon sampling from *Dendrodoa*. If they turn out to be reciprocally non-monophyletic (e.g., *Syncarpa* completely nested within paraphyletic *Dendrodoa*), these two genera can be synonymized so that it consists of both colonial and non-colonial species, just as the diazonid *Rhopalaea* Philippi, 1843.

A clade comprised of *Dendrodoa*, *Polycarpa*, and *Polyandrocarpa zorritensis* was recovered in Alié et al.'s (2018) phylogenomic analysis based on 4,908 genes, in which *Polyandrocarpa zorritensis* was sister to *Polycarpa aurata*, forming a clade sister to *Dendrodoa grossularia*.

Although the branch support values were generally poor, the resultant phylogenetic tree does not support the three-subfamily classification system: Styelinae consisting of solitary styelid species, Polyzoinae of colonial styelid species without system, and Botryllinae of colonial styelid species with system. Highly reliable molecular analyses and detailed morphological observations including *Syncarpa* would help understanding the systematics of Styelidae.

Chapter 3

First record of a deep-sea ascidian, *Fimbrora calsubia* (Ascidiacea: Phlebobranchia), in the North Pacific with an inference of its phylogenetic position

Introduction

The ascidiid genus *Fimbrora* Monniot and Monniot, 1991a is currently monotypic, consisting of the deep-sea carnivorous ascidian *Fimbrora calsubia* Monniot and Monniot, 1991a. The taxonomic identity of *Fimbrora* is not fully established because states of some characters used for distinguishing other ascidiid genera are not known for this taxon. Apart from *Fimbrora*, the family Ascidiidae also contains four genera: *Ascidia* Linnaeus, 1767; *Ascidiella* Roule, 1884; *Phallusia* Savigny, 1816; and *Psammascidia* Monniot, 1962. *Fimbrora* is supposed to be distinguished from the other ascidiid genera by a combination of three characteristics: *i*) the large, cup-shaped oral siphon with thin, uniformly long, and soft lobes, *ii*) two large blood vessels running on the oral-siphon wall, and *iii*) macrophagous feeding behavior (cf. Monniot and Monniot 1991a). The remaining four genera are distinguished from each other based on *i*) whether primary and/or secondary branchial papillae in the pharynx are present and *ii*) whether accessory openings of the neural gland are present (e.g., Kott 1985; Monniot et al. 1991; Rocha et al. 2012a). However, while the branchial papillae have been reported to be present in *Fimbrora* (Monniot and Monniot 1991a), whether they are primary and/or secondary was not mentioned in any of the previous literature (Monniot and Monniot 1991a; Monniot 1993; Monniot and López-Legentil 2017); also, the nature of the neural-gland opening (or, whether accessory openings are present) has not been stated in any of these works.

In addition to *Fimbrora*, three more ascidian taxa—the family Octacnemidae (with 26 species in 10 genera), as well as the two molgulid genera *Asajirus* Kott, 1989 (with eight species) and *Oligotrema* Bourne, 1903 (with five species)—are known to consist of carnivorous members (Table 5), while ascidians are generally suspension feeders that filter food particles such as phytoplankton from the surrounding seawater (Millar 1971). Of the 40 species of carnivorous ascidians, small crustaceans were discovered in gut contents from 10 species (and thus carnivory has been directly confirmed), while the other 30 species were supposed to be carnivorous based on

having a big siphon and a pharynx without cilia (Table 5). The carnivorous ascidians exclusively inhabit deep waters below 200 m with one exception, *Oligotrema psammites* Bourne, 1903, which is also distributed up to 90 m (Table 5). Morphological data earlier suggested that carnivorousness among ascidians has evolved convergently, probably due to difficulty in filter-feeding in the deep sea (Millar 1959). A recent molecular phylogenetic study confirmed this view with respect to the octacnemid *Megalodicopia* Oka, 1918 and the molgulid *Oligotrema* (Tatián et al. 2011), but *F. calsubia* has not been represented with any molecular sequence data.

So far, *F. calsubia* has been known from the South Pacific bathyal zone in three publications based on a total of 13 specimens: three specimens at a depth of 1865 m in New Caledonian waters (Monniot and Monniot 1991a), two specimens at about 1000 m in Indonesia (Monniot 1993), and eight specimens at 1000–1200 m in Papua New Guinea (Monniot and López-Legentil 2017). Meanwhile, during a biodiversity survey in an off-shore submarine nature conservation area around Nishi-Shichito Ridge in the western North Pacific, a 14th individual of *F. calsubia* was obtained. Here, I provide a morphological redescription and an inference of its phylogenetic position within the class Ascidiacea.

Methods

A single specimen of *F. calsubia* was collected near the south of Hoei Seamount (Fig. 7) with a manipulator of the manned submersible *Shinkai 6500* (Dive 1651) during the cruise YK22-17C of the R/V *Yokosuka*. The live animal was photographed with an OM-D E-M1X digital still camera (Olympus) attached to an M.Zuiko Digital ED 30 mm F3.5 Macro lens (Olympus). Two of the thread-like lobes of the specimen were dissected from the oral siphon; one was preserved in 99% ethanol for DNA extraction; the other in RNAlater (Thermo Fisher Scientific) for future analysis; the remaining body was fixed in 10% formalin seawater for morphological observation. For detailed examination, the pharynx was stained with hematoxylin. The voucher specimens have been deposited in the Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, with the catalog number JAMSTEC No. 111618 for the formalin-fixed specimen, JAMSTEC No. 111619 for the lobe in 99% ethanol, and JAMSTEC No. 111620 for the lobe in RNAlater.

Total DNA was extracted using a DNeasy Tissue Kit (Qiagen). For amplification, KOD One PCR Master Mix (TOYOBO) was used. Partial sequences of 18S and COI genes were PCR amplified from the total DNA; the primer pairs 1F/9R (Giribet et al. 1996) and dinF/Nux1R (Brunetti et al. 2017) were used for 18S and COI,

respectively. PCR was performed under the following conditions: For 18S: 94°C for 2 min; 35 cycles of 94°C for 45 sec, 52°C for 50 sec, and 72°C for 90 sec; then 72°C for 5 min. For COI: 94°C for 2 min; 35 cycles of 94°C for 40 sec, 50°C for 60 sec, and 72°C for 60 sec; then 72°C for 7 min. Purification of PCR products was conducted by enzymatic reaction with ExoSAP-IT (Thermo Fisher Scientific). The purified products were sequenced with an BigDye Terminator ver. 3.1 Cycle Sequencing Kit and a 3100 Avant Genetic Analyzer (Thermo Fisher Scientific), using the same primer pairs for amplification; for 18S, the internal primers 3F and 5R (Giribet et al. 1996), as well as a2.0 and bi (Whiting et al. 1997), were also used.

For phylogenetic analysis, 18S and COI sequences of 27 ascidian species and those of the lancelet *Branchiostoma floridae* Hubbs, 1922 were obtained from GenBank (Table 6). The dataset of 18S was aligned using MAFFT ver. 7.310 with *E-INS-I* strategy (Katoh and Standley 2013); the aligned 18S dataset was trimmed by using trimAl ver. 1.4.rev15 with gappyout command (Capella-Gutiérrez et al. 2009). An alignment of COI was obtained by using MEGA X (Kumar et al. 2018) following Hasegawa and Kajihara (2019). Then, the 18S and COI sequences were concatenated on MEGA X (Kumar et al. 2018).

For constructing phylogenetic trees, BI and ML analyses were performed; MrBayes ver. 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003: Ronquist et al. 2012) for BI and IQtree ver 2.1.4-beta (Minh et al. 2020) for ML. PartitionFinder ver. 2.1.1 (Lanfear et al. 2016) was used for selecting the best-fit substitution models, which suggested GTR + I + G for 18S and COI first codon position, and GTR + G for COI second and third codon positions. Markov chains were started from a random tree and run for 10^7 generations; trees were picked up every 100 generations from the chain. Burn-in was set at 25%. The “sumt” command was used for calculating a consensus of trees; the posterior probability (PP) for each branch was collected to assess the certainty of the inference. Run convergence was assumed based on the following values of variables: average standard deviation of split frequencies = 0.002002; average estimated sample size of all parameters > 200; and potential scale reduction factor for all parameters ≤ 1.008. For ML analysis, branch support was calculated with 1000 ultrafast bootstrap (Minh et al. 2013).

Results

Taxonomy

Order Phlebobranchia

Family Ascidiidae

***Fimbrora calsubia* Monniot and Monniot, 1991a** (Figs 8–10)

Fimbrora calsubia Monniot and Monniot, 1991a, p. 384, figs 1–6; Monniot 1993, p. 356; Monniot and López-Legentil 2017, p. 531, figs 1, 2.

Material examined. One individual, JAMSTEC No. 111618, collected by N. Hookabe and Y. Fujiwara on September 26, 2022, about 400 km off the Pacific coast of middle Honshu, Japan, 30°47.05′ N; 138°44.72′ E, at a depth of 2027 m (Fig. 7).

Description. Individual ca. 20 cm in length including oral siphon (Fig. 8A, B). Tunic opaque and gelatinous; blood vessels running on surface of tunic (Fig. 8B); fine warts scattered evenly over entire tunic. Body attached to substrate with its posterior end (Fig. 8A, B). Oral siphon enlarged, ca. 10 cm in diameter; single annular muscle strand running in outer edge of oral siphon; thread-like lobes, 52 in number, tightly arranged to each other on oral-siphon edge; single groove radially arranged on edge of oral siphon between base of each lobe; muscle strand associated to each lobe, running in inner wall of oral-siphon edge from lobe base for ca. 1 cm; beneath inner surface of oral siphon, neural cords radially running from neural ganglion (Fig. 8C). Oral aperture situated 2.5 cm anterior to neural ganglion. Atrial siphon 1.5 cm in diameter; 37 blood vessels longitudinally running on surface of atrial siphon (Fig. 8D).

Body wall attached to tunic on oral siphon, heart, and renal vesicles; irregular cavity existing between tunic and body wall; inner surface of tunic covered with epithelial tissue. Neural ganglion situated between oral siphon and atrial siphon. On base of oral siphon, 105 oral tentacles present, each being ca. 8 mm in length. Peripharyngeal band made of single lamina running in short distance posterior to oral tentacles, forming V-shape posterior to neural-gland ciliated aperture (Fig. 9A); latter being single in number, almost straight in shape (Fig. 9A), and opening at dorsal tubercle. Pharynx connected by mesenteries to peripharyngeal epithelium; mesenteries 0.5–3.0 mm in diameter (Fig. 9B). Smooth dorsal lamina running along midline on ventral side of pharynx (Fig. 9A, B). Longitudinal and transverse vessels running on inner surface of pharynx (Fig. 9C); 6–10 stigmata without lateral cilia per mesh (Fig. 9C). Secondary branchial papillae present on intersections of longitudinal and transverse vessels (Fig. 9C).

Digestive tract positioned on left side of body (Fig. 10A). Esophagus opening to left side of dorso-posterior part of pharynx. Stomach about 1.5 cm in length, having 10 folds, surrounded with renal vesicles (Fig. 10A); multiple crustaceans (probably copepods) found in stomach lumen (Fig. 10B). Intestinal loop S-shaped, having primary loop and secondary loop; intestine ca. 7 cm in length, ca. 5 mm in diameter (Fig. 10A).

Anus smoothly edged, opening close to atrial siphon (Fig. 10A).

Gonad situated proximally on intestinal loop (Fig. 10C). Ovaries surrounded with male testis (Fig. 10C, D). Oviduct and spermiduct running along secondary loop, opening close to anus (Fig. 10A). Eggs contained in ovaries and oviduct, up to 0.2 mm in diameter (Fig. 10D).

Habitat. The animal attached itself to a dead sponge in an area with accumulated sand and mud at a depth of 2027 m. An euplectellid sponge was also found attached to the same substrate (Fig. 8A).

Phylogeny

The clade comprising four genera in the family Ascidiidae, i.e., *Ascidia*, *Ascidiella*, *Fimbrora*, and *Phallusia*, received high support values (97% bootstrap; 1.00 posterior probability) (Fig. 11). In this clade, *F. calsubia* was sister to *Ascidia zara* Oka, 1935, though its branch had less-supported values (53% bootstrap; 0.68 posterior probability). The clade of *Ascidia* + *Fimbrora* + *Phallusia* was sister to the genus *Ascidiella*. The genus *Ascidia* was recovered as non-monophyletic.

The three carnivorous ascidians included in this analysis—*F. calsubia*, *Megalodicopia hians* Oka, 1918, and *Oligotrema lyra* Monniot and Monniot, 1973—were each positioned differently in the phylogenetic tree. As in previous analyses (Kurabayashi et al. 2003; Tatián et al. 2011), *M. hians* was sister to *Corella eumyota* Traustedt, 1882, while *O. lyra* was sister to *Molgula manhattensis* (De Kay, 1843).

Discussion

Previous studies posited that *Fimbrora* would belong to Ascidiidae (Monniot and Monniot 1991a; Monniot and López-Legentil 2017) and my phylogenetic analysis supported this view. The morphological characteristics that suggested *Fimbrora*'s familial affiliation were the longitudinal vessels having papillae and straight stigmata in the pharynx (Monniot and Monniot 1991a; Monniot and López-Legentil 2017), while Monniot and Monniot (1991a) noted the superficial resemblance of the genus with the family Octacnemidae in having an enlarged oral siphon. The more precise phylogenetic position of *Fimbrora* in the family would require the inclusion of additional ascidiid taxa in molecular analyses. One such to-be-included taxa is *Psammascidia*, which shares two characteristics with *Fimbrora*—having secondary branchial papillae on the longitudinal vessels and lacking primary and intermediate branchial papillae (Monniot and Monniot 1973), features that are not found in other ascidiid genera (cf. Kott 1985; Brunetti and Mastrototaro 2017). Future molecular analyses may show that these two

genera are closely related to each other.

Monniot and Monniot (1991a) suggested that *F. calsubia* is carnivorous based on the presence of copepods in its gut contents as well as the shape of the oral siphon. The presence of small crustaceans, likely copepods, in the stomach of the present specimen supports this assertion. The reports of *F. calsubia* from Indonesia (Monniot 1993) and Papua New Guinea (Monniot and López-Legentil 2017), however, did not provide any information on gut contents in their specimens.

While the convergent evolution of carnivorousness in *Megalodicopia* and *Oligotrema* has already been revealed by Tatián et al. (2011), my phylogenetic tree clearly shows that *Fimbrora* is also the case: this trait was acquired at least three times independently within the class Ascidiacea (Fig. 11).

The present study expanded the species' known distribution range for about 4000 km northward, representing the first record of the species from the North Pacific. The present material also represents the deepest record for the species with the known vertical distribution range being about 1000–2000 m (Monniot and Monniot 1991a; Monniot 1993; Monniot and López-Legentil 2017; present study).

Chapter 4

Graveyards of giant pandas at the bottom of the sea? A strange-looking new species of colonial ascidians in the genus *Clavelina* (Tunicata: Ascidiacea)

Introduction

The enterogonan ascidian genus *Clavelina* Savigny, 1816 belongs to the family Clavelinidae along with three genera, *Euclavella* Kott, 1990, *Nephtheis* Drasche, 1882, and *Pycnoclavella* Garstang, 1891. My enumeration indicates that 44 species have been reported in *Clavelina* from around the world (Table 7). All are distributed in tropical, subtropical, and temperate waters (e.g., Millar 1975; Tokioka and Nishikawa 1976; Kott 1990; Monniot and Monniot 1996; Rocha et al. 2012a; Seo and Rho 2015). Apart from a few exceptions, members in *Clavelina* are colonial, with zooids connected by stolons or basal tunic. Each species shows a unique zooid morphology with specific color patterns; after preservation in formalin or ethanol, however, the color rapidly fades away (Nishikawa and Tokioka 1976). This genus is characterized by the following set of characteristics: *i*) zooids having stalked body that is divided into two parts, the thorax and the abdomen; *ii*) both siphons without lobes; *iii*) no folds or papillae in the pharynx, and 8–20 stigmatal rows; *iv*) languets on the dorsal lamina; *v*) the abdomen possessing an intestinal loop and gonads; and *vi*) buds vegetatively produced at terminal ampullae of the vascular stolons (Savigny 1816; Kott 1990; Rocha et al. 2012a; Seo and Rho 2015).

From Japanese waters, nine species of clavelinids have been reported: *C. coerulea* Oka, 1934; *C. cyclus* Tokioka and Nishikawa, 1975; *C. elegans* (Oka, 1927); *C. miniata* Watanabe and Tokioka, 1973; *C. minuta* Tokioka, 1962; *C. obesa* Nishikawa and Tokioka, 1976; *C. polycitorella* (Tokioka, 1954); *C. robusta* (Kott, 1990); and *C. viola* Tokioka and Nishikawa, 1976 (Nishikawa and Tokioka 1976; Tokioka and Nishikawa 1976; Nishikawa 1995, 2017; Nishikawa and Namikawa 2018; Ota et al. 2020). Nishikawa and Tokioka (1976) reviewed Japanese clavelinid ascidians for the first time, which was supplemented by Tokioka and Nishikawa (1976). The Atlantic clade of *C. lepadiformis* (Müller, 1776) species complex *sensu* Turon et al. (2003) was subsequently recorded from Orido Bay (Nishikawa 2017) and Suruga Bay (Nishikawa and Namikawa 2018).

A colonial ascidian called *gaikotsu-panda-hoya* (literally meaning 'skeleton panda ascidian' in Japanese) in Kumejima Island (Fig. 12) became popular among a certain fraction of Japanese SCUBA divers in the last several years. Its appearance, especially the zooids' color pattern, appeared to be different from those of the other Japanese clavelinid ascidians. To uncover the taxonomic identity of this ascidian, I collected fresh samples of this species for detailed examination.

Methods

Four colonies were collected by SCUBA diving. These were photographed underwater with a Nikon COOLPIX W300 digital camera and in the laboratory with a Nikon D5600 digital camera. Live ascidians were anesthetized with menthol. For nucleic-acid extraction, a couple of zooids were removed from a colony and preserved in 99% ethanol and RNAlater (Thermo Fisher Scientific), respectively. For morphological observation, colonies were fixed in 10% formalin–seawater and subsequently transferred to 70% ethanol. One zooid from each colony was dissected and stained with hematoxylin for detailed examination. Measurements, counting, and means of metric and countable traits were made/calculated based on four zooids, one from each colony. All specimens have been deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan (Table 8).

DNA extraction, amplification, and sequencing were conducted following Hasegawa and Kajihara (2019). Total DNA was extracted from a piece of zooids' body-wall (ICHUM 5837 and 5838) preserved in 99% ethanol. A partial sequence of COI gene was amplified with the primer pair dinF/Nux1R (Brunetti et al. 2017). Conditions for PCR were 94°C for 2 min; 35 cycles of 94°C for 45 sec, 46°C for 50 sec, and 72°C for 80 sec; then 72°C for 5 min. Before sequencing, the PCR products were purified by a reaction combining two enzymes: 24 mU/μL of Exonuclease I (TaKaRa) and 4.9 mU/μL of Shrimp Alkaline Phosphatase (TaKaRa). Sequencing was performed with a BigDye Terminator ver. 3.1 Cycle Sequence Kit (Thermo Fisher Scientific) and 3730 Genetic Analyzer (Thermo Fisher Scientific) using the same primer pair as for PCR amplification. GeneStudio Professional Edition ver. 2.2.0.0 (GeneStudio) was used for base calling. The sequences have been deposited in the International Nucleotide Sequence Database Collaboration (INSDC) (Table 8). Kimura's (1980) 2-parameter (K2P) genetic distance between two sequences was computed by using MEGA X (Kumar et al. 2018).

To infer the phylogenetic position of the species in question among *Clavelina*, ML and BI analyses were performed, designating *Pycnoclavella* as the outgroup (cf.

Pérez-Portela and Turon 2008). For constructing a dataset, COI sequences of 20 clavelinid species were obtained from the INSDC (Table 9). The nucleotide sequences were manually aligned by using MEGA ver. 11.0.13 (Tamura et al. 2021) following Hasegawa and Kajihara (2019). The best partition scheme (GTR + I +G for the first and second codon position; GTR + G for the third codon position) was selected with PartitionFinder ver. 2.1.1 (Lanfear et al. 2016). The ML analysis was conducted by using IQ-TREE ver. 2.2.2.6 (Minh et al. 2020) with the ultrafast (UF) bootstrap method (Hoang et al. 2018). Branch support values were evaluated with 1,000 UF bootstrap replicates. The BI was performed with MrBayes ver. 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003: Ronquist et al. 2012). An initial tree was randomly selected for four Markov chains in two parallel runs with 3×10^6 generations; trees were sampled every 100 generations; the burn-in fraction was set to 0.25. A consensus tree was obtained by using the "sumt" command. Convergence was estimated with the average standard deviation of split frequencies reaching ca. 0.3%, the minimum estimated sample sizes for all parameters above 200, and the maximum potential scale reduction factor of 1.003.

Results

Taxonomy

Clavelina ossipandae sp. nov.

(Figs 13–15)

Diagnosis. A *Clavelina* with colony consisting of zooids extending from basal mass; zooids completely free, mean 15 mm long; in life, a white, laterally elongated white patch present between oral and atrial siphons; small black point present on anterior body wall between oral and atrial siphons; an elongated black band situated laterally to the small black point on each side, slightly curved along edge of the white patch; transverse vessels white; endostyle black; short mid-dorsal black line situated posterior to atrial siphon, spanning for about four transverse vessels; 10–14 stigmatal rows in pharynx; on each side of thorax, 10 or 11 very thin longitudinal muscle bands, of which two running to endostyle, 5–6 to branchial siphon, and 2–4 to dorsal side.

Material examined. Holotype: ICHUM 5837, colony with four zooids, of which two had been removed for nucleotide extraction. Paratypes: ICHUM 5838, 5839, 5840, colonies with single, two, and four zooids, respectively. All were collected by N. Hasegawa, A. Izeki, and I. Nakayoshi, on March 14, 2021, at depths of 10–20 m at a diving point (26°16′10″N, 126°49′9″E) around a huge rock—locally called Tonbara

(Fig. 12)—off the southeast coast of Kumejima Island in the Okinawa Islands, Japan.

Description. Colony consisting of 1–4 zooids (4 in holotype); each zooid with its own enclosing thin tunic completely free from adjoining ones, united only by its basal tunic (Fig. 13A, B; Table 8). Each zooid basally connected to each other with short vascular stolon. Tunic colorless, transparent, and gelatinous; tunic of thorax softer and thinner than that of abdomen. Zooid 7–14 mm (14 mm in holotype, mean 9.3 mm) (Fig. 13C, D). In life, zooid's main body shrinking easily upon stimulus; laterally elongated white patch present between oral and atrial siphons, width of patch almost same as that of pharynx; small black point present on anterior body wall between oral and atrial siphons; elongated black band situated laterally to the small black point on each side, positioned anterior to first stigmatal row, slightly curved along edge of white patch; transverse vessels white; endostyle black; short mid-dorsal black line situated posterior to atrial siphon, spanning for about four transverse vessels (Fig. 13A); after fixation in formalin, white color faded away, black point and bands remained.

In fixed condition, thorax and abdomen almost equal in length (Fig. 13C, D; Table 8). Siphons having no lobes, close to each other, and smooth-rimmed; diameter of oral siphon twice larger than atrial siphon (Fig. 13C, D). Ten oral tentacles, each approximately 0.5 mm in length (Fig. 14A, B). Longitudinal muscle bands 10 or 11 (11 in holotype) in number, running on each side of thorax; two of them running to endostyle, 5 or 6 (5 in holotype) to branchial siphon, 2–4 to dorsal side (4 in holotype) (Fig. 13B, C; Table 8). Peripharyngeal groove made up of single lamina. Ciliated aperture of dorsal tubercle forming longitudinal slit (Fig. 14B). Pharynx without folds, longitudinal vessels, and papillae. Stigmatal rows 10–14 in number (14 in holotype) (Table 8). Approximately 50 stigmata contained in each half-row. Dorsal languets present (Fig. 14A, B). Brood pouch black in color, situated in dextro-dorso-posterior position of thorax (Fig. 13B).

Esophagus opening to posterior end of pharynx. Stomach with five longitudinal folds, positioned almost in middle of abdomen (Fig. 13B, C). Post-stomach indistinct. Intestine isodiametric from pylorus to anus. Anus without lobes, lying about two-thirds position from anterior end of thorax.

Gonads attached on left side of intestinal loop, posterior to stomach (Figs 13C, 15A). Ovaries surrounded with testicular follicles, latter being 0.05–0.2 mm in long-axis length, ca. 20 in number (22 in holotype) (Figs 13C, 15A). Eggs and larvae brooded in brood pouch; one larva contained in ICHUM 5837 (holotype); 20 eggs and 12 embryos in ICHUM 5838 (paratype). Larvae approximately 1.25 mm in length, having ca. 0.5-mm trunk and ca. 0.75-mm tail; three protruding adhesive organs arranged in triangle on

frontal plate; ocellus and otolith present in dorsal side of trunk (Fig. 15B).

Etymology. The new species epithet, *ossipandae*, is a noun in the genitive case, a composite derived from *os* ('bone' in Latin) and *panda*, the latter is meant to be the giant panda, *Ailuropoda melanoleuca* David, 1869, and herein treated as a noun that is latinized. The species is so named because the white anterior portion of the zooid with the characteristic black markings resembles to the face of the giant panda and the white transverse vessels evoke the ribs of a skeleton.

Distribution. Currently, the new species is only known from a diving point, Tonbara, near Kumejima Island in the East China Sea (Fig. 12). It is difficult to access this point except in winter due to tidal currents and wind direction. The depth of this area is 10–20 m.

COI sequence. Partial sequences (810 bp) of the COI gene were determined from the holotype (ICHUM 5837) and one of the paratypes (ICHUM 5838). The K2P distance between the two specimens was 0.0126. Variations were found in 10 sites between the two: 60 (G in the holotype vs. A in the paratype), 213 (G vs. A), 225 (G vs. C), 246 (G vs. A) 340 (A vs. G), 348 (T vs. C), 474 (T vs. C), 477 (C vs. T), 594 (C vs. T), and 801 (A vs. G). There was no difference when they were translated into amino-acid sequences.

Phylogeny

The ML and BI trees showed a similar relationship to the phylogenetic analysis by Pérez-Portela and Turon (2008), in which *Clavelina* and *Pycnoclavella* form a separate clade and *Nephtheis* is included in the *Clavelina* clade (Fig. 16). In the phylogenetic tree, *C. ossipandae* turned out to be sister to *C. australis* (Herdman, 1899) with well-supported values (UF bootstrap value, 94; posterior probability, 1.00) (Fig. 16).

Discussion

The present new species can be regarded as a member of the genus *Clavelina* based on the following three morphological characteristics. First, the colony in *C. ossipandae* consists of free zooids, in contrast to the genera *Euclavella* and *Nephtheis*, where zooids are completely embedded within colonies. Second, the larvae of *C. ossipandae* do not possess tubular invaginated structures in the adhesive organs which is characteristic in ones of the genus *Pycnoclavella* (Kott 1990; Pérez-Portela et al. 2007; Rocha et al. 2012a). Thirdly, the rows of stigmata in *C. ossipandae* are 10–14 in number; according to Rocha et al. (2012a), these are 8–20 in *Clavelina* and 3–8 in *Pycnoclavella*. Moreover, my phylogenetic analysis supported that this species belongs not to

Pycnoclavella but to *Clavelina* (Fig. 16).

The color pattern of *C. ossipandae* is unique among congeners (Fig. 13A; Table 7). This species is similar to *C. moluccensis* (Sluiter, 1904) and *C. viola* in that they share a pattern consisting of a small point between the siphons and a pair of elongated bands lateral to the point (cf. Kott 1990; Ota et al. 2020). Each of the three species has a different coloration. The anterior part of the zooid is light blue in *C. moluccensis*, white in *C. ossipandae*, and yellow in *C. viola*; the points and the elongated bands are dark blue in *C. moluccensis*, black in *C. ossipandae*, and blue in *C. viola*. The transverse vessels are blue in *C. moluccensis*, white in *C. ossipandae*, and not pigmented in *C. viola*. The endostyle is colorless in *C. moluccensis*, black in *C. ossipandae*, and blue in *C. viola*. There is no dorsal line in *C. moluccensis*, but it is present in *C. ossipandae* (black) and *C. viola* (blue).

Clavelina ossipandae also differs from the two congeners *C. moluccensis* and *C. viola* in the number and arrangement of muscle bands in the thorax, which are useful discriminatory traits in preserved specimens (Tokioka and Nishikawa 1976; Kott 1990). In *C. ossipandae*, the number of the muscular bands running longitudinally to the endostyle is two, whereas it is six to seven in *C. viola* (Tokioka and Nishikawa 1976). In *C. moluccensis*, Kott (1990) reported all muscle bands are transverse based on material from Australia, whereas Sluiter (1904) did not mention anything about muscle bands in the type material from Indonesia.

The body coloration in living state is not known for the seven congeners *C. borealis* Savigny, 1816; *C. brasiliensis* (Millar, 1977); *C. concrescens* Hartmeyer, 1924; *C. fasciculata* Van Name, 1945; *C. kottae* (Millar, 1960); *C. michaelseni* Millar, 1982; and *C. simplex* Kott, 2006 (Table 7). However, *C. ossipandae* can be distinguished from all of them: by the greatest zooid length from *C. borealis* (160 mm), *C. brasiliensis* (75 mm), and *C. kottae* (120 mm) (vs 20 mm in *C. ossipandae*) (Table 7) (cf. Savigny 1816; Hartmeyer 1903; Monniot 2001; Brunetti and Mastrototaro 2017); by the colony form from *C. consrescens* and *C. simplex* (zooids partially embedded and grouped vs zooids completely free in *C. ossipandae*) (Table 7) (cf. Hartmeyer 1924; Van Name 1945; Kott 2006); by the number of zooid(s) from a single stalk from *C. fasciculata* (more than two vs one in *C. ossipandae*) (cf. Van Name 1945); and by the number of stigmatal rows from *C. michaelseni* (30 vs 10–14 in *C. ossipandae*) (cf. Millar 1982) (Table 7).

Prior to my study, there had been no record of *Clavelina* or other ascidians from Kumejima Island according to Tokioka (1963) and Nishikawa (1995). Further research is needed to understand the ascidian fauna of this region.

Chapter 5

From solitary to colonial with zooid miniaturization: ancestral-state reconstruction based on phylogenomic analysis of stolidobranch ascidians

Introduction

Ascidians, exhibit a wide range of life history strategies (Satoh 1994; Burighel and Cloney 1997; Davidson et al. 2004). Within Ascidiacea, the order Stolidobranchia has been recognized as an ideal group for exploring the evolutionary plasticity of coloniality (Zeng et al. 2006; Pérez-Portela et al. 2009; Brown and Swalla 2012; Alié et al 2021). This order encompasses three families, Molgulidae, Pyuridae, and Styelidae (cf. Kott 1985; Monniot et al. 1991). While both molgulid and pyurid ascidians are solitary, styelids comprise solitary and colonial species (cf. Kott 1985; Monniot et al. 1991). Kott (1985) classified Styelidae into three subfamilies: Botryllinae, which includes colonial forms with systems—zooids' arrangements around common cloacal cavities in a colony; Polyzoinae, which comprises colonial forms without systems; and Styelinae, which consists solely of solitary forms.

Among the colonial groups, a diverse range of coloniality has been observed (Fig. 17). In terms of the degree of colonial complexity, the polyzoine genus *Symplegma* Herdman, 1886 positions between other polyzoines and the highly integrated botryllines (Shirae et al. 1999; Gutierrez and Brown 2017). There is no morphological difference between the colonial genus *Syncarpa* Redikorzev, 1913 and the solitary genus *Dendrodoa* MacLeay, 1824 except forming colonies (Hasegawa and Kajihara 2019). The diversity of coloniality in Styelidae is actualized by various budding modes, which can be categorized based on the tissues from which buds (= daughter zooids) originate in mother zooids. Specifically, three budding modes are recognized: basal budding (Alié et al. 2018), peribranchial budding (Metschnikoff 1869; Pizon 1893; Ritter 1896; Selys-Longchamps 1917; Berrill 1940, 1948; Watanabe and Tokioka 1972; Mukai and Watanabe 1976; Kawamura and Watanabe 1981; Akhmadieva et al. 2007), and vascular budding (Giard 1872; Oka and Watanabe 1957; Sabbadin et al. 1975; Nakauchi 1982; Brunetti and Mastrototaro 2004; Brown et al. 2009; Kawamura and Sunanaga 2010; Gutierrez and Brown 2017).

Phylogenetic analyses of Stolidobranchia based on partial sequences of 18S

rRNA and mitochondrial cytochrome *c* oxidase subunit I genes have variably suggested that coloniality evolved once (Zeng et al. 2006; Tsagkogeorga et al. 2009; Hasegawa and Kajihara 2019) or seven times (Pérez-Portela et al. 2009), but these studies often yielded trees with less-supported nodes. Alié et al. (2018) suggested that peribranchial budding and basal budding evolved convergently, and that vascular budding was secondarily derived in an ancestor exhibiting peribranchial budding, based on a phylogenomic tree with high support values. However, their analysis (Alié et al. 2018) lacked some key taxa, such as *Symplegma* and *Syncarpa*, which are pivotal for discussions on the evolution of coloniality. While the phylogenetic relationships in Stolidobranchia have been inferred by many previous authors to elucidate how coloniality evolved, no attempt has been made to reconstruct the ancestral states of coloniality using statistical methods.

The aim of this study is to clarify the evolutionary relationship between zooid size and coloniality. A robust phylogenomic tree was constructed, encompassing key taxa in the Stolidobranchia essential for understanding the evolution of coloniality. Through Bayesian inference for ancestral-state reconstruction, I provide a clear depiction of trait evolution related to coloniality on the tree. Additionally, I propose a mathematical model suggesting that zooid miniaturization has been driven by spatial competition on substrates.

Methods

Sampling, fixation, and identification. A total of 17 ascidian species, including both colonial and solitary forms, were sampled in Israel and Japan from May 2021 to January 2023 (Table 10). I conducted taxon sampling without bias in body size. The animals were anesthetized with menthol. For solitary individuals, oral or atrial siphons were excised and preserved in RNAlater; the remaining bodies were preserved in 10% formalin. For colonial forms, a part of the colonies was cut off and preserved in RNAlater; another part was preserved in 10% formalin. The RNAlater-preserved specimens were used for total RNA extraction; the formalin-fixed specimens were used for morphological identification. For specimens collected in Israel, 1% borax was added to the formalin fixative solution.

RNA extraction, purification, and sequencing. The tissues preserved in RNAlater were homogenized in tubes containing 500 mL of Sepazol-RNA I Super G (Nacalai tesque, Kyoto). The homogenate was left at room temperature for 5 minutes and then mixed with 100 μL of chloroform. Then, the samples were centrifuged at 12,000 × g for 15 minutes. 250 μL of upper aqueous phase was mixed with 250 μL of

isopropanol and 0.5 μL of glycogen solution, followed by incubation at room temperature for 10 minutes. Subsequently, they were centrifuged at 12,000 × g for 10 minutes. The solution was removed from tubes, and 500 μL of 80% ethanol was added. After centrifugation at 12,000 × g for 5 minutes, the ethanol was carefully removed; then, pellets were air-dried. The pellets were dissolved in 200 μL of TE to obtain crude RNA extracts. The crude RNA solution (dissolved in 200 μL of TE) was added to 200 μL of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1). The samples were centrifuged at 13,000 rpm for 10 minutes at room temperature. Upper aqueous phase was transferred to a new tube; then, 200 μL of chloroform:isoamyl alcohol (24:1) was added. Once more, after centrifugation at 13,000 rpm for 10 minutes at room temperature, 180 μL of upper aqueous phase was collected. To this, 20 μL of 3M sodium acetate solution, 500 μL of 100% ethanol, and 0.5 μL of glycogen solution were added. The samples were then centrifuged at 15,000 rpm for 30 minutes at 4°C, and the supernatant was removed. Pellets were mixed with 700 μL of 80% ethanol, followed by centrifugation at 15,000 rpm for 5 minutes at 4°C. The supernatant was removed, and pellets were air-dried. The pellets were dissolved in 10 μL of TE to obtain total RNA solutions. Concentration of total RNA in the solution was measured using a NanoDrop (Thermo Fisher Scientific). The volume of the solution containing 5 μg of RNA was measured; then, pure water was added to adjust the total volume to 26.5 μL; for samples that did not meet the 5 μg threshold, the entire volume of the total RNA solution was added. For removing DNA from the samples, 3 μL of 10 × TURBO DNase buffer (Thermo Fisher Scientific) and 0.5 μL of TURBO DNase (Thermo Fisher Scientific) were added. The samples were incubated at 37°C for 30 minutes using a 2720 thermal cycler (Thermo Fisher Scientific). After incubation, 3 μL of DNase Inactivation Reagent (Thermo Fisher Scientific) was added, and the samples were incubated at room temperature for 5 minutes with tapping every 2 min. Subsequently, the samples were centrifuged at 10,000 × g for 2 minutes at room temperature, and 25 μL of supernatant was collected. To the supernatant, 75 μL of water, 10 μL of 3M sodium acetate solution, 0.5 μL of glycogen solution, and 275 μL of 100% ethanol were added. The samples were centrifuged at 15,000 rpm for 30 minutes at 4°C; then, the supernatant was removed. Pellets were mixed with 400 μL of 80% ethanol, followed by centrifugation at 15,000 rpm for 5 minutes at 4°C. Once more, supernatant was removed; the pellets were air-dried. The pellets were dissolved in 11 μL of pure water to obtain total RNA solutions free of genomic DNA. Concentration of RNA in the solutions was measured using a NanoDrop (Thermo Fisher Scientific). Absence of RNA degradation was confirmed by electrophoresis. cDNA library preparation and paired-end sequencing

were performed by Novogene; the Illumina NovaSeq 6000 was used for next-generation sequencing.

Phylogenomic analysis. For obtaining *de novo* transcriptomes, I mainly followed Alié et al. (2018). The following three types of Illumina reads were filtered by Novogene: reads containing adapter contamination, with more than 10% of bases that could not be determined throughout the entire length, and where more than 50% of bases had Q20 of Phred value (Ewing et al. 1998) in their entire length. The reads data was registered as the BioProject PRJNA880804 in Sequnce Read Archive (SRA). The filtered reads were *de novo* assembled with Trinity ver. 2.8.5 (Grabherr et al. 2011) using default parameters. Open reading frames (ORFs) with less than 90 amino acids were discarded by TransDecoder ver. 5.5.0 (https://github.com/TransDecoder/TransDecoder; last accessed May 2023). ORFs having similar nucleotide sequences were clustered using CD-HIT-EST ver. 4.8.1 (Li and Godzik 2006; Fu et al. 2012) with default parameters. The reads were mapped on the remaining contigs by using Kallisto ver. 0.46.2 (Bray et al. 2016). The contigs that had low expression levels were picked up by drawing density plots with R; the contigs were retained based on the threshold corresponding to the lower peak of the plot. To assess the accuracy of the sequencing, BUSCO ver. 5.3.2 (Simão et al. 2015; Waterhouse et al. 2017; Seppey et al. 2019; Manni et al. 2021) with metazoan datasets was used to examine the presence of core gene sets in the assembled sequences. Cross-contamination occurring in the process from the extraction to the RNA-seq was removed using CroCo (Simion et al. 2018). In addition to the *de novo* transcriptomes, 16 transcriptomes were downloaded from a GitHub repository, styelidae (Alié et al. 2018; https://github.com/AlexAlie/styelidae) for a phylogenomic analysis (Table 11); core proteomes of nine ascidian species were also downloaded from Ascidian Network for In Situ Expression and Embryological Data (ANISEED) (Table 11). I retained only the longest sequence for each gene in their respective proteomes from each of the nine species; CD-HIT ver. 4.8.1 (Fu et al. 2012) was used for dereplicating with -c *1.0* option, for excluding sequences consisting of less than 30 amino acids.

Multiple alignments were constructed for the phylogenomic analysis. Orthologous clusters were built based on the proteomes from 42 species using OrthoFinder ver. 2.5.4 (Emms and Kelly 2015) with -M *msa* and -S *blast* options. I downsampled the sequences within each cluster that had multiple sequences derived from same sample; the longest sequence was designated as the representative sequence for each species. From these downsampled clusters, I excluded the clusters that did not meet the following criteria: within each cluster, *i*) containing 30 or more sequences of

the total 33 species listed in Table 10 and from the GitHub repository styelidae (Table 11); and *ii*) containing seven or more sequences of the nine species from ANISEED (Table 11). For these two steps, I utilized a Python script in the GitHub repository Directionality_zooid_miniaturization (https://github.com/231007NHasegawa/Directionality_zooid_miniaturization). Each remained cluster was aligned by MAFFT ver. 7.520 (Katoh and Standley 2013) with *E-INS-i* strategy. The alignments were individually trimmed using TrimAl ver. 1.4. rev. 15 (Capella-Gutiérrez et al. 2009) with *strictplus* method. Then, the trimmed alignments were concatenated with each other by my original Python script (https://github.com/231007NHasegawa/Directionality_zooid_miniaturization).

Maximum Likelihood (ML) analysis and Bayesian inference (BI) were performed based on the concatenated alignment for inferring a phylogenetic relationship among Stolidobranchia. Prior to conducting the ML analysis, the substitution models corresponding to each partition in the multiple alignment were selected with ModelFinder program (Kalyaanamoorthy et al. 2017) referring partition models (Chernomor et al. 2016) implemented in IQ-TREE ver. 2.2.2.6 (Minh et al. 2020); then, the ML analysis was conducted by IQ-TREE ver. 2.2.2.6 (Minh et al. 2020) with ultrafast (UF) bootstrap method (Hoang et al. 2018). Branch support values were evaluated with 1,000 UF bootstrap replicates. BI analysis was performed using the MPI version of ExaBayes ver. 1.5.1 (Aberer et al. 2014). Two independent MCMC runs were parallelly run, started from a random tree; cold chain and heated chain with 0.5 of "heatFactor" option were executed per independent run. The runs were stopped that each chain was run for 500,000 generations; at that time, the average standard deviation of split frequencies reached 0.0%. Trees were sampled every 100 generations; the initial 25% of sampled trees were discarded as burn-in. Convergence was checked by postProcParam program implemented in ExaBayes.

Ancestral-state reconstruction. To determine values for matrix entries in transition probability matrices for each character—*i*) basal budding, *ii*) peribranchial budding, *iii*) vascular budding, *iv*) common cloacal cavity, and *v*) coloniality (colonial or solitary)—I initially computed a maximum likelihood estimate by using "MultiState" (Pagel et al. 2004) as implemented in BayesTraits ver. 4.0.1 (http://www.evolution.reading.ac.uk/BayesTraitsV4.0.1/BayesTraitsV4.0.1.html; last accessed September 30th, 2023) for a transition rate of each character state. The rates calculated as zero were simply adjusted to 0.01 in the matrices, indicating that such transitions were rare evolutionary events. Subsequently, I conducted Bayesian inferences (BI) using "MultiState" (Pagel et al. 2004) based on the ML tree with

10,000,000 iterations, a burn-in of 2,500,000, a sampling frequency of 1,000, and the "ScaleTrees" option set at 0.1 because a mean of branch lengths of the ML tree was ca. 0.1. I employed a uniform distribution as a non-informative prior, adjusting an interval such that the transition probability matched the mean of the uniform distribution. Expected a posteriori (EAP) was computed to represent the posterior probabilities (PPs) of the character states at each node.

For the ancestral-state reconstruction, I obtained literature-based data of the longest body length for each species based on taxonomic descriptions (Table 13). For *Botrylloides* sp., *Microcosmus* sp., and *Styela* sp., the body lengths were measured based on the formalin-fixed specimens. Information about the body length of *Polycarpa* sp. is lacking (Alié et al. 2018). Based on the phylogenomic tree, MCMC analysis was conducted selecting Brownian motion model by "Continuous: Random Walk" (Pagel 1999) with 10^8 iterations, 25% of burn-in, and the "ScaleTrees" option set 0.1. The body length of each node was represented by a mean of values sampled from every 1,000 generations.

Character correlation analysis. To test the correlation between coloniality and body length reconstructed on the tree nodes (whether or not there is any evolutionary tendency between these characters), I utilized the "Test trait correlations: continuous" and "Independent Contrast: Correlation" (Felsenstein 1973; Freckleton 2012) in BayesTraits version 4.0.1. Both MCMC approaches were adopted, employing a stones command, setting 100 stones with 1,000 iterations each to determine a marginal likelihood. Subsequent analyses were implemented using the "TestCorrel" command, which sets the covariance to zero, employing identical command parameters as the first analysis. Log Bayes Factor (Log BF) was calculated by taking twice the difference of log marginal likelihoods between the two analyses.

Detection of evolutionary direction. The resulting tree included a subclade solely consisting of members that undergo peribranchial budding (= Clade A, see below). To evaluate if the body size evolved directionally in the entire Stolidobranchia and this subclade, respectively, MCMC analyses were separately conducted based on whole of the ML tree and this subclade. First, an MCMC analysis was executed with the command selecting "Continuous: Random Walk (Model A)" (Pagel 1999) and a stones command estimating a marginal likelihood using 100 stones and 1000 iterations per stone. Second, another MCMC analysis was carried out with the "Continuous: Directional (Model B)" method (Pagel 1999), maintaining the same parameters for the stones command. Then, the Log BF was computed by subtracting the log marginal likelihood of the Model A and Model B. In addition, I calculated correlation coefficient

between body length and branch length for solitary and colonial forms.

Results

Phylogenomics. The number of Illumina reads after filtration obtained from 17 species ranged from 42 million to 137 million. Through the assembly process, 26,116–108,633 contigs were kept for each species (Table 12). These contigs, along with transcriptomes of 16 species from Alié et al. (2018) and proteomes of nine species from ANISEED, were clustered in 360,094 orthologous groups. The number of orthologous groups that met the filtration criteria were 1,883 out of 360,094. After alignment, trimming, and concatenation, the dataset comprised 1,039,648 aa including gaps of 42 ascidian species with 0.1% of missing data. The dataset was partitioned into 130 blocks; the best-fit model was selected in each partition.

Almost all the nodes in the tree received highly supported values (100% UF bootstrap; 1.00 PP); as an exception, the clade of *Eusynstyela tincta* + *E. latericius* + *E. misakiensis* has 85% UF bootstrap value (Fig. 18). The order Stolidobranchia was sister to the clade Aplousobranchia + Phlebobranchia. Phlebobranchia was non-monophyletic; in it, *Phallusia* was sister to Aplousobranchia. In Aplousobranchia, *Aplidium* formed a clade with *Pseudodistoma*. In Stolidobranchia, Molgulidae was inferred to have branched off first from the rest of this group; Styelidae was recovered as monophyletic, which turned out to be sister to a part of paraphyletic Pyuridae. In Styelidae, members that perform peribranchial budding—botryllines and part of polyzoines—formed a clade (hereafter Clade A); in Clade A, *Symplegma* was sister to Botryllinae (*Botrylloides* and *Botryllus*). *Syncarpa* grouped together with paraphyletic *Dendrodoa*.

Ancestral-state reconstruction. The EAPs for the coloniality-related character states—either *i*) budding from basal stolon, *ii*) budding from peribranchial epithelium, *iii*) budding from blood vessel, *iv*) with a common cloacal cavity, or *v*) non colonial (i.e., solitary)—exceeded 99% in every node (Figs 19–23).

Character correlation. The log marginal likelihoods were −240.18 and −239.98 using complex models in “Test trait correlations: continuous” and “Independent Contrast: Correlation”, respectively, for testing whether there is a correlation between the characteristic states of coloniality and body/zooid length; the log marginal likelihoods with simple models setting the covariance to zero were −242.32 and −242.26, respectively. The log BFs were 4.29 and 4.56, respectively; the values exceeded two, which means positive evidence of correlation between the character states in the two traits.

Evolutionary direction. The log marginal likelihood based on the entire

phylogenetic tree using Model A was −239.80. When using Model B, the value was −240.47. From these two values, the Log BF was calculated to be 1.34. This value is smaller than the threshold value of two, which does not support directionality in trait changes. Based on Clade A, the value using Model A was −109.33; it was −112.35 using Model B. The Log BF was 6.05; as it falls within 5–10, the presence of directionality was strongly supported.

The correlation coefficients between body lengths and branch lengths were 0.01 for solitary forms and −0.85 and colonial forms, respectively. The slopes of the regression lines for these two parameters were ca 2.8 in solitary form (coefficient of determination: $R^2 = 0.0002$) and ca. −46.3 in colonial form ($R^2 = 0.7292$), respectively (Fig. 24).

Discussion

This study indicates that zooids became gradually smaller once coloniality was acquired (Figs 23, 24). To explain this evolutionary pattern, I hypothesize that zooid miniaturization would shorten the period for daughter zooids to start budding (typically, as an animal's body size increases, the time it requires to reach maturity also extends; Blueweiss et al. 1978), leading to rapid expansion of the colony over substrates (Fig. 25). The marine substrates represent a significant limiting resource for sessile organisms, with living space being at a premium (Connell 1961; Pequegnat 1964; Dayton 1971; Paine 1971; Jackson 1977, 1985; Hughes 2005; Tyrrell and Byers 2007). Colonial invertebrates achieve this by continuously covering the substrate and exhibiting rapid lateral growth through asexual reproduction with a rate of expansion, which is unattainable through sexual reproduction alone (Jackson 1977).

The relationship between the substrate encrusting rate of a colony and the zooid size at maturity can be mathematically described as follows. Let $A_C(t)$ be the colony area at time t, V_Z be the zooid volume at maturity, and k be the zooid's volume–growth rate per unit time. The growth rate of colony area per unit time can be expressed by the following equation:

$$\frac{dA_C}{dt} = \frac{k \cdot A_C}{V_Z} \tag{1}$$

because the area of a colony increases proportionally to the number of zooids in the colony, which is proportional to the colony area. Solving Eq. (1) yields:

$$\int \frac{dA_C}{A_C} = \int \frac{k}{V_Z} dt \tag{2}$$

$$ln|A_C| = \frac{kt}{V_Z} + C \tag{3}$$

where C is an integration constant. Since $A_C > 0$, Eq. (3) can be rearranged as:

$$A_C = e^{\left(\frac{kt}{V_Z}+C\right)} \tag{4}$$

where e indicates Euler's number. Setting $B = e^C$ (B is a constant), we yield

$$A_C = Be^{\frac{kt}{V_Z}}. \tag{5}$$

Plugging $t = 0$ in Eq. (5), we obtain:

$$A_C = B. \tag{6}$$

The initial value of the colony area $A_C(0)$ is equal to the area of a single zooid that arises when the larva attaches to the substrate and then metamorphoses. If we denote the zooid area as A_Z, the initial condition $A_C(0)$ equals to A_Z. Plugging this into Eq. (6), we obtain:

$$A_C(t) = A_Z e^{\frac{kt}{V_Z}}. \tag{7}$$

By considering Eq. (7), the competition between two colonies with different zooid volumes and areas can be examined. Let us assume that Colony 1 possesses a larger zooid volume and area compared to Colony 2 (with $A_{Z1} > A_{Z2} > 0$ and $V_{Z1} > V_{Z2} > 0$), while their respective zooid's volume growth rates, denoted as k_1 and k_2 (each being a constant). Commencing at $t = 0$, both Colony 1 and Colony 2 originate from a single zooid, where the initial areas can be expressed as $A_{C1}(0) = A_{Z1}$ and $A_{C2}(0) = A_{Z2}$. The value of t at which the areas of both colonies become equivalent is determined by the following equation:

$$t = \frac{lnA_{Z2} - lnA_{Z1}}{\frac{k_1}{V_{Z1}} - \frac{k_2}{V_{Z2}}}. \tag{8}$$

In scenarios where k_2/V_{Z2} exceeds k_1/V_{Z1}, the implication is that Colony 2, with its smaller zooids, will eventually surpass the area of Colony 1, which comprises larger zooids (Fig. 25). Conversely, if k_2/V_{Z2} is less than k_1/V_{Z1}, the resultant negative value of t suggests that the area of A_{C2} will not overtake that of A_{C1}. Therefore, this model implies that Colony 1 must accelerate the growth rate of its zooids to achieve a faster colony expansion than Colony 2.

It is important to note here that the model merely discusses the advantages of smaller zooids in colony expansion rates and does not necessarily indicate that colonies with smaller zooids will invariably produce more offspring in subsequent generations. At least three disadvantages with smaller zooids can be conceived: *i*) the zooids would

become physically weaker, *ii*) they would be more susceptible to external environmental impacts on the body surface, and *iii*) the number of eggs or sperm per zooid would be reduced. The size of the zooids would be determined by a trade-off with these disadvantages. Moreover, even when the two curves intersect as depicted in Eq. (8) and Fig. 25, the lineage of Colony 1 does not necessarily go extinct if the colony succeeds to form a sufficiently large area to reproduce for the next generation before the time of intersection.

Coloniality was independently acquired at least three times in the evolutionary history of Stolidobranchia (Fig. 23). These results contrast with previous phylogenetic and phylogenomic analyses; acquisition of coloniality was inferred to occur only once (Zeng et al. 2006; Tsagkogeorga et al. 2009) or at least seven times independently (Pérez-Portela et al. 2009) based on 18S and COI, and at least twice based on transcriptome data (Alié et al. 2018). In addition, the phylogenomic tree, with high support values, supported the view that *Symplegma* would be an intermediate group between other polyzoines and botryllines, an idea earlier proposed based on morphological and immunological evidence (Shirae et al. 1999; Gutierrez and Brown 2017). These results indicate that part of descendants from the last common ancestor of the ascidians that undergo peribranchial budding sequentially acquired vascular budding and common cloacal cavity. The result that the genus *Syncarpa* did not form a clade with other colonial ascidians suggests the potential discovery of a new budding mode or convergent evolution of peribranchial/basal budding in the future; the budding mode has not been recorded in *Syncarpa* (cf. Redikorzev 1913; Tokioka 1951; Sanamyan 2000; Hasegawa and Kajihara 2019).

In the evolution of coloniality within Stolidobranchia, once traits related to coloniality were acquired, it seemed hard to lose them. The phylogenetic tree did not depict evolutionary pathways reverting from colonial forms back to solitary (Fig. 23); in addition, each budding mode and common cloacal cavity have never been lost after they were acquired. This tendency can also be observed broadly within Tunicata, with some species within the diazonid genus *Rhopalaea* being hypothesized to have undergone an evolutionary loss of budding (Alié et al. 2021). Likewise, very few species in Bryozoa have secondarily acquired solitary forms (Schwaha et al. 2020); no example of reversion from coloniality to solitary in hemichordates (cf. Cannon et al. 2013) and kamptozoans (cf. Fuchs et al. 2010). In contrast, reversal evolution from colonial to solitary forms (and vice versa) is not uncommon in cnidarians (e.g., McFadden et al. 2021).

Conclusions

In the systematic studies, the taxonomic status of the two species including the first record from the north-west Pacific were confirmed by being complemented their morphological information, as well as one new species was described. The regions targeted by this study were included the deep-sea area and Ryukyu Islands where the ascidian fauna have been less investigated in Japan. The knowledge given by this study, however, was a few for clarifying Japanese ascidian fauna. Further systematic study of Japanese ascidians is needed.

In the evolutionary study, the evolutionary direction of the zooid miniaturization was detected in the evolutionary process in Stolidobranchia. This is just a step forward in making clear the evolutionary process of the zooid miniaturization in colonial invertebrates. It is hoped that more progress will be made in evolutionary studies of body size in colonial organisms.

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Figures and Tables

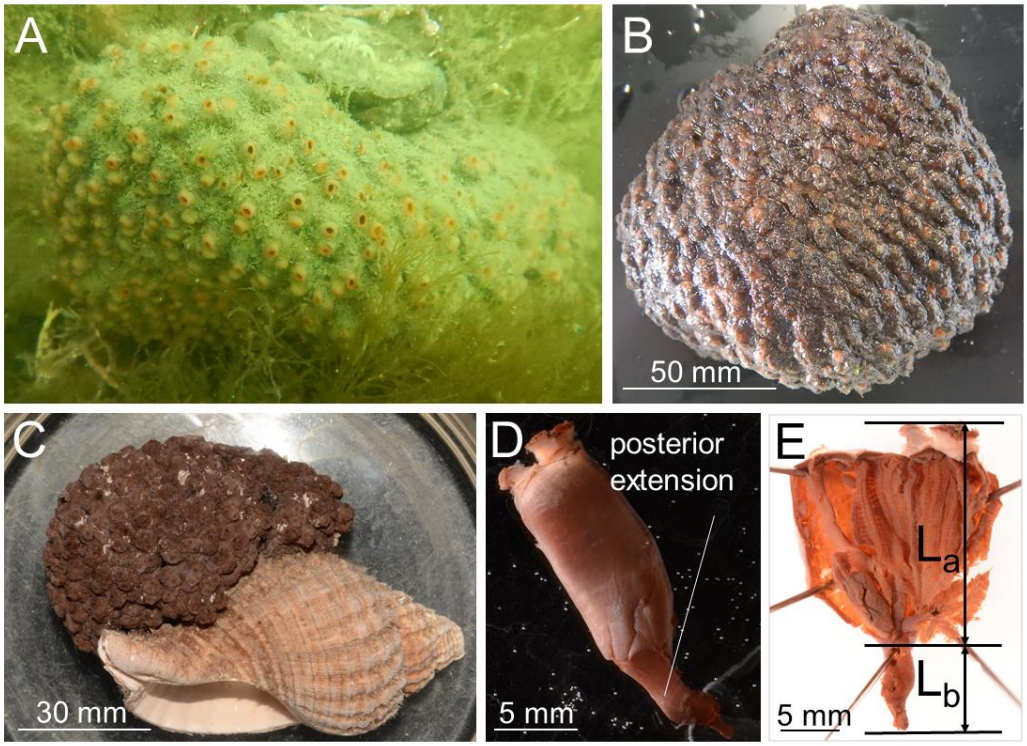

Figure 1. *Syncarpa composita* (Tokioka, 1951). A, B, D, E, ICHUM 5817; C, SMBL 104 (syntype). A, live colony; B, intact colony; C, preserved colony; D, intact zooid; E, zooid showing length from top of siphon to end of stomach (L_a) and from end of stomach to posterior end of zooid (L_b).

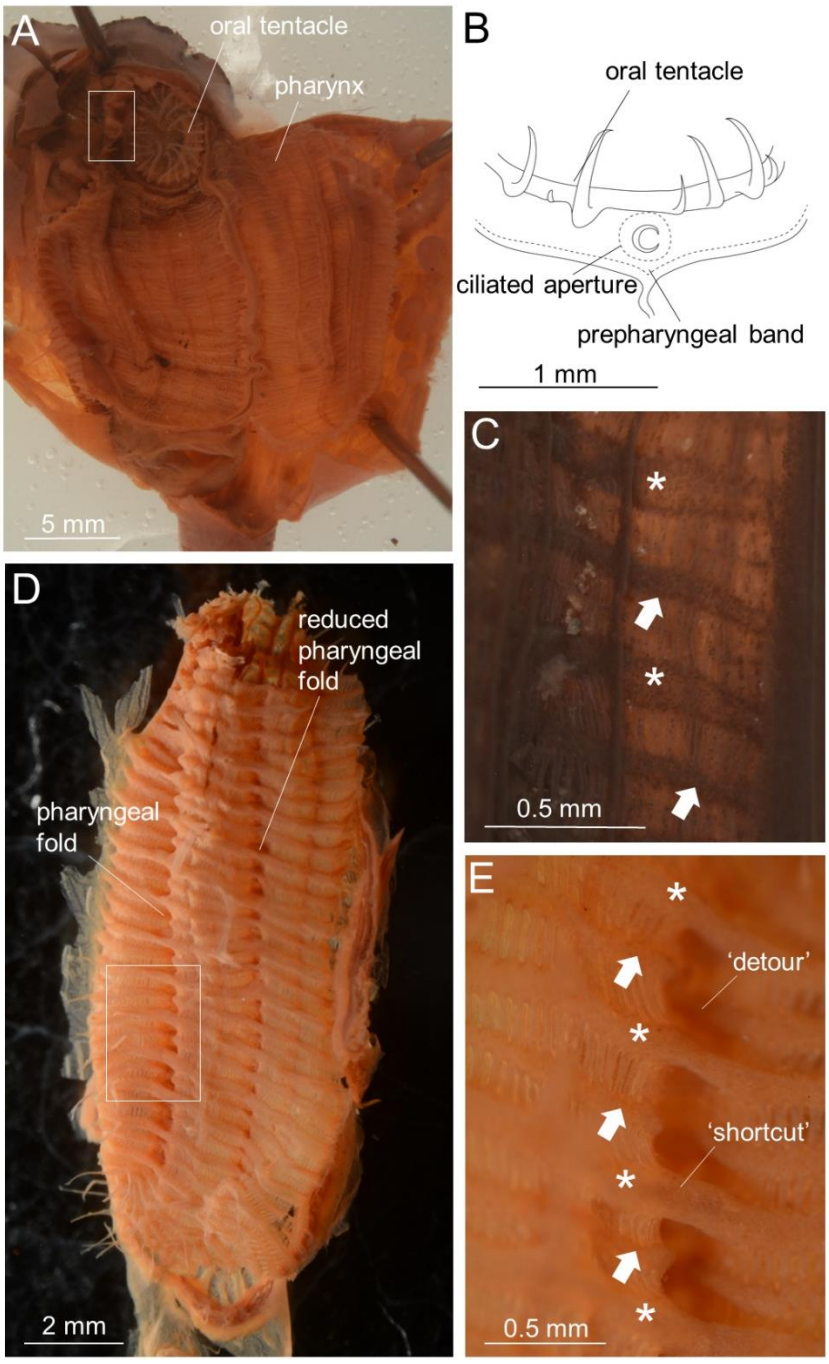

Figure 2. *Syncarpa composita* (Tokioka, 1951). A, B, D, E, ICHUM 5817; C, SMBL 104. A, zooid opened dorsally; B, ciliated groove (rotated 90 degrees anti-clockwise and enlarged view of the white square of A); C, magnification of inner surface of pharynx, showing large (indicated by an asterisk) and small (indicated by an arrow) transverse vessels; D**,** outer surface of pharynx, viewed from right side; E, magnification of white square in D, showing 'shortcut' of large transverse vessel (asterisk) above pharyngeal fold and 'detour' of small transverse vessel (arrowed) along pharyngeal fold.

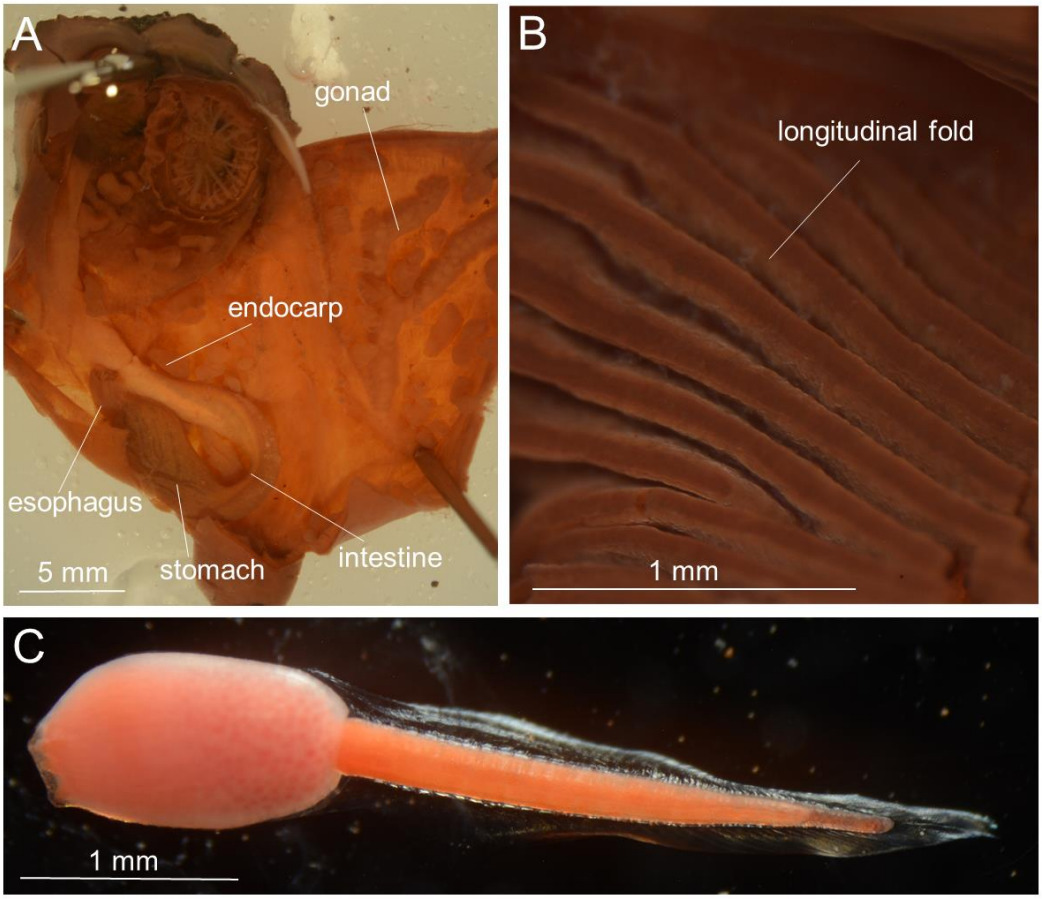

Figure 3. *Syncarpa composita* (Tokioka, 1951). A, B, ICHUM 5817; C, ICHUM 5824. A, zooid opened dorsally, with pharynx removed; B, stomach internal surface; C, tadpole larva.

Figure 4. *Syncarpa composita* (Tokioka, 1951), ICHUM 5824, cross section of a tadpole larva, showing photolith.

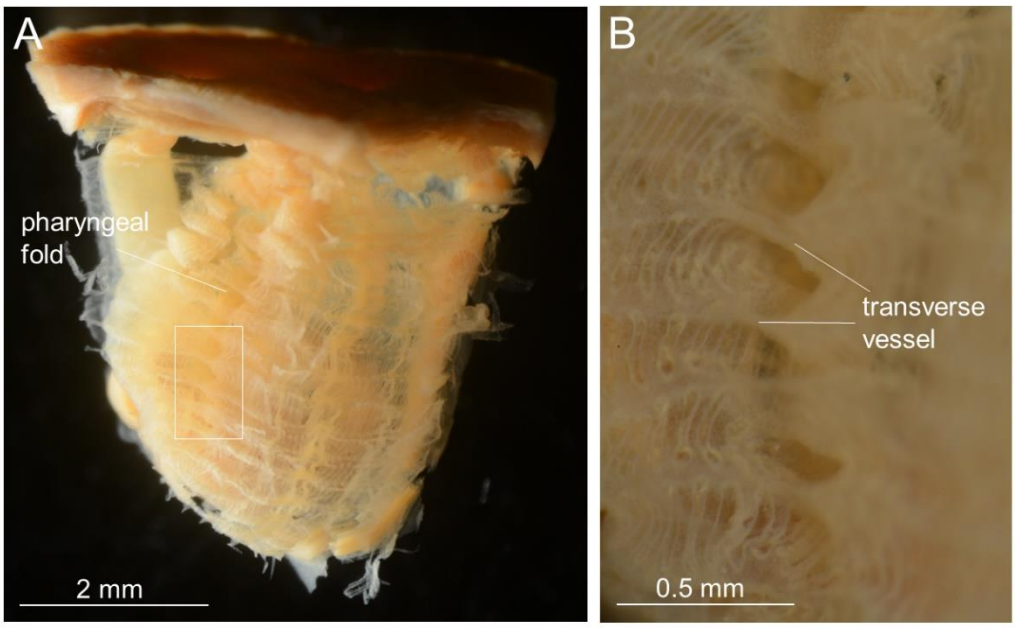

Figure 5. *Syncarpa oviformis* Redikorzev, 1913, ZIRAS 508-911 (syntype). A, outer surface of pharynx, viewed from right side; B, magnification of white square in A, showing that all transverse vessels make ‘shortcuts’ and bridge across the pharyngeal fold.

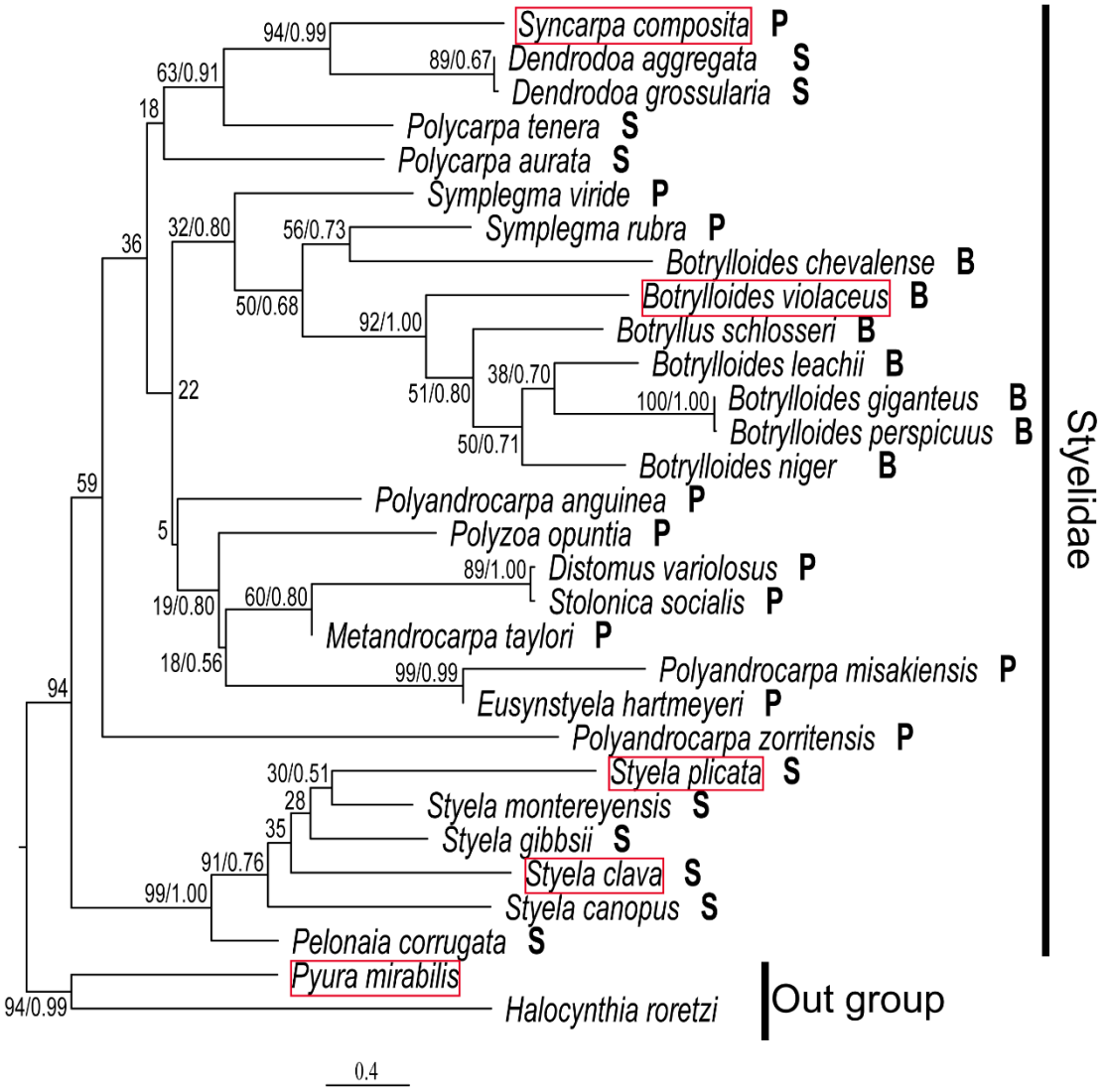

Figure 6. Maximum-likelihood tree for 28 styelid ascidians based on the concatenated sequences of 18S (1582 bp) and COI (686 bp). Numbers on branches indicate bootstrap values and, where applicable, posterior probabilities. Scale bar indicates number of substitutions per site. B, P, and S represented Botryllinae, Polyzoinae, and Styelinae. Red boxes showed the OTUs that I generated the sequences.

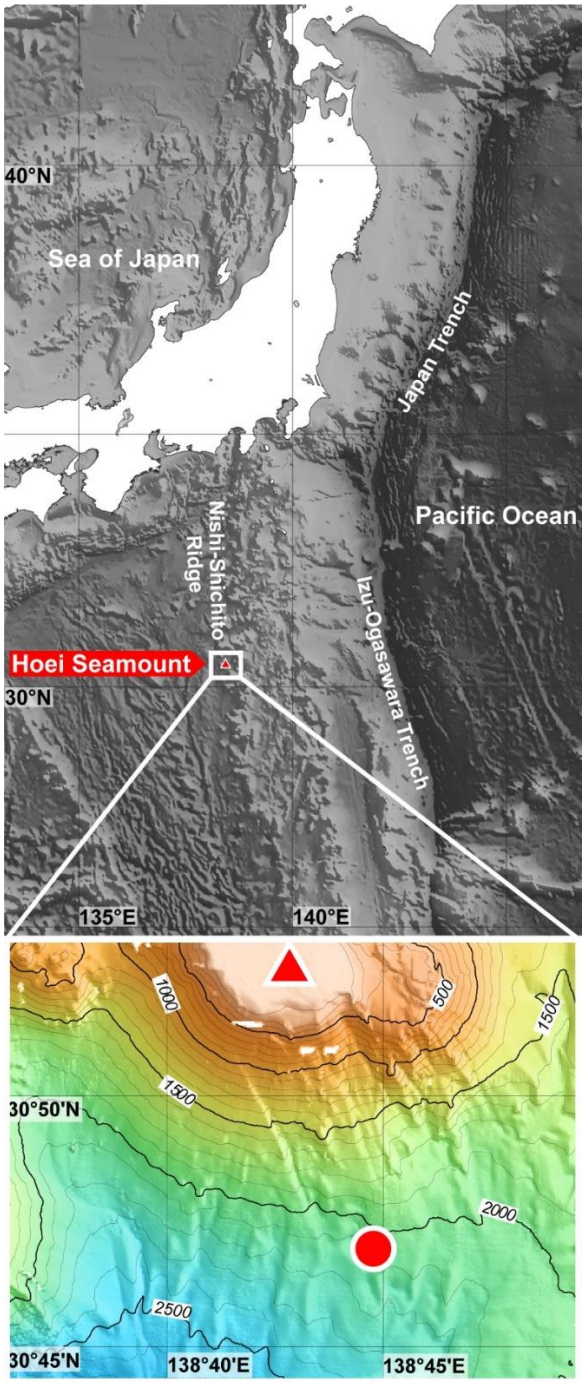

Figure 7. Maps showing the sampling site (red circle), south of Houei Seamount (of which the top is indicated with a red triangle). The images were generated by using GMT 6 (Wessel et al. 2019) based on grid data provided by the General Bathymetric Chart of the Oceans.

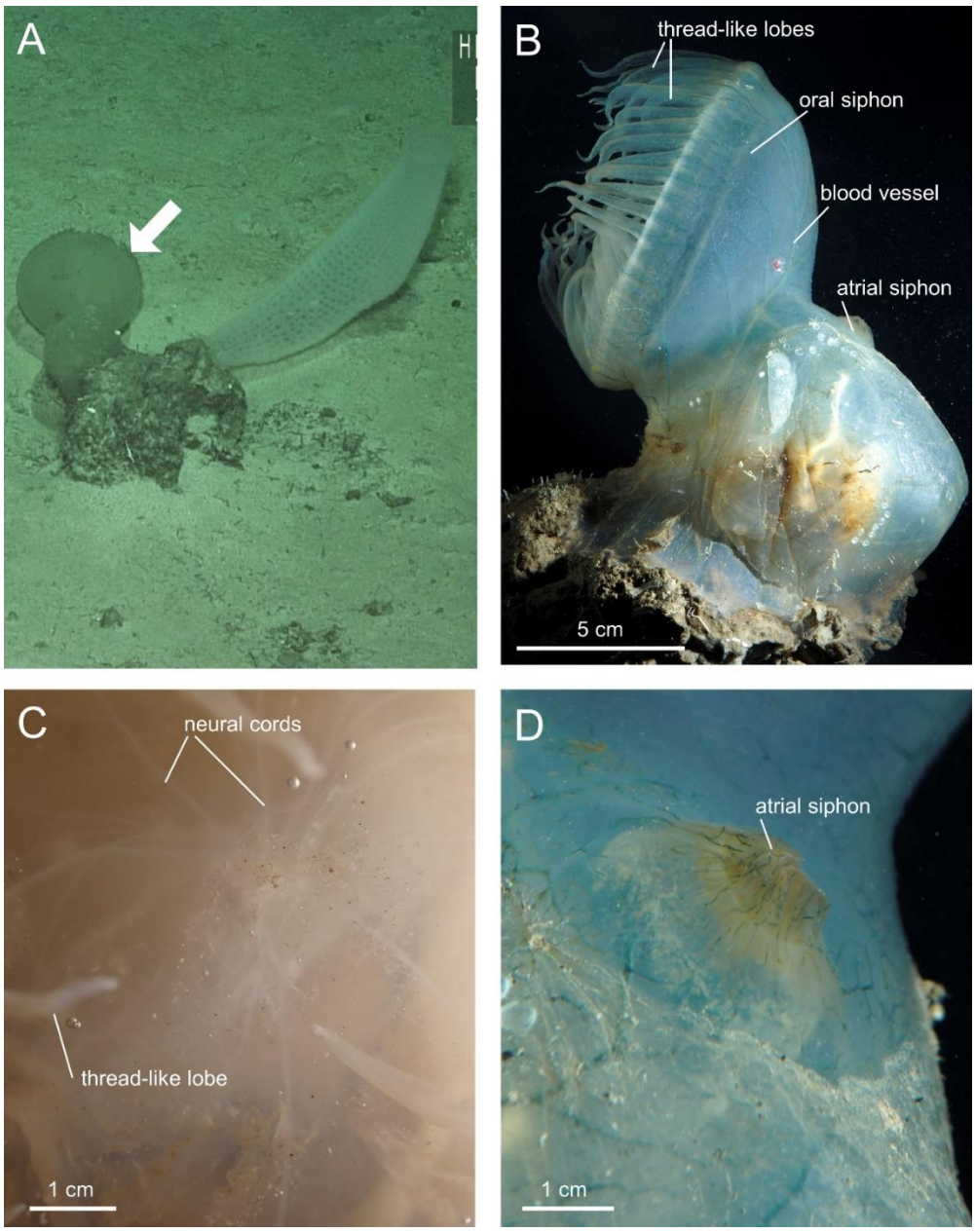

Figure 8. *Fimbrora calsubia* Monniot and Monniot, 1991a, photographs showing external appearance of JAMSTEC No. 111618. A, the individual *in situ* (indicated with an arrow), attaching to a dead sponge along with a euplectellid glass sponge; B, left view in life; C, inner surface of the oral siphon in fixed state; D, enlarged view of atrial siphon in life.

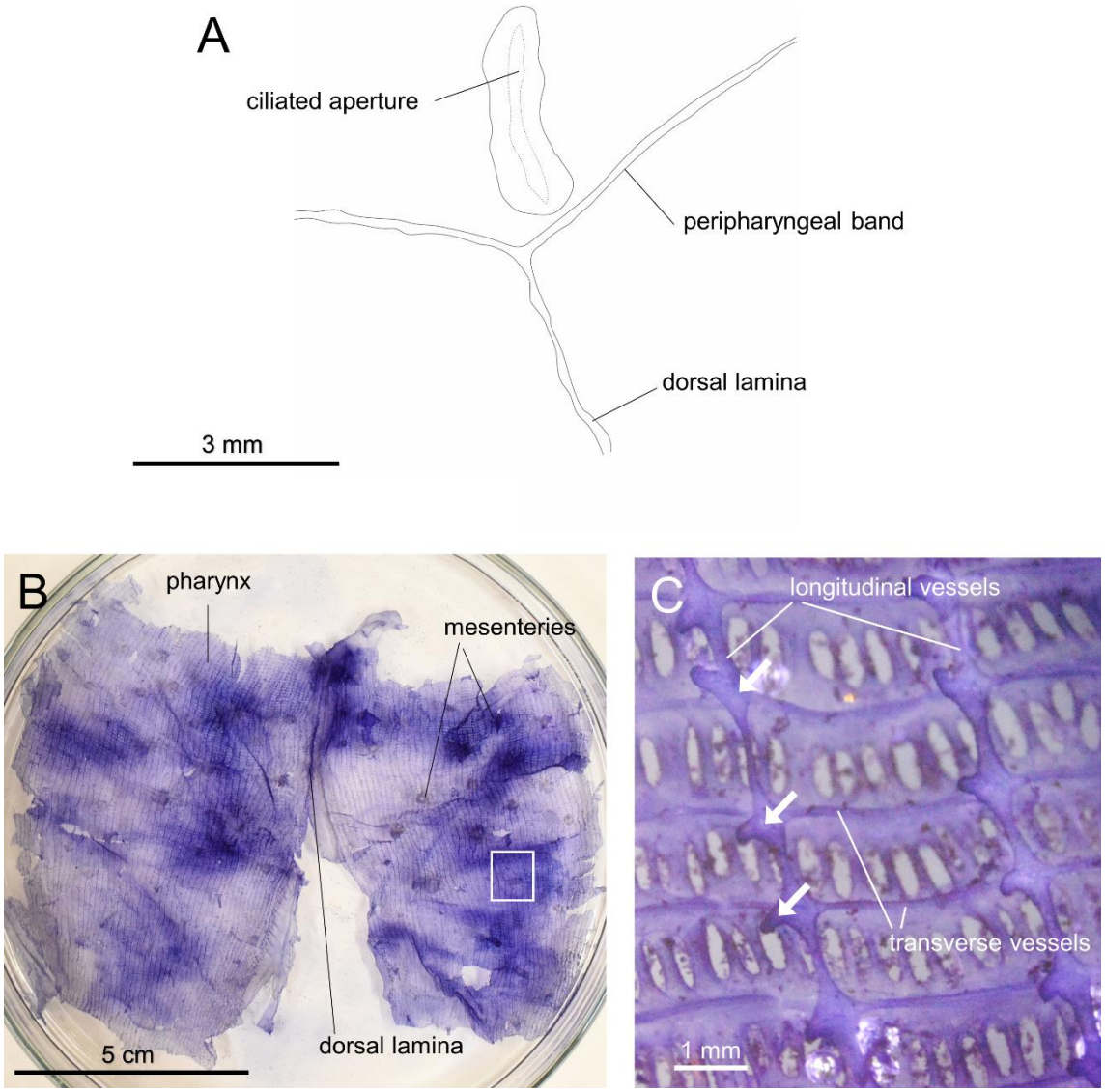

Figure 9. *Fimbrora calsubia* Monniot and Monniot, 1991a (JAMSTEC No. 111618). A, drawing of dissected specimen, showing the shape of neural-gland ciliated aperture, peripharyngeal band, and dorsal lamina; B, photograph of dissected pharynx cut opened from ventral side; C, magnification of the rectangle on B, showing the arrangement of longitudinal vessels, transverse vessels, stigmata, and secondary branchial papillae (indicated with arrows).

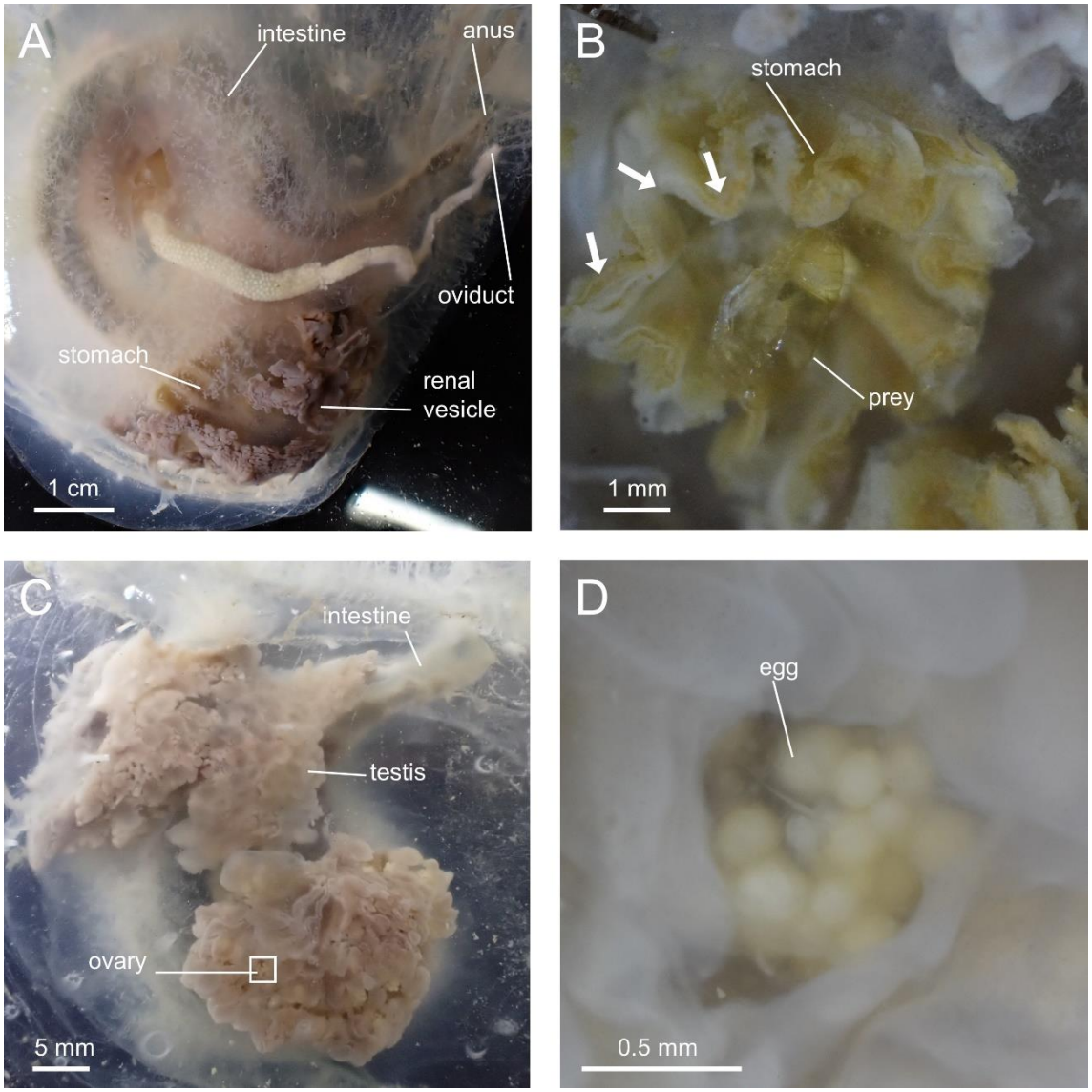

Figure 10. *Fimbrora calsubia* Monniot and Monniot, 1991a (JAMSTEC No. 111618), photographs of fixed specimen. A, sinistero-posterior portion of body, viewed from outside, showing alimentary canal and reproductive system; B, cross section of stomach, showing the prey crustacean (probably a copepod); arrows indicating stomach folds; C, gonads; D, magnification of the rectangle on C, showing an ovary containing multiple eggs.

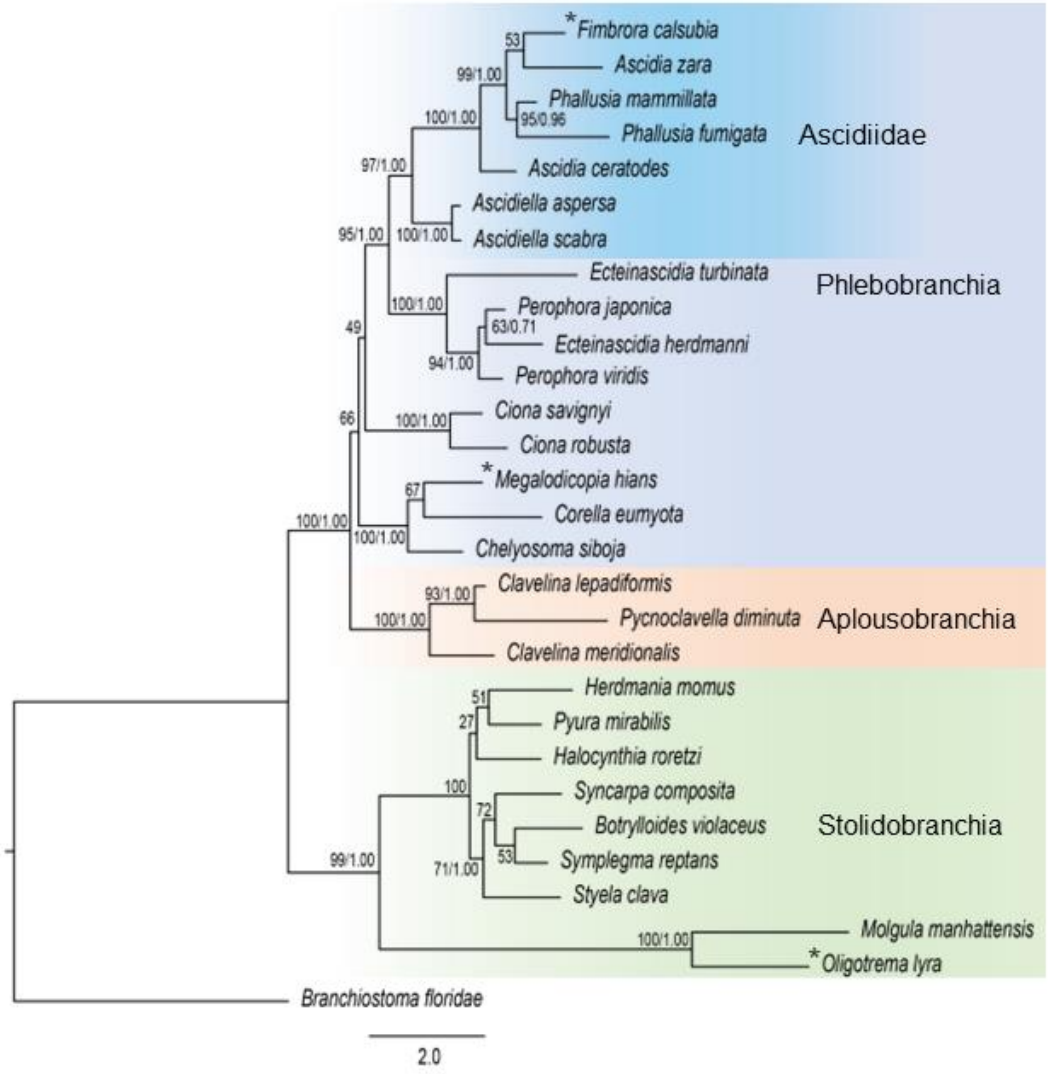

Figure 11. Maximum-likelihood tree for 28 ascidian species based on the concatenated sequences consisting of 18S rRNA (1676 bp) and COI (1136 bp) genes. Bootstrap values are indicated on each branch. Posterior probabilities are indicated if they are higher than 0.70. Carnivorous species are indicated with an asterisk (*).

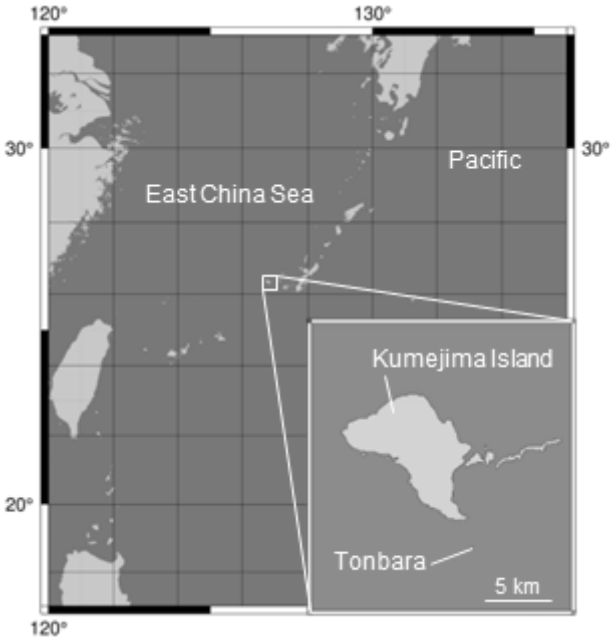

Figure 12. Map showing a sampling locality, Tonbara, off Kumejima Island, Japan.

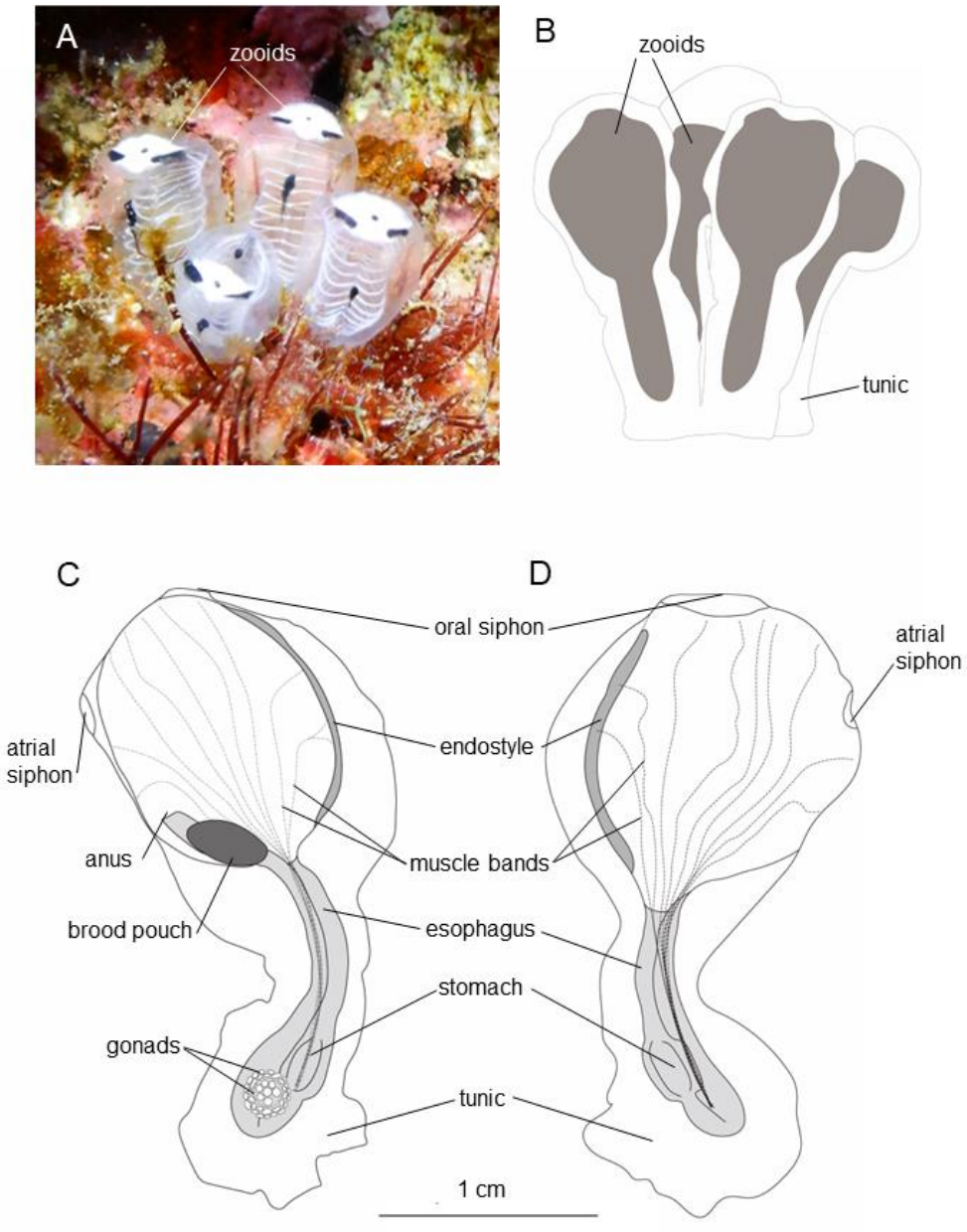

Figure 13. *Clavelina ossipandae* sp. nov., holotype, ICHUM 5837. A, *in situ* live colony with four zooids; B, a schematic depiction of the colony showing colony organization and zooid insertion; C, D, a single zooid detached from the colony, drawn from the right side (C) and the left side (D).

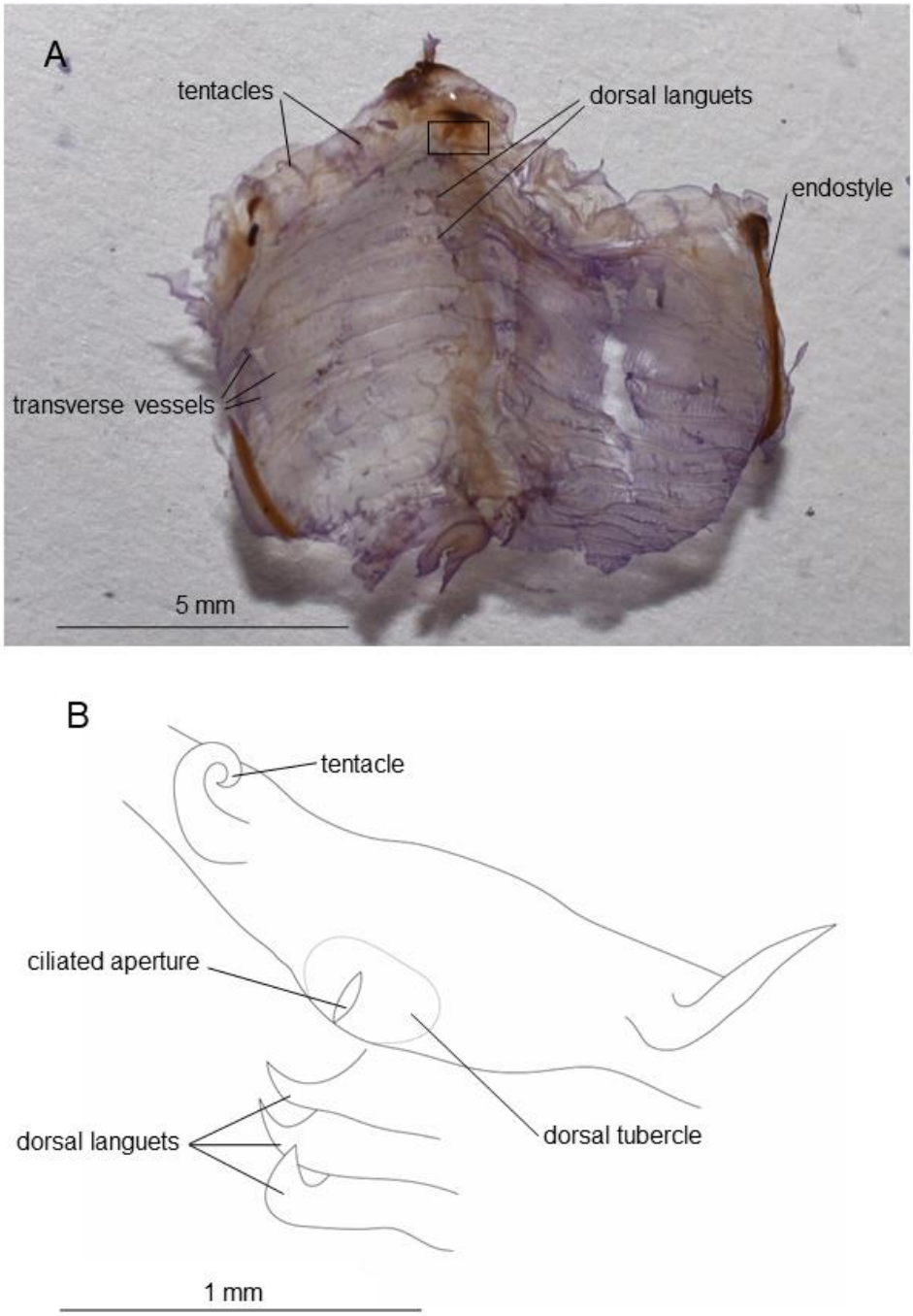

Figure 14. *Clavelina ossipandae* sp. nov., holotype, ICHUM 5837. A, pharynx opened ventrally; B, enlarged view of a square in A, showing dorsal part of the peripharyngeal area.

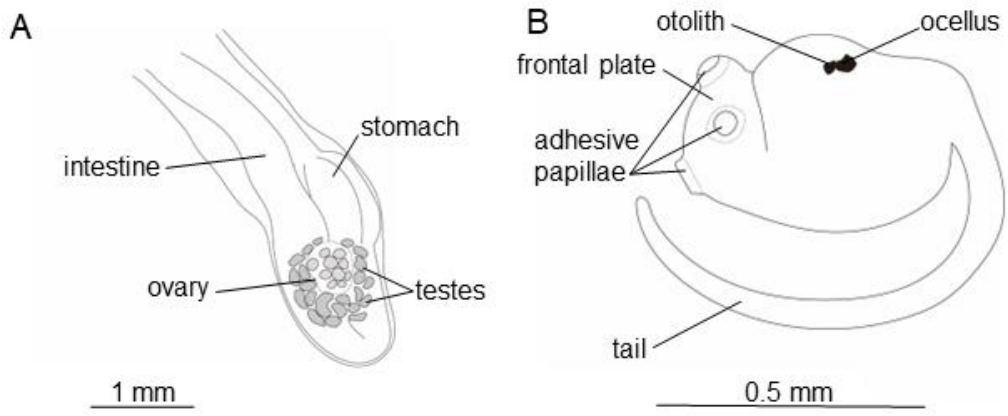

Figure 15. *Clavelina ossipandae* sp. nov., holotype, ICHUM 5837. A, intestinal loop and gonads, viewed from the right side; B, larva.

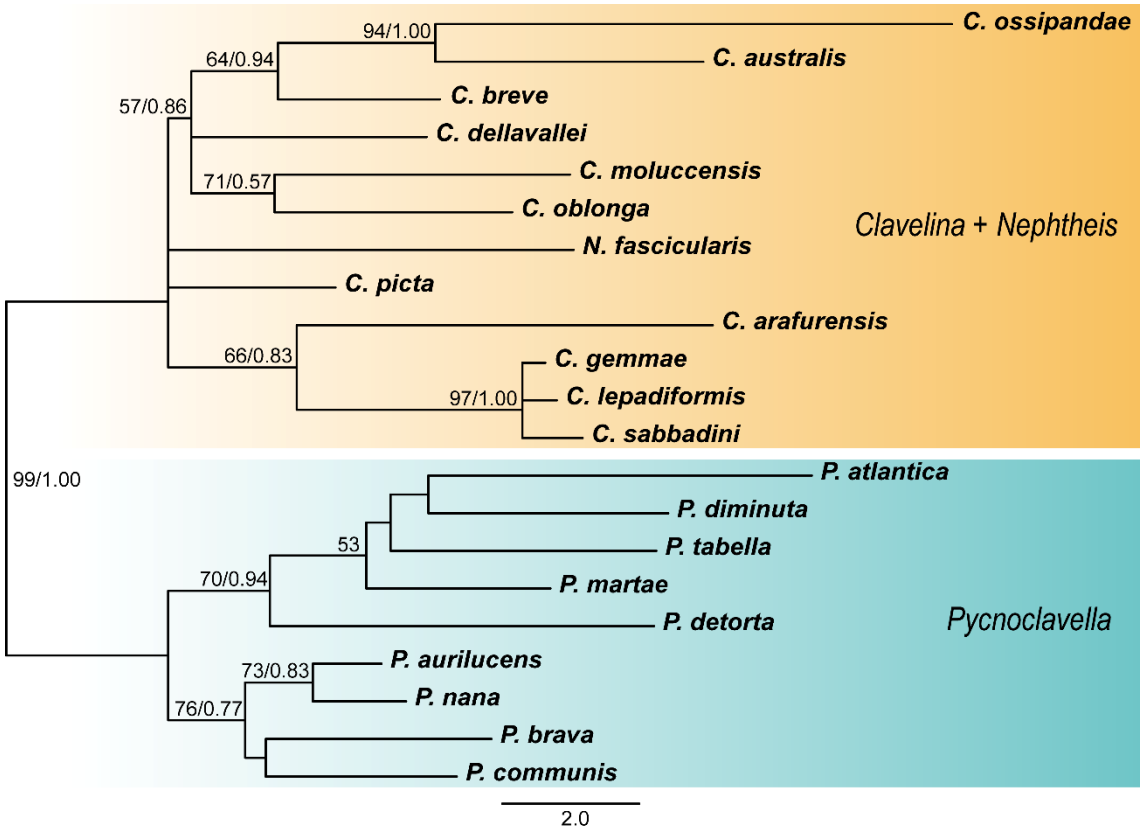

Figure 16. Maximum-likelihood tree based on COI sequences (690 bp), showing the phylogenetic position of *Clavelina ossipandae* sp. nov. among Clavelinidae. Numbers on branches indicate UF bootstrap values (≥ 50) and posterior probabilities (≥ 0.50).

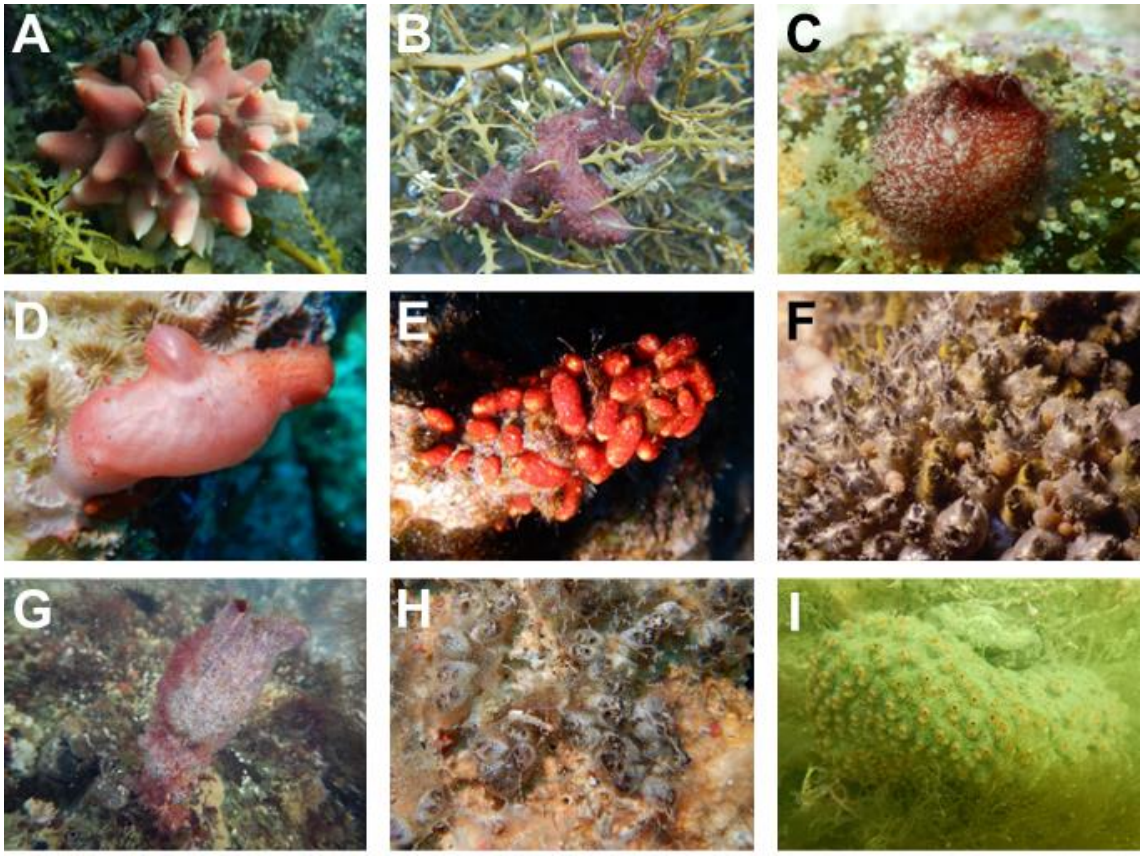

Figure 17. Solitary and colonial ascidians in Stolidobranchia. A, *Halocynthia roretzi*; B, *Botrylloides violaceus*; C, *Cnemidocarpa clara*; D, *Cnemidocarpa margaritifera*; E, *Eusynstyela latericius*; F, *Polyandrocarpa zorritensis* (photographed by Dr. Nishikawa); G, *Styela clava*; H; *Symplegma systematica*; I, *Syncarpa composita*.

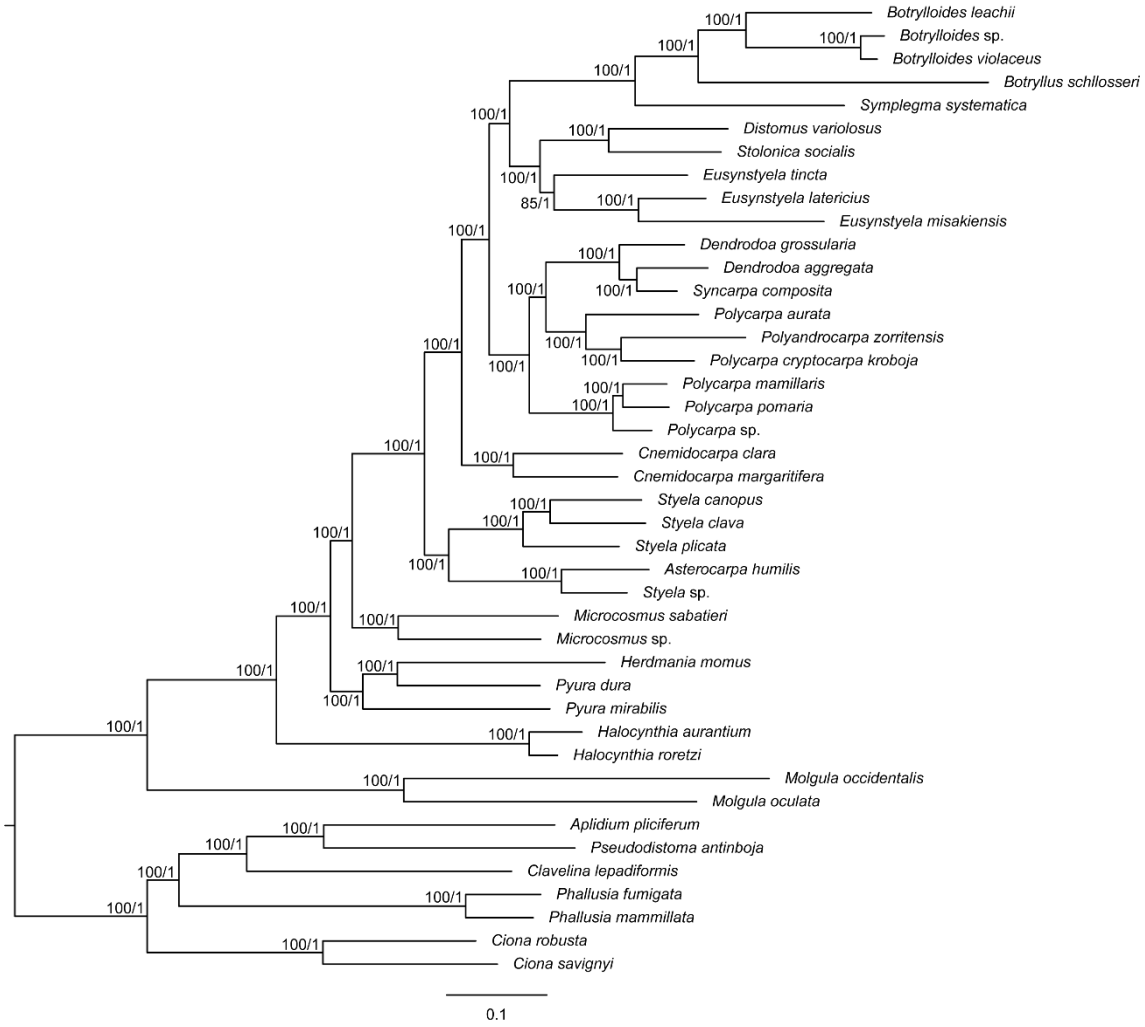

Figure 18. Maximum-likelihood tree based on 1,883 genes; the number on each branch indicates UF bootstrap value and PP.

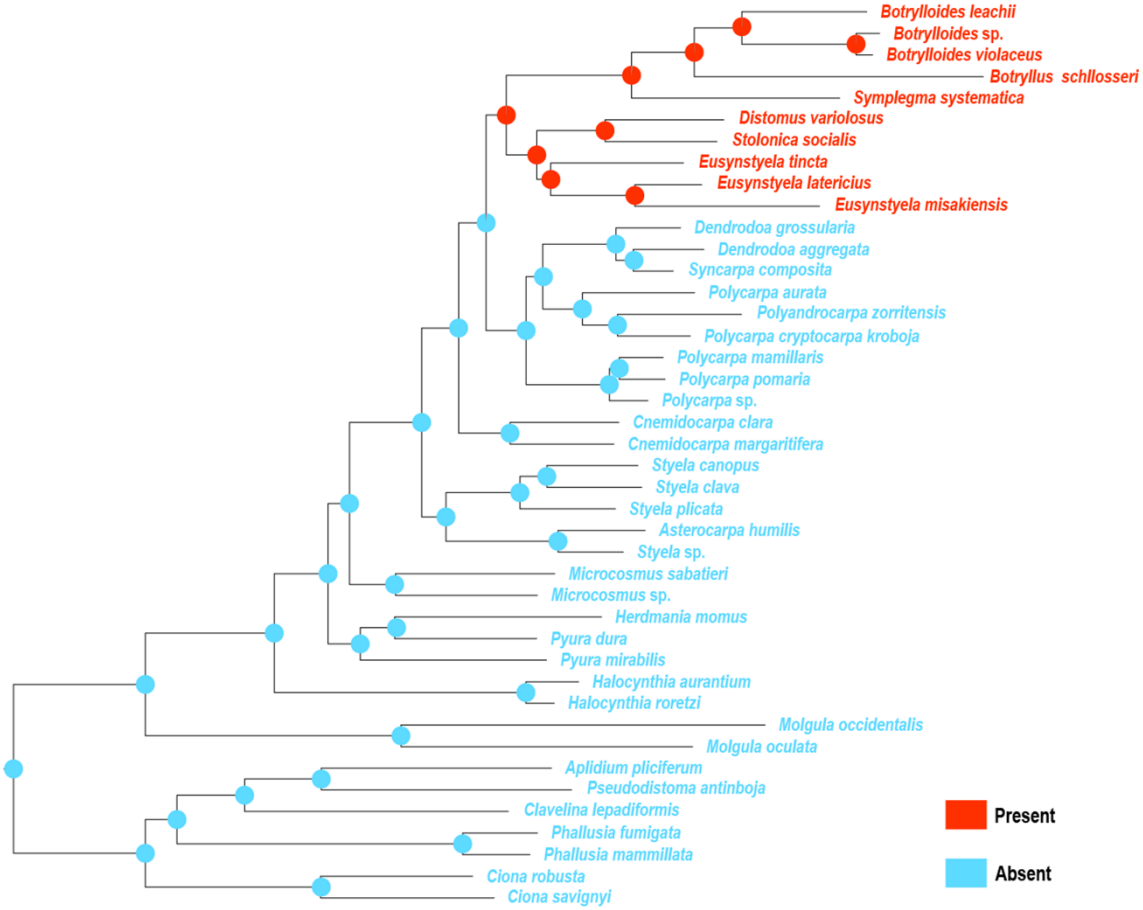

Figure 19. ML tree showing the presence of peribranchial budding. The pie charts indicate EAPs of the characteristics in the nodes.

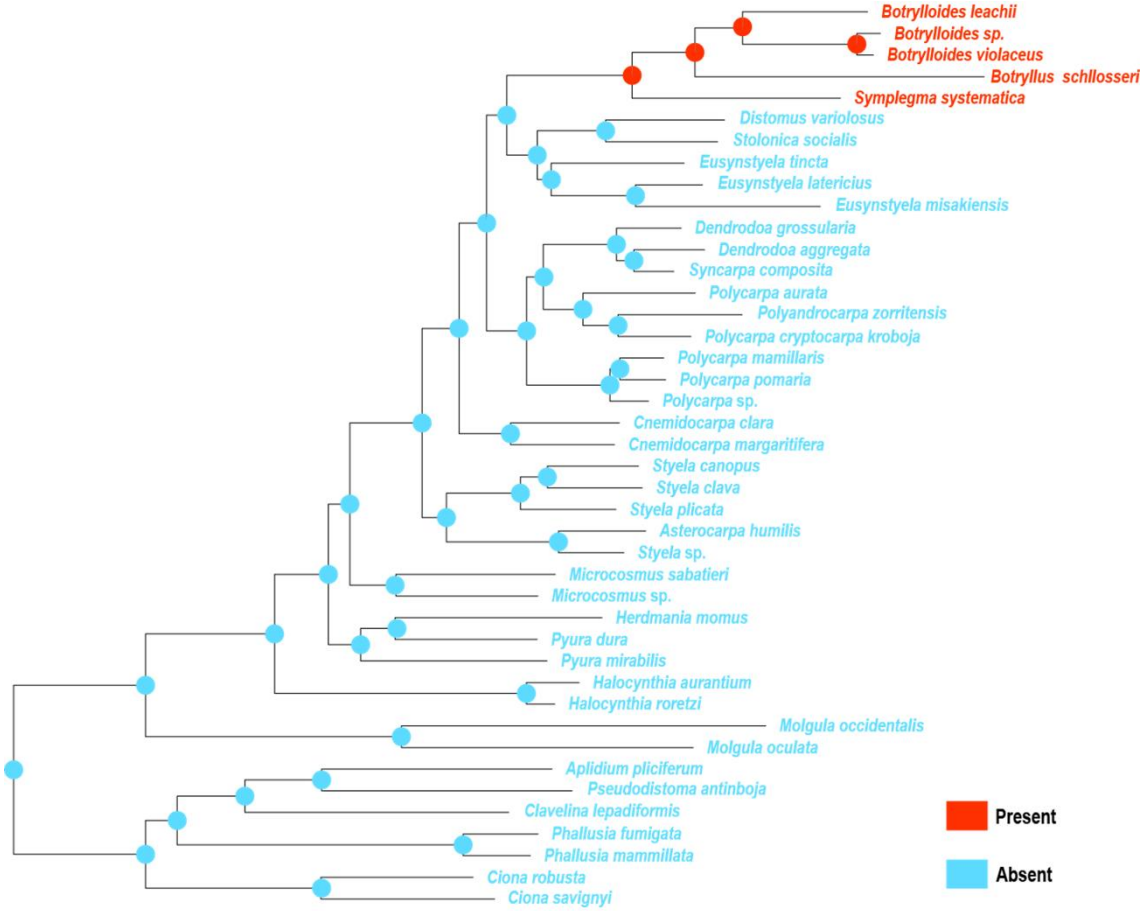

Figure 20. ML tree showing the presence of vascular budding. The pie charts indicate EAPs of the characteristics in the nodes.

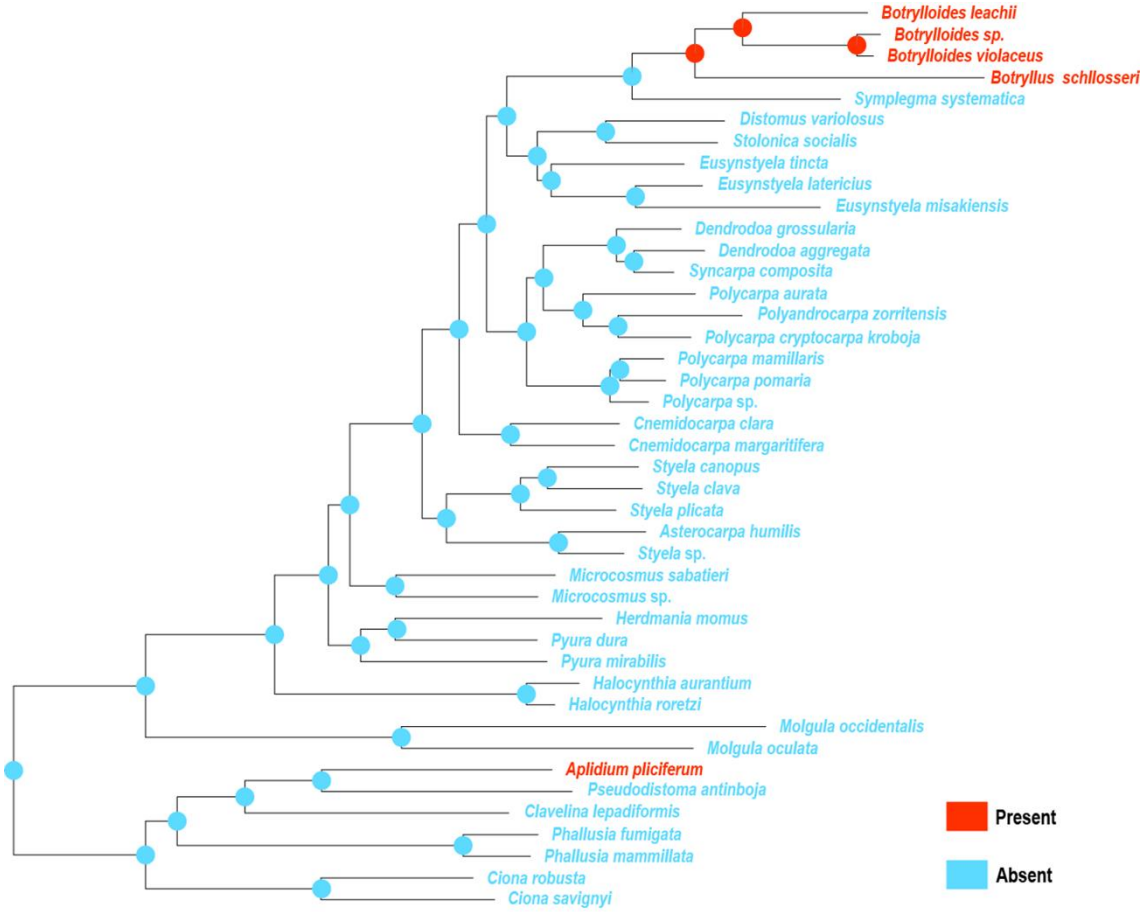

Figure 21. ML tree showing the presence of common cloacal cavity. The pie charts indicate EAPs of the characteristics in the nodes.

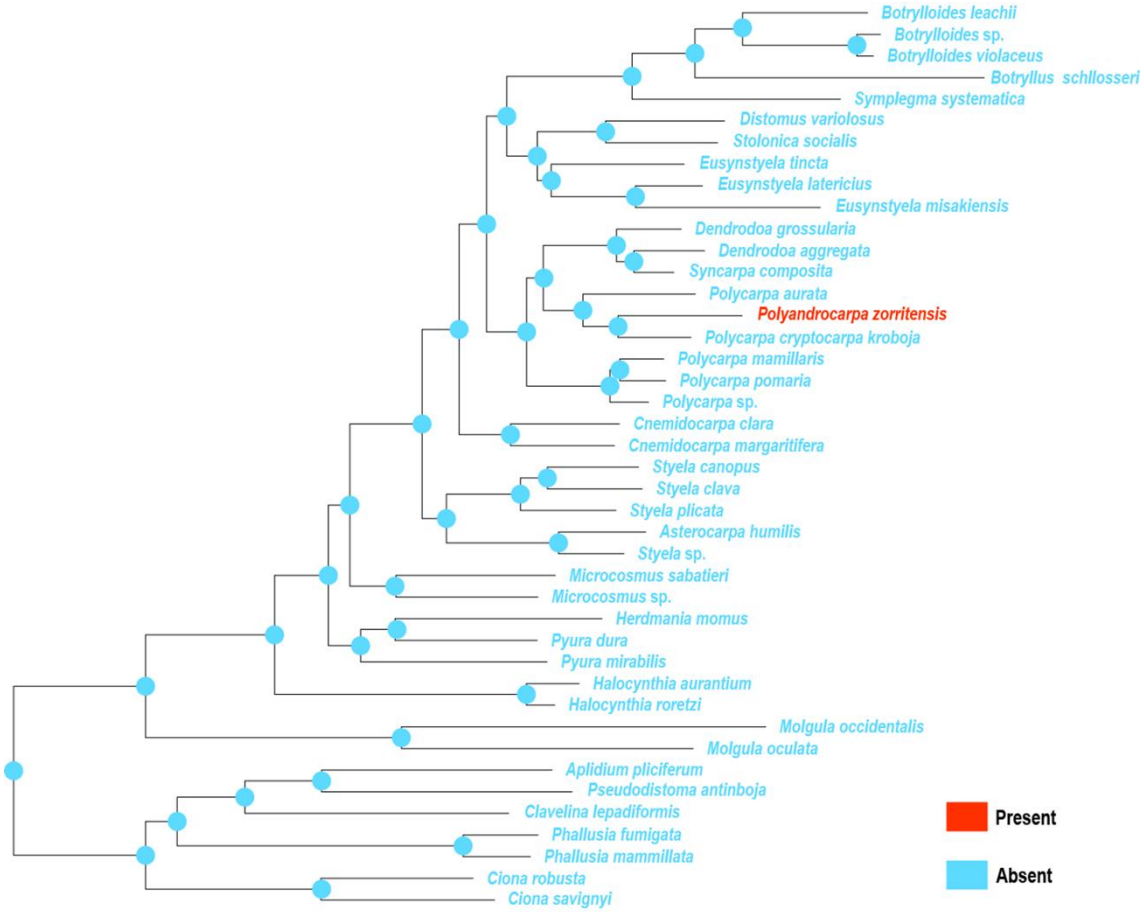

Figure 22. ML tree showing the presence of basal budding. The pie charts indicate EAPs of the characteristics in the nodes.

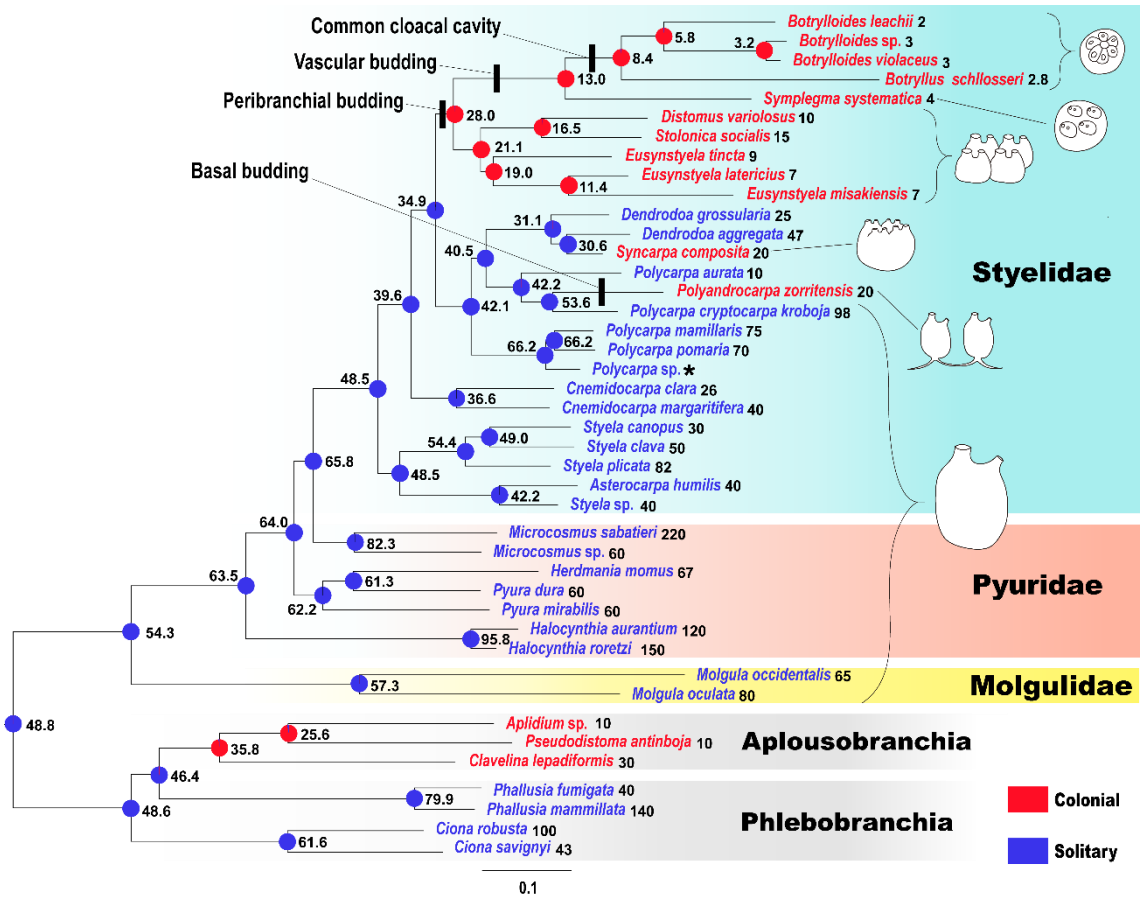

Figure 23. Evolutionary changes of coloniality and body length inferred by ancestral state reconstruction. Pie charts indicate EAPs that an ancestor at each node was colonial/solitary. The numbers show body length of ancestors and OTU; the body length of *Polycarpa* sp. was unknown, so it was indicated by asterisk (*).

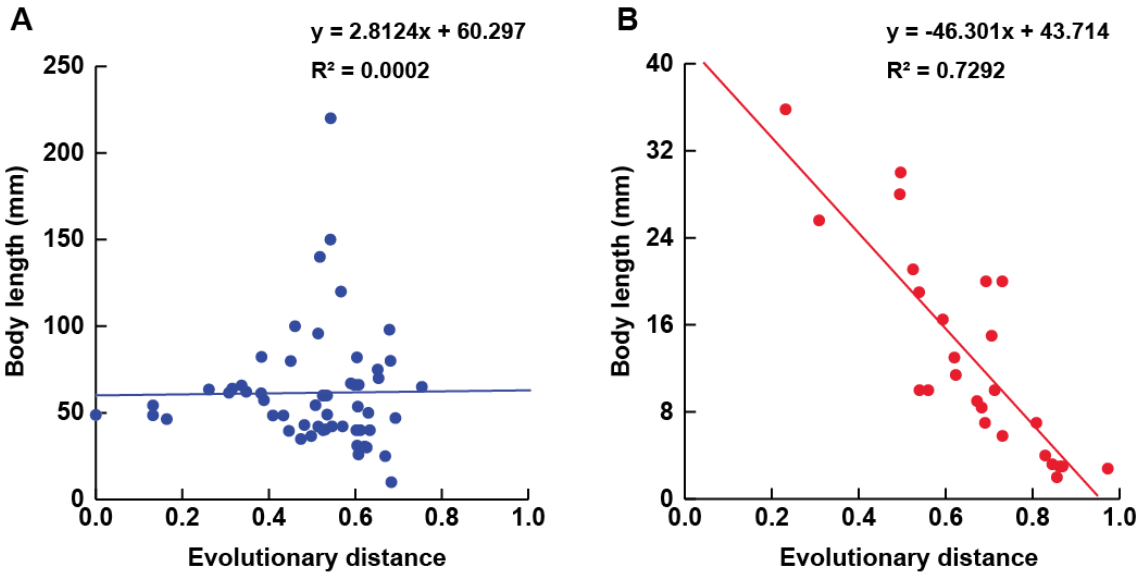

Figure 24. Relationship between body length and evolutionary distance in the OTUs and the nodes of the phylogenomic tree. A, solitary forms (n = 56); B, colonial forms (n = 25).

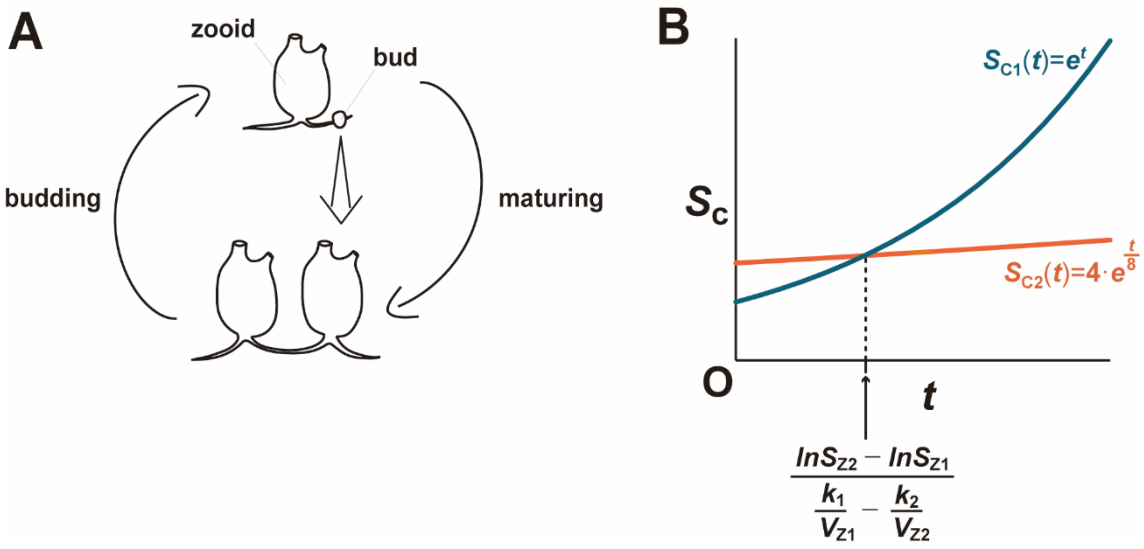

Figure 25. Illustrations of the relationship between zooid size and growth rate of a colony. A, asexual cycle of a budding colony; B, a graph representing two different colonies having similar geometrical structure (S_Z = 2 or 4; V_Z = 1 or 8; k = 1).

Table 1. List of specimens newly collected in this study with species, family, sampling date, sampling site, GenBank accession numbers and numbers of base pairs for 18S and COI sequences included in the analysis, and catalog numbers.

Species	Family	Sampling date	Sampling site	GenBank accession number		Number of base pairs		Catalog number
				18S	COI	18S	COI	
Botrylloides violaceus	Styelidae	30 March 2017	Oshoro Bay	LC432326	LC432331	1702	656	ICHUM 5826
Styela clava	Styelidae	26 August 2017	Shukutsu	LC432329	LC432334	1714	658	ICHUM 5827
Styela plicata	Styelidae	10 July 2017	Moroiso Bay	LC432328	LC432333	1711	657	ICHUM 5828
Syncarpa composita	Styelidae	25 June 2017	Akkeshi Bay	—	—	—	—	ICHUM 5815
Syncarpa composita	Styelidae	25 June 2017	Akkeshi Bay	—	—	—	—	ICHUM 5816
Syncarpa composita	Styelidae	2 August 2017	Akkeshi Bay	LC432325	LC432330	1716	435	ICHUM 5817
Syncarpa composita	Styelidae	7 September 2017	Akkeshi Bay	—	—	—	—	ICHUM 5818
Syncarpa composita	Styelidae	7 September 2017	Akkeshi Bay	—	—	—	—	ICHUM 5819
Syncarpa composita	Styelidae	7 September 2017	Akkeshi Bay	—	—	—	—	ICHUM 5820
Syncarpa composita	Styelidae	7 September 2017	Akkeshi Bay	—	—	—	—	ICHUM 5821
Syncarpa composita	Styelidae	7 September 2017	Akkeshi Bay	—	—	—	—	ICHUM 5822
Syncarpa composita	Styelidae	7 September 2017	Akkeshi Bay	—	—	—	—	ICHUM 5823
Syncarpa composita	Styelidae	13 July 2018	Akkeshi Bay	—	—	—	—	ICHUM 5824
Syncarpa composita	Styelidae	13 July 2018	Akkeshi Bay	—	—	—	—	ICHUM 5825
Pyura mirabilis	Pyuridae	21 June 2017	Oshoro Bay	LC432327	LC432332	1728	658	ICHUM 5829

Table 2. List of species obtained from GenBank included in the phylogenetic analysis with accession numbers for 18S and COI sequences.

Species	Family	GenBank accession number	
		18S	COI
Botrylloides chevalense	Styelidae	—	KX650764
Botrylloides giganteus	Styelidae	—	HF922627
Botrylloides leachii	Styelidae	MG009583	KY235402
Botrylloides niger	Styelidae	—	KP254541
Botrylloides perspicuus	Styelidae	—	KY235404
Botryllus schlosseri	Styelidae	FM244858	AY600987
Dendrodoa aggregata	Styelidae	AJ250774	—
Dendrodoa grossularia	Styelidae	L12416	FJ528650
Distoma variolosus	Styelidae	FM897308	FJ528652
Eusynstyela hartmeyeri	Styelidae	FM897309	—
Metandrocarpa taylori	Styelidae	AY903922	—
Pelonaia corrugata	Styelidae	L12440	—
Polyandrocarpa anguinea	Styelidae	—	KY111428
Polyandrocarpa misakiensis	Styelidae	AF165825	—
Polyandrocarpa zorritensis	Styelidae	FM897311	KX138505
Polycarpa aurata	Styelidae	FM897312	FJ528646
Polycarpa tenera	Styelidae	FM897313	FJ528655
Polyzoa opuntia	Styelidae	FM897314	FJ528647
Stolonica socialis	Styelidae	FM897317	—
Styela canopus	Styelidae	—	KU905887
Styela gibbsii	Styelidae	AY903923	HQ916447
Styela montereyensis	Styelidae	L12443	FJ528638
Symplegma rubra	Styelidae	FM897315	FJ528648
Symplegma viride	Styelidae	DQ346655	—
Halocynthia roretzi	Pyuridae	AB013016	AB024528

Table 3. Comparison of the posterior extension length and the ratios of L_a to L_b. Each zooid from two colonies of SMBL 104 was measured.

L_b / L_a	L_a (mm)	L_b (mm)	Catalog number
0.33	9	3	ICHUM 5817
0.36	11	4	ICHUM 5821
0.47	15	7	SMBL 104
0.5	12	6	ICHUM 5817
0.5	14	7	ICHUM 5820
0.56	9	5	ICHUM 5819
0.58	12	7	ICHUM 5821
0.64	11	7	SMBL 104
0.64	14	9	ICHUM 5818
0.79	19	15	ICHUM 5825
0.8	10	8	ICHUM 5822
0.82	11	9	ICHUM 5819
0.86	14	12	ICHUM 5818
1.05	22	23	ICHUM 5824
1.08	13	14	ICHUM 5823
1.32	19	25	ICHUM 5825
1.5	20	30	ICHUM 5824
1.54	13	20	ICHUM 5823
1.61	18	29	ICHUM 5820
1.83	12	22	ICHUM 5822

Table 4. Comparison of four species in *Syncarpa*. The number of size-classes of transverse vessels in *S. oviformis* (indicated by an asterisk*) was newly confirmed in this study. Sanamyan (2000) regarded that *S. corticiformis* and *S. longicaudata* were junior synonyms of *S. oviformis*.

Character	Species					
	S. composita		*S. corticiformis*	*S. longicaudata*	*S. oviformis*	
Source	Tokioka (1951)	present study	Beniaminson (1975)	Skalkin (1957)	Redikorzev (1913)	Sanamyan (2000)
Zooid length (mm)	12	12–50	15	40	10	10–30
Zooid width (mm)	8	8	5	7.5	4	4–8
Posterior extension of zooid long (+) or short (–)	–	– / +	–	+	–	–
Number of oral tentacles	30	30–35	20	30–35	20–25	20–25
Number of size-classes of transverse vessels	?	2	1	2	1*	?
Stomach internal wall present (+) or absent (–)	?	+	+	+	+	+
Intestinal loop	?	J-shaped	J-shaped	J-shaped	J-shaped	J-shaped
Number of anal lobes	0	0	2	0	2	2
Number of gonadal branches	2–5	2–5	4	3	2	2–4
Locality	Akkeshi Bay	Akkeshi Bay	Kunashiri Island	South Kuril Islands	Ul’banskij Bay	Sea of Okhotsk

Table 5. List of carnivorous species in Ascidiacea with information about family, species, depth, evidence for carnivorousness, and references.

Family	Species	Depth (m)	Evidence for carnivorousness*	References
Ascidiidae	*Fimbrora calsubia* Monniot and Monniot, 1991	1000–2027	m/c	Monniot and Monniot (1991a), Monniot (1993), Monniot and López-Legentil (2017), present study
Octacnemidae	*Benthascidia michaelseni* Ritter, 1907	399	m	Ritter (1907), Monniot (1998)
	Cibacapsa gulosa Monniot and Monniot, 1983	567	m/c	Monniot and Monniot (1983)
	Cryptia planum Monniot and Monniot, 1985	4930	m/c	Monniot and Monniot (1985a)
	Dicopia antirrhinum Monniot, 1972a	600–4300	m/c	Monniot (1972a), Monniot and Monniot (1974, 1985a), Sanamyan (2014)
	Dicopia fimbriata Sluiter, 1905	1210	m	Sluiter (1905a), Monniot and Monniot (1991b), Monniot and Lopéz-Legentil (2017), Sanamyan and Sanamyan (1999)
	Dicopia japonica Oka, 1913	4526–4609	m	Oka (1913), Millar (1988)
	Kaikoja globosa Monniot, 1998	1978	m	Monniot (1998)
	Kaikoja multitentaculata (Vinogradova, 1975)	4485–4520	m	Vinogradova (1975), Sanamyan and Sanamyan (2002)
	Megalodicopia hians Oka, 1918	200–5325	m/c	Oka (1918), Tokioka (1953), Kott (1969), Nishikawa (1991), Sanamyan (1998), Okuyama et al. (2002), Havenhand et al. (2006)
	Megalodicopia rineharti (Monniot and Monniot, 1989)	695–3970	m	Monniot and Monniot (1989), Sanamyan and Sanamyan (2002)
	Myopegma melanesium Monniot and Monniot, 2003	445–472	m/c	Monniot and Monniot (2003)
	Myopegma midatlantica Monniot, 2011	2087	m	Monniot (2011)
	Octacnemus alatus Monniot and Monniot, 1985	3344	m	Monniot and Monniot (1985b)
	Octacnemus bythius Moseley, 1876	1957–4087	m/c	Moseley (1876), Ritter (1906), Ihle (1935), Millar (1959), Monniot and Lopéz-Legentil (2017)

	Octacnemus ingolfi Madsen, 1947	640–4655	m	Madsen (1947), Monniot and Monniot (1973, 1976, 1985a, 1985b, 1985c, 1991b, 2003), Sanamyan (2014)
	Octacnemus kottae Sanamyan and Sanamyan, 2002	3700–3910	m	Sanamyan and Sanamyan (2002)
	Octacnemus vinogradovae Sanamyan and Sanamyan, 1999	5400	m	Sanamyan and Sanamyan (1999)
	Octacnemus zarcoi Monniot and Monniot, 1984	4260–4270	m/c	Monniot and Monniot (1984a), Sanamyan (2014)
	Polyoctacnemus patagoniensis (Metcalf, 1893)	1920	m	Metcalf (1893), Ihle (1935)
	Situla cuculli Monniot and Monniot, 1991	2040	m	Monniot and Monniot (1991b)
	Situla galeata Monniot and Monniot, 1991	1395–4891	m	Monniot and Monniot (1991b), Sanamyan and Sanamyan (1998)
	Situla lanosa Monniot and Monniot, 1973	1800–4990	m	Monniot and Monniot (1973, 1974, 1985a), Sanamyan (2014)
	Situla macdonaldi Monniot and Monniot, 1977	790	m	Monniot and Monniot (1977)
	Situla pelliculosa Vinogoradova, 1969	5035–8400	m	Vinogradova (1969)
	Situla rebainsi Vinogradova, 1975	3700–5651	m	Vinogradova (1975), Sanamyan and Sanamyan (2002)
	Situla rineharti Monniot and Monniot, 1989	695–3680	m	Monniot and Monniot (1989, 1991b)
Molgulidae	*Asajirus arcticus* (Hartmeyer, 1923)	905–1283	m	Hartmeyer (1923)
	Asajirus dichotomus (Monniot and Monniot, 1984)	3550	m	Monniot and Monniot (1984a, 1985a), Kott (1989)
	Asajirus eunuchus (Monniot and Monniot, 1976)	2000–5000	m	Monniot and Monniot (1976)
	Asajirus gulosus (Monniot and Monniot, 1984)	1800–2500	m	Monniot and Monniot (1984a), Kott (1989)
	Asajirus hemisphericus (Monniot and Monniot, 1990)	3680–3740	m	Monniot and Monniot (1990)
	Asajirus indicus (Oka, 1913)	800–5000	m/c	Oka (1913), Hartmeyer (1923), VanName (1945), Millar (1959, 1970), Kott (1957a, 1969, 1989), Monniot (1969, 1971), Monniot and Monniot (1968, 1970, 1973, 1974, 1976, 1982, 1984a, b, 1985a, b, 1990), Sanamyan and Sanamyan (2006), Maggioni et al. (2018, 2022)

Asajirus ledanoisi (Monniot and Monniot, 1990)	720–4829	m	Monniot and Monniot (1973, 1974, 1977, 1985b, 1990), Sanamyan (2014)
Asajirus ovirarus (Monniot and Monniot, 1990)	820–1900	m	Monniot and Monniot (1990, 2003)
Oligotrema lyra (Monniot and Monniot, 1973)	3360–4680	m/c	Monniot and Monniot (1973, 1974, 1984b, 1985a, 1990), Kott (1989), Sanamyan and Sanamyan (1999), Sanamyan (2014)
Oligotrema psammatodes (Sluiter, 1905)	1158	m	Millar (1969), Sluiter (1905a, b), Monniot and Monniot (1990)
Oligotrema psammites Bourne, 1903	90–4000	m	Bourne (1903), Monniot and Monniot (1990), Monniot (2022), Kott (1992, 2009)
Oligotrema sandersi (Monniot and Monniot, 1968)	2200–5020	m	Monniot and Monniot (1968, 1970, 1974, 1985a, 1990), Millar (1970), Kott (1989), Sanamyan (2014)
Oligotrema unigonas (Monniot Monniot, 1974)	2300–5500	m	Monniot and Monniot (1974, 1984b, 1985a, b, 1990), Kott (1989), Sanamyan (2014)

*****‘m’ indicates that the species was judged to be carnivorous based on morphological characteristics; ‘m/c’ indicates that gut contents were also observed in addition to morphological features.

Table 6. The GenBank accession numbers of 18S and COI sequences of *Fimbrora calsubia* Monniot and Monniot, 1991a, as well as 27 ascidian species and the lancelet *Branchiostoma floridae* Hubbs, 1922, used for phylogenetic analysis in this study.

Species	18S	COI
Ascidia ceratodes	L12378	MW872268
Ascidia zara	LC547325	KY235397
Ascidiella aspersa	LC547321	KF886702
Ascidiella scabra	AB811928	MN064599
Botrylloides violaceus	LC432326	LC432331
Chelyosoma siboja	AF165821	AB104867
Ciona robusta	AB013017	MF479417
Ciona savignyi	LC547329	MK512499
Clavelina lepadiformis	JN573225	AY603104
Clavelina meridionalis	FM244840	AM706470
Corella eumyota	FM244846	KU299765
Ecteinascidia herdmanni	FM244847	AY600968
Ecteinascidia turbinata	FM244848	MT873564
Fimbrora calsubia	LC777587	LC777585
Halocynthia roretzi	AB013016	HM151268
Herdmania momus	AF165827	KM411616
Megalodicopia hians	AB075543	AB104866
Molgula manhattensis	L12426	MT873565
Oligotrema lyra	JN565043	—
Perophora japonica	AB499607	MN064600
Perophora viridis	FM244849	OM912740
Phallusia fumigata	FM244844	KF309548
Phallusia mammillata	AF236803	MN064634
Pycnoclavella diminuta	KJ632948	KC017435
Pyura mirabilis	LC432327	LC432332
Styela clava	LC432329	LC432334
Symplegma reptans	AF165826	LS992553
Syncarpa composita	LC432325	LC432330
Branchiostoma floridae	M97571	AB478593

Table 7. Comparison of 45 species in *Clavelina* including *Clavelina ossipandae* sp. nov.

Species	Type of colony[a]	Color	Greatest zooid length (mm)	Number of stigmatal rows	Muscles[b]	Stomach surface[c]	Rim of the anal opening[d]	Embryonic development[e]	Reference
C. amplexa Kott, 2002	F	creamish-yellow	20	30	E	—	L	BP	Kott (2002)
C. arafurensis Tokioka, 1952	NF	pink	16	20	E	P	L	—	Tokioka (1952), Kott (1990)
C. auracea C. Monniot, 1997	F	white	50	5	E	—	—	PBC	Monniot (1997)
C. australis (Herdman, 1899)	F	yellow	40	14–18	E	—	—	BP and PBC	Herdman (1899), Kott (1990)
C. baudinensis Kott, 1957	NF	blue, white	30	17–20	E	S	L	PBC	Kott (1957b)
C. borealis Savigny, 1816	F	—	160	35	E	S	L	PBC	Savigny (1816), Hartmeyer (1903), Monniot (2001), Brunetti and Mastrototaro (2017)
C. brasiliensis (Millar, 1977)	F	—	75	17	—	P	—	PBC	Millar (1977), Monniot (2016)
C. breve C. Monniot, 1997	F	white	8	16	L	—	L	PBC	Monniot (1997)
C. coerulea Oka, 1934	F	blue	25	16–18	L	P	S	BP	Oka (1934), Nishikawa and Tokioka (1976), Nishikawa (1995)
C. concrescens Hartmeyer, 1924	NF	—	30	—	—	—	—	—	Hartmeyer (1924), Van Name (1945)
C. cyclus Tokioka and Nishikawa, 1975	NF	blue, white	26	15–19	L	P	—	BP	Tokioka and Nishikawa (1975), Nishikawa (1995)

C. cylindrica (Quoy and Gaimard, 1834)	F	blue	20	9–13	L	—	S	BP	Quoy and Gaimard (1834), MacDonald (1858), Bronn (1862), Caullery (1908, 1909), Hartmeyer (1911), Michaelsen (1930), Kott (1957b, 1972a, 1976, 1990) Millar (1960, 1963, 1966)
C. dagysa (Kott, 1957)	F	blue	40	24–30	E	P	L	PBC	Kott (1957b, 1990)
C. dellavallei (Zirpolo, 1925)	F	white	60	21	L	S/P	S	BP	Zirpolo (1925), Salfi (1927a, b), Pérès (1956), Rasotto and Colombera (1980), Brunetti (1987), Monniot (2001), Brunetti and Mastrototaro (2017)
C. detorta (Sluiter, 1904)	F	orange	25	6	E	S	L	PBC	Sluiter (1904), Millar (1975)
C. elegans (Oka, 1927)	F	white	27	24–26	L	P	L	BP	Oka (1927), Nishikawa and Tokioka (1976)
C. enormis Herdman, 1880	F	gray	50	19	—	—	—	OV	Herdman (1880), Millar (1975), Monniot and Monniot (1976)
C. fasciculata Van Name, 1945	F	—	14	16–20	E	P	—	—	Van Name (1945)
C. fecunda (Sluiter, 1904)	F	yellow, blue	10	16–20	E	P	—	BP	Sluiter (1904), Kott (1990)
C. gemmae Turon, 2005	F	pink	13	7–10	E	P	—	PBC	Turon (2005)
C. huntsmani Van Name, 1931	F	yellow, orange	40	16–20	E	P	—	PBC	Van Name (1931, 1945)
C. kottae (Millar, 1960)	F	—	120	27	E/L	P	S	—	Millar (1960)

C. lepadiformis (Müller, 1776)	F	white, yellow, pink	33	17	E	P	S	BP	Müller (1776), Milne Edwards (1841), Herdman (1891a), Millar (1966), Monniot (2001)
C. maculata F. Monniot and C. Monniot, 2001	F	dark blue	10	19	E	P	—	OV	Monniot and Monniot (2001)
C. meridionalis (Herdman, 1891)	F	green, yellow	200	35	E	P	L	BP	Herdman (1891a, 1899), Hartmeyer (1919), Hastings (1931), Kott (1957b, 1990)
C. michaelseni Millar, 1982	F	—	26	30	E	P	—	—	Millar (1982)
C. miniata Watanabe and Tokioka, 1973	F	vermilion	10	10	E	P	—	—	Watanabe and Tokioka (1973), Nishikawa and Tokioka (1976)
C. minuta Tokioka, 1962	F	orange-brown	7	4	E	P	—	—	Tokioka (1962)
C. mirabilis Kott, 1972	F	yellow-brown	40	15	E	—	—	—	Kott (1972b, 1990)
C. moluccensis (Sluiter, 1904)	F	blue	30	14–20	L	P	L	BP	Sluiter (1895, 1904), Kott (1957b, 1972a, b, 1975, 1976, 1990)
C. nigra Kott, 1990	F	grey, black, white	10	19	L	S	—	—	Kott (1990)
C. obesa Nishikawa and Tokioka, 1976	F	bluish white	21	14 or 15	L	P	L	BP	Nishikawa and Tokioka (1976), Nishikawa (1995)

C. oblonga Herdman, 1880	F	colorless	20	more than 15	—	S	L	BP	Herdman (1880, 1882, 1891a, b, 1899), Verrill (1900), Hartmeyer (1911, 1912), Pratt (1916, 1935), Salfi (1929), Berrill (1932), Van Name (1902, 1921, 1930, 1945), Ordóñez et al. (2016), Brunetti and Mastrototaro (2017)
C. oliva Kott, 1990	F	black, white, yellow, green	50	11–22	E	S	—	OV	Kott (1990)
C. ossipandae sp. nov.	F	black, white	20	10–14	E	P	S	BP	present study
C. ostrearium (Michaelsen, 1930)	F	blue	110	24–34	E	—	L	OV and PBC	Michaelsen (1930), Kott (1972b, 1990)
C. picta (Verrill, 1900)	F	purple, pinkish white	20	—	—	—	—	—	Verrill (1900), Seeliger (1907), Hartmeyer (1911, 1912), Van Name (1902, 1921, 1930, 1945), Berrill (1932), Monniot (1972b)
C. polycitorella (Tokioka, 1954)	F	grayish green	10	20	E	S	L	—	Tokioka (1954), Nishikawa and Tokioka (1976)

C. pseudobaudinensis (Kott, 1976)	NF	grey, white	10	18–20	E	—	L	BP	Millar (1966), Kott (1957b, 1972a, b, 1976, 1990)
C. puertosecensis Millar and Goodbody, 1974	F	blue	20	12–18	E	—	S	PBC	Millar and Goodbody (1974), Rocha et al. (2012a)
C. robusta Kott, 1990	F	dark blue, white	40	18–22	E	P	—	BP	Pizon (1908), Van Name (1928), Hastings (1931), Tokioka (1967), Tokioka and Nishikawa (1976), Kott (1990)
C. sabbadini Brunetti, 1987	F	blue-violet	15	13	E	P	L	BP	Brunetti (1987), Brunetti and Mastrototaro (2017)
C. simplex Kott, 2006	NF	—	30	—	—	—	—	—	Kott (2006)
C. steenbrasensis Millar, 1955	NF	blue	18	16	—	P	—	PBC	Millar (1955, 1962)
C. viola Tokioka and Nishikawa, 1976	F	blue, yellow	20	24–26	E	P	—	BP	Nishikawa and Tokioka (1976), Ota et al. (2020)

[a]F, zooids completely free, united only by basal tunic; NF, zooids not free, partially embedded or somewhat grouped in common tunic.

[b]E, extended to abdomen; L, limited to thorax.

[c]P, plicate; S, smooth.

[d]L, lobed or toothed; S, smooth.

[e]BP, in a brood pouch; OV, in the oviduct; PBC, in peribranchial cavity.

Table 8. List of the type material of *Clavelina ossipandae* sp. nov., indicating the catalogue number, type status, numbers of zooids, zooid length, thorax length, abdomen length, muscle formula, number of stigmatal rows, and INSDC accession numbers for partial sequences (810 bp) of the COI gene.

Catalogue number	ICHUM 5837	ICHUM 5838	ICHUM 5839	ICHUM 5840
Type status	holotype	paratype	paratype	paratype
Number of zooids	4	2	1	4
Zooid length (mm)*	14	7	9	7
Thorax length (mm)*	6	4	4	3
Abdomen length (mm)*	8	3	5	4
Muscle formula*	2E·5B·4D=11	2E·6B·2D=10	2E·6B·3D=11	2E·5B·4D=11
Number of stigmatal rows*	14	10	14	14
INSDC accession number	LC777586	LC777588	—	—

*Based on a single zooid randomly selected from each colony.

Table 9. List of COI sequences for each clavelinid species from the INSDC used for the construction of the phylogenetic trees.

Species	Accession number	Reference
Clavelina arafurensis Tokioka, 1952	AM706463	Pérez-Portela and Turon (2008)
Clavelina australis (Herdman, 1899)	AM706464	Pérez-Portela and Turon (2008)
Clavelina breve C. Monniot, 1997	AM706466	Pérez-Portela and Turon (2008)
Clavelina dellavallei (Zirpolo, 1925)	AY603105	Turon and López-Legentil (2004)
Clavelina gemmae Turon, 2005	AJ884573	Turon (2005)
Clavelina lepadiformis (Müller, 1776)	KF309638	López-Legentil et al. (2015)
Clavelina moluccensis (Sluiter, 1904)	AM706472	Pérez-Portela and Turon (2008)
Clavelina oblonga Herdman, 1880	MT637963	Streit et al. (2021)
Clavelina ossipandae sp. nov.	LC777586	present study
Clavelina picta (Verrill, 1900)	JN703740	Rocha et al. (2012b)
Clavelina sabbadini Brunetti, 1987	KF309645	López-Legentil et al. (2015)
Nephtheis fascicularis (Drasche, 1882)	AM706489	Pérez-Portela and Turon (2008)
Pycnoclavella atlantica Pérez-Portela et al., 2007	AM403685	Pérez-Portela et al. (2007)
Pycnoclavella aurilucens Garstang, 1891	AM403693	Pérez-Portela et al. (2007)
Pycnoclavella brava Pérez-Portela et al., 2007	AM403688	Pérez-Portela et al. (2007)
Pycnoclavella communis Pérez-Portela et al., 2007	AM746371	Pérez-Portela and Turon (2008)
Pycnoclavella detorta (Sluiter, 1904)	AM706473	Pérez-Portela and Turon (2008)
Pycnoclavella diminuta (Kott, 1957)	KJ632945	Mohamed et al. (unpublished)
Pycnoclavella martae Perez-Portela and Turon, 2008	AM706481	Pérez-Portela and Turon (2008)
Pycnoclavella nana (Lahille, 1890)	AM403702	Pérez-Portela et al. (2007)
Pycnoclavella tabella Kott, 1990	AM706488	Pérez-Portela and Turon (2008)

Table 10. Sample information including order, family, species name, sampling date, and sampling site.

Order	Family	Species	Sampling date	Sampling site
Aplousobranchia	Clavelinidae	*Clavelina lepadiformis* (Müller, 1776)	2021/5/12	Kushimoto, Japan
	Polyclinidae	*Aplidium* sp.	2022/3/9	Misaki, Japan
	Pseudodistomidae	*Pseudodistoma antinboja* Tokioka, 1949	2021/5/11	Koza, Japan
Stolidobranchia	Pyuridae	*Herdmania momus* (Savignyi, 1816)	2021/5/11	Koza, Japan
		Microcosmus sp.	2021/5/11	Koza, Japan
		Pyura mirabilis (Heller, 1877)	2021/11/1	Sugashima Island, Japan
	Styelidae	*Botrylloides* sp.	2022/3/15	Misaki, Japan
		Botrylloides violaceus Oka, 1927	2021/11/1	Sugashima Island, Japan
		Cnemidocarpa clara (Hartmeyer, 1906)	2021/11/1	Sugashima Island, Japan
		Cnemidocarpa margaritifera Michaelsen, 1918	2023/1/18	Eilat, Israel
		Eusynstyela latericius (Sluiter, 1904)	2023/1/16	Eilat, Israel
		Eusynstyela misakiensi (Watanabe and Tokioka, 1972)	2021/5/11	Koza, Japan
		Polycarpa cryptocarpa kroboja (Sluiter, 1885)	2021/11/1	Sugashima Island, Japan
		Dendrodoa aggregate (Rathke, 1806)	2022/6/10	Akkeshi, Japan
		Styela sp.	2022/3/15	Misaki, Japan
		Symplegma systematica (Sluiter, 1904)	2022/4/23	Irabujima Island, Japan
		Syncarpa composita (Tokioka, 1951)	2022/6/30	Akkeshi, Japan

Table 11. Sequence data obtained from the GitHub repository styelidae and ANISEED.

Order	Family	Species	Source
Phlebobranchia	Ascidiidae	*Phallusia fumigata* (Grube, 1864)	ANISEED
		Phallusia mammillata (Cuvier, 1815)	ANISEED
	Cionidae	*Ciona robusta* Hoshino and Tokioka, 1967	ANISEED
		Ciona savignyi Herdman, 1882	ANISEED
Stolidobranchia	Molgulidae	*Molgula occidentalis* Traustedt, 1883	ANISEED
		Molgula oculata Forbes, 1848	ANISEED
	Pyuridae	*Halocynthia aurantium* (Pallas, 1787)	ANISEED
		Halocynthia roretzi (Drasche, 1884)	ANISEED
		Microcosmus sabatieri Roule, 1885	styelidae
	Styelidae	*Asterocarpa humilis* (Heller, 1878)	styelidae
		Botryllus schlosseri (Pallas, 1766)	ANISEED
		Botrylloides leachii (Savigny, 1816)	styelidae
		Dendroda grossularia (Van Beneden, 1846)	styelidae
		Distomus variolosus Gaertner, 1774	styelidae
		Eusynstyela tincta (Van Name, 1902)	styelidae
		Polyandrocarpa zorritensis (Van Name, 1931)	styelidae
		Polycarpa aurata (Quoy and Gaimard, 1834)	styelidae
		Polycarpa mamillaris (Pallas, 1774)	styelidae
		Polycarpa sp.	styelidae
		Stolonica socialis Hartmeyer, 1903	styelidae
		Styela canopus (Savigny, 1816)	styelidae
		Styela clava Herdman, 1881	styelidae
		Styela plicata (Lesueur, 1823)	styelidae

Table 12. Number of reads and contigs of each species among each step of quality control.

Species	Number of reads	Trinity	Transdecoder	CD-HIT	min TPM	CroCo	ratio	Busco Index (%)
Clavelina lepadiformis	56682322	126660	122351	36843	36843	36843	29.1	96.3
Aplidium pliciferum	55774938	115622	95490	39219	39219	39219	33.9	94.1
Pseudodistoma antinboja	61465810	253926	130804	50694	50694	50694	20.0	94.9
Herdmania momus	42434406	88008	89631	26116	26116	26116	29.7	94
Microcosmus sp.	54163154	87164	106927	30896	30896	30896	35.4	95.6
Pyura mirabilis	43199584	83141	89898	29224	29224	29224	35.1	92.5
Botrylloides sp.	137362636	201787	137979	65432	65432	65432	32.4	96
Botrylloides violaceus	122384370	147738	112922	49286	49286	49286	33.4	95.8
Cnemidocarpa clara	43854848	114765	96283	34223	34223	34223	29.8	93.4
Cnemidocarpa margaritifera	44546458	72124	89219	27893	27893	27893	38.7	94.4
Eusynstyela latericius	124303040	344331	146190	74497	74497	74497	21.6	92.8
Eusynstyela misakiensi	127341340	327188	265280	108633	108633	108633	33.2	99.2
Polycarpa cryptocarpa kroboja	51953554	130749	122330	46481	46481	46481	35.5	95.3

Dendrodoa aggregata	46787562	172013	157965	71393	71393	71393	41.5	97.4
Styela sp.	56036346	140726	120963	43202	43202	43202	30.7	96.5
Symplegma systematica	126899150	314471	126874	64540	64540	64540	20.5	93.4
Syncarpa composita	134010390	501552	229526	99555	99555	99555	19.8	94.4

Table 13. The longest body length and coloniality of each species.

Order	Family	Species	Coloniality	Length (mm)	source of length
Aplousobranchia	Clavelinidae	*Clavelina lepadiformis*	colonial	30	this study
	Polyclinidae	*Aplidium pliciferum*	colonial	20	this study
	Pseudodistomidae	*Pseudodistoma antinboja*	colonial	10	this study
Phlebobranchia	Ascidiidae	*Phallusia fumigata*	solitary	40	Julin and Robert (1913)
		Phallusia mammillata	solitary	140	Millar (1970)
	Cionidae	*Ciona robusta*	solitary	100	Hoshino and Tokioka (1967)
		Ciona savignyi	solitary	43	Nishikawa (1991)
Stolidobranchia	Molgulidae	*Molgula occidentalis*	solitary	65	Van Name (1945)
		Molgula oculata	solitary	80	Monniot (1969)
	Pyuridae	*Halocynthia aurantium*	solitary	120	Nishikawa (1991)
		Halocynthia roretzi	solitary	150	Nishikawa (1991)
		Herdmania momus	solitary	67	Nishikawa (1991, 2002)
		Microcosmus sabatieri	solitary	220	Monniot (1962)
		Microcosmus sp.	solitary	60	this study
		Pyura dura	solitary	60	Heller (1877)
		Pyura mirabilis	solitary	60	Nishikawa (1991)
	Styelidae	*Asterocarpa humilis*	solitary	40	Kott (1985)
		Botrylloides leachii	colonial	2	Kott (1985)
		Botrylloides violaceus	colonial	3	Saito and Okuyama (2003)
		Botrylloides sp.	colonial	3	this study
		Botryllus schllosseri	colonial	2.8	Saito and Okuyama (2003)
		Cnemidocarpa clara	solitary	26	Nishikawa (1991)

Cnemidocarpa margaritifera	solitary	40	Monniot (1973)
Dendrodoa aggregata	solitary	47	Nishikawa (1991)
Dendrodoa grossularia	solitary	25	Berrill (1950)
Distomus variolosus	colonial	10	Millar (1970)
Eusynstyela latericius	colonial	7	Tokioka (1967), Kott (1985)
Eusynstyela misakiensis	colonial	7	Watanabe and Tokioka (1972)
Eusynstyela tincta	colonial	9	Van Name (1945)
Polyandrocarpa zorritensis	colonial	20	Van Name (1945)
Polycarpa aurata	solitary	10	Kott (1985)
Polycarpa cryptocarpa kroboja	solitary	98	Nishikawa (1991)
Polycarpa mamillaris	solitary	75	Vazques et al. (1995)
Polycarpa pomaria	solitary	70	Brunetti and Mastrototaro (2017)
Polycarpa sp.	solitary	—	—
Stolonica socialis	colonial	15	Berrill (1948)
Styela canopus	solitary	30	Nishikawa (1995)
Styela clava	solitary	50	Nishikawa (1991)
Styela plicata	solitary	82	Nishikawa (1991)
Styela sp.	solitary	40	this study
Symplegma systematica	colonail	4	Nishikawa (1984)
Syncarpa composita	colonail	20	Hasegawa and Kajihara (2019)