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Diversity of convolutid acoels (Convolutidae, Acoela) in Hokkaido, Japan, including a description of the symbiont-bearing *Amphiscolops ranshimensis* n. sp.

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ABSTRACT

This study used light and fluorescence microscopy, histology, and molecular phylogenetic analyses to catalogue the diversity of acoels within the family Convolutidae in northern Japan. Here, we examined two convolutid acoels in Hokkaido: *Sagittifera sagittifera* and *Amphiscolops ranshimensis* n. sp. This is the first report of *S. sagittifera* from Hokkaido and the first genetic data from this species. The novel convolutid, *A. ranshimensis* n. sp., shares typical features of the genus including a trilobed posterior end, ocelli, symbiotic algae, and paired parts of the female copulatory organ. It is uniquely distinguished by an accessory male stylet and prostatoid organs; features absent from all previously described species of *Amphiscolops*. It harbors both *Tetraselmis convolutae* and *Prorocentrum concavum*, representing the first reported case of *Prorocentrum* associating with acoels. Additionally, this is the first report of acoels harboring symbionts in Hokkaido. Morphological traits, together with concatenated 18S-28S rRNA gene data, support its designation as a new species. Phylogenetic analyses confirmed the placement of *A. ranshimensis* within *Amphiscolops*, and recovered *S. sagittifera* with other sagittocyst-bearing convolutids.

1. Introduction

Convolutid acoels form diverse symbiotic relationships with microalgae (Achatz et al., 2013; Riewluang and Wakeman, 2023; Hikosaka-Katayama et al., 2024). Some genera consist entirely of species with green algal symbionts (Antonius, 1968; Kostenko and Mamkaev, 1990a, b) while others include species with dinoflagellate symbionts (Haswell, 1905; Dörjes, 1970; Winsor, 1990). Within the convolutid genus *Amphiscolops* Graff, 1904, species form a variety of symbiotic relationships with microalgal symbionts. Some host exclusively dinoflagellates (*A. hamus* Ono and Kajihara, 2025a, *A. trifurcatus* Beltagi, 1983, and *A. bermudensis* Hyman, 1939), and others host dinoflagellates together with green algae (*A. oni* Asai, Miyazawa, Yanase, Inaba and Nakano, 2022, *A. potocani* Achatz, 2008, and *A. marinelliensis* Beltagi and Khafagi, 1984); there are also examples of lineages that do not contain microalgal symbionts (*A. japonicus* Kato, 1947, *A. cinereus* (Graff, 1874), *A. fuligineus* Peebles, 1913, and *A. zeii* Riedl, 1956). Identification of these microalgal symbionts has rarely been emphasized in past scientific literature (Lohner and Micoletzky, 1911; Yamasu, 1982; Hikosaka-Katayama et al., 2020), with most recorded only as zooxanthellae or zoochlorellae (Winsor, 1990; Asai et al., 2022). However, all green algae sequenced to date belong to the genus *Tetraselmis* F. Stein (Chlorodendrophyceae), and all dinoflagellates belong to the Symbiodiniaceae or *Amphidinium gibbosum* (Barneah et al., 2007; Hikosaka-Katayama et al., 2012; Kunihiro and Reimer, 2018; Riewluang and Wakeman, 2023).

Symbiotic convolutids are relatively well studied in Europe, with several taxa belonging to *Symsagittifera* Kostenko and Mamkaev, 1990a, b and *Amphiscolops* originally described in these temperate zones (36°N–58°N). *Convoluta convoluta* (Abildgaard, 1806), host to a diatom in the genus *Licmophora* (Licmophoraceae), has been reported as far North as Norway and Sweden (~60°N) (Westblad, 1948; Apelt, 1969; Karling, 1974; Stöhr, 2024). Surveys of symbiotic convolutids, and convolutids in general, at similar latitudes in northern Japan are limited. To date, only *Oxyposthia praedator* Ivanov, 1952, a non-symbiotic

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convolutid, has been reported from Hokkaido (Ono and Kajihara, 2025b). The majority of surveys on symbiotic convolutids in Japan have mainly focused on subtropical areas on the Pacific side of Honshu, as well as in the southern Islands of the Ryukyus Archipelago (Faubel et al., 2004).

To this end, we further examined the biodiversity of convolutid acoels in northern Japan, focusing on *Sagittifera sagittifera* (Ivanov 1952) and an exceptional occurrence of a novel species containing two types of microalgal symbionts. We examined their external morphology using light and fluorescence microscopy, as well as reconstruct their internal structures using histological sections. In addition, the 18S and 28S rRNA genes were sequenced to place this diversity into a molecular phylogenetic framework.

2. Materials and methods

2.1. Collection of host acoels and establishment of microalgal cultures

Brown macroalgae (*Sargassum* sp., Phaeophyceae) collected from Ranshima Beach, Hokkaido, Japan (43°12′16.6″N 140°51′23.9″E) in June 2024 were washed and strained through a 100 μm plankton net. The filtrate was examined under an Olympus SZ61 stereomicroscope (Olympus, Tokyo, Japan), and acoels were isolated into a clean Petri dish using a pulled transfer pipette. Two types of acoels were collected: one flat with ventrally enfolded sides and orange due to its rhabdoids; the other with a trilobed posterior and green due to its symbiotic microalgae. Individuals were relaxed with magnesium chloride solution isotonic to seawater (salinity of 35 ppm), pressed under a glass cover slip, and imaged under a Zeiss Axioskop 2 Plus (Zeiss, Oberkochen, Germany) connected to a Canon EOS Kiss X8i digital camera (Canon, Tokyo, Japan). Imaged acoels were then processed for culture establishment, DNA barcoding, or histology. Cultures were established by breaking apart acoels to release microalgal cells in a 24-well culture plate filled with half-concentration Daigo's IMK medium (Wako Pure Chemical Industries, Tokyo, Japan). Microalgal strains were further isolated by picking individual cells and washing them through a series of seawater-filled well slides using a fine, hand-drawn glass pipette. The 24-well culture plate was maintained in a Biotron Incubator LH-350 (NK Systems, Tokyo, Japan) at 20 °C (12:12, light:dark hours), where individual cells from established strains were imaged and sub-cultured into fresh media every 5 weeks.

2.2. DNA extraction, PCR amplification, and sequencing

Acoels were washed through a series of seawater-filled well slides and transferred into 1.5 mL Eppendorf tube filled with 150 μL of lysis buffer provided by the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, Wisconsin, USA). Genomic DNA was extracted from acoels as well as pelleted cultures of *Tetraselmis* and *Prorocentrum* following manufacturer protocol. Two cultures were established from acoel isolate H24R46. The 18S rRNA gene (18S) and 28S rRNA gene (28S) were targeted for amplification. The ITS region was pcr-amplified for microalgal cultures. All three regions were initially amplified using SR1–28-Gen3000R primer pair and KOD One® PCR Master Mix (Toyobo, Osaka, Japan). Thermocycler conditions used for the initial reaction were as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 52 °C for 5 s, extension at 68 °C for 25 s, and a final extension at 68 °C for 5 min. The products of the initial amplification were diluted 1:50 times in Ultrapure distilled water (Invitrogen, Grand Island, New York, USA) and used as the template for the nested reaction using general primer pairs designed for their respective genes: SR1b–18SRF and SR4–SR12 (18S), Lp1F1–25F1R (ITS region), and D1RF1–28-1483R (28S). Thermocycler conditions used for the nested reaction were as follows: initial denaturation at 94 °C for 1 min, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 56/55/52 °C (18S/ITS/28S) for 5 s, extension at 68 °C for 10 s, and a final extension at 68 °C for 5 min. Following the nested reaction, PCR products were purified with polyethylene glycol and sequenced using BrilliantDye Terminator v1.1 (Nimagen, Nijmegen, Gelderland, Netherlands), according to manufacturer protocol on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems, Waltham, Massachusetts, USA). An additional sequencing primer, 852R-70, was used to join 28S gene fragments. When symbiont sequences were amplified instead of host acoels, the nested reactions were re-done using acoel-specific primers designed to only amplify the host DNA (Table S1).

2.3. Phylogenetic analysis

Geneious Prime 2025.0.3 (https://www.geneious.com) was used to process Sanger sequencing reads. Sequences were assembled with the "De Novo Assemble" tool, trimming regions with more than 1% chance of error per base (Error Probability Limit: 0.01) at both ends. Three separate alignments were created. For acoels, a concatenated 18S-28S dataset was created based on similar sequences obtained via the Basic Local Alignment Search Tool (BLAST) together with publicly available sequences of other convolutids from the National Center for Biotechnology Information (NCBI) (Table S2). Six additional sequences from sister families, Dakuidae Hooge, 2003 and Mecynostomidae Dörjes, 1968, served as the outgroup. For the symbionts, *Tetraselmis* and *Prorocentrum*, concatenated 18S-ITS-28S datasets were created from sequences obtained from BLAST and combined with other sequences based on previous phylogenetic analyses used in their respective groups; a Chlorodendrophyceae alignment following Riewluang and Wakeman (2025) (Table S3), and a trimmed Prorocentrales alignment following Tillmann et al. (2023) (Table S4), respectively. To create the final alignment, single-gene datasets were aligned using MAFFT tool (Katoh et al., 2002) and trimmed using the 'Mask Alignment' tool (70% gaps) within Geneious Prime before combining. The length of the final alignment for convolutids, *Tetraselmis*, and *Prorocentrum* were 2994 bps, 3625 bps, and 3133 bps, respectively.

IQ-TREE v2.3.2 (Minh et al., 2020) was used to select the best-fit model for maximum-likelihood and Bayesian analyses. Under the Akaike Information Criterion with correction (AICc), the model GTR + F + I + R5 was selected for convolutids, TIM2e + I + R3 for *Tetraselmis*, and TIM + F + I + R3 for *Prorocentrum*. Bootstrap analysis was performed with 1000 pseudoreplicates. MrBayes 3.2.7a (Huelsenbeck and Ronquist, 2001) was used to run Bayesian analyses. Based on the Bayesian Information Criterion, four Markov Chain Monte Carlo (MCMC) chains were run with the general time reversible (GTR) substitution model, for 1.0×10^7 generations and sampled every 100th generation. The rate variation across sites was drawn from a gamma distribution with a proportion being invariable (lset rates = invgamma). Other parameters were set to the default setting.

Sagittifera sagittifera (GenBank Accessions: PX136612–PX136614 (18S) and PX136622–PX136624 (28S)), *Amphiscolops ranshimensis* n. sp. (GenBank Accessions: PX136606–PX136610 (18S) and PX136616–PX136620 (28S)), *Tetraselmis convolutae* strain C23R46 (GenBank Accessions: PX136615 (18S), PX136605 (ITS), and PX136625 (28S)), and *Prorocentrum concavum* strain C23R46 (GenBank Accessions: PX136611 (18S), PX136604 (ITS), and PX136621 (28S)) sequences were deposited to GenBank, NCBI.

2.4. Confocal microscopy

Specimens were relaxed with magnesium chloride solution isotonic to seawater (salinity of 35 ppm) before being fixed in 4% (w/v) paraformaldehyde in PBS for 1 h. Fixed specimens were rinsed with 1X PBS

and stained with Alexa Fluor 488 phalloidin (Invitrogen, Eugene, Oregon, USA) at 1:100 dilution for 2 h. Before viewing, stained specimens were rinsed with 1X PBS, mounted on a coverslip with Vectashield mounting medium (Vector Laboratories, Burlingame, California, USA), and sealed with nail polish. Images were obtained on a Zeiss LSM 980 laser scanning microscope (Zeiss, Oberkochen, Germany) with the accompanying software and image stacks were further processed with Fiji image analysis software (Schindelin et al., 2012).

2.5. Serial sectioning for histology

Acoels with visible copulatory organs were selected and transferred into a 1.5 mL Eppendorf tube filled with warm Stefanini's fixative. Fixed specimens were rinsed with autoclaved seawater until the wash solution was clear. Specimens were then post-fixed in 1% (v/v) osmium tetroxide for 1.5 h and rinsed again with autoclaved seawater and distilled water 3 times for 15 min each. Subsequently, fixed specimens were dehydrated and embedded in resin (Agar Scientific, Stansted, Essex, USA). Dehydration was done with an ethanol series of increasing concentration (30%, 50%, 70%, 80%, 90%, 100%, 100%, 100%) for 5 min at each step, followed by a 1:1 ratio of acetone:ethanol for 10 min, and two exchanges of 100% acetone for 10 min each. Embedding was done with exchanges of 1:1 acetone:resin for 1 h, followed by two 1 h exchanges of 100% resin, and polymerized at 68 °C for 48 h. Resin embedded samples were sectioned at a thickness of 1250 nm using a glass knife on an ultramicrotome (Leica, Wetzlar, Hesse, Germany). Serial sections were collected on glass slides and dried overnight at 60 °C on a HP-4530N hot plate (AS ONE, Osaka, Japan), before being stained with 1% Toluidine Blue O solution (Sigma–Aldrich, St. Louis, Missouri, USA) for 45 s. Stained sections were rinsed with distilled water twice for 5 min each and dried. Finally, serial sections were mounted with DPX (Sigma–Aldrich, St. Louis, Missouri, USA), viewed, and imaged (Supplementary material 1, 2).

3. Results

3.1. Phylogenetic analysis

Maximum-likelihood analysis based on concatenated 18S-28S rRNA gene dataset resolved a monophyletic Convolutidae (100% BS/1.00 BPP) (Fig. 7). *Sagittifera sagittifera* sequences (JP-H24R11, JP-H24R17, and JP-H24R21) generated in this study branched independently and formed a clade with other sagittocyst-bearing convolutids (97% BS/1.00 BPP) from the genera *Convolutriloba* Hendelberg and Akesson, 1988 and *Symsagittifera*. The sequences were sister to an unidentified Convolutidae sp. H22017 that harbored symbiotic *Tetraselmis*. *Amphiscalops ranshimensis* n. sp. sequences (JP-H24R1, JP-H24R9, JP-H24R44, JP-H24R46, and JP-H24R49) formed a clade of trilobed convolutids with *A. oni* and *A. potocani* (100% BS/1.00 BPP). The clade was sister to *Heterochaerus* Haswell, 1905 (99% BS/1.00 BPP) and *Waminoa* Winsor, 1990 (100% BS/1.00 BPP); the three clades were sister to all other convolutid sequences (100% BS/1.00 BPP).

Tetraselmis strain C24R46 and *Prorocentrum* strain C24R46 were established and sequenced from *A. ranshimensis* n. sp. isolate JP-H24R46. Analysis of the corresponding 18S-ITS-28S rRNA gene datasets placed *Tetraselmis* strain C24R46 in a clade with other *T. convolutae* strains (58% BS/0.93 BPP), including both free-living strains (e.g. BEA 1918B and KB CR05) and symbiotic strains (e.g. RCC 1563 from *Symsagittifera roscoffensis* (Graff, 1891), AW-2009 from *Convoluta schuelii* Achatz, 2008, and C23181 from Convolutidae sp. isolate H23181). *Prorocentrum* strain H24R46 formed a clade with *P. concavum* sequences (100% BS/1.00 BPP), all were free-living strains (PPAN04, BEGRL134, SKLMP S030, NCMA 1724, CCMP 1724) (Fig. 8).

3.2. Taxonomic summary

Sagittifera sagittifera Ivanov, 1952
Fig. 1

Materials Examined. Living specimens in squeeze preparation, a complete set of (515) serial sagittal sections (JP- H24R10), and 3 whole mounts for fluorescence microscopy (JP- H24R17, JP- H24R18, and JP-H24R19). Specimens collected by S. Riewluang, R.C. Manglicmot, S. Cunningham, and K.C. Wakeman from Otaru, Hokkaido, Japan (43°12′16.6″N 140°51′23.9″E) on brown macroalgae (*Sargassum* sp.).

DNA sequences. Partial 18S and 28S rRNA gene sequences of JP-H24R11 (GenBank Accession: PX136612 (18S) and PX136622 (28S)), JP- H24R17 (GenBank Accession: PX136613 (18S) and PX136623 (28S)), and JP- H24R21 (GenBank Accession: PX136614 (18S) and PX136624 (28S)).

Description. Mature animals flat with ventrally enfolded sides (Fig. 1A), ~0.5 mm in length and up to ~0.6 mm when fully extended. Epidermis ciliated. Orange-colored rhabdoids evenly distributed dorsally, resulting in orangish body color. Mouth central (40U). Frontal organ opens to terminal frontal pore at anterior tip. Symbionts absent. Statocyst (10U) and eye spots (10U) present (Fig. 1A). Ovaries and testes paired.

Male and female gonopores separate (Fig. 1B–E). Male gonopore positioned behind female gonopore, opens to short male antrum that leads to penis. Male copulatory organ consists of bulb surrounding male antrum and muscular, tubular penis lined with densely packed, needle-like sagittocysts (Fig. 1C–E). Seminal vesicle absent. Female gonopore opens to vagina with sphincter and leads to seminal bursa (Fig. 1B–E). Seminal bursa with single bursal nozzle with conspicuous central extensions of nozzle matrix cells (Fig. 1A–D, E).

Fan-shaped arrangements of sagittocysts present ventrally between male and female copulatory organs at the level of the female gonopore (Fig. 1A and B). Sagittocysts, perpendicular to the body midline, evenly distributed ventrally along enfolded sides. Smaller, needle-like sagittocysts surrounding the male bulb (Fig. 1C). Large sagittocyts often found posterior to the male copulatory organ.

Collection Locality and habitat. 43°12′16.6″N 140°51′23.9″E, on brown macroalgae (*Sargassum* sp.) from Ranshima Beach, Otaru, Hokkaido, Japan.

Remarks. *Sagittifera sagittifera* was identified by its enfolded sides, orange colored rhabdoid glands, separate male and female gonopores, absence of endosymbionts, and a unique fan-shaped arrangement of sagittocysts. We were surprised by the presence of *Prorocentrum* in some specimens as we initially interpreted it as an endosymbiont; however, upon further inspection, the *Prorocentrum* was determined to be food due to its irregular occurrence and localization in the digestive syncytium.

Sagittifera sagittifera was originally described in the Sea of Okhotsk (Ivanov, 1952). Yamasu (1991) reported finding *Praesagittifera shikoki* Kostenko and Mamkaev, 1990a,b in Chiba, Japan; however, Gschwentner et al., (1999) determined this was actually *S. sagittifera* based on Yamasu's description and figures. The descriptions and line drawings of both Ivanov (1952) and Yamasu (1991) depicted the unique fan-shaped arrangements of sagittocysts on either side of the female gonopore, which is also evident in our specimens. Here, light microscopy images of living specimens and a micrograph of a sagittal histological section showing the copulatory organs are provided for *S. sagittifera*. Some differences of note to previous descriptions are the presence of a male antrum leading from the male gonopore to the muscular penis (Fig. 1E), and a sphincter around the vagina (Fig. 1E).

Sagittifera sagittifera sequences based on partial 18S and 28S rRNA genes formed a highly supported clade with other sagittocysts-bearing

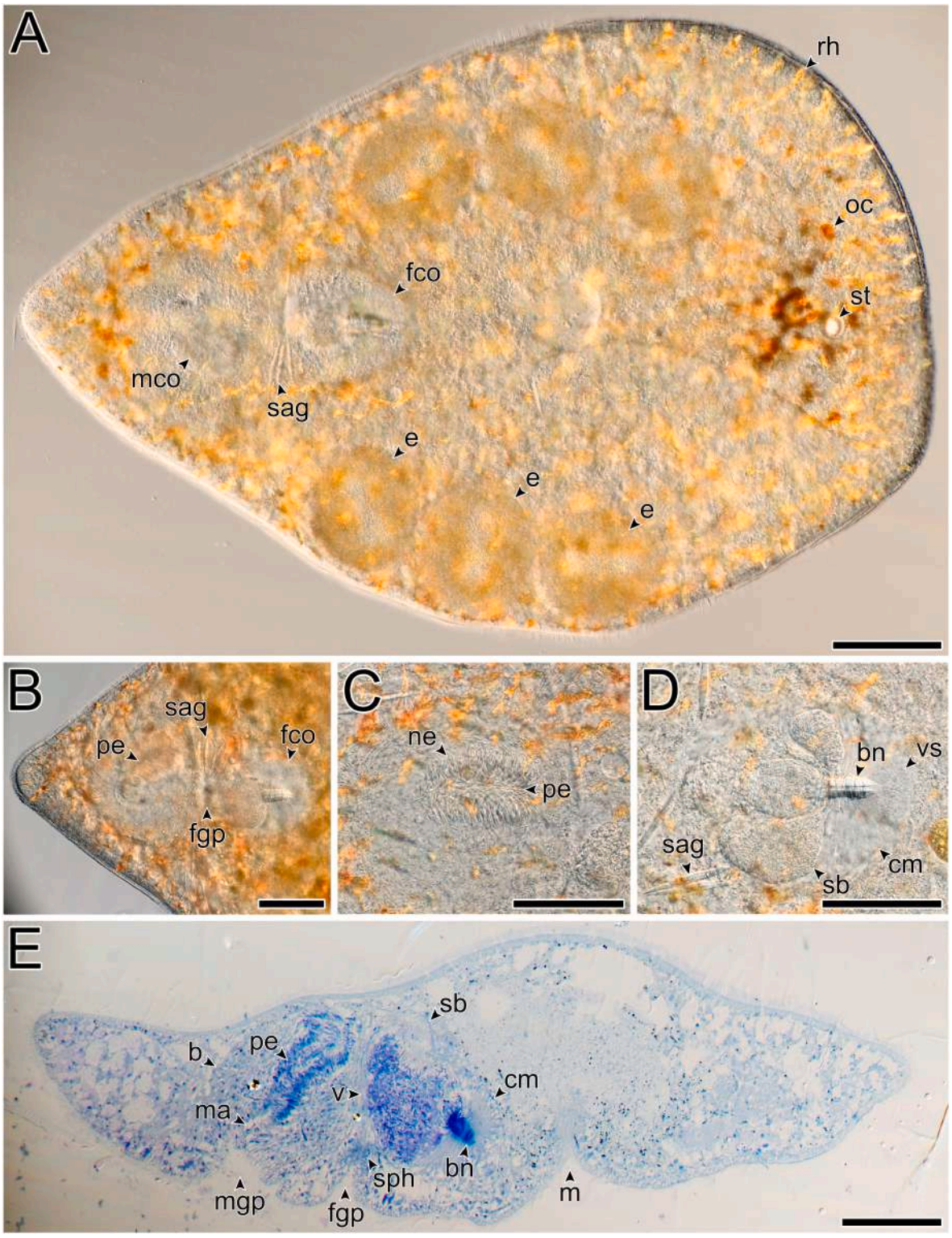

Fig. 1. *Sagittifera sagittifera*. **A** Pressed specimen showing the general morphology of *S. sagittifera* isolate H24R11. **B** Copulatory organs of isolate H24R13 showing the fan-shaped arrangement of sagittocysts. **C** Male copulatory organ of isolate H24R12. **D** Female copulatory organ of isolate H24R12. **E** Sagittal histological section of isolate H24R10 through the whole specimen showing the organization of internal organs. Scale bar: **A** = 50 μm; **B**–**D** = 25 μm; **e** = 50 μm. Abbreviations: **b**, bulb; **bn**, bursal nozzle; **cm**, central extensions of matrix cells; **e**, eggs; **fco**, female copulatory organ; **fgp**, female gonopore; **m**, mouth; **ma**, male antrum; **mco**, male copulatory organ; **mgp**, male gonopore; **ne**, needle-shaped sagittocyst; **oc**, ocelli; **pe**, penis; **rh**, rhabdoids; **sag**, sagittocyst; **sb**, seminal bursa; **sph**, sphincter; **st**, statocyst; **v**, vagina; **vs**, vestibulum.

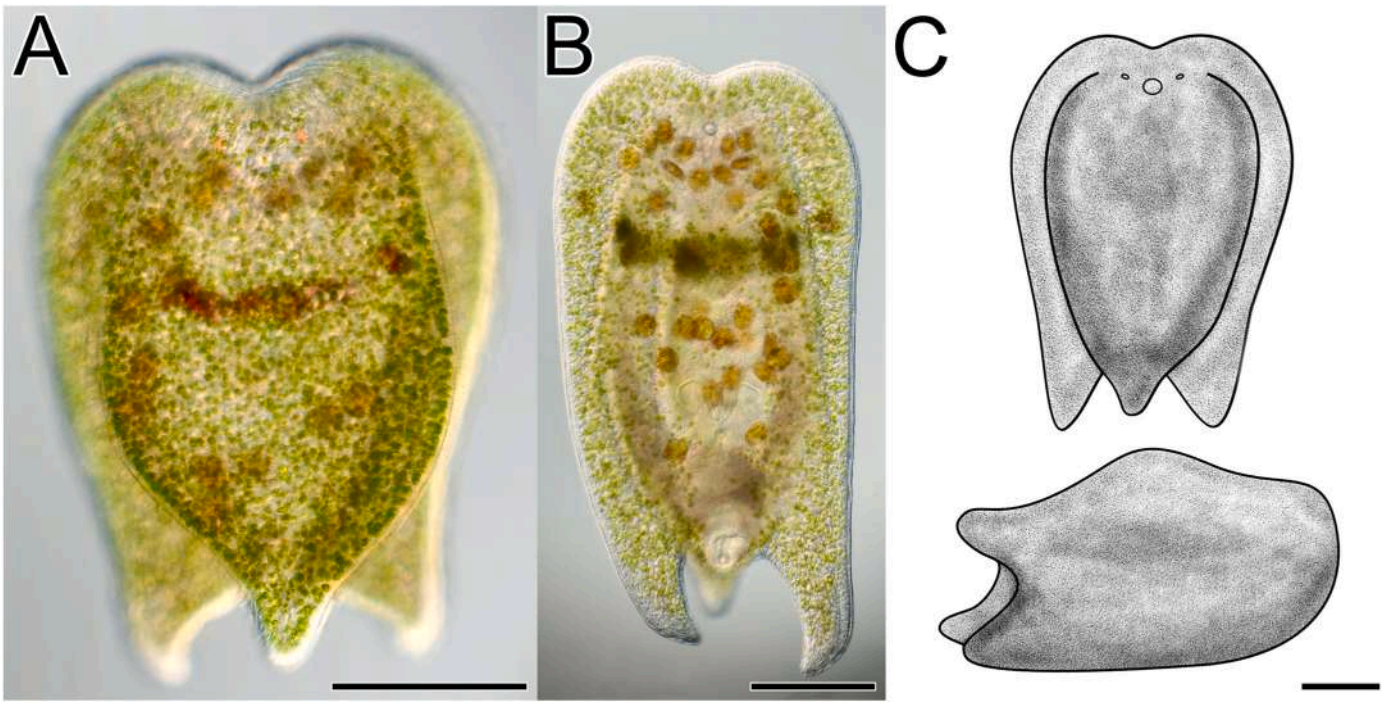

Fig. 2. Unpressed specimens of *Amphiscolops ranshimensis* n. sp. **A** Dorsal view of isolate JP-H24R48 showing the symbiotic dinoflagellate *Prorocentrum concavum* (brown) and the chlorophyte *Tetraselmis convolutae* (green). **B** Ventral view of isolate JP-H24R43. **C** Line drawing of the dorsal (top) and lateral view (bottom). Scale bars: **A**–**C** = 200 μm.

convolutids belonging to *Convolutriloba* and *Symsagittifera* (see Fig. 7). This corresponds to previous morphology-based groupings, based primarily on the presence of sagittocysts (Mamkaev and Kostenko, 1991; Achatz et al., 2010; Jondelius et al., 2011).

Amphiscolops Graff, 1904

Emended diagnosis. Convolutidae colored through pigment, concrement, symbiotic algae, or combination thereof. Posterior end rounded, bilobed, or trilobed, without filaments. Statocyst present. Frontal organ present. Brain insunk. Female copulatory organ with two or more bursal nozzles. Male copulatory organ with tubular penis and no caudal penis fold. Sagittocysts absent.

Remarks. Convolutidae includes eight genera with species that possess two or more bursal nozzles. *Amphiscolops* can be distinguished from other convolutids possessing two or more bursal nozzles based on the following characters: *Convolutriloba* (sagittocyst present), *Heterochaerus* (caudal penis fold present), *Oligochoerus* Beklemischev, 1963 (pharynx present), *Polychoerus* Mark, 1892 (caudal filaments present), *Thalassoanaperus* Hernández, 2018 (symbionts absent, typically with numerous prostatoid organs surrounding male copulatory organ), *Waminoa* (body discoid to obcordate and epizoic on corals), and *Wulguru* Winsor, 1988 (three gonopores present).

Amphiscolops ranshimensis n. sp. Riewluang and Wakeman, 2026
Figs. 2–6

Materials examined. Holotype: JP- H24R52, a complete set of (560) serial coronal sections. Paratypes: Living specimens in squeeze-preparation (JP-H24R40–JP-H24R70); partial set of serial frontal sections (JP-H24R40), and 5 whole mounts for fluorescence microscopy (JP- H24R53, JP- H24R54, JP- H24R55, JP- H24R61, and JP- H24R68). Collected by S. Riewluang and K.C. Wakeman, 22nd June 2024 from Otaru, Hokkaido, Japan (43°12′16.6″N 140°51′23.9″E) on brown macroalgae, *Sargassum* sp.

DNA sequences. Partial 18S rRNA gene and 28S rRNA gene of paratypes JP- H24R1 (GenBank Accession: PX136606 and PX136616), JP- H24R9 (GenBank Accession: PX136607 and PX136617), JP- H24R44 (GenBank Accession: PX136608 and PX136618), JP- H24R46 (GenBank Accession: PX136609 and PX136619), and JP- H24R49 (GenBank Accession: PX136610 and PX136620).

Diagnosis. *Amphiscolops* with two pronounced lateral lappets and trilobed posterior. Statocyst and eye spots present. Color and pattern resulting from two types of symbiotic algae, *T. convolutae* and *P. concavum*. Frontal organ with frontal pore. Central mouth. Separate male and female gonopores. Subterminal male gonopore opens to short male antrum that leads posteriorly to accessory male stylet and anteriorly to penis. Male copulatory organ consists of a seminal vesicle surrounding a muscular, tubular penis, paired glandular vesicles, and sperm mass. Female copulatory organ T-shaped, vagina with sphincter and medial pair of prostatoid organ, opens to paired seminal bursae with one bursal nozzle each, unconnected by bursal tissue.

Description. Dimensions of mature animals ~1.0 mm in length, ~0.5 mm in width, and ~0.6 mm in height. Body cylindrical with lateral lappets creating deep, medial crevice extending entire body length ending as two caudal lobes (Figs. 2 and 3). Medial lobe of equal length is present (Figs. 2 and 3A). Ventral mouth, located centrally (50U) in the crevice where lappets join. Animals uniformly green, due to *T. convolutae* symbionts, with variable brown patches, due to *P. concavum* symbionts (Fig. 2A and B). *Tetraselmis convolutae* (Fig. 3D) distributed evenly, found below body-wall and between rhabdoid gland cells and internal organs (Fig. 4C). *Prorocentrum concavum* (Fig. 3E) distributed near female copulatory organs, developing oocysts, and testes in varying densities (Fig. 4C). Statocyst (10U) and ocelli (8U) present (Fig. 2A, B, 3C). Colored rhabdoids absent.

Epidermis ciliated (Fig. 4C). Body-wall musculature consists of dorsal circular, longitudinal, and longitudinal cross-over muscle fibers, and ventral circular and longitudinal muscle fibers (Fig. 5). Frontal organ consists of elongated gland cells opening to frontal pore (Fig. 4A, B, 5A). Dense cyanophilic globules cluster around glands (Fig. 4). Rhabdoid glands organized, comprised of distinct, rectangular cells occupying space between body-wall and inner organs (Fig. 4); concentrated dorsally and extends to lateral lappets.

Ovaries and testes paired (Fig. 4A and B). Young oocytes separate,

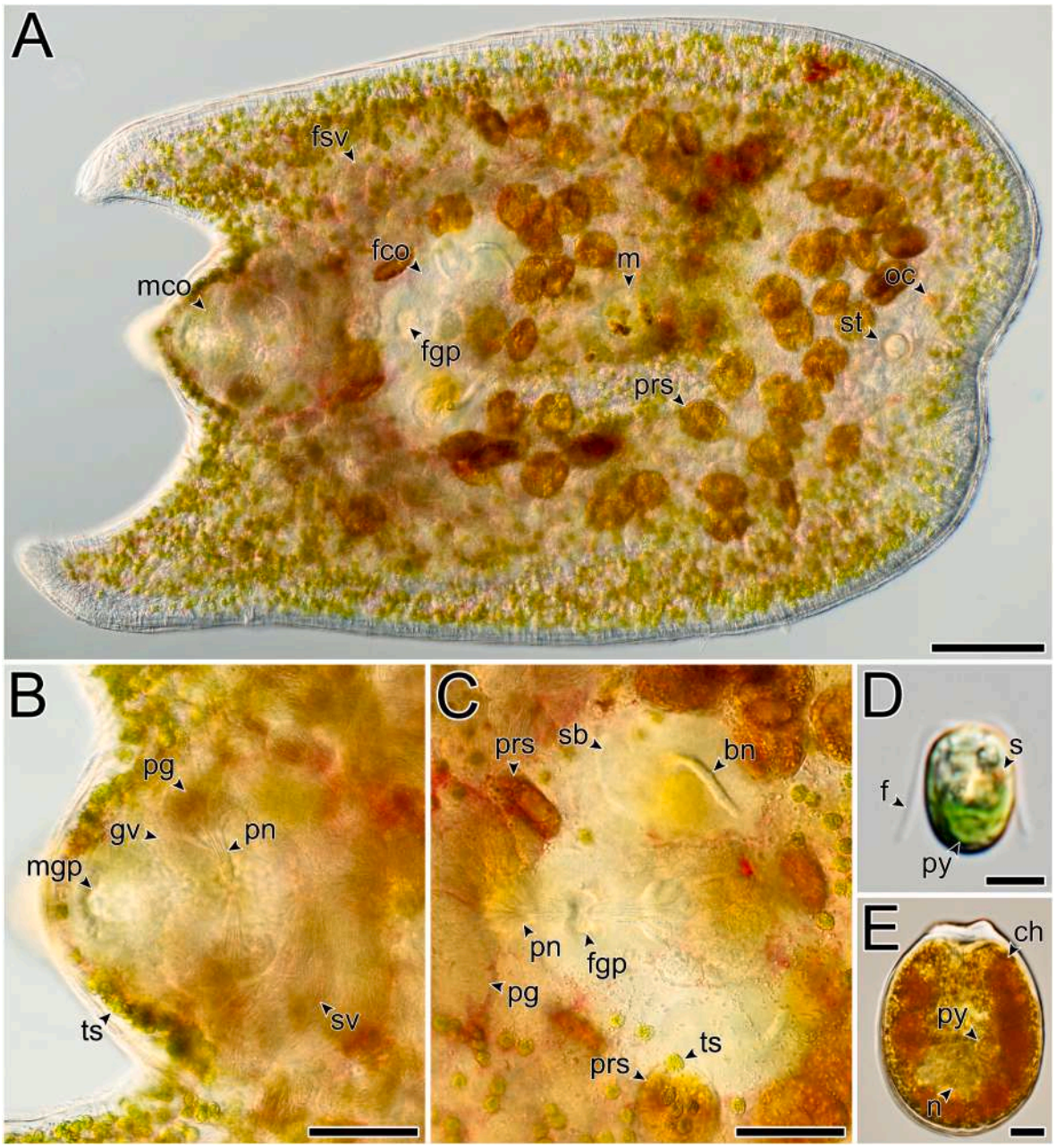

Fig. 3. *Amphiscolops ranshimensis* n. sp. isolate H24R42 and symbionts in culture. **A** General morphology of *Amphiscolops ranshimensis* n. sp. **B** Male copulatory organ. **C** Female copulatory organ. **D** *Tetraselmis convolutae* strain C24R46 in culture. **E** *Prorocentrum concavum* strain C24R46 in culture. Scale bars: **A** = 100 μm; **B**, **C** = 50 μm; **D** = 5 μm; **E** = 10 μm. Abbreviations: **bn**, bursal nozzle; **ch**, chloroplast; **f**, flagella; **fco**, female copulatory organ; **fgp**, female gonopore; **fsv**, false seminal vesicle; **gv**, glandular vesicle; **m**, mouth; **mco**, male copulatory organ; **mgp**, male gonopore; **n**, nucleus; **oc**, ocelli; **pg**, prostatoid gland; **pn**, prostatoid organ needles; **prs**, *Prorocentrum* symbiont; **py**, pyrenoid; **s**, stigma; **sb**, seminal bursa; **st**, statocyst; **sv**, seminal vesicle; **ts**, *Tetraselmis* symbionts.

extends from anterior fourth (25U) and joins posterior to mouth. Fully developed eggs dorsal. Ovaries do not extend posterior to seminal bursa. Testes separate in both lappets, extends from anterior fifth (20U), forms a false seminal vesicle, and joins seminal vesicle ventrally.

Male gonopore (Fig. 3B and 5B) and female gonopore (Fig. 3C and 5D) separate. Female gonopore (69U) opens to sphincter (Fig. 5D) and leads to T-shaped vagina (Fig. 6G), each lobe containing one bursa with associated, curved bursal nozzle (Fig. 3A–C, 5C, 6E, F). Seminal bursae unconnected by bursal tissue (Fig. 6G). Paired prostatoid organs medial to female gonopore, with 6 well developed glands and needles protruding at the level of sphincter (Fig. 3C, 4B and 5D, 6E–G).

Male gonopore (85U) opens to short male antrum that leads to two passages. Posterior passage with thick outer longitudinal muscles and inner circular muscles opens to accessory male stylet comprised of ~30 tightly packed stylet needles, proximal part muscular, inserted into glandular vesicle (Figs. 4 and 6A, B). Individual stylet needles containing glandular secretions, parallel with longitudinal muscles (Fig. 5F and 6B). Anterior passage opens to muscular, tubular penis (Figs. 5E and 6A, B). Penis musculature consists of thick, anastomosing outer longitudinal muscles, continuous with antrum, and inner circular muscles. A muscular seminal vesicle surrounds penis and paired glandular vesicles that surround the proximal portion of the penis (Fig. 5E and F). A mass of sperm enclosed by seminal vesicle caps the glandular vesicles (Fig. 4A). Proximal portion of penis with three openings; lateral openings to glandular vesicles, and central opening to sperm mass (Fig. 5E). Prostatoid organ needles located within each glandular vesicle, open to penis

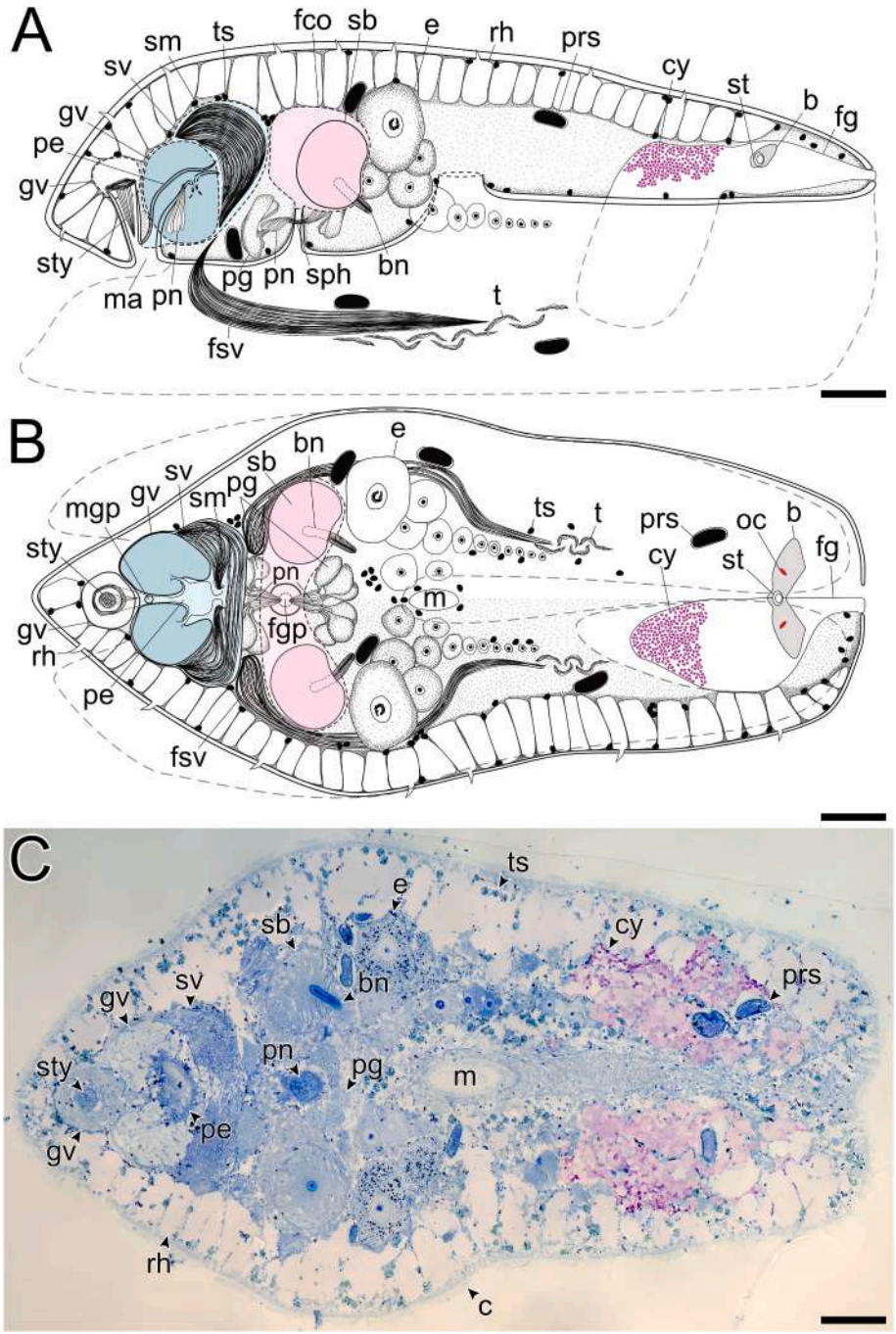

Fig. 4. Reconstruction of *Amphiscolops ranshimensis* n. sp. The male copulatory organ (blue) and female copulatory organ (pink) are highlighted. **A** Sagittal reconstruction of whole specimen. **B** Frontal reconstruction of whole specimen. *Amphiscolops ranshimensis* n. sp. is bilaterally symmetrical, but for clarity, only one side of the rhabdoid glands, frontal glands, and cyanophilic globules are shown. **C** Frontal histological section of *Amphiscolops ranshimensis* n. sp. isolate H24R52 stained with 1% Toluidine Blue O. Scale bars: **A**–**C** = 100 μm. Abbreviations: **c**, cilia; **cy**, cyanophilic globules; **b**, brain; **bn**, bursal nozzle; **e**, eggs; **fco**, female copulatory organ; **fg**, frontal gland; **fgp**, female gonopore; **fsv**, false seminal vesicle; **gv**, glandular vesicle; **m**, mouth; **ma**, male antrum; **mgp**, male gonopore; **oc**, ocelli; **pe**, penis; **pg**, prostatoid gland; **pn**, prostatoid organ needles; **prs**, *Prorocentrum* symbiont; **rh**, rhabdoid gland; **sb**, seminal bursa; **sm**, sperm mass; **sph**, sphincter; **st**, statocyst; **sty**, stylet needles; **sv**, seminal vesicle; **t**, testes; **ts**, *Tetraselmis* symbionts.

lumen proximally to the lateral openings to glandular vesicle (Fig. 4A).

Type Locality and Habitat. 43°12′16.6″N 140°51′23.9″E, on brown macroalgae (*Sargassum* sp.) from Ranshima Beach, Otaru, Hokkaido, Japan.

Etymology. Derived from Latin *ranshimensis*, referring to locality from which the material was collected: Ranshima Beach, Otaru, Hokkaido, Japan.

Remarks. *Amphiscolops* includes 13 valid species (Ono and Kajihara, 2025a), including two recently described species from southern Japan, *A. oni* and *A. hamus* (Asai et al., 2022; Ono and Kajihara, 2025a). Among the congeners, *A. ranshimensis* most closely resembles *A. potocani* in having a trilobed posterior, a statocyst, subterminal male gonopore, and two types of endosymbionts—a green alga and a dinoflagellate (Table 1). Both species have a T-shaped vagina with paired seminal bursae unconnected by bursal tissue; a morphological trait unique within *Amphiscolops*. However, *A. ranshimensis* has eyespots, a male

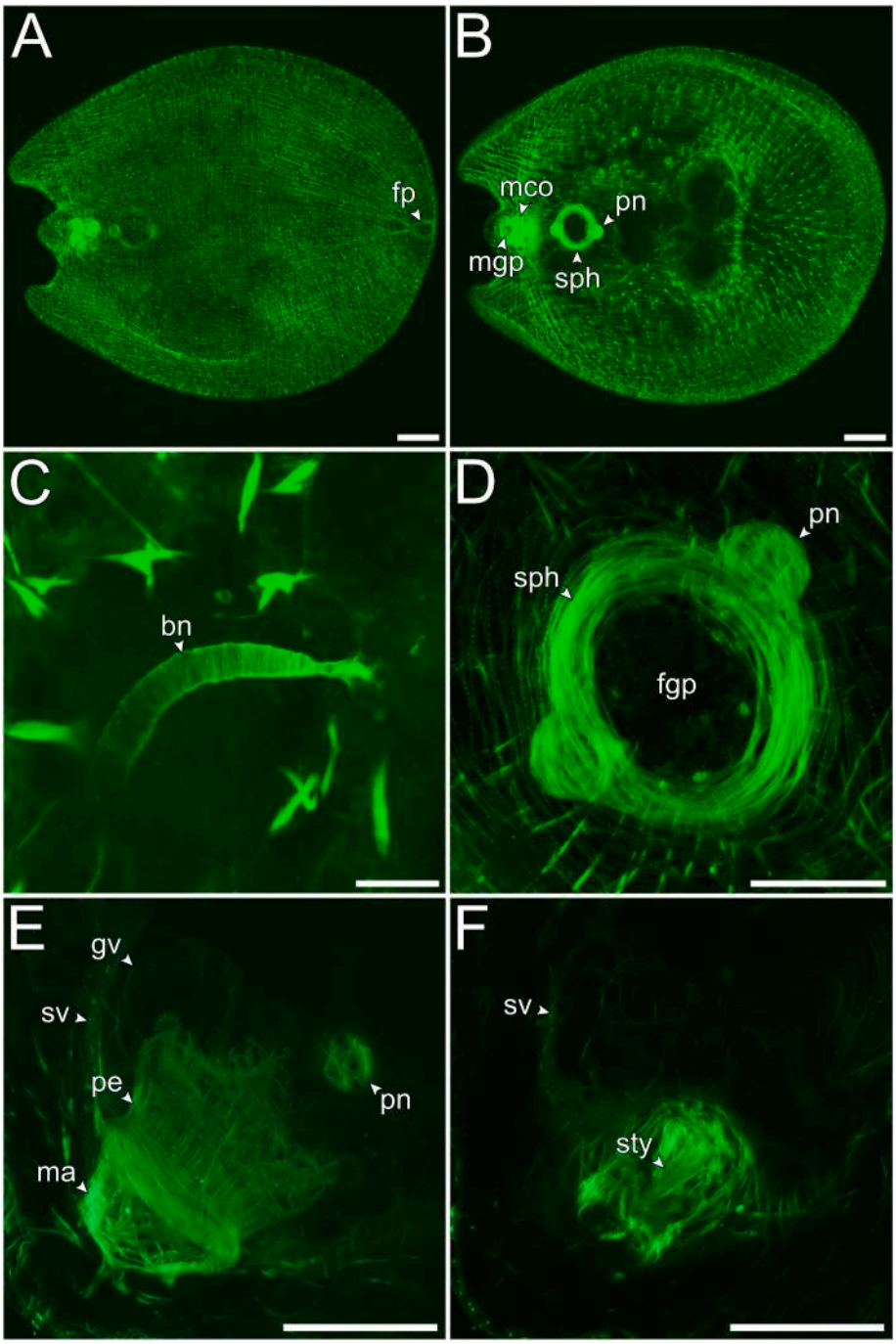

Fig. 5. *Amphiscolops ranshimensis* n. sp. stained with Alexa-488-labelled phalloidin. **A** Dorsal projection of body-wall musculature. **B** Ventral projection of body-wall musculature. **C** Bursal nozzle. **D** Female gonopore and prostatoid organs. **E** Male copulatory organ. **F** Accessory male stylet. Scale bars: **A**, **B** = 100 μm; **C** = 20 μm; **D** = 50 μm. Abbreviations: **bn**, bursal nozzle; **fgp**, female gonopore; **fp**, frontal pore; **gv**, glandular vesicle; **ma**, male antrum; **mco**, male copulatory organ; **mgp**, male gonopore; **pe**, penis; **pn**, prostatoid organ needles; **sph**, sphincter; **sty**, stylet needles; **sv**, seminal vesicle.

antrum, prostatoid organs, and an accessory male stylet, all of which are absent in *A. potocani*. The occurrence of both a green alga and a dinoflagellate as endosymbionts is also found in two other *Amphiscolops* species, *A. oni* and *A. marinelliensis*, both of which can be distinguished from *A. ranshimensis* based on external morphology alone. *A. oni* possesses a middorsal appendage and displays varied coloration (white, yellow, orange, light brown) not observed in *A. ranshimensis*, and *A. marinelliensis* has a forked posterior rather than trilobed as found in *A. ranshimensis*.

Amphiscolops ranshimensis is the only species in the genus with prostatoid organs and a sclerotized, accessory male stylet. The prostatoid organs are composed of composite needles strengthened with F-actin (labelled as prostatoid organ needles) that connects to 6 well developed glands (labelled as prostatoid glands). This structure resembles prostatoid organs observed in other species of convolutids, including *Achoerus pachycaudatus* Dörjes, 1968, *Conaperta cirrata* Achatz et al., 2007, and *Thalassoanaperus singularis* (Hooge and Smith, 2004). In contrast, the accessory male stylet is structurally distinct and to our knowledge, is unique not only within *Amphiscolops* but across the Convolutidae. Different from prostatoid organs, the tightly packed, secretion filled needles are connected to a glandular vesicle similar to those of the male copulatory organ. The positioning of the accessory stylet resembles

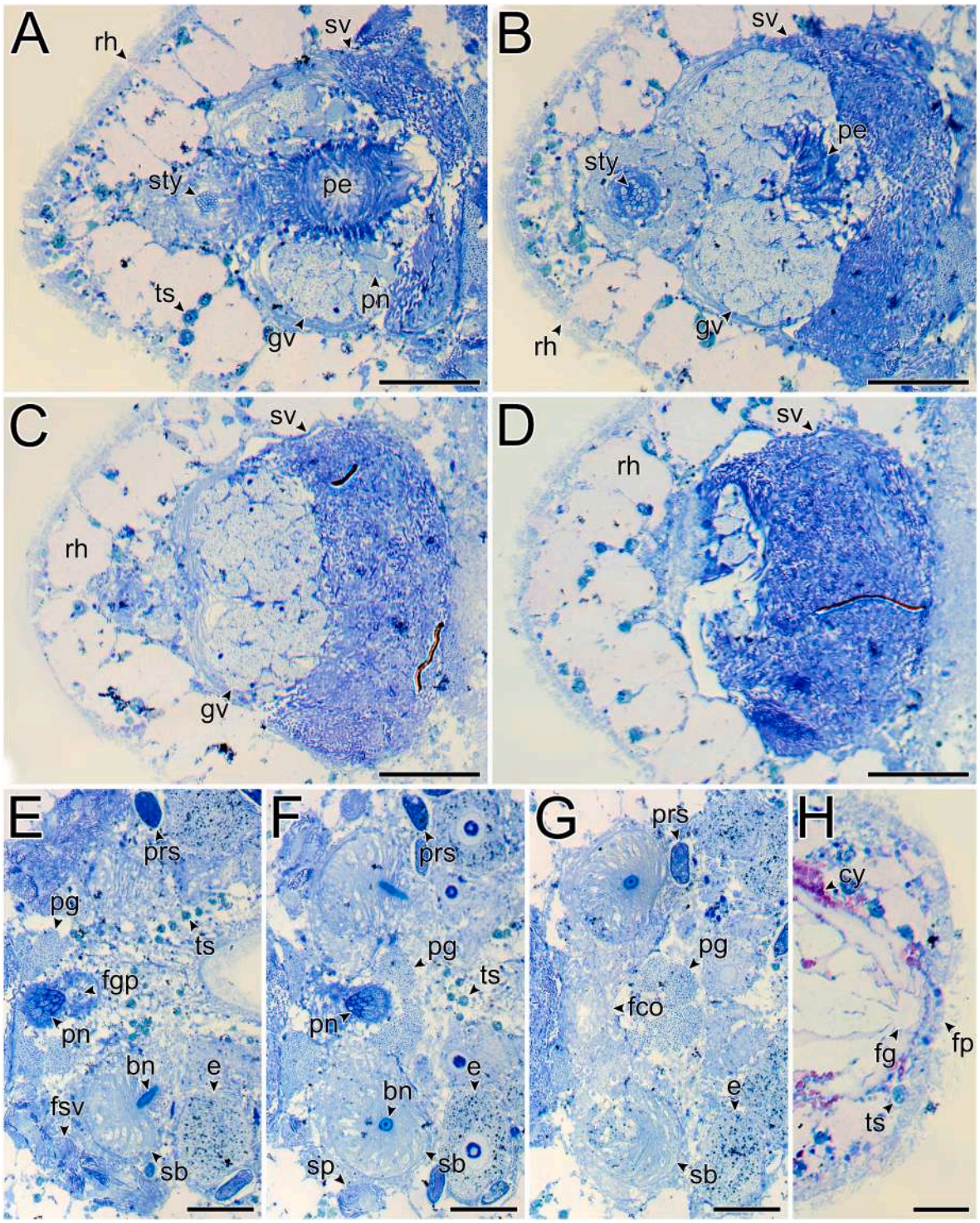

Fig. 6. Frontal histological sections of *Amphiscolops ranshimensis* n. sp. isolate H24R52 stained with 1% Toluidine Blue O. **A–D** A series of histological sections through the male copulatory organ, each section is 37.5 -µm apart. **E–G** A series of histological sections through the female copulatory organ, each section is 25 -µm apart. **H** Frontal glands opening to the frontal pore. Scale bars: **A**–**G** = 50 µm; **H** = 25 µm. Abbreviations: **bn**, bursal nozzle; **cy**, cyanophilic globules; **e**, eggs; **fg**, frontal glands; **fgp**, female gonopore; **gv**, glandular vesicle; **ma**, male antrum; **m**, mouth; **mgp**, male gonopore; **pe**, penis; **pg**, prostatoid glands; **pn**, prostatoid organ needles; **prs**, *Prorocentrum* symbiont; **rh**, rhabdoid gland; **sb**, seminal bursa; **sty**, stylet needles; **sv**, seminal vesicle; **ts**, *Tetraselmis* symbionts.

the childiid stylet-like penis, where it lies in a passage that opens on the ventral side of the animal, near the posterior end (Tekle et al., 2007). Unlike the stylets of childiids, the accessory male stylet of *A. ranshimensis* is not a penis, as it does not have a direct connection to the autosperm, nor is there a lumen with which to transfer sperm.

Phylogenetic analysis based on concatenated 18S-28S rRNA gene dataset further supports the placement of this species within *Amphiscolops* (see Fig. 7). *Amphiscolops ranshimensis* sequences formed a highly supported clade with *A. oni* and *A. potocani*. All three species have a trilobed posterior and harbor a green alga and dinoflagellate simultaneously. Another trilobed species, *A. bermudensis*, branched independent of this clade. Still, several species within this group have not been

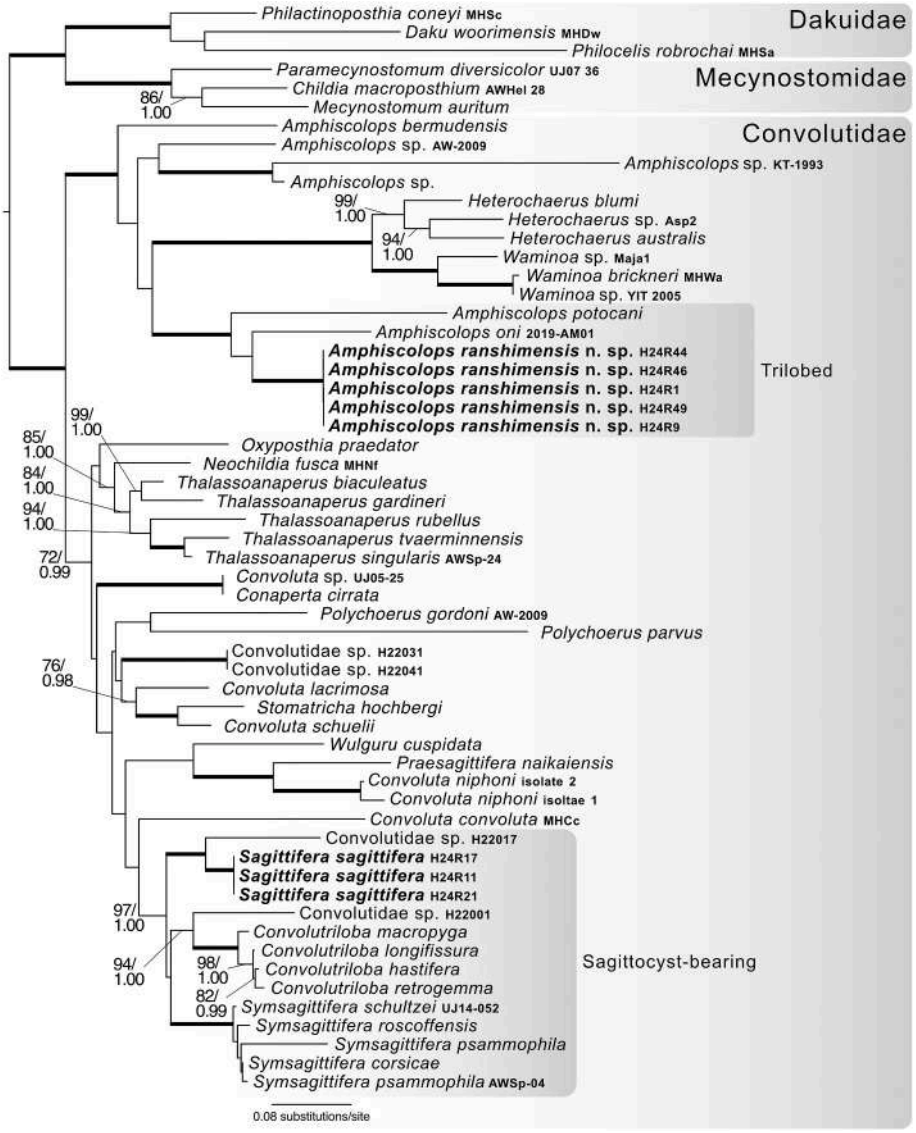

Fig. 7. Maximum-likelihood tree of the Convolutidae inferred from concatenated 18S rRNA and 28S rRNA genes. Bootstrap values (BS) < 70% and Bayesian Posterior Probability (BPP) values < 0.95 were omitted. Fully supported branches (100% BS/1.00 BPP) are represented by thick lines. Sequences generated in this study are in bold.

sequenced. A more diverse molecular dataset for species within this morphologically variable group would be helpful for evaluating taxonomically important morphological traits.

4. Discussion

4.1. Photosymbiosis in acoels in Hokkaido, Japan

Surveys in northern Japan are limited as most research has focused on subtropical areas in Honshu and the Ryukyu Islands (Faubel et al., 2004). From these locations, a relatively high diversity of symbiotic convolutids is represented. This includes *Amphiscolops* (a total of 8 species, 5 undescribed), *Heterochaerus* (1 species), *Haplodiscus* Weldon, 1889 (1 undescribed species), *Praesagittifera* (3 species), *Symsagittifera* (2 species) and *Waminoa* (2 undescribed species) (Faubel et al., 2004). *Amphiscolops ranshimensis* represents the first record of an acoel with symbionts from northern Japan in Hokkaido. Although notable, this is not the highest-latitude record of a symbiotic acoel globally, as *Convoluta convoluta* has been reported from Norway at approximately 60°N (Stöhr, 2024).

4.2. A new dimension to acoel-microalgae symbiosis in Amphiscolops

Amphiscolops ranshimensis highlights a novel form of acoel-microalgal association, simultaneously hosting *Tetraselmis convolutae* (Chlorodendrophyceae) and *Prorocentrum concavum* (Prorocentrales). The occurrence of two different species of microalgal symbionts within a single acoel host is relatively rare, previously documented only in the genera *Waminoa* (Ogunlana et al., 2005; Hikosaka-Katayama et al., 2012) and *Amphiscolops* (Beltagi and Khafagi, 1984; Achatz, 2008; Asai et al., 2022). All dinoflagellates sequenced from these symbiotic species to date belong to either the Symbiodiniaceae or *Amphidinium* (Ogunlana et al., 2005; Asai et al., 2022), marking *A. ranshimensis* as the first reported to host symbiotic *Prorocentrum*. To our knowledge, this also represents the first documented occurrence of the Prorocentrales as a symbiotic partner of any animal.

Several *Tetraselmis* species form symbiotic relationships with acoels (Riewluang and Wakeman, 2023), but the specificity of these associations appears to be driven by the host. For instance, *S. roscoffensis* selectively acquires *T. convolutae* (Bailly et al., 2014; Arboleda et al., 2018). Among *Tetraselmis*, *T. convolutae* appears to be broadly associated

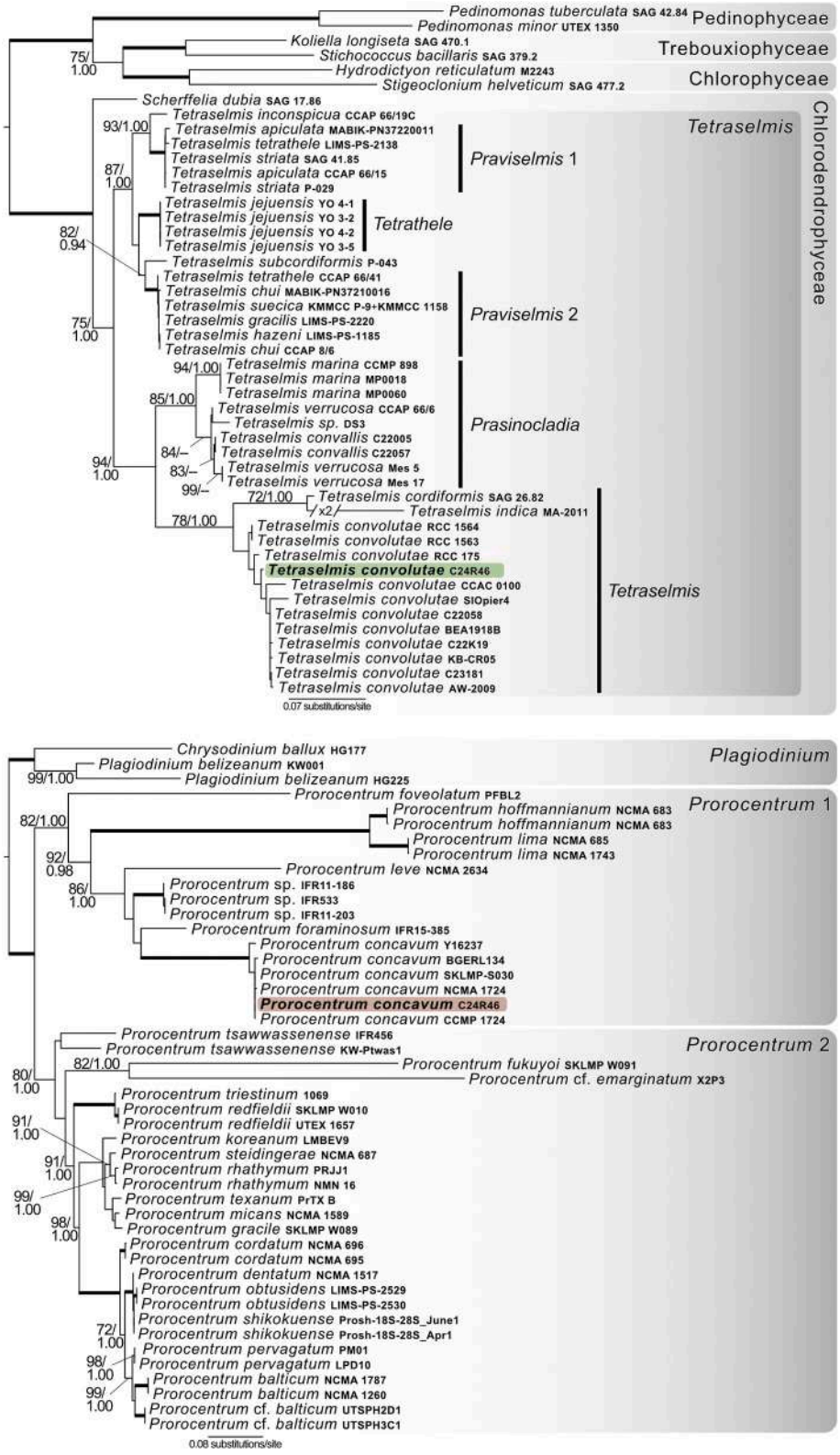

Fig. 8. Maximum-likelihood tree of *Tetraselmis* (top) and *Prorocentrum* (bottom) inferred from concatenated 18S rRNA gene, ITS region, and 28S rRNA gene. Bootstrap values (BS) < 70% and Bayesian Posterior Probability (BPP) values < 0.95 were omitted. Fully supported branches (100% BS/1.00 BPP) are represented by thick lines. Sequences generated in this study are highlighted and in bold.

among convolutids, being documented in the aforementioned *S. roscoffensis* from Europe (Parke and Manton, 1967), multiple undescribed convolutids from southern Japan (Asai et al., 2022; Riewluang and Wakeman, 2023), *Convoluta schuelii* from Thailand (Achatz et al., 2010), and now in northern Japan (present study). Considering the broad geographic range and host variety with which *T. convolutae* associates, it is possible that this particular *Tetraselmis* lineage represents a type of generalist-symbiont. This could be analogous to other algal-invertebrate systems such as coral. In these systems, Symbiodiniaceae also have lineages that are more general or specialized to a

Table 1
Morphological characteristics of species within the genus *Amphiscolops*.

Characters	*A. ranshimensis* n. sp.	*A. hamus* Ono & Kajihara, 2025	*A. oni* Asai et al., 2022	*A. potocani* Achatz, 2008	*A. marinelliensis* Beltagi & Khafagi, 1984	*A. trifurcatus* (Beltagi, 1983)	*A. zeii* Riedl, 1956	*A. japonicus* Kato, 1947	*A. bermudensis* Hyman, 1939	*A. fuligineus* Peebles, 1913	*A. cinereus* (Graff, 1874)
External Features											
Body Shape	Cone-shaped	Cone-shaped	Cone-shaped	Cone-shaped	Cone-shaped	Cone-shaped	Planular	Cone-shaped	Cone-shaped	Planular	Planular
Caudal appendages	Trilobed	Trilobed	Trilobed	Trilobed	Forked	Trilobed	Blunt	Forked	Trilobed	Single	Single
Statocyst	Present	Present	Absent	Present	Present	Present	Present	Present	Present	Present	Present
Eyes/Ocelli/Eye Fields	Present	Present	Absent	Absent	Absent	Absent	Absent	Present	Present	Present	Present
Frontal Organ	Present	Present	Present	Present	Present	Present	Undetermined	Present	Present	Present	Present
Coloration	Green with brown spots from symbiotic algae	Brown from dinoflagellates	Ranges from white, orange, yellow and light brown	Green with brown spots from symbiotic algae and white concrements (variable)	Greenish brown from symbiotic algae	Brown from symbiotic algae	Reddish-brown from pigments	Translucent white	Dark brown from symbiotic algae with refractive concrements (variable)	Distinct brown bands	Grayish blue/green
Mouth	Central	Central	Central	Central (63U)	Central	Anterior (~35U)	N/A	Central	Posterior (~65U)	Central (~40U)	Anterior (~25U)
Symbiotic Algae	*Tetraselmis convolutae* and *Prorocenturm concavum*	Dinoflagellates	*Tetraselmis convolutae* and dinoflagellates	Green algae and dinoflagellates	Green algae and dinoflagellates	Dinoflagellates	N/A	Absent	Dinoflagellates	Absent	Absent
Gonopores											
Male gonopore	Subterminal	Terminal	Subterminal	Subterminal	Subterminal	Subterminal	N/A	Subterminal	Subterminal	Terminal	Terminal
Female gonopore	Present	Absent	Present	Present	Present	Absent	N/A	Present	Present, Paired	Present	Present
Male Copulatory Organ											
Penis	Tubular with male antrum	Tubular, hook-shaped with male antrum	Tubular without male antrum	Tubular without male antrum	Tubular with male antrum	Tubular with male antrum	N/A	Tubular without male antrum	Tubular without male antrum	Tubular with male antrum	Tubular with male antrum
Male Bulb	Seminal vesicle with paired glandular vesicles	Muscular seminal vesicle	Muscular seminal vesicle	Muscular plug	Muscular seminal vesicle	Muscular seminal vesicle	Muscular seminal vesicle	Muscular plug	Muscular seminal vesicle	Penis sac	Muscular seminal vesicle
Accessory Male Organs	Prostatoid organs and accessory male stylet	Absent	Absent	Absent	Absent	Absent	N/A	Absent	Absent	Absent	Absent
Female Copulatory Organ											
Vagina	T-shaped	Absent	Tubular	T-shaped	Tubular	Absent	N/A	Tubular	Paired (lateral), tubular	Tubular	Tubular
Seminal bursa	Paired, unconnected by bursal tissue	Single	Single, bilobed	Paired, unconnected by bursal tissue	Single, bilobed	3–9 sperm balls, unwalled	Single	Single, bilobed	Single, bilobed	Single, bilobed	Single, bilobed
Bursal Nozzles	2	>20	9	2	2	3–9	2, corkscrew-shaped	2	2	2	2

particular coral host or environment (Lien et al., 2012; Hume et al., 2013; LaJeunesse et al., 2018).

Histological sections highlighted the localization of algal symbionts within the host acoel. *Tetraselmis convolutae* was distributed uniformly beneath the epidermis, like other convolutids (Achatz et al., 2007; Achatz, 2008; Asai et al., 2022). *Prorocentrum concavum* was irregular in distribution but was consistently found beneath the layer of rhabdoid cells and near internal structures such as the ovaries and female copulatory organ. The organization of dinoflagellates within *A. ranshimensis* is similar to *A. potocani* (Achatz, 2008), but contrasts with other convolutids such as *A. oni* (Asai et al., 2022), *Waminoa* spp. (Winsor, 1990; Ogunlana et al., 2005) and *Heterochaerus* spp. (Marcus, 1952; Achatz and Hooge, 2006; Achatz et al., 2007) in which all symbionts, regardless of their type, are situated just underneath the epidermal layer. Frontal sections of *A. ranshimensis* also revealed clusters of localized cyanophilic globules in the anterior portion of the specimens. We were unable to identify these structures, as they are not documented in *Amphiscolops* (or other acoel) literature. This raises the intriguing possibility of a third symbiont, possibly bacteria, though this remains ambiguous and warrants further investigation.

CRediT authorship contribution statement

Siratee Riewluang: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Regine C. Manglicmot:** Resources, Methodology, Investigation, Formal analysis. **Shaun Cunningham:** Writing – review & editing, Methodology, Investigation, Data curation. **Matthew D. Hooge:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation. **Rintaro Ono:** Conceptualization, Validation, Writing – review & editing. **Hiroshi Kajihara:** Conceptualization, Writing – review & editing. **Brian S. Leander:** Writing – review & editing, Supervision. **Kevin C. Wakeman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Ethics approval

No permits were required.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2026.02.010.

Data availability

Data will be made available on request.

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