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A New Species of Acoela Possessing a Middorsal Appendage With a Possible Sensory Function

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Acoels, belonging to Xenacoelomorpha, are small worms with a relatively simple body plan and are considered a critical clade for understanding the evolution of bilaterians. Despite acoels' importance, however, many undiscovered species are predicted to be present worldwide. Here, we describe a new marine acoel species, *Amphiscolops oni* sp. nov., based on materials collected from the intertidal and subtidal zones of rocky shores at several localities along the Japanese Pacific coast. The new species is approximately 3 mm long and shows typical characteristics of the family Convolutidae, such as the presence of eyespots, symbiosis with algae, position of the gonopores, morphology of the bursal nozzles, lack of central singlet microtubules in the axonemes of spermatozoa, and funnel-like posture of the anterior end. Based on morphology and the results of molecular phylogenetic analyses, we assign this species to the genus *Amphiscolops*. Interestingly, these worms show unique behaviors such as swimming by flapping the lateral sides and actively capturing prey by swinging the anterior funnel. Furthermore, they possess a dorsal appendage—a characteristic previously unreported in Xenacoelomorpha—representing an evolutionary novelty acquired by this species.

Key words: marine invertebrates, Xenacoleomorpha, Acoelomorpha, acoel, Convolutidae, *Amphiscolops*, taxonomy, Japan

INTRODUCTION

Acoels are a group of small aquatic worms with a very simple body plan, lacking a coelom, excretory organs, anus, and epithelial gut. There are approximately 400 reported species of acoels, but many undiscovered species may be present worldwide. For instance, approximately 10 species have been reported from Japan, but roughly 100 species are predicted to be present in its waters (Tajika and Yamasu, 2003). Acoels were originally regarded as an order within Platyhelminthes (Ehlers, 1986; Haszprunar et al., 1991), but they were subsequently suggested to form the clade Acoelomorpha together with Nemertodermatida, with Acoelomorpha being a sister group to all other bilaterians (Nephrozoa) (Ruiz-Trillo et al., 1999; Telford et al., 2003). Recent molecular phylogenetic analyses support the sister relationship of Acoelomorpha and Xenoturbellida, another group of marine worms once regarded as a member of Platyhelminthes (Hejnol et al., 2009; Philippe et al., 2011). Large-scale phylogenomic studies suggest that this clade, termed Xenacoelomorpha, is a member of the deuterostomes (Philippe et al., 2011, 2019; Kapli and Telford, 2020) or a sister group to the Nephrozoa (Hejnol et al., 2009; Cannon et al., 2016). Therefore, acoels are an important group of animals to questions of the origin and evolutionary history of bilaterians.

Despite their simple body plan, acoels exhibit rich morphological diversity in the digestive system, muscle fibers, and the nervous system (reviewed in Achatz et al., 2013). Traditionally, features of the reproductive system, namely the morphology, position, and components of the reproductive organs such as the gonopore, gonads, and male/female organs, have often been used as taxonomic characteristics (Hooge, 2001; Hooge and Tyler, 2005; Petrov et al., 2006; Achatz et al., 2010; Jondelius et al., 2011). However, these characteristics vary widely even within a family or genus, and some species may not fit the taxonomic definitions of a certain clade. The most extensive study to date, which used both morphology and molecular phylogeny of 126 species, supports the presence of at least nine families: Diopisthoporidae, Paratomellidae, Hofsteniidae, Solenofilomorphidae, Proporidae, Isodiametridae, Dakuidae, Mecynostomidae, and Convolutidae (Jondelius et al., 2011). However, the phylogenetic relationships among these families remain unresolved, and taxonomically credible synapomorphy has not been found in many clades (Jondelius et al., 2011). To solve

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this ambiguous phylogeny within acoels and to overcome the difficulties in determining the taxon of a newly discovered acoel species, more thorough sampling, including new species, and comprehensive analyses, utilizing both molecular and morphological studies, are essential. Furthermore, ecological and behavioral characteristics may offer new insights into the taxonomy of acoels.

We found acoels with a dorsal appendage at Nabeta Bay, Shimoda, Shizuoka, Japan, and subsequently at different locations along Japan's Pacific coast. Morphological observations revealed the presence of a statocyst and a pair of eyespots in juveniles. The sperm had two flagella incorporated within the cell body with axonemes lacking central microtubules (a 9+0 pattern). Symbiotic algae were found, but the worms also actively fed on small crustaceans by dynamically swinging their anterior end toward the prey. Upon stimulation, the animals would swim. We also studied development and performed molecular phylogenetic analyses. Based on these results, we report the collected individuals as a new acoel species, *Amphiscolops oni* sp. nov. The function and evolutionary implications of the protruding dorsal appendage—a feature that has never been reported in acoels—are also discussed.

MATERIALS AND METHODS

Field sampling

From March 2018 to November 2019, eight plastic plates (5 × 7.5 cm) were placed at a depth of approximately 2 m in Nabeta Bay, Shimoda, Shizuoka, Japan (34°39′59.6″N 138°56′18.2″E) (Fig. 1) and changed every month. The retrieved plates were checked under a stereomicroscope for acoel individuals.

Algae, including *Jania adhaerens* and *Halimeda* sp., were collected by hand, snorkeling, or scuba diving from the intertidal and subtidal zones in Shimoda (34°39′55.0″N 138°56′13.2″E), Miura (Kanagawa, 35°09′32.6″N 139°36′39.2″E), and Hachijojima (Tokyo, 33°05′53.5″N 139°46′17.2″E) between May 2018 and July 2019 (Fig. 1). The algae were washed in plastic trays or buckets, and the specimens were collected from these containers. At Shirahama (Wakayama, 33°41′33.2″N 135°20′01.1″E), the worms were collected by hand from rocky shores during low tide in February 2018.

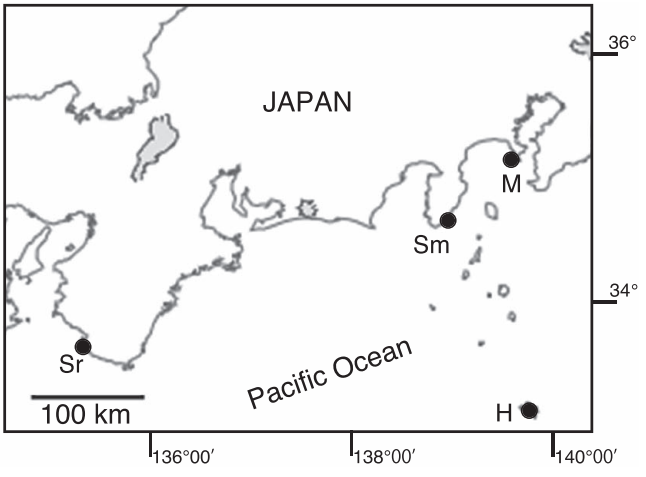


Fig. 1. Sampling locations of *Amphiscolops oni* sp. nov. M: Miura (Kanagawa), Sm: Shimoda (Shizuoka), Sr: Shirahama (Wakayama), H: Hachijojima (Tokyo). Scale bar: 100 km.

Type specimens were deposited in the Department of Zoology, the University Museum, the University of Tokyo (UMUT).

Observation of live specimens

The collected specimens were maintained in aquaria (20 × 13 × 12 cm) containing filtered seawater (FSW) at 23°C under continuous light. They were fed *Artemia* sp. (A&A Marine LLC, Salt Lake City, Utah) daily, and the water was changed every second day.

For observations of the external morphology and behavior, live specimens were placed in glass dishes (diameter, 8 cm; depth, 4 cm) or glass tanks (20 × 13 × 12 cm) filled with FSW, observed under a microscope, and recorded with a digital camera.

To observe the internal morphology of live specimens, droplets of FSW were prepared on glass slides, and individuals were placed in these droplets. After the animals settled on the glass, FSW was replaced with 3.5% $MgCl_2$ in FSW. After 10 min, coverslips were gently placed on the animals, and the slides were observed under a microscope and photographed with a digital camera.

Histological observations

Individuals were relaxed in 3.5% $MgCl_2$ in FSW for 10 min and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 23°C. After five washes with PBS, the samples were stored in PBS at 4°C or in 70% ethanol at –20°C.

Fixed individuals were stained overnight at 4°C with 0.5% eosin in 70% ethanol, dehydrated in a series of ethanol and xylene, and embedded in paraffin. Sections (8 μm) were prepared using a microtome, mounted on glass slides, and stained with hematoxylin and eosin.

For phalloidin staining, fixed individuals were washed five times with 0.5% Triton X in PBS and stained with 0.7% Acti-stain 488 phalloidin (Cytoskeleton Inc., Denver, Colorado) in PBS for > 2 h at 23°C in the dark. The samples were then washed three times with PBS and stored in PBS at 4°C in the dark until observation.

Sample preparation and observation were performed according to the methods described by Sasaki et al. (2019) for scanning electron microscopy and Jokura et al. (2019) for transmission electron microscopy.

Developmental observations

Egg masses found in the aquaria harboring adult specimens were transferred to plastic containers (8.5 × 5.5 cm) with FSW using paint brushes and dissecting needles. The eggs were incubated at 23°C under continuous light. Juveniles began hatching approximately 3 days after isolation. Juveniles from different egg masses were maintained in individual plastic containers (8.5 × 5.5 cm) in FSW at 23°C under continuous light, with daily water change. Starting at 1 week after hatching, the juveniles were fed every day with several species of microscopic benthic crustaceans collected from plastic plates (5 × 7.5 cm) placed in Nabeta Bay. Live juveniles were observed using the same methods as those used to observe adults.

Molecular phylogenetic analyses

Specimens for DNA extraction were fixed in 70% ethanol and stored at 4°C. To avoid contamination of probable symbiotic algal DNA, 10 juveniles hatched from a single egg mass were also fixed.

Specimens were homogenized using disposable pestles in 1.5 mL tubes. Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The *18S rRNA* gene (18S) was amplified using the primers 1F, 3F, 5R, 5F, 9R (Giribet et al., 1996), and bi (Giribet et al., 1999). The *28S rRNA* gene (28S) was amplified using the primers U178, L1642, U1148, 1200F, F1642, UJR2176, L3449, UJ2176 (Wallberg et al., 2007), F2093, R3182, R3239 (Jondelius et al., 2011), and two additional primers designed in the present study, namely 28S_JOIN_F (5′-ATCGTACCGATTACCGCATC-3′) and 28S_JOIN_R (5′-GTCTCACCCAGCCTTCAGAG-3′). The *COI*

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gene was amplified using the primers LCO1490 and HCO2198 (Folmer et al., 1994). PCR was performed as described by Wallberg et al. (2007) and Folmer et al. (1994) using Ex*Taq* and LA*Taq* (TaKaRa Bio, Kusatsu, Japan). The PCR products were purified using exonuclease I and alkaline phosphatase (Calf intestine) (Takara Bio) or the QIAquick PCR Purification Kit (Qiagen). The purified PCR products were sequenced by FASMAC (Atsugi, Japan). The sequences were deposited in GenBank under the accession numbers LC632956–LC632959.

Phylogenetic analysis was performed using concatenated alignment of the nucleotide sequences of 18S and 28S from 25 Acoelomorpha species (21 Convolutidae species, three Mecynostomidae species as outgroup, and the new species. Another analysis using concatenated alignment of 18S, 28S, and amino acid sequences of COI from 70 Acoelomorpha species (69 known species plus the new species) was also performed. Sequences were aligned with MAFFT (mafft-linsi) v7.017 (Katoh and Standley, 2013) with default parameters, and the ambiguous sites in the alignments were excluded using Gblocks, with the -b5 = a option (Castresana, 2000). Maximum likelihood trees were constructed using RAxML v8.2.12 with 100 bootstrap replicates and a partitioned model (Stamatakis, 2014). The GenBank accession numbers of the sequences used in the analyses are listed in Supplementary Table S1.

RESULTS

Taxonomy

Amphiscolops oni sp. nov.
(Figs. 2–8)
New Japanese name:
oni-muchou-uzumushi

Diagnosis

Amphiscolops oni sp. nov. is characterized by a rounded anterior end, three posterior caudal lobes, a frontal organ, and a middorsal appendage standing upright. Specimens with two middorsal appendages are present but rare. A single female gonopore is present posterior to the midventral mouth and the male gonopore is located near the median caudal lobe. About nine bursal nozzles are present in the seminal bursa.

Material examined

Holotype: UMUT_RW33800, whole specimen of an adult individual, fixed in 4% paraformaldehyde in PBS, collected on 24 April 2019 by MA. Type locality: Nabeta Bay, Shimoda, Shizuoka, Japan (34°39′59.6″N 138°56′18.2″E, 2 m depth). Paratypes: UMUT_RW33801-RW33804, collection data same as the holotype. Additional mate-

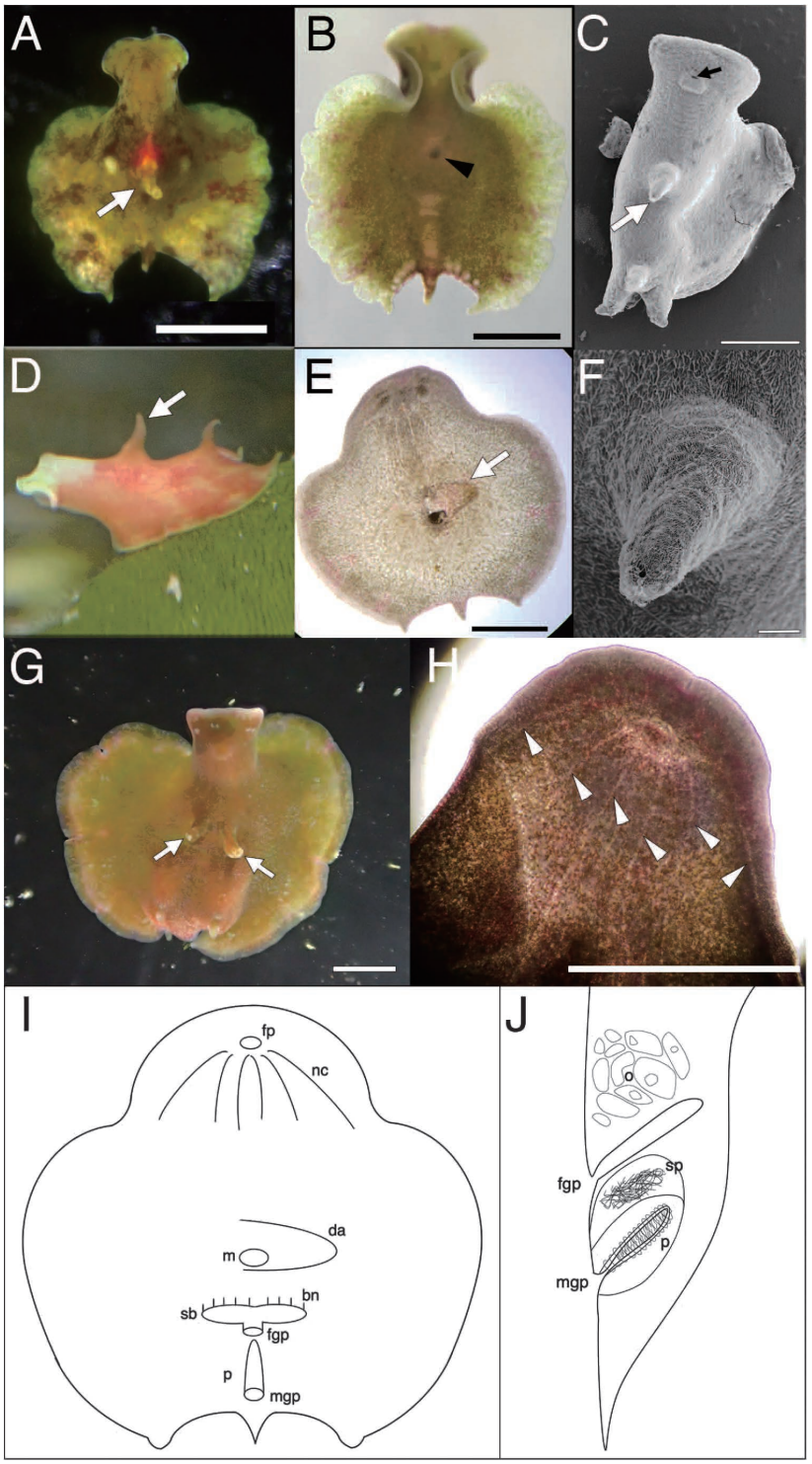


Fig. 2. Morphology of *Amphiscolops oni* sp. nov. **(A)** Dorsal view. **(B)** Ventral view. **(C)** Scanning electron micrograph, dorsal view. **(D)** Left view. **(E)** Dorsal view of an individual pressed under a coverslip. **(F)** Scanning electron micrograph of the dorsal appendage. **(G)** Dorsal view of an individual with two dorsal appendages. **(H)** Anterodorsal view of an individual pressed under a coverslip. White arrows: dorsal appendage; black arrow: frontal pore; black arrowhead: mouth; white arrowheads: longitudinal nerve cords. Schematic drawings showing **(I)** dorsal view of the arrangement of organs and **(J)** the reproductive organs at the median sagittal section. bn, bursal nozzle; da, dorsal appendage; fgp, female gonopore; fp, frontal pore; m, mouth; mgp, male gonopore; nc, nerve cord; o, oocytes; p, penis; sb, seminal bursa; sp, sperm. Scale bars: 1 mm **(A, B, E, G, H)**; 500 μm **(C)**; 50 μm **(F)**.

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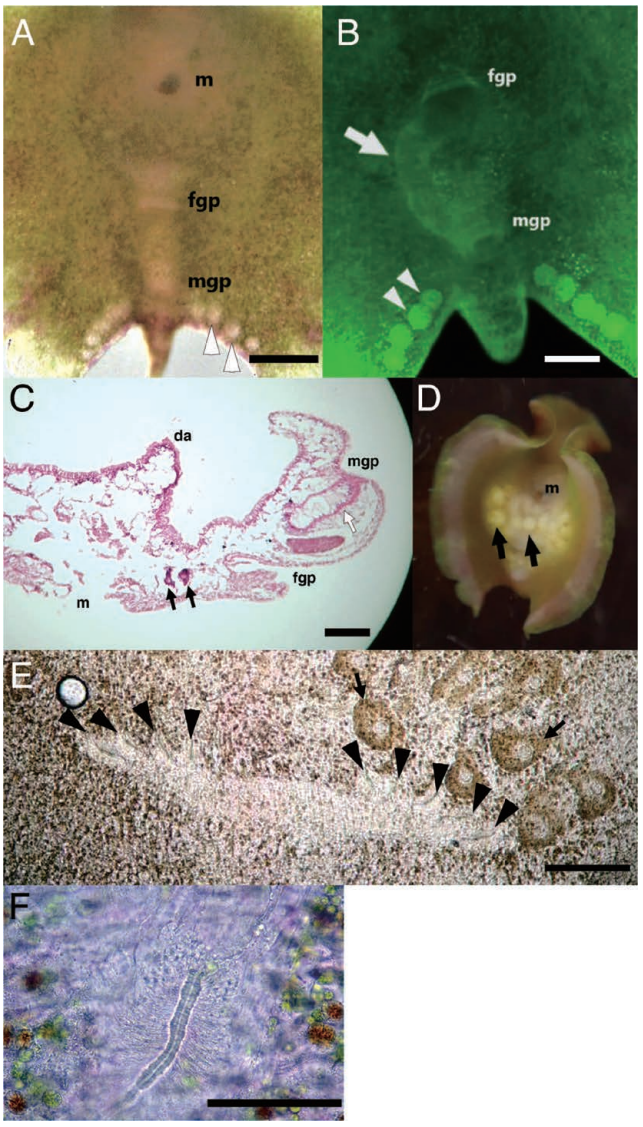


Fig. 3. Reproductive organs of *Amphiscolops oni* sp. nov. **(A)** Posteroventral view. **(B)** Internal structures revealed by phalloidin staining. **(C)** Longitudinal section of the midposterior part of an individual with the posterior tip curved toward the top of the image. **(D)** Ventral view of an individual whose mature eggs could be observed externally. **(E)** Bursal nozzles and **(F)** a close-up of one of the bursal nozzles of an individual pressed under a coverslip. da: dorsal appendage; fgp: female gonopore; m: mouth; mgp: male gonopore; white arrow: penis; white arrowheads: spherical structures; black arrows: eggs; black arrowheads: bursal nozzles. Scale bars: 200 μm **(A, B, C, E)**; 100 μm **(F)**.

rial: More than 50 specimens collected, fixed, and stored in 70% ethanol or PBS.

Etymology

The species is named after "oni", a *yokai* or monster with one or two horns in Japanese folklore.

Description

General morphology: Anterior end rounded. Body widens about one-third from the anterior end, with the posterior end divided into three caudal lobes (Fig. 2A, I). Body length,

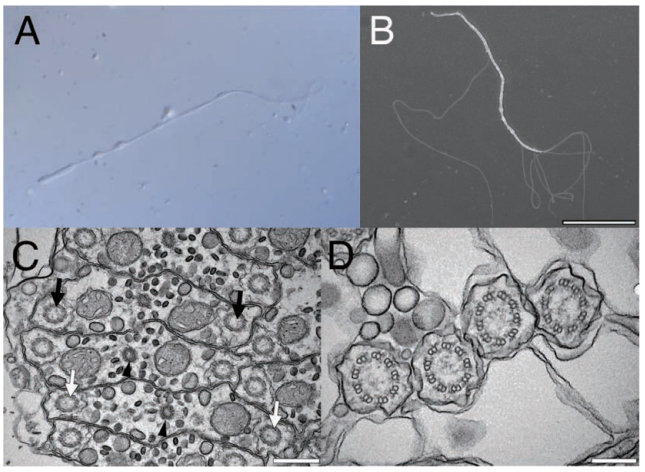


Fig. 4. Sperm of *Amphiscolops oni* sp. nov. **(A)** Light microcopy and **(B)** scanning electron microscopy of the sperm. **(C)** Transmission electron micrograph of the shaft section of a bundle of sperm. A pair of axonemes present in a single sperm is shown with black and white arrows, respectively. Black arrowheads: axial microtubules. **(D)** Transmission electron micrograph of axonemes of sperm. Lack of central singlet microtubules in the axonemes can be seen. Both outer and inner dynein arms are observed. Scale bars: 20 μm **(B)**; 500 nm **(C)**; 200 nm **(D)**.

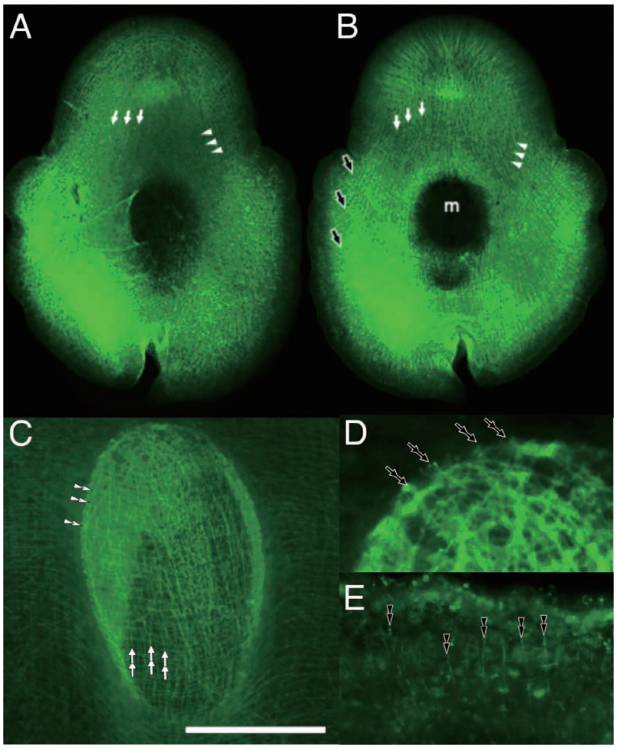
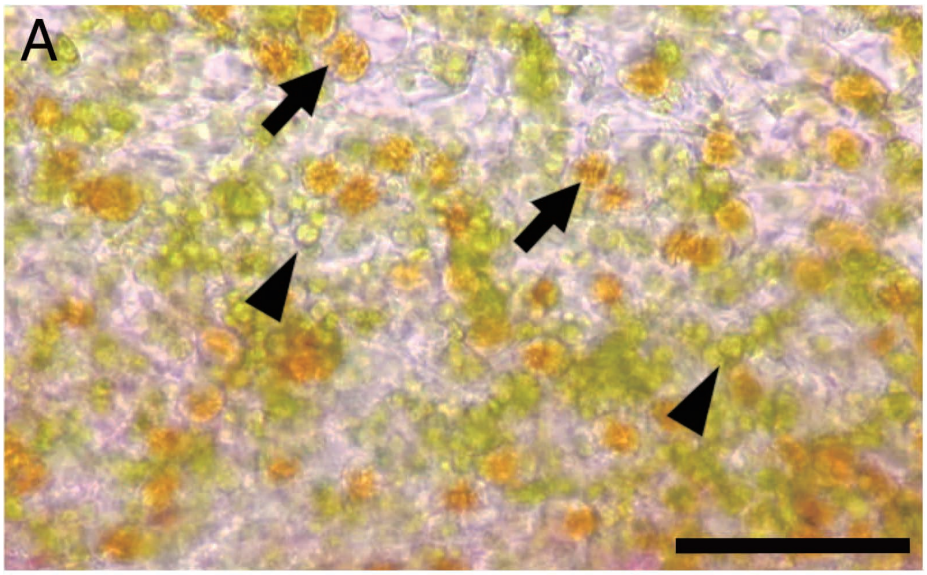


Fig. 5. *Amphiscolops oni* sp. nov. stained with phalloidin. **(A)** Dorsal and **(B)** ventral views. White arrows: longitudinal muscle fibers; white arrowheads: circular muscle fibers; black arrows: U-shaped muscle fibers enwrapping the mouth (m). **(C)** Dorsal appendage. Dense vertical (double white arrows) and horizontal (double arrowheads) muscle fibers are present. **(D)** A close-up of the dorsal appendage. Projections above the muscles of the body wall are present (double black arrows). **(E)** Close-up of the dorsal appendage of an individual pressed under a coverslip. Double black arrowheads: actin-rich collars of sensory receptors. Scale bar: 200 μm.

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B

Sequences producing significant alignments:

Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession
Tetraselmis sp. KB-CR05 small subunit ribosomal RNA gene	1243	1243	100%	0	100	MH055452.1
Tetraselmis sp. SIOpier4 small subunit ribosomal RNA gene	1242	1242	99%	0	100	MH071711.1
Uncultured Chlorophyta clone G0Esp_4_29 18S ribosomal RNA gene	1221	1221	100%	0	99.27	KC911760.1
Prasinophyte sp. Xmm38S5 18S ribosomal RNA gene	1216	1216	100%	0	99.13	KU561213.1
Uncultured marine eukaryote clone SA2_1E12 18S ribosomal RNA gene	1216	1216	100%	0	99.13	EF527125.1
Uncultured eukaryote clone T4A4_4 18S ribosomal RNA gene	1214	1214	99%	0	99.41	HQ394044.1
Uncultured marine eukaryote clone SA2_4E2 18S ribosomal RNA gene	1213	1213	100%	0	99.13	EF526921.1
Tetraselmis sp. KB-FL40 small subunit ribosomal RNA gene	1207	1207	100%	0	98.84	MH055449.1
Tetraselmis sp. KB-FL46 small subunit ribosomal RNA gene	1207	1207	100%	0	98.84	MH055445.1
Tetraselmis sp. KB-FL40 small subunit ribosomal RNA gene	1203	1203	100%	0	98.69	MH055448.1

Fig. 6. Symbionts of *Amphiscolops oni* sp. nov. **(A)** Epidermis of a live specimen pressed under a coverslip. Many green granules (arrowheads) and red-brown granules (arrows) are present. **(B)** BLAST search results of contaminating 18S rRNA sequences.

from the anterior tip to the tip of the lateral caudal lobe, 2.8 mm (± 0.3 mm, *n* = 5). Mouth present midventrally (Fig. 2B, I). Frontal organ present (Fig. 2C, I). Appendage 0.3–0.6 mm in length, present at the middorsal position, standing upright, and like the median caudal lobe, is an out-pocketing of the body wall (Figs. 2A, C–F, I; 3C). Specimens with two middorsal appendages were found but rare (Fig. 2G). Sensory receptors were observed in this structure, their actin-rich collars evident in phalloidin-labeled fluorescence preparations (Fig. 5D, E). Three pairs of lines, likely longitudinal nerve cord structures based on similarities to the structures of *Convolutriloba* (Sikes and Bely, 2008), extend from near the anterior end to approximately one-third of the body length (Fig. 2H, I). Eye spots and statocysts were not observed even in individuals pressed under coverslips.

Coloration: In life, basic body color ranges from white, orange, yellow, or light brown (Fig. 2A, D, G), with red, orange, or green patterns. Significant variation was observed in both basic body color and color pattern even among individuals collected from the same site.

Reproductive organs: At the ventral midline, the female gonopore is present posterior to the mouth, and the male gonopore is located more posteriorly, near the median caudal lobe (Figs. 2I, J; 3A–C). Between the gonopores, a penis with ciliated epithelium is present internally (Figs. 2I, J; 3B, C). Circular and longitudinal muscle layers are present in the penis. In large specimens, approximately 21–23 eggs were observed inside the body, posterior to the mouth (Fig. 3D). In specimens pressed under coverslips, seminal bursa was present posterior to the egg mass. The seminal bursa was bilobed, with about nine bursal nozzles present ventrally, four or five on each side (182.4 ± 11 µm, *n* = 9) (Figs. 2I, J; 3E, F).

Sperm was obtained from dissected adult individuals (Fig. 4A, B), and the internal structure was observed using transmission electron microscopy. Cross sections of sperm within the testes of adult individuals revealed an axial microtubule and two flagella incorporated into the cell body (Fig. 4C), with their axonemes showing a 9+0 pattern, lacking the central singlet microtubules typically present in metazoan flagella (Fig. 4D). Each outer doublet microtubule possesses both the outer and inner dynein arms, suggesting that the sperm flagella are motile (Fig. 4D).

Spherical structures: Near the lateral caudal lobes, four to five spherical structures on each side were observed in some specimens (Fig. 3A, B). As the ovaries are situated just posterior to the mouth (Fig. 3D), these spheres are not likely to be eggs or oocytes. To date, these spheres have not been reported in any xenacoelomorphs.

Muscles: Muscle fibers, running latitudinally, longitudinally, and diagonally, are present throughout the body in multiple layers, but are particularly dense at the anterior tip and along the lateral edges (Fig. 5A, B). Muscles surrounding the mouth are present (Fig. 5B). Many U-shaped muscle fibers are present ventrally and along the edges, posterior to the mouth (Fig. 5A, B). The dorsal appendage has muscle fibers running both vertically and horizontally (Fig. 5C), continuations of the circular and longitudinal muscle layers, respectively, of the body wall.

Symbiotic algae: When live specimens were pressed under coverslips, many green (diameter, 9.4 ± 0.7 µm, *n* = 5) and red-brown (19.8 ± 1.1 µm, *n* = 5) granules were observed in the epidermis (Fig. 6A). During 18S sequencing from adult DNA, contamination of the green alga *Tetraselmis* sp. was observed in multiple specimens (Fig. 6B).

Behavior: Animals glided on the substrate using their cilia

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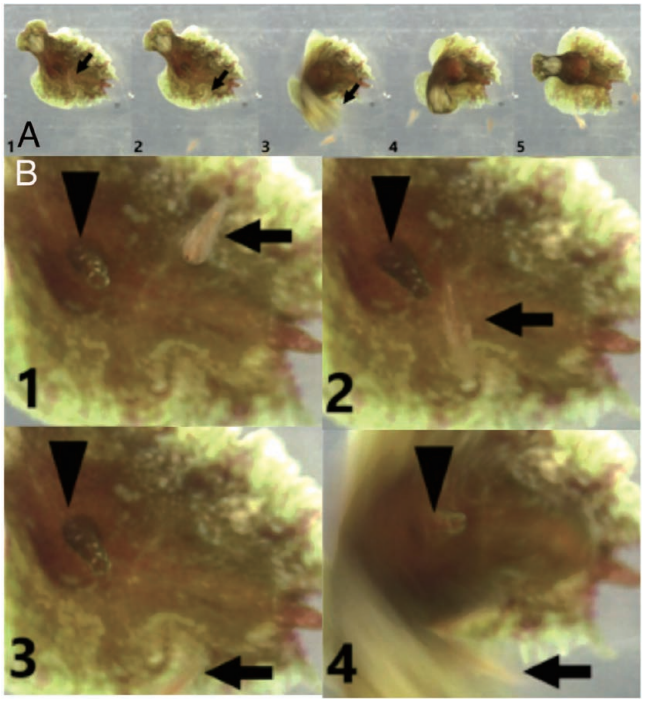


Fig. 7. Feeding behavior of *Amphiscolops oni* sp. nov. **(A)** Dorsal view and **(B)** middorsal section of a feeding individual obtained every 0.01 s. Arrow: captured *Artemia* sp; arrowhead: base of the dorsal appendage.

(see Supplementary Movie S1). When gliding, the anterior end and both lateral sides are in contact with the substrate, while the ventral midline is lifted from the substrate. The width of the anterior and posterior parts of the body is similar during gliding. The gliding motion is similar to that of *Convolutriloba macropyga* and *Convolutriloba longifissura* (Bartolomaeus and Balzer, 1997; Shannon and Achatz, 2007).

When attached to the substrate, the anterior end appeared fan-shaped, with approximately one-fourth of the body lifted from the substrate. The lifted part is shaped like a funnel, with the lateral sides folded inward (Fig. 2A–D). The wide posterior part is attached to the surface. The ventral midline is lifted, such that the body is shaped like a tunnel. Water flows from the anterior to the posterior end of the tunnel.

While attached to the substrate, the animals were able to capture *Artemia* sp. that swam close by or came in contact using the funnel-like structure at the anterior (Fig. 7A, and see Supplementary Movie S2). They could feed on prey swimming beside or behind their body by rapidly swinging the funnel-like structure to catch the prey while remaining attached to the substrate (Fig. 7A). Immediately prior to the funnel-swinging motion, the dorsal appendage moved slightly (Fig. 7B, and see Supplementary Movie S2).

When physically disturbed, the animals flattened their bodies and flapped their lateral sides like a butterfly, enabling them to swim in the water column (see Supplementary Movie S3).

Development

Egg masses were found on the walls and bottom of the aquaria or on algae within the aquaria harboring adult specimens (Fig. 8A, B). Egg masses were covered in mucus and had a diameter of 2.03 mm (± 0.22 mm, n = 3). Each egg mass contained approximately 30 white eggs (± 1, n = 3), with a diameter of 242 μm (± 3.12 μm, n = 32). Immediately after spawning, some eggs in the egg mass had already undergone cleavage, with some having at least four blastomeres (Fig. 8B).

Three days after spawning, the white embryos elongated and folded ventrally, and they began to rotate within

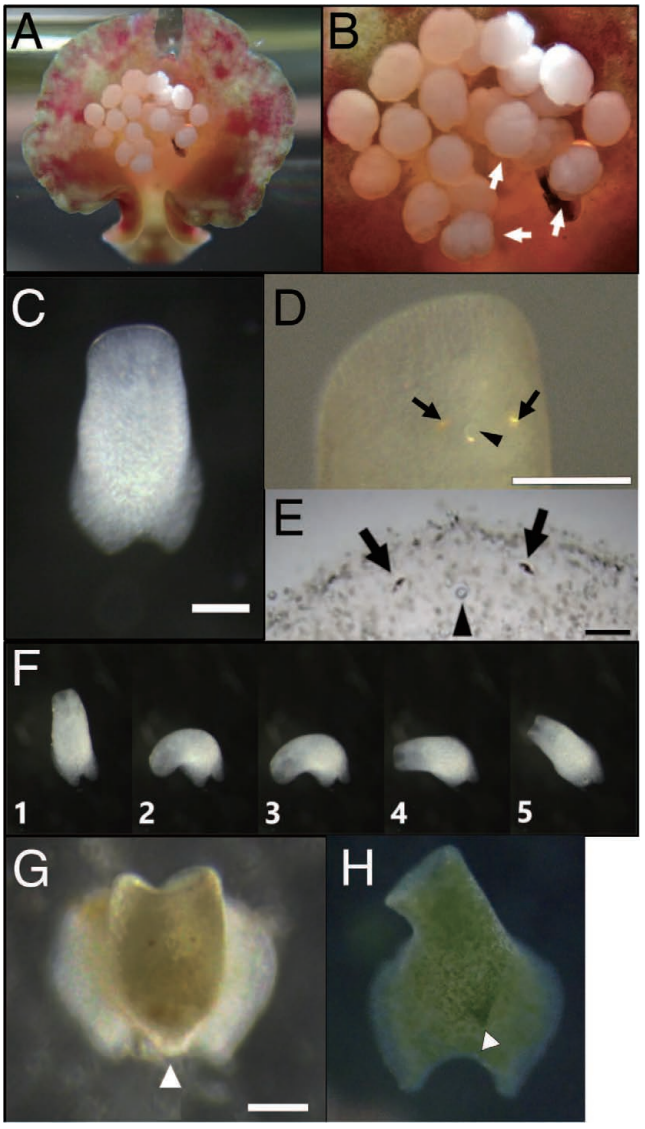


Fig. 8. Development of *Amphiscolops oni* sp. nov. **(A)** An adult individual just after spawning on the wall of an aquarium. The water surface is to the top of the photograph, and the individual is facing downward. **(B)** Spawned egg mass. Some of the embryos were already at the cleavage stage (white arrows). **(C–F)** Juvenile just after hatching. **(C)** Dorsal view of a juvenile settled to the bottom. **(D)** Antero-dorsal view and **(E)** anterior part of a juvenile pressed under a coverslip. Black arrows: eye spots; black arrowhead: statocyst. **(F)** A settled juvenile swinging the anterior part of the body toward animals that came close, obtained every 0.05 s. **(G–H)** Dorsal view of a juvenile settled to the bottom at 1 **(G)** and 3 **(H)** weeks after hatching. White arrowheads: dorsal appendage. Scale bars: 100 μm.

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the egg shell using their cilia.

Approximately 3–4 days after spawning, juveniles began to hatch. Juveniles could swim using their cilia, but soon settled on a substrate. When settled, the posterior one-third of the body was attached to the substrate and the anterior part lifted (Fig. 8C). At this stage, the body length was 448 μm (± 19.7 μm, $n = 5$) and the body width was 266 μm (± 16.6 μm, $n = 5$) when attached to the substrate. Immediately after hatching, juveniles presented a rounded anterior tip and a pair of caudal lobes. Their body was translucent white, with two light brown eye spots and a statocyst near the anterior tip (Fig. 8D, E). The dorsal surface was smooth with no humps or appendages (Fig. 8C). The settled juveniles could swing the anterior part of the body toward animals that came close (Fig. 8F). When disturbed, the individuals swam away or glided on the substrate, with the ventral midline lifted from the substrate in an arch-like manner.

One week after hatching, the juveniles reached approximately 500 μm in length and approximately 380 μm in width. A rudiment of the dorsal appendage was formed at the mid-dorsal position (Fig. 8G). From around this time, they could feed on small benthic crustaceans by swinging the anterior part of their body to catch the prey.

Two weeks after hatching, some individuals turned green. Three weeks after hatching, the green color darkened, and the body length reached approximately 1 mm. There were only two caudal lobes, but the dorsal appendage was apparent (Fig. 8H). These individuals showed behavior identical to mature adults: they fed on small crustaceans by swinging the anterior part of their body, which was lifted from the substrate, forming a funnel. All juveniles died by 4 weeks after hatching.

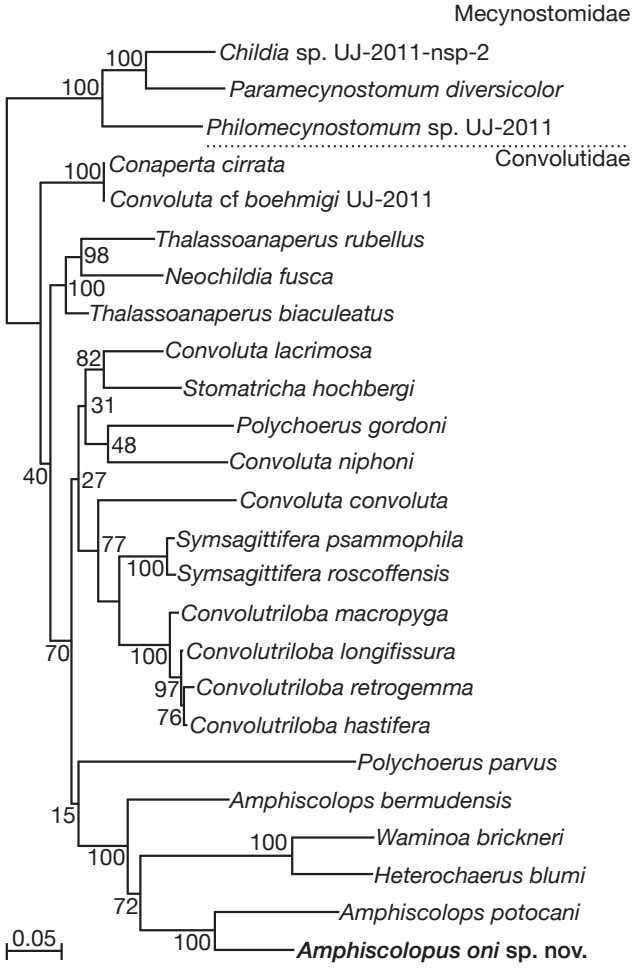


Fig. 9. Phylogenetic tree of the family Convolutidae inferred from the concatenated alignment of 18S and 28S sequences with species of Mecynostomida as outgroup. Bootstrap values from RAxML are shown at each node.

Phylogeny

Results of molecular phylogenetic analyses strongly supported the affiliation of *A. oni* sp. nov. to the Convolutidae (Fig. 9), a family whose monophyly was shown in previous studies (Jondelius et al., 2011; Achatz et al., 2010). Within the family, its placement in the previously reported monophyletic clade (clade F in Achatz et al., 2010) comprising three genera (*Amphiscolops*, *Heterochaerus*, and *Waminoa*), and a sister group relationship to *Amphiscolops potocani* were both supported with high bootstrap value (BP = 100) (Fig. 9). Analysis employing additional COI sequences did not improve the resolution of the phylogenetic position of *A. oni*, and addition of the new species did not largely affect the overall topology within Acoela in comparison with previous studies (Jondelius et al., 2011) (see Supplementary Figure S1).

DISCUSSION

Morphology

Dorsal appendage: Amphiscolops oni sp. nov. possesses a ciliated dorsal appendage with sensory receptors, a feature previously unknown in Xenacoelomorpha (Figs. 2A–G, I; 5D, E). The animals move this appendage before capturing *Artemia* sp., even when the prey does not touch the individual (Fig. 7). As individuals could sense their prey without being in direct contact, including the ones behind them, we believe that the dorsal appendage is a sensory organ that perceives the surrounding water flow using cilia. The dorsal appendage may improve the efficiency of feeding by extending the range in which the receptor cilia receive the stimuli upward and by enabling three-dimensional sensing of the prey while lying in ambush.

Eyespots/ocelli: Statocysts and eyespots were not observed in adult specimens but were prominent in juveniles (Fig. 8D). This is also reported in other convolutids, including several *Convolutriloba* species and *Waminoa brickneri* (Bartolomaeus and Balzer, 1997; Ogunlana et al., 2005; Shannon and Achatz, 2007).

In acoels, eyespots/ocelli are present in multiple Convolutidae species and two Isodiametridae species; however, their structure greatly differs between the two families (Hooge and Tyler, 2005). In Convolutidae, the eyespots lack cilia and comprise pigment cells that contain reflective platelets embedded within the epidermis (Yamasu, 1991). Conversely, in Isodiametridae, eyespots lack the platelets and comprise a region of ciliated pigmented epidermal cells (Lanfranchi, 1990). In *A. oni* sp. nov., the eyespots of juveniles were reflective and clearly embedded within the epidermis (Fig. 8C, D). Therefore, although morphological observation of platelets is awaited, the eyespots of *A. oni* sp.

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nov. are more similar to those of Convolutidae species than to those of Isodiametridae species.

Reproductive organs: The reproductive organs of acoels have traditionally been used as taxonomic characteristics (Dörjes, 1968; Jondelius et al., 2011) and a non-cellular seminal bursa with multiple bursal nozzles directed frontally have been reported as one of the features of *Amphiscolops* (Achatz et al., 2010). This was also observed in *A. oni* sp. nov (Fig. 3). On the other hand, a wide variety in the morphology of the reproductive organs has been reported within the genus *Amphiscolops* (Achatz et al., 2010). *Amphiscolops oni* sp. nov. possesses a single female gonopore posterior to the mouth and a subterminal male gonopore, both along the ventral midline (Figs. 2I, J; 3A–C), an arrangement similar to other congeneric species such as *A. potocani* (Achatz, 2008) and *Amphiscolops japonicus* (Kato, 1947), but different from *Amphiscolops bermudensis*, in which the female gonopore is paired (Hyman, 1939). Bursal nozzles are sclerotized ducts through which the sperm is transported to the oocytes and their number varies across species (Jondelius et al., 2011). *Amphiscolops oni* sp. nov. possesses about nine bursal nozzles (Fig. 3E), compared with two in *A. bermudensis* (Hyman, 1939), two in *A. potocani* (Achatz, 2008), four in *A. japonicus* (Kato, 1947), and eight in *Amphiscolops trifurcatus* (Beltagi, 1983).

Acoel spermatozoa possess two flagella incorporated within the cell body, and most species possess two central microtubules in the axonemes, presenting a 9+2 structure commonly observed in eukaryotes. However, Mecynostomidae species possess only a single central microtubule, resulting in a 9+1 structure, whereas Convolutidae species lack the central microtubules, resulting in a 9+0 structure (Achatz et al., 2010, 2013). The flagella of *A. oni* sp. nov. spermatozoa presented a 9+0 structure (Fig. 4C, D), strongly implying that the species belongs to Convolutidae.

Symbiotic algae

Within Acoela, the presence of symbiotic algae in the epidermal cells has been reported in multiple Convolutidae species, with most species being associated with a single symbiotic species (Achatz et al., 2010). *Waminoa litus* and *W. brickneri* are associated with two species of dinoflagellates within a single host, whereas several species in the genus *Amphiscolops* are associated with both a dinoflagellate and a green alga within a single host (Achatz et al., 2010). The symbiotic green alga present in the epidermal cells of *A. oni* sp. nov. is a *Tetraselmis* sp. (Fig. 6A, B), different from the *Tetraselmis* sp. previously reported to be a symbiont of *C. longifissura* (Bartolomaeus and Balzer, 1997). It would be interesting to compare the evolutionary history of the symbiont *Tetraselmis* spp. and their host acoels. Interestingly, based on light microscopy of the epidermis of *A. oni* sp. nov., a second symbiont of different color (red-brown) and size is present (Fig. 6A), and likely to be a dinoflagellate as in other *Amphiscolops* species. This dinoflagellate was not identified by gene sequencing, but the PCR conditions may not have been appropriate for its detection.

Behavior

When individuals were attached to a substrate, their anterior part was lifted, forming a funnel (Fig. 2A–D). This funnel-like structure has also been reported in several *Convolutriloba* species (Convolutidae) (Bartolomaeus and Balzer, 1997; Shannon and Achatz, 2007). However, in species of *Convolutriloba*, the funnel opens broadly at the anterior and is wider than the posterior part of the body (Bartolomaeus and Balzer, 1997; Shannon and Achatz, 2007). In *A. oni* sp. nov., the funnel was narrower than the posterior part of the body (Fig. 2A, B). There were also differences in the manner in which the funnel was used during feeding. In *C. macropyga*, *C. longifissura*, and *Polychoerus carmelensis* (Convolutidae), the funnel is pressed down to the substrate when the prey enters through the anterior opening, capturing the food between the body and the substrate (Bartolomaeus and Balzer, 1997; Shannon and Achatz, 2007). In *A. oni* sp. nov., the funnel is rapidly swung around toward the prey to capture it within the funnel itself without pressing the structure to the substrate (Fig. 7, and see Supplementary Movie S2).

Convolutriloba longifissura floats in the water column (Bartolomaeus and Balzer, 1997), but there have been no reports of convolutids that swim by flapping the sides of the body, similar to polyclad turbellarians, as observed in *A. oni* sp. nov. (see Supplementary Movie S3).

Only limited reports on acoel behaviors such as feeding are available, and these have not been widely used as characteristics for classification. The present study shows that some acoels exhibit unique and interesting behaviors, such as prey capturing and swimming, and implies that behavior may be useful for assisting taxonomic studies of acoels.

Development

Nearly all acoels reproduce by mating and internal fertilization. Most species then lay fertilized eggs in which cleavage has not started. However, in some species such as *Diopisthoporus brachypharyngeus*, cleavage begins within the adult body (Apelt, 1969). In *A. oni* sp. nov., the eggs were already at the cleavage stage immediately after spawning, suggesting that development begins within the adult body (Fig. 8A, B).

Immediately after hatching, juveniles of *C. macropyga* and *A. oni* sp. nov. showed similar morphology, but their behaviors were different. *Convolutriloba macropyga* juveniles constantly swim in the water column (Shannon and Achatz, 2007). In contrast, *A. oni* sp. nov. juveniles, despite being able to swim, remain attached to the substrate immediately after hatching and maintain the same posture as the adults (Fig. 8). Therefore, acoel juveniles may exhibit species-specific behaviors.

Phylogenetic considerations

In acoels, the eyespots/ocelli have been regarded as taxonomically relevant characteristics, reported only in Convolutidae and Isodiametridae. To distinguish between the two families, algal symbiosis has been proposed as a useful diagnostic feature for classification, being present only in the former (Hooge and Tyler, 2005). *Amphiscolops oni* sp. nov. possesses eyespots (Fig. 8D) and is symbiotically associated with the green alga *Tetraselmis* sp. (Fig. 6). Furthermore, it shares various characteristics with species in the Convolutidae, such as the lack of central singlet microtubules in the axonemes of spermatozoa, position of the

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gonopores, morphology of the bursal nozzles, and funnel-like posture of the anterior end (Figs. 2–4) (Achatz et al., 2013). Lastly, its characteristics are consistent with the family-specific diagnoses proposed for Convolutidae by Jondelius et al. (2011). Based on these observations, we regarded it as a member of the Convolutidae. This was also supported by the result of our molecular phylogenetic analysis (Fig. 9, and see Supplementary Figure S1), which further suggested its close relationship with *A. potocani*. However, *A. oni* sp. nov. possesses a dorsal appendage—a feature previously unknown in Xenacoelomorpha. Moreover, the number of bursal nozzles differs from that previously reported for Convolutidae species. Furthermore, it shows unique behaviors of swimming by flapping its lateral sides and actively capturing its food by swinging the anterior funnel. Therefore, we regarded this as a new species belonging to Convolutidae. Of the specific features, the dorsal appendage is of particular interest. Previously reported acoel sensory organs consist of only the statocyst, eyespots/ocelli, and single-celled monociliary receptors in the epidermis (Achatz et al., 2013), and the dorsal appendage represents an evolutionary novelty acquired by *A. oni* sp. nov.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

MA and HN conceived and designed the study. MA and HN performed the experiments. HM participated in molecular analyses. RY and KI contributed to electron microscopy. MA, HM, and HN prepared the figures and tables. MA and HN wrote the manuscript. All authors approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs210058)

Supplementary Table S1. List of acoelomorph species used in molecular phylogenetic analyses. Accession numbers and species names were obtained from GenBank, and their classifications were based on the World Register of Marine Species (WoRMS Editorial Board [2021]). Available from http://www.marinespecies.org at the VLIZ (Accessed 25 March 2021; doi:10.14284/170).

Supplementary Movie S1. Video of *Amphiscolops oni* sp. nov. gliding on a surface.

Supplementary Movie S2. Video of *Amphiscolops oni* sp. nov. attached to a surface and preying on *Artemia* sp.

Supplementary Movie S3. Video of *Amphiscolops oni* sp. nov. swimming.

Supplementary Figure S1. Phylogenetic tree inferred from the concatenated alignment of 18S, 28S, and COI sequences. Bootstrap values from RAxML are shown at each node. Family and order names are listed on the right. Asterisks show families that are not monophyletic.

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