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Flatworm cocoons in the abyss: same plan under pressure

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Abstract

While knowledge of early ontogeny in abyssal animals is highly limited in general, it was completely lacking for abyssal, free-living platyhelminths. We discovered flatworm egg-capsules (or "cocoons") on rocks collected at depths of 6176–6200 m on the abyssal slope of the Kuril-Kamchatka Trench, northwestern Pacific. The egg capsules were black and spherical, around 3 mm in diameter, and contained three to seven individuals (n = 4) at the same developmental stage, either the spherical (putative early embryo) or vermiform (putative late embryo) stages. A molecular phylogenetic analysis based on 18S and 28S rRNA sequences revealed that the flatworms belong in suborder Maricola in Tricladida and suggested that they may have colonized from shallow to deep waters. This study provides the deepest record for free-living flatworms and the first information on their early life stages in the abyssal zone, which were very similar to those in shallow-water forms. This similarity in development between the relatively benign shallow-water and the extreme abyssal environments suggests that triclads adapting to the latter faced primarily physiological and/or ecological adaptive challenges rather than developmental ones.

Keywords: deep sea, early development, egg capsule, Japan, ontogeny, Platyhelminthes

Introduction

The life cycles of most abyssal animals are largely unknown, despite their importance in understanding how animals have successfully colonized and adapted to the extreme conditions of the abyssal zone (3500–6500 m depth; [1]). This is especially true of early ontogeny, including embryonic and larval development. Only a few fragmentary studies have described the early life history of representatives of several animal groups at abyssal depths, including barnacles (e.g., [2]), molluscans (e.g., [3]), fishes (e.g., [4]), sponges

(e.g., [5]), hydrozoans (e.g., [6]), and digeneans (e.g., [7]).

Almost nothing is known about abyssal free-living platyhelminths, for which a “potential platyhelminth” found on sunken wood at depths of 5257–5236 m [8] may be the sole currently available information; to date, the deepest certain record is the polyclad *Oligocladus voightae* from 3232 m [9]. Due to their fragility, platyhelminths are unlikely to be collected with coarse sampling gear such as dredges and trawls [9], and information on their development at depths is lacking. Free-living flatworms can vary markedly in ontogeny [10]. In sexual reproduction, they generally produce single or multiple embryos (along with extra-embryonic yolk cells in neoophorans) enclosed in an egg capsule (or "cocoon") attached to a substrate by a secretion from the uterine glands. Embryonic development differs among groups; while polyclads undergo spiral cleavage, triclads and other neoophorans undergo irregular, dispersed cleavage [10]. Most free-living flatworms show direct development, except for several specific groups in Catenulida and Polycladida, and little is known of the later stages of development.

During a deep-sea faunal survey by R/V *Hakuho-maru* (Japan Agency for Marine-Earth Science and Technology; JAMSTEC) along the Kuril-Kamchatka and Japan Trenches, northwestern Pacific, we found many black spherical bodies on rock fragments. Most of these bodies were torn and empty, but several intact ones contained flatworms, indicating they were the egg capsules of abyssal flatworms. In this study, we identified the flatworms to the limit of currently available data using a molecular phylogenetic approach based on partial sequences for the 18S rRNA (18S), 28S rRNA (28S), and cytochrome *c* oxidase subunit I (COI) genes. Here we present these results and briefly discuss insights into their early ontogeny.

Materials and methods

Egg capsules attached to two rock fragments were collected with a beam trawl on 21 September 2023 during cruise KH-23-5 of R/V *Hakuho-maru*, at depths of 6176–6200 m at Station C5 (41°28.411′ N 146°06.803′ E to 41°28.519′ N 146°07.632′ E). Four intact egg capsules were detached from the rocks, and their contents were extracted by pipet, forceps, and a needle. The flatworms thus obtained were fixed in 70% ethanol, 99% ethanol, or Bouin’s fluid; some of them were photographed before fixation. The material studied was deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo under catalog numbers ICHUM8616 and ICHUM8617.

One specimen (ICHUM8616) fixed in 70% ethanol was dehydrated in an ethanol series, cleared in xylene, embedded in paraffin, and serially sectioned sagittally at 7 µm. Sections were mounted on five glass slides, stained with hematoxylin and eosin (HE), and sealed in Entellan New (Merck, Germany) under coverslips. The serial sections were photographed under an Olympus BX51 compound microscope.

DNA was extracted from the whole body of two flatworms, one spherical and one vermiform (for details, see Results and Discussion) by using a NucleoSpin Tissue XS Kit (Macherey–Nagel, Germany). For the COI gene, newly designed primers COI_MarF (CAAATTGGACATCCTGARGTTTATA) and COI_MarR (AATTAATAACGRCGAGGCAT) were used for PCR amplification and cycle sequencing. For the 18S gene, primers SR1 and SR12 [11] were used for amplification, and primers SR3, 18S-b3F, 18S-b4F, 18S-b4R, 18S-b5F, 18S-b6F, 18S-a6R, and 18S-b8F [11–15] for cycle sequencing. For the 28S gene,

primers 28S_1F and 28S_6R [16] were used for amplification, and primers 300F, 300R, 900F, 28S_Rd4.2b [17, 18] and 28S_b5F (TATCCGGTAAAGCGAATGATTAGA, newly designed in this study) for cycle sequencing. PCR amplification conditions for COI with TaKaRa Ex Taq DNA polymerase (TaKaRa Bio, Japan) were 94 °C for 1 min; 35 cycles of 98 °C for 10 s, 42 °C for 30 s, and 72 °C for 50 s; and 72 °C for 2 min. Conditions for 18S and 28S with KOD FX Neo (Toyobo, Japan) were 94 °C for 2 min; 45 cycles of 98 °C for 10 s, 65 °C (18S) or 60 °C (28S) for 30 s, and 68 °C for 1 min; and 68 °C for 2 min. All nucleotide sequences were determined with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). Fragments were concatenated by using MEGA7 [19]. The sequences we determined were deposited in the International Nucleotide Sequence Database (INSD) through the DNA Data Bank of Japan (DDBJ).

The results of a BLAST search [20] for our 18S sequence indicated that the flatworm belongs in Tricladida, and so a concatenated 18S+28S dataset that included both sequences for two of our flatworm specimens, 35 triclad species, and seven outgroup taxa (electronic supplementary material, table S1) was analyzed by maximum likelihood (ML) to infer the position of the abyssal flatworm within Tricladida. The 18S and 28S data were aligned independently by using the "Q-INS-i" strategy [21] in MAFFT ver. 7 [22] (electronic supplementary material, files S1, S2) and then trimmed with MEGA7 to match the shortest length for each gene. Alignment-ambiguous sites were removed with Gblocks ver. 0.91b [23] in NGPhylogeny.fr [24] under the "relaxed" parameters described in [25]. The dataset contained 1458 aligned positions for 18S, 982 for 28S, and 2440 in total (electronic supplementary material, file S3). Methods for selecting the optimal substitution model (GTR+F+R4 for 18S; GTR+F+R5 for 28S), the ML analysis, and drawing the tree were as described by [26].

Results and Discussion

One of the two rock fragments with attached black, spherical egg capsules is shown in figure 1*a*. Most egg capsules had been torn; among four intact egg capsules we observed, one (diameter 3.1 mm; figure 1*b*) contained three spherical-stage individuals (figure 1*c*), which may have been early embryos. The other three (one shown in figure 1*d*, *e*; two measured 3.3 mm in diameter, while the third was not measured) contained seven, four, and three vermiform-stage individuals (figure 1*f*). Serial sections of a vermiform individual (figure 1*g*) showed a posteriorly directed tubular pharynx, a mouth opening near the distal end of the pharynx, and a yolk-filled gut diverticulum, indicating vermiform individuals were late embryos. When we opened the egg capsules, a milky liquid (particulate emulsion?) that might have been yolk was observed along with the flatworms.

The 18S (1760 bp; LC783379 and LC783380) and 28S (1629 bp; LC783381 and LC783382) sequences we obtained were respectively identical between two individuals (one spherical, one vermiform). In the 18S+28S ML tree (figure 2), our flatworm lies in Maricola, a small triclad group containing about 80 described species [27]. Although exact depth information was unavailable for most of the representative maricolan individuals for which sequences were obtained from databases, all except our species in the Maricola clade are freshwater, brackish, or shallow-water taxa, suggesting that a habitat

expansion from coastal regions to the abyssal zone may have occurred in the clade.

The two COI (581 bp, encoding 193 amino acids; LC783383 and LC783384) sequences differed by two nucleotide substitutions, corresponding to intraspecific variation (cf. [28]). This indicates that (1) the spherical and vermiform individuals represent different developmental stages of a single species, and (2) the aggregations of egg capsules on a single rock fragment were laid by at least two adults. In addition, the fact that some egg capsules contained early embryos and others contained late embryos suggests that the egg capsules were laid over a period of time. Video footage recorded during trawling at the sampling site showed a muddy bottom overlain with a lot of rocks and gravel (Takuya Yahagi, The University of Tokyo, personal communication on 18 October 2023), with the hard substrates probably providing a favorable spawning site for the abyssal flatworms.

The egg capsules and early development of maricolans have not been well investigated [29]. Two types of egg capsules are known in the group: a spherical type attaching directly to a substrate (e.g., *Procerodes littoralis*; [30]) and an ellipsoid type attaching to a substrate by a stalk (e.g., *Ectoplana undata* and *Bdelloura candida*; [31, 32]). While the egg capsules we observed were of the former type, they were also relatively large, as spherical egg capsules are generally 0.7–1.7 mm in diameter [33]. The number of worms per egg capsule was within the previously reported range, from one to nine [29, 33]. The spherical early embryos and vermiform late embryos we observed in the egg capsules showed no obvious differences from the early developmental stages known in other triclads.

This study represents the deepest known record of free-living platyhelminths and the first report of early developmental stages in an abyssal free-living flatworm, which, superficially at least, are indistinguishable those in shallow-water forms. It indicates that, in both cases, the shell of the egg capsule tears open during hatching (cf. figure 1*d*), and adult-like juveniles emerge to begin a benthic mode of life. This similarity of early ontogeny between the relatively benign shallow-water and the extreme abyssal environments suggests that in adapting to the latter, flatworms faced primarily physiological and/or ecological challenges rather than developmental ones.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. The specimens studied were deposited in the ICHUM and nucleotide sequences in the INSD. Information related to the phylogenetic analysis is provided in the electronic supplementary material [XX].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. K.K.: conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing, resources, visualization, funding acquisition; A.T.: investigation, methodology, writing—original draft, writing—review and editing.

Both authors gave final approval for publication and agreed to be held accountable for the work

performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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Figure legends

Figure 1. Freshly collected egg capsules (or "cocoons") and flatworms. (*a*) Egg capsules on rock fragment (arrowhead, one egg capsule). (*b*) Partly opened egg capsule containing three spherical-stage flatworms. (*c*) Spherical-stage flatworm extracted from egg capsule. (*d*) Cracked egg capsule containing seven vermiform-stage flatworms (arrow, empty egg capsule). (*e*) Same, half of egg-capsule shell removed. (*f*) Vermiform-stage flatworm (ICHUM8616) extracted from egg capsule, in ventral view; anterior to the left. (*g*) Sagittal section of individual ICHUM8616, HE stained; an, anterior; do, dorsal; gd, gut diverticulum; mo, mouth opening; ph, pharynx; po, posterior; ve, ventral. Scale bars: 10 mm (*a*); 1 mm (*b*–*g*).

Figure 2. ML tree for triclad platyhelminths based on an 18S+28S dataset (2440 positions). Numbers near nodes are Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT; left of slash) and ultrafast bootstrap (UFBoot; right of slash) values as percentages; only values of SH-aLRT ≥70% and UFBoot ≥80% are shown. Scale at bottom indicates branch length in substitutions per site.

Electronic Supplementary Material legends

Table S1 from Flatworm cocoons in the abyss: same plan under pressure

Table S1. Information on the flatworms included in our phylogenetic analysis.

File S1 from Flatworm cocoons in the abyss: same plan under pressure

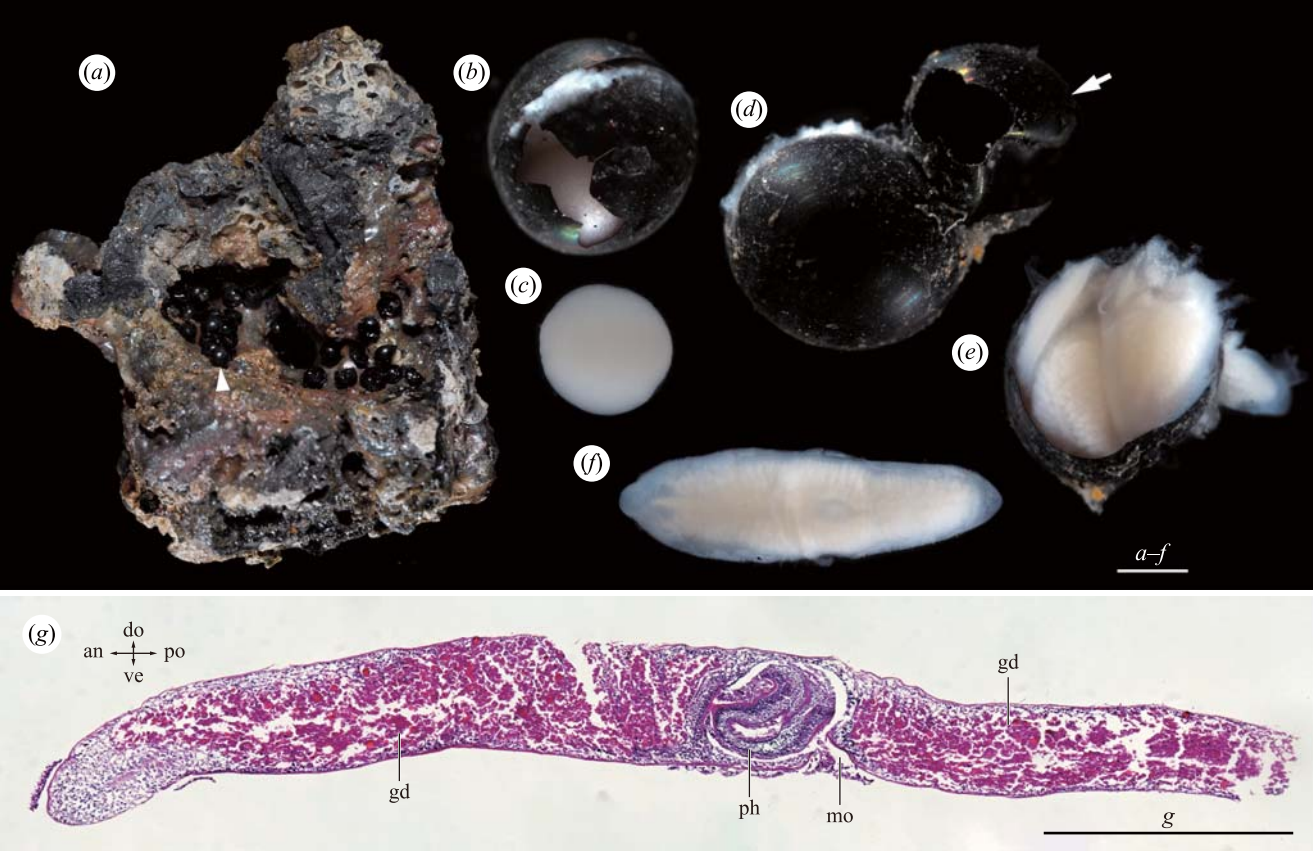
File S1. Aligned 18S sequences used for the maximum-likelihood analysis, trimmed in MEGA7 to the shortest length among the sequences.

File S2 from Flatworm cocoons in the abyss: same plan under pressure

File S2. Aligned 28S sequences used for the maximum-likelihood analysis, trimmed in MEGA7 to the shortest length among the sequences.

File S3 from Flatworm cocoons in the abyss: same plan under pressure

File S3. Concatenated 18S+28S sequences used for the maximum-likelihood analysis, reduced to 2440 positions (1–1458 for 18S; 1459–2440 for 28S) by removing alignment-ambiguous sites with Gblocks under "relaxed" parameters.


(a)
(b)
(c)
(d)
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a–f
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do
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ve
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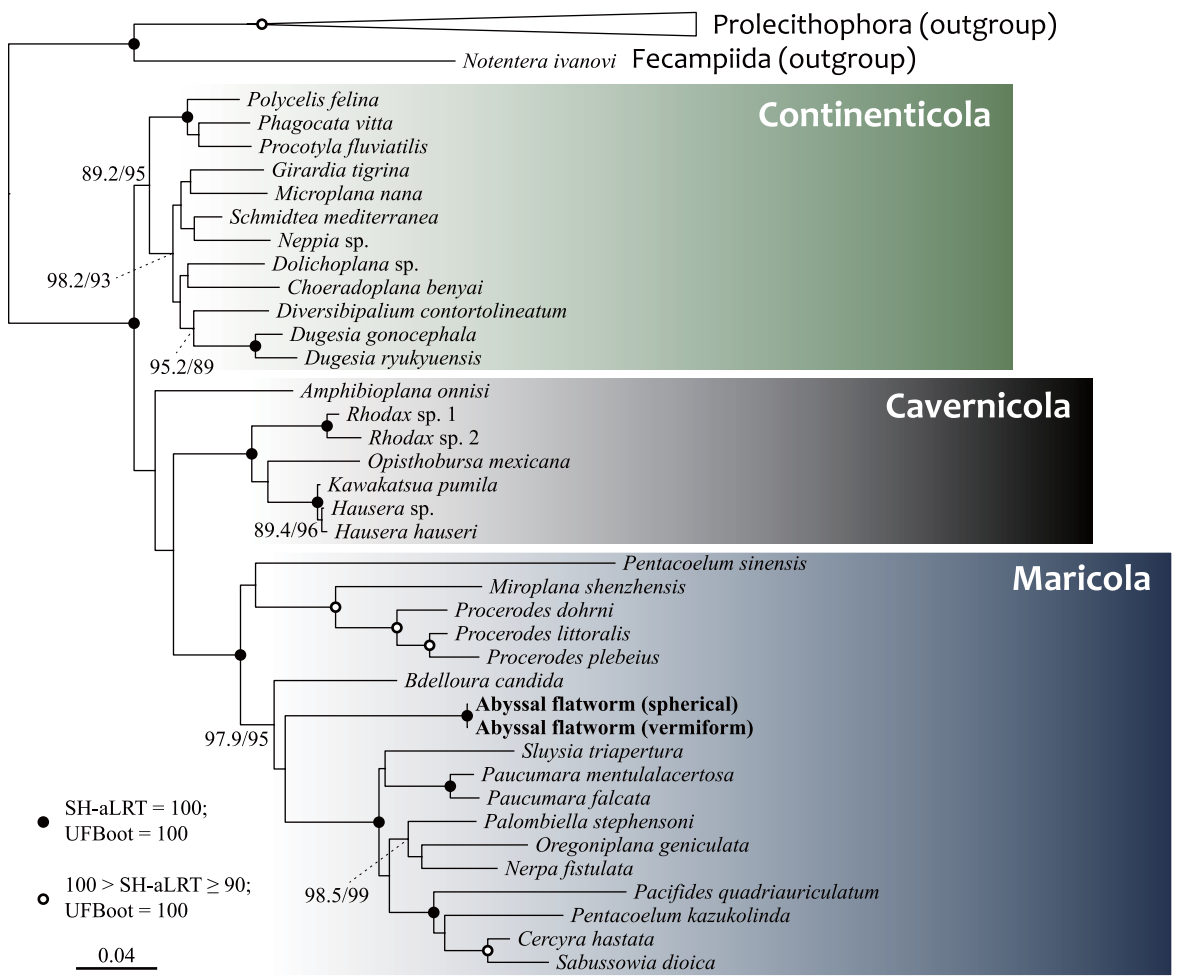
Prolecithophora (outgroup)
Fecampiida (outgroup)
Notentera ivanovi
Continenticola
Polycelis felina
Phagocata vitta
Procotyla fluviatilis
89.2/95
Girardia tigrina
Microplana nana
Schmidtea mediterranea
Neppia sp.
98.2/93
Dolichoplana sp.
Choeradoplana benyai
Diversibipalium contortolineatum
Dugesia gonocephala
95.2/89
Dugesia ryukyuensis
Cavernicola
Amphibioplana onnisi
Rhodax sp. 1
Rhodax sp. 2
Opisthobursa mexicana
Kawakatsua pumila
Hausera sp.
89.4/96
Hausera hauseri
Maricola
Pentacoelum sinensis
Miroplana shenzhensis
Procerodes dohrni
Procerodes littoralis
Procerodes plebeius
Bdelloura candida
Abyssal flatworm (spherical)
Abyssal flatworm (vermiform)
97.9/95
Sluysia triapertura
Paucumara mentulalacertosa
Paucumara falcata
Palombiella stephensoni
Oregoniplana geniculata
Nerpa fistulata
98.5/99
Pacifides quadriauriculatum
Pentacoelum kazukolinda
Cercyra hastata
Sabussowia dioica
SH-aLRT = 100;
UFBoot = 100
100 > SH-aLRT ≥ 90;
UFBoot = 100
0.04