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1 Article type: Research Article

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3 **Flatworm cocoons in the abyss: same plan under pressure**

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16
17 **Abstract**

18 While knowledge of early ontogeny in abyssal animals is highly limited in general, it was completely
19 lacking for abyssal, free-living platyhelminths. We discovered flatworm egg-capsules (or "cocoons") on
20 rocks collected at depths of 6176–6200 m on the abyssal slope of the Kuril-Kamchatka Trench,
21 northwestern Pacific. The egg capsules were black and spherical, around 3 mm in diameter, and contained
22 three to seven individuals (n = 4) at the same developmental stage, either the spherical (putative early
23 embryo) or vermiform (putative late embryo) stages. A molecular phylogenetic analysis based on 18S and
24 28S rRNA sequences revealed that the flatworms belong in suborder Maricola in Tricladida and suggested
25 that they may have colonized from shallow to deep waters. This study provides the deepest record for free-
26 living flatworms and the first information on their early life stages in the abyssal zone, which were very
27 similar to those in shallow-water forms. This similarity in development between the relatively benign
28 shallow-water and the extreme abyssal environments suggests that triclads adapting to the latter faced
29 primarily physiological and/or ecological adaptive challenges rather than developmental ones.

30
31 **Keywords:** deep sea, early development, egg capsule, Japan, ontogeny, Platyhelminthes

32
33 **Introduction**

34 The life cycles of most abyssal animals are largely unknown, despite their importance in understanding
35 how animals have successfully colonized and adapted to the extreme conditions of the abyssal zone (3500–
36 6500 m depth; [1]). This is especially true of early ontogeny, including embryonic and larval development.
37 Only a few fragmentary studies have described the early life history of representatives of several animal
38 groups at abyssal depths, including barnacles (e.g., [2]), molluscans (e.g., [3]), fishes (e.g., [4]), sponges

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

40 Almost nothing is known about abyssal free-living platyhelminths, for which a “potential
41 platyhelminth” found on sunken wood at depths of 5257–5236 m [8] may be the sole currently available
42 information; to date, the deepest certain record is the polyclad *Oligocladus voightae* from 3232 m [9]. Due
43 to their fragility, platyhelminths are unlikely to be collected with coarse sampling gear such as dredges and
44 trawls [9], and information on their development at depths is lacking. Free-living flatworms can vary
45 markedly in ontogeny [10]. In sexual reproduction, they generally produce single or multiple embryos
46 (along with extra-embryonic yolk cells in neophorans) enclosed in an egg capsule (or “cocoon”) attached
47 to a substrate by a secretion from the uterine glands. Embryonic development differs among groups; while
48 polyclads undergo spiral cleavage, triclads and other neophorans undergo irregular, dispersed cleavage
49 [10]. Most free-living flatworms show direct development, except for several specific groups in Catenuvida
50 and Polycladida, and little is known of the later stages of development.

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

58

59 **Materials and methods**

60 Egg capsules attached to two rock fragments were collected with a beam trawl on 21 September 2023
61 during cruise KH-23-5 of R/V *Hakuho-maru*, at depths of 6176–6200 m at Station C5 (41°28.411' N
62 146°06.803' E to 41°28.519' N 146°07.632' E). Four intact egg capsules were detached from the rocks, and
63 their contents were extracted by pipet, forceps, and a needle. The flatworms thus obtained were fixed in
64 70% ethanol, 99% ethanol, or Bouin’s fluid; some of them were photographed before fixation. The
65 material studied was deposited in the Invertebrate Collection of the Hokkaido University Museum
66 (ICHUM), Sapporo under catalog numbers ICHUM8616 and ICHUM8617.

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

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

77 primers 28S_1F and 28S_6R [16] were used for amplification, and primers 300F, 300R, 900F, 28S_Rd4.2b
78 [17, 18] and 28S_b5F (TATCCGGTAAAGCGAATGATTAGA, newly designed in this study) for cycle
79 sequencing. PCR amplification conditions for COI with TaKaRa Ex Taq DNA polymerase (TaKaRa Bio,
80 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
81 2 min. Conditions for 18S and 28S with KOD FX Neo (Toyobo, Japan) were 94 °C for 2 min; 45 cycles of
82 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
83 nucleotide sequences were determined with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer
84 (Life Technologies, USA). Fragments were concatenated by using MEGA7 [19]. The sequences we
85 determined were deposited in the International Nucleotide Sequence Database (INSD) through the DNA
86 Data Bank of Japan (DDBJ).

87 The results of a BLAST search [20] for our 18S sequence indicated that the flatworm belongs in
88 Tricladida, and so a concatenated 18S+28S dataset that included both sequences for two of our flatworm
89 specimens, 35 triclad species, and seven outgroup taxa (electronic supplementary material, table S1) was
90 analyzed by maximum likelihood (ML) to infer the position of the abyssal flatworm within Tricladida. The
91 18S and 28S data were aligned independently by using the “Q-INS-i” strategy [21] in MAFFT ver. 7 [22]
92 (electronic supplementary material, files S1, S2) and then trimmed with MEGA7 to match the shortest
93 length for each gene. Alignment-ambiguous sites were removed with Gblocks ver. 0.91b [23] in
94 NGPhylogeny.fr [24] under the “relaxed” parameters described in [25]. The dataset contained 1458 aligned
95 positions for 18S, 982 for 28S, and 2440 in total (electronic supplementary material, file S3). Methods for
96 selecting the optimal substitution model (GTR+F+R4 for 18S; GTR+F+R5 for 28S), the ML analysis, and
97 drawing the tree were as described by [26].

98

99 **Results and Discussion**

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

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

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

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

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

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

140

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

142

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

146

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

148

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

152

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

153 performed therein.

154

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

156

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163 evolutionary mechanisms of deep-sea animals in trenches in the northwestern Pacific” supported by
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165 technicians from Marine Work Japan and MOL Marine & Engineering, and researchers aboard for support
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167 information on the bottom type at the sampling site; and Matthew H. Dick for reviewing the manuscript
168 and editing the English.

169

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259 **Figure legends**

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

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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 $\geq 70\%$ and UFBoot $\geq 80\%$ are shown. Scale at bottom indicates branch length in substitutions per site.

273 **Electronic Supplementary Material legends**

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

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

276

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

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

280

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

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

284

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

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

289



