Trematode metacercariae parasitic in the estuarine crustacean *Cyathura muromiensis* Nunomura, 1974 (Peracarida: Isopoda: Anthuroidea)

Shoki Shiraki[a, *], Keiichi Kakui[b]

[a] *Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*

[b] *Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan*

* Corresponding author.

E-mail addresses: white-tree0703@eis.hokudai.ac.jp (Shoki Shiraki), keiichikakui@gmail.com (Keiichi Kakui).

ABSTRACT

This is the first report of trematodes parasitic in the estuarine isopod crustacean *Cyathura muromiensis*, and the fourth report from anthuroid isopods worldwide. From 52 of 54 host individuals collected qualitatively on the tidal flat of the Muromi River estuary, Fukuoka, Japan, 389 cysts of metacercariae were extracted (sample prevalence = 96.3%). Host individuals contained from one to 71 metacercarial cysts. The range in cyst diameter was 172.3–252.1 μm, and the distribution of cyst sizes contained only one component. Cysts

occurred in pereonites 2–7 and the pleon of the host, but not in the head, pereonite 1, or telson. There was no correlation between the number of cysts and host sex or size. Sequences of the nuclear "ITS1 region," from the 3′ region of 18S rRNA to the 5′ region of internal transcribed spacer I gene (ITS1), from five cysts ranging in size from nearly the lowest diameter to the greatest diameter in our sample showed p-distances of 0.0–0.2%, suggesting that all cysts obtained were conspecific. A phylogenetic reconstruction based on nuclear 28S rRNA gene sequences showed that the trematode belongs in the genus *Microphallus*. The definitive host of our trematode species is likely a bird, since the definitive hosts of microphallids are chiefly birds, and birds are known to prey on *Cyathura* isopods.

***Keywords**:* Anthuridae; Digenea; Japan; Microphallidae; Tidal flat; Platyhelminthes

Because the life cycles of parasitic trematode platyhelminths are generally complicated, they have not been fully elucidated for most trematodes. Trematodes typically utilize a definitive host and two intermediate hosts. One group that trematodes utilize as secondary intermediate hosts is the isopod superfamily Anthuroidea Leach, 1814, but among ca. 600 anthuroid species, only *Anthura gracilis* (Montagu, 1808), *Cyathura polita* (Stimpson, 1855), and *Cy. carinata* (Krøyer, 1847) have been reported in this role. *Levinseniella brachysoma* (Creplin, 1837) utilizes *A. gracilis* [1], unidentified *Gynecotyla* (sic; possibly the misspelling of *Gynaecotyla* Yamaguti, 1939) species often occurs in *Cy. polita* [2], and several microphallid species such as *Maritrema subdolum* Jägerskiöld, 1909 and *Microphallus claviformis* (Brandes, 1889) utilize *Cy. carinata* [3–8]. All the above-mentioned trematodes belong to Microphallidae Ward, 1901 and the definitive hosts of its members are mainly birds [9].

Here we report trematode cysts in *Cyathura muromiensis* Nunomura, 1974, corresponding the first report of trematodes from this species and the fourth report from anthuroid isopods worldwide. The isopods were collected on a brackish tidal flat in the Muromi River estuary (33°34'48.2"N 130°20'13.7"E), Fukuoka, Japan (the species' type locality) [10], on 7 March (52 specimens) and 9 March (two specimens) 2023. Bottom sediment was filtered with a 1 mm-mesh sieve, and isopods retained in the residue were recovered by hand; this process was carried out repeatedly. Some individuals collected on 7

March 2023 were fixed in 99% ethanol on the same day as collection. All other individuals collected on 7 and 9 March 2023 were brought back to our laboratory alive. Some of these were photographed, dissected to obtain live cysts, and fixed in 99% ethanol; the others were fixed in 99% ethanol. The head length of each *Cyathura* isopod was measured as a proxy for host size, since head length is linearly related to body length in congeneric *Cy. carinata* [11]. Fixed isopods were dissected with chemically sharpened tungsten wire needles under a Nikon SMZ1500 stereomicroscope to examine the trematode infection. The number of cysts and the host body segment where they were located (head, pereonites 1–7, pleon, and telson) were recorded. The diameters of the extracted cysts were measured.

An extracted, living cyst was opened with chemically sharpened tungsten wire needles in a 1.0% saline solution, and the metacercaria was removed, observed with an Olympus BX51 microscope under a cover glass, and subsequently fixed in 70% ethanol. Measurements of host head length, cyst diameter, and the length of excysted metacercaria and its body structures were made from digital images by using ImageJ (http://rsb.info.nih.gov/ij). *Cyathura* individuals were sexed according to the presence (males) or absence (females) of the appendix masculina on the endopod of pleopod 2. The methods of statistical analyses, DNA extraction and polymerase chain reaction (PCR), and molecular phylogenetic analysis were provided in Supplementary File S1. The sequences we obtained were deposited in the International Nucleotide Sequence Database (INSD) through the DNA Data Bank of Japan

(DDBJ), under accession numbers LC808145 (28S) and LC808146–LC808150 (ITS1 region; cysts with diameters 228.8, 206.9, 252.1, 172.6, and 205.0 μm, respectively).

In all, 389 cysts were obtained from 52 of 54 specimens of *Cy*. *muromiensis* (Fig. 1A, B; 50 were collected on 7 March 2023 and two on 9 March 2023); the sample prevalence was 96.3%. Hosts contained one to 71 cysts each (mean intensity = 7.5; median intensity = 4.0). The cysts (Fig. 1C) were the round, encysted metacercarial stage and were situated inside the host body (Fig. 1B). Description and measurements of excysted metacercariae based on two individuals were as follows. Excysted metacercaria (Fig. 1D) linguiform, flattened. Body length 567 μm and body width 276–323 μm under a cover glass. Oval oral sucker (length 40.4–43.8 μm, width 49.1–52.3 μm) larger than round ventral sucker (length 38.1 μm, width 33.0–34.9 μm). Prepharynx present but short, 7.4–36.9 μm. Pharynx oval, near oral sucker, length 22.3–24.1 μm and width 15.8–20.2 μm. Esophagus narrow, length 114–200 μm. Caecum bifurcating in center of body and terminating near lateral side of midbody. Testes oval, locating symmetrically, postovarian. Right testis (ovary side) length 70.9–86.6 μm and width 61.7–70.3 μm. Left testis length 71.8–86.4 μm and width 49.4–61.3 μm. Cirrus sac arched, locating between caeca and ventral sucker. Ovary oval, locating between cirrus sac and right testis, length 56.2–65.9 μm and width 47.8–48.9 μm.

Range in cyst diameter (N = 378) was 172.3–252.1 μm (mean = 211.1 μm; median = 210.6 μm; Fig. 2A). For both Dataset A containing data from 378 cysts extracted from 52

host individuals and Dataset S containing data from 70 cysts extracted from the single host individual (Fig. 2A, B; for detail, see Supplementary File S1), the model with the optimal BIC value (−2971.462 under both the equal and unequal models for Dataset A; –533.3162 under both models for Dataset S) had one component.

Cysts were found in host pereonites 2–7 and the pleon, with the higher occurrences in pereonites 3–6 (Fig. 2C). A similar distribution was evident among the 71 cysts extracted from the single host with the highest intensity (Fig. 2D). No cysts were found in the head, pereonite 1, or the telson. The head bears several appendages and has little space for parasites, and the telson is thin, which may explain the lack of cysts in these locations. No infections in pereonite 1 and low infection in pereonite 2 may be related to pereonites 1–2 and 2–3 being connected by a narrow joint in anthuroid isopods (cf. [12]: fig. 2A, E–G), which might prevent or impede parasites from entering pereonites 1 and 2 from adjacent segments. We found most cysts in pereonites 2–7, with a few in the pleon, a distribution similar to that observed in *Levinseniella* sp. and *Ma*. *subdolum*/*Mi*. *claviformis* parasitic in *Cy*. *carinata* under experimental conditions [8]. Interestingly, in a different experiment [7], most cysts of *Ma*. *subdolum* were found in the pleon of *Cy. carinata*. In these two experiments, individuals of *Cy. carinata* came from the same locality, perhaps indicating that the preferred location of the parasite can change with factors such as season, water temperature, or substrate.

Of 54 collected isopods, 40 and 7 individuals were female and male, respectively, while 7 individuals losing pleopods were not sexually identified. All the females were not ovigerous, 38 of which were parasitized (sample prevalence = 95%), with one to 71 cysts each (mean intensity = 8.1; median intensity = 5.5). All seven males were parasitized, with one to 18 cysts each (mean intensity = 7.0; median intensity = 4.0). The number of cysts was not normally distributed for females, but was for males with low p-value (Shapiro-Wilk test; $p < 0.001$ for females; $p = 0.05254$ for males). The number of cysts was not significantly different between host females and males (Fig. 2E; Mann–Whitney U test, $p = 0.7299$).

There was no correlation between host head length (HL) and the number of cysts (NC) among 47 sexed host individuals (Fig. 2F). The regression line was $NC = 7.4256HL + 0.4918$ ($0.736 \leq HL \leq 1.316$), but the coefficient of determination was quite low ($R^2 = 0.0052$). There was also no correlation between HL and NC when this was examined separately by host sex. The coefficients of determination were low for both female ($R^2 = 0.0114$; $NC = 13.74HL – 5.2645$) and male hosts ($R^2 = 0.001$; $NC = –1.1886HL + 8.2264$).

ITS1-region sequences (LC808146–LC808150; 865 bp) from five cysts (172.6, 205.0, 206.9, 228.8, and 252.1 μm in diameter) differed by 0–2 nucleotide substitutions, giving p-distances of 0–0.2%. In BLAST searches [13], the ITS1 sequence most similar to our ITS1 region (LC808146) was from an unidentified digenean parasite in *Cy. carinata* (AJ001831; identity score 76.85%, query cover 100%; [5]). As the p-distances were low and the model

fitted to the cyst-size distribution contained only one component, we concluded that all the metacercariae obtained were conspecific.

A 28S sequence (LC808145; 1694 bp) was determined from one cyst (diameter 228.8 μm). BLAST searches suggested that our 28S sequence (LC808145) was most similar to that of microphallid from the tanaidacean species (AB974360; identity score 97.05%, query cover 98%; [14]). In the optimal maximum-likelihood tree (Fig. 1E), our taxon is embedded in a clade containing only sequences from *Microphallus* Ward, 1901 (except for an unidentified microphallid), with high nodal support (SH-aLRT = 98.2%, UFBoot = 99%), indicating that our cysts belong to the genus *Microphallus*. Our 28S sequence differed by 3.1–7.4% from the other sequences in the *Microphallus* clade (Fig. 1E; Supplementary Table S1), much greater than the minimum interspecific genetic distance observed in our dataset, 0.2% between *Mi. minutus* Johnston, 1948 (KT355822) and *Mi. calidris* Belopolskaya & Rizhikov, 1963 (HM584124), and within the range of interspecific differences reported in *Microphallus* (e.g., 0.4–8.6%; [15]). The observed distances suggest that our trematode species is not conspecific with any of the *Microphallus* species included in the analysis. Interestingly, two metacercariae from peracarid hosts (our sequence, LC808145, from a *Cyathura* isopod and Microphallidae sp., AB974360, from a tanaidacean *Longiflagrum nasutus* [Nunomura, 2005]) formed a moderately supported clade (SH-aLRT = 94.4%, UFBoot = 91%), even though microphallids use various crustaceans as second intermediate hosts [15], such as decapods for

146 *Mi*. *minutus* [16] and *Mi*. *basodactylophallus* (Bridgman, 1969) [17,18]. This may imply a
147 single origin of the utilization of peracarids as secondary intermediate hosts in *Microphallus*.
148 That our ITS1 region sequence was most similar to the ITS1 sequence from a digenean
149 parasitic in *Cy*. *carinata* may support the idea. However, information on the second
150 intermediate host is lacking for most species, and more data on *Microphallus* from peracarids
151 are needed to test this hypothesis.

152 The definitive host for our trematode species may be birds, since microphallids
153 chiefly use birds as the definitive host [9]. Although information is lacking regarding
154 predators of *Cy*. *muromiensis*, the small sandpiper *Calidris alpina* (Linnaeus, 1758) preys on
155 the congener *Cy*. *carinata* [19]. The Muromi River estuary is rich in birds, and *Ca*. *alpina* has
156 been observed there [20]. A speculative but plausible life cycle for our trematode species
157 might involve a cercaria emerging from the first intermediate host (possibly a gastropod), the
158 cercaria entering a *Cy*. *muromiensis* isopod and giving rise to encysted metacercariae, and
159 (followed by predation of the isopod by the definitive host, possibly a shorebird), the
160 metacercariae becoming sexually mature adults.

161

162 **Ethical standards**

163 Not applicable.

164

Funding

This work was supported by a grant to SS from the Research Institute of Marine Invertebrates (FY 2022; KO2022-04) and by a Grant-in-Aid for JSPS Fellows (JP23KJ0063) to SS from the Japan Society for the Promotion of Science (JSPS).

CRediT authorship contribution statement

Shoki Shiraki: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Visualization, Writing – original draft, Writing – review and editing. **Keiichi Kakui:** Conceptualization, Formal analysis, Supervision, Writing – review and editing.

Declaration of Competing Interest

We declare no competing interests.

Acknowledgements

We thank Yuki Kita for help in observing and fixing excysted metacercaria; Leonard Tan Yi Herng for help with the Mann–Whitney U test; Matthew H. Dick for reviewing the manuscript and editing our English; and four anonymous reviewers for providing comments that improved our manuscript.

184

References

[1] M.A. Villot, Organisation et développement de quelques espèces de trématodes endoparasites marins (1), Ann. Sci. Nat. Zool. 8 (1879) 1–40.

[2] W.D. Burbanck, An ecological study of the distribution of the isopod *Cyathura polita* (Stimpson) from brackish waters of Cape Cod, Massachusetts, Am. Midl. Nat. 67 (2) (1962) 449–476.

[3] L. Reimer, Zur Verbreitung der Adulti und Larvestadien der Familie Microphallidae Viana, 1924 (Trematoda, Digenea) in der Mittleren Ostsee, Z. Parasitenkd. 23 (1963) 253–273, https://doi.org/10.1007/BF00259377.

[4] J.H.G. Schulenburg, J.W. Wägele, Molecular characterisation of digenetic trematodes associated with *Cyathura carinata* (Crustacea: Isopoda) with a note on the utility of 18S ribosomal DNA for phylogenetic analysis in the Digenea (Platyhelminthes: Trematoda), Int. J. Parasitol. 28 (9) (1998) 1425–1428, https://doi.org/10.1016/s0020-7519(98)00121-0.

[5] J.H.G. Schulenburg, U. Englisch, J.W. Wägele, Evolution of ITS1 rDNA in the Digenea (Platyhelminthes: Trematoda): 30 end sequence conservation and its phylogenetic utility, J. Mol. Evol. 48 (1999) 2–12, https://doi.org/10.1007/pl00006441.

[6] K.T. Jensen, S.M. Ferreira, M.A. Pardal, Trematodes in a *Cyathura carinata* population from a temperate intertidal estuary: infection patterns and impact on host, J. Mar.

Biol. Ass. U.K. 84 (2004) 1151–1158, https://doi.org/10.1017/S0025315404010598h.

[7] S.M. Ferreira, K.T. Jensen, M.A. Pardal, Infection characteristics of a trematode in an estuarine isopod: influence of substratum, Hydrobiologia 539 (2005a) 149–155, https://doi.org/10.1007/s10750-004-3917-0.

[8] S.M. Ferreira, K.T. Jensen, P.A. Martins, S.F. Sousa, J.C. Marques, M.A. Pardal, Impact of microphallid trematodes on the survivorship, growth, and reproduction of an isopod (*Cyathura carinata*), J. Exp. Mar. Biol. Ecol. 318 (2005b) 191–199, https://doi.org/10.1016/j.jembe.2004.12.018.

[9] R.A. Bray, D.I. Gibson, A. Jones, Keys to the Trematoda volume 3, CABI Publishing and the Natural History Museum, London, 2008.

[10] N. Nunomura, A new anthurid isopod from the estuary of the Muromi River, northern Kyushu, Japan, Bull. Osaka Mus. Nat. Hist. 28 (1974) 13–16.

[11] J.C. Marques, I. Martins, C. Teles-Ferreira, S. Cruz, Population dynamics, life history, and production of *Cyathura carinata* (Krøyer) (Isopoda: Anthuridae) in the Mondego estuary, Portugal, J. Crustacean Biol. 14 (2) (1994) 258–272, https://doi.org/10.1163/193724094X00254.

[12] K. Kakui, S. Shiraki, Presumptive stridulatory organs in *Paranthura* cf. *japonica* Richardson, 1909 (Isopoda: Cymothoida: Paranthuridae), J. Crustacean Biol. 41 (2) (2021) 1–5, http://dx.doi.org/10.1093/jcbiol/ruab026.

[13] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (1990) 403–410, https://doi.org/10.1016/s0022-2836(05)80360-2.

[14] K. Kakui, A novel transmission pathway: first report of a larval trematode in a tanaidacean crustacean, Fauna Ryukyuana 17 (2014) 13–22.

[15] K.V. Galaktionov, I. Blasco-Costa, *Microphallus ochotensis* sp. nov. (Digenea, Microphallidae) and relative merits of two-host microphallid life cycles, Parasitol. Res. 117 (4) (2018) 1051–1068, https://doi.org/10.1007/s00436-018-5782-1.

[16] O. Kudlai, S.C. Cutmore, T.H. Cribb, Morphological and molecular data for three species of the Microphallidae (Trematoda: Digenea) in Australia, including the first descriptions of the cercariae of *Maritrema brevisacciferum* Shimazu et Pearson, 1991 and *Microphallus minutus* Johnston, 1948, Folia Parasitol. 62 (53) (2015), https://doi.org/10.14411/fp.2015.053.

[17] J.F. Bridgman, Life cycles of *Carneophallus choanophallus* n. sp. and *C. baso dactylophallus* n. sp. (Trematoda: Microphallidae), Tulane Stud. Zool. 15 (3) (1969) 81–104.

[18] A.L.M. Gehman, M. Mahaffey, J.E. Byers, Influences of land use and ecological variables on trematode prevalence and intensity at the salt marsh-upland ecotone, Ecosphere 12 (8) (2021) e03723, https://doi.org/10.1002/ecs2.3723.

242 [19] C.D. Santos, J.P. Granadeiro, J.M. Palmeirim, Feeding ecology of dunlin *Calidris alpina*
243 in a southern European estuary, Ardeola 52 (2) (2005) 235–252.
244 [20] iNaturalist, Dunlin (*Calidris alpina*) (2022)
245 https://www.inaturalist.org/observations/142528126 (accessed 5 April 2024)

246

247

248 **Figure captions**

249 **Fig. 1.** Trematode metacercaria parasitic in the isopod *Cyathura muromiensis*, and its
250 phylogenetic position within Microphallidae. (A, B) Dorsal (A) and ventral (B) views of *Cy.*
251 *muromiensis*, living individual (arrowheads indicate cysts); scale, 5 mm. (C) Extracted
252 encysted metacercaria, fixed specimen; scale, 200 μm. (D) Excysted metacercaria, living
253 individual; scale, 200 μm; abbreviations: c, caecum; cs, cirrus sac; e, esophagus; o, ovary; os,
254 oral sucker; p, pharynx; t, testis; vs, ventral sucker. (E) Maximum likelihood phylogenetic
255 tree for microphallid trematodes based on 28S rRNA gene sequences (945 bp); numbers near
256 nodes are SH-aLRT/UFBoot support values; the portion of the tree containing species in
257 Microphallidae is outlined in green and the *Microphallus* clade is shaded orange; our taxon is
258 in bold font; *Maritrema* clade was collapsed (terminal triangles); the scale bar indicates
259 branch length in number of substitutions per site; Not-collapsed tree was provided as
260 Supplementary Figure S1.

261

262 **Fig. 2.** Number of cysts for each parameter. (A, B) Size frequency distributions of
263 metacercarial cysts from the host *Cyathura muromiensis* for 378 cysts extracted from 52 host
264 individuals (A) and for 70 cysts extracted from the single host individual (B). (C, D)
265 Frequency distributions of metacercarial cysts among segments of the host *Cyathura*
266 *muromiensis* for 389 cysts from 52 host individuals (C) and for 71 cysts from the single host

with the highest intensity (D). Abbreviations: H, head; P1–7, pereonites 1–7; Pl, pleon; T, telson. (E) Numbers of metacercarial cysts for female and male *Cyathura muromiensis* hosts. The boxplots show median values (thick, black bars), first and third quartiles (bottoms and tops of the boxes), outliers (circles), and minimum and maximum values excluding outliers (ends of the whiskers). The Mann–Whitney U test showed no significant difference between the sexes. (F) Relationship between host size and the number of cysts. Solid and open circles denote female and male hosts, respectively. The dotted line is the regression line.

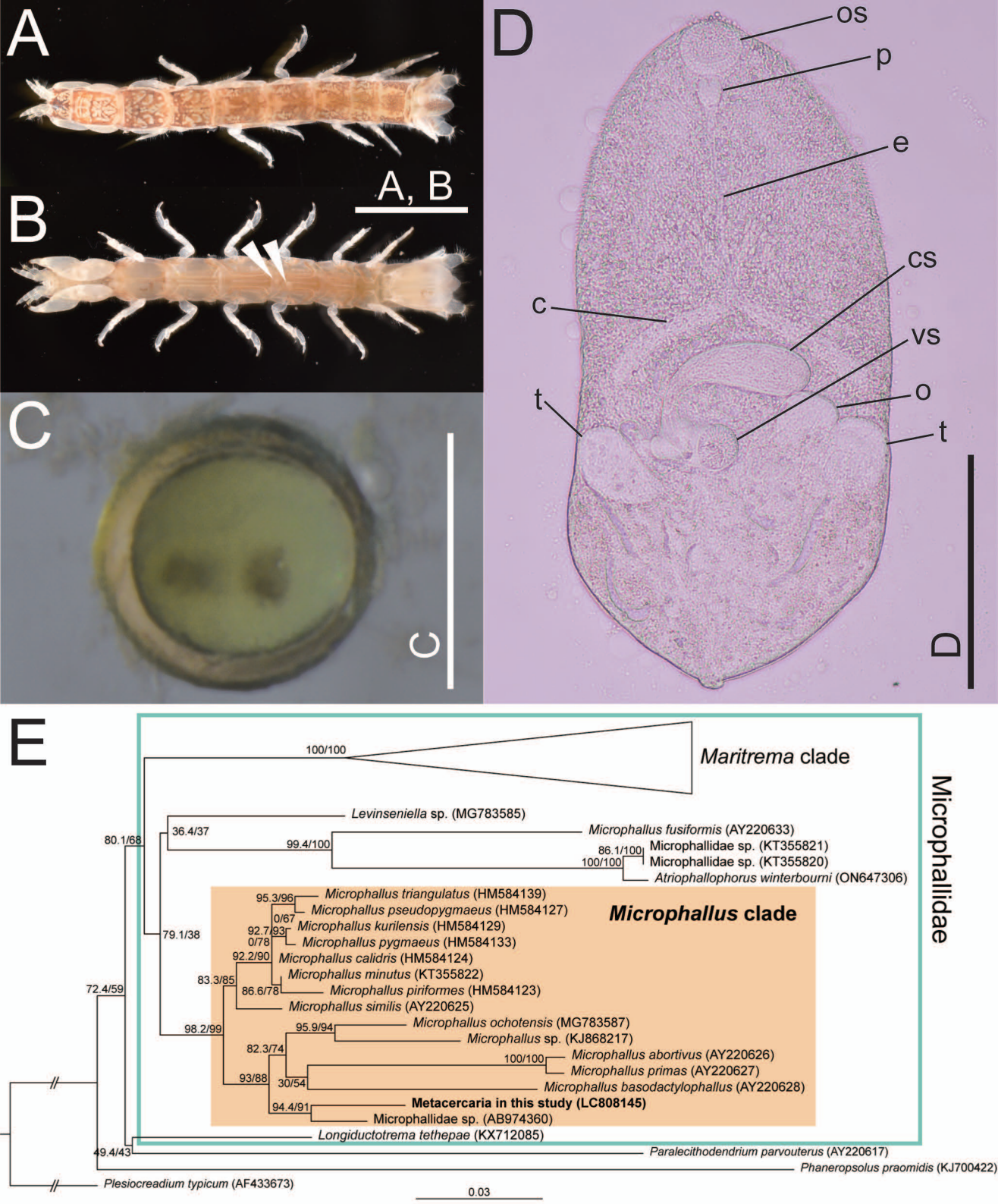
Appendix A. Supplementary data

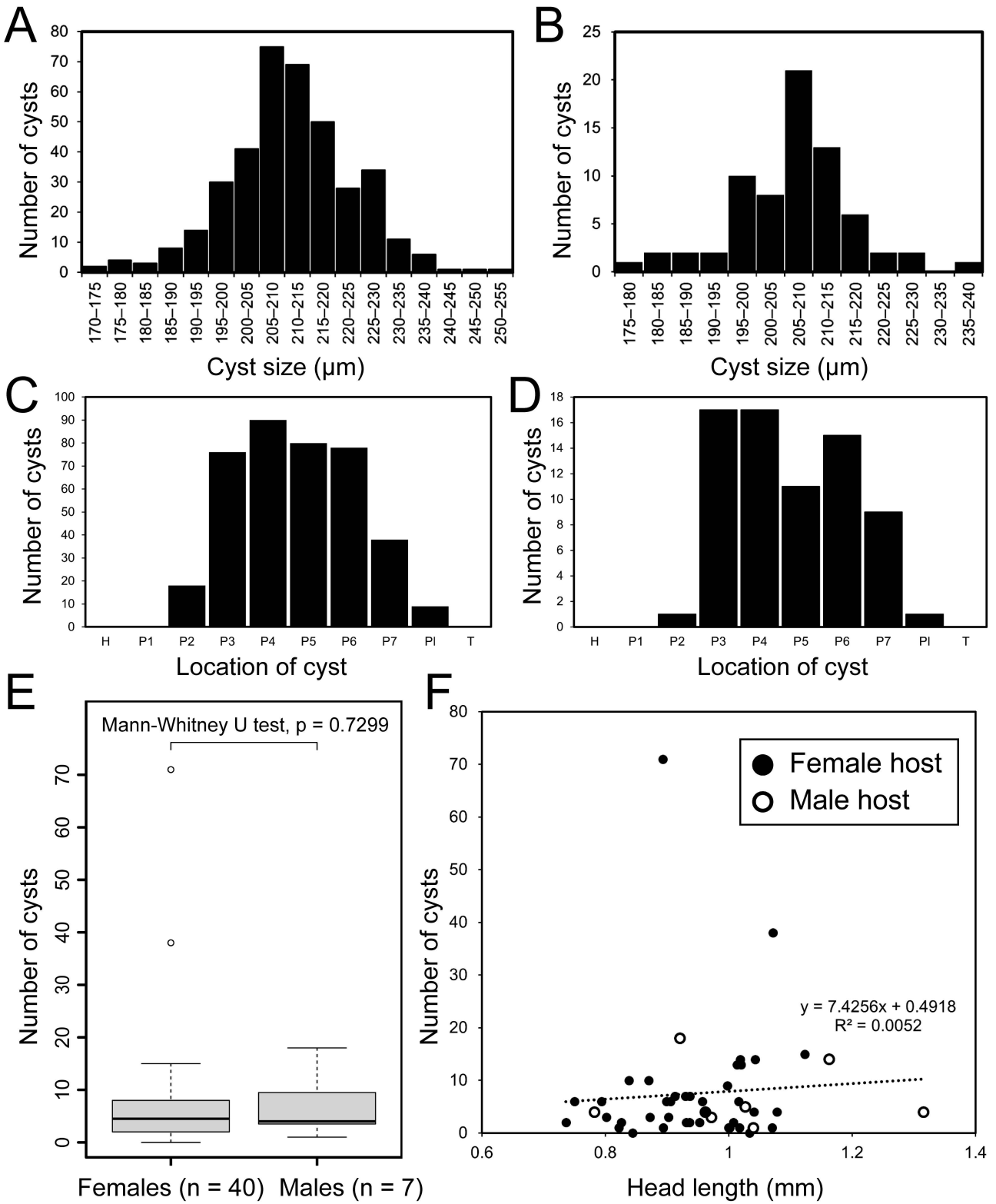
Supplementary Material

File S1. Methods for statistical analyses and molecular phylogeny.

Fig. S1. Maximum likelihood phylogenetic tree for microphallid trematodes based on 28S rRNA gene sequences (945 bp).

Table S1. P-distances (in percent) among partial 28S rRNA sequences (945 bp) from species in the *Microphallus* clade in the phylogenetic tree.


A
A, B
B
C
c
D
os
p
e
cs
c
vs
o
t
t
d
E
Maritrema clade
100/100
Levinseniella sp. (MG783585)
36.4/37
Microphallus fusiformis (AY220633)
80.1/68
99.4/100
86.1/100
Microphallidae sp. (KT355821)
100/100
Microphallidae sp. (KT355820)
Atriophallophorus winterbourni (ON647306)
95.3/96
Microphallus triangulatus (HM584139)
Microphallus pseudopygmaeus (HM584127)
0/67
Microphallus clade
92.7/93
Microphallus kurilensis (HM584129)
79.1/38
0/78
Microphallus pygmaeus (HM584133)
92.2/90
Microphallus calidris (HM584124)
Microphallus minutus (KT355822)
83.3/85
86.6/78
Microphallus piriformes (HM584123)
Microphallus similis (AY220625)
72.4/59
98.2/99
Microphallus ochotensis (MG783587)
95.9/94
Microphallus sp. (KJ868217)
82.3/74
100/100
Microphallus abortivus (AY220626)
Microphallus primas (AY220627)
93/88
30/54
Microphallus basodactylophallus (AY220628)
94.4/91
Metacercaria in this study (LC808145)
Microphallidae sp. (AB974360)
Microphallidae
Longiductotrema tethepae (KX712085)
49.4/43
Paralecithodendrium parvouterus (AY220617)
Phaneropsolus praomidis (KJ700422)
Plesiocreadium typicum (AF433673)
0.03


A
Number of cysts
Cyst size (μm)
170–175
175–180
180–185
185–190
190–195
195–200
200–205
205–210
210–215
215–220
220–225
225–230
230–235
235–240
240–245
245–250
250–255
B
Number of cysts
Cyst size (μm)
175–180
180–185
185–190
190–195
195–200
200–205
205–210
210–215
215–220
220–225
225–230
230–235
235–240
C
Number of cysts
Location of cyst
H
P1
P2
P3
P4
P5
P6
P7
PI
T
D
Number of cysts
Location of cyst
E
Mann-Whitney U test, p = 0.7299
Number of cysts
Females (n = 40)
Males (n = 7)
F
Female host
Male host
y = 7.4256x + 0.4918
R² = 0.0052
Number of cysts
Head length (mm)