New type of xiphidiocercariae (Digenea: Microphalloidea) from South Vietnam

Darya Krupenko¹, Anna Gonchar¹,³, Georgii Kremnev¹, Boris Efeykin¹,⁴ and Vladimir Krapivin¹,³,⁴

¹Saint Petersburg University, Department of Invertebrate Zoology, Saint Petersburg, Russia;
²Zoological Institute, Laboratory of Parasitic Worms and Protists, Russian Academy of Sciences, Saint Petersburg, Russia;
³Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia;
⁴The Russian-Vietnamese Tropical Scientific and Technological Centre, Southern Branch, Ho Chi Minh, Vietnam;

Abstract: We found unusual digenean intramolluscan stages, sporocysts and cercariae, in gastropods Sulcospira dautzenbergiana (Morelet) (Caenogastropoda: Pachychilidae) from Southern Vietnam and named them Cercaria cattieni 1. These cercariae have a stylet and thus belong to the Xiphidiata. However, such combination of characters as extremely large body size and I-shaped excretory bladder has not been found before in any other xiphidiocercariae. We obtained COI, ITS1, 5.8S + ITS2, and 28S rDNA sequences for C. cattieni 1. The latter allowed us to specify the phylogenetic position of the discovered cercariae: C. cattieni 1 falls within the superfamily Microphalloidea and is most closely grouped to Pachyphaloides irroratus (Rudolphi, 1819) (Pachyphaloidae), the sea turtle parasite. Information on the family Pachyphaloidae is limited. Judging from the molecular phylogeny, C. cattieni 1 might be the larva of the Pachyphaloidae, documented for the first time.

Keywords: Digenea, xiphidiocercariae, sporocysts, Xiphidiata, Microphalloidea, Pachyphaloidae, 28S rDNA, ITS1, ITS2, COI

MATERIALS AND METHODS

Gastropods Sulcospira dautzenbergiana were collected on 4–5 October 2018 in a stream in Cát Tiên National Park, Southern Vietnam (11.4305N, 107.4294E). Snails were dissected to detect digenean infection. Sporocysts and cercariae found were rinsed in freshwater, fixed in 70% ethanol and later transferred to 96% ethanol for further morphological and molecular analyses. Video of living cercaria was made with a dissecting microscope and a camera Canon A720.

For morphological description sporocysts and cercariae were stained either with Ehrlich’s haematoxylin or Heidenhain’s iron haematoxylin. Picric acid was used to destain worms after Heidenhain’s haematoxylin, and 70% ethanol with 0.1 M HCl after Ehrlich’s haematoxylin. Toluidine blue was used to identify mucoid substances that give metachromatic staining. After staining, samples were dehydrated in graded alcohols and mounted in Bio-Mount medium (Bio Optic, Milan, Italy).

Photographs of the whole-mounted cercariae and sporocysts were made using a compound microscope Leica DM 2500 (Leica Microsystems, Wetzlar, Germany) and a camera Nikon DS F1i in bright field and with differential interference contrast microscopy (DIC). Measurements were made in Fiji software (Schindelin et al. 2012). All measurements are in micrometres the range of values is followed by mean in parentheses.
For molecular analysis two sporocysts were removed from 96% ethanol and dried; DNA was extracted from each of them separately by incubation in 200 μl 5% Chelex® 100 resin (BioRad, Hercules, California, USA) solution with 0.2 mg/ml proteinase K at 56 °C overnight; 8 min at 90 °C; and centrifugation at 16,000 g for 10 min. Supernatant containing DNA was then transferred to a new tube and stored at -20 °C. Amplifications were performed in 25 μl reaction mixtures containing 17 μl Super-Q® water, 5 μl ScreenMix-HS reaction mix (Evrogen, Moscow, Russia), 0.5 μl of each forward and reverse primer (10 pmol/μl), and 2 μl of the DNA template. PCR products were stained with SYBR® Green (Invitrogen, Carlsbad, California, USA), size-separated by electrophoresis in a 1% agarose gel, and visualised using ChemiDoc MP (BioRad, USA). Primers that we used to amplify and sequence the 28S rDNA, ITS1, 5.8S rDNA+ITS2 and COI are listed in Table 1. Sequencing was performed on ABI PRISM 3500xl (Applied Biosystems, Foster City, California, USA).

Data were analysed using Geneious® 11.1.5 (https://www.geneious.com). BLAST was used for preliminary assessment of new sequences. Relevant 28S rDNA sequences from GenBank were used in alignments and phylogenetic reconstructions. The best substitution model was determined as GTR+I+G with BIC in jModelTest 2.1.10 (Darriba et al. 2012). MrBayes v.3.2.6 (Ronquist et al. 2012) was used to build a tree via the Bayesian inference method. Brachycladium goliath (van Beneden, 1858) (family Brachycladiidae) served as an outgroup. To estimate genetic distances, we used a Maximum Composite Likelihood model in MEGA 7 (Kumar et al. 2016); standard errors were obtained by a bootstrap procedure (1,000 replicates).

RESULTS

Of 41 S. dautzenbergiana dissected, one snail was infected with daughter sporocysts containing unidentified xiphidiocercariae – Cercaria cattieni 1.

Superfamily Microphalloidea Ward, 1901

Cercaria cattieni 1

First intermediate host: Sulcospira dautzenbergiana (Morelet, 1884) (Caenogastropoda: Pachychilidae).

Locality: Stream in Cát Tiên National Park, Southern Vietnam (11.4305N, 107.4294E).

Prevalence: 2% (n = 41).

Site of infection: Digestive gland.

Voucher specimens: Five slides from one gastropod specimen were placed in the collection of the Department of Invertebrate Zoology of Saint Petersburg University under numbers V2018F01-1–V2018F01-5.

Daughter sporocysts (measurements based on six fixed specimens; Figs. 1A, 2A):

Table 1. DNA sequences obtained for Cercaria cattieni 1.

| Fragment      | GenBank no. | Fragment length, bp | PCR and sequencing primers | References                  |
|---------------|-------------|---------------------|----------------------------|-----------------------------|
| 28S rDNA      | MN962960    | 1223                | digl2/1500R                 | Tkach et al. 1999           |
| ITS1          | MT657959    | 864                 | BD1/4S                     | Luton et al. 1992           |
| 5.8S rDNA+ITS2| MT648945    | 451                 | 3S/ITS2.2                  | Morgan and Blair 1995       |
| cox1          | MT653695    | 871                 | JB3/CO1-R trema             | Miura et al. 2005           |
Sporocysts white, elongate, 799–1,628 × 291–369 (1,124 × 336), contain cercariae at different stages of development. Cercariae (measurements based on 21 fixed specimens; Figs. 1B–E, 2B–I):

- Body 481–758 (594) long, 285–460 (369) wide, pear-shaped, narrow at fore end. Hindbody wide, with folded margins. Tail, 603–841 (697) long, 69–101 (93) wide at base, with smooth tegument. Oral sucker subterminal, spherical, 69–103 × 66–102 (86 × 85). Its rim armed with simple spines thicker than body spines, their size reduces posteriorly (Fig. 2D). Stylet 43–52 (48) long, 5–8 (7) wide at base, single-pointed, with anterior thickening (Figs. 1D,E, 2G). Ventral sucker spherical, 142–196 × 140–204 (160 × 165). 4–5 irregular rows of spines (thicker than body spines) lie at sucker rim, around opening, surrounded by smooth zone with 6 large papillae (probably, sensory receptors) (Fig. 2E).

- Prepharynx short; pharynx 28–47 × 20–41 (37 × 28); oesophagus very short. Caecal primordia composed of single row of cells (Fig. 2F), 3–6 (4) wide, extending nearly to level of excretory vesicle. Paired cephalic ganglia conspicuous, posterior to oral sucker, interconnected by dorsal commissure between pharynx and oral sucker. Dorsal and ventral pairs of longitudinal nerve chords visible along whole body. Penetration glands numerous, in forebody, anterolateral to ventral sucker (Fig. 2F). Ducts of penetration glands proceed along dorsal surface of oral sucker and open as two groups, lateral to stylet pouch (Fig. 2G,H).

- Excretory vesicle I-shaped, 164–232 × 34–67 (189 × 47). Main collecting ducts starting from excretory vesicle anterolaterally (Fig. 2I). Flame-cell formula not determined. Primordia of testes symmetrical, 12–15 (13) in diameter, anterolateral to excretory vesicle. Cirrus sac primordium anterior to ventral sucker. Primordia of ovary, Laurer’s channel and ovary also visible (Fig. 2I). Cercariae swim with their body contracted, thus taking saucer-shape; elongated tail beats vigorously (Supplementary material).

We obtained sequences of three fragments of the rDNA and the mitochondrial COI gene for C. cattieni (1) (Table 1). None of these sequences had any close BLAST hits. Preliminary 28S rDNA-based phylogenetic analysis (covering...
DISCUSSION

Tropical fauna of digeneans is apparently much understudied and no wonder that some findings may be strikingly unlike anything previously described. Among digeneans found in freshwater gastropods of South-East Asia (Sewell 1922, Ito et al. 1962, Ditrich et al. 1992, Dechruksa et al. 2007, Jayawardena et al. 2011, Besprozvannykh et al. 2013, Chontananarth and Wongsawad 2013, and others) nothing similar to *Cercaria cattieni* has been reported. Survey of the literature considering digenean larvae in other geographical regions and other types of environment (marine, terrestrial) produced the same result. According to the classification of Lühe (1909), *C. cattieni* has to be placed into the armate type of xiphidiocercariae. However, such combination of characters as I-shaped excretory bladder, extremely large body size and advanced primordium of the reproductive system is very unusual and has not been described previously for any xiphidiocercariae.

The process of mucoid formation in *C. cattieni* has similarities with other xiphidiocercariae. Numerous dendrites of mucoid glands were previously described in *Cercaria longistyla* McCoy, 1929 by Kruidenier (1953). Four pairs of mucoid glands were found in various xiphidiocercariae (Galaktionov and Malkova 1994, Shchenkov 2012, Shchenkov et al. 2019).

According to the results of the molecular genetic analysis, *C. cattieni* certainly belongs to the Microphalloidea, and within this superfamily to the clade comprising the Pachysolidae, Eucotylidae and Renicolidae with high nodal support. Close relationship between *C. cattieni* and *Pachysolus irroratus* gives us a clue to the systematic position of these cercariae. The family Pachysolidae was established for the only genus *Pachysolus* Looss, 1901 by Yamaguti (1958) with sexual adults inhabiting sea turtles and freshwater crocodilians (Blair 2008). No life cycles of the Pachysolidae have been elucidated. In the digenean phylogeny inferred by Olson et al. (2003) this family is a sister group to the Renicolidae + Eucotylidae, and our data confirm this general topology.

The member of the family Pachysolidae appears as the closest relative of *C. cattieni* so far. Some of the morphological characteristics support similarity of *C. cattieni* cercariae and adults of *Pachysolus* to a certain degree. The shared features are the tegument with spines, position of suckers, short oesophagus, position of testes, ovary and cirrus sac, and I-shaped excretory vesicle (Blair 2008; present data). However, they differ in the body shape and digestive system: species of *Pachysolus* lack a prepharynx and their caeca are inflated and have anterior diverticula. These comparisons should be treated critically because allometric growth during the development from cercaria to sexual adult may substantially affect morphology.
We suppose that the new type of xiphidiocercariae *C. cattieni* 1 is related or even belongs to the *Pachyp*-*solidae*. Another option is that it forms a separate branch within the clade comprising the Pachypsolidae, Eucotyliidae and Renicolidae. When sequences from more taxa are added to the tree in future, its topology, including the position of *C. cattieni* 1, may change. Recent discovery of new lineages within the Microphalloidea that cannot be placed in any existing family (Awharitoma and Enabulele 2018, Shchenkov et al. 2020) along with our data suggest that current understanding of the diversity in this superfamily is very limited, even at the high taxonomic level. To better resolve the position of *C. cattieni* 1, more molecular data on the Pachypsolidae are required.

**Supplementary material.** The video of living cercariae is available at DOI: 10.13140/RG.2.2.10264.62726

**Acknowledgements.** The authors are grateful to Frank Koehler (Australian Museum, Sydney) for his help with host identification. A.G. was supported by the research programme of the Zoological Institute of the Russian Academy of Sciences (project number AAAA-A19-119020690109-2). We thank the research resource centre “Molecular and Cell Technologies” of Saint Petersburg State University for providing sequencing facilities.

**REFERENCES**

Awharitoma A.O., Enabulele E.E. 2018: Molecular phylogenetic location of a digenean larva isolated from the African ampullarid snail, *Pila ovata*, from Obazuwa in Edo State, Nigeria. Nigerian J. Parasitol. 39: 189–193.

Besprozvannykh V., Ngo H., Ha N., Hung N., Rozhkov K., Ermolenko A. 2013: Descriptions of digenean parasites from three snail species, *Bitynia fuchsiensis* (Morelet), *Parasitology 2020, 67: 033*
afosarulus striatulus Benson and Melanoides tuberculata Müller, in North Vietnam. Helminthologia 50: 190–204.
BLAIR D. 2008: Family Pachyplosidae Yamaguti, 1958. In: D.I. Gibson, A. Jones, R.A. Bray (Eds.), Keys to the Trematoda, Vol 3. CAB International, Wallingford, and Natural History Museum, London, pp. 541–543.
BLASCO-COSTA L., POULIN R. 2017: Parasite life-cycle studies: a plea to resurrect an old parasitological tradition. J. Helminthol. 91: 647–656.
BRAY R.A. 2008: Introduction and key to superfamilies. In: R.A. Bray, D.I. Gibson, A. Jones (Eds.), Keys to the Trematoda, Vol 3. CAB International Wallingford and Natural History Museum, London, pp. 1–5.
CHONTANANARTH T., WONGSAWAD C. 2013: Epidemiology of cercarial stage of trematodes in freshwater snails from Chiang Mai province, Thailand. Asian Pacific J. Trop. Biomed. 3: 237–243.
CRIBB T.H., BRAY R.A., OLSON P.D., LITTLEWOOD D.T.J. 2003: Life cycle evolution in the Digenea: a new perspective from phylogeny. Adv. Parasitol. 54: 197–254.
DARRIBA D., TABOADA G.L., DOALLO R., POSADA D. 2012: jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9: 772.
DECHRUKS W., KRAILAS D., UKONG S., INKAPATANAKUL W., KOONCHORNBOON T. 2007: Trematode infections of the freshwater snail family Thiaridae in the Khek River, Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 38: 1016–1028.
DITRICH O., NAŚCINCOVÁ V., SCHOLZ T., GIBOA M. 1992: Larval stages of medicinally important flukes (Trematoda) from Vientiane province, Laos. Part H. Cercariae. Ann. Parasitol. Hum. Comp. 67: 75–81.
GALKOŤILOV K.V., MALKOVA I.I. 1994: The glands of trematode cercariae of the family Microphalidae Travassos, 1920. Int. J. Parasitol. 24: 595–604.
ITO J., PAPASARTHORN T., TONGKOOM B. 1962: Studies on cercariae from fresh water snails in Thailand. Jpn. J. Med. Sci. Biol. 15: 249–270.
JAYAWARDENA U., RAJAKARUNA R., AMERASINGHE P. 2011: Cercariae of trematodes in freshwater snails in three climatic zones in Sri Lanka. Ceylon J. Sci. Biol. Sci. 39: 95–108.
KRUIDENIER F.J. 1953: Studies on the formation and function of mucoids in cercariae: non-virgulate xiphidiocercariae. Am. Midl. Nat. 50: 382–396.
KUMAR S., STECHER G., TAMURA K. 2016: MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33: 1870–1874.
LÜHE M. 1909: Parasitische Plattwurmer. I. Trematoden. Süßwasserfauna Deutschlands 17: 1–217.
LUTON K., WALKER D., BLAIR D. 1992: Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). Mol. Biochem. Parasitol. 56: 323–327.
MIURA O., KURIS A.M., TORCHIN M.E., HECHINGER R.F., DUNHAM E.J., CHIBA, S. 2005: Molecular-genetic analyses reveal cryptic species of trematodes in the intertidal gastropod, Batillaria cumingi (Crosse). Int. J. Parasitol. 35: 793–801.
MORGAN J.A.T., BLAIR D. 1995: Nuclear rDNA ITS sequence variation in the trematode genus Echinostoma: an aid to establishing relationships within the 37-collar-spine group. Parasitology 111: 609–615.
OLSON P.D., CRIBB T.H., TKACH V.V., BRAY R.A., LITTLEWOOD D.T.J. 2003: Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int. J. Parasitol. 33: 733–755.
RONQUIST F., TESLENKO M., VAN derMARK P., AYRES D.L., DARLING A., HÖHNA S., LARGET B., LIU L., SUCHARD M.A., HUELSENBECK J.P. 2012: MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61: 539–542.
SCHINDELIN J., ARGÁNDARA-CARRERAS I., FRISE E., KAYNG V., LONGAIR M., PIETZCH T., PREIBISCH S., RUEDEN C., SAALFELD S., SCHIMID B., TINEVEZ J.Y., WHITE D.J., HARTENSTEIN V., ELICIERI K., TOMANCACK P., CARDONA A. 2012: Fiji: an open-source platform for biological-image analysis. Nat. Methods 9: 676–682.
SEWELL R.S. 1922: Cercariae indicas. Ind. J. Med. Res. 10: 1–370.
SCHENKOV S.V. 2012: [Mucoid glands of Cercaria baushii 6 (xiphidiocercariae: cercariae microcotylae)]. Parazitologiya 46: 314–319. (In Russian.)
SCHENKOV S.V., DENISOVA S.A., KREMEV G.A., DOBROVOLSKII A.A. 2020: Five new morphological types of virgulate and microcotylous xiphidiocercariae based on morphological and molecular phylogenetic analyses. J. Helminthol. 94: e94.
SCHENKOV S.V., DENISOVA S.A., SMIRNOVA A.D., SHUNATOVA N.N. 2019: [Mucoid glands of cercariae.] Invertebr. Zool. 16: 377–392. (In Russian.)
TKACH V., GRABA-KAZUBSKA B., PAWŁOWSKI J., SWIDERSKI Z. 1999: Molecular and morphological evidence for close phylogenetic affinities of the genera Macrodera, Leptophalus, Metaleptophalus and Paraleptophalus (Digenea, Plagiorchiata). Acta Parasitol. 44: 170–179.
YAMAGUTI S. 1958: Systema Helminthum. Volume I. The Digenean Trematodes of Vertebrates. Part I. Interscience Publishers, New York, 1575 pp.