Research Article

Molecular identification of morphologically similar microcercous cercariae of two trematode families, Paragonimidae and Troglotremaidae, concurrently found in the same snail species of the subfamily Triculinae

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Abstract: Microcercous cercariae possess a very short tail and are produced by digenean species of several families including medically important species, such as members of the genera Paragonimus Braun, 1899, Nanophyetus Chapin, 1927 and Troglotrema Odhner, 1914. During our survey of cercariae of Paragonimus spp. in Vietnam, we found microcercous cercariae from ten (0.29%) out of 3,400 snails of Triculinae gen. sp. 2. They were morphologically and molecularly analysed for species identification. The molecular analysis, based on ITS2 sequences, revealed two distinct species: four specimens were identical to Paragonimus proliferus Hsia et Chen, 1964 (Paragonimidae Dollfus, 1939), and the other six specimens were closest to members of the family Troglotremaidae Odhner, 1914 and were temporarily named Troglotremaidae gen. sp. Morphologically, cercariae of the two species found in this study are similar to each other in their gross characteristics but can be distinguished from one another by subtle morphological details. The cercaria of P. proliferus has an I-shaped excretory bladder and does not have mucous gland cells. In contrast, that of Troglotremaidae gen. sp. has a Y-shaped excretory bladder and mucous gland cells. Besides, the redia of P. proliferus is elongate with a short intestine and contains 5–6 cercariae whereas that of Troglotremaidae gen. sp. is more round with a longer intestine and harbours 3–4 cercariae. Our results have shown the importance of the shape of the excretory bladder and the presence/absence of mucous gland cells of the cercaria as well as the shape and size of the redia, and its intestinal length as valuable taxonomic characters of intramolluscan trematode larvae. In addition, the finding of similar microcercous cercariae of different species in the same snail species suggests that careful attention to morphological details is required in the differentiation of Paragonimus cercariae and those of closely related species.

Keywords: morphology, ITS2, digenean larvae, microcercariae, Vietnam.

Digenean trematodes have a complex life cycle with the involvement of at least two hosts. Of which, the obligatory first intermediate hosts are usually snails, where the development of larval stages, such as sporocyst, redia, and cercaria, takes place (Adams 2006). Thus, studies on trematode larvae in snail hosts allow understanding the life cycles of trematodes and also studying the evolutionary relationships between snail hosts and parasites (Wilke et al. 2000, Lockyer et al. 2004).

The traditional identification of trematode larvae is mainly based on the morphological characteristics of the cercaria (Schell 1970). Generally, it is possible to identify cercarial specimens to the family level, and occasionally to the genus level, using morphological criteria, but it is not easy to differentiate cercariae of related genera/species based on their morphology alone (Frandsen and Chris-tensen 1984). Alternatively, molecular techniques have been used to support the morphological identification (e.g., Bartoli et al. 2000, Pina et al. 2007, Chuboon and Wongsawad 2009, Kudlai et al. 2015, Blasco-Costa et al. 2016, Chontananarth et al. 2017, Doanh et al. 2018).

Among cercarial groups, microcercous cercariae possess a short ‘knoblike’ tail (Cable 1938, Schell 1970) and are produced by digenean species of several families including medically important species, such as members of the genera Paragonimus Braun, 1899 (causing paragonimiasis for animals and humans), Nanophyetus Chapin, 1927 (a common parasite of humans in East Siberia and a vector for a rickettsial disease of canids referred to as ‘salmon poisoning disease’ in North America), and Troglotrema Odhner, 1914 (associated with skull lesion in mustelids) (Adams 2006, Blair et al. 2008).

During our previous survey of snail hosts of Paragonimus spp. in a highly endemic area in central Vietnam (Doanh et al. 2013, 2016), we identified the cercaria of Paragonimus proliferus Hsia et Chen, 1964 in an individ-
ual snail of Triculinae gen. sp. 2 (Doanh et al. 2018). In the present study, we examined a large number of snails of Triculinae gen. sp. 2 and found the cercaria of *P. proliferus* of the family Paragonimidae Dollfus, 1939 and an unidentified species of the family Troglotrematidae Odhner, 1914. The morphological characteristics and molecular data on these cercariae were analysed and discussed herein.

**MATERIALS AND METHODS**

**Collection and examination of snails**

Freshwater snails, which were molecularly identified as Triculinae gen. sp. 2 of the subfamily Triculinae Annandale, were collected in the stream where crabs were heavily infected with metacercariae of *Paragonimus* spp., in Huong Son Commune, Huong Hoa District, Quang Tri Province (Doanh et al. 2016, 2018). Snails were examined for microcercous cercariae. In the laboratory, snails were washed with water and placed in 20 mm wide-mouth plastic containers with tap water for shedding cercariae. The containers were examined under a stereomicroscope for the presence of emerged cercariae at night and the next morning. If any microcercous cercariae were found, they were used for further study. Afterward, all molluscs were separately compressed between two glass slides and examined under a microscope to find cercariae that did not emerge, and other intra-molluscan stages, such as redia.

**Morphological data**

Live cercariae and rediae were observed under an Axio Lab A1 microscope (Carl Zeiss, Oberkochen, Germany). Then, live specimens were fixed in a hot 4% formaldehyde solution for morphological study and 96% ethanol for molecular study. Morphological characteristics of cercariae and rediae were studied from both live and fixed specimens. Measurements of cercariae and rediae were taken from fixed specimens. All measurements are in micrometers.

**Molecular analyses**

A fragment of the internal transcribed spacer 2 region (ITS2) was chosen for analyses because this region has been proven to be a reliable marker and has been commonly used to identify digenean species (Esteban et al. 2014, Blasco-Costa et al. 2016, Chai 2019). Total genomic DNA was extracted from cercariae isolated from infected snails using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). The ITS2 region was amplified using forward primer 3S and reverse primer A28 (Bowles et al. 1995), following the amplification protocol described in Doanh et al. (2018). PCR products were purified and directly sequenced by an Ab3730 sequencer using a Big-Dye Terminator Cycle Sequencing Kit.

We obtained ten ITS2 sequences of ten cercarial specimens from ten infected snails. The new sequences were deposited in the DNA Data Bank of Japan (DDBJ) with the accession No. LC506420-LC506429 (Table 1). In addition, 20 sequences of the Paragonimidae and Troglotrematidae, and an outgroup sequence (MK450527) of *Clonorchis sinensis* (Cobbold, 1875) (Table 1) were downloaded from GenBank and included in the phylogenetic analysis. These sequences were aligned using ClustalW in MEGA7.0 software (Kumar et al. 2016). The maximum likelihood phylogenetic tree was obtained using the best-fit Kimura 2-parameter model tested in MEGA 7.0. Branch support was estimated by bootstrap analyses with 1,000 replicates.

![Fig. 1. Microcercous cercariae from freshwater snails of Triculinae gen. sp. 2 collected in Huong Son Commune, Huong Hoa District, Quang Tri Province, Vietnam. A, B – cercaria and redia of *Paragonimus proliferus* Hsia et Chen, 1964; C, D – cercaria and redia of Troglotrematidae gen. sp.](image-url)
RESULTS

Prevalence of micro cercous cercariae and molecular identification

A total of 3,400 snails of Triculinae gen. sp. 2 were examined for cercariae. Micro cercous cercariae were found from ten snails (0.29%). The molecular analyses indicated two distinct sequences: four ITS2 sequences of four specimens were completely identical with each other and 464 bp in length. The other six ITS2 sequences from six specimens were also completely identical with each other and 481 bp in length. The BLAST search for highly similar sequences in GenBank revealed that four ITS2 sequences showed 100% identity to sequence (LC360503) of Paragonimus proliferus, while the other six sequences showed the highest similarity 89–93% to members of the family Troglo trematidae. They are temporarily named Troglo trematidae gen. sp. Four sequences of P. proliferus obtained in this study were clustered with that of P. proliferus (LC360503) in the clade of the Paragonimididae, while all six sequences of Troglo trematidae gen. sp. formed a distinct group in the clade of the Troglo trematidae.
Table 1. ITS2 sequences used in the present study with GenBank accession numbers, name of species and their geographical origin.

| Species                      | Location origin | GenBank No. | Reference                        |
|------------------------------|-----------------|-------------|----------------------------------|
| Clonorchis sinensis (Cobbold, 1875) (out-group) | China           | MK450527    | Qiu et al. 2019                  |
| Nanophyetidae gen. sp.       | Slovakia        | KM594165    | Heneberg et al. 2015             |
| Nanophyetidae gen. sp.       | Slovakia        | KM594150    | Heneberg et al. 2015             |
| Nanophyetus japonensis Saito, Saito, Yamashita, Watanabe et Sekikawa, 1982 | Japan           | LT796186    | Unpublished                      |
| Nanophyetus japonensis       | Russia          | LNN871823   | Unpublished                      |
| Nanophyetus salmincola (Chapin, 1926) | Russia        | LT745961    | Voronova et al. 2017             |
| Nanophyetus schlichthali Skjabin et Podyapolskaya, 1931 | Russia         | LT745962    | Voronova et al. 2017             |
| Paragonimus bangkokensis Miyazaki et Vrajuthra,1967 | Vietnam      | FJ615213    | Doanh et al. 2009                |
| Paragonimus harinaxatui Miyazaki et Vrajuthra, 1968 | Thailand       | AF159609    | Blair et al. 1999a               |
| Paragonimus heterotrema Chen et Hsia, 1964 | Vietnam     | LC360505    | Doanh et al. 2018                |
| Paragonimus macrorchis Chen, 1962 | Thailand       | AF159608    | Blair et al. 1999a               |
| Paragonimus miyazakii Kamo, Nishida, Hatsuhika et Tomimura, 1961 | Japan         | AB629937    | Unpublished                      |
| Paragonimus ohirai Miyazaki, 1939 | Japan        | U96911      | Blair et al. 1997                |
| Paragonimus proliferus Hisa et Chen, 1964 | Vietnam     | LC506420    | Present study                    |
| Paragonimus proliferus       | Vietnam         | LC506421    | Present study                    |
| Paragonimus proliferus       | Vietnam         | LC506422    | Present study                    |
| Paragonimus proliferus       | Vietnam         | LC506423    | Present study                    |
| Paragonimus proliferus       | Vietnam         | LC360503    | Doanh et al. 2018                |
| Paragonimus skjarabini Chen, 1959 | China          | AY618729    | Blair et al. 2005                |
| Paragonimus westermeni (Kerbert, 1878) | India       | JN656198    | Devi et al. 2013                |
| Paragonimus westermeni       | Vietnam         | LC360504    | Doanh et al. 2018                |
| Paragonimus vietnamensis Doanh, Shinohara, Horii, Habe, Nawa et Le, 2007 | Vietnam     | AB270689    | Doanh et al. 2007                |
| Troglotremitidae gen. sp.    | Vietnam         | LC506424    | Present study                    |
| Troglotremitidae gen. sp.    | Vietnam         | LC506425    | Present study                    |
| Troglotremitidae gen. sp.    | Vietnam         | LC506426    | Present study                    |
| Troglotremitidae gen. sp.    | Vietnam         | LC506427    | Present study                    |
| Troglotremitidae gen. sp.    | Vietnam         | LC506428    | Present study                    |
| Troglotremitidae gen. sp.    | Vietnam         | LC506429    | Present study                    |
| Troglotremitidae gen. sp.    | Japan           | AB521803    | Sato et al. 2010                 |
| Troglotremitidae gen. sp.    | Slovakia        | KM594158    | Heneberg et al. 2015             |

Morphological description of microcercous cercariae in snails of Triculinae gen. sp. 2

Cercaria of Paragonimus proliferus (Cobbold, 1875) (out-group)

Description (measurements based on 30 specimens)

Mature cercaria has a stylet and a short tail (Fig. 1A). Cercaria body elongate oval, measuring 285–320 × 115–155 (305 × 121). Oral sucker rounded, 60–75 × 60–70 (72 × 67), larger than ventral one, located at anterior extremity and accompanied with a stylet of 35–42.5 × 7.5–8.7 (38 × 7.7), with rounded, enlarged base and tapering to sharp point. Ventral sucker rounded, situated slightly posterior to middle of body, 35–50 × 35–50 (43 × 43). Prepharynx relatively long, pharynx about midway between suckers. Intestinal bifurcation slightly anterior to ventral sucker; caeca terminate at posterior end of body. Penetration gland cells 14 in total, arranged as submedian group of three anterior to ventral sucker and group of four anterolateral to ventral sucker on each side. Gland ducts extend forward along lateral margins of body, open on anterior sides of stylet. Excretory bladder U-shaped, situated between posterior end of ventral sucker and end of body. Tail ‘knob-shaped’, spinous, 25–30 × 25–27.5 (27 × 26).

Redia elongate, 900–1,100 × 200–300, intestine short, 180–220, about 20% of body length, containing 5–6 cercariae (Fig. 1B). Cercariae of P. proliferus were found in four (0.12%) snails.

Cercaria of Troglotremitidae gen. sp.

Description (measurements based on 30 specimens)

Mature cercaria with a stylet and short tail (Fig. 1C). Cercaria body elongate oval, measuring 210–240 × 90–98 (227 × 93). Oral sucker rounded, 65–75 × 60–70 (71 × 67), larger than ventral one, located at the anterior extremity and accompanied with a stylet of 35–42.5 × 7.5–8.7 (38 × 7.7) with rounded, enlarged base and tapering to a sharp point. Pharynx located just posterior to oral sucker, but esophagus and caeca not observed. Ventral sucker rounded, situated slightly posterior to middle of body, 40–47.5 × 40–47.5 (43 × 43). Penetration gland cells 16 in total, arranged in 4 clusters of 4 cells each at anterolateral to ventral sucker. Gland ducts extended forward along lateral margins of body and opening on anterior sides of stylet. Excretory bladder Y-shaped, situated between posterior end of ventral sucker and end of body. Adhesive (mucous) gland cells in ventral part of posterior end of body. Tail ‘knob-shaped’, 15–22.5 × 15–17.5 (18.5 × 16).

Redia oval, 440–500 × 360–410, intestine terminating at near posterior end of body, 240–260, about 50% of body length, containing 3–4 cercariae (Fig. 1D).

Cercariae of Troglotremitidae gen. sp. were found in six (0.18%) snails.

Remarks

Microcercous cercariae of two species found in this study are similar to each other in their gross morpholo-
gy with a stylet at the anterior end and a short tail (Fig. 1A,C). However, they are different from each other in the presence/absence of adhesive (mucous) gland cells and the shape of the excretory bladder. In addition, their redial stage is also distinguishable from each other in the shape and size of the body, the ratio of intestinal length and body length (20% vs. 50%), and the number of cercariae per redia (Fig. 1B, D). The morphological characters and measurements of cercariae from four infected snails are similar to those of *P. proliferus* previously described by Doanh et al. (2018), while microcercous cercariae from the other six infected snails do not correspond to any reported species.

**DISCUSSION**

As mentioned above, microcercous cercariae are produced by members of some digenean families (Cable 1938), including the Troglotremaeidae from which the Paragonimidae Dolfus, 1939 and Nanophyetidae Dolfus, 1939 were erected as distinct families. However, Blair et al. (2008) did not recognise the Nanophyetidae as distinct, and still placed them within the Troglotremaeidae. The molecular analyses in the present study supported this view, with sequences classified into the Nanophyetidae by Henegar et al. (2015) being intermingled with those of Troglotremaeidae in the phylogenetic tree.

In the cercarial stage, the presence/absence of a cluster of mucous gland cells arranged radially around the posterior-ventral end of the body is a unique feature to distinguish *Paragonimus*, the sole genus in the Paragonimidae, from genera of the Troglotremaeidae (see Blair et al. 2008). In agreement with this, we observed mucous gland cells in the cercaria of Troglotremaeidae gen. sp., but not in *Paragonimus proliferus*. In addition, we found that the differentiation between the shape of the excretory bladder, I-shaped in *P. proliferus* and Y-shaped in Troglotremaeidae gen. sp., is more easily recognised. Regarding this feature, I-shaped excretory bladder was reported in members of the Paragonimidae (e.g. Yamaguti 1943, Komiya and Ito 1950, Tomimura et al. 1989, Iwagami et al. 2009, Doanh et al. 2018), Y-shaped form was described from *Troglotrema acutum* (Leuckart, 1842) (see Vogel and Voelker 1978), and U- or V-shaped forms were recorded from species of *Nanophyetus* (see Bennington 1960, Filimonova 1963, Saito 1982, and *T. acutum* (see Bennington 1960, Filimonova 1963, Vogel and Voelker 1978, Saito 1985). Further studies are necessary to collect adult worms to assign this microcercous cercaria to a species.

To our best knowledge, this is the first record of the concurrence of cercariae of *Paragonimus* and a closely related species of the Troglotremaeidae in the same snail species, suggesting that the detailed examination of morphological characteristics and molecular analyses should be combined for accurate identification. The shape of the excretory bladder in addition to the presence/absence of the adhesive gland cells can be used as valuable taxonomic features for distinguishing these cercariae of two closely related families. Also, morphometrics (shape, size, ratio of length of intestine and body, and number of cercariae) of the redial stage provide additional traits for differentiation between genera/species.

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