MOLECULAR AND MORPHOLOGICAL CHARACTERISATION OF FLATWORM LARVAE PARASITISING ON FISH IN CAT TIEN NATIONAL PARK, VIETNAM

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Cat Tien National Park in southern Vietnam provides a unique opportunity to study the diversity of parasites associated with animals of the plains of tropical forests and lowland river basins in Southeast Asia. In this study we provide morphological description and phylogenetic analysis based on partial sequences of the 28S rRNA gene of metacercariae belonging to five species: Clinostomum sp., Posthodiplostomum sp. 1, Posthodiplostomum sp. 2, Crassiphialinae gen. sp. 1 and Crassiphialinae gen. sp. 2, collected in four species of freshwater fish (Rasbora paviana, Trichopodus trichopterus, Anabas testudineus and Channa straita). The digenean Clinostomum sp. was found to be phylogenetically close (possibly conspecific) to metacercaria of Clinostomum sp. recorded in Australia. There are no sequences in the GenBank database identical to any Posthodiplostomum spp. nor two other crassiphialine metacercariae found by us. Phylogenetic analysis supports the sister position of Crassiphialinae gen. sp. 1 to the Uvulifer spp. + Crassiphiala spp. group of crassiphialine trematodes. At the same time, phylogenetic relationships of Crassiphialinae gen. sp. 2 were poorly resolved. We also provide morphological description, cox1 and 28S rRNA genes-based reconstruction of phylogeny for the plerocercoid of solenophorid cestode Scyphocephalus sp. ex liver of Trichopodus trichopterus. Phylogenetic analyses unite plerocercoid of Scyphocephalus sp. in one group with solenophorid Duthiersia expansa, while the type species of the genus Scyphocephalus – S. bisulcatus – appears as a sister to Duthiersia fimбриata on the tree. Thus, phylogenetic data cast doubt on both the monotypy and the monophyly of the genus Scyphocephalus. This is the second record of plerocercoids of solenophorids and the first one of fish as second intermediate host of this cestode family.

Key words: Clinostomum, Crassiphialinae, fresh waters, metacercariae, plerocercoids, Posthodiplostomum, Scyphocephalus, tropics

Introduction

National parks and wildlife sanctuaries are essential for the preservation of native biodiversity of relevant natural communities. Untouched nature in the Cat Tien National Park in southern Vietnam provides a unique opportunity to study the diversity of parasites associated with animals of the plains of tropical forests and lowland river basins in Southeast Asia. Most of the Cat Tien National Park’s territory is covered by rain forest with a few large permanent water bodies (Tordoff et al., 2004). However, temporal streams and ponds that occur in the forest during the wet season are of no less interest in terms of metazoan parasites’ life cycle investigation. Temporary water bodies are the habitat of truly aquatic animals, and due to their shallow depth, are accessible to a wide range of terrestrial, near-water and amphibiotic hosts, which makes them an ideal environment for parasitic larvae distribution and the successful development of a fish-borne zoonosis.

The fauna of trematodes and cestodes parasitising in the metacercarial and metacestoid phases on fish in the oriental zoogeographical region is still poorly resolved, despite certain achievements in this field (e.g. Pandey & Agrawal, 2013; Gupta, 2016; Choudhary et al., 2017; Patarwut et al., 2020). Many species of metacercariae that parasitise on fish in this region are able to infect humans (Rim et al., 2008; De & Le, 2011; Hung et al., 2015), which also gives the study of their fauna practical significance.

The species identification of the larval stages of parasitic worms is a problem that all parasitologists encounter. Adequate identification of larval-like forms of helminths is possible only with a combination of morphological and molecular genetic approaches (Galazzo et al., 2002; Faltýnková et al., 2014). In this paper, we provide morphological and molecular data on metacercariae and plerocercoids collected from fish that inhabit both permanent and temporal water bodies in the Cat Tien National Park.
Material and Methods

Study area

Cat Tien National Park (11.35–11.80° N, 107.17–107.57° E) is located in District Tân Phú, Đồng Nai Province in South Vietnam (Tordoff et al., 2004). The topography of Cat Tien National Park varies greatly among the three sectors: Cat Loc, Nam Cat Tien, and Tay Cat Tien. The first one is rather hilly, although altitudes only reach 659 m a.s.l., the hills are relatively steep. The other two sectors (Nam Cat Tien and Tay Cat Tien) are situated in the lowlands. The topography of these sectors is characterised by low, gentle hills. The River Dong Nai, the second largest river in southern Vietnam, flows through the national park (Tordoff et al., 2004).

Cat Tien National Park is a well-developed protected area with a long history of efforts to preserve the pristine rainforest (Nguyen & Yen, 2013). The uniqueness of the ecosystem and the status of the protected area attract scientists who are actively studying the flora (Blanc et al., 2000), soil formation (Chernov et al., 2019), climate (Deshcherevskaya et al., 2013) and other aspects of tropical forest life.

Sample collection and morphological observations

All fish were caught between 16 November 2017 and 14 December 2017 during an expedition carried out within the framework of the Russian-Vietnamese Tropical Centre (Gordeev et al., 2018; Sokolov & Gordeev, 2019). Fish specimens in temporal water bodies (Nui Tuong ponds, the brook Da Brout and an unnamed pond) were caught using hand nets and fish net traps. Specimens from the Bao Sao (Crocodile) Lake were provided by employees of the Cat Tien National Park. Immediately after capture, specimens were dissected using standard methods (Bykhovskaya-Pavlovskaya, 1985; Klimpel et al., 2019). At all, 57 fish specimens were examined in this research: one Rasbora paviana Tirant, 1885 (total length (TL) 7.4 cm; weight 3.91 g) of the Cyprinidae, 49 Trichodorus trichopterus (Pallas, 1770) (TL 4.0–10.5 cm; weight 1.17–16.44 g) of the Osphronemidae, six Anabas testudineus (Bloch, 1792) (TL 6.4–10.6 cm; 5.84-37.46 g) of the Anabantidae, and one Channa striata (Bloch, 1793) (TL 14.9 cm; weight 30.55 g) of the Channidae. Worms collected for morphological study were taken out of the cysts using dissecting needles and fixed in hot 70% ethanol, stained with acetocarmine and mounted in Canadian balsam. All the measurements were made in micrometres based on one specimen. Ecological terms followed Bush et al. (1997). Host species were identified by Dr Ekaterina Vasilyeva (Zoological Museum of Lomonosov Moscow State University, Russia). Specimens destined for molecular analysis were fixed in 96% ethanol and stored at -18°C. The whole mounts were deposited in the Museum of Helminthological Collections at the Centre of Parasitology of the A.N. Severtsov Institute of Ecology and Evolution of RAS (IPEE RAS) in Moscow, Russia.

DNA extraction, amplification and sequencing

The total DNA was extracted from 96% ethanol-fixed metacercariae using a «hot shot» technique (Truett, 2006). The nuclear 28S rRNA gene of trematode larvae was amplified using the polymerase chain reaction (PCR) with the primers DIG12 (5′-AAGCATATCAC-TAAGCGG-3′) and 1500R (5′-GCTATCCT-GAGGAAACTTCG-3′), which were described earlier (Tkach et al., 2003). The initial PCR was performed in a total volume of 20 µl that contained 0.25 mM of each primer pair, 1 µl DNA in water, 1× Taq buffer, 1.25 mM dNTPs, 1.5 mM MgCl2 and 1 unit of Taq polymerase. The amplification was carried out in a GeneAmp 9700 (Applied Biosystems) with a 3-min. denaturation hold at 94°C, 40 cycles of 30 s at 94°C, 30 s at 55°C and 2 min. at 72°C, and a 7-min. extension hold at 72°C. Negative and positive controls were amplified using both primers. The PCR products were directly sequenced using the ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit, as recommended by the manufacturer, with the internal sequencing primers (Tkach et al., 2003). The PCR products were analysed using an Applied Biosystems 3130xl Genetic Analyser at the Russian Federal Research Institute of Fisheries and Oceanography. Unfortunately, an attempt to obtain sequences of ITS1-5.8S-ITS2 rDNA locus for all studied metacercariae using universal primers BD1 (5′-GTCGTAACAGTTTCCGTA-3′) and BD2 (5′-TATGCTTAARTTCAGCGGGT-3′) (Luton et al., 1992) was unsuccessful.

The total DNA was extracted from one 96% ethanol-fixed plerocercoid using Wizard SV Genomic DNA Purification System (Promega), as recommended by the manufacturer. The nuclear 28S rRNA gene of the cestode larva was amplified (PCR) with the primers ZX-1 (5′- ACCC-
GCTGAATTTAAGCATAT-3′), 1500R (5′-GC-TATCTGAGGAACCTTGG-3′), LSU_300F (5′-CAATGAGGAAAGTGGTTAATC-3′), 1090F (5′-TGAAACACCCCAACGACGG-3′), LSU_1200F (5′-CCCGAAAGATGGTGAACTATGC-3′), ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′), which were described by Waeschenbach & Littlewood (2017). The cox1 of the same specimen was amplified using the polymerase chain reaction (PCR) with the primers PBI-cox1F_PCR (5′-CATTTTGCTGCCGGTCA-3′), PBI-cox1R_PCR (5′-CTTTGTCGATACTGCCAA-3′), which were described by Waeschenbach & Littlewood (2017). The initial PCR was performed in a total volume of 20 µl that contained 0.25 mM of each primer pair, 1 µl DNA in water, 1× Taq buffer, 1.25 mM dinucleotide triphosphates (dNTPs), 1.5 mM MgCl2 and 1 unit of Taq polymerase. The amplification was carried out by Eurogen (Moscow) with a 3-min. denaturation hold at 94°C, 40 cycles of 30 s at 94°C, 30 s at 55°C (cox1 – 60°C) and 2 min. (cox1 – 1 min) at 72°C, and a 10-min. extension hold at 72°C. Negative and positive controls were amplified using all primers. The PCR products were directly sequenced using the ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit, as recommended by the manufacturer, with the PCR primers for 28S and with sequencing primers PBI-cox1F_seq (5′-CATTTTGCTGCCGGTCA-3′), PBI–cox1R_seq (5′-TAATGCATDGGRAAAAAAC-3′) for cox1 (Waeschenbach & Littlewood, 2017). The PCR products were analysed by Eurogen (Moscow).

Alignment and phylogenetic analysis

Partial sequence of the 28S rRNA and cox1 gene, used in our study to evaluate the phylogenetic relationships, were assembled using the Geneious ver. 10.0.5 software and aligned with sequences retrieved from the Genbank database (Electronic Supplement 1; Electronic Supplement 2) using the ClustalW DNA weight matrix within the MEGA 10.0.5 software alignment explorer (Kumar et al., 2018) selected using the BLAST search and Phylogenetic analysis of the nucleotide sequences was undertaken using the maximum likelihood (ML) and Bayesian (BI) methods. Phylogenetic trees using ML and BI methods were reconstructed using the MEGA 10.0.5 (Kumar et al., 2018) and MrBayes v. 3.6.2 software (Ronquist & Huelsenbeck, 2003), respectively. Best nucleotide substitution model for the dataset was estimated using jModelTest version 0.1.1 software (Posada, 2008). In both methods, the general time-reversible model GTR+G+I was used based on the Aikake Information Criteria (AIC). A Bayesian algorithm was performed using the Markov chain Monte Carlo (MCMC) option with ngen = 10 000 000, nruns = 4, nchains = 4 and sample-freq = 1000. The burn-in values were 2 500 000 for the «sump» and «sumt» options. The robustness of the phylogenetic relationship was estimated using bootstrap analysis with 1000 replications (Felsenstein, 1985) for ML and with posterior probabilities for BI (Ronquist & Huelsenbeck, 2003). The values of p-distance for solenophorid cestodes that are given in Table 1 were calculated using MEGA 10.0.5 (Kumar et al., 2018).

Results

Based on morphology, metacercariae and plecercoids were identified as *Clinostomum* sp., *Posthodiplostomum* sp. 1, *Posthodiplostomum* sp. 2, *Crassiphialinae* gen. sp. 1, *Crassiphialinae* gen. sp. 2 and *Scyphocephalus* sp.

**Clinostomum** sp.
Host: *Rasbora paviana*.
Site of infection: outer surface of an intestine.
Location: Bao Sao (Crocodile) Lake, 11.459444° N, 107.344167° E.
Prevalence and intensity: one of one host; two worms/host specimen.
Representative DNA sequences: partial 28S rRNA gene sequence was deposited in Genbank (NCBI) as MT406143.

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**Table 1.** Pairwise comparison of nucleotide sequence differences (p-distance, %) in the cox1 (above the diagonal) and 28S rRNA (below the diagonal) genes among solenophorid cestodes

| Species                | Scyphocephalus sp. | Scyphocephalus bisulcatus | Duthiersia expansa | Duthiersia fimbriata | Bothridium pithonis |
|------------------------|--------------------|---------------------------|--------------------|---------------------|--------------------|
| Scyphocephalus sp.     | –                  | 14.9                      | 2.7/8.4*           | 15.5                | 17.1               |
| Scyphocephalus bisulcatus | 3.6                | –                         | 14.8/15.7          | 13.2                | 16.6               |
| Duthiersia expansa     | 1.4                | 3.4                       | –                  | 15.9/15.9           | 17.1/16.5          |
| Duthiersia fimbriata   | 4.3                | 3.1                       | 3.9                | –                   | 15.7               |
| Bothridium pithonis    | 8.1                | 7.5                       | 7.5                | 8.1                 | –                  |

*Note:* * – sequences of cox1 gene with Genbank numbers KY552895/ KY552894.
Description: Body elongate-oval, 3256 × 999 μm (Fig. 1A). Tegument aspinose. Anterior end with weakly developed oral collar. Forebody 15.9% of body length. Oral sucker rounded, terminal, smaller than ventral sucker, 225 × 250 μm. Ventral sucker rounded, 500 × 438 μm. Oral sucker to ventral sucker width ratio 1:1.75 Prepharynx absent. True pharynx absent, but distinct pharyngeal chamber surrounded by dense pharyngeal gland present. Intestine bifurcating just posterior to pharyngeal chamber. Caeca extending to posterior end of body. Testes two, tandem, inter-testicular space wide. Anterior testis irregularly shaped, with irregular outline, 350 × 286 μm. Posterior testis almost triangular, slightly irregular in outline, 313 × 350 μm. Cirrus-sac ovoid, 225 × 123 μm, dextral to anterior testis. Genital pore in mid-level of body, dextro-submedian. Ovary small, rounded, entire, dextro-submedian, 125 × 125 μm. Anlage of oö-type inter-testicular, median. Uterus forming several loops in inter-testicular space, ascending around left margin of anterior testis, and distally forming tubular well developed uterine sac. Uterine sac extending anteriorly to short distance from posterior border of ventral sucker. Vitellarium undeveloped. Excretory vesicle V-shaped.

Phylogenetic data

A partial sequence of our specimen’s 28S rRNA (1313 bp) is identical to metacercaria of Clinostomum sp. (AY222175) ex Hypseleotris galii (Ogilby, 1898), Moggil Creek, Queensland, Australia. Unfortunately, no morphological information on this sample is available (Olson et al., 2003). On the phylogenetic tree (Fig. 2), both metacercariae are members of the Clinostomum spp. group that also includes Clinostomum complanatum (Rudolphi, 1814), C. cutaneum Paperna, 1964, and C. phalacrocoracis Dubois, 1931. This group is weakly supported by BI analysis and moderately supported by ML analysis, but it was poorly resolved internally.

Fig. 1. Metacercariae from fish of Cat Tien National Park. Designations: A – Clinostomum sp., whole view; B – Posthodiplostomum sp. 1 ex Anabas testudineus, whole view; C – Posthodiplostomum sp. 2, whole view; D, E – Crassiphialinae gen. sp. 1, whole view (D), mouth opening area with rows of large tegumental spines (E); F – Crassiphialinae gen. sp. 2, whole view. Scale bars: A – 1 mm; B – 0.4 mm; C – 0.3 mm; D, F – 0.15 mm; E – 0.05 mm.
**Posthodiplostomum** sp. 1

Hosts: *Trichopodus trichopterus* and *Anabas testudineus*.

Site of infection: liver.

Location: ponds Nui Tuong, 11.406667° N, 107.4075° E.

Prevalence and intensity: one of 25 hosts; one worm/host specimen (*T. trichopterus*) and one of six hosts; 26 worms/host specimen (*A. testudineus*).

Representative DNA sequences: two partial 28S rRNA gene sequences were deposited in Genbank (NCBI) as MT394050 (ex *A. testudineus*) and MT394051 (ex *T. trichopterus*).

Description: Body bipartite, with elongate, lancet-shaped forebody and subcylindrical or conical hindbody (Fig. 1B). Total length 1613 μm, maximal width 313 μm at level of holdfast organ. Forebody covered with small spines. Hindbody 375 μm in length, lodging primordial testes and ovary, and anlage of copulatory bursa with primordial fleshy prepuce. Oral sucker subterminal, 40 × 23 μm. Pseudosuckers absent. Ventral sucker rounded, 73 × 63 μm, at 875 μm from anterior extremity. Oral sucker to ventral sucker width ratio 1:2.71. Holdfast organ suboval, 182 × 149 μm, distinctly separated from ventral sucker. Proteolytic gland transverse-oval, at level of posterior edge of holdfast organ. Prepharynx very short. Pharynx 23 × 13 μm. Oesophagus 53 μm. Intestine bifurcating in anterior quarter of forebody. Caeca terminating blindly at midlevel of primordium of copulatory bursa.

**Posthodiplostomum** sp. 2

Host: *Channa striata*.

Site of infection: eye retina.

Location: unnamed pond, 11.432222° N, 107.407778° E.

Prevalence and intensity: one of one host; two worms/host specimen.
Representative DNA sequences: partial 28S rRNA gene sequence was deposited in Genbank (NCBI) as MT394045.

Description: Body bipartite, with foliate forebody and conical hindbody (Fig. 1C). Total length 900 μm, maximal width 413 μm at border between anterior and middle third of forebody. Forebody covered with small spines. Hindbody 238 μm in length, lodging primordial testes and ovary, and anlage of copulatory bursa with primordial fleshy prepuce. Oral sucker subterminal, 36 μm in width. Pseudosuckers absent. Ventral sucker rounded, 49 × 43 μm, at 438 μm from anterior extremity. Oral sucker to ventral sucker width ratio 1:1.18. Holdfast organ suboval, 125 × 92 μm, distinctly separated from ventral sucker. Proteolytic gland transverse-oval, at level of posterior edge of holdfast organ. Prepharynx not observed. Pharynx 26 μm in width. Oesophagus 66 μm. Intestine bifurcating in anterior quarter of forebody. Caeca terminating blindly at level of posterior third of primordium of copulatory bursa.

Crassiphialinae gen. sp. 1
Host: *Trichopodus trichopterus*.
Site of infection: liver.
Location: brook Da Brout, 11.441944° N, 107.428889° E.
Prevalence and intensity: four of 24 hosts; 2–8 worms/host specimen.

Representative DNA sequences: partial 28S rRNA gene sequence was deposited in Genbank (NCBI) as MT394053.

Description: Body bipartite, covered with small spines (Fig. 1D). Total length 613 μm, maximal width 213 μm at level of holdfast organ. Forebody elongate, lancet-shaped, slightly concave ventrally. Hindbody conical, 188 μm in length. Oral sucker subterminal, 59 × 43 μm, mouth opening surrounded by two rows of large spines (Fig. 1E). Pseudosuckers well developed, overlapping level of posterior part of oral sucker and anterior part of pharynx, penetrated by ducts of large gland cells lying in parenchyma between holdfast organ and anlage of gonads. Ventral sucker transverse-oval, 59 × 69 μm, at 300 μm from anterior extremity. Oral sucker to ventral sucker width ratio 1:1.61. Holdfast organ cylindrical, with deep median cavity, 63 × 63 μm, separated from ventral sucker. Proteolytic gland almost V-shaped, at level of posterior edge of holdfast organ. Prepharynx very short. Pharynx 23 × 16 μm. Oesophagus 83 μm. Intestine bifurcating at border between anterior and middle third of forebody. Caeca extending to posterior end of body. Anlage of gonads in middle part of hindbody.

Crassiphialinae gen. sp. 2
Host: *Trichopodus trichopterus*.
Site of infection: liver.
Location: brook Da Brout, 11.441944° N, 107.428889° E.
Prevalence and intensity: one of 24 hosts; eight worms/host specimen.

Representative DNA sequences: partial 28S rRNA gene sequence was deposited in Genbank (NCBI) as MT394052.

Description: Body bipartite, covered with small spines (Fig. 1F). Total length 588 μm, maximal width 200 μm at level of ventral sucker. Forebody elongate, foliate, slightly concave ventrally. Hindbody conical, 250 μm in length. Oral sucker subterminal, 56 × 36 μm. Pseudosuckers absent. Ventral sucker rounded, 46×40 μm, at 175 μm from anterior extremity. Oral sucker to ventral sucker width ratio 1:1.09. Holdfast organ cylindrical, with deep median cavity, 99 × 59 μm, just posterior to from ventral sucker. Proteolytic gland triangular, distinctly separated from posterior edge of holdfast organ. Prepharynx very short. Pharynx 23×16 μm. Oesophagus 83 μm. Intestine bifurcating at border between anterior and middle third of hindbody. Anlage of gonads at border between middle and posterior third of hindbody.

Phylogenetic data for crassiphialine metacercariae
The 28S rDNA gene-based phylogenetic analysis unites crassiphialines *Posthodiplostomum* sp. 1 and *Posthodiplostomum* sp. 2 into one well-supported group that is also including *Posthodiplostomum* sp. (AB693170), collected ex *Channa argus* (Cantor, 1842) in Japan (Nguyen et al., 2012) (Fig. 3). Within the group *Posthodiplostomum* sp. 2 and Japanese *Posthodiplostomum* sp. appears as a sister species. Crassiphialinae gen. sp. 1 is well-supported sister taxon to the *Uvulifer* spp. + *Crassiphiala* spp. group of trematodes, however the phylogenetic relationships of Crassiphialinae gen. sp. 2 were poorly resolved (Fig. 3).

*Scyphocephalus* sp.
Host: *Trichopodus trichopterus*.
Site of infection: liver.
Location: brook Da Brout, 11.441944° N, 107.428889° E.
Fig. 3. Phylogenetic position of crassiphialine metacercariae based on the analysis of 28S rRNA gene partial sequences (1003 bp). Nodal numbers are posterior probability values for BI/bootstrap values for ML. Clades with grey shading have different topology in the BI and ML trees.

Prevalence and intensity: one of 24 hosts; three worms/host specimen.

Representative DNA sequences: two partial 28S rRNA and cox1 genes sequences deposited in Genbank (NCBI) as MT408587 and MT375386, respectively.

Description: Body ovoid, 518 × 490 μm. Scolex cup-shaped, with deep apical cavity and two rudimentary bothria (Fig. 4A,B).

Phylogenetic data
The 28r rDNA gene-based phylogenetic analysis unites Scyphocephalus sp. in one group with the solenophorid cestode Duthiersia expansa Perrier, 1873 (KY552840) ex Varanus salvator (Laurenti, 1768), Vietnam (Fig. 5). The type species of the genus Scyphocephalus Riggenbach, 1898, S. bisulcatus Riggenbach, 1898 appears as a sister to another species of the genus Duthiersia Perrier, 1873, D. fimbriata (Diesing 1854). The cox1-based analysis (561 bp) also supports the close phylogenetic affinities of Scyphocephalus sp. to D. expansa; however, D. expansa appears as a paraphyletic taxon (Fig. 6). The values of p-distance for solenophorid cestodes are given in Table 1.

Fig. 4. Plerocercoid of Scyphocephalus sp., dorso-ventral (A) and apical views (B). Scale bar: 0.3 mm.
Discussion

Both Clinostomum Leidy, 1856 and Posthodiplostomum Dubois, 1936 are widespread genera of trematodes, the adult stages of which mainly parasite the piscivorous birds (Dubois, 1968; Locke et al., 2015). In regions with poorly studied fauna, species diagnostics of Clinostomum and Posthodiplostomum metacercariae, based on morphological characters, does not give an adequate and useful result, in view of cryptic diversity in these genera (e.g., Locke et al., 2015; Pérez-Ponce de León et al., 2016; Boone et al., 2018).

This is the first study of the genera Clinostomum and Posthodiplostomum metacercariae parasitising on fish in Vietnam, carried out using molecular methods. Previously, only four species of this genera were recorded in fish in Vietnam, namely Clinostomum complanatum, C. piscidium (Southwell & Prashad, 1918), Posthodiplostomum cuticula (Nordmann, 1832), and P. grayi (Verma, 1936) (see Arthur & Tê, 2006; Lysenko, 2013; Guseva et al., 2014). Our Clinostomum sp. was found to be phylogenetically close (possibly conspecific) to metacercaria of Clinostomum sp. recorded in Aus
tralia. Presently there are no sequences in the GenBank database identical to any of Posthodiplostomum sp. found by us. Also Posthodiplostomum sp. 1 and Posthodiplostomum sp. 2 differ from each other in body size, suckers ratio and utilisation of host tissues. Current phylogenetic analysis, like the data of our predecessors (e.g. Locke et al., 2010; López-Hernández et al., 2018), does not support the monophyly of the genus Posthodiplostomum.

Crassiphialinae gen. sp. 1 closely fits the morphology of Subuvulifer sabahensis (Fischthal & Kuntz, 1973), originally described in kingfisher Pelargopsis capensis javana (Boddart, 1783) in North Borneo, namely forebody shape, morphology of pseudo-suckers and holdfast organ (compare with Fischthal & Kuntz, 1973). However, the absence of molecular data on the genus Subuvulifer Dubois, 1952 and its life cycle does not allow us to verify this assumption. Crassiphialinae gen. sp. 2 differs from Crassiphialinae gen. sp. 1 by a set of significant morphological features, e.g. lack of pseudo-suckers, shape and position of holdfast organ that are a clear sign of a different generic affiliation. The position of Crassiphialinae gen. sp. 2 on the tree (Fig. 3) suggests that this trematode belongs to a separate and unknown genus.

The morphology of the studied plerocercoids’ scolexes is indicative of belonging to the genus Scyphocephalus (see Riggenbach, 1899; Wae-schenbach et al., 2017). According to the current concept of the genus Scyphocephalus, it is a monotypic taxon, and previously described nominal species S. secundus Tubangui, 1938 and S. longus Sawada & Kugi, 1973 are conspecific to the type species S. bisulcatus (see Schmidt & Kuntz, 1974; Vlnová, 2014). The values of p-distance in the 28S rRNA and cox1 genes in pairs Scyphocephalus sp./S. bisulcatus and Scyphocephalus sp./D. expansa ranged from 1.4–3.6% and 2.7–12.2%, respectively (Table 1). These values correspond to at least the interspecific level of differences in cestodes (e.g. Agusti et al., 2005; Zhang et al., 2014). Together with the topology of the trees (Fig. 5, Fig. 6) these data allow us to consider genera Duthiersia and Scyphocephalus as polyphyletic taxa. The question of the generic affiliation of cestodes included in the Duthiersia spp. + Scyphocephalus spp. clade has no clear solution yet. The taxonomic interpretation of the clade requires the union of all species into one genus or consideration as independent genera of Scyphocephalus bisulcatus, Scyphocephalus sp., Duthiersia fimbriata and all «D. expansa». Thus, the molecular data we obtained cast doubt on not only the monotypy, but also the monophyly of Scyphocephalus.

This study is the second record of solenophorid plerocercoid and the first on a fish as the second intermediate host of this cestode family. The plerocercoid stage was described earlier only in D. expansa. Pandey & Rajvanshi (1984) found this metacestode in the amphibian Hoplobatrachus tigerinus Daudin in India.

**Conclusions**

The species richness of helminths in the tropics remains a topical question of biogeography, emphasising the importance of obtaining primary faunistic data from different hosts and different regions of this climate zone (Dobson et al., 2008; Poulin, 2010). The short-term collection of fish parasites that we carried out in November and December 2017 in only four of many temporal and permanent water bodies located in the Cat Tien National Park resulted in several years of work to identify the numerous larvae of parasitic flatworms. Following the establishment of Asasco-trema vietnamiense Sokolov & Gordeev, 2019 in this paper we provide a morphological description and molecular data on the 28S rRNA gene for five species of metacercariae: Clinostomum sp., Posthodiplostomum sp. 1, Posthodiplostomum sp. 2, Crassiphialinae gen. sp. 1 and Crassiphialinae gen. sp. 2, collected from four species of fish. Of these five, only Clinostomum sp. was found to have an identical sequence in GenBank. We also provide a morphological description as well as cox1 and 28S rRNA genes-based phylogeny reconstruction of solenophorid cestode Scyphocephalus sp. Phylogenetic analysis casts doubt on both the monotypy and monophyly of the genus Scyphocephalus. Fish were recorded as the second intermediate host of solenophorids for the first time.

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Supporting Information

The dataset of 63 sequences of trematodes (Electronic Supplement 1: List of previously published trematode sequences used in the phylogenetic analysis), and 31 sequences of cestodes (Electronic Supplement 2: List of previously published cestode sequences used in the phylogenetic analysis) may be found in the Supporting Information here.

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МОЛЕКУЛЯРНАЯ И МОРФОЛОГИЧЕСКАЯ ХАРАКТЕРИСТИКА ЛИЧИНОК ПЛОСКИХ ЧЕРВЕЙ, ПАРАЗИТИРУЮЩИХ В РЫБАХ НАЦИОНАЛЬНОГО ПАРКА КАТТЬЕН, ВЬЕТНАМ

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Научное издание.

Национальный парк Каттьен (Южный Вьетнам) предоставляет уникальную возможность изучения разнообразия паразитов, связанных с животными равнинных тропических лесов и равнинных речных бассейнов Юго-Восточной Азии. В этом исследовании мы приводим морфологическое описание пяти видов метацеркарий Clinostomum sp., Posthodiplostomum sp. 1, Posthodiplostomum sp. 2, Crassiphialinae gen. sp. 1, Crassiphialinae gen. sp. 2, обнаруженных у четырех видов пресноводных рыб парка (Rasbora paviana, Trichopodus trichopterus, Anabas testudineus, Channa striata) и их филогенетический анализ, основанный на частичных последовательностях гена 28S рРНК. Трематода Clinostomum sp. оказалась филогенетически близкой (возможно, конспецифичной) к метацеркарии Clinostomum sp., ранее отмеченной в Австралии. Последовательности, идентичные двум Posthodiplostomum sp. и остальным, обнаруженным нами метацеркариям, в базе GenBank отсутствуют. Филогенетический анализ подтверждает близкородственную связь Crassiphialinae gen. sp. 1 с группой классифицированных трематод Uvulifer spp. + Crassiphiala spp. В то же время, филогенетические связи Crassiphialinae gen. sp. 2 были плохо разрешены. Мы также приводим морфологическое описание плероцеркоида соленофоридной цестоды Scyphocephalus sp. из печени Trichopodus trichopterus и реконструкцию филогении этого паразита, основанную на частичных последовательностях генов cox1 и 28S рРНК. Филогенетические анализы объединяют Scyphocephalus sp. в одну группу с Duthiersia expansa, тогда как типовой вид рода Scyphocephalus – S. bisulcatus – выступает в качестве сестринского таксона к виду Duthiersia fimbriata. Таким образом, филогенетические данные ставят под сомнение как монотипию, так и монофилию рода Scyphocephalus. Это вторая зарегистрированная находка плероцеркоидов соленофорид и первая в рыбe, как втором промежуточном хозяине цестод этого семейства.

Ключевые слова: Clinostomum, Crassiphialinae, Posthodiplostomum, Scyphocephalus, метацеркарии, плероцеркоиды, пресные воды, тропики