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Molecular identification of trematode parasites infecting the freshwater snail *Bithynia siamensis goniomphalos* in Thailand

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Research Paper

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Abstract

Digenetic trematodes are important parasites of humans and animals. They have complex life cycles and typically infect a gastropod as the first intermediate host. *Bithynia siamensis goniomphalos*, the first intermediate host of the liver fluke, *Opisthorchis viverrini*, harbours a wide variety of other trematode species. Morphological details of cercariae of 20 trematode taxa from *B. s. goniomphalos*, collected mainly in Thailand from 2009 to 2014, were provided in an earlier paper. Correct identification to the species or genus level based on morphology of these cercariae is generally not possible. Therefore, we used molecular data to improve identification and to investigate the diversity of the species of trematodes infecting *B. s. goniomphalos*. We were successful in extracting, amplifying and sequencing portions of the 28S ribosomal RNA (rRNA) gene for 19 of these 20 types of cercaria, and the internal transcribed spacer 2 region for 18 types. BLAST searches in GenBank and phylogenetic trees inferred from the 28S rRNA sequences identified members of at least nine superfamilies and 12 families. Only a few cercariae could be assigned confidently to genus or species on the basis of the sequence data. Matching sequence data from named adult trematodes will be required for definitive identification. There is clearly a great diversity of trematode species utilizing *B. s. goniomphalos* in Thailand.

Introduction

Digenean trematodes are an important group of parasites of humans and animals, responsible for many public health and livestock problems. They have complex life cycles, usually involving two or three different hosts, with molluscs playing a crucial role as first intermediate hosts. Any given trematode species can generally infect only one, or a few congeneric, species of mollusc (Blair *et al.*, 2001). In Thailand, opisthorchiasis is a major disease caused by *Opisthorchis viverrini* (Poirier, 1886) (also known as the small liver fluke). Cercariae emerging from freshwater snails penetrate cyprinid fish, in which they develop to become metacercariae that are infective to humans and animals. Cercariae of *O. viverrini* develop in snails of the genus *Bithynia* Leach, 1818, a genus occurring broadly across Eurasia and North Africa. In Thailand, *Bithynia funiculata* Walker, 1927, *Bithynia siamensis siamensis* Lea, 1856 and *Bithynia siamensis goniomphalos* (Morelet, 1886) are the species and subspecies utilized (Saijuntha *et al.*, 2019). In addition to *O. viverrini*, these snails are highly susceptible to infection with a variety of other trematodes (Kiatsopit *et al.*, 2016). Twenty different types of cercariae, identified by morphological characteristics, were found in *B. s. goniomphalos* from Sakon Nakhon Province, north-east Thailand and Vientiane Province, Lao PDR (Kiatsopit *et al.*, 2016).

Although it is usually possible to assign cercariae to superfamilies or families of trematodes based on the morphological category to which a cercaria belongs, identification to lower levels (genus and species) is often difficult or impossible (Żbikowska & Nowak, 2009). Molecular data (DNA sequences) will give finer resolution and, in some cases, likely permit identification to species where sequence data from taxonomically determined adult stages are already available in public databases. Sequences from the nuclear ribosomal second internal transcribed spacer 2 (ITS 2) region have been used for identification of various life-history stages (e.g. Wee *et al.*, 2021). However, ITS2 sequences are very variable in length and, apart from the

5' end, they are generally difficult to align across taxonomic categories higher than genus or family (Blair, 2006). Thus, this spacer region is most useful for distinguishing between congeneric species. At higher taxonomic levels, studies of phylogenetics and systematics of trematodes will continue to rely upon nuclear 28S ribosomal DNA (rDNA) sequences because a rich database already exists and is constantly growing (Littlewood *et al.*, 2015; Kostadinova & Pérez-del-Olmo, 2019).

Kiatsopit *et al.* (2016) listed previous surveys of cercariae in *Bithynia* species in Thailand. Supplementary fig. S1 is a reproduction of the figure from that paper, showing the morphology of the cercariae found. Since the publication of Kiatsopit *et al.* (2016), several additional papers on cercariae in *Bithynia* species in Thailand have appeared. Two of these (Sripa *et al.*, 2016; Kopolrat *et al.*, 2022) have been from the same research group as Kiatsopit *et al.* (2016) and did not provide additional morphological or molecular data. Other reports from Thailand will be mentioned in the discussion section. All of these studies assigned cercariae to broad morphological categories (morphotypes), without detailed anatomical descriptions of each cercaria. Several of these studies supplemented morphological identifications with DNA sequence data (nuclear ribosomal ITS2 regions or portions of the nuclear 28S ribosomal RNA (rRNA) gene) (listed in 'Concluding remarks'). However, no previous study in Thailand has utilized both ITS2 and 28S sequence data to identify cercariae, as we have done here.

From elsewhere in Southeast Asia, reports on cercariae in molluscs including *Bithynia* species have been published from Vietnam (Besprozvannykh *et al.*, 2013; Nguyen *et al.*, 2021). Most other publications concerning trematodes in *Bithynia* species have come from Russia and Europe. The most comprehensive of these include the checklist in Cichy *et al.* (2011) and a survey by Schwelm *et al.* (2020) (see also the historical account by Żbikowska & Nowak (2009) and references therein).

Here, we carried out phylogenetic analyses using DNA sequences from the nuclear 28S rRNA for each type of cercaria reported by Kiatsopit *et al.* (2016) in *B. s. goniomphalos* (Gastropoda: Bithyniidae) in Thailand. The specimens sequenced were among those collected for that study. The terminology employed by Kiatsopit *et al.* (2016) has been retained here (see table 1). Phylogenetic analyses generally provided evidence for the familial and sometimes for the generic affiliation of the cercariae found. We also obtained nuclear ITS2 sequences from most of these cercariae. This made possible refinement of identification, to the species level in a few cases. Where sequence data do not provide unambiguous identification at present, they nevertheless can assist by matching cercarial stages to later-discovered adults or metacercariae.

Materials and methods

Sample collection

Naturally infected snails were collected from a paddy field in Sakon Nakhon Province (17°21.818'N, 103°46.643'E), Thailand, by handpicking and dredging the sediment with a scoop during the years 2009–2014. The snails were identified according to the standard morphological criteria of Brandt (1974), Chitramvong (1992) and Upatham *et al.* (1983). In the laboratory, snails were placed individually into plastic containers with dechlorinated tap water and cercarial emergence was stimulated under a light source for 5 h. Living cercariae were identified as far as possible using the keys of Ito *et al.* (1962), Schell (1970), Yamaguti (1975) and Ditrich *et al.* (1997). After preliminary identification, about 30 cercariae per infected snail were washed with 0.85% normal saline solution and kept at −20°C for DNA isolation.

DNA extraction and DNA amplification

Genomic DNA of cercariae was extracted using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. Extracted genomic DNA was stored at −20°C until used. Amplification of trematode 28S rRNA gene fragments used primers listed in Lockyer *et al.* (2003) to amplify a region of ∼620 base pairs (bp). Polymerase chain reactions (PCRs) were performed in a total volume of 25 µl using 10× buffer containing 2.5 mM magnesium chloride ($MgCl_2$), 0.2 mM deoxynucleoside triphosphate (dNTP), 10 pmol of each primer, 50–100 ng of template DNA and Taq polymerase (1.5 U; iNtRON Biotechnology, Gyeonggi, Korea). The cycling conditions for 28 s rRNA were as follows: a first cycle of denaturation at 95°C for 8 min, annealing at 58°C for 2 min, elongation at 72°C for 3 min then 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, elongation at 72°C for 3 min, followed by a final denaturation at 94°C for 1 min, annealing at 58°C for 2 min, elongation at 72°C for 10 min. The ITS2 region was amplified using the forward primer 3S and the reverse primer A28 (Bowles *et al.*, 1995; Blair *et al.*, 1997). PCRs were performed in a total volume 25 µl of 10× buffer containing 2.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each primer, 50–100 ng of template DNA and Taq polymerase (1.5 U; iNtRON Biotechnology, Korea). The cycling conditions for ITS2 were as follows: initial denaturation at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 3 min and then a final 5 min elongation at 72°C. To confirm that the amplifications were successful, PCR products were run in 1.5% agarose gels stained with ethidium bromide (AMRESCO®, Solon, Ohio, USA). Each PCR amplicon was gel-excised and purified using a GeneJET Gel Extraction Kit (Thermo Scientific, Vilnius, Lithuania).

Molecular identification and phylogenetic tree analysis

Sequences were obtained successfully from 19 types of cercariae (table 1). PCR products were sent to a DNA-sequencing service (1st Base DNA Sequencing Service, Malaysia) and sequenced in both directions using the PCR primers as sequencing primers. Sequences were subjected to similarity searches in BLAST against other trematode sequences in the GenBank database (National Center for Biotechnology Information: NCBI). The partial sequences generated for the 28S rRNA, together with similar sequences selected from GenBank, were aligned using Clustal (Higgins & Sharp, 1988) with default parameters, and we manually adjusted, by eye, poorly aligned regions. Note that there are many sequences in GenBank from unidentified or tentatively identified trematodes, often cercariae. We generally avoided including these in our analysis, preferring to use sequences from named and characterized species. Phylogenetic relationships were reconstructed using Bayesian inference (BI) carried out in MrBayes 3.2 (Ronquist *et al.*, 2012) using Markov chain Monte Carlo (MCMC) searches on two simultaneous runs each of four chains and 10,000,000 generations, sampling trees every 2000 generations. The substitution model used (chosen using MEGA

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Table 1. List of morphological types of cercariae from field-infected *Bithynia siamensis goniomphalos* from Thailand, and GenBank accession numbers for partial 28S and ITS2 sequences.

Type of cercaria	28S	ITS2	Code[a]	Superfamily	Family	Likely definitive host group
Virgulate xiphidiocercaria 1	ON312618	ON312600	A	Microphalloidea	Lecithodendriidae	Bats, birds
Virgulate xiphidiocercaria 2	ON312619	ON312601	B	Microphalloidea	Pleurogenidae	Amphibians
Virgulate xiphidiocercaria 3	ON312620	ON312602	C	Microphalloidea	Pleurogenidae	Amphibians
Virgulate xiphidiocercaria 4	ON312621	ON312603	D	Microphalloidea	Pleurogenidae?	Birds?
Virgulate xiphidiocercaria 5	ON312622	ON312604	E	Microphalloidea	Prosthogonimidae	Birds
Amphistome cercaria	ON312629	ON312605	F	Paramphistomoidea	Gastrothylacidae or Paramphistomidae	Ruminant mammals
Pleurolophocercous cercaria (*Opisthorchis viverrini*)	ON312626	ON312606	G	Opisthorchioidea	Opisthorchiidae	Mammals (humans, dogs, cats)
Monostome cercaria	ON312628	ON312607	H	Pronocephaloidea	Notocotylidae	Birds
Parapleurolophocercous 1	ON312625	ON312608	I	Opisthorchioidea	Opisthorchiidae	Mammals, birds, fish
Parapleurolophocercous 2	ON312624	ON312609	J	?	?	Reptiles, amphibians, fish
Mutabile cercaria	ON312627	ON312610	K	Monorchioidea	Lissorchiidae	Fish
Ophthalmoxiphidiocercaria	ON312623	ON312611	L	?	?	Fish?
Cystophorous cercaria 1	ON312632	ON312612	M	Hemiuroidea	Derogenidae	Fish, reptiles
Cystophorous cercaria 2	ON312633	[b]	N	Hemiuroidea	Derogenidae	Amphibians, reptiles
Furcocercous cercaria 1	ON312636	ON312613	O	Schistosomatoidea	Aporocotylidae	Fish
Longifurcate-pharygeate 1	ON312635	ON312614	Q	Diplostomoidea	Cyathocotylidae	Birds
Longifurcate-pharygeate 2	ON312634	ON312615	R	Diplostomoidea	Diplostomidae	Birds, mammals
Echinostome cercaria 1	ON312630	ON312616	S	Echinostomatoidea	Echinochasmidae	Birds, mammals
Echinostome cercaria 2	ON312631	ON312617	T	Echinostomatoidea	Echinochasmidae	Birds, mammals

[a]Letter code used by Kiatsopit *et al.* (2016) and as shown in the supplementary fig. S1 reproduced from that paper.
[b]An ITS2 sequence was obtained for this cercaria but was not used because of its poor quality.

v.11: Tamura *et al.*, 2021) was the general time-reversible model with gamma-distributed rate variation across sites and allowing for a proportion of invariable sites. The first 25% of the sampled trees were discarded as burn-in for each data set, and the consensus tree topology and the nodal support were estimated from the remaining samples as posterior probability values (Huelsenbeck *et al.*, 2001).

No phylogenetic tree was constructed using ITS2 sequences. This is because these spacer sequences are difficult or impossible to align across families and higher taxa. The sequences we obtained include a portion of highly conserved 5.8S and 28S gene regions at the 5' and 3' ends, respectively. These conserved regions were removed and the remainder, constituting the actual spacer, was submitted for BLAST searches in GenBank. Being less conserved than the 28S, the ITS2 sequences provide resolution at lower taxonomic levels, making it easier to distinguish between congeners. Note that there can be some intra-specific variation in ITS2 and occasionally in the 28S gene (Blair, 2006). Searches with ITS2 sequences might fail to find any matches if no closely related taxa are represented in GenBank.

Results and discussion

Sequences were successfully obtained from 19 of the 20 cercarial types reported by Kiatsopit *et al.* (2016), the exception being 'furcocercous cercaria 2', coded with the letter 'P' in that paper. We have retained the name and code letter for each cercarial type that was used in Kiatsopit *et al.* (2016). PCR products for the 28S gene were around 620 bp in length (see supplementary fig. S2). The 28S alignment that we used for analysis included 637 sites. Lengths of individual sequences varied substantially, with those of cystophorous cercariae being the shortest (see supplementary fig. S2). Lengths of ITS2 PCR products also varied substantially, ranging from under 500 to over 800 bp (see supplementary fig. S3). In particular, the presence of repeats in the ITS2 sequences of the echinostome cercariae increased the lengths of these sequences.

We used the superfamily system as proposed by Olson *et al.* (2003), slightly modified by Littlewood *et al.* (2015), Pérez-Ponce de León & Hernández-Mena (2019) and others. The phylogenetic tree based on the 28S sequences and using BI is shown in fig. 1. Despite the fact that we used a shorter sequence alignment than Olson *et al.* (2003) and later workers, we nevertheless recovered a very similar phylogeny. The most derived group in fig. 1 represents the superfamily Microphalloidea including (amongst others) the families Prosthogonimidae, Pleurogenidae and Lecithodendriidae. Basal to this are the Opisthorchioidea and Monorchioidea. Within the former, a close affinity between the Opisthorchiidae and Heterophyidae is apparent, as has been noted by many others (e.g. Thaenkham *et al.*, 2012). Deeper in the tree, the Pronocephaloidea and Paramphistomoidea belong within a well-supported clade, the Pronocephalata of Olson *et al.* (2003), and the Diplostomidae and Cyathocotylidae are both within a clade forming the Diplostomoidea.

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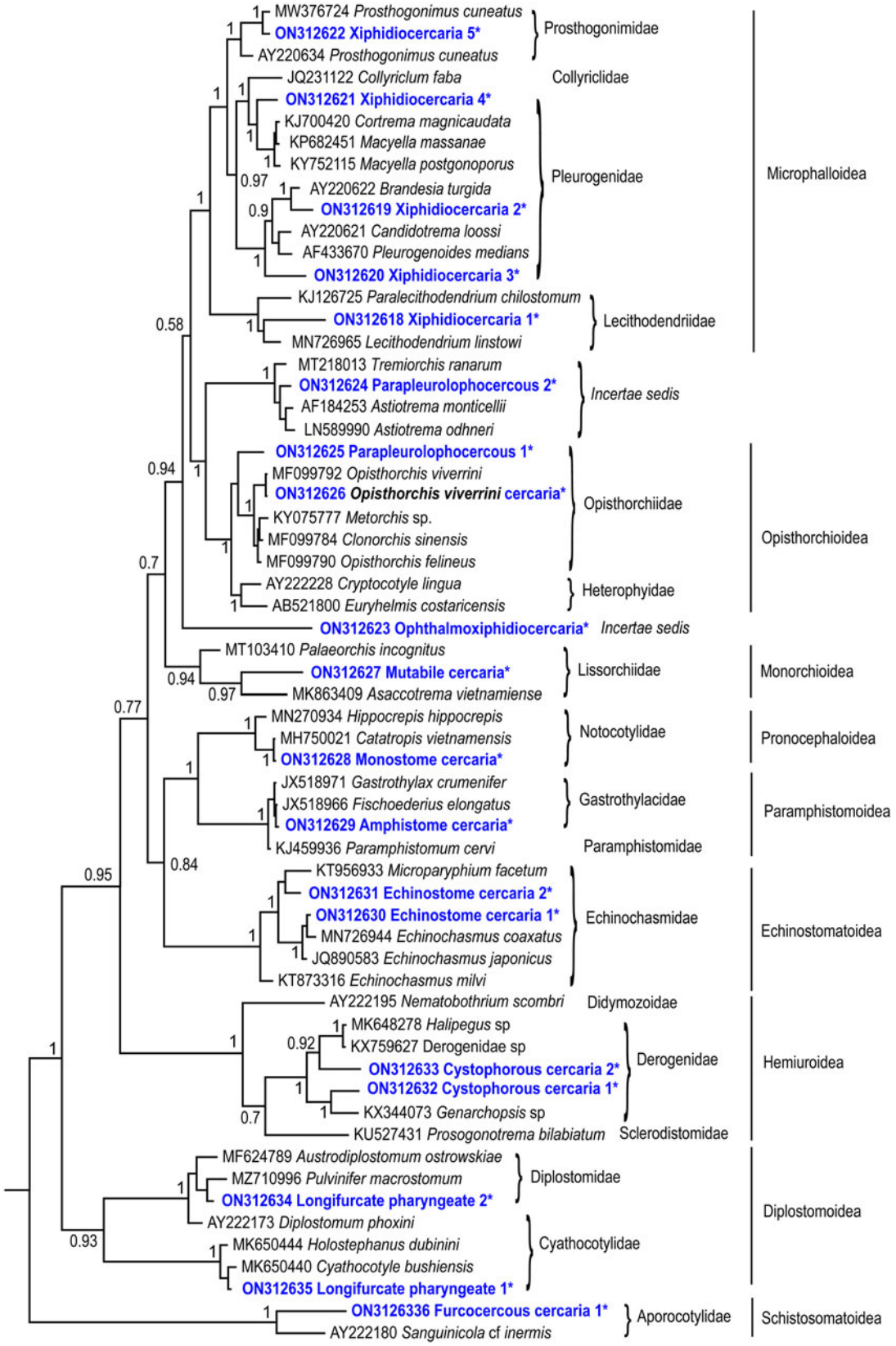

Fig. 1. Midpoint-rooted tree of partial 28S sequences from 19 types of cercariae recovered from *Bithynia siamensis goniomphalos* in Thailand (indicated in bold font and with an asterisk) and publicly available sequences from a range of related species of trematodes. Family and superfamily names have been added where appropriate. The tree was constructed using Bayesian analysis (see Methods section for details). Posterior probabilities are shown for most nodes, including all well-supported nodes.

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Our tree assigns the cercariae to family-level clades in most cases (table 1 and fig. 1). Thus, virgulate xiphidiocercaria type 5 clearly belongs within the Prosthogonimidae, types 2 and 3 within the Pleurogenidae and type 1 within the Lecithodendriidae. Inspection of the tree (fig. 1) will show similar clear affinities for most of the other cercariae. In a few cases, the family-level affinities of a cercaria are less clear given available data, or because of taxonomic debate. There is no firm rule as to the degree of similarity expected for a 28S sequence to be assigned to a particular family. But we noted that in the majority of cases where a cercaria was clearly assigned to a family, similarities of our partial 28S sequences with other sequences from that family exceeded 86% and were often over 90%. On the other hand, similarities with clearly unrelated families were generally between 73% and 85%. At the level of species and genus, it is worth noting that we obtained sequences from cercariae of *O. viverrini*, which matched sequences of this species in GenBank at 99.67–100% (28S) and 98.2–100% (ITS2) of sites. Our *O. viverrini* 28S sequence matched sequences from other *Opisthorchis* species at ≥97.55% of sites and from other opisthorchiid genera at ≥92.32% of sites. For the ITS2 region, the corresponding values were ≥95.12% and ≥84.32%, respectively.

Most of the following discussion is based on the 28S sequence data. Where informative, ITS2 sequences will also be mentioned. Note that BLAST searches using ITS2 sequences often find sequences with relatively high similarities but only short query coverage; these matches were with the conserved 5' region of the spacer and we did not discuss them.

Virgulate xiphidiocercariae (superfamily Microphalloidea Ward, 1901)

Virgulate xiphidiocercaria 1: likely family Lecithodendriidae Lühe, 1901, which are parasites of bats and occasionally of birds. There were many 28S matches in the range of 85–90.5% with various lecithodendriids. The best matches were with species of *Paralecithodendrium* Travassos, 1921, but these did not exceed 90.5%, suggesting that no close relative is represented in GenBank. The best match with our ITS2 sequence (ON312600) was with a likely lecithodendriid cercaria (96.52%), probably from *B. siamensis* collected in Central Thailand (Dunghungzin & Chontananarth, 2020). Kudlai *et al.* (2015) reported the cercaria of *Lecithodendrium linstowi* Dollfus, 1931 from *Bithynia tentaculata* (Linnaeus, 1758) from Lithuania. The 28S sequence of this cercaria (MN726965) was only 88.46% identical to our sequence. Cercariae belonging to Lecithodendriidae have been reported from *B. tentaculata* in western Russia by Shchenkov *et al.* (2019) and these also shared about 88% of sites with our 28S sequence from xiphidiocercaria 1.

Virgulate xiphidiocercaria 2: family Pleurogenidae Looss, 1899, adults likely in amphibians. The best matches with the 28S sequence from our cercaria (ON312619) were 95.74% with *Brandesia turgida* (Brandes, 1888) and 95.25% with *Prosotocus confusus* (Looss, 1894) (AY220623) (both from frogs). There were other matches over 90% with various unidentified pleurogenids, including cercariae from *B. tentaculata* in Europe (Schwelm *et al.*, 2020) and with at least three cercariae from the same host in western Russia (Shchenkov *et al.*, 2019).

Virgulate xiphidiocercaria 3: family Pleurogenidae, adults likely in amphibians. The 28S sequence from this cercaria (ON312620) had many matches with sequences of Pleurogenidae and Prosthogonimidae Lühe, 1909 in the range 85–90%.

Virgulate xiphidiocercaria type 4: probably family Pleurogenidae, and adults possibly in birds. The 28S sequence (ON312621) of this cercaria grouped in the tree with members of two small families, Cortrematidae Yamaguti, 1958 and Collyriclidae Ward, 1917, which, as adults, are parasites of birds. Kanarek *et al.* (2017) considered that Collyricloides Vaucher, 1968 (one of two genera in the Collyriclidae) was a junior synonym of *Macyellai* Neiland, 1951, and belongs within the Pleurogenidae, other members of which generally parasitize amphibians. The genus *Cortrema* Tang, 1951 also fell within the Pleurogenidae, and the family Cortrematidae was, therefore, a junior synonym of Pleurogenidae (Kanarek *et al.*, 2014, 2017). Although Kanarek and co-workers continued to recognize the family Collyriclidae with the single genus *Collyriclum* Kossack, 1911, molecular phylogenies did not provide strong support for this, nor is *Collyriclum faba* (Bremser in Schmalz, 1831) very distinct from pleurogenid species in our tree. The status of the family Collyriclidae requires further investigation. All these families fall within the Microphalloidea Ward, 1901 (see Heneberg & Literák, 2013). Schwelm *et al.* (2020) found a cercaria in *B. tentaculata* in Europe that occupied a similar position in a molecular phylogeny. However, their 28S sequence (MN726970) was slightly less similar to that of xiphidiocercaria 4 (93%) than were the related sequences we included in our tree (94%). Our ITS2 sequence (ON312603) had a similarity of around 91% with that of *C. faba* (JQ231122) and of 94.92% (96% coverage) with an unidentified pleurogenid cercaria (MN726997) from *B. tentaculata* from Germany. Little is known about the life cycle of any member of *Collyriclum*, *Macyella* or *Cortrema*. The last has been shown experimentally to infect freshwater pulmonate snails (Tang & Tang, 1981). *Collyriclum faba* uses *Bythinella austriaca* (Frauenfeld, 1857) (family Amnicolidae) in Europe (Heneberg *et al.*, 2015). *Leyogonimus polyoon* (Linstow, 1887) (a pleurogenid infecting birds) is among the close matches to our xiphidiocercaria 4 (not included in fig. 1, KY752116; 94.17%). Shchenkov *et al.* (2019) found a cercaria probably of *Leyogonimus* in *B. tentaculata* in western Russia.

Virgulate xiphidiocercaria 5: family Prosthogonimidae, with birds a likely final host. There was a 98.15% match with the 28S sequence from *Prosthogonimus cuneatus* (Rudolphi, 1809) from China (MW376724) and a 91.25% match with another digenean also identified as *P. cuneatus* (AY220634). It seems unlikely that these two sequences come from the same species. The ITS2 sequence (ON312604) was 99.55% (100% coverage) similar to that (OK044379) of a newly described *Prosthogonimus* species from the United Arab Emirates (Schuster *et al.*, 2022). Several cercariae described by Shchenkov *et al.* (2019) from western Russia utilize *B. tentaculata* and have 28S sequences similar to that of xiphidiocercaria 5 (92% identity), as do cercariae from the same host species in Germany (Schwelm *et al.*, 2020).

Ophthalmoxiphidiocercaria

The affinities of the ophthalmoxiphidiocercaria from *B. s. goniomphalos* are unclear. This type of cercaria is known so far only from the family Allocreadiidae and is typically found in bivalves, with adults typically in freshwater fish (Caira & Bogéa, 2005). The only previous exceptions to use of bivalves as intermediate hosts are from India, where Madhavi (1978, 1980) found ophthalmoxiphidiocercariae in freshwater bithyniid gastropods, from Thailand, where Ito *et al.* (1962) reported a cercaria of this type in *B. funiculata*, and from the USA, where Cable & Peters (1986) found them in freshwater pulmonate limpets (Ancylidae). Our ophthalmoxiphidiocercaria (ON312623) was

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only 82.2% similar to its closest 28S sequence matches in GenBank, which were from unidentified marine digeneans (published by Sokolov *et al*., 2019a). Matches of around 80% were found for various Acanthocolpidae Lühe, 1906, Deropristidae Cable & Hunninen, 1942, Psilostomidae Odhner, 1913, and others. It is unlikely that our ophthalmoxiphidiocercaria belongs to any of these families. BLAST searches using the ITS2 sequence (ON312611) returned no result. We cannot assign this cercaria to any family. Its adult form might be of considerable systematic interest.

Mutabile cercaria (superfamily Monorchioidea Odhner, 1911)

Mutabile cercaria: family Lissorchiidae Magath, 1917, adults (or progenetic metacercariae) in freshwater fish, usually cypriniforms. This type of cercaria, a cercariaeum, is found in the superfamily Monorchioidea, which consists of Monorchiidae Odhner, 1911, Lissorchiidae Magath, 1917 and Deropristidae Cable & Hunninen, 1942 (see Sokolov *et al*., 2020). The 28S sequence most similar to that of our cercaria from Thailand was from *Assacotrema vietnamiense* Sokolov & Gordeev, 2019, a lissorchiid from a cyprinid fish from Vietnam. Ito *et al*. (1962) recorded a cercaria of this type from *B. funiculata* in Thailand. Other previous reports of lissorchiid cercariae from bithyniid species include Petkevičiūtė *et al*. (2020) and Schwelm *et al*. (2020), both from Europe, and Besprozvannykh *et al*. (2012) in the Russian Far East. Wiroonpan *et al*. (2021) reported mutabile cercariae to be common in *B. s. siamensis* in the vicinity of Bangkok. Their ITS2 sequence for one of these (MW020049) had only partial coverage (72%) and low similarity (77%) with our mutabile sequence, suggesting that they are not conspecific.

Monostome cercaria (superfamily Pronocephaloidea Looss, 1899)

Monostome cercaria: family Notocotylidae Lühe, 1909, genus *Catatropis* Odhner, 1905, *Catatropis vietnamensis* Izrailskaia, Besprozvannykh, Tatonova, Nguyen & Ngo, 2019, with final host probably birds. Our 28S sequence (ON312628) had 99.84% identity with *C. vietnamensis*, from Vietnam as the name suggests. The snail host of *C. vietnamensis* was identified as *Melanoides tuberculata* (Müller, 1774) (family Thiaridae). Other species of *Catatropis* include bithyniids among their snail hosts (Izrailskaia *et al*., 2019). Sequences (28S) from other *Catatropis* species in GenBank had 96–98% similarity with ours, and other genera of notocotylids had similarities of over 94%. Our ITS2 sequence (ON312607) had a 100% match with that of *C. vietnamensis*. There was also a 100% match with a sequence (MT268104: Nguyen *et al*., 2021) from a cercaria from Vietnam (host unclear, but likely a bithyniid), and 100% match with a monostome cercaria from *B. s. siamensis* near Bangkok (Wiroonpan *et al*., 2021). Ito *et al*. (1962) recorded a monostome cercaria (which he identified rather tentatively as that of *Notocotylus attenuatus* Rudolphi, 1809) from *B. funiculata* from Thailand. Cercariae of the monostome type have often been noted from *Bithynia* species elsewhere (e.g. see checklist for Europe in Cichy *et al*., 2011).

Parapleurolophocercous cercariae (usually superfamily Opisthorchioidea Looss, 1899)

Parapleurolophocercous 1: family Opisthorchiidae Looss, 1899 or Heterophyidae Leiper, 1909, adults might occur in mammals, birds or even fish. Additional morphological information about this cercaria would be welcome but is not possible retrospectively: it might lack a ventral sucker and the fin folds on the tail might be dorsal and ventral rather than lateral, as is typical for most opisthorchioids (see Pinto, 2019). The 28S sequence from this cercaria (ON312625) had matches in the range 87–94% with many opisthorchiids and heterophyids. There was an 87% match with a parapleurolophocercous cercaria from northern Thailand (KU820965: Wongsawad *et al*., 2016), but the snail host cannot be identified. The ITS2 sequence showed similar levels of identity to several sequences in GenBank. It is not possible to assign this cercaria to any genus, but we tentatively place it within the Opisthorchiidae. In addition to *O. viverrini*, opisthiorchioid cercariae have often been reported from bithyniids (e.g. Schwelm *et al*., 2020 and references therein).

Parapleurolophocercous cercaria 2. This reflects an interesting discussion in the literature. The 28S sequence of our cercaria (ON312624) matched those of species in the genus *Astiotrema* Looss, 1900 and of *Tremiorchis ranarum* Mehra & Negi, 1926 (MT218013) at over 96% of sites. *Tremiorchis* Mehra & Nega, 1926 is often regarded as a junior synonym of *Astiotrema* (see Karar *et al*., 2021; Shinad *et al*., 2021). The next-best 28S matches were much lower (~87%) and were with campulids, brachycladiids and acanthocolpids, none of which is a likely home for this cercaria. The ITS2 region (ON312609) had a 98.59% similarity with that of a parapleurolophocercous cercaria from Vietnam (MT268100), reported by Nguyen *et al*. (2021), possibly from *Bithynia fuchsiana* Möllendorff, 1888 (host identity not very clear from the paper). No other full-length ITS2 matches were found using BLAST. Parapleurolophocercous cercariae are generally assumed to be produced by members of the Opisthorchioidea (see Pinto, 2019). The family placement of *Astiotrema* has been uncertain and the monophyly of the genus has been questioned. Typically, the genus has been placed within the Plagiorchioidea, usually within the Plagiorchiidae (discussed in Tkach, 2008). However, molecular studies generally place it close to the Opisthorchioidea (as also suggested by our fig. 1) (Tkach, 2008; Besprozvannykh *et al*., 2015; Sokolov & Shchenkov, 2017, amongst others). Responding to this uncertainty, Pojmańska *et al*. (2008) regarded the genus as *incertae sedis*. Besprozvannykh *et al*. (2015) experimentally completed the life cycle of *Astiotrema odhneri* Bhalerao, 1936 *sensu* Cho & Seo, 1977 and carried out molecular analyses (their partial 28S sequence – LN589990 – is represented in our fig. 1). The cercaria was a typical xiphidiocercaria. Besprozvannykh *et al*. (2015) provided evidence that an earlier study from Russia reporting a parapleurolophocercous cercaria from *Bithynia leachi* (Sheppard, 1823) in the life cycle of *Astiotrema monticellii* Stossich, 1904, was erroneous. However, given our finding of a close relationship between *Astiotrema* species and a parapleurolophocercous cercaria, the question needs to be revisited. Additional molecular data from the type species of *Astiotrema*, *A. reniferum* (Looss, 1898), will help to clarify the status and placement of the genus: at present only a partial 18S sequence is available for this species.

Amphistome cercaria (superfamily Paramphistomoidea Fischoeder, 1901)

Amphistome cercaria: family Gastrothylacidae Stiles & Goldberger, 1910 or Paramphistomidae Fischoeder, 1901. Adults probably occur in ruminant mammals. The 28S sequence from this cercaria (ON312629) had 99.51% identity with that of *Fischoederius elongatus* (Poirier, 1883) (Gastrothylacidae) from

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north-eastern India, but had almost the same degree of similarity (> 99%) with several other species in the Gastrothylacidae and Paramphistomidae. A similar situation was noted for the ITS2 sequence (ON312605): there was 100% identity with that of *Orthocoelium dicranocoelium* (Fischoeder, 1901) (MZ612015) and *Orthocoelium streptocoelium* (Fischoeder, 1901) (KJ630834, family Paramphistomidae) from Thailand. There was also 100% similarity with an amphistome cercaria from Vietnam, reported in Nguyen *et al.* (2021) from *B. fuchsiana*. Pérez-Ponce de León & Hernández-Mena (2019) and Alves *et al.* (2020) noted that Gastrothylacidae and Paramphistomidae merged in trees inferred from 28S sequences and that there was uncertainty as to the separate identities of these families according to molecular data. The high degree of conservation of nuclear ribosomal sequences in these paramphistomoids led Ghatani *et al.* (2014) to propose that mitochondrial sequences would be better for distinguishing between species. Mitchell *et al.* (2021) suggested that some sequences of paramphistomoids in GenBank were from incorrectly identified specimens.

Almost all amphistome cercariae known to date have been recovered from pulmonate snails (Tandon *et al.*, 2019). However, Ito *et al.* (1962) and Nithiuthai *et al.* (2002) have reported amphistome cercariae from *Bithynia* in Thailand (in the latter paper, the figure of an amphistome seems to show a metacercaria). Sewell (1922) recorded an amphistome cercaria from *Amnicola travancorica* (now recognized as *Gabbia travancorica* (Benson, 1860), a bithyniid) in India. *Stichorchis subtriquetus* (Rudolphi, 1814) (family Cladorchiidae Fischoeder, 1901) usually develops in pulmonates but is said to occasionally develop in *B. tentaculata* in Europe (Orlov, 1941 as cited in Flowers, 1996) – a situation that requires further investigation. Looss (1896) said that *Gastrodiscus aegyptiacus* (Cobbold, 1876) (Gastrodiscidae) develops in paludomid caenogastropods (*Cleopatra* species). An unidentified paramphistome cercaria was found in *B. fuchsiana* in Vietnam by Besprozvannykh *et al.* (2013). Several amphistomes have been reported from Thailand. Sey & Prasitirat (1994) listed species of *Gastrothylax* Poirier, 1883, *Fischoederius* Stiles & Goldberger, 1910 and *Orthocoelium* Stiles & Goldberger, 1910 from cattle and buffalo. Sripalwit *et al.* (2015) sequenced the ITS2 region of *O. streptocoelium* from Thailand. Watthanasiri *et al.* (2021) provided molecular evidence suggesting that *F. elongatus* in Thailand represents a cryptic species complex.

Echinostome cercariae (superfamily Echinostomatoidea Looss, 1899)

Echinostome cercaria 1: family Echinochasmidae Odhner, 1910, genus *Echinochasmus* Dietz, 1909, adults in birds or mammals. The 28S sequence of this cercaria (ON312630) had a 98.47% match with some *Echinochasmus* species including *E. coaxatus* Dietz, 1909 and *E. japonicus* Tanabe, 1926. Other *Echinochasmus* species were much less well matched, and other echinochasmid genera were nearly as close. Consistent with this, Tkach *et al.* (2016), Besprozvannykh *et al.* (2017) and Tatonova *et al.* (2020) all noted that *Echinochasmus* was not monophyletic in their molecular studies. Sequences of our cercaria and of *E. japonicus* and *E. coaxatus* fell within Cluster 1 of Tatonova *et al.* (2020). Cercariae of the two main clusters of *Echinochasmus* differ in morphology (Tatonova *et al.*, 2020). The ITS2 region (ON312616) had a 97.53% match with a number of sequences from *E. japonicus*, suggesting that our cercaria represents this species or a close relative, and a 96.67% match with *E. coaxatus*. In contrast, the ITS2 had <80% match with sequences from *Echinochasmus milvi* Yamaguti, 1939, a member of Tatonova's Cluster 2. Ito *et al.* (1962) reported the cercaria of *E. japonicus* from *B. funiculata* from Thailand (based on cercarial morphology). Besprozvannykh *et al.* (2013) found the snail host of *E. japonicus* in Vietnam to be *Parafossarulus striatulus* (Benson, 1842) (family Bithyniidae), whereas Nguyen *et al.* (2021) implied that *B. fuchsiana* is a host for *E. japonicus* in Vietnam, but that paper is a little unclear regarding the snail host. Besprozvannykh *et al.* (2013) cited earlier reports from European Russia that *B. tentaculata* is host for this species there.

Echinostoma cercaria 2: probably family Echinochasmidae, with adults in birds or mammals. The 28S sequence (ON312631) had 95.6% identity with the sequence (LC599528) from a cercaria of a probable *Microparyphium* species from the snail *Semisulcospira libertina* (Gould, 1859) (family Semisulcospiridae) in Japan (Nakao & Sasaki, 2021) and also with an echinochasmid cercaria from a *Juga* species (also Semisulcospiridae) in Oregon (Preston *et al.*, 2021). Matches with many other echinochasmids exceed 93%. For the ITS2 region (ON312617), matches with various echinochasmids were in the range 85–90%. It is unclear to which genus this cercaria should be assigned.

Cystophorous cercariae (superfamily Hemiuroidea Looss, 1899)

Cystophorous 1: family Derogenidae Nicoll, 1910, final hosts are likely to be fish or possibly reptiles. The 28S sequence (ON312632) had a 92% match with a sequence (KX344073; no associated publication) from a *Genarchopsis* species (Derogenidae) from the fish *Channa punctatus* (Bloch, 1793) from India. There was a slightly lower match (91%) with a sequence from *Genarchopsis chubuensis* Shimazu, 2015 (MH628311: Sokolov *et al.*, 2019b) from the fish *Rhinogobius flumineus* (Mizuno, 1960) from Japan. There were no close matches for the ITS2 sequence.

Cystophorous 2: family Derogenidae, final hosts are likely to be amphibians or possibly reptiles. The 28S sequence (ON312633) had a 91.86% match with a sequence (KX759627) from eggs of a derogenid from a chameleon imported from Africa to the USA (Collicutt *et al.*, 2017) and a 91.43% match with a sequence (MK648278) of a *Halipegus* sp. (Derogenidae) from a frog in Mexico (reported in Pérez-Ponce de León & Hernández-Mena, 2019). Other 28S matches were below 90% and included many derogenids. Although we obtained an ITS2 sequence for this cercaria, we decided not to use it, or to submit it to GenBank, because of concerns about its quality. Neither of these hemiuroid cercariae can be assigned to a genus with any confidence.

Longifurcate pharyngeate cercariae (superfamily Diplostomoidea Poirier, 1886)

Longifurcate pharyngeate 1: family Cyathocotylidae Mühling, 1898, genus *Cyathocotyle* Mühling, 1896, with adults probably in birds. The 28S sequence (ON312635) had ~98% identity with sequences from several *Cyathocotyle* species and a 97.5% match with *Holostephanus dubinini* Vojtek & Vojtkova, 1968 (also Cyathocotylidae). The ITS2 sequence (ON312614) had ~94% identity with *Cyathocotyle* species and slightly lower matches with *Holostephanus* species). Cyathocotylid cercariae have often been reported from *Bithynia* species. Records from *B. tentaculata* in Europe are particularly common (summarized in Cichy *et al.*, 2011 and Schwelm *et al.*, 2020). Besprozvannykh

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et al. (2013) reported cyathocotylid cercariae from *B. fuchsiana* in Vietnam. A cyathocotylid cercariae was reported from Thailand by Wongsawad *et al.* (2016), but it is not completely clear what the snail host was: their 28S sequence (KU820967) had a moderate match (~90%) with that of longifurcate pharyngeate cercaria 1.

Longifurcate pharyngeate 2: family Diplostomidae Poirier, 1886, adults probably in piscivorous birds or mammals. The 28S sequence (ON312634) had 95.84% identity with *Pulvinifer macrostomum* (Jagerskiold, 1900) (Diplostomidae) (MZ710996) and almost as good a match with other diplostomids in genera such as *Diplostomum* von Nordmann, 1832, *Austrodiplostomum* Szidat & Nani, 1951, *Tylodelphys* Diesing, 1850 and *Alaria* Schrank, 1788. These genera are widespread in freshwater habitats. The ITS2 sequence (ON312615) had at most about 83% similarity with other diplostomids represented in GenBank. In Europe, Sweeting (1976) demonstrated the life cycle of a diplostomid that infects *B. tentaculata*.

Furcocercous cercaria (superfamily Schistosomatoidea Stiles & Hassall, 1898)

Furcocercous Cercaria 1: probably family Aporocotylidae Odhner, 1912, adults in circulatory system of fish. The 28S sequence (ON312636) had poor matches and short query coverages in BLAST searches in GenBank. The only 100% coverage was with a sequence from *Sanguinicola* cf. *inermis* (AY222180) (84.75% identity). The ITS2 sequence (ON312613) had low query coverage (32% or less) and poor matches with all sequences in GenBank, except for the first, relatively conserved, portion of the spacer. Zhokhov *et al.* (2021) have summarized records of *Sanguinicola* Plehn, 1905 from snails, including *Bithynia*, mostly in Europe.

Concluding remarks

Most or all of the morphotypes of cercaria reported here and in Kiatsopit *et al.* (2016) have been found previously in *Bithynia siamensis* subspecies in Thailand (e.g. Anucherngchai *et al.*, 2016; Dunghungzin, 2017; Kulsantiwong *et al.*, 2017; Chantima *et al.*, 2018; Haruay & Piratae, 2019; Bunchom *et al.*, 2020; Chusongsang *et al.*, 2021). However, in the absence of detailed anatomical information and molecular data, it is impossible to say whether these represent the same trematode species as those for which we have provided sequence data. A further difficulty is that many of these papers report cercariae from several species of snail host and it is not always clear which cercaria emerged from which snail host. In some cases, the same morphological class occurred in several unrelated snail hosts. Given the high specificity of trematodes for their snail hosts, cercariae of the same morphological class from different families of snails are unlikely to represent the same species.

Where molecular data have been reported from cercariae in Thailand (e.g. Wongsawad *et al.*, 2016; Dunghungzin & Chontananarth, 2020; Wiroonpan *et al.*, 2021), it is noteworthy how infrequently such data matched closely to ours. In part, this might be because the identity of the snail host was not always explicitly stated in these papers. But the low frequency of close matches with our data does suggest that there is a much greater diversity of trematodes in *B. siamensis* than we have discovered. The only matches close enough to suggest conspecificity were all for ITS2 data (see above for parapleurolophocercous cercaria 2, xiphidiocercaria 5 and the monostome cercaria).

Given the wide range of molluscan taxa occurring in Thai freshwater systems (Sri-Aroon *et al.*, 2007 identified 39 species in one series of surveys), there must be a very large number of trematode species in the country, few of which have been studied. Schwelm *et al.* (2020) found little overlap in the trematode fauna of *B. tentaculata* in Europe with that of other families of freshwater snails. Thus, even greater taxonomic diversity of trematodes might be encountered in Thailand when the several other families of freshwater snails there are examined.

Apart from their acknowledged activities as causes of disease, trematodes in aquatic systems play several ecological roles, at least in temperate climates. The biomass of trematodes in streams may exceed that of other, more visible, taxa of invertebrates (Preston *et al.*, 2021) and they have an impact on food webs and nutrient cycling (Vannatta & Minchella, 2018; Schultz & Koprivnikar, 2021). The presence of a diverse assemblage of trematodes cycling within an ecosystem is evidence of good ecological health (Hudson *et al.*, 2006). As stated above, all these studies have been done on temperate systems. Nothing is known about the ecological functions of trematodes in tropical freshwater habitats.

A great strength of this kind of work is that it allows connection of larval stages with adult worms, identifying host species in the process. Sequence data are 'absolute', in that they are independent of taxonomic opinions and of host identity and parasite lifecycle stage. Sequence data can allow later workers to complete the picture with regard to life cycles and hosts. Blasco-Costa & Poulin (2017) called for a revival of life-history studies on trematodes: these should include not only molecular work, but also detailed morphological studies on cercarial stages, something that has been lagging around the world. We add our call for additional life-history and morphological studies on this diverse group of parasites in Thailand. In particular, there is a need for taxonomists to determine the trematodes present in Thailand as adults: this is a long-neglected area.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X22000402

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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