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Prevalence of cercarial infections in freshwater snails and morphological and molecular identification and phylogenetic trends of trematodes

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ABSTRACT

Objective: To investigate the prevalence of cercarial infections in freshwater snails from several water sources in Nakhon Nayok, Nonthaburi, and Pathum Thani provinces of Central Thailand, and to reconstruct a phylogenetic tree for improved understanding of the relationships in the cercarial stage.

Methods: The snail specimens were collected from 34 total sampling sites and investigated for cercarial infections using the crushing method. The cercarial specimens were classified and used for the phylogenetic tree analysis using the Internal Transcribed Spacer 2 (ITS2).

Results: A total of 1 921 snail specimens were classified into five families and seven species. The results showed that four snail species were identified as intermediate hosts of the larval stages of trematodes, with an overall prevalence of infection of 2.45% (47/1 921). The infected snail specimens included five groups of the cercarial type: cercariaeum cercariae, echinostome cercaria, megalurous cercaria, parapleurolophocercous cercaria, and xiphidiocercariae. This is particularly true of xiphidiocercariae, which was found to be the dominant type among cercarial infections in bithyniid snails by approximately 38.00%. With regard to molecular identification, the phylogenetic tree was reconstructed using the neighbor-joining method with 10 000 bootstraps and separated the trematodes into three clades: Echinostomatoidea, Microphalloidea and Opisthorchioidea.

Conclusions: The study reveals a high prevalence of cercarial infection for each cercarial type and maturation into a definite trematode genus and delineates morphological characteristics and evolutionary trends among each larval trematode in Nakhon Nayok, Nonthaburi and Pathum Thani provinces. In addition, the ITS2 sequence data of cercariae could be used to examine classification of these species at the family level.

KEYWORDS: Cercaria; Freshwater snails; Molecular identification; Phylogenetic tree; Prevalence; Thailand

1. Introduction

Trematode infection is one of public health problems commonly found in Thailand, in which several trematode species have been involved as a cause of various diseases such as heterophyiasis, echinostomiasis, and opisthorchiasis[1–4]. The heterophyid trematode is a food- and snail-borne disease that has been constantly reported in several areas with moderate to high prevalence, including the Chiang Mai (cyprinoid fish, 87.52%)[5], Chiang Rai (freshwater snails, 41.20%)[6], Lamphun (humans, 22.00%)[2], Khon Kaen (cyprinoid fish, 81.62%)[7], Sakon Nakhon (cyprinoid fish, 2.60%)[8], Mukdahan (cyprinoid fish, 1.94%)[8], Udon Thani (cyprinoid fish, 1.59%)[8], Lop Buri (cyprinoid fish, 52.83%)[9], and Nakhonnayok (freshwater snails, 15.85%)[10] provinces. These parasites were acquired by the consumption of raw or undercooked food, which was contaminated at the infective stage. It usually has a damaging effect on several types of economically important animals, such as cattle, birds, and aquatic animals due to the lack of an effective surveillance system and animal health care[8,11].

The life cycle of the digenetic trematodes is relatively complex, as they require freshwater snails to act as primary intermediate hosts for maturation to the intramolluscan stages (sporocyst, redia, and cercaria)[12,13]. The cercarial stage is a free-living stage which resides in the snail and emerges when fully mature. Various

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snail families including Bithyniidae, Lymnaeidae and Thiariidae have been reported as intermediate hosts of blood, intestinal, and liver trematodes[13–17]. The freshwater snails in Thailand make up a diverse ecological niche, especially in large rivers such as Chao-Phraya River, where a high diversity of snails has been observed[18,19]. Snail control was thus necessary to control the food- and water-borne diseases transmitted by freshwater snails that may be present across freshwater resources, such as agricultural areas, dams, irrigation canals, rivers, and streams. Snail-borne diseases have had a harmful impact on public health and socio-economic factors due to a lack of effective control or health care systems. Parasitic diseases could be carefully monitored throughout the area, particularly in the Nakhon Nayok, Nonthaburi, and Pathum Thani regions, situated on the Chao Phraya River areas of Central Thailand[10,19]. These areas have different activities that influence freshwater resources and are associated with densely populated settlements due to their access to these resources, including fishing communities along the rivers, rice paddy agriculture, irrigation canal for villages, and farming. In addition, these conditions also contribute to the abundance and diversity of intermediate hosts leading to trematodes becoming widespread throughout the area.

The traditional methods used to examine cercarial infections in freshwater snails are crushing[16] or shedding[17], which can be time-consuming. These methods also require a high level of skill and make it difficult to identify the cercarial type at the species level due to their morphological characteristics, which are very similar in the larval stage. Molecular techniques are the most efficient and accurate tools for the identification of trematodes[10,19,20–24]. Several DNA regions and genes selected and used for the identification of various stages (cercariae, metacercariae and adults) in intermediate and/or definitive hosts can also be used for phylogenetic relationship construction, especially the Internal Transcribed Spacer 2 (ITS2) in nuclear genes[17,20,23,24]. Previous studies applied molecular techniques to identify cercarial stages into more specific levels such as echinostome cercaria/echinostomatid[10,19], gymnocephalous cercaria/fasciolid[17,22–24], parapleurolophocercous and pleurolophocercous cercaria/heterophyid[19,22–24], parapleurolophocercous cercaria/opisthorchiid[10,22], amphistome cercaria/paramphistomatid[18], megalourous cercaria/philophthalmid[10,19,21], and furcocercous cercaria/schistosomatid[20].

The purpose of this study was to study the larval stage of trematode infection among freshwater snails in the Nakhon Nayok, Nonthaburi, and Pathum Thani provinces in Central Thailand and to construct a phylogenetic relationship based on ITS2 sequence data for molecular identification and clustering of each cercarial type into a specific level. These results are intended to obtain current epidemiological

data for describing infection rates and to help develop effective control systems that could be applied in these locations.

2. Materials and methods

2.1. Ethical approval

All experimental procedures involving animals were approved by the Biological Experiment Animals Committee in the Faculty of Science at Srinakharinwirot University (Project No. 071/2562) and conducted in accordance with the guidelines approved by the Institute for Animals for Scientific Purpose Development (IAD) and the National Research Council of Thailand (NRCT).

2.2. Study sites

In each sampling site, all available snail taxa were collected by hand picking. The 50 specimen of each snail taxa were randomly selected and examined the cercarial infections[25]. Specimens were obtained from rice paddies, rivers, marshes and irrigation canals (34 total sampling sites) located in Nakhon Nayok, Nonthaburi and Pathum Thani provinces from January to November 2018. Collections from each sampling site were performed using a stratified random sampling method[26] and based on areas with trematode-related public health problems. The geographical coordinates for each collecting sample site were recorded using a global positioning system (GPS) with UTM unit (Figure 1).

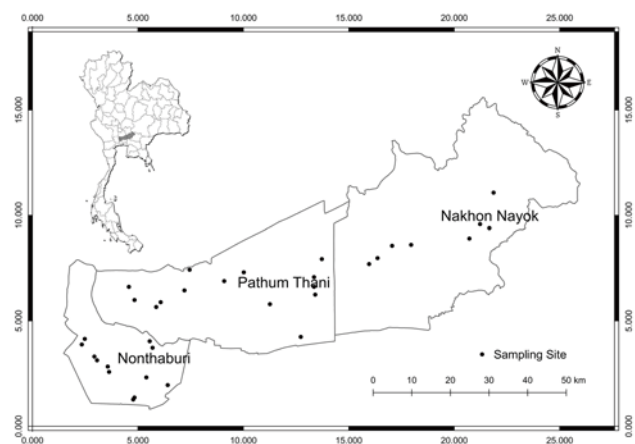


Figure 1. Map of the location sampling sites that were investigated in this study.

2.3. Snail classification and cercarial infections

Freshwater snail specimens were classified following the taxonomic keys of Brandt[27]. Each individual snail was investigated

for cercarial infections using the crushing method[16] under a light microscope. Then, the living cercarial specimens were classified according to morphological characteristics as described previously by Ito[28] and Schell[29]. The cercarial specimens were stained with Delafield's hematoxylin, dehydrated using an ethyl alcohol gradient, cleared with xylene, and mounted in permount. The cercariae were classified to the family level to record information on morphological characteristics. In addition, each type of cercaria from each sampling site was stored with 70% ethanol until use for molecular approaches[8,10,14,20,30].

2.4. DNA extraction and molecular identification

Genomic DNA was extracted from all types of cercariae from this study using the commercial NucleoSpin® tissue kit (Macherey-Nagel, Germany) according to the instructions provided by the manufacturer to be a highest producer. All extracted genomic DNA was stored at -20 °C to retain DNA integrity prior to use. Samples were polymerized using the PCR method.

The ITS2 region was amplified using a pair of primers as described by Barber *et al*[20]. The primers consisted of a forward primer with ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and a reverse primer with ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR conditions were as follows: a 4 min pre-denaturation step at 94 °C; 35 cycles consisting of 1 min of denaturation at 94 °C, 30 sec of annealing at 50 °C, and 45 sec of extension at 72 °C; and a final 7 min extension at 72 °C. The ITS2 PCR products were analyzed by gel electrophoresis using 1.5% agarose gel and separated with 80 amps for 50 min. PCR products were subsequently purified using the Cleanup PCR kit and directly sequenced in both formations by Ward Medic Ltd., Part. Obtained sequences were confirmed using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information database. Sequences were aligned using the ClustalW algorithm (MEGA7 program).

2.5. Phylogenetic tree analysis

The phylogenetic tree of ITS2 sequence data was reconstructed using MEGA7 program. The data were analyzed with the maximum likelihood (ML) method based on the Kimura 2 parameter model with a discrete gamma distribution (+G) following the Bayesian information criterion scores. The statistics for confirmed phylogenetic tree branches were evaluated using bootstrap resampling with 10 000 replicates. The related sequence of parasites acquired from the NCBI database were used to construct the phylogenetic tree (Table 1).

Table 1. List of ITS2 sequences data used for constructed phylogenetic tree analysis.

Species of trematodes	Families	Accession member
<i>Cyclocoelum mutabile</i>	Cyclocoelidae	KU877906.1
<i>Cyclocoelum mutabile</i>	Cyclocoelidae	KX097822.1
<i>Cyclocoelum mutabile</i> (Cercariaeum cercariae)	Cyclocoelidae	MN432599*
<i>Echinostoma revolutum</i>	Echinostomatidae	AY168930.1
<i>Echinostoma revolutum</i>	Echinostomatidae	KF894682.1
<i>Echinostoma revolutum</i>	Echinostomatidae	U58102.1
<i>Echinostoma revolutum</i> (Echinostome cercaria)	Echinostomatidae	MN432601*
<i>Echinostoma revolutum</i> (Echinostome cercaria)	Echinostomatidae	MN432604*
<i>Centrocestus formosanus</i>	Heterophyidae	KX430149.1
<i>Centrocestus formosanus</i>	Heterophyidae	KX430150.1
<i>Haplorchis pumilio</i>	Heterophyidae	HM004161.1
<i>Haplorchis pumilio</i>	Heterophyidae	KP165437.1
<i>Haplorchis taichui</i>	Heterophyidae	KJ630831.1
<i>Haplorchis taichui</i> (Parapleurolophocercous cercaria)	Heterophyidae	MN432600*
<i>Haplorchis taichui</i> (Parapleurolophocercous cercaria)	Heterophyidae	MN432607*
<i>Haplorchoides</i> sp.	Heterophyidae	KU753595.1
<i>Metagonimus yokogawai</i>	Heterophyidae	KJ631739.1
<i>Stellantchasmus falcatus</i>	Heterophyidae	HM004170.1
<i>Ganeo tigrinus</i>	Lecithodendriidae	KU378109.1
<i>Ganeo tigrinus</i> (Xiphidiocercariae)	Lecithodendriidae	MN432602*
<i>Lecithodendrium</i> sp.	Lecithodendriidae	KJ126724.1
<i>Lecithodendrium</i> sp. (Xiphidiocercariae)	Lecithodendriidae	MN432605*
<i>Lecithodendrium</i> sp. (Xiphidiocercariae)	Lecithodendriidae	MN432606*
<i>Mehraorchis ranarum</i>	Lecithodendriidae	KU727159.1
<i>Mehraorchis ranarum</i> (Xiphidiocercariae)	Lecithodendriidae	MN432598*
<i>Mehraorchis ranarum</i> (Xiphidiocercariae)	Lecithodendriidae	MN432603*
<i>Philophthalmus gralli</i>	Philophthalmidae	KF986198.1
<i>Philophthalmus gralli</i>	Philophthalmidae	JX121229.1
<i>Philophthalmus gralli</i> (Megalurous cercaria)	Philophthalmidae	MN432608*
<i>Philophthalmus lucipetus</i>	Philophthalmidae	KX925604.1
<i>Paragonimus heterotremus</i>	Troglotrematidae	AB827364.1

*Sequences from this study.

3. Results

3.1. Cercarial infection in freshwater snails

A total of 1 921 snail specimens were collected from 34 sites in Nakhon Nayok, Nonthaburi and Pathum Thani provinces, Central Thailand. The snail specimens were classified into five families and eight taxa, consisting of the following: *Bithynia* (*B.*) *siamensis*, *Clea helena*, *Filopaludina* (*F.*) *martensi*, *Filopaludina* (*F.*) *sumatrensis* *polygramma*, *Lymnaea auricularia*, *Melanoides* (*M.*) *tuberculata* and *Tarebia* (*T.*) *granifera*. With regard to cercarial infection, the overall prevalence of infection was 2.45% (47/1 921) in four snail

Table 2. The number of snail specimens and cercarial infections in each snail species.

Snail families	Snail taxa	No. of snails	Type of cercariae					Total
			Cer	Ech	Meg	Par	Xip	
Family Bithyniidae	<i>Bithynia siamensis</i>	650	3	-	-	3	14	20
Family Buccinidae	<i>Clea helena</i>	50	-	-	-	-	-	-
Family Lymnaeidae	<i>Lymnaea auricularia</i>	121	-	-	-	-	-	-
Family Thiaridae	<i>Melanooides tuberculata</i>	100	-	-	-	-	-	-
	<i>Tarebia granifera</i>	300	-	-	13	6	-	19
Family Viviparidae	<i>Filopaludina martensi</i>	100	-	2	-	-	-	2
	<i>Filopaludina sumatrensis polygramma</i>	600	-	2	-	-	4	6
Total		1 921	3	4	13	9	18	47

Cer: cercariaeum cercariae, Ech: echinostome cercaria, Meg: megalurous cercaria, Par: parapleurolophocercous cercaria and Xip: xiphidiocercariae.

taxa (*M. tuberculata*, *B. siamensis*, *F. martensi* and *F. sumatrensis polygramma*) (Table 2). The prevalence values of each infected snail species were 19.00% (19/100), 3.08% (20/650), 2.00% (2/100) and 1.00% (6/600), respectively.

The Nakhon Nayok province had the highest prevalence of cercarial infection (7.00%), with cercarial infections in *B. siamensis* (20.00%), *F. sumatrensis polygramma* (2.40%) and *M. tuberculata* (19.00%), followed by the Pathum Thani province (1.22%) with *B. siamensis* (3.20%) and *F. martensi* (2.00%) infections. The Nonthaburi province had the lowest prevalence (0.33%), with infections found only in *B. siamensis* (0.57%).

Furthermore, five types of cercarial stages were identified, including cercariaeum cercariae, echinostome cercaria, megalurous cercaria, parapleurolophocercous cercaria, and xiphidiocercariae. The proportion of each cercarial type revealed that the xiphidiocercaria were the dominant cercarial type infecting freshwater snails at 38.30% (18/47), followed by megalurous cercaria (27.66%), parapleurolophocercous cercaria (19.15%), echinostome cercaria (8.51%), and cercariaeum cercariae (6.38%), respectively.

Classification of cercarial stages was based on morphological differentiation according to Ito[28] and Schell[29] (position and numbers of suckers, internal organ arrangement, numbers of adhesive organs and tail features). The cercaria types were consequently described as follows.

3.1.1. Cercariaeum cercariae

Intermediate host: *B. siamensis*. Morphological appearances: The large body is tapered at both ends with yellowish-brown pigment granules. The oral suckers are located on the sub-terminal part of the anterior of the body. The pharynx is observable and esophagus is separated to bifurcate. The ventral sucker is medial and its size is equal to the oral sucker. The tail of cercariaeum cercariae does not appear at the posterior tip of the body (Figure 2A).

3.1.2. Echinostome cercaria

Intermediate hosts: *F. martensi* and *F. sumatrensis polygramma*.

Morphological appearances: The body of this cercarial type is elliptical with white pigment granules. An oral sucker presents at the sub-terminal section of the body, circular in shape and enclosed with a collared spine. The prepharynx is long and the pharynx is present. The esophagus is present with a bifurcated intestine and located between the pharynx and ventral sucker. A ventral sucker presents at the mid-ventral position of the body and the penetration glands are absent. The tail is long and slender with a thicker tip and a similar body length (Figure 2B).

3.1.3. Megalurous cercaria

Intermediate host: *M. tuberculata*. Morphological appearances: The body is flat and long with a yellowish-brown pigment and numerous granules. The oral sucker is globular in shape and located at the terminal part of the body. The esophagus is Y-shaped. The intestine is long and bifurcated. The ventral sucker appears at the mid-body, round-shaped and larger than the oral sucker. The tail of cercaria has adhesive gland cells at the terminus. It is narrow, slender, and approximately the same length (Figure 2C).

3.1.4. Parapleurolophocercous cercaria

Intermediate host: *M. tuberculata*. Morphological appearances: The body is transparent, ovoid, and pear-shaped with yellowish-orange pigment. All parts of the body are covered with minute spines. The oral sucker is small, outstanding, and located at the end of the body. The pharynx is small and the intestine is bifurcated. There are one pair of eye spots with a rectangular shape, present on the lateral side of the pharynx. There are seven pairs of penetration glands arranged in two lines. The ventral sucker is smaller than the oral sucker and takes up approximately two-thirds of the posterior body. The tail is flexible, slender and longer than the body part. The lateral fin folds near the dorsal ends of the body and a broad dorso-ventral fin lines approximately two-thirds the length of the tail (Figure 2D).

3.1.5. Xiphidiocercariae

Intermediate hosts: *B. siamensis* and *F. sumatrensis polygramma*.

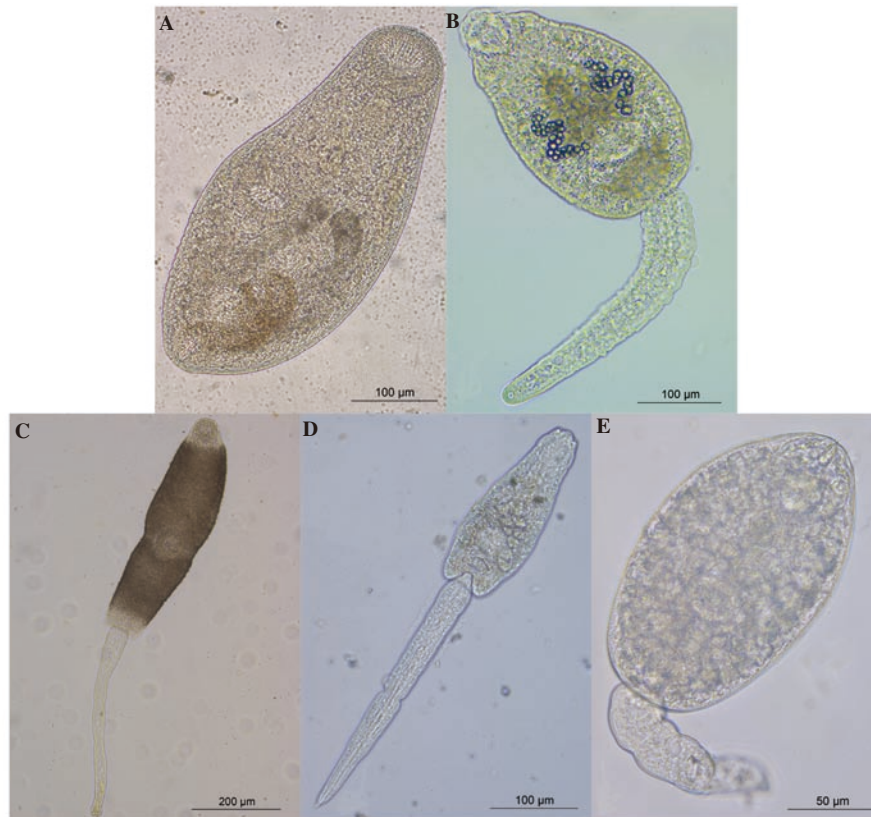


Figure 2. Morphological characteristics of five types of cercariae: (A) cercariaeum cercariae; (B) echinostome cercaria; (C) megalurous cercaria; (D) parapleurolophocercous cercaria; and (E) xiphidiocercariae.

Morphological appearances: The body of cercaria is small, flat, oblate-shaped, and unpigmented. The entire body is covered with spines. The oral sucker is small and circular, while a ventral sucker is present in the middle of the body. It is globular and smaller than the oral sucker. There is a stylet on the oral sucker on the anterior part of the body. The pharynx is short. The intestine is divided to bifurcate next to the pharynx and three pairs of penetration glands take up approximately two-thirds of the body. The tail is short, slender, and flexible (Figure 2E).

3.2. Molecular identification and phylogenetic analysis

The partial sequence data from PCR fragments of the ITS2 region were used to identify phylogenetic relationships. The length of these fragments was approximately 480-500 base pairs. The cercarial sequences were compared to sequence databases and the statistical significance was calculated by BLAST. The results showed that the sequences of NA1BS (xiphidiocercariae) (MN432603), NA2FP (echinostome cercaria) (MN432604), NA3BS (xiphidiocercariae) (MN432605), NA4FP (xiphidiocercariae) (MN432606), NA5MT (parapleurolophocercous cercaria) (MN432607), NA6MT (megalurous cercaria) (MN432608), NT1BS (xiphidiocercariae) (MN432598), PT1BS (cercariaeum cercariae) (MN432599), PT2BS (parapleurolophocercous cercaria) (MN432600), PT3FM

(echinostome cercaria) (MN432601) and PT4BS (xiphidiocercariae) (MN432602) were aligned with *Mehraorchis ranarum* (96%), *Echinostoma revolutum* (96%), *Lecithodendrium* sp. (91%-98%), *Lecithodendrium* sp. (92%-99%), *Haplorchis* (*H.*) *taichui* (100%), *Philophthalmus gralli* (99%), *Mehraorchis ranarum* (90%), *Cyclocoelum mutabile* (100%), *H. taichui* (96%), *Echinostoma revolutum* (95%) and *Ganeo tigrinus* (95%), respectively.

The phylogenetic tree of ITS2 sequence data was constructed using maximum likelihood (ML) method with most suitable model, which the lowest Bayesian information criterion scores are considered to describe the substitution algorithm the best. All sequences appeared in a monophyletic tree using the sequence of *Paragonimus heterotremus* (Troglorematidae) as an outgroup. This study revealed that the cercarial specimens can be divided into three clades according cercarial stage as well as separated from their classification (superfamily). The first clade is separated into three families of trematodes comprised of (A) Echinostomatidae/echinostome cercaria, (B) Philophthalmidae/megalurous cercaria and (C) Cyclocoelidae/cercariaeum cercariae. This group can be classified as Echinostomatoidea. The second clade showed that (D) Lecithodendriidae/xiphidiocercariae can be classified as Microphalloidea and the last group in this phylogenetic tree (E) Heterophyidae/parapleurolophocercous cercaria, can be classified as the Opisthorchioidea (Figure 3).

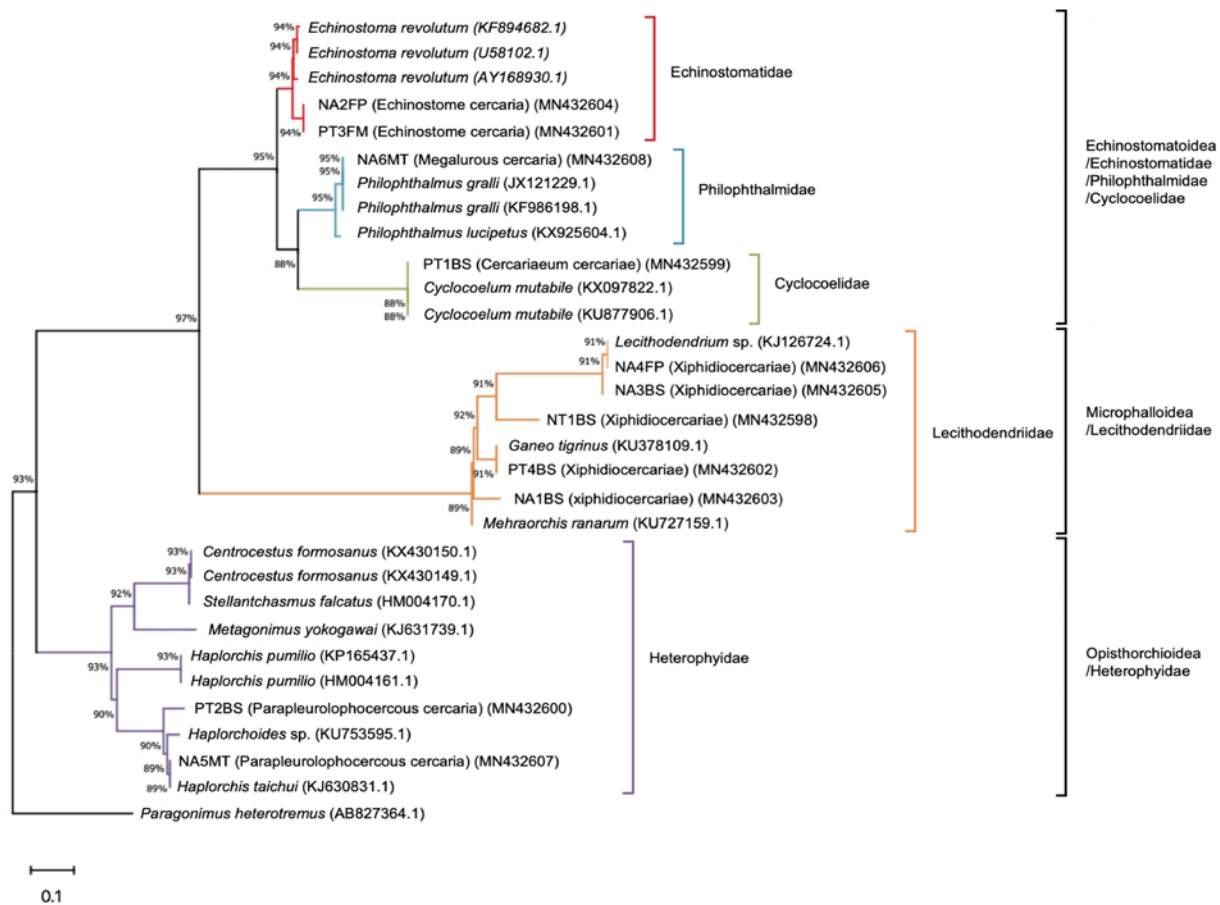


Figure 3. The rooted phylogenetic relationship from partial ITS2 sequences of each cercarial type using the maximum likelihood (ML) method based on the Kimura two-parameter with discrete gamma distribution (K2+G) model. Bootstrap values were computed independently for the purposes of 10 000 replicates.

4. Discussion

These results reveal that there is a diversity of numerous freshwater snails from the Nakhon Nayok, Nonthaburi and Pathum Thani provinces of Central Thailand. Four out of seven observed species of snail acted as intermediate hosts of trematodes: *B. siamensis*, *M. tuberculata*, *F. martensi* and *F. sumatrensis polygramma*. The overall prevalence of cercarial infection observed in this study was 2.45% lower than that of a previous study in freshwater snails from Chao-Phraya Basin in Central Thailand, which identified a prevalence of 5.90%[19]. This result is also promoted by the particular ecological conditions of the freshwater resources in Central Thailand, which maintains a complex and diverse freshwater ecosystem and with high species diversity of freshwater snails[18,19]. The prevalence of cercarial infection was highest in Nakhon Nayok, reaching approximately 7.00%; this was higher than the previous report of 4.70% prevalence of cercarial infection in freshwater snails in the same area[10]. This demonstrated that this area was suitable for cercarial infection and contained agriculture, farming, and fishery activities. The Pathum Thani and Nonthaburi provinces displayed prevalence rates of cercarial infection of 1.22% and 0.33%, respectively. The probable occurrence was the snails and parasites

may be widely distributed in the area of the Central Thailand *via* the Chao-Phraya River, which is *via* the confluence with the Chao-Phraya and Nakhon Nayok River at Pathum Thani, also known as Rangsit Canal. Moreover, the Chao-Phraya and Nakorn Chai Sri River at Nonthaburi, is also called the Bang Bua Thong Canal. In this context, ecological and biological conditions of the freshwater resources in the Chao-Phraya Basin may be suitable for cercariae reproduction and their maturation in intermediate hosts. These conditions are similar to the highly diverse water ecosystems in some areas of Asia such as Northern and Northeast Thailand[4–6,8,11], Central Myanmar[31], Northern Vietnam[12,13,30,32], Korea[33,34], Iran[35], and India[17,35].

The *M. tuberculata* snail, which can be found worldwide in fresh water and is especially abundant in ponds in Thailand[6,13], is an important intermediate host for heterophyid trematodes; in which we found that the most common type of cercarial infection was parapleurolophocercous cercaria[6,13,27]. In this study, *M. tuberculata* showed the highest susceptibility for megalurous and parapleurolophocercous cercaria infections. The previous report concluded that *M. tuberculata* was infected by megalurous cercariae, which have the same morphological characteristics, belonging to the genus *Philophthalmus*. The adult *Philophthalmus* lacked

an integumental spine, a prepharynx, and an esophagus. This trematode is typically known as an eye trematode of birds and has been known to infect humans[11,19,21,30]. Also, *M. tuberculata* serve as intermediate hosts for the parapleurolophocercous cercaria, and have been reported to harbor the larval stage of intestinal trematodes in the Heterophyidae family[14,19,20] such as *H. taichui*, *H. pumilio*, *Centrocestus caninus*, *Centrocestus formosanus*, *Stellantchasmus falcatus*, and *Metagonimus yokogawai*. This type of cercarial infection of various species of vertebrates, including humans, has led to significant public health problems in different countries such as Thailand, Vietnam, Korea, and India[30,32,34,35].

In addition, this study found that *B. siamensis* demonstrated a high susceptibility for cercarial infections and served to represent suitable intermediate hosts for harboring several types of cercarial stages, including cercariaeum cercariae, parapleurolophocercous cercaria, and xiphidiocercariae. According to previous studies, the parapleurolophocercous cercaria belong to the Heterophyidae family[10,19]. The xiphidiocercariae developed within the bithyniid snail and encysted in fish (secondary intermediate host), before maturing into the adult stage of Lecithodendriidae[4,10,19].

Over the past few years, an investigation of the prevalence of cercarial infections in freshwater snails from Central Thailand discovered several species of snails that serve as the intermediate hosts of important trematodes. For example, *B. siamensis* has been reported to be an intermediate host of Strigeidae (furcocercous cercaria), Plagiorchiidae (xiphidiocercariae)[10,33,36,37] and Opisthorchiidae[4,38]; *F. sumatrensis polygramma* and *Lymnaea auricularia* are intermediate hosts of Echinostomatidae (echinostome cercaria)[10,13,19]. Additionally, *M. tuberculata* is an intermediate host of Heterophyidae (parapleurolophocercous cercaria)[10,11,19,39] and Philophthalmidae (megarulous cercaria), and *T. granifera* is an intermediate host of Philophthalmidae (megarulous cercaria)[10,19,39]. The results of this study were in accordance with other reports on thiarid snails *M. tuberculata* and *T. granifera* and *Thiara scabra* in Thailand[5,11,14].

Regarding the investigation of cercarial diversity, the xiphidiocercariae revealed the highest prevalence of infection among *B. siamensis* and *F. sumatrensis polygramma*. It has been previously reported that[5,10,11,19] and present the adult stage of the Plagiorchiidae superfamily[10,33,36,37]. In contrast, the echinostome cercaria infected only viviparid snails. This type of cercaria infected the gastrointestinal tract, especially in the *Hypoderaeum* genus and the *Echinostoma* genus[6,10,15,19,32]. It has been known to heavily infect domestic ducks and humans[34,35]. The cercariaeum cercariae infected only one snail species. *B. siamensis* is an intermediate host of Cyclocoelidae (trematode of the respiratory tracts of birds) and Monorchidae (intestinal trematode of fish). It appears that this type of cercaria has a high specificity against infection in the *B. siamensis*

host.

In terms of molecular identification, this study analyzed the sequence data in order to establish a phylogenetic tree. The tree contains three clades following the specificity of cercarial type including cercariaeum cercariae, echinostome cercaria, megarulous cercaria, parapleurolophocercous cercaria, and xiphidiocercariae, which were found to be able to develop into the adult stage as Cyclocoelidae[10], Echinostomatidae[13,15], Philophthalmidae[10,19], Heterophyidae[10,13,39], and Lecithodendriidae[10,19], respectively. The monophyletic tree showed that the cercarial sequence data could be divided into three superfamilies of trematode comprised of Opisthorchioidea (Heterophyidae), Microphalloidea (Lecithodendriidae), and Echinostomatoidea (Echinostomatidae, Philophthalmidae, and Cyclocoelidae). The data sequences revealed that the Heterophyidae clade were separated from the other clades (Echinostomatidae, Philophthalmidae, Cyclocoelidae and Lecithodendriidae). This family has been reported as intestinal trematodes of birds and mammals[10,19,22,23]. The Lecithodendriidae were grouped closely with Echinostomatoidea (Echinostomatidae, Philophthalmidae and Cyclocoelidae) and has been identified as resulting in trematode infections of insectivorous vertebrates[4,10,19]. The Echinostomatoidea superfamily can develop into the trematodes of both birds and mammals, including the Echinostomatidae (intestine trematode)[10,19,13], Philophthalmidae (eye trematode)[11,19,21,40] and Cyclocoelidae (respiratory tract trematode).

In conclusion, the freshwater snails in this survey showed a high level of significance in terms of public health including biomedical, clinical, and veterinary medicine effects. This work revealed a high prevalence of cercarial infection for each cercarial type and maturation into a definite trematode genus. Moreover, this study confirms that the sequence data of ITS2 could be applied to investigate the molecular identification and evolutionary trends among different trematode families, leading to increased epidemiological knowledge and development of an accurate control program for water-borne trematode infections in humans and animals that are present in the Nakhon Nayok, Nonthaburi, and Pathum Thani provinces of Central Thailand.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Authors' contributions

TC contributed, designed and participated in all parts of study, collected and identified the samples. CD conducted the experiment in the laboratory and analyzed the data. TC and DC were working together for writing the manuscript. All authors have approved this manuscript.

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