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Epidemiological situation and molecular identification of cercarial stage in freshwater snails in Chao-Phraya Basin, Central Thailand

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ABSTRACT

Objective: To investigate the prevalence of cercarial trematode infection in snails and to examine the reconstruction of the phylogenetic relationship to explain the molecular system of cercarial stage trematodes to estimate the infection rate of in the definite host from the Chao-Phraya Basin.

Methods: The snails were collected from 10 provinces of the Chao-Phraya Basin, Thailand by stratified sampling method. The snails were examined for cercarial infection by the crushing method. All DNA specimens were amplified with internal transcribed spacer 3 (ITS3) and ITS4 primer based on PCR technique. The sequence data were aligned and used to reconstruct the phylogenetic tree by unweighted pair-group method with arithmetic means with 10 000 bootstraps.

Results: The overall rate of cercarial infection was found to be 5.90% (122/2 067). Snails in the family Thiaridae were found to be in the highest prevalence followed by Lymnaeidae, Bithyniidae, Planorbidae, Viviparidae, and Ampullariidae, respectively, while the Buccinidae family (*Clea helena*) did not reveal any infections. The frequently found species of cercariae were parapleurolophocercous cercariae, cercariae and megarulous cercariae. The monophyletic tree separated the snails into five groups comprised of Heterophyidae, Strigeidae, Lecithodendriidae, Philophthalmidae and Echinostomatidae using the sequence of *Angiostrongylus cantonensis* as an out-group.

Conclusions: This study was the first to report on cercarial infection in the Chao-Phraya Basin, Thailand. This revealed that a high variety of freshwater snails were infected by cercariae stage trematodes with a high prevalence. The sequence data of ITS2 can be used to investigate the phylogenetic relationships of trematodes at the family level and in each clade of different families separated by the definitive hosts.

1. Introduction

Digenetic trematodes are widely distributed [1–5] and continue to be an important public health problem in the Greater Mekong Subregion including Vietnam, Myanmar, Cambodia, Laos and Thailand [6]. The life cycle of the trematodes is very complex as they require an intermediate host such as snails or fish for maturation to the infective stage, while the definitive host is often infected by eating raw or half-cooked like fermented fish dishes (pla-ra and pla-som) [6,7]. All digenetic trematodes have been implicated as a cause of various parasitic diseases such as heterophyiasis which often result in significantly high rates of eosinophilic, diarrhea, abdominal pain for the patients who are infected by heterophyid trematodes [8], which are wildly distributed throughout Northern and Northeastern Thailand. Furthermore, the infective larvae stage of blood flukes in the family Schistosomatidae has been known to cause schistosomiasis in humans by penetrating the skin, after which, mild dermatitis

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and Katayama fever would appear [9,10]. Moreover, the human liver fluke, *Opisthorchis viverrini* (*O. viverrini*) that is known to cause opisthorchiasis, is currently reported to have infected about 6 million people in Thailand who have been diagnosed with hepatobiliary diseases and cholangiocarcinoma by chronic infection [11]. The agricultural area located in the central plain of Thailand is one of areas that produce extensive amounts of rice for export [12]. As a result, the activities of agriculturists in this area may produce and discharge waste into the water resources including rivers, irrigation canals, and reservoirs over a long period of time. This is a reason for the widespread occurrence of many trematodes and the high prevalence of cercarial infection in Thailand [4,8,13–16], especially in the Chao-Phraya Basin area. This location has a diverse ecological system comprised of paddy fields, forests and a variety of water resources. This ecological system is suitable for many freshwater snails that play an important role as the intermediate hosts of various trematodes [4,13,14]. Therefore, the current data on the prevalence of cercarial infection in snails have been usefully applied to predict the epidemiological situation of trematode infections in definitive hosts like mammals, Aves, reptiles and humans for the purposes of developing preventative applications in the future.

The classic method used to identify cercaria considered only the morphological characteristics. However, this method consumes more time and requires a high level of experienced-based skills. Nevertheless, the morphology of the larval stage might not be accurately distinguishable other than by a specially trained diagnostic researcher. Moreover, difficulties arise because cercaria are small and soft and also possess only a few stable morphological characteristics and are subject to host-induced phenotypic variations [17]. Therefore, molecular biological methods are the most efficient and accurate tools for the identification of numerous organisms including trematodes [13,18–20]. The internal transcribed spacer 2 (ITS2) of the 18S rDNA gene was selected and used for the identification of various stages and for studying the life cycles of heterophyid trematode (cercaria, metacercaria and adult stages) infections in freshwater fish [21,22]. The sequences of the ITS2 region have been used as potential marker for species or population level [23]. Phan *et al.* [7] separated larval and adult stages of *Haplorchis taichui* (*H. taichui*) and *Haplorchis pumilio* (*H. pumilio*) using the same target gene. Conventional PCR methods have been widely used because the nuclear DNA method is highly accurate, sensitive and can be rapidly applied. Therefore, this sequence data have proven to be beneficial for the purposes of studying species identification, geographical distribution, phylogenetic relationships particularly for *Schistosoma haematobium* and *Schistosoma bovis* [24], *O. viverrini*, *Clonorchis sinensis*, *H. pumilio* and *H. taichui* [25]. In addition, previous reports have used the ITS2 region to characterize *Paragonimus westermani*, *Fasciolopsis buski* and *Fasciola gigantica* collected from Northeast India [19].

Regarding phylogenetic reconstruction, rDNA in the genomes of animals are involved with the evolutionary process and can produce mutations in populations or species. For example, the ITS2 region is a conservative region that appears between 5.8S, and 28S rDNA and becomes homogenized within a given organism and helps the researcher to differentiate between species [26]. Furthermore, this region was used to construct the phylogenetic tree of several organisms. For the example, Prasad *et al.* [19] revealed the relationship of many trematodes in India. A recent experiment conducted by Tang *et al.* [27] involved the construction of the phylogeny tree of the parasitic protozoa *Trypanosoma*. Therefore, this region was deemed to be suitable for use in phylogenetic relationship analysis. Consequently, to identify the species level of cercarial infection in snails, this investigation applied the molecular technique while considering the morphological characteristics for higher levels of accuracy in the results.

The purpose of this study was to investigate the prevalence of each type of cercarial infection present in snails and to reconstruct the phylogenetic tree showing the overall relationship using specific analytical methods based on the PCR technique and focused on the ITS2 region of cercariae found in freshwater snails to estimate the infection rate among the definitive hosts in the Chao-Phraya Basin and to identify beneficial prevention techniques for future investigation.

2. Materials and methods

2.1. Collected samples and cercarial infection

The snail specimens were collected by stratified sampling method [28] during the period of February 2014 to October 2014 from 10 provinces located in the Chao-Phraya Basin, which were Nakhon Sawan, Chai Nat, Sing Buri, Ang Thong, Suphan Buri, Ayutthaya, Nakhon Nayok, Pathum Thani, Nonthaburi and Bangkok (Figure 1). The coordinates for all the collection sites were recorded using the global positioning system. The specimens were classified using a taxonomic key and then they were separated by species level [29].

The cercarial infection was examined in freshwater snails and identified manually under a high magnification stereomicroscope. The living cercariae were vitally stained with 0.5% neutral red dye and identified according to morphological classification as previously described [30]. In addition, the cercarial specimens were stained with Delafield's hematoxylin or acetone orcine, dehydrated in an ethyl alcohol series, cleared with xyline, and mounted in permount. Using a camera lucida,

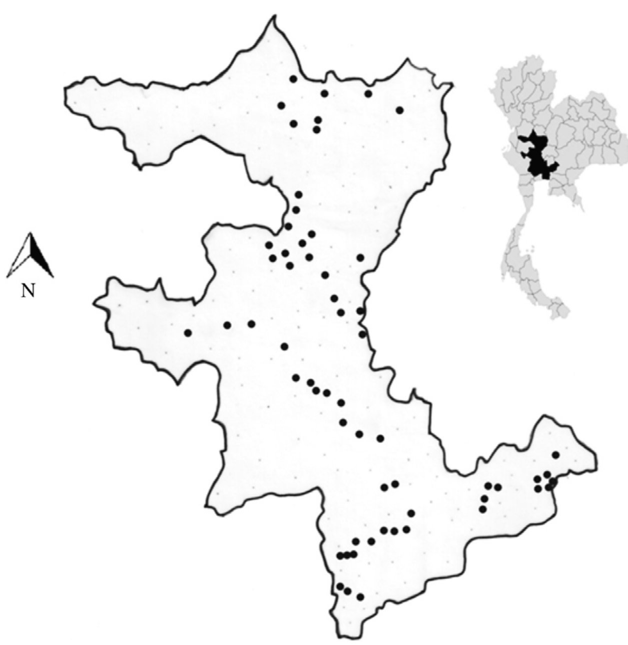

Figure 1. The location points in the Chao-Phraya Basin where snails were collected for this investigation.

illustrations were made to record information for the study of the morphological characteristics. The cercariae were identified at the family level, and in some cases, identification was possible at the genus level.

2.2. DNA extraction and ITS2 amplification

Genomic DNA of cercariae was extracted using commercial GF-1 DNA extraction kit (Vivantis, Malaysia) following the GF-1 protocol form provided by the manufacturer to achieve highest yield. After that, specimens were stored in −20 °C to maintain DNA integrity. The samples were polymerized using the PCR method. We applied the primer following Barber *et al.* [24]. The forward primer was ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′), and the reverse primer was ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) which were applied to get ITS2 as the target [24]. The PCR conditions were achieved by pre-denaturing the specimens at 94 °C for 4 min and denaturing at 94 °C for 1 min, while the annealing time was at 30 s at 50 °C with the extension at 72 °C for 45 min and the final extension was at 72 °C for 7 min. About 50 μL of each PCR reaction was comprised of 0.75 μL *Taq* polymerase, 2 μL $MgCl_2$, 20 pmol in 1.25 μL of each primer, 5 μL buffer, 1 μL dNTP and 2.5 μL of the DNA template. The amplicons were separated with gel electrophoresis using 1.4% agarose gel and separation was achieved at 90 V for 45 min. PCR amplicons were approximately 480 bps in length.

2.3. Molecular identifications and phylogenetic tree analysis

The sequence data of each DNA sample were confirmed PCR target using standard nucleotide basic local alignment search tool with megablast from the NCBI database. Consequently, the sequence data were aligned and used to construct the phylogenetic trees through the use of the Mega6® program using the unweighted pair-group method with arithmetic means with 10 000 bootstrap tests. The related sequence data acquired from the NCBI database were used for this analysis (Table 1).

Table 1

List of ITS2 sequence data used for phylogenetic tree analysis.

Species of trematodes	Families	References
Euryhelmis zelleri	Heterophyidae	KM594177.1
Cryptocotyle lingua	Heterophyidae	KJ641524.1
Opisthorchis felineus	Heterophyidae	EF688141.1
Haplorchoides sp.	Heterophyidae	KJ630832.1
H. pumilio	Heterophyidae	KP165437.1
Lecithodendrium spathulatum	Lecithodendriidae	JF784192.1
Lecithodendrium linstowi	Lecithodendriidae	KJ934792.1
P. gralli	Philophthalmidae	KF986200.1
Echinoparyphium recurvatum	Echinostomatidae	AY168931.1
Hypoderaeum conoideum	Echinostomatidae	KF894683.1
Echinostoma robustum	Echinostomatidae	GQ463132.1
Echinostoma trivolvis	Echinostomatidae	GQ463127.1
Echinostoma malayanum	Echinostomatidae	JF412727.1
Angiostrongylus cantonensis	Metastrongylidae	HQ540551.1

P. gralli: *Philophthalmus gralli*.

3. Results

3.1. Cercarial infection in snail intermediate hosts

The total number of 2 067 snail samples were classified into 7 families and 14 species, comprising 320 *Bithynia siamensis* (*B. siamensis*), 147 *Clea helena* (*C. helena*), 19 *Filopaludina filosa* (*F. filosa*), 1 *Filopaludina javanica*, 96 *Filopaludina martensi*, 520 *Filopaludina polygramma* (*F. polygramma*), 109 *Indoplanorbis exustus* (*I. exustus*), 271 *Lymnaea auricularia* (*L. auricularia*), 218 *Melanoides tuberculata* (*M. tuberculata*), 9 *Pila ampullacea*, 160 *Pomacea diffusa* (*P. diffusa*), 10 *Sinotaia* sp., 130 *Tarebia granifera* (*T. granifera*) and 57 *Thiara scabra*. The overall prevalence of cercarial infection was found to be 5.90% (122/2 067).

According to the results, the proportion of each snail family revealed that the snails in the family Thiaridae had the highest prevalence (14.32%), followed by Lymnaeidae (9.59%), Bithyniidae (8.13%), Planorbidae (5.50%), Viviparidae (0.79%) and Ampullariidae (0.59%), respectively. While snails in the family Buccinidae (*C. helena*) did not reveal any infection at the cercarial stage (Table 2). The proportions of cercarial infection by each type of cercaria are shown in Figure 2. Nine types of cercariae were reported, namely parapleurolophocercous cercaria, furcocercous cercaria, megarulous cercaria, pleurolophocercous cercaria, cercariae, xiphidiocercaria cercaria, virgulate cercaria, monostome cercaria and echinostome cercaria (Figure 3). The criterion for divided the type of cercaria involved the stylet, the tail of the cercaria, internal organ arrangement, place and number of sucker virgulate organ, *etc*. All of the cercaria types were described as follows.

3.1.1. Cercariae

The snail hosts of cercariae were *B. siamensis*, *L. auricularia* and *M. tuberculata*. The body of cercaria is fusiform. A big oral sucker is present on the sub-terminal section of the body part. A ventral sucker is near the middle of portion of the body and the size is same as the oral sucker, its pharynx is big and the esophagus is divided to bifurcate next to the pharynx. Most of the body, especially the outer zone, reveals the brown round-pigment. The tail is absent in this type (Figure 3A).

3.1.2. Echinostome cercaria

The snail hosts of echinostome cercaria were *I. exustus* and *L. auricularia*. The body of the cercaria is elongated in shape. Half of the bifurcate esophagus reveals dark granules. The oral sucker is circular in shape and located on the sub-terminal part of the body and has collar spines. The ventral sucker is located approximately two-thirds down the posterior through the body. The tail is slender and almost the same length as the body (Figure 3B).

3.1.3. Furcocercous cercaria

The snail hosts of furcocercous cercaria were *B. siamensis*, *F. polygramma* and *M. tuberculata*. The body of this cercarial type is long, flat-like and oval shaped. Its oral sucker is located on the terminal section of the body. The pharynx is quite small but it has two pairs of large penetration glands. The esophagus is long and narrow. The eyespots are globularly shaped and located in the anterior position near the pharynx. The ventral sucker is present at two-thirds of its body-length. The tail is longer than the body and divided into two furca (Figure 3C).

Table 2

The number of cercarial infections in each snail family.

Family of snails	Type of snails	Type of cercariae									Total in families
		Par	Fur	Meg	Ple	Cer	Xip	Vir	Mon	Ech	
Bithyniidae	*B. siamensis*	1	3	0	1	2	8	4	7	0	26
Buccinidae	*C. helena*	0	0	0	0	0	0	0	0	0	0
Viviparidae	*F. filosa*	0	0	0	1	0	0	0	0	0	5
	F. polygramma	0	1	3	0	0	0	0	0	0	
Planorbidae	*I. exustus*	0	0	0	0	0	0	0	1	5	6
Lymnaeidae	*L. auricularia*	4	0	0	0	19	0	0	0	3	26
Ampullariidae	*Pila ampullacea*	0	0	0	0	0	0	0	0	0	1
	P. diffusa	0	0	0	0	0	0	0	1	0	
Thiaridae	*M. tuberculata*	14	4	9	2	1	0	0	0	0	58
	T. granifera	0	0	28	0	0	0	0	0	0	

Par: Parapleurolophocercous cercaria; Fur: Furcocercous cercaria; Meg: Megarulous cercaria; Ple: Pleurolophocercous cercaria; Cer: Cercariae; Xip: Xiphidiocercaria cercaria; Vir: Virgulate cercaria; Mon: Monostome cercaria; Ech: Echinostome cercaria.

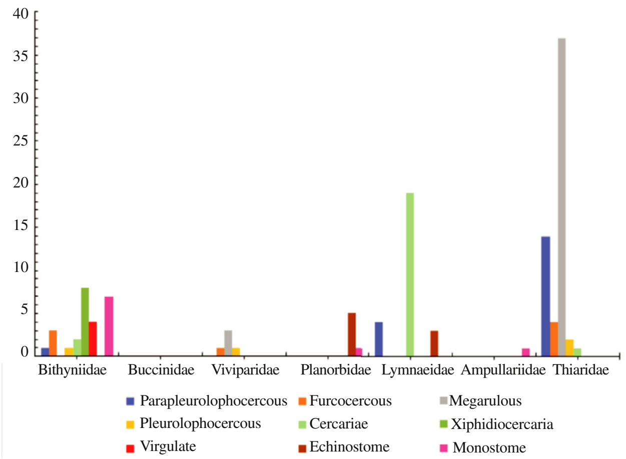

Figure 2. Proportion of cercarial infection according to each type of cercaria among freshwater snails collected from the Chao-Phraya Basin, Thailand.

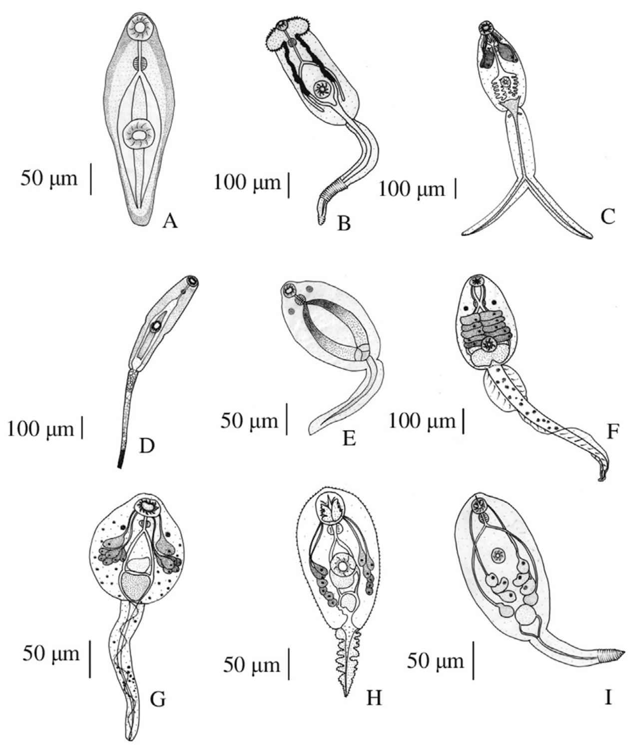

Figure 3. Morphological characteristics of each cercarial type of infection in snails.

3.1.4. Megarulous cercaria

The snail hosts of megarulous cercaria were *F. polygramma*, *M. tuberculata* and *T. granifera*. The body of this cercarial type is elongated with granules. The tail is slender and a little shorter than the body. It has a sub-terminal oral sucker. The esophagus is bifurcated and located in between the ventral sucker and the pharynx. Its ventral sucker appears approximately in the middle section of the body. The tip of the tail carries many adhesive gland cells to enable this type of cercariae to be encysted after 1 h in the Petri-dish.

3.1.5. Monostome cercaria

The snail hosts of monostome cercaria were *B. siamensis*, *I. exustus* and *P. diffusa*. The body of this cercarial type is oval-shaped. Most areas of the body are transparent, but the bifurcate esophagus reveals a dark-brown pigment. Pairs of big round eye-spots are on the side of the pharynx. The oral sucker is circular in shape and located on the sub-terminal portion of the body. The ventral sucker is absent. The tail is thick and shorter than the body (Figure 3E).

3.1.6. Parapleurolophocercous cercaria

The snail hosts of parapleurolophocercous cercaria were *L. auricularia*, *B. siamensis* and *M. tuberculata*. The body of the cercarial type is pear-shaped. It has a circular oral sucker located in the sub-terminal region and the ventral sucker is located near the posterior of the body. The small pharynx is situated behind the bifurcate esophagus. Two globular big eyespots are located on each side of the pharynx. Five pairs of penetration glands are present between the pharynx and the posterior segment of the body. Its excretory vesicle is butterfly-shaped and has brown granules. The tail of this cercaria is longer than the body. It possesses a lateral fin at one-third of its body length and a dorso-ventral fin widen at about two-thirds of the length of its tail (Figure 3F).

3.1.7. Pleurolophocercous cercaria

The snail hosts of pleurolophocercous cercaria were *B. siamensis*, *F. filosa* and *M. tuberculata*. The body of this cercarial type is oval-shaped. Its oral sucker is located on the sub-terminal section of the body. The ventral sucker is absent. The pharynx is situated next to the oral sucker and has seven pairs of penetration glands. A pair of eye-spots is located near the end of the lower third of the body. The esophagus and

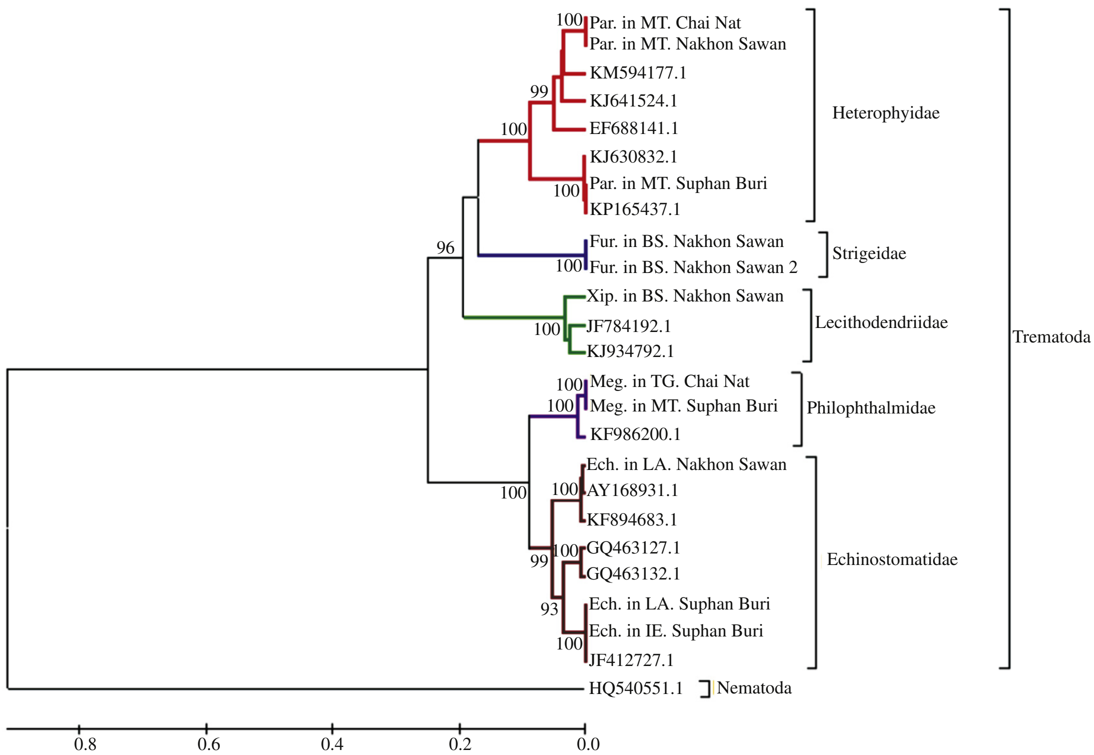

Figure 4. The phylogenetic relationship of each cercarial infection in freshwater snails.
MT: *M. tuberculata*; BS: *B. siamensis*; TG: *T. granifera*; LA: *L. auricularia* and IE: *I. exustus*.

intestines are bifurcate and extend posteriorly to the excretory vesicles. The slender tail is a little longer than the body length and has dorso-ventral finfolds (Figure 3G).

3.1.8. Virgulate cercaria

The snail host of virgulate cercaria was *B. siamensis*. The cercaria in this type is small in size. It has a stylet and a virgulate organ on its oral sucker. The pharynx is situated close to the oral sucker. The esophagus is bifurcate; the virgulate organ is complex in shape. It has six pairs of penetration glands. The ventral sucker is smaller than oral sucker. The tail of this cercaria is shorter than its overall body length (Figure 3H).

3.1.9. Xiphidiocercaria cercaria

The snail host of xiphidiocercaria cercaria was *B. siamensis*. This cercaria has a small body that is elongated, oval-shaped and colorless. The oral sucker is globularly shaped and there is a stylet on the anterior end of body. Three pairs of penetration glands appear on about two-thirds of the body. The small virgulate occurs around the oral sucker. The esophagus is bifurcate. The ventral sucker is located in the middle of the body and the genital primordial is spherically shaped. The tail is shorter than the body and is slender (Figure 3I).

3.2. Molecular identification and phylogenetic tree analysis

The full length of the ITS2 nucleotide sequence that was amplified by PCR was comprised of approximately 480 base pairs. All sequences were removed, particularly near the sequencing primer site and toward the end of the longer sequence, which is run partially to the ITS2 by trimming for the purposes of accuracy in constructing a representation of the phylogenetic relationships. All cercarial sequences appeared in a monophyletic tree by applying the sequence of *Angiostrongylus cantonensis* (HQ540551.1) as an out-group. The cercarial sequences were separated into five groups following the descriptions of the families of cercariae, and are comprised of Heterophyidae (parapleurolophocercus cercariae in *M. tuberculata* in Chai Nat, Nakhon Sawan and Suphan Buri), Strigeidae (furcocercous cercariae in *B. siamensis*), Lecithodendriidae (xiphidiocercariae in *B. siamensis*), Philophthalmidae (megarulous cercariae in *T. granifera* and *M. tuberculata*) and Echinostomatidae (echinostome cercariae in *L. auricularia* and *I. exustus*). The data sequences revealed that the Philophthalmidae groups were grouped closely with the Echinostomatidae groups and these groups were separated from the other groups (Heterophyidae, Strigeidae and Lecithodendriidae) (Figure 4).

4. Discussion

The present study is the first known report on cercarial infection in the Chao-Phraya Basin, Thailand. The study revealed a high variety of freshwater snails that were infected by cercariae that was present in 6 families, namely Bithyniidae, Viviparidae, Planorbidae, Lymnaeidae, Ampullariidae and Thiaridae. Total prevalence of cercarial infections found in the Chao-Phraya Basin (5.90%) was similar to that of the previous report by Mard-arhin *et al.* [31] for Northern Thailand, which illustrated the prevalence of cercariae infection at 6.20%. It is likely that the snails and parasites may be discharged from the north to the central area of Thailand via the Ping and Nan Rivers and via the confluence with the Chao-Phraya River at Nakhon Sawan (also called Pak Nam Pho). This conclusion is also supported by the ecological conditions of the water resources reported in the Chao-Phraya Basin, which were found to be complex and to present a highly diverse fresh water ecosystem in the north of Thailand. Moreover, this ecosystem has been found to be suitable for cercariae reproduction and their development in intermediate hosts. From this result, the morphology of the cercarial was similar to those that were described in the previous report [15].

In this investigation, the freshwater snails found in the Chao-Phraya Basin served as the intermediate host of various species of zoonotic trematodes with a high prevalence value (5.90%). This was particularly true for snails in the family Thiaridae including *M. tuberculata* and *T. granifera* which were found to be highest in terms of being infected by parapleurolophocercous cercaria. According to a number of recent reports, this snail family has displayed high susceptibility for heterophyid cercariae infection [15,32]. Various reports have indicated the presence of parapleurolophocercous cercaria at the cercarial stage of the intestinal trematode in the family Heterophyidae, such as *H. taichui*, *H. pumilio*, *Centrocestus caninus* [syn. *Centrocestus formosanus* (*C. formosanus*)], *Stellantchasmus falcatus* and *Metagonimus yokogawai*. A recent study has reported on the *Stellantchasmus falcatus* infection in Korean patients [33]. In Lao PDR, many patients have been infected by *H. taichui* with 99.8% of the trematode specimens coming from 10 patients [34]. In addition, Chai *et al.* [35] have reported that 7 patients were infected by *C. formosanus* in Lao PDR. All patients that were infected with *C. formosanus* experienced abdominal pain, indigestion and diarrhea [35]. Moreover, Chung *et al.* [36] reported the first case in Korean in which patients were infected with *H. pumilio*. Additionally, in the northeast of Thailand, the human liver fluke *O. viverrini*, was found to be the major cause of cholangiocarcinoma [6]. These reports have revealed the widespread presence of Heterophyidae infection among humans and this situation is considered an important and continuing public health problem in many countries.

Regarding the highest prevalence of the cercarial types, in this investigation, megarulous cercaria revealed the highest prevalence and has been morphologically characterized as belonging to the genus *Philophthalmus*. This parasite is commonly known as the oriental avian eye fluke and it has been known to infect humans [37]. The cercariae in this study were identified by 98% to *P. gralli* in accordance with Mukaratirwa *et al.* [38] who reported on *P. gralli* that was found in *M. tuberculata* and *T. granifera* in Zimbabwe. In general, this cercarial type can be found in *M. tuberculata* [39,40], but some studies have reported that there were also other snail species present, namely *Fagotia* spp., *Amphimelania holandri* and *Melanopsis praemorsa* [41], *Semisulcospira libertina* [42] and *Zeacumantus subcarinatus* [43]. This species has been known to contaminate chickens or ducks in poultry farms resulting in diseased livestock. This can be a significant financial problem for the rancher. In addition, if this parasite is not controlled, the poultry industry in Thailand would be negatively affected, as well.

With regard to other types of cercariae, the echinostome cercaria was observed to occur in *I. exustus* and *L. auricularia* in this study. This type of cercaria developed in many important trematodes that had been infected in the gastrointestinal tract, particularly *Hypodereaum conoideum*, and *Echinostoma malayanum*. However, *Echinostoma revolutum* is known to be the medically important zoonotic intestinal parasite in humans and commonly occurs in Southeast Asia [44]. Moreover, it has been known to heavily infect domestic ducks, which play a major role in egg and meat production in Eastern Asia [45].

Many types of cercaria examined in this study were analyzed in order to reconstruct the phylogenetic tree. The relationship was shown in the monophyletic tree and the cercaria specimens were divided into two groups that were separated by their definitive hosts. The first group is separated into three families of trematodes comprised of Heterophyidae, Strigeidae and Lecithodendriidae. This group has been reported as the trematodes that typically infect mammals. Schistosomiasis remains a public health problem with a high level of prevalence in Egypt, China, Indonesia and Philippines [46,47]. The infection of *Phaneropsolus bonnei* and *Prosthodendrium molenkampi* (Lecithodendriidae) in human intestinal tracts has been reported in Lao PDR [48] and many instances of heterophyid infection have also been recorded. The second group in this tree is the group that is classified as the common infection in Aves. This group is comprised of Philophthalmidae and Echinostomatidae.

Conflict of interest statement

We declare that we have no conflict of interest.

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