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Molecular confirmation of trematodes in the snail intermediate hosts from Ratchaburi Province, Thailand

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ABSTRACT

Objective: To analyze the prevalence of trematode infections in the snails intermediate host *Tarebia granifera* and *Melanoides tuberculata* collected from Ratchaburi Province by a molecular approach in order to construct a dendrogram to identify species at the cercarial stage.

Methods: Specimens were collected from nine districts located in Ratchaburi Province using the stratified sampling method. The samples were examined for cercarial infection using the crushing method. All specimens were amplified by region of internal transcribed spacer 2 (ITS2) based on the PCR technique, and the sequence data were aligned in order to construct the dendrogram by maximum likelihood with 10000 bootstrap replicates.

Results: Five cercarial types were observed comprising parapleurolophocercous cercaria, xiphidiocercaria, megarulous cercaria, furcocercous cercaria and transversotrema cercaria. The overall prevalence of cercarial infection was found to be 7.92% (63 / 795). The parapleurolophocercous cercaria was found in the highest level of prevalence followed by xiphidiocercaria, megarulous cercaria, furcocercous cercaria and transversotrema cercaria, respectively. The parapleurolophocercous cercaria samples were identified and separated into *Haplorchis taichui* and *Haplorchis pumilio*, while xiphidiocercaria were identified as Lecithodendriidae and megarulous cercaria were identified as *Philophthalmus*. The dendrogram separated the cercariae into five groups, which were comprised of parapleurolophocercous cercaria, xiphidiocercaria, megarulous cercaria, furcocercous cercaria and transversotrema cercaria using the sequence of *Angiostrongylus cantonensis* as an out-group.

Conclusions: This is the first study to report on the prevalence of cercarial infection in Ratchaburi Province, Thailand. The high prevalence of cercariae was revealed by the infection in *Tarebia granifera* and *Melanoides tuberculata*. Thus, the sequence data of ITS2 can be used to investigate the dendrogram of trematodes and can help to separate the collected samples at the species level. This information can be used to create the prevention program for parasite infection from intermediate hosts in the future.

1. Introduction

Digenetic trematodes, particularly intestinal and liver flukes, are widely distributed in many Asian countries including Korea, Vietnam, Myanmar, Cambodia, Laos and Thailand, and remain a public health problem throughout the region[1-7]. The heterophyid trematode and some liver flukes can infect mammals and humans who eat raw or uncooked infected-fish species such as *Haplorchis taichui* (*H. taichui*) and *Haplorchis pumilio* (*H. pumilio*)[2,8,9]. They have been implicated as a cause of heterophyiasis which can result in significantly high incidences of eosinophilic, abdominal pain, diarrhea[10], and in some cases if the patients have been infected with a high number of trematodes, the trematodes would invade the mucosa and pass their eggs on through the blood vessels and spread the infection to the viscera including the heart and brain, and can lead to heart failure[11]. Moreover, the *H. taichui* infection can lead to mucosal ulceration, mucosal and submucosal haemorrhages, fusion and shortening villi, chronic inflammation, fibrosis of the submucosa[12], and possibly etiologic agent irritable bowel syndrome (IBS)-like symptoms, as well[13]. The life cycle of these trematodes is very complex, as they require two intermediate hosts to reach maturation and to complete their life cycle. Freshwater snails and fish typically serve as the intermediate host in most instances[1,6,14,15]. These trematodes are endemic throughout the Southeast Asian region, including Thailand, Lao PDR, Vietnam,

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Korea and the Philippines[1,2,5,7,10,15], with a high incidence of mixed infections involving small liver trematodes and other minute intestinal trematodes[16,17]. The distribution of trematodes requires specific suitable ecological conditions, typically involving certain water resources such as those located in agricultural areas as well as rivers and irrigation canals. Consequently, the western province of Ratchaburi in Thailand is a geographical area that possesses the suitable ecological conditions for trematode growth and development. This province has the major river known as "Mae Klong" which flows through the center of town. This river is the primary source for several types of freshwater ecosystems, including irrigation canals, paddy fields and waterfalls. These water bodies provide suitable conditions for cercaria and metacercaria development[1,18].

Molecular biological methods have been acknowledged as the most efficient and accurate tools that can be used for the identification of several parasitic organisms including trematodes[1,14,19-21]. The internal transcribed spacer 2 (ITS2) of the 18S rDNA gene was selected and used in this study. This region can be used for the purposes of identification of various stages of minus intestinal trematode (cercaria, metacercaria and adult stages) infections in their intermediate and definitive hosts[1,22,23]. Moreover, the sequences of the ITS2 region have been used as a potential molecular marker at the species or population level[24]. Conventional PCR methods have been widely applied for the purposes of identification because the DNA method is very sensitive, highly accurate and can be rapidly applied. Therefore, the sequence data have proven to be helpful in the study of species identification and geographical distribution for numerous species such as *Paragonimus westermani*, *Fasciolopsis buski* and *Fasciola gigantica*[20], *Explanatum explanatum*, *Paramphistomum epiclitum* and *Calicophoron calicophorum*[25].

Hence, the purpose of this study was to investigate the prevalence of cercarial infection in certain freshwater snails like *Tarebia granifera* (*T. granifera*) and *Melanoides tuberculata* (*M. tuberculata*), and to amplify and sequencing the ITS2 regions. The sequencing data were used to reconstruct the dendrogram for separating cercarial species that infect the freshwater snails *T. granifera* and *M. tuberculata* both found in Ratchaburi Province, Thailand, and to identify a putative preventive technique which warranted future investigations.

2. Materials and methods

2.1. Study area and snail specimens

Snail specimens were collected by stratified sampling method during the period of August 2015 to January 2016 from various sites of nine districts located in Ratchaburi Province, which were Mueang Ratchaburi (MR), Chom Bueng (CB), Suan Phueng (SP), Damnoen Saduak (DS), Ban Pong (BP), Bang Phae (BPh), Photharam (PR), Pak Tho (PT) and Ban Kha (BK) (Figure 1). The coordination of each collection site in this study was recorded using the global positioning system (GPS). Snail specimens were collected and classified using a taxonomic key[26] and separated by species level when possible.

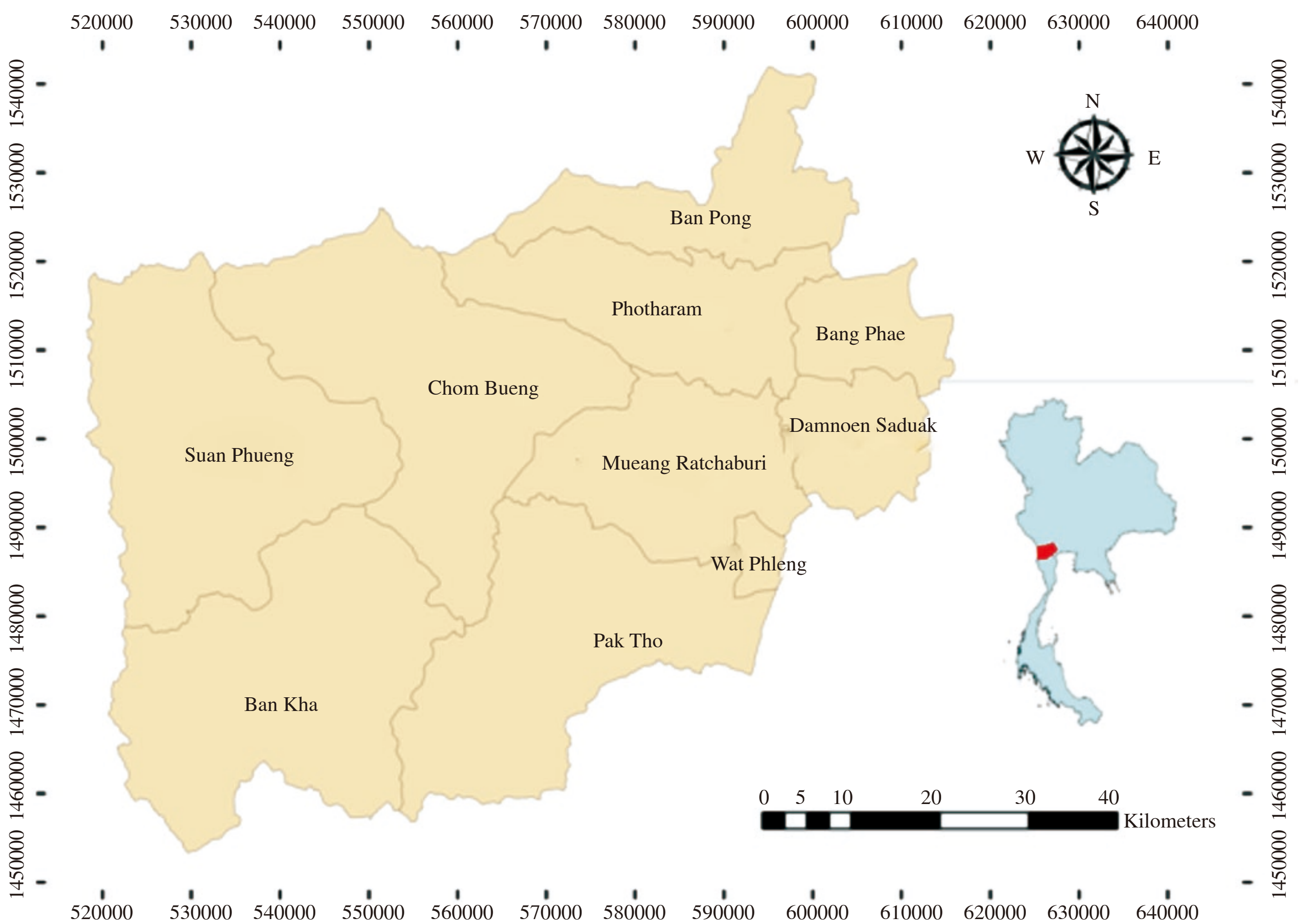

Figure 1. Districts in Ratchaburi Province - the nine districts in the study area included Mueang Ratchaburi, Chom Bueng, Suan Phueng, Damnoen Saduak, Ban Pong, Bang Phae, Photharam, Pak Tho and Ban Kha.

2.2. Cercarial infections

Freshwater snails were crushed by crushing method[27] and identified by cercarial type under a high magnification stereo-microscope. The prevalence value was then calculated. The living cercariae were vitally stained with 0.5% neutral red dye and identified according to morphological classification as previously described[1,18]. Furthermore, the cercariae were stained with Delafield's haematoxylin and dehydrated in an ethyl alcohol series, cleared with xylene and mounted in permount. Afterwards, the permanent slides were illustrated using a camera lucida to record pertinent information of their morphological characteristics.

2.3. DNA extraction and ITS2 amplification

Genomic DNA of cercariae from *T. granifera* and *M. tuberculata* were extracted using the comercial DNA extraction kit NucleoSpin® Tissue (MACHEREY-NAGEL, Germany) following manufacturer instructions. In order to maintain the integrity of the DNA, it was stored in –20 °C. Specimens were amplified for detection of the region of ITS2 using the PCR method following the primer of Barber *et al*.[28]. The forward primer was recorded as ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') and the reverse primer was recorded as ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR conditions were achieved by pre-denaturing the specimens at 94 °C for 4 min. After that, ITS2 regions were polymerized by denaturing at 94 °C for 1 min, while the annealing time was at 30 s at 50 °C with an extension time of 45 s at 72 °C. The PCR was replicated 35 times and the final extension was made at 72 °C for 7 min. 50 µL of each PCR reaction contained 0.75 µL *Taq* polymerase, 2 µL $MgCl_2$ and 20 pmol in 1.25 µL of each primer, 5 µL buffer, 1 µL dNTP and 2.5 µL of the DNA template. PCR amplicons were separated with agarose gel electrophoresis using 1.5% agarose gels, and separation was achieved at 90 V for 45 min. Amplicons were approximately 450 bps in length.

2.4. Molecular identification and dendrogram construction

Each amplicon was sequenced by 1st Base® Laboratories, SdnBhd (Malaysia) and the PCR target was confirmed by using the standard nucleotide Basic Local Alignment Search Tool (BLAST) with megablast from the National Center for Biotechnology Information search (NCBI) database. Consequently, all data were aligned and the dendrogram was constructed to present the related cluster of cercariae by Mega7® program using the maximum-likelihood method and the general time reversible model with maximum parsimony as the initial tree, and the process involved 10 000 bootstrap tests. The relevant sequence data was acquired from the NCBI database and was used for this analysis (Table 1).

Table 1
List of ITS2 sequence data used for constructing the dendrogram.

Species of parasites	Families	References
H. taichui	Heterophyidae	KJ630831.1
H. pumilio	Heterophyidae	KP165437.1
Tetracotyle xenentodoni	Diplostomatidae	KU316948.1
P. gralli	Philophthalmidae	JX121231.1
Lecithodendrium linstowi	Lecithodendriidae	KJ934792.1
Transversotrema polynesiae	Transversotrematidae	KF765501.1
A. cantonensis	Metastrongylidae	FJ965594.1

A. cantonensis: *Angiostrongylus cantonensis*; *P. gralli*: *Philophthalmus gralli.*

3. Results

3.1. Cercarial infection in freshwater snails

A total of 795 snail samples were collected, comprising 591 *T. granifera* and 204 *M. tuberculata*. The overall prevalence of cercarial infection was 7.92% (63/795). According to these results, the proportion of each snail species revealed that *M. tuberculata* had a higher level of prevalence (21.08%) than *T. granifera* (3.38%), and the highest prevalence was found in Suan Phueng (7.04% in *T. granifera* and 22.92% in *M. tuberculata*) (Figures 2 and 3).

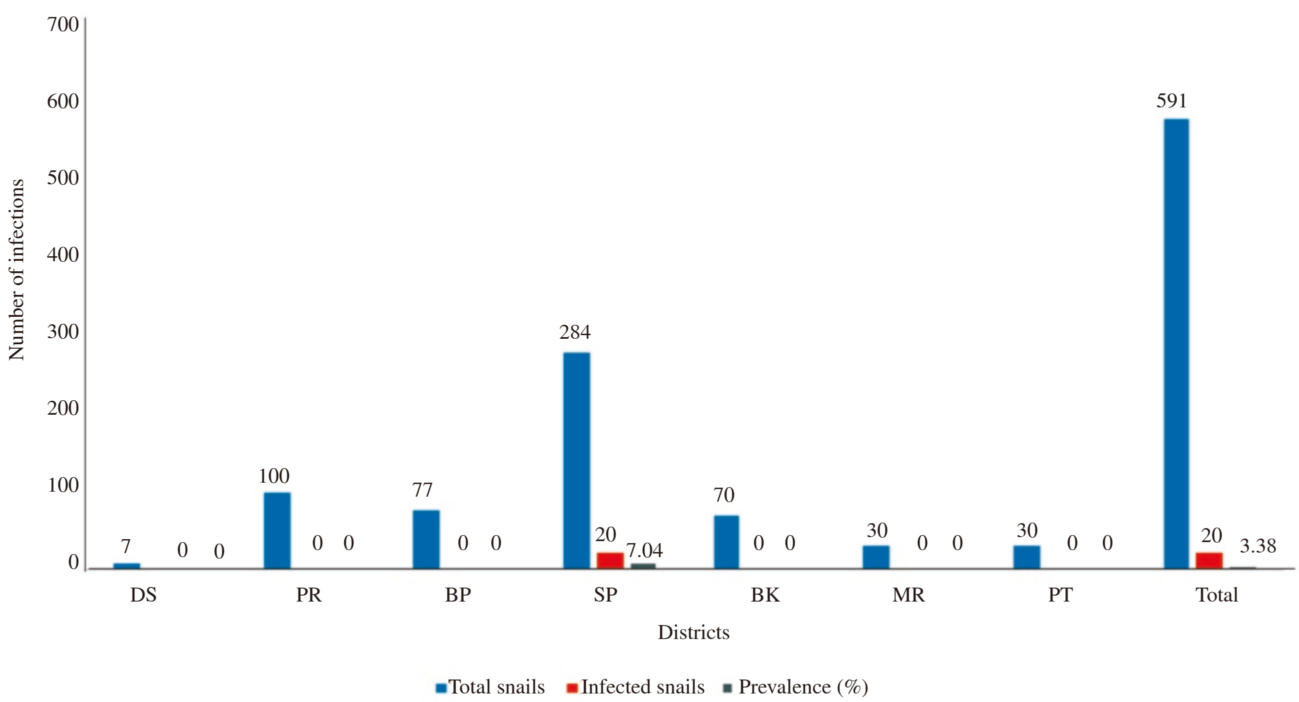

Figure 2. Prevalence of cercarial stage infection in *T. granifera*.

To assess the prevalence of each cercarial type, different types of cercaria were studied. They included parapleurolophocercous cercaria, megarulous cercaria, and xiphidiocercaria in *T. granifera*, which showed the high prevalence values of 65% (13/20), 20% (4/20) and 15% (3/20), respectively. *M. tuberculata* was infected by parapleurolophocercous cercaria, megarulous cercaria, xiphidiocercaria, furcocercous cercaria and transversotrema cercaria with prevalence values of 39.53% (17/43), 2.33% (1/43), 51.16% (22/43), 4.65% (2/43) and 2.33% (1/43), respectively.

3.2. Morphological characteristics of each cercarial type

3.2.1. Parapleurolophocercous cercaria

Hosts for this cercaria are *M. tuberculata* and *T. granifera*. This cercaria has a body pear-shaped, concaved at one-third. It has two suckers, one oral sucker located in the sub-terminal region at the anterior, and one ventral sucker located four-fifths down the body. It has seven pairs of penetration glands and one pair of eyespots. This cercaria has a unique tail with two finfolds, including a dorsal finfold located about one-third down the length of the tail, and a lateral finfold located at about two-thirds of the tail's length (Figure 4A).

3.2.2. Megarulous cercaria

Host for this cercaria are *M. tuberculata* and *T. granifera*. The body is elongated with granules. An oral sucker is located at the anterior terminal next to the pharynx. A ventral sucker is located in the middle of the body. The bifurcate esophagus is located between the ventral sucker and the pharynx. A unique characteristic of this cercarial type is the presence of many adhesive gland cells located at the tip of its tail. This gland enables to be encysted at the infective stage (metacercaria) in a Petri dish (Figure 4B).

3.2.3. Xiphidiocercaria

Host for this cercaria are *M. tuberculata* and *T. granifera*. This cercaria type is relatively small. The body is oval-shaped and colorless. An oval-shaped oral sucker is located two-thirds down the length of the anterior with a unique "stylet" on its body. A ventral sucker is located in the middle of the body. Two pairs of penetration glands appear at about the middle to two-thirds of the body's length. The tail of this cercarial type is shorter than the body and has tiny colorless finfolds on it (Figure 4C).

3.2.4. Furcocercous cercaria

This type of cercaria is only found in *M. tuberculata*. This cercaria is elongated, slender and tiny. Its body is shorter than its tail. The oral sucker is located at the end of the anterior part. It has a small pharynx and prepharynx. A ventral sucker is located three-fourths down the length of the body. This cercarial type has a unique tail that is divided into two furca. This cercarial type is representative of the infection stage of the blood fluke (Figure 4D).

3.2.5. Transversotrema cercaria

This cercarial type is only found in *M. tuberculata*. The cercarial body is short, flat and semi-circular in shape. The body has a yellowish-brown pigment. The pharynx is large while the oral sucker is absent. The ventral sucker of this cercaria is located in the middle of its body. The tail is longer than the body and appears to be thicker than those of other types. Furthermore, its tail has arm-like processes at the anterior end of the tail and the tip of the tail is separated into two furca-like furcocercous cercaria (Figure 4E).

3.3. Molecular identifications and dendrogram construction

The full length of the ITS2 region was amplified by PCR with a length of approximately 450 bp. The sequences were shortened by trimming for accuracy in constructing the dendrogram. All specimen sequences were recorded in the monophyletic group by applying the sequence of *A. cantonensis* (FJ965594.1) as an out group. The cercarial sequences were separated into five groups following the descriptions of different types of cercariae

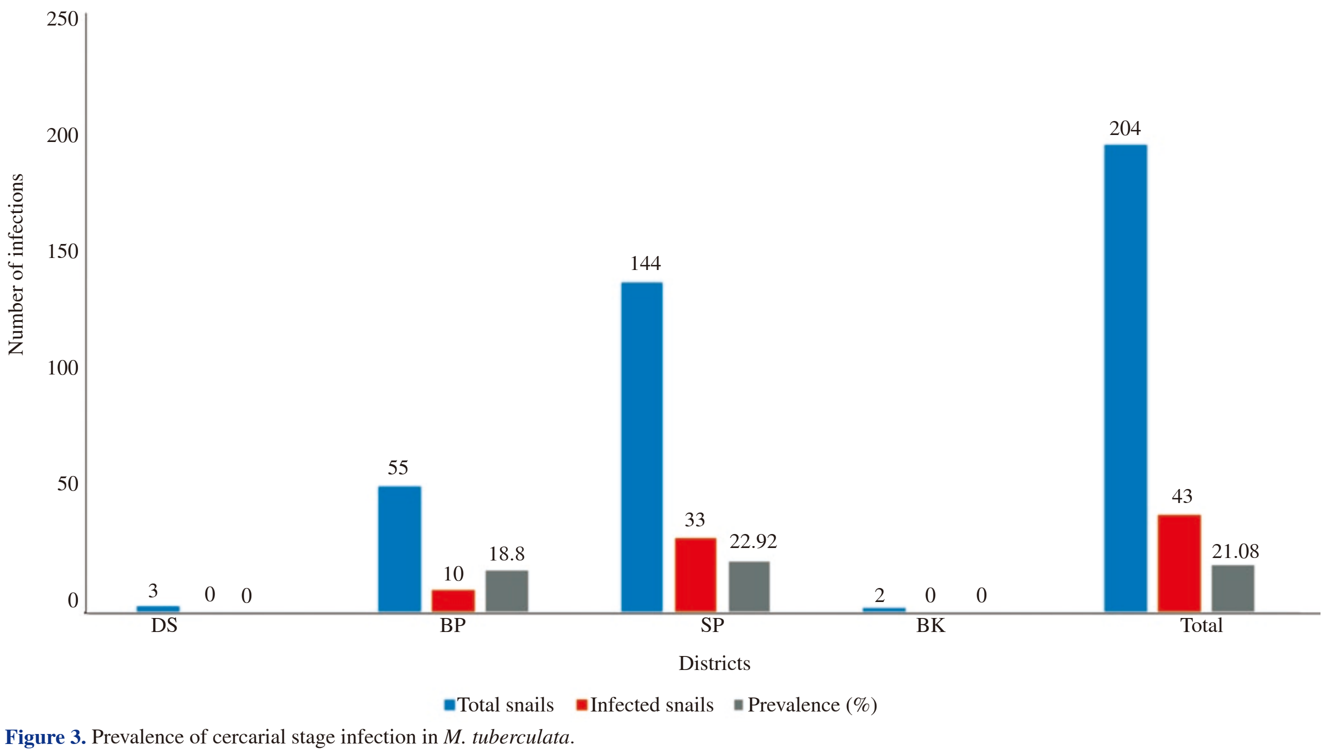

Figure 3. Prevalence of cercarial stage infection in *M. tuberculata*.

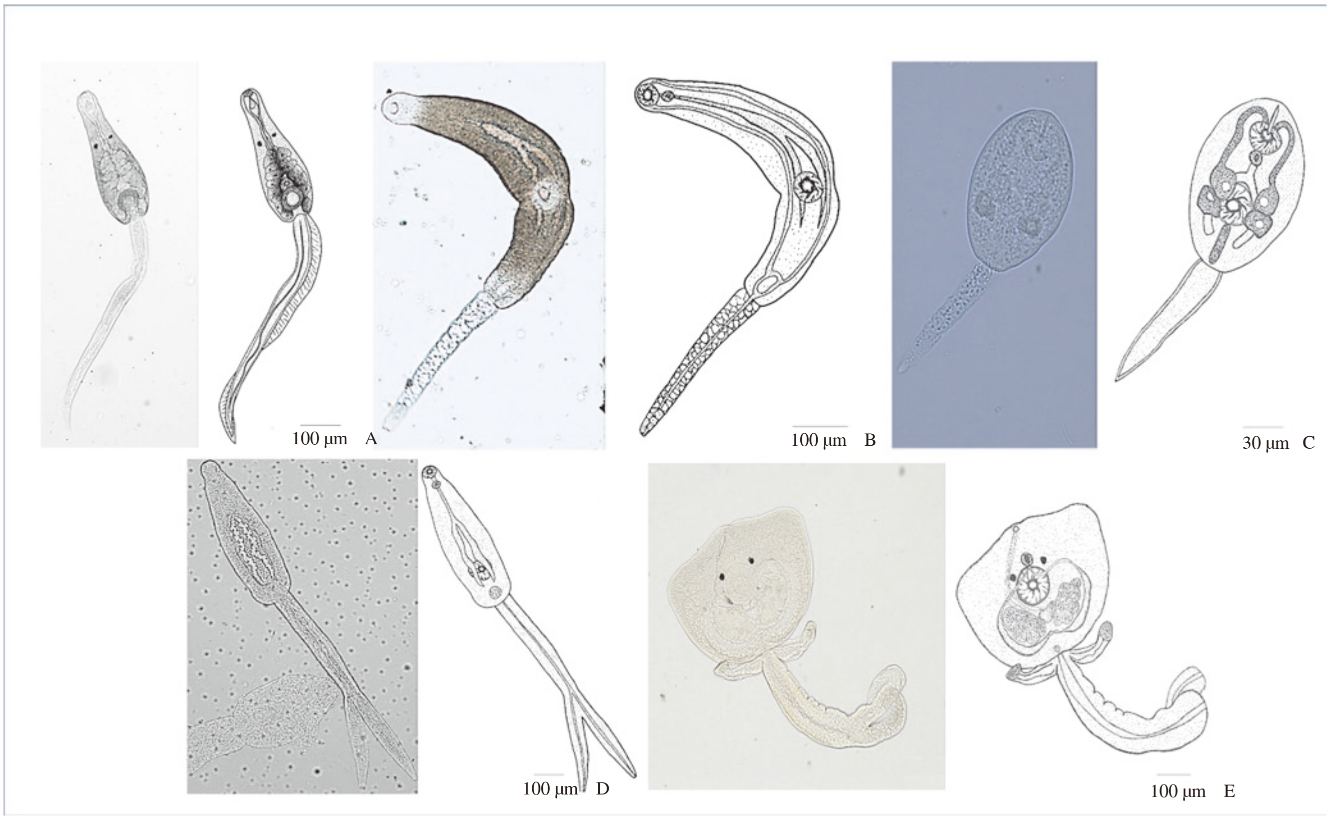

Figure 4. The illustrations of each cercarial type were infected in thiarid snails in this study.
A: Parapleurolophocercous cercaria; B: Megarulous cercaria; C: Xiphidiocercaria; D: Furcocercous cercaria; E: Transversotrema cercaria.

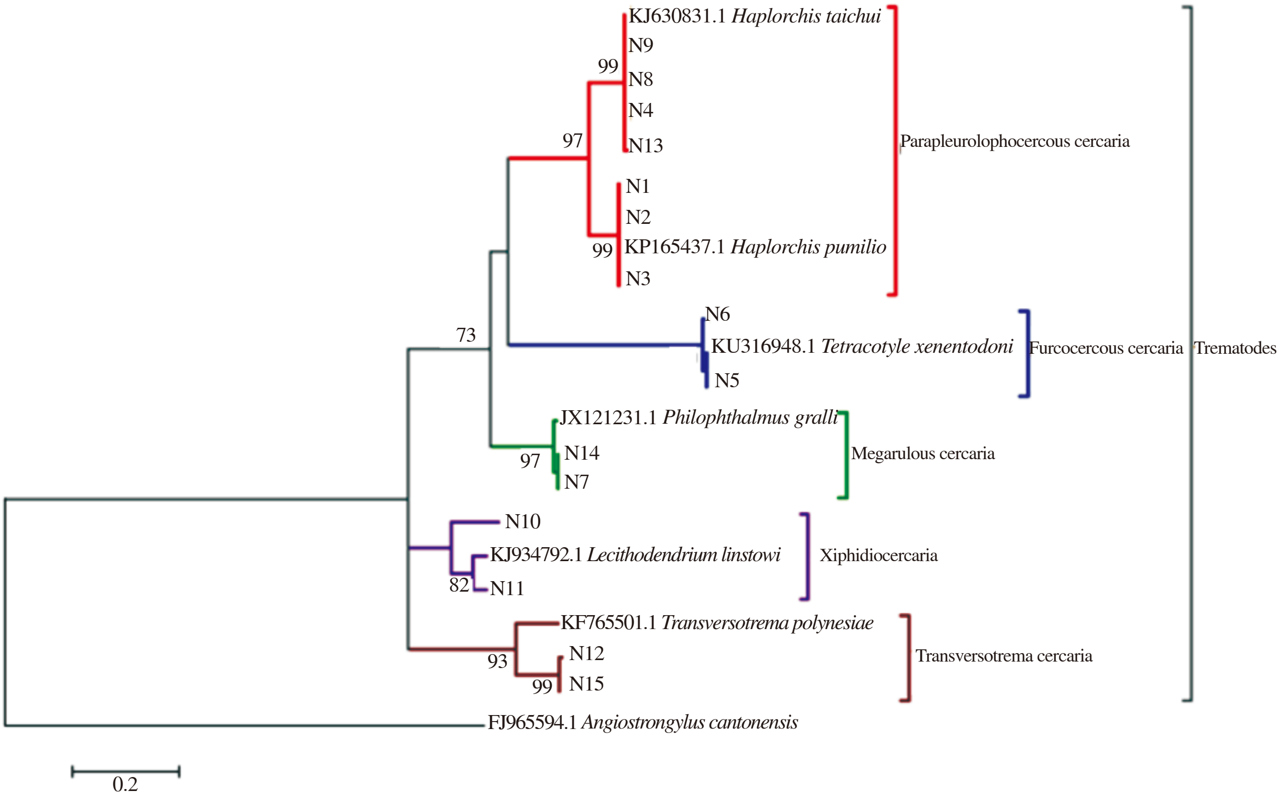

Figure 5. Dendrogram of each cercarial type infecting freshwater snails.

including parapleurolophocercous cercaria, megarulous cercaria, xiphidiocercaria, furcocercous cercaria and transversotrema cercaria (Figure 5). The data sequences revealed that the parapleurolophocercous cercaria in this study can be separated into two species of the family Heterophyidae including *H. taichui* and *H. pumilio*. The group of megarulous cercaria has developed into Philophthalmidae, the group of xiphidiocercaria has developed into Lecithodendriidae, the furcocercous cercaria group has developed into strigeids and the last group, transversotrema cercaria has developed into Transversotrematidae.

4. Discussion

This study is the first reporting the cercarial infection in *T. granifera* and *M. tuberculata* from Ratchaburi Province, Thailand. *T. granifera*and *M. tuberculata* have been found to be infected with a high variety of cercarial types from five trematode families: Heterophyidae, Philophthalmidae, Lecithodendriidae, strigeids and Transversotrematidae. The overall prevalence found in this study (7.92%) was higher than the observed in previous studies conducted in the Chao-Phraya Basin, Thailand, which detected a prevalence of 5.90%[1]. Furthermore, the overall prevalence found in this study is higher than that recorded in a previously published report on Northern Thailand, with a prevalence of cercariae infection of 6.20%[29]. It should be mentioned that trematodes and their intermediate hosts may be originated from the northern and central areas of Thailand to the down steam area of Ratchaburi via the Mae Klong River, which merges with an early portion of the Khwae-Yai River in Tak Province. Our finding are also supported by the ecological conditions of the water resources that are present in Ratchaburi Province. This area contains a diversity of freshwater ecosystems including rivers, waterfalls, paddy fields, irrigation canals and streams. The suitable conditions of these ecosystems allow cercariae to infect snails and lead to the development to the adult stage in definitive hosts. According to this study, the morphology of the cercaria was found to be similar to that which was described in previous reports[1,15,18]. In recent studies, *T. granifera* and *M. tuberculata* have been noted as having a high level of susceptibility for heterophyid cercariae infection[18,30].

Two species of heterophyid trematodes have been identified in this study, namely, *H. taichui* and *H. pumilio*, which are known to infect humans and other mammals, and are widely distributed in many provinces of Thailand such as Chiang Mai, Phitsanulok, Mae Hong Son, Kamphaeng Phet, and Tak[10,23,30], as well as in other close countries, like Lao PDR, Korea and Vietnam[2,5,7,16]. A recent study conducted in Lao PDR revealed that many patients were infected with *H. taichui*, with a 99.8% of the trematode specimens identified in patients[2]. Moreover, a report conducted in Savannakhet of the Lao PDR revealed the presence of *H. taichui* and *H. pumilio* fecal specimens collected from various Mekong River-side villages[31]. In addition, a high prevalence of infection of *H. taichui* and *H. pumilio* was recorded in Xieng Khouang Province in Lao PDR[32]. Moreover, *H. taichui* was reported as a possible etiologic agent of irritable bowel syndrome (IBS)-like symptoms and can lead to abdominal illnesses that have been associated with excessive gas or flatulence, tiredness, and loose feces[13]. Furthermore, some reports have shown *H. taichui* infection as a pathogenic parasite in three human cases through microscopic examinations and mucosal ulceration, mucosal and submucosal haemorrhages, fusion and shortening villi, chronic inflammation, and fibrosis of the submucosa were identified in these patients[12].

With regard to another type of cercariae, the megarulous cercaria has been genetically characterized as belonging to the genus *Philophthalmus*. This parasite is commonly referred to as the oriental avian eye trematode and in some cases, the accidental infection in humans was identified[1]. Here we detect in 96% of megarulous cercariae the trematode species *P. gralli*. This result is in accordance with Mukaratirwa *et al*., who reported *P. gralli* in *M. tuberculata* and *T. granifera* in Zimbabwe[33]. This parasite species may lead to diseases in the conjunctiva sac of the eyes of chickens or ducks in poultry farms, and may result in significant economic and veterinary problems for the ranchers if not controlled. If this were the case in Thailand, the poultry industry would be negatively affected. With regard to *Transversotrema* cercaria, this is typically observed as ectoparasite in fish. This parasite infects the skin, mucus, muscle, fin and tail of the fish and adversely influences the growth rate and development of fish. It may also negatively affect the aquarium fish farms that are commonly found in Ratchaburi Province. Regarding the xiphidiocercaria and furcocercous cercaria, these are parasites that are known to be prevalent in livestock and other animals of economic importance in Thailand such as various fish and anuran species. These are known to be pathogenic parasites associated with various veterinary diseases. For instance, previous reports of strigeid trematodes in the genus *Tetracotyle* sp. have reported on the high prevalence and intensity of this species in sardines[34] and chicken[35].

We constructed a dendrogram of cercariae with the use of the ITS2 sequences of the identified cercariae found in the intermediate hosts *T. granifera* and *M. tuberculata*. The tree was used to identify and separate the five different types of cercariae, which also separated the genus *Haplorchis* into two different species.

Our preliminary data indicate that cercarial infection of *T. granifera* and *M. tuberculata* can be separated and accurately identified by using a molecular method with the internal transcribed spacer 2 region sequences. Moreover, *T. granifera* and *M. tuberculata* could be important factors in monitoring public health. Notably, the intestinal trematodes of the genus *Haplorchis* is known to play an important role in bowel diseases such as irritable bowel syndrome, diarrhea and submucosal haemorrhages. Furthermore, the avian eye trematode *Philophthalmus gralli* infection may cause catastrophic veterinary health problems in poultry farms. Further studies should be conducted to create an effective prevention program for parasitic infection transmitted by freshwater snails in Ratchaburi Province, as well as in other areas in the future.

Conflict of interest statement

We declare that we have no conflict of interest.

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