Prevalence of *Haplorchis taichui* Infection in Snails from MaeTaeng Basin, Chiang Mai Province, by Using Morphological and Molecular Techniques

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Abstract

The investigation of biological diversity of intestinal trematode in snails which the report concerned is rarely and not extended in current status. Especially in minute intestine trematode, *Haplorchis taichui* Witenberg, 1930, family of Heterophyidae, which was found in the small intestine of mammals including human, and causes serious clinical problem worldwide. So, this study was aimed to investigate *H. taichui* infection in freshwater snails from Mae Taeng basin, Chiang Mai, Thailand. Total of 1,836 snails were collected during April 2008 to August 2012. Cercarial infection was examined by crushing method. Molecular identication of *H. taichui* were conducted by a DNA specic primer which amplied the mCOI gene. The PCR product of mCOI were sequenced and conrmed by BLAST program. Six types of cercariae were found viz. megalurous, furcocercous, monostome, pleurolophocercous, parapleurolophocercous, and virgulate cercaria. The parapleurolophocercous cercaria in Melanoides tuberculata, Tarebia granifera, and Thiara scabra were larval stages of *H. taichui* which yield the specic fragment of 256 bp. Overall of *H. taichui* infection of snails was 42.71%. The mCOI sequences had 99% similarity with the 29 isolated gene references of the *H. taichui* in Genbank data base. The molecular method had suitability as an epidemiological tool for suitable control programs against the dissemination of trematodes.

Keywords : Intestinal trematodes Prevalence Molecular identication mCOI gene

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ค่าความชุกของการติดพยาธิใบไม้ชนิด *Haplorchis taichui* ในหอยเดียวจากลุ่มแม่น้ำแม่แตง จังหวัดเชียงใหม่ โดยเทคนิคทางสัญฐานวิทยาและอณูชีววิทยา

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บทคัดย่อ

การศึกษาความหลากหลายทางชีวภาพของพยาธิใบไม้ในลำไส้ (intestinal trematode) มีรายงานน้อยมากและยังไม่ครอบคลุม โดยเฉพาะพยาธิใบไม้ในลำไส้ขนาดเล็ก *Haplorchis taichui* Witenberg, 1930 ในวงศ์ Heterophyidae เป็นปรสิตในสัตว์เลี้ยงลูกด้วยนมรวมถึงมนุษย์ การติดเชื้อพยาธิใบไม้ก่อให้เกิดปัญหาที่สำคัญทางสาธารณสุข และมีรายงานการระบาดในประเทศไทยอย่างต่อเนื่อง ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการติดเชื้อพยาธิใบไม้ *H. taichui* ในหอยฝาเดียวจากลุ่มแม่น้ำแม่แตงจังหวัดเชียงใหม่ โดยทำการเก็บหอยฝาเดียวทั้งหมด 1,836 ตัว ระหว่างเดือนเมษายน 2008 ถึง เดือนสิงหาคม 2012 เพื่อตรวจสอบการติดตัวอ่อนพยาธิใบไม้โดยวิธี crushing สำหรับการระบุอัตลักษณ์ของพยาธิใบไม้ชนิด *H. taichui* จะถูกศึกษาโดยเทคนิคทางอณูชีววิทยาด้วยไพร์เมอร์แบบจำเพาะและถูกเพิ่มปริมาณดีเอ็นเอของยีน mCOI (mitochondrial cytochrome c oxidase subunit I) เพื่อหาลำดับเบสและตรวจสอบด้วยโปรแกรม BLAST จากผลการศึกษาพบเซอร์คาเรียทั้งหมด 6 รูปแบบ คือ megalurous cercaria furcocercous cercaria monostome cercaria pleurolophocercous cercaria parapleurolophocercous cercaria และ virgulate cercaria การตรวจสอบการติดพยาธิ *H. taichui* ระยะเซอร์คาเรียในหอยโดยเทคนิคทางอณูชีววิทยาพบว่าเซอร์คาเรียรูปแบบ parapleurolophocercous cercaria ให้ผลการทดสอบเป็น positive result โดยสามารถเกิดแถบดีเอ็นเอขนาด 256 bp ที่มีความจำเพาะต่อพยาธิ *H. taichui* มีค่าความชุกรวมร้อยละ 42.71 ส่วนลำดับเบสของยีน mCOI มีความคล้ายคลึงเท่ากับร้อยละ 99 จาก 29 ไอโซเลทของ *H. taichui* ในฐานข้อมูลของ Genbank การศึกษาในครั้งนี้ชี้ให้เห็นว่าเทคนิคทางอณูชีววิทยาสามารถนำมาประยุกต์เป็นเครื่องมือในการศึกษาด้านระบาดวิทยาเพื่อใช้ในการวางแผนควบคุมการกระจายตัวของพยาธิใบไม้อย่างมีประสิทธิภาพยิ่งขึ้น

คำสำคัญ : พยาธิใบไม้ ค่าความชุก การระบุชนิดเชิงโมเลกุล ยีนไซโคโครมซี ออกซิเดส

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Introduction

Mae Taeng basin is located in Chiang Mai province, northern Thailand (Figure 1). It is serves as an important area for both aquatic culture and fisheries. Much of this area is in Mae Ngad Somboonchon Dam irrigating channels which have high biodiversity of aquatic animals that are associated in the completion of trematode life cycles (1, 2). There have been several reports the heterophyid trematode infections in freshwater animals in Chiang Mai (3), especially, the minute intestinal trematode, *Haplorchis taichui*, Witenberg, 1930 which have a high prevalence of infection in cyprinoid fishes (4-6).

The life cycle of *H. taichui*, involves freshwater snails as the first intermediate host, cyprinoid fishes as second host, and with fish-eating mammals as definitive hosts (7, 8). The pleurolophocercous and parapleurolopho cercous cercariae have been reported heterophyid trematodes including *H. taichui*, *H. pumilio, Stellantchasmus falcatus*, and *Centrocestus caninus*, which are abundantly recovered from freshwater snails in northern Thailand (9-11).

The traditional method of *H. taichui* detection in snails is usually performed by exposing the snails to light or by dissection, in which cercariae may be observed. These methods are difficult to detect specific parasite stages to the species because of similar morphological traits in closely related species the Heterophyidae. An accurate identification of cercarial stage is very important for understanding the epidemiological situation

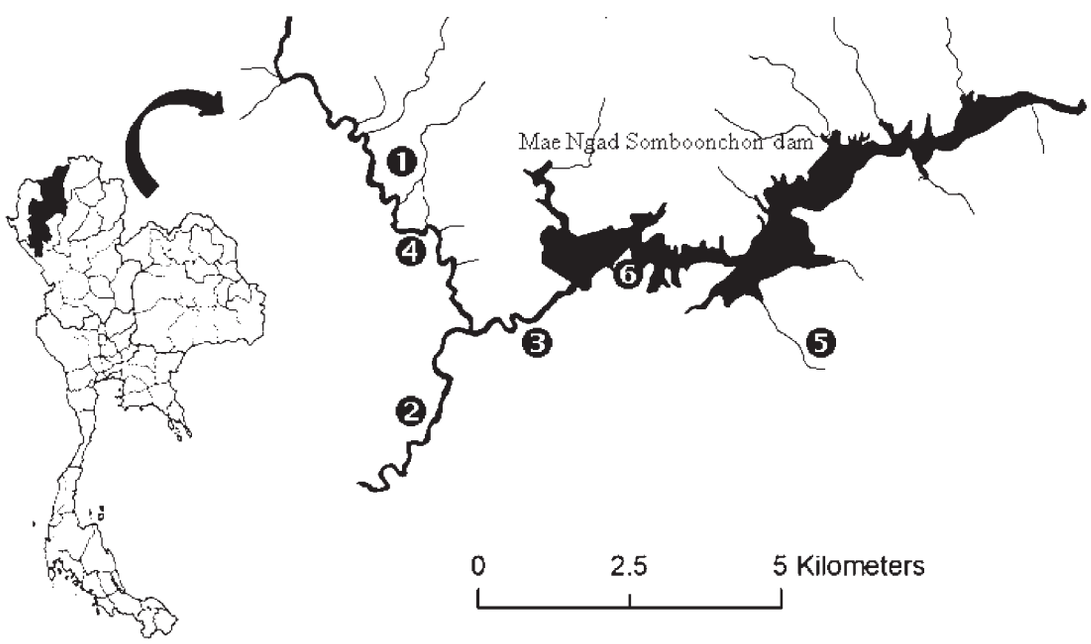

Figure 1 A map of Thailand showing the collecting sites in Mae Taeng basin, Chiang Mai province, Thailand. (1) irrigation canal (2-3) rice paddys (4-5) Mae Taeng River (6) Mae Ngad Somboonchon Dam.

and for developing effective control measures.

Various PCR methods have been developed for detection of trematode species in intramolluscan stages namely, Amplified Fragment Length Polymorphism (AFLP), High Annealing Temperature – Random Amplified Polymorphic DNA (HAT-RAPD), Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP), and gene sequencings (12-14). The development of aspecific DNA primer for *H. taichui* detection has been rarely successful. Some report have been established specific primers for the detection of *H. taichui* by generating the 256 bp specific fragment as a positive result (6).

Data from mitochondrial-based PCR have been extensively used in studies of identification, evolution, phylogeny, biogeography, population genetics and systematic because the mitochondrial genomes are readily adundantly copied and not strikingly different in related species (15). Lee et al. compared the phylogenetic relationship of these heterophyid trematodes, *viz. Metagonimus yokogawai, M. takahashii*, and *M. miyatai*, using 28S rDNA and cytochrome c oxidase subunit I (mCOI) gene. The results showed that the phylogenetic tree from 28S ribosomal DNA was similar with mCOI sequence data (16).

Our study provides data on the prevalence of *H. taichui* infections in freshwater snails from Mae Taeng basin, Chiang Mai, Thailand by morphological and PCR-based methods. This is the first step in providing new data on the recent distribution and prevalence of trematode infections and for developing effective control measures.

Method

Study sites

Six sites were selected based on areas with public health problems with trematode infections in and around the Mae Ngad Somboonchon Dam, Mae Taeng district, Chiang Mai province. These areas include an irrigation canal (N 21//12.864/ E 0.50//08.488/), rice paddies (N 21//13.134/ E 0.50//07.982/ and N2116.486 E 0.409.359), Mae Taeng River (N 21//12.992/ E 0.50//07.862/ and N2112.992 E0.5007.862), and the dam (N2119.725E0. 5003.645) (Figure 1).

Snail samples and cercarial infections

Snail specimens were collected by hand during April 2008 to June 2010. After collection, the snails were identified by using Brandt (17) and were examined for cercarial infection using a crushing method. Each groups of cercaria were stained in Delafield's haematoxylin, dehydrated in an alcohol series, cleared with xylene, and mounted in permount. The cercariae were then identified to family level and in some cases to genus (18, 19). The pleurolophocercous and parapleurolo phocercous cercariae were preserved in 70% alcohol for DNA extraction since these cercarial types have been reported as minute intestinal heterophyid trematodes (20).

Genomic DNA extraction and PCR amplification

The genomic DNA was extracted and purified individually from both cercarial types found in each snail using a commercial GF-1 tissue DNA extraction kit (Vivantis, Malaysia) according to the manufacturer's instructions.

Molecular identification by DNA specific primer

PCR was performed to detect the *H. taichui* infections in snails. The protocol followed Wondsawad et al. (6). Sequences of the primers used in this PCR amplification were Forward 5′-GGCCAACGCAATCGTCATCC -3′ and Reverse 5′-GCGTCGGGTTTCAGACA TGG-3′. Using these primers, genomic DNA was amplified in 20 μl final volume, including 50 ng of DNA template, 50 pM/ l of each primer (forward and reverse), 1.5 mM of MgCl2, 200 μ of dNTPs and 0.5 unit of Taq DNA polymerase, and volume made to 20 μl with deionized water.

Amplification was perform in a thermocycles with the initial denaturation step at 95°C for 5 minutes, then 35 cycles including denaturation at 95°C for 45 seconds, primer annealing at 68°C for 45 seconds, extension at 72°C for 1 minutes, and final extension at 72°C for 7 minutes. PCR products were analyzed through 1.8% agarose gels electrophoresis separation at 50 volts for 40 minutes in TBE buffer, stained with ethidium bromide, and visualized with a Kodak digital camera (Gel Logic 100).

The genomic DNA of positive results, which gave an amplification of the 256 bp fragment, were amplified the barcoding region mCOI gene by a pair of primers according to Yu et al. (21). It included (JB3) 5′ TTTTTTGGGCATCC TGACGTTTAT 3′, as a forward primer, and (JB 4,5) 5′ TAAAGAAAGAACATAATGAAAATG 3′, as a reverse primer.

The specific DNA fragment of mCOI were direct sequenced. And confirmed by BLAST (Basic Local Alignment Search Tool) program in the NCBI (National Center for Biotechnology Information).

Results

Prevalence of infection

1,836 snails of 11species were collected, *viz. Bithynia siamensis, Clea helena, Eyriesia eyriesi, Filopaludina doliaris, F. martensi, F. sumatrensis, Lymnaea auricularia, Melanoides tuberculata, Pila polita, Tarebia granifera*, and *Thiara scabra*. The results showed that 315 out of 1,836 snails had cercarial infection, with an overall prevalence of 17.16% (Table 1). *M. tuberculata, L. auricularia,* and *T. granifera* were the most infected and susceptible hosts for cercarial infections with a prevalence of 35.71%, 33.33%, and 30.50%, respectively. *C. helena, F. sumatrensis, F. doliaris,* and *P. polita* were not infected.

Six groups of cercariae were detected: megalurous, furcocercous, monostome, pleurolophocercous, parapleurolophocercous, and virgulate cercaria. They were subdivided into 7 types based on morphological characteristics and internal organ arrangement. They are described here (Figure 2).

Table 1 Prevalence of cercarial infections in snails from the Mae-Taeng basin, Chiang Mai, Thailand

Snail species	Snail samples	Infected samples	Prevalence (%)	Type of cercariae	Prevalence of cercarial types (%)
Family Bithyniidae					
Bithynia siamensis	150	16	10.67	Notocotylidae	7.33
				Haplorchis sp.	1.33
				Strigeidae	0.68
				Lecithodendriidae	1.33
Family Buccinidae					
Clea helena	2	0	0.00	-	-
Family Viviparidae					
Eyriesia eyriesi	80	2	2.50	*Haplorchis* sp.	2.50
Filopaludina doliaris	150	0	0.00	-	-
Filopaludina martensi	296	2	0.68	Strigeidae	0.34
				Lecithodendriidae	0.34
Filopaludina sumatrensis	120	0	0.00	-	-
Family Lymnaeidae					
Lymnaea auricularia	30	3	33.33	Heterophyidae	33.33
Family Thiaridae					
Melanoides tuberculata	448	160	35.71	Distome	2.01
				Lecithodendriidae	3.12
				Haplorchis sp.	30.56
				Strigeidae	0.22
				Heterophyidae	8.48
Tarebia granifera	377	125	33.16	Philophtthalmidae	0.53
				Haplorchis sp.	28.64
				Lecithodendriidae	0.53
				Notocotylidae	0.16
				Heterophyidae	0.82
Thiara scabra	93	7	7.22	Heterophyidae	5.38
				Lecithodendriidae	1.08
				Transversotrema sp.	1.08
				Haplorchis sp.	4.30
Family Ampullariidae					
Pila polita	90	0	0.00	-	-
Total	1,836	315	17.16		

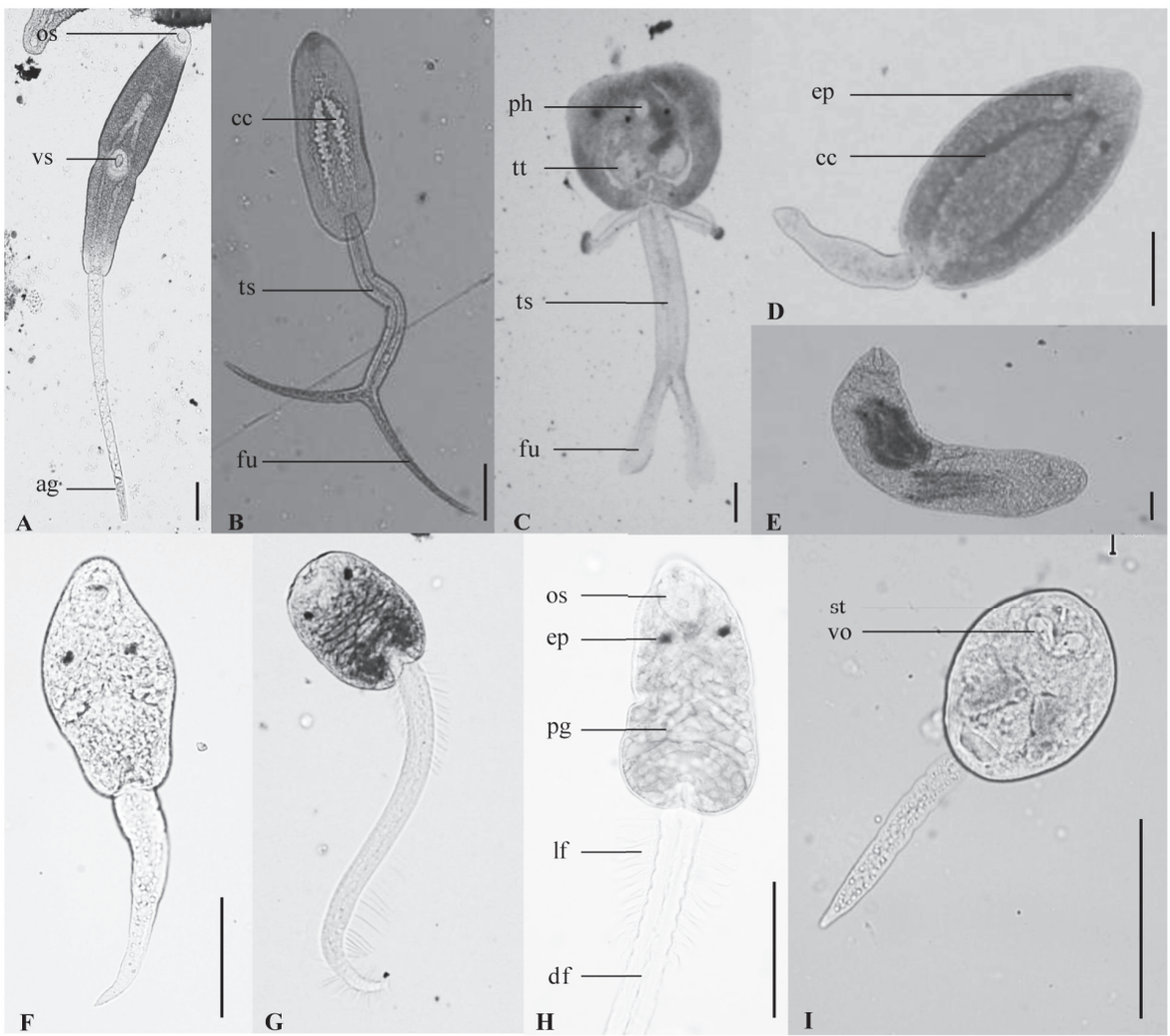

Figure 2 Seven types of cercaria were found infect to snails. **A** – distome cercaria: Philophthalmidae cercaria, **B** – furcocercouse cercaria, Strigeidae cercaria, **C** – furcocercous cercaria: *Transversotrema sp.*, **D** – monostome cercaria: Notocotylidae cercaria, **E** - redia of Notocotylidae, **F** – pleurolophocercous cercaria, Heterophyidae cercaria, **G** – parapleurolophocercous cercaria: Haplorchis sp. cercaria (body), **H** - anterior part of *Haplorchis sp.*, **I** –virgulate cercaira: Lecithodendriidae cercaria (ag - adhesive gland, df - dorsoventral nfold, ep - eye spots, fu - furca, lf - lateral nfold, os - oral sucker, pg - penetrated gland, ph - pharynx, st - stylet, ts – tail stem, tt – testis, vo - virgulate organ, vs – ventral sucker) (scale bar 0.1 mm).

Megalurous cercaria: Philophthalmidae

This parasite only infected *T. granifera*. The megalurous cercaria is ovoid with yellowish-brown granules. The body surface is covered with longitudinal and fine traverse wrinkles. Minute spines cover the surface of the posterior half of the body. The esophagus bifurcated midway between the pharynx and the ventral sucker, and the intestinal ends blindly near the posterior end of body. The ventral sucker is slightly larger than the oral sucker, and is located medially on the the body. The tail is as long as the body, elastic, and slender. Long tail tips numerous adhesive gland cells and lacks an excretory canal in the tail base (Figure 2A).

Furcocercous cercaria: Strigeidae

This parasite was found in *B. siamensis, F. martensi*, and *M. tuberculata*. The cercaria body is ovoid and the body surface is covered with minute spines. The oral sucker is subterminal. The pharynx is very small and circular. The large intestine is composed of two ceca. The tail is forked and longer than the body. The tail stem is longer than the furca, and each furca has a dorsal and ventral finfold (Figure 2B).

Furcocercous cercaria: *Transversotrema* sp.

Transversotrema cercaria was found only in *T. scabra*. The cercaria body is short, flat, discord, and with yellowish-brown pigmentation. The body surface is covered with scale-like spines. The pharynx is very large and the esophagus is short and narrow. There are one pair of eyespots which are large spherical, located posterior to the pharynx. An oral sucker is absent while a ventral sucker is present medially. Testes are symmetrical and within the intestinal ring, while the genital pore is located medially. The tail is longer than the body and with arm like processes at the anterior end of the tail-stem (Figure 2C).

Monostome cercaria: Notocotylidae

Notocotylidae cercariae were found only in *B. siamensis* and *T. granifera*. The cercaria body is ovoid and the anterior quarter of the body is highly pigmented, especially around the eyespots. There are three black pigmented eyespots arranged in the form of a triangle in the anterior dorsal region of the mature cercaria (Figure 2D).

Pleurolophocercous cercaria: Heterophyidae

This type of cercaria was found in *B. siamensis, L. auricularia, M. tuberculata, T. granifera*, and *T. scabra*. The cercaria is ovoid and entirely covered with minute spines. A pair of conspicuous eyespots rectangular are found at the end of the anterior third of the body. The pharynx lies just behind the oral sucker. Intestinal ceca bifurcate and extend posteriorly to the excretory vesicle level. Seven pairs of penetration glands are arranged in 2 bundles anterolaterally to the genital premordium, which is a relatively large triangular mass of cells located just anterior to the excretory vesicle. The tail is almost the as long as body, slender, and usually attached to the dorso-ventral finfolds. A brownish pigment is dispersed throughout the body except in the oral sucker (Figure 2F).

Parapleurolophocercous cercaria : *Haplorchis* sp.

This type of cercaria was found in B. siamensis, *E. eyriesi*, *M. tuberculata, T. granifera*, and *T. scabra*. The cercaria is ovoid and is entirely covered with minute spines. The small pharynx lies just behind the oral sucker. Two square eyespots are located on each side above the pharynx. The anterior part of the body is covered with fine spines. Seven pairs of penetration glands are located between the pharynx and the posterior part of the body, surrounding mainly the genital premordium and arranged into two columns with the ventral sucker. The excretory vesicles are elongate. The tail is slender about 3 times the body length with two lateral finfolds extending along the anterior third of the tail A dorsoventral finfolds extends along the posterior two- thirds and around tip of the tail (Figure 2 G).

Virgulate cercaria: Lecithodendriidae

This carcaria was found in *B. siamensis, F. martensi, M. tuberculata, T. granifera*, and *T. scabra*. The cercaria is small shaped with a prominent stylet and develops in sporocysts. The oral sucker extends to a pharynx; oesophagus bifurcating preacetabularly into an intestinal caeca which encircles the acetabulum, terminating postacetabularly. The acetabulum is smaller than the oral sucker. The virgulate organ is butterfly–shaped, situated below the stylet. There are three pairs of penetration glands, the two anterior pairs have fine granules and the posterior pair with coarser granules medially. The tail is shorter than the body (Figure 2I).

***H. taichui* infection in snails**

H. taichui specific primers were performed to amplify a 256 bp fragment in the PCR technique for the identification of *H.taichui* cercariae infecting snails. Amplification of *H. taichui* cercariae could detect only the 256 bp specific fragment. Genomic DNA of adults from this parasite were used as a positive control, which also gave 256 bp specific fragment.

As shown in Table 2, 199 of 1,836 snails were infected by parapleurolophocercous cercaria including *B. siamensis*, *E. eyriesi, M. tuberculata, T. granifera*, and *T. scabra*.

Table 2 Prevalence of *H. taichui* infections, using specic PCR.

Snail species	Number infected with parapleurolocercouse cercaria	Positive	Prevalence (%)
Bithynia siamensis	2	0	0.00
Eyriesia eyriesi	2	0	0.00
Melanoides tuberculata	98	42	42.86
Tarebia granifera	93	42	45.16
Thiara. scabra	4	1	25.00
Total	199	85	42.71

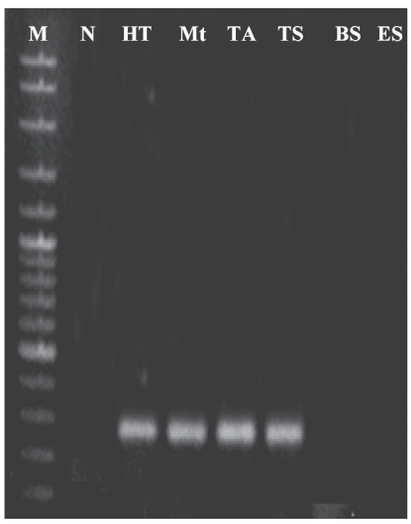

Figure 3 Gel electrophoresis proles of parapleurolophocercous cercariae isolated from thiarid snails. Lane M: molecular size maker, Lane N: negative control, Lane Ht: adult of *H. taichui*, DNA samples of parapleurolophocercous cercariae form snails (Lane Mt: *Melanoides tuberculata*, Lane Ta: *Tarebia granifera*, Land Ts : *Thiara scabra*, Lane Bs: *Bithynia siamensis*; Lane Ee: *Eyriesia eyriesi*).

1AGTGAGACACATTTGTAGGACGTTAACAAATAAAGATTCCTTGTTTGGTT
ATGGGGGTTTAGTTCTTGCTATGTTTTCTATAGTCTGTTTGGGGAGTGTT
GTTTGAGCTCATCATATGTTTATGGTTGGGTTGGATGTTAAGACGGCTGT
TTTTTTTAGTTCTGTGACTATGGTTATAGGAGTCCCCACAGGTATAAAGG
TTTTTTCTTGGCTGTATATGTTGGCGGGAAGTCGGGGCCGGTTTTGGGAT
CCGATAATGTGGTGGATATTGGGTTTTATTGTCCTATTTACTATCGGGGG
GGTGACCGGGATTGTGTTGTCTTCTTCCTATAATGGATA 340

Figure 4 Sequence of mCOI nucleotides of *Haplorchis taichui*.

After testing with *H. taichui* specific primers, only three species were infected this trematode, viz. M. tuberculata, *T. granifera*, and *T. scabra* (Figure 3). The overall prevalence of *H. taichui* infections based on PCR detection was 42.71%. T. granifera was the most susceptible snail host for *H. taichui* infections (Table 2).

The length of partial mCOI nucleotide sequence data was 340-bp. The mCOI sequence showed 99% similarity with the reference sequence of the 29 isolated *H. taichui* in the Genbank data base (Figure 4).

Discussion

Eleven snail species from the Mae Taeng basin are intermediate hosts of digenetic trematodes. We found, 7cercarial types in 4 snail families, viz. Thiaridae, Viviparidae, Bithyniidae, and Lymnaeidae) from 7 snail species (Table 1). The prevalence of cercarial infections found in our study (17.16%) was higher than Mard-arhin et al. found in five provinces in northern Thailand (6.20 %) (22). Dechruruksa et al. reported that the prevalence of cercarial infections in thiarid

snails from the Khea River, Phitsanulok province, Thailand was only 0.9% (11). The *Transversotrema sp*. cercaria infected only *T. scabra* which agrees with Wongsawad and Kumchoo (23).

In our study, parapleurolophocercous and pleurolophocercous cercariae were dominant. They have been reported previously as heterophyid trematodes, viz. *C. caninus, H. taichui, H. pumilio*, and *S. falcatus* (11, 13, 24). Our findings agree with Radomyos et al. who reported that heterophyid trematodes are widely distributed and with high prevalence in northern Thailand (25). Kumchoo et al. reported a high prevalence of *H. taichui* infections (91.4%) in cyprinoid fish from Mae Taeng district, Chiang Mai province (5). Nithikathkul and Wongsawad (2008) indicated a high prevalence of *H. taichui* and Haplorchoides sp. infections (83.90%) in Chiang Mai province (26).

For the PCR-based assay, parapleurolo phocercous cercariae were confirmed to be *H. taichui*. Overall *H. taichui* infections based on specific PCR amplification was 42.71% of all parapleurolophocercous cercariae populations. The prevalence of the morphological method was higher than the molecular method because the PCR-based method is more exact specific than the identification by using only morphology. Pleurolophocercous and parapleurolo phocercous cercariae can develop to be several heterophyid species. Our study provides significant evidence for the identification and epidemiological situation of larval trematodes in their intermediate hosts. The high similarity of mCOI with 29 isolates of *H. taichui* from Vietnam and Thailand indicated that the mCOI sequence products good to species level and applicable for molecular identification. According to Thaekham et al. investigated the PCR-RFLP patterns of cytochrome c oxidase subunit I (mCOI) gene amplification to distinguish O. viverrini from *H. taichui* (12).

Our results established that three species of snails (*M. tuberculata, T. granifera*, and *T. scabra*) serve as the first intermediate hosts of *H. taichui*. Dechruksa et al. reported that thiarid snails were infected with intestinal and blood fluke larvae (11). The snails, *M. tuberculata* and *T. granifera* are medically important since they can serve as the first intermediate host of minute intestinal flukes (13, 24).

The PCR-based method is a more effective approach for the identification of *H. taichui* cercariae including other medically important species, which can be used for epidemiological studies, and developing a control program.

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