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Research Note

Morphological and molecular characterization of *Haplorchoides mehrai* Pande and Shukla 1976 (Digenea: Heterophyidae) from Chiang Mai province

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Summary

Cyprinoid fish in Chiang Mai province has been reported the presence of a large number of metacercariae, particularly the metacercariae of *Haplorchoides* and those not identified to species. This study aims to investigate morphological and molecular characteristic of the minute intestinal fluke *H. mehrai* metacercariae in two cyprinoid fish species from Chom Thong district, Chiang Mai province, Thailand: the Tinfoil barb (*Barbonymus schwanenfeldii*) and the White eye barb (*Cyclocheilichthys repasson*). A total of 180 fish (90 from *B. schwanenfeldii* and 90 from *C. repasson*) were collected over three seasons: cool, hot and the rainy season (December 2015 to August 2016). Fish were examined for *H. mehrai* metacercariae infection, including areas such as muscle and the inner side of body scales, by using a light microscope. The prevalence of *H. mehrai* metacercariae in *B. schwanenfeldii* and *C. repasson* was 73.33 % and 100 % respectively. *Haplorchoides* metacercariae were identified as *H. mehrai* based on the morphological characteristics; the position of the acetabulum and the number and arrangement of the acetabular spines. Phylogenetic analysis based on Cytochrome c Oxidase subunit I (COI) gene showed that *H. mehrai* metacercariae from *B. schwanenfeldii* and *C. repasson* were the same species as the adult stage of *H. mehrai* from *Hemibagrus nemurus* and *Mystus multiradiatus*. Both morphological and molecular characteristic could indicate that *Haplorchoides* metacercariae originated from this study were *H. mehrai*. Furthermore, it is a new record of the minute intestinal fluke *Haplorchoides mehrai* in Chiang Mai Province.

Keywords: *Haplorchoides mehrai*, *Barbonymus schwanenfeldii*, *Cyclocheilichthys repasson*, Metacercariae, COI, Chiang Mai province

Introduction

Haplorchoides mehrai is a minute intestinal fluke, first described by Pande and Shukla (1976). The *Haplorchoides* genus belongs to the subfamily Haplorchiinae, family Heterophyidae (Chen, 1949; Pearson & Ow Yang, 1982; Yamaguti, 1958). Freshwater fish, particularly cyprinoid fish served as the second intermediate host of *H. mehrai* metacercaria (Scholz *et al*., 1991; Manpratum *et al*., 2017). The adult stage of *H. mehrai* have been first recorded in the small intestines of *Mystus vittatus* from India (Pande & Shukla, 1976). Some previous studies, *H. mehrai*, adult stages have been reported from Yellow catfish, *Hemibagrus nemurus* in Khon Kaen Province, Northeast Thailand (Manpratum *et al*., 2017). In the Northern Thailand, the high prevalence of *Haplorcoides* spp. metacercariae in cyprinoid fish have been recorded in Phitsanulok (Noikong *et al*., 2011) and Chiang Mai province (Saenphet *et al*., 2001; Nithikathkul & Wongsawad, 2008). Moreover, the adult stage of *Haplorchoides* spp. have been reported to infect the Yel-

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low catfish, *H. nemurus* in Chiang Mai (Wongsawad *et al*., 2004) and Chiang Rai province (Purivirojkul & Areechon, 2008). The Ping River is an important river in Chiang Mai province, containing many aquatic animals, particularly cyprinoid fish which act as the second intermediate hosts for *Haplorchoides* spp. The Ping river flows through the Chom Thong district, Chai Mai province an area that supports a large amount of agriculture and many fisheries. Kumchoo *et al.* (2005) recorded that cyprinoid fish, *Barbonymus schwanenfeldii* and *Cyclocheilichthys repasson* were infected with *Haplochoides* sp. metacercaria in Chom Thong district.

Mitochondrial DNA based Polymerase Chain Reaction (PCR) methods have been effective for identification and studying the phylogenetics of trematodes in the family Heterophyidae (Chontananarth *et al*., 2014). A COI primer was used in the differentiation of COI fragments from Heterophyidae (*Haplorchis taichui*) with fragments from Opisthorchiidae (*Opisthorchis viverrini*) by Thaenkham *et al*. (2007). The COI gene is useful for assessing the genetic variation in *H. taichui* (Dung *et al*., 2013).

This study aimed to examine the prevalence of *Haplorchoides mehrai* metacercariae in *B. schwanenfeldii* and *C. repasson* from Chom Thong district, Chiang Mai province, Thailand. A phylogeny based on the COI gene of *H. mehrai* and other heterophyid trematodes was also reconstructed. This data provides useful information for the control and prevention of *H. mehrai* infection in Chiang Mai province and for Thailand in general.

Materials and Methods

Parasite specimens

A total of 180 fish (90 from *B. schwanenfeldii* and 90 from *C. repasson*) were collected in the same river area (N18.403918, E98.702038) from Chom Thong district, Chiang Mai province, Thailand. Fish were collected over 3 seasons: cool (n = 30), hot (n = 30) and the rainy season (n = 30), from December 2015 to August 2016. Fish were transferred to the laboratory at the Department of Biology, Faculty of Science, Chiang Mai University. Standard length (cm) and weight (g) of the fish were recorded. The fish were individually examined, which included an examination of their body scales (30 scales per fish) and meat (2g). The scales were directly examined for metacercariae under light microscope. The fish meat was ground by blender, then mixed with pineapple juice and incubated at 37 °C for 1 – 2 hours. The processed meat was filtrated with graded sieves to remove large particles, rinsed twice with water and examined under light microscope.

Adults of *Haplorchoides mehrai* were collected from Yellow catfish (*Hemibagrus nemurus*), Asian redtail catfish (*Hemibagrus wyckioides*) and Iridescent mystus (*Mystus multiradiatus*).

For the species identification, encysted and excysted metacercariae and adults of *Haplorchoides mehrai* were fixed and flattened in 4 % formalin for preparation of permanent slides. The trematodes were stained with Delafield' hematoxylin, then dehydrated in alcohol series, cleared in xylene and permanently mounted in permount. Specimens on permanent slides were illustrated using a compound microscope with a drawing tube. Measurements were obtained using an ocular micrometer and expressed in micrometers (µm). The identification was based on morphology according to Pande and Shukla (1976) and Shameem and Madhavi (1988). The prevalence of infection was calculated based on the equation of Margolis *et al*. (1982).

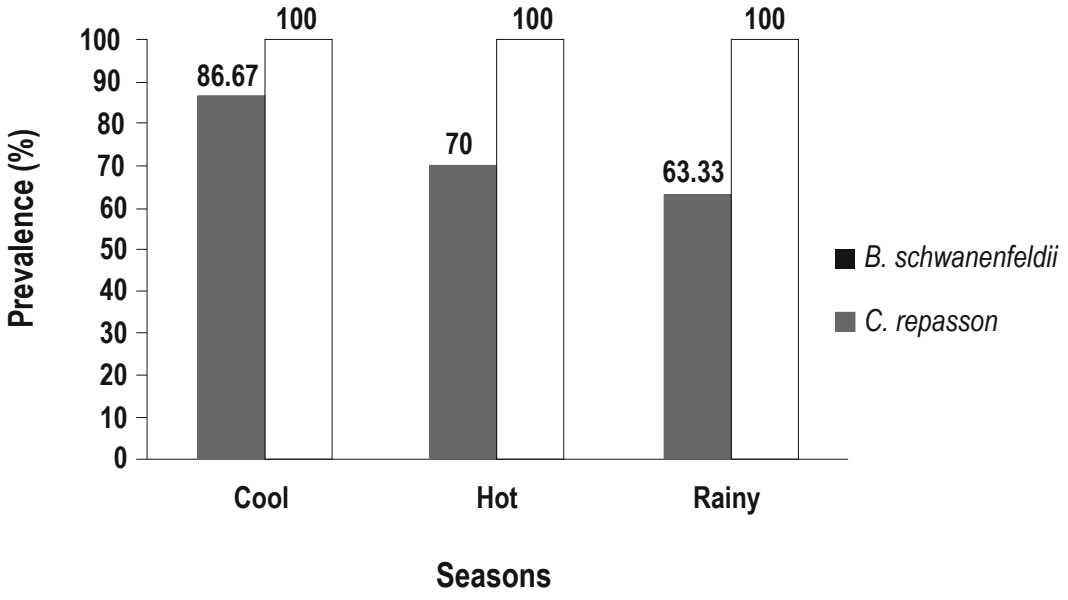

Fig. 1. Prevalence of *Haplorchoides mehrai* metacercaria during 3 seasons.

Table 1. Prevalence of *Haplorchoides mehrai* metacercariae in Cyprinoid fish from Chiang Mai province.

Cyprinoid fish	No. of examined fish	No. of infected fish	Prevalence	Intensity
Barbonymus schwanenfeldii	90	66	73.33	14.86
Cyclocheilichthys repasson	90	90	100	129.85

Genomic DNA extraction

Genomic DNA of all parasites was extracted from both adults and metacercariae based on the Chelex method used by Caron *et al.* (2010). The genomic DNA of the flukes was stored at -20 °C until used.

COI PCR

The PCR amplification of Cytochrome c Oxidase subunit I (COI) was followed the methods described in Chontananarth *et al.* (2014). It consists of a pair of primers: forward primer (JB3) 5' TTTTTTGGGCATCCTGACGTTTAT 3' and reverse primer (JB 4,5) 5' TAAAGAAAGAACATAATGAAAATG 3'. The final volume of 20 µl PCR product mixture consisted of 1.0 µl genomic DNA, 2.0 µl PCR buffer, 2.0 µl (10 mM) of dNTPs, 0.7 µl (50mM) of $MgCl_2$, 1 µl of primer and 0.3 µl of *Taq* polymerase. PCR amplification followed an initial denaturation of 3 min at 95 °C, followed by 40 cycles, which consisted of denaturation for 1 min at 95 °C, 1 min of annealing at 50 °C, 1 min of elongation at 72 °C and a final elongation step for 7 minutes at 72 °C. The COI PCR product was checked using DNA Dye Non Tox (AappliChem) staining and separated on 1.4 % TBE agarose gel electrophoresis. All COI PCR products were subjected for purify and sequencing.

Phylogenetic tree construction

Phylogenetic trees were constructed using the program Mega version 6.06, and molecular data were analyzed using Maximum likelihood (ML) and Neighbor joining (NJ) methods. The reliability of internal branches in both methods was estimated using 1000 bootstrap replications. Sequences of the fluke *Fasciola gigantica* (Fasicolidae) were used as an outgroup for phylogenetic analysis.

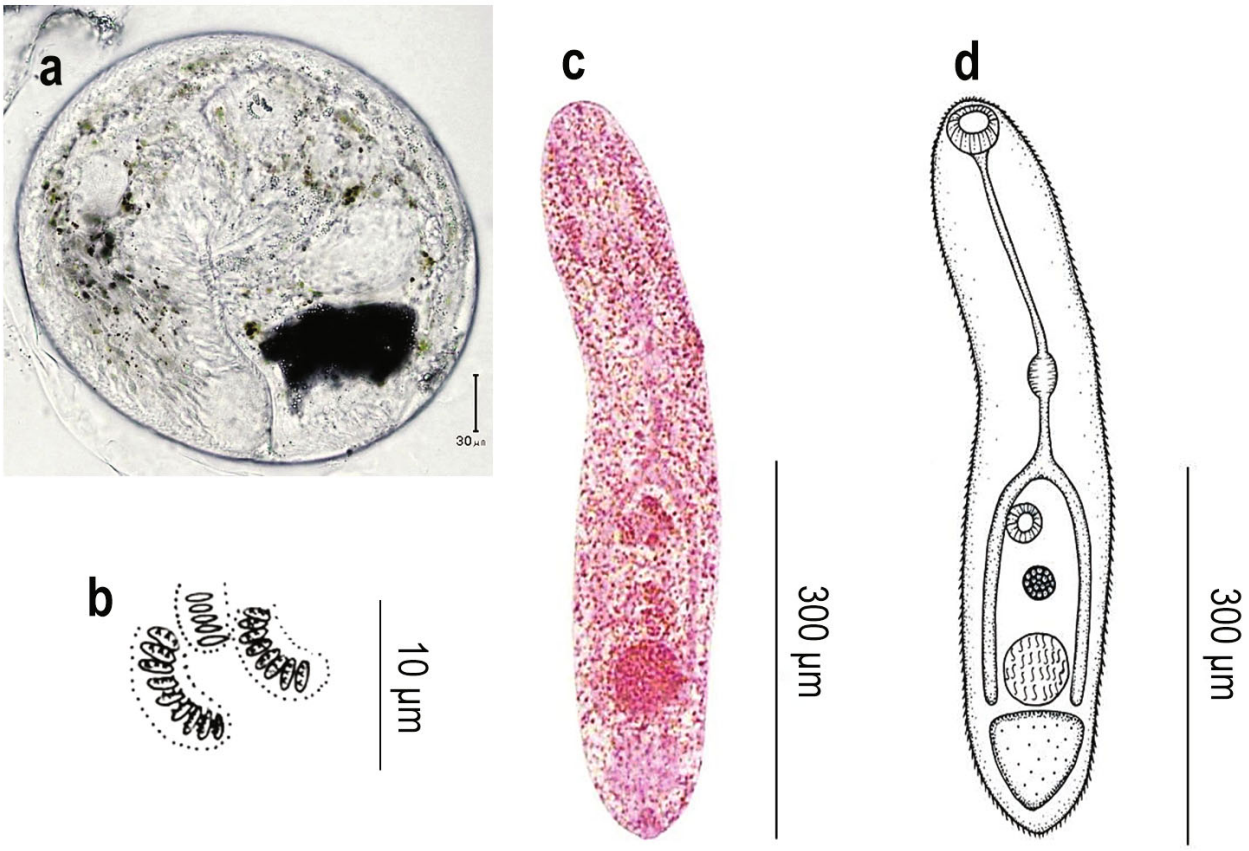

Fig. 2. Encysted metacercariae of *H. mehrai* from *B. schwanenfeldii* (a) Acetabular spine (b). Excysted metacercariae stained Delafield' hematoxylin (c,d).

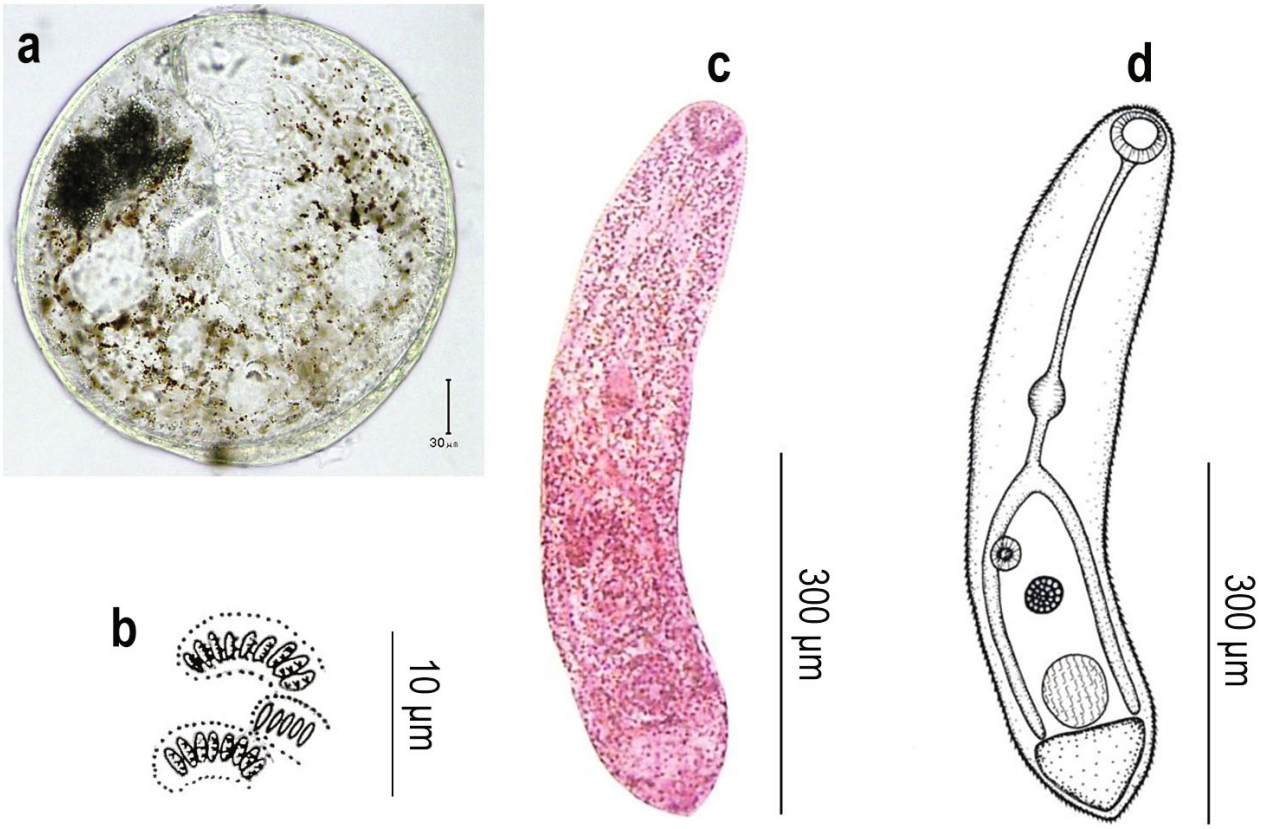

Fig. 3. Encysted metacercariae of *H. mehrai* from *C. repasson* (a) Acetabular spine (b). Excysted metacercariae stained Delafield' hematoxylin (c,d)

Ethical Approval and/or Informed Consent

There are no the use of animal for experimentation but use only for surveyed research. We have animal use license number of U1-07209-2560 that issued by the Institute of Animal for Scientific Purpose Development (IAD), Thailand. However, this research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

Prevalence of infection

The results revealed that the prevalence of *H. mehrai* metacercaria infection (Table 1) in *Barbonymus schwanenfeldii* was 86.7 % in the cool, followed by 70 % in the hot and 63.3 % in the rainy season (Fig. 1). The average prevalence and intensity across all three seasons was 73.3 % (66/90) and 14.86 respectively. Prevalence in *Cyclocheilichthys repasson* was 100 % for all three seasons (90/90) (Fig. 1) with an intensity of 129.85. The metacercariae of *H. mehrai* were recovered from the inner side of body scales and the general muscular tissue of *B. schwanenfeldii* and *C. repasson*. Body scales and fish meat of *B. schwanenfeldii* respectively contained 17.21 % and 10 % of all metacercariae found, whereas the body scales and fish meat of *C. repasson* contained 43.39 % and 16.66 % of all metacercariae respectively.

Morphological analysis

Metacercariae of *Haplorchoides mehrai* from *Barbonymus schwanenfeldii* and *Cyclocheilichthys repasson*

Encysted metacercariae of *H. mehrai* from *B. schwanenfeldii* (Fig. 2) and *C. repasson* (Fig. 3) are nearly spherical, with a double layered cystic wall. Both excysted metacercariae of *H. mehrai* from *B. schwanenfeldii* (Fig. 2c, 2d) and *C. repasson* (Fig. 3c, 3d) have lance-shaped bodies, with a scale like spine on the body surface. Oral sucker subterminal. Prepharynx longer than esophagus. Caeca extends slightly beyond posterior border of testes. Acetabulum submedian, located near intestinal bifurcation. Acetabulum with spines present in three groups. Testes rounded, median, located between the caecal ends and posterior body. Ovary spherical, pre-testicular, median. Excretory bladder saccular, post-testicular. The measurements of the excysted metacercariae were shown in Table 2.

Table 2. Comparing the Organs size (in μm) of *H. mehrai* excysted metacercariae from *B. schwanenfeldii* and *C. repasson.*

Organs	Previous study	This study	
	H. mehrai (Pande and Shukla,1976)	*H. mehrai* from *B. schwanenfeldii*	*H. mehrai* from *C. repasson*
Body length	225 – 565	499.6 (355 – 630)	494.4 (390 – 620)
Body width	90 – 180	120.3 (90 – 160)	130.3 (110 – 160)
Number of acetabular spines	15 – 32	19 – 27	19 – 27
Anterior group	5 – 14	5 – 7	5 – 7
Median group	5 – 9	7 – 10	7 – 10
Posterior group	5 – 9	7 – 10	7 – 10
Oral sucker length	25 – 50	41.6 (32.0 – 49.4)	41.3 (32.5 – 52.0)
Oral sucker width	32 – 54	46.6 (39.0 – 54.6)	48.3 (41.0 – 59.8)
Acetabulum length	18 – 40	27.9 (20.8 – 36.4)	27.8 (20.8 – 38.8)
Acetabulum width	14 – 40	27.4 (20.8 – 34.0)	27.1 (20.8 – 36.4)
Prepharynx length	14 – 94	139.8 (70.2 – 187.2)	136.6 (78.0 – 195.0)
Pharynx length	25 – 47	34.5 (23.4 – 44.2)	31.2 (25.0 – 44.2)
Pharynx width	14 – 36	31.5 (20.9 – 44.2)	30.6 (25.0 – 43.2)
Esophagus length	11 – 58	44.2 (23.4 – 78.0)	40.5 (23.4 – 65.0)
Ovary length	11 – 47	27.7 (20.8 – 36.4)	26.8 (15.6 – 36.4)
Ovary width	18 – 47	28.2 (20.8 – 39.0)	28.2 (18.2 – 39.0)
Testis length	29 – 58	46.1 (26.0 – 67.6)	44.0 (28.6 – 62.4)
Testis width	29 – 90	51.1 (33.8 – 70.2)	47.9 (28.6 – 62.4)

( ) = average value

Adult of *Haplorchoides mehrai* from *Hemibagrus nemurus* and *Mystus multiradiatus*

H. mehrai from *H. nemurus* (Fig. 4a, 4b) and *M. multiradiatus* (Fig. 4c, 4d) have a small body size. Body is lance-shaped. The body tegument has a scale like spine. Oral sucker subterminal. Prepharynx longer than esophagus. Caeca extend slightly beyond posterior border of testes. Acetabulum small, submedian, located near intestinal bifurcation. Acetabulum with spines in three groups. Seminal vesicle with two-chambers, behind intestinal bifurcation. Testes rounded, median, between caecal ends posterior to the body. Ovary spherical, pretesticular. Seminal receptacle pretesticular, lateral of ovary. Vitelline follicles around testes. Eggs small, numerous, operculate, with fully embryonated. The measurements of adult stages were shown in Table 3.

Molecular analysis

Our COI sequence data revealed the partial size of 396 bp. in all specimens. Phylogenetic trees were constructed using the Neighbor joining method and the Maximum likelihood method (Fig 5). Bootstrap values were computed independently for 1000 replications. Both methods revealed the monophyletic group of *Haplorchoides* which separated from related group (*Haplorchis* and *Metagonimus*). In *Haplorchoides* group, *Haplorchoides* metacercariae originated from *B. schwanenfeldii* and *C. repasson* were clustered with the *H. mehrai* from *Hemibagrus nemurus*, *H. wyckioides* and *Mystus multiradiatus*, with high bootstrap support. The *H. mehrai* group in this study was separated from *Haplorchoides* sp. from previous studies with high bootstrap support.

Discussion

In this study, a high prevalence of *H. mehrai* metacercaria infection was found in *C. repasson*. This result was similar to Kumchoo *et al*. (2005), in which 100 % of *C. repasson* was infected by *Haplorchoides* sp. metacercariae, whereas the prevalence in *B. schwanenfeldii* was much lower. However, this result is quite different from Noikong *et al*. (2011), which reported 76.23 % and 56.26 % prevalences of *Haplorchoides* sp. metacercaria infection in *C. re-*

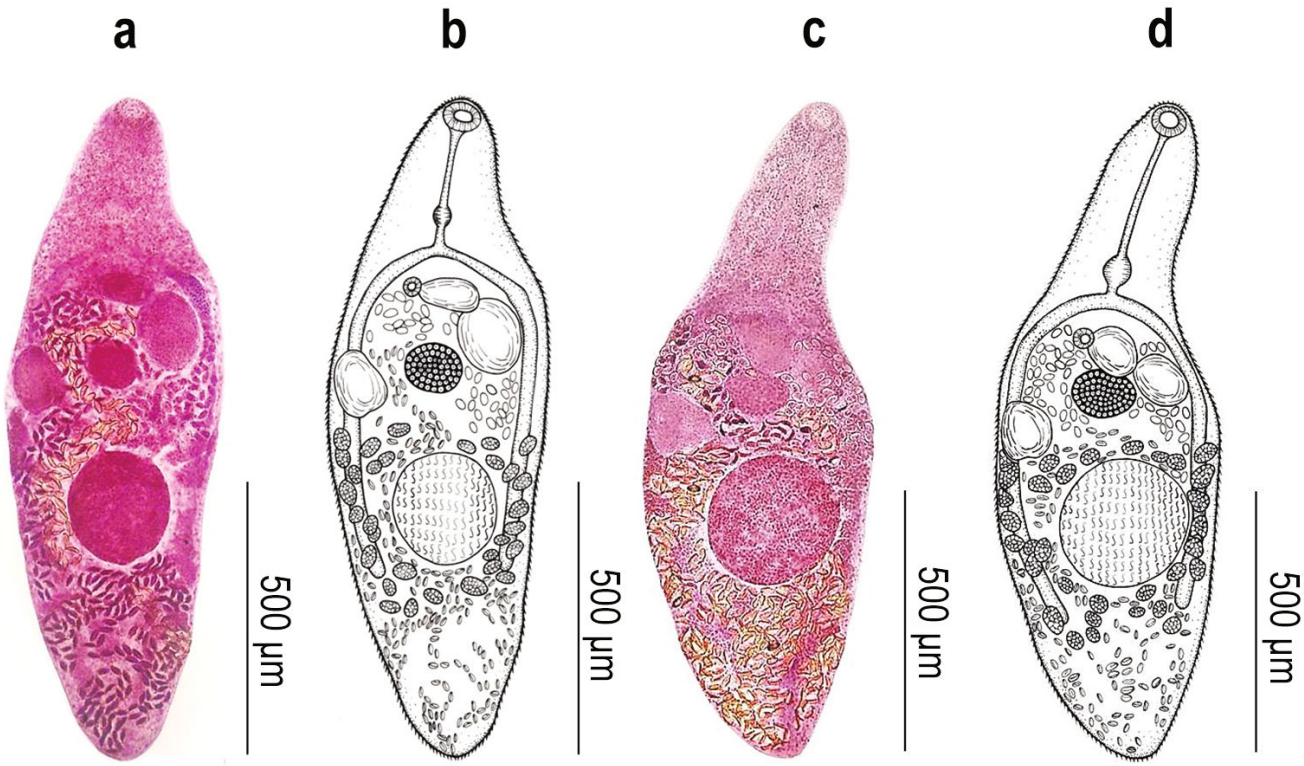

Fig. 4. Adult of *H. mehrai* from *H. nemurus* (a,b) and adult of *H. mehrai* from *M. multiradiatus* (c,d).

passon and *B. Schwanenfeldii* from the Kwae Noi Bamroongdan dam, Wat Bot district, Phitsanulok province, northern Thailand, respectively. The prevalence of infection of *H. mehrai* metacercariae in this study was higher than Noikong *et al*. (2011). However, prevalences over the three seasons were similar to Noikong *et al.* (2011); *H. mehrai* metacercariae were most prevalent in cool, followed by the hot and the rainy season respectively. Metacercariae of *H. mehrai* were found on the inner side of body scales and in muscle, which is in concordance with previous studies, such as Namue *et al*. (1998) Saenphet *et al*. (2001) and Kumchoo *et al*. (2005). *Haplorchoides* spp. metacercariae are found in common species of freshwater fish particularly cyprinoid fish. In Chiang Mai, they are often found together with metacercariae of *Haplorchis taichui* (Namue *et al*., 1998; Boonchot & Wongsawad, 2005; Nithikathkul & Wongsawad, 2008; Wongsawad *et al*., 2013).

In the morphological analysis, excysted *H. mehrai* from *B. schwanenfeldii* and *C. repasson* were similar to the adult stage of *H. mehrai* from *Hemibagrus nemurus* and *M. Multiradiatus* collected from Chiang Mai province, Thailand and other countries (Pande & Shukla, 1976; Shameem & Madhavi, 1988; Manpratum *et al*., 2017). They show the same position of ceaca, acetabulum and the same number of acetabular spines in three groups. However, the numbers of acetabular spines in *H. mehrai* from the four different fish species in this study were also some out of range at posterior and median group. The prepharynx length of both excysted and adult stage *H. mehrai* were longer than described in previous studies by Pande and Shukla (1976) and Shameem and Madhavi (1988). The body size of adult *H. mehrai* in this study was bigger than reported for *H. mehrai* in Northeast Thailand by Manpratum *et al*. (2017).

In previous studies, the High Annealing Temperature Random Amplified Polymorphic DNA (HAT-RAPD) technique was used to identify *Haplorchoides* spp. and other heterophyid species (Chuboon & Wongsawad, 2009; Wongsawad *et al*., 2013). HAT-RAPD was also used to compare metacercariae of *Haplorchoides* sp. from cyprinoid fish with the adult stage of *Haplorchoides* sp., which infect the same fish as those used in this study, such as the Yellow catfish, *Hemibagrus nemurus.* (Wongsawad & Wongsawad, 2011). Likewise, the COI gene can be used to identify *H. mehrai* metacercariae originated from this study. Phylogenetic trees using Neighbor joining and Maximum likelihood methods showed the monophyletic group of *Haplorchoides*. *H. mehrai* metacercariae clustered with *H. mehrai* adults and separated from *Haplorchoides* sp. originated from previous study (Chontananarth *et al*., 2014), with high bootstrap support. The COI gene can also be used to distinguish *H. mehrai* from other trematodes in family Heterophyidae. Our study could indicate that *H. mehrai* metacercariae originated from *B. schwanenfeldii* and *C. repasson* tended to be

Table 3. Comparing the organs size (in μm) of adult *H. mehrai* from *H. nemurus* and *M. multiradiatus*.

Organs	Previous study		This study	
	H. mehrai (Pande and Shukla, 1976)	*H. mehrai* (Shameen and Madhavi, 1988)	*H. mehrai* from *H. nemurus*	*H. mehrai* from *M. multiradiatus*
Body length	255 – 720	928 – 1360	1,243.8 (830 – 1,975)	1,285 (910 – 1,625)
Body width	75 – 390	384 – 512	319 (250 – 470)	327.0 (230 – 450)
Number of acetabular spines	15 – 32	18 – 26	19 – 27	19 – 27
Anterior group	5 – 14	6 – 10	5 – 7	5 – 7
Median group	5 – 9	6 – 8	7 – 10	7 – 10
Posterior group	5 – 9	6 – 8	7 – 10	7 – 10
Oral sucker length	25 – 54	72 – 80	49.7 (33.8 – 67.6)	49.6 (31.2 – 65.0)
Oral sucker width	36 – 65	80 – 96	59.6 (44.2 – 72.0)	56.8 (41.6 – 70.2)
Acetabulum length	19 – 65	48 – 56	36.3 (27.0 – 46.8)	38.3 (31.2 – 47.0)
Acetabulum width	17 – 40	56 – 80	37.1 (27.0 – 42.6)	40.3 (26.0 – 52.0)
Prepharynx length	11 – 65	80 – 96	236.4 (91 – 350)	252.1 (122.2 – 400)
Pharynx length	14 – 54	52 – 56	42.6 (31.2 – 52.0)	46.8 (36.4 – 57.2)
Pharynx width	14 – 40	46 – 48	42.8 (28.6 – 59.8)	43.0 (31.2 – 56.9)
Esophagus length	108	?	69.2 (27.0 – 117.0)	54.9 (13.0 – 132.6)
Seminal vesicle 1 length	36 – 79	96 – 128	74.7 (31.2 – 119.6)	83.7 (41.6 – 113.0)
Seminal vesicle 1width	29 – 90	80 – 112	50.8 (28.6 – 80.6)	60.0 (31.2 – 91.0)
Seminal vesicle 2 length	29 – 72	64 – 88	88.4 (52.0 – 153.4)	90.1 (57.2 – 140.4)
Seminal vesicle 2 width	25 – 79	48 – 56	61.5 (20.8 – 140.2)	73.7 (31.2 – 127.4)
Seminal receptacle length	32 – 72	92 – 112	87.0 (46.8 – 128.2)	98.1 (59.8 – 132.6)
Seminal receptacle width	25 – 72	90 – 110	71.7 (44.2 – 120.5)	84.9 (46.8 – 148.2)
Ovary length	25 – 76	96 – 112	93.9 (52 – 135.2)	101.4 (75.4 – 130.0)
Ovary width	36 – 90	96 – 128	103.8 (49.4 – 140.4)	105.5 (62.4 – 135.2)
Testis length	72 – 126	250 – 264	202.5 (124.8 – 280)	207.8(111.8 – 265)
Testis width	65 – 252	248 – 304	201.1 (130.0 – 270)	206.7 (101.4 – 266)
Egg length	30.6 – 37.7	36 – 38	28.0 (25.0 – 31.2)	28.1 (25.0 – 31.2)
Egg width	17 – 21.4	20	17.7 (15.0 – 19.5)	17.7 (15.0 – 19.5)

( ) = average value

H. mehrai associated with the similar morphology (three groups of acetabular spines).

In conclusion *Haplorchoides mehrai* metacercariae were found on the inner side of body scales and in the muscle of the cyprinoid fish, *Barbonymus schwanenfeldii* and *Cyclocheilichthys repasson* from Chom Thong district Chiang Mai province, Thailand. Both the prevalence and intensity of infection was high. Therefore, this is a high-risk area for *H. mehrai* infection in freshwater animals. This study revealed new records of both *H. mehrai* metacercaria (from *B. schwanenfeldii* and *C. repasson)* and adult stage (from *Hemibagrus nemurus* and *Mystus multiradiatus)* in Chiang Mai province, Northern Thailand.

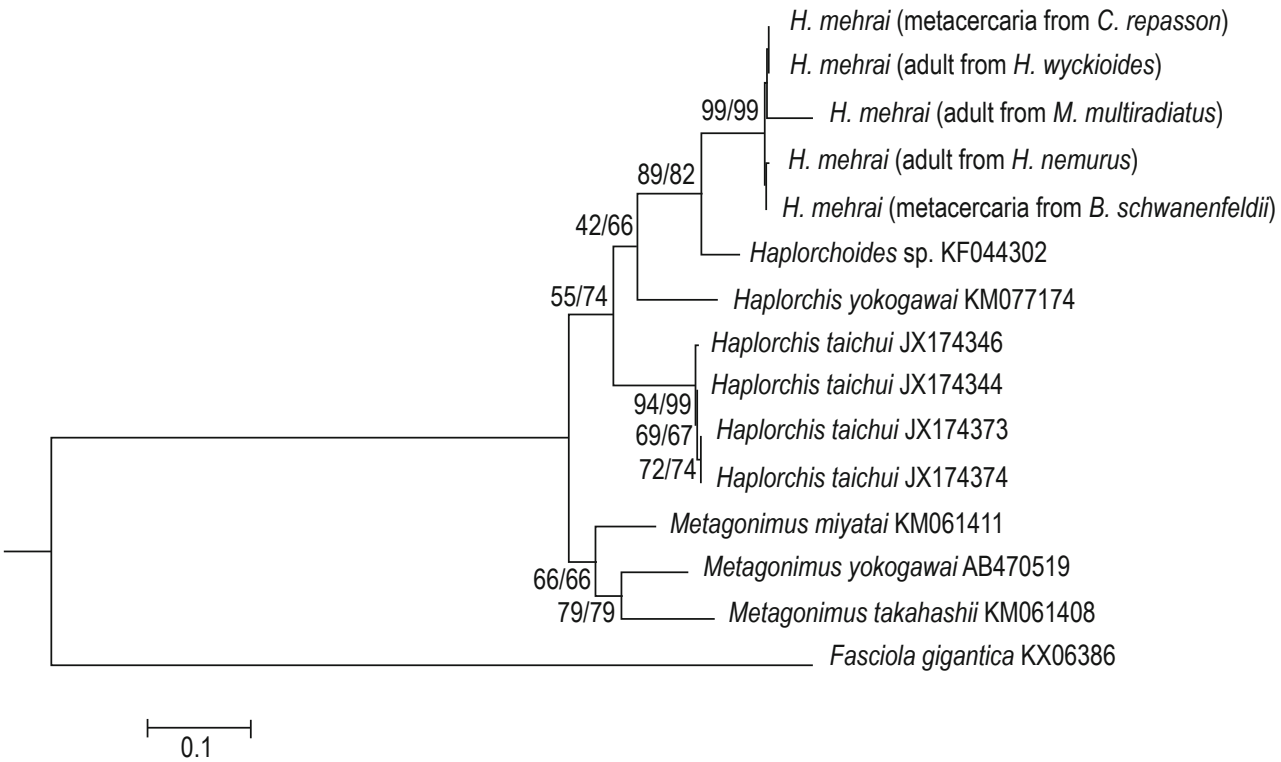

Fig. 5. Phylogenetic tree of *Haplorchoides* spp. and related groups constructed using Neighbor joining (NJ) and Maximum likelihood (ML) (Tamura-Nei model for ML method) analysis of COI gene, with 1,000 bootstrap replicates. Statistic support values for individual nodes are shown on the tree (based on NJ/ML method).

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

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