MOLECULAR CHARACTERIZATION OF FISH-BORNE TREMATODE METACERCARIAE INFECTING FRESHWATER FISH IN RICE FIELD OF CENTRAL THAILAND

Saiwasan Buathong[1], Bonnie Lee Webster[2], Saovanee Leelayoova[3],
Mathirut Mungthin[3], Toon Ruang-areerate[3], Tawee Naaglor[3], Paanjit Taamasri[3],
Picha Suwannahitatorn[3], Sommai Janekitkarn[4] and Peerapan Tan-ariya[1]

[1]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand; [2]Parasite and Vectors Division, Wolfson Wellcome Biomedical Laboratories, Life Sciences, The Natural History Museum, London, UK; [3]Department of Parasitology, Phramongkutklao College of Medicine; [4]Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand

Abstract. Fish-borne trematode (FBT) infection is the major parasitic infection in Asia. In Thailand, a high prevalence of FBT infection in humans has been reported in the north and northeast regions; however, there is no study on the distribution and prevalence of FBT infection in the central part of the country. Hence, we identified trematode metacercariae in freshwater fish from central Thailand. From rice fields of three villages in a rural area of central Thailand, 218 freshwater fish were morphologically identified and their tissues digested to isolate metacercariae. Morphologically identical metacercariae from each fish were pooled in groups of 2-3 and their species and phylogenetic relationships were determined using DNA sequences of nuclear ITS2 and mitochondrial *cox1*. Eleven fish were infected with, in all, 60 metacercariae and from 20 pooled samples, fish from Na Ngam Village were found to be infected with metacercariae closely related to *Haplorchis taichui* and two unidentified genotypes, and fish from Na Yao Village were infected with NayaoMeta1 (12 metacercariae) and NayaoMeta2 (17 metacercariae). More studies on the prevalence of these infections are required to assess whether the FBT infections have an impact on human health in these areas of Thailand.

Keywords: *cox1*, cyprinoid fish, fish-borne trematode, ITS2 nucleotide sequence, metacercariae, Thailand

INTRODUCTION

Fish-borne trematodes (FBT) infections are a public health problem worldwide (Chai *et al*, 2005). Approximately 600 million people are at risk of FBT infections (Chai *et al*, 2005), with more than 40 million people infected annually (WHO, 2002; Tantrawatpan *et al*, 2014). FBT infections constitute an important group of neglected tropical diseases (NTDs) caused by digenetic trematodes.

Thailand is an endemic area for FBT especially in the north and northeast

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Correspondence: Professor Peerapan Tan-ariya, Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand.
Tel: +66 (0) 2201 5524; Fax:+66 (0) 2644 5411
E-mail: peerapan.tan@mahidol.ac.th

regions where local people still consume undercooked infected fish (Nithikathkul and Wongsawad, 2008; Krailas *et al*, 2016; Onsurathumab *et al*, 2016). The most common FBT in Thailand belongs to three taxonomic families, namely, Echinostomatidae (medium intestinal fluke), Heterophyidae (minute intestinal fluke) and Opisthorchiidae (human liver fluke) (Keiser and Utzinger, 2009). They all have complex life cycles involving two intermediate hosts, fresh water snails and fish. Humans, dogs and cats are recorded as the main definitive hosts and become infected through consuming raw and/or undercooked fish that contain trematode metacercariae (Chai *et al*, 2005; Dung *et al*, 2007; Andrews *et al*, 2008; Skov *et al*; 2009). About 11 species of the family Heterophyidae and three species of the family Echinostomatidae have been identified as human and animal intestinal flukes (Chai *et al*, 2005; Sorvillo, 2008; Sohn, 2009; Noikong *et al*, 2014). *Haplorchis taichui* and *Haplorchis pumilio* are the most common small intestinal flukes frequently reported in humans from northern Thailand and Vietnam (Kaewkes *et al*, 1991; Dung *et al*, 2007; Krailas *et al*, 2016).

FBT metacercariae have been identified in various species of freshwater fish collected in rice fields in Vietnam as well as in water reservoirs in Lao PDR, namely, *Centrocestus formosanus, H. pumilio, H. taichui, Opisthorchis viverrini*, and *Stellantchasmus falcatus* (Rim *et al*, 2008). Metacercariae of *Clonorchis sinensis* have been found in freshwater cyprinoid fish in southern China, of which 132 species of freshwater fish serve as the second intermediate hosts (Lun *et al*,2005). In addition, metacercariae of *O. viverrini* in cyprinoid fish have been reported in Cambodia (Touch *et al*, 2009), and Thailand (Waikagul, 1998). In Korea, FBT metacercariae were identified as *Centrocestus armatus, C. sinensis, Echinostoma hortense* and *Metagonimus* spp (Sohn *et al*, 2015).

In Thailand, *O. viverrini* metacercariae are commonly detected in *Cyclocheilichthys, Puntius* and *Hampala* cypriniod fish (Vichasri *et al*, 1982; Srisawangwong *et al*, 1997; Waikagul, 1998). However, in northern Thailand, freshwater fish in water reservoirs are mostly infected with *H. taichui* and *Haplorchoides* spp. These findings show the prevalence of FBT in fish constitutes a significant food-borne infection among people in Thailand (and elsewhere) who prefer consuming raw or inadequately cooked fish.

Molecular tools having high sensitivity and specificity are commonly employed in the identification of FBT at different life-cycle stages, including adult worm, egg, sporocyst, cercaria, and metacercaria (Duenngai *et al*, 2008; Sato *et al*, 2009; Traub *et al*, 2009; Kaewkong *et al*, 2013). The most commonly used molecular markers used to identify FBT are the internal transcribed spacer (ITS) region of ribosomal DNA and mitochondrial genes cytochrome oxidase subunit one *(cox1)* and NADH dehydrogenase subunit one *(nad1)* (Wongratanacheewin *et al*, 2001; Le *et al*; 2006, Sithithaworn *et al*, 2007; Thaenkham *et al*, 2007; Duenngai *et al*, 2008; Lovis *et al*, 2009; Sato *et al*, 2009; Wongsawad and Wongsawad, 2012).

In Thailand, most studies have focused on human FBT infections in northern and northeastern regions where the prevalence is highest (15.7% and 19.5%, respectively) (Sripa *et al*, 2010). Fewer studies have focused on human FBT infections in central Thailand where prevalence ranges from 3.8% to 21.3% (Jongsuksuntigul and Imsomboon, 2003). This study focused on the determination of prevalence of secondary intermediate host, fresh water fish, of FBT in the central Thailand and the identification of infective FBT metacercariae. The results will contribute to the development of prevention and control programs of FBT infection in this region of the country.

MATERIALS AND METHODS

Study area

Freshwater fish were collected from rice fields of three villages (Na Yao, Na Ngam and Thung Heang) in a rural area of Sanam Chai Khet District (latitude is 13° 37′ 34″ N and longitude is 101° 39′ 17″ E), Chacheongsao Province, central Thailand, located 228 km east of Bangkok. Because most villagers are farmers, during working hours they always prepare their lunch meals using uncooked freshwater fish caught from the rice fields. In this area, rice fields are flooded and contain free standing water during the rainy season until a few weeks before harvesting.

Freshwater fish and metacercariae collection

In total, 218 freshwater fish were collected using fishing nets in September 2014 and February 2015. Fish were collected in rice fields nearby homes. Identification of fish species was conducted according to Nelson (2006). In brief, whole body of fish including gills and fins was sliced to approximately 200-mg pieces and each piece was digested in 1 ml aliquot of digestion solution [1 ml of 12.1 M HCl, 1 g of pepsin, 99 ml of 0.85% (w/v) NaCl] at 37 ºC for 1-2 hours (Nithikathkul and Wongsawad, 2008; Kim *et al*, 2013). One ml aliquot of the digest was placed in a 96-well flat bottom plate (Thermo Fisher Scientific, Waltham, MA) and metacercariae observed under an inverted microscope (10x magnification). Morphologically identical metacercariae were pooled in groups of two to three, washed twice with phosphate-buffered saline solution pH 7.2 (Sigma-Aldrich, St Louis, MO), centrifuged at 8,000*g* for 1 minute and stored at -20 ºC until used.

PCR assay

Stored metacercariae were homogenized by repeated passage through a microfuge pipette tip and DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). PCR assay employed 5 primer sets (Table 1). Reaction mixture (50 μl) contained DNA template, 12.5 pmol of each primer, 200 μM dNTPs, 2 mM $MgCl_2$, 1X PCR buffer (Kapa Biosystems, Wilmington, MA), and 1 U KAPA2GTM Robust Hotstart polymerase (Kapa Biosystems). Thermocycling was performed in Mastercycle® personal (Bio-Rad, Hercules, CA) as follows: 95 ºC for 5 minutes; 40 cycles of 95 ºC for 30 seconds, annealing temperature (see Table 1) for 30 seconds and 72°C for 60 seconds; with a final step of 72°C for 7 minutes. Amplicons were separated by 1.5% agarose gel-electrophoresis, stained with SYBR® safe DNA Gel Stain (Invitrogen, Waltham, MA), purified using QIAquick PCR Purification Kit (Qiagen) and directly sequenced in both directions (1st BASE and BST Scientific, Singapore). Nucleotide sequences of ITS2 and *cox1* were independently aligned using BioEdit version 7.1.9 (Hall, 2013) and Crimson Editor version 3.72 (The Emerald Editor Community, 2008), then analyzed using NCBI BLAST search (Altschul *et al*, 1990) for species identification. Sequences were deposited at GenBank with accession numbers indicated in Fig 1.

Phylogenetic tree construction

Phylogenetic relationships of ITS2 and *cox1* sequences were constructed based on maximum likelihood estimation (1,000 bootstrap replications) using

Table 1
Primers used in the study

Primer	Sequence	Target gene	Reference	Annealing temperature	Amplicon size (bp)
Uni18S	Uni18s_F: 5′-GCTTGTCTCAGATTAAGCC-3 Uni18s_R: 5-ACGGAAACCTTGTTACGA-3	Trematode 18S rDNA (SSU)	Dzikowski *et al* (2004)	50 °C	360
28S	LSU5: 5-TAGGTCGACCCGCTGAAYTTAAGCA-3 1500R: 5-GCTATCCTGAGGGAAACTTCG-3	Trematode 28S rDNA (LSU)	Littlewood *et al* (2000), Tkach *et al* (2003)	56 °C	1,290
Asmit	Asmit1: 5-TTTTTTGGGCATCTGAGGTTTAT-3 Asmit2: 5-TAAAGAAAGAACATAATGAAAATG-3	Tremarode *cox1*	Stothard and Rollinson (1997)	45 °C	437 and 970
ITS-2	ITS2-F: 5-CTTGAACGCACATTGCGGCCATGGG-3 ITS2-R: 5-GCGGGTAATCACGTCTGAGCCGAGG-3	ITS2 *Opisthorchis*-like egg	Sato *et al* (2009)	60 °C	380 and 530
RTFluke	RTFlukeFa: 5′-CTTGAACGCACATTGCGGCC-3′ RTFlukeRa: 5′-CACGTTTGAGCCGAGGTCAG-3′.	ITS2 *Opisthorchis*-like egg	Traub *et al* (2009)	60 °C	380 and 529

Seaview Program (Galtier *et al*, 1996). Published ITS2 sequences of *H. taichui* (KJ630831.1), *Heterophyes herophyes* (KU674952.1), *Apophallus donicus* (MF44672.1) and *O. viverrini* (AY584735.1) from GenBank database were included in the alignment and analysis. In addition, *cox1* sequences of *H. taichui* (KF214770.1), Opecoelidae gen. sp. (FJ765503.1) and *O. viverrini* (JF739555.1) from GenBank database were used in the analysis.

RESULTS

Prevalence of FBT infection in freshwater fish of central Thailand

Among the 218 freshwater fish collected from the rice fields in central Thailand, six families (Ambassseidae, Anabantidae, Bagridae, Cyprinidae, Nandidae, and Osphronomidae) consisting of 20 species were identified, among which 11 (five species family Cyprinidae) were infected with trematode metacercariae (n = 201) (Table 2). Size of fish collected ranged 6-13 cm in length. Prevalence of FBT infection was 5%. In all, 55 pooled morphologically identical metacercariae specimens were obtained, 13, 20 and 22 pooled samples from Na Yao, Na Ngam and Thoong Heang Village, respectively (Table 3).

PCR-based identification of pooled metacercaria samples

Using an array of five primer pairs PCR amplicons were successfully generated from 20, 12 and 0 pooled (2-3 metacercariae) samples isolated from freshwater fish of Na Ngam, Na Yao and Thoong Heang village, respectively (Table 3). All

Table 2
Numbers of water-borne trematode infected freshwater fish species and PCR-based identified metacercaria species collected from rice fields of Na Ngam, Na Yao and Thung Heang Villages, Sanam Chai Khet District, Chacheongsao Province, central Thailand, September 2014 and February 2015.

Family	Species	Number of fish	Number of infected fish	Number of metacercariae per fish species	Number of metacercariae used for PCR assay (number of PCR samples)	Metacercaria sp (number of PCR samples)[a]
Cyprinidae	*Barbonymus gonionotus*	13	1	86	60 (20)	*Haplorchis taichui* (20)
			1		8 (3)	NA
			1		6 (2)	NA
Cyprinidae	*Cyclocheilicthys armatus*	25	1	50	21 (7)	NayaoMeta1 (4) NayaoMeta2 (2)
			1		9 (3)	NA
			1		8 (3)	NA
Cyprinidae	*Rasbora paviana*	14	1	20	17 (6)	NayaoMeta1 (2) NayaoMeta2 (4)
Cyprinidae	*Puntius aurotaenia*	22	1	9	9 (3)	NA
Cyprinidae	*Puntius brevis*	64	1	36	12 (4)	NA
			1		8 (3)	NA
			1		5 (2)	NA
	Total	138	11	201	167 (62)	

[a]From Fig 1. NA, negative PCR amplification.

samples from Na Ngam Village produced only the 400-bp ITS2 fragment using primers derived from *Opisthorchis*-like eggs and 12 samples from Na Yao Village only the 437- and 970-bp trematode mitochondrial *cox1* fragments, seven samples producing 437-bp amplicon, three 970-bp amplicon and two both amplicons.

Phylogenetic analysis

A phylogenetic tree constructed using a maximum-likelihood method (1,000 replications) of the 20 ITS2 metacercariae sequences showed strong support (86%) for a monophyletic clade of *H. taichui* (Fig 1A). Whereas, the phylogenetic tree constructed using *cox1* sequences formed two distinct monophyletic clades containing four and six isolates, the former named NayaoMeta1 clade (consisting of NY1, NY2, NY9, and NY10) obtained from two fish and the latter named NayaoMeta2

Table 3
PCR detection of trematode genes from two to three pooled metacercariae of infected freshwater fish collected from rice fields of three villages, Sanam Chai Khet District, Chacheongsao Province, central Thailand, September 2014 and February 2015.

Village	Number of samples	Gene									
		Trematode 18S rDNA (SSU)		Trematode 28S rDNA (LSU)		Trematode *cox1*		*Opisthorchis*-like egg ITS2[a]		*Opisthorchis*-like egg ITS2[b]	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Na Ngam	20	0	20	0	20	-	-	20	0	0	20
Na Yao	13	0	13	0	13	12	1	0	13	0	13
Thung Heang	22	0	22	0	22	0	22	0	22	0	22

[a]ITS-2 primer pair (Table 1). [b]RTFluke primer pair (Table 1). Neg, negative; Pos, positive.

clade (consisting of NY3, NY4, NY5, NY6, NY7, and NY8) collected also from two fish (Fig 1B). NayaoMeta1 clade would appear to be a separate species from that of *H. taichui* and NayaoMeta2 clade was distinct from Opecoelidae gen. sp.

DISCUSSION

In the study region, prevalence among humans of FBT infection (from *Opisthorchis*-like eggs) ranged from 17.4% to 21.3% (Rangsin *et al*, 2009), much higher than the average prevalence (3.8%) in humans of *O. viverrini* in central Thailand (Jongsuksuntigul and Imsomboon, 2003). The occupation of villagers in the study areas involved mainly farming and agriculture. The villagers migrated from the northeast region of the country and have retained the custom of eating undercooked freshwater fish freshly caught from rice fields, local ponds or water reservoirs. Five species of freshwater fish of four genera, namely, *Barbonymus*, *Cyclocheilicthys*, *Puntius* and *Rasbora*, identified in our study have been reported as secondary intermediate host for *O. viverrini* and small intestinal flukes (Sithithaworn *et al*, 1997; Sukontason *et al*, 1999; Sayasone *et al*, 2007; Wongsawad and Wongsawad, 2011).

Pooling fish according to species for digestion in an initial screening for presence of metacercariae was not performed in the current study, and individual fish was examined to minimize false negative findings stemming from low density of metacercariae infection. Although fish were collected at two periods, June and September, considered months of high prevalence (Sithithaworn *et al*, 1997; Sayasone *et al*, 2007), only 5% of the freshwater fish were infected with metacercariae. Clearly further studies involving larger areas of central Thailand are required.

Although pooled 2-3 morphologically identical metacercariae were employed to increase PCR sensitivity, negative amplification results were still obtained despite using five primer pairs. In particular, no amplifications were achieved with 22 pooled metacercariae samples from Thung Heang Village. Reasons for these negative

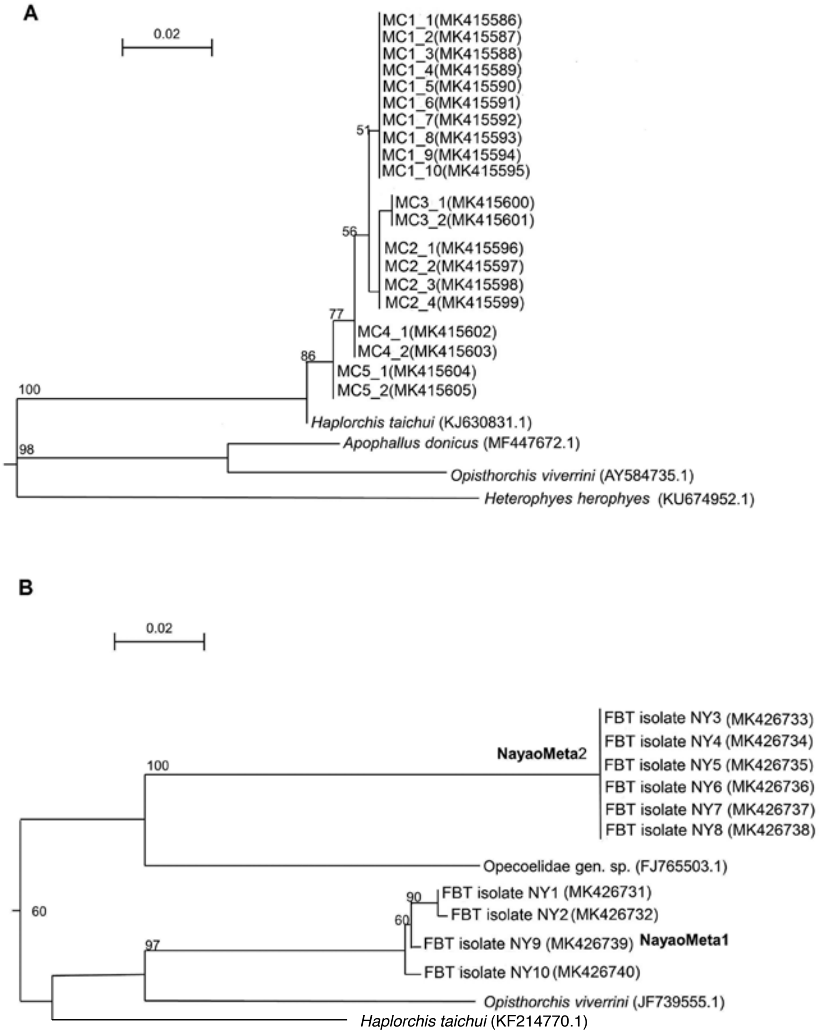

Fig 1-Maximum likelihood phylogenetic trees based on ITS2 (A) and *cox1* sequences (B) of metacercariae from infected freshwater fish collected from rice fields of central Thailand, September 2014 and February 2015. Trees were constructed from partial gene fragments and *Opisthorchis viverrini* was used as an outgroup. Percent similarity (1,000 bootstrap replicates) is shown at branch node. (A) ITS2 sequences (MC1-5, $n = 20$) and 4 reference species of fish-borne tematodes. (B) *cox1* sequences (NayaoMeta1, $n = 4$ and NayaoMeta2, $n = 6$) and 3 reference species of fish-borne tematodes. GenBank accession number is shown in parenthesis. Scale bar indicates number of nucleotide substitutions per site. FBT, fish-borne trematode.

results need further studies. Nevertheless, FBT metacercariae were identified as *H. taichui* and two new genotypes, named NayaoMeta1 and NayaoMeta2.

H. taichui is the most common small intestinal fluke found in northern Thailand and Lao PDR (Sukontason *et al*, 1999; Noikong *et al*, 2014; Sohn *et al*, 2014; Sato *et al*, 2015) but we now demonstrate the presence of *H. taichui* in freshwater fish from central Thailand. The fish were collected from rice fields, which could possibly have been contaminated with fish-eating wild bird feces, thus heterophyid eggs were mostly identified in this study area (Chai and Jung, 2005; Wongsawad *et al*, 2013). However, in this study, no *O. viverrini* metacercariae were found among the fish collected. Thus, other collection sites including ponds and canals located nearby the villages should be surveyed for presence of heterophyid and opisthorchiid metacercariae.

Mitochondrial *cox1* provides an informative molecular marker for investigations of genetic diversity, intra- and inter-species relationships and characterization of *Fasciola* spp (Martinez-Valladares and Rojo-Vazquez, 2014), *O. viverrini* (Tesana *et al*, 2007) and *Paragonimus* spp (Ryu *et al*, 2000). In order to confirm the discovery the two new genotypes, amplifications of 18S and 28S rDNA region are necessary, but were unsuccessful. Further studies using other DNA markers, *eg nad1* and microsatellites need to be conducted. In addition, we also used the morphology of metacercariae as one of the criteria to identify FBT; the morphology of metacercariae looked similar only to those of Opecoelidae gen. sp. and *H. taichui*. Because the prevalence of FBT infection remains high in Thailand, further molecular investigations of FBT in freshwater snails and fish are required to elucidate any links or effects with human or animal health.

This study reports a 5% prevalence of fish-borne trematode metacercariae in freshwater fish collected from three-villages in a rural community in central Thailand. Three distinct genotypes of fish-borne trematodes were detected, one closely related to *H. taichui* and two new genotypes, NayaoMeta1 and NayaoMeta2, closely related to Opecoelidae gen. sp. based on phylogenetic analysis of *Opisthorchis*-like egg ITS2 and trematode *cox1* fragments. This is the first report of potential zoonotic trematode metacercariae in freshwater fish in central Thailand and warrants further studies on their life cycle, intermediate host specificities and threat to humans and animals.

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