ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 51, No. 6: 677-681, December 2013
http://dx.doi.org/10.3347/kjp.2013.51.6.677

▣ ORIGINAL ARTICLE

Molecular Variation in the *Paragonimus heterotremus* Complex in Thailand and Myanmar

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Abstract: Paragonimiasis is an important food-borne parasitic zoonosis caused by infection with lung flukes of the genus *Paragonimus*. Of the 7 members of the genus known in Thailand until recently, only *P. heterotremus* has been confirmed as causing human disease. An 8th species, *P. pseudoheterotremus*, has recently been proposed from Thailand, and has been found in humans. Molecular data place this species as a sister species to *P. heterotremus*, and it is likely that *P. pseudoheterotremus* is not specifically distinct from *P. heterotremus*. In this study, we collected metacercariae of both nominal species (identification based on metacercarial morphology) from freshwater crabs from Phetchabun Province in northern Thailand, Saraburi Province in central Thailand, and Surat Thani Province in southern Thailand. In addition, we purchased freshwater crabs imported from Myanmar at Myawaddy Province, western Thailand, close to the Myanmar-Thailand border. The DNAs extracted from excysted metacercariae were PCR-amplified and sequenced for ITS2 and *cox1* genes. The ITS2 sequences were nearly identical among all samples (99-100%). Phylogenies inferred from all available partial *cox1* sequences contained several clusters. Sequences from Indian *P. heterotremus* formed a sister group to sequences from *P. pseudoheterotremus*-type metacercariae. Sequences of *P. heterotremus* from Thailand, Vietnam, and China formed a separate distinct clade. One metacercaria from Phitsanulok Province was distinct from all others. There is clearly considerable genetic variation in the *P. heterotremus* complex in Thailand and the form referred to as *P. pseudoheterotremus* is widely distributed in Thailand and the Thai-Myanmar border region.

Key words: *Paragonimus heterotremus*, *Paragonimus pseudoheterotremus*, molecular epidemiology, internal transcribed spacer 2 (ITS2), cytochrome *c* oxidase subunit 1 (*cox1*)

INTRODUCTION

Paragonimiasis is an important food-borne zoonosis, especially in Asian countries, caused by infection with lung flukes of the genus *Paragonimus*. More than 50 species of *Paragonimus* have been described from Asia, America, and Africa [1]. Seven species, namely, *P. heterotremus*, *P. westermani*, *P. siamensis*, *P. bangkokensis*, *P. harinasutai*, *P. paishuihoensis*, and *P. macrorchis* - have been reported from Thailand. Of these, only *P. heterotremus* was regarded until recently as causing human disease in Thailand [2,3], as it also does in India [4-6], Vietnam [7-9], and Laos [10]. Recently, an 8th nominal species, *P. pseudoheterotremus*, was described from Thailand [11], and human pulmonary paragonimiasis caused by this species has been identified using DNA sequence data of the internal transcribed spacer 2 (ITS2) and cytochrome c oxidase subunit 1 (*cox1*) from eggs in the sputum of a patient [12]. This species was distinguished from *P. heterotremus* by the small size of its metacercariae and by details of adult surface spination. *P. pseudoheterotremus* appears, on the basis of DNA sequence data, to be a very close sister species to *P. heterotremus*. In Vietnam, metacercariae resembling those of both of these species have been found, but cannot be separated using molecular data (Doanh, pers. comm.). *P. pseudoheterotremus* may thus not be specifically distinct from *P. heterotremus*. However, both names will be used in this paper, simply to indicate the size differences in metacercariae.

The discovery of a human case attributed to *P. pseudohetero-*

• Received 30 April 2013, revised 3 August 2013, accepted 11 October 2013.
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tremus [12] prompted us to study the distributions and relationships of metacercariae of *P. heterotremus* and *P. pseudoheterotremus* types in freshwater crabs in Thailand. Moreover, neither of these types were reported from Myanmar, which shares a long border with Thailand. Here, we report ITS2 and *cox1* gene sequences of metacercariae of *P. heterotremus* and *P. pseudoheterotremus* types from several regions in Thailand and an area in Myanmar, and place these in a phylogenetic context using new and published sequences.

MATERIALS AND METHODS

Collection of *Paragonimus* metacercariae

Paragonimus metacercariae (Fig. 1) were collected from naturally infected freshwater crabs which were collected in 4 provinces of Thailand (Fig. 2); Phitsanulok Province and Phetchabun Province in the north, Saraburi Province in the center, and Surat Thani Province in the south of Thailand. In addition, crabs were also collected in Myawaddy Province, Myanmar, bordering western Thailand. The crabs were digested with artificial gastric juice (1% pepsin in HCl solution). Digested samples were filtered through wire sieves (pore size; 300 and 500 μm), and the filtrate was allowed to stand for sedimentation. The sediments were examined under a stereomicroscope. The metacercariae found were excysted using a method previously described [13]. Each freshly excysted *Paragonimus* metacercaria was morphologically identified using microscopy before DNA extraction.

Fig. 2. Locations of provinces in Thailand and Myanmar from which freshwater crabs were sampled for this study. Myawaddy: Myanmar (1; present study), Thailand: Phitsanulok (2; present study), Phetchabun (3; present study), Saraburi (4; present study and Thaenkham and Waikagul, 2008), and Surat Thani (5; present study). In addition, provinces from which *P. pseudoheterotremus* has been reported in previous studies are shown: Kanchanaburi (6; Thaenkham and Waikagul, 2008) and Loei (7; Intapan et al., 2012).

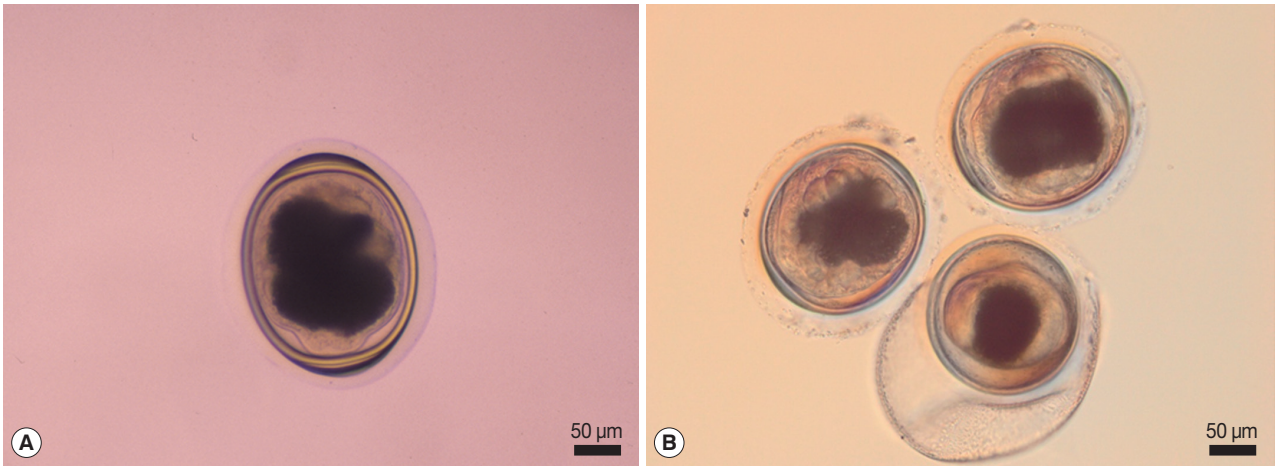

Fig. 1. Representative metacercariae morphologically resembling those of *P. heterotremus* (A) and *P. pseudoheterotremus* (B). The *P. heterotremus*-type metacercaria (A) was collected in Phisanulok Province, northern Thailand, whereas the *P. pseudoheterotremus*-type metacercariae (B) were collected in Surat Thani Province, southern Thailand.

DNA extraction

To extract genomic DNA, each excysted *Paragonimus* metacercaria was homogenized separately with a disposable polypropylene pestle (Bellco Glass Inc., Vineland, New Jersy, USA). Then, DNA was extracted using a NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co., Düren, Germany). The DNA was eluted in 100 µl of distilled water and stored at -20℃ until used.

PCR, sequencing, and molecular phylogenetic analysis

The ITS2 sequence was amplified using primers PhITS2-F (5′-CTG TGT GAA TTA ATG TGA ACT GC-3′) and PhITS2-R (5′-AGT GAT ATG CTT AAG TTC AGC G-3′) [12], which were designed from the known ITS2 sequence of *P. heterotremus* from northeastern India (GenBank accession no. AB308377). A fragment of the *cox1* gene was amplified using primers, PphCOI-F (5′-CCG GGT TTG GTG TTG TG-3′) and PphCOI-R (5′-ACA ACG AAC CAA GTG TCA TG-3′) [12], which were designed from a region of *P. pseudoheterotremus cox1* gene (GenBank accession no. EF446315).

PCR was conducted using a GeneAmp® PCR System 9700 (Applied Biosystems, Singapore). The reaction was carried out in a 25 µl volume containing PCR 1× FastStart High Fidelity Reaction Buffer (Roche Applied Science, Mannheim, Germany), 1.8 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, 0.2 µM of each primer, and 0.625 units of FastStart High Fidelity Enzyme Blend (Roche Applied Science). The DNA template was initially denatured at 94℃ for 5 min. The amplification procedure comprised 35 cycles at 95℃ for 30 sec (denaturation), 55℃ for 30 sec (annealing), and 72℃ for 30 sec (extension), with a final extension at 72℃ for 10 min. The amplified product was subjected to electrophoresis on a 1.5% agarose gel. The PCR product was then cut and purified for DNA sequencing, which was performed using the MegaBACE 1,000 DNA Analysis System (GE Healthcare, Piscataway, New Jersy, USA).

The partial ITS2 and *cox1* gene sequences of individual *Paragonimus* metacercariae from each locality were deposited in GenBank under accession numbers presented in Table 1. They were analyzed using the BLAST-N search (National Center for Biotechnology Information, Bethesda, Maryland, USA). Published *cox1* sequences from *P. heterotremus* and *P. pseudoheterotremus* were aligned with our new sequences (alignment length 309 bp when trimmed to the length of the shortest sequence) using ClustalW [14] and a maximum likelihood tree constructed using MEGA 5.2 [15]. The best-fit substitution model was determined in MEGA to be the Hasegawa-Kishino-Yano (HKY) model with uniform rates among sites, but assuming a proportion (0.65) of invariant sites. Sequences from *P. westermani* were used to provide an outgroup.

Table 1. Accession numbers for new *Paragonimus* sequences deposited in GenBank

Country (Province)	*cox 1*		ITS2	
	Sequence ID	GenBank No.	Sequence ID	GenBank No.
Myanmar: (Myawaddy, MW)	PhCO1MW4	KC859926	PhI2MW4	KC894640
	PphCO1MW5	KC859936	PphI2MW5	KC894649
	PphCO1MW6	KC859937	PphI2MW6	KC894650
Thailand: (Phitsanulok, PL)	PhCO1PL2	KC859931	PhI2PL2	KC894644
Thailand: (Phetchabun, PB)	PhCO1PB3	KC859927	PhI2PB3	KC894641
Thailand: (Saraburi, SB)	PhCO1SB1	KC859933	PhI2SB1	KC894646
	PhCO1SB3	KC859935	PhI2SB3	KC894648
Thailand: (Surat Thani, ST)	PphCO1ST1	KC859938	PphI2ST1	KC894651
	PphCO1ST2	KC859939	PphI2ST2	KC894652
	PphCO1ST3	KC859940	PphI2ST3	KC894653
	PphCO1ST4	KC859941	PphI2ST4	KC894654
	PphCO1ST5	KC859942	PphI2ST5	KC894655
	PphCO1ST6	KC859943	PphI2ST6	KC894656
	PphCO1ST7	KC859944	PphI2ST7	KC894657
	PphCO1ST8	KC859945	PphI2ST8	KC894658
	PphCO1ST9	KC859946	PphI2ST9	KC894659

RESULTS

The partial ITS2 sequences of all metacercariae from differ-

Table 2. Pairwise percentage differences in range (mean) between clusters on the tree

	(2)	(3)	(4)	(5)
P. heterotremus (excl. India) (1)	7.8-9.1 (8.1)	8.7-11 (9.5)	7.8-9.1 (8.3)	13-20 (16.3)
P. heterotremus (India) (2)		4.5-6.5 (5.5)	8.1-9.1 (8.5)	14-20 (16.8)
P. pseudoheterotremus (3)			9.1-10 (9.9)	14-21 (16.7)
P. heterotremus Phitsanulok (4)				16-21 (18.1)
P. westermani (5)				

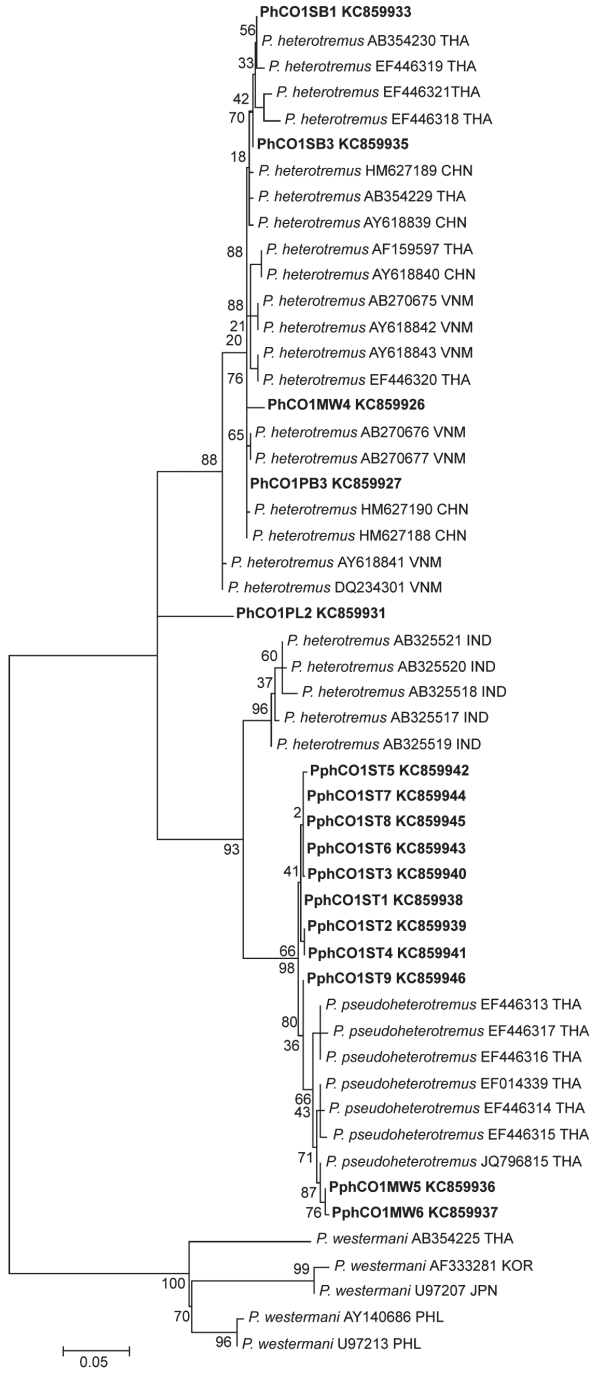

Fig. 3. Maximum likelihood tree based on partial cytochrome *c* oxidase subunit I (*cox1*) gene sequences. Sequences of *P. heterotremus*, *P. pseudoheterotremus*, and *P. westermani* (outgroup) obtained from GenBank are indicated with accession number and country code (ISO 3166-1 alpha-3 codes). *Paragonimus* sequences of this study are presented in bold (KC859926, KC859936, KC859937, KC859931, KC859927, KC859933, KC859935, KC859938-KC859946). The sequences were deposited in GenBank numbers as shown in Table 1.

ent localities in Thailand and Myanmar were almost completely (98-99%) identical. Phylogenies inferred from all available partial *cox1* sequences contained several well-supported clusters (Fig. 3). Sequences from Indian *P. heterotremus* formed a sister group to sequences from *P. pseudoheterotremus*-type metacercariae. Sequences of *P. heterotremus* from Thailand, Vietnam, and China formed a separate distinct clade. A metacercaria from Phitsanulok Province was distinct from all others. Table 2 shows the ranges and means of percentage differences between these clusters, and that *P. heterotremus* from northeastern India are closest to sequences from metacercariae of the *P. pseudoheterotremus*-type from Thailand.

DISCUSSION

In this study, we explored the molecular relationships of metacercariae corresponding morphologically with those described for *P. heterotremus* and *P. pseudoheterotremus*. Samples used were collected in different parts of Thailand including the Thai-Myanmar border area. Metacercariae corresponding to the *P. heterotremus* type were found in northern and central parts of Thailand and Myawaddy Province, Myanmar. Those of the *P. pseudoheterotremus* type were found in Surat Thani Province, Thailand and in Myawaddy Province, Myanmar. Members of this complex are clearly widespread in Thailand and the Thai-Myanmar border region, and this must alert us to possible human infections in those areas. The ITS2 sequences were near-identical among all metacercariae sampled, and with sequences available from GenBank. This reflects the situation seen in the *P. skrjabini* complex, which exhibits far less variation in ITS2 sequences than in mitochondrial genes [16].

Although *P. pseudoheterotremus* was proposed as a species distinct from *P. heterotremus*, the phylogeny in Fig. 3, based on mitochondrial *cox1* sequences, shows that the true situation is complicated. *P. heterotremus* from northeastern India is sister to Thai metacercariae of the *P. pseudoheterotremus* type, and 1 metacercaria from Phitsanulok Province in Thailand is distinct from all others. The considerable variation in *cox1* sequences found in Thailand indicates that further work is required to demonstrate the full diversity of *P. heterotremus* and related forms in Thailand.

ACKNOWLEDGMENTS

This research was funded by grants from the National Science

and Technology Development Agency (Discovery Based Development Grant); the Higher Education Research Promotion National Research University Project of Thailand, Office of the Higher Education Commission through the Health Cluster (SHep-GMS), Thailand; the Faculty of Medicine, Khon Kaen University. Wanchai Maleewong, Pewpan M. Intapan, and Tongjit Thanchomnang are supported by TRF Senior Research Scholar Grant, Thailand Research Fund grant no. RTA5580004. We wish to acknowledge the support of the Khon Kaen University Publication Clinic, Research and Technology Transfer Affairs, Khon Kaen University, for their assistance.

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