RESEARCH NOTE

MOLECULAR PHYLOGENETIC RELATIONSHIP OF *PARAGONIMUS PSEUDOHETEROTREMUS*

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Abstract. A part of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and the nuclear ribosomal DNA second internal transcribed spacer 2 (ITS2) of a newly described lung fluke, *Paragonimus pseudoheterotremus*, were sequenced and compared with *P. heterotremus*, the species with a similar morphology. Pairwise distance of COI sequences revealed a genetic difference between *P. heterotremus* and *P. pseudoheterotremus* with a nucleotide difference of COI sequences between these two species of 10.6%. The constructed phylogenic tree with high bootstrap proportion suggested that *P. pseudoheterotremus* is a sister species of *P. heterotremus.*

INTRODUCTION

The genus *Paragonimus* Braun, 1899 contains more than 40 species, which are divided into two main groups. One group consists of *P. westermani* and *P. siamensis* and the other all the remaining species from Asia and America (Blair *et al*, 1998). The latter group shows *P. skrjabini* and *P. macrorchis* are well separated (Blair *et al*, 1999). *P. skrjabini* is very closely related to *P. miyazakii* from Japan (Blair *et al*, 1997) and this relationship is called *P. skrjabini* complex (Blair *et al*, 2005). *P. hokuoensis* was proposed as a sister species to *P. skrjabini* complex. The *P. ohirai* group consists of *P. iloktsuenensis* and *P. sadoensis*. *P. harinasutai* is closely related to *P. ohirai* (Blair *et al*, 1998). Although *Euparagonimus cenocopiosus* has been recognized as a distinct taxon, it seems that this species should not be placed in a distinct genus as *E. cenocopiosus* is nested among species assigned to the genus *Paragonimus* and *Pagumogonimus* (Blair *et al*, 1999).

Recently, a new species of *Paragonimus* similar to *Paragonimus heterotremus* has been described as *Paragonimus pseudoheterotremus* (Waikagul, 2007). The adult worm is similar to *P. heterotremus* in morphology of the ovary and testes, and in the ratio of suckers and spination. However, it is distinctly different to *P. heterotremus* in the sizes of metacercariae and adults in the definitive hosts. *P. pseudoheterotremus* metacercaria is smaller in size and has a thicker cyst wall. The adult is smaller but the integument spines are bigger. The rat is considered a definitive host, while *P. heterotremus* is unable to become fully mature in the rat (Waikagul, 2007). In this paper, partial cytochrome *c* oxidase subunit I (COI) gene and the nuclear ribosomal DNA second internal transcribed spacer 2 (ITS2) DNA sequences of *P. pseudoheterotremus* were analyzed and compared with *P. heterotremus* to determine the phylogenetic position of *P. pseudoheterotremus* within the genus *Paragonimus*.

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MATERIALS AND METHODS

Metacercariae of *P. pseudoheterotremus* and *P. heterotremus* were recovered from freshwater crabs (*Larnaudia larnaudii*) collected from Kanchanaburi Province, western Thailand and Saraburi Province, central Thailand respectively (Table 1). Fresh metacercariae were individually broken on sterile glass slide with sterile cover slip. The genetic material on the slide was then washed with 5 μl of sterile double distilled water. Genetic material of homogenized metacercariae was added to 5 μl of mineral oil and then boiled at 95ºC for 10 minutes. Genomic DNA was directly used in PCR. Adult worms of *P. pseudoheterotremus* were obtained from the lungs of the experimentally infected rats 2 months post-infection and kept in absolute ethanol at -20ºC until used. Genomic DNA of adult worms was extracted following a tissue protocol (QIAamp DNA Mini kitit, QIAGEN). All animal studies were conducted following ethical clearance from Faculty of Tropical Medicine, Mahidol University.

For PCR amplification of the COI region, the primers used were designed from the complete sequence of mitochondrial DNA (mtDNA) of *P. westermani* in GenBank (NC_002354). Primers for COI amplification were COI-1 (5′ T(CT)T TTG GGC ATC CGG AGG TG 3′) (forward) and COI-2 (5′ AG(AT) GA(CA) A(AG)(GA) AC(GA) TAA TGA AAA TG 3′) (reverse). Primers for ITS2 amplification were 3S (5′ CGG TGG ATC ACT CGG CTC GT 5′) (forward) and A28 (5′ CCT GGT TAG TTT CTT TTC CTC CGC 3′) (reverse) (Bowles *et al*, 1995). Each PCR reaction mixture, in a total volume of

Table 1
Samples used in the study.

Sample code	DNA source	Species	Locality	COI accession number	ITS2 accession number
GBph-A	Adult	*P. heterotremus*	Thailand	AF159597	AF159603
Sph-M1	Metacercaria	*P. heterotremus*	Thailand	EF446318	EF446322
Sph-M2	Metacercaria	*P. heterotremus*	Thailand	EF446319	EF446323
Sph-M3	Metacercaria	*P. heterotremus*	Thailand	EF446320	-
Sph-M4	Metacercaria	*P. heterotremus*	Thailand	EF446321	-
Kpp-A1	Adult	*P. pseudoheterotremus*	Thailand	EF014339	EF014340
Kpp-A2	Adult	*P. pseudoheterotremus*	Thailand	EF446317	-
Kpp-M1	Metacercaria	*P. pseudoheterotremus*	Thailand	EF446313	-
Kpp-M2	Metacercaria	*P. pseudoheterotremus*	Thailand	EF446314	-
Kpp-M3	Metacercaria	*P. pseudoheterotremus*	Thailand	EF446315	-
Kpp-M4	Metacercaria	*P. pseudoheterotremus*	Thailand	EF446316	-
		P. skrjabini	China	AY618806	U9613
		P. kellicotti	USA	-	AF159606
		P. hokuoensis	China	AY618838	-
		P. miyazakii	China	AY618834	AY618742
		P. macrorchis	Thailand	AF159598	AF159608
		P. ohirai	Japan	AF008189	U96911
		P. sadoensis	-	AF008190	-
		P. iloktsuenensis	-	U97205	-
		P. harinasutai	Thailand	AF159600	AF159609
		P. siamensis	Thailand	AF159599	AF159605

50 μl, contained genomic DNA from 5 μl of boiled genetic material, 25 mM $MgCl_2$, 2.5 mM dNTPs, 40 pmol of each primer, and 1 x of *Taq* polymerase buffer and 1 unit of *Taq* polymerase. PCR products were sequenced directly by cycle sequencing method using ABI PRISM 3100 automated sequencer. PCR primers were used as sequencing primers.

DNA alignment analyses were conducted using CLUSTALW program version 1.83 (Thompson *et al*, 1994). Both COI and ITS2 sequences were determined on the transition/ transversion ratio (ti/tv) and percent GC contents within *P. heterotremus* and *P. pseudoheterotremus* and between these two species using MEGA3 program (Kumars *et al*, 2004). Genetic distances were calculated using Felsenstein (1984) (F84) model by DNADIST program in PHYLIP package (Felsenstein, 1993). Both COI and ITS2 nucleotides were assembled in 1,000 replications of pseudosamples using SEQBOOT program in PHYLIP package (Felsenstein, 1993). Neighbor-joining tree (NJ) (Saitou and Nei, 1987) and bootstrap consensus of NJ tree were reconstructed by NEIGHBOR and CONSENSE programs in the PHYLIP package (Felsenstein, 1993) and the bootstrap consensus of maximum parsimony (MP) tree (Stewart, 1993) were reconstructed by using MEGA3 program (Kumars *et al*, 2004). The nucleotide sequences of several species of *Paragonimus* used in this study are listed in Table 1.

RESULTS

The studied COI sequences comprised of 380 nt, and the transition/ transversion (ti/tv) ratio between *P. pseudoheterotremus* and *P. heterotremus* was 5.9 ± 1.2. Percent nucleotide difference between *P. pseudoheterotremus* and *P. heterotremus* was 10.6%. For ITS2 sequences, the percent nucleotide difference between *P. pseudoheterotremus* and *P. heterotremus* was between 0 and 0.3%. The GC content of COI sequence of *P. heterotremus* and *P. pseudoheterotremus* was 39.2 ± 0.3% and 39.9 ± 0.3% respectively, and of ITS2 sequence 54.8% and 54.7% respectively. Genetic distances were estimated using pairwise comparison method. The F84 distance of partial sequence of COI within *P. heterotremus* and *P. pseudoheterotremus* was 0.016±0.010 and 0.0110 ± 0.009 respectively, and of ITS2 sequence 0.0007 ± 0.00140.

The NJ and MP method showed *P. pseudoheterotremus* shares a derived trait with *P. heterotremus* when the partial COI sequence of *P. siamensis* is used as an outgroup (Fig 1). Congruent topology between NJ and MP methods of COI showed *P. heterotremus* and *P. pseudoheterotremus* were not grouped into the same clade at ≥50% of bootstrap proportion value. The NJ and MP methods using ITS2 sequences were also reconstructed (Fig 2), but the congruent phylogenetic tree between the two methods for ITS2 could not reveal any difference in their genetic relationships.

DISCUSSION

The present study using COI and ITS2 sequences revealed the phylogenetic position of *P. pseudoheterotremus* in the genus *Paragonimus*. ITS2 sequence alignment between *P. pseudoheterotremus* and *P. heterotremus* was nearly identical. Percent of nucleotide difference and F84 distance from COI sequences between these species indicated distinct genetic difference between the two. Ti/tv ratio of the two species showed that transition is favored over transversion, especially for *P. pseudoheterotremus.* The congruent phylogenetic trees (between NJ and MP method) from COI data revealed *P. pseudoheterotremus* is separated from *P. heterotremus*.

A small metacercaria very similar to *P. heterotremus* found in Yenbai Province, Vietnam (Doanh *et al*, 2007) was previously

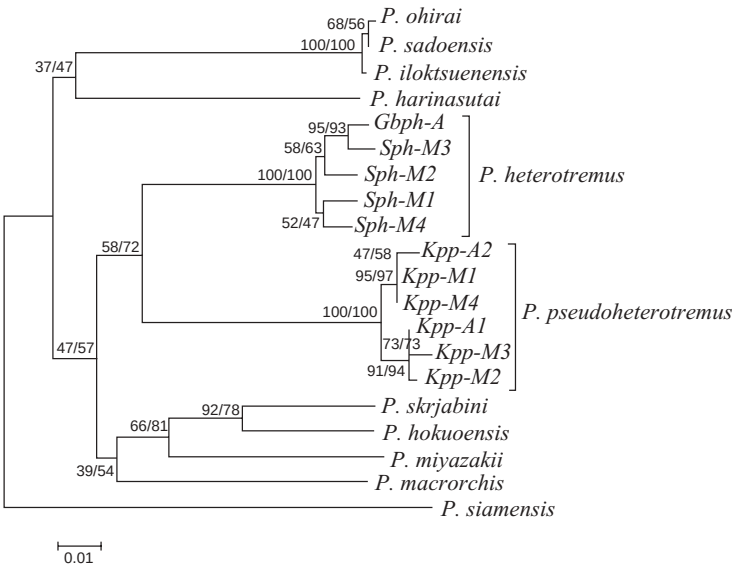

Fig 1–Congruent phylogenetic tree between neighbor-joining and maximum parsimony reconstructed from COI gene sequence. Bootstrap proportions of maximum parsimony neighbor-joining trees are presented in each node. The sample codes of nucleotide sequences are presented in Table 1.

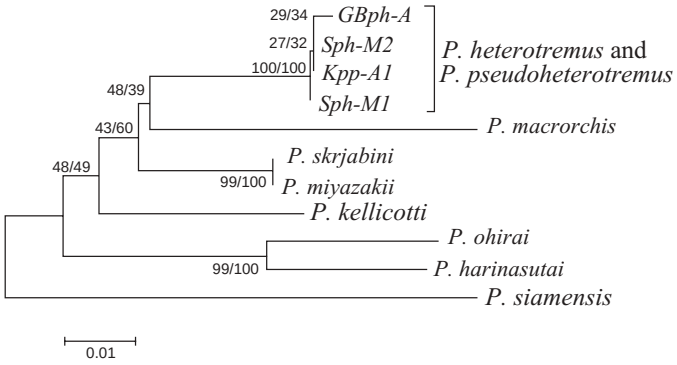

Fig 2–Congruent phylogenetic tree between neighbor-joining and maximum parsimony reconstructed from ITS2 sequence. Bootstrap proportions of maximum parsimony neighbor-joining trees are presented in each node. The sample codes of nucleotide sequences are presented in Table 1.

reported as being *P. heterotremus*. The size of metacercaria was the only morphological difference. The phylogenetic relationship of the small metacercaria was grouped with *P. heterotremus* when using ITS2 sequence, while the relationship between *P. heterotremus* and the small metacercaria could not be placed in a monophyletic group using COI sequence (Doanh *et al*, 2007). Our study showed that the small metacercaria is a closely related species of *P. heterotremus* and is therefore named as *P. pseudoheterotremus*.

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