

# MOLECULAR SYSTEMATICS OF A NEW FORM OF *PARAGONIMUS WESTERMANI* DISCOVERED IN THAILAND

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**Abstract.** This study aimed to clarify evolutionary relationships of *P. westermani*-like with other members of *Paragonimus* in Asia. The parsimony method was employed in molecular analyses of the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA and the partial cytochrome c oxidase subunit I (COI) region of mitochondrial DNA. A single most parsimonious tree obtained from the ITS2 region revealed two important groups within *P. westermani* complex that is based on geographical origins. From this study, it is evident that *P. westermani*-like is either placed well within the *P. westermani* complex or is located close to the complex. Since a significant genetic variation was observed between Thai *P. westermani* and *P. westermani*-like, further investigation on the specificity of first intermediate hosts should be carried out to determine a proper taxonomic status of *P. westermani*-like.

## INTRODUCTION

*Paragonimus westermani* is widely distributed in Asia (Miyazaki, 1991). In Thailand, *P. westermani* metacercariae were reported in the central and southern parts of the country (Miyazaki, 1982; Kawashima *et al*, 1989). During our field survey, a new form of *P. westermani* metacercariae was discovered. The metacercariae obtained were almost identical to *P. westermani* metacercariae, except the size was smaller; thus, they were provisionally named *P. westermani*-like. Studies concerning the morphology of adult worms and susceptibility of feline hosts to *P. westermani*-like carried out by Sugiyama *et al* (2007) indicated that the adult worms resembled a diploid-type *P. westermani*, but the susceptibility in cats differed between *P. westermani* and *P. westermani*-like. This present study aimed to characterize genetically *P. westermani*-like as well as to clarify its phylogenetic relationships with other members of *Paragonimus* in Asia using nucleotide sequences of the ITS2 region of nuclear ribosomal DNA and a portion of the mitochondrial COI gene.

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## MATERIALS AND METHODS

### Parasite materials

Metacercariae of *P. westermani* and *P. westermani*-like were obtained from the waterfall crab, *Phricotelphusa aedes*, which were collected in Phanom District, Surat Thani Province. The metacercariae of other Thai *Paragonimus* species were harvested as follows: *P. bangkokensis* from *Ranguna smalleyi* (Phanom District, Surat Thani Province); *P. harinasutai* and *P. heterotremus* from *Larnaudia larnaudii* (Kaeng Khoi District, Saraburi Province) and *P. siamensis* from *Sayamia germaini* (Na Di District, Prachin Buri Province).

### DNA sequencing and amplification

Total genomic DNA was prepared from individual metacercariae following Sugiyama *et al* (2002). The ITS2 region of nuclear ribosomal DNA and a portion of the mitochondrial COI gene were amplified by PCR and sequenced using primers 3S (forward: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3'; Bowels *et al*, 1995), A28 (reverse: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'; Blair *et al*, 1997), JB3 (forward: 5'-TTTTTTGGGCATCCTGAGGTTTAT-3'; Bowels *et al*, 1995) and JB 4.5 (reverse: 5'-TAAAGAAAGAACATAATGAAAATG-3'; Bowels *et al*, 1995), respectively. The PCR cycle consisted of three major steps: 98° C for 5 seconds to denature DNA, 55° C for 10 seconds

for primer annealing, and 72° C for 10 seconds for primer extension. The cycle was repeated 30 times, followed by a final extension at 72° C for 1 minute.

### Sequence and phylogenetic analyses

Sequence alignments were carried out using Clustal X program (Jeanmougin *et al.*, 1998) with additional sequences of *Paragonimus* species and *Fasciola hepatica* (outgroup) from GenBank database. The GenBank accession numbers of all sequences employed are shown in Table 1. Phylogenetic trees were reconstructed using maximum parsimony analysis with a branch-and-bound algorithm. Alignment gaps were treated as missing data; all characters were assigned equal weight. The reliability of internal branches of the trees was assessed using the bootstrap method (Felsenstein, 1985), with 1,000 replicates. All phylogenetic analyses were performed using PAUP\* version 4.0b (Swofford, 1998).

## RESULTS

Metacercariae of *Paragonimus* species employed in this study were shown in Fig 1.

### Sequence characteristics

**ITS2.** The actual length range of the ITS2 region of the ingroup was 359-363 bp. The alignment of this region of 15 taxa of *Paragonimus* species and its outgroup was 378 bp in length, with 10 sites of insertion or deletion. Out of 378 total characters, 230 (60.8%) were constant, 92 (24.4%) were parsimony-uninformative and 56 (14.8%) were parsimony-informative. Sequence divergence between ingroup and outgroup taxa obtained from pairwise distance analysis ranged from 37.6-41.9% but within the ingroup the sequence divergence range was 0-13.7%. The mean G+C content of all taxa was 55.5%, and transition/transversion ratio was 2.60.

**COI.** The actual length of the partial COI region of the ingroup was 381 bp. The alignment of this region for all 15 taxa under study was 384-bases long, with only one site of deletion. From 384 characters, 241 (62.8%) were constant, 38 (9.9%) were parsimony-uninformative, and 105 (27.3%) were parsimony-informative. The sequence divergence between ingroup and outgroup taxa was computed using pairwise distance analysis, and ranged from 22.2-34.7%;

Table 1  
GenBank accession numbers of *Paragonimus* and *Fasciola* used in this study.

Species	Origin	ITS2	COI
<i>P. westermani</i>	Hyogo, Japan	U96907	U97205
	Minchin, China	U96907 <sup>a</sup>	AY140681
	Haenam, Korea	AF333278	AF333281
	Karapai, Taiwan	U96908	AY140673
	Philippines	U96910	U97213
	Malaysia	U96909	U97211
	Central Thailand	AF159604	U97212
	Southern Thailand	AB354216	AB354224
	Thailand	AB354218	AB354225
<i>P. westermani</i> -like	Thailand	AF159608	AF159598
<i>P. macrorchis</i>	Thailand	AB354221	AB354229
<i>P. heterotremus</i>	Thailand	AB354220	AB354226
<i>P. harinasutai</i>	Thailand	AB248091	Ab354228
<i>P. bangkokensis</i>	Thailand	AB354222	AB354231
<i>P. siamensis</i>	Australia	AB207148	AF216697
<i>Fasciola hepatica</i>			

<sup>a</sup>sequence identical to *P. westermani* from Hyogo, Japan (Blair *et al.*, 1997).

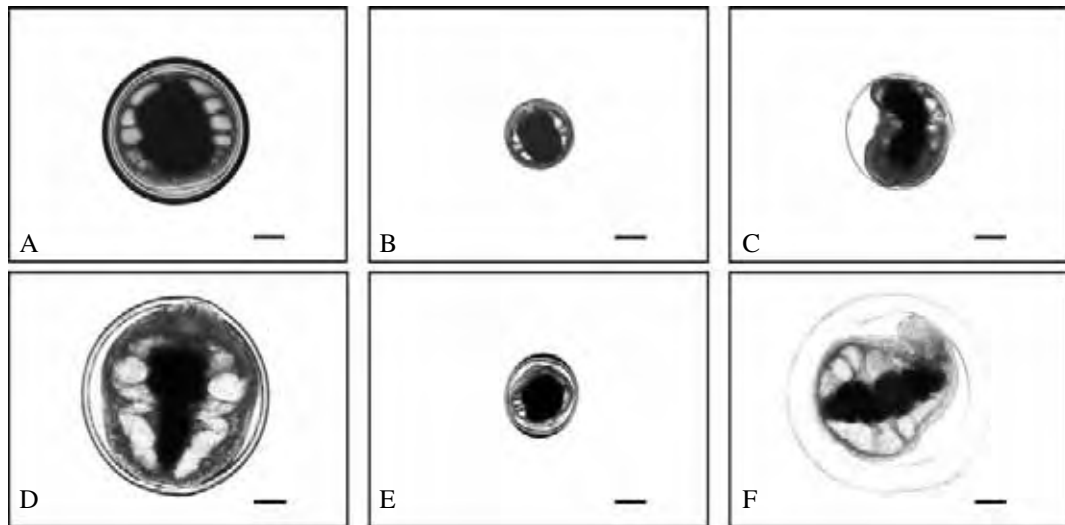


Fig 1- Metacercariae of *Paragonimus* species A: *P. westermani*, B: *P. westermani*-like, C: *P. bangkokensis*, D: *P. harinasutai*, E: *P. heterotremus* and F: *P. siamensis*. Scale bar indicates 100  $\mu$ m.

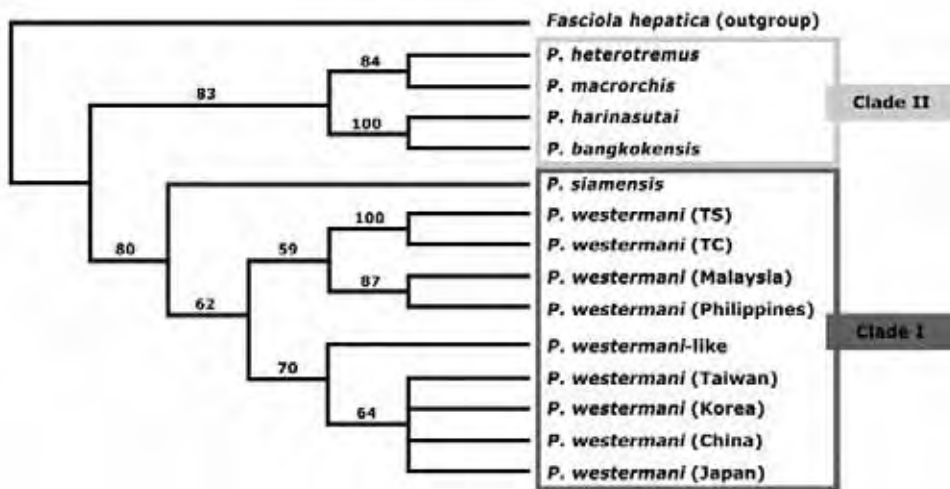


Fig 2- Single most parsimonious tree of length 191 steps based on parsimony analysis of the informative characters of the ITS2 region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. *P. westermani* (TS) = *P. westermani* from southern Thailand. *P. westermani* (TC) = *P. westermani* from central Thailand.

whereas, within the ingroup, it ranged from 0.3-25.3%. The mean G+C content was 44.0%, and transition/transversion ratio was 3.85.

### Phylogenetic analyses

**ITS2.** A single most parsimonious tree (Fig 2) of length 191 steps was obtained based on parsimony analysis of the informative characters with 1,000 bootstrap replicates. Fit measures of the tree were as follows: consistency index (CI) =

0.9058, homoplasy index (HI) = 0.0942, retention index (RI) = 0.8393, and rescaled consistency index (RC) = 0.7602. The phylogenetic tree comprised two clades: clade I, including the *P. westermani* complex and *P. siamensis* (bootstrap value (BS) = 62%), and clade II, including other Thai *Paragonimus* species (BS = 83%). Within the *P. westermani* complex, two groups of organism can be obtained based on geographical distribution. The first group

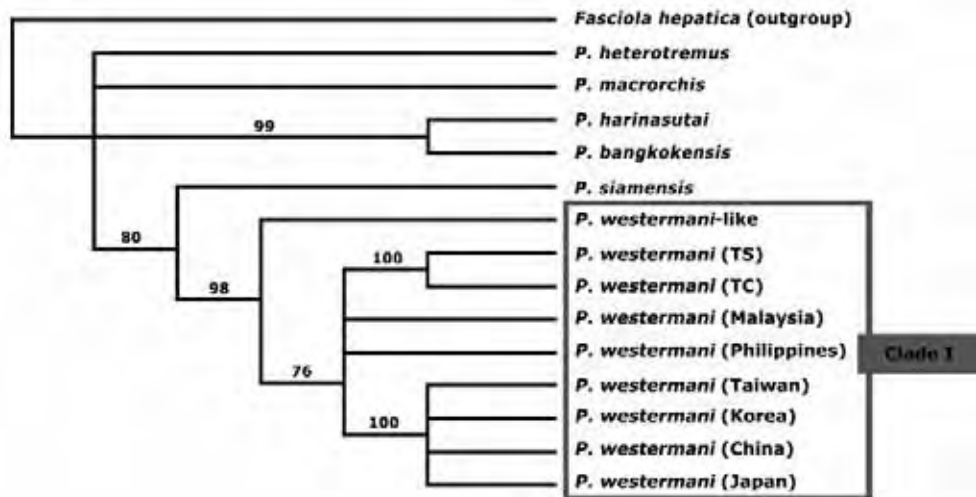


Fig 3- Strict consensus tree derived from 10 equally parsimonious trees of length 319 steps based on parsimony analysis of the informative characters of the partial COI region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. *P. westermani* (TS) = *P. westermani* from southern Thailand. *P. westermani* (TC) = *P. westermani* from central Thailand.

contains *P. westermani* from Southeast Asia (BS = 59%), while the second group contains *P. westermani* from East Asia and *P. westermani*-like from Thailand (BS = 70%).

**COI.** A strict consensus tree (Fig 3) was derived from 10 equally parsimonious trees of 319 steps long, based on parsimony analysis with 1,000 bootstrap replicates. Fit measures of the tree were as follows: CI = 0.6364, HI = 0.3636, RI = 0.6822, and RC = 0.4341. The tree inferred from the partial COI region showed a single clade with strong bootstrap support of 98%. This clade forms a complex of *P. westermani* from Southeast and East Asia (BS = 76%). *Paragonimus westermani*-like is excluded from the complex and designated as a sister group.

## DISCUSSION

The alignment of the ITS2 region of *Paragonimus* species and its outgroup was 378 bp in length which was similar to those of other digeneans such as *Schistosoma* (398 bp; Bowles *et al*, 1995) and *Fasciola* (364 bp; Mas-Coma *et al*, 2001). The level of sequence variation between *P. westermani*-like and *P. westermani* (1.39-4.0%) was close to the intraspecific variation within *P.*

*westermani* from different geographical origins (0-3.41%). Intraspecific variation in the ITS2 region was also observed in other digeneans, including *Schistosoma* (Agatsuma *et al*, 2001) and *Fasciola* (Adlard *et al*, 1993).

The numbers of the variable characters of the partial COI (143 characters) and the ITS2 (148 characters) sequences were almost equal. However, this region of the COI gene exhibited approximately two-times more informative characters (27.3%) than the ITS2 region (14.8%). Nonetheless, a remarkably large amount of homoplasy was observed in the COI data (HI = 0.3636) as compared to the ITS2 data (HI = 0.0942).

From this study, the phylogenetic tree inferred from the ITS2 region showed that *P. westermani* formed a complex of cryptic species and could be divided into two groups as previously reported (Blair *et al*, 1997, 1998). The first group comprises *P. westermani* from Southeast Asia (Thailand, Malaysia, and the Philippines), and the second group composes of *P. westermani* from East Asia (Taiwan, Korea, China, and Japan), which was closely related to *P. westermani*-like. In contrast to the ITS2 tree, the phylogenetic tree reconstructed from the COI region revealed that *P. westermani*-like is excluded from the complex and

designated as a sister group. Thus, it is evident that *P. westermani*-like is either well placed within the *P. westermani* complex (ITS2 data), or it is located close to the complex (COI data). However, since the protein-coding gene (COI) is under selective constraint while the non-coding ITS region is not, this suggests that the spacer is free to diverge and evolve with a rate that is close to the neutral rate of sequence evolution. In addition, due to such a high level of homoplasious characters present in the COI data, the tree inferred from the ITS2 data would be more reliable. This result of *P. westermani*-like being classified as one of the members of the *P. westermani* complex was strongly supported by the morphological characters of the adult worms (Sugiyama *et al.*, 2007).

Since the susceptibility of feline hosts to *P. westermani*-like was found to be different from that of Thai *P. westermani* (Sugiyama *et al.*, 2007) and a significant genetic variation was also observed between them, further investigation on the specificity of first intermediate hosts should be carried out to determine the proper taxonomic status of *P. westermani*-like.

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