APPLICATION OF MULTIPLEX PCR FOR SPECIES DISCRIMINATION USING INDIVIDUAL METACERCARIAE OF *PARAGONIMUS* OCCURRING IN THAILAND

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Abstract. A total of 6 lung fluke species have been documented in Thailand, of which *P. heterotremus* is the most important, since it affects humans. Although *P. westermani* is found as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed. To accurately discriminate between the individual metacercariae of these two species, we established a multiplex PCR method. Using this method, two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp). In contrast, 520-bp products alone were found to be generated from the DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, 3 other species of lung flukes known to occur in Thailand. Digestion of these 520-bp products with the restriction enzyme *Scr*FI could unequivocally discriminate species by the number and size of the produced band(s): 3 bands (ca. 60, 210 and 250 bp) for *P. harinasutai*, 2 bands (ca. 250 and 270 bp) for *P. bangkokensis*, and an uncut band (520 bp) for *P. siamensis*. The established multiplex PCR used in combination with restriction enzyme digestion (PCR-RFLP with *Scr*FI) is effective for discriminating the 5 different species of lung flukes occurring in Thailand, even at the metacercarial stage.

INTRODUCTION

Six lung fluke species have been documented in Thailand (Srisont *et al*, 1997; Blair *et al*, 1999): *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis* and *P. harinasutai*. Of them, *P. heterotremus* is the most important, since it affects humans. Although *P. westermani* occurs as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed (Blair *et al*, 1998). In order to accurately discriminate between individual metacercariae of these two species, we established a multiplex PCR method (Sugiyama *et al*, 2005). In this study, we further evaluated the usefulness of the previously established multiplex PCR for species-level discrimination among *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, 3 other species of lung flukes known to occur in Thailand.

MATERIALS AND METHODS

Parasite samples and DNA isolation

The metacercariae of *P. siamensis* (Fig 1) were harvested from the freshwater crab, *Sayamia germaini*, captured in paddy fields in Prachin Buri Province, Thailand (Srisont *et al*, 1997). The metacercariae of *P. bangkokensis* (Fig 2) were harvested from the freshwater crab, *Ranguna smalleyi*, captured in a mountain stream in Surat Thani Province, Thailand (Rangsiruji *et al*, personal communication). The metacercariae of *P. heterotremus*, *P. westermani* (Thailand strain) and *P. harinasutai* (Fig 3) were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al*, 1989). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al*, 2002).

DNA amplification and sequencing

For multiplex PCR amplification (Sugiyama *et al*, 2005), the *P. heterotremus*-specific forward primer (PhTF1; 5 -TTCCCCAACGTGGCCTTGTGT-3 , alignment positions 184 to 204 for the *P. heterotremus* second internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA (rDNA)) and a newly designed *P. westermani*-specific forward primer (PwTF3; 5 -GTCTGCGTTCGATGCTGACCTACG-3 , alignment positions 367 to 390 for the *P. westermani* ITS2 region) were used in combination with an interspecies-conserved primer pair, 3S (forward, 5 -G GTACCGGTGGATCACTCGGCTCGTG-3) and A28 (reverse, 5 -GGGATCCTGGTTAGTTTCTTTTCCTC CGC-3) (Bowles *et al*, 1995). These primers were all

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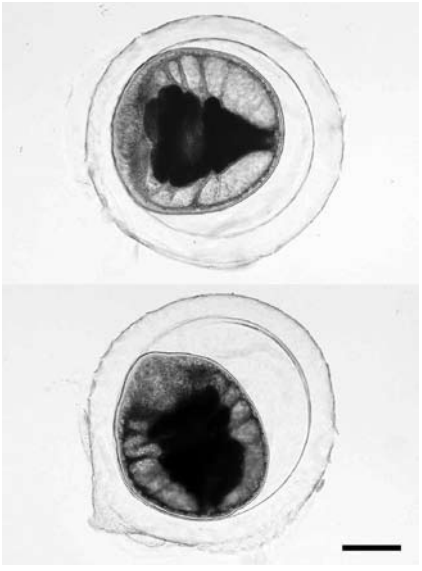

Fig 1- Photomicrograph of fresh *P. siamensis* metacercariae. The metacercariae were encysted by a thick wall and had a spherical shape. The wall thickness averaged 94 μm. The diameter of the cyst ranged from 668 to 736 μm with an average of 701 μm. Bar is 150 μm.

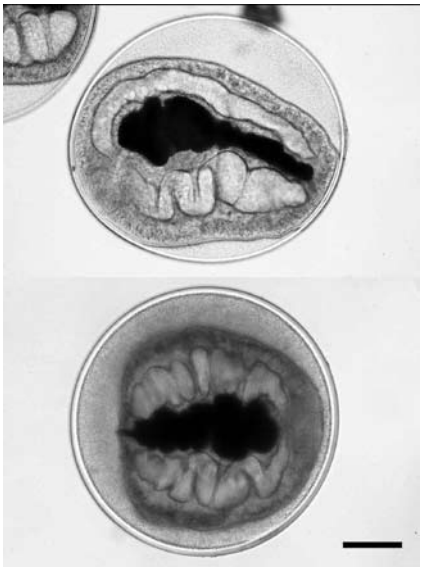

Fig 2- Photomicrograph of fresh *P. bangkokensis* metacercariae. The metacercariae were encysted by a wall and had a spherical-to-suboval shape. The wall thickness averaged 13 μm. The longitudinal and transverse diameters of the cyst ranged from 379 to 521 μm and 365 to 469 μm, respectively, with average dimensions of 437 x 422 μm. Bar is 150 μm.

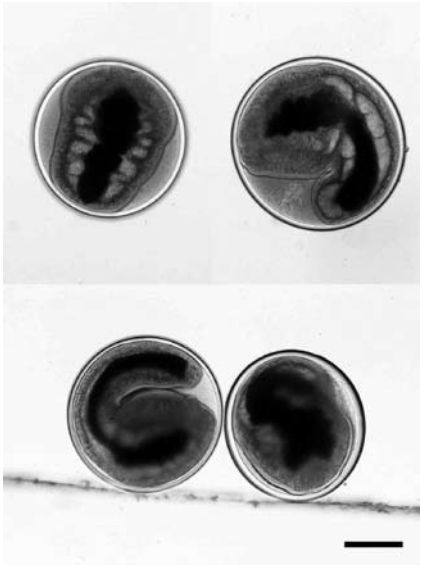

Fig 3- Photomicrograph of fresh *P. harinasutai* metacercariae. The metacercariae were encysted by a wall and exhibited a spherical-to-suboval shape. The wall thickness averaged 14 μm. The longitudinal and transverse diameters of the cyst ranged from 570 to 748 μm and 534 to 724 μm, respectively, with average dimensions of 655 x 634 μm. Bar is 150 μm.

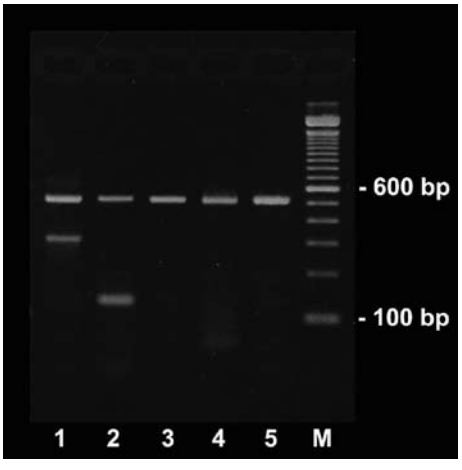

Fig 4- Results of multiplex PCR amplification of the metacercarial DNA samples from *P. heterotremus* (lane 1), *P. westermani* (lane 2), *P. siamensis* (lane 3), *P. bangkokensis* (lane 4) and *P. harinasutai* (lane 5). Two PCR fragments were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp). A single 520-bp fragment was produced for *P. siamensis*, *P. bangkokensis* and *P. harinasutai*. A 100-bp DNA ladder was used to estimate the sizes of the bands (lane M).

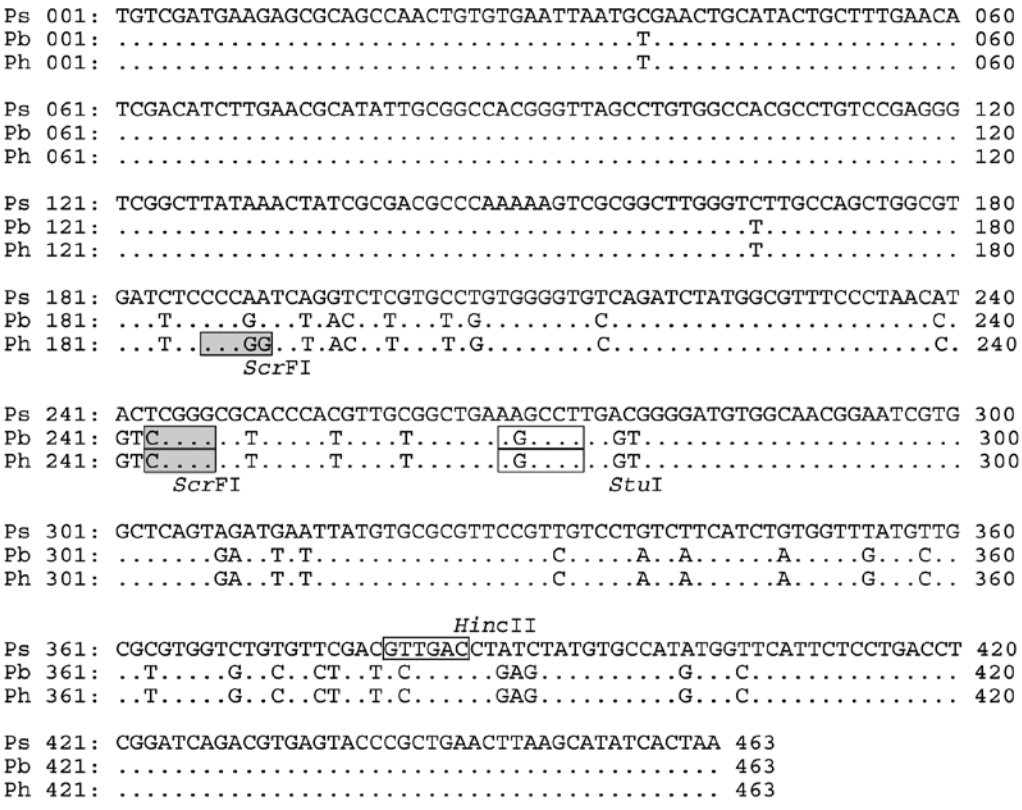

Fig 5- Aligned sequences of the ITS2 region from *P. siamensis* (Ps), *P. bangkokensis* (Pb) and *P. harinasutai* (Ph) metacercariae. A dot in the *P. bangkokensis* and *P. harinasutai* sequences indicates identity with the *P. siamensis* sequence. The recognition sites of the *Hinc*II (GTT/GAC), *Stu*I (AGG/CCT) and *Scr*FI (CC/CGG and CC/GGG) restriction enzymes are enclosed in boxes. The numbers refer to the lengths of the nucleotide sequences.

incorporated into a single-tube reaction. The multiplex PCR amplification was performed as previously described (Sugiyama *et al*, 2004) using 0.1 μM of PhTF1 and PwTF3 primers, 0.5 μM of 3S and A28 primers, 2.5 units of the Taq polymerase (Invitrogen, USA) and 10 ng of the DNA template. The resultant PCR products were separated by electrophoresis on 2% (w/v) agarose gels.

The amplified products were extracted from agarose gels and sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated sequencer (ABI310, Applied Biosystems). Sequence alignment and comparison were completed using the GENETYX-WIN (ver. 7.0, Software Development Co, Japan) program.

Restriction enzyme digestion of the multiplex PCR products [PCR-linked restriction fragment length polymorphism (PCR-RFLP)]

The amplified products (4 μl) were also treated with five units of the restriction enzyme *Hinc*II, *Stu*I or *Scr*FI (New England Biolabs, USA) at 37°C for 12 hours. The treated samples were then separated by electrophoresis on 3% (w/v) agarose gels.

RESULTS

Based on the established multiplex PCR method (Sugiyama *et al*, 2005), we confirmed that two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp) (Fig 4). On the other hand, 520-bp products alone were generated from the metacercarial DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai* (Fig 4). Sequence analysis of the amplified products revealed that the aligned ITS2 region was 463 bp in length in each of the latter 3 species (Fig 5).

Similarity searches of the GenBank/EMBL/DDBJ nucleotide databases revealed that the *P. siamensis*

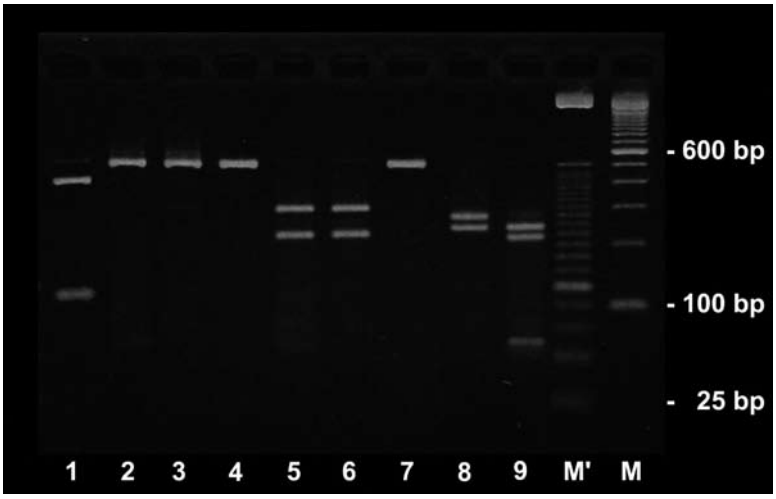

Fig 6- Results of RFLP analysis of the ITS2 products amplified from *P. siamensis* (lanes 1, 4 and 7), *P. bangkokensis* (lanes 2, 5, and 8) and *P. harinasutai* (lanes 3, 6, and 9) metacercarial DNA samples. The PCR product of *P. siamensis* (ca. 520 bp) was cleaved with *Hin*cII, producing two bands (ca. 110 and 410 bp, lane 1). The PCR products of the two other species remained uncut (lanes 2 and 3). In contrast, the PCR product of *P. siamensis* remained uncut with *Stu*I (lane 4), while those of the two other species were cleaved to produce two bands (ca. 220 and 300 bp, lanes 5 and 6). Digestion with *Scr*FI discriminated *P. harinasutai* based on the restriction pattern of three distinctive bands (ca. 60, 210 and 250 bp, lane 9). The PCR products of *P. bangkokensis* were cleaved with *Scr*FI to produce two distinctive bands (ca. 250 and 270 bp, lane 8), while those of *P. siamensis* remained uncut (lane 7). Both 25-bp and 100-bp DNA ladders were used to estimate the sizes of the bands (lanes M and M, respectively).

and *P. harinasutai* ITS2 sequences were identical to the sequences deposited under accession numbers AF159605 and AF159609, respectively. However, there was no sequence data in the GenBank/EMBL/DDBJ for *P. bangkokensis*; therefore, we deposited the ITS2 region sequence under accession number AB248091.

Pairwise comparisons between *P. siamensis* and each of *P. bangkokensis* and *P. harinasutai* revealed 33 (7.1%) or 34 (7.3%) nucleotide differences, respectively. In contrast, only one (0.2%) nucleotide difference was found between *P. bangkokensis* and *P. harinasutai*.

For species discrimination by PCR-RFLP, the restriction enzymes, *Hin*cII, *Stu*I and *Scr*FI, were selected on the basis of the theoretical restriction maps generated from the ITS2 sequences of *P. siamensis*, *P. bangkokensis* and *P. harinasutai* (Fig 5). Digestion with *Hin*cII discriminated *P. siamensis* by the restriction pattern of two distinctive bands of about 110 and 410 bp in size, while the 520-bp amplification products of the two other species remained uncut (Fig 6). In contrast, the PCR product of *P. siamensis* remained uncut by *Stu*I, while those of the other two species were cleaved to produce two bands of about 220 and 300 bp. Digestion with *Scr*FI could unequivocally discriminate *P. siamensis*, *P. bangkokensis* and *P. harinasutai* by the number and size of the produced band(s): 3 bands (ca. 60, 210 and 250 bp) for *P. harinasutai*, 2 bands (ca. 250 and 270 bp) for *P. bangkokensis*, and an uncut band (520 bp) for *P. siamensis* (Fig 6).

DISCUSSION

The phylogenetic relationships of the *Paragonimus* species occurring in Thailand have been demonstrated using genetic markers in the ITS2 region of the rDNA (Blair *et al*, 1998; Iwagami *et al*, 2000). In these studies, the ITS2 sequences were amplified by PCR with the primer pair 3S and A28, from DNA samples prepared from adult worms. Using this primer pair with two other species-specific primers in the previously established multiplex PCR method (Sugiyama *et al*, 2005), we demonstrated that 520-bp ITS2 sequences alone were generated from the individual metacercariae of *P. siamensis*, *P. bangkokensis* and *P. harinasutai*. Through pairwise comparisons of the sequences of the amplified products, these species were unequivocally discriminated from one another. We then utilized nucleotide differences to select the restriction enzymes *Hin*cII, *Stu*I and *Scr*FI for PCR-RFLP analysis, which

allowed development of a more rapid and labor-saving discrimination method. Of the restriction enzymes examined, we confirmed that *Scr*FI allowed the most efficient discrimination among these species, based on the number and size of the produced band(s). It is noteworthy that analysis with this enzyme could unequivocally discriminate between *P. bangkokensis* and *P. harinasutai*, which have only a single base difference in the ITS2 region.

In this study, we demonstrated that the previously established multiplex PCR method (Sugiyama *et al*, 2005), when used in combination with restriction enzyme digestion, is effective for discriminating the 5 different species of lung flukes occurring in Thailand, even at the metacercarial stage. This method may be applicable to *Paragonimus* occurring in other Asian countries, *eg*, China and India, where sets of *Paragonimus* species that have not yet been studied occur. Further collaborative studies, including evaluation of the usefulness of this method, are now in progress in these areas using locally obtained parasite samples.

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