MOLECULAR DISCRIMINATION BETWEEN INDIVIDUAL METACERCARIAE OF *PARAGONIMUS HETEROTREMUS* AND *P. WESTERMANI* OCCURRING IN THAILAND

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Abstract. To accurately discriminate between individual metacercariae of *Paragonimus heterotremus* and *P. westermani* occurring in Thailand, polymerase chain reaction (PCR)-based molecular methods were established and subjected to an evaluation. We first amplified and sequenced the second internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA of the two species. Based on their nucleotide differences, *P. heterotremus* and *P. westermani* were unequivocally discriminated from each other. These nucleotide differences were further utilized to select the *Apa*L1 endonuclease site for PCR-restriction fragment length polymorphism (PCR-RFLP) analyses and to design species-specific primers for multiplex PCR reactions. Both PCR-RFLP and multiplex PCR methods allowed a more rapid and labor-effective species discrimination. Furthermore, the multiplex PCR method enabled the most efficient discrimination because species identification involved a single round of PCR in a single tube. In Thailand, *P. heterotremus* is the only species affecting humans. Thus, the methods established in the present study can be used as reliable tools to identify the lung fluke metacercariae that cause human disease.

INTRODUCTION

Six lung fluke species have been documented to date in Thailand: *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis* and *P. harinasutai* (Srisont *et al*, 1997; Blair *et al*, 1999). Identification of the metacercariae of these lung flukes to the species level requires careful morphological observation of not only the fresh metacercariae, but also the adult flukes from experimentally infected animals (Miyazaki, 1991). This process, however, can be laborious and time-consuming.

We recently reported the successful development of molecular methods for the unequivocal species discrimination between individual metacercariae of the Japanese lung flukes, *P. westermani* and *P. miyazakii*, which cause human infection (Sugiyama *et al*, 2002, 2004). Previously established methods included the direct cycle sequencing of polymerase chain reaction (PCR) products, PCR-restriction fragment length polymorphism (PCR-RFLP), and direct PCR-amplification using consensus and/or species-specific primers. All of these methods utilize nucleotide differences in the second internal transcribed spacer (ITS2) of the nuclear ribosomal DNA (rDNA) for dicrimination between the two species. In the present study, we focused on the lung flukes occurring in Thailand and applied the methods for species discrimination between individual metacercariae of *P. heterotremus* and *P. westermani*.

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MATERIALS AND METHODS

Parasite material and DNA isolation

The metacercariae of *P. heterotremus* and *P. westermani* (Figs 1 and 2) 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, restriction digestion and sequencing

The rDNA region spanning the ITS2 from individual metacercariae of the two species was amplified by PCR using the primers, 3S (forward, 5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and A28 (reverse, 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'). These primers were designed on the basis of the conserved rDNA sequences of the *Schistosoma* species (Bowles *et al*, 1995) and were previously used as consensus primers for discriminating between the Japanese species of

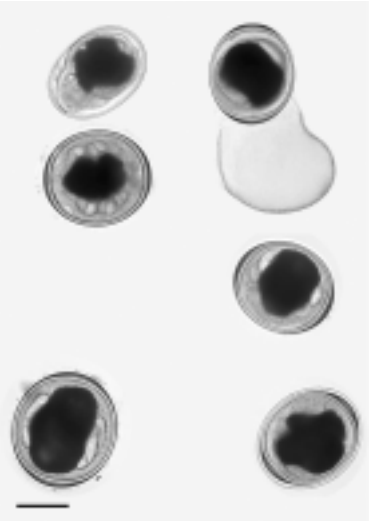

Fig 1- Photomicrograph of fresh *P. heterotremus* metacercariae. The metacercariae were encysted with a wall and exhibited a suboval shape. The thickness of the wall on the side (9.5 μm on average) gradually increased at both poles (21.6 μm on average). The longitudinal and transverse diameters of the cyst averaged 302 by 232 μm, respectively. Bar 150 μm.

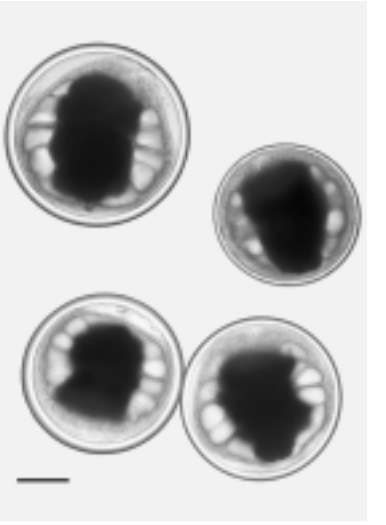

Fig 2- Photomicrograph of fresh *P. westermani* metacercariae. The metacercariae were encysted with a wall and exhibited a spherical shape. The wall thickness averaged 14.6 μm. The diameter of the cyst ranged from 410 to 570 μm with an average of 475 μm. Bar 150 μm.

Paragonimus (Sugiyama *et al*, 2002, 2004). In the present study, 0.5 μM of each primer, 2.5 units of DNA polymerase (TaKaRa Z-Taq, Takara Shuzo, Japan) and 10 ng of the DNA samples were added to each PCR reaction (final reaction volume, 100 μl). The resultant PCR products (10 μl) were restricted with five units of the *Apa*LI endonuclease (New England Biolabs, USA) at 37°C for 12 hours, then separated by electrophoresis through 2% (w/v) agarose gels. The intact PCR products were also electrophoresed and excised from the gels and sequenced using the corresponding primer 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 (version 5.0, Software Development, Japan) program.

Design of specific primers and amplification by (multiplex) PCR

To establish a more direct PCR procedure for species discrimination, we designed species-specific forward primers; PhTF1 for *P. heterotremus* (5'-TTCCCCAACGTGGCCTTGTGT-3', nucleotide positions 184 to 204 for the ITS2 region) and PwTF1 for *P. westermani* (5'-GTTCATGTTGCGCGTGGT CTGCGTTC-3', nucleotide positions 351 to 376) (Fig 3). The species-specific primer(s) as well as the consensus primer(s) were incorporated into single tubes. The multiplex PCR amplification was performed under the conditions described above using 0.5 μM of the consensus primers and 0.1 μM of the species-specific primers.

RESULTS

The ITS2-PCR products were amplified from DNA samples of individual *P. heterotremus*- and *P. westermani*-metacercariae using the consensus primers, 3S and A28. Agarose gel electrophoresis showed that the generated products were about 520 bp in size for both species. Sequence analysis of the products revealed that the aligned ITS2 region of *P. heterotremus* and *P. westermani* was 463 bp in length. A pairwise comparison of the sequences showed 36 (7.8%) nucleotide differences consisting of two deletions/insertions and 34 substitutions (Fig 3). Similarity searches of the nucleotide databases revealed that the ITS2 and flanking regions were identical to those deposited in the GenBank/EMBL/DDBJ nucleotide databases, accession numbers AF159603 for *P. heterotremus* and AF159604 for *P. westermani*.

The *Apa*LI endonuclease was selected for species discrimination by PCR-RFLP based on the theoretical

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Ph 001:TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA 060
Pw 001:.....C..... 060

Ph 061:TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pw 061:..... 120

Ph 121:TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT 180
Pw 121:.....C..... 180

          PhTF1-->
Ph 181:GATTTCCCCAACGTGGCCTTGTGTCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAA 240
Pw 181:...C.....TC...T.....C.....A.....T 240

Ph 241:ATCCGGGCGTATCCATGTTGTGCCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTG 300
Pw 241:.CT..C...C.C...C.....C.....A..... 300

                          ApaLI
Ph 301:GCTCAGTGAATGATTTATGTGCACGTTCCGCTGTCCCGTCATCATCTATGGTTGAAGTTG 360
Pw 301:.....A.....G....T.....T...T.....G.....C.T..... 360
                                                    PwTF1-->

Ph 361:CGCGTGGTGTG--TCCGATGCTGACCTATATATGTGCCATGTGGCTCATTTTCCTGACCT 418
Pw 361:.....C..CG.T.....CG.....TC....C.T..... 420

Ph 419:CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA 461
Pw 421:....T..... 463
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Fig 3- Aligned nucleotide sequences of the ITS2 region from *P. heterotremus* (Ph) and *P. westermani* (Pw) metacercariae. A hyphen indicates an alignment gap. A dot in the *P. westermani* sequence indicates a nucleotide identical to that in *P. heterotremus*. The 5' and 3' ends of the sequences include the 5.8S rDNA and 28S rDNA, respectively. The recognition site of the *Apa*LI endonuclease (G/TGCAC) is boxed. The locations of the *P. heterotremus*-specific forward primer (PhTF1; 5'-TTCCCCAACGTGGCCTTGTGT-3') and *P. westermani*-specific forward primer (PwTF1; 5'-GTTCATGTTGCGCGTGGTCTGCGTTC-3') are underlined. Numbers refer to the actual length of the nucleotide sequences.

restriction maps generated from the ITS2 sequences of the two species (Fig 3). When the enzyme was applied, expected results were obtained. The PCR product of *P. heterotremus* (520 bp) was cleaved, thereby producing two fragments with sizes about 350 and 170 bp (Fig 4). In contrast, the PCR product of *P. westermani* remained unrestricted (Fig 4).

For species discrimination by direct PCR, the *P. heterotremus*-specific and *P. westermani*-specific primers (PhTF1 and PwTF1, respectively) were designed on the basis of the nucleotide differences in the ITS2 region. The specificity of these primers was evaluated as to whether they could amplify different sized species-specific fragments only from the respective DNA samples in combination with the primer A28. As expected, the PhTF1-A28 primer set amplified a PCR product of about 310 bp from *P. heterotremus* DNAs, but not from *P. westermani* DNAs. In contrast, PwTF1-A28 amplified a product of about 140 bp from *P. westermani* DNAs, but not from *P. heterotremus* DNAs (figure not shown).

Having demonstrated the species-specificity of the PhTF1 and PwTF1 primers, both were incorporated

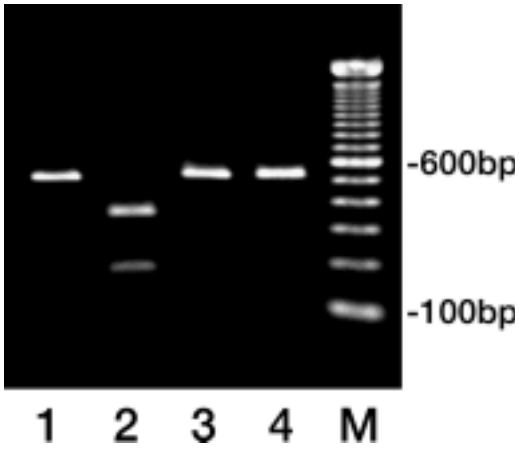

Fig 4- PCR amplification products of the ITS2 region from *P. heterotremus* (lanes 1 and 2) and *P. westermani* (lanes 3 and 4) metacercarial DNAs. The ITS2-PCR products were then restricted with the *Apa*LI endonuclease (lanes 2 and 4). The 100-bp DNA ladder marker was used to estimate the size of the fragments (lane M).

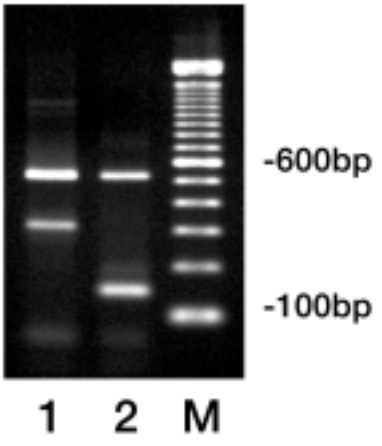

Fig 5- Results of multiplex PCR amplifications from *P. heterotremus* (lane 1) or *P. westermani* (lane 2) metacercarial DNAs. For amplification, two species-specific primers, PhTF1 and PwTF1, were incorporated into single tubes containing the two consensus primers, 3S and A28. A 100-bp DNA ladder was used to estimate the size of the fragments (lane M).

into single tubes with the two consensus primers, 3S and A28, and then a multiplex PCR amplification was carried out. As a result, two PCR products of about 520 bp and 310 bp were amplified from the *P. heterotremus* DNAs and products of about 520 bp and 140 bp were amplified from the *P. westermani* DNAs (Fig 5). The amplified PCR products were sequenced, which confirmed that they corresponded to the ITS2 region of the rDNA from their respective species.

DISCUSSION

The phylogenetic relationships of the *Paragonimus* species occurring in Thailand have been studied using genetic markers in the ITS2 region of rDNA (Blair *et al*, 1998; Iwagami *et al*, 2000). In these studies, the ITS2 sequences were generated from DNA samples prepared from adult worms using the consensus PCR primers, 3S and A28. Using these primers, we demonstrated that the ITS2 sequences were generated from the individual metacercariae of *P. heterotremus* and *P. westermani*. By pairwise comparison of the generated products, the two species were unequivocally discriminated from each other. These nucleotide differences were utilized to select the *Apa*LI endonuclease for the PCR-RFLP analyses and to design species-specific primers for the multiplex PCR reactions, both of which allowed the more rapid and labor-effective discrimination between *P. heterotremus* and *P. westermani* at the metacercarial stage.

In Thailand, the metacercariae of *P. heterotremus* and *P. westermani* have been detected in the same crab species (Kawashima *et al*, 1989; Blair *et al*, 1998) but only the former is known to affect humans (Srisont *et al*, 1997; Blair *et al*, 1998). Thus, the discrimination methods established in the present study can be used as reliable tools to identify the lung fluke metacercariae that cause human disease.

Of the molecular methods examined, we confirmed that the multiplex PCR method allowed the most efficient discrimination because only a single-round PCR was required. By this method, species-specific products of different sizes were produced from the *P. heterotremus* (310 bp) and *P. westermani* (140 bp) DNA samples. At the same time, 520 bp-products were also generated from the DNA samples of both species, which could serve as sets of internal controls to confirm the integrity of the PCR reactions and might function as diagnostic reagents for the (genus and) species identification(s) (Sugiyama *et al*, 2004). In fact, we found that the 520-bp product alone was generated from DNA samples of *P. harinasutai* metacercariae whose crab host is identical to that of *P. heterotremus* and *P. westermani* (data not shown). Further studies are in progress to evaluate the usefulness of the established multiplex PCR method for species discrimination and/or identification using DNA samples from the remaining three species of lung flukes occurring in Thailand (*P. siamensis*, *P. bangkokensis* and *P. macrorchis*).

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