MOLECULAR DISCRIMINATION BETWEEN *PARAGONIMUS HETEROTREMUS* AND TWO FORMS OF *P. WESTERMANI* OCCURRING IN THAILAND

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Abstract. In areas of central Thailand where paragonimiasis is endemic, metacercariae of *Paragonimus westermani* (large metacercarial form) and *P. heterotremus* have been detected in a single crab species. Of these two species, only the latter has been confirmed to infect humans. In southern Thailand, we have previously identified another form of *P. westermani* (small metacercarial form) in another crab species, which also acts as host for *P. westermani* (large metacercarial form). In this study, we established a new multiplex PCR method and evaluated its applicability for discriminating between *P. heterotremus* and two forms of *P. westermani* at the metacercarial stage. We found that multiplex PCR in combination with restriction enzyme digestion (PCR-RFLP with *Bsa*HI) was effective for the discrimination.

INTRODUCTION

During an intensive field survey for lung flukes in southern Thailand, we found two forms of *Paragonimus westermani* metacercariae in a single crab species, *Phricotelphusa aedes* (Binchai *et al*, 2007; Sugiyama *et al*, 2007). Metacercariae of these two forms had the same shape, but were of different sizes: the diameter of metacercarial cysts of the large form is about twice that of the small one. As the nuclear ribosomal DNA (rDNA) second internal transcribed spacer (ITS2) sequences obtained from the large metacercarial form were identical to those of *P. westermani*, whose sequence was deposited in the GenBank/EMBL/DDBJ nucleotide databases under the accession number of AF159604 (referred to as *P. westermani* strain Thailand), we referred to the small metacercarial form as *P. westermani*-like for descriptive purposes.

In Thailand, human infections with *P. westermani* have not been confirmed, although *P. heterotremus* is known to affect humans (Srisont *et al*, 1997; Blair *et al*, 1998). The metacercariae of these two species have been detected in the same crab host in paragonimiasis-endemic areas (Miyazaki, 1991). Therefore, we had developed methods that could be used as reliable tools for discriminating these two lung fluke species. We demonstrated that multiplex PCR method was the most efficient because species identification involved a single round of PCR in a single tube (Sugiyama *et al*, 2005). In this study, we modified the previously established multiplex PCR method and evaluated its applicability for discriminating between *P. heterotremus* and two forms of *P. westermani* at the metacercarial stage.

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MATERIALS AND METHODS

Parasite material and DNA isolation

Metacercariae of *P. heterotremus* were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al*, 1989). Metacercariae of *P. westermani* and *P. westermani*-like were isolated from the freshwater crab, *Phricotelphusa aedes*,

captured in a mountain stream in Surat Thani Province, Thailand (Sugiyama *et al*, 2007). This study also includes *P. siamensis*, the species known to be closely related to *P. westermani* (Blair *et al*, 1998). Metacercariae of *P. siamensis* were harvested from the freshwater crab, *Sayamia germaini*, captured in paddy fields in Prachin Buri Province, Thailand (Srisont *et al*, 1997). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al*, 2002).

DNA amplification and sequencing

For multiplex PCR amplification, we constructed new species-specific forward primers based on the respective rDNA ITS2 sequences in order to generate the products that would remain uncut by further restriction enzyme digestion. The sequences (and alignment positions) (Fig 1) of the primers used for the multiplex PCR are as follows:

1) Interspecies-conserved forward primer (3S): 5′ GGTACCGGTGGATCACT CGGCTCGTG 3′;

2) Interspecies-conserved reverse primer (A28): 5′GGGATCCTGGTTAGTTTCTTTTC CTCCGC 3′ (Bowles *et al*, 1995);

3) *P. heterotremus*-specific forward primer (PhTF2): 5′ CAAATCCGGGCGTAT CCATGTTGTG 3' (positions 238 to 262);

4) *P. westermani*-specific forward primer (PwTF4): 5′ TCTGCGTTCGAT GCTGACCTACG 3′ (positions 368 to 390, a sequence common between the two forms of *P. westermani*).

These four primers were included in a single-tube reaction. Multiplex PCR amplification was performed using 0.1 μM of PhTF2 and PwTF4 primers, 0.5 μM of 3S and A28 primers, 2.5 units of the *Taq* polymerase (Invitrogen, USA) and 10 ng of DNA template. The resulting PCR products were separated by electrophoresis in 3% (w/v) agarose gels.

The amplicons were extracted from agarose gels and sequenced using the corresponding primers and BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) in an automated sequencer (ABI310, Applied Biosystems). The sequence alignment and comparison were conducted using GENETYX-WIN (ver. 7.0, Software Development, Japan) program.

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

Amplicons (4 to 10 μl) were also digested with five units of *Bsa*HI (New England Biolabs, USA) at 37 °C for 1 hour. The samples were then separated by electrophoresis in 3% (w/v) agarose gels.

RESULTS

Using multiplex PCR method with the new species-specific primers, two products were amplified from each of the metacercarial DNA samples of *P. heterotremus* (ca. 520 bp and 250 bp), *P. westermani* (ca. 520 bp and 125 bp), and *P. westermani*-like (ca. 520 bp and 125 bp) (Fig 2, lanes 1 to 3). However, a single 520-bp product was generated from the DNA samples of *P. siamensis* (Fig 2, lane 4). Sequence analysis of the amplification products (520 bp and others) revealed that the products corresponded to the rDNA ITS2 region of the respective species (Fig 1).

For species discrimination by RFLP using the multiplex PCR products, we selected restriction enzyme *Bsa*HI based on the putative restriction maps generated from ITS2 region sequences (Fig 1). Digestion of multiplex PCR products of *P. westermani*-like produced three fragments (ca. 270, 170 and 90 bp) from the 520-bp amplicon (Fig 2, lane 7). However, the 520-bp amplicons of the other 3 species (*P. heterotremus*, *P. westermani* and *P. siamensis*) produced two fragments (ca. 350 and 170 bp; Fig 2, lanes 5, 6 and 8). Multiplex PCR products of less than 520 bp in size (250-bp product for *P. heterotremus* and 125-bp products for *P.*

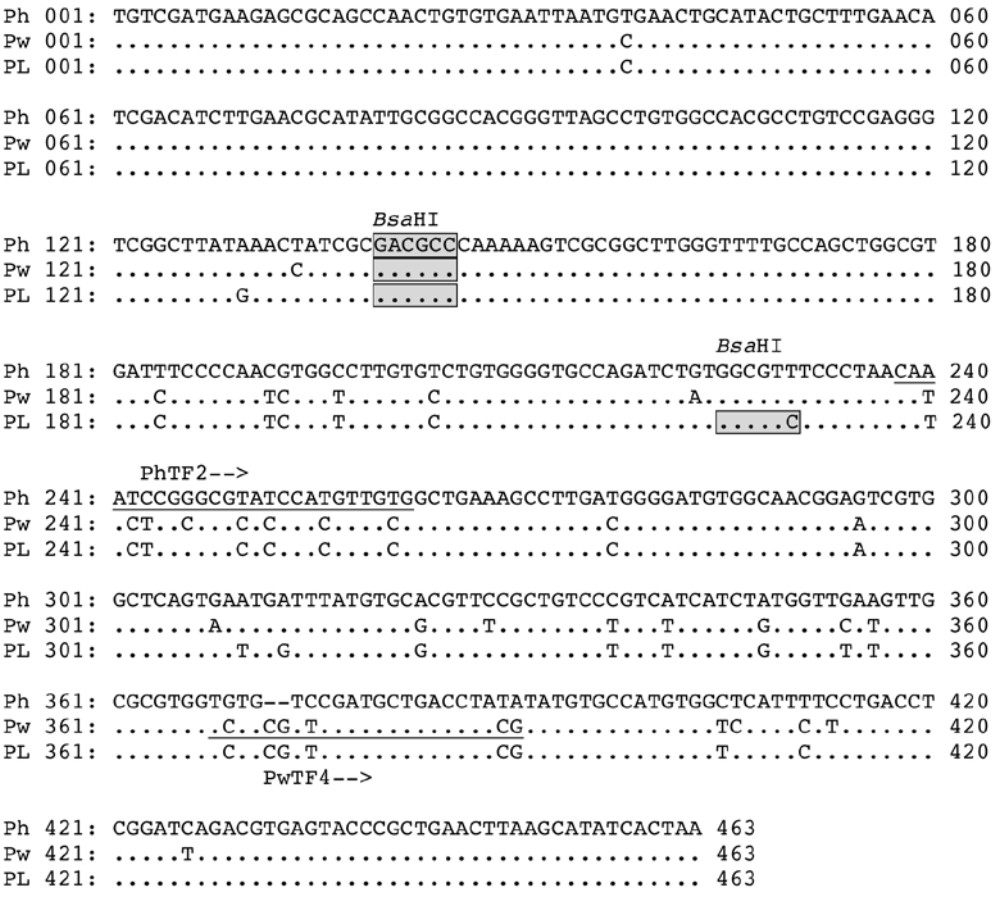

Fig 1- Sequences alignment of the ITS2 region from *P. heterotremus* (Ph), *P. westermani* (Pw) and *P. westermani*-like (PL) metacercariae. The 5′ and 3′ ends of the sequences include 5.8S rDNA and 28S rDNA, respectively. A dot in the *P. westermani* and *P. westermani*-like sequences indicates identity with *P. heterotremus* sequence. The locations of the *P. heterotremus*-specific forward primer (PhTF2; 5′ CAAATCCGGGCGTATCCATGTTGTG 3′) and *P. westermani*-specific forward primer (PwTF4; 5′ TCTGCGTTCGATGCTGACCTACG 3′) are underlined. The recognition sites of *Bsa*HI (GR/CGYC) are located in boxes. The numbers refer to the alignment positions.

westermani and *P. westermani*-like) remained undigested by *Bsa*HI.

DISCUSSION

We previously reported that the multiplex PCR method we developed (Sugiyama *et al*, 2005) was effective for discriminating among the five *Paragonimus* species occurring in Thailand when used in combination with *Scr*FI digestion (Sugiyama *et al*, 2006). However, this method was not applicable for discriminating among *P. heterotremus* and two forms of *P. westermani* because the latter two forms showed identical PCR-RFLP patterns. Therefore, in this study, we treated the amplicons with *Bsa*HI chosen based on the sequence differences between these two forms of *P. westermani*. In addition, new species-specific primers were constructed to generate products that would remain uncut by *Bsa*HI digestion. The improved method was shown to be effective in discriminating among *P. heterotremus* and two forms of *P. westermani*.

Two forms of *P. westermani* were found

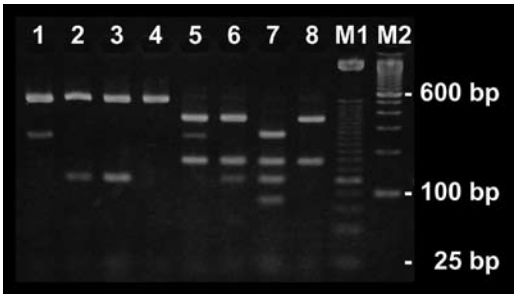

Fig 2- Multiplex PCR and multiplex PCR plus PRLP analysis of ITS2 amplification products from the metacercarial DNA samples of *P. heterotremus* (lanes 1 and 5), *P. westermani* (lanes 2 and 6), *P. westermani*-like (lanes 3 and 7) and *P. siamensis* (lanes 4 and 8). After digestion of the multiplex PCR products with *Bsa*HI, three bands were observed for *P. heterotremus* (ca. 350, 250 and 170 bp, lane 5), three bands for *P. westermani* (ca. 350, 170 and 125 bp, lane 6), four bands for *P. westermani*-like (ca. 250, 170, 125 and 90 bp, lane 7) and two bands for *P. siamensis* (ca. 350 and 250 bp, lane 8). Both the 25-bp and 100-bp DNA ladders were used to estimate the sizes of the bands (lanes M1 and M2, respectively).

to occur in Surat Thani, southern Thailand, and they both used a single crab species as the second intermediate host (Sugiyama *et al*, 2007). Possible discovery of *P. westermani*-like metacercariae were reported from crabs occurring not only in Surat Thani (Shibahara *et al*, 1995) but Nakhon Si Thammarat (Tsuzuki *et al*, 1995), the neighboring province of Surat Thani. However, little attention has been paid to *P. westermani*-like and its infection of humans has not been determined. To obtain accurate epidemiological information about the prevalence of the lung fluke species and forms in Thailand, various methods for identification are needed on parasitological materials obtained from host animals. The method developed in this study has the potential for this purpose.

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