

# A MULTIPLEX PCR FOR DISCRIMINATION BETWEEN *PARAGONIMUS WESTERMANI* AND *P. MIYAZAKII* AT THE METACERCARIAL STAGE

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**Abstract.** A multiplex polymerase chain reaction (PCR) system was developed for accurate species discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. The interspecies-conserved and species-specific primers designed from the sequences of the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA (rDNA) were all incorporated into single tubes and PCR amplification was carried out. The method allowed us to identify *P. westermani* and *P. miyazakii*, and discriminate them from *P. ohirai* at the metacercarial stage in a single tube reaction.

## INTRODUCTION

The lung flukes, *Paragonimus westermani* and *P. miyazakii*, are of known medical importance as pathogens causing human paragonimiasis in Japan. The metacercariae of these species are found in the same freshwater crab species and are morphologically quite similar (Miyazaki, 1991). Therefore, the development of sensitive and objective diagnostic methods is required for accurate species discrimination and identification of the individual metacercariae of these species. These methods could be used for epidemiological investigations of the prevalence of the metacercariae in the crab host, and thus have important implications for controlling lung fluke disease.

We recently reported the establishment of molecular methods based on the use of polymerase chain reaction (PCR) for accurate discrimination of individual metacercariae of *P. westermani* and *P. miyazakii* (Sugiyama *et al.*, 2002). The methods included direct cycle sequencing of the PCR products, PCR-restriction fragment length polymorphism (RFLP) and direct PCR-amplification using species-specific primers. All of these methods utilize nucleotide differences in the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA (rDNA) for discrimination. Of these methods, direct PCR-amplification provides a more rapid differential identification of species, because only a

single-round PCR is required. However, this method limits identification to a single species and requires concurrently-run controls. In the present paper, we report the development of a multiplex PCR utilizing interspecies-conserved and species-specific primers designed from the sequences of the ITS2 region, which allows species discrimination of the individual metacercaria in a single tube reaction.

## MATERIALS AND METHODS

### Parasite material and DNA isolation

Metacercariae of *P. westermani* (the diploid type) and *P. miyazakii* were harvested from the freshwater crab, *Geothelphusa dehaani*, and used for DNA isolation (Sugiyama *et al.*, 2002). DNA was also extracted from the metacercariae of *P. ohirai*, which were isolated from the brackish water crab, *Chiromantes dehaani*, collected in Tokyo, Japan (Sugiyama *et al.*, 2004).

### Primers and amplification by PCR

The *P. westermani*-specific forward primer (PwF1; 5'-GTTTATGTTGCGCGTGGTCTGCTTTC-3', alignment positions 351 to 376 for *P. westermani* ITS2 region) and *P. miyazakii*-specific forward primer (PmF1; 5'-TTCCCCAACCTGGCCTCGTGG-3', alignment positions 184 to 204 for *P. miyazakii* ITS2 region) were newly designed in this study to target the 3'-terminal and the central portion of the ITS2 sequences of the corresponding species, respectively (Fig 1). In combination with the species-specific primers, consensus primers, 3S: 5'-GGTACCGTGGATCACTCGGCTCGTG-3' (forward) and/or A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCGC-3' (reverse), which were designed based on the conserved sequences of the 5.8S and 28S genes (Bowles *et al.*, 1995), were used.

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Pw 1: TGTCGATGAAGAGCGCAGCCAACCTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA  
Pm 1: ..... T .....

Pw 61: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG  
Pm 61: .....

Pw 121: TCGGCTTATAAACTATCGCGACGCCAAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT  
Pm 121: .....

Pw 181: GATCTCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAT  
Pm 181: ... T..... C... C... C... G.....  
PmF1→

Pw 241: ACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG  
Pm 241: . T C ..... T ..... T ..... T .....

PwF1-->

Pw 301: GCTCAGTGAATGATTTATGTGCGCGTTCGCTGTCCTGTCTTCATCTGTGGTTTATGTTG  
Pm 301: ..... T ..... - - - - - ..... A ..... A ..... A ..... G ..... A ..... G .....

Pw 361: CGCGTGGTCTGCTTTTCGATGCTGACCTACGTATGTGCCATGTGGTTCATTCTCCTGACCT  
Pm 359: ..... G .. T G C C ..... T ..... C .....

Pw 421: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA :463  
Pm 419: .. :461

Fig 1- Aligned nucleotide sequences of an ITS2 region from *P. westermani* (Pw) and *P. miyazakii* (Pm) metacercariae. A hyphen indicates an alignment gap. A dot in the *P. miyazakii* sequence indicates a nucleotide identical to that in *P. westermani*. The 5' and 3' ends of the sequences include 5.8S rDNA and 28S rDNA, respectively. The locations of the *P. westermani*-specific forward primer (PwF1; 5'-GTTTATGTTGCGCGTGGTCTGCTTTC-3') and *P. miyazakii*-specific forward primer (PmF1; 5'-TTCCCAACCTGGCTCGTGG-3') are underlined. The numbers refer to alignment positions.

PCR amplification was performed as described previously (Sugiyama *et al*, 2002) using 0.25 µm of each primer and 2.5 units of Taq polymerase (Invitrogen, USA). In the present study, 1 ng of the DNA was added to each PCR reaction (final reaction volume, 100 µl); 1 ng of the DNA was equivalent to about 1/250, 1/400 and 1/100 of the DNA isolated from a single *P. westermani*, *P. miyazakii* and *P. ohirai* metacercaria, respectively. The resultant PCR products were separated by electrophoresis through 2% (w/v) agarose gels. The amplified PCR products were also excised 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).

RESULTS

In the first step, the species-specificity of the newly designed forward primers, PwF1 and PmF1, was evaluated as to whether they could amplify different

sized species-specific fragments from metacercarial DNA by PCR in combination with the consensus reverse primer A28. As was expected, the primer set PwF1-A28 amplified a PCR product of about 140 bp from *P. westermani* DNA, but not from *P. miyazakii* DNA (Fig 2). In contrast, PmF1-A28 amplified a product of about 300 bp from *P. miyazakii* DNA, but not from *P. westermani* DNA (Fig 2).

Having demonstrated the species-specificity of the primers PwF1 and PmF1, these two primers were mixed and incorporated into single tubes with the consensus primer A28 for the PCR reaction. As a result, a PCR product of about 140 bp was amplified from *P. westermani* DNA and that of about 300 bp was from *P. miyazakii* DNA (Fig 3). The same PCR amplification was carried out using DNA extracted from *P. ohirai* metacercariae but no product was amplified (Fig 3).

We previously reported that PCR products of the same size (520 bp) were amplified from *P. westermani* and *P. miyazakii* metacercarial DNAs using the

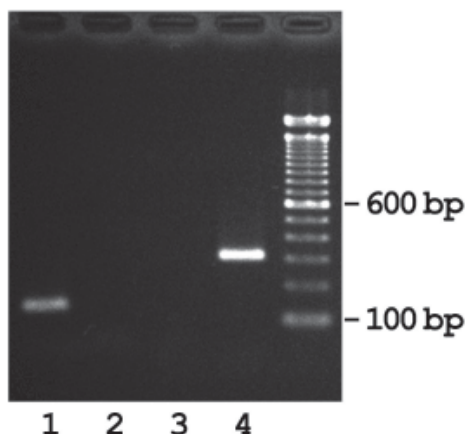


Fig 2- Results of PCR amplification of DNA from *P. westermani* (lanes 1 and 2) or *P. miyazakii* (lanes 3 and 4) metacercariae using the *P. westermani*-specific PwF1 (lanes 1 and 3) and *P. miyazakii*-specific PmF1 (lanes 2 and 4) primers. A 100-bp DNA ladder was used to estimate the size of the fragments.

consensus primer set 3S-A28 (Sugiyama *et al.*, 2002). These two consensus primers as well as the two species-specific primers, PwF1 and PmF1, were all incorporated into single tubes, and PCR amplification was then carried out. As a result, two PCR products of about 520 bp and 140 bp were amplified from *P. westermani* DNA and products of about 520 bp and 300 bp were amplified from *P. miyazakii* DNA (Fig 4). In addition, we found that only the 520-bp product was amplified from *P. ohirai* DNA (Fig 4). The amplified PCR products were sequenced, and we confirmed that they corresponded to the ITS2 regions of rDNA from the respective species.

The sensitivity of the established multiplex PCR system was tentatively determined by titration of the DNA. The lowest concentration of *P. westermani*, *P. miyazakii* and *P. ohirai* DNAs that produced PCR products was estimated to be 0.001 ng (figure not shown). This is equivalent to approximately  $10^{-5}$  of the total DNA isolated from a single metacercaria.

#### DISCUSSION

In the present study, we developed a multiplex PCR system that allowed us to identify *P. westermani* and *P. miyazakii*, and discriminate them from other *Paragonimus* species at the metacercarial stage in a single tube reaction. By this system, species-specific bands of different sizes were produced from *P. westermani* DNA (140 bp) and *P. miyazakii* DNA (300 bp). At the same time, interspecies-conserved bands (520 bp) were generated from DNAs prepared from

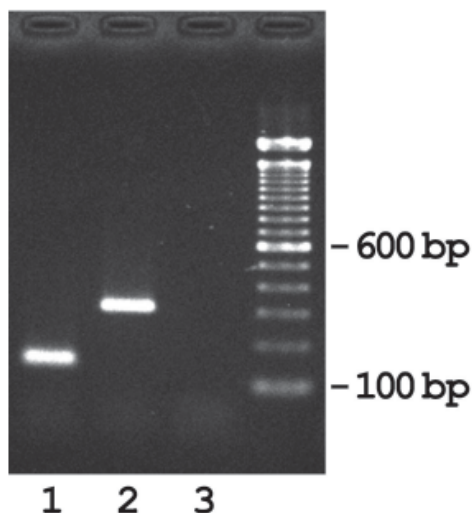


Fig 3- Results of multiplex PCR amplification of DNA from *P. westermani* (lane 1), *P. miyazakii* (lane 2) or *P. ohirai* (lane 3) metacercariae using the two species-specific primers, PwF1 and PmF1, in combination with the consensus reverse primer, A28. A 100-bp DNA ladder was used to estimate the size of the fragments.

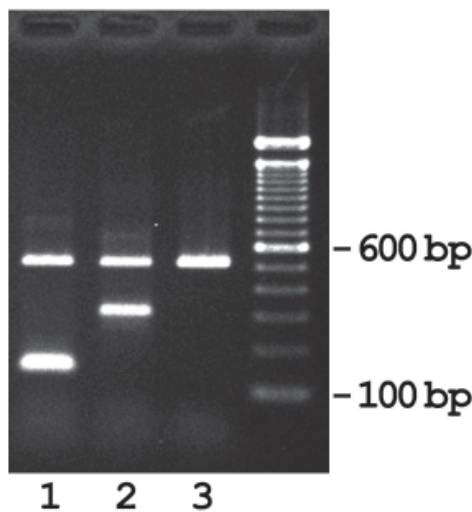


Fig 4- Results of multiplex PCR amplification of DNA from *P. westermani* (lane 1), *P. miyazakii* (lane 2) or *P. ohirai* (lane 3) metacercariae using the two species-specific primers, PwF1 and PmF1, combined with the consensus primer set, 3S and A28. A 100-bp DNA ladder was used to estimate the size of the fragments.

metacercariae of all the three species examined, *P. westermani*, *P. miyazakii* and *P. ohirai*. The PCR products of about 520 bp could serve as an internal control for the integrity of the PCR reaction and might

function as a diagnostic reagent for genus identification. The consensus primers, 3S-A28, were previously used to amplify the ITS2 region from *P. ohirai* adult DNA (Blair *et al*, 1997).

From the high sensitivity of the multiplex PCR system, we speculated that it has the potential to be used for species identification and discrimination of eggs of lung flukes, the life cycle stages with particularly small sizes. In addition, the eggs of *P. westermani* (the diploid type) and *P. miyazakii* are quite similar in size and shape (Miyazaki, 1991), and therefore it is quite difficult to differentiate the species of individual eggs using morphological characters. As the eggs in the feces or sputa from patients represent the parasite stage for which diagnostic tests are most often requested, studies are underway to elucidate the established multiplex PCR system for this purpose.

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