

## MOLECULAR IDENTIFICATION OF *PARAGONIMUS OHIRAI* AND *P. WESTERMANI* FROM ANHUI PROVINCE, CHINA

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### Summary :

Nucleotide sequences of the internal transcribed spacer 2 (ITS2) region were determined from seven adults of species *Paragonimus* collected from Jinde and Xiuning Counties, Anhui Province, China. Among these, the nucleotide sequence obtained from one *Paragonimus* adult (Jinde County) was identical to the ITS2 sequence of *P. ohirai* previously reported. In order to confirm the result, partial regions of mitochondrial cytochrome C oxidase I (COI) and NADH dehydrogenase 1 (ND1) from the putative *P. ohirai* sample were further sequenced. They showed a high level of similarity with those of *P. ohirai*, COI (99.7 %) and ND1 (99.5 %), supporting the result obtained from the ITS2. In addition to this, we designed *P. ohirai*- and *P. westermani*-specific primers (BDW and BD2OH) from ITS2 to identify *P. westermani* and *P. ohirai* easily and rapidly. After testing utility of the primers, they were applied to identify seven unidentified *Paragonimus* samples collected from Jinde and Xiuning Counties, China. All the examined samples showed *P. westermani* band pattern, and it was reconfirmed by sequencing their ITS2 regions that they are *P. westermani*. This result indicates that the two newly designed specific primers could be quite helpful for easily identifying *P. westermani* and *P. ohirai*, that most of *Paragonimus* in Jinde and Xiuning Counties consist of *P. westermani*, and that *P. ohirai* exists in Jinde County with minority.

**KEY WORDS :** *Paragonimus ohirai*, *Paragonimus westermani*, Anhui Province, China, internal transcribed spacer 2 (ITS2).

### Résumé : IDENTIFICATION MOLÉCULAIRE DE *PARAGONIMUS OHIRAI* ET *P. WESTERMANI* DE LA PROVINCE D'ANHUI, CHINE

Les séquences nucléotidiques de la région ITS2 ont été déterminées à partir de sept *Paragonimus* adultes provenant des régions de Jinde et Xiuning de la province d'Anhui en Chine. La séquence nucléotidique obtenue à partir d'un *Paragonimus* adulte (région de Jinde) était identique à la séquence ITS2 de *P. ohirai* précédemment décrite. Afin de confirmer ceci, il a été réalisé un séquençage partiel portant sur le cytochrome C oxydase I (COI) et sur la deshydrogénase NADH1 (ND1) de l'exemplaire supposé être un *P. ohira*. Il a été observé un haut degré de similitude avec celles de *P. ohira* – COI (99,7 %) et ND1 (99,5 %) – en faveur du résultat obtenu avec ITS2. De plus, nous avons repéré des zones spécifiques d'ITS2 (BDW et BD2OH) de *P. ohira* et *P. westermani* afin de les distinguer plus facilement et rapidement. Elles ont été utilisées pour identifier sept *Paragonimus* provenant des régions de Jinde et de Xiuning. Tous les exemplaires ont été identifiés en tant que *P. westermani*. Ceci confirme que ces deux nouvelles séquences spécifiques peuvent être utile à une identification aisée de *P. ohirai* et *P. westermani*, et que la plupart des *Paragonimus* des régions de Jinde et Xiuning sont des *P. westermani*, et que *P. ohirai* est minoritaire dans la région de Jinde.

**MOTS CLÉS :** *Paragonimus ohirai*, *Paragonimus westermani*, province d'Anhui, Chine, ITS2.

## INTRODUCTION

*Paragonimus* (lung fluke) has been described in Asia, Africa and America. The intermediate host is a snail in fresh and brackish waters, and definitive hosts are usually infected by eating freshwater crustaceans containing metacercariae.

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To distinguish among morphologically similar parasites, a variety of molecular markers, including nucleotide sequences, are increasingly being used (Blair *et al.*, 1997a; Morgan & Blair, 1998). In particular, hypervariable genes evolving quickly, such as ITS1 and 2, and mitochondrial protein coding genes have been preferred for this purpose (Hwang & Kim, 1999). In the genus *Paragonimus*, the synonymy of *P. ohirai*, *P. iloktsuenensis* and *P. sadoensis*, previously considered to be separate species based on morphological characteristics, was recently proved using the sequence analysis of ITS2 and mitochondrial COI genes (Blair *et al.*, 1997b). Additionally, the geographical genetic structure within the human lung fluke, *P. westermani*, from Japan, China, Korea, Taiwan, the Philippines, peninsular Malaysia and Thailand, was examined based on ITS2 and COI sequences (Blair *et al.*, 1997a). The original purpose of this study was to elucidate the distribution pattern of *P. westermani* in Anhui province, and to estimate the genetic differences among those

from Anhui and between those in Anhui and those from other regions in China, Korea, Japan and other parts of Asia. Interestingly, we found *P. ohirai* in samples collected from Jinde County, a species not previously described in Anhui province. Here, we focused on the confirmation of *P. ohirai* based on ITS2, COI, and ND1 sequences, and also discussed the distribution of *P. westermanni* and *P. ohirai* in Jinde and Xiuning Counties (Anhui province). In addition to this, we presented *P. ohirai*- and *P. westermanni*-specific primers designed from ITS2 to identify *P. westermanni* and *P. ohirai* easily and rapidly.

## MATERIALS AND METHODS

### COLLECTION OF *PARAGONIMUS*

Freshwater crabs (*Sinopotamon yangtsekiense*) were collected in a small stream in Jinde and Xiuning Counties, Anhui Province, China in 1997-1999. The crabs were crushed in a small bowl with tap water. The crushed tissues of the crabs were suspended in artificial digestive solution (0.2 g pepsin, 0.7 ml HCl, sterile water 100 ml) for 2 hr at 37°C and then filtered once through a mesh screen. We isolated *Paragonimus* metacercariae from the filtered sediments under a dissecting microscope with micropipette. Three dogs were each fed with 50 metacercariae. After four months, *Paragonimus* adults were collected from the sacrificed dog lungs. The adult *Paragonimus* were stored in 70 % ethanol or 3 % glutaraldehyde for nucleotide analysis and ultrastructural observation, respectively.

### GENOMIC DNA EXTRACTION

A single adult *Paragonimus* stored in 70 % ethanol was suspended in lysis buffer (10 mM Tris, pH 8, 100 mM EDTA, 0.5 % SDS) and lysed with homogenizer (IKA, German). The lysate was incubated overnight at 37°C. Following consequential treatment of RNase (20 µg/ml) and proteinase K (100 µg/ml), DNA was extracted with phenol:chloroform, precipitated with 2 vol. of 100 % EtOH and 1/10 vol. of 3 M sodium acetate, rinsed with 70 % ethanol, and dissolved in TE buffer (Sambrook *et al.*, 1989).

### PCR AMPLIFICATION AND CLONING

The complete ITS2 and partial COI and ND1 genes were amplified using polymerase chain reaction (PCR) with Taq DNA polymerase. Primers suitable for PCR and sequencing were designed from evolutionarily conserved regions of the COI and NDI sequence previously published for *Fasciola hepatica* (Garey & Wolstenholme, 1989). The complete ITS2 amplification of *Paragonimus* was carried out with 3S (5'-

GGTACCGGTGGATCACTCGGCTCGTG-3', Blair, 1993) and BD2 (5'-TATGCTTAAATTCAGCGGGT-3', Bowles *et al.*, 1995). For amplifying the partial COI and ND1 genes of *P. ohirai*, JB3 corresponding to the positions 2575-2598 of *Fasciola hepatica* (5'-TTTTTTGGG-CATCCTGAGGTTTAT-3') and JB4 corresponding to the positions 3021-3044 (5'-TAAAGAAAGAACATAAT-GAAAATG-3'), JB11 (5'-AGATTCGTAAGGGGCC-TAATA-3', matched to the positions 311-331) and JB12 (5'-ACCACTAATACTAATTCACCTTC-3', matched to the positions 840-864) were employed, respectively. The reaction mixture was 5 µl of 1.5 mM Mg<sup>++</sup> containing buffer, 200 µM of each dNTP, 0.5 µM of the designed primers, 1 ng of template DNA in 50 µl. PCR amplification was performed for 30 cycles: 94°C for 30 sec, 57°C (ND1 and ITS2) and 52°C (COI) for 30 sec, 72°C for 30 sec. The amplified products were purified by extracting from an agarose gel using QiaXII (QIAGEN Co.). The purified PCR products were ligated into the pGEM T easy vector system (Promega Co.). A ligation mixture was used to transform CaCl<sub>2</sub>-competent *E. coli* TOP10. The selection of clones was executed by the Blue/White screening method. Plasmid DNA was isolated from selected clones using the alkaline method (Sambrook *et al.*, 1989).

### DNA SEQUENCE ANALYSIS

DNA sequencing was conducted with a Li-COR automatic DNA sequencer (model 4200) using T7 forward and SP6 reverse primers. Final DNA sequence construction, alignment, and comparison were facilitated using the GeneJockey II (BIOSOFT Co.), Clustal X (Thompson *et al.*, 1997), and MacClade ver 3.0 (Sinauer Associates, Inc.) programs. *Paragonimus* ITS2, COI, and ND1 sequences deposited into the EMBL data bank were retrieved and analyzed with the present data.

### *P. WESTERMANI*- AND *P. OHIRAI*-SPECIFIC PRIMER

We designed *P. westermanni*- and *P. ohirai*- specific primers from variable regions of ITS2 in order to easily and rapidly distinguish between them using PCR; BD2W (*westermanni*-specific primer; 5'-CCCACAGG-CACAAGACC-3', position 119-135) and BD2OH (*ohirai*-specific primer; 5'-CCCACCAACACATGGTC-3', position 119-135). Each specific primer was used for species-specific PCR amplification with the 3S primer.

## RESULTS

The nucleotide sequences (363bp) of the internal transcribed spacer 2 (ITS2) regions were determined in seven adults of species *Paragonimus* collected from Jinde and Xiuning Counties, Anhui Pro-

**(A)**

U96911 atattgCGGCCacgggttagcctgtggccacgcctgtccgagggtcggcttataaactat  
 Jinde atattgCGGCCacgggttagcctgtggccacgcctgtccgagggtcggcttataaactat

U96911 cGCGacGcccaaaaagtcGCGgcttgggttttGccagctggcgtgatttccccaatctga  
 Jinde cGCGacGcccaaaaagtcGCGgcttgggttttGccagctggcgtgatttccccaatctga

U96911 **ccatgTgttggTgggT**gccagatctatggcgtttccctaacctaaccgggCGTaccat  
 Jinde **ccatgTgttggTgggT**gccagatctatggcgtttccctaacctaaccgggCGTaccat

U96911 gttTgtgctgaaggccttggTggggtgTggcaacggaatcgtggctcagTggattattt  
 Jinde gttTgtgctgaaggccttggTggggtgTggcaacggaatcgtggctcagTggattattt

U96911 atgtGcGcttccgttGtcctatcatcatctatggttgatgctGcGcatggtGtGcGtcc  
 Jinde atgtGcGcttccgttGtcctatcatcatctatggttgatgctGcGcatggtGtGcGtcc

U96911 gacGccaacctacgtatgggCGctgTggctcattctcctgacctcggatcagacgtgagT  
 Jinde gacGccaacctacgtatgggCGctgTggctcattctcctgacctcggatcagacgtgagT

U96911 acc  
 Jinde acc

**(B)**

AF008189 tttgatgactctaactaataaagattcgTtGtttggTtattatggattggTgttGctA  
 Jinde tttgatgactctaactaataaagattcgTtGtttggTtattatggattggTgttGctA

AF008189 tggggCGattgtGtGtttgggaagTtGtctgggcccacatagtttatggTtggTt  
 Jinde tggggCGattgtGtGtttgggaagTtGtctgggcccacatagtttatggTtggTt

AF008189 tggatgttaagactGcagTatTTTTcagatctGttacgggggtgataggTattcctacag  
 Jinde tggatgttaagactGcagTatTTTTcagatctGttacgggggtgataggTattcctacag

AF008189 ggattaaggTTTTcttggTtattatgTtaggggtactcGtctacggTTTTgggatc  
 Jinde ggattaaggTTTTcttggTtattatgTtaggggtactcGtctacggTTTTgggatc

AF008189 ctgtaatatgatgaattcttgggttatctTTTTgtttacaataggggtgtaactggaa  
 Jinde ctgtaatatgatgaattcttgggttatctTTTTgtttacaataggggtgtaactggaa

AF008189 ttatcttGtcttctctatTTTTggacagTtattGcatgacacttggTtGttGt  
 Jinde ttatcttGtcttctctatTTTTggacagTtattGcatgacacttggTtGttGt

**(C)**

AF063787 tacagaagtttGcagatcttttgaagTtggTtataaagTtaaggTtcttcccttcagG  
 Jinde tacagaagtttGcagatcttttgaagTtggTtataaagTtaaggTtcttcccttcagG

AF063787 gTcGtagttggcttcttGatgaggggtggTgTcttggTtcttGggTtGtcttatt  
 Jinde gTcGtagttggcttcttGatgaggggtggTgTcttggTtcttGggTtGtcttatt

AF063787 gTgTcttcttcttctttagTtattctggtttgaggagaagaaatagagctcttGgtGt  
 Jinde gTgTcttcttcttctttagTtattctggtttgaggagaagaaatagagctcttGgtGt

AF063787 tggTcattactagTttacagggtatagTctttaaaggataggttgggTtcttataaaa  
 Jinde tggTcattactagTttacagggtatagTctttaaaggataggttgggTtcttataaaa

AF063787 agtatGcttTgttGagatgttTgcGatctGcGtttggTtctGtGacttTgaagcctgtt  
 Jinde agtatGcttTgttGagatgttTgcGatctGcGtttggTtctGtGacttTgaagcctgtt

AF063787 ttatgtGtattgttttattgttggctatagTtaggggatttattcattaggtccttGtt  
 Jinde ttatgtGtattgttttattgttggctatagTtaggggatttattcattaggtccttGtt

AF063787 tggag  
 Jinde tggag

Fig. 1. – Sequence comparisons of ITS2 (A), COI (B), and ND1 (C) gene regions of a *Paragonimus* sample from Jinde and *P. obirai* retrieved from EMBL data base (accession number U96911, AF008189, and AF063787, respectively). Bold letters indicate the site of *P. obirai*-specific primer (BD2OH). From the same position, *P. westermani*-specific primer was also designed.

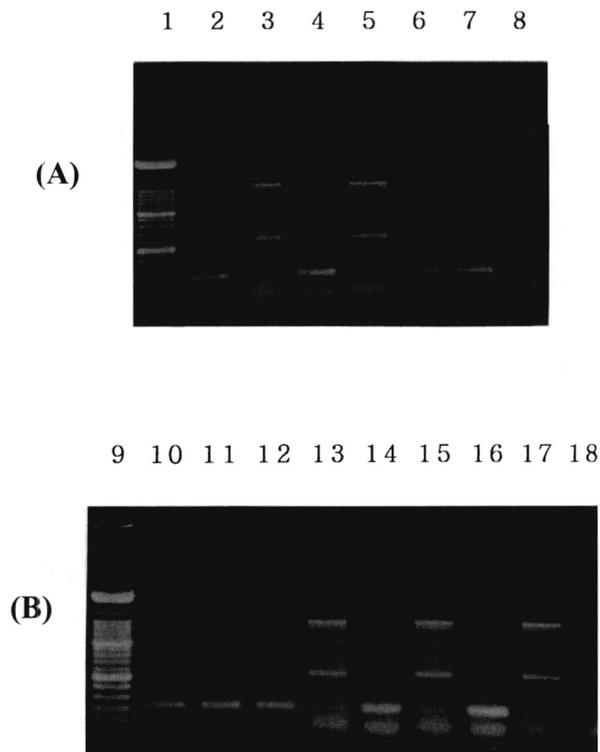


Fig. 2. – PCR identification of *Paragonimus westermani* and *P. obirai* using specific primers designed from ITS2 region. (A) Lane 1, DNA size marker 100 bp ladder; lanes 2 and 3, *P. westermani* from Bogil-do, Korea; lanes 4 and 5, *P. westermani* from Tsushima, Japan; lanes 6 and 7, *P. iloktsuenensis* from Hadong, Korea; lane 8, negative control performed without DNA. (B) Lane 9, DNA size marker 100 bp ladder; lanes 10 and 11, *P. obirai* from Jinde County, China; lanes 12 and 13, *P. westermani* from Jinde County, China; lanes 14 to 17, two individuals of *P. westermani* from Xiuning County, China; lane 18, negative control performed without DNA. Except for lanes 1, 8, 9, and 18, *P. obirai*-specific primer was used in every odd number lanes and *P. westermani*-specific primer in every even number lanes.

vince, China. Among these, six demonstrated 100 % similarity with that of Chinese *P. westermani* (accession number U96907) deposited in EMBL data base (data not shown). Only one *Paragonimus* adult (Jinde County) was completely identical to that of *P. obirai* reported (accession number U96911). To confirm this result, partial COI (355 bp) and ND1 (365 bp) from the putative *P. obirai* sample were further sequenced. A high level of similarity with those of *P. obirai*, COI (99.7 %) and ND1 (99.5 %) was observed, supporting the result obtained from the ITS2. The sequence alignments of ITS2, COI, and ND1 are shown in Figure 1. The alignments are 363, 355, and 365 in length, respectively, which have no alignment gaps.

*P. obirai*- and *P. westermani*-specific primers (BD2OH and BDW) from ITS2 sequences were designed to easily and rapidly identify *P. obirai* and *P. westermani*. First, we tested the utility of the newly designed primers (Fig. 2). Using *P. westermani* DNAs extracted

from 1 Bogil-do (Korea), 1 Tsushima (Japan), 1 Jinde, 2 Xiuning (China) samples as templates, PCR carried out with BDW and 3S showed the expected band size (230 bp). However, PCR performed with BD2OH and 3S did not produce the expected band size (only non-specific bands were observed). On the other hand, in PCR experiments using DNA extracted from one *P. iloktsuenensis* (Hadong-gun, Kyongsang nam-do, Korea) and one *P. obirai* (Jinde, China) samples as templates, PCR fragments with 230 bp were observed regardless of the kinds of specific primer employed. After examining the primer utility, the specific primers were applied to seven unidentified *Paragonimus* samples collected from Jinde and Xiuning Counties (China). As a result, PCR band pattern of *P. westermani* were observed, and it was reconfirmed by sequencing their ITS2 regions that they are *P. westermani* (data not shown).

## DISCUSSION

Since *Paragonimus obirai* was first discovered by Miyazaki (1943) at the mouth of Yangtze river in China, it has been known that *P. obirai* was distributed along the coastal region from Canton (Guangdong) to Liaoning provinces in China (Blair *et al.*, 1999). The present study proved that *P. obirai* is also distributed in Jinde County, Anhui Province on the basis of a high sequence similarity with those of the reported. *P. obirai*, ITS2 (100 %), COI(99.7 %), and ND1 (99.5 %). No report in relation to *P. obirai* in Jinde County has been published so far. The results obtained from previous surveys related with *Paragonimus* have suggested that there exist *P. westermani*, *Euparagonimus cenocopiosus*, *Paragonimus asymmetricus* in Anhui (Xu *et al.*, 2000; Blair *et al.*, 1999).

*P. westermani* is a typical hermaphroditic trematode (Digenea), which causes a human infection. They are distributed throughout China, Japan, Korea and Taiwan (Herwerden *et al.*, 1999). In this study, ITS2 sequences of six *P. westermani* from Jinde and Xiuning Counties, Anhui Province were completely identical. Through a BLAST search, we found that they have the same sequences as the Chinese *P. westermani* ITS2 sequence retrieved from the EMBL data base (accession number U96911, Tanegashima, Japan). Such 100 % identity among their ITS2 sequences reflects that *P. westermani* ITS2 is highly conserved. Recently, it has been demonstrated that spacers such as ITS2 are found to be highly conserved within species and display between 1.1 and 19.2 % sequence divergence between species (Morgan & Blair, 1998). Blair *et al.* (1997a) also insisted upon a similar viewpoint. According to their results, the ITS2 sequences of *P. westermani* from China, Japan,

and Korea were almost the same, showing the highly conserved property of ITS2 in *P. westermani*.

To easily and rapidly identify *Paragonimus* samples, *P. obirai*- and *P. westermani*-specific primers (BDW and BD2OH) designed from ITS2 could successfully distinguish between *P. westermani* and *P. obirai*. In the PCR experiments conducted with BDW and 3S, however, PCR fragments with same size (230bp) were observed using *P. obirai* (or *P. iloktsuenensis*) DNA as well as using *P. westermani* DNA as a template. Because it was in the same region (position 119-135) that we designed BDW and BD2OH, it is possible to happen if specificity of BDW is low. In the primer-designed sites (17 bp), only six bases are different between those of *P. westermani* and *P. obirai*. Thus, it may be needed to design other *P. westermani*-specific primer having higher specificity. Despite the relatively lower capacity of BDW to distinguish between *P. westermani* and *P. obirai*, simultaneous use of BDW and BD2OH primers makes it possible to identify *P. obirai* and *P. westermani* and to distinguish between them. The identification method using the two specific primers was applied to seven unidentified *Paragonimus* samples collected from Jinde and Xiuning Counties (China) (data not shown). It appeared that all the examined samples are *P. westermani*. The result indicated that the two newly designed specific primers could be quite helpful for easily identifying *P. westermani* and *P. obirai*, that most of *Paragonimus* in Jinde and Xiuning Counties consist of *P. westermani*, and that *P. obirai* exists in Jinde County with minority.

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