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PCR-RFLP patterns of three kinds of *Metagonimus* in Korea

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Abstract: We tried to compare the three kinds of *Metagonimus* species, *M. yokogawai*, *Metagonimus* Miyata type, and *M. takahashii*, which were known to be distributed in Korea with polymerase chain reaction based-restriction fragment length polymorphism (PCR-RFLP) patterns. We amplified the internal transcribed spacer 1 (ITS1) site of ribosomal RNA and mitochondrial cytochrome *c* oxidase I (mCOI) gene. The restriction patterns of ITS1 gene with *Rsa* I, *Alu* I and *Msp* I showed multiple fragmented bands of different sizes between three kinds of *Metagonimus*. In case of mCOI gene, *Rsa* I and *Alu* I enzymes produced differentially fragmented band patterns. According to the parsimony analysis of PCR-RFLP patterns, the estimated genetic divergence between *M. yokogawai* and *Metagonimus* Miyata type was 0.034880, between *Metagonimus* Miyata type and *M. takahashii* was 0.028098, between *M. yokogawai* and *M. takahashii* was 0.018179. It is suggested that *Metagonimus* Miyata type may be separate species and evolutionize at the older time than the other two species.

Key words: *Metagonimus yokogawai*, *Metagonimus* Miyata type, *M. takahashii*, PCR-RFLP

INTRODUCTION

The infection rate of *Metagonimus yokogawai* in Korea was 0.3% through the nationwide survey in 1992 (MHSA and KAH, 1992). It means that *M. yokogawai* is the third prevalent human parasite in Korea. It is quite surprising that the small village in Chungchongbuk-do has as much as 20% of infected residents with *Metagonimus* species (Yu *et al.*, 1994). From the aspects of taxonomy, *Metagonimus* species have many controversies yet. Saito (1984) proposed 4 types of *Metagonimus* species based on the morphology and biology. But other scientists disagree on his suggestion because there were many artificial factors inducing morphological differences (Koga, 1938; Kogame, 1939; Takabayashi, 1953; Ito, 1964).

Chai *et al.* (1993) reported *Metagonimus* Miyata type and *M. takahashii* at the upper reaches of Namhangang (River). In this area, *Zacco platypus* and *Carassius carassius* were reported as the second intermediate hosts of Miyata type and *M. takahashii*, respectively. However, *M. yokogawai* could not be found in this region. So, we are curious whether the three kinds of *Metagonimus* are taxonomically valid species. Recently, Bowles and McManus (1993) tried polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) method to discriminate

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Echinococcus species rapidly. They recommended it as a good method to differentiate the worms in confusing status. In this study, we observed the PCR-RFLP patterns of the three kinds of *Metagonimus* to determine their taxonomic significance.

MATERIALS AND METHODS

Parasites collection

Metacercariae (Mc) of *M. yokogawai* were collected by artificial digestion of *Plecoglossus altivelis* caught at Oshipcheon (Stream), Samchok-gun, Kangwon-do. Mc of *Metagonimus* Miyata type and *M. takahashii* were collected from *Z. platypus* and *C. carassius*, respectively, caught at Talchongang, Chungju. Three kinds of adult worms were obtained from experimental rats (Sprague Dawley) through oral infection. Collected worms were stored at -20°C until being used for DNA extraction.

DNA extraction

The adult worms of three species were pulverized using a liquid nitrogen-cooled mortar and pestle. Approximately 1/10 volume of pulverized tissue was placed into a 1.5 ml tube containing lysis buffer (10 mM Tris-Cl, pH 8.0, 100 mM EDTA, 0.5% SDS). These samples were thoroughly mixed and incubated overnight at 37°C. Samples were incubated for 2 hrs at 37°C with proteinase K (100 μg/ml) and RNase (20 μg/ml), subsequently extracted with an equal volume of phenol:chloroform: isoamyl alcohol. Total genomic DNA was finally precipitated overnight with addition of 1/20 volume 5 M NaCl and 2 volume 99% ethanol at -70°C. Genomic DNA pellet was obtained by centrifugation at 10,000 *g* for 10 min and resuspended in 50 μl of TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) buffer. RNA in the sample was digested by the addition of 0.1 volume RNase A (10 mg/ml) and incubated for 1 hr at 37°C. Extraction using phenol:chloroform: isoamyl alcohol was done as before. Residual phenol was removed by an chloroform extraction. Total genomic DNA was precipitated as before, resuspended in 25 μl of TE buffer and stored at 4°C.

Table 1. The sequences of primers used for PCR reaction

primers	sequences
BD1	5' GTCGTAACAAGGTTCCGTA 3'
4S	5' TCTAGATGCGTTCGAAGTGTCGATG 3'
3S	5' GGTACCGGTGGATCACTCGGCTCG 3'
JB3	5' TTTTTTGGGCATCCTGAGGTTTAT 3'
JB4.5	5' TAAAGAAAGAACATAATGAAAATG 3'

PCR amplification

Primers BD1 (forward), 4S (reverse) and 3S (forward), designed by Bowles and McManus (1993) to hybridize to the conserved sequences between 18S rRNA and 5.8S rRNA gene, were used (Bowles and McManus, 1993). Internal transcribed spacer 1 (ITS1) of the rDNA repeat unit was obtained by PCR using BD1 and 4S. For the PCR product of ITS1 which included 5.8S rRNA gene, 4S and 3S were used to make a probe for ITS1. For amplifying mitochondrial COI (mCOI) gene, JB3 and JB4.5 primers designed as general primer to amplify mCOI gene based on mCOI gene of *Fasciola hepatica* were used (Bowles and McManus, 1994). The seqences of used primers were shown in Table 1. A 100 μl volume mixture containing sample DNA (90 ng), 2.5 mM $MgCl_2$, 100 μM of each dNTP (Promega), 25 pmol of each of primers and 2.5 units of *Thermus aquaticus* DNA polymerase (Promega) was amplified by PCR (Perkin Elmer). PCR DIG probe synthesis kit (Boehringer Manheim) was used for making probe (5.8S rRNA gene) detecting ITS1. PCR for ITS1 was programed as follows: 95°C for 45 sec (denaturation), 51°C for 1 min (annealing), and 72°C for 90 sec (extension). PCR program for mCOI gene was as follows: 94°C for 1 min (denaturation), 50°C for 1 min (annealing), and 72°C for 2 min (extension). Both cycles were repeated 45 times.

Southern blotting

PCR products of BD1 and 4S were electrophoresed through 1% (w/v) agarose LE (BM) gels (Tris-acetate EDTA). DNA fragments were transferred to nylon membrane (Amersham) by traditional method (Darling and Brickell, 1994), which were probed by digoxigenin-labelled 5.8S rRNA gene. DIG nucleic acid detection kit (BM) was used to detect the band.

Enzyme digestion

PCR products from both templating genes were digested for overnight with the restriction endonucleases *Rsa* I, *Alu* I, *Msp* I, *Cfo* I (10 U, 37°C) and *Taq* I (10 U, 65°C) using buffers recommended by the manufacturer (BM). Restriction fragments were analysed by electrophoresis through 3% (w/v) Tris-acetate EDTA (TAE) agarose gel and detected by staining with ethidium bromide. Restriction patterns were analyzed using RESTSITE. The estimated genetic convergence was calculated using CALCD and the dendrogram was constructed using UPGMA (Miller, 1994).

RESULTS

The PCR products for ITS1 fragments were shown as two bands from all three species (Fig. 1). But the smaller band (872 bp) was thicker than larger one (1,605 bp). Both bands were confirmed to be ITS1 by southern blotting using 5.8S ribosomal DNA probe (Fig. 1). These PCR products were digested by *Rsa* I, *Alu* I and *Msp* I. The fragmented patterns of the three kinds of worms were different. The fragmented band pattern was the same between *Metagonimus* Miyata type and *M. takahashii*, but different from *M. yokogawai* by *Rsa* I digestion (Fig. 2). There was one more band (289 bp) in *M. yokogawai* than the other. More various sized bands were produced by *Alu* I digestion. *M. yokogawai* and *M. takahashii* have two more bands (442 bp and 250 bp) on *Alu* I digestion, and three more bands (776 bp, 450 bp and 267 bp) than in *Metagonimus* Miyata type on *Msp* I digestion (Fig. 2). All three kinds of *Metagonimus* were not digested by *Cfo* I and *Taq* I.

The sizes of PCR products for mCOI gene

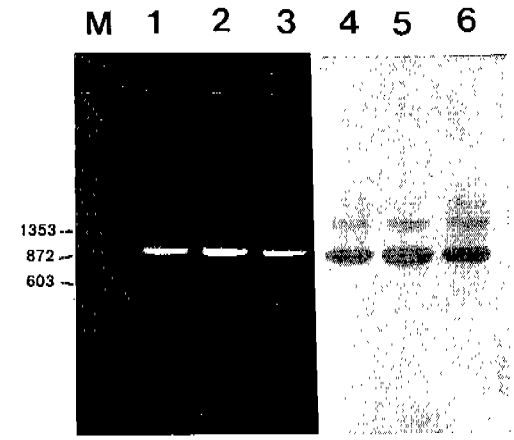

Fig. 1. PCR products for the ITS1 region of rDNA of three *Metagonimus* spp. Lane 1, *Metagonimus* Miyata type; lane 2, *M. yokogawai*; lane 3, *M. takahashii*; lane 4-6, southern blotting using DIG labeled 5.8S rDNA probes. M, marker.

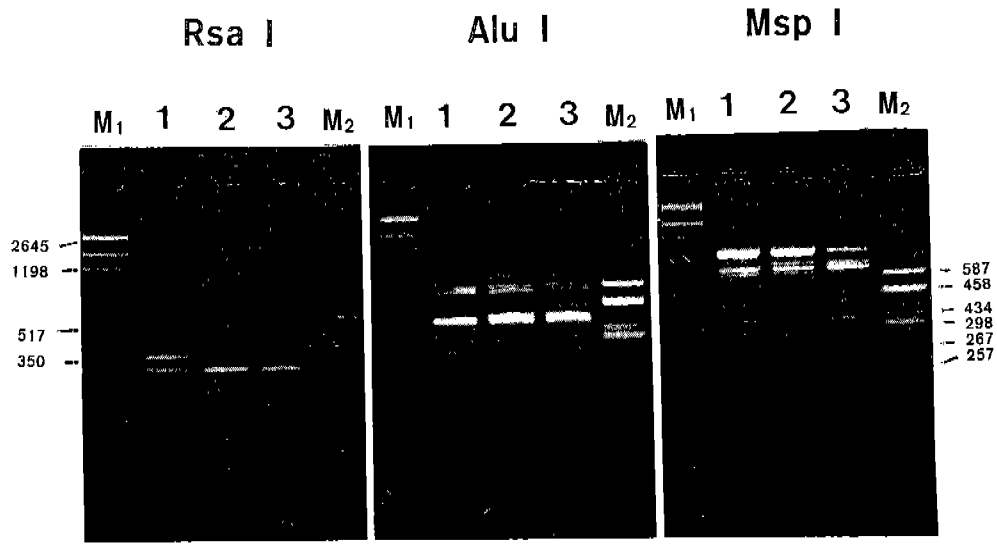

Fig. 2. PCR-RFLP products of the ITS1 gene digested with *Rsa* I, *Alu* I, and *Msp* I enzymes. Lane 1, *Metagonimus* Miyata type; lane 2, *M. yokogawai*; lane 3, *M. takahashii*. M_1, M_2, marker.

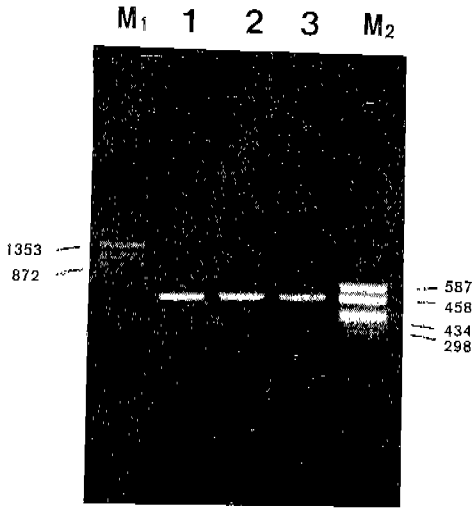

Fig. 3. PCR products for the mCOI gene of three kinds of *Metagonimus* spp. Lane 1. *Metagonimus* Miyata type; lane 2. *M. yokogawai*; lane 3. *M. takahashii*. M_1, M_2, marker.

from three kinds of *Metagonimus* were the same (458 bp) (Fig. 3). But all three species showed different bands when digested with *Rsa* I and *Alu* I (Fig. 4). With *Cfo* I digestion, PCR products of *Metagonimus* Miyata type and *M. takahashii* were not fragmented, but that of *M. yokogawai* splited as two bands (Fig. 4). With *Msp* I digestion, *Metagonimus* Miyata type was not digested but the others were fragmented in the same pattern (Fig. 4). Proportions of homologous fragments and estimated genetic convergence was shown in Table 2. The dendrogram of the three kinds of *Metagonimus* was shown in Fig. 5.

Table 2. Proportion of homologous fragments (upper triangle) and estimated genetic divergence (lower triangle)

	Metagonimus Miyata type	*M. yokogawai*	*M. takahashii*
Metagonimus Miyata type		17/34	11/34
M. yokogawai	0.034880		
M. takahashii	0.028098	0.018179	

DISCUSSION

Taxonomically the genus *Metagonimus* has five species such as *M. yokogawai* (Katsurada, 1912), *M. takahashii* (Suzuki, 1930), *M. minutus* (Katsurada, 1932), *M. katsuradai* (Izumi, 1935) and *M. otsurui* (Saito and Shimizu, 1968). Among them, *M. yokogawai* and *M. takahashii* were still unclear in that they are true separate species. Furthermore, since *Metagonimus* Miyata type had been newly

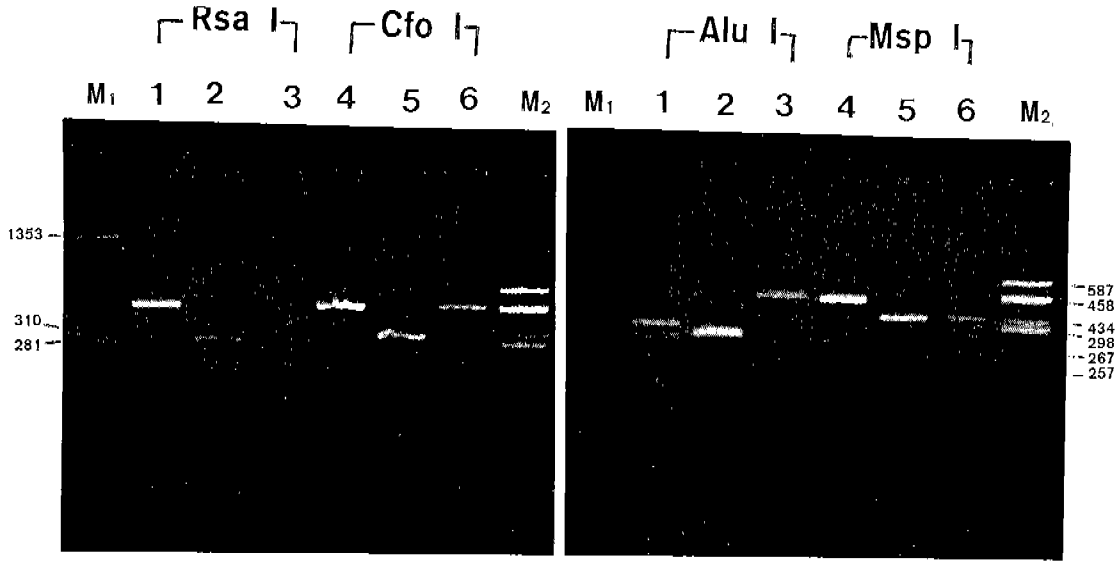

Fig. 4. PCR-RFLP products of the mCOI gene digested with *Rsa* I, *Cfo* I, *Alu* I, and *Msp* I. Lane 1 & 4, *Metagonimus* Miyata type; lane 2 & 5, *M. yokogawai*; lane 3 & 6: *M. takahashii*. M_1, M_2, marker.

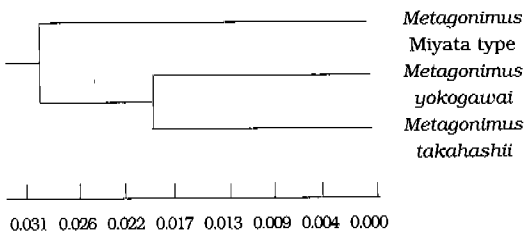

Fig. 5. Dendrogram of estimated genetic divergence of the three kinds of *Metagonimus* using program UPGMA v2.0.

described by Saito (1984), these three kinds of *Metagonimus* became confusing complex.

Morphological differential points were described as follows: 1) the location of end point of testes, 2) distribution of uterine tubule, 3) endmost position of vitelline follicles and 4) egg sizes (Chai *et al.*, 1993). Geographical distribution is also different. Although Chai and Lee (1990) described the endemic areas of metagonimiasis as confined to the eastern coastal and southern riverside areas, inland areas where sweetfish don't reside were also evaluated as endemic areas of metagonimiasis after that study (Chai *et al.*, 1993; Yu *et al.*, 1994). Biologically the second intermediate host of *M. yokogawai* is known as *P. altivelis* and *Tribolodon taczanowskii*, that of *Metagonimus* Miyata type and *M. takahashii* as *Z. platypus* and *C. carassius*, respectively (Chai *et al.*, 1993).

In this study, we tried to evaluate the differential points in DNA level between three *Metagonimus* species by PCR-RFLP study which was already proved to be effective to discriminate the parasites in taxonomically confusing status. Ribosomal RNA genes are very strongly conserved coding regions separated by relatively poorly conserved non-coding spacer regions (Long and Dawid, 1980). Transcribed spacers are present in the primary gene transcript, and generally found to be less variable in length and sequence than the non-transcribed spacers (Botchan *et al.*, 1977). Because of the highly conserved nature of the rRNA genes, most of the polymorphism detected between strains of *Echinococcus* results from sequence dissimilarity in the ITS1 and ITS2 spacer regions (Bowles and McManus, 1993). Bowles and McManus (1993) recommended PCR-RFLP analysis as a simple and rapid method to distinguish parasites. They also succeeded in discriminating parasites in taxonomically confusing status such as taeniid cestodes and *Schistosoma* species as well as *Echinococcus granulosus* (Bowles and McManus, 1993 & 1994; Bowles *et al.*, 1994). The results of PCR-RFLP of ITS1 digestion in this study showed that all three kinds of *Metagonimus* have different PCR-RFLP patterns. And also the result of PCR-RFLP of mCOI gene showed differences. The primers used in this study, designed by Bowles *et al.* (1993), were thought to be as suitable to *Metagonimus* spp. as to *E. granulosus* or *Taenia* species. So, it could be thought that these three species have different sequences in ITS1 and mCOI genes. According to the dendrogram, *Metagonimus* Miyata type may be evolutionized at the older time than the other two worms. We added another evidence that both *Metagonimus* Miyata type and *M. takahashii* are genetically distinct parasites from *M. yokogawai*.

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=초록=

국내에 존재하는 세 종류 메타고니무스속 흡충의 RCR-RFLP 반응양상

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메타고니무스속 흡충의 형태학적인 차이점은 잘 알려져 있으나 이러한 미세한 형태학적 차이로 종을 분류할 수 있을 지에 대해서는 의문시되어 왔다. 이 연구는 비교적 유전자 염기서열이 잘 보존되어 있어 종간 또는 strain간의 차이를 밝힐 수 있는 리보솜리보핵산 유전자 중 ITS1 유전자와 사립체 COI 유전자를 중합효소반응으로 증폭시킨 후 제한효소로 소화시켜 나타나는 밴드의 차이를 관찰하였다. 요코가와흡충 (*M. yokogawai*)의 피낭유충은 삼척산 은어에서, 미야타흡충 (*Metagonimus* Miyata type)은 충주산 피라미에서, 타카하시흡충 (*M. takahashii*)은 충주산 붕어에서 분리하여 사용하였다. 세 종류 충체에서 얻은 ITS1 유전자 증폭산물은 제한효소 *Rsa* I, *Alu* I 및 *Msp* I에 의해 서로 다른 크기의 밴드로 소화되었다. 세 종류 충체의 사립체 COI 유전자 증폭산물도 *Rsa* I과 *Alu* I에 의해 서로 다른 양상으로 잘라졌다. 추정 유전자 차이 (estimated genetic divergence)는 미야타흡충과 요코가와흡충이 0.034880, 요코가와흡충과 타카하시흡충이 0.018179, 미야타흡충과 타카하시흡충이 0.028098 이었다. 이 결과로 보면 미야타흡충은 별개의 종으로 볼 수 있으며, 다른 충체보다 이른 시기에 진화하였음을 알 수 있다.

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