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Sequence comparisons of 28S ribosomal DNA and mitochondrial cytochrome c oxidase subunit I of *Metagonimus yokogawai, M. takahashii* and *M. miyatai*

Soo-Ung LEE[1], Sun HUH[1]*, Woon-Mok SOHN[2] and Jong-Yil CHAI[3]

[1]*Department of Parasitology, College of Medicine, Hallym University, Chuncheon 200-702,*
[2]*Department of Parasitology, Institute of Health Sciences,*
Gyeongsang National University College of Medicine, Jinju 660-751, and
[3]*Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine,*
Seoul 110-799, Korea

Abstract: We compared the DNA sequences of the genus *Metagonimus*: *M. yokogawai*, *M. takahashii*, and *M. miyatai*. We obtained 28S D1 ribosomal DNA (rDNA) and mitochondrial cytochrome c oxidase subunit I (mtCOI) fragments from the adult worms by PCR, that were cloned and sequenced. Phylogenetic relationships inferred from the nucleotide sequences of the 28S D1 rDNA and mtCOI gene. *M. takahashii* and *M. yokogawai* are placed in the same clade supported by DNA sequence and phylogenic tree analysis in 28S D1 rDNA and mtCOI gene region. The above findings tell us that *M. takahashii* is closer to *M. yokogawai* than to *M. miyatai* genetically. This phylogenetic data also support the nomination of *M. miyatai* as a separate species.

Key words: *Metagonimus*, Heterophyidae, polymerase chain reaction, sequence analysis, DNA, classification

INTRODUCTION

Metagonimiasis is an endemic intestinal trematode infection in Korea with 0.3% egg positives in the general population, i.e. 130,000 infected people (Ministry of Health and Social Welfare, and Korea Association of Health, 1997). Human infection with the three *Metagonimus* (Digenea: Heterophyidae) species is due to eating of raw fishes (intermediate hosts), and is regarded as a parasitic disease of importance to public health in the riverside areas of the southern and eastern coasts of Korea (Yu et al., 1994; Chai et al., 2000b; Lee et al., 2002). Three species of the genus *Metagonimus* are known in Korea (Chai et al., 1991): *M. yokogawai* (Katsurada, 1912), *M. takahashii* (Suzuki, 1930), and *M. miyatai* (Miyata, 1941, 1944; Saito et al., 1984, 1997). These three species are differentiated by their morphology (Chai et al., 1991, 1998, 2000a), as they have different fish species as hosts (Rim et al.,

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*Corresponding author (e-mail: shuh@hallym.ac.kr)

1996), different geographical distributions (Kim et al., 1987; Chai et al., 1993; Yu et al., 1994; Lee et al., 2002). In addition, they have different polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) patterns and random amplified polymorphic DNA (RAPD) patterns (Yu et al., 1997a, 1997b), and simple sequence repeat anchored polymerase chain reaction (SSR-PCR) patterns of genomic DNA of ribosomal RNA and mitochondrial cytochrome c oxidase 1 (mtCOI) gene (Yang et al., 2000). However, few studies have been conducted on the DNA sequences of *Metagonimus* spp. In the present study, we compared the 28S D1 rDNA and mtCOI gene sequences of the three species of *Metagonimus* distributed in the Republic of Korea, to determine their molecular phylogenies.

MATERIALS AND METHODS

Genomic DNA purification from worms

The adult specimens of *M. takahashii*, *M. miyatai* and *M. yokogawai* species were obtained 7 days post-infection from rats (Sprague-Dawley, 4 to 6-wk-old). Metacercariae of each species were collected from Korean freshwater fishes, *Carassius auratus*, *Zacco platypus*, and *Plecoglossus altivelis*, respectively (Saito et al., 1997; Yu et al., 1997b). Two adult trematodes of family Heterophyidae, *Pygidiopsis summa* and *Stellantchasmus falcatus* obtained from experimentally infected animals were included and *S. falcatus* was used as an outgroup to infer phylogeny. Adult worms were stored at –70°C until used. DNA was extracted using the phenol/chloroform method and precipitated in ethanol, as reported by Sambrook and Russell (2001).

28S D1 rDNA and mtCOI PCR

PCR was conducted using a mixed solution of extracted DNA as a template (0.01 $\mu g/\mu l$), primer, and ExTaq enzyme (TaKaRa Ex Taq Kit, TAKARA Shuzo Co., LTD. Japan) in a GeneAmp PCR System 9600 (Perkin Elmer, USA). The PCR reaction cycle consisted of 40 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds followed by a final extension of 6 minutes. The forward primer (JB10, 5′ GATTACCCGCTGAACTTAAGCATA 3′) consisted of the conserved region from the 21st-45th base pairs of 28S rDNA sequence of mouse. The reverse primer (JB9, 5′ GCTGCATTCACAAACACCCCGACTC 3′) was obtained from the 278th-302nd base pairs of the same gene (Hassounna et al., 1984; Qu et al., 1988; Bowles and McManus, 1994). The PCR of mtCOI were performed using the above described method, but with different primers. The forward primer was JB3 (5′ TTTTTTGGGCATCCTGAGGTTTAT 3′) (2575), and the reverse primer was JB4.5 (5′ TAAAGAAAGAACATAATGAAAATG 3′) (3021). The numbers in brackets refer to the position of the 5′ end of the primer from the nucleotide sequences of *Fasciola hepatica* (Bowles and McManus, 1994). The PCR condition used was the same as that described above, except for that annealing was done at 48°C for 30 seconds.

Cloning and sequencing of 28S D1 rDNA and mtCOI

The PCR products amplified with the primer sets were purified by gel extraction (QIAEX DNA Gel extraction Kit, QIAGEN Co., Germany) and were subcloned into the EcoRV site of a pT7Blue T-vector Kit plus ligase (Novagen Co., USA), according to the protocol from the supplier. For transformation, NovaBlue competent cells were used as a host cell (Novagen Co., WI, USA). The recombinant plasmid was screened using isopropyl-β-thiogalactoside (IPTG) and 5-bromo-4 chloro-3-indolyl-β-D-galactoside (X-gal). The cloned fragments in the recombinant plasmids were digested with *Bam*HI and *Hind*III enzyme and purified from agarose gel using a QIAprep spin plasmid kit (QIAGEN Co.). DNA sequencing was performed by dideoxy chain termination method (Sanger et al., 1977) using a Sequenase kit (ABI prism dye terminator cycle sequencing core kit, Perkin Elmer) and an automated DNA sequencer (Applied Biosystems model 373A, Perkin Elmer) on both strands with T3 and T7 primers. At least three clones were sequenced per sample with additional clones sequenced as necessary to resolve ambiguous sites.

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M.takahashii    CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTTAGTAACGGCGAGTGAACAGGGA   60
M.yokogawai     CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTAAGTAACGGCGAGTGAACAGGGA   60
M.miyatai       CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTTAGTAACGGCGAGTGAACAGGGA   60
P.summa         CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTCAGTAACGGCGAGTGAACAGGGA   60
S.falcatus      CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTCAGTAACGGCGAGTGAACAGGGA   60
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M.takahashii    AAAGCCCAGCACCGAAGCCTGTGACCATTTGGTTACTAGGCAATGTGGTGTTCAGGTCGT  120
M.yokogawai     AAAGCCCAGCACCGAAGCCTGTGACCATTTGGTTACTAGGCAATGTGGTGTTCAGGTCGT  120
M.miyatai       AAAGCCCAGCACCGAAGCCTGTGACCATTTGGTTACTAGGCAATGTGGTGTTCAGGTCGT  120
P.summa         AAAGCCCAGCACCGAAGCCTGTGGCCAATTGGTCACTAGGCAATGTGGTGTTCAGGTCGT  120
S.falcatus      AAAGCCCAGCACCGAAGCCTGTGGCCAATTGGTCACTAGGCAATGTGGTGTTCAGGTCGT  120
                ********************** *** ***** **************************

M.takahashii    TCCGCGAAGGTGCTGCTCCATTCCAAGTCCAGCAATGAGTACGGTAATGCTGACATGGCC  180
M.yokogawai     TCCGCGAAGGTGCTGCTCCATTCCAAGTCCAGCAATGAGTACGGTAATGCTGACATGGCC  180
M.miyatai       TCCGCGAAGGTGCTGCTCCATTCCAAGTCCAGCAATGAGTACGGTAATGCTGACATGGCC  180
P.summa         TCCGCGGAGGTGCTGCTCCATTCCAAGTCCAGCAATGAGTACGGTAATGCTGACATGGCC  180
S.falcatus      TCCGCGGAGGTGCTGCTCCATTCCAAGTCCAGCAATGAGTACGGTAATGCTGACATGGCC  180
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M.takahashii    CAAAGAGGGTGAAAGGCCCGTTGGGGTGGAGGTGCAAAAATGTCAGTGCCTTCCTGGGTT  240
M.yokogawai     CAAAGAGGGTGAAAGGCCCGTTGGGGTGGAGGTGCAAAAATGTCAGTGCCTTCCTGGGTT  240
M.miyatai       CAAAGAGGGTGAAAGGCCCGTTGGGGTGGAGGTGCAGAAATGTCAGTGCCTTCCTGGGTT  240
P.summa         CAAAGAGGGTGAAAGGCCCGTTGGGGTGGAGAGGCAGAAATGACAGCACCTTCCTGGAT-  239
S.falcatus      CAGAGAGGGTGAAAGGCCCGTTGGGGTGGAGAGGCAGAAATGACAGCACCTTCCTGGAT-  239
                ** ***************************  *** ***** ***  ********* *

M.takahashii    AGACCTTG    248
M.yokogawai     AGACCTTG    248
M.miyatai       AGACCTTG    248
P.summa         AGACCTTG    247
S.falcatus      AGACCTTG    247
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Fig. 1. Sequences alignment of 28S D1 rDNA region of *Metagonimus* species. An asterisk (*) denotes an identical nucleotide position, and alignment gaps are indicated by a hyphen. Sequences for each species have been deposited in the GenBank databases (GenBank accession number: *M. miyatai*: AF095333, *M. takahashii*: AF095332, *M. yokogawai*: AF095331, *P. summa*: AF181885, *S. falcatus*: AF181886).

Sequence analysis and alignment

For the analysis of DNA sequences, NCBI (National Center for Biotechnology Information) databases were used for homology analysis (BLAST2). We also calculated the fractional GC content of nucleic acid sequences using an EMBOSS GEECEE program in Sanger Institute, Cambridge, U.K. (http://analysis.molbiol.ox.ac.uk/pise_html/geecee.html). All obtained sequences were aligned automatically using CLUSTAL W program (version 1.82, CLUSTAL W WWW Service at the European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw) for multiple sequence alignments while alignment gaps were treated as missing data (Higgins et al., 1994, European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw/). Sequence format was Pearson (Fasta) (Pearson and Lipman, 1988); sequence type was

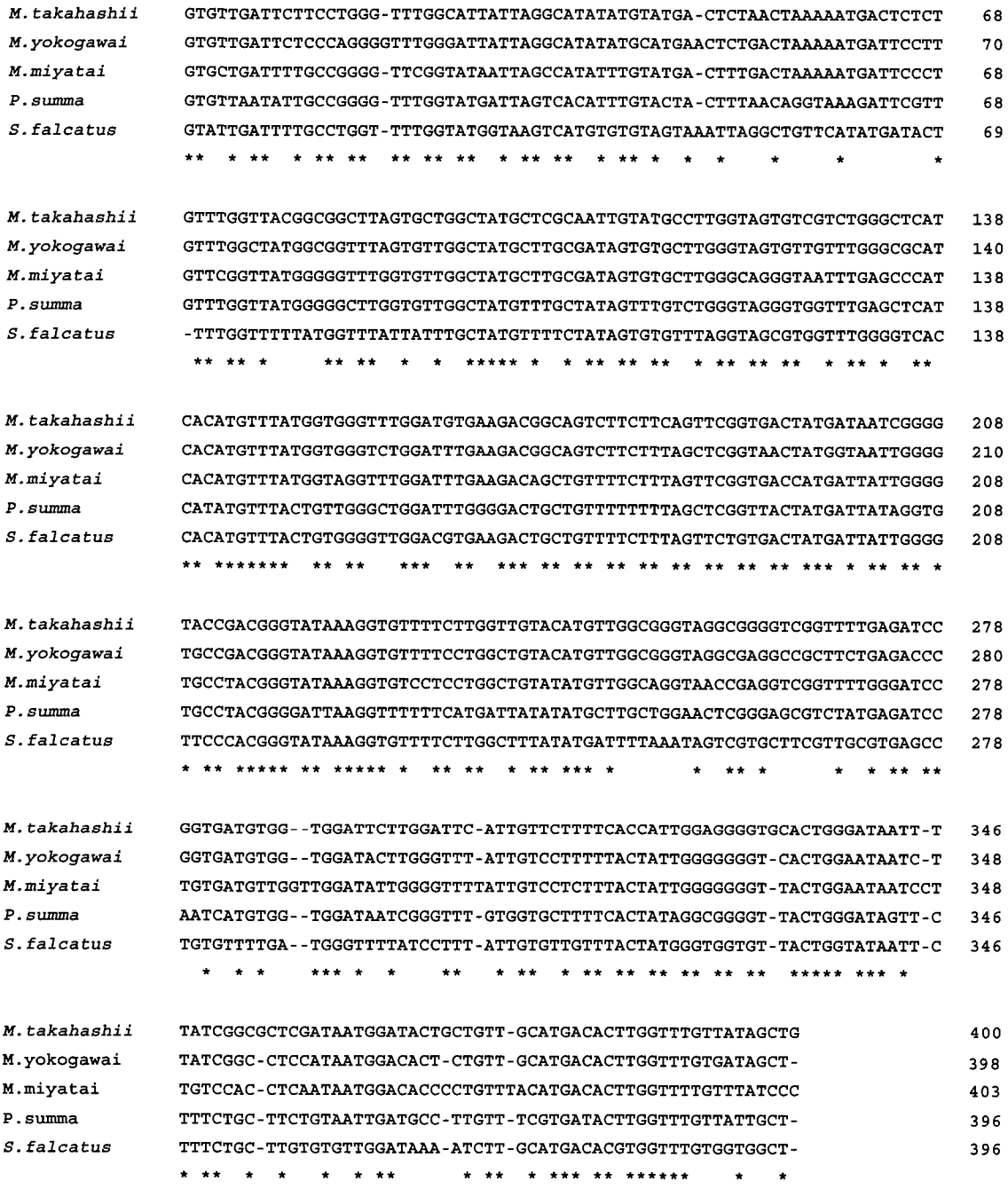

Fig. 2. Sequences alignment of mtCOI region of *Metagonimus* species. An asterisk (*) denotes an identical nucleotide position, and alignment gaps are indicated by a hyphen. Sequences for each species have been deposited in the GenBank databases (GenBank accession number: *M. miyatai*: AF096232, *M. takahashii*: AF096231, *M. yokogawai*: AF096230, *P. summa*: AF181884, *S. falcatus*: AF181887).

nucleotide (nt). The sequences were submitted to GenBank data base to get the accession numbers.

Phylogenetic tree

Phylogenetic trees were generated by TREEVIEW tree drawing software for windows (version 1.6.6, Page, 1996) from aligned nucleotide sequence by CLUSTAL W program. To observe the tree type, we used Neighbor-Joining (NJ) as distance method (Saitou and Nei, 1987), phylogeny inference package

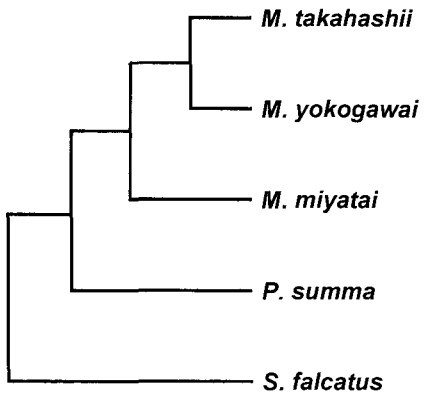

Fig. 3. Phylogenetic trees of *Metagonimus* species inferred from the 28S rDNA D1 gene nucleotide sequences by TREEVIEW program with the neighbor-joining and parsimony methods. The phylogenetic trees were outgroup rooted using the 28S D1 gene nucleotide sequence of *Stellantchasmus falcatus*.

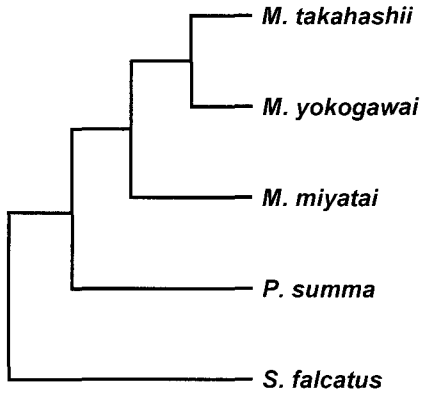

Fig. 4. Phylogenetic trees of *Metagonimus* species inferred from the mtCOI gene nucleotide sequences by TREEVIEW program with the neighbor-joining and parsimony methods. The phylogenetic trees were outgroup rooted using the mtCOI gene nucleotide sequence of *Stellantchasmus falcatus*.

(PHYLIP) as parsimony method (version 3.4., Felsenstein, 1989, 1993). Alignment gaps were treated as missing data (Hendy and Penny, 1982, Hillis et al., 1996). The correction for nucleotide distance was done by the Kimura's 2 parameter method (Kimura, 1980). The phylogenetic trees were outgroup rooted using the 28S D1 rDNA and mtCOI gene nucleotide sequences of *S. falcatus*, because it represented a sister taxon.

RESULTS

The length of the 28S D1 region rDNA sequence of the three *Metagonimus* species was 248 bp (adjusted for missing data) and its G+C content was 52% (*M. takahashii, M. yokogawai* and *M. miyatai*). Nucleotide sequence differences among *Metagonimus* species were less than 0.8% (2/248 bp), and that between the species, 0.4% (1/248 bp). There was no sequence gap in 28S D1 rDNA region (Fig. 1). The length of the mtCOI sequence averaged 400 bp (398-403 bp, adjusted for missing data) with a G+C content ranged from 44% (*M. miyatai*), 46% (*M. takahashii*) to 47% (*M. yokogawai*) (data not shown). Nucleotide sequence differences between species were 23.0% (92/400 bp) between *M. miyatai* and *M. takahashii*, 16.2% (65/400 bp) between *M. miyatai* and *M. yokogawai*, 13.2% (53/400 bp) between *M. takahashii* and *M. yokogawai* adjusted for missing data (Fig. 2). Nucleotide gaps sequence differences were 2.5% (10/400 bp) between *M. miyatai* and *M. takahashii*, 2.7% (11/400 bp) between *M. miyatai* and *M. yokogawai*, and 2.4% (11/400 bp) between *M. takahashii* and *M. yokogawai* (Fig. 2). The aligned sequences of three *Metagonimus* species showed high similarities with other comparative human intestinal trematodes (*P. summa* and *S. falcatus*) for 28S D1 rDNA (95.0%, 234/248 bp and 94%, 233/248 bp) and the mtCOI gene (68.5%: 274/400 bp and 78.0%: 312/400 bp) from nucleotide BLAST database program in NCBI (Figs. 1, 2). *M. takahashii* and *M. yokogawai* placed in the same clade supported by DNA sequence and phylogenetic tree analysis for 28S D1 rDNA and mtCOI gene by neighbor-joining and parsimony method. *M. miyatai* placed in different clade from two other *Metagonimus* species (Figs. 3, 4).

DISCUSSION

Our data coincided with the previously reported data, i.e., general morphology, PCR-RFLP, PCR-RAPD and SSR-PCR patterns of the three *Metagonimus* species. Rim et al. (1996) reported that the general morphology of the three species of *Metagonimus* was distinct and *M. miyatai* type was distinctive from other

species of *Metagonimus*. Yu et al (1997a & 1997b) suggested that the *M. miyata* type had different DNA sequences from *M. yakogawai* by RAPD and RFLP patterns of ITS1 and mtCOI gene. Yang et al. (2000) showed that the three species of *Metagonimus* had different genotypes. Our data supported the suggestions of previous genetic studies. However, sequence data did not coincide with the results of the chromosome analyses of these three *Metagonimus* species. Lee et al. (1999) also suggested that the karyology of *Metagonimus* species had closer relationship between *M. miyatai* and *M. takahashii* (both 2n = 18) rather than between *M. miyatai* and *M. yokogawai* (2n = 32). This kind of discrepancy may be seen between karyologic and sequencing studies. We add a genetic evidence on the distinctness of *M. miyatai* from *M. yokogawai* and *M. takahashii*.

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