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Genetic comparison between *Spirometra erinacei* and *S. mansonoides* using PCR-RFLP analysis

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Abstract: The only observed morphological difference between *Spirometra erinacei* and *S. mansonoides* is the uterine shape of the mature proglottid. Two species of worms are thought to be evolutionarily closely related. Biomolecular comparison of the two worms by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was conducted to observe the genetic distance. The 28S rDNA, mitochondrial cytochrome *c* oxidase subunit I (mCOI), and ribosomal internal transcribed spacer 1 (ITS1) fragments were obtained from the worms by PCR. The PCR products were cleaved by 5 four-base pair restriction enzyme combinations (*Msp* I, *Hae* III, *Alu* I, *Cfo* I, *Rsa* I), electrophoresed and analyzed with PAUP 3.1.1. The fragment patterns of 28S rDNA and ITS1 demonstrated that two worms were in identical systematic tree with bootstrap number 94 and 100, respectively. As for mCOI, bootstrap number was 74 in a different tree. Above results are indicative of recent common ancestry between *S. erinacei* and *S. mansonoides*.

Key words: *Spirometra erinacei*, *Spirometra mansonoides*, polymerase chain reaction-restriction fragment length polymorphism analysis, molecular biology, phylogeny

INTRODUCTION

Spirometra erinacei Faust, Campbell *et* Kellog, 1929 (=*S. mansoni*, =*S. erinaceieuropei*) and *S. mansonoides* [Mueller, 1935] Wardle, McLeod *et* Stewart, 1947 are pseudophyllidean cestodes. Their plerocercoid larva (sparganum) causes sparganosis in man which is manifested as tumor or abscess of the subcutaneous tissue, brain, scrotum, breast, spinal cord or eye. Adults of *Spirometra* inhabit the intestine of cats or dogs (Lee *et al.*, 1984). *S. erinacei* is found in Asia, Africa, Australia, South America, and Europe, while *S. mansonoides* is found in North America. Only observed morphological difference between the two is the uterine shape of the mature proglottid: piled in *S. erinacei* and C-shaped in *S. mansonoides*. Iwata (1972) argued that the difference is due to differences in the developmental stages. Mueller (1974) separated the two species based on the morphological differences. However, they may be closely related species, since their biological, physiological or epidemiological characteristics are similar.

For phylogenetic analysis, genetic variation may be investigated using the mitochondrial or nuclear genome. Mitochondrial DNA is useful for the discrimination of closely related organisms because of its relatively rapid rate of evolution. The nuclear ribosomal RNA gene (rDNA) repeat unit has regions evolving at varying rates and also has been used exten-

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sively to study variation and phylogeny at a number of taxonomic levels. The rDNA internal transcribed spacer (ITS) gene family consists of regions of highly conserved gene sequences that is flanked by transcribed and non-transcribed spacer regions, which may contain variations that can be useful for identifying the relatedness of species. This study was conducted to determine the genetic distance between the two worms using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the mitochondrial and nuclear genomes.

MATERIALS AND METHODS

Genomic DNA purification from worms

S. erinacei was obtained from the intestine of a cat infected with spargana of a snake (*Rhadophis tigrinus tigrinus*, Boie, 1826) in Korea. *S. mansonoides* was obtained from the intestine of a cat infected with spargana, provided by the Department of Biochemistry and Molecular Biology, University of Nebraska, USA. For controls, *Diphyllobothrium latum* and *Taenia asiatica* (man), *T. taeniaeformis* (cat) and *Hymenolepis diminuta* (*Rattus norvegicus*) were also included. Adult worms were stored at -70°C until used. Frozen worms were lyophilized and lysed with lysis buffer, proteinase K, and RNAase. DNA was extracted in phenol/chloroform and precipitated in ethanol as reported by Sambrook *et al.* (1989).

28S rDNA D1 PCR-RFLP

PCR was conducted using a mixed solution of extracted DNA as a template (0.01 μg/μl), primer and TaKaRa Ex Taq Kit (TAKARA Shuzo Co., LTD., Japan) in a GeneAmp PCR System 9600 (Perkin Elmer, USA). PCR reaction cycles consisted of denaturing at 95°C for 20 sec, annealing at 55°C for 30 sec, extending at 72°C for 30 sec, followed by a final extension of 6 min at the end of 40 cycles. The forward primer (JB10, 5' GATTACCCGCTGAACTTAAGCATA 3') consisted of the conserved region, 21st-45th site of 28S rDNA sequence of *Schistosoma mansoni*. The reverse primer (JB9, 5' GCTGCATTCACAAACACCCCGACTC 3') was the 278th-302nd site of same gene (Qu *et al.*, 1988; Bowles and McManus, 1994). The PCR products were digested by restriction enzymes (*Msp* I, *Hae* III, *Alu* I, *Cfo* I, *Rsa* I, product of Promega, USA) for 1-2 hrs at 37°C. The digested products were electrophoresed through 2% agarose (Metaphor®, FMC BioProducts, Rockland, ME, USA) gel in 1 × Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0).

Mitochondrial cytochrome *c* oxidase I (mCOI) PCR-RFLP

mCOI PCR-RFLP was done with above method using 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 *Fasciola hepatica* sequence (Garey *et al.*, 1989; Bowles and McManus, 1994). PCR condition was the same as described above, except that annealing was done at 48°C for 30 sec.

rDNA Internal transcriber spacer 1 (ITS1) PCR-RFLP

rDNA ITS1 PCR-RFLP was conducted as described for 28S rDNA, except using different primers. The forward primer was BD1 (5' GTCGTAACAAGGTTCCGTA 3') and the reverse primer was 4S (5' TCTAGATGCGTTCGAA (G/A)TGTCGATG 3') (Bowles and McManus, 1993).

Analysis of PCR-RFLP

For the analysis of the PCR-RFLP, gels were photographed. Then the number of bands and their size were calculated, and each alignment was statistically analyzed (Power Macintosh 6100/66, MacClade V 3.0., Clustal V and PAUP Ver.3.1.1.) (Maddison *et al.*, 1992; Swofford, 1993; Higgins *et al.*, 1994; Hills *et al.*, 1996).

RESULTS

S. erinacei and *S. mansonoides* 28S rDNA D1 PCR products were digested with *Hae* III. There was close resemblance between *S. erinacei* and *S. mansonoides* since a large portion of the restriction fragments comigrated (Fig. 1).

S. erinacei mCOI PCR products were not digested but those of *S. mansonoides* were digested with *Hae* III (Fig. 2). *S. erinacei* and *S. mansonoides* 28S rDNA ITS1 PCR products were digested with all enzymes. There was close resemblance between the two species due to a high proportion of the restriction fragments comigrated (Fig. 3). For the PCR-RFLP analysis, *D. latum*, a pseudophyllidean tapeworm, was selected as an outgroup. For ITS1, the two are in an identical tree topology with a bootstrap number of 100. For 28S rDNA, they are in an identical tree with a bootstrap number of 94. For mCOI, the bootstrap number was 74 in a different tree topology. Except for *H. diminuta*, the other cyclophyllidean tapeworms, are in different trees than *Spirometra* spp. based on analysis of ITS1, 28S rDNA and mCOI. *H. diminuta* was in the identical tree topology with *S. mansonoides* for mCO1 with a bootstrap number of 93 (Fig. 4).

DISCUSSION

For the phlyogenetic classification of helminths, base sequencing of specific DNA (Bowles and McManus, 1994), fragment pattern by endonuclease (Fukumoto *et al.*, 1992), PCR-random amplified polymorphic DNA (RAPD) (Bowditch *et al.*, 1993) and PCR-single strand conformational polymorphism (SSCP) (Orita *et al.*, 1989) are used for auxiliary methods for the verification of the debating taxa.

S. erinacei and *S. mansonoides* have differ-

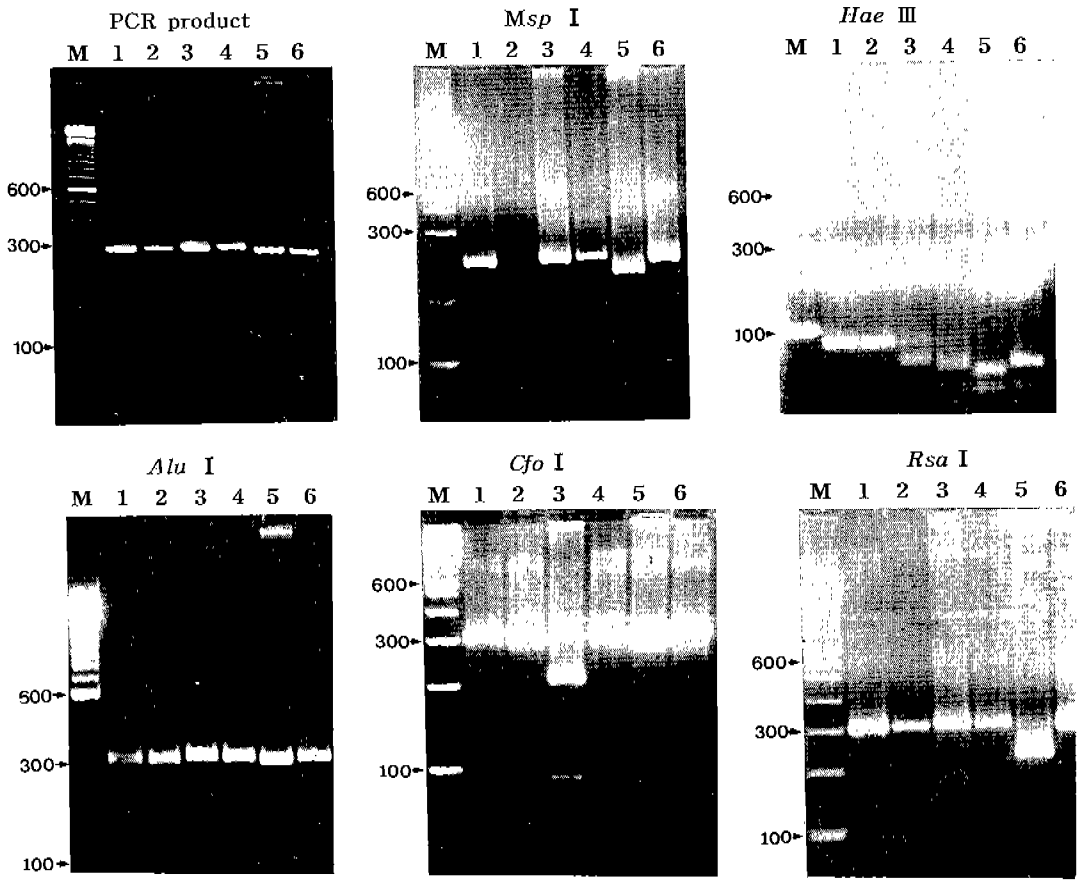

Fig 1. Two percent agarose gel electrophoresis of PCR-RFLP for 28S rDNA D1 using the restriction endonuclease. Lane M, molecular weight markers; lane 1, *S. erinacei*; lane 2, *S. mansonoides*; lane 3, *T. asiatica*; lane 4, *T. taeniaeformis*; lane 5, *H. diminuta*; lane 6, *D. latum*.

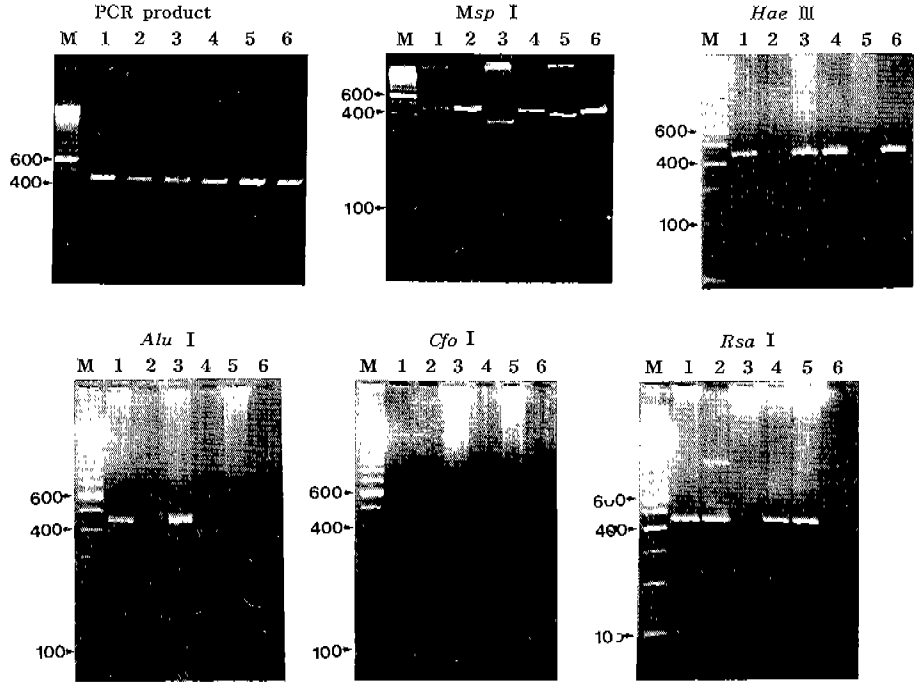

Fig 2. Two percent agarose gel electrophoresis of PCR-RFLP for mCOI using the restriction endonuclease. Lane M, molecular weight markers; lane 1, *S. erinacei*; lane 2, *S. mansonoides*; lane 3, *T. asiatica*; lane 4, *T. taeniaeformis*; lane 5, *H. diminuta*; lane 6, *D. latum*.

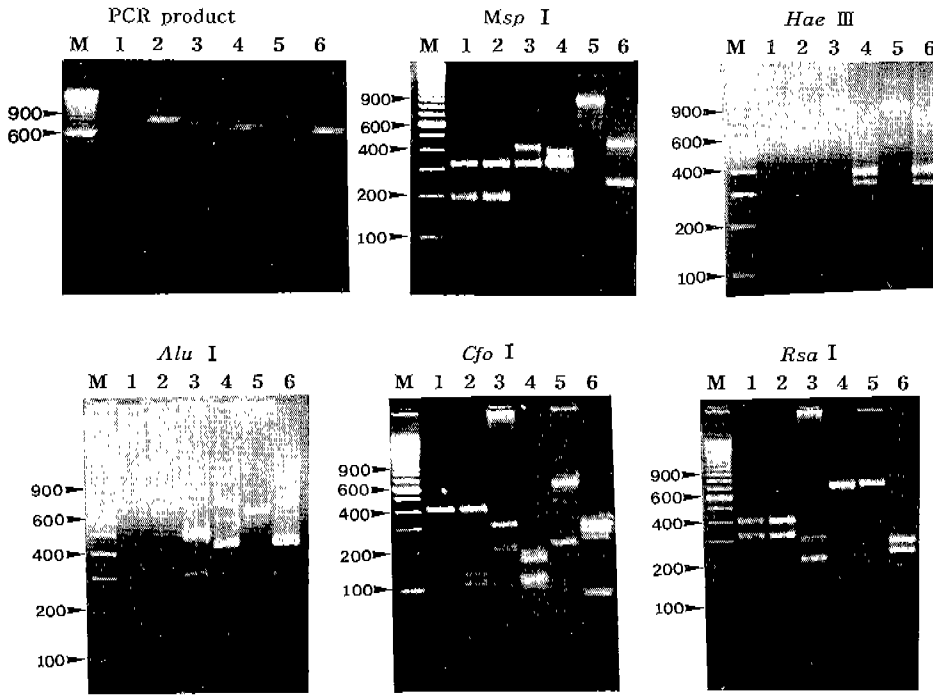

Fig 3. Two percent agarose gel electrophoresis of PCR-RFLP for rDNA ITS1 using the restriction endonuclease. Lane M, molecular weight markers; lane 1, *S. erinacei*; lane 2, *S. mansonoides*; lane 3, *T. asiatica*; lane 4, *T. taeniaeformis*; lane 5, *H. diminuta*; lane 6, *D. latum*.

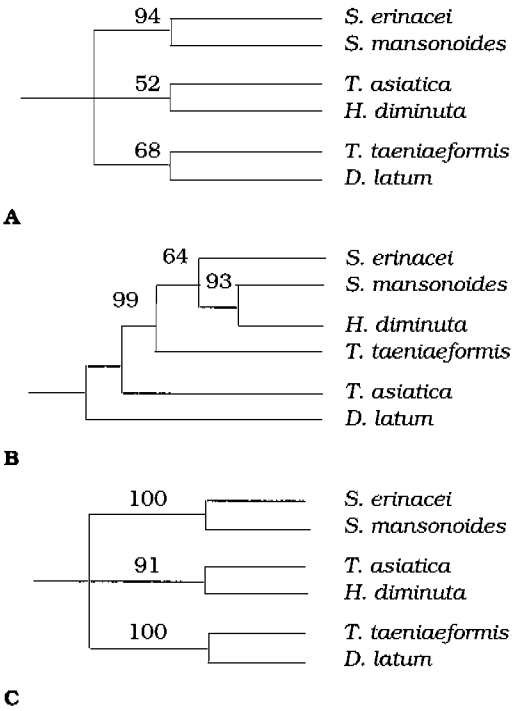

Fig 4. Molecular phylogenetic analyses of PCR-RFLP obtained using 28S rDNA, mCOI and the rDNA ITS1 gene. Branch lengths and ranges of bootstrap values were obtained using statistical package (Power Macintosh 6100/66, MacClade V 3.0., Clustal V and PAUP Ver.3.1.1). Bootstrap values are given as a percent of the above corresponding branch. A, 28S rDNA; B, mCOI; C, rDNA ITS1 gene.

ent isozyme patterns (Fukumoto *et al.*, 1992). The PCR-RFLP method, described here, allows *Spirometra* species isolates to be easily and rapidly identified using the size of the nuclear genomic 28S rDNA, ITS1 and mCOI regions as genetic markers. These results on the phylogeny of *Spirometra* species demonstratd a close relationship between the two species. It is indicative of the common ancestry between *S. erinacei* and *S. mansonoides*, and close evolutionary relationship.

Nuclear DNA may be more reliable for genetic comparisons than mitochondrial DNA in the pseudophyllidean tapeworm taxa. For the PCR-RFLP, the nuclear DNA, *i.e.*, ITS1 and 28S rDNA, is more conserved than mCOI. Mitochondrial DNA is the result of maternal inheritance in heterosexual organisms. Since cestodes are hermaphroditic species, the similarities of mCOI restriction patterns between morphologically distinct species, *i.e.*, *S. mansonoides* and *H. diminuta*, and not between *S. erinacei* and *S. mansonoides*, can be explained from two points. First, the evolutionary closeness between *S. mansonoides* and *H. diminuta* may be a result of gene transfer between the two species. When we place *H. diminuta* as an outgroup, *D. latum* and *S. mansonoides* were in an identical tree topology (data not shown). Therefore, the first explanation is hard to accept. The second possibility is that there was an insertion of intron in the mCO1 gene product. In this connection, the gene sequence should be analyzed for the evidence.

As a phylogenetic tool, PCR-RFLP has limitations in interpreting the data, since there are two kinds of problems in handling the data. First, it violates the assumption of independence among characters. Secondly, insertions or deletions are difficult to interprete. Thus, application of this method for measuring genetic divergence among the species is rather difficult. For a more straightforward phylogenic analysis, sequence data is required. The sequence data for specific genes, *i.e.*, nuclear ribosomal DNA, mitochondrial DNA, nuclear protein-coding genes, NADH dehydrogenase 1, between two species of *Spirometra* would be necessary.

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= 초록 =

만손열두조충과 북미열두조충의 중합효소연쇄반응-마디길이여러꼴 분석법을 이용한 유전 형질 비교

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만손열두조충과 북미열두조충 (*Spirometra mansonoides*)의 형태 차이점은 성숙편절의 자궁의 형태가 전자는 차곡차곡 쌓인 꼴이고, 후자는 알파벳 씨 자 (C) 형태이라는 점이다. 이 비슷한 형태의 두 종의 조충이 유전학적으로는 얼마나 차이가 있는 지를 알기 위하여 중합효소연쇄반응-마디길이여러꼴 분석법 (polymerase chain reaction-restriction fragment length polymorphism analysis)을 이용하여 유전 형질을 비교하였다. 충체로부터 28S 리보솜 리보핵산 (28S rDNA), 사립체 cytochrome *c* 산화효소 아단위 I (mitochondrial cytochrome *c* oxidase subunit I, mCOI) 및 리보솜 내부 전사된 영역 1 (ribosomal internal transcribed spacer 1, ITS1)에 대한 중합효소반응 산물을 구하였다. 이 산물은 *Msp* I, *Hae* III, *Alu* I, *Cfo* I, *Rsa* I 의 4 염기 제한 효소로 잘라서, 전기영동하여 결과를 PAUP 3.1.1을 이용하여 분석하였다. 28S 리보솜 리보핵산과 리보솜 내부 전사된 영역 1 유전자에서는 두 충체가 동일한 분류가지에 묶였고, 홀로서기수 (bootstrap number)는 94, 100 이었다. 사립체 cytochrome *c* 산화효소 아단위 1에서는 다른 분류가지에 묶였고, 홀로서기수가 74이었다. 위 결과로 두 조충이 같은 조상에서 유래함과 진화 단계에서 매우 가까운 위치에 있음을 알 수 있었다.

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