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Research Note

Characterization of Genetic Divergence Among Species within the Genus *Fasciola* by PCR-SSCP

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In the genus *Fasciola*, *F. hepatica* and *F. gigantica* are now recognized as taxonomically varid species. *Fasciola* species in Japan, however, has been categorized neither as *F. hepatica* nor as *F. gigantica*, because of its intermediate morphological and ecological characteristics (Watanabe and Iwata, 1954; Itagaki and Akane, 1959; Oshima *et al.*, 1968; Akahane *et al.*, 1970). For the identification of Japanese *Fasciola* species, further studies should be needed to provide additional taxonomical criteria.

Several genes in mitochondrial DNA, especially those encoding NADH dehydrogenase subunit I (NDI) and cytochrome *c* oxidase subunit I (COI), have been providing useful markers for characterizing genetic variability in closely related parasitic helminths (Despres *et al.*, 1991; Bowles *et al.*, 1992; Bowles and McManus, 1993; Bowles and McManus, 1994). Therefore, we thought it would provide new criteria on identification of Japanese *Fasciola* species to compare genetic characteristics of mitochondrial DNA. In the present study, we used polymerase chain reaction – single strand conformational polymorphism (PCR-SSCP) to examine genetic diversities in the regions of the mitochondrial NDI and COI genes among three species of *Fasciola*. PCR-SSCP is a method to detect nucleotide differences in PCR-amplified DNA as mobility shifts caused by conformational changes in a native polyacrylamide gel (Orita *et al.*, 1989) and is successfully used to screen mutations in various oncogenes (Cottrell *et al.*, 1992) and to identify genotype of virus associated disease (Lin *et al.*, 1993).

F. hepatica, *F. gigantica* and Japanese *Fasciola* species were obtained from bile ducts of naturally infected cattle in Montevideo (Uruguay), Lusaka (Zambia) and Iwate (Japan), respectively. Additionally, three laboratory isolates of Japanese species were used; Shiwa and Tenpoku isolates were originated from naturally infected cows slaughtered in Iwate Prefecture and Hokkaido, Japan, respectively, and Nara isolate from naturally infected deer in Nara Prefecture, Japan. These isolates were maintained for several generations in Wistar rats and snail host, *Lymnaea ollula*, in our laboratory. Alcohol- or frozen-preserved worms of the three species of *Fasciola* were chopped up by a scissor, and then homogenized in an extraction buffer consisting of 50 mM Tris-HCl (PH 7.9), 100 mM EDTA, 200 mM NaCl, 0.5% SDS, 50 μg/ml RNase A and 150 μg/ml proteinase K. The homogenates were incubated for 5 hours or overnight at 65°C. Total DNA including mitochondrial one was prepared by the standard

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phenol extraction/ethanol precipitation procedure. Two DNA fragments of 187 bp and 446 bp corresponding to the mitochondrial NDI and COI genes, respectively, were amplified by PCR. The primers used were; 5'-AAGGATGTTGCTTTGTCGTGG-3' (sense), and 5'-AGGCCCCTTACGAATCTG-CAT-3' (antisense) for a region of NDI gene, and 5'-TTTTTTGGGCATCCTGAGGTTTAT-3' (sense) and 5'-TAAAGAAAGAACATAATGAAAATG-3' (antisense) for a region of COI gene. The 5'-nucleotides of these primers correspond to nucleotide positions 141 and 327 (NDI), and 2575 and 3020 (COI), according to the published data for *F. hepatica* (Gray and Wolstenholme, 1989). Amplification reactions were carried out in a final volume of 50 μl containing 25 pmol of each primer, 200 μM each of dATP, dCTP, dTTP, and dGTP, 3 μCi ^{32}P dCTP (3000 Ci/mmole), 2.5 units *Taq* polymerase, and 100 ng *Fasciola* total DNA in 10 mM Tris-HCl buffer, pH 8.3, containing 1.5 mM $MgCl_2$ and 50 mM KCl. PCR condition was as follows: 94°C for 0.5 min initially, then for 1.5 min at the start of each cycle; 53°C for 2.0 min; 72°C for 2.0 min and for 10 min at the end of all 30 cycles. The PCR products were extracted with chloroform/isoamyl alcohol (24:1), precipitated with ethanol and resuspended in 20 μl H_2O. Nucleotide length of the PCR products was confirmed by autoradiography after electrophoresis on a 5% polyacrylamide gel. All the PCR products had sizes comparable to those expected from the published nucleotide sequences (data not shown). For PCR-SSCP analysis, aliquotes of the PCR products were mixed with four volumes of a denaturing solution consisting of 0.3 M NaOH, 10% glycerol, 0.04% bromophenol blue and 0.04% xylene cyanol, and then applied to a 6% polyacrylamide gel containing 5% glycerol. After electrophoresis overnight at constant voltage of 250 V, the gels were vacuum-dried on Whatman 3MM paper and exposed to X-ray film with an intensifying screen at –80°C. Each PCR product from NDI gene was separated into two bands (Fig. 1). Band patterns with the same electrophoretic mobilities were observed within either individuals of *F. hepatica* (lanes 1–7) or Japanese *Fasciola* species (lanes 15–21), indicating the pattern is species-specific. However, the PCR products from *F. gigantica* showed different pattern; two individuals (lanes 8 and 13) exhibited distinct band pattern from other five individuals (lanes 9–12, and 14). One individual (lane 13) showed the same pattern as those of Japanese *Fasciola* species. On the other hand, the PCR products of COI gene were separated into four bands by PCR-SSCP analysis (Fig. 2). The reason for the appear-

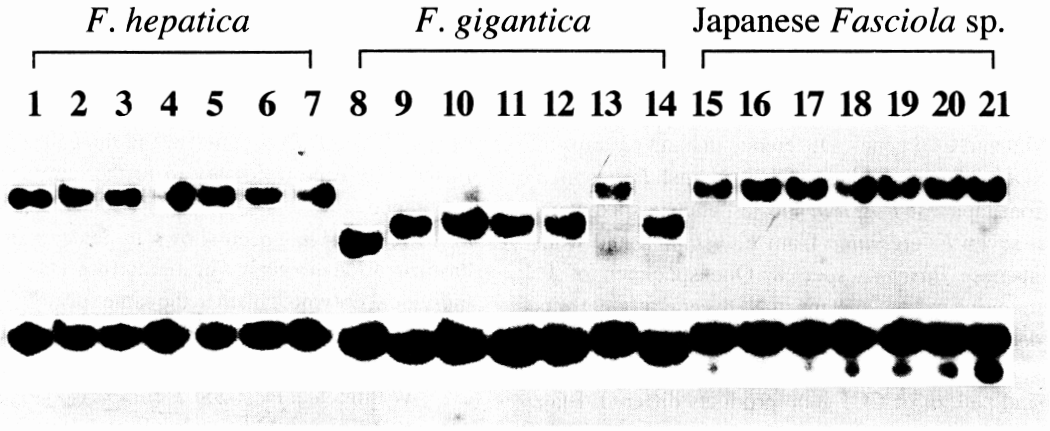

Fig. 1 PCR-SSCP analysis of a 187bp segment of the mitochondrial NDI gene among three species of *Fasciola*. Lanes 1–7 and 8–14 show 7 individuals of *F. hepatica* and *F. gigantica*, respectively, obtained from naturally infected cattle. Lanes 15, 16–17, and 21 show individuals of Tenpoku, Shiwa and Nara isolates, respectively, of Japanese *Fasciola* species obtained from experimentally infected rats. Lanes 18–20 show three individuals of Japanese *Fasciola* species obtained from naturally infected cattle.

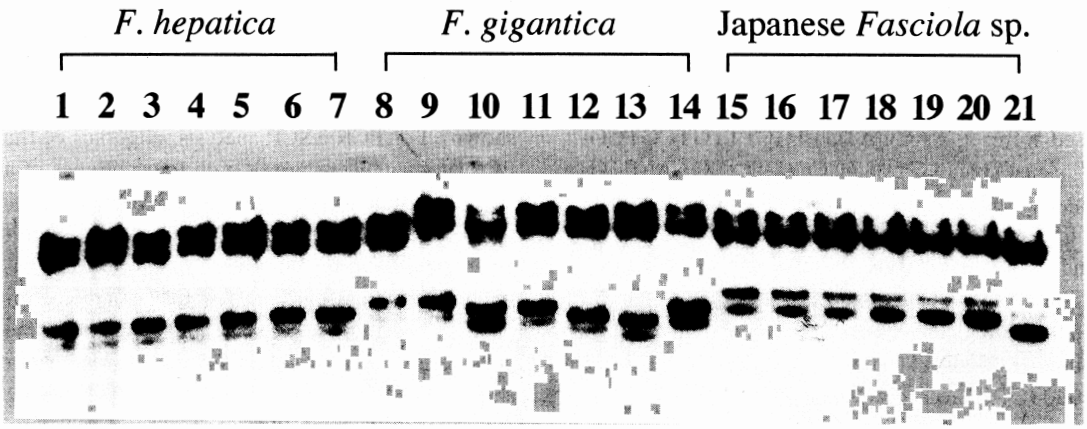

Fig. 2 PCR-SSCP analysis of a 446bp segment of the mitochondrial COI gene among three species of *Fasciola*. Lanes 1–7 and 8–14 show 7 individuals of *F. hepatica* and *F. gigantica*, respectively, obtained from naturally infected cattle. Lanes 15, 16–17, and 21 show individuals of Tenpoku, Shiwa and Nara isolates, respectively, of Japanese *Fasciola* species obtained from experimentally infected rats. Lanes 18–20 show three individuals of Japanese *Fasciola* species obtained from naturally infected cattle.

ance of additional two bands are not clear. Nevertheless, it is apparent that band patterns of *F. hepatica*, *F. gigantica*, and Japanese species are similar among individuals of the same species but are different among the species. Only one individual in each of *F. hepatica* (lane 3) and Japanese species (lane 21) had different pattern from others within the species. In contrast, all individuals in *F. gigantica* examined here exhibited different pattern.

The present study suggested that *F. hepatica*, *F. gigantica* and Japanese *Fasciola* species had different genetic characteristics in the regions within the NDI and COI genes. Difference in band patterns of NDI products enabled us to distinguish *F. hepatica* from Japanese *Fasciola* species, and six specimens of seven *F. gigantica* from *F. hepatica* and from Japanese *Fasciola* species. One specimen of *F. gigantica*, whose pattern in NDI gene product was identical to that of Japanese *Fasciola* species, could be distinct from Japanese *Fasciola* species, since the band pattern of COI gene products differed. Blair and McManus (1989), and Adlard *et al.* (1993) discriminated *F. hepatica* from *F. gigantica* by the differences of restriction enzyme map and nucleotide sequence in the regions of ribosomal DNA. In these reports, they showed that the restriction map and the nucleotide sequence of Japanese *Fasciola* species were nearly identical to those of *F. gigantica*. However, the results presented here showed that in both PCR products of NDI and COI genes, band patterns of Japanese *Fasciola* species had apparent difference as compared to that of *F. gigantica*, but subtle difference when compared to that of *F. hepatica*. Thus, it is suggested that nucleotide sequence in NDI and COI genes of Japanese *Fasciola* species is similar to that of *F. hepatica* rather than *F. gigantica*, since Lin *et al.* (1993) reported that subtle changes of band patterns shown by PCR-SSCP reflect minor base changes. Nucleotide sequencing of the NDI and COI genes in the three species of *Fasciola* will be necessary to further confirm the difference.

PCR-SSCP has been shown to detect a single-base mutation in a gene. The method detects even an individual polymorphism in the same species. Thus, application of the method to genetic divergence among the species seems to rather difficult. However, we think that PCR-SSCP comparison together of several loci would be a simple and rapid procedure for elucidation of genetic difference among parasite species.

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