A Epidemiology Study on the Molecular Phylogeny of Japanese Fasciola species in Asia

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要旨
Japanese Fasioraの分類学的地位を解明するために、オーストラリアFasciola hepatica、
マレー産F.giantica、日本産Fasioraの全ミトコンドリアDNAを、Hinfl、MspI、rsaiの3種の4
塩基切断endonucleasesを用いて消化させた。得られた消化サンプルより、地理的に異なる各株に
対して各酵素はいくつかの特異的なカットを示し、JapaneseFasioraとF. hepaticaよりF.gi-
nanticaに対して共通するカットを多く有することが明らかにされた。また、それぞれの核
リボソームRNAクラスターの第2内縁転写スパーク（ITS2）とミトコンドリア転写サブユニットI（COI）の2つ
の領域の塩基配列の比較も行った。その結果Japanese FasioraがF.giganticaであることが
が示唆された。

キーワード:ミトコンドリアDNA、核DNA、日本産肝吸虫
key words:MitochondrialDNA, NuclearDNA, Japanese Fasciola species

Introduction
Controversy concerning the taxonomic status of the Japanese species of Fasciola continues despite the
numerous studies conducted over the years (e.g., Itagaki and Akane 1959; Ueno and Watanabe 1960; Watanabe
1965; Oshima et al. 1969). There is some morphological variation among Japanese isolates. In addition, chromo-
some studies found that three types (diploid, triploid, and “mixoploid”) existed even in a single host and that
all these types had little sperm, if any, indicating parthenogenetic reproduction (Sakaguchi and Nakagawa
1975; Moriyama et al. 1979; Sakaguchi 1980; Terasaki et al. 1982). This contrasts strikingly with the situation
in Fascioloids from other parts of the world, all of which are diploids possessing numerous sperm (Sakaguchi and
Ueno 1977; Terasaki et al. 1982). Faced with this un-
certainty about the identity of the Japanese liver fluke, most workers prefer to refer to it simply as Fasciola sp.
Using restriction maps of the ribosomal genes, Blair
and McManus (1989) demonstrated that a *Fasciola* isolate from Japan was identical to *F. gigantica* but different from *F. hepatica*. Adlard et al. (1993) determined partial nucleotide sequences of the nuclear ribosomal second internal transcribed spacer region (ITS2) for several isolates of *F. hepatica*, *F. gigantica*, and *Fasciola* sp. from Japan. Again, the Japanese *Fasciola* sp. was almost indistinguishable from *F. gigantica*. Using allozymes in an investigation of triploid individuals of the Japanese *Fasciola* species (Agatsuma et al. 1994a), we have found a diagnostic allele distinguishing between Japanese *Fasciola* sp. and *F. hepatica* at the Got locus. Whether this allele also occurs in *F. gigantica* is unknown.

Recently, Itagaki et al. (1995) examined the mitochondrial nicotinamide adenine dinucleotide phosphate (NADH) subunit I (NDI) and cytochrome c oxidase subunit I (COI) genes among the three fasciolids using the polymerase chain reaction–single-stranded conformational polymorphism (PCR-SSCP) method. The patterns observed showed more similarity between *F. hepatica* from Uruguay and *Fasciola* sp. from Japan, implying more similarity between their nucleotide sequences. This finding seems to contradict several of the studies mentioned above.

In the present study, restriction-fragment-length polymorphism (RFLP) patterns were analyzed for whole mitochondrial DNA after digestion with three four-base-cutting enzymes (Hinfl, MspI, and RsaI). In addition, nucleotide sequences of the nuclear ribosomal second internal transcribed spacer (ITS2) and of the mitochondrial COI gene were determined for *Fasciola* sp. from Japan, *F. hepatica* from Australia, and *F. gigantica* from Malaysia to evaluate the phylogenetic relationships among them and the specific status of *Fasciola* sp. from Japan.
Materials and methods

Two specimens each were obtained of the Japanese *Fasciola* sp. [from slaughterhouses in Kochi City (JK10) and Tosa City (JT6) in Kochi Prefecture], *F. hepatica* [(A2, A4) from cattle imported from Australia to the slaughterhouse in Fukuoka], and *F. gigantica* [(M7, M8) obtained from a slaughterhouse in Kuala Lumpur, Malaysia, and carried over to Japan on dry ice].

For RFLP analysis, each frozen specimen was homogenized in 500 µl of extraction buffer [0.25 M sucrose, 30 mM TRIS-HCl (pH 7.5), 10 mM ethylenediaminetetraacetic acid (EDTA)] and centrifuged at 800 g to remove nuclei and cell debris. The supernatant recovered was recentrifuged at 10,000 g for 10 min at 4 °C to pellet mitochondria. The resultant pellet was suspended in 50 µl of 10 mM TRIS-EDTA buffer (0.15 M NaCl and 10 mM EDTA, pH 8.0). To this was added 100 µl of lysis buffer [0.18 N NaOH and 1% sodium dodecyl sulfate (SDS)] and, subsequently, 75 µl of neutralizing buffer (3 M potassium and 5 M acetate solution). The suspension mixture was centrifuged at 12,000 g for 5 min at 4 °C and the supernatant was extracted twice with phenol/chloroform and once with chloroform. The mitochondrial DNA (mtDNA) was precipitated with ethanol and then resuspended in an appropriate volume of sterile distilled water. The mtDNA prepared by this method was of sufficient purity for restriction-enzyme analysis. The mtDNAs purified were digested with three four-base-cutting enzymes: HinfI, MspI, and Rsal. Digested DNA samples were size-fractionated on 4–15% gradient polyacrylamide gels. After electrophoresis at a constant voltage of 200 V for 2 h, gels were silver-stained according to the manufacturer’s instructions (Wako).

For PCR analysis, genomic DNA was extracted from whole worms. Worms were minced with a razor and incubated in extraction buffer (Nalgene extraction kit) containing SDS and proteinase K either overnight or until the tissues were solubilized. The solubilized liquids were treated with a buffered phenol solution three times, and the extracted DNAs were ethanol-precipitated. Gene regions of interest were amplified. The PCR conditions were as follows: 94 °C for 3 min, 50 °C for 1 min, and 72 °C for 3 min for 30 cycles. Amplification reactions were performed in a final volume of 50 µl containing primers (3.2 pmol), deoxynucleoside triphosphates (dNTPs, 0.2 mM), and Taq polymerase (2.5 U/reaction). As primers we used 5'-CGGTGGATCTCGGCTCGT-3' (3S) as a forward primer and 5'-CCTGGTTAGTTCTTCTTCCGC-3' (A28) as a reverse primer for the ITS2 region and 5'-TTTTTGGGATCCCTGAGCTTA-3' (FH5: forward) and 5'-TAAAGAAGAGACATAATGAAAATAATC-3' (FH3: reverse) for the COI region. The PCR products were treated with chloroform and purified using high-performance liquid Chromatography (HPLC). The DNA fraction obtained was precipitated with ethanol and resuspended in 20 µl of distilled water, and aliquots were sequenced using the PRISM kit (ABI). The reactions were purified
according to the manufacturer's instructions (ABI) and applied to
an ABI sequencer (373A).

Homology and alignment analyses were done using the pro-
grams CLUSTAL V (Higgins and Sharp 1988) and GENETYX-
MAC V 6.0 (Software Development Co., Tokyo, Japan). Trees
were constructed using distance and parsimony methods in PHY-
LIP (Felsenstein 1989). Amino acid sequences of the mitochon-
drial COI gene were inferred using the codon tables of Garey and
Wolstenholme (1989).

Results

RFLP of mitochondrial DNA

As shown in Figs. 1–3, RFLP patterns obtained, for
each of the three four-base-cutting enzymes included
multiple bands. Specimens from within each geo-
graphical region showed little variation. However, dis-
tinct differences in patterns were observed between
geographical isolates. The Japanese Fasciola sp. and
Malaysian F. gigantica were more similar and shared
more bands in each case.

Nuclear ribosomal ITS2

The alignment of 537 bp included a part of the 5.8S gene
and a part of the 28S gene (Fig. 4). The end point of the
5.8S rRNA gene was determined by comparative align-
ment with the sequences of the Schistosoma species
published by Bowles et al. (1993). A length of 362 bp
was estimated for the whole ITS2 gene, as was that of

127 bp for a part of the 5.8S gene and that of 48 bp for
the 28S gene. The average G + C content was close to
51%. No variation was found in the region of 5.8S and
28S genes. The few substitutions noted in the ITS2 were
transitions in all cases. No variation was observed be-
tween worms from the same geographical location.
However interlocation variations were observed. F. he-
patica differed from the other two isolates at six nu-
cleotide sites (one of these was an deletion), whereas no
difference was observed between F. gigantica and the
Japanese Fasciola sp.
Mitochondrial COI gene

The COI alignment consisted of 395 bp (Fig. 5). The G + C content was close to 37%. The average transition/transversion ratio was 3.21. Intraspecific variation was found at one nucleotide site in the Malaysian *F. gigantica*. The Australian *F. hepatica* differed from the Japanese *Fasciola* sp. and *F. gigantica* at 25–28 sites; however, *F. gigantica* and the Japanese *Fasciola* sp. differed at only 4–5 sites. Substitutions were observed at 5 of the 131 sites in the inferred amino acid sequence alignment (Fig. 6). The Australian *F. hepatica* differed from the other two geographical isolates at four sites, whereas the Malaysian *F. gigantica* and the Japanese *Fasciola* sp. differed at only one amino acid site.

**Discussion**

RFLP patterns have been useful tools for taxonomic studies and species identification for *Paragonimus* species (Agatsuma et al. 1994b). In the present study the mtDNA digestion patterns differed markedly between geographical isolates of fasciolids. Given the variation observed within the short region of the COI gene sequenced, there are likely to be considerable differences in RFLP patterns even between quite closely related forms. However, more bands appeared to be shared between the Malaysian *F. gigantica* and the Japanese *Fasciola* sp. than between either of these and the Australian *F. hepatica*.

The ITS2 region was highly conserved. Our ITS2 sequences of *F. hepatica* (A2 and A3) contained two insertions, each of a single base, relative to the sequences reported by Adlard et al. in 1993 (Fig. 7). More substantial differences (at nine sites) were noted between our sequences and that reported for *F. hepatica* by Michot et al. (1993) as shown in Fig. 7. These were found especially at the 3' end of the sequence at sites that were invariant among or within geographical isolates in the present study. Gel misreading by Michot et al. (1993) is a possible explanation for these discrepancies. In our study, all six variable sites supported a grouping of the
Japanese *Fasciola* sp. with *F. gigantica*. Similar sequence conservation in the ITS2 region has been found in various species of digeneans (Despres et al. 1992; Luton et al. 1992; Bowles et al. 1995; Morgan and Blair 1995) as well as in *Fasciola* species (Adlard et al. 1993).

As compared with the COI sequence reported for *F. hepatica* by Garey and Wolstenholme (1989), our *F. hepatica* (A2 and A3) differed at two nucleotide sites, but no amino acid difference was found. Our COI data agreed with the conclusions drawn from ITS2 data: the Japanese *Fasciola* sp. is very close to *F. gigantica*. The only findings arguing against the identity of the Japanese *Fasciola* sp. with *F. gigantica* come from work by Itagaki et al. (1995). Their PCR-SSCP experiments using NDI and COI genes produced banding patterns from the Japanese *Fasciola* species that were different from those of *F. gigantica* but rather similar to those of *F. hepatica*. This implies corresponding differences in nucleotide sequences. However, the primers we used for amplifying and sequencing the COI gene were the same as those used by Itagaki et al. (1995). The results of Itagaki et al. (1995) are therefore misleading, perhaps due to some artifact of the PCR-SSCP technique. In conclusion, the present results suggest that the Japanese species of *Fasciola* represents a strain of *F. gigantica*.

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References


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Fig. 1. RFLP patterns obtained from 4-15% gradient polyacrylamide gel electrophoresis of Fasciola hepatica from Australia, F. gigantica from Malaysia, and Fasciola sp. from Japan after restriction enzyme digestion with Hinf I.

Fig. 2. RFLP patterns resulting from 4-15% gradient polyacrylamide gel electrophoresis of F. hepatica from Australia, F. gigantica from Malaysia, and Fasciola sp. from Japan after restriction enzyme digestion with Msp I.

Fig. 3. RFLP patterns produced by 4-15% gradient polyacrylamide gel electrophoresis of F. hepatica from Australia, F. gigantica from Malaysia, and Fasciola sp. from Japan after restriction enzyme digestion with Rsa I.

Fig. 4. Nucleotide sequences of a region of the 18S rDNA of F. hepatica from Australia, F. gigantica from Malaysia, and Fasciola sp. from Japan. The presumed beginning and end of the actual spacer region are marked by asterisks. The 3' end of the sequence is 5.8S origin, whereas a small portion of 28S sequences is shown at the 3' end.
Fig. 5 Nucleotide sequences of a region of the COI gene of mitochondrial DNA of *F. repens* from Australia, *F. gigantea* from Malaysia, and *F. excisa* sp. from Japan

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Fig. 6 Amino acid sequences of a region of the COI gene of mitochondrial DNA of *F. repens* from Australia, *F. gigantea* from Malaysia, and *F. excisa* sp. from Japan

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Fig. 7 Comparison of a part of the 16S rRNA region between the sequences described by Midhop et al. (1993) and the present sequences

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