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

アジアにおける日本住血吸虫類の種分化に関する疫学的研究

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要旨

Japanese Fasioraの分類学的地位を解明するために、ｵｰｽﾄﾘｱ産Fasiola hepatica, ﾏﾚｰｼｱ産F. gigantica、Japanese Fasioraの全ﾐﾄｺﾝﾄﾞﾘｱDNAを、HinfI、MspI、rsaI の３種の４塩基切断ｴﾝﾄﾞﾇｸﾚｱｰｾﾞ を用いて消化させた。得られた消化ﾊﾟﾀｰﾝより、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ﾊﾞﾝﾄﾞ を示し、JapaneseFasioraはF. hepaticaよりF. gigantica に対して共通するﾊﾞﾝﾄﾞ を多く有することが明らかにされた。また、それぞれの核ﾘﾎﾞｿﾞｰﾑRNAｸﾗｽﾀｰ の第２内部転写ｽﾍﾟｰｻｰ (ITS2) とﾐﾄｺﾝﾄﾞﾘｱﾁﾄｸﾛｰﾑCｵｷｼﾀﾞｰｾﾞｻﾌﾞﾕﾆｯﾄ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. giganntica であることが示唆された。

ｷｰﾜｰﾄﾞ:ﾐﾄｺﾝﾄﾞﾘｱDNA、核DNA、日本産肝ﾋﾞﾛ

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, chromosome 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 fasciolids 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 uncertainty 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 (HinfI, 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 m*M* TRIS-HCL (pH7.5), 10 m*M* ethylenediaminetetraacetic acid (EDTA)] and centrifuged at 800 *g* to remove nulei 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 m*M* TRIS-EDTA buffer (0.15 *M* NaCl and 10 m*M* 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 RsaI. 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 m*M*), and Taq polymerase (2.5 U/reaction). As primers we used 5′-CGGTGGATCACTCGGCTCGT-3′ (3S) as a forward primer and 5′-CCTGGTTAGTTTCTTTTCCTCCGC-3′ (A28) as a reverse primer for the ITS2 region and 5′-TTTTTTGGGCATCCTGAGGTTTA-3′ (FH5: forward) and 5′-TAAAGAAAGAACATAATGAAAATAATC-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 programs 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 PHYLIP (Felsenstein 1989). Amino acid sequences of the mitochondrial 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 geographical region showed little variation. However, distinct 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 alignment 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 between worms from the same geographical location. However interlocation variations were observed. *F. hepatica* differed from the other two isolates at six nucleotide 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*.

Acknowledgements The authors wish to thank Dr. Terasaki, St. Mary's Junior College, Kurume, Japan, for the supply of Australian flukes.

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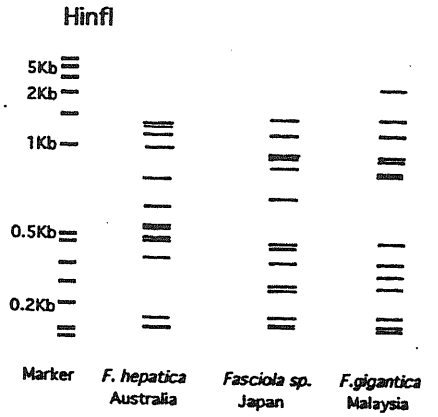

Fig. 1 RFLP patterns obtained from 4–15% gradient polyacrylamide gel electrophoresis of *Fasciola hepatica* from Australia, *F. gigantica* from Malaysia, and *Fasciola* sp. from Japan after restriction-enzyme digestion with Hinf I

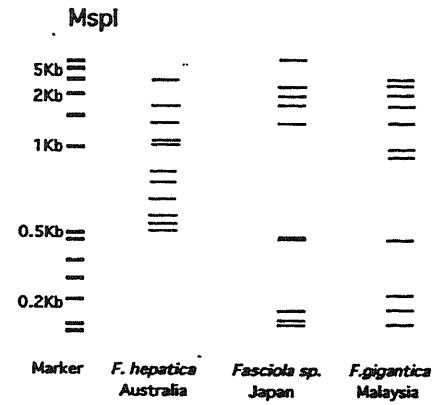

Fig. 2 RFLP patterns resulting from 4–15% gradient polyacrylamide gel electrophoresis of *F. hepatica* from Australia, *F. gigantica* from Malaysia, and *Fasciola* sp. from Japan after restriction-enzyme digestion with Msp I

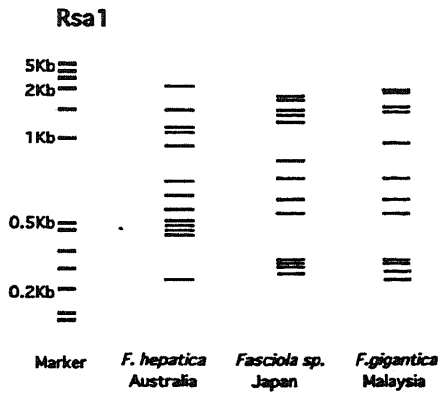

Fig. 3 RFLP patterns produced by 4–15% gradient polyacrylamide gel electrophoresis of *F. hepatica* from Australia, *F. gigantica* from Malaysia, and *Fasciola* sp. from Japan after restriction-enzyme digestion with Rsa I

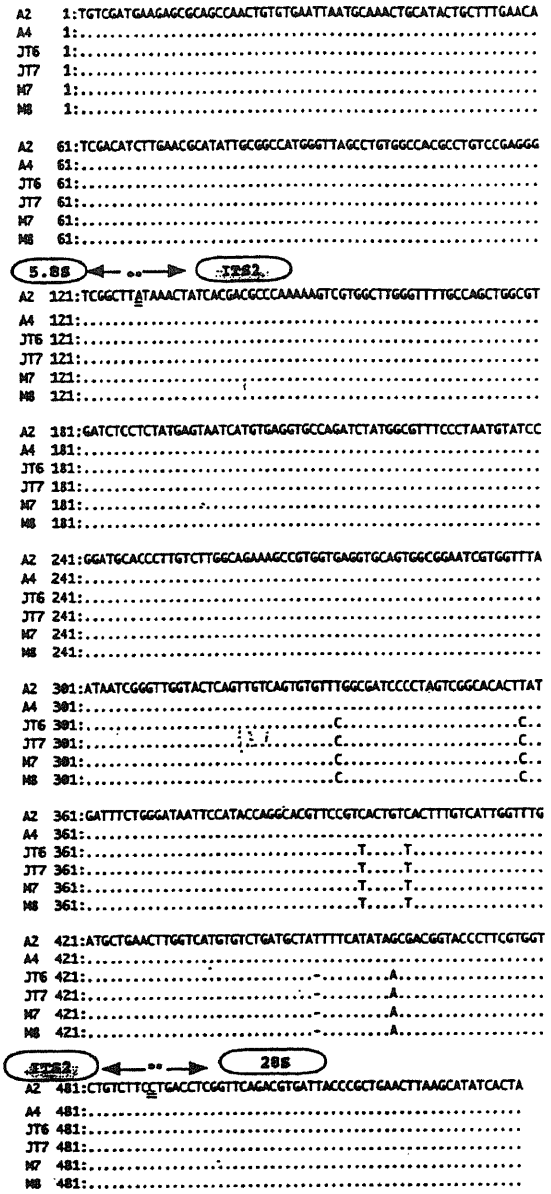

Fig. 4 Nucleotide sequences of a region of the ITS2 gene of nuclear ribosomal DNA of *F. hepatica* from Australia, *F. gigantica* from Malaysia, and *Fasciola* sp. from Japan. The presumed beginning and end of the actual spacer region are marked by *asterisks*. The 5′ end of the sequence is of 5.8S origin, 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. hepatica* from Australia, *F. gigantica* from Malaysia, and *Fasciola* sp. from Japan

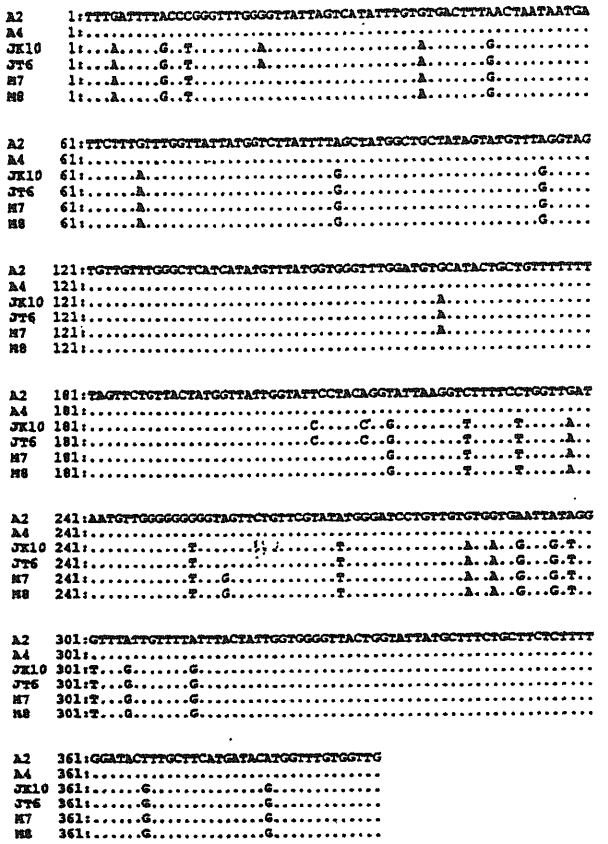

Fig. 6 Amino acid sequences of a region of the COI gene of mitochondrial DNA of *F. hepatica* from Australia, *F. gigantica* from Malaysia, and *Fasciola* sp. from Japan

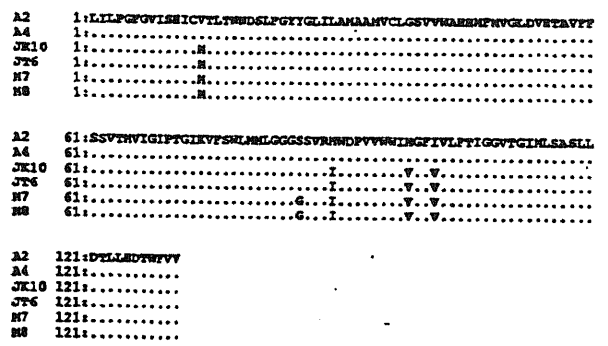

Fig. 7 Comparison of a part of the ITS2 region between the sequences described by Michot et al. (1993) and the present sequences