FULL PAPER *Parasitology*

Molecular Characterization of Parthenogenic *Fasciola* sp. in Korea on the Basis of DNA Sequences of Ribosomal ITS1 and Mitochondrial NDI Gene

Tadashi ITAGAKI[1)], Masayuki KIKAWA[1)], Kunio TERASAKI[2)], Toshiyuki SHIBAHARA[3)] and Koichi FUKUDA[4)]

[1)]Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, Ueda 3–18–8, Morioka 020–8550, [2)]St. Mary's Junior College, Kurume 830–8558, [3)]Division of Laboratory Animal Science, Research Center for Bioscience and Technology, Tottori University, Yonago 683–8503 and [4)]Center for Laboratory Animal Science, National Defense Medical College, Tokorozawa 359–8513, Japan

(Received 27 April 2005/Accepted 7 July 2005)

ABSTRACT. Nucleotide sequences of ribosomal internal transcribed spacer (ITS1) and mitochondrial NADH dehydrogenase I (NDI) gene were analyzed to genetically characterize aspermic *Fasciola* forms in Korea. From the difference in ITS1 sequences, Korean flukes were divided into 3 haplotypes represented by Kor1, Kor2 and Kor1/2, which had nucleotides identical to *F. hepatica, F. gigantica* and those overlapped between the two species, respectively. NDI sequences also showed that Korean flukes could be classified into 3 distinct haplotypes (Kor1: *F. hepatica*-type, Kor2a and Kor2b: *F. gigantica*-type). The sequences of Kor1 and Kor2a were 100% identical to those of the haplotypes Fsp1and Fsp2, respectively, which are major *Fasciola* forms in Japan. These findings strongly suggest that aspermic *Fasciola* forms in Korea and Japan originated from same ancestors and have recently spread throughout both countries.
KEY WORDS: *Fasciola* sp., haplotype, ITS1, Korea, NDI.

J. Vet. Med. Sci. 67(11): 1115–1118, 2005

Fasciola hepatica and *F. gigantica* are recognized as the causative agents of fascioliasis in domestic animals and humans. Although the two species are classified by morphological characters, especially body length and width, it is difficult to accurately discriminate between the two species because of many variations in size depending on such factors as age of the fluke, species of host and technical difficulty in fixation of the flukes [8]. Furthermore, aspermic *Fasciola* forms have been found in Asian countries, including Japan and Korea [17]. These aspermic forms have not been identified yet because they include worms showing a wide range of morphological variations [3, 6, 12] and parthenogenetic diploid, triploid and mixoploid [10, 13, 14].

Molecular approaches based on DNA analysis have been employed to solve the problems of identification and genetic characterization of morphologically similar parasites [1, 4, 9]. Recently, at least five haplotypes that differ in the sequences of ribosomal ITS1 and ITS2 and mitochondrial NDI and COI genes have been found in Japanese *Fasciola* forms [7]. The purpose of the present study was to genetically characterize Korean *Fasciola* forms closely resembling Japanese *Fasciola* in morphological and chromosomal features.

MATERIALS AND METHODS

Fasciola individuals and chromosome observation: Twelve adults of *Fasciola* sp. were obtained from the bile ducts of 5 infected cattle slaughtered in Seoul, Korea (Table 1) and kept at –80°C or in 70% ethanol until DNA extraction. In two of the flukes, a small piece of the testis was cut off and fixed in freshly prepared ethanol-acetic acid (3:1) before freezing or fixation with 70% ethanol, and chromosomes were observed by using a squashing method [15].

DNA extraction and amplification: Total DNA was extracted from individual flukes by using an E.Z.N.A. mollusk DNA kit (Omega Bio-tek, Doraville, U.S.A.). DNA fragments of the ITS1 region and NDI gene were amplified by PCR using 1.25 units of Taq polymerase (Promega, Madison, U.S.A.), 0.4 mM each of dATP, dTTP, dCTP and dGTP, 2 mM $MgCl_2$, each primer set (50 pmol/25 μl reaction mixture), and PCR buffer. The primer sets used were Ita 10 (5'-AAGGATGTTGCTTTGTCGTGG-3') and Ita 2 (5'f-GGAGTACGGTTACATTCACA-3') for NDI, and ITS1-F (5'-TTGCGCTGATTACGTCCCTG-3') and ITS1-R (5'-TTGGCTGCGCTCTTCATCGAC-3') for ITS1. Reaction cycles consisted of an initial denaturing step at 94°C for 90 sec, followed by 30 cycles at 94°C for 90 sec, 53°C for 90 sec and 72°C for 120 sec with a final extension at 72°C for 10 min using a GeneAmp PCR System 2400 (Applied Biosystems, Tokyo, Japan). PCR products were precipitated with ethanol/sodium acetate and dissolved in MQ.

Sequencing analysis: PCR fragments were directly sequenced using ABI Prism Big Dye terminator v. 1.0 ready reaction cycle sequencing kits (Applied Biosystems) with the use of the same primers as those used in PCR. At least two fragments amplified for individual flukes were sequenced for each target region in both directions using forward and reverse primers. The sequencing reactions were run on a PE Applied Biosystems 310 automated sequencer. Sequence data were analyzed by Factura software (Applied Biosystems) to detect heterozygotes. The sequence data were aligned by Clustal X program v. 1.53b [19]. Phylogenetic analyses were conducted by neighbor-joining (NJ) and maximum parsimony (MP) using PAUP 4.0b10 [16] with *F. hepatica* (accession nos. AB207154, AB207155), *F. gigantica* (AB207157 - AB207167) and Japanese haplotypes, Fsp1 (AB207169) and Fsp2 (AB207168), and a lung fluke, *Paragonimus westermani*

(AF219379), designed as an outgroup. All characters were run unordered and equally weighted. Alignment gaps were treated as missing data. A heuristic search with tree-bisection-reconnection (TBR)-branch swapping was used in MP analysis to infer the shortest trees. The length, consistency index excluding uninformative characters (C. I.) and retention index (R.I.) of the most parsimonious trees were recorded. A bootstrap analysis using 1,000 repricates was conducted using heuristic searches and TBR branch swapping with the Multrees option in order to determine the relative support for clades of the consensus tree. The following Genbank data were also used for comparison of ITS1 sequences: *F. hepatica* (accession nos. AB207139-AB207141) and *F. gigantica* (AB207142-AB207144).

RESULTS

The number of chromosomes in 2 flukes was counted in well-spread primary spermatocytes. One fluke was found to have 30 univalent chromosomes (3n=30, triploid), and the other had 20 univalent chromosomes (2n=20, diploid) (Table 1).

The sequences of the ITS1 region in Korean flukes consisted of 600 bps including complete ITS1, partial 18S and 5.8S rDNA and had 6 variable nucleotide positions of which 1 was located in 18S and 5 were located in ITS1. From the differences in the 6 positions, Korean flukes were divided into 3 haplotypes represented by Kor1 (accession no. AB211236), Kor2 (AB211238) and Kor1/2(AB211237), which had nucleotides identical to *F. hepatica* (accession nos. AB207139–AB207141), and *F. gigantica* (AB207142–AB207144) and those overlapped between the two species, respectively (Table 2).

Table 1. DNA haplotypes of 12 Korean *Fasciola* sp. used in the study

Specimen code	Host code	Ploidy	Haplotype	
			ITS1	NDI
SG 4	A	ND	Kor 1	Kor 1
HS 1	B	3n	Kor 1	Kor 2
HS 3	B	ND	Kor 1/2	Kor 2
HS 4	C	ND	Kor 1/2	Kor 2
HS 5	C	ND	Kor 2	Kor 2
HS 8	D	ND	Kor 1/2	Kor 1
HS 9	D	ND	Kor 1/2	Kor 1
HS 11	D	ND	Kor 1/2	Kor 1
HS 14	E	ND	Kor 1/2	Kor 1
HS 17	E	ND	Kor 1	Kor 2
HS 20	E	ND	Kor 1	Kor 1
HS 21	E	2n	Kor 1	Kor 1

"2n" and "3n" mean diploid and triploid, respectively.
"ND" means no data.

Partial NDI sequence (535 bps) was determined for Korean *Fasciola* and was found to include 46 variable sites. On the basis of the sequences, Korean flukes were classified into 3 distinct haplotypes (Kor1, Kor2a, Kor2b; accession nos. AB211239–AB211241). Kor1 and Kor2a differed from each other at 45 of the 46 sites, and Kor2a and Kor2b differed from each other in 1 of the 46 sites. Kor1 was very similar to the sequence of *F. hepatica*, and Kor2a and Kor2b were similar to the sequence of *F. gigantica*. In addition, the sequences of Kor1 and Kor2a coincided with those of the haplotypes, Fsp1(accession no. AB207169) and Fsp2 (AB207168), respectively, found in Japanese *Fasciola* forms. Seven, 3 and 2 of 12 Korean flukes showed Kor1, Kor2a and Kor2b, respectively. MP analysis resolved most parsimonious trees for NDI (length 518; CI=0.8958; RI=0.9004). Tree topologies from MP and NJ analyses were similar to each other. Kor1, *F. hepatica* and Fsp1 belonged to the same clade, which clearly differed from the clade of Kor2a , Kor2b, *F. gigantica* and Fsp2 (Fig. 1A and B).

DISCUSSION

Aspermic *Fasciola* forms in Korea and Japan have common characteristics: they include worms morphologically resembling *F. hepatica*, *F. gigantica* and their intermediate type [3, 6, 12] and cytologically showing parthenogenic diploid, triploid and mixoploid [10, 13, 14]. The present study showed that both forms were also similar to each other in molecular characters: Korean forms consisted of ITS1 and NDI haplotypes of *F. hepatica*-type, *F. gigantica*-type and

Table 2. Comparison of the nucleotides at 6 variable sites in ITS1 region among 3 Korean haplotypes, *F. hepatica* and *F. gigantica*

Species	Locality, haplotype	Nucleotide sites of ITS1 region					
	Accession no.	48	175	265	359	437	457
F. hepatica	Uruguay (AB207139)	T	C	A	C	T	C
	Australia (AB207140)	T	C	A	C	T	C
	N. Ireland (AB207141)	T	C	A	C	T	C
F. gigantica	Indonesia (AB207143)	C	T	T	T	A	T
	Thailand (AB207144)	C	T	T	T	A	T
	Zambia (AB207142)	C	T	T	T	A	T
Fasciola sp.	Korea ; Kor 1	T	C	A	C	T	C
	Korea ; Kor 2	C	T	T	T	A	T
	Korea ; Kor 1/2	T/C	C/T	A/T	C/T	T/A	C/T

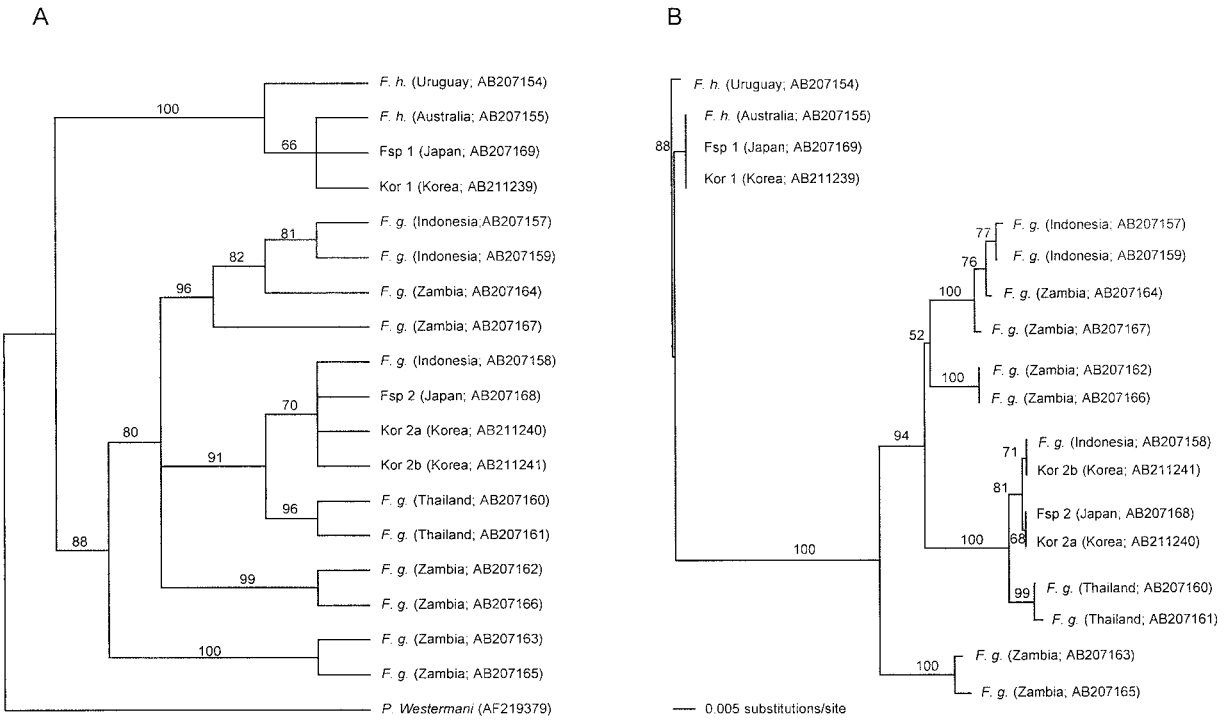

Fig. 1. Phylogenetic relationships between Korean haplotypes and *Fasciola* spp. inferred from the nucleotide sequences of partial NDI. A and B show MP and NJ trees, respectively.The abbreviations are as follows: Fsp1 and Fsp2 show the haplotypes of Japanese *Fasciola* sp., and *F. h.* and *F. g.* show *F. hepatic*a and *F. gigantica*, respectively. Parentheses represent locality and accession no. of individual flukes. The numbers of the branches indicate bootstrap support values (≥ 50, 1000 replications).

their intermediate type as well as Japanese forms, although Agatsuma *et al.* [2] reported that the mitochondrial NDI and COI sequences of 5 Korean *Fasciola* specimens were all *F. gigantica*-type. Furthermore, the NDI sequences of Korean haplotypes, Kor1 and Kor2a, were 100% identical to those of Fsp1 and Fsp2, respectively, which are major haplotypes of Japanese *Fasciola* forms. These findings strongly suggest that *Fasciola* forms in Korea and Japan originated from same ancestors and have recently spread throughout both countries with movement of infected animals (probably cattle). In fact, Japanese native cattle are thought to have been introduced into Japan via the Korean Peninsula in about the second century AD [11]. Recently, Terasaki *et al.* [18] have suggested that aspermic (parthenogenic) diploid *Fasciola* forms originated from spermic (sexual) *F. hepatica* and *F. gigantica* that had acquired asynapsis of chromosomes in the primary spermatocytes and mitotic division in the primary oocytes due to gene mutations and that parthenogenic triploid forms were derived from hybrids between sexual diploid and parthenogenic diploid forms. According to this hypothesis, the origin of parthenogenic *Fasciola* would not be in Korea as well as in Japan, because sexual *F. hepatica* and *F. gigantica* have not been found in Korea [17]. Molecular phylogeny using *Fasciola* individuals from other Asian counties is needed to elucidate the evolutionary origin(s) and route of spread throughout Asia of parthenogenic *Fasciola* forms.

In the present study, a heterozygous haplotype (Kor1/2) that had nucleotides overlapped between the ITS1sequences of *F. hepatica* and *F. gigantica* was found in 6 Korean flukes. This intermediate genotype has been also found in Korean *Fasciola* flukes using D2 sequences of 28S rDNA [2], in Chinese flukes using ITS2 [5] and in Japanese specimens using ITS1 and ITS2 [7]. Furthermore, two Korean flukes that were Kor1 in ITS1and Kor2 in NDI were also detected in this study. Although these aspermic *Fasciola* forms may be progenies of hybrids between the two Fasciola species, additional information on DNA sequences in other genes is needed to clarify the interspecific hybridyzation.

ACKNOWLEDGEMENT. This study was supported in part by a Grant-in-Aid for Scientific Research (C2) (no. 14560243) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Adlard, R.D., Barker, S.C., Blair, D. and Cribb, T.H. 1993. Comparison of the second internal transcribed spacer (ribosomal DNA) from populations and species of Fasciolidae (Digenea). *Int. J. Parasitol.* **23**: 423–425.
2. Agatsuma, T., Arakawa, Y., Iwagami, M., Honzako, Y., Cahyaningsih, U., Kang, S-Y. and Hong, S-J. 2000. Molecular evidence of natural hybridization between *Fasciola hepatica* and

F. gigantica. *Parasit. Int.* **49**: 231–238.
3. Akahane, H., Harada, Y. and Oshima, T. 1970. Patterns of variation of the common liver fluke (*Fasciola* sp.) in Japan. III. Comparative studies on the external form, size of egg and number of eggs in the uterus of flukes in cattle, goats and rabbits. *Jpn. J. Parasitol.* **19**: 619–627 (in Japanese with English summary).
4. Blair, D. and McManus, D.P. 1989. Restriction enzyme mapping of ribosomal DNA can distinguish between fasciolid (liver fluke) species. *Mol. Biochem. Parasitol.* **36**: 201–208.
5. Huang, W. Y., He, B., Wang, C. R. and Zhu, X. Q. 2004. Characterisation of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. *Vet. Parasitol.* **120**: 75–83.
6. Itagaki, H. and Akane, S. 1959. Morphological study on the Japanese liver fluke, compared with the African specimens. *Bull. Azabu Vet. Coll.* **6**: 115–123.
7. Itagaki, T., Kikawa, M., Sakaguchi, K., Shimo, J., Terasaki, K., Shibahara, T. and Fukuda, K. 2005. Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology* (in press).
8. Kendall, S. B. 1965. Relationships between the species of *Fasciola* and their molluscan hosts. *Adv. Parasitol.* **3**: 59–95.
9. McManus, D.P. and Bowles, J. 1996. Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. *Int. J. Parasitol.* **26**: 687–704.
10. Moriyama, N., Tsuji, M. and Seto, T. 1979. Three karyotypes and their phenotypes of Japanese liver flukes (*Fasciola* sp.). *Jpn. J. Parasitol.* **28**: 23–33 (in Japanese with English summary).
11. Mukai, F., Tsuji, S., Fukazawa, K., Ohtagiki, S. and Nambu, Y. 1989. History and population structure of a closed strain of Japanese Black cattle. *J. Anim. Breed. Genet.* **106**: 254–264.
12. Oshima, T., Akahane, H. and Shimazu, T. 1968. Patterns of the variation of the common liver fluke (*Fasciola* sp.) in Japan. I. Variations in the sizes and shapes of the worms and eggs. *Jpn. J. Parasitol.* **17**: 97–105 (in Japanese with English summary).
13. Rhee, J. K., Eun, G. S. and Lee, S. B. 1987. Karyotype of *Fasciola* sp. obtained from Korean cattle. *Kor. J. Parasitol.* **25**: 37–44 (in Korean with English Summary).
14. Sakaguchi, Y. 1980. Karyotype and gametogenesis of the common liver fluke, *Fasciola* sp., in Japan. *Jpn. J. Parasitol.* **29**: 507–513.
15. Snow, R. 1963. Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. *Stain Technol.* **38**: 9–13.
16. Swofford, D. L. 2001. PAUP*. Phylogenetic analysis using parsimony and other methods ver. 4.0beta. Sinauer Associates, Sunderland, Massachussetts.
17. Terasaki, K., Akahane, H. and Habe, S. 1982. The geographical distribution of common liver flukes (the Genus *Fasciola*) with normal and abnormal spermatogenesis. *Jpn. J. Parasitol.* **44**: 223–231.
18. Terasaki, K., Noda, Y., Shibahara, T. and Itagaki, T. 2000. Morphological comparisons and hypotheses on the origin of polyploids in parthenogenetic *Fasciola* sp. *J. Parasitol.* **86**: 724–729.
19. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res.* **24**: 4876–4882.