NOTE *Parasitology*

DNA Types of Aspermic *Fasciola* Species in Japan

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ABSTRACT. In order to reveal DNA types of aspermic *Fasciola forms* in Japan, *Fasciola* specimens obtained from eight prefectures that had not been previously reported were analyzed for DNA of ribosomal internal transcribed spacer 1 (ITS1) and mitochondrial NADH dehydrogenase 1 (ND1) gene. Five combinations in DNA types of both ITS1 and ND1 were revealed from the results of this study and previous studies. The DNA type Fsp2, which is identical to that of *F. gigantica* in both ITS1 and ND1, was the most predominant in Japan, followed by Fsp1, which is the same DNA type as that of *F. hepatica*. *Fasciola* forms with Fsp1 mainly occurred in the northern region of Japan and those with Fsp2 were mainly in the western region. The founder effect related to migration of definitive host and susceptibility of intermediate host snail might play an important role in both geographical distribution and frequency of DNA types in Japanese *Fasciola* specimens.

KEY WORDS: DNA type, geographical distribution, ITS1, Japanese aspermic *Fasciola*, ND1.

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Although *Fasciola hepatica* and *F. gigantica* are recognized as the causative agents of fasciolosis in domestic animals and humans, aspermic *Fasciola* specimens in Japan are difficult to accurately identify [9]. Molecular approaches based on DNA analysis have been employed for genetic characterization and identification of morphologically similar parasites, including *Fasciola* species [1, 2, 13]. Regarding ribosomal and mitochondrial DNA characterization of Japanese aspermic *Fasciola* specimens, Itagaki *et al.* [9] reported that the two major *Fasciola* forms represented by Fsp1 and Fsp2 that closely resemble *F. hepatica* and *F. gigantica*, respectively, occurred in Japan with distinct geographical distributions. In order to confirm the distribution of *Fasciola* forms in Japan, we investigated DNA types of *Fasciola* specimens obtained from eight prefectures that had not been reported previously.

Twenty-six adults of *Fasciola* sp. were obtained from the bile ducts of 17 infected cattle in Niigata, Gunma, Kyoto, Okayama, Shimane, Yamaguchi, Kagawa and Ehime Prefectures and kept in 70% ethanol until use. The anterior parts including the seminal vesicles of the fixed flukes were cut off and stained with Hematoxylin-Carmin solution. Then they were observed with an optical microscope for checking the presence of sperm within the seminal vesicle. The posterior parts of the flukes, not including reproductive organs, were used for DNA extraction. Total DNA was extracted from individual flukes using an E.Z.N.A. mollusc DNA kit (Omega Bio-tek, Doraville, GA, U.S.A.) according to the manufacturer's instructions. DNA samples were stored at –20°C until use. DNA fragments of each target region were amplified by polymerase chain reaction (PCR) according to Itagaki *et al.* [9]. The primer sets used to amplify the fragments were ITS1-F and ITS1-R for the internal transcribed spacer 1 (ITS1) region and Ita 10 and Ita 2 for the NADH dehydrogenase 1 (ND1) gene. A restriction fragment length polymorphism of amplified DNA (PCR-RFLP) method [3] was used for analysis of DNA types of the ITS1 region. PCR amplicons of the ITS1 region were digested by the restriction enzyme *Rsa* I, and DNA types were distinguished by the difference in fragment pattern detected on agarose gel. On the other hand, PCR amplicons of the ND1 gene were directly sequenced in both directions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) with use of a forward primer, Ichi1 (5'-AGGTGTTGGGTTATATGCA-3'), and a reverse primer Ita2. The sequencing reactions were run on an ABI PRISM 3100-Avant Genetic Analyzer. The sequence data were aligned by Clustal X program v. 2.0. [12], and DNA types were distinguished by the difference of sequences.

None of the 26 *Fasciola* specimens contained sperms in the seminal vesicles and they were all comfirmed to be aspermic (Table 1). In DNA type of the ITS1 region, 24 and 2 *Fasciola* specimens were found to be Fsp2 (accession No. AB207146) and Fsp1/2 (AB207147), respectively, which were reported previously [9], since the Fsp2 and Fsp1/2 types showed the same restricted fragments (about 360, 170 and 60 bp) as *F. gigantica* and both fragments (about 360, 170, 100, 60 bp) of *F. hepatica* and *F. gigantica*, respectively (Fig. 1, Table 1). In DNA types of the ND1 gene (535 bp), 2 and 24 specimens were Fsp1 (accession No. AB207169) and Fsp2 (AB207168), respectively, which were the haplotypes reported previously [9].

This study showed the occurrence of two distinct *Fasciola* forms: one form (24 specimens) with Fsp2 in both ITS1 and ND1 and the other (2 specimens from Kyoto) with Fsp1/2 in ITS1 and Fsp1 in ND1. In order to summarize DNA types of aspermic *Fasciola* sp. in Japan, data for DNA types

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Table 1. Locality and DNA types of 26 Japanese *Fasciola* sp. used in this study

Specimen code	Cattle code	Locality	Sperm in the seminal vesicle	DNA type	
				ITS1	ND1
1	A	Niigata	–[a)]	Fsp2	Fsp2
2	A	Niigata	–	Fsp2	Fsp2
3	A	Niigata	–	Fsp2	Fsp2
4	B	Gunma	–	Fsp2	Fsp2
5	B	Gunma	–	Fsp2	Fsp2
6	B	Gunma	–	Fsp2	Fsp2
7	C	Kyoto	–	Fsp2	Fsp2
8	C	Kyoto	–	Fsp2	Fsp2
9	D	Kyoto	–	Fsp1/2	Fsp1
10	D	Kyoto	–	Fsp1/2	Fsp1
11	E	Okayama	–	Fsp2	Fsp2
12	F	Okayama	–	Fsp2	Fsp2
13	G	Okayama	–	Fsp2	Fsp2
14	H	Okayama	–	Fsp2	Fsp2
15	I	Shimane	–	Fsp2	Fsp2
16	J	Shimane	–	Fsp2	Fsp2
17	K	Shimane	–	Fsp2	Fsp2
18	L	Shimane	–	Fsp2	Fsp2
19	M	Yamaguchi	–	Fsp2	Fsp2
20	M	Yamaguchi	–	Fsp2	Fsp2
21	M	Yamaguchi	–	Fsp2	Fsp2
22	M	Yamaguchi	–	Fsp2	Fsp2
23	N	Kagawa	–	Fsp2	Fsp2
24	O	Ehime	–	Fsp2	Fsp2
25	P	Ehime	–	Fsp2	Fsp2
26	Q	Ehime	–	Fsp2	Fsp2

a) No sperm in the seminal vesicle.

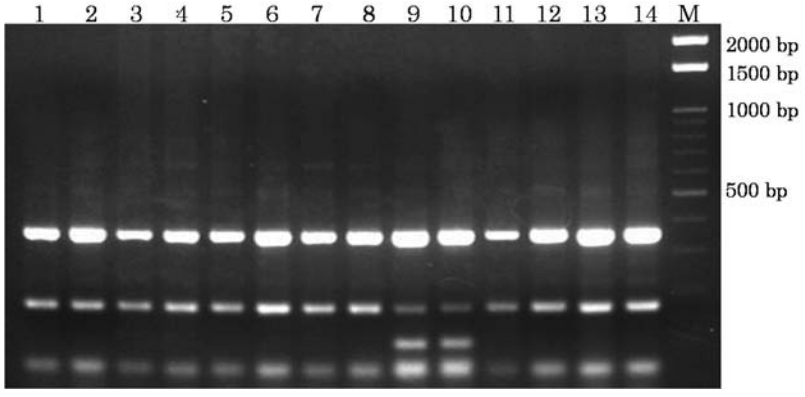

Fig. 1. Restriction fragments of the PCR-RFLP method performed in this study. Lanes 1–8 and 11–14 indicate Fsp2, Lanes 9–10 indicate Fsp1/2 (see Table 1.), M: 100 bp DNA ladder.

reported by Itagaki *et al.* [9] were added to the data obtained in this study (Table 2). The DNA types of Japanese *Fasciola* specimens consisted of five combinations in both ITS1 and ND1, except for a combination of Fsp2 in ITS1 and Fsp1 in ND1. The frequency of the combinations differed, and the combination of Fsp2 in both ITS1 and ND1 was predominant, followed by that of Fsp1 in both ITS1 and ND1. Interestingly, *Fasciola* specimens having the two combinations of DNA types showed different geographical distributions: the specimens with Fsp1 were mainly in the northern region of Japan and those with Fsp2 were mainly in western region (Fig. 2).

Although *Fasciola* specimens in Japan have been considered to be of the same origin as that of aspermic *Fasciola* specimens in Korea, China and Vietnam [7–9, 16], the frequency of DNA type combinations in Japanese specimens apparently differs from that in those three countries (e.g., the combination of Fsp1/2 in ITS1 and Fsp2 in ND1 is conspic-

Table 2. The frequency of DNA types of *Fasciola* sp. in Japan

		ITS1 types			Total
		Fsp1	Fsp2	Fsp1/2	
ND1 types	Fsp1	12	0	2	14 (23.3%)
	Fsp2	1	42	3	46 (76.7%)
	Total	13 (21.7%)	42 (70.0%)	5 (8.3%)	60[a)]

a) Numbers include the data of Itagaki *et al.* [9].

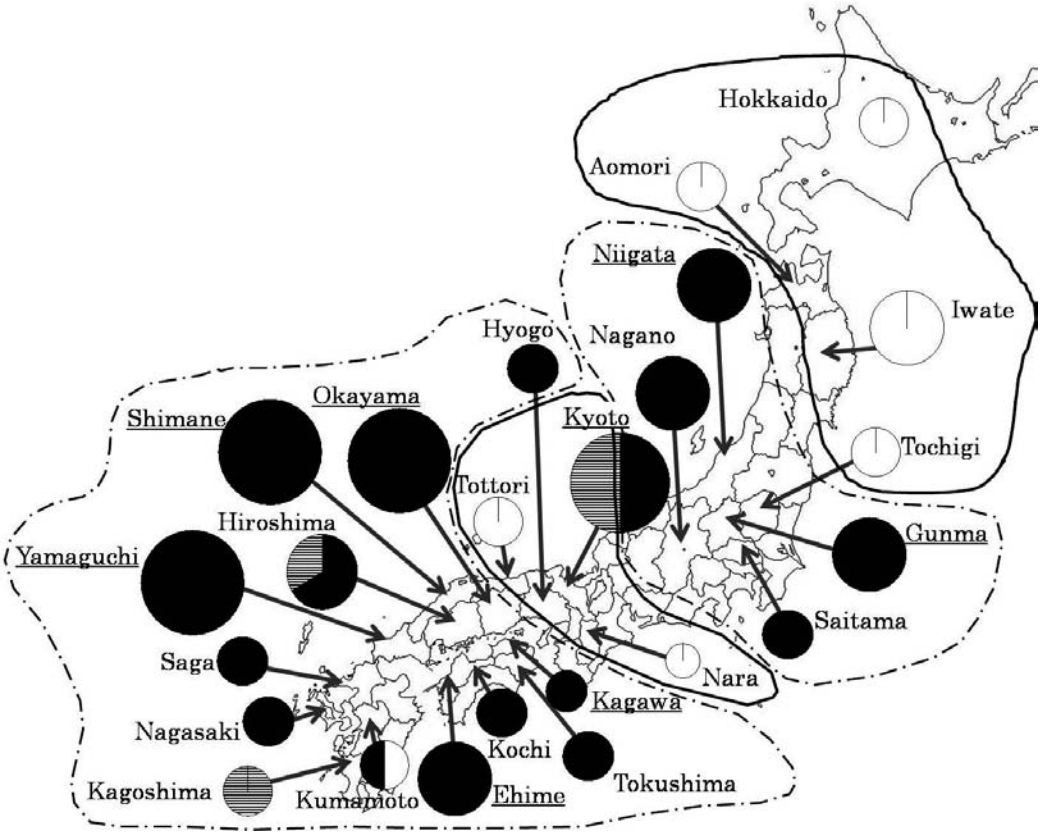

Fig. 2. Distribution of DNA types of *Fasciola* sp. in Japan. White, black and border in circles represent Fsp1, Fsp2 and Fsp1/2 in ITS1 types, respectively. Size of each circle corresponds to the number of *Fasciola* samples. Circles surrounded by solid lines and broken lines indicate Fsp1 and Fsp2 in ND1 types, respectively. The results of 8 prefectures underlined were obtained in this study, and those of the other prefectures were based on the previous study [9].

uously dominant in China and Vietnam). The difference between frequencies of DNA type combinations in the *Fasciola* population in Japan and in *Fasciola* populations in neighboring countries might be the result of a founder effect when cattle, the final host of the flukes, were introduced into Japan. It is thought that aspermic *Fasciola* species seem to have entered Japan together with domestic cattle introduced via the Korean Peninsula around the second century [9, 15]. Moreover, it is speculated that limited numbers (breeds) of primary immigrated cattle spread to several areas of Japan in a short period [14, 17]. Therefore, it is reasonable to assume that *Fasciola* flukes having DNA types of Fsp1 and Fsp2 extended their distribution with the process of the cattle expansion.

Furthermore, geographical settlement of aspermic *Fasciola* flikes would be related to susceptibility to their intermediate snail hosts. Although *Lymnaea ollula* is the most suitable host for *Fasciola* sp. in Japan and is distributed throughout the country, the snail host has higher susceptibility to *F. gigantica* than to *F. hepatica* [5, 6, 11, 18]. Additionally, *L. truncatula*, a major intermediate host of *F. hepatica* in Europe, is abundantly distributed in eastern and northern Honshu and Hokkaido [4], and the snail host of *Fasciola* sp. in Hokkaido was experimentally defined to be

L. truncatula [10]. This snail susceptibility to *Fasciola* forms with Fsp1 and Fsp2, which genetically resemble *F. hepatica* and *F. gigantica*, respectively, also might have an important role in the geographical distribution of the two *Fasciola* forms in Japan.

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