

Molecular characterization of *Fasciola hepatica*, *Fasciola gigantica*, and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA

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Abstract Parthenogenic *Fasciola* forms as well as bisexual *Fasciola hepatica* and *Fasciola gigantica* in mainland China have been identified on the basis of their spermatogenesis and genotypes in nuclear ribosomal internal transcribed spacer 1 (ITS1) and mitochondrial NADH dehydrogenase I (NDI). The Chinese aspermic *Fasciola* would include forms originating in interspecific hybrids between *F. hepatica* and *F. gigantica*, since they showed the genotype of ITS1-Fh/Fg that had mixed sequences of the two *Fasciola* species or heterogeneous genotypes in ITS1 and NDI. Additionally, there were Chinese aspermic flukes in which the sequences of ITS1 and NDI genotypes completely coincided with those in aspermic forms from Japan, Korea, and Vietnam, suggesting that the aspermic forms from these four countries are offspring with a common provenance. The Fh-C4 haplotype in NDI was detected in both aspermic specimens and *F. hepatica*, indicating that aspermic forms showing the haplotype might come into

existence in China. The ratio of body length and width in aspermic *Fasciola* specimens showed intermediate values between those of *F. hepatica* and *F. gigantica*.

Introduction

Fasciola species that mainly cause fasciolosis in animals and humans are *Fasciola hepatica* and *Fasciola gigantica*. The two *Fasciola* species have been classified by morphological characters such as body length and width, and they have been shown to be spermic diploid and meiotically functional in cytogenetical characteristics (Sanderson 1953; Reddy and Subramanyam 1973). The two species can also be discriminated by DNA sequences of nuclear ribosomal internal transcribed spacer 1 (ITS1), ITS2, and 28S rRNA genes (Adlard et al. 1993; Itagaki and Tsutsumi 1998; Marcilla et al. 2002; Itagaki et al. 2005a) and of mitochondrial NDI and COI genes (Itagaki et al. 2005a). *F. hepatica* mainly occurs in Europe, the Americas, and Oceania, while *F. gigantica* exists in Africa and Asia (Torgerson and Claxton 1999).

On the other hand, *Fasciola* specimens intermediate between *F. hepatica* and *F. gigantica* in morphology have been found in Asian countries, including Japan (Watanabe and Iwata 1954; Itagaki and Akane 1959; Oshima et al. 1968), India (Varma 1953), Korea (Chu and Kim 1967), the Philippines (Kimura et al 1984), and Iran (Ashrafi et al. 2006). These intermediate forms are difficult to identify accurately. Moreover, Asian *Fasciola* forms include aspermic diploid and triploid specimens that are meiotically dysfunctional and gynogenic (Moriyama et al. 1979; Sakaguchi 1980; Terasaki et al. 1998). Aspermic specimens from Japan, Korea, and Vietnam exhibited heterogeneity

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between *F. hepatica* and *F. gigantica* in nuclear ribosomal and mitochondrial DNA, suggesting that their origin may be hybridization between the two species (Itagaki and Tsutsumi 1998; Agatsuma et al. 2000; Itagaki et al. 2005a, b; Itagaki et al. 2009). These findings indicate that Asian *Fasciola* specimens that are diversified in biological nature should be characterized by spermatogenesis and genetic differentiation as well as morphology.

In China, *F. hepatica* has mainly occurred in the north, and *F. gigantica* has mainly occurred in the south (see Huang et al. 2004). Diploid and triploid specimens of *Fasciola* have also been found in China (Yin and Ye 1990). Molecular analysis using ITS1 and ITS2 sequences has recently shown the existence of *Fasciola* specimens intermediate between *F. hepatica* and *F. gigantica* in China (Huang et al. 2004; Lin et al. 2007), although their spermatogenesis was unfortunately not elucidated in those studies. The present study was designed to characterize *Fasciola* forms in China based on spermatogenesis, morphology, and DNA data of nuclear ribosomal ITS1 and mitochondrial NDI gene.

Materials and methods

Fasciola specimens, measurements, and spermatogenesis

Forty-five adults of *Fasciola* species were obtained from the bile ducts of 17 infected cattle and three infected yak at slaughterhouses in Hohhot, Urumqi, Xinin, Fuzhou, and Guiyang, China in 2006 (Table 1). The flukes were fixed in 70% ethanol between two slide glasses with slight pressure, and then measurements of body length and width were carried out. Thereafter, the anterior parts including the vesicula seminalis of the fixed flukes were cut, stained with hematoxylin–carmin solution, and observed for the presence of sperm within the vesicula seminalis. The posterior parts, not including reproductive organs such as the uterus in which sperm of another individual may be present, were used for genomic DNA extraction. The data of the measurements were statistically analyzed using Student's *t* test, and $P < 0.05$ was considered significant.

DNA extraction and amplification

Total DNA was extracted from individual flukes using an E. Z.N.A. mollusk DNA kit (Omega Bio-tek, Doraville, USA) according to the manufacturer's instructions. DNA fragments of each target region were amplified by polymerase chain reaction (PCR) using 1.25 units of *Taq* polymerase (Promega, Madison, USA), 0.4 mM each of dATP, dTTP, dCTP, and dGTP, 2 mM MgCl₂, each primer set (50 pmol/25 μl reaction mixture), and PCR buffer. The primer sets

used to amplify the fragments were ITS1-F and ITS1-R for the ITS1 region and Ita 10 and Ita 2 for NDI (Itagaki et al. 2005a). Reaction cycles consisted of an initial denaturing step at 94°C for 90 s, followed by 30 cycles at 94°C for 90 s, 55°C for 90 s, and 72°C for 120 s with a final extension at 72°C for 10 min using GeneAmp PCR Systems 2700 (Applied Biosystems, Tokyo, Japan). PCR amplicons were precipitated with ethanol/sodium acetate and dissolved in MilliQ water.

Sequence analysis

PCR amplicons were directly sequenced using ABI Prism Big Dye terminator v. 3.0 ready reaction cycle sequencing kits (Applied Biosystems) with the use of an additional reverse primer, Ita 4 (Itagaki et al. 2005a), for NDI together with the same primers as those used in PCR. At least two amplicons for individual flukes were sequenced for each target region in both directions using forward and reverse primers except for NDI fragments (Ita 2 and Ita 4). The sequencing reactions were run on a PE Applied Biosystems 3100 automated sequencer. The sequence data were aligned by Clustal X program v. 1.53b (Thompson et al. 1997). When heterogeneous nucleotides were detected at a nucleotide position, they were represented as a symbol, “/”: “T/C” and “C/T” mean the nucleotide T or C, and the nucleotide coinciding with that of *F. hepatica* and *F. gigantica* were at the upper left and at the lower right of the symbol, respectively. Phylogenetic analyses based on NDI sequence data were conducted by neighbor-joining using PAUP 4.0b10 (Swofford 2001) with a lung fluke, *Paragonimus westermani* (accession no. AF219379) designated as an outgroup. Additional sequences of *F. hepatica* from Uruguay (AB207154), Australia (AB207155), and Ireland (AB207156), *F. gigantica* from Zambia (AB207162, AB207164, AB207166, AB207167), Thailand (AB207160, AB207161), and Indonesia (AB207157, AB207158, AB207159), and parthenogenic *Fasciola* sp. from Japan (Fsp1: AB207169, Fsp2: AB207168) and Korea (Kor1: AB211239, Kor2a: AB211240, Kor2b: AB211241) were used to compare the phylogenetic relationships among them. All characters were run unordered and equally weighted. Alignment gaps were treated as missing data. Bootstrap analyses were conducted using 1,000 replicates.

Results

Spermatogenesis

All of the 18 *Fasciola* specimens from Hohhot, Urumqi, and Xinin had many normal spermatozoa in the seminal vesicles (spermic), suggesting that they have the ability for

Table 1 Profile of *Fasciola* specimens used and their spermatogenesis, DNA types, and measurements

Specimen code	Location	Host code	Sperm in the seminal vesicle	DNA type		Measurements of body			Species
				ITS1	ND1	Length (mm)	Width (mm)	Length/width	
H1-1	Hohhot	Cattle #1	+	Fh	Fh-C1	2	0.82	2.43	<i>F. hepatica</i>
H2-1	Hohhot	Cattle #2	+	Fh	Fh-C2	2.4	1.2	2	<i>F. hepatica</i>
H2-2	Hohhot	Cattle #2	+	Fh	Fh-C2	3.2	1.3	2.46	<i>F. hepatica</i>
H2-3	Hohhot	Cattle #2	+	Fh	Fh-C2	2.8	1.4	2	<i>F. hepatica</i>
H3-1	Hohhot	Cattle #3	+	Fh	Fh-C1	2.5	1.03	2.5	<i>F. hepatica</i>
H3-2	Hohhot	Cattle #3	+	Fh	Fh-C1	2.56	1.23	2.08	<i>F. hepatica</i>
H4-1	Hohhot	Cattle #4	+	Fh	Fh-C3	2.4	1	2.4	<i>F. hepatica</i>
U1-1	Urumqi	Cattle #5	+	Fh	Fh-C4	3.5	1.56	2.24	<i>F. hepatica</i>
U1-2	Urumqi	Cattle #5	+	Fh	Fh-C5	2.6	1.16	2.24	<i>F. hepatica</i>
U2-1	Urumqi	Cattle #6	+	Fh	Fh-C4	2.42	1	2.42	<i>F. hepatica</i>
U3-1	Urumqi	Cattle #7	+	Fh	Fh-C6	2.68	1.04	2.5	<i>F. hepatica</i>
U3-2	Urumqi	Cattle #7	+	Fh	Fh-C1	2.4	0.9	2.5	<i>F. hepatica</i>
Q1-1	Xinin	Yak #1	+	Fh	Fh-C4	2	0.8	2.5	<i>F. hepatica</i>
Q1-2	Xinin	Yak #1	+	Fh	Fh-C4	2.6	0.9	2.89	<i>F. hepatica</i>
Q2-1	Xinin	Yak #2	+	Fh	Fh-C4	3.2	1.2	2.67	<i>F. hepatica</i>
Q2-2	Xinin	Yak #2	+	Fh	Fh-C4	3.37	1.47	2.29	<i>F. hepatica</i>
Q3-1	Xinin	Yak #3	+	Fh	Fh-C1	2.63	1.3	2.02	<i>F. hepatica</i>
Q3-2	Xinin	Yak #3	+	Fh	Fh-C1	2.5	1.21	2.07	<i>F. hepatica</i>
B1-1	Fuzhou	Cattle #8	–	Fh/Fg	Fg-C2	3.13	1.4	2.24	<i>Fasciola</i> sp.
B1-2	Fuzhou	Cattle #8	–	Fh/Fg	Fg-C2	3.33	1.06	3.14	<i>Fasciola</i> sp.
B2-4	Fuzhou	Cattle #9	–	Fh/Fg	Fh-C4	4.09	1.33	3.08	<i>Fasciola</i> sp.
B3-1	Fuzhou	Cattle #10	–	Fh/Fg	Fg-C2	4.56	1.75	2.61	<i>Fasciola</i> sp.
B3-2	Fuzhou	Cattle #10	–	Fh/Fg	Fg-C2	3.12	1.12	2.78	<i>Fasciola</i> sp.
B4-1	Fuzhou	Cattle #11	–	Fh/Fg	Fg-C3	3.4	1.1	3.09	<i>Fasciola</i> sp.
B4-2	Fuzhou	Cattle #11	–	Fh/Fg	Fg-C3	3.7	1	3.7	<i>Fasciola</i> sp.
B5-1	Fuzhou	Cattle #12	–	Fh/Fg	Fg-C4	2.8	1	2.8	<i>Fasciola</i> sp.
B5-2	Fuzhou	Cattle #12	–	Fh/Fg	Fg-C2	3.07	1	3.07	<i>Fasciola</i> sp.
G1-1	Guiyang	Cattle #13	–	Fh/Fg	Fg-C2	3.84	1.15	3.34	<i>Fasciola</i> sp.
G1-2	Guiyang	Cattle #13	–	Fh/Fg	Fg-C2	2.84	1.38	2.06	<i>Fasciola</i> sp.
G2-1	Guiyang	Cattle #14	–	Fh/Fg	Fg-C2	3.75	1.25	3	<i>Fasciola</i> sp.
G2-2	Guiyang	Cattle #14	–	Fh/Fg	Fg-C2	4.65	1.35	3.44	<i>Fasciola</i> sp.
G2-4	Guiyang	Cattle #14	–	Fh/Fg	Fh-C4	4.75	1.35	3.52	<i>Fasciola</i> sp.
G3-1	Guiyang	Cattle #15	+	Fg	Fg-C1	4.41	1	4.41	<i>F. gigantica</i>
G3-2	Guiyang	Cattle #15	–	Fh	Fg-C2	2.93	0.86	3.41	<i>Fasciola</i> sp.
G3-5	Guiyang	Cattle #15	+	Fg	Fg-C5	2.9	0.8	3.63	<i>F. gigantica</i>
G3-6	Guiyang	Cattle #15	+	Fg	Fg-C1	4.1	1	4.1	<i>F. gigantica</i>
G3-7	Guiyang	Cattle #15	+	Fg	Fg-C1	3.1	0.8	3.88	<i>F. gigantica</i>
G3-8	Guiyang	Cattle #15	–	Fh	Fg-C2	3.7	1.4	2.64	<i>Fasciola</i> sp.
G3-9	Guiyang	Cattle #15	+	Fg	Fg-C1	2.7	0.9	3	<i>F. gigantica</i>
G4-1	Guiyang	Cattle #16	+	Fg	Fg-C1	4.13	0.89	4.64	<i>F. gigantica</i>
G4-2	Guiyang	Cattle #16	+	Fg	Fg-C1	3.63	1.03	3.52	<i>F. gigantica</i>
G4-3	Guiyang	Cattle #16	–	Fg	Fg-C6	4.6	1.2	3.83	<i>Fasciola</i> sp.
G4-4	Guiyang	Cattle #16	–	Fh/Fg	Fg-C2	4.4	1.3	3.18	<i>Fasciola</i> sp.
G5-1	Guiyang	Cattle #17	–	Fh/Fg	Fg-C2	4	1.5	2.67	<i>Fasciola</i> sp.
G5-2	Guiyang	Cattle #17	–	Fh/Fg	Fg-C2	5.07	1.15	4.41	<i>Fasciola</i> sp.

“+” and “–” show plenty of and no or a few sperms in the seminal vesicle, respectively

spermatogenesis and bisexual reproduction (Terasaki et al. 1982). The nine flukes from Fuzhou contained no or only a few sperms with or without round rosetted cells in the vesicles (aspermic), indicating that they seem to have abnormal spermatogenesis and parthenogenic reproduction (Terasaki et al. 1982) (Table 1). Seven of the 18 specimens from Guiyang were spermic, and 11 were aspermic, and both spermic and aspermic flukes were obtained from identical hosts (e.g., cattle #15, #16).

Genotypes of ITS1 region

The Chinese *Fasciola* specimens showed three different genotypes represented by ITS1-Fh, ITS1-Fg, and ITS1-Fh/Fg based on sequences of the ITS1 region. ITS1-Fh and ITS1-Fg had sequences identical to those of *F. hepatica* and *F. gigantica*, respectively, which differed from each other in six variable nucleotide positions of ITS1 region sequences (600 bp), and the ITS1-Fh/Fg genotype had nucleotides overlapped between the two *Fasciola* species in the six positions (Table 2). All of the 18 spermic specimens from Hohhot, Urumqi, and Xinin showed ITS1-Fh, and the nine aspermic specimens from Fuzhou showed ITS1-Fh/Fg (Table 1). In the specimens from Guiyang, the seven spermic specimens were all ITS1-Fg, while the remaining 11 aspermic flukes were ITS1-Fh/Fg for eight flukes, ITS1-Fh for two flukes, and ITS1-Fg for one fluke. The sequences of the ITS1 region determined were submitted to DNA Data Bank of Japan as accession nos. AB477352–AB477356.

Haplotypes of NDI gene

Partial NDI sequences (535 bp) of the Chinese flukes contained 54 variable sites and yielded 12 haplotypes represented by Fh-C1~C6 and Fg-C1~C6 (accession nos. AB477357–AB477369; Table 1), which phylogenetically

belonged to the same clade as *F. hepatica* and *F. gigantica*, respectively (Fig. 1). The haplotype Fh-C4 was detected in both spermic and aspermic specimens, and the nucleotide sequence was completely identical to that of the haplotypes Fsp1 (AB207169) and Kor1 (AB211239) obtained from Japanese and Korean aspermic *Fasciola* forms, respectively. The sequence of Fg-C1, which was only detected in spermic flukes, completely coincided with the sequence of the haplotype ND1-Fg2 (AB385616) of *F. gigantica* from Vietnam, while Fg-C2~C6 were detected only in aspermic specimens. The sequence of Fg-C2 was identical to the sequences of haplotypes Fsp2 (AB207168), Kor2a (AB211240), and NDI-Fg5 (AB385619) in aspermic Japanese, Korean, and Vietnamese *Fasciola* forms, respectively, and the sequence of Fg-C3 detected in aspermic specimens coincided with that of the Kor2b haplotype (AB211241) detected in aspermic Korean *Fasciola* specimens and of *F. gigantica* from Indonesia.

Species identification and measurements

Spermic *Fasciola* specimens that showed genotypes of ITS1-Fh and ITS1-Fg were identified as *F. hepatica* and *F. gigantica*, respectively, and the identification was also supported by the fact that they belonged to the same clades as *F. hepatica* and *F. gigantica*, respectively, in the phylogenetic tree based on the NDI sequence. It was impossible to accurately identify aspermic *Fasciola* specimens because they showed ITS1-Fh/Fg or genotypes of opposite species in ITS1 and NDI, except for one specimen (sample code, G4-3), as well as an intermediate phenotype as described below.

The Chinese specimens of *F. hepatica* measured 2.653 ± 0.421 cm (mean \pm S.D.) in body length, 1.140 ± 0.220 cm in width, and 2.345 ± 0.248 in length/width, while the specimens of *F. gigantica* measured 3.567 ± 0.675 cm in body length, 0.917 ± 0.096 cm in width, and 3.883 ± 0.559 in

Table 2 The nucleotides at six variable sites in the ITS1 region of Chinese *Fasciola* specimens in comparison with *F. hepatica* and *F. gigantica*

Species	Locality and genotype	Nucleotide sites in the ITS1 region						Accession no.
		48	175	265	359	437	457	
<i>F. hepatica</i>	Uruguay	T	C	A	C	T	C	AB207139
	Australia	T	C	A	C	T	C	AB207140
	N. Ireland	T	C	A	C	T	C	AB207141
<i>F. gigantica</i>	Indonesia	C	T	T	T	A	T	AB207143
	Thailand	C	T	T	T	A	T	AB207144
	Zambia	C	T	T	T	A	T	AB207142
<i>Fasciola</i> sp.	China; ITS1-Fh	T	C	A	C	T	C	AB385611
	China; ITS1-Fg	C	T	T	T	A	T	AB385612, AB385614
	China; ITS1-Fh/Fg	T/C	C/T	A/T	C/T	T/A	C/T	AB385613

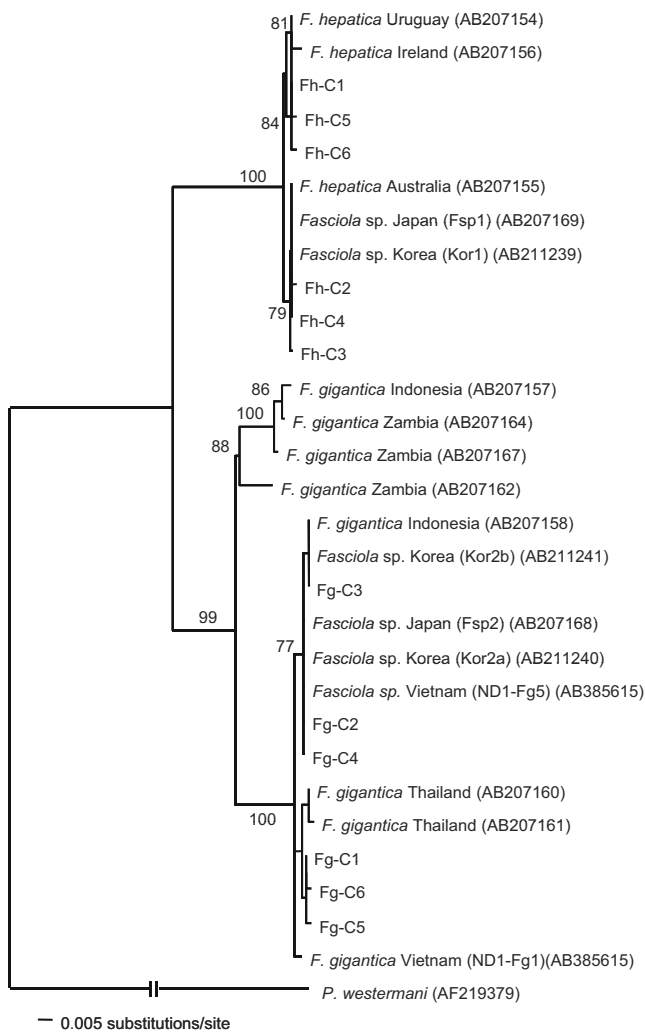


Fig. 1 A phylogenetic tree of *Fasciola* spp. inferred from the nucleotide sequences of partial NDI and the neighbor-joining method. Fh-C1~C6 and Fg-C1~C6 show the haplotypes of *Fasciola* specimens in China. Parentheses represent the accession no. or the haplotypes (Itagaki et al. 2005a, b, 2009) of *Fasciola* spp. used in the analysis. Numerals indicate bootstrap values (%) from 1,000 replicates

length/width. *F. hepatica* and *F. gigantica* showed significant differences in the three measurements ($p < 0.01$). The aspermic specimens were 3.787 ± 0.706 cm in body length, 1.233 ± 0.221 cm in width, and 3.101 ± 0.549 in length/width. There were significant differences in length and length/width ($p < 0.01$) between aspermic *Fasciola* specimens and *F. hepatica* and between aspermic specimens and *F. gigantica*.

Discussion

Although the origin and evolutionary history of flukes in the genus *Fasciola* have remained unclear, major factors that played an important role in geographical spread of *Fasciola*

species are thought to be artificial movements of domestic animals, especially cattle, that are the main definitive hosts of the flukes. This can be explained by the fact that the geographical distributions of taurine cattle (*Bos taurus*) and zebu cattle (*Bos indicus*), which are now the most dominant cattle species in the world, almost correspond to those of *F. hepatica* and *F. gigantica*, respectively: taurine cattle are raised mainly in Europe, western and northern Asia, the Americas, and Oceania, while zebu cattle are raised mainly in southern Asia and in central and southern Africa. In China, the occurrence of *F. hepatica* in the north and *F. gigantica* in the south (see Huang et al. 2004) is also consistent with the fact that cattle breeds of taurine and zebu type are dominant in the north and south, respectively (Cai et al. 2007). The present study showed the same distribution of the two *Fasciola* species in China as reported previously, since *F. hepatica* was detected in Hohhot, Urumqi, and Xinin in the north, and *F. gigantica* was detected in Guiyang in the south.

Although the NDI genotypes shown in the Chinese specimens of *F. hepatica* had a high level of diversity (six haplotypes for 18 specimens), they belonged to either of the two phylogenetically distinguishable clades, as did the flukes of *F. hepatica* from Eastern Europe and Western Asia (Semyenova et al. 2006). It is impossible to accurately calculate the time of divergence of the two lineages because no data on nucleotide substitution rates in mitochondrial DNA of *Fasciola* are available. The expansion of the two lineages, however, would also be due to the breeding and expansion of taurine cattle, as supported by the fact that multiple genotypes were detected in specimens obtained from identical city and cattle hosts. Accumulation of fossil information on *F. hepatica* is needed to clarify the origin and divergence of the species.

On the other hand, only two haplotypes were detected from the Chinese specimens of *F. gigantica*. The number of *Fasciola* samples used, however, was very few, and further analysis using more specimens from different localities will be needed to clarify the extent of mitochondrial DNA diversity of *F. gigantica* in China.

The present study firstly showed that aspermic *Fasciola* forms have occurred in mainland China (Guiyang and Fuzhou), although aspermic specimens have been found in Taiwan (Terasaki et al. 1982) and triploid specimens have been reported in Fuzhou (Yin and Ye 1990). Aspermic *Fasciola* forms have become widely distributed in Asian countries, including Japan, Korea, and Vietnam (Terasaki et al. 1982), and some aspermic forms from Japan, Korea, and Vietnam seem to be hybrids between *F. hepatica* and *F. gigantica* or offspring originating in the hybrids (Itagaki and Tsutsumi 1998; Agatsuma et al. 2000; Itagaki et al. 2005a, b; Itagaki et al. 2009). The Chinese aspermic *Fasciola* would also include forms originating in the

hybrids because they showed the ITS1-Fh/Fg type which had mixed sequences of the two *Fasciola* species or heterogeneous genotypes in ITS1 and NDI (e.g., sample codes G3-2 and G3-8). Additionally, there were Chinese aspermic flukes in which the sequences of ITS1 and NDI genotypes completely coincided with those in aspermic forms from Japan, Korea, and Vietnam, suggesting that the aspermic forms from these four countries are offspring with a common provenance. The aspermic *Fasciola* forms would have come into existence from spermic *F. hepatica* or *F. gigantica* when they acquired the ability for parthenogenic production probably due to mutation and hybridization. Therefore, aspermic forms and their ancestral *F. hepatica* or *F. gigantica* should have close relations to each other in genetic inheritance, and they should coexist in regions where aspermic forms came into existence. The Fh-C4 haplotype was detected in both aspermic specimens and *F. hepatica*, and the aspermic forms showing the haplotype might therefore have originated in China. Furthermore, since the aspermic forms showed ITS1-Fh/Fg, they might be hybrids or their offspring between maternal *F. hepatica* with the Fh-C4 haplotype and paternal *F. gigantica*. There is a geographical hybrid zone of *B. taurus* and *B. indicus* in the central area of China (Cai et al. 2007). Thus, interspecific hybridization between *F. hepatica* and *F. gigantica* might also have occurred in this area. Further analysis using more *Fasciola* samples from Asian countries, including China, should clarify whether the origin of aspermic *Fasciola* sp. is China.

The ratio of body length and width (BL/BW) has been considered to be one of the useful criteria for discrimination between *F. hepatica* and *F. gigantica*. Watanabe (1958) reported that the BL/BW value was about 2 in *F. hepatica* and more than 3 in *F. gigantica* and Periago et al. (2007) have recently shown, using specimens from Europe and Africa, that the appropriate ranges of BL/BW values were 1.29–2.80 in *F. hepatica* and 3.40–6.78 in *F. gigantica*. The Chinese specimens of *F. hepatica* and *F. gigantica* identified in this study showed BL/BW values of the two *Fasciola* species reported previously, while the aspermic specimens had BL/BW values intermediate between those of *F. hepatica* and *F. gigantica*. These findings regarding phenotype such as BL/BW values also suggest that the aspermic forms originated from interspecific hybridization between the two *Fasciola* species. Intermediate forms of *Fasciola* have recently been found in Iran and Egypt (Ashrafi et al. 2006; Periago et al. 2007), although their spermatogenesis has unfortunately not been clarified in those studies. These intermediate forms may also be aspermic *Fasciola* sp. originating from hybridization between the two *Fasciola* species.

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