Nuclear and mitochondrial DNA analysis reveals that hybridization between *Fasciola hepatica* and *Fasciola gigantica* occurred in China

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SUMMARY

The well-known pathogens of fasciolosis, *Fasciola hepatica* (Fh) and *Fasciola Gigantica* (Fg), possess abundant mature sperms in their seminal vesicles, and thus, they reproduce bisexually. On the other hand, aspermic *Fasciola* flukes reported from Asian countries, which have no sperm in their seminal vesicles, probably reproduce parthenogenetically. The aim of this study was to reveal the origin of aspermic *Fasciola* flukes. The nuclear single copy markers, *phosphoenolpyruvate carboxykinase* and *DNA polymerase delta*, were employed for analysis of *Fasciola* species from China. The hybrid origin of aspermic *Fasciola* flukes was strongly suggested by the presence of the Fh/Fg type, which includes DNA fragments of both *F. hepatica* and *F. gigantica*. China can be regarded as the cradle of the interspecific hybridization because *F. hepatica* and *F. gigantica* were detected in the northern and southern parts of China, respectively, and hybrids flukes were distributed between the habitats of the two species. The Chinese origin was supported by the fact that a larger number of mitochondrial *NADH dehydrogenase subunit 1* (*nad1*) haplotypes was detected in Chinese aspermic *Fasciola* populations than in aspermic populations from the neighbouring countries. Hereafter, 'aspermic' *Fasciola* flukes should be termed as 'hybrid' *Fasciola* flukes.

Key words: *Fasciola*, geographical origin, China, hybridization, *pepck*, *pold*, *nad1*.

INTRODUCTION

Fasciolosis caused by liver flukes of the genus *Fasciola* (Trematoda: Fasciolidae) is a major problem in the livestock industry. Humans are also susceptible to *Fasciola* spp., and the World Health Organization (WHO) estimates that 180 million people are currently at risk worldwide (Mas-Coma *et al*. 2009). *Fasciola hepatica*, which occurs mainly in Europe, the Americas and Oceania, and *Fasciola gigantica*, which occurs mainly in Africa and Asia, are well-known pathogens that cause fasciolosis (Torgerson and Claxton, 1999). Both the species have normal spermatogenetic ability and reproduce bisexually by cross-or self-fertilization. The presence of abundant mature sperms in the seminal vesicle, the male reproductive organ for temporary storage of self-produced sperm, is a prominent feature of both the species (Terasaki *et al*. 2001). On the other hand, aspermic *Fasciola* flukes that lack sperm in their seminal vesicles have been reported throughout Asia (Terasaki *et al*. 1982; Hayashi *et al*. 2015). Aspermic *Fasciola* flukes exhibit abnormal spermatogenetic ability, and are therefore thought to reproduce by parthenogenesis (Terasaki *et al*. 1998, 2000, 2001). So far, internal transcribed spacer 1 (ITS1) genotype of nuclear ribosomal DNA has been used for molecular characterization for *Fasciola* flukes (Itagaki *et al*. 2005*a*). Three ITS1 genotypes were observed among aspermic flukes, namely *F. hepatica* (Fh) type, *F. gigantica* (Fg) type and a mixed genotype of Fh and Fg types (Fh/Fg). The existence of Fh/Fg type suggests that natural hybridization occurred between *F. hepatica* and *F. gigantica* (Itagaki *et al*. 2005*a*). However, when *Fasciola* flukes display Fh or Fg type in ITS1, species identification could not be precisely performed if spermatogenetic status of the fluke remains unclear. On the other hand, phylogenetic relationships among *Fasciola* flukes have been analysed by using nucleotide sequences of mitochondrial *NADH dehydrogenase subunit 1* (*nad1*). Aspermic *Fasciola* flukes show the two major *nad1* haplotypes; one of them belongs to the *F. hepatica* clade, whereas the other belongs to the *F. gigantica* clade (Itagaki *et al*. 2005*a*), indicating that the maternal ancestors of aspermic *Fasciola* flukes are either *F. hepatica* or *F. gigantica* with these *nad1*

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haplotypes (Itagaki *et al*. 2005*b*). Even though spermatogenic status, ITS1, and *nad1* were employed for species identification, there were confusions in some of our previous studies (Ichikawa *et al*. 2011; Mohanta *et al*. 2014). Indeed, three aspermic *Fasciola* flukes from Myanmar and Bangladesh displayed Fg type in ITS1 and showed *nad1* haplotypes belonging to an *F. gigantica* haplogroup instead of an aspermic *Fasciola* haplotype. They were therefore identified as *F. gigantica* that had temporally lost their spermatogenic ability, probably because of ageing. Furthermore, one spermic *Fasciola* fluke from Bangladesh, displaying Fg type in ITS1, carried an identical *nad1* haplotype to that of the aspermic *Fasciola* flukes.

As ribosomal DNA contains hundreds of copies organized as tandem repeats, the ITS1 region are highly recombinogenic and unstable (Miyazaki and Kobayashi, 2010). Because of this nature, ribosomal DNA cannot provide definitive evidence for interspecific hybridization. Therefore, novel nuclear single-copy markers, *phosphoenolpyruvate carboxykinase* (*pepck*) and *DNA polymerase delta* (*pold*), were recently developed for precise discrimination of *Fasciola* spp. (Shoriki *et al*. 2015). They were considered adequate to detect the interspecific hybridization between *F. hepatica* and *F. gigantica*, because aspermic flukes exclusively displayed Fh/Fg type even though they displayed Fh or Fg type in ITS1 (Shoriki *et al*. 2015).

China should be regarded as a candidate for the cradle of the hybridization because geographical distributions of *F. hepatica* and *F. gigantica* appear to overlap in the country (Torgerson and Claxton, 1999). It was reported that *F. hepatica*- and *F. gigantica*-like flukes were distributed in the northern and southern parts of mainland China, respectively, and that the *Fasciola* flukes with the Fh/Fg type in ITS1 and ITS2 of nuclear ribosomal DNA also existed in China (Huang *et al*. 2004; Lin *et al*. 2007). Our previous study (Peng *et al*. 2009), on the basis of spermatogenetic status, ITS1 genotype, and *nad1* haplotype, revealed that *F. hepatica, F. gigantica* and aspermic *Fasciola* flukes were distributed in China; however, the number of samples and locations analysed was insufficient to reveal the cradle of the interspecific hybridization. Therefore, the present study aimed to analyse *Fasciola* flukes throughout mainland China by using the nuclear *pepck* and *pold* instead of ITS1 genotype to precisely detect the evidence of hybridization between *F. hepatica* and *F. gigantica*.

MATERIALS AND METHODS

Collection of Fasciola flukes and inspection of seminal vesicles

A total of 211 Chinese *Fasciola* flukes were used in this study (Table 1). In addition to 44 flukes that were analysed in the previous study (Peng *et al*. 2009), 167 were newly collected from bile ducts of slaughtered cattle, water buffaloes and yaks. The geographical origins of the flukes were 13 locations in mainland China (Fig. 1). The flukes were fixed in 70% ethanol between two glass slides under mild pressure. The anterior parts, including the seminal vesicle, were removed, stained with haematoxylin–carmine solution, and observed under an optical microscope to examine the presence of sperm within the seminal vesicles.

DNA extraction, multiplex PCR, PCR–RFLP, sequencing

Nuclear *pepck* and *pold* genes were analysed for all of the 211 flukes, whereas mitochondrial *nad1* was analysed for only 167 flukes that were newly included in this study. To exclude sperms from other flukes, only the posterior parts of the flukes without the uteri were used for DNA extraction. Total DNA was extracted from individual flukes using either the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek, Doraville, GA, USA) or the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany).

DNA fragments of *pepck* were analysed using the multiplex PCR method with Fh-pepck-F, Fg-pepck-F and Fcmn-pepck-R primers (Shoriki *et al*. 2015). DNA fragments of *pold* were analysed using the PCR–RFLP method with Fasciola-pold-F1 and Fasciola-pold-R1 primers. Subsequently, PCR amplicons were digested by the restriction enzyme, *Alu*I (Roche) (Shoriki *et al*. 2015). ITS1 genotype was also analysed by using the PCR–RFLP method as described previously (Ichikawa *et al*. 2011).

DNA fragments of *nad1* were amplified using Ita 10 and Ita 2 primers (Itagaki *et al*. 2005*a*). Partial nucleotide sequences of the *nad1* gene (535 bp) were directly determined in both the directions using the BigDye Terminator v3·1 Cycle Sequencing Kit and ABI Prism 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The partial *nad1* sequences were aligned using GENETYX version 10·0·2 (Genetyx, Tokyo, Japan), and different haplotypes were distinguished.

Phylogenetic analyses

Our results were compared with relevant reference data from Japan, Korea, Vietnam, Myanmar, Thailand, Bangladesh, Nepal and India (Itagaki *et al*. 2005*a*, *b*, 2009; Ichikawa *et al*. 2010, 2011; Chaichanasak *et al*. 2012; Ichikawa and Itagaki, 2012; Mohanta *et al*. 2014; Shoriki *et al*. 2014; Hayashi *et al*. 2015), and 575 samples were used for phylogenetic analyses. Median-joining (MJ) networks were constructed using Network 4·6·1·3 software (Bandelt *et al*. 1999) to elucidate the phylogenetic relationships among the *nad1* haplotypes detected in *Fasciola* flukes

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Table 1. Profiles for 211 *Fasciola* samples from China

Location and year	Number of livers and burden of flukes	Number of flukes	Sperm in seminal vesicle	*pepck* and *pold*	ITS1	*nad1*	Species
Hohhot	Cattle 4	3	+	Fh	Fh	Fh-C1	*F. hepatica*
2006	1–3 flukes/liver	3	+	Fh	Fh	Fh-C2	*F. hepatica*
		1	+	Fh	Fh	Fh-C3	*F. hepatica*
Subtotal		*7*					
Urumqi	Cattle 3	1	+	Fh	Fh	Fh-C1	*F. hepatica*
2006	1–2 flukes/liver	2	+	Fh	Fh	Fh-C4	*F. hepatica*
		1	+	Fh	Fh	Fh-C5	*F. hepatica*
		1	+	Fh	Fh	Fh-C6	*F. hepatica*
Subtotal		*5*					
Xining	Yak 3, Cattle 6	19	+	Fh	Fh	Fh-C1	*F. hepatica*
2006, 2009	2–6 flukes/liver	13	+	Fh	Fh	Fh-C4	*F. hepatica*
		1	+	Fh	Fh	Fh-C7	*F. hepatica*
		1	+	Fh	Fh	Fh-C8	*F. hepatica*
		1	+	Fh	Fh	Fh-C10	*F. hepatica*
		1	+	Fh	Fh	Fh-C11	*F. hepatica*
Subtotal		*36*					
Fuzhou	Cattle 5	1	–	Fh/Fg	Fh/Fg	Fh-C4	hybrid
2006	1–2 flukes/liver	5	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
		2	–	Fh/Fg	Fh/Fg	Fg-C3	hybrid
		1	–	Fh/Fg	Fh/Fg	Fg-C4	hybrid
Subtotal		*9*					
Guiyang[a]	Cattle 5	6	+	Fg	Fg	Fg-C1	*F. gigantica*
2006	2–7 flukes/livers	1	+	Fg	Fg	Fg-C5	*F. gigantica*
		1	–	Fg	Fg	Fg-C6	*F. gigantica*
		2	–	Fh/Fg	Fh	Fg-C2	hybrid
		7	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
Subtotal		*17*					
Changchun	Cattle 11	2	+	Fh/Fg	Fh	Fg-C2	hybrid
2009	1–3 flukes/liver	3	–	Fh/Fg	Fh	Fg-C2	hybrid
		1	–	Fh/Fg	Fg	Fg-C2	hybrid
		21	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
Subtotal		*27*					
Yanji	Cattle 2	5	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
2007	2–4 flukes/liver	1	–	Fh/Fg	Fh/Fg	Fg-C17	hybrid
Subtotal		*6*					
Yushu	Yak 3	14	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
N.D.	2–6 flukes/liver						
Subtotal		*14*					
Wuhan	Cattle 25	5	+	Fh/Fg	Fg	Fg-C2	hybrid
2007	1–2 flukes/liver	1	+	Fh/Fg	Fh/Fg	Fg-C2	hybrid
		4	–	Fh/Fg	Fh/Fg	Fh-C4	hybrid
		1	–	Fh/Fg	Fh/Fg	Fh-C9	hybrid
		25	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
Subtotal		*36*					
Changsha	Cattle 7	1	+	Fg	Fg	Fg-C1	*F. gigantica*
2009	1–3 flukes/liver	1	+	Fg	Fg	Fg-C9	*F. gigantica*
		1	+	Fh/Fg	Fh/Fg	Fg-C2	hybrid
		1	–	Fh/Fg	Fg	Fg-C2	hybrid
		12	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
		1	–	Fh/Fg	Fh/Fg	Fg-C18	hybrid
Subtotal		*17*					
Kunming	Cattle 14, Buffalo 1	3	+	Fg	Fg	Fg-C9	*F. gigantica*
2007	1–2 flukes/liver	1	+	Fg	Fg	Fg-C15	*F. gigantica*
		1	+	Fg	Fg	Fg-C16	*F. gigantica*
		2	+	Fh/Fg	Fh/Fg	Fg-C2	hybrid
		15	–	Fh/Fg	Fh/Fg	Fg-C2	hybrid
Subtotal		*22*					
Guangzhou	Buffalo 5	3	+	Fg	Fg	Fg-C1	*F. gigantica*
2007	1–5 flukes/liver	1	+	Fg	Fg	Fg-C7	*F. gigantica*
		2	+	Fg	Fg	Fg-C8	*F. gigantica*
		3	+	Fg	Fg	Fg-C9	*F. gigantica*
		2	+	Fg	Fg	Fg-C10	*F. gigantica*
		1	+	Fg	Fg	Fg-C11	*F. gigantica*
		1	+	Fg	Fg	Fg-C12	*F. gigantica*

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Table 1. (*Cont.*)

Location and year	Number of livers and burden of flukes	Number of flukes	Sperm in seminal vesicle	*pepck* and *pold*	ITS1	*nad1*	Species
		1	+	Fg	Fg	Fg-C13	*F. gigantica*
		1	+	Fg	Fg	Fg-C14	*F. gigantica*
Subtotal		*15*					
Total		211					

N.D. means no record.
[a] One fluke (G2–4) previously reported by Peng *et al*. (2009) was excluded because DNA solution had been used up.

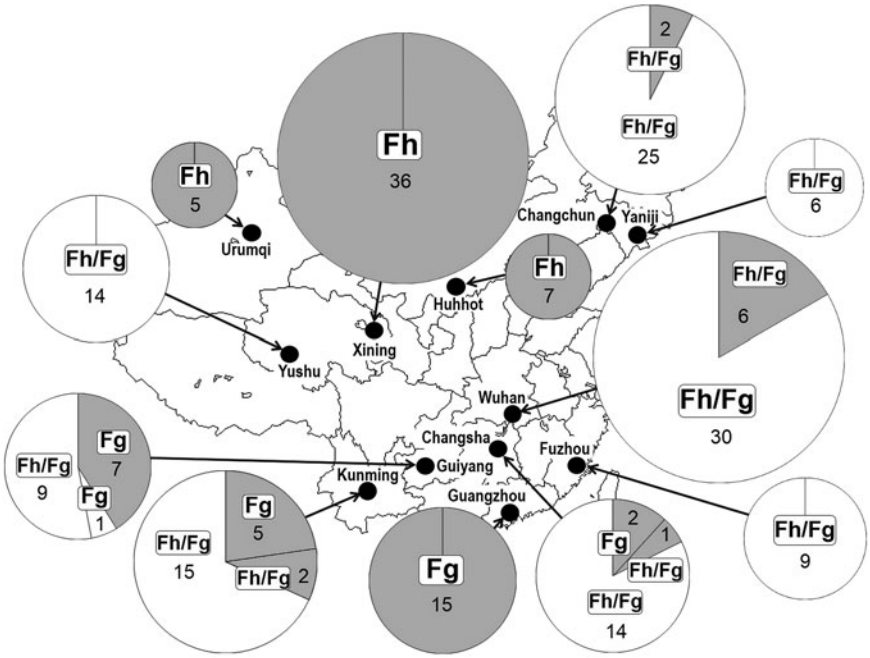

Fig. 1. Combined data of spermatogenesis and the nuclear *phosphoenolpyruvate carboxykinase* (*pepck*) and *DNA polymerase delta* (*pold*) for Chinese *Fasciola* flukes from the 13 geographical locations. Numeric characters in circles indicate the number of samples. Spermic and aspermic flukes are shown as grey and white, respectively.

from China and those from neighbouring reference countries. Haplotype diversity (Hd) and nucleotide diversity (π) were calculated using DnaSP 5·10·01 (Librado and Rozas, 2009).

RESULTS

Sperm in seminal vesicles

A spermic fluke possessed abundant mature sperms in at least half of the seminal vesicle, whereas an aspermic fluke possessed no sperm or abnormal sperms (a few sperms or rosette cells) in the seminal vesicle. Among the 211 Chinese *Fasciola* flukes, 88 and 123 flukes were found to be spermic and aspermic, respectively (Table 1, Fig. 1).

Nuclear pepck and pold

Fragment patterns of Fh and Fg types, and a mixed fragment pattern of both the species (Fh/Fg type) (Shoriki *et al*. 2015) were detected in nuclear *pepck* and *pold*. The results of the two markers were consistent with each other. All the spermic *Fasciola* flukes from Urumqi, Xining and Hohhot were identified as *F. hepatica*, whereas all of those from Guangzhou were *F. gigantica* because they displayed Fh and Fg types, respectively. *Fasciola gigantica* was also found from Changsha, Guiyang and Kunming. Notably, 122 of 123 Chinese aspermic *Fasciola* flukes possessed the Fh/Fg type. The remaining one fluke from Guiyang was regarded as *F. gigantica* because this fluke possessed Fg type. On the other hand, 11 spermic flukes from Changchun, Wuhan, Changsha and Kunming were found to have Fh/Fg type (Table 1, Fig. 1). These findings revealed that *F. hepatica* and *F. gigantica* were detected in the northern and southern parts of China, respectively, and *Fasciola* flukes that possess the Fh/Fg type were distributed between the habitats of the two species. Moreover, the *Fasciola* flukes with Fh/Fg type were predominant (63·0%) in China (Table 1, Fig. 1).

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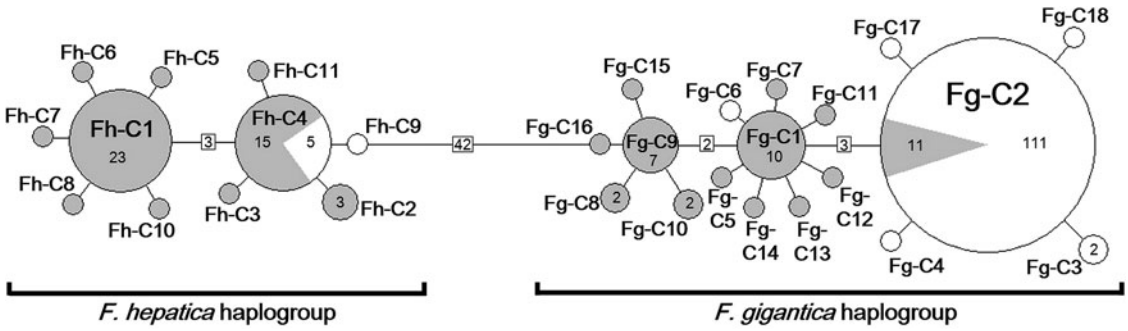

Fig. 2. MJ networks constructed for the *NADH dehydrogenase subunit 1* (*nad1*) haplotypes of *Fasciola* flukes from China. A circle represents a haplotype. The haplotype name is labelled adjacent to the circle. Numeric characters in circles are the number of samples. The haplotypes found in spermic and aspermic *Fasciola* flukes are shown in grey and white, respectively. The number of nucleotide substitutions between haplotypes is labelled on the nodes. In case the number is one, there is no label.

Comparison of pepck and pold with ITS1

Fh, Fg and Fh/Fg types were detected in ITS1. Interestingly, the results of ITS1 were inconsistent with those of *pepck* and *pold* in total of 14 flukes from Guiyang, Changchun, Wuhan and Changsha. These flukes possessed Fh or Fg type in ITS1, however, all of them displayed Fh/Fg type in *pepck* and *pold* (Table 1).

Mitochondrial nad1 haplotypes

Among the 211 Chinese *Fasciola* flukes, 11 haplotypes belonged to *F. hepatica* haplogroup designated as Fh-C1 to Fh-C11, whereas 18 haplotypes were included in *F. gigantica* haplogroup designated as Fg-C1 to Fg-C18 (Table 1, Fig. 2) (accession nos. AB477357–AB477369, AB604926–AB604942, AB605772, AB697063–AB697065). In the *F. hepatica* haplogroup, 10 *nad1* haplotypes were found in Chinese *F. hepatica* where eight haplotypes were derived from the numerically predominant haplotype Fh-C1 or Fh-C4 with a single nucleotide mutation. Fh-C4 and Fh-C9 were found in Chinese aspermic *Fasciola* flukes. It is noteworthy that Fh-C4 was detected in both *F. hepatica* and aspermic flukes (Table 1, Fig. 2). Among the 13 *nad1* haplotypes found in Chinese *F. gigantica*, 11 were derived from the major haplotype Fg-C1 or Fg-C9 with a single nucleotide mutation (Fig. 2). Fg-C6 was included here and was constituted by one aspermic *Fasciola* fluke from Guiyang with Fg type in *pepck* and *pold* (regarded as *F. gigantica*) (Table 1, Fig. 2). A centrally positioned, predominant haplotype in Chinese aspermic *Fasciola* flukes belonging to the *F. gigantica* haplogroup (Fig. 2) was Fg-C2, from which the derivative haplotypes Fg-C3, Fg-C4, Fg-C17 and Fg-C18 coalesced with a single nucleotide mutation (Fig. 2). All the 11 spermic flukes with Fh/Fg type in *pepck* and *pold* were included in Fg-C2 (Table 1, Fig. 2). Moreover, Fh-C4 displayed identical sequence to the *nad1* haplotypes detected in aspermic *Fasciola* flukes from Japan and Korea, whereas Fg-C2 displayed identical sequence to the *nad1* haplotypes detected from aspermic flukes from Korea, Japan, Vietnam, Myanmar, Thailand, Bangladesh, Nepal and India (Fig. 3). Again, Fg-C3, one of the derivative haplotypes of Fg-C2, displayed the identical sequence to that of aspermic *Fasciola* flukes detected in Korea (Fig. 3). As a result of these phylogenetically close relationships among the aspermic *Fasciola* haplotypes, the values of Hd and π in the Chinese aspermic populations were much lower than those in *F. hepatica* and *F. gigantica* populations from China (Table 2). Chinese aspermic flukes possessed the largest number of haplotypes in the aspermic populations, and Hd and π values were higher in Chinese aspermic populations than in the neighbouring aspermic populations, except the Korean aspermic populations of *F. gigantica* haplogroup, which showed the highest values because of the frequency of Fg-C3 (Table 2, Fig. 3).

DISCUSSION

In this study, both the nuclear markers, *pepck* and *pold*, aided in precise discrimination of *F. hepatica*, *F. gigantica* and their hybrids. On the other hand, the ITS1 genotype of the 14 flukes was Fh or Fg type even though they had Fh/Fg type in *pepck* and *pold* (Table 1). This strongly indicates *pepck* and *pold* should be used to detect interspecific hybridization instead of ITS1. One aspermic fluke from Guiyang was characterized as *F. gigantica* not only because it displayed Fg type in the nuclear markers, but also because it possessed Fg-C6 in the *nad1* gene (Table 1), one of the derivatives of the major *F. gigantica* haplotype, Fg-C1 (Fig. 2). This *F. gigantica* fluke appeared to lose the mature sperm due to ageing or some other unknown reason. On the other hand, 122 aspermic and 11 spermic *Fasciola* flukes were considered to be derived from interspecific hybridization between *F. hepatica* and

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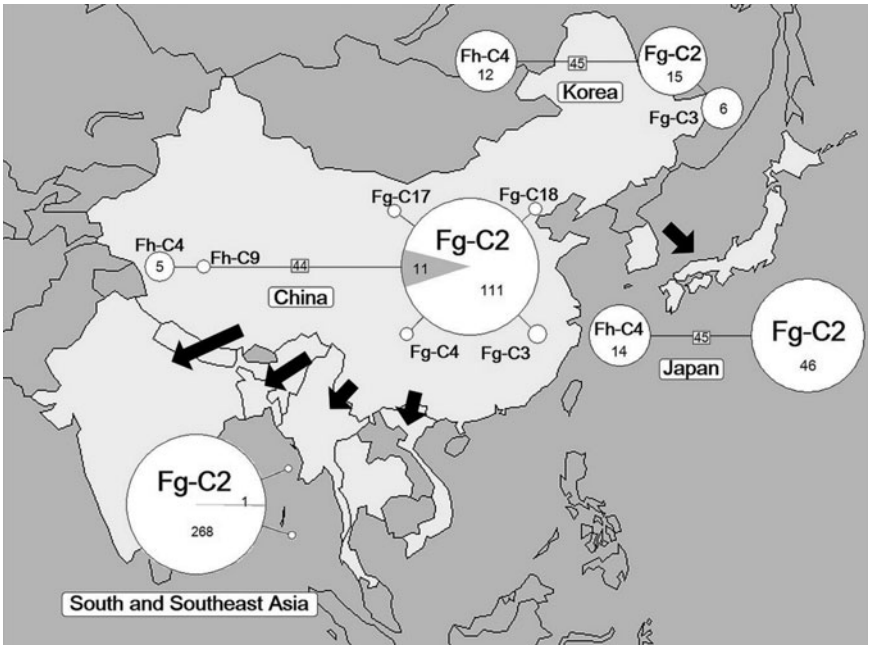

Fig. 3. MJ networks constructed for the *NADH dehydrogenase subunit 1* (*nad1*) haplotypes of the aspermic *Fasciola* lineages across Asia. The haplotype name is labelled adjacent to the circle. When the haplotype identical to the Chinese one is found from the neighbouring countries, it is labelled as well. Numeric characters in circles indicate the number of samples. The haplotypes found in spermic and aspermic *Fasciola* flukes are shown in grey and white, respectively. The number of nucleotide substitutions between haplotypes is labelled on the nodes. In case the number is one, there is no label. South and Southeast Asian populations include the aspermic lineage found from Vietnam, Myanmar, Thailand, Bangladesh, Nepal and India. For South and Southeast Asian population, one spermic fluke with Fg-C2 was excluded from population genetics calculations because the species of the fluke could not be precisely identified in the previous studies (Mohanta *et al*. 2014). The arrows indicate the dispersal direction of the aspermic *Fasciola* lineage.

Table 2. Haplotype diversity (Hd) and nucleotide diversity (π) of *Fasciola* flukes used in this study

Haplogroup	Category	Populations	*n*	Hn	Hd ± S.D.	π ± S.D.
Fasciola hepatica haplogroup	*F. hepatica* aspermic flukes	China	48	10	0·680 ± 0·051	0·00355 ± 0·00027
		China	6	2	0·333 ± 0·215	0·00062 ± 0·00040
		Korea	12	1	0	0
		Japan	14	1	0	0
Fasciola gigantica haplogroup	*F. gigantica* aspermic flukes	China	30	13	0·844 ± 0·050	0·00350 ± 0·00034
		China	127	5	0·077 ± 0·033	0·00015 ± 0·00006
		South and Southeast Asia[a]	270	3	0·015 ± 0·010	0·00003 ± 0·00002
		Korea	21	2	0·429 ± 0·089	0·00080 ± 0·00017
		Japan	46	1	0	0
Total			574			

'n' and 'Hn' are the number of samples and of haplotypes, respectively.
[a] South and Southeast Asia includes Vietnam, Myanmar, Thailand, Bangladesh, Nepal and India. One fluke was excluded because it could not precisely be identified in the previous studies (see Fig. 3).

F. gigantica because they possessed Fh/Fg type in both the nuclear markers. They possessed Fg-C2, the predominant haplotype of aspermic flukes (Table 1, Fig. 2), and were therefore included in the aspermic population without exception. These findings strongly support the hybrid origin of aspermic *Fasciola* lineages. Hereafter, we suggest that they should be termed as 'hybrid' *Fasciola* flukes rather than 'aspermic' flukes, since the 11 flukes retain sperm in the seminal vesicle. Spermic hybrids seem to be rare, nevertheless, they are found from the wide area. One spermic fluke found from Bangladesh were reported to have Fg type in ITS1 and Fg-C2 in *nad1* (Mohanta *et al*. 2014). This fluke was regarded as a hybrid because it possessed Fh/Fg type in *pepck* and *pold* (unpublished). More recently, spermic hybrids were found also in Japan (unpublished). Further study is needed to reveal the reproductive mechanism of hybrids *Fasciola* flukes.

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Some vertebrates displaying parthenogenetic reproductive patterns have been considered descendants originated through hybridization of the two related species, exhibiting sympatric geographical distribution, and carry nuclear genomes of both the related species (Avise *et al*. 1992). Similarly, the coexistence of *F. hepatica* and *F. gigantica* in mainland China might have allowed them to undergo interspecific hybridization (Fig. 1). The coexistence was observed exclusively in China and not in neighbouring South and Southeast Asian countries (Itagaki *et al*. 2009; Ichikawa *et al*. 2011; Chaichanasak *et al*. 2012; Mohanta *et al*. 2014; Shoriki *et al*. 2014; Hayashi *et al*. 2015). The maternal progenitors of the hybrids were thought to be *F. hepatica* with Fh-C4 or *F. gigantica* with Fg-C2, which are the common *nad1* haplotypes of aspermic flukes detected from Asian countries (Fig. 3). Although *F. hepatica* with Fh-C4 existed in China, *F. gigantica* with Fg-C2 appeared to become extinct in the country (Table 1). The hybrids seemed to have superior fecundity, which might cause extinction of one of their progenitors. As a result, the coexistence of *F. hepatica, F. gigantica* and the hybrids was not found in any of the location in this study. This speculation was manifested by the predominant distribution of the hybrids (63·0%) between the habitats of *F. hepatica* and *F. gigantica* in China (Fig. 1).

Chinese hybrid *Fasciola* flukes had the largest number of haplotypes and possessed higher Hd and π values compared with most of the neighbouring populations (Table 2). Hybrid *Fasciola* flukes with the derivative haplotypes of Fh-C4 and Fg-C2 probably emerged through single nucleotide mutations (Fig. 2). This fact also supports the Chinese origin of the hybrid flukes, because genetic diversity in modern populations generally decreases with the distance from the geographical origin (Troy *et al*. 2001; Beja-Pereira *et al*. 2006; Chen *et al*. 2010). Actually, the Korean aspermic population of *F. gigantica* haplogroup showed the highest Hd and π values (Table 2). However, this result was probably inconsistent with the actual history, because Fg-C3 of the Korean aspermic population was one of the derivative haplotypes of Fg-C2 and was also observed in the Chinese population (Fig. 3).

Extremely low values of both Hd and π in the hybrid *Fasciola* populations (Table 2) indicate the quite recent emergence of the two hybrid *Fasciola* lineages; these findings also suggest that these lineages rapidly spread into neighbouring countries (Fig. 3). Interestingly, the worldwide distribution of *F. hepatica* and *F. gigantica* almost corresponds to that of domestic definitive hosts, in particular, taurine cattle (*Bos taurus*) and zebu cattle (*Bos indicus*), respectively (Phillips, 1961; Torgerson and Claxton, 1999). In China, the taurine types are found mainly in the northern part and zebu types in the southern part; hybrid breeds between the two types (for example, yellow cattle) are found in the central part (Cai *et al*. 2007). Therefore, distribution of cattle breeds in China completely corresponds with that of *Fasciola* spp. (Fig. 1). Taurine cattle are believed to have been introduced into central and northern China between 5000 and 4000 years before present (YBP) (Flad *et al*. 2007), whereas zebu cattle spread to southern China approximately 2500 YBP (Higham, 1996). Water buffalo (*Bubalus bubalis*), another important domestic definitive host for *F. gigantica*, was distributed in southern China at least 3400 YBP (Chang, 1976). *Fasciola hepatica* and *F. gigantica* were assumed to be introduced into China, along with migration of these domestic ruminants, facilitating the hybridization between the two *Fasciola* species; thereafter, the emergence of the hybrid *Fasciola* flukes was ultimately triggered by the dispersal of the domestic hosts throughout China. The time of emergence could not be estimated accurately because the precise mutation rate has not yet been established for this genus. Nonetheless, the hybrid lineages might have been introduced into Korea, with the migration of domestic cattle from China, after their emergence. Around the second century (approximately 1800 YBP), the lineages were further introduced into Japan along with domestic cattle from Korea (Mukai *et al*. 1989; Itagaki *et al*. 2005*a*, *b*; Ichikawa and Itagaki, 2012) (Fig. 3).

The high prevalence rate of hybrid *Fasciola* flukes in China suggests that they have adapted to the environment, and that asexuality could be a successful evolutionary strategy. A possible explanation of the superiority of the hybrids is 'heterosis', the improved or increased function of any biological quality in the hybrids. The biological feature of the hybrids should be carefully investigated to elucidate a possibility of erecting a new species for hybrid *Fasciola* flukes in the future. Furthermore, adequate and effective control strategies against hybrid *Fasciola* flukes should be developed to limit their further distribution.

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