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NOTE

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Hybrid origin of Asian aspermic *Fasciola* flukes is confirmed by analyzing two single-copy genes, *pepck* and *pold*

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ABSTRACT. Nuclear gene markers, phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*), have been developed for precise discrimination of *Fasciola* flukes instead of internal transcribed spacer 1. In this study, these two genes of 730 *Fasciola* flukes from eight Asian countries were analyzed. The results were compared with their mitochondrial NADH dehydrogenase subunit 1 (*nad1*) lineages for obtaining a definitive evidence of the hybrid origin of aspermic *Fasciola* flukes. All the flukes categorized into the aspermic *nad1* lineages possessed both the fragment patterns of *F. hepatica* and *F. gigantica* (mixed types) in *pepck* and/or *pold*. These findings provide clear evidence for the hybrid origin of aspermic *Fasciola* lineages and suggest that "aspermic *Fasciola* flukes" should hereafter be called "hybrid *Fasciola* flukes".

KEY WORDS: Asia, *Fasciola*, hybrid, *pepck*, *pold*

Fasciolosis is a parasitic disease responsible for liver disorders in ruminant hosts and leads to a reduction in livestock productivity. The two well-known causative agents of fasciolosis are *Fasciola hepatica* and *Fasciola gigantica*. The former species is distributed mainly in Europe, America and Oceania, while the latter is distributed mainly in Asia and Africa [19]. The two species reproduce bisexually thorough fertilization. Mature spermatozoa of the species are ejaculated from their seminal vesicles, which serve as temporary storage for self-produced sperm [18]. In addition to the two species, aspermic *Fasciola* flukes, which contain only few or no spermatozoa in their seminal vesicles, have been reported in Asian countries [3, 18]. Not only diploid but also triploid flukes were reported from aspermic *Fasciola* flukes [11].

The nucleotide sequence of the ribosomal internal transcribed spacer 1 (ITS1) has been employed so far for molecular characterization of *Fasciola* flukes [1, 3, 5–7, 9–11, 14, 15]. Three ITS1 types (ITS1-Fh, Fg and mixed type) were distinguished using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method [4]. The Fh and Fg types have identical fragment patterns to *F. hepatica* and *F. gigantica*, respectively. Therefore, the spermic species have been identified based on their ITS1 types [1, 3, 4, 6, 7, 10, 14, 15]. However, species identification cannot be performed using ITS1 when the spermatogenic status of a fluke is unclear because both the ITS1-Fh and ITS1-Fg types are found also in aspermic flukes [4]. The ITS1-mixed type has both the fragment patterns of the two species that were detected in aspermic *Fasciola* flukes [5–7, 9–11, 14, 15], suggesting that the aspermic flukes are hybrids between *F. hepatica* and *F. gigantica* [4]. However, the ITS1 has hundreds of copies organized as tandem repeats with highly recombinogenic and unstable characteristics [13], and it is therefore, unsuitable to provide reliable evidence of natural hybridization.

Phylogenetic studies based on the nucleotide sequence of the mitochondrial NADH dehydrogenase subunit 1 (*nad1*) have revealed that the *nad1* haplotypes of *F. hepatica* and *F. gigantica* are well diversified [8]. In contrast, the *nad1* haplotypes of aspermic *Fasciola* flukes displayed uniform characteristics, which originated from the two different maternal lineages, *F. hepatica* and *F. gigantica* [11]. The two maternal lineages, which are described as "aspermic Fh" and "aspermic Fg" in this study, contain respective major haplotypes together with some derivative haplotypes. This indicates the maternal ancestors of aspermic flukes

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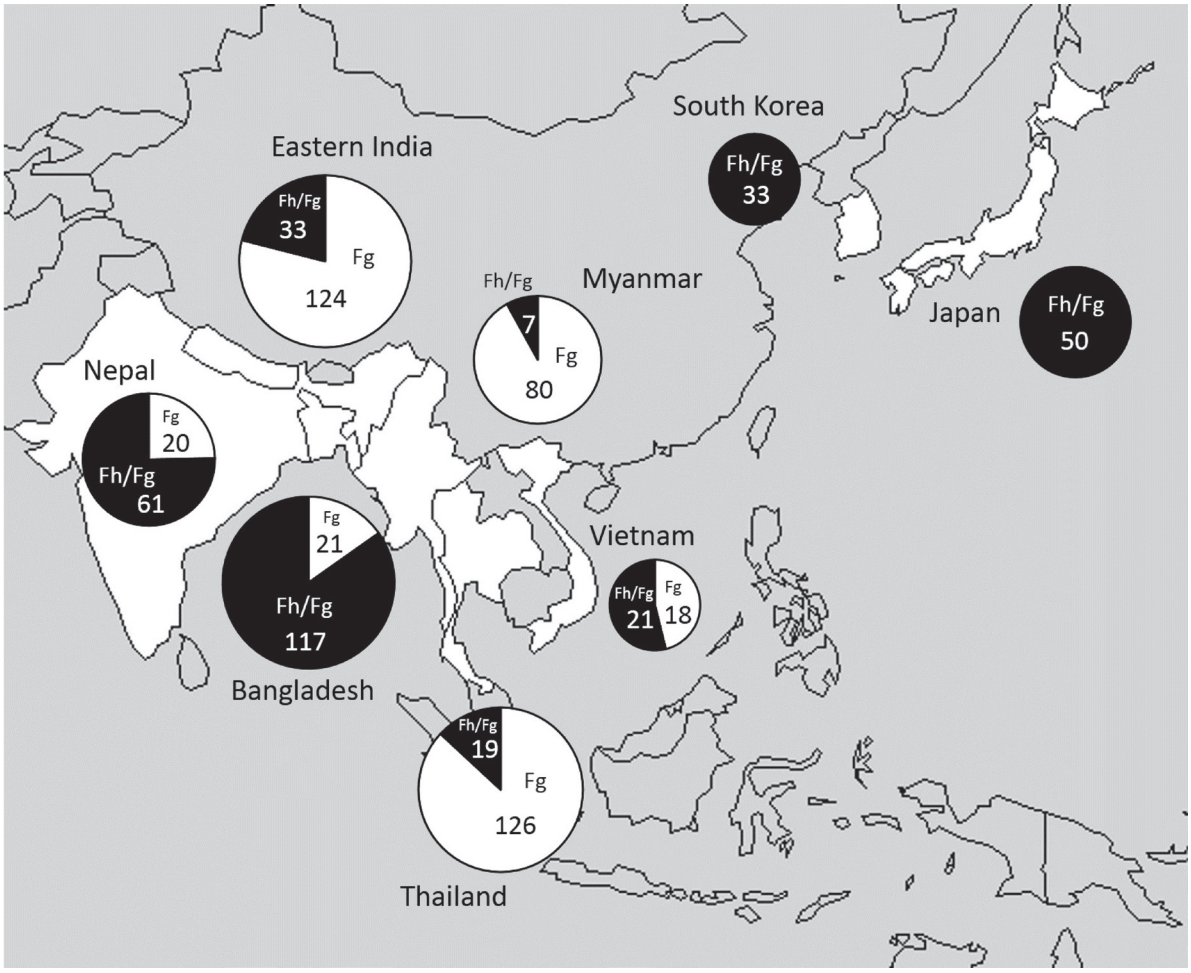

Fig. 1. Species discrimination of *Fasciola* flukes from eight Asian countries based on the analysis of *pepck* and *pold* genes. White and black circles denote *F. gigantica* and hybrid *Fasciola* flukes, respectively. The circle sizes are proportional to the number of flukes, and the actual numbers are labeled in the circles.

were *F. hepatica* or *F. gigantica* with the major haplotypes [8]. So far, the spermatogenic status, ITS1 type and *nad1* haplotypes have been reported for 730 *Fasciola* flukes from eight Asian countries [1, 3, 5–7, 9, 14, 15]. However, contradictions concerning species discrimination were observed for 11 flukes from Eastern India, Myanmar and Bangladesh [3, 7, 14], because inconsistent results of the *nad1* lineages and spermatogenic status were of the flukes could not be resolved by analyzing ITS1.

Recently, the single-copy nuclear gene markers, *pepck* and *pold*, which encode phosphoenolpyruvate carboxykinase and DNA polymerase delta, respectively were developed [16]. The fragment patterns of *F. hepatica*, *F. gigantica* and the mixed fragment of both the species were distinguished by both *pepck* and *pold* based on multiplex PCR and PCR-RFLP methods, respectively [16]. In this study, the fragment patterns of *F. hepatica* in *pepck* and *pold* were described as "pepck-Fh type" and "pold-Fh type", respectively. Similarly, the fragment patterns of *F. gigantica* and those of both the species were described as "pepck-Fg type", "pold-Fg type", "pepck-mixed type" and "pold-mixed type". Single-copy genetic markers are suitable for detecting evidence of hybridization. Interestingly, all the aspermic *Fasciola* flukes analyzed previously by *pepck* and *pold* were determined as hybrids even though they possessed the ITS1-Fh or ITS1-Fg type [16]. However, the number of samples was insufficient to draw a conclusion regarding the hybrid origin in the aspermic flukes. In this study, 730 *Fasciola* flukes from eight Asian countries [1, 3, 5–7, 9–11, 14, 15] were reanalyzed using *pepck* and *pold* markers to obtain a definitive evidence for the origin of aspermic *Fasciola* flukes as well as to confirm the reliability of the markers.

A total of 730 *Fasciola* flukes from eight Asian countries (Japan, Korea, Vietnam, Thailand, Myanmar, India, Bangladesh and Nepal) were examined in this study (Fig. 1). The spermatogenic status, ITS1 types, and mitochondrial *nad1* haplotypes of the flukes were reported in previous studies [1, 3, 5–7, 9–11, 14, 15] (Table 1 and S1). Ploidy of the 42 flukes from Japan and Vietnam were also reported in the previous studies (Table S1) [10, 11]. Unfortunately, ploidy of the remaining flukes could not be analyzed in the previous studies because the flukes were not fixed in ethanol-acetic acid, essential for the ploidy analysis.

The *pepck* region was amplified from genomic DNA by using a multiplex PCR with Fh-pepck-F (5′-GATTGCACCGTTAGGTTAGC-3′), Fg-pepck-F (5′-AAAGTTTCTATCCCGAACGAAG-3′) and Fcmn-pepck-R

Table 1. Profiles of *Fasciola* flukes from eight Asian countries

Country	Species[a)]	Number of flukes	Sperm in seminal vesicles[a)]	Nuclear DNA types			Mitochondrial *nad1*[a)] lineages
				pepck	*pold*	ITS1[a)]	
Japan	Aspermic *Fasciola* flukes	5	-	mixed	mixed	Fh	aspermic Fh
		1	-	mixed	mixed	Fh	aspermic Fg
		4	-	mixed	N.D.	Fh	aspermic Fh
		1	-	N.D.	mixed	Fh	aspermic Fh
		33	-	mixed	mixed	Fg	aspermic Fg
		2	-	mixed	N.D.	Fg	aspermic Fg
		2	-	mixed	mixed	mixed	aspermic Fh
		2	-	mixed	mixed	mixed	aspermic Fg
Subtotal		50[b)]					
Korea	Aspermic *Fasciola* flukes	1	-	mixed	mixed	Fh	aspermic Fh
		4	-	mixed	mixed	Fh	aspermic Fg
		2	-	mixed	mixed	Fg	aspermic Fg
		10	-	mixed	mixed	mixed	aspermic Fh
		1	-	N.D.	mixed	mixed	aspermic Fh
		15	-	mixed	mixed	mixed	aspermic Fg
Subtotal		33					
Vietnam	Aspermic *Fasciola* flukes	2	-	mixed	mixed	Fh	aspermic Fg
		1	-	mixed	mixed	Fg	aspermic Fg
		1	-	mixed	N.D.	Fg	aspermic Fg
		12	-	mixed	mixed	mixed	aspermic Fg
		5	-	mixed	N.D.	mixed	aspermic Fg
	F. gigantica	15	+	Fg	Fg	Fg	*F. gigantica*
		3	+	Fg	N.D.	Fg	*F. gigantica*
Subtotal		39[b)]					
Thailand	Aspermic *Fasciola* flukes	17	-	mixed	mixed	Fg	aspermic Fg
		2	-	mixed	N.D.	Fg	aspermic Fg
	F. gigantica	122	+	Fg	Fg	Fg	*F. gigantica*
		4	+	Fg	N.D.	Fg	*F. gigantica*
Subtotal		145[b)]					
Myanmar	Aspermic *Fasciola* flukes	7	-	mixed	mixed	mixed	aspermic Fg
	F. gigantica	79	+	Fg	Fg	Fg	*F. gigantica*
		1[c)]	-	Fg	Fg	Fg	*F. gigantica*
Subtotal		87[b)]					
Eastern India	Aspermic *Fasciola* flukes	29	-	mixed	mixed	Fg	aspermic Fg
		4	-	mixed	N.D.	Fg	aspermic Fg
	F. gigantica	115	+	Fg	Fg	Fg	*F. gigantica*
		6[c)]	-	Fg	Fg	Fg	*F. gigantica*
		3	+	Fg	N.D.	Fg	*F. gigantica*
Subtotal		157					
Bangladesh	Aspermic *Fasciola* flukes	86	-	mixed	mixed	Fg	aspermic Fg
		29	-	mixed	mixed	mixed	aspermic Fg
		1[c)]	+	mixed	mixed	Fg	aspermic Fg
		1[c)]	+	mixed	mixed	mixed	aspermic Fg
	F. gigantica	19	+	Fg	Fg	Fg	*F. gigantica*
		2[c)]	-	Fg	Fg	Fg	*F. gigantica*
Subtotal		138[b)]					
Nepal	Aspermic *Fasciola* flukes	61	-	mixed	mixed	mixed	aspermic Fg
	F. gigantica	20	+	Fg	Fg	Fg	*F. gigantica*
Subtotal		81					
Total		730					

"Fh" and "Fg" represent *F. hepatica* and *F. gigantica* band patterns, respectively. "mixed" represents a both band pattern for Fh and Fg types. "aspermic Fh" and "aspermic Fg" represent nad1 haplotypes of aspermic *Fasciola* flukes whose maternal ancestry is *F. hepatica* and *F. gigantica*, respectively. N.D., not detected. a) Spermatic status, ITS1 types and *nad1* haplotypes were analyzed in previous studies; Japan [7, 9], Korea [5], Vietnam [11], Thailand [1], Myanmar [6], Eastern India [3], Bangladesh [14] and Nepal [15]. b) The numbers for some of the subtotals do not completely match those from previous reports because some DNA samples were exhausted. c) Flukes possessing inconsistent characters for spermatogenesis status, ITS1 genotypes and *nad1* haplotypes.

(5′-CGAAAATTATGGCATCAATGGG-3′) primers, and the fragment patterns were distinguished on 1% agarose gel [16]. The *pold* region was analyzed by PCR-RFLP [16]. Briefly, the *pold* products amplified by Fasciola-pold-F1 (5′-GCTAACTTATCTGCTTACACGTGGACA-3′) and Fasciola-pold-R1 (5′-ATCGCATTCGATCAAAGCCCTCCCATG-3′) primers were digested with the *Alu*I restriction enzyme (Roche, Mannheim, Germany), and then the fragment patterns were distinguished on 1.8% agarose gel.

In this study, the *pepck* genotyping yielded two DNA types, namely pepck-Fg type (*F. gigantica*) and pepck-mixed type (hybrid between *F. hepatica* and *F. gigantica*). Similarly, *pold* genotyping yielded pold-Fg type, and pold-mixed type. The newly obtained results of the *pepck* and *pold* genotyping were combined with the previous results of the spermatogenic status, ITS1 types and mitochondrial *nad1* haplotypes (Table 1) [1, 3, 5–7, 9–11, 14, 15]. The *nad1* haplotypes are divided into three haplogroups, "*F. gigantica*", "aspermic Fh" and "aspermic Fg" [8]. The first haplogroup consists of haplotypes detected from populations of *F. gigantica*, whereas the second and third haplogroups include haplotypes of aspermic *Fasciola* flukes whose maternal ancestries are *F. hepatica* and *F. gigantica*, respectively. Detailed information about each fluke is summarized in Supplementary Table (Table S1).

Although the ITS1 region for all the flukes was adequately amplified in the previous studies [1, 3, 5–7, 9–11, 14, 15], the *pepck* and *pold* regions for two and 28 of the flukes, respectively were not amplifiable in the present study (Table 1). This observation is probably related to differences in the copy numbers of the target genes since ITS1 is a multi-copy gene, it was more easily amplified than *pepck* and *pold*, single-copy genes in many eukaryotic species [2, 12]. Additionally, the small sizes of the *pepck* amplicons (241 bp and 509 bp or 510 bp) made them more readily generated than the *pold* amplicons (844 bp) [16].

The discrimination of *Fasciola* species based on spermatogenic status, ITS1 type and *nad1* haplotype has produced contradictory results for 11 *Fasciola* flukes analyzed in previous studies. Indeed, nine aspermic flukes from Myanmar, eastern India and Bangladesh appeared to have no spermatogenic ability and were actually thought to be *F. gigantica* because their *nad1* haplotypes were not included in "aspermic Fg" but were included in "*F. gigantica*" [3, 7, 14] (Table1). In the present study, these flukes showed the pepck-Fg type and the pold-Fg, and were confirmed as *F. gigantica* (Table 1). Additionally, one fluke from Bangladesh, appeared to retain its spermatogenic ability and possessed the ITS-Fg type, was regarded as an aspermic *Fasciola* fluke because it was included in the "aspermic Fg" haplogroup in the *nad1* gene [14]. Here, this fluke displayed the pepck-mixed type and pold-mixed type (Table 1). Similarly, another fluke from Bangladesh, appeared to retain its spermatogenic ability and possessed the ITS1-mixed type and the *nad1* haplotype of "aspermic Fg" [14], also showed the mixed types in both *pepck* and *pold*. In summary, all the *Fasciola* flukes belonging to the "aspermic Fh" or "aspermic Fg" haplogroups in *nad1* showed the mixed types in both *pepck* and *pold* regardless of their ITS1 types (Table 1). These results strongly suggest that Asian aspermic *Fasciola* flukes originated through the hybridization between *F. hepatica* and *F. gigantica*, and should now be called "hybrid *Fasciola* flukes" instead of "aspermic *Fasciola* flukes" as having been proposed by Ichikawa-Seki *et al.* [8]. "Aspermic" seems no longer an adequate term because the two hybrid flukes from Bangladesh [14] (Table 1) retained sperm in their seminal vesicles.

Although the ploidy of almost all the flukes was unknown in this study, the 25 triploids as well as the 1 diploid aspermic flukes from Japan and Vietnam showed the mixed types in both *pepck* and *pold* (Table S1). Since triploid flukes can never occur in a single hybridization, the most possible origin of a triploid may be through the fertilization of a parthenogenetically produced egg (diploid) by the sperm of a male from the bisexual ancestor [17]. According to this theory, triploid would also be called as "hybrid" in a broad sense. Actually, triploid flukes were successfully produced by an experimental hybridization between hybrid diploid and *F. hepatica* (unpublished results).

In this study, all the *Fasciola* flukes belonging to "*F. gigantica*" haplogroup in *nad1* displayed pepck-Fg type and pold-Fg type, and were therefore confirmed as *F. gigantica*. These findings revealed that the results of the *pepck* and *pold* analyses were completely consistent with those of the *nad1* lineages. Therefore, *pepck* and *pold* were proved as potential markers for precise discrimination of *F. hepatica*, *F. gigantica* and hybrid *Fasciola* flukes. Accurate discrimination of *Fasciola* flukes is very important because hybrid *Fasciola* flukes are thought to have superior fecundity to *F. gigantica* [14]. Hybrid *Fasciola* flukes were predominant in Nepal (75.3%), Bangladesh (84.8%), South Korea (100%), Japan (100%) and Vietnam (53.8%) (Fig. 1), and are therefore needed a further attention to monitor their dispersal route in Asian countries.

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