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Genetic variation in the triploids of Japanese *Fasciola* species, and relationships with other species in the genus

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Abstract

Twelve enzymes (encoded by 14 loci) in liver flukes of *Fasciola* species originating from Japan (parthenogenetic triploids), Korea (parthenogenetic diploids), the United States of America (USA) and Australia (all sexual diploids) were analysed using starch gel electrophoresis. Variation in electrophoretic patterns between samples was detected at five enzyme loci (*Ak*, *Got*, *Gpi*, *6-Pgd* and *Pgm*-2). Japanese worms (31, of which six were established as uniparental laboratory strains), which reproduce by parthenogenesis, exhibited three different isozyme patterns. This indicates that triploidy has arisen more than once in Japanese flukes. Japanese *Fasciola* sp. can be separated into three types on morphological grounds. For the six laboratory strains of Japanese worms, the parental morphological type was known. Each of the three isozyme patterns observed was restricted to one morphological type. Most alleles detected in the Japanese triploids were also found in diploid worms from the other countries: the only alleles not represented elsewhere were four at the *Got* locus and two at the *Pgm* locus. Flukes from a laboratory strain derived from a single Korean diploid worm resembled the Japanese worms in genotype more closely than did American (seven uniparental laboratory strains) or Australian (30 worms) specimens. Worms from the last two countries were closely related.

Introduction

The taxonomic identity of Japanese species of *Fasciola* is still controversial despite numerous studies, mostly using morphological characters (Itagaki & Akane, 1959; Ueno & Watanabe, 1960; Oshima *et al.*, 1969).

Chromosome studies, which have often been conducted without reference to fluke morphology, have showed that diploids, triploids and mixoploids exist in Japan and can occur in the same geographical location and host (Sakaguchi & Nakagawa, 1975; Moriyama *et al.*, 1979; Terasaki *et al.*, 1982). All these Japanese forms have little or no sperm and presumably undergo parthenogenetic reproduction. In contrast, individuals of *F. hepatica* from Australia are diploid, possessing numerous sperm (Sakaguchi & Ueno, 1977; Terasaki *et al.*, 1982).

We have previously demonstrated isozyme variation within Japanese populations of *Fasciola* sp. (Agatsuma & Suzuki, 1980; Agatsuma, 1981). We now extend this work to survey additional enzymes and assess the relationship between the Japanese forms and other species of *Fasciola*. We report the electrophoretic analysis of 12 enzymes (encoded by 14 loci) from liver flukes of the genus *Fasciola* originating from four different countries.

Materials and methods

Japanese samples (34 worms) were obtained from cattle at slaughter-houses in Fukuoka and Kochi. A single Korean

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Table 1. Electrode and gel buffer used for starch gel electrophoresis of liver flukes of the genus *Fasciola*.

Systems	Electrode buffer		Gel buffer
AC	0.067 M aminopropyl-morpholine 0.04 M citrate (pH 6.0)		Dilute 50 ml of electrode buffer to 1 litre (pH 6.0).
Poulic	0.3 M boric acid 0.06 M NaOH (pH 8.3)		0.076 M Tris 0.005 M citrate (pH 9.2)
S18	0.1 M Tris 0.01 M Na_2EDTA 0.13 M NaOH	0.1 M maleic acid 0.01 M $MgCl_2$ (pH 7.4)	Dilute 100 ml of electrode buffer to 1 litre (pH 7.4).
TC (pH 6.0)	0.25 M Tris 0.17 M citrate (pH 6.0)		Dilute 100 ml of electrode buffer to 1 litre (pH 6.0)
TC (pH 7.5)	0.25 M Tris 0.08 M citrate (pH 7.5)		Dilute 100 ml of electrode buffer to 1 litre (pH 7.5)

fluke, whose species name has not been identified, came from cattle at a slaughter-house in Seoul, Korea. American (7) and Australian (30) *F. hepatica* specimens came from cattle imported from those countries to the slaughter-house in Fukuoka. Fourteen uniparental laboratory strains were set up using the single fluke from Korea, all seven American flukes, and six flukes from Fukuoka, Japan. *Lymnaea viridis* was used as the snail host, and adults were raised in rats. Japanese flukes selected to start laboratory strains were chosen from the three morphologically distinct types recognized by one of us (Terasaki). Three flukes were of type 1, resembling *F. hepatica* in having a short body and wide shoulders. One fluke was of type 2, resembling *F. gigantica* in having a long body and no shoulders. The remaining two flukes were of type 3, an intermediate form with a pointed tail and probably representing the *F. indica* type.

Representatives of each laboratory strain were checked for karyotype, using the air-dry method of Terasaki (1977).

Electrophoresis was carried out using Connaught starch according to the methods of Agatsuma & Habe (1986). Electrode buffer systems and electrophoretic conditions are shown in tables 1 and 2.

The twelve enzymes (encoded by 14 loci; both GPT and PGM possess two loci) analysed in this study are as follows; adenylate kinase (AK, EC: 2.7.4.3), diaphorase (DIA, ECL 1.6.2.2), esterase (EST, EC: 3.1.1.1), glutamic dehydrogenase (GDH, EC: 1.1.1.47), glutamic-oxaloacetic transaminase (GOT, EC: 2.6.1.1), glucosephosphate isomerase (GPI, EC: 5.3.1.9), glutamic-pyruvate transaminase (GPT, ECL 2.6.1.2), glucose-6-phosphate dehydrogenase (G6PD, EC: 1.1.1.49), malate dehydrogenase (MDH, EC: 1.1.1.37), malic enzyme (ME, EC: 1.1.1.40), 6-phosphogluconate dehydrogenase (6PGD, EC: 1.1.1.44), and phosphoglucomutase (PGM, EC: 2.7.5.1).

Results

All six uniparental strains of Japanese flukes were triploid with no normal sperm formation. Offspring of the single Korean worm were diploid without normal sperm. Strains originating from the seven American worms were all diploid with sperm. Karyotypes of the 28 Japanese flukes from Kochi were not checked. However, previous studies (Terasaki *et al.*, 1982) have indicated that all flukes from Kochi are triploid and lack sperm. Similarly, the 30 Australian flukes were not checked, but were assumed to be diploids (Terasaki *et al.*, 1982).

There were no electrophoretic variations in any enzymes examined within clones. However among the different clones as well as different fluke populations, variations were observed at five loci (*Ak, Got, Gpi, 6-Pgd* and *Pgm-2*). The remaining nine enzyme loci revealed no variations in all the samples surveyed. Electrophoretic profiles of the five enzymes (AK, GOT, GPI, 6PGD, and PGM) are shown in figs 1 and 2. Table 3 shows genotype frequencies at these five polymorphic loci in the *Fasciola* fluke populations from four different countries. Allelic frequencies are shown in

Table 2. Electrophoretic conditions for 12 enzymes in *Fasciola* spp. from cattle of four different countries.

Systems	Enzymes	Conditions
AC	G6PD MDH ME PGM	30 mA current const. 6 h
Poulic	DIA EST GOT	150 V voltage const. 5 h
S18	GPI	50 mA current const. 15 h
TC (pH 6.0)	AK	50 mA current const. 4 h
TC (pH 7.5)	GDH GPT 6PGD	50 mA current const. 4 h

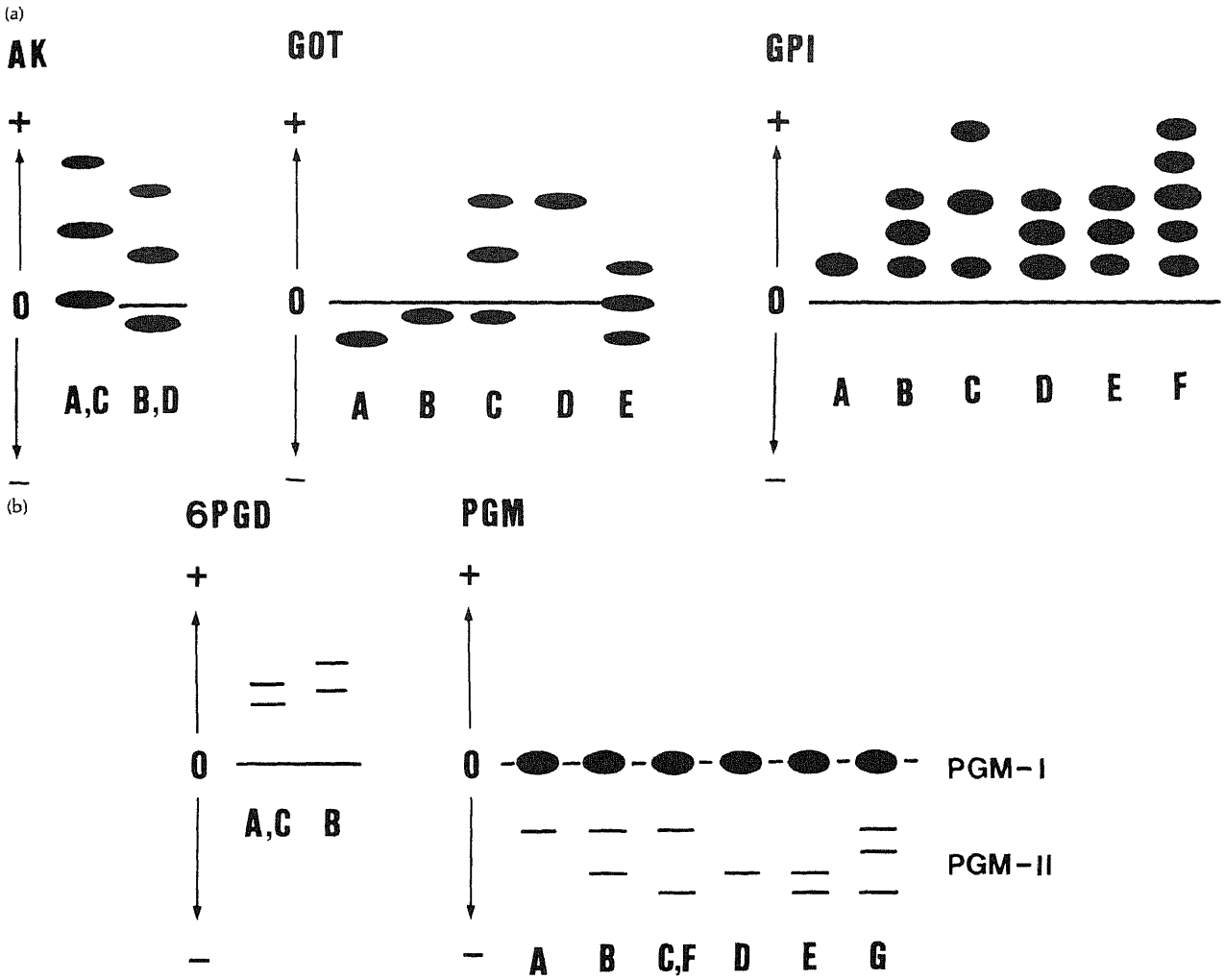

Fig. 1. Electrophoretic profiles of five enzymes AK, GOT, GPI, 6PGD and PGM in the liver flukes of the genus *Fasciola* derived from four different countries. Fig. 1a shows the profiles of AK, GOT and GPI, and fig. 1b, 6PGD and PGM. Letters show tentative genotypes at the five loci as follows:
Ak A: 1/1, B: 2/2, C: 1/1/1, D: 2/2/2 *Got* A: 1/1, B: 2/2, C: 2/3, D: 3/3, E: 1/1/4
Gpi A: 1/1, B: 1/2, C: 1/3, D: 1/1/2, E: 1/2/2, F: 1/2/3 *6Pgd* A: 1/1, B: 2/2, C: 1/1/1
Pgm-2 A: 1/1, B: 1/3, C: 1/4, D: 3/3, E: 3/4, F: 1/1/4, G: 1/2/4.

table 4. The following are descriptions of the five polymorphic enzymes in the *Fasciola* flukes.

AK formed sub-bands running in the anodal direction, probably due to polymerization of the enzyme molecules (Agatsuma & Suzuki, 1980). Two genotypes were observed for AK. Both patterns were found in diploids and in triploids.

Five genotypes of GOT were observed. Heterozygotes were triple-banded, indicating dimeric enzymes. Both alleles detected in Japanese flukes (of which one also occurred in the Korean fluke) were absent from Australian and American flukes. A further two alleles were seen in works from Australia and America.

Six genotypes of GPI were observed. The allele 2 was only observed in Oriental flukes (Korea and Japan).

At the *6Pgd* locus, only the allele 1 was found in Australian and Oriental flukes. The allele 2 was the only allele in American flukes. This is the only locus providing a diagnostic feature for distinguishing between American and Australian flukes on the one hand, and between American and Oriental flukes on the other.

Two zones of staining activity were observed for PGM. This suggests two loci might be involved. Seven genotypes derived from four alleles were detected for *Pgm*-2, the only variable locus. All four alleles were seen in Australian samples, and subsets of these in the other samples.

Discussion

Electrophoretic variants of AK and PGM were observed by Agatsuma & Suzuki (1980) among Japanese *Fasciola* species from Kochi. We have now found polymorphism in further three enzymes, GOT, GPI and 6PGD.

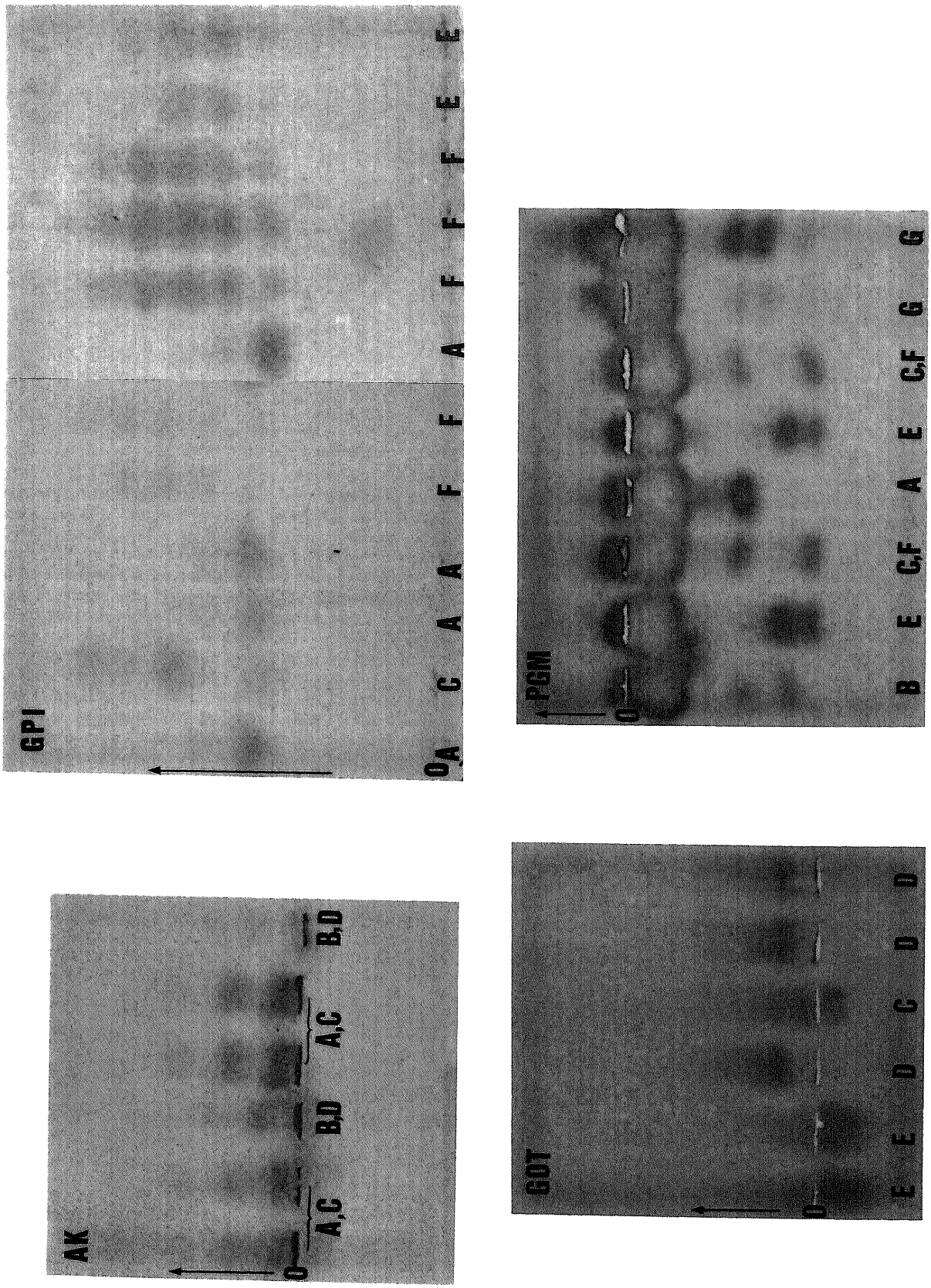

Fig. 2. Photographs of examples of electrophoretic patterns of four enzymes, AK, GOT, GPI and PGM in the liver flukes of the genus *Fasciola*. See fig. 1 for letters on the gel.

Table 3. Genotype frequencies at polymorphic enzyme loci of *Fasciola* flukes from Japan, Korea, USA and Australia.

Loci	Genotype	Sources and numbers of samples					
		Japan			Korea	USA	Australia[a]
	type[e]	1	2	3	1	7	30
	N[f]	3	8	23			
Ak	1/1					7	23
	1/1/1[b]	3					
	2/2				1		7
	2/2/2[cd]		8	23			
Got	1/1				1		
	2/2					1	
	2/3					3	11
	3/3					3	19
	1/1/4[bcd]	3	8	23			
Gpi	1/1					7	29
	1/2				1		
	1/3						1
	1/1/2[c]		8				
	1/2/2[d]			23			
	1/2/3[b]	3					
6Pgd	1/1				1		30
	1/1/1[bcd]	3	8	23			
	2/2					7	
Pgm-2	1/1				1	2	9
	1/3					4	4
	1/4					1	9
	3/3						2
	3/4						6
	1/1/4[b]	3					
	1/2/4[cd]		8	23			

[a]A sample consists of a single worm specimen, or of any number of worms of a laboratory strain derived from a single ancestor.
[b]This genotype seen in a strain derived from *F. hepatica*-like worms from Fukuoka.
[c]This genotype seen in a strain derived from one *F. gigantica*-like worm from Fukuoka.
[d]This genotype seen in two uniparental strains of *F. indica*-like worms from Fukuoka.
[e]See text for 'type'.
[f]Total number examined.

Japanese flukes reproduce by parthenogenesis, regardless of their ploidy, because of their abnormal gametogenesis (Sakaguchi, 1980). In the present study, the apparent non-Mendelian distribution of alleles among Japanese worms helps confirm this. However, three different fixed genotypes were observed among Japanese isolates, indicating that these parthenogenetic lines have arisen independently of each other.

There is a correlation between morphological type and genotype among the uniparental triploid strains established from Japanese works. Genotype 1, to which belong three uniparental strains of *F. hepatica*-like worms, possessed two alleles (1 at the *Ak* locus and 3 at the *Gpi*) not seen in the other genotypes. Genotype 2 (one strain, *F. gigantica*-like) and genotype 3 (two strains, *F. indica*-like) both possessed two alleles (2 at the *Ak* locus and 2 at the *Pgm*) not seen in genotype 1. Genotypes 2 and 3 possessed the same alleles and differed only in the gene dosage of alleles at the *Gpi* locus.The *F. hepatica*-like worms were therefore clearly distinct from the other two genotypes. All 28 worms from Kochi were morphologically of the *F. gigantica* type and belonged to genotypes 2 or 3.

Descendants of the single diploid worm from Korea, also a parthenogen as inferred by its abnormal gametogony, resembled genotypes 2 and 3 more closely than genotype 1. However, they lacked the allele 4 at the *Got* locus, and the alleles 2 and 4 at the *Pgm* locus seen in genotypes 2 and 3. A larger sample of Korean worms is needed. The Korean form also differed from American and Australian *F. hepatica* by its possession, shared with Japanese worms, of the allele 1 at the *Got* and the allele 2 at the *Gpi* locus.

American and Australian strains of *F. hepatica* had similar patterns, except for the *6Pgd* locus, where different alleles were fixed in each strain. Both strains were found to be Mendelian populations because of the occurrence of allelic segregation at the *Got* and *Pgm*-2 loci. Diagnostic alleles distinguishing between Oriental *Fasciola* sp. and *F. hepatica* occurred only at the *Got* locus.

The independent origins of parthenogenetic strains in Japan might have occurred through independent hybridization events between strains. The existence of such hybrids would explain the continuing confusion among scientists with respect to the taxonomic status of the Japanese liver flukes.

Recently, Blair & McManus (1989) found that a single specimen of the Japanese fluke yielded a ribosomal DNA restriction map identical to that of *F. gigantica*. The possibility of the Japanese *Fasciola* sp. being a hybrid between *F.*

Table 4. Total number of alleles at each locus observed among *Fasciola* spp. from different countries[a].

Loci Alleles	Japan			Korea	USA	Australia
	1	2	3			
Ak						
1	9				14	46
2		24	69	2		14
Got						
1	6	16	46	2		
2					5	11
3					9	49
4	3	8	23			
Gpi						
1	3	16	23	1	14	59
2	3	8	46	1		
3	3					1
6Pgd						
1	9	24	69	2		60
2					14	
Pgm-2						
1	6	8	23	2	9	31
2		8	23			
3					4	14
4	3	8	23		1	15

[a]Numbers in the table are numbers of alleles over all worm. Thus a single triploid fluke with genotype 1/1/2 at the *Ak* locus, for example, would score 2 for allele 1 and 1 for 2.

gigantica and *F. hepatica* must be considered. If this is the case, a survey of alleles in Asian populations of *F. gigantica* might find the alleles (1 and 4 at the *Got* and 2 at the *Pgm*) not detected in *F. hepatica* during this study.

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