



## Molecular analysis reveals expansion of *Fasciola hepatica* distribution from Afghanistan to China

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### ARTICLE INFO

#### Keywords:

*Fasciola hepatica*  
Afghanistan  
*Pepck*  
*Pold*  
*nad1*

### ABSTRACT

Recently, phosphoenol pyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) were established as reliable nuclear markers for species identification of *Fasciola* spp. in multiplex polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism-based assays, respectively. Currently, little is known about *Fasciola* species distribution in Central Asia. Therefore, the objective of the present study was to perform precise molecular species identification of liver flukes from Afghanistan and to reveal their dispersal route(s) via phylogenetic analysis based on mitochondrial *nad1* haplotypes. Ninety-two *Fasciola* flukes collected from sheep in Kabul, Afghanistan, were identified as *F. hepatica* based on *pepck* and *pold* screening. Although the *pepck* fragment pattern obtained via multiplex PCR analysis could not distinguish the species of the seven *Fasciola* flukes, the *pepck* nucleotide sequence data confirmed that they were *F. hepatica*. The 20 *nad1* haplotypes detected among the Afghani liver flukes were closely related to those from China and Egypt, with the  $F_{ST}$  value ( $-0.003$ ,  $P = .41$ ) between the *F. hepatica* populations from Afghanistan and China confirming a very close relationship. Nucleotide diversity was greater in the population from Afghanistan compared with that from China, indicating that the Afghani population was older, and that the dispersal direction of *F. hepatica* was from Afghanistan to China. The results of the present study contribute to our understanding of the dispersal of *F. hepatica* from its predicted origin, the Fertile Crescent.

### 1. Introduction

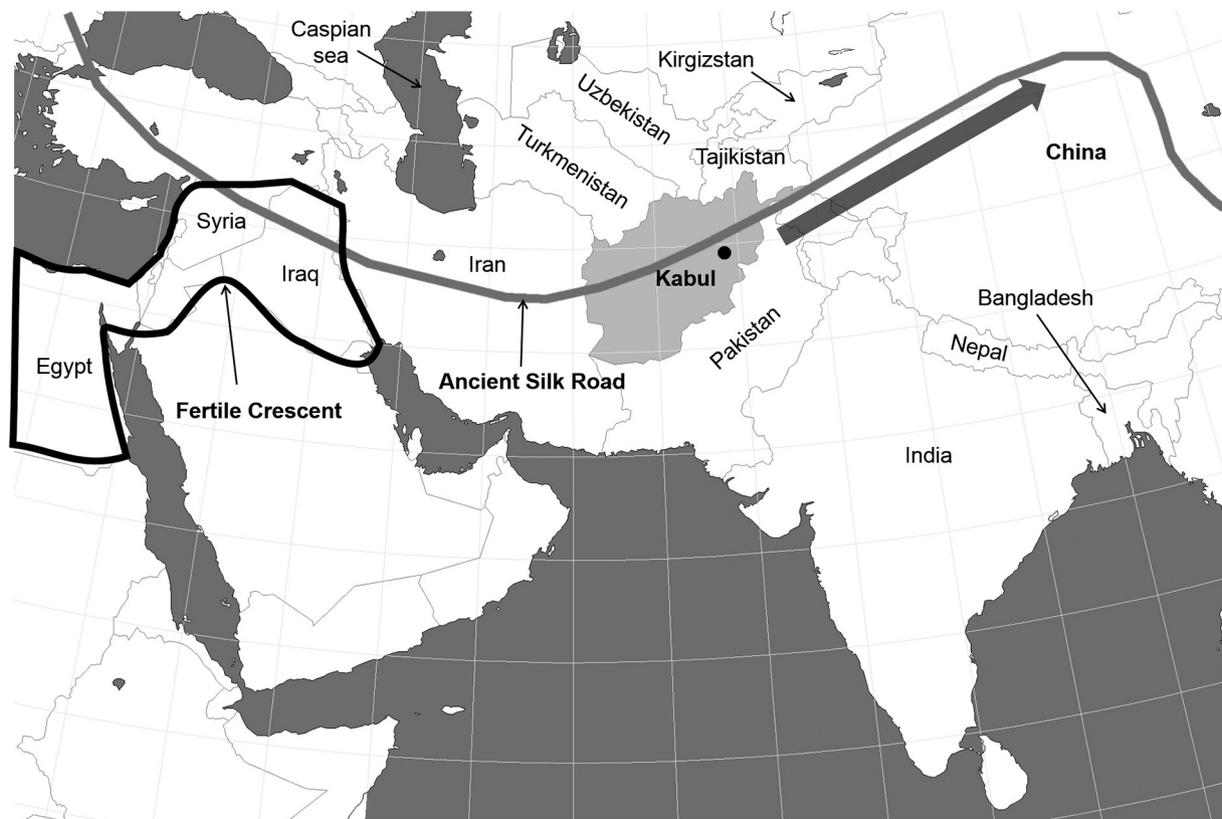
Fasciolosis is a globally-distributed zoonotic disease caused by the trematode parasites *Fasciola hepatica* and *F. gigantica*. The former species mainly occurs in Europe, the Americas, Oceania, and northern parts of Asia, while the latter is distributed in Africa and southern Asia [1,2]. In addition to these two better-known species, hybrid *Fasciola* flukes originating from interspecific hybridization [3] can be found throughout East, South East, and South Asian countries [3–14]. However, the distribution of hybrid *Fasciola* flukes in the region between Central Asia and the Middle East has never been investigated to our knowledge. Several previous reports [15–18] attempted to identify the species of *Fasciola* flukes from Iran using morphological and morphometric features as well as molecular markers (nuclear internal transcribed spacer 1 and 2); however, these results have not been validated using more recently-established and reliable nuclear gene markers.

Recently, novel nuclear markers phosphoenol pyruvate

carboxykinase (*pepck*) and DNA polymerase delta (*pold*) were found to be useful for the precise discrimination of *F. hepatica*, *F. gigantica*, and hybrid *Fasciola* flukes [3,19]. In addition, the nucleotide sequences of mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) and cytochrome oxidase subunit 1 (*cox1*) genes have been used to analyze intraspecific phylogenetic relationships among *Fasciola* spp. [4]. Phylogenetic relationships as well as nucleotide diversity within the *nad1* gene were then used in the previous studies [4–14] to determine the dispersal routes of *Fasciola* spp. in many Asian countries.

Previous reports from Iran [15–18] analyzed the nucleotide sequences of *nad1* and *cox1*; however, frequency data for each haplotype could not be obtained from the reports. As such, based on currently available data, it is impossible to infer the dispersal route(s) of *Fasciola* flukes in Central Asia. Therefore, the objectives of the current study were to precisely identify *Fasciola* species in sheep from Kabul, Afghanistan, based on the nuclear *pepck* and *pold* genes, and to analyze their phylogenetic relationship with *Fasciola* populations from different parts

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**Fig. 1.** The small black circle indicates the approximate collection site (Kabul, Afghanistan). The Fertile Crescent, the predicted origin of *Fasciola hepatica*, as suggested by Mas-Coma et al. [27], is highlighted by the bold black line. The Silk Road via Afghanistan to China is shown by the gray line, and the bold arrow indicates the dispersal direction of *F. hepatica*.

of the world based on mitochondrial *nad1* gene sequences to reveal the dispersal route(s) of *Fasciola* flukes in the area.

**2. Materials and methods**

**2.1. Collection of *Fasciola* flukes and DNA extraction**

*Fasciola* flukes were collected from the bile ducts of 46 sheep in Kabul, Afghanistan (Fig. 1), between January and September 2017. They were mainly Hazaragi local breed. Hazaragi local breed is a breed of sheep originated from Hazara ethnic group. The ethnic group mainly resides in central Afghanistan called Hazarajat. However, there was no detailed information available regarding the geographical origin, and herds of the slaughtered sheep. The flukes were fixed in 70% ethanol and transported to the laboratory for further analysis. Two *Fasciola* flukes were randomly selected from each sheep for molecular analysis; thus, a total of 92 flukes were examined in this study (Table 1).

A small number of vitelline glands from the posterior section of each fluke were used for DNA extraction. Total DNA was extracted using a NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) in accordance with the manufacturer's protocols and stored at -20°C until use.

**2.2. Species identification using nuclear DNA markers**

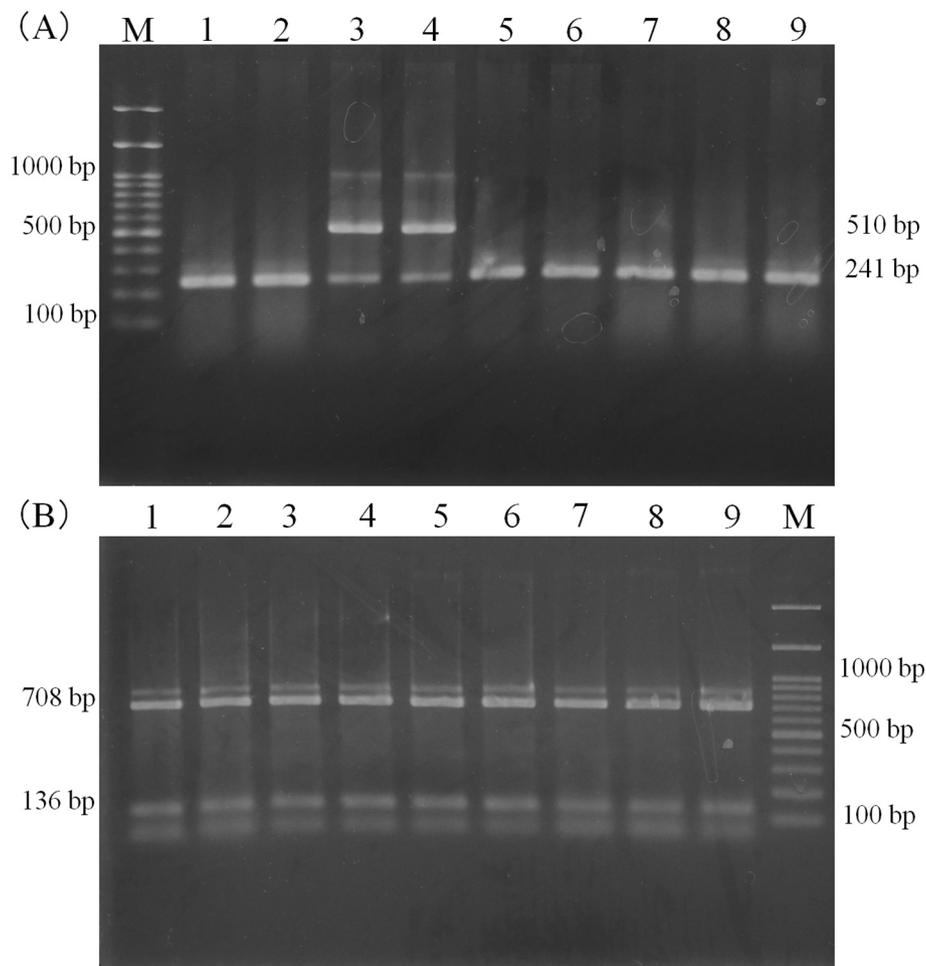
Nuclear DNA was analyzed for molecular species identification according to the method of Shoriki et al. [19]. Briefly, *pepck* was amplified via a multiplex polymerase chain reaction (PCR) approach using primers Fh-*pepck*-F, Fg-*pepck*-F, and Fcmn-*pepck*-R. The PCR amplicons were electrophoresed on 1.8% agarose gels and then stained with ethidium bromide solution (10 mg/ml) for 30 min to visualize and identify *F. hepatica*, *F. gigantica*, or both fragment patterns (the hybrid

**Table 1**

Molecular profiles of *Fasciola* flukes from Kabul, Afghanistan, examined in this study.

Mitochondrial <i>nad1</i> haplotype	Nuclear DNA types		Number of flukes	Accession number for <i>nad1</i> haplotypes
	<i>pepck</i>	<i>pold</i>		
AFG- <i>nad1</i> Fh1	Fh	Fh	35	LC436788
AFG- <i>nad1</i> Fh2	Fh	Fh	29	LC436806
AFG- <i>nad1</i> Fh2	Fh/Fg	Fh	5	LC436806
AFG- <i>nad1</i> Fh3	Fh	Fh	1	LC436797
AFG- <i>nad1</i> Fh4	Fh	Fh	2	LC436791
AFG- <i>nad1</i> Fh5	Fh	Fh	1	LC436792
AFG- <i>nad1</i> Fh6	Fh	Fh	1	LC436795
AFG- <i>nad1</i> Fh7	Fh	Fh	1	LC436804
AFG- <i>nad1</i> Fh8	Fh	Fh	1	LC436798
AFG- <i>nad1</i> Fh9	Fh	Fh	1	LC436789
AFG- <i>nad1</i> Fh10	Fh/Fg	Fh	1	LC436794
AFG- <i>nad1</i> Fh11	Fh	Fh	1	LC436800
AFG- <i>nad1</i> Fh12	Fh	Fh	1	LC436802
AFG- <i>nad1</i> Fh13	Fh	Fh	2	LC436799
AFG- <i>nad1</i> Fh14	Fh	Fh	1	LC436790
AFG- <i>nad1</i> Fh15	Fh	Fh	1	LC436805
AFG- <i>nad1</i> Fh16	Fh	Fh	1	LC436796
AFG- <i>nad1</i> Fh17	Fh	Fh	1	LC436807
AFG- <i>nad1</i> Fh18	Fh	Fh	2	LC436803
AFG- <i>nad1</i> Fh19	Fh/Fg	Fh	1	LC436793
AFG- <i>nad1</i> Fh19	Fh	Fh	1	LC436793
AFG- <i>nad1</i> Fh20	Fh	Fh	2	LC436801
Total			92	

“Fh” indicates the *Fasciola hepatica* fragment pattern as determined by multiplex polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism analysis. “Fh/Fg” indicates that both the *Fasciola hepatica* and *Fasciola gigantica* fragment patterns were observed in the multiplex PCR.



**Fig. 2.** The representative electrophoresis results of (A) multiplex PCR for *pepck* and (B) PCR-RFLP for *pold*. The samples on the gels (lanes 1 to 9) are coincided with each other. (A) Lanes 1, 2, and 5 to 9: *F. hepatica* fragment pattern, Lanes 3 and 4: both the *F. hepatica* and *F. gigantica*-like fragment patterns. (B) Lanes 1 to 9: *F. hepatica* fragment pattern.

fragment pattern). *Pold* was analyzed using a PCR-restriction fragment length polymorphism (RFLP)-based assay. Amplicons generated using the *Fasciola-pold-F1* and *Fasciola-pold-R1* primers were subsequently digested with *AluI* (Toyobo, Osaka, Japan) at 37 °C for 3 h. Then, the resultant DNA fragments were electrophoresed as described above.

The *pepck* sequences (925 or 980 bp) from the seven *Fasciola* flukes which had contradictory results between *pepck* and *pold* were analyzed to confirm the results of the multiplex PCR. The sequencing was also performed for the representative two flukes which had the consistent result in *pepck* and *pold*. The primer set *Fasciola-pepck-F1/Fasciola-pepck-R1* [19] was used for gene sequencing. PCR assays were performed using a 25- $\mu$ l reaction mixture containing 100 ng of template DNA, 0.4 mM of each dNTP, 0.3  $\mu$ M of each primer, and 1 U of KOD FX Neo (Toyobo, Osaka, Japan). Thermal cycler conditions included an initial denaturing step at 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 s and 68 °C for 30 s. PCR amplicons were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's protocol. Since direct sequencing could not analyze the heterozygous fragments, the purified amplicons were cloned into the pUC118 *HincII*/BAP vector (Takara Bio Inc., Japan) and then directly sequenced from both directions. The resultant sequences were aligned to construct a maximum likelihood (ML) tree using MEGA 10.0.5 [20]. In the ML tree construction, complete deletion was selected in Gaps/Missing Data Treatment. The *pepck* nucleotide sequences from *F. hepatica*, *F. gigantica* and hybrid *Fasciola* flukes (GenBank accession numbers between LC061148 and LC061171) [19] were used as

reference sequences. This analysis was conducted to determine whether the resultant *pepck* sequences belong to *F. hepatica* or *F. gigantica* clade.

The 95% confidence including continuity correction interval for the frequency of hybrid *Fasciola* flukes in Afghanistan was calculated using the website for statistical computation (VassarStats: <http://www.vassarstats.net>).

### 2.3. *nad1* sequencing and phylogenetic analysis

The mitochondrial *nad1* gene was amplified using primers Ita 10 and Ita 2 [4] and purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer's instructions. The *nad1* amplicons were then sequenced by Eurofin Genomics K.K. (Tokyo, Japan). The resultant DNA sequences were assembled using ATGC ver. 6.0.3 (Genetyx Co., Tokyo, Japan), and haplotypes were identified using GENETYX ver. 10.0.2 (Genetyx Co.).

The obtained sequences were aligned with each other and with reference sequences using DNA Alignment ver. 1.3.3.2 (Fluxus Technology, Clare, England). A median-joining (MJ) network was constructed using Network 5.0.0.3 software (Fluxus Technology) [21] to identify the phylogenetic relationships among the *nad1* haplotypes. Reference *F. hepatica nad1* haplotypes from China [14], Peru [22], Egypt [23], and Spain [24] were obtained from GenBank, and haplotype frequencies were determined.

### 2.4. Genetic diversity

The number of flukes (*N*), number of variable sites (*S*), number of haplotypes (*h*), and nucleotide diversity ( $\pi$ ) were calculated for the Afghani *Fasciola* population and for reference populations from Spain, Peru, Egypt, and China using DnaSP ver. 6.11.01 [25]. The dataset used for these calculations was the same as that used for the MJ network. Tukey's test, implemented in GraphPad Prism 7.04 (GraphPad Software Inc., San Diego, CA, USA), was used to identify significant differences in  $\pi$  values among the populations.

Pairwise fixation index ( $F_{ST}$ ) values between *Fasciola* populations from Afghanistan and reference countries (Egypt, China, Peru, and Spain) were calculated using Arlequin version 3.5.2.2 [26].  $F_{ST}$  values approaching 1 indicate extreme genetic differentiation between the two populations.

## 3. Results

### 3.1. Species identification based on *pepck* and *pold*

While all 92 *Fasciola* flukes from Afghanistan showed the *F. hepatica* fragment pattern following PCR-RFLP analysis of the *pold* (Table 1, Fig. 2), only 85 flukes displayed the *F. hepatica* (Fh) fragment pattern following multiplex PCR analysis of the *pepck*. Using this assay, the remaining seven flukes displayed both the *F. hepatica* and *F. gigantica* (Fg)-like fragment patterns (Table 1, Fig. 2), consistent with hybrid *Fasciola* flukes.

The four *pepck* clones were analyzed for each of the seven flukes, resulting in 28 sequences (Table 2). In total, 14 genotypes (AFG-*pepck* Fh1 to Fh14) were identified among the 28 sequences and have been deposited in the GenBank database under accession numbers LC436808–LC436821 (Table 2). A ML tree (Fig. 3) constructed from the nucleotide sequences of the 14 genotypes showed that all 14 genotypes belonged to the *F. hepatica* clade. However, AFG-*pepck* Fh1 to Fh6, detected from Fg-like fragments of the seven flukes (Table 2) were

separated from the reference *F. hepatica* clade. Multiple alignment analysis of *pepck* gene sequences (Fig. 4) revealed that multiplex PCR primer Fg-*pepck*-F amplified the variant *F. hepatica* sequences (i.e., AFG-*pepck* Fh1 to Fh6), with the resultant fragment size identical to that of the *F. gigantica* fragment.

Again, the *pepck* sequences of the two flukes which had the Fh fragment pattern both in *pepck* and *pold* were included in the reference *F. hepatica* clade (data not shown).

Therefore, all *Fasciola* flukes from sheep in Kabul examined in this study were definitively identified as *F. hepatica* using nuclear markers *pepck* and *pold*. No hybrid *Fasciola* fluke was detected in this study, and the 95% confidence interval (CI) of the frequency of hybrid *Fasciola* flukes were calculated as 0–5.0%.

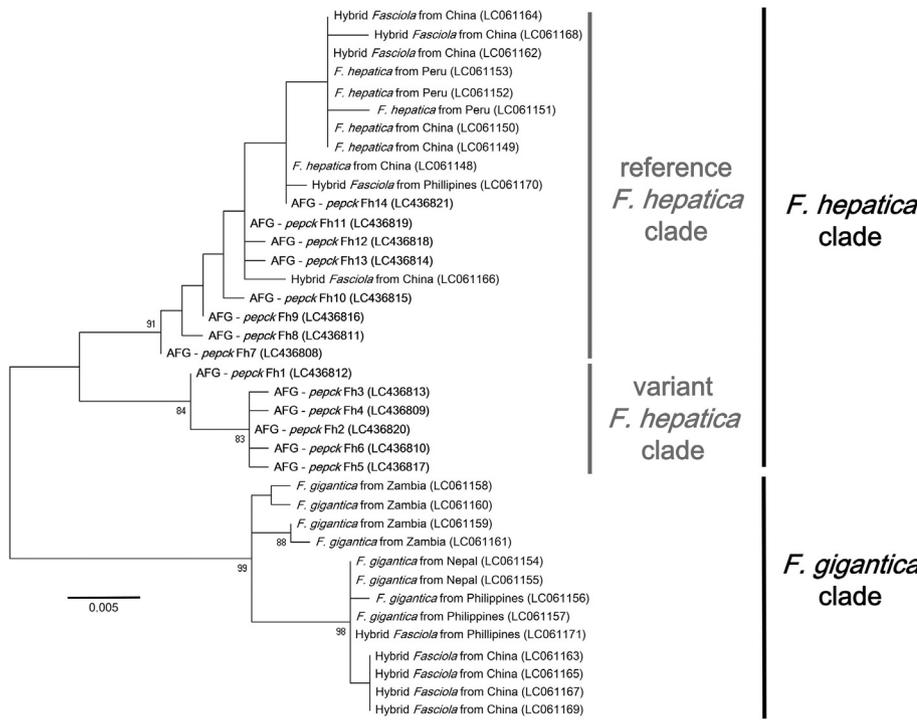
### 3.2. Mitochondrial *nad1* haplotypes

Among the 92 flukes examined in the current study, 11 substitution sites were identified in the *nad1* nucleotide sequence (535 bp), corresponding to 20 different haplotypes. These were designated AFG-*nad1*Fh1 to AFG-*nad1*Fh20 (Table 1). MJ network analysis (Fig. 5) revealed that AFG-*nad1*Fh1 and AFG-*nad1*Fh2 were the predominant haplotypes of the examined *F. hepatica* flukes and were present at almost the same frequency (Table 1, Fig. 5). The AFG-*nad1*Fh1 haplotype sequence was identical to *F. hepatica* haplotypes from China (Fh-C1) [14] and Egypt (LC076241) [23], whereas AFG-*nad1*Fh2 was identical to haplotypes from China (Fh-C4), Egypt (LC076271), and Peru (ND1-P7) [22]. Ten (AFG-*nad1*Fh3 to AFG-*nad1*Fh12) and seven (AFG-*nad1*Fh13 to AFG-*nad1*Fh19) satellite haplotypes were derived from the AFG-*nad1*Fh1 and AFG-*nad1*Fh2 haplotypes, respectively, with each containing one or two nucleotide substitutions. In particular, AFG-*nad1*Fh3 and AFG-*nad1*Fh14 showed identical sequences to *F. hepatica* haplotypes Fh-C5 and Fh-C3, respectively, from China [14]. In addition, the AFG-*nad1*Fh20 haplotype had an identical nucleotide sequence to *F. hepatica* haplotypes from Peru (ND1-P3) [22] and Egypt (LC076228) [23].

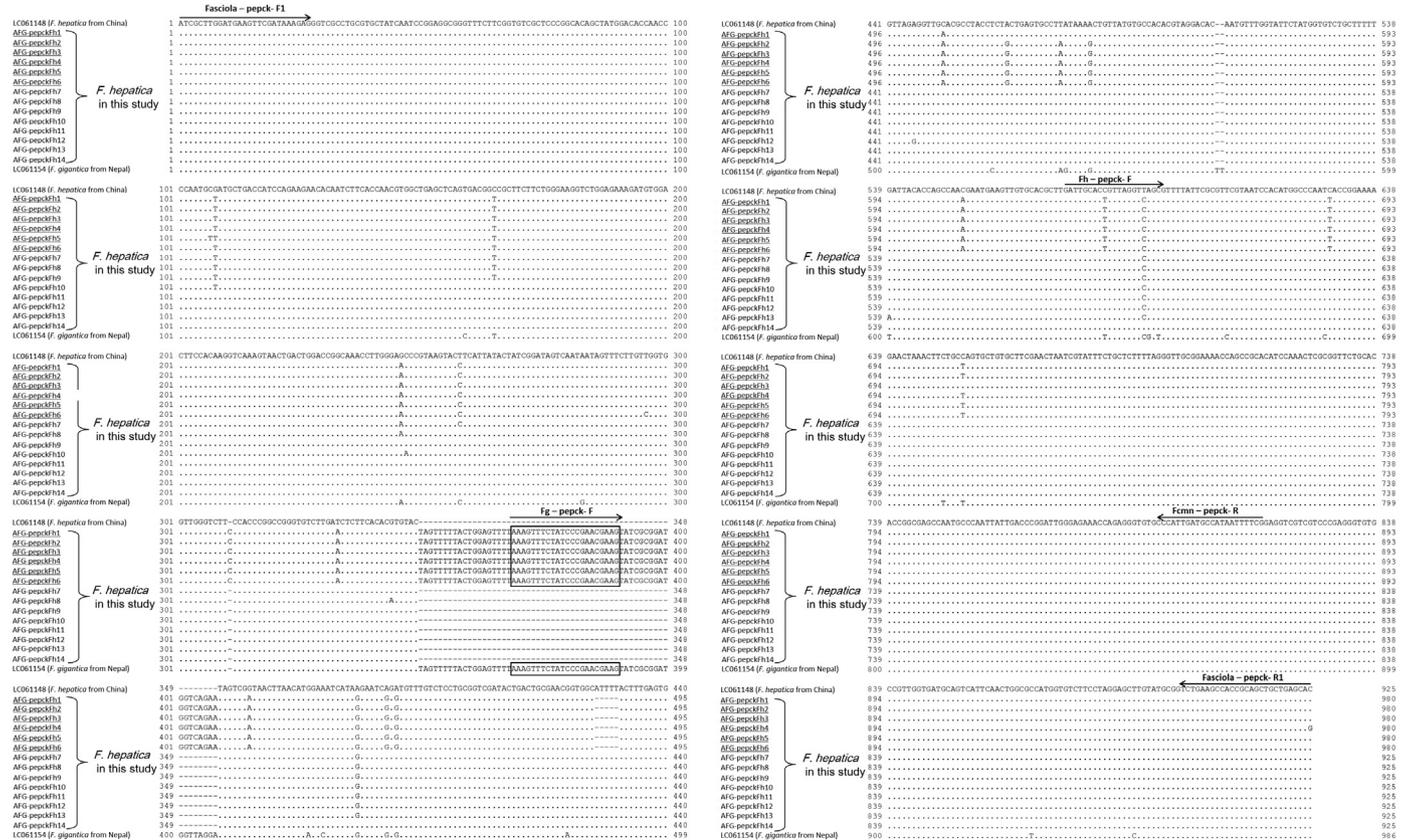
**Table 2**  
Pepck genotypes of the seven *Fasciola* flukes from Afghanistan.

Mitochondrial <i>nad1</i> haplotype	Number of flukes	Multiplex PCR fragment pattern	<i>pepck</i> genotypes of the four clones	Accession number for <i>pepck</i> genotypes
AFG- <i>nad1</i> Fh2	1	Fh	AFG- <i>pepck</i> Fh7	LC436808
		Fg-like	AFG- <i>pepck</i> Fh11	LC436819
	1	Fh	AFG- <i>pepck</i> Fh4	LC436809
			AFG- <i>pepck</i> Fh2	LC436820
		Fg-like	AFG- <i>pepck</i> Fh13	LC436814
			AFG- <i>pepck</i> Fh10	LC436815
	1	Fh	AFG- <i>pepck</i> Fh1	LC436812
			AFG- <i>pepck</i> Fh3	LC436813
		Fg-like	AFG- <i>pepck</i> Fh11	
			AFG- <i>pepck</i> Fh9	LC436816
	1	Fh	AFG- <i>pepck</i> Fh2	LC436817
			AFG- <i>pepck</i> Fh5	LC436821
		Fg-like	AFG- <i>pepck</i> Fh14	
			AFG- <i>pepck</i> Fh14	
1	Fh	AFG- <i>pepck</i> Fh2		
		AFG- <i>pepck</i> Fh2		
	Fg-like	AFG- <i>pepck</i> Fh14		
		AFG- <i>pepck</i> Fh2		
AFG- <i>nad1</i> Fh10	1	Fh	AFG- <i>pepck</i> Fh11	LC436811
		Fg-like	AFG- <i>pepck</i> Fh8	
	1	Fh	AFG- <i>pepck</i> Fh2	LC436810
			AFG- <i>pepck</i> Fh6	LC436818
AFG- <i>nad1</i> Fh19	1	Fh	AFG- <i>pepck</i> Fh12	
			AFG- <i>pepck</i> Fh11	
		Fg-like	AFG- <i>pepck</i> Fh2	
Total	7			

The four *pepck* clones were analyzed for each of the seven flukes.



**Fig. 3.** Maximum likelihood tree inferred from the *pepck* sequences of the seven *Fasciola* flukes from Afghanistan and the reference countries. Bootstrap values higher than 80% are shown on the tree node. The *pepck* genotypes obtained in this study are highlighted in bold font. The reference sequences were retrieved from the previous study [19]. *F. hepatica* (GenBank accession numbers LC061148 to LC061153), *F. gigantica* (LC061154 to LC061161), and hybrid *Fasciola* flukes (*F. hepatica* fragment: LC061162, LC061164, LC061166, LC061168, LC061170, *F. gigantica* fragment: LC061163, LC061165, LC061167, LC061169, LC061171). No suitable outgroup was available in GenBank.



**Fig. 4.** The *pepck* nucleotide sequences from the seven *Fasciola* flukes from Afghanistan analyzed in this study. The *pepck* genotypes corresponded to those shown in Table 2. Dots in the alignment indicate that the sequence is identical to that of *Fasciola hepatica* from China (LC061148 in Fig. 3) and *Fasciola gigantica* from Nepal (LC061154 in Fig. 3). Horizontal bars in the sequences indicate alignment gaps, while arrows and boxes indicate the positions and directions of the primers. AFG-*nad1*Fh1 to Fh6, indicated by underlining, are the variant sequences of *F. hepatica* that are amplified using primer Fg-*pepck*-F.

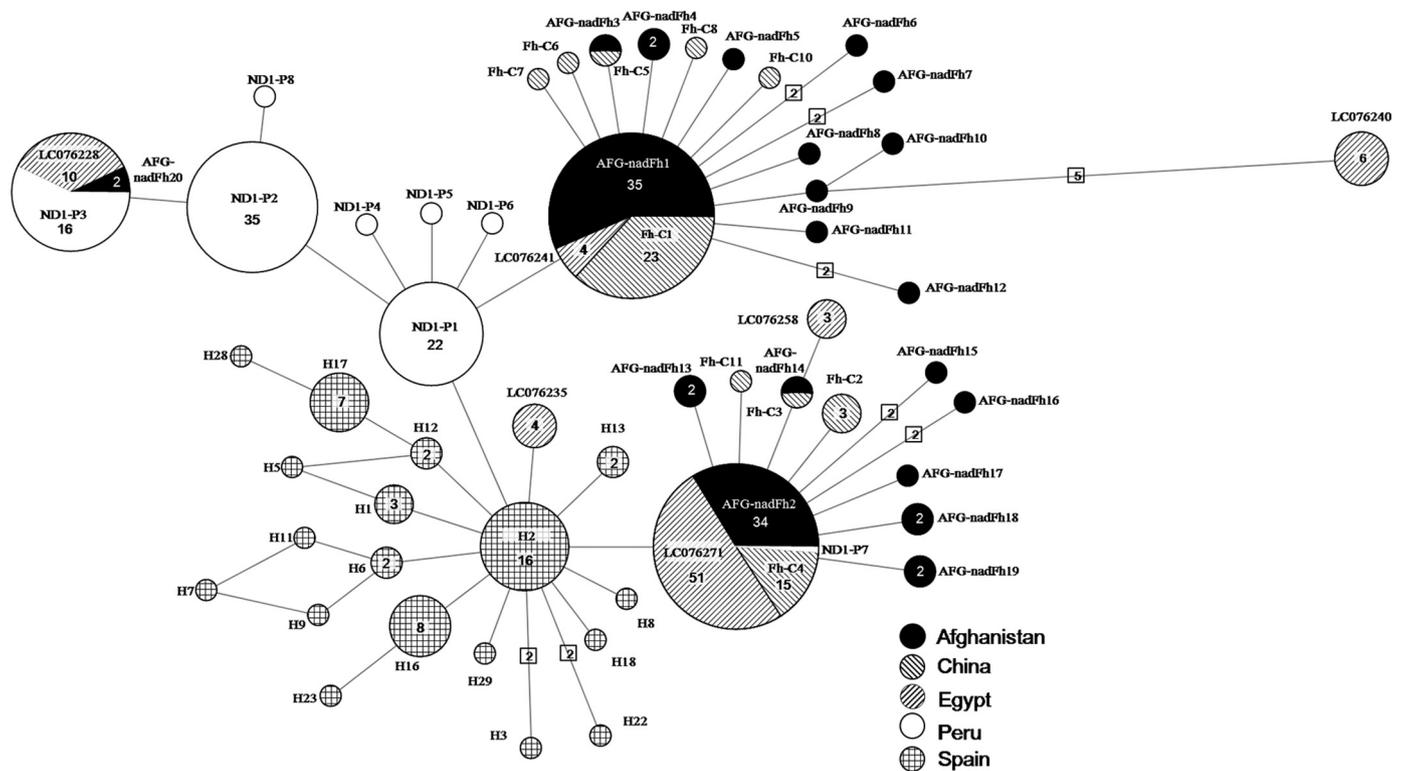


Fig. 5. Median-joining network based on the mitochondrial *nad1* haplotypes of *Fasciola hepatica*. The *Fasciola* flukes from Afghanistan are shown in black. Each circle indicates a haplotype. The haplotype codes are shown within or adjacent to the circles. Numbers on each circle and on the node indicate the number of flukes and the number of substitution sites, respectively. No label indicates only one fluke and one substitution site.

**Table 3**  
Diversity indices of *Fasciola hepatica* flukes examined in this study based on *nad1* nucleotide sequence data.

Populations	N	S	h	$\pi$	Standard deviation
Afghanistan	92	26	20	0.00408	0.00024
China	48	11	10	0.00355	0.00027
Egypt	78	14	6	0.00488	0.00079
Spain	51	16	18	0.00340	0.00035
Peru	78	8	8	0.00175	0.00017

N: number of flukes assessed; S: number of variable sites; h: number of haplotypes;  $\pi$ : nucleotide diversity.

All values were significantly different from each other, except for the comparison between the populations from China and Spain ( $P < .05$ ).

### 3.3. Genetic diversity

Diversity indices for the *nad1* haplotypes were calculated to compare the haplotypes from Afghanistan with those from the reference countries. The highest  $\pi$  value was calculated for the *F. hepatica* population from Egypt, followed by populations from Afghanistan, China, and Spain. The Peruvian population had the lowest  $\pi$  value. Significant differences were observed between all populations ( $P < .05$ ), except for between those from China and Spain ( $P = .96$ ) (Table 3).

The  $F_{ST}$  values for the *F. hepatica* populations from Afghanistan and China were not significant ( $P = .41$ ), indicating that genetic flow between the two populations has been maintained at a high level. In contrast, significant differences in  $F_{ST}$  values ( $P < .0001$ ) were observed between other populations, suggesting that they were genetically differentiated (Table 4).

### 4. Discussion

Although molecular species identification was performed on the

**Table 4**  
Pairwise fixation index ( $F_{ST}$ ) values among *Fasciola hepatica* populations from Afghanistan and reference countries.

Populations	Afghanistan	Egypt	China	Peru
Afghanistan	-			
Egypt	0.10618	-		
China	-0.003*	0.15519	-	
Peru	0.41001	0.42809	0.46669	-
Spain	0.28931	0.21873	0.33656	0.52142

\* Statistically non-significant ( $P = .41$ ), all other values are statistically significant ( $P < .0001$ ).

basis of nuclear *pepck* and *pold* genes, there were contradictions between the results of the two markers. While 85 flukes examined in the current study showed Fh fragment pattern at both the *pepck* and *pold*, the seven flukes displayed the Fh/Fg pattern at the *pepck* but the Fh pattern at the *pold*. However, the ML tree constructed using the *pepck* nucleotide sequences demonstrated that the six *pepck* genotypes (AFG-*pepck* Fh1 to Fh6) detected from Fg-like fragment of the seven flukes (Table 2) were included in the variant *F. hepatica* clade (Fig. 3). The sequence analysis revealed that the primer Fg-*pepck*-F, designed for *F. gigantica* in a previous study [19], amplified divergent *F. hepatica* sequences (Fig. 4). Therefore, when a mismatch is observed between *pepck* and *pold* fragment patterns, sequence analysis should be employed to confirm the results. Nonetheless, *pepck* is still a reliable marker for species identification as sequence analysis can precisely identify the species. However, sequencing for the fragment in *pepck* is costly and time-consuming, and therefore, one more simple technique based on another nuclear marker instead of *pepck* is required in near future.

All 92 *Fasciola* flukes from Kabul, Afghanistan, were identified as *F. hepatica* based on analysis of nuclear genes *pepck* and *pold*. This molecular species identification was supported by the MJ network generated for the mitochondrial *nad1* gene (Fig. 5). All *nad1* haplotypes detected

among the Afghani samples were related to *F. hepatica* haplotypes from Egypt, China, Spain, and Peru. Therefore, these findings suggest that hybrid *Fasciola* flukes have not disseminated westwards to Kabul, Afghanistan, from China, Bangladesh, Nepal, and India, where they are commonly identified [9–11,13,14]. However, since the population size examined in this study seemed not enough to conclude the absence of hybrid *Fasciola* flukes in Afghanistan (95% CI: 0–5.0%), further sampling from different locations in Afghanistan is required in the future.

*Nad1* haplotypes from the Afghani, Egyptian, and Chinese *F. hepatica* populations were closely related in the MJ network (Fig. 5), with all three populations sharing two main haplotypes, represented by AFG-*nad1*Fh1 and AFG-*nad1*Fh2. Among the three countries, the *F. hepatica* population from Egypt appeared to be the oldest, followed by those from Afghanistan and China, as the greater genetic diversity ( $\pi$ ) values indicate more ancient populations (Table 3). In contrast, the *F. hepatica* populations from Spain and Peru were distinct from those from Egypt, Afghanistan, and China, and showed significant  $F_{ST}$  values (Table 4).

*F. hepatica* populations from Afghanistan and China shared not only the same main haplotypes, but also the same satellite haplotypes. The continuity of the *F. hepatica* populations from the two countries was statistically confirmed by the non-significant  $F_{ST}$  value ( $P = .41$ ) (Table 4). Considered with the higher genetic diversity values of the Afghani population compared with the Chinese population, these results suggest that *F. hepatica* likely disseminated through Central Asia to East Asia, i.e., from Afghanistan to China. This dispersal direction agrees with the conclusions of Mas-Coma et al. [27], who suggested that *F. hepatica* dispersed northwards from the Fertile Crescent to East Asia via the Caspian Sea, and also eastwards through Iran, Turkmenistan, Uzbekistan, Tajikistan, and Kirgizstan to China, Mongolia, and the Far East [27]. Afghanistan lies along this dispersal route (Fig. 1). This dispersal route is almost coincided with the Silk Road, and therefore, it is speculated that the livestock trade along the Silk Road caused the migration of *F. hepatica* into China. Mas-Coma et al. [27] also suggested another dispersal route eastward through the Himalayan chain to South Asia, but cautioned that insurmountable climatic barriers, along with the distribution of intermediate hosts, need to be considered [27]. Supporting this, *F. hepatica* has yet to be found in any of the countries located in South and South East Asia [5, 7–13].

This study suggested that *F. hepatica* is present in Afghanistan and appears to have dispersed in an eastward direction to China. However, to confirm the dispersal route between Egypt and Afghanistan, further *F. hepatica* samples from neighboring countries, including those in the Middle East, are needed.

## Funding

This work was supported by a Grant-in-Aid for Scientific Research (B) from MEXT KAKENHI (Grant Number16H05806) and a Training Program for Asian Veterinarians (TP-FAVII) by Japan Veterinary Medical Association (JVMA).

## Declarations of interest

none.

## Acknowledgments

We thank Tamsin Sheen, PhD, from Edanz Group ([www.edanzediting.com/ac](http://www.edanzediting.com/ac)) for editing a draft of this manuscript.

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