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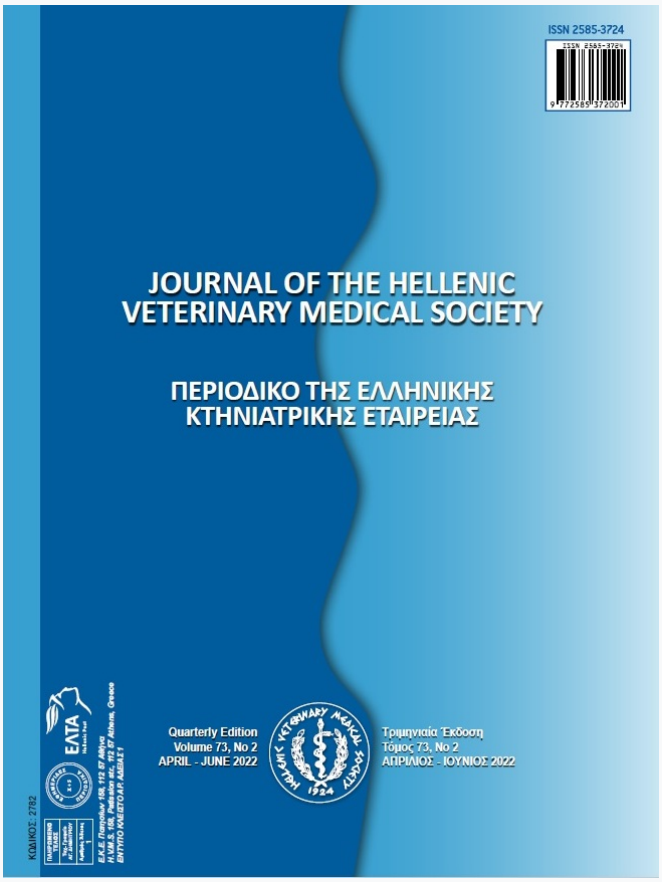

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Research article
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A Comprehensive in silico Analysis of mt-CO1 gene of *Fasciola hepatica*

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ABSTRACT: This study was conducted for the aim of comprehensive evaluation of the genetic diversity and phylogenetic relations of *F. hepatica* among various hosts. In the current work, published mt-CO1 gene sequences belonging to final hosts of *F. hepatica* were used to create the dataset. First of all, 478 sequences were obtained with PubMed search. Then, some shorter sequences were removed after alignment, and 319 sequences which included cattle (n=242), sheep (n=46), goat (n=5), donkey (n=8), bison (n=7), buffalo (n=7), human (n=2) and camel (n=2) sequences were examined in the MEGA X. The existence of 72 haplotype groups was detected. The most polymorphic sites (n=42) containing 31% (13/42) parsimony informative sites were detected in Iranian isolates. The mt-CO1 network involved of 35 haplotypes, 80% out of which were geographically unique. However, a main haplotype consisting of 39.2% of the total isolates was formed. The bison isolates of *F. hepatica* showed the maximum haplotype variety within the final host isolates, followed by the sheep, buffalo, cattle, goat and donkey isolates. The genetic varity of *F. hepatica* in different hosts and countries revealed the possibility of new strains appearing in the future.

Keywords: *Fasciola hepatica*, in silico analysis, mt-CO1, haplotype.

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INTRODUCTION

Fasciola hepatica is a flatworm which is seen in many parts of the world (Mas-Coma *et al.*, 2009). It is transmitted by the intake of herbs with contaminated metacercariae and can infect many mammals. Ruminants are the main definitive hosts causing significant economic losses (Robinson and Dalton, 2009). Nevertheless, various other mammals such as deer, horses and humans may also be infected with the worm (Ichikawa-Seki et al., 2017; Mendes et al., 2008). In the past two decades, fascioliasis has been recognized as a attentioned disease. It is recently classified as a food-borne trematodiasis, frequently gained by uptake of metacercaria encysted on greens (Mas-Coma et al., 2005). Low-income countries have a high prevalence of human fascioliasis due to their permanent close contacts with livestock and agriculture. WHO has estimated that more than 2 million people are infected with fasciolosis, and 180 million are under the risk mostly in developing countries (Mehmood et al., 2017). Remarkable economic losses happen regarding animal fascioliasis due to liver contamination at slaughterhouses, as well as reduced milk yield and mortality. The global losses in animal production were estimated as more than US$3.2 billion/year by fascioliasis (Nyindo and Lukambagire, 2015).

Fascioliasis has been defined in more than 70 countries and is still the most widespread food-borne helminthic infection in all around the globe (Mas-Coma, 2005; Mas-Coma et al., 2009). Although *F. hepatica* originated from Europe, its global circulation has enlarged over the last a few centenary because of the worldwide colonisations by Europeans and livestock exportation (Mas-Coma, 2003). The prevalence of *F. hepatica* was 8.4% (in cow) in the eastern part of Switzerland (Schweizer et al., 2003),11.2 to 25.2% (in cattle) in France (Mage et al., 2002), 72% (in dairy herd) (seroprevalence) in the United Kingdom (McCann et al., 2010), 61.6% (in sheep) in Ireland and 7.9% (in sheep) in Italy (Rinaldi et al., 2015). Besides, it was reported to be between 6.5% and 18.2% in sheep and 9.1% and 27% in cattle from Algeria (Mekroud et al., 2004; Ouchene-Khelifi et al., 2018). Alongside the prevalence data, there are many reports about molecular characterization of *F. hepatica* isolates. After the complete sequence of the mitochondrial genome of the parasite (Le et al., 2001), many studies (Ai et al., 2011; Dosay-Akbulut et al., 2005; Walker et al., 2011; Walker et al., 2007) have been performed on mt-DNA as an indicator for population diversity.

Although the significance of *F. hepatica* for human and animal health is known, data on its genetic diversity and population structure are still limited.The main purpose of the currentwork was to comprehensively evaluate the genetic diversity of *F. hepatica* among various hosts, as well as the dispersion of this genetic diversity in both phylogenetic and geographical contexts.

MATERIAL AND METHODS

Data Composition

In this study, the mt-CO1 gene fragment sequences were amplified belonging to the final hosts of *F. hepatica* were used to create the dataset. The sequences had been submitted to the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) until March 24th, 2019. As a consequence of the sequence information search, totally 478 sequences were obtained. However, after trimming from both ends of the sequences, we obtained 377-bp-long sequences. Sequences shorter than 377 bp were omitted, and finally, 319 sequences which included cattle (n=242), sheep (n=46), goat (n=5), donkey (n=8), bison (n=7), buffalo (n=7), human (n=2) and camel (n=2) sequences were used for analyses.

Data and Phylogenetic analysis

Each mt-CO1 sequences were taken from GenBank and moved to the MEGA X programme (Kumar et al., 2018). ClustalW was used to examine the sequences of diverse sizes and harvest results using a number of output setups like FASTA suitable for following examination. All aligned sequences were examined, and 377-bp-long sequences were selected, comprising the mt-CO1 gene fragment without indel complications. A total of 319 sequences were downloaded then all of them trimmed from both ends, and the sizes of the DNA sequences were matched. Subsequently, the sequences were examined in the MEGA X by using Akaike Information Criterion (AIC) and Bayesian Information Criterion analysis, the most appropriate base alterations were found, and the phylogenetic tree was constructed with the Tamura-Nei, Gamma distribution (TN93+G) models (Kumar et al., 2018). Statistical maintenance for exact clades was acquired via 1000 bootstrap repeats of the *F. gigantica* sequences (AB385622, MF287791, MG987198).

Haplotype Network and Population Genetics Analysis

All the sequence information were moved to DnaSP 6 (Rozas et al., 2017). The population varie-

ty guides (numbers of haplotypes (h), haplotype (Hd) and nucleotide varieties (π)), the neutrality indices (Tajima's D (Tajima, 1989) and Fu's statistics (Fu, 1997)), Fu and Li's D and F tests (Fu and Li, 1993), and to guess the grade of population diversity, the pairwise fixation index (Fst) (Salzburger et al., 2011), were measured by Dna SP 6 (Rozas et al., 2017). It was used to produce results using a series of output formats, such as NEXUS, which permits the user to improve extra info for the following analysis. NEXUS files in a Traits blocks were included the environmental, phenotypic or other characters correlated with the sequences (Maddisonet al., 1997). Following, the minimum spanning networks (MSN) were used for the subsequent networks, which comprises all the sides noticeable in the lowest distance tree of arrays, through the PopART-1.7 (Bandelt et al., 1999) (http://popart.otago.ac.nz).

RESULTS

In the currentwork, a data set of the mt-CO1 sequences of the adult *F. hepatica* isolates obtained NCBI was used for bioinformatics. After alignment of all the sequences, 377-bp-long sequences were selected for further analyses. Finally, a total of 319 sequences which included cattle (n=242), sheep (n=46), goat (n=5), donkey (n=8), bison (n=7), buffalo (n=7), human (n=2) and camel (n=2) sequences were obtained. The existence of 72 haplotype groups was revealed according to the haplotype network calculated in all sequences (Supplemental Table 1). The entire list of isolates and their geographical and host dispersions are shown in Table 1.

Table 1. *Fasciola hepatica* isolates derived from final hosts used in this study

Host/Geographical origin	No. of mt-CO1 Isolates	Accession number
CATTLE		
Uruguay	1	AB207170
Australia	1	AB207103
Brazil	75	MK838613-MK838687
Iran	56	MK447938- MK447941, MK447946, MK447948- MK447951, MK447956-MK447958, MK447961,MK447962, MK447965, MK447966, MK447968, MK447973, MK447974, MK447981- MK447988, GQ398051, GQ398053, GQ398056, MG987177- MG987180, MG987184, MG987185, MG987190-MG987192 KF992216, KX712312, KX712313, KX063832, KX036352, KX021290- KX021297, KX021273, KX021274, KX021278, KX021279
Algeria	20	LC485089- LC485108
Ecuador	89	LC273025- LC273113
SHEEP		
Iran	34	MG987176, MG987175, GQ398052, GQ398054, GQ398055, KF992218, KF992217, MG987181-MG987183, MG987186-MG987189, MG987193, MK447942-MK447945, MK447952-MK447955, MK447959, MK447960, MK447963, MK447969, MK447970, MK447975-MK447980
UK	2	KR422388, KR422383
Egypt	10	AB553812- AB553821
DONKEY		
Iran	8	MF537583-MF537590
BISON		
Poland	7	KR422380-KR422387
BUFFALO		
Egypt	7	AB553810, AB553811, AB553822-AB553826
GOAT		
Iran	5	MK447990, MK447989, MK447972, KF992220, KF992219
CAMEL		
Iran	2	FJ895606, FJ895605
HUMAN		
Iran	2	MK447991, FJ895604

Table 2. Diversity and neutrality indices for *Fasciola hepatica* isolates from various geographical regions using nucleotide data of the cytochrome oxidase subunit 1 (CO1) mitochondrial gene (377 bp)

Geographical origin	n	H	hd ± SD	πd ± SD	Tajima's D	p value	Fu's Fs	p value	FLD	p value	FLF	p value
Iran	107	35	0,819±0,033	0,00606±0,00077	-2,36077	P < 0.01	-32,711	0,000	-5,3585	P < 0.02	-4,9597	P<0.02
Ecuador	89	9	0,628±0,041	0,00241±0,00036	-1,66540	0.10 > P > 0.05	-3,604	0,018	-1,5955	P > 0.10	-1,9250	0.10 > P > 0.05
Brazil	75	10	0,475±0,070	0,00212±0,00049	-1,86913	P < 0.05	-5,841	0,002	-1,4887	P > 0.10	-1,922	0.10 > P > 0.05
Algeria	20	16	0,947±0,044	0,00623±0,00103	-2,12423	P < 0.05	-15,182	0,000	-2,3317	0.10 > P > 0.05	-2,6391	P < 0.05
Egypt	17	11	0,949±0,033	0,01240±0,00199	-0,47709	P > 0.10	-2,792	0,040	-0,6410	P > 0.10	-0,6870	P>0.10
Poland	7	6	0,952±0,096	0,00909±0,00143	0,26266	P > 0.10	-1,929	0,109	-0,0757	P > 0.10	0,0000	P>0.10

n: Number of isolates, *h*n: number of haplotypes; *h*d: haplotype diversity; *π*d: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F test statistic.

Table 3. Diversity and neutrality indices for *Fasciola hepatica* isolates from various hosts using nucleotide data of the cytochrome oxidase subunit 1 (CO1) mitochondrial gene (377 bp)

Host	n	H	hd ± SD	πd ± SD	Tajima's D	p value	Fu's Fs	p value	FLD	p value	FLF	p value
Cattle	242	51	0,765±0,027	0,00659±0,00043	-2,19006	P < 0.01	-51,219	0,000	-8,00561	P < 0.02	-6,45927	P < 0.02
Sheep	46	24	0,905±0,034	0,00980±0,00170	-2,00717	P < 0.05	-14,365	0,000	-3,11166	P < 0.05	-3,23583	P < 0.05
Donkey	8	3	0,607±0,164	0,00341±0,00158	-0,72673	P > 0.10	0,671	0,336	-0,92081	P > 0.10	-0,96287	P > 0.10
Bison	7	6	0,952±0,096	0,00909±0,00143	0,26266	P > 0.10	-1,929	0,109	-0,07573	P > 0.10	0,00000	-
Buffalo	7	4	0,810±0,130	0,00531±0,00182	-0,93141	P > 0.10	-0,132	0,292	-1,09691	P > 0.10	-1,15490	P > 0.10
Goat	5	2	0,600±0,175	0,00637±0,00186	1,64070	P > 0.10	3,022	0,232	-7,89062	P < 0.02	-6,22762	P < 0.02

n: Number of isolates, *h*n: number of haplotypes; *h*d: haplotype diversity; *π*d: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F test statistic.

Country-based nucleotide polymorphism, diversity and neutrality indices and haplotype networks of *Fasciola hepatica*

The diversity and neutrality indices for mt-CO1 sequences (377 bp) of *F. hepatica* isolates from Iran, Ecuador, Brazil, Algeria, Egypt and Poland are given in Table 2. The Poland isolates of *F. hepatica* demonstrated the maximum haplotype differences among the countries, followed by the Egypt, Algeria, Iran, Ecuador and Brazil isolates. Tajima's D and Fu's Fs were meaningfully negative for almost all of the isolates in the zones. In Iranian isolates the Fu's Fs values were at least 2, 6, 10 and 15-fold that were seen in the Algeria, Brazil, Ecuador and Egypt isolates, respectively.This was an indicator of the existence of matchless single haplotype features in the Iranian isolates of *F. hepatica*.

The most polymorphic sites (n=42) containing 31% (13/42) parsimony informative sites were determined in the Iranian isolates. The mt-CO1 database involved of 35 haplotypes, 80% (28/35) of which were geographically unique. However, a main haplotype consisting of 39.2% (42/107) of total isolates was formed. As the second main haplotype, there was a haplotype that covered 15% (16/107) of all isolates.

The Algeria isolates showed 19 of polymorphic areas comprising 26.3% (5/19) parsimony useful spots. It was observed that 93.7% (15/16) of the data network consisted of 16 geologically distinctive haplotypes, and 25% (5/20) of the all isolates were resided in a single multiple haplotype site. Moreover, the Egypt isolates had 17 polymorphic regions containing 58.8% (10/17) parsimony informative sites. Besides, 54.5% (6/11) of the data network involved of 16 geographically sole haplotypes.

Furthermore, 12 polymorphic points containing 58.3% (7/12) parsimony informative sites were determined in both the Ecuador and Brazil isolates. It was observed that 44.4% (4/9) of the data network consisted of nine geographically distinctive haplotypes, 54% (48/89) of all isolates were positioned in a central haplotype area, and a second main haplotype area was covered by 39.3% (35/89) of the total isolates in Ecuador, while 50% (5/10) of the data network consisted of 10 geographically matchless haplotypes, and 72% (54/75) of the total isolates were resided in a primary haplotype dot in the Brazil samples. Eight polymorphic points containing 50% (4/8) parsimony

informative sites were detected in the Poland samples with the least among all the analysed haplotypes. 80% (5/6) of the haplotypes contained of six geographically sole haplotypes.

Nucleotide polymorphism, diversity and neutrality indices and haplotype networks of *Fasciola hepatica* based on definitive host

The diversity and neutrality indices for the mt-CO1 sequences (377 bp) of *F. hepatica* from the cattle, sheep, donkey, bison, buffalo, goat, camel and human isolates are shown in Table 3. The bison isolates of *F. hepatica* represented the maximum haplotype differences, followed by the sheep, buffalo, cattle, goat and donkey isolates. Tajima's D and Fu's Fs were negative for almost all the isolates in the countries. The highest negative value was determined for the cattle isolates of Fu's Fs, followed by sheep, which was a consideration of the existence of sole singleton haplotype properties to the cattle isolates. The *F. hepatica* isolates used in this study initiated from varied hosts with bison, buffalo, goat, donkey, camel and human hosts.

When haplotype analysis was performed among the cattle, sheep, donkey, bison, buffalo and goat isolates, 50 polymorphic sites were determined, of which 30% (15/50) were parsimony informative in the cattle isolates. The network had 51 haplotypes settled within a star-like shape around a central haplotype, discreted from the other haplotypes by 1-12 mutational facts which comprised 45.9% (111/242) of the total isolates. This main haplotype covered 54 (48.6%) of the cattle isolates of Brazil. Secondly, the main haplotype contained 48 (43.2%) of the cattle isolates of Ecuador, followed by Algeria (n=5), Iran (n=3) and Uruguay (n=1). It was found that the second main haplotype covered 10.7% (26/242) of the total isolates. This haplotype was located in the Ecuador (n=25) and Algeria (n=1) isolates. Thirdly, another haplotype containing 10.3% (25/242) of the total isolates was determined. This haplotype contained the cattle isolates of Iran (n=19), Brazil (n=3), Ecuador (n=2) and Algeria (n=1). There were 76.5% (39/51) unique singleton haplotypes within the mt-CO1 haplotype network with the highest reported from Iran (n=19), followed by Algeria (n=12), Ecuador (n=4) and Brazil (n=4).

***Fasciola hepatica* populations: for geographical regions, definitive host diversity and neutrality indices**

A total of 319 mt-CO1 sequences (377 bp) of the *F. hepatica* isolates from the NCBI database from Uruguay, Australia, Brazil, Iran, Algeria, Ecuador, UK, Poland and Egypt were used to define the haplotype and molecular relations. The hosts of these isolates were determined as cattle, sheep, donkey, bison, buffalo, goat, camel and human (Table 1). In total, we identified 72 polymorphic sites for the mt-CO1 sequences, and 38.9% (28/72) of them were parsimony informative. Great haplotype and low nucleotide varieties were determined in the examined gene piece (Table 4). Tajima's D value was detected minus for the sequences signifying population expansion and/or purifying selection. The highly negative Fu's Fs values identified for the sequences pointed out the existence of uncommon haplotypes forecasted from a final population expansion. The presence of matchless sole haplotypes for the mt-CO1 (53/72) sequences of *F. hepatica* could be inferred from the formation of the haplotype networks.

Haplotype networks

The mt-CO1 of the *F. hepatica* haplotype network had 72 haplotypes arranged within a star-like formation with a central haplotype which covered 37.3% (Hap01: 119/319) of the whole isolates (Supplemental Table 1). This main haplotype contained 45.4% (54/119) from Brazil, 40.3% (48/119) from Ecuador, 6.8% (8/119) from Iran, 1.7% (2/119) from Egypt, 0.8% (1/119) from Uruguay and 0.8% (1/119) from Poland. Moreover, the second main haplotype included 16.6% (Hap09: 53/319) of the total isolates. 79.2% (42/53) isolates belonging to this haplotype were from Iran, 5.7% (3/53) were from Brazil, 5.7% (3/53) were from Egypt, 3.8% (2/53) were from Ecuador, 3.8% (2/53) were from Poland, and 1.9% (1/53) were from Algeria. The third main haplotype included 8.1% (Hap36: 26/319) of the total isolates. This haplotype

Table 4. Diversity and neutrality indices obtained by using nucleotide data of *Fasciola hepatica* mt-CO1 gene

mtDNA	n	H	hd ± SD	πd ± SD	Tajima's D	p value	Fu's Fs	p value	FLD	p value	FLF	p value
CO1	319	72	0,824±0,018	0,00805±0,00035	-2,27578	P < 0.01	-34,252	0,000	-7,89062	P < 0.02	-6,22762	P < 0.02

n: Number of isolates, *h*n: number of haplotypes; *h*d: haplotype diversity; *π*d: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F test statistic.

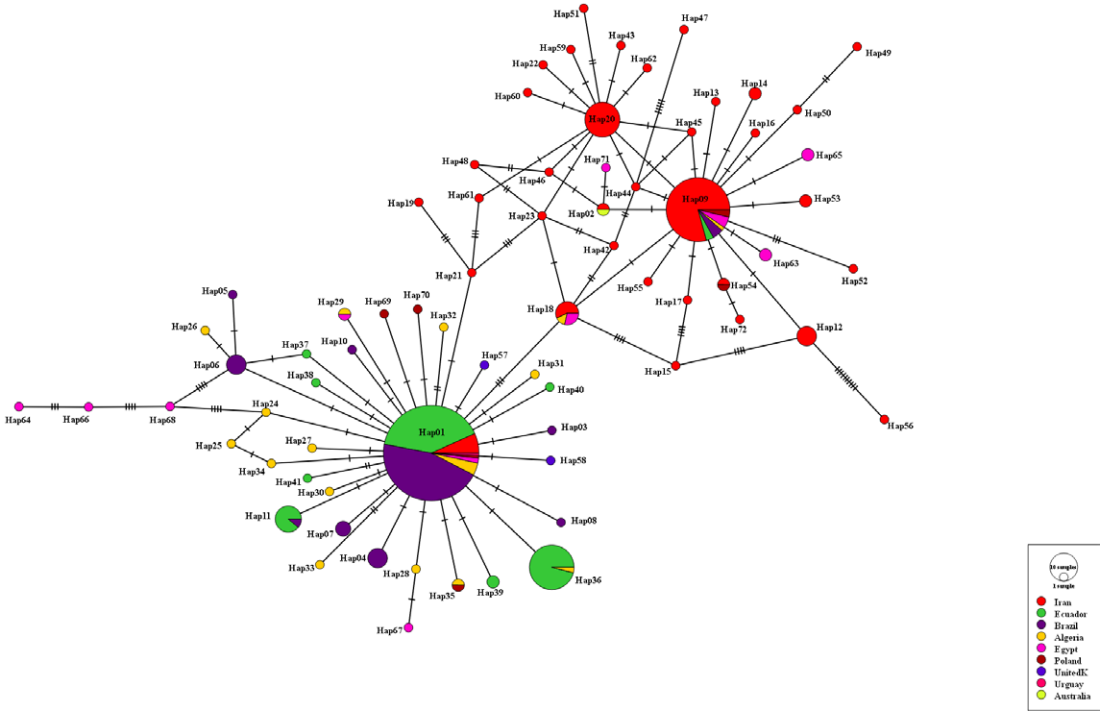

Fig.1. The haplotype network for *Fasciola hepatica* isolates compared with those from different geographic zones using the sequence data of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene. Different colors pointed out the haplotype distributions according to the country. The sizes of the circles are associated with the haplotype frequency. The number of mutations that different haplotypes are shown by screening marks.

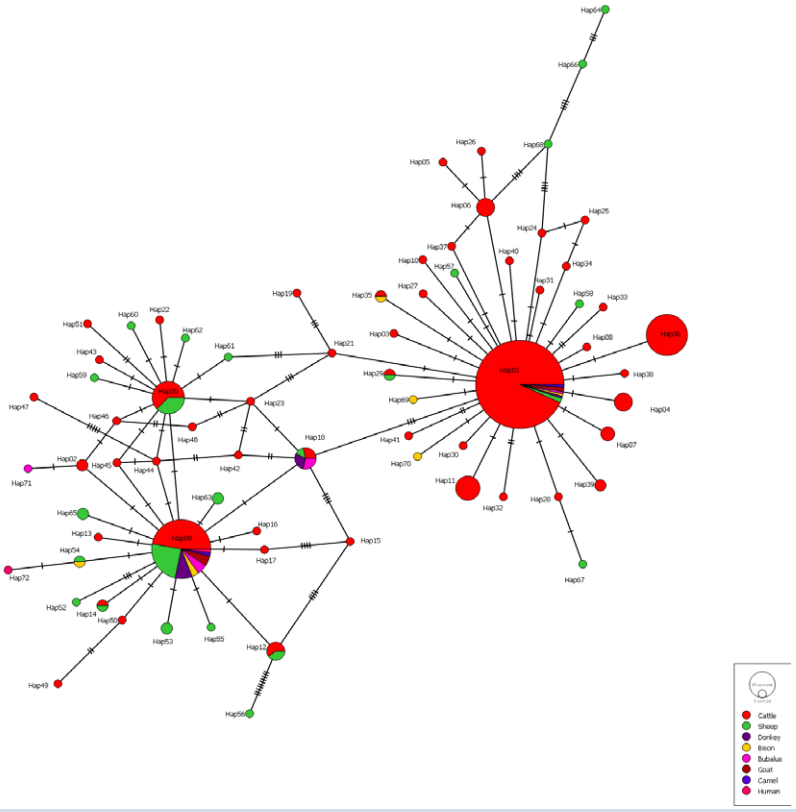

Fig.2. The haplotype network for *Fasciola hepatica* isolates compared with those from varied hosts using the sequence data of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene. Different colors indicated the haplotype distributions. The size of the circles is related to the haplotype frequency. The number of mutations that different haplotypes are shown by screening marks.

consisted of isolated by 3.8% (1/26) from Algeria and 96.1% (25/26) from Iran. Finally, the fourth multiple haplotypes contained 9.7% (Hap20: 16/319) of the total isolates. All of the isolates in this haplotype were of Iranian origin. In the mt-CO1 haplotype network analysed in this study, there were 73.6% (53/72) sole haplotypes, and this ratio was the highest for the Iran (n=26), followed by the Algeria (n=10), Egypt (n=5), Brazil (n=4), Ecuador (n=4), UK (n=2) and Poland (n=2) isolates (Fig.1).

We grouped the same sequences based on their final host, and the haplotype analysis results are shown in Fig.2. In this case, it constituted 37.3% (Hap01: 119/319) of the total sequences resided in the main haplotype. 93.2% (111/119) of this main haplotype contained cattle isolates, 1.7% (2/119) were from sheep, 0.8% (1/119) was from donkey, 0.8% (1/119) was from bison, 0.8% (1/119) was from buffalo, 1.7% (2/119) were from goat, and 0.8% (1/119) was from camel isolates. The second main haplotype covered 16.6% (Hap09: 53/319) of the total isolates. 47.1% (25/53) of the cattle isolates resided in the second main haplotype, while 24.5% (13/53) of the sheep, 9.4% (5/53) of the donkey, 3.8% (2/53) of the bison, 5.7% (3/53) of the buffalo, 5.7% (3/53) of the goat, 1.9% (1/53) of the camel and 1.9% (1/53) of the human isolates were inside the second main haplotype. Additionally, the third main haplotype consisted of 8.1% (Hap36: 26/319) of the whole isolates. All isolates in this haplotype were of cattle origin. In the mt-CO1 haplotype network analysed in this study, there were 73.6% (53/72) unique single haplotypes, and this ratio was mostly observed in the cattle (n=36) isolates, followed by the sheep (n=13), bison (n=2), buffalo (n=1) and human (n=1) isolates.

Phylogenetic tree

Since the number of *F. hepatica* sequences were very high, a reference sequence representing each haplotype was chosen, and then, a phylogenetic tree was constructed between the sequences of each host. As a result, MK447953 (Hap56-sheep-Iran) and AB553819 (Hap64-sheep-Egypt) were identified as two most distant isolates due to 19 nucleotide differences from the others. Besides, MK447953 (Hap56-sheep-Iran) affected the second uttermost branching to AB553816 (Hap66-sheep-Egypt) due to 18 nucleotide alterations. Essentially, the isolates in the Hap01 set and the isolates in the Hap09 group moulded two distinct sets. The Hap18 isolate resided at their midpoint (Fig.3) (Supplemental Table 1).

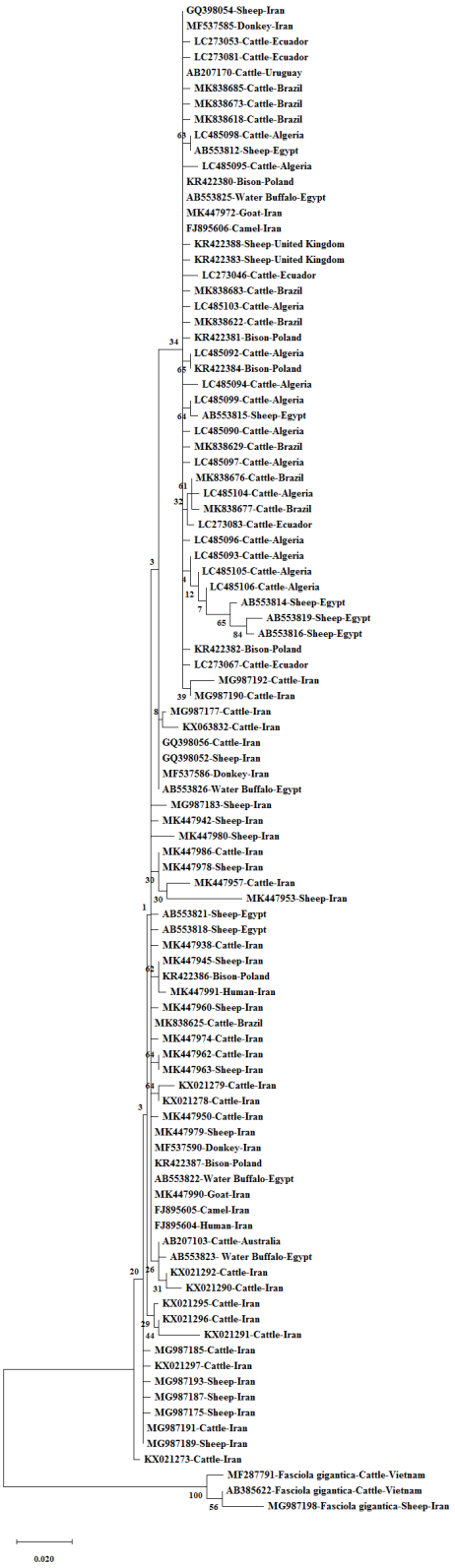

Fig. 3. Phylogenetic tree view of mt-CO1 gene (377 bp) of *F. hepatica* isolates. A reference sequence representing each haplotype was selected for establishing of the tree. MEGA X was used to construct a Maximum Likelihood tree based on the TN93+G model. The reliability of the tree was assessed by 1000 bootstrap replications. *Fasciola gigantica* sequences (AB385622, MF287791, MG987198) were used as closely related sequences

Supplemental Table 1. Grouping haplotypes of *F. hepatica* mt-CO1 sequences and accession numbers of isolates forming groups

Haplotype name	No of isolates	Accession Numbers
Hap01	119	AB207170-CATTLE-URUGUAY MK838687-CATTLE-BRAZIL MK838686-CATTLE-BRAZIL MK838684-CATTLE-BRAZIL MK838682-CATTLE-BRAZIL MK838681-CATTLE-BRAZIL MK838680-CATTLE-BRAZIL MK838679-CATTLE-BRAZIL MK838678-CATTLE-BRAZIL MK838670-CATTLE-BRAZIL MK838668-CATTLE-BRAZIL MK838667-CATTLE-BRAZIL MK838666-CATTLE-BRAZIL MK838665-CATTLE-BRAZIL MK838664-CATTLE-BRAZIL MK838663-CATTLE-BRAZIL MK838661-CATTLE-BRAZIL MK838660-CATTLE-BRAZIL MK838659-CATTLE-BRAZIL MK838658-CATTLE-BRAZIL MK838657-CATTLE-BRAZIL MK838656-CATTLE-BRAZIL MK838655-CATTLE-BRAZIL MK838654-CATTLE-BRAZIL MK838653-CATTLE-BRAZIL MK838652-CATTLE-BRAZIL MK838651-CATTLE-BRAZIL MK838650-CATTLE-BRAZIL MK838649-CATTLE-BRAZIL MK838648-CATTLE-BRAZIL MK838647-CATTLE-BRAZIL MK838645-CATTLE-BRAZIL MK838644-CATTLE-BRAZIL MK838643-CATTLE-BRAZIL MK838641-CATTLE-BRAZIL MK838640-CATTLE-BRAZIL MK838639-CATTLE-BRAZIL MK838638-CATTLE-BRAZIL MK838637-CATTLE-BRAZIL MK838636-CATTLE-BRAZIL MK838635-CATTLE-BRAZIL MK838634-CATTLE-BRAZIL MK838633-CATTLE-BRAZIL MK838632-CATTLE-BRAZIL MK838631-CATTLE-BRAZIL MK838630-CATTLE-BRAZIL MK838626-CATTLE-BRAZIL MK838624-CATTLE-BRAZIL MK838623-CATTLE-BRAZIL MK838620-CATTLE-BRAZIL MK838619-CATTLE-BRAZIL MK838616-CATTLE-BRAZIL MK838615-CATTLE-BRAZIL MK838614-CATTLE-BRAZIL MK838613-CATTLE-BRAZIL MK447982-CATTLE-IRAN MK447946-CATTLE-IRAN KF992216-CATTLE-IRAN LC485102-CATTLE-ALGERIA LC485101-CATTLE-ALGERIA LC485100-CATTLE-ALGERIA LC485091-CATTLE-ALGERIA LC485089-CATTLE-ALGERIA LC273112-CATTLE-ECUADOR LC273109-CATTLE-ECUADOR LC273108-CATTLE-ECUADOR LC273107-CATTLE-ECUADOR LC273105-CATTLE-ECUADOR LC273104-CATTLE-ECUADOR LC273103-CATTLE-ECUADOR LC273102-CATTLE-ECUADOR LC273101-CATTLE-ECUADOR LC273093-CATTLE-ECUADOR LC273092-CATTLE-ECUADOR LC273091-CATTLE-ECUADOR LC273090-CATTLE-ECUADOR LC273089-CATTLE-ECUADOR LC273088-CATTLE-ECUADOR LC273087-CATTLE-ECUADOR LC273086-CATTLE-ECUADOR LC273085-CATTLE-ECUADOR LC273084-CATTLE-ECUADOR LC273082-CATTLE-ECUADOR LC273079-CATTLE-ECUADOR LC273078-CATTLE-ECUADOR LC273077-CATTLE-ECUADOR LC273076-CATTLE-ECUADOR LC273074-CATTLE-ECUADOR LC273072-CATTLE-ECUADOR LC273070-CATTLE-ECUADOR LC273069-CATTLE-ECUADOR LC273068-CATTLE-ECUADOR LC273066-CATTLE-ECUADOR LC273065-CATTLE-ECUADOR LC273062-CATTLE-ECUADOR LC273061-CATTLE-ECUADOR LC273060-CATTLE-ECUADOR LC273054-CATTLE-ECUADOR LC273051-CATTLE-ECUADOR LC273050-CATTLE-ECUADOR LC273049-CATTLE-ECUADOR LC273039-CATTLE-ECUADOR LC273038-CATTLE-ECUADOR LC273036-CATTLE-ECUADOR LC273034-CATTLE-ECUADOR LC273033-CATTLE-ECUADOR LC273032-CATTLE-ECUADOR LC273030-CATTLE-ECUADOR LC273027-CATTLE-ECUADOR LC273026-CATTLE-ECUADOR LC273025-CATTLE-ECUADOR GQ398054-SHEEP-IRAN AB553813-SHEEP-EGYPT MF537585-DONKEY-IRAN KR422380-BISON-POLAND AB553825-WATER BUFFALO-EGYPT MK447972-GOAT-IRAN KF992220-GOAT-IRAN FJ895606-CAMEL-IRAN
Hap02	2	AB207103-CATTLE-AUSTRALIA GQ398053-CATTLE-IRAN
Hap03	1	MK838685-CATTLE-BRAZIL
Hap04	5	MK838683-CATTLE-BRAZIL MK838669-CATTLE-BRAZIL MK838662-CATTLE-BRAZIL MK838646-CATTLE-BRAZIL MK838642-CATTLE-BRAZIL
Hap05	1	MK838677-CATTLE-BRAZIL
Hap06	5	MK838676-CATTLE-BRAZIL MK838675-CATTLE-BRAZIL MK838674-CATTLE-BRAZIL MK838672-CATTLE-BRAZIL MK838671-CATTLE-BRAZIL
Hap07	3	MK838673-CATTLE-BRAZIL MK838628-CATTLE-BRAZIL MK838627-CATTLE-BRAZIL
Hap08	1	MK838629-CATTLE-BRAZIL
Hap09	53	MK838625-CATTLE-BRAZIL MK838621-CATTLE-BRAZIL MK838617-CATTLE-BRAZIL MK447988-CATTLE-IRAN MK447987-CATTLE-IRAN MK447985-CATTLE-IRAN MK447984-CATTLE-IRAN MK447983-CATTLE-IRAN MK447981-CATTLE-IRAN MK447968-CATTLE-IRAN MK447966-CATTLE-IRAN MK447965-CATTLE-IRAN MK447958-CATTLE-IRAN MK447951-CATTLE-IRAN MK447949-CATTLE-IRAN MK447948-CATTLE-IRAN MK447941-CATTLE-IRAN MK447940-CATTLE-IRAN MK447939-CATTLE-IRAN MK447961-CATTLE-IRAN GQ398051-CATTLE-IRAN MG987180-CATTLE-IRAN LC485108-CATTLE-ALGERIA LC273058-CATTLE-ECUADOR LC273055-CATTLE-ECUADOR MK447979-SHEEP-IRAN MK447977-SHEEP-IRAN MK447976-SHEEP-IRAN MK447975-SHEEP-IRAN MK447970-SHEEP-IRAN MK447959-SHEEP-IRAN MK447955-SHEEP-IRAN MK447952-SHEEP-IRAN MK447944-SHEEP-IRAN MK447943-SHEEP-IRAN GQ398055-SHEEP-IRAN KF992218-SHEEP-IRAN KF992217-SHEEP-IRAN MF537590-DONKEY-IRAN MF537589-DONKEY-IRAN MF537588-DONKEY-IRAN MF537587-DONKEY-IRAN MF537583-DONKEY-IRAN KR422387-BISON-POLAND KR422385-BISON-POLAND AB553822-WATER BUFFALO-EGYPT AB553811-WATER BUFFALO-EGYPT AB553810-WATER BUFFALO-EGYPT MK447990-GOAT-IRAN MK447989-GOAT-IRAN KF992219-GOAT-IRAN FJ895605-CAMEL-IRAN FJ895604-HUMAN-IRAN
Hap10	1	MK838622-CATTLE-BRAZIL
Hap11	9	MK838618-CATTLE-BRAZIL LC273113-CATTLE-ECUADOR LC273111-CATTLE-ECUADOR LC273110-CATTLE-ECUADOR LC273100-CATTLE-ECUADOR LC273097-CATTLE-ECUADOR LC273057-CATTLE-ECUADOR LC273056-CATTLE-ECUADOR LC273052-CATTLE-ECUADOR
Hap12	5	MK447986-CATTLE-IRAN MK447973-CATTLE-IRAN MK447956-CATTLE-IRAN MK447978-SHEEP-IRAN MK447969-SHEEP-IRAN
Hap13	1	MK447974-CATTLE-IRAN
Hap14	2	MK447962-CATTLE-IRAN MK447963-SHEEP-IRAN
Hap15	1	MK447957-CATTLE-IRAN
Hap16	1	MK447950-CATTLE-IRAN
Hap17	1	MK447938-CATTLE-IRAN
Hap18	7	GQ398056-CATTLE-IRAN LC485107-CATTLE-ALGERIA GQ398052-SHEEP-IRAN MF537586-DONKEY-IRAN MF537584-DONKEY-IRAN AB553826-WATER BUFFALO-EGYPT AB553824-WATER BUFFALO-EGYPT
Hap19	1	MG987192-CATTLE-IRAN

Hap20	16	MG987191-CATTLE-IRAN MG987184-CATTLE-IRAN MG987179-CATTLE-IRAN MG987178-CATTLE-IRAN KX712313-CATTLE-IRAN KX712312-CATTLE-IRAN KX036352-CATTLE-IRAN KX021294-CATTLE-IRAN KX021293-CATTLE-IRAN KX021274-CATTLE-IRAN MG987189-SHEEP-IRAN MG987188-SHEEP-IRAN MG987186-SHEEP-IRAN MG987182-SHEEP-IRAN MG987181-SHEEP-IRAN MG987176-SHEEP-IRAN
Hap21	1	MG987190-CATTLE-IRAN
Hap22	1	MG987185-CATTLE-IRAN
Hap23	1	MG987177-CATTLE-IRAN
Hap24	1	LC485106-CATTLE-ALGERIA
Hap25	1	LC485105-CATTLE-ALGERIA
Hap26	1	LC485104-CATTLE-ALGERIA
Hap27	1	LC485103-CATTLE-ALGERIA
Hap28	1	LC485099-CATTLE-ALGERIA
Hap29	2	LC485098-CATTLE-ALGERIA AB553812-SHEEP-EGYPT
Hap30	1	LC485097-CATTLE-ALGERIA
Hap31	1	LC485096-CATTLE-ALGERIA
Hap32	1	LC485095-CATTLE-ALGERIA
Hap33	1	LC485094-CATTLE-ALGERIA
Hap34	1	LC485093-CATTLE-ALGERIA
Hap35	2	LC485092-CATTLE-ALGERIA KR422384-BISON-POLAND
Hap36	26	LC485090-CATTLE-ALGERIA LC273106-CATTLE-ECUADOR LC273099-CATTLE-ECUADOR LC273098-CATTLE-ECUADOR LC273096-CATTLE-ECUADOR LC273095-CATTLE-ECUADOR LC273094-CATTLE-ECUADOR LC273080-CATTLE-ECUADOR LC273075-CATTLE-ECUADOR LC273073-CATTLE-ECUADOR LC273071-CATTLE-ECUADOR LC273064-CATTLE-ECUADOR LC273063-CATTLE-ECUADOR LC273059-CATTLE-ECUADOR LC273048-CATTLE-ECUADOR LC273047-CATTLE-ECUADOR LC273045-CATTLE-ECUADOR LC273044-CATTLE-ECUADOR LC273043-CATTLE-ECUADOR LC273041-CATTLE-ECUADOR LC273040-CATTLE-ECUADOR LC273037-CATTLE-ECUADOR LC273035-CATTLE-ECUADOR LC273031-CATTLE-ECUADOR LC273029-CATTLE-ECUADOR LC273028-CATTLE-ECUADOR
Hap37	1	LC273083-CATTLE-ECUADOR
Hap38	1	LC273081-CATTLE-ECUADOR
Hap39	2	LC273067-CATTLE-ECUADOR, LC273042-CATTLE-ECUADOR
Hap40	1	LC273053-CATTLE-ECUADOR
Hap41	1	LC273046-CATTLE-ECUADOR
Hap42	1	KX063832-CATTLE-IRAN
Hap43	1	KX021297-CATTLE-IRAN
Hap44	1	KX021296-CATTLE-IRAN
Hap45	1	KX021295-CATTLE-IRAN
Hap46	1	KX021292-CATTLE-IRAN
Hap47	1	KX021291-CATTLE-IRAN
Hap48	1	KX021290-CATTLE-IRAN
Hap49	1	KX021279-CATTLE-IRAN
Hap50	1	KX021278-CATTLE-IRAN
Hap51	1	KX021273-CATTLE-IRAN
Hap52	1	MK447980-SHEEP-IRAN
Hap53	2	MK447960-SHEEP-IRAN MK447954-SHEEP-IRAN
Hap54	2	MK447945-SHEEP-IRAN, KR422386-BISON-POLAND
Hap55	1	MK447942-SHEEP-IRAN
Hap56	1	MK447953-SHEEP-IRAN
Hap57	1	KR422388-SHEEP-UNITED
Hap58	1	KR422383-SHEEP-UNITED
Hap59	1	MG987193-SHEEP-IRAN
Hap60	1	MG987187-SHEEP-IRAN
Hap61	1	MG987183-SHEEP-IRAN
Hap62	1	MG987175-SHEEP-IRAN
Hap63	2	AB553821-SHEEP-EGYPT, AB553820-SHEEP-EGYPT
Hap64	1	AB553819-SHEEP-EGYPT
Hap65	2	AB553818-SHEEP-EGYPT, AB553817-SHEEP-EGYPT
Hap66	1	AB553816-SHEEP-EGYPT
Hap67	1	AB553815-SHEEP-EGYPT
Hap68	1	AB553814-SHEEP-EGYPT
Hap69	1	KR422382-BISON-POLAND
Hap70	1	KR422381-BISON-POLAND
Hap71	1	AB553823-WATER BUFFALO-EGYPT
Hap72	1	MK447991-HUMAN-IRAN

Genetic differentiation

By means of the mt-CO1 sequences, low trivial Fst values were detected when the *F. hepatica* sequences from the UK were matched in a pairwise method to those derived from Brazil (0.01020) and Algeria (0.01196), pointed out that these populations were not genetically diversed. However, a limited number of sequences (n=2) data from UK were analysed should be noticed. On the contrary, when the Iran isolates were compared in pairs to the Ecuador (0,66150) and Brazil (0,65508) samples, they had highly significant Fst values (Table 5).Utilisation of the mt-CO1 sequences, low non-significant Fst values were observed when the *F. hepatica* cattle isolates were compared in a pairwise manner to those derived from camel (-0.27881) and bison (0.00057), indicating that these populations were not genetically diversed. On the contrary, when the cattle samples were compared in pairs to the human (0.51055) and donkey (0.40734) samples, they had quite significant Fst values (Table 6).

DISCUSSION

Fasciolosis is a major economic issue for livestock farming owing to big losses and for public health due to its globally distributed zoonotic condition (Mas-Coma et al., 2009). Molecular analysis-based trainings on the phylogenetic variety of *F. hepatica* using nuclear and mt-DNA have determined genetically distinct fluke populations around the globe. After achievement of the mitochondrial genome sequences of *F. hepatica* (Leet al., 2001), many scientists (Ai et al., 2011; Dosay-Akbulut et al., 2005; Walker et al., 2011; Walker et al., 2007) have used mt-DNA as a display for inhabitants variety. mt-DNA has a great alteration rate (Blair et al., 1996) and can be used to classify alterations between two strictly related people (Semyenova et al., 2006).

This in-silico study was designed for the analysis of published *F. hepatica* mt-CO1 sequence data which were collected from different hosts and geographic areas. *Fasciola hepatica* is one of the prevalent trematode parasites, and it seems to have distributed around the globe for many years through human movement and animal transfer. With this study, the phylogenetic relationships among the mt-CO1 gene isolates of *F. hepatica* from diverse geographies were studied by in-silico analysis, and thus, on the one hand, the perpendicular spread of the extremely conserved mt-CO1 gene sequences was presented, and besides, the horizontal spread was shown geographically.

While Tajima D concentrates on ancient mutations that can reveal population actions for a long time and all sequences representing population extension and/or purifying variety, Fu's Fs value is primarily sensitive to the latter mutation, and the statistics by Fu and Li are frequently negative when new mutations are widespread. Commonly, neutrality tests such as Tajima's D, Fu's Fs have been used to test the neutral-

Table 5. Pairwise fixation index (Fst) for *Fasciola hepatica* isolates as compared to those from various geographical regions using nucleotide data of the mt-CO1 gene

Geographical origin	Iran	Ecuador	Brazil	Algeria	Egypt	Poland
Ecuador	0,66150					
Brazil	0,65508	0,00300				
Algeria	0,52819	0,03951	0,14286			
Egypt	0,12683	0,34748	0,32810	0,22665		
Poland	0,20442	0,21085	0,17514	0,08938	0,01782	
UK	0,59862	0,17391	0,01020	0,01196	0,31627	0,17391

Iran n=107, Ecuador n=89, Brazil n=75, Algeria n=20, Egypt n=17, Poland n=7, UK n= 2.

Table 6. Pairwise fixation index (Fst) for *Fasciola hepatica* isolates as compared to those from various hosts using nucleotide data of the mt-CO1 gene

Host	Cattle	Sheep	Donkey	Bison	Buffalo	Goat	Camel
Sheep	0,29112						
Donkey	0,40734	0,00539					
Bison	0,00057	0,10637	0,14286				
Buffalo	0,33488	-0,00497	-0,12195	0,10135			
Goat	0,11028	-0,1106	-0,05306	-0,10870	-0,06944		
Camel	-0,27881	-0,18350	-0,32143	-0,44444	-0,31250	-0,60000	
Human	0,51055	0,06476	0,06122	0,26923	0,06667	0,15385	0,00000

Cattle n=242, Sheep n=46, Donkey n=8, Bison n=7, Buffalo n=7, Goat n=5, Camel n= 2, Human n=2.

ity of values such as nucleotide variability and population extension (Ramos-Onsins and Rozas, 2002). Tajima's D (-2,27578) was negative, and the highest significantly negative Fu's Fs (-34,252) values were observed for all the sequences of mt-CO1, indicating the occurrence of sporadic haplotypes anticipated from a new population enlargement or hitchhiking.

It was detected that the central haplotype stated for the mt-CO1 gene region of *F. hepatica* was extensive. Truly, as a result of the in silico analysis achieved with this study, it was shown that the sequences in only seven countries which were Uruguay (n=1), Brazil (n=54), Iran (n=8), Algeria (n=5), Ecuador (n=48), Egypt (n=2) and Poland (n=1) exist in the central haplotype. At the same time, the cattle, sheep, goat, donkey, bison, buffalo, and camel isolates were in this main haplotype, which supported the situation.This showed that there were no high genetic differences among the *F. hepatica* isolates in terms of hosts in a wide geographic region.

The maximum haplotype variety in this study was recognised in the bison isolates, followed by sheep. Six haplotypes were identified in seven bison samples. On the other hand, 24 haplotypes were identified in 46 sheep sequences. However, the numbers of the isolates for the plurality of these hosts were small. Greater sample amounts of adult *F. hepatica* from final hosts are essential before we may strongly comment on these apparent variances.

Interestingly, Hap18 was the most significant link with the other main haplotypes (Hap01, Hap09 and Hap20), while the other small link was Hap21. Hap 18 was included in the sheep, cattle and donkey isolates from Iran, the cattle isolate from Algeria and the buffalo isolates from Egypt. It is suggested that this group also forms a common haplotype between countries and species. This may indicate that there may be a new haplotypic variation in the Middle East and North Africa regions for the mt-CO1 gene of *F. hepatica*.

The total haplotype and nucleotide diversities were relatively low for the isolates of the donkey hosts.These results showed that the *F. hepatica* haplotypes were not genetically distinct in donkeys. In this instance, it was not amazing that 5 out of the 8 isolates in the second main haplotype belonged to the donkey isolates of Iranian origin.

Fasciola hepatica is more widespread in sheep than cattle across countries, and the number of samples are high, while similarly, the total haplotype and nucleotide diversities in the cattle isolates were relatively lower than those in the sheep isolates. These results indicated that haplotypes belonging to the cattle isolates of *F. hepatica* were not highly diverse. This was supported by the finding that 111 out of the 119 isolates of cattle origin resided in the main haplotype. Considering that 53.9% (48/89) of the Ecuador and 72% (54/75) of the Brazil cattle isolates were accommodated in the main haplotype, South America regions had lower genetic diversity in the cattle isolates of *F. hepatica*. This was supported by the fact that they had the lowest Fst values (intergroup mean distance was 0.003 for the Brazil / Ecuador).

The total haplotype variety and nucleotide diversity were found to be relatively low for the Brazil isolates, showing that the Brazil *F. hepatica* haplotypes were not distinct. Thus, it was not amasing that 54 out of the 119 isolates that constituted the main haplotype belonged to the cattle isolates of Brazilian origin. The genetic diversity within the mt-CO1 gene found in the Brazil isolates was lower than those recorded in the other countries. Since nucleotide substitutions were rare, it was assumed that there was not enough time to create many nucleotide substitutions for the ancestral haplotypes. Additionally, the exclusive haplotypes found in the Brazil samples generally contained only one substitution compared to the more frequent haplotypes (Schwantes et al., 2020).

In addition to animal hosts, some human samples were also included in the study. Two different haplotypes were found in the human isolates. One of them was in the main haplotype group (Hap01), while the other human sequence formed a single unique haplotype (Hap72). This indicated that there may be new genetic changes in the human isolates. However, this needs more comprehensive sequence data.

According to the country-based analyses, the highest haplotype diversity was identified in the Poland isolates, followed by the Egypt, Algeria and Iran isolates. Six haplotypes were identified in 7 Poland isolates, while 11 in 17 in Egypt, 16 in 20 in Algeria and 35 haplotypes in 107 in the Iran isolates were identified. Therefore, the Poland (5 out of 6), Egypt (6 out of 11), Algeria (15 out of 16) and Iran (28 out of 35) isolates were accommodated in unique (single) haplotypes. Helminths have great genomes with the possibility of high genetic mutation, and the presence of triploidy in some populations of *F. hepatica* offer an even greater potential for genetic diversity (Fletcher et al., 2004).

CONCLUSION

F. hepatica is a quite adaptive helminth parasite and has a high evolutionary potential due to host variation and selection pressures. Because of a higher recombination and genetic variety, *F. hepatica* can more rapidly adapt to different circumstances. Its life cycle includes hermaphroditic, sexual or parthenogenic reproduction in the final host and asexual proliferation in the intermediate host. This complexity in its life cycle permits the quick spread of polymorphisms that are useful for survival and adaptation to new condition as a result of climate changes. Besides, the wide geographic range of the parasite may suggest that it is able to adapt to different climatic conditions, and this is why it may also be able to respond to climate changes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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