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Molecular phylogenetic and genetic variability of *Fasciola gigantica* in Kermanshah province, western Iran with an overview to understand haplotypes distribution in Asia and Africa

Mohammad Bagher Rokni[1], Homayoon Bashiri[2], Saber Raeghi[3], Aref Teimouri[4], Vahid Shojaeimotlagh[5], Mohammad Reza Shiee[1], Arezoo Bozorgomid[2]*

[1] Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; [2] Infectious Diseases Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran; [3] Department of Laboratory Sciences, Maragheh University of Medical Sciences, Maragheh, Iran; [4] Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran; [5] Department of Medical Surgical Nursing, Khoy University of Medical Sciences, Khoy, Iran.

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Abstract

Over the last decade, diagnostic tools to detect and differentiate *Fasciola* species have improved, but our understanding of the distribution of haplotypes and population structure of this parasite is less clear. This study was designed to survey this gap in the *F. gigantica* epidemiology in Kermanshah province, western Iran from 2015 to 2017. Sixty-eight *Fasciola* isolates were collected from slaughterhouses from this province. We evaluated the PCR-RFLP assay of the ITS1 genes for the identification of *Fasciola* species using the RsaI enzyme. After *Fasciola* species identification, the partial sequence of mitochondrial NADH dehydrogenase subunit 1 (ND1) gene of *F. gigantica* was used for subsequent construction of the phylogenetic tree and network analysis. Based on the PCR-PRFLP profile, one (6.25%) of sheep isolates and 19 (39.60%) of cattle isolates were detected as *F. gigantica*, whereas 93.75% of sheep isolates, 60.40% of cattle isolates and all of the goat isolates were *F. hepatica*. In the 20 analyzed flukes, five ND1 haplotypes were detected. Statistically significant genetic differentiation was demonstrated between the Iran population and all the other populations. Evidence is presented for the existence of two well-separated populations: African and West Asian *gigantica* flukes and East Asian *gigantica* flukes. Genetic relationships among haplotypes were associated with geographical divisions. Also, our results have heightened our knowledge about the genetic diversity of *F. gigantic*, providing the first evidence for the existence of two well-separated populations of this parasite.

Introduction

Fasciolosis is a food-borne parasitic zoonosis caused by liver fluke species of the genus *Fasciola*, *F. hepatica* and *F. gigantica*.[1,2] The *F. hepatica* is found in Europe, Africa, Asia, Oceania, and the Americas, while *F. gigantica* is endemic in tropical areas of the Old World.[1,2] Fasciolosis causes economic losses through liver condemnation, cost of therapy and reduced milk and meat.[3] Humans are accidental hosts by eating aquatic plants or drinking water contaminated with metacercariae.[4]

The accurate identification of *Fasciola* species is a crucial requirement to improve prevention and control programs especially in areas with an overlapping distribution of both species. In the past, morphological characteristics have been the foundation for trematode classification.[5] Nevertheless, molecular data have illustrated that morphological differences do not necessarily related to genetic distance.[6]

Phylogenetic studies based on both mitochondrial and nuclear DNA genes are widely used for trematodes.[7,8] To date, there are a large amount of partial NADH dehydrogenase subunit 1 (ND1) sequences for *Fasciola* species from a wide range of animals and humans in GenBank. Iran lies in the Middle East region, where both species of *Fasciola* are present.[9,10] The epidemiology and economic

***Correspondence:**

Arezoo Bozorgomid, PhD
Infectious Diseases Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
E-mail: arezoobozorgomid@yahoo.com

losses of fasciolosis were reviewed by several researchers,[11,12] however, the population structure and haplotypes circulating of this species from Iran have been scarcely investigated.

To gain a better picture of the haplotypes dispersal and association with ancestral lineages of *F. gigantica*, the genetic diversity of ND1 fragment was analyzed in the flukes present in this study and compared to other flukes from Asia and Africa.

Materials and Methods

Study area. The location of the sampling was Kermanshah province, Western Iran (34.3176°N, and 47.0869°E), located within the Zagros Chain of a moderate and mountainous climate. It is one of the major livestock husbandry and pastoralism areas of the country due to sufficient rainfall and fertile soil.

Parasites collection. A total of 68 isolates from ruminants (sheep n = 16, cattle n = 48, and goat n = 4) were collected over two years (April 2015-July 2017) during randomly repeated visits of slaughterhouses. The flukes were preserved in 70.00% ethanol and transferred to the Helminthology Laboratory, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran for molecular studies.

DNA extraction and sequencing. The posterior part of each fluke (without uterus) was used for DNA extraction using DNGTM-plus Kit (CinnaGen, Tehran, Iran) following the manufacturer's recommendations and stored at – 20.00 ˚C until use. The ITS1 region (representing the complete sequence of ITS1 and partial sequence of both 18 S and 5.8 S rDNA regions) was amplified according to Bozorgomid *et al*.[13] The PCR-RFLP was used to specifically determine the *Fasciola* species by fast digestion of ITS1 region, using RsaI enzyme.[13] To phylogenetic analysis of *F. gigantica*, the ND1 gene fragment of approximately 500 bp was amplified using primers Ita10-5´ AAGGATGTTGCTT TGTCGTGG -3' and Ita2-3´ GGAGTACGGTTACATTCACA 5'. The reaction was run at 95.00 ˚C for 5 min, followed by 35 cycles of 95.00 ˚C for 60 sec, 60.00 ˚C for 60 sec and 72.00 ˚C for 75 sec and a final extension at 72.00 ˚C for 10 min. The PCR amplicons of the mitochondrial ND1 gene were directly sequenced by Bioneer Company (Bioneer Co. Ltd., Daejeon, Korea) using the same primers.

Phylogenetic analysis. Data obtained from a sequence in this study were trimmed and edited with 35 previously published ND1 sequences from *F. gigantica* isolates in BioEdit v.7.2 software.[14] Multiple sequence alignment was done with the muscle algorithm. The maximum-likelihood phylogram (ML) was constructed in MEGA (version 6.0; Biodesign Institute, Tempe, USA)[15] using the Hasegawa-Kishino-Yano model[16] which was chosen as the best-fitting substitution model. Node support was assessed with 1000 bootstrap replicates.

Genetic differentiation and haplotype network analysis. DnaSP software (version 5.10) was used to calculate population diversity indices (haplotype diversity and nucleotide diversity), number of segregating sites, and neutrality value (Tajima's *D* test).[17] Pairwise fixation index values obtained from *Fasciola* populations were calculated using Arlequin (version 3.5.2.2).[18] The haplotype network inferred using haplotypes of ND1 regions was constructed by PopART software and median-joining algorithm.[19]

Genetic population structure. The Bayesian clustering method implemented in STRUCTURE 2.3.4 software[20] was performed to identify subpopulations of *F. gigantica*. Ten independent runs were carried out for different numbers of genetic clusters (K = 1–4), with a burn-in period of 100,000 iterations and 1,000,000 Markov chain Monte Carlo iterations, under an admixture model and independent allele frequencies. STRUCTURE HARVESTER v0.6.94 was used to select the most probable number of clusters by calculating the ΔK value. The software CLUMPAK was used to summarize and visualize the STRUCTURE outputs.[21] The ND1 sequences from other geographic sites were obtained from the NCBI GenBank: For Bangladesh, n = 11: AB894362- AB894372[22], for Nepal, n = 12: AB894337- AB894348[23], for Egypt, n = 9: AB554151-AB554159[24] and for Iran, n = 3: KX036360, KX063829, KX063830.[25]

Results

The PCR-RFLP analysis. Based on the PCR-PRFLP profile, one (6.25%) of sheep isolates and 19 (39.60%) of cattle isolates were *F. gigantica*, whereas 93.75% of sheep isolates, 60.40% of cattle isolates and all of the goat isolates were *F. hepatica* (Fig. 1).

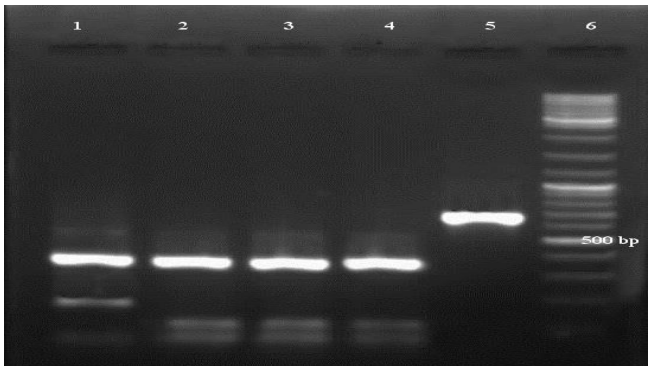

Fig. 1. The PCR-RFLP pattern of *Fasciola* after digestion with Rsa I restriction enzyme. Lane 1: *Fasciola gigantica* from cattle after digestion with Rsa I restriction enzyme; Lanes 2, 3 and 4: *Fasciola hepatica* from sheep, cattle, and sheep after digestion with Rsa I restriction enzyme, respectively; Lane 5: *Fasciola gigantica* from cattle; Lane 6: 50bp DNA ladder.

The NADH dehydrogenase subunit 1 gene analysis. Nucleotide sequences for each haplotype were deposited in GenBank under the following accession numbers:

MF428464 (one replicate), MF428465 (one replicate), MF428466 (one replicate), MF428467 (five replicates) and MF428468 (12 replicates; Fig. 2). For the 20 Kermanshah flukes (*F. gigantica*), genetic diversity indices revealed a total of 23 variable sites and five haplotypes as well as a total haplotype diversity of 0.101 and nucleotide diversity of 0.00826. In addition, Tajima's *D* test showed value of -1.44 ($p > 0.10$).

Fig. 2. Alignment of NADH dehydrogenase subunit 1 sequence of *Fasciola hepatica* (NC_002546.1) and *Fasciola gigantica* (NC_024025.1) deposited in GenBank with *Fasciola gigantica* (MF428464.1 - MF428468.1) of Kermanshah province, Western Iran.

Statistically significant genetic differentiation ($p < 0.05$) was demonstrated between the Iran population and all the other populations (Table 1). East Asia seemed to have no genetic differentiation.

Table 1. Pairwise fixation index between different *Fasciola gigantica* populations calculated from the nucleotide data set of NADH dehydrogenase subunit 1 gene.

Population	Population			
	Iran	Egypt	Nepal	Bangladesh
Iran	0.00000	-	-	-
Egypt	0.14576 *	0.00000	-	-
Nepal	0.63430 *	0.85049 *	0.00000	-
Bangladesh	0.65149 *	0.86541 *	-0.02597	0.00000

* Asterisks indicate statistically significant differences at $p < 0.05$.

The ML tree was formed with two major distinctive clusters in monophyletic clades, which was formed based on the high similarity between the sequences (Fig. 3). Although almost more the original sequences of the present study occurred within the cluster A, MF428464 and MF428464 exhibited wide variations in the composition and were associated with the East Asia sequences (Nepal and Bangladesh). The cluster C represented intermediate forms of *F. gigantica*, whereas no hybrid forms were detected in the present study.

The haplotype network inferred by using haplotypes of ND1 regions was constructed (Fig. 4). The network showed a moderately diversified topology. The network consisted of three main star-like features in the entire population with

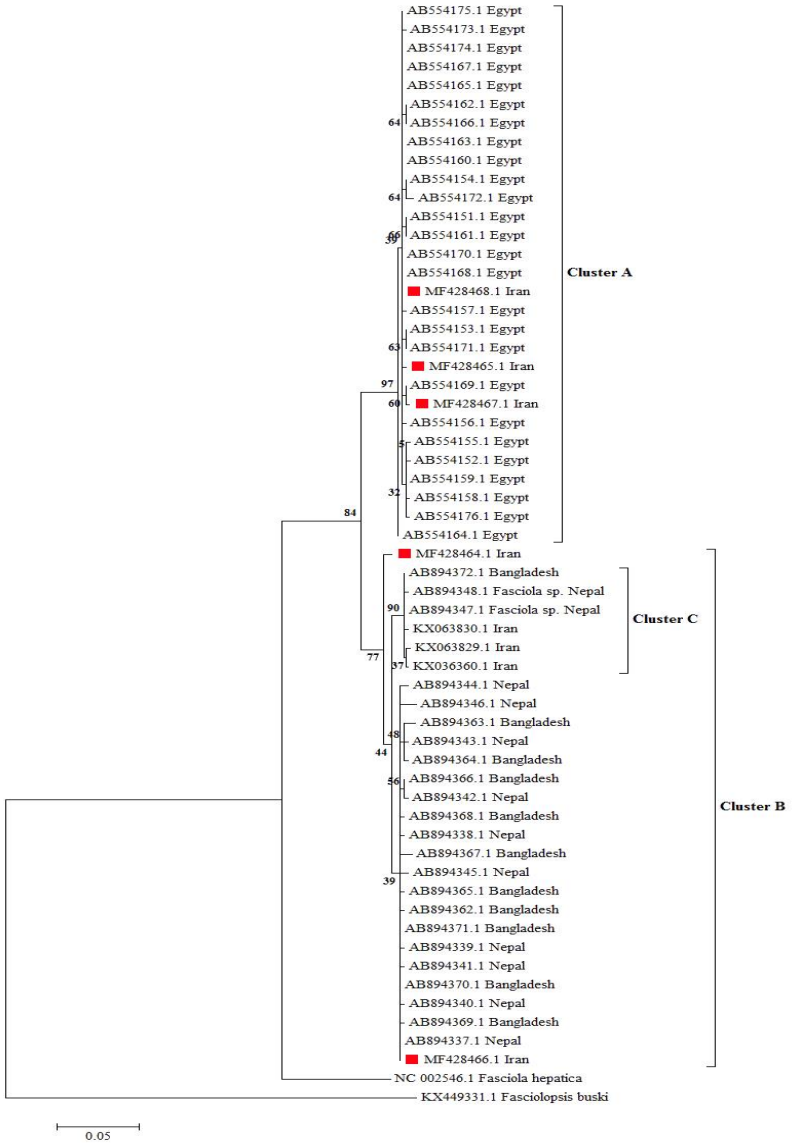

Fig. 3. Phylogenetic relationship of the haplotypes of *Fasciola gigantica* identified in this study and known haplotypes previously published in GenBank as inferred by maximum-likelihood analysis of NADH dehydrogenase subunit 1 sequence calculated by Hasegawa-Kishino-Yano model. The numbers on the branches are percent bootstrap values from 1000 replicates. Haplotypes of Kermanshah *Fasciola* flukes detected in this study are highlighted with the red square.

satellites. Forty-two ND1 haplotypes were detected including 18 in haplogroup 3 (Iran, Egypt). The haplotypes were separated by one to four mutational steps. Haplogroup 1 represented inter mediate forms of *F. gigantica.*

The results of STRUCTURE analysis using an independent allele frequency model are shown in Figure 5. This result suggested that the most appropriate population number of *F. gigantica* was 2.

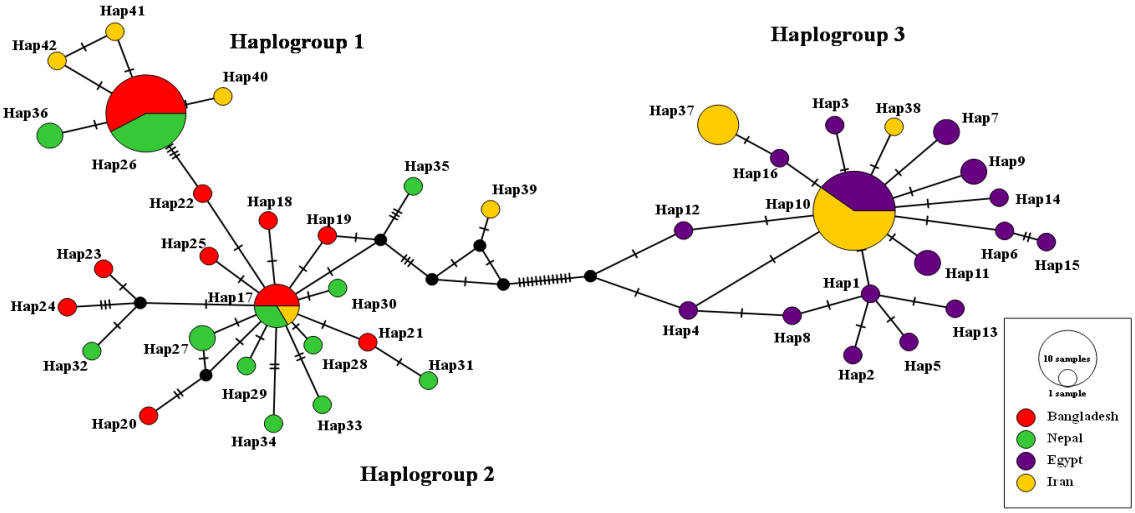

Fig. 4. Haplotype network inferred by used haplotypes of NADH dehydrogenase subunit 1 constructed by PopART software and median-joining algorithm. Each circle represents a unique haplotype, the circle size reflects frequency and colors indicate the region of origin. The mutational steps are indicated by the short marks crossing the connection lines.

Fig. 5. Population structure of 42 haplotypes of *Fasciola gigantica*, according to ΔK, the most probable number of genetic clusters is two.

Discussion

The PCR-RFLP is a suitable method for differentiation among *Fasciola* species.[26] In this study, ITS1 region was analyzed by PCR-RFLP to differentiate and identify the *Fasciola* species in Kermanshah province, Western Iran. Our results showed that the prevalence of *F. hepatica* was higher than *F. gigantica.* The *F. gigantica*, *F. hepatica*, and intermediate forms have been reported from livestock in Iran.[27] Kermanshah province is located in Western Iran dividing into three distinct portions including warm, moderate, and cold climates with suitable pastures for traditional rearing of domestic animals. Previous studies have shown that *F. hepatica* and *F. gigantica* mainly exist in moderate and tropical areas, respectively.[13] However, genetic diversity and expansion of *Fasciola* species could be influenced by the intermediate and definitive hosts and climate.[28]

In the present study, adult isolates of *F. gigantica* infecting different definitive hosts (cattle and sheep) from Kermanshah province, Western Iran were characterized by sequencing ND1 region. The ND1 gene provided reliable genetic markers to reveal genetic inter-relationships and the accurate differentiation of *Fasciola*.[29]

In our study, the nucleotide diversity of 0.00826 differences/site was lower than *F. gigantica* populations in Asia (0.01004),[23] while it was higher than *F. gigantica* populations from Bangladesh (0.00345) and Nepal (0.00366).[22,23] In general, genetic diversity was significantly lower in recently colonized areas.[30] However, the speculation is that the *F. gigantica* had originated from the East African regions and moved into Asia during the domestication of bovine and their use in agriculture.[31]

We have used previously published *F. gigantica* isolates along with the *F. gigantica* of the present study to provide more information about the genetic variation of the ND1 locus. We found five distinct haplotypes from ND1 sequences recovered from all individuals sequenced from Kermanshah province including Fg-H17, Fg-H10, Fg-H37, Fg-H38, and Fg-H39 (Fig 4). Haplogroup 2 with the dominant haplotype (Hap 17) is mainly distributed in East Asia, but also in Iran. This shows that, although the flukes from Iran were derived from three well-defined clusters, most of the flukes from Iran belonged to Haplogroup 3. The Fg-H10 haplotype has been found as the predominant form across wide geographical areas of Kermanshah shared with Egypt.

The analysis of the ND1 gene allowed us to compare and differentiate populations of *F. gigantica* from Asia and Africa regions (Fig. 3). East Asian populations are genetically distinct from West Asian and African ones. Against expectations, there was considerable genetic variation among populations of *F. gigantica* from Iran and Egypt. This may be explained by the rise of trade exchanges of livestock allowing the global range expansion of *Fasciola* from Southeast Asia to Western Asia. Nevertheless, Iran exhibits a large variety of ecological conditions that have been demonstrated to play a role in *the Fasciola* population structure from the tropical or temperate climate as well as intermediate and definitive host diversity. Increasing the number of samples in future studies could help to make hypotheses about the circulation of haplotypes from the world of different regions and the dispersal pathway of *F. gigantica*.

The present study provides evidence for the existence of two different genetic population types from hosts located in geographically and climatically different environments. We believe that natural selection may lead to the evolution of other adaptive differences between the lineages. These can result in many different biological characteristics such as different intermediate host preferences, tolerance to high or low temperatures, and sensitivity to anti-helminthic drugs, all of which could be of importance for geographical expansion and control of fasciolosis. We recommend assessed genetic inter-relationships of global *F. gigantica* populations by multilocus population genetic markers which can provide more detailed population structuring.

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Conflict of interest

The authors declare that there is no conflict of interest.

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