



## Molecular Characterization of Animal *Fasciola* Spp. Isolates from Lorestan Province, Western Iran

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### Abstract

**Background:** We aimed to detect the genetic diversity of samples identified morphologically as *Fasciola* spp. from sheep, cattle and goat from Lorestan Province, western Iran using PCR-RFLP method. Besides, we evaluated the genetic diversity indices, sequencing and phylogenetic analysis using mitochondrial gene (*ND1* and *CO1*).

**Methods:** PCR-RFLP analysis of ribosomal *ITS1* fragment by *RvaI* restriction enzyme to investigate the genetic characteristics of *Fasciola* species obtained from different hosts (18 sheep, 21 cattle, and 17 goats) was conducted. The samples were sequenced. Sequences were evaluated using BLAST software and the parasite species were identified with similarity percentage and overlap with the species registered in the gene bank. Then similarity and diversity of intra-species and inter-species diversity of *Fasciola* species were calculated.

**Results:** In Lorestan, based on RFLP pattern, 93% (52) of the *Fasciola* spp. isolates had a RFLP pattern related to *F. hepatica* and 7% (4) were *F. gigantica*. No hybrid forms were detected. The *CO1* gene could clarify 19 haplotypes against *ND1* gene that found 22 haplotypes among livestock. Sequencing results of the mtDNA showed intra-species identity 98.5%-100% and Intra-species-diversity: 0-1.5% compared to the GenBank sequences.

**Conclusion:** Using PCR-RFLP method, two species of *F. hepatica* and *F. gigantica*, were present in Lorestan Province, but *F. hepatica* was more prevalent. Mitochondrial genes could better test variability indices in different hosts than ribosomal genes, consequently among mitochondrial genes, the *ND1* gene could better examine differences and similarities than *CO1*.

**Keywords:** Fascioliasis; *Fasciola*; Polymerase chain reaction; Iran

## Introduction

Fascioliasis as a neglected zoonosis is a well-known veterinary and public health problem. This disease in livestock and humans is caused by two

liver fluke parasites: *Fasciola hepatica* and *F. gigantica* (1, 2).



It is estimated that 2.4 million, up to 17 million people suffer from fascioliasis worldwide with 180 million at-risk people. *F. hepatica* has a wide distribution in Europe, Africa, Asia, Oceania and the Americas, while *F. gigantica* restricted to Asia and Africa (3). The distributions of these two species overlap in many regions of Africa and Asia.

Due to different control strategies, the differentiation of *Fasciola* species is essential in these regions (4). Although the *Fasciola* species can generally be differentiated by morphometric methods, the morphological characteristics of adult worms are affected by different factors such as parasite's age, type of the host and intensity of infection leading to considerable taxonomic confusion and are unable to differentiation accurately (5, 6). Therefore, the accurate identification of causative species seems necessary to prevent this disease.

Nowadays a variety of molecular methods based on DNA analyses have been used for the differentiation of *Fasciola* species (7-10). PCR-RFLP assay is very rapid, simple, inexpensive, easy to perform and an appropriate method for differentiation of *F. hepatica* from *F. gigantica* (11-15).

The prevalence of fascioliasis in various livestock in Iran has been reported at 0.1% to 91.4% (16). To determination of the fascioliasis status in Iran in addition to genotypic and phylogenetic characterization of the parasite, the differentiation between causative species in different geographical regions and determine the distribution of each species is important.

Lorestan Province is considered as a high-risk area for fascioliasis due to, a neighborhood with Kermanshah Province as an endemic area, the prevailing rural lifestyle in this province, close contact of inhabitants with the livestock through animal husbandry, which is the occupation of most inhabitants in this area and the presence of snails as an intermediate host.

We aimed to identify and differentiate *Fasciola* flukes by PCR-RFLP of the *ITS1* gene in Lorestan Province, western Iran and to analyze their phylogenetic relationship with population from other parts of the world using mitochondrial *ND1* and *COX1* gene.

## Material and Methods

### Sample collection

A total of 56 confirmed infected livers to *Fasciola* species (21 cattle, 18 sheep and 17 goats) were collected from slaughterhouses of Lorestan Province, western Iran during 2016 (Fig. 1). An adult worm was isolated from each liver and washed in phosphate buffer saline (PBS) solution twice and stored in 70% ethanol for subsequent molecular methods.



**Fig. 1:** Iran map: Location of Lorestan Province in Iran

### Genomic DNA extraction and amplification

Genomic DNA was extracted from the collected flukes using DNGTM-plus Kit (CinnaGen, Iran) following manufacturer's instruction. The *ITS1* region was amplified using two set of primers (Table 1). PCR amplifications were performed in 25  $\mu$ L reactions containing 12.5  $\mu$ L Master Mix (Amplicon, Odense, Denmark), 1  $\mu$ L of each primer (10 pmol of forward and reverse primers), 5  $\mu$ L genomic DNA, and 5.5  $\mu$ L distilled water. The reactions were performed as follows: pre-denaturation at 95  $^{\circ}$ C for 5 min, 25 cycles of denaturation at 94  $^{\circ}$ C for 30 sec, annealing at 62  $^{\circ}$ C for 30 sec, elongation at 72  $^{\circ}$ C for 30 sec, followed by a final extension at 72  $^{\circ}$ C for 5 min. The PCR products were run on 2% agarose gel.

**Table 1:** Gens and sequences of the primers used in this study

<i>Gene</i>	<i>Sequence</i>
ITS1	F: 5'- TTGCGCTGATTACGTCCCTG -3' R: 5'- TTGGCTGCGCTCTTCATCGAC -3'
ND1	F: 5'-AAGGATGTTGCTTTGTCTGG-3' R: 5'-GGAGTAC GGTTACATTCACA-3'
CO1	F: 5'-ACGTTGGATCATAAGCGTGT-3' R: 5'-CCTCATCCAACATAACCTCT-3'

ITS1= Internal transcribed spacer 1, ND1= Nicotiamide adenine dinucleotide dehydrogenase subunit I, CO1= Cytochrome oxidase subunit I

### **PCR-RFLP analysis**

A PCR-RFLP method was used on PCR product of ITS1 gene (700 bp) for differentiation of *F. hepatica* and *F. gigantica*. The amplicons were subsequently digested using the RsaI restriction enzyme. By this method, two species of *Fasciola* genus will be differentiate with different bands pattern, that way *F. hepatica* will have fragment bands 28, 54, 59, 68, 104 and 367 bp, and in *F. gigantica* fragment bands 28, 54, 59, 68, 172 and 367 bp will be evident. To confirm the results of PCR-RFLP, a fragment of 700 bp from the ITS1 gene was sequenced for seven cases. Sequencing was performed in forward direction using Sanger sequencing (Macrogen Inc. Korea, ABI3730XL).

### **Genetic Diversity Indices, Sequencing and phylogenetic analysis**

The PCR products of *ND1* and *CO1* were amplified using the primers (Table 1) and sequenced by Sanger sequencing method (Macrogen Inc. Korea, ABI3730XL). Sequences were aligned using Bioedit Sequence Alignment Editor (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>), version 7.0.9 (17). Then obtained sequences were analyzed and compared with those of available in GenBank using BLAST 2.0 (<http://www.ncbi.nlm.nih.gov/>) to find out the closest entries related to *Fasciola* species. Phylogenetic analysis based on *ND1* and *CO1* sequence data were conducted by Maximum Likelihood (ML) algorithm using the Tamura 3-parameter model by sequences recovered from this study along with GenBank reference sequences using the MEGA 7.0 software (18).

Bootstrap analysis was performed using 1000 replicates. Genetic variability for mitochondrial marker was evaluated by the number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity ( $\pi$ ) and number of polymorphic sites, using DnaSP v.5.0 (19). Finally, the similarity and difference rates of sequences determined with DNA Star Laser MegAlign program (DNASTAR, Madison, WI, USA).

### **Ethics approval**

This research has been registered with the ethics code IR.TUMS.REC.1395.2404 by the Ethics Committee of Tehran University of Medical Sciences.

## **Results**

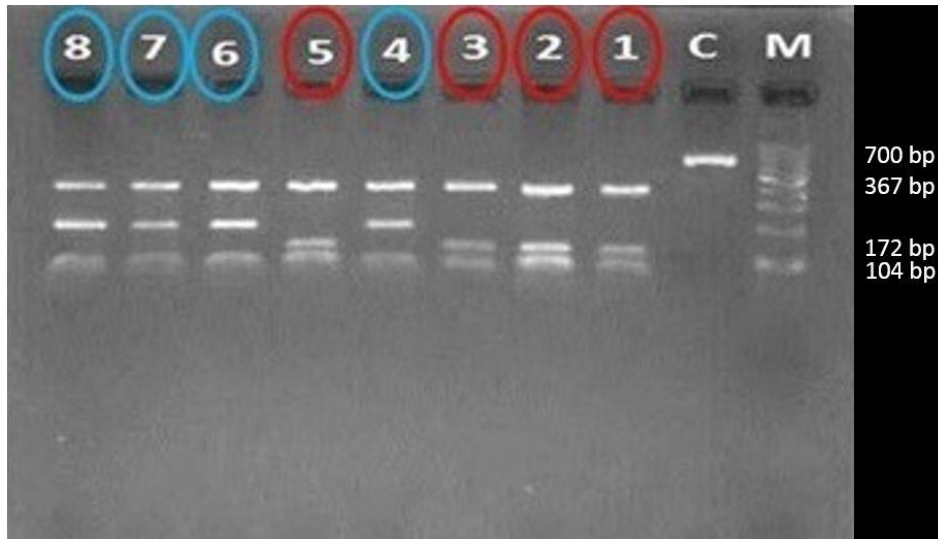
### **PCR-RFLP findings**

Based on RFLP pattern, 93% (52) of the *Fasciola* spp. isolates had a RFLP pattern related to *F. hepatica* and 7% (4) of them were *F. gigantica* (Fig. 2). *ND1* fragments (approximately 1074 bp) and *CO1* fragments (approximately 974 bp) were amplified for several specimens. Partial sequences of *CO1* and *ND1* showed 26 and 11 variable sites, respectively, and yielded eight haplotypes in both genes and high diversity indices in *ND1* gene (Table 2). The nucleotide sequences for every haplotype were deposited in GenBank under following accession numbers: KX021280-KX021299. Phylogenetic analyses based on *ND1* and *CO1* sequence data were conducted by neighbor-joining (NJ) using MEGA 7.0 with *Fascioloides magna* designated as an outgroup

showed in Figs. 2,3 and 4 respectively. Pairwise fixation index (Fst values) between different *F. hepatica* populations calculated from the nucleotide data group.

According to the haplotype analysis in *CO1* gene, the most haplotypes diversity and number of haplotypes were detected in cattle and goat, therefore the most nucleotide diversity was found in goat (0.01567). The most haplotypes diversity observed in goat and cattle,  $0.657 \pm 0.138$  and  $0.652 \pm 0.01583$  respectively. The number of polymorphic sites in cattle and goat found 7 sites, while in sheep detected 6 sites. The results of

haplotype analysis in *ND1* gene was clarified the most haplotypes diversity observed in goat ( $0.912 \pm 0.059$ ), meanwhile the least haplotypes diversity shown in cattle ( $0.588 \pm 0.135$ ). The number of polymorphic sites in cattle and goat showed 8 and 7 sites respectively, while in sheep was 19 sites. The *CO1* gene could clarified 19 haplotypes against *ND1* gene that found 22 haplotypes among livestock (Table 2). Sequencing results of the mtDNA showed intra-species identity 98. 5%-100% and Intra-species-diversity: 0-1.5% compared to the GenBank sequences.

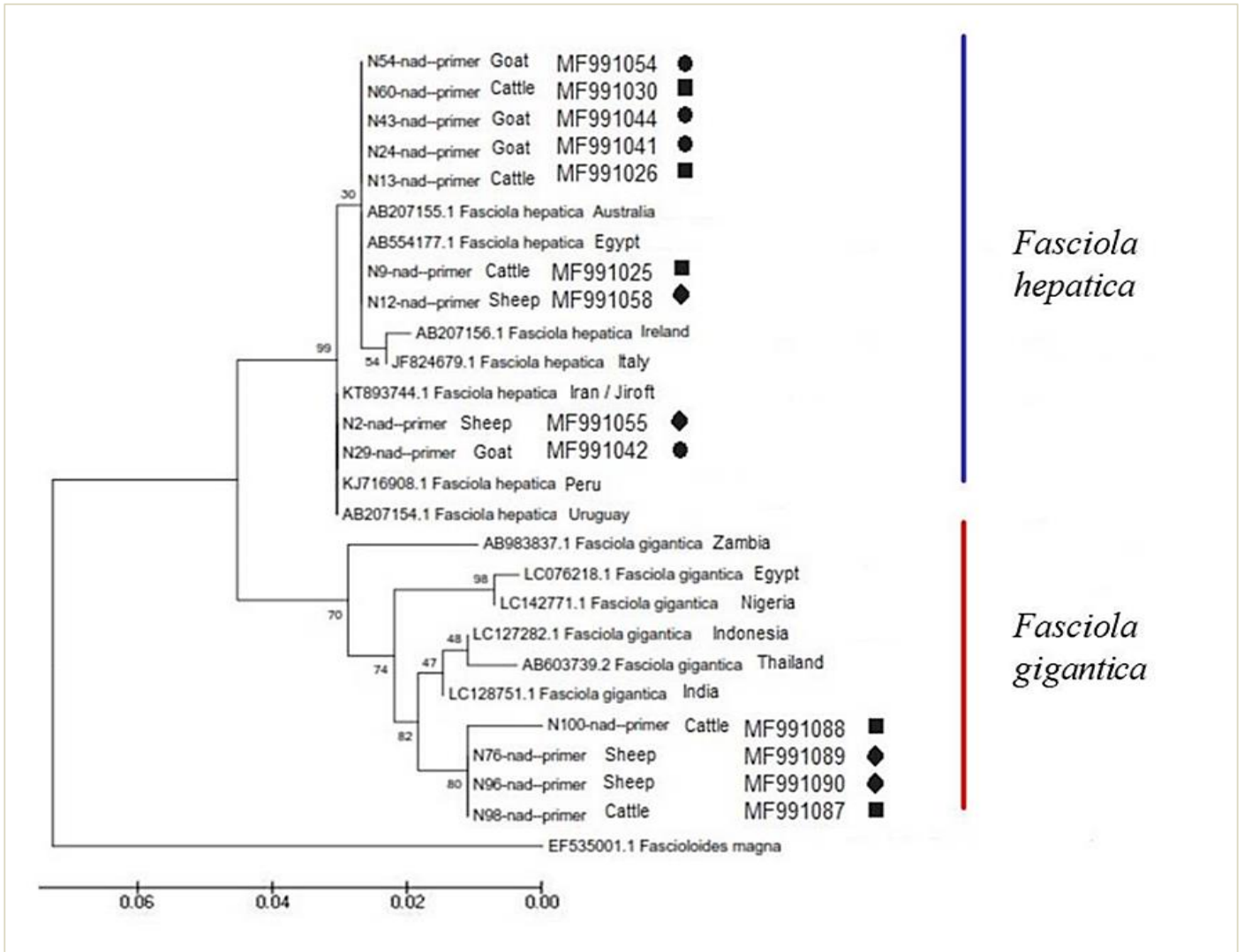


**Fig. 2:** The pattern of amplified *ITS* gene after digestion with *RsaI*: Lane M 100-bp DNA marker, Lane C: intact amplified *ITS1* gene as control, Lanes 1–3 and 5 are *F. hepatica*, Lanes 4 and 6-8 are *F. gigantica*

**Table 2:** Diversity and neutrality indices of *Fasciola hepatica* isolated from various hosts based on nucleotide sequences of the *COX1* and *ND1* genes

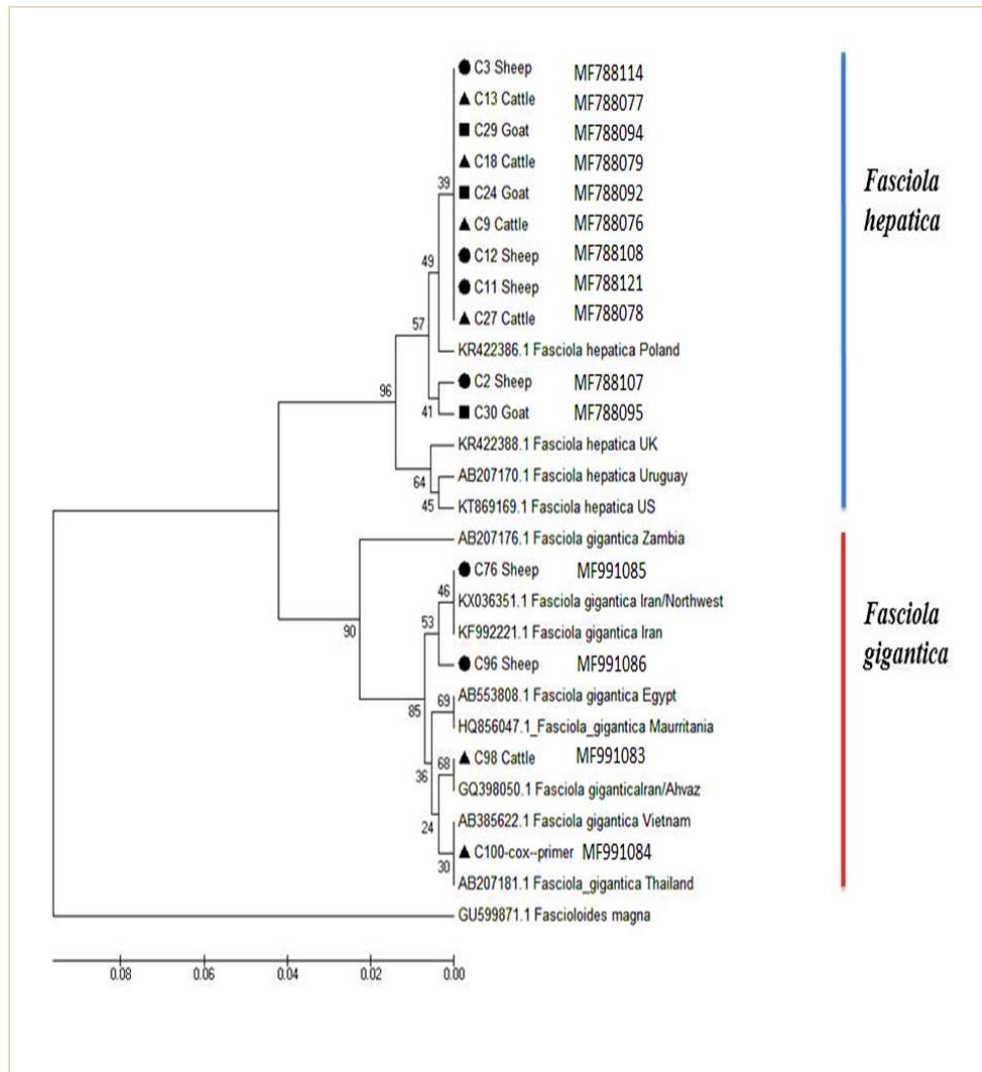
Gene	Host	n	Hn	Hd ± SD	No. of polymorphic sites	Nd (π) ± SD
<i>Cox1</i>	Cattle	17	7	$0.652 \pm 0.01583$	7	$0.00368 \pm 0.00339$
	Goat	14	7	$0.657 \pm 0.138$	7	$0.01567 \pm 0.01306$
	Sheep	16	5	$0.450 \pm 0.151$	6	$0.00286 \pm 0.00314$
<i>ND1</i>	Cattle	17	6	$0.588 \pm 0.135$	8	$0.00359 \pm 0.00250$
	Goat	14	9	$0.912 \pm 0.059$	7	$0.00486 \pm 0.00231$
	Sheep	13	7	$0.795 \pm 0.795$	19	$0.00443 \pm 0.00304$

n: number of isolates; Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity



**Fig. 3:** Phylogenetic trees based on *NDI* gene sequences of isolated *Fasciola spp.* obtained by using MEGA 7.0 (Tamura 3- parameter model) with bootstrap values of 1000 replicates set for neighbor-joining. *Fascioloides magna* was used as outgroup





**Fig. 4:**Phylogenetic trees based on *Co1* gene sequences of isolated *Fasciola Spp.* obtained by using Mega 7.0 (Tamura 3- Parameter Model) with bootstrap values of 1000 replicates Set for neighbor-joining. *Fascioloides Magna* was used as out group

## Discussion

The differentiation of *Fasciola* species and epidemiology patterns of the parasites is crucial for prevention of disease in the country. Both species of the *F. hepatica* and *F. gigantica* are reported in different parts of Iran (5, 15, 20), so accurate survey is necessary to distinguish the species in the country.

Differentiation of *Fasciola* species based on morphometric methods has some pitfalls such as morphological changes of the parasite by parasite

age, type of host or intensity of the infection (5, 6). Therefore, differentiation of the fascioliasis causative agents using molecular methods, especially in the regions where both *F. hepatica* and *F. gigantica* are present, are suitable methods to determine the dominant species in the region.

One of the most appropriate molecular methods for the differentiation of *Fasciola* spp. is PCR-RFLP (6, 12, 13, 15, 21-24). The results of this study using PCR-RFLP method on *ITS1* nuclear gene revealed that two species of *F. hepatica* and *F. gigantica* are present in Lorestan Province and

*F. hepatica* is more prevalent than *F. gigantica*. This finding is consistent with the studies carried out in other parts of Iran and the world (12, 25-29), although in some parts, *F. gigantica* have been reported as dominant species (10, 30-32). These results also confirm previous reports that *F. hepatica* is found mainly in temperate regions while *F. gigantica* mainly observed in tropical regions (11). The result of the present study showed a sheep isolate had simultaneous infection of *F. hepatica* and *F. gigantica*, aligned with foundlings in other parts of Iran and different parts of world such as Egypt, Japan, South Korea countries (33-36). The phylogenetic analysis performed in the present study based on *COI* and *ND1* gene sequences, clarified that all *F. hepatica* and *F. gigantica* isolates were placed in two branches with other specimens of different parts of the world. Our study was designed based on the *RsaI* enzyme to *ITS1* gene for differentiation of the two species. Three bands of 59, 172 and 367 bp fragments to *F. gigantica* and 59, 104 and 360bp fragments to *F. hepatica* were observed. Based on our results the enzyme could identify two species of *Fasciola* easily in the region, also the similar outcome obtained from Myanmar, Egypt and Thailand (22, 37-39). In Turkey, the enzyme *CO1* gene had no effect on *F. hepatica* (40).

According to the findings, the enzyme in ribosomal gene region, specific *ITS1* gene is exclusive, while in the mitochondrial genes may be non-specific. Therefore, it probably that PCR-RFLP on nuclear genes could be used as beneficial and effective method in rapid diagnosis or even primary detection of the *Fasciola* species. Since the host specificity, drug resistance or sensitive, differences in parasitic virulence could be manifested in molecular variations (41), in the present study, genetic indices including the number of haplotypes, haplotype diversity, nucleotide diversity and the number of polymorphic sites were surveyed for ribosomal gene (*ITS1*) and mitochondrial genes (*ND1* and *CO1*) in livestock to determine the appropriate gene as for analysis of the genetic indicators.

Mitochondrial genes compare to ribosomal genes are better to study genetic diversity indices in dif-

ferent hosts and result of our study is consistent with other studies (27, 37, 42-45). Moreover, among mitochondrial genes, *ND1* gene compared to *CO1* gene, with minor difference could be appropriate to determined genetic diversity and nucleotide mutation. This result also was consistent with the results of similar studies (35, 46, 47). Therefore, *ND1* gene can be used for inter-species and intra-species of *Fasciola* spp. genetic differences and similarities.

## Conclusion

Using PCR-RFLP method and the *ITS-1* nuclear gene, two species, *F. hepatica* and *F. gigantica*, are present in the current study. *F. hepatica* is more prevalent in Lorestan Province. The current study confirms that those mitochondrial genes can better test variability indices in different hosts than ribosomal genes, and that among mitochondrial genes, the *ND1* gene can better examine differences and similarities than *CO1*. Therefore, this gene can be used in inter-species and intra-species diversity of *Fasciola* species.

## Ethics Journalism considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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## Conflict of Interest

The authors declare there is no conflict of interest.

## References

- Mas-Coma S, Valero MA, Bargues MD (2019). Fascioliasis. In: *Digenetic Trematodes*. Ed(s): Springer, pp. 71-103.
- WHO (2013). *Sustaining the drive to overcome the global impact of neglected tropical diseases: second WHO report on neglected diseases*. ed. World Health Organization.
- Torgerson PR, de Silva NR, Fèvre EM, et al (2014). The global burden of foodborne parasitic diseases: an update. *Trends Parasitol*, 30:20-26.
- Mas-Coma S, Bargues MD, Valero M (2005). Fascioliasis and other plant-borne trematode zoonoses. *Int J Parasitol*, 35:1255-1278.
- Ashrafi K, Valero M, Panova M, Periago M, Massoud J, Mas-Coma S (2006). Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan, Iran. *Parasitol Int*, 55:249-260.
- Aryaeipour M, Bozorgomid A, Kazemi B, Behnia M, Azizi H, Rokni MB (2017). Molecular and morphometrical characterization of *Fasciola* species isolated from domestic ruminants in Ardabil Province, Northwestern Iran. *Iran J Public Health*, 46:318-325.
- Alasaad S, Soriguer RC, Abu-Madi M, et al (2011). A TaqMan real-time PCR-based assay for the identification of *Fasciola* spp. *Vet Parasitol*, 179:266-271.
- Ali H, Ai L, Song H, Ali S, Lin R, Seyni B, Issa G, Zhu X (2008). Genetic characterisation of *Fasciola* samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. *Parasitol Res*, 102:1021-1024.
- Hosseini-Safa A, Rokni MB, Mosawi SH, Heydarian P, Azizi H, Davari A, Aryaeipour M (2019). High-resolution melting analysis as an appropriate method to differentiate between *Fasciola hepatica* and *F. gigantica*. *Iran J Public Health*, 48:501-507.
- Itagaki T, Tsutsumi K-I, Ito K, Tsutsumi Y (1998). Taxonomic status of the Japanese triploid forms of *Fasciola*: comparison of mitochondrial ND1 and COI sequences with *F. hepatica* and *F. gigantica*. *J Parasitol*:445-448.
- Aryaeipour M, Rouhani S, Bandehpour M, Mirahmadi H, Kazemi B, Rokni MB (2014). Genotyping and phylogenetic analysis of *Fasciola* spp. isolated from sheep and cattle using PCR-RFLP in Ardabil province, northwestern Iran. *Iran J Public Health*, 43:1364-1371.
- Bozorgomid A, Nazari N, Rahimi H, et al (2016). Molecular characterization of animal *Fasciola* spp. isolates from Kermanshah, Western Iran. *Iran J Public Health*, 45:1315-21.
- Hasanpour H, Falak R, Naddaf SR, et al (2020). Molecular characterization of *Fasciola* spp. From some parts of Iran. *Iran J Public Health*, 49:157-166.
- Mahami-Oskouei M, Dalimi A, Forouzandeh-Moghadam M, Rokni M (2011). Molecular identification and differentiation of *Fasciola* isolates using PCR-RFLP method based on internal transcribed spacer (ITS1, 5.8 S rDNA, ITS2). *Iran J Parasitol*, 6:35-42.
- Rokni MB, Mirhendi H, Mizani A, et al (2010). Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method. *Exp Parasitol*, 124:209-213.
- Ashrafi K (2015). The status of human and animal fascioliasis in Iran: a narrative review article. *Iran J Parasitol*, 10:306-328.
- Hall TA (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. *Nucleic acids symposium series*. [London]: Information Retrieval Ltd., c1979-c2000., pp. 95-98.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*, 28:2731-39.
- Librado P, Rozas J (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25:1451-52.
- Shahnazi M, Ebadi M, Abbaspoor Z, Hajjalilo E, Javadi A, Heydarian P, Saraci M, Alizadeh S (2020). Molecular characterization of *Fasciola*



- and *Dicrocoelium* species isolated from ruminant livestock in Qazvin, Iran. *Infectious Disorders Drug Targets*, 20(5):737-742.
21. Anh DN, Anh LT, Tuan LQ, et al (2018). Identification of *Fasciola* species isolates from Nghe An province, Vietnam, based on ITS1 Sequence of ribosomal DNA using a simple PCR-RFLP method. *J Parasitol Res*, 2018: 2958026.
  22. Ichikawa M, Itagaki T (2010). Discrimination of the ITS1 types of *Fasciola* spp. based on a PCR-RFLP method. *Parasitol Res*, 106:757-761.
  23. Sedighe M, Dabirzadeh M, Rokni MB, Aryaeipour M, Shahraki MK, Azizi H (2019). Identification and Phylogenetic Classification of *Fasciola* species Isolated from Sheep and Cattle by PCR-RFLP in Zabol, in Sistan and Baluchistan Province, Southeast Iran. *Iran J Public Health*, 48:934-942.
  24. Siribat P, Dekumyoy P, Komalamisra C, Sumruayphol S, Thaenkham U (2018). Molecular identification of *Fasciola* spp. representative samples from Thailand based on PCR-RFLP. *J Trop Med Parasitol*, 41:1-7.
  25. Farjallah S, Sanna D, Amor N, et al (2009). Genetic characterization of *Fasciola hepatica* from Tunisia and Algeria based on mitochondrial and nuclear DNA sequences. *Parasitol Res*, 105:1617.
  26. Rahimi P, Ghavami M, Haniloo A, Nourian A, Biglari A (2009). Identification of *Fasciola* Species by PCR-RFLP Assay. *ZUMS Journal*, 16:41-48.
  27. Shafiei R, Sarkari B, Sadjjadi SM, Mowlavi GR, Moshfe A (2014). Molecular and morphological characterization of *Fasciola* spp. isolated from different host species in a newly emerging focus of human fascioliasis in Iran. *Vet Med Int*, 2014.
  28. Shahbazi A, Akbarimoghaddam M, Izadi S, Ghazanchaïi A, Jalali N, Bazmani A (2011). Identification and genetic variation of *Fasciola* species from Tabriz, North-Western Iran. *Iran J Parasitol*, 6:52-59.
  29. Sharifiyazdi H, Moazeni M, Rabbani F (2012). Molecular characterization of human *Fasciola* samples in Gilan province, Northern Iran on the basis of DNA sequences of ribosomal and mitochondrial DNA genes. *Comp Clin Path*, 21:889-894.
  30. Agatsuma T, Arakawa Y, Iwagami M, et al (2000). Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*. *Parasitol Int*, 49:231-238.
  31. Amor N, Farjallah S, Salem M, Lamine DM, Merella P, Said K, Slimane BB (2011). Molecular characterization of *Fasciola gigantica* from Mauritania based on mitochondrial and nuclear ribosomal DNA sequences. *Exp Parasitol*, 129:127-136.
  32. Chaudhry U, Van Paridon B, Shabbir M, Shafee M, Ashraf K, Yaqub T, Gilleard J (2016). Molecular evidence shows that the liver fluke *Fasciola gigantica* is the predominant *Fasciola* species in ruminants from Pakistan. *J Helminthol*, 90:206-213.
  33. Amer S, ElKhatam A, Zidan S, Feng Y, Xiao L (2016). Identity of *Fasciola* spp. in sheep in Egypt. *Parasites & Vectors*, 9:623.
  34. Ashrafi K, Massoud J, Holakouei K, et al (2004). Evidence suggesting that *Fasciola gigantica* might be the most prevalent causal agent of fascioliasis in northern Iran. *Iran J Public Health*, 33:31-37.
  35. Itagaki T, Kikawa M, Terasaki K, Shibahara T, Fukuda K (2005). Molecular characterization of parthenogenic *Fasciola* sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene. *J Vet Med Sci*, 67:1115-1118.
  36. Terasaki K, Moriyama-Gonda N, Noda Y (1998). Abnormal spermatogenesis in the common liver fluke (*Fasciola* sp.) from Japan and Korea. *J Vet Med Sci*, 60:1305-1309.
  37. Chaichanasak P, Ichikawa M, Sobhon P, Itagaki T (2012). Identification of *Fasciola* flukes in Thailand based on their spermatogenesis and nuclear ribosomal DNA, and their intraspecific relationships based on mitochondrial DNA. *Parasitol Int*, 61:545-549.
  38. Dar Y, Amer S, Mercier A, Courtioux B, Dreyfuss G (2012). Molecular identification of *Fasciola* spp.(digenea: Fasciolidae) in Egypt. *Parasite*, 19:177-82.
  39. Ichikawa M, Bawn S, Maw NN, et al (2011). Characterization of *Fasciola* spp. in Myanmar on the basis of spermatogenesis status and nuclear and mitochondrial DNA markers. *Parasitol Int*, 60:474-479.
  40. Simsek S, Utuk A, Balkaya I (2011). Molecular differentiation of Turkey cattle isolates of

- Fasciola hepatica* and *Fasciola gigantica*. *Helminthologia*, 48:3-7.
41. Constantine CC (2003). Importance and pitfalls of molecular analysis to parasite epidemiology. *Trends Parasitol*, 19:346-348.
  42. Ichikawa-Seki M, Ortiz P, Cabrera M, Hobán C, Itagaki T (2016). Molecular characterization and phylogenetic analysis of *Fasciola hepatica* from Peru. *Parasitol Int*, 65:171-174.
  43. Ichikawa-Seki M, Tokashiki M, Opara MN, et al (2017). Molecular characterization and phylogenetic analysis of *Fasciola gigantica* from Nigeria. *Parasitol Int*, 66:893-897.
  44. Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T, Fukuda K (2005). Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology*, 131:679-685.
  45. Reaghi S, Haghghi A, Harandi MF, Spotin A, Arzamani K, Rouhani S (2016). Molecular characterization of *Fasciola hepatica* and phylogenetic analysis based on mitochondrial (nicotinamide adenine dinucleotide dehydrogenase subunit I and cytochrome oxidase subunit I) genes from the North-East of Iran. *Vet World*, 9:1034-38.
  46. Carnevale S, Malandrini JB, Pantano ML, et al (2017). First genetic characterization of *Fasciola hepatica* in Argentina by nuclear and mitochondrial gene markers. *Vet Parasitol*, 245:34-38.
  47. Rouhani S, Raeghi S, Mirahmadi H, Harandi MF, Haghghi A, Spotin A (2017). Identification of *Fasciola* spp. in the east of Iran, based on the spermatogenesis and nuclear ribosomal DNA (ITS1) and mitochondrial (ND1) genes. *Arch Clin Infect Dis*, 12:e57283.