Original Article

Molecular Differentiation of *Fasciola* Species and Characterization of Genetic Diversity of *F. gigantica* Using NADH Dehydrogenase I (ND1) Gene in the Endemic Areas of Iran

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**Abstract**

**Background:** *Fasciola hepatica* and *F. gigantica* are the causative agents of fasciolosis in domestic animals and humans. Based on the morphometric criteria, differential diagnosis between them is problematic. In addition, intermediate forms of *Fasciola* have been found in Iran, which makes the differentiation more difficult. The aim of the present study was to provide molecular evidence for the existence of *F. gigantica* in Iran using sequencing analysis of ND1 and PCR-RFLP analysis of ITS2 regions and to study the intraspecies variations of *F. gigantica* based on mitochondrial ND1 gene polymorphism.

**Methods:** Forty *Fasciola* spp. samples collected from four distinct provinces (Fars, Khuzestan, Gilan, Khorasan Razavi) in Iran were collected for morphological and molecular characterization. In molecular method, PCR-RFLP analysis of ITS2 using *pag1* restriction enzyme was used as a screening approach for *F. gigantica* differentiation. Then mitochondrial DNA sequence variations in the ND1 gene were used for phylogenetic analysis.

**Results:** Based on the morphometric criteria and RFLP analysis, 14 parasitic samples were initially identified to be *F. gigantica*. Phylogenetic results showed that there are at least 10 different genotypes of *F. gigantica* in Iran, which are different from those existing in the GenBank. Twenty-six points out of 410 base pairs of sequenced ND1 gene in 10 varieties of *F. gigantica* were diagnosed to be polymorphic. From 26 points of polymorphism, only eight resulted in the post-translational amino acid changes in ND1 gene product structure.

**Conclusion:** Data revealed noticeable genetic diversity (up to 4.63%) between different varieties of *F. gigantica* in Iran.

**Keywords:** *Fasciola gigantica*, NADH dehydrogenase I (ND1) gene, Genetic diversity, Iran

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Introduction

Infestation with the liver flukes *Fasciola hepatica* and *Fasciola gigantica*, has traditionally been associated with substantial production and economic losses in livestock, particularly sheep and cattle (1).

Several studies have shown that *F. hepatica* occurs in temperate areas, while *F. gigantica* occurs mainly in tropical zones, and both species overlap in subtropical areas (2-4). *Fasciola hepatica* and *F. gigantica* were previously reported from a range of domestic animals (bison, cattle, sheep, and goat) and humans based on morphological and some molecular features in Iran (5, 6).

The differential diagnosis between *F. hepatica* and *F. gigantica* infection is very important because of their different transmission and epidemiological characteristics. *Fasciola hepatica* and *F. gigantica* can generally be distinguished based on their morphology (3). However, it is usually difficult to discriminate accurately between *F. hepatica* and *F. gigantica* because of the high variations in their morphological characteristics. The two species can be discriminated by DNA sequences of nuclear ribosomal internal transcribed spacer 1 (ITS1), ITS2, and 28S rDNA regions and of mitochondrial NADH dehydrogenase I (ND1) and cytochrome C oxidase I (CO1) genes (7, 8). As intraspecific genetic variations among liver flukes may reflect differences in virulence, host specificity and drug susceptibility or resistance (9), the population genetics of *Fasciola* parasites in Iran needs to be studied.

The genetic variation within *F. hepatica* sequences of the mitochondrial DNA and rosomal regions has been reported previously from Iran and several other countries (5, 6, 9-12). However, limited information is available about intraspecific genetic diversity among Iranian *F. gigantica* isolates. ITS1, ITS2, 18S rDNA and CO1 regions have been used as markers for genetic characterization of *Fasciola* flukes from Iran (9, 13, 14). In previous studies, there was little information available on the characterization of Iranian *F. gigantica* existing genotypes based on variations in NADH dehydrogenase subunit 1 (ND1) DNA sequences.

The present study was designed to molecularly differentiate *Fasciola* species and characterize genetic diversity of *F. gigantica* using ND1 gene in the endemic areas of Iran.

Materials and Methods

Parasites

*Fasciola* specimens (n=40) were collected at local abattoirs during post-slaughter inspection from livers of naturally infected sheep (n=15), cattle (n=15), buffalos (n=5) and camels (n=5) from four distinct areas in Iran including Fars (29°37’N, 52°31’E), Khorasan Razavi (36°17’N, 59°36’E), Gilan (37°16’N, 49°34’E) and Khuzestan (32°22’N 48°25’E) provinces. Figure 1 shows the Iran’s map and the geographic locations where the parasitic flukes were collected. Fresh adult worms were washed thoroughly in physiological saline, fixed in 70% ethanol and stored at -20°C until further use.

Fig. 1: The geographic locations where the parasitic flukes (*Fasciola* spp.) were collected from different hosts in Iran.

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**Morphometric examination**

Individual worms were pressed slightly between two slides and were subjected to computerized morphometric examination using computer image analysis system (Image Analyzer® version 1.34). Body length (BL), body width (BW), body area (BA) and body perimeter were measured and BL/BW ratio was calculated. The initial differentiation between *F. hepatica* and *F. gigantica* species was made based on the morphometric criteria according to standard taxonomic keys (15).

**DNA extraction and amplification**

Samples were transferred from fixative solution to sterile microtubes and left until complete evaporation of ethanol. DNA was extracted from a small part of the parasite apical zone, to avoid inclusion of female genitalia likely to include foreign sperm. DNA extraction was carried out using a commercial extraction kit (DNeasy Blood & Tissue Kit®, Qiagen, Germany) in accordance to the manufacturer’s instructions. The purity of the extracted DNA samples was evaluated using spectrophotometric method (Eppendorf® Germany). DNA samples were stored at −20°C until further use.

**Primer selection**

The mitochondrial DNA was amplified using specific primers for NADH dehydrogenase 1 (ND1) (JB11: 5'-AGA TTC GTA AGG GGC CTA ATA-3' and JB12: 5'-ACC ACT AAC TAA CTA ATTCAC TT C-3') (16, 17). The primers for amplification of the ITS2 region (5'-TCT TGA ACG CAT ATT GCG GC-3' and 5'- AGT TCA GCG GGT AAT CAC GT-3') were used as described by Gha- vami and others (11). Primers were synthesized by CinnaGen® Company (Tehran, Iran). The expected PCR amplicons are fragments of 534 bp from ND1 gene and fragments of 456 bp from ITS2 region.

**PCR**

Reactions were carried out in a final volume of 50μl containing 5μl and 4μl of genomic DNA for ND1 and ITS2 assays, respectively. Each ITS2 PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH=9.0), 1 mM MgCl₂, 200 μM dNTPs, 0.4 μM of each primer, and 2.5 U Taq DNA polymerase (Fermentas, USA). Each ND1 PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH=9.0), 1.5 mM MgCl₂, 100 μM dNTPs, 0.48 μM of each primer, and 2.6 U Taq DNA polymerase (Fermentas, USA). PCR amplification was performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 45 s (ND1) and at 60°C for 45 s (ITS2), extension at 72°C for 45 s, and a final synthesis at 72°C for 7 min, in the Mastercycler Gradient Eppendorf® Thermal Cycler (Germany). Sterile distilled water was used instead of DNA samples as blank controls.

5μl of each reaction product was subjected to electrophoresis in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) using 1% agarose gels, stained with ethidium bromide, (0.5μg/ml) and visualized on a UV transilluminator. Images were captured on a computer and printed.

**Restriction fragment length polymorphism (RFLP) analysis**

RFLP analysis for ITS2 products was performed as previously described (11). Briefly, ten microliters of PCR products were incubated with 2 μl of *papI* restriction endonuclease (ER1282, 00022771, Fermentas, USA) and 2 μl of supplied buffer at 37°C for 16 hours. The final reaction volume was adjusted to 20 μl by adding enough sterile distilled water. The digested DNA was analyzed by electrophoresis in 2% agarose gels in TAE buffer, visualized by ethidium bromide staining, and photographed. Lengths of resulting restriction fragments from *F. hepatica* and *F. gigantica* were predicted by means of the Gene Runner software v. 3.05. Based on computerized cutting, this enzyme has no cutting site on ITS2 PCR amplicons for *Fasciola hepatica* and one cutting
site for *F. gigantica*, producing fragments of 165 and 291bp.

**Sequence analysis of NDI in *F. gigantica***
The PCR products of NDI for *F. gigantica* parasites (characterized by morphological data and RFLP analysis of ITS2) were directly sequenced using capillary DNA analyzer (ABI 3730, Applied Biosystems, Foster City, CA, USA) after sequencing reactions with a Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). Forward and reverse nucleic acid sequence data were used to construct a continuous sequence of inserted DNA. Further comparison of the continuous NDI sequences was made with previously available sequences in National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) and compared with other homologues by means of neighbor joining and maximum parsimony trees reconstructed from Kimura 2-parameter distances from 1,000 bootstrap replicates of multiple sequence alignments created using CLUSTALW, as implemented by MEGA version 4.0 (18).

**Ethics considerations**
This experiment was performed under the approval of the state Committee on Animal Ethics, Shiraz University, Shiraz, Iran. In addition, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the protection of animals used for experimental purposes, were considered.

**Results**
Based on the morphometric criteria, 14 parasitic samples were initially identified to be *F. gigantica* while 26 samples were diagnosed to be *F. hepatica*. Morphometric data of the *F. gigantica* samples from different geographical locations and host origins captured in Iran are present in Table 1.

| Sample codes | Host | Locality      | Body length (BL) (mm) | Body width (BW) (mm) | BL/BW | Body area (mm²) | Body perimeter (mm) |
|--------------|------|---------------|-----------------------|----------------------|-------|----------------|---------------------|
| 1M           | Sheep | Fars          | 4.29                  | 0.87                 | 4.95  | 3.267          | 9.55                |
| 2M           | Cow   | Fars          | 4                     | 0.98                 | 4.09  | 3.32           | 9.27                |
| 3M           | Cow   | Fars          | 6.04                  | 1.37                 | 4.4   | 8.115          | 13.76               |
| 4M           | Sheep | Khorasan Razavi | 4.9                  | 0.58                | 8.47  | 4.102          | 10.91               |
| 5M           | Cow   | Khorasan Razavi | 5.27                 | 0.82                | 6.38  | 4.208          | 11.53               |
| 6M           | Sheep | Khuzestan     | 7.03                  | 1.53                | 4.6   | 10.895         | 16.16               |
| 7M           | Sheep | Gilan         | 5.43                  | 1.16                | 4.68  | 6.84           | 12.71               |
| 8M           | Buffalo | Khuzestan | 4.82                  | 0.96                | 5.03  | 5.323          | 11.08               |
| 9M           | Buffalo | Khorasan Razavi | 3.72                 | 1.02                | 3.65  | 2.99           | 8.77                |
| 10M          | Cow   | Fars          | 4.4                   | 1.3                 | 3.38  | 4.49           | 10.6                |
| 11M          | Cow   | Gilan         | 5.44                  | 1.17                | 4.65  | 6.85           | 12.72               |
| 12M          | Cow   | Khuzestan     | 4.2                   | 1.2                 | 3.5   | 3.95           | 9.14                |
| 13M          | Camel | Fars          | 5.01                  | 1.12                | 4.47  | 6.37           | 11.92               |
| 14M          | Camel | Fars          | 5.36                  | 1.05                | 5.1   | 6.45           | 12.43               |

*Table 1: Morphometric data of adult liver flukes of *Fasciola gigantica* form different geographical locations and host origins used for genotyping in Iran*
The expected PCR products of 456bp were produced in all morphologically like *F. gigantica* samples using ITS2 gene primers independently from the different geographical origins and the different host species. Restriction fragment length polymorphism (RFLP) patterns of parasitic flukes were obtained after digestion of the PCR products with *pag*I enzyme, in order to differentiate two fluke species (Fig. 2B). Based on sequence differences of amplified portions of the ITS2 gene between *F. gigantica* and *F. hepatica*, the restriction enzyme *pag*I was expected to have one cutting site on the ITS2 PCR amplicons from *F. gigantica*. Accordingly, all of the ITS2 gene PCR amplicons of 456 bp from *F. gigantica* flukes were cut by the *pag*I endonuclease to fragments of 165 and 291bp. None of ITS2 PCR products from *F. hepatica* like samples was digested by the restriction enzyme treatment (Fig. 2B). Based on RFLP analysis, all samples confirmed to be *F. gigantica* (n=14) were subjected to PCR amplification of ND1 gene. The expected PCR products of 534 bp were produced in all reactions from *F. gigantica* samples (Fig. 2A). In the current study, sequencing results of the ND1 PCR products were used to characterize the genotypic diversity of the *F. gigantica* flukes obtained from endemic areas of Iran. Comparison of partial sequencing results of ND1 gene (410 bp) with previously available sequences in NCBI using BLAST showed that 13 out of 14 studied *Fasciola* samples belonged to *F. gigantica*. Interestingly, one fluke sample (12M: KF356181) that had been classified as *F. gigantica* according to morphometric criteria and ITS2 PCR-RFLP pattern, belonged to *F. hepatica* based on ND1 nucleotide sequence. This type of *Fasciola* considered as an intermediate form of the parasite.

The phylogenetic analysis of the ND1 sequence data revealed no host and geographic specificity among genotypes (Fig. 3). Twenty-six sites out of 410 base pairs of analyzed ND1 gene in 10 isolates of *F. gigantica* were diagnosed to be polymorphic denoting noticeable genetic diversity between different isolates of the parasite in Iran. Nucleotide sequence variation among the Iranian *F. gigantica* isolates for ND1 analyzed in this study ranged from 0% to 4.63%. From 26 polymorphism sites, only eight ones were leded to the post-translational amino acid changes in ND1 gene product (Fig. 4). The nucleotide sequence data obtained in this study have been deposited in the GenBank under accession numbers KF356168 to KF356181.

![Fig. 2: Agarose gel electrophoresis; (A) ND1 PCR products from *Fasciola* samples compared with the molecular 100 bp weight marker. (B) PCR-RFLP for ITS2 products using *pag*I (*F. hep* = *F. hepatica*, Lane 4 and *F. gig* = *F. gigantica*, Lanes 2, 5 and 6), Lane 3=Blank control](http://ijpa.tums.ac.ir)
Fig. 3: The NDI phylogenetic analysis, constructed according to maximum parsimony (MP) method to show the position of Fasciola spp. isolates in sheep, camel, cow and water buffalo in various locations in Iran.
Fig. 4: Partial Alignment of the ND1 amino acid sequences for *Fasciola gigantica* (n=13 isolates) obtained from different geographical locations and host origins in Iran. Six locations of amino acid substitutions were detected.

**Discussion**

The species of genus *Fasciola* (Platyhelminthes: Digenea) are the etiological agents of hepatic damage in a wide range of vertebrate hosts worldwide (4), resulting in profound economic losses (19) and public health challenge (9, 20). The present study characterized the genetic diversity of *F. gigantica* obtained from different hosts and different geographic locations in Iran. Intraspecific genetic variations among liver flukes may reflect differences in virulence, host specificity and drug susceptibility or resistance (9). The differential diagnosis between *F. hepatica* and *F. gigantica* infection is very important because of their different transmission and epidemiological characteristics. Due to the limitations of morphological methods, various molecular approaches have been developed and used successfully for the identification and differentiation of *Fasciola* species (21). PCR analyses of ribosomal DNA genes have been widely used for molecular identification of fasciolides. Previous studies have confirmed that the first and second internal transcribed spacers (ITS1 and ITS2) of rDNA located between the nuclear small and large subunit rDNA genes can provide genetic markers for species-level identification of *Fasciola*. Several studies on species identification of *Fasciola* flukes based on ITS2 sequence as molecular marker have been conducted (10, 12, 22-25). In the current study, the application of PCR-RFLP analysis of the ITS2 sequence was successful to differentiate *Fasciola* species and results were in agreement with morphological classification of two fluke species. However, it seems that the accurate characterization of some types of intermediate form of *Fasciola* needs both mitochondrial ND1 and ribosomal ITS sequence data. The mitochondrial markers have been extensively used for phylogenetic studies and population differentiation because of its relatively rapid rate of evolution, importance in differentiation and discrimination of closely related organism, maternally inherited and does not undergo any
recombination (26). For example, NADH dehydrogenase subunit 1 gene (ND1) have been shown to be well suited for investigating the population genetics of trematodes, i.e., *F. hepatica* (27), *Clonorchis sinensis* (28) and *Opisthorchis viverrini* (29).

In the present study, mitochondrial ND1 gene was amplified using PCR from all *F. gigantica* samples and partial sequencing results were compared with previously available sequences in NCBI using BLAST and compared with other homologues by means of maximum parsimony trees. ND1 gene sequence showed a considerable genetic variability among samples obtained from the different geographical origins and the different host species in Iran. All of the isolates were classified as *F. gigantica* based on both ITS2 and ND1 gene sequences except for one sample, which categorized as *F. hepatica* based on ND1 gene sequence. The presence of intermediate forms of *F. gigantica* and *F. hepatica* has been reported from several countries. Previous studies has confirmed the existence of mixed infections of the both fluke species in the same animal, which imply that hybridization and/or introgression phenomena are possible cause of formation of intermediate forms (3, 6). Phylogenetic analysis revealed that it was impossible to identify accurately genotype using a host, suggesting that host associations are not likely to be useful characters for *Fasciola* genotype classification. For example, genotypes of A and J were reported from different hosts. Similarly, no associations were found between geographic location and phylogenetic lineage, indicating that these characters were not useful for classification of *F. gigantica*. In addition, the results of this study showed heterogeneity might also exist in one location. For example, Fars samples showed 5 different genotypes (Fig. 3). The existence of the heterogeneity in one area may be due to traditional shepherding in Iran, which includes regional movements between different pastures as well as transportation of sheep during slaughtering process.

The amino acid substitutions identified in the ND1 peptide sequence of *F. gigantica* suggests that this polymorphism may exhibit considerable functional heterogeneity in ND1, which may be of importance to the parasite biological activities. Some authors believe that the capacity of fasciolids to adapt rapidly to new definitive hosts species and environments is most probably related to the high genetic variability of this parasite (30).

The mitochondrial genetic variations among the Iranian *F. gigantica* isolates (0% to 4.63%) were higher than those previously reported for *F. hepatica* in Iran were. Formerly, Moazeni et al. (2101) showed a limited genetic variation using mitochondrial CO1 sequence (0% to 0.98%) among 19 different isolates of *F. hepatica* from cattle and sheep in different areas of Iran (5). These authors only defined four different CO1 haplotypes-based on five nucleotide substitutions- among this trematode isolates.

**Conclusion**

The number of different ND1 genotypes obtained in this study demonstrates that, the mitochondrial gene is a suitable marker to show variability within *F. gigantica* isolates. Additional studies involving more isolates and other mitochondrial polymorphic genes, such as that for cytochrome c oxidase gene (CO1), may help us to clarify genetic divergence of *F. gigantica* worldwide.

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