Tehran University of Medical Sciences Publication
http:// tums.ac.ir

Iran J Parasitol

Open access Journal at
http:// ijpa.tums.ac.ir

Iranian Society of Parasitology
http:// isp.tums.ac.ir

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

Shabnam TADAYON [1], *Hassan SHARIFIYAZDI [2], Mohammad MOAZENI [1], Mohammad Reza DIVAR [2]

1. *Dept. of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*
2. *Dept. of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

Received 04 Jun 2014
Accepted 25 Nov 2014

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

***Correspondence**
Email:
sharifiy@shirazu.ac.ir

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 *pag*I 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.

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 (buffalo, 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 ribosomal 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 ND1gene 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° until further use.

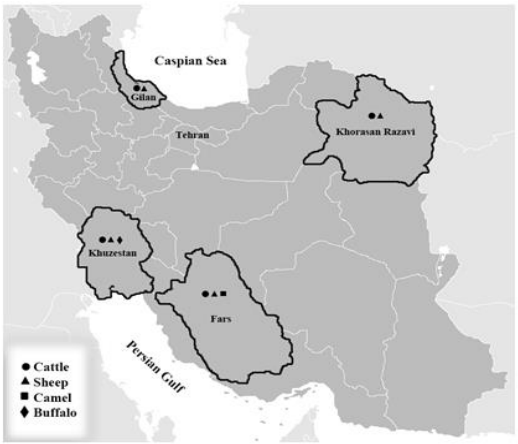

Fig. 1: The geographic locations where the parasitic flukes (*Fasciola* spp.) were collected from different hosts in Iran

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 according 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 TTT 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 Ghavami and others (11). Primers were synthetized 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 NDI and ITS2 assays, respectively. Each ITS2 PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH=9.0), 1 mM $MgCl_2$, 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 $MgCl_2$, 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 *pag*I 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.

Table 1: Morphometric data of adult liver flukes of *Fasciola gigantica* form different geographical locations and host origins used for genotyping in Iran

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	Khuzestan	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

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 leaded 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.

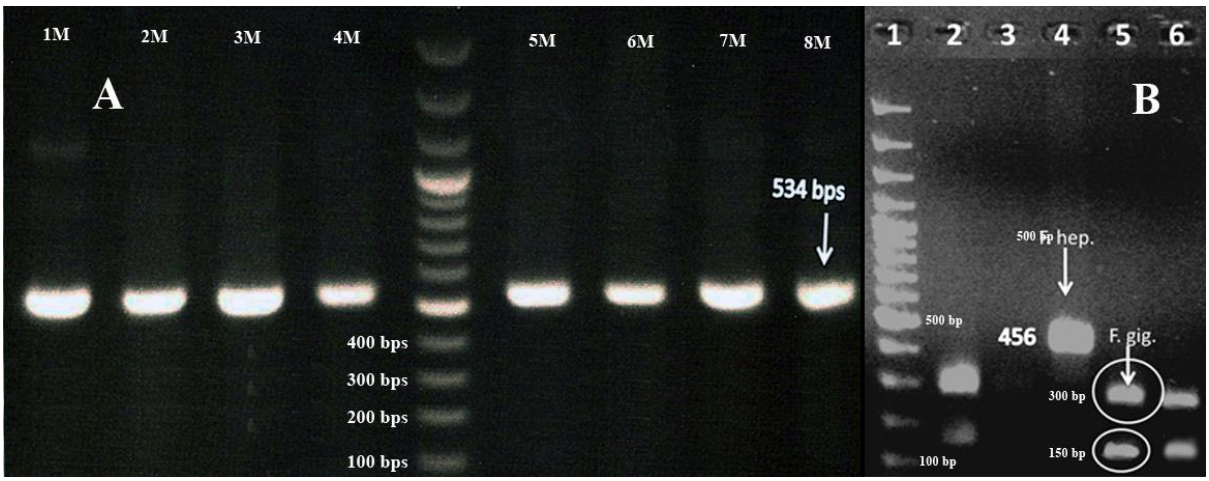

Fig. 2: Agarose gel electrophoresis; (A) NDI PCR products from *Fasciola* samples compared with the molecular 100 bp weight marker. (B) PCR-RFLP for ITS2 products using pagI *(F.hep* = *F. hepatica*, Lane 4 and *F. gig* = *F. gigantica*, Lanes 2, 5 and 6), Lane 3=Blank control

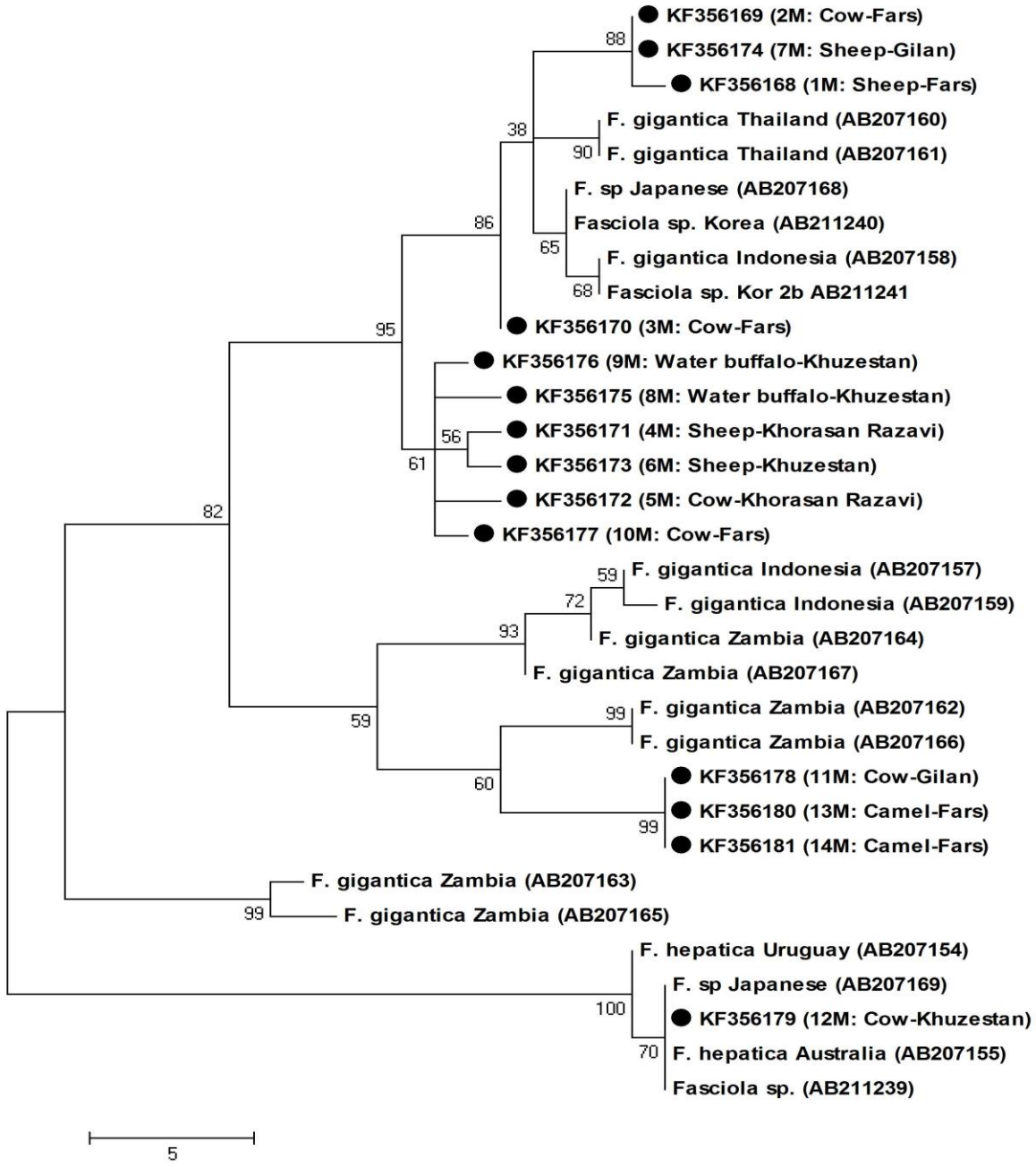

Fig. 3: The NDI phylogenetic analysis, constructed according to maximum parsimony (MP) method to show the position of *Fasciola* spp. isolates in sheep, camel, cow and water buffalo in various locations in Iran

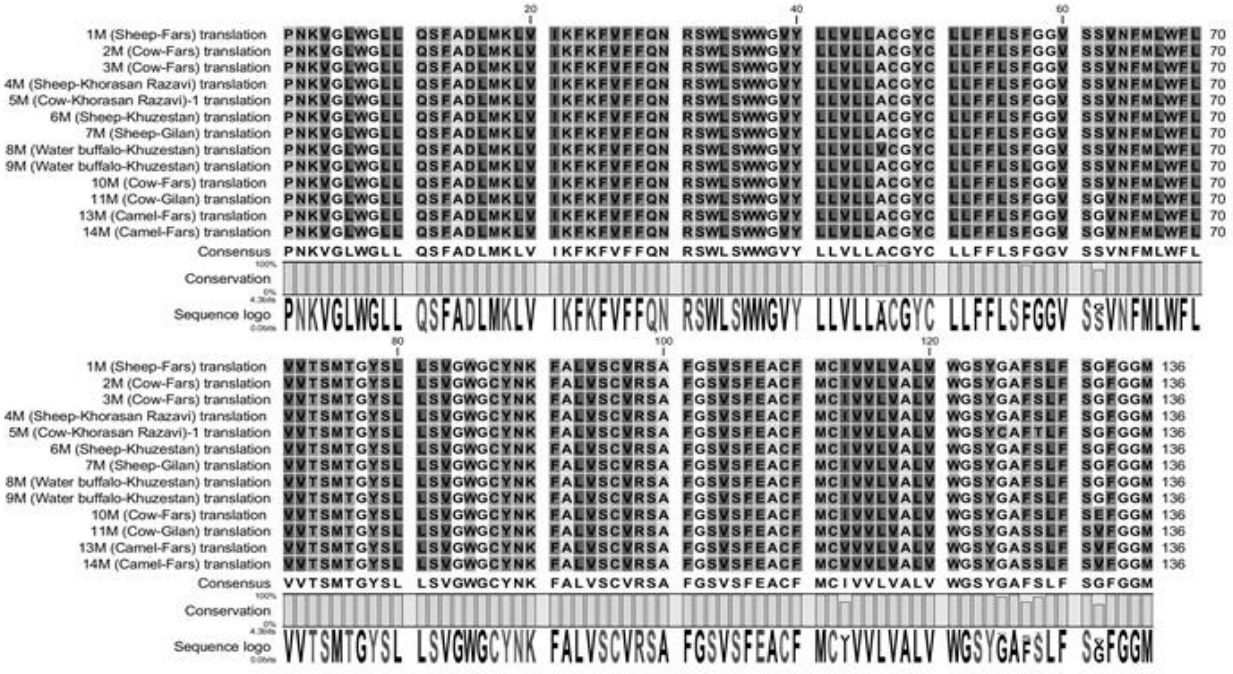

Fig. 4: Partial Alignment of the ND1 amino acid sequences for *Fasciola gigantica* (n=13 isolates) obtained from different geographical locations and host origins in Iran. Six locations of amino acid substitutions were detected

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 NDI and ribosomal ITS sequence data. The mitochondrial markers have been extensively used for phylogenetic studies and population differentiation because of its relativity 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.

Acknowledgements

This study was funded totally by Shiraz University. The authors are very grateful to Amir Moutabi- Alavi for his technical assistance. The authors declare that they have no conflicts of interest.

References

1. Mas-Coma S, Esteban J, Bargues M. Epidemiology of human fascioliasis: a review and pro-

posed new classification. Bull World Health Organ. 1999; 77 (4): 340-46.
2. Amor N, Halajian A, Farjallah S, Merella P, Said K, Ben Slimane B. Molecular characterization of *Fasciola* spp. from the endemic area of northern Iran based on nuclear ribosomal DNA sequences. Exp parasitol. 2011; 128 (3): 196-204.
3. Ashrafi K, Valero MA, Panova M, Periago MV, Massoud J, Mas-Coma S. Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan. Iran J Parasitol. 2006; 55 (4): 249-60.
4. Mas-Coma S, Valero M, Bargues M. Effects of climate change on animal and zoonotic helminthiases. Rev Sci Tech. 2008; 27 (2): 443-57.
5. Moazeni M, Sharifiyazdi H, Izadpanah A. Characterization of *Fasciola hepatica* genotypes from cattle and sheep in Iran using cytochrome C oxidase gene (CO1). Parasitol Res. 2012; 110 (6): 2379-84.
6. Sharifiyazdi H, Moazeni M, Rabbani F. Molecular characterization of human *Fasciola* samples in Gilan Province, Northern Iran based on DNA sequences of ribosomal and mitochondrial DNA genes. Comp Clin Path. 2012; 21 (5): 889-94.
7. Itagaki T, Tsutsumi K-i. Triploid form of *Fasciola* in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantica* determined by ITS-2 sequence of nuclear rDNA. Int J Parasitol. 1998; 28 (5): 777-81.
8. Marcilla A, Bargues MD, Mas-Coma S. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. Mol cell probe. 2002; 16 (5): 327-33.
9. Amer S, Dar Y, Ichikawa M, Fukuda Y, Tada C, Itagaki T, Nakai Y. Identification of *Fasciola* species isolated from Egypt based on sequence analysis of genomic (ITS1 and ITS2) and mitochondrial (NDI and COI) gene markers. Parasitol Int. 2011 60 (1): 5-12.
10. Ali H, Ai L, Song H, Ali S, Lin R, Seyni B, Issa G, Zhu XQ. Genetic characterization of *Fasciola* samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. Parasitol Res. 2008; 102 (5): 1021-4.
11. Ghavami M, Rahimi P, Haniloo A, Mosavinasab S. Genotypic and phenotypic analysis of *Fasciola* isolates. Iran J Parasitol. 2009; 4 (3): 61-70.
12. Peng M, Ichinomiya M, Ohtori M, Ichikawa M, Shibahara T, Itagaki T. Molecular characterization of *Fasciola hepatica*, *Fasciola gigantica*, and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA. Parasitol Res. 2009; 105 (3): 809-15.
13. Rokni M, Mirhendi H, Behnia M, Jalalizand N. Molecular characterization of *Fasciola hepatica* isolates by RAPD-PCR and ribosomal ITS1 sequencing. Iran Red Crescent Med J. 2010; 12 (1): 27-32.
14. Ashrafi K, Massoud J, Holakouei Nainei K, Jo-Afshani MA, Mahmoodi M, Ebadati N, Rezvani SM, Artigas P, Bargues MD, Mas-Coma S. Nuclear ribosomal DNA ITS-2 sequence characterization of *Fasciola hepatica* and Galba truncatula. Iran J Public Health. 2007; 36: 42-9.
15. Yamaguti S. *Systerma helminthum*. Vol. I. *The digenetic trematodes of vertebrates. Parts II*. Interscience Publishers, New York; 1958
16. Bowles J, Hope M, Tiu WU, Liu X, McManus DP. Nuclear and mitochondrial genetic markers highly conserved between Chinese and Philippine *Schistosoma japonicum*. Acta Trop. 1993; 55 (4): 217-29.
17. Van Herwerden L, Blair D, Agatsuma T. Multiple lineages of the mitochondrial gene NADH dehydrogenase subunit 1 (ND1) in parasitic helminths: implications for molecular evolutionary studies of facultative anaerobic eukaryotes. J Mol Evol. 2000; 51 (4): 339-52.
18. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007; 24 (8): 1596-9.
19. Spithill TW, Dalton JP. Progress in development of liver fluke vaccines. Parasitol Today. 1998; 14 (6): 224-8.
20. Haseeb AN, el-Shazly AM, Arafa MA, Morsy AT. A review on fascioliasis in Egypt. J Egypt Soci Parasitol. 2002; 32 (1): 317-54.
21. Itagaki T, Kikawa M, Terasaki K, Shibahara T, Fukuda K. 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. 2005; 67 (11) :1115-8.
22. Erensoy A, Kuk S, Ozden M. Genetic identification of *Fasciola hepatica* by ITS-2 sequence of

nuclear ribosomal DNA in Turkey. Parasitol Res. 2009; 105 (2): 407-12.
23. Huang WY, He B, Wang CR, Zhu XQ. Characterization of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. Vet Parasitol. 2004; 120 (1-2): 75-83.
24. Prasad P, Tandon V, Biswal D, Goswami L, Chatterjee A. Molecular identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on the ribosomal internal transcribed spacer regions. Parasitol Res. 2008; 103 (6): 1247-55.
25. Semyenova SK, Morozova EV, Vasilyev VA, Gorokhov VV, Moskvin AS, Movsessyan SO, Ryskov AP. Polymorphism of internal transcribed spacer 2 (ITS-2) sequences and genetic relationships between *Fasciola hepatica* and *F. gigantica*. Acta Parasitol. 2005; 50 (3): 240-3.
26. McManus DP, Thompson RC. Molecular epidemiology of cystic echinococcosis. Parasitology. 2003; 127 (S3): 7-51.
27. Semyenova SK, Morozova EV, Chrisanfova GG, Gorokhov VV, Arkhipov IA, Moskvin AS, Movsessyan SO, Ryskov AP. Genetic differentiation in eastern European and western Asian populations of the liver fluke, *Fasciola hepatica*, as revealed by mitochondrial nad1 and cox1 genes. J Parasitol. 2006; 92 (3): 525-30.
28. Park GM. Genetic comparison of liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, based on rDNA and mtDNA gene sequences. Parasitol Res. 2007; 100 (2): 351-7.
29. Thaenkham U, Nuamtanong S, Sa-nguankiat S, Yoonuan T, Touch S, Manivong K Vonghachack Y, Sato M, Waikagul J. Monophyly of *Opisthorchis viverrini* populations in the lower Mekong Basin, using mitochondrial DNA nad1 gene as the marker. Parasitol Int. 2010; 59 (2): 242-7.
30. Mas-Coma S. Epidemiology of fascioliasis in human endemic areas. J Helminth. 2005; 79 (3): 207-16.