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Identification of *Fasciola* spp. in the East of Iran, Based on the Spermatogenesis and Nuclear Ribosomal DNA (*ITS1*) and Mitochondrial (*ND1*) Genes

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Abstract

Background: Fascioliasis is a neglected zoonotic disease with worldwide distribution. Phylogenetic analysis of *Fasciola* species is critical because of their different epidemiology.
Objectives: The current study aimed at identifying *Fasciola* spp. based on spermatogenesis and phylogenetic analysis of mitochondrial (*ND1*) gene.
Methods: One hundred and fifty adults with fascioliasis were selected from the Eastern provinces of Iran, based on spermatogenetic ability, measurement criteria, and ITS (nuclear ribosomal DNA internal transcribed spacers) 1 gene restriction fragment length polymorphism (RFLP) pattern. Then, their genetic diversity indices and phylogenetic relationships were analyzed using mitochondrial DNA marker of *Fasciola* population spread in the East of Iran.
Results: Aspermic *F. gigantica* was observed in the eastern part of Iran. Partial sequences of mtDNA showed new haplotypes in both species. Pairwise fixation index between different *F. gigantica* populations calculated from the nucleotide data set of *ND1* gene were statistically significant and showed the genetic differences in pairwise population.
Conclusions: The results of the study showed that *F. hepatica*, lives in this region of Iran, had different genetic structures compared with the other *Fasciola* populations in the world. *Fasciola gigantica* present in the East of Iran had different genetic structures, compared with the other *Fasciola* population, based on genetic index.

Keywords: Spermatogenesis, Phylogenetic, *ND1*, Iran, *Fasciola* spp

1. Background

Fascioliasis is one of the most important parasitic zoonosis causing public health problems in human, and economically the main disease and infection of domestic livestock especially by the reduction in productivity due to liver disorder (1). Two different reports estimated the rate of global human fascioliasis as 2.4 to 17 million, whereas the population at risk was estimated 180 million (2).

Fascioliasis emerged as a main health problem in Guilan province, North of Iran. This province experienced 2 outbreaks of human fascioliasis in 1987 and 1997, affecting more than several thousands of people (3). Moreover, many cases of human fascioliasis were reported from other provinces of Iran (4). Animal fascioliasis is quite frequent in grazing animals generally in most areas of the state and the prevalence reaches about 50% in certain provinces (5).

Morphological criteria such as body size and shape are among the traditional and important methods to distinguish between the 2 species, but these methods are not commonly trusted because of the variable range in different species (6).

The species of *Fasciola* are meiotically functional diploid and can produce sperm and temporarily store the produced sperms in the seminal vesicles named spermic fluke. This male reproductive organ is the common predominant characteristic of both species (7). On the other

hand, intermediate *Fasciola* that have morphological characteristics intermediate between *F. hepatica* and *F. gigantica* with no sperm (aspermic fluke) in their seminal vesicles are found in Asian countries (8, 9).

One of the main problems in anthelmintic resistance of this parasite is due to aspects of biology and population structure and pathogenicity, and the control of fascioliasis depends on genetic diversity (10, 11). Both of these species may be identified by applying DNA sequences of nuclear ribosomal DNA internal transcribed spacers (ITS), and sequences of *CO1* and *ND1* as mitochondrial genes to analyze intraspecific phylogenetic relationship of *Fasciola* spp. (12-14).

There are some reports from Iran animal fascioliasis, particularly in cattle, based on geographical and climatic variations (3, 15, 16). Also, there are no rich and valuable surveys on the molecular and spermatogenetic ability of *Fasciola* spp. from Iran.

2. Objectives

The current study aimed at identifying *Fasciola* spp. based on spermatogenesis ability and nuclear *ITS1* marker in the East of Iran, and also analyzing their phylogenic relationship with population from other parts of the world using mitochondrial *ND1* gene.

3. Methods

3.1. Study Population

The current cross sectional study was performed on *Fasciola* spp. isolated from natural hosts (cattle) in the eastern provinces of Iran from January 2015 to January 2016. Sistan and Baluchistan province is noted for its unique culture and extremely dry climate, bordering with Pakistan and Afghanistan. Khorasan region includes 3 provinces with the climatic conditions similar to that of Sistan and Baluchistan province, bordering Afghanistan and Turkmenistan (Figure 1). The mentioned provinces are the poles of livestock in Iran.

3.2. Sampling of Fasciola spp. and Morphological Analysis of the Spermatogenic Status

One hundred and fifty adult flukes were collected from the bile ducts of 70 naturally infected cattle at abattoirs located in the Eastern provinces of Iran. Almost 2 flukes were randomly isolated from each host, washed with saline solution, fixed in 70% ethanol between 2 slides, and taken to the parasitology lab; then, the measurement of morphological criteria such as body length and width were carried out. The whole body of *Fasciola* spp. including seminal vesicle in the anterior part of the flake was dyed with hematoxylin carmine and observed under an optical microscope to examine the existence of sperm (17). Prior to staining, a small posterior part of the fluke was used for DNA extraction (Table 1).

3.3. DNA Extraction and Amplification

Total DNA was extracted from a sample of *Fasciola* sp. using high pure PCR template preparation kit (Dynabio®, Takapouzist, Iran), according to the manufacturer's instructions, and stored at -20°C until use. The *ITS1* region, as a nuclear marker, was amplified with primers named *ITS1*-F and *ITS1*-R and fragments of mitochondrial target (*ND1*) by polymerase chain reaction (PCR) (18, 19). Total volume of the reaction was 40 μL containing 4 μL DNA template, 14 μL distilled water, 10 pmol of each primer, and 20 μL master mix (amplicon®). Reaction cycles consisted of an initial denaturation at 94°C for 90 seconds, followed by 35 cycles at 94°C for 90 seconds, 53°C (*ITS1*) or 55°C (*ND1*) for 90 seconds and 72°C for 120 seconds, with a final extension step at 72°C for 10 minutes using a gradient thermocycler. DNA fragments were visualized and analyzed by 2% agarose gel electrophoresis (20).

3.4. PCR-RFLP Method

The *ITS1* marker was used to identify *Fasciola* spp. in PCR-RFLP (restriction fragment length polymorphism). According to the manual, DNA was digested using restriction enzyme RsaI (Cinagen®, Iran) with reaction buffer under in vitro conditions, and then, analyzed by gel electrophoresis (21).

3.5. Genetic Diversity Indices, Phylogenetic Analysis, and Sequences

Products of *ND1* gene of specimens sequenced using the same primers in PCR reaction by Bioneer Company. The sequences were aligned and compared with available and existing sequences from the region in the GenBank, related to *Fasciola* spp. using BLAST 2.0 and ClustalW with default parameters. Phylogenic analyses based on *ND1* sequence data were conducted by maximum likelihood (ML) using MEGA6 (22). Diversity indices and neutrality indices were estimated by Dnasp software package version 5.10 (23). The degree of gene flow (gene migration) among the populations was evaluated using a pairwise fixation index (Fst) (24).

4. Results

4.1. Microscopic Observation

Both spermic and aspermic *Fasciola* spp. were detected in the studied geographical region. One group of flukes

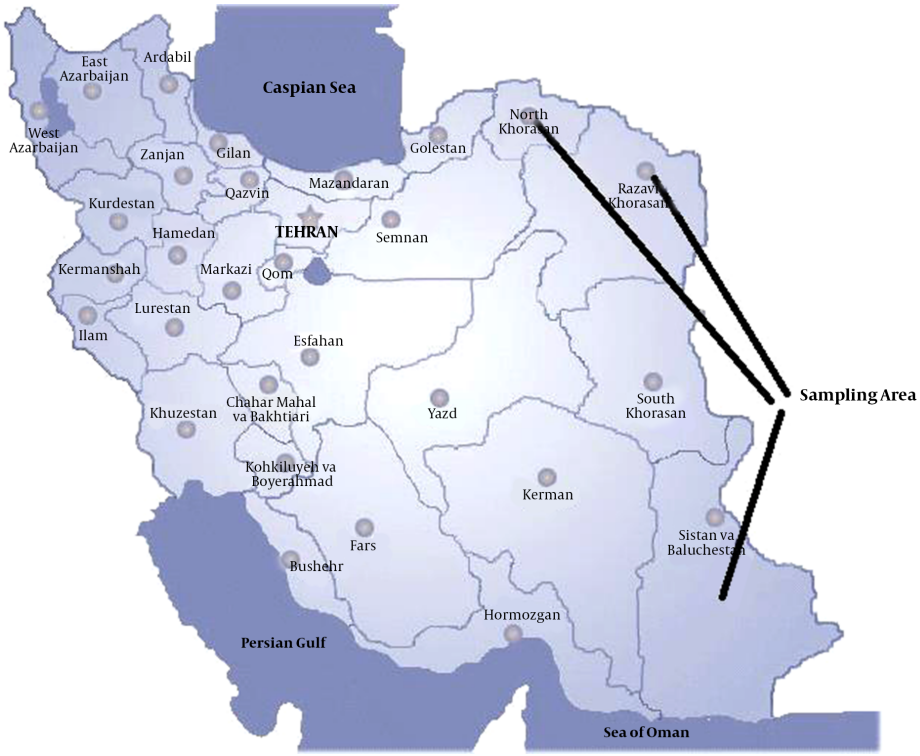


Figure 1. Sampling Area of *Fasciola* spp. in the East of Iran

Table 1. Profiles of *Fasciola* spp. From the East of Iran

Fasciola spp. Fluke Population			Number of Flukes			DNA Types		
Province	**District**	**Number of Infected Cattle**	**Total**	**Spermic**	**Aspermic**	***ITS1***	***ND1***	***CO1***
Khorasan Razawi	Ghochan and Mashhad	10	20	20	-	F.g	F.g	F.g
North Khorasan	Bojnurd	8	16	16	-	F.h[a] F.g	F.h F.g	F.h F.g
North Khorasan	Maneh	8	16	16	-	F.h[a] F.g	F.h F.g	F.h F.g
North Khorasan	Shirvan	4	12	12	-	F.g	F.g	F.g
Sistan and Baluchistan	Zahedan	16	48	48	-	F.g	F.g	F.g
Sistan and Baluchistan	Zahedan (2)	11	22	22	-	F.g	F.g	F.g
Sistan and Baluchistan	Saravan	3	15	12	3	F.g	F.g	F.g

Abbreviations: F.g, *F. gigantica;* F.h, *F. hepatica*.
[a] Coexistence of *F. hepatica* and *F. gigantica* in most cattle.

from imported cattle in Sistan and Baluchistan province were aspermic and the remaining were spermic (Table 2). Length to width ratio in the spermic *F. hepatica* and spermic *F. gigantica* as a morphological criteria showed a significant difference ($P < 0.05$).

4.2. Molecular Findings

The amplicons of *ITS1* (approximately 680 bp) obtained from all of the spermic and aspermic flukes were cut using RsaI endonuclease digestion. RFLP patterns for *F. gigantica* and *F. hepatica* were 360 170 and 60, and 360 100 and 60, re-

Table 2. Haplotype Diversity and Nucleotide Diversity of *Fasciola* spp. in the East of Iran, Based on NDI Gene

Species	Population	Diversity Indices					Neutrality Indices	
		n	Hn	S	Hd ± SD	π	Tajima's D	Fu's Fs Statistic
F. hepatica	East of Iran	60	9	17	1 ± 0.052	0.01668	-1.26770[a]	-3.138
F. hepatica	Egypt	19	14	NC	0.978 ± 0.027	0.08217	-2.50218	1.298
F. hepatica	China	6	6	NC	1 ± 0.096	0.00586	0.12841	-3.178
F. gigantica	East of Iran	90	4	22	0.867 ± 0.129	0.02689	0.74707	3.366
F. gigantica	Eastern Indiaa	91	32	NC	0.751 ± 0.050	0.00242	NC	NC
F. gigantica	Nepala	20	10	NC	0.753 ± 0.101	0.00366	NC	NC
F. gigantica	Bangladesh[b]	20	15	NC	0.832 ± 0.075	0.00362	NC	NC

Abbreviations: Hd, haplotype diversity; Hn, number of haplotypes; NC, not calculated; Nd, nucleotide diversity; S, number of variable sites.
[a]Statistical significance: not significant, P > 0.1.
[b]Cited from Hayashi et al. (9).

spectively. The *ND1* fragments (approximately 535 bp) were amplified for all specimens.

4.3. Phylogeny and Genetic Diversity

Haplotype diversity, nucleotide diversity, and variable sites of *Fasciola* spp. in the East of Iran, based on *ND1* gene, were compared with those of other countries are shown in Table 3. The nucleotide sequences for each haplotype were deposited in GenBank under the following accession numbers: KX021280-KX021299 and KX063827- KX063836. Phylogenetic analyses based on mtDNA (*ND1*) sequence data were conducted by ML with lung fluke; Paragonimus westermani designated as outgroup is shown in Figure 2. Fst values between various populations of *F. gigantica* were calculated by Dnasp5 software package with the nucleotide data set of *ND1* gene. This index was statistically significant in different *F .gigantica* populations and showed the genetic difference in pairwise population (Table 3). In addition, this index was statistically significant between different *F. hepatica* populations, except in Asia and East of Iran (Table 4 and Figure 3).

5. Discussion

The differentiation of *Fasciola* species is crucial because of their epidemiology patterns. Fascioliasis is one the most important global concerns both for public and veterinary health. All the specimens of the current study were obtained only from the cattle that were traditionally nurtured.

Both *F. hepatica* and *F. gigantica* were prevalent in the East of Iran. The existence of both species from cattle in the North East of Iran owed to the coexistence of sheep and cattle.

Periago et al. demonstrated that the ratio of body length and width (BL/BW) is one of the useful criteria for diagnosing of *Fasciola* species (25), however, the current study used molecular methods to discriminate the species. A morphological report from North of Iran indicated the existence of intermediate forms of *Fasciola* (6), but the morphological findings of the current study did not show the existence of the intermediated form in the East of Iran, although aspermic *F. gigantica* was detected.

The current study aimed at finding the aspermic forms of *Fasciola* app. because of bordering Pakistan and Afghanistan. The study found aspermic *Fasciola* spp. in 2 infected cattle from Saravan in Sistan and Baluchistan province. Some reports showed the *Fasciola* sp. taxa in South West of Asia, in India and Bangladesh, near the studied region of Iran, but The Fg type of these flukes was detected in aspermic *Fasciola* sp. using ITS-RFLP, and also the phylogenetic study with *ND1* gene showed that they were placed in *F. gigantica* complex (8, 9). These flukes were probably considered as abnormal *F. gigantica* with oligozoospermia, which might have occurred because of the aging of flukes. This finding was also shown in the research by Mohanta from Bangladesh (17). Studies showed that molecular phylogeny with mitochondrial DNA can be effectively used for appropriate differentiation of haplotypes (12, 18, 20).

Iran is a vast country and multiple factors may affect the haplotypes. The existence of several haplotypes in this region of Iran demonstrated the variables of ecology and climate, and the needs to provide other studies from multiple regions of Iran.

Genetic diversity in this region of Iran is high. Genetic diversity may have occurred due to free trade of cattle and transporting them through the borders of Iran with other neighboring countries. Further studies on larger populations are needed to find the reasons.

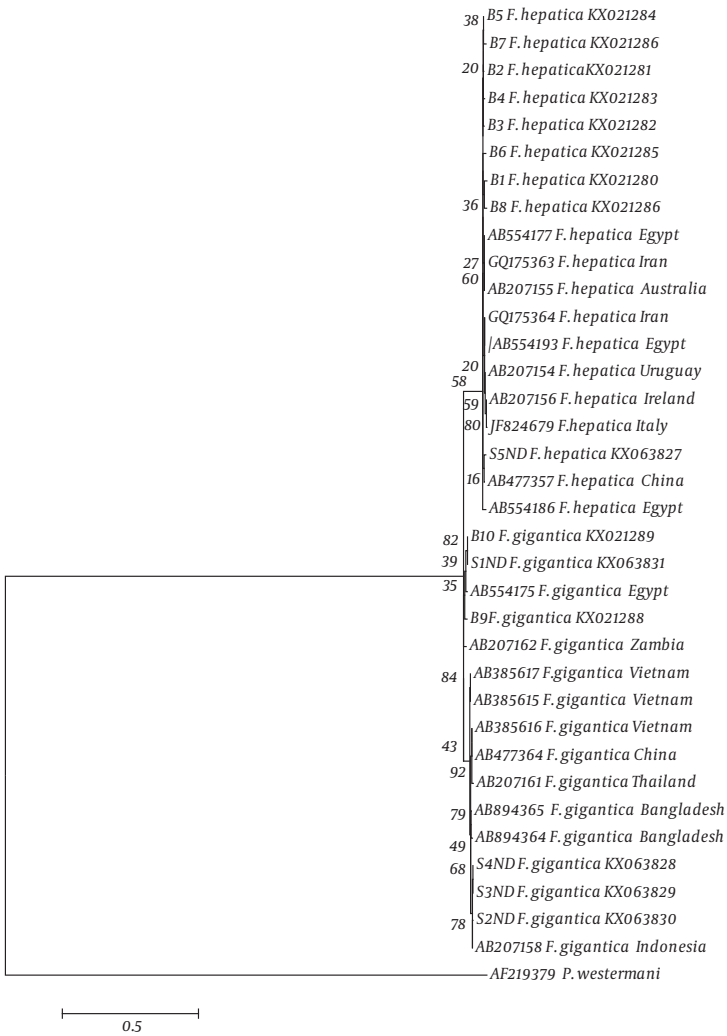
In the current study, the Fst values among 5 populations of *F. gigantica* ranged from 0.54111 to 0.99599, which

Table 3. Pairwise Fixation Index Between Different *Fasciola gigantica* Populations Calculated From the Nucleotide Data set of NDI Gene[a]

Population	Population				
	East of Iran	Egypt	Bangladesh	Zambia	Vietnam
East of Iran	-				
Egypt	0.98798	-			
Bangladesh	0.99075	0.99599	-		
Zambia	0.99133	0.98401	0.53823	-	
Vietnam	0.99133	0.99704	0.68867	0.54111	-

[a] All values are statistically significant (P > 0.05).

Figure 2. Phylogenetic Relationship Based on NDI Sequences of *Fasciola* spp. from East of Iran



The tree was constructed by MEGA6 using maximum likelihood analysis. Scale bars indicated nucleotide substitutions per site. Paragonimus westermani was used as outgroup.

suggested that *F. gigantica* populations were genetically differentiated (Table 3). These results could be related to

Table 4. Pairwise Fixation Index Between Different *Fasciola* hepatica Populations Calculated From the Nucleotide Data Set of NDI Gene[a]

Population	Population				
	East of Iran	Egypt	Peru	Asia[b]	Europe[c]
East of Iran	-				
Egypt	0.98329	-			
Peru	0.98516	0.98892	-		
Asia*	0.89128	0.89282	0.01768	-	
Europe**	0.78107	0.78042	0.00250	-0.06162	-

[a] All values are statistically insignificant (P > 0.05).
[b] Asia: China, Thailand, Japan.
[c] Europe: Italy, Poland.

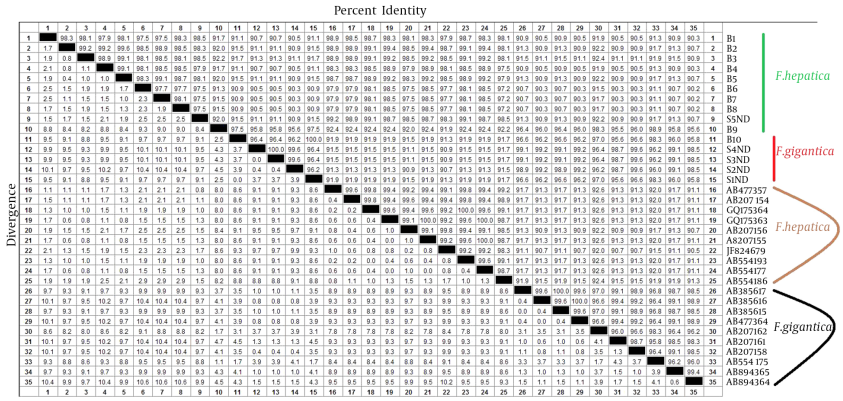


Figure 3. Percent Identity and Divergence of *Fasciola* spp. from the East of Iran, Compared with Other Sequences Submitted to GenBank.

the presence of several diverse haplotypes of the studied populations and no transfer of alleles from one population to another through the immigration of natural hosts in the region.

The Fst values showed that F. hepatica population in 3 continents was genetically different from each other, based on *ND1* sequence (Table 4).

In conclusion, the current study illustrated that *F. gigantica* in the East of Iran has different genetic structures from the other *Fasciola* populations according to genetic index, but to identify the genetic diversity, further molecular studies should be performed in other regions of Iran.

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Footnote

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References

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. *Int J Parasitol.* 2005;**35**(11-12):1255–78. doi: 10.1016/j.ijpara.2005.07.010. [PubMed: 16150452].
2. Organization WH . Control of foodborne trematode infections: report of a WHO study group. ; 1995.
3. Ashrafi K. The Status of Human and Animal Fascioliasis in Iran: A Narrative Review Article. *Iran J Parasitol.* 2015;**10**(3):306–28. [PubMed: 26622287].
4. Shafiei R, Sarkari B, Sadjjadi SM, Mowlavi GR, Moshfe A. 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;**2014**:405740. doi: 10.1155/2014/405740. [PubMed: 25018891].
5. Rokni MB. The present status of human helminthic diseases in Iran. *Ann Trop Med Parasitol.* 2008;**102**(4):283–95. doi: 10.1179/136485908X300805. [PubMed: 18510809].

6. 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. *Parasitol Int.* 2006;**55**(4):249–60.
7. Sanderson AR. Maturation and probable gynogenesis in the liver fluke, Fasciola hepatica L. *Nature.* 1953;**172**(4368):110–2. [PubMed: 13072592].
8. Hayashi K, Ichikawa-Seki M, Allamanda P, Wibowo PE, Mohanta UK, et al. Molecular characterization and phylogenetic analysis of Fasciola gigantica from western Java, Indonesia. *Parasitol Int.* 2016;**65**(5 Pt A):424–7. doi: 10.1016/j.parint.2016.06.004. [PubMed: 27266482].
9. Hayashi K, Ichikawa-Seki M, Mohanta UK, Singh TS, Shoriki T, Sugiyama H, et al. Molecular phylogenetic analysis of Fasciola flukes from eastern India. *Parasitol Int.* 2015;**64**(5):334–8. doi: 10.1016/j.parint.2015.04.004.
10. Beesley NJ, Williams DJ, Paterson S, Hodgkinson J. Fasciola hepatica demonstrates high levels of genetic diversity, a lack of population structure and high gene flow: possible implications for drug resistance. *Int J Parasitol.* 2017;**47**(1):11–20. doi: 10.1016/j.ijpara.2016.09.007. [PubMed: 27940066].
11. Hodgkinson J, Cwiklinski K, Beesley NJ, Paterson S, Williams DJ. Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant Fasciola hepatica. *Parasitology.* 2013;**140**(12):1523–33. doi: 10.1017/S0031182013000528. [PubMed: 23721579].
12. Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T, et al. Genetic characterization of parthenogenic Fasciola sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology.* 2005;**131**(5):679–85.
13. Ichikawa M, Iwata N, Itagaki T. DNA types of aspermic Fasciola species in Japan. *J Vet Med Sci.* 2010;**72**(10):1371–4.
14. Ichikawa-Seki M, Peng M, Hayashi K, Shoriki T, Mohanta UK, Shibahara T, et al. Nuclear and mitochondrial DNA analysis reveals that hybridization between Fasciola hepatica and Fasciola gigantica occurred in China. *Parasitology.* 2017;**144**(2):206–13.
15. Bozorgomid A, Nazari N, Rahimi H, Beigom Kia E, Hajjaran H, Mohebali M, et al. Molecular Characterization of Animal Fasciola spp. Isolates from Kermanshah, Western Iran. *Iran J Public Health.* 2016;**45**(10):1315–21. [PubMed: 27957438].
16. Mahami-Oskouei M, Dalimi A, Forouzandeh-Moghadam M, Rokni M. Molecular Identification and Differentiation of Fasciola Isolates Using PCR- RFLP Method Based on Internal Transcribed Spacer (ITS1, 5.8S rDNA, ITS2). *Iran J Parasitol.* 2011;**6**(3):35–42. [PubMed: 22347295].
17. Mohanta UK, Ichikawa-Seki M, Shoriki T, Katakura K, Itagaki T. Characteristics and molecular phylogeny of Fasciola flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. *Parasitol Res.* 2014;**113**(7).
18. 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.
19. Galavani H, Gholizadeh S, Hazrati Tappeh K. Genetic Characterization of Fasciola Isolates from West Azerbaijan Province Iran Based on ITS1 and ITS2 Sequence of Ribosomal DNA. *Iran J Parasitol.* 2016;**11**(1):52–64. [PubMed: 27095969].
20. Reaghi S, Haghighi A, Harandi MF, Spotin A, Arzamani K, Rouhani S. Molecular characterization of Fasciola hepatica and phylogenetic analysis based on mitochondrial (nicotiamide adenine dinucleotide dehydrogenase subunit I and cytochrome oxidase subunit I) genes from the North-East of Iran. *Vet World.* 2016;**9**(9):1034–8. doi: 10.14202/vetworld.2016.1034-1038. [PubMed: 27733809].
21. Ichikawa M, Itagaki T. Discrimination of the ITS1 types of Fasciola spp. based on a PCR-RFLP method. *Parasitol Res.* 2010;**106**(3):757–61. doi: 10.1007/s00436-010-1724-2. [PubMed: 20107839].
22. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol.* 2013;**30**(12):2725–9. doi: 10.1093/molbev/mst197. [PubMed: 24132122].
23. Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics.* 2003;**19**(18):2496–7. [PubMed: 14668244].
24. Reynolds J, Weir BS, Cockerham CC. Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics.* 1983;**105**(3):767–79. [PubMed: 17246175].
25. Periago MV, Valero MA, El Sayed M, Ashrafi K, El Wakeel A, Mohamed MY, et al. First phenotypic description of Fasciola hepatica/Fasciola gigantica intermediate forms from the human endemic area of the Nile Delta, Egypt. *Infect Genet Evol.* 2008;**8**(1):51–8. doi: 10.1016/j.meegid.2007.10.001. [PubMed: 18006385].