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Original Article

Genetic Characterization of *Fasciola* Isolates from West Azerbaijan Province Iran Based on ITS1 and ITS2 Sequence of Ribosomal DNA

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Abstract

Background: Fascioliasis, caused by *Fasciola hepatica* and *F. gigantica*, has medical and economic importance in the world. Molecular approaches comparing traditional methods using for identification and characterization of *Fasciola* spp. are precise and reliable. The aims of current study were molecular characterization of *Fasciola* spp. in West Azerbaijan Province, Iran and then comparative analysis of them using GenBank sequences.

Methods: A total number of 580 isolates were collected from different hosts in five cities of West Azerbaijan Province, in 2014 from 90 slaughtered cattle (n=50) and sheep (n=40). After morphological identification and DNA extraction, designing specific primer were used to amplification of ITS1, 5.8s and ITS2 regions, 50 samples were conducted to sequence, randomly.

Result: Using morphometric characters 99.14% and 0.86% of isolates identified as *F. hepatica* and *F. gigantica,* respectively. PCR amplification of 1081 bp fragment and sequencing result showed 100% similarity with *F. hepatica* in ITS1 (428 bp), 5.8s (158 bp), and ITS2 (366 bp) regions. Sequence comparison among current study sequences and GenBank data showed 98% identity with 11 nucleotide mismatches. However, in phylogenetic tree *F. hepatica* sequences of West Azerbaijan Province, Iran, were in a close relationship with Iranian, Asian, and African isolates.

Conclusions: Only *F. hepatica* species is distributed among sheep and cattle in West Azerbaijan Province Iran. However, 5 and 6 bp variation in ITS1 and ITS2 regions, respectively, is not enough to separate of *Fasciola* spp. Therefore, more studies are essential for designing new molecular markers to correct species identification.

Introduction

Fascioliasis has been recognized as a water- and food-borne parasitic zoonosis caused by *Fasciola hepatica* and *F. gigantica* (1-5). "*F. hepatica* occurs in temperate areas and *F. gigantica* in tropical zones, but both species may overlap in subtropical areas" (6-8). Human fascioliasis accounts a serious threat to public health, and the transmission of this disease occurs when healthy individuals consume uncooked aquatic vegetables or drinking fresh water contaminated with immature parasite larvae (4, 9). The Highlands of South America, the Nile Valley, the Caspian Sea Basin as well as East and Southeast Asia have been considered the areas with high transmission of this infection (10). Fascioliasis also exist in Asian countries, such as Pakistan, Saudi Arabia, Iraq, Vietnam, Turkey, China, Korea, Japan, Thailand, India, Yemen, and especially Iran (3, 6, 9, 11-18). Indeed, this zoonotic infection is found in Kurdistan, Zanjan, Kermanshah, Mazandaran, Tehran, Azerbaijan, Guilan, Fars, and Khuzestan Provinces of Iran(8). In recent 26 yr, two large outbreaks of fascioliasis have been occurred in the Northern provinces of the country around the Caspian Sea with about several thousand deaths in each outbreak (19, 20).

Fasciola species can be characterized using morphometric values, such as body length, body width, cephalic cone length, and length of the area behind the testes (9, 21). However, different molecular markers and techniques are needed to identify accurately the inter- and intra-species of *Fasciola* (22). Several molecular studies have reported *F. hepatica*, *F. gigantica*, and their intermediate forms from different countries including Iran (8, 9, 15, 16, 23-30). Nevertheless, there is no report from West Azerbaijan Province of Iran, located in northwestern corner of the country, bordering with Iraq, Turkey, East Azerbaijan, and Armenian countries.

Therefore, this study was undertaken to haplotype analysis of ITS1 and ITS2 rDNA isolated from liver of cattle and sheep in West Azerbaijan Province.

Materials and Methods

Parasites

Adult trematodes (n=580) were collected from the 90 liver of slathered cattles (n= 335) and sheep (n=245) in Salmas (n=65 cattle, 45 sheep), Makou (n=50 cattle, 50 sheep), Urmia (n=90 cattle, 70 sheep), Mehabad (n=80 cattle, 50 sheep), and Bukan (n=50 cattle, 30 sheep) districts as well as West Azerbaijan Province, Iran (Fig. 1). All samples were washed in normal saline, fixed in 70% ethanol, and then kept at room temperature until DNA extraction. All morphological measurements of adults were made according to methods described for *Fasciola* (9, 21, 31). The morphometric values such as, body length (BL), body width (BW), cephalic cone length (CL) and length of area behind the testes, were obtained using a microscope and calibrated ocular micrometer.

Fig. 1: Location of the sites where *Fasciola* isolates were collected in West Azerbaijan Province, Iran

DNA extraction

DNA was extracted using Collin's method (32) with a slight modification (33). Briefly, one part of the parasite fixed in 70% ethanol was added to 50 µl Lysis buffer (8 µL Nacl, 10 µL Tris-Hcl, 6 µL EDTA, 2.5 µL SDS, 16 µL sucrose, and 7.5 µL deionized distilled water) and mixed in a 1.5-ml Eppendorf tube. The mixture was then homogenized by using a pestle and incubated at 65 ºC for 60 min. After incubation, 16.68 µl of 3MNaAc (sodium acetate) was added to the Eppendorf tube, and the tube was then placed on ice for 60 min and centrifuged at 12,000 rpm for 10 min in room temperature. DNA was pelleted by -20 ºC ethanol and centrifuged for further 15 min. The dried pellet was rehydrated in 50µl ddH_2O and stored at 4 ºC until use.

Primer designing

To amplify a 1081-bp DNA fragment of the ITS region (ITS1, 5.8s, and ITS2) in *F. hepatica*, *F. gigantica* and their intermediate form, forward (5'GCTGAGAAGACGACCAAAC3') and reverse primers (5'AGTTCAGCGGGTAATCAC3') designed using Gene Runner (version 3.05, 1994, Hastings Software Inc.) and BLAST (http://www.ncbi.nlm.nih.gov/blast) software. The primers were synthesized by Genfanavaran Company (Iran).

PCR amplification

All PCR reactions were performed in 25-µL volumes, containing 12.5 µL PCR master mix (Cinnagen, Iran), 1 µL each primer (forward and reverse), 1 µL extracted genomic DNA, and 9.5 µL deionized distilled water. The amplification profile was carried out at 95 ºC for5 min, followed by 35 cycles including denaturation at 95 ºC for 1 min, annealing at 57 ºC for 75 s, and extension at 72 ºC for 1 min with a final extension at 72 ºC for 10 min. PCR products (5 µL) were visualized with ethidium bromide on a 1.5% agarose gel. In total, 50 PCR products (10 from each study area) were sequenced randomly using an ABI377 automated sequencer by Takapou Zist Company (Iran).

Sequence analysis

rDNA ITS1 and ITS2 sequences used in phylogenetic analysis were obtained from the current study and extracted from the GenBank using "*Fasciola*", "ITS1", "ITS2", and "Iran" keywords. Most of the obtained sequences included partial 18s, 5.8s, and 28s. ITS1 and ITS2 sequences were annotated according to the previously submitted sequences using the ITS2 annotation tool (34). Both sequences of *Fasciola* species were aligned using ClustalW (35) and MEGA5 (36). Nucleotide sequences are available in the GenBank, European Molecular Biology Laboratory (EMBL), and DNA Data Bank of Japan (DDBJ) databases [GenBank: KF531639 to KF531788].

Sequence and phylogenetic analyses in the current study were performed based on the methods used for Anophelines species in Iran (37). Briefly, the phylogenetic tree was constructed using neighbor-joining method (38). Evolutionary analysis was also conducted using MEGA5 software (36). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is indicated next to the branches (39). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions including gaps and missing data were considered complete deletion, and the numbers of nucleotide substitutions per site were estimated.

Results

A total number of 580 collected isolates from 90 slathered cattle (n=50) and sheeps (n=40) were morphologically identified as *F. hepatica* (n=575) and *F. gigantica* (n=5). The 1081-bp fragments of 110 samples from both hosts were successfully amplified, and 50 samples (30 from cattle and 20 from sheep) were randomly subjected to direct sequencing.

```
F.hepatica.WestAzarbaijan   ATCATTACCTGAAAATCTACTCTCACACAAGCGATACACGTGTGACCGTC 50
JF708027.F.hepatica.China   ------ACCTGAAAATCTACTCTCACACAAGCGATACACGTGTGACCGTC 44
JF432073.F.gigantica.Iran   -----------------TACTCTTACACAAGCGATACACGTGTGACCGTC 33
                                             ****** **************************

F.hepatica.WestAzarbaijan   ATGTCATGCGATAAAAATTTGCGGACGGCTATGCCTGGCTCATTGAGGTC 100
JF708027.F.hepatica.China   ATGTCATGCGATAAAAATTTGCGGACGGCTATGCCTGGCTCATTGAGGTC 94
JF432073.F.gigantica.Iran   ATGTCATGCGATAAAAATTTGCGGACGGCTATGCCTGGCTCATTGAGGTC 83
                            **************************************************

F.hepatica.WestAzarbaijan   ACAGCATATCCGAACACTGATGGGGTGCCTACCTGTATGATACTCCGATG 150
JF708027.F.hepatica.China   ACAGCATATCCGAACACTGATGGGGTGCCTACCTGTATGATACTCCGATG 144
JF432073.F.gigantica.Iran   ACAGCATATCCGATCACTGATGGGGTGCCTACCTGTATGATACTCCGATG 133
                            *************:************************************

F.hepatica.WestAzarbaijan   GTATGCTTGCGTCTCTCGGGGCGCTTGTCCAAGCCAGGAGAACGGGTTGT 200
JF708027.F.hepatica.China   GTATGCTTGCGTCTCTCGGGGCGCTTGTCCAAGCCAGGAGAACGGGTTGT 194
JF432073.F.gigantica.Iran   GTATGCTTGCGTCTCTCGGGGCGCTTGTCCAAGCCAGGAGAACGGGTTGT 183
                            **************************************************

F.hepatica.WestAzarbaijan   ACTGCCACGATTGGTAGTGCTAGGCTTAAAGAGGAGATTTGGGCTACGGC 250
JF708027.F.hepatica.China   ACTGCCACGATTGGTAGTGCTAGGCTTAAAGAGGAGATTTGGGCTACGGC 244
JF432073.F.gigantica.Iran   ACTGCCATGATTGGTAGTGCTAGGCTTAAAGAGGAGATTTGGGCTACGGC 233
                            ******* ******************************************

F.hepatica.WestAzarbaijan   CCTGCTCCCGCCCTATGAACTGTTTCATTACTACATTTACACTGTTAAAG 300
JF708027.F.hepatica.China   CCTGCTCCCGCCCTATGAACTGTTTCATTACTACATTTACACTGTTAAAG 294
JF432073.F.gigantica.Iran   CCTGCTCCCGCCCTATGAACTGTTTCATTACTACAATTACACTGTTAAAG 283
                            ***********************************:**************

F.hepatica.WestAzarbaijan   TGGTACTGAATGGCTTGCCATTCTTTGCCATTGCCCTCGCATGCACCCGG 350
JF708027.F.hepatica.China   TGGTACTGAATGGCTTGCCATTCTTTGCCATTGCCCTCGCATGCACCCGG 344
JF432073.F.gigantica.Iran   TGGTATTGAATGGCTTGCCATTCTTTGCCATTGCCCTCGCATGCACCCGG 333
                            ***** ********************************************

F.hepatica.WestAzarbaijan   TCCTTGTGGCTGGACTGCACGTACGTCGCCCGGCGGTGCCTATCCCGGGT 400
JF708027.F.hepatica.China   TCCTTGTGGCTGGACTGCACGTACGTCGCCCGGCGGTGCCTATCCCGGGT 394
JF432073.F.gigantica.Iran   TCCTTGTGGCTGGACTGCACGTACGTCGCCCGGCGGTGCCTATCCCGGGT 383
                            **************************************************

F.hepatica.WestAzarbaijan   TGGACTGATAACCTGGTCTTTGACCATACGTACAACTCTGAACGGTGGAT 450
JF708027.F.hepatica.China   TGGACTGATAACCTGGTCTTTGACCATACGTACAACTCTGAACGGTGGAT 444
JF432073.F.gigantica.Iran   TGGACTGATAACCTGGTCTTTGACCATACGTACAACTCTGAACGGTGGAT 433
                            **************************************************

F.hepatica.WestAzarbaijan   CACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATG 500
JF708027.F.hepatica.China   CACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATG 494
JF432073.F.gigantica.Iran   CACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATG 483
                            **************************************************

F.hepatica.WestAzarbaijan   CAAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCC 550
JF708027.F.hepatica.China   CAAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCC 544
JF432073.F.gigantica.Iran   CAAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCC 533
                            **************************************************

F.hepatica.WestAzarbaijan   ATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATC 600
JF708027.F.hepatica.China   ATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATC 594
JF432073.F.gigantica.Iran   ATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATC 583
                            **************************************************

F.hepatica.WestAzarbaijan   ACGACGCCCAAAAAGTCGTGGCTTGGGTTTTGCCAGCTGGCGTGATCTCC 650
JF708027.F.hepatica.China   ACGACGCCCAAAAAGTCGTGGCTTGGGTTTTGCCAGCTGGCGTGATCTCC 644
JF432073.F.gigantica.Iran   ACGACGCCCAAAAAGTCGTGGCTTGGGTTTTGCCAGCTGGCGTGATCTCC 633
                            **************************************************

F.hepatica.WestAzarbaijan   TCTATGAGTAATCATGTGAGGTGCCAGATCTATGGCGTTTCCCTAATGTA 700
JF708027.F.hepatica.China   TCTATGAGTAATCATGTGAGGTGCCAGATCTATGGCGTTTCCCTAATGTA 694
JF432073.F.gigantica.Iran   TCTATGAGTAATCATGTGAGGTGCCAGATCTATGGCGTTTCCCTAATGTA 683
                            **************************************************

F.hepatica.WestAzarbaijan   TCCGGATGCACCCTTGTCTTGGCAGAAAGCCGTGGTGAGGTGCAGTGGCG 750
JF708027.F.hepatica.China   TCCGGATGCACCCTTGTCTTGGCAGAAAGCCGTGGTGAGGTGCAGTGGCG 744
JF432073.F.gigantica.Iran   TCCGGATGCACCCTTGTCTTGGCAGAAAGCCGTGGTGAGGTGCAGTGGCG 733
                            **************************************************

F.hepatica.WestAzarbaijan   GAATCGTGGTTTAATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTTGGC 800
JF708027.F.hepatica.China   GAATCGTGGTTTAATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTTGGC 794
JF432073.F.gigantica.Iran   GAATCGTGGTTTAATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTTGGC 783
                            **************************************************

F.hepatica.WestAzarbaijan   GATCCCCTAGTCGGCACACTTATGATTTCTGGGATAATTCCATACCAGGC 850
JF708027.F.hepatica.China   GATCCCCTAGTCGGCACACTTATGATTTCTGGGATAATTCCATACCAGGC 844
JF432073.F.gigantica.Iran   GATCCCCTAGTCGGCACACTCATGATTTCTGGGATAATTCCATACCAGGC 833
                            ******************** *****************************

F.hepatica.WestAzarbaijan   ACGTTCCGTCACTGTCACTTTGTCATTGGTTTGATGCTGAACTTGGTCAT 900
JF708027.F.hepatica.China   ACGTTCCGTCACTGTCACTTTGTCATTGGTTTGATGCTGAACTTGGTCAT 894
JF432073.F.gigantica.Iran   ACGTTCCGTTACTGTTACTTTGTCATTGGTTTGATGCTGAACTTGGTCAT 883
                            ********* ***** **********************************

F.hepatica.WestAzarbaijan   GTGTCTGATGCTATTTTCATATAGCGACGGTACCCTT-CGTGGTCTGTCT 949
JF708027.F.hepatica.China   GTGTCTGATGCTATTTTCATATAGCGACGGTACCCTT-CGTGGTCTGTCT 943
JF432073.F.gigantica.Iran   GTGTCTGATGCTATTTT-ATATAACGACGGTACCCTTTCGTGGTCTGTCT 932
                            ***************** *****.************* ************

F.hepatica.WestAzarbaijan   TCC----------- 952
JF708027.F.hepatica.China   TCC----------- 946
JF432073.F.gigantica.Iran   TCCTGACCTCGGTT 946
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Fig. 2: Multiple sequence alignments of the rDNA ITS1, 5.8s, and ITS2 regions of *F. hepatica* (JF708027) and *F. gigantica*(JF432073) as well as representative *F. hepatica* sequences of the current study [KF531639 (ITS1), KF531689 (5.8s), KF531739 (ITS2)]. Bold sequences belong to 5.8s region flanking to ITS1 and ITS2

The sequence analysis showed that ITS1, 5.8s, and ITS2 had a length of 428, 158, and 366 bp, i.e. 952 bp in total (Fig. 2). The BLAST analysis of rDNA ITS1, 5.8s, and ITS2 sequences showed 100% similarity with *F. hepatica* and 98% with *F. gigantic* (Fig. 2). There were 11 mismatches in positions 24, 114, 208, 286, 306, 821, 860, 866, 918, 924, and 938, including seven transitions, two transversions, one insertion, and one deletion (Fig. 2). The sequence compositions of ITS1, 5.8s, and ITS2 regions were 51.87% (GC) and 48.13% (AT), 53.17% (GC) and 46.83% (AT) as well as 48.63% (GC) and 51.37% (AT), respectively. Based on morphological characteristics, 0.86% (5 samples) was identified as *F. gigantica*. In addition, ITS1, 5.8s, and ITS2 sequences of these samples showed 100% similarity with *F. hepatica* (Fig. 2).

Of 107 sequences of *Fasciola* species submitted to the GenBank from Iran, 62.6% belonged to *F. hepatica*, 24.3% to *F. gigantica*, and the remaining 13.1% was recorded as *Fasciola* spp. Registered sequences in GenBank are related to18s, ITS1, 5.8s, ITS2, 28s, ND1 (mitochondrial NADH dehdrogenase1), COI (cytochrome C oxidase I), and CatL1 (Cathepsin L1) regions. In sum, 39 fragments were identified in ITS1 region of *F. hepatica* (24) and *F. gigantica* (15).

Table 1: Details of *Fasciola* rDNA ITS1 sequences from Iran and other countries used for phylogenetic tree construction

Species	Location	Accession number	Reference
F. hepatica	Italy	JF824666	(8)
F. hepatica	Andorra	AM707030	(46)
F. hepatica	Egypt	AB553690	(30)
F. hepatica	China	JF708028	Chen (2011) Direct submission
F. hepatica	Iran	JN828959	(43)
F. hepatica	Iran	JF432078	(43)
F. hepatica	USA	JF708031	Chen (2011) Direct submission
F. hepatica	Spain	JF708037	Chen (2011) Direct submission
F. hepatica	Saudi Arabia	HE972273	Shalaby et al.(2012) Direct submission
F. hepatica	France	JF708034	Chen (2011) Direct submission
F. hepatica	Iran	JN828960	(43)
F. hepatica	Iran	JF432072	(43)
F. hepatica	Iran	JF432076	(43)
F. hepatica	Iran	HM746786	(43)
F. hepatica	Iran	HM746785	(43)
F. gigantica	China	AJ628425	(47)
F. gigantica	Kenya	EF612472	(48)
F. gigantica	Egypt	EF612471	(48)
F. gigantica	Egypt	EF612470	(48)
F. gigantica	China	AJ628043	(47)
F. gigantica	Egypt	AB553672	(30)
F. gigantica	Iran	JN828958	(43)
F. gigantica	Vietnam	JN828960	(6)
F. gigantica	Niger	AB211238	(24)
F. gigantica	Burkina Faso	AM900371	(49)
F. gigantica type	South Korea	AB385614	(24)
Fasciola sp.	South Korea	AB385613	(24)
Fasciola sp.	Vietnam	AB211237	(6)
Fasciola jaksoni	Sri Lanka	EF612473	(48)
Fascioloides magna	USA	EF534991	(50)

Moreover, ITS2 fragments of *F. hepatica* and *F. gigantica* were 26 and 13, respectively. Primary sequence analysis showed 95.1%-100% similarity within ITS1 fragment of *F. hepatica*, 94.85%-100% within F. gigantica, while 98.63%-100%, and 98.43%-100% identity was observed among ITS2 fragments of *F. hepatica* and *F. gigantica*, respectively.

A total of 50 rDNAITS1 and ITS2 sequences of *F. hepatica* constructed in the current study and 57 sequences of the same regions from other studies were aligned using MEGA5 (36). The details of sequences used in this study are presented in Table 1 and 2.

ITS1 and ITS2 sequences of *Fasciola jaksoni* (GenBank ID: EF612473 and EF612486) and *Fascioloides magna* (GenBank ID: EF534991 and EF534995) were used as out-groups. In ITS1 region, the similarity of sequences among *F. hepatica* was in a range of 97.39%-100%, but among *F. gigantica* was 100%. In addition, the identity between *F. hepatica* and *F. gigantica* was 97.39%-98.84%. Recently, a study on molecular characterization of *Fasciola* species from Northern Iran has recognized *F. jaksoni* and *F. magna* as out-groups (8). The similarity of *F. jaksoni* to *F. hepatica* and *F. gigantica* was 96.35%-96.5% and 94.79%-95.84%, respectively, while that of *F. magna* to both species was 91.47%-93.22% and 91.71%-93.49%, respectively.

In ITS2 region, the similarity of sequences among *F. hepatica* and *F. gigantica* was 98.07%-100% and 98.89-100%, respectively, but between the two species was in a range of 96.95%-98.9%.

Table 2: Details of *Fasciola* rDNA ITS2 sequences from Iran and other countries used for phylogenetic tree construction

Species	Location	Accession number	Reference
F. hepatica	France	AJ557567	(29)
F. hepatica	Niger	AM900370	
F. hepatica	Turkey	JN585288	Yazar (2011) Direct submission
F. hepatica	Egypt	AB553734	(8)
F. hepatica	USA	JF708031	Chen (2011) Direct submission
F. hepatica	France	JF708034	Chen (2011) Direct submission
F. hepatica	Spain	JF708037	Chen (2011) Direct submission
F. hepatica	Australia	EU260058	(26)
F. hepatica	Andorra	AM707030	(46)
F. hepatica	Iran	JN828959	(43)
F. hepatica	Iran	JF432076	(43)
F. hepatica	Iran	JF432072	(43)
F. hepatica	Iran	EU391424	(41)
F. hepatica	France	AJ557567	(29)
F. hepatica	Iran	HM746786	(43)
F. gigantica	Niger	AM900371	(49)
F. gigantica	China	AJ557569	(29)
F. gigantica	India	EF027103	(51)
F. gigantica	Vietnam	EU260078	(26)
F. gigantica	China	EU260079	(26)
F. gigantica	Burkina Faso	AJ853848	Bargues (2008) Direct submission
F. gigantica	Kenya	EF612484	(48)
F. gigantica	Iran	JN828957	(43)
F. hepatica/gigantica	South Korea	HQ821457	(16)
Fasciola sp	Japan	AB207153	(24)
Fasciola jaksoni	Sri Lanka	EF612486	(48)
Fascioloides magna	USA	EF534995	(50)

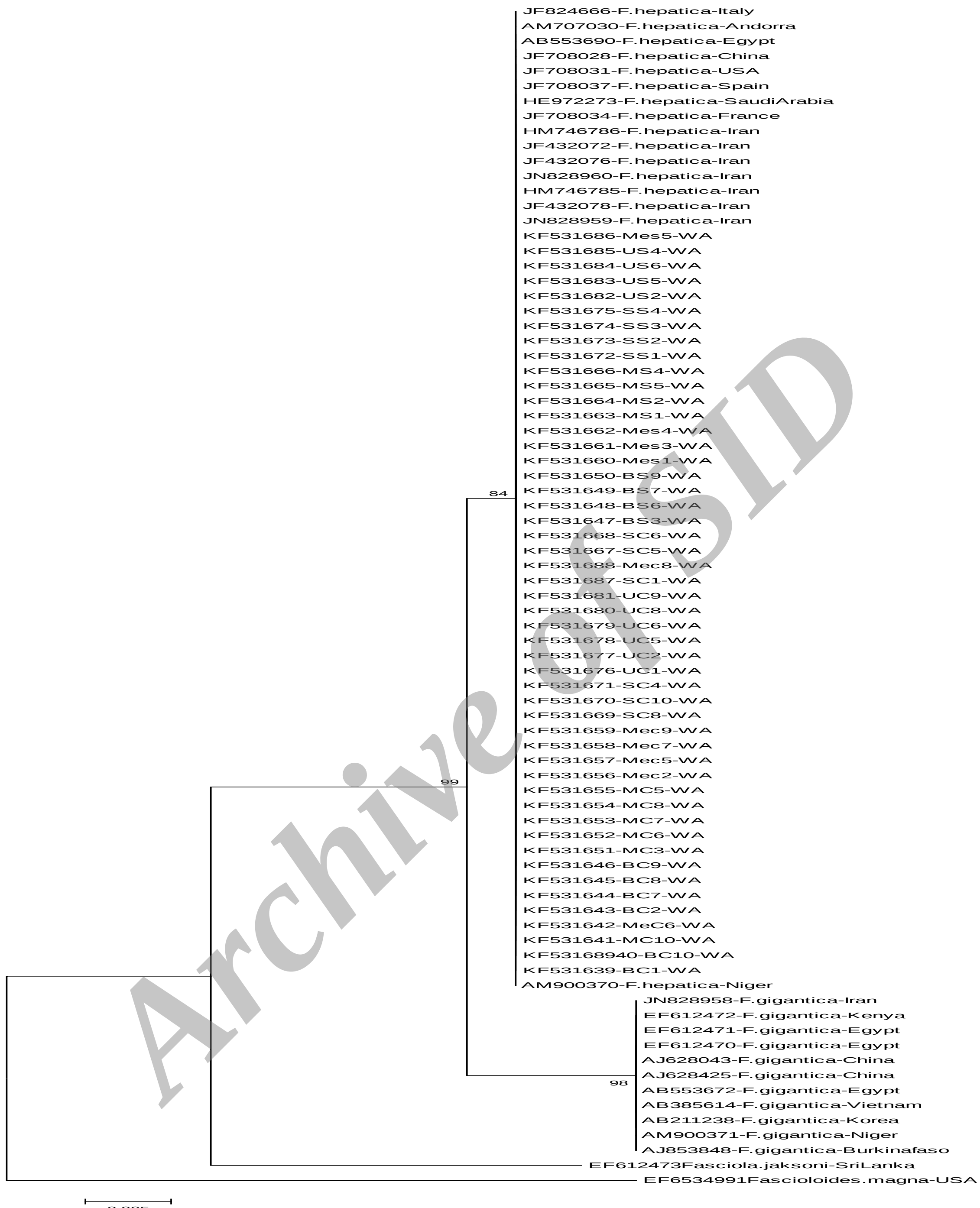

Fig. 1: The phylogenetic relationship of the *Fasciola* spp. isolates collected from cattle and sheep in West Azerbaijan Province of Iran and other *Fasciola* spp. isolates in different locations based on ITS1 sequence estimated by neighbor-joining algorithms

Fig. 2: The phylogenetic relationship of the *Fasciola* spp. isolates collected from cattle and sheep in West Azerbaijan Province of Iran and other *Fasciola* spp. isolates in different locations based on ITS2 sequence estimated by neighbor-joining algorithms

Moreover, the identity of *F. jaksoni* with *F. hepatica* and *F. gigantica* was 89.78%-90.66% and 90.86%-91.46%, respectively, whereas that of *F. magna* with both species was 88.09%-89.46% and 88.37%-88.98%, respectively.

Phylogenetic trees were constructed by comparing ITS1 and ITS2 sequences of this study with those of other Fasciolids species deposited in the GenBank (Table 1 and 2). When neighbor-joining algorithms were used to construct phylogenetic tree, a tree with similar topology was created that showed single differences in bootstrap values based on ITS1 sequences (Fig. 2). *F. hepatica* and *F. gigantica* were separated in two clusters. Reliable grouping among ITS1 sequences of *F. hepatica* from the current study and those from Iran, Asia (China and Saudi Arabia), Europe (Italy, Spain, and France), Africa (Egypt, Andorra, and Niger), and the USA are shown in Fig. 2.
Additional sequence analysis showed that the nucleotide frequencies of ITS1 and ITS2 regions were A=19.54%, T/U=27.42%, C=26.71%, G=26.33% as well as A=20.37%, T/U=31.21%, C=21.58%, and G=26.84%, respectively. For estimating ML values, a user-specified topology was used.

Discussion

Morphological differentiation among *F. hepatica* and *F. gigantica* species and their intermediate forms are difficult or even impossible (8, 40). In this investigation, less than one percent of isolates was morphologically characterized as *F. gigantica*. Based on morphometric criteria, such as body length, body weight, cephalic cone length, and length of area behind the testes, the intermediate form was not identified. However, phenotypic analysis of *Fasciola* isolates from Zanjan Province (Iran) showed remarkable differences in morphometric indices of animals (41). Using indices of body length and body weight, Ghavami and Rahimi identified *F. hepatica* (31%), *F. gigantica* (7%), and their intermediate forms (62%), but ITS2-RFLP genotypes and sequencing of the ITS2 confirmed that all of them were *F. hepatica* (41). "Simple, traditional microscopic measurements may be sufficient for morphometric characterization of Fasciolids, even in areas where the intermediate forms are present" (9). Due to the overlapping of morphometric indices between the two species, morphometric criteria are not sufficient for accurate differential diagnosis of the *Fasciola* species.

DNA-based molecular methods in comparison with other diagnostic methods for detection of isolates of *Fasciola* species are accurate and reliable (22). Therefore, various DNA markers are needed to identify *Fasciola* species, such as ITS1, 5.8s, ITS2, COI, and ND1 (2, 8, 28, 42, 43). The PCR-RFLP results of ITS1 region revealed that both *F. hepatica* and *F. gigantica* exist in Tabriz, Northwestern Iran (42). Recently, different molecular studies have shown that both *F. hepatica* and *F. gigantica*, and their intermediate forms prevail in Iran (8, 44). Notably, 150 sequences (50 sequences from each of ITS1, 5.8s, and ITS2) constructed in the current study showed 100% similarity with *F. hepatica*, indicating that this species is dominant between cattle and sheep in West Azerbaijan Province, Iran. Therefore, the absence of *F. gigantica* and the intermediate form may be due to different animal hosts or less prevalence in summer (cross-sectional sampling time in the current study). Rokni et al. also reported the presence of *F. hepatica* isolates in buffalo (eight isolates) and goat (one isolate) using ITS1 in Urmia (45). The origin of their samples was from Urmia district, whereas our samples were collected from five districts, including Urmia in center, Makou and Salmas in north, and Bukan and Mehabad in south of West Azerbaijan Province.

ITS1 and ITS2 sequences of *F. hepatica* from West Azerbaijan province showed no nucleotide variation, but the comparison with *F. gigantica* showed 1-2% nucleotide differences. The differences were because of five mismatches in ITS1 and six mismatches in ITS2 regions. When these differences were conducted to construct phylogenetic tree, *F. hepatica* and *F. gigantica* were differentiated into two separate clades by using both neighbor-joining algorithm. Interestingly, in tree deduced from ITS2, different topology was obtained and different algorithms were used. In neighbor-joining tree, sequences named as *F. gigantica* from Niger (GenBank ID: AM900371) and China (GenBank ID: AJ557569) were placed near *F. hepatica* clusters. These differences may be because of initial morpho-taxonomical mis-identification of a species.

Sequence variation in both regions was ranged from 435 bp to 470 bp in ITS1 and 361 to 362 bp in ITS2 region of *Fasciola* species from Iran (8, 41-43, 45). Variation between the size of ITS1 (428 bp) and ITS2 (366bp) in this study and the above-mentioned studies was because of using the ITS2 annotation tool for sequence analysis in the current study.

Conclusion

F. hepatica and *F. gigantica* are very similar species based on morphological and molecular data. Therefore, the present markers are insufficient to explain population genetic structure of *Fasciola* species. Moreover, accurate identification of *Fasciola* species is necessary for diagnosis and control of this parasite. Undoubtedly, this investigation would provide useful data and help improve new markers for species-specific characterization.

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