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Original Article

Morphological and Molecular Discrimination of *Fasciola* Species Isolated From Domestic Ruminants of Urmia City, Iran

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Abstract

Background: The trematodes of the genus *Fasciola* (the liver flukes) are among the well-known instances of food-borne parasites worldwide. Differentiation of *Fasciola* species is important because of their different transmission and epidemiological characteristics. The current study was undertaken to discriminate *Fasciola* species in the domestic ruminants of Urmia city, Iran.

Methods: Adult flukes were isolated from the naturally infected livers of the slaughtered water buffaloes and sheep. The flukes were initially identified based on morphological and morphometric parameters. A 618-bp-long fragment of the 28SrRNA gene of *Fasciola* was amplified by polymerase chain reaction (PCR). The amplified fragment was digested by *Dra*II or *Ava*II enzymes for a restriction fragment length polymorphism (RFLP) analysis and sequenced for the phylogenetic tree construction.

Results: Based on the morphometric examination, the flukes belonged to *F. hepatica*, *F. gigantica* and an intermediate *Fasciola* form. The PCR-RFLP analysis was able to differentiate *F. hepatica* from *F. gigantica*. While the phylogenetic reconstruction justified, to some extent, the morphological diagnosis, it failed to segregate *F. hepatica* from *F. gigantica* identified in this and the previous studies.

Conclusion: To resolve fully the problem of taxonomy and evolution in *Fasciola* species, employing a broad range of molecular and morphological approaches is necessary. This is crucial for epidemiological surveys and successful clinical management of their infection.

Introduction

Fasciolosis has traditionally been considered as an important human and animal disease caused by the taxonomically valid species of *Fasciola hepatica* (Linnaeus, 1758) and *F. gigantica* (Cobbold, 1856) (Trematoda: Fasciolidae) (1). Prevalence of fasciolosis in domestic animals has long been perceived in a wide range of geographical areas (2), while human fasciolosis has also been reported from 51 countries in the five continents (1). Nevertheless, *Fasciola* species seem to have uneven geographic distribution. *F. hepatica* is present in Europe, Africa, Asia, the Americas and Oceania, whereas the major infected areas by *F. gigantica* are the tropical regions of Africa and several Asian countries including Uzbekistan, Turkmenistan, Iran, Iraq, India and Pakistan (3, 4).

In Iran, sporadic cases of human fasciolosis have been documented by the late 1980s. However, fasciolosis has become an important zoonotic disease during the 1990s, when several large epidemics were explored in Gilan, north of Iran (5- 9). Fasciolosis has also been reported in the domestic animals from several parts of Iran, having higher prevalence rates in the southern part of the country compared to its northern regions (10).

Differentiation between the two common flukes, *F. hepatica* and *F. gigantica*, in the definitive hosts is essential because of their different transmission and epidemiological characteristics. However, their accurate recognition is generally awkward because of the substantial variations in their morphological features. In addition, hybridization between different *Fasciola* genotypes can give rise to the generation of new forms (11) and as a result, to more taxonomic complexities in the genus. Classically, the distinction between the *Fasciola* species has been made based on their morphological, i.e. morphometric and morphoanatomic, attributes. However, it has generally been accepted that specific differentiation of the liver flukes cannot be achieved solely by morphological examination (12), and their detailed genetic analysis is necessary (13). Application of molecular techniques permits the identification of parasitic trematodes at species or strain level, and at any stage of their life history (14-17).

This study aimed to characterize the frequent fasciolid taxa infecting some of the domestic ruminants of Urmia city, Iran.

Materials and Methods

Sampling and morphological examination

A total of 86 adult flukes of the genus *Fasciola* were isolated from the naturally-infected livers of the slaughtered water buffaloes (n=23) and sheep (n=63) at the abattoir of Urmia City, NorthWest Iran. The flukes were washed several times in 0.01M phosphate buffer saline (PBS, pH=7.2), stained by asetocarmine, examined under light microscope at 100× magnification, and identified based on the morphological and morphometric parameters (18- 21) (Table 1).

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from the *Fasciola* specimens by a modified phenol-chloroform method using cetyltrimethylammonium bromide (CTAB) (22). A fragment of the 28S ribosomal RNA (rRNA) gene of the *Fasciola* was amplified using two primers (forward: 5ˈ-ACGTGATTACCCGCTGAACT-3ˈ and reverse: 5ˈ-CTGAGAAAGTGCACT GACAAG-3ˈ) (13). The PCR was carried out by 25µl reaction containing 2µl of the genomic DNA (diluted 1:30), 2.5U of *Taq* DNA polymerase (Fermentas, Germany), 50µM of each dNTPs (CinnaGen, Iran), 2mM $MgCl_2$, 2.5µl PCR reaction buffer (10×) and 0.5µM of each primer. The reaction was performed in a Bioer XP thermal cycler and comprised an

initial DNA denaturation step at 94 ºC for 3min, followed by 30 cycles of DNA denaturation at 94 ºC for 30s, primer annealing at 60 ºC for 30s and primer extension at 72 ºC for 60s, and finally, an extension step at 72 ºC for 5min. A volume of 10 µl of each of the PCR products along with the positive (i.e. the PCR mixture including the known DNA samples of *F. hepatica* and *F. gigantica*) and negative (i.e. the PCR mixture excluding the DNA) controls were analysed by electrophoresis on 1.5% agarose gel for about 1.5h at 90V and visualized by staining with 1% ethidium bromide.

Table 1: Morphometric measures (Mean ± SD) of the liver flukes isolated from domestic ruminants of West Azarbaijan, Iran (n=86)

Host animal	Identified flukes	No. of flukes	Body size (cm)						
			BL	BW	DBS	LCC	WCC	DBVE	BL/BW
Buffalo (23)	*F. hepatica*	13	4.07±0.49	2.3±0.23	0.63±0.03	0.67±0.03	1.04±0.57	2.34±0.25	1.67-2.43
	F. gigantica	10	5.4± 0.19	0.9±0.16	0.33±0.03	0.35± 0.02	0.34±0.03	2.59±0.31	4.01-7.93
	Fasciola sp.	31	2.52±0.20	1.±0.11	0.26±0.06	0.22± 0.18	0.26±0.11	2.25±0.22	2.55-3.57
Sheep (63)	*F. hepatica*	15	2.11±0.2	1.2±0.15	0.28±0.08	0.41±0.07	0.23± 0.05	1.9±0.18	1.46-2.09
	F. gigantica	12	3.41±0.4	0.9±0.01	0.29±0.03	0.31± 0.06	0.37±0.06	2.37±0.42	3.77-6.28
	Fasciola sp.	26	2.82±0.3	1.04±0.09	0.23±0.03	0.25± 0.08	0.24±0.04	2.08±0.17	2.11-3.19

BL: body length; BW: body width; BL/BW: ratio of body length to body width; DBS: distance between suckers; LCC: length of cervical cone; WCC: width of cervical cone; DBVE: distance between ventral sucker and posterior end of body

Restriction fragment length polymorphism (RFLP) analysis

A PCR-RFLP procedure was developed to distinguish specifically *F. hepatica* and *F. gigantica*. The definite restriction enzymes were selected and lengths of the resulting restriction fragments were predicted by BioEdit software package (http://www.mbio.ncsu.edu). For restriction digestion, a total volume of 15µl of the digestion reaction containing 5 µl of the PCR product, 1µl of the restriction enzyme (*Ava*II or *Dra*II), 1.5 µl of enzyme buffer (Fermentas, Germany) and 7.5 µl of ddH_2O was prepared. The mixture was incubated at 37 ºC for 16 h and the digested DNA was run on 2% agarose gel, and visualized by ethidium bromide staining.

Phylogenetic analysis

The amplified gene fragments of the selected individuals belonging to both *F. hepatica* and *F. gigantica* were sequenced by Bioneer (South Korea) using an automated sequencer (3730xl/Bioneer 3730xl).

Table 2: Origin and GenBank accession numbers of the gene sequences used in our phylogenetic analysis

Fluke species	Accession no.	Country
	HM369289.1	Bulgaria
	HM369289.1	Bulivia
	HM369290	India
	AJ440788.1	Egypt
	AJ439738.1	Spain
F. hepatica	JQ999965	Iran[a]*
	JN811688.1	Iran[b]*
	JQ999966	Iran[c]*
	JQ999967	Iran[d]*
	JQ999969	Iran[e]*
	HM776945.1	India
	AB674553	Iran
	AJ440786.1	Egypt
F. gigantica	AY222245.1	Thailand
	JN811689.1	Iran[f]*
	JQ999968	Iran[g]*

* Sequenced in this study: A, WAP1; b, WAP2; c, WAP3; d, WAP4; e, WAP5; f, WAP; g, WAP2; WAP, West Azarbaijan Province.

Seven new sequences of Iranian *Fasciola* species obtained in this study were added to the Genbank. The sequences together with nine reference sequence data of *Fasciola* isolates from water buffaloes and sheep worldwide (Table 2) were aligned manually using BioEdit alignment editor. Phylogenetic tree was constructed using the 16-nucleotide sequences according to maximum likelihood (ML) method and Kimura 2-parameter model (23) by MEGA 5.0 software package (24). All positions containing gaps and missing data were eliminated, so that there were 536 nucleotide sites in the final dataset. The statistical significance of branching orders was calculated by the bootstrap resampling process (1000 replicates).

Results

Morphological comparisons

The morphometric measures (mean ± standard deviation) of the examined flukes are shown in Table 1. Based on the variations observed in their morphometric dimensions, the flukes were grouped into *F. hepatica*, *F. gigantica* and an intermediate form assigned as *Fasciola* sp. As can been seen in the table, all measured parameters were differing considerably among the three *Fasciola* forms isolated from both the host animals, i.e., 57 specimens had the intermediate morphological features between those of *F. hepatica* and *F. gigantica*. The differences in the body measures among the three-fasciolid forms are more obvious in those isolated from buffalos, while the body dimensions of the three forms obtained from sheep showed high level of similarity. However, body length and length of the cervical cone were the parameters differing considerably among the three *Fasciola* forms isolated from both the host animals.

PCR and RFLP patterns

The PCR amplified a portion of 618bp in length of the 28SrRNA gene of both *F. hepatica* and *F. gigantica* (Fig. 1).

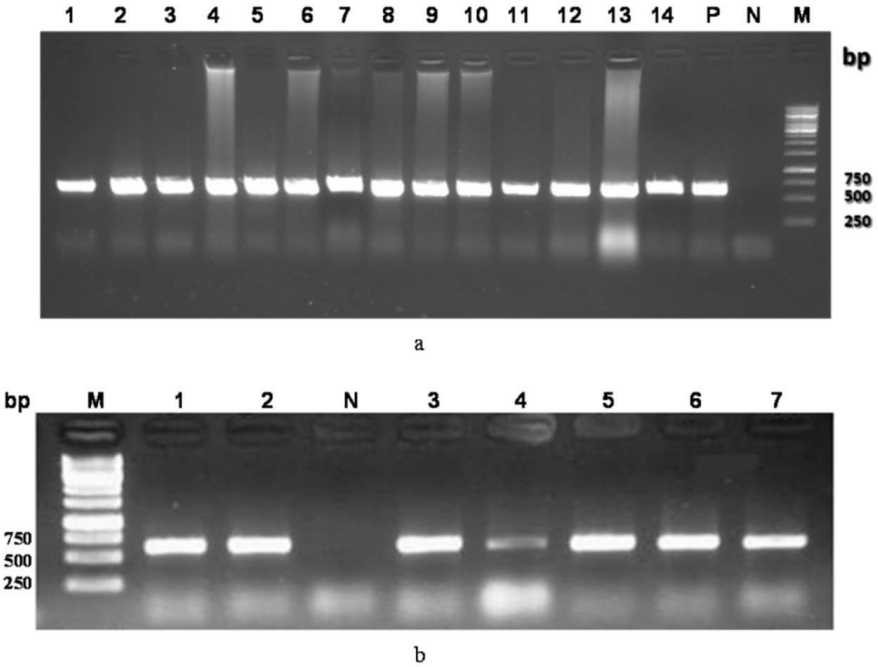

Fig.1: Agarose gel electrophoresis of 28SrRNA gene of the liver flukes. (a) Lanes 1-7, *F. hepatica* and lanes 8-14, *F. gigantica* from water buffaloes. (b) Lanes 1-2, *F. hepatica* and lanes 3-7, *F. gigantica* from sheep. Lane P, positive control; Lane N, negative control; Lane M, 250bp DNA size marker

Based on the RFLP analysis, restriction of the PCR products by the enzyme *Ava*II produced two fragments of 529 and 62bp for *F. hepatica* and 322 and 269bp for *F. gigantica* isolates (Fig. 2). *Dra*II enzyme digested the PCR product of *F. hepatica* in one position generating a fragment of 529bp in length, while the enzyme was unable to digest the PCR product of *F. gigantica* (Fig. 3).

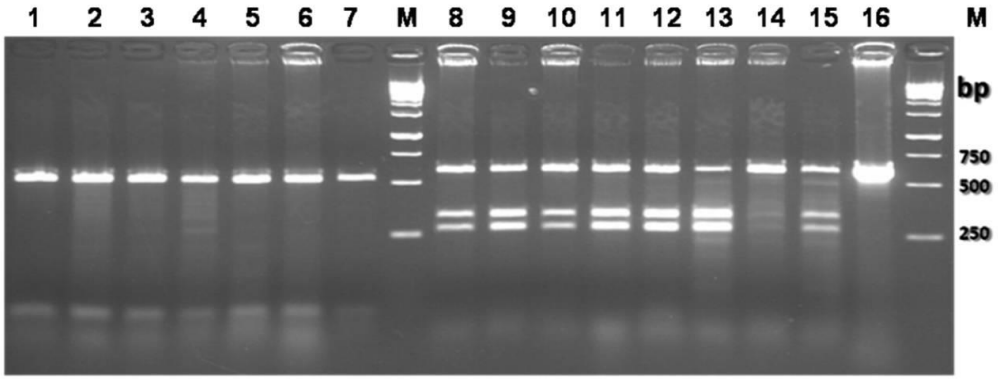

Fig. 2: Restriction fragment length polymorphism (RFLP) pattern of the PCR products of the liver flukes after digestion with *Ava*II restriction enzyme. Lanes 1-6, *F. hepatica*; Lanes 8-15, *F. gigantica*. Lanes 7 and 16, 618-bp-long PCR products of *F. hepatica* and *F. gigantica*, respectively; Lane M, 250bp DNA size marker

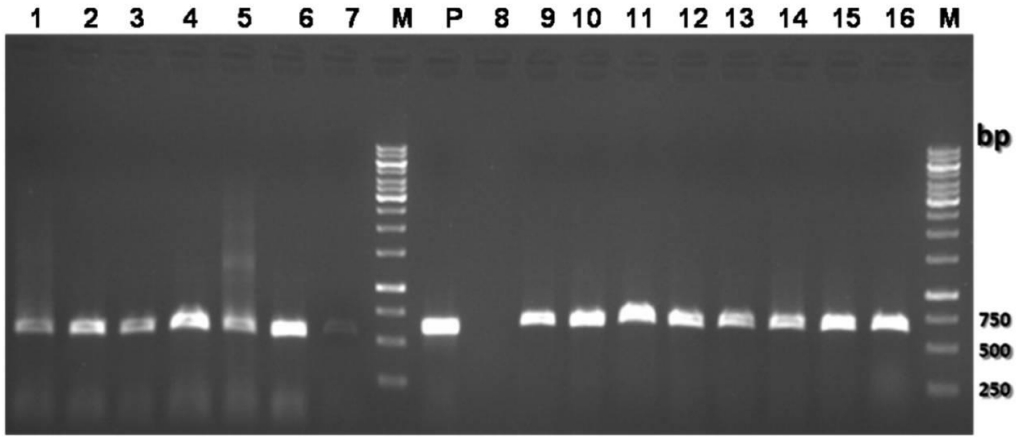

Fig. 3: Restriction fragment length polymorphism (RFLP) pattern of the PCR products of the liver flukes after digestion with *Dra*II restriction enzyme. Lanes 1-7, PCR products of *F. hepatica*; Lanes 9-16, PCR products of *F. gigantica*. Lane P: 618-bp-long PCR product of *F. gigantica*; Lane M, 250bp DNA size marker

Phylogenetic analysis

Phylogenic tree constructed by using 28SrRNA gene sequences of the *Fasciola* species is shown in Fig. 4. Based on the obtained topology, a large and highly supported (bootstrap value 99%) monophyletic group has been formed comprising all but two of the examined fasciolid taxa. Both *F. hepatica* and *F. gigantica*, including those isolated in this study, had polyphyletic positions on the tree. A group of the Iranian *Fasciola*, mostly belonging to *F. hepatica*, is located on the top of the tree, while a distinct sister group has been formed by both the species from this study in its basal part. In the uppermost clade, *F. hepatica* and *F. gigantica* from West Azerbaijan made a monophyletic group with a high statistical support (94%). In the middle of the tree, several *F.*

hepatica isolates from different countries including those from our study made sister groups but with moderate bootstrap supports. While isolates of *F. gigantica* from various localities (i.e. Iran, India, Egypt and Thailand) were sister taxa by high bootstrap support (99%), they also clustered with the groups consisting of *F. hepatica* isolates with a considerable replication rate (93%).

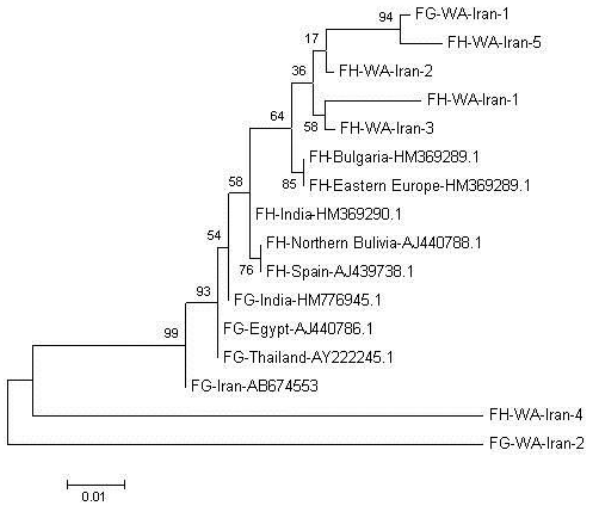

Fig. 4: Phylogenetic tree constructed by maximum likelihood (ML) method and based on the Kimura 2-parameter model using 16 nucleotide sequences of *Fasciola hepatica* and *F. gigantica*. The tree with the highest log likelihood (-1475.6886) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

Discussion

Characterization of the definite cause of fasciolosis in each geographic region has been a major global concern from both veterinary medicine and public health points of view. For several years, the disease was considered to be caused by either of the two-fasciolid species, *F. hepatica* and *F. gigantica*. Later, it was found that at least one intermediate form or likely yet-undescribed taxonomic units within the genus *Fasciola* are also responsible for fasciolosis worldwide (2). *Fasciola* is currently known as a genus comprising a range of the morphological forms with different ploidy levels (4), so that application of the biological species concept to whom has been problematic (2). Different *Fasciola* forms can also vary morphologically depending on their host species, so that the differences among the specimens from different hosts are even far greater than those usually observed between species of other flatworms (25).

Identification of fasciolid trematodes has traditionally been accomplished by their morphology and measurement of their morphometric dimensions. Based on our morphological examinations, all the three described fasciolid forms, i.e. *F. hepatica*, *F. gigantica* and their intermediate entity, were present in the studied water buffaloes and sheep. Periago et al. (26) showed that body length and roundness, the ratio of body length to body width (BL/BW) and the length of ventral sucker and the posterior end of the body were the main criteria discriminating *F. hepatica* from *F. gigantica* isolated from Egypt. The measure of BL/BW has been considered as one of the useful indices for differentiating *F. hepatica* from *F. gigantica* (27). Different BL/BW values have been reported for the two *Fasciola* species; however, Itagaki et al. (28) recommended that the BL/BW values of 1.29-2.80 and 3.40-6.78 more accurately match *F. hepatica* and *F. gigantica*, respectively. In the present study, 28 and 12 specimens had the BL/BW values in the range of those reported for *F. hepatica* and *F. gigantica*, respectively, while 57 specimens showed having intermediate values between the two size ranges. Both *Fasciola* species and their intermediate form have phenotypically been characterized from Iran (29, 30). Moghaddam (29) by considering the same morphological and morphometric criteria as those of our study identified the three forms of *Fasciola* in Mazandaran Province, northern Iran. Morphometric and allometric analyses have also confirmed the existence of the intermediate form of *Fasciola* in another northern province of Iran, Gilan (30).

The intermediate/hybrid *Fasciola* have also been reported from several other Asian countries including India (31), Korea (32, 33), Japan (34) and the Philippines (35). Occurrence of these intermediate creatures has not only raised the taxonomic complexity in the genus *Fasciola*, but has also made it more difficult to recognize the particular cause of fasciolosis in a certain region (36).

The invention of molecular techniques has provided more opportunities for the investigators to study species-specific epidemiology of fasciolosis and to explore its accurate prevalence rates in different localities (2, 25, 37, 38). The PCR-restriction enzyme patterning is a simple, rapid, and reliable method for differentiating *Fasciola* species in the areas where the species distributions overlap (39, 40). In the current study, the identities of two morphologically described fasciolid species, i.e. *F. gigantica* and *F. hepatica*, were verified by the RFLP analysis. The digestion patterns obtained in this analysis were identical to those in the study of Marcilla et al. (13), confirming the suitability of this method for discrimination of *Fasciola* isolates from distinct geographical locations. Furthermore, it was found that there was no host-specific restriction pattern for both *Fasciola* species. Shahbazi et al. (41) could differentiate *F. hepatica* from *F. gigantica* isolated from sheep and cattle in Tabriz, northwest Iran, by PCR-RFLP analysis of the first internal transcribed spacer (ITS1) of ribosomal DNA (rDNA). Mahami-Oskouei et al. (42) by application of PCR-RFLP analysis of a fragment of rDNA realized that the *Fasciola* samples isolated from sheep and cattle of three different geographical regions in Iran (Khorasan, Fars and East Azerbaijan provinces) belonged to either *F. hepatica* or *F. gigantica*.

Phylogenetic analysis using DNA sequence data is considered as a powerful approach for construction of the evolutionary relationships among different groups of organisms including the parasitic trematodes (17, 43, 44). However, the utility of this method seems to be dependent on the target DNA fragment and the rates of sequence divergence among the organisms in question (25). The phylogenetic tree constructed by using the sequences of 28SrDNA of the selected *Fasciola* species from diverse localities failed to fractionate properly *F. hepatica* from *F. gigantica*, as none of these two species formed a highly supported monophyletic assemblage. This can be attributed to the weakness of the 28SrRNA gene fragment for resolving the taxonomic problem in the genus *Fasciola*. One other potential reason for this 'taxonomic failure', which is worth noting, is that at least part of the recorded *Fasciola* isolates worldwide might have erroneously been identified. Such misidentification is not necessarily due to the technical errors, but more importantly, can be because the conventional criteria for discriminating the fasciolid trematodes have limited validity. Ashrafi et al. (45) compared the sequences of the second internal transcribed spacer of the rDNA (ITS2) and found that *F. hepatica* from Iran was genetically identical to those present in Spain and the Northern Bolivian Altiplano. Ghavami et al. (46) by phylogenetic and RFLP analyses of the ITS2 gene region discovered that all the three morphologically-different adult fasciolids isolated from sheep and cattle of Zanjan Province, Iran were in fact *F. hepatica*. Amor et al. (47) has recently demonstrated the existence of an endemic intermediate *Fasciola* form in northern Iran based on the phylogenetic analysis of the nuclear rDNA.

Employing a number of molecular analyses has met with considerable success in characterizing *F. hepatica*, *F. gigantica* and their intermediate forms. These analyses included PCR-linked single strand conformation polymorphism (PCR-SSCP) and sequencing of ITS1 rDNA (2), sequence-related amplified polymorphism (SRAP) (48), sequencing of ITS2 (33), ITS1 and mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nd*-1) (49) genes and microsatellite loci (37). Walker et al. (22) could also discriminate two *Fasciola* groups by comparison of their

28rDNA and entire mitochondrial genome. Therefore, choosing an appropriate genetic marker (i.e., portion of DNA with highest intrageneric divergence) can be of eminent importance for studying the taxonomy of liver flukes. Such marker should also be able to identify clearly the hybrid/intermediate forms, which are bearing nuclear and mitochondrial genome of both *F. hepatica* and *F. gigantica* (4).

Conclusion

The traditional morphometric indices are insufficient for the aim of species-specific diagnosis of *Fasciola* isolates, especially when the existence of their larval stages is to be detected in the intermediate hosts. Thus, discovering new discriminative morphological criteria and their application together with a combination of molecular tools can be helpful for unraveling the problem of *Fasciola* taxonomy and correct estimation of its epidemiological patterns.

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References

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol. 2005; 35:1255-78.
2. Lin RQ, Dong SJ, Nie K, Wang CR, Song HQ, Li AX, Huang WY, Zhu XQ. Sequence analysis of the first internal transcribed spacer of rDNA supports the existence of the intermediate *Fasciola* between *F. hepatica* and *F. gigantica* in mainland China. Parasitol Res. 2007; 101(3): 813-7.
3. Mas-Coma S. Human fascioliasis. In: Waterborne Zoonoses: Identification, Causes and Control. Cotruvo JA, Dufour A, Rees G, Bartram J, Carr R, Cliver DO, Craun GF, Fayer R, Gannon VPJ, editors. World Health Organization (WHO), London, UK: IWA Publishing; 2004.
4. Raina OK, Jacob SS, Sankar M, Bhattacharya D, Bandyopadyay S, Varghese A, Chamuah JK, Lalrinkima H. Genetic characterization of *Fasciola gigantica* from different geographical regions of India by ribosomal DNA markers. J Parasit Dis. 2013; doi: 10.1007/s12639.013.0276.7.
5. Massoud J. Fascioliasis outbreak of man and drug test (Triclabendazol) in Caspian littoral, northern part of Iran, 1989. Bull Soc Fr Parasitol. 1990; 8 (Suppl 1): 438.
6. Pourtaghva M, Shafi A, Saberi A, Bahar K, Solaymanlou F. Fasciolase en Iran. Bull Soc Fr Parasitol. 1990; 8(Suppl. 1): 404.
7. Yadegari D, Forghanparast K, Assmar M. Investigation of an epidemic of fascioliasis in north Iran. Bull Soc Fr Parasitol. 1990; 8 (Suppl2): 868.
8. Assmar M, Milaninia A, Amir-Khani A, Yadegari AD, Forghanparast K, Nahravanian H, Piazak N, Esmayli A, Hovanesian A, Valadkhani Z. Seroepidemiological investigation of fascioliasis in northern Iran. Medical Journal of the Islamic Republic of Iran 1991; 5, 23-7.
9. Forghanparast K. Clinicoepidemiological study of human fascioliasis in Guilan. Journal of the Guilan University of Medical Sciences. 1993; 2: 63-4.
10. Yakhchali M, Ghobadi K. A survey on helminthes infection of liver and economic loss in slaughtered sheep of Urmia slaughterhouse, Iran. Iranian Veterinary Journal. 2005; 11: 60-5. (in Persian with English abstract)
11. Mas-Coma S, Funatsu IR, Bargues MD. *Fasciola hepatica* and Lymnaeid snails occurring at very high altitude in South America. Parasitol. 2001; 123: 115-27.
12. Periago MV, Valero MA, El Sayed M, Ashrafi K, El Wakeel A, Mohamed MY, Desquesnes M, Curtale F, Mas-Coma S. 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: 51-8.
13. Marcilla A, Bargues MD, Mas-Coma S. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. Mol Cell Probes. 2002; 16:327-33.

14. Lockyer AE, Olson PD, Littlewood DTJ. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the *Neodermata* (Platyhelminthes): implications and a review of the cercomer theory. Biol J Linn Soc. 2003; 78: 155-71.
15. Tkach VV, Littlewood DT, Olson PD, Kinsella JM, Swiderski Z. Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). Syst Parasitol. 2003; 56(1): 1-15.
16. Wilson WD, Johnson PT, Sutherland DR, Moné H, Loker ES. A molecular phylogenetic study of the genus *Ribeiroia* (Digenea): trematodes known to cause limb malformations in amphibians. Parasitol. 2005; 91(5): 1040-5.
17. Choudhury A, Rosas Valdez R, Johnson RC, Hoffmann B, De León GP. The phylogenetic position of allocreadiidae (Trematoda: Digenea) from partial sequences of the 18S and 28S ribosomal RNA genes. Parasitol. 2007; 93(1): 192-6.
18. Yamaguti S. Systema helminthum: The digenetic trematodes of vertebrates. Vol. I. New York, USA: Interscience; 1958.
19. Sahba GH, Arfaa F, Farahmandian I, Jalali H. Animal fascioliasis in Khuzestan, southwestern Iran. J Parasitol. 1972; 58**:** 712**-**6.
20. Soulsby EJL. Helminths, Arthropods and Protozoa of domesticated animals. 7th ed. London, UK: Baillière Tindall; 1982.
21. Salahimoghaddam A. Epidemiology of human fascioliasis in Iran. Journal of Kerman University of Medical Sciences. 2009; 16(4): 385-98. (in Persian with English abstract)
22. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. New York, USA: Cold Spring Harbor Laboratory Press; 2002.
23. Walker SM, Prodöhl P, Hoey EM, Fairweather I, Hanna RE, Brennan G, Trudgett A. Substantial genetic divergence between morphologically indistinguishable populations of *Fasciola* suggests the possibility of cryptic speciation. Int J Parasitol. 2012; 42(13-14): 1193-9.
24. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0., Molecular Biology and Evolution. 2007; 24: 1596-9.
25. Dosay-Akbuluta M, Trudgettb A, Stanhope M. Understanding genetic diversity of the liver fluke *Fasciola hepatica*. Z Naturforsch. 2005; 60: 774-8.
26. Valero MA, Panova M, Mas-Coma S. Phenotypic analysis of adults and eggs of *Fasciola hepatica* by computer image analysis system. J Helminthol. 2005; 79: 217-25.
27. Itagaki T, Sakaguchi K, Terasaki K, Sasaki O, Yoshihara S, Van Dung T. Occurrence of spermic diploid and aspermic triploid forms of *Fasciola* in Vietnam and their molecular characterization based on nuclear and mitochondrial DNA. Parasitol Int. 2009; 58: 81-5.
28. Moghaddam AS, Massoud J, Mahmoodi M, Mahvi AH, Periago MV, Artigas P, Fuentes MV, Bargues MD, Mas-Coma S. Human and animal fascioliasis in Mazandaran province, northern Iran. Parasitol Res. 2004; 94: 61-9.
29. 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: 249-60.
30. Varma AK. On *Fasciola indica* n. sp. with some observations on *F. hepatica* and *F. gigantica*. J Helminthol. 1953; 27: 185-98.
31. Chu JK, Kim YK. Taxonomical study on the Fasciolidae in Korea. Parasitol. 1967; 5: 139-46.
32. Choe SE, Nguyen TT, Kang TG, Kweon CH, Kang SW. Genetic analysis of *Fasciola* isolates from cattle in Korea based on second internal transcribed spacer (ITS-2) sequence of nuclear ribosomal DNA. Parasitol Res. 2011; 109 (3): 833-9.
33. Oshima T, Akahane H, Shimazu T. Patterns of the variation of the common liver fluke (*Fasciola* sp.) in Japan. I. Variations in the sizes and shapes of the worms and eggs. Jpn J Parasitol. 1968; 17: 97-105. (in Japanese with English abstract)
34. Kimura S, Shimizu A, Kawano J. Morphological observation on liver fluke detected from naturally infected carabaos in the Philippines. Sci Rept Fac Agr Kobe Univ. 1984; 16: 353-7.
35. Mas-Coma S, Bargues MD. Human liver flukes: a review. Res Rev Parasitol. 1997; 57:145-218.
36. Vilas R, Vázquez-Prieto S, Paniagua E. Contrasting patterns of population genetic structure of Fasciola hepatica from cattle and sheep: implications for the evolution of anthelmintic resistance. Infect Genet Evol. 2012; 12(1): 45-52.
37. 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.
38. Rokni MB, Mirhendi H, Behnia M, Fasihi Harandi M, Jalalizand N. Molecular charac-

terization of *Fasciola hepatica* isolates by RAPD-PCR and Ribosomal ITS1 sequencing. Iranian Red Crescent Medical Journal. 2010a; 12(1): 27-32.
39. Rokni MB, Mirhendi H, Mizani A, Mohebali M, Sharbatkhori M, Kia EB, Abdoli H, Izadi S. Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR restriction enzyme method. Exp Parasitol. 2010b; 124: 209-13.
40. Shahbazi A, Akbarimoghaddam M, Izadi S, Ghazanchaii A, Jalali N, Bazmani A. Identification and Genetic Variation of *Fasciola* Species from Tabriz, North-Western Iran. Iran J Parasitol. 2011; 6(3): 52-9.
41. Mahami-Oskouei M, Dalimi A, Forouzandeh-Moghadam M, Rokni MB. Molecular identification and differentiation of *Fasciola* isolates using PCR-RFLP method based on Internal Transcribed Spacer (ITS1, 5.8S rDNA, ITS2). Iranian J Parasitol. 2011; 6(3): 35-42.
42. Tkach VV, Littlewood DT, Olson PD, Kinsella JM, Swiderski Z. Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). Syst Parasitol. 2003; 56(1): 1-15.
43. Wilson WD, Johnson PT, Sutherland DR, Moné H, Loker ES. A molecular phylogenetic study of the genus *Ribeiroia* (Digenea): trematodes known to cause limb malformations in amphibians. Parasitol. 2005; 91(5): 1040-5.
44. Ashrafi K, Massoud J, Holakouei Nainei K, Jo-Afshani MA, Mahmoodi M, Ebedati N. Nuclear ribosomal DNA ITS-2 sequence characterization of *Fasciola hepatica* and *Galba truncatula*. Iran J Publ Health. 2007; 36: 42-9
45. Ghavami MB, Rahimi P, Haniloo A, Mosavinasab SN. Genotypic and phenotypic analysis of *Fasciola* isolates. Iran J Parasitol. 2009; 4(3): 61-70.
46. Amor N, Halajian A, Farjallah S, Merella P, Said K, Slimane BB. Molecular characterization of *Fasciola* spp. from the endemic area of northern Iran based on nuclear ribosomal DNA sequences. Exp Para. 2011; 128: 196-204.
47. Li QY, Dong SJ, Zhang WY, Lin RQ, Wang CR, Qian DX, Lun ZR, Song HQ, Zhu XQ. Sequence-related amplified polymorphism, an effective molecular approach for studying genetic variation in *Fasciola* spp. of human and animal health significance. Electrophoresis. 2009; 30(2): 403-9.
48. Chaichanasak P, Ichikawa M, Sobhon P, Itagaki T. Identification of *Fasciola* flukes in Thailand based on their spermatogenesis and nuclear ribosomal DNA, and their intraspecific relationships based on mitochondrial DNA. Parasitol Int. 2012; 61(4): 545-9.
49. Itagaki T, Tsutsumi K, Ito K, Tsutsumi Y. Taxonomic status of the Japanese triploid forms of *Fasciola*: comparison of mitochondrial ND1 and COI sequences with *F. hepatica* and *F. gigantica*. J Parasitol. 1998; 84: 445-8.