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Research Article
Spermatogenic and Phylo-molecular Characterizations of Isolated *Fasciola* Spp. From Cattle, North West Iran

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Abstract

Background and Objective: Fascioliasis is economically important to the livestock industry that caused with *Fasciola hepatica* and *Fasciola gigantica.* The objective of this study was to identify these two species *F. hepatica* and *F. gigantica* by using nuclear and mitochondrial markers (ITS1, ND1 and CO1) and have been employed to analyze intraspecific phylogenetic relations of *Fasciola* spp. **Materials and Methods:** Approximately 150 *Fasciola* specimens were collected, then stained with haematoxylin-carmine dye and observed under an optical microscope to examine for the existence of sperm. The ITS1 marker was used to identify different *Fasciola* and phylogenetic analysis based on ND1 and CO1 sequence data were conducted by maximum likelihood algorithm. **Results:** *Fasciola* samples were separated into 2 groups. Almost all specimens had many sperms in the seminal vesicle (spermic fluke) and one fluke did not contain any sperm in the seminal vesicle. The aspermic sample had *F. gigantica* RFLP pattern with ITS1 gene. Phylogenetic analysis based on NDI and COI sequence data were conducted by maximum likelihood showed a similar topology of the trees obtained particularly for *F. hepatica* and *F. gigantica.* **Conclusion:** This study demonstrated that aspermic *Fasciola* found in this region of Iran has same genetic structures through the spermic *F. gigantica* populations in accordance to phylogenetic tree.

Key words: *Fasciola* spp., spermatogenesis, nicotinamide adenine dinucleotide dehydrogenase subunit I, cytochrome oxidase subunit I, cattle, phylogenetic, aspermic

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The *Fasciola* species are found in domestic ruminants and may occasionally affect humans. Fascioliasis is economically important to the livestock industry. It is estimated that economic losses is over two billion US dollars annually. Human infection has been reported worldwide and is serious health problem[1]. About 180 million people are at risk of infection worldwide in endemic areas[2].

The causative species of the disease are *Fasciola hepatica* and *Fasciola gigantica*. *Fasciola hepatica* is distributed worldwide, whereas *F. gigantica* is restricted to tropical regions and has been found in Africa as well as South and Southeast Asia[3].

Fasciola hepatica and *F. gigantica* can be differentiated based on morphological criteria such as body size, ratio of body length to width and shape; however, these criteria are not always reliable because of the morphological diversity within the species[4].

These species are meiotically functional diploid and therefore they have abundant mature sperm (spermic fluke) in the seminal vesicles[5]. This male reproductive organ is the common predominant characteristic of both species[6]. On the other hand intermediate *Fasciola* flukes, which have morphological characteristic intermediate between *F. hepatica* and *F. gigantica* and no sperm (aspermic fluke) in their seminal vesicles have been found in Asian countries[7]. *Fasciola* flukes reproduce bisexually and aspermic *Fasciola* probably reproduce parthenogenetically. The hybrid origin of aspermic *Fasciola* flukes was strongly suggested by the presence of the *F. hepatica*/*F. gigantica* type, which includes DNA fragments of both *F. hepatica* and *F. gigantica*.

These two species can be identified using nucleotide sequences of the nuclear ribosomal internal transcribed spacer 1 (ITS1) and 2 (ITS2)[8,9]. Additionally, DNA sequences of mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit I (ND1) and cytochrome c oxidase subunit I (CO1) genes have been employed to analyze intraspecific phylogenetic relations of *Fasciola* spp[10,11].

There are some reports from Iran ruminant's fascioliasis particularly in cattle and buffaloes based on geography and climate variability[12,13]. Also, there have been no valuable reports on molecular and spermatogenic characterization of *Fasciola* flukes. The objective of this study was to characterized the *Fasciola* flukes from North West of Iran for first time based on spermatogenic status to identify of probably hybrid of *Fasciola* sp. in Iran and PCR-RFLP method in ribosomal ITS1 and mitochondrial ND1 and CO1 sequences for phylogenetic analysis.

MATERIALS AND METHODS

Sample collections and spermatogenic status assessment: One hundred and fifty *Fasciola* specimens were collected from the bile ducts of 4 buffaloes, 38 cattle at slaughter house located in 7 different geographical locations in the North West of Iran (Azerbaijan) from January-September, 2015 (Table 1). This region of Iran borders Iraq, Turkey and Armenia with different mountain climate and biosphere reserve. *Fasciola* flukes were collected, washed in 0.9% saline solution, fixed in 70% ethanol between two glass slides and transported to the laboratory for further studies. The whole body of *Fasciola* including seminal vesicle in the anterior part of worm was stained with haematoxylin-carmine dye and observed under an optical microscope to examine for the existence of sperm[14]. Prior to staining, a small posterior part of fluke was used for DNA extraction.

Table 1: Profiles of *Fasciola* haplotypes and their accession no. in North West of Iran

	Samples			Sperm in seminal		Haplotype existence	
Locations	Host	No.	No. of flukes	vesicles	ITS1	ND1	CO1
Urmia	Cattle	6	30	+	*F. gigantica*	Fg-N1 (KX021268)	Fg-C1 (KX021275)
						Fg-N2 (KX021269)	Fg-C2 (KX021276)
				+	*F. hepatica*	Fh-ND1 (KX021266)	Fh-CO1 (KX021274)
Maragheh and Bonab	Cattle	12	39	+	*F. gigantica*	Fg-N1	Fg-C1
						Fg-N2	Fg-C2
				+	*F. hepatica*	Fh-ND2 (KX021267)	Fh-CO2 (KX021274)
						Fh-ND3 (KX021271)	Fh-CO3 (KX021278)
Azarshahr and Gogan	Buffalo	4	9	+	*F. hepatica*	Fg-N1	Fg-C1
	Cattle	5	17	-	*F. gigantica*	Fg-N3 (KX021270)	Fg-C3 (KX021278)
Miandoab and Mahabad	Cattle	15	55	+	*F. gigantica*	Fg-N1	Fg-C1
						Fg-N2	Fg-C2
				+	*F. hepatica*	Fh-ND4 (KX021272)	Fh-CO4 (KX021279)

Fg: *Fasciola gigantica*, Fh: *Fasciola hepatica*

DNA extraction and amplification: Total DNA was extracted from each fl uke with a high pure PCR template preparation Kit (Dynabio®, Takapouzist, Iran), according to the manufacturer's instructions and stored at -20°C until use. DNA fragments of each target regions (nuclear ITS1 region and mitochondrial ND1 and CO1 regions) were amplified by Polymerase Chain Reaction (PCR) using a pair primer. Total volume of reaction was 15 µL containing 1.5 µL DNA template, 5 µL distilled water, 10 p mol of each primers (Forward and Reverse) and 7.5 µL master mix (Amplicon®). The primer sets used were ITS1-F (5-TTGCGCTGATTACGTCCCTG-3) and ITS1-R (5-TTGGCTGCGCTCTTCATCGAC-3) for ITS1 region and Ita 10 (5-AAGGATGTTGCTTTGTCGTGG-3) and Ita 2 (5-GGAGTAC GGTTACATTCACA-3) for ND1 region and Ita 8 (5-ACGTTG GATC ATAAGCGTGT-3) and Ita 9 (5-CCTCATCCAAC ATAACC TCT-3) for CO1. Reaction cycles consisted of an initial denaturing step at 94°C for 90 sec, followed by 35 cycles at 94°C for 90 sec, 53°C (ITS1) or 55°C (ND1 and CO1) for 90 sec and 72°C for 120 sec, with a final extension at 72°C for 10 min using a Eppendorf Mastercycler gradient thermocycler. The DNA fragments were analyzed by 1.5% agarose gel electrophoresis.

Restriction fragment length polymorphism of amplified DNA (PCR-RFLP): The ITS1 marker was used to identify different *Fasciola*. Briefly, the reaction volume of 10 µL contained 5 µL of PCR products with approximately 680 bp fragments, 1 U of the *Rsa*I restriction enzyme and 1 µL of manufacturer-supplied reaction buffer (Cinagen®, Iran). After incubation at 37°C for 1 h and heat inactivation of *Rsa*I at 65°C for 20 min, the digestions were exposed to 1.5% agarose gel electrophoresis and visualized by with ethidium bromide. The fragment data were recorded with UV illumination (UVITEC).

Sequencing and phylogenetic analysis: The products of ND1 and CO1 were directly sequenced by Bioneer Company using the same primers which were used in the PCR. The sequences were aligned and compared with those of existing sequences from the region, related to *Fasciola* spp. Available in the GenBank, using the chromas and multiple alignments were performed with data related to *Fasciola* spp. From Iran and other countries deposited in GenBank. Phylogenetic analysis based on ND1 and CO1 sequence data were conducted by Maximum Likelihood (ML) algorithm using MEGA6 software. All characters were run unordered and equally weighted. Alignment gaps were treated as missing data. Bootstrap analysis were conducted using 1000 replicates.

RESULTS

Spermatogenesis: *Fasciola* samples were separated into 2 groups consisting of flukes with spermatogenic ability based on the presence of sperm in the seminal vesicles (Table 1). Almost all specimens had many sperms in the seminal vesicle (spermic fluke) and one fluke did not contain any sperm in the seminal vesicle (aspermic fluke).

ITS1 types and species identification: The RFLP pattern of 149 spermic *Fasciola* isolates showed RFLP pattern of *F. hepatica* and *F. gigantica.* The aspermic sample had *F. gigantica* RFLP pattern.

ND1 and CO1 haplotypes and phylogeny: The ND1 fragments (approximately 535 bp) and CO1 fragments (approximately 438 bp) were amplified for all specimens. Partial sequences of NDI and COI showed 45 and 36 variable sites and also yielded 6 and 8 haplotypes respectively, which were represented by Accession No. KX021266 to KX021279 and deposited in the GeneBank (Table 1). Phylogenetic analysis based on NDI and COI sequence data were conducted by maximum likelihood using MEGA6 with lung fluke, *Paragonimus westermani* (Accession no. AF219379) designated as an out group branch showed in Fig. 1 and 2.

DISCUSSION

This is the first study on the basis of spermatogenesis characterization of *Fasciola* sp. in Iran. This study has shown that both spermic and aspermic *Fasciola* existed in the North West of Iran on the basis of the staining (haematoxylin-carmine dye).

The differentiation of *Fasciola* species is crucial due to their epidemiological patterns. All of the specimens in this study obtained from cattle (*Bus taurus*) that traditionally were nurtured. Cattle are the host for both *Fasciola* species in this region of Iran. The ratio of body length and width (BL/BW) has been considered to be one of the useful criteria for discrimination of both species in *Fasciola*[15]. There is only one aspermic sample in this region of Iran and the size of this fluke is similar in range with *F. gigantica*.

Some reports from the North West of Iran were detected *Fasciola* spp., with only ITS-RFLP method and constructed phylogenetic trees using nuclear rDNA but this method is not

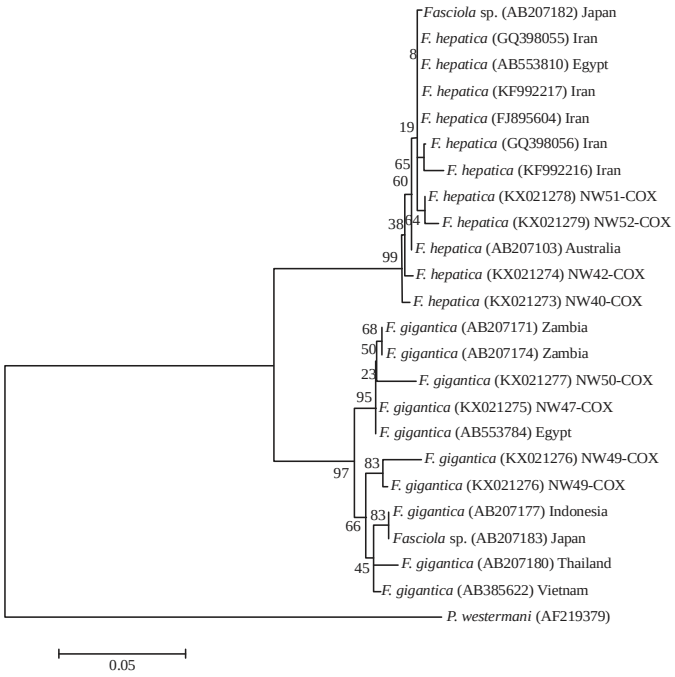

Fig. 1: A phylogenetic tree of *Fasciola* spp. based on ND1 gene in North West of Iran

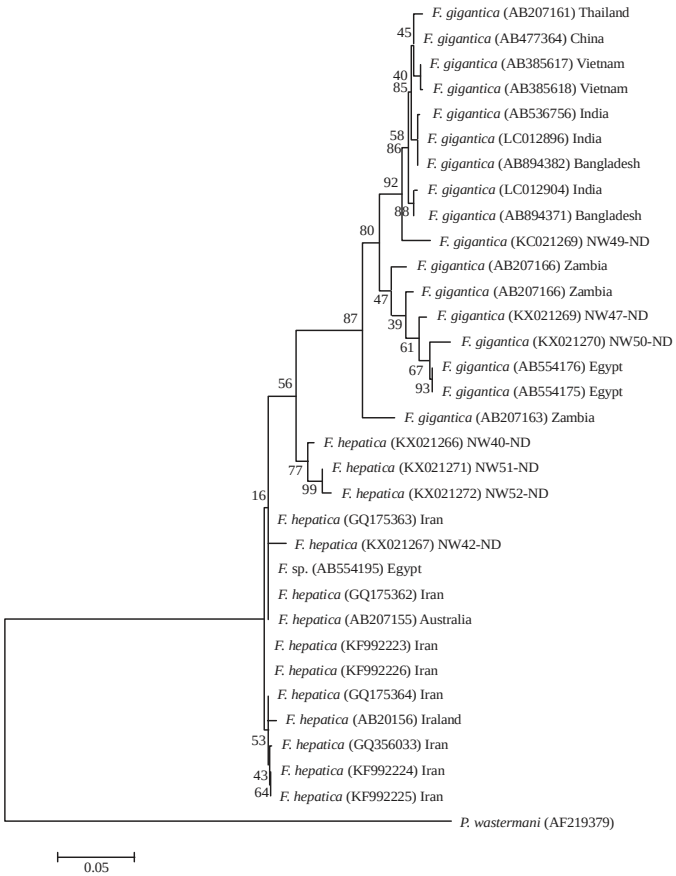

Fig. 2: A phylogenetic tree of *Fasciola* spp. based on CO1 gene in North West of Iran

sufficient for *Fasciola* spp. taxonomic characterization[16-18]. The existence of an endemic intermediate *Fasciola* form in Northern Iran based on the phylogenetic analysis of the nuclear rDNA[19]. Base on morphological study and phenotypic analysis of adults *Fasciola* flukes, there are *F. hepatica*, *F. gigantica* and intermediate forms in the endemic region of Gilan, Iran[4]. Although both species can generally be distinguished on the basis of their morphology but the use of molecular methods and markers are often necessary for species confirmation and to identification of the intermediate forms[15].

Previous studies have shown that molecular phylogeny with mtDNA, including ND1 and CO1 can be effectively used for proper differentiation haplotypes and intermediate forms *Fasciola* sp[10,20]. Molecular analysis of the intermediate forms are mainly performed in the Far East Asian countries such as China, Japan, Korea and Vietnam[14]. Existence of several haplotype in North West of Iran demonstrated the effect of ecology and climate which indicates further need for studies in other regions of Iran. These differences showed in North East of Iran before[12].

Iran is a vast country with numerous climate regions which may indicate variable *Fasciola* haplotypes. The phylogenetic analysis using Neighbor Joining as well as maximum likelihood methods showed separate position on both trees (Fig. 1, 2), which supported with different bootstrap values (%) from 1000 replicates probably reflecting the prominent nucleotide that may be attributed to host range and genetic migration. Also *P. westermani* (AF219379) was used as outgroup. Host specificity, drug susceptibility or resistance and differences in virulence may be influences molecular variation[21].

CONCLUSION

It can be concluded that *F. hepatica* found in this region of Iran is closely related to *F. hepatica* found throughout of Iran and Asian countries like India and Bangladesh. Also, in this region of Iran, new aspermic haplotypes of *F. gigantica* are similar to African countries such as Egypt and Zambia and this aspermic *Fasciola* belonged to *F. gigantica* clades. Further genetic studies of *Fasciola* parasite obtained from hosts in different regions are necessary to show the diversity of *Fasciola* spp. and help the miserable hybrid of *Fasciola* sp. This is the first report of existence spermic and aspermic *Fasciola* in the North West of Iran.

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SIGNIFICANCE STATEMENTS

Fasciola hepatica and *Fasciola gigantica* possess abundant mature sperms in their seminal vesicles and thus, they reproduce bisexually. On the other hand, aspermic *Fasciola* flukes reported from Asian countries, which have no sperm in their seminal vesicles, probably reproduce parthenogenetically. The hybrid origin of aspermic *Fasciola* flukes was strongly suggested by the presence of the *F. hepatica*/ *F. gigantica* type, which includes DNA fragments of both *F. hepatica* and *F. gigantica*.

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