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Original Article

Rapid Detection and Identification of *Fasciola* spp. and *Dicrocoelium* spp. Isolated from the Ruminant Livestock of Northwest Iran Using High-Resolution Melting Analysis (HRM)

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Abstract

Background: The liver flukes of the *Fasciola* species and *Dicrocoelium* spp. are recognised as parasites of domestic and wild herbivores. Both species of *F. hepatica* and *F. gigantica* as well as *D. dendriticum* are distributed in Iran. The present study aimed to identify *Fasciola* spp. and *Dicrocoelium* spp. using mitochondrial *Cox1* (cytochrome c oxidase I) gene by HRM method.

Methods: Totally, thirty infected liver specimens were collected from the sheep (n:23) and cattle (n:7) at the abattoirs of Qazvin Province, northwest Iran in 2022. DNA extraction and PCR amplification of *Cox1* gene were conducted by HRM technique. DnaSP v.5.0 was used for compression of diversity indices of ribosomal *28S rDNA* and mitochondrial *Cox1* markers of *Dicrocoelium* spp. The taxonomic status of *Dicrocoelium* spp. was performed by sequencing and phylogenetic analysis.

Results: Overall, 26 and 4 isolates were identified as *F. hepatica* and *F. gigantica*, respectively. *D. dendriticum* was the sole infecting species of *Dicrocoelium* revealed by HRM analysis. Genomic analysis showed a moderate (*28S rDNA* genes: 0.600±0.215) to high (*Cox1*: 0.733±0.155) haplotype diversity for *D. dendriticum*.

Conclusion: The parasite-dependent mitochondrial gene (*Cox1*) could identify a higher genetic diversity of *D. dendriticum* compared to nuclear *28S rDNA* gene. HRM technique in the present study found to be a reliable technique for identification and genetic diversity of liver flukes but more comprehensive and in-depth studies in different parts of the country are needed.

Keywords: High-resolutionmelting (HRM); *Fasciola hepatica*; *Fasciola gigantica*; *Dicrocoelium dendriticum*; CoxI

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Introduction

Digenetic helminthes species of *Fasciola* and *Dicrocoelium* are often infect the ruminants in different parts of the world, although they are also observed in the bile ducts of definitive hosts (1).

Fascioliasis mainly caused by two species including *F. hepatica* and *F. gigantica*; a clinical condition considered as a global zoonotic disease (2). Both species of *Fasciola* are distributed and overlapped in Asia and Africa (3), therefore, Iran and in particular the northern provinces of the country is regarded as endemic areas for human fasciolosis (4-6). Human infections in endemic regions demonstrate variable intensities ranging from low to very high in acute and chronic stages (7, 8). Fasciolosis of domestic ruminants is diagnosed by diarrhea, weight loss, and decreased milk yield (9).

The main causative agent of dicrocoeliasis is *D. dendriticum*, a lancet shape parasite also known as a lancet fluke. The pathogenicity of the fluke is mild but several clinical signs such as anemia, edema, weight loss, and decreased milk yield are attributed to infection by this parasite (10). Human infection is generally mild in which the disease could appear with chronic diarrhea, abdominal pain, cramping, nausea, enlarged liver, eosinophils in peripheral blood, or even bile duct obstruction (11).

Dicrocoeliasis and fasciolosis are two important burdens for the economic disadvantages in the societies. Accurate and rapid detection of the parasite are vital for the prevention and control of the disease. Currently, the emergence of molecular approaches provides precise detection of helminths at species level and make it possible to identify the worms in definitive and intermediate hosts. In this regard, several molecular methods have been used as detection techniques including conventional PCR, PCR-RFLP, multiplex PCR, RAPD PCR, PCR-SSCP, and quantitative and qualitative real-time PCR (qRT-PCR) (12-19). Among all methods, the real-time PCR has shown to be highly sensitive and superior to other detecting protocols. High-resolution melting (HRM) analysis is a post-PCR analysis with the potential to provide rapid screening test for detecting of the target species (16). Right now, the HRM method is mostly applied in human clinical studies to diagnose a number of parasitic protozoa (20-25). As this method is generally restricted to be used in the diagnosis of helminths species, it was specifically used in two studies carried out in northwest and west of the country to survey *Fasciola* spp. (13, 26). Moreover, another study used HRM to diagnose *Trichostrongylus* spp. in central Iran (Isfahan province) (27).

There are extensive studies that have been performed on genetic markers of ITS (internal transcribed spacer of nuclear ribosomal DNA) and mitochondrial DNA for phylogenetics and species identification of platyhelminths (18, 19, 28-31).

The results of these studies imply that the coding mitochondrial genes are more suitable than the non-coding ribosomal gene sequences and that the mtDNA sequences of platyhelminths nucleotide substitutions could be identified much faster than the ITS. Therefore, mtDNA could present differences better than ITS among different populations (31). In the present study, we identified *Fasciola* spp. and *Dicrocoelium* spp. by using HRM method for mitochondrial *Cox1*(cytochrome c oxidase I) gene and further compared the mitochondrial and nuclear genes of *Dicrocoelium*.

Materials and Methods

Sample collection

Thirty infected liver specimens were collected from the slaughtered livestock at the abattoirs of Qazvin province, northwest Iran in 2022. The province is located in the northern margin of central Iran. Adult trematodes of *Fasciola* spp. and *Dicrocoelium* spp. were collected from the bile ducts of infected sheep (n: 23) and cattle (n: 7).

Ethical approval of the study was obtained from the Medical Ethics Committee of Qazvin University of Medical Sciences (IR. QUMS. REC.1397.189).

DNA extraction

Flukes were washed in a saline solution for three times before DNA extraction. Genomic DNA of adult flukes was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol (18). The specimens were stored at -20 °C for further analysis.

DNA amplification and HRM

Fragments of the *Cox1* gene were amplified with the specific primers designed for *F. hepatica,* sense primer (F.h:5´-AGATTTGGGCTTTGGTTGCTCGG-3´) and anti-sense primer (F.h:5´-GACAAACAAACACAAGCAGGCAAT-3´), that amplified a 440-bp DNA fragment. The specific primers for *Cox1* region designed for *F. gigantica*, (F.g: 5´-GCTTTGAGTGCTTGGTTGTTGC-3´) and anti-sense primer (F.g: 5´-TATATGACGACCAGTACCCTCGC-3´) amplified a 240-bp DNA fragment. All primers pair were designed by Beacon Designer8.12, PREMIER BIOSOFT software and rechecked by software PRIMER BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) from the consensus sequence obtained by multiple alignments. The amplification of *Cox1* gene used for the detection of *Dicrocoelium* species, resulted in generation of a 169bp partial DNA fragment (32).

PCR amplification was done in twenty-microliter reaction volumes containing10 µL master mix (Type-it HRM PCR Kit; Qiagen, Hilden, Germany), 4 µL of DNA template, 5.2 µL distilled water, and 0.4 µL of each primers. Thermal cycling conditions were conducted in the following order, initial denaturation step at 95 °C for 10 min followed by denaturation step at 95 °C for 25 sec, annealing at 63.5 °C for 30 sec in case of *Fasciola* spp. and 58 °C for 30 sec for *Dicrocoelium* spp., extension step at72 °C for 30 sec, and a final extension step at 72 °C for 5 min. The number of amplification cycles performed for *Fasciola* spp. and *Dicrocoelium* spp. were 30 and 40, respectively. The HRM analysis was conducted by raising the temperature from 70 °C to 90 °C during the following process in which the amplicons obtained from the PCR were denatured prior to the development of melting curves in the inflexion point, where the changes in the fluorescence level compared to the changes in the temperature (Df/Dt), were recorded with a ramp of 0.3 °C/sec. Fluorescence dye signaling was measured after each cycle. The kit contained the novel double stranded DNA-binding fluorescent dye, EvaGreen, and an optimized HRM PCR master mix buffer, consisting of HotStar Taq plus DNA polymerase, Q-Solution, and dNTPs. Real-time PCR was done with the Mini Opticon real-time PCR detection system (Applied Biosys-tems Step One Plus Inc., CA, USA). The real-time amplification results and Tm analysis were obtained using the Step One PlusTM software ver. 2.3 (Life technologies[@]).The Tm analysis in each run was performed in triplicate to confirm the repeatability of the Tm assay by estimating the Tm variation within a PCR amplification (intra-assay), and between the PCR amplifications (inter-assay). The coefficient of variation (CV) was calculated by dividing the standard deviation (SD) to the arithmetic mean of the measured values of Tm (CV=SD[Le, 2012 #11]/mean value). Furthermore, to check the uniformity of temperature in the cycler block, a number of samples were reamplified at different positions of the cycler block during the same amplification cycle. The intra-assay CVs represent the mean CVs of the results obtained from the replications of *Fasciola* spp. and *Dicrocoelium* spp.

Genetic differentiation

Six isolates of *Dicrocoelium* spp. were randomly selected and sequenced by the gene region of *28S rDNA* and compared with mitochondrial gene regions (18). Genetic compression of ribosomal *28S rDNA* and mitochondrial *Cox1* markers were evaluated by the number of haplotypes (Hn),

haplotype diversity (Hd), nucleotide diversity Nd (π), and the number of variable sites (S) using the DnaSP v.5.0 (Librado2009). Phylogenetic analysis was performed for *Dicrocoelium* spp. based on the ML (Maximum Likelihood) method using the Tamura 2-parameter model by the sequences obtained in this study along with those on GenBank reference sequences using the MEGA 5.0 software. To demonstrate the percent identity and intra-species diversity among the aligned sequences of *28S rDNA* gene, the pairwise distances matrix was built using the DNASTAR's MegAlign program.

Results

The adult flukes were collected from thirty infected liver samples including 7 cattles and 23 sheep hosts at the slaughterhouses of Qazvin Province, Iran. Real-time PCR and HRM analysis procedure were performed for all of specimens. Tm analysis was repeated three times in each reaction to confirm of the Tm assay (Table 1).

Table 1: Mean T_m, SD, and CV calculated based on intra- and inter-assay of gene sequences *Cox1* of *Fasciola* Spp. and *Dicrocoelium* spp.

Gene	***Mean Tm (°C)***	***SD***	***Intra-assay CV* (%)***	***Inter-assay CV (%)***
Cox1				
F. hepatica	82.2 °C	0.1	0.08	0.11
F. gigantica	82.9 °C	0.11	0.06	0.13
Dicrocoelium spp.	80.8 °C	0.13	0.04	0.11
Dicrocoelium variation	79.8 °C	0.14	0.07	0.12

*CV, coefficient of variation

The melting curve and HRM curve analysis of the *Fasciola* spp. and *Dicrocoelium* spp. were identified and are presented in Fig. 1 and 2. The result of HRM analysis showed that 26 and 4 isolates were identified as *F. hepatica* and *F. gigantica*, respectively. *Dicrocoelium dendriticum* was the sole infecting species of the genus *Dicrocoelium* identified by HRM analysis. The phylogenetic tree generated by the *28S rDNA* sequences of *Dicrocoelium* comfirmed the accuracy of identified isolates of *D. dendriticum* (Accession numbers; MH298630, MH298633, MH298636, MH298640, MH299967 and MH299971) placed in their specific clade is showed in Fig. 3. Three isolates of *Dicrocoelium* (MH298636), (MH299971), and (MH298633) demonstrated various peaks (Fig. 2). Haplotype (genetic) diversity of *Cox1* (0.733±0.155) and *28S rDNA* genes (0.600±0.215) of *D. dendriticum* indicated that the *Cox1,* as a mithodondrial marker, could precisely clarify the differences compared to nuclear gene (Table 2).

Table 2: The comparison of diversity indices between nuclear (28S rDNA) and mitochondrial (*Cox1*) genes of *D.dendriticum* isolated from livestock of Qazvin, Iran

Area	***Parasite (Gene)***	***Diversity indices***				
		N	Hn	Hd± SD	S	Nd (π)
Qazvin, Iran	*Dicrocoelium dendriticum* (28S rDNA)	6	3	0.600±0.215	2	0.00529
	Dicrocoelium dendriticum(Cox1)	6	3	0.733± 0.155	4	0.00232

N: number of sequences; Hn: number of haplotypes; Hd: haplotype diversity; S: number of variable sites; Nd (π): nucleotide diversity

The pairwise distances matrix (percent of diversion and identity) is presented in Fig. 4 between the new identified *D. dendriticum* isolates and the selected references' sequences circulating globally from the GenBank database, showed a low intra species-divergency of 0-0.2% and high intra-species identity of 99.8-100%.

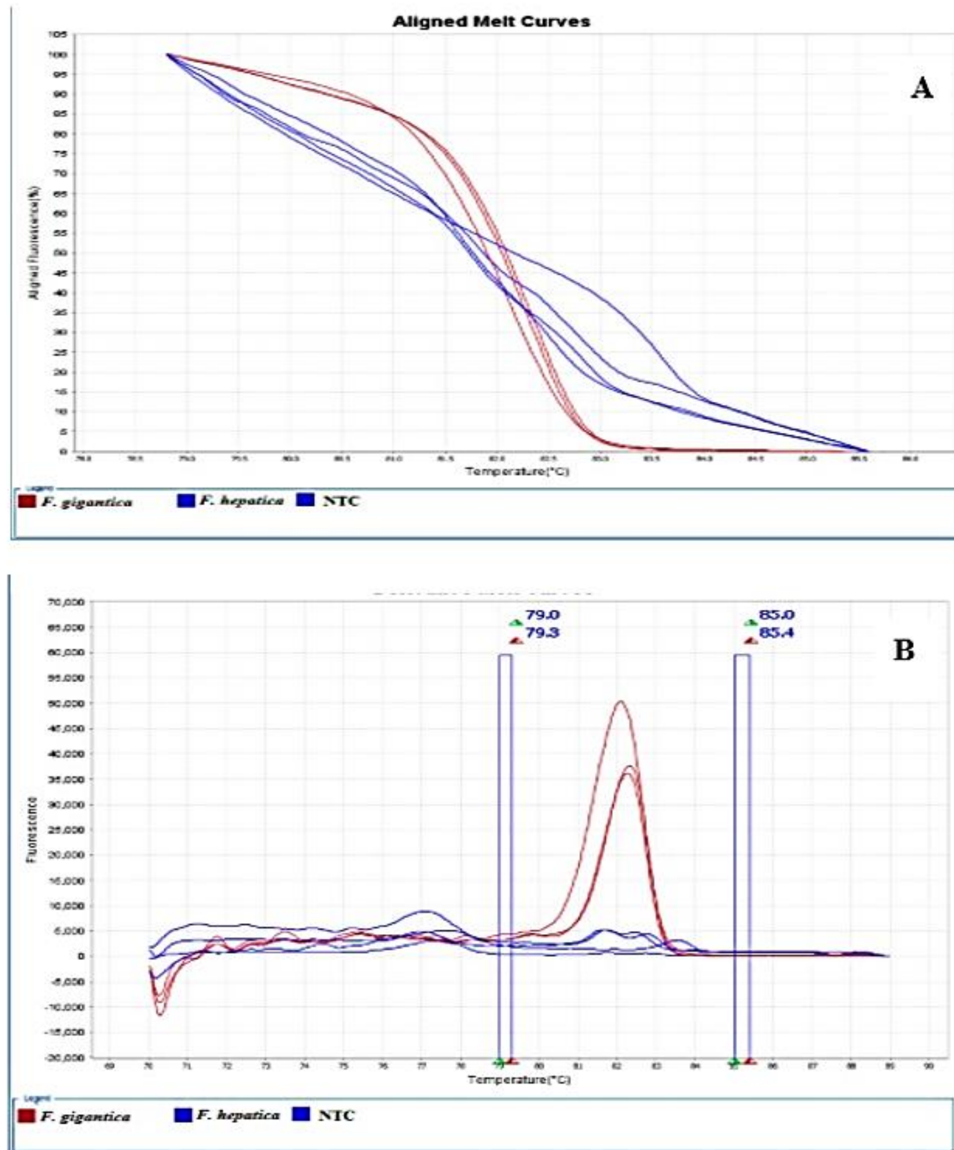

Fig. 1: HRM plots (A) and derivative melt curve (B) for *Cox1* gene amplicon showing melt curves analyses of *F. gigantica* in slaughtered livestock samples

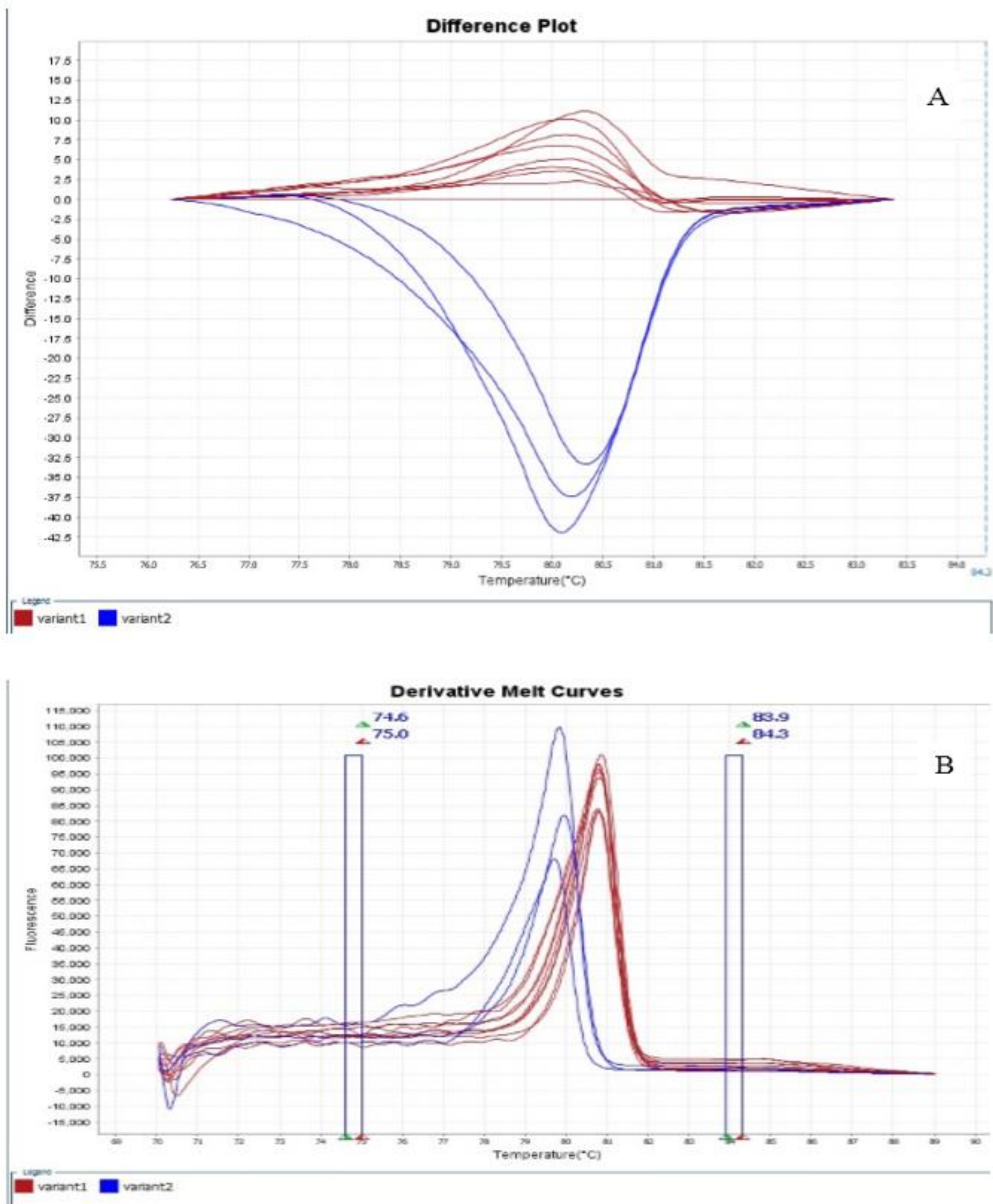

Fig. 2: HRM plots (A) and derivative melt curve (B) for *Cox1* gene amplicon showing melt curves analyses of *D. dendriticum* and variation in slaughtered livestock samples

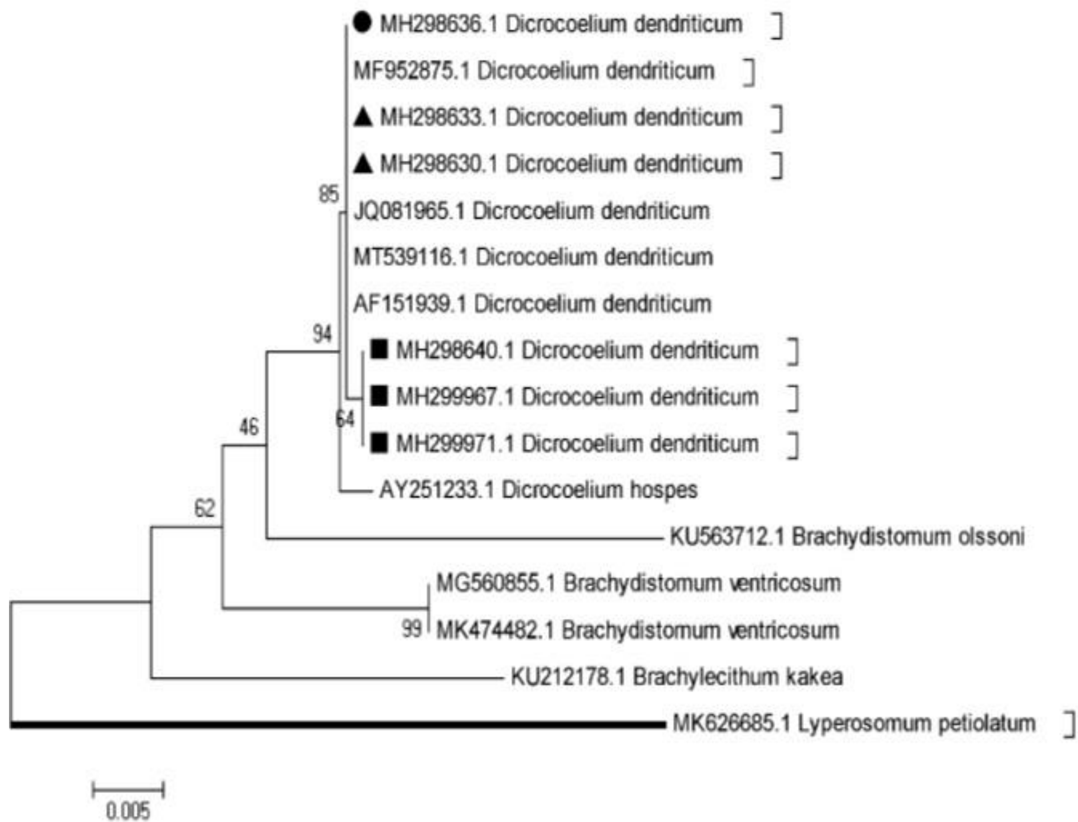

Fig. 3: Phylogenetic tree of isolates of *Dicrocoelium* spp. obtained in the present study and other isolates of *Dicrocoelium* retrieved from GenBank. The tree was designed by using the Maximum-Likelihood algorithm and the Tamura 2-parameter model as implemented in the MEGA5. 0 software. *Lyperosomum petiolatum* was considered as an out group branch. The characterized isolates marked by geometric shapes

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10		*Dicrocoelium dendriticum*	
1		100.0	100.0	99.8	99.8	99.8	100.0	100.0	100.0	100.0	**1**	MH298630*	Qazvin,Iran
2	0.0		100.0	99.8	99.8	99.8	100.0	100.0	100.0	100.0	**2**	MH298633*	Qazvin,Iran
3	0.0	0.0		99.8	99.8	99.8	100.0	100.0	100.0	100.0	**3**	MH298636*	Qazvin,Iran
4	0.1	0.1	0.1		100.0	100.0	99.8	99.8	99.8	99.8	**4**	MH298640*	Qazvin,Iran
5	0.1	0.1	0.1	0.0		100.0	99.8	99.8	99.8	99.8	**5**	MH299967*	Qazvin,Iran
6	0.1	0.1	0.1	0.0	0.0		99.8	99.8	99.8	99.8	**6**	MH299971*	Qazvin,Iran
7	0.0	0.0	0.0	0.1	0.1	0.1		100.0	100.0	100.0	**7**	MF952875	Mazandaran, Iran
8	0.0	0.0	0.0	0.1	0.1	0.1	0.0		100.0	100.0	**8**	MT539116	Kurdistan, Iran
9	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.0		100.0	**9**	AF151939	Ukraine
10	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0		**10**	JQ081965	East Azerbijan, Iran
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**			

Fig. 4: The pairwise distance between sequences (divergence and percent identity) of *D. dendriticum* isolates inferred by *28S rDNA* gene

Discussion

In the current study, the *Cox1* mitochondrial genes of *F. hepatica, F. gigantica,* and *D. dendriticum* were investigated by real-time PCR and HRM techniques. The HRM method is reliable and a suitable protocol for identification of flukes (33). Two species of the genus *Fasciola* including *F. hepatica* and *F. gigantica* have overlapping distribution in different parts of Iran (34-36), so the detection of differences between these species with accurate methods are extremely important to preventive and control programs of the country. Some restricted studies have detected the species with HRM technique in Iran. The HRM technique by targeting *Cox1* is as a powerful, rapid and sensitive method to differentiate into *Fasciola hepatica* and *F. gigantica* in the northwest (Ardabil) and southeast (Zahedan) of Iran. Another study was conducted to detect *F. hepatica* and *F. gigantica* by HRM analysis of mDNA including *Cox1*, *Cox3*, and *ND6* in western Iran and concluded that the method is simple, fast and reliable (26).
Considering the limited number of studies performed based on HRM technique in Iran, more studies with larger sample size within a wider geographical area in the country, to be performed to better judge the outcomes of this technique.
The result of HRM analysis clarified the presence of *F. hepatica* (n: 26) and *F. gigantica* (n: 4) isolates among the specimens. As the findings of our previous study, based on nuclear gene, were consist with those found in the present study, the mithochondrial (*Cox1*) gene and the nuclear gene (*ITS2*) demonstrate similar accuracy in differentiating the both species of *Fasciola* (18). Several studies used mitochondrial and nuclear genes for diagnostic and phylogenetic surveys of the flukes (37-39). Overall, the mitochondrial (mt) genomic materials are frequently used in the evolutionary relationships, phylogenetic and taxonomic levels of helminthes (40-43). In addition, mtDNA has been introduced as a useful molecular marker for accurate identification and differentiation, within and between species, of the flukes such as *Dicrocoelium* and *Fasciola* species (44-46).
Although, the diagnosis of *Dicrocoelium* spp. based on conventional PCR shows high specificity yet the current study emphasizes on the HRM technique for its simplicity and the accuracy by which the species could be identified. Several studies distinguished the *Dicrocoelium* spp. based on mtDNA, including *D. chinensis* and *D. dendriticum* (46-48). So far, no *Cox1* analysis of *Dicrocoelium* species was conducted in the country and the present study is the first of its type to achieve such experimental results.
Our current findings showed that the parasite-dependent mitochondrial gene (*Cox1*) could identify a higher genetic diversity of *D. dendriticum* than the nuclear *28S rDNA* gene. Among the mitogenome markers tested, the *Cox1,* due to its low repetitive sequences in the length of genome, haploidy nature, and semi-conserved structure, is extensively considered as one of the well-known evolutionary marker for differentiation of genetic structure of platyhelminth populations (49). Only a few gene sequences of *Cox1* led to design the phylogenetic tree for *Dicrocoelium* based on *28S rDNA* sequences. Phylogenetic analysis of the *28S rDNA* sequences clarified that *D. dendriticum* is circulating among different parts of Iran, Ukraine, and USA.

Conclusion

The *F. hepatica and F. gigantica* flukes were successfully identified by real-time PCR-HRM technique targeting *Cox1* gene. Moreover, for the first time, HRM analysis of *Cox1* developed in the present study as a powerful, rapid and sensitive technique for molecular identification of *D. dendriticum* in Iran.
Our finding proved that the mitochondrial gene (*Cox1*) could identify a higher genetic diversity of *D. dendriticum* compared with nuclear *28S rDNA* gene. Future studies should expand and focus on haplotype diversity and deep genetic evaluation

of the flukes based on nuclear and mitochondrial genes in other endemic areas of Iran.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of interest

The authors declare no conflicts of interest.

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