



Morphological and Molecular Characterization of *Dicrocoelium* spp. Isolates from Sheep, Goat and Cattle in the West of Iran



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DICROCELIOSIS is a prevalent parasitic disease in ruminants and in humans and is accidental caused by different species of *Dicrocoelium* spp. This study aimed to evaluate the morphological characteristics and molecular parameters of *Dicrocoelium* spp. isolated from livers of slaughtered sheep, goat, and cattle in three provinces in west of Iran from Feb 2018 to Feb 2019. In the present study, 45 infected livers with *Dicrocoelium* were collected (each province 15 and each host 5 infected liver). After morphological examination, the molecular evaluation was carried out for the ribosomal DNA (partial 5.8S, complete ITS2 and partial 28S genes) using PCR and PCR-RFLP techniques. Morphological analysis revealed that all specimens belonged to *Dicrocoelium dendriticum*. There were profound morphometric variations among *D. dendriticum* from different hosts and sampling areas, but no statistically significant differences was found ($P > 0.001$). The sequencing analysis of obtained fragments in GeneBank showed 96% to 100% similarity within homogeneous fragments in the database. The obtained sequences were also registered in Gene Bank with the accession numbers, MN831473, MN831474 and MN831475. Additionally, all PCR-RFLP products revealed two similar fragments (319bp and 271 bp) in all examined samples. According to the present study, haplotype A was shown to be the predominant haplotype of *D. dendriticum* in the west of Iran.

Keywords: *Dicrocoelium*, ITS2, Iran, Morphology, PCR-RFLP.

Introduction

Dicrocoeliosis, 'the lancet fluke' is a world-wide parasitic disease that caused by *Dicrocoelium* spp. This parasite is an important hepatic trematode, affecting domestic and wild ruminant worldwide [1, 2]. *Dicrocoelium* is found in the bile ducts and gall bladder of domestic and wild ruminants [1, 3]. Clinical signs of the disease giving rise to various mild to severe symptoms [4].

Three species of the *Dicrocoelium* genus, namely, *D. dendriticum*, *D. hospes*, and *D. chinensis* are recognized as the causative agents

of the dicrocoeliosis. *D. dendriticum* has been reported in Europe, Asia, northern Africa, and North America, whereas *D. hospes* is distributed in Africa, and *D. chinensis* in Eastern Asia and Europe [4-7].

Dicrocoeliosis has great economic and public health burden [1, 3, 8]. This disease causes high direct and indirect economic losses in the livestock industry in Iran and other affected countries. The direct losses are caused by liver condemnation and the indirect losses are associated with the reduced meat and milk production, as well as, the costs associated with anthelmintic treatments [2, 8, 9]. Different studies have been conducted

to determine the prevalence of *Dicrocoeliosis* in domestic ruminants, which all have reported *D. dendriticum* is the common liver flukes in ruminants, in Iran [10-12]. Accordingly, the highest rates of infection have been reported in the north and north-west of Iran [11-15].

Many studies have evaluated some morphometric and molecular aspects of *Dicrocoelium* spp. in Iran and other regions [13, 16]. However, there are little data about ribosomal sequencing of *Dicrocoelium* spp. in the west of Iran. Therefore, the aim of this study was to investigate the morphometric and molecular characteristics of *Dicrocoelium* spp. gained through the ribosomal sequencing, in Ilam, Kermanshah and Kurdistan provinces, which covering a broad area in the west of Iran

Materials and Methods

Morphological study

Forty-five infected livers with *Dicrocoelium* were collected from slaughtered cattle (n=15), sheep (n=15) and goat (n=15) in three western provinces of Iran, including Ilam, Kermanshah and Kurdistan provinces (15 infected livers from each province).

A total of 845 adult *Dicrocoelium* flukes were separated through necropsy from these 45 infected livers. All samples were washed in normal saline and fixed in 70% ethanol. Morphological measurements of *Dicrocoelium* spp. were conducted using a microscope and calibrated ocular micrometer according to Yamaguti [17]. After staining in 10% carmine, the morphological characteristics such as, body length and width, oral and ventral sucker diameters, length and width of testis, ovary and vitelline glands length were assessed using light microscope at 10 × magnifications.

DNA extraction

Genomic DNA of all *Dicrocoelium* spp. was extracted using a commercial DNA extraction Kit of Molecular Biological System Transfer institute (MBST, Iran) according to the manufacturer's instructions. Briefly, the adult worms were washed three times using phosphate buffer saline (PBS) to remove ethanol. For each sample three parasites were first added into the separate tubes and followed by lysing in 180 µl lysis buffer and

protein degradation with 20 µl proteinase K for 10 min at 55 °C. After adding 360 µl binding buffer and incubation for 10 min at 70°C, 270µl ethanol (100%) was added to the solution, followed by rapid vortex. Finally, the mixture was transferred to the MBST-column. The MBST-column was then centrifuged followed by washing twice with 500 µl wash buffer. DNA was eluted from the MBST-column in 100 µl elution buffer and then stored at -20°C until experiments.

Molecular analysis

The ribosomal DNA fragment (about 590 bp) including complete ITS2, partial 5.8S and 28S ribosomal DNA was amplified by polymerase chain reaction (PCR) assay. In order to amplify this fragment, a primer pair was designed for the ITS2 gene of *D. dendriticum* (accession no; NC 025280). The sequences of designed primer pair were 5'-ATATTGCGGCCATGGGTTAG-3' (F, forward primer) and 5'-ACAAACAACCCGACTCCAAG-3' (R, reverse primer). Approximately, 10 ng DNA (1 µl) was applied for the PCR amplification, in a final volume of 100 µl, containing 2 µl (20 µM) of each primer (Sinnagen, Iran), 2 µl (200 µM) of dNTP (Fermentas), 3 µl (1.5 mM) MgCl₂, 10 µl PCR buffer 10x, 0.5 µl (2.5 U) Taq polymerase (Cinnagen, Iran) and 79.5 µl Nuclease-free water. Then, PCR was performed on an automated thermocycler (MWG Biotech Primus, Germany) with the following thermal cycling program: 5 min incubation at 95 °C to denature double-strand DNA, followed by 34 cycles of 45 s at 94 °C (denaturation), 45 s 58 °C (annealing), and 45 s at 72 °C (extension) and an additional extension step at 72 °C for 5 min.

Totally, 18 PCR products (each province 6 and each host 2 samples) were selected randomly and sent to Takapozist Biotech Company in Iran for sequencing in both directions using the same forward and reverse primers as those used in the PCR assay.

PCR-RFLP

Restriction fragment length polymorphism method was performed as described [18, 19]. Briefly, the PCR products were digested by the *TruI* I restriction enzyme for 1 hour at 65°C in a total volume of 15 µl, containing 0.5 µl of *TruI* I enzyme and buffer R (Termo scientific, Lithuania). The restriction fragments were electrophoretically

assessed on 1.2% (w/v) agarose gel. The observed fragments by the documentation gel system were found to be 319 and 271 bp.

Data analysis

In the present study, the obtained fragments were compared with recorded homogeneous sequences of *D. dendriticum* in NCBI Gene Bank and also aligned by Bio Edit 7.0.5.3. Furthermore, the phylogenetic tree was designed by the maximum likelihood method based on the version 6 Mega software package.

Statistical analysis

SAS statistical software version 1.2 was used for analysis. The data were analyzed according to the principles of variance analysis with factorial 3×3 experiment and twenty observations.

Results

In this study, 845 *Dicrocoelium* spp. were isolated from 45 affected livers of slaughtered cattle, sheep and goat in the west of Iran. Morphological analysis of adult *Dicrocoelium* worms showed that all specimens were *D. dendriticum* using light microscope at 10 × magnifications. Morphometric

variations were observed among *D. dendriticum* from different hosts and areas, but no statistically significant difference between them ($P > 0.001$). The morphometric data for *D. dendriticum* are depicted in (Tables 1, 2 and 3). PCR assay was carried out on 45 extracted DNA of *D. dendriticum* to amplify ribosomal DNA (partial 5.8S, complete ITS2 and partial 28S gene). The observed PCR amplicons in all samples were 590 bp (Fig 1).

In the current study, no differences were observed in our fragments in terms of the studied hosts and locations. The obtained sequences data were registered in Gene Bank database under the accession numbers (MN831473, MN831474 and MN831475). To generate a phylogenetic analysis, the obtained sequences of ribosomal DNA were compared with the homologous sequences in Gene Bank reference. The findings indicated the presence of 96% to 100% identity. Also, the endonuclease digestion of PCR products showed two distinct fragments of 319 bp and 271 bp, respectively (Fig 2). According to the results, haplotype A was the predominant haplotype of *D. dendriticum* in the west of Iran. The output of phylogenetic tree has been shown in (Fig. 3).

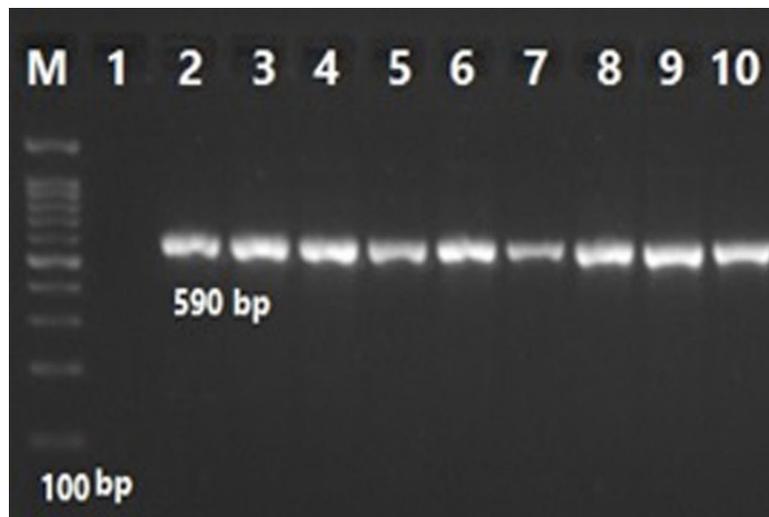


Fig. 1. Electrophoresis of the PCR products of ribosomal DNA in *Dicrocoelium*.

M: 100 bp marker. Lane 1: negative control. Lane 2, 5 and 8: *D. dendriticum* isolated from cattle in Ilam, Kermanshah, and Kurdistan. Lane 3, 6 and 9: *D. dendriticum* isolated from sheep in Ilam, Kermanshah, and Kurdistan. Lane 4, 7 and 10: *D. dendriticum* isolated from goats in Ilam,

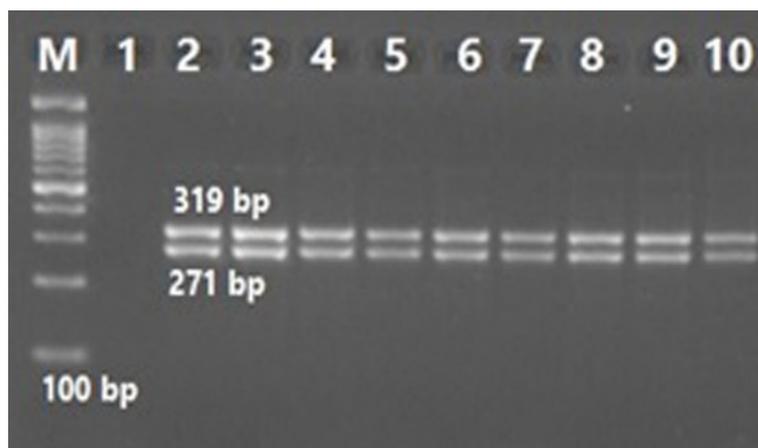


Fig. 2. Electrophoresis of the PCR products of ribosomal DNA after digestion with the *TruI* I restriction enzyme.

M: 100 bp marker. Lane 1: negative control. Lane 2, 5 and 8: *D. dendriticum* isolated from cattle in Ilam, Kermanshah, and Kurdistan. Lane 3, 6 and 9: *D. dendriticum* isolated from sheep in Ilam, Kermanshah, and Kurdistan. Lane 4, 7 and 10: *D. dendriticum* isolated from goats in Ilam, Kermanshah,

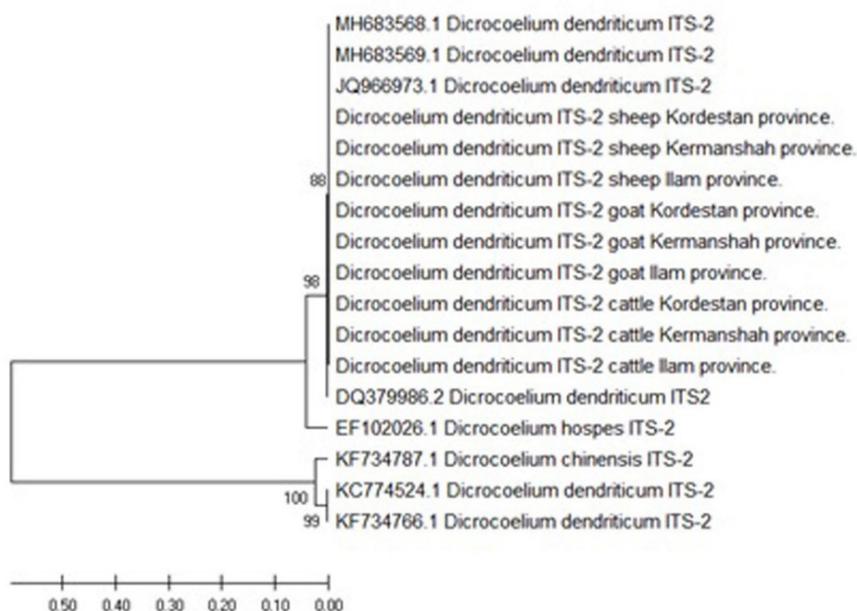


Fig. 3. The phylogenetic tree of obtained fragments in comparison with homologous sequences registered in the GeneBank database. It is designed by Mega v.6 program using maximum likelihood method, supported by 2000 bootstrap replicates.

TABLE. 1. Morphometric indexes of *D. dendriticum* in the Ilam province.

	Morphometric characteristics	Measurement index	Cattle	Sheep	Goats
1	Worm length	mm	10.7	7.9	7.6
2	Worm width	um	1894.32	1638.42	1700.12
3	Oral sucker diameters	um	469.88	414.90	389.64
4	Ventral sucker diameters	um	563.32	434.69	451.97
5	Anterior testis length	um	943.29	930.91	931.69
6	Width Anterior testis	um	882.71	874.41	897.01
7	Posterior testis length	um	1000.67	933.23	939.20
8	Posterior testis Width	um	957.72	890.11	905.35
9	Ovary length	um	427.10	403.05	381.79
10	Ovary Width	um	361.47	327.41	313.19
11	Vitelline glands length	um	2000.09	1867.89	1789.66

TABLE. 2. Morphometric indexes of *D. dendriticum* in the Kermanshah province.

	Morphometric characteristics	Measurement index	Cattle	Sheep	Goats
1	Worm length	mm	10.2	7.6	7.15
2	Worm width	um	1884.74	1677.40	1618.56
3	Oral sucker diameters	um	404.68	373.78	351.31
4	Ventral sucker diameters	um	500.48	430.51	429.79
5	Anterior testis length	um	891.39	825.37	831.92
6	Width Anterior testis	um	826.68	760.00	768.55
7	Posterior testis length	um	960.05	863.96	868.67
8	Posterior testis Width	um	919.48	795.29	790.79
9	Ovary length	um	403.36	373.17	363.67
10	Ovary Width	um	352.10	294.40	295.42
11	Vitelline glands length	um	2000.32	1812.37	1714.60

TABLE. 3. Morphometric indexes of *D. dendriticum* in the Kurdistan province.

	Morphometric characteristics	Measurement index	Cattle	Sheep	Goats
1	Worm length	mm	10	7.3	7.2
2	Worm width	um	1872.77	1663.41	1707
3	Oral sucker diameters	um	435.36	371.13	358.95
4	Ventral sucker diameters	um	489.51	428.94	427.52
5	Anterior testis length	um	848.85	807.46	813.29
6	Width Anterior testis	um	751.65	726.78	737.94
7	Posterior testis length	um	887.59	842.12	854.97
8	Posterior testis Width	um	805.86	777.15	760.70
9	Ovary length	um	402.10	370.34	354.23
10	Ovary Width	um	328.06	298.38	284.31
11	Vitelline glands length	um	2009.63	1778.78	1727.43

Discussion

Dicrocoeliosis is an important disease in the livestock industry. This disease has a zoonotic potential and can be transmitted to humans by eating ants infected with metacercaria. Dicrocoeliosis is associated with weight loss and reduced milk production, anemia, reduced product quality, digestive disorders, liver failure, leading to indirect economic losses in ruminants [20]. In Iran, the prevalence of *D. dendriticum* infection, based on the slaughter house inspections, have been declared to be from 0.34% to 1.79% in sheep, 0.69% to 1.47% in cattle and 0.1% to 2.1% in goats in the province of Fars [10]. Oryan *et al.* reported that the prevalence of Dicrocoeliosis in sheep and cattle is 4.54% and 11.03%, respectively, in Khorasan province, Eastern Iran [21]. So far, different studies have been done to reveal morphological and molecular evidences differentiating *Dicrocoelium* species [4, 6, 8, 16, 20, 22]. However, studies regarding phenotypic and genetic diversity of *Dicrocoelium* spp. are limited, especially, in the western parts of Iran. In the current study, no significant intraspecific differences were recorded among morphometric and genetic characteristics of isolates obtained from different hosts and different provinces, in western of Iran. Gorjipoor *et al.* observed the minor phenotypic variations among adult flukes with different hosts in Iran based on the *nad1* and ITS2 sequences, where they exhibited eight and one polymorphic sites, respectively. Aforementioned study indicated that six silent site and two nucleotide substitutions were responsible for amino acid alterations in the *nad1* [13]. In a study carried out by Arbabi *et al.* in the northern parts of Iran, the morphological characteristics of the parasite in various provinces were found to be significantly different. Differences between the morphological findings may be related to the age, nutritional conditions, infection severity, geographical distribution, developmental stage, and method of measurement, as well as sample size, study period and study area [16, 23]. In our study, ribosomal DNA (partial 5.8S, complete ITS2 and partial 28S gene) of collected *Dicrocoelium*'s were first amplified. Then, the RFLP technique was applied (by *TruI* I restriction enzyme) to differentiate the parasite species. Finally, PCR products from hosts and

different areas were characterized molecularly via sequencing. The results of sequencing showed that all specimens were *D. dendriticum*, where no difference was found between sequences corresponding to parasites from all isolates. It is noteworthy that similar results have been reported in Iran [13, 21]. The obtained fragments have a high resemblance with some sequences available in Gene Bank. For example, sequences in our study had high alliance with those obtained by Gorjipoor *et al.* in Shiraz province in which they reported 0–0.42% genetic diversity for ITS2 of *D. Dendriticum*. In their study, a phylogenetic analysis of the sequence data demonstrated that host associations and geographical localities are not likely useful markers for *D. dendriticum* haplotype classification [13]. In conclusion, we characterized the partial ribosomal DNA (partial 5.8S, complete ITS2 and partial 28S gene) of *D. dendriticum* in the western parts of Iran and determined the genetic variations of obtained fragments via homogeneous sequences from different hosts and geographical localities. Our findings provided basic information for further molecular examinations therefore, this data would provide an insight in to control of spread of *D. dendriticum* infection in the western areas of Iran.

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Conflict of interest

The authors declare that there is no conflict of interest.

Funding statement

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Ethical consideration

This manuscript was conducted on slaughtered ruminant and does not contain clinical or experimental studies and patient data.

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