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Original Article

Molecular Characterization of Animal *Fasciola* Spp. Isolates from Lorestan Province, Western Iran

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Abstract

Background: We aimed to detect the genetic diversity of samples identified morphologically as *Fasciola* spp. from sheep, cattle and goat from Lorestan Province, western Iran using PCR-RFLP method. Besides, we evaluated the genetic diversity indices, sequencing and phylogenetic analysis using mitochondrial gene (*ND1* and *CO1*).

Methods: PCR-RFLP analysis of ribosomal *ITS1* fragment by *RsaI* restriction enzyme to investigate the genetic characteristics of *Fasciola* species obtained from different hosts (18 sheep, 21 cattle, and 17goats) was conducted. The samples were sequenced. Sequences were evaluated using BLAST software and the parasite species were identified with similarity percentage and overlap with the species registered in the gene bank. Then similarity and diversity of intra-species and intra-species diversity of *Fasciola* species were calculated.

Results: In Lorestan, based on RFLP pattern, 93% (52) of the *Fasciola* spp. isolates had a RFLP pattern related to *F. hepatica* and 7% (4) were *F. gigantica*. No hybrid forms were detected. The *CO1* gene could clarify 19 haplotypes against *ND1* gene that found 22 haplotypes among livestock. Sequencing results of the mtDNA showed intra-species identity 98. 5%-100% and Intra-species-diversity: 0-1.5% compared to the GenBank sequences.

Conclusion: Using PCR-RFLP method, two species of *F. hepatica* and *F. gigantica*, were present in Lorestan Province, but *F. hepatica* was more prevalent. Mitochondrial genes could better test variability indices in different hosts than ribosomal genes, consequently among mitochondrial genes, the ND1 gene could better examine differences and similarities than CO1.

Keywords: Fascioliasis; *Fasciola*; Polymerase chain reaction; Iran

Introduction

Fascioliasis as a neglected zoonosis is a well-known veterinary and public health problem. This disease in livestock and humans is caused by two liver fluke parasites: *Fasciola hepatica* and *F. gigantica* (1, 2).

It is estimated that 2.4 million, up to 17 million people suffer from fascioliasis worldwide with 180 million at-risk people. *F. hepatica* has a wide distribution in Europe, Africa, Asia, Oceania and the Americas, while *F. gigantica* restricted to Asia and Africa (3). The distributions of these two species overlap in many regions of Africa and Asia.

Due to different control strategies, the differentiation of *Fasciola* species is essential in these regions (4). Although the *Fasciola* species can generally be differentiated by morphometric methods, the morphological characteristics of adult worms are affected by different factors such as parasite's age, type of the host and intensity of infection leading to considerable taxonomic confusion and are unable to differentiation accurately (5, 6). Therefore, the accurate identification of causative species seems necessary to prevent this disease.

Nowadays a variety of molecular methods based on DNA analyses have been used for the differentiation of *Fasciola* species (7-10). PCR-RFLP assay is very rapid, simple, inexpensive, easy to perform and an appropriate method for differentiation of *F. hepatica* from *F. gigantica* (11-15).

The prevalence of fascioliasis in various livestock in Iran has been reported at 0.1% to 91.4% (16). To determination of the fascioliasis status in Iran in addition to genotypic and phylogenetic characterization of the parasite, the differentiation between causative species in different geographical regions and determine the distribution of each species is important.

Lorestan Province is considered as a high-risk area for fascioliasis due to, a neighborhood with Kermanshah Province as an endemic area, the prevailing rural lifestyle in this province, close contact of inhabitants with the livestock through animal husbandry, which is the occupation of most inhabitants in this area and the presence of snails as an intermediate host.

We aimed to identify and differentiate *Fasciola* flukes by PCR-RFLP of the *ITS1* gene in Lorestan Province, western Iran and to analyze their phylogenic relationship with population from other parts of the world using mitochondrial *ND1* and *COX1* gene.

Material and Methods

Sample collection

A total of 56 confirmed infected livers to *Fasciola* species (21 cattle, 18 sheep and 17 goats) were collected from slaughterhouses of Lorestan Province, western Iran during 2016 (Fig. 1). An adult worm was isolated from each liver and washed in phosphate buffer saline (PBS) solution twice and stored in 70% ethanol for subsequent molecular methods.

Fig. 1: Iran map: Location of Lorestan Province in Iran

Genomic DNA extraction and amplification

Genomic DNA was extracted from the collected flukes using DNGTM-plus Kit (CinnaGen, Iran) following manufacturer's instruction. The *ITS1* region was amplified using two set of primers (Table 1). PCR amplifications were performed in 25 µL reactions containing 12.5 µL Master Mix (Amplicon, Odense, Denmark), 1 µL of each primer (10 pmol of forward and reverse primers), 5 µL genomic DNA, and 5.5 µL distilled water. The reactions were performed as follows: predenaturation at 95 ºC for 5 min, 25 cycles of denaturation at 94 ºC for 30 sec, annealing at 62 ºC for 30 sec, elongation at 72 ºC for 30 sec, followed by a final extension at 72 ºC for 5 min. The PCR products were run on 2% agarose gel.

Table 1: Gens and sequences of the primers used in this study

Gene	*Sequence*
ITS1	F: 5'- TTGCGCTGATTACGTCCCTG -3'
	R: 5'- TTGGCTGCGCTCTTCATCGAC -3'
ND1	F: 5'-AAGGATGTTGCTTTGTCGTGG-3'
	R: 5'-GGAGTAC GGTTACATTCACA-3'
CO1	F: 5'-ACGTTGGATCATAAGCGTGT-3'
	R: 5'-CCTCATCCAACATAACCTCT-3'

ITS1= Internal transcribed spacer 1, ND1= Nicotiamide adenine dinucleotide dehydrogenase subunit I, CO1= Cytochrome oxidase subunit I

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PCR-RFLP analysis

A PCR-RFLP method was used on PCR product of ITS1 gene (700 bp) for differentiation of *F. hepatica* and *F. gigantica*. The amplicons were subsequently digested using the RsaI restriction enzyme. By this method, two species of *Fasciola* genus will be differentiate with different bands pattern, that way *F. hepatica* will have fragment bands 28, 54, 59, 68, 104 and 367 bp, and in *F. gigantica* fragment bands 28, 54, 59, 68, 172 and 367 bp will be evident. To confirm the results of PCR-RFLP, a fragment of 700 bp from the ITS1 gene was sequenced for seven cases. Sequencing was performed in forward direction using Sanger sequencing (Macrogen Inc. Korea, ABI3730XL).

Genetic Diversity Indices, Sequencing and phylogenetic analysis

The PCR products of *ND1* and *CO1* were amplified using the primers (Table 1) and sequenced by Sanger sequencing method (Macrogen Inc. Korea, ABI3730XL). Sequences were aligned using Bioedit Sequence Alignment Editor (https://bioedit.software.informer.com/7.2/), version 7.0.9 (17). Then obtained sequences were analyzed and compared with those of available in GenBank using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/) to find out the closest entries related to *Fasciola* species. Phylogenetic analysis based on *ND1* and *CO1* sequence data were conducted by Maximum Likelihood (ML) algorithm using the Tamura 3-parameter model by sequences recovered from this study along with GenBank reference sequences using the MEGA 7.0 software (18). Bootstrap analysis was performed using 1000 replicates. Genetic variability for mitochondrial marker was evaluated by the number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity (π) and number of polymorphic sites, using DnaSP v.5.0 (19). Finally, the similarity and difference rates of sequences determined with DNA Star Laser MegAlign program (DNASTAR, Madison, WI, USA).

Ethics approval

This research has been registered with the ethics code IR.TUMS.REC.1395.2404 by the Ethics Committee of Tehran University of Medical Sciences.

Results

PCR-RFLP findings

Based on RFLP pattern, 93% (52) of the *Fasciola* spp. isolates had a RFLP pattern related to *F. hepatica* and 7% (4) of them were *F. gigantica* (Fig. 2). *ND1* fragments (approximately 1074 bp) and *CO1* fragments (approximately 974 bp) were amplified for several specimens. Partial sequences of *CO1* and *ND1* showed 26 and 11 variable sites, respectively, and yielded eight haplotypes in both genes and high diversity indices in *ND1* gene (Table 2). The nucleotide sequences for every haplotype were deposited in GenBank under following accession numbers: KX021280-KX021299. Phylogenic analyses based on *ND1* and *CO1* sequence data were conducted by neighbor-joining (NJ) using MEGA 7.0 with *Fascioloides magna* designated as an outgroup

showed in Figs. 2,3 and 4 respectively. Pairwise fixation index (Fst values) between different *F. hepatica* populations calculated from the nucleotide data group.

According to the haplotype analysis in *CO1* gene, the most haplotypes diversity and number of haplotypes were detected in cattle and goat, therefore the most nucleotid diversity was found in goat (0.01567). The most haplotypes diversity observed in goat and cattle, 0.657±0.138 and 0.652±0.01583 respectively. The number of polymorphic sites in cattle and goat found 7 sites, while in sheep detected 6 sites. The results of haplotype analysis in *ND1* gene was clarified the most haplotypes diversity observed in goat (0.912±0.059), meanwhile the least haplotypes diversity shown in cattle (0.588±0.135). The number of polymorphic sites in cattle and goat showed 8 and 7 sites respectively, while in sheep was 19 sites. The *CO1* gene could clarified 19 haplotypes against ND1 gene that found 22 haplotypes among livestock (Table 2). Sequencing results of the mtDNA showed intra-species identity 98. 5%-100% and Intra-species-diversity: 0-1.5% compared to the GenBank sequences.

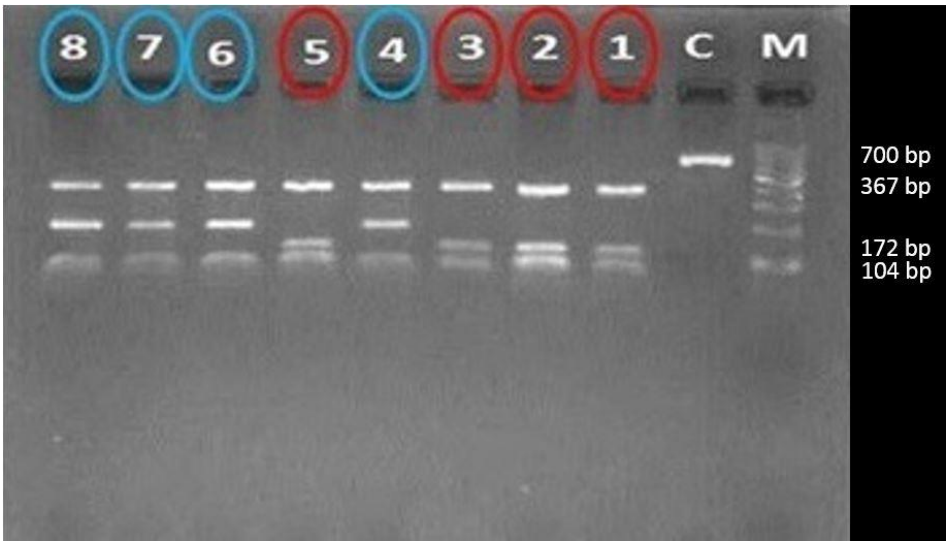

Fig. 2: The pattern of amplified *ITS* gene after digestion with *RsaI*: Lane M 100-bp DNA marker, Lane C: intact amplified *ITS1* gene as control, Lanes 1–3 and 5 are *F. hepatica*, Lanes 4 and 6-8 are *F. gigantica*

Table 2: Diversity and neutrality indices of *Fasciola hepatica* isolated from various hosts based on nucleotide sequences of the *COX1* and *ND 1*genes

Gene	***Host***	***n***	***Hn***	***Hd± SD***	***No. of polymorphic sites***	***Nd (π) ± SD***
Cox1	Cattle	17	7	0.652±0.01583	7	0.00368± 0.00339
	Goat	14	7	0.657±0.138	7	0.01567± 0.01306
	Sheep	16	5	0.450±0.151	6	0.00286±0.00314
ND1	Cattle	17	6	0.588±0.135	8	0.00359± 0.00250
	Goat	14	9	0.912±0.059	7	0.00486± 0.00231
	Sheep	13	7	0.795±0.795	19	0.00443±0.00304

n: number of isolates; Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity

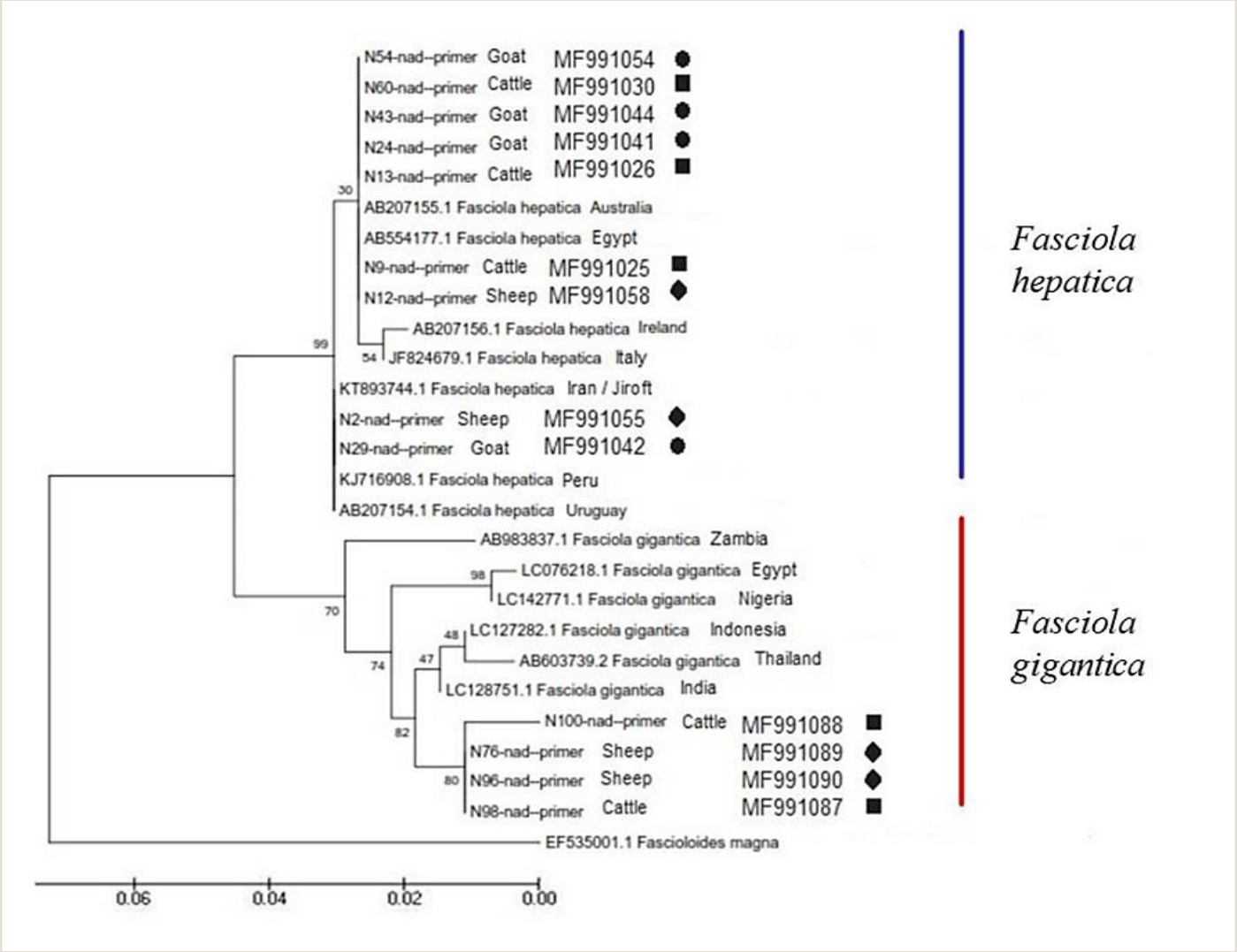

Fig. 3: Phylogenetic trees based on *NDI* gene sequences of isolated *Fasciola spp.* obtained by using MEGA 7.0 (Tamura 3- parameter model) with bootstrap values of 1000 replicates set for neighbor-joining. *Fascioloides magna* was used as outgroup

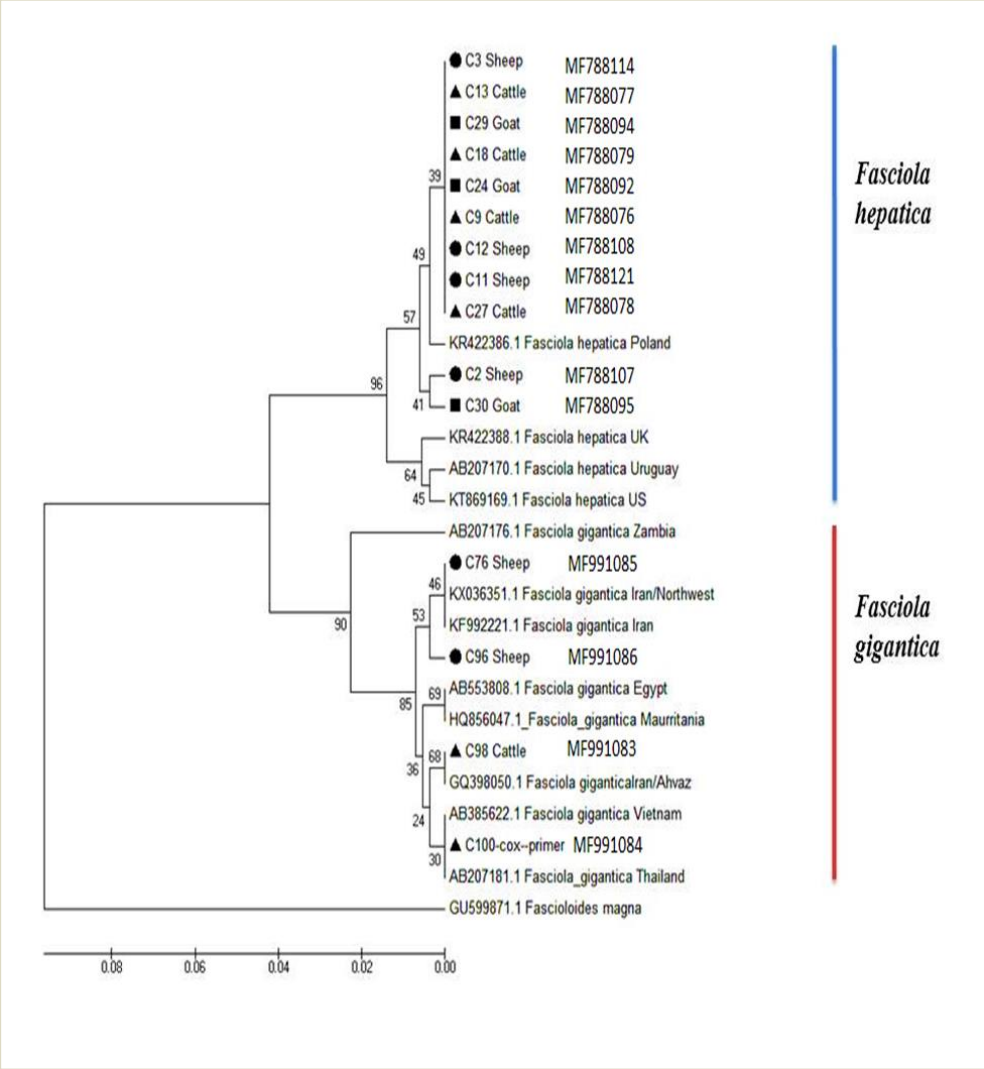

Fig. 4: Phylogenetic trees based on *Co1* gene sequences of isolated *Fasciola Spp.* obtained by using Mega 7.0 (Tamura 3- Parameter Model) with bootstrap values of 1000 replicates Set for neighbor-joining. *Fascioloides Magna* was used as out group

Discussion

The differentiation of *Fasciola* species and epidemiology patterns of the parasites is crucial for prevention of disease in the country. Both species of the *F. hepatica* and *F. gigantica* are reported in different parts of Iran (5, 15, 20), so accurate survey is necessary to distinguish the species in the country.

Differentiation of *Fasciola* species based on morphometric methods has some pitfalls such as morphological changes of the parasite by parasite age, type of host or intensity of the infection (5, 6). Therefore, differentiation of the fascioliasis causative agents using molecular methods, especially in the regions where both *F. hepatica* and *F. gigantica* are present, are suitable methods to determine the dominant species in the region.

One of the most appropriate molecular methods for the differentiation of *Fasciola* spp. is PCR-RFLP (6, 12, 13, 15, 21-24). The results of this study using PCR-RFLP method on *ITS1* nuclear gene revealed that two species of *F. hepatica* and *F. gigantica* are present in Lorestan Province and

F. hepatica is more prevalent than *F. gigantica*. This finding is consistent with the studies carried out in other parts of Iran and the world (12, 25-29), although in some parts, *F. gigantica* have been reported as dominant species (10, 30-32). These results also confirm previous reports that *F. hepatica* is found mainly in temperate regions while *F. gigantica* mainly observed in tropical regions (11).

The result of the present study showed a sheep isolate had simultaneous infection of *F. hepatica* and *F. gigantica,* aligned with foundlings in other parts of Iran and different parts of world such as Egypt, Japan, South Korea countries (33-36). The phylogenetic analysis performed in the present study based on *COI* and *ND1* gene sequences, clarified that all *F. hepatica* and *F. gigantica* isolates were placed in two branches with other specimens of different parts of the world. Our study was designed based on the *RsaI* enzyme to *ITS1* gene for differentiation of the two species. Three bands of 59, 172 and 367 bp fragments to *F. gigantica* and 59, 104 and 360bp fragments to *F. hepatica* were observed. Based on our results the enzyme could identify two species of *Fasciola* easily in the region, also the similar outcome obtained from Myanmar, Egypt and Thailand (22, 37-39). In Turkey, the enzyme *CO1* gene had no effect on *F. hepatica* (40).

According to the findings, the enzyme in ribosomal gene region, specific ITS1 gene is exclusive, while in the mitochondrial genes may be non-specific. Therefore, it probably that PCR-RFLP on nuclear genes could be used as beneficial and effective method in rapid diagnosis or even primary detection of the *Facsiola* species. Since the host specificity, drug resistance or sensitive, differences in parasitic virulence could be manifested in molecular variations (41), in the present study, genetic indices including the number of haplotypes, haplotype diversity, nucleotide diversity and the number of polymorphic sites were surveyed for ribosomal gene (*ITS1*) and mitochondrial genes (*ND1* and *CO1*) in livestock to determine the appropriate gene as for analysis of the genetic indicators.

Mitochondrial genes compare to ribosomal genes are better to study genetic diversity indices in different hosts and result of our study is consistent with other studies (27, 37, 42-45). Moreover, among mitochondrial genes, *ND1* gene compared to CO1 gene, with minor difference could be appropriate to determined genetic diversity and nucleotide mutation. This result also was consistent with the results of similar studies (35, 46, 47). Therefore, *ND1* gene can be used for inter-species and intra-species of *Fasciola* spp. genetic differences and similarities.

Conclusion

Using PCR-RFLP method and the ITS-1 nuclear gene, two species, *F. hepatica* and *F. gigantica*, are present in the current study. *F. hepatica* is more prevalent in Lorestan Province. The current study confirms that those mitochondrial genes can better test variability indices in different hosts than ribosomal genes, and that among mitochondrial genes, the *ND1* gene can better examine differences and similarities than *CO1*. Therefore, this gene can be used in inter-species and intra-species diversity of *Fasciola* species.

Ethics Journalism 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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Conflict of Interest

The authors declare there is no conflict of interest.

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