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Molecular Identification and Phylogenetic Analysis of Fasciola Hepatica Isolates From Cattle and Sheep in Golestan Province, Northeast of Iran.

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Abstract

Background: Fascioliasis in livestock and humans, caused by *Fasciola hepatica* and *Fasciola gigantica*, has socioeconomic importance worldwide. Iran is one of the important endemic foci for fascioliasis. Molecular approaches can be precise and reliable for the identification and characterization of *Fasciola spp*. The aims of this study were molecular identification of *Fasciola hepatica* isolates in Golestan province, north of Iran, and then comparative analysis of results using GenBank sequences.

Material and Method: *Fasciola* flukes were isolated from the livers of infected livestock. DNA was extracted from samples using the phenol-chloroform technique. RFLP-PCR using TasI restriction enzyme was performed along with morphometric evaluations for the detection of *F. hepatica* species. PCR was performed to amplify partial fragments of the internal transcribed spacer 1 (ITS1), cytochrome c oxidase sub.1 (CO1), and NADH dehydrogenase sub.1 (ND1) genes for samples that were confirmed as *F. hepatica.* Twenty-eight PCR products were sequenced. The phylogenetic tree with MEGA 7 software was drawn for all three genes.

Results: Out of 271 flukes collected from sheep and cattle, 126 confirmed as *F. hepatica* by the PCR-RFLP method. Results based on PCR-RFLP analysis were confirmed by sequence analysis. Only one haplotype for the CO1 gene and four different haplotypes for the ND1 gene were identified. Seven sequences of each gene registered on GenBank and accession numbers were received.

Conclusions: This study showed that *F. hepatica* is widely distributed among livestock in Golestan province. It was also found that genetic diversity among the ND1 region in isolates of our study was considerably higher than the CO1 sequence region.

Introduction

Fascioliasis is one of the most important diseases in domestic livestock and humans caused by the genus *Fasciola*. The disease causes significant economic loss and health problems in several regions of the world. *Fasciola hepatica* and *Fasciola gigantica* were thought to be the main causes of this disease worldwide (Mas-Coma 2005). Cattle and sheep are the primary hosts of the *Fasciola* spp. and intermediate hosts comprising lymnaeid snails such as *Galba* and *Fossaria* (Mas-Coma et al. 2009).

It is estimated that 2.4–17 million people contracted fascioliasis in more than 70 countries, and 91.1 million people living at risk of infection. In most areas where animal cases have been reported, human cases also exist. So no continent is free of Fascioliasis. (WHO, 2014; Ashrafi et al. 2015; Cwiklinski et al. 2019).

This zoonotic infection exists in some Asian countries including Iran, China, Turkey Iraq, Korea, Japan, Thailand, Vietnam, India, Bangladesh, Nepal, Yemen, Saudi Arabia, and Israel (Mahdi and Al-Baldawi 1987; Morel and Mahato 1987; Mas-Coma et al. 2005; Itagaki et al. 2005a; Do et al. 2007; Rokni 2008; Galtier et al. 2009; Ai et al. 2011; Salahi-Moghaddam et al. 2011; Ashrafi et al. 2015). Recently, some studies about the prevalence of animal fascioliasis have been done in several parts of Iran, such as Mazandaran, Guilan, Azerbaijan, Khuzestan, Kurdistan, Golestan, and Tehran (Moghaddam et al. 2004; Ashrafi et al. 2006; Sarkari et al. 2017; Halakou et al. 2017). Concerning the health, financial and veterinary problems of fascioliasis in

Iran, it is important to detect the species of the genus of these helminths to do follow-up work, control, and treatment of the disease.

Due to the several variations in morphological specifications, abnormal ploidy (diploidy, triploidy, and mixoploidy), morphological methods cannot guarantee the accurate differentiation between *Fasciola* species (Itagaki et al. 1998; Mas-Coma et al. 1999). Therefore, several studies, especially to genotyping *Fasciola spp.* have been done in different parts of Iran (Aryaeipour et al. 2014; Bozorgomid et al. 2016).

Over the last two decades, to overcome the limitation of the morphologic methods, several PCR-based methods, including specific PCR assays, PCR-RFLP, and PCR-SSCP, have been developed for the identification and differentiation of *Fasciola spp.* PCR-RFLP technique is the one in which many researchers expressed its importance (Hashimoto et al. 1997; Marcilla et al. 2002; Aryaeipour et al. 2014; Cwiklinski et al. 2018).

This technique has been used in some studies based on internal transcribed spacer (ITS1) or ITS2, 28s rRNA, 18s rRNA, etc. (Marcilla et al. 2002; Huang et al. 2004; Rokni et al. 2010). ITS1 sequence was an appropriate genetic marker for genotyping and phylogenetic studies of helminths (Prasad et al. 2008). PCR-RFLP analysis of ITS1 using some restriction enzyme was used as a simple and reliable method for *F. hepatica* and *F. gigantica* differentiation (Rokni et al. 2010).

Another marker to differentiate *Fasciola spp.* is mitochondrial DNA (mtDNA). The mitochondrial genome, due to its faster evolving and elevated mutation rate in comparison with the nuclear genome, is a suitable target for discrimination among individuals from the same population and closed related species (Boore 1999; Le et al. 2001; Semyenova et al. 2006; Ladoukakis and Zouros 2017).

Cytochrome b oxidase subunit 1 (CO1) and NADH dehydrogenase subunit 1 (ND1) are the main mitochondrial genes used for genetic variability (Itagaki et al. 2005b, a). This discrimination could be more useful, especially at the level of species or sub-species (Galtier et al. 2009). Garey and Wostenholme described mitochondrial DNA genes, including ND1 and CO1 of *F. hepatica* for the first time (Garey and Wolstenholme 1989). Walker et al. showed that mitochondrial DNA shows polymorphism in *F. hepatica* populations of cattle and sheep, which causes genetic diversity and provides clues for the existence of different mitochondrial lineages within infra-populations of *F. hepatica* (Walker et al. 2007).

Since Golestan province is one of the endemic areas for animal and human fascioliasis in Iran (Halakou et al. 2017; Bahram et al. 2020), the present study was designed for molecular identification and phylogenetic analysis of *F. hepatica* using ITS1 ribosomal and CO1/ND1 mitochondrial DNA genes.

Materials And Methods

Study area and sample collection

In this field study, from December 2014 to July 2016, 271 samples were collected in Golestan Province (Halakou et al. 2017). Liver flukes were collected from infected cattle and sheep in several abattoirs of Gorgan, Gonbad Kavus, Ramian, Aliabad, Aq Qala, Bander Turkman, and Kalaleh in Golestan province in the northeast of Iran (Fig. 1). The liver worms were identified to the species based on morphometric criteria

according to standard taxonomic keys (Yamaguti 1958; Sahba et al. 1972) and the posterior parts (excluding the uterus) of worms' bodies were cut and put in ethanol 70% and stored at -80 °C until further use.

DNA extraction and PCR

Samples were washed three times with PBS buffer. Small posterior parts of the worms were subjected to DNA extraction using a conventional phenol-chloroform method with slight modifications (Sambrook et al. 1989) and extracted DNA samples were stored at -20°C. PCR for *partial* sequences of ITS1, CO1, and ND1 genes was performed (Itagaki et al. 2005a) in 15 µl reactions containing 1µL (1ng) DNA template, 1µL primers (10 pmol of each primer), 5.5 µL distilled water, and 7.5µL master mix (Ampliqon, Denmark). Table 1 shows the sequences of primers, PCR profiles, numbers of cycles, and sizes of amplicons that were determined for *F. hepatica* ITS1, CO1, and ND1 genes. 5µL of each PCR product was examined in a 1.5% agarose gel and stained with Ethidium Bromide (EtBr) and visualized under a UV transilluminator.

Table 1. Primers and profiles used for amplification of ITS1, CO1, and ND1 genes.

Primer	Sequence	Denaturing (temp (°C) /time (sec))	Annealing (temp (°C) /time (sec))	Extension (temp (°C) /time (sec))	Product Size (bp)	Cycles
ITS1	F(ACCGGTGCTGAGAAGACG) R(CGACGTACGTGCAGTCCA)	94/30	60/30	72/30	463	30
CO1	F(ACGTTGGATCATAAGCGTGT) R(CCTCATCCAACATAACCTCT)	94/45	57/45	72/45	438	30
ND1	F(AAGGATGTTGCTTTGTCGTGG) R(GGAGTACGGTTACATTCACA)	94/30	58/45	72/30	535	30

Restriction Fragment Length Polymorphism (RFLP) analysis

PCR-RFLP analysis was used to differentiate *F. hepatica* from *F. gigantica* in the ITS1 gene with TasI enzyme (Fermentas, Canada). PCR-RFLP producing the two expected restriction fragment sizes of 312 and 151bps for *F. hepatica,* and three fragment sizes of 215,151, and 93 bps for *F. gigantica* (Halakou et al. 2017).

A total volume of RFLP reaction contained 0.5 µL of the TasI enzyme, 1.5 µL of enzyme buffer, 9mL distilled water, and 5µL ITS1-PCR product was incubated at 65° C for 2.5 h and 80° C for 20 minutes. 5µL of each reaction product mixed with 2µL of loading buffer was subjected to the gel electrophoresis on 2.5% agarose gel in TAE buffer and were stained with Ethidium Bromide.

Sequencing and Phylogenetic Tree Analysis

Due to budget constraints, 14 samples of RFLP confirmed *F. hepatica* samples were sequenced for ITS1, CO1, and ND1 genes. About 25µL of PCR products were sent to Bioneer Company (South Korea). The sequencing results were edited by Chromas software version 2.22 and analyzed with Nucleotide Blast. Then, all sequenced samples were compared with different samples studied in Iran and some other parts of the world registered in the GenBank.

A maximum-likelihood (ML) phylogram based on ITS1, CO1, and ND1 genes was constructed using MEGA 7. In addition to the ITS1, ND1, and CO1 haplotypes of the *Fasciola* flukes from Golestan province, those of *F. hepatica* from other parts of Iran and some countries were included as references (Figure 4-6). Nucleotide sequences of *Fasciolopsis buski* (GenBank:EF612477), and *Fasciola gigantica* (GenBank:AB385622, AB385617) were used as outgroups.

Results

The primers used in this study successfully amplified a fragment of 463 bp in all *Fasciola spp.* samples for the ITS1 gene (Figure 2). PCR products of all ITS1 samples were digested with TasI enzyme. TasI has one cutting site for *F. hepatica* and two cutting sites for *F. gigantica*. Samples identified as *F. hepatica* were selected for following sequencing analysis, and the rest samples (*F. gigantica* samples) were stored at -20℃ for further eventual studies. Figure 3 shows the PCR-RFLP pattern after digestion with the TasI enzyme. Table 2 shows the number of *Fasciola spp.* infected sheep and cattle confirmed by RFLP-PCR.

Table 2. Species identified based on the host in Golestan province (PCR-RFLP technique)

Sheep			**Cattle**		
Total	***Fasciola gigantica***	***Fasciola hepatica***	**Total**	***Fasciola hepatica***	***Fasciola gigantica***
168	44	124	103	2	101
100%	26.19%	73.8%	100%	1.94%	98.05%

Due to the similarity of ITS1 sequences, only seven isolates of 14 *F. hepatica* species were registered in GenBank (Table 3). Primers for amplifying mitochondrial genes successfully amplified fragments of 438 bp and 535 bp for CO1 and ND1 genes, respectively. Results of sequencing for CO1 and ND1 genes for the seven samples were the same as ITS1. Alignment of our results with NCBI Blast databases revealed that PCR product samples on both CO1 and ND1 were *F. hepatica*. Also, all seven samples were sequenced for mitochondrial genes. All results are presented according to the host and location in Table 3. All CO1 and ND1 sequences were checked for identical recorded haplotypes using BLAST. This analysis showed that FhC2 was the only single haplotype for CO1 while ND1 had four several haplotypes (Table 4).

Table 3. Genbank accession numbers of ITS1, CO1, and ND1 genes obtained in this study

ND1 Accession Number	CO1 Accession Number	ITS1 Accession Number	Parasite	host	town	Number
MN527604	MN527597	KX577794			Aliabad	1
MN527602	MN527599	KX577795			Aqqala	2
MN527603	MN527596	KX577797			Bandar Turkman	3
MN527605	MN527595	KX577798			Gonbad Kavus	4
MN594514	MN527594	KX577799			Gorgan	5
MN597600	MN527593	KX577800			Gorgan	6
MN594515	MN527598	KX577801	*Fasciola hepatica*	Sheep	Ramian	7

Table 4. CO1 and ND1 gene haplotypes according to sequence similarity using BLAST.

number	GenBank Accession numbers	location	host	CO1 haplotypes	ND1 haplotypes
1	CO1:MN527597 ND1:MN527604	Aliabad		FhC2	Not found
2	CO1:MN527599 ND1:MN527602	Ramian		FhC2	FhN21, FhN2
3	CO1:MN527596 ND1:MN527603	Bandar Turkman	Sheep	FhC2	Not found
4	CO1:MN527595 ND1:MN527605	Gonbad Kavus		FhC2	Fh20
5	CO1:MN527594 ND1:MN594514	Gorgan		FhC2	Fh04
6	CO1:MN527593 ND1:MN527600	Gorgan		FhC2	Fh27, Fh26, Fh18, Fh08, Fh01
7	CO1:MN527598 ND1:MN594515	Ramian		FhC2	Fh27, Fh26, Fh18, Fh08, Fh01

To construct a phylogenetic tree, we used MEGA 7 computer software (Kumar et al. 2016). Phylogenetic trees were obtained by comparing the sequences of *F.hepatica* from Golestan province with available ITS1, CO1,

and ND1 genes from other parts of the world (Figs. 4, 5, and 6). The phylogenetic analysis of ITS1 sequences revealed that all isolates from the present study were very similar to each other and closely related to the references from Uruguay, Japan, Australia, the United Kingdom, and also some other parts of Iran. The results showed that all samples from this Golestan province were composed of 100% homologous sequences, and only the Bandar Turkman sample was recorded with 82% homology compared to other samples. (Figure 4). All isolates of *F. hepatica* positive for CO1 gene in the present study were closely related to the references from Ecuador, Poland, and some other parts of Iran (Figure 5). Results of the phylogenetic tree analysis for *F.hepatica* ND1 indicated that isolates from Gonbad Kavus, Gorgan, and Ramian were similar to two isolates from Iran (GenBank GQ175362-63), and two isolates from Egypt (GenBank LC076257 and AB554177). Isolates from Bandar Turkman and Aliabad were very similar to each other. Finally, an isolate from Gorgan (GenBank MN594514) was very similar to a sequence from China (Figure 6).

Discussion

Fasciola species, including *F. hepatica* and *F. gigantica,* were considered as a critical veterinary problem until the end of the 1980s, mainly due to considerable economic losses which these parasites cause in livestock (Mas-Coma et al. 1999). Meanwhile, human infections were rare and sporadic (Cook 1996). Rising cases of human fascioliasis over the 1970-90 years in 42 countries convinced the World Health Organization to consider fascioliasis as an important human disease (Mas-Coma et al. 1999). Both *Fasciola* species including *F. hepatica* and *F. gigantica* can infect humans and animals. Many techniques including morphometry and molecular-based methods have been developed to differentiate these species (Mas-Coma and Bargues 1997; Itagaki et al. 1998; Marcilla et al. 2002; Ashrafi et al. 2006; Amer et al. 2011). DNA-based techniques like PCR-RFLP are the most appropriate methods for differentiation *Fasciola* flukes in comparison with other diagnostic methods (Marcilla et al. 2002). Moreover, sequence analysis of both ribosomal and mitochondrial DNA of *Fasciolaspp*. is a reliable method for genotyping, phylogenetic studies, and inter/intra-species investigation (Boore 1999; Le et al. 2001; Itagaki et al. 2005b; Semyenova et al. 2006; Prasad et al. 2008; Rokni et al. 2010; Ladoukakis and Zouros 2017)

In the present study, the TasI restriction enzyme was used for the RFLP technique based on the 463 bp sequence of the ITS1 gene. These *F. hepatica* sequences revealed two fragments of 151 and 312 bp, while *F. gigantica* producing three fragments of 151, 219, and 93 bp. Our samples based on the hosts showed that both species of *Fasciola* exist in Golestan province, and the majority of samples isolated from sheep were *F. hepatica* (69.65%). Many studies using PCR-RFLP have been done for detecting *Fasciola* spp. by targeting the ITS1 gene in Iran (Yamaguti 1958; Mas-Coma et al. 1999; Marcilla et al. 2002; Itagaki et al. 2005a; Kumar et al. 2016). Morphometric and PCR-RFLP results in the present study were consistent with some of the mentioned studies (Marcilla et al. 2002). In 2015, Yakhchali et al. used RFLP for identification and sequencing for the phylogenetic tree construction. They reported that all species of *Fasciola* worms found in West Azerbaijan were *F.hepatica* (Yakhchali et al. 2015). The RFLP-PCR and sequencing results of the present study using the ITS1 gene showed that most of the sheep were infected with *F. hepatica*, while the predominant species in cattle was *F. gigantica*.

ITS1 sequence analysis comparing to other registered sequences on GenBank databases showed that almost all of the isolates of the present study and selected isolates from different parts of the world were 100%

identical. The only exception was a sample from Bandar Turkman, which showed 82% identity to other selected isolates. High similarity between the isolates in this region may cause a great variation even due to one or two nucleotide change.

Results obtained from the sequencing of ITS1, CO1, and ND1 genes confirmed that *F. hepatica* is the main fluke of sheep in the Golestan province in the north of Iran. Sarkari et al. (2017) showed that 78% of flukes isolated from sheep in Talesh county located at Guilan province (neighboring province in Northern Iran) were *F. hepatica* using PCR-RFLP assay. Comparing CO1 sequences of *F. hepatica* isolated from the present study with other sequences revealed that all isolates of our study showed 100% identity with some recorded sequences from Iran, Ecuador, and Poland. Based on our findings, there were no differences between CO1 sequences in our studied isolates. According to comparing our isolates sequences with the GenBank database using the BLAST tool, the main haplotype of the CO1 gene was FhC2.

According to the results of the phylogenetic tree, *F. hepatica* ND1 sequences of isolates from Aqqala showed 85% identity with sequences from Gonbad Kavus, Gorgan, Ramian, Egypt, and some other parts of Iran which had 100% identity with each other their selves. Two isolates from Bandar Turkman and Aliabad showed 100% identity with each other and over 80% identity with isolates from Aqqala, Gonbad Kavus, Gorgan, Ramian, Egypt, Iran, Uruguay, Ireland, and Italy. One reminded isolate from Gorgan showed 100% identity with an isolate from China. The Findings of the present study represent substantial differences between sequences of ND1 *F. hepatica* isolates from various regions of Golestan province. These findings were far different from results in which Sarkari et al. (2017) presented about substantial diversity of CO1 sequences compared to ND1 sequences. Based on the GenBank database and sequence similarity of ND1 gene, haplotypes Fh20 from Gonbad Kavus, haplotypes Fh04 and (Fh27/Fh26/Fh18/Fh08/Fh01) from Gorgan, and haplotypes (FhN21/FhN2) and (Fh27/Fh26/Fh18/ Fh08/Fh01) from Ramian were found (Table 4). Haplotypes that are separated with a slash (/) had completely identical sequences, and it is not clear why authors selected different names for them.

Sharifiyazdi et al. (2010) used ribosomal ITS1 and mitochondrial DNA (CO1 and ND1 genes) for genetic characterization of two *Fasciola* samples directly isolated from humans in Guilan province, North of Iran. Both samples were confirmed as *F. hepatica* and showed 100% identity to an isolate from a Japanese patient (Sharifiyazdi et al. 2012).

Sarkari et al. (2015), using ITS1, ITS2, ND1, and CO1 genes, showed that over 87% of liver flukes that were isolated from sheep in Guilan province, were *F. hepatica*. They indicated that CO1 region sequences exhibit considerable variation among different isolates (Sarkari et al. 2017). The present study shows substantial diversity over the ND1 region instead. Raeghi et al. (2016) used ND1 and CO1 sequence regions for phylogenetic analysis of 90 *F. hepatica* flukes collected from 30 cattle in the North-East of Iran. Their study revealed significant genetic differences of the ND1 region among the analysed isolates (Raeghi et al. 2016). The overall result of the present study shows a correlation with what they concluded.

In a study by Shafiei et al. (2014) on different *Fasciola* hosts in Kohgilouyeh and Boyer-Ahmad province, in the southwest of Iran, ribosomal ITS1, ITS2, mitochondrial ND1, and CO1 sequencing were used for the analysis of genetic variations within and between isolates. ITS1 sequences showed six single base

substitutions for *F. hepatica* isolates. Also, CO1 sequences showed seven DNA polymorphic sites for *F. hepatica* samples.

Aghayan et al. (2019) used 28s rRNA an ND1 for evaluating the genetic diversity of *Fasciolaspp*. in livestock from central Armenia. They found 29 haplotypes of the *F. hepatica* ND1 gene among 55 flukes and Fh01was the dominant haplotype (Aghayan et al. 2019).

Conclusion

Overall, the findings of the present study suggested that *F. hepatica* is the main liver fluke of the sheep population of Golestan province, North of Iran. This study indicated that the sequence differences between isolates among ND1 were much more than CO1 individuals. All samples were identified by morphometric and ITS1 RFLP-PCR evaluations, confirmed by ITS1, CO1, and ND1 genes sequencing as *F. hepatica*. Sequencing analysis of the ITS1, CO1, and ND1 regions showed no intermediate form of *Fasciola* in our research.

Declarations

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Figures

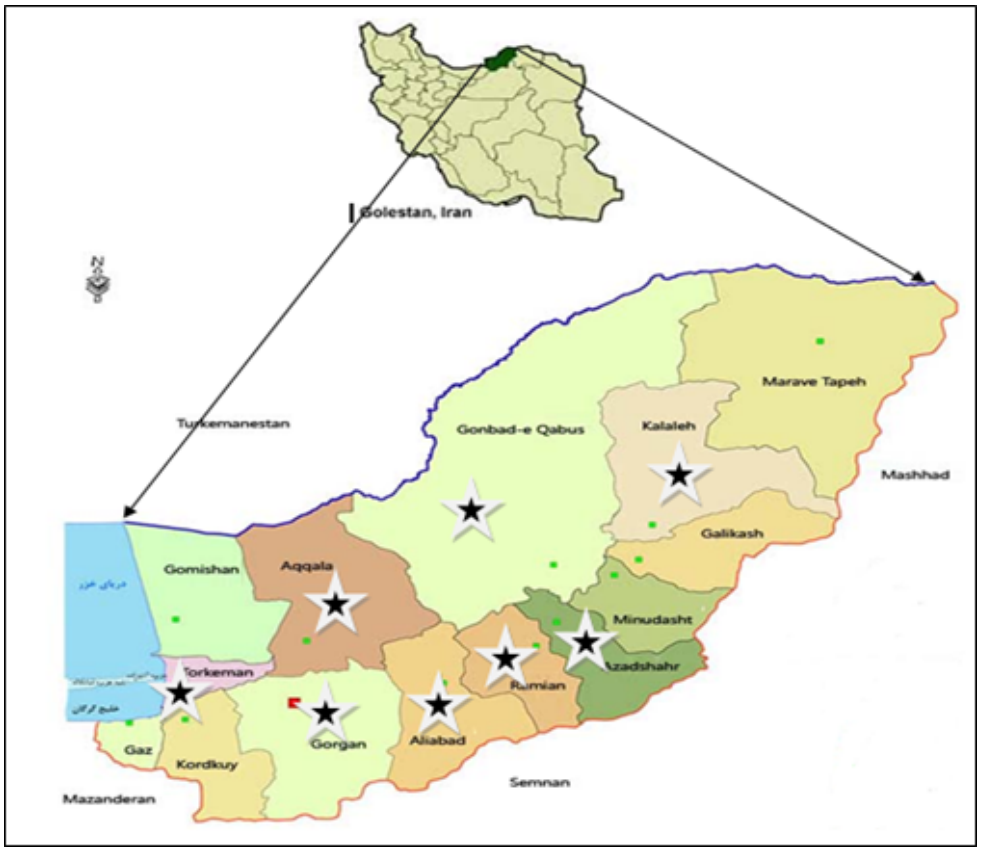

Figure 1

Golestan province (North-East of Iran) and locations where the samples were collected Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

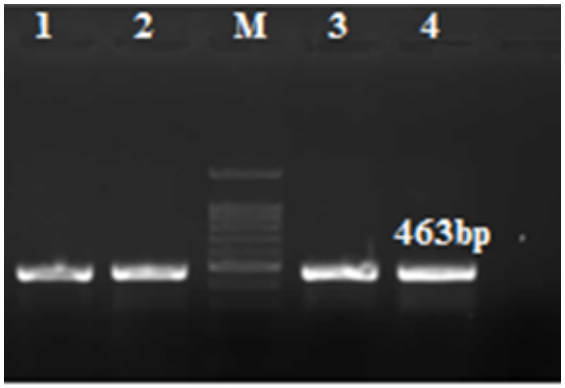

Figure 2

gel electrophoresis of Fasciola. ITS1–PCR products. Lanes1 -4: Fasciola spp; Lane M: 100 bp DNA ladder

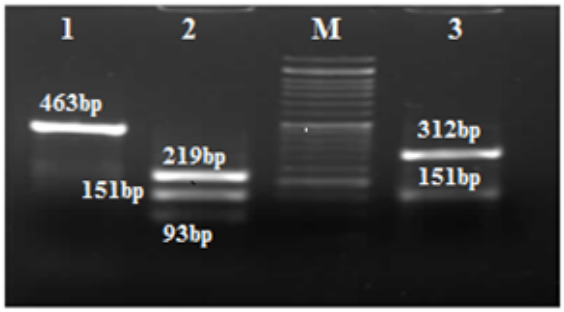

Figure 3

PCR-RFLP pattern of Fasciola after digestion with TasI enzyme. Lane1: PCR product Fasciola before enzymatic digestion; Lane 2 Fasciola gigantica from cattle; Lane M: 50 bp DNA ladder; Lane 3: F. hepatica from sheep.

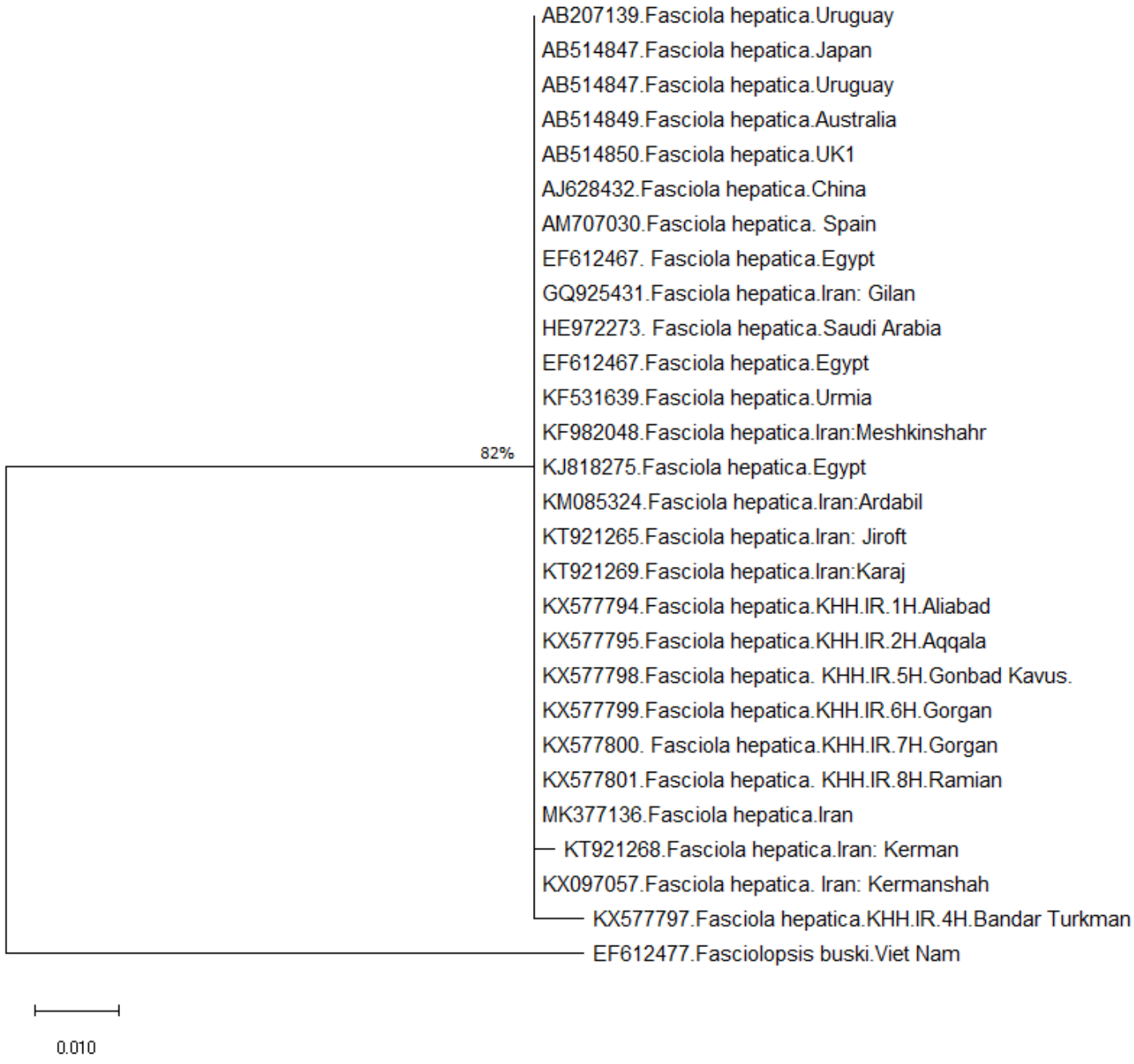

Figure 4

Maximum-likelihood tree of F. hepatica ITS1 gene from Golestan Province of Iran. The nucleotide sequence of the F. buski (GenBank accession no EF612477) detected in Vietnam was considered outgroup.

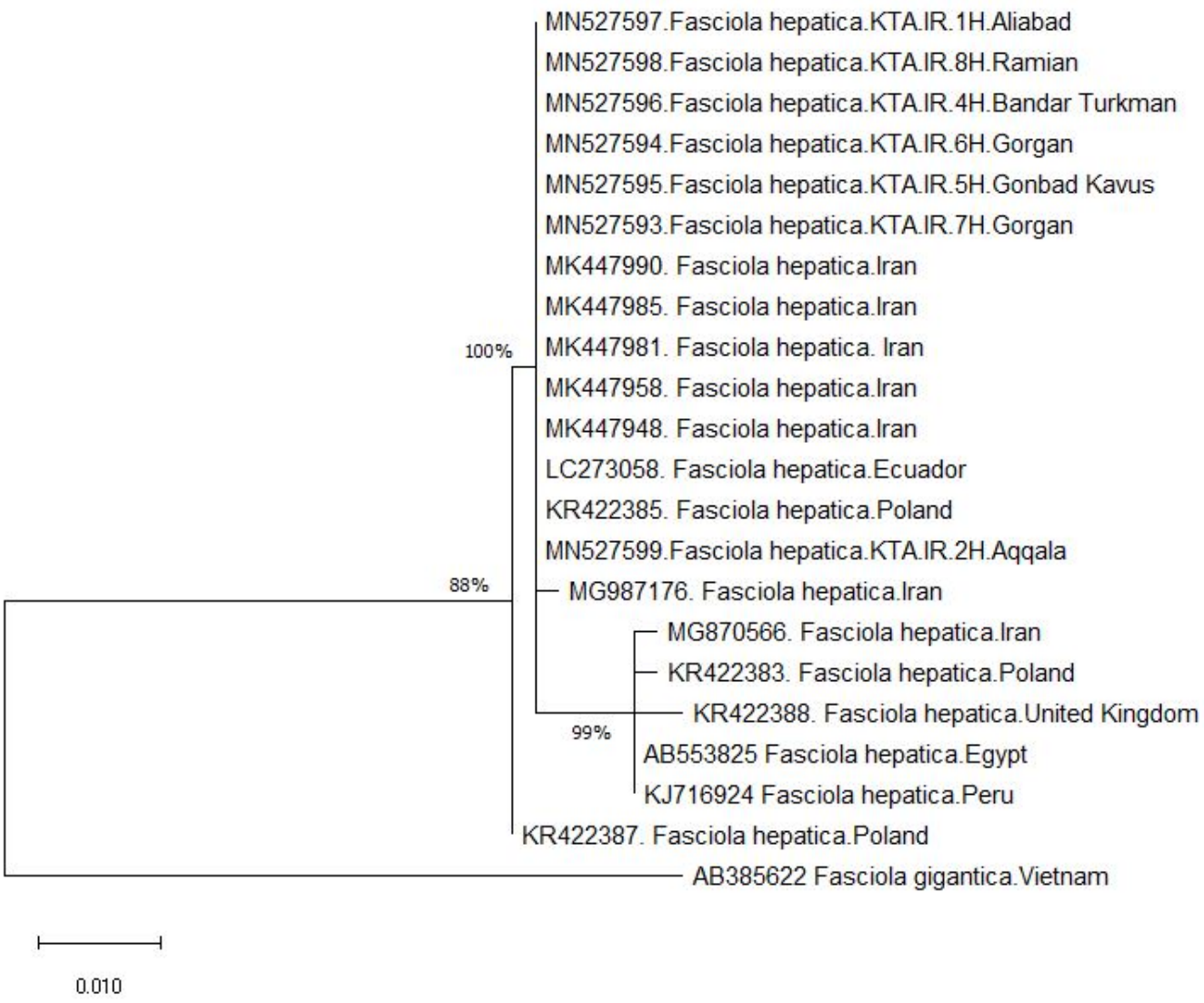

Figure 5

Maximum-likelihood tree of F. hepatica CO1 gene from Golestan Province of Iran. The nucleotide sequence of the F. gigantica (GenBank AB385622) detected in Vietnam was considered as an outgroup

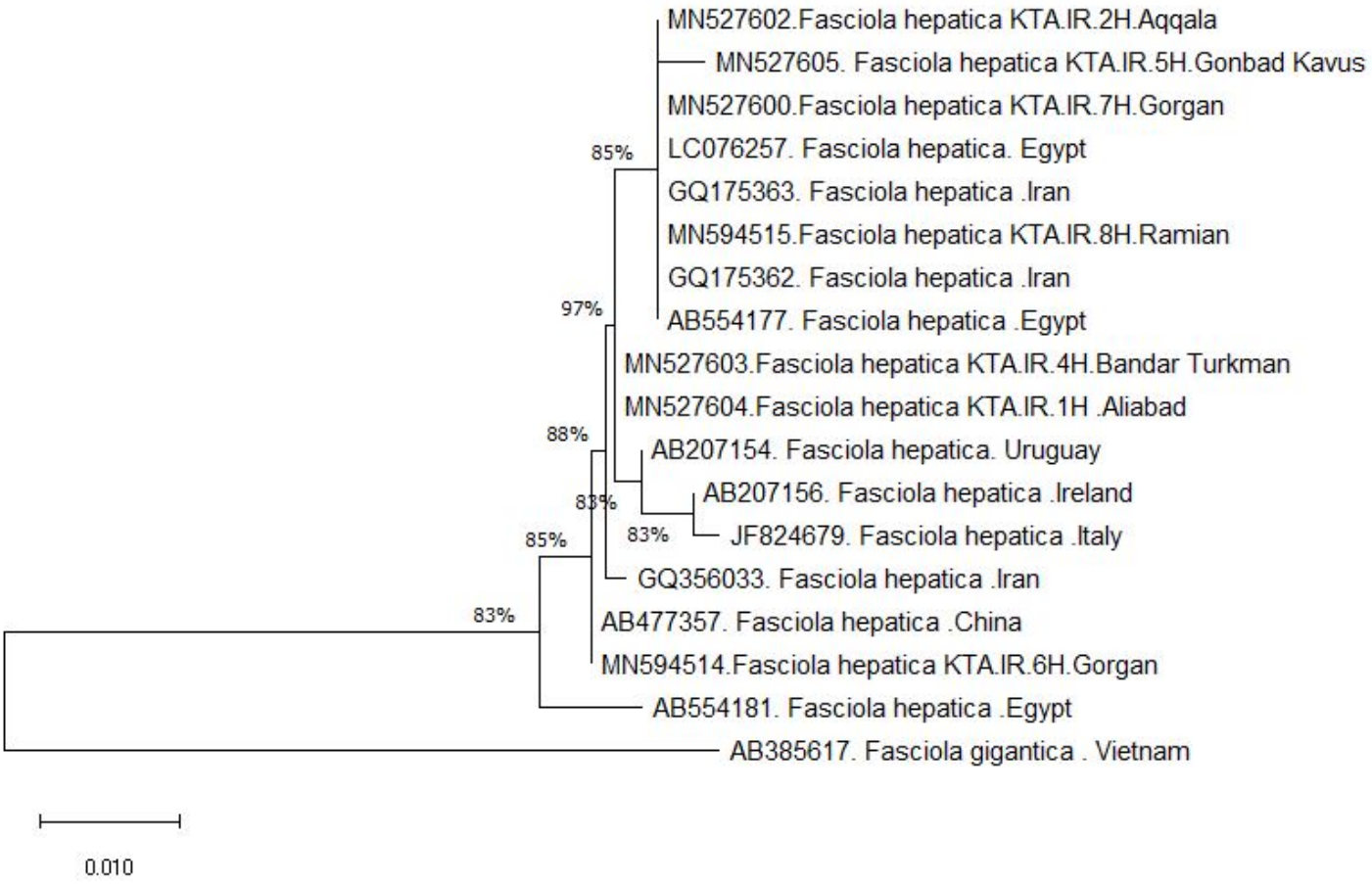

Figure 6

Maximum-likelihood tree of Fasciola hepatica ND1 gene from Golestan Province of Iran. The nucleotide sequence of the F. gigantica (GenBank AB385617) detected in Vietnam was considered as an outgroup