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ORIGINAL ARTICLE

Genetic diversity of *Fasciola* spp. isolates from northern part of Iran: comparison with southwestern isolates

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Abstract Animal and human fascioliasis is a health and economic problem in few of tropical and subtropical areas of the world, including Iran. The present study aimed to determine the genotype diversity of *Fasciola* isolates in different hosts from Gilan province, northern Iran, and compare it with those isolates from southwestern Iran. Forty-eight adult *Fasciola* spp. were collected from cattle, sheep, and goats from slaughterhouse in Talesh, north of Iran. DNA was extracted from each fluke and PCR-RFLP was used to find out the species of the isolates. The ribosomal ITS1 and ITS2, and mitochondrial genes of NDI and COI from individual *Fasciola* isolates of each host were PCR-amplified and the PCR products were sequenced. Genetic variation within and between the isolates was evaluated by comparing the sequences of ribosomal and mitochondrial genes. For analysis of phylogenetic diversity of the flukes, phylogenetic trees were constructed, using ITS1, ITS2, NDI, and COI sequences of the isolates. Based on PCR-RFLP profile, 5 (22.7%) of the total of sheep isolates and 18 (90%) of cattle isolates were identified as *F. gigantica* and other remaining samples from sheep, cattle and goats were identified as *F. hepatica*. Based on ITS1 and ITS2 sequences, six and seven nucleotide polymorphism were respectively noted in the isolates. On the other hand, CO1 region sequences showed considerable variation, which laid Talesh (north) isolates in a separate cluster. Findings of the study showed that the sequences of CO1 isolates from north and southwest have substantial differences mainly in CO1 region.

Keywords Genotype · Fasciola · Iran · Ribosomal · Mitochondrial · DNA sequences

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Introduction

Fasciola, is one of the most important food and waterborne parasitic zoonoses and a major public health problem in some of tropical and subtropical countries (Ashrafi et al. 2015; Hosseini et al. 2015; Mas-Coma et al. 2009; Sarkari et al. 2012). The two species *F. hepatica* and *F. gigantica* are considered as causative agents of human and animal fascioliasis. While *F. hepatica* is dominantly in temperate areas, *F. gigantica* is usually in tropical areas (Dar et al. 2012). Moreover, presence of intermediate forms of the parasite has been recognized in some Asian endemic areas including Iran, Thailand, Myanmar, Japan, Vietnam and Korea (Shoriki et al. 2014).

Human and animal fascioliasis is serious health and veterinary problems in Iran (Ashrafi et al. 2015; Hosseini et al. 2015; Moazeni et al. 2012; Sarkari et al. 2012; Mohammadi-Ghalehbin et al. 2012). Northern Province of Gilan in Iran is one of the most important endemic regions of the disease where two massive outbreaks of human

fascioliasis occurred in 1987 and in 1997 and affected more than 10,000 people (Ashrafi et al. 2015; Rokni 2008).

The evaluation of *Fasciola* genotypes and increasing of the knowledge of genetic diversity in the northern part of Iran is necessary due to the presence of intermediate form and both species of *Fasciola* (Ashrafi et al. 2006). The most frequently used methods for taxonomical studies and differentiation of two *Fasciola* species have been morphological approaches, although differences in isoenzymatic and also in the electrophoretic patterns of two species are reported (Abdolahi Khabisi and Sarkari 2016; Sarkari et al. 2016). Nowadays, morphometric methods considered inappropriate for differentiation of the two *Fasciola* species. Molecular techniques, using mitochondrial markers including ND1 and CO1 and ribosomal DNA markers including ITS1 and ITS2, are recently utilized to find out the genetic variation of *Fasciola* species (Itagaki et al. 2005; Peng et al. 2009; Shafiei et al. 2013, 2014; Shoriki et al. 2014). The present study was conducted to determine the genotype diversity of *Fasciola* isolates in different hosts from Gilan province, northern Iran, and compare it with those isolates from southwestern Iran (Shafiei et al. 2014), which has a different geographical and ecological features.

Fig. 1 Map of Iran and study area (Talesh County in Gilan province)

Materials and methods

Study area

Fasciola isolates were collected from Talesh abattoir located in Gilan province, 140 km north-west of Rasht, the capital of Gilan province, on the southwest coast of the Caspian Sea (Fig. 1). Talesh is a mountainous wonderful region covering an area of 2373 square kilometers, about one quarter of surface area of Gilan province.

Parasites collection

Adult *Fasciola* (n = 48) were collected from liver of sheep (n = 22), goats (n = 6) and cattle (n = 20) from Talesh in Gilan province. The worms were washed four times, in PBS, and then preserved in 70% ethanol until use.

DNA extraction and PCR amplification

DNA extraction and PCR amplification was performed as previously described (Shafiei et al. 2014). Briefly, a portion of the apical and lateral zone of adult flukes was removed and homogenized, using 500 µL of lysis buffer (50 mL of Tris–HCl (100 Mm), pH 8; 1 mM of EDTA; 0.1% of SDS) in tissue grinder. 8 µL of proteinase K (20 mg/mL) was added and the sample was incubated overnight at 37 °C. DNA was extracted with phenol–chloroform method. PCR was performed to amplify the target genes, CO1, ND1, ITS1 and ITS2, using four pairs of primers as shown in Table 1 (Itagaki et al. 2005).

Table 1 Sequences of the primers used in PCR reaction

Target	Primers	Sequence
CO1	Ita 8	5-ACGTTGGATCATAAGCGTGT-3
	Ita 9	5-CCTCATCCAACATAACCTCT-3
ND1	Ita 2	5-GGAGTACGGTTACATTCACA-3
	Ita 10	5-AAGGATGTTGCTTTGTCGTGG-3
ITS1	ITS1-F	5-TTGCGCTGATTACGTCCCTG-3
	ITS1-R	5-TTGGCTGCGCTCTTCATCGAC-3
ITS2	ITS2-F	5-TGTGTCGATGAAGAGCGCAG-3
	ITS2-R	5-TGGTTAGTTTCTTTTCCTCCGC-3

PCR-PFLP

PCR-RFLP was used to specifically determine the *Fasciola* species by fast digestion of ITS1 region, using *RsaI* enzyme. To do that, in 10 µl of PCR products, 17 µl of water nuclease free, 2 µl of fast digest green buffer (10×) and 1 µl of fast digest enzyme were mixed and incubated at 37 °C for 10 min. The product was loaded on 2.5% agarose gel.

DNA sequencing

PCR products of ITS1, ITS2, COI, and NDI of isolates from each host (cattle, sheep, and goat) were purified, using Vivantis purification kit (Vivantis Technologies Sdn. Bhd., Malaysia) and sequenced from both directions using the same primers which were used in the PCR.

Phylogenetic analysis

The sequences were aligned and compared with those of previously available sequences from our previous study in southwest of Iran and from other regions of the world, available in the GenBank, using the BLAST program of GenBank. Maximum likelihood tree was constructed, using the MEGA 5.0 software.

Results

PCR-RFLP analysis

Based on PCR-PRFLP profile, five (22.7%) of sheep isolates and 18 (90%) of cattle isolates were *F. gigantica* whereas 77.3% of sheep isolates, 10% of cattle isolates and all of the goat isolates were *F. hepatica.*

Genotype analysis based on ITS1 sequences

Sequences of ITS1 region (600 bp) of *F. hepatica* and *F. gigantica* were aligned with those of available sequences of both *F. gigantica* and *F. hepatica* from southwestern area of Iran. Between these isolates, six DNA variable sites were observed in which single-base substitution at different sites was occurred.

Genotype analysis based on ITS2 sequences

Multiple alignments between sequences of *F. gigantica* and *F. hepatica* isolates showed five variable sites. Aligned sequences of ITS2 region (505 bp) of *Fasciola* isolates form north of the country with those from southwest area showed seven variable sites which single-base substitution were occurred.

Genotype analysis based on CO1 sequences

Alignment of CO1 region (438 bp) sequences of *F. gigantica* and *F. hepatica* isolates showed 34 variable sites. Substitutions were observed at different nucleotide sites. Isolates from north area showed considerable nucleotide site substitutions in comparison with southwest isolates.

Genotype analysis based on ND1 sequences

Alignment between partial ND1 (535 bp) sequences of *F. hepatica* isolate showed only one variable site. However, 33 variable nucleotide sites were detected between north and southwest isolates.

Phylogenetic analysis

Phylogenetic trees were constructed, using ITS1, ITS2, NDI, and COI sequences of *F. gigantica* and *F. hepatica* from the present study along with sequences from the southwest region in our previous study, and also from other regions of the world. Phylogenetic analysis of ITS1 and ITS2 of northern isolates in comparison to southwest isolates and other identified isolates from different geographical regions showed two clusters of *Fasciola* species (Figs. 2, 3).

In CO1, north and southwest isolates separated into two different clusters (Fig. 4) demonstrating considerable differences between Iranian *Fasciola* isolates from north and south of the country. In ND1, highly relationship was observed between north isolates and southwest isolates (Fig. 5).

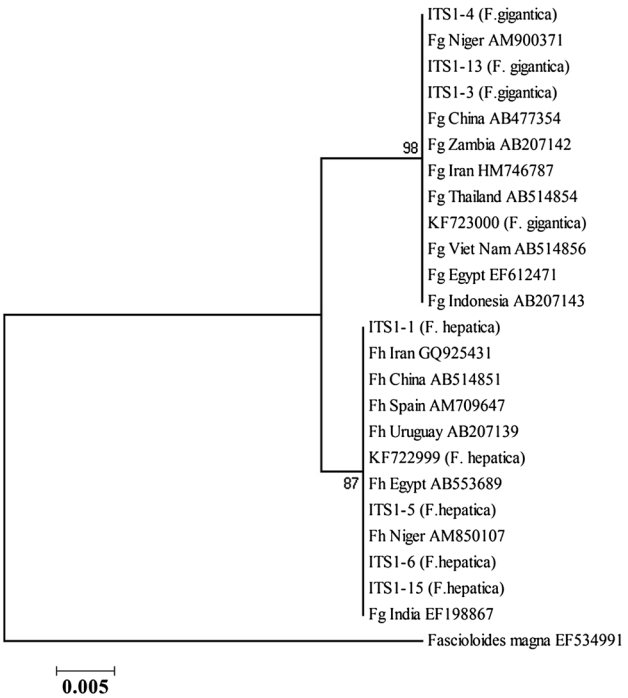

Fig. 2 Phylogenetic association between ITS1 sequences of north (*1*, *3*, *4*, *5*, *6*, *13*, *15*) and southwest isolates (KF) of Iran and identified isolates from different geographical regions, using likelihood method

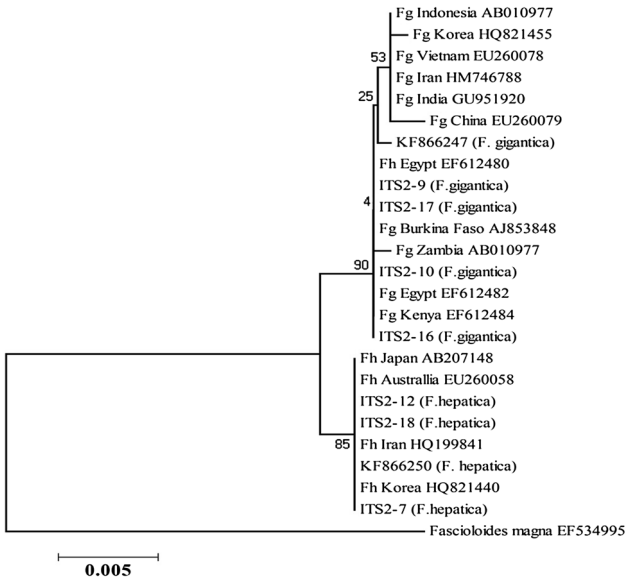

Fig. 3 Phylogenetic association between ITS2 sequences of north (*7*, *9*, *10*, *12*, *16*, *17*, *18*) and southwest isolates (KF) of Iran and identified isolates from different geographical regions, using likelihood method

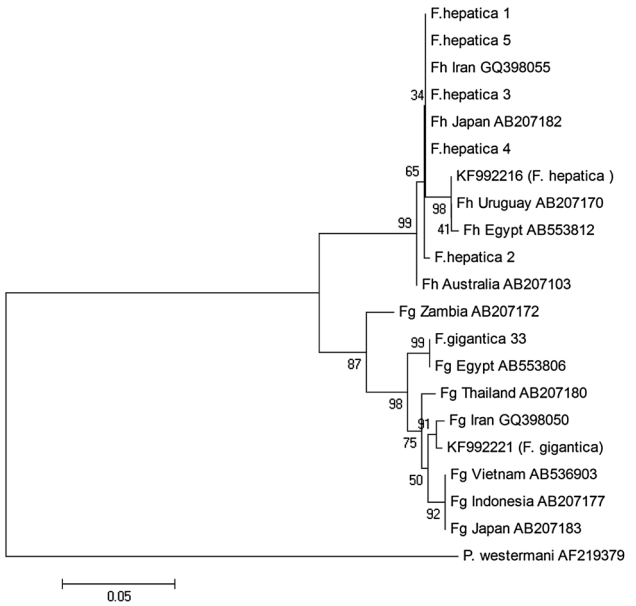

Fig. 4 Phylogenetic association between CO1 sequences of north (*1*, *2*, *3*, *4*, *5*, *33*) and southwest isolates (KF) of Iran and identified isolates from different geographical regions, using likelihood method

Discussion

In the present study, molecular characterization of *Fasciola* isolates was determined and the findings was compared with those available data from the isolates from southwest of the country. Based on phylogenetic analysis of the two species, ITS1 region showed six variable nucleotide sites

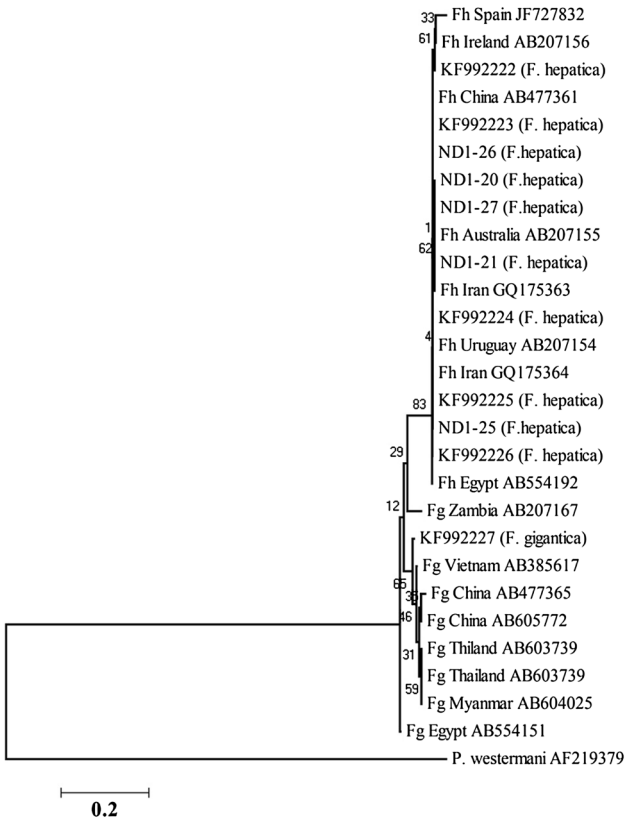

Fig. 5 Phylogenetic association between ND1 sequences of north (*20*, *21*, *25*, *26*, *27*) and southwest isolates (KF) of Iran and identified isolates from different geographical regions, using likelihood method

which are compatible with the findings of previous studies (Itagaki et al. 2009; Shafiei et al. 2014).

F. hepatica and *F. gigantica* isolates were in separate clusters, based on ITS1 region. Nevertheless, close relationship between two species of north isolates and isolates from other geographical regions was observed.

Comparison of sequences of ITS2 region of north isolates and other isolates showed seven variable nucleotide sites among *Fasciola* species. Such variation was reported in previous studies as well (Choe et al. 2011; Erensoy et al. 2009; Shafiei et al. 2013; 2014).

Findings of the current study demonstrated a close association between north and southwest isolates regarding the ND1 region. As already shown in phylogenetic tree, north and southwest isolates are sit in one cluster.

Fasciola isolates of southwest of Iran and Asian zones showed genetic variation in mitochondrial genes including ND1 and CO1 regions in a few studies (Itagaki et al. 2005; Semyenova et al. 2009). Considering the findings of current and other related studies, ND1 region is a suitable marker for evaluation of genetic diversity of *Fasciola*. Having said that, in our study, this region showed only one nucleotide variation among north isolates and high relationship was

observed between the ND1 sequence of north and southwest isolates.

CO1 sequences of *F. hepatica* isolated from goat and sheep from north showed 100% identity with KF992217.1 (from southwest Iran), AB553810.1 (from Egypt), GQ398051.1 (from Iran) and FJ895604.1 (from north of Iran) isolates. In addition, samples isolated from cattle showed 100% identity with HQ857101 (from Mauritania) and AB553784 (from Egypt).

CO1 mitochondrial gene has been suggested as an approperiate region for evaluation of genetic diversity in different hosts and geographical areas for *Fasciola* and also other helminthes including *Echinococcus* (Sarkari et al. 2015; Shafiei et al. 2014). Our results showed that the sequences of CO1 isolates from north and southwest have substantial differences. Genetic diversity in north and south isolates of *Fasciola* might be linked to the host species, including intermediate and final hosts. In keeping with these findings, previous studies on Iranian isolates of *Fasciola* showed high level of diversity in CO1 region (Moazeni et al. 2012).

Taken together, findings of the present study based on RFLP-PCR and sequencing of ribosomal and mitochondrial DNA, presented only two species of *Fasciola* in the studied area. However, in this study, Iranian isolates presented less variation in ITS1, ITS2 and ND1 regions. Based on CO1 marker, high genetic variation was noted in north isolates in comparison with isolates from southwest of Iran and also other geographical regions of the world.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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