# *Fasciola gigantica* of Ruminants: The phylogenetic analysis based on COX1 sequence

**Jahani, Z., Meshgi, B.*, Amininia, N.**

*Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran (Center of Excellent of Ecosystem and Ultrastructural Changes of Helminthes)*

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**Correspondence**
Meshgi, B.
Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran (Center of Excellent of Ecosystem and Ultrastructural Changes of Helminthes)
Tel: +98(21) 61117070
Fax: +98(21) 66933222
Email: Bmeshgi@ut.ac.ir

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## Abstract:

**BACKGROUND:** Fasciola species are parasitic trematode with world wide distribution that infects wild and domesticated herbivores, particularly ruminants. **OBJECTIVES:** The aim of the present study was to investigate the intra species variations of *F. gigantica*, from goats and buffalo isolates in two common geographic climates of Iran. **METHODS:** *Fasciola* species were collected from goat, buffalo, sheep, and cattle in different regions. Cytochrome c oxidase I (COX1) of mitochondrial DNA (mt-DNA) was amplified from individual trematodes by polymerase chain reaction (PCR), using universal primers, and the amplicons were consequently sequenced and sequencing data were analyzed, using Clutal W software against the GenBank database. **RESULTS:** A monomorphic DNA segment of approximately 499bp was seen in Fasciola isolates. The results of the amino acid sequence alignment defined strictly conserved amino acid residues in buffalo isolates of *F. gigantica* and partially conserved residues for goat isolates of *F. gigantica*. There are four tandem amino-acid replacements in the goat isolates at the position of 135-138, where Leucine (L), F (Phenylalanine), T (Threonine), and D (Aspartate) sequences changed into S (Serine), L (Leucine), H (Histidine), and L (Leucine), respectively. Furthermore, a replacement in the sequence of amino acid was found in isolates from buffalo at the position of 154, where Serine (S) was transformed into Leucine (L). **CONCLUSIONS:** The findings of our study indicate that the variants of goat and buffalo can be responsible for persistence of Fasciola infection in the endemic areas of Iran. It seems that biological differences could occur by considering a variety of *F. gigantica*-hosts in Iran. Thus, suitable approaches are required for effective treatments and useful control strategies.

## Introduction

Fasciolosis is one of the most common zoonotic diseases in different regions of Iran (16). It has been reported that 32% of sheep, 17% of cattle, and 50% buffalos are infected by *Fasciola* spp. in Bandar Anzali, as well as 9.5%, 32.5%, and 50% of sheep, cattle, and horses in Guilan province, north of Iran, respectively (8, 13). Two species, *F. hepatica* and *F. gigantica*, are common causative agents of fasciolosis in ruminants

and humans. *F. hepatica* is found in Europe, the Americas, as well as Oceania, while *F. gigantica* is distributed in Africa and Asia (14, 15). Although morphological characterization clearly determines the differentiation between *F. hepatica* and *F. gigantica* and separates them in to two distinct species, it is difficult to accurately differentiate the two species due to the numerous variations in their morphological parameters (10). Previous studies indicated that the ribosomal and mitochondrial DNA sequences are useful for phylogenetic studies and analysis (7, 10). However, some researchers suggested that r-DNA sequences are suitable for molecular identification of plathyhelminthes (3). It is likely that cytochrome c oxidase 1 subunit could be a potential candidate for taxo-molecular studies such as *D. dendriticum*. Therefore, the mitochondrial genome was applied to discriminate between the sub species or strains, and COX1 provided valuable information for the identification of Fasciola strains in Egypt (2). The second nuclear internal transcribed spacer (ITS-2) rDNA of Schistosoma japonicum has been previously applied to determine the genetic diversity (20). The findings of the mentioned studies indicated that ITS2 region is not a suitable marker for assessing inter- and intra- population variation in S. japonicum, while mitochondrial genes are suitable resources for molecular taxonomic studies. Recent investigations have shown that mt-genome can be considered as a useful molecular marker in the population genetic and phylogenic studies of trematoda such as *S. japonicum* (21) and *F. hepatica* (18). Nowadays, the drug treatment against fasciolosis in Iran is carried out with various anthelminthes combinations such as triclabendazol and albendazol, as well as monitoring of performance which is based on fecal examination for EPG (egg per gram) and slaughterhouse inspection. The purpose of this study was to determinate the molecular characterization of Iranian *F. gigantica* from goats buffalos, sheep and cattle isolates using PCR on COX1-mtDNA fragment.

## Materials and Methods

**Parasite collection:** Adult flukes of Fasciola species were obtained from livers of naturally infected cattle, sheep, buffalos, and goats from local slaughterhouses of two geographical origins, Guilan and Tehran Provinces, Iran. Fresh worms were washed in phosphate-buffered saline (pH=7.2).

Two Fasciola species were differentiated from each other based on their morphological and morphometric characteristics. The samples were labeled and preserved immediately in a 70% ethanol solution until DNA extraction. The hosts and localities of the specimens are shown in Table 1.

**DNA extraction, Amplification, and Sequencing:** One hundred adult *F. gigantica* and *F. hepatica* from four hosts and two geographical origins were considered in the present molecular study. Total genomic DNA was extracted from individual flukes, using a DNA extraction kit (MBST, Iran) according to the manufacturer's instructions

DNA was extracted from a small portion of anterior end of the worm that lacked eggs (16). DNA fragments of mitochondrial COX1 gene were amplified by the specific primers. The primer sets which were employed in amplifying the fragments were F-COX: (forward; 5́-ACGTTGGATCATAAGCGTG-3́) and (reverse; 5́-CCTCATCCAACATAACCTC-3́). The polymerase

chain reaction (100 µl) was performed using master mix (Amplicon, USA), 2 µl of each forward and reverse primers and 4 µl of genomic template in an automated thermocycler (Biorad-Italia) with the following procedure: 95°C for 5 min, 37 cycles of denaturation at 95°C for 45 sec, annealing at 54°C for 45 sec, extension at 72°C for 45 sec, and final extension at 72°C for 5 min as the final step. Samples without any genomic DNA were included as negative controls in each PCR run. All amplicons were subjected to 1.5% agarose gel containing Sybersafe staining (Cinaclone, Iran) and were visualized under UV, and their size was compared with 100 bp DNA ladder (Vivantis- Malaysia) as well. Consequently, the PCR products were purified, using a quick PCR purification kit (MBST, Iran) according to the manufacturer's protocol and 10 PCR products from goat (Guilan), 10 PCR products from buffalo (Guilan), 10 PCR products from cattle (Guilan) for *F. gigantica* and 10 PCR products from cattle (Tehran) for *F. hepatica* were considered for sequencing analysis (Takapouzist, Iran). The obtained sequences were then analyzed using the Chromas software (version 2.1.1) and subsequently aligned with the ClustalW software for nucleotide and T-COFFEE software for amino acid (version-11). Sequences available in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) were included in the final alignment for comparing the obtained sequences. The aligned sequences were analyzed with Neighbor Joining Analysis, using MEGA 7 software (version 7.0).

## Results

**PCR amplification:** DNA amplification of COX1-mtDNA showed a single fragment of 499 bp in all Fasciola species from different isolates. Moreover, the negative controls produced no bands in any experiments. Agarose gel electrophoresis of COX1 PCR product of *F. gigantica* and *F. hepatica* from different hosts and localities are shown in Figs 1 and 2, respectively.

Table 1. *Fasciola* species in Iran based on isolates and origin Subtitle: FGGG: *F. gigantica*-Goat Guilan isolate, FGBG: *F. gigantica*-Buffalo Guilan isolate, FGCG: *F. gigantica*-Cattle Guilan isolate, FGST: *F. gigantica*-Sheep Tehran isolate, FGCT: *F. gigantica*-Cattle Tehran isolate, FGGT: *F. gigantica*-Goat Tehran isolate, FHGG: *F. hepatica*-Goat Guilan isolate, FHCG: *F. hepatica*-Cattle Guilan isolate, FHSG: *F. hepatica*-Sheep Guilan isolate, FHCT: *F. hepatica*-Cattle Tehran isolate, FHST: *F. hepatica*-Sheep Tehran isolate, FHGT: *F. hepatica*-Goat Tehran.

| Species (host) | Trematode code | N. of worms | Isolate origin |
|---|---|---|---|
| *F. gigantica* (goat) | FGGG | 38 | Gilan |
| *F. gigantica* (buffalo) | FGBG | 26 | Gilan |
| *F. gigantica* (cattle) | FGCG | 26 | Gilan |
| *F. gigantica* (sheep) | FGST | 33 | Tehran |
| *F. gigantica* (cattle) | FGCT | 21 | Tehran |
| *F. gigantica* (goat) | FGGT | 14 | Tehran |
| *F. gigantica* (goat) | FHGG | 40 | Gilan |
| *F. hepatica* (cattle) | FHCG | 30 | Gilan |
| *F. hepatica* (sheep) | FHSG | 19 | Gilan |
| *F. hepatica* (cattle) | FHCT | 33 | Tehran |
| *F. hepatica* (sheep) | FHST | 29 | Tehran |
| *F. hepatica* (goat) | FHGT | 12 | Tehran |

**Sequence analysis and phylogenetic:** Sequences of 499 bp COX1 of the Fasciola species were aligned with those of available sequences in GenBank (acces-

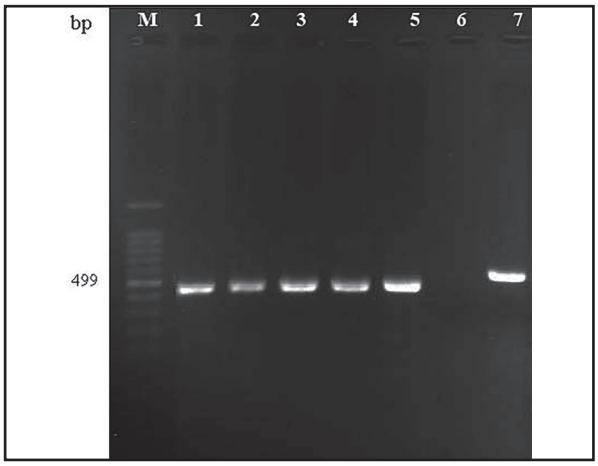


Figure 1. Agarose gel electrophoresis of COX1 PCR product of *F. gigantica* from Goats- Guilan (Lane 1), Buffalos-Guilan (Lane 2), Cattle- Guilan (Lane 3), Cattle-Tehran (Lane 4), Sheep-Tehran (Lane 5), Negative DNA control (Lane 6), Goats-Tehran (Lane 7), M: DNA marker.

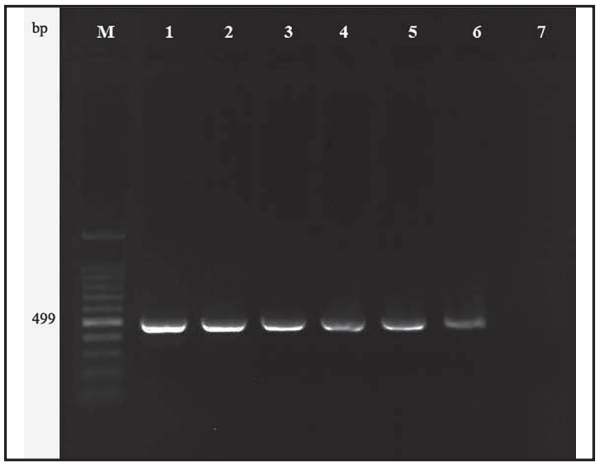


Figure 2. Agarose gel electrophoresis of COX1 PCR product of *F. hepatica* from Cattle- Guilan (Lane 1), Sheep-Guilan (Lane 2), Goats- Guilan (Lane 3), Cattle-Tehran (Lane 4), Sheep-Tehran (Lane 5), Goats-Tehran (Lane 6), Negative DNA control (Lane 7), M: DNA marker.

sion no. GQ398051.1, AB536898.1 and AB385622.1 for *F. hepatica* from cattle, Iran; *Fasciola* spp. from cattle-Vietnam and *F. gigantica* from cattle,Vietnam, respectively). Although COX1 nucleotid sequences of different isolates of *F. gigantica* showed 96-100% similarity, findings demonstrated 91% similarity between isolates of goat and cattle isolates from Iran ,while 81% similarity was observed between the goat isolates of Iran and cattle isolates from Vietnam.

The sequences were sub¬mitted to GenBank (accession no. KX423725 for *F. gigantica*- Guilan cattle isolate, KX395645 for *F. gigantica*- Guilan buffalo isolate, KX458034 for *F. gigantica*- Guilan goat isolate and KX423726 for *F. hepatica*-Tehran cattle isolate,).

Furthermore, nucleotide sequence was translated into amino acids. Consequently, *F. hepatica* and *F. gigantica* nucleotide sequences were aligned with sequences released in GenBank, which were similar to each other. In addition, host diversity was observed in *F. gigantica*.

Phylogenetic tree was plotted based on partial sequences of COX1 gene of Fasciola isolates in Iran using neighbor joining analysis. This phylogenetic tree indicates that *F. gigantica* isolates clustered in a separate cluster, in accordance with the specific host variations. *F. gigantica* isolates of goats are placed in the separate cluster, while those of buffalos and cattle are placed in the same cluster and different branches. Moreover, *F. gigantica* isolates of cattle in two different geographical regions of Tehran and Guilan are placed in the same cluster (Fig. 3).

Our findings suggested that the isolate of *F. gigantica* and *F. hepatica* from Iran (GQ398050.1 and GQ398056.1, respectively) are located at the same cluster together with Fasciola species.

Amino acids sequence of *F. gigantica* isolates from goats showed noticeable difference as compared to submitted sequences of *F. hepatica* in GenBank (Accession numbers: AB300704, AB207170, KJ716921, GQ398054.1, GQ398056.1, and FJ895606.1).

Amino acid sequence of *F. gigantica* isolates from buffalo and the sequence of *F. gigantica* isolated from cattle in GenBank were quite similar. The sequences of *F. gi-*

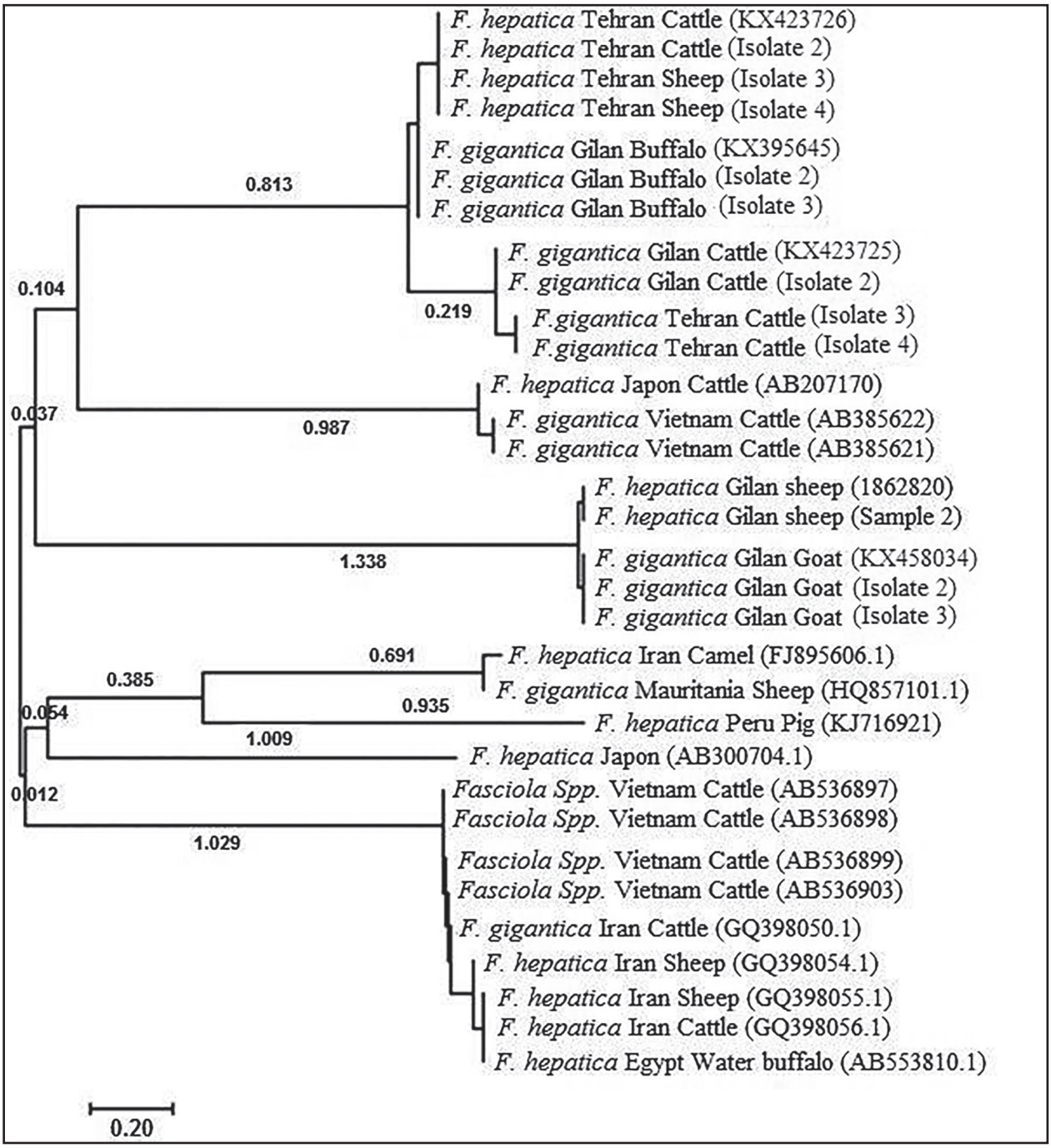


Figure 3. Phylogenetic relationships of Iranian *Fasciola* species derived from different isolates based on COX1 amino acids sequences in comparison with other isolates (Phylogenetic analyses were conducted in MEGA 7).

*gantica* isolates from goats were not in GenBank database and they had the least difference from *F. gigantica* isolates of cattle in four amino acids. Amino acids of Serine, Leucine, Histidine, and Leucine in the sequence of GenBank changed into Leucine, Phenylalanine, Threonine, and Aspartate in Iranian goats isolates (Fig. 4).

## Discussion

The liver flukes of *F. hepatica* and *F. gigantica* are the two most important helminthes, causing substantial economic loss in ruminants worldwide mainly due to loss of weight, fertility, and liver condemnation

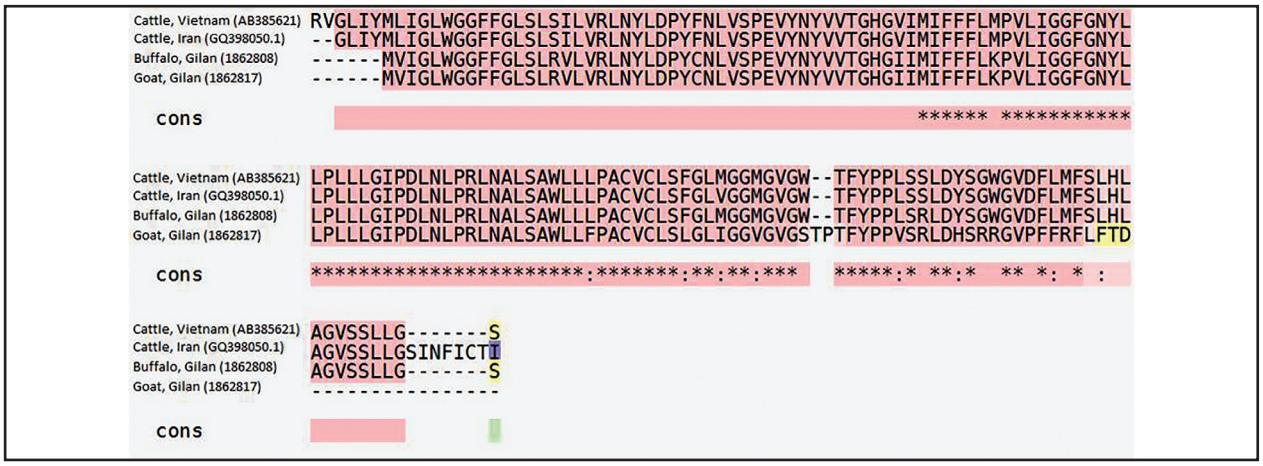


Figure 4. Amino acid alignment of Fasciola species by T-COFFEE software.

(9).

*F. hepatica* and *F. gigantica* infections have generally occurred in temperate and tropical zones, respectively, both species may overlap in subtropical areas (6, 11). Both species have been reported in cattle, sheep, goats, buffalos, and camels in Iran (5, 9, 16).

During the past fifty years, high prevalence rate of fasciolosis has been documented, especially in southern and northern areas of Iran. Recent studies indicated that prevalence rates of fasciolosis in northern provinces of Guilan and Mazandaran are higher compared with other parts of the country. On the other hand, the prevalence rate of fasciolosis in livestock has decreased in the southern part of Iran. It is worth noting that two *Fasciola* species, *F. hepatica* and *F. gigantica*, are prevalent in both animal and human. Recent studies determined that *F. hepatica* transmission occurs in the mountainous area of Guilan and *F. gigantica* transmission takes place in the lowlands. However, overlap distribution of *F. hepatica* and *F. gigantica* are involved in other parts of Iran (4).

Molecular approaches are the powerful tools to determine the diversity of inter and intra-species as well as intermediate forms of micro and macro organisms, particularly in helminth populations. Among all targets of DNA makers, genomic sequencing of different regions is a useful technique for specific identification of species and molecular variation studies for a wide range of parasitic flat worm.

Mitochondrial and nuclear DNA sequences have been previously used for molecular studies of Fasciola species (2, 10). The molecular characterization of human Fasciola samples from Guilan, the coastal Caspian Sea of Iran, based on ITS1 and COX1 genes, indicated that human Fasciola specimens were represented as *F. hepatica* (19). It has been previously reported that host association and geographical origin are not likely to be useful indicators for Fasciola molecular classification based on COX1 fragment (17).

Itagaki et al. (2005) studied genetic characterization of parthenogenic Fasciola on the basis of the sequences of ribosomal and mitochondrial DNA (12, 14). Their findings suggested that 2 major and 3 minor distinct forms of Fasciola occurred in Japan. Heterozygosis in mitochondrial DNA of Japanese form may be originated from interspecific cross hybridization between paternal *F. hepatica* and maternal *F. gigantica*.

Previous study by Agatsuma et al. (2000) reported that COX1 and NADH dehydrogenase subunit 1 (Nicotinamide adenine dinucleotide) fragments are monomorphic in the Korean fasciolid and similar to those of *F. gigantica* (1).

Most of the molecular variations of *F. gigantica* have been explained by specific variation within host-associated populations of ruminants. In other words, *F. gigantica* tends to be relatively host-specific in ruminants. However, it is not an individual variation related to common geographic location. Semyenova et al. (2006) revealed that single cattle may be concurrently harbored with more than one genotypes of the trematode (18). It must be said that intermediate forms of two Fasciola species are present in Iran. But in the present study, the used samples were quite identifiable in terms of morphological characteristics.

In the present study, genomic DNA was extracted from *F. gigantica* isolates in goats, buffalos, cattle, and sheep in two geographical locations of Guilan and Tehran Provinces, Iran. COX1 fragments were amplified from each sample, using universal primers, and fragments with 499 bp in length were amplified and consequently sequenced. The sequencing results showed that goat isolates are different from the other amino acid sequences in a consecutive sequence with 4 amino acids. Goat sequence was LFTD (Leucine: hydrophobic-nonpolar, Phenylalanine: hydrophobic-nonpolar, Threonine: hydrophilic-nonpolar and Aspartate: hydrophilic-negatively charged polar) but all other sequences contained SLHL (Serine: hydrophilic-nonpolar, Histidine: hydrophilic-positively charged polar) and were similar to each other. The amino acid sequences of buffalo isolates were different from cattle isolates only in one amino acid. Amino acid of S was replaced with L in cattle isolates of Iran, but it was similar to cattle isolates from Vietnam that had S at this position. The identification of amino acid substitutions showed the least variability in the buffalo isolates. The results of phylogenetic tree defined the separate cluster that was associated with particular host species. *F. gigantica* of goat isolates were placed in separate cluster, while buffalo and cattle isolates were clustered in the same cluster, although they were placed in the separate branches.

## Acknowledgments

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# فاسیولا ژیگانتیکا نشخوارکنندگان: تجزیه و تحلیل شجره‌شناسی بر اساس ترادف COX۱

**زهرا جهانی  بهنام مشگی*  نرگس امینی‌نیا**

**گروه انگل‌شناسی دانشکده دامپزشکی دانشگاه تهران، تهران، ایران (قطب علمی بوم‌سازگان و تغییرات فراساختاری در کرم‌ها)**

(دریافت مقاله: ۶ تیر ماه ۱۳۹۵،  پذیرش نهایی: ۲۷ مرداد ماه ۱۳۹۵)

## چکیده

**زمینه مطالعه:** گونه‌های فاسیولا بعنوان ترماتودهای دیژنه‌آ با پراکندگی جهانی توصیف شده که باعث آلوده شدن علفخواران بخصوص نشخوارکنندگان می‌شوند. **هدف:** هدف از بررسی حاضر تنوع درون گونه‌ای فاسیولا ژیگانتیکا از دو جدایه بز و گاومیش، مربوط به دو منطقه جغرافیایی ایران بود. **روش کار:** جمع‌آوری نمونه‌ها در بررسی کشتارگاهی از دو منطقه تهران و گیلان انجام گرفت. نمونه‌ها بر اساس مشخصات ریختی بصورت اولیه و براساس کلید تشخیص شناسایی گردید. در بخش مولکولی، واکنش زنجیره‌ای پلیمراز به منظور تکثیر توالی ژن COX۱ انجام شد و محصول PCR پس از خالص‌سازی، تعیین توالی گردید و درخت شجره‌ایی ترسیم شد. ترادف اسیدهای آمینه نیز صورت پذیرفت. سپس ترادف‌های حاصل با استفاده از نرم افزارهای مربوط تحت تجزیه و تحلیل قرار گرفتند. **نتایج:** الگوی PCR در همه جدایه‌ها با وجود باندی به اندازه ۴۹۹ جفت باز قابل تشخیص بود. نتایج تعیین توالی اسیدهای آمینه مشخص کرد، بین دو جدایه بز و گاومیش از این حیث اختلاف وجود دارد. در جدایه بز ۴ اسیدآمینه از شماره ۱۳۵ تا ۱۳۸ بترتیب شامل لوسین، فنیل‌آلانین، ترئونین و اسپارتات به سرین، لوسین، هیستیدین و لوسین تغییر پیدا کرده‌اند. علاوه بر این در اسیدآمینه شماره ۱۵۴ جدایه گاومیش لوسین جایگزین سرین شده است. **نتیجه‌گیری نهایی:** نتایج بدست آمده نشان داد که جدایه‌های بز و گاومیش می‌توانند مسؤل بقا ابتلا به فاسیولا در مناطق بومی آلودگی باشند. بنظر می‌رسد تنوع موجود بین فاسیولا ژیکانتیکا و میزبان می‌تواند منجر به اختلافات زیستی در انگل گردد و لذا رهیافت‌های مناسبی بعنوان سیاست‌های کنترلی و درمانی مورد نیاز است.

**واژه های کلیدی:** COX۱، فاسیولا ژیگانتیکا، شجره شناسی، نشخوارکنندگان، ترادف

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*) نویسنده مسؤول: تلفن: ۶۱۱۱۷۰۷۰ (۲۱)۹۸+  نمابر: ۶۶۹۳۳۲۲۲ (۲۱)۹۸+  Email: Bmeshgi@ut.ac.ir