Neuro-Cell Molecular Research

Review Article

Neuro-Cell Mol Res 2025;2(1):21-26

# Gene Regions and Methods Used in Molecular Characterization of *Fasciola* Species

**Sedat Kavak[1], Milad Afşar[2], Selahattin Aydemir[2*]**

[1]Ardahan University Vocational School of Health Services, Ardahan, Türkiye
[2]Van Yüzüncü Yıl University Faculty of Medicine, Department of Parasitology, Van, Türkiye

***Corresponding Author:**
**Selahattin Aydemir**
Van Yüzüncü Yıl University Faculty of Medicine, Department of Parasitology, Van, Türkiye.
**E-mail:** saydmr23@gmail.com
**Orcid ID:** 0000-0002-0941-2779

**DOI: 10.5281/zenodo.15311805**

**Received: 3 March 2025**
**Accepted: 18 April 2025**
**Published: 30 April 2025**

The author(s) - Available online at **www.neurocellmolres.com.tr**



## ABSTRACT

*Fasciola* species are zoonotic trematodes that cause fascioliasis in a wide range of hosts, including humans and ruminants such as camels, cattle, sheep and goats. The parasite causes significant economic losses in the livestock sector. Although its effect on human health has previously been understated, recent cases of fascioliasis have sparked increased interest among researchers globally. These situations make it essential to precisely characterize species of *Fasciola*. However, available phenotypic approaches fail to represent the diversity of *Fasciola* species in a summarized manner. In this context, the use of molecular approaches offers important advantages for the characterization of *Fasciola* species. Current molecular techniques greatly facilitate the elucidation of the biology, epidemiology and genetics of the parasite. In this article, the molecular markers used for *Fasciola* species, the properties of these markers, their intended use and the molecular techniques used to analyze these markers are discussed.

**Keywords**: *ITS, nad, cox1, F. hepatica, F. gigantica*

**Cite this article as:** Kavak S, Afşar M, Aydemir S. Gene Regions and Methods Used in Molecular Characterization of Fasciola Species. Neuro-Cell Mol Res. 2025;2(1):21-26. doi:10.5281/zenodo.15311805

## INTRODUCTION

*Fasciola* species are zoonotic trematodes that cause fasciolasis by colonizing the liver and bile ducts of their final hosts. They require a final host and an intermediate host in their life cycle. Their final hosts are various species such as cattle, sheep, goats, buffaloes and humans. Intermediate hosts are *Lymnaea* snails where the larval stages develop and reproduce. Transmission occurs by consuming a carrier (e.g. aquatic plants) to which the metacercariae attach [1, 2]. There are currently two known species. While *F. hepatica* has a cosmopolitan spread, *F. gigantica* is common in tropical and subtropical zones of Africa and Asia. In addition, recent studies have reported a new species of *Fasciola*, described as a hybrid of *F. hepatica* and *F.* gigantica [3].

Morphology, epidemiology, behavioral features, host and geographical spread, cross-breeding works, and physiology and biochemistry works were used in the species characterization process. However, in digeneans, the vast majority of species have been described solely on the basis of morphological characteristics of the adults, the hosts they infect, and their geographic distribution. However, many species cannot be identified with certainty on the basis of morphological characteristics alone. Some factors that make digenean identification difficult include: the small size of the adults and the paucity of taxonomic characters, uncertainty about the validity of the characters to be considered; high morphological similarities between closely related species; the significant time lag between initial genetic divergence and structural change; phenotypic plasticity, and the lack of distinguishing morphological characters for many digenean life cycle stages, such as cercariae and metacercariae, making it impossible to associate them with the adult form of the parasite on morphological characters alone [4].

Developments in the field of molecular biology have increased the utilization of various molecular techniques and genetic markers in molecular phylogeny. Genetic markers are segments of DNA in the genome that can provide molecular knowledge for species characterization. The use of DNA regions as genetic markers has demonstrated to be beneficial not only for species identification and detection of new species, but also for revealing relationships between groups of organisms in taxonomic works [5].

### Gene Used in Molecular Characterization

Genetic markers are widely used in molecular studies and their suitability for the type of application depends on the degree of conservation of the nucleotide sequence. As a result, determining the suitability of a genetic marker for any given study is a complex process [5].

Genetic markers can be constructed from distinct DNA sequences of the nuclear or mitochondrial genomes. The utility and specificity of each genetic marker greatly depends on the degree of sequence variation of the marker [5].

Four key characteristics are suggested for a genetic marker for the molecular characterization of parasitic helminths: 1. It should show sequence diversity across species; 2. Reference sequences in the database should be of appropriate length; 3. It should be simple to align sequences over a wide phylogenetic range; and 4. It should be simple to construct universal primers [5].

Although *F. hepatica* is an important parasite, little is known about its genetic variation and population structure. Most of the studies on *Fasciola* species are based on interspecific differences. Nuclear ribosomal DNA (rDNA)-derived internal transcribed spacer 1 (ITS1), ITS2, ITS1-5.8S-ITS2 and mitochondrial DNA-derived 28S ribosomal RNA (rRNA), NADH dehydrogenase 1 (nad1), cytochrome c oxidase 1 (cox1) and cytochrome b gene (cytb) gene regions have been used as molecular markers for *Fasciola* species [6]. Apart from these, nuclear DNA-derived phosphoenolpyruvate carboxykinase (pepck) and DNA polymerase delta (pold) genes are also used as molecular markers for more precise differentiation of *F. hepatica, F. gigantica* and aspermic *Fasciola* parasites (Table 1) [7].

### Mitochondrial DNA (mtDNA) Regions

As genetic markers, mitochondrial gene regions have several important features. Their large number of copies in the cell and haploid structure facilitate their amplification. They also have a high mutation rate due to the absence of a molecular repair mechanism [8,9], thus producing a higher degree of sequence variation.

Liu et al. [10] used sliding-window analysis for mitochondrial genomes of *Fasciola* species to provide an estimate of nucleotide diversity ($\pi$) in mitochondrial genes. The study identified conserved regions within the nad1, nad6, cytb and cox1 genes. When the results of the study were evaluated, it was found that the most conserved regions among the investigated gene regions were nad1 and cox1 protein coding genes, while the least conserved regions were nad6, nad5 and nad4 gene regions [10].

***Table 1.*** *Molecular markers used for Fasciola and their properties*

| Molecular Marker | Location | Intended Use | Method | Reference |
|---|---|---|---|---|
| **ITS1 - 5.8S - ITS2** | Nuclear rDNA | Species identification | PCR-RFLP (*Tsp509I*) | [22] |
| **ITS1** | Nuclear rDNA | Haplotype analysis, species distinction, population structure | PCR-RFLP (*RsaI*), sequence | [16,20] |
| **ITS2** | Nuclear rDNA | Haplotype analysis, species distinction, population structure | PCR-RFLP (*NlaIII*), sequence | [16, 20] |
| **28S rRNA** | Mitochondrial rRNA | Species distinction, intraspecific and interspecific variation and phylogenetic studies | Sequence | [18] |
| **Cox1** | Mitochondrial DNA | Haplotype analysis, DNA barcoding, species discrimination, population structure | PCR-RFLP (*Hpy*188III), sequence | [16, 25] |
| **Nad1** | Mitochondrial DNA | Haplotype analysis, species determination, phylogenetic analysis, | Sequence | [24] |
| **Cytb** | Mitochondrial DNA | Highly accurate species-specific detection between *F. gigantica* eggs and other *F. gigantica*-like eggs | PCR | [6] |
| **Pepck** | Nuclear DNA | Definitive differentiation for *F. hepatica*, *F. gigantica* and aspermic *Fasciola* | Multiplex PCR, PCR-RFLP (*AccII)* | [7] |
| **Pold** | Nuclear DNA | Definitive differentiation for *F. hepatica*, *F. gigantica* and aspermic *Fasciola* | PCR-RFLP (*Alu*I) | [7] |

## Cytochrome c Oxidase 1 and NADH dehydrogenase 1 Gene

The cytb [6], cox1 and nad1 genes, which encode important components of the cellular respiratory system, and the 12S and 16S ribosomal RNA (rRNA) genes are genetic markers located in mitochondrial DNA (mtDNA) [5,11]. Although 12S and 16S are considered suitable for use as genetic markers, their use in *Fasciola* studies has not been found in the literature.

The cox1 gene region originating from mitochondrial DNA, which is considered as a "universal'' DNA barcoding marker for metazoans, is used as a typical genetic marker for many animal phyla. Its conserved structure in aeorobic organisms and its heterogeneous structure consisting of a number of different functional units have made the cox1 gene an important marker for use in classification studies [8].

The cox1 and nad1 mitochondrial gene regions are utilized as genetic markers to investigate genetic variation and phylogenetic relationships among *Fasciola* species [12,13]. Cox1 is a useful marker not only for the identification of *Fasciola* species, but also for understanding the origin and source of infection [14]. To investigate genetic variation and phylogenetic relationships among *Fasciola* species, DNA sequences of cox1 and nad1 genes can be obtained by sequence analysis [15]. In *Fasciola* species, species distinction can also be made by using the cox1 gene region restriction fragment length polymorphism (RFLP) molecular technique. When the cox1 gene region is exposed to *Hpy188*III, a restriction enzyme, using the RFLP technique, *F. hepatica* and *F. gigantica* as well as the aspermic *Fasciola* can be distinguished [16].

## Cytochrome b Gene

The cytb contains conserved nucleotide sequences that contain information about which genus or species the organism will be grouped into. It has been reported that these

conserved nucleotide sequences are used in the taxonomic classification of some animal species. The fact that more commonly used molecular markers such as cox1, nad1, 28S rDNA and ITSs become useless in the presence of various inhibitors that can affect PCR reactions in stool and reduce the detection limit of the PCR reaction makes cytb more successful in identifying eggs in stool samples compared to these markers. The cytb gene region is also a useful marker for identifying degraded DNA, such as in processed foods and forensic samples. It has also been suggested to be used as a marker gene due to its species-specific regions. Therefore, it has the necessary potential to create species-specific primers in *Fasciola* egg samples [6].

## Mitochondrial 28S rRNA Gene

Molecular techniques such as transcriptomics (RNA-Seq), RT-qPCR and RNA microarrays, which are useful in determining gene expression levels, are important tools for understanding the biology of parasites. All of these techniques require high-quality RNA extraction and analyze the extracted total RNA with high sensitivity and precision. In most of the research laboratories, this assessment is based on the completeness of 18S and 28S ribosomal RNAs (rRNAs) [17].

Using fragmented sequences of the mitochondrial 28S rRNA gene, it was used as a genetic marker for the precise identification, genotyping, intraspecific and interspecific variations and phylogenetic studies of Fas*ciola* [18].

## Nuclear DNA Regions

mtDNA is more susceptible to mutation compared to NDNA (especially nuclear rRNA) genes. Nuclear rRNA genes are highly conserved genes, making them a potentially useful source of genetic markers for resolving high taxonomic levels for organisms. In NDNA, ITS regions have a higher degree of sequence variation than nuclear rRNA genes due to a higher rate of nucleotide exchange. Although more than one type of genetic marker is appropriate for molecular systematics and characterization goals, the varying characteristics of genetic markers make selection difficult for relevant applications [5,11].

## Internal Transcribed Spacer (ITS)

Ribosomes, the intracellular and molecular machines found in all living organisms, play important roles in protein synthesis and gene expression. The rRNA gene and spacer regions comprehensively provide phylogenetic information in prokaryotes and eukaryotes and are collectively known as rDNA; it consists of coding regions (18S, 5.8S and 28S) and the non- coding region of two internal transcribed spacers (ITS1, ITS2) and one non-transcribed spacer (NTS) [19].

The availability of molecular approaches has facilitated the identification and genetic characterization of morphologically similar parasites. However, the search for reliable molecular markers suitable for low-level phylogenetic analysis remains a challenging problem [19].

Coding regions are highly conserved sequences in living organisms and can be used to reveal phylogenetic relationships between major phyla, while non-coding regions are highly variable and have great potential for studying relationships between closely related genera or species due to the faster evolutionary rate. ITS regions are widely used as molecular markers for taxonomy and phylogenetic analyses. The reasons for the preference of ITS over other non- coding regions are the following: ( 1) multiple copies of rRNA genes; ( 2) suitable for PCR amplification with a variety of universal primers for different organisms; (3) average sequence length for sequencing; and (4) high degree of variation at the generic and species level due to frequent nucleotide polymorphisms or insertions/deletions in the sequences [19]. These features have made the internal transcribed spacer (ITS) region the most common marker for distinguishing *F. gigantica* from *F. hepatica* and for identifying the intermediate form *Fasciola* [12].

Current studies have shown that ITS1 and ITS2 can be used as genetic markers for the identification of *Fasciola* at the species level [20,21]. In addition, ITS1, 5.8S rDNA and ITS2 regions were amplified as a whole by PCR and used as genetic markers [22].

For ITS1, ITS2 and ITS1 - 5.8S - ITS2 regions, species distinction can be made using PCR-RFLP technique. When looking at the literature, it was seen that it was possible to distinguish both *Fasciola hepatica*, *Fasciola gigantica* and aspermic *Fasciola* using restriction enzymes such as RsaI for ITS1, NlaIII for ITS2 [16] and Tsp509I for ITS1–5.8S–ITS2[22]. ITS1 and ITS2 regions can be sequenced and used for haplotype analysis, phylogenic analysis, species distinction and population structure [20].

## Pepck and Pold

Because ribosomal DNA contains hundreds of copies arranged in tandem repeats, the ITS1 region is highly recombinogenic and unstable. Because of this nature, ribosomal DNA cannot provide conclusive evidence for interspecies hybridization. Therefore, new nuclear single copy markers, pepck and pold have recently been developed for the precise differentiation of *Fasciola* species. These were

considered sufficient to detect interspecies hybridization between *F. hepatica* and *F. gigantica*, since ITS1 can be inaccurate in distinguishing aspermic *Fasciola* [23].

For both gene regions, species distinction can be made using PCR-RFLP molecular technique. By using *Acc* II restriction enzymes for pepck and *Alu* I restriction enzymes for pold as restriction enzymes, definite distinction can be made between *F. hepatica* and *F. gigantica* and aspermic *Fasciola*. In addition, by using the Multiplex PCR molecular method for *pepck*, a definite distinction can be made between the 3 species [7].

## CONCLUSION

Fascioliasis is a major global concern for both veterinary and public health. Molecular studies have contributed significantly to the understanding of *Fasciola* and the fight against through the development of diagnostic tests that enable earlier detection of fascioliasis, phylogenetic analyses that provide important information on epidemiology and species, information obtained in studies on the molecular basis of drug resistance, and its role in vaccine development research.

In the future, *Fasciola* species, mechanisms of drug resistance and host-parasite interactions can be analyzed in more depth with next-generation sequencing (NGS) technologies such as whole genome sequencing (WGS) and RNA-seq. In addition, the integration of gene editing approaches such as CRISPR-Cas9 into *Fasciola* studies can provide important advances.

In conclusion, advances in the understanding of *Fasciola* species will ensure lasting success in the fight against fascioliasis.

### Acknowledgements

The authors declared that this study received no financial support.

### Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

### Conflicts of Interest

There is no conflict of interest for the publication of this article.

### Referee Evaluation Process

Externally peer-reviewed

## REFERENCES

1. Hoang Quang V, Levecke B, Do Trung D, et al. *Fasciola spp*. in Southeast Asia: A systematic review. PLoS Negl Trop Dis. 2024;18(1):e0011904. https://doi.org/10.1371/journal.pntd.0011904
2. Drescher G, Vasconcelos TCB, Belo VS, et al. Serological diagnosis of fasciolosis (Fasciola hepatica) in humans, cattle, and sheep: a meta-analysis. Front Vet Sci. 2023;10:1252454. https://doi.org/10.3389/fvets.2023.1252454
3. Hammami I, Ciuca L, Maurelli MP, et al. First morphometric and molecular characterization of *Fasciola* spp. in Northwest Tunisia. Parasitol Res. 2023;122(11):2467–76. https://doi.org/10.1007/s00436-023-07933-0
4. Nolan MJ, Cribb TH. The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. Adv Parasitol. 2005;60:101-63. https://doi.org/10.1007/s00436-023-07933-0
5. Chan AHE, Chaisiri K, Saralamba S, et al. Assessing the suitability of mitochondrial and nuclear DNA genetic markers for molecular systematics and species identification of helminths. Parasites Vectors. 2021;14:233. https://doi.org/10.1186/s13071-021-04737-y
6. Chontananarth T, Parawat J. Development of cytochrome B, a new candidate gene for a high accuracy detection of *Fasciola* eggs in fecal specimens. Vet Parasitol. 2019;274:108922. https://doi.org//10.1016/j.vetpar.2019.108922
7. Shoriki T, Ichikawa-Seki M, Suganuma K, et al. Novel methods for the molecular discrimination of *Fasciola spp*. on the basis of nuclear protein-coding genes. Parasitol Int. 2016;65(3):180-3. https://doi.org/10.1016/j.parint.2015.12.002
8. Rach J, Bergmann T, Paknia O, et al. The marker choice: Unexpected resolving power of an unexplored CO1 region for layered DNA barcoding approaches. PLoS One. 2017;12(4):e0174842. https://doi.org/10.1371/journal.pone.0174842
9. Elliott T, Muller A, Brockwell Y, et al. Evidence for high genetic diversity of NAD1 and COX1 mitochondrial haplotypes among triclabendazole resistant and susceptible populations and field isolates of *Fasciola hepatica* (liver fluke) in Australia. Vet Parasitol. 2014;200(1-2):90-6. https://doi.org/10.1016/j.vetpar.2013.11.019
10. Liu GH, Gasser RB, Young ND, et al. Complete mitochondrial genomes of the 'intermediate form' of *Fasciola* and *Fasciola gigantica*, and their comparison with *F. hepatica*. Parasites Vectors. 2014;7:150. https://doi.org/10.1186/1756-3305-7-150
11. Ai L, Chen MX, Alasaad S, et al. Genetic characterization, species differentiation and detection of *Fasciola spp*. by molecular approaches. Parasites Vectors. 2011a;4:1-6. https://doi.org/10.1186/1756-3305-4-101
12. Ai L, Weng YB, Elsheikha HM, et al. Genetic diversity and relatedness of *Fasciola spp*. isolates from different hosts and geographic regions revealed by analysis of mitochondrial DNA sequences. Vet Parasitol. 2011b;181(2–4):329–34. https://doi.org/10.1016/j.vetpar.2011.03.057
13. Itagaki T, Tsutsumi KI, Ito K, et al. Taxonomic status of the Japanese triploid forms of **Fasciola**: comparison of mitochondrial ND1 and COI sequences with *F. hepatica* and *F. gigantica*. J Parasitol. 1998;84(3):445-8. https://doi.org/10.2307/3284510
14. Moazeni M, Sharifiyazdi H, Izadpanah A. Characterization of *Fasciola hepatica* genotypes from cattle and sheep in Iran using cytochrome C oxidase gene (CO1). Parasitol Res. 2012;110:2379-84. https://doi.org/10.1007/s00436-011-2774-9
15. Asadpour M, Sharifiyazdi H, Moazeni M, et al. Molecular characterization of *Fasciola spp*. from a donkey (*Equus asinus*) using partial sequencing of cox1 and nad1. Iran J Parasitol. 2020;15(4):549-58. https://doi.org/10.18502/ijpa.v15i4.4860

16. Siribat P, Dekumyoy P, Komalamisra C, et al. Molecular identification of *Fasciola spp*.-representative samples from Thailand based on PCR-RFLP. J Trop Med Parasitol. 2018;41(1): 1-7.
17. Hachariz O, Sayers G. *Fasciola hepatica* - Where is 28S ribosomal RNA? Exp Parasitol. 2013;135(2):426-9. https://doi.org/10.1016/j.exppara.2013.07.026
18. Alajmi RA. Molecular characterization of *Fasciola* flukes using mitochondrial 28S rRNA gene in Naimi Saudi sheep. Saudi J Biol Sci. 2019;26(1):112-7. https://doi.org/10.1016/j.sjbs.2017.06.010
19. Choudhary K, Verma AK, Swaroop S, et al. A review on the molecular characterization of digenean parasites using molecular markers with special reference to ITS region. Helminthologia. 2015;52(3):167-72. https://doi.org/10.1515/helmin-2015-0031
20. Amer S, Dar Y, Ichikawa M, et al. Identification of *Fasciola* species isolated from Egypt based on sequence analysis of genomic (ITS1 and ITS2) and mitochondrial (ND1 and COI) gene markers. Parasitol Int. 2011;60(1):5-12. https://doi.org/10.1016/j.parint.2010.09.003
21. Shu FF, Lv RQ, Zhang YF, et al. Characterization of *Fasciola* samples by ITS of rDNA sequences revealed the existence of *Fasciola hepatica* and *Fasciola gigantica* in Yunnan Province, China. J Parasitol. 2012;98(4):889-90. https://doi.org/10.1645/GE-2974.1
22. Mahami-Oskouei M, Dalimi A, Forouzandeh-Moghadam M, et al. Molecular identification and differentiation of *Fasciola* isolates using PCR-RFLP method based on internal transcribed spacer (ITS1, 5.8S rDNA, ITS2). Iran J Parasitol. 2011;6(3):35-42
23. Ichikawa-Seki M, Peng M, Hayashi K, et al. Nuclear and mitochondrial DNA analysis reveals that hybridization between *Fasciola hepatica* and *Fasciola gigantica* occurred in China. Parasitology. 2017;144(2):206-13. https://doi.org/10.1017/S003118201600161X
24. Tadayon S, Sharifiyazdi H, Moazeni M, et al. Molecular differentiation of *Fasciola* species and characterization of genetic diversity of *F. gigantica* using NADH dehydrogenase I (ND1) gene in the endemic areas of Iran. Iran J Parasitol. 2015;10(1):9-18
25. Semyenova SK, Morozova EV, Chrisanfova GG, et al. Genetic differentiation in eastern European and western Asian populations of the liver fluke, *Fasciola hepatica*, as revealed by mitochondrial nad1 and cox1 genes. J Parasitol. 2006;92(3):525-30.