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Original Article

Genotyping and phylogenetic analysis of *Fasciola* species in animals from Iraq using the *ITS1* marker

Abdullah A Hama[1,2]

[1] *Medical Laboratory Department, College of Health and Medical Technology, Sulaimani Polytechnic University, Iraq*
[2] *Medical Laboratory Science, College of Health Science, University of Human Development, Kurdistan, Iraq*

Abstract

Introduction: *Fasciola* species are trematodes primarily infecting the liver and bile ducts of animals and humans, and causing serious lesions. They have significant medical and economic impacts, leading to chronic illness and reduced productivity in livestock. This study aimed to assess the genetic diversity of liver flukes isolated from domestic ruminants in Sulaimani province in Iraq.

Methodology: A total of 100 fecal samples were collected from animals living in local farms, including sheep (n = 44), goats (n = 36), and cattle (n = 20). Additionally, 42 liver flukes were obtained from 21 slaughtered animals (10 sheep, 6 cattle, and 5 goats) at the Sulaimani abattoir; 2 flukes per host were collected. DNA was extracted from sedimentation-positive fecal samples and from fluke tissue. Molecular characterization was performed by polymerase chain reaction (PCR) of the *internal transcribed spacer 1* (*ITS1*), and subsequent restriction fragment length polymorphism (RFLP) using *RsaI* and *Tsp509I* endonucleases. Genetic diversity was assessed through sequence comparison and phylogenetic analysis.

Results: RFLP analysis revealed 3 distinct patterns among liver flukes. DNA sequencing and phylogenetic analysis revealed 3 main clusters, primarily consisting of *Fasciola hepatica*, *Fasciola gigantica*, and *Fasciola intermediate*.

Conclusions: The study demonstrates that PCR-RFLP of *ITS1* with *RsaI* is effective for distinguishing *F. hepatica* from *F. intermediate*, while *Tsp509I* is useful for differentiating *F. hepatica* from *F. gigantica*. Additionally, PCR-RFLP of the *ITS1* is a simple, fast, and reliable method for species identification of liver flukes present in fecal samples of animals, and directly from fluke tissue.

Key words: liver fluke; *ITS1*; RFLP; *Rsal; Tsp509I.*

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Introduction

Fasciola species belong to the subclass Digenea; and primarily affect the liver and bile ducts of animals and humans which act as definitive hosts. Lymnaeid snails are the intermediate hosts; they are commonly found in freshwater habitats and have remarkable ability to adapt to mountain environments [1]. Definitive hosts (humans and animals) are commonly infected by drinking contaminated water or eating aquatic plants such as watercress, which harbor the metacercaria of these flukes [2]. The larval stage of *Fasciola* species migrates extensively through the liver parenchymal cells causing traumatic lesions and inflammation [3]. In addition, the adult forms mostly dwell in the bile ducts, and may sometimes block the bile ducts and cause jaundice. The severity of the infection varies depending on the species of liver fluke and the parasite load [4].

Human fasciolosis is widespread, particularly in the Middle East, South America, Africa, and Asia [5,6]; though a few cases have also been reported in Europe [7]. Nevertheless, clinical manifestations, other than in severe cases or bile duct obstruction, are commonly misunderstood. Additionally, there are limited effective medications available globally. Therefore, fasciolosis was classified as one of the most ignored tropical diseases by the World Health Organization (WHO) [8].

Accurate diagnosis of liver fluke infections poses significant challenges for both laboratory technicians and physicians. This is primarily because the parasite inhabits the liver and bile ducts, making it difficult to access directly. Additionally, detecting the parasite's eggs in fecal samples is often problematic due to their low concentration and intermittent shedding. These factors complicate accurate diagnosis and require advanced diagnostic techniques or supplementary testing to confirm the presence of the infection.

Genotyping of liver fluke populations to understand their genetic diversity is of significant medical importance, and offers valuable insights for diagnosis,

treatment, and disease control. Each species and genotype of liver fluke has distinct evolutionary traits, and specific intermediate hosts within the Lymnaeid snail family, which play a crucial role in the transmission of the disease [9]. However, conventional diagnostic methods, e.g., coprological and immunological techniques, do not allow the identification of *Fasciola* species. Two main species of liver flukes have been identified morphologically: *F. gigantica* and *F. hepatica*. Although adults of these species are similar in many ways, there are some key differences: *F. gigantica* is larger, with a proportionally shorter cephalic cone compared to *F. hepatica,* and its body tends to be more leaf-like in shape [10]. More accurate identification of liver fluke populations was achieved using molecular methods that are sensitive and applicable [11,12]. Polymerase chain reaction (PCR), quantitative PCR (qPCR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and microsatellite markers have been used for genetic variation analysis, genotyping, and species identification of liver flukes [13–15]. Three species of *Fasciola* can be identified: *F. hepatica, F. gigantica,* and *Fasciola intermediate* [16]. The target genes used for genetic characterization and phylogenetic analysis include *ribosomal DNA* (*28S rDNA*), *cytochrome oxidase one* (*COX1*), *internal transcribed spacer 1* (*ITS1*), and *ITS2* [5,14]. *ITS1* is situated inside the ribosomal RNA gene cluster, and is a well-known genetic marker that has made it possible to monitor the genetic diversity and relationships among various *Fasciola* species and strains [17–19].

Therefore, the aim of the present study was to identify species of liver flukes isolated from domestic animals and to assess their genetic variation using molecular markers and PCR-RFLP targeting *ITS1*, along with nucleotide sequencing in order to construct the phylogenetic tree. Two restriction enzymes *Tsp509*I (which recognizes ^AATT) and *RsaI* (which recognizes the sequence GT^AC) were utilized to produce fragments which reflected genetic variation. This is a recently documented use of these specific enzymes for species discrimination. Additionally, the protocol was optimized by reducing the enzyme incubation time, which had no effect on the precision and dependability of the outcomes. This work contributes to both methodological advancements and the recent molecular-based identification and genetic analysis of *Fasciola* species in this region where their distribution had not been previously well-defined.

Methodology

Sample collection

A total of 100 fecal samples (44 sheep, 36 cattle, 20 goats) were collected from Sulaimani farms in Iraq and examined using direct wet mount and sedimentation techniques to identify liver fluke eggs. In addition, 42 adult flukes were collected from 21 slaughtered animals including cattle (n = 6), sheep (n = 10), and goats (n = 5) at the Sulaimani slaughterhouse after visual inspection of the liver; two flukes per animal were collected. Flukes (n = 42) and coprology-positive stool samples (n = 2) were preserved in 70% ethanol for further molecular analysis.

DNA extraction and molecular analysis

Genomic DNA was extracted from 200 mg of fecal samples and adult fluke tissue using a commercial DNA stool mini kit (Geneaid Biotech, New Taipei city, Taiwan) and a DNA tissue extraction kit (AddPrep Genomic DNA extraction kit, ADDBIOINC, Daejeon, Korea), respectively. Adult flukes were first rinsed with nuclease-free water to remove the residual ethanol, and a piece of the posterior and or mid-body region of the worm was cut into small parts. This region contains less parts of the digestive system which minimizes PCR inhibition. The extracted genomic DNA was stored in Tris EDTA (TE) buffer at – 20 °C until further use. A specific primer set designed to amplify a 680 bp fragment of the *ITS1* region was used [12]. A 50 µL reaction mixture was prepared for PCR amplification, consisting of 25 µL of 2X master mix (0.5 U/1 µL *Taq* polymerase, 200 µM dNTPs, and 1.5 mM $MgCl_2$; 2x GS Taq Master mix (Genesand Biotech Co., Beijing, China), 0.2 µM of each primer, and 2 µL of DNA sample. The thermocycler conditions were as follows: initial denaturation at 96 °C for 5 minutes; followed by 40 cycles of denaturation at 96 °C for 45 seconds, annealing at 56 °C for 30 seconds, and extension at 72 °C for 1 minute; with a final extension at 72 °C for 5 minutes. The PCR product (3 µL) was loaded on a 1.5% agarose gel, stained with 5 µL of Biolit Safe Dye (Sisco Research Laboratories Pvt., Mumbai, India), electrophoresed at 80 V for 50 minutes, and visualized under UV light using BK-AG100 – Automatic Gel Imaging and Analysis System (BIOBASE, Shandong Province, China).

RFLP and DNA digestion

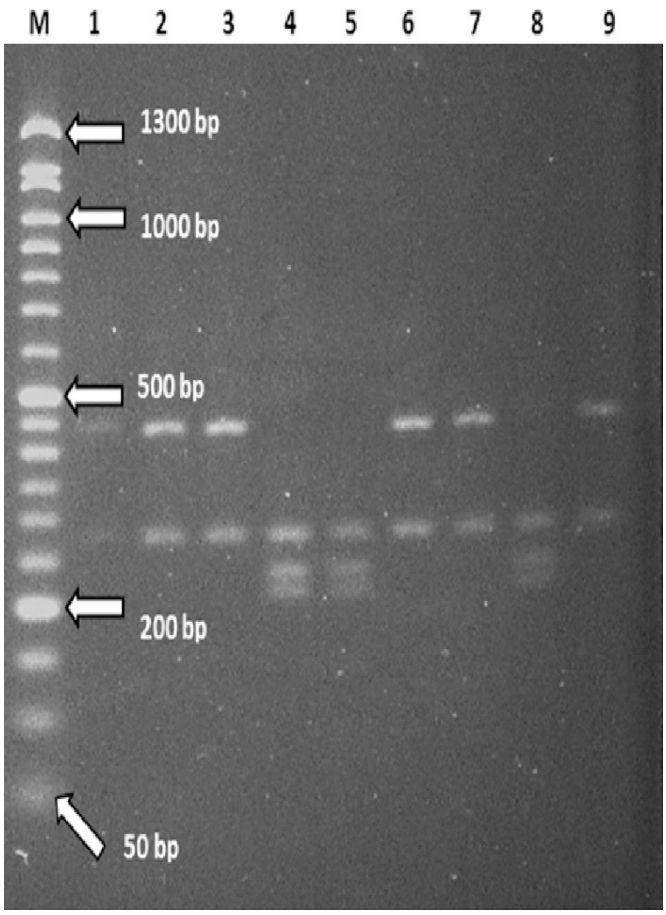
The restriction enzymes *Tsp509 I* and *Rsa I* (New England BioLab Inc, Massachusetts, United States) were used for RFLP analysis. For DNA digestion, 5 µL of the 1X rCutSmart™ Buffer (BioLab Inc,

Massachusetts, United States), 5 µL of the PCR product, 2 µL of each restriction enzyme, and 38 µL of nuclease-free water were added. The *Tsp509I* digestion mixture was incubated at 60 °C for 1 hour, while the *RsaI* digestion mixture was incubated at 37 °C for 1 hour, following the manufacturer's instructions. After incubation, the enzymes were inactivated by heating at 65 °C for 20 minutes. A 5 µL aliquot of the enzyme-digested PCR product was then loaded on a 2% agarose gel, electrophoresed at 84 V for 90 minutes, stained with 4 µL of Safe Dye (Sisco Research Laboratories Pvt., Mumbai, India), and visualized under UV light.

Sequence and phylogenetic analysis

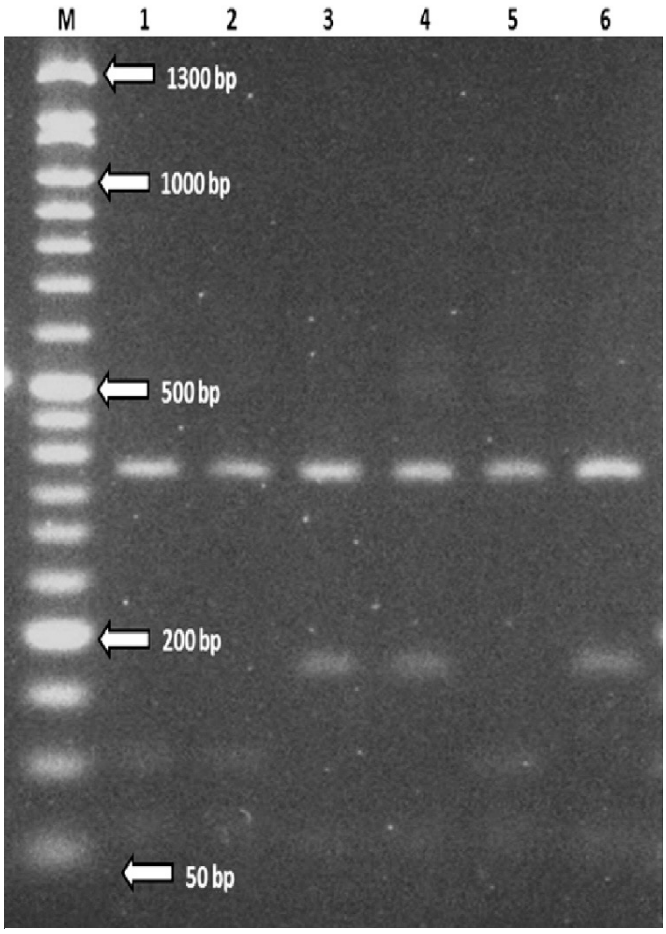
The PCR-amplified *ITS1* were sent to Macrogen Inc. (Seoul, South Korea) for nucleotide sequencing. Bidirectional Sanger sequencing was performed, and the chromatograms were manually edited in BioEdit [20] to resolve ambiguities. The DNA sequences were analyzed, aligned, and compared against previously recorded sequences in GenBank. The phylogenetic tree was constructed using partial *ITS1* sequences. The sequences obtained from the collected specimen were deposited in GenBank under the following accession numbers; PP590002-PP590004 and PP590006-PP590009 for *F. hepatica*, PP590010-PP590012 for *F. gigantica*, and PP590005 for the genetically distinct form potentially representing *F. intermediate*. Additionally, ITS1 sequences were retrieved from GenBank, including MK37715, MT644667, MF969015, MZ614968, and MW842577 for *F. gigantica*; and PP108836 and KJ818275 for *F. hepatica*. A sequence of *Echinococcus* spp. (AJ237775) was used as an outgroup to root the tree. Multiple sequence alignments were carried out using MUSCL [21], and the maximum likelihood method was used to construct the phylogenetic tree, using the MEGA software (version 7.1).

Figure 1. PCR-RFLP pattern of *ITS1* amplicon (680 bp) digested by *Rsa1* restriction enzyme.



Lane M: 50 bp DNA ladder; lanes 1, 2, and 5: *F. intermediate*; lanes 3, 4, and 6: *F. gigantica*.

Figure 2. PCR-RFLP pattern of *ITS1* amplicon (680 bp) digested by *Tsp509I* restriction enzyme.



Lane M: 50 bp DNA ladder; lanes 1, 2, 3, 6, 7, and 9: *F. hepatica*; lanes 4, 5, and 8: *F. gigantica*.

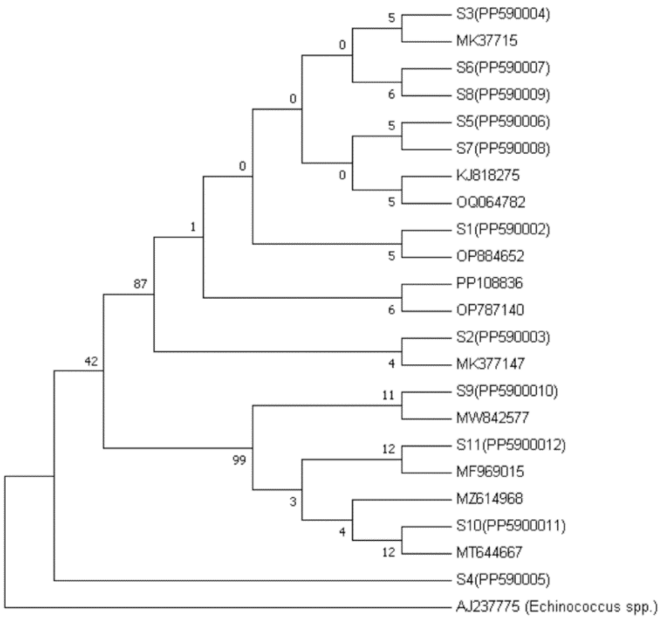
Results

The microscopical observations concluded that *Fasciola* eggs were detected in 2 out of 100 (2%; 1 sheep, 1 cattle) of the fecal samples, and no other trematode eggs were observed. The molecular results indicated that all tissue of fluke worms (n = 21) samples were positive, while only two fecal samples were positive.

The RFLP analysis with both restriction enzymes (*Rsa1* and *Tsp5091*) revealed two distinct patterns for each enzyme, indicating genetic variation among the liver fluke isolates (Figures 1 and 2). PCR product (*ITS1*) digestion with *Rsa1* produced a fragment pattern consistent with *F. gigantica* and *Fasciola* sp. being genetically distinct, while *Tsp5091* digestion patterns corresponded to *F. hepatica* and *F. gigantica*. These findings were supported by nucleotide sequencing of the partially amplified *ITS1* region which confirmed the genetic variation among the isolates. BLAST analysis of the nucleotide sequences discriminated three groups which were recorded in GenBank with the following accession numbers: *F. hepatica* (PP590002, PP590003, PP590004, PP590006, PP590007, PP590008, and PP590009), *F. gigantica* (PP590010, PP590011, and PP590012), and one genetically distinct *Fasciola* species (PP590005) (Table 1).

The phylogenetic analysis based on *ITS1* nucleotide sequences showed 3 main clusters corresponding to *F. hepatica*, *F. gigantica*, and a genetically distinct *Fasciola* sp. The first cluster consisted of 7 of the study samples (PP590002, PP590003, PP590004, PP590006, PP590007, PP590008, and PP590009), which clustered closely with known *F. hepatica* reference sequences (PP108836, KJ818275). This grouping was confirmed as *F. hepatica* through the high bootstrap value. The second clade comprised 3 study samples (PP590010, PP590011, and PP590012) which were clustered with *F. gigantica* reference sequences (MT644667, MZ614968, MW842577). These clusters also showed strong bootstrap support, indicating that these samples were *F. gigantica*. Finally, one sample (PP590005) did not cluster with *F. hepatica* and *F. gigantica*; it formed a separate well-supported branch. This isolate represents a genetically distinct form (*F. intermediate*) (Figure 3).

Figure 3. Phylogenetic tree constructed from nucleotide sequences of partial *ITSI* gene of *Fasciola* species isolated from animals using neighboring pairwise.



Discussion

In this study, *ITS1*-RFLP and phylogenetic analysis were applied to identify and discriminate between *Fasciola* species isolates from livestock. The RFLP results of *Rsa1* and *Tsp509I* revealed two different digestion patterns indicating genetic variation among isolates. This finding was confirmed by sequencing *ITS1*, followed by phylogenetic analysis of the sequences, which identified *F. hepatica* as a common species in the study region. *F. gigantica* was the second most common species, and the presence of the genetically distinct *Fasciola* sp. that may correspond to *F. intermediate* (PP590005) was limited. This finding

Table 1. Restriction fragment length polymorphism (RFLP) patterns of *ITS1* PCR products digested with *RsaI* and *Tsp509I*.

Host	Restriction enzyme	PCR product	Restriction fragment (size)	Accession no. of *ITS1* DNA sequences	*Fasciola* species
Sheep	*Rsal*	~680 bp	367, 172, 55, 58,28 bp	PP590012	*F. gigantica*
Cattle	*Rsal*	680 bp	367, 172, 55, 58, 28 bp	PP590011	*F. gigantica*
			367, 172, 103, 58 bp	PP590005	*F. intermediate*
Goat	*Rsal*	680 bp	367, 172, 55, 58,28 bp	PP590011	*F. gigantica*
Sheep	*Tsp509I*	680 bp	440, 260 bp	PP590004 PP590002	*F. hepatica*
			240, 250, 20 bp	PP590012	*F. gigantica*
Cattle	*Tsp509I*	680 bp	440, 260 bp	PP590007	*F. hepatica*
			240, 250, 20 bp	PP590010	*F. gigantica*
Goat	*Tsp509I*	680 bp	440, 260 bp	PP590008	*F. hepatica*
			240, 250, 20 bp	PP590011	*F. gigantica*

highlights the complex epidemiology of fasciolosis and suggests possible hybridization or novel variants in circulation. These results demonstrate the value of molecular tools in accurate species identification and genetic diversity analysis of *Fasciola* species in endemic areas [20]. Direct parasitological methods, involving the finding of eggs in animal feces, duodenal samples, and bile fluid for human infections, are among the most widely employed diagnostic techniques. In addition, biomarkers such as eosinophilia should be considered for early diagnosis [23,24]. Microscopic examination of eggs remains a widely used technique due to providing results with high specificity and sensitivity. However, an expert laboratory technician is required for the identification of liver fluke eggs [25]. In contrast, molecular approaches are preferred due to their high sensitivity and specificity; therefore, the *ITS1*-RFLP technique was used in this study.

The detection of intraspecific polymorphisms allows for the characterization of genetic diversity and population structure among liver flukes isolates from different geographic regions or host species. Researchers can infer evolutionary relationships, gene flow patterns, and demographic dynamics within *Fasciola* populations by analyzing their RFLP profiles [25]. In the present study, PCR-RFLP of the *ITS1* region and phylogenetic analysis were utilized to discern the genetic polymorphism and phylogenetic ancestry of *Fasciola* species isolated from animals in the Sulaimani province of Iraq. The findings of this study somewhat differ from those of Raoof *et al*. [11] in Sulaimani province (Iraq) since they used the mitochondrial 28S rRNA gene and identified two species, *F. hepatica* and *F. gigantica*. The findings in this study indicate more genetic diversity, and the genetically distinct *Fasciola* species which may correspond to *F. intermediate* was identified. These differences reveal the high resolution of the *ITSI* region compared to the mitochondrial gene. A study conducted by Othman *et al.* [13] supports the findings of this study that PCR-RFLP of *ITS1* is more reliable in finding genetic diversity among liver flukes. They used the mitochondrial gene *COX1* and 28S rDNA for species identification of liver flukes, finding two species of *Fasciola* (*F. hepatica* and *F. gigantica*). Furthermore, studies by Mohammed *et al.* [16] and Koyee *et al.* [18] used different DNA markers (*COXI* and 28S rDNA) for species identification in Erbil province (Iraq). They reported the presence of *F. hepatica* and *F gigantica*, depending on the nucleotide sequencing of *COXI* and 28S rDNA. However, they did not identify *F. intermediate*, as in this study. These results suggest that the *ITS1* region is a more efficient marker for liver fluke species identification and detection of genetic variation.

Compared to traditional morphological methods, molecular techniques such as the PCR-RFLP of the *ITS1* region provide rapid and accurate species identification without the need for specialized expertise in parasite taxonomy. However, PCR-RFLP of the *ITS1* region may have limitations in detecting cryptic species or hybrid forms that exhibit similar RFLP patterns, necessitating complementary molecular markers or sequencing approaches for comprehensive characterization. The continued application of PCR-RFLP for *ITS1* analysis, combined with advances in next-generation sequencing and bioinformatics, holds promise for further elucidating the diversity and evolutionary dynamics of *Fasciola* species. Further investigations in this context might target tailoring RFLP protocols, increasing the number of reference databases, and combining molecular data with ecological and epidemiological research to shape the approaches for disease control and prevention.

The DNA sequencing results indicate that genetic variation exists among the isolated samples, indicating the complexity within the liver fluke populations. This finding can be explained by the assumption of genetic diversity among trematode parasites because of reasons such as the different geographical distribution, the chosen host, and evolutionary processes [26]. Understanding genetic variability is essential for multiple domains of parasitology, such as epidemiology, taxonomy, and disease management. Three species of liver flukes *F. hepatica, F. gigantica,* and the genetically distinct *Fasciola* species that may correspond to *F. intermediate* were identified using nucleotide sequence analysis. This discovery aligns with other research using molecular methods to confirm the fluke species [13,25]. Accurate identification of the species of live samples using DNA sequences is a strong evidence in support for molecular methods such as RFLP.

Moreover, submission of nucleotide sequences to the GenBank database under specific accession numbers (e.g., PP590002–PP590012) is a great way to facilitate data sharing and ensure their availability for future research. Accession numbers were introduced as distinct molecular signatures of nucleic acids to enable researchers all over the globe to include the data for comparative analyses, phylogenetic studies, and other applications. This allows transparency, reproducibility, and collaboration within the scientific community; which is very interesting particularly where unique genetic variation is observed, as in the case of the

PP590005 sequence, which was recorded as *Fasciola* species. This sequence's divergence from previously identified *F. hepatica* and *F. gigantica* sequences implies the existence of a genetically distinct entity that might be considered an intermediate form of *Fasciola*. The use of intermediate forms or cryptic species points at how important molecular techniques are in unravelling the problems of taxonomy and evolutionary questions arising within parasite taxa [27].

Phylogenetic analysis revealed three main clusters: the first group aligned with *F. hepatica,* the second cluster aligned with *F. gigantica*, and the third cluster was the genetically distinct isolate, that may correspond to the *F. intermediate* form. This evolutionary relationship is supported by reference sequences for *F. hepatica* and *F. gigantica,* the presence of genetic variation suggesting a hybrid or novel variant, and the similar findings in China and Turkey where overlapping distributions facilitated introgression [28,29]. These results indicate the efficiency of the *ITS1* marker for detecting genetic variation and species identification, even beyond the resolution of mitochondrial markers [30,31].

While *ITS1*-RFLP is a valuable tool for genetic diversity and species identification; it has limitations, particularly when it comes to identifying closely related forms. It is essential to use it alongside other markers like DNA sequencing and different molecular tools to get a clear and complete picture.

Conclusions

PCR-RFLP of *ITS1* is a valuable and informative tool for species identification and genetic variation analysis of the *Fasciola* population. RFLP with *RsaI* is a fast and reliable method for differentiation between *F. gigantica* and *F. intermediate*; while the restriction enzyme *Tps509I* can be used for distinguishing *F. hepatica* from *F. gigantica*. Species identification is essential for advancing our understanding of the parasite and informing efforts to control fasciolosis. DNA sequencing of *ITS1* promotes more accurate species classification and provides an essential source of data on genetic differences and evolutionary lineage within the *Fasciola* genus. The combination of molecular techniques with other analytical models will continue to deepen our knowledge of parasite biology; which in turn, will support the development of more effective measures for disease control and prevention.

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Ethical approval

This project was approved by the Ethics Committee of the College of Health and Medical Technology, Sulaimani Polytechnic University.

Corresponding author

Abdullah Hama, PhD.
Zewar Street, No. 44, Chwarchra Campus, Sulaymaniyah, Kurdistan Region, Iraq
Tel: 009647501119333
Email: abdullah.hama@spu.edu.iq

Conflict of interest

No conflict of interest is declared.

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