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Molecular Epidemiology and β-Tubulin Gene Characterisation of Fasciola Species in Cattle Revealing Benzimidazole Resistance-Associated Mutations

Basim A. Ali

basim.ali@univsul.edu.iq

University of Sulaimani

Research Article

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Abstract

Faciola, resulting in substantial economic losses worldwide and posing an emerging zoonotic threat to human health. The present study aimed to determine the molecular occurrence and species distribution of *Fasciola* spp. in cattle and to investigate genetic variations in the β-tubulin gene associated with benzimidazole resistance. A total of 100 faecal samples were collected from cattle and analysed using polymerase chain reaction (PCR)-based assays targeting conserved genetic markers. Among the examined samples, 39% (39/100) were positive for *Fasciola* spp. Molecular characterisation based on species-specific markers identified the presence of *Fasciola hepatica*, *Fasciola gigantica*, and *Fascioloides magna.* Partial sequencing of the β-tubulin isotype 3 gene was performed on selected isolates to evaluate genetic diversity and detect resistance-associated mutations. Phylogenetic analysis demonstrated that the local isolates clustered closely with corresponding reference sequences deposited in GenBank, confirming species identity and indicating limited genetic divergence. Sequence analysis revealed the presence of the F200Y mutation in *F. hepatica* isolates, whereas codons 167 and 198 remained conserved. The F200Y substitution is widely recognised as a molecular marker associated with reduced susceptibility to benzimidazole anthelmintics. Notably, the detection of *Fascioloides magna* was confirmed by sequencing, representing the first molecular evidence of this species in cattle in Iraq. These findings demonstrate the coexistence of multiple fasciolid species in cattle and provide molecular evidence of a benzimidazole resistance-associated mutation in local *F. hepatica* populations. Furthermore, this study represents the first sequence-based characterisation of the β-tubulin gene in *Fasciola* isolates from Iraq, providing valuable baseline data for future surveillance and control programs.

Introduction

Fasciolosis is a globally important food-borne trematode infection caused primarily by *Fasciola hepatica* and *Fasciola gigantica*, two liver flukes that infect a wide range of domestic and wild mammals, including cattle, sheep, goats, and humans [1–3]. The disease is recognised as one of the most economically significant parasitic infections affecting livestock production worldwide due to its detrimental effects on animal health, productivity, and reproduction. It is estimated that more than 300 million cattle and 250 million sheep and goats are exposed to fasciolosis globally, resulting in annual economic losses exceeding US$3 billion through reduced weight gain, decreased milk production, impaired fertility, treatment costs, and liver condemnation at slaughter [4, 5].

The prevalence and distribution of fasciolosis vary considerably across geographical regions and are strongly influenced by climatic and environmental factors that affect the survival of both the parasite and its intermediate snail hosts. Recent epidemiological studies have reported prevalence rates ranging from 6.2% in Iran to 28.02% in Ethiopia, while prevalence exceeding 90% has been documented in highly endemic areas [7, 8]. Warm temperatures, high humidity, and the presence of suitable freshwater habitats facilitate the development and transmission of *Fasciola* spp., contributing to the persistence of the disease in tropical and subtropical regions [8].

Accurate diagnosis is essential for effective disease management and epidemiological surveillance. Traditional coprological methods remain widely used; however, their sensitivity is often limited, particularly during early infection and in animals with low parasite burdens. Consequently, molecular diagnostic approaches have gained increasing importance due to their superior sensitivity, specificity, and ability to differentiate closely related species [9, 10]. Molecular markers such as ribosomal RNA genes and mitochondrial genes, including cytochrome c oxidase subunit 1 (*cox1*), have been extensively employed for species identification, population genetic studies, and phylogenetic analyses of fasciolid parasites [10, 11]. Continued improvements in DNA extraction and amplification protocols are further enhancing the applicability of molecular tools for routine diagnosis and surveillance of fasciolosis in both veterinary and public health settings [11].

Control of fasciolosis relies predominantly on the strategic administration of anthelmintic drugs combined with management practices aimed at reducing exposure to infected snail habitats [12]. Benzimidazole compounds, including triclabendazole and related derivatives, constitute a major class of anthelmintics used for the treatment of fasciolid infections. These compounds exert their antiparasitic activity through selective binding to β-tubulin, disrupting microtubule formation and impairing essential cellular functions within the parasite [13, 15, 16]. Despite their widespread use and effectiveness, increasing reports of benzimidazole resistance have emerged from several countries, raising concerns regarding the long-term sustainability of current control strategies [13, 14].

Resistance to benzimidazole drugs has been associated with specific point mutations in the β-tubulin gene, particularly at codons 167, 198, and 200. These mutations alter the drug-binding site and reduce the affinity of benzimidazole compounds for β-tubulin, thereby diminishing treatment efficacy [15, 16]. Molecular characterisation of β-tubulin genes has therefore become an important approach for monitoring the emergence and spread of anthelmintic resistance in parasite populations. Furthermore, understanding the genetic diversity and phylogenetic relationships among local and global isolates can provide valuable insights into parasite evolution, transmission dynamics, and the dissemination of resistance-associated alleles [17].

Although fasciolosis remains endemic in Iraq, molecular data regarding species distribution and genetic markers of drug resistance are limited. In particular, information concerning β-tubulin polymorphisms associated with benzimidazole resistance in local *Fasciola*

populations is largely lacking. Therefore, the present study aimed to determine the molecular occurrence and species composition of fasciolid parasites infecting cattle using PCR assays targeting the 28S rRNA and mitochondrial *cox1* genes. In addition, partial sequencing of the β-tubulin isotype 3 gene was conducted to investigate genetic diversity, evaluate phylogenetic relationships with global isolates, and identify mutations potentially associated with benzimidazole resistance. The study also sought to provide molecular evidence for the occurrence of fasciolid species in Iraq, including the potential detection of previously unreported species.

Materials and Methods

Study Area and Sample Collection

This cross-sectional study was conducted in Sulaymaniyah Province, Kurdistan Region, Iraq (figure 1). Fresh faecal samples were collected from cattle raised on different farms located across the province between March 2025 – May 2026

A total of 100 faecal samples were obtained directly from the rectum of cattle using sterile disposable gloves to minimise environmental contamination. Approximately 10–20 g of faecal material was collected from each animal and transferred into sterile, labelled plastic containers. Samples were transported to the laboratory in insulated containers maintained at approximately 4°C and processed within 24–48 h of collection.

DNA Extraction

Genomic DNA was extracted from faecal samples using the Stool DNA Extraction Kit (Genaid Biotech Ltd., Republic of Korea) according to the manufacturer's instructions with minor modifications to enhance DNA yield and purity. Briefly, approximately 200 mg of faecal material was suspended in lysis buffer and homogenised thoroughly. Samples were subjected to proteinase K digestion followed by purification through silica membrane spin columns. Purified DNA was eluted in 100 µL of elution buffer and stored at −20°C until further molecular analysis.

DNA concentration and purity were assessed spectrophotometrically using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA), and DNA integrity was evaluated by electrophoresis on 1% agarose gels.

Molecular Identification of Fasciolid Species

Molecular identification of fasciolid parasites was performed using PCR assays targeting multiple genetic markers. A partial fragment of the 28S rRNA gene was used for the detection of *Fasciola* spp., while the internal transcribed spacer 2 (ITS2) region was employed for the specific identification of *Fascioloides magna*. Species differentiation between *F. hepatica* and *F. gigantica* was achieved using species-specific primers targeting the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene. Additionally, a fragment of the β-tubulin isotype 1 gene was amplified for genetic characterisation and screening of mutations associated with benzimidazole resistance. All primers were selected from previously published studies and synthesised commercially (Macrogen Inc., Seoul, South Korea). Primer sequences, target genes, expected amplicon sizes, and references are shown in **Table 1**. Working primer solutions were prepared at a concentration of 10 pmol/µL.

Table 1. Primers used for the molecular identification of Fasciola spp**.**

Primer name	Primer sequence	Amplicon size bp	Target gen	detection	Ref.
F.ssp1 **F.spp2**	ACGTGATTACCCGCTGAACT CTGAGAAAGTGCACTGACAAG	618	Mitochondria28S rRNA	F. genus	[18]
FH-f **FH-r**	ACGTGATTACCCGCTGAACT CTGAGAAAGTGCACTGACAAG	440	Cox1	F. hepatica	[19]
FG.f **FG.r**	AGATTTGGGCTTTGGTTGCTCGGACAAACAAACACAAGCAGGCAT	244	COX1	F. gigantica	[19]
GA1 BD2	AGA ACATCGACATCTTGAAC TAT GCTTAAATTCAG CGGGT	500	ITS2	F. magna	[20]
β-tub295f **β-tub1230r**	AYAAYTGGGCYAARGGNCAYTA TCRGTRAAYTCCATYTCRTCCAT	935	β-tubulin isotype 1	F. hepatica benzimidazole resistance	[21]

Polymerase Chain Reaction (PCR)

PCR amplification was performed in a final reaction volume of 20 µL containing 10 µL of 2× PCR Master Mix (containing Taq DNA polymerase, $MgCl_2$, and dNTPs), 1 µL each of forward and reverse primers (10 pmol/µL), 5 µL of genomic DNA template, and 3 µL of nuclease-free water.

Amplification reactions were carried out in a thermal cycler (Bio-Rad, USA) under the following cycling conditions: an initial denaturation step at 95°C for 5 min, followed by 40 amplification cycles consisting of denaturation at 95°C for 30 s, annealing at 58–60°C (depending on the primer pair) for 30 s, and extension at 72°C for 30–45 s. A final extension step was performed at 72°C for 5 min. Positive and negative controls were included in each PCR run to validate amplification results and monitor potential contamination.

Agarose Gel Electrophoresis

PCR products were analysed by agarose gel electrophoresis. Briefly, 1.5% agarose gels were prepared by dissolving 1.5 g of agarose in 100 mL of 1× Tris–acetate–EDTA (TAE) buffer. After complete dissolution and cooling to approximately 55°C, ethidium bromide was added to a final concentration of 0.5 µg/mL.

Following gel solidification, electrophoresis was performed in 1× TAE running buffer. Five microliters of each PCR product were mixed with loading dye and loaded into individual wells. A 100-bp DNA ladder (GeneDireX, Taiwan) was included as a molecular size marker.

Electrophoresis was conducted at 100 V for approximately 45–60 min. Amplified DNA fragments were visualised under ultraviolet illumination using a gel documentation system (UVtec, United Kingdom), and fragment sizes were estimated by comparison with the molecular weight marker.

DNA Sequencing and Bioinformatics Analysis

Representative PCR products corresponding to the 28S rRNA, *cox1*, ITS2, and β-tubulin genes were purified using a PCR purification kit and subjected to bidirectional Sanger sequencing (Macrogen Inc., Seoul, South Korea).

Raw sequence chromatograms were manually inspected and edited to remove low-quality regions. Consensus sequences were generated and aligned using the ClustalW algorithm implemented in MEGA version 11 software [22,23]. Sequence identities were confirmed using the Basic Local Alignment Search Tool (BLAST) available through the National Centre for Biotechnology Information (NCBI) database [24].

Reference sequences representing different fasciolid species were retrieved from GenBank and included in comparative analyses. Multiple sequence alignments were performed to identify nucleotide polymorphisms and assess genetic variation among isolates. Particular attention was given to codons 167, 198, and 200 of the β-tubulin gene, which have been implicated in benzimidazole resistance in trematode and nematode parasites.

Phylogenetic Analysis

Phylogenetic relationships among local isolates and reference sequences were inferred using the Neighbor-Joining (NJ) method implemented in MEGA version 11 [22]. Evolutionary distances were calculated using the Kimura two-parameter model. The robustness of phylogenetic groupings was evaluated through bootstrap analysis based on 1,000 replicates. Bootstrap values greater than 70% were considered indicative of strong branch support.

Sequence Submission and GenBank Accession Numbers

Representative nucleotide sequences generated in the present study were deposited in the NCBI GenBank database. The accession numbers assigned to the obtained sequences were as follows:

- **28S rRNA gene:** *Fascioloides magna* (PZ177365)
- **cox1 gene:** *Fasciola hepatica* (PZ177371–PZ177373)
- **cox1 gene:** *Fasciola gigantica* (PZ177374–PZ177375)
- β-tubulin gene: (PZ379053-PZ379054).

The deposited sequences are publicly available and can be accessed through the GenBank database for future comparative and phylogenetic studies.

Statistical Analysis

Descriptive statistics were used to determine the prevalence of *Fasciola* spp. Infection among the examined cattle population. Infection rates were calculated as percentages of PCR-positive samples relative to the total number of samples analysed. Sequence similarities and genetic distances were calculated using MEGA version 11. Statistical significance was considered at $P < 0.05$ where applicable.

Ethical Standards: This study was a retrospective analysis using samples already collected by veterinarians during standard medical procedures. Since the researchers did not perform any experiments on live animals or collect new samples directly, formal ethical approval for animal experimentation was deemed unnecessary. However, the researchers did obtain permission from the participating clinics to use the anonymized data for research.

Results

3.1 Molecular Detection and Species Identification of Fasciolid Parasites

A total of 100 faecal samples collected from cattle were screened for the presence of fasciolid parasites using PCR amplification of a partial fragment of the 28S rRNA gene. Of the examined samples, 39 (39.0%) yielded the expected 618-bp amplicon and were considered positive for *Fasciola* spp. (Figure 2).

Subsequent species-specific PCR assays targeting the mitochondrial *cox1* gene and the ITS2 region as shown in (Figure 3) were performed to identify the fasciolid species present in the positive samples,. Among the 39 positive samples, *Fasciola hepatica* was the most frequently detected species, accounting for 20 cases (20.0% of total samples; 51.3% of positive samples), followed by *Fasciola gigantica* with 12 cases (12.0% of total samples; 30.8% of positive samples). In addition, seven samples (7.0% of total samples; 17.9% of positive samples) were identified as *Fascioloides magna* based on amplification of the ITS2 marker and subsequent sequence confirmation (Table 2).

The detection of three distinct fasciolid species demonstrates the coexistence of multiple liver fluke species in cattle within the study area. The predominance of *F. hepatica* suggests that environmental conditions in Sulaymaniyah Province may be particularly favourable for the maintenance and transmission of this species (Figure 2).

Table 2. Prevalence of fasciolid species and association with age among examined cattle.

Category	Subcategory	Number	Percentage (%)	Mean Age (years)	OR (95% CI)	P-value
Prevalence	Positive	39	39.0	–	–	–
Prevalence	Negative	61	61.0	–	–	–
Prevalence	Total	100	100.0	–	–	–
Species	*F. hepatica*	20	20.0	–	–	–
Species	*F. gigantica*	12	12.0	–	–	–
Species	*Fascioloides magna*	7	7.0	–	–	–
Age comparison	Positive animals	–	–	5.21	–	0.035
Age comparison	Negative animals	–	–	4.29	–	–
Logistic regression	Age	–	–	–	1.345 (1.056–1.712)	0.016

- Mann–Whitney U test: **p = 0.035**

PSPP software was used to do the statictical tests.The mean age of infected cattle (5.21 years) was significantly higher than that of non-infected animals (4.29 years) (*P* = 0.035). Logistic regression analysis further demonstrated a positive association between age and infection status, indicating that the odds of fasciolid infection increased by approximately 34.5% with each additional year of age (OR = 1.345; 95% CI: 1.056–1.712; *P* = 0.016). These findings suggest cumulative exposure to infective metacercariae over time and support age as a significant risk factor for fasciolid infection.

PCR amplification of the 28S rRNA marker generated the expected 618-bp fragment in all positive samples (Figure 3), confirming the presence of fasciolid DNA in the examined faecal specimens.

Species-specific PCR assays further differentiated positive samples into *F. hepatica*, *F. gigantica*, and *Fascioloides magna*. Amplification of the *cox1* gene produced fragments of 440 bp and 240 bp for *F. hepatica* and *F. gigantica*, respectively, while amplification of the ITS2 region generated the expected 500-bp fragment for *Fascioloides magna* (Figure 4).

3.2 Amplification of the β-Tubulin Gene

To investigate genetic variation associated with benzimidazole resistance, PCR amplification of the β-tubulin isotype 3 gene was performed on representative *F. hepatica* isolates. The assay successfully amplified the expected 935-bp fragment in selected samples, confirming the presence of the target gene and enabling downstream sequencing analysis (Figure 5).

3.3 Sequence Analysis and BLAST Identification

Representative PCR products from the 28S rRNA, *cox1*, ITS2, and β-tubulin genes were subjected to Sanger sequencing. BLAST analysis demonstrated high sequence similarity (98–100%) between the obtained sequences and corresponding reference sequences available in the GenBank database. The *F. hepatica* isolates exhibited the highest similarity to previously reported isolates deposited under accession numbers KU946979.1 and MW867325.1. Similarly, *F. gigantica* isolates shared high nucleotide identity with reference sequences KU376410.1 and OR717610.1. The *Fascioloides magna* isolate showed strong similarity to sequences OQ689976.1 and KU232369.1, confirming species identification. Notably, sequencing confirmed the presence of *Fascioloides magna*, representing the first molecularly verified detection of this species in cattle from Iraq.

3.4 Phylogenetic Analysis of Fasciolid Species

Phylogenetic analysis based on partial *cox1*, ITS2, and 28S rRNA sequences was performed to investigate the evolutionary relationships of local isolates with globally reported strains.

The Neighbor-Joining phylogenetic tree clearly separated the sequences into three well-supported clades corresponding to *F. hepatica*, *F. gigantica*, and *Fascioloides magna* (Figure 5). Field isolates generated in the present study clustered within their respective species groups with high bootstrap support.

The *F. hepatica* isolates (BAA/2/2025, BAA/3/2025, and BAA/4/2025) clustered closely with GenBank reference sequences KU946979.1 with highest identity 100-99.74%. Likewise, *F. gigantica* isolates (BAA/5/2025 and BAA/6/2025) grouped with reference sequences OR717610.1. and highest identities 99.31% with them The *Fascioloides magna* isolate (BAA/1/2025) formed a distinct cluster with reference sequences

KU232369.1 and DQ683545.1 idnetitites 100-99.13 respectivly, clearly separated from both *F. hepatica* and *F. gigantica*. These findings confirm the taxonomic identity of the recovered isolates and indicate relatively low genetic divergence from previously reported international strains (Table 3).

Table 3: Highest Nucleotide Sequence Identity Between Field and Reference Strains

Accession no.	Field strain	species	Target gene	Ref. seq.	Identities
PZ177365	BAA/1/2025	F. Magna	28S rRNA	KU232369.1	100
PZ177365	BAA/1/2025	F. Magna	28S rRNA	DQ683545	99.13
PZ177371	BAA/2/2025	F. hepatica	Cox1	KU946979.1	100
PZ177372	BAA/3/2025	F. hepatica	Cox1	MT862417	99.76
PZ177373	BAA/4/2025	F. hepatica	Cox1	KU946979.1	99.52
PZ177374	BAA/5/2025	F.gigantica	Cox1	OR123716	98.31
PZ177375	BAA/6/2025	F.gigantica	Cox1	OR123716	98.31
PZ379053	BAA/7/2025	F. hepatica	β-tubulin	MK248794.1	99.51
PZ379054	BAA/8/2025	F. hepatica	β-tubulin	AM933587.2	99.02

3.5 Phylogenetic Analysis of the β-Tubulin Gene

Phylogenetic analysis of partial β-tubulin isotype 3 gene sequences demonstrated that the local *F. hepatica* isolates (BAA/7/2025 and BAA/8/2025) clustered within the *F. hepatica* clade together with previously reported isolates from different geographical regions (Figure 7).

The field isolates showed close genetic identities to reference sequences MK248794.1, and AM933587.2, 99.51-99.02% that indicaing high level of conservation within the analysed β-tubulin region (Table 3). Bootstrap support values confirmed the robustness of the observed clustering pattern.

3.6 Detection of Benzimidazole Resistance-Associated Mutations

Multiple sequence alignment of deduced β-tubulin amino acid sequences from field isolates and representative reference sequences revealed a high degree of conservation across the analysed region (Figure 8). However, detailed examination of the known benzimidazole resistance-associated codons identified a single amino acid substitution at codon 200.

Specifically, a phenylalanine-to-tyrosine substitution (F200Y) was detected in the analysed *F. hepatica* isolates. In contrast, codon 167 remained unchanged as phenylalanine (F), and codon 198 remained conserved as glutamic acid (E) in all examined sequences.

The F200Y mutation has been widely recognised as a molecular marker associated with reduced susceptibility to benzimidazole anthelmintics in helminth parasites. The detection of this mutation suggests the possible emergence of benzimidazole resistance within local *F. hepatica* population and highlights the importance of continued molecular surveillance of anthelmintic resistance markers.

3.7 GenBank Submission of Sequences

Representative sequences generated during this study were deposited in the NCBI GenBank database. The accession numbers assigned were PZ177365 for *Fascioloides magna* (28S rRNA), PZ177371–PZ177373 for *Fasciola hepatica* (*cox1*), and PZ177374–PZ177375 for *Fasciola gigantica* (*cox1*). These sequences provide the first publicly available molecular data describing fasciolid species and β-tubulin-associated genetic variation in cattle from Iraq.

Discussion

The present study provides molecular evidence of fasciolid infections in cattle from Sulaymaniyah Province, Iraq, and represents one of the few investigations in the region to combine species identification with the molecular characterisation of β-tubulin gene mutations associated with benzimidazole resistance. The overall prevalence of *Fasciola* Species infection was 39%, indicating that fasciolosis remains a significant parasitic disease affecting cattle in the study area. This prevalence is comparable to reports from several endemic regions where environmental conditions favour the development of lymnaeid snail intermediate hosts and the transmission of infective metacercariae [7,8].

Molecular characterisation revealed the presence of three fasciolid species, namely *Fasciola hepatica*, *Fasciola gigantica*, and *Fascioloides magna*. Among these, *F. hepatica* was the predominant species, accounting for more than half of all positive cases. This finding is consistent with previous studies conducted in temperate and semi-temperate regions where *F. hepatica* is the dominant liver fluke infecting cattle and other ruminants [1,2]. The coexistence of *F. hepatica* and *F. gigantica* has been documented in several countries located within transitional climatic zones, where environmental conditions support the transmission of both species [29,30]. The detection of multiple fasciolid species within the same geographical area highlights the complexity of fasciolosis epidemiology and emphasises the need for species-specific surveillance programs.

A particularly noteworthy finding of the present study was the molecular detection and sequencing confirmation of *Fascioloides magna*. Although *F. magna* is traditionally considered a parasite of cervids and wildlife reservoirs, increasing evidence suggests its capacity to infect domestic ruminants under suitable ecological conditions [31]. To the best of our knowledge, this study represents the first molecularly confirmed report of *F. magna* in cattle in Iraq. The identification of this species expands the known geographical distribution of the parasite and raises important questions regarding its epidemiological significance, transmission dynamics, and potential impact on livestock production in the region. Further studies involving larger sample sizes and wildlife hosts are warranted to determine whether *F. magna* has become established in local ecosystems.

Age was identified as a significant risk factor for infection in the present study. Infected cattle exhibited a significantly higher mean age than non-infected animals, and logistic regression analysis demonstrated that the probability of infection increased with age. These findings support the hypothesis of cumulative exposure, whereby older animals experience prolonged contact with contaminated grazing environments and infective metacercariae over time. Similar observations have been reported in previous epidemiological studies, which demonstrated that age is an important determinant of fasciolosis prevalence due to repeated exposure and the chronic nature of infection [32,33]. Consequently, control programs should prioritise strategic treatment schedules and monitoring of older animals, which may act as important reservoirs for parasite transmission.

The molecular methods employed in this study proved highly effective for the detection and differentiation of fasciolid species. Amplification of the 28S rRNA gene enabled sensitive detection of fasciolid DNA, while species-specific analysis of the *cox1* and ITS2 markers provided accurate discrimination among *F. hepatica*, *F. gigantica*, and *F. magna*. The reliability of these markers has been demonstrated extensively in previous phylogenetic and epidemiological investigations [10,11,34]. Furthermore, sequencing analysis confirmed species identity and eliminated potential ambiguity associated with morphological identification, particularly in areas where multiple fasciolid species may coexist.

Phylogenetic analyses based on the *cox1*, ITS2, and β-tubulin gene sequences demonstrated a high degree of genetic similarity between the local isolates and previously reported international strains. The clustering of Iraqi isolates with reference sequences from GenBank confirmed species assignments and indicated relatively limited genetic divergence within the analysed genomic regions. Similar findings have been reported in molecular studies of *Fasciola* populations from Asia, Europe, and the Middle East, where conserved mitochondrial and nuclear markers have been widely used to assess genetic diversity and population structure [35,36]. The observed phylogenetic relationships suggest that the isolates identified in the present study belong to globally distributed lineages rather than representing highly divergent local variants.

One of the major objectives of this investigation was the assessment of genetic variations in the β-tubulin gene associated with benzimidazole resistance. Benzimidazoles remain among the most widely used anthelmintic drugs for the treatment and control of helminth infections due to their ability to bind β-tubulin and disrupt microtubule assembly within parasite cells [13,15]. However, increasing reports of treatment failure and reduced drug efficacy have raised concerns regarding the emergence of resistance in several helminth species [14,16].

Single nucleotide polymorphisms (SNPs) occurring at codons 167, 198, and 200 of the β-tubulin gene have been strongly associated with benzimidazole resistance in numerous nematode and trematode parasites [25,26]. These mutations result in amino acid substitutions, including F167Y, E198A/E198L, and F200Y, which alter the interaction between benzimidazole compounds and β-tubulin, thereby reducing drug binding affinity and treatment efficacy [27,28]. Among these mutations, the F200Y substitution has been most consistently associated with resistance phenotypes and is frequently used as a molecular marker for monitoring emerging anthelmintic resistance [28].

In the present study, sequence analysis of the partial β-tubulin gene identified the F200Y substitution in the examined *F. hepatica* isolates, whereas codons 167 and 198 remained completely conserved. The detection of this mutation suggests the presence of a genetic marker potentially associated with reduced susceptibility to benzimidazole treatment in local parasite populations. The absence of mutations at codons 167 and 198 may indicate that resistance evolution in these isolates is currently limited to alterations at codon 200, which has been proposed as an early molecular indicator of developing resistance in helminth populations [27,28].

Comparable findings have been reported in studies investigating benzimidazole resistance in *F. hepatica* populations from Europe, South America, and Asia, where the F200Y mutation has been detected in isolates exhibiting reduced drug susceptibility [37–39]. Although the identification of the mutation does not alone confirm phenotypic resistance, its presence highlights the necessity for continued surveillance and integrated monitoring programs combining molecular diagnostics with in vivo and in vitro efficacy testing. Such approaches are essential for determining the actual clinical significance of resistance-associated polymorphisms and for guiding evidence-based parasite control strategies.

The present study has several strengths, including the use of multiple molecular markers for species identification, sequence confirmation of all major findings, and phylogenetic analysis of both species-specific and resistance-associated genes. Nevertheless, certain limitations should be acknowledged. The sample size was relatively modest and was restricted to a single geographical region. In addition, phenotypic assessment of benzimidazole resistance through faecal egg count reduction tests or laboratory bioassays was not performed. Therefore, while the detected F200Y mutation suggests the possibility of reduced drug susceptibility, definitive conclusions regarding clinical resistance require further investigation.

Overall, the findings of this study demonstrate the circulation of multiple fasciolid species in cattle in Iraq, provide the first molecular evidence of *Fascioloides magna* in the country, and identify a β-tubulin F200Y mutation potentially associated with benzimidazole resistance in local *F. hepatica* populations. These results contribute valuable baseline data for future epidemiological surveillance, molecular monitoring of anthelmintic resistance, and the development of more effective fasciolosis control programs in Iraq and neighbouring regions.

Conclusion

This study demonstrated a 39% prevalence of fasciolid infection in cattle from Sulaymaniyah Province and confirmed the presence of *Fasciola hepatica*, *Fasciola gigantica*, and *Fascioloides magna* using molecular techniques. The molecular identification of *F. magna* represents the first sequence-confirmed report of this species in Iraq. Phylogenetic analyses revealed high genetic similarity between local isolates and global reference strains. Importantly, sequence analysis of the β-tubulin gene identified the F200Y mutation, a well-recognised marker associated with benzimidazole resistance, while codons 167 and 198 remained conserved. These findings highlight the value of molecular surveillance for accurate species identification and early detection of resistance-associated mutations. Continued monitoring using larger sample sizes and phenotypic resistance assays is recommended to evaluate the epidemiological significance of these findings and to support sustainable fasciolosis control strategies in Iraq.

Declarations

Data Access

the complete dataset is not available to the general public, because of privacy regulations and institutional policies. Interested parties may request relevant data from the corresponding author, provided they have a legitimate reason and the participating clinics grant permission.

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Author Contribution

B.A. conceived and designed the study, collected the samples, performed the laboratory experiments, analyzed and interpreted the data, prepared all figures and tables, wrote the manuscript, and approved the final manuscript.

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Figures



Figure 1

Geographical location of Sulaymaniyah province on the map, where fecal samples were collected from cattle for PCR tests. https://www.mapchart.net/asia-detailed.html.

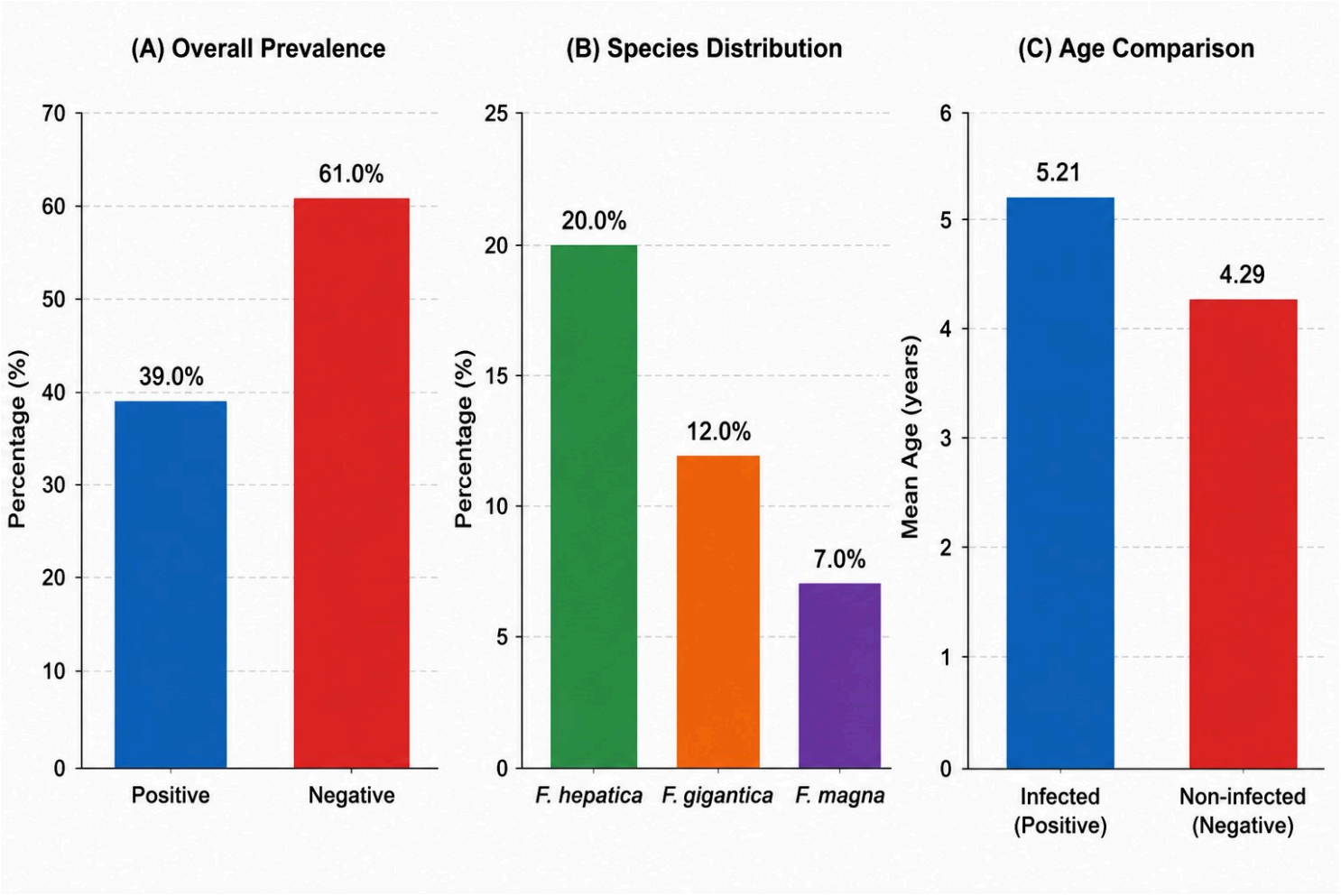


Figure 2

Overall prevalence of fasciolid infection, species distribution, and comparison of age between infected and non-infected cattle.

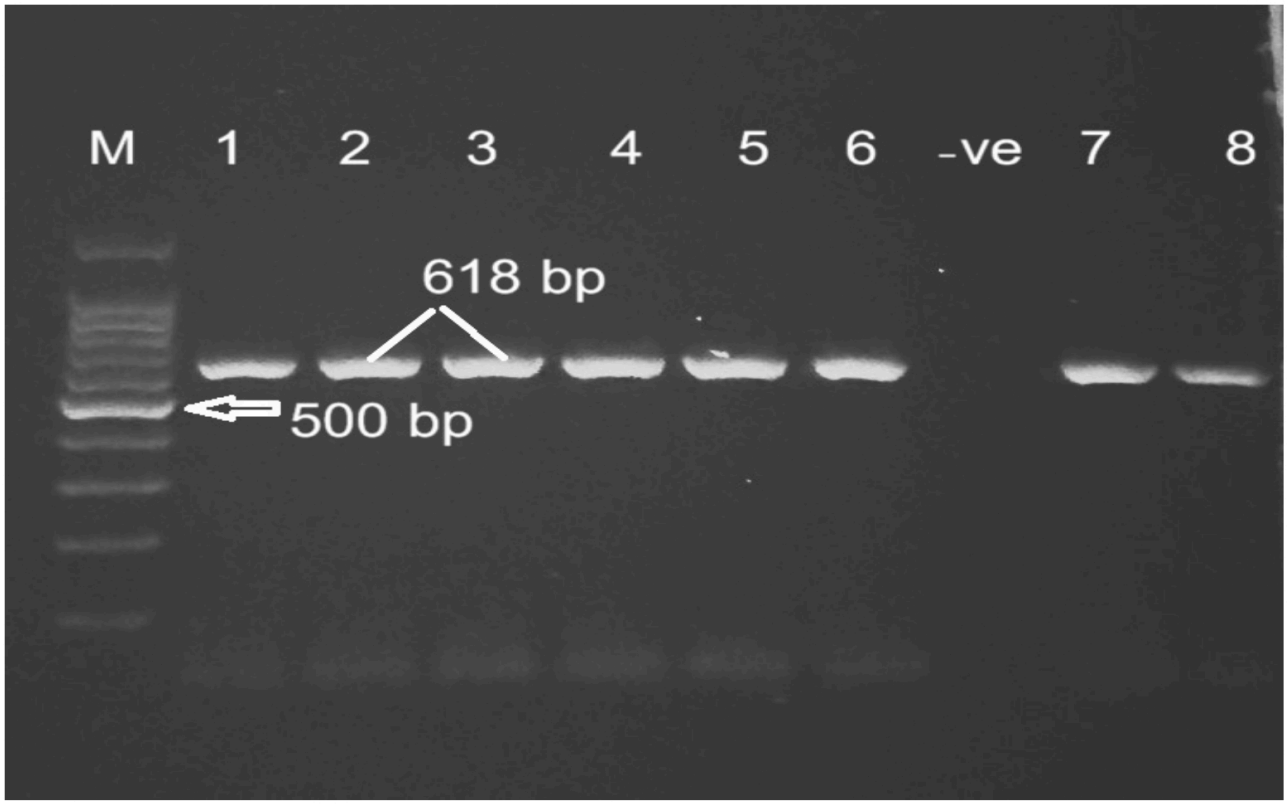


Figure 3

Agarose gel electrophoresis of PCR-amplified partial 28S rRNA gene (618 bp) of *Fasciola* spp.

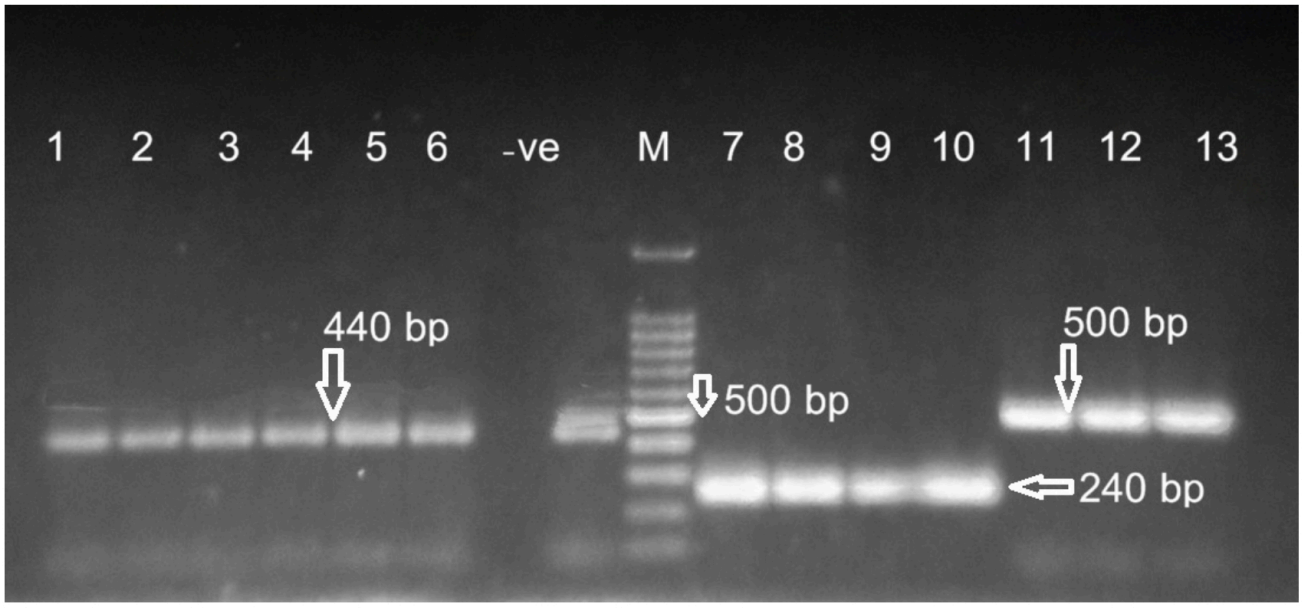


Figure 4

Species identification of *F. hepatica*, *F. gigantica*, and *Fascioloides magna* by PCR amplification of *cox1*and ITS2 gene fragments.



Figure 5

Agarose gel electrophoresis showing amplification of the β-tubulin isotype 3 gene (935 bp) from representative *F. hepatica* isolates.

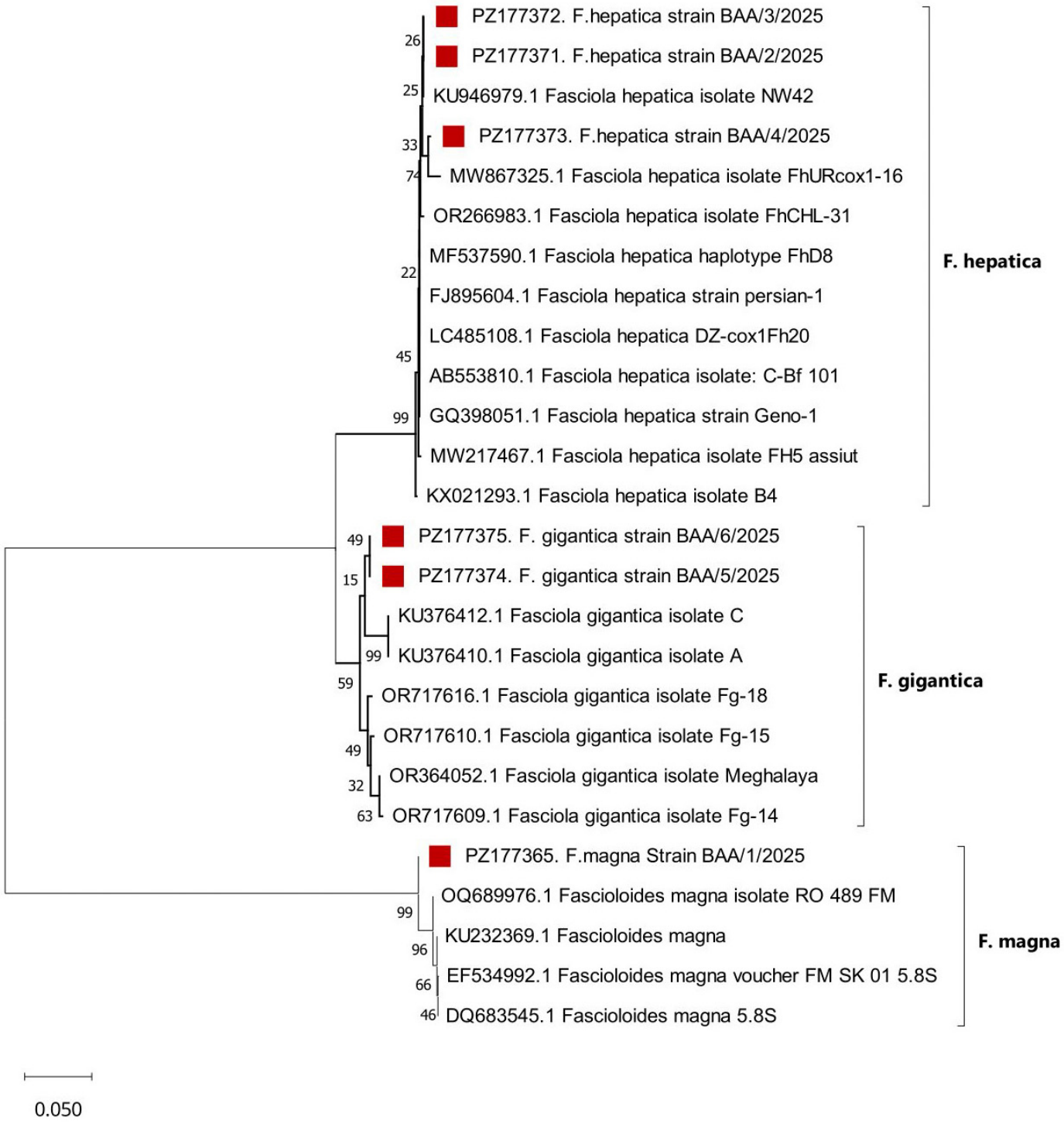


Figure 6

Neighbor-Joining phylogenetic tree based on partial ITS1 sequences showing the genetic relationships among six field isolates obtained in this study (red squares) and representative reference sequences retrieved from GenBank. Three distinct clusters corresponding to *Fasciola hepatica*, *Fasciola gigantica*, and *Fascioloides magna* were observed. Bootstrap values (>50%) based on 1,000 replicates are shown at the branch nodes

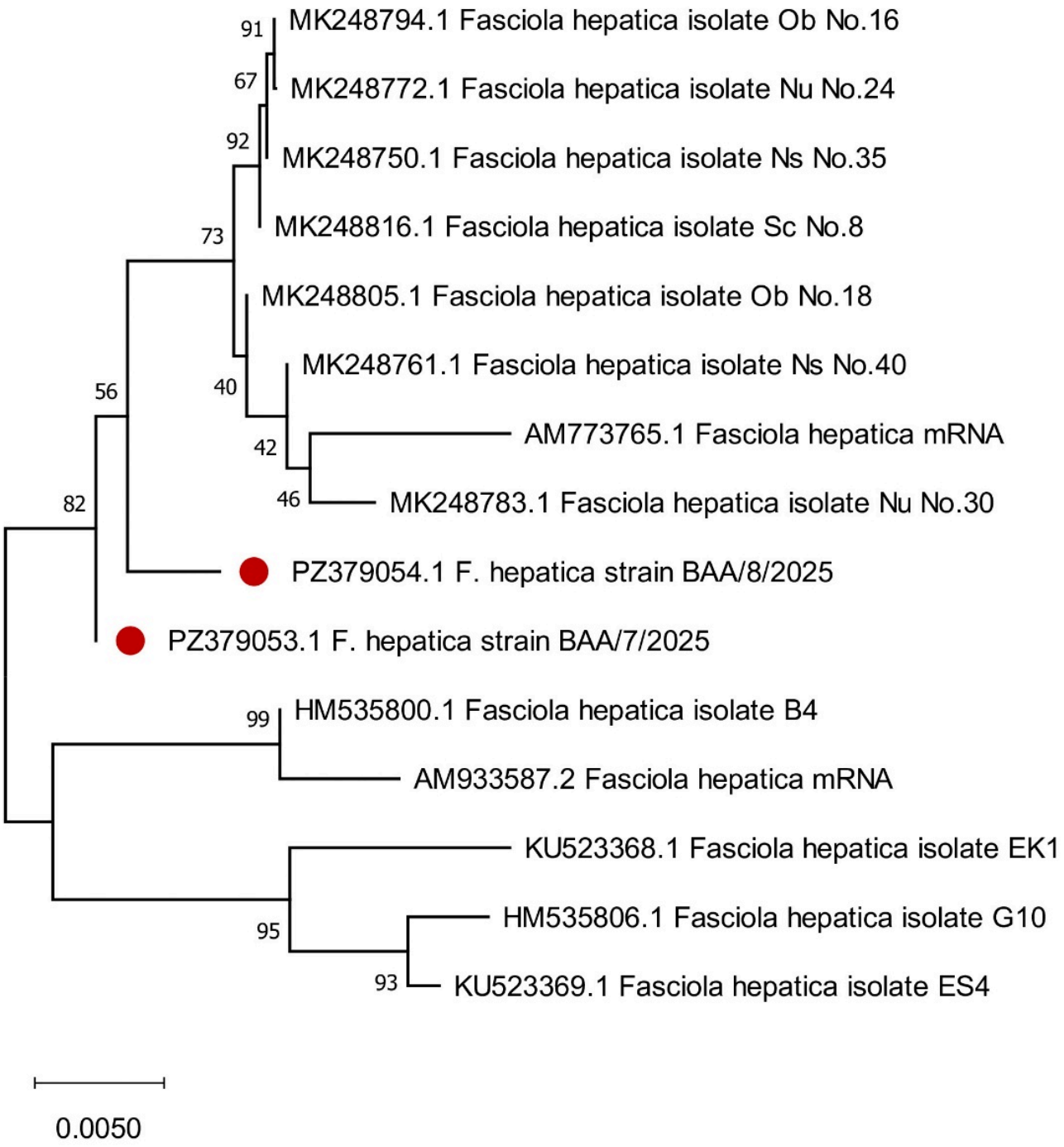


Figure 7

Neighbor-Joining phylogenetic tree based on partial β-tubulin gene sequences of *Fasciola hepatica*. The field isolates generated in the present study (BAA/7/2025 and BAA/8/2025; red circles) are compared with representative *F. hepatica* β-tubulin sequences retrieved from GenBank. Bootstrap values (>50%) based on 1,000 replicates are shown at the branch nodes.

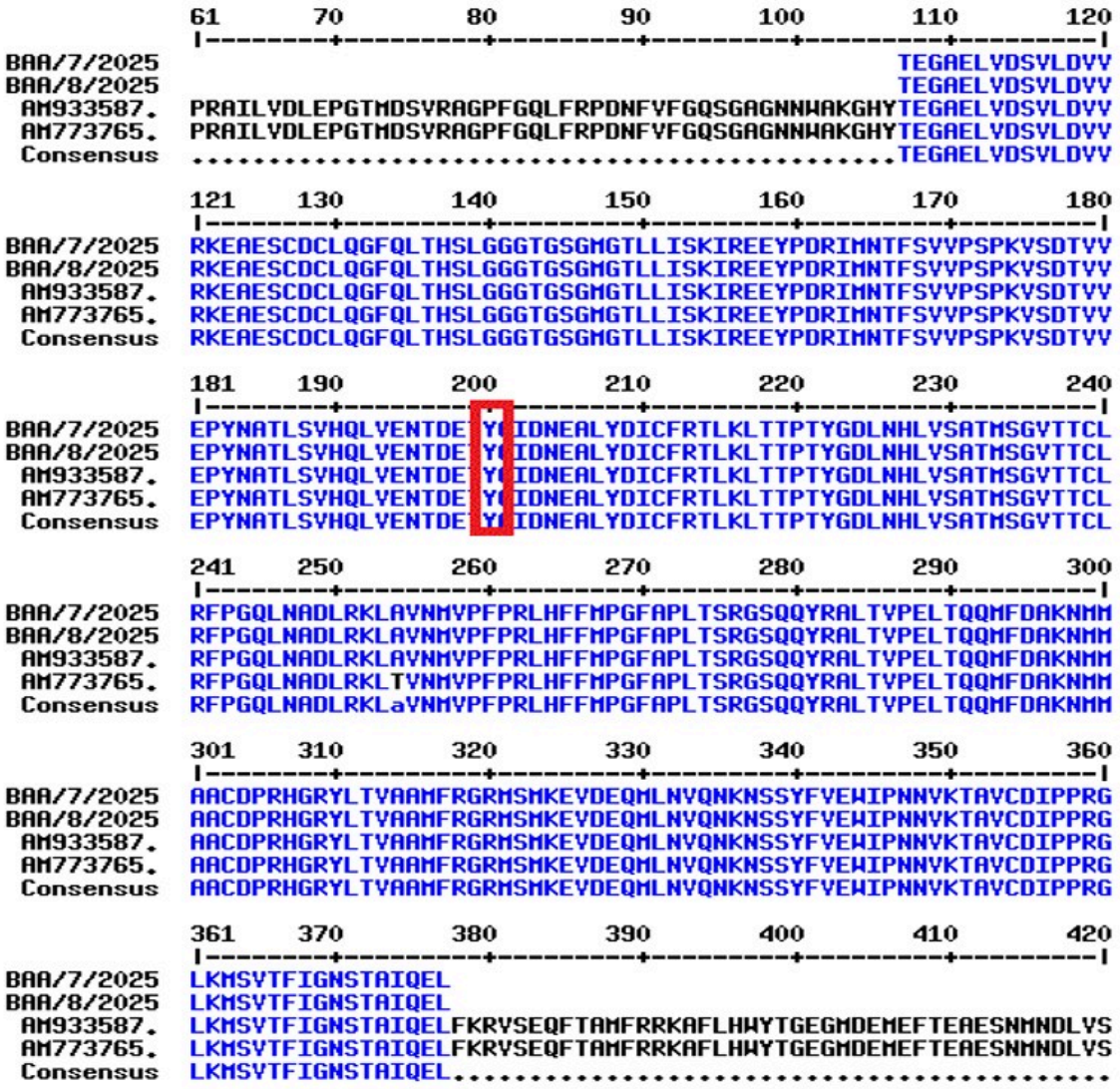


Figure 8

Amino acid alignment of β-tubulin sequences from *Fasciola hepatica* field isolate (BAA/7/22025, and BAA/8/2025) and reference sequences (AM933587, and AM773765) showing a single mutation at codon 200 (F→Y), while codons 167 and 198 remain conserved.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- figure3age.png
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- figure2species.png
- figure4logistic.png
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