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# MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF *FASCIOLA* SPECIES ISOLATED FROM SHEEP AND CATTLE USING THE ITS2 rDNA MARKER IN DUHOK PROVINCE, KURDISTAN REGION, IRAQ

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## ABSTRACT

**The aims of the current study were molecular identification and phylogenetic analysis of *Fasciola species* isolated from infected cattle and sheep using the ITS2 region of rDNA as a molecular marker in Duhok province, Kurdistan region, Iraq. DNA was extracted from 40 adult flukes isolated from the livers of infected sheep (23) and cattle (17) slaughtered at the abattoirs of Duhok Province. The amplification of the rDNA ITS2 region of these 40 specimens was performed by PCR using designed primers. ITS2 fragments of the rDNA with a length of 314 bp were successfully amplified. From these isolates, 5 amplicons of ITS2 from both species (sheep and cattle) were selected for sequencing. The blast alignment of NCBI-GenBank revealed four sequences of five amplicons belonging to *Fasciola hepatica* and one belonging to *Fasciola gigantica*, both with 100% similarity. The genetic distance between *F. hepatica* and *F. gigantica* was 0.0127. Phylogenetic analysis clustered the sequences of this study into two main clades (*F. hepatica* and *F. gigantica)* in comparison with the corresponding sequences of *Fasciola* species worldwide. The study concluded that ITS2 rDNA sequences were appropriate to identify both *Fasciola* species isolated from the infected animals, which were identical to those of neighboring countries (Iran and Turkey). The primers for the ITS2 rDNA region that were designed for the current study can be useful for the diagnosis of animal and human fasciolosis.**

***KEYWORDS:*** *ITS2, PCR, Fasciola species, Phylogenetic analysis, Ruminants, Iraq*

## INTRODUCTION

Fasciolosis is a common helminthic infection of livestock and humans caused by *Fasciola hepatica*, and *Fasciola gigantica* (El-Rahimy *et al.* 2012). These parasites have a worldwide distribution, particularly in tropical, temperate, and subtropical climates (Khanjari *et al.* 2014). East and Southeast Asia, the Caspian Sea Basin, as well as the Nile Valley, has been identified as hotspots for the transmission of *Fasciola* infection (WHO 2013). In Asian countries, fasciolosis occurs in Pakistan, Vietnam, China, Korea, Japan, Thailand, and India, as well as in the Middle East, such as Yemen, Saudi Arabia, Iraq, Turkey, and Iran (Mas-Coma *et al.* 2009; Qureshi *et al.* 2005; Turhan *et al*. 2006; Galavani *et al*. 2016).

Domestic animals are infected with *Fasciola* spp. all over the world, and it causes significant economic losses (Soulsby, 1987; Khan, 2013). Fasciolosis has a negative impact on public health and the ruminant's industry through causing morbidity, mortality in sheep and goats in addition to reducing the productive performance of ruminants, condemned livers, and the cost of treatments (Mas-Coma *et al.* 1999; Oskoui 2011; Amer *et al.* 2016).

Morphometric measurements have been used for the identification of *Fasciola* species (Lotfy *et al*., 2002; Ashrafi *et al*., 2006). However, in subtropical areas where both species of *Fasciola* (*F. hepatica* and *F. gigantica*) exist, it is difficult to distinguish between them by morphometric methods because such measurements alone are insufficient or unreliable due to the possibility of forming hybrid forms (Mas-Coma and Bargues 1997; Abdulwahed and Al-Amery 2019).

Both species of *Fasciola,* including *F. hepatica* and *F. gigantica,* can overlap in subtropical areas, and many African and Asian countries have demonstrated the overlapping

distribution of both causal agents (Mas-Coma *et al*., 2005). In addition, several Asian countries, including China, Korea, Japan, Vietnam (Hasanpour *et al*., 2020), Iran (Amor *et al*., 2011), as well as Egypt in Africa (Periago,*et al*., 2008), have reported the presence of intermediate forms. Most areas of Iraq have somewhat of a subtropical climate, and preliminary studies suggest that both species are present in different parts of the country (Abass *et al*. 2018; Raoof *et al*. 2020; Nerway *et al*. 2021). Therefore, in these areas, it is required to use molecular techniques and genetic markers for identification instead of morphometric methods. Ribosomal DNA (rDNA) regions are important markers in genomic research because they contain abundant polymorphic and conserved sequences (Rokni *et al*., 2010; Amor *et al.,* 2011). Most studies have focused on the nuclear ITS1 and ITS2 regions of rDNA for interspecific variation in the identification of *Fasciola* species (Huang *et al*., 2004; Mahami-Oskouei *et al*., 2011; Abdulwahed and Al-Amery, 2019).

Few molecular studies on the identification of *Fasciola* species in ruminants have been conducted in Iraq, especially in Duhok province. Therefore, this study was aimed to identify the species of *Fasciola* infecting the slaughtered sheep and cattle based on the rDNA ITS2 sequences and to determine the genetic relationships with other sequences of *Fasciola* spp. worldwide.

## MATERIALS AND METHODS

### PARASITES

This study was conducted on sheep and cattle infected with fasciolosis in Duhok province, north of Iraq, during November 2020–October 2021. Forty adult *Fasciola* flukes were isolated individually from the livers of slaughtered sheep (23) and cattle (17) at the Duhok and Zakho abattoirs. The adult flukes were placed in normal saline (0.9%), washed several times with phosphate buffer solution (PBS) pH 7.2, stored in 70% ethanol, and maintained frozen at -20 ˚C for use in genomic DNA extraction.

### PHENOTYPING OF PARASITES

After washing the adult parasites with PBS, each fluke was placed between two slides and photographed by a digital microscopic camera (Dino-Lite). And then the morphometric measurements were taken by Dino Capture 2.0 (version 1.4.0B) software, a computer program.

### DNA EXTRACTION

Approximately 20–25 mg from the posterior end of each fluke was cut. The samples were left at room temperature until the ethanol was removed, and then they were washed with PBS solution (pH 7.2). The DNA of prepared samples was extracted using a DNA extraction kit (Add-bioInc., Korea), proteinase K to enzymatically digest non-nucleic acid cellular components and proteins. The concentration and purity of the extracted DNA were evaluated by the NanoDrop Spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc., USA) and the quality of isolated DNA samples was estimated by gel electrophoresis (1.5%). All the extracted DNA samples were preserved in a deep freezer at -20 °C until further analysis.

### PRIMER'S DESIGN AND SYNTHESIS

To amplify the DNA fragment of ITS2 regions of *Fasciola* spp. isolates, a pair of primers was designed through the Geneious Prime software 2016.3.4 (https://www.geneious.com), from the consensus sequences obtained by the multiple alignments, with an expected amplicon size of 314 bp of the ITS2 sequence. These primers were named FITS2-F as the forward primer (5′-ATGTGAGGTGCCAGATCTATGG-3′) and FITS2-R as the reverse primer (5′-GGTAATCACGTCTGAACCGAGG-3). The primers were synthesized by the Macrogen Company in South Korea.

### PCR AMPLIFICATION

Polymerase chain reaction (PCR) amplification of the rDNA ITS2 region of 40 specimens (DNA concentration above 50 μg/μL) was conducted using the designed primers FITS2-F and FITS2-R. All PCR reactions were done in 20 μL volumes, with each reaction consisting of 10 μL of hot start PCR Master Mix (2X), 1 μL of each primer (forward and reverse primer), 3 μL of nuclease-free water, and 5 μL of template DNA. The PCR amplification was performed using a thermocycler (Gene AMP PCR System 9700) under the cycling conditions as follows: a preliminary denaturation step at 95 °C for 10 min, then 35 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec; finally, one cycle of final extension at 72 °C for 5 min, and hold at 4 °C. PCR products were separated using 1.5 % agarose gels in 100 ml of 1X TAE buffer, stained with RedSafe dye, and visualized using a UV transilluminator.

## SEQUENCING AND PHYLOGENETIC ANALYSIS.

Five samples of PCR products representing *Fasciola* species were selected based on phenotype and parasites host, then they were sent to the Macrogen Company in Korea for DNA sequencing (Sanger sequencing technology). The BioEdite software program was used for editing and cleaning the obtained ITS2 sequences. The identity of *Fasciola* species was identified by the basic local alignment search tool (BLAST) program in the NCBI database. Geneious Prime 2016.3.4 and MEGA 11 software (Tamura *et al.,* 2021) were used to estimate pairwise identity percentage and genetic distance (proportion distance), respectively. The sequences were entered into the MEGA 11 software in order to construct a phylogenetic tree (evolutionary history) using the Neighbor-Joining method (Saitou *et al*., 1987).The Kimura 2-parameter method (Kimura, 1980) was used to determine the evolutionary distances between the sequences of the phylogenetic tree.

## RESULT

Figure (1) shows the adult flukes isolated from the livers of slaughtered sheep and cattle at abattoirs in Duhok Province. Morphologically, the adult flukes in images (A) and (C) represent *F. hepatica* and *F. gigantica,* respectively, while the fluke in image (B) resembles both *Fasciola* species (*F .hepatica x F. gigantica*) and lookalike an intermediate form. When compared to *F. hepatica, F. gigantica* is longer but narrower, with smaller shoulders; the ventral sucker is larger; and the cephalic cone is shorter. Figure (2) shows the different morphological characteristics of the adult *Fasciola* species isolated from sheep and cattle, which include: Image A represents the two adult flukes isolated from cattle liver that look like an intermediate form or hybrid species; image B shows the different shapes, sizes, and colors of *F. hepatica* in the same infected sheep liver; and image C is *F. gigantica* isolated from cattle with a length of about 51 mm. The differences are clear between images A and C in figure (1) and B and C in figure (2), but sometimes, due to unknown reasons, some morphological deviations occur in these flukes, as shown in figure 1 (A) and figure 2 (A, B). Therefore, molecular identification is mandatory for their identification. Figure (3) depicts adult flukes of *Fasciola* species after being removed from 70% ethanol and prepared for DNA extraction. These *Fasciola* specimens were used for sequencing and phylogenetic analysis. The adult flukes in images A, B, and C look like the hybrid form based on the morphological characteristics. But after the sequencing, the blast alignment of NCBI revealed that the adult flukes in images A, B, C, and D belong to *F. hepatica,* while E belongs to *F. gigantica,* and none of them belong to the intermediate form.

DNA was successfully extracted from 40 adult fluke of *Fasciola* isolates using the extraction kit (Add-bioInc). The concentrations of extracted DNA ranged from 40 to 285 ng/ µl with a purity of 1.8 to 2.15 measured at A260/A280 nm, using NanoDrop instrument, and clear mono bands of DNA were apparent in agarose gel electrophoresis. ITS2 fragments of approximately 314 base pairs (bp) from *Fasciola* isolates' rDNA were amplified through the PCR technique using designed primers (FITS2-F and FITS2-R) as shown in Figure (4). Five PCR products of the ITS2 segment were successfully sequenced, and after their editing and cleaning, five sequences with lengths ranging from 241- to 460 bp were obtained. A Blast alignment of these sequences revealed that four out of the five were identical as *F. hepatica*, while one sequence was identical to *F. gigantica*, and these sequences were then submitted to GenBank with accession numbers: OM936011, OM936012, OM936013, OM936015 for *F. hepatica*, and OM936014 for *F. gigantica*, as shown in table (1) and their morphological features are shown in figure (3).

Figure (5) illustrates the alignments of *Fasciola* species ITS2 sequences (242 bp) of this study (OM936011 and OM936014) and other isolates from different countries deposited in GenBank. There were differences in the nucleotides between the ITS2 sequences of *Fasciola* species when compared interspecifically; at three positions (148, 187, and 193) between *F. hepatica* and *F. gigantica*; at four sites between *F. hepatica* and *F.* sp. from China and Vietnam; and at five sites between *F. hepatica* and *F.* sp. from Japan. The ITS2 sequence of *F. nyanzae* from Zimbabwe differs from that of *F. hepatica* and *F. gigantica* at one and two nucleotide sites, respectively. In contrast, no differences in the nucleotides were found in the ITS1 sequences of each *Fasciola* species when compared intraspecifically (within the same species). The substitutions of T to C at the 148th position, C to T at the 187th and 193th

positions were sufficient to differentiate *F. gigantica* from *F. hepatica*.

Table (2) shows the pairwise identity percentage and genetic distance of ITS2 sequences of *Fasciola* species isolated from slaughtered sheep and cattle in Duhok province, which were compared with other ITS2 sequences from different countries deposited in GenBank. The pairwise identity percentage between the ITS2 sequences of *F. hepatic*a and *F. gigantica* (interspecific) from the current study and others from GenBank references was 98.8% with a genetic distance (p-distance) of 0.0127, whereas these values were higher between the sequences of *F. hepatica* group and *F.* sp. from China, Vietnam, and Japan, which were 99.2%, 98.4%, and 98.% with 0.008, 0.016, and 0.02 of p-distance value, respectively, and they were lower between the sequences of *F. gigantica* group and *F.* sp. group. The pairwise identity % of ITS2 sequences between *F. nyanzae* and other *Fasciola* species was 99.6 %, 99.2 % with p-distance 0.004, and 0.008 for *F. hepatica* and *F. gigantica,* respectively. The ITS2 sequences were 100 % identical within the same species (intraspecific) for each of *F. hepatica* and *F. gigantica* from this study isolates and others from Iraq, Iran, Turkey, Pakistan, Egypt, Ireland, Switzerland, and Kenya with a value of 0.00 for genetic distance, indicating there was no genetic diversity between them.

The phylogenetic relationships tree was constructed using the neighbor-joining method, and it was based on a comparison of ITS2 sequences from *Fasciola* species (n = 5) obtained from this study with corresponding sequences from different countries published in the NCBI database (n = 19), with *Fasciola jacksoni* (MN970006) used as an out group. Figure 6 shows three major clades of *Fasciola* species clustered together. The first was the *F. hepatica* clade, which included eleven nucleotide sequences (NS) with (0.00) evolutionary distance (p-distance) and 100% similarity; the second was the intermediate form, which included three subgroups, one of which included three nucleotide sequences from Zimbabwe (2 *F. nyanzae* and one *Fasciola sp.*) and others were *Fasciola sp*. from China and Egypt.; and the third was the *F. gigantica* clade, including seven nucleotide sequences (NS) with (0.00) evolutionary distance and similarity of 100%.The results of the phylogenetic analysis indicated the presence of polymorphism between the interspecies (different species) of *Fasciola* while it wasn't present within the intraspecies (same species).

The sequences of the *F. nyanzae* group were separated from the *F. hepatica* and *F. gigantica* groups by a value of 0.004 and 0.008 in genetic distance, respectively. The genetic distance between *F. hepatica* and *F. nyanzae* is lower when compared with the distance value between both *F. gigantica* and *F. Nyanza* and the last (*F. nyanzae*) is located between these two groups. Therefore, this species is considered an intermediate form, as illustrated in Table (2) and Figure (6).

**Fig. (1):** The adult fluke of *Fasciola* species with their morphometric values that were isolated from infected animals in Duhok province.

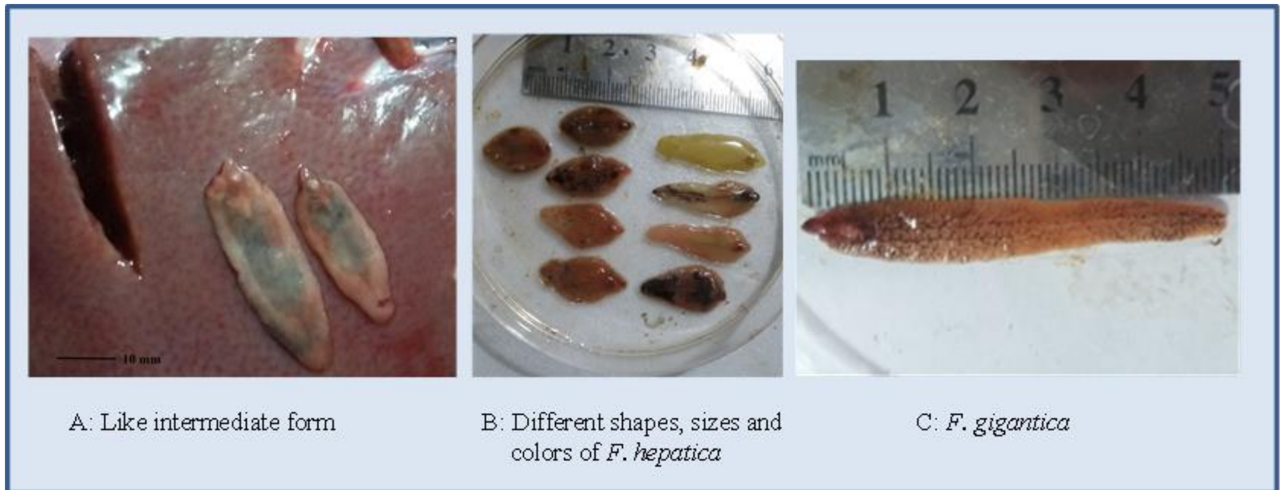


**Fig. (2):** The adult fluke of *Fasciola* species isolated from sheep and cattle; A: two adult flukes from sheep liver that look like intermediate forms; B: different shapes, sizes, and colors of *F. hepatica* from the same infected cattle liver; C: *F. gigantica* isolated from sheep liver.

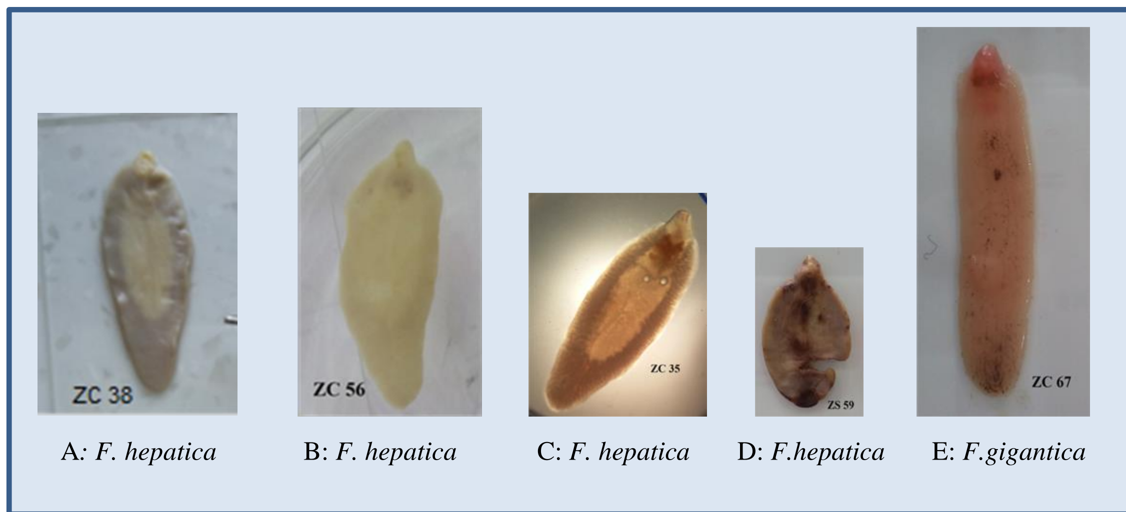


**Fig. (3)**: Adult flukes of *Fasciola* spp. isolated from sheep and cattle in Duhok province after removing them from ethanol (70%). Amplicons of these isolates were used for sequencing and phylogenetic analysis.

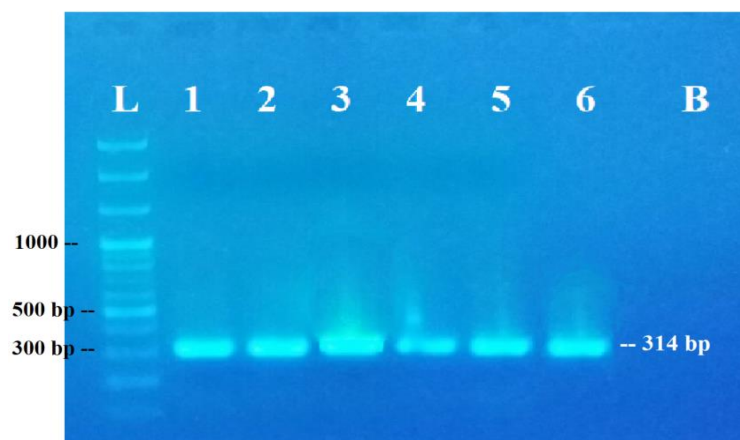


**Fig. (4)**: Gel electrophoresis of *Fasciola* spp. rDNA ITS2 bands isolated from sheep and cattle in Duhok province. Lane L: DNA ladder (100+ bp); Lanes 1-6: amplified samples of rDNA ITS2 region (314 bp); Lane B: Control (blank).

**Table (1)**: Isolate code, GenBank accession numbers, host and species of published ITS2 sequences of *Fasciola* isolates from sheep and cattle

| Image code* | Isolate code | Accession numbers | Host | Species |
|---|---|---|---|---|
| A | ZC38 | OM936011 | Cattle | *F. hepatica* |
| B | ZC56 | OM936012 | Cattle | *F. hepatica* |
| C | ZS35 | OM936013 | Sheep | *F. hepatica* |
| D | ZS59 | OM936015 | Sheep | *F. hepatica* |
| E | ZC67 | OM936014 | Cattle | *F. gigantica* |

*= image code of the adult *Fasciola* spp. flukes as shown in figure (3)

**Fig. (5):** Comparative alignment of ITS2 sequences of *Fasciola* species isolates that include: (OM936011 and OM936014) from this study and others from different countries available in GenBank.

**Table (2):** Pairwise identity percentage (%) and genetic distance (proportion distance) of *Fasciola* species isolates from Duhok province and NCBI-GenBank references based on ITS2 region of rDNA.

| S. No. | Code, country & Acc. No. | Pairwise identity percent % | | | | | Genetic distance ( P-distance) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** | **1** | **2** | **3** | **4** | **5** |
| 1 | **Fg ZC67* (OM936014)** | | | | | | | | | | |
| 2 | **Fh ZC38* (OM936011)** | **98.8** | | | | | **0.0127** | | | | |
| 3 | **Fh ZS59* (OM936015)** | **98.8** | **100** | | | | **0.0127** | **0** | | | |
| 4 | **Fh ZS35* (OM936013)** | **98.8** | **100** | **100** | | | **0.0127** | **0** | **0** | | |
| 5 | **Fh ZC56* (OM936012)** | **98.8** | **100** | **100** | **100** | | **0.0127** | **0** | **0** | **0** | |
| 6 | Fh Iraq (MW084349) | 98.8 | 100 | 100 | 100 | 100 | 0.0127 | 0 | 0 | 0 | 0 |
| 7 | FhTurkey (JN585288) | 98.8 | 100 | 100 | 100 | 100 | 0.0127 | 0 | 0 | 0 | 0 |
| 8 | Fh Iran (KT033698) | 98.8 | 100 | 100 | 100 | 100 | 0.0127 | 0 | 0 | 0 | 0 |
| 9 | Fh Ireland(AB973396) | 98.8 | 100 | 100 | 100 | 100 | 0.0127 | 0 | 0 | 0 | 0 |
| 10 | Fh Swit. (MK321600) | 98.8 | 100 | 100 | 100 | 100 | 0.0127 | 0 | 0 | 0 | 0 |
| 11 | Fh Egypt (KX470585) | 98.8 | 100 | 100 | 100 | 100 | 0.0127 | 0 | 0 | 0 | 0 |
| 12 | Fh Kenya (MZ396926) | 98.8 | 100 | 100 | 100 | 100 | 0.0127 | 0 | 0 | 0 | 0 |
| 13 | Fg Iraq (MW082589) | 100 | 98.8 | 98.8 | 98.8 | 98.8 | 0 | 0.0127 | 0.0127 | 0.0127 | 0.0127 |
| 14 | Fg Turkey(KY613944) | 100 | 98.8 | 98.8 | 98.8 | 98.8 | 0 | 0.0127 | 0.0127 | 0.0127 | 0.0127 |
| 15 | Fg Iran (JF432073) | 100 | 98.8 | 98.8 | 98.8 | 98.8 | 0 | 0.0127 | 0.0127 | 0.0127 | 0.0127 |
| 16 | Fg pakistan (KM259917) | 100 | 98.8 | 98.8 | 98.8 | 98.8 | 0 | 0.0127 | 0.0127 | 0.0127 | 0.0127 |
| 17 | Fg Egypt (MT423006) | 100 | 98.8 | 98.8 | 98.8 | 98.8 | 0 | 0.0127 | 0.0127 | 0.0127 | 0.0127 |
| 18 | Fsp China (MN310034) | 99.4 | 99.2 | 99.2 | 99.2 | 99.2 | 0.006 | 0.008 | 0.008 | 0.008 | 0.008 |
| 19 | Fsp Viet. (MT429182) | 99.6 | 98.4 | 98.4 | 98.4 | 98.4 | 0.004 | 0.0158 | 0.0158 | 0.0158 | 0.0158 |
| 20 | Fsp japan (AB010979) | 99.2 | 98 | 98 | 98 | 98 | 0.008 | 0.02 | 0.02 | 0.02 | 0.02 |
| 21 | Fn Zimbabwe (MT893595) | 99.2 | 99.6 | 99.6 | 99.6 | 99.6 | 0.008 | 0.004 | 0.004 | 0.004 | 0.004 |
| 22 | Fn Zimbabwe (MT909821) | 99.2 | 99.6 | 99.6 | 99.6 | 99.6 | 0.008 | 0.004 | 0.004 | 0.004 | 0.004 |

Fh = *Fasciola hepatica* , Fg = *Fasciola gigantica*, Fsp.= *Fasciola species* , Fn = *Fasciola nyanzae*

Bold writing (1-5) represent the specimens of this study , 6-22= Specimens from NCBI-GenBank

S. No.= Serial Number, (Acc. No.) = Accession numbers, *= Isolate code

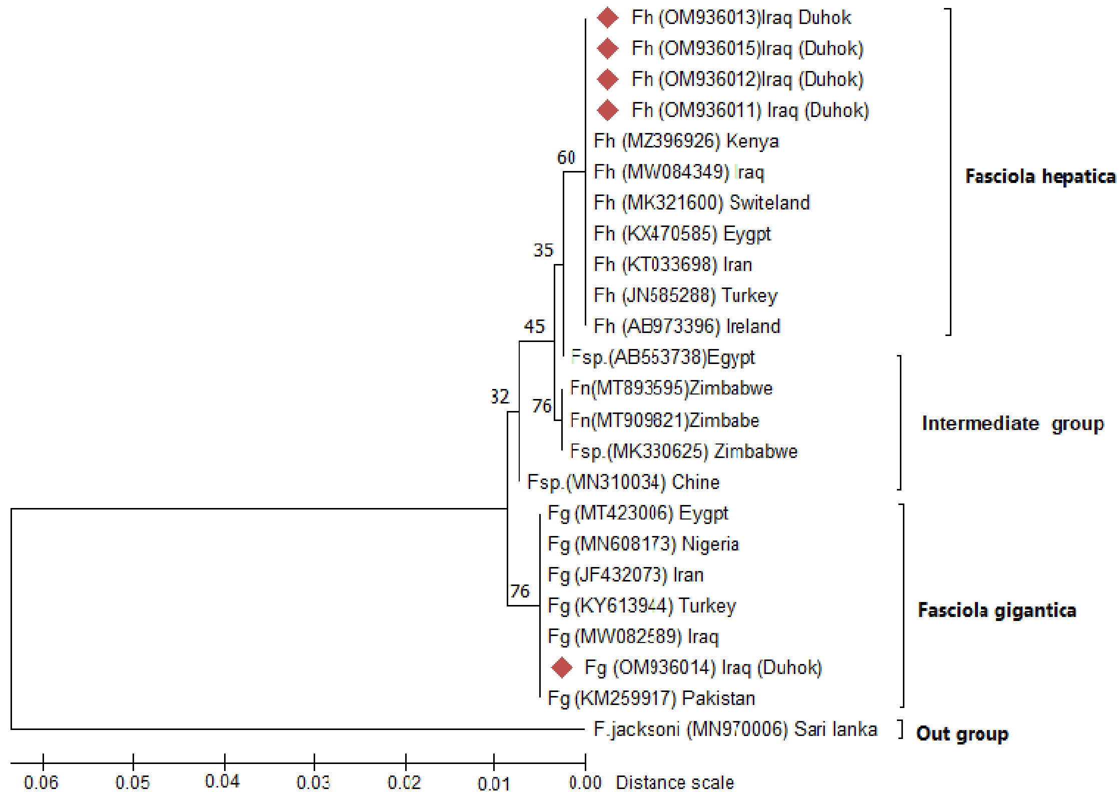


**Fig. (6):** The phylogenetic tree (the evolutionary history) of *Fasciola* species isolates was inferred using the Neighbor-Joining method based on the ITS2 sequences from this study and NCBI-GenBank. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches (Felsenstein, 1985). *Fasciola jacksoni* (MN970006) was used as the out group. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 24 nucleotide sequences: 5 from this study, which are highlighted by a red color marker and 19 from GenBank. Evolutionary analyses were conducted in MEGA11.

## DISCUSSION

*Fasciola* species can be initially discriminated by morphometric characterizations such as body measurements (Lotfy and Hillier, 2003). Many previous studies used morphometric values to differentiate *Fasciola* species (Lotfy *et al*., 2002; Ashrafi *et al*., 2006). However, the results of the studies conducted by Peng *et al*. (2009), Nguyen *et al.* (2018), and Haridwal *et al*. (2021) failed to distinguish between the two species of *Fasciola* (*F. hepatica* and *F. gigantica*) using the morphometric characters, due to the presence of intermediate forms. Furthermore, in areas where the possibility of the existence of intermediate and hybrid forms exists, it is necessary to use molecular characterization and evaluation to distinguish between species (; Karimi, 2008, Saki and Yousefi, 2011; Mir *et al.,* 2019,). In this study, the adult flukes that look like hybrid forms are hardly distinguishable by morphological methods; therefore, they must be distinguished by molecular approaches as described by Ai *et al*. (2011). For this reason, the current study was carried out using molecular techniques, especially amplification of the ITS2 region of ribosomal DNA sequences through the PCR reaction using primers (FITS2-F and FITS2-R) designed by the authors. were used to identify *Fasciola* species. These findings were in line with the studies of Amer *et al*. (2006), Lotfy *et al*. (2008), and Alasaad *et al*. (2008), who used nuclear-ITS2 of rDNA to distinguish both *Fasciola* species.

To accurately distinguish between and within *Fasciola* species (inter- and intra-species), various molecular markers and techniques are

needed (Marcilla *et al*., 2002). Previous studies have suggested that the rDNA-ITS2 sequence is an appropriate genetic marker for species identification, phylogenetic analysis, and confirming the morphometric results (Huang *et al*., 2004; Prasad *et al.*, 2008; Ghavami *et al.,* 2009).

Genotypic and phylogenetic analysis revealed that *F. hepatica* and *F. gigantica* were the two main *Fasciola* species found in infected sheep and cattle in Duhok province, as described by other researchers like Ramadhan (2021) and Mohammed *et al.* (2022) in this area, Raoof *et al*. (2020) in Sulaymaniyah province, Simsek *et al.* (2011) in Turkey, and Mahami-Oskouei (2011) in Iran. Their results confirm that both species (*F. hepatica* and *F. gigantica)* are present in the mentioned areas.

The ITS2 sequences of *F. hepatica* and *F. gigantica* from Duhok province revealed no genetic variability or nucleotide variation with a 100% similarity rate. While the comparison between interspecies (*F. hepatica* and *F. gigantica*) showed 3 nucleotide differences with 0.0127 of genetic distance (P-distance) and 98.6% similarity, this is in line with the study of Galavani *et al*. (2016).

The phylogenetic tree showed that the *Fasciola* flukes of this study were scattered as pure *F. hepatica* and *F. gigantica* clades, proposing that two genotypes of *Fasciola* can infect ruminants in Duhok province*,* these results agree with those of Shafiei *et al.* (2014) and Mir *et al.* (2019) in Iran. Furthermore, the present results showed the presence of a higher genetic diversity in the nuclear ITS2 sequence between *F. hepatica* and *F. gigantica* than that between them and *F. nyanzea* (Dinnik and Dinnik 2009) and both *Fasciola* species (*F. hepatica* and *F. gigantica*). The phylogenetic tree showed that the *F. nyanzea* clade was positioned between the *F. hepatica* clade and the *F. gigantica* clade; these results agree with the study of Bargues *et al*. (2022). As a result, this species contains genetic variation from both *F. hepatica* and *F. gigantica*, confirming that *F. nyanzea* is a hybrid species formed by the hybridization of *F. hepatica* and *F. gigantica.*

## CONCLUTIONS

This study revealed that fasciolosis in sheep and cattle in Duhok province was caused by both species of *Fasciola (F. hepatica* and *F. gigantica*). There was a strong genetic relationship between the *Fasciola* species from this study and those from neighboring countries (Iran and Turkey), as well as those from Pakistan, Switzerland, Ireland, Egypt, Kenya, and Nigeria, without the presence of intra-sequence variations in either species. No hybrid forms were found in the current study. The genotypes of these species are closely related to the genotype of *F. nyanzea* that was identified in Zimbabwe. The molecular techniques are good tools for studying the epidemiology, systematics, and ecology of liver fluke. Furthermore, the primers designed for this study can be used for the diagnosis of infections in animals and humans, which contributes to enhanced control and prevention efforts for these parasites.

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## التشخيص الجزيئي والتحليل التطوري الجيني للمتورقات الكبديه (الفاشيولا) المعزوله من الأغنام ووالابقار المصابه في محافظة دهوك اعتمادا على قطعة ITS2 للحمض النووي الريبوسومي.


**الخلاصة**

اجريت هذه الدراسة لتحديد انواع المتورقات الكبدية (*Fasciola* spp.) المعزولة من الأغنام والابقار المصابة في محافظة دهوك بالعراق بواسطة التوصيف الجزيئي وايضا لتحليل التطور الجيني للتسلسلات ومقارنتها مع التسلسلات الموجودة والمثبتة في بنك الجينات باستخدام مقطع (ITS2) من الحمض النووي الريبوسومي (rDNA) كعلامة جينية. استخرج الحمض النووي الجينومي من 40 متورقة كبدية بالغة عزلت من أكباد 23 راسا من الاغنام 17 راسا من الابقارالمصابة طبيعيا والمذبوحة في مسالخ محافظة دهوك. بعد ذلك تم تضخيم قطعة ال rDNA ITS2 بواسطة تقنية الPCR وباستخدام بادئات (FITS2-F) و(FTS2-R) المصممة لهذه التجربة. تضخيم قطعة ITS2 (314bp) للحمض النووي الرايبوسومي بواسطة تقنية PCR تمت بنجاح وانتجت اشرطة احادية ومنفردة لكل عزلة من هذه المتورقات .ومن هذه العزلات ، تم اختيار 5 من نواتج PCR ل ITS2 (2 من الاغنام و3 من الابقار) للتسلسل الجيني، وقارنت التساسلات المستحصلة بتلك الموجودة في بنك الجينات (NCBI-GenBank) للتنميط الجيني والتحليل الوراثي. كشفت نتائج (BLAST NCBI-GenBank) ان أربعة من هذه التسلسلات تنتمي إلى نوع (*F. hepatica*) وتسلسل واحد كان ينتمي إلى (*F. gigantica*) ، مع تشابه بنسبة 100٪. وكانت المسافة الجينية بين *F. hepatica* و*F. gigantica* هي 0.0127. اظهرالتحليل الجيني أن تسلسلات هذه الدراسة جمعت في مجموعتين رئيسيتين هما (*F. hepatica* و*F. gigantica*). استنتجت هذا الدراسة على أن تسلسلات rDNA ITS2 تمكنت من تحديد أنواع *F. hepatica* و *F. gigantica* بين الأغنام والابقار المصابة في محافظة دهوك ، والتي كانت متطابقة بشكل رئيسي مع تلك الموجودة في البلدان المجاورة (إيران وتركيا). علاوة على ذلك ، كانت هذه العزلات متطابقة مع العزلات الأخرى من الأغنام والماشية المصابة في باكستان وسويسرا وأيرلندا ومصر وكينيا ونيجيريا مع عدم وجود اختلافات وراثية بين تسلسلات نفس النوع لكل من *F. hepatica* و *F. gigantica* .