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Molecular characterization of *Fasciola* spp. from ruminants in Duhok province using the ITS1 ribosomal DNA marker

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Abstract

This study aimed to characterize and identify the genotypes of *Fasciola* spp. isolated from sheep, goats, and cattle in Duhok province based on the ITS1 region of rDNA. About 54 adult *Fasciola* flukes were individually isolated from the livers of naturally infected ruminants. After morphological identification, the genomic DNA of 54 isolated *Fasciola* spp. was successfully extracted, and the ITS1segment (518 bp) of rDNA was amplified. The amplicons were confirmed by gel electrophoresis and yielded mono cleared bands. Five amplicons from these samples (2 sheep, 2 cattle, and 1 goat) were selected for sequencing and then compared with NCBI-GenBank sequences for genotyping and phylogenetic analysis. Sequencing analysis and the BLAST results revealed that 3/5 of the resultant sequences were *F. hepatica* and 2/5 were *F. gigantica*. The ITS1 sequences were submitted to NCBI-GenBank with accession numbers: OM920533, OM920534, OM948733, OM948683, and OM918714. Alignment analysis of the current study and GenBank ITS1 sequences showed the presence of nucleotide variations between *F. hepatica* and *F. gigantica* species (interspecific), which were enough to separate them. At the same time, they were not observed within the same species of *Fasciola* (intraspecific). The pairwise identity percentage of intraspecific and interspecific *Fasciola* isolates was 100% and 99.2-99.6%, respectively. Phylogenetic analysis of the ITS1 sequences demonstrated that the *Fasciola* isolates of this study were clustered into two clades (*hepatica* and *gigantica* clades). The present study concluded that both *Fasciola* spp. (*F. hepatica and F. gigantica*) existed among the infected ruminants in Duhok province, and are closely related to intraspecific *Fasciola* isolates from different countries in the Middle East, Asia, Europe, and Africa.

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Introduction

Fascioliasis is a severe parasitic disease that affects ruminants (sheep, goats, and cattle), humans, and other mammals, caused by infection with *F. hepatica* and *F. gigantica* (1,2). These flukes cause severe public health issues and critical economic losses in livestock production (3,4). Fascioliasis in domestic ruminants leads to economic loss because of reduced production of milk, meat, and wool; reduced herd productivity; condemned livers; and raised mortality rates of animals (5,6). *Fasciola gigantica* is prevalent in tropical zones, while *F. hepatica* is in temperate areas, and both species can exist in subtropical regions (7,8). Previous studies have demonstrated that both species are common in the Middle East, particularly in Iran, Turkey, Saudi Arabia, and Iraq (9,10). The distribution of *Fasciola* species depends on the specific intermediate snail host, local plants, and environmental conditions on which their cercariae encyst (11,12). Duhok province consists of different geographical and climatic regions. There are some

areas, especially in the highlands, which are suitable habitats for amphibian snails, such as *Lymnaea truncatula*, the intermediate host for *F. hepatica* (13), and others are aquatic environments for the distribution of aquatic snails like *Lymnaea Auricularia* (*Radix Auricularia*), the intermediate host of *F. gigantica* (14). Historically, morphological differences have been used to distinguish these two species (15). Compared to *F. gigantica*, adults of *F. hepatica* are smaller, with a broader shoulder, a smaller ventral sucker, and a more extended cephalic cone (16). Traditional morphological approaches were the only way to identify *Fasciola* species for a long time. Due to the limitations of morphological identification of *Fasciola* species, various molecular tools can be used to identify and differentiate *Fasciola* species (17-19). Furthermore, molecular methods and markers are required for species confirmation and distinguishing intermediate forms (20,21). Sequences of the first and second internal transcribed spacers of the rDNA (ITS1 and ITS2) can distinguish the two species and their intermediate forms (22,23). Siribat *et al.* (24) confirmed that the ITS1 region of rDNA is the most effective genetic marker for identifying *Fasciola* species.

However, so far, limited studies have been conducted in Iraq for molecular characterization and genotyping of *Fasciola* spp. Hence, this study aims to use molecular markers to characterize and identify *Fasciola* spp. isolated from different hosts in different localities of Duhok province based on the first internal transcribed spacer (ITS1) rDNA sequence and to find the phylogenetic relationships between the isolated specimens of the present study and other isolates from various parts of the world.

Materials and methods

Samples and data collection permit

Approval was obtained from the Veterinary Directorate in Duhok Province (No. 1017, dated 9/13/2020) to examine and collect liver samples of slaughtered ruminants at Duhok Province Abattoirs infected with *Fasciola* spp.

Samples (parasites) collection

Adult *Fasciola* spp. flukes were collected from the livers of 54 naturally infected local ruminants (sheep, goats, and cattle) slaughtered at various abattoirs in Duhok province from November 2020 to October 2021. The flukes were isolated from infected livers using forceps and placed into Petri dishes containing 0.9% normal saline solution, washed with PBS (pH 7.2), preserved in 70% ethanol, and kept frozen at -20° C to be used for genomic DNA extraction.

Morphological identification

Each fluke was placed between two slides for morphological identification of the species of liver flukes and identified according to the descriptions of (25,26).

DNA extraction

All individual worms were washed three times with PBS before DNA extraction to remove the ethanol, and then a small piece of the posterior region of an adult fluke was cut and weighted (about 20-25 mg). Total DNA was extracted from 54 adult flukes individually (one adult fluke was taken for each case of infection) using an AddPrep Genomic DNA Extraction Kit (Add-bioInc., Korea) and according to the manufacturer's reference protocols. The DNA concentration and purity of the extracted DNA were evaluated by the NanoDrop Spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc., USA), and the samples were stored at -20 °C for further analysis.

Primer's design and preparation:

To amplify the DNA fragment of ITS1 regions of *Fasciola* spp. isolates, a pair of primers were designed using Geneious (version: 2016.3.4), primer BLAST software [available at], from the consensus sequence obtained by the multiple alignments, and with an expected amplicon size of 518 bp of ITS1 sequence. These primers were named FITS1-F as the forward primer (5-CATTGAGGTCACAGCATATCCG-3) and FITS1-R as the reverse primer (5-GGCGTCGTGATAGTTTATAAGCC-3). The primers were synthesized in Germany.

DNA amplification using PCR

DNA fragments of ITS1 regions were amplified by the FITS1-(F and R) primers through the polymerase chain reaction (PCR). The PCR reaction was carried out in a 20 µl reaction volume that included 10 µl of Hot Start DNA Master Mix (2X), 5 µl of DNA template, 1 µl of each forward and reverse primer (= 2 µl), and 3 µl of nuclease free deionized water. A thermocycler (Gene AMP PCR System 9700 Thermocycler) was used to perform the amplification. The PCR cycling protocol for ITS1 was as follows; an initial denaturation step at 95 ˚C for 10 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec; followed by one cycle of final extension at 72˚C for 5 min, and hold at 4˚C. The amplification of the ITS1 fragments by PCR was confirmed by electrophoresis on a 1.5% agarose gel in 100 ml of 1X TAE buffer, subsequent staining with RedSafe Dye, and visualization with a UV Transilluminator.

DNA sequencing and phylogenetic analyses

Five amplicons (PCR products) of *Fasciola* isolates were selected depending on different hosts (2 sheep, 2 cattle, and 1 goat) and localities (Duhok, Zakho, Akre). The selected samples with the amplifying primers as sequencing primers were sent to Macrogen Company (Korea) for DNA sequencing. All sequences were cleaned up and aligned by BioEdite and Geneious software programs. The Basic Local Alignment Search Tool (BLAST) program in NCBI-GenBank was applied to identify and compare the obtained

nucleotide sequences based on the ITS1 region [available at]. Pairwise identity percent of isolated *Fasciola* species sequences from this study and blast hits were aligned and analyzed using the ClustalW method (27) and Geneious (version: 10.2.3.) software. The sequences were entered into MEGA 11 (28) software for constructing a phylogenic tree using the Neighbor-Joining method (29), and for the calculation of evolutionary distance, the Kimura 2-parameter method was used (30). Also, the pairwise genetic distances (evolutionary divergence) between the ITS1 sequences were conducted using the proportion distance (p-distance) method in MEGA 11 software and used as percentage values. The details of *Fasciola* species rDNA ITS1 sequences from different countries available in NCBI-GenBank that were used for comparison of similar rates and constructing a phylogenic tree with sequences of current study are shown in table 1.

Table 1: Details of *Fasciola* species rDNA ITS1 sequences from different countries available in NCBI-GenBank that were used for phylogenic analysis

Species	Country	Accession numbers	Host	Isolate code	Locality
F. heptica	Iran	MN784625	Cow	SanandajV2	
F. hepatica	India	KX198628	Sheep	8772	Kashmir
F. hepatica	Chine	KJ789331	Cattle	YW2014.5.4	
F. hepatica	Spain	MG569981		FhH2ASpain	La corunya
F. hepatica	Switzerland	MK321601	Cattle	ChE 3-4	
F. hepatica	Tunisia	GQ231547		Ari 11	
F. hepatica	Aljeria	MK212150	Bos taurus	F12-Algeeria	
F. hepatica	North Africa	MN559387			
F. gigantica	Iran	MN784632	Sheep	AhavzV2	
F. gigantica	Pakistan	KF638561	Buffalo	bdnfgPKI	
F. gigantica	India	KX198619	Cattle	76555	Kashmir
F. gigantica	China	KJ789336		YW2014.5.4.5	
F. gigantica	Kenya	MZ396913		60_ITS1_CO8	
F. sp.	China	MN310034	*Ovis aries*	HLJI	Heilongjiang
F. sp.	Zimbabwe	MK330624	*P. Columella*		
F. nyanzae	Zimbabwe	MW046870	*R. Natalensis*		
F. nyanzae	Zimbabwe	MT893595	*R. Natalensis*		

F = *Fasciola*, P = Pseudosaccinea, R = Rax.

Results

Figure 1 shows *Fasciola gigantica* in the bile duct of the infected local cattle slaughtered at Zakho abattoir. Calcification, dilatation, and hyperplasia appear in the biliary duct. The morphology and shape of *F. hepatica* and *F. gigantica* are shown in (Figures 2 and 3). *F. gigantica* is longer than *F. hepatica*, while its shoulders are wider. The width of the *F. hepatica* is irregular along the length of the parasite's body; the posterior part is pointed and has prominent shoulders, while in *F. gigantica*, the width is regular along the length of the parasite's body with no protruding shoulders.

Figure 3 shows the adult flukes of *Fasciola* ssp. that were selected for rDNA ITS1 sequencing and used for phylogenetic analysis. These flukes were identified according to their morphology and shape before the DNA extraction. The flukes of the figures A and C were similar to *F. heatica*, and those in figures D and E were similar to *F. gigantica*, while the fluke of figure B looks like a hybrid or intermediate form. Their details are demonstrated in table 2.

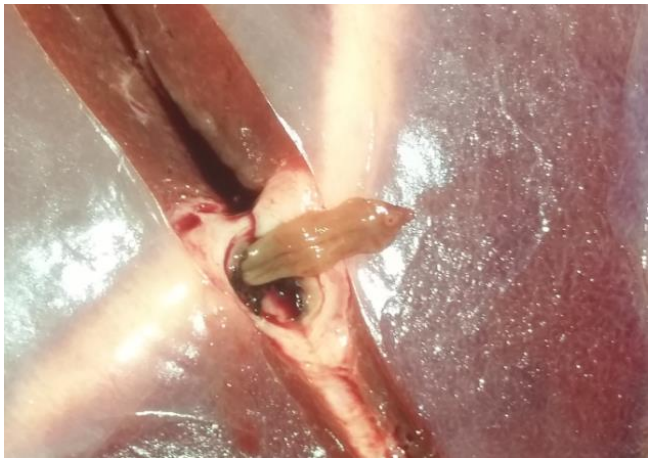

Figure 1: *Fasciola gigantica* in the bile duct of an infected cattle liver.

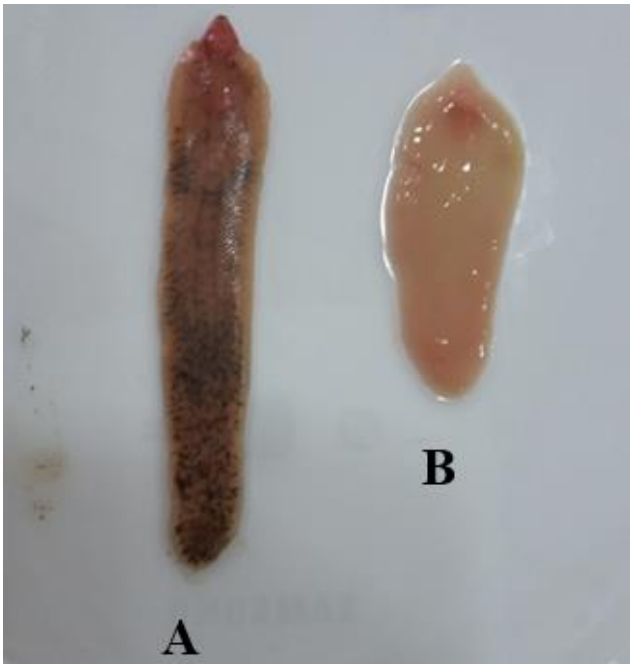

Figure 2: *Fasciola* spp. isolated from the liver of infected ruminants in Duhok province (A: *F. gigantica*, B: *F. hepatica*).

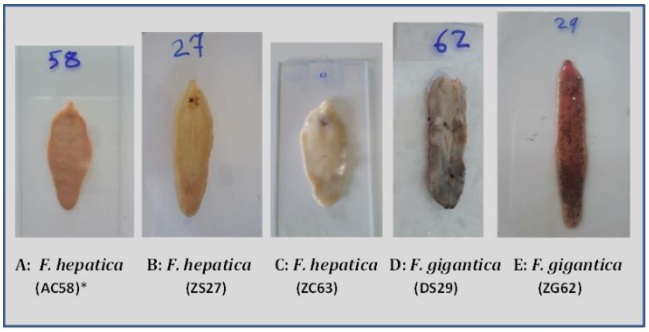

Figure 3: Adult flukes of *Fasciola* spp. isolated from ruminants in Duhok province (A, B, C, D represent stored parasites after removing them from ethanol 70%, and E is an unpreserved parasite). Amplicons of these isolates were selected for sequencing. (*= Isolate code).

The DNA of all isolates (54 samples) of the *Fasciola* was successfully extracted. The DNA concentration ranged between 35 and 310 ng/µl with a purity of 1.85 to 2.1 measured at A260/A280 nm. In almost all DNA isolated samples, a clear band of DNA with no smearing was visible in agarose gel electrophoresis, indicating that it had not been degraded. Designed primers FITS1-(F&R) successfully amplified the ITS1 segment of ribosome DNA of all *Fasciola* specimens isolated from local domestic ruminants (sheep, goats, and cattle) in different locations of Duhok province. A single band (a monomorphic DNA fragment) from approximately 518 bp was apparent in all amplified ITS1 sequence lanes, as shown in figure 4.

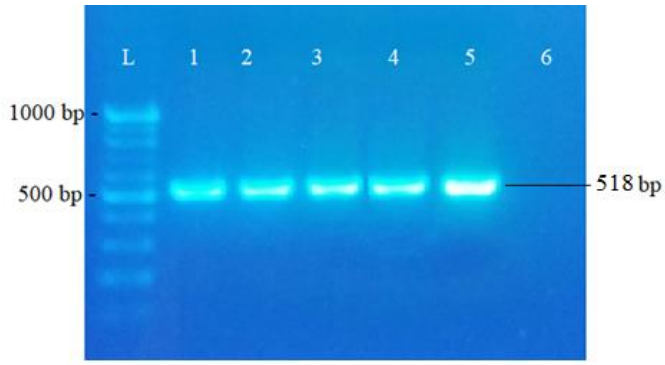

Figure 4: Gel electrophoresis of *Fasciola* spp. rDNA ITS1 segments isolated from Duhok province. Lane L: DNA ladder (100+ bp); Lanes 1-5: amplified samples of rDNA ITS1 region (518 bp); Lane 6: blank.

Five PCR product samples with both forward and reverse primers were sent to the Macrogen Company for sequencing. The PCR products of the ITS1 segment were successfully sequenced in the resulting forward and reverse sequences for all the selected *Fasciola* isolates. Five sequences with a length ranging from 477 minimum to 495 maximum base pairs (bp) were obtained. GenBank's sequence alignment and the blast hit results for each of the obtained ITS1 sequences revealed that three isolates belonged to *F. hepatica* and two to *F. gigantica*. These five ITS1 sequences were deposited in NCBI-GenBank under Accession Numbers: OM920533; OM920534; OM948733 for *F. hepatica* and OM948683; OM918714 for *F. gigantica*, as demonstrated with their information in table 2. The variable sites of nucleotides (nitrogen bases) between *F. hepatica* and *F. gigantica* ITS1 sequences of the current study were 4 bases located at positions 13, 107, 185 and 205. In contrast, no nucleotide variation was detected in the ITS1 within intraspecific (within the same species) of each *Fasciola* species. The substitutions A to T at the 13th position, C to T at the 107th and 205th positions, and T to A at the 185th position clearly distinguished *F. gigantica* from *F. hepatica* and were sufficient to differentiate both species, as shown in table 2.

Table 3 shows the pairwise identity percentage and the genetic distance (p-distance) based on ITS1 sequences (targeting 495 bp) of the *Fasciola* isolates and sequences of other isolates' that are available in NCBI-GenBank. The pairwise identity percent of ITS1 sequences within the same species of *Fasciola* (intraspecific) from this study isolates and others from Iran, India, China, Pakistan, Spain, and Kenya was 100% with a 0.00 p-distance value and no genetic diversity. While the pairwise identity percent of ITS1

sequences between the *F. hepatica* and *F. gigantica* was 99.2 %, and the genetic distance (p-distance) was 0.082. The results indicate that there was close (identity) similarity between the ITS1 sequences of the same species (intraspecific) of *Fasciola*, which shows the purity of the isolates in this study with the *Fasciola* species.

The phylogenetic relationship tree was constructed using the Neighbor-Joining method based on a comparative study of ITS1 sequences of *Fasciola* spp. (n = 5) obtained in the present study with closely related sequences from different countries submitted to GenBank (n = 19). 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 result showed that three main groups of *Fasciola* species were clustered. The first was the *F. hepatica* group, including twelve nucleotide sequences with 0.00 evolutionary distance (p-distance) and a similarity of 100%; the second was the *F. gigantica* group, including seven nucleotide sequences (NS) with (0.00) evolutionary distance and similarity of 100%; the third was an intermediate group that was generated into two subgroups; the first was *Fasciola* spp. sequence from China and the second was composed of three sequences from Zimbabwe. The ITS1 sequences clustered into *F. hepatica* and *F. gigantica* groups were genetically highly homogeneous, and no polymorphism was observed within the same species (intraspecific) while it was present between the different species (interspecific). The sequences of the *F. hepatica* group were separated from the intermediate and *F. gigantica* groups by a value of 0.8 and 0.4% in genetic distance, respectively. The genetic distance between *F. hepatica* and the intermediate form is lower when compared with the distance value between *F. hepatica* and *F. gigantica*. Therefore, the intermediate group is located between these two groups, as illustrated in figure 5.

Table 2: Details and comparison of *Fasciola* species rDNA ITS1 sequences from Duhok province

Species	Country	Accession numbers	Variable sites in the ITS1						Host	Isolate code	Locality
			13	90	107	185	205	341			
F. hepatica	Iraq/ Duhok	OM948733	-	A	C	T	C	A	Cattle	AC58	Akre
F. hepatica	Iraq/ Duhok	OM920534	-	A	C	T	C	A	Sheep	ZS27	Zakho
F. hepatica	Iraq/ Duhok	OM920533	A	A	C	T	C	A	Cattle	ZC63	Zakho
F. gigantica	Iraq/ Duhok	OM948683	T	A	T	A	T	A	Sheep	DS29	Duhok
F. gigantica	Iraq/ Duhok	OM918714	T	A	T	A	T	A	Goats	ZG62	Zakho

Table 3: Pairwise identity percentage (%) of *Fasciola* spp. ITS1 sequences of this study and compared with NCBI-GenBank references (in left lower matrix), and genetic distance only for this study's ITS1 sequences (in suitable upper matrix)

No.	Code (Acc. No.)	1	2	3	4	5
		Fh ZC63	Fh ZS27	Fh AC58	Fg ZG62	Fg DS29
1	Fh ZC63 (OM920533)		0	0	0.082	0.082
2	Fh ZS27 (OM920534)	100		0	0.065	0.065
3	Fh AC58 (OM948733)	100	100		0.065	0.065
4	Fg ZG62 (OM918714)	99.2	99.4	99.4		0
5	Fg DS29 (OM948683)	99.2	99.4	99.4	100	
6	Fh Iran (MN784625)	100	100	100	99.2	99.2
7	Fh India (KX198628)	100	100	100	99.2	99.2
8	Fh Spain (MG569981)	100	100	100	99.2	99.2
9	Fh.Chine (KJ789331)	100	100	100	99.2	99.2
10	Fg Iran (MN784632)	99.2	99.4	99.4	100	100
11	Fg India (KX198619)	99.2	99.4	99.4	100	100
12	Fg Chine (KJ789336)	99.2	99.4	99.4	100	100
13	Fg Kenya (MZ396913)	99.2	99.4	99.4	100	100

Acc. No. = Accession numbers. Fh= *Fasciola hepatica*, Fg= *Fasciola gigantica*. 1-5 represent the isolates from this study (bold writing).

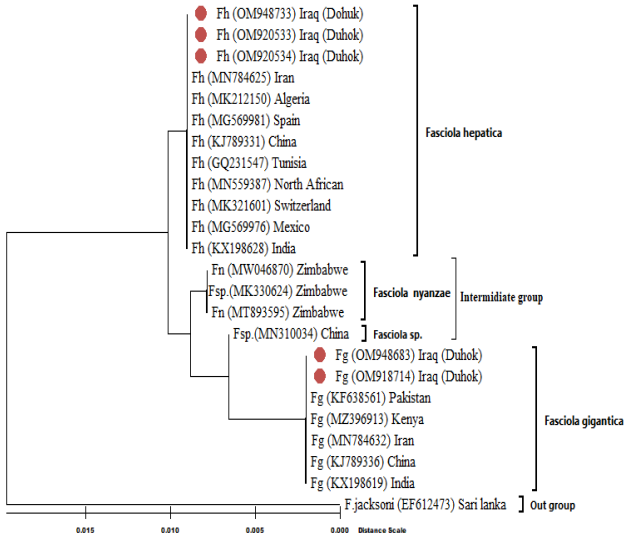

Figure 5: Phylogenetic relationship tree based on the ITS1 sequences of *Fasciola* species isolates from Duhok province and references in the GenBank database using the Neighbor-Joining method. *Fasciola jacksoni* (AN: EF612473) was used as the out-group. This analysis involved 24 nucleotide sequences: 5 from this study highlighted by a red color marker and 19 from GenBank. The phylogenetic tree was conducted in MEGA11.

Discussion

The morphology of *F. hepatica* and *F. gigantica* can generally be used for distinguishing between them, but for detecting and distinguishing intermediate forms between *Fasciola* species, one need to use molecular methods, and genetic markers (20). Aryaeipour *et al.* (31) concluded that the PCR-RFLP method was a more reliable method than morphology for the differentiation of *Fasciola* species and the morphological methods were inefficient for determining genetic diversity. The first and the second internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA (rDNA) were used as genetic markers for genetic characterization and differential between the species of *Fasciola* that have been confirmed by several previous studies (32-34). These markers are non-coding regions between the 18S, 5.8S, and 28S coding regions and have been used for diagnostic purposes at the species level (35-38). In the Middle East, many studies relied on the ITS1 marker to identify *Fasciola* species (39,40). In this research, *Fasciola* spp. were diagnosed and characterized using the ITS1 region, which is in line with the previous studies conducted by other researchers (41-46).

In the present study, a 518 bp segment (encompassing ITS1 and 5.8 RNA gene) was amplified using primers ITS1, and five amplicons were sequenced by Macrogen company (using the Sangar method). According to the findings of the current study, *F. hepatica* and *F. gigantica* are the only two *Fasciola* species isolated from infected ruminants (sheep, goats, and cattle) in the Duhok province as described by other research in the Erbil, like Mohammed *et al.* (47) in Duhok province based on ITS1 and ITS2 markers, Raoof *et al.* (48) in Sulaymaniyah province based on the mitochondrial 28S rRNA gene, and others from different parts of Iraq. Previous studies in neighboring countries stated that both *Fasciola* species (*F. hepatica* and *F. gigantica*) were present in domestic ruminants from different geographical and climatic areas (49,50).

Alignment analysis revealed very close similarity among the nucleotide sequences, and genetic variation was not observed within the same species (no intraspecific variation within species) of the current study and BLAST hits in GenBank. On the other hand, significant genetic variability was detected between *F. hepatica* and *F. gigantica*, and these results agree with those of Shaldoum *et al.* (51) and Mir *et al.* (2), which used the same marker (ITS1) to distinguish between the two *Fasciola* species.

The sequence variation between *F. hepatica* and *F. gigantica* was not related to the geographical origins and host species of isolates, as reported by Alasaad *et al.* (32). The sequences of the ITS1 region from nuclear ribosome DNA are reliable genetic markers for systematic molecular studies of *Fasciola* spp. and interspecific variations as suggested by Huang *et al.* (43).

The presence of hybrid and intermediate form genotypes of *Fasciola* were not observed in this study using ITS1 sequences. This result is in line with Shaldoum *et al.* (51) from Egypt and Rokni *et al.* (39) from Iran, who also identified *Fasciola* spp. as *F. gigantica* and *F. hepatica*.

Phylogenetic analyses support understanding species information, population differentiation, and ecological adaptation (46). According to the phylogenetic tree depended on the ITS1 sequence of rDNA, isolated *Fasciola* species from slaughtered ruminants in Duhok province were clustered into two clades (*hepatica* and *gigantica* clade) with a remarkable genetic divergence between them. Phylogenic analysis indicated that the ITS1 sequences of this study were identical to those from Iran, India, China, Spain, Switzerland, Tunisia, Algeria, and North Africa for the *F. hepatica* as reported by Muhammad *et al.* (52) in Erbil province. Results were also identical to those from Iran, Pakistan, India, China, Nigeria, and Kenya for *F. gigantica* as reported by Hamoo *et al.* (53) in Duhok province (in Aqrah city), and also this close relationship between the same species of *Fasciola* has been shown by Nguyen *et al.* (54). The analysis of the rDNA ITS1 sequence of *Fasciola* species was commonly reported in Asia, Europe, and African countries (2,18,49). The present study results and those of other studies demonstrate the diagnostic power of the ITS1 region in differentiating the *Fasciola* species (39,55).

Conclusion

The designed primers that were used for amplifying ITS1 nuclear rDNA of *Fasciola* spp. in the present study can be used to detect and diagnose *Fasciola* species. The genotypic and phylogenic analysis indicated that *F. hepatica* and *F. gigantica* were the two *Fasciola* species isolated from infected ruminants (sheep, goats, and cattle) in Duhok province. The genotype of these species is closely related to the genotypes of the same species identified in other countries in the Middle East, Asia, Europe, and Africa. In this study, hybrid forms of *Fasciola* were not detected, and the nucleotide variation of ITS1 sequences between the two species was enough to separate them.

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Conflict of interest

There is no conflict of interest in the current study.

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التوصيف الجزيئي لأنواع المتورقات الكبدية المعزولة من المجترات المذبوحة في محافظة دهوك باستخدام فواصل النسخ الداخلية الأول للحمض النووي الريبوسومي

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الخلاصة

صممت هذه الدراسة لتصنيف وتحديد الأنماط الجينية لأنواع المتورقات الكبدية المعزولة من أكباد الأغنام والماعز والأبقار المذبوحة في محافظة دهوك باستخدام علامة فواصل النسخ الداخلية الأول من الحمض النووي الريبوسومي. عزلت ٥٤ متورقه كبديه بالغة من أكباد المجترات المحلية المذبوحة والمصابة طبيعيا. بعد التعرف الشكلي، استخلص الحمض النووي لجميع هذه العزلات من المتورقات بنجاح، وتم تضخيم قطعة فواصل النسخ الداخلية الأول (بوزن ٥١٨ قاعدة نتروجينيه) للحمض النووي الرايبوسومي بواسطة تقنية تفعل السلسلة المتبلمرة وثم تأكيدها بواسطة طريقة الهجرة الكهربائية الهلامية التي ظهرت على شكل اشرطه أحادية ومنفردة لكل عزلة من هذه المتورقات. اختيرت خمسة نواتج لتفاعل السلسلة المتبلمرة من هذه العينات (٢ من الأغنام، ٢من الأبقار، ١ من الماعز) لغرض تثبيت التسلسل والتنميط الجيني وأيضا التحليل الجيني التطوري. اظهر كشف تحليل التسلسل الجيني في بنك الجينات أن ٥/٣ من التسلسلات الناتجة لديدان الكبد و ٥/٢ من نوع ديدان الكبد العملاقة. سجلت هذه التسلسلات في بنك الجينات تحت الأرقام OM920533, OM920534 OM948733, OM948683, OM918714. أظهر تحليل المحاذاة لتسلسلات فواصل النسخ الداخلية الأول للدراسة الحالية والتسلسلات المتطابقة والموجودة في بنك الجينات وجود اختلافات في القواعد النيتروجينية بين أنواع ديدان الكبد، والتي كانت كافية لفصلهما، في حين لم يتم ملاحظة أي اختلاف بين أفراد نفس النوع من المتورقة الواحدة. كانت النسبة المئوية لتطابق التسلسلات المزدوجة لعزلات المتورقة داخل النوع الواحد وبين الأنواع هي ١٠٠٪ و ٩٩,٢ - ٩٩,٦٪ على التوالي. أظهر التحليل الجيني لتسلسلات فواصل النسخ الداخلية الأول أن عزلات ديدان المتورقات في هذه الدراسة. نستنتج من هذه الدراسة أن كلا النوعين من المتورقات الكبدية موجودتان بين الأغنام والماعز والأبقار المصابة في محافظة دهوك وكانت تسلسلات فواصل النسخ الداخلية الأول لهذه الأنواع من المتورقات متطابقة جينيا ١٠٠٪ مع تسلسلات الدول المختلفة في الشرق الأوسط وآسيا وأوروبا وأفريقيا لنفس النوع والمنشورة والمثبتة في بنك الجينات.