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Molecular and Phylogenic Characterization of *Fasciola hepatica* from Assiut, Egypt based on nuclear ribosomal DNA sequences

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ABSTRACT
Background: *The species of liver fluke of the genus Fasciola are obligatory parasites that inhabit the biliary ducts of herbivorous animals as well as human. Understanding genetic structure and status of genetic variation of F. hepatica populations has important implications for epidemiology and effective control of fasciolosis. Aim: To genetically characterize Fasciola isolates from different hosts from Assiut, Egypt using sequence analysis of the first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA).*
Methods: *Three adults F. hepatica were isolated from naturally infected sheep and fragments of Fasciola spp. were extracted from three human cases by ERCP (Endscopic Retrograde Cholangio-Pancreatography). Genomic DNA was extracted from preserved flukes. Conventional polymerase chain reaction (PCR) with a set of arbitrary primers was used to estimate genetic variation within the species. Ribosomal ITS-1 and ITS-2 regions of the isolates were amplified. The amplicons were sequenced at ITS-1 and ITS-2.*
Results: *Both regions were amplified successfully for all samples and bands ranged between 400 bp and 650 bp were shown in all cases. Comparison of the obtained ITS sequences to those from known Fasciola species circulating globally and retrieved from GenBank revealed that the present worms were genetically identical (100%) with F. hepatica. Different isolates did not show any significant genetic variations in rDNA-ITS-1 and ITS-2 as all the sequences showed to be 100% identical.*
Conclusions: *These findings have implications for studying the population genetics, epidemiology, diagnosis and control of fasciolosis especially in human.*
Keywords: *Molecular, Phylogeny, Fasciola, Human, Sheep, Egypt*

Introduction

Fascioliasis is considered one of the most important food borne parasitic zoonoses caused by members of genus *Fasciola* which transmitted by freshwater lymnaeid snails. Two major species, *Fasciola hepatica* and *Fasciola gigantica* are the causative agents of fascioliasis in ruminants and humans worldwide [1, 2]. Actually, fascioliasis is considered the most important helminth infection

of ruminants causing considerable socioeconomic problems in tropical countries [3]. More importantly, human infection with *Fasciola* spp. represents a significant human health problem in a number of countries, and millions of people are estimated to be infected throughout the world [4].

Infection with *Fasciola* is considered a major animal and human health problem in many parts of Africa, such as Egypt, Zambia, Kenya, Algeria, Zimbabwe, Tanzania [5, 6]. *F. hepatica* has a wider range than its tropical counterpart, *F. gigantica*, but their geographical distribution overlaps in many African and Asian countries and sometimes in the same country, although in such cases the ecological requirement of the flukes and their snail host are distinct [7, 8]. *F. hepatica* typically occurs worldwide in temperate regions. *F. gigantica* causes outbreaks in tropical areas of southern Asia, Southeast Asia, and Africa. Infection is most prevalent in regions with intensive sheep and cattle production [9]. The two species have been traditionally classified based on their morphological features, such as body length and width. Because of variations in size of these two species, the discrepancy of morphological features, and the presence of intermediate forms, it might be difficult to distinguish the two species, solely based on these characters [10]. The presence of a hybrid and intermediate forms of *Fasciola* complicates the problem of identification and subsequently misaddressing of exact epidemiological information on fascioliasis. Advances in molecular biology, in particular, the amplification of specific DNA regions via the PCR and improved direct sequencing techniques, may allow closely related species to be distinguished [11]. The nuclear ribosomal DNA is particularly useful for molecular studies because of its repetitive sequence and the presence of variable regions flanked by more conserved regions [12]. ITS-1 and ITS-2 of nuclear ribosomal DNA (rDNA) which occur between the 18S, 5.8S, and 28S coding regions respectively, have been used for molecular identification purposes at the level of species [13-15]. Indeed, the ITS sequences mitochondrial cytochrome oxidase subunit I (COI) and *NADH* dehydrogenase subunit I (NDI) genes have also been used as a specific markers to characterize the liver flukes from different geographical regions [16, 17]. Several studies have characterized *Fasciola* using molecular genetic techniques [14, 18, 19]. In Egypt few of such molecular studies have been conducted for genotyping of *Fasciola* spp. isolated from different hosts [20, 21]. Therefore the objective of the present study was to perform molecular characterization of *Fasciola* isolates from human and sheep in Assiut, Upper Egypt by sequence and phylogenetic analyses of ITS-1 and ITS-2 of ribosomal DNA genes.

Material and Methods

Parasitic samples

Sheep *Fasciola* worms were recovered from the biliary tract of the liver of freshly slaughtered sheep at local abattoirs in Assiut Governorate of Upper Egypt. Fragments of human *Fasciola* samples were obtained from patients complaining of obstructive jaundice and underwent ERCP at Assiut University Hospital, Assiut, Egypt. Morphologically sheep *Fasciola* were identical to *F. hepatica*. The collected specimens were washed thoroughly in physiological saline and preserved by freezing prior to extraction of genomic DNA.

Extraction of genomic DNA

To avoid inclusion of female genitalia that are likely to include foreign sperms, genomic DNA was extracted and purified from a small portion of anterior or posterior margin of ethanol preserved flukes (approximately 50 mg was used) using QIAamp DNA Mini Tissue Extraction Kit (Qiagen, USA), according to manufacturer's instructions. DNA concentration (ng/µl) was estimated using a NanoDropTM 1000 (V3.5) Spectrophotometer at 260 nm (NanoDrop Technologies Inc, USA). All extracted genomic

DNA were diluted to a working concentration of 50 ng/µl and stored at -20°C until used.

DNA amplification and sequencing

Amplification of the ITS-1 and ITS-2 regions of rDNA were carried out using the universal trematode primers following Bowles *et al.* [22]. The following primer pairs were used for ITS-1 gene, (BD1-F) 5′-GTCGTAACAAGGTTTCCGT-A-3′ as a forward primer, and (4S-R) 5′-TCTAGATGCGTTCGAATGTCGA TG-3′ as a reverse primer. The pair of primers for *ITS-2* gene was, (3S-F) 5′-GGTACCGGTGGATCACTCGG-CTCGTG-3′ as a forward primer, and (A28-R) 5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′ as a reverse primer.

The PCR amplification was performed following the standard protocol[23] with minor modifications. Briefly 12.5 µl PCR Master Mix "Promega", 1 µl forward primer (conc. 10 p/mol.), 1 µl reverse primer (conc.10 p/mol) and 50 ng of genomic DNA were added and completed to 25µl reaction volume with nuclease free water. The DNA was preheated at 94°C for 5 min and added to each PCR reaction. The PCR reaction was amplified in My cycler TM Thermal Cycler (Bio-Rad, USA) according to the following conditions; 35 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 1 min. followed by a final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gels, and visualized by ethidium bromide using UV trans-illuminator with GELSMART 7.0 software (Clara Vision). The size of each band was determined by a 100-bp plus ladder molecular weight marker.

ITS-1 and ITS-2 Sequencing and phylogenic tree (data) analysis

For DNA sequencing the PCR products were purified using Qiagen QIAquick PCR Purification kit according to manufacturer's recommendation. The purified products of ITS-1 and ITS-2 were directly sequenced utilizing the same PCR forward primers using an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer) using the BigDye® Terminator v3.1 Sequencing kit (Applied Bio-systems) following manufacturer instructions. The sequences were visually inspected. The identities of the sequences were confirmed using Blast function from NCBI, applying the default parameter provided by the online tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE= Blast Search & LINK_LOC=blasthome). Fourteen additional sequences of *F. hepatica* and *F. gigantica* from different geographical regions were retrieved from GenBank for the purpose of sequence alignment and phylogenetic tree construction. Clustal W multiple alignment tool from EBI (http://ebi.ac.uk/Tools /msa/clustalw2) and MEGA6 [24] were used for sequence alignment and phylogenic tree construction respectively. All characters were run unordered and equally weighted. Alignments gaps were treated as missing data. Bootstrap analyses were conducted using 1,000 replicates. The phylogenic trees were conducted by comparing the current *F. hepatica* ITS-1 and ITS-2 with those previously published in GenBank, *Fascioloides magna* designated as outgroup. New sequences were deposited in GenBank with accession numbers KP215281 and KJ818275 for ITS-1 and KP21582 and KJ818276 for ITS-2 of human and sheep *F. hepatica* respectively.

Ethical consideration: The study protocol was approved by the Research and Ethic Committee of Faculty of Medicine, Assiut University. an informed signed consent was taken from all the patients before performing ERCP (Endscopic Retrograde Cholangio-Pancreatography) and for using their samples in the study .

Results

The PCR amplification of ITS-1 using BD1 (forward) and 4S (reverse) primers yielded a fragment of 400 and 600 bp (Fig. 1) while

amplification of ITS-2 using 3S and A28 primers yielded a fragment of 431 and 650 bp (Fig. 2) for human and sheep *Fasciola* samples respectively. The identity of the sequences was confirmed. The sheep retrieved ITS-1 sequence in the current study represented the whole sequence of ITS-1, 3' end of 18S and the 5' end of 5.8S rDNA regions and that of ITS-2 represented the whole sequence of ITS-2, the 3' and the 5' ends of 5.8S and 28S rDNA regions, respectively. Alignment of obtained sequences revealed that the pattern of sheep and human fluke samples belonged to *F. hepatica* type.

The examined ITS-1 and ITS-2 sequences of *F. hepatica* samples showed 99-100% similarity to sequences ITS-1 and ITS-2 of *Fasciola* isolates from different geographical regions and different hosts. Both multiple sequence alignment and phylogenic analysis based on ITS-1 sequences of the examined *F. hepatica* samples and isolates from other geographical regions showed that there is 99-100% similarity between the examined *F. hepatica* ITS-1 and those retrieved from GenBank, while the examined *F. hepatica* ITS-1 sequences was differed from *F. gigantica* ITS-1 in 5 variable nucleotides as shown in (Table 1). However the *ITS-2* sequence of the examined *F. hepatica* samples showed two base differences from *F. hepatica ITS-2* from France at positions of 466 and at 467; while it was differed from F. *gigantica ITS-2* in 4-5 nucleotides (Table 2).

The phylogenic trees were conducted by comparing the current *F. hepatica ITS-1* and *ITS-2* with those previously published in GenBank, *Fascioloides magna* designated as outgroup. Phylogenic analysis using Neighbor-Joining method based on ITS-1 and ITS-2 regions showed the close relation between the examined *F. hepatica* sequences and the other isolates (Fig. 3 and 4 respectively).

Table 1: Estimates of Evolutionary Divergence between Sequences of examined F. hepatica ITS-1 and other isolates of *F. hepatica* and F. gigantica from different geographical regions.

	***Fasciola* isolates**	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	***F.hepatica*.ITS1.human.Assiut**														
2	***F.hepatica*.ITS1.sheep.Assiut**	0.0													
3	***F.hepatica*(DQ385828.1)sheep.Egypt**	0.0	0.0												
4	***F.hepatica*(AB514848.1)Bos.Taurus.Uruguay**	0.0	0.0	0.0											
5	***F.hepatica*(AB477354.1)Bos.Taurus.China**	0.0	0.0	0.0	0.0										
6	***F.hepatica*(GQ231547.1)Tunisia**	0.0	0.0	0.0	0.0	0.0									
7	***F.hepatica*(AM709643.1)Spain**	0.0	0.0	0.0	0.0	0.0	0.0								
8	***F.hepatica*(KF866248.1)sheep.Iran**	0.0	0.0	0.0	0.0	0.0	0.0	0.0							
9	***F.hepatica*.ITS1.sheep.Italy**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0						
10	***Fasciola.sp*(AB536911.1)cattleViet_Nam**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0					
11	***F.gigantica*(JF295000.1)Bos_Taurus.Egypt**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	0.0				
12	***F.gigantica*(KF866250.1)cattle.Iran**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	0.0	0.0			
13	***F.gigantica*(JF295000.1)Cameron**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	0.0	0.0	0.0		
14	***Fascioloides.magna*(EF534991.1)USA**	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	25.0	25.0	25.0	25.0	

Table 2: Estimates of Evolutionary Divergence between Sequences of examined F. hepatica ITS-2 and other isolates of *F. hepatica* and *F. gigantica* from different geographical regions.

	***Fasciola* isolates**	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	***F.hepatica*.ITS1.human.Assiut**														
2	***F.hepatica*.ITS1.sheep.Assiut**	0.0													
3	***F.hepatica*(DQ385828.1)sheep.Egypt**	0.0	0.0												
4	***F.hepatica*(AB514848.1)Bos.Taurus.Uruguay**	0.0	0.0	0.0											
5	***F.hepatica*(AB477354.1)Bos.Taurus.China**	0.0	0.0	0.0	0.0										
6	***F.hepatica*(GQ231547.1)Tunisia**	0.0	0.0	0.0	0.0	0.0									
7	***F.hepatica*(AM709643.1)Spain**	0.0	0.0	0.0	0.0	0.0	0.0								
8	***F.hepatica*(KF866248.1)sheep.Iran**	0.0	0.0	0.0	0.0	0.0	0.0	0.0							
9	***F.hepatica*.ITS1.sheep.Italy**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0						
10	***Fasciola.sp*(AB536911.1)cattleViet_Nam**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0					
11	***F.gigantica*(JF295000.1)Bos_Taurus.Egypt**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	0.0				
12	***F.gigantica*(KF866250.1)cattle.Iran**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	0.0	0.0			
13	***F.gigantica*(JF295000.1)Cameron**	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	0.0	0.0	0.0		
14	***Fascioloides.magna*(EF534991.1)USA**	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	25.0	25.0	25.0	25.0	

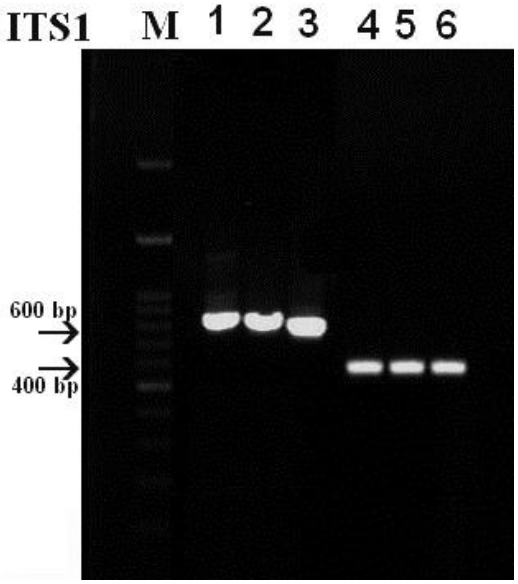

Figure 1: Agarose gel electrophoresis of amplified *ITS-1* ribosomal region. Lanes 1-3 denote sheep and lanes 4-6 denote human fluke samples amplified as a single band of 600 bp and 400 bp; respectively. M is 100 bp, molecular weight marker.

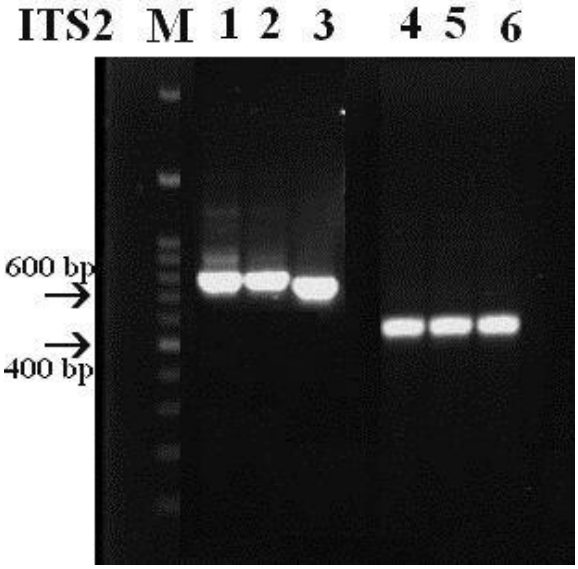

Figure 2: Agarose gel electrophoresis of amplified *ITS-2* ribosomal region. Lanes 1-3 denote human and lanes 4-6 denote sheep fluke samples amplified as a single band of 431 bp and 650 bp; respectively. M is 100 bp molecular weight marker.

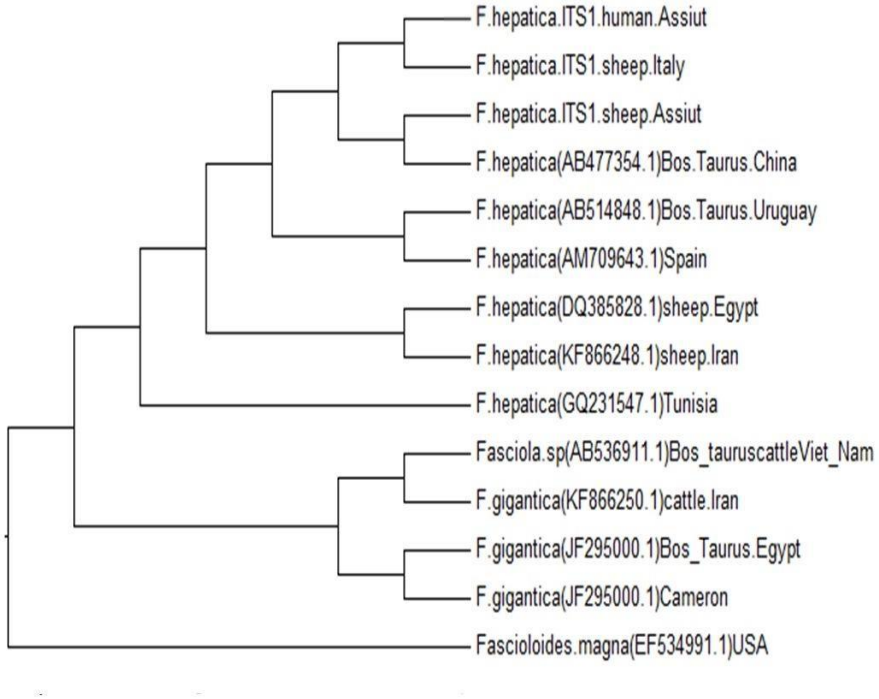

Figure 3: Neighbor-Joining phylogenic tree, the bootstrap test (1000 replicates), based on *ITS-1* gene of *F. hepatica*, *Fascioloides magna* (EF534991.1) designated as outgroup. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 338 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

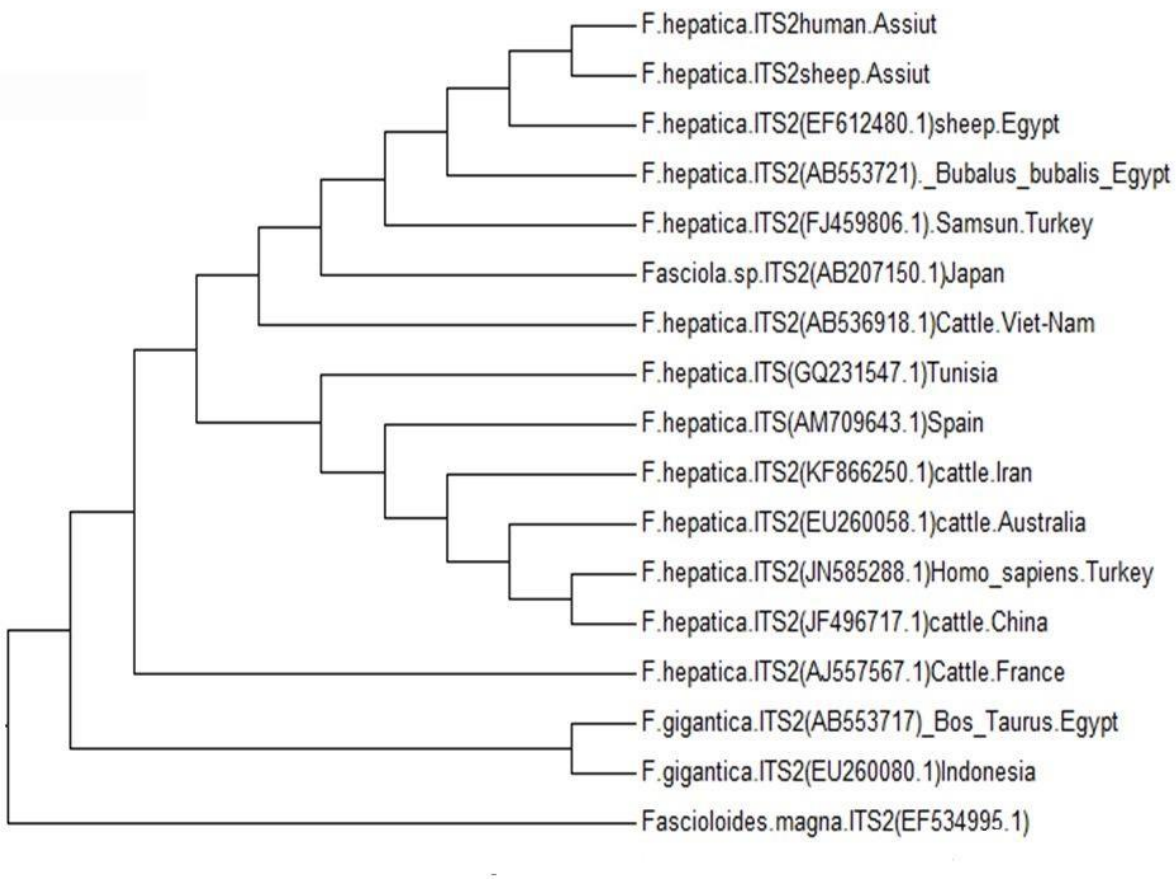

Figure 4: Neighbor-Joining phylogenic tree based on *ITS-2* gene of *F. hepatica*, the bootstrap test (1000 replicates), *Fascioloides magna* (EF534991.1) designated as outgroup. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 282 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Discussion

Investigating the genetic variability within and between populations of *Fasciola* has several implications for epidemiology, control and diagnosis of fasciolosis [25]. In the present study, adult specimens of *F. hepatica* from sheep and fragments of *Fasciola* specimens from human patients were characterized by sequencing of the ITS regions. Sequencing of ITS-1 and ITS-2 regions of the worms recovered both specimens revealed complete identity (100%) with *F. hepatica* reported from many different geographical localities. Analysis of the examined *F. hepatica* ITS-1was identical to previously published sequences from different localities such as Egypt, Uruguay, Tunisia, China, Iran, Spain and Italy [25-30]. This finding is in concordant with results of previous studies in which analyses of the parasites' ITS-1 genes have confirmed that *F. hepatica* from South America was identical to *F. hepatica* from Western Europe [31]. Farjallah *et al.* [32] reported that there was no nucleotide variation in the ITS-1 and 5.8S rDNA among 65 *F. hepatica* samples from Tunisia and Algeria, indicating no impact of the locality on the sequence of the *ITS-1* region. Also, no sequence variations were identified in ITS-1 region that could be attributed to variation in the host. Rokni *et al.* [25] reported that *F. hepatica* samples collected from sheep, cattle, buffaloes and goat in Iran from two geographical regions showed exactly 100% similarity with the available *ITS-*

sequence data of *F. hepatica* in GenBank. The same was noted by Shalaby *et al.* [33] who reported a uniform sequence pattern with no sequence variation detected in *F. hepatica* ITS-1 sequences in Saudi Arabia. This indicates highly conserved nature of rDNA internal transcribed spacers in *F. hepatica* (12). This showed the ITS-1 as a valuable tool for fluke identification in Assiut locality.

ITS-1 of *F. hepatica* sequences of the present study differed from that of *F. gigantica*; in five polymorphic nucleotide sites. This was the same noted by Amor *et al.* [29] and by Farjallah *et al.* [30]. The present study demonstrated that there was no nucleotide variation detected when the examined *F. hepatica* ITS-2 compared with those previously published from Egypt, Turkey, Japan, Vietnam, Tunisia, Spain and Iran. Meanwhile there was 2 nucleotide differences between the examined *F. hepatica* ITS-2 and the isolate recovered from France. The sequences of the ITS rDNA reported in the present study match with the most frequent allele (FhITS-H1) of *F. hepatica*. In fact, the most frequent ITS-2 allele (FhITS-2-H1) showed a widespread distribution, indicating that this is the main allele involved in the spread of *F. hepatica* from different localities worldwide including Egypt [20]. The second most frequent ITS-2 allele of *F. hepatica* (FhITS-2-2) differed by a transition in position 282 of the alignment of the two species, but appeared to be less common, being reported from few parts of the world and not from Egypt [32]. These findings suggest that the above mentioned variants of *F. hepatica*, occurring in isolated countries, may have a common origin, and that they have spread recently throughout these countries because of movement of infected animals. Moreover, it is interesting to note that the *F. hepatica* sequences from France and China include two additional transversions near the 3' end of ITS-2 gene, inverted relatively to all other available sequences that are claimed by original authors as a sequencing error [16, 26]. Comparing the ITS-2 of *F. hepatica* sequences revealed in the present study with *F. gigantica* ITS-2 revealed four to five polymorphic nucleotide sites. These finding came in line with the results revealed by Amor *et al.* [29].

In conclusion; the genetic characterization of *F. hepatica* present in Egypt is useful to achieve the basic information necessary for the field control of this parasite and may have implications for the diagnosis and control of the disease. To better understand the genetic variability and population genetic structure of *F. hepatica* in Egypt and in other neighboring areas a wide range of isolates from different hosts and geographical localities and the use of more variable genetic markers are needed; in fact, studies applying rDNA and mtDNA markers are necessary to the understanding of a disease which causes important public health problems worldwide and that involves very heterogeneous epidemiological situations and transmission patterns. Such molecular epidemiology baseline will help in designing global control measures and local interventions.

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Disclosure of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

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