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Original paper

Genetic characterization and phylogenetic analysis of *Fasciola* species based on ITS2 gene sequence, with first molecular evidence of intermediate *Fasciola* from water buffaloes in Aswan, Egypt

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ABSTRACT. Fasciolosis is an important food and water-borne parasitic infection caused by the two trematode species, *Fasciola hepatica,* and *F. gigantica*. The present study aimed to identify the phenotypic features and genetic characterization of adult fasciolid that infecting buffaloes were studied in Aswan, Egypt. The genetic identity of *Fasciola* species was investigated by the analysis of forward and reverse sequences of the ITS-2 of the rDNA gene. The *Fasciola* isolates were obtained from sheep, buffaloes, and cows in the regions of Aswan. The sequence of ITS2 gene isolates obtained from the present investigation were compared with GenBank reference sequences of *F. hepatica*, *F. gigantica*, and intermediate *Fasciola*. The obtained results were based on morphometric and genetic data which revealed the existence of *F. gigantica, F. hepatica*, and an intermediate form of *Fasciola*. Several variable sites were encountered among the investigated isolates in the Aswan, that were compared with the *Fasciola* species acquiesced in Gene Bank. Furthermore, the relationships between Egyptian *Fasciola* and *Fasciola* spp. from various other nations were discussed in the study.

Keywords: *Fasciola* species, genetic identity, phylogenetic analysis, ITS2 gene

Introduction

Fasciolosis is a zoonotic parasitic infection infecting humans and livestock, which is caused by species of trematodes, *Fasciola hepatica* and *F. gigantica* that primarily affect the liver [1]. The disease is familiar mostly as a veterinary issue due to their distribution worldwide [2]. The disease-causing both worms are leaf-shaped, outsized to visible by a naked eye. The adult *F. hepatica* is normally measured to be 20–30 mm×13 mm, whereas adult *F. gigantica* is measured to be 25–75 mm×12 mm [3]. This parasitic disease is widely distributed as longitudinal, latitudinal, and

altitudinal and infected up to 17 million people.

Recently, World Health Organization has incorporated fasciolosis as a significant disease in the human among neglected infections, with clinical cases largely in various states of Europe, Africa, the Americas, Oceania, and Asia [1–3]. The climatic conditions appear to have aggravated the clinical conditions by intensifying the geographic distribution of such helminthic parasites [4]. Therefore, fasciolosis is recognized as a zoonoitic disease of primary global and regional significance. Studies have demonstrated that the geographic dissemination of *F. hepatica* and *F. gigantica* differ based on the availability of their in-between snail hosts [1,3,5,6]. *F. hepatica* is largely distributed globally and however *F. gigantica* is largely present in tropic and sub-tropical regions of Asia and Africa [7].

Studies showed that some of the *Lymnaea* species are suitable as the intermediate hosts for digenetic trematodes *F. gigantica* and *F. hepatica* [7–10]. In several African and Asian countries showed overlapping the distribution of these species [1,9]. In Egypt, *F. gigantica* is widespread in ruminants along the Nile Valley [6,7]. *F. hepatica* is reported in imported sheep and cattle [8,9,11] whereas; both digenetic trematodes *F. hepatica* and *F. gigantica* co-exist with native livestock animals in Egypt [5,6,12].

Animal fasciolosis is relatively more common in livestock in most regions of the nations including Egypt and their occurrence ranges up to 60% in some states [7,13]. In Egypt, the occurrence of fasciolosis is endemic causing epidemiological and medical health issues [13]. The understanding of the epidemiology, causes, factors influencing the occurrence rate are highly required to provide states of arts in which effective prevention and control measures can be generated [14].

Both species of *Fasciola* have been generally categorized according to their morphology, including the length and width of the body. However, due to the dissimilarities in the size and morphological features, there has been an intermediate form, based on their diverse characters [3]. The intermediate forms generally have parthenogenesis, aberrant gametogenesis, mixoploidy, diploidy, and triploidy and the trials of hybridization among various genotypes [1,4,15]. However, multidisciplinary investigations have strongly that *F. hepatica* and *F. gigantica* can be measured effective species, although their ability to crossbreed and provide 'intermediate forms' in such overlapping regions [4].

Recently, this overlying dissemination of either species has developed a long debate based on their taxonomic identification of *Fasciola* species locating in far East Nations, particularly China, Hong Kong, Japan, Korea, Combodia, Taiwan, Myanmar, and the Philippines. In these countries, a varied assortment of morphological types was documented [16,17]**.** Various studies demonstrated that both *F. hepatica* and *F. gigantica* or their hybrid forms have been reported in various nations [12,18–23]. in Egypt, It is not precisely known whether *Fasciola* spp. belongs to a single or several species or it may be a hybrid of the two species, Lotfy et al. [12] used morphological, morpho-anatomical, and morphometric analysis to identify of *Fasciola* species. Accurate identification of *Fasciola* remains challenging because of their morphological variations [5,24].

Molecular studies have also confirmed that both species can be aptly differentiated by mitochondrial DNA sequencing of NDI and COI or nuclear ribosomal of internal transcribed spacers (ITS) 1 and 2 [25–28]. Studies in Thailand have also confirmed that *Fasciola* intermediate forms occurred in the livestock liver that has been recognized as hybrids according to the sequences of ITS1 and ITS2 [29,30]. For molecular and genetic characterization of *F. gigantica*, *F. hepatica*, and *Fasciola* intermediate forms, ITS1, and ITS2 have been renowned and more efficient genetic markers [29].

Moreover, in Egypt, researchers found a third species hybrid form that has a morphometric character owing between both species [31]. The modern techniques of PCR and DNA sequencing ease the species identification, strain clarification, and inherent populations [32–34]. The selected gene or sequence must be ordinary, exceptionally moderated inside, and adequately disparate between taxa. In a perfect world, the variable areas ought to have neighboring preserved areas so that „global" oligonucleotide primers may be chosen [17]. Therefore, this study aimed to determine the sequence analysis of ITS2 of rDNA and a highly repetitive DNA of the- genotype analysis of *Fasciola* population obtained from the hosts of buffalo, cattle, and sheep.

Materials and Methods

Study area

The current study was performed in different

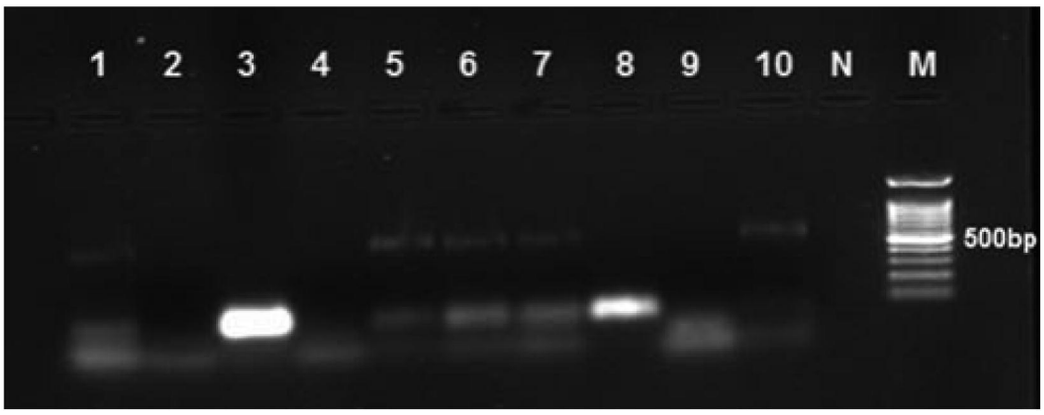

Figure 1. Illustrated the ITS2 fragments of PCR RFLP Profiles that identified three groups of *Fasciola* spp. Agarose gel electrophoresis of ITS2 of respective *Fasciola* isolates obtained from the Aswan governorate. Lane M: 100bp DNA ladder, Lanes N: Negative control, Lanes 1, 5, 6, 7, 10: *F. hepatica*, Lanes 2, 4, 9: F. intermediate form, Lanes 3, 8: *F. gigantica*

slaughtered houses of the Aswan region in the south of Egypt. This study was approved by the Department of Parasitology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.

Collection of the parasites

The *Fasciola* species were isolated from the individual animal of buffalo, cattle, and sheep that were achieved during the regular post mortem inspection of the liver and gall bladder. The obtained worms were washed separately using saline water (0.9%) and repeated washing at least thrice to eliminate the debris, maintained in ethanol (70%), and kept at – 80°C for the extraction of genomic DNA.

Morphometric analysis

The morphometric analysis was acheived based on the phenotypic distinction among *F. hepatica*, *F. gigantica* or *Fasciola* sp. The phenotypic comparison of adult *Fasciola* flukes were obtained from infected buffaloes. The width and breath of adult worms were measured based on earlier designated methods [9]. The populations of Fasciolid have been categorized based on the minimum and maximum scores of morphological difference were previously established [28].

Extraction of genomic DNA and PCR

The genomic DNA extraction was achieved from individual adult worms using (Easy –DNATM) Kit, following the manufacturer's instructions. The ITS2 fragment using a set of forward and reverse primers as follows [22,36]:

3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3'

A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'.

The digested DNA isolates were analyzed by gel electrophoresis [9].

Analysis of gene sequencing and phylogenetic

The products of ITS2 of isolates sequenced using the same primers, which were found in the PCR. Alignment of Sequence data and compared with those of the current sequences connected to *Fasciola* spp. accessible in the GenBank, Phytogenic analyses performed on ITS2 sequence data were carried out by Neighbour-joining using MEGA7 and were achieved by BLAST algorithms and National Center for Biotechnology databases.

Sequence results were analyzed by MEGA10 software. The sequences were compared with related sequences by the BLAST program. Phylogenetic analysis predicated on the IST2 sequence data was conducted using the method of Neighbor-joining (NJ) based on Tamura's model using MEGA7. Bootstrap analysis was carried out with 1500 replications.

Results

Fasciolid populations from Egypt have been categorized based on the maximum and minimum scores of variable measurement of morphology. These findings support the *Fasciola* intermediate forms that occur in Aswan, Egypt. However, it is value stating that the trials from Egypt overlay with standard populations of *F. hepatica* and *F. gigantica.* These results were confirmed by conducting sequences for ITS2 genes, which confirmed the

Table 1. *Fasciola* species morphometry recovered from water buffaloes (n=6 for each species)

Parameters (mm)	*Fasciola hepatica*	*Fasciola gigantica*	Intermediate *Fasciola*
Body length (BL)	17.95–27.5 (23.8)	35.80–53 (39.7)	23.60–40 (31.6)
Body width (BW)	8.40–13.2 (10.6)	7.1–11.9 (9.9)	7.4–12.2 (10.3)
Cone length (CL)	1.34–2.75 (2.1)	2.57–3.48 (3.1)	1.85–3.2 (2.8)
Cone width (CW)	2.00–3.07 (2.60)	3.1–4.2 (3.8)	2.70–3.8 (3.3)
Oral sucker diameter	0.81–1.18 (0.98)	0.96–1.15 (1.11)	86–1.12 (1.01)
Ventral sucker diameter	1.22–1.82 (1.42)	1.78–2.01 (1.95)	1.55–1.92 (1.82)
Pharynx length (PhL)	0.47–1.15 (0.86)	0.66–1.1 (0.91)	0.65–1.2 (0.90)
Pharynx width (PhW)	0.34–0.71 (0.46)	0.39–1.19 (0.56)	0.35–0.81 (0.51)
Distance between suckers (OS-VS)	1.09–2.37 (1.8)	1.39–2.31 (1.82)	1.01–2.2 (1.7)

morphometric outcomes (Table 1, Figure S1, and S2). Based on RFLP fragment patterns, from 10 *Fasciola* isolates, five isolates were corresponding to *F. hepatica*, and three isolates were related to *Fasciola* intermediate form. In comparison, two isolates were complementary to *F. gigantica*.

The nucleotide sequences for the three haplotypes of ITS2 isolates were kept in the GenBank using the consent numbers of *F. hepatica* (MT025519), *F. gigantica* (MT025356), and *Fasciola* intermediate (MT025436) (Table 2). Comparison with other sequences from GenBank was shown the three groups of *Fasciola* isolates belonged to *F. gigantica* (AB553718.1, AB553719.1) and *F. hepatica* (Japan, Austria, and Egypt with Accession number LC056929.1, LC056930.1 Japan, AB207148.1 DQ683546.1 Austria, and AB510492.1 Egypt). Whereas the present sequence hybrid between *F. hepatica* and *F. gigantica* were called *Fasciola* intermediate form, also it was found closely related to *Fasciola* sequences from GenBank with Accession number (AB536918.1 Egypt, AB553737.1 Egypt, AB553738.1 Egypt, KF543341.1 China) under *Fasciola* species. The final aligned sequences of 486 base pairs included 06 variable positions, and 06 were singletons. The pairwise distances between three groups of *Fasciola* spp. from different livestock animals were low, ranging from 0.004 to 0.01 with an overall mean of 0.008 (Table 3).

The analysis of Neighbour-Joining (NJ) was performed according to the Jukes-Cantor parameter with 1000 replicates (Figure 2). Based on the analysis, two primary clusters were yielded. The initial cluster comprised of *F. gigantica* from various animals, the present sequence was closed relation with the other sequences from GenBank, with a high bootstrap confidence level of 83%. The next cluster had high support (84%), which was allocated into two core sub-clusters with the reasonable provision, without any apparent genetic connections to the host (GenBank MT025356.1), representing *F. gigantica* (from buffalo in this study) grouped in this cluster. Next monophyletic cluster comprised of two sub-clusters, which branched into terminal sub-clusters that the *F. hepatica* (MT025519.1) (from a cow in this study) was corresponding with the same species from GenBank (JF432075) but from different hosts, and the *F.* intermediate (MT025436.1) (from sheep and buffalo) was corresponding with the *F. hepatica* (KJ818276.1) and *Fasciola* spp. (AB536918.1) from GenBank. On the other hand, according to the Maximum Parsimony analysis, agree with the NJ tree revealing two major clusters. one cluster closely related to *F. gigantica* while the other made up of two sequences from different populations were near associated with *F. hepatica* and *Fasciola* spp. have an 80% support, higher than on the NJ tree as illustrated in Figure 3.

Table 2. Comparison of ITS-2 Genes of *Fasciola* species obtained from variable nations with the data associated in GenBank

Secies	Country	Identity %.	Variable sites																				Accession number.
			1,2,3	16	22,23,24	166	249	283	328	338	351	353	356	379	380	413	416	422	429	438	462	519	
F.g.	Aswan (buffalo)		TGA	A	TGA	A	C	A	T	C	T	A	A	G	G	T	A	A	T	T	G	C	MT025356
F.g.	Egypt	100	TGA	A	TGA	A	C	A	T	C	T	A	A	G	G	T	A	A	T	T	G	C	AB553718.1
F.g.	Egypt	100	TGA	A	TGA	A	C	A	T	C	T	A	A	G	G	T	A	A	Y	T	G	C	AB553719.1
F.g.	India	99	ATC	gap	ATG	A	C	A	T	C	T	A	A	G	G	T	A	A	C	T	C	C	KC535542.1
F.g.	India	99	TGA	A	TGA	A	G	A	C	C	T	A	A	G	G	T	A	A	T	T	G	T	KF667377.1
F.g.	India	99	TGA	A	TGA	A	C	A	C	A	T	A	A	G	G	T	A	A	T	T	G	T	KF667382.1
F.g.	India	99	TGA	A	TGA	A	C	A	C	C	gap	A	A	G	G	T	A	A	T	T	G	T	JF930346.1
F.g.	India	99	TGA	A	TGA	A	C	A	C	C	T	G	A	G	G	T	A	A	T	T	G	T	KF667380.1
F.g.	India	98	TGA	A	TGA	A	C	A	C	C	T	A	A	G	G	T	A	A	T	T	G	T	KF667381.1
F.g.	India	99	TGA	A	TGA	A	C	A	C	C	T	A	A	G	G	T	G	G	T	C	G	T	KF667378.1
F.g.	India	98	TGA	A	TGA	G	C	G	C	C	T	A	G	G	G	C	A	A	T	T	G	T	KF667379.1
F.g.	China	99	TGA	A	TGA	A	C	A	C	C	T	A	A	C	G	T	A	A	T	T	G	C	JF496714.1
F.g,	China	99	TGA	A	TGA	A	C	A	C	C	T	A	A	C	G	T	A	A	T	T	G	C	JF496711.1
F.g.	China	98	TGA	A	TGA	A	C	A	C	C	T	A	A	C	G	T	A	A	T	T	G	C	JF496715.1
F.g.	China	99	TGA	A	TGA	A	C	A	C	C	T	A	A	G	gap	T	A	A	T	T	G	C	JF496713.1
F.g.	China	99	TGA	A	TGA	A	C	A	C	C	T	A	A	G	G	T	A	A	T	T	G	C	KF543340.1
F.g.	China	99	TGA	A	TGA	A	C	A	C	C	T	A	A	G	G	T	A	A	T	T	G	C	JF496712.1
			1	3	315	387	384	436	443	495-496	498-506	50-8	511-512	514									
F.i.	Aswan(sheep)	100	C	A	T	C	C	T	G	TA	CCGCTGAAC	T	GC	T									MT025436
*F.*sp	Japan	99	A	gap	T	C	C	T	G	TA	CCGCTGAAC	T	GC	T									AB536918.1
*F.*sp	Japan	99	C	A	T	Y	Y	T	R	TA	CCGCTGAAC	T	GC	T									AB536917.1
*F.*sp.	Japan	99	C	A	Y	Y	Y	gap	R	TA	CCGCTGAAC	T	GC	T									AB536920.1
*F.*sp.	Egypt	99	C	A	T	Y	Y	T	R	TA	CCGCTGAAC	T	GC	T									AB553738.1
*F.*sp.	Egypt	99	C	A	T	Y	Y	T	G	TA	CCGCTGAAC	T	GC	T									AB553737.1
*F.*sp.	China	100	C	A	T	C	C	T	G	AC	TACAATTTA	A	AA	A									KF543341.1
			1-7	17	19	22	28-29	40	479	502	516-525	526-528	529	530-533									
F.h.	Aswan(cattle)	99	GTCGATGA	G	C	C	GA	A	G	T	CCGCTGAACT	TAA	C	CATA									MT025519
F.h.	Egypt	99	gaps	gap	gap	gap	TG	gap	T	T	CCGCTGAACT	TAA	G	CATA									AB510492.1
F.h.	Japan	99	gaps	gap	gap	gap	GA	A	G	T	CCGCTGAACT	TAA	G	CATA									AB207148.1
F.h.	Ireland	99	gaps	C	A	gap	GA	A	G	C	CCGCTGAACT	TAA	G	CATA									AB973395.1
F.h.	Egypt	99	CGTCGATG	G	C	C	GA	A	G	T	CCGCTGAACT	TAA	G	CATA									KJ818276.1
F.h.	Austria	99	GTCGATGA	G	C	C	GA	A	G	T	CCGCTGAACT	gaps	gap	gaps									DQ683546.1
F.h.	Japan	99	GTCGATGA	G	C	C	GA	A	G	T	CCGCTGAACT	TAA	G	CATA									LC056929.1
F.h.	Japan	99	GTCGATGA	G	C	C	GA	A	G	T	CCGCTGAACT	TAA	G	CATA									LC056930.1
F.h	Iran	99	GTCGATGA	G	C	C	GA	A	G	T	gaps	gaps	gap	gaps									JF430072.1

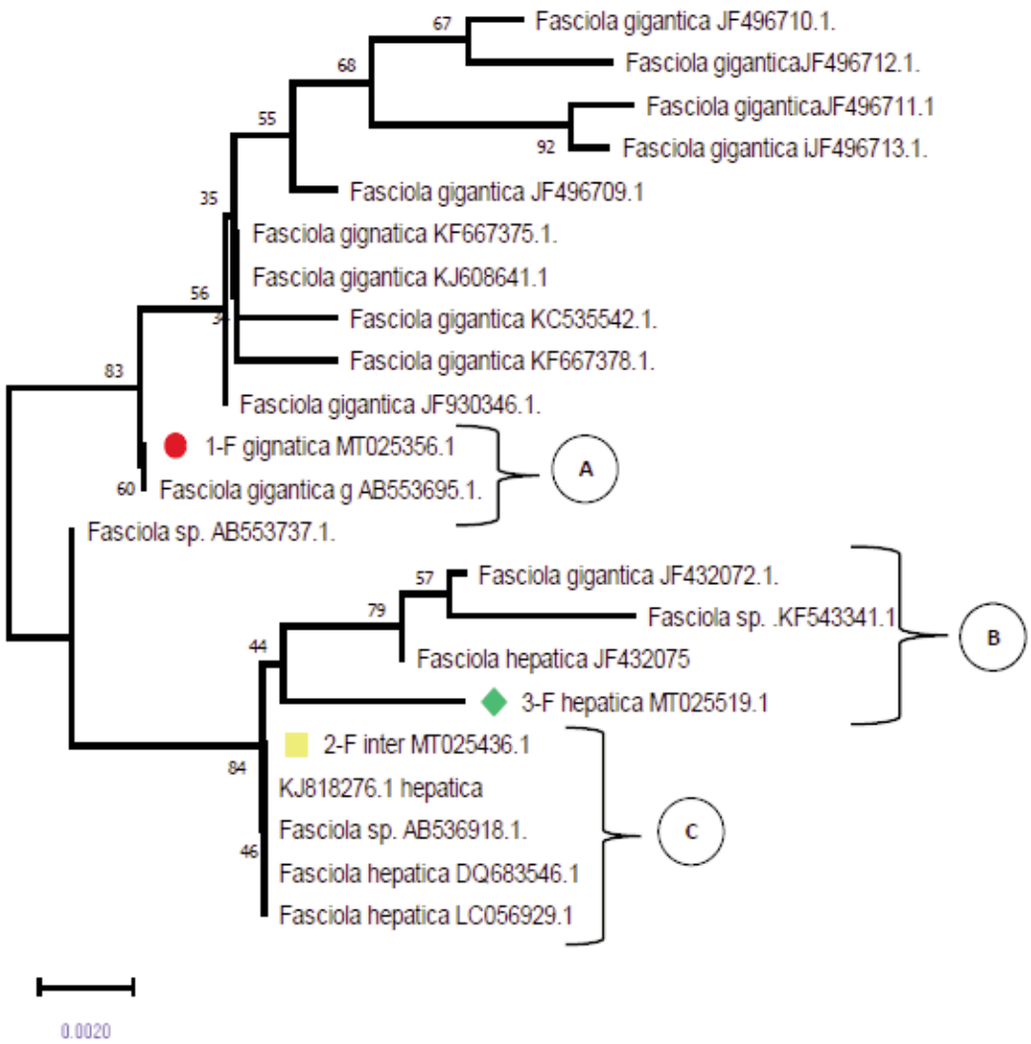

Figure 2. Neighbour-joining phylogenetic tree of *F. species* haplotypes from various animals ITS2 gene with 1000 bootstrap replications. A. *Fasciola hepatica*, B. *Fasciola gigantica*, C. *Fasciola* intermediate form.

Discussion

The current pilot investigation on fasciolids infecting buffaloes from Aswan, Egypt shows the occurrence of *Fasciola* intermediate forms (Table 1, Figures 3 and 4). Similarly, earlier investigations demonstrated the occurrence of intermediate forms of morphology [6]. Various studies showed that genotype analysis and molecular phylogeny with the ITS2 gene could be appropriate tools for differentiation of *Fasciola* species as well as origin and causes of the diseases [1,9,20,35]. However, according to Bowles et al. [36], the whole ITS2 gene gives a length of 560 pb and 563 pb. In this existing investigation, the occurrence of three different patterns of RFLP was performed by using agarose gel electrophoresis. The analysis of RFLP was established by sequence analysis of the respective sample, where it was confirmed that there were three species of *Fasciola* in Aswan governorate.

Molecular characterization of the *Fasciola* species were detected based on partial sequences of

Table 3. Mean pairwise genetic distance between three groups of *Fasciola* spp.

	F. gigantica	*Fasciola* intermediate sp.	*F. hepatica*
F. gigantica	0.00		
F. (intermediate)	0.008		
F. hepatica	0.013	0.004	0.00

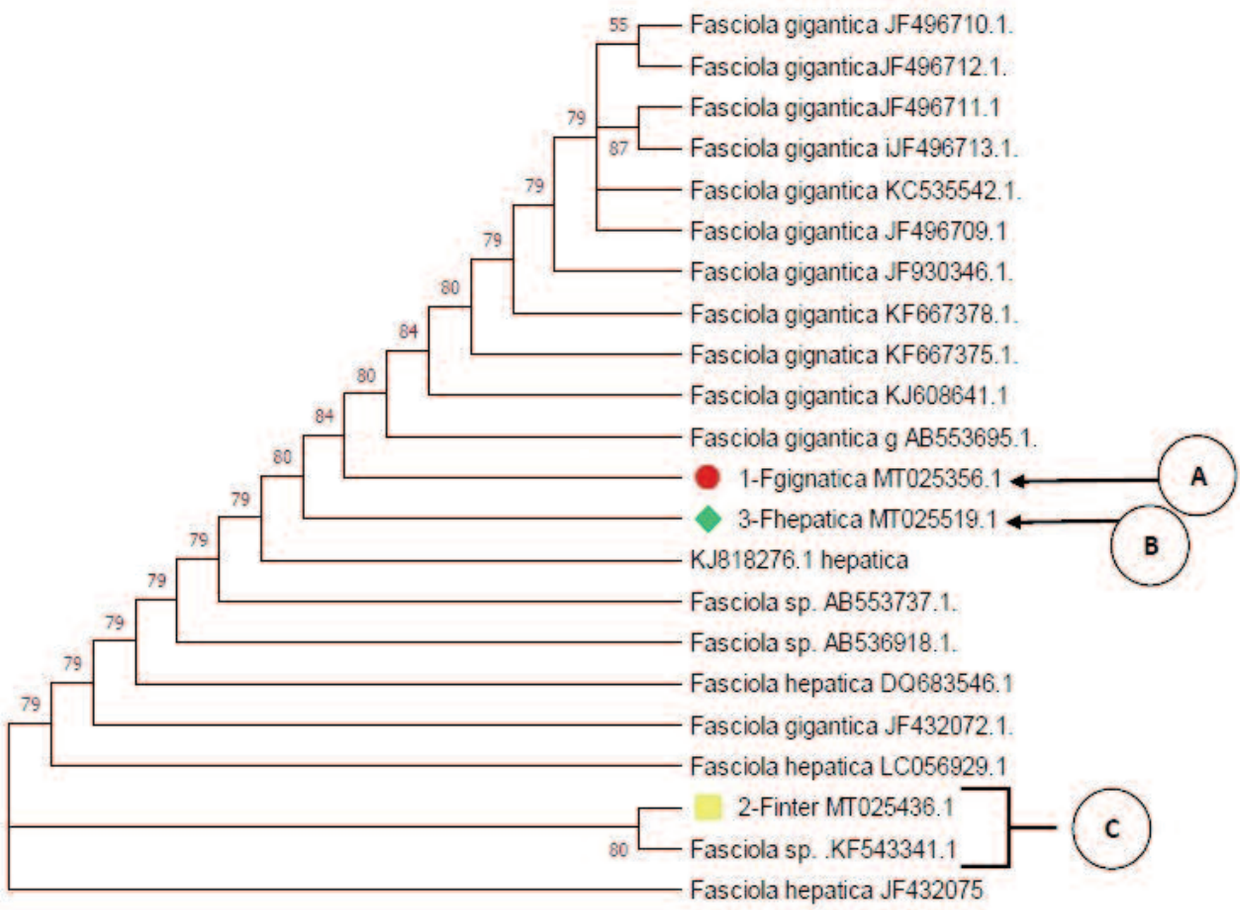

Figure 3. Maximum Parsimony phylogenetic tree of *F. species* haplotypes from various animals ITS2 gene with 1000 bootstrap replications. A. *Fasciola hepatica*, B. *Fasciola gigantica*, C. *Fasciola* intermediate form

ITS2 rDNA and the studies showed that the *Fasciola* spp. sequences from diverse hosts are almost matching to those of earlier available sequences. It has been shown that three *Fasciola* species are existing in Aswan, Egypt, while the hybrid form was recorded for the first time at south valley university. These outcomes meet the full agreement with those described by Amer et al. [28], who recorded the presence of the three *Fasciola* species in Egypt including the intermediate form of *Fasciola* sp. while the present result differs from that result recorded by Arafa et al. [37] Egypt, Cairo who reported pure *F. gigantica* (isolate from cow and buffalo). Whereas, *Fasciola* species isolated from sheep which had a different sequence variation in many sites from both *F. hepatica* and *F. gigantica*. However, El-Tahawy et al. [38] from Kalyobia, Egypt recorded that the *Fasciola* in Egypt concerning the application of PCR for differentiation of two species of *Fasciola* by using specific primers of *F. hepatica*, they recorded that eight samples had positive bands related to *F. hepatica* and 2 negative bands representing *F. gigantica*.

The outcomes of the existing work established that *F. hepatica* was more dominant in a cow in the Aswan governorate, in addition to an intermediate form of *Fasciola* found in sheep and buffalo. This result disagrees with the results of Amer et al. [28] who reported that *F. hepatica* was predominant in sheep compared to other hosts. Earlier studies recorded the presence of different *Fasciola* species in African nations based on the phylogenetic position and interspecific variation, using ITS2 sequences [20,21,39,40].

Several investigations have studied that these ITS2 gene sequence can give consistent genetic markers for the real identities of *Fasciola* spp. [1,3,6,10,18,20,21,26,41]. Moreover, this pilot investigation exposed profound genetic heterogeneity in various species of *Fasciola* at the inhabitants of Egypt. The outcome of PCR strongly suggested that hybridization might be a dynamic mechanism in liver flukes based on the diverse

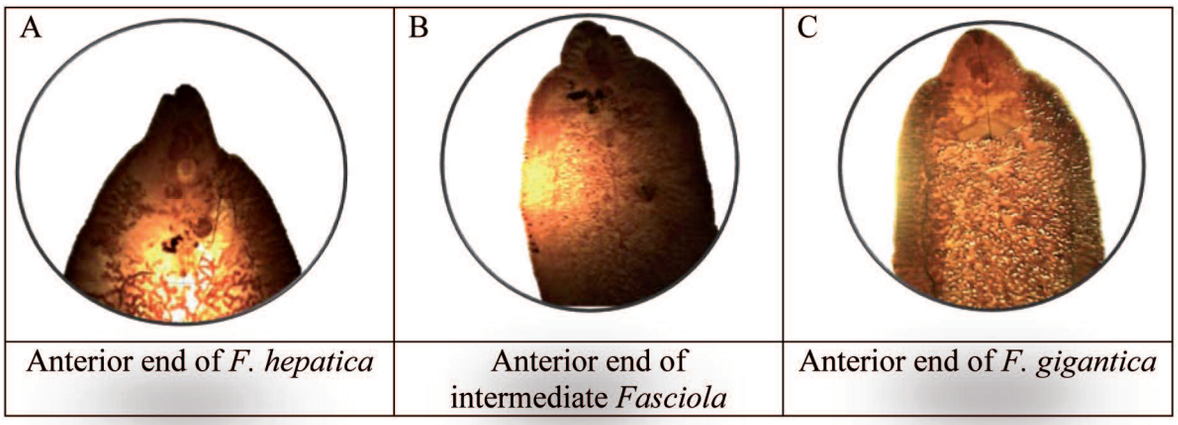

Figure S1. The anterior end of different *Fasciola* sp. shows the variances in the cone length and width

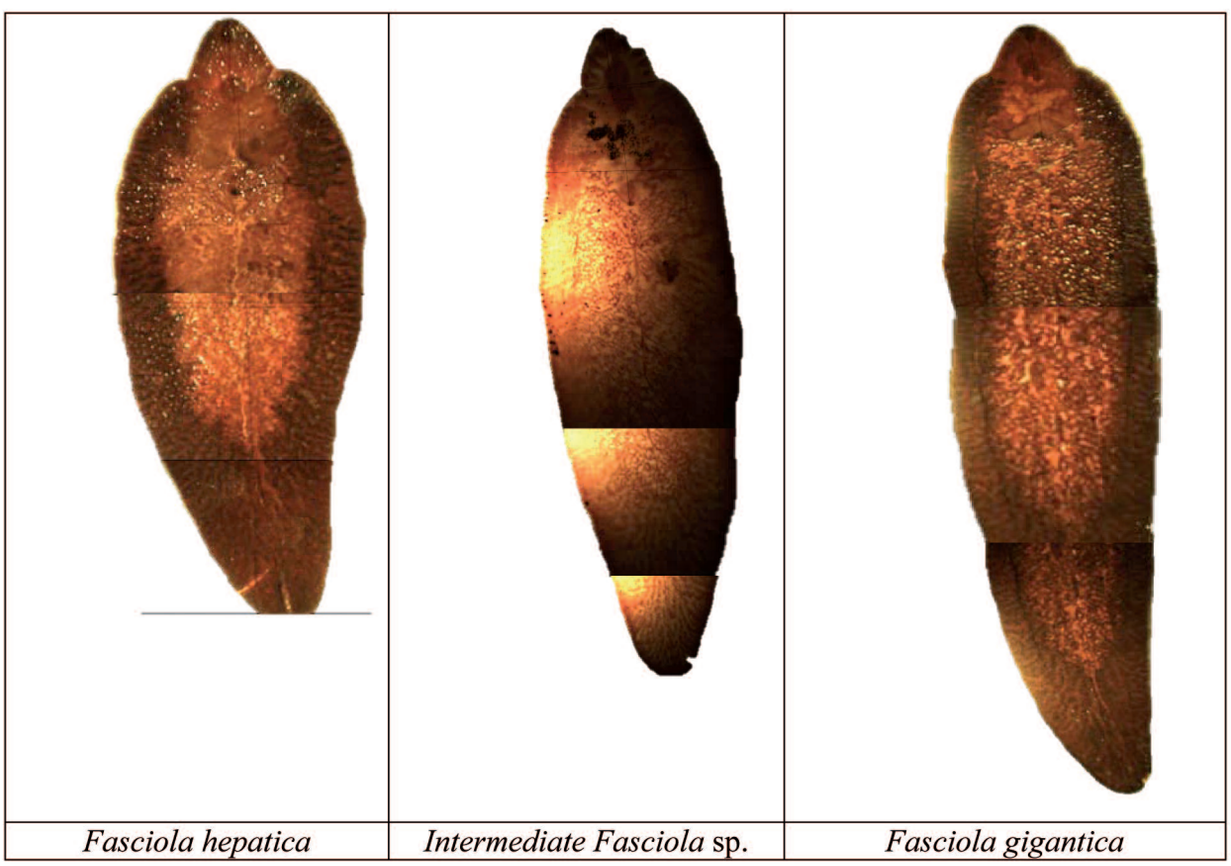

Figure S2. Adult worm of *Fasciola* sp. shows clear variations in body length, width

infections and the occurrence of cross-fertilization among *F. hepatica* and *F. gigantica*. It was found that buffalo host harboring *F. gigantica* only by using morphological analysis while through PCR technique; it was located that buffalo host harboring *F. gigantica* and *F. intermediate* form. In conclusion, the present PCR-based assays aided to show as a useful method of distinguishing *Fasciola* species; accordingly, one can confirm that PCR is a simple, rapid, and accurate tool for differentiation of the species of *Fasciola* as compared with those of morphological, pathological, or immunological techniques.

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