Advanced and classical diagnosis of *Fasciola spp.* in Egypt

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Abstract: Objective: The current study aimed to find out the morphometric and genotypic divergences of *Fasciola spp* isolated from different hosts in Egypt. **Methods:** Total number of 112 Adult flukes was collected; 13 from Cow, 71 from sheep and 28 from Buffalo at Al- Basateen slaughter house (Cairo, Egypt), in time period ranged from November 2013 until May 2014. Morphometric characteristics of flukes were measured by assessed Lineal biometric characters and Ratios. Genomic DNA was extracted from the flukes and polymerase chain reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) was used to characterize the isolates. The ITS1 from individual liver flukes were amplified and the amplicons were sequenced. A fragment approximately 680 bp in all of the *Fasciola* samples were amplified and then digested with the *Rsa*1 restriction endonuclease. **Result:** From the total of 112 isolates;68 (60.7%) were identified as *Fasciola gigantica*; Body length (BL)= 43.14 ± 6.54 mm, Body width(BW)= 8.77 ± 1.58 mm, Ratio between body length and body width (BL/BW)= 4.94 ± 0.18 mm and Distance between ventral sucker and posterior end (VS-P)=40.13 ± 6.2 mm, while 44 (39.3%)isolates were identified as *Fasciola hepatica*; BL= 20.53 ± 4.75mm, BW =10.78±2.49 mm, BL/BW =1.905 ± 0.06 mm and 17.71 ± 4.2 mm, depending on morphometric characteristics. Two types of patterns were found by genotyping using *Rsa*1; the first pattern composed of three bands of 360, 100 and 60 bp in size, whereas the second was 360, 170, and 60 bp in size for *F. hepatica* and *F. gigantic* respectively. The PCR products were followed by Internal transcribed spacer - 1Ribosomal Deoxyribonucleic acid (ITS-1 rDNA) selective sequencing provided an accurate identification of *Fasciola spp.* Alignment of the sequences of ITS1 showed six DNA variable sites in which nucleotides base in one type at the position of 48, 175, 265, 359, 437, and 457 were substituted by another type resulting in segregation of the specimens into two different groups (genotypes); *F. gigantica and F. hepatica*. **Conclusion**: This study has shown that simple, traditional microscopic measurements may be sufficient for the morphometric characterization of fasciolids but the new PCR-RFLP assay using *Rsa1*restriction enzyme provides a simple, practical, fast and more accurate method for identification and differentiation of *Fasciola* isolates.
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1. Introduction

Fasciolasis is a disease caused by digenean trematodes, commonly referred to as liver flukes. The two etiological agents of Fasciolasis are *F. hepatica* (**Linnaeus, 1758**) and *F. gigantica* (**Cobbold, 1855**; **Kimura *et al.*, 1984; Haseeb *et al.*, 2002; Ashrafi *et al.*, 2006).**

Animal Fasciolasis (AF) is clinical and epidemiological public health problems. According to the Egyptian Academy of Scientific Research and Technology Report, losses due to AF in Egypt, were estimated at 190 million Liver annually (**Lotfy and Hillyer, 2003; El-Shazly *et al.*, 2006)**. Fasciolasis causes significant economic loss, as valued by animal productivity, estimated at approximately 3.2 billion US dollars per annum to the global agricultural community with 600 million animals infected **(Mas-Coma, 2005; McManus and Dalton, 2006)**.

Human Fasciolasis (HF) has now been found in almost all governorates of the Delta region, in some governorates of Upper Egypt and in the reclaimed desert land. The population at risk in Egypt is considered to be 27 million (**Lotfy and Hillyer, 2003).** *F. hepatica* has the widest distribution compared to *F. gigantica*. *F. hepatica* is prevalent in almost all temperate regions where sheep and other ruminants are raised. It originated in the European continent and gradually migrated to other continents (**Boray, 1969**). *F. gigantica* is restricted mainly to tropical and subtropical areas such as Africa, South America, Southeast Asia, Southern Europe and Hawaii and also in the former USSR **(McCarthy and Moore 2000; Esteban *et al.*, 2003; Ashrafi *et al.*, 2004; Moghaddam *et al.*, 2004; Mas-coma 2005).** The two species of flukes show a wide distribution in African and Asian Continents and have common characteristics.

The two species have been traditionally classified based on their morphological features, such as body length and width. The adult mature and

gravid fluke is flat with its body shaped like a leaf. The size range is 25 to 30 mm and 8 to 15 mm in length and width respectively, depending upon the species (**Andrews and Dalton, 1999**). Fasciolids are identified primarily on differences in body shape and size of adults, with the smaller *F. hepatica* exhibiting wide and defined shoulders compared to the slender *F. gigantica* having less defined shoulders and shorter cephalic cones (**Kimura *et al*., 1984; Merck Veterinary Manual, 2008**).There are many variations in morphological characteristics, presence of intermediate forms, exact distinguishing of *Fasciola* species is usually difficult by simple traditional microscopic measurements and, therefore, the morphometric characterization may be insufficient for the species identification**(Valero *et al*., 2001 and Gherbawy *et al.,* 2013)**.

More recently studies on prevalence and species identification have been extensively conducted in different parts of the world using molecular methods. These recent molecular studies demonstrated that the two species can be properly distinguished by DNA sequencing of ITS1 and ITS2 and also mitochondrial genes of NDI and COI (**Peng*et al*., 2009; Tamura *et al*., 2011).** ITS-1 and ITS-2 sequences of flukes from Japan, Korea, China, Spain, India and Turkey, Egypt, were characterized to differentiate between *F. hepatica* and *F. gigantic* (**Semyenova *et al.,* 2005; Alasaad *et al.,* 2007; Erensoy*et al.,* 2009; Prasad *et al.,* 2009; Mohammad *et al.,* 2012**). Genomic DNA from the flukes and PCR-RFLP was used to characterize the isolates. Comparison of the ITS1 and ITS2 sequences showed six and seven singlebase substitutions, resulting in segregation of the specimens into two different genotypes. The sequences of COI markers showed seven DNA polymorphic sites for *F. hepatica* and 35 DNA polymorphic sites for *F. gigantic* **(Shafiei *et al*., 2014).**

2. Material and Methods

Study Area

Egypt is located in northern Africa. Its topography consists mainly of desert plateau but the eastern part is cut by the Nile River valley. Egypt's total area of 386,662 square miles (1,001,450 sq. km) makes it the 30th largest country in the world. The altitude of Egypt ranges from 133 m (436 ft.) below sea level in the Libyan Desert to 2,629 m (8,625 ft.) above in the Sinai Peninsula. South of Cairo, Egypt has a hot desert climate, the climate is generally dry. The temperatures are hot or very hot in summer days and warm or mild in winter days.

Sample collection

The adult/mature liver flukes used in this study were recovered from the examined hosts included the cow (*Bos Taurus*), the buffaloes (*Bubalus bubalis*), the sheep (*Ovis arises*), in Al- Basateen automated slaughterhouse in Cairo town, Egypt, from November 2013 until May 2014. The bile ducts were incised longitudinally through the gall bladder and the parasites were removed with the help of fine forceps, taking all necessary precautions to avoid any damage to the parasite. Each parasite was thoroughly washed individually 2 to 3 times in a 0.9% saline solution to remove debris and contamination. The samples were then kept in 70% ethanol and were carried to the laboratory where they were stored at 4ºC.

Morphometric Measurement

All morphological measurements of adults were made according to methods described for *Fasciola* by **(Valero *et al*., 1996, 2005 and Periago *et al.,* 2006, 2008).** The stained adult worms were examined under applied to a stereomicroscope (for adult studies) and dimensions of the body were assessed using a microscope and calibrated ocular micrometer (OSM-4, Olympus).

Staining of the flukes

The recovered flukes were thoroughly washed with 0.9% saline solution to remove debris and contamination. Staining was done according to protocol suggested by **(Bukhary, 1988),** with slight modifications in dehydration time which related to thickness of specimen flukes.

Statistical Analysis

We used Student's T-test to compare the mean of different variables between two *Fasciola spp*.(*F. hepatica* and *F. gigantica*) and one-way analysis of variance (ANOVA) was used to determine whether there are any significant differences between the means of morphometric values in flukes isolated from different hosts (Buffaloes, sheep and cow).

Genomic DNA isolation and Purification DNA extraction: Genomic DNA was isolated from adult worms using Thermo Scientific Gene JET Genomic DNA Purification Kit (Cat. No. #K0721) according to the manufacturer's protocol.

Polymerase Chain Reaction (PCR) Amplification of the ITS1: The DNA region the 1st (ITS-1) Internal Transcribed Spacers, gene of nuclear ribosomal DNA (rDNA) sequences was amplified by PCR using primers as **(Itagaki *et al*., 2005a)**.A standard PCR reaction was carried out on the thermo-cycler (7300 Real Time PCR System, Applied Bio-systems) at the following conditions, The reaction was done in a total volume of 25 µl containing of 12.5 µl Qiagen Multiplex, 1 µl of each primer (0.3 µM), 1 µl genomic DNA, and 9.5 µl H2O. Reaction cycles consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 90 sec, annealing at 53 °C for 30 sec and extension at 72 °C for 60 sec, followed by a final

extension step at 72 °C for 10 min. The electrophoresis of amplified DNA fragments was conducted in 1.5 % Agarose gel in 1xTBE-buffer and stained in ethidium bromide to verify that they represented single bands. These bands compared with the fragments of GeneRuler 100bp Plus DNA Ladder, Ready-to-Use 100 to 3000 bp (Thermo Scientific).

PCR-RFLP reaction was performed: PCR-RFLP method was used to specifically distinguish *F. hepatica* from *F. gigantica* in ITS1with *Rsa1* enzyme **(Ichikawa and Itagaki, 2010).**

DNA Purification: All purification steps carried out at room temperature by Thermo Scientific Gene JET PCR Purification Kit, according to the provided protocol. The purified products were run on 1.5% agarose gel electrophoresis to check for bands and only clear products were sent for sequencing to a service provider.

DNA Sequencing: For confirmation of RFLP reaction, ITS1 amplicons of representative samples of Egyptian *Fasciola* Sequencing to the PCR product made by(GATC Biotech Company, Germany) using ABI 3730xl DNA Sequencer. The purified PCR products were sequenced in both directions (forward and reverse) to resolve any potentially ambiguous sites.

Data analysis: The obtained sequences forward and reverse were assembled by CAP3 a DNA sequence assembly program online (**Huang and Madan, 1999**). The program uses forward–reverse constraints to correct assembly errors and link Contig. By using BioEdit program unresolved 'noisy' nucleotide sites at both ends of the sequence removed. Sequence alignments were conducted using the program Clustal W within MEGA v.6.0 **(Kumar *et al.,* 2013)** and showed by jalview v.2.8.1 **(Waterhouse *et al.,* 2009).** The phylogenetic analyses were conducted using MEGA v.6.0 **(Kumar *et al*., 2013).** Representative sequences were deposited in the GenBank under the accession numbers of KP099942, KP099943.

3. Results

Morphological identification of *Fasciola* spp.:

At present study, a total of 112 worms have been recovered from livers of several animal hosts (buffalo, sheep and cow). Out of this number, 68 (60.7%) were identified as *Fasciola gigantica* (*F. gigantica*) while44 (39.3%) were *Fasciola hepatica* (*F. hepatica*) using morphological characteristics (Table 1). The fresh liver fluke of Egyptian *F. hepatica* and *F. gigantica* exhibit a grayish buff to brown fleshy color (Fig. 1A, B). Both of them have flat and leaf-like bodies and the body has two lateral straight edges (Fig. 2). A distinct cephalic cone gives a characteristic shouldered appearance; while it is more distinct and relatively shorter in *F. hepatica* the cephalic cone is less prominent in *F. gigantica* as their body gradually tapers toward the anterior extremity (Fig.1A, B).

Morphometric criteria taken up in this study consisted of 12 different parameters, which are known to be suitable for the differentiation of both *Fasciola* species, based on lineal biometric characters, and ratios (Table1).Of these parameters, body length and body width are the most obvious ones. *F. gigantica* has morphology similar to *F. hepatica* but is much larger measuring 43.14±6.54 mm in length while in *F. hepatica* measuring 20.53±4.75 mm. the anterior conical structure is similar, but the widening of the body is not as distinct as *F. hepatica*. In *F. gigantica* width is 8.77±1.58 mm while in *F. hepatica* width is 10.78±2.94 mm.

Analysis of morphometric features, with ANOVA, demonstrated that the differences in parameters including body length and width, cone length and width, distance between the ventral sucker and the posterior end of the body, Oral sucker maximum, Ventral sucker minimum, ratio of Body Length to Body width, in both *F. gigantica* and *F. hepatica* from different hosts were statistically significant, $P< 0.05$ (Table 1).

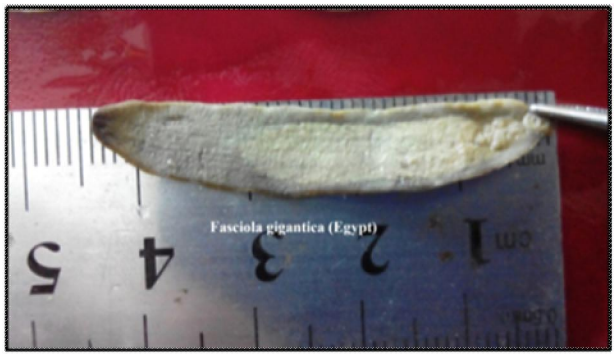
Molecular analysis

RT-PCR: In this study the gel picture PCR of the Egyptian species of 7 *Fasciola* (Fig. 3A, B) recovered from several hosts (sheep, Buffalo and cow) showed the same band pairs, suggesting no difference in the species of *Fasciola*. Hence this step has been followed by another one to assure species similarity and/or differences.

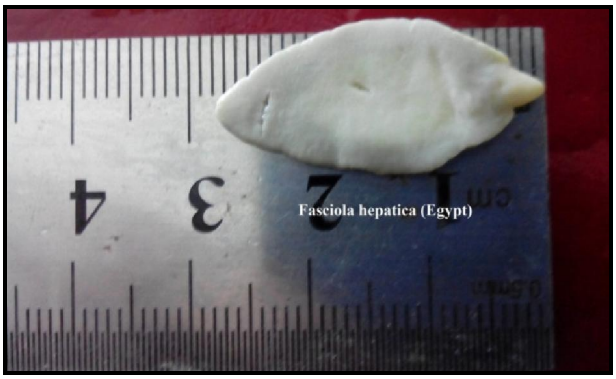
PCR-RFLP: The PCR products from the first step were subsequently subjected to digestion by *Rsa*1restriction enzyme. Electrophoresis of the digested products revealed two different bands patterns, regardless of their host origins. The first pattern composed of three bands of 360, 100, and60 bp in size in case of *F. hepatica*, whereas the second was 360, 170, and60 bp in size in *F. gigantica* (Fig. 3C).

Genotype analysis based on the ITS1 ribosomal DNA: Complete sequences of 680 bp ITS1of the two types of flukes were aligned with each other (Fig. 4). Alignment of the sequences ofITS1 showed six DNA variable sites in which nucleotides at the position of 48, 175, 265, 359, 437, and 457 were single-base substituted resulting in segregation of the specimens into two different groups (genotypes). We have found that the main differences between *F. gigantica* and *F. hepatica* were the single-base substitution of T<->C at nucleotide site of 48, C<->T at 175, 359, and 457, A<->T at 265, and T<->A at 437. Sequences of ITS1 of the two flukes, from this study, were deposited in

GenBank (accession numbers: KP099942 to *F. hepatica* and KP099943 to *F. gigantica*).



(A)



(B)

Fig 1: Dimensions of *Fasciola gigantica* (A) and *Fasciola hepatica* (B), Egypt

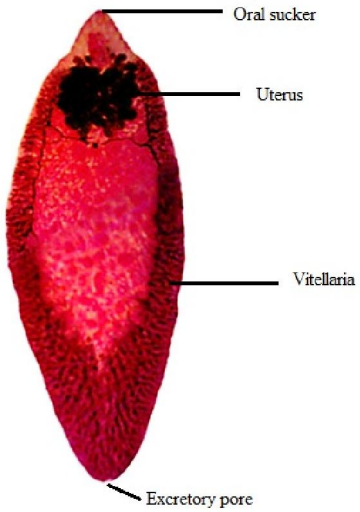


Fig 2: *Fasciola gigantica* stained with (H&E)

Phylogenetic analyses: The phylogenetic trees comparing the sequences of *F. gigantica, F. hepatica* and available ITS-1 sequences of other fasciolid species are shown in (Fig. 5) Phylogenetic tree of *F. gigantica* ITS-1 constructed by Bayesian methods Shows similarity of *F. gigantica* ITS1 (Egypt) with the *F. gigantica* ITS1 Species in Zambia, Indonesia and Thailand (Accession no.:AB207142, AB207143 and AB207144). This Phylogenetic tree shows also similarity of *F. hepatica*ITS1 (Egypt) with the *F. hepatica* ITS1 Species in Australia, Ireland and Uruguay (Accession no.: AB207140, AB207141 and AB207139).

Table 1: Morphometric variations between 112 liver flukes; *Fasciola hepatica* (44) and *Fasciola gigantica* (68) isolated from several animal hosts (Cairo, Egypt).

Parameter (mm)	***Fasciola spp.* (112)**	
	***Fasciola gigantica* (68)** Mean ± SD	***Fasciola hepatica*(44)** Mean ± SD
BL	43.14±6.54 *	20.53±4.75 *
BW	8.77±1.58 *	10.78±2.94 *
VS-P	40.13±6.2 *	17.71±4.2 *
BL/BW	4.94±0.18 *	1.905±0.06*
CL	3.16±0.435 *	1.96 ±0.57 *
CW	3.84±0.405 *	2.59 ±0.49 *
OS max	0.952±0.070 *	0.77±0.104 *
VS min	1.34±0.089 *	1.067±0.216*
OS min	0.75±0.047	0.579±0.01
VS max	1.46±0.11	1.27±0.26
A-VS	2.89±0.35	2.81±0.5
OS-VS	2.005±0.37	1.99±0.44

*$p \leqq 0.05$ significant

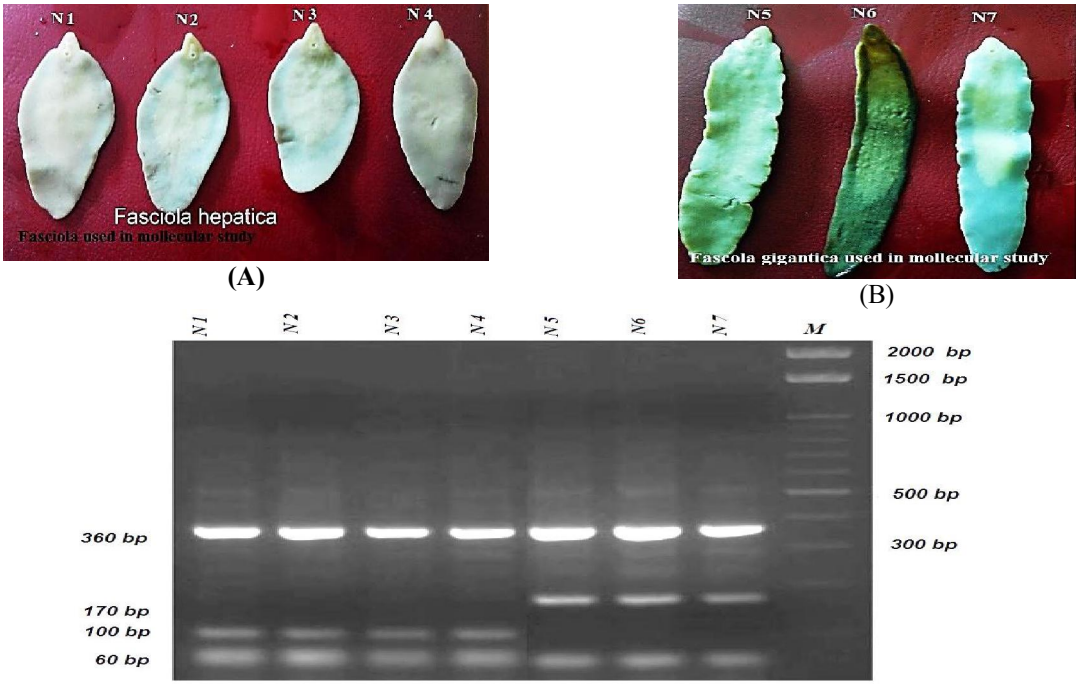


Fig 3: *F. hepatica* samples used in molecular study (A), *F. gigantica* samples used in molecular study (B) and Gel electrophoresis Showing the PCR-RFLP for both *F. hepatica* and *F. gigantica* (C).

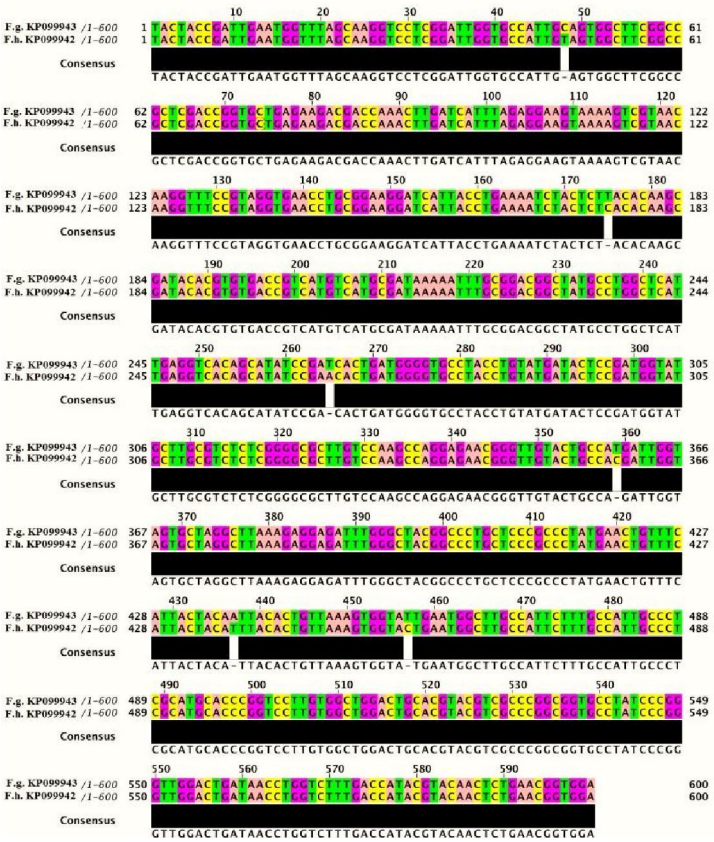


Fig 4: Alignment of the ITS-1 sequences (5'to3') of *Fasciola hepatica* accession numbers: KP099942 and *Fasciola gigantica* accession number KP099943, according to the current study.

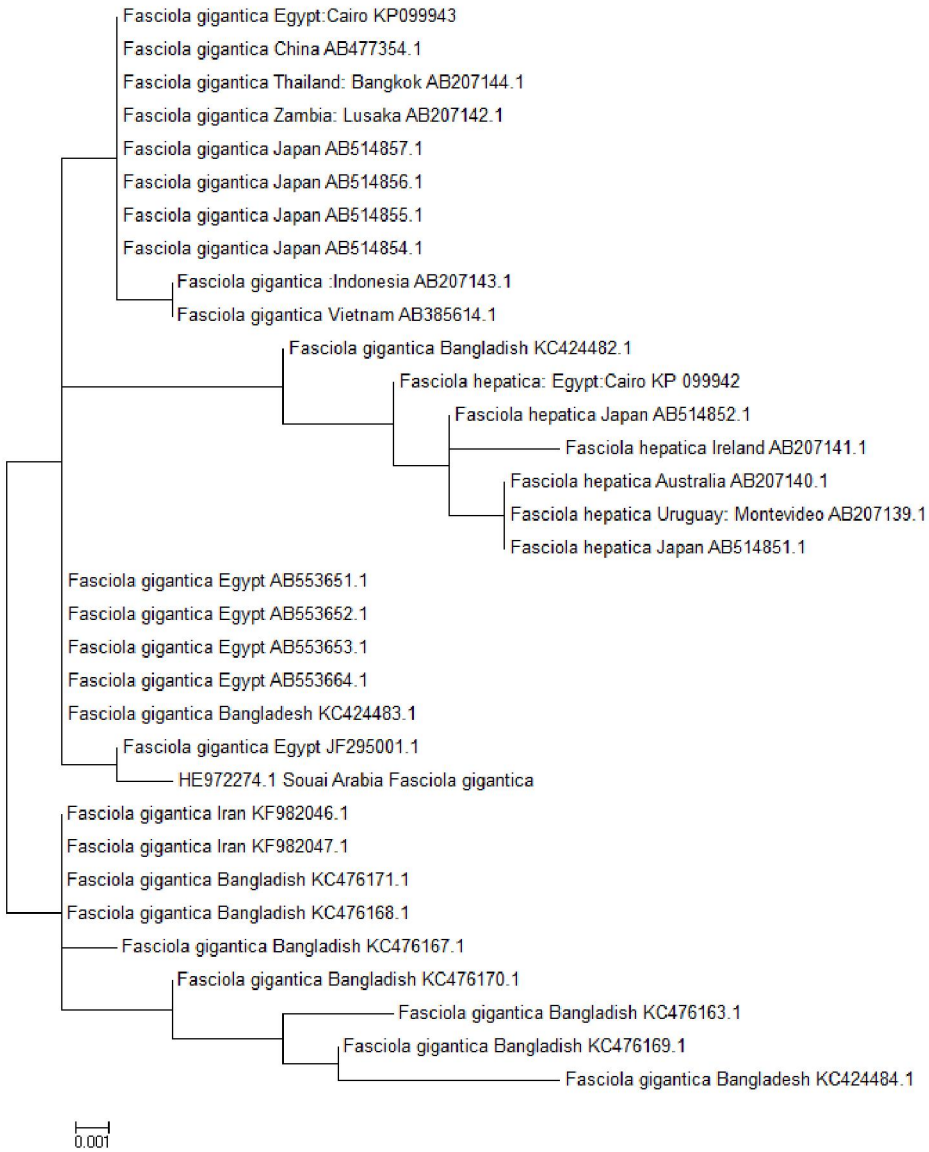


Fig5: Phylogenetic relationships of *Fasciola* parasites based on ITS1 sequences. *Fasciola hepatica, Fasciola gigantica* (Kp099942, p099943) was used as out group Evolutionary relationship of 31 taxa was inferred using Maximum Parsimony (MP) method Phylogenetic analyses were conducted in MEGA 6.0

4. Discussion

This study demonstrated that Classical indices can be used for differential diagnosis of the two *Fasciola* species. These indices include external morphology, morphometric parameters, especially the size and shape of the body. Some other authors have outlined useful morphometric descriptions for the specific differentiation of the two species **(Ashrafi *et al*., 2006)**. Several other studies however have shown that it is difficult, and in certain cases even impossible, to differentiate morphologically between *F. hepatica and F. gigantica* (**Kimura *et al.,* 1984**), so that several specimens have been considered as intermediate forms (**Moghaddam *et al.,* 2004**), contrary with our study which not indicated intermediate species in Egypt.

The main difference between *F. gigantica* and *F. hepatica* in this study is the larger size of the worm in addition to an elongated body regardless of their host origins. Other differences include the shape of the posterior end which may be narrow (**Kimura *et al.,* 1984).** Earlier studies however suggest conflicting data on the size of the liver fluke hosted in different animals, with infrequent explanation signifying that flukes from different hosts may differ in size, but no valid conclusions have yet been finalized (**Panaccio and Trudgett, 1999**).

Species identification in the present study was done by emphasizing BL, BW, VS-P, indices and BL/BW ratios as suggested by previous studies (**Valero *et al.,* 2001; Lotfy, *et al.,* 2002 and Ashrafi *et al.,* 2006;Dube *et al.,* 2014** and **Shafiei *et al.,* 2014**). It is observed that *Fasciola* in this study to a large extent resembled the *F. hepatica* and *F. gigantica* spp. from Bolivia and Burkina Faso (**Periago *et al.,* 2006**). They tend to be very similar to those from Thailand (**Srimuzipo *et al.*, 2000**). Species in this study are longer and wider than both *F. hepatica* and *F. gigantica* from Philippine (**Claveria *et al.,* 2011).** *F. gigantica* in this study is longer but not wider than *F. gigantica* in Zimbabwe **(Dube *et al.,* 2014)**. Both of *Fasciola gigantica* and *Fasciola hepatica* are shorter than *F. gigantica* and/or *F. hepatica* in Iran **(Shafiei *et al*., 2014)**.

The difference in the body length and width of the *F. gigantica* and *F. hepatica* between the present and earlier works may be geographically-influenced. There are also some of the factors that may affect the Fixing and mounting of specimens may affect some parameters. Also, the fixation of individual flukes in the earlier surveys between glass slides or between a glass slide and cover slip as against the use of a relaxant in the other studies may have also unnaturally overstretched or distended the worms (**Biu *et al.,* 2103**).

In the present study, adult specimens of *Fasciola spp*. infecting buffaloes, sheep and cow from Egypt were characterized by sequences of the ITS1-rDNA. These sequences revealed a few nucleotide differences between the two species. At the same time, there were no intraspecific variations within species (**Marcilla *et al.,* 2002; Shafiei et *al*., 2014**). Various DNA markers have been considered to identify *Fasciola* species, as the ITS region (ITS1, the 5.8S and the ITS2) of nuclear rDNA (**Tsutsumi, 1998; Marcilla *et al*., 2002; Periago *et al.,* 2004; Itagaki *et al*., 2005a, b; Farjallah *et al*., 2009; Itagaki and Rokni *et al*., 2010**).

The presence of intermediate genotypes of *Fasciola* has not been shown in the current study using sequences of the ITS1 PCR–RFLP. This result is in agreement with (**Marcilla *et al.,* 2008; Rokni *et al.,* 2010)** who have identified *Fasciola* as either *F. hepatica* or *F. gigantica* from Iran using ITS1 PCR–RFLP patterns, stressing the lack of mixed patterns. This lack of mixed pattern is in contrast with **(Karimi, 2008)** who is the first to report the molecular evidence of an intermediate genotype of *Fasciola* in the Fars province in Iran using 18S rDNA–RFLP and sequencing.

This study based on 680 bp ITS1 using RsaI as restriction enzyme. Different six bands have been obtained differentiating between *F. hepatica* and *F. gigantica*. These findings is consistent with most previous studies used Rsa1 (**Itagaki, *et al.,* 2005a, b; Itagaki *et al.,* 2009; Peng*et al.,* 2009; Mohammed *et al.,* 2012 and Shafiei *et al.,* 2014)**. Another study based on 263 and 356 bp fragments of 18s rDNA using DraI and BfrI restriction enzymes showed that there were no difference in bands patterns using BfrI restriction enzyme while DraI restriction enzyme revealed bands differences and hence can be used to discriminate the two species of *Fasciola* **(Karimi 2008)**. In another research work based on 463 bp region of the ITS1 sequence with restriction enzyme TasI, *Fasciola* samples from Tehran, West Azerbaijan and Khuzestan provinces were identified by PCR-RFLP method **(Rokni, *et al*., 2010**). Other studies digested both *F. hepatica* and *F. gigantica* samples with Tsp509I restriction enzyme, which showed different RFLP patterns (**Ghavami *et al.,* 2009; Dalimi *et al.,* 2011; and Saki *et al.,* 2011**).

Findings of Complete sequences in the present work, regarding the genotypes of ITS1 region, demonstrated also six variable nucleotides sites between two species of *Fasciola*. These data is consistent with previous studies **(Itagaki, *et al.,* 2005a; Itagaki *et al.,* 2009; Peng *et al.,* 2009 and Shafiei *et al*., 2014**).

Concerning the phylogenetic trees a close relationship of Egyptian *Fasciola* isolates from this study with those isolates from different regions of the world has been shown (**Itagaki, *et al.,* 2005a, b).**

This study confirmed, thus leaving no room for doubt, that there are two well-known digenean trematodes, *F. hepatica* and *F. gigantica* in ruminant (buffalo, cow and sheep) in Egypt. The advanced method PCR-RFLP assay using *Rsa1* restriction enzyme provides a simple, practical, faster, more accurate, and reliable method for identification and differentiation of *Fasciola* isolates than Classical method.

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