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Molecular Characterization of *Fasciola* spp. in Egypt on the Basis of certain rDNA fragments and Highly Repetitive DNA Sequences

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ABSTRACT

The genetic identity of *Fasciola* species in Egypt has been investigated by carrying out the sequence analysis of the ITS-2 region of rDNA gene. Also, highly repetitive sequences of genomic DNA (124 bp) were determined and compared. Three isolates of *Fasciola* recovered from Egyptian local breeds (sheep, Buffaloes and Cows) were used. Sequence comparison including ITS-2 sequences of isolates from this study and reference sequences from *Fasciola hepatica* and *F. gigantica* and intermediate *Fasciola* in Genbank was done. The results revealed several variable sites encountered among the investigated isolates in comparison with species of *Fasciola* submitted in Genebank. The relationship between Egyptian *Fasciola* and *Fasciola* spp. from different countries were discussed.

INTRODUCTION

Fasciola hepatica Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 (Platyhelminthes: Trematoda: Digenea) are two common species causing fascioliasis in domestic animals and humans. Several studies have revealed that *F. hepatica* is prevalent in temperate areas, while *F. gigantica* is widespread in tropical zones, and both species interfere in subtropical areas (Bargues and Mas-Coma, 2005; Mas-Coma *et al*., 2005; Ashrafi *et al*., 2006; Mas-Coma *et al*., 2009). This overlapping distribution of both species has even become the basis of an already long controversy on the taxonomic identity of *Fasciola* species occurring in Far East countries, especially Japan, Taiwan, the Philippines and Korea. In these countries a wide range of morphological types was detected (Mas-Coma and Bargues, 1997).

These different forms indicated the necessity of applying other techniques, in addition to the morphology as an approach to the taxonomy of *Fasciola* species. In several earlier studies, the two species and their intermediate forms have been discriminated by sequence analysis of the first (ITS1), the 5.8S, and second Internal Transcribed Spacers (ITS2) of the nuclear ribosomal DNA (rDNA), 28S ribosomal ribonucleic acid (rRNA) (Adlard *et al.,* 1993; Itagaki and Tsutsumi, 1998; Marcilla *et al*., 2002; Itagaki *et al*., 2005a; Le *et al.,* 2008; Ichikawa & Itagaki, 2010), 18S rRNA (Karimi, 2008), mitochondrial NADH dehydrogenase I (NDI) and Cytochrome C Oxidase I (COI) genes (Hashimoto *et al*., 1997; Itagaki *et al.,* 2005b). Molecular analyses of the intermediate forms were mainly done in the Far East Asian countries such as China, Japan, Korea and Vietnam.

These studies have detected individuals that have nuclear DNA related to one species, while their mitochondrial DNA is typical of the other species. In addition, individuals might have nuclear ribosomal genes with divergent copies apparently derived from both *Fasciola* species (Bandelt *et al*., 1999; Itagaki *et al.,* 2005; Ashrafi *et al.,* 2006; Semyenova*et al.,* 2006; Ichikawa *et al*., 2010).

In Egypt, fascioliasis posed a public health problem as the infections dramatically increased among human populations (Farag *et al*., 1979; Hassan *et al*., 1995; El Shazly *et al*., 2009). It is not exactly known whether *Fasciola* spp. is belonging to a single or several species or it may be a hybrid of the two species, Lotfy *et al.* (2002) used morphological, morphoanatomical and morphometric analysis to identify the identity of *Fasciola* species in Egypt. They also used isoelectrofocusing assay to confirm their findings. However, further studies are needed to provide additional taxonomic criteria for the identification of *Fasciola* species in Egypt. Lotfy and Hillyer (2003) in their review of *Fasciola* species in Egypt recommended studying the molecular characterization of *Fasciola* species in Egypt. Recently, Periago *et al.* (2008) claimed the existence of *F. hepatica/F. gigantica* intermediate forms in Egypt utilizing morphometric criteria. Therefore, Amer *et al*. (2011) molecularly ascertain the nature of *Fasciola* population derived from different hosts and different geographic locations in Egypt. They used sequence analysis of ribosomal first and second internal transcribed spacer (ITS1 and ITS2) in addition to mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit I (NDI) and cytochrome C oxidase subunit I (COI) as target gene markers. They revealed profound genetic heterogeneity in the *Fasciola* sp. populations in Egypt and existence of hybrid form.

The present study aimed to use sequence analysis of the ITS2 region of rDNA and a highly repetitive DNA sequences to determine the identity and heterogeneity among *Fasciola* isolated from buffalo, cow and sheep hosts.

MATERIALS AND METHODS

Parasites collection

Three isolates of adult worms of *Fasciola* spp. were obtained from bile ducts of three Egyptian natural hosts: Buffaloes, cows and sheep. The worms were washed several times in 0.85% NaCl to remove adherent host cells and empty the intestinal caeca, and then stored at –70 °C until used.

DNA Isolation

Genomic DNA was extracted from flukes by the CTAB precipitation technique as described by Yap and Thompson (1987) and Mostafa *et al*. (2002). Briefly, the adult worms were homogenized individually in 1.5 ml of lysis buffer (8% Triton X-100, 0.25 M sucrose, 50mM Tris-HCl, 50 mM EDTA, pH 7.5). After homogenization, freshly prepared proteinase K (1mg/ml) was added. The homogenate was incubated at 65ºC in water bath for 2 hrs. To precipitate the genomic DNA, 1.0 ml of a sterile 2% CTAB solution was added to the homogenate and centrifuged at 1500 rpm. The supernatant was discarded and the precipitate was dissolved in 0.5 ml of 2.5 M NaCl, 10mM EDTA, pH 7.7 and diluted with 1.0 ml of 40 mM Tris-HCl, 2 mM EDTA, pH 7.7. Two volumes of chloroform-isoamyl was added to the mixture and centrifuged at 12000 rpm for 10 min. The aqueous phase was isolated, and the DNA was precipitated by two volumes of cold absolute ethanol. The DNA pellet was dissolved in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

PCR amplification

Fragments spanning 5.8S, ITS-2, and 28S in the rRNA gene region were amplified by PCR using the forward primer, 5′-TGTGTCGATGAAGAGCGCAG-3′ and the reverse primer, 5′-TGGTTAGTTTCTTTTCCTCCGC-3′.
Amplification reactions were performed in a final volume of 50 µl containing 10mM HCl buffer, pH8.3, 25 pmol of each primer,

200µM each of dATP, dCTP, dTTP. and dGTP, 2.5 units of *Taq* DNA polymerase, 100ng *Fasciola* DNA, 1.5mM-$MgCl_2$ and 50 mM-KCl. The PCR protocol required 94°C for 0.5 min initially, then for 1.5 min at the start of each cycle, 53°C for 2 min, 72°C for 2 min and 10 min at the end of all 30 cycles.

PCR amplification of highly repetitive sequence

PCR reaction was carried out in a total volume of 50µl containing 5µl of 15 mM MgCl2, 1 µl of 10 mM dNTPs, 2.5 µl of 50 pm of the two specific primers (Forward 5′-ATTCACCCATTTCTGTTAGTCC-3′; reverse 5′-ACTAGGCTTAAAGGCGTCC-3′). The design of the primers is based on 124 base-pair (bp) highly repeated sequence of DNA specific for *Fasciola* spp. (Kaplan *et al*., 1995 and Kramer and Schnieder, 1998). The PCR reaction was performed in DNA thermal cycler (T-personal, Biometra) under the following conditions: 1x94ºc for 3 min., 35x (94ºc for 1 min., denaturing, 65ºc for 1 min. annealing, 72ºc for 1 min. extension); followed by a single incubation at 72ºc for 10 min.

ITS-2 Sequencing

The PCR products were purified using an ultra clean DNA purification kit (Qiagen) according to the manufacturer's protocol. Sequencing reactions were carried out with Big Dye 3.3 terminator cycle sequencing kit (Applied Biosystems, Foster City, A) using the PCR conditions recommended by the manufacturer. Samples were then analysed on 6% polyacrylamide gel on an applied Biosystems model 3100 Automated DNA Sequencing S-ystem at Genetic Engineering research Center, Ain Shams University-Cairo-Egypt.

Beside ITS region, the smallest band of the highly repetitive sequence of amplified products (approximately 124bp) were sequenced using a Big Dye terminator cycle sequencing kit (Applied Bio System, Foster City, A). Sequencing products were purified by using Centri-Sep Spin columns (Princeton separations, Adelphia, NJ) and were resolved on an applied Biosystems model 3100 automated DNA sequencing system at Vacsera Authority.

Phylogenetic Analysis

NCBI (National Center for Biotechnology Information) databases of *Fasciola* spp. Collected from various localities were used for sequence similarity analysis. Sequence of *Fasciolopsis buski* obtained from Gen Bank data base was used as outgroup.

The maximum likelihood tree was built using RAxML version 7.2.8 algorithm (Stamatakis *et al*., 2005) as proposed on the Black Box portal (http://phylobench.vital-it.ch/raxml-bb/) using the GTR+Γ+I model. Model parameters were estimated in RAxML over the duration of the tree search. Two simultaneous MCMC chains were performed, and 500,000 generations. The generations were added until the standard deviation of split frequencies fell below 0.01 according to the manual of Mr Bayes 3.1. For every 100th generation. The tree with the best likelihood score was saved, resulting in 5000 trees. The burn in value was set to 25%. Trees were viewed using Fig Tree (a program distributed as part of the BEAST package (http://tree.bio.ed.ac.uk/software/figtree/).

The divergences between sequences were calculated using the program BioEdit software (Hall, 1999).

RESULTS

Structure of the ITS2 gene

A completeITS-2, a part of 5.8S and a part of 28S gene fragment were amplified from each sample and expected to be approximately 500-538 bp in length. The sequenced ITS2 fragment was ca. 525 bp for *Fasciola* sp. isolated from sheep, (accession number in Gen Bank: DQ385828), 509 bp for *Fasciola* sp. isolated from buffaloes (accession number DQ383512) and 538 bp for *Fasciola* spp. Isolated from cows (accession number DQ385829).

The ITS2 coding region was subjected to direct sequencing giving products approximately 343 bp. The average GC contents were nearly 48-50% in the three

isolates. Few gaps were inserted in the most variable areas of the alignment. As demonstrated in Table (1), among the three samples under study, two individuals possessed ITS-2 sequences identical to that of pure *F. gigantica*. (isolate of cow & buffalo) On the other hand, *Fasciola* isolate of sheep had a different sequence variation in many sites from both *F. hepatica* and *F. gigantica* (Table 1).

Table 1: Comparison of ITS-2 variable sites in different Fasciola isolates.

		Varaible sites of sequence bases																				
Sp.	**Country**	**18**	**19**	**26**	**62**	**65**	**146**	**151**	**165**	**172**	**178**	**195**	**207**	**218**	**259**	**264**	**282**	**286**	**291**	**301**	**307**	Accession number
F.g	Egypt (cow)	G	A	G	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	DQ385829
F.g.	Egypt (Buffalo)	G	A	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	DQ383512
F.g.	Egypt (sheep)	C	C	T	A	A	C	C	T	C	A	C	T	T	T	A	T	G	G	T	C	DQ385828
F.g.	Iran	C	C	T	C	T	T	T	C	T	T	T	C	T	G	T	G	T	T	A	T	HM746788.1
F.g.	Boirkina Faso	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AJ853848.2
F.g.	Boirkina Faso	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AJ853848.2
F.g.	Boirkina Faso	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AJ853848
F g	Spain	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AM709616.1
F g	Tunsia	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	HQ197359.1
F g	India	C	C	T	C	T	T	T	C	T	T	T	C	T	G	T	G	T	T	A	T	EF027103.1
F g	Egypt3	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AB553695.1
F g	Egypt3	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AB553696.1
F g	China4	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	HQ700438.1
F g	Vitname4	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EU260080.1
F g	Kenya	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EF612482.1
F g	Indonesia3	C	C	T	C	T	T	T	C	T	T	T	C	T	G	T	G	T	T	A	T	EU260080.1
F h	10	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EF612480.1
F h	10	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EF612481.1
Fh	Turky9	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	FJ467927.1
F h	France	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AJ557567.1
F h	Australia3	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EU260058.1
F h	Iran 3	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EF612481.1
F h	Niger4	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AM900370.1
F h	Tunisia4	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	GQ231546.1
F h	Algeria4	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	GQ231547.1
F h	Egypt4	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EF612479.1
F h	Spain	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	AM709648.1
F h	Fh c4	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	JF432078.1
F h	Iran	C	C	T	C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	EU391420.1
F sp	Japan4	C	C	T	C	T	T	T	C	T	T	T	C	C	G	T	G	T	T	A	T	AB010979.1
F sp	Hiroshima	C	C	T	C	T	T	T	C	T	T	T	Y	Y	G	T	G	T	T	A	T	AB207153.1
F sp	Khan Vitname7	C TC		C	T	T	T	C	T	T	T	T	T	G	T	G	T	T	A	T	T	AB536925.1

F.g.: *F. gigantica*, F. h.: *F. hepatica*

Also, many sites substitutions can be noticed in Egyptian *Fasciola* (cow and buffaloes isolates) as compared with *F. gigantica* and *F. hepatica* of different countries. Whereas, in sheep isolates there are many substitution sites that are different from all compared sequences.

Phylogenetic position of the studied species

The topologies of phylogenetic trees inferred from maximum likelihood and Bayesian inference were identical (Fig. 1). The tree revealed the existence of three main clades: A, B and C. Clade Acomprises sequences of *F. gigantica* and it receives the maximum support value (BS =100% /1.00=PP), clade B comprises representatives of *F. Hepatica* and it receivesa high support value (BS=75%/1.00=PP), while clad C comprises sequences of *Fascioledes magna* and *F. jaksoni* and it receives BS=85%/1.00=PP.

Our obtained partial ITS2 gene sequences fall within clade A, the obtained sequences of *Fasciola* isolates of buffalo and cow receive high support value (BS=100%/1.00=PP), while the obtained sequence of *Fasciola* obtained from sheep received weak support value. The phylogenetic tree revealed that *F. gigantica* has a high genetic diversity in comparison to *F. hepatica*. The intra-clade genetic variability within *F. gignatica* group (A) ranged between 0.3%-2%, while in *F. hepatica* group (B) is less than 0.7%. The phylogenetic tree also suggests a possible pattern of geographical origin with *F. gigantica* group.

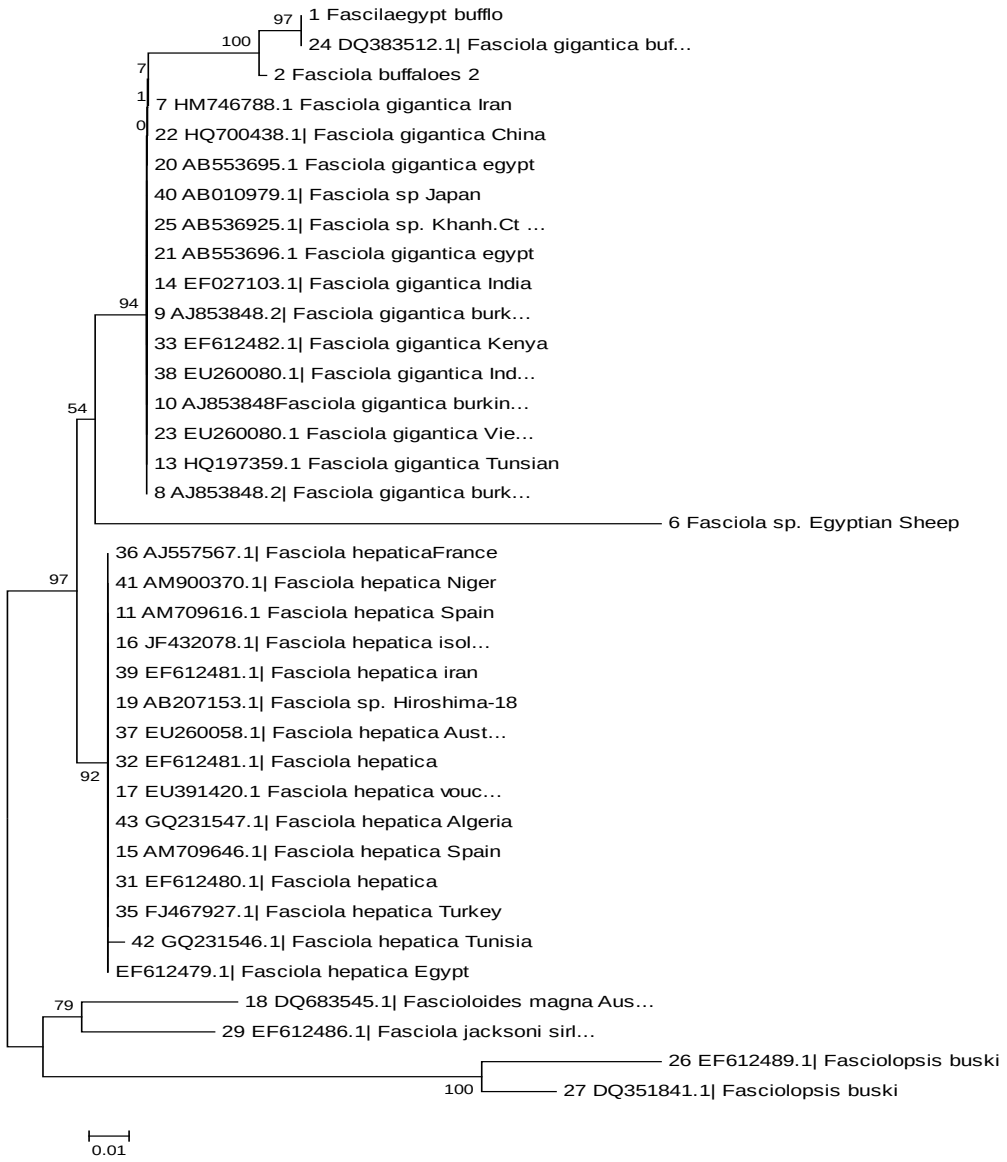

Fig.1: The phylogenetic relationships of the *Fasciola* spp. collected from buffalo, cow and sheep in Egypt and other representative isolates (*F. gigantica*, *F. hepatica* and *Fasciola* spp.) in different localities in the world based ITS2 region.

Concerning the results of the highly repeated fragments of DNA in the three isolates, the size of the smallest one is ~ 114 bp. Also the purified fragments were visualized on the agarose gel electrophoresis (Fig.2). No band variations were noticed among the three isolates of *F. gigantica*. The smallest fragment was subjected to direct sequencing, as described in Fig. (3). The obtained results revealed sequence variations among the three isolates and other *Fasciola* of various countries with a percentage of 90%.

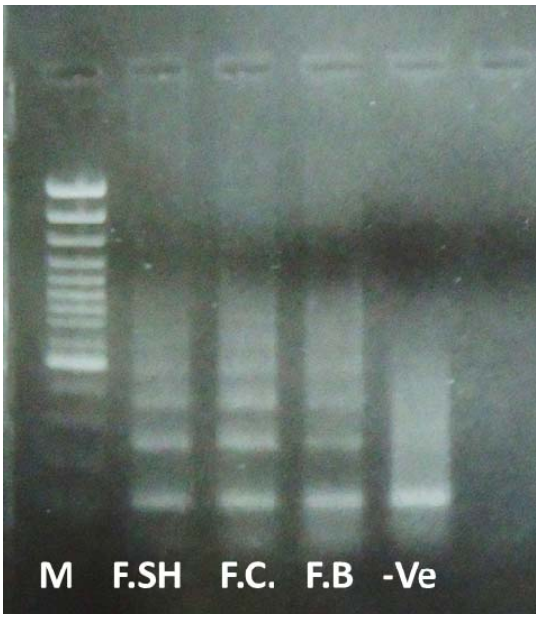

Fig. 2: 1.5% agarose gel electrophoresis of PCR products of highly repetitive sequences of *Fasciola* sp recoverd from sheep (F.SH), cow (F.C) and buffalo (F.B). the last lane represent the uninfected liver as negative control. M: 100bp DNA ladder.

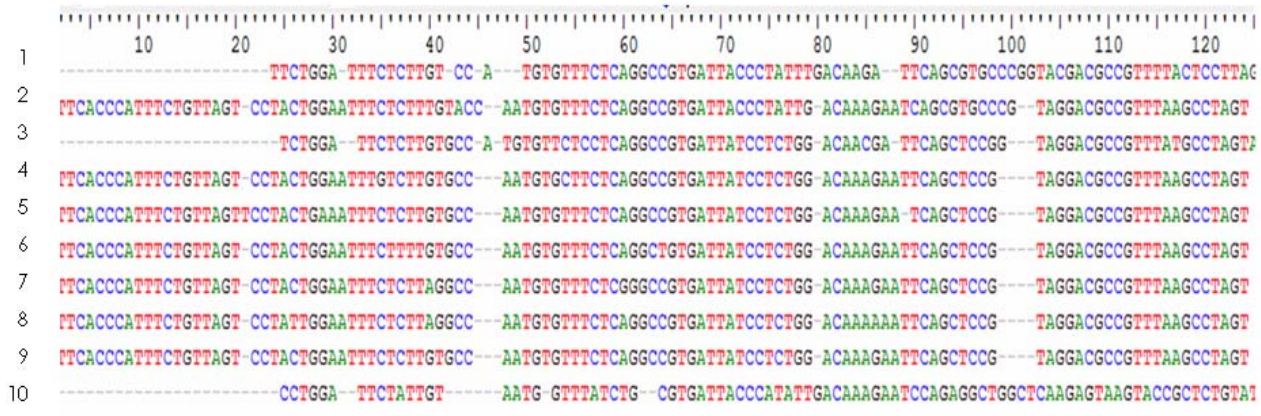

Fig. 3: 124 bp repetitive DNA sequences of *Fascioloa* spp. *recovered* from the three isolates in comparison to *Fasciola* spp. from other countries: 1, 2,3 are *Fasciola* of buffaloes, cows and sheep in Egypt respectively,4: *F. gigantica* from China, 5: *F. gigantica* from Sudan, 6: *F. hepatica* from Netherland , 7: *F. hepatica* Australia , 8: *F. hepatica* from Cuba , 9: *F. hepatica* from China, 10: *F. hepatica* from Germany.

DISCUSSION

Fasciola species are considered as geographically variable in different countries. In the current study, isolates of *F. gigantica* infecting sheep, cows and buffaloes from Egypt were molecularly identified on the basis of partial sequences of ITS2 rDNA and a repetitive sequence regions. The analyses revealed that the sequences of *Fasciola* spp. From different host species are almost identical to those of previously published sequences.

Several previous studies have investigated that these sequences can give reliable genetic markers for the accurate identities of *Fasciola* spp. (Itagaki and Tsutsumi, 1998; Agatsuma *et al*., 2000; Huang *et al*., 2004; Itagaki *et al*., 2005 a,b 2009; Mas-Coma *et al*., 2009).The results of the present work confirmed that *F. gigantica* is the main species exists in Egypt, in addition to an intermediate form of *Fasciola* found in sheep. This disagree with the results of Amer *et al.* (2011) who reported that *F.*

hepatica was more dominant in sheep than in other hosts.

Earlier studies recorded the presence of other species of *Fasciola* in African countries based on the phylogenetic position and interspecific variation, using ITS2 sequences (Adlard *et al*., 1993; Hashimoto *et al*., 1997; Itagaki and Tsutsumi, 1998; Itagaki *et al*., 2005 and Semyenova *et al*., 2005).

Differentiation of the ITS2 gene among populations and species has been reported to be dependent upon many factors, including genetic drift, the relative number and size of repeats, rates of unequal crossover, gene conserving, immigration, and the number of loci and now biogeographical distribution (Levinson and Gutman, 1987). In the present study, the nucleotide sequences of ITS2 were determined in three worms of *Fasciola* recovered from Egyptian livestock (buffaloes, cows and sheep).

Nowadays the genetic diversity of Egyptian *Fasciola* species is underestimated. Previously, *F. gigantica* was the only species reported in animals. When human fascioliasis emerged, it was reported to be due to *F. hepatica* and importation of infected animals was considered the source of propagation of infection (Lotfy *et al*., 2002). However, the absence of *Lymnaea truncatula* in an area endemic for human fascioliasis, posed a question about the possibility of local transmission.

In the present study, three isolates of the Egyptian *Fasciola* were characterized using well-defined ITS-2 sequence which emphasize the suggestions of earlier investigators that this rDNA sequence provides reliable genetic markers for the accurate differentiation and identification of *Fasciola* species. Previously published data (Adlard *et al*., 1993; Hashimoto *et al*., 1997; Itagaki and Tsutsumi, 1998; Agatsuma *et al*., 2000), demonstrated that *Fasciola* from buffalo from Guangxi represented *F. gigantica*. The ITS-2 sequences of *Fasciola* from sheep and cows were quite different, where ITS-2 sequences of *Fasciola* species from Koyoto has not been identified as *F. hepatica* nor *F. gigantica*, so the isolates of *Fasciola* from cows and sheep could represent an intermediate genotype or even a "hybrid" between *F. hepatica* and *F. gigantica*.

So far, for the best of our knowledge, this is the first time that molecular evidence have suggested the possible existence of an "intermediate genotype" of *Fasciola* in Egypt, in addition to *F. hepatica* and *F. gigantica*. This was confirmed by Agatsuma *et al*. (2000) who suggested the existence of natural hybridization between *F. hepatica* and *F. gigantica*, co-existing. This is the case of *Fasciola* spp., in Japan and Korea and perhaps, in Egypt, where *F. hepatica* and *F. gigantica* overlap. In Japan and Korea a triploid *Fasciola* spp. has been the subject of intense study to define its speciation. In a series of articles, Itagaki and collaborators (1998) showed the complexity of this issue. First, they compared the mitochondrial NADH dehydrogenase subunit 1 (ND1) and cytochrome oxidase subunit 1 (CO1) gene sequences *of F. hepatica* (Uruguay), *F. gigantica* (Zambia) and the triploid form of Japanese *Fasciola* spp. They found that intraspecific variation in the sequences was generally low, but the sequences of the Japanese triploid form were identical to those found in *F. gigantica* but were different from those of *F. hepatica*. This leads to the conclusion that Japanese *Fasciola* can be categorized as *F. gigantica*.

Second, they examined the ITS2 sequence of the nuclear RNA using the fluke population described above and concluded that the Japanese triploid worms were divided into two distinct types, one of which was almost identical to *F. hepatica* and the other to *F. gigantica* (Itagaki and Tsutsumi, 1998). Third, Itagaki *et al*. (2001) examined the mitochondrial DNA polymorphism detected by restriction fragment length polymorphism (RFLP) using 8 restriction enzymes and detected 3 different types in 76 *Fasciola* flukes studied. The authors concluded that the *Fasciola* Japanese triploids are composed of two lineages

having a genetic structure identical to *F. hepatica* and *F. gigantica* and hybrids between the two species. Whether a similar situation is occurring in the Nile delta of Egypt remains to be ascertained. Further investigations are warranted to clarify the phylogenetic position of Egyptian *Fasciola* spp. using other molecular markers.

Previous studies documented a high level of genetic diversity of Egyptian *Fasciola* at both NDI and COI (Walker *et al.,* 2007; Peng *et al*., 2009). In the present study it was observed that Egyptian *F. gigantic* has a separate position on both trees which supported by high bootstrap values reflecting the prominent nucleotide differences from homologous sequences in data base especially those isolated from sheep. As stated by Constantine (2003) and Criscione *et al*. (2005), these differences may be attributed to the host range, long persistence of infection in the country. Moreover, regular import strategies of cattle from different countries may have an impact on the stability and consistency of the parasite lineages. As the molecular variation may reflect differences in virulence, host specificity and drug susceptibility/resistance population genetics of *Fasciola* parasites in Egypt needs close surveillance

In conclusion, the present study revealed profound genetic heterogeneity in the *Fasciola* spp. populations in Egypt and provided the first molecular clue on existence of hybrid form. The results indicated that hybridization might be an active process in liver flukes due to the mixed infection.

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