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Molecular and Phylogenetic Status of *Fasciola* sp., of Cattle in Qena, Upper Egypt

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Abstract: The species of liver fluke of the genus *Fasciola* (phylum platyhelminthes, order Digenea, Family Fasciolidae) are obligatory parasites that inhabit the large biliary ducts of herbivore animals as well as man. Reports on the species of *Fasciola* present in the Nile Delta, Egypt, appear controversial. In the current study a precise identification of *Fasciola* isolates from cattle in Qena province, Upper Egypt was done based on examination of the second Internal Transcribed Spacer (ITS2) and the mitochondrial cytochrome oxidase subunit I (COI). Amplification, sequencing and phylogenetic examination revealed that the collected *Fasciola* isolates represent only one species which is *Fasciola hepatica*.

Key words: *Fasciola*, cattle, identification, molecular, phylogenetic

INTRODUCTION

Fascioliasis is an important disease caused by members of genus *Fasciola*, transmitted by freshwater lymnaeid snails and infects many livestock species as well as man. Identification of *Fasciola* species depends mainly on morphological examination (Itagaki and Tsutsumi, 1998; Amer *et al*., 2011). *Fasciola hepatica* and *F. gigantica* thought to be the main causes of fascioliasis allover the world, the overlapping distribution both species has led to a long ranging controversy on the taxonomic identity of *Fasciola* species (Mas-Coma, 2004). The existence of a hybrid form of *Fasciola* complicates the problem of identification and subsequently misaddressing of exact epidemiological information on fascioliasis.

DNA studies and using of molecular tools aid in the exact identification of parasites. In ribosomal DNA (rDNA) the Internal Transcribed Spacer 2 (ITS2) gene have proven useful for diagnostic purposes at the level of species (Morgan and Blair 1995; Leon-Regagnon *et al*., 1999). ITS2 sequences have also been used to characterize and identify different *Fasciola* spp., (Huang *et al*. 2004; Amer *et al*., 2011). While, mitochondrial DNA (mt DNA) genes proves to be excellent markers to differentiate in-between closely-related species and testing the polymorphism of *Fasciola* species (Amer *et al*., 2011).

In Egypt, *F. hepatica* and *F. gigantica* are prevalent among livestock's in the Nile delta (Lotfy and Hillyer, 2003) results showed that identification of Egyptian *Fasciola* based only on morpho-metric criteria is not a countable. Dar *et al*. (2012) confirmed the presence of *F. hepatica*, *F. gigantica* besides the hybrid form in Egypt. The pervious Egyptian studies focused on *Fasciola* isolates collected from Cairo and Delta region, with no information about situation in Upper Egypt.

So that in the present study, nucleotide sequences of the mitochondrial DNA, cytochrome oxidase subunit 1 (CO1) and Internal Transcribed Spacer 2 (ITS2) of the ribosomal RNA gene were used to identify *Fasciola* species that infects cattle in Qena, Upper Egypt.

MATERIALS AND METHODS

Parasitic samples: During visits to Qena city slaughter house, Upper Egypt (26°1012, N 32°4337 E), adult *Fasciola* specimens were obtained alive from biliary ducts of freshly slaughtered cattle. The collected specimens were washed thoroughly in physiological saline, preserved in ethyl alcohol 70% prior to genetic study.

Nucleic Acid extraction and amplification: Genomic DNA was extracted from two flukes by using a QIAGEN genomic DNA kit according to manufacturer protocol. The rDNA region spanning the ITS2 and a partial fragment of the mitochondrial CO1 gene were amplified by Polymerase Chain Reaction (PCR). The following universal trematode primer sets were used following Bowles *et al*. (1995) as follows for CO1 region: FH5 (forward):

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5'-TTTTTTGGGCATCCTGAGGTTTA-3' and FH3 (reverse): 5'-TAAAGAAAGAACATAATGAAAAT AATC-3' and for ITS2 region: 3S (forward): 5'-GGTACCG GTGGATCACTCGGCTCGTG-3' and A28 (reverse): 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'.

The PCR amplification was performed following the standard protocol (White, 1993) with minor modifications as described elsewhere (Prasad *et al*., 2007). For DNA sequencing the PCR products were purified using the Geneclean2 PCR purification kit according to manufacturer protocol and sequenced in both directions using PCR primers.

Phylogenetic analysis: For phylogenetic analysis, the sequences were aligned using ClustalW multiple alignments W, trees were constructed using MEGA4 (Higgins and Sharp, 1988; Tamura *et al*., 2007). Amino acid sequences of the mitochondrial COI gene were inferred using the codon tables of Garey and Wolstenholme (1989).

RESULTS AND DISCUSSION

To identify and study the phylogenetic relationship of *Fasciola* sp., collected from cattle in Qena, Upper Egypt. Amplification and sequencing of ITS2 rDNA and COI mt DNA genes was done. ITS2 of rDNA of our isolate was successfully obtained, Genbank accession number is (AB510492.). ITS2 fragments of were estimated to be 481 bp long. For comparative purposes, the ITS2 sequences of fasciolids from various hosts and geographical regions were obtained from GeneBank. No difference was observed between the examined *Fasciola* samples and *F. hepatica* from other hosts from Delta of Egypt and other parts of the world (Fig. 1). Also, Upper Egypt *Fasciola* isolate differs than ITS2 of *F. gigantica* by percentage agrees as previously reported by Adlard *et al*. (1993). The phylogenetic tree based on ITS2 region showed a close relationship with *F. hepatica* from Egypt, Iran and Turkey (Fig. 2). It was remarkable that *F. giagnatica* "hybrid form" (EU260069) from human case in Vietnam clustered with *F. hepatica*.

Mitochondrial CO1 gene of our *Fasciola* isolate was successfully amplified and sequenced. Its Genbank accession number is (AB510491).The CO1 alignment consisted of 421bp. The G+C content were close to 37%. 'Variation' between sequences of Upper Egypt *Fasciola* sp. and *F. hepatica* from Genbank sequences was found at only one nucleotide site. On the other hand, *F. gigantica* deposited sequences differed from the

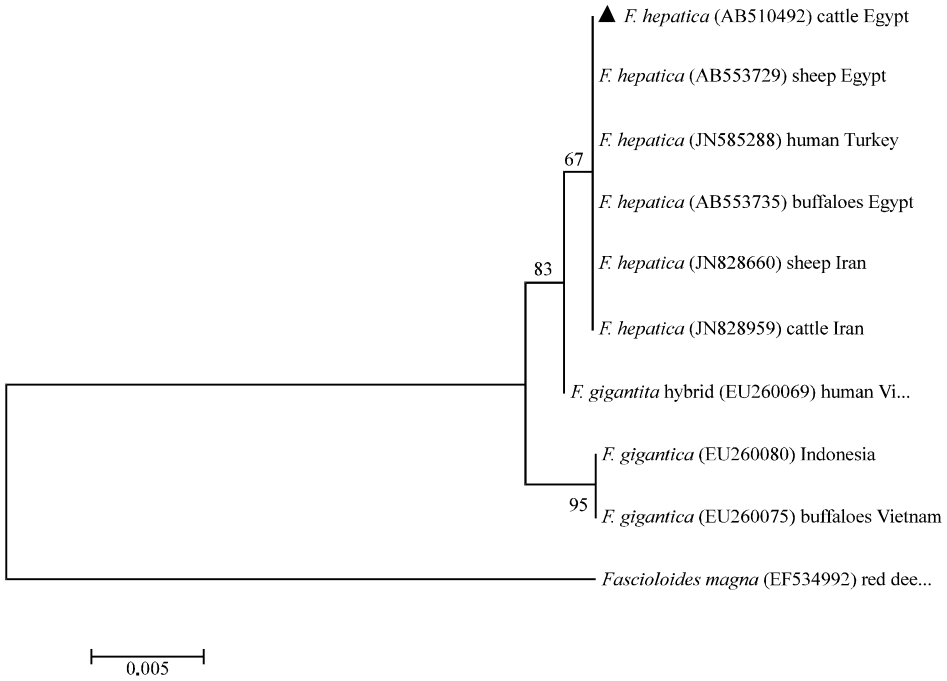

Fig. 1: Neighbor-Joining phylogenetic tree (1,000 bootstraps) based on ITS2gene sequences of *Fasciola* spp., *Fascioloides magna* (Accession No. EF534992) used as outgroup. Tree was constructed using MEGA4. Bootstrap values showed on internal nodes. The scale bar indicates the proportion of sites changes along each branch. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 347 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4

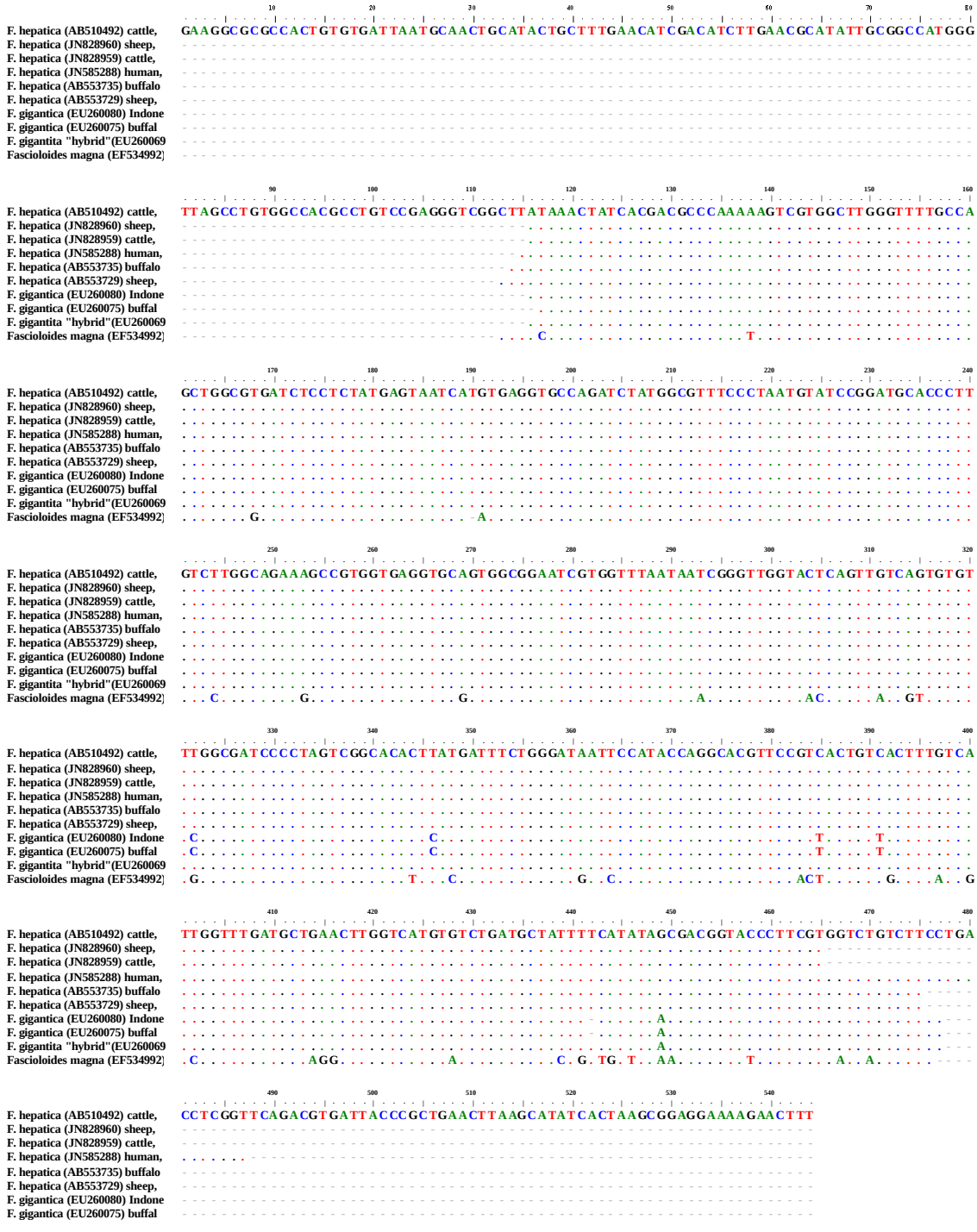

Fig. 2: Multiple alignments done of ITS-2 sequences of *Fasciola* spp.

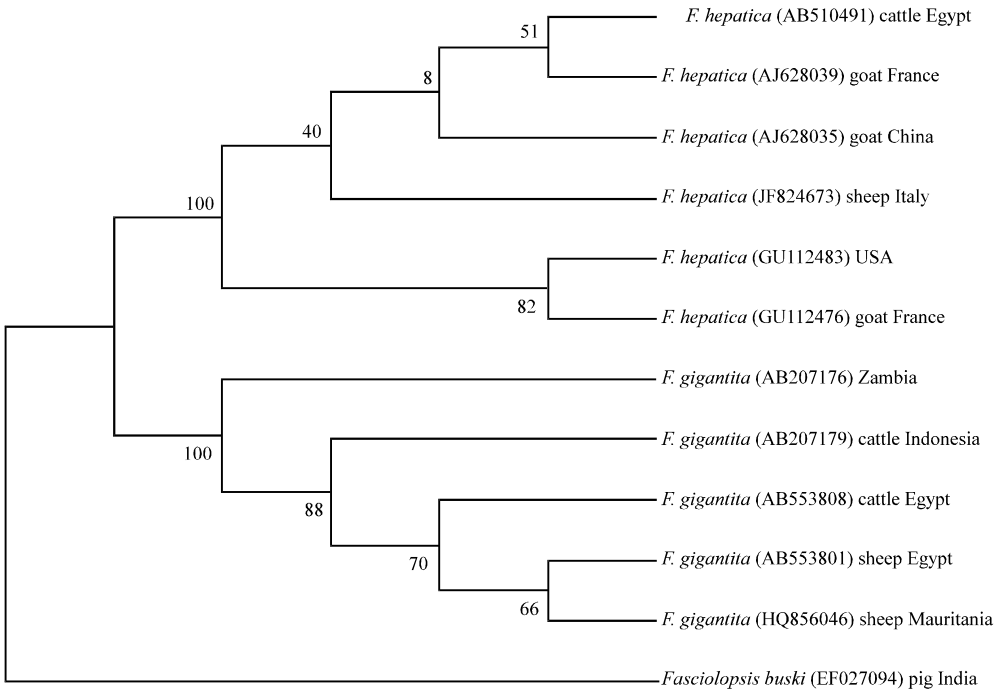

Fig. 3: Maximum parsimony phylogenetic tree (1, 00 bootstraps) based on COIgene sequences of *Fasciola* spp., *Fasciolopsis buski* (Accession No. EF027094) used as outgroup. Tree was constructed using MEGA4. Bootstrap values showed on internal nodes. The scale bar indicates the proportion of sites changes along each branch. Codon positions included were 1st+2nd+3rd+Noncoding. Complete deletion option was used. There were a total of 310 positions in the final dataset, out of which 162 were parsimony informative. Phylogenetic analyses were conducted in MEGA4

examined *Fasciola* samples at 28±50 sites. Thus, our specimens of *Fasciola* sp., represent *F. hepatica*. Comparison of the newly obtained CO1 sequence with other *Fasciola* sequences on Genbank through BLAST indicates that this new isolate is pure *F. hepatica*. The inferred maximum parsimony phylogenetic tree based on CO1 sequences showed that our samples are *F. hepatica* (Fig. 3). When compared with the COI sequence reported for *F. hepatica* by Garey and Wolstenholme (1989). Our *Fasciola* sp., isolate differed at seven nucleotide sites with *F. gigantica* from Indonesia (AB207179). The phylogenetic analysis based on CO1 gene agreed with the conclusions drawn from ITS2 data.

In the present study, sequence analysis revealed a close relationship between the query sequence from Upper Egypt and those of *F. hepatica* from Egypt and other parts of the world. In Egypt molecular approaches (e.g., Dar *et al*., 2012; El-Rahimy *et al*., 2012) have been utilized to specify *Fasciola* species. The results are also in line with these results that confirm the presence of *F. hepatica* in Egypt. At the same time we note that our isolates differ from *F. gigantica* from Egypt, Japan and Indonesia in more than six sites (Adlard *et al*., 1993) and also differs that the hybrid form. According to ITS2, the *Fasciola* sp., collected from cattle in Qena, Upper Egypt showed itself to be almost a perfect match with *F. hepatica*.

CONCLUSION

The results represented herein demonstrate that the species of *Fasciola* infects cattle in Qena, Upper Egypt is *F. hepatica*. Sequencing and amino acids analysis is a precise tool for identification of *Fasciola*. Despite the limitation of hosts included in this study, it will be a basic stone in the future works about fascioliasis in Egypt which is strongly engorged.

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