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Research Article
Molecular Characterization of *Fasciola hepatica* Infecting Cattle from Egypt Based on Mitochondrial and Nuclear Ribosomal DNA Sequences

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Abstract

Background and Objective: *Fasciola hepatica* is a zoonotic liver fluke infects wide range of hosts (all ruminant animals, rabbits, horses and human). It is commonly prevalent where the intermediate snail host is present. The main objective of this study was to provide molecular characterization of *F. hepatica* using ribosomal DNA cluster (rDNA) of more conserved Internal Transcribed Spacer (ITS) regions and mitochondrial cytochrome oxidase subunit I (COI) gene. **Materials and Methods:** Adult fluke samples were collected from livers of infected cattle hosts. All flukes were subjected to DNA extraction for polymerase chain reaction. **Results:** A 421 bp fragment of COI and 603 bp ITS2 genes were amplified, purified and sequenced. The obtained sequence was compared to the corresponding sequences available in the GenBank. Phylogenetically, *F. hepatica* resembles closely the other members of family Fasciolidae, showing considerable, expectation value in the alignment. **Conclusion:** This study corroborate that the mitochondrial COI and ITS2 sequences could be used as species specific markers for characterization of *F. hepatica*.

Key words: *Fasciola hepatica*, COI, ITS2, phylogeny

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fasciola hepatica is a liver fluke which mostly found in Egypt and neighboring areas. The fluke is the main causative agent of the disease (Fascioliasis). The infection occurs due to the ingestion of raw food grasses soiled with the infective stage (encysted metacercariae). In endemic places, reservoir hosts are ruminant, horses and rabbits. The infection rate of fascioliasis in Egypt reaches to 50.6% in cattle and 32.3% in buffaloes (Bazh *et al*., 2012). The clinical findings varied; emaciation, diarrhea, moderate icterus, bitter taste of milk, decreased milk production and anestrum animals (Bazh *et al*., 2012). The economic losses reaches to be 2 billion US $ per year (Rokni *et al*., 2010).

Molecular biology, especially the DNA amplification by using Polymerase Chain Reaction (PCR) and other techniques of sequencing have been used to support the taxonomy of various helminthes depending on DNA genetic markers of nuclear ribosomal DNA (rDNA) and special mitochondrial DNA (Prasad *et al*., 2007). The PCR amplification process enhances many templates for initial priming that allows the primers to anneal the identified conserved regions to amplify across unknown variable regions. In metazoan parasites, the nuclear rDNA internal transcribed spacers (ITS1 and ITS2), that occur between the following code regions (18S, 5.8S and 28S) were helpful for diagnosis of species (Nolan and Cribb, 2005). Among different *Fasciola* spp. isolates from several geographical districts phylogeny and/or intra-specific variations have been categorized according to ITS2 and COI sequences (Omar *et al*., 2013).

The objective of this study were to amplifies the internal transcribed spacer (ITS2) rDNA and cytochrome oxidase subunit I (mitochondrial COI gene) regions of *F. hepatica*, which considered advanced and clear identification of *F. hepatica* sp. in this study area where DNA from adult stages were extracted and assessed their potential for molecular characterization. Also was to determine the sequences of the specific molecular markers by amplifying the ITS2 and mitochondrial COI gene for cytochrome oxidase subunit I, regions of the parasite DNA from adult flukes.

MATERIALS AND METHODS

Parasite sample: Liver adult *F. hepatica* flukes were collected from the bile duct or liver tissue of freshly slaughtered cattle at local slaughter houses in El-Behira province, Egypt. The collected flukes were represented the geographical isolates from El-Behira province, region of Western Egypt. Firstly, after collection they washed thoroughly in physiological saline, then preserved at -20°C till be used for the DNA extraction purpose. This study protocol was confirmed by the animal welfare and ethics committee, Faculty of Veterinary Medicine, Damanhour University.

DNA extraction: Genomic DNA was extracted from tissue samples of each adult flukes using a DNA extraction GeneJET genomic DNA purification kit (Thermo scientific kits, Germany) according to manufacture's instructions. The DNA quality was assessed on 1.5%. Agarose gel then examined in the UV transilluminator and bands were visualized and photographed.

PCR amplification: Genomic DNA of *F. hepatica* was amplified using two primers (Table 1) for the rDNA region of the partial segment of mitochondrial cytochrome oxidase subunit 1 (COI) gene and internal transcribed spacer (ITS2) regions by polymerase chain reaction. PCR was performed in a reaction volume of 25 µl according to (White, 1993) with some modifications.

The reaction consists of 2.5 µL of 10x Dream Taq Green buffer (Thermo scientific, Germany), 1.5 µL primer each primer forward and reverse (10 pmole), 0.5 µL of (10 mM) dNTP (Thermo scientific, Germany), 0.5 µL of Dream Taq DNA polymerase (Thermo scientific, Germany) and 16.5 µL ddH_2O which finally added to 2 µL genomic DNA (20 ng μL^{-1}). The reactions were done in a thermal cycler (Sure cycler 8800, Malaysia) and thermal cycling program denaturizing at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, annealing temperature as shown in Table 1 for 45 sec and extension at 72°C for 45 sec, final step is the extension at 72°C for 10 min.

The PCR product of each sample (10 µL) and 100 bp DNA ladder (Thermo scientific, Germany) were loaded in 2% (w/v) agarose gels in tris-borate-EDTA (TBE) buffer staining using

Table 1: Showing the primer name, sequence and annealing temperature

Primer name	Sequences	Annealing temperature	References
COI	F:5′-TTTTTTGGGCATCCTGAGGTTTA-3′	45°C	Bowles *et al*. (1995)
	R:5′-TAAAGAAAGAACATAATGAAAATAATC-3′		
ITS2	F:5′-GGTACCGGTGGATCACTCGGCTCGTG-3	55°C	
	R:5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3		

ethidium bromide. The electrophoresis was carried out for 45 min at 100 V. The electrophoresis gel was examined on an UV transilluminator and bands were visualized and photographed.

Sequencing and analysis of PCR product: Purification of the PCR products were done using GeneJET PCR purification Kit (Thermo scientific kits, Germany) and sequenced using PCR primers on an automated sequencer by DNA sequencing services of LGC, Germany. The DNA sequences undergo to further analysis by using bioinformatics tools, basic local alignment search tool (BLAST http://www.ncbi.nlm.nih.gov/blast) and nucleotide alignment using ClustalW (http://www.ebi.ac.uk/clustalw) for each DNA sequence query. Amino acid sequences of the partial fragment of the COI gene were done by ncbi http://www.ncbi.nlm.nih.gov/blast GenBank submission tools.

Molecular phylogenetic analysis: Methods of phylogenetic tree-building consider as particular evolutionary models, during interpretation of the results, it was used different models to determine possible explanations. Unique sequences were used in tree construction. Sequences of COI and ITS2 were entered in the MEGA6 programme (Tamura *et al*., 2013) for the phylogenetic trees construction by using neighbor joining methods and maximum parsimony distance methods. The distance methods were used so as to enhance maximum parsimony because they are less to give inaccuracy when trees contain long branches (Blair and Barker, 1993).

RESULTS

PCR amplification of COI and ITS2 regions and its analysis: The PCR amplification of COI mitochondrial and ITS2 rDNA genes of the isolated *F. hepatica* was done using the primer as mentioned above and presented in Fig. 1.

Mitochondrial COI gene of *F. hepatica* El-Behera isolate was amplified giving a PCR product 421 bp (Fig. 1) and sequenced. The COI gene sequence was submitted to the genbank with accession number is gb |KX470584|. The COI gene alignment consisted from 404 bp and its similarity with GenBank accessions was 99% similarity after BLAST on GenBank (NCBI). The BLAST results showed that the query-COI *F. hepatica* are more similar to the other sequences of *F. hepatica* which derived from the nucleotide sequence databases of NCBI in different countries using neighbor joining tree and maximum parsimony of mega 6 programme indicating that phylogenetic tree based on COI sequence showed that our isolate close to different isolates from Tunisia and Spain (Fig. 2, 3).

The ITS2 fragment of were estimated to give 603 bp long by PCR (Fig. 1) and this fragment was sequenced. Our isolate ITS2 rDNA was obtained; genbank accession number is gb |KX470585|.

The nucleotide sequences of COI and ITS2 of rDNA adult *F. hepatica* were compared with other species sequences of different parts of the world get up from GenBank revealed that, there were an identity between our isolated sample and *F. hepatica* from other hosts in Egypt and other part of the world (Fig. 4, 5). The results showed that the query-ITS2 *F. hepatica* sequence is more similar to the sequence of

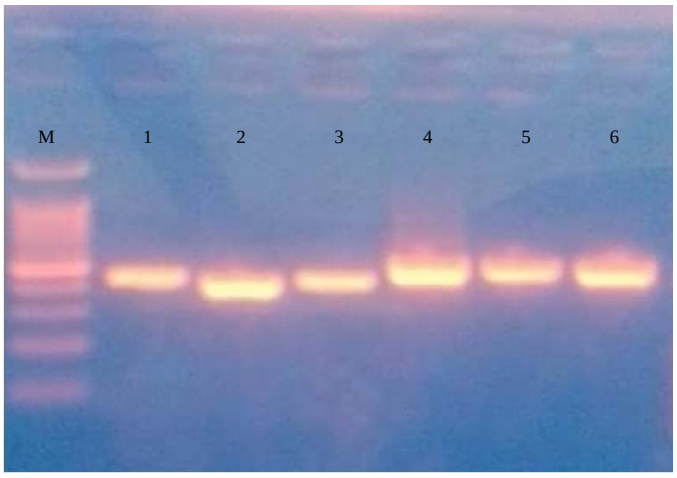

Fig. 1: Agarose gel stained with ethidium bromide showing the PCR products of *Fasciola hepatica*, Lane M DNA marker 100 bp, Lanes 1-3: PCR products generated by COI gene and Lanes 4-6: PCR products generated by ITS2 gene

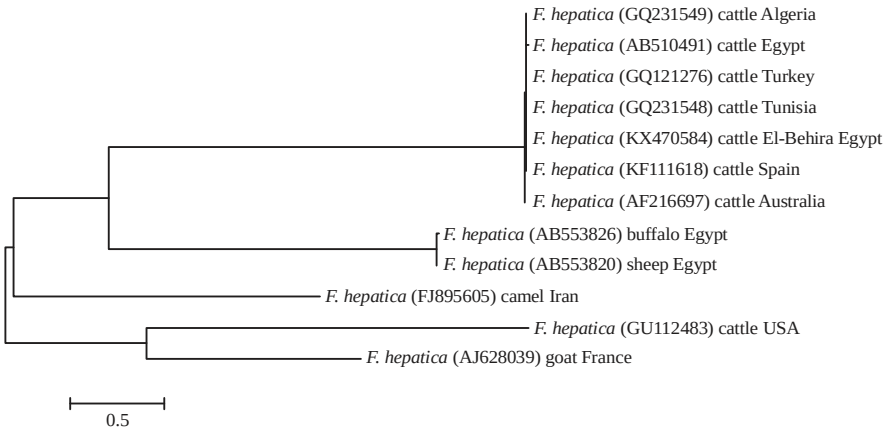

Fig. 2: Phylogenetic tree showing relationship among *Fasciola hepatica* and others from different animal species and regions as inferred from COI data by neighbor-joining method showing bootstrap values using MEGA6

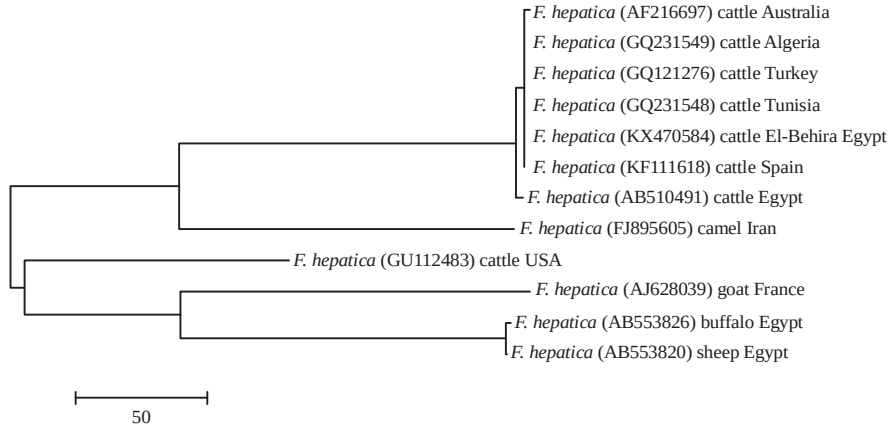

Fig. 3: Phylogenetic tree showing relationship among *Fasciola hepatica* and others from different animal species and regions as inferred from COI data by using maximum parsimony tree of MEGA6

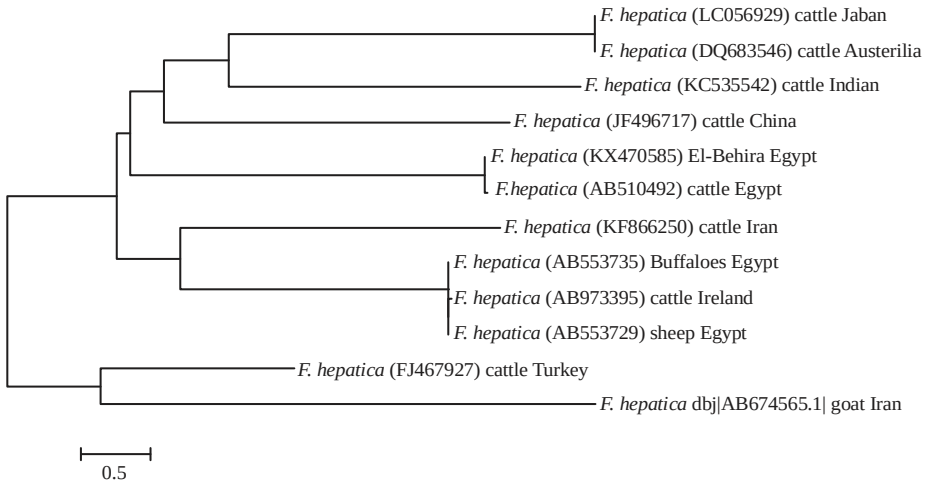

Fig. 4: Phylogenetic tree showing relationship among *Fasciola hepatica* and others from different animal species and regions as inferred from ITS2 data by neighbor-joining method showing bootstrap values using MEGA6

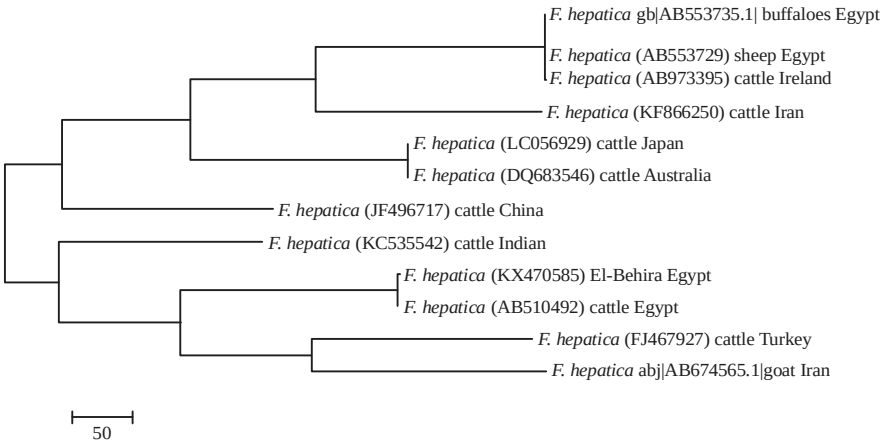

Fig. 5: Phylogenetic tree showing relationship among *Fasciola hepatica* and others from different animal species and regions as inferred from ITS2 data by using maximum parsimony tree of MEGA6

F. hepatica different strains from different regions all over the world. The phylogenetic tree using either neighbor-joining tree or maximum parsimony of ITS2 gene of *F. hepatica* showed close relationship with *F. hepatica* from cattle Egypt and Turkey (Fig. 4, 5).

Phylogenetic trees: In this study, the isolated *F. hepatica* sequences and the available COI and ITS2 sequences for other *F. hepatica* species from different region were compared. Phylogenetic analyses by using the different distance methods and character state method like neighbor-joining of both primers COI and ITS2 were carried out (Fig. 2, 4). Maximum parsimony showed that, the topology of the obtained trees is similar (Fig. 3, 5). The sequences boot strapping with neighbor-joining tree showed a significant support (99%) for the clade that containing *F. hepatica* and others. The value of above the boot strap test of phylogenetic accuracy indicates reliable genetic relation among different members of *F. hepatica*. This study helps in more advanced identification of *F. hepatica* in this study area by using advanced techniques such as polymerase chain reaction and DNA sequencing techniques.

DISCUSSION

Fascioliasis is a major zoonotic parasite in the tropical region (Torgerson and Claxton, 1999) and cause great health problem in Egypt. It is often difficult to identify species of trematodes based on the egg morphology. So, now ordinary techniques used in parasitology diagnosis are complemented by molecular ones to help in support of the parasite taxonomy which may be needed to describe new parasite species depending on the basis of phenotypic analysis (Thompson *et al*., 2004). Few data on genotypic analysis of *F. hepatica* flukes in Egypt makes it necessary to make phylogeographic analysis of flukes concerning the origin of regional populations. The taxonomy of *F. hepatica* based mainly on morphological data, fecal examination is complemented with biochemical changes of liver function tests (Bazh *et al*., 2012). The PCR techniques utilizing the ITS sequences were found to be a trusted tool of identification of the different trematodes species and also the phylogenetic relationships between them (Prasad *et al*., 2007).

In this study for molecular markers for *F. hepatica*, it was characterized the mitochondrial COI and ITS2 rDNA regions. The sequences showed close resemblance with the other *F. hepatica* in different sites of the world. From the phylogenetic trees constructed, results showed that the bootstrap values is almost 99% among the trees obtained and the COI and ITS2 sequences of *F. hepatica* resemble others in different sites. The nucleotide sequence divergence for ITS2 among the isolates was found to be negligible or nil, such as (Shafiei *et al*., 2014; Galavani *et al*., 2016) from Iran. Also, (Erensoy *et al*., 2009; Simsek *et al*., 2011) from Turkey. Likewise, no variation was observed between majority of *F. hepatica* populations from different regions in Turkey, Iran, India even Australia. Compared to COI, the ITS2 sequences in our study showed a higher bootstrap value of 99% confirming that it is the most conserved monophyletic group. This is in accordance with other studies (Omar *et al*., 2013). The sequences of the PCR products from adult stages of the fluke in the present study were showed that no genetic variation in *F. hepatica* collected from cattle of El-Behera region. These observations indicate that the different trematode life cycle

stages do not alter the applicability of the method and corroborate that the sequences of ITS2 are not stage specific and are conserved through different stages of the different fluke development (Sugiyama *et al*., 2002).

CONCLUSION

In conclusion, *F. hepatica* accession number gb |KX470584| and gb |KX470585| was identified morphologically and molecularly by using mitochondrial and nuclear ribosomal DNA genes to show its situation with others allover the world. The COI and ITS2 gene act as effective DNA genetic markers for molecular characterization of *F. hepatica*. However, to ascertain the variations of any population structure, various geographical isolates of *F. hepatica* from different regions and hosts need to be studied more with the usage of more molecular markers. This gives hopeful approach to make diagnosis, control and treatment of *F. hepatica*.

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