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ORIGINAL PAPER

Molecular identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on the ribosomal internal transcribed spacer regions

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Abstract The species of liver flukes of the genus *Fasciola* (Platyhelminthes: Digenea: Fasciolidae) are obligate parasitic trematodes residing in the large biliary ducts of herbivorous mammals. While *Fasciola hepatica* has a cosmopolitan distribution, the other major species, i.e., *Fasciola gigantica* is reportedly prevalent in the tropical and subtropical regions of Africa and Asia. To determine the phylogenic location of *Fasciola* sp. of Assam (India) origin based on rDNA molecular data, ribosomal ITS regions were sequenced and compared with other species of trematodes in the family Fasciolidae. NCBI databases were used for sequence homology analysis using BLAST and ClustalW programs. The phylogenetic trees constructed based upon the ITS (1 and 2) sequences revealed a close relationship with isolates of *F. gigantica* from China, Indonesia, Japan, Egypt, and Zambia, the isolate from China with significant bootstrap values being the closest. Using the novel approach of molecular morphometrics that is based on ITS2 secondary structure homologies, phylogenetic relationships of the various isolates of fasciolid species have also been discussed. While comparing ITS1, the sequence of another Indian isolate designated as *F. gigantica* (accession no. EF198867) showed almost absolute match with *F. hepatica*. Hence, this particular isolate should be identified as *F. hepatica* and not *F. gigantica*.

The nucleotide sequence data reported in this paper have been submitted to the GenBank data with the accession numbers EF027103 and EF027104.

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Introduction

The trematode flukes of the genus *Fasciola* (the sheep liver fluke) are parasites of herbivores and infect humans accidentally causing fascioliasis worldwide. The parasite is very cosmopolitan in distribution being found throughout all regions of the world, both temperate and tropical. *Fasciola hepatica* is the causative agent of fascioliasis or 'liver rot' in ruminants where it may be an important pathogen. Human infections with *F. hepatica* are found in areas where sheep and cattle are raised and where humans consume raw watercress, including Europe, the Middle East, and Asia (Mas-Coma et al. 1999). Infection with *Fasciola gigantica*, on the other hand, is found more commonly in tropical regions of the world, areas affected including Africa, Asia, many Pacific islands including Hawaii (where it has been reported in man), the Middle East, Southern Europe, and the south of the USA (Hammond 1974). Both fasciolids follow a similar two-host life cycle in which freshwater snails of the family Lymnaeidae act as intermediate or vector host and a broad spectrum of mammals including humans, mainly herbivorous large-sized species, act as definitive host (Mas-Coma et al. 2005).

Identification of closely related species based on morphological characters alone can be difficult. The rDNA,

which codes for structural components of ribosomes, is particularly useful for genetic studies because it is highly repeated and contains variable regions flanked by more conserved regions (Hillis and Dixon 1991). The nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2), which occur between the 18S, 5.8S, and 28S coding regions, have proven useful for diagnostic purposes at the level of species (Morgan and Blair 1995; Leon-Regagnon et al. 1999; Tkach et al. 2000; Kostadinova et al. 2003; Scholz et al. 2004; Prasad et al. 2007 and Tandon et al. 2007). *Fasciola* spp. and isolates of *Fascioloides magna*, another member of the same family, from different geographical regions have been discriminated on the basis of ITS sequences (Adlard et al. 1993). The ITS2 sequences have also been used to characterize the liver flukes from mainland China, which include *F. hepatica*, *F. gigantica*, and an intermediate genotype, including polymorphism among ITS2 copies within the same fluke individual (Huang et al. 2004). ITS1, however, has been used for only relatively few numbers of species, though the ITS1 studies confirm the results and conclusions previously reached with ITS2 (Mas-Coma et al. 2001).

Most phylogenetic studies using current methods have focused on primary DNA sequence information. However, RNA secondary structures are particularly useful in systematics because they include characteristics not found in the primary sequence that give 'morphological' information (Caetano-Anollés 2002). The novel approach of molecular morphometrics that relies both on traditional morphological comparison and on molecular sequence comparison by measuring the structural parameters of the ITS2 secondary structure homologies (geometrical features, bond energies, base composition, etc.) is recently being used to study the phylogenetic relationships of various species (Billoud et al. 2000). This method allows one to take into account the regions where multiple alignments are barely reliable because of a large number of insertions and deletions. This method is based on the assumption that secondary structure can be phylogenetically as significant as primary sequence. It is well-known that rRNA is highly conserved throughout evolution. Thus, the secondary structure elements of the RNA molecule, i.e., the helices, loops, bulges, and separating single-stranded portions, can be considered phylogenetic characters (Zwieb et al. 1981, Schultz et al. 2005, Grajales et al. 2007).

In the present study, our main objective was to describe the molecular identification of the Indian liver fluke (the Assam isolates) on the basis of ITS sequences of the parasite collected from ruminant hosts. We also aimed at determining the species prevalent in the northeastern region of the country by comparing these sequences by primary sequence analysis and molecular morphometrics data.

Materials and methods

Parasite material and DNA isolation

Adult *Fasciola* were obtained in live form from hepatic biliary ducts of freshly slaughtered cow, *Bos indicus*. The worms recovered from these hosts represented the geographical isolates from Assam, Northeast India and morphologically resembled *F. gigantica* (deposition number of paratypes at Zoological Survey of India, Kolkata=W7787/1). Eggs were obtained from mature adult flukes by squeezing between two glass slides. For the purpose of DNA extraction, adult flukes were processed singly; eggs recovered from each of these specimens were also processed separately. The adult flukes were first immersed in digestion extraction buffer (containing 1% SDS, 25 mg Proteinase K) at 37°C overnight. DNA was then extracted from lysed individual worms by standard ethanol precipitation technique (Sambrooke et al. 1989) and also extracted on FTA cards using Whatman's FTA Purification Reagent as described elsewhere (Prasad et al. 2007). DNA from the eggs was extracted only with the FTA card technique.

DNA amplification, sequencing, and its analysis

The rDNA region spanning the ITS regions was amplified from DNA obtained from the fluke by polymerase chain reaction (PCR). We used the universal primers considered to be the general primers for trematodes and are designed based on conserved ITS sequences of *Schistosoma* species following Bowles et al. (1995) as detailed below:

1. ITS2 region—3S (forward): 5′-GGTACCGGTGGAT CACTCGGCTCGTG-3′ and A28 (reverse): 5′-GGG ATCCTGGTTAGTTTCTTTTCCTCCGC-3′.
2. ITS1 region—BD1 (forward): 5′-GTCGTAACA AGGTTTCCGTA-3′ and 4S (reverse): 5′-TCTAGA TGCGTTCGAA (G/A) TGTCGATG-3′.

The PCR amplification was performed following the standard protocol (White 1993) with minor modifications as described elsewhere (Prasad et al. 2007; Tandon et al. 2007). The resultant PCR products were separated by electrophoresis through 1.6% (*w/v*) agarose gels in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light, and then photographed. The known size fragments of Phi X 174 DNA/*Hae*III digest in agarose gel were used as marker. For DNA sequencing, the PCR products were purified using the Genei Quick PCR purification kit and sequenced in both directions using PCR primers on an automated sequencer by the DNA sequencing services of TCGA, New Delhi and Bangalore Genei.

The DNA sequences were put to further analysis by using various bioinformatics tools including similarity

search BLAST (http://www.ncbi.nlm.nih.gov/blast) and phylogenetic prediction by ClustalW (http://www.ebi.ac.uk/clustalw) for each query DNA sequence.

Molecular phylogenetic analysis

Initially, the sequences were aligned using ClustalW multiple alignment (Thompson et al. 1994) with the default gap and extension penalties used by this program. Phylogenetic tree-building methods presume particular evolutionary models. Therefore, while interpreting the results obtained, we considered different tree-building models to entertain possible explanations. Only unique sequences were used in tree construction. ITS sequences were entered in the MEGA for construction of the phylogenetic trees using maximum parsimony and distance methods, namely, neighbor-joining, UPGMA, and minimum evolution. Branch support was given using 1,000 bootstrap replicates in MEGA (Hillis and Bull 1993).

Phylogenetic analysis was also carried out using the Bayesian approach with combined datasets using Mrbayes 3.1 (Huelsenbeck and Ronquist 2001), wherein each data partition is allowed to have a different evolution rate.

Predicted ITS2 RNA secondary structures and analyses

Secondary structures of ITS2 sequences of various fasciolid species were reconstructed by aligning their sequences using BioEdit (Hall 1999). The acquired structures with restrictions and constrains were submitted in MFOLD (Zuker 2003). RNA was folded at a fixed temperature of 37°C, and the structure chosen from different output files was the desired six-helicoidal ring or the one with the highest negative free energy if various similar structures were obtained.

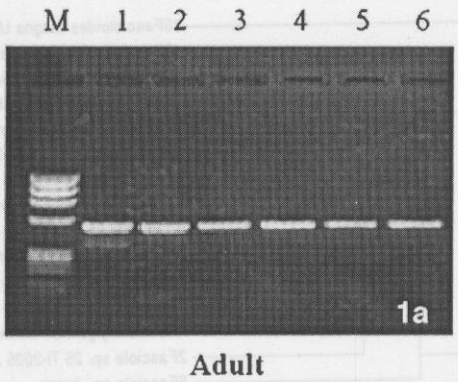

Fig. 1 PCR products of *Fasciola* sp. adult DNA using primer set 3S–A28 for ITS2, marker Phi X 174 DNA/*Hae*III digest

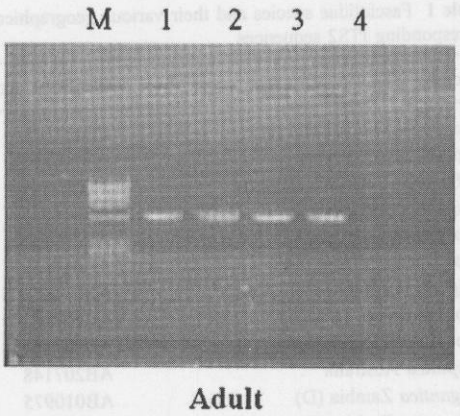

Fig. 2 PCR product of *Fasciola* sp. adult DNA using primer set BD1–4S for ITS1, marker Phi X 174 DNA/*Hae*III digest

Results

PCR amplification of ITS regions and its analysis

The PCR-amplified products were successfully obtained using the primers as mentioned above and are depicted in Figs. 1 and 2. The nucleotide sequences were obtained for ITS1 and ITS2 of rDNA of *F. gigantica* and were compared with sequences of other fasciolid species obtained from GenBank by the ClustalW tree-building method (Fig. 3). The fragments of amplified DNA were estimated to be 480–550 bp long. No intraspecific variations in length or composition of the sequence were observed, and the ITS sequences of both adult and egg origin were found to be identical in length as well as composition. For comparative purpose, the ITS2 sequences of fasciolids from

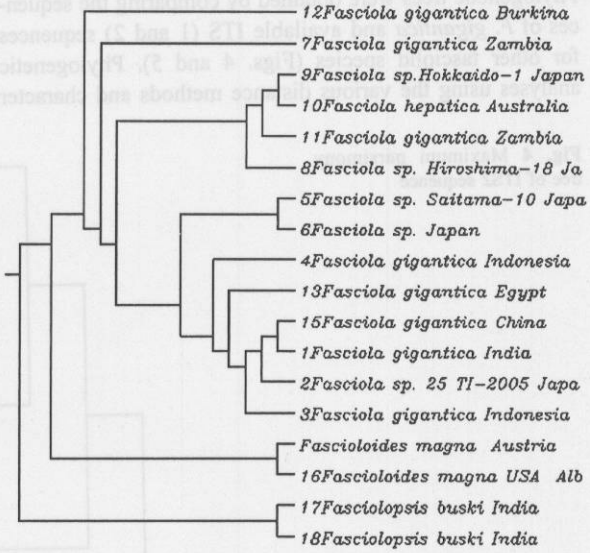

Fig. 3 ClustalW tree for ITS2 sequence

Table 1 Fasciolidae species and their various geographical isolates used in this study with the respective GenBank accession numbers for the corresponding ITS2 sequences

Species	GenBank accession no.	Sequence length (bp)	Classification
Fasciola gigantica India (B)	EF027103	720	Digenea: Fasciolidae
F. sp. 25 TI-2005 Japan	AB207152	505	Digenea: Fasciolidae
F. gigantica Indonesia (E)	AB207149	505	Digenea: Fasciolidae
F. gigantica Indonesia	AB010977	505	Digenea: Fasciolidae
F. sp. Saitama-10 Japan	AB207151	505	Digenea: Fasciolidae
F. sp. Japan	AB010979	505	Digenea: Fasciolidae
F. gigantica Zambia	AB010976	505	Digenea: Fasciolidae
F. sp. Hiroshima-18 Japan	AB207153	506	Digenea: Fasciolidae
F. sp. Hokkaido-1 Japan	AB207150	506	Digenea: Fasciolidae
F. hepatica Australia	AB207148	506	Digenea: Fasciolidae
F. gigantica Zambia (D)	AB010975	505	Digenea: Fasciolidae
F. gigantica Burkina Faso	AJ853848	950	Digenea: Fasciolidae
F. gigantica Egypt (A)	DQ383512	509	Digenea: Fasciolidae
Fasciola gigantica China (C)	AJ557569	361	Digenea: Fasciolidae
Fascioloides magna Austria	DQ683545	538	Digenea: Fasciolidae
F. magna USA: Albany	EF051080	2920	Digenea: Fasciolidae
Fasciolopsis buski India	DQ351842	498	Digenea: Fasciolidae
F. buski India	DQ351841	481	Digenea: Fasciolidae

B sequence generated as part of the present study, *A*, *C*, *D*, and *E* sequences from different geographical isolates of *F. gigantica* used for the construction of secondary structures

various geographical regions were obtained from GenBank (Table 1). The BLAST hit results show that the query ITS sequences were more similar to the sequences of various geographical isolates of *Fasciola* sp., *F. hepatica* and *F. gigantica* besides *Fasciolopsis buski* and *F. magna* (both belonging to the same family, i.e., Fasciolidae).

Phylogenetic trees

Phylogenetic trees were obtained by comparing the sequences of *F. gigantica* and available ITS (1 and 2) sequences for other fasciolid species (Figs. 4 and 5). Phylogenetic analyses using the various distance methods and character method like maximum parsimony showed that the topology is similar among the trees obtained. Bootstrapping of the sequences with neighbor-joining revealed significant support for the clade containing *F. hepatica*, *F. gigantica*, *F. buski*, and *F. magna*. The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of fasciolids. The phylogenetic trees constructed based upon the ITS (1 and 2) sequences by multiple tree-building methods in MEGA revealed a close relationship with isolates of *F. gigantica* from China, Indonesia, Japan, Egypt, and Zambia. While comparing the ITS1 sequences through BLAST search (ClustalW alignment), the sequence of another Indian isolate designated as *F. gigantica* (accession no. EF198867) from

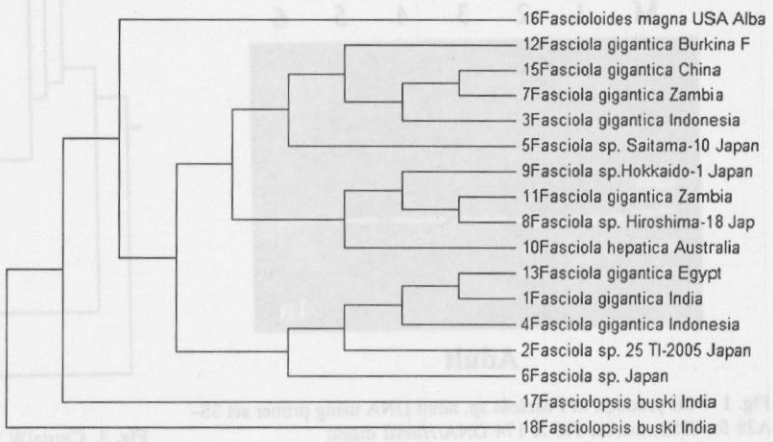

Fig. 4 Maximum parsimony tree of ITS2 sequence

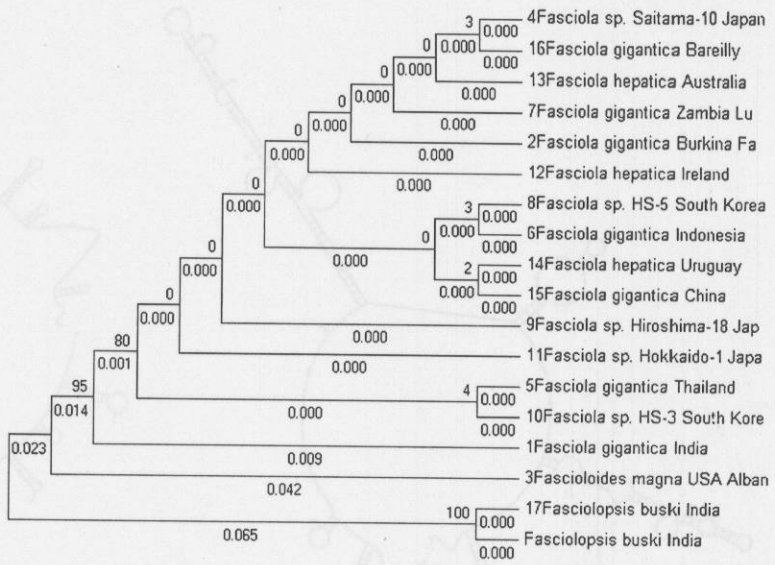

Fig. 5 Neighbor-joining tree of ITS1 sequence showing bootstrap values and distance

the Indian Veterinary Research Institute, Bareilly showed almost absolute match with *F. hepatica*.

Secondary structure analysis

Five predicted RNA secondary structures were reconstructed from the unique sequences with the highest negative free energy of *F. gigantica* to provide the basic information for phylogenetic analysis (Figs. 6, 7, 8, 9, and 10). The ITS2 plus flanking regions of the nuclear region ranged from 720 bp in *F. gigantica* India to a minimum length of 361 bp in *F. gigantica* China. The secondary structural features of

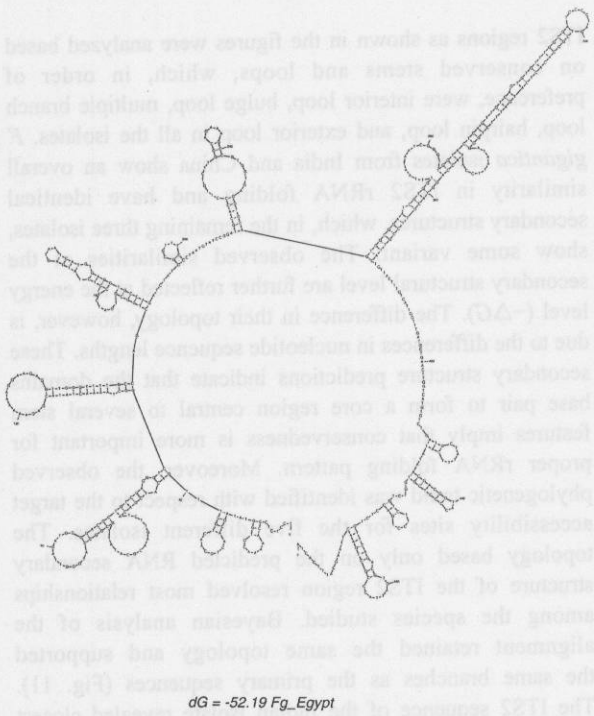

Fig. 6 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

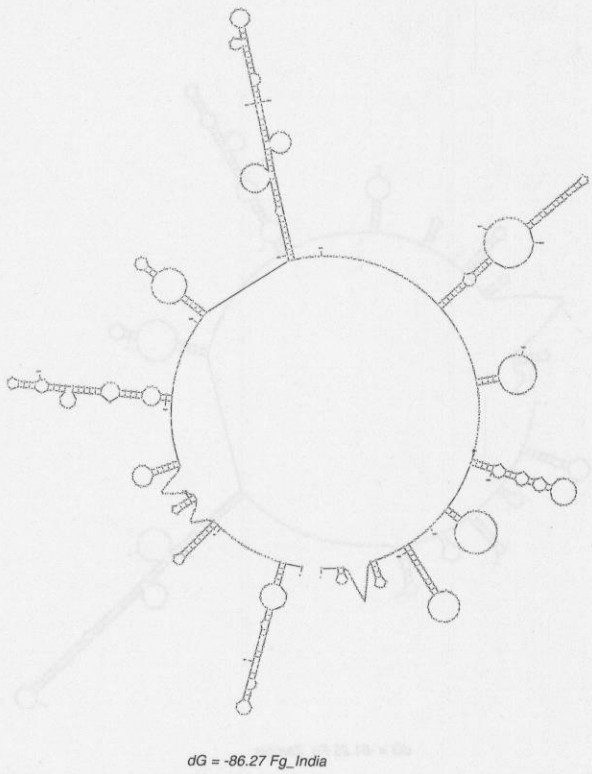

Fig. 7 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

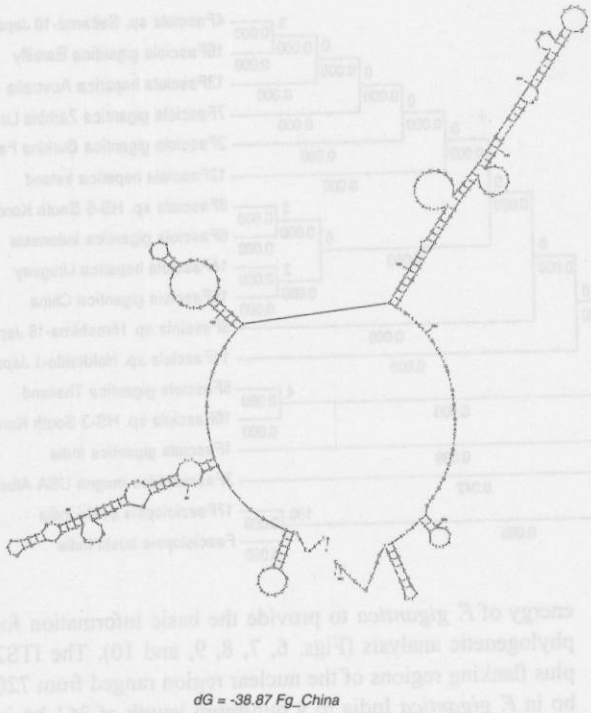

Fig. 8 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

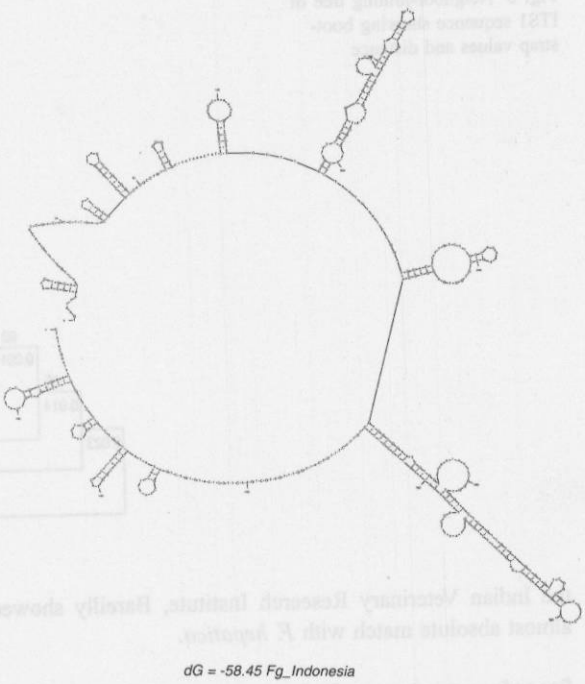

Fig. 10 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

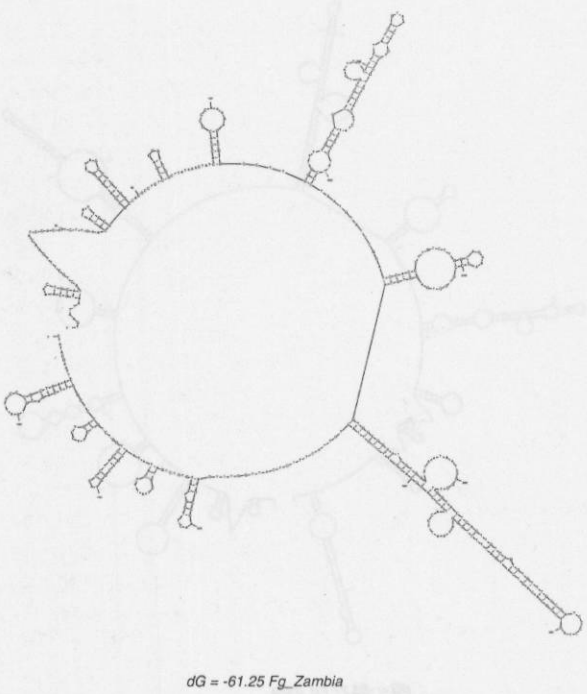

Fig. 9 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

ITS2 regions as shown in the figures were analyzed based on conserved stems and loops, which, in order of preference, were interior loop, bulge loop, multiple branch loop, hairpin loop, and exterior loop in all the isolates. *F. gigantica* isolates from India and China show an overall similarity in ITS2 rRNA folding and have identical secondary structures, which, in the remaining three isolates, show some variant. The observed similarities at the secondary structural level are further reflected at the energy level ($-\Delta G$). The difference in their topology, however, is due to the differences in nucleotide sequence lengths. These secondary structure predictions indicate that the domains base pair to form a core region central to several stem features imply that conservedness is more important for proper rRNA folding pattern. Moreover, the observed phylogenetic trend was identified with respect to the target accessibility sites for the five different isolates. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences (Fig. 11). The ITS2 sequence of the Indian isolate revealed closest similarity with the isolate from China with significant bootstrap value.

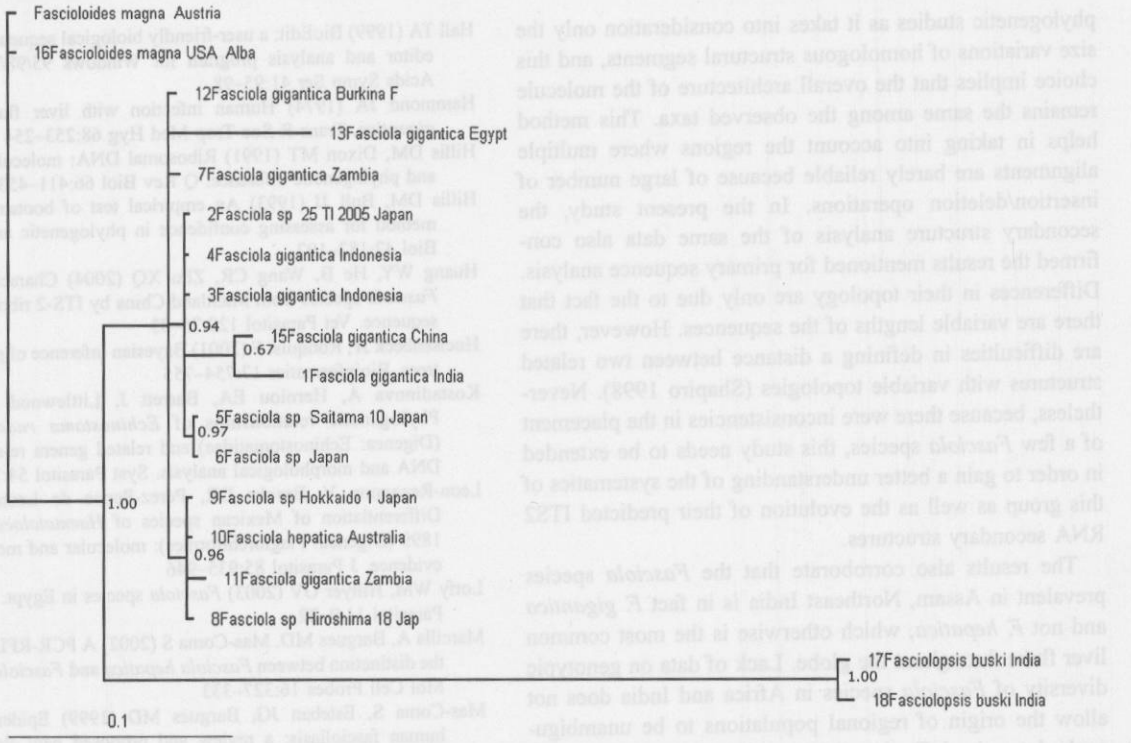

Fig. 11 Phylogenetic relationships between members of family Fasciolidae. This tree shows hypothetical Bayesian analysis phylogeny based on the secondary structure alignment data of the ITS2 region. The numbers are equivalent to bootstrap percentages

Discussion

The taxonomy of *Fasciola* spp. has been based mainly on morphological data complemented with ecological, cytological, and pathological results as well as clinical manifestations. Morphological differences found on stained and mounted adult specimens have been widely used to discriminate between platyhelminth species (Miyazaki 1974). It is possible to distinguish between adult *F. hepatica* and *F. gigantica* on the basis of morphology, but much variation exists. Differentiating between two species is not possible on the basis of clinical, pathological, or immunological findings and their eggs are morphologically very similar (Lotfy and Hillyer 2003). Consequently, where both species occur concurrently or in overlapping geographical distribution, it is not possible to be certain as to which species is responsible for the disease. The low number of records of infection with *F. gigantica* may well be due to the lack of good tools to distinguish this species from *F. hepatica* (Marcilla et al. 2002).

The comparison of ITS sequences from worms of different hosts and of different countries indicates that there exists a high species-specific homogeneity. In the present study, primary sequence analysis revealed a close relationship between the query sequence (from Northeastern region of India) and isolates of *F. gigantica* from China, Indonesia, Japan, Egypt, and Zambia (Adlard et al. 1993). The phylogenetic trees constructed showed that the groups of multiple closely related genotypes of *F. gigantica* from Asia are broadly sympatric. Such a pattern is expected for species with high gene flow whose populations have not been sundered by long-term biogeographic barriers (Avise 2000). The sequence of another Indian isolate, designated as *F. gigantica* (accession no. EF198867) from the Indian Veterinary Research Institute, Bareilly showed almost absolute match with *F. hepatica*. Hence, on the basis of molecular similarity, this particular isolate should be identified as *F. hepatica* and not *F. gigantica*.

In this study, we present a new approach of molecular morphometrics in which the measurable structural parameters of the molecules are directly used as specific characters to construct a phylogenetic tree. These structures are inferred from the sequence of the nucleotides, often using energy minimization (Zuker 1994). Several patterns of predicted secondary structures of RNA were constructed from unique ITS sequences from different geographical isolates of *F. gigantica*, which provided us with the additional information for the correct identification of the species prevalent in the region. Molecular morphometrics appears to be complimentary to classical primary sequence analysis in

phylogenetic studies as it takes into consideration only the size variations of homologous structural segments, and this choice implies that the overall architecture of the molecule remains the same among the observed taxa. This method helps in taking into account the regions where multiple alignments are barely reliable because of large number of insertion/deletion operations. In the present study, the secondary structure analysis of the same data also confirmed the results mentioned for primary sequence analysis. Differences in their topology are only due to the fact that there are variable lengths of the sequences. However, there are difficulties in defining a distance between two related structures with variable topologies (Shapiro 1998). Nevertheless, because there were inconsistencies in the placement of a few *Fasciola* species, this study needs to be extended in order to gain a better understanding of the systematics of this group as well as the evolution of their predicted ITS2 RNA secondary structures.

The results also corroborate that the *Fasciola* species prevalent in Assam, Northeast India is in fact *F. gigantica* and not *F. hepatica*, which otherwise is the most common liver fluke throughout the globe. Lack of data on genotypic diversity of *Fasciola* species in Africa and India does not allow the origin of regional populations to be unambiguously determined. Further studies with additional molecular markers are needed to determine the population structure and divergence between the two closely related species of this genus.

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