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ORIGINAL PAPER

PCR-based determination of internal transcribed spacer (ITS) regions of ribosomal DNA of giant intestinal fluke, *Fasciolopsis buski* (Lankester, 1857) Looss, 1899

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Abstract *Fasciolopsis buski*, the zoonotic intestinal flukes of pigs in South and Southeast Asia, is commonly prevalent in regions across Northeast India. Populations of the fluke collected from different parts of the region exhibit variations in morphology. The main objective of our study was to provide molecular characterization of the parasite so as to supplement morphological criteria, using ribosomal DNA cluster (rDNA), which is flanked by more conserved internal transcribed spacer (ITS) regions. We describe herein the ITS sequences of the parasite collected from swine hosts of Assam region. The ITS sequences of both egg and adult origins were found to be identical in length and composition. Phylogenetically, *F. buski* resembles closely the other members of family Fasciolidae, showing significant expectation value in the alignment. The results corroborate that the ITS sequences are not stage specific and are conserved through different stages of development of the fluke, and thus could be used as species markers.

Nucleotide sequence data reported in this paper have been submitted to the Genbank data with the accession numbers DQ351841–DQ351844.

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Introduction

The giant intestinal fluke, *Fasciolopsis buski* (Trematoda: Fasciolidae), is widely distributed in India and neighboring countries of the continent in South and Southeast Asia (Roy and Tandon 2003). The fluke is the etiological agent of the disease commonly known as fasciolopsosis. The infection occurs by ingestion of raw aquatic vegetation or food plants that are contaminated with the infective encysted larvae, the metacercariae. In endemic zones, pigs, dogs, and rabbits act as reservoir of infection. In India, the parasite has been reported from different states including those in the Northeast. Variations in the morphology of the fluke have been observed when collected from different geographical regions (Roy and Tandon 1993).

Identification of closely related species based on morphological characters alone can be difficult. This is particularly so in the case of soft-bodied animals parasites such as digenean trematodes. However, recent advances in molecular biology, in particular the amplification of specific DNA regions via the polymerase chain reaction (PCR) and improved sequencing techniques, have been employed to resolve taxonomic issues related to various helminth parasites by comparing their DNA, utilizing genetic markers in nuclear ribosomal DNA (rDNA) and mitochondrial DNA in particular (Blair et al. 1996). The rDNA cluster, 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). It enhances PCR amplification because many templates are available for initial

priming and allows primers to be designed to anneal the known conserved regions to amplify across unknown variable regions. In context of metazoan parasites, 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; Nolan and Cribb 2005). These sequences are often assumed to be homogenized within individuals and populations of the same species by concerted evolution (Dover 1982; Hillis and Davis 1988). ITS2 sequences have been shown to be a sensitive marker at the species level in trematodes, whereas the sequences in the ITS1 might be less conserved than those in the ITS2 (Luton et al. 1992). Among the fasciolid digeneans, isolates of *Fasciola* spp. and of *Fascioloides magna* from different geographical regions have been discriminated on the basis of ITS sequences (Adlard et al. 1993). Studies on phylogeny and/or intraspecific variations in *Paragonimus* species have recently been done using second ITS (ITS2) sequences (Blair et al. 1997, 1999; Iwagami et al. 2000). The usefulness of the ITS2 region for species discrimination has also been demonstrated in nematodes, for example, *Strongylus*, *Trichostrongylus* species, larvae of *Dictyocaulus viviparus* and *D. eckerti* (Campbell et al. 1994; Hoste et al. 1995; Samson-Himmelstjerna et al. 1997).

In the present study, we amplified the ITS regions of *Fasciolopsis* (DNA from egg and adult stages) and assessed their potential for discriminating between species and genera within the group. Our main objectives were to determine whether the sequences are stage specific and conserved or not and to find out the species-specific molecular markers by amplifying the ITS (I and II) regions of the parasite DNA both from adult and egg stages.

Materials and methods

Parasite material and DNA isolation

Live adult *F. buski* were obtained from the intestine of freshly slaughtered pig, *Sus scrofa domestica* at local abattoirs. The worms recovered from these hosts represented the geographical isolates from Assam region of Northeast India. Eggs were obtained from mature adult flukes by squeezing between two glass slides. For the purpose of DNA extraction, adult flukes collected from different host animals 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% sodium dodecyl sulfate (SDS), 25 mg Proteinase K] at 37°C for overnight.

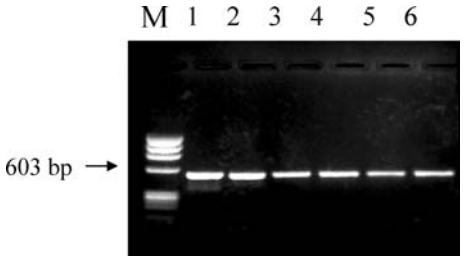

Fig. 1 Agarose gel stained with ethidium bromide showing the PCR products of *Fasciolopsis buski* genomic DNA from adult fluke using the primer set 3S-A28 for ITS2 (*lanes 1–6*). *M* Molecular weight marker (∅×174 DNA/HaeIII Digest)

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. The FTA cards were allowed to dry for 1 h at room temperature before punching; two to three sample discs of 1.2-mm size were taken from the desired spot using coring device assuming a 25-µl reaction and placed in PCR amplification tube. The discs, then washed with FTA Purification reagent and TE Buffer, were allowed to dry at room temperature for 1 h to make them ready for PCR. 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 egg and adult stages of the fluke by PCR. We used the universal primers based on conserved ITS sequences of *Schistosoma* species following (Bowles et al. 1995) as detailed below:

1. ITS2 region-3S (forward), 5′GGTACCGGTGGATC ACTCGGCTCGTG-3′ and A28 (reverse), 5′-GGGATC CTGGTTAGTTTCTTTTCCTCCGC-3′
2. ITS1 region-BD1 (forward), 5′GTCGTAACAAGG TTTCCGTA-3′ and 4S (reverse), 5′TCTAGATGCG TTCGAA (G/A) TGTCGATG-3′

The PCR amplification was performed following the standard protocol (White 1993) with minor modifications in 100 mM Tris HCl (pH 9.0), 500 mM KCl, 1.5 mM $MgCl_2$,

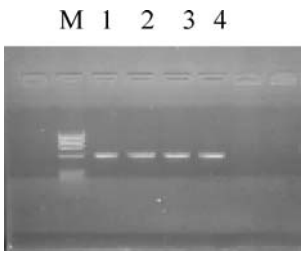

Fig. 2 Agarose gel stained with ethidium bromide showing the PCR products of *Fasciolopsis buski* genomic DNA from adult fluke using the primer set BD1-4S for ITS1 (*lanes 1–4*). *M* Molecular weight marker (∅× 174 DNA/HaeIII Digest)

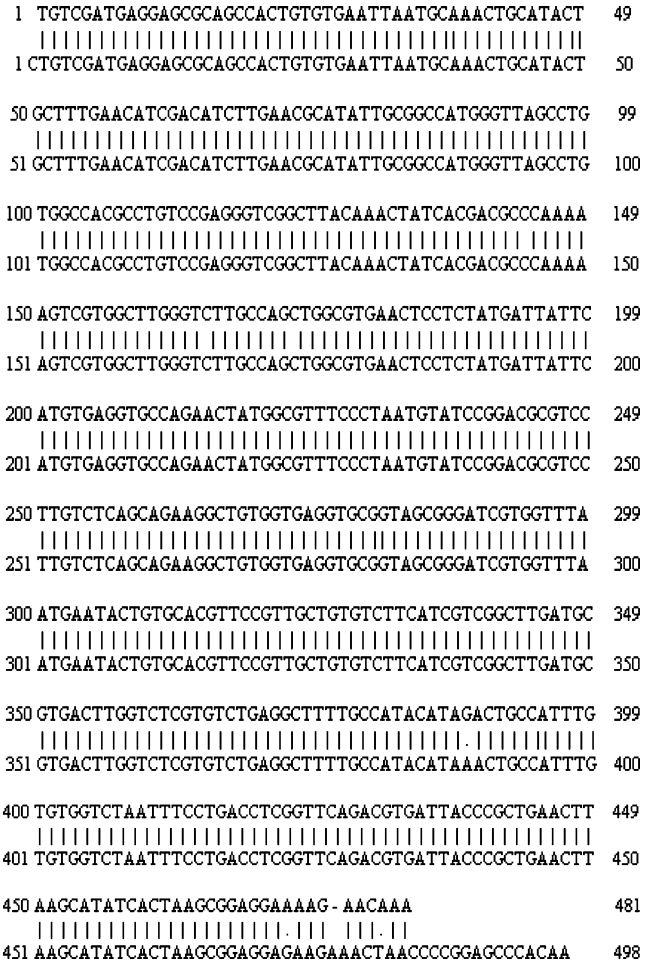

Fig. 3 Pair-wise alignment of ITS2 sequence of egg and adult stages of *Fasciolopsis buski*

and 0.2 mM deoxynucleotide triphosphates each of dATP, dGTP, dCTP, dTTP, 0.25 mM of each primer, and 2.5 units of Taq polymerase (Bangalore Genei Pvt., India). The DNA was preheated at 94°C for 5 min and added to each PCR reaction. The PCR cocktail (final reaction volume 25 μl) was amplified with the following conditions: 26 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 38 s and extention at 72°C for 42 s followed by a final extention at 72°C for 10 min. The resultant PCR products were separated by electrophoresis through 1.6% (*w*/*v*) agarose gels in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide, transilluminated under ultraviolet light and then photographed. The known size fragments of Phi X 174 DNA/HaeIII Digest in agarose gel were used as marker. For DNA sequencing, the PCR products were purified using Genei Quick PCR purification Kit and sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of Bangalore Genei.

The DNA sequences were put to further analysis by using various Bioinformatics tools including similarity search basic local alignment search tool (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

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 the neighbor-joining, UPGMA and minimum evolution. The distance methods were used so as to augment maximum parsimony because they are less likely to give errors when trees contain long branches (Blair and Barker 1993).

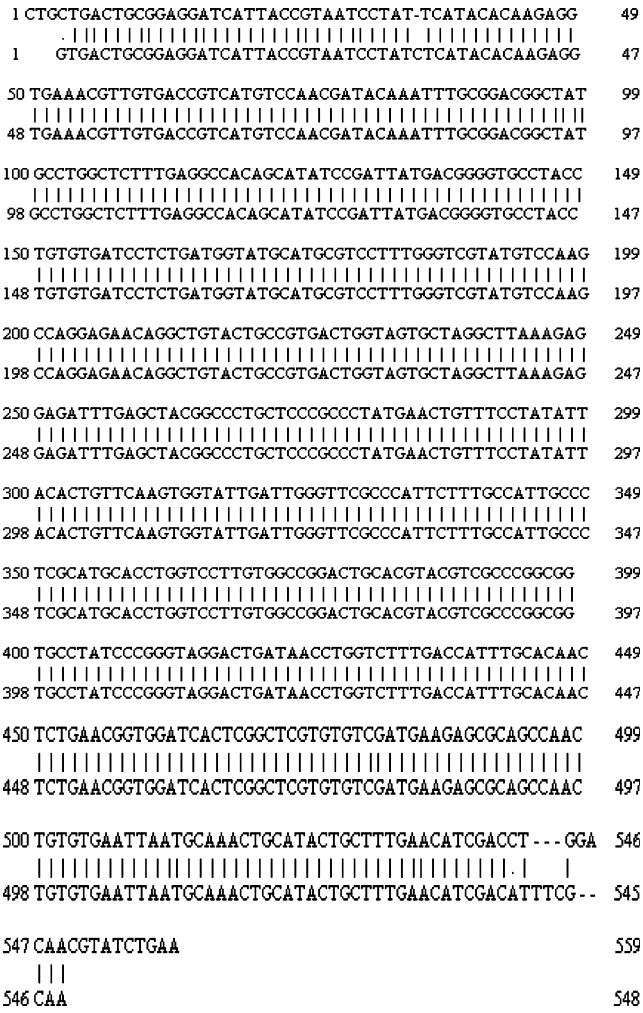

Fig. 4 Pair-wise alignment of ITS1 sequence of egg and adult stages of *Fasciolopsis buski*

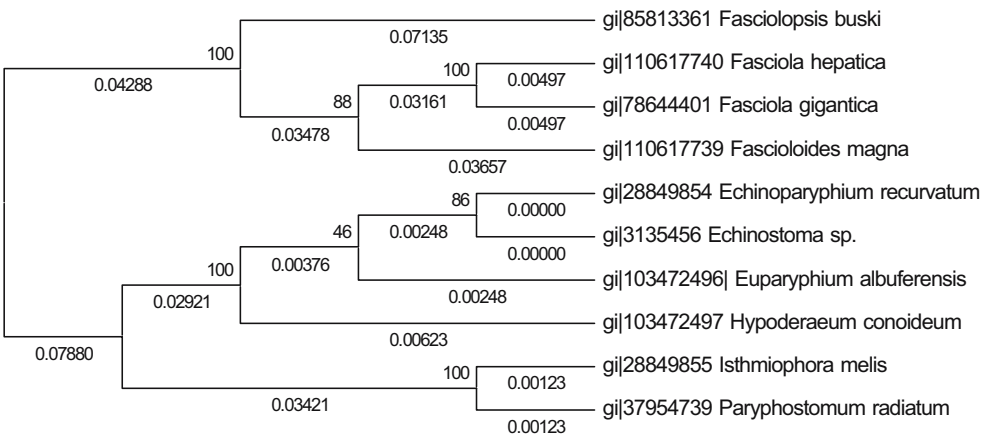

Fig. 5 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS2 data by neighbor-joining method showing bootstrap values

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 adult and egg stages of *F. buski* and were compared with sequences of other trematode species obtained from GenBank. The fragments of amplified DNA were estimated to be 480–550 bp long. Sequence analysis of the ITS PCR products revealed that the alignments of the rDNA region spanning ITS2 were 481 and 498 bp for forward primer and 559 and 548 bp for forward primer of ITS1, respectively, in adult and egg. No intra-specific 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 and composition (Figs. 3, 4).

The BLAST hit results show that the query-ITS2 *F. buski* forward and reverse sequences are more similar to the sequences of the species *Fasciola gigantica*, *Echinostoma revolutum*, *Isthmiophora melis*, *Echinostoma* sp., *Paryphostomum radiatum*, *Echinostoma trivolvis*, *Echinostoma paraenei*, *Fasciola* sp., *Fasciola hepatica*, and *Petasiger phalacrocorasis* obtained from the nucleotide sequence databases of NCBI. These results also showed that the query-ITS1 *F. buski* forward sequence is more similar to the sequence of the species *F. gigantica*, *Fasciola* sp., *F. hepatica*, *E. paraenei*, *E. revolutum*, *E. trivolvis*, and *Echinostoma caproni*.

Phylogenetic trees

Phylogenetic trees were obtained by comparing the sequences of *F. buski* and available ITS (I and II) sequences for other digenean trematodes including fasciolid species. Phylogenetic analyses using the various distance methods and character state method like maximum parsimony show that the topology is similar among the trees obtained (Figs. 5, 6, 7, and 8). Boot strapping of the sequences with neighbor-joining revealed significant support (100%) for the clade containing *F. buski*, *F. hepatica*, *F. gigantica*, and *F. magna*. The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of fasciolids.

Discussion

Morphological differences found in adult specimens have been widely used to discriminate between platyhelminth

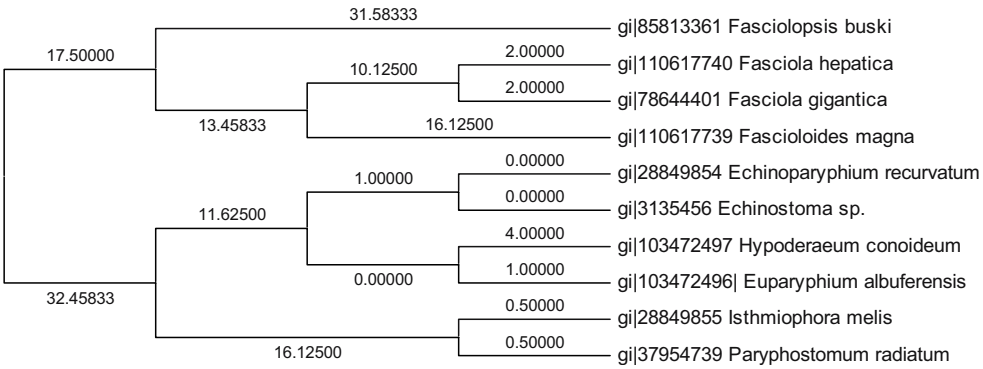

Fig. 6 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS2 data by maximum parsimony method

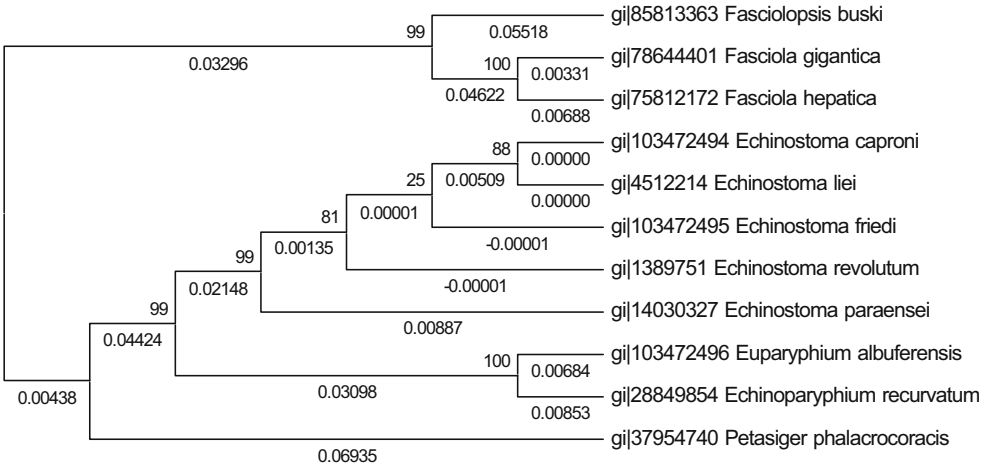

Fig. 7 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS1 data by neighbor-joining method

species (Miyazaki 1974). However, traditional diagnostic techniques in parasitology are now complemented by a variety of molecular tools to help in resolving the taxonomic issues associated with describing new species or strains on the basis of phenotypic characteristics (Thompson et al. 2004). Lack of data on genotypic diversity of fasciolid flukes in India makes it difficult to perform fine-scale phylogeographic analysis of populations and does not allow the origin of regional populations to be umambiguously determined. The taxonomy of *F. buski* has been based mainly on morphological data complemented with ecological, cytological, and pathological results as well as clinical manifestations. PCR-based techniques utilizing the ITS sequences have proven to be a reliable tool to identify the various lung fluke species and their phylogenetic relationships (Blair et al. 1999; Iwagami et al. 2000). In respect of *F. buski*, the 18S rRNA sequence is known (Blair and Barker 1993, accession number L06668) but the sequences from ITS markers are not described so far. In search for molecular markers for this species, we characterized the ITS region of rDNA.

The sequences showed close resemblance with the members of families Fasciolidae and Echinostomatidae (Echinostomatinae). From the phylogenetic trees constructed, sequences of two distinct clusters are revealed, one of *Fasciola* spp. and the other comprising the genera *Echinostoma*, *Paryphostomum*, and *Isthmiophora*. Our results showed that the bootstrap values is almost 100% among the trees obtained, and the ITS sequences of *F. buski* resemble other members of Fasciolidae. In closely related fasciolids, *F. hepatica* and *F. gigantica*, intraspecific nucleotide sequence divergence for ITS2 among the isolates from Mexico was found to be negligible or nil (Adlard et al. 1993). Likewise, no variation was observed between most of the populations of liver fluke species from different regions in East Europe, Central Asia, and Caucasus; while only one specimen of *F. hepatica* from Armenia showed 0.3% variation, three out of 11 populations of *F. gigantica* differed at four nucleotide transitions only (Semyenova et al. 2005).

The evolutionary distance between organisms is indicated by horizontal branch lengths, which reflect the number of nucleotide substitutions per site along the branch from the node to the end points. Compared to ITS1, the ITS2 sequences in our study showed a higher bootstrap value of 100% confirming that it is a highly conserved monophyletic

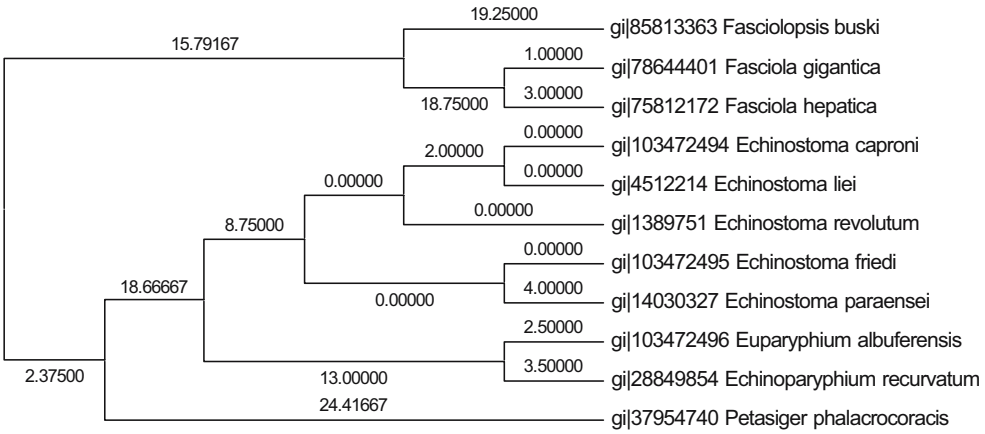

Fig. 8 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS1 data by maximum parsimony method

group. This is in accordance with other studies on trematodes, which indicate that sequences in the ITS1 might be less conserved then those in the ITS2 region (Luton et al. 1992).

The sequences of the PCR products from adult and egg stages of the fluke in the present study were found to be identical in length and composition and showed no genetic variability in the worms collected from pigs of the Assam region. These findings indicate that the different life cycle stages do not alter the applicability of the method and corroborate that the ITS sequences are not stage specific and are conserved through different stages of the development of the fluke (Sugiyama et al. 2002).

In conclusion, as has already been demonstrated for other parasitic helminths, ITS can serve as an effective genetic marker for molecular identification. However, to ascertain intra-specific strain variations, if any, and to determine the population structure, different geographical isolates of *F. buski* from the region need to be studied with the use of additional molecular markers.

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