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Molecular characterization of pouched amphistome parasites (Trematoda: Gastrothylacidae) using ribosomal ITS2 sequence and secondary structures

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Abstract

Members of the family Gastrothylacidae (Trematoda: Digenea: Paramphistomata) are parasitic in ruminants throughout Africa and Asia. In north-east India, five species of pouched amphistomes, namely *Fischoederius cobboldi*, *F. elongatus*, *Gastrothylax crumenifer*, *Carmyerius spatiosus* and *Velasquezotrema tripurensis*, belonging to this family have been reported so far. In the present study, the molecular phylogeny of these five gastrothylacid species is derived using the second internal transcribed spacer (ITS2) sequence and secondary structure analyses. ITS2 sequence analysis was carried out to see the occurrence of interspecific variations among the species. Phylogenetic analyses were performed for primary sequence data alone as well as the combined sequence-structure information using neighbour-joining and Bayesian approaches. The sequence analysis revealed that there exist considerable interspecific variations among the various gastrothylacid fluke species. In contrast, the inferred secondary structures for the five species using minimum free energy modelling showed structural identities, in conformity with the core four-helix domain structure that has been recently identified as common to almost all eukaryotic taxa. The phylogenetic tree reconstructed using combined sequence–structure data showed a better resolution, as compared to the one using sequence data alone, with the gastrothylacid species forming a monophyletic group that is well separated from members of the other family, Paramphistomidae, of the amphistomid flukes group. The study provides the molecular characterization based on primary sequence data of the rDNA ITS2 region of the gastrothylacid amphistome flukes. Results also demonstrate the phylogenetic utility of the ITS2 sequence–secondary structure data for inferences at higher taxonomic levels.

Introduction

Amphistomes are parasitic in the digestive tracts of many vertebrates (from fishes to mammals). In ruminant animals the adult flukes are conspicuously present in the stomach and the immature ones in the small intestine, causing the disease amphistomiasis. This disease poses a major obstacle to livestock production in ruminants worldwide, especially in young animals where it causes high morbidity and mortality (Phiri *et al.*, 2007). More than 70 species of amphistomes have been reported from animals of food value from different parts of the globe (Roy & Tandon, 1995). Members of the amphistomid

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family Gastrothylacidae (Trematoda: Digenea: Echinostomida) are parasitic in ruminants throughout Africa and Asia and are characterized by the presence of a deep or shallow ventral pouch, the latter being an invagination of the genital atrium (Jones, 2005). In north-east India, five species of pouched amphistomes belonging to this family have been recorded so far, namely *Fischoederius cobboldi*, *F. elongatus*, *Gastrothylax crumenifer*, *Carmyerius spatiosus* and *Velasquezotrema tripurensis*.

The taxonomy of amphistomes is based mainly on morphological data; histological examination of median sagittal sections of adult amphistomes using features of pharynx, terminal genitalium and acetabulum has been used to discriminate them (Sey *et al.*, 1997), which involves long and cumbersome procedures. In recent times, 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 utilizing genetic markers in nuclear ribosomal DNA (rDNA) and mitochondrial DNA (Blair *et al.*, 1996). The second internal transcribed spacer (ITS2) region of rDNA is particularly valuable and is one of the more frequently utilized regions for phylogenetic analyses at the genus and species levels (Itagaki *et al.*, 2003). Recent studies have also shown the utility of the ITS2 region, in addition to its role for low-level phylogenetic analyses, in inferring phylogenies at higher taxonomic levels, due to the highly conserved core secondary structure of this region; the secondary structures have thus proven to be additional tools for use in systematic and taxonomic studies (Coleman, 2003, 2007, 2009; Schultz *et al.*, 2006; Selig *et al.*, 2008).

In the present study, we aimed to infer a molecular phylogeny using ITS2 sequence as well as secondary structures of the five gastrothylacid fluke species, which commonly prevail in the bovine livestock in north-east India.

Materials and methods

Parasite material and DNA isolation

Adult flukes were collected from the rumen of freshly slaughtered host animals, *Bos indicus*, at local abattoirs from Tura (25.52°N 90.22°E), Shillong (25.57°N 91.88°E) and Dharmanagar (24.37°N 92.17°E), District Headquarters in the state of Meghalaya and Tripura, north-east India. Collected materials were first preserved in 70% ethanol and then processed for DNA isolation. Total genomic DNA was isolated from single flukes individually. Flukes were first immersed in digestion extraction buffer (containing 1% sodium dodecyl sulphate (SDS), 25 mg proteinase K) at 37°C overnight. DNA was then extracted from lysed individual worms by the standard ethanol precipitation technique (Sambrook *et al.*, 1989).

DNA amplification and sequencing

The rDNA region spanning ITS2 was PCR-amplified from total genomic DNA obtained from the flukes following the standard protocol with minor modifications, as described elsewhere (Prasad *et al.*, 2007). We used universal primers for trematodes, designed based on sequences of the 5.8S and 28S genes of *Schistosoma* species (Bowles *et al.*, 1996):

3S (forward): 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′

A28 (reverse): 5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′

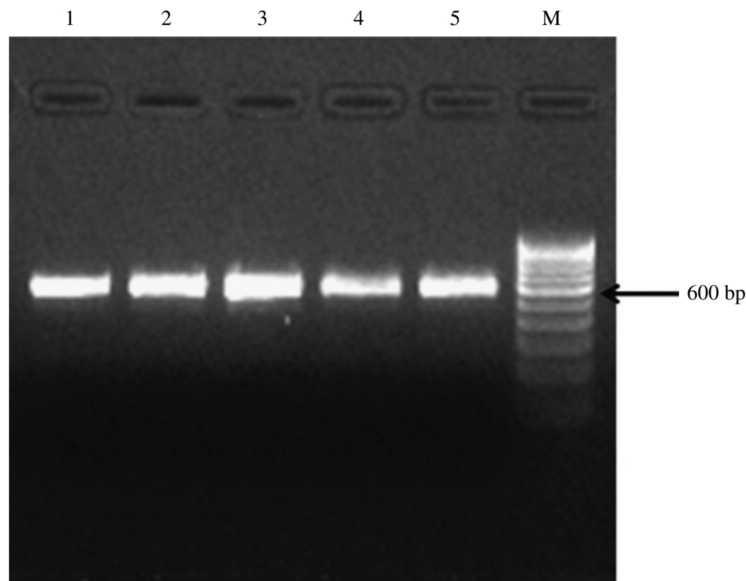

Fig. 1. PCR products obtained from the five gastrothylacid species using ITS2 primers. Lanes: 1, *Fischoederius cobboldi* (477 bp); 2, *F. elongatus* (520 bp); 3, *Carmyerius spatiosus* (464 bp); 4, *Gastrothylax crumenifer* (463 bp); 5, *Velasquezotrema tripurensis* (482 bp); M, molecular weight marker (100 bp ladder).

Table 1. Gastrothylacid species and other digenetic trematode species used in this study with their respective GenBank accession numbers for corresponding ITS2 sequences and their classification.

Species	GenBank Accession No.	Annotated sequence length (bp)	Final host	Classification
*Fischoederius cobboldi**	GU133060	288	Cattle**	Echinostomida: Gastrothylacidae
*F. elongatus**	GU133062	288	Cattle**	Echinostomida: Gastrothylacidae
*Gastrothylax crumenifer**	HM159382	288	Cattle**	Echinostomida: Gastrothylacidae
*Carmyerius spatiosus**	HM159381	287	Cattle**	Echinostomida: Gastrothylacidae
*Velasquezotrema tripurensis**	HM159383	287	Cattle**	Echinostomida: Gastrothylacidae
Gastrodiscoides hominis	EU887295	293	Pig***	Echinostomida: Paramphistomidae
Homalogaster paloniae	AB042190	293	Cattle***	Echinostomida: Paramphistomidae
Orthocoelium streptocoelium	AB042189	292	Cattle**	Echinostomida: Paramphistomidae
C. microbothriodes	AB056570	292	Cattle**	Echinostomida: Paramphistomidae
Echinostoma caproni	AJ564382	434	Birds***	Echinostomida: Echinostomatidae
E. revolutum	AF067850	434	Birds***	Echinostomida: Echinostomatidae
E. trivolvis	AF067851	434	Birds***	Echinostomida: Echinostomatidae
Hypoderaeum conoideum	AJ564385	436	Birds***	Echinostomida: Echinostomatidae
Echinoparyphium recurvatum	AY168931	439	Birds***	Echinostomida: Echinostomatidae

*Sequence generated as part of the present study; **rumen; ***intestine.

For DNA sequencing, PCR products were purified using the Genei Quick PCR purification kit (Bangalore Genei, Bangalore, India) and sequenced in both directions using the above-mentioned primers on an automated sequencer (Applied Biosystems 3130 Genetic Analyzer, California, USA).

ITS2 sequence analysis

The sequenced ITS2 rDNA plus flanking 5.8S and 28S sequences (ITS2+) were first annotated in order to retrieve the exact ITS2 sequences for subsequent analysis. The annotation was carried out using the 'Annotate' feature (default settings) on the ITS2 database (Koetschan *et al.*, 2010). This tool uses HMMer (Eddy, 1998) to annotate eukaryote ITS2 sequences with Hidden Markov Models (HMMs) by identifying a 25-nucleotide interaction of the 5′ 5.8S rDNA subunit end with 25 nucleotides of the 28S rDNA subunit 3′ end (Keller *et al.*, 2009).

Sequence identity was calculated for annotated ITS2 sequences of the five gastrothylacid species with Bioedit software version 7.0.9.0 (Hall, 1999) using 'Sequence Identity Matrix' from the 'Alignment' menu to see the occurrence of inter-specific variations among the ITS2 sequences.

Predicted ITS2 RNA secondary structures

Secondary structures of annotated ITS2 sequences were reconstructed using free energy folding algorithms with MFOLD software version 3.2 (Zuker, 2003). The ITS2 sequences were treated as linear and the folding temperature was set at 37°C for analysis. The structure with the highest negative free energy was chosen from different output files.

Phylogenetic analysis using sequence data

Multiple sequence alignment of the ITS2 sequences was carried out using ClustalW of Bioedit software. The resulting sequence alignment from Bioedit in FASTA format was exported to the program ProfDistS for computing phylogenetic trees (Friedrich *et al.*, 2005; Wolf *et al.*, 2008). The phylogenetic tree was constructed using the neighbour-joining (NJ) distance method with the settings Bootstraps = 1000, Distance Correction Model = Jukes Cantor. NjPlot was set as default viewer for visualizing the tree.

Bayesian phylogenetic analysis

DNA sequences were aligned using ClustalX 2.0.7 and the alignment file in NEXUS format was generated. The alignment file was imported into the program jModelTest v 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) to determine the best-fit model of nucleotide substitution using the Akaike Information Criterion (AIC). The program returned GTR+G as the best-fit model. Bayesian phylogenetic analysis was carried out using MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001) with settings according to the GTR+G model.

Table 2. Sequence identity matrix of the gastrothylacid amphistome species generated using Bioedit (numbers indicate percentage identities/differences (PI/PD); ID = identical).

Sequence PI/PD →	*F. cobboldi*	*F. elongatus*	*G. crumenifer*	*C. spatiosus*	*V. tripurensis*
F. cobboldi	ID	98.6/1.4	98.9/1.1	96.5/3.5	97.5/2.5
F. elongatus	98.6/1.4	ID	98.2/1.8	97.2/2.8	96.8/3.2
G. crumenifer	98.9/1.1	98.2/1.8	ID	96.1/3.9	97.2/2.8
C. spatiosus	96.5/3.5	97.2/2.8	96.1/3.9	ID	95.1/4.9
V. tripurensis	97.5/2.5	96.8/3.2	97.2/2.8	95.1/4.9	ID

Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed with a combinatorial run of four incrementally heated chains for 5,000,000 generations with sampling frequency set to 100. The phylogram containing information on the Bayesian posterior probabilities indicated by the branch were generated and subsequently visualized in TreeViewV1.6.6 (Page, 1996). In addition to the sequence data set used in the previous analysis, *Schistosoma mansoni* sequence was included and chosen as the outgroup taxon.

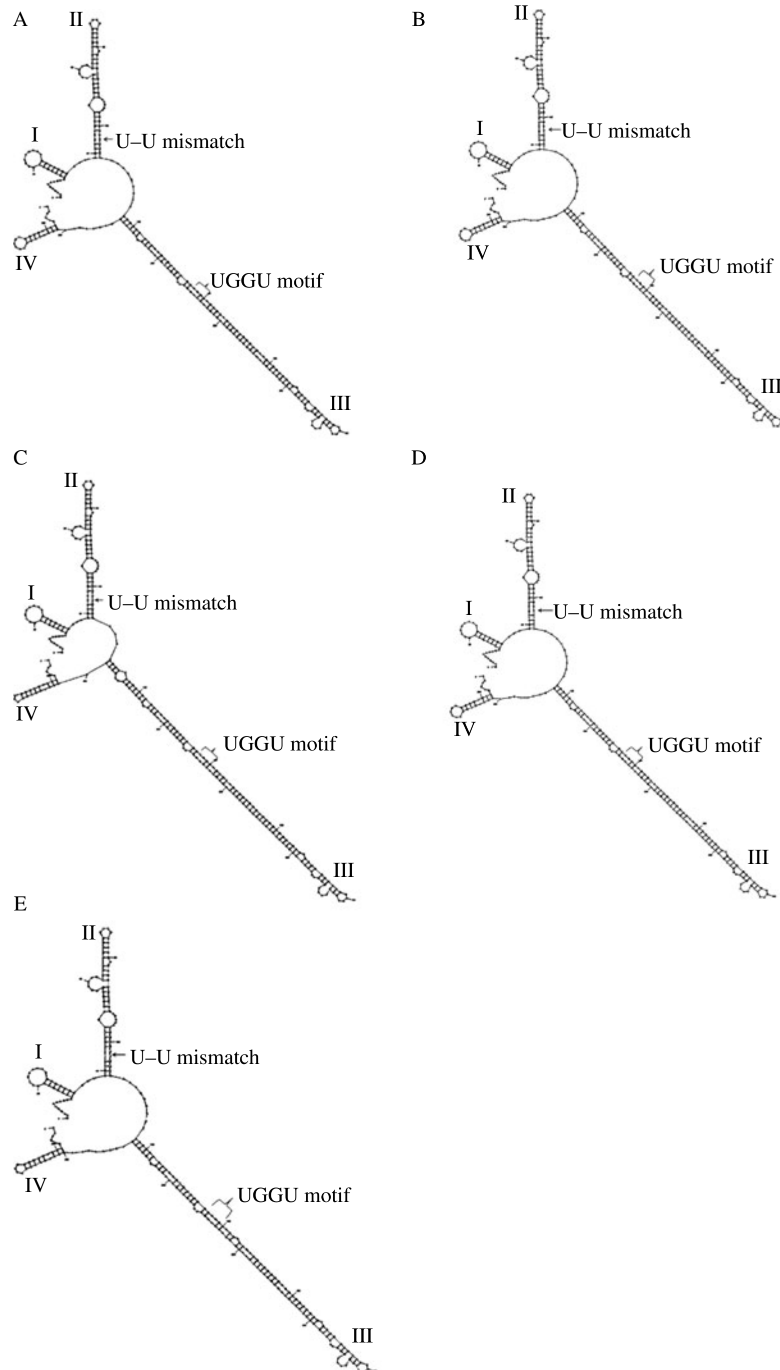

Fig. 2. Inferred secondary structure of the ITS2 rDNA region based on minimum free-energy modelling using Mfold software. (A) *F. cobboldi* (dG = − 120.50 kcal/mol); (B) *F. elongatus* (dG = − 115.70 kcal/mol); (C) *C. spatiosus* (dG = − 123.60 kcal/mol); (D) *G. crumenifer* (dG = − 118.70 kcal/mol); (E) *V. tripurensis* (dG = − 117.20 kcal/mol). Helices I through IV are indicated. The U–U mismatch in helix II and UGGU motif in helix III are also indicated.

Phylogenetic analysis using sequence–structure data

ITS2 RNA sequences with homologous structures in Vienna format from MFOLD were synchronously aligned using the program 4SALE (Seibel *et al*., 2006, 2008). The alignment with structural information was exported with the resultant filename changed to a filename with the extension '.xfasta' to ProfDistS for tree construction.

The alignment output file with the '.xfasta' extension from 4SALE was imported into ProfDistS. 'RNA/DNA structure Profile Neighbour Joining' was selected from the 'Run' menu with the settings Bootstraps = 1000, Distance Correction Model = General Time Reversible, Ratematrix Q = Q_ITS2.txt (an ITS2 specific substitution model included as a supplemental file in ProfDistS). The resultant tree file with node strengths was viewed in ProfDistS by setting NjPlot as the default viewer.

Results

DNA amplification and sequencing

Universal primers, as mentioned above, successfully amplified the desired region spanning ITS2 (ITS2+) with sequenced product sizes of 477 bp, 520 bp, 463 bp, 464 bp and 482 bp in the case of *F. cobboldi*, *F. elongatus*, *G. crumenifer*, *C. spatiosus* and *V. tripurensis*, respectively (fig. 1). The sequences obtained were submitted to GenBank and their accession numbers acquired, as shown in table 1.

ITS2 sequence analysis

The sequences showed a 5.8S–28S rRNA gene interaction which is evidenced by the ability to produce an HMM-based annotation. The annotated ITS2 sequences were found to be 288 bp in the case of both *Fischoederius* and *Gastrothylax* species (*F. cobboldi*, *F. elongatus* and *G. crumenifer*) and 287 bp for those of the genera *Carmyerius* and *Velasquezotrema* (*C. spatiosus* and *V. tripurensis*).

Sequence identity matrix data revealed a considerable sequence divergence among the five gastrothylacid species, with inter-specific variations ranging from 1.4 to 4.9% (table 2). A total of two insertions/deletions, six transversions and ten transitions were found amongst the ITS2 sequences of these species.

Secondary structure analysis

Predicted secondary structures for the ITS2 region of all the five species in the present study, analysed using Mfold, resulted in a four-helix model with calculated minimum free energies of −120.50 kcal/mol, −115.70 kcal/mol, −123.60 kcal/mol, −118.70 kcal/mol and −117.20 kcal/mol, respectively (fig. 2). Helices I and IV of this model were found to be relatively short, each comprising fewer than 30 nucleotides; helix III was the longest, consisting of about 135 nucleotides, and contained a UGGU motif 5′ to the apex; a U–U mismatch was found in the second helix.

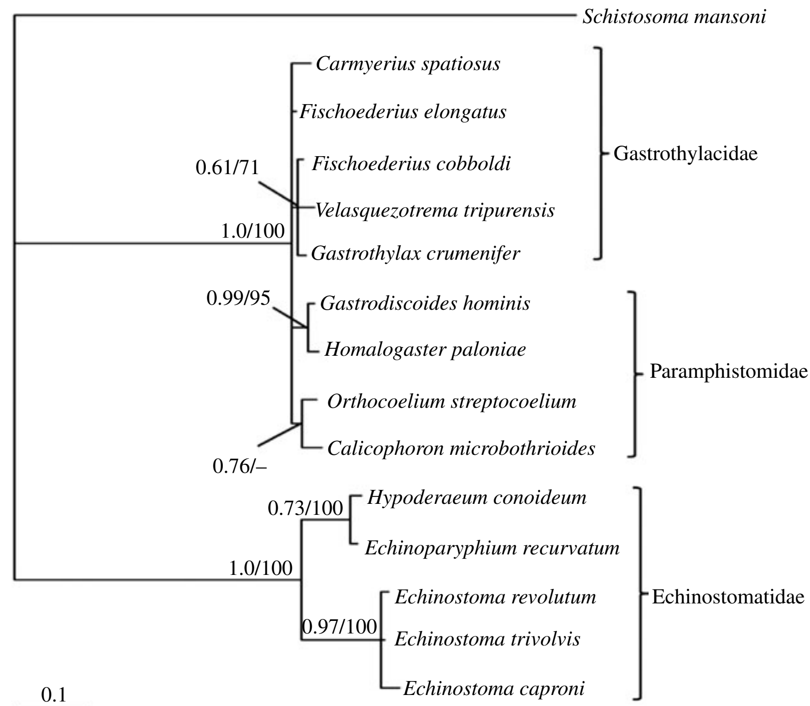

Fig. 3. Phylogram obtained based on ITS2 primary sequence data. Numbers represent Bayesian posterior probabilities/bootstrap values from 1000 replicates (neighbour joining). Bootstrap values of <50% are not shown.

Phylogenetic analyses

For phylogenetic analyses, annotated ITS2 sequences of other digenetic trematodes (all belonging to the Order Echinostomida) already available in the ITS2 database were retrieved (table 1). Tree topology obtained based on the sequence data alone using the neighbour-joining method showed two distinct clades: one of the gastrothylacid species forming a well-supported clade with members of the other amphistomid family Paramphistomidae (100% bootstrap value) and the other of the family Echinostomidae (fig. 3). However, the amphistome clade was not well resolved, with the two families Gastrothylacidae and Paramphistomidae clumping together. The phylogenetic tree obtained using the Bayesian phylogenetic approach also resulted in a similar topology, retaining same grouping (fig. 3). However, the tree reconstructed on the basis of sequence–structure data (fig. 4) showed a better resolution within the amphistome clade, with gastrothylacid species forming a monophyletic group well separated from the family Paramphistomidae, as indicated by high bootstrap values (>70%).

Discussion

In morphology-based generic and species identification of gastrothylacid flukes, several criteria are regarded as having high taxonomic value. These include: the extent of the ventral pouch (terminating in the mid-region or extending posteriorly up to or near to the level of the testes), uterine loops (extending from one side of the body to the other, or in the mid-line throughout) and the position of testes (symmetrical or tandem) (Jones, 2005). However, the field diagnosis of infection is conventionally based on detection of eggs in the stool samples. These transmission stages of most paramphistomoid fluke species are not morphologically distinguishable from one another. DNA-based methods thus provide supplementary tools in authentic identification of taxa (Blair *et al.*, 1996); hence the present study.

The ITS2 sequence analysis revealed a considerable sequence divergence, with inter-specific variations ranging from 1.4 to 4.9% among the gastrothylacid species, suggesting the usefulness of the ITS2 region for discriminating closely related species. As has been already demonstrated, differences of as little as one nucleotide change in the ITS2 sequence alone can be used as an effective genetic marker for low-level analyses to distinguish closely related species of digeneans (Nolan & Cribb, 2005).

In secondary structure analysis using minimum free-energy modelling, the inferred secondary structure for all the five gastrothylacid species was revealed to corroborate with the core four-helix domain structure (with helix

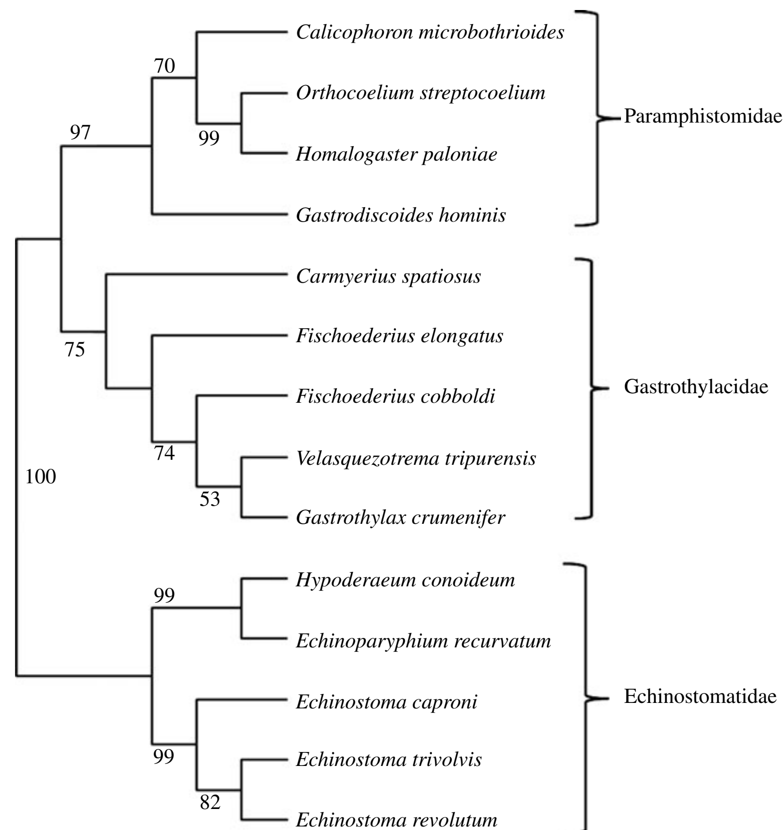

Fig. 4. Profile neighbour-joining tree obtained by ProfDistS synchronously calculated on ITS2 sequence and secondary structure information. Bootstrap support values from 1000 pseudoreplicates are shown.

III being the longest, containing a UGGU motif, and a U–U mismatch in helix II) that has been recently identified as common to almost all eukaryote taxa (Coleman, 2003; Schultz *et al.*, 2005). The optimal secondary structures inferred herein also agree with the four-domain model of the ITS2 rDNA region for other digeneans as well (Morgan & Blair, 1998). This structurally identical nature of the ITS2 region can be attributed to the functional similarity of rRNA biogenesis among eukaryotes, where the folding pattern of the ITS2 region has been shown to play an important role in the correct processing of mature rRNA (Van Nues *et al.*, 1995; Joseph *et al.*, 1999).

Inclusion of secondary structures in phylogenetic reconstructions has been shown to improve the robustness and accuracy of phylogenetic trees (Keller *et al.*, 2010). Morever, the utility of ITS2 sequence–structure data in reliable taxonomic inferences has been demonstrated (Shylla *et al.*, 2011). In the present study, the phylogenetic tree obtained based on sequence data alone, using both neighbour-joining and Bayesian methods, could not resolve the constituent families within the amphistome clade. This reflects the ability of the ITS2 sequence, which is highly variable, as an effective marker for discriminating species at the genus and species level only, and therefore it cannot be applied to higher-level phylogenetic analyses. On the contrary, the highly conserved nature of the ITS2 secondary structure makes it a suitable candidate for phylogenetic analyses at higher taxonomic levels. This is reflected here by the tree reconstructed using sequence data combined with secondary structure information, which showed a better resolution, with the family Gastrothylacidae forming a monophyletic group well separated from Paramphistomidae, as indicated by high bootstrap values. This result conforms to the classification of the Paramphistomata group based on morphological observations (Jones, 2005; Sey, 2005). More importantly, it also showed the applicability of the ITS2 sequence–secondary structure information in deriving phylogenetic relationships at higher taxonomic levels, which in this case is the inter-family level, i.e. between the families Gastrothylacidae and Paramphistomidae.

The present study provides the first molecular characterization of the gastrothylacid fluke species. The study also demonstrates and reiterates the phylogenetic utility of the ribosomal ITS2 region both for low-level phylogenetic analysis, as indicated by the sequence variation among the gastrothylacid species studied, and for inferences at higher taxonomic levels, as shown by the conservation of the secondary structures and the tree reconstructed using sequence–structure data.

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References

Blair, D., Campos, A., Cummings, M.P. & Laclette, J.P. (1996) Evolutionary biology of parasitic platyhelminths: the role of molecular phylogenetics. *Parasitology Today* **12**, 66–71.

Bowles, J., Blair, D. & McManus, D.P. (1996) A molecular phylogeny of the human schistosomes. *Molecular Phylogenetics and Evolution* **4**, 103–109.

Coleman, A.W. (2003) ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends in Genetics* **19**, 370–375.

Coleman, A.W. (2007) Pan-eukaryote ITS2 homologies revealed by RNA secondary structure. *Nucleic Acids Research* **35**, 3322–3329.

Coleman, A.W. (2009) Is there a molecular key to the level of 'biological species' in eukaryotes? A DNA guide. *Molecular Phylogenetics and Evolution* **50**, 197–203.

Eddy, S.R. (1998) Profile hidden Markov models. *Bioinformatics* **14**, 755–763.

Friedrich, J., Dandekar, T., Schultz, J. & Müller, T. (2005) ProfDist: a tool for the construction of large phylogenetic trees based on profile distances. *Bioinformatics* **21**, 2108–2109.

Guindon, S. & Gascuel, O. (2003) A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology* **52**, 696–704.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755.

Itagaki, T., Tsumagari, N., Tsutsumi, K. & Chinoni, S. (2003) Discrimination of three amphistome species by PCR-RFLP based on rDNA ITS2 markers. *Journal of Veterinary Medical Science* **65**, 931–933.

Jones, A. (2005) Family Gastrothylacidae Stiles & Goldberger, 1910. pp. 337–341 *in* Jones, A., Bray, R.A. & Gibson, D.I. (*Eds*) *Keys to the Trematoda, Volume 2*. London, CABI Publishing and The Natural History Museum.

Joseph, N., Krauskopf, E., Vera, M.I. & Michot, B. (1999) Ribosomal internal transcribed spacer 2 (ITS2) exhibits a common core of secondary structure in vertebrates and yeast. *Nucleic Acids Research* **27**, 4533–4540.

Keller, A., Schleicher, T., Schultz, J., Müller, T., Dandekar, T. & Wolf, M. (2009) 5.8S–28S rRNA interaction and HMM-based ITS2 annotation. *Gene* **430**, 50–57.

Keller, A., Forster, F., Müller, T., Dandekar, T., Schultz, J. & Wolf, M. (2010) Including RNA secondary structures improves accuracy and robustness in reconstruction of phylogenetic trees. *Biology Direct* **5**, 4.

Koetschan, C., Förster, F., Keller, A., Schleicher, T., Ruderisch, B., Schwarz, R., Müller, T., Wolf, M. & Schultz, J. (2010) The ITS2 Database III – sequences

and structures for phylogeny. *Nucleic Acids Research* **38**, D275–D279.

Morgan, J.A.T. & Blair, D. (1998) Trematode and monogenean rRNA ITS2 secondary structures support a four-domain model. *Journal of Molecular Evolution* **47**, 406–419.

Nolan, M.J. & Cribb, T.H. (2005) The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. *Advances in Parasitology* **60**, 101–163.

Page, R.D.M. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357–358.

Phiri, A.M., Chota, A. & Phiri, I.K. (2007) Seasonal pattern of bovine amphistomosis in traditionally reared cattle in the Kafue and Zambezi catchment areas of Zambia. *Tropical Animal Health and Production* **39**, 97–102.

Posada, D. (2008) jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* **25**, 1253–1256.

Prasad, P.K., Tandon, V., Chatterjee, A. & Bandhopadhyay, S. (2007) PCR-based determination of internal transcribed spacer (ITS) regions of ribosomal DNA of giant intestinal fluke, *Fasciolopsis buski* (Lankester, 1857) Looss, 1899. *Parasitology Research* **101**, 1581–1587.

Roy, B. & Tandon, V. (1995) *Calicophoron shillongensis* sp. n. (Trematoda: Paramphistomidae), a new parasite from the goat (*Capra hircus* L.) in India. *Acta Parasitologica* **40**, 193–197.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. 2nd edn. 69 pp. Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Schultz, J., Maisel, S., Gerlach, D., Müller, T. & Wolf, M. (2005) A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. *RNA* **11**, 361–364.

Schultz, J., Müller, T., Actziger, M., Seibel, P.N., Dandekar, T. & Wolf, M. (2006) The internal transcribed spacer 2 Database – a web server for (not only) low level phylogenetic analyses. *Nucleic Acids Research* **34**, 704–707.

Seibel, P.N., Müller, T., Dandekar, T., Schultz, J. & Wolf, M. (2006) 4SALE – a tool for synchronous RNA sequence and secondary structure alignment and editing. *BMC Bioinformatics* **7**, 498.

Seibel, P.N., Müller, T., Dandekar, T. & Wolf, M. (2008) Synchronous visual analysis and editing of RNA sequence and secondary structure alignments using 4SALE. *BMC Research Notes* **1**, 91.

Selig, C., Wolf, M., Müller, T., Dandekar, T. & Schultz, J. (2008) The ITS2 Database II: homology modelling RNA structure for molecular systematics. *Nucleic Acids Research* **36**, D377–D380.

Sey, O. (2005) *Keys to the identification of the taxa of the amphistomes (Trematoda, Amphistomida)*. Veszprém, Pécs, Regional Centre of the Hungarian Academy of Sciences (MTA VEAB), University of Pécs.

Sey, O., Prasitirat, P., Romratanapun, S. & Mohkaew, K. (1997) Morphological studies and identification of rumen flukes of cattle in Thailand. *Rivista di Parassitologia* **2**, 247–256.

Shylla, J.A., Ghatani, S., Chatterjee, A. & Tandon, V. (2011) Secondary structure analysis of ITS2 in the rDNA of three Indian paramphistomid species found in local livestock. *Parasitology Research* **108**, 1027–1032.

Van Nues, R.W., Rientjes, J.M.J., Morre, S.A., Mollee, E., Planta, R.J., Venema, J. & Raue, H.A. (1995) Evolutionarily conserved structural elements are critical for processing of internal transcribed spacer 2 from *Saccharomyces cerevisiae* precursor ribosomal RNA. *Journal of Molecular Biology* **250**, 24–36.

Wolf, M., Ruderisch, B., Dandekar, T., Schultz, J. & Müller, T. (2008) ProfDistS: (profile-) distance based phylogeny on sequence–structure alignments. *Bioinformatics* **24**, 2401–2402.

Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31**, 3406–3415.