

## Molecular characterization of *Gastrodiscoides hominis* (Platyhelminthes: Trematoda: Digenea) inferred from ITS rDNA sequence analysis

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Received: 15 January 2009 / Accepted: 20 January 2009 / Published online: 6 February 2009  
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**Abstract** *Gastrodiscoides hominis* (Digenea: Paramphistomata: Gastrodiscidae) is an amphistomid intestinal fluke of pigs causing gastrodiscoidiosis. With the use of molecular tools assisting the conventional diagnostic procedures, we aimed at finding out molecular characterization of *G. hominis* using PCR amplifications of rDNA ITS (1, 2) sequences. The sequences obtained (GenBank accession numbers EF027096, EF027097, EF027098, EU887294, and EU887295) were compared with available sequences of other digenean parasites, particularly those having a zoonotic potential in the northeastern region of India. The BLAST search revealed a close similarity with members of the family Paramphistomidae, showing maximum similarity with the amphistome, *Homalogaster paloniae* (subfamily Paramphistominae). Based on various tree construction methods, phylogeny of *G. hominis* is discussed.

been reported from American muskrat (*Ondatra zibethica*) in Volga Delta, Russia (Ivanov and Semenova 2000). Human infection was reported in a 7-year-old girl in Nigeria, diagnosed by eggs in stools (Dada-Adegbola et al. 2004). The exact life cycle is unknown but probably similar as in other species of Gastrodiscidae involving aquatic vegetations as the second intermediate environment that is used for the encystment of the metacercarial infective stage (Zablotski 1964; Dutt and Srivastava 1972; Mas-Coma et al. 2007). Gastrodiscoidiosis has symptoms similar to diarrhea and, if untreated, might kill the patient, mostly children (Kumar 1980). Eggs are voided in feces, but species identification and diagnosis of infection from eggs alone may be problematic, since most amphistomid eggs have similar morphology.

*G. hominis* has a wide distribution throughout India including the states of Assam, Bengal, Bihar, Uttar Pradesh, Madhya Pradesh and Orissa (Shrivastav and Shah 1970; Murty and Reddy 1980). Apart from India, it is widely distributed in countries like Pakistan, Burma, Thailand, Vietnam, Philippines, China, Kazakhstan, and Russia (Ahuwalia 1960; Buckley 1939; Kumar 1980; Harinasuta et al. 1987; Yu and Mott 1994; Ivanov and Semenova 2000). Buckley (1964) reported high prevalence of *Gastrodiscoides* in humans, mostly children (around 41%), in Kamrup district of Assam, India. In a later study carried out in Meghalaya (India), *G. hominis* was shown to have seasonal prevalence (Roy and Tandon 1992).

The application of DNA methods to studies on the systematics and population genetics of platyhelminth parasites has become widespread in recent years, since identification based on morphology is not always unequivocal. With the use of PCR and sequencing approaches, taxonomic issues can be resolved quite accurately by comparing the DNA of the taxa, utilizing genetic markers in nuclear ribosomal DNA in particular (Blair et al. 1996; Hust et al. 2004). The ribosomal

### Introduction

*Gastrodiscoides hominis*, a digenean trematode, is commonly found in cecum and colon regions of pig and human where pig is a normal host species (Ahuwalia 1960; Kumar 1980, 1999) and is the only amphistomid parasite of man (Mas-Coma et al. 2005). Apart from pig, some other animals are also found to be infected such as Napu mouse deer (*Tragulus napu*), common field rat (*Rattus brevicaudatus*), rhesus monkey (*Macaca mulatta*), and some other species of monkey (Buckley 1964; Fox and Hall 1970; Herman 1967). Easwaran et al. (2003) reported a wild pig also infected by this parasite in Thekaddy forest, South India. *G. hominis* has

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non-coding DNA internal transcribed spacer regions (ITS1 and ITS2) can be utilized for species level diagnosis, as these are the most conserved genes in eukaryotic cells showing an extreme sequence similarity even between distant phylogenetic taxa (Hillis and Dixon 1991). ITS sequences were found to be useful in characterizing the species of *Fasciolopsis* and *Paragonimus* that occur as potential zoonoses in the northeastern regions of India (Prasad et al. 2007; Tandon et al. 2007). With regard to amphistomid trematodes, the published information about ITS2 sequences is so far limited to only a few species, namely *Calicophoron calicophorum*, *C. daubneyi*, *C. microbothrioides*, *Orthocoelium streptocoelium* and *Homalogaster paloniae* (Itagaki et al. 2003).

As the eggs pose a greater challenge in species level diagnosis than the much larger adults, the present study aimed at amplifying the ITS1 and ITS2 regions of (rDNA derived from egg and adult) *G. hominis*, yet another trematode parasite of zoonotic implications in the region; the information emerging from the study would be useful in differentiating this species from other trematode-borne potential zoonoses viz. *Paragonimus westermani*, *Fasciolopsis buski*, *Fasciola gigantica*, *Clonorchis sinensis* and *Opisthorchis viverrini* for all of which ITS (1 and 2) regions have been characterized and will be further used for assessing the extent of genetic diversity, if any, of the parasite.

## Materials and methods

### Parasite material and DNA isolation

Live adult *G. hominis* flukes were obtained from the intestine of freshly slaughtered pigs of Assam and Megha-

laya origin, *Sus scrofa domestica*, at local slaughter houses. To isolate DNA, five adult worms each collected from different host animals of the two geographical locations were processed singly; they 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 phenol chloroform technique (Sambrook et al. 1989). Eggs were taken out from the mature adult fluke by squeezing between two slides and placed on the FTA cards (Whatman Biosciences Ltd.). The cards were dried for 1 h, punched, and two sample discs were taken from each card for one PCR reaction. Sample discs were washed with Whatman's FTA purification reagent and TE buffer, and allowed to dry for 1 h at room temperature, after which they were ready for PCR.

### DNA amplification, sequencing, and its analysis

The rDNA region spanning the ITS regions was amplified from genomic DNA obtained from the adult or egg stage, by PCR. We used the universal primers based on conserved ITS sequences of *Schistosoma* species (Bowles et al. 1995) as detailed below:

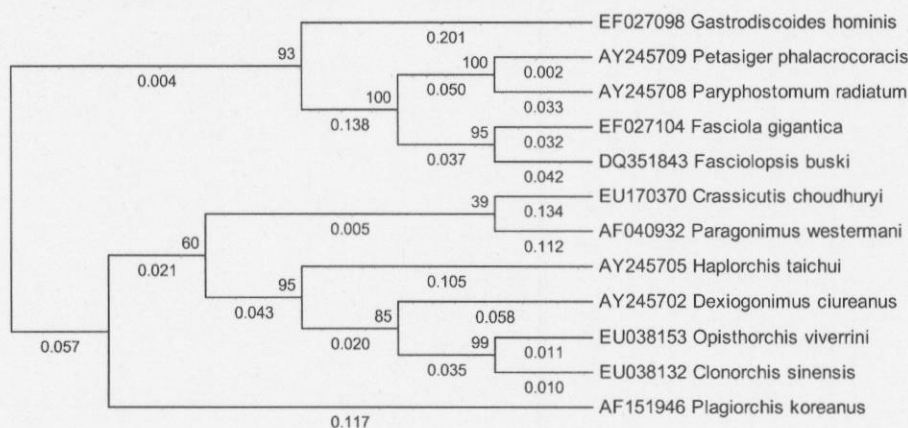
1. ITS1 region—BD1 (forward): 5'-GTCGTAACAAG GTTTCCGTA-3' and 4S (reverse): 5'-TCTAGATGCG TTCGAA (G/A) TGTCGATG-3'
2. ITS2 region—3S (forward): 5'-GGTACCGGTGGATC ACTCGGCTCGTG-3' and A28 (reverse): 5'-GGGATC CTGGTTAGTTTCTTTTCCTCCGC-3'

The PCR amplification was performed following the standard protocol (White 1993) with minor modifications as described elsewhere (Prasad et al. 2007; Tandon et al.

**Fig. 1** Pair-wise alignment of ITS2 sequence of egg and adult stages of *G. hominis*

<b>Adult</b>	51	TACTGCTTTGAACATCGACATCTTGAACGCATATGCGGCCACGGGTTTT	100
<b>Egg</b>	49	TACTGCTTTGAACATCGACATCTTGAACGCATATGCGGCCACGGGTTTT	98
<b>Adult</b>	101	CCTGTGGCCACGCCTGTCCGAGGGTCGGCTATAAACTATCAGCAGCCCC	150
<b>Egg</b>	99	CCTGTGGCCACGCCTGTCCGAGGGTCGGCTATAAACTATCAGCAGCCCC	148
<b>Adult</b>	151	AAAAAGTCGTGGCTTGGAACTCGCCAGCTGGCGTGAITTCCTCTGTGGTT	200
<b>Egg</b>	149	AAAAAGTCGTGGCTTGGAACTCGCCAGCTGGCGTGAITTCCTCTGTGGTT	198
<b>Adult</b>	201	CGCCACGTGAGGTGCCAGATCTATGGCGTTTTCTTAATGTCTCCGGACAC	250
<b>Egg</b>	199	CGCCACGTGAGGTGCCAGATCTATGGCGTTTTCTTAATGTCTCCGGACAC	248
<b>Adult</b>	251	AACCGGCTTGTCTGGTAGCGCAGACGAGGGTGTGGCGGTAGAGTCGT-G	299
<b>Egg</b>	249	AACCGGCTTGTCTGGTAGCGCAGACGAGGGTGTGGCGGTAGAGTCGTGG	298
<b>Adult</b>	300	CTCAATGTAATGTAIGTGGTATCACGCTCTTCTGTTGGCCTTTGTTAGT	349
<b>Egg</b>	299	CTCAATGTAATGTAIGTGGTATCACGCTCTTCTGTTGGCCTTTGTTAGT	348

**Fig. 2** Neighbor-Joining tree of ITS1 sequences of *G. hominis* and other zoonotic trematodes showing bootstrap values and distance



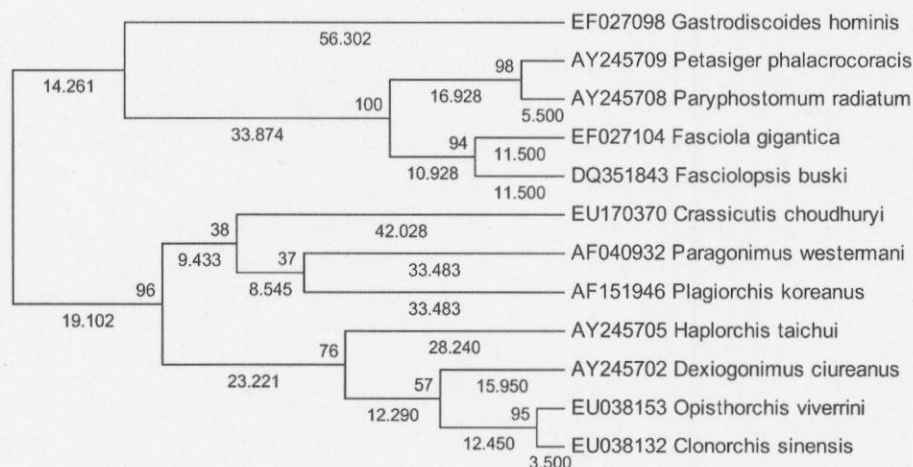
2007). The PCR cocktail (final reaction volume 25  $\mu$ l) was amplified with the following conditions for ITS1—initial denaturation at 94°C for 5 min, then 35 cycles including denaturation at 94°C for 60 s, annealing at 54°C for 50 s, extension at 72°C for 80 s, followed by final extension for 7 min at 72°C. For amplification of ITS2 region, the conditions were as follows—initial denaturation at 94°C for 5 min, then 26 cycles including denaturation at 94°C for 30 s, annealing at 55°C for 38 s, extension at 72°C for 42 s, followed by final extension for 10 min at 72°C. The resultant PCR products were separated by electrophoresis through 1.5% (w/v) agarose gel in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light, and then photographed. The known size fragments of 100 bp ladder in agarose gel were used as marker. For DNA sequencing, the PCR products were purified using Genei Quick PCR purification kit, sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of The Centre for Genomic Applications, New Delhi, India, and submitted to GenBank

under the accession numbers EF027096 (ITS2, adult), EF027097 (ITS2, egg), EF027098 (ITS1, adult), EU887294 (ITS1, adult), and EU887295 (ITS2, adult).

The ITS1 and ITS2 sequences were compared using nucleotide BLAST (blastn) with default settings (URL <http://www.ncbi.nlm.nih.gov/blast>), and phylogenetic prediction was done by CLUSTALW (URL <http://www.ebi.ac.uk/clustalw>) for each query DNA sequence. Phylogenetic reconstructions were done using the MEGA 4.0 package (Tamura et al. 2007). ITS sequences were entered in MEGA for construction of the phylogenetic trees using maximum parsimony and distance methods, namely the Neighbor-Joining, UPGMA, and Minimum Evolution.

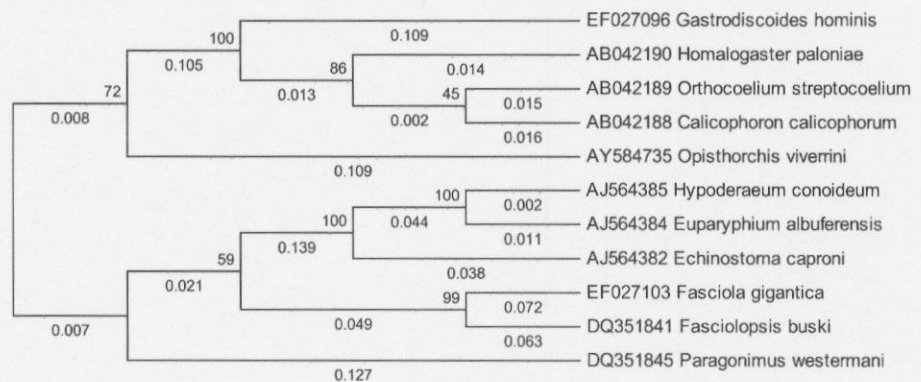
## Results

The ITS1 and ITS2 regions of *G. hominis* DNA of adult fluke and egg origin were successfully amplified by using the abovementioned trematode primers. The size of the



**Fig. 3** Maximum parsimony tree of ITS1 sequences of *G. hominis* and other zoonotic trematodes showing bootstrap values and distance

**Fig. 4** Neighbor-Joining tree of ITS2 sequences of *G. hominis* and other zoonotic trematodes showing bootstrap value and distance



amplified DNA from the adult parasite was 841 bp for ITS1 and 494 bp for ITS2 with flanking regions. The ITS2 sequences of the egg and adult stage DNA of the parasite were aligned and found to be absolutely similar in both (Fig. 1), thus revealing no stage-specific difference.

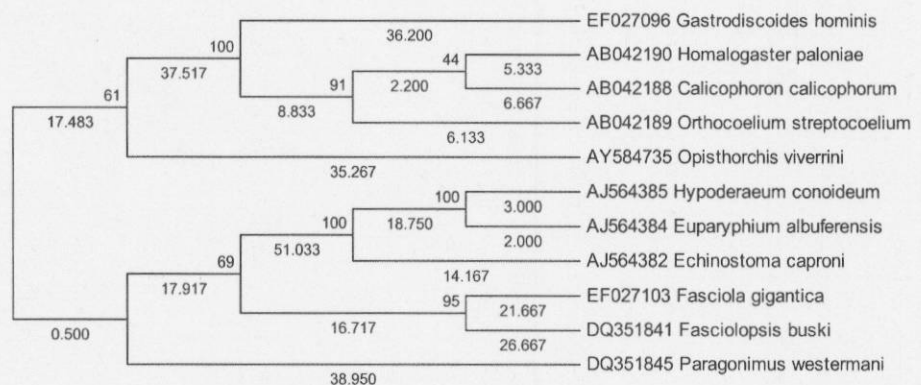
The sequences were aligned in BLAST. Sequences generated from both the geographical isolates (of Assam and Meghalaya origin) were found to be identical. Phylogenetic analyses using the various distance methods and character state method like maximum parsimony show that the topology is similar among the trees obtained. Since the ITS1 sequences of paramphistomid trematodes are not available in the GenBank database so far, the sequences available for other digenean parasites were used for alignment. Phylogenetic trees constructed showed that the query ITS1 *G. hominis* forward and reverse sequences stand close to and are similar in descending order to the sequences of *Petasisiger phalacrocoracis*, *Paryphostomum radiatum*, *Fasciola gigantica*, *Fasciolopsis buski*, *Crassicutis choudhuryi*, *Paragonimus westermani*, *Haplorchis taichui*, *Dexiogonimus ciureanus*, *Opisthorchis viverrini*, *Clonorchis sinensis* and *Plagiorchis koreanus* (Figs. 2 and 3). The ITS2 sequences of *G. hominis* were aligned with those available for other members of the family Param-

phistomidae, Echinostomata, and also of other families having zoonotic potential in the region and neighboring countries. The phylogenetic trees based on ITS2 sequences showed close resemblance with *Homalogaster paloniae*, *Orthocoelium streptocoelium*, *Calicophoron calicophorum*, *Opisthorchis viverrini*, *Hypoderaeum conoideum*, *Euparyphium albuferensis*, *Echinostoma caproni*, *Fasciola gigantica*, *Fasciolopsis buski* and *Paragonimus westermani* (Figs. 4 and 5), maximum similarity being shown with *H. paloniae* with significant bootstrap value. The values 70% and above in the bootstrap test of phylogenetic accuracy indicated reliable grouping among different members of Paramphistomidae.

## Discussion

Morphological differences found in adult specimens have been widely used for plathyhelminth discrimination (Miyazaki 1974). However, traditional diagnostic techniques are now being 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). PCR-

**Fig. 5** Maximum parsimony tree of ITS2 sequences of *G. hominis* and other zoonotic trematodes showing bootstrap value and distance



based techniques using the ITS sequences have been 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; Tandon et al. 2007). In the phylogenetic analysis of the ITS1 sequence and comparison with the available sequences of other Digenea, *G. hominis* shows close resemblance with members of Paramphistomidae and Fasciolidae due to lack of sequences for amphistomes. In case of ITS2 sequences, *G. hominis* stood close to the members of families Paramphistomidae and Echinostomatidae. Based upon the trees constructed, two distinct clusters are revealed: one of Paramphistomidae and other for Echinostomatidae. The ITS2 sequences in the present study showed a higher bootstrap value confirming that it is a highly conserved monophyletic group compared to ITS1, the results being in accordance with other studies on trematodes (Luton et al. 1992; Prasad et al. 2007).

The present study herewith provides the first molecular characterization of *G. hominis* using ITS1 and ITS2 sequences. However, to ascertain intra-specific strain variations, if any, and to determine the population structure and genetic variability, different geographical isolates of *G. hominis* from the region need to be studied with the use of additional molecular markers.

**Acknowledgements** This study was carried out under the Department of Biotechnology-sanctioned project to VT & AC and partly supported by DSA (UGC-SAP) program in the Department of Zoology and UPE-Biosciences program in the School of Life Sciences at NEHU, Shillong. We are also thankful to the Coordinator, Bioinformatics Centre NEHU, for allowing access to its facility.

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