Utilizing ribosomal DNA gene marker regions to characterize the metacercariae (Trematoda: Digenea) parasitizing piscine intermediate hosts in Manipur, Northeast India

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Abstract Freshwater fishes in Manipur, Northeast India frequently harbour several types of metacercariae, which based on morphological criteria were identified as *Clinostomoides brienii*, *Euclinostomum heterostomum* (Clinostomidae) and *Polylekithum* sp. (Allocreadiidae). Molecular techniques utilizing PCR amplification of rDNA regions of larger subunit (LSU or 28S), smaller subunit (SSU or 18S) and inter transcribed spacers (ITS1, 2) were used for molecular characterization of these types. Sequences generated from the metacercariae were compared with their related sequences available in public databases; an analysis of the identity matrices and phylogenetic trees constructed was also carried out, which confirmed their identification. Similarly, the sequences generated from *Polylekithum* sp. were found to be highly similar to the species of the same genus. The rDNA ITS2 secondary structure provided additional confirmation of the robustness of the molecular marker as a tool for taxon-specific characterization.

Keywords Molecular · rDNA ITS2 · 28S · 18S · Metacercaria · Fish hosts · Clinostomidae · Allocreadiidae · India

Introduction

With the advent in recent molecular techniques, the DNA-based PCR amplification methods in particular have helped

resolve many taxonomic issues related to various helminth parasites, by isolating many target sequences and analysing them (Hillis and Dixon 1991). Among these sequence regions, the nuclear ribosomal DNA (rDNA) coding regions and their non-coding spacers, namely the highly conserved larger subunit (LSU or 28S), the smaller subunit (SSU or 18S) and highly variable inter transcribed spacer (ITS1 and ITS2) regions have proven usefulness in answering systematic questions at many taxonomic levels (Hillis and Davis 1986). The ITS2, flanked by more conserved 5.8S and 28S genes, evolves comparably fast and thus has a wider application for taxonomic issues at the species and genus levels relating to trematode taxa (Morgan and Blair 1995; Rinaldi et al. 2005; Goswami et al. 2009; Shylla et al. 2011; Ghatani et al. 2012). The secondary structure of this region aids in alignment of ITS2 sequences at taxonomic levels above that of the genus due to highly conserved secondary structure core (Michot et al. 1993; Morgan and Blair 1995; Coleman 2003).

Fish serves as second intermediate host for a large numbers of trematodes (Nguyen et al. 2009; Phan et al. 2010). The trematode genera *Clinostomum*, *Euclinostomum* (family Clinostomidae) and *Polylekithum* sp. (family Allocreadiidae) are common parasites found infecting the piscivorous birds in many parts of the globe including India as well (Verma 1936; Vidyarthi 1938; Jaiswal 1957; Ukoli 1966, Yamaguti 1971, Jhansilakshmi and Madhavi 1997, Kanev et al. 2002). From Northeast India, Athokpam and Tandon (2013) reported the metacercariae of *E. heterostomum* and *Polylekithum* sp. from the snakehead fish, *Channa punctata* and *Clinostomoides brienii* from *Heteropneustes fossilis* in the state of Manipur. In the present communication we provide molecular characterization and reliable identification of these taxa using rDNA ITS2, 28S and 18S gene marker regions.

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Table 1 Primers used for the amplification of rDNA marker regions

Primer sequences		References
ITS2	3S (forward): 5'-GTACCGGTGGATCACTCGGCTCGTG-3' A28 (reverse): 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'	Bowles et al. (1995)
28S	dig12 (forward): 5'-AAGCATATCACTAAGCGG-3' 1500R (Reverse): 5'-GCTATCCTGAGGGAACTTCG-3'	Tkach et al. (2000)
18S	EukA (forward): 5'-AACCTGGTTGATCCTGCCAGT-3' EukB (reverse): 5'-TGATCCTTCTGCAGGTTACCTAC-3'	Diez et al. (2001)

Table 2 List of Clinostomidae taxa included in the sequence analysis along with their host and accession numbers of marker genes

Sr. No.	Parasite name	Host name	Locality	Genes		
				ITS2	28S	18S
1.	<i>Clinostomum complanatum</i>	<i>Squalius cephalus</i>	Italy	FJ609420.1	FJ609420.1	FJ609420.1
2.	<i>C. complanatum</i>	<i>Squalius cephalus</i>	Croatia	–	JX235337.1	–
3.	<i>C. complanatum</i>	<i>Squalius cephalus</i>	Israel	–	–	AY245701.1
4.	<i>C. marginatum</i>	<i>Pimephales notatus</i>	Canada	JF718643.1	–	–
5.	<i>C. marginatum</i>	<i>Perca flavescens</i>	Canada	JF718642.1	–	–
6.	<i>C. marginatum</i>	<i>Perca flavescens</i>	USA	–	–	AY245760.1
7.	<i>C. marginatum</i>	<i>Perca flavescens</i>	Canada	JF718641.1	–	–
8.	<i>C. marginatum</i>	<i>Ardea herodias</i>	Canada	JF718636.1	–	–
9.	<i>C. tataxumui</i>	<i>Petenia splendida</i>	Mexico	JX631140.1	–	–
10.	<i>C. phalacrocoracis</i>	<i>Oreochromis niloticus</i>	Kenya	FJ609422.1	FJ609422.1	–
11.	<i>C. piscidium</i>	<i>Colisa fasciata</i>	India	–	–	FJ970655.1
12.	<i>C. giganticum</i>	<i>Channa punctatus</i>	India	–	–	FJ970654.1
13.	<i>Clinostomum</i> sp.	<i>Hypseleotris galii</i>	Australia	–	AY222175.1	AY222094.1
14.	<i>Clinostomum</i> sp.	<i>Barbus barbus</i>	Italy	JF718621.1	–	–
15.	<i>Clinostomum</i> sp.	<i>Rana catesbeiana</i>	USA	–	AY222176.1	–
16.	<i>Clinostomid</i> sp.	<i>Rana catesbeiana</i>	USA	–	AY858877.1	AY829252.1

Table 3 List of the Alloeceadidae taxa and details of their host and gene accession numbers used in the study

Sr. No.	Parasite name	Host name	Locality	Genes	
				ITS2	28S
1.	<i>Alloeceadium isoporum</i>	<i>Alburnus alburnus</i>	Russia	FJ874921.1	GU462126.1
2.	<i>A. lobatum</i>	<i>Semotilus corporalis</i>	USA	–	EF032693.1
3.	<i>A. neotenicum</i>	<i>Hydroporus rufifrons</i>	UK	–	JX977132.1
4.	<i>Bunodera luciopercae</i>	<i>Sphaerium rivicola</i>	Lithuania	FJ874916.1	GU462116.1
5.	<i>B. luciopercae</i>	<i>Sphaerium rivicola</i>	Ukraine	FJ874915.1	GU462111.1
6.	<i>Crepidostomum illinoiense</i>	<i>Hiodon alosoides</i>	USA	–	HQ833705.1
7.	<i>C. cornutum</i>	<i>Lepomis gulosus</i>	USA	–	EF032695.1
8.	<i>Creptotrema funduli</i>	<i>Fundulus notatus</i>	USA	JQ425256.1	JQ425256.1
9.	<i>Paracreptotrematina limi</i>	<i>Umbra limi</i>	USA	HQ833706.1	HQ833706.1
10.	<i>Polylekithum ictaluri</i>	<i>Ictalurus punctatus</i>	Canada	–	DQ189999.1
11.	<i>P. catahoulensis</i>	<i>Ictalurus furcatus</i>	USA	EF032698.1	EF032698.1
12.	<i>P. ictaluri</i>	<i>Ictalurus furcatus</i>	USA	EF032697.1	EF032697.1

Table 4 Sequence identity matrix (%) among the ITS2 (a), 28S (b) and 18S (c) sequences of the various Clinostomidae taxa as retrieved from GenBank

(a)										
Sequences	1	2	3	4	5	6	7	8	9	10
1. gblKF781298 <i>Clinostomoides brienii</i> , India*	ID									
2. gblKF781297 <i>Euclinostomum heterostomum</i> , India*	91.0	ID								
3. gblJF718641.1 <i>Clinostomum marginatum</i> , Canada	78.7	72.9	ID							
4. gblJF718636.1 <i>C. marginatum</i> , Canada	78.0	72.0	98.5	ID						
5. gblFJ609420.1 <i>C. complanatum</i> , Italy	95.3	88.5	77.3	76.5	ID					
6. gblJF718621.1 <i>Clinostomum</i> sp., Italy	23.1	22.8	28.0	28.4	23.5	ID				
7. gblJF718643.1 <i>C. marginatum</i> , Canada	76.3	70.5	96.1	97.6	74.8	29.1	ID			
8. gblJF718642.1 <i>C. marginatum</i> , Canada	51.7	48.9	64.0	64.9	52.4	43.7	66.5	ID		
9. gblJX631140.1 <i>C. tataxumui</i> , Mexico	85.8	80.0	88.2	87.1	83.4	25.6	85.7	57.3	ID	
10. gblFJ609422.1 <i>C. phalacrocoracis</i> , Kenya	96.3	88.8	77.9	77.0	96.0	23.2	75.3	51.3	83.8	ID
(b)										
Sequences	1	2	3	4	5	6	7			
1. gblKF781299 <i>Clinostomoides brienii</i> , India*	ID									
2. gblFJ609420.1 <i>Clinostomum complanatum</i> , Italy	32.7	ID								
3. gblJX235337.1 <i>C. complanatum</i> , Croatia	16.1	40.1	ID							
4. gblFJ609422.1 <i>C. phalacrocoracis</i> , Kenya	32.3	98.1	39.2	ID						
5. gblAY222175.1 <i>Clinostomum</i> sp., Australia	46.6	18.6	32.3	18.5	ID					
6. gblAY222176.1 <i>Clinostomum</i> sp., USA	46.9	18.9	32.1	18.8	96.5	ID				
7. gblAY858877.1 <i>Clinostomid</i> sp., USA	45.1	18.0	33.8	17.8	95.2	92.1	ID			
(c)										
Sequences	1	2	3	4	5	6	7	8	9	10
1. gblKF781300 <i>Clinostomoides brienii</i> , India*	ID									
2. gblFJ609423.1 <i>Clinostomum phalacrocoracis</i> , Kenya	95.8	ID								
3. gblAY245701.1 <i>C. complanatum</i> , Israel	95.5	98.2	ID							
4. gblFJ609422.1 <i>C. phalacrocoracis</i> , Kenya	95.9	99.9	98.1	ID						
5. gblFJ970655.1 <i>C. piscidium</i> , India	21.0	22.1	22.4	22.1	ID					
6. gblFJ970654.1 <i>C. giganticum</i> , India	29.9	30.8	31.0	30.8	67.3	ID				
7. gblAY245760.1 <i>C. marginatum</i> , USA	96.3	98.3	98.0	98.3	22.0	30.8	ID			
8. gblFJ609420.1 <i>C. complanatum</i> , Italy	96.0	98.9	99.2	98.9	22.3	30.8	98.5	ID		
9. gblAY222094.1 <i>Clinostomum</i> sp., Australia	95.6	98.2	99.1	98.2	23.0	31.3	97.9	98.5	ID	
10. gblAY829252.1 <i>Clinostomid</i> sp., USA	86.6	88.7	89.0	88.7	25.1	34.2	88.4	89.0	89.5	ID

* Query sequence generated from the study; highest value marked in bold

Materials and methods

Parasite material

Metacercariae representing the parasites namely, *Clinostomoides brienii*, *Euclinostomum heterostomum* and *Poly-lekithum* sp. [identified based on morphological criteria following standard literature (Yamaguti 1971; Gibson et al. 2002; Jones et al. 2005)] were recovered from the snake-head *Channa punctata* and the singhi fish *Heteropneustes fossilis* from various localities in Manipur and made to

excyst by artificial digestion as reported earlier (Athokpam and Tandon 2013). The excysted metacercariae were fixed in 70 % ethanol for further molecular study.

Molecular study

DNA isolation, amplification and sequencing

Total genomic DNA was extracted from the fixed specimens using a QIAamp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's

Fig. 1 Phylogenetic trees constructed for Clinostomidae: **a** ITS2; **b** 28S; **c** 18S (Asterisk Query sequence generated from the study)

instructions. The rDNA genes under consideration were amplified with different sets of primers as detailed below (Table 1).

The PCR-amplified products were purified using Genei Quick PCR purification kit for DNA and sequenced using automated sequencing services provided by Macrogen Service Center, Seoul, Korea.

For the 28S and 18S genes, as the full length could not be obtained through one direction sequencing, the contigs were created assembling both the forward and reversed sequences in DNA Baser v3.5.3 (<http://www.dnabaser.com/>). The sequences generated were submitted to GenBank (NCBI) and their accession numbers acquired.

Sequence and phylogenetic analyses

For the sequence similarity search the Basic Local Alignment Search Tool (BLAST), available at <http://www.ncbi.nlm.nih.gov/vblast>, was used. Multiple sequence alignment was done and sequence identity matrices were constructed

using Bioedit software version 7.0.9.0 (Hall 1999) for each of the amplified markers for all metacercaria species under study, using sequences of their closely related taxa as available in the public domain.

The output fasta format files from Bioedit were then entered into MEGA5 (Tamura et al. 2011) for phylogenetic tree construction deducing the distance-based Neighbour Joining (NJ) and the character-based Maximum likelihood (ML) methods.

Predicted ITS2 RNA secondary structures

The ITS2 sequences generated were annotated using the Hidden Markov Models (HMM)-based annotation (available at <http://its2.bioapps.biozentrum.uni-wuerzburg.de/>) to retrieve the exact sequence of the region (Keller et al. 2009); their secondary structures were constructed using MFOLD software version 3.2, using free energy folding algorithms (Zuker 2003). The output Vienna format was

Table 5 Sequence identity matrix (%) among the ITS2 (a) and 28S (b) gene sequences of the various Allocreadiidae taxa as retrieved from GenBank

(a)														
	Sequences	1	2	3	4	5	6	7	8					
1.	<i>Polylekithum</i> sp., India*	ID												
2.	<i>P. catahoulensis</i> , USA	73.1	ID											
3.	<i>P. ictaluri</i> , USA	72.7	98.0	ID										
4.	<i>Allocreadium isoporum</i> , Russia	49.0	50.1	49.8	ID									
5.	<i>Bunodera luciopercae</i> , Lithuania	57.7	59.4	58.9	80.0	ID								
6.	<i>B. luciopercae</i> , Ukraine	58.2	59.8	59.3	79.5	99.4	ID							
7.	<i>Creptotrema funduli</i> , USA	61.2	61.0	60.7	71.1	79.5	80.0	ID						
8.	<i>Paracreptotrematina limi</i> , USA	60.7	61.8	61.3	66.6	77.0	77.5	80.3	ID					
(b)														
	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13
1.	<i>Polylekithum</i> sp., India*	ID												
2.	<i>P. ictaluri</i> , Canada	67.0	ID											
3.	<i>P. catahoulensis</i> , USA	91.1	72.6	ID										
4.	<i>P. ictaluri</i> , USA	90.9	73.2	98.9	ID									
5.	<i>Allocreadium isoporum</i> , Russia	82.0	59.7	82.3	82.2	ID								
6.	<i>A. lobatum</i> , USA	83.0	60.2	82.9	83.0	95.9	ID							
7.	<i>A. neotenicum</i> , UK	83.1	60.3	83.0	83.1	95.9	99.7	ID						
8.	<i>Bunodera luciopercae</i> , Lithuania	83.0	60.7	83.6	83.4	92.1	92.5	92.7	ID					
9.	<i>B. luciopercae</i> , Ukraine	83.0	60.7	83.6	83.4	92.1	92.5	92.7	100.0	ID				
10.	<i>Crepidostomum illinoiense</i> , USA	83.6	60.6	82.9	82.9	92.1	93.1	93.3	94.1	94.1	ID			
11.	<i>C. cornutum</i> , USA	83.9	61.1	83.4	83.4	92.2	93.1	93.3	94.2	94.2	97.8	ID		
12.	<i>Creptotrema funduli</i> , USA	82.3	60.1	82.2	82.2	90.9	92.2	92.2	92.8	92.8	95.9	96.9	ID	
13.	<i>Paracreptotrematina limi</i> , USA	87.3	63.6	86.7	86.8	82.5	83.8	83.8	83.7	83.7	84.2	84.3	83.3	ID

* Query sequence generated from the study; highest value marked in bold

then imported to 4SALE to view the secondary structure (Seibel et al. 2006).

Results

The sequences generated from the three metacercaria species were analysed for their sequence identity with their closely related sequences from the families Clinostomidae and/or Allocreadiidae as retrieved from NCBI (Tables 2, 3, 4). For phylogenetic analysis the trees constructed with ML and NJ methods showed almost the same topology of taxa; therefore, only the ML tree is shown herein. In all trees *Fasciolopsis buski* was used as the outgroup. The species-wise results of analyses are detailed below.

Clinostomoides brienii

The rDNA ITS2, 28S and 18S sequences deposited in GenBank (under accession numbers: KF781298,

KF781299 and KF781300) are 430, 2546 and 1907 bp in length, respectively. For sequence analysis sequences of the various Clinostomidae taxa available in the public domain were used (Table 2). The metacercaria under the present study comes close to *Clinostomum* spp, showing 96.3 % sequence similarity for ITS2 (with *C. phalacrocoracis*) as well as 18S (with *C. marginatum*); however, with regard to 28S the sequence similarity with *Clinostomum* sp. was less than 50 % (Table 4a–c). In phylogenetic analysis, the query sequence *C. brienii* claded with clinostomid species, but with bootstrap values of 65–99 % (Fig. 1a–c). In all trees, *Fasciolopsis buski*, the outgroup, formed a separate clade.

Euclinostomum heterostomum

The ITS2 sequence (accession number: KF781297) has a length of 415 bp. There being no sequence available for *Euclinostomum* species in public database, the sequence analysis was done comparing with *Clinostomum* spp.

Fig. 2 Phylogenetic trees constructed for Allocreadiidae: **a** ITS2; **b** 28S (Asterisk Query sequence generated from the study)

(Table 2). In sequence identity matrix, the metacercaria under the present study stands very close to *Clinostomoides brienii* sequence, with a sequence identity of 91.0 % (Table 4a); in phylogenetic tree it forms a separate branch from other Clinostomidae taxa (Fig. 1a).

Polylekithum sp.

The rDNA ITS2 and 28S sequences (accession numbers: KF738449 and KF738452, respectively) deposited in GenBank have a length of 459 and 985 bp, respectively. The metacercaria under the present study stands very close to *Polylekithum catahouensis*, with sequence identities of 73.1 % (ITS2) and 91.1 % (28S) (Table 5). In phylogenetic analysis *Polylekithum* sp. claded within the genus *Polylekithum*, with significant bootstrap value of 87–99 % (Fig. 2).

ITS2 rDNA secondary structures

The secondary structure of ITS2 of both *C. brienii* and *E. heterostomum* shows deviation from the 4-helical structure

model and consists of only one helix having two branches: Helix 1a and 1b, the latter (Helix 1b) again showing three sub branches—Helix 1b.i, ii and iii (Fig. 3a, b). However, in contrast to clinostomatids the ITS2 secondary structure of *Polylekithum* sp. comprises three helices—Helix 1, Helix 2 (with two sub-branches—2a and 2b) and Helix 3; Helix 2 is the longest and Helix 3 shows UGG motifs (Fig. 3c).

Discussion

The sequences of the rDNA marker regions have been successfully used, as in the present study, for molecular characterization of the trematode taxa including the clinostomids and allocreadiids. The 18S gene was used for validating *C. marginatum* (Dzikowski et al. 2004). Caffara et al. (2011) also supplemented the studies in morphological difference among various *Clinostomum* spp. utilizing the rDNA ITS and 18S regions, and mitochondrial

Fig. 3 Inferred secondary structure of ITS2 rDNA region (based on minimum free energy modelling using Mfold); **a** *C. brieni* India: Northeast, dG = -94.80 kcal/mol; **b** *E. heterostomum* India: Northeast, dG = -90.00 kcal/mol; **c** *Polylekithum* sp. India: Northeast, dG = -107 kcal/mol

cytochrome oxidase 1 (mtCO1) regions. From India, only three sequences from the genus *Clinostomum* are available in the public domain—28S partial sequence (GQ925911.1) (Singh and Chaudhary, unpublished) and two 18S partial sequences (FJ970655.1 and FJ970654.1, derived from *Clinostomum piscidium* found infecting the host *Colisa fasciatus* and *Clinostomum giganticum* found infecting the fish *Channa punctatus*, respectively (Agrawal et al., unpublished). Our present study provides for the first time the sequence data for *C. brieni* and *E. heterostomum*. With regard to the various Alloeceadiidae taxa, Curran et al. (2006) characterized *Polylekithum* sp. utilizing the rDNA (ITS2 and 28S) marker regions. Petkevičiūtė et al. (2010) also used these marker sequences for discrimination of host-specific subspecies of the alloeceadiid trematode species, and to clarify species relationships among these taxa. Our results of the phylogenetic analysis indicating a high bootstrap value also supported the sequence analysis; as

generally accepted a bootstrap value of $\geq 70\%$ for a given interior branch of a phylogenetic tree is indicative of an authentic analysis (Hillis and Bull 1993; Tandon et al. 2007).

The ITS2 secondary structure can provide additional informations related to taxonomic characters, which otherwise cannot be explained by primary sequence alone (Caetano-Anollés 2002; Grajales et al. 2007; Krüger and Gargas 2008). Schultz et al. (2005) showed a common core of secondary structure of ITS2 throughout the Eukaryota. High sequence variation with conservedness in its secondary structure have made ITS2 useful in various evolutionary comparison studies (Coleman 2003). The secondary structures generated from the ITS2 regions of *E. heterostomum* and *C. brieni* in our study do not display the typical four-helix model. Generally RNA secondary structure prediction is based on the minimum free energy at 37 °C and variations in topology are attributed to

differences in their nucleotide length (Prasad et al. 2009). Only 17.9 % of the available ITS2 sequences in the public databases show the four-domain structure (Wolf et al. 2005), though three-helix models are also available (Subbotin et al. 2005). Schultz et al. (2005) attributed several reasons for the absence of the four-helix structure—firstly, the chosen consensus structure may be very conservative to avoid false positives; secondly, the low abundance of the consensus structure could be caused by the fold prediction itself as only the minimal energy structure was considered and this might not represent the biological active form.

In conclusion, the present study is the first to provide the molecular characterization of the clinostomatiid/allocreadiid metacercariae that occur in freshwater fishes in the region. The study also corroborates the utility of molecular tools in validating the species identification.

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