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ORIGINAL ARTICLE

Molecular identification of two cestodes species parasitizing freshwater fishes in India

Philayung Zimik[1] · **Bishnupada Roy**[1]

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Abstract In the present study, molecular identification of two species of cestodes, *Lytocestus indicus* and *Senga lucknowensis* infecting freshwater fishes *Clarias magur* and *Channa punctata*, respectively in Manipur is carried out. To ascertain the taxonomic status of these helminth parasites, 18S gene marker was used. Phylogenetic analysis of 18S of *Lytocestus* sp. showed that it claded with *L. indicus* from Indian Isolate with a sequence similarity index of 99%. In case of *Senga* sp., the phylogenetic analysis revealed that it formed a separate clade with *S. lucknowensis* and *Senga vishakapatnamensis*, and the sequence similarity index showed maximum homogeneity with *S. lucknowensis* i.e., 99.8%. Thus, molecular characterization revealed that the two species of cestodes belong to *L. indicus* and *S. lucknowensis.*

Keywords *Lytocestus indicus* · *Senga lucknowensis* · 18S · Molecular characterization

Introduction

Fishes are believed to be medicinal in some parts of India and it is traditionally favoured among children, the elderly, pregnant women, immuno-compromised or ill people (Debnath 2011). The nutrient content in fish and its important role in human health is one of the reasons dieticians and medical practitioners advised in prescribing diet chart for human population (Islam et al. 2013; Bogard et al. 2015; Paul et al. 2015).

Parasitic infection due to helminths belonging to genus *Lytocestus* and *Senga* in *Clarias magur* and *Channa punctata* respectively, are common in India (Tandon et al. 2005; Bhure et al. 2010; Sawarkar 2012; Solunke et al. 2012). The comparative histopathologicl study on different hosts parasitized by cestode parasites of the genus *Lytocestus* and *Senga* showed high pathological effects which might lead to mortality of the hosts (Chakravarty and Tandon 1989; Ahmed and Sanaullah 1979; Reddy and Benarjee 2014; Kaur 2014; Reddy et al. 2017)

It is a well-known fact that certain species of parasites are more successful in establishing an infection as compared to other species of the same genus (Procop 2009). It is also true in terms of treatment where some strains showed sensitivity to certain drugs while the other strain is resistant to the same drug (Chaijaroenkul et al. 2005). Hence, proper identification of organisms up to molecular level to ascertain their taxonomic status is important. Molecular identification has been extensively used for delineating species, especially between cryptic and sister species (Nadler and Perez-Ponce de Leon 2011). Thus, molecular methods have aided modern taxonomy of parasitic species using various genetic markers (Sharma et al. 2016; Janssen et al. 2017).

The utility of 18S rRNA in delineating, identification and phylogenetic inference is well elucidated in different forms of organisms like nematodes, digeneans and cestodes (Kodedova et al. 2000; Ndeda et al. 2013; Umbers et al. 2015) The objective of this study is to ascertain the identity of *Senga* sp. and larval forms of *Lytocestus* up to the species level using 18S rRNA.

✉ Bishnupada Roy
bishnuroy12@rediffmail.com

[1] Department of Zoology, North-Eastern Hill University, Shillong, Meghalaya 793022, India

Materials and methods

DNA isolation, amplification and sequencing

For DNA extraction, the samples were washed in Phosphate buffered saline (PBS) and then crushed in 1.5 ml microtubes. About 500 µl of TNE (*Tris*-HCl; NaCl; EDTA) buffer was added, homogenized and incubated at 37 °C overnight in the lysis buffer [containing 1% sodium dodecyl sulphate (SDS), 25 mg proteinase K]. Then, equivalent amount of phenol chloroform-isoamyl (PCI) alcohol was added, centrifuged at 13,000 rpm for 10 min for three times. The DNA was precipitated with 100% ethanol, washed with 70% ethanol, centrifuged, dried and dissolved in 25 µl pH 8.0 TE buffer (Sambrook and Russell 2001). The rDNA-18S region was amplified by PCR using primers set worm A (5′-CGAATGGCTCATTAAATCAG-3′) and worm B (5′-CTTGTTACGACTTTTACTTCC-3′) (Littlewood et al. 1999). The thermal gradient of this marker region was done under the following conditions: initial denaturation at 94 °C (5 min), 35 cycles including denaturation at 94 °C (1 min), annealing at 52 °C (1 min), extension at 72 °C (2 min), followed by final extension for 10 min at 72 °C. The PCR products were purified and sequenced in both directions on an automated sequencer (Macrogen sequencing services, South Korea). The generated sequences were submitted to NCBI-GenBank and the accession numbers acquired.

Sequence and phylogenetic analysis

The generated sequences, along with sequences of the other related helminth species were retrieved from GenBank for analyses (Tables 1, 2). The sequences were aligned using the ClustalW program in MEGA6 (Tamura et al. 2013). The aligned sections were then imported to BioEdit (Hall 1999) for generation of sequence similarity matrix. Phylogenetic trees were constructed using Bayesian Inference (BI) in MrBayes (Ronquist et al. 2012) taking *Djombangia penetrans* and *Bothriocephalus cuspidatus* as an outgroup species for *Lytocest*us sp. and *Senga* sp. respectively. Branch support for MrBayes was given using Bayesian posterior probabilities (Bpp) that was computed using the Metropolis-Coupled Markov Chain (MCMC) method. The analysis was run for 500,000 generations and sampled every 1000 generations, with the first 25% of the trees being discarded as the 'burn-in' phase.

Result

The multiple sequence alignment of the 18S gene of *Lytocestus* taxa shows the presence of numerous gaps and mismatches (Fig. 1). The sequence similarity index matrix generated revealed maximum homology with *Lytocestus indicus* (Table 3) and the comparison of the genetic variations between our sequence with the rest revealed lowest in *L. indicus* and highest in *L. birmanicus* and *L. heteropneustii*. But the interspecific variation between *L. bimanicus* and *L. heteropneustii* is relatively low. Similar

Table 1 18S sequence of *Lytocestus* species used for sequence analysis and phylogenetic inference

S. no.	Species	Accession no.	Locality	Host
1	*Lytocestus indicus*	KX758631[a]	India	*Clarias magur*
2	*L. indicus*	KC332243	India	*Clarias magur*
3	*L. birmanicus*	KC332244	India	*Clarias magur*
4	*L. heteropneustii*	KC332245	India	*Heteropneustus fosillis*
5.	*Djombangia penetrans*	JQ034142	India	*Clarias magur*

[a]Sequence generated for the study

Table 2 18S sequences of *Senga* species used for sequence analysis and phylogenetic inference

Species	Accession no.	Locality	Host
1. *Senga lucknowensis*	KU761847[a]	India	*Channa punctata*
2. *S. lucknowensis*	KR780938	Vietnam	*Mastacembelus armatus*
3. *S. vishakapatnamensis*	KR780937	India	*Channa punctata*
4. *S. magna*	KR780960	Russia	*Siniperca chautsi*
5. *Bothriocephalus cuspidatus*	KR780955	USA	*Sander vitreus*

[a]Sequence generated for the study

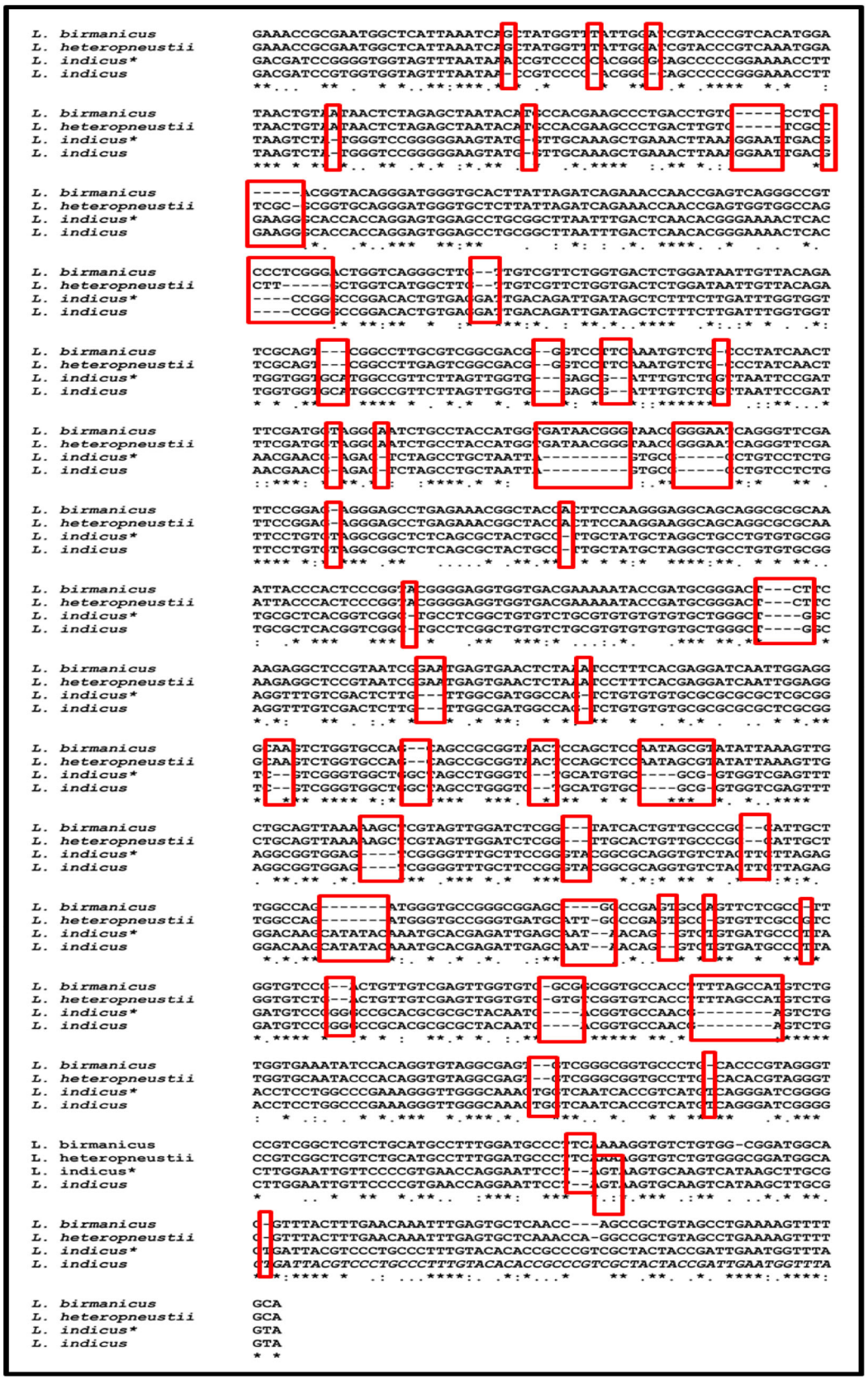

Fig. 1 Multiple sequence alignment of 18S gene of *Lytocestus* sp. showing gap in highlighted (red) boxes and mismatches [asterisks (*) just below the nucleotide base pairs shows well aligned sequences whereas the space between the asterisks shows mismatches] (color figure online)

Table 3 Sequence identity matrix for 18S gene with values indicating % identities/% differences among the various species of *Lytocestus*

	1	2	3	4
1. *L. indicus**	ID			
2. *L. indicus*	**99.4**/0.6	ID		
3. *L. birmanicus*	40.0/60	40.0	ID	
4. *L. heteropneustii*	40.7/60	40.7	94.1	ID

* sequence generated for the study

The numbers in **bold** indicate the highest value

ID identical

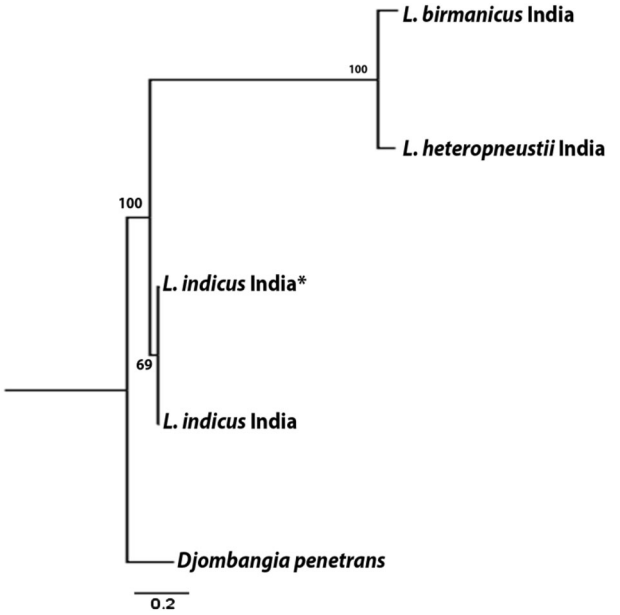

Fig. 2 Phylogenetic tree of *Lytocestus* species inferred via Bayesian Inference in MrBayes using 18S gene regions. Numbers against the nodes indicate Bayesian posterior-probability values and the scale bar represents number of substitutions per site (*sequence generated for the study)

result was also depicted in the phylogenetic tree. *Djombangia penetrans* was taken as the outgroup (JQ034142) for constructing the phylogenetic tree. The Bayesian Inferred tree of 18S for the species of *Lytocestus* is well resolved and the nodes were supported by Bpp values. The 18S inferred phylogeny showed that the species collected from Manipur claded with *L. indicus*, of Indian isolate whereas *L. birmanicus* and *L.. heteropneustii* were erected separately with a long branch length (Fig. 2).

The multiple sequence alignment showed gaps in three sites in sequence of *Senga lucknowensis* (Fig. 3). The similarity index matrix revealed that the sequence of our species to be highly identical to *S. lucknowensis* of Vietnamese isolate and *Senga vishakapatnamensis* of Indian isolate (Table 4). The variation among them is almost negligible, i.e., 0.1%. This similarity between the species is also observed in the phylogenetic tree. The Bayesian inferred phylogenetic tree is well resolved with 98% Bpp values. The tree depicted that our isolate, *S. lucknowensis* of Vietnamese isolate and *S. vishakapatnamensis* of Indian isolate formed a separate clade just as the similarity index result had depicted (Fig. 4).

Discussion

The adult forms of Lytocestidae could be identified through morphological studies and many species have been identified using such methods since the reproductive organs are well developed in adults (Tandon et al. 2005; Bhure et al. 2010). But, larval stages are not distinguishable up to species level by studying their morphological features. During our collection, metacestodes were sampled and therefore molecular and bioinformatic tools were used to specify its taxonomic position. The molecular information gathered in our study revealed that there is a high inter-specific variation. The similarity index matrix showed that the species of our study is 99.4% identical to *L. indicus* of Indian isolate. Similar result is inferred in phylogenetic tree where our species is claded with *L. indicus* with nodal support of 100% Bpp values. Therefore, present species is confirmed as *L. indicus*.

The 18S sequence generated from *Senga* sp. was first checked in BLAST, NCBI and it matched with the taxa *Senga*, where the result follows in accordance with the classification of Bothriocephalidae based on morphological traits (Kuchta et al. 2008). The molecular analysis of the sequence query showed an interesting result. It was observed that the inter-specific variation among *Senga* is very less which means they are very similar to one another. The similarity index matrix showed that our *Senga* sp. is identical to *S. lucknowensis* of Vietnamese isolate (99.8%) and well as *S. vishakapatnamensis* (99.7%) with a variation between them in just 0.1. Moreover, in the phylogenetic tree, all the three species were clustered together in one clade with Bpp value. The slight difference could be because of the geographical variation. It has been clearly

Fig. 3 Multiple sequence alignment of 18S gene of *Senga sp.* showing gap (red) in highlighted boxes and mismatches [asterisks (*) just below the nucleotide base pairs shows well aligned sequences whereas the space between the asterisk shows mismatches] (color figure online)

Table 4 Sequence identity matrix for 18S gene with values indicating % identities/% differences among the various species of *Senga*

	1	2	3	4	5
1. *Senga lucknowensis**	ID				
2. *S. lucknowensis*	**99.8**/0.2	ID			
3. *S. vishakapatnamensis*	99.7/0.3	10	ID		
4. *S. magna*	99.4/0.6	99.5	99.5	ID	
5. *Bothriocephalus cuspidatus*	96.5/3.5	96.6	96.6	96.5	ID

* sequence generated for the study

The numbers in **bold** indicate the highest value

ID identical

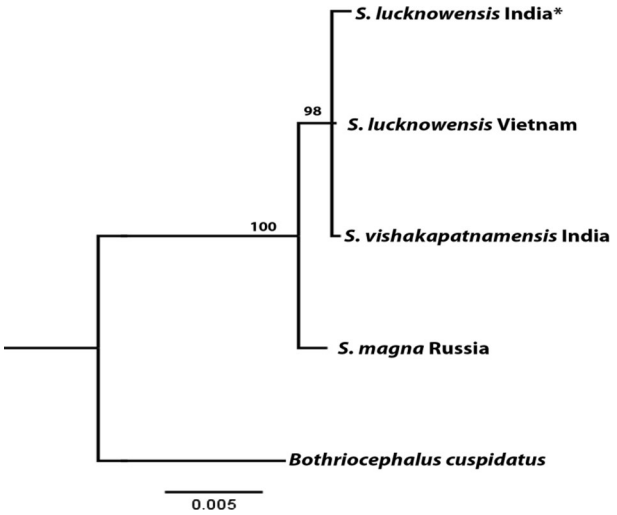

Fig. 4 Phylogenetic tree of *Senga* species inferred via Bayesian Inference in MrBayes using 18S gene regions. Numbers against the nodes indicate Bayesian posterior probability values and the scale bar represents number of substitutions per site (*sequence generated for the study)

indicated from all the molecular information that the species of our study belongs to *S. lucknowensis*.

The present study provides the molecular characterization and identification of *L. indicus* and *S. lucknowensis*. This study also proves the benefit and utility of molecular tools in delineating and identification of parasite having medicoveterinary importance.

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Author contributions BR framed and designed the experiment and wrote the manuscript. PZ carried out the experiment and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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