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Utilization of rDNA ITS2 Region to Characterized the Metacercaria of *Paragonimus heterotremus* (Family: Paragonimidae) Parasitizing Crab Host in Manipur, India

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Authors' contributions

This work was carried out in collaboration between both authors. Author VT designed and analyzed the work. Author VDA carried out experimental work, analyzed and prepared the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Freshwater crabs, *Potamiscus manipuriensis* in Manipur, Northeast India harbour trematode metacercariae of family Paragonimidae which was identified as *Paragonimus* sp. based on morphological criteria. Molecular characterization by PCR amplification of rDNA regions of inter

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transcribed spacers 2 was only used for molecular characterization of this metacercaria. The sequence generated from the metacercariae was compared with other species of trematode in the family Paragonimidae. The data extracted from the public domain NCBI, were analysed using BLAST and ClustalW embedded in Bioedit. The phylogenetic tree constructed based on the ITS2 marker revealed a close relationship with *P. heterotremus* isolates with a significant bootstrap value of 99. Hence the particular metacercaria confirmed the identification as *Paragonimus heterotremus*.

Keywords: Crabs; paragonimus; rDNA ITS2; metacercaria; India.

1. INTRODUCTION

With the development of molecular techniques, especially the PCR amplification of ITS2 rDNA has solved many taxonomic issues associated with helminth parasites. As the region is evolving at a faster rate, thus it contained variable regions flanked by more conserved 5.8S and 28S genes [1]. This gene region is proven as potential candidate for phylogenetic analysis and for answering many systematic issues at different taxonomic level [2,3,4,5]. These molecular tools diagnosed all the larval stages of the trematode [6]. The gene has been successfully used for species characterization for Paragonomid taxa [7,8,9].

Crustacean serves as a potent second intermediate host for large numbers of trematodic fluke thus harbouring their infective metacercarial larval stage; thus completing their complex life cycle [10]. The trematode flukes of the family Paragonimidae Dollfus, 1939, is an important zoonotic disease causing public health problem worldwide, and endemic in many parts of Asia, South America, Cameroon and Nigeria of Africa with the cases are centering in Asia [11,12,13,14]. In India due to the increasing reports of the human paragonimiasis, the endemicity is expanding in the northeastern states of Manipur, Arunachal Pradesh and Nagaland [12,15,16]. In Manipur, Northeast India, two commonly edible fresh water crab species namely, *Potamiscus manipurensis* and *Barytelpusa lugubris* was found to be infected with metacercaria of *Paragonimus* spp [17,18]. In the present communication we provide molecular characterization and identification of the taxa at metacercarial stage using rDNA ITS2 gene marker.

2. MATERIALS AND METHODS

2.1 Parasite Material and DNA Isolation

Metacercaria of *P. heterotremus* was found infecting the freshwater crab host *Potamiscus manipuriensis* in the region Manipur, Northesat India [18]. So, collection of respective hosts was made from the susceptible foci. Metacercariae were isolated from the host muscle using artificial gastric digestion technique [19], and were fixed in 70% alcohol for further molecular work. The total genomic DNA was extracted from the recovered metacercaria on FTA cards using Whatman's FTA Purification Reagent as described elsewhere [9].

2.2 DNA Amplification, Sequencing, and Its Analysis

The rDNA ITS2 region was amplified from the DNA extracted from the metacercaria by polymerase chain reaction (PCR) using the universal primers for ITS2: 3S (forward): 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' and A28 (reverse): 5'- GGGATCCTGGTTAG TTTCTTTTCCTCCGC-3' [20]. The PCR amplification was performed following the standard protocol [21] with minor modification. The amplified products were view and separated by gel electrophoresis with an agarose concentration of 1.6% (w/v) in TAE buffer, stained with ethidium bromide and then photographed during ultraviolet light transillumination.

The PCR-amplified products was purified using Genei Quick PCR purification kit. Then the amplified products was sequenced in both directions using automated sequencing services provided by Macrogen Service Center, Seoul, Korea. The generated DNA sequence was analyse further by using various bioinformatics tools including similarity search Basic Local Alignment Search Tool (BLAST), (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3 Molecular Phylogenetic Analysis

Sequence identity matrices was constructed and multiple sequence alignment was done using Bioedit software version 7.0.9.0 [22] for the amplified ITS2 region for the metacercaria

recovered under study, using related sequences from the same family retrieved from the public domain. Phylogenetic tree was constructed from the output files from Bioedit in fasta format using MEGA11 [23] deducing the distance-based Neighbour Joining (NJ) methods. Boostraping was done with 1000 bootstrap replicates while constructing tress [24].

3. RESULTS

3.1 PCR Amplification of ITS Regions and Its Analysis

The selected marker rDNA- ITS2 amplicon had an estimate length of ~500bp and the PCR-amplified products were obtained (Fig. 1). The amplicon size for rDNA-ITS2 of *P. heterotremus* is 509 bp in length. This generated sequence was deposited in GenBank (under accession numbers: KF781293). The sequence was subsequently compared and analysed with their closely related sequences from the family Paragonimidae retrieved from the GenBank. A total of 24 accession numbers for various *Paragonimus* spp pertaining to rDNA-ITS2 regions were used during the analysis (Table 1). The BLAST hit results show that the query ITS2 sequences were more similar to the sequences of *Paragonimus heterotremus* (Fig. 2).

For phylogenetic analysis, the tree was constructed with NJ methods. In the tree, *Opisthorchis viverrini* was used as the outgroup.

From the Sequence identity matrix, it was found that the metacercaria recovered under the present study stay closely related to *P. heterotremus* isolates from North-east India (Manipur, Arunachal Pradesh and Nagaland) with highest sequence similarity of 94% with isolates from Arunachal Pradesh, India (Table 2). The phylogenetic tree constructed for the marker gene depicted the topology in which the query sequence claded in a single clade with isolates of *P. heterotremus* with a significant bootstrap values of 90-99%. Different species forming different clades with their isolate. In the tree constructed, the outgroup, *Opisthorchis viverrini* formed a separate clade (Fig. 3).

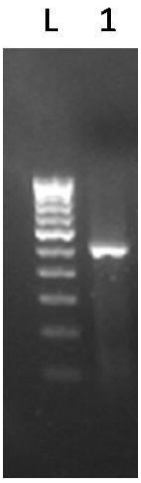

Fig. 1. PCR products of *Paragonimus* sp metacercaria (L- Marker (100 bp ladder), Lane 1 – ITS2 from the metacercaria)

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Paragonimus heterotremus internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and interna...	Paragonimus het...	941	941	100%	0.0	100.00%	509	KF781293.1
Paragonimus heterotremus from India 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, ...	Paragonimus het...	872	872	98%	0.0	98.20%	508	DQ836248.1
Paragonimus heterotremus isolate LC small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Paragonimus het...	843	843	98%	0.0	97.20%	7661	OP081040.1
Paragonimus heterotremus genes for 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, type2	Paragonimus het...	817	817	91%	0.0	98.49%	461	AB308377.1
Paragonimus heterotremus genes for 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, type1	Paragonimus het...	811	811	91%	0.0	98.28%	461	AB308376.1
Paragonimus heterotremus Cr7-10 genes for contains 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and ...	Paragonimus het...	791	791	91%	0.0	97.42%	462	LC360505.1

Fig. 2. Blast Result showing the query sequence showing similarity to *P. heterotremus* isolates

Table 1. List of Paragonimidae taxa included in the sequence analysis along with their accession numbers for the corresponding ITS2 sequence

Sl. No.	Name of species	Host Species	Locality	ITS2
1	*P. westermani*	-	India: Arunachal Pradesh (AP)	DQ836246.1
2	"	*Maydelliathelphusa lugubris*	India: Arunachal Pradesh (AP), East Siang	JN656206.1
3	"	"	India: Assam	JN656199.1
4	"	-	Thailand	AB354214.1
5	"	*Cambarooides* sp.	China	AB713404.1
6	"	*Cambarooides similis*	South Korea	AF333278.1
7	"	-	Japan	U96907.1
8	"	-	Taiwan	U96908.1
9	"	-	Philippines	U96910.1
10	"	-	Malaysia	U96909.1
11	*P. heterotremus*	*Potamiscus manipurensis*	India: Manipur	AB308378.1
12	"	*Potamiscus manipurensis*	India: Manipur	AB308377.1
13	"	Homo sapiens	India: Arunachal Pradesh (AP)	DQ836248.1
14	"	-	India: Nagaland	AB456559.1
15	"	-	Thailand	AB354221.1
16	"	-	China	HM627192.1
17	"	*Potamon lipkei*	Laos	AB370343.1
18	"	*Potamiscus tannanti*	Viet Nam	AB270688.1
19	*P. siamensis*	*Sartoriana spinigera*	India: Assam	JQ322635.1
20	"	*Sayamia germaini*	Thailand	AB354222.1
21	*P. bangkokensis*	*Ranguna smalleyi*	Thailand	AB248091.1
22	*P. proliferus*	-	China	EU401805.1
23	*P. proliferus*	Potamiscus tannanti	Viet Nam	AB663676.1
24	*P. skrjabini*	*Potamiscus manipurensis*	India: Manipur	AB325516.1

Table 2. Sequence identity matrix (%) of ITS2 sequences of various Paragonimus spp as retrieved from GenBank

Seq->	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1.*P.heterotremus*,India:Manipur;Metacercaria*	ID																								
2.*P.westermani*,India:AP,East-Siang	87	ID																							
3.*P.westermani*,India:Assam	89	96	ID																						
4.*P.westermani*,India:AP	87	94	96	ID																					
5.*P.westermani*,Thailand:Saraburi	82	91	91	88	ID																				
6.*P.westermani*,China	83	35	92	89	97	ID																			
7.*P.westermani*,South Korea	64	73	71	69	76	78	ID																		
8.*P.westermani*,Philippines	63	71	71	69	76	76	98	ID																	
9.*P.westermani*,Japan	64	73	71	69	76	78	100	98	ID																
10.*P.westermani*,Taiwan	64	73	71	69	76	78	99	97	99	ID															
11.*P.westermani*,Malaysia	64	72	71	69	76	77	98	99	98	98	ID														
12.*P.heterotremus*,India:Manipur;Meta	89	85	85	83	90	91	71	70	71	71	70	ID													
13.*P.heterotremus*,India:Manipur;Egg	89	85	85	83	90	91	71	70	71	71	70	100	ID												
14.*P.heterotremus*,Thailand:Saraburi	88	87	87	84	91	93	71	70	71	71	71	98	98	ID											
15.*P.heterotremus*,China	80	78	79	76	76	78	78	77	78	78	77	83	83	84	ID										
16.*P.heterotremus*,India:AP	94	89	89	90	81	83	64	63	64	64	63	90	90	88	81	ID									
17.*P.heterotremus*,India:Nagaland	89	85	85	83	90	91	71	70	71	71	70	100	100	98	83	90	ID								
18.*P.heterotremus*,Laos	69	67	67	64	70	71	91	90	91	91	90	77	77	78	85	70	77	ID							
19.*P.heterotremus*,Vietnam	68	66	66	64	69	71	90	89	90	90	89	76	76	77	85	69	76	99	ID						
20.*P.siamensis*,India: Assam	80	89	89	86	89	90	75	75	75	75	75	84	84	85	80	80	84	70	69	ID					
21.*P.siamensis*,Thailand	81	90	90	87	95	96	74	74	74	74	74	89	89	91	76	81	89	69	69	93	ID				
22.*P.bangkokensis*,Thailand	84	86	87	84	91	92	71	70	71	71	70	92	92	93	78	83	92	71	71	85	90	ID			
23.*P.proliferus*,Vietnam	86	88	88	85	92	93	72	71	72	72	71	944	94	95	80	85	94	74	73	86	92	94	ID		
24.*P.proliferus*,China	86	87	87	84	92	93	72	71	72	72	71	94	94	96	80	85	94	74	73	86	92	94	99	ID	
25.*P.skrjabini*,India: Manipur	86	87	87	84	92	93	72	71	72	72	71	93	93	95	80	85	93	73	73	86	91	94	98	98	ID

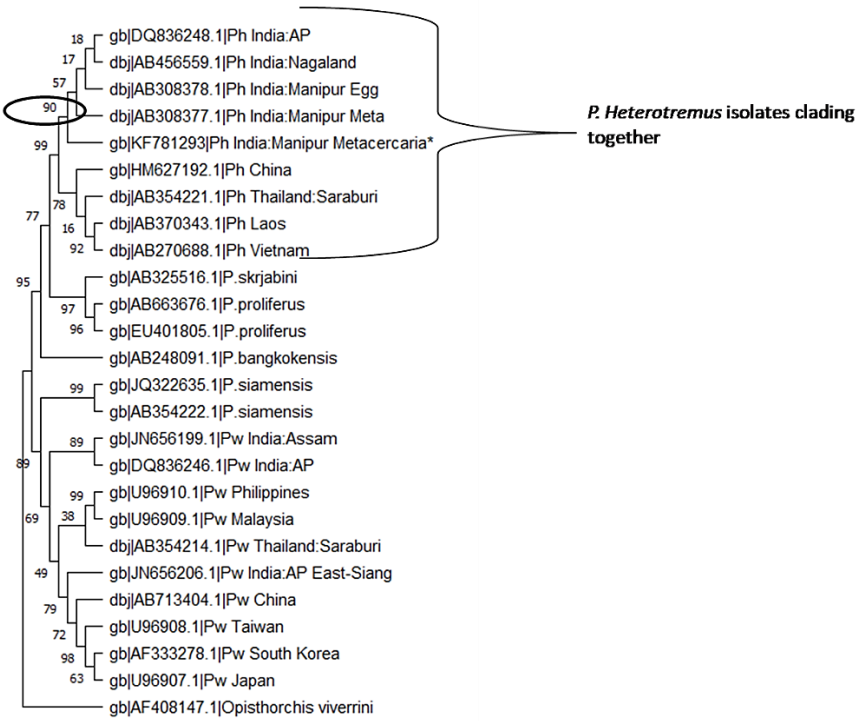

Fig. 3. Phylogenetic trees using NJ method inferred from the ITS2 sequence data of various Paragonimus species. The bootstrap values are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

[- Query sequences: Ph- Paragonimus heterotremus; Pw- Paragonimus westermani]*

4. DISCUSSION

Characterization of trematode species with morphological parameters has become insufficient to solve many taxonomic issues involving various animal phyla including platyhelminth especially for morphologically similar but genetically distinct species and also for their different larval stages. Also the traditional methods fail to identify the adult species with damaged internal organs or deformed structures due to the insufficient characters for the identification of species. Nevertheless with the development of new molecular tools and technique, especially PCR based amplification of rDNA has been used successfully used to supplement the morphological characterization of various taxa [25,26,27,28,29].

During the first epidimiological research on Paragonimiasis which was carried out in Manipur, during 1986- 1987, have identified many endemic areas in the region [17]. From the study, two fresh water carb species namely *Potamiscus manipurensis* and *Barythelphusa lugubris* was found to serves as a potential intermediate host for *Paragonimus* spp. *P. manipuriensis* found in the hill streams of Manipur was found harbouring the metacercaria of *P. hueitu'ngensis*, *P. heterotremus*, and *P. skrjabini* [17,29] supporting our present study. Many endemic areas for P. heterotremus infection were also reported in different parts of the states Nagaland, Arunachal Pradesh [30]. So, *P. heterotremus* was reported as causative agent for human pulmonary paragonimiasis in Manipur and Arunachal Pradesh, Northeast India [12,15,17,31,32]. In phylogenetic analysis based on the PCR amplification of rDNA-ITS2 marker, our query sequence closely claded with *P. heterotremus* of the family Paragonimidae with a significant boostrap value of more than 70% as generally accepted indicating an authentic phylogenetic analysis [19,24]. So, the technique has also provided the accurate discrimination between distinct species as in the phylogenetic tree the different species claded among their geographical isolates.

5. CONCLUSION

The present study provides the molecular characterization of the *Paragonimus*

heterotremus metacercariae that infect freshwater crab host *Potamiscus manipuriensis* in the region. The study also provides the utility of molecular tools in taxonomic identification of the species. Thus the technique can be utilized for epidemiological survey for finding prevalence of any infective helminth parasite and possible intermediate host in the region, if any.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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