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Molecular identification of a proteocephalid cestode, *Gangesia* sp., infecting *Mystus bleekeri* in Manipur, India using 28S ribosomal DNA

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Abstract
This study identifies a proteocephalid cestode of the genus *Gangesia* inhabiting the freshwater catfish *Mystus bleekeri*. Molecular characterization was conducted via PCR amplification of the 28S ribosomal DNA (rDNA) region, with the resulting sequences compared against related taxa from the NCBI database. Phylogenetic analysis using Maximum Likelihood revealed a high bootstrap value (80–88%), placing the specimen in a clade with *G. vachai* and suggesting they belong to a species complex. Notably, this represents the first documented report of *Gangesia* sp. from Manipur, India.

Keywords: Manipur, 28s rDNA, fish hosts, *Gangesia*, India

Introduction
Advances in the field of molecular techniques have revolutionized the identification of helminth parasites, by isolating and analysing specific rDNA markers now solving many taxonomic issues, that morphological study alone might miss (Álvarez & Wendel, 2003 [1]; Müller *et al*., 2007; Wickramasinghe *et al*., 2009; Yan *et al*., 2013) [14, 15]. Among these marker regions, the large subunit ribosomal DNA (28S rDNA) is valuable with mixed variable and conserved fragments, proven highly effective in resolving phylogenies of digeneans (Athokpam & Tandon, 2014) [2]. Molecular data for the genus *Gangesia* remain limited. There is a research gap considering the catfish, carnivorous in nature and typically harbor high helminth load (Nguyen *et al*., 2009; Phan *et al*., 2010). In the Indian context, information's available in the public domain for the genus *Gangesia* (family Proteocephalidae) are sparse; with *G. pseudotropii* and *G. agraensis* (Verma, 1928) [12], *Gangesia bendsurensis* n. sp. (Reddy *et al*., 2011) [10], *Gangesia shivajiraoi* Sp. Nov (Dhole *et al*., 2012) [5], *Gangesia orientalis* Sp. Nov. (Deshmukh *et al*., 2016) [4], *Gangesia punjabensis* sp. nov. (Jasrotia & Kaur, 2017) [7], *Gangesia pashupatii* sp.n, *Gangesia puriensis* sp. n. (Banerjee *et al*., 2019), respectively.
From the region, there is no report available on the infection of freshwater fish species with cestode of the genus *Gangesia*. This study aims to provide the first molecular characterization of a *Gangesia* species infecting *M. bleekeri* from Manipur. By utilizing 28S rDNA sequencing, the study not only identifies the parasite but also contributes to the molecular database of fish parasites for North East India.

Materials and methods
Parasite material
The cestode parasite was recovered from small intestine of fish host genus *Mystus bleekeri* (Ngasep: Local name) from different collection sites in Manipur. The recovered specimens were processed for whole mount preparation, stained with acetocarmine stain following standard protocol. The specimen is characterized as *Gangesia* sp. by the presence of four scoleces, armed rostellum (Verma, 1928) [12] (Fig 1). They were fixed in 70% ethanol for further molecular study.

Molecular study
The specimen fixed in 70% ethanol was further processed for genomic DNA extraction by using a QIAamp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany) following manufacturer's instructions. The 28S rDNA region was PCR amplified following the standard protocol of White (1993) [13] with minor modifications by using PCR primer sets dig12 (forward): 5‟-AAGCATATCACTAAGCGG-3‟ and 1500R (Reverse): 5‟-GCTATCCTGAGGGAAACTTCG-3‟ (Tkach *et al*., 2000). The thermal gradient for the reaction with an initial denaturation at 94 ºC (5min), annealing at 58 ºC (30sec) and final extension at 72 ºC (10 min) respectively. The amplified products were sequenced and submitted to NCBI GenBank (National Center for Biotechnology information) and the accession number is acquired.

Sequence and phylogenetic analyses
Basic Local Alignment Search Tool (BLAST) was used to conduct similarity search in the public domain and retrieved highly similar sequences for analysis and are presented in Table 1. Multiple sequence alignment was constructed using Bioedit software version 7.0.9.0 (Hall, 1999) and file was saved in 'fasta' format. The saved 'fasta' file was open in MEGA12 (Kumar *et al*., 2024) [8] for Maximum Likelihood (ML) phylogenetic tree construction. Adaptive boostrapping was done with 1000 replicates. The values are shown next to the branches (Hillis & Bull, 1993). The analytical procedure encompassed 24 coding nucleotide sequences using 1st, 2nd, 3rd, and non-coding positions with 1,652 positions in the final dataset.

Table 1: List of various *Gangesia* spp with corresponding genetic markers 28S rDNA region used in sequence analysis along with their host, geographical location and accession numbers

Sr. No	Name of the species	Accession number	Host	Geographical location
1	*Gangesia vachai*	JX477432.1	*Wallago attu*	Bangladesh: Durgapur
2	*G. vachai*	JX477437.1	*Mystus tengara*	India: Siliguri, West Bengal
3	*G. bengalensis*	JX477438.1	*Wallago attu*	India: Berhampur, West Bengal
4	*G. bengalensis*	JX477427.1	*Wallago attu*	India: Rishra, West Bengal
5	*G. bengalensis*	JX477429.1	*Wallago attu*	India: Rishra, West Bengal
6	*G. macrones*	JX477433.1	*Sperata seenghala*	Bangladesh: Mymensingh
7	*G. macrones*	JX477446.1	*Sperata seenghala*	India: Godavari River, Maharashtra
8	*G. macrones*	JX477444.1	*Sperata seenghala*	India: Godavari River, Maharashtra
10	*G. agraensis*	JX477435.1	*Wallago attu*	India: Balurghat, West Bengal
11	*G. agraensis*	JX477430.1	*Wallago attu*	India: Balurghat, West Bengal
12	*G. agraensis*	JX477443.1	*Wallago attu*	India: Siddeshwar reservoir, Maharashtra
13	*G. agraensis*	JX477440.1	*Wallago attu*	India: Guwahati, Assam
14	*G. agraensis*	JX477439.1	*Wallago attu*	India: Guwahati, Assam
15	*G. parasiluri*	AF286935.1	*Silurus asotus*	Japan
16	*G. oligonchis*	JX477451.1	*Tachysurus fulvidraco*	Russia: Ilistaya River, Far East
17	*G. polyonchis*	ON181449.1	*Silurus asotus*	Russia
18	*G. polyonchis*	ON181453.1	*Silurus asotus*	Russia
19	*G. polyonchis*	ON181447.1	*Silurus asotus*	Russia
20	*G. oligonchis*	JX477452.1	*Tachysurus fulvidraco*	Russia: Ilistaya River, Far East
21	*G. oligonchis*	JX477451.1	*Tachysurus fulvidraco*	Russia: Ilistaya River, Far East
22	*G. oligonchis*	MH665421.1	-	China: hubei
23	*G. oligonchis*	MH665422.1		China: hubei

Results and Discussion

The sequence generated from cestode species (Fig. 1) were analysed for systemic position with their closely related isolates from the family Proteocephalidea retrieved from public domain listed in Table 1. The amplified and sequenced rDNA 28S region was deposited in GenBank under accession numbers: PP079917 with amplicon size of 1456 base pair in length. Phylogenetic trees was constructed using the rDNA 28S datas retrieved from GenBank (Table 1). Evolutionary analysis by the Maximum Likelihood method showed with the highest likelihood of (-4,234.76) which was drawn to scale, number of substitutions per site measured the branch lengths. The parasite under study cladded with *G. vachai* showing species complex with a significant bootstrap value of 80-88 respectively (Fig. 2). Bootstrap value of ≥70% in a phylogenetic analysis is generally accepted as an authentic analysis (Hillis &Bull 1993; Tandon *et al.,* 2007). In the phylogenetic tree, *Fasciolopsis buski*, the outgroup, formed a seperate clade. So, molecular analysis characterized the cestode harbouring in the intestine of *Mystus bleekeri* as *Gangesia* sp. Zehnder and Mariaux (1999) have analysed 53 proteocephalidean cestodes (Eucestoda) based on nuclear rDNA sequences providing better systematics analysis among Proteocephalidea. Jasrotia & Kaur (2017) has also utilized LSUrDNA (28S rDNA) to identify a new species *Gangesia punjabensis* sp. nov. So, this molecular technique of amplifying rDNA region as a marker provides accurate characterization of the species at different taxonomic level.

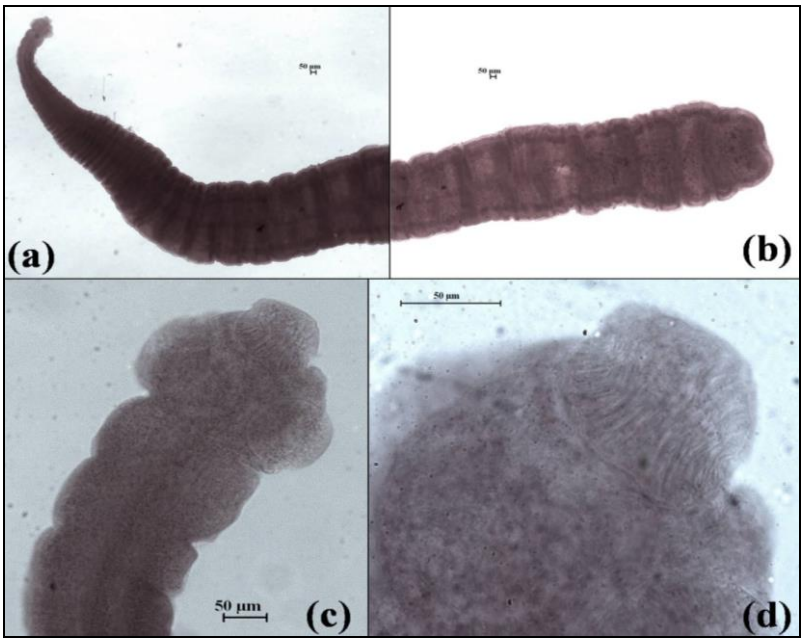

Fig 1: Light microscopy of the *Gangesia* sp. a. Anterior portion b. Posterior showing gravid proglottids, c & d. Magnified head showing rostellum and sucker

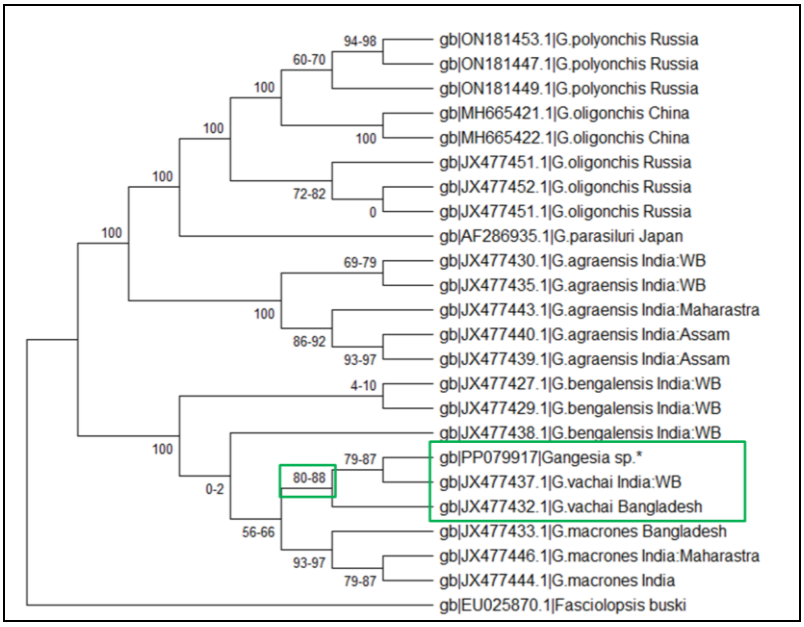

Fig 2: Phylogenetic trees from 28S rDNA sequence data of various *Gangesia* species. The analysis was inferred using Maximum Likelihood method. Bootstrap values are shown next to the branches. * query sequence

Conclusion

This study focuses on the molecular identification of *Gangesia* sp., a parasite found in the intestine of the freshwater catfish *Mystus bleekeri* within this region. By utilizing 28S rDNA regions as genetic markers, the research demonstrates the precision of molecular tools in taxonomic classification—especially in cases where traditional morphological identification proves difficult or prone to error. These findings highlight a broader need to investigate other regional freshwater fish species for potential parasitic infections.

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