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Molecular Identification of *Pallisentis Ophiocephali* (Acanthocephala) Parasitizing Fresh Water Garfish *Xenentodon Cancila* in Bangladesh

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ABSTRACT

The diversity of parasites in Freshwater fish has attracted significant attention from researchers due to its ecological and economic implications. This study introduces the first molecular example of *Pallisentis ophiocephali* (Acanthocephala), a parasite typically found in the garfish *Xenentodon cancila*, which inhibits the freshwater ecosystems of Bangladesh. The identity of the parasite has been confirmed through morphological analysis and molecular techniques, specifically PCR amplification of the mitochondrial Cytochrome C Oxidase I (COI) gene sequencing. After thorough phylogenetic analysis, *P. ophiocephali* has been classified into the phylum Acanthocephala. This research enhances our understanding of host-parasite relationships within the freshwater ecosystems of Bangladesh and highlights the importance of molecular tools in parasitic identification.

Keywords: *Pallisentis Ophiocephali; Xenentodon Cancila*; Acanthocephala; Molecular Identification; Bangladesh Freshwater Parasites

Introduction

The diversity in Freshwater fish in Bangladesh serves as a suitable habitat for various parasitic organisms. Amongst these, the Acanthocephalans, also known as thorny-headed worms, which attract interest due to their complex life cycles involving vertebrates as definite hosts. One such acanthocephalan is the parasite *Pallisentis ophiocephali*, commonly found in freshwater environment across Asia. Helminth infection caused by the acanthocephan parasite *Pallisentis ophiocephali* in *Xenentodon cancila*, is prevalent in Bangladesh (Bashirullah [1-3]). Studies on Acanthocephalans across Southeast Asia prominently highlight the vast diversity of species in the genus Palisentis, with 28 out of the 33 species reported from India (Gautam, et al. [4]). Research on *Pallisentis roparensis* n. sp. (Acanthocephala: Quadrigyridae) has been conducted in India, focusing on morphology, molecular description and epidemiology (Khushboo Rana, et al. [5]). Histopathological studies on different hosts infected by various helminth parasites have shown significant pathological effects, which might lead to host mortality (Chakravarty, et al. [6-8]; Ahmed, et al. 1979).

It is well-established that certain parasite species are more capable of causing disease than others within the same genus (Procop [9]). Consequently, some species exhibit greater sensitivity to certain drugs, while others demonstrate resistance to the same treatments (Chaijaroenkul, et al. [10]). Therefore, proper identification of organisms at the molecular level to ascertain their taxonomic status is crucial. Molecular identification techniques can significantly expedite

the process of identifying unknown organisms, allowing for species identification regardless of their physical state. This typically involves the use of a -DNA based marker known as a molecular marker; characterization using this marker is referred to as molecular characterization. Molecular markers, especially mitochondrial DNA cytochrome oxidase I (mt-COI) are non-recombinant and independent of environmental factors, making them preferable for species distinction (Layton [11]). Molecular markers serve as convenient tools for intricate taxonomic identification, especially when morphological characteristics may be confusing (Douek, et al. [12,13]).

The technique has been extensively used to describe and differentiate species, particularly between closely related and sister species (Nadler, et al. [14,15]). Such methods contribute significantly to the current taxonomy of parasites through various genetic markers (Sharma, et al. [16,17]). The utility of COI in outlining, identifying and inferring phylogenetic relationships is well established across different organisms, including nematodes, digeneans, cestodes and acanthocephalans (Garcia-Varela, et al. [18]). Acanthocephalans, as spiny- headed worms, represent the phylum *Acanthocephala*, which comprises four classes: *Archiacanthocephala, Eoacanthocephala, Palaeacanthocephala* and *Polyacanthocephala* (Amin, et al. [19-21]). Although several authors have endeavored to study the phylogenetic relationships between these classes, such relationships remain unresolved (Garcia-Varela, et al. [22]). Recently, the mitochondrial genome, has been sequenced in acanthocephalans, specifically Pallisentis celatus (Ting Shuang Pan, et al. [23]), and examined alongside mt genomes from other acanthocephalans to assess phylogenetic relationships within the Syndermata.

Despite the presence of acanthocephalan parasites in various fish species, Bangladesh still lacks molecular data necessary for their identification. The freshwater garfish *Xenentodon cancila*, despite being commercially abundant and a common host for various parasites, has not had any molecular data reported for *P. ophiocephali* in this host species. Therefore, the objectives of this study were to

1) Identify *P. ophiocephali* both morphologically and molecularly, and
2) Understand the phylogenetic positioning of the species within Acanthocephala.

Justification of the Work

Despite previous records of acanthocephalan parasites in various fish species, molecular data for their identification remain limited in Bangladesh. The study aims to enhance the understanding of parasite-host interactions in *X. cancila* and highlight the importance of molecular tools in accurately identifying the acanthocephalan parasite *Pallisentis ophiocephali*.

Materials and Methods

Sample Collection

Specimens of freshwater garfish (*Xenentodon cancila*) were collected from the Meghna River in Bangladesh during a field survey conducted from March to December 2021. The fish were immediately transported to the laboratory in aerated containers for parasitological examination. Dissections were performed under sterile conditions to retrieve endoparasites from the gastrointestinal tract.

Morphological Identification

The extracted parasites were rinsed in physiological saline and examined under a light microscope. Morphological characteristics were documented using established taxonomic keys for Acanthocephala. Key identifying features, such as body size, proboscis structure, and trunk supination, were carefully noted.

Molecular Techniques

DNA Extraction: The parasite specimens were preserved in 70% ethanol until molecular analysis. DNA extraction was performed using a commercial DNA extraction kit (Qiagen, Germany) following the manufacturer's protocol.

PCR Amplification: The Cytochrome c Oxidase I (COI) gene was targeted for amplification using polymerase chain reaction (PCR). PCR is a common molecular technique used to amplify specific regions of DNA, generating thousands to millions of copies of a particular DNA sequence. For PCR amplification, we used short DNA fragments called primers to define a 432 bp region of the COI gene to be copied. The following primers were used:

Forward primer (COI P1-F: TTTTTTGGGCATCCTGAGGTTTAT)

Reverse primer (COI P2-R: TAAAGAAAGAACATAATGAAAATG)

PCR reactions were conducted in a 25 µl mixture consisting of 2.5µl of 10x PCR buffer, 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 unit of Taq DNA polymerase (Thermo Fisher Scientific), and 2 µL of template DNA. The cycling conditions for COI included an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds, 50 °C for 45 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes.

Sequencing and Phylogenetic Analysis: Purification of amplified PCR products was carried out using a PCR purification kit (Thermo Fisher Scientific), and the products were sequenced by Sanger sequencing. The sequences were aligned and compared to those in the NCBI GenBank database using BLAST. A phylogenetic tree was constructed using the neighbor-joining method with MEGA X software to determine the evolutionary relationships of *P. ophiocephali* with other acanthocephalan species (Kumar, et al. [24]).

Results

Morphological Identification

The parasites were identified as *Pallisentis ophiocephala* based on unique morphological characteristics, including a robust trunk with spines, a sac-like body shape, and a cylindrical proboscis featuring 16 longitudinal rows of hooks. The proboscis hooks are arranged in four circles, with the hook sizes gradually decreasing from the first to the fourth circle. The vulva is located posterio-ventrally, and a long tube opens into the vagina, continuing into the basal portion of the funnel-shaped uterine bell. Key measurements include a body length of 7.01-8.39 mm, a body breadth of 0.36-0.58 mm, and a proboscis length and breadth of 0.48-0.54 mm by 0.13-0.15 mm. The hook lengths are as follows: first circle: 0.08-0.07 mm; second circle: 0.081-0.084 mm; third circle: 0.064-0.07 mm; fourth circle: 0.034-0.04 mm. These characteristics are consistent with earlier descriptions of *P. ophiocephali*.

Molecular Identification

The COI gene amplified by PCR produced products of the anticipated size (approximately 432 bp for COI). The nucleotide composition of the gene was found to be 19% for A, 37% T, 16% C and 28% G. The nucleotide composition yielded 20% for A, 39% T, 13% C and 28% G. GC content is an important parameter for a particular gene, and it was analyzed for the species as shown in Table 1. The resulting sequences were uploaded to GenBank with the accession numbers OM679999, OM680000 and OM680001. However, during the experiment, no accurate match for *P. ophiocephala* was found, although a related match, *Pallisentis celatus* was identified. This represents the first report of *P. ophiocephali* in NCBI. Genetic divergence (K2P distance %) between intra-species and inter-species was calculated. In addition to the three specimens of *Pallisentis ophiocephali* in this study, additional COI sequences of one related species (*Pallisentis celatus*) were downloaded from GenBank, NCBI. Upon calculation of K2P with these four COI sequences from two species, genetic divergence increased as expected with higher taxonomic rank- 0% to 2% within species, and 22% between species. The K2P distance results indicate that intra-species divergence was significantly lower than inter-species divergence, confirming the effectiveness of DNA barcoding as a powerful tool for species identification and differentiation (Figure 1) (Table 2).

Table 1: GC (%) content for seven specimens of acanthocephalans.

Species	Number of the sequenced specimen (BD)	Overall (% GC)	Overall (% AT)
Pallisentis ophiocephali	1	44	56
P. ophiocephali	1	44	56
P. ophiocephali	1	44	56
Pallisentis celatus	1	41	59
Neoechinorhynchus emyditoides	1	34	66
Neoechinorhynchus emyditoides	1	34	66

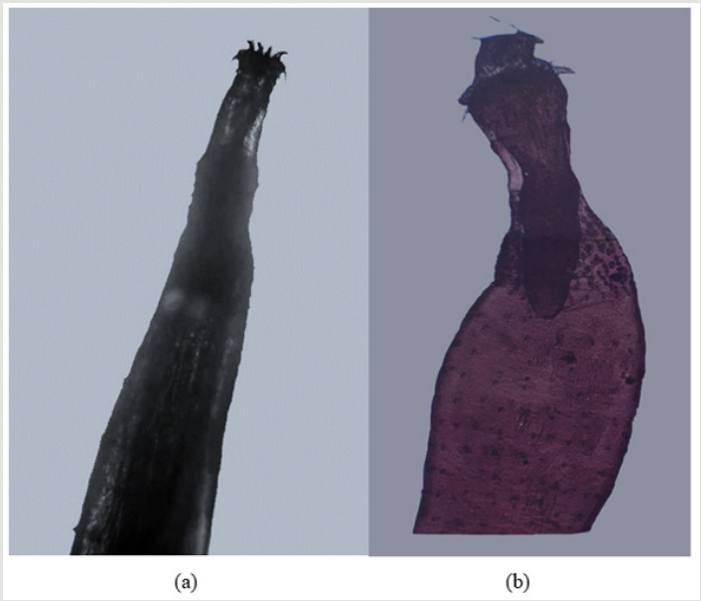

Figure 1: (a - b): *Pallisentis ophiocephali* (Anterior Portion).

Table 2: Minimum, maximum, mean K2P (%) within and between species.

	Min	Max
Intra species *(P. ophiocephali)*	0	2
Interspecies *(P. celatus)*	-	22
Intra species *(N.emyditoides)*	-	7
Interspecies *(N. mexicoensis)*	-	26

Phylogenetic Analysis

A phylogenetic tree was constructed using COI sequences with three statistical methods: UPGMA, Neighbor Joining and Maximum Likelihood. The monophyletic clade of intraspecific sequences in the phylogenetic tree confirmed the effectiveness of COI in species delimitation, with no taxonomic deviations at the species level. All sequences of *P. ophiocephali* and *P. celatus* clustered under a monophyletic clade with strong support in the evolutionary tree. The phylogenetic tree based on COI sequences positioned *P. ophiocephali* within the family Quadrigyridae, closely related to other *Pallisentis* species reported in Asia (Figure 2). The bootstrap values for key nodes indicated strong support for the clustering of *P. ophiocephali* with other acanthocephalans.

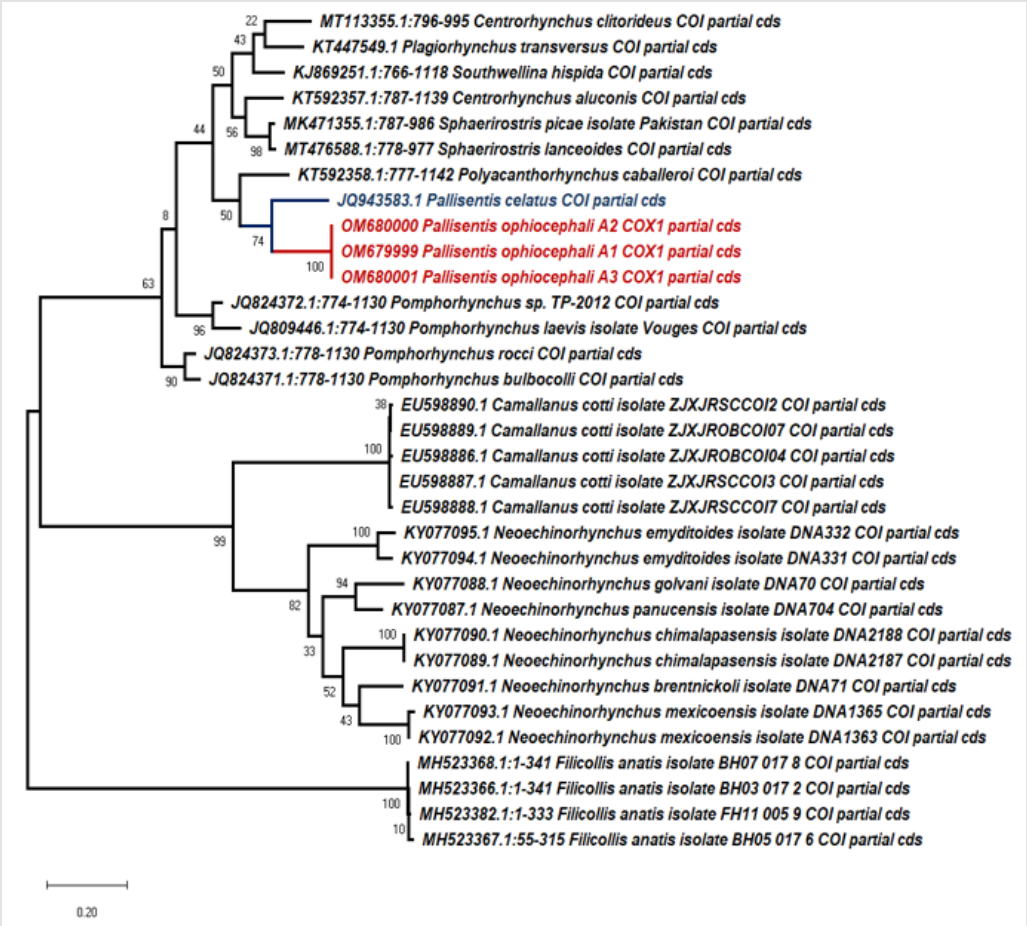

Figure 2: Phylogenetic tree of acanthocephalans based on partial sequence of COI gene. Sequences amplified in this study were designated as BD. Neighbor-Joining (NJ) was chosen as the statistical method (500 bootstrap replicates were done and the values were included).

Discussion

The mitochondrial (mt) COI gene of *Pallisentis ophiocephali*, an acanthocephalan from the class Eoacanthocephala, was sequenced in the present study, which providing the first molecular evidence of *P. ophiocephali* infection in *Xenentodon cancila*. The degree of similarity between the GenBank and COI sequences supports the accuracy of molecular methods in identifying parasitic species. Phylogenetic analysis aligns with previous studies, placing *P. ophiocephali* within the Quadrigyridae family. The COI sequence of *P. ophiocephali* formed a monophyletic clade with the sequence of *P. celatus* from China, with 78% bootstrap support. The utility of molecular identification for distinguishing acanthocephalan species, such as *P. celatus*, has been documented in previous studies (Tin Shuang Pan, et al. [23]) based on partial COI sequences. Due to the recent surge in the description of many new species of the genus Pallisentis (Gupta, et al. [25,26]) molecular characterization of species is essential. The description of some species, either with overlapping characters of more than one subgenus or not fitting into any sub-genus, necessitated the revision of the genus. This study contributes to revising the taxonomic status of *Pallisentis ophiocephai* in Bangladesh and adds to the global DNA barcode database. The findings underscore the ecological significance of *X. cancila* as a host for acanthocephalans and raise concerns about potential health effects on this economically important fish species. Furthermore, this study highlights the critical need to combine morphological and genetic methods for precise parasite identification, especially in regions like Bangladesh, where parasitic biodiversity is not well understood.

Conclusion

Pallisentic ophiocephali in Bangladeshi garfish *Xenentodon cancila* was identified molecularly, providing crucial insights into the freshwater parasite biodiversity in the region. This study extends the known range of *P. ophiocephali* and establishes a foundation for further research on the ecological and health impacts of parasitic infections within the Bangladeshi freshwater ecosystem.

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