MOLECULAR IDENTIFICATION OF SIX NEW BENTHIC FAUNA RECORDS IN THE ANDHARMANIK RIVER, BANGLADESH

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ABSTRACT

DNA barcoding has proven to be an effective alternative to traditional morphological methods for species identification. In this study, we employed a molecular approach to identify benthic fauna collected from the Andharmanik River estuary in Bangladesh. Mitochondrial cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) genes were used as genetic markers for species delineation. A total of six benthic species—*Cerebratulus lacteus*, *Maculaura magna*, *Maculaura cerebrosa*, *Perinereis cultrifera*, *Paralacydonia weberi*, and *Phyllodoce medipapillata*—were reported for the first time in Bangladesh in this study. The mean interspecific genetic divergence, calculated using the Kimura 2-Parameter (K2P) model, was 39.72 ± 0.07% for 16S rRNA and 29.72 ± 0.03% for COI, indicating sufficient resolution for accurate species discrimination. Phylogenetic relationships were inferred using the Maximum Likelihood method, and individuals belonging to the same species consistently formed species-specific clades. This study presents the first DNA barcoding-based identification of polychaete and nemertean species in Bangladesh.

KEYWORDS: Andharmanik River, Benthos, Nemertean, New records, Polychaete, DNA Barcoding.

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Introduction

Benthic organisms, collectively known as benthos, represent a diverse array of invertebrate taxa, including Arthropoda, Annelida, Mollusca, and various minor phyla that inhabit the bottom substrates of aquatic ecosystems. These organisms perform crucial ecological functions, serving as a vital link between primary producers, decomposers, and higher trophic levels. Through their involvement in nutrient cycling and energy flow, benthic fauna plays a foundational role in maintaining the health and productivity of aquatic environments (Mustafa et al., 2015). Certain benthic species, such as polychaete worms, are extensively used as feed in shrimp farming and aquaculture, while others, including nemertean worms, may engage in parasitic or symbiotic relationships with fish species.

Beyond their ecological functions, benthic organisms are increasingly recognized as reliable bioindicators in environmental monitoring and ecological assessments. Their sensitivity to environmental changes makes them valuable in assessing the impacts of pollution, habitat alteration, and climate change. However, the diversity and ecological roles of benthic communities in Bangladesh's coastal and estuarine systems remain inadequately documented, posing a challenge to the effective management and conservation of these ecosystems.

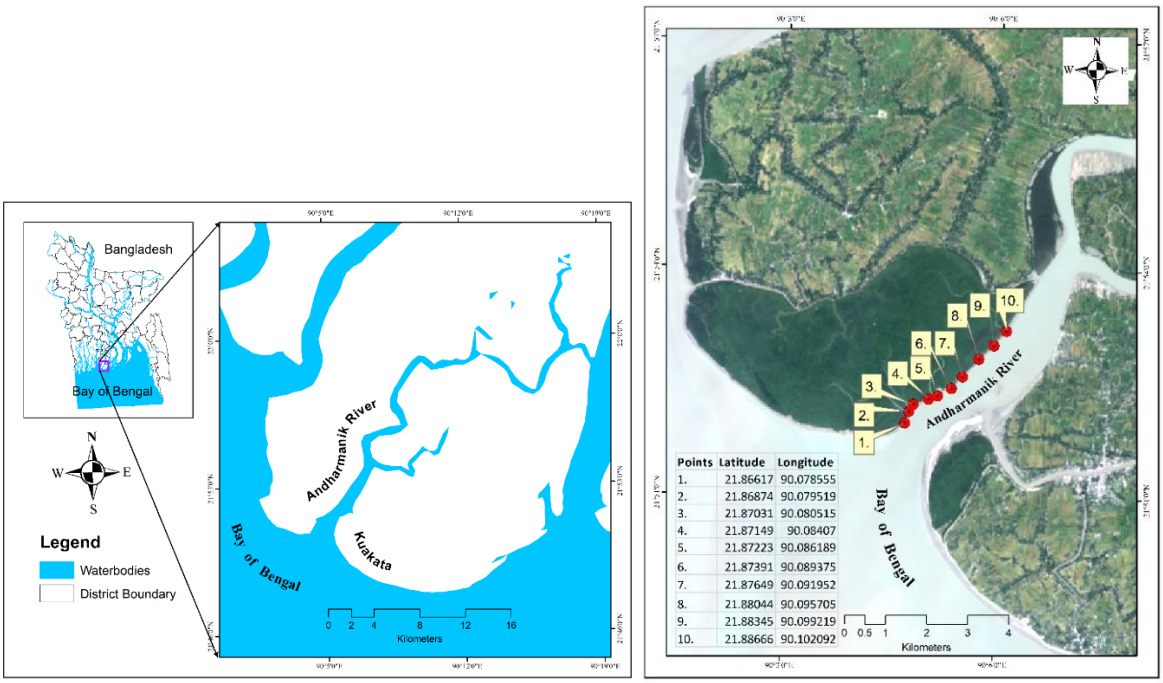
The Andharmanik River, a coastal river in the southern region of Bangladesh and part of the Ganges-Padma river system, flows into the Bay of Bengal (Mohsin et al., 2014). This river has been declared a fish sanctuary due to its high ichthyofaunal diversity and ecological importance (Roy et al., 2022). The estuarine and intertidal zones of the river present a unique habitat that supports diverse benthic communities, yet scientific knowledge of their composition and ecological roles is scarce.

In this study, we conducted a comprehensive survey of benthic species in the Andharmanik River using an integrative taxonomic approach that combines both morphological and molecular techniques. Morphological identification, while foundational, often poses challenges due to cryptic diversity and morphological plasticity. To address these limitations, molecular markers- specifically the mitochondrial 16S rRNA and cytochrome c oxidase subunit I (COI) genes were employed to enhance the accuracy of species identification (Hebert et al., 2003; Maturana et al., 2011). This is the first study in Bangladesh to apply molecular techniques to identify benthic fauna, thus providing valuable baseline data for future ecological assessments and conservation planning.

Materials and Methods

Sample Collection and Identification

In 2022, samples were collected from ten distinct estuarine locations along the Andharmanik River, where tidal flows facilitate the mixing of seawater from the Bay of Bengal with freshwater from the river (Table 1). Using an Ekman dredge, sediment was gathered from benthic habitats along the riverbed. The sample organisms were separated from the bulk sediment and sieved through a 0.5-mm mesh sieve. Immediately after collection, the specimens were fixed in 70% alcohol and transported to the Advanced Fisheries and DNA Barcoding Laboratory, Department of Zoology, University of Dhaka, for morphometric and molecular identification. The specimens were identified following Rouse & Pleijel (2001), Moore (1909), Horst (1923), Kinberg (1865), Leidy (1852) and, Hiebert & Maslakova (2015).



A **`B**

Figure 1. Map of Bangladesh showing (A) the Andharmanik River and (B) the sampling locations along the river.

Table 1. Water parameters and coordinates of sampling area.

Stations	Water Temperature (°C)	pH	Salinity (ppt)	Latitude	Longitude
1	26.0	8.80	10.0	21.866167	90.078555
2	25.5	9.21	10.0	21.868740	90.079519
3	26.0	8.80	09.5	21.870305	90.080515
4	26.0	8.90	09.5	21.871493	90.084070
5	25.4	8.80	10.0	21.872225	90.086189
6	25.6	8.90	10.0	21.873908	90.089375
7	26.0	8.80	10.0	21.876497	90.091950
8	25.4	9.15	08.0	21.880437	90.095705
9	25.7	9.00	09.5	21.883454	90.099219
10	25.8	9.18	08.5	21.886662	90.102092

DNA Extraction and Quantification

Without any fatty layer, 20–30 mg of tissues was placed into collection tubes. DNA was extracted from fresh tissues, and the remaining samples were stored in pure alcohol at -20°C for future use. Using a Qiagen DNeasy Blood & Tissue kit, genomic DNA was extracted from the tissue following the protocol. The amount of DNA in the extracted sample was measured using a NanoDrop spectrophotometer. The samples with DNA concentrations greater than 20 µl/µg were selected for PCR. In order to reduce the concentration and proceed with PCR amplification, samples with an excessively high DNA concentration of 200 µl/µg or more were diluted.

PCR amplification and DNA sequencing

The analysis included the amplification of two mitochondrial genes for species identification: COI and 16S ribosomal RNA. Universal primers 16Sar and 16Sbr were utilized to amplify 16S rRNA genes (Palumbi et al., 1991). Another universal primer LCO 1490 and HCO 2198 was employed to amplify the COI barcode region (Folmer et al., 1994). Polymerase chain reaction (PCR) amplifications were performed in a 25 µL of reaction volume consisting of 12.5 µL Taq Polymerase, 8.5 µL

Nanopure water, 1 µL forward primer and 1 µL reverse primer. PCR cycling conditions consisted of an initial denaturation at 97°C for 10 minutes (3 minutes for COI), followed by 40 cycles of denaturation at 94°C for 1 minute (30 seconds for COI), annealing at 48.5°C for 1 minute for 16S rRNA (48°C for 30 seconds for COI), and extension at 72°C for 2 minutes (5 minutes for COI). The final extension was carried out at 72°C for 13 minutes (5 minutes for COI). Amplified products were visualized by agarose gel electrophoresis. After purifying the PCR products, the DNA Sequencing was done from external sources by commercial Sanger sequencer.

Bioinformatics Analysis

The obtained sequences were assembled and aligned using MUSCLE (Edgar, 2004). Species identification was confirmed via BLASTn searches against the NCBI nucleotide database. Verified sequences were then submitted to GenBank. To further support identification, additional sequences of the same species were retrieved from GenBank for comparative analysis. Genetic distances were calculated using Kimura-2-parameter (K2P) model (Kimura, 1980) in MEGA 11 (Tamura et al., 2021). Neighbor-Joining (NJ) phylogenetic trees for both COI and 16S rRNA genes were constructed in MEGA 11 using 1,000 bootstrap replicates to assess tree robustness.

Results and Discussion

A total of 13 benthic fauna were identified in this study through both morphological and molecular analyses. Additionally, one specimen (*Perinereis* sp.) was identified to the genus level. Among the identified taxa, six benthic worm species—comprising three polychaetes and three nemerteans—were confirmed as new records for Bangladesh (Table 2). Species identification was conducted using two molecular markers: six species were identified using the 16S rRNA gene, while four were identified using the COI gene (Table 2). Notably, only three species were confirmed by both markers. Based on an integrated assessment of morphometric and molecular data, six species representing five different genera were recorded as new additions to the benthic fauna of Bangladesh.

Table 2. List of identified benthos species accompanied with voucher ID and GenBank Accession Numbers.

	Order	Species	Voucher ID	Accession number	
				16S	COI
Polychaeta	Phyllodocida	*Perinereis cultrifera*	DUZM_BT_011	OQ626666	
		Paralacydonia weberi	DUZM_BT_008 DUZM_BT_003	OQ626663	OQ626347
		Phyllodoce medipapillata	DUZM_BT_001		OQ626345
Nemertea	Heteronemertea	*Cerebratulus lacteus*	DUZM_BT_002 DUZM_BT_005 DUZM_BT_007	OQ626660 OQ626662	OQ626346
		Maculaura magna	DUZM_BT_004 DUZM_BT_010	OQ626665	OQ626348
		Maculaura cerebrosa	DUZM_BT_009	OQ626664	

Common identifying morphometric characteristics of studied polychaetes:

Long, Slender, numerously segmented, dorso-ventrally flattened and posteriorly tapering body. A thin, wet cuticle covers the body. Distinct head with tentacles and palps. The head has paired appendages and two parts: a prostomium (part in front of the mouth opening) and a peristomium (part around the mouth) palps, antennae and cirri. Head tentacles and lateral parapodia are made of flesh. Lateral parapodia have many setae.

Key identifying morphometric characteristics of studied polychaetes:

Phyllodoce medipapillata (Moore, 1909)

Description

Length 90 mm, width 2 mm. Brownish yellow in color. Unique and wide prostomium. Head tentacles and lateral parapodia are made of flesh.

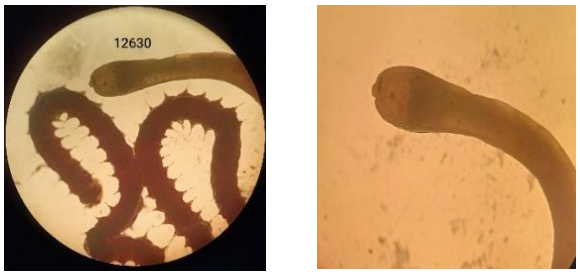
A **B** **C**

Figure 2. *Phyllodoce medipapillata* (A) External morphology, (B) anterior part and (C) segmented dorsal body part

Paralacydonia weberi (Horst, 1923)

Description

Length 200 mm, width 1.5 mm brownish in color. The prostomium is conical. Many little brownish spots around the body and on the prostomium including parapodia.



A **B**

Figure 3. *Paralacydonia weberi* (A-B) External morphology

Perinereis cultrifera (Kinberg, 1865)

Description

Length 120 mm and body width around 8 mm. Yellowish in color. Parapodia are paddle-shaped.

Figure 4. External morphology of *Perinereis cultrifera*

Common identifying morphometric characteristics of studied nemerteans:

Flattened body, covered by thin epidermis. Distinct head, trunk and tail region. Head somewhat broader than rest of body.

Key identifying morphometric characteristics of studied nemerteans:

Maculaura magna (Hiebert & Maslakova, 2015)

Description

Length 170 mm, dusty pink in color. The mid-body region is flattened dorso-ventrally. Mouth is ventral and quite long. Caudal cirrus suddenly terminates the body's posterior area. Transition from foregut to intestinal region is met with a thickening of the circular musculature.

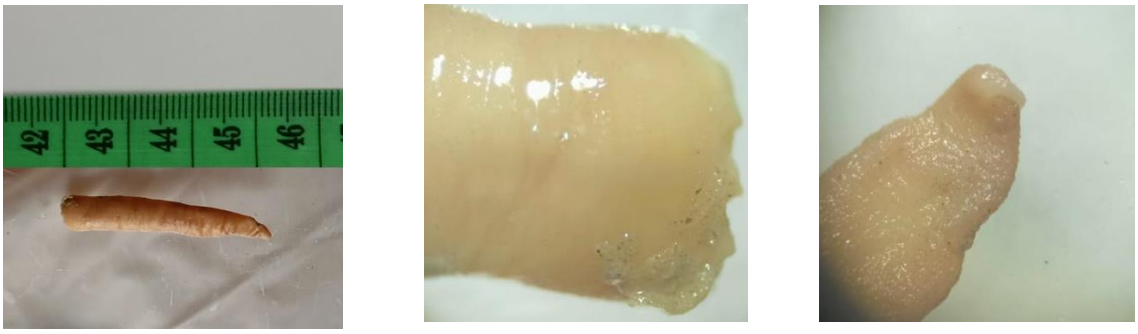
A **B** **C**

Figure 5. *Maculaura magna* (A) External morphology, (B) anterior and (C) posterior body part

Maculaura cerebrosa (Hiebert & Maslakova, 2015)

Description

Length 33 mm, width 4 mm. Color pale whitish. Short but broad flattened body. *Maculaura cerebrosa* is distinguishable from other *Maculaura* species by its smaller size, body color, having a distinctly pink brain which is visible through the body wall and short caudal cirrus gradually tapering.



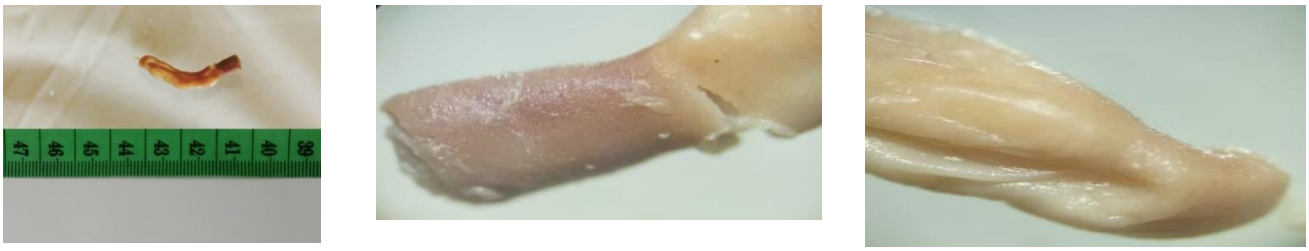
A **B** **C**

Figure 6. *Maculaura cerebrosa* (A) External morphology, (B) anterior and (C) posterior body part

Cerebratulus lacteus (Leidy,1851)

Description

Length 28 mm and a width of 2 mm. Milky white in color, with orange or red marking along its length. The head dorso-ventrally flattened and indistinguishable from the trunk. The body progressively tapers at the back before ending in a fine caudal cirrus.



A **B** **C**

Figure 7. *Cerebratulus lacteus* (A) External morphology, (B) anterior and (C) posterior body part

Molecular analysis

A total of 10 DNA sequences were obtained for molecular analysis. Of these, four sequences were generated using the COI marker, while the remaining 6 were amplified using the 16S rRNA marker. All 10 partial sequences, derived from specimens collected in Bangladesh, have been submitted to GenBank for the first time in this study.

Nucleotide content analysis

The average nucleotide composition of the 16S rRNA gene for polychaete and nemertean benthic species was as follows: adenine (A) 34.20%, thymine/uracil (T/U) 28.97%, guanine (G) 17.90%, and cytosine (C) 18.93%. For the COI gene, the average composition was: A 25.26%, T/U 43.89%, G 21.01%, and C 21.17%. In terms of GC content across codon positions in the COI gene, the following pattern was observed: 1st codon position (46.93%) > 2nd codon position (43.70%) > 3rd codon position (23.96%).

Genetic Divergence Analysis

Interspecies genetic divergence was calculated based on the Kimura 2-Parameter (K2P) model for both the 16S rRNA and COI genes. For the 16S rRNA gene, interspecies divergence ranged from a minimum of 11.43% to a maximum of 50.62%, with an average of 39.72 ± 0.07% (Table 3 and 5). In comparison, the COI gene showed slightly narrower divergence values, ranging from 13.32% to 36.95%, with an average of 29.72 ± 0.03% (Table 3 and 4). These values reflect a high level of genetic differentiation among the species analyzed, supporting the utility of both markers for species delimitation. The higher average divergence in 16S rRNA suggests it may offer better resolution at deeper taxonomic levels, while COI remains a reliable marker for distinguishing closely related species.

Table 3. Comparative Genetic Divergence (K2P distance %)

Comparison	16S rRNA			COI		
	Min	Max	Average±SE	Min	Max	Average±SE
Interspecies	11.43	50.62	39.72± 0.07	13.05	34.74	28.19± 0.03

Table 4. Estimates of Evolutionary Divergence over Sequence Pairs between Groups using COI gene.

	P. weberi	*P. medipapillata*	*C. lacteus*	*M. magna*
P. weberi		0.03	0.03	0.03
P. medipapillata	31.51		0.02	0.03
C. lacteus	33.99	27.05		0.02
M. magna	34.74	28.79	13.05	

Standard error estimate(s) are shown above the diagonal

Table 5. Estimates of Evolutionary Divergence over Sequence Pairs between Groups using 16S rRNA gene.

	P. cultrifera	*P. weberi*	*C. lacteus*	*M. magna*	*M. cerebrosa*
P. cultrifera		0.06	0.03	0.10	0.10
P. weberi	46.04		0.05	0.06	0.06
C. lacteus	23.93	40.14		0.09	0.09
M. magna	40.59	41.00	46.49		0.01
M. cerebrosa	50.62	50.62	46.39	11.43	

Standard error estimate(s) are shown above the diagonal

Phylogenetic Tree analysis

Phylogenetic trees were constructed using the Maximum Likelihood method and the relationships among identified species were established where individuals belonging to same species were grouped within the same clade. Individuals related to the same species were categorized under the same clade and no errors were not found in the phylogenetic connection among the identified benthic worms. All interspecific sequences formed a monophyletic clade in both evolutionary trees, with strong bootstrap support (82-100), validating the molecular characterization of the species.

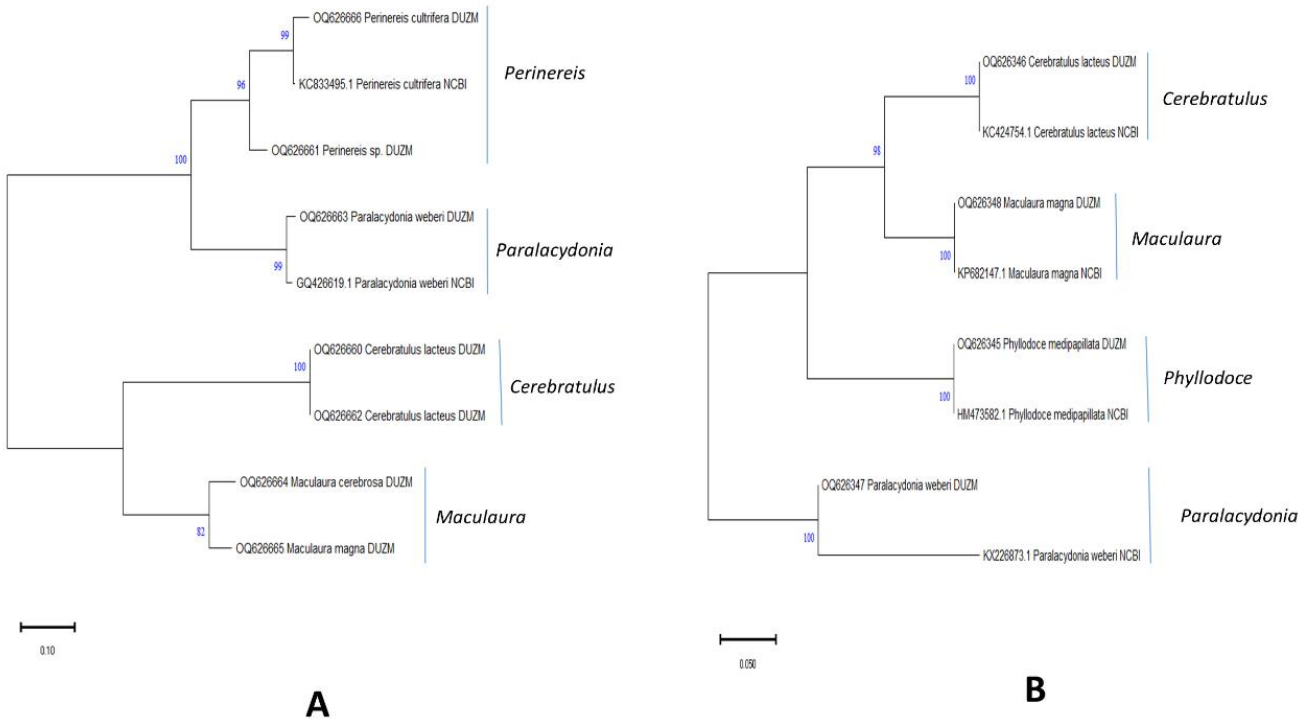


Figure 8. Maximum Likelihood phylogenetic trees of benthic worms constructed using (A) 16S rRNA gene sequences and (B) COI gene sequences. Sequences generated in the present study are indicated by the prefix DUZM.

Conclusions

Morphological identification of the specimens often led to confusion, as some observed features did not clearly match with known traits. Previous studies primarily listed polychaete and nemertean species and their abundance but did not provide new taxonomic keys for species identification. In this study, benthic polychaete and nemertean species were identified using both genetic and morphological methods for the first time in Bangladesh. Further research is recommended to genetically characterize the remaining benthic fauna and develop a comprehensive DNA barcode reference library, which would support biodiversity conservation and management efforts.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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