DNA BARCODING AND PHYLOGENETIC ANALYSIS OF SOME GASTROPOD MOLLUSCS (CLASS-GASTROPODA) FROM THREE ECOLOGICAL HABITATS OF BANGLADESH

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Mysha Mahjabin[1], Zerin Tasnim[1], Sujan Kumar Datta[1], Wahida Haque[2] and Md. Sagir Ahmed[1]*

[1]Department of Zoology, University of Dhaka, Dhaka 1000, Bangladesh
[2]Department of Fisheries, University of Dhaka, Dhaka 1000, Bangladesh

ABSTRACT

An attempt has been made for molecular characterization of gastropods from three different ecological habitats (marine, freshwater and terrestrial) in Bangladesh. A total of eight species of gastropod (*Lissachatina fulica*, *Pila globosa, Pila virens, Brotia costula, Nerita articulata, Nerita balteata, Macrochlamys indica* and *Telescopium telescopium*) were identified belongs to seven genera and six families. These species were confirmed through morphological studies and by adopting DNA barcoding using mitochondrial cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) gene. The average GC content 39.53% and 33.40% were found for COI and 16S rRNA, respectively. Genetic divergence increased as predicted with higher taxonomic rank, ranging from 0% to 2.84% with an average 1.45% within species and 3.57% to 35.23% between species with a DNA barcoding gap of 0.73 for 16S. This study revealed that gastropod species can be discriminated using both COI and 16S rRNA gene. Phylogenetic relationships among identified species were established where individuals belonging to the same species were grouped under the same clade. Molecular characterization of remaining molluscan species of Bangladesh remains to be completed.

KEYWORDS: DNA Barcoding, gastropod, Mollusca,16S rRNA, COI.

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CORRESPONDING AUTHOR: Dr. Md. Sagir Ahmed
Department of Zoology, University of Dhaka, Dhaka 1000, Bangladesh.
Email: sagir@du.ac.bd

Introduction

Molluscs are a large and diversified group of invertebrates with a large number of species ranging from 80,000 to 135,000, of which about 500 are freshwater, 14,000 to 35,000 are terrestrial and 31,000 to 100,000 are marine (Abott, 1950). From Bangladesh, about 20 terrestrial molluscs, 22 freshwater and 437 marine belonging to 210 genera, 105 families, 23 orders under four classes (Siddique *et al.*, 2007) were documented. Molluscs are usually soft-bodied, triploblastic, bilaterally symmetrical and non-segmented invertebrates and have colorful ornamented shells that protect the soft body. Size and shape are also varied from species to species. Gastropods are a soft-bodied, single-valve class of animals of the Phylum Mollusca (Galan et al., 2015). Among all the classes of Mollusca, gastropod is one of the largest classes. There are about 80,000 living gastropods around the world and about 318 are found in Bangladesh of which 16 freshwater, 20 land, 282 marine. Widespread occurrences of gastropods are vivid evidence of their significant adaptation to various environments.

For biodiversity conservation and assessment, accurate identification of organisms is a pivotal component. Few research works on morphological identification of mollusc have been published recently from Bangladesh (Antu et al., 2022; Sultana et al., 2021; Hossain et al., 2014). Sometimes this traditional morphological identification is not capable of distinguishing similarly looking or damaged specimens. In this case, molecular identification is able to distinguish cryptic or closely related species. Molecular markers are useful tools for complex taxonomic identification whereas morphological characteristics are ambiguous (Douek et al., 2002; Westheide et al., 2003). Most scientists use a fragment of COI gene (658 bp) for species identification in DNA barcoding. Because of short length and robust universal primers, it is easy to amplify and sequence (Folmer et al., 1994; Zhang and Hewitt, 1997; Simmons and Weller, 2001). 16S rRNA is also used more frequently than COI in many taxa of amphibians and reptiles. In this study, we use two distinct gene markers to attempt molecular characterization of gastropod from three different ecological habitat *viz.* marine, freshwater and terrestrial.

Materials and Methods

Sample collection

Specimens were collected from Tangail Sadar upazila (24.26°N, 89.86°E), Sakhipur upazila (24.31°N, 90.17°E), Delduar upazila (24.16°N,89.96°E), Keranigonj (23.70°N,90.39°E), Sonadia (21.49°N,91.89°E), Cox's Bazar

(21.47°N, 91.96°E), Maheshkhali (21.51°N, 91.97°E), Sundarbans (21.94°N,89.18°E) and adjacent areas from their natural habitat and opportunistic catch, between July 2019 to June 2020. Digital photograph of all gastropod molluscs were taken. Fresh specimens were spot examined for particular morphological features following the guideline of Abbott (1950) and Siddiqui et al., (2007). Then the voucher specimens were tagged with DUZM voucher ID.

DNA extraction, PCR amplification and sequencing

DNA was obtained from a 5 mg tissue sample of each specimen using the Invitrogen PureLink™ Genomic DNA Mini Kit according to the manufacturer's instructions. The amount and quality of the isolated DNA were assessed using a NanoDropTM spectrophotometer. COI and 16S rRNA gene sequences were amplified by polymerase chain reaction with the primer LCO-1490 (forward) and HCO-2198 (reverse) (Folmer et al. 1994); 16Sar (forward) and 16Sbr (reverse) (Palumbi et al. 1991), respectively. The PCR was carried out in 25 µl volumes comprising 23 µl of PCR Master Mix and 2 µl of DNA sample, which were combined and spun for 30 seconds to homogenize the mixture. 12.5 µl Taq Polymerase, 8.5 µl Nano Pure water, 1 µl forward primer, and 1 µl reverse primer were added in PCR Master. The annealing temperature for both COI and 16S rRNA was 48°C to 54°C for 30 seconds. The PCR amplifications were carried out as follows: an initial denaturation at 95 °C for 5 minutes, followed by 41 cycles of 95 °C for 30 seconds, 48-54 °C for 30 seconds, 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes. The PCR products were visualized in a 1% agarose gel and purified with the PureLinkTM PCR purification kit. Sequencing was performed for high-quality purified PCR products with DNA concentrations greater than 10 ng/ µl.

Bioinformatics analysis

MUSCLE was employed to align all of the COI and 16S rRNA sequences, and those were then manually corrected (Edgar 2004). CHROMAS software was used to inspect raw sequences, and after removing unnecessary bases, the sequences were converted to FASTA file format. For the distance-based technique, genetic pairwise divergence for each marker was analyzed by MEGA 11 (Tamura et al., 2021) and the Kimura two parameter distance (Kimura, 1980). Phylogenetic trees for COI and 16S rRNA sequences were built using Mega 11 and the Neighbor-Joining statistical approach, with clustering robustness evaluated by bootstrap analysis with 1000 repetitions.

Results and Discussion

About 24 sequences were generated (17 COI and 7 16S rRNA) from eight species of gastropods belonging to seven genera and six families (Table 1 and Figure 1). Molecular characterization using both COI and 16S rRNA sequences validated morphologically identified species. The sequences were blast and after confirmation deposited in the NCBI GenBank with assigned accession number (Table 1).

Table 1. List of identified gastropod species along with their GB (GenBank) accession number

Order	Family	Species name	GB Accession number		Place of Collection*
			16 S rRNA	COI	
Stylommatophora	Achatinadae	*Lissachatina fulica*	MW685664 MW685662 MW685663	MW659082 MW659083 MW847632	TS
Mesogastrropoda	Ampullariidae	*Pila globosa*	MW685666 MW685667 MW685669	MW659084 MW847633	DU, TS
		Pila virens	MW685668 MW685670 MW685672		TS
	Thiaridae	*Brotia costula*	MW685673		KD
Archaeogastropoda	Neritidae	*Nerita articulata*	MW685671		SI
		Nerita balteata	MW832720 OM760860 OM760861 OM760862	MW847634	ST, DC,
Stylommatophora	Ariopantidae	*Macrochlamys indica*	MW685665		DU
Mesogastropoda	Potamididae	*Telescopium telescopium*	MW847805	MW847635	ST

*TS-Tangail sadar; DU- Delduar upazila; KD-Keraniganj, Dhaka; ST-Satkhira; SI-Sonadia Island, DC-Dublar Char

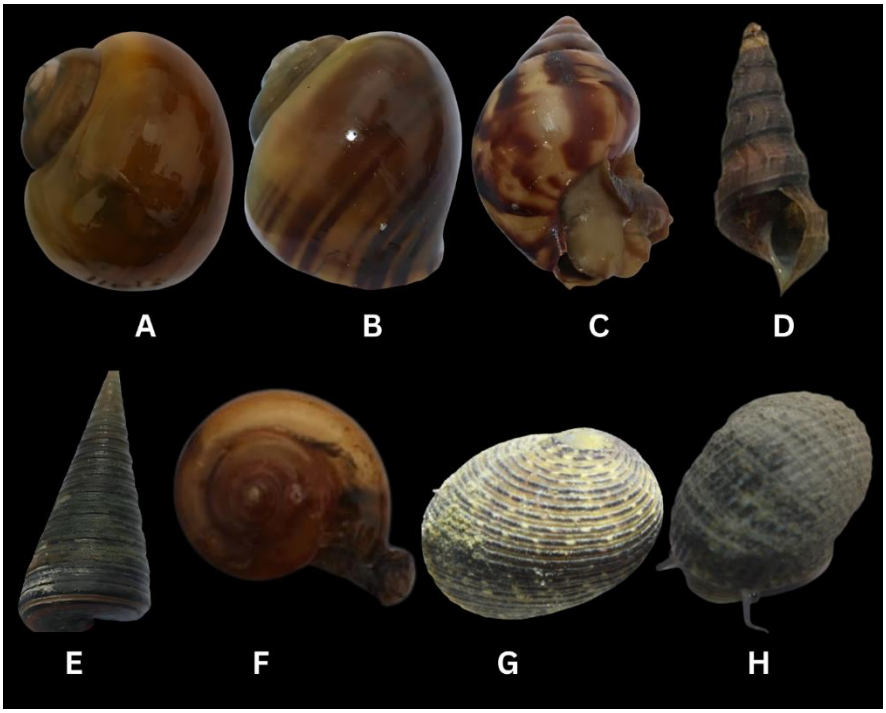

Figure 1. A. *Pila globosa*; B. *Pila virens*; C. *Lissachatina fulica;* D. *Brotia costula;* E. *Telescopium telescopium;* F. *Macrochlamys indica;* G. *Nerita balteata* and H. *Nerita articulata*

The mean length of the generated sequences were 590 and 478 bp for COI and 16S rRNA, respectively. Average nucleotide percentage were A 33.20%, T 31.46%, G 19.86%, C 15.48% for 16s RNA and A 22.27%, T 38.49%, G 22.63%, C 16.61% for COI region. The average GC content 35.34% for 16S rRNA and 39.24% for COI gene sequences. Genetic divergence was calculated by using Kimura two parameter (K2P) distance model (Kimura, 1980). Average genetic divergence was observed as 1.45 within species and to be increased as expected with higher taxonomic rank 0% to 2.84% within species and 3.57% to 35.23% between species with a barcoding gap of 0.73 in 16S (Table 2). In case of COI, mean genetic divergence was found 0.81 and 35.07 within and between species, respectively (Table 2).

Table 2. Genetic divergence (K2P Distance %) within and between species for COI and 16S ribosomal RNA gene

Comparison	**K2P Distance (%)**					
	16S rRNA			**COI**		
	Min	**Max**	**Mean**	**Min**	**Max**	**Mean**
Intraspecies	0	2.84	1.45±0.005	0	1.62	0.81±0.004
Interspecies	3.57	35.23	29.45 ±0.066	29.26	44.58	35.07±0.073

Phylogenetic analyses were accomplished to ensure the molecular identification and taxonomic classification of species. For phylogenetic analysis, we have constructed Neighbor-joining (NJ) tree in the present study. The Neighbor-Joining method was used to understand the evolutionary history. In general, all the species belonging to the same family were grouped under similar clade (neighbor-joining tree) (Figure 2-3).

DNA barcoding has evolved into a highly effective tool for identifying existing species and their phylogenetic relationship. The current study shown that gastropod species can be easily discriminated through DNA barcode. Further, and extensive studies are needed involving all the available groups of gastropod molluscs for establishing a rich database containing morphological as well as genetic information for better conservation and management.

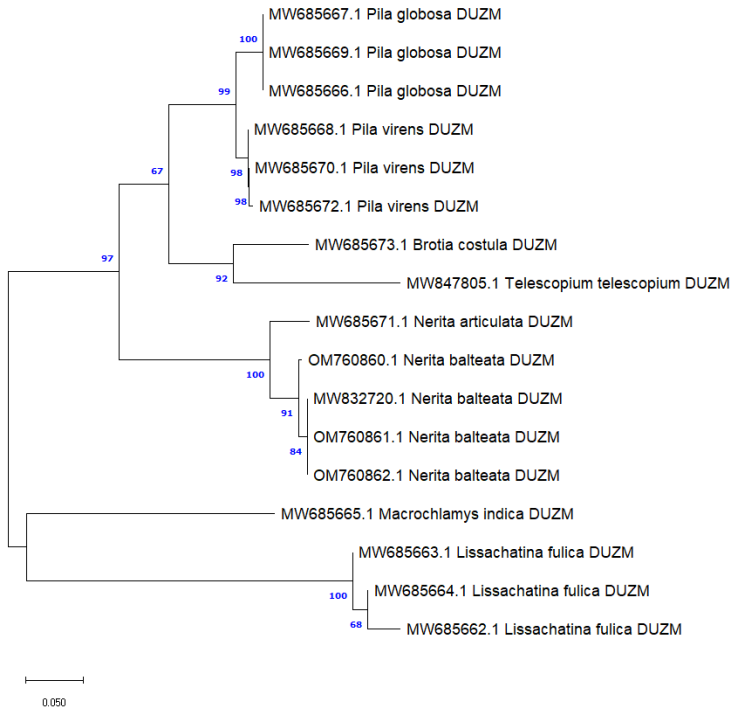

Figure 2. Neighbor-joining (NJ) phylogenetic tree constructed based on 16S rRNA sequences

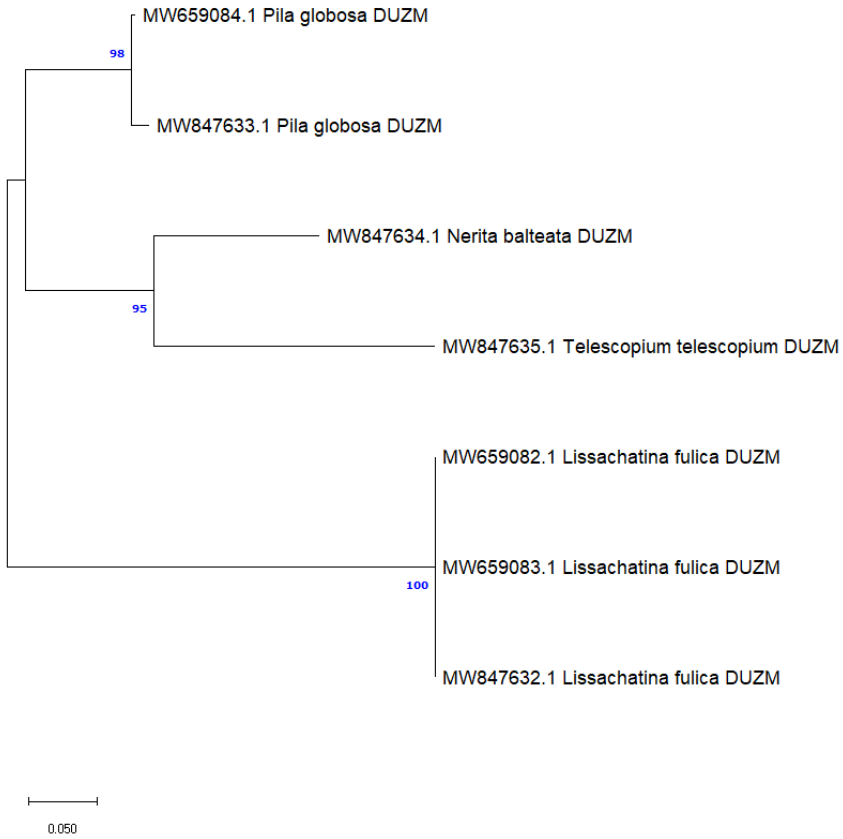

Figure 3. Neighbor-joining (NJ) phylogenetic tree constructed based on COI sequences

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