MORPHOMETRIC AND DNA-BASED IDENTIFICATION OF HORSESHOE CRAB *CARCINOSCORPIUS ROTUNDICAUDA* (LATREILLE, 1802) AND *TACHYPLEUS GIGAS* (MÜLLER, 1785) FROM BANGLADESH COAST

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ABSTRACT

Horseshoe crabs are the living fossils of xiphosurans that have survived for over 200 million years without morphological change and records fossil dating back 500 million years. A study was conducted on morphometric and molecular characterization of horseshoe crab *Carcinoscorpius rotundicauda* (Latreille, 1802) and *Tachypleus gigas* (Müller, 1785) from Bangladesh waters. A total of ten samples of *C. rotundicauda* and one sample of *T. gigas* were collected from Dublar Char, Sundarbans and Cox's bazar from June 2016 to May 2017 and February 2024, respectively. Among them, four specimens were male and seven female. Morphometric measurements were taken from all the collected samples and differentiated male and female based on the second pair of legs, the pedipalps. The species were genetically identified using mitochondrial cytochrome c oxidase subunit I (COI) gene. An average length of about 670.5 bp nucleotide sequences were obtained. Average percentage of nucleotide frequencies were T (30.3), C (16.3), A (34.4) and G (19.0) for *C. rotundicauda* and T (34.45), C (22.01), A (27.91) and C (15.63) for *T. gigas*. GC content analysis showed that average GC 36.20% and AT 63.80%. The average GC content at the 1st, 2nd and 3rd codon position were 38.82%, 37.87% and 31.92% respectively. This is the first report of DNA barcoding of a living fossil *C. rotundicauda* and *T. gigas* from Bangladesh and will serve as baseline information for future research, conservation and management of this invaluable primitive creature.

KEYWORDS: Horseshoe crab, *Carcinoscorpius rotundicauda, Tachypleus gigas,* DNA Barcoding, COI

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Introduction

Horseshoe crab, a chelicerate arthropod belonging to the class merostomata is such an amazing creature, considered to be the oldest 'living fossil'. The archaeal animal body has not shown any significant phenotypic change even after a span of millions of years (Itow *et al.*, 2004). They are more closely related to spiders and scorpions than to crabs. Based on their physical traits and distribution, they are classified into two subfamilies (Pocock, 1902). The waters around the east coast of North America are home to the Limulidae family, which includes *Limulus polyphemus* (Linnaeus, 1758), and the Tachypleinae family, which includes *T. tridentatus* (Leach, 1819), *T. gigas* (Müller, 1785) and *C. rotundicauda* (Latreille, 1802), is restricted to the waters around the southeast and east coasts of Asia. Among them only *C. rotundicauda* and *T. gigas*, are found in coastal waters of Bangladesh. The status of this species is vulnerable in Bangladesh (IUCN, 2015).

Horseshoe crabs is an important component of the macro-benthos community in the coastal waters with fine sand or mud substrate. It is essential to the biomedical sector because they produce copper-based blue blood that contains a substance called "*Limulus/Tachypleus* Amebocyte Lysate (LAL/TAL)" (Dybas, 2019). It was even used for the testing of coronavirus vaccine (Arnold, 2020; Waycott, 2020). Many fish species including sharks, sea turtles, alligators, and horse conchs, consume the eggs of horseshoe crabs (Gibson *et al.*, 2010).

DNA barcoding is a taxonomic method that goal is not to determine patterns of relationship but to identify an unknown sample in terms of a preexisting classification (Hebert *et al.*, 2003). It allows non-experts to objectively identify species – even from small, damaged, or industrially processed material. The most commonly used barcode region for animals and protists is a segment of approximately 648 base pairs of the mitochondrial gene cytochrome c oxidase subunit I (COI) (Hebert *et al.*, 2003). COI is proving highly effective in identifying gastropods (Remigio and Hebert, 2003), birds (Hebert *et al.*, 2004), butterflies, springtails (Hogg and Hebert, 2004), fish (Ahmed *et al.*, 2021a; Ward *et al.*, 2005; Wong *et*

al. 2011), flies, crustacean (Ahmed *et al.*, 2021b, 2022; Costa *et al*., 2007) and many other animal groups. DNA barcoding can help with conservation policy by speeding up local biodiversity assessments, prioritizing conservation areas, and evaluating conservation actions. It also provides information on evolutionary histories and phylogenetic diversity.

The present study was the first attempt to genetically identify the horseshoe crab from Bangladesh waters.

Materials and Methods

Collection and preservation of specimen

Specimens were collected from the coastal areas of Bangladesh especially Cox's Bazar and the Sundarbans where most of the species are distributed. After collecting the crabs, photos were taken immediately. The specimens were finally preserved in ice. A total of 11 specimens were collected and chosen for morphometric and meristic study.

Then the samples brought to the Advanced Fisheries and DNA Barcoding Laboratory at the Department of Zoology, University of Dhaka for further analysis. In the laboratory, the specimens were placed on tray and measured them properly and identified those scientifically using crab identification keys. Crabs were identified as follows with the help of key of Alcock (1900), Cumberlidge and Sachs (1989), Chowdhury and Hafizuddin (1980), Itow et al. (2004) and with the description of Ahmed *et al.*, (2008) and Nandi and Pramanik (1994). Tissue from each of the sample were collected from the ventral side of the crab especially from claws for DNA extraction.

Genomic DNA extraction and PCR amplification

Genomic DNA was isolated using the conventional Proteinase K/Phenol-Chloroform-isoamyl alcohol technique from the muscle tissue samples (Sambrook and Russell, 2001; Chowdhury *et al.*, 2016). For amplifying COI genes, primer namely LCO1490: 5´-GGTCAACAAATCATAAAG ATATTGG-3´ and HCO2198: 5´-TAAACTTCAGGGTGACCAAAAAATC-3´ were used (Folmer *et al.*, 1994). The PCR reaction was set with an initial denaturation temperature of 94 ºC for 1 min for denaturation, 50 ºC for 45 seconds, 72 ºC for 45 seconds for extension primer annealing for 30 cycles followed by 72 ºC for 10 minutes for final extension using gradient thermal cycler (Applied Bio system, Inc. USA). The amplified gene were further visualized through 1% agarose gel electrophoresis. Then they were purified using PureLink™ PCR purification kit and were sent for sequencing in Malaysia using ABI PRISM 3730xl Genetic Analyzer exploiting the BigDye® Terminator v3.1 cycle sequencing kit chemistry.

Sequence analysis

The sequences of the sample were transferred to FASTA format and using multiple sequence alignment tool MUSCLE, all the gene sequence were aligned (Edgar, 2004). Sequences were identified using BLAST search within the reserved sequences of NCBI. Phylogenetic relationship among the identified crab was conducted on Neighbor-Joining method using MEGA 11 (Tamura *et al.*, 2021). The identified individual sequence was submitted to the NCBI GenBank for accession number.

Results

Systematic account

Order: Xiphosura

Family: Limulidae

Genus: *Carcinoscorpius*

Carcinoscorpius rotundicauda **(Latreille, 1802)** Mangrove horseshoe crab

Diagnosis

Telson circular in cross-section; opisthosomatic spines quite small and rudimentary; no spur on fourth segment of sixth prosomatic appendage; inner branches of genital operculum extend distally to tips of outer branches (Table 1).

Description

Body is horse shoe-shaped, consists of three parts: prosoma, opisthosoma and telson (Fig 1. A, B). Prosoma is dome-shaped and frontier part of the body. The middle spinous part is called the opisthoma and spike like rear extension is the telson, which is known as the tail. Hard and dark brown carapace protects the whole body. It has two compound eyes. Besides, it has two median eyes and an endoparietal eye on its carapace that function as light-sensing organ and two ventral eyes located on the underside by the mouth that may aid orientation of the animal swimming. It also has a hinge which spans the width of the body, functioning as a mobile joint between the head and segmental abdomen. Each individual possesses six pairs of appendages. Chelicerae, the first pair, are utilized to place food in its mouths. The next pair serve as walking legs and are known as pedipalps. Others legs are known as pusher legs and used for locomotion. Book gills are typically used to propel in swimming and exchange gases during respiration. They are situated behind the legs. Because of the abundance of barnacles, worms and sponges affixed to the shells, they are also referred to as "walking museums." (Chowdhury and Hafizuddin, 1980). Digestive tract begins with the bristly mouth, continuing into a cuticle-lined esophagus and the proventriculus. The morphometric measurement was shown in Table 2.

Table 1. Distinguishing characteristics of *Carcinoscorpius rotundicauda* and *Tachypleus gigas*

Characteristics	***Carcinoscorpius rotundicauda***	***Tachypleus gigas***
Margin of Carapace		
Front of Carapace		
Cross section of Telson		
Opistosomal marginal spines		
Genital Operculum		

A B

Figure 1. *Carcinoscorpius rotundicauda* A) Dorsal view B) ventral view; Voucher ID: DUZM-CR-001, Date of Collection: 27-Jan-2016, Place: Dublar Char, Sundarbans

A B

Figure 2. *Tachypleus gigas* A) Dorsal view B) ventral view; Voucher ID: DUZM-CR-004, Date of Collection: 27 February 2024, Place: Maheshkhali, Cox's Bazar

Table 2. The morphometric measurement of *Carcinoscorpius rotundicauda* and *Tachypleus gigas*

Characteristics	*Carcinoscorpius rotundicauda*				*Tachypleus gigas*	
	Measurement (cm)		% to carapace length		Measurement (cm)	% to carapace length
	Male (n=4)	Female (n=6)	Male	Female	Female (n=1)	Female
Carapace length	11.5-15.0	13.5-16.0			23.8	
Prosomal length	6.3-8.6	7.80-9.0	54.8-57.3	56.3-57.8	15.3	64.29
Prosomal width	13.7-16.5	15.1-17.9	110.0-119.1	111.8-111.9	24.0	100.84
Opisthosomal length	5.1-6.2	5.60-7.10	41.3-44.3	41.5-44.4	8.5	35.71
Caudal spines length	13.1-17.3	14.1-17.1	113.9-115.3	104.4-106.9	20.7	86.97

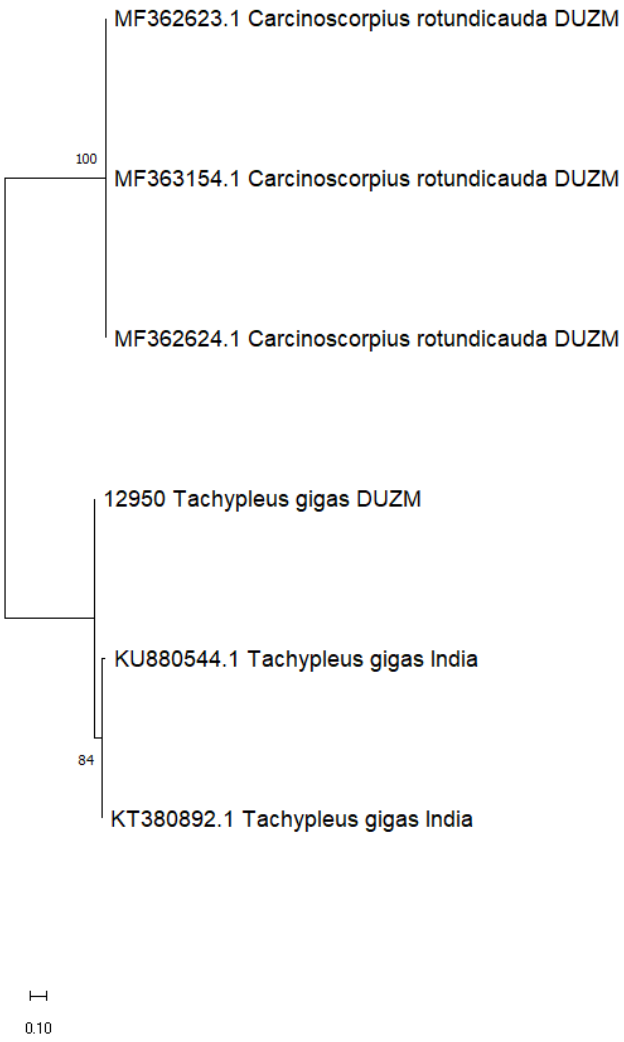

Figure 3. Cladogram of the *Carcinoscorpius rotundicauda* and *Tachypleus gigas* sequences amplified in this study and retrieved from NCBI using NJ method

Distribution

This crab is found in tropical or subtropical region of the eastern part of Bangladesh coast such as Chittagong, Cox's Bazar, Teknaf and St. Martin's Islands and western part of Bangladesh coast such as the Sundarbans (Siddique and Zafor, 2002). In addition, according to Sekiguchi et al. (1978), it is found in Singapore, Thailand, the Philippines, Malaysia, Indonesia, India, and Hong Kong.

Systematic account

Order: Xiphosura
Family: Limulidae
Genus: *Tachypleus*

***Tachypleus gigas* (Müller, 1785)** Indo-Pacific horseshoe crab

Diagnosis

Post-anal tail or telson crested dorsally, concave ventrally and round in cross – section; the opisthosoma has lengthy lateral spines, the inner branches of the genital operculum do not extend distally to the ends of the outer branches, and the fourth segment of the sixth prosomatic appendage has a moveable spur.

Description

The chitinous exoskeleton sage-green in color. Carapace made up of two parts, similar to other horseshoe crabs: the prosoma, which is larger and spinous, and the opisthosoma, which is smaller and less spiny. Six pairs of prosomal appendages with a small frontal pair in front and five larger walking legs on both side of the mouth. On the underside of the opisthosoma are the book gills. The telson, a long, spiky tail, is characteristic of them. The tail with a nearly triangular cross section due to its dorsal crest and ventral concavity. Male can be distinguished from female readily because the prosomal appendages two and three on their front pairs of walking legs are adorned with hooks. In females, however, they resemble to scissor.

Distribution

One of the three extant species of horseshoe crabs in Asia is *T. gigas*; the other two are *T. tridentatus* and *C. rotundicauda*. *T. gigas* is widely distributed throughout tropical South and Southeast Asia, extending from the Bay of Bengal to the South China Sea, according to data from India, Malaysia, Singapore, Indonesia, Thailand, Vietnam, and the Philippines (Vestbo *et al.*, 2018).

Molecular analyses

Sequences obtained from the amplified fragment showed a length of about 670.5 nucleotides. The blast results with NCBI GenBank data showed 99% similarity with the COI gene sequence of *C. rotundicauda* in the GeneBank database KM350551 and the submitted sequences assigned GenBank Accession No MF362623, MF362624 and MF363154. Another species *T. gigas* showed 98.7% similarity with pre-existing sequence (KU880543). Average percentage of nucleotide frequencies were T (32.40), C (19.19), A (31.40) and G (17.01). GC content analysis showed that average GC 36.20% and AT 63.80%. Average GC content in 1st, 2nd and 3rd codon position was 38.82%, 37.87% and 31.92% respectively. Neighbor-Joining tree was constructed using the four sequences of present study with other two sequences downloaded from NCBI GenBank.

Discussion

Bangladesh is regarded as hot spot of biodiversity. The horseshoe crab *C. rotundicauda* and *T. gigas* are living fossil in the sea and coast of Bangladesh. It plays important role in food chain and regard as biological indicator of water quality. It also plays an important role in the ecological equilibrium of coastal environments. It contributes in other ways to human health providing the pharmaceuticals and food industry.

Among the collected 11 specimens, there were 4 males and 7 females. Male and female are easily differentiated by the size (males are smaller than females) and the second pair of legs, the pedipalps. The tarsus of pedipalps is modified as a grasping appendage which allows the males to clasp the female during spawning. While the female has all similar legs known as "pusher" legs. Total body length (excluding caudal spine) of male is 11.7-15.1 cm and female is 13.6-16.1 cm (Chowdhury and Hafizuddin, 1980; Itow *et al.*, 2004). Morphometric measurement was done separately according to their sex (Table 2). The obtained morpho-meristic result was similar to the taxonomic data analysis of Chowdhury and Hafizuddin (1980) and Itow *et al* (2004). The blast results with NCBI GenBank data showed 99% similarity with the COI gene sequence of *C. rotundicauda* in the GeneBank database (KM350551) and *T. gigas* showed 98.7% similarity with pre-existing sequence (KU880543). The base composition analysis of the COI sequences revealed AT content (64.7%) to be higher than GC content (35.3%), similar to the pattern observed in crustacean species (Ahmed et al., 2021b, 2022; Costa *et al.*, 2007). The GC contents in the first, second, and third codon positions were 38.82%, 37.87%, and 31.92%, respectively which follow the 1st>2nd>3rd codon position. At the first codon position, percentage of A (31.33%) was the highest, and the usages of the other bases were 29.84%, 19.31%, and 19.50% for T, C, and G, respectively. At the second codon position, the content of T (36.34%) was highest, and other bases were C: 19.50%, A: 31.72% and G: 12.41. At the third codon position, the base A (31.13%) was highest where C (18.77%) was lowest and other was T: 31.00%, and G: 19.09%. Phylogenetic analyses of two species showed a distinctive clade with 100% bootstrap value in the Neighbor-Joining (NJ) phylogenetic tree (Fig. 3). Thus morpho-meristic result with molecular approaches confirmed the species *C. rotundicauda* and *T. gigas.* Further research is needed for the conservation of this exclusive prehistoric creature in the largest mangrove heritage and the coastal areas in Bangladesh.

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