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NEW RECORD OF SLIPPER LOBSTER *THENUS INDICUS* LEACH, 1816 (CRUSTACEA: DECAPODA: SCYLLARIDAE) FROM BANGLADESH WATERS

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Abstract

Slipper lobster *Thenus indicus* Leach, 1816 (Crustacea: Decapoda: Scyllaridae) has been recorded for the first time from Cox's Bazar coast of the Bay of Bengal. The species was taxonomically identified by using the traditional morphometric method which was further validated by molecular approach based on partial cytochrome c oxidase subunit I (COI) and 16S rRNA gene sequences (DNA barcodes). The key characteristics of this species are spotless pereiopods, 1st pereiopod merus width less than 7% of carapace length, and 3rd pereiopod merus length more than 45% of carapace length.

Introduction

Lobsters fetch a high price in domestic and international markets. In Bangladesh, six species of lobsters are documented; scalloped spiny lobster *Panulirus homarus*, ornate spiny lobster *P. ornatus*, mud spiny lobster *P. polyphagus* and painted spiny lobster *P. versicolor* under the family Palinuridae and two slipper Lobster; flathead lobster *Thenus orientalis* and scaled slipper lobster *Scyllarus depressus* under the family Scyllaridae[1,2]. Palinurid lobsters are more popular than slipper lobster of the family Scyllaridae in Bangladesh.

The family Scyllaridae includes four subfamilies, 19 genera, 88 species, and two subspecies worldwide[3-7]. The four subfamilies are Arctidinae, Ibacinae, Scyllarinae, and Theninae. The subfamily Arctidinae contains two genera (*i.e.*, *Arctides* and *Scyllarides*), and the subfamily Theninae only one genus (*i.e.*, *Thenus)*. All slipper lobster species are bottom-dwelling, prefer sandy and muddy habitats and rest in extremely shallow water to a depth of more than 484 m[4]. *Thenus* considered a monotypic genus, with only species *i.e.*, *T. orientalis* Lund 1793[4,8,13]. Burton and Davie (2007) revised the genus throughout its geographic range by using the morphological, morphometric and molecular approaches. Basically, the genus *Thenus* is a complex of five species: *T. indicus*, *T. orientalis*, *T. unimaculatus*, *T. australiensis* and *T. parindicus*[9].

T. indicus occurred in Pakistan, India, the Gulf of Thailand, Singapore, Indonesia and Taiwan[9]. Previously, *T. orientalis* reported from the coastal waters of Bangladesh[1-2]. This

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study describes the presence of *Thenus indicus* based on morphometric and molecular (DNA barcoding) approaches.

Materials and Methods

Sampling and morphological analysis: A single specimen of *Thenus indicus* was collected on 10 December 2020 from BFDC fish landing center of Cox's Bazar, (21°27'6·15" N, 91°58'5.77" E) Bangladesh. It was caught by fishermen during fishing in the off coast of Cox's Bazar. Immediately after collection, the specimen was preserved in ice and transported to the DNA Barcoding lab, Department of Zoology, University of Dhaka. The specimen was kept frozen (−18 °C) until further use. Taxonomic identification of the specimens was performed based on morphometric and meristic characteristics following the guidelines of Burton and Davie (2007) and Chan (1998). The morphometric measurements were done in centimeter (cm) scale. The specimen was tagged with voucher ID (DUZM_CR_145B) and kept at Dhaka University Zoology Museum (DUZM).

Genomic DNA extraction and amplification by PCR: DNA was extracted from a 5mg tissue sample of the specimen using Invitrogen™ PureLink™ Genomic DNA Mini Kit, following the manufacturer's protocol. The quality and quantity of the extracted DNA were measured using NanoDrop™ spectrophotometer. COI and 16S rRNA gene sequences were amplified by polymerase chain reaction with the primer LCO (forward) 5′-TCAACAAATCATAAGGACATTGG-3′ and HCO (reverse) 5′- TAAACTTCAGGGTGTCCAAAGAATCA-3′ for COI[10] and primer 16Sar (forward) 5′-CGCCTGTTTATCAAAAACAT-3′ and 16Sbr (reverse) 5′-CCGGTCTGAACTCAGATCATGT-3′ for 16S rRNA[11] genes. The PCR was conducted in 25 µl volumes containing 23 µl of PCR Master Mix and 2 µl of DNA sample, mixed and spun for 30s for homogenization of the mixture. PCR Master Mix consists of 12.5 µl Taq Polymerase, 8.5 µl Nano Pure water, 1 µl forward primer and 1 µl reverse primer. The PCR amplifications were performed following the conditions: initial denaturation at 95 ℃ for 5 min followed by 35 cycles of 94 ℃ for 45s, 50 ℃ (COI) and 42 ℃ (16S rRNA) for 30s, 72 ℃ for 45s, and a final extension at 72 ℃ for 10 min. The PCR products was kept at room temperature for 15 min, and then stored at -26 ℃ until further downstream application. PCR products were separated in 1% agarose gel and purified using PureLink™ PCR purification kit. The good quality purified PCR products of DNA concentration >10ng/µl were sent to First BASE laboratories, Malaysia for sequencing. Sequencing was done by Sanger dideoxy sequencing technology using ABI PRISM 3730xl Genetic Analyzer exploiting the BigDye R Terminator v3.1 cycle sequencing kit chemistry.

Sequencing analysis: The assembled contigs of each gene were prepared by the CAP3 DNA assembly program using bioinformatics software Unipro Ugene[12]. The sequences were confirmed via BLASTn against the best match sequences of the nucleotide database

(identity cut off ≥ 99%) and deposited in the NCBI GenBank. Our analysis includes DUZM sequences, along with sequences of three other *Thenus* species *T. parindicus, T. australiensis, T. orientalis* and *T. unimaculatus* retrieved from the NCBI GenBank database. *T. orientalis was* not included in the 16S rRNA sequences analysis due to the lack of similarity between existing sequences. All the COI and 16S rRNA sequences were aligned automatically using MUSCLE and then adjusted manually[13]. For the distance-based method, genetic pairwise divergence was determined by calculating Kimura-2-parameter (K2P)[14] distance using MEGA X[15]. Phylogenetic trees were constructed for COI and 16S rRNA sequences using Mega X based on the Maximum Likelihood (ML) statistical method. The phylogenetic construction uses the K2P substitution model with Gamma distributed rates. The robustness of clustering was determined by bootstrap analysis with 1000 replicates.

Results and Discussion

Diagnosis: Body markedly depressed and dark brown in color. Carapace trapezoid and narrowing posteriorly. Eyes small and subspherical; orbits situated at anterolateral angles of the carapace. Antennae broad flattened and plate-like. Five teeth present in second antennal segment. Antennules short and slender. Pereiopods and telson are yellowish in color without any spots. Rostral processes are sharply directed anteriorly and upward. Maxillipeds 1 and 3 lacks flagella (Fig. 1). A small spine present in the merus of the 3rd maxilliped. The inner margin of the ischium was prominently dentate along the total length. Abdominal segments expanded downward concealing the pleopods. The morphometric ratio of 1st pereiopod merus width and carapace length is 0.06 and 3rd pereiopod merus length and carapace length is 0.46 (Table 1).

Table 1. Morphological measurement ratios *Thenus indicus* following Burton and Davie (2007).

Morphometric characters	Present Study, n=1		Burton and Davie (2007)
	Measurement (cm)	Ratio with CL	
Carapace Length (CL)	6.3		
Carapace Width (CW)	8.1	1.27	
Length of the 3rd merus (ML3)	2.9	0.46	> 0.45
Width of the 1st merus (MW1)	0.4	0.06	< 0.07
Telson Length (TL)	0.85	0.13	
Telson Width (TW)	2.7	0.43	
Length of the 1st (and 2nd) propodus (PL1)	1.7	0.27	
Width of the 1st propodus (PW1)	0.35	0.05	

Remarks: T. indicus is closely related and often confused with the other species *T. orientalis.* The most obvious differences to distinguish this species is *T. indicus* tends to be brown whereas, *T. orientalis* is more reddish in body coloration. Spotless pereopods and telson; five teeth in the second antennal segment in *T. indicus* whereas four in *T. orientalis*[16]

and a spine on the merus of third maxilliped[17]. Abdominal segments, each with lateral margins expanded downward concealing the pleopods. The morphometric ratio of 1st pereiopod merus width and carapace length is less than 0.07 and 3rd pereiopod merus length and carapace length is more than 0.45 CL[9, 18-19].

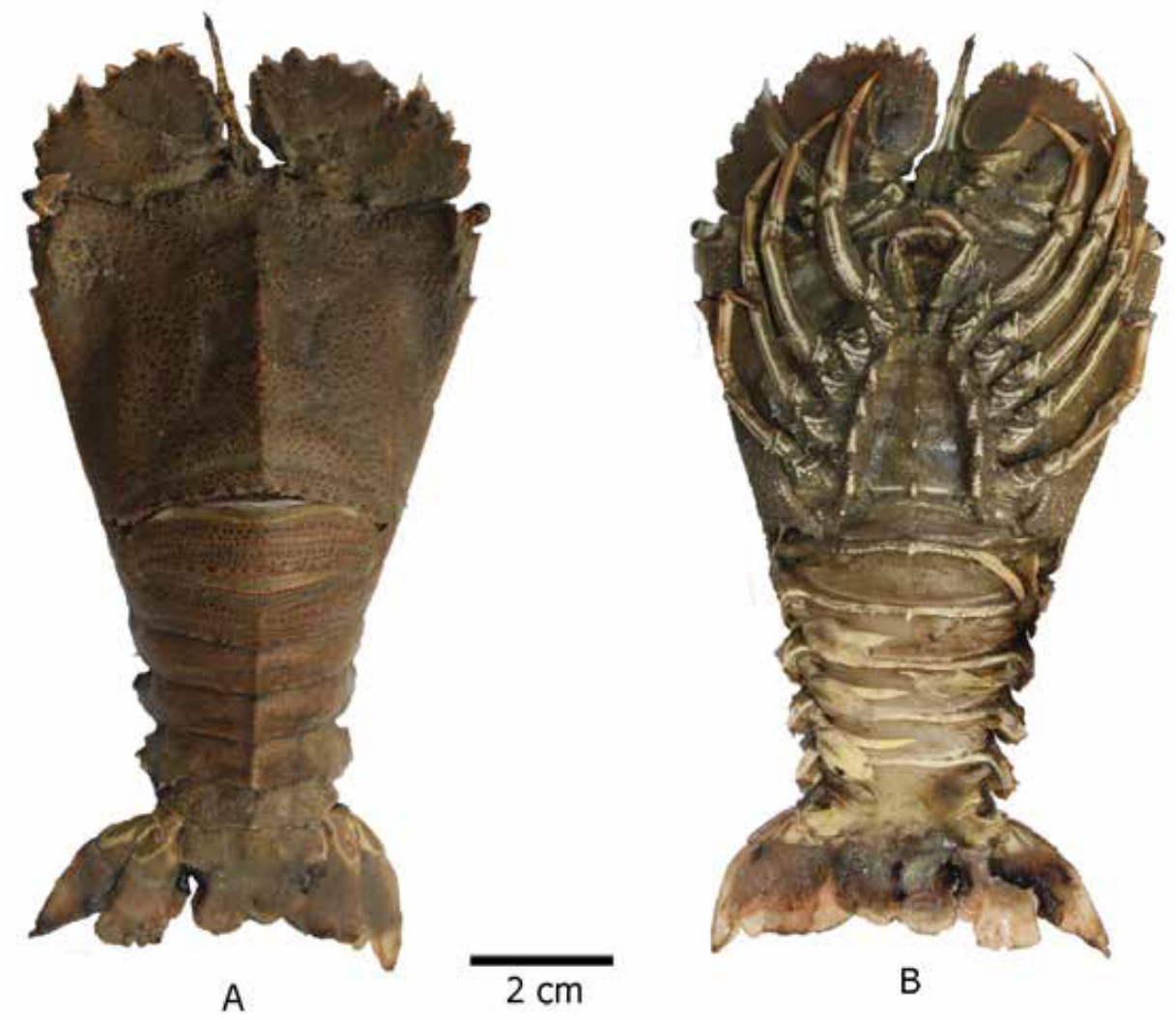

Fig. 1. *Thenus indicus* A. Dorsal view; B. Ventral view.

Molecular characterization: The accession numbers of the partial sequences of COI and 16S rRNA genes obtained are MW514207, MW504993, respectively. The acquired 630 bp COI sequence showed 99.21% species identity with 100% query cover with the *Thenus indicus* from India (JQ229892). The sequence was also in monophyly with the other species sequences from different Asian countries. The 463 bp long 16S rRNA sequence showed 100% identity and query coverage with the species from India (JQ229878) and Singapore (MT704568).

The average K2P distance between *T. indicus* and three other congeneric species sequences was calculated to understand their genetic relationships (Tables 2-3). Our species showed the lowest genetic divergence with the *T. parindicus,* with a genetic distance of 11.54% for COI and 4.234% for 16S rRNA genes. For both the genes, the distance was greater than the standard threshold for species identification of 3%[20]. The wide genetic divergence between COI sequences confirms the organism collected to be different from the compared *Thenus* species. On the other hand, the fewer genetic divergence between 16S rRNA sequences and the distinct morphological characteristics confirms the accuracy in delimiting the genus *Thenus*. The other species sequences compared showed the highest mean divergence with *T. unimaculatus* of 22.09% for COI and *T. australiensis* of 11.80% for 16S rRNA sequences.

Table 2. The genetic divergence (K2P distance %) between T. *indicus* (DUZM) and *T. parindicus, T. australiensis, T. orientalis* and *T. unimaculatus* based of COI gene (Burton and Davie 2007).

Species (COI)	*T. parindicus*	*T. australiensis*	*T. orientalis*	*T. unimaculatus*
Mean	11.54±0.286	15.01±0.582	14.13±0.687	15.68±1.071
Range	11.04-12.06	13.74-16.06	13.06-15.24	14.01-18.30
No. of comparison	25	20	25	35

Table 3. The genetic divergence (K2P Distance %) between *T. indicus* (DUZM) and *T. parindicus, T. australiensis* and *T. unimaculatus* based on 16S rRNA gene (Burton and Davie 2007).

Species (16S rRNA)	*T. parindicus*	*T. australiensis*	*T. unimaculatus*
Mean	4.234±0.649	5.686±0.806	5.221±1.034
Range	3.490-5.271	4.465-6.835	3.437-6.326
No. of comparison	15	12	12

Maximum likelihood (ML) tree was constructed for phylogenetic analysis where the lineage support was interpreted based on the bootstrap percentage (BP). The sequences of each species formed monophyletic clade, and our species clustered with the pre-existing *T. indicus* of the GenBank database. *Ibacus peronii* was taken as an out group as the species has morphological similarity with the *Thenus* species[21]. The monophyly within *T. indicus* was supported with a strong bootstrap value of 94% in the COI evolutionary tree (Fig. 2). Among the four *Thenus* species compared, *T. indicus* shared the common ancestor with *T. parindicus* with strong clade support of 96% BP. The 16S rRNA sequence of our species was grouped with the *T. indicus* from India with 95% BP support (Fig. 3). Thus, proving the effectiveness of the COI and 16S rRNA in species delimitation.

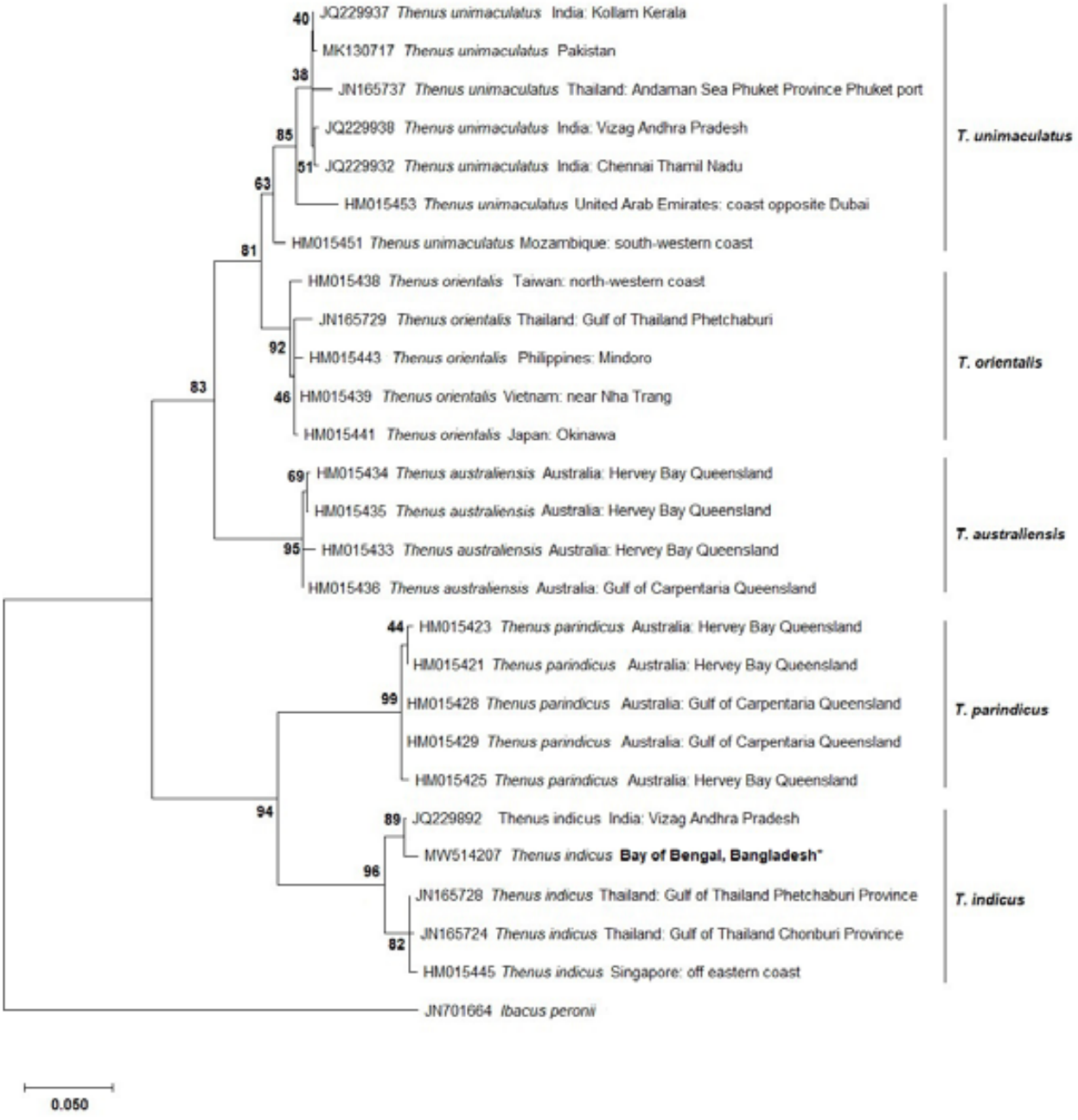

Fig. 2. Maximum Likelihood tree of the COI sequences showing the relationships among *Thenus indicus* with the pre-existing sequences of *Thenus* species of the NCBI GenBank. *Ibacus peronii* was taken as an outgroup.

Fig. 3. Maximum Likelihood tree of the 16S rRNA sequences showing the relationships among *Thenus indicus* with the pre-existing sequences of *Thenus* species of the NCBI GenBank. *Ibacus peronii* was taken as an outgroup.

Conclusion

The morphological characterization and the overall sequence analysis based on two marker genes supports the identification of the species as *T. indicus*. It confirms the species occurrence for the first time reported in Cox's Bazar, Bay of Bengal, Bangladesh. Thus, extending the geographical distribution range of the species.

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Research article

Characterization of spiny lobsters from Bangladesh waters using morphology, COI and 16S rRNA sequences

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HIGHLIGHTS

- Four Lobster species, *Panulirus homarus, P. ornatus, P. polyphagus* and *P. versicolor,* were morphologically described.
- Molecular characterization was confirmed based on two markers, COI and 16S rRNA.
- This is the first comprehensive taxonomic description of spiny lobsters from Bangladesh.

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ABSTRACT

This study aims to taxonomically identify and characterise the phylogenetic relationships of spiny lobsters based on mitochondrial cytochrome c oxidase I (COI) and 16S rRNA genes from Bangladesh waters. A total of 19 barcode sequences (10 partial COI sequences and 9 partial 16S rRNA) were successfully generated from 12 collected spiny lobster samples representing four species belonging to the family Palinuridae. The average genetic distances within and between species were 0.834 ± 0.427 and 17.810 ± 0.830, respectively, in COI and 0.107 ± 0.255 and 8.401 ± 2.547, respectively, in 16S rRNA genes. The successful amplification rate of 16S rRNA was higher than that of the COI marker. In the maximum likelihood (ML) tree, the sequences of the same species were clustered together under a single clade for both COI and 16S rRNA, which supports the efficacy of both marker genes in differentiating lobster species.

1. Introduction

Lobsters are one of the most valuable and highly priced crustaceans in domestic and international markets. There are approximately 149 species of lobsters around the world (Holthuis, 1991). Most lobsters fall under one of the two families, Palinuridae, known as spiny lobsters, and Scyllaridae, known as slipper lobsters. The family Palinuridae consists of 47 species of the genus *Palinurus* (Holthuis, 1991), and the Scyllaridae family includes 88 species, 19 genera and two subspecies worldwide (Holthuis, 1991; Carpenter and Niem, 1998; Chan, 2010; Yang et al., 2011; Yang and Chan, 2012). Only four species of spiny lobsters (*Panulirus homarus, P. ornatus, P. polyphagus* and *P. versicolor*) and two species of slipper lobsters (*Thenus orientalis* and *Scyllarus depressus)* have been documented thus far from Bangladesh under the families Palinuridae and Scyllaridae, respectively (Ahmed et al., 2008; IUCN Bangladesh, 2015). Although *Scyllarus depressus* is a Western Atlantic species, it has been recorded from shallow rocky substrates and coral reefs of St. Martins Island, Naf River mouth (Teknaf) and the coast of Bangladesh (Ahmed et al., 2008; IUCN Bangladesh, 2015). Slipper lobster species, on the other hand, are bottom-dwelling lobster species that prefer sandy and muddy habitats and rest in extremely shallow water, as reported off the coast of Cox's Bazar (Carpenter and Niem, 1998; IUCN, 2015). Due to the high market price, spiny lobsters are more highly exploited than slipper lobsters in Bangladesh.

Holthuis (1991) explored the morphological characteristics of adult lobsters in detail of nearly all marine lobsters known up to the early 1990s. Chan (2010) updated the valid species list with several newly described taxa and organized all living marine lobsters into four

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infraorders: Astacidea, Glypheidea, Achelata and Polychelida. He has recognized six families, 55 genera, 248 species and four subspecies of marine lobsters. However, DNA barcoding develops traditional taxonomy methods and is useful for distinguishing cryptic or polymorphic species of marine lobsters (Chow et al., 2006); moreover, it also allows us to discriminate the species from processed and commercial products (Jeena et al., 2016).

DNA barcoding is a universal method designed to identify species by using partial sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene (Hebert et al., 2003). The high mutation rate of the gene between interspecific sequences and conserved regions among conspecifics can distinguish even closely related species (Hebert et al., 2003). Another mitochondrial marker, 16S ribosomal RNA, is also considered a candidate marker. As a conserved gene, it can reliably measure the true divergences between closely related organisms. It can be easily amplified and successfully sequenced across various animals, distinguishing specific species (Sinniger et al., 2008; Zheng et al., 2013; Chakraborty and Iwatsuki, 2006; Lee et al., 2014; Vences et al., 2005). The combination of conserved and variable regions within the same gene makes 16S rRNA one of the most popular genes for reconstructing animal phylogenies.

There is no published work thus far on the detailed taxonomic description and molecular characterization of spiny lobsters from Bangladesh. The present study aims to validate the morphologically identified lobster species based on mitochondrial cytochrome c oxidase I (COI) and the 16S rRNA gene.

2. Materials and methods

2.1. Sampling and morphological analysis

The target lobster specimens were collected as dead from Teknaf (20°46′37.6″N 92°15′20.0″E), Cox's Bazar (21°24′15.3″N 91°53′10.1″E), and St. Martin Island (20°36′49.3″N 92°19′51.6″E) of Bangladesh from December 2020 to March 2021. Immediately after collection, the specimens were preserved in ice and transported to the laboratory for morphological identification. Taxonomic identification of the specimen was performed based on morphometric and meristic characteristics following the guidelines of Ahmed et al. (2008), Carpenter and Niem (1998), and Burton and Davie (2007). Tissue samples were excised and stored in 90% ethanol. Voucher specimens were fixed with 10% formalin and then transferred to 70% ethanol solution for preservation. The species were tagged with a Dhaka University Zoology Museum (DUZM) voucher ID and kept in the institutional museum.

2.2. Genomic DNA extraction and amplification by PCR

DNA was extracted from a 5-mg tissue sample collected from the lower abdomen of the specimen using a Promega Wizard® Genomic DNA Purification kit. The quality and quantity of the extracted DNA were measured using a NanoDrop™ spectrophotometer. COI and 16S rRNA gene sequences were amplified by polymerase chain reaction with the primers LCO-1490 (forward) 5′-TCAACAAATCATAAGGACATTGG-3′ and HCO-2198 (reverse) 5′- TAAACTTCAGGGTGTCCAAAGAATCA-3′ for COI (Folmer et al., 1994) and the primers 16Sar (forward) 5′-CGCCTGTTTATCAAAAACAT-3′ and 16Sbr (reverse) 5′-CCGGTCTGAACTCAGATCATGT-3′ for 16S rRNA (Palumbi et al., 1991) genes, respectively. PCR was conducted in 25-μl volumes containing 23 μl of PCR Master Mix (12.5 μl GoTaq® Green Master Mix, 8.5 μl of Nano Pure water, 1 μl of forward primer and 1 μl of reverse primer) and 100 ng of DNA sample and mixed and spun for 30 s for homogenization of the mixture. The amplification conditions included initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 42 °C (COI) and 50 °C (16S rRNA) for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were kept at room temperature for 15 min and then stored at -26 °C until further downstream application. Amplified gene bands were visualized in a 1% agarose gel and purified using a

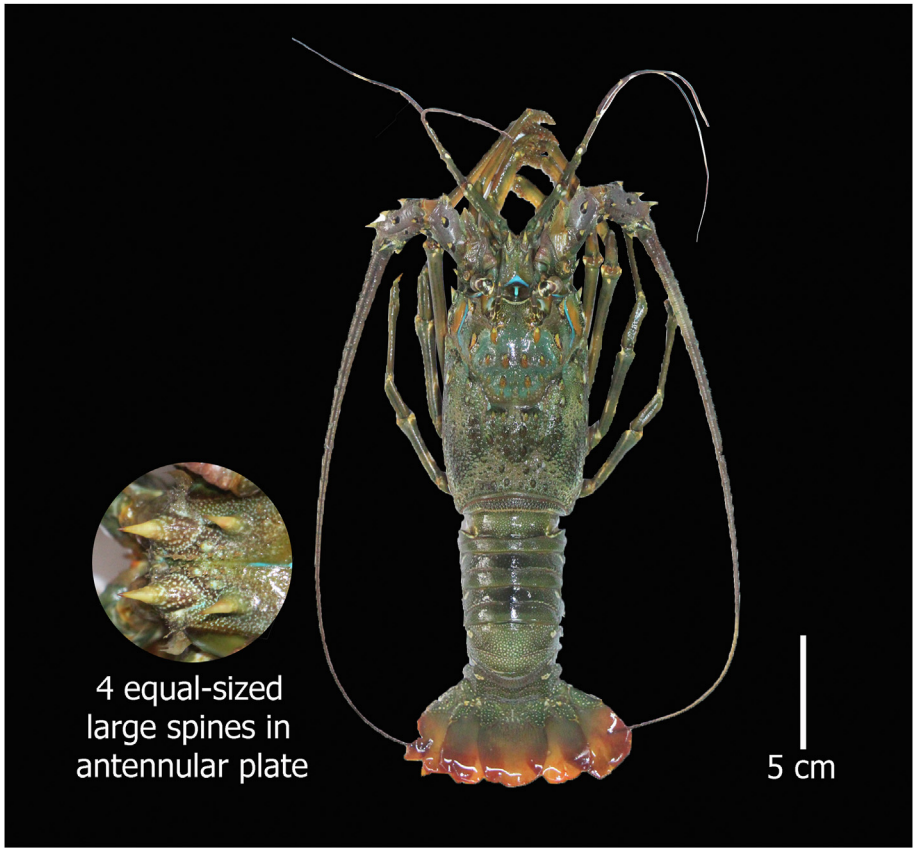

Figure 1. *Panulirus homarus*, Family Palinuridae, voucher ID DUZM_CR_144B, collection date: 28-November-2020, place: Teknaf.

PureLink™ PCR purification kit. The purified PCR products with DNA concentrations >10 ng/μl were sent to First BASE laboratories, Malaysia, for sequencing. Sequencing was performed by Sanger dideoxy sequencing technology using an ABI PRISM 3730xl Genetic Analyser exploiting the BigDye R Terminator v3.1 cycle sequencing kit chemistry.

2.3. Bioinformatics analysis

The assembled contigs of the gene sequences were prepared by the CAP3 DNA assembly program using Unipro Ugene (Okonechnikov et al., 2012). Each sequence was confirmed via BLASTn against the best match sequences of the nucleotide database (identity cut off ≥99%) and deposited in NCBI GenBank. Our analysis includes sequences of the collected species, along with sequences of the identical species retrieved from the NCBI GenBank database. All the COI and 16S rRNA sequences were aligned automatically using MUSCLE (Edgar, 2004). For the distance-based method, genetic pairwise divergence was determined by calculating the Kimura-2-parameter (K2P) (Kimura, 1980) distance using MEGA X (Kumar et al., 2018). The genetic divergence within and between species was illustrated as a box plot distribution using RStudio (RStudio Team, 2015). Phylogenetic trees were constructed for COI and 16S rRNA sequences using Mega X based on the maximum likelihood (ML) statistical method and K2P substitution model with gamma distribution rates. The robustness of clustering was determined by bootstrap analysis with 1000 replicates.

3. Results

A total of 12 specimens were examined during the study from the Bangladesh coast: Teknaf, Cox's Bazar and St. Martin's Island. Morphometric identification and molecular characterization confirmed four species of spiny lobsters, *Panulirus homarus, P. ornatus, P. polyphagus* and *P. versicolor*.

3.1. Morphological analyses

3.1.1. Panulirus homarus (Linnaeus, 1758)

English Name: Scalloped spiny lobster

Material examined: Two females, Bangladesh, Teknaf, the southernmost point in mainland Bangladesh. 20°46′37.6″N 92°15′20.0″E, ID DUZM_CR_144B- DUZM_CR_144B.2

Habitat: Inhabits shallow waters among rocks, often in the surf zone. Maximum depth of 90 m (Chan, 1998).

Characteristics: Round shape carapace having numerous spines of different sizes; antennular peduncles are smaller than antennular flagella; absence of rostrum; anterior margin armed with 4 frequently spaced large spines apart from the frontal horns; height of the eye is 2 times smaller than the height of the frontal horn; antennular plate bearing 4 properly separated principal spines and few small spinules; every abdominal segment with a transverse groove, occasionally interrupted in the centre, its anterior margins formed into superficial scallops; legs 1 to 4 are without pincers (Figure 1).

Colour: Carapace is darkish green to reddish brown in colour. Very small white spots were present on the head and especially distinct on the posterior half of the abdomen. All the legs are darkish green in colour. The stripes of the leg are coloured white. A white and green colour band is present on the antennules.

3.1.2. Panulirus ornatus (Fabricius, 1798)

English Name: Ornate spiny lobster

Material examined: One female, Bangladesh, St. Martin's Island, northeastern part of the Bay of Bengal. 20°36′49.3″N 92°19′51.6″E, ID DUZM_CR_142

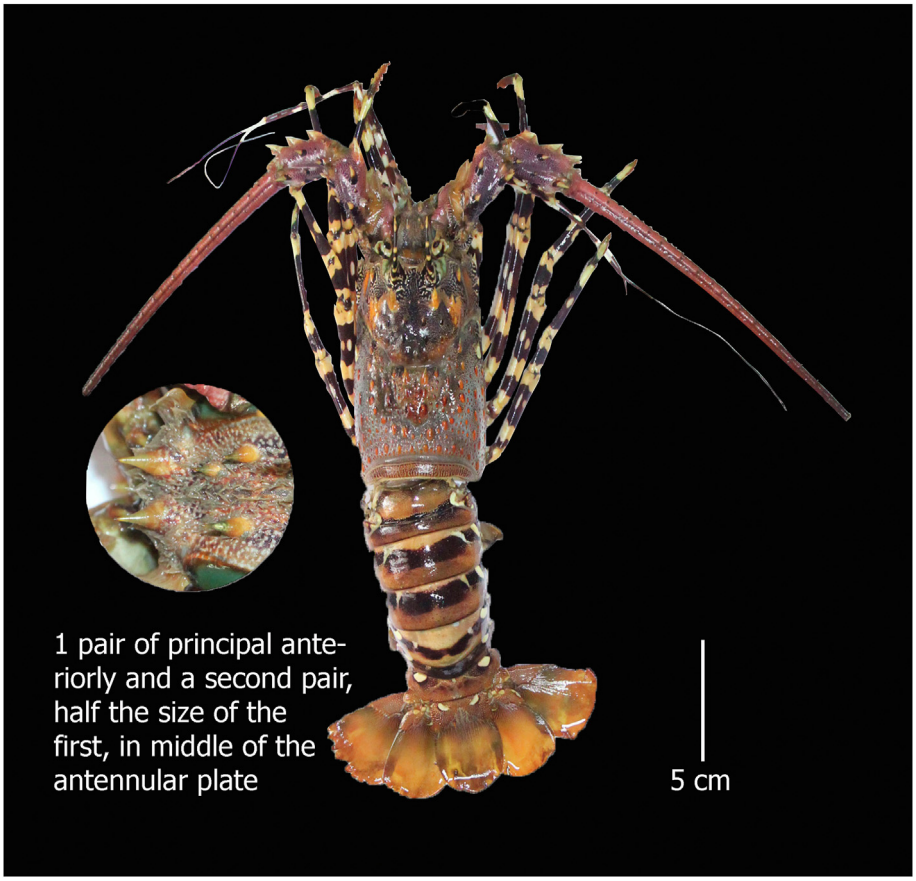

Figure 2. *Panulirus ornatus*, family Palinuridae, voucher ID DUZM_CR_142, collection date: 2-March-2021, place: St. Martin's Island.

Figure 3. *Panulirus polyphagus*, Family Palinuridae, voucher ID DUZM_CR_143.3, collection date: 17-January-2020, place: Cox's Bazar.

Habitat: Inhabits shallow, sometimes slightly turbid coastal waters usually on sand and mud substrates but also on coral reefs and rocky bottoms. Maximum depth of 8 m (George, 1968; Chan, 1998).

Characteristics: Carapace rounded and covered with several spines and tubercles of various sizes; absence of rostrum, and antennular peduncles are smaller than antennular flagella; a broad antennular plate separates the bases of the antennae. The antennar plate has a pair of principal spines anteriorly and a second pair that are half the size of the first and a small spine in between two pairs at the right side of the plate

Figure 4. *Panulirus versicolor*, family Palinuridae, voucher ID DUZM_CR_144.3, collection date: 10-February-2021, place: St. Martin's Island.

(Figure 2). Smooth abdominal segment without a transverse groove; pincers are absent in the legs.

Colour: On the yellow carapace, bluish or greenish spines are present. The anterior part of the carapace possesses a vermicular pattern of pale and dark lines near the bases of the frontal horns and the anterior spines. The abdomen has a broad, dark transverse band over the middle of the segments. On the sides, each segment with a large pale spot and an extra elongate mark higher up on the 2nd, 3rd and 4th segments at an angle. Transverse white bands are not present along the posterior margin of the segments. Brightly coloured flagella are present and anteriorly banded. Legs having distinct, sharply defined dark and pale blotches.

3.1.3. *Panulirus polyphagus (Herbst, 1793)*

English Name: Mud spiny lobster

Material examined: Three females and two males; Bangladesh, Cox's Bazar, southeastern coast of Bangladesh; 21°24′15.3″N 91°53′10.1″E and Bangladesh, St. Martin's Island, northeastern part of the Bay of Bengal. 20°36′49.3″N 92°19′51.6″E; ID DUZM_CR_143- DUZM_CR_143.5

Table 1. GenBank Accession number of the COI and 16S rRNA sequences of Lobsters.

Sl. No.	Family	Name of the species	GB Accession No	
			COI	16S rRNA
1	Palinuridae	*Panulirus homarus*	MW514210-11	MW504995
2		*Panulirus ornatus*	–	MW832555
3		*Panulirus polyphagus*	MW514205-06 MW514208-09 MW832713	MW504991-92 MW504994 MW504996 MW832554
4		*Panulirus versicolor*	MW832710-12	MW832552-53

Table 2. Genetic divergence (percentage, K2P distance) within various taxonomic levels.

Taxonomic rank	COI (%)			16S rRNA (%)		
	Mean	Min	Max	Mean	Min	Max
Intra species	0.834 ± 0.427	0	1.43	0.107 ± 0.255	0	0.857
Inter species	17.810 ± 0.830	14.97	19.67	8.401 ± 2.547	4.90	12.00

Habitat: Mainly found on muddy bottoms (sometimes also on rocky bottoms) in turbid waters near river mouths at depths from 3 to 90 m but usually less than 40 m deep (Morgan, 1980; Holthuis, 1991).

Characteristics: Round shape carapace having numerous spines of different sizes; the height of the eye is 2 times larger than the height of the frontal horn; antennular peduncles are smaller than antennular flagella; absence of rostrum; a broad antennular space bearing a single pair of principal spines separates the bases of antennae; no transverse grooves on the abdominal segments; 1 to 4 pereiopods without pincers (Figure 3).

Colour: Body dull green in colour, carapace with yellowish brown spines, and eyes are black–brown. The orbital margin and posterior marginal groove are yellowish white in colour. Antennular peduncle alternated with yellowish white and pale green bands. Yellowish white and dark brown bands are present on the flagella. Pereiopods have yellowish white blotches and are a light brown colour. The abdomen is covered by small pale dots. Each abdominal segment has a yellowish white band with brown margins near the posterior border.

3.1.4. Panulirus versicolor (Latreille, 1804)

English Name: Painted spiny lobster

Material examined: Two females and one male, Bangladesh, St. Martin's Island, northeastern part of the Bay of Bengal. 20°36′49.3″N 92°19′51.6″E, ID DUZM_CR_144- DUZM_CR_144.3

Habitat: Shallow waters, from the sub–littoral zone down to 15 m, on coral reefs, often on the seaward edges of the reef plateau; nocturnal (Holthuis, 1991; Chan, 1998; Wahyudin et al., 2017).

Characteristics: Round-shaped carapace having numerous spines of different sizes; anterior margin with 4 regularly spaced large spines other than the frontal horns; the height of the eye is 3 times smaller than the height of the frontal horn; the antennular peduncles are smaller than antennular flagella, rostrum absent; antennular plate bearing 2 pairs of unequal and separated principal spines and separates the base of the antennae; abdominal segments without transverse grooves; 1 to 4 pereiopods without pincers (Figure 4).

Colour: The green–blue coloured carapace contains a distinguished pattern of blue–black patches and white lines. A transverse white band presents each abdominal segment, which is bordered by 2 black lines. Legs and antennules are covered by longitudinal stripes. Bases of the antennae contain a bright pink colour except for the antennular plate.

3.2. Identification key

Guide to families

a. The first pair of pereiopods are large, and the third pair of pereiopods have chela……Nephropidae
b. The pair of pereiopods are simple, and the third pair of pereiopods are without chela……Palinuridae

Guide to identify species of the family palinuridae

a. 4 equal–sized large spines in the antennular plate……*Panulirus homarus*

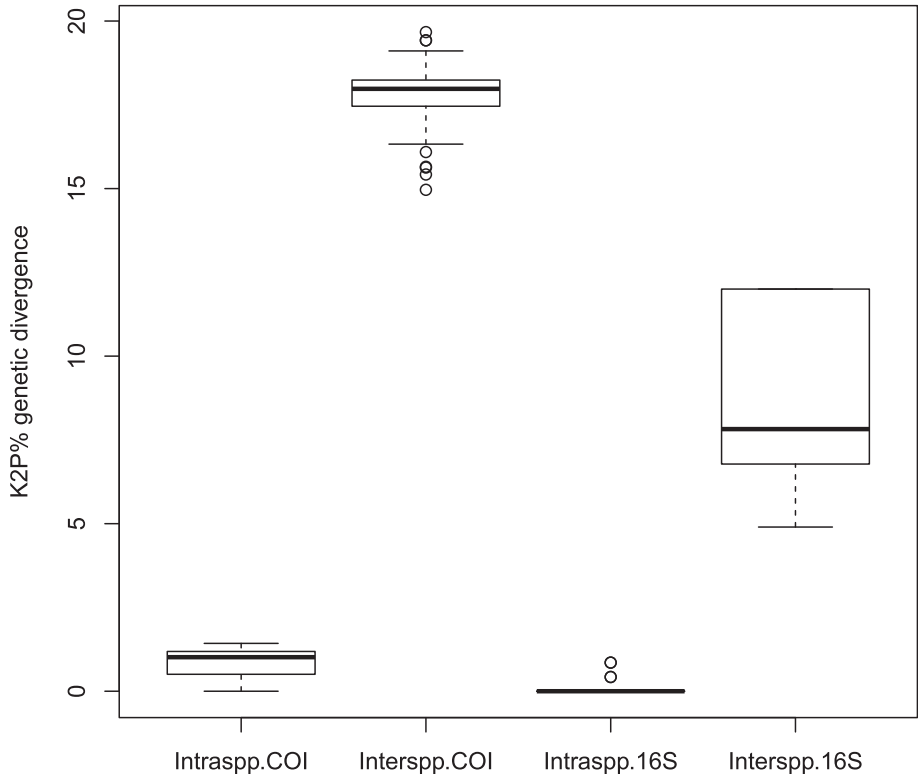

Figure 5. The box plot distribution of the K2P % genetic divergence within and between *Panulirus* based on COI and 16S rRNA genes.

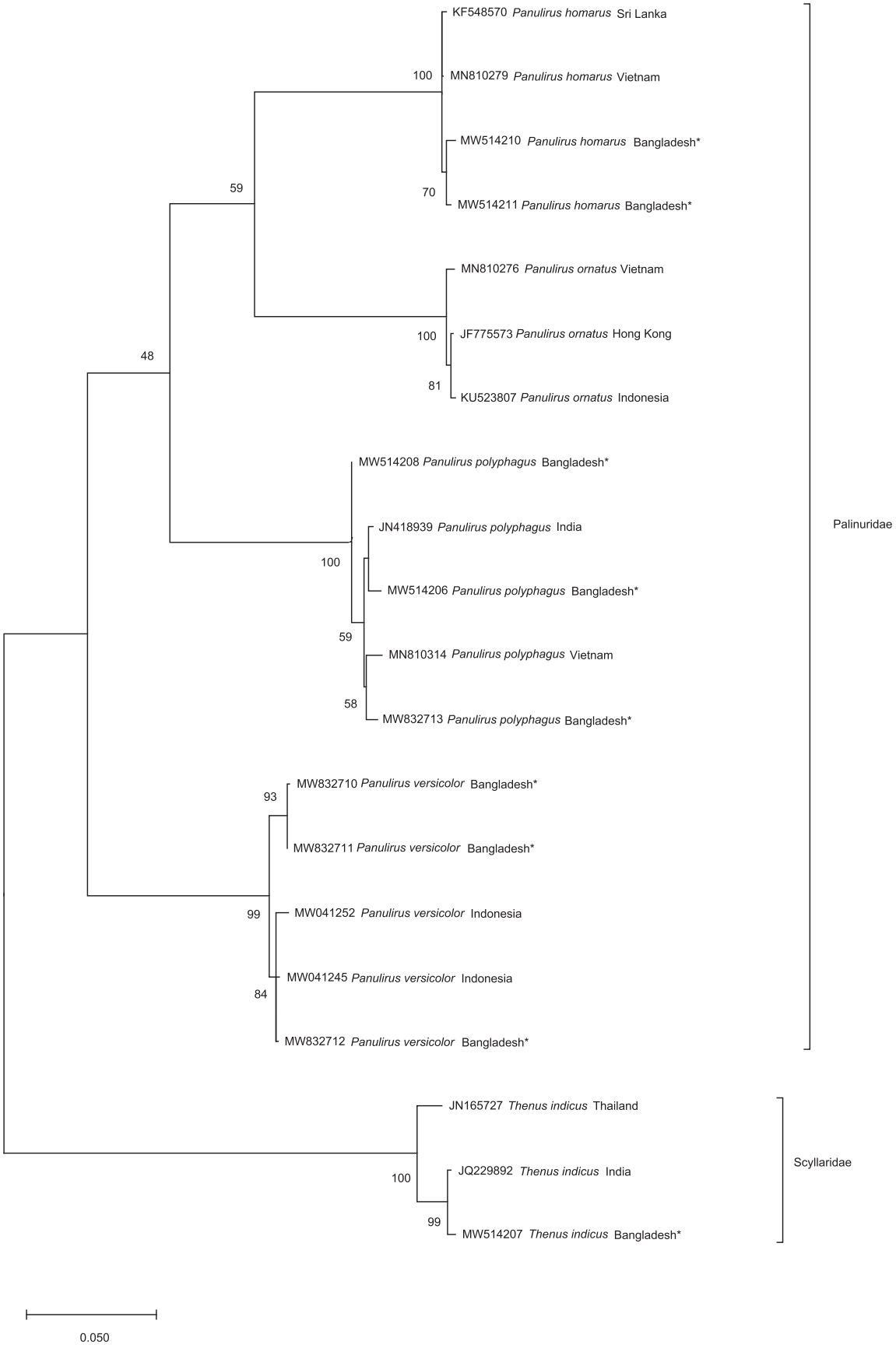

Figure 6. Maximum Likelihood (ML) tree showing the relationships among *Panulirus* based on COI sequences analyzed in present study with the pre-existing sequences of NCBI GenBank. (The sequences generated in this study marked as Bangladesh*).

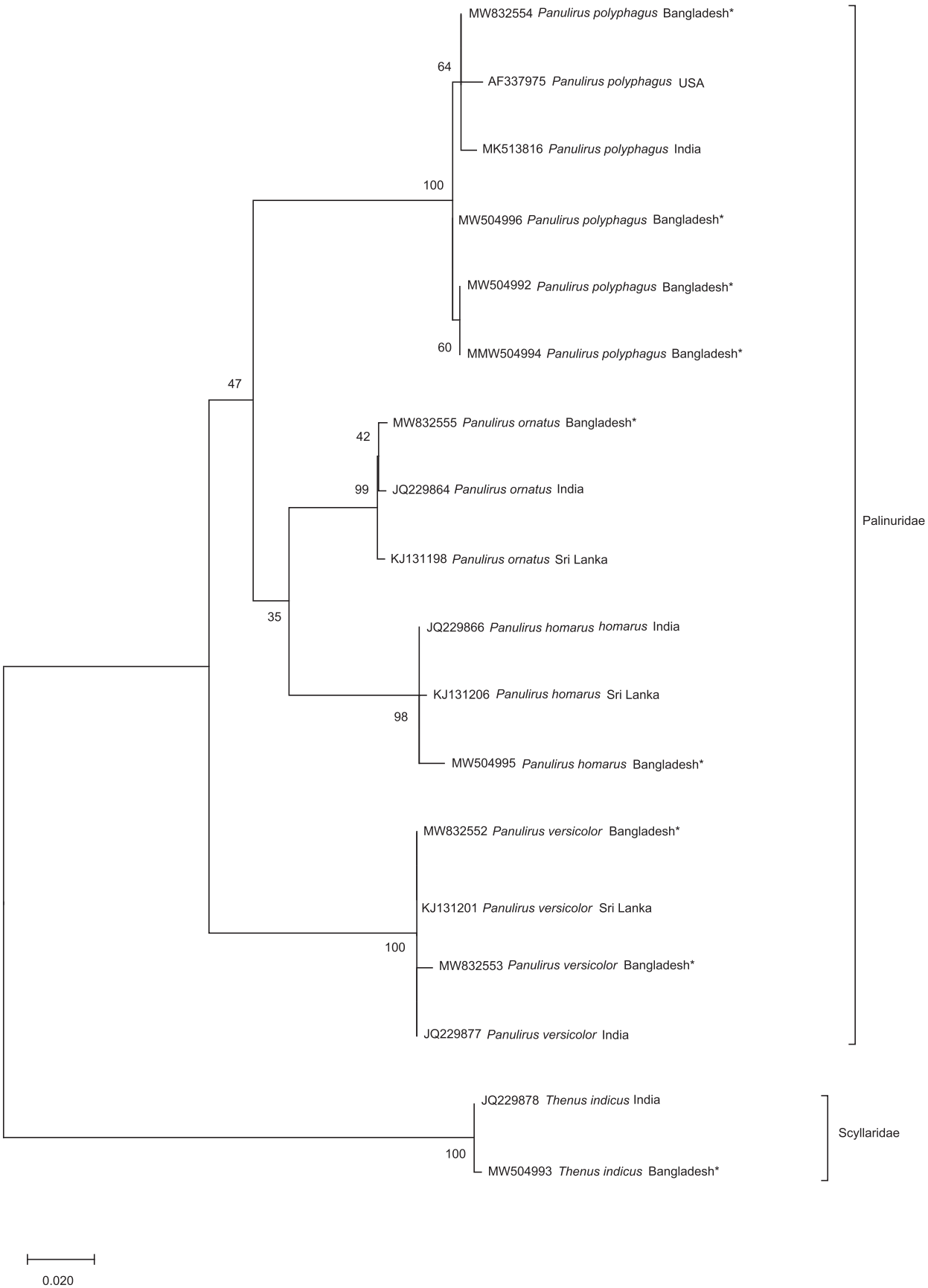

Figure 7. Maximum Likelihood (ML) tree showing the relationships among *Panulirus* based on 16S rRNA sequences analyzed in present study with the pre-existing sequences of NCBI GenBank. (The sequences generated in this study marked as Bangladesh*).

b. 1 pair of principal spines anteriorly and a second pair that is half the size of the first in antennular plate……*P. ornatus*
c. Only 1 pair of equal sized large spines in the antennular plate……*P. polyphagus*
d. 2 pairs of unequal and separate principal spines in the antennular plate……*P. versicolor*

3.3. Molecular analyses

We generated barcodes consisting of 19 partial COI and 16S rRNA sequences of the 4 *Palinurus* species belonging to the family Palinuridae (Table 1). The average 614 base pair COI sequences ranging from 410 to 679 bp had nucleotide frequencies of 32.25% T/U, 22.23% C, 25.81% A and 19.71% G. The alignment matrix of the mean 476-bp 16S rRNA ranged from 275 to 567 bp with nucleotide frequencies of 33.97% T/U, 12.96% C, 31.70% A and 21.37% G. The mean genetic divergence within and between species is summarized in Table 2 and illustrated as a box plot distribution (Figure 5). Wide barcoding gaps of 13.54% and 4.043% were observed in COI and 16S rRNA, respectively, revealing that both genes could successfully discriminate against lobster species. The maximum likelihood (ML) phylogenetic tree analysis produced three major clades, where the first group consisted of *P. polyphagus, P. homarus* and *P. ornatus*. *P. versicolor* formed a second clade, and the clade of *T. indicus* was the outgroup. Lineage support was interpreted based on bootstrap percentage (BP) [BP: 100% maximal clade support, 95% to <100% strong clade support, 75% to <95% moderate clade support, 50% to <75% weak clade support and <50% negligible clade support].

4. Discussion

Morphometric key characteristics clearly identified spiny lobsters collected from the coastal region of Bangladesh (Figures 1, 2, 3, and 4), which is consistent with previous literature (Holthuis, 1991; Chan, 1998; Wahyudin et al., 2017). Among the four species of identified lobsters, *P. homarus, P. ornatus,* and *P. polyphagus* have been nationally assessed as vulnerable (VU) with *P. versicolor* assessed as endangered (EN) (IUCN Bangladesh, 2015). However, globally, all of them are considered the least concerning (LC) (IUCN, 2021). Moreover, *P. ornatus, P. polyphagus* and *P. versicolor* are listed in Schedule I of Wildlife (Conservation and Protection) Act 2012 as 'protected animals' (Bangladesh Gazettes, 2012). *P. polyphagus* is frequently observed in the muddy and turbid coastal belt and river mouths of Bangladesh, as this is the most suitable habitat for this species (Holthuis, 1991). Unfortunately, we observed that all of these species are indiscriminately harvested from the coastal region and trade transpired due to high prices in the domestic and overseas markets, leading this invaluable resource to be threatened with extinction.

The morphological identifications were further validated by utilizing the partial sequences of mitochondrial COI and 16S rRNA genes. The mean length of partial sequences of COI and 16S rRNA sequences generated showed AT bias, *i.e.*, high AT content in Palinuridae, similar to that in a previous study (Matzen da Silva et al., 2011). The mean K2P divergence within and between species was found to be 0.83 and 17.81, respectively, for the COI gene. In the case of Indian lobster species, the K2P divergence within and between species ranged from 0.30 to 0.70 and 15.00 to 26.80 (Jeena et al., 2016). In contrast, for 16S rRNA, the divergence within and between species was 0.11 and 8.40, respectively, which was quite similar to the value observed by Jeena et al. (2016). The calculated K2P divergence showed a significant barcoding gap, and the progressive increment in genetic divergence at a higher taxonomic level supports a marked change in genetic divergence at the species boundary. The lowest genetic divergence was observed between *P. homarus* and *P. ornatus* with a 14.97% distance in COI and 4.9% in 16S rRNA sequences. The minimum interspecific divergence greater than the 2% threshold also indicates the efficiency of markers in differentiating congeneric species. The highest divergence was found between intergenus species with 19.67% divergence between COI sequences of *P. versicolor* and *P. ornatus* from Indonesia and 12% divergence between 16S rRNA sequences of *P. polyphagus* and *P. versicolor* (Table 2).

The monophyletic clade of the intraspecific sequences in the phylogenetic tree constructed based on each marker gene proved the effectiveness of the COI and 16S rRNA in species delimitation. Additionally, no taxonomic deviation at the species level confirmed the authenticity of their recognition. Among the four species compared, *P. homarus* and *P. versicolor* formed close associations within species with strong clade support of 98%–100% BP in both evolutionary trees. However, the COI sequences of *P. polyphagus* formed monophyly with sequences from Vietnam and Indian sequences with 58% and 72% BP support, respectively (Figure 6), and the 16S rRNA sequences of the species grouped with the sequences from the USA and India with 64% BP (Figure 7). Additionally, the 16S rRNA sequences of *P. ornatus* formed a negligible clade with 42% BP support with species from India. The utility of molecular identification for delineating spiny lobster species found in Sri Lanka has been previously recorded by Senevirathna and Munasinghe (2013) based on partial COI sequences. The present study enables revision of the taxonomic status of *Panulirus* species residing in the geographical region of Bangladesh and their molecular characterization using partial sequences of two identification marker genes, COI and 16S rRNA, hence contributing to the global DNA barcode database.

This is the first comprehensive taxonomic description of spiny lobsters from Bangladesh, which has been further validated by two marker genes, COI and 16S rRNA. This baseline integrative approach would substantiate further taxonomic research on lobsters to understand their distribution, diversity and ecological importance in Bangladesh waters.

Declarations

Author contribution statement

Md. Sagir Ahmed: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Anindita Barua & Sujan Kumar Datta: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tonmoy Saha & Durjoy Raha Antu: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sumaiya Ahmed: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data associated with this study has been deposited at NCBI Gen-Bank databases (https://www.ncbi.nlm.nih.gov/genbank/) under accession number MW514205-06, MW514208-11, MW832710-13, MW504991-92, MW504994-96, and MW832552-55.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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GEOGRAPHICAL RANGE EXTENSION OF TWO PENAEID SHRIMP *PENAEUS PULCHRICAUDATUS* STEBBING, 1914 AND *KISHINOUYEPENAEOPSIS INCISA* (WANG & LIU *IN* LIU & WANG, 1987) IN BANGLADESH WATERS

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Keywords: DNA barcoding, *Kishinouyepenaeopsis incisa,* new records, Penaeid Shrimp, *Penaeus pulchricaudatus*

Abstract

Shrimps are among the most intriguing groups within the Decapoda order. In Bangladesh, penaeid shrimps hold significant economic value as a major export commodity. This study aimed to analyze the morphometric and molecular characteristics of penaeid shrimps in Bangladesh. Two species of penaeid shrimp, *Penaeus pulchricaudatus* and *Kishinouyepenaeopsis incisa* were identified as new records in Bangladesh waters. A distinguishing feature of *P. pulchricaudatus* is its dark brown transverse bands that do not extend to the lower half of the carapace. The identification of these species was confirmed using both morphometric characteristics and DNA barcoding techniques. Two sequences were generated for these species utilizing the 16S rRNA gene marker. The findings reveal two new records of shrimp species in Bangladesh, expanding the known diversity of shrimp in the region and highlighting the presence of previously undocumented species.

Introduction

Crustacean fisheries represent a crucial resource for Bangladesh, including commercially important species like shrimp, prawns, lobsters, and crabs. These species are vital to the food chain within tropical marine ecosystems. Worldwide, more than 30,000 marine crustacean species have been recorded(1). The Penaeidae family, commonly referred to as penaeid shrimp or prawn, encompasses 48 recognized genera, 23 of which are known solely from fossils. In Bangladesh, a total of 64 prawn and shrimp species from 8 families have been documented, with the Penaeidae family alone comprising 24 shrimp species, primarily originating from marine and coastal environment(2).

Shrimp is a major export commodity for Bangladesh. The total production of shrimp and prawn, including capture, has increased from 140,000 MT to 2,61,154 MT over the past 20 years, from 2000-01 to 2020-21(3). Among them 47,606 MT are marine production and the

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penaeid shrimp[3]. In 2020, Bangladesh exported 30,036.18 MT of frozen shrimp, generating approximately BDT 2,948.94 crore in revenue[4]. The prawn and shrimp sector accounts for 74.05% of the total export earnings from fisheries products[4]. The industry employs 1.2 million people directly in production, with an additional 4.8 million household members involved in the sector.

The Penaeidae family is distinguished by a range of sizes from small to large and features five pairs of well-developed legs, with the first three pairs forming pincers, none of which are notably large. The abdomen's posterior pleura overlap the anterior pleura of the next segment. Males have a prominent copulatory organ on the first pair of pleopods, known as the petasma, while females have a copulatory organ on the posterior thoracic sternites, called the thelycum. Eggs are released directly into the water and are not retained by the females. This paper presents the first documentation of two penaeid shrimp species, *Penaeus pulchricaudatus* Stebbing, 1914, and *Kishinouyepenaeopsis incisa* (Wang & Liu in Liu & Wang, 1987), from the marine waters of Bangladesh based on both morphological and molecular characteristics.

Materials and Methods

Sampling and Morphological Analysis

A specimen of *Penaeus pulchricaudatus* was collected on November 28, 2020 from Teknaf, (20.728 N 92.351 E) and another one specimen of *Kishinouyepenaeopsis incisa* on May 4, 2018 from Kuakata (21.847875 N 90.059220 E). Specimens were caught as a bycatch during pelagic fishing in the Bay of Bengal. After the collection, samples were immediately preserved in ice and transfer it to the Advanced Fisheries and DNA Barcoding lab, Department of Zoology, University of Dhaka. Fresh condition photographs were taken before the samples were stored in a refrigerator at -18°C for further analysis. Taxonomic identification of the specimen was conducted following Liu and Wang (1987)[5] and Tsoi et al., (2014)[6]. A portion of tissue (20mg) was transfer to a vial for genetic analysis, tagged the specimen as DUZM_CR_107B *(Kishinouyepenaeopsis incisa)* and DUZM_CR_094BS *(Penaeus pulchricaudatus)* and deposited at Kazi Zaker Hossain Zoological Museum.

Extraction and PCR amplification of genomic DNA

Using a Qiagen® Dneasy Blood & Tissue Kits (USA) and the manufacturer's instructions, DNA was extracted. A NanoDrop spectrophotometer was used to assess the extracted DNA's quality and quantity. The contigs were amplified using polymerase chain reaction (PCR) with the primers 16Sar (forward) 5′-CGCCTGTTTATCAAAAACAT-3′ and 16Sbr (reverse) 5′-CCGGTCTGAACTCAGATCATGT-3′[7]. The amplification protocol consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 94°C for 45 seconds, 48°C for 30 seconds, 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. The amplified gene bands were visualized on a 1% agarose gel. PCR purification and sequencing were carried out by Celemics Inc., Korea (outsourcing company).

Bioinformatics analysis

The quality of the generated sequences was assessed using CHROMAS software. Sequence confirmation was performed by conducting a BLASTn search against the best-matching sequences in the nucleotide database, and the sequences were subsequently deposited in the NCBI GenBank. A phylogenetic tree was constructed using the neighbor-joining (NJ) statistical method with gamma distribution rates, employing bootstrap analysis with 1000 replicates in MEGA 11[8] and iTOL v5[9].

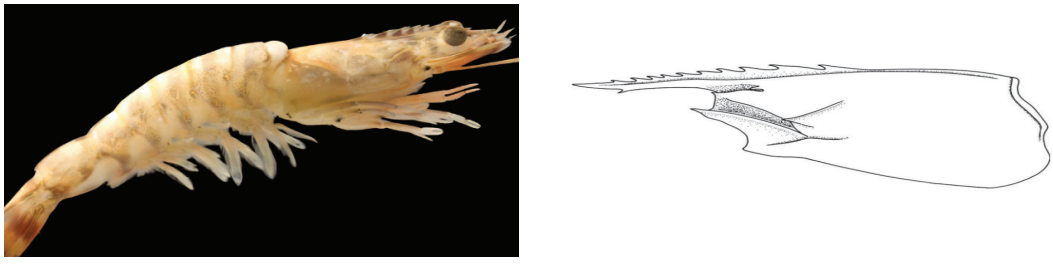
Results and Discussion

Taxonomy

Penaeus pulchricaudatus Stebbing, 1914

Material examined

Teknaf, Bangladesh, 20.728 N 92.351 E, 28 November, 2020, carapace length 37.2 mm, DUZM_CR_094BS (deposited at the Kazi Zaker Hossain Zoological Museum), 16S GenBank accession number MW483130



A B

Fig. 1. *Penaeus pulchricaudatus* Stebbing, 1914, A. A specimen from Teknaf, Bangladesh B. Carapace[10]

Description

The carapace features well-developed ridges and grooves, devoid of longitudinal and transverse sutures. The rostrum has 9 dorsal teeth, with 3 on the carapace and 1 ventral tooth (Fig. 1 A,B). The integument is smooth. The postrostral carina nearly extends to the posterior carapace, featuring a deep median groove along its length. The adrostral groove is as wide as the postrostral carina and extends close to the posterior carapace. There is no postocular sulcus. The gastrofrontal groove is distinct, with the posterior end divided into two. The cervical carina is sharp, accompanied by a well-marked groove. The hepatic spine is very pronounced, and the hepatic carina is well-marked, curved, and ventrally inclined at the anterior part. The ischial spine of the first pereiopod is either absent or barely visible. The sixth abdominal somite lacks a dorsolateral groove and has three cicatrices. The thelycum is double-tubed and pouch-like, opening anteriorly. The spermatophore is deposited on the thelycum as a large subtriangular wing-like process. The telson has three sets of movable lateral spines. The morphometric measurement was shown in Table 1.

Table 1. Biometry of *Penaeus pulchricaudatus* and *Kishinouyepenaeopsis incisa* (in mm and % to TL)

Characteristics	*Penaeus pulchricaudatus*		*Kishinouyepenaeopsis incisa*	
	Measurement (mm)	**% to total length**	**Measurement (mm)**	**% to total length**
Total length	137.7		77.5	
Ocular/Body length	122.2	88.74	65.4	84.39
Abdomen length	85.0	61.73	47.9	61.81
Carapace length	37.2	27.02	17.5	22.58
Rostrum length	15.5	11.26	12.1	15.61
Telson length	15.2	11.04	8.1	10.45
Rostral formula				

Color

The body is pale yellowish, marked with dark brown transverse bands. These bands extend from the top to about the middle of the carapace, with the rearmost band on abdominal somite VI being interrupted. The eyes are black-brown. The scaphocerite has a somewhat greenish hue with white tips, while the antennal flagella range from reddish-brown to yellowish-brown. The pereiopods are whitish to yellowish, and the pleopods are yellowish to reddish, featuring brown and white spots at the bases.

P. pulchricaudatus is almost identical to *P. japonicus*, but can be differentiated by the coloration of the ventrolateral carapace. In *P. pulchricaudatus*, the dark brown transverse bands do not extend to the lower half of the carapace, whereas they do in the latter species.

Kishinouyepenaeopsis incisa (Wang & Liu in Liu & Wang, 1987)

Material examined

Kuakata, Bangladesh, 21.847875 N 90.059220 E, 4 May, 2018, carapace length 17.5 mm, DUZM_CR_107B (deposited at the Kazi Zaker Hossain Zoological Museum), 16S GenBank accession number ON264685.

Fig. 2. K*ishinouyepenaeopsis incisa* (Wang & Liu in Liu & Wang, 1987), A specimen from Kuakata, Bangladesh

Description

The body is slender and setose. The rostrum is mostly straight, with a slight upward curve at the tip, bearing 7-8 dorsal teeth except for a short toothless distal portion (Fig. 2). It does not extend beyond the third antennular segment of the peduncle. The antennular flagella are equal in length and shorter than the carapace, while the antennal flagellum is larger and exceeds the total body length. The distolateral projections of the petasma are longer than the distomedian projections, slender, horn-like, diverging at the base and curving inward at the tips. The anterior plate of the thelycum is rectangular with rounded corners, fused to the posterior plate by a broad posteromedian process. The posterior plate features a pair of lateral depressed regions, and a tuft of long hairs is located behind the thelycum. All five pairs of legs are well developed, with the first three pairs forming pincers. The morphometric measurement was shown in Table 1.

Color

The body is pale greenish, marked with numerous minute dark spot. Eyes are black-brown. The scaphocerite has a somewhat greenish hue with white tips, while the antennal flagella range from reddish-brown to black-brown. The pereiopods are whitish to yellowish, and the pleopods are yellowish to reddish, featuring brown and greenish spots at the bases.

Molecular Analysis

Two partial ribosomal RNA sequences were generated, with lengths of 475 and 478 base pairs for the two identified species. The aligned partial sequences were deposited in GenBank with accession numbers ON264685 for *K. incisa* and MW483130 for *P. pulchricaudatus.* In the

BLAST search results, *P. pulchricaudatus* and *K. incisa* showed 100% query coverage, with similarity percentages of 100% and 97.56%, respectively, compared to existing sequences from India and China. The nucleotide base frequencies for *P. pulchricaudatus* were A: 32.43%, T: 32.85%, C: 13.39%, and G: 21.34%. For *K. incisa*, the frequencies were A: 30.74%, T: 33.68%, C: 13.47%, and G: 22.11%. Both sequences exhibited a strong AT bias, with percentages of 64.42% for K. incisa and 65.28% for *P. pulchricaudatus*. A Neighbor Joining (NJ) phylogenetic tree was constructed using a total of 7 sequences from 7 penaeid shrimp species (Fig. 3).

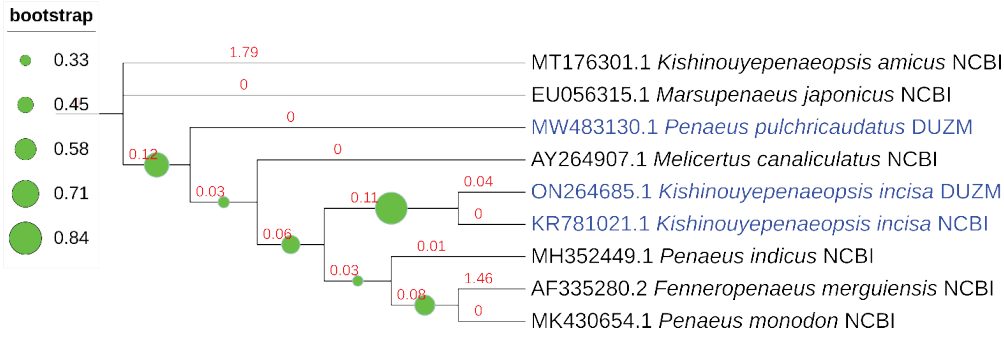


Fig. 3. Phylogenetic analysis of penaeid shrimp species by the Neighbor-joining method in MEGA 11 and iTOL v5. The values in red color denote the branch length and round circle bootstrap values (33-83%). DUZM represents the generated sequence of the present study.

The sequences from the present study are denoted as DUZM, while the remaining sequences were retrieved from the NCBI database. In the phylogenetic tree, each species forms a distinct clade (Fig. 3). *P. pulchricaudatus* is almost identical to *P. japonicus* which was referred to as I and II. They were distinguished by diagnostic color banding patterns on the carapace, previously considered merely a color variant in taxonomic studies[11-12]. Despite the lack of differences in other morphological traits or morphometric parameters[12], phylogenetic analyses using mitochondrial (mt) DNA markers consistently show that while these two forms are closely related to each other , they are genetically distinct[10].

The results reveal two new records of shrimp species from Bangladesh. These findings expand the known diversity of shrimp in the region and highlight the presence of previously undocumented species.

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