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Taxonomic Clarification of Mud Crab Species of Genus *Scylla* (Brachyura: Portunidae) Available in the Coastal Regions of Bangladesh

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Abstract

Taxonomy of mud crabs under the genus *Scylla* that are found in the coastal regions of Bangladesh has become a major concern due to the lack of taxonomic identification of the species. In this study 371 samples collected randomly from the seven locations along the coastal areas of Bangladesh were used to study the taxonomy of mud crabs based on morphological characteristics, statistical analysis of morphometric ratios and genetic analysis. Partial sequences of one mitochondrial gene, 12S rRNA, in populations of *Scylla* spp. obtained from the study areas were used in the genetic analysis. Morphological characteristics, morphometric ratios and sequence data of the samples observed in this study provided sufficient evidence to conclude that the common mud crab species which is being caught from the coastal regions of Bangladesh is *Scylla olivacea* .

Introduction

Mud crab (genus *Scylla* De Haan 1833; Crustacea: Decapoda: Brachyura: Portunidae) is a common inhabitant of mangrove areas, brackish or coastal waters along the shorelines, ponds and intertidal swamps in the coastal regions (Hoq 2008) and is a fast growing exportable commodity in Bangladesh. The coastal ecosystem of Bangladesh including its canals, rivers and estuaries provides a muddy substrate, which acts as a significant habitat, shelter and nursery ground for commercially important mud crab species (Acharya and Kamal 1994).

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The taxonomic classification of the genus *Scylla* was controversial worldwide for a long time. Estampador (1949) studied external morphology and gametogenesis of mud crabs collected from the Philippines and classified those specimens into three species and one subspecies, namely *Scylla serrata* (Forskal 1775), *Scylla oceanica* (Dana 1852), *Scylla tranquebarica* (Fabricius 1798) and *Scylla serrata* var. *paramamosain* (Estampador 1949).Serene (1952) categorised the mud crab into two species from four forms in Vietnam. Stephenson and Campbell (1960) suggested only a single species for this genus i.e. *S. serrata*. Joel and Raj (1983) and Kathirvel and Srinivasagam (1992) reported the existence of *S. serrata* and *S. tranquebarica* from India on the basis of morphological characteristics. Keenan et al. (1998) extensively revised the taxonomy of the genus *Scylla* considering morphology, morphometric ratios and allozyme variability and classified this genus into four distinct species: *S. serrata, S. olivacea, S. tranquebarica* and *S. paramamosain* Estampador 1950. Subsequently, several researchers around the world who dealt with the taxonomy of mud crabs used either morphological characteristics (Jirapunpipat et al. 2008; Ogawa et al. 2012) or genetic characterization (Imai et al. 2004; Ma et al. 2012; Mandal et al. 2014).

In Bangladesh, the mud crab species has always been considered as *S. serrata*. Authors in all grey and scientific literature use this nomenclature while referring to the mud crab species found in the coastal regions of Bangladesh. For example, the Department of Forest in Bangladesh, the responsible authority for crab fisheries management in Sundarban Reserve Forest (SRF) publishes annual reports where *S. serrata* is recognised as the single species found in the SRF. A large numbers of researchers used the name *S. serrata* in their studies. These studies include stock assessment (Chantarasri 1994), growth, recruitment and economic performance (Zafar et al. 2006), fattening technology (Kamal et al. 2007), harvesting technique (Hossain and Ahmed 2006), culture and bio-economics (Ferdoushi and Xiang-Guo 2010) of mud crab. It is anticipated that most of the researchers name the crabs based on previous reports without taxonomic identification of the species. Keenan et al. (1998) also indicated that *S. olivacea* is the species that is most of the time identified as *S. serrata* by many researchers. Various taxonomic studies, however, revealed ambiguity in the taxonomy and identification of *Scylla* species around the world (Keenan et al. 1998; Padate et al. 2013). Detailed taxonomic study has yet to be conducted to identify and confirm the specific *Scylla* species available in Bangladesh. Hence, the present study was conducted to identify the mud crab species in the coastal regions of Bangladesh using morphological characteristics, statistical analysis of morphometric ratios and genetic analysis using mitochondrial DNA marker.

Materials and Methods

Sample collection

Wild mud crab samples were collected from eight major locations on the basis of crab availability in the coastal zone of Bangladesh. Three hundred and seventy one fresh wild samples were collected directly from commercial crab collectors during July to December 2014.

Sampling was done from the various locations of western, mid and eastern regions along the coast of the Bay of Bengal (Fig.1). The western region, which is adjacent to the Sundarbans mangrove forest, includes Mongla (22.3°N, 89.3°E; n=33), Sharankhola (22.1°N, 89.5°E; n=27), Shamnagar (22.2°N, 89.0°E; n=58) and Paikgacha (22.3°N, 89.1°E; n=32). The mid region includes Noakhali (22.5°N, 91.0°E; n=66) and Patuakhali (22.0°N, 90.1°E, n=52). The eastern region comprises Cox's Bazar (21.2°N, 92.0°E; n=56) and Chakoria Sundarbans (21.4°N, 92.0°E; n=47). The collected samples were preserved in 95% ethanol. Soon after, an exhaustive morphological and morphometric studies were carried out in the laboratory of the Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, Bangladesh.

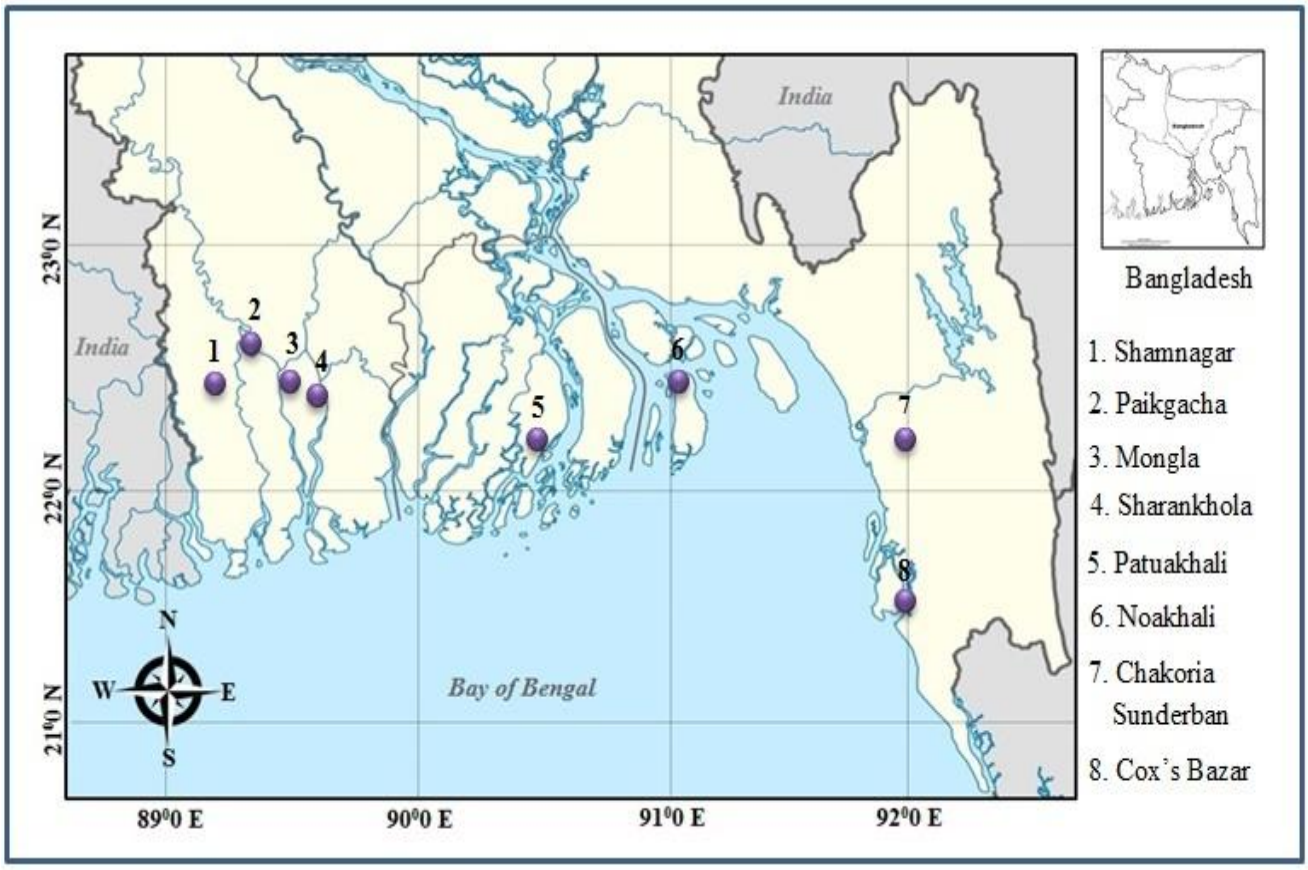

Fig. 1. Mud crab sampling locations in the coastal areas of Bangladesh.

Morphological analysis

Three hundred and seventy one male and female mud crabs were used for this study. The shape and number of carpus spine were considered for the preliminary identification process. Phenotypic features such as the colour of the carapace and chelipeds, frontal lobe spine height and polygonal patterning were examined based on the standard key by Keenan et al. (1998) and Jirapunpipat et al. (2008) to identify and distinguish the findings with other species of *Scylla*.

Morphometric analysis

Only large (carapace length > 8.0 cm) male mud crabs (n = 80) with right appendages were considered for morphometric study to preclude the chances of confounding the influence of juvenile ontogenetic changes. Twenty three morphometric characteristics include internal carapace width (ICW), carapace width (CW), carapace width at 8^{th} spine (8CW), carapace length (CL), carapace posterior width (PWC), 9^{th} lateral spine height (LSH), frontal median spine height (FMSH), distance between frontal median spines (DFMS), distance between frontal lateral spines (DFLS), carapace frontal width (FW), abdomen width (AW), sternum width (SW), dactyl length (DL), merus length (ML), propodus length (PL), propodus width (PW), propodus depth (PD), inner propodus spine (IPS), outer propodus spine (OPS), inner carpus spine (ICS), outer carpus spine (OCS), 5^{th} periopod dactyl width (5PW), 5^{th} periopod dactyl length (5PL) and 3^{rd} periopodmerus length (3PML) were measured by using digital calipers to the nearest 0.01 mm.

Each measurement was taken three times and the averages were considered in this study. Subsequently, 27 morphometric ratios were also calculated as described by Keenan et al. (1998). Keenan et al. (1998) emphasised on eight morphometric ratios (ICS/OCS, FMSH/ICW, ML/PL, AW/SW, PL/ICW, FW/ICW, FMSH/FW and IPS/PL) and Jirapunpipat et al. (2008) considered five ratios (ICS/OCS, PWC/FW, OPS/PL, DFMS/FW and LSH/ICW) to distinguish *Scylla* species. The present study considers seven important morphometric ratios i.e. LSH/ICW, FW/ICW, PWC/FW, DFMS/FW, OPS/PL, ICS/OCS and FMSH/FW for the differentiation of the *Scylla* species.Student t-test was carried out to decide on the compliance of the observed values of morphometric measurements with the mean values mentioned by Keenan et al. (1998) and Jirapunpipat et al. (2008).

The observed data were transformed into square root format for improving the normality of the data and the analysis was conducted at 1% significance level. One way ANOVA was also conducted to address the comparison of morphometric ratios among the samples obtained from three sampling regions following 0.1% significance level.

Genetic analysis

DNA isolation

Muscle tissue from each specimen selected for morphometric study (n = 80) was removed from a single claw and stored at -80°C prior to DNA extraction. Genomic DNA was extracted from muscle tissue using DNAZOL® Reagent (Invitrogen Life Technologies, USA),ethanol and sodium hydroxide (Difco Laboratories, MI, USA) and stored at -20°C until being used.

Amplification and sequence analysis of mitochondrial 12S rRNA genes

A 605-base pair (bp) length of the 12S rRNA excluding primers was amplified by PCR using two specific primers designed from the alignment sequences of insect (GenBank accession number NC_001322.1) and *Scylla* species (FJ827760.1, FJ827758.1, FJ827759.1 and FJ827761.1): MTD_12SF (5'-TAG CTC TGC TTT GAT TCT CCC TG-3') as well as MTD_12SR (5'-TTG CGG TTA TAC TCT AA GGC TAA-3') corresponding to 14130 and 14763 locations of the *Drosophila yakuba* mtDNA sequence, respectively (Clary and Wolstenholme 1985).

PCRs were performed in 25 µL total volume that included 0.4 mM of each primer, 0.2 mM each of dNTP, 10X PCR buffer 0.75 unit Blend Taq Plus DNA polymerase (Toyobo Co. Ltd., Osaka, Japan) and approximately 100 ng template DNA under the following conditions: one cycle of denaturation at 94°C for 5 min; 35 cycles of 0.5 min at 94°C; 0.5 min at a primer-specific annealing temperature 53°C, and 1.5 min of extension at 72°C, using a Bio-Rad PCR system T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).

As a final step, products were extended for 10 min at 72°C. The resulting PCR products were tested by electrophoresis through 1.0% agarose gel and stained with ethidium bromide solution for photography. PCR products were purified by means of magnetic silica beads of MagExtractor kit (Toyobo Co. Ltd.) and sequenced using similar primers as for PCR of 12S RNA on ABI 3730XL sequencing machines (Applied Biosystems, Foster City, CA, USA). Sequences were edited using the software BioEditver 7.0.1 (Hall 1999) and deposited into the GenBank database (the accession number is KT984200). The sequences were aligned with the sequences from four mud crabs and one fruit fly from National Center for Biotechnological Information (NCBI) using ClustalW. The un-weighted pair-group method with arithmetic mean (UPGMA) tree was constructed from the maximum likelihood distance using MEGA 6.0 (Tamura et al. 2013).

Results

Morphological analysis

Carapace was observed to be smooth with prominent transversal ridges, broader than long and with strong H-shaped groove on the gastric zone. Carapace colour varied from greenish brown to brownish green on the surface (Fig. 2A). The ventral surface of the male abdomen was of cream colour with light brown shade (Fig. 2B). The apex of the male first gonopod was long and narrow. The female abdomen was dark to light green or brownish with transverse band and devoid of polygonal marking. The antero-lateral margin was broader than postero-lateral margin and divided into nine similar sized broad spines projecting outwards obliquely. The frontal margin possessed rounded spines with shallow interspaces (Figs. 2A and 2C).

The periopods were flattened into swimming appendages. Among the four pairs of periopods, the first three pairs were similar and the fourth pair was natatorial (Fig. 2D). The chelipeds were smooth, robust and longer than the legs. Three and two well-developed spines were present on the anterior and posterior margin of the merus respectively. The propodus of the cheliped shad a prominent spine on the inner margin and a small blunt spine on the outer margin (Fig. 2E). Carpus contained a short blunt spine on the outer margin and the inner margin was devoid of spine (Fig. 2F). Polygonal marking on carapace, abdomen, chelipeds and periopods were absent in adults (Figs. 2A, 2D and 2E). Sometimes juvenile and sub adult display light cream colour patterns on the surface or epibranchial region of carapace and chelipeds.

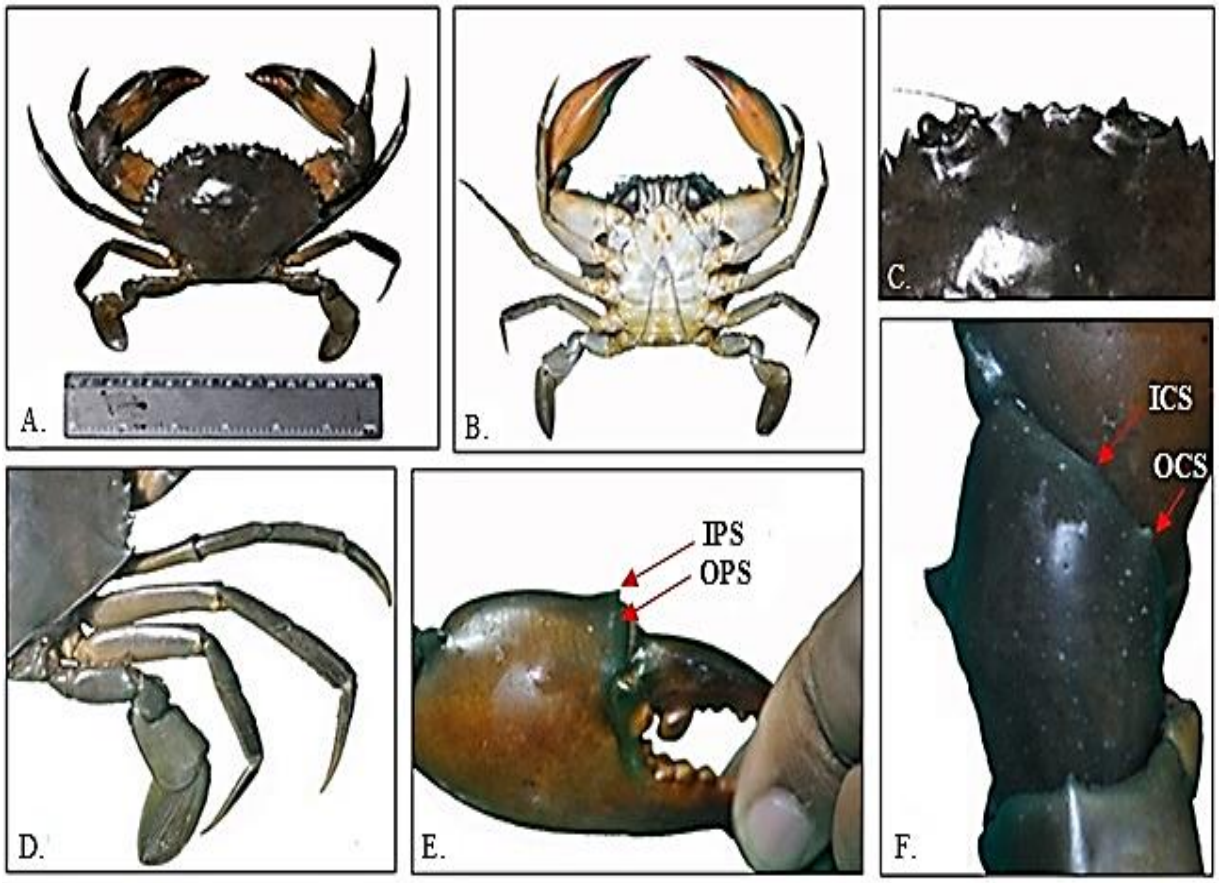

Fig. 2. Male *Scylla* species observed in the present study; (A) Dorsal view; (B) abdomen; (C) Frontal margin; (D) Swimming legs *(without polygonal patterning)*; (E) Propodus spine *(reduced)* and (F) Carpus spine *(ICS absent and OCS reduced)*.

Morphometric analysis

Mean, standard deviation and range of the morphometric ratios obtained from the present observation are presented in Table 1.

Table 1. Morphometric ratios of mud crabs obtained from the coastal regions of Bangladesh.

Morphometric ratios	Mean±SD	Range	Morphometric ratios	Mean±SD	Range
LSH/ICW	0.021±0.004	0.014-0.032	**DL/PL**	0.516±0.279	0.441-1.996
CW/8CW	0.996±0.008	0.980-1.014	**PW/PL**	0.312±0.210	0.242-1.424
CL/ICW	0.703±0.008	0.686-0.720	**PD/PL**	0.411±0.044	0.336-0.528
PWC/ICW	0.314±0.008	0.292-0.326	**(PW*PD*0.7854)/PL**	0.584±0.199	0.341-1.061
FW/ICW	0.444±0.011	0.418-0.464	**IPS/PL**	0.018±0.005	0.008-0.036
PWC/FW	0.706±0.025	0.649-0.758	**OPS/PL**	0.004±0.001	0.001-0.007
FMSH/FW	0.025±0.005	0.017-0.034	**IPS/OPS**	4.371±3.069	1.800-15.330
FMSH/DFMS	0.217±0.048	0.141-0.323	**ICS/PL**	0.000	0.000
DFMS/FW	0.116±0.007	0.103-0.138	**OCS/PL**	0.008±0.006	0.004-0.036
DFLS/FW	0.131±0.005	0.126-0.136	**ICS/OCS**	0.024±0.052	0.000-0.201
DFMS/DFLS	0.887±0.047	0.772-1.031	**ML/PL**	0.677±0.499	0.178-1.176
SW/ICW	0.536±0.035	0.354-0.561	**5PW/5PL**	0.478±0.102	0.552-3.319
AW/SW	0.629±0.174	0.473-1.543	**3PML/ICW**	0.422±0.034	0.247-0.457
PL/ICW	0.754±0.106	0.235-0.866			

One way ANOVA was carried out to determine the differentiation of morphometric ratios among the samples obtained from the three sampling stations and the results are depicted in Table 2. The data showed that there was no significant difference ($P \geq 0.001$) in the morphometric ratios (like LSH/ICW, FW/ICW, PWC/FW, DFMS/FW, OPS/PL, and ICS/OCS except FMSH/FW) among the samples collected from the three regions. The result therefore indicates that a single species exists in the coastal water of Bangladesh.

Table 2. Statistical comparison among the crab samples collected from three sampling regions following the values of seven morphometric ratios.

Inferential analysis	Morphometric ratios						
	LSH/ICW	**FW/ICW**	**PWC/FW**	**DFMS/FW**	**OPS/PL**	**ICS/OCS**	**FMSH/FW**
F value	2.184	0.118	2.143	1.241	2.068	0.421	12.023
Significance	0.133	0.889	0.138	0.306	0.147	0.661	0.000

Note: Significance level 0.001and Degree of freedom= 297

Genetic analysis

A blast search of *Scylla* spp. 12S rRNA sequence against sequence submitted to NCBI showed 99.5% identity with *S. olivacea* (accession nos. FJ827760) followed by *S. tranquebarica* (51.4%), *S. serrata* (49.9%), *S. paramamosain* (49.2%), and *Drosophila yakuba* (39.3%).

The phylogenetic analysis of 12S rRNA, mud crabs, and fruit fly is illustrated in Fig. 3. The *Scylla* spp. of this study formed a cluster with *S. olivacea*, which were phylogenetically closely related to the other three mud crabs. The degree of identity was lower with *D. yakuba*. The constructed phylogenetic tree based on mitochondrial 12S rRNA gene indicated that the *Scylla* species obtained in this study was *S. olivacea*.

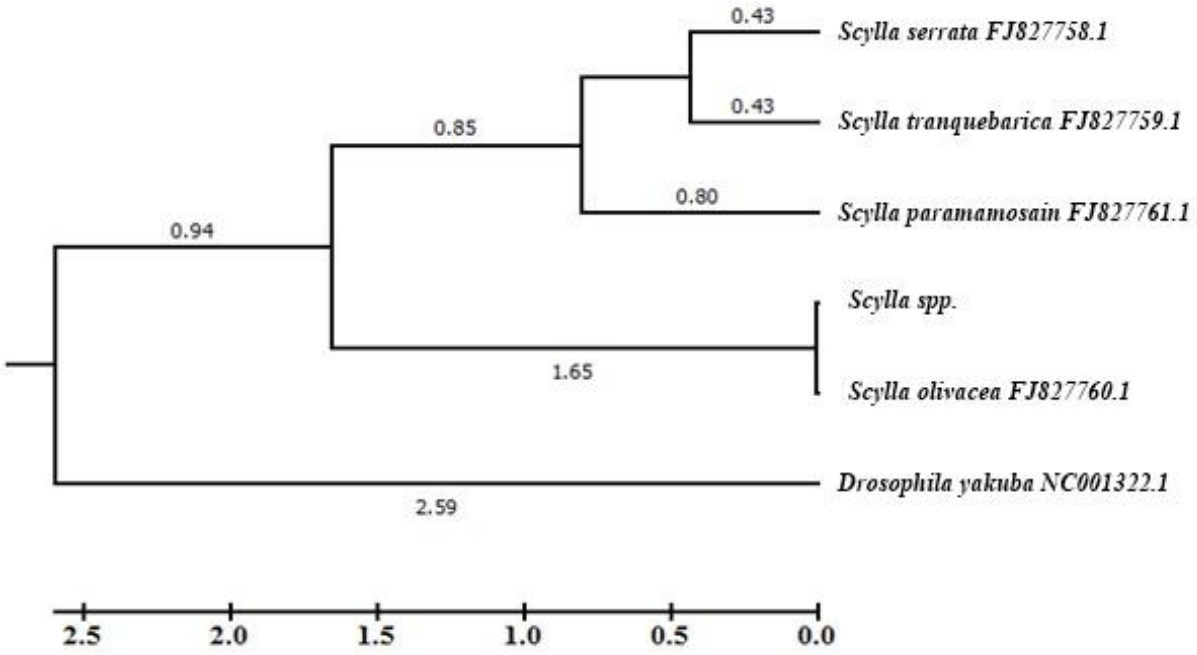

Fig.3. UPGMA phylogram of 12S rRNA, mud crabs,and fruit fly. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Discussion

Among the four known *Scylla* species, *S. serrata* and *S. olivacea* were reported from the neighbouring countries like India (Mandal et al. 2014) and Thailand (Jirapunpipat et al. 2008). The other two species *S. tranquebarica* and *S. paramamosain* were conspicuously not available in India (Mandal et al. 2014) and rarely available in Thailand (Jirapunpipat et al. 2008). This study therefore, attempted to focus on *S. serrata* and *S. olivacea* considering the geographical proximity of their distribution in neighbouring countries.

Distinguishing morphological characteristics among the specimens observed in the present study and that of *S. serrata* and *S. olivacea* as reported by Keenan et al. (1998) and Jirapunpipat et al. (2008) are presented in Table 3. The carapace colour of the mud crabs observed in this study was brownish green to brown which was identical with *S. olivacea.* However, Keenan et al. (1998) mentioned that carapace colour may vary within the same species due to habitat differences and is not considered as a distinguishing character. The frontal lobe spines of the specimen in this study displayed rounded shape with rounded interspaces.

According to Padate et al. (2013), the frontal lobe spines were rounded with rounded interspaces in *S. olivacea* whereas the spines were steep, with 'V' shaped interspaces in *S. serrata*. Polygonal marking on the chelipeds and periopods were very noteworthy distinguishing characters because adult *S. serrata* revealed strong pattern on carapace, chelipeds and periopods whereas *S. olivacea* was devoid of such marking (Jirapunpipat et al. 2008). Specimens considered in this study showed no polygonal marking on the carapace, chelipeds and periopods. The long and narrow apex of the male first gonopod is significantly distinctive in *S. olivacea* which was observed in the collected specimens. *S. olivacea* has a brownish red colour patch just below the apex in fresh specimens (Joel and Raj 1980). Transverse bands with dark to light green or brownish colour were present on the female abdomen without polygonal marking (Figs. 4A and 4B). From the comparative morphological characteristics the mud crab in the present study resembles S. olivacea instead of S. serrata, which has been reported from Bangladesh in the past.

Table 3. Comparison of morphological characteristics among the specimens observed in the present study and that of S. serrata and S. olivacea as reported by Keenan et al. (1998) and Jirapunpipat et al. (2008).

Morphological Characters	***Scylla serrata***	***Scylla olivacea***	**Observed mud crab**
Carapace colour	Dark green/ or grayish green*	Rusty brown or dark brown*	Brownish to brownish green, olive
Chelae colour	Blue green to dark green*	Red brown to dark brown*	Red brown with greenish shade on upper side
Polygonal pattern	Strong on carapace, chelipeds and peripods*	Absent*	Absent
Frontal lobe spine pattern	Shape-bluntly pointed**	Shape-rounded**	Shape-rounded
	Height-high**	Height-low**	Height-low
Cheliped Pattern (Carpus spines)	Both obvious**	Inner absent outer reduced**	Inner absent outer reduced
Cheliped Pattern (Propodus spines)	Obvious**	Reduced**	Both present but reduced

Note: * Jirapunpipat et al. 2008 and ** Keenan et al. 1998

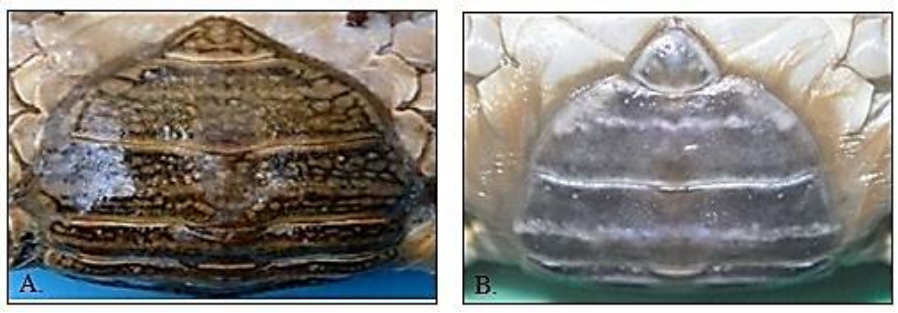

Fig. 4. Female gonopod; (A) *Scylla*. *serrata* (source: Jirapunpipat et al. 2008); and (B) *Scylla olivacea* (present study).

Using box plot diagram, the median, quartile and range of the seven major morphometric ratios for the specimens observed in the present study were compared with the identical *Scylla* species from Thailand reported by Jirapunpipat et al. (2008) and are shown in Fig. 5. Morphometric ratios such as LSH/ICW, ICS/OCS, OPS/PL, DFMS/FW, FMSH/FW and PWC/FW reported in the present study showed positive compliance with *S. olivacea* instead of *S. serrata.*

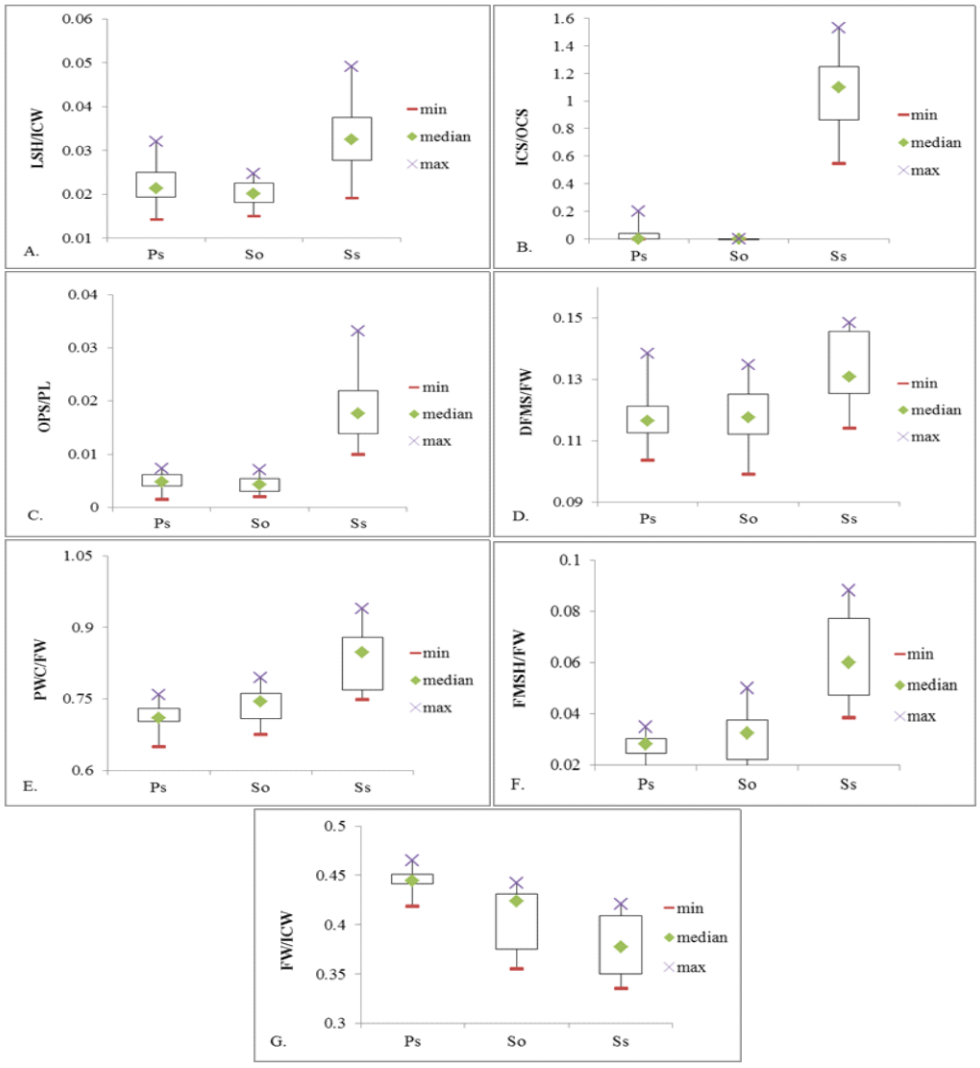

Fig. 5. Morphometric ratios of mud crab found in the present study (Ps) with the respective ratios mentioned in Jirapunpipat et al. (2008) for *Scylla olivacea* (So) and *Scylla. serrata* (Ss).

Comparison of the mean of selective morphometric ratios presented in Keenan et al. (1998) and Jirapunpipat et al. (2008) with the observed value of respective ratios are shown in Tables 4 and 5.The results of the student t-test showed significant differences ($p < 0.01$) between the present observations and the *S. serrata* recorded in Keenan et al. (1998) and Jirapunpipat et al. (2008) while no significant differences ($p > 0.01$) was noticed with *S. olivacea* based on LSH/ICW, OPS/PL, ICS/OCS and DFMS/FW. This analysis clearly indicates that the specimen examined in this study was different from *S. serrata* and was similar to *S. olivacea*.

Table 4. Statistical analysis of the observed values of three morphometric ratios with the mean of respective value provided by Keenan et al. (1998) for *Scylla serrata* and *Scylla olivacea*.

Ratios	***Scylla serrata***			***Scylla olivacea***		
	Test value	**Calculated T value**	**P value**	**Test value**	**Calculated T value**	**P value**
LSH/ICW	0.031	-12.395	0.000	0.022	-1.090	0.285
DFMS/FW	0.019	-10.306	0.000	0.006	-0.223	0.825
ICS/OCS	0.940	-25.173	0.000	0.006	1.503	0.144

Note: Significance level = 0.01 and Degree of freedom = 29

Table 5. Statistical analysis of the observed values of three morphometric ratios with the mean of respective value provided by Jirapunpipat et al. (2008) for *Scylla serrata* and *Scylla. olivacea*.

Ratios	***Scylla serrata***			***Scylla olivacea***		
	Test value	**Calculated T value**	**P value**	**Test value**	**Calculated T value**	**P value**
LSH/ICW	0.034	-16.164	0.000	0.021	0.166	0.870
DFMS/FW	0.130	-9.930	0.000	0.118	-1.080	0.289
ICS/OCS	1.119	-111.915	0.000	0.000	1.503	0.144

Note: Significance level = 0.01 and Degree of freedom = 29

Keenan et al.(1998) mentioned that *S. serrata* is dominant in oceanic waters where the surface salinity reaches above 34ppt whereas the other species can be found in areas where salinity is less than 33ppt. For example, *S. olivacea* is a stenohaline species and are common in places where salinity level falls below 31 ppt (Keenan et al. 1998). Walton et al. (2006) also mentioned that *S. olivacea* prefers reduced salinity and mangrove as their habitat. In the coastal zone of Bangladesh, from where the crab samples were collected, the salinity ranges from 5 to 30 ppt (Hussain 2014). Considering the salinity preference range, from where the mud crab samples were obtained and the taxonomic studies confirms the species as *S. olivacea*. Beside that *S. serrata* might also be found off the coast where salinity level is above 30 ppt.

Conclusion

Considering the morphology, morphometric ratios and genetic analysis, it is clearly evident that the common mud crab species which is being caught from the coastal regions of Bangladesh is *S. olivacea* instead of *S. serrata* that has been reported in the past. Henceforth, it is hoped that researchers will use the proper species name to describe the mud crabs in the coastal regions of Bangladesh.

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