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

A Thesis

By

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Examination Roll No: MS-140613

Session: 2013-14

The thesis submitted to the Fisheries and Marine Resource Technology Discipline in partial fulfillment of the requirements for the degree of

Master of Science

in

Coastal and Marine Science

October, 2015

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

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Declaration

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The results presented in the thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it being concurrently submitted for any degree

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October, 2015

Dedicated to...

My Beloved Parents

Acknowledgement

At first, all of my admiration goes to the supreme creator and ruler of the universe, almighty God, whose mercy keeps me alive and enables me to pursue my education in Fisheries & Marine Resource Technology Discipline and to complete my thesis for the degree of M.Sc. in Coastal and Marine Science.

Then, I would like to express my heartiest gratitude, sincere appreciation and indebtedness to my honorable teacher and supervisor **Muhammad Abdur Rouf, *Ph.D.*** Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna for his wise guidance, scholastic advice, useful comment and unfailing enthusiasm throughout the whole period of my thesis.

I am very glad to express my sincere appreciation and profound respect to my honorable teacher

Md. Ayaz Hasan Chisty, *Ph.D.* Professor and Head, of Fisheries and Marine Resource Technology Discipline, Khulna University for providing me the opportunity for this study.

I would like to recall with great respect the cordial guidance and support provided by Dean and Professor **Md. Nazmul Ahsan, *Ph.D.*** and Professor **Md. Golam Sarower, *Ph.D.***, Lecturer **Md. Shahin Parvez** of Fisheries and Marine Resource Technology Discipline and Professor **Md. Mahbubur Rahman, *Ph.D.*** Biotechnology and Genetics Engineering Discipline, Khulna University towards completion of my thesis.

I would also like to express my heartiest gratitude to my intimate friends Babu Kumar Roy and Md. Nazmul Hasan Zilani who have helped me in various ways to complete this study.

Finally, I am indebted to my beloved parents, for their immeasurable blessings, continuous inspiration and financial support throughout the study.

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October, 2015.

Abstract

Taxonomic ambiguity of mud crabs under genus *Scylla* available in the coastal regions of Bangladesh has become a major concern due to lack of proper identification of specific species. An attempt was made to resolve this taxonomic ambiguity for the first time in Bangladesh considering genetic analysis followed by external morphology and morphometric characters. A total of 371 samples were collected randomly from seven representative stations. The sampling was done two times in a month from July to December, 2014. An effective PCR-Restriction Fragment Length Polymorphism (RFLP) method was developed based on targeted sequence of 12S rRNA gene by digestion with three restriction endonucleases (*Aan*I, *Hind*III and *Dra*I) to identify specific species of mud crab. The results clearly indicate the existence of having only one species of mud crab under genus *Scylla* along the coastal regions of Bangladesh and is identified as *Scylla olivacea*.

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List of Abbreviations

Abbreviation	Elaboration
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
bp	Base pair
ng	Nano gram
µl	Micro liter
mg	Mili gram
ml	Mili liter
mM	Mili mole
PCR	Polymerase chain reaction
rpm	Rotation per minute
pM	Pico mole
DNTPs	Deoxynucleotide triphosphates
mtDNA	Mitochondrial deoxyribonucleic acid
UV	Ultra violet
w/v	Weight per volume
mg	Mili gram
RFLP	Restriction fragment length polymorphism
TAE	Tris acetate ethylenediaminetetraacetic acid

Chapter 1

Introduction

1. Introduction

1.1 Background of the study

Mud crab (genus *Scylla* de Haan, 1833; Crustacea: Decapoda: Brachyura: Portunidae) is a usual inhabitants of mangrove areas, brackish or coastal waters along the shorelines, ponds and intertidal swamps in the coastal regions (Hoq 2008; Marichamy and Rajapackian 2001; Chiou and Huang, 2003) and is a fast growing exportable commodity of Bangladesh. The coastal ecosystem of Bangladesh including its canals, rivers, estuaries and the coastline of Bay of Bengal provides muddy substrate, which is significant as habitat, shelter and nursery ground for commercially important mud crab species (Overton and Macintosh 1997; Acharya and Kamal, 1994; Shafi and Quddus, 1982).

The taxonomic classification of genus *Scylla* was controversial worldwide for long time. Estampador (1949) studied external morphology and gametogenesis of mud crabs collected from Philippine and classified those specimens into three species and one subspecies, namely *Scylla serrata*, *S. oceanica*, *S. tranquebarica* and *S. serrata* var. *paramamosain*. Serene (1952) categorized mud crab into two species from four forms in Vietnam. Stephenson and Campbell (1960) suggested only a single species name 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 characters. Keenan et al. (1998) extensively revised the taxonomy of 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*. Afterwards, several researchers dealt with mud crab around the world considering either morphology (Macintosh et al. 2002; Sangthong and Jondeung 2006; Jirapunpipat et al. 2008; Ogawa et al. 2012) or genetic approaches (Imai and Numachi 2002, Imai et al. 2004, Ma et al. 2012; Mandal et al. 2014).

In general, people used to consider *Scylla serrata* as a common mud crab species in Bangladesh. When people think or talk about mud crab, a common species i.e. *S. serrata* come into their thought and do not even think of other species names under the genus *Scylla*. Such as the department of forest in Bangladesh, responsible authority for crab fisheries management in Sundarban Reserve Forest (SRF) publishes their annual report every year where single name *Scylla serrata* is used. A large numbers of researchers (Khan and Alam 1992; Azam et al. 1998; Zafar et al. 2006; Hoq 2008) used the name *S. serrata* and a few

researchers (Kamal et al. 2004; Kamal et al. 2007) used *S. olivacea* in their studies. It is anticipated that most of the researchers used these names without prior identification of the specific species. Keenan et al. (1998) also addressed that *Scylla olivacea* is the species that is most of the time identified as *Scylla serrata* by many researchers. Various taxonomic studies, however, revealed ambiguity in the taxonomy and identification of *Scylla* species around the world (Joel and Raj 1980; Keenan et al. 1998; Padate et al. 2013). A three days International seminar-workshop on 'mud crab aquaculture and fisheries management' was held in India on April, 2013. The workshop was also highlighted the ambiguity in identifying the specific mud crab species in Asian countries. Food and Agriculture Organization (FAO) in Bangladesh even showed their concern regarding the identification of mud crab species in the coastal regions of Bangladesh.

Various researches regarding mud crab had been carried out in Bangladesh. The researches include taxonomy (Shafi and Quddus 1982), 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), production, marketing and transportation (Kamal et al. 2004), culture and bio-economics (Khan and Alam 1992; Ferdoushi and Xiang-Guo 2010) of mud crab. Detailed taxonomic study however, yet not conducted to identify and address the specific *Scylla* species available in Bangladesh. Present study therefore, aimed to identify and address the specific *Scylla* species available in the coastal regions of Bangladesh through PCR-Restriction Fragment Length Polymorphism (RFLP) method based on 12S rDNA marker combined with the morphological and morphometric features.

1.2 Specific objectives of the study

The intended aim of the research is to investigate the possibility of having various species under genus *Scylla* and to identify the specific species that exists in coastal zone of Bangladesh. To achieve that aim following are the specific objectives to be accomplished;

- To determine the morphological characters and morphometric features of the existing specimen.
- Finally, PCR-RFLP method based on 12S rDNA marker will be taken to ensure the specific species.

Chapter 2

Review of Literature

2. Review of Literature

This chapter reviews the past studies and remarks of experts having relevance to this investigation. Majority of the literature presented in this chapter was directly related with mud crabs' lifespan, habitat, distribution, behavior, taxonomy and identification process of the *Scylla* species.

2.1 Age, size and lifespan

This large crab can exceed 18 cm in carapace width (Eldredge and Smith 2001). The FAO species identification and data program suggests a maximum male carapace width of 25-28 cm and a maximum weight of 2-3 kg.

2.2 Size at which juvenile mud crabs recognized in the field

Heasman (1980) and Knuckey (1996) found that *S. serrata*, maturity occurs between 90 and 110 mm and all crabs are mature at 120 mm in Australia. Keenan et al. (1998) stated that smaller sizes at maturity have been noted in the literature; there may be problems with species identification and leading towards some misreporting of species. Based on aquaculture work it appears that at 50 mm it is relatively simple to discriminate juvenile *S. serrata* from other crab species and by 80 mm to sex individuals. Work done on other *Scylla* species (*S. paramamosain*) has shown that males and females may recruit to the fishery at different sizes and there is massive variability in the time it takes them to reach maturity (Moser et al. 2004) and the size at maturity (Walton et al. 2006).

2.3 Size based habitat distribution

Several researchers identified that juveniles were evident in the intertidal zone ranging from mangrove communities to the sea-grass and algal beds. The findings from Hill et al. (1982) demonstrated that there appeared to be size and spatial distribution of juvenile mud crabs (<100 mm), and -

- Over a two year period over 300 juvenile crabs in the size range 20 to 80 mm were collected under tiles placed in the intertidal zone with a large component found on large intertidal flats

- In warmer months over 900 crabs, mainly between 80 to 130 mm, were caught using nets with an average 19 crabs each set
- The majority of crabs caught in pots inter-tidally were sub adult.

Chandrasekaran and Natarajan (1994) worked in south east India on seasonal abundance and distribution of *S. serrata*, showed that the greatest catch of juvenile mud crabs was found in the post monsoon period, in muddy substrates in shallower water with areas of rich sea-grass and algal community, more so than areas of mangrove roots. Walton et al. (2006) used a range of methods to identify settlement of *Scylla sp.* (mainly, *S. paramamosain*) and ascertain the significance of mangrove and mudflats as nursery grounds for mud crab.

Webley and Connolly (2007) worked on vertical movement of *S. serrata* megalopa, in response to light, showed that megalopa tended to adopt a benthic habit and may therefore not be found in surface waters. The literature showed little result for the extensive amount of work undertaken in trying to catch juvenile *S. serrata*. However, in most literature it was consistent that juveniles are expected to be most commonly found in-

- muddy areas near the mouths of estuaries
- near shore areas rich in seagrass, algal communities and macrophytes
- areas that have pneumatophores or other forms of shelter.

2.4 Seasonal abundance of mud crab

Seasonal abundance of genus *Scylla* from various published literature is shown in Table 1.

Table 1. Month based abundance of mud crab genus *Scylla* found around the world.

Species	Month of abundance	Area	References
Scylla serrata	June - September	Lawele Bay, Indonesia	Sara 2010
Scylla olivacea	April - June	Setiu Wetland Areas, Malaysia	Zaidi et al. (2011)

S. serrata population in Lawele bay distributed throughout a year, the relative abundance of adult and sub adult are considered for major peaks in the month of July; where the juvenile as minor peaks in the month of February (Sara 2010).

2.5 Time of recruitment

The literature was assessed with a view to determining whether there was a clear seasonal timing when juvenile crabs may be more abundant. Most authors based their work on the premise that juveniles would be more prevalent during the post wet period. A summary of time of recruitment is presented in Table 2.

Table 2. Peak recruitment month of *Scylla* species.

Name of Juvenile	Zone & Country	Peak recruitment months		References
Scylla serrata	Back waters, India	May to October		Kathirvel and Srinivassagam 1992
	Mangrove, India	January to February		Kathirvel and Srinivassagam 1992
	Lawele Bay,	January to February		Sara 2010
	Chakaria Sundarban and Moheskhiand Kutubdia channel,	Male	May to August	Zafar et al. (2006)
		Female	May(pre wet) & August to	

Most of the researchers indicated that the recruitment pattern and abundance depends on the tidal steam, current, nutrient or food potential or seasonal variation. Some of them are highlighted right below:

1. During the post monsoon period, there is evidence that greater number of juvenile recruitment occurs but zero or no evidence at monsoon (Chandrasekaran and Natarajan 1994)
2. The period of spawning becomes more prominent with decreasing latitude (Walton et al. 2006)
3. There are three peaks of zoeal profusion (commencement of dry, dry season and wet) but peaks were predictable due to the periods of high nutrient and food concentration related to monsoon (Sara 2010)
4. Most authors believed the majority of juveniles would be prevalent during the post wet period
5. During monsoon no juveniles are caught.

6. Young crabs of *S. olivacea* enter into the population twice in a year, correlated to monsoon activity (Zaidi et al. 2011)
7. Positive current is necessary for successful recruitment to the nursery ground (Moser et al. 2004)
8. It is supposed that favorable tidal stream may be significant for larval dispersion, settlement and consequently recruitment of juvenile (Sara et al. 2006)
9. There was no evidence of seasonal influx in *S. serrata* (there may be a continuous recruitment to the fishery in other *Scylla* species)
10. It is believed that spawning periods become more pronounced with decreasing latitude (this is possibly related to water quality and monsoonal influences)
11. Few or no juveniles were caught during the monsoon
12. The majority of juveniles would appear to be caught in the post wet
13. Due to the low numbers caught, there is really no real clear cycle identified for *S. serrata.*

2.6 Response to physical and chemical variables

In many instances researchers believed that a range of chemical or physical cues may stimulate distribution and recruitment. By identifying these variables, the distribution and timing of recruitment to the fishery could possibly be determined. Adult and sub-adult *Scylla serrata* are broadly eurythermal, while larvae exhibit a somewhat narrower tolerance. It was reported that an impressive tolerance range of 3-45°C for *Scylla serrata* in the Karnafully River estuary, Bangladesh was found. In Bangladesh, successful pen culture of the species could be carried out in waters that ranged seasonally between 25°C and 36°C. Hill (1974), however, noted considerable larval mortality at temperatures above 25°C. The author also reported larval tolerance of temperatures as low as 5°C, although individuals become inactive below 10°C. *S. serrata* are considered to be less tolerable of freshwater than other *Scylla sp.* (Walton et al. 2006, Moser et al. 2004) with some juveniles of other species able to tolerate salinities as low as 2 ppt, but generally preferring around 34 ppt. Based on work undertaken in South Africa (Hill 1974) growth was faster at salinities of 20 to 22%. Freshwater run may be an important cue to allow juveniles to identify and locate estuary systems (Robertson 1996). Chandrasekaran and Natarajan (1994) found that after the monsoon juveniles moved into areas as salinity increased and reestablished populations. Hill (1974) showed that larvae could not tolerate water temperatures above 25^0C in South Africa, but in northern Australia,

the water temperatures often exceed that. From the references it was unclear what physical or chemical cues may drive recruitment, settlement and distribution of juvenile crabs, but the following were considered as possibilities;

- Increasing salinities
- Freshwater run
- Depth or pressure
- Chemicals (sediment traces, mangroves etc.)
- Temperature.

2.7 Length based maturation and differentiation

1. The first step of maturation in male *Scylla serrata* occurs when they reach to the carapace width (CW) of 90-110 mm
2. In between 140-160 mm of carapace width (CW), male *Scylla serrata* develops their characteristics "large claw"
3. The male and female differential shape of abdomen can be used to determine the sex of *Scylla serrata* over the carapace width (CW) of at 30 mm. Various Australian crab studies say that maturation of *Scylla serrata* occurs in between 90-110 mm of CW.
4. Darwin aquaculture center's crab research states that it is very easy to differentiate juvenile *Scylla serrata* from other *Scylla* species at 50 mm and sex individuals at 80 mm
5. Recent studies in northern Australia have shown that the transition of immature crabs to physiological maturity probably occurs between 90-110 mm carapace widths (Knuckey 1996).

2.8 Taxonomic study

Keenan et al. (1998) found considerable confusion regarding the taxonomy of species in the genus *Scylla*, commonly known as mud or mangrove crabs. To resolve this confusion they collected material from the red sea (the original type of locality of *S. serrata*), and from many other locations throughout the Indo-Pacific. According to genetic and morphological data of that study he confirmed that there are species of mud crab found all over the world, recognized as *S. serrata* (Forskal 1775), *S. olivacea* (Herbst 1796), *S. tranquebarica*

(Fabricius 1798) and *S. paramamosain* (Estampador 1949). According to morphological study of Keenan et al. (1998):

Species - *S. serrata* (Forskal 1775)

Diagnosis - frontal lobe spines high (mean height c. 0.06 times frontal with measured between medial orbital suture), bluntly pointed with tendency to concave margins and rounded interspaces. Anterolateral carapace spines narrow, with outer margin straight or slightly concave. Carpus of chelipeds with two obvious spines on distal half of outer margin, palm of cheliped with a pair of distinct spines on dorsal margin behind insertion of the dactyl. Chelipeds and legs all with polygonal patterning of both sexes on abdomen of female only. Male first gonopod as in Fig. 3a. Color variable from purple through green to browny/black depending on habitat. Means and standard deviations of the most important morphological ratios for species discrimination are presented in Table 3.

Remarks - *S. serrata* can be separated from its congeners using the characters presented in Tables 3 and 4 and Figs. 1 and 2.

Habitat - Associated with mangrove forests inundated with full salinity oceanic water for the greatest part of the year and can tolerate reduced salinity.

Distribution - samples have been positively identified for the study from a wide range of locations in the Indo-West Pacific. Indian Ocean: Red Sea; Richard's Bay; South Africa; Mauritius; Broome; Western Australia; the Arafura Sea; Darwin, Northern territory; Gulf of Carpentaria; and Kupang, Timor, Indonesia. From the Pacific Ocean: Fiji; Solomon Islands; New Caledonia; Western Samoa; Panay Island, Philippines; Okinawa, Japan; Southern Taiwan; and the east coast of Australia.

Table 3. Means and standard deviations of the three most useful morphological ratios for discriminating between the four species of mud crab (source: Keenan et al. 2008).

Species	ICS/OCS	FMSH/FW	FW/ICW
S. serrata	0.940±0.233	0.061±0.010	0.371±0.016
S. tranquebarica	0.980±0.251	0.043±0.006	0.412±0.016
S. paramamosain	0.352±0.235	0.058±0.012	0.377±0.007
S. olivacea	0.006±0.035	0.029±0.005	0.415±0.017

Table 4. Morphological characters useful in determining species identify of adult mud crabs (source: Keenan et al. 2008).

Species	Frontal lobe spine		Chelipeds	
	shape	**height**	**Carpus spine**	**Propodus spine**
S. serrata	blunt point	high	both obvious	obvious
S. tranquebarica	blunted	moderate	both obvious	obvious
S. paramamosain	triangular	moderately high	inner absent, outer reduced	obvious
S. olivacea	rounded	low	inner absent, outer reduced	reduced

This is the most widespread *Scylla* species. It occurs naturally throughout the Indo-Pacific, from South Africa to Tahiti, north to Okinawa and south to Port hacking in Australia, and the Bay of Islands, New Zealand. There is also a report of the species from the South Atlantic Ocean off Brazil, although there is no evidence that it has no viable population.

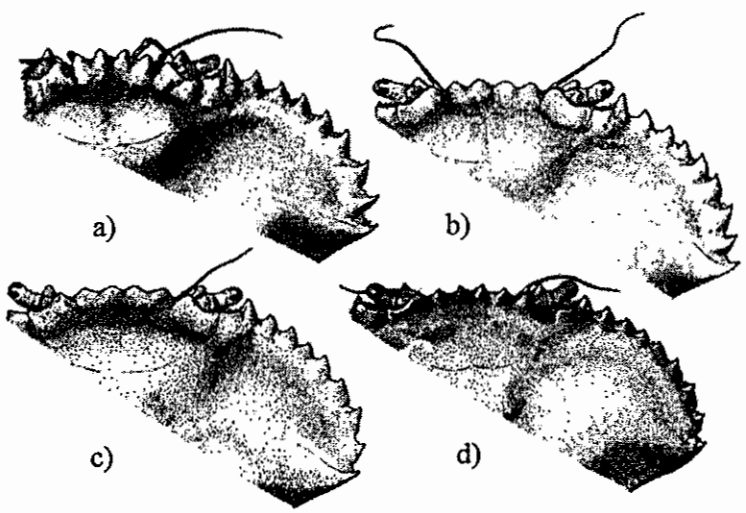

Fig. 1. Drawing of part carapace of adult male *Scylla* species showing diagnostic taxonomic features; a) *S. serrata* b) *S. tranquebarica* c) *S. paramamosain* d) *S. olivacea* (source: Keenan et al. 2008).

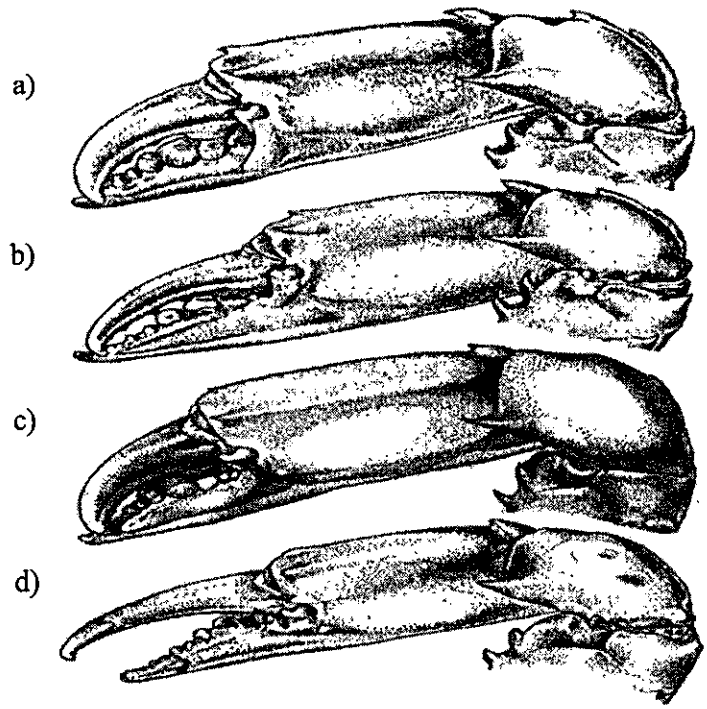

Fig. 2. Drawing of right cheliped adult male *Scylla* species showing diagnostic taxonomic features; a) *S. serrata* b) *S. tranquebarica* c) *S. paramamosain* d) *S. olivacea* (source: Keenan et al. 2008).

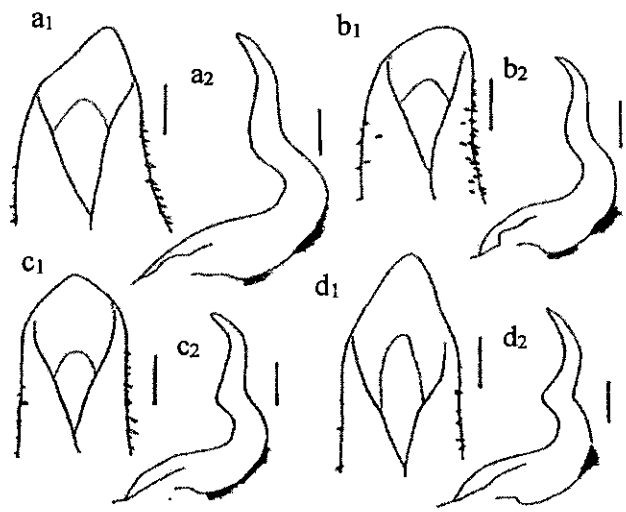

Fig. 3. Drawing of left gonopods of adult male *Scylla* species; a) *S. serrata* b) *S. tranquebarica* c) *S. paramamosain* d) *S. olivacea*; showing (1) detail of tip, (2) whole view. Scale (1) = 0.5mm; (2) = 5.0mm (source: Keenan et al. 2008)

Species - *Scylla tranquebarica* (Fabricius 1798)

Diagnosis - Frontal lobe spines of moderate height (mean height c. 0.04 times frontal width measured between medial orbital sutures) blunted with rounded interspaces. Anterolateral carapace spines broad, with outer margin convex. Carpus of chelipeds with two obvious spines on distal half of outer margin, palm of cheliped with a pair of distinct spines on dorsal margin behind insertion of the dactyl. Polygonal patterning weak on chelipeds and first two pairs of leg; last two pairs of legs with stronger patterning for both sexes; patterning variable on abdomen of female, absent on male. First male gonopod as in Fig 3b. Color variable, similar to *S. serrata*. Means and standard deviations of the most important morphological ratios for species discrimination are presented in Table 3.

Remarks - *S. tranquebarica* can be separated from its congeners using the characters presented in Tables 3 and 4 and Figs. 1 and 2. Following our lectotype designation this species corresponds with the *Scylla tranquebarica.*

Habitat - Associated with mangrove forests and coastlines inundated with reduced salinity seawater for part of the year.

Distribution - A widespread species of *Scylla*, *S. tranquebarica* is commonly found from the South China Sea but also occurs in specific locations around the Indo-Pacific. It is often associated with *S. olivacea.*

Samples have been positively identified for this study from the Indian Ocean: Karachi, Pakistan; Penang, Malaysia. From the Pacific Ocean; Panay Island, Philippines. From the South China Sea: Sarawak, Malaysia; Singapore.

Species - *Scylla paramamosain* (Estampador 1949)

Diagnosis - Frontal lobe spines high (mean height c. 0.06 times frontal width measured between medial orbital sutures), typically triangular with straight margins and angular interspaces. Anterolateral carapace spines broad, with outer margin convex. Carpus of chelipeds with one small blunt prominence (spinous in juveniles) ventro-medially on outer margin. On juveniles, reduced second spine may be present dorso-distally. Palm of chelipoeds with a pair of distinct spines on dorsal margin behind insertion of the dactyl, followed by ridges running posteriorly. Chelipeds and legs with weak polygonal patterning

for both sexes. Male first gonopod as in Fig. 3c. Color varies from purple through green to browny/black depending on habitat. Means and standard deviations of the most important morphological ratios for species discrimination are presented in Table 3.

Remarks - *S. paramamosain* can be separated from its congeners using the characters presented in Tables 3 and 4 and Figs.1 and 2.

Habitat - Associated with shallow denuded coral (reef rubble) in Singapore, shallow subtidal flats and estuarine ponds in central java; and in mangrove forests in the Lower Mekong Delta, Vietnam.

Distribution - Samples have been positively identified for this study from the South China Sea: Xiamen, China; Hong kong; Beihai, South China; Kaohsing, South Taiwan; Labrador Beach, Singapore; and Trat, Cambodia. Also from the Java sea: Banjarmasin, South Kalimantan; and Central Java, Indonesia.

Species - *Scylla olivacea* (Herbst 1796)

Diagnosis - Frontal lobe spines low (mean height c. 0.03 times frontal width measured between medial orbital sutures) rounded with shallow interspaces. Anterolateral carapace spines broad, with outer convex. Carpus of chelipeds with usually one small blunt prominence (may be spinous in juveniles) ventro-medially on outer margin; reduced second spine may be present dorso-distally in juvenile and young adults. Palm of the chelipeds usually with a pair of blunt prominences on dorsal margin behind insertion of dactyl, inner larger than outer; may be spinous in juvenile and young adults. Chelipeds, legs and abdomen all without obvious polygonal patterning for both sexes. Male first gonopod as in Fig. 3d. Color varies from red through brown to browny/black depending on habitat. Means and standard deviations of the most important morphological ratios for species discrimination are presented in Table 3.

Remarks - *S. olivacea* can be separated from its congeners using the characters presented in Tables 3 and 4 and Figs.1 and 2. This is the species that has most often been identified as *S. serrata.*

Habitat - Associated with mangrove forests and coastlines inundated with reduced salinity seawater during the wet season. In Australia, distribution is limited to embayment where

salinity is reduced e.g. Albatross Bay, off Weipa, North-Western Queensland, and King Sound, Derby, Western Australia..

Distribution - Samples have been positively identified for the study from the Indian Ocean: King Sound, Western Australia; Phuket, Thailand; Karachi, Pakistan. From the Pacific Ocean: Panay Island, Mindanao Island, Negros Island, Philippines. From the South China Sea: Bangkok, Thailand; Singapore; Vietnam; Sarawak, Malaysia; Beihai, Southern China; Kaohsiong, Southern Taiwan. From the Arafura Sea: Kupang, Timor, Indonesia; Weipa, Gulf of Carpentaria. A moderate widespread species of *Scylla*, *S. olivacea* is commonly found from the South China Sea, but also occurs in specific locations across the Indo-West Pacific. It is often associated with *S. tranquebarica.*

2.9 Morphological and morphometric study

Jirapunpipat et al. (2008) conducted a similar research to distinguish mud crabs genus *Scylla* found the coastal area of Thailand. In that study samples of the genus *Scylla* were collected from Klong Ngao mangrove, Ranong Province, Thailand, for identification purposes and to determine the most useful characters for discriminating between the mud crab species. A morphological study with the use of a key to the species of *Scylla* indicated that four species can be recognized from the area: *Scylla olivacea*, *S. paramamosain*, *S. tranquebarica*, and *S. serrata.* The results supported that the ratio of the inner carpus spine (ICS) and outer propodus spine (OCS) was the main character to distinguish *S. olivacea* and *S. paramamosain* from *S. tranquebarica* and *S. serrata.* However, it was difficult to apply only morphometric data to identify the species of *Scylla* in Klong Ngao. In that study, the phenotypic characteristics as well as the color of the carapace and chelae of the crab specimens were recommended as good characters to distinguish the four species. In particular, the coloration and pattern of chelae and swimming legs were especially useful in discriminating between adult *S. tranquebarica* and *S. serrata.*

Padate et al. (2013) found that taxonomic study on mud crabs (*Scylla*) in India reveal considerable ambiguity in species identification owing to overlap of morphological characters. That study revealed a new record of *S. olivacea* along with a comparative diagnosis with *S. serrata* from the region. Further, minor phenotypic variations in the present *S. olivacea* specimens could be attributed to geographical isolation from existing populations.

3.0 Genetic study

Keenan et al. (1998) employed two independent genetic methods, allozyme electrophoresis and sequencing of two mitochondrial DNA genes, cytochrome oxidase I (COI) and 16S rRNA gene to discriminate species. The genetic data showed that there are at least four distinct *Scylla* species.

Mandal et al. (2014) worked with a title named 'Molecular Markers Reveal Only Two Mud Crab Species of Genus *Scylla* (Brachyura: Portunidae) in Indian Coastal Water'. They investigated the taxonomic ambiguity of the Indian mud crab (genus *Scylla* de Hann 1833). they made the first attempt to resolve the taxonomic uncertainty of the mud crab commonly available in Indian coastal waters using first internal transcribed spacer (ITS-1) and sequencing of cytochrome oxidase subunit I (COI) gene following with morphometric study.

Ma et al. (2012) also conducted a research on molecular identification of genus *Scylla* based on DNA barcoding and polymerase chain reaction. In that study, they first examined the utility of COI sequences as DNA barcoding for identification of genus *Scylla*. They found that the mean intraspecific Kimura 2 parameter (K2P) distances were 0.003 for *S. paramamosain*, 0.014 for *S. serrata*, 0.017 for *S. olivacea* and 0.006 for *S. tranquebarica*. The interspecific K2P distances were higher than the intraspecific distances: the minimum interspecific distance (0.092) was between *S. paramamosain* and *S. tranquebarica* while the maximum interspecific distance (0.196) was between *S. paramamosain* and *S. olivacea*.

According to Imai et al. (2004) the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA and mitochondrial DNA of 16S rRNA were amplified by polymerase chain reaction (PCR) using genomic DNA extracted from adult tissue of four species of *Scylla* spp. and the first zoeal stages of *S. serrata, S. paramamosain* and *S. olivacea* as template. Using the ITS-1 region, variation in product fragment length was found to be useful for distinguishing *S. serrata* and *S. Olivacea* from two other species. The other two species (*S. paramamosain and S. tranquebarica*) could be identified using the restriction endonuclease *Hha*I. Using 16S rDNA, all four species were identified by double digestion with *Dra*I and *Hind*III.

Chapter 3

Materials and Methods

3. Materials and Methods

3.1 Sample collection

Wild mud crab samples were collected from 8 major locations in coastal zone of Bangladesh, covering all the areas previously reported. Three hundred and seventy one (371) fresh wild samples were obtained directly from commercial crab collectors. Sampling was done from various locations of western, mid and eastern regions along the coast of Bay of Bengal (Fig. 4). Western region includes Mongla (22.3°N, 89.3°E; n=33), Sharankhola (22.2°N, 89.4°E; n=27), Shamnagar (22.2°N, 89.1°E; n=58) and Paikgacha (22.3°N, 89.2°E; n=32); four different places adjacent to Sundarban mangrove forest. Mid region includes Noakhali (22.2°N, 91.0°E; n=66) and Patuakhali (22.1°N, 90.3°E, n=52). Eastern region comprises Cox's Bazar (21.3°N, 92.0°E; n=56) and Chakoria Sunderban (22.1°N, 92.0°E; n=47). Sampling was done two times in a month for each station from July to December, 2014. The collected samples were preserved temporarily in ice. Soon after an exhaustive morphological and morphometric study was carried out in the laboratory of Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna.

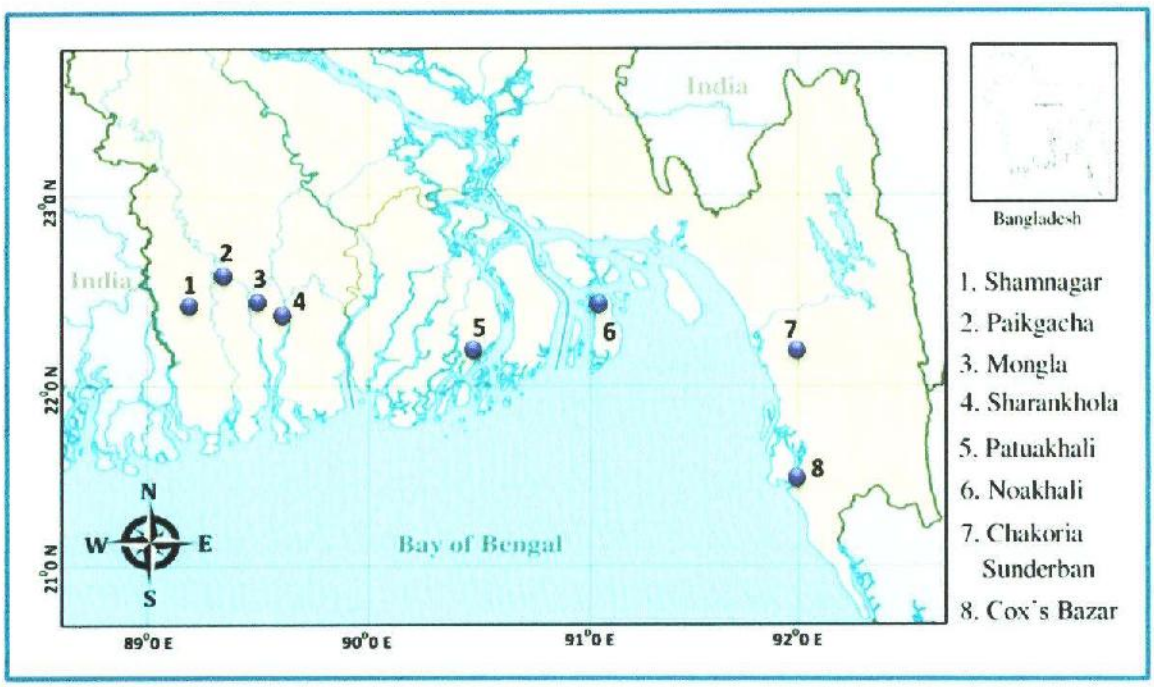

Fig 4. Sampling locations in the coastal zone of Bangladesh.

3.2 Morphological analysis

Three hundred and seventy one (371) male and female mud crabs were considered for this study. The carpus spine shape and number was considered for the preliminary identification process of our study. Phenotypic features such as color of carapace and chelipeds, frontal lobe spine height, polygonal patterning, and male's first gonopod were observed and identified on the basis of standard key mentioned by Keenan et al. (1998), Estampador (1949) and Jirapunpipat et al. (2008) to distinguish the species of *Scylla*.

3.3 Morphometric analysis

Only large male mud crabs (carapace length>8.0 cm) with right appendages were considered for this study to preclude the chances of confounding the influence of juvenile ontogenetic changes. Twenty three (23) morphometric characters from carapace, frontal spines, chelipeds, pereiopods and sternum were measured by using digital calipers to the nearest 0.01 mm. Each measurement was taken for three times and the averages were considered in this study. Subsequently, twenty seven (27) morphometric ratios were also calculated as described by Keenan et al. (1998). One way ANOVA was conducted to address the comparison of morphometric ratios among three sampling regions. ANOVA was conducted at 0.1% significance level. Student's t-test was also carried out to decide the compliance of the observed values of morphometric measures with the mean of respective measures mentioned by Keenan et al. (1998) and Jirapunpipat et al., (2008). The observed data was transformed into square root format for improving the normality of the data and the analysis was conducted at 1% significance level.

3.4 Genetic analysis

3.4.1 Extraction of genomic DNA from mud crab samples

- ✓ An amount of 25-30 mg of adult crab leg tissue was dissected from periopod using sterile blade and placed into 1.5ml sterile microcentrifuge tube
- ✓ 500 µl of lysis buffer (GeneReach Biotecnology Corp., Taiwan) was added to the tube, ensuring the tissue was completely immersed in the buffer and the sample was homogenized with a disposable grinder

- ✓ After homogenization, the tube was incubated at 65^0C in a heat block for 20 minutes with occasional vortexing
- ✓ The lysate was centrifuged at a maximum speed of 12000 rpm for 5 minutes.
- ✓ About 350 µl of clear supernatant was transferred to a new sterile 1.5 ml microcentrifuge tube
- ✓ 700 µl (two times of the supernatant) of absolute ethanol was added to the tube. Sample was slowly mixed by inversion and was kept at room temperature for 4-5 minutes
- ✓ The tube was centrifuged at 12000 rpm for 3 minutes and the ethanol was decanted
- ✓ 500 µl of 70% ethanol was added into the tube and centrifuged at 8000 rpm for 5 minutes. Finally, ethanol was removed by pipetting or decanting carefully
- ✓ DNA pellet was air dried briefly by keeping tubes open at room temperature for 7-10 minutes
- ✓ 100 µl of distilled water was inserted into the tube for the solubilisation of the DNA precipitate by passing slowly through a pipette tip
- ✓ Samples were run on a mini gel to test for the presence of high molecular DNA mass
- ✓ Finally, those samples were stored in freezer at -20°C until PCR was performed.

3.4.2 Quantification of genomic DNA

The quantity of DNA isolated from mud crab tissue samples was assessed by using a UV Spectrophotometer (U 2910, spectrophotometer, Hitachi, Japan) at 260 nm. A vortex shaker for approximately 30 minutes before measurements was taken to confirm the complete sample homogeneity that is critical when measuring genomic DNA concentration with this instrument. The following protocol outlines the steps involved in measuring DNA concentration.

At first the spectrophotometer is turn on and set the wavelength to 260 nm. Transfer 2000 µl of 10mM Tris-Cl, pH 8.0 to each of the two quartz cuvettes. Insert the cuvettes into the spectrophotometer and zero the instrument. Remove one cuvette and discard Tris-Cl solution. Then add 20 µl of the DNA sample and 1980 µl of 10mM Tris-Cl in this cuvette. Mix well the sample by inverting several times. Place cuvette in the spectrophotometer and record the absorbance at 260 nm. For calculation of the concentration of RNA free DNA, the following

conversion factor is used: 1unit OD260 = 50 mg of DNA/ml. DNA concentration in ng/µl was calculated as follows;

$$\text{DNA Concentration (ng/µl)} = OD_{260} \times A_{260} \times \text{Dilution Factor}$$

3.4.3 Primer design

A pair of primers were designed from partial sequence of 12S rRNA gene of mitochondrial DNA (mtDNA). GenBank database of 12S rRNA gene sequence for four mud crab species: *S. serrata* (accession no. FJ827758), *S. paramamosain* (accession no. FJ827761), *S. tranquebarica* (accession no. FJ827759), and *S. olivacea* (accession no. FJ827760) were considered to select specific primer set. The sequences of the primers used and their sizes are presented in Table 5.

Table 5. The nucleotide sequence of primer with their size and melting point.

Nucleotide sequence of primer	M.T (°C)	Size (bp)
12S rRNA F: 5'-TCCAGGCTCACTTTCCAGTA-3'	57.0	20
12S rRNA R: 5'-GCAACTGCTGGCACAATATTAATTAGG-3'	57.0	27

F=Forward; **R**=Reverse; **M.T**=Melting Temperature; **bp** = Base pair.

3.4.4 DNA amplification and detection

The specific sequence of 12S rRNA of mitochondrial DNA (mtDNA) was amplified using the following PCR technique. PCR reaction was performed on a Thermal Cycler (BIO-RAD, series C1000^{TM}, USA). The selected region was amplified in a 25µl reaction mixture volume containing 1 µl of template DNA, 2.5µl each of 10 pM primers, 0.65µl of 10 mM dNTPs, 2.5µl of 5X Go Taq® reaction buffer (with $MgCl_2$) and 0.25 units of Go Taq® DNA polymerase (Go Taq®,USA)and 18.1 µl of nuclease free water. Thirty two PCR cycles were carried out under the following conditions: denaturation at 95^{0}C (45 s), annealing at 58^{0}C (35 s) and extension at 72^{0}C (45 s). The PCR reactions were started after initial denaturation at 95^{0}C (1 min) and stopped after a final extension at 72^{0}C (5 min). The PCR products were separated on 1.5% agarose gels to check the success of the PCR reaction. A 2µl of 100 bp DNA ladder was used to specify the position of amplified PCR bands. PCR conditions to amplify specific length of 12S rRNA gene is given in Fig. 5.

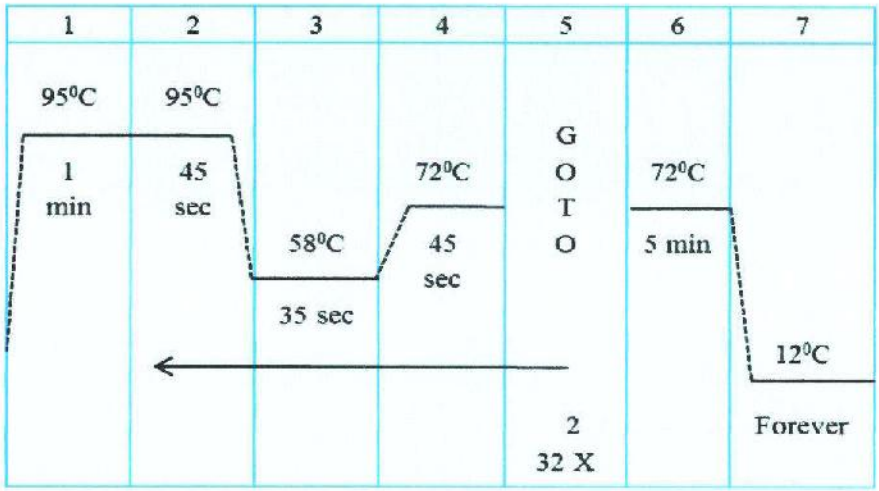

Fig. 5. PCR conditions to synthesize species specific sequence of 12S rRNA gene.

3.4.4 Restriction endonuclease digestion

Three restriction endonucleases, *Hind*III, *Dra*I, and *Aan*I (Thermo-scientific, USA) were selected from restriction sites on the GenBank database of 12S rRNA sequences of the four species. Preparation of restriction endonuclease mixture for ten (10) samples were made by mixing 1 µl of restriction endonuclease, 10 µl of enzyme buffer and 90 µl of nuclease free water. After PCR amplification, 10 µl of the PCR products were digested with 10 µl of restriction endonuclease through incubating at 37°C for overnight. The name of three restriction endonucleases, sites of digestion and length of the expected fragments on digestion are shown in Tables 6 and 7.

Table 6. Name of three restriction endonucleases with its sites of digestion.

Restriction endonucleases	Sites of digestion
***Hind*III**	5'…A^AGCT_T…3'
***Dra*I**	5'…TTT_^AAA…3'
***Aan*I**	5'…TTA_^ATT…3'

Table 7. The restriction endonucleases, its cutting sites and length of the expected fragments on digestion to identify specific mud crab species.

Species with amplified product size (bp)	**Cutting site and expected fragments**	**Restriction endonucleases**		
		***Hind*III**	***Aan*I**	***Dra*I**
S. serrata	*Cutting site*	315	-	415, 472
(670 bp)	*Fragments*	315, 355	-	57, 198, 415
S. tranquebarica	*Cutting site*	-	518	154, 347, 413, 471, 535
(665 bp)	*Fragments*	-	147, 518	58, 64, 66, 130, 154, 193
S. olivacea	*Cutting site*	104, 308	-	154, 408, 467
(652 bp)	*Fragments*	104, 204, 344	-	59, 154, 185, 254
S. paramamosain	*Cutting site*	-	579	154, 348, 414, 472, 534
(665 bp)	*Fragments*	-	86, 579	58, 62, 66, 133, 154, 192

Chapter 4

Results

4. Results

4.1 Morphological Study

Mud crab specimens were initially examined on the basis of morphological key characters suggested by Keenan et al. (1998) are mentioned below.

4.1.1 Key to *Scylla* species as mentioned by Keenen et al. (1998)

1. Carpus of chelipeds with two obvious spines on distal half of outer margin..... 2
- Carpus of chelipeds without two obvious spines on distal half of outer margin.....3

2. Frontal lobe spines high (mean height c. 0.06 times frontal width measured between medial orbital sutures), bluntly pointed with tendency to concave margins and rounded interspaces. Anterolateral carapace spines narrow, with outer margin straight or slightly concave. Chelipeds and legs all with polygonal patterning for both sexes and on abdomen of female only. ..... *Scylla serrata*
- Frontal lobe spines of moderate height (mean height c. 0.04 times frontal width measured between medial orbital sutures), blunted with rounded interspaces. Anterolateral carapace spines broad, with outer margin convex. Polygonal patterning weak on chelipeds and first two pairs of legs; last two pairs of legs with stronger patterning for both sexes; patterning variable on abdomen of female, absent on male. ..... *Scylla tranquebarica*

3. Frontal lobe spines high (mean height c. 0.06 times frontal width measured between medial orbital sutures), typically triangular with straight margins and angular interspaces. Palm of cheliped with a pair of distinct spines on dorsal margin behind insertion of the dactyl, followed by ridges running posteriorly. Chelipeds and legs with weak polygonal patterning for both sexes. ..... *Scylla paramamosain*
- Frontal lobe spines low (mean height c. 0.03 times frontal width measured between medial orbital sutures), rounded with shallow interspaces. Palm of chelipeds usually with a pair of blunt prominences on dorsal margin behind insertion of the dactyl, inner larger than outer; may be spinous in juveniles and young adults. Chelipeds, legs and abdomen all without obvious polygonal patterning for both sexes.....*Scylla olivacea*

Noticeably, *S. tranquebarica* and *S. paramamosain* were not reported from India (Mandal et al. 2014) and *S. tranquebarica* was rarely available in Thailand (Jirapunpipat et al. 2008). This study was therefore, attempted to focus on *S. serrata* and *S. olivacea* recorded in Thailand and India considering the geographical proximity of *Scylla* species distribution.

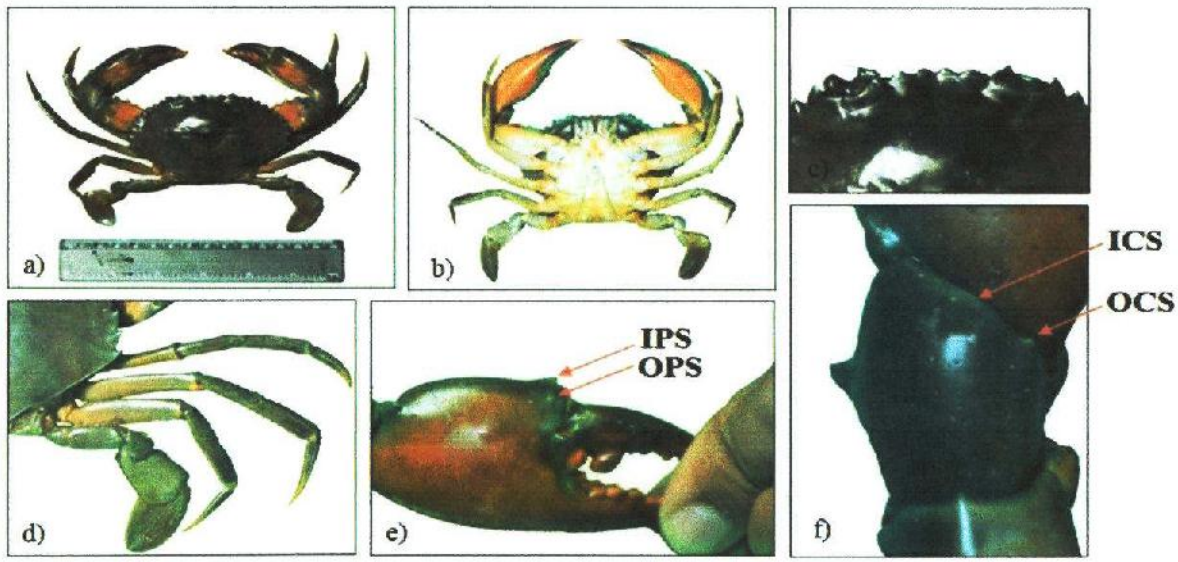

Fig. 6. Photographs of male *Scylla* species observed in the present study; a) Dorsal view; b) abdomen; c) Frontal margin; d) Swimming legs *(without polygonal patterning)*; e) Propodus spine *(reduced)* and (f) Carpus spine *(ICS absent and OCS reduced).*

4.1.2 Morphological characters of the specimen observed in the present study

Carapace smooth with prominent transversal ridges, broader than long and with strong H-shaped groove on the gastric zone. Carapace color varied from the greenish brown to brownish green on the surface. Ventral surface of male abdomen cream colored with light brown shade (Figs. 6a and 6b). Transverse bands with dark to light green or brownish color present on female abdomen (Fig. 6b). Antero-lateral margin broader than postero-lateral margin and divided into nine similar sized broad spines projected outwards obliquely. Frontal margin with rounded spines and shallow interspaces (Figs. 6a and 6c). Periopods flattened into swimming appendages. Among four pairs of periopods, first three pairs similar and fourth pair natatorial (Fig. 6d). Chelipeds smooth, robust and longer than legs. Three and two well-developed spines present on anterior and posterior margin of merus respectively. Carpus contained a short blunt spine on the outer margin and the inner margin devoid of spine (Fig. 6f). Propodus of chelipeds with a

prominent spine on inner margin and a small blunt spine on outer margin (Fig. 6e). Polygonal patterning on carapace, abdomen, chelipeds and periopods absent in adult (Figs. 6a, 6d and 6e). Sometimes juvenile and sub adult display light cream colored patterning on the surface or epibranchial region of carapace chelipeds and periopods. Transverse bands with dark to light green or brownish color present on female abdomen without polygonal marking (Fig. 7b). Distal tip of first gonopod bluntly pointed, long and slender with convex outer margin (Fig. 8b).

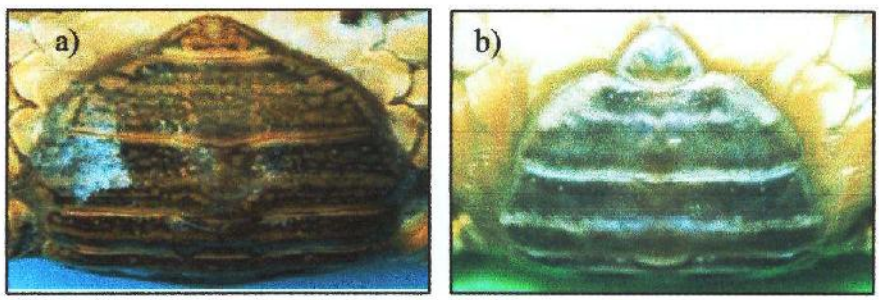

Fig 7. Female gonopod; a) *S. serrata* (source: Jirapunpipat et al. (2008)) b) *S. olivacea* (present study).

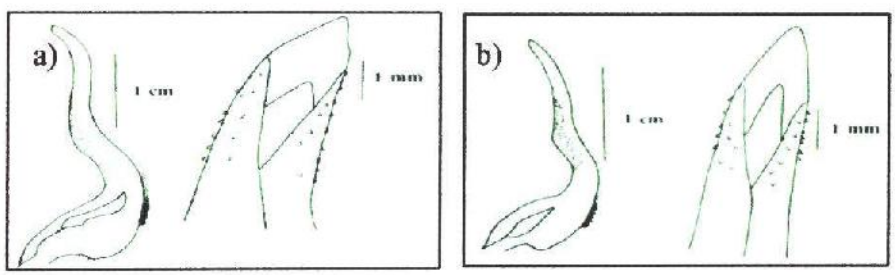

Fig. 8. Male's first gonopod; a) *S. serrata* b) *S. olivacea* (Source: Jirapunpipat et. al. 2008).

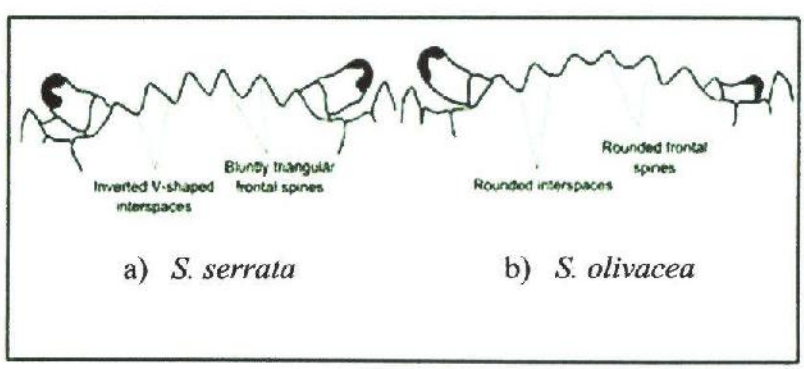

Fig. 9. Frontal margin of carapace; a) *Scylla serrata;* (b) *Scylla olivacea* (Source: Padate et al. 2013).

4.2 Morphometric study

Twenty seven (27) morphometric ratios followed by twenty three (23) morphometric measurements were measured according to Keenan et al. (1998). Positions of the various measured points are presented in Fig. 10.

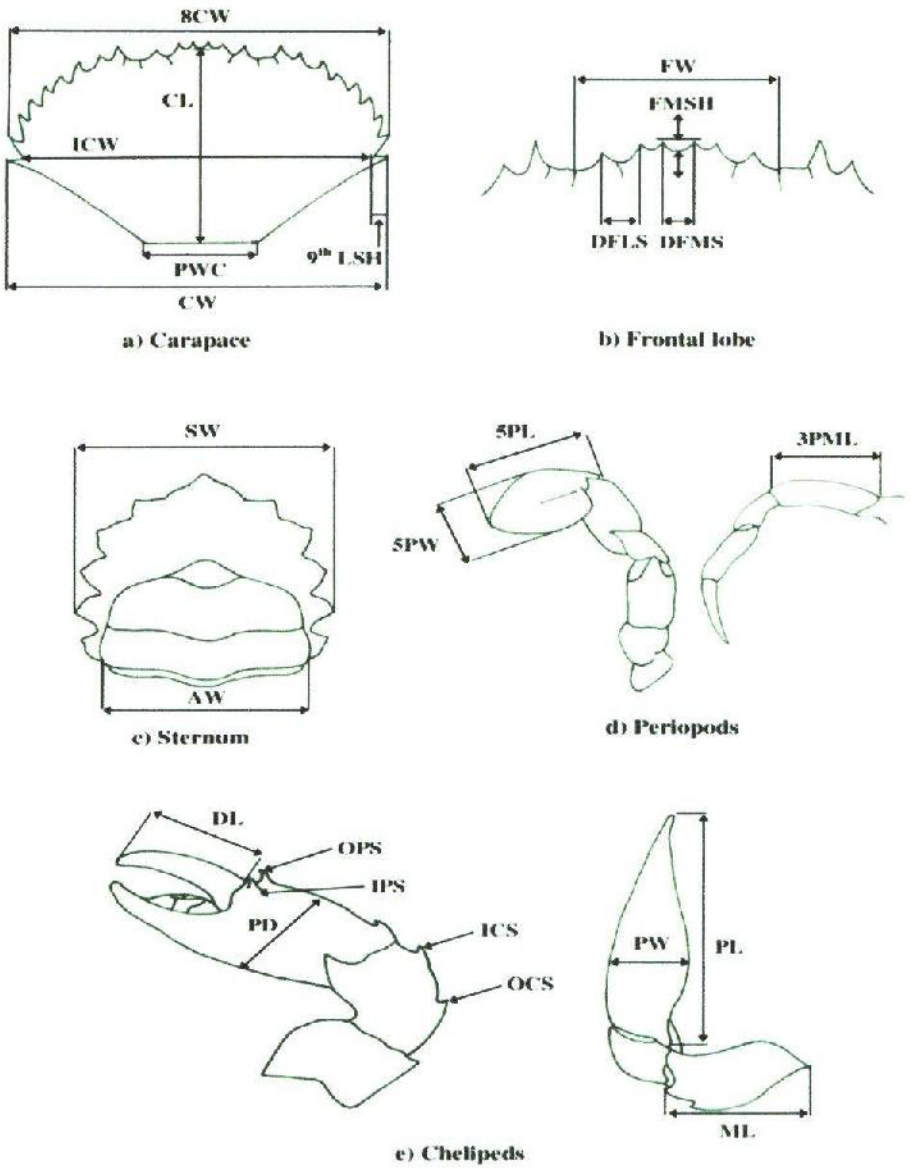

Fig. 10. Position of the morphometric measurement points in; a) Carapace, b) Frontal lobe, c) Sternum, d) Periopods and e) Chelipeds. (Abbreviations used on figures: ICW, internal carapace width; CW, carapace width; 8CW, carapace width at 8th spine; CL, carapace length; PWC, carapace posterior width; LSH, 9th lateral spine height; FMSH, frontal median spine height; DFMS, distance between frontal median spines; DFLS, distance between frontal lateral spines; FW, carapace frontal width; AW, abdomen width; SW, sternum width; DL, dactyl length; ML, merus length; PL, propodus length; PW, propodus width; PD, propodus depth; IPS, inner propodus spine; OPS, outer propodus spine; ICS, inner carpus spine; OCS, outer carpus spine; 5PW, 5th periopod dactyl width; 5PL, 5th periopod dactyl length; and 3PML, 3rd periopod merus length).

Keenan et al. (1998) emphasized on seven morphometric ratios which were considered as significant in differentiation under the four species of genus *Scylla* collected from indo pacific and red sea. These ratios were ICS/OCS, FMSH/ICW, ML/PL, AW/SW, PL/ICW, FW/ICW and IPS/PL. But finally he concluded with three ratios i.e. ICS/OCS, FMSH/FW and FW/ICW as key ratios to differentiate *Scylla* species. Jirapunpipat et al. (2008) were considered five ratios to distinguish *Scylla* species in Thailand. These were ICS/OCS, PWC/FW, OPS/PL, DFMS/FW and LSH/ICW. The present study addressed seven important morphometric ratios i.e. LSH/ICW, FW/ICW, PWC/FW, DFMS/FW, OPS/PL, ICS/OCS, FMSH/FW considering the above mentioned two references. Mean, standard deviation and range of those morphometric ratios obtained from the present observation was compared with the respective ratios for *S. serrata* and *S. olivacea* mentioned in Keenan et al. (1998) and are presented in Table 8.

Table 8. Comparison of morphometric ratios of mud crab found in the present study with ratios mentioned in Keenan et al. (1998).

Morphometric ratio		*Scylla serrata*	*Scylla olivacea*	Present study
LSH/ICW	Mean±SD	0.031±0.006	0.022±0.005	0.021±0.004
	Range	0.018-0.046	0.015-0.037	0.014-0.032
FW/ICW	Mean±SD	0.371±0.016	0.415±0.017	0.444±0.011
	Range	0.335-0.406	0.371-0.451	0.418-0.464
PWC/FW	Mean±SD	0.892±0.075	0.762±0.059	0.706±0.025
	Range	0.724-1.058	0.671-0.928	0.649-0.758
DFMS/FW	Mean±SD	0.145±0.009	0.130±0.012	0.116±0.007
	Range	0.127-0.165	0.112-0.165	0.103-0.138
OPS/PL	Mean±SD	0.019±0.006	0.006±0.005	0.004±0.001
	Range	0.009-0.033	0.000-0.018	0.001-0.007
ICS/OCS	Mean±SD	0.940±0.233	0.006±0.035	0.024±0.052
	Range	0.500-1.583	0.000-0.250	0.000-0.201
FMSH/FW	Mean±SD	0.061±0.010	0.029±0.005	0.025±0.005
	Range	0.041-0.095	0.018-0.037	0.017-0.034

The mean and range data of the morphometric ratios for the specimens in present study was complied with the respective mean and range of morphometric ratios mentioned by Keenan et al. (1998) for addressing more clear compliance level among the species. Median, quartile and range of the seven major morphometric ratios for the species observed in the present study and *Scylla* species from Thailand reported in Jirapunpipat et al. (2008) is presented in Fig. 11 using box plot diagram.

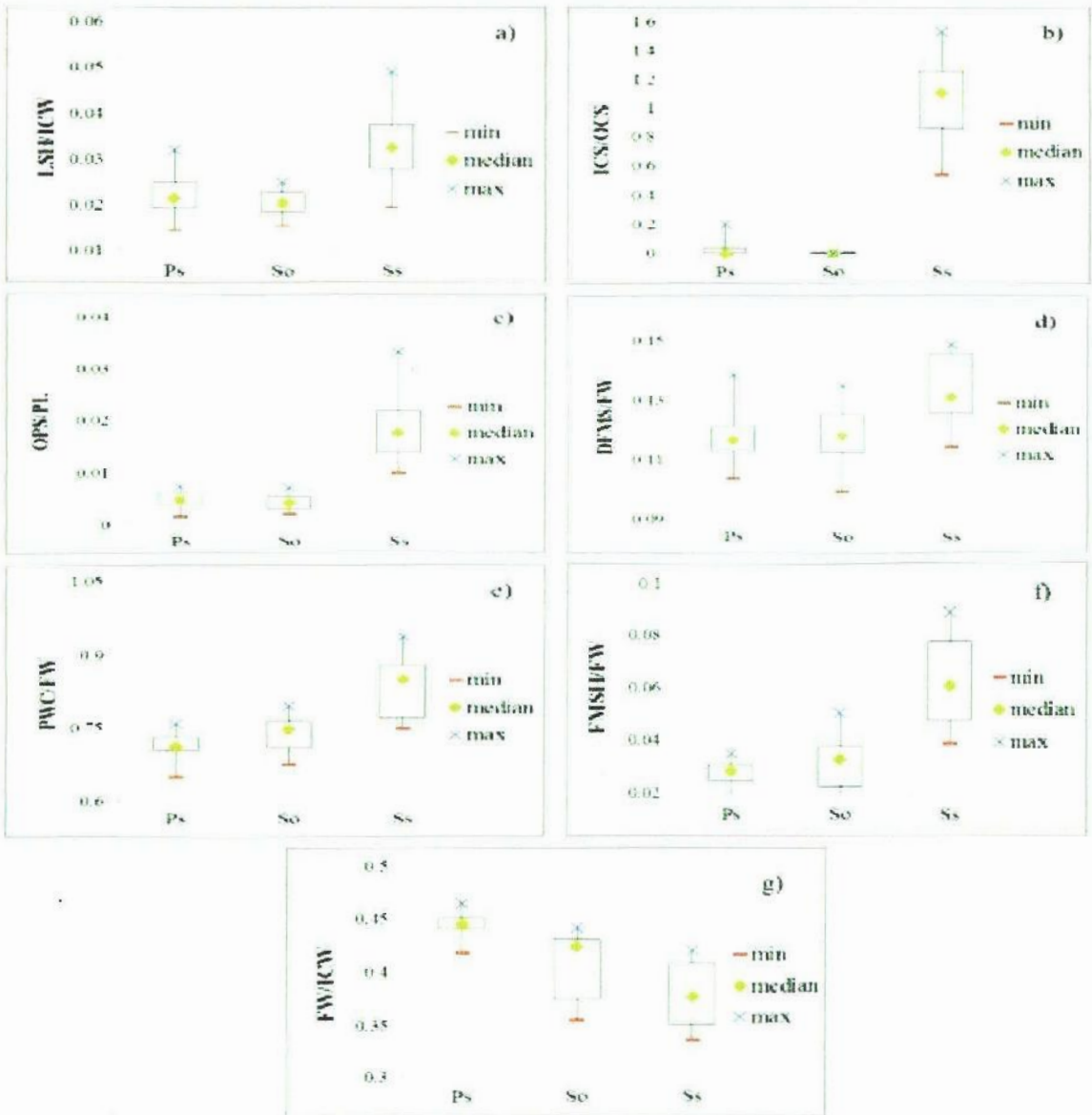

Fig. 11. Comparison of morphometric ratios of mud crab found in the present study (Ps) with the respective ratios mentioned in Jirapunpipat et al. (2008) from *S. olivacea* (So) and *S. serrata* (Ss). Here, triangle indicates median, lower and upper line shows 1st and 3rd quartile respectively and shaded box and cross represents maximum and minimum value respectively.

Morphometric ratios such as LSH/ICW, ICS/OCS, OPS/PL, DFMS/FW, FMSH/FW and PWC/FW of present study showed positive compliance with *S. olivacea* reported from Thailand. As a result, *Scylla* species of the present study complied with *S. olivacea* instead of *S. serrata.*

4.2.1 One way ANOVA

To determine the possibility of having differentiation of morphometric ratios between the sampling stations, one way ANOVA was carried out and the results are depicted in Table 9.

Table 9. Comparison between the crab samples collected from three sampling regions following selective morphometric ratios.

Ratios		Degree of freedom	F value	Significance
LSH/ICW	Between Groups	2	2.184	0.133
	Within Groups	297		
	Total	299		
FW/ICW	Between Groups	2	0.118	0.889
	Within Groups	297		
	Total	299		
PWC/FW	Between Groups	2	2.143	0.138
	Within Groups	297		
	Total	299		
DFMS/FW	Between Groups	2	1.241	0.306
	Within Groups	297		
	Total	299		
OPS/PL	Between Groups	2	2.068	0.147
	Within Groups	297		
	Total	299		
ICS/OCS	Between Groups	2	0.421	0.661
	Within Groups	297		
	Total	299		
FMSH/FW	Between Groups	2	12.023	0.000
	Within Groups	297		
	Total	299		

Significance level 0.001

The data considered in this study provide sufficient evidence to show that there was no significant difference ($P \geq 0.001$) among the samples collected from three stations considering the morphometric ratios like LSH/ICW, FW/ICW, PWC/FW, DFMS/FW, OPS/PL, and ICS/OCS except FMSH/FW. Therefore, it may conclude that the mud crab samples collected from different coastal regions were a single species.

4.2.2 Student's t-test

Student's t-test was carried out to compare the morphometric ratios obtained from present study with the mean of respective ratios reported in previous works. Measurements of thirty samples were taken randomly from the total collected samples for the test. Comparison of the mean of selective ratios presented in Keenan et al. (1998) and Jirapunpipat et al. (2008) with the observed value of respective ratios are shown in Tables 10 and 11.

Table 10. Comparison of the observed value with the mean of selective ratios provided by Keenan et al. (1998).

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
OPS/PL	0.019	-10.306	0.000	.006	-0.223	0.825
ICS/OCS	0.94	-25.173	0.000	.006	1.503	0.144

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

Table 11. Comparison of the observed value with the mean of selective ratios provided by Jirapunpipat et al. (2008).

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	0.13	-9.930	0.000	.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

The student's t-test (Tables 10 and 11) suggested significant differences ($p < 0.01$) between *S. serrata* recorded in Keenan et al. (1998) and Jirapunpipat et al. (2008) and our observation. Similar significant differences ($p < 0.01$) was also noticed with *S. paramamosain* 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 identically different from *S. serrata* and was similar to *S. olivacea*.

4.3 Genetic study

4.3.1 Genomic DNA extraction

Genomic DNA was extracted from ~25 mg of leg tissue from each specimen selected for morphometric study. Extracted DNA was tested by electrophoresis through a 1% agarose gel containing a nonmutagenic fluorescent DNA dye (0.1µl/ml; Ethidium Bromide) to analyse the yield and quality. Genomic DNA sample gel electrophoresis (1% agarose) is shown in Fig. 12.

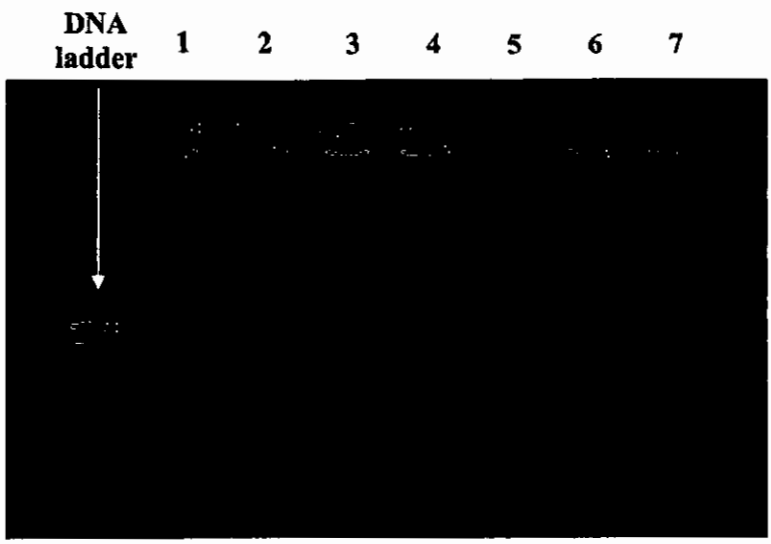

Fig. 12. Gel electrophoresis (1% agarose) for genomic DNA samples of mud crab. (DNA Sample no. 1 to 7)

4.3.2 PCR product amplification and detection

The targeted sequence of 12S rRNA gene was amplified by using the appropriate pair of primers and other PCR reaction program parameters. The obtained PCR product size was

visualized in 1.5% agarose gel (Fig. 13) and compared with 100 bp DNA ladder (Bioneer, USA). After comparing with the molecular marker, it was observed that in 652 bp band was amplified for every sample.

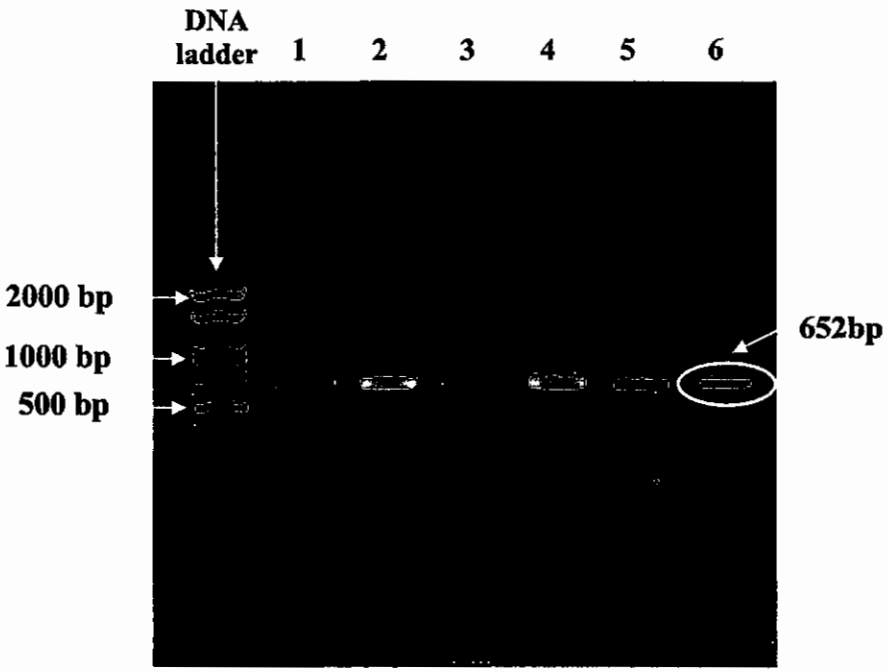

Fig. 13. PCR product of targeted sequence of 12S rRNA 1.5% agarose gel, Lane 1, 100 bp Molecular marker, lane 2-6, PCR amplified product.

4.3.3 Restriction endonuclease digestion and detection

The PCR amplified products were digested with three restriction endonucleases i.e. *Hind*III, *Dra*I and *Aan*I (Thermo-scientific, USA) and it was observed that the *Aan*I showed no digestion (Fig. 14). Here, digestion with *Hind*III produced three individual bands (104 bp, 204 bp and 344 bp) and digestion with *Dra*I showed four individual bands (59 bp, 154 bp, 185 bp, 244 bp) for each sample (Fig. 14). Digested fragments were separated using 3% agarose gel (Fig. 14).

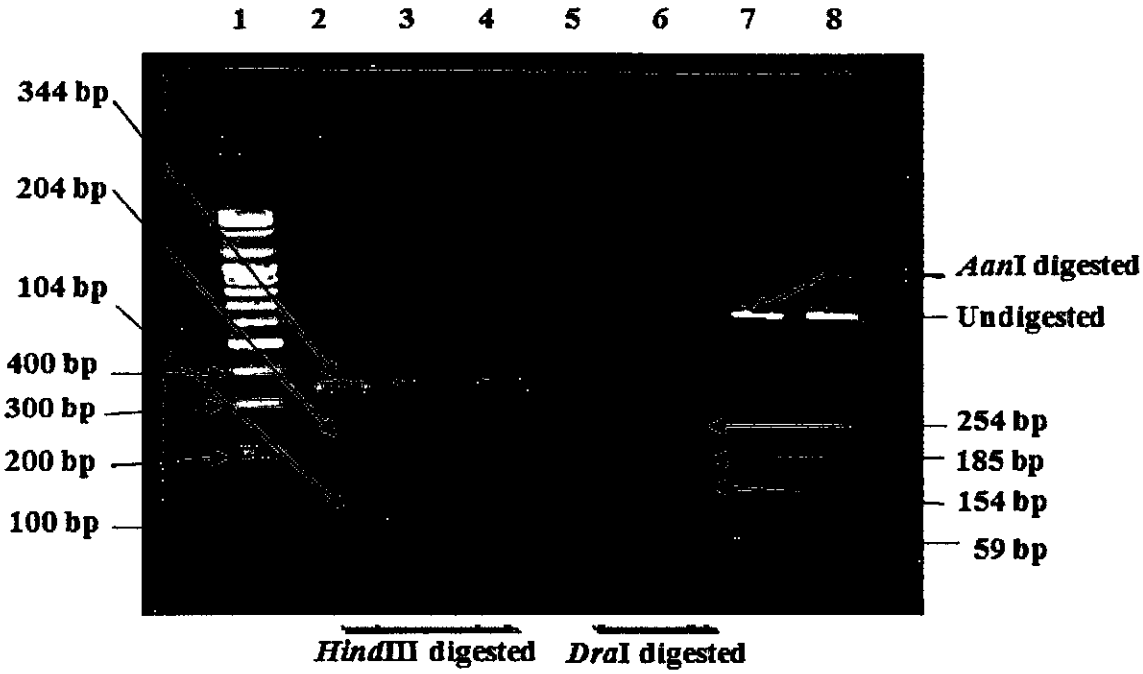

Fig. 14. Restriction endonuclease digestion in 3% agarose gel. Lane 1, DNA ladder; lane 2-4, digestion with *Hind*III, lane 5-6, digestion with *Dra*I, lane 7, digestion with *Aan*I and lane 8, undigested PCR product.

Chapter 5

Discussion

5. Discussion

Carapace color of the mud crabs observed in this study was brownish to brownish green which was identical with *S. olivacea.* However, Keenan et al. (1998) addressed that the color of carapace may vary within the same species due to habitat differences and is not considered as 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. serrate* (Fig. 9). Polygonal patterning on chelipeds and periopods were very noteworthy distinguishing character because adult *S. serrata* revealed strong pattern on carapace, chelipeds and periopods whereas *S. olivacea* was devoid of such patterning (Jirapunpipat et al. 2008); specimen considered in this study showed no polygonal patterning on carapace, chelipeds and periopods. The long and narrow apex of male first gonopod is significantly distinctive in *S. olivacea* which was observed in collected specimens. *S. olivacea* possess brownish red color just below the apex in fresh specimen which was reported by Joel and Raj (1980). From the comparative morphological characters mentioned in the Table 1 as well as Key to *Scylla* species as mentioned by Keenen et al. (1998), it can be anticipated that observed mud crab in present study mostly comply with *S. olivacea* instead of *S. serrata*. Distinguishing morphological characters among the specimens observed in present study and *Scylla* species particularly *S. serrata* and *S. olivacea* reported by Keenan et al. (1998) and Jirapunpipat et al. (2008) are presented in Table 12.

It was noticed that some morphological characters of *S. olivacea* particularly at juvenile and sub adult stages displayed partially distinct character than the adult. *S. olivacea* at juvenile stage exhibited olive green or darkish green coloration with indistinct polygonal marking on the surface of epibranchial regions of carapace and chelipeds while at adult stage exhibited dark brown coloration without any polygonal marking. Frontal lobe spine height was quiet higher in juvenile than adult. *S. olivacea* had obvious spine on outer margin and a small projection on inner margin of carpus in juvenile but spine on inner margin was absent in sub-adult and adult stages. *S. olivacea* showed obvious spines on propodus in juvenile and reduce as it become sub adult or adult. These variations of morphological characters of at different stages of *S. olivacea* can mislead to identify the specific species.

Table 12. Comparison of morphological characters in identifying adult mud crab.

Morphological characters		***Scylla serrata***	***Scylla olivacea***	**Present study**
Carapace color*		dark green/ or grayish green	rusty brown or dark brown	brownish to brownish green, olive
Chelae color*		blue green to dark green	red brown to dark brown	red brown with greenish shade on upper side
Polygonal patterning*		strong polygonal patterning present on carapace, chelipeds and peripods	absent	absent
Male's first gonopod		distal portion, comparatively broader	distal portion, comparatively narrower	distal portion narrower
Frontal lobe spine pattern **	**Shape**	bluntly pointed	rounded	rounded
	Height	High	low	low
Cheliped Pattern **	**Carpus spines**	both obvious	inner absent outer reduced	inner absent outer reduced
	Propodus spines	Obvious	reduced	both present but reduced

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

From one way ANOVA, it was found that a single species is therefore, available in coastal water of Bangladesh. Keenan et al. (1998) mentioned that *S. serrata* is dominant in oceanic water where the surface salinity reaches above 34 ppt whereas the other species can be found in areas where salinity is less than 33ppt. Particularly, *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. Salinity in the coastal zone of Bangladesh, especially from where the crab samples were collected, ranges from 5 to 30 ppt (Hussain 2014 and Rahaman et al. 2013). Considering

the recommended salinity preference range, the mud crab samples collected from the study areas ought to be *S. olivacea*. Beside that *S. serrata* can be found off the coast where salinity level is above 35ppt. Several genetic approaches were made identify mud crab species under genus *Scylla*.

The desired fragment of 12S rRNA gene was successfully amplified for each sample with a length of 652 bp band (Fig. 6). PCR amplified product was purified using PCR product purification kit (Accuprep® PCR purification kit, Korea) to increase the concentration. As *Aan*I had cut site in the targeted sequence of *S. paramamosain* and *S. tranquebarica* and no cut site was present in the targeted sequence of *S. serrata* and *S. olivacea* (Table 2); digestion results with *Aan*I indicated that these specimens considered in this study would have either *S. olivacea* or *S. serrata* but neither *S. paramamosain* nor *S. tranquebarica*. For S. *serrata*, the digestion fragments of targeted PCR product should be 315 bp and 355 bp with *Hind*III and 57 bp, 198 bp and 415 bp with *Dra*I. No such fragments were found in this experiment when digestion was performed with *Hind*III and *Dra*I. These digested fragments length observed in this experiment were analogous to the fragment length of *Hind*III and *Dra*I respectively for *S. olivacea*. The digestion fragments pattern confirmed that the mud crab species that are available in the coastal water of Bangladesh is *Scylla olivacea*.

Chapter 7

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Annex 1

Standardized morphometric data of mud crab used for descriptive and inferential analysis (Keenan et al. 1998).

No.	Standardized morphometric data	Abbreviation
	Carapace data	
1	9th Lateral Spine Height/ Internal Carapace Width Where, LHS = (CW-ICW)/2	**LSH/ ICW**
2	Carapace Width/ Carapace Width at 8th Spine	**CW/8CW**
3	Carapace Length)/ Internal Carapace Width	**CL/ICW**
4	Carapace Posterior Width/ Internal Carapace Width	**PWC/ICW**
5	Carapace Frontal Width/ Internal Carapace Width	**FW/ICW**
6	Internal Carapace Width/ Carapace Frontal Width	**PWC/FW**
7	Frontal Median Spine Length/ Carapace Frontal Width	**FMSH/ FW**
8	Frontal Median Spine Length/ Distance Between Frontal Median Spine	**FMSH/DFMS**
9	Distance Between Frontal Median Spine/ Carapace Frontal Width	**DFMS/FW**
10	Distance Between Frontal Lateral Spine /Carapace Frontal Width	**DFLS/FW**
11	Distance Between Frontal Median Spine/ Distance Between Frontal Lateral Spine	**DFMS/DFLS**
12	Sternum Width/ Internal Carapace Width	**SW/ICW**
	Cheliped Data	
13	Abdomen Width /Sternum Width	**AW/SW**
14	Propodus Length/ Internal Carapace Width	**PL/ICW**
15	Dactyl Length/Propodus Length	**DL/PL**
16	Propodus Width /Propodus Length	**PW/PL**
17	Propodus Depth /Propodus Length	**PD/PL**
18	Propodus Width x Propodus Depth x0.7854/Propodus Length (0 = no spine)	**PWxPDx0.7854/ PL**
19	Inner Propodus Spine /Propodus Length	**IPS/PL**
20	Outer Propodus Spine /Propodus Length	**OPS/PL**
21	Inner Propodus Spine/Outer Propodus Spine	**IPS/OPS**
22	Inner Carpus Spine /Propodus Length	**ICS/PL**
23	Outer Carpus Spine /Propodus Length	**OCS/PL**
24	Inner Carpus Spine /Outer Carpus Spine	**ICS/OCS**
25	Merus Length/Propodus Length	**ML/PL**
	Pereiopod data	
26	5th Peripod Dactyl Width/5th Peripod Dactyl Length	**5PW/5PL**
27	3rd Peripod Merus Length /Internal Carapace Width	**3PML/ICW**

Annex 2

Visualization of PCR products

PCR amplification products were visualized by means of agarose gel electrophoresis in order to allow for size estimation and thus confirmation of amplification of the desired genomic target region. Molecular ruler (100 bp) was used for size estimation of PCR amplification products, which served as confirmation that amplification of the desired genomic target region had occurred, as well as for quantification of PCR products prior to restriction enzyme digestion reactions. All agarose and Polyacrylamide gels were visualized under ultraviolet (UV) light and photographed with a Gel Documentation and Analysis System.

Gel electrophoresis

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose gel electrophoresis of DNA was used to determine the presence and distinguish the type of nucleic acids obtained after extraction and to analyze digestion products. Desired DNA fragments can be physically isolated for various purposes such as sequencing, probe preparation, or for cloning fragments into other vectors. Agarose gels are used for DNA analysis. Typically, agarose gels are used for most purposes and polyacrylamide gels are used when small fragments, such as digests of 16S reran genes, are being distinguished. Regular agarose gels may range in concentration from 0.6 to 3.0%.

Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatine-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current.

Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA in the gel will be visualized by the use of Ethidium Bromide,

added to the gel. Ethidium bromide binds to DNA and illuminates when exposed to ultraviolet light, causing the DNA to 'glow'. All PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gel at 80 volts.

Agarose gel electrophoresis procedure

All agarose gels were made with and resolved in 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer, which was made and stored as a 10X stock solution and diluted to the required working concentration as was needed. In order to facilitate the visualization of DNA within the agarose gel under UV light, 1 µg of ethidium bromide (EtBr) per ml agarose solution was added -i.e. 0.01% EtBr stock solution (10 mg/ml).

A. Casting a gel

1. An appropriate volume of 1X Tris-acetate-EDTA (TAE) buffer with an appropriate amount of agarose (these values are determined based on the gel dimensions and the desired percentage of agarose) was mixed in a conical flask. The flask was swirled to evenly distribute the agarose.
2. The solution was then heated in the microwave oven for 1 minute. Protective gloves were worn and the flask was removed from the microwave oven (before it boiled over), swirled, and reheated while keeping constant watch to be sure it did not boil over. When it started to boil, boiling was stopped and swirled again repeating the process until all of the agarose went into solution.
3. The flask was allowed to cool. The gel was poured when the temperature of the solution was 55-65° C.
4. The gel apparatus was prepared for casting the gel while the agarose was cooling.
5. Prior to pouring the gel, Ethidium bromide was added to the dissolved agarose and swirled to mix.
6. The gel was poured into the casting tray and the comb was adjusted to keep the wells perpendicular. The gel was allowed to cool and was hardened (20-30 minutes) prior to use.

B. Preparing the gel for electrophoresis

1. A few ml of 1X TAE buffer was added to the well area of the gel and the comb was carefully removed by pulling straight up.
2. The electrophoresis tank was filled with buffer solution (1X TAE) and the gel was placed (In the casting tray) on the tank platform.

C. Preparing samples for loading/running the gel

1. An appropriate volume of loading dye (6X) was added to the sample (1 µl of 6X sampledye for every 5 µl of sample).
2. The sample was loaded using a 5µl micropipette. The marker was also loaded at Lane-1.
3. After the gel had been loaded, the cover was gently placed on the apparatus and the power leads were hooked up. The power was adjusted to 80 volts (constant voltage). The gel was run until the first dye front (bromophenol blue) had migrated about two-thirds the length of the gel and the second dye front (xylene cyanol) had migrated approximately one-third of the length of the gel.
4. The power was turned off before removing the gel for photographing.
5. The gel was placed on the UV transilluminator to visualize the DNA.