INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 10, No. 2, p. 1-15, 2017
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

On the systematics of genus *Scylla* De Haan,1833 of cochin backwaters, a South Indian estuary

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Article published on February 14, 2017

Key words: Systematics, *Scylla*, Mud crabs, Cochin Backwaters, Taxonomy

Abstract

The present work is an attempt to describe the *Scylla* spp collected from Cochin backwaters, a South Indian estuary, for a period of two years from June 2010- May 2012. Identification and description of *Scylla* spp. was carried out based on the conventional taxonomic tools viz., morphological characters including the description of the first and second male gonopods and the third maxillipeds; morphometry as well as the molecular methods viz., sequencing of CO1 gene and the amplification of ITS-1 region. The present study confirms the occurrence of two species of *Scylla,* from Cochin backwaters, namely *Scylla serrata and Scylla olivacea*. The study also rules out the existence of *Scylla tranquebarica* in Cochin backwaters. The smaller species being identified as *S. serrata* is *S. olivacea* and the larger one being identified as *S. tranquebarica* is *S. serrata*.

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Introduction

Mud crabs belonging to the genus *Scylla* inhabit brackish waters, such as estuaries and mangrove areas. They are widely distributed throughout the Pacific and Indian Ocean, from Tahiti, Australia and Japan to Southern Africa (Chhapgar, 1957; Hill, 1975; Sakai, 1976). In India, mud crabs are reported from the estuaries of the rivers of Ganga, Mahanadi, Krishna and Cauvery and the brackish water lakes viz., Chilka and Pulicut on the east coast, the estuaries of Narmada and Tapti and the brackish waters of Kerala on west coast. They are also found to inhabit the mangrove regions of Andaman and Nicobar Islands, Andhra Pradesh, Tamil Nadu and Kerala (Anil, 1997).

Mud crabs are commercially important due to their large size and taste. Hence, they are of great demand in the domestic as well as foreign market. India earns about US$ 18 million as foreign exchange from the export of live mud crabs (MPEDA, 2011). The export of live mud crabs from India to countries like Singapore, Malaysia and Hong Kong stimulated increased the exploitation of mud crabs from their natural habitats during the past few years. The attractive prices offered for live crabs in the export market encouraged farmers to culture mud crabs in some parts of India as practiced in a more organized manner in South East Asian countries (Suseelan *et al*., 1995).

Despite of the importance of mud crabs in both coastal aquaculture and artisanal fisheries, studies conducted on the Indian mud crab population is very scanty. In India, studies on mud crabs have been dealt with by Kathirvel (1981), Radhakrishnan and Samuel (1982), Joel and Sanjeevaraj (1983) and Kathirvel and Srinivasagam (1992). Kathirvel (1981) recorded two species of *Scylla* from Cochin backwaters, larger species being *Scylla oceanica* and smaller one being *Scylla serrata* based on the morphology. Later in 1982, Radhakrishnan and Samuel reported the occurrence of a subspecies *Scylla serrata serrata* from Cochin backwaters on the basis of morphological characters.

Joel and Sanjeevaraj (1983) studied the taxonomy of *Scylla* from Pulicut lake and reported the occurrence of two species, namely *S. tranquebarica* and *S. serrata*. Taxonomy of mud crabs from India were then critically analysed by Kathirvel and Srinivasagam (1992) and stated that two species are found to occur in Indian waters, *S. serrata* and *S. tranquebarica*, which are characterized by the differences in size, spines on the outer border of the carpus of the cheliped and habitat preferences. In contrast to their findings, Shaji *et al.* (2006) stated that *S. serrata* and *S. olivacea* are the most common species occurring in India, based on the taxonomic identification of Keenan *et al.* (1998).

Knowledge on taxonomy is important for the development of a more successful aquaculture industry and management of wild stock. There is a clear need to identify the mud crab species in India using molecular tools, as morphological diagnostic characteristics of mud crabs are rather weak or specific to life stages or sex. It is crucial to investigate which species is dominant in coastal aquaculture in India, as an incorrect name application can affect the success of aquaculture industry, and it can lead to the farming of a wrong species (Wowor and Ng, 2007), or utilizing the fund to develop a technology for the aquaculture of an economically or biologically unsuitable species (Balasubramanian *et al*., 2014).

Quite recently, Mandal *et al.* (2014 a) dealt with the taxonomic uncertainty of mud crabs commonly available in the Indian coastal waters using molecular genetic markers, ITS-1 and sequencing of COI gene combined with traditional morphometry. A similar study was conducted by Balasubramanian *et al.* (2014) on the basis of two mitochondrial genes, 16S rRNA and COI of *Scylla* populations collected from different locations along the Indian coast. Mandal *et al.* (2014 b) identified the *Scylla* species from the Indian waters using RAPD and PCR-RFLP markers.

The present work is an attempt to describe the *Scylla* spp from Cochin backwaters on the basis of morphological characters including the description of the male gonopods and the third maxillipeds, morphometry as well as molecular tools.

Materials and methods

Sample collection

The study was conducted on Cochin backwaters, a positive tropical estuarine system located at the south west region of the Indian sub continent. 15 sampling stations were fixed along the study area on the basis of salinity, habitat type, presence of mangroves and availability of crabs; namely Thanneermukkom, Vaikom, Aroor, Valanthakad, Kumbalanghi, Kandakkadavu, Thevara, Thoppumpady, Barmouth, Marine Science Jetty, Varappuzha, Vallarpadam, Vypin, Munambam and Azheekode (fig.1).

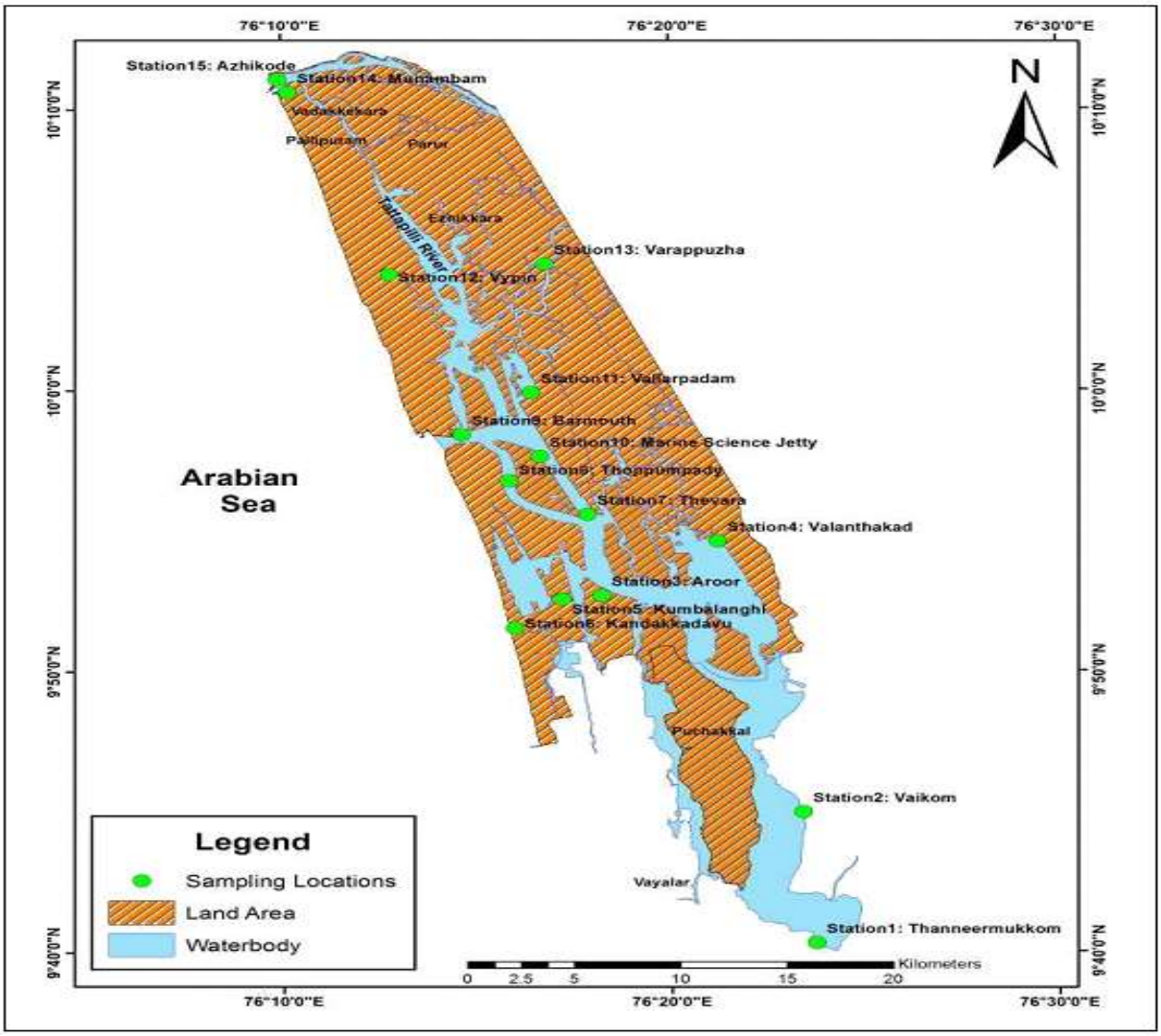


Fig. 1. Map showing the sampling sites in Cochin Backwaters.

Sampling of crabs was conducted monthly for a period of two years from June 2010 to May 2012.

Live mud crabs with a size range of 80 mm- 128 mm carapace width (100.8±10.17), collected from local market, local fishermen, Chinese dip net operators and other collectors were brought to the laboratory.

Morphological study

The morphological characters of the crabs such as the colour and shape of the carapace, shape of the frontal lobes, spination of the anterolateral borders and the chelae and the presence of hexagonal markings were noted. The crabs were then identified using the identification key provided by Keenan *et al*. (1998), which has been widely accepted by crab taxonomists as well as by FAO (Ng, 1998).

The specimens were immobilized by freezing and then preserved in 10% formaldehyde (Ajmal Khan and Ravichandran, 2009). The first and second pleopods from the male specimens with a size range of 95- 128 mm carapace width (110.94±10.2) and the third maxillipeds were cut carefully using a forceps and scissors and examined under a binocular microscope. The pleopods and the maxillipeds were then drawn using a camera lucida.

Morphometric study

24 morphometric characters (Fig.2) were recorded for 365 mud crabs in total (211 males and 154 females) using vernier calipers to the nearest 0.1mm and the characters were then size standardized through the creation of 27 ratios (Table.1) (Keenan *et al*., 1998).

Step wise Discriminant function analysis using SPSS ver. 20.0 was conducted using the 27 ratios to determine the characters that best discriminate the morphologically recognized species (Keenan *et al*., 1998).

For Discriminant Function Analysis, only adult crabs with an internal carapace width 95mm were used in order to avoid the juvenile ontogenic changes. Crabs with broken or missing appendages and spines were avoided to obtain a reliable data.

Molecular study

DNA isolation

Muscle tissue from the third thoracic appendage of live mud crabs were dissected and considered for DNA isolation. Total genomic DNA was isolated from 10 mg of muscle tissue. Tissue was digested by incubating with proteinase K/SDS solution at 37°C for two hours. DNA isolation was carried out following standard phenol: chloroform extraction and ethanol precipitation technique (Sambrook *et al*., 1989). Purity and quality of DNA was checked on 0.7% agarose gel. The concentration of dissolved DNA was estimated using UV spectrophotometer at 260 nm. DNA was diluted so as to obtain a final concentration of 75 ng/µl. (Mandal *et al*. 2014a).

Amplification of First Internal Transcriber Spacer (ITS-1) region

The ITS-1 region was amplified using the primer sets designed by Rajiv Gandhi Centre for Aquaculture and following the protocol described by Imai *et al*. (2004)

Amplification and sequencing of cytochrome oxidase c- oxidase sub unit I (COI) gene

PCR amplification was carried out to obtain sequences of the partial mitochondrial COI gene, in a total of 25 µl volume containing 1x standard Taq buffer (10 mMTris-HCl, 50 mMKCl, pH 8.3), 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer, 1U Taq DNA polymerase (Fermentas, Inc.) and 1µl DNA template (100ng). Amplification was done using Species specific primer (Keenan primer), designed by Central Genetics Lab, Rajiv Gandhi Centre for Aquaculture, Sirkazhi, Tamil Nadu. The thermal profile used was 94°C for 5 min followed by 35 cycles of 94 °C for 15 sec, 50°C for 30 sec and 72°C for 30 sec and a final extension at 72°C for 10 min. 10 µl of the amplified PCR product was analyzed by electrophoresis in 1.5 % agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light. PCR products were purified using Isopropanol precipitation technique and the purified PCR products were sequenced with CO1 primers using ABI Prism Sequencing kit (BigDye Terminator Cycle). The homologue searching of the nucleotide sequence (using blastn suite) was performed with the Basic Local Alignment Search Tool (BLAST) through National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/blast).

Results

Morphological Study

The initial examination of the morphological characters, viz., shape of the frontal lobes, spination of the carpus of the chelae and the pattern of hexagonal markings indicates the presence of three morphotypes of *Scylla* in the Cochin Backwaters, viz., *Scylla serrata*, *Scylla tranquebarica* and *Scylla olivacea* based on the identification key provided by Keenan *et al*. (1998), Devi and Joseph (2013).

The first morphotype , which is identified to be *S. serrata* exhibits pale green to olive green colouration and the dactylus of the chelae pale orange. H-shaped gastric groove is found to be deep. The frontal margin has bluntly pointed spines, with rounded interspaces. Anterolateral margin of the carapace has 9 narrow teeth. Chelipeds are large and sturdy with its propodus inflated.

Table. 1. Size standardized mud crab morphometric data used for discriminant function analysis.

	A. Carapace Data
1.	9th Lateral spine height (LSH)/ Internal Carapace width (ICW), where LSH = (CW/ICW)/2
2.	Carapace width (CW)/ Carapace width at 8th spine (8CW)
3.	Carapace length (CL)/ Internal Carapace width (ICW)
4.	Posterior carapace width (PCW)/ Internal Carapace width (ICW)
5.	Carapace Frontal Width (FW)/ Internal Carapace width (ICW)
6.	Posterior carapace width (PCW)/ Carapace Frontal Width (FW)
7.	Frontal median spine height (FMSH)/ Carapace Frontal Width (FW)
8.	Frontal median spine height (FMSH)/ Distance between frontal median spines (DFMS)
9.	Distance between frontal median spines (DFMS) / Carapace Frontal Width (FW)
10.	Distance between frontal lateral spines (DFLS) / Carapace Frontal Width (FW)
11.	Distance between frontal median spines (DFMS) / Distance between frontal lateral spines (DFLS)
12.	Sternam width (SW)/ Internal Carapace width (ICW)
13.	Abdomen width/ Sternam width
	B. Cheliped Data
14.	Propodus Length (PL)/ Internal Carapace width (ICW)
15.	Dactyl Length (DL)/ Propodus Length (PL)
16.	Propodus Width (PW)/ Propodus Length (PL)
17.	Propodus Depth (PD)/ Propodus Length (PL)
18.	(Propodus Width (PW)* Propodus Depth (PD) * 0.7854) / Propodus Length (PL)
19.	Inner propodus spine (IPS)/ Propodus Length (PL)
20.	Outer propodus spine (OPS)/ Propodus Length (PL)
21.	Inner propodus spine (IPS)/ Outer propodus spine (OPS)
22.	Inner carpus spine (ICS)/ Propodus Length (PL)
23.	Outer carpus spine (OCS)/ Propodus Length (PL)
24.	Inner carpus spine (ICS)/ Outer carpus spine (OCS)
25.	Merus Length (ML)/ Propodus Length (PL)
	C. Periopod Data
26.	5th periopod Dactyl width (5PW)/ 5th periopod Dactyl length (5PL)
27.	3rd periopod merus width (3PML)/ Internal carapace width (ICW)

The distinguishing character of the species is the presence of two prominent sharp spines on the distal half of outer margin of the carpus of the cheliped (fig.3). The limbs of the crabs exhibited conspicuous hexagonal patterning in both male and female. This is found to be the largest among the three *Scylla* species.

The second morphotype, which is assigned to be *S. tranquebarica* is grayish green in colour and the limbs have a pale orange tinge. The propodus of the chelae and the last segment of the paddle legs exhibited orange colouration. The frontal margin of the carapace has blunt spines, with round interspaces. Anterolateral margin of the carapace has 9 broad teeth. Chelipeds are large with propodus inflated. The carpus of the cheliped possesses two spines on the distal half of outer margin (fig.4). Hexagonal markings are weak and inconspicuous on the chelae and paddle legs.

The third morphotype, identified to be *S. olivacea* is grayish green in colour, while the propodus of the chelae and the last segments of the walking legs are pale orange. The frontal margin is cut into rounded lobes. Anterolateral margin of the carapace cut into 9 broad teeth. Chelipeds are large and the propodus are inflated.

Table 2. Discriminant Function Analysis (DFA) table showing the 7 morphometric characters which contribute most in discriminating the *Scylla* spp.

Step	Entered	Wilk's Lambda						
		Statistic	df1	df2	df3	Exact F		
						Statistic	df1	df2
1	Ratio 24	0.042	1	2	291.000	3360.491	2	291.000
2	Ratio 16	0.024	2	2	291.000	794.852	4	580.000
3	LSH	0.020	3	2	291.000	582.026	6	578.000
4	Ratio 13	0.018	4	2	291.000	464.317	8	576.000
5	Ratio 25	0.016	5	2	291.000	392.092	10	574.000
6	Ratio 23	0.015	6	2	291.000	342.486	12	572.000
7	Ratio 27	0.014	7	2	291.000	297.829	14	570.000

The species is characterized by the presence of single spine on the distal half of outer margin of the carpus of the cheliped. Palm of the cheliped exhibits a pair of prominences on the dorsal margin behind the insertion of the dacytyl. The inner prominence is larger than the outer one (fig.5). Hexagonal markings are weak or absent.

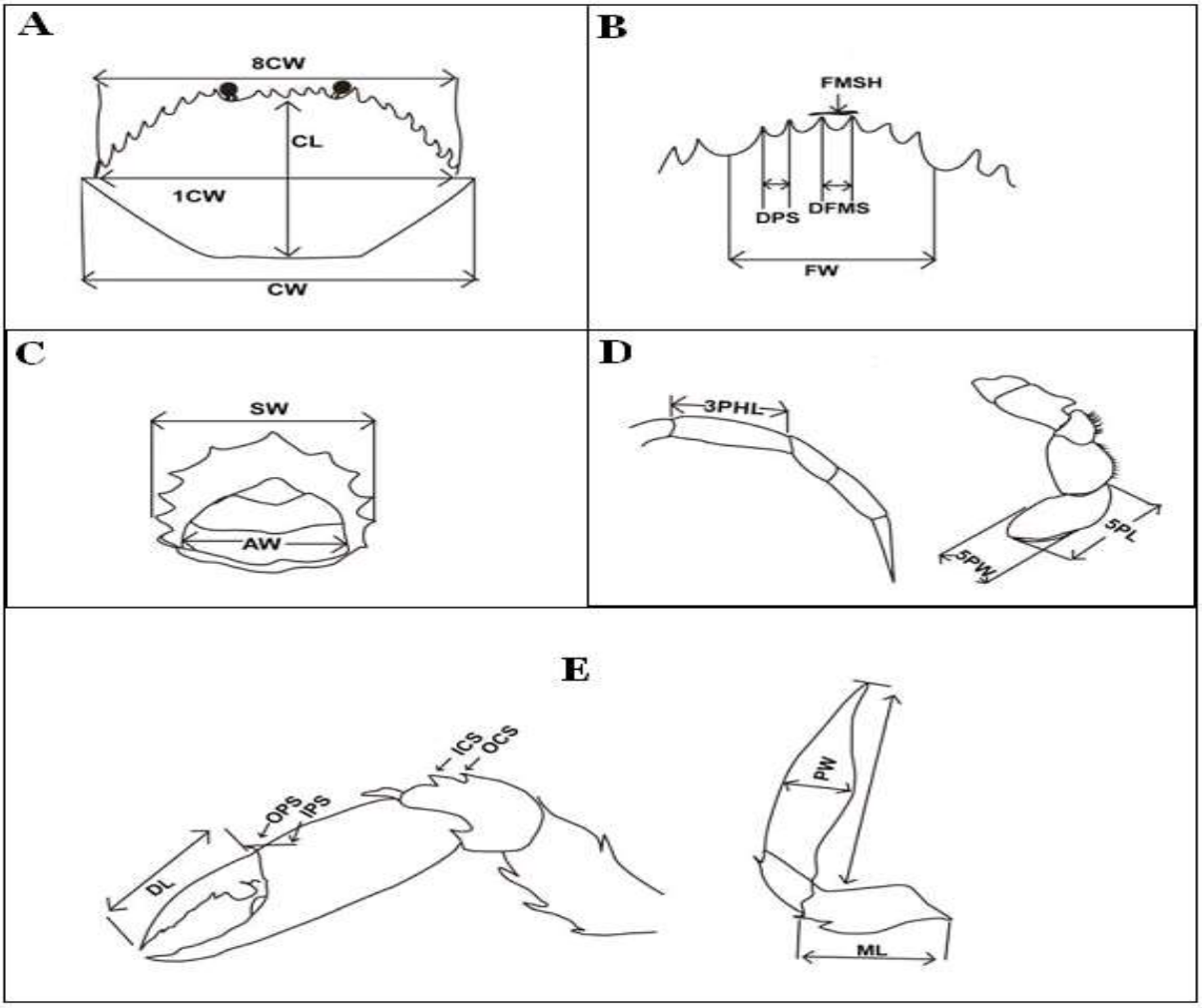


Fig. 2. Details of the morphological characters considered for morphometric analysis; (A) Carapace, (B) Frontal Lobe, (C) Abdomen (D) Periopods and (E) Chelipeds.

Description of the Third maxillipeds and the first and second male pleopods

The camera lucida study of the first and second male pleopods exhibited variations among the three morphotypes, while the third maxillipeds was found to be similar (fig.6). The third maxilliped has a broad and flattened ischium and are lined with thick hairs on its inner side.

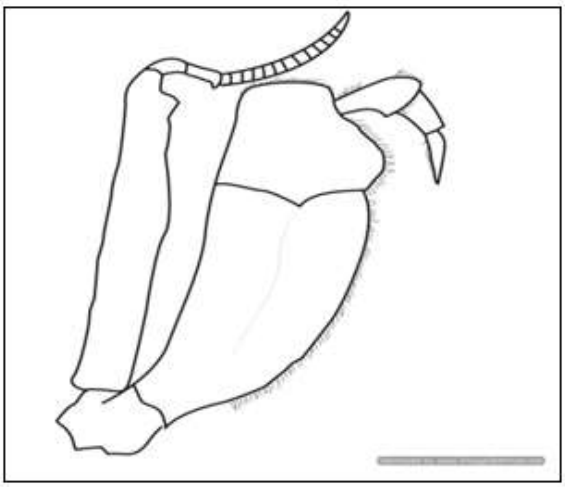


Fig. 3. Third maxillipeds of *Scylla* spp – characterized by broad and flattened ischium, lined with thick hairs on its inner side.

The male pleopods of the three morphotypes were similar in shape, but showed variation in the pattern of setation. The first male pleopod of the first morphotype (assigned to be *S. serrata)* exhibited two patches of thin and inconspicuous setae on the inner margin on its posterior end. The second male pleopod is smaller than the first pleopod. The tip of the second male pleopod is bilobed and the pleopod lacks any setation (fig.7).

The first male pleopod of the second morphotype (assigned to be *S. tranquebarica*) has two patches of setae on the inner margin on its posterior end. Unlike the first morphotype, the setation is thick and conspicuous. The tip of the second male pleopod is bilobed and the pleopods lack setation, similar to the first morphotype (fig.8).

The first male pleopod of the third morphotype (assigned to be *S.olivacea)* exhibited a single patch of setae, which is thick and prominent. The second male pleopod is bilobed at its tip. Unlike, the first and second morphotype, the second pleopods are found to possess setation on the inner margin on its posterior region (fig.9).

Morphometric study

The forward stepwise Discriminant Function Analysis gave two canonical discriminant functions incorporating 7 characters, which together provided 94.9% of discrimination between the three morphotypes of *Scylla*.

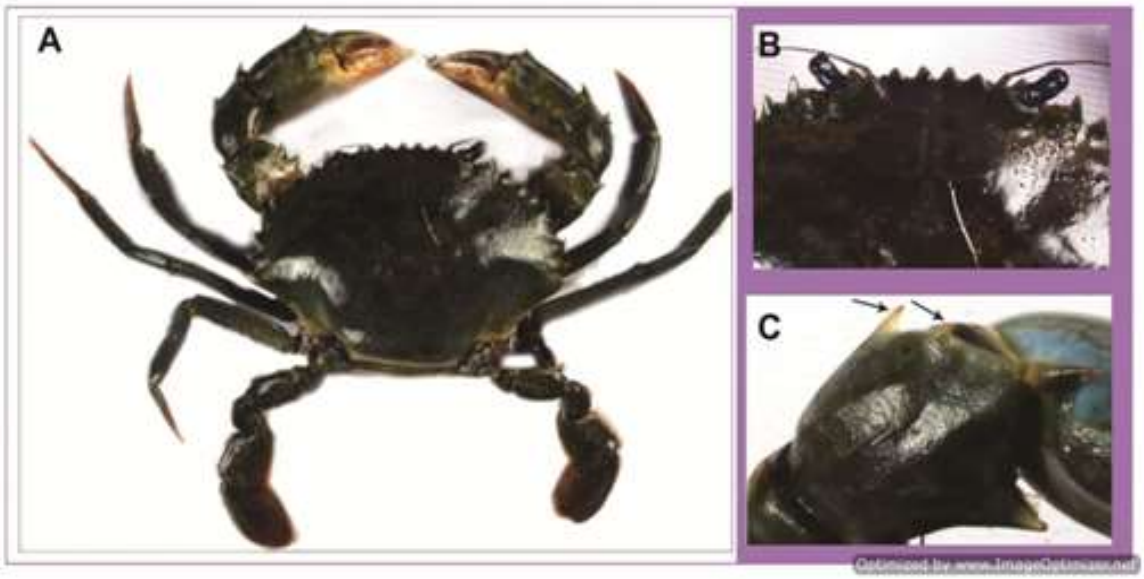


Fig. 4. Morphology of first morphotype (assigned to be *S. serrata)* male, 117.23mm- A, closer view of the pointed frontal lobes and the narrow anterolateral spines- B, two obvious carpal spines-C.

The 7 characters contributing the most to discriminate between the species are ICS/OCS, PW/PL, LSH, AW/SW, ML/PL, OCS/PL and 3PML/ICW (Table.2). The group centroid reveals that the first morphotype (*S. serrata*) is entirely different from the other two morphotypes viz., *S. tranquebarica* and *S. olivacea*. A mixing between the second morphotype *S. tranquebarica*) and third morphotype (*S. olivacea*) is clearly visible (fig. 10).

Molecular Study

First Internal Transcribed Spacer (ITS-1)

The ITS-1 marker produced only two types of band when tested with three morphotypes.

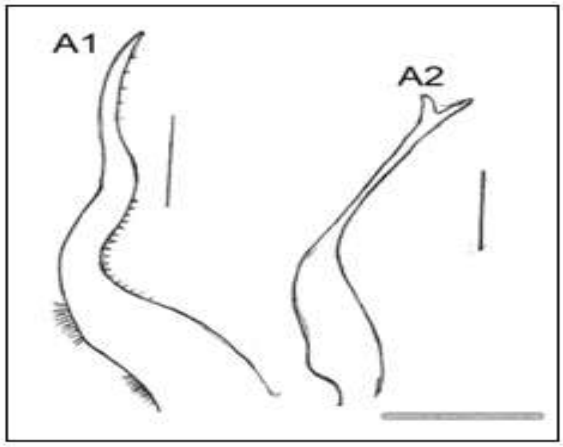


Fig. 5. Male gonopods of first morphotype (assigned to be *S. serrata)*- male, 117.23mm- First pleopods showing two tufts of thin and inconspicuous setae on the inner margin-A1, second pleopods showing its bilobed tip-A2. Scales (1) = 10 mm; (2) = 0.25 mm.

The sample identified to be *S. serrata* (with two prominent carpus spines) produced a 1,474bp band, whereas the sample identified to be *S. olivacea* (with single carpus spine) and *S. tranquebarica* (with two carpus spines, one bud like and the other prominent spine like) produced a 1,282 bp band (Fig. 11), analogous to *S. serrata* and *S. olivacea* respectively. No amplified product of 1,618 bp was found to match the band reported for *S. tranquebarica* (Imai *et al*., 2004).

The amplification of ITS-1 region confirms the occurrence of two species of *Scylla* viz., *Scylla serrata* and *S. olivacea* in Cochin Backwaters. The sample identified as *S. tranquebarica* came out to be *S. olivacea* in the molecular study.

Cytochrome oxidase c- oxidase sub unit I (COI) gene

When the COI region of the DNA samples isolated from the three morphotypes were amplified and sequenced (using the species specific pimers), a partial COI readable sequence of 658bp were obtained for *S. serrata* and *S. olivacea* (Fig.12). BLAST analysis of the nucleotide sequence of *S. olivacea* showed 98 % similarity to *Scylla olivacea*(GenBank ID KC154078) and *S. serrata* showed 99% similarity to *Scylla serrata* (GenBank ID KC154082). The nucleotide sequences obtained were submitted to GenBank database (*Scylla serrata* **Gen** Bank ID AB861521and*Scylla olivacea* Gen Bank ID AB861522).

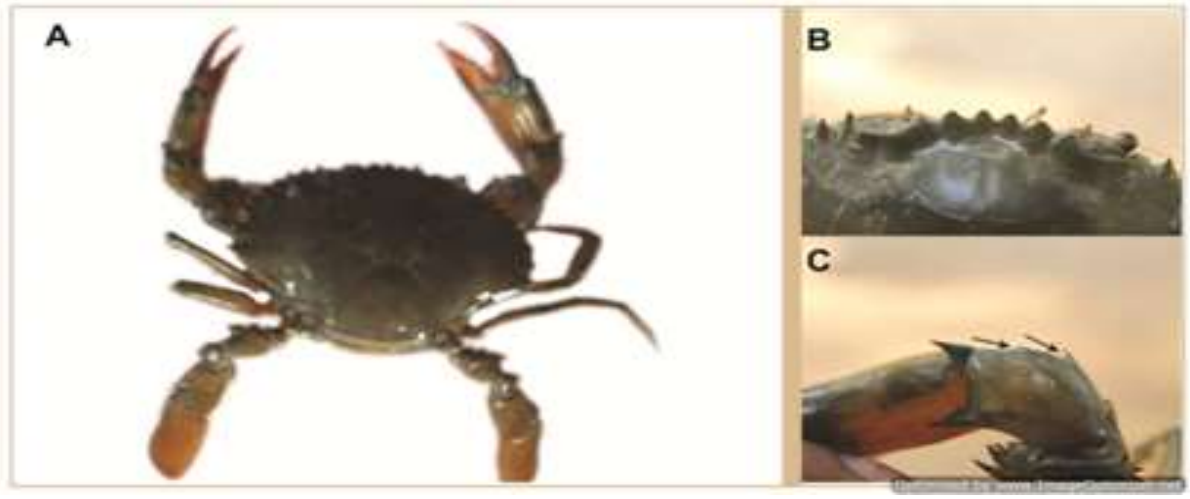
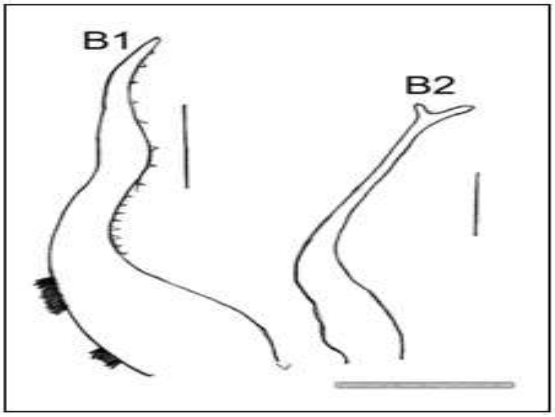


Fig. 6. Morphology of second morphotype (assigned to be *S. tranquebarica)*, male, 97.85mm-A; closer view of the bluntly pointed frontal lobes and the broader anterolateral spines- B, two carpal spines-C.

Discussion

Attempts to identify *Scylla* species have led to much confusion because of the subtle morphological differences between the species. Estampador (1949) classified the mud crabs into three species, *S. serrata*, *S. oceanica*, *S. tranquebarica* and one variety, and *S. serrata* var. *paramamosain,* based on its external morphology. In accordance with Estampador's findings, Serene (1952) also recognized the existence of four forms in Vietnam; which were further categorized into the marked and unmarked forms.



Scales (1) = 10 mm; (2) = 0.25 mm

Fig. 7. Male gonopods of second morphotype (assigned to be *S. tranquebarica)*- male, 97.85mm- First pleopods showing two tufts of thick and conspicuous setae on the inner margin-B1, second pleopods showing its bilobed tip-B2.

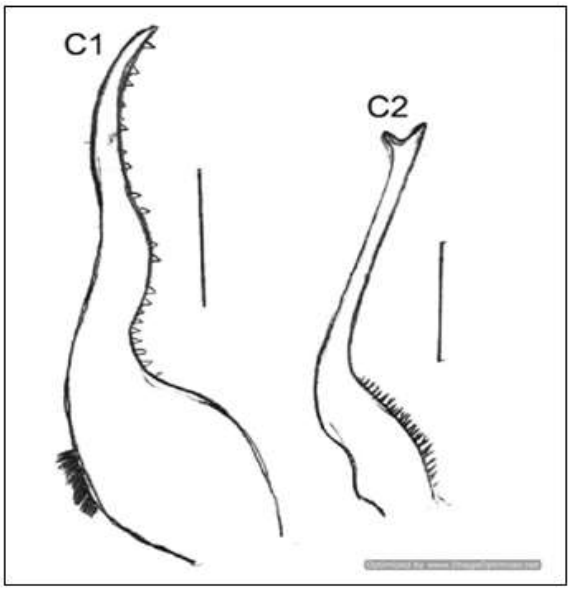
The marked form comprises a single species *S. oceanica* and its variety *S. oceanica tranquebarica*, while the unmarked form comprises *S. serrata* and its variety *S. serrata paramamosain*. However, Stephenson and Campbell (1960) considered the four forms as a single species, *S. serrata* and suggested that the morphological differences may be the result of environmental changes. Fushimi (1983) reported the presence of three forms of mud crabs, viz., *S. serrata*, *S. tranquebarica* and *S. oceanica* in Hamana Lake, Japan. Later on Oshiro (1988) also recognized three species of mud crabs from Japan, which is in agreement with the findings of Fushimi (1983).

The genetic variability analysis was carried out by Fuseya and Watanabe (1996) employing horizontal starch gel electrophoresis and they confirmed the existence of three species of mud crabs namely, *S. serrata*, *S. tranquebarica* and *S. oceanica* in Japan.

Keenan *et al.* (1998) made a revision of the genus *Scylla* using specimens collected from the Red Sea and throughout the Indo-Pacific region. Aside from the morphometry and morphological characters, molecular method was used for the identification of four species of *Scylla* viz., *S.serrata*, *S. tranquebarica*, *S. olivacea* and *S. paramamosain*.

Fig. 8. Morphology of third morphotype (assigned to be *S. olivacea)*- male, 97.11mm- A; closer view of the rounder frontal lobes and the broader anterolateral spines, C- single carpal spine, D- the pair of prominences on the dorsal margin behind the insertion of the dacytyl; inner prominence larger than the outer one.

Kathirvel (1981) identified a larger and a smaller species of mud crab from Cochin backwaters and designated them as *S. oceanica* and *S. serrata* respectively. Subsequently, Radhakrishnan and Samuel (1982) described the occurrence of a sub species in the Cochin backwaters, which was observed to possess a convex carapace, with blunt frontal teeth and a single spine on the outer margin of the carpus and this subspecies was designated as *S. serrata serrata*. Joel and Sanjeevaraj (1983) reported the existence of two species of genus *Scylla*, viz., *S. serrata* and *S. tranquebarica* from Pulicut lake. The findings of Radhakrishnan and Samuel (1982) were critically analysed by Kathirvel and Srinivasagam (1992) and stated that *S. serrata serrata* described by them could be *S. serrata* only.



Scales (1) = 10 mm; (2) = 0.25 mm.

Fig. 9. Male gonopods of third morphotype (assigned to be *S. olivacea),* male, 97.11mm - First pleopods showing single tuft of thick setae on the inner margin-C1, second pleopods showing its bilobed tip and the setae-C2.

In the present study three morphotypes of *Scylla,* viz. *S. serrata*, *S. tranquebarica* and *S. olivacea* has been recorded in the Cochin Backwaters based on the morphological details provided in the identification key of Keenan *et al.* (1998). The *Scylla* species with a single spine on the outer margin of the carpus of the cheliped are considered to be *S. serrata* and that with two spines are considered to be *S.tranquebarica* by Joel and Sanjeevaraj (1983), Kathirvel and Srinivasagam (1992), Fushimi and Watanabe (1998). However, according to Keenan *et al.* (1998), Sangthong and Jondeung (2006), S. *serrata* is characterized with bluntly pointed frontal lobes, two spines on the outer margin of the carpus of the cheliped and conspicuous hexagonal markings on chelipeds and limbs. *S. tranquebarica* are also found to possess two spines on the carpus of cheliped, but they can be distinguished with their blunt frontal lobes with rounded interspaces and the pattern of hexagonal markings, i.e weak and inconspicuous markings on the chelipeds and first two pairs of limbs and stronger markings on the last two pairs. *S. olivacea* and *S. paramamosain* are characterized by single spine on the outer margin of the carpus of the cheliped (Keenan *et al.*, 1998; Sangthong and Jondeung, 2006). The observations of Keenan *et al.* (1998) is generally accepted by crab taxonomists as well as by FAO (Ng, 1998), since he has given more concrete evidences on the basis of morphological, morphometric and molecular analysis. Hence, in the present study, three morphotypes of genus *Scylla,* viz. *S. serrata*, *S. tranquebarica* and *S. olivacea,* has been observed in Cochin backwaters, a South Indian estuary as reported earlier by Devi and Joseph (2013). The first and second pairs of male pleopods and the third maxillipeds have been recognized as of taxonomical value by several carcinologists. (Stephenson and Campbell, 1960; Joel and Sanjeevaraj, 1983; Fuseya, 1998; Keenan *et al.*; 1998). The first male pleopods were found to vary in *S. serrata* and *S. tranquebarica* (Joel and Sanjeevaraj, 1983). Fuseya (1998) examined the first and the second male pleopods of *S. serrata*, *S. tranquebarica* and *S. oceanica* and found these clearly distinguishable. Keenan *et al.* (1998) stated that the shapes of first male pleopods are similar in the *Scylla* spp. examined, however there exist some minor variations which are not clear enough to distinguish between the species easily. According to his observations, the first male pleopods showed variations in the apex region and the pattern of setation.

The first male pleopods of *Scylla olivacea* exhibited long and slender apex, while it is more sinuous in the other three species. As per the illustrations provided by Keenan *et al*. (1998) the first male pleopods of *S. serrata* and *S. tranquebarica* exhibited double setation pattern, while *S olivacea* and *S. paramamosain* exhibited single setae on the inner margin, which is in accordance with the observations of the present study. In the present study also, the first male pleopods of the three species were found to be similar in shape, but variations were observed in the pattern of setation (Keenan *et al*., 1998). The first and second morphotypes assigned to be *S. serrata* and *S. tranquebarica* showed two patches of setae on the inner margin on its posterior end, which is thin and inconspicuous in the former , while thick and conspicuous in the latter.

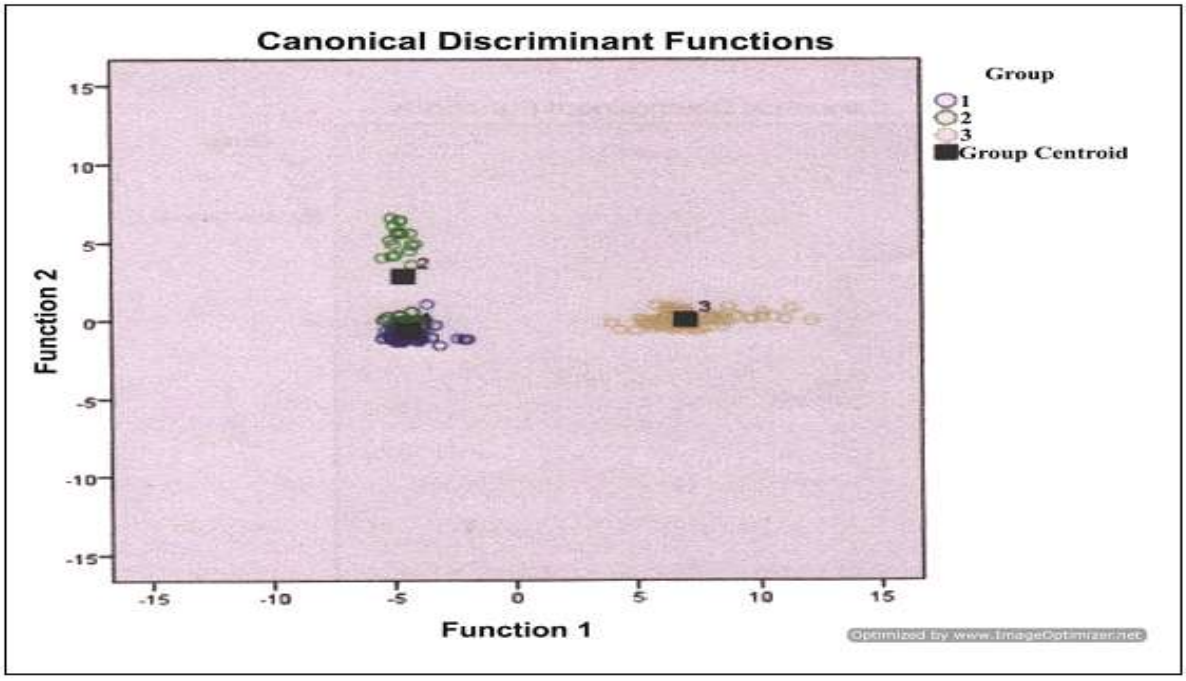


Fig. 10. Diagram showing the group centroid of the morphometric characters of the three morphotypes of genus *Scylla*.

The third morphotype identified to be *S. olivacea* was found to possess only a single tuft of thick setae on the inner margin of the first male pleopod. The second male pleopods were similar in the first and second morphotypes, while found to be varied in the third morphotype. The second male pleopods are found to possess setation on the inner margin on its posterior region of the third morphotype (*S. olivacea*), which is totally absent in the first and second morphotype (*S. serrata* and *S. tranquebarica* respectively).

The third maxillipeds were observed to be an important taxonomic tool in the identification of closely related species of crabs.

However, in the present study, no significant variations were observed among the three morphotypes of genus *Scylla,* found in the Cochin backwaters. It was observed that the third maxillipeds of three morphotypes of *Scylla* were characterized by broad and flattened merus and ischium, lined with thick hairs on the inner margin (Fig.6).

The morphometric study reveals that 7 characters contributes mostly to discriminate between the species are ICS/OCS, PW/PL, LSH, AW/SW, ML/PL, OCS/PL and 3PML/ICW (Table.2). Keenan *et al*. (1998) provided seven useful characters to distinguish between the species viz., ICS/OCS, FMSH/FW, FW/ICW, ML/PL, AW/SW, PL/ICW and IPS/PL.

However, no single characters can be considered to provide clear discrimination information between the species Keenan *et al.* (1998). The group centroid reveals that the first morphotype (*S. serrata*) is entirely different from the other two morphotypes viz., *S. tranquebarica* and *S. olivacea*. A mixing between the second morphotype (*S. tranquebarica*) and third morphotype (*S. olivacea*) is clearly visible. Hence the morphometric study reveals that the two morphotypes assigned to be *S. tranquebarica* and *S. olivacea* are found to be very similar to each other and at the same time, those are clearly different from the first morphotype, which is assigned to be *S. serrata*.

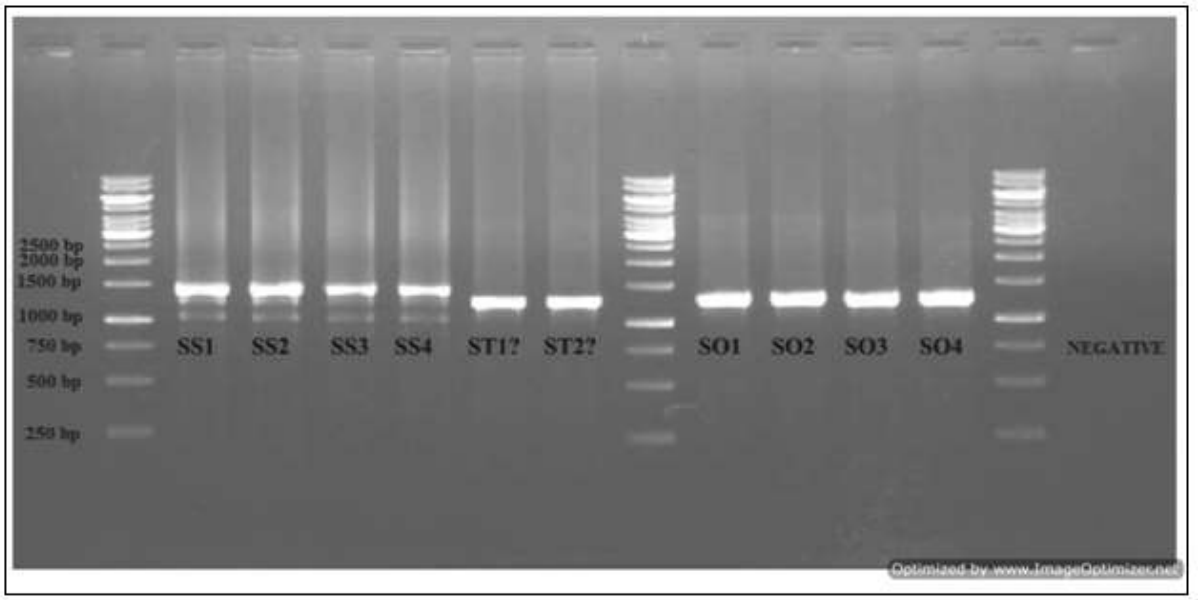


Fig. 11. Electrophoretic pattern from the ITS-1 region of three morphotypes of genus *Scylla* collected from Cochin Backwaters, Lane 1: Blank. Lane 2,9 & 14: 100bp ladder. Lane 3,4,5,6 : First morphotype assigned to be *Scylla serrata*. Lane 7&8: Second morphotype assigned to be *Scylla tranquebarica*. Lane 10, 11,12,13: Third morphotype assigned to be *Scylla olivacea*. Lane 14: Negative control.

The molecular analysis clearly indicate the possibility of the existence of only two species of *Scylla* viz., *S. serrata* and *S. olivacea* and in turn rules out the occurrence of *S. tranquebarica* in the study area. The amplification of ITS-1 region produced only two genotypes, one with a fragment size 1,474 bp and the other with a fragment of 1,282 bp which have concordance with *S. serrata* and *S. olivacea* respectively, as reported by Imai *et al.* (2004).

The samples identified as *S. tranquebarica* produced that same band as *S. olivacea*. No amplified product of 1,618 bp was found to match *S. tranquebarica* as reported by Imai *et al.* (2004). The results is in total agreement with the revelation of Mandal *et al.* (2014a) and Balasubramanian *et al.* (2014).

The amplification and sequencing of CO1 gene also confirms the existence of only two species of *Scylla*, *S. serrata* and *S. olivacea* in the Cochin Backwaters. When the COI region of the DNA samples isolated from the three morphotypes were amplified and sequenced (using the species specific pimers), a partial COI readable sequence of 658bp were obtained for *S. serrata* and *S. olivacea* (Fig.12). BLAST analysis of the nucleotide sequence of *S. olivacea* showed 98 % similarity to *Scylla olivacea*(GenBank ID KC154078) and *S. serrata* showed 99% similarity to *Scylla serrata* (GenBank ID KC154082).The sequences produced with *S. serrata* and *S. olivacea* samples in the present study show enough barcoding gap required for interspecific divergence between the two species.

Normally, the barcoding gap between interspecific species is demonstrated to be larger than 0.03 (3% threshold) in more than 98% of closely related lepidopteran species pairs (Hebert *et al*., 2003) using a CO1- based identification system. Keenan *et al*. (1998) reported less than 2% sequence difference within and more than 8% between mud crab species over a wide geographic range to conclude the existence of four mud crab species.

Hence the present study confirms the occurrence of two species of mud crab namely, *S. serrata* and *S. olivacea* in the Cochin Backwaters. The study strongly indicates the non-existence of *S. tranquebarica* in the Cochin Backwaters. Shaji *et al*. (2006) also stated that *S. serrata* and *S. olivacae* are the most common species occurring in India, based on the taxonomic identification of Keenan *et al*. (1998).

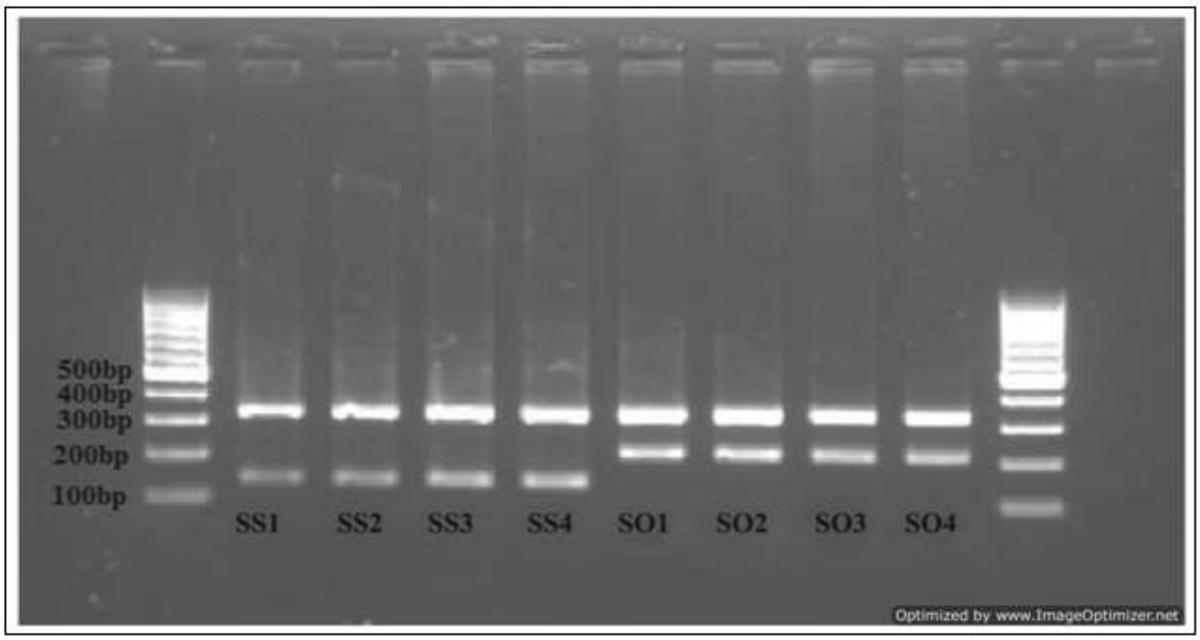


Fig. 12. Electrophoretic pattern from the CO-1 region (Keenan Primer) of three morphotypes of genus *Scylla* collected from Cochin Backwaters. Lane 1: Blank. Lane 2& 11: 100bp ladder. Lane 3,4,5,6 : First morphotype assigned to be *Scylla serrata*. Lane 7, 8, 9,10: Third morphotype assigned to be *Scylla olivacea*.

The present work is in total agreement with Mandal *et al* (2014 a,b) and Balasubramanian *et al* (2014). Mandal *et al* (2014a) attempted to resolve the taxonomic uncertainty of mud crabs available in Indian coastal waters using molecular genetic markers ITS-1 and sequencing of CO1 gene. Their study clearly indicates that the green morph of Indian mud crab is *S. serrata* and the brown mroph is *S. olivacea*. Molecular Identification of mud cabs using RAPD- PCR-RFLP markers also indicates that only two species of mud crabs *S. serrata* and *S. olivacea* are commonly present in the Indian coastal waters (Mandal *et al*., 2014b). Balasubramanian *et al*. (2014) rules out the existence of *S. tranquebarica* in Indian waters based on the 16s rRNA and CO1 gene analysis.

Conclusion

The study confirms the occurrence of two species of *Scylla* from Cochin Backwaters, *S. serrata* and *S. olivacea*. The species being identified as *S. serrata* is *S. olivacea* and that being identified as *S. tranquebarica* is *S. serrata*. It is possible that those specimens which had been identified as *S. tranquebarica* may be the juveniles of *S. olivacea*.

Acknowledgements

The first author is thankful to Kerala State Council for Science, Technology and Environment, Sastra Bhavan, Pattom, Trivandrum for the KSCSTE Research Fellowship as well as Rajiv Gandhi Centre for Aquaculture, Sirkazhi, Nagapattinam, tamil Nadu

for the permitting to make use of the facilities of their Central Genetics Lab for the molecular analysis. The authors are thankful to Dept. of Marine Biology, Microbiology & Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Cochin, Kerala for providing the necessary facilities to carry out this work.

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