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Molecular Markers Reveal Only Two Mud Crab Species of Genus *Scylla* (Brachyura: Portunidae) in Indian Coastal Waters

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Abstract The taxonomic ambiguity of the Indian mud crab (genus *Scylla* de Hann 1833) is still a cause of concern as several papers have been published with misleading identification. This is the first attempt to resolve the taxonomic uncertainty of the mud crab commonly available in Indian coastal waters using molecular genetic markers (*ITS-1* and sequencing of *COI* gene) combined with traditional morphometry. Additionally, we developed a PCR method by which Indian mud crab species can be identified rapidly and effectively. The results clearly indicate that the green morph of the Indian mud crab is *Scylla serrata* and the brown morph is *S. olivacea*. The *S. serrata* commonly mentioned in the literature from India is *S. olivacea;* the *S. tranquebarica* noted by many Indian researchers should belong to *S. serrata*. Caution should be taken when interpreting or implementing the biological, molecular, and aquaculture data in the literature.

Keywords *Scylla* spp. · Taxonomic ambiguity · Species identification · Molecular markers

Introduction

Mud crabs (genus *Scylla* de Haan 1833; Crustacea: Decapoda: Brachyura: Portunidae) are economically important crab species in India and an attractive alternative to shrimp farming within coastal areas (Overton et al. 1997). They are

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widely distributed from southeastern and eastern Africa to Southeast Asia and Indo-Pacific regions, inhabiting brackish waters, such as mangrove areas and estuaries (Fushimi and Watanabe 1999). These crabs present many variations in coloration, size, spination, habitat, and other characteristics, thereby contributing to much confusion in their identification (Joel and Raj 1983), which is a major constraint on stock assessment and the future development of their aquaculture and fisheries management (Macintosh et al. 2002).

The taxonomy for the genus *Scylla* has long been controversial worldwide. Estampador (1949) classified mud crabs from the Philippines based on gametogenesis and external morphology into three species and one variety: *Scylla serrata* (Forskål 1775), *S. oceanica* (Dana 1852), *S. tranquebarica* (Fabricius 1798), and *S. serrata* var. *paramamosain* (Estampador 1949). Serene (1952) divided the four Vietnamese forms into two categories, recognizing *S. oceanica* as the only species for the marked kind, with its variety *S. oceanica tranquebarica*, and *S. serrata* as the only valid species for the unmarked kind, with its variety *S. serrata paramamosain*. Fushimi (1983) recognized at least three species present in Japan, whereas Fuseya and Watanabe (1996) discriminated these species (*S. serrata*, *S. tranquebarica*, and *S. oceanica*) based on genetic variability at allozyme loci. Keenan et al. (1998) revised the taxonomy of the genus using external morphology, genetic variations, and multivariate analysis of morphometric characters; they identified the four distinct nonhybridizing species *S. serrata* (Forskål 1775), *S. tranquebarica* (Fabricius 1798), *S. olivacea* (Herbst 1796), and *S. paramamosain* (Estampador 1949) based on samples collected from many parts of the Indo-Pacific region, excluding India. Subsequently, several researchers (Macintosh et al. 2002; Sangthong and Jondeung 2006; Ma et al. 2006; Jirapunpipat et al. 2008; Ogawa et al. 2012) revised the taxonomic status of local mud crabs in various parts of the world based on either morphological keys or molecular techniques following Keenan et al. (1998).

Several workers have dealt with mud crabs from India, and most refer only to *Scylla serrata* (Forskål 1775). Serene (1952), however, pointed out that the specimens in Indian museums identified as *S. serrata* consist of at least two forms and that the species commonly found in Madras (now Chennai) markets was *S. tranquebarica*. Joel and Raj (1983) also reported the presence of two species, *S. serrata* and *S. tranquebarica,* from Pulicat Lake near Chennai based on morphological characters. Kathirvel and Srinivasagam (1992) reported two species of Indian mud crabs collected from Cochin backwaters in Kerala, a dark green morph (*S. tranquebarica*) and a greenish brown morph (*S. serrata*). Many recent publications from India (Mohanty et al. 2006; Vartak et al. 2008) follow the Kathirvel and Srinivasagam (1992) classification, and Jithendran et al. (2010) follows the same with a cautionary note. Lakshmi and Joseph (2013) report that three species (*S. serrata, S. tranquebarica,* and *S. olivacea*) are present in Cochin backwaters, Kerala, based on morphological characteristics. The limitations inherent in morphology-based identification systems and the dwindling pool of taxonomists signal the need for a new approach to identify closely related species, especially when morphological features are misleading (Hebert et al. 2003b).

In this study we collected mud crab samples from various locations in the coastal waters of India and used a combination of morphological and molecular genetic markers to develop species-specific molecular markers for the identification of mud crab species commonly found in Indian coastal waters.

Materials and Methods

Sample Collection

Mangrove crab samples were collected from 21 major locations in western, eastern, and Andaman coastal waters of India (Fig. 1), covering all the areas previously reported. Fresh wild samples (1,204) were obtained from commercial crab collectors from various locations of western and southwestern coastal regions including Jamnagar, Gujarat (22.4°N, 70.0°E; $n = 33$), Mumbai (18.9°N, 72.8°E; $n = 62$) and Ratnagiri, Maharashtra (16.9°N, 73.3°E; $n = 31$), Madgaon, Goa (15.3°N, 73.9°E; $n = 73$), Mangalore, Karnataka (12.8°N, 74.8°E; $n = 40$), Kannur (11.8°N, 75.3°E; $n = 46$), Thrissur (10.5°N, 76.2°E; $n = 19$), and Cochin, Kerala (9.5°N, 76.4°E; $n = 82$), and Nagercoil, Tamil Nadu (8.1°N, 77.4°E; $n = 28$). On the Coromandel coast the locations were Karaikal, UT, of Puducherry (10.9°N, 79.8°E, $n = 96$), Thirumullaivasal (11.2°N, 79.8°E, $n = 134$), and Pulicat Lake, Chennai, Tamil Nadu (13.4°N, 80.3°E, $n = 80$). Sites on the coast of Andhra Pradesh included Nellore (14.4°N, 79.9°E, $n = 62$), Nagayalanka (15.9°N, 80.9°E; $n = 57$), Machilipatnam (16.1°N, 81.1°E, $n = 70$), Kakinada (16.9°N, 82.2°E, $n = 32$), and Visakhapatnam (17.0°N, 83.0°E, $n = 49$). Samples were collected from the Orissa coast at Chilika Lake, Balugaon, Odisha (19.6°N, 85.4°E; $n = 77$); from the West Bengal coast at Canning, Sundarban, West Bengal (21.9°N, 88.8°E; $n = 35$); and from the Andaman coast at Port Blair, South Andaman (12.5°N, 92.7°E; $n = 47$), and Diglipur, North Andaman (13.2°N, 92.9°E; $n = 51$). The fifth pereopod of each specimen was detached using hot forceps, and the dissected muscles were preserved in 95% ethanol.

Initial Classification

Specimens were broadly assigned to *S. serrata* ($n = 605$), *S. tranquebarica* (15), *S. paramamosain* (71), and *S. olivacaea* (513) based on morphological characters provided by Estampador (1949), Kathirvel and Srinivasagam (1992), and Keenan et al. (1998). The carpus spine number was considered for primary identification (two for *S. serrata* and *S. tranquebarica*; one for *S. paramamosain* and *S. olivacaea*). Differences in the shape of the frontal lobe spine (comparatively moderate height and blunted in *S. tranquebarica*) and differences in polygonal patterning (weak on chelipeds and first two pairs of legs in *S. tranquebarica*) were considered for differentiating *S. tranquebarica* from *S. serrata*. Propodus spine (a pair of distinct spines) and weak polygonal patterning on chelipeds and legs were considered for *S. paramamosain* to differentiate it from *S. olivacaea* for preliminary assignment. The number of samples assigned to *S. tranquebarica* was very small compared with other species because the differences in shape of the frontal lobe

Fig. 1 Collection sites (*solid black circles*) in Indian coastal waters of mud crabs used in the present study

spine and in polygonal patterning as mentioned by Keenan et al. (1998) were obtained very rarely and were mostly confusing. The number of *S. paramamosain* was large enough because in many cases we obtained crabs with a pair of distinct propodus spine and weak polygonal patterning during collection.

Morphometric Analysis

Morphometrics were analyzed with 179 selected samples depending on the size and initial identification (60 *S. serrata* samples, 12 *S. tranquebarica*, 47 *S. paramamosain*, and 60 *S. olivacaea*, $n = 60$). As suggested by Oshiro (1991), an internal carapace width (ICW) greater than 80 mm was the criterion to select the animals for morphometric analysis, to preclude the chances of confounding the influence of juvenile ontogenetic changes. A total of five measurements, including ICW, frontal median spine height (FMSH), frontal width (FW), outer carpus spine (OCS), and inner carpus spine (ICS), were recorded with Sontax digital calipers to the nearest 0.1 mm. The ratios FMSH/FW, FW/ICW, and ICS/OCS were calculated from the recorded data, as they contributed most to discrimination among species as described by Keenan et al. (1998). Morphometric data were analyzed with one-way ANOVA (Soper 2013). A box-and-whisker diagram was prepared using MS-Excel (2007) software, displaying the differences between species by graphically

depicting groups of numerical data through their five-number summaries: the smallest observation (sample minimum), lower quartile, median, upper quartile, and largest observation (sample maximum).

DNA Extraction

Total genomic DNA was extracted from ~25 mg of alcohol-preserved muscle tissue from each specimen selected for morphometric study ($n = 179$) using the traditional proteinase K and phenol–chloroform extraction protocol described by Sambrook et al. (1989) with some modifications. To analyze the yield and quality, extracted DNA was tested by electrophoresis through a 0.7% agarose gel containing a nonmutagenic fluorescent DNA dye (0.1 µl/ml; EZ Vision In-Gel Solution, Amresco, USA). The final DNA concentration was estimated by optical density reading (BioPhotometer Plus, Eppendorf) at 260 nm. Most of the extracted DNA samples were concentrated; therefore, samples were diluted with sterile double-distilled water to reach appropriate concentrations (75 ng/µl) for PCR.

PCR Amplification of First Internal Transcribed Spacer (*ITS-1*)

The *ITS-1* region was amplified using the primer sets L *SP-1-3′* (5′-ATTT AGCTGCGGTCTTCATC-3′) and H *SP-1-5′138* (5′-CACACCGCCCGTCGCT ACTA-3′) and following the protocol described by Imai et al. (2004). The amplification was carried out in a Veriti Thermal Cycler (Applied Biosystems) in a 25 µl reaction volume. The resulting products were tested by electrophoresis through 1.0% agarose gel, stained with EZ Vision solution for photography. The gels were imaged using a gel documentation system (G-Box, Syngene, UK). A Supermix DNA ladder (GeNei Bangalore) was used as the size standard. The sizes of major bands were determined by the Syngene Gene Tool Software on the gel documentation system.

Amplification and Sequence Analysis of Cytochrome c-Oxidase Subunit I Gene (*COI*)

The *COI* gene was amplified from all the selected samples using two sets of primers. The first set, described by Folmer et al. (1994), was *LCO1490* (5′-GGTCAACAAA TCATAAAGATATTG G-3′) and *HCO2198* (5′-TAAACTTCAGGGTGACCAAA AAATCA-3′). The second set combined two specific primers described by Roehrdanz (1993), *mtd-10* (5′-TTGATTTTTTGGTCATCCAGAAGT-3′), and by Gopurenko et al. (1999), *C/N-2769* (5′-TTAAGTCCTAGAAAATGTTGRGGG A-3′), specially designed for insects and *Scylla* species. Many researchers use the first set of primers as universal *COI* primers for DNA barcoding of species, whereas Keenan et al. (1998) and Fratini and Vannini (2002) used the second set of primers in their studies. Amplification was performed following the protocols described by the authors. The amplified products were purified with a gel purification kit (GeneJET PCR purification kit, Thermo Scientific, EU-Lithuania) after confirming the correct amplification. Purified PCR products were sequenced in forward and

reverse directions using a Genetic Analyzer 3500 (Applied Biosystems). Sequences were aligned, edited, and analyzed using ClustalW and Mega software version 5 (Tamura et al. 2011). The genetic distance between the species was calculated under the assumptions of the Tajima-Nei model (Tajima and Nei 1984), and the standard error estimate was obtained by a bootstrap procedure (1,000 replicates). The evolutionary history was inferred using the neighbor-joining method, exploiting sequence data from GenBank and sequence data from the present study to determine the phylogenetic relationship among all four mud crab species. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The unique sequences generated in this study were submitted to NCBI GenBank.

Development of a PCR-Based Species Identification Method

Both segments of the *COI* gene were aligned, and a combined sequence of 1,229 bp was obtained for each genotype. Based on the conserved sequence present in the two haplotypes, a pair of conserved primers, *Scy-F* (5′-GATACSCGAGCTTAYT TTACATC-3′) and *Scy-R* (5′-TAGGATTAAGRGAYAAACCTGTAAA-3′), was designed and synthesized following Ma et al. (2012) that can amplify a PCR product of 325 bp for each species. This product could be considered a positive control to confirm the authenticity of PCR. Additionally, two reverse primers, *ScyS-R* (5′-AATAAATCCTAAAGCCCATAATATA-3′) and *ScyO-R* (5′-GTGTCATGTAGG ATAATATCGATG-3′), were designed according to the species-specific nucleotides near the 3′ end of the primer binding region for species-specific amplification. *ScyS-R* was designed to produce PCR products of 138 bp specifically for *S. serrata* when paired with the conserved forward primer *Scy-F*. Similarly, *ScyO-R* was designed to produce PCR products of 212 bp exclusively for *S. olivacea* when paired with *Scy-F*. We used a total of 48 samples (12 each of four initially assigned species) for the PCR, and the amplified products were separated on 1.5% agarose gels with a comigrating 100 bp DNA ladder.

Results

Morphometric Analysis

Variations in five morphometric characters and three morphological ratios among the four initially assigned mud crab morphs were analyzed (Table 1). The mean ratios of ICS/OCS, FMSH/FW, and FW/ICW of *S. serrata* were similar to those of *S. tranquebarica,* corresponding to the values of *S. serrata* described by Keenan et al. (1998). Likewise, the mean ratios for *S. olivacea* were similar to those of *S. paramamosain,* matching the values of *S. olivacea* reported by Keenan et al. (1998). When morphometric data were analyzed with one-way ANOVA (Soper 2013), the three ratios of ICS/OCS ($F = 673.2$), FMSH/FW (387.7), and FW/ICW (262.4) contributed more to discriminate between *S. serrata* and *S. olivacea* (all $P < 0.01$). For *S. serrata* and *S. tranquebarica,* the ratios indicated that the differences between the

Table 1 Morphometric characters and ratios for four morphotypes of mangrove crabs collected from Indian coastal waters

Scylla species (*N*)	Source	Morphometric character (mm)					Ratio		
		ICS	OCS	FMSH	FW	ICW	ICS/OCS	FMSH/FW	FW/ICW
Ss (60)	Present study								
	Mean ± SD	4.027 ± 1.425	4.047 ± 1.249	2.203 ± 0.621	37.298 ± 6.121	101.40 ± 14.66	1.027 ± 0.261	0.059 ± 0.010	0.367 ± 0.011
	Range	2.6–8.6	2.3–6.9	1.6–3.8	31.2–54.9	83.0–137.6	0.623–1.609	0.046–0.077	0.349–0.404
	Keenan et al. (1998)								
	Mean ± SD	NA	NA	NA	NA	138.4 ± 23.2	0.940 ± 0.233	0.061 ± 0.010	0.371 ± 0.016
	Range	NA	NA	NA	NA	95.5–191.7	0.500–1.583	0.041–0.095	0.335–0.406
So (60)	Present study								
	Mean ± SD	0.232 ± 0.247	2.143 ± 0.561	1.195 ± 0.280	39.598 ± 4.010	97.77 ± 12.42	0.103 ± 0.091	0.030 ± 0.005	0.405 ± 0.014
	Range	0.0–0.9	1.5–3.5	0.8–1.6	32.0–49.2	80.1–120.2	0.000–0.273	0.021–0.037	0.386–0.428
	Keenan et al. (1998)								
	Mean ± SD	NA	NA	NA	NA	107.5 ± 10.1	0.006 ± 0.035	0.029 ± 0.005	0.415 ± 0.017
	Range	NA	NA	NA	NA	95.0–133.9	0.000–0.250	0.018–0.037	0.371–0.451
St (12)	Present study								
	Mean ± SD	4.53 ± 1.386	4.43 ± 1.188	2.32 ± 0.656	38.59 ± 7.120	104.45 ± 17.40	1.025 ± 0.143	0.060 ± 0.011	0.369 ± 0.012
	Range	2.9–7.4	2.7–6.8	1.7–3.7	31.0–54.4	82.5–137.0	0.806–1.259	0.047–0.081	0.346–0.397
	Keenan et al. (1998)								
	Mean ± SD	NA	NA	NA	NA	113.7 ± 11.4	0.980 ± 0.251	0.043 ± 0.006	0.412 ± 0.016
	Range	NA	NA	NA	NA	97.1–137.8	0.467–1.429	0.031–0.053	0.383–0.443

Table 1 continued

Scylla species (*N*)	Source	Morphometric character (mm)					Ratio		
		ICS	OCS	FMSH	FW	ICW	ICS/OCS	FMSH/FW	FW/ICW
Sp (47)	Present study								
	Mean ± SD	0.225 ± 0.215	2.112 ± 0.550	1.177 ± 0.271	39.255 ± 3.959	96.53 ± 12.33	0.106 ± 0.084	0.030 ± 0.005	0.404 ± 0.012
	Range	0.0–1.0	1.4–3.6	0.7–1.6	32.2–44.5	80.2–110.0	0.000–0.278	0.019–0.037	0.386–0.434
	Keenan et al. (1998)								
	Mean ± SD	NA	NA	NA	NA	114.7 ± 9.0	0.352 ± 0.235	0.058 ± 0.012	0.377 ± 0.007
	Range	NA	NA	NA	NA	104.8–134.1	0.188–0.941	0.040–0.081	0.364–0.386

Species codes *Ss Scylla serrata*, *So S. olivacea, St S. tranquebarica*, *Sp S. paramamosain*; Character codes *N* sample size, *ICS* inner carpus spine, *OCS* outer carpus spine, *FMSH* frontal median spine height, *FW* frontal width, *ICW* internal carapace width, *NA* data not available

two species are insignificant ($F = 0.001$ for ICS/OCS, 0.083 for FMSH/FW, and 0.162 for FW/ICW; $P = 0.98$, 0.77, and 0.69, respectively). A similar conclusion can be drawn in the case of *S. olivacea* and *S. paramamosain* ($F = 0.027$, 0.0001, and 0.276; $P = 0.87$, 0.98, and 0.60, respectively). A box plot diagram (Fig. 2) generated using morphometric ratios clearly represents two groups, one comprising *S. serrata* and *S. tranquebarica* and the other involving *S. olivacea* and *S. paramamosain*.

First Internal Transcribed Spacer (*ITS-1*)

The *ITS-1* marker produced only two types of bands when tested with all four morphotypes. Samples assigned to *S. serrata* and *S. tranquebarica* (with two spines on carpus of chelipeds) produced a 1,474 bp band, whereas *S. olivacea* and *S. paramamosain* (with one spine in the carpus of chelipeds) produced a 1,282 bp band (Fig. 3), analogous to *S. serrata* and *S. olivacea,* respectively, as described by Imai et al. (2004). No amplified product of 1,618 bp was found to match the band reported for *S. tranquebarica* and *S. paramamosain* by Imai et al. (2004).

Amplification and Sequence Analysis of *COI*

Amplified products of 709 and 597 bp were obtained using the first and second set of primers, respectively, and only two unique sequences were generated with the first set of primers (one with the *S. serrata* and *S. tranquebarica* group and another with the *S. olivacea* and *S. paramamosain* group of samples). A Blast search of the *S. serrata* group sequence against sequences submitted to NCBI showed 99% identity with *S. serrata* (GenBank accession nos. FJ827758 and JN085429-36). Similarly, the sequence of the *S. olivacea* group matched the *S. olivacea* complete mitochondrion genome, with a maximum identity of 99% (GenBank acc. no. FJ827760). The sequences (one each from *S. serrata* and *S. olivacea*) were submitted and published in the NCBI GenBank (acc. nos. KC200562 and KC200563). The sequences had a GC content of 36.0 and 37.7%, respectively. The first set of the COI primer region of *S. serrata* differed from *S. olivacea* by 0.148 ± 0.017. The second set of primers also produced only two unique sequences (one with the *S. serrata* and *S. tranquebarica* group and another with the *S. olivacea* and *S. paramamosain* group of samples). The *S. serrata* group sequence matches the NCBI submitted sequences of *S. serrata* with 99% identity (acc. nos. FJ827758, EF203948, and FJ011456). The *S. olivacea* group sequence matches the *S. olivacea* sequences with maximum identity of 99% (acc. nos. FJ827760, FJ011463-67). The sequences were submitted and published in the NCBI GenBank (acc. nos. KC200564 and KC200565). These sequences had a GC content of 33.4 and 36.5%, respectively. The calculated genetic distance between *S. serrata* and *S. olivacea* was 0.172 ± 0.02.

The neighbor-joining tree (Fig. 4) shows clear clustering of *S. serrata* and *S. olivacea* sequences generated in this study (GenBank acc. nos. KC200562 and KC200563) with the respective species when analyzed with the NCBI submitted sequences. The second set of primers with Indian mud crabs (acc. nos. KC200564 and KC200565) form clusters with *S. serrata* and *S. olivacea,* respectively, with the sequences submitted by the Keenan group (He, Keenan, and Sha; acc. nos. FJ011452

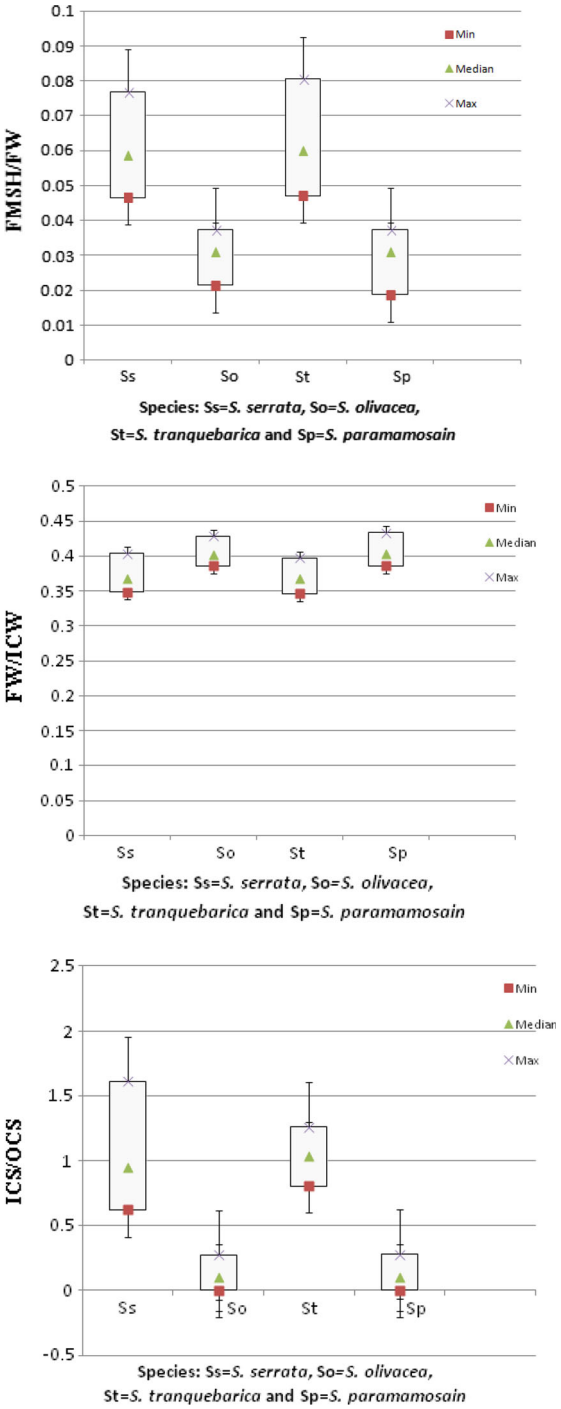

Fig. 2 Summary of morphometric ratios for mud crab species from Indian coastal waters. (*top*) Frontal median spine height (FMSH)/frontal width (FW). (*center*) FW/internal carapace width (ICW). (*bottom*) Inner carpus spine (ICS)/outer carpus spine (OCS). *Box plots* with whiskers indicate median ratio (triangle), lower and upper quartiles, and smallest and largest observations (sample minimum and maximum). Species codes *Ss Scylla serrata*, *So S. olivacea, St S. tranquebarica*, and *Sp S. paramamosain*

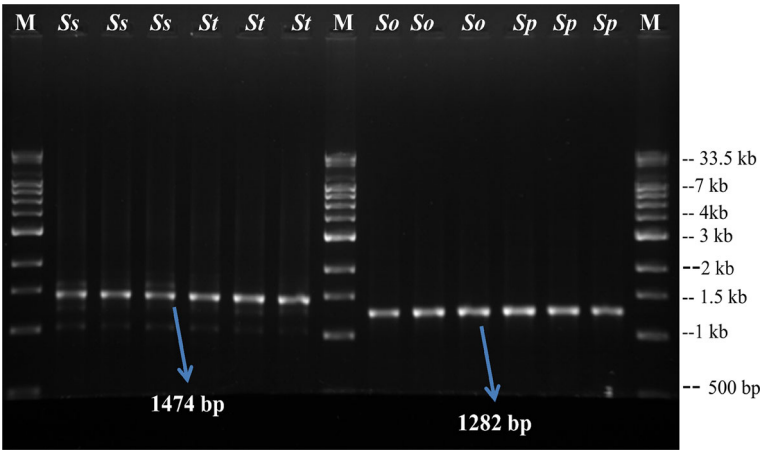

Fig. 3 *ITS-1* fragment profile of mud crab species collected from Indian coastal waters. *Lanes M* Supermix DNA ladder. Species codes as in Fig. 2

and FJ011466), further confirming the species status of Indian mud crabs (Fig. 5). We find 11 mud crab *COI* sequences submitted to GenBank from Indian samples, collected by one research group from a single mangrove area, for DNA barcoding using the first set of primers (acc. nos. JN085428-JN085436, JN688964, and JN688965). The identity of the species was not authenticated, as all of these mud crab *COI* sequences were direct submissions with an unpublished tag. After proper alignment of these sequences, we find that many of them seem to be identical (acc. nos. JN085428, JN085429, and JN085434; JN085432 and JN085433; JN085435 and JN085436). We are not sure about the *S. tranquebarica* (JN688964) and *S. olivacea* (JN688965) sequences, because although they seem to be generated with the same primers and show a product size similar to the *S. serrata* sequences, none of them properly matches the *S. serrata* (KC200562) or *S. olivacea* (KC200563) sequences generated in the present study using the same primer set. Instead, they match sequences of *S. tranquebarica* (FJ011460) and *S. olivacea* (FJ011466) submitted by Keenan et al. (1998) and generated with the second set of primers. We observed a total of 154 different sites between the two species (Fig. 6) when the sequences obtained by the two sets of primers were combined and compared. We estimated the time of divergence between *S. serrata* and *S. olivacea* by applying the substitution rate of 1.66–2.33%/Myr (previously used for the *COI* gene for different crab species by Schubart et al. 1998) and found that the evolutionary separation between the two lineages of the genus *Scylla* started about 6.38 ± 0.8 to 7.42 ± 0.9 Myr ago during the early Miocene epoch of the Neogene period.

PCR-Based Species Identification Method

The pair of conserved primers (*Scy-F* and *Scy-R*) produced a product of 325 bp in all the samples, as expected. The two species-specific reverse primers (*ScyS-R* and *ScyO-R*), when paired with the forward primer *Scy-F*, amplified only in their

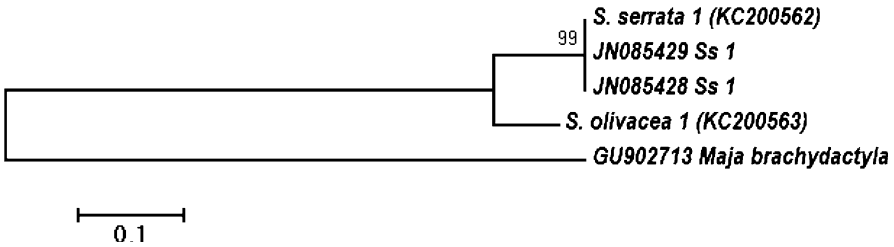

Fig. 4 Phylogenetic relationship of *Scylla serrata* and *S. olivacea,* two mud crab species commonly found in Indian waters, and other mud crab species. *Ss* 1 indicates *S. serrata* sequences generated with the first set of primers (references taken from sequences submitted to GenBank). KC200562 and KC200563 are Indian mud crab sequences generated during the present study. *Maja brachydactyla* sequence used as an outgroup. Neighbor-joining tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

respective species, producing a band of 212 bp for *S. olivacea* and 138 bp for *S. serrata*. All the samples (12 each of four initially assigned species) amplified with either *S. serrata* or *S. olivacea* specific primers with the proper product size (Fig. 7).

Discussion

Most of the previous taxonomic studies of mud crabs from India were based on external morphology, with very few samples collected even from a single location. Jirapunpipat et al. (2008) reported the difficulties of assigning a mud crab sample unambiguously at the species level on the basis of color and other morphometric characters. This is the first study of Indian mud crab species to examine a large number of samples collected from the entire stretch of Indian coastal waters, including Andaman waters, to solve taxonomic ambiguity using a combination of morphometry and molecular markers.

The mean ratios of ICS/OCS, FMSH/FW, and FW/ICW for *S. serrata* and *S. tranquebarica* and for *S. olivacea* and *S. paramamosain* were similar (Table 1), corresponding to the values of *S. serrata* and *S. olivacea* reported by Keenan et al. (1998). The results clearly indicate the possibility of the existence of only two species (*S. serrata* and *S. olivacea*) of mud crabs in Indian waters. Similarly, the *ITS-1* marker produced only two common genotypes (Genotype A with a fragment of 1,474 bp and Genotype B with a fragment of 1,282 bp), which have concordance with *S. serrata* and *S. olivacea* as reported by Imai et al. (2004). No amplified product of 1,618 bp was found to match the *S. tranquebarica* and *S. paramamosain* amplified product reported by Imai et al. (2004), indicating the possible nonexistence of these species in Indian coastal waters.

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. 2003a) using a *COI*-based

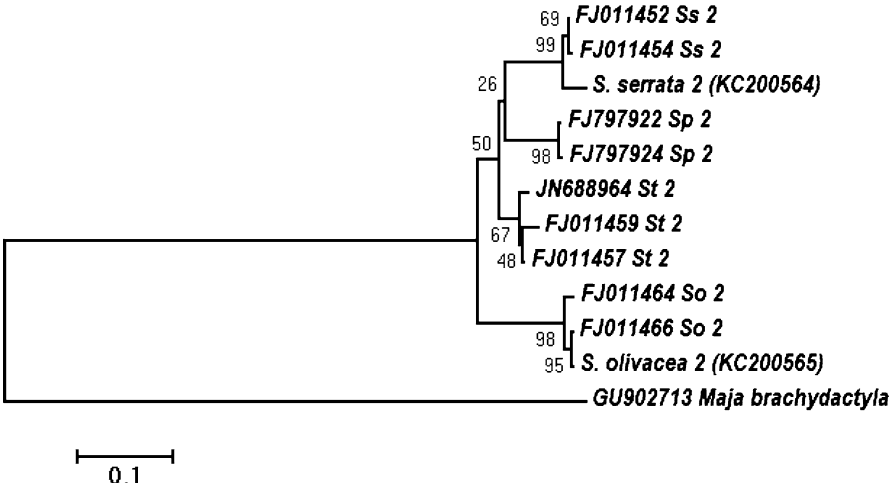

Fig. 5 Phylogenetic relationship of *Scylla* species commonly found in Indian waters. Ss 2, St 2, Sp 2, and So 2 indicate sequences generated with the second set of primers for *S. serrata, S. tranquebarica*, *S. paramamosain,* and *S. olivacea,* respectively (references taken from sequences submitted to GenBank). KC200564 and KC200565 are Indian mud crab sequences generated during the present study. *Maja brachydactyla* sequence used as an outgroup. Neighbor-joining tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

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 at least four mud crab species. Ma et al. (2012) reported the maximum interspecific distance (0.196) between *S. paramamosain* and *S. olivacea*. In the present study the genetic distance calculated between *S. serrata* and *S. olivacea* was 0.148 ± 0.017 and 0.172 ± 0.02, respectively. Our results confirmed that the *COI* gene sequence can provide distinct species-level divergence between *S. serrata* and *S. olivacea* and strongly indicated the nonexistence of *S. tranquebarica* and *S. paramamosain* in Indian coastal waters.

The results of gene sequencing and other molecular markers along with morphometric analysis used in the study clearly indicate that the green morph of the Indian mud crab is *S. serrata* and the brown morph is *S. olivacea*. The *S. tranquebarica* described by many Indian researchers should belong to *S. oceanica,* which is presently treated as a synonym of *S. serrata* by Keenan et al. (1998). Therefore, caution should be taken when interpreting or implementing the biological, molecular, and aquaculture data published in those papers, as the species quoted may no longer be accurate. In some cases, investigations may have been undertaken on a number of species of *Scylla* but were assumed to have just one species.

The present study supports the possibility of the existence of only two species (*S. serrata* and *S. olivacaea*) of mud crabs in Indian coastal waters. For most of the published literature concerning mud crabs from India, species under the name *S. serrata* (brown morph) should be considered to be *S. olivacea* and species called *S. tranquebarica* (green morph) should be treated as *S. serrata*. The number of spines

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S.serrata     TAC TCT ATA TTT CAT CTT TGG AGC ATG ATC TGG AAT AGT AGG GAC TTC ATT AAG TCT AAT TAT CCG TGC TGA ATT AGG ACA GCC AGG TAC  90
S.olivacea    *** AT* *** *** T** *** *** *** *** *** T** *** G** A** C** CC* *** *T* *** C** T** A** *** *C* *** T** A** *** *** 
              
S.serrata     ACT TAT TGG CAA CGA TCA AAT CTA TAA TGT TGT TAC CGC TCA TGC TTT TGT TAT AAT CTT TTT CAT AGT TAT ACC AAT TAT AAT TGG 180
S.olivacea    *** *** *** T** *** *** *** *** *** *** C** *** T** C** *** *** C** *** *** T** C** T** *** *** *** *** *** 180
              
S.serrata     AGG ATT TGG TAA TTG ATT AGT TCC ACT TAT ACT AGG AGC TCC TGA TAT AGC TTT TCC TCG TAT AAA TAA TAT AAG ATT CTG ACT TTT ACC 270
S.olivacea    *** *** C** A** *** *** *** *** T** *** *T* *** *** *** *** *** *** C** C** *** A** G** *** *** *** *** C** *** 270
              
S.serrata     TCC ATC TCT AAC TCT ATT ATT AAT AAG AGG TAT AGT AGA AAG AGG AGT TGG TAC AGG TTG AAC TGT TTA TCC ACC TTT AGC AGC AGC TAT 360
S.olivacea    *** T** *** *** C** TC* T** *** *** *** *** *** *** G** *** *** A** *** *** A** *** *** C** C** *** *** *** *** 360
              
S.serrata     TGC CCA TGC AGG TGC TTC AGT CGA CCT TGG TAT TTT TTC GCT CCA TCT TGC AGG TGT CTC TTC AAT CCT TGG TGC AGT TAA TTT TAT AAC 450
S.olivacea    C** *** C** *** G** C** *** T** T** *** G** *** *** T** *** *** *** *** *** A** C** *** *** A** *** *** *** *** 450
              
S.serrata     TAC TGT AAT TAA TAT ACG ATC TTT CGG TAT AAG AAT AGA CCA AAT ACC TTT ATT CGT TTG ATC TGT TTT CAT TAC GGC AAT TCT TCT TCT 540
S.olivacea    A** *** *** *** *** *** *** *** *** *** *** *** *** *** *** A** *** T** *** *** *** T** *** A** G** *** *T* A** 540
              
S.serrata     TTT ATC CCT ACC AGT TCT AGC AGG AGC AAT CAC TAT ACT TTT AAC CGA CCG AAA TCT TAA TAC ATC ATT TTT TGA CCC TGC TGG TGG TGG 630
S.olivacea    *** *** T** C** *** *T* *** *** *** C** T** A** *** CC* T** T** T** C** A** *** *** C** *** T** *** C** A** *** 630
              
S.serrata     AGA CCC TGT CTT ATA TCA ACA CTT ATT CTG ATT TTT TGG TCA CCC TGA AGT TTA CAT TCT TTT TTT ACC AGC ATT CGG TAT AAT TTC CCA 720
S.olivacea    *** *** C** TC* C** C** *** T** *** *** *** *** *** *** *** *** *** *** *** *A* C** *** *** *** T** *** *** *** A** 720
              
S.serrata     CAT TGT GAG ACA AGA ATC GGG AAA AAA AGA ATC ATT CGG AAC CCT GGG TAT AAT CTA CGC TAT AAT GGC TAT TGG TAT TCT AGG ATT TAT 810
S.olivacea    *** *** A** C** *** *** A** *** *** *** *** *** T** AT* A** *** *** *** T** *** *** C** *** *** CT* G** *** C** 810
                                                                                    Scy-F
S.serrata     TGT CTG AGC TCA CCA CAT ATT TAC AGT GGG AAT AGA CGT TGA TAC CCG AGC TTA TTT TAC ATC GGC AAC AAT AAT TAT TGC TGT TCC CAC 900
S.olivacea    *** *** *** *** T** *** *** *** A** *** T** *** *** *** *** *** G** *** *** C** *** *** A** *** *** *** *** C** *** 900
                                                                                                                                Scy-S-R
S.serrata     AGG AAT TAA AAT TTT TAG ATG ACT TAG AAC TCT CCA TGG AAC ACA AAT TAA TTA TAG GCC TTC TAT ATT ATG GGC TTT AGG ATT TAT TTT 990
S.olivacea    G** T** C** *** *** *** *** *** *** *** C** T** *** *** *** T** *** C** *** C** C** A** GC* T** A** C** *** T** *** *** 990
                                                                                Scy-O-R
S.serrata     CTT ATT TAC TGT TGG TGG TCT TAC TGG GGT TGT TTT AGC TAA TTC GTC AAT TGA TAT TAT TCT TCA TGA CAC ATA TTA TGT TGT AGC CCA 1080
S.olivacea    T** *** C** *** A** *** *** *** C** A** *** *C* *** *** *** C** A** C** C** *** *** C** A** *** *** *** *** T** 1080
                                                                                                                                Scy-R
S.serrata     TTT CCA TTA TGT TCT TTC TAT AGG AGC TGT ATT TGG TAT TTT TGC AGG TAT TGC ACA TTG ATT CCC TCT TTT TAC AGG TTT ATC TCT TAA 1170
S.olivacea    C** *** C** C** GT* A** *** *** *** C** *** C** *** *** C** C** C** *** C** C** *** T** *** *** *** *** G** C** *** 1170
              
S.serrata     TCC TAA ATG AAT AAA AAT TCA TTT TTC TAT TAT GTT TGC TGG AGT GAA CAT TAC TTT CT                                        1229
S.olivacea    *** *** *** *** *** *** *** *** *** *** A** C** *** *** A** C** A** *** T** *** A** **                                1229
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◀ **Fig. 6** Comparative combined sequences of mitochondrial *COI* gene generated using two sets of primers for commonly available mud crab species (*Scylla serrata* and *S. olivacea*) from Indian coastal waters. *Asterisk* Common site. Highlighted areas used to design species-specific primers

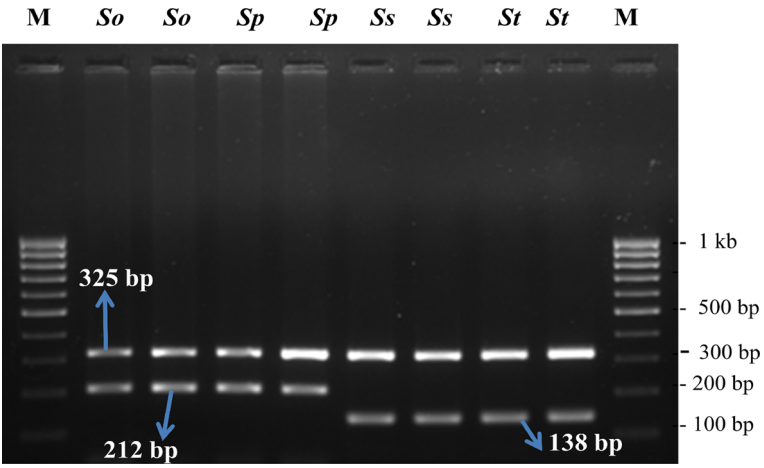

Fig. 7 PCR-based species identification of mud crab species commonly found in Indian coastal waters. *Lanes M* 100 bp DNA ladder. Species codes *So Scylla olivacea, Sp S. paramamosain* (as initially assigned), *Ss S. serrata,* and *St S. tranquebarica* (as initially assigned)

at the outer margin of the carpus of chelipeds may be considered a major identification key instead of the polygonal patterning, differences in the size of the frontal lobe spine, and the contour of the propodus spine, which may be misleading. We believe that the morphological description is insufficient for species identification of the genus *Scylla.* It is necessary to confirm the identification of these species with molecular genetic markers because accurate identification is crucial to the success of stock enhancement and breeding programs of domesticated stock.

Diagnostic Characteristics of the Common Mud Crab Species Found in Indian Coastal Waters

Scylla serrata (Forskål 1775)

1. Carapace color mostly greenish to dark greenish, which may vary depending on the habitat.
2. Chelipeds and all legs with polygonal patterning, but in some cases partially absent or may not be obvious.
3. Frontal lobe spines usually pointed sharp and with rounded interspaces.
4. Carpus of chelipeds with two sharp spines at the outer margin.
5. Palm of chelipeds with a pair of distinct spines (propodus spines).

Scylla olivacaea (Herbst 1796)

1. Carapace color predominantly red to metallic brown or dark brown, depending on habitat.

2. Chelipeds, legs, and abdomen all without obvious polygonal marking.
3. Frontal lobe spines low, rounded with shallow interspaces.
4. Carpus of chelipeds usually with one small blunt prominence (may be spinous in juveniles) ventro-medially present on outer margin.
5. Palm of chelipeds usually with a pair of blunt prominences, inner larger than outer; may be spinous in juveniles and young adult.

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