Identification of two mud crab species (genus *Scylla*) using restriction fragment length polymorphism

Mud crabs belonging to the genus *Scylla* are large portunids with high commercial value. Since the 1900s, the biology, ecology and culture of these crabs have been intensively studied in many parts of the world[1]. These species are commonly found in shallow coastal waters, lagoons, brackishwater lakes, estuaries, intertidal swamp and mangrove areas. The demand for crabs resulted in overfishing in many parts of the region. About 4–5 tonnes of live crabs are being air-lifted from India daily[2].

The uncertainty of genetic relationships and taxonomic details of the genus *Scylla* is a primary constraint to the management of the wild fishery and development of aquaculture[3]. While it is widely recognized that the mud crabs of the Indo-West Pacific region belong to more than one morph of the genus *Scylla*, there is considerable confusion about the taxonomic nomenclature[4] and the identification of species. Some authorities have not accepted the justification of Estampador[5] for the classification of members of the genus *Scylla* into different species and varieties. All morphs were placed in synonymy by Stephenson and Campbell[6], a move supported by Ong[7]. Several genetic studies to determine relationships between these different forms have been completed[8]. Keenan *et al.*[9] have examined and revised the taxonomy of the species within the genus.

Recently, increasing interest in this genus has led to studies of population structure[10], interspecific genetic variability and seed stocking with genetic tags[11]. Klinbunga *et al.*[12] reported species-specific markers for three adult mud crabs (*S. oceanica*, *S. serrata* and *S. tranquebarica*) using randomly amplified polymorphic DNA in Thailand. Allozyme analysis has been shown to be useful for discriminating between species. However, this technique has problems with respect to data analysis, as some researchers have not included allozyme electrophoresis data in their published reports[9]. Furthermore, different results have been reported using the same gel buffer systems for alleles at the same locus (i.e. aspartate aminotransferase, esterase and superoxide dismutase)[13]. The identification of larval and juvenile *Scylla* sp. based on morphological characteristics alone is problematic. The ability to identify larval and juvenile mud crabs would therefore be useful for various studies in biology and fisheries science, and for stock management of these species. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA and the first internal transcribed spacer (ITS-1) have been used to identify fish and shellfish species[14]. In the present study, we used RFLP to identify the species of *S. tranquebarica* and *S. olivacea*.

Male crabs of *S. tranquebarica* and *S. olivacea* were collected from Rajiv Gandhi Centre for Aquaculture (RGCA) hatchery, brought to laboratory and stored at –80°C for further study. To extract DNA from the adult right chelipedes of the two species (*S. tranquebarica* and *S. olivacea*), samples (10 mg) were dissected in 50 µl of TNES 8 M urea buffer and treated with 10 µl of 20 mg/ml proteinase K (Sigma–Aldrich). The mixture was then incubated for 3 h at 37°C. Then 5 µl of 10 mg/ml ribonuclease A (Sigma–Aldrich) was added and the sample was maintained at constant temperature for 30 min. The extract was then mixed with 65 µl of phenol–chloroform isoamyl alcohol (25 : 24 : 1), and centrifuged for 10 min at 10,000 *g*. The supernatant was removed and added to 100 µl of diethyl ether. The sample was then centrifuged for 10 s at 3000 *g*. The diethyl ether extraction procedure was repeated. DNA was precipitated with 5 µl of 5 M NaCl and 200 µl of 99% ethanol. The sample was centrifuged for 10 min at 10,000 *g* at 0°C, the supernatant removed, and the DNA pellet washed with 200 µl of 70% ethanol. After drying, the DNA pellet was suspended in 10–30 µl TE buffer. All samples were run on agarose gel (1.0%) to test for the presence of high molecular mass DNA.

The yield of DNA was calculated by the following formula: 50 × OD 260 of sample = concentration of DNA (µg/ml). That is, when OD 260 of the sample is 1, concentration of DNA will be approximately 50 µg/ml. The purity of DNA was checked by the method of Davis *et al.*[15]. The ratio of measurements at two wavelengths 260 and 280 nm was taken as the protein has more absorbance at 280 nm and RNA and DNA at 260 nm. The purity of DNA = OD 260/OD 280.

For RFLP, standard methodology followed by Sambrook and Russell[16] was adapted in the present study with two restriction enzymes, namely *Hin*dIII and *Eco*RI. The reaction mixture was prepared by dissolving 5 µl of sample DNA, 1 µl of enzymes (0.5 µl of *Hin*dIII and 0.5 µl of *Eco*RI), and 1 µl of enzyme buffer in 3 µl of distilled water. At the beginning the DNA isolated from 0.5 g of *S. tranquebarica* and *S. olivacea* tissue, stored and frozen was thawed quickly brought on ice and the restriction enzymes were diluted before use to a concentration of 1 unit/µl. (After taking micrograms of DNA, it is necessary to bring it on ice.) The contents in each tube were mixed thoroughly. All the

Table 1. Yield and purity of DNA

Sample	Yield	Purity
Scylla tranquebarica	1.65	1.08
Scylla olivacea	1.60	1.39

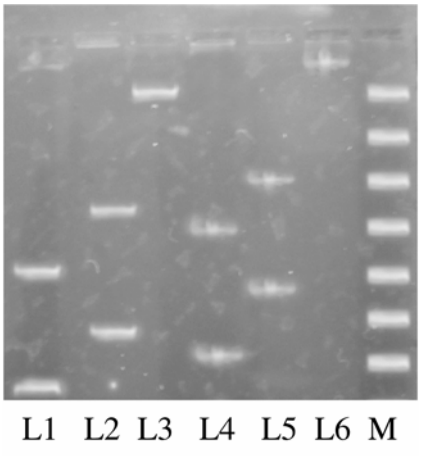

Figure 1. RFLP of *Scylla olivacea* and *Scylla tranquebarica*. Lane 1, *Hin*dIII digested DNA of *S. olivacea*; lane 2, *Eco*R1 digested DNA of *S. olivacea*; lane 3, Total DNA of *S. olivacea*; lane 4, *Hin*dIII digested DNA of *S. tranquebarica*; lane 5, *Eco*R1 digested DNA of *S. tranquebarica*; lane 6, Total DNA of *S. tranquebarica*, and lane M, Marker.

tubes were centrifuged in a microfuge for 2 s for the contents to settle at the bottom of the tubes. Then the tubes were incubated at 37°C in a water bath for 1 h. The digestion was terminated by heating the mixture at 65°C for 120 min. Again the contents were mixed thoroughly and centrifuged. Equal volumes of each digest and the samples were loaded in 1.0% agarose gel and stained with 1% ethidium bromide. After the run the bands were observed under UV illumination chamber and compared with molecular marker (200–5000 bp) (Genei, India).

DNA gel electrophoresis of *S. tranquebarica* and *S. olivacea* showed only one band lying between 2500 and 3000 bp, which confirms their correct placement in one and the same family. The banding pattern of total DNA and the two restriction enzymes on the two crabs along with molecular weight calibrations are depicted in Figure 1.

Using the RFLP double digestion with *Eco*RI and *Hin*dIII, adult *S. olivacea* (genotype A; 4005 bp) and *S. tranquebarica* (genotype B; 4322 bp) are clearly distinguishable from one another, based on species-specific fragment lengths (Figure 1). *S. olivacea* and *S. tranquebarica* can be divided into haplotypes 1 (3034 bp and 1841 bp), 2 (2443 bp and 1100 bp), 3 (2656 bp and 1349 bp) and 4 (2004 bp and 721 bp).

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