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Karyological and PCR-RFLP Studies of the Mud Crabs - *Scylla serrata* and *Scylla tranquebarica*

G. Gopikrishna* and M.Shashi Shekhar

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ABSTRACT

Karyology and Restriction Fragment Length Polymorphism (RFLP) of 12s and 16s rRNA mitochondrial PCR products were carried out in an attempt to differentiate the two species of mud crabs - *Scylla serrata* and *Scylla tranquebarica*. Testicular tissues from the two species were used for obtaining cells. A total of 60 cells each in both the species were examined for metaphase spreads. A colchicine level of 2 μg/g body weight for a period of 5-6 hours was found to be effective in obtaining clear metaphase spreads. Karyological examination revealed that the modal haploid chromosome number was 53 in *Scylla serrata* and 51 in *Scylla tranquebarica*. PCR of 12s and 16s rRNA mitochondrial genes resulted in amplification of 415 bp and 520 bp products respectively for both species of mud crabs. Restriction pattern profile was observed to be similar in both species on using Cla I and Mbo I restriction enzymes with 12s rRNA mitochondrial PCR product. Similarly, no difference in both the species was observed with restriction pattern of Mbo I restriction enzyme with 16s rRNA mitochondrial PCR product. However, RFLP of 16s rRNA mitochondrial PCR product using Hind III restriction enzyme showed a clear distinction in the restriction pattern between the two mud crab species.

Key words: *Crab, Karyology, PCR-RFLP, rRNA Mitochondrial genes.*

INTRODUCTION

Mud crabs, *Scylla* spp. is considered a valuable fisheries commodity especially in Asian countries. Mud crabs are found all along the east and west coasts of India. The taxonomic status of *Scylla* has not been well documented which has led to a lot of difficulties and uncertainty in species identification thereby necessitating an urgent need to focus research on refining its taxonomical status (Camacho and Aypa; 2001, Vay, 2001). The history of species selection and identification dates back when three species and one variety of *Scylla* was described by Estampador (1949), which was later revised to only one species *Scylla serrata* by Stephenson and Campbell (1960). Keenan *et al.*, (1998), very recently, based on allozyme electrophoresis, mitochondrial DNA sequencing and morphometric analysis categorized mud crabs into four species of *S.serrata, S. tranquebarica, S. olivacea* and *S. paramamosain*. However, the taxonomy of Scylla is still under confusion as Ronquillo *et al.*, (1999) observed inconsistency with morphological characters of neotypes reported by Keenan *et al.*, (1998) when compared to holotypes of *C.serrata* by Forsskal, *C.olivaceus* by Herbst, *S.serrata var.paramamosain* by Estampador (cited in William and Primavera, 2001).

As an aid to comprehend the different species a combination of several approaches

Genetics and Biotechnology Unit, Central Institute of Brackishwater Aquaculture, 75, Santhome High Road, R.A.Puram, Chennai- 600028, India
* Corresponding author (Email: gknani@yahoo.com)

are required. Karology of the mud crabs is one such approach. Another modern approach is by PCR-Restriction Fragment Length Polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) rRNA genes. The advantages with mtDNA lie with its high rate of evolution, maternal mode of inheritance and lack of recombination (Moritz *et al*., 1987). In the present study, karyology and PCR-RFLP of 12s and 16s rRNA mitochondrial genes has been carried out to differentiate between the two species of mud crabs (*S. serrata* and *S. tranquebarica*). The classification followed in the present study is that of Kathirvel and Srinivasagam (1992).

MATERIALS AND METHODS

Chromosome study

Mature male crabs in each of the two species were obtained from Pulicat lake near Chennai. The crabs were brought to the wet laboratory of the Muttukadu Experimental Station of Central Institute of Brackishwater Aquaculture, Chennai, and were maintained individually in 100 l FRP tanks containing well-aerated seawater (average salinity of 28.6 ppt; temperature about 28.2°C). The crabs were fed clam meat *ad libitum*. The carapace width in males was > 14 cm in *Scylla tranquebarica* and > 9 cm in *Scylla serrata*. The methods of Kligerman and Bloom (1977) and Campos Ramos (1997) were modified to obtain chromosome spreads. Colchicine was dissolved in filtered seawater and injected intramuscularly @ 2 μg/g body weight. Four to six hours after the injection, the crabs were sacrificed and the testes dissected out. Immediately the testes was cut into small pieces and introduced into a hypotonic solution containing 0.9% sodium citrate dissolved in distilled water, for about 20 minutes. Thereafter, the testes pieces were fixed using freshly prepared Carnoy's fixative (3 parts of methyl alcohol and 1 part of acetic acid). Five changes of fixative each of 10 min duration was carried out. The fixed tissue pieces were then placed on a piece of filter paper to absorb the remnants of the fixative and later introduced into a cavity block containing few drops of 50% acetic acid. The tissue pieces were then gently macerated with a fine-tipped forceps. The suspension containing cells was drawn out using a Pasteur pipette and dropped onto ethanol-cleaned warm glass slides. The slides after drying at room temperature were stained overnight using giemsa stain diluted in Sorenson's buffer. The slides were washed gently under distilled water, dried and microphotographed. A total of 60 cells each in the two species were examined. The haploid chromosome number was arrived at by computing the mode.

Mitochondrial DNA extraction

Mitochondrial DNA was extracted from eight mud crabs each in both species as described by Bouchon *et al.,* (1994), except that muscle tissue was not ground in liquid nitrogen. Approximately, 100 mg of muscle tissue was homogenized in 1.5 ml eppendorf tube using 500 µl of homogenization buffer (30 mM Tris-HCl, 30 mM EDTA, 15 mM NaCl, pH 7.8) containing 100 µg/ml proteinase K.

Amplification of mitochondrial 12s and 16s rRNA genes

Polymerase chain reaction for mitochondrial 12s and 16s rRNA genes was carried out using primers and procedure as described by Bouchon *et al*., (1994).

Restriction enzyme analysis

Amplified PCR products of 12s rRNA gene of both species was digested with Cla I, Mbo I, and Hinc II restriction enzymes, whereas, the 16s rRNA gene PCR products was digested with Hind III, Mbo I and Hinc II restriction enzymes. Agarose gel analysis was carried out after 4 h of incubation with restriction enzymes.

RESULTS AND DISCUSSION

Chromosome study

The initial work in this study was to obtain the combination of level of colchicine and the time required for obtaining good spreads. Various levels of colchicine were used: 0.01,0.03,0.04, 0.05, 0.08 and 0.1%. The time was also varied from 1 to 6 hrs. The spreads from 0.05% colchicine were of average quality. Thereafter, it was decided to administer the colchicine depending upon the body weight. Four levels of 1, 1.5, 2 and 2.5 µg were chosen for varying periods of time. The combination that gave good spreads was 2µg per gm body weight for a period of 5-6 h.

A series of short exposures to the fixative using Carnoy's fixative lasting 10 min at room temperature was found to fix the cells effectively.

The preparation of slides was also done at room temperature. However, the slides to be used have to be extremely clean, dry and warm. The cell suspension should invariably be dropped onto the slides immediately after maceration in 50% acetic acid. Staining with giemsa overnight results in the chromosomes retaining the stain reasonably well. The number of haploid metaphases counted and the chromosome number are shown in Table 1. The haploid modal chromosome number was found to be 53 in *Scylla serrata* and 51 in *Scylla tranquebarica*. Niiyama (1942) reported a haploid chromosome number of 53 (2n = 106) in *S. serrata* (cited in Murofushi and Deguchi, 1989). The chromosome spreads of *Scylla serrata* and *Scylla tranquebarica* are presented in Fig. 1 and Fig. 2. respectively.

PCR-RFLP of 12s and 16s rRNA mitochondrial genes

The PCR products of 415bp and 520 bp size were observed for 12s and 16s rRNA mitochondrial genes respectively for both *S. serrata* and *S. tranquebarica*. The 12s rRNA mitochondrial PCR product of both species on digesting with Cla I restriction enzyme, resulted in restriction fragments of approximately 275 bp and 140 bp size. The same PCR product when digested with Mbo I restriction enzyme, yielded two restriction fragments of approximately 255 bp and 160 bp size. The 16s rRNA mitochondrial PCR product when digested with Mbo I restriction enzyme, resulted in approximately 375 bp and 145 bp restriction fragments for both the species. The Hinc II restriction enzyme was non informative for both 12s and 16s rRNA PCR products of both species. (Fig. 3 and Fig. 4).

However, a clear distinction could be observed in the restriction pattern between *S. serrata* and *S. tranquebarica* with the RFLP of 16s rRNA mitochondrial PCR product using Hind III restriction enzyme. Approximately, 300 bp and 220 bp restriction fragments were generated with 16s rRNA PCR product of *S. serrata* (Lane 5, Fig. 3) whereas, 270 bp and 250 bp restriction fragments were generated with 16s rRNA PCR product of *S. tranquebarica* (Lane 5, Fig. 4).

Keenan *et al*., (1998), on sequencing mitochondrial 16 rRNA and cytochrome oxidase subunit I PCR products, observed a sequence difference of more than 8% between the mud crab species. In the present study, it was observed that Hind III restriction profile of 16s rRNA mitochondrial PCR product could perhaps be

Table 1. The number of haploid metaphases counted and the chromosome number.

Number of Chromosomes	48	49	50	51*	52	53*	54	55	Total cells
S. serrata	1	5	9	3	7	18	15	2	60
S. tranquebarica	6	8	13	13	11	4	5	0	60

* The modal haploid number in *S. serrata* is 53 and in *S. tranquebarica* is 51.

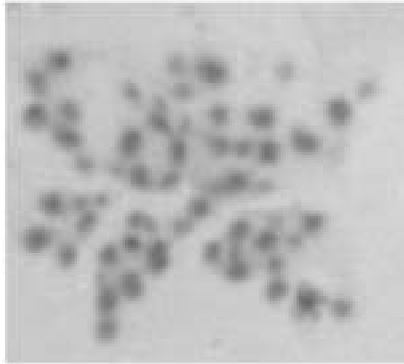

Fig. 1

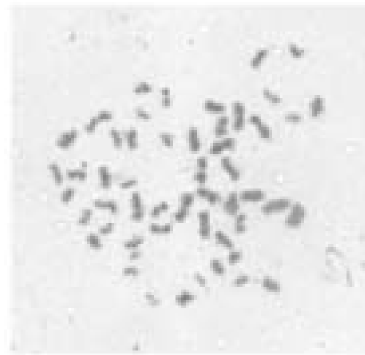

Fig. 2

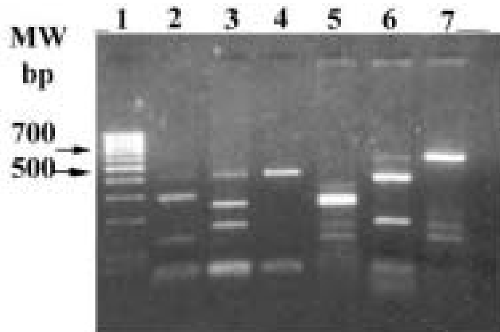

Fig. 3

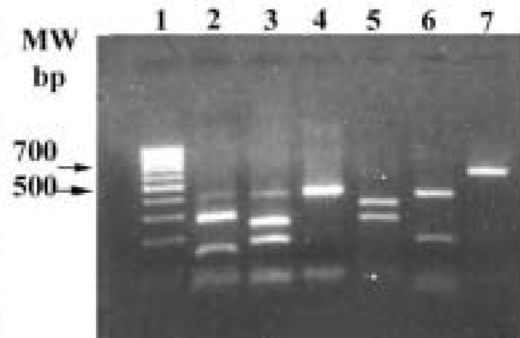

Fig. 4

Fig. 1. Chromosomes of *S. serrata*

Fig. 2. Chromosomes of *S. tranquebarica*

Fig. 3. RFLP of 12s and 16s rRNA mitochondrial PCR products of *S. serrata*. Lane 1: 100 bp marker; Lane 2-4: 12s rRNA digested with Cla I, Mbo I, Hinc II. Lanes 5-7: 16s rRNA digested with Hind III, Mbo I, Hinc II

Fig. 4. RFLP of 12s and 16s rRNA mitochondrial PCR products of *S. tranquebarica*.. Lane 1: 100 bp marker; Lane 2-4: 12s rRNA digested with Cla I, Mbo I, Hinc II. Lanes 5-7: 16s rRNA digested with Hind III, Mbo I, Hinc II.

used to differentiate *S. serrata* and *S. tranquebarica* mud crabs.

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兩種蟹 *Scylla serrata* 及 *S. tranquebarica* 細胞核學及 PCR-RFLP 的研究

G. Gopikrishna* and M. Shashi Shekhar

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本研究以細胞核學及限制片段長度多形質法(RFLP) 12s及 16s rRNA 粒腺體 PCR 產物來嘗試區分兩種蟹類 *Scylla serrata* 及 *S. tranquebarica*。所使用的細胞來自睪丸組織。每種蟹檢視了 60 個細胞分裂中期的細胞絲長度。秋水仙素相對於體重 2 μg/g 的濃度在 5~6 小時可有效地獲得分裂中期細胞絲。細胞核檢視顯示單倍體染色體型量數目在 Scylla serrata 為 53，而在 *S. tranquebarica*.為 51。12s 及 16s rRNA 粒腺體基因 PCR 的結果其放大量則分別為 415bp 及 520bp。以 12s rRNA 粒腺體 PCR 產物 Cla Ⅰ 及 Mbo Ⅰ 限制酶的限制型圖譜在這兩種蟹則相類似。同樣的 16s rRNA PCR 產物的 Mbo Ⅰ 限制酶也無差異。然而以 16s rRNA 粒腺體 PCR 產物的 RFLP 的 Hind Ⅲ 限制酶則顯示這兩種蟹的限制型圖譜有明確的區別。

關鍵詞：螃蟹，細胞核學，PCR- RFLP，rRNA 粒腺體基因。

Genetics and Biotechnology Unit, Central Institute of Brackishwater Aquaculture, 75, Santhome High Road, R.A. Puram, Chennai- 600028, India

* Corresponding author (Email: gknani@yahoo.com)