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NOTE

Identification of four mud crab species (genus *Scylla*) using ITS-1 and 16S rDNA markers

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Abstract – The first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA and mitochondrial DNA 16S rRNA were amplified by Polymerase chain reaction (PCR) using genomic DNA extracted from adult tissue of four species of *Scylla* spp. and the first zoeal stages of *S. serrata*, *S. paramamosain* and *S. olivacea* as template. Using the ITS-1 region, variation in product fragment length was found to be useful for distinguishing *S. serrata* and *S. olivacea* from two other species. The other two species (*S. paramamosain* and *S. tranquebarica*) could be identified using the restriction endonuclease *Hha* I. Using 16S rDNA, all four species were identified using PCR-restriction fragment length polymorphism (RFLP) by double digestion with *Dra*I and *Hin*dIII. These genetic markers can be used for hybridization breeding studies and in field studies of larval and juvenile mud crabs of the genus *Scylla*.

Key words: Larval stage / Species identification / PCR-RFLP / Mud crab / *Scylla* spp.

1 Introduction

Mud crabs of the genus *Scylla* are an important crustacean resource for commercial fisheries and aquaculture industries in the Indo-West Pacific region. The mud crab fishery is an important contributor to the total value of crustacean fisheries in this region. Three species of mud crab (*S. serrata*, *S. paramamosain,* and *S. olivacea*) are distributed widely in Japan (Oshiro and Imai 2003). A recent revision of the genus *Scylla* identified four distinct non-hybridizing species (Keenan et al. 1998). Recently, increasing interest in this genus has led to studies of population structure (Fratini and Vannini 2002; Gopurenko et al. 1999; Harris and Crandall 2000; Gopurenko and Hughes 2002; Gopurenko et al. 2002), interspecific genetic variability (Imai and Numachi 2002), and seed stocking with genetic tags (Imai et al. 2002). Klinbunga et al. (2000) 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 (Fuseya and Watanabe 1996; Keenan et al. 1998; Sugimoto 1990) however, this technique has problems with respect to data analysis, since some researchers have not included allozyme electrophoresis data in their published reports (Keenan et al. 1998). 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) (Fuseya and Watanabe 1996; Sugimoto 1990). The identification of larval and juvenile *Scylla* spp. 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) has been used to identify fish and shellfish species (Bouchon et al. 1994; Chow et al. 1993; Fernandez et al. 2001; Masuda et al. 1995). Murphy and Goggin (2000) reported a high divergence rate in the ITS-1 region among species of *Sacculina carcini* (Cirripedia, Rhizocephala), and Chu et al. (2001) demonstrated that the PCR product from the ITS-1 region exhibits a high degree of length polymorphism among crustaceans.

In the present study, we used genetic markers of nuclear and mitochondrial DNA to identify species origin of broodstock and larvae of *Scylla* species.

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Table 1. Sampling sites and sampling size. Asterisk marks are larvae samples.

Species	Sample size	Sampling site	Date
Scylla serrata	30	Shizuoka, Japan	April-June 1995
	10	Tungkang, Taiwan	May 2003
	10	Maputo, Mozambique	February 2003
	10	Grafton, Australia	June 1997
	10*	Okinawa, Japan	August 1999
S. olivacea	36	Shizuoka, Japan	April-July 1995
	10*	Shizuoka, Japan	September 1999
	5	Tungkang, Taiwan	February 2003
	10	Cilacap, Indonesia	January 1995
S. paramamosain	30	Shizuoka, Japan	April-August 1995
	10*	Kochi, Japan	April 1998
	10	Kochi, Japan	October 1995
	10	TungKang, Taiwan	May 2003
S. tranquebarica	5	TungKang, Taiwan	June 1999
	5	Philippine	January 2003

Table 2. Sequences of the primers used.

L primer	Sequences	H primer	Sequences
SP-1-3'	5'-ATTTAGCTGCGGTCTTCATC-3'	SP-1-5'138	5'CACACCGCCCGTCGCTACTA-3'
16sar-L	5'-CGCCTGTTTATCAAAAACAT-3'	16sbr-H	5'GGTTTGAACTCAGATCATGT-3'

2 Materials and methods

2.1 Sample collection

From 1995 to 2003, a total of 171 adult individuals of four species of genus *Scylla* were collected from seven sampling sites: Shizuoka, Kochi and Okinawa in Japan, Tungkang in Taiwan, Grafton in Australia, Cilacap in Indonesia and Philippine (Table 1). Identification of the species using morphological characteristics was based on the descriptions by Keenan et al. (1998). Larval samples (first zoeal stage) were obtained from ovigerous female specimens of *S. serrata*, *S. paramamosain,* and *S. olivacea* collected at Okinawa, Kochi, and Shizuoka, respectively, in Japan, in 1999, and fixed with 95% ethanol.

2.2 DNA extraction

Genomic DNA was extracted from 10 mg of adult leg tissue using a Get *pure* DNA Kit - Tissue (DOJINDO Molecular Technologies). To extract DNA from the first zoeal stage larvae of the three species (*S. serrata*, *S. paramamosain* and *S. olivacea*), samples were dissected in 50 μl of TNES 8 M urea buffer (Asahida et al. 1996), and treated with 10 μl of 20 mg ml^{-1} Proteinase K (Sigma-Aldrich). The mixture was then incubated for 3 h at 37 °C. Five microliters of 10 mg ml^{-1} 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 a mini gel to test for the presence of high molecular mass DNA.

2.3 DNA amplification, restriction endonuclease digestion and detection

The ITS-1 region was amplified using a two-temperature PCR with the primer sets: SP-1-3' and SP-1-5'138 (Table 2) (Chu et al. 2001). Amplification products were compared to detect species-specific fragment lengths. The PCR reaction was performed in a 25 μl volume, using *Z-Taq*™ (Takara Shuzo). A thermal cycler GeneAmp® 9700 (Perkin-Elmer) was used with the following parameters: denaturation at 94 °C (60 s), followed by 25 cycles of denaturation at 98 °C (5 s) and annealing-extension at 72 °C (20 s). Five microliters of the amplified PCR product was tested on a 1% electrophoretic TreviGel™ 500 (Trevigen), and stained with ethidium bromide for photographing.

A 562 bp fragment of the 16S rRNA gene of mitochondrial DNA (mtDNA), was amplified using the following PCR technique. The PCR primers used were 16sar-L and 16sbr-H (Table 1) (Palumbi et al. 1991). The selected region was amplified in a 25-μl reaction mixture volume containing 2.5 μl of 2.5 mM dNTPs, 2.5 μl each of 25 pM primers, 1 μl of template DNA, 2.5 μl of recombinant Ex buffer and 0.5 units of *ExTaq*™ DNA polymerase (Takara Shuzo). Thirty PCR cycles were carried out under the following conditions: denaturation at 94 °C (60 s), annealing at 45 °C (30 s) and extension at 72 °C (90 s). The PCR reactions were started after initial denaturation at 94 °C (2 min) and stopped after a final extension at 72 °C (10 min). Two restriction endonucleases,

Table 3. Distribution of mtDNA haplotypes in mud crabs sampled from seven sampling site. Abbreviations: So, Shizuoka; Kc, Kochi; On, Okinawa; Tk, Tungkang; Mt, Maputo; Gf, Grafton; Asterisk marks is larvae samples.

haplotype	*S. serrata*					*S. olivacea*				*S. paramamosain*				*S. tranquebarica*	
	So	Tk	Mt	Gf	On	So	So	Tk	Cp	So	Kc	Kc	Tk	Tk	Pp
1														5	5
2	30	10	10	10	10*										
3						36	10*	5	10						
4										28	10	10*	9		
5										2	0	0	1		

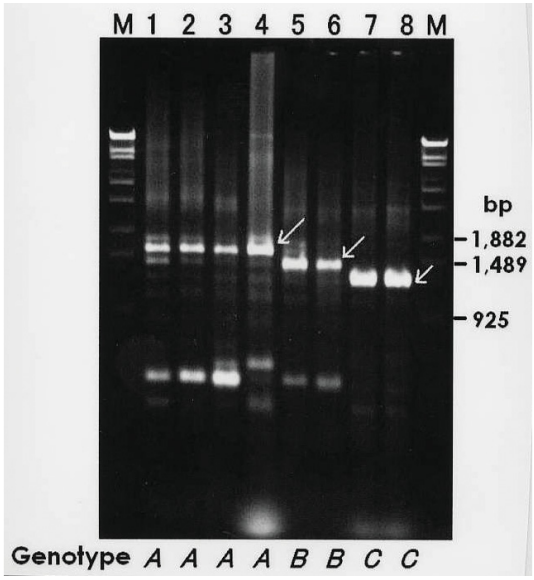

Fig. 1. Electrophoretic patterns of the ITS-1 PCR products from four mud crab species. Lane M: molecular marker. Lane 1, 2: *Scylla tranquebarica*; 3, 4: *S. paramamosain*;. 5, 6: *S. serrata*; 7, 8: *S. olivacea*. The numbers shown at the right are molecular size of markers (bp).

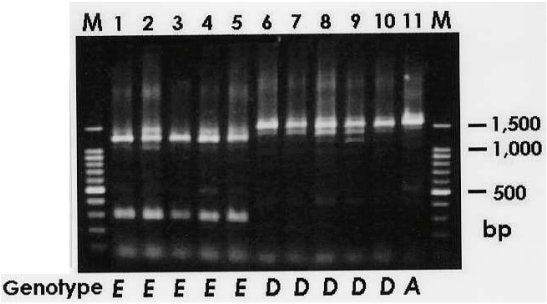

Fig. 2. Electrophoretic fragment patterns from the ITS-1 region for *Scylla paramamosain* and *S. tranquebarica* obtained by digestion with *Hha* I. Lane M: molecular marker. Lanes 1-5: *paramamosain*; 6-10: *S. tranquebarica*; 11: undigested *S. paramamosain*. The numbers at the right are molecular size of markers (bp).

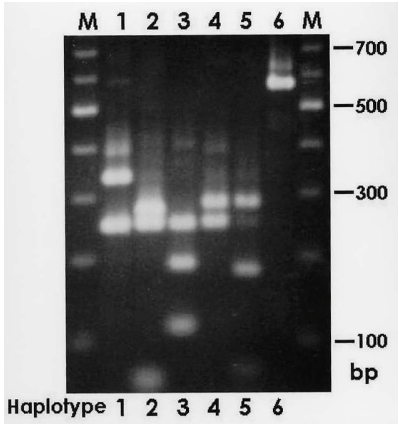

Fig. 3. Electrophoretic fragment patterns of 16S rRNA produced by double digestion with *Dra* I and *Hin*d III. Lane M: molecular marker. Lane 1: *Scylla tranquebarica*; 2: *S. serrata*; 3: *S. olivacea*; 4, 5: *S. paramamosain*; 6: no digestion. The numbers on the right are molecular size of markers (bp).

*Dra*I and *Hin*dIII were selected from restriction sites on the DDBJ/ EMBL/ GeneBank database of 16S rRNA sequences of the four species (*S. serrata* (AF109318), *S. paramamosain* (AF109319), *S. tranquebarica* (AF109320), and *S. olivacea* (AF109321)). Products were tested by electrophoresis using a 2% gel under the same conditions as for mtDNA analysis.

3 Results and discussion

Using the ITS-1 PCR technique, adult *S. serrata* (Genotype *B*; 1474 bp) and *S. olivacea* (Genotype *C*; 1282 bp) were clearly distinguishable from the other two species, based on species-specific fragment lengths (Fig. 1). Although non-specific amplification bands were also found along with the target ITS-1 gene segment, these bands did not interfere the ability to identification among species. Thirty restriction endonucleases were tested in the preliminary screening using one adult individual of each of the other two species (*S. paramamosain* and *S. tranquebarica*). Figure 2 shows the distinctions between *S. paramamosain* (Genotype *E*; 1283 bp and 330 bp) and *S. tranquebarica* (Genotype *D*; 1558 bp), based on results of Genotype *A* (1618 bp) digested with the restriction endonuclease *Hha* I (Takara Shuzo). These ITS-1 markers was successfully amplified 100% of investigated all specimens.

Figure 3 show that all four adult mud crab species could be identified using PCR-RFLP of double digestion with *Dra*I and *Hin*dIII (Takara Shuzo). These 16S rDNA markers was successfully amplified 100% of investigated all specimens (Table 3). It was found that *S. tranquebarica*, *S. serrata*, *S. olivacea*, and *S. paramamosain* could be divided into haplotypes 1 (325 bp and 238 bp), 2 (268 bp, 268 bp and 55 bp),

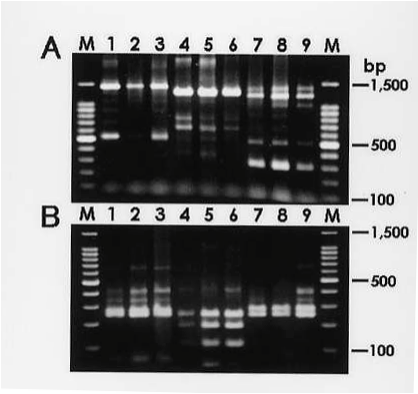

Fig. 4. Electrophoretic fragment patterns of undigested ITS-1 from 2 different species (Lanes 1–6), *Hha* I digested ITS-1 of *Scylla paramamosain* (Lanes 7–9) (A) and the 16S rRNA product produced by double digestion with *Dra* I and *Hin*d III (Lanes 1–9) (B) from the first zoeal stage samples. Line M: molecular marker. Lane 1–3: *S. serrata*; 4–6: *S. olivacea*; 7–9: *S. paramamosain*. The numbers on the right are molecular size of markers (bp).

3 (238 bp, 188 bp and 111 bp), and 4 (277 bp and 238 bp) + 5 (277 bp, 174 bp and 64 bp), respectively. Polymorphisms of fragment pattern haplotype 5 were observed in three individuals of *S. paramamosain*. This technique was found to unambiguously identify all four species of mud crab.

The genotypes and haplotypes of the first zoeal stages of *S. serrata*, *S. olivacea*, and *S. paramamosain* could be distinguished (Fig. 4).

The results of this study showed that the four mud crab species could be distinguished using genetic markers. The ability to identify these species has several applications for field studies of the distribution range of larvae and the environmental factors that affect the growth and survival of juveniles, and for hybridization breeding studies.

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