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PRIMER NOTE

Isolation and characterization of 23 polymorphic microsatellite markers for diversity and stock analysis in tiger shrimp (*Penaeus monodon*)

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Abstract

The tiger shrimp (*Penaeus monodon*) is an important marine crustacean in terms of biological diversity and aquaculture resource. The shrimp is widespread across the Indo-Pacific region and shows apparent genetic differentiation among geographical populations. It is common practice to transport female brooders between different countries to seed the shrimp farms, posing potential problems of unwanted population admixture. We developed 23 polymorphic microsatellites for *P. monodon* (average H_E = 0.936) and these microsatellites were applicable for studying population differentiation, identifying valid stocks and tagging nonindigenous farmed shrimps.

Keywords: microsatellite, molecular tagging, Penaeus monodon, stock

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The tiger shrimp (*Penaeus monodon*) is an important marine species for two reasons. First, the species is widely distributed across the Indian and Pacific Oceans, suitable for genetic analyses that have a broad implication for the evolution of biodiversity in marine environments (Duda & Palumbi 1999; Benzie *et al*. 2002). Second, the tiger shrimp is one of the most economically important crustaceans in the world, subjected to fishery exploitation and intense aquaculture practice (Bailey-Brook & Mass 1992; Lucien-Brun 1997). The shrimp aquaculture industry still relies on wild broods to seed farmed shrimp populations. Consequently, the gravid female brooders have been transported between regions of high aquaculture intensity, in particular southeast Asian countries and Australia. The exchange of female brooders creates a situation that is prone to the admixture of local and introduced populations once the cultured shrimps escape. We developed microsatellite primers for *P. monodon* in an attempt to: (i) investigate the genetic diversity of natural tiger shrimp populations; (ii) identify valid stocks or management units (MU; *sensu* Moritz 1994); (iii) tag the introduced shrimps for monitoring purposes; and (iv) increase the number of available markers for genetic mapping purposes.

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Genomic DNA for constructing the partial libraries was prepared according to the procedures in Sambrook *et al*. (1989). Genomic DNA was digested with *Sau*3AI and fractioned on a 1% agarose gel. DNA (size range 600–1200 bp) was isolated, purified with a GFX™ Band Purification kit (Amersham) and ligated into plasmids PUC18/*Bam*HI/BAP (Amersham) according to the manufacturer's protocols. Ligated plasmids were transformed into the competent ECOS 101 cells (Yeastern Biotech). Recombinant clones containing inserts were transferred to Hybond-N^+ nylon membranes (Amersham), which were hybridized to a set of oligonucleotide probes, including $(AC)_{15}$, $(AT)_{15}$, $(AG)_{15}$, and $(AAG)_{10}$. Probes were labelled with a DIG Oligonucleotide 3′-End Labeling Kit (Roche). Hybridization was performed at 55 °C for 16 h in a standard hybridization buffer consisting of 5 × SSC, 0.1% sodium *N*-lauroylsarcosine, 0.2% sodium dodecyl sulphate (SDS), and 1% Blocking Reagent (Roche). The membranes were washed twice, each for 5 min at 55 °C with a solution of 2 × SSC and 0.1% SDS, and then twice, each for 15 min at 65 °C with a solution of 0.1 × SSC and 0.1% SDS. Chemiluminescent detection was performed with a DIG Luminescent Detection Kit (Roche). A total of 832 positive clones was sequenced on an automated sequencer (MegaBACE 500; Molecular Dynamics) and 393 contained dinucleotide, trinucleotide or other types of repeats. We designed primers only for those

microsatellite clones that contained flanking sequences of more than 50 bp at both ends. The on-line program PRIMER 3.0 (Rozen & Skaletsky 2000) was used to design primers from flanking regions of microsatellite DNA loci.

Individual genotypes were determined by the polymerase chain reaction (PCR). Each PCR reaction totalled 10 µl, containing 50–100 ng template DNA, 0.5 U *Taq* DNA polymerase (Promega), 1.5 mM Mg^{2+}, 0.1 mM dNTP, 10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton X-100, 0.3 µM of each primer, with the forward primer being end-labelled with fluorescent dye (FAM, HEX or TAMRA). Amplification was carried out by the thermal profile: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, optimal annealing temperature (Table 1) for 30 s, 72 °C for 30 s, and a final

Table 1 Characteristics of 23 polymorphic microsatellite loci in *Penaeus monodon* (n = 30)

Locus	Repeat motif	GenBank Accession no.	Primer sequences	T_a (°C)	Size range (bp)	No. of alleles	H_O	H_E
PM138	$(GT)_{47}$	AY500853	F: 5′-ACGGAGTGGGTAGAGACATA R: 5′-ACAAGCGAAGTGAAGAGG	56	268–338	22	0.833	0.951
PM205	$(AG)_{23}$	AY500854	F: 5′-AGGAATGATGGGAGGGAAAG R: 5′-AAGCTCAGGCAAGCGTGTAT	56	153–209	17	0.867	0.876
PM528	$(AC)_{44}TC(AC)_{14}$	AY500855	F: 5′-GTGTTATTTTCCACGGGTGC R: 5′-GCTGCAGGAAGTGTAGGGAG	56	245–325	21	0.767	0.947
PM580	$(AG)_{28}$	AY500856	F: 5′-AACTGCCTACAGTGTGTGCG R: 5′-GAATGGAGCCTGTTGGTTTG	56	250–340	23	0.4	0.953
PM1091	$(GT)_{28}$ … $(GCGTGT)_7(GT)_{10}$	AY500857	F: 5′-TTCACGACCCAGTATGTCCA R: 5′-CAGGTCGCAGGCTCATATTT	50	232–406	29	0.633	0.963
PM1713	$(CT)_{44}$	AY500858	F: 5′-GTTGCGACGGGTTGATTC R: 5′-TTTATGGCTATGGCTGACAC	60	261–433	27	0.433	0.962
PM2334	$(GT)_{16}$	AY500859	F: 5′-TAATTTCTCTGCAACGTCCT R: 5′-CTGCTCATTATCCAGTCCAT	50	268–330	20	0.533	0.789
PM2345	$(TC)_{42}$	AY500860	F: 5′-GATATTTCAAGGAATGCTCG R: 5′-TAATTCGTGCCTTACCTCAT	56	143–229	20	0.333	0.918
PM3538	$(AC)_{12}$	AY500861	F: 5′-GAACGTCGGGGGATTTACTT R: 5′-ACTATCACACCGAGGCTTGG	56	371–441	17	0.5	0.932
PM3852	$(TG)_{15}$ … $(TG)_{27}$	AY500862	F: 5′-TAATGGGCGTAAGTCTTCGG R: 5′-TGAAAGGAGTCGGGATATGC	56	193–303	31	0.533	0.972
PM3854	$(GT)_{16}$ … $(GT)_{33}$	AY500863	F: 5′-TCTTGGTCGGAATGGGTAAG R: 5′-TTCTGAGAAGGCACACATGC	56	184–316	27	0.6	0.954
PM3945	$(GT)_{50}$	AY500864	F: 5′-TTTGGACTTCACAATCGGTG R: 5′-CGGCTGAACAGGTCTGAAAT	52	234–312	25	0.7	0.96
PM4018	$(AC)_{27}$	AY500865	F: 5′-GTTCCAAGCGACAGACGAGT R: 5′-CGAATGCACTGCCTGTATGT	54	177–255	20	0.733	0.941
PM4089	$(CA)_{44}$	AY500866	F: 5′-CTTTTTGAAATCGCCCTGTT R: 5′-CATTCATCCCGCTCTTCTGT	56	243–377	25	0.7	0.964
PM4505	$(CA)_{34}$	AY500867	F: 5′-CTTCTAGCGCCATTTCAAGG R: 5′-TCCTTCCAGTGTTCGGAGTT	56	333–393	22	0.5	0.954
PM4793	$(GA)_6$ … $(GA)_6$	AY500868	F: 5′-CATTCACACACGTTCACCTG R: 5′-CCCAGTTCACGTATCGTGTG	56	227–335	24	0.633	0.959
PM4798	$(TG)_{32}$ … $(TG)_{16}$	AY500869	F: 5′-GCTTGCGTGTGTGCATACTT R: 5′-GTTCCCCTCGTGTTTACGAA	52	275–431	30	0.5	0.967
PM4858	$(AC)_{16}$	AY500870	F: 5′-GCCTTGTTACGGTGGAGGTA R: 5′-CGGCCTATAACTGTCTGCCT	56	215–295	23	0.833	0.955
PM4868	$(GT)_{10}$ … $(GT)_{16}$ … $(GT)_{19}$	AY500871	F: 5′-CCAGCATACCGCTTACCAAT R: 5′-GTCCCTTAACCTCTCCTGCC	56	266–356	19	0.567	0.835
PM4927	$(CA)_{25}$	AY500872	F: 5′-GGGGAATTAATCTGCCCATT R: 5′-AATGGCACAAGCAAAAGGAC	54	296–362	26	0.667	0.954
PM5213	$(AT)_6$ … $(CA)_{19}$	AY500873	F: 5′-TGGACTGAGGTATGCAGCAC R: 5′-TCCTTGTTTGGAACCCTTTG	53	231–283	15	0.4	0.889
PM5271	$(AC)_{23}$	AY500874	F: 5′-AAAACACTCAGGGGAACACG R: 5′-CGTGAGCCATAGCTGTAGCA	53	222–400	29	0.6	0.967
PM5625	$(CT)_{16}$	AY500875	F: 5′-AAAAGCCAGAGGAAACGTG R: 5′-ACAGTGCACGTACCCACAAA	52	172–246	24	0.933	0.961

T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity.

extension step at 72 °C for 20 min. The PCR products were run on linear polyacrylamide gels with a MegaBACE 500 automated sequencer. The ET-400 Size Standard (Amersham) was used as a size marker to determine the allele sizes. Most of the allelic PCR products differed in multiples of their repeat motifs. The individuals with ambiguous genotypes were amplified and scored at least twice to determine the allele sizes.

Twenty-three microsatellites were found to be polymorphic among 30 wild tiger shrimps from three disparate localities in Taiwanese waters (Table 1). The number of alleles per locus ranged from 15 to 31. The observed and expected heterozygosities ranged from 0.4 to 0.933 and from 0.789 to 0.972, respectively. The observed genotypes deviated from Hardy–Weinberg expectation at 20 of the 23 loci ($P < 0.05$), resulting from heterozygote deficiency. If the shrimps from the three localities are representatives of different stocks (MUs), the deficiency may be due to combining samples of the three different capture localities (i.e. different stocks). However, null alleles cannot be ruled out.

We tested for fluorescent multiplex genotyping with the loci of the same annealing temperature. An equal proportion of primer pairs was applied and the conditions for PCR were as described above. Four sets of loci (labelled dye) gave satisfactory results: (i) PM205 (FAM), PM528 (HEX), and PM4868 (TAMRA); (ii) PM4089 (FAM) and PM4858 (TAMRA); (iii) PM3852 (FAM), PM4505 (FAM), and PM4793 (TAMRA); and (iv) PM3945 (FAM) and PM4798 (TAMRA). For set (iii), two pairs of primers (PM3852 and PM4505) were labelled with the same dye but the alleles at the respective locus were readily discernable by size. In addition, all the 30 shrimps tested could be assigned a unique composite genotypic 'bar code' by applying any of the four sets of multiplex PCR. These results show prospective applications with these microsatellite molecular tags for tiger shrimps.

As many microsatellite loci for *P. monodon* have been published for various purposes (e.g. Xu *et al*. 1999; Wuthisuthimethavee *et al*. 2003), we searched in existing relevant databases to detect redundant loci. None of our primer sequences were overlapping with existing ones. In addition, three of the 23 loci yielded positive results, two with *P. monodon* (GenBank accession nos. AF077583 and AY188966) and one with *P. vannamei* (GenBank accession no. AY466256). Nonetheless, neither of the two matches with *P. monodon* could be aligned with our loci. The single match with *P. vannamei* could be aligned with 56% identity and could be a homologous locus between the two shrimp species. The 23 microsatellite loci reported here will merge with the existent microsatellite data for *P. monodon*, leading to an attempt to construct a genetic map for the species.

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