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ISOLATION AND CHARACTERIZATION OF NOVEL MICROSATELLITE LOCI IN GREEN TIGER SHRIMP (*PENAEUS SEMISULCATUS*)

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ABSTRACT

The green tiger shrimp (*Penaeus semisulcatus*) is an economically important species among native Iranian Shrimps. Microsatellite loci were developed for population genetic assessment of this species in Iranian waters. A total of eight polymorphic microsatellite loci were developed and tested on 40 individuals. The number of alleles per locus ranged from 5 to 12 with average of 7.38 per locus. The values of the observed and expected heterozygosities ranged from 0.100 to 0.800 and 0.534 to 0.869 respectively. These novel microsatellite markers provide useful tools to assess genetic variation and genetic structure in the populations of *P. semisulcatus* to facilitate a selective breeding program.

Key words : Microsatellite, green tiger shrimp, *P. semisulcatus*

INTRODUCTION

The green tiger prawn, *Penaeus semisulcatus* De Haan has wide geographical distribution, ranging from the waters of south and east Africa to India and Sri Lanka, including the Red Sea, the Persian Gulf and western Madagascar (Holthuis LB, 1980). Its distribution extends as far as Korea Peninsular, Japan, the Philippines, New Guinea and northern Australia; the species has also entered the eastern Mediterranean through the Suez Canal and now forms the basis of an important fishery in that region (Holthuis LB, 1980).

Shrimp farming in Iran began 20 years ago with the activity seen as a good way to develop the otherwise unproductive salty coastal flats. The major cultured shrimp species in Iran include *P. indicus* and *P. monodon*. Presently, *P. semisulcatus* is being investigated as the future aquaculture shrimp species by the Iranian Fisheries Research Organization (IFRO).

Assessment of diversity and population structure is of enormous importance to understanding in order to ensure sustainability of fishery resources. Such assessments are also an important process in the selection of broodstock for breeding programs since different broodstocks may well differ in growth rate, disease resistance, or other characteristics (Lester LJ and Pante MJR, 1992).

Many microsatellite markers have been characterized for commercially important marine shrimps e.g., *P. vannamei* (Bagshaw JC and Buckholt MA, 1997), *P. japonicas* (Moore SS et al. 1999), and *P. monodon* (Xu Z et al. 1999). Xu Z et al. (2001) successfully employed microsatellite DNA to study the genetic structure of wild *P. monodon* populations in the Philippines and its association with mangrove status and shrimp culture systems. Vanavichit A et al. (1998) used microsatellites as genetic markers in *P. monodon*, and suggested that microsatellites are suitable for shrimp genome study and broodstock management.

This study was performed in order to isolate and characterize microsatellite markers from *P. semisulcatus* to facilitate population studies for populations from Iranian waters. As noted above, *P. semisulcatus* has been identified as a future shrimp species for mass-breeding in Iran. For this reason, assessment of genetic diversity is urgently needed to enable broodstock selection and selective breeding of the species. The microsatellite markers described here offer a valuable tool for the assessment and understanding of levels of genetic variation for a breeding program.

MATERIALS AND METHODS

Sample collection and DNA extraction

Genomic DNA was isolated from pleopod tissues of a single individuals using the phenol-chloroform method (Taggart et al. 1992). Total genomic DNA was extracted from 40 individuals collected from Hormoz (Persian Gulf). 0.1- 0.2 g of frozen pleopod tissues were mixed with 600µl DNA extraction buffer (10mM Tris-HCl, pH 7.5, 100mM EDTA, 1µg/ml proteinase K and 0.05 µg/ml RNase) and subsequently ground using a mortar and pestle. The resultant homogenate was incubated for 12h at 55ºC. DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1), and precipitated in 100% cold ethanol. Extracted genomic DNA was diluted to a working concentration of 50 ng/µl in deionized water and stored in -20ºC.

Development of microsatellite libraries

Genomic DNA libraries enriched for microsatellites were constructed based on Edwards KJ et al. (1996) with modifications as per Nguyen T et al. (2007). The genomic DNA (250 ng) was digested extensively with RsaI enzyme followed by ligation using Mlu1 oligo-adaptor and T4 DNA ligase enzyme. The ligated DNA was then denatured by boiling for 5 minute and hybridized in 500 µL hybridization buffer [50 % formamide, 3x standard saline citrate (SSC; 45 mM sodium citrate, pH 7·0, 450 mM NaCl), 25 mM Na-phosphate, pH 7·0 and 0·5 % sodium dodecyl sulphate (SDS)] containing 1 µg of the 21-mer oligonucleotide (Oligo1). The DNA solution was incubated at 50 °C for ~ 48 h and hybridized on single hybond N+ membrane with bound oligo nucleotides including of 5-14 repeats of di-, tri-, and tetra nucleotides motifs including GACA, GATA, AAAT, GATG, CAA, CA, GT, AAT, CT, and AAG. Fragments which contained microsatellite motifs were directly ligated into 10 ng of a modified pGEM-T Easy vector (Promega, Madison, Wisconsin, USA).

The ligation mixture was transformed into JM109 *Esherichia coli* competent cells (Promega) and plated onto LB-agar containing 10 mg/mL ampicillin. To allow for blue- white selection, the plates were spread with X-Gal (40 µL of 50 mg ml-1 stock solution) plus IPTG (20 µL of 100 mM stock solution). Colonies were transferred onto micro-plates after overnight incubation at 37 °C. After blue – white screening, 96 positive colonies were amplified at 30 ºC for 16 h using TempliPhi DNA Sequencing Kit (GE Healthcare). The plate was sent to 1st BASE Laboratories (Malaysia) for direct sequencing in both directions conducted under BigDyeTM terminator cycling conditions. 34 primer pairs were designed for the flanking regions of each microsatellite loci using Primer3 plus software (Rozen S and Skaletsky HJ, 2000). Fluorescently labeled primers were synthesized by the 1st BASE Laboratories (Malaysia).

Amplification and characterization of microsatellite loci

Compound microsatellite primers were labeled with fluorescent dyes 6-FAM, HEX, ROX or TAMRA (1st BASE) and used for PCR amplification, with their respective primers. Amplification of microsatellite loci was carried out in a 25 µL reaction volume, containing 100 ng DNA template, 0.6 µM of forward and reverse primers, 25 mM of MgCl2, 2 mM of dNTPs, 5X PCR buffer and 5 U/µl *Taq* DNA polymerase (Promega USA).

All loci were amplified on a MJ Research PTC-200, Peltier Thermal Cycle (Waltham, USA). The cycling profile was as follows: initial denaturation of 94º C for 4.5 min, followed by 40 cycles of denaturinge at 94ºC for 30 s, annealing with temperature indicated for each primer (Table 1), and extension at 72ºC for 90 s with a final extension of at 72ºC for 5 min. Results were analyzed using the allele size standard GeneScan-

500 LIZ, and Peak Scanner v1.0 (Applied BioSystems).

Statistical analysis

The number of alleles observed (N_A), expected heterozygosity (H_E), observed heterozygosity (H_O) and test for deviation from Hardy-Weinberg equilibrium were performed using GENALEX version 6.4 (Peakal R and Smouse PE, 2006). MICRO-CHECKER (Van Oosterhout C, 2004) was used to perform a null allele analysis. Arlequin ver. 3 was used to check significant linkage disequilibrium among loci (Excoffier L et al. 2005).

Of the 96 clones which revealed presence of insert DNA, 34 were chosen for primer design and subsequently used for evaluation of microsatellite polymorphisms in *P. semisulcatus*. The remainder were eliminated either because they contained inserts that were too small (< 10 repeats) or had a very short flanking sequence between the repeat sequences. The numbers of repeats in 34 clones which were chosen for primer design ranged from 11 to 43. (AG) n dinucleotide repeats formed the majority, and trinucleotide repeats the minority with only one clone for (TGT) n, (GAT) n, (CAA) n and (ATT) n. Information on all microsatellite loci is summarized in Table 1.

RESULTS AND DISCUSSIONS

Table 1. Characterizations of eight microsatellite loci in P. semisulcatus. Number of individuals examined (n), annealing temperature (Ta), number of alleles (Na), observed heterozygosity (Ho) and expected heterozigosity (He) are listed for each locus.

Locus	sequence	Repeat units	Ta(°C)	Size Range (bp)	Na	Ho	He	P-value	Accession No
E2	F: TGCGTGGAGTAACTGTGAGC R: TGTGGAATTGTGAGCGGATA	$(GT)_{13}$	48	161-183	7	0.700	0.534	0.495	HQ877820
H9	F: CGGGAATTCGATTTAGTCCA R: CTCAAGCTATGCATCCAACG	$(CA)_{11}$	48	215-239	6	0.800	0.726	0.039	HQ877823
C9	F: ATGCTGTGCTTATGCCATGT R: AGCGCAGGGTCTTTTGATTA	$(GT)_{17}$	48	270-316	8	0.100	0.780	****	HQ877819
C6	F: AAGGGAAGATTGGATTGGAGA R: AGTGGGTGTTTCCGATTACG	$(AG)_{22}$	56	400-442	7	0.375	0.556	0.007	HQ877818
B5	F: ATGCGAGCAGTGTGCTCTT R: CTCAAGCTATGCATCCAACG	$(TG)_{13}$	56	170-220	12	0.375	0.831	****	HQ877822
F11	F: AATTGTCCTTCGCAAAAGGT R: CTCAAGCTATGCATCCAACG	$(GAT)_{13}$	58	231-277	9	0.250	0.869	****	HQ877821
B9	F: TGACAGGCTATCAGGCAGAG R: GACTGCAGTGAAGGGGTGT	$(CA)_{11}$	56	150-206	5	0.500	0.546	0.204	HQ877816
F5	F: GGCCGGCAATTATTTAGTCA R: AGGTCGCAGGTCACTGCTAT	$(TC)_{11}$	65	124-170	5	0.750	0.689	****	HQ877817

Note: Accession is GenBank ID derived from NCBI
**** for P-value <0.0001

Only eight of the 34 primers designed produced good amplified PCR products and polymorphisms. The remainders of the primers were not easily amplified, or monomorphic or produced stutter bands. Of the succesfull eight primers, seven were dinucleotide repeats and one a trinucleotide repeat. The eight primer pairs were used to amplify 40 *P. semisulcatus* individuals to test for usefulness in population genetic study. A total of 59 alleles were recorded from the 40 individuals. The number of alleles ranged from 5-12 per locus. Allele sizes ranged from 124 to 422 bp across eight microsatellite loci. Observed heterozygosity ranged from 0.100 to 0.800, while the expected heterozygosity ranged from 0.534 to 0.869, respectively.

MICRO-CHECKER was used to identify the most likely causes of deviation from HWE. Micro-Checker analysis showed no sign of scoring error owing to stuttering, or from large allele dropout. However, Micro-Checker analysis showed signs of null alleles in four microsatellite loci (B5, C6, C9 and F11), maybe owing to non-random sampling. The significant of linkage disequilibrium was detected in loci B5 and F11.

Investigation in this study on the eight loci based on Hardy Weinberg equilibrium showed that the observed heterozygosity was less than the expected heterozygosity in five loci (C9, C6, B5, F11 and F5). Loci E2 and B9 showed significant deviation from HWE equilibrium ($P<0.05$). These observed deficiencies may be regarded as owing to low variation through inbreeding, genetic drift or the existence of null alleles.

All eight loci used were highly polymorphic, exhibiting between 5-12 alleles at each locus. The high polymorphism (100%) observed in all eight microsatellite loci was comparable to studies by Brooker AL et al. (2000) and Xu Z et al. (2001). Ball AO et al. (1998) found 83% of microsatellite loci used in *P. setiferus* were polymorphic. Other researchers have also illustrated a high number of polymorphic alleles per microsatellite locus e.g., 14–28 alleles reported for two loci in *P. monodon* from Thailand (Tassanakajon A et al. 1998) and also 4–24 loci identified for *P. japonicus* (Moore SS et al. 1999).

These novel microsatellite markers will provide useful tools to assess genetic variation and genetic structure in the populations of *P. semisulcatus*. These results could assist to study genetic diversity of natural and farm populations, as well as for resource conservation and future management plans.

CONCLUSIONS

A total of eight novel polymorphic microsatellite loci were successfully isolated and characterized for *P. semisulcatus*. These primers were proved useful for future genetic diversity assessment and population structure studies for the species.

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