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Full Length Research Paper

Isolation and characterization of polymorphic microsatellite loci from cDNA library of *Scylla paramamosain*

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In this study, we reported 36 novel polymorphic microsatellite markers derived from a cDNA library of *Scylla paramamosain*. The polymorphisms of these markers were detected in a sample of 24 individuals. A total of 159 alleles were detected with an average of 4.4 alleles per locus. The number of alleles per locus ranged from two to eight. The observed and expected heterozygosity per locus ranged from 0.100 to 1.000 and from 0.097 to 0.819, with an average of 0.679 and 0.625, respectively. Five loci significantly deviated from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.0014$) and no significant linkage disequilibrium between pairs of loci was found. These loci will provide useful information for the studies on genetic diversity and structure, construction of genetic linkage maps and the effectively management of *S. paramamosain*.

Key words: *Scylla paramamosain*, microsatellite markers, cDNA, polymorphism.

INTRODUCTION

Scylla paramamosain is mainly distributed in the southeastern coastal region of China, as well as in the tropical and warm temperate zones in the Pacific and Indian Oceans (Keenan, 1999). This species is a commercially important species because of its market value and taste. The aquaculture of *S. paramamosain* has been carried out for many years in southern China and other Asian countries (Cowan, 1985), but the wild-caught seed was the main source of *S. paramamosain* farming (Keenan, 1999). Moreover, the wild resource of *S. paramamosain* has decreased sharply under the pressure of overfishing and pollution. In order to conserve and sustainably exploit this crab resource, study on its population genetic structure is very necessary. For population structure and genetic background of *S. paramamosain*, some reports are available based on mitochondrial DNA (Ma et al., 2006; Obata et al., 2006). However, few research based on microsatellite markers are reported for this species.

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Microsatellites are short tandem repeats DNA with a length of 1-6 bp, widely existing along the eukaryotic genome (Weber and May, 1989; O'Reilly and Wright, 1995). Because of high polymorphism, ease of genotyping and co-dominant inheritance, microsatellite markers are very popular for the study of molecular phylogeography and population genetics (Sun et al., 2009). However, null alleles and stutter bands of microsatellite markers may have an effect on the genotyping of microsatellite. Isolation of microsatellite markers has been carried out in many important species (Chistiakov et al., 2004; Ma et al., 2009; Ruan et al., 2011). Moreover, polymorphic markers have been applied in the research of fishery science, such as genetic diversity and differentiation (Beacham et al., 2000; Spruell et al., 2003; Sawadogo et al., 2010; Ji et al., 2011), individual identification and phylogenetic relationships (Ortega-Villaizan et al., 2005, 2006) and construction of genetic map (Shimoda et al., 1999; Waldbieser et al., 2001; Chistiakov et al., 2008). Currently, a few microsatellite markers have been isolated from genomic DNA libraries in this crab species (Takano et al., 2005; Xu et al., 2009; Ma et al., 2010; Cui et al., 2010). However, these microsatellite markers are

limited and not enough for further studies, such as the evaluation of population genetic structure, construction of genetic linkage maps, and molecular marker-assisted selection (MAS) in *S. paramamosain*. The cDNA was a valuable resource for microsatellites (Chen et al., 2005; Nonneman and Waldbieser, 2005). Currently, no cDNA-derived microsatellites have been reported. In this study, 36 novel polymorphic microsatellite markers were isolated in the cDNA library of *S. paramamosain,* and they are useful for thoroughly understanding the genetic population structure, genetic diversity and resource history of this crab species.

MATERIALS AND METHODS

Samples collection and DNA extraction

A total of 24 individuals of *S. paramamosain* were collected from landing center in Hainan, China. Genomic DNA was extracted from the muscle tissue using traditional phenol-chloroform extraction protocols as described by Ma et al. (2010). The DNA was adjusted to 100 ng/µl and stored at -20℃ until use.

Microsatellite primer design and PCR

Microsatellite sequences were screened from cDNA library constructed from *S. paramamosain* in our laboratory. Microsatellite sequences were screened from a total of 1709 ESTs in the library using the software SSRHUNTER 1.3 (Li and Wan, 2005). Microsatellite primers were designed using Primer Premier 5.0 software (http: //www. premierbiosoft.com/primerdesign/). The major parameters for primer design were set as follows: primer length from 19 to 25 nucleotides, the size of PCR product from 100 to 350 bp, and annealing temperature at 50-65℃. The primers were synthesized by Sangon Company (Shanghai).

Polymorphism assessment for primers

The polymorphisms of microsatellite primers were tested in 24 individuals of *S. paramamosain*. The PCR amplification was performed in 25 µl volume containing 1×PCR buffer, 0.4 µM of each primer, 0.2 mM dNTP mix, 1U *Taq* polymerase (TaKaRa) and 50 ng of template DNA. After denaturation at 94℃ for 5 min, amplification was proceeded for 35 cycles (94℃ for 30 s, annealing temperature for each pair of primers (Table 1) for 40 s, 72℃ for 45 sec) and a final step at 72℃ for 5 min. The PCR products were separated on a 6% denaturing polyacrylamide gel and visualized by silver staining. The ranges of allele size were determined by referring to the pBR322/*Msp* I marker (TianGen Biotech CO., LTD).

Data analysis

Genetic diversity indexes including observed number of alleles (N_a), observed heterozygosity (Ho) and expected heterozygosity (He), and Chi-square tests for Hardy-Weinberg equilibrium (HWE) were calculated using software POPGENE version 1.31 (Yeh et al., 1999). Significance values for all multiple tests were corrected by sequential Bonferroni procedure (Rice, 1989). The null allele frequency was estimated by software MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004). The algorithm of null allele frequency is listed as: $r = A + \sqrt{(A^2 + B)} / 2(1 + He)$,in which A = He (1 + N) - Ho, and B = 4N(1 - He^2). Here, the observed heterozygosity (Ho) was measured from the data as n2 / (n1 + n2), where n1 is the number of one-banded individuals and n2 is the number of two-banded individuals in the sample. The expected heterozygosity (He) was calculated as the sum of the product of all observed allele frequencies ($\Sigma p_i p_j$, with $i \neq j$).

RESULTS AND DISCUSSION

The microsatellite markers have many advantages for understanding population genetics and evolution by sequencing alleles among species and within populations. Identification of microsatellites can be isolated from EST library (Sha et al., 2010); it has been widely used in many species such as turbot (Chen et al. 2007) and small yellow croaker (Wang et al., 2009). In this study, a total of 1710 ESTs were screened of which 291 ESTs contained microsatellite sequences. 162 primer pairs could be designed using Primer Premier 5.0 software. The polymorphism of these primers was evaluated using 24 individuals of *S. paramamosain*. Although the PCR conditions were optimized, 36 pairs of primers were found to be polymorphic and the remaining ones had either amplified single PCR products of the expected size or smears. The image results of the two polymorphic primers are shown in Figure 1.

The details (GenBank Accession numbers, primer sequences, repeat motifs and so on) of these 36 pairs of primers are listed in Table 1. A total of 159 alleles were identified in 24 individuals. The allele size was between 99 and 301 bp. The number of alleles per locus ranged from two to eight, with an average of 4.4. The average value of N_a was relatively lower than that detected in the studies of *S. paramamosain* (Takano et al., 2005; Xu et al., 2009; Ma et al., 2010; Cui et al., 2010).

Gene heterozygosity is a good criterion to assess the genetic diversity of a species (Ma et al., 2010). In previous reports about *S. paramamosain* evaluated by microsatellite markers, the average observed heterozygosity was higher than 0.70. In this study, the observed and expected heterozygosity per locus ranged from 0.100 to 1.000 and from 0.097 to 0.819, with an average of 0.679 and 0.625, respectively. Significant deviation from Hardy-Weinberg equilibrium at five microsatellite loci (SP04, SP12, SP17, SP33 and SP34) was detected after Bonferroni correction (P<0.0014), which may be due to the small sample size or the presence of null alleles (Van Oosterhout et al., 2004), and the MICRO-CHECKER analysis showed no evidence for scoring error or technical or statistical artifacts. None of the 630 possible pairwise comparisons between the 36 loci showed significant genotypic linkage disequilibrium (LD) after Bonferroni correction (P>0.0014).

In conclusion, we isolated 36 novel polymorphic microsatellite loci from 24 individuals of *S. paramamosain*. These loci will provide useful information for studying the genetic diversity and structure, construction of genetic linkage maps and the effective management of *S.*

Table 1. Locus name, repeat sequence, primer sequences, annealing temperature (T_a), number of observed (N_a) alleles, allele size range, observed (Ho) and expected heterozygosity (He), Chi-square tests for Hardy-Weinberg equilibrium (HWE) after after Bonferroni correction (adjusted *P* value = 0.0014) and GenBank accession number for the 36 polymorphic microsatellite loci in *Scylla paramamosain*.

Locus	Repeat sequence	Primer sequences (5' - 3')	N_a	T_a (℃)	Allele size range(bp)	Ho	He	*P*	GenBank accession no.
SP01	(TGC)6	F:ACGCCAGTAATCCTCTCCTCTTT R:TTACATAAACATCATCACTTTGCCTT	4	50	240-252	0.684	0.730	0.5600	JF715385
SP02	(TGG)6	F:GCTGGGACTGATGTAAACACTGG R: ATGTTCAGTACAGGGAGAGTGGC	5	60	127-139	0.778	0.716	0.3048	JF715386
SP03	(TG)7	F:TGAAAGGGAGAGGGAAGATGTTAC R:TTTTTTGAGACTGGTGCAAGAGTA	4	55	145-159	0.917	0.608	0.0032	JF715387
SP04	(CTA)13	F:CTAACTCACCCTTCTAAATGGAC R:GTGGAAACAATACTTGAGTGACC	5	55	215-241	0.750	0.676	0.0012*	JF715388
SP05	(AGT)10	F:AGGAGGGCTGCTGGTAGTGG R:GGAGTATCTGAGTCTGTGTATGCTTT	4	58	240-256	0.667	0.635	0.0017	JF715389
SP06	(TCC)5	F: GAAGACTTGGGTGATGTGAGAG R:TTCAAGCCTAAGAATCACGACT	2	55	114-122	0.409	0.511	0.3399	JF715390
SP07	(CA)12T(AC)5	F:AAGAGAGACATTGTGTTTACCC R:AGTTATGTCTATCAGCACAGTTATTA	6	50	186-202	0.913	0.718	0.2610	JF715391
SP08	(AC)14	F:GACGAAGGGAAGGTGGAGAGAAA R:CGTCATTCATTTAATATTTTTCTGTCTCT	4	55	265-281	0.524	0.612	0.3290	JF715392
SP09	(GAA)4…(TG)5	F:CAAGTGGCTACATCCAAGAAGGCT R:CGATTTCACATTCAGCATAACACATT	5	55	168-180	0.692	0.609	0.0022	JF715393
SP10	(CT)9	F: CTGTTTATGTGCGTCCAGTCTAG R:AAAAGTTGTGTCTGTGTGAAGGT	4	55	241-253	0.714	0.616	0.2100	JF715394
SP11	(AG)10	F:CTGTTTCCCCCCGAGATAGAGAC R:GCTCCACCTCTCCGCCCATC	5	60	252-268	0.609	0.704	0.6372	JF715395

Table 1. Continue.

SP12	(TTA)6	F:ACTGTAGCCAGGGATGGAAGG R:CCGCAATAGGCTCTAACTTAAAAT	2	55	242-246	0.917	0.507	0.0001*	JF715396
SP13	(TG)11	F:AGCGGGAGGTCTCACGGAT R:GACAAATGAAACCAGTACAGTATAATC	4	57	193-207	0.667	0.652	0.8609	JF715397
SP14	(TG)7G(GT)4GC(GT)7	F:TGGTTGATTACAGTGCCGAGC R: ATGCGGGGGCGTGACTGT	5	58	243-261	0.714	0.738	0.0273	JF715398
SP15	(GA)8	F:GTGTGGGTCACCAGGCGAGAG R:TGTGATGAGGCTGCTGCGTTG	5	60	170-188	0.824	0.624	0.0709	JF715399
SP16	(TG)4…(GA)4	F:GAAACAAGAGCGGGAGAATAAAA R:ATTTCACTTATTTTCCGCCTTCA	4	50	247-259	0.333	0.301	0.9914	JF715400
SP17	(AC)5T(AC)8	F:TTCATGGGCTGTACATAGAGATC R:AGAGTTTTTAAGCGTAAGTGGTA	8	50	143-167	0.579	0.677	0.0002*	JF715401
SP18	(TG)6…(GA)8…(TC)7	F:AAAGGACCAAAAATTCTGTTAAA R: GCAGCACAAGATGGGAGG	5	50	198-214	0.609	0.706	0.4852	JF715402
SP19	(CCA)4A(CAA)5	F:GCAAACCAGGGAAAACGGAAG R:CAGGCAGCGAAATGGAACGAG	4	58	163-175	0.900	0.703	0.0708	JF715403
SP20	(TGG)6	F:AGCAAGGAATAATGTCTTCTAACCC R:TCTGGAGTTGCCTCAGTAGTGTAGT	6	56	120-140	0.824	0.724	0.0074	JF715404
SP21	(GT)18	F:TGAAGAGAAATGTTTGAAAATAAGAA R:ACAGCTATGAATTACAACAAAGTGC	3	52	163-175	0.773	0.517	0.0433	JF715405
SP22	(GT)7	F:TTTTATCACGACCCAGCTCTCG R:CTATCTGTCATCCCTTTCCCCC	6	58	202-218	0.438	0.516	0.1156	JF715406
SP23	(TG)5CGC(GT)4	F:TCAATAGAATGGAATGAAATGTC R: TTTAAATAGTTTGTTTCTCTGCA	5	50	179-193	0.600	0.763	0.1097	JF715407
SP24	(TCC)4(CCG)5	F:GCGTTCAAAGACCCGAGTGG R:GTCAGAGGCGGTGGCGATACA	4	60	151-161	1.000	0.686	0.0053	JF715408

Table 1. Continue.

SP25	(ACC)6	F:TGTTGATGGCTGCTGCGTTAGAG R:GAGTCGTGGTAGATGGCCTGGT	2	62	191-199	0.417	0.431	0.9012	JF715409
SP26	(TTA)18T3(TTA)4	F:TATCTATGAACACACTGAAGCAC R:TAAAGCAAACAAAAAAGGTCC	7	50	163-181	0.632	0.812	0.1023	JF715410
SP27	(TG)10	F:CACTTCCCCTCCAGTCTATTCCAT R: GTCCCCCCATAATCAGCCTCTTT	4	58	247-259	0.750	0.559	0.3965	JF715411
SP28	(GA)19	F:ATAGTCAACTCCATCCTTCAAAT R:CGTAGAACAAGTAAAGTAAGAACATAA	4	52	269-287	0.722	0.722	0.2518	JF715412
SP29	(TG)9	F:AAGAAGCAGAGAAAAGATTGAGA R:GCGCTGCAATACTTTATTATAAG	4	50	181-193	0.842	0.620	0.0726	JF715413
SP30	(TGC)4…(TG)4	F:CTCCACTTATGATGACTTGAACC R:CCATTAAGAAAGTATACAAAGCATT	3	53	99-111	0.750	0.675	0.1344	JF715414
SP 31	(TG)9	F:GCCAGAGATACTGAAAAGAAGAA R:GTAAAAAGAACAGATACACCACAG	4	52	130-144	1.000	0.639	0.0204	JF715415
SP32	(CAA)11	F:CTCTTTTTTCCTTCCGTTTACTTTT R:TATAGCACAACGATTAGGCTCTAGC	4	53	208-222	0.542	0.694	0.0241	JF715416
SP33	(TG)16	F:AGTGGGAAAAACATACCCTTGGA R:AGCACAGTTGTGGCACCCTACAT	6	56	195-212	0.611	0.656	0.0005*	JF715417
SP 34	(GT)5A(TG)13	F:GGATGTGCTGGATGAAAAATAA R:TACACAGAGGTGATATGAATGACAT	6	52	157-173	0.750	0.819	0.0007*	JF715418
SP35	(TGA)9	F:AAAGAATGACTTCAGAACTCCAGAG R:GAAGGAGATACATCTTCAAAAGCTG	4	52	258-276	0.478	0.521	0.2950	JF715419
SP36	(CT)9	F:ACTAATCTGTTCCTCACCCTCCTTT R:GTAAATGAACACACGCCTGACCAAT	2	56	287-301	0.100	0.097	0.8694	JF715420
Mean	-	-	4.4	-	-	0.679	0.625	-	

* Show significant deviation from HWE after Bonferroni correction.

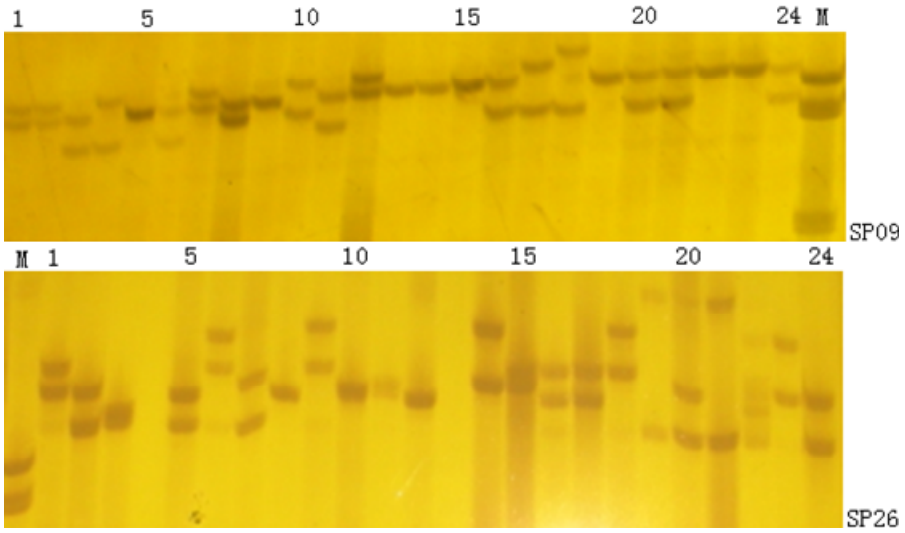

Figure 1. Image results of polymorphic primers (SP09 and SP26).

paramamosain.

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