



# Isolation and characterization of polymorphic microsatellite loci in the swimming crab *Portunus trituberculatus* (Portunidae)

L.P. Ren<sup>1</sup>, Y. Qin<sup>1</sup>, X.C. Li<sup>2</sup>, Y.N. Sun<sup>1</sup> and R.X. Wang<sup>1</sup>

<sup>1</sup>Laboratory for Marine Living Resources and Molecular Engineering, College of Marine Science, Zhejiang Ocean University, Zhoushan, China

<sup>2</sup>Key Laboratory of East China Sea and Oceanic Fishery Resources Exploitation, Ministry of Agriculture, Shanghai, China

Corresponding author. R.X. Wang  
E-mail: wangrixin1123@126.com

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**ABSTRACT.** The Swimming crab *Portunus trituberculatus* (Portunidae) is an important economically food species. To provide molecular markers for *P. trituberculatus*, we isolated and characterized polymorphic microsatellite markers. We developed a 5'-anchored genomic library of *P. trituberculatus* DNA, and derived 45 positive clones. We designed 30 pairs of primers from the sequences of these clones, and 10 of which were polymorphic. The loci were screened in 31 *P. trituberculatus* individuals; the number of alleles ranged from 2 to 5. The observed heterozygosity ranged from 0.20 to 0.49 and the expected heterozygosity ranged from 0.50 to 0.78. The polymorphism information content per locus ranged from 0.37 to 0.75. These polymorphic microsatellite markers may provide some useful information for the population genetic studies of swimming crab and other *Portunus* species.

**Key words:** *Portunus trituberculatus*; Microsatellite; Polymorphism; Molecular markers

## INTRODUCTION

The swimming crab, *Portunus trituberculatus* (Crustacea, Decapoda, Brachyura) is widely distributed in the coastal waters of Korea, Japan, China, and Southeast Asia (Dai et al., 1986). It inhabits estuaries and coastal waters and has a life span of about 1 to 3 years. Mating season of this species varies with geographic locations and the age of the individual (Dai, 1977; Xue et al., 1997), and they can spawn one to three times during each spawning season (Song et al., 1988). This swimming crab also has a capacity for long distance dispersal species, the patterns of seasonal wind and water currents may influence the dispersal distance in this species (Shen and Liu, 1965).

In China, *P. trituberculatus* is not only a major edible crab species but also important for aquaculture (Yu et al., 2003a). Owing to its rapid growth and lucrative breeding, it has become an important breeding variety in the coastal areas of China. In addition, *P. trituberculatus* has been under intense fishing pressure gradually from 1950s across most of its Chinese distributions (Yu et al., 2003b, 2004). Due to the high commercial value of this species, *P. trituberculatus* supports a large crab fishery and aquaculture in China. In order to ensure the sustainability of this resource, artificial propagation has been promoted along the coastal waters of China (Sun, 1984). In general, the crab industry has relied on wild populations, both for harvest and to provide seed stock for aquaculture (Wu et al., 1996).

Mortality and life history parameters of this species had been widely studied (Yeon, 1999; Yeon et al., 1998). Knowledge of population genetic structure has become significant to the development of effective strategies for the fishing industry. In particular, determining levels of genetic diversity and histories of divergence has been significant for selective breeding in crab aquaculture (Imai et al., 1999). The population genetic diversity of this species was first surveyed in the coastal waters of Okayama Prefecture (Japan) by using mitochondrial DNA (mtDNA) RFLP markers. SSR markers are simple sequence repeats of one to six nucleotides, which are distributed widely and abundantly in the genome. These microsatellite markers have some unique characteristics, such as allelic co-dominance of alleles, high allelic diversity and the reproducibility of the relatively simple PCR-based screening methods; they have been considered to be one of the most powerful Mendelian markers (Jarne and Lagoda, 1996). As a result, these features have been advantageous for genetic mapping, quantitative trait locus association, population genetics and evolutionary studies (Hu et al., 2010; Xu et al., 2011). At present, microsatellite sequences have mainly been isolated by two ways: direct genomic sequencing or by generating DNA libraries by enrichment for the microsatellite sequences. The latter also has been achieved by two methods: the FIASCO method (Zane et al., 2002) and the 5'-anchored PCR method (Fisher et al., 1996). The anchored PCR method has been applied widely because of its high efficiency of isolation. In this study, we applied a 5'-anchored method to develop the microsatellite loci for this species.

## MATERIAL AND METHODS

### DNA samples

*P. trituberculatus* individuals were collected from the Zhoushan coastal (Zhejiang, China). Total DNA was isolated from samples using the standard phenol-chloroform method

(Sambrook and Russell, 2001). The quality of the isolated DNA was assessed by agarose gel electrophoresis and then stored at  $-20^{\circ}\text{C}$  for future use.

### Development of microsatellite library

An enrichment library containing sequences with different microsatellite motifs was constructed by using a 5'-anchored PCR technique (Fisher et al., 1996). In brief, we selectively amplified by a single degenerate primer that anchors at the 5'-end of a microsatellite motif by a sequence of seven degenerate bases ( $\text{PCT}_6$ : 5'-KKVVRV(CT) $_6$ , K = G/T, V = G/C/A, R = G/A). The PCR amplification was carried out in a 25- $\mu\text{L}$  reaction volume, containing 1X PCR buffer, 0.2 mM dNTPs, 0.2  $\mu\text{M}$  of the degenerate primer, and 1.5 U *Taq* polymerase. The reaction conditions of the PCR were as follows: an initial denaturation of  $95^{\circ}\text{C}$  for 5 min; 5 cycles of  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s, and an extension for 1 min at  $72^{\circ}\text{C}$ ; a further 30 cycles of  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and a 1-min extension at  $72^{\circ}\text{C}$ ; and a final extension step of  $72^{\circ}\text{C}$  for 10 min. The PCR products were assessed on a 1.5% agarose gel. Amplicons of 300-800 bp were extracted from the gel, purified and were then cloned into pMD19-T plasmid vectors (TaKaRa) and used to transform competent *Escherichia coli* DH5 $\alpha$  cells. Positive clones, which appeared as single distinct bands on agarose gels, were selectively amplified by using the M13-F/R universal primers. Finally, the sequences were detected on the ABI PRISM 3730 Genetic Analyzer. Thirty pairs of specific primers were designed using the Primer Premier 5.0 software (PREMIER Biosoft International, USA).

### Microsatellite genotyping

Microsatellite loci were characterized based on all the 31 *P. trituberculatus* different individuals. PCR amplifications were carried out with a 15- $\mu\text{L}$  reaction volume containing 50 ng template DNA, 0.2  $\mu\text{M}$  primer (each), 1 U *Taq* polymerase (Tiangen), 0.15 mM of each dNTP, and 1.5  $\mu\text{L}$  10X PCR buffer. The reaction conditions of the PCR were as follows:  $95^{\circ}\text{C}$  for 5 min; 35 cycles of  $95^{\circ}\text{C}$  for 30 s, annealing temperature for 30 s, and  $72^{\circ}\text{C}$  for 40 s and a final extension at  $72^{\circ}\text{C}$  for 10 min. The amplified products were denatured for 8 min at  $95^{\circ}\text{C}$ , then separated on 6% denaturing polyacrylamide gels (19:1 acrylamide:bis-acrylamide) and visualized by silver staining. The allele sizes were calibrated using a pBR322 DNA/*Msp*I molecular weight marker (Tiangen).

The Popgene 32 program was used to determine the number of alleles per locus, the effective number of alleles, the expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosities, and to test for departures from Hardy-Weinberg equilibrium (HWE) (Yeh and Boyle, 1997). Arlequin 3.11 was used to test for genotypic linkage disequilibrium between the loci (Excoffier and Lischer, 2010). All P values were corrected for multiple testing using a Bonferroni's correction (Rice, 1989).

## RESULTS AND DISCUSSION

Among 30 pairs of microsatellite primers designed, 26 pairs were successfully amplified and 10 (38.5%) of these loci were found to be polymorphic. Details of these polymorphic microsatellite loci in *P. trituberculatus* and their variability are summarized in Table 1. The

number of alleles found at each locus ranged from 2 to 5, with a mean of 3.50.  $H_O$  and  $H_E$  ranged from 0.20-0.49 and from 0.50-0.78, respectively. The PIC values of the microsatellites ranged from 0.37 to 0.75 and all loci were highly polymorphic (PIC > 0.5).

**Table 1.** Characteristics of 10 polymorphic microsatellite loci in the swimming crab.

Locus	GenBank	Primer sequence (5'-3')	Tm (°C)	Repeat motif	Size range (bp)	$N_A$	$N_E$	HWE (P)	PIC	Null	$H_O/H_E$
Potr-11	KC470116	F: TCATCACCACCATTAGTACG R: CTCCTCCTTCTTTATCCC	55	(GT) <sub>33</sub>	110-123	4	3.90	0.00*	0.67	0	0.24/0.74
Potr-14	KC470117	F: GCTCTCTCTCTGGAACACTT R: GCTGCTGCCGCTCATATCAACC	55	(CT) <sub>6</sub>	118-123	2	2.00	0.23	0.37	0	0.49/0.50
Potr-10	KC470114	F: AACGACAACAATGATCCCTT R: CAGTTAGTTATTTGATTTGCTTTA	52	(CT) <sub>12</sub>	149-158	3	2.85	0.25	0.57	0	0.34/0.65
Potr-15	KC470118	F: GCTCTCTCTCTGGAACACTT R: CATA CGGCAACAGTCCACCTCT	56	(CT) <sub>6</sub>	104-110	3	2.87	0.02	0.58	0	0.33/0.65
Potr-9	KC470114	F: GGAGGAACTCTCTCTCTGTG R: TCTTTAGGTGTCATTTACTTGT	46	(CT) <sub>6</sub> (GT) <sub>33</sub> (AG) <sub>7</sub>	110-123	4	3.66	0.03	0.68	1	0.25/0.73
Potr-27	KC470122	F: TACAGTAAAATACAGCAACGAC R: AGCTAAGAATAAGAATCCTCCG	58	(GT) <sub>7</sub> (GA) <sub>41</sub>	147-155	3	2.82	0.98	0.57	0	0.34/0.65
Potr-26	KC470121	F: TACAGTAAAATACAGCAACGAC R: TAAGAATAAGAATCCTCCGCAC	58	(GT) <sub>7</sub> (GA) <sub>41</sub>	113-117	3	2.93	0.79	0.58	0	0.33/0.66
Potr-29	KC470123	F: GAAACTAACCAACTTACCTACAT R: TTCTTTAGGTGTCATTTACTTGT	58	(CT) <sub>6</sub> (GT) <sub>33</sub> (AG) <sub>7</sub>	145-157	5	4.57	0.30	0.75	0	0.20/0.78
Potr-18	KC470119	F: CACTAGGAAAATAATAAGCATAA R: GGA ACTCTCTCTCTGTGGGA	58	(CT) <sub>6</sub>	115-123	3	2.93	0.00*	0.58	0	0.33/0.66
Potr-25	KC470120	F: TACAGTAAAATACAGCAACGAC R: CAAGCTAAGAATAAGAATCCTC	56	(GT) <sub>7</sub> (GA) <sub>41</sub>	133-151	5	4.24	0.23	0.73	0	0.22/0.76

$N_A$  = number of alleles;  $N_E$  = effective number of alleles;  $H_E/H_O$  = expected and observed heterozygosities; HWE = Hardy-Weinberg equilibrium and GenBank accession number of 10 polymorphic microsatellite loci. \*Significant deviation from HWE (P < 0.004).

Among the 10 novel loci, 2 loci (potr-11 and potr-18) were found to deviate significantly from HWE in the sampled population, after Bonferroni's correction. The locus (potr-9) was found to exhibit null alleles. There were no stuttering errors and no evidence of allelic dropout in any of the loci analyzed by Micro-Checker (Van Oosterhout et al., 2004).

The 10 polymorphic microsatellite loci developed in this study will assist studies of genetic variation, population structure, and conservation genetics and the molecular-assisted selective breeding of this species of swimming crab.

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