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Short Communication

Development of Expressed Sequence Tag-Single Nucleotide Polymorphism Markers in Swimming Crab, *Portunus trituberculatus*

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ABSTRACT

In this study, sixteen SNP (single nucleotide polymorphism) markers were developed from EST (expressed sequence tag) database of *Portunus trituberculatus*. Polymorphism evaluation was tested on 30 wild individuals of *P.trituberculatus* collected from Xiangshan, Zhejiang province, China. The minor allele frequency ranged between 0.292 and 0.500, with an average of 0.384. The expected and observed heterozygosities (H_e and H_o) ranged from 0.422 to 0.545 and from 0.000 to 1.000 respectively. Four loci were found deviate significantly from Hardy-Weinberg equilibrium. Blast results give significant hits for nine confirmed SNP-associated sequences, some of these genes are associated with important immunological functions. These EST-derived SNP markers will be useful tools for fisheries management and conservation programme of *P.trituberculatus*.

The swimming crab (*Portunus trituberculatus*), which belongs to typical euryhaline crab species, is widely distributed in the coastal waters of Korea, Japan, China, and Southeast Asia (Dai *et al*., 1986). It is also one of the important fishery resources in China. Population analysis based on microsatellite molecular marker has been initiated to facilitate the protection of the natural resources of *P. trituberculatus* (Guo *et al*., 2013). Also, studies on the marker assisted selection (MAS) and aquaculture technology have been conducted to promote the production of this species (Liu *et al*., 2012; Mu *et al*., 2014; Jin *et al*., 2015; Liu *et al*., 2015).

Single nucleotide polymorphisms (SNPs) are the most common class and the smallest unit of genetic variation present in genomes. Because of their high density/frequency, lower mutation rate compared to microsatellite markers, and amenable to high-throughput automated analysis, SNP markers provide a powerful resource for the study of population structure (Morin *et al*., 2004). Moreover, because SNPs tend to occur in functional genomic regions, they are particularly valuable for characterizing genes associated with complex traits, therefore, they are suitable for genetic evaluation and strategies that employ molecular genetics for selective breeding (Glenn *et al*., 2005; Sauvage *et al*., 2007; Salem *et al*., 2012; Houston *et al*., 2014; Leitwein *et al*., 2017). In this study, we report a set of 16 SNP markers derived from expressed sequence tag (EST) database of *P. trituberculatus*, these novel EST-derived SNP markers should be useful complement to currently available genetic markers of this species.

Material and methods

A total of 14,340 *P. trituberculatus* EST sequences were downloaded from GeneBank. The EST dataset was aligned and assembled using SeqMan Pro sequence assembly software (DNASTAR Inc., Madison, WI, USA). The contigs that contained four or more sequences were identified for searching candidate SNPs upon visual inspection. In total, 176 sequences with sufficient flanking regions were selected for primer design with PRIMER 5.0 program (http://www.premierbiosoft. com/).

Polymorphism evaluation was tested using 30 wild individuals of adult *P. trituberculatus* collected randomly from Xiangshan, Zhejiang province, China. Genomic DNA was extracted from the muscle tissue by using a

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Table I. Characterization of 16 SNP markers in swimming crab, *Portunus trituberculatus*.

Primer ID	Primer sequences(5'-3')	Amplicon length (bp)	Locus ID	SNP	H_o	H_e	Minor allele frequency (MAF)	P_{HWE}	Genbank accession number	Predict function
PtSNP1	CATATGTCTGACACCCAGAAGCAGCGCGA	362	PtSNP1a	A/C	0.667	0.485	C (0.333)	0.033	EF110536.1	Hemocyanin
	GCGGCCGCTTAGTGGTGGTGGTGGTGGTG-CACGTGTTGTGTCAGAGGA		PtSNP1b	A/G	0.667	0.545	A/G (0.500)	0.030		
			PtSNP1c	C/G	0.833	0.530	G (0.417)	0.031		
PtSNP2	CATGATTTCGTCCCAGTGTT	323	PtSNP2a	A/C	0.583	0.454	A (0.333)	0.202	KJ631745.1	Lectin 3 gene
	TCGTGGGTAGTGTCGTGATT		PtSNP2b	A/T	0.583	0.422	T (0.292)	0.128		
PtSNP3	TTTTCATCCATACCACCTAG	236	PtSNP3	G/T	0.769	0.509	G/T (0.500)	0.016	AB093006.1	Mitochondrial DNA
	TACCTCACCGTAAACCTTTG									
PtSNP4	TTTCTGTTGTTTGGGAATGGG	510	PtSNP4	G/T	0.000	0.462	T (0.346)	0.000*	AB093006.1	Mitochondrial DNA
	TATCGAAGCCGAGGTAGTGTA									
PtSNP5	CACAGGTTGGGTATTCAGGG	539	PtSNP5a	G/T	0.308	0.483	T (0.385)	0.098	EF101999.1	Cuticle protein
	TCTATGGAGATTGGAGGTCA		PtSNP5b	G/A	0.308	0.483	G (0.385)			
PtSNP6	TTACATAACTCCCACTAACGAA	228	PtSNP6	A/T	0.030	0.506	T (0.470)	0.000*	AB093006.1	Mitochondrial DNA
	CATTGACCGTAGAATAATCCAG									
PtSNP7	CCTATCCATGATTTCGTCCCA	494	PtSNP7a	A/T	0.600	0.429	A (0.300)	0.063	FN434137.1	5S rRNA genes
	TTGAATGCACGGCTTGTTAT		PtSNP7b	G/T	0.520	0.429	T (0.300)	0.373		
PtSNP8	TGACAGTCGCCTTGTTGGTGCT	398	PtSNP8a	C/T	0.846	0.507	C (0.462)	0.001*	JQ728424.1	Crustin
	CCCTCAATCCCTCTTAGTTTCTC		PtSNP8b	T/G	1.000	0.510	T/G (0.500)	0.000*		
PtSNP9	ACGAGGAGGAGAAAGAGGATA	451	PtSNP9	A/T	0.083	0.431	T (0.292)	0.010	HM627758.1	Anti-lipopolysaccharide factor isoform
	CAAGTCTTACGGCTATTACCAT									
PtSNP10	AGGTGCTCGCTGCCTTATTTCC	551	PtSNP10	A/T	0.250	0.454	A (0.333)	0.056	GT555737.1	Unknown
	CTCCAGGTTCTTCATGCTTTCT									

H_o: observed heterozygosity; H_e: expected heterozygosity; * Significant deviation from HWE after Bonferroni correction ($P < 0.05$).

genomic DNA extraction kit (Bio Teke, Beijing, China) following the manufacturer protocols. Polymerase chain reaction (PCR) was performed in 10-μL volumes containing 2×Power Taq PCR Master Mix (Bio Teke, Beijing, China) 5μL, 1μM of each primer set, and about 100ng template DNA. PCR was performed on a Mastercycler gradient thermal cycler (Eppendorf) with the following program: 3 min at 94 °C; 35 cycles of 1 min at 94°C, annealing at 55°C for 1 min, 72°C for 1 min per cycle; followed by 5 min at 72°C. Amplification products were resolved via 2% agarose gel, DL2000 DNA Marker (Takara, Dalian, China) was used as a reference marker for allele size determination. PCR products of clear bands and predicted length were then sequenced in both directions with forward and reverse primers using Sanger technology on the ABI3730 platform (Applied Biosystems).

Alignment of the sequenced fragments was performed using Vector NTI 10.0 (Invitrogen, Carlsbad, CA), and putative SNPs were checked manually. Minor allele frequency (MAF), expected and observed heterozygosities (H_e and H_o, respectively) were calculated with the software CERVUS 3.0 (Kalinowski *et al*., 2007). Test for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium at each locus were performed using GENEPOP 4.0.10 (Raymond and Rousset, 1995). Sequential Bonferroni corrections (Rice, 1989) were applied for all multiple tests (P<0.05). The putative functions of SNP-associated sequences were searched against the NCBI database (http://www. ncbi.nlm.nih.gov) with E-value of <1.00 E^{-7} using BLASTX.

Results and discussion

SNP markers provide a powerful resource for genetic researches of genome-wide linkage disequilibrium and association studies, population structure estimation, marker-assisted breeding, individual identification and parentage analysis. In this study, 176 primer pairs were designed. Among them, 39 primer pairs provided readable sequences, and 10 sequences containing 16 polymorphic SNPs were confirmed successfully (Table I).

The minor allele frequency ranged between 0.292 and 0.500, with an average of 0.384. The expected and observed heterozygosities (H_e and H_o) ranged from 0.422 to 0.545 and from 0.000 to 1.000 respectively. Significant departure from HWE was found at four loci after Bonferroni correction for multiple tests. Significant pairwise linkage disequilibrium was detected in SNPs from the same sequences (PtSNP1a and PtSNP1b, PtSNP2a and PtSNP2b, PtSNP5a and PtSNP5b, PtSNP7a and PtSNP7b), which should be considered when used for population genetics and parentage studies.

To date, genetic markers for population studies of *P. trituberculatus* have been generally limited to mitochondrial DNA gene and microsatellites (Xu *et al*., 2009; Guo *et al*., 2013). By taking advantage of EST database, EST-derived SNPs can be easily discovered, which possess a number of advantages for the study of population structure (Morin *et al*., 2004). Here, we report 16 SNP markers in *P. trituberculatus* which will provide a useful complement to currently available genetic markers.

Analysis of gene-based single nucleotide polymorphisms (SNPs) is one of the efficient approaches for discovery of markers that can be used for MAS. In aquatic species, association between SNP in functionally important genes and immune response was reported in many species (Yu *et al*., 2011; Li *et al*., 2013; Hao *et al*., 2015; Santos *et al*., 2018). In this study, blast results give significant hits for nine confirmed SNP-associated sequences, some of these genes are associated with important immunological functions, such as hemocyanin, lectin and anti-lipopolysaccharide factor, which provide useful resources for MAS programs of *P. trituberculatus*.

In conclusion, these polymorphic EST-derived SNP markers we developed in the present study were expected to be valuable for researches involving population genetic diversity and marker assisted selection programs of *P. trituberculatus*.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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