*Pakistan J. Zool., vol. 54(2), pp 969-972, 2022* **DOI**: https://dx.doi.org/10.17582/journal.pjz/20181220171259

## Short Communication

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

**Shaokun Lu[1,2], Ronghua Li[1,2]*, Chunlin Wang[1,2], Changkao Mu[1,2] and Weiwei Song[1]**

[1]*Key Laboratory of Applied Marine Biotechnology, Ministry of Education, Ningbo University, Ningbo 315211, China*
[2]*Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture, Ningbo University, Ningbo 315211, China*

**Article Information**
**Received 20 December 2018**
**Revised 21 May 2019**
**Accepted 07 September 2019**
**Available online 19 May 2021 (early access)**
**Published 27 January 2022**

**Authors' Contribution**
**RL and CW designed the study. SL conducted the study with the help of CM and WS. SL analyzed the data and wrote the article.**

**Key words**
**SNP, Single nucleotide polymorphism, Swimming crab, *Portunus trituberculatus***

## 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

* Corresponding author: lironghua@nbu.edu.cn
0030-9923/2022/0002-0969 $ 9.00/0

**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*.

*Acknowledgements*

This work was supported by the Grants from Ministry of Agriculture of China & China Agriculture Research System (no: CARS-48), Natural Science Foundation of Zhejiang Province (LY17C190005), Major Program of Ningbo (2017C110007, 2016C10037), Open Fund of Zhejiang Provincial Top Key Discipline of Aquaculture in Ningbo University (xkzsc1505) and KC Wong Magana Fund in Ningbo University.

*Statement of conflict of interest*

Authors have declared no conflict of interest.

*References*

Dai, A.Y., Yang. S.L. and Song, Y.Z., 1986. *Marine crabs in China Sea*. Marine Publishing Company, Beijing, pp. 213-221.

Glenn, K.L., Grapes, L., Suwanasopee, T., Harris, D.L., Li, Y., Wilson, K. and Rothschild, M.F., 2005. *Anim. Genet*., **36:** 235-236. https://doi.org/10.1111/j.1365-2052.2005.01274.x

Guo, E., Cui, Z., Wu, D., Hui, M., Liu, Y. and Wang, H., 2013. *Biochem. Syst. Ecol.*, **50:** 313-321. https://doi.org/10.1016/j.bse.2013.05.006

Hao, G., Lin, F., Mu, C., Li, R., Yao, J., Yuan, X., Pan, X., Shen, J. and Wang, C., 2015. *Aquaculture*,

**442:** 125-131. https://doi.org/10.1016/j.aquaculture.2015.02.007

Houston, R.D., Taggart, J.B., Cézard, T., Bekaert, M., Lowe, N.R., Downing, A., Talbot, R., Bishop, S.C., Archibald, A.L., Bron, J.E., Penman, D.J., Davassi, A., Brew, F., Tinch, A.E., Gharbi, K. and Hamilton, A., 2014. *BMC Genomics*, **15:** 90. https://doi.org/10.1186/1471-2164-15-90

Jin, M., Wang, M.Q., Huo, Y.W., Huang, W.W., Mai, K.S. and Zhou, Q.C., 2015. *Aquaculture*, **448:** 1-7. https://doi.org/10.1016/j.aquaculture.2015.05.021

Kalinowski, S.T., Taper, M.L. and Marshall, T.C., 2007. *Mol. Ecol.*, **16:** 1099-1106. https://doi.org/10.1111/j.1365-294X.2007.03089.x

Leitwein, M., Gunand, B., Pouzadoux, J., Desmarais, E., Berrebi, P. and Gagnaire, P.A. 2017. *G3*, **7:** 1365–1376. https://doi.org/10.1534/g3.116.038497

Li, X., Cui, Z., Liu, Y., Song, C., Shi, G. and Wang, C., 2013. *Fish Shellf. Immunol.*, **34:** 1560-1568. https://doi.org/10.1016/j.fsi.2013.03.373

Liu, L., Li, J., Liu, P., Zhao, F.Z., Gao, B.Q. and Du, Y., 2012. *Aquaculture*, **344:** 66-81. https://doi.org/10.1016/j.aquaculture.2012.01.034

Liu, L., Li, J., Liu, P., Zhao, F.Z., Gao, B.Q. and Du, Y., 2015. *Aquacul. Res*., **46:** 850-860. https://doi.org/10.1111/are.12239

Morin, P.A., Luikar,t G., Wayne, R.K. and TSW Group, 2004. *Trends Ecol. Evol.*, **19:** 208-216. https://doi.org/10.1016/j.tree.2004.01.009

Mu, C., Song, W., Li, R., Chen, Y., Hao, G. and Wang, C., 2014. *Aquaculture*, **426–427:** 148-153. https://doi.org/10.1016/j.aquaculture.2014.01.006

Morin, P.A., Luikart, G., Wayne, R.K. and SNP Workshop Group, 2004. *Trends Ecol. Evol.*, **19:** 208-216. https://doi.org/10.1016/j.tree.2004.01.009

Raymond, M. and Rousset, F., 1995. *J. Hered.*, **86:** 248-249. https://doi.org/10.1093/oxfordjournals.jhered.a111573

Rice, R.W., 1989. *Evolution*, **43:** 223-225. https://doi.org/10.1111/j.1558-5646.1989.tb04220.x

Salem, M., Vallejo, R.L., Leeds, T.D., Palti, Y., Liu, S., Sabbagh, A., Rexroad, C.E 3rd. and Yao, J., 2012. *PLoS One*, **7:** e36264-e36264. https://doi.org/10.1371/journal.pone.0036264

Santos, C.A., Andrade, S.C.S. and Freitas, P.D., 2018. *Peer J.*, **6:** e5154. https://doi.org/10.7717/peerj.5154

Sauvage, C., Bierne, N., Lapegue, S. and Boudry, P. 2007. *Gene*, **406:** 13-22. https://doi.org/10.1016/j.gene.2007.05.011

Xu, Q.H., Liu, R.L. and Liu, Y., 2009. *J. Exp. Mar. Bio. Ecol.*, **371:** 121-129. https://doi.org/10.1016/j.jembe.2009.01.014

Yu, H., He, Y., Wang, X., Zhang, Q., Bao, Z. and Guo, X., 2011. *Fish Shellf. Immunol.*, **30:** 757–762. https://doi.org/10.1016/j.fsi.2010.12.015