SNP E4-205 C/T in C-type lectin of *Portunus trituberculatus* is association with susceptibility/resistance to *Vibrio alginolyticus* challenge

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**ARTICLE INFO**

Article history:
Received 17 December 2014
Received in revised form 4 February 2015
Accepted 6 February 2015
Available online 14 February 2015

Keywords:
*Portunus trituberculatus*
C-type lectin
Polymorphism
Susceptibility/resistance
High resolution melting (HRM) analysis

**ABSTRACT**

C-type lectins play an important role in innate immunity of invertebrates, especially in defending them against bacterial pathogens. In this study, the full-length genomic DNA of C-type lectin (designated as CTL gene) was isolated from the swimming crab *Portunus trituberculatus*. The genomic DNA of CTL consists of 1473 bp, containing four exons and three introns. Polymorphisms of the CTL gene were identified to explore their association with susceptibility/resistance to *Vibrio alginolyticus* infection. Four sites of single nucleotide polymorphisms (SNPs) and one site of insertion/deletion (ins/del) polymorphism were identified in CTL. Three of these SNPs were synonymous and one was non-synonymous. The distribution of these polymorphisms in the susceptible and resistant stocks was identified, according to the survival time after *V. alginolyticus* challenge. The non-synonymous SNP E4-205 C/T, a dimorphism caused from C to T transition that resulted in a Threonine to Isoleucine substitution, was located at position 152 in the peptide of CTL protein, showed significant difference between the two stocks according to Chi-squared test ($P < 0.05$). The SNP site has three genotypes that are C/T, C/C and T/T. The T/T genotype frequency was 6.1% in susceptible stock, and 21.2% in resistant stock, with a significant difference between them ($P = 0.022$). Therefore, the T/T genotype was suggested to be associated with increased resistance to *V. alginolyticus* ($P < 0.05$). The presumption was confirmed by another challenge experiment, in which high resolution melting (HRM) analysis was used to analyze the three genotypes distribution. The cumulative mortality of genotype T/T (57.1%) was significantly lower than C/C (82%) and T/C (81.4%) ($P = 0.001$). These results suggested that the E4-205 genotype T/T is a potential marker for selection of swimming crabs resistant to *V. Alginolyticus* challenge.

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1. Introduction

*Portunus trituberculatus* is widely distributed along the coast of China, Japan, Korea, and other Southeast Asian countries. This species is one of the most common edible marine crabs found in China, and has also been artificially cultured since the 1990s. However, the uncontrolled expansion in the scale of farming, increase in stocking density, and the polluted breeding environment, all result in the frequent outbreaks of diseases. Some of the most recurrent of these are the infections caused by the *Vibrio* family, such as *Vibrio metschnikovii* (Wan et al., 2011), and *Vibrio alginolyticus* (Liu et al., 2007). The “emulsification disease” caused by *V. alginolyticus* has brought considerable economic losses, and has seriously restricted the sustained and healthy development of crab aquaculture (Wang et al., 2006). Select breeding of new strains resistant to *V. alginolyticus* is a very important step towards controlling this disease.

Traditional breeding techniques are expensive, time-consuming, and easily influenced by the environment (Collard et al., 2005). One of the methods that could be used to improve breeding strategies is marker assisted selection (MAS), a molecular method successfully used in the improvement of agricultural population (Dekkers and Hospital, 2002). It is a method where a marker is used for the indirect selection of the target trait. The marker used is closely linked to the target trait. Some studies have reported on the use of DNA markers in MAS of livestock in order to cultivate novel disease-resistant varieties (Mallard et al., 2003; Masoudi et al., 2007). However, the use of MAS on *P. trituberculatus* is strategically difficult, as there are very few markers associated with specific traits (Liu et al., 2012; Lee et al., 2013). Therefore, it is important to identify the marker associated with resistance to *V. alginolyticus* in the swimming crab to develop strategies for MAS.

Immune-related genes are considered as the optimal candidates for the selection of markers associated with resistance to pathogens. Gene
polymorphism could improve the genetic quality or quantity, enhancing the ability of the immune system in protecting itself against infection. In recent years, polymorphisms in some immune-related genes have been reported to affect pathogen or disease resistance in aquatic animals. Examples of such genes include the lysozyme gene in Zhikong scallop (Chlamys farreri) (Li et al., 2009), the superoxide dismutase gene family in the bay scallop (Argopecten irradians) (Bao et al., 2010), the serine protease inhibitor gene in the eastern oyster (Crassostrea virginica Gmelin) (He et al., 2012; Yu et al., 2011), the MDA5 gene in grass carp (Wang et al., 2012), and the mannose binding lectin (MBL) in Zebra fish (Danio rerio) (Jackson et al., 2007). Polymorphism in these immune genes yielded different genotypes, which exhibited immunity phenotypes. Many genes involved in immune response of swimming crab, such as the C-type lectin (Kong et al., 2008), C-type lysozyme (Pan et al., 2010), crustins (Yue et al., 2010), prophenoloxidase (Chen et al., 2010) and anti-lipopoly saccharide (Y. Liu et al., 2011, 2012), were reported. However, polymorphisms in these genes, and the correlation between these gene polymorphisms and disease susceptibility and resistance are not yet well understood (Li et al., 2013).

The C-type lectins play an important role in pattern recognition and innate immune response in invertebrates (McGreal et al., 2004). They generally contain one or more carbohydrate recognition domains. In the presence of Ca2+, they specifically recognize the oligosaccharides expressed on the cell surface of exogenous microbes, and initiate an immune reaction against the invasion of pathogenic microorganisms (Wang and wang, 2013). A number of C-type lectins have been identified in crustaceans, most of which display anti-bacterial and anti-viral properties (Huang et al., 2014; Wang et al., 2013; Wei et al., 2012). In P. trituberculatus, the cDNA of a C-type lectin-like domain (CTLD)-containing protein with one carbohydrate recognition domain (CRD) was cloned and designated as PtLP (CTLD-containing protein) (Kong et al., 2008). In our previous study, a high level of PtLP transcripts was found in the hemocytes and hepatopancreas of P. trituberculatus (Wang et al., 2013). In our previous study, a high level of PtLP transcripts was found in the hemocytes and hepatopancreas of P. trituberculatus (Wang et al., 2013). In our previous study, a high level of PtLP transcripts was found in the hemocytes and hepatopancreas of P. trituberculatus (Wang et al., 2013). In our previous study, a high level of PtLP transcripts was found in the hemocytes and hepatopancreas of P. trituberculatus (Wang et al., 2013). In our previous study, a high level of PtLP transcripts was found in the hemocytes and hepatopancreas of P. trituberculatus (Wang et al., 2013).

2. Materials and methods

2.1. Crab breeding and V. alginolyticus challenge

One hundred and sixty swimming crabs with an average weight of 25 ± 3 g were collected from a commercial farm (Xiangshan, China) and acclimatized in aerated seawater at 25 °C for a week before processing. During the entire period of the experiment, the crabs were fed with clam meat once every night, and seawater changed before processing. During the entire period of the experiment, the crabs were fed with clam meat once every night, and seawater changed before processing. During the entire period of the experiment, the crabs were fed with clam meat once every night, and seawater changed before processing.

For the bacterial challenge experiment, the crabs were divided into four groups (40 crabs in each group). These were cultured in four cement pools (2 m × 5 m × 0.9 m, width × length × depth). Crabs from the bacteria-challenged groups were injected of 200 μL live V. alginolyticus suspended in 0.01 mol/L Phosphate Buffer Saline (PBS) (pH 7.2, 7.8 × 10^7 cfu/mL), and the injection site is the arthrodial membrane of the last walking leg. The control group crabs received an injection of 200 μL PBS. All the crabs were observed every hour for the first 24 h, followed by observation every 3 h after 120 h post-challenge. The crabs that died in the first 24 h were classified as the susceptible stock, and the crabs that survived 120 h post-challenge were regarded as being relatively resistant to V. alginolyticus (resistant stock). Muscles in the last walking leg of all crabs from these two stocks were removed and stored at −80 °C for DNA extraction.

2.2. Acquiring of CTL DNA sequence

Genomic DNA was extracted from the muscle of P. trituberculatus using Genomic DNA Purification Kit (Axygen Biosciences, Corning, NY, USA). The primers CTL-F1 and CTL-R1 (Table 1) were designed, according to the full-length cDNA of PtLP in P. trituberculatus (GenBank accession no. EU477491.1), to acquire the DNA sequence [21]. The reaction was carried out in a 50 μL reaction mixture, containing 5 μL of 10 × LA PCR buffer (TakaRa), 8 μL of dNTP (2.5 mM), 1 μL each of forward primer and reverse primer (10 μM), 0.5 μL (2.5 U) of LA Taq (TakaRa), 3 μL of target DNA and 31.5 μL of double distilled water. The reaction was performed in a Tprofessional Thermocycler (Biomatra, Goettingen, Germany) under the following conditions; 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 2.5 min, and a final extension at 72 °C for 10 min. The PCR products were purified by the manufacturer’s protocol of PCR gel purification kit (Axygen) and cloned into pMD19-T simple vector (TakaRa Bio Inc., Otsu, Japan). The vector was transformed into the Trans1-T1 phage resistant chemically competent cell (TransGen, Beijing, China), and the positive recombinants identified through anti-amp selection and PCR screening using M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced using an ABI3730 Automated Sequencer (Applied Biosystems, Carlsbad, CA, USA).

2.3. Identification of polymorphic loci in the CTL

One pair of gene specific primer CTL-F2 and CTL-R2 (Table 1) was designed based on the obtained CTL DNA sequence to amplify the whole CTL gene fragment. The reaction was carried out in a 50 μL reaction mixture, containing 5 μL of 10 × PCR buffer (TakaRa), 6 μL of dNTP (2.5 mM), 1 μL each of forward primer and reverse primer (10 μM), 0.5 μL (1.25 U) of PrimeSTAR HS DNA Polymerase (TakaRa), 3 μL of target DNA and 33.5 μL of double distilled water. The reaction was performed following conditions; one cycle of 94 °C for 4 min, followed by 35 cycles of 94 °C for 50 s, 55 °C for 50 s, 72 °C for 1.5 min, and a final 10 min extension at 72 °C.

The PCR products from three susceptible and resistant crabs each were analyzed on 1% agarose gels and purified. The objective fragments were cloned into pMD19-T vector (TakaRa) and at least three clones were sequenced from every sample. The objective nucleotide sequence of CTL was aligned using the Vector NT1 Suite 9 (Life Technologies, Carlsbad, CA, USA). The polymorphisms in CTL gene were detected from the sequence alignments of different crabs.

2.4. Analysis of SNPs in the CTL and association with V. alginolyticus resistance

Three pairs of gene-specific primers (CTL-F3/R3, CTL-F4/R4 and CTL-F5/R5) (Table 1) were designed based on the sites of polymorphism

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>PCR objective</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13-47</td>
<td>CGCGCGGTTTCCTCAGTGCACC</td>
<td>Sequencing</td>
</tr>
<tr>
<td>RV-M</td>
<td>GAGCGGCTACATGTTCCCCAGG</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CTL-F1</td>
<td>TCTCGTCTGCACTCCACCAA</td>
<td>Obtaining DNA sequence</td>
</tr>
<tr>
<td>CTL-R1</td>
<td>CTGTCGGCCGTCAGAAGAT</td>
<td>Obtaining DNA sequence</td>
</tr>
<tr>
<td>CTL-F2</td>
<td>AGCCACGACGATATATGACACC</td>
<td>Finding SNPs</td>
</tr>
<tr>
<td>CTL-F3</td>
<td>AAGCGCTTATGCACATACCAC</td>
<td>Finding SNPs</td>
</tr>
<tr>
<td>CTL-R3</td>
<td>TCAAATTTTACATTTTCCTTGG</td>
<td>Sequencing SNPs in two stocks</td>
</tr>
<tr>
<td>CTL-F4</td>
<td>CAAATTTTACATTTTTCCTTGG</td>
<td>Sequencing SNPs in two stocks</td>
</tr>
<tr>
<td>CTL-R4</td>
<td>TAAATGAGTCCTCCATAGGAAA</td>
<td>Sequencing SNPs in two stocks</td>
</tr>
<tr>
<td>CTL-R5</td>
<td>CAAATTTTACATTTTTCCTTGG</td>
<td>Sequencing SNPs in two stocks</td>
</tr>
<tr>
<td>CTL-R6</td>
<td>AATTCTCCCTATCTCCTACAAATCG</td>
<td>Genotyping amplification</td>
</tr>
</tbody>
</table>
in the CTL gene to amplify the genomic DNA obtained from the two stocks. Specific PCR products were purified and sequenced. The genotype of every SNP from each sample was determined based on the sequencing chromatograms. Statistical analysis was carried out using the SPSS 11.0 software (IBM, Armonk, NY, USA). Significance analysis of genotype, and the allele frequencies between susceptible and resistant stocks were calculated by Chi-squared test. Results were considered as a significant difference when \( P < 0.05 \), and an extremely significant difference when \( P < 0.01 \).

2.5. High resolution melting (HRM) for E4-205 locus genotyping

The significant SNP locus E4-205 was selected for genotyping with high resolution melting (HRM) assay. HRM analysis, which included the primer design target DNA template concentration, prediction of melting temperatures, and other parameters, was performed according Protocol of Rotor Gene Q (Qigaoen, Venlo, Netherlands). This analysis is based on the amplification of a short DNA fragment, for which the specific primers CTI-HRM-F (R) were designed (Table 1). PCR mixture consisted of 1 \( \mu \)L genomic DNA (20 ng), 10 \( \mu \)L SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA, USA), 0.8 \( \mu \)L each of the forward and reverse primer (10 \( \mu \)M), and 7.4 \( \mu \)L of double distilled water, making up a volume of 20 \( \mu \)L. PCR cycling and HRM analysis were performed on the Rotor-Gene 6000 real-time PCR system (Qigaoen) under the following conditions: one cycle of 98 °C for 2 min, followed by 40 cycles of 98 °C for 5 s, 58 °C for 20 s, 72 °C for 20 s, and a final 10 min extension at 72 °C. For melt curve analysis, the PCR products were heated from 65 °C to 85 °C uniformly, and the fluorescent signal collected at every 0.1 degree rise in temperature. Normalized and difference graphs were generated using the High Resolution Melting Analysis software provided with the Rotor-Gene 6000.

2.6. Confirmation of significant SNP by challenge test of large sample

The association between the E4-205 locus and the susceptibility or resistance of swimming crab to V. alginolyticus was investigated by a challenge experiment. The bacterial challenge experiment was performed on 300 crabs. Muscle was collected from all the crabs (dead and surviving) and stored at \(-80 °C\) for DNA extraction. Genotype of each sample was determined by HRM assay (protocol as described in the previous section). The mortality and survival rate for each genotype were compared by Chi-squared analysis, and the statistical significance was calculated using SPSS 11.0 (IBM).

3. Results

3.1. Sequence characterization of CTL

The full length cDNA of PtLP is 923 bp containing a 138 bp-long 5'-UTR and 290 bp 3'-UTR (Fig. 1A). The genomic DNA fragment between positions 14 and 866 of PtLP cDNA was amplified. The obtained amplicon was designated as CTL gene. The sequence and length (1473 bp) of CTL genomic DNA were deposited in GenBank under the accession number JX195096.1. The exon–intron boundaries of CTL were determined by alignment with the corresponding cDNA sequence. Three introns were found in the open reading frame region of CTL containing typical intron–exon junction structures, in which, donor and acceptor (GT and AG) dinucleotide sequences were found (Figs. 1A and 2).

3.2. Analysis of crabs infected with live bacteria

In the challenge group, the first dead crab was observed 6 h after infection. The deaths increased gradually and reached a peak at 60 h. The crabs that died in the first 24 h (n = 33) were classified as the susceptible stock, and the 33 crabs that survived the challenge experiment were regarded as being relatively resistant to V. alginolyticus (resistant stock). No dead crabs were found in the control group.

3.3. CTL gene polymorphisms

A 1320 bp fragment was amplified from three susceptible and resistant crabs each using the CTL–F2 (R2) primer. This primer amplifies all the exons and introns of CTL gene. 18 positive clones of each fragment from the six crabs were sequenced. One insertion/deletion (ins/del) polymorphism and four SNPs were found in the amplified CTL fragments. Four polymorphisms were located in introns; I2-309 T-A, I3-89 A-C, I3-101 A-G, and I3-104 CAA insertion/deletion. One nonsynonymous substitution in the fourth exon, E4-205 C-T (Fig. 2), resulted in the substitution of Threonine (ACT) to Isoleucine (ATT).

3.4. Association between gene polymorphisms and susceptibility/resistance to V. alginolyticus

Genomic DNA from the 33 susceptible and resistant crabs each was amplified via PCR. The SNP genotype isolated from each sample was determined through sequencing chromatograms. Table 2 shows the allele and genotype frequencies for CTL in the resistant and susceptible stocks. According to the results of the Chi-squared \((\chi^2)\) test, three SNPs (I2-309 T-A, I3-89 A-C, I3-101 A-G), and one ins/del polymorphism (I3-104 CAA ins-del) exhibited significant \((P < 0.05)\) difference in genotype frequencies, but no difference in these allele frequencies was found between the resistant and susceptible crabs. However, the SNP (E4-205) showed significant difference in both genotype and allele frequencies between the two stocks \((P < 0.05)\). The T allele frequency was 33.3% in susceptible stock, and 53% in resistant stock. In addition, the T/T genotype frequency was 6.1% in susceptible stock, and 21.2% in resistant stock, with a demonstrated significant difference between them \((P = 0.022)\).

3.5. SNP E4-205 genotyping by HRM

The genotypes of SNP E4-205 were determined by the C/T, T/T and C/C sequencing chromatograms (Fig. 3). We have established the HRM
method for the detection of the three genotypes, by screening of primers and optimizing other parameters. Normalized graph and difference graph were generated with the aid of the High Resolution Melting Analysis software provided with the Rotor-Gene 6000. Genotypes of each sample were automatically indicated when the known genotypes (identified by sequencing) were included as positive controls. Fig. 4 shows a clear separation of the three genotypes.

3.6. Relation of susceptibility/resistance phenotypes to genotypes at significant SNPs

To determine whether variation at CTL is associated with V. alginolyticus resistance, 300 crabs were infected with the bacteria, and the SNP at E4-205 genotyped by HRM assay. Table 3 shows the distribution of the three SNP genotypes in the 300 crabs. C/T genotype is observed in 46.7%, T/T genotype in 16.3%, and C/C in 37% of the total number of samples. The cumulative mortality of E4-205 T/T SNP was 57.1%, which was extremely significantly lower than that of C/T (81.4%) and C/C (82%). However, no significant difference was detected between samples expressing C/T and C/C polymorphism (P > 0.05) (Fig. 5).

4. Discussion

V. alginolyticus has been responsible for considerable economic losses in the raising of swimming crabs over the past few years (Liu et al., 2007; Wang et al., 2006). The selective culture of swimming crabs by traditional methods in our laboratory has revealed that some crabs were disease resistant (Mu et al., 2012). However, little is known about the molecular mechanism of disease resistance in crabs. Therefore, research conducted on the identification of genes, and its SNPs related to disease resistance, could help improve our understanding of disease resistance in crabs, and may beneficial to cultivate and develop disease-resistant crabs.

Polymorphisms affecting gene expression, playing an important role in resistance and susceptibility to diseases, is seen in humans (Mardan-Nik et al., 2014; Sun et al., 2007), scallops (Bao et al., 2010; Li et al., 2009), and oysters (Yu et al., 2011). In the present study, genomic DNA of C-type lectin is identified and one SNP is found to be related to susceptibility/resistance to V. alginolyticus in P. trituberculatus. To our knowledge, this is the first study to identify an association between nucleotide polymorphisms of CTL genes and bacterial resistance. We have analyzed the temporal expression change of CTL gene in hemolymph and hepatopancreas after V. alginolyticus challenge in ordinary crabs and the disease-resistant stock cultured by traditional methods in our laboratory (unpublished data). The results demonstrated that the CTL gene was upregulated in both stocks, higher expression levels of the gene being observed in the disease-resistant stock, following V. alginolyticus challenge. The combined results of these studies support the theory that polymorphisms in the CTL gene are the causative mutations affecting V. alginolyticus-resistance in swimming crab.

Among all the polymorphisms identified in the CTL gene, only the SNP at the E4-205 locus is significantly different in both genotype and allele frequencies between the two stocks of crabs (P < 0.05). The E4-205 SNP is a non-synonymous variation, and its polymorphism results in the substitution of Threonine (ACT) to Isoleucine (ATT). The T/T SNP frequency was determined to be 6.1% in susceptible stock, and 21.2% in resistant stock, displaying a significant difference of P = 0.022. This indicated that E4-205 T/T was associated with the resistance of the swimming crabs to V. alginolyticus. Similar results have been reported in the P. trituberculatus anti-lipopolysaccharide factors (Li et al., 2013), and the SOD family of A. irradians (Bao et al., 2010). Non-synonymous genetic variations, which improve crab’s immune resistance, may cause considerable differences in the composition and structure of the affected protein, thus changing its biological traits and functions. Therefore, the identification of the different functional
Table 2
Distribution of CTL polymorphisms in susceptible and resistant stocks.

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Position</th>
<th>Genotype</th>
<th>Genotype no.</th>
<th>(n = 33)</th>
<th>$x^2$</th>
<th>$P$</th>
<th>Base type</th>
<th>Allele no.</th>
<th>$x^2$</th>
<th>$P$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Susceptible</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>I2-309</td>
<td>T/A</td>
<td>22(0.667)</td>
<td>29(0.879)</td>
<td>4.227</td>
<td>0.040*</td>
<td>T</td>
<td>22(0.333)</td>
<td>29(0.439)</td>
<td>1.566</td>
</tr>
<tr>
<td>2</td>
<td>I3-89</td>
<td>A/A</td>
<td>11(0.333)</td>
<td>4(0.121)</td>
<td>4.227</td>
<td>0.040*</td>
<td>A</td>
<td>44(0.667)</td>
<td>37(0.561)</td>
<td>1.566</td>
</tr>
<tr>
<td>3</td>
<td>I3-101</td>
<td>A/G</td>
<td>22(0.667)</td>
<td>29(0.879)</td>
<td>4.227</td>
<td>0.040*</td>
<td>A</td>
<td>44(0.667)</td>
<td>37(0.561)</td>
<td>1.566</td>
</tr>
<tr>
<td>4</td>
<td>I3-104</td>
<td>CAA/CAA</td>
<td>11(0.333)</td>
<td>4(0.121)</td>
<td>4.227</td>
<td>0.040*</td>
<td>ins</td>
<td>44(0.667)</td>
<td>37(0.439)</td>
<td>1.566</td>
</tr>
<tr>
<td>5</td>
<td>E4-205</td>
<td>C/T</td>
<td>18(0.545)</td>
<td>21(0.636)</td>
<td>6.564</td>
<td>0.038*</td>
<td>T</td>
<td>22(0.333)</td>
<td>35(0.530)</td>
<td>5.218</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>2(0.061)</td>
<td>7(0.212)</td>
<td></td>
<td></td>
<td>C</td>
<td>44(0.667)</td>
<td>31(0.470)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>13(0.394)</td>
<td>5(0.152)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I*-intron, E-exon, and the number in position indicate number of base pairs. $P$ values less than 0.05 are shown in bold.

* Indicates significant difference between resistant and susceptible stocks.

Fig. 3. The sequencing chromatograms of E4-205 SNP. The arrow shows that the genotype of E4-205 is (A) C/T, (B) T/T, and (C) C/C.
proteins resulting due to the polymorphism at E4-205, which could be responsible for disease resistance, could help elucidate the molecular mechanism of CTL gene action. Clearly, there is a need for further research, in order to better evaluate and investigate this association, and the possible mechanisms of this association.

There are many exiting methods that can be used for SNP genotyping, including allele specific amplification and single-base extension, restriction enzyme digestion (in cases where the gene expresses enzyme loci), sequence-specific conformational polymorphism, and thermal (Tm) shift assay (Svyänen, 2001; Wang et al., 2005). High resolution melting (HRM) is an enhanced version of the traditional DNA melting analyses for PCR products (Montgomery et al., 2007). This technology displays such characteristics as high throughput analysis, low cost, high sensitivity, specificity and closed tube operation preventing pollution (Liew et al., 2004; Reed and Wittwer, 2004; Seipp et al., 2008) have demonstrated that HRM can be a powerful tool for the discovery of novel SNPs, finding 11 novel SNPs to distinguish between two species of trout (McGlaflin et al., 2010). In addition, the HRM assay can detect small changes in the melting curve caused by SNPs in a short amplicon, so, it can be used for SNP genotyping (Norambuena et al., 2009). Yu et al. successfully applied the HRM assay to determine the three genotypes of SNP198 in the serine protease inhibitors cvSI-1, associated with Dermo resistance in eastern oyster (Yu et al., 2011). In this study, HRM analysis was used for SNP genotyping to confirm the association between genotype and resistance. Our results show that HRM assay can accurately genotype the SNPs in the P. trituberculatus. The HRM assay for SNP E4-205 genotyping produced clear and reliable results, which were supported by the results of sequencing (Figs. 2 and 3).

Table 3
Distribution of the three genotypes at the E4-205 locus, and its mortality and survival rate in 300 crabs infected with V. alginolyticus.

<table>
<thead>
<tr>
<th>Position</th>
<th>Genotype</th>
<th>Mortality no. (%)</th>
<th>Mortality no. (%)</th>
<th>Survival no. (%)</th>
<th>Total (%)</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4-205</td>
<td>C/T</td>
<td>114 (81.4)</td>
<td>26 (18.6)</td>
<td>140 (46.7)</td>
<td>14.233</td>
<td>0.001</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>28 (57.1)</td>
<td>21 (42.9)</td>
<td>49 (16.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>91 (82.0)</td>
<td>20 (18.0)</td>
<td>111 (37.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Indicates significant difference between different genotypes.
In conclusion, polymorphisms in the CTL gene were investigated and significant association between the E4-205 SNP and V. alginolyticus susceptibility/resistance was established in P. trituberculatus. The developed HRM assay provides a useful rapid method for SNP genotyping. The E4-205 T/T SNP variant might potentially be applied as the gene marker for selection of swimming crabs with enhanced resistance to V. alginolyticus.

Acknowledgements

The authors would like to thank Yanfei Wang, Chen Chen, and Shaokun Lu for technical assistance in crab breeding and collection. This research was supported by the National High Technology Research and Development Program of China (No. 2012AA10A408), the Zhejiang Major Special Program of Breeding (2012C22507-3), the Zhejiang Provincial Natural Science Foundation of China under Grant No. LY12C109009, the National Natural Science Foundation of China (41106120), Ningbo Natural Science Foundation (2014A610183) and K.C. Wong Magna Fund by Ningbo University.

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