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Characterization of novel EST-SNP markers and their association analysis with growth-related traits in the Pacific oyster *Crassostrea gigas*

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Abstract The Pacific oyster *Crassostrea gigas* is one of the most commercially important and worldwide cultured fishery animals. To facilitate marker-assisted selection in genetic improvement of this species, 108 novel polymorphic single-nucleotide polymorphism (SNP) markers were developed from expressed sequence tags (ESTs) by high-resolution melting analysis. The association between the markers and growth traits in a fast-growing strain and validation in two cultured populations was analyzed. For the 108 SNP loci, the observed heterozygosity varied from 0.011 to 0.625 and the expected heterozygosity varied from 0.031 to 0.501. By selective genotyping method, special allele frequencies of nine SNP markers showed significant difference between the fast-growing strain and the commercial control population ($P < 0.0001$). The nine SNP loci were further validated in extreme phenotype tails of the commercial control population and an independent cultured population. Three markers, jl027, ji090, and jl615, were found to be significantly associated with growth traits in oysters ($P < 0.01$). The proportion of additive genetive variance explained by the three confirmed SNPs ranged from 0.27 to 2.23%. The putative functions of the ESTs containing the jl090 and jl615 markers indicated that they might be related to growth of *C. gigas*. The three confirmed SNPs associated with growth traits would have potential applications in future genetic improvement of *C. gigas*.

Keywords *Crassostrea gigas* · EST-SNP · High-resolution melting · Selective genotyping · Marker-trait association

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Introduction

The Pacific oyster *Crassostrea gigas*, originating from north-eastern Asia, has been introduced to many countries for aquaculture purposes since 1940 (Ruesink et al. 2005). Although it has been one of the most economically important marine bivalve species, some problems have limited the development of oyster industry within recent decades, including the decline in growth (Wang et al. 2013) and mass mortalities in both juvenile and adult *C. gigas* populations caused by the infection of *Ostreid herpesvirus 1* (Serracca et al. 2016). So far, several directional selection programs based on family selection (Langdon et al. 2003; Evans and Langdon 2006; Dégremont et al. 2010) and mass selection (Li et al. 2011) have been conducted for improving body weight, growth rate, and survival. However, the traditional selection breeding method is expensive, time-consuming, and easily influenced by the environment. Alternatively, marker-assisted selection (MAS) provides a more accurate and efficient approach for genetic improvement of economic traits (Dirlewanger et al. 2004; Jena and Mackill 2008). Through MAS, selection for the interested traits can be achieved by the markers tightly linked underlying genes or that have been development from the actual gene sequences (Xu and Crouch 2008). Marker-assisted selection programs have been achieved successfully in several fishery animals such as Japanese flounder *Paralichthys olivaceus* (Fuji et al. 2007) and Atlantic salmon *Salmo salar* (Moen et al. 2009), demonstrating the validity of the methodology (Ozaki et al. 2012). Thus, it is essential to combine with MAS to enhance the efficiency of breeding programs in oysters.

Single-nucleotide polymorphisms (SNPs), as the third generation of molecular markers, have great advantages in genomics and genetic studies when compared to other markers. They are the most abundant heritable variation in genomes and can be detected and genotyped automatically and high-throughput (Kim and Misra 2007). To date, SNPs have been identified in many aquatic species, such as Atlantic cod (Hubert et al. 2010), rainbow trout and steelhead (Hansen et al. 2011), Nile tilapia (Xia et al. 2014), mud crab (Feng et al. 2014), hard clam (Jing et al. 2015), and the Pacific oyster (Sauvage et al. 2007; Zhong et al. 2013, 2014; Jin et al. 2014; Lapegue et al. 2014). However, the number of available SNP markers is still limited for further applications in constructing high density linkage maps and marker-assisted selection. With the development of next-generation sequencing technologies, the sharply increased expressed sequence tags (ESTs) in public database provide abundant resources for developing SNPs. EST-SNPs originate from the highly conserved transcribed regions of genomes; they are especially valuable for identification of genes that associated with certain important functions and transferable to relative species in genus *Crassostrea* (Gupta et al. 2003; Poncet et al. 2006).

During the last decade, a great many of accurate and rapid technologies have been developed for SNP detection (Kim and Misra 2007). High-resolution melting (HRM) analysis is based on the real-time fluorescent quantitative PCR. As it monitors exactly the decreasing fluorescence of intercalating saturated dye in the process of dissociation of double-stranded DNA, we can obtain melting curves to analyze the variances in PCR products based on curve differences (Wittwer et al. 2003; Reed et al. 2007). The technology has been applied for rapid mutation screening and SNP genotyping (Lehmensiek et al. 2008; Garritano et al. 2009; Huang et al. 2014).

Quantitative traits, such as growth and salt-tolerance of oysters, show continuous variation in progeny. Each quantitative trait is controlled by several genes with relatively small effects, leading to the relation between genotype and phenotype that is difficult to be detected (Yano

and Sasaki 1997; Van Ooijen 1999). The primary approach for identifying quantitative trait loci (QTL), linkage mapping, is time-consuming to construct an experimental population with a significant phenotypic difference for the quantitative traits (Edwards et al. 1992; Charcosset and Gallais 1996; Gupta et al. 2005). Several factors would affect the power and accuracy of detecting QTLs by linkage analysis, such as the size of mapping families, the heritability of target traits, and the statistical threshold used for analysis (Yano and Sasaki 1997; Stella and Boettcher 2004). Moreover, QTLs detected in one mapping population are often difficult to apply to different genetic backgrounds; thus, the general applicability for MAS is limited (Lu et al. 2013).

Selective genotyping is an alternatively powerful approach to detect the linkage between markers and QTLs. It is carried out by genotyping only individuals from the high and/or low phenotypic traits of the entire sample population and comparing the differences in marker allele frequencies between the extreme phenotype groups (Lander and Botstein 1989; Darvasi and Soller 1992; Lu et al. 2013). The change in marker allele frequencies results from the association between marker loci and QTLs controlling traits under selection (Foolad et al. 1997). As a marker-trait association analysis, various statistical methods have been proposed and used to detect QTLs for both bidirectional and unidirectional selective genotyping in plants and animals (Slatkin 1999; Moreau et al. 2004; Coque and Gallais 2006; Gallais et al. 2007; Huang and Lin 2007; Nie et al. 2013).

In the present study, we validated 108 novel SNPs from EST database using HRM method, analyzed their association with QTLs for growth traits in a fast-growing selected strain and a control population of *C. gigas*, and further validated the identified markers in the control population and an independent cultured population.

Materials and methods

Animals and DNA extraction

One fifth-generation selective strain for fast growth of *C. gigas* (FGS) (Cong 2014) and one commercial cultured population (CCP) collected from Rongcheng were used to identify SNPs associated with growth traits. Ninety-six oysters randomly sampled from 145 individuals of the FGS and 96 oysters randomly sampled from the CCP were collected when they were 24 months old in 2014.

The CCP and another cultured population collected from Rushan (RS), Shandong province, were used for validating the markers identified in the initial association analysis. For the CCP, 48 individuals with high phenotype values (CCP_H) and 48 individuals with low phenotype values (CCP_L) (16% for shell height and total weight from the extreme phenotype tails) were selected, respectively. Similarly, 48 individuals with high phenotype values (RS_H) and 48 individuals with low phenotype values (RS_L) (16% for shell height and total weight from the extreme phenotype tails) were selected from the RS when they were 18 months old in 2015 (Fig. 1). The shell height, shell length, shell width, total weight, and soft-tissue weight were measured to compare the differences of phenotype values between the FGS and CCP populations, CCP_H and CCP_L, RS_H and RS_L groups by *t* test.

Genomic DNA was isolated from adductor muscle using phenol-chloroform method described by Li et al. (2002). The quality and concentration of samples DNA were checked using Nano Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE USA). DNA

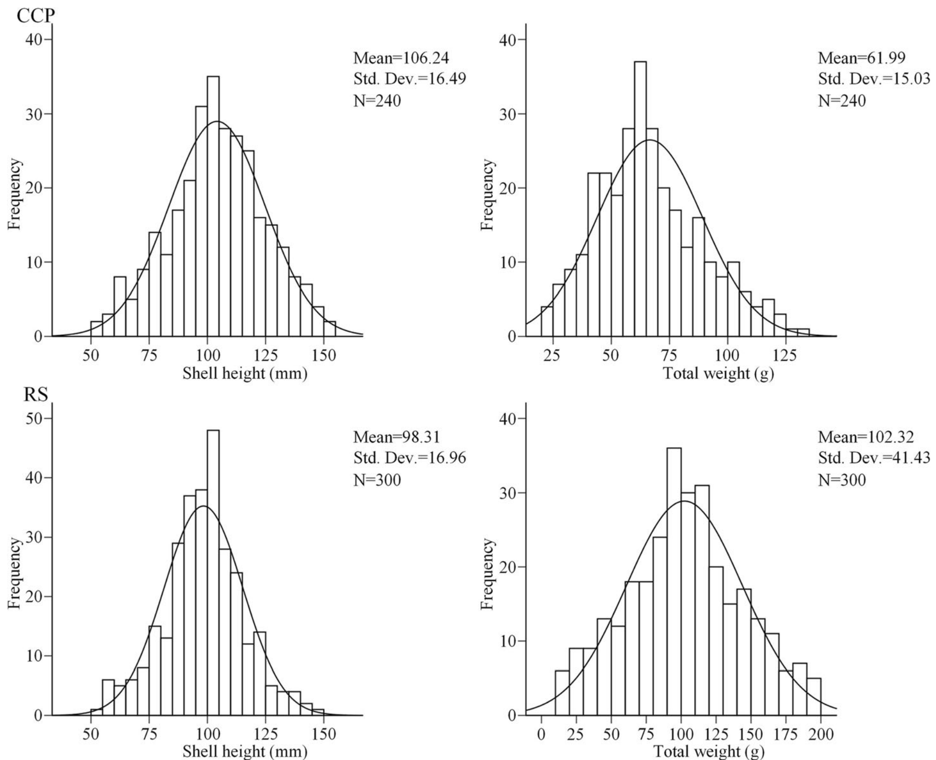

Fig. 1 The distributions of shell height and total weight for CCP and RS populations

templates were diluted to 10 ng μl^{-1} with ultrapure water and stored at −30 °C before polymerase chain reaction (PCR).

EST-SNP mining and validation

A total of 46,171 EST sequences of the Pacific oyster were downloaded from GenBank public database in our previous study (Zhong et al. 2013). Putative SNPs were derived after the sequences clustered and assembled into contigs. A single-base mutation that occurred in four or more ESTs was chosen for further validation. In this study, 407 putative SNPs were randomly selected to screen for polymorphic SNPs.

Primer Premier 5.0 program (PREMIER Biosoft International, Palo Alto, CA, USA) was used for designing primer pairs with parameters as follows: annealing temperature at 58~62 °C, product size within 60~150 bp, and the composition of GC should be 40~60%. Primers with significantly stable structure of hairpin, primer dimer, or duplex formation were excluded.

Amplification and genotyping

Using the polymorphic SNPs validated above, all of the sample individuals collected from the FGS, CCP, and RS were genotyped by HRM analysis on a LightCycler®480 real-time PCR

instrument (Roche Diagnostics Burgess Hill, UK). PCR were performed in a 10-μl reaction mixture: 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 μM forward and reverse primers, 10 ng template DNA, 0.25 U *Taq* DNA polymerase (Takara, Dalian, China), and 5 μM SYTO®9 (Invitrogen, Foster City, CA, USA). The thermal cycling procedure consisted of initial denaturation at 95 °C for 5 min, 45–50 cycles of denaturation at 95 °C for 10 s, annealing temperatures at 60 °C for 10 s, then extension at 72 °C for 10 s. Melting curves were generated by collecting fluorescence data between 60 to 90 °C after amplification. Then, the data were analyzed using the Gene Scanning and Tm Calling programs within LightCycler®480 Software 1.5 (Roche Diagnostics).

Genetic analysis and function prediction

For each SNP locus, the allele frequencies, polymorphic information content (PIC), observed heterozygosity (*H*o), and expected heterozygosity (*H*e) were calculated using CERVUS 3.0 (Kalinowski et al. 2007). Sequences containing the SNPs were annotated using BlastX with the homology *E* values less than 1.0×10^{-6} on NCBI database (http://www.ncbi.nlm.nih.gov/). The Open Reading Frame Finder (ORF finder) was used to determine whether SNPs were synonymous, non-synonymous, or from untranslated regions (UTRs).

Marker-QTL association analysis

The method described by Gallais et al. (2007) was used to analyze the effect of selection on the frequency of a marker linked to a QTL associated with growth traits; the significance of the change was approached by a χ^2 test ($P < 0.0001$) with sequential Bonferroni-like correction (Rice 1989):

$$\chi^2 = (\hat{p}_1 - \hat{p}_2)^2 / \mathrm{var}\, p$$

where $\hat{p}_1$ is the observed allele frequency after selection for the FGS, $\hat{p}_2$ is the observed allele frequency for the CCP, and var*p* is the expected variance of allele frequency due to sampling. The equation to compute var*p* is as follows:

$$\mathrm{var}\, p = \hat{p}_2 (1 - \hat{p}_2)[1 - (1 - 1/N)(1 - 1/Ns)]$$

N refers to the individuals collected at random from the FGS ($N = 145$) and the *N*s refers to the individuals collected randomly from the *N* individuals for association analysis ($Ns = 96$).

Validating the candidate SNPs associated with growth

To validate the SNP markers identified in the initial association analysis between FGS and CCP populations, the allele frequencies of these markers in CCP_H and CCP_L, RS_H and RS_L were further examined by Pearson's Chi-square test ($P < 0.01$). Only the markers that showed significantly different allele frequency both in the CCP and RS groups can be considered as SNPs associated with growth performance.

The part of genetic variation explained by the validated SNPs (EPV) was calculated by the equation as follows (Falconer and Mackay 1996):

$$\mathrm{EPV} = \left[2pq(\alpha + \delta(q-p))^2\right]/V_A$$

Briefly, p and q are the frequency of the major and minor allele, respectively, α is the additive effect, δ is the dominance effect, and V_A is the total additive genetic variance of the trait.

Results

SNP development and characterization

In this study, 407 primer pairs were designed for validating putative SNPs in 32 wild oysters. Among them, 21 (5.2%) were monomorphic while 108 loci (26.5%) were polymorphic and their melting curves can be genotyped distinctly (Fig. 2). In 17 cases, the size of amplicon was larger than expected probably due to introns that exist in the PCR productions. The PIC ranged from 0.03 to 0.374 with an average of 0.237. The observed heterozygosity varied from 0.011 to 0.625 (mean 0.277 ± 0.150) and the expected heterozygosity varied from 0.031 to 0.501 (mean 0.286 ± 0.118). Among the polymorphic SNPs, 17 were located in the UTR and 91 in the coding region. Forty-one of the 91 SNPs were synonymous and 50 were nonsynonymous. The classification is putative because ORFs were identified by choosing the longest possible translation into amino acid sequence. Information about the 108 polymorphic markers is summarized in Supplementary Table 1.

Description of phenotypic variability in the experiment populations

The mean shell height, shell length, shell width, total weight, and soft tissue weight of the FGS strain were 111.21 ± 1.06 mm, 59.96 ± 0.69 mm, 33.61 ± 0.89 mm, 80.53 ± 1.92 g, and 21.88 ± 0.93 g, respectively, significantly superior to those of the CCP population ($P < 0.01$)

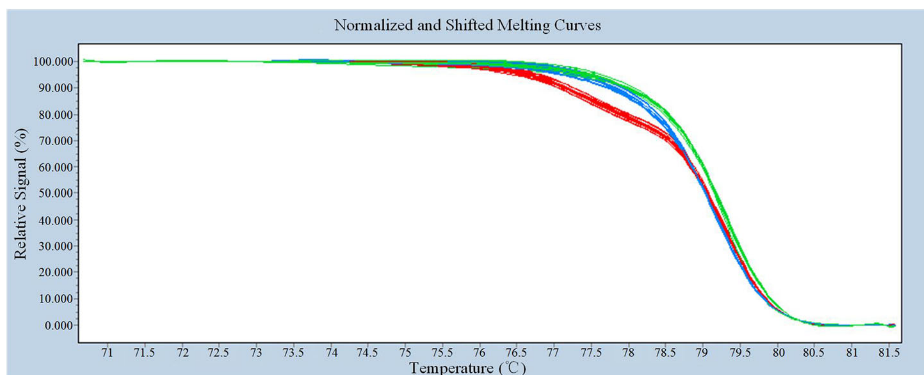

Fig. 2 Normalized and shifted melting curves of jl090 in the FGS. *Green curves* indicate the homozygote of GG, *blue curves* indicate the homozygote of AA, *red curves* indicate heterozygote AG

Table 1 Growth traits (means ± SE) of the fast-growing strain and commercial control population

Groups	Shell height (mm)	Shell length (mm)	Shell width (mm)	Total weight (g)	Soft tissue weight (g)
FGS	111.21 ± 1.06[a]	59.96 ± 0.69[a]	33.61 ± 0.89a	80.53 ± 1.92a	21.88 ± 0.93a
CCP	106.24 ± 1.59[b]	53.61 ± 0.90[b]	28.91 ± 0.71b	61.99 ± 1.45b	15.07 ± 0.60b

Different letters in the same column mean significant difference between the two populations ($P < 0.01$)

(Table 1). For the validation groups, the mean shell height, shell length, shell width, total weight, and soft tissue weight of the CCP_H were 135.23 ± 1.09 mm, 66.25 ± 2.15 mm, 35.28 ± 0.35 mm, 104.50 ± 1.50 g, and 24.05 ± 1.11 g, while those of RS_H 119.03 ± 2.72 mm, 59.72 ± 0.87 mm, 36.04 ± 0.61 mm, 141.67 ± 3.48 g, and 48.10 ± 1.91 g, respectively, significantly superior to those of the CCP_L and RS_L ($P < 0.01$) (Table 2).

Identify and confirmation of SNPs associated with growth

By association analysis, the specific alleles of 9 SNP markers (jl006, jl027, jl090, jl162, jl169, jl331, jl469, jl514, and jl615) showed significantly higher frequency in the FGS strain than that in the CCP population ($P < 0.0001$) (Table 3). Individuals selected from the extreme phenotype tails of the CCP (CCP_H and CCP_L) and RS (RS_H and RS_L) were used to confirm the growth-associated markers identified in the initial analysis based on the FGS and CCP populations. As a result, three common markers of jl027, jl090, and jl615 showed significantly different allele frequency between the high tails and low tails of the CCP and RS. These three markers were confirmed to be associated with QTLs that contributed to growth at $P < 0.01$ (Table 4). In addition, the allele frequency of two markers (jl331 and jl469) only showed significantly different in the CCP_H and CCP_L but not in the RS_H and RS_L.

For the three validated loci, G, C, and C were the favorable alleles that were associated with superior growth performance, respectively. The evaluation of the additive genetic variation explained by the SNPs showed that they could explain a small proportion of the total phenotypic variance in shell height and total weight (0.27–2.23%) with a mean of 0.93%.

Function annotation of the associated SNPs

The putative function of the ESTs containing the jl027, jl090, and jl615 was predicted by BlastX searching (Table 5). The EST containing the jl090 matched to guanine nucleotide-

Table 2 Growth traits (means ± SE) of the extreme phenotype tails of the CCP and RS populations

Traits	CCP		RS	
	CCP_H	CCP_L	RS_H	RS_L
Shell height (mm)	135.23 ± 1.09a	71.13 ± 1.15b	119.03 ± 2.72a	79.55 ± 1.55b
Shell length (mm)	66.25 ± 2.15a	44.29 ± 0.38b	59.72 ± 0.87a	45.29 ± 0.88b
Shell width (mm)	35.28 ± 0.35a	21.99 ± 0.47b	36.04 ± 0.61a	26.31 ± 0.63b
Total weight (g)	104.50 ± 1.50a	35.63 ± 0.88b	141.67 ± 3.48a	50.56 ± 1.99b
Soft-tissue weight (g)	24.05 ± 1.11a	8.25 ± 0.43b	48.10 ± 1.91a	18.80 ± 0.82b

Different letters mean significant difference between the two groups ($P < 0.01$)

Table 3 The summary statistics of the association analysis between the fast-growing strain and the commercial control population

Marker/allele	$\hat{p}_1$	$\hat{p}_2$	var p_1	χ^2
jl006/T	0.3085	0.0990	0.0015	28.5638*
jl027/G	0.3385	0.0781	0.0012	54.6139*
jl090/C	0.2708	0.0469	0.0008	65.1135*
jl162/T	0.6368	0.2263	0.0030	55.8253*
jl169/G	0.4105	0.1667	0.0024	24.8336*
jl331/G	0.4789	0.1875	0.0026	32.3388*
jl469/A	0.9632	0.6579	0.0039	24.0138*
jl514/A	0.6146	0.3053	0.0037	25.0451*
jl615/C	0.4468	0.1563	0.0023	37.1417*

*$P < 0.0001$, significant difference of the allele frequencies between the two populations

binding protein G (i) subunit alpha while the jl0615-contained EST sequence was homologous to SH2 domain-containing protein 3C. The EST containing the jl027 was annotated as uncharacterized protein. The putative function of this marker remains to be further researched.

Discussion

As there are differences in the EST sequence and the genome sequence, it may increase the difficulty and cost during the process of SNP validation. However, for most non-model organisms, it is still an important and effective way to develop SNPs from EST database. We designed 407 primer pairs and successfully validated 108 (26.5%) SNPs by HRM method. The validation ratio is lower than 37% that is reported by Jin et al. (2014). The *C. gigas* is typically highly heterozygous with one SNP per 60–113 bp by mining EST database (Sauvage et al. 2007; Zhong et al. 2013). Such a high density of SNP due to large effective population sizes (Sauvage et al. 2007) and bad quality of flanking sequences may affect primer design and PCR amplification, leading to the low validation rate. It is essential to select primers as close to the target SNP as possible for obtaining amplicons containing only one SNP. The short amplicons are more likely to show the effects of small sequence changes, which is beneficial for differentiating genotypes using HRM analysis. In addition, insufficient sample size,

Table 4 The summary statistics of the Pearson's Chi-square test based on the extreme phenotype tails of the CCP and RS populations and the explained part of variance (EPV) of the confirmed loci

SNP	Allele	Number		EPV (%)		χ^2	Number		EPV (%)		χ^2
		CCP_H	CCP_L	Shell height	Total weight		RS_H	RS_L	Shell height	Total weight	
jl027	G	59	26	0.43	0.38	22.99*	68	44	0.54	0.27	12.34*
	A	37	70				28	52			
jl090	C	61	38	0.68	1.41	11.03*	77	52	2.23	0.55	14.77*
	T	35	58				19	44			
jl615	C	65	35	1.62	0.74	18.78*	63	36	0.92	1.37	15.20*
	T	31	61				33	60			

*$P < 0.01$, significant difference of the allele frequencies between the two groups

Table 5 Characterization of the three SNP-containing ESTs associated with growth traits in *C. gigas*

SNP	Putative function	GenBank accession no.	*E* value
jl027	uncharacterized protein LOC105320876	XP_011417306.1	$1e^{-19}$
jl090	guanine nucleotide-binding protein G (i) subunit alpha	XP_011447788.1	$2e^{-21}$
jl615	SH2 domain-containing protein 3C	EKC25450.1	$5e^{-18}$

presence of selective pressures, and sampling individuals from genetically structured population also may affect the efficiency of SNP validation (Jin et al. 2014).

In order to measure the informativeness of the EST-derived SNP markers, the polymorphism information content was calculated based on a wild population. Fifty-five loci are reasonably informative ($0.5 > PIC > 0.25$), and 53 only slightly informative ($PIC < 0.25$) with a mean PIC value of 0.237 (Botstein et al. 1980). By comparison, a high level of polymorphism was reported by microsatellites with an average PIC of 0.811 (Li et al. 2010). The characteristic of biallelic for SNPs leads to the polymorphism information at a lower level when compared to the multi allelic marker microsatellites (Aitken et al. 2004).

So far, five linkage maps and 19 QTLs of economic traits have been reported for *C. gigas* (Hubert and Hedgecock 2004; Li and Guo 2004; Hedgecock et al. 2006, 2007; Sauvage et al. 2010; Guo et al. 2012; Zhong et al. 2014). These genetic maps were mainly constructed based on amplified fragment length polymorphism (AFLP) and microsatellite markers. Low coverage and large interval between markers limit the maps to be used for QTL fine mapping and further application in marker-assisted selection for the oyster. Therefore, the progress of QTL mapping for *C. gigas* moved forward slowly by linkage analysis, and more studies are still needed to detect the markers associated with various important economic traits. In this study, the fifth-generation of fast-growing selected strain provides us a valuable material for detecting the association between markers and QTLs by selective genotyping. If QTLs controlling growth performance exist, selection for fast growth would result in an increase in the frequency of favorable alleles in the selected high phenotype tail and a decrease in the frequency of favorable alleles in the selected low phenotype tail (Stuber et al. 1980; Lander and Botstein 1989). By testing changes of marker allele frequency, nine SNP markers showed significant difference in allele frequencies between the FGS and CCP, and three of them were further validated in the CCP and an independent RS population with significantly different phenotypic values. The allele G of jl027, allele C of jl090, and allele C of jl615 showed significantly higher frequencies again in the high phenotypic values groups CCP_H and RS_H than that in the low phenotypic value groups CCP_L and RS_L.

The efficiency and power of detecting QTLs by selective genotyping depend on several factors, such as heritability of the trait, intensity of the selection, and the distance between the marker locus and the QTL(s) (Foolad et al. 2001). One of the main interests of the selective genotyping is that the researchers can detect markers associated with QTLs by only genotyping individuals from one or two tails (Gallais et al. 2007). However, this would lead to overestimate the expectation of detected QTL effects (Beavis 1994, 1998). Meanwhile, it would also cause a relatively high type I error (Moreau et al. 1998). For the FGS strain which was constructed by five generations of successive selection breeding, the favorable alleles for growth had got efficient accumulation during the selection process. In contrast, the cultured CCP and RS populations were constructed by mass spawning without intense selective pressure, and their power to detect the markers associated with growth was limited. This may be the reason why four and six of the nine SNPs identified in the initial association did not

show significantly different allele frequencies between the CCP_H and CCP_L, RS_H and RS_L groups, respectively. In addition, the differences in allele frequencies between the FGS and CCP populations may not result from their association with growth but from the neutral genetic history in the studied populations (e.g., genetic drift or initial allele frequencies in the funding population of the selected strain) (Kimura 1968, 1983). As the growth traits were affected by environment significantly, the CCP and RS populations that cultured in different areas would get distinguishing results in the marker-trait association. Therefore, more different populations should be applied to validate the nine EST-SNPs before initiating a marker-assisted selection program.

According to the result of BlastX, two identified SNP (jl090 and jl615)-involved fragments were homologous to genes encoding guanine nucleotide-binding, protein G (i) subunit alpha and SH2 domain-containing protein 3C. Guanine nucleotide binding proteins regulate many biological processes, including protein synthesis, various transport processes, and cell growth and differentiation (Cockcroft and Gomperts 1985; Vetter and Wittinghofer 2001). SH2 domains are regulated by receptor protein and they take part in the process of normal signaling and cellular transformation (David et al. 1995). Thus, the two genes containing the verified SNPs may participate in the process of growth of *C. gigas*, and the two markers can be used in the production practice for obtaining fast-growing oysters. The function of jl027-contained genes is unknown and still remains to be further studied.

In summary, we developed and validated 108 EST-SNPs using the HRM method. These new derived markers can be used for parentage assignment, genetic diversity, and comparative mapping studies. Three SNPs of them were confirmed to be associated with growth traits of *C. gigas*, which may have potential applications in future genetic improvement of this important aquatic species.

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