

Identification of Single-Locus PCR-Based Markers Linked to Shell Background Color in the Pacific Oyster (*Crassostrea gigas*)

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Abstract A number of Pacific oyster (*Crassostrea gigas*) with golden shell background color were obtained which show great potential to develop a niche market. To improve the selective breeding progress of true-breeding strains with complete golden oysters, research was conducted to identify genetic markers linked to the shell color locus. An F₁-segregating population was obtained by crossing two oysters with golden shell and white shell. Genomic DNA from eight progenies with golden shell and eight progenies with white shell were equally pooled for amplified fragment length polymorphism (AFLP) screening. In bulked segregant analysis, six out of 225 selective primer pair combinations produced seven polymorphic fragments tightly linked to shell color across the segregating population. The seven AFLP markers were all derived from the golden dam and mapped onto a single linkage group flanking the shell color locus. In conversion of the AFLPs into single-locus PCR-based markers, a sequence-characterized amplified region (SCAR) marker, named SCARJ8-2, a single nucleotide polymorphism (SNP) marker, named SNPL2-4, and a simple sequence repeat (SSR) marker, named SSRO11-2, were obtained. These markers obtained in this study will be useful for marker-assisted selection of the Pacific oyster.

Keywords Pacific oyster · Shell color · AFLP · SCAR · SSR · SNP

Introduction

The shells of Mollusca with their diversity in beautiful forms and colors have attracted the interest of naturalists and collectors. Mollusca shell color is known to be inheritable and affected by environmental factors, such as lights, salinity, and substratum (Heath 1975; Lindberg and Pearse 1990; Sokolova and Berger 2000). Several studies have demonstrated that shell colors were genetically controlled by a relatively simple genetic basis (Cole 1975; Innes and Haley 1977; Adamkewicz and Castagna 1988; Wada and Komaru 1990; Winkler et al. 2001; Kobayashi et al. 2004; Liu et al. 2009; Zheng et al. 2012). However, little is known about the molecular mechanisms for shell coloration.

The Pacific oyster (*Crassostrea gigas*) is now the most widely farmed oyster in the world, ranking first in production among all other cultured animals (FAO 2014), and its coloration is of interest to the whole oyster industry (Brake et al. 2004; Kang et al. 2013). Consumers are willing to pay more for seafood with specific color, such as rich red salmon (Alfnes et al. 2006). Consumer preference for Pacific oysters may also be influenced by shell coloration. The oysters with golden shell coloration are rarely seen in the market and are sold at much higher prices than others (Nell 2001). In our selective breeding practice of the Pacific oyster, we obtained a number of golden shell variants and established a set of full-sib families using these golden shell oysters as parents. Cross-mating study has demonstrated that the background color was controlled by one locus with the allele for golden background being dominant to the allele for white (Ge et al. 2015). However, the molecular mechanisms for golden shell

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coloration in *C. gigas* are still unknown, and no strains with complete golden oysters have been established via the traditional selection.

The development of molecular markers linked to shell color can help us to determine the genotype of shell colors and therefore enables marker assisted selection (MAS) for the monitoring of similarity/dissimilarity between homozygous and heterozygous golden individuals. This can significantly reduce the cost of producing breeding lines and can accelerate the breeding program greatly. Several genetic markers associated with shell color have been identified in aquaculture species such as bay scallop (Qin et al. 2007), Pacific lion-paw scallop (Petersen et al. 2012), and noble scallop (Yuan et al. 2012). A SCAR marker which was well correlated with Pacific oyster shell pigmentation of light and dark was also identified, suggesting a major locus that is responsible for foreground pigmentation (Ge et al. 2014). However, molecular markers linked to shell background color in the Pacific oyster have not been identified.

There are several molecular marker technologies available for MAS in aquatic animal breeding (Liu and Cordes 2004). The amplified fragment length polymorphism (AFLP) technique is a powerful marker system (Vos et al. 1995), which has been used extensively for linkage mapping and gene/QTL mapping in marine organisms (Yu and Guo 2003; Qin et al. 2007; Guo et al. 2012; Yuan et al. 2012). The bulked segregant analysis (BSA), developed by Michelmore et al. (1991), has been proved to be an effective method for detecting markers that are tightly linked with genes in many aquatic species (Nakamura et al. 2001; Araneda et al. 2005, 2009; Felip et al. 2005; Liu et al. 2011; Ge et al. 2014; Wang et al. 2014). Nakamura et al. (2001) obtained four AFLP markers linked to the dominant albino locus in rainbow trout after a screening of 64 AFLP primer combinations. Felip et al. (2005) identified 19 AFLP markers linking to the sexuality of rainbow trout after a screening of 486 AFLP primer combinations. And in a previous study, seven AFLP markers linking to a major locus for light/dark shell foreground pigmentation of the Pacific oyster were obtained utilizing the BSA method (Ge et al. 2014).

In the present study, BSA in combination with AFLP technique was used to identify associated fragments of the shell background color, and tightly linked AFLP fragments were converted into single-locus markers. Our aim was to identify potential markers that may be helpful in selective breeding for golden shell color in the Pacific oyster.

Materials and Methods

Oyster Materials

A segregating population was obtained by mating a golden shell female with a white shell male in May 2011. The parents

were selected from two full-sib families, which were established in 2010. The rearing of the larvae, spat, and adults was carried out with standard practices. In August 2012, a total of 110 progenies were obtained by random sampling and verifying their descent at seven microsatellite loci: CgEJ040, CgEJ053, CgEJ074, CgEJ110, CgEJ133, CgEJ195, and CgEJ224 (Table S1).

BSA and AFLP Analysis

The DNA was extracted from adductor muscle by standard proteinase K digestion, phenol-chloroform extraction, and DNA precipitation. DNA concentration was adjusted to 100 ng/ μ l. One golden shell bulk (GB) and one white shell bulk (WB) were constructed for bulked segregant analysis (Michelmore et al. 1991) by pooling equal quantities of DNA from eight oysters with golden background and eight oysters with white background.

The AFLP procedure was carried out as described by Li et al. (2009), which was originally derived from Vos et al. (1995). One hundred nanograms of genomic DNA was digested with an *EcoRI/MseI* combination and then ligated to double-stranded adaptors. The adaptor-ligated DNA was diluted ten times with TE_{0.1} (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), and 2.0 μ l was used as template DNA for the pre-amplification step of the AFLP procedure. After determining that the amplifications were successful using 1.5 % agarose gel electrophoresis, the pre-amplification products were diluted 1:10 and used for selective amplification. The selective PCR products were separated on a 6 % denaturing polyacrylamide gel. AFLP patterns were then visualized by silver staining. A 10-bp DNA ladder (Invitrogen) was used as a reference marker for fragment size determination.

Cloning of AFLP Markers

AFLP bands of interest were excised from the dried polyacrylamide gel, and the DNA from the gel bands was isolated following Cho et al. (1996). The excised bands were dissolved in 30 μ l TE_{0.1}, boiled for 15–30 min, and centrifuged at 10,000 rpm for 1 min. The supernatant was used for selective amplification, using the same conditions as the original AFLP reaction. The products were purified using an agarose gel DNA fragment recovery kit (TaKaRa), cloned into the PMD-19T vector (TaKaRa), and sequenced using a BigDye Terminator Cycle sequencing kit and an ABI PRISM 3730 Genetic Analyser (Applied Biosystems). DNA sequences were assembled and edited using the DNASTar SeqMan program (DNASTar Inc.).

Table 1 Numbers of AFLP primer combination tested and polymorphisms observed among the golden and white shell parents and two DNA bulks

These of marker development	Number
AFLP primers screened	225
Total bands scored	6078
Polymorphic bands between parents	1580
Primers showing polymorphic bands between parents and bulks	69
Polymorphic bands between parents and bulks	86
Polymorphic primers after individual test	6
Polymorphic bands after individual test	7
Bands converted into SCAR/SSR/SNP	3

Conversion of AFLP Markers into Single-Locus Markers

Homology of the shell color-associated fragments to known sequences was assessed using BLAST searching in the *C. gigas* genome database (<http://oysterdb.cn/blast.html>). Based on the AFLP sequences, internal locus-specific primers were designed to amplify genomic DNA of the parents. In the cases that no polymorphic bands generated between two parents, external primers based on their flanking sequences were designed and used to amplify genomic DNA of the parents. If still no polymorphic bands generated by external primers, PCR products were cloned and sequenced on four progenies (2 golden-shell and 2 white-shell progenies) in order to reveal molecular causes for the AFLP polymorphisms and determine the strategies for marker conversion. All locus-specific primers were designed using the Primer Premier 5.0 program (<http://www.premierbiosoft.com/>).

Polymerase chain reaction (PCR) amplification was carried out in a 10- μ l mixture consisting of 0.5 U *Taq* DNA polymerase (TaKaRa), 1 \times PCR buffer, 0.8 mM dNTP mix, 0.5 μ M of each primer pair, 1.5 mM MgCl₂, and about 50–100-ng template DNA. PCR conditions

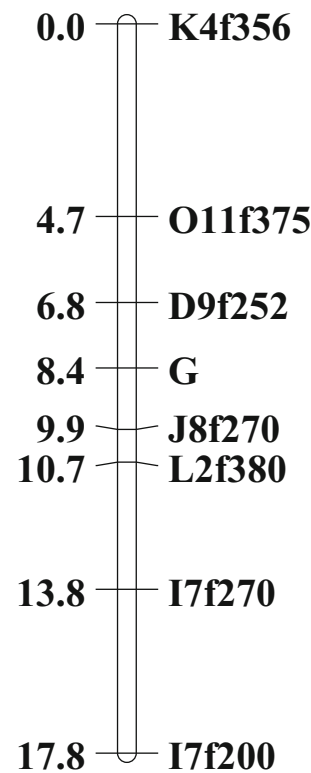
Table 2 Characteristics of the seven AFLP markers linked to shell color of the Pacific oyster

Marker ^a	Primer combinations ^b	Fragment size (bp)	Origination	Golden ^c (n=61)	White ^c (n=49)
D9f252	<i>E</i> -ATC/ <i>M</i> -CGT	252	Dam	60	3
I7f200	<i>E</i> -AAG/ <i>M</i> -CTT	200	Dam	7	46
I7f270	<i>E</i> -AAG/ <i>M</i> -CTT	268	Dam	5	46
J8f270	<i>E</i> -AAT/ <i>M</i> -CGA	271	Dam	55	3
K4f355	<i>E</i> -ATT/ <i>M</i> -CTA	356	Dam	56	5
L2f380	<i>E</i> -ATA/ <i>M</i> -CAG	379	Dam	2	47
O11f375	<i>E</i> -AGA/ <i>M</i> -CTG	376	Dam	58	2

^a *Eco*RI- and *Mse*I-selective primers were coded by letters and numbers, respectively. AFLP markers are named with the codes followed by a letter f (fragment) and the size of fragment in base pairs

^b *E Eco*RI and *M Mse*I; after “-,” the three selective nucleotides used

^c The number of oysters exhibiting the AFLP band in the 61 offspring of golden background shell and 49 offspring of white background shell, respectively

**Fig. 1** Linkage map of the AFLP markers linked to the locus for shell background color. *G* indicates the shell background color gene

were 94 °C for 5 min, followed by 35 cycles of DNA amplification (94 °C, 45 s; 55 °C, 45 s; 72 °C, 45 s), and a final incubation at 72 °C for 10 min. This thermal profile was standardized according to the specific T_m of the primer pair. Sequence-characterized amplified region (SCAR) markers were directly separated on a 1.5 % agarose gel to detect polymorphisms, and simple sequence repeat (SSR) markers were separated on a 6 % denaturing polyacrylamide gel. Single nucleotide polymorphism (SNP) detection was performed on a LightCycler 480[®] real-time PCR instrument and analyzed by high-

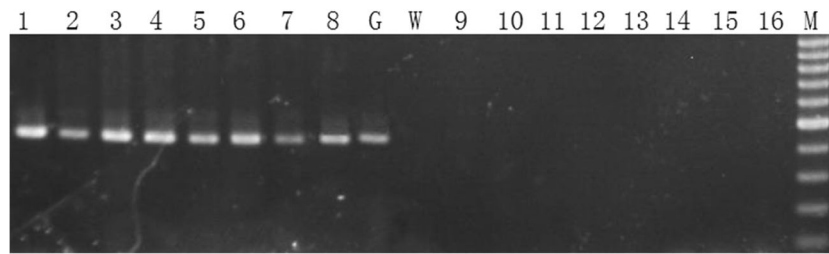


Fig. 2 Specific amplification result with the SCAR marker SCARJ8-2 within the bulks. *G* indicates the female parent, *W* the male parent, *M* the 100-bp DNA marker, lanes 1–8 the eight golden shell individuals, and lanes 9–16 the eight white shell individuals

resolution melting (HRM) following Zhong et al. (2013). All converted single-locus markers were firstly tested in the individuals within the bulks and then in all F_1 individuals.

Data and Linkage Analysis

AFLPs are scored as dominant markers and recorded as “1” for band presence and “0” for band absence. Only markers presenting in one of the two parents and also presenting in one of the two bulks were selected for individual AFLP validation. AFLP markers which displayed no recombinants in the 16 individuals within the bulks were validated. The validated markers were further analyzed on all other F_1 individuals, and linkage analysis was conducted using Joinmap 3.0 (Van Ooijen and Voorrips 2001) based on the genotype data of these markers. The phenotypic data of shell background color was involved in the linkage analysis to locate the color locus. The backcross model, i.e., the population type: BC1 in Joinmap 3.0, was used, and the data was formatted according to this model. Considering that the linkage phase of markers is unknown, the data were entered in the program in both normal and reverse position, to also allow the linkage among markers in repulsion phase. The most likely map order was determined at the minimum LOD=3.0, and the maximum recombination of 0.4. Map distances in centiMorgans (cM) was calculated with Kosambi mapping function (Kosambi 1944).

Results

Of the 110 progenies, 61 were golden shell background, and 49 were white shell background. The chi-square test showed that shell background color segregated in a ratio of 1:1 ($\chi^2=1.309$, $P=0.253$).

A total of 225 primer-pair combinations were used to screen the polymorphism between the two parents and the two bulks (Table 1). Each AFLP primer combination amplified ~20–40 fragments among 100–500 bp. A total of 6078 bands were scored, and 26 % show polymorphisms between the two parents. Finally, 86 AFLP fragments which were amplified from 69 primer-pair combinations showed polymorphisms between the parents and the two bulks. These primer combinations subsequently were analyzed on each individual within the bulks. As a result, seven AFLP fragments originating from the golden parent displaying no recombinants in the 16 individuals within the bulks were obtained. These markers were further analyzed on all the F_1 individuals to confirm the marker-trait association. All seven AFLP markers were almost specific to one group and segregated in the ratio of 1:1 expected for a dominant marker in the full-sib family (Table 2). The seven AFLP markers were mapped onto a single linkage group that spanned a region of 17.8 cM (Fig. 1). Three markers (D9f252, O11f375, K4f355) were on one side of the shell background color gene (*G*), while the other four markers (L2f380, J8f270, I7f270, I7f200) were found on the other side. The two flanking markers (D9f252 and J8f270) closest to the gene had a genetic distance of 1.6 and 1.5 cM, respectively.

Table 3 Characteristics of the single-locus PCR-based markers derived from AFLP fragments linking to shell color of the Pacific oyster

Marker name	Original marker	Primer sequences (5′–3′)	T _m (°C)	Product length (bp)
SCARJ8-2	J8f270	F: ATGCTTTAAGAATCTTGACACACT R: TCCTGAACGAAATGACTGTATAAGT	59	409
SNPL2-4	L2f380	F: CATCTTGTCAAAGTTGGTAGCAGC R: AATCAATGACAACAAAGAAGCTCAG	60	56
SSRO11-2	O11f375	F: ATCGGTCTTTGTCAGTTCCTTC R: GACTTTGTTTTGATTGACTCCAGA	60	232/237

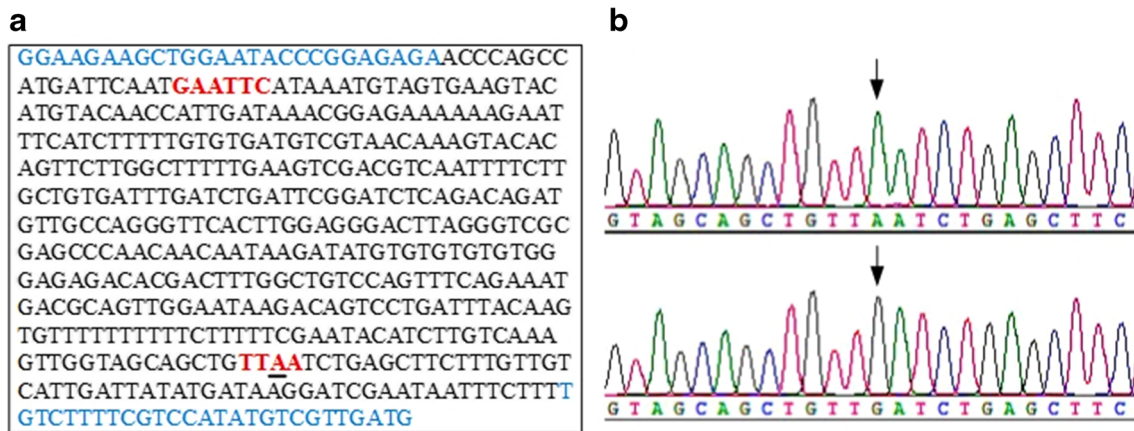


Fig. 3 Characterization of the AFLP marker L2f380. **a** AFLP marker and its flanking sequences. Primer sequences used for the PCR amplification are marked in blue color. Both *EcoRI* and *MseI* cut sites are marked in red

color. A mutant site is *underlined*. **b** An A/G mutation is detected in the AFLP fragment, located in the *MseI* cut site. (color figure online)

To develop PCR-based markers, all the confirmed bands were excised, cloned, and sequenced. Based on the sequence information (Table S2), locus-specific primers were designed as SCAR primers for PCR amplification. However, all the primers displayed the same amplification between the two parents and two pools and therefore failed to be directly converted into SCAR markers. Blast searching revealed that all of the AFLP sequences were highly homologous with known sequences in the oyster genome database excepted for K4f355 (Table S2). The D9f252 hit more than ten scaffolds which was likely part of the repeated sequences and thus discarded for further analysis.

The I7f200 and I7f270 hit scaffold363 and scaffold461, respectively. External primers based on the corresponding flanking sequence amplified no polymorphism bands between two parents. Sequencing analysis on four progenies showed that there was no mutation in the *MseI* cut sites, but the *EcoRI* cut sites were mutated in the same form in the two golden-shell offspring and two white-shell offspring. Consequently, no mutation was detected that was responsible for the AFLP polymorphism.

The J8f270 hit two scaffolds (Scaffold268 and Scaffold1062). A pair of primers based on the flanking sequence in Scaffold268 amplified a dominant pattern in the

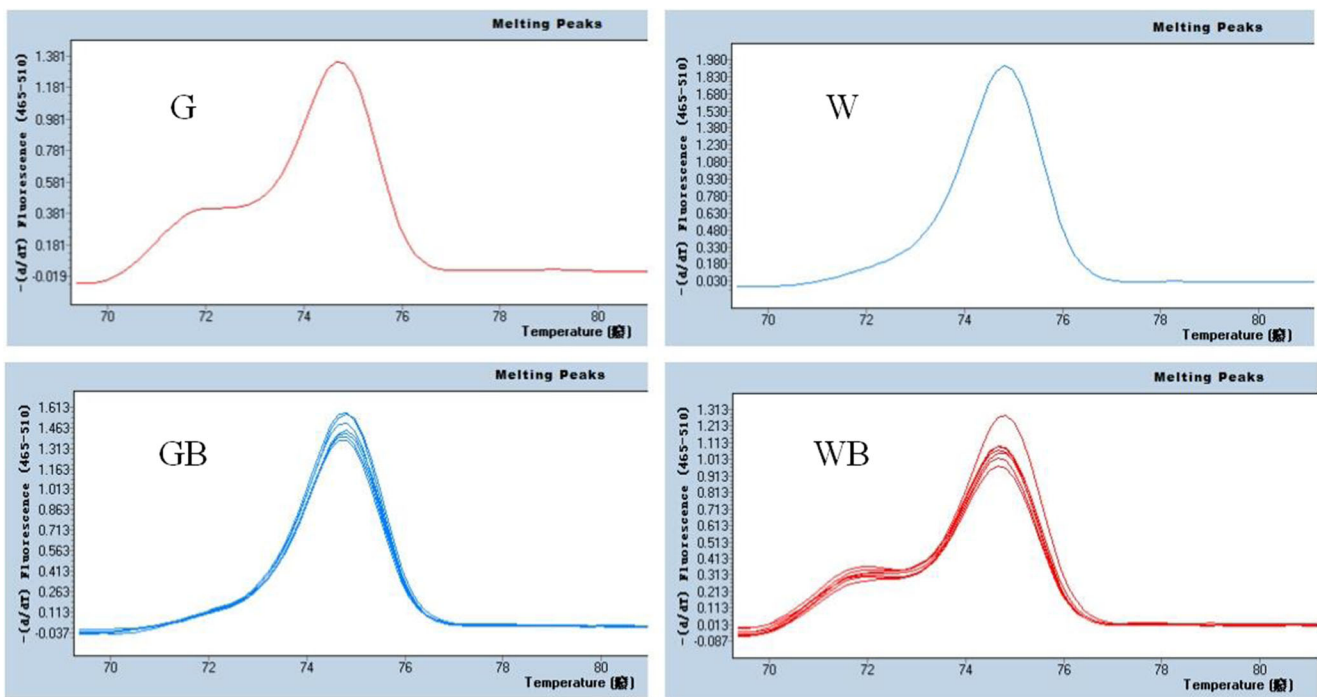


Fig. 4 Specific amplification result with the SNP marker SNPL2-4 within the bulks. *G* indicates the female parent, *W* the male parent, *GB* the eight golden shell individuals, and *WB* the eight white shell individuals

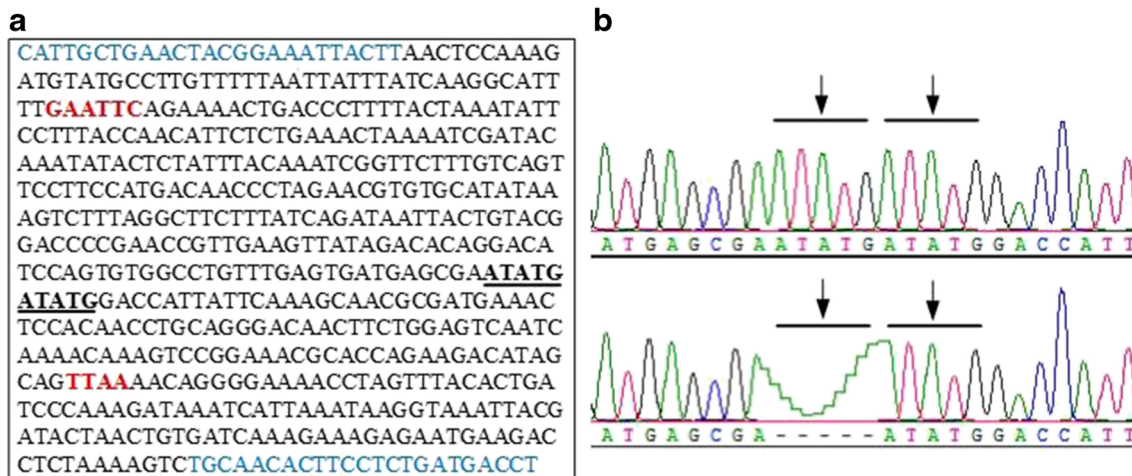


Fig. 5 Characterization of the AFLP marker O11f375. **a** AFLP marker and its flanking sequences. Primer sequences used for the PCR amplification are marked in blue color. Both *EcoRI* and *MseI* cut sites

are marked in red color. A simple sequence repeat (SSR) is underlined. **b** A simple sequence repeat is detected in the AFLP fragment. (color figure online)

golden female parent and golden shell individuals and cosegregated with the corresponding AFLP marker (Fig. 2). Thus, a SCAR marker was obtained and defined as SCARJ8-2 (Table 3). The primers based on Scaffold1062 amplified no bands or displayed bands of the same size between the two parents and thus failed to be converted into SCAR markers.

The L2f380 marker sequence and its flanking sequence with external primers designed to further characterize the AFLP fragment is illustrated in Fig. 3. Both *EcoRI* and *MseI* restriction enzymes make cuts for a fragment of 357 bp, which corresponded to this AFLP marker of 380 bp identified in the gel when an extra adaptor sequence of 22 bp was included. Sequencing analysis of the amplified products on four progenies revealed a single nucleotide polymorphism (SNP) at the *MseI* cut site, which certainly is responsible for the AFLP polymorphism at this locus, but nothing at the *EcoRI* cut site (Fig. 3). Another pair of primers just flanking this SNP locus was designed. The amplified results were the same as the sequence results and also the AFLP marker (Fig. 4). Thus, an SNP marker was obtained and defined as SNPL2-4 (Table 3).

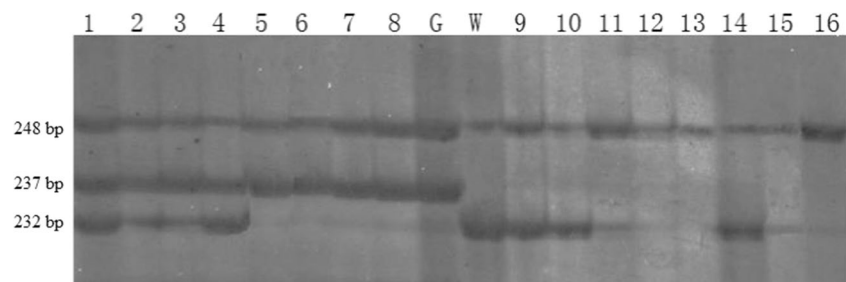
Figure 5 illustrates both the O11f375 marker sequence and its flanking sequence with external primers designed to further characterize the AFLP marker. Both *EcoRI* and *MseI* restriction enzyme recognition sites were clearly identified in the

fragment (Fig. 5), which spans a sequence of 354 bp in length. By adding 22 bp of adaptor sequence to the enzyme cut fragment, the total length exactly matched the AFLP size of 375 bp. Sequencing analysis of PCR products on four progenies failed to show any mutations at either *EcoRI* or *MseI* cut sites. Instead, a 5-base simple sequence repeat (SSR) was found, which certainly caused the AFLP polymorphism at the locus (Fig. 5). Another pair of primers just flanking this SSR locus was designed and named as SSRO11-2 (Table 3). The amplified results were the same as the sequence results and also the AFLP marker (Fig. 6). A 237-bp band only presented in the golden shell female and golden progenies, while a 232-bp (237–5 bp) band presented in the white shell male and separated into both the white shell progenies and golden shell progenies. The 248-bp band was shown to be nonspecific amplification by sequence alignment with the original AFLP sequence.

Discussion

Results of the present study are in agreement with our previous study that shell background color in the Pacific oyster is under one-locus system with the allele for golden being dominant to the allele for white (Ge et al. 2015). The observation

Fig. 6 Specific amplification result with the SSR marker SSRO11-5 within the bulks. *G* indicates the female parent, *W* the male parent, lanes 1–8 the eight golden shell individuals, and lanes 9–16 the eight white shell individuals



that all seven tightly linked markers were originated from the golden female (instead of the white male) and observation of a roughly 1:1 ratio of golden and white progenies suggested that the test family was a cross between a golden heterozygote and a white homozygote. The relationship of genotype and phenotype is hypothesized to be Gw golden and ww white.

A SCAR marker (SP-170) which was well correlated with Pacific oyster shell pigmentation of light and dark was identified in our previous study (Ge et al. 2014). The SP-170 marker was tested in the segregating population but did not display linkage with the seven AFLP markers and three single-locus markers (data not shown). This suggests that golden shell coloration may be controlled by a separate genetic locus and supports the hypothesis that golden coloration follows a different pattern from black pigmentation (Ge et al. 2015).

AFLP is a robust and reliable molecular assay that can detect variety of genetic polymorphisms in the genome (Liu and Cordes 2004). In the present study, for the AFLP marker L2f380, an A/G substitution was detected within the restriction enzyme recognition, and for the AFLP marker O11f375, a 5-base simple sequence repeat within the AFLP fragment was confirmed by sequencing four progenies. It has also been demonstrated that the AFLP assay can detect polymorphisms within the restriction enzyme recognition sites as well as the surrounding areas where the selective primer can reach (Xiao et al. 2007a) and any deletion/insertion with the AFLP fragment can be detected by the technique (Savelkoul et al. 1999).

The generation of AFLP markers is often expensive and labor intensive, and thus, it is inconvenient for large-scale application in marker-assisted selection. Therefore, AFLP markers need to be converted into simple PCR-based markers. In the present study, we demonstrated that making the best use of available database resources could save significant labor in AFLP conversion. We identified seven AFLP markers linked to shell background color locus and converted markers J8f270, O11f375, and L2f380 into a dominant SCAR marker, an SSR marker and a codominant SNP marker, respectively. In the conversion of AFLPs into PCR-based markers, flanking sequences for external primers designing were all derived from the oyster genome database instead of using inverse PCR or genome walking method in other studies (Bradeen and Simon 1998; Xiao et al. 2007b). Utilizing this strategy saved us considerable time and labor in laboratory.

Previous studies have introduced various strategies for the conversion of dominant AFLP markers into single-locus-specific markers (Brugmans et al. 2003; Piao et al. 2004; Shahinnia and Sayed-Tabatabaei 2009; Zhang et al. 2014). Locus-specific primers could be designed on the ends of the AFLP fragments or nearby with optimum GC content, if the amplified products are scored as present/absent on the gel, a dominant SCAR marker was obtained (Piao et al. 2004). If the variation exists within a restriction site belonging to a set of

cost-effective restriction enzymes, a codominant CAPS marker can be developed by digesting the PCR products from each genotype (Brugmans et al. 2003). If the polymorphism does not alter a restriction site, existing among the selective bases, a dCAPS marker can be developed (Shahinnia and Sayed-Tabatabaei 2009). In the present study, an SNP marker was developed and detected by the high-resolution melting (HRM). Compared with CAPS method, HRM is a very attractive, fast, and cost-effective SNP genotyping technology because it is simple, nondestructive, and amendable to high-throughput on 96- or 384-well plates (Wittwer 2009). Therefore, with the continuous development of molecular biotechnology and the increase in publicly available genome sequences, AFLP conversion has become easier than ever (Zhang et al. 2014).

The most significant result of this study is the identification of genetic markers linked to the Pacific oyster shell color locus. The SCAR marker, SSR marker, and SNP marker could be detected more simple than original AFLP markers and transferable across populations. Additionally, based on these AFLP sequences, homology analysis was conducted to locate the genome region for shell background color, and homologous scaffolds were identified in the oyster genome database. The gene for shell color was primarily mapped on the Pacific oyster genome, which provided a start point for further mapping and map-based cloning of this gene. In the future, fine mapping of this gene could be conducted with the availability of a complete oyster sequence map, sufficient molecular markers, and a larger segregating population.

In conclusion, three single-locus PCR-based markers linking to the gene controlling the shell background color were identified for the Pacific oyster. These markers can potentially be used for marker-assisted selection of golden oyster strains. A further characterization of this locus may ultimately help in the positional cloning of the shell color gene and the better understanding of shell color polymorphisms in this species.

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