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Association between novel EST-SNPs and commercial traits in *Pinctada fucata martensii*

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ABSTRACT

To facilitate marker-assisted selection for genetic improvement of the pearl oyster *Pinctada fucata martensii*, two novel single nucleotide polymorphism (SNP) markers (A376C and A316C) were screened from two shell matrix genes (prismalin-14 and N19 gene), and genotyped to investigate their correlations to five commercial traits, including shell width (SW), shell length (SL), shell height (SH), hinge length (HL) and total weight (TW) in 160 pearl oysters. Both of the two loci showed AC heterozygous. The highest trait values of the five commercial traits measured were the CC genotype in the A316C SNP locus and the AC genotype in the A376C SNP locus, respectively. Significant differences in the trait values were detected between AA- and AC-type individuals ($P = 0.005$ for SW, $P = 0.037$ for SH, $P = 0.031$ for HL and $P = 0.007$ for TW), but not for SL ($P = 0.057$) in the A376C locus. These results indicated that AC-type at locus A376C from prismalin-14 gene could be used as a preferred genotype for future selection in breeding program of pearl oyster.

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1. Introduction

The melting temperature (Tm)-shift single nucleotide polymorphism (SNP) genotyping assay is considered as a promising method because of its high-throughput and without the need for developing expensive labeled probes (Wang et al., 2005; Feng et al., 2014a). It relies on two allele-specific (AS) primers. One of the AS primer is a long 14 bp 5′ GC sequence (5′ – GCGGGCAGGGCGGC- 3′), while a short 6 bp 5′ GC tail (5′ –GCGGGC- 3′) was attached to the other allele-specific primer. Generally, the long tail to an AS primer has the higher Tm base (G or C), and the short tail to the other AS primer has the lower Tm base (A or T). According to GC tails, the PCR product has a distinct Tm. Therefore, genotypes at the SNP could be identified from the melting curve on a real-time PCR instrument. A common reverse primer that amplified both alleles was also designed for each SNP (Wang et al., 2005; Jiang et al., 2010, 2011; Derzelle et al., 2011). The homozygous genotype with lower melting temperature curve presented that SNP locus was the last base of lower Tm primer. The last base of higher Tm primer was resulting in a melting peak at a higher temperature. While, heterozygous genotype generated two melting peaks at both high and low temperatures, respectively.

The pearl oyster *Pinctada fucata martensii* is one of the most important bivalves in marine pearl production worldwide (Southgate and Lucas, 2008) and more than 90% marine pearl production in China is from this species (Gu et al., 2009). However, the pearl oyster has recently suffered from slow growth, mass mortality, pearl quality decline and worm infestation, and the yield of pearl has reduced dramatically (Qiu et al., 2014; Wada and Komaru 1994, 1996; He et al., 2008). Traditional animal improvement methods have had some appropriate traits which can be easily measured among candidates for selection (Wada and Jerry, 2008), but they are not particularly effective for complex commercial traits selection because these commercial traits are usually difficult to be measured and are governed by multiple genes (Jerry et al., 2012; Jones et al., 2013). There are lots of limitations in the selection of animals with high commercial traits using phenotype data. Gene markers are more precisely targeted and can detect variation in or close to genes of known or inferred function that relates directly or indirectly to the trait of interest (Lynch and Walsh, 1997; Tao and Boulding, 2003). Associated with commercial traits could provide useful information for the genetic improvement of *P. fucata martensii*.

Shell matrix genes secreted from mantle tissues play important roles in crystal nucleation, regulation of crystal growth, crystal morphology and microstructure in shell and pearl formation (Marin et al., 2008; Wilt et al., 2003; Zhang and He 2011). Accordingly, a number of shell matrix genes have been cloned and characterized

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in *P. fucata martensii*, such as nacrein (Miyamoto et al., 1996, 2005), extracellular EF-hand calcium-binding protein (EFCBP) (Huang et al., 2007), msi60 and msi31 (Sudo et al., 1997), N16 (Samata et al., 1999), msi7 (Zhang et al., 2003), prismalin-14 (Suzuki et al., 2004), amorphous calcium carbonate-binding protein (ACCBP) (Ma et al., 2007), nacre protein of 19 kDa (N19) (Yano et al., 2007), aspein (Tsukamoto et al., 2004), calmodulin (CaM) (Li et al., 2004) and KRMP (Zhang et al., 2006).

The shell of the pearl oyster consists mainly of two mineralized layers, the inner nacreous layer and the outer prismatic layer. Prismalin-14 and N19 can be found in prismatic layer and nacreous layer, respectively (Zhang and He 2011). Previous reports concentrated on understanding the molecular mechanisms of shell formation in the pearl oyster by analyzing expression pattern of shell matrix genes in mantle tissue (Shi et al., 2013a,b; Zhan et al., 2015; Gu et al., 2014), and few studies focused on SNP polymorphisms of shell matrix genes.

In the present study, two novel EST-SNP markers were developed by screening the EST sequences of prismalin-14 and N19 shell matrix genes in the pearl oyster and these two SNP markers showed polymorphism in 160 pearl oysters by Tm-shift analysis. Association between SNP markers and five phenotypic traits were calculated. Locus A376C in prismalin-14 gene was significantly associated with four shell commercial traits. These results will be important for the selective breeding of the pearl oyster.

2. Materials and methods

2.1. Sample collection, genomic DNA preparation and commercial traits measurement

Pearl oysters were collected from Li' an Lagoon in Hainan, China. A total of 160 individuals were randomly selected from a cultured stock. Shell width (SW), shell length (SL), shell height (SH) and hinge length (HL) were measured by Vernier Caliper (0.01 mm accuracy). Total weight (TW) was weighed using an electronic balance (0.01 g accuracy). Adductor muscles were preserved in 95% alcohol, and genomic DNAs were extracted using high-salt method (Dong et al., 2012).

2.2. Polymorphism SNP loci screening

EST sequences of two shell matrix genes (prismalin-14 and N19) were downloaded from GenBank Database (GenBank accession number, prismalin-14 AB159512.1; N19, AB332326.1). Each of the two EST sequences was aligned with the whole genomic DNA sequence of *P. fucata martensii* (http://marinegenomics.oist.jp/pinctada_fucata) to design primers in the coding regions.

All the primers were used to screen possible SNP loci in 5 individuals, which were randomly selected from the 160 pearl oysters. PCR were performed in a 50 μL reaction volume, containing 5 μL 10 × PCR Buffer, 3.5 μL dNTP (2.5 mM), 4 μL forward primer (10 μM), 4 μL reverse primer (10 μM), 1 μL ExTaq DNA polymerase (5U/μL) (TaKaRa), 250–500 ng genome DNA as template, add ddH_2O to 50 μL reaction volume. PCR profiles included an initial denaturation at 94 °C for 4 min, 2 circles of 94 °C for 45 s, 60 °C for 30 s, 72 °C for 45 s, another 2 circles of 94 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s, then followed by 26 circles of 94 °C for 45 s, 48 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR fragments were sequenced by ABI3730 sequencer (Applied Biosystem).

2.3. SNP genotyping by Tm-shift analysis

PCR fragments showed polymorphism among the five individuals were used to design primers for Tm-shift analysis and genotyped in the 160 pearl oysters. PCR reaction mixture contained genome DNA 50–100 ng, 10 × Buffer 2 μL, 2.5 mM dNTP 1.6 μL, 10 μM forward primer with long tail 0.2 μL, 10 μM forward primer with short tail 0.4 μL, 10 μM reverse primer 0.4 μL, ExTaq 2 U, 20 × EvaGreen (BIOTIUM, US) 1 μL, add ddH_2O to 20 μL volume. PCR program was 4 min at 94 °C, followed by 35 circles of a 3-step amplification profile of 30 s at 94 °C, 30 s at 55 °C, and 72 °C for 1 min. Melting curve analysis of PCR products were performed by Eppendorf 5400 (Eppendorf, German). The procedure was conducted by the on board software of the PCR instrument.

2.4. Data analysis

Observed heterozygosity (*Ho*), expected heterozygosity (*He*) and Hardy-Weinberg equilibrium (HWE) for SNP loci were calculated using POPGENE version 32 software (Yeh and Boyle, 1997). The 160 pearl oysters were grouped according to their genotypes. For the five commercial traits, including SW, SL, SH, HL, and TW, the mean values and standard deviations were calculated for each genotype group. Potential associations between the marker genotypes and phenotypic variables (SW, SL, SH, HL and TW) were analyzed by one-way analysis of variance (ANOVA) with a post-hoc test. In all tests, *p*-values lower than 0.05 were considered significant.

3. Results

3.1. Polymorphism SNP loci

One primer pair was designed for prismalin-14 gene and two pairs of PCR primers were designed for N19 gene. All the three primers were successfully yield products with expected sizes. Two of the three products were showed polymorphism and found candidate SNP loci in the tested 5 pearl oysters. The two candidate SNP

Table 1
Two pairs of primers for Tm-shift analysis.

LocusName	Annotation	Forward primer	Size(bp)	SNP type	Location	*Ho*	*He*	P_{HWE}
A376C	prismalin-14	Long tail forward primer: GCGGGCAGGGCGGCTTACGGATACAATCCC Short tail forward primer: GCGGGCTTACGGATACAATCCA Reverse primer: GCCATCATCGTCACCA	55	CCA- > CCC Pro	376	0.2938	0.3666	0.011[a]
A316C	N19	Long tail forward primer: **GCGGGCAGGGCGGC**GTTGCCTATCTGGACC Short tail forward primer: **GCGGGC**GTTGCCTATCTGGACA Reverse primer: GTTGTATCTAACCTTCG	102	AAA- > CAALys- > Gln	316	0.3312	0.4873	0.000[a]

Ho: observed heterozygosity; *He*: expected heterozygosity; P_{HWE}: p value for Hardy-Weinberg equilibrium test.
[a] Denotes significant departure from HWE.

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