# Development of coding single nucleotide polymorphic markers in the pearl oyster *Pinctada fucata* based on next-generation sequencing and high-resolution melting analysis

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Genet. Mol. Res. 15 (4): gmr15049054
Received August 3, 2016
Accepted September 19, 2016
Published November 3, 2016
DOI http://dx.doi.org/10.4238/gmr15049054



**ABSTRACT.** The pearl oyster *Pinctada fucata* is an important commercial marine shellfish that is cultured for producing saltwater pearls. In this study, 468 single nucleotide polymorphisms (SNPs) were screened from *P. fucata* transcriptome data, and 119 polymorphic SNPs were successfully isolated by a two-step small-amplicon high-resolution melting assay. Of these, 88 were annotated with BLAST in the Nr database and 90 were in the open reading frame, including 16 non-synonymous SNPs and 74 synonymous SNPs; 12 SNPs were in the 3'-untranslated region (UTR) and 1 was in the 5'-UTR. Twenty-five SNPs were randomly chosen to test the genetic diversity of 40 wild

individuals from Liusha Bay, China. All of the loci had two alleles. The observed and expected heterozygosities ranged from 0.0417 to 0.6042 and from 0.2945 to 0.5053, respectively. Minor allele frequencies ranged from 0.1771 to 0.5000, and the polymorphism information content ranged from 0.2516 to 0.3750. These novel SNP markers can contribute to *P. fucata* genetics and breeding studies.

**Key words:** SNP; *Pinctada fucata*; Transcriptome sequencing; High-resolution melting

## INTRODUCTION

The pearl oyster, *Pinctada fucata*, is an important commercial marine shellfish that is cultured for producing saltwater pearls in China, Japan, and Australia (Yu and Chu, 2006). It is also an important animal model for investigating biomineralization (i.e., scientific, medical, and commercial applications) and evolutionary biology (Jones et al., 2013). Pearl quality has recently decreased in both China and Japan. One possible reason is that the growth performance of *P. fucata* is hampered by inbreeding during aquaculture (Wada and Komaru, 1996; Qiu et al., 2014).

Genetic markers are powerful genetics study tools, particularly for genetic mapping and trait improvement (Huang et al., 2014a). Because of their abundance, value, and efficiency, single nucleotide polymorphisms (SNPs) have become the most powerful marker system for genetic research (Gomez-Uchida et al., 2014). Compared to non-coding genomic markers, SNPs developed from functional genes may be responsible for traits of commercial interest in this species, such as growth, reproduction, and resistance (Gao et al., 2013; Klinbunga et al., 2015; Ranjan et al., 2015). Transcriptome sequencing with next-generation sequencing technologies could provide extensive resources for large-scale gene-associated SNP mining (Grabherr et al., 2011). High-resolution melting (HRM) has proven to be a simple, low-cost, and highly sensitive technique to detect SNPs, and to profile genetic variation within polymerase chain reaction (PCR) amplicons (Cui et al., 2013).

In this study, the genetic diversity and structure of a wild population of *P. fucata* from South China were examined. A total of 119 polymorphic SNPs from the transcriptome sequence were successfully isolated by HRM analysis, which can contribute to *P. fucata* genetics and breeding studies.

## MATERIAL AND METHODS

### DNA extraction

Forty-eight wild adult individuals of *P. fucata* (shell length, 3-4 cm) were obtained from Liusha Bay, Zhanjiang, Guangdong province, China (109°49'E, 20°26'N). Each adductor muscle was cut and stored in 95% ethanol. Genomic DNA was extracted using a Marine Animals DNA Kit (Tiangen, China) according to the manufacturer specifications. DNA integrity and purity were determined by agarose gel (1%) electrophoresis and spectrophotometry (NanoDrop™ 2000; Thermo Fisher Scientific, USA).

## Primer design

A total of 468 putative SNPs with no other predicted SNPs in the 30-bp neighboring regions were randomly chosen from *P. fucata* transcriptome data (Yu DH and Fan SG, unpublished data). The primers were designed by Primer Premier 5.0 (Premier Biosoft International, USA). Amplicon lengths ranged from 40 to 100 bp, primer lengths from 20 to 30 bp, the GC content was 40-60%, and the melting temperatures were 50°-60°C. The sequence and amplicon size of primers were shown in Table 1. Two unblocked double-stranded oligonucleotides were used as high- and low-temperature internal controls to calibrate the temperature variation between reactions (Table 2) (Seipp et al., 2007). All of the primers were synthesized and purified by Sangon Biotech (Shanghai, China).

## Amplification of candidate SNPs

PCR amplification was performed in a 25-μL volume containing 1.25 U rTaq polymerase (TaKaRa, Japan), 1X PCR buffer ($MgCl_2$), 0.2 mM dNTPs, 0.2 μM of each primer, and 20-50 ng genomic DNA. The PCR conditions were as follows: pre-incubation at 95°C for 5 min, followed by 30 cycles at 94°C for 20 s, 55°C or 50°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. All of the PCR products were verified by 8% non-denaturing polyacrylamide gel electrophoresis (PAGE). Only primer pairs that produced a clear target band on the gel were selected for subsequent HRM analysis.

## SNP validation and polymorphism detection by HRM analysis

SNP genotyping was performed using the two-step HRM method described by Wang et al. (2013, 2015), with small modifications. Genomic DNA from eight *P. fucata* individuals was used as amplification templates. After PCR amplification, 8.9 μL PCR product, 0.1 μL of each internal control (10 μM), 0.7 μL LC Green (Idaho Technology Inc., USA), and 20 μL mineral oil (Sigma, USA) were added to BLK/WHT 96-well plates (Bio-Rad, USA). After centrifuging at 2000 *g*/min for 30 s, the mixture was denatured at 95°C for 10 min using a thermal cycler (Hamburg, Germany). A LightScanner™ instrument (Idaho Technology Inc., USA) was used for the HRM analysis. Fluorescence intensity data were collected over 55°-98°C at a thermal transition rate of 0.1°C/s. The HRM system software was used to analyze the melt curve peaks and genotypes.

## Functional annotation

All of the unigene-obtained polymorphic SNPs were BLASTx searched in the Nr database with an e-value cutoff of 1e-5. SNP positions were determined using open reading frame (ORF) Finder (https://www.ncbi.nlm.nih.gov/orffinder/). SNP mutation type was analyzed using Primer Premier 5.0.

## Genetic diversity

Twenty-five polymorphic loci were randomly chosen to examine the genetic diversity of a wild population of *P. fucata* from Liusha Bay.

**Table 1.** Summary of 119 single nucleotide polymorphism (SNP) markers in *Pinctada fucata*.

| Locus ID | Primer sequence (5'–3') | Amplicon size (bp) | SNP type and location | Gene annotation | Amino acid change |
|---|---|---|---|---|---|
| PF_SNP1 | TAGTCGCTAACACTGCCCATTAA | 59 | A/T 2121 | Universal stress protein A-like protein (*Crassostrea gigas*) | TT: act→aca |
| | ACTGGATGTAGAGGATTGGGAAC | | | | |
| PF_SNP2 | CTGAGGTATGAGAATGGAAGGGAC | 81 | C/T 404 | Splicing factor, arginine/serine-rich 4 (*Crassostrea gigas*) | DD: gac→gat |
| | TGGTGCTCTGCGTGGGTT | | | | |
| PF_SNP4 | AGATAGTCCAATCAGGTGTTCAG | 86 | T/A 1860 | Exocyst complex component 1 (*Crassostrea gigas*) | 3'-UTR |
| | CAAAACTTTCTACAAGCAGGTC | | | | |
| PF_SNP5 | TTTGTCCATTTGTTCAGCTG | 80 | A/T 2251 | Retinal rod rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit delta-like (*Crassostrea gigas*) | II: ata→att |
| | CTCGTGTTCCCAAGAAGATAC | | | | |
| PF_SNP6 | ACCTTGTGAACAGGCGATGC | 78 | A/G 597 | Transcription factor HES-1 (*Crassostrea gigas*) | SS: tca→tcg |
| | GGTCGCAAGTAGTCCGCAAAT | | | | |
| PF_SNP9 | GGACCAAATTCCTCTTGTTCTT | 47 | C/G 556 | Death-associated protein 1 (*Crassostrea gigas*) | TT: acc→acg |
| | GAACCTGCTCCAGCTCAAAC | | | | |
| PF_SNP13 | CCACTGCCTCTTTCATTACATCT | 89 | C/T 1404 | Nucleolar protein 56 (*Nasonia vitripennis*) | AA: gct→gcc |
| | ACAGCAGGTAGAAGACAGATTGA | | | | |
| PF_SNP14 | TCATCGGTGCTGCCTCA | 85 | G/A 718 | Nucleoprotein TPR (*Crassostrea gigas*) | GG: ggg→gga |
| | ACAACCCTCCCAGTCATTCC | | | | |
| PF_SNP18 | GAGGATTTGGCATCAGACTATTCA | 70 | C/A 1669 | Patched domain-containing protein 3 (*Crassostrea gigas*) | QP: cag→ccg |
| | CGTTCCTTCCCTTTGTTCTTC | | | | |
| PF_SNP26 | GGTGAAGAGGGCTGTATTTGG | 55 | C/A 1089 | Repressor of RNA polymerase III transcription MAF1 homolog isoform X1 (*Crassostrea gigas*) | SS: tcc→tca |
| | CCAGTTTGCGATTGTAGAAGAAG | | | | |
| PF_SNP31 | ATGGACATGAGACTTGCGATCT | 60 | T/C 122 | Putative signal peptidase complex subunit SPC25 (*Crassostrea ariakensis*) | AA: gcc→gct |
| | CAATAAGGCAAACAGTGAGAACC | | | | |
| PF_SNP33 | TCGTTGTTGGAGTTTGAAGG | 72 | A/T 1448 | GPI mannosyltransferase 1 (*Crassostrea gigas*) | II: ataatt |
| | GATCAGGCAAAACATAATGGA | | | | |
| PF_SNP34 | GTTGATACTGATGAGTCGTTTG | 70 | A/T 729 | Unknown | Unknown |
| | CACTGCCCCTATCTTACCTAT | | | | |
| PF_SNP38 | TGTGGAAGGAGGGTAGATGT | 91 | T/A 304 | Unknown | Unknown |
| | CTTGTATCTTCAAAACTGTGCTC | | | | |
| PF_SNP39 | ATGGGAAGATAAACAGCAGGTA | 91 | T/C 1562 | Hypothetical protein CGI_10011359 (*Crassostrea gigas*) | AA: gcc-gct |
| | CCTATTTGGTATCTCATCCTCATT | | | | |
| PF_SNP45 | TTCGTACGTCAAGGTTCCCG | 94 | C/T 773 | Succinate-CoA ligase GDP-forming alpha subunit (*Oncorhynchus mykiss*) | II: atc-att |
| | GCCTGGAGAATGTAAGATTGGTAT | | | | |
| PF_SNP50 | CGCTTTTCTGTGCGAGTTG | 100 | A/G 6516 | Uncharacterized protein LOC105335671 (*Crassostrea gigas*) | VI: gtt-att |
| | GGGATCGGAATCCTTGTTAA | | | | |
| PF_SNP52 | CATTCTAGCTCATTCTTGATCCCC | 67 | G/A 1348 | Wiskott-Aldrich syndrome protein family member 3 (*Crassostrea gigas*) | VV: gtg-gta |
| | GGATAGTAGAGCCGATCAACGTAAG | | | | |

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**Table 1.** Continued.

| Locus ID | Primer sequence (5'–3') | Amplicon size (bp) | SNP type and location | Gene annotation | Amino acid change |
|---|---|---|---|---|---|
| PF_SNP53 | ATTGGGAAACATATCACTGGG | 68 | T/C 903 | 28S ribosomal protein S35, mitochondrial-like isoform X1 (*Aplysia californica*) | SS: tce-tct |
| | CATCTGCTGTATAATGGAGACTACA | | | | |
| PF_SNP54 | GCGGCGTTTTAATCATCTC | 100 | G/T 877 | Fatty acid-binding protein (*Procambarus clarkii*) | 3'-UTR |
| | GGCATCGATCATTACCTTTCA | | | | |
| PF_SNP55 | CCAGTCTTTGTCTGCTTTATTAA | 73 | C/T 621 | Hypothetical protein CGI_10014470 (*Crassostrea gigas*) | II: atc-att |
| | ACATCCATCTCACATCCAACA | | | | |
| PF_SNP57 | TTCACGTAATCGACCATACAAGC | 69 | G/A 275 | Cytochrome c oxidase assembly factor 4 homolog, mitochondrial-like (*Strongylocentrotus purpuratus*) | TA: act-gct |
| | CCACGGAGACTGGAGAAAATG | | | | |
| PF_SNP58 | CTTTGGATGTCATTTCCTCTGG | 64 | G/A 1550 | Protein arginine N-methyltransferase 1 (*Crassostrea gigas*) | EE: gag-gaa |
| | GCAGATGCTCCACCTAAGGA | | | | |
| PF_SNP60 | TTCCCGCATGGGTCACA | 56 | C/T 857 | Double-stranded RNA-binding protein Staufen-like protein 2 (*Crassostrea gigas*) | HH: cat-cac |
| | GAAACAAGAACAGGATCTGGCTA | | | | |
| PF_SNP61 | GCCAGAGGTTTAGAGCAAGG | 82 | G/C 686 | Structural maintenance of chromosomes protein 5-like (*Crassostrea gigas*) | LL: ctg-ctc |
| | CTTGTTCTAAGCGGGCATT | | | | |
| PF_SNP62 | GAAATCAAGGGAAACGAAGAG | 58 | G/T 1602 | Leucine-rich repeat and fibronectin type III domain-containing protein 1-like protein (*Crassostrea gigas*) | KN: aag-aat |
| | CGGCTGCTTGAATAATAACG | | | | |
| PF_SNP64 | CCGTGTGCAATAATTTCTCCTCT | 55 | G/A 2544 | Cell division cycle 5-like protein (*Crassostrea gigas*) | RR: cgg-cga |
| | GGTATTAAGAAAACAGAATGGAGCC | | | | |
| PF_SNP66 | ATATGACTACGAGATTCTCAGCAAG | 74 | T/A 982 | N-alpha-acetyltransferase 40-like isoform X2 (*Crassostrea gigas*) | PP: cct-cca |
| | ATTCTCCAGCGGGTTTAGG | | | | |
| PF_SNP67 | GGAGGAAACAAATGGAGGA | 61 | A/G 716 | RNA polymerase-associated protein RTF1-like protein (*Crassostrea gigas*) | KK: aaa-aag |
| | ACCAAGTCTGTAAGTGCTGAGA | | | | |
| PF_SNP68 | TGTCAGTACTAGCTCCCCTCAT | 82 | T/C 1466 | Sister chromatid cohesion protein PDS5 homolog B-like (*Meleagris gallopavo*) | SS: tct-tcc |
| | TCTCGGGGTCGTCCAAC | | | | |
| PF_SNP69 | CGTGATGTTTGTGGATTTGG | 54 | A/T 2069 | Sister chromatid cohesion protein PDS5 homolog B-like (*Meleagris gallopavo*) | LL: cta-ctt |
| | GCTGTCTGTGATATTGCCCTAG | | | | |
| PF_SNP70 | CTCGTATCATAACCATTGACGT | 80 | T/C 2984 | Cullin-3-B (*Crassostrea gigas*) | AA: gca-gcg |
| | AGCAGCTCTGAACAACAACTTT | | | | |
| PF_SNP71 | ACAGCTTGACAGCGCCTCT | 72 | G/T 232 | 28S ribosomal protein S5, mitochondrial (*Crassostrea gigas*) | QK: cag-aag |
| | CAAAACAAAACGAAAAGTTCCTAT | | | | |
| PF_SNP73 | CAGGCAGGAGAATGTGAGA | 100 | T/C 376 | Unknown | Unknown |
| | TGCTACACTGAAGGCTTTATGA | | | | |
| PF_SNP75 | CCATCCATAGCCCTGCGTTTT | 89 | C/A 1800 | Tumor necrosis factor receptor-associated factor 6 (*Pinctada martensii*) | GG: ggc-gga |
| | TCCCCTTGCGGCATCCAC | | | | |

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**Table 1.** Continued.

| Locus ID | Primer sequence (5'–3') | Amplicon size (bp) | SNP type and location | Gene annotation | Amino acid change |
|---|---|---|---|---|---|
| PF_SNP77 | TCCTTCGCACCTAGTTTCCC | 87 | A/G 1585 | Phosphoinositide 4-kinase beta (*Crassostrea gigas*) | TA: aca-gca |
| | CCTGTAGCATCTGACCTTGACCT | | | | |
| PF_SNP78 | AAGATATTATCCAAGGAGCGACC | 79 | T/A 3056 | Manganese-transporting ATPase 13A1-like (*Crassostrea gigas*) | PP: cct-cca |
| | CCGCAAACTGTAAAACTACTGTGAGT | | | | |
| PF_SNP82 | ATTGCCTGGAGGAGGTTCG | 53 | T/C 397 | HBS1-like protein (*Crassostrea gigas*) | LL: tta-eta |
| | GACTTGTTCCGGGACAGTTTCT | | | | |
| PF_SNP83 | GCGAGGACTACAAACAAGATATG | 93 | C/A 2545 | Enhanced at puberty protein 1-like protein B (*Crassostrea gigas*) | 3'-UTR |
| | CCACGATTTCCAAACCGAG | | | | |
| PF_SNP84 | GCATCCGCACAGACCATT | 94 | G/A 2122 | Hypothetical protein CGI_10021394 (*Crassostrea gigas*) | 5'-UTR |
| | TTAGCATCCAGAAGGACTCGA | | | | |
| PF_SNP85 | TAACTTCCTTCCCTGCAACTGG | 98 | A/G 2748 | Unknown | Unknown |
| | AAGTCCCTCTATCACAGCAAATCAG | | | | |
| PF_SNP88 | ATGTTGCTTAGCACGAGCCC | 78 | G/A 1066 | CD63 antigen-like (*Crassostrea gigas*) | VV: gtg-gta |
| | CCTGTCCCCGTCTAGTGTTGT | | | | |
| PF_SNP92 | AGAGGAGGGGAAAGCCAA | 52 | A/G 588 | Heterochromatin protein 1-binding protein 3 (*Crassostrea gigas*) | SS: tca-tcg |
| | TGGCATTATCCTCAGACTTCC | | | | |
| PF_SNP95 | AACGATTCCCAGGGCGTAC | 77 | T/C 361 | NADH dehydrogenase (ubiquinone) iron-sulfur protein 3, mitochondrial-like isoform X1 (*Crassostrea gigas*) | RR: cge-cgt |
| | GAAAGGATAGTCATAGAGCCTGTAGAA | | | | |
| PF_SNP98 | TCTAATACCGACCAGGCTTCACA | 66 | A/C 2163 | Calcium-responsive transcription factor-like (*Aplysia californica*) | II: ata-atc |
| | CAGATCCGTACACGAGTCTACCATAC | | | | |
| PF_SNP103 | CTGAACTGGAAAGGGAAAT | 61 | A/T 779 | Unknown | LQ: ctg-cag |
| | GATGCCCATTAGAAATCTTC | | | | |
| PF_SNP105 | ACAGCATTCCGCCATGTTTGG | 88 | A/C 2043 | Bromodomain adjacent to zinc finger domain protein 2B (*Crassostrea gigas*) | PP: cca→ccc |
| | CGGGTGACGACGACGAAGATAGA | | | | |
| PF_SNP132 | CTCTGCCTTTCTAGCTCCTCTTGC | 81 | T/C 1904 | Unknown | KK: aaa-aag |
| | TGCCCGTGAAGCCTTGGAT | | | | |
| PF_SNP134 | CGAGCGTACCGTAGTAAATGAAGC | 61 | A/G 3671 | Ubiquitin carboxyl-terminal hydrolase 25 isoform X3 (*Chrysemys picta bellii*) | 3'-UTR |
| | TCAGACATTAGCCAGCGAGACAA | | | | |
| PF_SNP138 | GGCTCTAAGTACCGTCCTCACC | 58 | A/G 2011 | Unknown | SS: tcg-tca |
| | GCAACAGAATGCCCACAACA | | | | |
| PF_SNP141 | GGGTGTCCGTCAAACTTCTT | 79 | A/G 993 | AP-2 complex subunit alpha-2 (*Crassostrea gigas*) | QQ: caa-cag |
| | TCTGTGAGTCTGGTTCTTACTGC | | | | |
| PF_SNP142 | AGCTGTAGCCGAGGAGAAG | 93 | G/A 2079 | AP-2 complex subunit alpha-2 (*Crassostrea gigas*) | KK: aag-aaa |
| | TGTGGAGTAGGAGGATGGTTA | | | | |
| PF_SNP147 | AACGATTATTTGGCACTGGA | 54 | C/T 1129 | RNA-binding protein PNO1-like (*Crassostrea gigas*) | EE: gag-gaa |
| | AATTGACAGGAGGAAAGTCAGA | | | | |

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**Table 1.** Continued.

| Locus ID | Primer sequence (5'–3') | Amplicon size (bp) | SNP type and location | Gene annotation | Amino acid change |
|---|---|---|---|---|---|
| PF_SNP155 | CATGGGTAGTGTTCACTCTGTGA | 72 | C/T 2215 | Pre-rRNA-processing protein TSR1-like protein (*Crassostrea gigas*) | VV: gtc-gtt |
| | AATGGGTAACCACTAAGGACGA | | | | |
| PF_SNP156 | TGAAAGAAAATGGGACAGGT | 94 | A/G 195 | F-box only protein 8 (*Crassostrea gigas*) | NS: aat-agt |
| | TCGTCAAGTCGGGGAA | | | | |
| PF_SNP157 | ACATTCCGGCAGACTCAAC | 90 | G/T 81 | Membrane magnesium transporter 1-like (*Crassostrea gigas*) | AA: gct-gcg |
| | TGGGCAGCAGAATATGCA | | | | |
| PF_SNP164 | CGCAAAGCATATCGTTAAGTGAGAA | 86 | T/A 203 | SRY-related HMG-domain containing transcription factor 9 (*Pinctada fucata*) | 3'-UTR |
| | TGGGGCTGATTCCTTATGG | | | | |
| PF_SNP168 | TTTTGTTCAGTTGGCGGAGA | 78 | G/A 1079 | Uncharacterized protein LOC105333005 isoform X2 (*Crassostrea gigas*) | TA: act-gct |
| | ACCTACTGCCTCTGTTAGTTCTCC | | | | |
| PF_SNP189 | TGCTCGCTTCCATCAAC | 94 | A/T 3855 | Hypothetical protein CGI_10025135 (*Crassostrea gigas*) | 3'-UTR |
| | GTCACTTAGGACATCTTCACG | | | | |
| PF_SNP206 | CAGGTGGGGAAAATGAGAA | 73 | A/C 267 | Unknown | NK: aac-aaa |
| | GGTATACTTGCATAATGTCCGTAC | | | | |
| PF_SNP208 | GTTAGAACAGTTGAATGACGAGTC | 85 | A/G 1457 | Unknown | KK: aaa-aag |
| | TCTGTCAGCATCCTCCTCAAT | | | | |
| PF_SNP212 | ATGAGTTCCACGCCCAGTGA | 97 | T/G 2415 | Unknown | SS: tct-tcg |
| | GGAATGTACTGCTTGGTTCGTTAT | | | | |
| PF_SNP213 | TCCATTAGTACTCGCCAGTTTAGC | 94 | G/A 6265 | Unknown | LL: ttg-tta |
| | CAACTGTCGGGTATCAAAGGAA | | | | |
| PF_SNP214 | TTATTGTCCCTGGTAGGCTTCT | 48 | G/T 699 | Unknown | Unknown |
| | GCTTGCACGATTAACTAGGATGA | | | | |
| PF_SNP215 | ATGCCATAGCCTCCAACCC | 78 | A/T 805 | Unknown | PP: cca-cct |
| | CGGCAACCGTTCGTGAAA | | | | |
| PF_SNP219 | AGGCAGATGAGTCTACCACCAGG | 96 | G/A 4887 | Unknown | PP: ccg-cca |
| | AGAGTGAGGGGACATCAGGAG | | | | |
| PF_SNP221 | TGTCAGACCTCTACGGCTAAA | 59 | T/C 283 | RNA polymerase II elongation factor ELL (*Gallus gallus*) | 3'-UTR |
| | GGATTGAGATAACCGAGTGCT | | | | |
| PF_SNP228 | GTACATACAATTTGCTCGCTAG | 86 | G/A 832 | Glutaryl-CoA dehydrogenase, mitochondrial (*Crassostrea gigas*) | 3'-UTR |
| | TGTGATACTCAGAATGTCAAGC | | | | |
| PF_SNP229 | AAAACGACTAGGTCTGTAGCTGA | 80 | A/G 294 | Unknown | QQ: cag-caa |
| | GGATTTCCAAACTTGGACTCTT | | | | |
| PF_SNP231 | CTTCGTCGAGGTGAGCTAAA | 97 | C/A 167 | Unknown | TK: aca-aaa |
| | TTGATTGCTGAGTGATAGGCT | | | | |
| PF_SNP235 | CTATGGTAAACATAGTCGCCATAT | 73 | G/C 132 | Unknown | Unknown |
| | ACTAAAGGGGCAAGGAGGTAT | | | | |

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**Table 1.** Continued.

| Locus ID | Primer sequence (5'–3') | Amplicon size (bp) | SNP type and location | Gene annotation | Amino acid change |
|---|---|---|---|---|---|
| PF_SNP245 | TCCCAAGCTGTAACGTCTATCC | 72 | C/T 1884 | Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit alpha isoform (*Crassostrea gigas*) | AV: gec-gtc |
| | GGGGCTCTATACTATCGAATGTG | | | | |
| PF_SNP246 | CTATAAGTGCTACATGCACCAG | 73 | T/A 109 | Unknown | Unknown |
| | GTCAAGGTCAGATTTCAATAGTC | | | | |
| PF_SNP251 | AACTGTTCATCCCCATCATCTG | 83 | A/G 583 | Histidine triad nucleotide-binding protein 1 (*Crassostrea gigas*) | --:tag-taa |
| | CCCCAAGCTCCTACTCATTTTC | | | | |
| PF_SNP289 | GCTGAAACAAAACAAGCCAT | 53 | T/G 77 | Unknown | Unknown |
| | CCGTCCTTACCAAATTCTATCT | | | | |
| PF_SNP294 | CCATGAACAGAATGAGACCAT | 72 | 4312 C/T | Laminin subunit alpha (*Crassostrea gigas*) | YY: tac-tat |
| | TCAACTCTAAGGAATCCGACA | | | | |
| PF_SNP299 | CCGAACAAGAATTACGCA | 78 | A/G 285 | Unknown | Unknown |
| | TCACATCCTGATTTTGCCT | | | | |
| PF_SNP308 | GCAGGCTAAAGCAGTAGGAAAGA | 85 | C/A 844 | Ubiquitin carboxyl-terminal hydrolase 14 (*Crassostrea gigas*) | TT: aca-acc |
| | ATCATCAGGGAACCAATAGGGA | | | | |
| PF_SNP310 | TGGGGTGTCCATCGTGAA | 80 | C/T 1488 | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (*Crassostrea gigas*) | LL: tta-cta |
| | AAAAGAAGACAGAGGAACTGAAGAC | | | | |
| PF_SNP312 | ATCTGGATCGGTGAACTTGA | 54 | T/C 271 | JNK1-associated membrane protein (*Harpegnathos saltator*) | LL: ttg-ctg |
| | GTTCCGCTCCCCGTTAT | | | | |
| PF_SNP316 | TCAGCTCAAGTTCCTCCAGTTC | 89 | 644 A/G | Polyglutamine-binding protein 1 (*Crassostrea gigas*) | SS: tcg-tca |
| | TTTGGAGTCATCGTCATAGCG | | | | |
| PF_SNP319 | TTCTTATGTCTGACTAAGGGCTCG | 86 | 443 G/A | Mitochondrial ribosomal protein S11 (*Nilaparvata lugens*) | PP: cca-ccg |
| | GACACTTGTGCGATGATACTGG | | | | |
| PF_SNP320 | CAAGTCAGGTTGGGGCATT | 87 | 615 T/C | Stress-70 protein, mitochondrial (*Crassostrea gigas*) | FF: ttt-ttc |
| | GATAGTTTTCTGCGGTTTCTTTC | | | | |
| PF_SNP322 | GGATGTTATTCTGGTCGGAGG | 100 | 1251 A/G | Stress-70 protein, mitochondrial (*Crassostrea gigas*) | GG: ggg-gga |
| | ATCGGGGTTCACAGCCT | | | | |
| PF_SNP326 | TAACCATCCACAGACACCAAGTA | 51 | 420 T/A | Small nuclear ribonucleoprotein F (*Crassostrea gigas*) | IN: atc-aac |
| | GCTGAAGTGGGGAATGGAATA | | | | |
| PF_SNP332 | CAGAAAGATAACAACTGTGGGG | 41 | 1672 T/C | Homologue of Sarcophaga 26, 29 kDa proteinase (*Periplaneta americana*) | VV: gtt-gtc |
| | AAGGTCGGATTGGTGGC | | | | |
| PF_SNP333 | ATGCACCACCTACTCAAAGACA | 82 | 790 G/A | Putative sodium/potassium-transporting ATPase subunit beta-2 (*Crassostrea gigas*) | 3'--UTR |
| | GACTCAGTATCAAAACAGAAGCAG | | | | |
| PF_SNP337 | AGCCTAACGAGTTACCCCAGT | 78 | 915 C/T | Pre-mRNA-processing-splicing factor 8 (*Crassostrea gigas*) | DD: gac-gat |
| | CCCATGATGGATTGTCGG | | | | |
| PF_SNP341 | CAAGTAGATACCAATGAGCAGCA | 85 | 1392 C/G | Histone-lysine N-methyltransferase PRDM9 (*Crassostrea gigas*) | PP: ccg-ccc |
| | CCACATATTGGACAACGTAGGTG | | | | |

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**Table 1.** Continued.

| Locus ID | Primer sequence (5'–3') | Amplicon size (bp) | SNP type and location | Gene annotation | Amino acid change |
|---|---|---|---|---|---|
| PF_SNP343 | AATGACGGAGGAGCGTTACA | 65 | 2048 A/G | RAD50-interacting protein 1-like (*Aplysia californica*) | QQ: ca**a**-ca**g** |
| | TCCCCGAATAGAGGAAAGAG | | | | |
| PF_SNP344 | ACATCCCTTGAGATGTGAGGG | 73 | 606 A/G | Arrestin domain-containing protein 2 (*Crassostrea gigas*) | PP: cc**a**-cc**g** |
| | GCGGGGAAAACAGACTTGG | | | | |
| PF_SNP347 | TTCACCTGACCGCTGTTCC | 100 | 1292 C/G | Protein rogdi-like (*Crassostrea gigas*) | VV: g**t**g-g**t**c |
| | GGAGATTTCCACATAAACCAGG | | | | |
| PF_SNP357 | ATCCAGGGAGAATATCGGG | 43 | 256 T/C | Cullin-4A (*Crassostrea gigas*) | VV: g**t**t-g**t**c |
| | TTCAGTCATTTGTGGCTCTTC | | | | |
| PF_SNP369 | GCCCGTTTGTTCTACCATCG | 88 | 1804 T/C | ATP-binding cassette sub-family D member 3 (*Crassostrea gigas*) | VA: g**t**g-g**c**g |
| | GGTACATGAATCCTTCTACATCTACAC | | | | |
| PF_SNP374 | CAACCTTGGCTAGAGCAACA | 81 | 2109 A/G | Protein disulfide-isomerase A3 (*Crassostrea gigas*) | GR: g**g**a-a**g**a |
| | GCAAAAGATTAGCCCCTGAG | | | | |
| PF_SNP375 | GATGCTCTGGCAAAGCTACA | 93 | 505 C/T | Ras-related GTP-binding protein C (*Crassostrea gigas*) | NN: aa**c**-aa**t** |
| | GCCGTCTACTTTATGTATGAACAC | | | | |
| PF_SNP376 | CCTACCTGTATGGCCTACATCC | 90 | 1158 T/C | Dynein beta chain, ciliary (*Crassostrea gigas*) | NN: aa**t**-aa**c** |
| | GCTGCATCTCAAACACGGTC | | | | |
| PF_SNP383 | TCGTCCCATTCTTCACCG | 72 | 299 T/C | Wiskott-Aldrich syndrome protein family member 3 (*Crassostrea gigas*) | PL: c**c**g-c**t**g |
| | TGACATGAGGCGTAAAGCTG | | | | |
| PF_SNP399 | CCAAATGGAAATTCCGTTGA | 65 | 208 A/G | Plancitoxin-1 (*Crassostrea gigas*) | VV: g**t**a-g**t**g |
| | GCTTTATTCTTGGTGTTCTCAGGTAG | | | | |
| PF_SNP416 | AGCAGACCTACCACATCGTT | 70 | 657 A/G | Unknown | Unknown |
| | TTGTCCATGTAACAGTCCTATCA | | | | |
| PF_SNP425 | GTTCTGACTCCTCATTTATAGGGT | 93 | 2497 C/T | Unknown | Unknown |
| | CCATAAATAGACTGACTGAGGCT | | | | |
| PF_SNP426 | TCAGGTACACGTCGATAACA | 78 | 231 G/C | Unknown | Unknown |
| | TATTGCGCCAGTAACTACAT | | | | |
| PF_SNP427 | GATCCCTATAATCGTGTCGCC | 77 | 1039 C/T | Unknown | HH: ca**t**-ca**c** |
| | GGCTAATTACAGGGACAAATACCA | | | | |
| PF_SNP433 | AGCATACTTCAATGATTCCCAGA | 62 | 655 C/T | Heat shock protein 70 (*Pinctada fucata*) | AA: g**c**t-g**c**c |
| | TGAGACCAGCGATTGTGCC | | | | |
| PF_SNP437 | AAGAATTTGGTGCAAGATGTG | 56 | 1900 A/T | Unknown | Unknown |
| | AAAGCGACATTTCCCAGA | | | | |
| PF_SNP441 | GCCTTATTGGCATATCTACTATG | 89 | 248 T/A | Unknown | Unknown |
| | GAGTCGGCTTTTACAAATGAT | | | | |
| PF_SNP444 | TTTCGCCCTCGGCAACAA | 48 | 1280 C/T | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (*Crassostrea gigas*) | LL: ct**c**-ct**t** |
| | CCAATCAGAAGACGGACCAAGAA | | | | |
| PF_SNP445 | GACCAAAGGTCATGTGACCAAGG | 84 | 183 C/A | Unknown | ED: ga**a**-ga**c** |
| | GGATAAAGCACGGACTGGAATGT | | | | |

Continued on next page

**Table 1.** Continued.

| Locus ID | Primer sequence (5'–3') | Amplicon size (bp) | SNP type and location | Gene annotation | Amino acid change |
|---|---|---|---|---|---|
| PF_SNP448 | TGCACCTTTAGACACTGTTTGTAC | 92 | 1699 C/T | Zinc finger CCCH domain-containing protein 15 (*Crassostrea gigas*) | LL: ct**g**-ct**a** |
| | AGCAACAGGAGGCTAAGAAAA | | | | |
| PF_SNP454 | ACGCATCAATAACTCAGTCTTCG | 78 | 2897 G/A | Ribose-phosphate pyrophosphokinase 1 (*Crassostrea gigas*) | TT: ac**a**-ac**g** |
| | GGAGCTTGTTTCATTCTTTCCT | | | | |
| PF_SNP459 | TCCCCGTTTGATGCGTC | 99 | 1737 A/G | ADP-ribosylation factor-binding protein GGA1-like isoform X1 (*Crassostrea gigas*) | LL: tt**g**-tt**a** |
| | GGCCGTTGACTGTGGTGA | | | | |
| PF_SNP471 | CCTCCTTCTAGGCATAAATTGAC | 100 | 1717 A/G | Unknown | 3 UTR |
| | TCTCCTCAAAAGACGACACTACTC | | | | |
| PF_SNP474 | ACCCGGTTACTGTTTTGGG | 83 | 3323 G/A | Protein phosphatase 1E (*Crassostrea gigas*) | Unknown |
| | TGACTTTCGTGCGTGTTCC | | | | |
| PF_SNP480 | CGAAACGGACAGTAAGAAAGAA | 100 | 906 C/A | Hypothetical protein CGI_10022149 (*Crassostrea gigas*) | TT: ac**c**-ac**a** |
| | ATGGGTTTACGATTGGCAC | | | | |
| PF_SNP482 | GCCTTCCATAGATGTAGAGTATTCAG | 96 | 842 G/A | Uncharacterized protein LOC105327635 (*Crassostrea gigas*) | LL: ct**a**-ct**g** |
| | CATAACCTATCCTTTCACATCCC | | | | |
| PF_SNP484 | TGCCAAGGACGGAGATG | 88 | 573 T/C | Fidgetin-like protein 1 (*Crassostrea gigas*) | 3'-UTR |
| | TCAAAGAAAGGTGAGAATAGCC | | | | |
| PF_SNP485 | TATGACATCTATCCAATGGCAAG | 92 | 215 A/T | Troponin T (*Mizuhopecten yessoensis*) | 3'-UTR |
| | TCCCCTATTCTTTTGTAGCGTA | | | | |
| PF_SNP488 | TCTCGTGATCCCAACGAAGTAGC | 84 | 553 T/G | T-complex protein 1 subunit alpha-like (*Crassostrea gigas*) | SS: tc**t**-tc**g** |
| | AACGGTTGTTCCGGGAGGT | | | | |
| PF_SNP489 | GAGGACTGGTTCATTTGTTGTG | 66 | 1060 T/G | Unknown | Unknown |
| | TGTCAGCGCCCTTATTCC | | | | |

UTR, untranslated region.

The PCR process and HRM analysis were performed as described above. The number of alleles per locus, effective number of alleles, observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), and minor allele frequency (MAF) were assessed using the POPGENE 32 software (Yeh et al., 2000), and the polymorphism information content (PIC) was calculated using the PICcalc online software (Nagy et al., 2012).

**Table 2.** Sequences and predicted and observed melting temperatures of internal temperature controls.

| Name | Forward/reverse sequence (5'-3')* | Predicted temperature (ºC) | Observed temperature (ºC) |
|---|---|---|---|
| High-temperature sequences | F:GCGGTCAGTCGGCCTAGCGGTAGCCAGCTG CGGCACTGCGTGACGCTCAG | 90.02 | 90.08 |
| | R:CTGAGCGTCACGCAGTGCCGCAGCTGGCTACCGC TAGGCCGACTGACCGC | | |
| Low-temperature sequences | F:ATCGTGATTTCTATAGTTATCTAAGTAGTTGGCAT TAATAATTTCATTTT | 68.5 | 68.5 |
| | R:AAAATGAAATTATTAATGCCAACTACTTAGATAA CTATAGAAATCACGAT | | |

*All of the sequences were blocked with a phosphate at the 3'-end.

## RESULTS AND DISCUSSION

Small-amplicon HRM assays (SA-HRMAs) provide a rapid, inexpensive, and high-throughput closed-tube method for genotyping (Smith et al., 2010). To ensure SA-HRMA accuracy, we used three criteria: 1) SA-HRMA amplicons were no more than 100 bp long, which ensured that homozygous genotypes of alleles were easily distinguished; 2) only one SNP was present in each amplicon; and 3) high- and low-temperature controls were added for each amplicon, which decreased melting temperature variations attributable to the instrument or solution chemistry and corrected melting profiles (Seipp et al., 2007). An improved two-step SA-HRM method for Pacific oyster (*Crassostrea gigas*) SNP validation has been shown to be efficient and economical (Wang et al., 2013, 2015), and this method was successfully used to validate 119 polymorphic SNPs from *P. fucata* transcriptome data, demonstrating that it is feasible in shellfish.

A subset of 468 primers was randomly designed to validate the SNP predictions. No amplification products were seen in 66 sets of primers, and introns were found in genomic DNA but not the transcriptome. If the primer flanked, or was located in, an intron, the intervening fragment could not be amplified. A total of 173 sets of primers amplified multiple bands, and 229 amplified a clear target band on PAGE. The ratio of primer screening was 48.93%, which is higher than previously reported values of 41.67% (Zhang et al., 2015) and 28.10% (Huang et al., 2014b).

All of the SNP-containing unigenes were annotated with the corresponding top best BLASTx hits, and 88 SNPs were annotated though BLASTx in the Nr database (Table 1). Of these, heat-shock protein 70 is expressed in response to changes in temperature, bacterial infection, or pH. Its main function is to promote protein folding, and thereby prevent the cellular accumulation of non-native proteins (Mymrikov et al., 2011). F-box proteins are an expanding family of eukaryotic proteins, characterized by an approximately 40-amino-acid motif (Cenciarelli et al., 1999). F-box proteins were first characterized as components of SCF ubiquitin-ligase complexes, in which they bind substrates for ubiquitin-mediated proteolysis (Kipreos and Pagano, 2000). Fatty acid-binding proteins participate in lipid uptake, transport, and homeostasis (Bayır et al., 2015). Sox9 (SRY-related HMG-domain-containing transcription factor 9) and cullin-3-B play important roles in testis development (Bergstrom et al., 2000; Lu et al., 2005). Among the 229 well-amplified SNPs, 119 (51.97%)

were polymorphic in 8 *P. fucata* individuals, according to the SA-HRMA (Table 1). Seventy-five SNPs were genotyped as transitions, including 40 A/G and 35 C/T, and 44 were genotyped as the transversions 11 A/C, 18 A/T, 6 C/G, and 9 G/T. According to ORF Finder, 90 SNPs were located in the ORF, including 16 non-synonymous SNPs and 74 synonymous SNPs; 12 SNPs were located in the 3'-untranslated region (UTR), and 1 was located in the 5'-UTR. SNPs within a coding sequence may change a protein's amino acid sequence and structure, thus influencing its functions (Gao et al., 2014; An et al., 2015). The post-transcriptional regulation of gene expression is crucial for many physiological processes. SNPs within UTRs may have consequences for gene splicing, expression, and regulation (Malodobra-Mazur et al., 2016; Xu et al., 2016). SNPs developed from functional genes may be used in association studies, which could genetically improve species. For example, some SNPs are associated with growth traits in the pearl oyster (Shi et al., 2014), and SNPs screened from the myostatin gene are associated with growth traits in the scallop and carp (Wang et al., 2010; Guo et al., 2011; Liu et al., 2012; Sun et al., 2012). All of the annotation unigenes and their SNPs may be useful for studying the commercial traits of *P. fucata*, such as growth, resistance, and reproduction.

Twenty-five SNPs were successfully used to test the genetic diversity of 40 wild *P. fucata* from Liusha Bay, China (Table 3). All of the SNP loci had intermediate PIC values ($0.25 < PIC < 0.5$), with a mean of 0.3336. The $H_O$ was 0.0417-0.6042 and the $H_E$ was 0.2945-0.5053. Li et al. (2016) used SNP loci to analyze the genetic diversity of *P. fucata* individuals from three families, and obtained PIC values of 0.2435, 0.2479, and 0.2977. Huang et al. (2014a) used SNP loci to study the genetic diversity of a wild *P. fucata* population in Shenzhen, China, and reported MAF, $H_O$, and $H_E$ values of 0.0642-0.4375, 0.1282-0.4872, and 0.1215-0.4984, respectively. These findings indicate that the Liusha population genetic diversity is higher than that in culture or in the Shenzhen population.

**Table 3.** Summary of 25 single nucleotide polymorphisms in wild *Pinctada fucata* individuals.

| Locus | $N_E$ | $H_O$ | $H_E$ | MAF | PIC |
|---|---|---|---|---|---|
| PF_SNP1 | 1.7041 | 0.5000 | 0.4175 | 0.2917 | 0.3270 |
| PF_SNP9 | 1.9321 | 0.6042 | 0.4875 | 0.4062 | 0.3668 |
| PF_SNP18 | 1.9965 | 0.5417 | 0.5044 | 0.4792 | 0.3746 |
| PF_SNP31 | 1.8221 | 0.3125 | 0.4559 | 0.3438 | 0.3481 |
| PF_SNP33 | 1.7771 | 0.1458 | 0.4419 | 0.3229 | 0.3405 |
| PF_SNP52 | 1.9459 | 0.4167 | 0.4912 | 0.4167 | 0.3685 |
| PF_SNP55 | 1.6265 | 0.3542 | 0.3893 | 0.2604 | 0.3108 |
| PF_SNP58 | 1.9991 | 0.6042 | 0.5050 | 0.4896 | 0.3749 |
| PF_SNP64 | 1.4113 | 0.2292 | 0.2945 | 0.1771 | 0.2516 |
| PF_SNP67 | 1.9584 | 0.4792 | 0.4945 | 0.4271 | 0.3700 |
| PF_SNP68 | 1.9965 | 0.2500 | 0.5044 | 0.4792 | 0.3746 |
| PF_SNP69 | 1.8824 | 0.4583 | 0.4737 | 0.3750 | 0.3589 |
| PF_SNP70 | 2.0000 | 0.4583 | 0.5053 | 0.5000 | 0.3750 |
| PF_SNP71 | 1.5463 | 0.2917 | 0.3570 | 0.2292 | 0.2915 |
| PF_SNP73 | 1.8000 | 0.4583 | 0.4491 | 0.3333 | 0.3444 |
| PF_SNP75 | 1.8633 | 0.1875 | 0.4682 | 0.3646 | 0.3546 |
| PF_SNP77 | 1.6000 | 0.2500 | 0.3789 | 0.2500 | 0.3047 |
| PF_SNP82 | 1.9692 | 0.5000 | 0.4974 | 0.4375 | 0.3714 |
| PF_SNP83 | 1.8432 | 0.0417 | 0.4623 | 0.3542 | 0.3515 |
| PF_SNP84 | 1.5463 | 0.2500 | 0.3570 | 0.2292 | 0.2915 |
| PF_SNP88 | 1.4922 | 0.1667 | 0.3333 | 0.2083 | 0.2768 |
| PF_SNP92 | 1.7041 | 0.5833 | 0.4175 | 0.2917 | 0.3270 |
| PF_SNP95 | 1.5463 | 0.2917 | 0.3570 | 0.2292 | 0.2915 |
| PF_SNP98 | 1.4922 | 0.1667 | 0.3333 | 0.2083 | 0.2768 |
| PF_SNP103 | 1.6528 | 0.4167 | 0.3991 | 0.2708 | 0.3165 |
| Average | 1.7643 | 0.3583 | 0.4310 | 0.3350 | 0.3336 |

$H_E$, expected heterozygosity; $H_O$, observed heterozygosity; MAF, minor allele frequency; $N_E$, effective number of alleles; PIC, polymorphism information content.

HRM technology can directly distinguish between different genotypes based on melting peak profiles (Smith et al., 2010). Figure 1a and b show the melting curve analyses of PF_SNP9 and PF_SNP98, respectively.

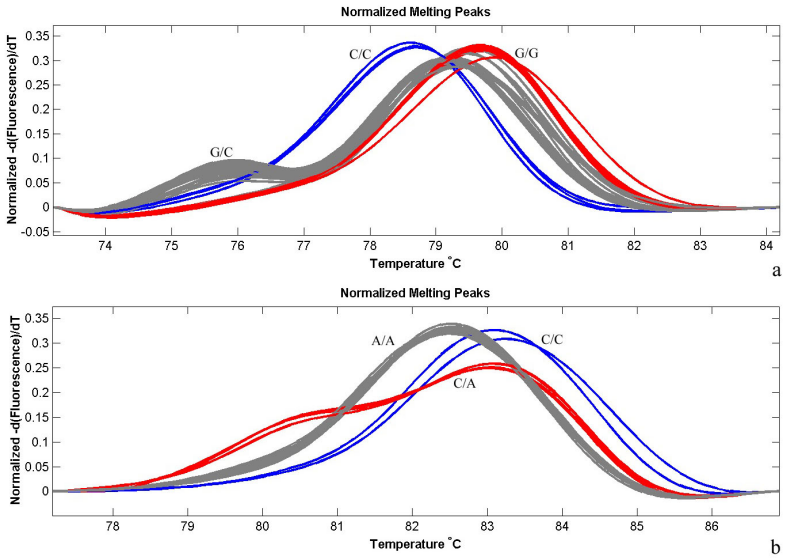


**Figure 1.** Genotyping results using high-resolution melting with a small amplicon. **a.** PF_SNP9, homozygotes (GG and CC) and heterozygotes (GC) are represented by red, blue, and gray curves, respectively. **b.** PF_SNP98, homozygotes (AA and CC) and heterozygotes (CA) are represented by gray, blue, and red curves, respectively.

In conclusion, 119 polymorphic SNPs were successfully isolated by SA-HRMA, thus contributing to our understanding of *P. fucata* genetics and breeding.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

Research supported by the PhD Start-Up Fund of Natural Science Foundation of Guangdong Province (#2014A030310237), the Natural Science Foundation Program of Hainan Province (#20154180), the Basic Scientific Research Fund for the Central Institutes of Public Welfare (South China Sea Fisheries Research Institute, #2014TS08), the Special Fund for Marine Fisheries Research and Extension of Guangdong Province (#A201401A07, #Z2015010), the National Natural Science Foundation of China (#31372525), and the Earmarked Fund for China Agriculture Research System (#CARS-48).

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