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ONLINE RESOURCES

Identification of twenty novel polymorphic microsatellite DNA markers from transcripts of the pearl oyster *Pinctada fucata* using next-generation sequencing approach

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Introduction

The pearl oyster, *Pinctada fucata* (Gould 1850) is a commercially important marine shellfish cultured for producing saltwater pearls mainly in China and Japan (Yu and Chu 2006). It is common in most areas of tropical and subtropical oceans and seas in the Pacific and Indian regions. In 1965, this specie was successfully propagated and reared under artificial conditions in Guangxi province in southern China and expanded rapidly to the neighbouring Guangdong and Hainan provinces subsequently (Meng *et al.* 1996). The pearls produced by the animals are referred to as 'South China Sea Pearl', accounting for over 90% production of the total marine pearls produced in China. For the last few years, some traits of *P. fucata* appear to have degenerated, due to overfishing, coastal water pollution and artificial propagation of years without recording their background, which hampered the advance of the pearl industry. Genetic improvement and culture of elite varieties should be carried out to prevent slowdown of the growth rate because of inbreeding depression and deterioration of the pearl quality.

Microsatellite DNA markers have proved to be a useful tool for evaluating the level of genetic variation of natural populations in many fishery animals because of the high polymorphism, abundance, neutrality and codominance (Liu and Cordes 2004). Polymorphic microsatellite loci have been frequently applied in the analysis of genetic diversity of populations. In spite of some microsatellite loci in this species were reported (Tong *et al.* 2007; Kuang *et al.* 2009; Shi *et al.* 2009; Qu *et al.* 2010; You *et al.* 2012; Wu *et al.* 2013), the amount is beyond the requirement and more polymorphic loci are required for population genetics study and genetic improvement of this species.

During the last decade, deep RNA sequencing (RNA-seq) technologies and the downstream applications provided large molecular markers such as microsatellite DNAs and SNPs (Davey *et al.* 2011). As a kind of RNA-seq technique, the Illumina sequencer can produce large amounts of data with longer reads (>75 bp) at lower cost (Kawahara-Miki *et al.* 2011; Ward *et al.* 2012). In this study, a new set of 20 cDNA-SSRs of *P. fucata* was developed and characterized from the Illumina sequencing data.

Materials and methods

In this study, *P. fucata* were collected from Marine Biology Research Station of South China Sea Research Institute at Lingshui, Hainan province, China. Total RNAs of the gill, digestive gland and mantle tissues dissected from *P. fucata* were extracted using NucleoSpin RNA II kit (Macherey-Nagel, Germany) following the instructions of manufacturers. After submitting for library construction, three cDNA libraries from different tissues were sequenced in BGI-Shenzhen (Beijing Genomics Institute) using Illumina HiSeq^{TM}2000 (San Diego, USA). Then, all reads produced from three libraries were assembled into unigenes together.

All unigenes data were screened for dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide microsatellite motifs with MicroSAtellite (MISA, http://pgrc.ipk-gatersleben.de/misa). To avoid PCR primers designed locating between two exons and thus resulting in

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Table 1. Characterization of 20 microsatellite loci in the pearl oyster, *Pinctada fucata.*

Locus/ accession no.	Primer sequence (5′–3′)	T_a(°C)	Repeat motif	Range (bp)	*A*	H_o	H_e	*P* value for HWE test	Annotation
Pf01	F: TCAACAAAAGGAGACGAAGC R: CTTGGAGTAGATGTTCCCTGAT	53	$(TGA)_7$	240–270	4	0.4000	0.4288	0.3152	Hypothetical protein
Pf02	F: GCTTGCCAGTGACTTCCTC R: GGCCTGAGCTATCACAAGTC	55	$(AT)_8$	80–100	3	0.6250	0.5750	0.4373	Signal recognition particle 68 kDa protein
Pf03	F: GGAGGACCATTACTATTCTACAC R: TTCCGCACGACTGGTCTA	53	$(GATG)_5$	200–230	5	0.0625	0.7178	0.0000*	Unknown
Pf04	F: TATTTCACAAACCCAGGATTACC R: GAGTTCAAAGTGGAGCAGACG	53	$(GTCT)_5$	160–190	4	0.4000	0.5816	0.0028	Unknown
Pf05	F:GTATCAAGGAAGGACCGAAGA R: AAAAGGACCAACACGCTCTAC	53	$(GT)_8$	220–240	4	0.1500	0.4500	0.0004*	RNA polymerase-associated protein RTF1-like protein
Pf06	F: TATTTTCCCCTGAACAGCA R: CCATCAAACTTGCAGGGA	50	$(GTTAA)_4$	160–210	6	0.4250	0.7424	0.0001*	Unknown
Pf07	F: CTGACAGACGTAAGCGTTACAAA R: TACCAGCAACTCTACCAGACCATC	57	$(GATCA)_4$	160–210	6	0.3000	0.5370	0.004	EH domain-containing protein 1
Pf08	F: AACCCTGGTGGACACTCTT R: GTAGCTTCGGCTTCTTGC	53	$(TAA)_5$	120–140	5	0.5641	0.7409	0.023	Unknown
Pf09	F: CTATGACGACGCTGTACTGATC R: ACTATGTACCTGTACGGGTTGTC	55	$(AC)_6$	70–90	3	0.1892	0.2458	0.1315	Unknown
Pf10	F: GTCCGTCTTATCTAACACCACT R: ATTTACTAACCTGTGCCCTCTG	53	$(TA)_9$	170–150	3	0.4250	0.6130	0.0024*	F-box/LRR-repeat protein 20
Pf11	F: TGCTGTACCCAAAGGGCTTATT R: TGGCGATTTTCTTGTATGTGAC	53	$(AT)_6$	130–100	6	0.5263	0.7323	0.0117	Unknown
Pf12	F: TGTGCGATTATACTCGGAACTGA R: TTTTAGGGGTAACATGCTCTGC	53	$(AT)_6$	180–200	4	0.0263	0.6839	0.0000*	Unknown
Pf13	F: GTCCAAACGGAAGAACCAATTA R: AGTGGTAGTTGATGGTTCTGGC	55	$(AAC)_5$	100–140	5	0.6750	0.6022	0.1915	Hypothetical protein
Pf14	F: GGTGCCGTGTAGTAAACCGTAA R: ACTTTCCTCCGCGTATTGATAT	55	$(TA)_6$	160–210	5	0.7179	0.6344	0.0408	Unknown
Pf15	F: TCATTTACAAGGGCACATAACTCCG R: GGACTCCTTGAATGGGCATCTTT	55	$(TCAAA)_4$	140–160	2	0.2250	0.2022	0.6039	Prostaglandin E2 receptor EP4 subtype
Pf16	F: TTCAGCAGTCACATCCTCCATT R: ATACAGATAGAGGAAGTGGAGGACAC	57	$(CTC)_5$	140–160	2	0.0513	0.0506	0.9867	Rho guanine nucleotide exchange factor 17
Pf17	F: GTGGATGGCTGAGTGAATAGTGCC R: TTCATCAAGCAGCAGGGTGGAG	59	$(GGA)_6$	100–120	5	0.0263	0.6663	0.0000*	Ubiquitin-protein ligase E3A
Pf18	F:TCGACGTAGAGGTCAAGTTGGTGT R: AGGCGTTCATTCTCCGTTAGCA	57	$(TCC)_5$	200–230	5	0.3333	0.6344	0.0029	Hypothetical protein
Pf19	F: TACGGCAAAACTTGATAGTCAG R: CACAAGTCAAAACAGGAGGTAC	53	$(TA)_7$	190–250	3	1.0000	0.5366	0.0000*	Unknown
Pf20	F: GATTTGTCGTTTGATTGCTCTG R: CTGAGAATGTAGGCTGAAAGGA	53	$(TGATT)_7$	190–240	4	0.2105	0.6137	0.0000*	Unknown

A, number of alleles, H_o, observed heterozygosity, H_e, expected heterozygosity. T_a, annealing temperature.
* Indicates significant deviation from HWE after Bonferroni correction.

no fragment, a part of a unigene containing SSR-motif(s) was aligned with draft genome data of *P. fucata* Takeuchi *et al* 2012 by local blast search. Sixty-nine sequences obtaining SSR-motifs, which did not contain introns, were used to design flanking primer pairs with the Primer Premier 5 (Lalitha 2000).

Polymorphisms were evaluated in a population of 40 individuals collected from Daya Bay in Shenzhen, Guangdong province, China. Following DNA extraction, PCR was performed on a Mastercycler (Eppendorf, Germany) in a volume of 25 μL containing 1 U of *Taq* polymerase (Takara, Japan), 1$\times$ PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer and 50–100 ng of total DNA. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, a primer-specific annealing temperature for 45 s and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were examined with electrophoresis on 8% nondenaturing polyacrylamide gel and visualized with silver staining. A 20 bp DNA ladder (Takara) was used for assessing the sizes of the DNA fragments.

Number of alleles per locus, expected heterozygosity and observed heterozygosity were calculated by PopGen 3.2 (Yeh and Boule 2000). Test of Hardy–Weinberg equilibrium (HWE) was analysed using Genepop 4.2 (Raymond and Rousset 1995).

Results and discussion

Among the 69 pairs of primers, 12 failed to amplify any PCR products, eight produced multi bands or complicated patterns, 29 generated a single band, and the other 20 showed polymorphism for the sample tested. These 20 loci included varied nucleotide repeats (table 1). The allele numbers of the polymorphic markers ranged from two to six per locus, with the average of 4.2. Observed heterozygosities varied from 0.0263 to 1.0000 with an average of 0.3666, while expected values for these loci ranged from 0.0506 to 0.7424 with an average of 0.5494 (table 1). After Bonferroni correction (adjusted $P = 0.0025$), deviation from HWE was observed in eight loci, which usually occurred in pearl oysters (Evans *et al.* 2006; Kuang *et al.* 2009; Qiu *et al.* 2013), Pacific oyster (Li *et al.* 2011) and abalone (Sekino *et al.* 2012). Besides, nine couples of loci (pf10& pf11, pf5& pf13, pf01& pf14, pf14 & pf17, pf11& pf18, pf12 & pf18, pf17& pf18, pf10 & pf05 and pf18 & pf20) were found significant linkage disequilibrium (LD) ($P < 0.01$). Additionally, among the 20 cDNA-SSR sequences seven matched to genes of known functions at E values less than 10^{-5} through comparing with the GenBank data by BLASTn and BLASTx. The putative functions of these cDNA -SSR sequences were related to signal recognition particle 68 kDa protein, RNA polymerase-associated protein RTF1-like protein, hypothetical protein (table 1). Obviously, the loci isolated in this research would be valuable in genetic improvement of *P. fucata*.

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