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# Isolation and screening of microsatellite markers from the pearl oyster, *Pinctada fucata* using FIASCO method

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Pearl oyster, *Pinctada fucata* is an important commercial species that is cultured for pearl in China (Meng *et al*. 1996). Pearls produced by this species are well-known worldwide and called 'South China Sea Pearl'. In China, artificial reproduction of this species was successful in 1965. Since then, hundreds of hatcheries have been established (Meng *et al*. 1996), and currently the industry of pearl production relies mainly on hatchery produced spats. Each year, the hatcheries use cultured stocks as the brood stocks to produce the next generation for culture. This operation has been continued for more than 40 years which lead to some degeneration of growth traits, such as miniaturization of body size, reduction of resistance to pathogens and adverse environments, and decrease of cultured pearl quality. Thus, it is desirable to investigate the genetic variability of this species to provide background information for its genetic improvement. Microsatellite DNA is one of the most popular molecular markers, because of its high level of polymorphism and codominant nature it is increasingly being used for identity test, population genetics and gene mapping (Evans *et al*. 2006; Herbinger *et al*. 2006). Here, we report nine polymorphic microsatellite loci that would be useful in genetic studies in the pearl oyster.

In a total of 32 individuals, samples for construction of enriched library were collected from Sanya Bay, Hainan Province, and samples for screening and characterization of polymorphic microsatellite loci were collected from Beibu Bay, Guangxi Province. Genomic DNA was extracted from the adductor muscle by proteinase K digestion, phenol–chloroform protocol. The enriched genomic library was constructed following the protocol of the fast isolation by AFLP of sequences containing repeats (FIASCO) as described by Zane *et al*. (2002). DNA was simultaneously digested with *Mse*I and ligated to *Mse*I-adaptor. The digestion ligation mixture was diluted (1:10) and directly amplified with AFLP adaptor-specific primers. The PCR product was then hybridized with a biotinylated $(CA)_{15}$ probe. DNA molecules hybridized with these probes were selectively captured by streptavidin-coated beads. Nonspecific DNA was removed by three nonstringent washes and three stringent washes. After two denaturation steps using TE, specific DNA was separated from the beads-probe complex. This specific DNA was PCR-amplified, purified and ligated with the pMD20-T vector and then transformed into Top10 competent cells. There were almost 2000 solitary colonies in the enriched library. The second PCR screening was performed using the primer of $(CA)_{15}$ probe and T vector's primers, and 357 positive clones were obtained. Sequencing results showed that 297 clones contained microsatellite repetitive units. By alignment, 280 special microsatellite clones were obtained finally, including 479 microsatellite domains. Overall, 49 pairs of primers were synthesized out of 219 pairs that were designed using Primer Premier 3.0 (Rozen and Skaletsky 1998), and 31 pairs were effectively amplified.

PCR conditions were optimized for these primers and screening of polymorphic loci was performed on 32 individuals of *P. fucata*. The optimal amplification conditions are as follows: final volume of 20 $\mu$L, containing 10× PCR buffer, 40 ng genomic DNA, 10 mM dNTP, 25 mM $MgCl_2$, 5 $\mu$M for each primer and 1 U *Taq* DNA polymerase. The amplification profile was: predenaturation at 94°C for 5 min; followed by 3 cycles including denaturation at 94°C for 30 s, annealing temperature at 47–52°C (table 1) for 30 s and elongation at 72°C for 1 min; and a final extension step at 72° for 10 min. Amplified fragments were separated on 5% denaturing polyacrylamide gels running at 110 W for about 120 min and visualized following $AgNO_3$ staining according to Bassam *et al*. (1991).

For each locus, the number of alleles (A), expected heterozygosity (He), observed heterozygosity (Ho), and the $\chi^2$ test of Hardy–Weinberg equilibrium were calculated using

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**Table 1.** Primer sequences, motif characteristics and amplification conditions of nine polymorphic loci for the pearl oyster *P. fucata*.

| Locus | Motif | Primer sequences (5′–3′) | Size range (bp) | Annealing temp. (°C) |
|---|---|---|---|---|
| M2 | $(GT)_6(GA)_{22}$ | F: TCTTCCGACAAACTAAACA<br>R: CTGAATTTTGAAATCCACA | 168 | 47 |
| M3 | $(GT)_7 \ldots (GTT)_6$ | F: CCAAGAAAGTCGATCTACCA<br>R: ACAATCCTGACAAGCATAAA | 147 | 50 |
| M7 | $(GT)_3 \ldots (GT)_{12}$ | F: GGACCAGACGTGTTGTCATT<br>R: TGATTCCTTCTCCCTTTCTC | 169 | 52 |
| M12 | $(CA)_3 \ldots (CA)_3$ | F: TAATAAGTACTGTGGATAGGC<br>R: CTCCATTGTTATGTCTTTATC | 140 | 50 |
| M113 | $(GT)_8$ | F: TGCAGTCATTTGTTCGTG<br>R: TTGCTTTGTCTCCTATGCTATT | 241 | 52 |
| M114 | $(GA)_{25}(GT)_9$ | F: GATGCTCAAATTCTGCTTTA<br>R: TCTTGGGTTGTTCCTCTT | 224 | 50 |
| M213 | $(GT)_7 \ldots (GT)_3$ | F: AGTTGACATAACCAGGGTG<br>R: CCATACAAACAGACAGCAT | 242 | 52 |
| M247 | $(GT)_7$ | F: ATGCTCCCGCGAACAAA<br>R: CAGCGTCTGGACAAGAAGTGA | 106 | 52 |
| M287 | $(GT)_9$ | F: TTAGACCCAATGAAAATCTG<br>R: TTGAAGTTGAACATAGCCAC | 226 | 50 |

**Table 2.** Statistics of genetic parameters in Guangxi wild population of *P. fucata*.

| | Parameter | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Locus | Sample size | $A$ | $A_e$ | $H_o$ | $H_e$ | PIC | $P_{HW}$ |
| M2 | 32 | 3 | 1.839 | 0.600 | 0.464 | 0.403 | 0.161 |
| M3 | 32 | 7 | 2.041 | 0.300 | 0.519 | 0.488 | 0.006 |
| M7 | 32 | 5 | 3.136 | 0.433 | 0.693 | 0.633 | 0.009 |
| M12 | 32 | 3 | 1.873 | 0.367 | 0.474 | 0.403 | 0.016 |
| M113 | 32 | 2 | 2.000 | 0.333 | 0.509 | 0.375 | 0.055 |
| M114 | 32 | 9 | 3.888 | 0.467 | 0.755 | 0.714 | 0.001 |
| M213 | 32 | 4 | 2.975 | 0.467 | 0.675 | 0.598 | 0.089 |
| M247 | 32 | 8 | 5.863 | 0.567 | 0.844 | 0.809 | 0.042 |
| M287 | 32 | 3 | 1.805 | 0.200 | 0.454 | 0.404 | 0.000 |
| Average | 32 | 4.889 | 2.824 | 0.412 | 0.598 | 0.536 | 0.042 |

$A$, number of alleles; $A_e$, number of effective alleles; $H_o$, observed heterozigosity; $H_e$, expected heterozygosity; PIC, polymorphic information content; $P_{HW}$, $P$ value of significant test on HWE at 0.05 level.

Popgene Gene 1.32 (Yeh and Boyle 1997). The polymorphic information content (PIC) was calculated according to Botstein *et al.* (1980). Nine pairs of primers showed polymorphism as tested by a wild population (table 2). The proportion of polymorphic loci was only 29.0% which was much lower in shellfish than in other fishery animals. One of the possible reasons was the high degree of genetic variation in individuals which might result in a high frequency of the null allele. Thus, primers designed according to one individual may not be amplified in other. An alternative explanation could be that the chromosomal rearrangement during the evolutionary process of the shellfish caused a complex structure of genome (Wang and Guo 2004). In eight loci, the observed heterozygosity was lower than the expected heterozygosity. Only three of the nine loci conformed approximately to HWE (M2, M113 and M213), suggesting the common occurrence of null alleles. In this study, four of the nine loci were highly polymorphic while five were moderately polymorphic. The average PIC was 0.536 which indicates that these microsatellite markers were competent for the research on genetic diversity and construction of genetic map.

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