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Full Length Research Paper

Genetic characteristics of hybrid populations derived by crossing Chinese and Indian pearl oysters, *Pinctada fucata*, based on AFLP markers

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In selective breeding, hybrid populations of pearl oysters *Pinctada fucata* were made by crossing Indian pearl oysters with Chinese ones. Genetic characteristics of 2 hybrid populations, Indian(♀) × Sanya(♂) and Sanya(♀) × Indian(♂) and 2 pure populations, Indian × Indian and Sanya × Sanya, were investigated using AFLP markers. A total of 230 loci were generated by 3 pairs of primer combinations. Indian-Sanya hybrid showed the highest proportion of polymorphic loci (99.6%) and the largest number of dominant, monomorphic loci (38). Gene diversity was 0.422 for total, 0.364 for average, ranging from 0.360 to 0.372. The level of gene diversity in Indian-Sanya (0.372) was significantly higher than those in the other populations ($P < 0.001$). Significant and high levels of overall and pairwise genetic differentiation were observed among the 4 populations as revealed by G_{ST} and AMOVA. These results suggested that both the hybrid and pure populations were characterized by high levels of genetic diversity and genetic differentiation at the beginning of selection, implying that artificial reproduction could have more impact on genetic differentiation than on genetic diversity at the first stage of selective breeding. Yet the high level of genetic divergence among populations suggested that monitoring changes of genetic variation of selected strains is necessary and critical for rational management of brood stocks in selective breeding practice.

Key words: AFLP, gene diversity, genetic differentiation, pearl oyster, hybrid population.

INTRODUCTION

The pearl oyster, *Pinctada fucata* (Gould) is common in south China and is the most important species that is cultured for production of marine pearls (Meng et al., 1996). This species has been cultured over 40 years in China. In recent years severe mortality in culture practice occurred frequently, resulting in great economic losses and threatening aquaculture efforts. Selective breeding has been shown to be effective in improving the performance of many aquaculture species, such as fast-growth and disease-resistance (Sheridan, 1997; Davis and Barber, 1999; Nell et al., 1999; Preston et al., 2004; Zheng et al., 2004; Donato et al., 2005), including the pearl oyster *P. fucata martensii*, which is synonymous with *P. fucata* (Yu and Chu, 2006a; Yu et al., 2006). The traits of growth, shell color and pearl quality have been improved through selective breeding (Wada, 1984, 1986a, b; Wada and Komaru, 1994). Thus developing traits-improved strains of pearl oysters is a major task for both the scientific and aquaculture community.

To breed new strains of the pearl oyster *P. fucata*, a selective breeding project granted by National Hi-Tech Research and Development (863) program has been initiated in 2002 in China. In this project, a strategy of intraspecific hybrid breeding was carried out using an Indian *P. fucata* stock crossing with local pearl oysters in Sanya, Hainan Province, China. The Indian base population was characterized by larger body size but lighter shell weight while Sanya population presented smaller body size but heavier shell weight. The bigger the shell size, the bigger the pearls (Meng et al., 1996) and the heavier the shell weight, the heavier the pearls (Wada and Komaru, 1996). Hence hybridizing Indian and Sanya

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Table 1. Number of dominant (recessive), monomorphic loci (fragment frequency = 100%) (on diagonal) within populations and pairwise shared number of dominant, monomorphic loci (below diagonal) among populations of pearl oyster *P. fucata*.

Population (sample size, n)	Indian pure	Indian♀ × Sanya♂ hybrid	Sanya♀ × Indian♂ hybrid	Sanya pure
Indian pure (44)	21(0)			
Indian♀ × Sanya♂ hybrid (36)	16	38(0)		
Sanya♀ × Indian♂ hybrid (31)	8	13	20(1)	
Sanya pure (49)	12	15	8	26(2)

stocks may combine the 2 desirable traits, large and heavy shells together. Under this hypothesis, 4 F_1 populations, among others, were established, namely, 2 pure populations and 2 hybrid populations. Among them, the Indian (female) × Sanya (male) hybrid population was increased by 6.8 - 20.6% in shell length and 9.9 - 63.5% in shell weight, while Sanya (female) × Indian (male) population increased by 16.3 - 28.0% in shell length and 21.5 - 74.0% in shell weight, as compared with Sanya pure population (for details please see Wang et al, 2005). To provide genetic baseline and to monitor genetic changes in selective breeding in the near future, investigation of genetic characteristics of breeding strains are desirable. In previous studies phenotypic performances were often closely monitored in selective breeding programs but genetic differentiation and genetic diversity among selected strains was sparsely reported (Wada, 1986b; English et al., 2001; Yu and Guo, 2005). The availability of these pure and hybrid pearl oyster populations in the current study provides a good opportunity to study genetic diversity and differentiation among selected strains.

Combining the advantages of the 2 kinds of markers - random amplified polymorphic DNA (RAPD) and restricted fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) have higher resolution and sensitivity than RAPD or RFLP analysis and are powerful in revealing genetic polymorphism and differentiation within and among populations (Barker et al., 1999; Mueller and Wolfenbarger, 1999; Liu and Cordes, 2004; Bensch and Åkesson, 2005). Now AFLP technique has been widely applied in studies of genetic differentiation, genetic diversity and the construction of genome maps in aquaculture species (Seki et al., 1999; Yu and Guo, 2003, 2005; Liu and Cordes, 2004; Wang et al., 2004) including *P. fucata* (Yu et al., 2007; Wang et al., 2007). In the present study, we used AFLP markers to investigate the genetic characteristics of the four *P. fucata* populations mentioned above. The aims of this investigation are to understand the genetic impacts of selective breeding on genetic variation of offspring populations of pearl oysters and to provide a genetic baseline for monitoring changes of genetic variation during the course of selection in the future.

MATERIALS AND METHODS

Animals

2 hybrid and 2 pure populations from Sanya and /or Indian source populations were analyzed in this study. All source populations were from hatchery-produced stocks. The 2 hybrid populations were crosses of Indian (♀) × Sanya (♂) and Sanya (♀) × Indian (♂). The 2 pure populations were Indian × Indian and Sanya × Sanya crosses. The reproduction practice was carried out as described by Meng et al. (1996). The hatchery experiments were conducted in February, 2002 in Sanya Experimental Station (Hainan), South China Sea Fisheries Research Institute. The numbers of parents were five males and ten females for each group, fertilizing by mass spawning. Individuals of one year old offspring were randomly taken from each group and the adductor muscle of each oyster was isolated and preserved in 70 % alcohol. The numbers used for genetic analysis were given in Table 1.

DNA extraction

Total DNA was extracted from the adductor muscle by phenol - chloroform routine method (Sambrook and Russell, 2001). Briefly, tissue (25 mg) was cut into tiny pieces (as small as possible) and then digested by lysis buffer TEN 9 (Tris-Cl 50 mmol/l, pH 9.0 EDTA 100 mmol/l NaCl 200 mmol/l) with addition of 2% SDS and 20 mg/ml proteinase K. DNA was purified by phenol, chloroform and isoamyl alcohol, precipitated by absolute ethanol and sodium acetate (3 mol/l, pH 5.2) and then dissolved in 200 ul ddH_2O after washing twice using 70% ethanol.

AFLP analysis

Procedures of AFLP analysis were conducted as described by Vos et al. (1995) with minor modifications (Yu and Chu, 2006b). Briefly, 100 ng DNA was double digested by 2.5 u *Eco*RI and 2.5 u *Mse*I enzymes (New England BioLabs) in a reaction of 20 µl at 37℃ for 3 h and then at 70℃ for 20 min. The restricted fragments were ligated with the *Eco*RI and *Mse*I adapters by adding 5 µl ligation mix containing 40 u T4 DNA ligase (New England BioLabs) and incubating at 37℃ for 3 h. Pre-selective PCR reaction was performed in a volume of 20 µl containing 1 µl ligased template DNA, 1 × PCR buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l each of dNTPs, 0.17 µl each of *Eco*RI (37 µmol/l) and *Mse*I (33 µmol/L) pre-selective primers and 0.5 u of *Taq*DNA polymerase (Promega). The PCR profile was as follows: 2 min at 94℃, 20 cycles of 30 s at 94℃, 30 s at 53℃ and 1 min at 72℃.

The PCR products were diluted ten folds and subject to selective amplification. Amplifications were carried out in 20 µl reaction with the same components as those in pre-amplification except that DNA templates and primers were replaced with 5.0 µl of diluted pre-

amplified product, 0.5 µl each of *Eco*RI (10 umol/l) and *Mse*I (10 umol/l) selective primers. The selective PCR profile was as follows: 2 min at 94℃, 30 s at 65℃, 1 min at 72℃ 10 cycles of 30 s at 94℃, 30 s at 65 - 56℃ (1 reduction per cycle) and 1 min at 72℃ and 28 cycles of 30 s at 94℃, 30 s at 56℃ and 1 min at 72℃ and a final extension at 72℃ for 5 min. Amplified products were then separated on 5% polyacrylamide gels with a 30 - 330 bp DNA ladder (Invitrogen), and visualized by silver staining.

Multilocus profiles were scored for the presence '1' or absence '0' of fragments and assembled in a binary data matrix table. Only the present bands that could be scored unambiguously by naked eye were analyzed. 3 selective primer pairs (E-AGG/M-CTT, E-AGG/M-CAA and E-AAG/M-CGA) were used after primer screening and all AFLP markers produced were combined into one raw data matrix for analysis.

Data Analysis

Treating the locus without band amplified as recessive homozygous locus, the allelic frequency of the recessive alleles in each locus was estimated using the Bayesian approach proposed by Zhivotovsky (1999) for diploid species, assuming Hardy-Weinberg equilibrium and a non-uniform prior distribution of allelic frequencies among loci. This method can produce nearly unbiased estimates of allelic frequencies for dominant markers (Krauss, 2000). For genetic diversity, we determined the proportion of polymorphic loci at 5% level ($P_{0.05}$), the total gene diversity (H_T, by pooling all individuals together), the within-population gene diversity (h_S) for each population and the average within-population gene diversity (H_S) by using AFLP-SURV 1.0 (Vekemans et al., 2002) based on estimated allelic frequencies. Variance of h_S was partitioned into the variance due to sampling of loci (*Var*L) and the variance due to sampling of individuals (*Var*I). For genetic differentiation, overall and pairwise proportion of genetic differentiation (G_{ST}) and overall genetic difference (D_{ST}) were computed. The significance of G_{ST} was tested by comparing the observed G_{ST} with a distribution of simulated G_{ST} under the null hypothesis of no genetic structure, obtained by conducting 1,000 random permutations of individuals among groups.

In addition, a hierarchical AMOVA (Excoffier et al., 1992) was performed using ARLEQUIN 2.0 (Schneider et al., 2000) to estimate how variation was partitioned within and among populations. The significance of the resultant statistics and variance components was tested with 10,000 random permutations. Besides, number of monomorphic bands (fragment frequency = 100%) was counted based on fragment frequency.

RESULTS

AFLP amplifications

In total, 160 individuals were studied and 230 loci were generated by 3 pairs of selective primers. The primer pairs E-AGG/M-CTT, E-AGG/M-CAA and E-AAG/M-CGA produced 66, 75 and 89 loci, respectively. The length of fragments scored ranged from 80 to 300 base pairs (bp). The number of monomorphic dominant ('1') loci (fragment frequency of '1' = 100%) was much higher in Indian-Sanya hybrid population, while the number of monomorphic recessive ('0') loci was very low, only presenting in Sanya-Indian hybrid and Sanya pure populations (Table 1, on diagonal). The shared monomorphic dominant loci were higher in the 2 pairs of Indian pure and Indian-Sanya hybrid, and Sanya pure and Indian-Sanya hybrid populations than in the other pairs (Table 1, below diagonal). Only 4 monomorphic dominant loci were shared by all populations. Fragment frequency shifts of dominant '1' were observed at about 20 loci among the 4 populations (Table 2).

Genetic variation within and among populations

The proportion of polymorphic loci ($P_{0.05}$) ranged from 96.5% (Sanya pure) to 99.6% (Indian-Sanya hybrid) (Table 3). The total gene diversity (H_T) was 0.422, and the average within-population gene diversity (H_S) was 0.364 ± 0.003. The gene diversity within each population (h_s) varied from 0.360 to 0.372 (Table 3). Among the four populations, Indian-Sanya hybrid population has a significant higher gene diversity ($t = 2.9$, $P < 0.001$). The variance attributed to difference among individuals (*Var*I) was higher in hybrid populations than that in pure populations (Table 3). Sanya pure population had the least *Var*I. In general, the variance due to sampling of loci (*Var*L) was larger that that due to sampling of individuals (*Var*L).

Genetic differentiation among populations

The overall genetic difference (D_{ST}) among the 4 populations was 0.059 ± 0.005. The overall proportion of genetic differentiation (G_{ST}) was 0.139 ± 0.078, significantly higher than values obtained after 10,000 random permutations at 1% level. The pairwise G_{ST} values ranged from 0.099 to 0.173 (Table 4, below diagonal) and they were also significant as indicated by permutation test at 1% level.

The analysis of molecular variance (AMOVA) demonstrated that 19.9% of the total genetic variance was attributed to difference among populations on average while 80.1% due to difference within populations. The overall fixation index ($\phi_{ST} = 0.199$) and pairwise ϕ_{ST} (0.156 - 0.249) (Table 4, above diagonal) were observed significant ($P < 0.0001$) among the four or in each pair of populations based on 10,000 permutations.

DISCUSSION

In selective breeding, the most concerns for hatchery-propagated populations are inbreeding and loss of genetic variation (Hedgecock and Sly, 1990; Gaffney et al., 1992; Hedgecock et al., 1992). In contrast high genetic diversity was detected for both hybrid and pure populations in the present study. A possible explanation may be that the selective breeding in this study is just in its early stage. In details, genetic variation attributed to hybrid individuals was higher than that attributed to individuals within pure populations as indicated by *Var*I. One of the hybrid populations, Indian-Sanya, had the highest *Var*I value as well as proportion of polymorphic

Table 2. Fragment frequency shift of dominant '1' at some loci among the four populations of pearl oyster *P. fucata*

Loci	Indian pure	Indian-Sanya hybrid	Sanya-Indian hybrid	Sanya pure
L020	0.39	1.00	0.23	0.59
L022	0.73	1.00	0.19	0.84
L023	0.64	1.00	0.19	0.84
L025	0.64	1.00	0.19	0.51
L028	0.32	0.56	0.00	0.41
L042	0.09	0.83	0.16	0.31
L047	0.09	0.50	0.13	0.47
L049	0.27	0.81	0.19	0.00
L070	0.50	0.78	0.13	0.06
L072	0.98	0.97	1.00	0.04
L075	0.68	1.00	0.84	0.04
L087	0.55	0.64	0.32	0.00
L123	0.61	0.89	0.16	0.14
L124	0.61	0.86	0.06	0.14
L137	0.32	0.47	0.61	0.02
L141	0.50	0.44	0.10	0.10
L152	0.02	0.69	0.45	0.16
L167	0.07	0.11	0.42	0.84
L181	0.57	0.78	0.13	0.65
L198	0.18	0.86	0.13	0.43

Table 3. Genetic diversity of Indian, Sanya and their hybrids populations of pearl oyster *P. fucata*.

Population (n)	$P_{0.5}$ (%)[1]	$h_S \pm$ S.E.	*Var*I (%)[2]	*Var*L (%)[3]
Indian pure (44)	97.8	0.360 ± 0.008	19.6	80.4
Indian♀ × Sanya♂(36)	99.6	0.372 ± 0.008	25.6	74.4
Sanya♀ × Indian♂(31)	97.0	0.361 ± 0.008	22.7	77.3
Sanya pure (49)	96.5	0.361 ± 0.009	12.4	87.6

[1] Percentage of polymorphism at 5% level
[2] Variance due to sampling of individuals
[3] Variance due to sampling of loci

Table 4. The observed pairwise proportion of genetic differentiation (*G*ST, below diagonal) and pairwise fixation index (ϕST) (above diagonal, as indicated by AMOVA) of *P. fucata*

Population	Indian pure	Indian×Sanya	Sanya×Indian	Sanya pure
Indian pure	-	0.176†	0.156†	0.185†
Indian♀ × Sanya♂	0.129*	-	0.249†	0.243†
Sanya♀ × Indian♂	0.099*	0.163*	-	0.190†
Sanya pure	0.131*	0.173*	0.134*	-

* significant at 1% level based on 1000 random permutation tests
$P < 0.0001$

loci. Furthermore, gene diversity (h_S) in Indian-Sanya hybrid population was significantly higher than that in pure populations. The growth performance of the 2 hybrid populations has also been improved (Unpublished data). High level of genetic diversity is beneficial for selective breeding as lowered genetic variability will limit response to selection and may lead to lowering of fitness (Amos and Harwood, 1998; Amos and Balmford, 2001). Thus

the hybrid populations produced in this study are useful for the selective breeding program. No significant reduction in genetic diversity was also reported in cultured populations or selected strains of pearl oyster *P. fucata martensii* (= *P. fucata*) (Wada, 1986b) as well as the other bivalves when compared with the corresponding wild populations in previous studies (Gosling, 1982; Hedgecock and Sly, 1990; Durand et al., 1993; Mgaya et al., 1995; Benzie and Williams, 1996; Gaffney et al., 1996; English et al., 2001; Yu and Guo, 2005). Even higher heterozygosity (Li et al., 1985) and genetic diversity (Yu and Chu, 2006c) in cultured *P. fucata* populations were observed than the wild populations. High genetic diversity was also observed in the first generation of 3 families constructed by Indian, Sanya and Indian × Sanya pearl oyster parents, respectively (Wang et al., 2007). Yet it is necessary to monitor the changes of genetic diversity in next generations since it was observed that there were 17 and 18% of the alleles in 18 protein loci disappeared in 3 successive generations of hatchery black pearl oyster, *P. margaritifera* in Japan (Durand et al., 1993).

In selective breeding of aquatic organisms, more and more attentions are being paid to genetic differentiation among selected strains (Yu and Guo, 2005). In this study, significant genetic differentiation among the 4 populations was observed as revealed by both G_{ST} and AMOVA. Overall genetic differentiation among populations accounts for 13.9% (G_{ST}) or 19.9% (AMOVA) of the total variation, ranging from 9.9 - 17.3% (G_{ST}) or 15.6 - 24.9% (AMOVA). Values of genetic differentiation between Indian and hybrid populations were slightly lower than the other pairs, respectively. Similar levels of divergence were also observed in selected strain of *C. virginica* ($ø_{PT}$ for AFLP: 6.2 - 25.5%, Yu and Guo, 2005). In the present study, marked shifts of fragment frequencies can be found at about 20 loci (out of 230 loci) in hybrid populations as compared with pure populations. In the eastern oyster *C. virginica*, four selected strains also showed frequency shifts at some loci as detected by microsatellite and AFLP markers (Yu and Guo, 2005). Thus, monitoring genetic divergence among selected strains is very important for rational management of broodstocks in selective breeding program.

To conclude, both the hybrid and pure populations are characterized by high levels of genetic diversity and genetic differentiation at the beginning of selection. Using small number of broods has more impact on genetic differentiation than on genetic diversity for pearl oysters at the beginning of selective breeding. Yet this is just a starting point to study on changes of genetic variation of pearl oysters in selective breeding. It needs to further investigate in successive generations. This study just provides a baseline for monitoring the genetic changes in selection thereafter.

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