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Genetic Variation Assessed with Microsatellites in Mass Selection Lines of the Pacific Oyster (*Crassostrea gigas*) in China

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Abstract Four successive mass selection lines of the Pacific oyster, *Crassostrea gigas*, selected for faster growth in breeding programs in China were examined at ten polymorphic microsatellite loci to assess the level of allelic diversity and estimate the effective population size. These data were compared with those of their base population. The results showed that the genetic variation of the four generations were maintained at high levels with an average allelic richness of 18.8–20.6, and a mean expected heterozygosity of 0.902–0.921. They were not reduced compared with those of their base population. Estimated effective population sizes based on temporal variances in microsatellite frequencies were smaller to that of sex ratio-corrected broodstock count estimates. Using a relatively large number of broodstock and keeping an equal sex ratio in the broodstock each generation may have contributed to retaining the original genetic diversity and maintaining relatively large effective population size. The results obtained in this study showed that the genetic variation was not affected greatly by mass selection progress and high genetic variation still existed in the mass selection lines, suggesting that there is still potential for increasing the gains in future generations of *C. gigas*. The present study provided important information for future genetic improvement by selective breeding, and for the design of suitable management guidelines for genetic breeding of *C. gigas*.

Key words Pacific oyster; genetic variation; microsatellite; mass selection line; effective population size

1 Introduction

In aquaculture breeding programs, a challenging problem is how to avoid the loss of genetic diversity over ensuing generations, particularly for fecund aquaculture organisms where relatively few animals can form the contributing parents of the next generation. Across time, the decrease in diversity is expected within breeding programs as the selection intensity and inbreeding need to be balanced (Bentsen and Olesen, 2002). Loss of genetic variation can be accompanied by inbreeding depression, as well as concordant loss of performance and fitness (Waples, 1991; Ferguson *et al*., 1995; Evans *et al*., 2004). In addition, a sufficient level of genetic variability is essential to maintain a sustained response from long-term selection for economically important traits. Therefore, the monitoring and maintenance of genetic diversity is crucial during the implementation of genetic improvement programs.

Native in Northeast Asia, the Pacific oyster (*Crassostrea gigas*) has now been introduced massively into many parts of the world. The ability of tolerating a variety of different salinities and temperatures, in conjunction with high fecundity, has made the Pacific oyster a commercially important aquaculture species worldwide. In 2013, the global production of this species was 555994 metric tons valued at more than US $ 1 billion (FAO, 2015). In many countries including China, the Pacific oyster industry is mainly hatchery-based, which means that it is in a position to benefit from the development of genetically improved strains. During the past decade, selection programs for the Pacific oyster have been strikingly successful, particularly in improving growth rates (Langdon *et al*., 2003; Ward *et al*., 2005; Li *et al*., 2011). A selective breeding program for the Pacific oyster was initiated in 2007 in China (Li *et al*., 2011), which has concentrated on the establishment of selected lines by mass selection for faster growth. However, there is few investigation on whether there is a decrease of genetic diversity in the mass selection lines (Jiang *et al*., 2013). The effective population size (*Ne*) is a crucial parameter for determining the extent of genetic diversity in a population (Lande and Barrowclough, 1987). During the artificial selection processes, changes in *Ne* need to be evaluated to predict the accumulation of inbreeding and assess the potential of future genetic improvement in the population. It is concerned whether the effective population size may have decreased within the mass selection lines.

In current study, to evaluate the impact of mass selec-

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tion process on the genetic properties in successive selection strains, ten polymorphic microsatellites were used to examine the genetic variation among one base population and four successive mass selection lines of *C. gigas*.

2 Materials and Methods

2.1 Sample Collections

In 2007, two-year-old Pacific oysters from one base population (G0) in Rushan, Shandong Province, China (36.4˚N, 121.3˚E), were used to establish the first-generation selection line (G1) for fast growth. The oysters derived from natural seed were collected on local coast, and were cultured on ropes suspended from rafts along the coastal regions. In 2008–2010, the second-generation (G2), third-generation (G3) and fourth-generation (G4) selective lines were constructed, respectively. In each generation, males and females were separated. Gametes were then rinsed into separate buckets by stripping the gonads. Rearing of the larvae, spats and adults was carried out using standard practices.

The number of the parents used to produce the four mass generation lines was summarized in Table 1. We genotyped oysters taken randomly from the base population and four generations of oysters mass-selected for growth rate (Table 1).

Table 1 Sample sizes of cultured mass selection lines of *C. gigas*

Population	Number of parents	N_f	N_m	Date of sampling	Sample size
Base population (G0)	–	–	–	01/2007	50
First-generation selected line (G1)	123	63	60	10/2008	48
Second-generation selected line (G2)	61	31	30	10/2009	55
Third-generation selected line (G3)	90	40	50	10/2010	59
Fourth-generation selected line (G4)	71	42	35	10/2011	60

Notes: N_f, number of female broodstock; N_m, number of female broodstock.

2.2 DNA Extraction and Microsatellite Analysis

Genomic DNA was extracted from frozen adductor muscles according to a modified version of the standard phenol-chloroform procedure described by Li *et al.* (2006). After extraction, the DNA samples were preserved in TE buffer at −30℃. Ten microsatellite loci *ucdCg129*, *ucdCg130*, *ucdCg134*, *ucdCg138*, *ucdCg148*, *ucdCg149*, *ucdCg151*, *ucdCg160*, *ucdcg198* and *ucdCg200* (Li *et al*., 2003) were selected for polymerase chain reaction analysis according to the conditions described therein. Amplification products were resolved *via* 6% denaturing polyacrylamide gel, and visualized by silver-staining. A 10-bp DNA ladder (Invitrogen, USA) was used as a reference for allele size determination. To avoid any inaccuracy in scoring due to differences in gels, a control DNA sample was included in each set of samples for each gel.

2.3 Genetic Variability Analysis

MICROCHECKER v.2.2.3 (Van Oosterhout *et al*., 2004) was first used to examine null alleles with the Oosterhout algorithm. As the analysis with MICROCHECKER indicated strong evidence of null alleles at all loci, the null allele frequencies and allele frequencies for each locus and population were estimated by the expectation maximization algorithm (Dempster *et al*., 1977) implemented in the software FreeNA (Chapuis and Estoup, 2007). To minimize the bias of the genetic diversity statistics induced by null alleles, the estimated false homozygous genotypes XX caused by null alleles were systematically changed to X999 (Chapuis and Estoup, 2007; Sun *et al*., 2012). Further analysis of data used both the adjusted allele frequency and raw data to assess the effect of null alleles on results. Number of alleles per locus (N), the expected and observed heterozygosities (H_e and H_o, respectively) were calculated using MICROSATELLITE ANALYSER v. 4.0 (Dieringer and Schlötterer, 2003). The inbreeding coefficiency (F_{is}; (Weir and Cockerham, 1984)) for each population and allelic richness (R_S) were calculated by FSTAT v. 2.9.3 (Goudet, 2001). A nonparametric analysis of variance (Kruskal-Wallis test) was performed to test the differences of the average number of alleles and mean H_e among populations. Deviations from Hardy-Weinberg equilibrium (Fisher's exact test) for each locus and linkage disequilibrium within populations were tested using GENEPOP v. 4.0 (Raymond and Rousset, 1995; Rousset, 2008). Significance levels in GENEPOP were determined after 500 batches of 5000 iterations.

To detect the genetic differentiation among different generations, a modified statistic F_T analogue to F_{ST} was used, as suggested by Sandoval-Castellanos (2010). The F_T was calculated as

$$F_T = F_{ST} - \overline{F}^S,$$

where $\overline{F}^S$ was the mean of gene drift, which was the average of F_{ST} among successive generations. Following Chapuis and Estoup (2007), we used the *ENA* (excluding null alleles) correction method to efficiently correct the positive bias induced by the presence of null alleles on F_{ST} estimation. $F_{ST}^{\{ENA\}}$ values were calculated using the software FreeNA. In calculating F_T, the value of F_{ST} was replaced by the value of $F_{ST}^{\{ENA\}}$. For the analysis of molecular variance (AMOVA; Excoffier, Smouse and Quattro 1992), components of variance were estimated using ARLEQUIN v. 3.5 (Excoffier and Lischer, 2010). The total variance was divided into variance among groups (G0 *vs* the four mass-selected populations), variance among populations within groups, and variance within populations.

In all cases with multiple tests, corrections of the significance level were performed following the sequential Bonferroni procedure (Rice, 1989).

2.4 Effective Population Size

The effective breeding size of each generation, N_e, was

estimated from the numbers of males and females used to produce that generation

$$N_e = 4N_m N_f /(N_m + N_f)$$ (Falconer *et al*., 1996).

In addition, the method based on temporal variances in allele frequencies was particularly suited in aquaculture broodstock to estimate the effective breeding size (Pollak, 1983; Waples, 1989). It was also used to estimate the effective breeding size (N_{em}). Briefly, the adjusted F_k values (F_a) of the sample size were used to compare allele frequency varies between samples, which was calculated using a formula

$$F_a = F_k - 1/S,$$

where S was the harmonic mean of sample size in two populations. Given the presence of null alleles, the frequencies of alleles were first adjusted using software FreeNA. Then N_{em} was calculated as

$$N_{em} \approx t/[2(F_k - 1/S)],$$

where F_k was the standardized variance of the allele frequency change, and t was the number of generations between generations. G0 population was the founder generation at time $t=0$. Allele frequencies in the G1 population were estimated at time $t=1$, in the G2 population at time $t=2$, in the G3 population at time $t=3$ and in the G4 population at time $t=4$. The 95% confidence intervals were calculated using χ^2 approximation (Waples, 1989).

3 Results

3.1 Genetic Variability

The number of alleles (N), the observed (H_o) and expected (H_e) heterozygosities calculated by raw data and corrected data for the ten microsatellite loci were summarized in Table 2. The ten microsatellite loci were all highly polymorphic, while the degree of variability was different at each locus (Kruskal-Wallis test, $df=9$, $P<0.01$), *ucdCg149* had the highest number of alleles in the G1 population, while the maximum number of alleles at *ucdCg129*, *ucdCg130*, *ucdCg134*, *ucdCg138*, *ucdCg148*, *ucdcg149*, *ucdCg151*, *ucdCg160*, *ucdCg198*, *ucdCg200*

Table 2 Genetic diversity measures estimated using ten microsatellites in the selected trains of *C. gigas*

Loci	Parameter	G0	G1	G2	G3	G4
	N	23	21	19	19	17
	R_s	22.3	20.5	18.8	19	16.9
	${}^{R}H_o/{}^{C}H_o$	0.778/0.889	0.532/0.936	0.527/0.891	0.643/0.911	0.569/0.845
ucdCg129	${}^{R}H_e/{}^{C}H_e$	0.955/0.957	0.921/0.914	0.930/0.925	0.914/0.926	0.889/0.905
	F(null)	0.086	0.202	0.207	0.134	0.172
	${}^{R}F_{is}/{}^{C}F_{is}$	0.188/0.061	0.425/−0.036	0.436/0.028	0.298/0.008	0.362/0.058
	${}^{R}P/{}^{C}P$	0.000*/0.438	0.000*/0.152	0.000*/0.217	0.000*/0.225	0.000*/0.119
	N	13	14	16	14	16
	R_s	12.7	13.9	15.8	20	16
	${}^{R}H_o/{}^{C}H_o$	0.643/0.905	0.362/0.915	0.481/0.926	0.737/0.912	0.722/0.870
ucdCg130	${}^{R}H_e/{}^{C}H_e$	0.908/0.921	0.910/0.878	0.907/0.901	0.911/0.912	0.927/0.935
	F(null)	0.131	0.284	0.218	0.092	0.101
	${}^{R}F_{is}/{}^{C}F_{is}$	0.295/0.006	0.605/−0.054	0.471/−0.037	0.192/0.000	0.222/0.060
	${}^{R}P/{}^{C}P$	0.013/0.397	0.000*/0.144	0.000*/0.062	0.001*/0.166	0.000*/0.129
	N	22	18	20	23	21
	R_s	20.3	17.7	19.6	22.7	20.5
	${}^{R}H_o/{}^{C}H_o$	0.750/0.886	0.574/0.915	0.782/0.873	0.780/0.898	0.864/0.898
ucdCg134	${}^{R}H_e/{}^{C}H_e$	0.940/0.946	0.919/0.922	0.929/0.936	0.938/0.943	0.924/0.927
	F(null)	0.09	0.177	0.068	0.078	0.013
	${}^{R}F_{is}/{}^{C}F_{is}$	0.204/0.052	0.377/−0.003	0.160/0.059	0.170/0.039	0.065/0.022
	${}^{R}P/{}^{C}P$	0.000*/0.069	0.000*/0.243	0.000*/0.204	0.001*/0.021	0.060/0.124
	N	25	19	26	27	29
	R_s	25	18.5	25.5	26.8	28.6
	${}^{R}H_o/{}^{C}H_o$	0.806/0.889	0.667/0.938	0.836/0.836	0.603/0.914	0.912/0.912
ucdCg138	${}^{R}H_e/{}^{C}H_e$	0.941/0.943	0.934/0.938	0.939/0.939	0.954/0.948	0.956/0.956
	F(null)	0.065	0.132	0.047	0.175	0.021
	${}^{R}F_{is}/{}^{C}F_{is}$	0.145/0.044	0.288/−0.010	0.110/0.101	0.369/0.028	0.046/0.037
	${}^{R}P/{}^{C}P$	0.020/0.352	0.000*/0.074	0.229/0.288	0.000*/0.228	0.032/0.088
	N	26	27	26	23	29
	R_s	25.5	26	25.7	22.9	29
	${}^{R}H_o/{}^{C}H_o$	0.625/0.700	0.708/0.833	0.667/0.852	0.421/0.965	0.685/0.907
ucdCg148	${}^{R}H_e/{}^{C}H_e$	0.960/0.961	0.956/0.958	0.947/0.953	0.939/0.897	0.944/0.948
	F(null)	0.169	0.125	0.141	0.266	0.128
	${}^{R}F_{is}/{}^{C}F_{is}$	0.352/0.263	0.261/0.121	0.298/0.097	0.554/−0.085	0.276/0.034
	${}^{R}P/{}^{C}P$	0.000*/0.081	0.000*/0.117	0.000*/0.267	0.000*/0.163	0.000*/0.189

(*to be continued*)

(*continued*)

Loci	Parameter	G0	G1	G2	G3	G4
	N	27	34	31	24	23
	R_s	25.7	32.8	30.8	24	22.5
	${}^{R}H_o/{}^{C}H_o$	0.659/0.850	0.809/0.875	0.731/0.944	0.607/0.946	0.561/0.930
ucdCg149	${}^{R}H_e/{}^{C}H_e$	0.961/0.953	0.967/0.956	0.956/0.944	0.945/0.940	0.889/0.906
	F(null)	0.153	0.076	0.112	0.168	0.164
	${}^{R}F_{is}/{}^{C}F_{is}$	0.317/0.097	0.165/0.075	0.238/−0.010	0.359/−0.016	0.371/−0.035
	${}^{R}P/{}^{C}P$	0.000*/0.055	0.000*/0.208	0.000*/0.259	0.000*/0.124	0.000*/0.189
	N	16	16	15	12	15
	R_s	15.5	16	14.8	11.9	14.8
	${}^{R}H_o/{}^{C}H_o$	0.913/0.913	0.929/0.929	0.796/0.796	0.864/0.864	0.917/0.917
ucdCg151	${}^{R}H_e/{}^{C}H_e$	0.920/0.920	0.899/0.899	0.887/0.887	0.892/0.892	0.903/0.903
	F(null)	0.004	0.009	0.036	0.004	0.008
	${}^{R}F_{is}/{}^{C}F_{is}$	0.007/−0.004	−0.033/−0.045	0.103/0.093	0.031/0.023	−0.015/−0.024
	${}^{R}P/{}^{C}p$	0.137/0.138	0.533/0.488	0.019/0.083	0.632/0.663	0.760/0.723
	N	28	29	25	23	22
	R_s	26.1	27.7	24.8	22.9	21.5
	${}^{R}H_o/{}^{C}H_o$	0.511/0.936	0.208/0.958	0.396/0.925	0.431/0.966	0.603/0.948
ucdCg160	${}^{R}H_e/{}^{C}H_e$	0.960/0.931	0.945/0.847	0.930/0.901	0.928/0.894	0.872/0.900
	F(null)	0.224	0.375	0.274	0.256	0.138
	${}^{R}F_{is}/{}^{C}F_{is}$	0.471/−0.016	0.781/−0.143	0.576/−0.036	0.538/−0.090	0.310/−0.063
	${}^{R}P/{}^{C}P$	0.000*/0.254	0.000*/0.170	0.000*/0.003*	0.000*/0.305	0.000*/0.000*
	N	16	14	16	11	13
	R_s	14.5	13.5	16	11	12.9
	${}^{R}H_o/{}^{C}H_o$	0.700/0.940	0.625/0.979	0.608/0.922	0.898/0.898	0.862/0.862
ucdCg198	${}^{R}H_e/{}^{C}H_e$	0.889/0.912	0.882/0.894	0.908/0.901	0.875/0.875	0.885/0.882
	F(null)	0.086	0.135	0.153	0.003	0.004
	${}^{R}F_{is}/{}^{C}F_{is}$	0.214/−0.041	0.294/−0.053	0.332/−0.017	-0.027/−0.035	0.026/0.014
	${}^{R}P/{}^{C}P$	0.034/0.288	0.000*/0.104	0.000*/0.216	0.437/0.428	0.868/0.763
	N	13	11	14	13	9
	R_s	11.8	11	13.8	12.8	9
	${}^{R}H_o/{}^{C}H_o$	0.796/0.878	0.479/0.854	0.582/0.909	0.542/0.949	0.586/0.845
ucdCg200	${}^{R}H_e/{}^{C}H_e$	0.856/0.869	0.875/0.889	0.867/0.886	0.858/0.878	0.829/0.852
	F(null)	0.009	0.2119	0.144	0.163	0.129
	${}^{R}F_{is}/{}^{C}F_{is}$	0.071/−0.021	0.455/0.029	0.331/−0.035	0.370/-0.091	0.294/0.068
	${}^{R}P/{}^{C}P$	0.057/0.366	0.000*/0.058	0.000*/0.194	0.000*/0.000*	0.000*/0.083
	N	20.9	20.3	20.8	18.9	19.4
	R_s	19.9	19.8	20.6	18.8	19.2
Mean of all loci	${}^{R}H_o/{}^{C}H_o$	0.718/0.888	0.589/0.923	0.641/0.898	0.653/0.926	0.728/0.893
	${}^{R}H_e/{}^{C}H_e$	0.929/0.931	0.921/0.910	0.920/0.919	0.915/0.903	0.902/0.911
	${}^{R}F_{is}/{}^{C}F_{is}$	0.226/0.034	0.362/−0.028	0.306/0.014	0.285/−0.026	0.196/0.010

Notes: N, number of alleles; R_S, allelic richness; ${}^{R}H_o$, heterozygosity observed calculated by raw data; ${}^{C}H_o$, heterozygosity observed calculated by corrected data; ${}^{R}H_e$, heterozygosity expected calculated by raw data; ${}^{C}H_e$, heterozygosity expected calculated by corrected data; *F(null)*, frequency of null allele; ${}^{R}F_{is}$, inbreeding coefficient calculated by raw data; ${}^{C}F_{is}$, inbreeding coefficient calculated by corrected data; ${}^{R}P$, P value for testing HWE using raw data; ${}^{C}P$, P value for testing HWE using corrected data. Table-wide significance levels were applied using the sequential Bonferroni technique (Rice, 1989; $k=10$). * Significant at $P<0.05$.

were 23, 16, 23, 29, 29, 34, 16, 29, 16, 14, respectively. Average numbers of alleles per locus ranged from 18.9 in the G3 population to 20.9 in the G0 population. There was no significant difference in the average numbers of alleles among the 5 populations (Kruskal-Wallis test, $df=4$, $P=0.406$). Average allelic richness ranged from 18.8 in G3 population to 20.6 in the G2 population. The frequencies of null alleles per locus per population ranged from 0.000 to 0.375 (only in one case was higher than 0.3, Table 2).

Allele frequency distribution at ten microsatellites in each population are shown in Fig.1. A total of 316 alleles were detected in the five populations, ranging from 19 for locus *ucdCg130* to 45 for locus *ucdCg160*. In the five populations, most of the alleles were at low frequencies (< 0.05). Contingency table analysis showed that there were no difference among generations (Pearson's chi-square test, $P>0.05$).

The observed genotype frequencies were tested for agreement with Hardy-Weinberg equilibrium (HWE). Significant departures from HWE were observed in 36 of the 50 single-locus tests after an adjustment of the P values across the ten loci using the sequential Bonferroni method. There was a strong overall heterozygote deficit, with an average F_{is} ranging from 0.196 to 0.362. The average expected heterozygosity (H_e) was relatively con-

stant ranging from 0.902 to 0.929. There was no significant difference in the average expected heterozygosity among the 5 populations (Kruskal-Wallis test, df=4, P= 0.406). The average observed heterozygosity was relatively low compared to average H_e, ranging from 0.589 to 0.728. No linkage disequilibrium between pair of loci within each generation was detected (275 tests). After correcting the data set for null alleles, the average observed and expected heterozygosity values were higher than the raw data ranging from 0.888 to 0.926 and 0.903 to 0.931, respectively (Table 2); No significant heterozygote deficiency was detected, with the corrected average F_{is} values ranging from −0.028 to 0.034, which was much lower than the values calculated with raw data. In the 50 locus-population combinations, there were only 3 cases deviating from HWE due to heterozygote excess (G2 at *ucdcg160*, G4 at *ucdcg160* and G3 at *ucdcg200*). This phenomenon suggested that the presence of null allele is one of the main causes for departure from HWE.

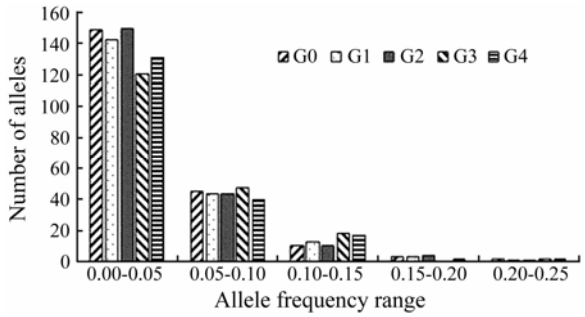

Fig.1 Allele frequency distribution at ten microsatellite loci in each population.

3.2 Genetic Differentiation Among Five Populations

Matrices of pairwise F_T are given in Table 3. The values are all highly significant from zero (P<0.005 after Bonferroni correction). The pairwise F_T values (0.0114–0.0230) among five populations were low (F_T < 0.05). Analysis of molecular variances (AMOVA) of microsatellites revealed that variation within populations, among populations within groups and among groups were 98.3%, 1.83%, and −0.13%, respectively (Table 4).

Table 3 F_T estimation of temporal genetic differentiation during the closed breeding of *C. gigas*

Population	G0	G1	G2	G3
G1	0.0114			
G2	0.0131	0.0131		
G3	0.0152	0.0155	0.0131	
G4	0.0181	0.0199	0.0230	0.0149

Table 4 Analysis of molecular variances (AMOVA) of microsatellites among the five populations

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation
Among groups	1	6.905	−0.00333	−0.13
Among populations within groups	3	23.575	0.04777	1.83
Within populations	539	1384.235	2.56815	98.3
Total	543	1414.715	2.6126	

3.3 Effective Population Sizes

All populations showed that the estimated effective numbers of broodstock corrected for sex ratio (N_e) was lower than the actual numbers of parents, indicating that the biologically effective number of broodstock is generally less than the actual number of spawned individuals. The temporal estimated effective population size (N_{em}) of G1-G4 populations was 27.3%, 20.5%, 38.5%, 16.6%, lower than N_e from G1-G4, respectively (Table 5).

Table 5 The effective population size in the selected strains of *C. gigas*

Population	F_k	t	N_{em}	N_e
G0-G1	0.02771	1	89.3 (60.4–138.9)	122.9
G0-G2	0.04144	2	48.5 (34.8–70.0)	61.0
G0-G3	0.04756	3	54.7 (40.7–74.8)	88.9
G0-G4	0.04391	4	63.7 (46.1–89.3)	76.4

Notes: F_k, standardized variance of allele frequency change; t, generation between samples; N_{em}, temporal estimates of effective population size (95% CI given in parenthesis); N_e, estimated effective numbers of broodstock corrected for sex ratio.

4 Discussion

The main objective of this study was to use classical population genetics to set up appropriate guidelines for the proper monitoring and management of the stocks for the implementation of selective breeding programs for *C. gigas* in China. Loss of genetic variation has been well documented in aquatic organisms under selection of closed populations (Appleyard and Ward, 2006; Loughnan *et al*., 2013; Vela Avitúa *et al*., 2013; Knibb *et al*., 2014; In *et al*., 2016). Genetic variation is related to the sustainability of selective breeding programs, while allelic diversity and heterozygosity are both measures of genetic variation. To achieve accurate measure of these parameters is paramount for monitoring the genetic variation of the selected lines. In this study, mean allelic richness and expected heterozygosity within populations ranged from 18.8 to 20.6, and from 0.902 to 0.929, with an average of 19.7 and 0.917, respectively. Compared with the base population, expected heterozygosity in the G4 population decreased 2.9%, which was much lower than that (17.4%) observed by Appleyard and Ward (2006).

All the populations analyzed showed disagreement with Hardy-Weinberg equilibrium, as shown by significant difference between expected and observed heterozygosities. However, contrary results were obtained for the corrected genotype data set. After correcting the data set for null alleles, significant heterozygote deficiency could not be detected. In this study, according to the estimate of the null allele frequencies following the EM algorithm, heterozygote deficiency caused by null alleles may be the main factor contributing to the departure from HWE. Actually, null alleles have been known to occur in oysters (Magoulas *et al*., 1998; McGoldrick *et al*., 2000; Li *et al*., 2003; Hedgecock *et al*., 2004). As

Hedgecock *et al*. (2004) reported, 51% of microsatellite markers in three surveyed families showed evidence of null alleles. In principle, other causes for deviation from HWE could be genotyping errors, sampling effects and inbreeding. However, these could contribute little to the deviations, as the base population also showed significant differences in H_o (0.718) and H_e (0.929) in this case.

The estimated effective population size based on temporal variances in allele frequencies (N_{em}) was smaller than that corrected for sex ratio (N_e). A number of studies on oysters have shown that the effective size of a population is generally smaller than the number of spawned individuals (Hedgecock and Sly, 1990; Boudry *et al*., 2002). The discrepancy can be explained by large variances in reproductive success, which can be detected by varying number of offspring of their parents (Hedgecock *et al*., 1992). The N_{em} obtained in this study (49–89) is similar to that (N_e: 44–90) in the white shrimp *Penaeus vannamei* from a mass selection over 12 generations (De Donato *et al*., 2005), but higher than that reported in mass selection programs for other farmed aquatic species such as Gilthead seabream *Sparus aurata* (N_e: 14–18) (Brown *et al*., 2005), and Barramundi *Lates calcarifer* (N_e: 10–17) (Loughnan *et al*., 2013). Appleyard and Ward (2006) also used temporal variance in microsatellite frequencies to estimate the effective population in mass selection program for *C. gigas*, and found that N_{em} (11–22) was one half of that corrected using sex ratio (21–42).

N_e can be depressed due to a variety of factors, including small initial population size, unequal sex ratio, and variance in family size (Franklin, 1980). The relatively large N_{em} obtained here may be attributed to relatively large number of parents, nearly equal sex ratio, gamete contribution, and no culling of small individuals during larval and grow-out stages in each generation. By computer simulations, it was concluded that increasing the number of broodstock from 4 to 50 pairs or more could decrease the rate of inbreeding from 6%–8% to about 1% per generation if the sex ratio is maintained 1:1 (Bentsen and Olesen, 2002). A minimum population size (*i.e*., 50–100) could constrain inbreeding 1% per generation in selection programs (Bijma *et al*., 2000; Ponzoni *et al*., 2010). The N_e of the G1–G4 populations (61.0–122.9) falls within what has been indicated as minimum population sizes to constrain inbreeding, suggesting that the mass-selected populations are not currently experiencing inbreeding depression.

In summary, despite four successive generations of mass selection for fast growth in *C. gigas*, the high genetic variability was still maintained through the generations in the breeding program. Estimated effective population sizes based on temporal variances in microsatellite frequencies were smaller than those of sex ratio corrected broodstock count estimates. Using a relatively large number of broodstock and keeping an equal sex ratio of the broodstock used in each generation may have contributed to retaining the original genetic diversity of the selected lines and maintaining relatively large effective population size. The results obtained in this study provided important information for future genetic improvement by selective breeding and for the design of suitable management guidelines for genetic breeding of *C. gigas*.

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