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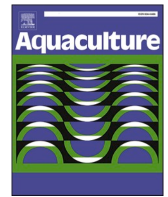

Genetic diversity in a genetically improved line of the Pacific oyster *Crassostrea gigas* with orange shell based on microsatellites and mtDNA data

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ABSTRACT

The shell color is of interest to the shellfish industry and has been regarded as a target trait for a better commercial value. Based on an inbred variant line of the Pacific oyster *Crassostrea gigas* with orange shell obtained previously, a genetically improved orange-shell line was formed through hybridization and subsequent mass selection. In the present study, the genetic diversity among the base population (G0) and two successive mass-selected generations of the improved orange-shell line of *C. gigas* (G1 and G2) were assessed by both 15 microsatellites and mitochondrial cytochrome oxidase I sequences (mtCOI) compared to four outbred populations and three progenitor selected strains. The microsatellite results showed that the genetic variation over two generations of mass selection were maintained since no significant reduction in average number of alleles (N_α: 4.87–5.40), expected heterozygosity (H_e: 0.59–0.61) and expected heterozygosity (H_o: 0.48–0.54) compared with those of G0. The number of different alleles in the generations of improved orange-shell line ranged from 73 to 81 alleles, which was approximate 55–64% less than the 176–204 alleles found in the oysters from wild populations. The N_α in G0-G2 were obviously higher than that of the inbred orange-shell line without improvement. The differences in alleles in the improved orange-shell line compared to four wild populations is likely due to small broodstock size resulting in genetic drift over successive generations. Effective population size of G1 and G2 calculated by linkage disequilibrium methods was 63.2 and 89.8, respectively. On a population level, pairwise F_{ST} value (F_{ST}: 0.14–0.21) along with AMOVA analysis indicated medium to high genetic differentiation between the improved orange-shell line and outbred populations and progenitor selected strains. Overall, the results of microsatellite suggested that the genetic diversity of the improved orange shell line was not affected greatly by mass selection during the process of improvement. This study provides insight into utilization of shell color variants and future genetic improvement through selective breeding in aquaculture.

1. Introduction

Among the enormous number of agricultural breeds raised around the world, many are regarded as rare germplasm resources owing to small population size or owing to their low intrapopulation genetic diversity (Lauvie et al., 2011; Rischkowsky and Pilling, 2007). In aquatic breeding programs, most rare breeds have peculiar traits and can be valorized economically through proactive development. In some countries such as China, the shellfish industry is mainly hatchery-based, which means that the development of genetically improved rare variant strains will provide economical varieties for farming and bring profitability (Wang et al., 2016).

Mass selection is an effective and common approach that has been applied to genetical improvement in shellfish species worldwide. For instance, in oyster species, this truncation selection method relies on high fecundity making it possible to use a high selection intensity to improve certain traits quickly (e.g., growth rate, survival, and disease resistance) (Degremont et al., 2015; Gjedrem and Baranski, 2010; Li et al., 2011; Nell and Perkins, 2006). Nevertheless, the loss of genetic diversity may be arise in mass selection due to the selection of just a few outstanding broodstock without pedigree information, or due to non-random mating between individuals which have high variance in reproductive success (Appleyard and Ward, 2006; Boudry et al., 2002). What's more, inbreeding depression caused by the loss of genetic diversity is concordant with a recession in performance and fitness which can ultimately hamper the future selection (Boudry et al., 2002; Evans

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et al., 2004; Hartl et al., 1997). Losses of genetic diversity in mass selection or hatchery strains have been reported in many fishery animals, such as fish (Sawayama and Takagi, 2016), shrimp (Knibb et al., 2014) and shellfish (In et al., 2016; Rhode et al., 2014).

Nowadays, in an effort to preserve genetic diversity in mass-selected shellfish stocks, several options have been suggested as precautionary methods. First, methods to increase genetic diversity during spawning included using a large number of broodstock and balanced sex ratio and undertaking artificial spawning were applied to increase effective population size (Xu et al., 2019). Next, various follow-up procedures such as keeping pedigree records, subdividing lines and then synthesizing sublines to store rare alleles and introducing wild individuals from long distances were demonstrated to be valid (In et al., 2016; Knibb et al., 2014; Chen et al., 2017). In addition, crossbreeding separately selected strains have gradually been considered as a more cost-effective means which is not only easier to obtain without the expenses of sampling, transportation and quarantine, but also having accumulated favorable traits, thus accelerating genetic gains (Knibb et al., 2020; Olesen et al., 2015). A sufficient level of genetic variation is crucial for maintaining a sustained response from long-term selection. It therefore should be a major priority to carefully monitor the change in allelic diversity and population structure so as to timely design pertinent schemes in future breeding selection.

The Pacific oyster, *C. gigas*, is one of the most widely farmed shellfish species in the world. With the rise of within-shell oyster market, shell color trait is increasingly of interest to the breeding industry and has been a target trait for selection (Kang et al., 2013; Song et al., 2016). In our previous breeding process, a rare orange-shell line of *C. gigas* has been established based on four orange-shell variants. But this typical inbred line is faced with the problems of reduction in genetic diversity over successive mass selection, in addition to inbreeding depression such as low adaptive capacity at early stage (Han and Li, 2020; Han et al., 2019). Through the hybridization between the orange-shell line and two selected strains, a genetically improved orange-shell line was established and two successive mass selection were implemented subsequently. However, the impact of mass selection process on the improved orange-shell line remains undocumented. This study aimed to evaluate the genetic consequences of mass selection on genetic diversity in an improved orange-shell line compared with outbred and progenitor populations of *C. gigas*. Fifteen microsatellites and mitochondrial COI sequences were used to assess if and how genetic diversity changed over two successive generations of mass selection.

2. Materials and methods

2.1. Selection, sample collections and DNA extraction

In 2011, the first generation of an orange-shell line (SO) of *C. gigas* was constructed by only four orange-shell variants (two males and two females) selected from the cultured population of *C. gigas* in Rushan, Shandong province, China. Next, two consecutive generations of family selection and three generations of mass selection were established from 2012 to 2016 to fix the shell color traits and enhance the growth performance (Han et al., 2019). Two Lines selected for black-shell (SB) (Xu et al., 2019) and white-shell (SW) (Xing et al., 2017) were originated from natural seed with relatively black and white shell color in Rushan, Shandon, China. Then, four generations of family selection for shell color and three generations of mass selection for fast growth were implemented from 2010 to 2016 (Fig. 1.).

Due to the reduction in genetic diversity and the effects of inbreeding such as low adaptive have been observed in the orange-shell line (SO), a breeding improvement program was initiated by introducing two fast-growth strains (SB and SW). In May 2017, a total of 27 F_1 families with 9 experimental crosses and three replicates were produced by a complete 3 × 3 diallel cross among oysters with equal numbers of SO and two selected strains. The next year, 18 s-generation (F_2) families were produced by single-pair mating between F_1 crosses and selected as the base population to start an improved orange-shell line. Since 2019, selection for shell height (top 10% of the largest individuals selected) and orange shell color (some orange stripes and/or bigger proportion of orange color) was initiated to construct the first generation of mass selections (G1) using individuals of families from the F_2 as broodstock. Similarly, truncation selections were implemented for the next successive generations of mass selection (G2) in 2020. The number of broodstock and sex ratio for G0-G2 are shown in Table 1. Around one month before the breeding period, the selected broodstock were transferred from the aquaculture sea area to the hatchery for temporary cultivation. After gonadal maturity, males and females were selected according to

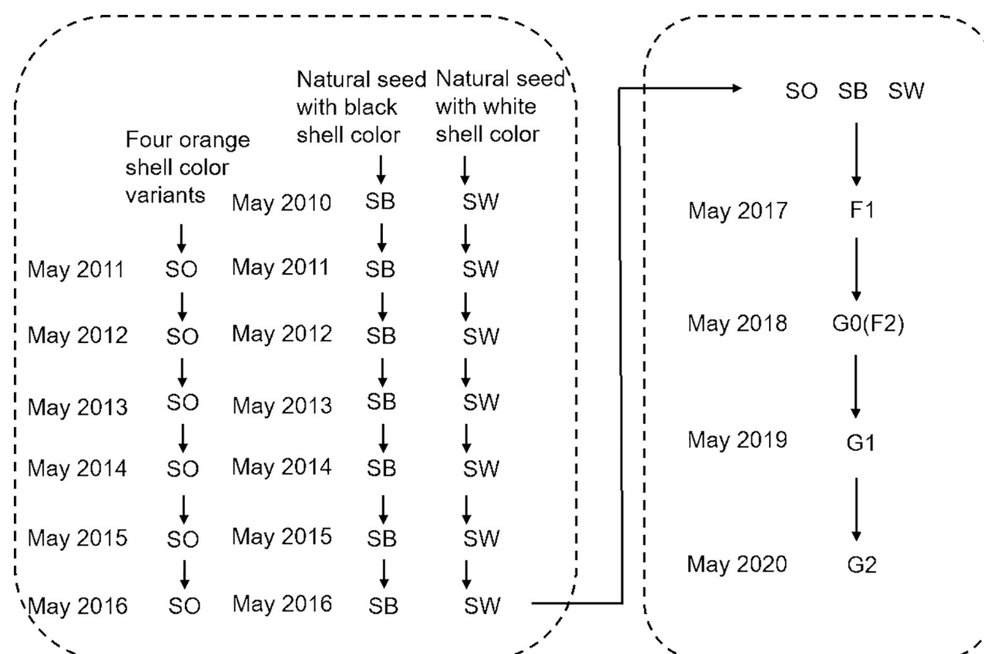

Fig. 1. Diagrammatic representation of the improved orange-shell line and three progenitor selection lines.

Table 1
List of sample information and the effective population size (N_e) in the breeding populations of *C. gigas*.

Population	Number of parents		Sample time	N_e-linkage disequilibrium	
	Female	Male		$N_{e\text{-lin}}$	95% CI (lower-upper)
G0	–	–	06/2018	122.2	75.0–278.3
G1	14	44	05/2020	63.2	38.2–134.1
G2	50	50	12/2020	89.8	60.2–173.2
SO	50	50	12/2020	–	–
SB	50	50	12/2020	–	–
SW	50	50	12/2020	–	–
RC	–	–	12/2020	–	–
QD	–	–	12/2020	–	–
LYG	–	–	12/2020	–	–
ZS	–	–	12/2020	–	–

the aforementioned criteria (shell color and shell height) and dissected to collect gametes into separate containers. To provide equal mating chances for each parent, equal amounts of eggs from each female were mixed after estimating oocytes concentrations by a microscope as well as sperm from each male. After 24 h of incubation, the D-shaped larvae were collected into 24-m^3 rearing tanks. The daily management of larva, spat and adult oysters were done as described by Li et al. (2011).

We randomly took up to 480 samples from six breeding populations and four wild populations. The breeding populations consisted of three generations of the improved orange-shell line (G0, G1 and G2) and three selected strains included black-shell line (SB), white-shell line (SW), and orange-shell line (SO) located in the cultured sea area of Rongcheng, Shandong province. Four wild populations were collected from Rongcheng (RC, 37.1°N, 122.5°E), Qingdao (QD, 36.1°N, 120.3°E), Lianyungang (LYG, 34.9°N, 119.3°E) in the Yellow Sea, China, and Zhoushan (ZS, 30.1°N, 121.1°E) in the Donghai Sea, China respectively. The sample size of each studied population is 48 and the corresponding sample time were shown in Table 1. The adductor muscle was separated and conserved at -30 °C for the molecular experiment. The genomic DNA of each oyster was extracted according to the phenol-chloroform method as described in Li et al. (2006) and preserved in 1× TE buffer.

2.2. Microsatellite analysis

Multi-locus genotypic data were obtained by amplifying the following 18 microsatellite markers in six multiplex PCRs: ucdCg-117, ucdCg-120, ucdCg-198, ucdCg-146, ucdCg-140, ucdCg-112, ucdCg-200, ucdCg-152(Li et al., 2003), Crgi3, Crgi39, Crgi4, Crgi45(Sekino et al., 2003), uscCgi-210(Yamtich et al., 2005), Cgsili57(Sauvage et al., 2009), otgfa0_0129_E11, otgfa0_408293, otgfa0_0139_G12, otgfa0_0007_B07(Qi et al., 2009). After the quality of PCR products was checked, genotyping was performed on all 480 individuals by ABI 3130 Automated DNA Sequencer (Applied Biosystems).

We used GeneMapper software v.4.0 (Applied Biosystems) to convert the genotyping results into alleles size. To ensure the accuracy of subsequent analysis, Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004) was used to examine scoring errors and detect the null alleles. Exact probability tests were performed in Genepop v.3.4 to test whether each locus in each population conformed to Hardy-Weinberg equilibrium (Rousset, 1995). The above results were again corrected after Bonferroni correlation by Myriads v.1.2 (Carvajal-Rodríguez, 2018).

Basic parameters of genetic variability containing: number of alleles (N_α), observed and expected heterozygosity (H_o and H_e respectively), inbreeding coefficient (F_{is}), Shannon Wiener index (I) were estimated using GenALEx v.6.501 (Peakall and Smouse, 2012). A non-parametric analysis of variance (Kruskal-Wallis test) among populations and generations was performed to test the difference of the above statistical results.

To gain an insight into the population level, pairwise F_{ST} values were calculated by Genetix v4.05, and analysis of molecular variance (AMOVA) was performed in Arlequin v3.5.2.2 (Excoffier and Lischer, 2010). A pairwise population matrix was constructed using *Nei's* unbiased genetic distance (*Nei's D*) (Hedrick, 2011). Genetic relationship among populations and individuals from ten populations was estimated by principal coordinates analysis on Structure 2.2 (Pritchard et al., 2003). A neighbor-joining tree was reconstructed utilize the *Nei's D* by Mega v5.0 (Tamura et al., 2011).

Effective population size (N_e) estimates of breeding populations were calculated based on linkage disequilibrium methods by NeEstimator v.2.0 (Do et al., 2014). N_e values were generated omitting alleles with frequencies <0.05 and 95% confidence intervals (95% CI) are reported.

2.3. Mitochondrial COI analysis

Mitochondrial cytochrome C oxidase subunit I (mtCOI) sequences were amplified using universal primers (LCO1490 and HCO2198) (Vrijenhoek, 1994). Twenty oysters were randomly selected for PCR amplification in each population. The PCR products were sequenced by the Personal Company (Shanghai, China) for both directions. The sequences received required further processed, including using SeqMan to remove the primer-binding regions and ambiguous bases at the 5′ and 3′ end and aligning by Mega. The number of haplotypes (N_h), haplotype diversity (H_d), and nucleotide diversity (P_i) were calculated by DNASP v.5.10 (Librado and Rozas, 2009).

3. Results

3.1. Genetic diversity

Overall, 480 oysters from six breeding populations and four wild reference populations were genotyped by 18 microsatellite loci. No evidence of scoring error due to stuttering and large allele dropout was detected in all loci. The frequencies of null alleles per locus ranged from 0.001 to 0.204 (ucdCg-146 and ucdCg-140 were higher than 0.2). The polymorphic information content (PIC) of the markers were above 0.5 except otgfa0_0139_G12, Crgi4 and Crgi45. The three markers were removed in the subsequent analysis (Supplementary Table 1). There were 204 alleles detected in total and 81 alleles in the generations of improved orange-shell line.

This trend of loss of genetic diversity was apparent from the wild populations to G0-G2, which was consistent with the outcome that there were significant reductions in N_α (54.0–64.2%), H_o (27.0–40.5%) and H_e (26.8–34.1%) between the two groups (Table 2). When compared with progenitor selected lines, the values of N_α, H_o and H_e in the improved orange-shell line increased by 12.1–24.1%, 20.5–38.5% and 3.7–13.1% respectively in comparison to SO population. However, N_α for G0-G2 were lower than SB and SW populations. Although N_α in two selection generations (G1-G2) were slightly smaller than G0, there was no significant difference in N_α among the G0 and two successive generations of mass selection. Within G1-G2, no significant difference in N_α (4.87–5.40) and H_e (0.59–0.61) were observed ($P < .05$). Lower genetic diversity in G1-G2 compared to wild populations was also observed in Shannon's Wiener index values (I), which were higher for all wild populations and ranked as a similar pattern with alleles numbers within G1-G2.

Mean expected heterozygosity (H_e) and mean inbreeding coefficient values (F_{is}) highlighted heterozygote deficiency in all populations except for SW, which exhibited heterozygote excess. Deviations from Hardy-Weinberg expectations were seen in all populations after Bonferroni correction, and there were more deviations in the breeding populations (Table 2).

We also examined mtDNA sequence variation in the cytochrome oxidase subunit I gene in 200 oysters from all 10 populations. After sequence alignment, the 599-bp fragment was used for further analysis. There were 19 mtCOI haplotypes in all the populations studied

Table 2
Genetic parameters within breeding populations and wild populations based on 15 microsatellite loci.

Population	N	N_α	I	H_o	H_e	F_{is}	dHW
Breeding populations							
G0	80	5.40 ± 0.51^{cd}	1.15	0.54 ± 0.05^{b}	0.61 ± 0.03^{bc}	0.11	5
G1	77	5.13 ± 0.49^{cd}	1.13	0.47 ± 0.06^{b}	0.56 ± 0.05^{bc}	0.16	5
G2	73	4.87 ± 0.46^{cd}	1.12	0.48 ± 0.05^{b}	0.59 ± 0.04^{bc}	0.19	8
SO	65	4.35 ± 0.56^{d}	1.01	0.39 ± 0.05^{c}	0.54 ± 0.06^{bc}	0.28	5
SB	95	6.33 ± 0.68^{c}	1.32	0.48 ± 0.04^{b}	0.64 ± 0.03^{b}	0.24	5
SW	96	6.41 ± 0.85^{c}	1.37	0.53 ± 0.06^{b}	0.66 ± 0.04^{b}	0.21	5
Wild populations							
RC	176	11.73 ± 1.53^{b}	2.02	0.75 ± 0.04^{a}	0.82 ± 0.02^{a}	0.07	4
QD	194	12.93 ± 1.70^{ab}	2.08	0.78 ± 0.03^{a}	0.82 ± 0.02^{a}	0.04	2
LYG	204	13.60 ± 1.56^{a}	2.23	0.74 ± 0.03^{a}	0.85 ± 0.05^{a}	0.01	2
ZS	179	11.93 ± 1.52^{ab}	2.06	0.79 ± 0.05^{a}	0.83 ± 0.02^{a}	0.04	2

N: number of alleles, N_α: average number of alleles, I: Shannon Wiener index, H_o: observed heterozygosity, H_e: expected heterozygosity, F_{is}: inbreeding coefficient, *dHW*: number of loci deviating from Hardy-Weinberg equilibrium; Means in the same column superscripted by different letters were significantly different ($P < .05$).

(Table 3). One of the haplotypes (haplotype 1) was dominant and detected in all 10 populations with a prevalence of 86.5% (173/200). All sampled individuals in G0-G2 only had haplotype 1. The highest value of haplotype diversity (H_d) and nucleotide diversity (P_i) were 0.763 (QD) and 0.00368 (QD) respectively in all sampled populations.

3.2. *Effective population size (N_e)*

Among the improved orange-shell line, the effective population size calculated by linkage disequilibrium method ($N_{e\text{-lin}}$) were ranged from 63.2 (G1) to 122.2 (G0) (Table 1). A higher $N_{e\text{-lin}}$ value was observed in G2 (89.8) compared with G1.

3.3. *Genetic differentiation*

Analysis of molecular variance (AMOVA) revealed that the global F_{ST} for breeding populations was 0.13 ($P < .01$), while that of the generations of improved orange-shell line was only 0.02 ($P < .01$) (Table 4). Similarly, within G0-G2, pairwise F_{ST} and *Nei's D* (F_{ST} range 0.022–0.041; *Nei's D* range 0.038–0.057) values were relatively low and gradually increasing between subsequent generations. Pairwise F_{ST} and *Nei's D* showed that the highest genetic differentiation was distributed between the G0-G2 and wild population (F_{ST} range from 0.156–0.210; *Nei's D* range from 0.524–0.701) (Table 5).

Genetic relationships among populations and individuals of ten populations were estimated using genetic distance matrixes (Structure2.2). The overall impression is that the PCoA analysis separated the 10 populations into two main clusters (Fig. 2). The results also can be confirmed by the neighbor-joining tree (Fig. 3). Ten populations fell into two clusters: one cluster included three generations of the improved orange-shell line and three selected strains, and the other consisted of four wild populations. The G0-G2 generations formed a small subcluster and differentiated with subsequent generations. The limited variation detected in the mtDNA did not allow further analysis of genetic difference.

4. Discussion

Prior studies have noted the importance of maintaining a sufficient level of genetic variability during long-term selection (Davis and Hetzel, 2000). The main objective of this study was to utilize microsatellites and mtDNA data to gain an insight into the proper monitoring and management for the selective breeding programs of *C. gigas*. In this study, levels of genetic diversity were evaluated over two successive generations of an improved orange-shell line of *C. gigas* and compared with three progenitor selected strains and four wild populations. The main finding was that there was no obvious genetic diversity loss in generations of improved orange-shell line after mass selection based on microsatellite data. However, a decreasing trend in number of alleles suggesting the potential for losses of genetic diversity. This study provides pertinent guidance for future selection.

Table 3
Haplotype frequencies of mtCOI sequences observed among breeding populations and wild populations.

Haplotype	Wild populations				Breeding populations						Total
	LYG	QD	RC	ZS	G0	G1	G2	SB	SW	SO	
1	12	10	16	18	20	20	20	17	20	20	173
2	1										1
3	1	1									2
4	3			1							4
5	1	1									2
6	2	1									3
7								3			3
8		1									1
9		1									1
10		1									1
11		1									1
12		1									1
13		1									1
14		1									1
15			1								1
16			1								1
17			1								1
18			1								1
19				1							1
N_h	6	11	5	3	1	1	1	2	1	1	
H_d	0.632 ± 0.113	0.763 ± 0.103	0.368 ± 0.135	0.205 ± 0.119	0	0	0	0.268 ± 0.113	0	0	
P_i	0.00160 ± 0.00048	0.00368 ± 0.00187	0.00083 ± 0.00036	0.00053 ± 0.00034	0	0	0	0.00045 ± 0.00019	0	0	

N_h: number of haplotypes, H_d: haplotype diversity, P_i: nucleotide diversity.

Table 4
Analysis of molecular variances (AMOVA) for generations of the improved orange-shell line and breeding populations based on 15 microsatellite loci.

Source of variance	d.f.	Variance components	Percentage of variation	F-statistics*
Among generations of the improved orange-shell line				
Among populations	2	0.11044	2.42	F_{ST} = 0.02416*
Among individuals within populations	141	0.60339	13.20	F_{IS} = 0.13526*
Within individuals	144	3.85764	84.39	F_{IT} = 0.15615*
Total	287	4.57147		
Among breeding populations				
Among populations	5	0.68813	12.92	F_{ST} = 0.12922*
Among individuals within populations	282	0.94790	17.80	F_{IS} = 0.20441*
Within individuals	288	3.68924	69.28	F_{IT} = 0.30722*
Total	575	5.32526		

* Significant at $P < .01$.

In previous studies, microsatellites and mitochondrial COI sequences were reliable tools and widely used in combination to evaluate the level of genetic diversity and population structure (Han et al., 2019; Xu et al., 2019). Here, as a result of mtCOI analysis, we found three generations of the improved orange-shell line had only one haplotype (Table 3). Though the limited variation detected in the mtDNA did not indicate if the genetic diversity reduced, it is evident that the breeding populations had less genetic diversity than wild populations. For microsatellite data, no significant reduction in the N_{α}, H_o and H_e and an increase in F_{is} were observed in G1-G2. Compared with the base population (G0), the N_{α} in G2 population decreased 9.8%, which was much lower than that (44.2%) observed by Xu et al. (2019). Although the reductions in genetic diversity of this magnitude were unremarkable, it could lead to lower adaptability for future generations as the rare alleles lost (Straus et al., 2015).

Overall level of genetic variation of a population is often assessed by comparing with reference populations. The three generations of improved orange-shell line of *C. gigas* significantly showed a 54.0–64.2%, 27.0–40.5% and 26.8–34.1% reduction in the N_{α}, H_o and H_e compared with wild populations. The reduction in allele number relative to wild populations has been found in other aquatic species microsatellite such as dusky kob, *Argyrosomus japonicus* (Jenkins et al., 2020) and mussel, *Mytilus edulis* (Gurney-Smith et al., 2017). As studies before, the initial founding effect of the improved orange-shell line from which the base population originated followed by genetic drift, may be causes for the loss of genetic diversity (Allendorf, 1986; Hedgecock and Sly, 1990; Yu and Li, 2007). Compared with the three progenitor populations, the N_{α} in G0-G2 were lower than SB and SW populations and more loci were out of HW equilibrium. Xu et al. (2019) and Xing et al. (2017) reported that the base population of SB and SW were selected from wild population which had high levels of genetic variation. In comparison, a rare variant line which was established by four orange-shell variants was used to form G0. When implementing selective breeding, a base population should possess high levels of genetic variation, which stores large numbers of genotypes like a "gene bank", to lay the foundation for genetic diversity and guarantee genetic gains during following artificial selection (Gjedrem and Robinson, 2014). The differences in N_{α} between two progenitor selected strains and improved orange-shell line may indicated the significance of capturing as much of genetic variation as possible at the beginning of breeding programs.

Maintaining a sufficiently large N_e is of great importance to prevent inbreeding depression and loss of genetic diversity while improving performance by selective breeding (Hillen et al., 2017). Hence, it is important to monitor and enhance N_e when implementing mass spawning. The depression in N_e can be attributed to small broodstock size, biased sex ratio, variance in contribution of gametes, inbreeding and different viability of gametes (Li et al., 2007). In the current study, the $N_{e\text{-lin}}$ for G1 and G2 was 63.2 and 89.8, respectively (Table 1). First, the G1 generation was produced by a small number of broodstock from several families added the probability that selected the best offspring produced by few parents which resulted in high variance of reproduction success of broodstock (Boudry et al., 2002). Gjerde et al. (1996) found that the less balanced the sex ratio, the larger the number of parents needed when maintaining the rate of inbreeding at 1% level. Thus, a balancing sex-ratio for broodstock plays a part in keeping a sufficient effective population size. The baised sex-ratio (almost 3:1) could also be a cause for low $N_{e\text{-lin}}$. In addition, the selection of rare orange shell line oysters in this case may impose bottleneck, those were the two reasons for low $N_{e\text{-lin}}$ in G1. The increased $N_{e\text{-lin}}$ in subsequent generation can also be found in previous study (Xu et al., 2019).

Investigating population differentiation is an effective approach to understanding aquaculture practices, as it can provide the origin and composition of the aquaculture stocks and guide the selection of new broodstock (Hillen et al., 2017). The significant pair F_{ST} values and AMOVA analysis within G0-G2 were consistent with changes in genetic diversity over two successive generations of mass selection. A gradual increase in genetic differentiation with subsequent generations were detected within G0-G2, which was result from mass selection in combination with founder effects and genetic drift and consistent with the neighbor-joining tree topology. Furthermore, it is revealed by the pairwise F_{ST} as well as *Nei's D* that genetic differentiation between improved orange-shell line and progenitor selected lines were large (F_{ST}:0.13–0.15; *Nei's D:* 0.285–0.397). The results supported the effects of founder effects and genetic drift once again (Hillen et al., 2017). Differentiation between cultured populations and progenitor populations resulting from founder effects have been reported in many aquaculture species, including Eastern oyster, *crassostrea virginiva* (Varney and Wilbur, 2020), dusky kob (Jenkins et al., 2020) and

Table 5
Estimated pairwise F_{ST} (lower diagonal) and *Nei's D* (upper diagonal) values of *C. gigas* based on 15 microsatellite loci.

	G0	G1	G2	RC	QD	LYG	ZS	SO	SB	SW
G0		0.042	0.038	0.604	0.587	0.628	0.699	0.285	0.397	0.351
G1	0.029		0.057	0.617	0.621	0.672	0.701	0.309	0.352	0.377
G2	0.022	0.041		0.546	0.524	0.611	0.674	0.261	0.330	0.288
RC	0.160	0.192	0.158		0.114	0.242	0.301	0.431	0.409	0.397
QD	0.158	0.190	0.156	0.020		0.198	0.258	0.601	0.475	0.436
LYG	0.159	0.193	0.169	0.034	0.032		0.264	0.523	0.432	0.391
ZS	0.174	0.210	0.178	0.047	0.043	0.038		0.639	0.475	0.407
SO	0.135	0.151	0.153	0.172	0.174	0.164	0.178		0.378	0.293
SB	0.144	0.134	0.141	0.126	0.125	0.135	0.147	0.165		0.246
SW	0.139	0.154	0.144	0.117	0.112	0.114	0.129	0.153	0.110	

All F_{ST} values were significantly different from zero ($P < .05$).

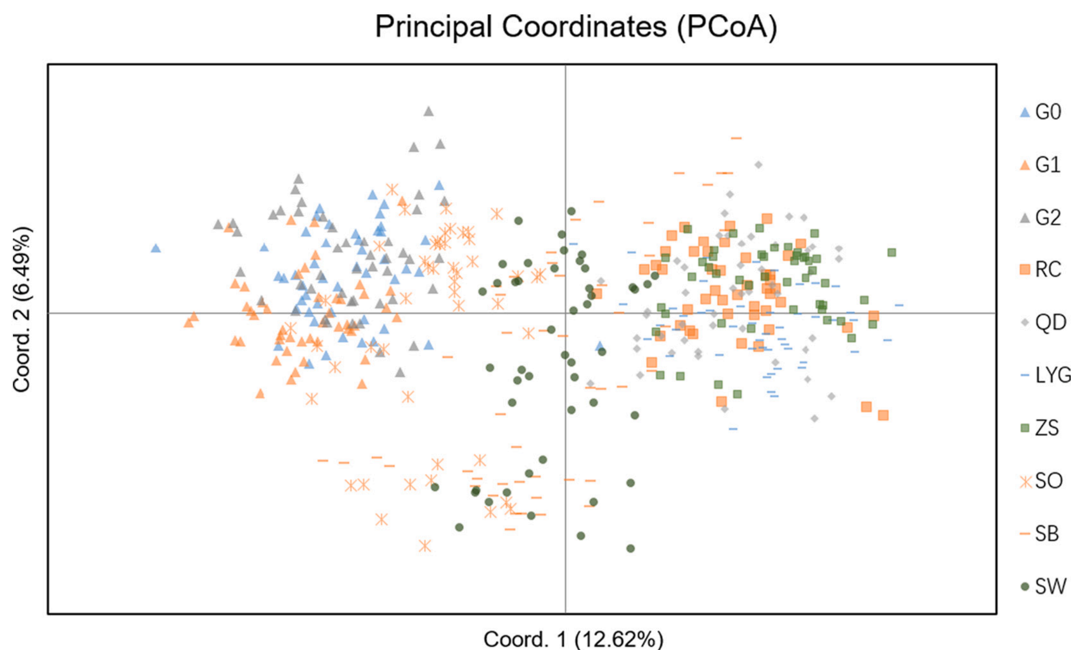

Fig. 2. Genetic relationships among individuals of ten populations from principal coordinates analysis (PCoA) using genetic distance matrices. The different populations are indicated by different colours and symbols, respectively. "Coordinate 1 (12.62%)" means the difference between samples located on the right and the left sides of the vertical coordinate 1 line is 12.62%; "Coordinate 2 (6.49%)" is 6.49% difference between samples distributed on the upper and lower sites of the horizontal Coordinate 2 lines.

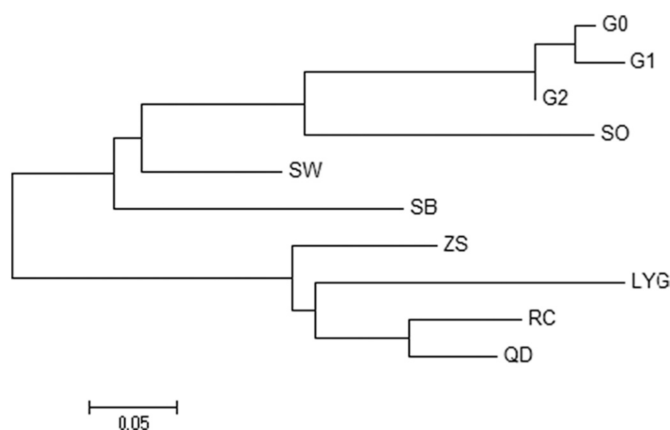

Fig. 3. Neighbor-joining tree of all ten populations based on *Nei's* unbiased genetic distance.

European seabass, *Dicentrarchus labrax* (Hillen et al., 2017).

The economic value of shell color variants as well as the requirement to implement improvement selective breeding led to the question of how to capture and maintain genetic diversity was worth studying. Many methods have been applied to capture and conserve genetic variation (Chen et al., 2017; Evans et al., 2004; In et al., 2016; Wang et al., 2016). In the current study, the N_{α} in the generations of improved orange-shell line were apparently higher than the inbred orange-shell line (SO) without improvement. The successful improvement of the inbred line (SO) can be attributed to hybridization between SO and two selected strains (SB and SW), which could capture high levels of genetic variation while conserving QTL alleles that have been selected indirectly. Based on the genetic monitoring, clearly, the breeding operations used in current study would have been effective to maintain genetic variability in the two generations of mass selection. However, it is noteworthy that N_{α} in G1 and G2 is still inferior than two fast-growth selected strains (SB and SW) (Table 2), which have been selected over multi-generations. Moreover, the loss in allele number and the $N_{e\text{-lin}}$ below the actual size indicated the potential for losses of genetic diversity. It has been suggested that genetic variation losses can result in reduced performance in aquaculture (Evans et al., 2004). Accordingly, it remains to continue monitoring any molecular variations and investigate the relationship between breeding practices and changes in genetic diversity.

5. Conclusions

No significant loss of genetic diversity was detected in the improved orange-shell line of *C. gigas* in two successive generations of mass selection based on microsatellite results. There was, however, a decreasing trend in number of alleles. Overall, it remains to study scientific breeding strategies which can better balance the capture and sustainability of genetic diversity by genetic monitoring. Finally, our study may provide insight into utilization of shell color variants and future genetic improvement through selective breeding in aquaculture.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737791.

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