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Genetic variability of an orange-shell line of the Pacific oyster *Crassostrea gigas* during artificial selection inferred from microsatellites and mitochondrial COI sequences

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ABSTRACT

Rare breeds represent a valuable resource for current or future market demands, but their low census compromises the genetic variability and future utilization of these breeds. Whether genetic variability of rare breeds with low initial genetic variation can be maintained during an intense mass selection becomes the key to applying mass selection to the genetic improvement of these breeds. The genetic variability among three generations of successive mass selection of the orange-shell line of *Crassostrea gigas* (MS1–MS3) were evaluated by both 20 nuclear microsatellite loci and mitochondrial cytochrome oxidase I sequences (mtCOI) compared to four wild populations. In this study, the orange-shell line exhibited very low genetic variability. Only one mtCOI haplotype was detected in all individuals of MS1–MS3. Significant reductions in average number of alleles (N_a: 69.55–76.92%), allelic richness (A_r: 68.17–74.91%) and expected heterozygosity (H_e: 34.21–39.24%) as well as increased mean pairwise genetic relatedness (R: 6.87–25.79 times) were observed in MS1–MS3 when compared to wild populations. However, the genetic variability of orange-shell line with very low initial variation successfully maintained during three generations of mass selection. No significant difference in N_a (3.60–4.40), A_r (3.51–4.08) and H_e (0.48–0.50) occurred among MS1–MS3. Pedigree reconstructions (no full-sib group from MS1–MS3 was larger than 16% of the whole group) revealed artificial spawning used in this study has a better control over contribution of gametes than traditional mass spawning. In addition, effective population size of MS1–MS3 calculated by linkage disequilibrium methods increased from 29.3 to 67.0 indicating the linkage disequilibrium decays over time. This study provides important insights in the genetic consequences of a rare variant line of *C. gigas* with very low genetic variation over generations of mass selection. This will provide a reference for carrying out genetic improvement programs on rare breeds where small populations are inevitable.

1. Introduction

Rare breeds, as valuable germplasm resources, always exhibit some specific agricultural traits that meet current or future market demands. However, their low census compromises the genetic variability and future of these breeds (Cervantes et al., 2016). This makes it difficult to use the rare resources to achieve sustainable profitability, especially when some rare breeds need further improvement in production performance. Genetic variability is the fundamental resource on which stock improvements rely. It therefore should be a major priority for breeding operations to not only capture, but also maintain, as much of genetic variation as possible within domesticated populations (Lind et al., 2009).

Achieving this, however, can be problematical in some breeding practices, especially in a small population, such as a breeding program with rare breeds as founder population. Firstly, at the beginning of the breeding program, the founding stock size is limited and difficulty are faced in sourcing wild germplasm lead to non-random mating between individuals that are closely related (inbreeding) and consequently to a reduction of genetic variation. Secondly, most marine invertebrates and fish are highly fecund, allowing stringent selection and faster gains (Gjedrem, 2012). However, the great fecundity is always accompanied by high variance in reproduction success of such organisms (Boudry et al., 2002; Hedgecock et al., 2007). These characteristics of aquatic species could result in inbreeding and loss of potentially beneficial alleles and net additive genetic variation during successive selection, especially without individual tagging and pedigree records, and when there are high selection intensities (In et al., 2016). The effects of

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traditional selection processes upon the genetics of aquatic populations is a topic that has received substantial research attention. Many studies have shown that genetic variation typically declines over successive generations of domestication, such as shrimp (Dixon et al., 2008), fish (Sawayama and Takagi, 2016) and shellfish (Fu et al., 2017; In et al., 2016; Rhode et al., 2014). Decreasing genetic variation will not only cause significant negative effect on stock performance and production traits, but also limit the potential for genetic gains and response to selection (Bentsen and Olesen, 2002; Zhang et al., 2010).

It is because of these factors mentioned above that breeders are not optimistic about the selection of rare breeds. Indeed, there are few reports or examples of reactions to effects of artificial selection on the genetic variability of rare breeds. Recently, in the case of an increasing understanding of the cause of loss of genetic diversity during selection, some measures to prevent losses have been proposed. Some of the easy-to-operate and low-cost measures have been applied to the oyster breeding process, such as artificial spawning, balanced sex ratio and large size of broodstock, and successfully maintained the genetic diversity during artificial selections in two strains of oyster selected for growth (Wang et al., 2016; Xu et al., 2019). Artificial spawning permits a better control of the number, sex ratio and contribution of gametes of real parents who provide gametes than the traditional mass spawning mentioned above. Therefore, mass selection is promising for the genetic improvement of rare breeds, if the genetic variability of small populations can be maintained by improved selection process. This information will be helpful in the sustainable use of rare breeds where small populations are inevitable.

The Pacific oyster (*Crassostrea gigas*) is one of the most widely farmed aquaculture species worldwide (FAO, 2016). A rare orange-shell variant of *C. gigas* (Fig. 1) was obtained through family selection in our breeding practice. Since orange-shell variant not only has a unique shell color, but also its soft tissue has twice the zinc content of commercial population of *C. gigas* with a common shell color (Zhu et al., 2018), it may become a valuable germplasm resources for further market demands. To further improve the growth performance of the orange-shell line, successive three-generation improved mass selection with a selection intensity of about 1.9 has been conducted since 2014 (Table 1). Rare variant, as typical small populations, provide an opportunity to assess whether genetic variability of rare breeds can be maintained over generations of intense selection with improved mass selection. This study aims at (1) assess the current level of genetic diversity of the orange-shell line compared to wild populations of *C. gigas*, (2) evaluating whether the genetic variability is maintained in orange-shell line over mass selection generations using nuclear microsatellite loci as well as mitochondrial cytochrome oxidase I sequences.

2. Materials and methods

2.1. Selection, sample collections and DNA extraction

Three generations of the orange-shell selection and four wild populations of *C. gigas* were surveyed in this study (Table 1). Four orange-shell individuals (about 0.2%) were found in the offspring of purple-black shell color individuals, which were produced by crossing females with black shell color and males with purple shell color selected from the cultured population of *C. gigas* in Rushan, Shandong province, China. These four individuals (two males and two females) with orange left and right shell color were collected and used to produce two full-sib families as the first generation. Next, two consecutive generations of family selection were established from 2012 to 2013 to fix the shell color. After three generations of family selection, we obtain genetically stable orange-shell line. To enhance the growth performance of the orange-shell line, truncation selection for shell height was initiated in 2014 to construct the first generation of mass selections (MS1) using individuals of families from the third generation with greatest shell height as broodstock. Similarly, truncation selections were implemented for the next two successive generations of mass selection (MS2 and MS3) in 2015 and 2016, respectively. The number of parents, truncation point and selection intensity for each generation are shown in Table 1. The eggs and sperm were collected from the matured males and females by dissection. For the purpose of providing equal mating chances for each parent, equal amounts of eggs and sperm from each female and male were mixed well after estimating concentrations using a microscope. Samples of the MS1 (14 months), MS2 (12 months) and MS3 (12 months) were collected randomly at harvest. Four wild populations were collected from Dongying (DY), Qingdao (QD), Penglai (PL) and Rushan (RS), Shandong province, China between 2013 and 2017 (Fig. 2). The sample size and sample time of each studied populations are shown in Table 1. Samples for mtDNA analysis were collected from the same individuals used for nuclear DNA analysis. The adductor muscle was collected from fresh specimens and immediately stored at −30 °C until DNA preparation. Genomic DNA was extracted

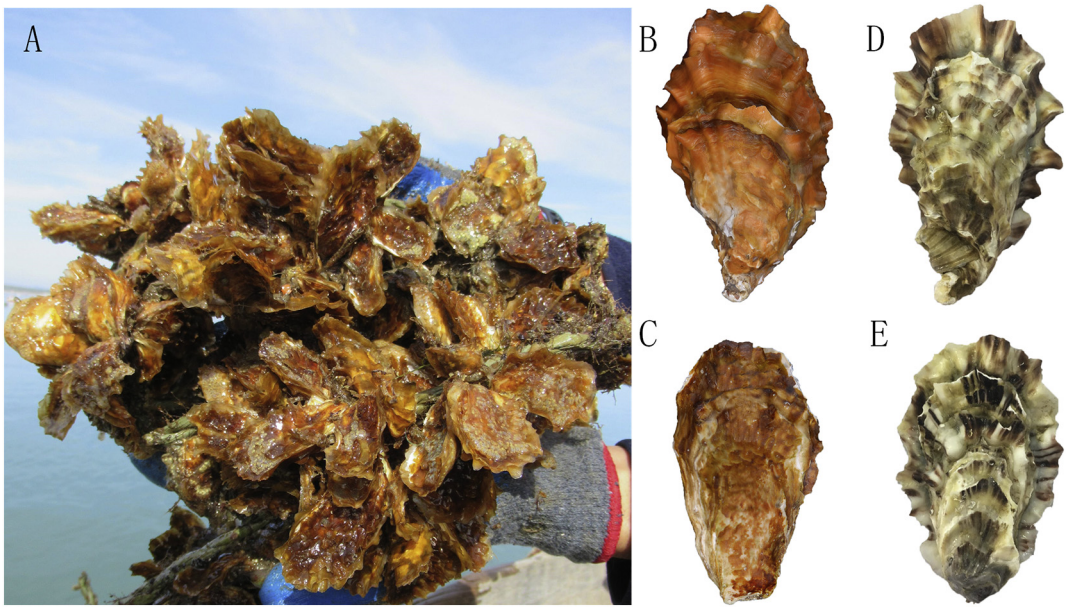

Fig. 1. Phenotypes of the orange-shell variant (A: living bodies, B: left shell, C: right shell) and wild-type (D: left shell, E: right shell) of *C. gigas*.

Table 1
List of sample information.

Population	Number of parents		Truncation point (mm)	Selection intensity	Sample time	Sample size	
	Female	Male				Microsatellite	mtCOI
RS	–	–			2016.09	48	20
PL	–	–			2013.02	50	20
DY	–	–			2013.07	51	20
QD	–	–			2017.11	50	20
MS1	60	60	51.45	1.87	2015.10	50	20
MS2	50	50	62.23	1.95	2016.06	48	20
MS3	50	47	62.50	1.98	2017.06	50	20

RS, Rushan wild population; PL, Penglai wild population; DY, Dongying wild population; QD, Qingdao wild population; MS1, 1st mass selected generation; MS2, 2st mass selected generation; MS3, 3st mass selected generation.

from approximately 100 mg of muscle tissue according to the phenol-chloroform method as described in Li et al. (2006) and preserved in 1 × TE buffer. DNA samples were quantified by Nanodrop 2000 and diluted to 100 ng/μl for PCR.

2.2. Microsatellites analysis

Six multiplex PCRs containing 17 microsatellite loci (ucdCg-117, ucdCg-120, ucdCg-146, ucdCg-152, ucdCg-170, ucdCg-198, ucdCg-199, ucdCg-200, uscCgi-210, Crgi3, Crgi4, Crgi39, Crgi45, otgfa0_0007_B07, otgfa0_0129_E11, otgfa0_408293, and otgfa0_0139_G12) (Liu et al., 2017) and three additional loci (ucdCg-140, ucdCg-153 and ucdCg-177) (Li et al., 2003) were used to genotype 347 individuals in total.

Alleles size was performed utilizing GeneMapper software v.4.0 (Applied Biosystems). Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004) was used to assess genotypic errors caused by stuttering or large-allele dropout. The presence of null alleles was tested using FREENA software (Chapuis and Estoup, 2007), in which loci with estimated frequencies of null alleles above 0.2 were potentially problematic for calculations (Napora-Rutkowski et al., 2017). Fisher's exact test of deviations from Hardy-Weinberg equilibrium for each locus was tested using Genepop v.4.0 (Raymond and Rousset, 1995). Number of alleles per locus (N_a), observed heterozygosity (H_o), excepted heterozygosity (H_e) and fixation indices (F_{is}) were calculated using GenAlEX v.6.5 (Peakall and Smouse, 2012). Allelic richness (A_r: the number of alleles adjusted for the smallest sample size) was calculated using FSTAT v.2.9.3.2 (Goudet, 1995).

To understand the genetic similarities among individuals in a population, mean pairwise relatedness estimate (R) of each population in this study was calculated using the methods of Queller and Goodnight (1989) and Lynch and Ritland (1999). To standardize the range of Lynch and Ritland (1999) estimators (range of −0.5 to 0.5) with Queller and Goodnight estimators, Lynch and Ritland (1999) estimators were multiplied by 2 to give a maximum value of 1 and minimum of −1. Pedigree reconstruction (Number of iterations: 1000000; Full-sib Constraint: 0 and 1; Temperature: 10; Weight: 1; Seed: −1) on three generations of the orange-shell selection populations was performed with PEDIGREE 2.2 (online, http://herbinger.biology.dal.ca:5080/Pedigree), which aimed at reconstructing full pedigree in a group of individuals base on their genotype data in the absence of parental information. The three selected populations and four wild populations were analysed with full-sib (Full-sib Constraint = 1) and kin group (Full-sib Constraint = 0) partition algorithm, and the setting of weight and temperature is based on the highest score.

Effective population size (N_e) of MS1–MS3 and four wild populations was calculated using linkage disequilibrium methods implemented in NeEstimator v.2.0 (Do et al., 2014). This method was shown to perform well in estimating N_e in non-ideal populations with a skewed sex ratio or non-random variance in reproductive success (Waples, 2006). N_e estimates were generated excluding alleles with frequencies < 0.05 to reduce bias related to rare alleles and 95% confidence intervals (95% CI) are reported. Beside the estimates based on genetic data, the unequal sex ratio correction method was used to calculate theoretical effective population size of each generation as: $N_e = 4N_mN_f/(N_m + N_f)$, with N_f and N_m the number of females and males broodstock respectively (Falconer and MacKay, 1996).

Pairwise F_{st} estimation (significance testing: 1000 permutations at 5% nominal level) and hierarchical analysis of molecular variance (AMOVA significance testing: 1000 permutations at 5% nominal level) were utilized to assess population differentiation and partition the genetic variance within/among populations. Also, a pairwise matrix assessing allele frequency heterogeneity among samples was constructed

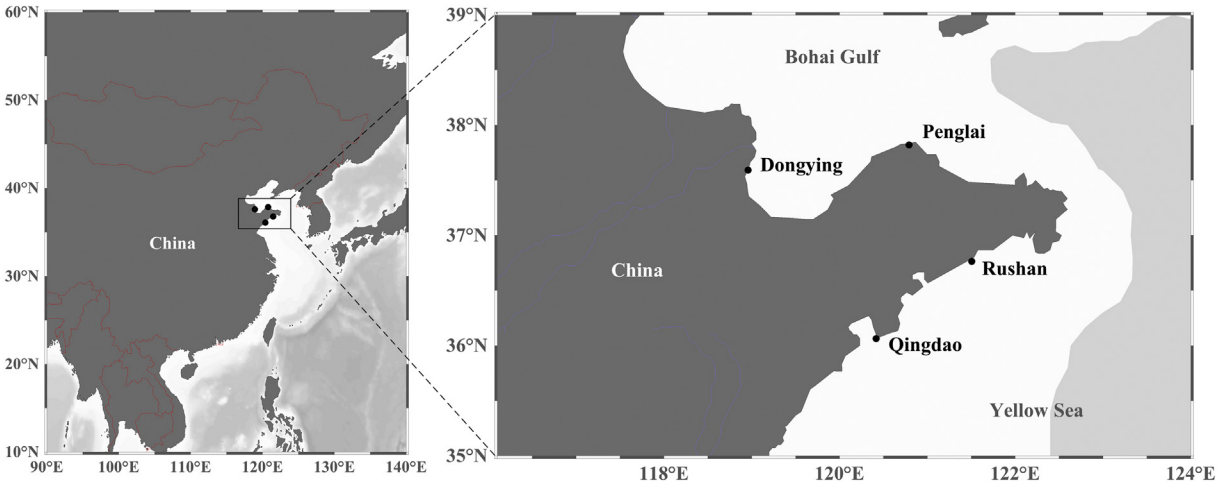

Fig. 2. Map of sampling sites of four wild populations of *C. gigas*.

using *Nei's* unbiased genetic distance (*Nei's D*) (Hedrick, 2000). These analyses were also conducted in GenAlEX v.6.5 (Peakall and Smouse, 2012). Then *Nei's D* was used to contrast neighbor-joining tree by Mega v.5.0 (Tamura et al., 2011).

2.3. Mitochondrial DNA sequencing and analysis

The mitochondrial cytochrome C oxidase subunit I (COI) was amplified using LCO1490 and HCO2198 universal prime (Folmer et al., 1994). The sequences were edited and aligned using Mega v.5.0 (Tamura et al., 2011). DNASP v.5.10.01 (Librado and Rozas, 2009) was used to calculate the total number of haplotypes, haplotype diversity and nucleotide diversity.

3. Results

3.1. Genetic variability

No evidence of stuttering error or large allele dropout was identified by the Micro-checker. Only the locus ucdcg-199 in MS3 (0.2227) and loci Crgi4 (0.35547) and ucdcg-152 (0.29782) in QD showed estimated frequencies of null alleles above 0.2. However, inclusion or exclusion of these loci did not qualitatively change the outcome, hence analysis was performed based on all loci.

There were no significant differences in average number of allele (N_a), allelic richness (A_r), observed (H_o) and expected heterozygosity (H_e) and fixed index (F_{is}) among the four wild populations (Table 2). However, significant reductions in N_a (69.55–76.92%), A_r (68.17–74.91%) and H_e (34.21–39.24%) were observed in MS1–MS3 comparing with the wild populations ($P < .05$). Within MS1–MS3, no significant difference in N_a (3.60–4.40), A_r (3.51–4.08), H_o (0.60–0.62), H_e (0.48–0.50) and F_{is} was observed, except that the A_r reduced slightly from 4.08 (MS1) to 3.51 (MS3) with successive mass selection.

Observed heterozygosity did not differ markedly across populations overall, with mean H_o of all seven populations ranged between 0.60 (MS1 and MS3) and 0.87 (RS). F_{is} for all seven populations ranged between −0.23 (MS1) and 0.15 (QD). Only DY and QD populations have positive F_{is}, while other populations have negative F_{is} values. Similarly, each of DY and QD had two loci deviating from Hardy-Weinberg equilibrium (HWE), while the number of loci deviating from HWE of other five populations ranged between 5 (PL) and 10 (MS2 and MS3).

A total of 599-bp fragments of mtCOI gene generated from 140 specimens were used for the analysis. There were 20 mtCOI haplotypes considering all seven populations in this study (Table 3). The conspicuous unique high-frequency haplotype (B) was observed in all seven populations and had a prevalence of 85.7% (120/140) in all individuals. All sampled individuals in MS1–MS3 only have haplotype B. Thus, no variation was detected within the orange-shell line. Besides haplotype B, each wild population had another four or five private haplotypes which were not shared with each other. Average haplotype diversity (H_d) of the four wild populations ranged from 0.442 to 0.447, while the average nucleotide diversity (P_i) of the four wild populations ranged from 0.083% to 0.127%.

3.2. Relatedness and pedigree reconstruction

Mean pairwise relatedness coefficients (R), a measure of genetic similarity relative to the population mean, across all seven populations ranged from 0.019 (RS) to 0.490 (MS1) (Fig. 3A). R of MS1–MS3 calculated by the method of Queller and Goodnight (1989) (R_{QG}: 0.446–0.490) turned larger mean estimates than that from Lynch and Ritland (1999) (R_{LR}: 0.268–0.272), while R of the wild populations calculated by both methods turned similar estimators (R_{QG}: 0.019–0.047; R_{LR}: 0.032–0.039). For both methods, R of MS1–MS3 was about 6.87–25.79 times that of the wild populations.

For the MS1–MS3 populations, the best full-sib partition (score: 6841.67–7367.49) identified 19–20 groups (Fig. 3B). No full-sib group from MS1–MS3 was larger than 16% of the whole group. Typically, kin groups contain mixture of full-sib groups and half-sib groups (Fig. 3C). The best kin group partition (score: 5828.81–7602.64) revealed 14–17 groups for MS1–MS3 populations. The kin groups from MS1–MS3 did not exceed 16% of the whole group except that one kin group from MS1 comprised 24% of the entire population. For four wild populations, the best kin group partition (score: 27198.86–32,695.33) identified 45–47 groups, and no kin group from wild populations was larger than 5% of the whole group (data not shown).

3.3. Effective population size

The effective population size estimates calculated by linkage disequilibrium methods (N_e-lin) of four wild populations were ranged from 478.4 (PL) to 1258.2 (RS). Compared to the N_e-lin of the four wild populations, the N_e-lin of the orange-shell lines were lower. The N_e-lin of each generation was in the following order: MS1 (N_e-lin: 29.3, 95% CI: 21.8–40.9), MS2 (N_e-lin:47.1, 95% CI: 31.5–80.0), and MS3 (N_e-lin:67.0, 95% CI: 41.1–141.8). The actual number of breeders used in each generation is shown in Table 1. The effective population size estimates calculated by sex ratio correction ($N_{e\text{-sex}}$) of both MS1 (120) and MS2 (100) were same as actual number of breeders. Only the $N_{e\text{-sex}}$ of MS3 (96.9) was smaller than the actual number breeder (97), because of the unequal female (50) and male (47) parents used in MS3.

3.4. Genetic differentiation

Analysis of molecular variance (AMOVA) revealed that the global F_{st} for four wild populations was 0.028 ($P < .001$), while that of MS1–MS3 was 0.029 ($P < .001$) (Table 4). Similarly, pairwise F_{st} and *Nei's D* showed that most genetic differentiation was distributed between the MS1–MS3 and wild populations (F_{st} range 0.166–0.197; *Nei's*

Table 2
Genetic parameters within mass selected orange-shell lines and wild populations based on 20 microsatellite loci.

Population	N_a	Ar	H_o	H_e	F_{is}	dHW
Wild populations						
DY	15.60 ± 8.78^{b}	13.81 ± 7.42^{b}	0.72 ± 0.21^{ab}	0.77 ± 0.18^{b}	0.08 ± 0.18^{bc}	2
QD	15.55 ± 7.81^{b}	13.99 ± 6.78^{b}	0.68 ± 0.24^{ab}	0.79 ± 0.14^{b}	0.15 ± 0.27^{c}	2
PL	14.45 ± 8.26^{b}	12.82 ± 7.17^{b}	0.76 ± 0.20^{ab}	0.76 ± 0.19^{b}	−0.02 ± 0.24^{abc}	5
RS	14.85 ± 7.90^{b}	13.36 ± 6.97^{b}	0.87 ± 0.10^{b}	0.79 ± 0.15^{b}	−0.14 ± 0.26^{abc}	9
Orange-shell lines						
MS1	4.40 ± 1.90^{a}	4.08 ± 1.72^{a}	0.60 ± 0.28^{a}	0.48 ± 0.16^{a}	−0.23 ± 0.36^{ab}	7
MS2	3.95 ± 1.93^{a}	3.72 ± 1.63^{a}	0.62 ± 0.26^{a}	0.50 ± 0.14^{a}	−0.21 ± 0.37^{ab}	10
MS3	3.60 ± 1.82^{a}	3.51 ± 1.64^{a}	0.60 ± 0.28^{a}	0.49 ± 0.18^{a}	−0.21 ± 0.41^{ab}	10

N_a: number of alleles, A_r: allelic richness, 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
Haplotype frequencies of mtCOI sequences observed among mass selected orange-shell lines and wild populations.

Haplotype	Wild populations				Orange-shell lines			Total
	DY	QD	PL	RS	MS1	MS2	MS3	
A				1				1
B	15	15	15	15	20	20	20	120
C			1					1
D			1					1
E			1					1
F			1					1
G				1				1
H		2						2
I		1						1
J	1							1
K	1							1
L	1							1
M	1							1
N	1							1
O				1				1
P		1						1
Q				1				1
I		1						1
S				1				1
T			1					1
N_h	6	5	6	6	1	1	1	
H_d	0.447 ± 0.137	0.442 ± 0.133	0.447 ± 0.137	0.447 ± 0.137	0	0	0	
P_i	0.00083 ± 0.00029	0.00127 ± 0.00044	0.00115 ± 0.00043	0.00083 ± 0.00029	0	0	0	

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

D range 0.434–0.641), while differentiation within MS1–MS3 (F_{st} range 0.009–0.047; *Nei's D* range 0.009–0.058) and within wild populations (F_{st} range 0.009–0.046; *Nei's D* range 0.029–0.192) was relatively low (Table 5). Within MS1–MS3, pairwise F_{st} and *Nei's D* values between adjacent generations were gradually increasing. All the pairwise F_{st} were significantly different from zero ($P < .05$).

Neighbor-joining tree generated from the *Nei's D* is shown in Fig. 4. Seven populations fell into two clusters: one cluster includes four wild populations, and the other includes MS1–MS3. The limited variation detected in the mtDNA did not allow further analysis of genetic difference.

4. Discussion

Genetic variation is the fundamental resource on which stock improvements rely, thus capturing and maintaining as much of genetic variation as possible within domesticated populations should be the major priority for breeding operations (Lind et al., 2009). However, founding stock size is limited and difficulty are faced in sourcing wild germplasm when using rare breeds as based population. Therefore, whether genetic variability can be maintained during the mass selection with low initial diversity and high selection intensity becomes the key to applying mass selection to the genetic improvement of rare breeds. This study is the first to report genetic change of rare variant of oysters over several generations of mass selection.

Low census in rare breeds compromises the genetic variability (Cervantes et al., 2016). The current level of genetic variability of orange-shell line was assessed in reference to four outbred wild populations in China. As a result of mtDNA analysis, only one haplotype was detected in all individuals of MS1–MS3, while five or six haplotypes were detected in wild populations. Also, significant reductions in both of average allelic richness (A_r: 68.17–74.91%) and average expected heterozygosity (H_e: 34.21–39.24%) were observed in the whole orange-shell line. Reduced genetic variation in domesticated stocks is common when compared with outbred wild populations. However, the N_a of the orange-shell lines ranging from 3.60 to 4.40 was lower than that of most other strains of mollusk (about 6–26) in previous studies (Chen et al., 2017; Fu et al., 2017; Rhode et al., 2014; Wang et al., 2016; Xu et al., 2019; Zhang et al., 2018). There was no study so far on the analysis of the effects of mass selection on the genetic variability of mollusk population with such a low level of genetic variation.

Despite the large drop in N_a, A_r, and H_e between wild populations and orang-shell lines, no significant differences in observed heterozygous and fixation index were observed among all seven populations in this study. Both heterozygote deficiency (positive F_{is}) and heterozygote excess (negative F_{is}) observed in the wild populations indicate non-randomly mating in these populations (Dixon et al., 2008), probably because large-scale hatchery populations in those locations have diluted or deteriorated the natural gene pool of *C. gigas* or sampling effects (Zhang et al., 2010). Meanwhile, heterozygosity excesses were prevalent in the orange-shell lines. This pattern that allele reduction is not accompanied by a decrease in heterozygosity has also been reported in other mass selected lines of *C. gigas* (Zhang et al., 2018) and other cultured populations of aquatic species (Hillen et al., 2017; Lind et al., 2009), supporting the view that heterozygosity is not as susceptible to decline as alleles in the immediate term (Lind et al., 2009). Furthermore, heterozygotes excesses will break the direct correlation between heterozygosity and inbreeding, and F_{is} will be temporally deflated (Rhode et al., 2014).

Molecular-based relatedness estimates, which can be interpreted as the likelihood of recent coalescence for a pair of individuals relative to a reference population, may provide a suitable proxy for inbreeding (Hillen et al., 2017; Lind et al., 2009; Rhode et al., 2014). The mean genetic relatedness estimate (R) among individuals within MS1–MS3 was about 6.87–25.79 times that of the wild populations, consistent with the large reduction of alleles in MS1–MS3. Like many studies using R in place of F_{is} in selected lines and cultured populations in aquatic species (Hillen et al., 2017; Lind et al., 2009; Rhode et al., 2014; Straus et al., 2015), we also found that R can more pertinently reflect the inbreeding level of population with heterozygotes excesses.

In aquaculture, how to maintain the maximum level of genetic variability over intense selection generations is a core objective for breeders (Xu et al., 2019). The fact that hatchery-propagation can lead to reduction of genetic variability in oysters is well documented and some measures against the reduction of genetic variability have also been investigated, such as artificial spawning, balanced sex ratio and

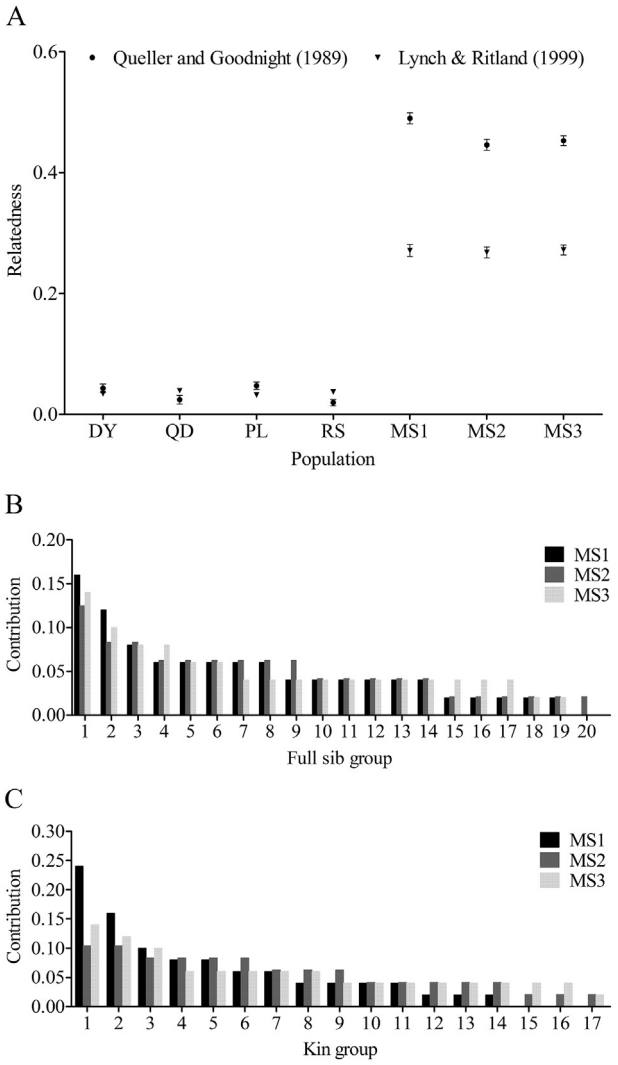

Fig. 3. A: means relatedness values (*R*) for all seven populations calculated according to the methods of Queller and Goodnight (1989) and Lynch and Ritland (1999), B: relative contributions of full-sib groups for orange-shell lines, C: relative contributions of Kin groups (mixture of full-sib group and half-sib group) for orange-shell lines.

Table 4
Analysis of molecular variance (AMOVA) for mass selected orange-shell lines and wild populations based on 20 microsatellite loci.

Source of variance	*d.f.*	*F*-statistic*	*P*
Among wild populations			
Among populations	3	0.03 (F_{st}: 0.028)	< .001
Among individuals/within population	195	0.03 (F_{is}: 0.032)	< .001
Within individuals	199	0.94 (F_{it}: 0.059)	< .001
Among generations of the orange-shell lines			
Among populations	2	0.03 (F_{st}: 0.029)	< .001
Among individuals/within population	145	0.00 (F_{is}: −0.077)	.998
Within individuals	148	0.97 (F_{it}: −0.047)	.972

Significance levels based on 9999 permutations, *percent of variation.

large size of broodstock. These measures have been applied in some strains of *C. gigas* and achieved good results (Wang et al., 2016; Xu et al., 2019), but this study is the first to apply them to a small population with such low levels of variability. No significant differences occurred in N_a, A_r, H_o, H_e and F_{is} among three generations of the orange-shell line, implying that subsequent selection for enhancing growth rate with a selection intensity of about 1.9 (Table 1) did not significantly reduce genetic variability. The reconstruction of putative full-sib and half-sib partitions provided deeper investigation into genetic variability within orange-shell line. For each outbred wild population in this study, no kin group (mixture of full-sib group and half-sib group) was larger than 5% of the whole group. However, the large skew in full-sib family representations always showed in traditional mass spawn population of oyster, in which one full-sib group can comprise up to 79% of the entire cohort (Lallias et al., 2010; Lind et al., 2009). In traditional practice of mass spawning and natural matting, whereby typically all potential broodstock are placed in a single tank and fertilization occurs at random as gametes are released (Lind et al., 2009). High fecundity and the stochastic nature of larval viability create an opportunity for relatively few contributors can dominate cohorts. However, no full-sib group from MS1–MS3 was larger than 16% of the whole group in this study. Even considering the contribution of half-sib group, no kin groups from MS1–MS3 exceed 16% of the whole group except that one kin group from MS1 comprised 24% of the entire population. This demonstrated that artificial spawning has a better control of contribution of gametes than traditional mass spawning. In addition, it can be seen from the increase of the $N_{e\text{-lin}}$ with generations that the linkage disequilibrium decay over time, conforming that the gamete contribution has been relatively well controlled during the breeding process.

In the present study, 27 of the 60 population-locus tests deviated from Hardy-Weinberg equilibrium (HWE) in the orange-shell lines, while 18 of the 80 population-locus tests deviated from HWE in the wild populations. As HWE is based on random mating in a population, it is expected that Hardy-Weinberg disequilibrium will occur in natural or artificially selected populations (Dixon et al., 2008). Similar result has been recorded in cultured and artificially selected populations of *C. gigas* (Li et al., 2006; Zhang et al., 2018).

Large genetic differentiation ($F_{st} > 0.15$) between wild populations (DY, QD, PL and RS) and orange-shell lines (MS1–MS3) and low level of genetic differentiation ($F_{st} < 0.05$) within the wild populations and the orange-shell lines were revealed by the AMOVA and pairwise F_{st} analysis. Similarly, the Neighbor-joining tree topology illustrated a clear division between the wild populations and the orange-shell lines. Genetic difference between them is most likely the result of three generations of family selection in combination with founder effects and genetic drift (Hillen et al., 2017). Furthermore, it is revealed by the pairwise F_{st} as well as *Nei's* genetic distance between the orang-shell lines that the differentiation between adjacent generations increased with the processes of mass selection. Because of small population size and genetic drift in the orange-shell line, the allele frequencies throughout generations fluctuate randomly. Similar results were also reported in other mass selected lines (Dixon et al., 2008; Chen et al., 2017; Zhang et al., 2018).

Inbreeding depression of low census populations is a problem that cannot be ignored. For aquatic species with high fecundity, inbreeding depression at an early stage could have been hidden by culling practices commonly used in hatcheries (Taris et al., 2007). Although the genetic variability of this orange line were maintained over generations of intense mass selection, evidence of inbreeding depression was observed in the line at early life stage (Han and Li, 2018). Overcoming problem of inbreeding depression of rare breeds is important for the sustainable use of these rare germplasm resources.

In conclusion, the genetic variability of orange-shell line with very low initial variation successfully maintained during three generations of mass selection with high selection intensity. Pedigree reconstructions revealed artificial spawning used in this study have a better control over the contribution of gametes than traditional mass spawning. This will provide a reference for carrying out genetic improvement programs on rare breeds where small populations are inevitable.

Table 5
Pairwise F_{st} (lower diagonal) and *Nei's D* (upper diagonal) values between mass selected orange-shell lines and wild populations based on 20 microsatellite loci.

	DY	QD	PL	RS	MS1	MS2	MS3
DY		0.041	0.118	0.133	0.569	0.547	0.534
QD	0.013		0.179	0.192	0.641	0.614	0.581
PL	0.030	0.046		0.029	0.460	0.452	0.434
RS	0.031	0.045	0.009		0.508	0.483	0.457
MS1	0.192	0.197	0.177	0.184		0.009	0.058
MS2	0.178	0.184	0.166	0.170	0.009		0.034
MS3	0.180	0.185	0.166	0.169	0.047	0.028	

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

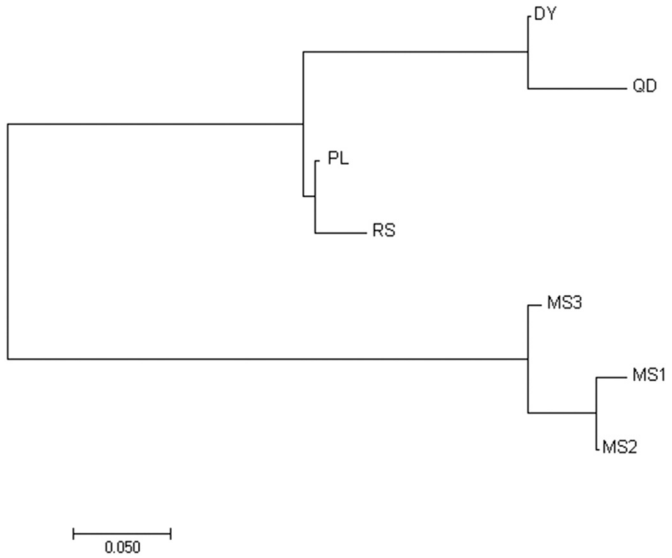

Fig. 4. Neighbor-joining tree of all seven populations based on *Nei's* unbiased genetic distance.

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