Aquaculture 500 (2019) 338–346

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Genetic diversity and effective population size in successive mass selected generations of black shell strain Pacific oyster (*Crassostrea gigas*) based on microsatellites and mtDNA data

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ARTICLE INFO

Keywords:
Crassostrea gigas
Black shell strain
Genetic diversity
Effective population size
Mass selection
Artificial breeding strategy

ABSTRACT

The Pacific oyster (*Crassostrea gigas*) is one of the highly fecund marine aquaculture species and has a large variance in reproductive success, these characteristics can result in loss of genetic diversity and inbreeding especially when conducting mass selection. In the current study, we investigated the level of genetic diversity and effective population size over three successive mass-selected generations in the black shell strain of *C. gigas* assessed by 11 microsatellites as well as mitochondrial COI region. The results showed that the genetic variation over three generations were maintained since no detectable depression of expected heterozygosity (H_e = 0.647–0.681), number of alleles (N = 5.6–6.0) and haplotypes (N_h = 2). The difference in alleles and haplotypes number between the base population (BP) and three mass selective generations (M5-M7) could be mainly due to the loss during preceding family selection process. Pair-wise F_{ST} values along with AMOVA analysis from both markers indicated unremarkable differentiation within each generation including BP. There was no deleterious effect on genetic diversity and population structure for mass selected generations imposed by our artificial breeding practice. The N_{eb} estimation for M5, M6 and M7 was 29.8, 40.7 and 52.7, respectively. The different number of broodstocks used for each generation implied that using a balanced sex ratio and large size of broodstock as well as low selection pressure would help to increase the effective population size and avoid high level of inbreeding. The estimated linkage disequilibrium-based effective breeders size (N_{eb}) was significantly lower than sex-ratio correction effective population size (N_e), which indicated high variance in family contribution. It is suggested during mass spawning to simply undertake several mini-spawn groups and then pool the embryos to produce next generation, which can be a hedge to the high variance in reproductive success, thus, the loss of effective populations size and diversity. Other practical strategies against the loss of genetic diversity were also discussed, however, it remains to be investigated how diverse selection and follow-up procedures will benefit retaining genetic variability. This study will provide an insight into the level of genetic diversity and effective population size within mass-selected black shell line and enable a better understanding of how efficient current breeding practices are at maintaining genetic variation. This information can be applied for future selective breeding program and in the design of suitable management guidelines for sustainable breeding of *C. gigas*.

1. Introduction

For almost all selective breeding programs in aquaculture, it's required to close the population by just selecting the offsprings from the initial broodstocks (base population) without adding wild animals into breeding nucleus in the following breeding processes. Wild animals are genetically lagged and may degrade genetic selection response of the line. However, wild population, from which the base populations are selected, is assumed to be large randombred populations possessing high levels of genetic diversity (Frankham, 1996; In et al., 2016). If without advisable precautions throughout the culture procedure, this high genetic diversity of natural population accumulated in thousands of years can be eroded even in as little as a single generation (Jackson et al., 2003; Lind et al., 2009; Porta et al., 2007).

In artificial selected populations, intensified processes of genetic drift such as founder effects and differential family survival as well as

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https://doi.org/10.1016/j.aquaculture.2018.10.007
Received 20 April 2018; Received in revised form 16 September 2018; Accepted 5 October 2018
Available online 07 October 2018
0044-8486/

non-random mating can lead to loss of genetic diversity, which can impact availability of desirable genes and increase the risk of inbreeding depression, as well as concordant overall decline in performance and fitness (Evans et al., 2004; Hartl and Clark, 1989; Wang et al., 2002). Loss of variation can be observed even without directive selection since effective population sizes (N_e) can be diminished during mass selection resulting from the large variances of reproductive success in highly fecund and broadcast spawning species like oysters and shrimp (Boudry et al., 2002; Goyard et al., 2003), where the broodstocks can be contributed by relatively few individuals. Maintaining a large N_e is essential to minimize the effect of inbreeding and loss of genetic variability while selecting for better industrial performance. The loss in genetic diversity and effective population size have been widely reported in long term mass selection or hatchery lines, including finfishs (Frost et al., 2006), crustaceans (Sbordoni et al., 1986; Vela-Avitúa et al., 2013) and molluscs (Chen et al., 2017; Lind et al., 2009). It's a primary concern as well as challenging problem for aquaculture industry as how to best avoid loss of genetic diversity over successive breeding generations. A sufficient level of genetic diversity within population not only will enhance its adaptation to new environments and withstand to disease outbreaks (Gamfeldt and Källström, 2007), but also is critical for the continuous genetic gains and improvements in sustainable breeding program.

The Pacific oyster *Crassostrea gigas* is one of the most cosmopolitan marine species, having been introduced from northwest Pacific to dozens of countries due to its high fecundity, fast growth rate and strong acclimatization. A number of breeding programs have been initiated over the years because of its commercial importance (Dégremont et al., 2010; Evans and Langdon, 2006; Hershberger et al., 1984; Li et al., 2011). In addition to growth rate, yields and disease resistance, its coloration is also of interest to the whole oyster industry and has been a target trait for selection (Kang et al., 2013; Nell, 2001; Song et al., 2016) as well as regarded as a new high potential trait for a better commercial value (Ward et al., 2000; Brake et al., 2004). For example, Pacific oyster with black mantle are favored by consumer and valued 20% higher price in Korea (Kang et al., 2013), and a small niche market for “golden” oysters has been developed in Asia (Nell, 2001), which indicated that black shell strain oyster also has potential to alter consumer preference and marketable value.

In our selective breeding practice of black shell strain, four generations of family selection following by three generations of mass selection were established (Fig. 1). There is considerable additive genetic variance in growth and shell color traits in this strain (Xu et al., 2017), which will make it feasible to improve these traits genetically through breeding scheme to bring about potential economic benefits. However, the overlook of avoiding the loss of valuable genetic variability over ensuing generations would limit the potential for continuous genetic gain and response to selection, which haven't been investigated in artificial breeding black shell strain of Pacific oyster. This study is to aim at evaluating whether the genetic diversity is maintained in black shell line over mass selection generations assessed by genomic microsatellite as well as mitochondrial DNA markers.

Fig. 1. A typical individual from M6 generation of black shell strain *C. gigas* compared to an unselected cultured oyster.

2. Materials and method

2.1. Selection, spawning and breeding protocols

In 2010, *C. gigas* from natural seed with relatively black shell color (some black stripes and/or darker shell color and/or bigger proportion of black color) in Rushan, Shandon, China were selected as base population (BP) to start this line. In order to fix the black shell trait, four consecutive generations of family selection (F1–F4) were established from 2010 to 2013. Briefly, one hundred individuals (50 sire and 50 dam) from BP were selected to found 50 full-sib families as the first generation (F1). Only the offsprings with greater black proportion in shell from families which have distinct black shell color, without shell color separation and with obvious growth were used as broodstocks to establish next generation. Under the same criterions, 41, 38 and 32 families were bred from F2 to F4 generations, respectively. Stable inheritance of shell color and pure black color in whole shell were achieved after four generations of family selection, whereas there was no superior productivity observed. Therefore, truncation selections for shell height were initiated to construct mass selection generations for both rapid growth and black shell color. In the summer of 2014, five families from F4 generations were preferable and individuals with greatest shell height as well as black color in whole shell from these families were used as broodstocks for the first generation of mass selection (M5, Wang et al., 2016a). In a similar fashion, truncation selections were implemented for the next two successive generations of mass selection (M6 and M7) in 2015 and 2016, respectively. The number of the parents used to produce each generation of selected lines are shown in Table 1. Selection intensity for each generation were 6.0% for M5 with truncation point of 60.31 mm in shell height (Wang et al., 2016a), 12.0% for M6 with truncation point of 62.00 mm in shell height and 13.3% for M7 with truncation point of 62.04 mm in shell height (Xu et al., unpublished data) respectively. Around one month prior to spawning, broodstock candidates were collected into a concrete tank and supplied with mixed algal diet of *Isochrysis galbana*, *Nitzschia closterium* and *Chaetoceros calcitrans*, and stimulated to spawn by manipulating water temperature. After gonadal maturation, males and females were selected and stripped to collect gametes into separate buckets. For the purpose of providing equal mating chances for each parent, equal amounts of oocytes from each female were mixed well

Table 1
Sample sizes of black shell strain mass selection lines and wild populations of *C. gigas*.

Populations	Number of parents	N_m	N_f	Date of sampling	Sample size for microsatellites	Sample size for COI
BP	–	–	–	06/2010	47	20
M5	60	30	30	08/2015	48	20
M6	75	23	52	01/2016	48	20
M7	90	40	50	01/2017	45	20
WP	–	–	–	03/2016	48	20
WR	–	–	–	03/2016	48	20

Notes: BP, Base population; M5, first-generation mass selection line; M6, second-generation mass selection line; M7, third-generation mass selection line; WP, wild population from Penglai; WR, wild population from Rushan; N_m, number of female broodstock; N_f, number of female broodstock.

after estimating concentrations using a microscope as well as sperm from each male. Then the mixture of oocytes and sperm were used for fertilization. After 24-h incubation, D-larvae were randomly collected from hatching tank and stocked into a 24-m^3 larval rearing tanks. The nursery and grow-out of the larvae, spat, and adults was carried out using standard practices as per Li et al. (2011). The spats were cultured on ropes suspended from rafts along the coastal regions of Rushan Bay in Yellow Sea, Shandong, China.

2.2. Sampling and DNA extraction

We randomly collected oysters from the base population (BP), three generations of mass selection and two wild populations from Penglai (37.7°N, 121.0°E) in Bohai Sea (WP) and Rushan (36.8°N, 121.6°E) in Yellow Sea (WR) respectively. Genomic DNA of each oyster was extracted from adductor muscle by standard protocol of proteinase K digestion, phenol-chloroform extraction and DNA precipitation (Li et al., 2006), and then dissolved in 1× TE buffer storing at −30 °C until further analysis.

2.3. Microsatellite analysis

In our study, we applied a fast and cost-efficient protocol which enables scoring of 11 microsatellite loci in 4 multiplex PCR sets developed by (Liu et al., 2017). Panel 1 (ucdCg-120, ucdCg-198, and ucdCg-117), Panel 2 (Crgi3, ucdCg-146, and uscCgi-210), Panel 4 (otgfa0_0129_E11 and otgfa0_0007_B07) and Panel 6 (otgfa0_408293, otgfa0_0139_G12 and ucdCg-20) were selected for polymerase chain reaction analysis according to the conditions described therein. For genotyping, PCR products were electrophoresed on capillary sequencing-based ABI PRISM 3130 Automated DNA Sequencer (Applied Biosystems) with GeneScan LIZ 500 (Applied Biosystems) as internal size standard. Allele size scoring was performed automatically by GeneMapper v.4.0 (Applied Biosystems) and adjusted manually.

For each population and locus, the number of alleles (N), expected heterozygosity (H_e) and observed heterozygosity (H_o) were calculated using MICROSATELLITE ANALYSER v.4.05 (Dieringer and Schlötterer, 2003). Allelic richness(A_r) as a standardized index of the number of alleles independent of sample size was computed by FSTAT v.2.9.3 (Goudet, 2001). The inbreeding coefficient F_{is} (Weir and Cockerham, 1984) was calculated to address the Hardy-Weinberg equilibrium (HWE) deviation with the same program. A nonparametric analysis of variance (Kruskal-Wallis test) was performed to test the differences of the number of alleles and H_e among generations and populations. Fisher's exact test for deviations from HWE were performed in GENEPOP v.4.0 (Rousset, 2008). The polymorphism information content (*PIC*) were derived by CERVUS v.3.0 (Kalinowski et al., 2007). The null allele frequencies for each locus and population were estimated by the EM (expectation maximization) algorithm (Dempster et al., 1977) implemented in the software FreeNA (Chapuis and Estoup, 2007), which was also used to efficiently correct possible bias caused by the presence of null alleles in F_{ST} estimation conducting ENA (excluding null alleles) correction method. The analysis of molecular variance (AMOVA), assessed with exact tests based on 10,000 permutations, was conducted to estimate components of genetic variance within and among groups (BP vs three mass-selected generations) by ARLEQUIN v.3.5 (Excoffier and Lischer, 2010).

2.4. Effective population size

The unequal sex ratio correction method was used to calculate the effective population size of each generation as: $N_e = 4\,N_m N_f/(N_m + N_f)$ (Falconer and MacKay, 1996), where the N_m and N_f are the number of males and females broodstocks respectively. We also estimated number of effective breeders (N_{eb}) based on linkage equilibrium method in NeEstimator 2.1 (Do et al., 2014). This method was shown to performs well in estimating N_e in non-ideal populations with skewed sex ratio or non-random variance in reproductive success (Waples, 2006). The 95% confidence intervals (CIs) were also given. Additionally, the rate of inbreeding (ΔF) was calculated as $\Delta F = 1/2(N_e)$ (Falconer and MacKay, 1996).

2.5. Mitochondrial COI analysis

The mitochondrial cytochrome C oxidase subunit I (COI) was amplified using universal primers (LCO1490 and HCO2198) in 30 μl reactions at the condition specified in (Folmer et al., 1994). PCR products were checked on agarose gel, extracted in slices from the gel, purified and then sequenced on an ABI 3730XL (Applied Biosystems) automatic sequencer by the Sangon Biotech Limited Company (Shanghai, China) for both directions. The sequences obtained were edited with Bioedit (Hall, 1999) and aligned using Clustal W (Larkin et al., 2007).

DNASP v.5.10 (Librado and Rozas, 2009) was used to estimate the number of haplotypes (N_h), haplotypic diversity (h) and percent nucleotide diversity (π). Hierarchical F_{ST} and AMOVA were used to assess the genetic differentiation among selected generations within Arlequin v.3.5 (Excoffier and Lischer, 2010). The groups assignment for AMOVA analysis was the same as microsatellite, i.e. BP versus three selection generations. The significance of each pairwise comparison was tested with 10,000 permutations. The evolutionary relationships among mtDNA haplotypes were represented by a median-joining (MJ) networks (Bandelt et al., 1999) constructed in Popart 1.7 (Leigh and Bryant, 2015). For all cases of multiple tests, the significance levels were adjusted with the sequential Bonferroni correction (Rice, 1989).

3. Results

3.1. Microsatellite allele diversity

Number of alleles (N), allelic richness (A_r), observed (H_o) and expected (H_e) heterozygosity for the 11 microsatellite loci are summarized in Table 2. By using 11 genomic microsatellite loci, a total of 218 alleles were detected, while the degree of diversity was different at each locus (Kruskal-Wallis test, $df = 10$, $P = 0.019$). Average numbers of alleles per locus ranged from 5.6 in the M7 generation to 14.6 in the WR population. The allele number for three mass-selected generations was significantly lower than that in BP and two wild populations respectively ($df = 3$, $P = 0.02$; $df = 4$, $P < 0.01$). Mean H_o and H_e values in all populations ranged from 0.583 to 0.731 and from 0.647 to 0.785, respectively. Although the average H_e values in all three selection generations were slightly smaller than BP, there was no significant difference in H_e among the BP and three mass-selected generations ($df = 3$, $P = 0.18$). Within three successive selection generations (M5-M7), no significant decline in number of allele ($df = 2$, $P = 0.89$) was detected as well as the expected heterozygosity ($df = 2$, $P = 0.79$). There was no significant difference in N, H_o, H_e, and A_r between BP and two wild populations, although BP have smaller values than wild populations. The frequencies of null alleles per locus ranged from 0.008 to 0.145 with an average of 0.050 (only otgfa0_0007_B07 was higher than 0.1, Table 2).

The observed genotype frequencies were tested for agreement with HWE after adjusting the P values across the 11 loci using the sequential Bonferroni correction. Twenty out of the 66 locus-population combinations deviated from HWE, especially for locus otgfa0_0007_B07, where 5 out of 6 population showed deviation from HWE. In all 20 deviated combinations, 15 cases indicated heterozygote deficiency with a mean null frequency of 13.8%, which suggested that the presence of null allele is one of the main causes for departure from HWE.

3.2. Effective population size

Numbers of male and female broodstock used to produce each

Table 2
Summary of the statistics for eleven microsatellite loci in the black shell strain mass selection lines and wild populations of *C. gigas*.

Multiplexes of microsatellite	Loci	Parameter	BP	M5	M6	M7	WP	WR
Panel1	ucdCg-120	*N*	7	3	3	3	13	13
		H_o	0.617	0.708	0.500	0.600	0.708	0.729
		H_e	0.640	0.524	0.552	0.521	0.777	0.752
		A_r	6.6	3.0	3.0	3.0	12.1	11.8
		F_{is}	0.036	−0.358	0.095	−0.153	0.089	0.031
		F(null)	0.000	0.000	0.016	0.000	0.033	0.001
		PIC	0.568	0.404	0.479	0.404	0.739	0.709
	ucdCg-198	*N*	15	7	8	8	20	16
		H_o	0.702	0.917	0.792	0.733	0.729	0.833
		H_e	0.860	0.829	0.838	0.842	0.911	0.923
		A_r	14.1	6.8	7.8	8.0	18.5	15.5
		F_{is}	0.176	**−0.108**	0.056	0.131	**0.202**	0.098
		F(null)	0.081	0.000	0.021	0.046	0.092	0.051
		PIC	0.826	0.795	0.807	0.811	0.894	0.907
	ucdCg-117	*N*	24	9	9	9	28	30
		H_o	0.609	0.854	0.792	0.867	0.851	0.913
		H_e	0.944	0.834	0.857	0.828	0.963	0.965
		A_r	22.7	8.7	9.0	8.7	27.1	28.4
		F_{is}	**0.358**	**−0.024**	0.077	−0.047	0.117	0.054
		F(null)	0.169	0.020	0.029	0.000	0.054	0.012
		PIC	0.930	0.802	0.841	0.799	0.951	0.952
Panel2	Crgi3	*N*	8	4	4	3	12	11
		H_o	0.447	0.625	0.667	0.467	0.896	0.417
		H_e	0.438	0.458	0.532	0.539	0.784	0.526
		A_r	7.6	3.9	4.0	3.0	11.114	10.1
		F_{is}	−0.021	−0.370	−0.256	0.135	**−0.144**	0.209
		F(null)	0.000	0.000	0.000	0.053	0.000	0.085
		PIC	0.420	0.383	0.454	0.469	0.747	0.501
	ucdCg-146	*N*	18	9	9	9	22	19
		H_o	0.787	0.917	0.771	0.844	0.771	0.854
		H_e	0.929	0.797	0.826	0.862	0.946	0.929
		A_r	17.0	8.8	9.0	9.0	21.1	18.2
		F_{is}	**0.154**	**−0.152**	0.068	0.021	**0.187**	0.081
		F(null)	0.067	0.002	0.015	0.000	0.090	0.029
		PIC	0.913	0.762	0.799	0.838	0.933	0.913
	uscCgi-210	*N*	6	5	4	4	9	7
		H_o	0.617	0.792	0.688	0.667	0.646	0.583
		H_e	0.679	0.661	0.635	0.675	0.704	0.641
		A_r	6.0	4.9	4.0	4.0	8.7	6.7
		F_{is}	0.092	−0.199	−0.083	0.013	0.084	0.091
		F(null)	0.061	0.000	0.000	0.000	0.037	0.031
		PIC	0.622	0.595	0.564	0.605	0.649	0.578
Panel4	otgfa0_0129_E11	*N*	9	7	7	7	12	17
		H_o	0.723	0.563	0.771	0.705	0.813	0.958
		H_e	0.843	0.586	0.708	0.740	0.798	0.856
		A_r	8.8	6.8	7.0	6.9	11.7	15.4
		F_{is}	**0.143**	0.041	−0.09	0.049	−0.018	−0.121
		F(null)	0.072	0.056	0.000	0.000	0.031	0.000
		PIC	0.814	0.547	0.674	0.703	0.769	0.832
	otgfa0_0007_B07	*N*	8	7	6	5	7	10
		H_o	0.630	0.500	0.333	0.237	0.458	0.511
		H_e	0.801	0.666	0.691	0.660	0.584	0.636
		A_r	7.5	6.8	5.8	5.0	6.5	9.4
		F_{is}	**0.214**	**0.251**	**0.520**	**0.644**	**0.217**	0.199
		F(null)	0.100	0.119	0.212	0.255	0.082	0.104
		PIC	0.762	0.630	0.647	0.608	0.543	0.602
Panel6	otgfa0_408293	*N*	11	6	6	5	13	13
		H_o	0.851	0.521	0.333	0.444	0.875	0.875
		H_e	0.841	0.534	0.389	0.408	0.849	0.863
		A_r	10.8	5.7	5.7	4.8	12.3	12.7
		F_{is}	−0.012	0.024	0.144	−0.101	−0.031	−0.014
		F(null)	0.000	0.025	0.081	0.000	0.000	0.005
		PIC	0.812	0.469	0.360	0.391	0.822	0.839
	otgfa0_0139_G12	*N*	5	5	5	5	10	11
		H_o	0.745	0.255	0.229	0.400	0.500	0.542
		H_e	0.561	0.536	0.506	0.750	0.441	0.529
		A_r	5.0	4.6	4.9	5.0	8.9	10.2
		F_{is}	−0.333	**0.527**	**0.550**	**0.470**	−0.135	−0.024
		F(null)	0.000	0.173	0.198	0.193	0.000	0.040
		PIC	0.513	0.479	0.463	0.696	0.418	0.507
	ucdCg-200	*N*	8	4	4	4	14	14
		H_o	0.596	0.813	0.604	0.444	0.792	0.729
		H_e	0.857	0.694	0.729	0.664	0.875	0.887
		A_r	8.0	4.0	4.0	4.0	12.9	13.4

(*continued on next page*)

Table 2 (*continued*)

Multiplexes of microsatellite	Loci	Parameter	BP	M5	M6	M7	WP	WR
		F_{is}	**0.307**	**−0.173**	0.172	**0.333**	0.096	0.180
		F(null)	0.133	0.000	0.071	0.122	0.036	0.081
		PIC	0.829	0.632	0.670	0.602	0.852	0.866
Aeverage		*N*	10.8	6.0	5.9	5.6	14.5	14.6
		H_o	0.666	0.679	0.589	0.583	0.731	0.722
		H_e	0.763	0.647	0.660	0.681	0.785	0.773
		A_r	10.4	5.8	5.8	5.6	13.7	13.8
		F_{is}	0.101	−0.049	0.114	0.136	0.060	0.071
		F(null)	0.062	0.036	0.058	0.061	0.041	0.040
		PIC	0.728	0.591	0.614	0.630	0.756	0.746

Notes: *N*, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; A_r, allelic richness; F_{is}, inbreeding coefficient; *F (null)*, null allele frequency; *PIC*, polymorphic information content; Probability of significant deviation from HWE are given for each population per locus, bold type indicates significant deviations after Bonferroni correction ($P < 0.01/11$).

Table 3
The effective population size (N_e) and inbreeding rate (ΔF) in the selected black shell strains of *C. gigas*.

Population	N_e-sex ratio correction		Ne-linkage disequilibrium		
	N_e	ΔF	N_{eb}	95% CI (lower–uper)	ΔF
BP	–	–	199.2	116.1–583.6	0.25%
M5	60.0	0.83%	29.8	23.3–39.1	1.68%
M6	63.8	0.78%	40.7	30.4–57.5	1.23%
M7	88.9	0.56%	52.7	36.0–87.4	0.95%

Notes: N_e-sex ratio correction, the estimated effective numbers of broodstock corrected for sex ratio by $4N_mN_f/(N_m + N_f)$.

generation is given in Table 1. The effective population size (N_e), number of effective breeders (N_{eb}) and rate of inbreeding (ΔF) are shown in Table 3. The unequal female and male parents used in M6 and M7 makes that the estimated N_e corrected for sex ratio (63.8 for M6 and 88.9 for M7 respectively) were lower than the actual numbers of parents (75 for M6 and 90 for M7 respectively). The N_{eb} calculated by linkage disequilibrium method of M7 (N_{eb} = 52.7) was greater than M5 (N_{eb} = 29.8) and M6 (N_{eb} = 40.7) generations due to a larger number and more balance sex ratio of parents. The ΔF for M5-M7 generated by N_{eb} was 1.68%, 1.23% and 0.95%, respectively.

3.3. mtDNA haplotype diversity

A 606-bp fragment of the COI region was obtained for 120 individuals in all 6 populations. Overall, 10 polymorphic sites defined 10 haplotypes (Table 4). All of haplotypes were deposited into the Genbank database (accession numbers: MH160012-MH160021). Number of haplotypes per population ranged from 2 to 5 with haplotype diversity (*h*) ranged from 0.279 to 0.479. Seven haplotypes were found to be private which are present only in one population, while the haplotype H2 are dominant and shared by all six populations, which was also the dominant one within each population. There was no loss of haplotypes in mass-selected generations, which all have 2 haplotypes, although that number is smaller than that in BP (3 haplotypes) and two wild populations (5 haplotypes). Median-joining network of haplotypes indicated that the haplotypes H2 and H3 were shared by BP and three selection generations (Fig. 2), while the haplotype H1 in BP might be lost during family selection from F1 to F4. The range of the percentage nucleotide diversity (π) for six populations varied from 0.048% (BP) to 0.083% (WP).

3.4. Genetic differentiation among populations

Matrices of pairwise F_{ST} of all populations derived by microsatellites and COI region are given in Table 5 respectively. For microsatellite, overall values of F_{ST} was 0.071 ($P < 0.01$). The F_{ST} between BP and three mass-selected generations are moderate and varied from 0.070 (between BP and M7) to 0.094 (between BP and M5). However, there was low genetic differentiation within M5, M6 and M7 generations with F_{ST} ranged from 0.006 to 0.034. For mitochondrial COI, the F_{ST} value revealed no subdivision between these selection generations (Table 6), in accordance with the result from mitochondrial AMOVA analysis. The AMOVA analysis indicated no significant genetic differentiation at all levels (among groups, among populations within groups, and within populations) for COI datasets, while results for the nuclear microsatellite datasets indicated significant amount of variance at two levels (among populations within groups and within populations). Both microsatellite and COI datasets indicated a majority of the total molecular variance was distributed within populations, which suggested that there was low to no variation among BP and selection generations as well as within selected generations.

4. Discussion

In aquaculture, how to maintain the maximum level of genetic variability in consecutive breeding process for long period is a core objective for breeders. It is generally believed that there is a trend of reduction in genetic diversity of cultured stocks undergone artificial breeding in high fecundity marine organisms like oyster (Hedgecock, 1994; Hedgecock et al., 1992). Hedgecock and Pudovkin (2011) regard the reproductive success in highly fecund shellfish as a sweepstakes event due to a combined effect of high fecundity and the stochastic nature of larval viability. Successful reproduction in these shellfish significantly vary among individuals because it requires success at each step of a complex chain of events including gamete maturation, fertilization, larval development, settlement, recruitment to the adult spawning population, etc. This high variance in reproductive success combined with high genetic load in hatchery-spawned oysters can result in severe inbreeding depression (Bierne et al., 1998; Evans et al., 2004; Launey and Hedgecock, 2001; Plough, 2016; Plough et al., 2016), which may severely limit the long-term viability of selective breeding. Thus, the level of genetic diversity and size of effective population breeders in breeding lines of oyster are extremely important for its sustainability.

4.1. Genetic diversity reveled by microsatellites and mtDNA COI region

In this study, we investigated the genetic variability of three successive mass-selected generations in black shell strain using both nuclear and mitochondrial DNA markers. Microsatellites and mitochondrial COI sequences are widely used in combination to estimate genetic variability, population genetic structure and demographic history in many bivalves (Cordero et al., 2017; In et al., 2016; Xue et al., 2014).

Table 4
Genetic diversity of selected strain and wild populations of *C. gigas* at mtDNA COI region.

Population	N_h	Haplotypes										h	π(%)
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		
BP	3	2	17	1								0.279 ± 0.123	0.048 ± 0.022
M5	2		15	5								0.395 ± 0.010	0.065 ± 0.017
M6	2		14	6								0.442 ± 0.087	0.073 ± 0.014
M7	2		13	7								0.479 ± 0.072	0.079 ± 0.012
WP	5		16				1		1	1	1	0.368 ± 0.135	0.083 ± 0.036
WR	5		16		1	1	1	1				0.368 ± 0.135	0.066 ± 0.027
overall	10	2	91	19	1	1	2	1	1	1	1	0.402 ± 0.026	0.075 ± 0.011

Notes: N_h, number of haplotypes; Haplotypes, number of individuals for each haplotypes; h, haplotypic diversity (± standard deviations); π, percent nucleotide diversity (± standard deviations).

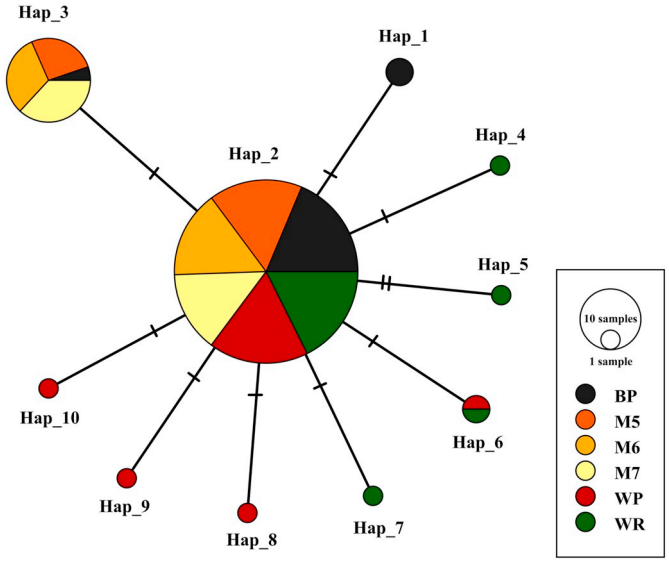

Fig. 2. Median-joining (MJ) networks of 10 COI haplotypes of selection lines and wild populations of *C. gigas*. The number of substitutions separating two haplotypes was indicated by the vertical bars on the line.

Table 5
Estimated pairwise F_{ST} values of *C. gigas* based on microsatellite makers (below diagonal) and mtDNA COI (above diagonal).

	BP	M5	M6	M7	WP	WR
BP	–	0.088	0.129	0.174	0.013	0.015
M5	0.094*	–	−0.046	−0.028	0.106	0.117
M6	0.087	0.006*	–	−0.047	0.144*	0.158
M7	0.070*	0.034	0.024	–	0.185*	0.201*
WP	0.055	0.129*	0.125*	0.110*	–	−0.011
WR	0.013*	0.091*	0.090*	0.073*	0.026	–

Table-wide significance levels were applied using the sequential Bonferroni correction (Rice, 1989).

* Significant at $P < 0.05/15$.

Our results indicated that there was no remarkable decline in heterozygosity, number of alleles as well as haplotypes among this mass selection line. Moreover, the lack of a significant level of pair-wise F_{ST} and hierarchical F_{ST} values from both markers suggest no detectable differentiation in the generations over the time period examined. There was no deleterious effect on genetic diversity and population structure for the three successive mass selected generations imposed by our artificial breeding practice. The reduction of alleles and haplotypes numbers from the base population (BP) to mass-selected generations (M5, M6 and M7) may mainly occurred at the previous family selection generations (F1–F4) which were focus on fixing the black shell color.

Table 6
Analysis of molecular variances (AMOVA) of microsatellites and mtDNA COI among the base population and selection generations of *C. gigas*.

Source of variation	*df*	Variance components	Percentage of variation	F-statistics
For microsatellites				
Among groups	1	0.29853	7.24	F_{CT} = 0.07239
Among populations within groups	2	0.08204	1.99	F_{SC} = 0.02145*
within populations	372	3.74341	90.77	F_{ST} = 0.09228*
Total	375	4.12398		
For mtDNA COI				
Among groups	1	0.02208	10.32	F_{CT} = 0.10321
Among populations within groups	2	−0.00747	−3.49	F_{SC} = −0.03892
within populations	76	0.19934	93.17	F_{ST} = 0.06831
Total	79	0.21396		

* Significant at $P < 0.01$.

However, there was no significant loss of heterozygosity among BP and M5-M7 generations. Consistent with other researches, we found that the loss of alleles was generally more easily observed than decline of heterozygosity in selected strains and cultured stocks of aquatic species (Appleyard and Ward, 2006; Dillon Jr and Manzi, 1987; Yu and Guo, 2004). As for HWE, 20 of 60 population-locus cases deviated from the expectations in total, which can be mainly attributed to the presence of null alleles because of the evidence that all 15 homozygote excess cases have an average null allele frequency of 0.138. The high prevalence of null alleles has been observed previously in studies of mollusk species especially oysters (Astanei et al., 2005; In et al., 2016; Li et al., 2003; Reece et al., 2004). In the study of Astanei et al. (2005), after correcting the data for null alleles, the number of departures from HWE decreased dramatically (also see in Wang et al., 2016b). Besides, other factors like non-random mating, Wahlund effect, artificial and nature selection during seed production and cultivation can also result in deviation to HWE.

There were relatively few different haplotypes in all populations including two wild populations (five haplotypes for both) in this study. Relatively low levels of haplotypic diversity (2–9 haplotypes) of mitochondrial COI sequences were also reported in natural *C. gigas* populations by Li et al. (2015), in spite of numerous sampling sites (12 locations) in the northwestern Pacific including China, Korea and Japan. Accordingly, the parsimonious reasoning is that the low number of haplotypes is probably not an evidence of inbreeding in the black shell lines per se, but a reflection of the overall level of COI haplotype diversity in the species (also see an example in In et al., 2017).

4.2. Effective population size

In general, the effective breeder population size is conditioned by farming constraints, resulting in limited number of parents contributed

to the next generation. N_e can be depressed because of insufficient broodstocks, biased sex ratio, unequal contribution of gametes and different viability of gametes (Li et al., 2007). Alternatively, the N_e can be restricted simply by selecting individuals based on best performance of certain valuable traits (like fast-growing) which may just from a few outstanding families. In this study, we combined both the fast growth and black shell as target traits jointly, which can be a hedge against just selecting a few families. The using of broodstocks chose from low N_e cohorts, which are more likely to be close related, can lead to inbreeding depression and lower the response to selection (Bentsen and Olesen, 2002). Besides, it will also impact the production performance, for example, Evans et al. (2004) estimated that there would be 8.8% decrease in body weight, 4.26% decrease in survival and 12.23% decrease in yield when inbreeding coefficient increase 10% in *C. gigas*.

We use linkage disequilibrium (LD) method to estimate the number of effective breeders, which thought to be more reliable than the heterozygous excess (HE) estimates, especially within a limited generational interval and small sample size (Waples and Do, 2010). Also, the HE-based methods are prone to bias given that heterozygote deficiencies and null alleles are reported to show in marine bivalves natural populations (Lind et al., 2009). In this study, the LD-based N_{eb} for M5, M6 and M7 was 29.8, 40.7 and 52.7, respectively (Table 3). The broodstock of M5 generation was from several families in F4 generations, which might have related genetic relationships. In addition, the selection pressure of M5 generation was as high as 6.0% (Wang et al., 2016a), those were the two primary reasons for low N_{eb} in M5. The selection pressure for M6 and M7 are 12.0% and 13.3%, respectively (Xu et al., unpublished data). The tradeoff between selection pressure and number of broodstock is critical for the success of an artificial breeding program. The higher selection intensity and smaller breeder size will increase the genetic gains, but likewise, it will intensify sibship among broodstocks and consequently result in inbreeding and loss of genetic diversity. The latter in return might impede resilience to environmental fluctuations of population and potential for future respond to selection. Bentsen and Olesen (2002) found that for traits affected by inbreeding depression, the response to selection will be further reduced with designs based on low numbers of broodstock pairs. It means that with the given certain inbreeding rate, lower number of broodstock will cause more severe recession of response to selection than higher number of broodstock (example within Bentsen and Olesen, 2002). Besides, due to insufficient males for broodstock, there were remarkable bias sex ratio for M6 generation. In a simulation study, Gjerde et al. (1996) fund that in mass selection designs, it's required to have 50–100 matings at a ratio of 1 male:2 females and 150–250 matings at a ratio of 1 male:10 females to keep the rate of inbreeding at 1% level. Thus, a balancing sex ratio for broodstock is also recommended for keeping a sufficient level of effective population size. The M7 generation has the highest N_{eb} compared to M5 and M6, which may be primarily benefitted by using a large breeder size, more balanced sex-ratio of broodstocks and less selection pressure and has an acceptable inbreeding level of 0.95%. In addition, equal proportions of oocytes and sperm from each parent were used to provide equal mating opportunities.

Nonetheless, even in M7 generation, the N_{eb} was 41.4% lower than the actual number of spawned individuals which most likely reflects the high variance in family contribution commonly observed in mass spawning marine species, for example, barramundi (*Lates calcarifer*) (Domingos et al., 2014), Nile tilapia (*Oreochromis niloticus*) (Ponzoni et al., 2010) and several oyster species (Gaffney et al., 1992; Hedgecock et al., 1992; In et al., 2016; Launey et al., 2001; Lind et al., 2009). The outcome of large skews in family size in oysters may be attributed to different survival rates between different families as well as the loss of slow growing families from grow-out system-when sieve sizes are increased (Beaumont and Hoare, 2003). But more importantly, the decline of effective population size compared to the actual number of broodstocks can be mainly due to large variance in reproductive success mentioned above. The Pacific oyster is amenable to strip-spawning by cutting the oyster open and teasing out the gametes which can provides more possibility to manipulate reproduction. However, there is no guarantee that stripped gametes from all individuals are fully ripe and able to fertilize despite the certainty about the numbers of individuals providing gametes for a mass spawning (Beaumont and Hoare, 2003). One simply and practical solution against this variance in reproductive success is to carry out several separate mini-spawnings between even numbers of males and females, and then combine the resulting embryos, which can decrease the chance that few broodstock generate most of the offspring and increases the certainty that the early larvae are derived from as many broodstock pairs as possible. Robinson et al. (2010) suggested the practices of spawning animals in isolated small groups (e.g. one female and two to three males per tank) and pooling equal quantities of larvae from spawning events can overcome potential inequality in parental contribution. Gaffney et al. (1992) found that the expedient of pooling the progeny of multiple small group spawns can reduce the discrepancy between nominal and estimated N_e in eastern oyster (*C. virginica*). In the study of Lind et al. (2009), they separated each female for spawning and then combined the embryos after fertilization, however, the reduction on allelic richness and effective population sizes were still observed, which may mainly due to relative small size of broodstocks and unbalance sex ratio (20 males and 2 or 5 females) in their study. Therefore, it is suggested to combine the mini-spawning practice and control of number of broodstocks to fight for the loss of effective population size and genetic diversity.

4.3. Other strategies against the loss of genetic diversity and effective population size

In addition to these two strategies discussed above, in recent years, there were increasing concerns in aquaculture about how different strategies can help to avoid decrease of genetic diversity in artificial breeding lines. In et al. (2016) proposed that having full pedigree is most likely the only way to maintain diversity for long term. With full pedigree information, the matings of related individuals can be avoided to better manage the accumulation of inbreeding effectively. However, it is a costly exercise associated with technical challenges such as tagging small animals, thus impractical for most commercial aquaculture facilities. Genetic tools, on the other hand, can be used to assess the levels of inbreeding, relatedness and genetic diversity of the stock without direct information about the parents (Kardos et al., 2015). One variation of pedigree-based selection is proposed as walk back selection, where only selected fish are genotyped as a way to minimize the cost as well as risks of inbreeding depression (Robinson and Jerry, 2009; Robinson et al., 2010). In this selection method, comparing to have full pedigree, only relative few individuals need to be genotyped until sufficient numbers of unrelated broodstock candidates are selected to keep an acceptable inbreeding level. However, it can be impractical and cost prohibitive to capture the best performing individuals from all families considering skewed family contributions, which is common for high fecundity species, would requires greater genotyping efforts. Domingos et al. (2014) achieved a balance between genotyping effort and the proportion of genetic diversity captured in broodstock. They found that genotyping the top 1.5% would not only significantly reduce sampling effort but would also speed progress towards selective improvement. Although there were few real studies work on walk back selection, sampling schemes that minimize genotyping cost and efforts are highly desirable in any case.

There were also other strategies discussed elsewhere to best preserve genetic variation without pedigree information. Evans et al. (2004) considered to avoid deleterious effects of inbreeding depression by crosses among distantly related lines or even the wild animals in bred bivalve species. The reintroducing the genetically unimproved wild populations may degrade genetic gains already achieved in breeding nucleus and bring the risks of pathogens infection. Another method suggested by Knibb et al. (2014) and In et al. (2016) was to

subdivide their breeding nucleus and keep multiple different lines independently over generations. In spite of loss of variation within each sublines, there were comparable genetic diversity between combined sublines and their ancestral line or wild population even over generations' mass selection. On the other hand, the economic, logistical and infrastructure costs of the effort to keep multiple sublines should be considered in practical production. In addition, Hillen et al. (2017) could not point to a major loss of genetic diversity in their study, where they thought it could be explained by the use of factorial matings which are more efficient at maintaining genetic variation than other types of matings. In summary, there were many strategies discussed to retain population diversity when implementing mass selection, however, it remains to be investigated how diverse selection and follow-up procedures will benefit avoiding the loss genetic diversity.

4.4. Conclusion

In conclusion, there was no detectable loss of genetic diversity and population differentiation over three mass-selected generations of black shell strain of *C. gigas* revealed by microsatellites and mitochondrial COI. The reduction in number of alleles and haplotypes from base population to mass selection generations may occur during previous family selection generations. A large size of broodstock and balanced sex-ration along with lower selection pressure would increase effective population size. It is suggested to intervene in mass spawning by undertaking several separate mini-spawnings and then combining the embryos as a whole to be a hedge against loss of effective breeder size and diversity. It is indicated that the problem of genetic diversity loss in black shell line has not been overlooked and the approaches towards ameliorating it are effective. The monitoring of genetic diversity is still noteworthy in coming continued long-term selective breeding of this line, given that oyster is highly fecund and has high variance in reproductive success.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (31772843), Taishan Scholars Seed Project of Shandong, and Shandong Province (2016ZDJS06A06).

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