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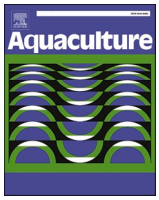

Heterosis and genetic diversity of intraspecific hybrids crosses between two selected lines of the Pacific oyster *Crassostrea gigas*

Yuanxin Liang [a], Chengxun Xu [a], Qi Li [a,b,*]

[a] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

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ABSTRACT

For aquatic species, long-term intense artificial selection inevitably leads to decreased genetic diversity and increased genotypic purity in the population, which may lead to a reduction in the adaptation of populations to their environment. Consequently, how to restore a high level of population genetic diversity becomes a critical issue in the breeding process. In this study, a complete diallel crosses was performed utilizing "Haida No. 1", which had been selected for 10 generations, and the Orange-shell line of the Pacific oyster (*Crassostrea gigas*), which had been selected for 8 generations. The growth and survival traits of the crossed and self-crossed groups were systematically evaluated during the larval and grow-out stages. Meanwhile, the population genetic diversity of the four combinations was also assessed using 18 microsatellite loci and mitochondrial cytochrome oxidase I sequences (mtCOI). The growth and survival of two reciprocal groups were significantly larger than those of two parental lines. Meanwhile, the average allelic richness (*Ar*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and number of alleles (*Na*) of the hybrids was considerably larger than two purebreds. Moreover, significant reduction in average inbreeding coefficient *Fis* was detected in hybrids when compared to purebreds. The results indicate that crossbreeding between selected lines could not only obtain phenotypically superior descendants, but also increase the genetic diversity of *C. gigas*.

1. Introduction

Selective breeding and crossbreeding are two traditional breeding methods, which were commonly used in genetic improvement (Hallauer et al., 2010; Han et al., 2020). In marine shellfish, selective breeding can efficiently improve characteristics with high heritability, such as shell height (Li et al., 2011), living weight (Langdon et al., 2003; Evans and Langdon, 2006; de Melo et al., 2016), shell color (Brake et al., 2004; Evans et al., 2009; Wan et al., 2017), survival (Dégremont et al., 2007; Chi et al., 2021; Dégremont et al., 2010), and resistance to pathogens (Dégremont et al., 2020). However, the genetic diversity of the selected population is commonly reduced during artificial selection due to insufficient number of parents, genetic drift and non-random mating, which in turn leads to the decline of adaptive traits in the species (Han et al., 2019; Zhang et al., 2018; Lind et al., 2009). In addition, directional selection often leads to genetic purification of the target trait, resulting in inbreeding depression, which is detrimental to aquaculture. Crossbreeding, however, can combine superior traits of both parents and increase genetic heterozygosity in the offspring, considerably improving traits such as meat quality, growth and survival. For example, survival and production of the Eastern oysters (*Crassostrea virginica*) can be significantly improved by crossing between lines (Rawson and Feindel, 2012). Hybridization employing the Kumamoto oysters (*C. sikamea*) from different geographic populations can yield offspring with high survival and fast growth (Ma et al., 2022). Furthermore, crossbreeding can markedly improve the traits in other aquatic organisms, such as fish (Altinok et al., 2020), scallops (Wang and Côté, 2012; Wang et al., 2011; Cruz and Ibarra, 1997), and abalones (Dang et al., 2011; Liang et al., 2018). However, little is known about the genetic variation of these hybrids at the molecular level.

The Pacific oyster (*Crassostrea gigas*) has been introduced to many countries for its adaptability, fast growth rate and high survival, and is also one of the key economic shellfish in China. The characteristics of oysters can be improved by crossing different geographical populations, strains and species. For example, the survival and growth rate of hybrids were efficiently improved by crossing between a sixth-generation orange shell strain, a seventh-generation black shell strain and a seventh-generation white shell strain of *C. gigas* (Han et al., 2020). Offspring bred

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China.
E-mail address: qili66@ouc.edu.cn (Q. Li).

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through crossing between inbred lines demonstrated significantly heterosis in yield (Hedgecock et al., 1995; Hedgecock and Davis, 2007). While crossbreeding can dramatically improve phenotypic and survival traits in shellfish, the effect on genetic diversity of the hybrids is not well documented.

In this study, a complete diallelic cross was constructed using "Haida No.1" and Orange-shell lines as the parents. The objectives were (1) to analyze the growth and survival traits of the different combinations and assess whether varieties with favorable traits could be produced by intra-line crosses; and (2) to compare the differences in population genetic diversity between the self-bred and cross-bred groups and assess whether hybridization increased the population genetic diversity of *C. gigas*.

2. Materials and methods

2.1. Experimental design and offspring rearing

In 2007, two-year-old oysters from cultured stocks in Rushan (36.45°N, 121.42°E) in Shandong Province, China, were utilized to produce first-generation "Haida No. 1" (H) of *C. gigas* (Li et al., 2011). Afterwards, to improve the growth rate of this line, we have targeted the selection for shell height and living weight. Up to 2018, 11 generations of "Haida No. 1" line have been successfully conducted through mass selection, with each generation used >50 sires and 50 dams, respectively. In 2011, four oysters with solid orange left and right shells were accidentally identified from the hybrids of purple-black shell color individuals of *C. gigas*, and been employed to generate the first generation of Orange-shell line (O). The shell color and growth were improved through two consecutive generations of family selection and five generations of mass selection from 2012 to 2018 (Han et al., 2019; Han et al., 2020). Moreover, a high selection intensity (top 10%) was applied in the two parental lines during successive mass selection. In April 2019, oysters from the "Haida No.1" and the Orange-shell line were sampled from Rushan, Shandong Province, China. Then, all broodstocks were temporarily reared in the hatchery for three weeks.

For each line, eggs from one female were obtained by dissection and examined under microscope to verify that no uncontrolled fertilization had taken place. Afterwards, the eggs were divided into two equal parts and each part was fertilized with one male from each of "Haida No.1" and the Orange-shell line respectively at a sperm: egg ratio of 30–50:1. The experiment was conducted in triplicates using three sets of parents. Therefore, artificial crosses were performed with the following four different combinations: HH (H♀ × H♂), HO (H♀ × O♂), OH (O♀ × H♂) and OO (O♀ × O♂). Subsequently, the fertilized eggs from each group were pooled separately into a 100-L bucket for hatching with gentle aeration.

Larvae and juveniles rearing methods were according to the standard procedure described by Li et al. (2011). Briefly, twenty-four hours after fertilization, D-larvae from each cross, HH, HO, OH and OO, were collected and reared separately. Larvae were fed with *Isochrysis galbana* at the D-larvae stage and a mixture of *Isochrysis galbana* and *Platymonas* at the umbo-stage and eyed-stage. The sea water was kept at 23–25 °C and 30–31 psu, and replaced every 2 days. The density of larvae in each bucket was initially set at 2–4 larvae ml^{-1} during the D-larvae stage and decreased to 0.5 larvae ml^{-1} during the umbo-stage and eyed-stage. When 30% of larvae developed an eyespot, strings of scallop shells were placed in the bucket as a settlement substrate. Larvae set within a week, and newly settled spat were nursed in an outdoor pond for another two weeks to prevent contamination from wild spat. Subsequently, all spats were transferred to Rongcheng, Shandong Province, China (37.11°N, 122.35°E).

2.2. Sampling and measurements

The growth-related paraments (D-larvae size, shell height, shell length and living weight at 5, 10, 15, 20, 90, 180, 270 and 360 days of age) and survival rate of each group were determined according to Xu et al. (2019). Briefly, at larval stage, the shell height of 30 larvae from each group were measured using Image-ProPlus 6.0 software. Larval survival was calculated as the ratio between the number of larvae in 100 ml at the sampling day and the number of larvae on the first day after hatching.

The shell height and living weight of 30 oysters from each group were measured using vernier calipers (0.01 mm accuracy) and electronic scales (0.01 g accuracy), respectively, at day 90, 180, 270 and 360. The survival rate was calculated using the following equation (Jiang et al., 2021):

$$Z_t\ (\%) = (N_t/N_0) \times 100$$

Where Z_t is the survival rate of each group at time t; N_t is the number of live oysters in each lantern at time t; N_0 represents the total number of oysters in each lantern net in July 2019.

2.3. Statistical analyses

Results are given as means ± standard deviations (SD). To improve the normality and homoscedasticity, survival rate and the growth-related data were arcsine-transformed and logarithmic-transformed with a base of 10, respectively, prior to analysis. All statistical analyses were calculated using SPSS 26.0. Moreover, the bivariate correlation between microsatellite data (*Na*, *Ar* and *Ho*) and each phenotypic performance (growth parameters) in purebred and crossbreed were also analyzed. Differences in shell height and survival rate among four groups were analyzed by one-way ANOVA and Turkey multiple comparison tests. Tests were considered to be significant at $P < 0.05$ level.

The mid-parental heterosis (*M*) was calculated based on the following formula (Hallauer et al., 2010):

$$M_{F1}\ (\%) = [(F1 - MP) \times 100]/MP$$

Where F1 and MP are the mean growth-related traits values (shell height, survival rate or living weight) of hybrids and purebred groups, respectively. M_{F1} represents the mid-parent heterosis of two reciprocal hybrids.

The high-parent heterosis (*H*) was calculated using the following equation (Wang et al., 2011):

$$H_{(F1/HH)}\ (\%) = (X_{F1} - X_{HH}) \times 100/X_{HH}$$

Here, X_{F1} is the mean phenotypic value (shell height, survival rate, etc) of the hybrid F1 (HO or OH) groups, X_{HH} indicates the mean phenotypic value (shell height, survival rate, etc) of "Haida No. 1".

A two-way ANOVA was used to compare the effects of egg origin (H vs. O) and mating strategy (intra- vs. interline crosses) on growth and survival of oysters (Cruz and Ibarra, 1997; Zhang et al., 2007):

$$Y_{ijk} = \mu + EO_i + MS_j + (EO \times MS)_{ij} + e_{ijk}$$

Here, Y_{ijk} represents the mean phenotypic value (shell height, survival rate or living weight) of offspring at the k-th replicate for the j-th mating strategy and the i-th egg origin, EOi represents the effect of egg origin on offspring phenotypic values ($i = 1, 2$); MSj represents the effect of mating strategy on offspring phenotypic values ($j = 1, 2$); (EO × MS)ij represents the interaction effect of egg origin and mating strategy on offspring phenotypic traits; eijk represents random observation error ($k = 1, 2, 3$).

2.4. Genomic DNA extraction

48 oysters were randomly selected from each group for genetic diversity analysis at day 360. After the phenotypic traits (shell length, shell height, shell width and living weight) of each individual were measured, adductor muscles from four groups were taken and fixed in

95% alcohol for DNA extraction. The DNA extraction procedure was based on the phenol/chloroform method described by Li et al. (2003).

2.5. Microsatellite analysis

Microsatellite were amplified referring to the multiplex PCR reaction flow developed by Liu et al. (2017). The reaction system included six sets of multiplex PCR combinations for a total of 18 microsatellite loci (Table 1). The genotyping results were converted into allele size through GeneMapper software v.4.0 (Applied Biosystems). The presence of null alleles was tested using Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004), in which loci with estimated frequencies of null alleles above 0.2 were potentially problematic for calculations (Napora-Rutkowski et al., 2017). We used MICROSATELLITE ANALYSER v.4.05 to calculate basic parameters of genetic variability, such as, the observed heterozygosity (*Ho*), expected heterozygosity (*He*) for each locus (Dieringer and Schlötterer, 2003). The number of alleles (*Na*) and *Nei's* unbiased genetic distance (*Dc*) between populations for each locus were computed by GenAlEx v.6.502 (Peakall and Smouse, 2012), and a neighbor-joining tree was reconstructed utilize the *Dc* by Mega v5.0 (Tamura et al., 2011). Genetic relationship among populations was estimated by performing principal coordinates analysis (PCoA) implemented in GenAlEx 6.5 (Peakall and Smouse, 2012). The structure analysis was obtained by STRUCTURE v.2.3 (Falush et al., 2003) and Structure Harvester software (Evanno et al., 2005).

Allelic richness (*Ar*), inbreeding coefficient (*Fis*) for each population and genetic differentiation index (*Fst*) between two populations were estimated by FSTAT v.2.9.3.2 (Goudet, 2001). Deviations from Hardy-Weinberg equilibrium (HWE) at each locus were subjected to Fisher's exact test using the GENEPOP software2 v.4.0 (Rousset, 2008; Raymond and Rousset, 1995).

2.6. Mitochondrial DNA analysis

The Mitochondrial cytochrome C oxidase subunit I (COI) sequences were amplified using universal primers (LCO1490 and HCO2198) (Vrijenhoek, 1994). Mega v.5.0 were employed to edit and align the sequences (Tamura et al., 2011). The processed sequences were then sent to DNASP v.5.10 to calculate the number of haplotypes (*Nh*), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) (Librado and Rozas, 2009).

3. Results

3.1. Phenotypes, growth and survival of purebred and hybrid oysters

3.1.1. Phenotypes

Both the left and right shells of the orange shell line used in this study have a solid orange color (Fig. 1). The "Haida No.1" line had/does not contain a dark radial stripe on the left shell. Furthermore, the hybrids had two types in terms of color pattern: 60% had radial stripes on the left shell but no specific color. 40% of the oysters presented purple shell color (ranging from light purple to dark purple).

3.1.2. Growth

As for the D larvae, a significant difference was noted in shell height among the four experimental groups ($P < 0.05$) (Fig. 2). Two reciprocal hybrids exhibited slighter heterosis in shell height, with a mid-parental heterosis ranged from 5.74% to 14.76%. The shell heights of two hybrid groups were greatly larger than those of two purebred lines at day 20 ($P < 0.05$), with a high-parent heterosis $H_{(HO/HH)}$ of 5.81% and $H_{(OH/HH)}$ of 1.92%, respectively (Table 2).

The shell height and living weight of hybrids were significantly higher than those of purebred groups during grow-out stage ($P < 0.05$)

Table 1
18 microsatellite loci of *C. gigas* used in this study.

Set	Locus	Primer sequence(5′to3′)	Size(bp)	Ta (°C)
1	ucdCg-117F	CCAAGCTTGCACTCACTCAA	290	58
	ucdCg-117R	GAGTGTTCTGGTGTGCCAAAT		
	ucdCg-120F	GGGTGAGATTTAGGGGGAGA	152	58
	ucdCg-120R	CTCCATCAAACCTGCCAAAC		
	ucdCg-198F	GAAAGACACGACCGGAGAGA	230	58
	ucdCg-198R	CTGATGATGTCCCACACCTG		
2	ucdCg-146F	CGCTCTGGTCTTTGTTCCAT	218	58
	ucdCg-146R	ACCCCAACAGATCACAATCC		
	Crgi3F	TAGGATGAGGCTGGCACCTTGGA	161–173	58
	Crgi3R	GCCTGCCTTGCCTTTGAGGAATA		
	uscCgi-210F	TTCACAATGAAGATGACAGTGC	345–348	58
	uscCgi-210R	CCTCCTCTGCCTCCATATCA		
3	ucdCg-170F	TGGTGGTCAGTGAATGTGAGA	276	58
	ucdCg-170R	CGGACAGTAGCCTTTTAACACA		
	ucdCg-156F	AGCAGACCTTGGCAAATACG	325	58
	ucdCg-156R	CCGTCATCAGGTCCTGTTTT		
	ucdCg-199F	GGGAAGAGTTGAATTCTGCAA	270	58
	ucdCg-199R	AAACCGAGGCTCAGGAAAAT		
4	otgfa0_0007_B07F	TATCATCGCGGCAATTCGTG	279–295	50
	otgfa0_0007_B07R	GCAACTTAGCTGGTCGTTCC		
	otgfa0_0129_E11F	TGACTGTTCTTCGTACCCATCA	155–165	50
	otgfa0_0129_E11R	AGGTGGAACGAGATTGCCTTT		
	Crgi4F	CCAAAACACGATAAGATACACTTTC	235,238	50
	Crgi4R	GATCAGTCCCTCACATCTTTCCTC		
5	ucdCg-152F	TGGTTTTGGAGCTTGGCTTA	257	50
	ucdCg-152R	TCAAGCAAAGAAAGTCACCTCA		
	Crgi39F	TTCCAAGTCCGTTTTGTCATCGT	190–214	50
	Crgi39R	GTGCACAAACCCACCATCAGCTC		
	Crgi45F	GAGTCACCATGAAGAGTATCTGAA	158–164	50
	Crgi45R	ATGATTACATAACTCTGACCCAAT		
6	ucdCg-200F	AAAGTTGCTTTGCTGTCGTC	254	54
	ucdCg-200R	CGCTAACGTGCTTCATTCAA		
	otgfa0_408293F	ACCCTGGTTTGATCTGAGAAATG	118–122	54
	otgfa0_408293R	TCTAAGGAGTGTTGAGTGTTAGTAG		
	otgfa0_0139_G12F	GTGCTTCAGGGTATCTCTTTCC	169–173	54
	otgfa0_0139_G12R	AGCTACTGCATGGACACGATT		

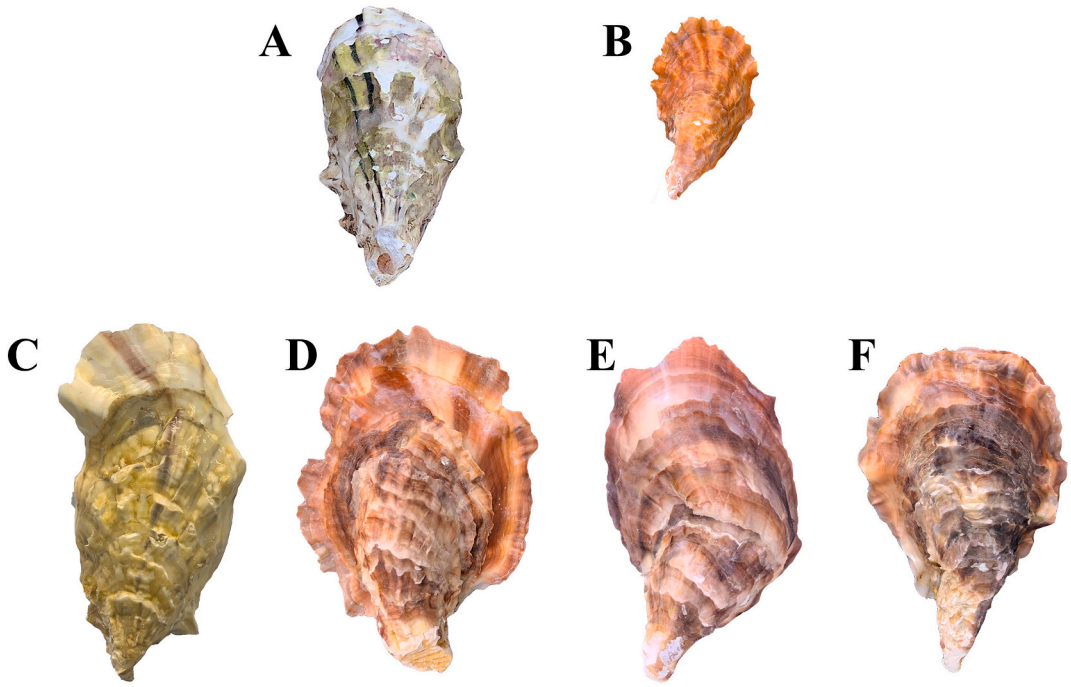

Fig. 1. Phenotypes of the four groups. A: the left shell of "Haida No. 1" line; B: the left shell of orange shell line; C: the left shell of hybrid offspring with normal shell color; D-F: the left shell of hybrid offspring with purple shell color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

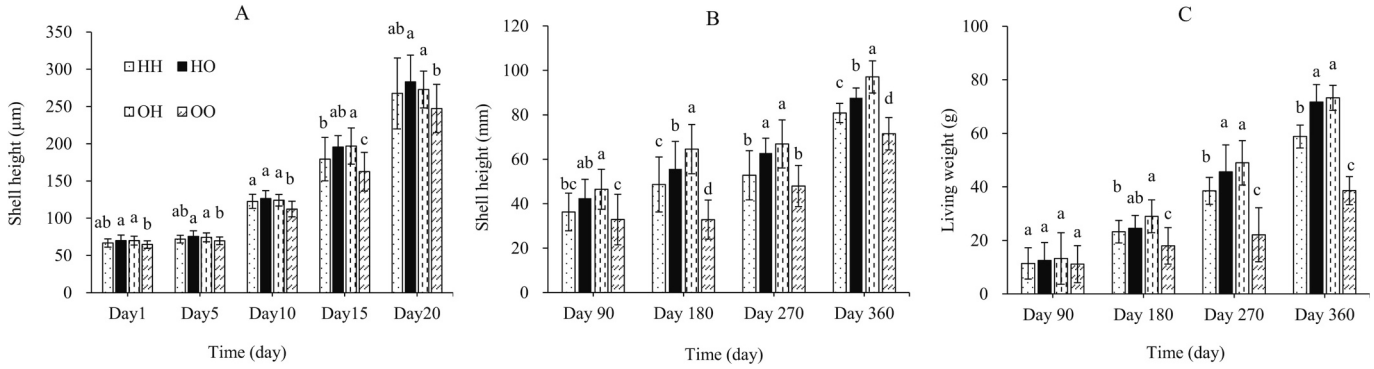

Fig. 2. Shell height and living weight for two purebred groups (HH and OO) and two hybrid groups (HO and OH). A: the shell height of four groups at larval stage; B: the shell height of four groups during grow-out stage; C: the living weight of four groups during grow-out stage Different superscript letters in the same day indicate significant difference ($P < 0.05$) among four groups.

Table 2
Heterosis (M and H) for survival rate and shell height in "Haida No. 1" and Orange-shell lines, and their reciprocal hybrids during larval stage.

Traits	Heterosis (%)	Day 1	Day 5	Day 10	Day 15	Day 20
Survival rate	M_{F1}	/	3.96	15.54	53.35	62.08
	$H_{(HO/HH)}$	/	2.14	14.55	28.57	39.87
	$H_{(OH/HH)}$	/	3.20	18.64	28.06	45.10
Shell height	M_{F1}	6.31	5.74	6.62	14.76	7.95
	$H_{(HO/HH)}$	4.65	4.79	3.12	9.00	5.81
	$H_{(OH/HH)}$	4.67	3.23	1.16	9.73	1.92

(Fig. 2). At day 180, the sizes of HO and OH were larger than those of the two parental groups and were significantly different from both ($P < 0.05$). At day 360, the growth trend of all the progeny was similar to the day 180. Moreover, the mid-parental heterosis and high-parental heterosis ($H_{(HO/HH)}$, $H_{(OH/HH)}$) at day 360 was 21.30%, 8.48% and 20.06% for shell height and 48.81%, 21.89% and 24.61% for wet weight, respectively (Table 3).

3.1.3. Survival

During the larval stage, the heterosis for survival rate of two hybrid

Table 3
Heterosis (M and H) for shell height, living weight and survival rate in "Haida No. 1" and Orange-shell lines, and their reciprocal hybrids at grow-out stage.

Traits	Heterosis (%)	Day 90	Day 180	Day 270	Day 360
Shell height	M_{F1}	28.75	47.50	28.76	21.30
	$H_{(HO/HH)}$	17.18	14.33	18.97	8.48
	$H_{(OH/HH)}$	28.19	32.74	26.80	20.06
Living weight	M_{F1}	14.53	29.68	56.03	48.81
	$H_{(HO/HH)}$	10.07	5.29	18.39	21.89
	$H_{(OH/HH)}$	16.30	24.60	27.15	24.61
Survival rate	M_{F1}	−1.36	29.69	45.61	45.61
	$H_{(HO/HH)}$	−3.82	11.87	23.13	23.13
	$H_{(OH/HH)}$	−1.91	15.53	36.25	36.25

groups was gradually increased while the larvae grew. At day 5, the survival rate of all the four groups exceeded 90%, no significant difference was existed among four groups ($P > 0.05$) (Fig. 3). The survival rate of the Orange-shell line was remarkably lower than the other three groups. At day 20, two hybrid groups exhibited heterosis in survival, with M_{F1}, $H_{(HO/HH)}$ and $H_{(OH/HH)}$ were 62.08%, 39.87% and 45.10%, respectively (Table 2).

Two hybrid groups exhibited considerably heterosis in survival from 180 days of age (Fig. 3). Moreover, the heterosis for survival increased steadily with the growth of spats. Notably, compared with survival of the "Haida No. 1", M_{F1}, $H_{(HO/HH)}$ and $H_{(OH/HH)}$ were 45.61%, 23.13% and 36.25%, respectively, at day 360 (Table 3). Meanwhile, two-factor ANOVA showed that growth and survival traits of *C. gigas* were dramatically influenced by egg origin and mating strategy ($P < 0.05$) (Table 4).

3.2. Microsatellite diversity

Overall, 192 oysters from "Haida No.1", the Orange-shell line and their reciprocal hybrids were genotyped with 18 microsatellite loci. No evidence of scoring error due to stuttering and large allele dropout was detected in all loci. Hence analysis was performed based on all loci.

Significant differences in the average number of alleles (*Na*), allelic richness (*Ar*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) were detected among the four groups ($P < 0.05$) (Table 5). The *Na* and *Ar* in HO and OH were higher than that of HH and OO. The mean *Ho* were lower than *He* in all four groups. However, higher *Ho* and *He* were being viewed in the cross groups (HO and OH), rather than in the self-cross groups (HH and OO). In addition, bivariate correlations proved that growth traits (shell height and living weight) were positively correlated with population genetic diversity (*Na*, *Ho*, *He*) (Table 6). The inbreeding coefficient (*Fis*) values were positive at all the loci in all the four groups. Moreover, mean *Fis* decreased by 51.62% and 54.84% for HO and OH, respectively, compared to the OO group. Besides, each of HH and HO had three loci deviating from Hardy-Weinberg equilibrium (HWE), while the number of loci deviating from HWE of the other two groups ranged between 4 (OH) and 5 (OO).

The genetic differentiation between the two parental lines and their reciprocal hybrids was proved by the pairwise *Fst* and *Nei's* unbiased genetic distance (*Dc*) analysis, separately. AMOVA revealed that the global *Fst* for OO and OH was 0.115, and that of HH and HO was 0.134. However, pairwise *Fst* and *Dc* suggested that most genetic differentiation was distributed between the "Haida No.1" and the Orange-shell line (*Fst*: 0.198; *Dc*: 0.725), while differentiation within HO and OH (*Fst*: 0.093; *Dc*: 0.0228) was lower. Furthermore, potentially genetic relationships between populations and individuals were further illuminated by

Table 4
Two-factor analysis of variance (ANOVA) showing the egg origin (EO) and mating strategy (MS) effects for shell height, living weight and survival rate of each experimental group.

Source	Parameter	d. *f*	*F*-value	*P*-value
MS	Shell height	1	91.297	0.000***
	Living weight	1	304.131	0.000***
	Survival rate	1	18.248	0.003**
EO	Shell height	1	0.000	0.999
	Living weight	1	46.549	0.000***
	Survival rate	1	127.182	0.000***
MS × EO	Shell height	1	30.409	0.000***
	Living weight	1	63.933	0.000***
	Survival rate	1	18.248	0.003**

* indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; MS indicates mating strategy; EO indicates egg origin.

Table 5
Genetic parameters within "Haida No. 1" and Orange-shell lines, and their reciprocal hybrids based on 18 microsatellite loci.

Line	*Na*	*Ar*	*Ho*	*He*	*Fis*	*dHW*
HH	5.176 ± 0.537^{c}	4.865 ± 0.537^{c}	0.534 ± 0.048^{b}	0.684 ± 0.056^{b}	0.21	3
HO	8.135 ± 0.874^{b}	7.464 ± 0.874^{b}	0.636 ± 0.064^{a}	0.785 ± 0.067^{a}	0.14	3
OH	8.583 ± 0.832^{a}	8.583 ± 0.819^{a}	0.652 ± 0.068^{a}	0.831 ± 0.066^{a}	0.15	4
OO	3.529 ± 0.529^{d}	3.529 ± 0.785^{d}	0.359 ± 0.063^{c}	0.430 ± 0.066^{c}	0.31	5

Na: number of alleles, *Ar*: allelic richness, *Ho*: observed heterozygosity, *He*: expected heterozygosity, *Fis*: inbreeding coefficient, *dHW*: number of loci deviating from Hardy-Weinberg equilibrium; Values with different small letter superscripts in the same column indicate significant difference ($P < 0.05$).

Table 6
Bivariate correlation between microsatellite data (Na, Ar and Ho) and each phenotypic performance (shell height and living weight) in purebred and crossbreed.

Items	Traits	Pearson's r	*P*-value
Na	Shell height	0.954	0.046*
	Living weight	0.966	0.034*
Ho	Shell height	0.938	0.062
	Living weight	1.000	0.000***
He	Shell height	0.995	0.005**
	Living weight	0.942	0.058

* indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$.

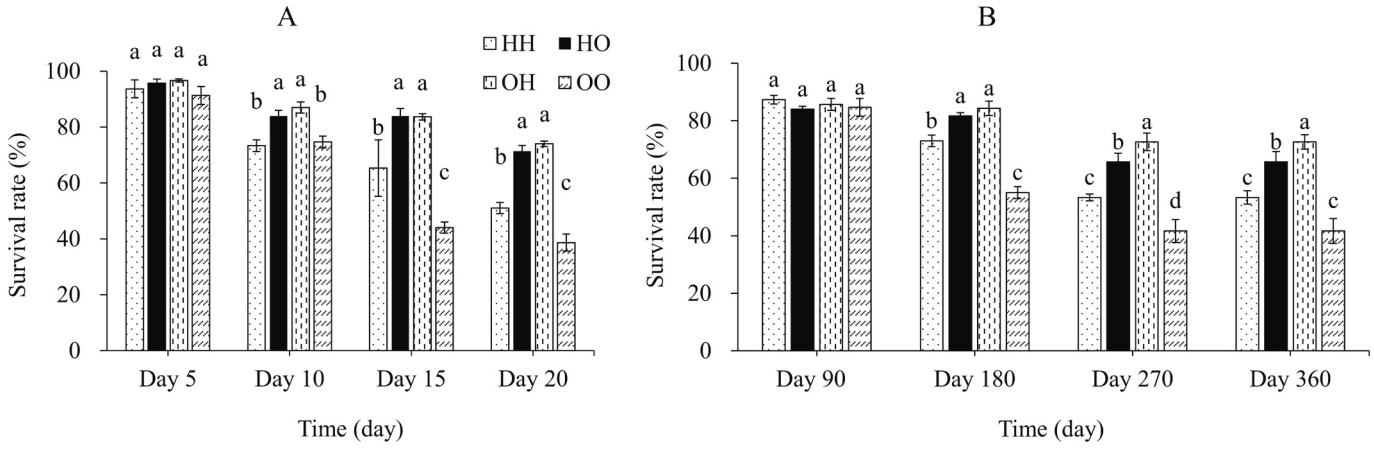

Fig. 3. Survival rate for "Haida No.1", Orange-shell line and their reciprocal hybrids. A: the survival rate of four groups at larval stage; B: the survival rate of four groups during grow-out stage. Different superscript letters in the same day indicate significant difference ($P < 0.05$) among four groups.

principal coordinate analysis (PCoA) based on genetic distance matrix (Fig. 4). The three principal components accounted for 44.83% of the total molecular variation, with Coordinate axis 1 and 2 explaining 26.64% and 11.62%, respectively. Clustering analysis of four groups totaling 192 oysters allowed the classification of the study sample optimally into four theoretical groups, as the parametric curve of ΔK showed a strongly significant peak at K = 4. The genetic composition of each cluster was clearly delineated, with very low genetic mixing at the individual level (Fig. 5).

3.3. mtDNA haplotype diversity

The variation in the cytochrome oxidase subunit I gene in 80 oysters from all 4 populations were examined through mtDNA sequence. A 637-bp fragment of the COI region was obtained after sequence alignment. Seven mtCOI haplotypes were shared among all the studied populations (Table 7). One of the haplotypes (haplotype 2) was conspicuous unique high-frequency and observed in all four populations with a prevalence of 88.75% (71/80) in all individuals. The haplotype diversity of HO and OH were higher than HH and OO.

4. Discussion

4.1. Genetic differences among the four groups

In this study, the number of alleles of the "Haida No.1" was higher than that of the Orange-shell line, and the genetic differentiation index and *Nei's* unbiased genetic distance value (*Fst*: 0.198, *Dc*: 0.752) between the two lines were largest, indicating that the genetic information of the two parental populations differed greatly, which may be due to the different genetic background of the two strains. The base population of "Haida No. 1" originated from cultured stocks in Rushan and has been bred by mass selection for rapid growth from 2007 (Li et al., 2011), while the Orange-shell line was constructed from only four oysters (two females and two males) with pure orange shells on both the left and right shells by family selection and mass selection (Han et al., 2019). Artificial selection for breeding traits (shell height, shell color, etc.) may lead to loss of genetic information in aquaculture (Bentsen and Olesen, 2002), which is the main reason for the difference in population genetic diversity between "Haida No. 1"and the Orange-shell line. Besides, the mean number of alleles and observed heterozygosity of the Orange-shell line were 3.528 and 0.359, respectively, which were lower than other *C. gigas* lines (Han et al., 2019; Xu et al., 2019). The greatest reason for the low variability was that the Orange-shell line was a highly inbred line and that the genetic diversity might have been lost during the artificially directed selection (Han et al., 2019). However, when this line was crossed with "Haida No. 1" strain, the mean allele number and observed heterozygosity of the offspring were improved, signifying that the genetic diversity of *C. gigas* could be restored to a higher level by crossing between different strains. Moreover, differences in genetic diversity were observed between the two hybrid groups, and especially the mean *Na* and *Ar* were significantly higher in OH than in HO, and differences in genetic diversity of the two reciprocal hybrids have been reported in clam (Lu et al., 2012) and scallop (Hu et al., 2015). However, the genetic diversity of OO was lower than that of HH, suggesting that, in addition to maternal effects, crosses caused other factors to influence the genetic diversity of the offspring.

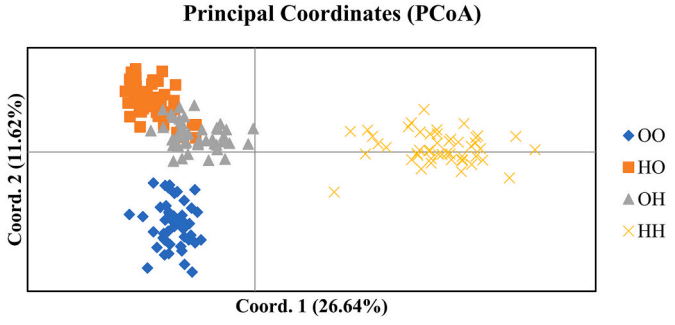

Fig. 4. Principal coordinates analysis (PCoA) of 192 individuals from four groups based on genetic distance matrices. Coordinate axis 1 explains 26.64% of the variation, coordinate axis 2 explains 11.62% of the variation, and Coordinate axis 3 (not shown) explains 6.57%. The different groups are indicated by different colors and symbols, respectively.

Mean number of alleles, allelic richness and expected heterozygosity of the two hybrid populations were considerably higher than those of the two purebred populations. Accordingly, we concluded that crosses could improve the genetic heterozygosity and population genetic diversity of *C. gigas*. Hybridization altering the genetic diversity of offspring has also been described in other aquatic animals, such as clam (*Meretrix meretrix*) (Lu et al., 2012), and scallop (*Argopecten purpuratus* × *A. irradians irradians*) (Hu et al., 2015). Moreover, heterozygosity has been documented positively correlate with growth and survival in shellfishes (Zouros and Mallet, 1989; Sheridan, 1981; Mitton and Grant, 1984). In this study, the hybrids of "Haida No.1" and Orange-shell line, which exhibited higher heterozygosity than their parental lines, have also been shown to possess significantly heterosis in both growth and survival rate.

In this study, 15 of the 60 population-locus tests deviated from Hardy-Weinberg equilibrium in the four groups, indicating that it could be due to the presence of null alleles (Zhang et al., 2018). Furthermore, heterozygous deletion, small base population size, non-random mating, Wahlund effect and artificial or natural selection occurring during breeding and culturing may be other reasons causing loci deviation from HWE to occur (Lu et al., 2012; McGoldrick, 2000; Li et al., 2003; Hedgecock et al., 2004; Li et al., 2006; Wachirachaikarn et al., 2009). Moreover, a relatively high selection intensity (top 10%) was applied in the two parental lines during successive mass selection, which increased the possibility of nonrandom mating, and could result in the deviation from HWE in the two parental lines.

The inbreeding coefficient *Fis* values of the four populations ranged from 0.14 to 0.31, demonstrating the presence of heterozygote deficiency within the populations (Lu et al., 2012), as well as the existence of differing degrees of inbreeding, null alleles, and non-random mating in each population (Xu et al., 2019). Besides, the gene conversion from heterozygous state to homozygous state at some loci during the process of selection of parental oysters was probably another important factor. Meanwhile, the *Fis* value of the Orange-shell line was highest among four groups, indicating that this population was an inbreeding line compared to the other three groups and its genotypes were at a highly pure level (Han et al., 2019). In addition, the *Fis* values of both two reciprocal hybrids were lower than those of the two purebred populations, emphasizing that crossbreeding can increase the genetic heterozygosity of *C. gigas*. Similar results have been found in *M. meretrix* (Lu et al., 2012). Although a higher genetic diversity in the reciprocal cross populations was generated by hybridization, an existing heterozygote deficiency was indicated by the *Fis* values in the two populations.

The population genetic differentiation index value and *Nei's* unbiased genetic distance are two important parameters for judging the degree of genetic differentiation of populations. In this study, The *Dc* value between the two parental populations was the largest (0.198). This showed that a high level of genetic differentiation occurred between the two parental population as a result of long-term artificial selection, which also supported by the pair *Fst* values ($0.15 < 0.198 \leq 0.25$). The significant genetic differences between the two parents may be caused by founder effects and genetic drift in the selection process (Hillen et al., 2017). Besides, the genetic differentiation in the two lines could provide a genetic basis for heterosis in their hybrids.

Throughout the whole experiment, we kept the four groups strictly separated. Thus, there was no possibility of mutual contamination between the groups. The occurrence of haplotypes in the offspring that

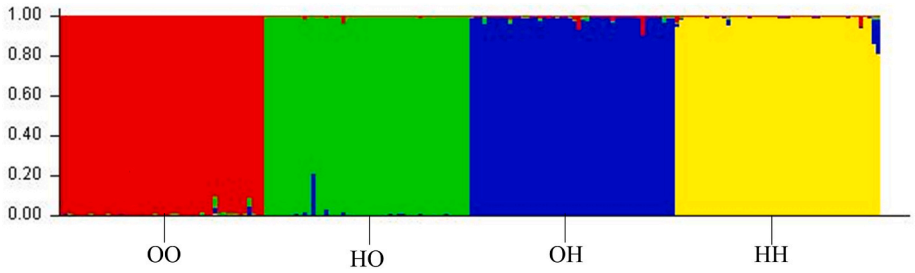

Fig. 5. Clustering analysis from STRUCTURE by 18 microsatellites. Each color represents a unique cluster detected by STRUCTURE.

Table 7
Haplotype frequencies of mtCOI sequences observed among the four groups.

Haplotype	Hybrid lines		Purebred lines		Total
	HO	OH	HH	OO	
1	19	17	17	18	71
2		3	1		4
3	1				1
4			1		1
5			1		1
6				1	1
7				1	1
Nh	2	2	4	3	
Hd	0.574 ± 0.090	0.268 ± 0.113	0.195 ± 0.115	0.1 ± 0.088	
Pi	0.00106 ± 0.00132	0.00047 ± 0.00132	0.00041 ± 0.00044	0.00016 ± 0.00044	

Nh: number of haplotypes, Hd: haplotype diversity, Pi: nucleotide diversity.

were not present in the parents or the failure of parental haplotypes to be fully inherited by the offspring has also been reported in fish (Cao, 2020; Zhou et al., 2012) and shellfish (Hu et al., 2022; Xu et al., 2019). Zhou et al. (2012) assumed that this might be due to individual differences in its original parent COI gene or artificial breeding resulting in the point mutations. Additionally, the smaller number of samples tested may also lead to different haplotype types in the parents and offspring (Cao, 2020).

4.2. Heterosis

The traits such as shell height and living weight of the hybrid groups were considerably higher than those of the purebred groups, with 24.13% to 42.15%, 6.72% to 20.85%, and 11.76% to 30.16% for mid-parental heterosis H_{F1}, high-parental heterosis $H_{(HO/HH)}$ and $H_{(OH/HH)}$, respectively. The results based on 18 microsatellite loci analyses as well showed that both *He* and *Ho* were higher in the hybrid group than in the two parental groups, being consistent with the statement that heterosis in marine shellfish shows a positive correlation with heterozygosity (Zouros and Mallet, 1989; Sheridan, 1981). While there were differences in phenotypic traits between the two hybrid groups, especially the shell length of the OH group being greatly higher than that of the HO group, indicating that there might be a maternal effect, but the phenotypic traits of the OO group were significantly lower than those of the HH group. Therefore, the phenotypic traits were also influenced by other factors. The different performance of the two reciprocal hybrids has also been reported in other aquatic animals, such as razor clams *Sinonovacula constricta* (Xue et al., 2020) and brown trout *Salmo trutta* (Altinok et al., 2020). In addition, there are significant effects of egg source and mating strategy on the growth of offspring. Therefore, when commercial breeding is performed, detailed give advices need to be given to the selection of maternal parents and maternal sources in order to maximize heterosis.

4.3. Prospects for the application of genetic diversity analysis in aquaculture

It is widely documented that the genetic diversity of cultured populations tends to decline after several generations of artificial selection in highly fertile shellfish. (Hedgecock et al., 1992). Consequently, it has become a central goal for breeders to maintain a high level of population genetic diversity while conducting an intensive successive selection process (Xu et al., 2019). In addition to measures such as increasing the number of parents, reducing the intensity of selection and balancing the parental sex ratio (Zhang et al., 2018), genetic variation in the selected population can be restored to a high level by crossing strains selected over multiple generations. In addition, genetic diversity analysis of target populations not only provides insight into genetic differences between populations, but can also further guide breeding efforts based on the results. Wachirachaikarn et al. (2009) analyzed the genetic diversity differences among four populations of African catfish (*Clarias gariepinus*) and descendant with increased immune response were obtained by crossing between the two populations with the greatest genetic differences. In addition, the magnitude of genetic distance between populations can be used to predict heterosis to avoid the laborious work associated with blind crosses (Singh et al., 2018; Singh et al., 2019).

5. Conclusions

In this study, a complete diallelic cross were conducted between the "Haida No. 1" and the Orange-shell line of the Pacific oyster. The results revealed that the phenotypic characteristics of the hybrid groups were significantly larger than those of the purebred groups. In addition, the genetic diversity parameters such as the mean number of alleles *Na*, expected heterozygosity *He* and observed heterozygosity *Ho* of the oysters could be improved through crossbreeding between the two lines. Meanwhile, significant reduction in inbreeding coefficient *Fis* was detected in hybrids when compared to purebreds. Thus, we considered crossbreeding between selected lines of the Pacific oyster can not only obtain offspring with favorable phenotypic characteristics, but also restore the population genetic diversity to a high level, which provides an effective way for the genetic improvement of *C. gigas*.

Credit author statement

Yuanxin Liang: Software, writing - original draft and investigation. Qi Li: Writing- review & editing and conceptualization. Chengxun Xu: Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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