Microsatellite-based genetic and growth analysis for a diallel mating design of two stocks of the clam, *Meretrix meretrix*

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Abstract

To determine the potential for productive efficiency and genetic improvement in the clam (*Meretrix meretrix*), four populations were produced from a diallel mating of two different geographical stocks (SD and JS). The genetic parameters at nine novel microsatellite loci indicated that the numbers of alleles, observed heterozygosity and polymorphic information content of the reciprocal cross populations (SDJS and JSSD) were larger than those of the pure populations (JSJS and SDSD). The values of Nei’s unbiased genetic distance and $F_{ST}$ revealed that the largest genetic divergence was between the two pure populations ($D_C = 0.2993$, $F_{ST} = 0.1438$) and the smallest was between the two reciprocal cross populations ($D_C = 0.1093$, $F_{ST} = 0.0583$). In addition, the mean shell lengths of the reciprocal cross populations were significantly larger than that of the pure populations by 1.577 mm ($P < 0.05$), and the same trend was observed in the other traits. A significant maternal effect was revealed after analysis of the effects of egg origin and mating strategy on the four traits. Heteroses for all the traits were detected in the reciprocal cross populations. Our research implies an extensive development potential in productive efficiency and genetic improvement for *M. meretrix*.

Keywords

microsatellite, *Meretrix meretrix*, reciprocal cross, heterosis, maternal effect

Introduction

The clam, *Meretrix meretrix*, is a commercially important bivalve that is distributed naturally along the coastal and estuarine areas of South and Southeast Asia (Tang, Liu, Wang, Zhang & Xiang 2006). The cultivation of *M. meretrix* has become an important industry in China. Along with the increasingly enlarged cultivation scale, intensive catching of natural seeds has threatened the germplasm resources of this clam. Thus, the lack of clam seeds with high performance has become a bottleneck to the development of the industry. Consequently, it is extremely important to carry out researches on population genetics in *M. meretrix*, which is practically important for the proper utilization of stock resources and sustainable development of cultivation. Genetic improvement based on the estimation of stock resources has become a significant requirement.

With the rapid development of molecular technology, genetic analysis based on molecular markers has become a powerful tool for genetic resource evaluation and genetic improvement. Many molecular markers have been developed, and they were increasingly available in many kinds of species (Westman & Kresovich 1997). In breeding of the clam, *M. meretrix*, the application of molecular markers lags because of a late start. So far, only a few dominant markers have been applied to examine the genetic background of different geographical natural
populations of clam, such as RAPD (Shen, Zhu, Ding, Chen, Lu, Zhu & Xu 2003; Du, Deng, Ye & Wang 2004), AFLP (He, Cong, Ge, Liu & Zhou 2008; Lin, Dong, Chai & Liu 2008) and ITS2 (Li, Zhang, Su, Li, Liu, Lin & Cai 2006), indicating that a high genetic variation exists in different geographical populations.

Co-dominant markers are more useful than dominant markers in genetic structure analysis and tracing traits. Among the co-dominant genetic markers, microsatellite markers are the most widely used in genetic diversity and population structure studies (Greg, Denise & Acacia 1997). Microsatellite, also known as single sequence repeat (SSR), has the advantages of high polymorphism and comparability, great reproducibility and low expense. In aquaculture studies, SSR markers are very useful in population genetics analysis (Davies, Villablanc & Roderick 1999), kinship inference (Blouin 2003), parentage assignment, pedigree tracing of hatchery populations (Hara & Sekino 2003; Vandeputte, Kocour, Mauger, Dupont-Nivet, Guerry, Rodina, Gela, Vallod, Chevassus & Linhart 2004; Jerry, Evans, Kenway & Wilson 2006) and for identification of families (MacAvoy, Wood & Gardner 2007). The combination of microsatellite markers and genetic improvement in breeding programmes has enabled considerable progress in marine organisms, such as the oyster Ostrea edulis (Bierne, Launey, Naciri-Graven & Bonhomme 1998) and Crassostrea gigas (Hedgecock, McGoldrick, Manahan, Vavra & Appelmans 1996; Hubert & Hedgecock 2004), the mussel Perna perna (Holland 2001), the abalone Haliotis discus hannai (Selvamani, Degnan, Paetkau & Degnan 2000; Li, Park, Kobayashi & Kijima 2003), the shrimp Penaeus stylirostris (Bierne, Beuzart, Vonau, Bonhomme & Bedier 2000) and the salmon Oncorhynchus tschawytscha (Banks, Blouin, Baldwin, Rashbrook, Fitzgerald, Blankenship & Hedgecock 1999). However, little information on the genetic diversity of M. meretrix populations based on microsatellite markers is currently available.

Diallel mating is an important method in plant and animal breeding programmes to obtain productive efficiency and genetic improvement. To determine the potential for productive efficiency and genetic improvement in M. meretrix, four populations were produced from a diallel mating of two different geographical stocks (SD and JS). Next, the genetic difference of the four populations, as well as the growth difference, was analysed using nine novel polymorphic microsatellite markers.

Materials and methods

Experiment design and offspring culture

Two cultured stocks of M. meretrix, which originated from Shandong province, China (SD), and Jiangsu province, China (JS), were cultivated in the hatchery for several generations separately, and they could be distinguished by morphological characteristics, such as the yellow background on the shells of the JS stock and black stripes in the SD stock. In June 2007, males and females with typical shell colour were selected from both stocks, and each of them was induced to spawn in separate containers after being exposed to air for 4 h. After spawning, the eggs or sperms of 20 spawned females or males from each stock were pooled together respectively. Two reciprocal crosses and pure populations were produced using the following mating design: SD × JS (SDJS), SD × JS (SSD), JS × JS (JSJS), SD × JS (SDJS) and JS × SD (SSSD).

At the larval period, each population was separately reared in a 100 L plastic barrel at a density of 5 individuals mL⁻¹, fed with Dicrateria inornata three times a day and at increased concentration with larval development. After settlement, each population was transferred to a 500 L plastic barrel whose bottom was covered with sand of 0.5 cm thickness, fed with a plentiful diet of mixed Chaetoceros muelleri and D. inornata. After about 2 months, the four populations were transferred to four isolated ponds in a blargerigger culture pond ensuring a common environment at the hatchery of Zhejiang Marine Culture Research Institute (Wenzhou, China).

Development of microsatellite markers

Microsatellite markers were developed following the enrichment procedure described by Glenn and Schable (2005), with some modifications. Briefly, 2 µg genomic DNA was digested by 20 U Sau3AI (Takara, Japan), and then the fragments were ligated to 50 pmol phosphorylated linker, which was generated by annealed SauLA (5'-GGGTAACCCGGGAA GCTTGG-3') with SauLB (5'-GATCCCAAAGCTTCGGGTTACCGC-3'), using 40 U T4 DNA ligase (Takara). The ligated fragments that ranged from 500 to 1000 bp in size were excised from an agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Düsseldorf, Germany). Three groups of biotinylated oligonucleotides (Invitrogen, Carlsbad, CA, USA) were used to construct partial genomic libraries of M. meretrix-enriched di- or trinucleotide
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microsatellite sequences. Group 1 is (TC)_{15}, group 2 is (CA)_{15} and group 3 is [(AAC)_{12} and (AGT)_{12}]. The purified 500–1000 bp ligated fragments were hybridized with biotinylated oligonucleotide groups. Then the fragments that were hybridized to biotinylated oligo-nucleotides were captured with M280 streptavidin-fragments that were hybridized to biotinylated oligonucleotide groups. Then the fixed 500–1000 bp ligated fragments were hybridized with biotinylated oligonucleotide groups. The captured DNA was amplified using SauLA as the primer in a 50 μL polymerase chain reaction (PCR) system and purified using a QIAquick PCR purification kit (Qiagen). The fragments were ligated into a pMD18-T vector (TaKaRa) and transformed into host bacteria (*Escherichia coli*, DH5α).

The recombinants were identified through blue/white selection on ampicillin plates, and the white clones were further randomly selected to verify with PCR reactions using SauLA as the primer. From the three libraries, 100 positive clones with 500–900 bp inserts were used to sequence for microsatellites. Twenty primers were designed from the flanking regions of the microsatellites using PRIMER PREMIER 5.0. After optimizing the PCR conditions, a pool of five individuals was used to amplify the microsatellites, and PCR products were examined on a 2% agarose gel for 30 min. The amplifiable primer pairs were used to genotype a larger sample of individuals, and the ones with visible polymorphisms were selected for further analysis, whereas monomorphic ones were excluded. Nine polymorphic primers were detected.

**Sampling and data collection**

In April 2009, 30 individuals of each population were collected randomly from the different areas of the culture pond for genetic analysis with nine polymorphic microsatellites. Wet weight, shell length, shell width and shell height were measured for each selected individual, and foot tissue was dissected separately from fresh specimens and fixed immediately in 95% ethanol. Genomic DNA was extracted using the standard phenol/chloroform method (Sambrook, Fritsch & Manitaris 1989).

**Genotyping and fragment analysis**

Thirty randomly selected individuals from each population and nine polymorphic microsatellite loci (MM02, MM07, MM09, MM10, MM11, MM14, MM15, MM16 and MM17) were used for genetic analysis. Polymerase chain reaction amplification was carried out in a 20 μL reaction mixture containing 1 × PCR buffer (Promega, Madison, WI, USA), 2.5 μmol MgCl₂, 100 μmol each dNTP, 0.5 μmol of each primer, 0.5 U Taq DNA polymerase (Promega) and approximately 30 ng of template DNA. Thermal cycling was carried out in an MJ PCR-200 thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation for 4 min at 94 °C, 32 cycles of denaturation at 94 °C for 50 s, anneal at temperature (Table 1) for 40 s, and extension at 72 °C for 40 s and a final extension step at 72 °C for 10 min. The size of the alleles was determined in 8% non-denaturing acrylamide gels with pBR322 (MBI, Glen Burnie, MD, USA) as a standard DNA marker. For acrylamide gels, bands were stained with ethidium bromide and visualized under UV light. Individual diploid genotypes were identified manually using QUANTITYONE version 4.5 software (Bio-Rad).

**Statistical analyses**

Genetic diversity

The genetic variability of markers and populations was analysed to determine the genetic diversity for each population. The number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He) and polymorphic information content (PIC) in each population were calculated using CERVUS version 3.0 (Kalinowski, Taper & Marshall 2007). Tests for Hardy–Weinberg equilibrium (HWE) at each locus for each population were calculated based on Fisher’s exact test using the Markov-chain method (Markov-chain length, 100,000; dememorization, 10,000) using GENEPOP version 4.0.10 (Raymond & Roussset 1995; available at http://genepop.curtin.edu.au). Nei’s (1978) unbiased genetic distances among the populations were calculated using TFPGA programme version 1.3 (Miller 1997).

The inbreeding coefficient (FIS; Weir & Cockerham 1984) and the measure of genetic differentiation among populations (FST; Weir & Cockerham 1984) were calculated using GENEPOP version 4.0.10. The resultant FST values were explained based on the qualitative guidelines of FST values suggested by Wright (1978).

**Growth traits**

In order to ensure more accurate results, the mean growth traits of each population were estimated using
Table 1 Characteristics of the nine polymorphic microsatellite loci isolated from clam Meretrix meretrix

| Locus | Forward primers | Reverse primers | Repeat unit | Size (bp) | \( T_a (^\circ C) \) | GenBank |
|-------|-----------------|-----------------|-------------|----------|----------------|---------|
| MM02  | GACAAATAGCAGGACGATTATA | CCCTTGAAGTAACTGCAA | (CA)_{14} | 249–334 | 48 | GU250832 |
| MM07  | AAA ATGCCAACACACATGC | GTACAGGTTGCTAAGATAG | (CA)_{28} | 218–271 | 51 | GU250837 |
| MM09  | GCCGTTCGGAAACGGCAT | AAGGACAAACGTATAGGA | (CA)_{12}(CG)_{10}(CA)_{15} | 240–330 | 53 | GU250838 |
| MM10  | GTGCTAAATGGCTATCAGA | CACCTCTGAACCCCAAGAG | (TG)_{18} | 227–324 | 51 | GU250845 |
| MM11  | GTGACATCTCCCTAAAGCG | ATGTCTGACCTTGGTTGGGG | (AAC)_{13} | 242–340 | 54 | GU250846 |
| MM14  | TCAGGGATAACCGTGACAAAT | GCAATGACCCACACAGGAA | (GGT)_{b}(GTT)_{9} | 226–315 | 58 | GU250841 |
| MM15  | TCTGATAGCTGCTGAAACA | AGGGATACCGTGACATTG | (AGT)_{10}(ATC)_{30} | 152–291 | 57 | GU250842 |
| MM16  | TACCATAACCAACACAT | AAATTCGAAATCTGTTATCC | (ATC)_{10} | 169–250 | 50 | GU250843 |
| MM17  | TCGGATAGTCGCTTACAC | ATTCCTTGTTCTATCC | (AGC)_{25}(ATC)_{30} | 178–284 | 49 | GU250844 |

\( T_a \) annealing temperature.

the bootstrap method. In this method, the original data of the measurements of each population were placed in a pool, and then 30 values were sampled with replacement 1000 times. The mean values were calculated and exported to Excel format in its generated order for each bootstrap sample. These resampled data of four traits were transformed to logarithms to ensure normality and homoscedasticity (Neter, Wasserman & Kutner 1985). The differences in the four traits among the populations were analysed using one-way analysis of variance (ANOVA), followed by multiple comparison tests (Tukey method).

Besides of the possible heterotic effects resulting from the expected increased heterozygosity in the reciprocal crosses, the effect from maternal differences was also considered. To determine the effects of egg origin (SD vs. JS dams) and mating strategy (inter- vs. intra-population crosses) on the four traits, a two-factor ANOVA model was used (Cruz & Ibarra 1997):

\[
Y_{ijk} = u + EO_i + MS_j + (EO \times MS)_{ij} + e_{ijk}
\]

where \( Y_{ijk} \) is the mean shell length (or other three traits) of the \( k \) replicate from the \( i \) egg origin and the \( j \) mating strategy estimated using the bootstrap method; \( u \) is the overall constant; \( EO_i \) is the egg origin effect on shell length (or other three growth traits); \( MS_j \) is the mating strategy effect on length (or other three growth traits); \( (EO \times MS)_{ij} \) is the interaction effect between egg origin effect and mating strategy; and \( e_{ijk} \) is the random observation error (\( k=1,2,3,\ldots,1000 \)).

Heterosis (\( H \)) was calculated following the method of Van Vleck, Pollak and Oltenacu (1987) and Cruz and Ibarra (1997):

\[
H(\%) = \frac{[(F1 - P) \times 100]}{P}
\]

where \( F1 \) is the mean shell length (or other three growth traits) of reciprocal crosses; \( P \) is the mean shell length (or other three growth traits) of pure populations (intra-population crosses).

All of the statistical analyses above were performed using software SAS version 8.0, and significance was set at \( P<0.05 \).

**Relationship between marker heterozygosity and growth performance**

To gain a better understanding of relationship between marker heterozygosity and growth performance, all the individuals of the four populations were divided into two groups (big and small group) based on the phenotypic values of the four traits respectively. The large group comprised individuals with larger phenotypic values than the mean value of each trait of the four populations, and the remaining individuals comprised the small group. Accordingly, the numbers of heterozygote and homozygote at each locus of each trait for the large and the small group were counted respectively. The difference in the heterozygosity of the two groups at each microsatellite locus was evaluated using a \( \chi^2 \)-test.

**Results**

**Microsatellite locus characterization**

From the 100 sequenced clones from the three libraries, 79 clones, containing microsatellite regions with flanking regions for primer design, were isolated. From these, 20 shorter-repeat microsatellites were designed primer pairs to amplify with genomic DNA of *M. meretrix*, as larger-repeat microsatellites have a tendency to yield 'stuttering bands', possibly resulting from the mis-scoring of alleles (O’Connell & Wright 1997). Of these 20, five primer
pairs did not amplify products and three primer pairs appeared as non-specific bands on agarose electrophoresis. The remaining 12 loci were tested for polymorphism using 8% non-denaturized acrylamide gels, and the results indicated that three loci were monomorphic, leaving nine polymorphic markers for further analysis (Table 1). The nine polymorphic markers were from the three libraries.

Genetic variance of populations

According to the nine microsatellite loci amplified in the analysed populations, genetic variations of the four *M. meretrix* populations were evaluated, which are summarized in Table 2. In this analysis, a total of 146 alleles observed from the nine microsatellite loci were identified in 120 individuals of *M. meretrix*. Across all the populations, the number of alleles observed across the microsatellite loci ranged from 9 to 23. The overall mean numbers of alleles in SDJS and JSSD (6.778 and 5.556) were higher than that of JSJS and SDSD (5.111 and 5.112). The observed heterozygosities (H₀) were lower than the expected heterozygosity values (Hₑ) in all the populations. However, the observed heterozygosities of SDJS and JSSD (0.656 and 0.663) were higher than that of JSJS and SDSD (0.624 and 0.606) (Table 2). The expected heterozygosities of SDJS and JSSD (0.694 and 0.710) were higher than that of SDSD (0.665) but lower than JSJS (0.707). The mean PIC of SDJS and JSSD (0.655 and 0.654) were higher than that of JSJS and SDSD (0.647 and 0.608).

Positive inbreeding coefficient (Fₛ) values were detected in each locus and population (Table 2), indicating a heterozygote deficiency in all populations. The populations deviated significantly from HWE (P < 0.05) at some microsatellite loci through exact tests after sequential Bonferroni's correction (Table 2). The JSJS, SDSD, SDJS and JSSD populations had three, three, one and two microsatellite loci that deviated from HWE respectively.

The smallest value of Nei’s unbiased genetic distance among the four populations was between the two reciprocal cross populations (SDJS-JSSD, Dₙₑ = 0.1093), and the largest one was between

| Locus | JSJS | SDSD | SDJS | JSSD | Locus | JSJS | SDSD | SDJS | JSSD |
|-------|------|------|------|------|-------|------|------|------|------|
| MM02  | Na 4 | 5     | 8    | 7    | MM14  | Na 6 | 6     | 10   |
| H₀    | 0.600 | 0.739 | 0.667 | 0.750 | H₀    | 0.806 | 0.742 | 0.759 | 0.844 |
| Hₑ    | 0.657 | 0.726 | 0.723 | 0.818 | Hₑ    | 0.796 | 0.765 | 0.829 | 0.865 |
| PIC   | 0.578 | 0.671 | 0.718 | 0.776 | PIC   | 0.751 | 0.728 | 0.817 | 0.835 |
| P     | 0.661 | 0.210 | 0.332 | 0.020*| P     | 0.553 | 0.065 | 0.064 | 0.130 |
| MM07  | Na 4 | 3     | 3    | 3    | MM15  | Na 7 | 8     | 6    |
| H₀    | 0.316 | 0.384 | 0.533 | 0.516 | H₀    | 0.679 | 0.700 | 0.719 | 0.742 |
| Hₑ    | 0.570 | 0.564 | 0.472 | 0.566 | Hₑ    | 0.795 | 0.768 | 0.827 | 0.799 |
| PIC   | 0.469 | 0.492 | 0.419 | 0.518 | PIC   | 0.749 | 0.731 | 0.789 | 0.755 |
| P     | 0.020*| 0.017*| 0.262 | 0.543 | P     | 0.047*| 0.178 | 0.060 | 0.081 |
| MM09  | Na 4 | 4     | 8    | 6    | MM16  | Na 7 | 5     | 8    |
| H₀    | 0.667 | 0.609 | 0.784 | 0.806 | H₀    | 0.700 | 0.484 | 0.719 | 0.633 |
| Hₑ    | 0.701 | 0.629 | 0.801 | 0.811 | Hₑ    | 0.796 | 0.520 | 0.726 | 0.690 |
| PIC   | 0.744 | 0.545 | 0.770 | 0.766 | PIC   | 0.774 | 0.484 | 0.709 | 0.614 |
| P     | 0.282 | 0.897 | 0.368 | 0.051 | P     | 0.306 | 0.169 | 0.211 | 0.113 |
| MM10  | Na 5 | 5     | 4    | 6    | MM17  | Na 3 | 3     | 5    |
| H₀    | 0.609 | 0.682 | 0.438 | 0.677 | H₀    | 0.520 | 0.500 | 0.483 | 0.364 |
| Hₑ    | 0.757 | 0.723 | 0.497 | 0.702 | Hₑ    | 0.529 | 0.520 | 0.629 | 0.561 |
| PIC   | 0.698 | 0.657 | 0.434 | 0.647 | PIC   | 0.458 | 0.447 | 0.544 | 0.498 |
| P     | 0.086 | 0.883 | 0.001*| 0.036*| P     | 0.587 | 0.002*| 0.025*| 0.053 |
| MM11  | Na 6 | 5     | 6    | 4    | Mean  | Na 5.111 | 5.112 | 6.778 | 5.556 |
| H₀    | 0.742 | 0.655 | 0.800 | 0.633 | H₀    | 0.624 | 0.607 | 0.656 | 0.663 |
| Hₑ    | 0.769 | 0.770 | 0.741 | 0.708 | Hₑ    | 0.707 | 0.665 | 0.694 | 0.710 |
| PIC   | 0.724 | 0.714 | 0.693 | 0.642 | PIC   | 0.647 | 0.608 | 0.655 | 0.654 |
| P     | 0.016*| 0.003*| 0.054 | 0.072 | Fₛ    | 0.128 | 0.095 | 0.018 | 0.065 |

*Significant after Bonferroni’s correction (P < 0.05).
Na, number of observed alleles per locus; H₀, observed heterozygosity; Hₑ, expected heterozygosity; PIC, polymorphism information content; P, Hardy–Weinberg equilibrium test by P-value; Fₛ, inbreeding coefficient.
two pure populations (JSJS-SDSD, $D_L = 0.2993$) (Table 3). However, the genetic distance values between the pure populations and the reciprocal cross populations were very close to each other, ranging from 0.1381 to 0.1508. The analysis of Wright’s $F_{ST}$ values of population pairwise comparison showed moderate differentiation ($0.05 < F_{ST} < 0.15$) among these four populations (Table 4). The highest genetic differentiation was detected between the two pure populations, and the lowest genetic differentiation was between the reciprocal cross populations.

### Growth difference of populations

The results of one-way ANOVA for the growth traits of the four populations are summarized in Table 5. From Table 5, we can see that the same trend is shared by the four traits. Take shell length for example: the mean shell length of the reciprocal crosses (46.124 mm) was significantly larger than that of the pure populations (44.547 mm) ($P < 0.05$), indicating hybridization could improve the growth traits of the clam. In the reciprocal cross populations, the mean shell length of JSSD (46.274 mm) was larger than that of SDJS (45.974 mm), but not significantly ($P = 0.13$).

In addition, significance of the egg origin and mating strategy for the four traits was observed in the present study (Table 6). When the SD population was used as the female parent, the phenotypic data of the offspring were obviously higher, indicating the effects of egg origin.

The estimated heteroses of the four traits are shown in Fig. 1. The four traits in the two reciprocal cross populations obviously showed heterosis but there was a difference among the tested traits. Heterosis for SDJS ranged from 4.3% (shell width) to

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**Table 3** Nei’s unbiased genetic distance among the four populations of *Meretrix meretrix*

| Population | JSJS | SDSD | SDJS | JSSD |
|-----------|------|------|------|------|
| JSJS      | –    | 0.2993 | 0.1381 | 0.1439 |
| SDSD      | 0.2993 | –    | 0.1414 | 0.1508 |
| SDJS      | 0.1381 | 0.1414 | –    | 0.1093 |
| JSSD      | 0.1439 | 0.1508 | 0.1093 | –    |

**Table 4** $F_{ST}$ values of pairwise comparison among the four populations of *Meretrix meretrix* at nine microsatellite loci

| Population | JSJS | SDSD | SDJS | JSSD |
|-----------|------|------|------|------|
| JSJS      | –    | 0.1438 | 0.0907 | 0.1101 |
| SDSD      | 0.1438 | –    | 0.1086 | 0.0583 |
| SDJS      | 0.0907 | 0.1086 | –    | –    |
| JSSD      | 0.1101 | 0.0583 | –    | –    |

**Table 5** Mean growth traits of (a) each population and (b) the crosses or pure populations of *Meretrix meretrix*

| Group/trait | Shell length (mm) | Shell height (mm) | Shell width (mm) | Wet weight (g) |
|-------------|-------------------|-------------------|------------------|----------------|
| (a)         |                   |                   |                  |                |
| SDSD        | 47.057*            | 39.269*           | 23.314*          | 27.576*        |
| JSSD        | 46.274*            | 38.977*           | 23.281*          | 27.097*        |
| SDJS        | 45.974*            | 38.505*           | 23.082*          | 26.801*        |
| JSSD        | 42.037b            | 35.046b           | 21.149b          | 20.996b        |
| (b)         |                   |                   |                  |                |
| JSSD and SDJS | 46.124*           | 38.741*           | 23.182*          | 26.949*        |
| JSJS and SDSD | 44.547*           | 37.158*           | 22.232b          | 24.286b        |

Within each column, means with different superscript letters are statistically different ($P < 0.05$).
12.2% (wet weight), and heterosis for JSSD ranged from 5.4% (shell width) to 13.9% (wet weight). The results suggested that different amounts of heterosis could be obtained for different traits by the mating strategy of hybridization.

### Relationship between marker heterozygosity and growth performance

The $\chi^2$-tests for the heterozygosity difference between the large and the small group at each locus of the four traits are shown in Table 7. The heterozygosity at locus MM14 in the large group was significantly higher than that of the small group for all the four traits. Furthermore, for shell length, shell width, shell height and wet weight, the heterozygosity at loci MM11, MM1 and MM16, MM15 and MM11 in the large group was significantly higher than that of the small group respectively. However, the heterozygosities at other loci for the four traits were not different between the two groups. The data suggest that the growth performance is dependent on higher heterozygosity at some microsatellite loci.

### Discussion

Intra-specific hybridization is an effective way to increase the productive efficiency of many species, and the method is based on the theory of heterosis. Heterosis is displayed when the offspring performance exceeds the average performance of their parents, especially when the parents are more genetically different (Shikano & Taniguchi 2002). Diallel mating design is an important tool in breeding programmes to obtain heterosis. In marine mollusks, the application of crossbreeding as an important means of genetic improvement was later than that in agriculture animal and plant species. However, considerable progress in mollusks has been achieved through intra-specific hybridization since the 1990s. For instance, Hedgecock, McGoldrick and Bayne (1995) reported that two inbred lines of the Pacific oyster

![Figure 1](image-url)  
**Figure 1** Results for difference in heterosis on the four traits for the reciprocal crosses of *Meretrix meretrix*. SL, shell length; SH, shell height; SW, shell width; WW, wet weight.

![Table 7](table-url)  
**Table 7** The $\chi^2$-tests for the heterozygosity difference between big and small group at each locus of the four traits

| Trait     | MM14 | MM15 | MM16 | MM17 | MM11 | MM12 | MM13 | MM14 |
|-----------|------|------|------|------|------|------|------|------|
| Shell length | 9.88 | 2.8  | 0.00 | 1.99 | 8.74 | 1.97 | 1.30 | 0.14 |
| d.f.      | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| $P$       | $<0.01^*$ | >0.05 | >0.05 | >0.05 | $<0.01^*$ | >0.05 | >0.05 | >0.05 |
| Shell width | 9.96 | 0.94 | 7.33 | 2.39> | 9.21 | 1.15 | 0.38 | 0.32 |
| d.f.      | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| $P$       | $<0.01^*$ | >0.05 | $<0.01^*$ | >0.05 | $<0.01^*$ | >0.05 | >0.05 | >0.05 |
| Shell height | 9.88 | 9.69 | 0.12 | 3.38 | 1.21 | 1.17 | 0.86 | 0.15 |
| d.f.      | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| $P$       | $<0.01^*$ | $<0.01^*$ | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| Wet weight | 9.55 | 3.79 | 1.35 | 3.02 | 13.54 | 3.13 | 1.93 | 1.78 |
| d.f.      | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| $P$       | $<0.01^*$ | >0.05 | >0.05 | >0.05 | $<0.01^*$ | >0.05 | >0.05 | >0.05 |

*Significant between the two groups. $\chi^2$; value of the $\chi^2$-test; d.f., degree of freedom.
were crossed in a $2 \times 2$ fashion to produce two inbred and two hybrid crosses, and both hybrid crosses had significantly lower mortalities and higher shell height than inbred populations. Zhang, Liu, Zhang and Wang (2007) carried out a complete diallel cross between two geographical bay scallop populations and found that the shell lengths of the two reciprocal crosses were significantly greater than those of pure populations. Deng, Liu, Wu and Zhang (2008) built a $2 \times 2$ factorial cross between two geographical populations of Pacific abalone and heteroses for all the analysed traits were detected.

In the previous studies, researchers have studied the genetic background of different geographical natural populations of *M. meretrix* using some molecular or morphological methods. For example, Du et al. (2004) analysed the genetic diversity of seven natural clam populations from two geographical locations using morphological parameters and RAPD markers. They detected that shell-morphological characteristics and the growth rates of Guangxi populations were significantly larger than those of Guangdong populations, and they also found that the genetic distance positively correlated to geographical distance between the populations. In addition, Lin et al. (2008) studied the genetic diversity of four different geographical natural populations using morphological parameters and AFLP markers. They suggested that shell-morphological characteristics of the Guangxi population were significantly different from that of Shandong and Jiangsu populations, and there were genetic diversities among the four natural populations. All these researches suggested that different geographical natural populations of *M. meretrix* showed differences in the morphological parameters, growth rates and genetic background, which have provided the probability for increasing the productive efficiency through crossbreeding among different geographical populations of *M. meretrix*. In the present work, our deduction was validated by the analysis on the genetic variance, using nine novel microsatellite markers, based on a diallel mating of two geographical populations (JS and SD).

In this study, Nei’s unbiased genetic distance revealed that there was genetic divergence between JSJS and SDSD, ($D_c = 0.2993$), which was also supported by the $F_{ST}$ values ($0.05 < 0.1438 < 0.15$). The genetic parameters indicated that the number of alleles, observed heterozygosity and PIC of the reciprocal crosses (SDJS and JSSD) were higher than those of the pure populations (JSJS and SDSD). We believe that the higher genetic diversity of reciprocal crosses resulted from the effect of hybridization, as has been reported by Tapio, Tapio, Grislis, Holm, Jeppsson, Kantanen, Miceikiene, Olsaker, Vinalass and Eythorsdottir (2005). This suggested that genetic diversity could be increased through hybridization between different geographic populations of *M. meretrix* with high genetic variance.

Although a higher genetic diversity in the reciprocal cross populations was generated by hybridization, an existing heterozygote deficiency was indicated by the $F_{IS}$ values in the two populations. null alleles probably represented one important factor for heterozygote deficiency. It has been reported that null alleles are probably a major cause of heterozygote deficiency observed in microsatellite marker analysis of populations (Holm, Loeschke & Bendixen 2001), and many studies have revealed that null alleles were common in bivalve mollusks (Hedgecock, Li, Hubert, Bucklin & Ribes 2004). The gene homogenization at some loci during the process of selection of parental clams was probably another important factor. However, genotype errors produced at the different steps of molecular marker genotyping, including non-specific amplification, or human mistakes in data acquisition (Pemberton, Slate, Bancroft & Barrett 1995; Brinkmann, Klintzchar & Neuhuber 1998; Bonin, Bellemain, Bronken Eidesen & Pompanon 2004) could not be excluded.

Some microsatellite loci in the four populations deviated from HWE significantly ($P < 0.05$). The deviation from HWE was observed universally in marine commercial fish (Kuang, Tong, Xu, Yin & Sun 2009) and mollusks (Sekino & Hara 2001; Sekino, Hamaguchi, Aranishi & Okoshi 2003; Reece, Ribeiro, Gañey, Bellemain, Bronken Eidesen & Pompanon 2004). The HWE deviations in our study may be due to a small population scale, non-random mating and the occurrence of selection in breeding. Furthermore, heterozygosity deficiency was an important factor resulting in deviation from HWE. Consequently, a large mating population and optimal mating strategy should be adopted in further breeding programmes for *M. meretrix* to avoid significant gene frequency and genotype frequency deviation from HWE.

In the present study, the mean growth traits in the reciprocal cross populations was found to be significantly larger than that of the pure populations, indicating an improvement in the growth was obtained by hybridization. As higher genetic diversity was found in the reciprocal cross populations, we deduce that higher growth performance is related to higher genetic diversity, which was supported by higher het-
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erozygosity at some loci in the large group. This result is consistent with the results observed by Syed and Chen (2005), who found that heterosis was related to the enhancement in heterozygosity.

In the reciprocal cross populations, the mean growth traits of JSSD were larger than those of SDJS. Our analysis indicated that a significant maternal effect existed, which led to JSSD showing higher growth performance. It seems that the offspring were obviously larger when the SD population was used as the female parent than when the JS population was used. Maternal effects are also common in other marine mollusks, such as the oyster *Crassostrea gigas* (Hedgecock et al. 1995; Hedgecock & Davis 2007) and *Crassostera virginica* (Mallet & Haley 1984) and the scallop *Argopecten irradians* (Zhang et al. 2007). These results suggested the importance of examining the performance of both the male and the female parent populations before a diallel mating for commercial seed production of *M. meretrix*.

In conclusion, compared with pure populations, the higher genetic diversity and the existing heterosis observed in reciprocal cross populations indicated that hybridization between different geographical populations of *M. meretrix* with high genetic variance is a proper method for genetic improvement and productive efficiency. The significant maternal effect for the growth traits suggested that the selection of mating populations and strategy in a breeding programme should also be considered.

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