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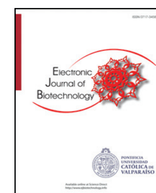
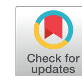


Research article

Genetic diversity and differentiation of nine populations of the hard clam (*Meretrix petechialis*) assessed by EST-derived microsatellites



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ABSTRACT

Background: *Meretrix petechialis* is one of the commercially important marine bivalves. In this study, we selected six highly polymorphic EST-derived microsatellite markers to assess the genetic diversity and population differentiation on nine wild populations of *Meretrix petechialis*.
Results: The number of alleles detected per locus ranged from 4 to 30 (mean $N_A = 27.5$) with a total of 165 alleles. The mean value of observed and expected heterozygosities varied from 0.717 to 0.861 and from 0.797 to 0.856, respectively. Meanwhile, the result of Neighbor-joining and overall $F_{ST} = 0.214$ ($P < 0.01$) reveled that *M. petechialis* populations from GX are the farthest populations, a certain degree of genetic variation among individuals in each population and the genetic differentiation is significant.
Conclusions: GX population has high genetic diversity among individual, and there are certain differences in genetic characteristics among different populations. This study will provide a basis for the domestication and cultivation of genetic diversity of *M. petechialis* population and the protection of clam germplasm resources.
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1. Introduction

The clam *Meretrix petechialis* is one of the most important economic marine mollusks, which is widely distributed in China, Japan and Korea [1]. In recent years, however, *M. petechialis* resources have declined dramatically due to habitat destruction, climate change, environmental pollution and overfishing, resulting in their genetic diversity is fragile [2,3]. To effectively utilize resources when breeding and proliferating marine bivalves, it is usually improved according to genetic diversity, so that excellent varieties can be obtained for reproduction. However, according to years of farming experience, if the progeny produced by a large number of parents continue to be cultivated as seedlings, the genetic diversity of the offspring may be affected, so that the offspring may not be able to resistant to adverse environment due to genetic defects [4].

In a complex environment of the coastal areas, it is a challenge for organisms to adapt to the changing climate and environment of the intertidal if they maintain a single genetic feature. Therefore, it is necessary for those organisms to have complex and diverse mutations and resulting in high genetic variations [5]. The protection and management of wild species play a pivotal role in determining which populations should be protected primarily [6]. By using population genetic analysis, effective population size, genetic structure, and so on can be analyzed and studied, so it can be said that it is of great significance for exploring population genetic resources and protecting population diversity [7].

Microsatellite markers or (Simple sequence repeats) SSRs have been widely used in genetic linkage maps, species identification, and molecular marker–assisted construction of a variety of marine shellfishes due to its high level of allelic variation, polymorphism, codominant inheritance, transferability, and good reproducibility [8,9,10]. With the continuous development of science and technology, microsatellite markers have become an effective means to assess population mutations and are widely used in the study of population diversity and population heredity [11]. In recent years, several studies have been conducted to develop the microsatellite markers of the *M. petechialis* [2,8,12,13]. So far, however, there have been few studies on *M. petechialis* genetic structure [14,15]. In order to genetically improve this species and promote the clam industry and sustainable

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Table 1
Sample details for the nine populations of the hard clam *Meretrix petechialis*.

Population	Abbreviation	Collection date	Geographical coordinates	*n*
Fujian	FJ	March, 2017	25°46′ N,119°01′ E	30
Guangxi	GX	March, 2017	21°49′ N,109°13′ E	30
Panjin	PJ	May, 2017	41°13′ N,122°08′ E	30
Shandong	SD	April, 2017	37°20′ N,122°07′ E	30
Dandong	DD	May, 2017	40°01′ N,124°36′ E	30
Hainan	HN	June, 2017	18°26′ N,109°52′ E	30
Dalian	DL	June, 2017	39°69′ N,122°97′ E	30
North Korea	NK	July,2017	40°00′ N,127°00′ E	30
Jiangsu	JS	September,2017	31°99′ N,120°89′ E	30

n number of individuals per site.

development, we need to analyze and investigate the genetic variation of *M. petechialis* to figure out its genetic diversity and population differentiation [16].

In this study, six highly polymorphism were selected from EST-derived SSRs of *M. petechialis* to assess the genetic characteristics of the species and in-depth studies and analyses were conducted on the differentiation of the nine *M. petechialis* populations. The present work shed light on the genetic conservation of *M. petechialis* populations and provides the basic knowledge and management methods for the selective cultivation of the *M. petechialis* population.

2. Materials and methods

2.1. Collection of samples and extraction of DNA

M. petechialis were sampled from different tidal flats and obtained a total of 270 samples from nine locations. The picture of *M. petechialis* was shown in Fig. S1. *M. petechialis* were collected from nine wild populations of Fujian (FJ), Guangxi (GX), Panjin (PJ), Shangdong (SD), Dandong (DD), Hainan (HN), Dalian (DL), North Korea (NK), and Jiangsu (JS) (Table 1; Fig. 1). The samples were dug out manually from the mudflats, then sealed (no water) and put into several ice bags shipped to Dalian, and cultivated in Research Center of Shellfish Breeding in Dalian Ocean University one week at room temperature. Extracting each tissue of samples and storing in 100% ethanol solution, the Genomic DNA of adductor muscle was extracted by using TIANamp Marine Animals DNA Extraction Kit (TIANGEN, Beijing, China). DNA quality and concentration was evaluated using 1% agarose gel electrophoresis, Preparing the TE buffer and storing the extracted DNA in it, then perform PCR after the quality test was completed. The concentration was diluted to 100 ng/μl and used for subsequent experiments.

2.2. SSR marker analysis

Due to the large number of alleles in the species and the consideration of the heterozygosity of the species, the 6 loci with the highest polymorphism were selected from the 10 polymorphic microsatellite markers verified by Zheng et al. [17] to study the genetic diversity of *M. petechialis*, and the primer information used in the simple repeat sequences used herein is detailed in Table 2, so as to obtain the data needed for this study. The parameters for PCR were as follows: 1 × PCR buffer; dNTP mixture was 0.2 mM; Taq DNA polymerase was produced in Takara, Japan, content was 0.25 U; $MgCl_2$ concentration was 1.5 mM, and template DNA was 100 ng. The PCR reaction volume was 10 μl. The reaction conditions were: 5 min at 94°C, 35 cycles, 30 s at 94°C, an annealing temperature of 30 s, then 30 s at 72°C, an extension temperature of 72°C, and an extension time of 7 min. After the completion of the PCR, detection was carried out by denaturing polyacrylamide gel electrophoresis, and in order to ensure the accuracy of results, a control is required for each test.

2.3. Genetic diversity analysis of data

By analyzing and calculating the data results using MICROSELLITE ANALYZER (MSA; [18]), include the observed heterozygosity (H_O), expected heterozygosity (H_E), allele size range, and the number of alleles (N_A). Heterozygotes were studied and analyzed by calculating the F-statistic. In the experiments conducted in this paper, the accuracy of the experimental results in this paper was ensured by using GENEPOP 4.0 software [19,20]. GENEPOP 4.0 software uses the Markov chain method to calculate the significant deviation of Hardy–Weinberg equilibrium (HWE) according to the parameters. The parameter values are: iterations = 1000, s = 500 and dememorization = 10,000. Rice [21] uses the sequential Bonferroni programs significantly adjust the number of simultaneous trials. Using Fisher exact detection method and linkage disequilibrium was

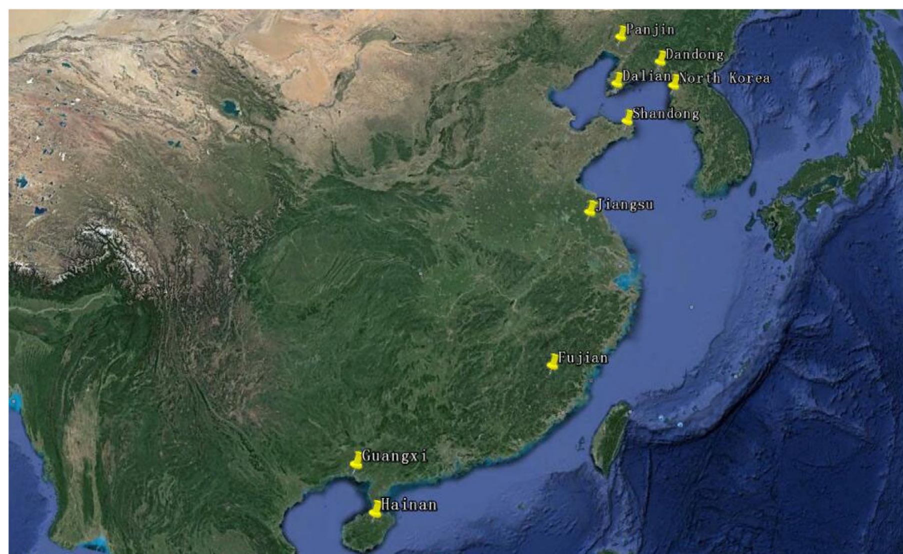

Fig. 1. Maps showing locations of the nine populations of *M. petechialis* sampled (FJ, GX, PJ, SD, DD, HN, DL, NK and JS).

Table 2
Characteristics of six EST-SSR markers in the hard clam *Meretrix petechialis*.

Locus	GenBank accession number	Repeat motif	Primer sequence (5′-3′)	T_a (°C)
Mmt31	GR211629	(AC)6	F:ATGATGATTTTGGTCTGCCATAGTA R:CGCTTACAACCATCGTAAATGATAT	60
Mmt34	GR211485	(AAC)8	F: CAGTGATTGAGTAAATAATTGTAGA R: ACGATTTTATTAAAGAGCTAGTATG	52
Mmt36	GR211423	$(TGA)_8$	F: AGACCACTCATCCCAAGACA R: GCCAAGGTTATAGTATTTATTTCTG	57.2
Mmt42	GR211338	$(CAA)_6$	F: TTCACAACTACGCACATACC R: TGATCTTCCCAATTACAAAG	50.4
Mmt45	GR211094	$(GT)_7$	F: AATCTTTACTATGTGAGAAATCGTT R: AGAGTTCAAAAGTAGTTGGAGATCA	57.2
Mmt47	GR210978	$(TTC)_{12}$	F: ACCAAAAAATATCTCAACTTAGCACT R: CTCGTGTTATTTGTGTTACAGTTCA	50.4

T_a, annealing temperature. * indicates significant departure ($P < 0.05$) from expected Hardy–Weinberg equilibrium conditions after correction for multiple tests ($k = 12$).

evaluated. The same procedure was used to test the linkage imbalance between loci pairs. Van Oosterhout et al. [22] use Micro-Checker software to estimate microsatellite site null alleles.

2.4. Cluster analysis of sample population

In order to analyze and study the genetic diversity of the sample, we measured population subdivision using Weir and Cockerham's [23] unbiased estimator of Wright's F statistics. Microsatellite Markers Reveal a Spectrum of Population Structures in the nine of *M. petechialis* populations (MSA [18]). F_{ST} values statistics were performed in 1000 permutations, and their significance was tested. To ensure accurate results, the results were calibrated using the Bonferroni program [21]. A suitable algorithm was constructed using the software MAGA 7.0 by the calculation of the pitch D_C.

3. Results

3.1. Population genetic parameter analysis

In the results of this study, we found the level of polymorphism is different in six microsatellite loci. The total number of alleles was detected 165 in six microsatellite loci, with an average of 27.5 alleles per locus. Among them, the microsatellite with the most polymorphism detected 55 alleles, which was *Mm47*, and the least polymorphism *Mmt31* microsatellite detected a total of 15 alleles. Estimated fragment size at each locus was between 126 and 297 bp (Table 3).

The Genotype locus data of nine hard calm populations were used to calculate the parameters of N_A, H_O, and H_E for assessing the genetic diversity and population level at the molecular level (Table 3). For nine populations, the average observed and expected heterozygosities

Table 3
Genetic diversity of nine populations of *Meretrix petechialis*.

EST-SSR		FJ	GX	PJ	SD	DD	HN	DL	NK	JS	Total
Mmt31	N_A	7	8	5	8	10	9	7	10	8	15
	S	128–135	126–138	126–134	128–137	126–135	128–140	128–134	127–136	132–140	126–140
	H_O	0.867	0.690	0.633	0.800	1.000	0.967	0.967	0.867	0.964	0.783
	H_E	0.766	0.785	0.726	0.790	0.790	0.772	0.769	0.807	0.840	0.828
	HWE	0.8855	0.9988	0.8465	0.6339	0.0005*	0.0006*	0.0019*	0.3360	0.0246*	
Mmt34	N_A	9	16	19	11	12	11	9	12	10	24
	S	142–154	139–159	135–155	142–153	136–147	136–148	141–151	141–152	138–154	135–159
	H_O	1.000	0.967	0.967	1.000	1.000	1.000	1.000	0.933	1.000	0.838
	H_E	0.757	0.894	0.900	0.849	0.873	0.771	0.860	0.861	0.774	0.922
	HWE	0.0000*	0.1614	0.2079	0.0060*	0.0066	0.0000*	0.0095*	0.4460	0.0000*	
Mmt36	N_A	10	6	14	8	9	12	14	9	10	22
	S	207–217	209–217	201–216	207–215	199–210	200–217	196–211	202–216	207–216	196–217
	H_O	0.733	0.345	0.633	0.700	0.500	0.500	0.533	0.433	0.655	0.840
	H_E	0.853	0.740	0.868	0.823	0.771	0.890	0.914	0.849	0.847	0.921
	HWE	0.9896	1.0000	1.0000	0.9357	0.9953	1.0000	1.0000	1.0000	0.9870	
Mmt42	N_A	7	5	7	6	7	5	12	9	4	20
	S	184–210	190–210	190–199	190–196	190–199	190–199	187–201	196–204	190–199	184–210
	H_O	0.586	0.333	0.400	0.269	0.533	0.275	0.667	0.267	0.467	0.700
	H_E	0.801	0.616	0.698	0.773	0.573	0.594	0.782	0.817	0.645	0.808
	HWE	0.9989	0.9998	1.0000	1.0000	0.5255	0.9993	0.9109	1.0000	0.9953	
Mmt45	N_A	18	13	9	12	8	11	4	9	12	29
	S	168–186	170–184	172–184	181–198	178–186	177–192	182–188	179–188	178–191	168–198
	H_O	1.000	0.964	1.000	1.000	0.897	1.000	1.000	1.000	1.000	0.829
	H_E	0.949	0.917	0.829	0.845	0.817	0.812	0.647	0.871	0.773	0.915
	HWE	0.2020	0.2141	0.0056*	0.0052*	0.2040	0.0000*	0.0000*	0.0210	0.0000*	
Mmt47	N_A	21	17	30	19	24	26	22	16	16	55
	S	224–294	250–292	246–295	260–296	255–296	258–290	249–286	262–282	269–297	224–297
	H_O	0.967	1.000	1.000	1.000	0.966	1.000	1.000	1.000	0.966	0.946
	H_E	0.924	0.936	0.973	0.951	0.955	0.964	0.954	0.932	0.921	0.972
	HWE	0.1635	0.1879	0.5531	0.2262	0.6621	0.5271	0.2083	0.1395	0.2753	
Mean	N_A	12	10.8	14	10.7	11.7	12.3	11.3	10.8	10	
	H_O	0.859	0.717	0.772	0.795	0.816	0.790	0.861	0.750	0.842	
	H_E	0.842	0.815	0.832	0.839	0.797	0.801	0.821	0.856	0.800	

Note: N_A, observed number of alleles; S, allele size range; H_O, observed heterozygosity; H_E, expected heterozygosity.
* Significant departure from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.05$).

Table 4
Pairwise F_{ST} values (below diagonal) and Cavalli-Sforza and Edwards chord distance (D_C, above diagonal) between nine *M. petechialis* populations.

	FJ	GX	PJ	SD	DD	HN	DL	NK	JS
FJ	0	0.011	0.005	0.074	0.005	0.004	0.004	0.004	0.004
GX	0.075	0	0.010	0.075	0.010	0.010	0.010	0.010	0.010
PJ	0.016	0.070	0	0.072	0.005	0.004	0.004	0.005	0.004
SD	0.894	0.901	0.881	0	0.071	0.069	0.070	0.071	0.069
DD	0.018	0.071	0.018	0.867	0	0.004	0.003	0.002	0.003
HN	0.010	0.067	0.013	0.860	0.010	0	0.003	0.003	0.002
DL	0.014	0.070	0.014	0.862	0.006	0.006	0	0.003	0.002
NK	0.014	0.070	0.017	0.867	0.004	0.009	0.003	0	0.003
JS	0.012	0.067	0.012	0.856	0.006	0.004	0.005	0.005	0

Permutations test proved all the F_{ST} were significant ($P < 0.01$).

ranged from 0.717 (GX) to 0.861 (DL) and from 0.797 (DD) to 0.856 (NK), respectively. The number of alleles ranged from 4 to 30 per locus, with the mean maximum and minimum value were 14 (PJ) and 10 (JS), respectively.

3.2. Hardy–Weinberg equilibrium

In this study, the value of Hardy–Weinberg equilibrium (HWE) showed that there was different degrees deviation in each locus of 9 populations (25.9%) after Bonferroni correction for multiple comparisons (Table 3). Three microsatellite loci deviated significantly from HWE, and *Mmt34* and *Mmt45* is high significantly at FJ, HN, JS and HN, DL, JS, respectively ($P < 0.05$). In general, these loci are suitable for the analysis of this study. Two of the three loci that deviated from HWE detected the null allele by Micro-Checker analysis and the lack of heterozygotes was found in seven populations.

3.3. Genetic variation and cluster among populations

The data of 6 microsatellite loci was employed to calculated the Pairwise F_{ST} values and Cavalli-Sforza and Edwards chord distance (D_C) between nine populations involved in the experiments described herein (Table 4). The range of paired F_{ST} changed from 0.003 to 0.901 indicated the minimum differentiation was between DL and NK, the maximum was between GX and SD population, respectively. Between these populations with the lowest genetic distance of D_C is the following three pairs of populations, namely DL and JS, HN and JS, DD and NK (0.002), the largest D_C genetic distance is between SD and GX population (0.075). The unweighted pair method (Neighbor-Joining) based on D_C (Fig. 2) indicated. It can be observed from the analysis that the nine populations were clustered into three groups. First, the two categories separately included PJ, JS, FJ, HN and NK, DL, SD, DD, and then clustered with the GX population.

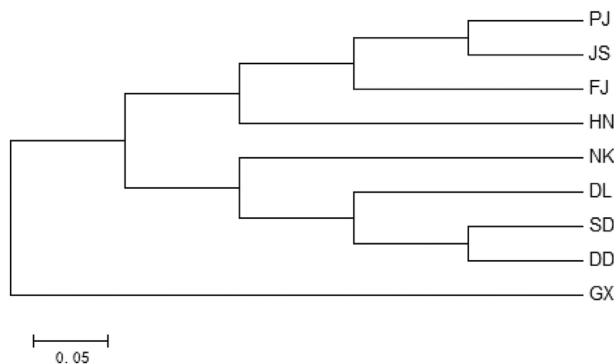

Fig. 2. The unweighted pair group method with the arithmetic mean (Neighbor-Joining) dendrogram based on D_C distance among the nine populations of *M. petechialis* (FJ, GX, PJ, SD, DD, HN, DL, NK and JS).

4. Discussion

4.1. Marker informativeness

In the past decades, a large amount of relevant information indicating that microsatellites have been characterized from marine aquaculture species and have been used extensively in the identification of variety strains in molecular marker-assisted selection, population genetics, genetic linkage maps, and quantitative traits loci (QTL) identification [24,25,26,27]. Studies have showed that population analysis of these collinear multi-labels is more efficient [28]. In the research experiments described in this paper, the genetic diversity of this population was demonstrated by SSR markers of clam populations. Compared with the results reported previously [29,30], the current experimental results described herein demonstrate higher genetic diversity and significant genetic differentiation in hard clam. Because marine organisms, especially bivalves, have high mutation rates in their microsatellites and populations, this result is in line with expectations [31].

4.2. Genetic diversity of M. petechialis

Heterozygosity refers to the ratio of heterozygote to microsatellite loci, and N_A, H_O, H_E are the optimal parameters to measure the genetic differences of a population at multiple loci. In the present study, the six polymorphism microsatellite loci were adopted to study and analyze the heterozygosity and allelic diversity of the populations. The number of polymorphic alleles in each locus (varied from 15 to 55, with an average of 27.5 per locus) is higher than those previous studies by Wang et al. [32] (ranging from 2 to 5, mean $N_A = 2.96$) and Dong et al. [8] (ranging from 2 to 8, mean $N_A = 4.19$). It indicated that the genetic diversity of different loci is different. Meanwhile, the mean value of N_A, H_O, H_E at 9 populations is ranged from 10 to 14, 0.717 to 0.861 and 0.797 to 0,856, respectively, which were similar with other studies of bivalves using microsatellite markers [33,34,35,36,37]. The observed heterozygosity was lower than the expected heterozygosity, indicating that there was heterozygote deficiency and excess of homozygotes in 9 wild populations of *Meretrix petechialis*. In the present study, all nine populations (overall $H_E = 0.823$) have high level of genetic diversity, indicating the germplasm resources of *Meretrix petechialis* are abundant and there is still enough breeding potential to carry out breeding, the reason may be related to high nucleotide mutations rates [36,37]. Null alleles are generally thought to be caused by variations in bases in the primers [38,39]. According to the result of Hardy–Weinberg equilibrium, it may be caused by natural evolution, mutations, ineffective alleles etc. [38].

4.3. Genetic differentiation characteristics of population

Genetic diversity plays an important role in selective breeding and the development of disease-resistant populations. The extent of population differentiation can be determined by using F_{ST} values in studying the genetic diversity of the *M. petechial* population. According to the existing research and treatment, the most effective distance measurement of the tree topology can be obtained by D_C value [40]. Relevant research data indicate four qualitative guiding effects of F_{ST} values [41], which are the largest degree of genetic differentiation is 0.25, and the great degree of genetic differentiation is 0.15, the maximum is 0.25, and moderate genetic differentiation The minimum is 0.05, the maximum is 0.15, and the least genetic differentiation is less than 0.15. Combine the result of the pairwise Fst, Dc and dendrogram, *M. petechialis* populations from GX have a certain degree of genetic variation among individuals in each population. Recently, Xu et al. [42] and Zheng et al. [43] reported that *M. petechialis* have high degree of genetic diversity and the potential of further breeding with excellent germplasm resources. In the present study, the F_{ST} value is 0.214 (>0.15), indicating that the population

studied in this study has a large degree of genetic differentiation. The cause might be the accumulation of mutations over time in different generations, which are probably leads to a high level of genetic variation in *M. petechialis* populations. The results showed that the germplasm resources of *M. petechialis* are abundant, and there is sufficient genetic diversity and potential to continue breeding.

5. Conclusion

This study showed that microsatellite markers are an effective approach to monitor the genetic diversity of *M. petechialis* populations. Among the 9 populations analyzed in this work, GX population has high genetic diversity among individual, and there are certain differences in genetic characteristics among different populations. Clam seed transplants between provinces in China to increase populations may result in relatively close genetic relationships among the original populations and reduced genetic variation. Further management and preservation of the results of this study can provide reference for other researchers to study genetic diversity and population differentiation in the future, and provide guidance for solving genetic problems.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

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Supplementary material

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