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Microsatellite marker analysis reveals the distinction between the north and south groups of hard clam (*Meretrix meretrix*) in China

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ABSTRACT. *Meretrix meretrix* is one of the important commercial bivalves in China. A total of 198 individual clams were collected from 5 locations characteristic of the clam's 5 main natural habitats in China, that is, Shandong, Jiangsu, Fujian, Guangdong, and Guangxi. Ten polymorphic microsatellite markers were selected to examine the genetic diversity and identify genetic differences between the 5 populations. A total of 183 alleles across 10 loci were detected in the individual clams. The observed heterozygosity and expected heterozygosity ranged from 0.197 to 0.7026 and from 0.6264 to 0.9408, respectively. The genetic diversity within samples was high (8.6-11.2 alleles per locus, observed heterozygosity = 0.25-0.875 and expected heterozygosity = 0.6848-0.9259). Most of the genotype distributions significantly deviated from Hardy-Weinberg equilibrium. Genetic structure analysis showed that

the 5 populations could be divided into 2 groups, the north and south groups. Neighbor-joining analysis revealed a clear distinction between the north group (Shandong and Jiangsu) and the south group (Fujian, Guangdong, and Guangxi). Locus MM1031 was used to distinguish between groups. Our results can be used for population identification and crossbreeding of *M. meretrix*.

Key words: *Meretrix meretrix*; Geographical differentiation; Population genetic structure; Microsatellite

INTRODUCTION

Meretrix meretrix is a widely distributed commercial species of hard clam present along the coastal and estuarine regions of China (Tang et al., 2006). This hard clam has become the main maricultured shellfish in China. Imbalanced larval distribution and high market demands have led to overexploitation of natural stocks of this species, severely diminishing its genetic diversity (Dong et al., 2013). Increasing the clam's productivity is necessary to allow for its continued use as a commercial species (Liu et al., 2006).

Various breeding programs, such as mass selection, family selection, and hybridization between different geographic populations, have been used for *M. meretrix* (Wang et al., 2011a). However, direct hybridization in accordance with phenotype is time-consuming, is labor-intensive, and has a high resource requirement (Lu et al., 2012a). Population genetics may offer an alternative approach for selecting combinations with distinct genetic backgrounds that are suitable for hybridization.

Morphological and molecular traits of *M. meretrix* from different geographic habitats have been analyzed previously (Lin, 2007). Rapid amplification polymorphic DNA analysis revealed genetic variations between different geographic regions (Shen et al., 2003). The internal transcribed spacer gene (Lin et al., 2009) and the mitochondrial cytochrome oxidase subunit I gene (Dong et al., 2011) showed distinct variations between the Guangxi population and other geographic populations. Morphological trait analysis, amplification fragment length polymorphism (AFLP), and fluorescent AFLP (fAFLP) analysis showed similar results (Lin et al., 2008, 2009). These studies indicated that there are high levels of genetic variation between different geographic populations of *M. meretrix*. However, these data used techniques that could not distinguish between a homozygote and a heterozygote; thus, additional population genetic studies involving codominant markers are needed.

Many techniques, such as allozyme analysis, mitochondrial DNA analysis, and microsatellite marker analysis, can be used to examine the population genetics of marine species. Microsatellite markers have been widely used because of their hypervariability, ubiquitous presence, neutrality, abundance, and co-dominance (Tautz, 1989; Weber and May, 1989; An et al., 2012; Ma et al., 2012). Simple sequence repeat loci (microsatellites) identified from the expressed sequence tag database (EST-SSR markers) are generally related to a particular function, such as resistance or growth. Microsatellite marker analysis has been applied in genetic studies of a number of different species (An et al., 2012; Dong et al., 2012; Lu et al., 2012a). When combined with growth analysis related to the diallel mating design of 2 different geographic stocks, hybridization was confirmed to improve the quality of *M. meretrix* (Lu et al., 2012b). Three EST-SSR quantitative trait loci for growth have also been identified in a

fast-growing population (Lu et al., 2013).

In this study, 10 highly polymorphic EST-SSR markers were used to assess genetic differentiation in the hard clam from 5 habitats in China: Shandong Province (SD), Jiangsu Province (JS), Fujian Province (FJ), Guangdong Province (GD), and Guangxi Province (GX). Our genetic analysis will aid in the protection and utilization of *M. meretrix*.

MATERIAL AND METHODS

Sample collection

Samples of the hard clam *M. meretrix* were collected from Dongying (SD), Rudong (JS), Changle (FJ), Zhanjiang (GD), and Beihai (GX) in China (Table 1).

Clams were dissected to harvest the adductor muscles, which were then stored at -80°C. The phenol-chloroform method was used to extract the genomic DNA from the muscle samples. Genomic DNA was detected by electrophoresis on a 1% agarose gel.

Table 1. Collection details for *Meretrix meretrix* samples.

Geographic populations (abbreviation used)	Sample location (longitude and latitude)	Sample size	Collection time
SD	Dongying (37°26N, 118°40E)	40	November, 2012
JS	Rudong (32°19N, 121°10E)	40	November, 2012
FJ	Changle (25°57N, 119°31E)	40	August, 2012
GD	Zhanjiang (21°16N, 110°21E)	38	August, 2012
GX	Beihai (21°19N, 109°06E)	40	May, 2012

Microsatellite genotyping

A total of 198 clams from 5 locations were genotyped at 10 microsatellite loci. The microsatellite alleles were amplified by polymerase chain reaction (PCR). PCR was performed in 20 μL volumes, which included 0.5 U *Taq* DNA polymerase (TaKaRa, Shiga, Japan), 1X Taq DNA polymerase PCR buffer (TaKaRa), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM each forward and reverse primers (Table 2), and approximately 100 ng template DNA.

PCR was performed in a thermal cycler (Eppendorf, Hamburg, Germany), under the following conditions: denaturation for 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at the locus-specific annealing temperature, and 45 s at 72°C, followed by a final extension for 7 min at 72°C.

Microsatellite allele sizes were assessed on 8% non-denaturing polyacrylamide gels, which were stained with ethidium bromide and visualized under ultraviolet light in a Bio-Rad Gel Doc XR + gel imaging analysis system (Hercules, CA, USA). A 10-bp DNA ladder (MBI) was used to determine allele sizes at each locus and genotype for each individual. Each microsatellite locus in the 5 populations was examined using MICROCHECKER v.2.2.3 for genotyping errors caused by stuttering or large allele dropout and occurrence of null alleles (Van Oosterhout et al., 2004).

Table 2. Characterization of the 10 EST-SSR primers used in this study.

Locus	Primer sequence (5'-3')	Repeat motif	Size (bp)	Ta (°C)	TSA accession No.
MM8105[a]	F: AGTTGCCTTGAAGTAAAGTCC R: CATGATCAATCATTGGTTACA	$(TGAT)_n$	135-164	55	JI265669
MM1031[b]	F: GGTTGTGAAAGACAATTGAGA R: TGCCCATTAAATGTTTCTTAAC	$(CAT)_n$	152	55	JI258639
MM573[b]	F: TTAACAAACTTGCATTTCCTC R: ATCATCTTCAACTCACCA	$(TGA)_n$	146	55	JI258183
MM5358[a]	F: TTCTACTGACCTAAGCTGCTG R: CCATATGTGTCATTGGAAGTT	$(AATC)_n$	91-170	55	JI262933
MM12736[a]	F: GTCAGCGAAGATTTTAACAAA R: TCATCATCTTCAACTCACCAT	$(TGA)_n$	114-172	55	JI270267
MM8476[c]	F: CTTCATCTAT GCTTTCGTA TTCG R: CTGCTGGCTA TGAATCAAGTG	$(TCA)_n$	155-168	58.7	JI266036.1
MM3923[a]	F: TTTTCGTCTTAATGAGGGTTA R: GTTTGTGAAATAGTGCTCTGC	$(AATC)_n$	78-157	55	JI261510
MM12295[a]	F: AAATGGTTCCTAACGTTCATT R: AAGCTTGTATAAGTGCACGAG	$(ACA)_n$	105-161	55	JI269829
MM26715[a]	F: ACATCATCATCTCAACTCACC R: GTCAGCGAAGATTTTAACAAA	$(ACT)_n$	118-188	57	JI284180
MM8706[c]	F: AGC CATTAGTTTT TCTTGCC R: CTTG GTAGAGGTCC AGTAGGT	$(CTCC)_n$	200-230	61.1	JI266266.1

Ta, annealing temperature. [a]Loci from Lu et al. (2012a), [b]Loci from Wang et al. (2011b), [c]Loci from Dong et al. (2013).

Statistical analysis

The number of alleles (N_A), effective alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), genetic identity, and genetic distance (D) were calculated using the POPGENE VERSION 1.31 software. Pairwise genetic differentiation F-statistics (F_{ST}) were computed using FSTAT (Goudet, 2001) version 2.9.3. Polymorphic information content was calculated using Cervus 3.0 (Table 1). Hardy-Weinberg (HWE) equilibrium analysis was performed with GENEPOP 4.0 (available at http://genepop.curtin.edu.au). A phylogenetic tree was constructed for each replicated genetic distance matrix using the neighbor-joining tree method of the MEGA 5.10 program. A Mantel Z-test was performed on matrices of pairwise F_{ST} values and geographical distances of populations using the IBDWS software (Jensen et al., 2005) (http://ibdws.sdsu.edu/~ibdws/).

RESULTS

Genetic diversity of the five populations

Ten polymorphic microsatellite markers were used to analyze the genetic diversity of the 5 populations of *M. meretrix*. A total of 183 alleles were detected by genotyping. Genotyping errors were examined using MICROCHECKER v.2.2.3 and showed no evidence of large allele dropout at any of the 10 loci; however, stuttering in some loci (e.g., MM5358, MM8476, MM26715, and MM8706 loci) may have resulted in scoring errors. Null alleles may have been present at some loci in each stock (e.g., MM5358, MM8476, MM26715, and MM8706 loci). N_A varied from 12-24 per locus. Locus MM3923 (24 alleles) was the most polymorphic, while MM8706 (12 alleles) showed the lowest N_A. N_E (in an ideal group, the allele number required to produce the same heterozygosity with the actual group in 1 locus)

ranged from 5.75 to 7.81, with the GD and JS populations showing the lowest and highest values, respectively.

The highest average N_A value (11.2) and the highest average H_E value (0.8717) were found in the SD population. The lowest average N_A value (8.6) and the lowest average H_E value (0.7553) were calculated for the GD and GX populations, respectively. An allele equilibrium status test for each population and locus was performed using HWE analysis (Table 3). The result of the GENEPOP analysis, after sequential Bonferroni's correction (Rice, 1989), showed that 78% of the combinations significantly deviated from HWE.

Table 3. Summary of genetic diversity in 5 *M. meretrix* populations.

Locus		Populations					
		SD	JS	FJ	GD	GX	Overall
MM8105	N_A	9	9	13	8	9	15
	N_E	5.8288	6.4257	8.7163	5.7645	5.0314	8.3121
	H_O	0.525	0.5	0.4359	0.5789	0.625	0.533
	H_E	0.8389*	0.8551*	0.8968*	0.8375*	0.8114*	0.8819
MM1031	N_A	12	11	1	1	1	14
	N_E	7.1749	7.3059	1	1	1	2.6657
	H_O	0.725	0.25	0	0	0	0.197
	H_E	0.8715*	0.8741*	0	0	0	0.6264
MM573	N_A	10	15	11	12	12	22
	N_E	5.7658	11.6788	6.7301	8.8464	7.9012	9.8028
	H_O	0.475	0.6	0.6923	0.5278	0.725	0.6051
	H_E	0.837*	0.9259*	0.8625	0.8995*	0.8845	0.9003
MM5358	N_A	8	8	9	9	14	17
	N_E	4.8855	5.7345	6.5339	6.5311	7.0244	8.2045
	H_O	0.325	0.5	0.5526	0.6471	0.5833	0.5163
	H_E	0.8054*	0.8372*	0.8582*	0.8595*	0.8697*	0.8805
MM12736	N_A	9	11	9	9	12	18
	N_E	5.5363	8.2687	4.8057	4.9422	7.1663	10.7855
	H_O	0.55	0.5	0.5128	0.7297	0.6053	0.5773
	H_E	0.8297*	0.8902*	0.8022*	0.8086	0.8719*	0.9096
MM8476	N_A	8	7	10	10	14	19
	N_E	5.6637	4.7059	5.4889	7.5208	7.7108	10.803
	H_O	0.45	0.575	0.725	0.6842	0.8	0.6465
	H_E	0.8339*	0.7975*	0.8282	0.8786*	0.8813	0.9097
MM3923	N_A	14	13	14	11	11	24
	N_E	11.0727	9.4395	10.0662	6.0176	7.6923	12.8802
	H_O	0.35	0.775	0.8649	0.4865	0.825	0.6598
	H_E	0.9212	0.9054*	0.913*	0.8452*	0.881	0.9247
MM12295	N_A	12	17	15	10	7	21
	N_E	9.1954	11.0727	10.2894	6.8599	5.3581	16.2396
	H_O	0.675	0.875	0.75	0.7368	0.4595	0.7026
	H_E	0.9025*	0.9212*	0.9142*	0.8656	0.8245*	0.9408
MM26715	N_A	12	13	10	9	10	21
	N_E	8.0808	8.1013	7.3574	4.9942	5.9651	9.7852
	H_O	0.55	0.5	0.4857	0.4167	0.5946	0.5106
	H_E	0.8873*	0.8877*	0.8766*	0.811*	0.8438*	0.9002
MM8706	N_A	8	8	3	7	4	12
	N_E	5.5556	5.3275	2.8322	5.049	3.0888	7.8274
	H_O	0.375	0.4615	0.3636	0.5789	0.5	0.4579
	H_E	0.8304*	0.8228*	0.6569*	0.8126*	0.6848*	0.8745
MEAN	N_A	10.2	11.2	9.5	8.6	9.4	18.3
	N_E	6.8759	7.8061	6.382	5.7526	5.7939	9.7306
	H_O	0.5	0.5537	0.5383	0.5387	0.5718	0.5406
	H_E	0.8558	0.8717	0.7609	0.7618	0.7553	0.8749
	PIC	0.8477	0.8270	0.7332	0.7375	0.7334	0.8743

N_A = allele number; N_E = effective allele number; H_O = observed heterozygosity; H_E = expected heterozygosity; *PIC* = polymorphic information content; *Significant deviations from the Hardy-Weinberg equilibrium ($P < 0.05$).

Genetic differentiation and relationship among populations

F_{ST} values (Table 4) indicated the levels of pairwise genetic differentiation between the 5 populations. The lowest F_{ST} value was observed between the JS and SD populations (F_{ST} = 0.029), while the F_{ST} values between south and north populations were relatively high.

A neighbor-joining tree (Figure 1) was constructed according to D (Table 5). The JS and SD populations formed 1 group, while the FJ and GX populations were clustered together and formed the 2nd group with the GD population. The tree demonstrated the clustering of groups within the 5 populations, further illustrating genetic differentiation.

The isolation-by-distance (Figure 2) explanation of genetic divergence between populations predicts that a positive correlation will be observed between increases in pairwise geographical distance and the enhancement of D (F_{ST}). This results from decreased gene flow or connectivity.

Table 4. F_{ST} estimates for pairwise comparisons of 5 *Meretrix meretrix* populations.

Populations	JS	SD	FJ	GD	GX
JS	-				
SD	0.029	-			
FJ	0.118	0.122	-		
GD	0.121	0.101	0.07	-	
GX	0.143	0.132	0.067	0.067	-

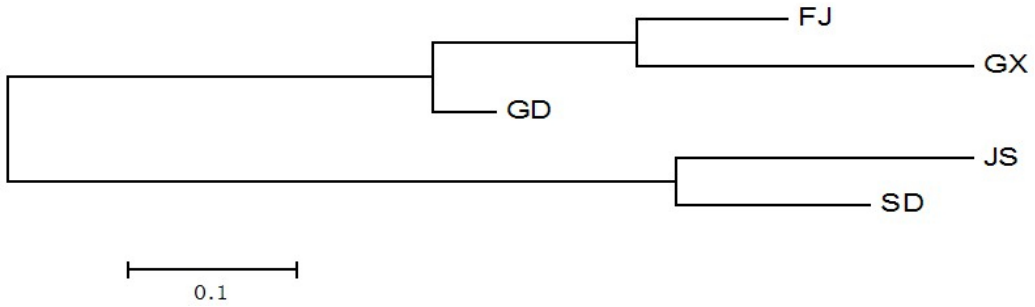

Figure 1. Dendrogram (NJ tree) based on Nei (1978) genetic distance between 5 *Meretrix meretrix* populations.

Table 5. Genetic distance and genetic similarity of 5 populations in *Meretrix meretrix*.

Populations	JS	SD	FJ	GD	GX
JS	-	0.971	0.849	0.858	0.834
SD	0.029	-	0.843	0.866	0.841
FJ	0.151	0.157	-	0.904	0.914
GD	0.142	0.134	0.096	-	0.914
GX	0.166	0.159	0.086	0.086	-

Genetic distance is below the diagonal, and genetic identity is above the diagonal.

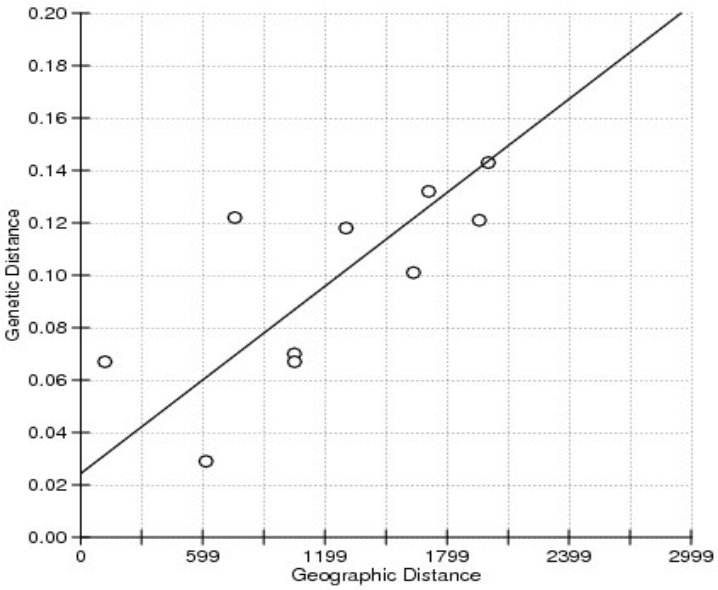

Figure 2. Pairwise geographical distance (km) against F_{ST} values to assess isolation by distance among *Meretrix meretrix* populations, r = 0.7180, P = 0.1210 from 1000 randomizations.

DISCUSSION

Genetic polymorphism and genetic variation

Marine bivalves, such as *Crassostrea gigas* and *Placopecten magellanicus*, generally show relatively high levels of genetic diversity (Li et al., 2003; Kenchington et al., 2006). High mutation rates during DNA replication and large population sizes may contribute to this diversity (Callen et al., 1993; Launey and Hedgecock, 2001). The dispersal capability and extended larval phases of marine bivalves, together with marine currents, can result in low geographical differentiation (Arnaud et al., 2000; Vadopalas et al., 2004; Zhan et al., 2008; Yan et al., 2011). However, opposite trends were observed in some studies (Ridgeway, 2001; Luttikhuizen et al., 2003). Anthropogenic activities, such as larval transportation, can affect the diversity of the natural population. In this study, all populations showed a high level of genetic differentiation when a number of alleles per locus, H_O, and H_E values were assessed. This demonstrates that genetic exchange between these populations, facilitated by the relatively long planktonic larval phase, is not sufficient to nullify genetic drift and selection.

In most cases, the observed genotype distributions deviated significantly from HWE. Such deviations may be caused by heterozygote deficiency (Zhan et al., 2008), which is commonly observed in microsatellite loci (Sato et al., 2005; Zhan et al., 2008; Lu et al., 2012a), and may be caused by sample size, non-random mating because of water flow (Ma et al., 2012), or null alleles (Callen et al. 1993; Zhan et al., 2008). Population genetic analyses of HWE may be compromised because of the existence of false homozygotes. Our results suggested that the gene frequency and genotype distribution were unstable.

Molecular distinction and relationships among the 5 populations

Genotyping using polyacrylamide gels demonstrated a specific phenomenon at locus MM1031, indicating that the south group was monomorphic (a unique band), while the north group was polymorphic. Thus, MM1031 can be used to distinguish the south group from the north group and is a potential indicator of gene flow (Slatkin, 1985). We also detected other loci that failed to amplify in individuals from the south group, but these loci were not listed because they lacked statistical meaning. However, these data indicate that it is possible to characterize the 2 groups using molecular markers.

Early genetic population studies on *M. meretrix*, using rapid amplification polymorphic DNA and AFLP analysis, yielded similar results for geographical differentiation (Shen et al., 2003; Lin et al., 2009). In this study, F_{ST} values were 0.029-0.143, which is high compared with other fishery species (Li et al., 2006; Zhan et al., 2008; Ma et al., 2012). Long periods of geographical and ecological isolation, in addition to random mating within the natural habitat, may explain the genetic differentiation of *M. meretrix* (Lin, 2007). The neighbor-joining tree revealed a clear separation of the 2 north populations from the 3 south populations (Figure 1). D value indicated that a slight genetic difference had already formed.

Isolation-by-distance results from limited gene flow (Slatkin, 1993). Populations are connected by continuous migration, but the geographical distance can weaken the effect of migration until it is no longer strong enough to counter the effect of random genetic drift (Lind et al., 2007). However, oceanographic factors, biogeographical factors, or even anthropogenic activities (larval release) can complicate the isolation-by-distance model. Generally, the observed genetic differentiation was consistent with the isolation-by-distance model.

The crossbreeding program of *M. meretrix*, between the SD and JS populations, has achieved the preliminary effect and heterosis, as evidenced by the growth rate and genetic parameters (Xiao et al., 2010; Lu et al., 2012b). Establishing the population genetic background will help to determine suitable hybridization methods. The quality of offspring is mainly determined by additive genetic factors (Liu and Cordes, 2004). Highly abundant genetic variation contributes to the survival of populations as an adaptation to environmental change (Ma et al., 2012). In this study, the SD and JS populations showed the least variation. The other combinations were more suited for hybridization and could maintain higher genetic diversity.

In summary, our study revealed the genetic variation of different geographic populations of *M. meretrix*. Populations with numerous genetic differences should be used for crossbreeding for genetic improvement of stocks of this hard clam species. In addition, adaption to salinity and temperature should be taken into consideration.

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