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Genetic characterizations of *Mactra veneriformis* (Bivalve) along the Chinese coast using ISSR-PCR markers

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Abstract

The genetic structure of seven different populations of the surf clam *Mactra veneriformes* along the coast of China was investigated by Inter-Simple Sequence Repeats (ISSR) fingerprinting of 210 individual clams. Of the 240 ISSR loci tested, 235 (97.9%) were polymorphic. We found a clear tendency for higher F_{ST} values and lower gene flow levels between the populations with increasing geographical separation. The seven different geographic populations can be divided into three subgroups: Liaoning, Qingdao/Lianyungang, and Ningbo based on the UPGMA dendrogram of Nei's genetic distance. These results indicated that isolation of geographic distance played an important role in population differentiation.

Keywords: *Mactra veneriformis*; Genetic structure; Gene flow; Differentiation; ISSR-PCR

1. Introduction

The surf clam, *Mactra veneriformis* Reeve (1854), is an important commercially bivalve in China, Korea and Japan. These clams are extensively utilized as seafood, raw materials for manufacturing flavoring materials and live feed at various aquaculture farms (Ryou, 1997; Lovatelli, 1988). About 50 thousand tons of surf clams are harvested in China per year, which dramatically reduces the natural resource of these clams.

The surf clam is a widely distributed species along the mud-sandy coasts of the Bohai Sea, the Yellow Sea and the South China Sea. Traditionally, for commercial purposes, culturists often purchase a large number of juvenile clams from one location and then transport them to different locations and culture them there in mariculture farms. This method of operation leads to gene flow between the local clam populations and the introduced populations. In addition, the veliger larva of *M. veneriformes* can migrate between the nearby populations assisted by the water currents. The population genetic structures of clams from different locations and the level of gene flow among surf clams from different locations along the Chinese sea coasts, is currently unknown.

Inter-Simple Sequence Repeats (ISSR) amplifies inter-microsatellite sequences at multiple loci throughout the genome (Salimath et al., 1995; Li and Xia, 2005). An ISSR molecular marker technique permits the detection of polymorphism in microsatellites and inter-microsatellite loci without previous knowledge of DNA sequences (Zietkiewicz et al., 1994). This technique has

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been widely used to investigate genetic diversity and population genetic structure (Reddy and Nagaraju, 1999; Li and Xia, 2005) because of its advantages in overcoming limitations of allozyme and RAPD techniques (Wolfe et al., 1998; Ratnaparkhe et al., 1998; Esselman et al., 1999). We can amplify Inter-Simple Sequence Repeat (ISSR) with oligonucleotide primers based on simple sequence repeats anchored at either the 3′ or 5′ end in the study. The aim of the present study is to use the ISSR technique to clarify the population genetic structure, to estimate the level of gene flow, and genetic differentiation of seven different geographic populations of *M. veneriformis*.

2. Materials and methods

2.1. Clam samples and DNA extraction

The 210 clam samples were collected from Zhuanghe (Liaoning Prov. ZH), Pikou (Liaoning Prov. PK), Donggang (Liaoning Prov. DG), Yantai (Shandong Prov. YT), Qingdao (Shandong Prov. QD), Lianyungang (Jiangsu Prov. LY) and Ningbo (Zhejiang Prov. NB), as shown in Fig. 1.

DNA was extracted according to the protocols of Pradeep et al. (2005) and Hou et al. (2003). The foot muscle of each sample was homogenized in cold buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and treated with 20 mg/ml proteinase K in 10% SDS at 55 °C over night. The homogenate was then extracted once with phenol: chloroform, twice with phenol: chloroform: isopentanol, once with chloroform, and then precipitated using 1/20 volume 3 M NaAc and 2 volumes ethanol. Precipitated DNA was lifted from the solution using a toothpick and washed in 75% ethanol, dried, and dissolved in 100 μl of TE buffer at 4 °C. DNA quality and quantity were determined in 0.8% agarose gels and an ultraviolet spectrophotometer.

2.2. ISSR Primers and ISSR PCR Amplification

The sequences of 20 ISSR primers are listed in Table 1 (some of the primer sequences from Wolfe and Liston (1998) were referenced). The primers were commercially synthesized by Shenggong Inc (Shanghai, China).

The PCR reactions took place in a final volume of 20 μl containing buffer (2.0 μl), 0.2 mM dNTP (2.0 μl), 10 pM primer (1.0 μl), 1 unit of Taq polymerase, and 10–100 ng of template DNA. DNA amplification was performed on an Express II Thermohybaid, and commenced with 5 min at 94 °C was followed by 40 cycles of 94 °C denaturation for 1 min, 43 °C–57 °C annealing for 1 min (according to different primer, see Table 1), and a 72 °C extension for 2 min. Amplification cycles were followed by a final 7 min extension at 72 °C. Amplification products were electrophoresed on 2.0% agarose gels run at 100 V in 1×TAE, visualized by staining with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using DL2000 DNA marker (Takara Inc). Gel-Pro Analyzer Version 3.1 software was used to score ISSR profiles. Specific amplified products (bands) were reproduced in successive amplifications and identified as marker bands defined by their molecular weights estimated from size standards.

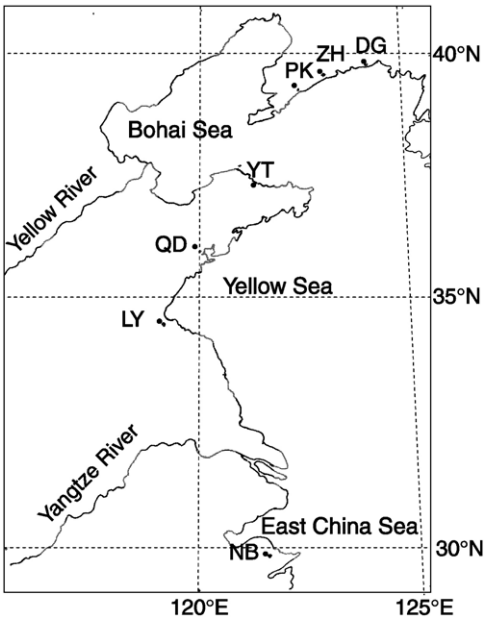

Fig. 1. Map illustrating the locations of seven *Mactra veneriformis* populations studied. ZH: Zhuanghe (Liaoning Prov.), PK: Pikou (Liaoning Prov.), DG: Donggang (Liaoning Prov.), YT: Yantai (Shandong Prov.), QD: Qingdao (Shandong Prov.), LY: Lianyungang (Jiangsu Prov.), and NB: Ningbo (Zhejiang Prov.).

2.3. Data Analysis

The survey of data was according to the presence/absence criterion (1=band; 0=no band). The data obtained were used to construct a data matrix that was elaborated utilizing Popgene1.32 software (Yeh and Yang, 1999). The data matrix of ISSR was analyzed for genetic structure, genetic differentiation, gene flow and diversity. Nei's (1978) genetic distances (*D*) between different geographical populations were calculated. Measurements of diversity including gene diversity

Table 1
List of primers used for ISSR amplification, GC content, annealing temperature (Tm.), total number of loci, the level of polymorphism and size range of fragments

Primer	Sequence (5′~3′)	Primer type	GC (%)	Tm. (°C)	Total number of loci	Number of polymorphic loci	Percentage of polymorphism (%)	Size range of fragments (bp)
ISSR-1	B*DB$(TCC)_5$	Tri-repeat	66.7	57	16	15	93.8	350–2000
ISSR-2	$(TCC)_5$RY	Tri-repeat	70.6	55	17	16	94.1	250–1900
ISSR-3	VBV$(CA)_8$	Di-repeat	57.9	54	14	14	100.0	480–1500
ISSR-4	VDV$(GT)_8$	Di-repeat	52.6	51	15	14	100	280–1900
ISSR-5	$(AG)_8$T	Di-repeat	47.1	48	13	13	100	200–1900
ISSR-6	HVH$(TG)_7$ T	Di-repeat	44.4	51	12	12	100	200–1900
ISSR-7	$(CT)_8$A	Di-repeat	47.1	49	14	13	92.9	250–1900
ISSR-8	$(AC)_8$T	Di-repeat	47.1	45	17	17	100	200–2000
ISSR-9	$(AC)_8$G	Di-repeat	47.1	45	9	9	100	250–2000
ISSR-10	$(TG)_8$GT	Di-repeat	50.0	48	16	16	100	180–2000
ISSR-11	$(AG)_8$TG	Di-repeat	50.0	54	15	15	100	280–2100
ISSR-12	$(TC)_8$C	Di-repeat	52.9	52	11	11	100	450–1100
ISSR-13	$(TG)_8$G	Di-repeat	52.9	53	9	9	100	450–1200
ISSR-14	$(CA)_6$R	Di-repeat	53.8	45	8	8	100	500–1500
ISSR-15	$(CA)_6$RY	Di-repeat	57.1	44	7	7	100	250–2000
ISSR-16	$(GT)_6$YR	Di-repeat	57.1	44	8	8	100	250–2000
ISSR-17	$(GT)_6$AY	Di-repeat	50.0	43	7	7	100	150–2000
ISSR-18	$(ACTG)_4$	Tetra-repeat	50.0	52	8	8	100	250–2000
ISSR-19	$(GACA)_4$	Tetra-repeat	50.0	48	12	11	91.7	150–1980
ISSR-20	$(CAC)_6$	Tetra-repeat	66.7	57	12	12	100	450–1100

*B=C, G, T; Y=C, T; R=A, G; H=A, C, T; V=A, C, G; D=A, G, T.

(*H*), observed number of alleles (N_a), effective number of alleles (N_e) and Shannon's information index (*SI*) were estimated by Popgene1.32 software. F_{ST} values for all possible pairs of the seven populations and gene flow (N_m) were calculated by RAPDFST4.0.1 software (Black, 1997). To test the correlation between N_m, F_{ST} and geographic distances, linear regression of relevant pair-wise logarithm F_{ST}, N_m values and logarithm of geographical distance (km) were plotted by SPSS11.5 software (Statistics Package for Social Science, SPSS Inc. http://www.spss.com). The dendrogram was constructed by Phylip3.5c neighbor software (Phylogeny Inference Package by Joseph Felsenstein, 1993) based on Nei' genetic distance and the dendrogram was plotted by Phylip 3.5c and TreeView1.66 softwares (Page, 1996). Significance of F_{ST} was tested for multiple comparisons using sequential Bonferroni Correction (Rice, 1989) (Table 3).

3. Results

3.1. ISSR polymorphism

Twenty pairs of ISSR primers were used to screen 210 randomly selected individual clams from 7 different geographic populations. A total of 240 ISSR loci were detected. The amplified PCR fragment sizes ranged from 100 bp to 2.1 kb with the scorable region being

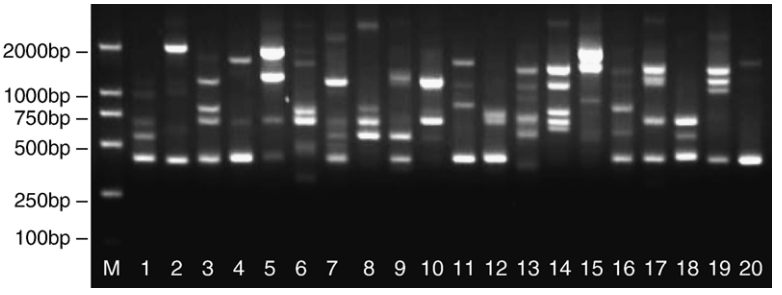

Fig. 2. ISSR PCR fingerprints of seven *Mactra veneriformis* populations using the ISSR-1 primer. The lanes 1–3 are ISSR patterns of Donggang (DG) populations, lanes 4–6: Zhuanghe (ZH), lanes 7–9: Pikou (PK), lanes 10–12: Yantai (YT), lanes 13–15: Qingdao (QD), lanes 16–18: Lianyungang (LY) and lanes 19–20: Ningbo (NB) population. The lane marked M shows the 2-kb DNA ladder.

Table 2
Summary of genetic variation statistics for all loci of ISSR

Populations	Sample size	N_a*	N_e	H	SI	No. of polymorphic loci (percentage)
DG	30	1.750±0.434	1.362±0.325	0.225±0.174	0.349±0.247	180 (75.00%)
PK	30	1.750±0.434	1.360±0.323	0.224±0.174	0.347±0.246	180 (75.00%)
ZH	30	1.725±0.447	1.336±0.317	0.212±0.171	0.331±0.245	174 (72.50%)
YT	30	1.804±0.398	1.399±0.328	0.246±0.171	0.374±0.238	193 (80.42%)
QD	30	1.800±0.401	1.394±0.332	0.242±0.173	0.374±0.240	192 (80.00%)
LY	30	1.917±0.277	1.498±0.304	0.303±0.150	0.461±0.199	220 (91.67%)
NB	30	1.967±0.180	1.567±0.267	0.342±0.122	0.514±0.155	232 (96.67%)
Mean	210	2.000±0.000	1.487±0.258	0.307±0.119	0.476±0.146	235 (97.92%)

*N_a=Observed number of alleles.
N_e=Effective number of alleles [Kimura and Crow (1964)].
H=Nei's (1973) gene diversity.
SI=Shannon's Information index [Lewontin (1972)].
Mean=N_a, N_e, H and SI of allover loci of seven populations.

from 150 bp to 2.1 kb. The number of bands generated by the primers ranged from 7 to 17 with an average of 12 (Table 1). Among these 20 primers, ISSR-1, ISSR-2, ISSR-4, ISSR-7 and ISSR-19 revealed 5 monomorphic loci existed in the all of the seven populations, and 235 (97.9%) polymorphic loci. ISSR-fingerprint patterns are shown for primer ISSR-1 on Fig. 2. The high reproducibility of ISSR markers may be due to the use of longer primers and higher annealing temperatures than those used for RAPD. We have adjusted the annealing temperature of each primer to avoid producing faint fragments. The annealing temperature in the present study ranged from 43 °C to 57 °C. The primers used for ISSR amplification, GC content, annealing temperature (Tm.), total number of loci, the level of polymorphism and size range of fragments were shown in Table 1. The mean observed number of alleles (N_a) ranged from 1.72 at ZH to 1.97 at LY which was closely followed by NB with a value of 1.92. Values of N_e were less than those for N_a in every population and ranged from 1.34 at ZH to 1.57 at NB. The mean Nei's gene diversity (H) ranged from 0.21 at ZH to 0.34 at NB. Ningbo (NB) displayed the highest value of mean Nei's gene diversity, which demonstrated that there were 21.2 to 34.2% heterozygosis within the population of *M. veneriformis* (Table 2).

3.2. Genetic differentiation

Pairwise F_{ST} values ranged from 0.058 (ZH/PK) to 0.367 (YT/NB), with the mean F_{ST} value being 0.202 (Table 3). Gene flow (N_m) estimates ranged from 0.43 (YT/NB) to 4.06 (ZH/PK). There was a clear pattern of higher F_{ST} values between populations separated by greater geographical distances (Fig. 4). The estimated genetic distance (D) among these seven populations of *M. veneriformis* ranged between 0.099 (PK/NB) and 0.011(LY/QD) (Table 3). The pairwise F_{ST} values in Table 3 also indicated significant differentiation among the seven populations (Fig. 3). No significant differences in F_{ST} values were observed between populations within the three geographically separated population groups observed in the UPGMA diagram (DG/PK/ZH, YT/QD/LY, NB), but significant differences were

Table 3
Pairwise F_{ST} values (above diagonal) and genetic distance (below diagonal) based on ISSR data

Populations	DG	PK	ZH	YT	QD	LY	NB
DG	____	0.089	0.093	0.132	0.153	0.173	0.209*
PK	0.069	____	0.058	0.264*	0.271*	0.281*	0.298*
ZH	0.063	0.045	____	0.176	0.289*	0.299*	0.300*
YT	0.075	0.095	0.077	____	0.101	0.086	0.367*
QD	0.073	0.087	0.073	0.018	____	0.139	0.311*
LY	0.066	0.081	0.073	0.029	0.011	____	0.155
NB	0.085	0.099	0.092	0.063	0.058	0.017	____

*Asterisk indicates significant genetic differentiation test ($P<0.05$) (Bonferroni correction).

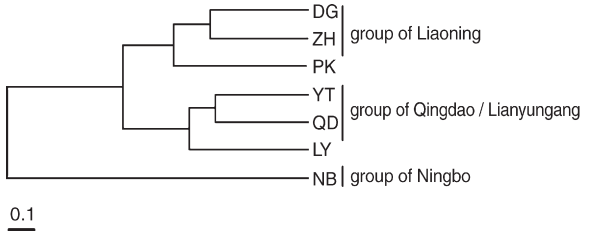

Fig. 3. The UPGMA dendrogram of D (Nei' genetic distance) based on ISSR data of seven *Mactra veneriformis* populations. ZH: Zhuanghe (Liaoning Prov.), PK: Pikou (Liaoning Prov.), DG: Donggang (Liaoning Prov.), YT: Yantai (Shandong Prov.), QD: Qingdao (Shandong Prov.), LY: Lianyungang (Jiangsu Prov.), and NB: Ningbo (Zhejiang Prov.).

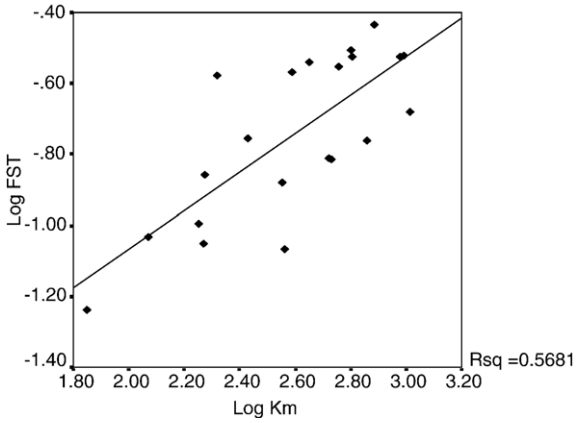

Fig. 4. Regression line based on $\log_{10} F_{ST}$ values and $\log_{10}$ km (geographical distance) pairwise between seven populations of *M. veneriformis*. There is a significant correlation of *t*-test of regression coefficient (t=4.9992, P=0.0001). The regression equation is y=0.5433x–2.1533, R^2=0.5681.

observed between most populations from the different groups (Fig. 4).

3.3. Population genetic structure

The pairwise H_t value (Nei's gene diversity among populations) and pairwise H_s value (Nei's gene diversity within subpopulations) of different geographic subpopulations and mean value of H_t, H_s of seven subpopulations showed that H_t value was the highest between PK and YT (0.275), and the lowest value was 0.241 between YT and QD. The mean value of H_t from seven different geographic subpopulations was 0.309. This means that about 30.9% of genetic variation came from within the population. The mean value H_s of 7 groups was the highest between DG and NB groups (0.287), and the lowest value was 0.265 between YT and QD groups. The mean value of H_s from 210 individuals was 0.256, which shows that about 25.6% of genetic variation came from within populations.

4. Discussion

ISSR-PCR has been successfully employed to reveal genetic variation in silkworm (Pradeep et al., 2005) , aphids (Abbot, 2001), Japanese flounder (*Paralichthys olivaceus*) (Liu et al., 2006) and shrimp (*Fenneropenaeus chinensis*) (Wang and Kong, 2002), to characterize genomic diversity (Yang et al., 1996), and to determine the origin of hybrids (Wolfe et al., 1998). As the primers are anchored at their 3′ end or 5′ end, to ensure that the annealing of the primer occurs only at the 3′ end or 5′ end of the microsatellite motif, thus avoiding internal priming and smear formation. The anchor also allows only a subset of the targeted inter-repeat regions to be amplified, thereby reducing a high number of PCR products expected from the priming of dinucleotide inter-repeat regions to a set of about 10–50 easily resolvable bands. Pattern complexity can be tailored by applying different primer lengths and sequences (Zietkiewicz et al., 1994). Based on its unique characters, ISSR technique can detect more genetic loci than isozyme and has higher stability than RAPD. Our work is the first application of this method to a wild marine bivalve (*M. veneriformis*). The experimental results of the present study provide evidence for the reliability and usefulness of ISSR markers, the high genetic diversity within and between *M. veneriformis* populations from China.

Higher F_{ST} value indicates a lower level of gene flow (N_m) among populations and higher genetic differentiation in populations. In our study, F_{ST} values increase with a more pronounced geographical separation of the populations concerned, implying isolation by distance. The F_{ST} values (0.058–0.367) of *M. veneriformis* are higher than those for the clam *Donax serra* [0.016–0.089, (Laudien et al., 2003)]. However, F_{ST} values for other bivalve molluscs with meroplanktonic larvae cover a wide range of F_{ST} [*Donax deltoides*, F_{ST}=0.009 (Murray-Jones and Ayre, 1997); *Ostrea edulis*, F_{ST}=0.062 (Saavedra et al., 1993); *Pinctada maxima*, F_{ST}=0.104 (Johson and Joll, 1993)]. The genetic similarities of different bivalves populations have been related to their degree of connectedness by current systems [*Chlamys opercularis* (Macleod et al., 1985)]; *Choromytilus meridionalis* (Lombard and Grant, 1986); *D. deltoids* (Murray-Jones and Ayre, 1997). It is notable that F_{ST} values were lower between ZH and PK (0.058), ZH and DG (0.093), DG and PK (0.089), YT and QD (0.101) and YT and LY (0.086) suggesting that higher levels of gene flow (N_m=4.060, 2.44, 2.560, 2.225 and 2.657) exist between these populations. A possible cause of a higher gene flow among these populations is the transfer of juvenile clams by farmers who trade clams between each other between these areas. Clam larvae from the DG, PK and ZH populations could also migrate between each other on areas by currents. Moreover, there is a higher genetic differentiation between Liaoning, Qingdao/Lianyungang and Ningbo groups. The higher F_{ST} values between the Liaoning, Qingdao/Lianyungang and Ningbo groups result from their greater geographical isolation, different water temperature and reproductive season.

We estimated the level of gene flow of populations using the formula: $F_{ST}=1/(4N_m+1)$, and found that the value of N_m (N_m=0.431–4.060) is higher among closely

related populations. The migration may have changed the genetic structure of the population and other environmental parameters, such as temperature, salinity and surf may also play a role in the genetic structure of *M. veneriformis*. When the geographical distance was farther and the gene flow (N_m) levels among these populations reduced, results in genetic isolation by a possible gene flow barrier. Conversely, as near geographic distances among populations of DG, ZH and PK, the F_{ST} values were lower, revealing the presence of higher level gene flow among these populations, so there are lower level differentiation among these populations. As for questions above, Fig. 2 showed the relationship between F_{ST} and geographic distances (km): a clear tendency for higher F_{ST} value with larger geographical distances (km) revealed lower gene flow (N_m) levels with larger geographical distance (km).

The values of Nei' D, F_{ST}, N_m (gene flow) and H revealed a lower degree of genetic divergence among subpopulation, especially among PK, ZH and DG, QD and YT. There was a lower genetic differentiation among subpopulations as demonstrated by a F_{ST} significance test (Bonferroni correction test, $P<0.05$, Table 3). There was an exception that higher genetic differentiation existed between NB and others, this also resulted from longer geographic distances which effectively interrupted the gene flow from other subpopulations to the NB population. The close relationship among PK, ZH and DG populations of *M. veneriformis*, may be due to the human activities for aquaculture, which brings a large number of migration of larvae from different populations into the same geographical location. Comparing values of H_t (0.309) and H_s (0.256) of the seven populations, we came to the conclusion that genetic variation came from overall population mainly, not from subpopulations. Population genetic structure and genetic differentiation of subpopulation from Japan and Korea need further study.

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