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Genetic differentiation between natural and hatchery populations of Manila clam (*Ruditapes philippinarum*) based on microsatellite markers

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ABSTRACT. Manila clam (*Ruditapes philippinarum*) is one of the major aquaculture species around the world and supports an important segment of the aquaculture industry in China. In this study, we used ten microsatellite markers to detect genetic diversity within six *R. philippinarum* populations and genetic differentiation between them. A total of 109 alleles were detected across all loci. Compared to wild populations (N_A = 8.4-9.1 alleles/locus, H_E = 0.75-0.77, H_O = 0.67-0.73), hatchery stocks showed less genetic variation as revealed in lower number of alleles and lower heterozygosity (N_A = 7.4-7.5 alleles/locus, H_E = 0.72-0.75, H_O = 0.68-0.70), indicating that a bottleneck effect has occurred in hatchery history. Significant genetic differentiation was observed between cultured stocks ($P < 0.05$), and between cultured and wild populations ($P < 0.05$). Phylogenetic analysis showed a clear separation of the northern three populations and the southern three populations, suggesting that

geographically separated populations of *R. philippinarum* could be genetically differentiated with limited genetic information exchanged between them. The information obtained in this study indicates that the northern and southern populations of *R. philippinarum* should be managed separately in hatchery practices for the preservation of genetic diversity in wild populations.

Key words: *Ruditapes philippinarum*; Microsatellite; Genetic diversity; Wild population; Hatchery stock; Genetic differentiation

INTRODUCTION

The Manila clam *Ruditapes philippinarum* is a bivalve mollusk of the family Veneridae native to the Indo-Pacific region. China has cultivated the Manila clam for a thousand years, with current annual production of 1.8 million tons, accounting for about 80% of mudflat fishery production in China (Zhang and Yan, 2006). *R. philippinarum* has also been used for marine environmental monitoring through the 'mussel watch' program in China. The popularity of this organism as a sentinel derives from several aspects, such as its wide distribution, sessile life style, and filter-feeding habit that accumulates contaminants from seawater. Therefore, *R. philippinarum* has been the focus of research in genetics, biochemistry, physiology, and ecology. Despite its commercial and ecological importance, little information is available about the genetic diversity and population structure of *R. philippinarum* in China.

An important challenge for the culture of any species is to control the loss of genetic variability, commonly caused by the founder effect (Marchant et al., 2009). The high fecundity (usually more than 1 x 10^6 eggs per female) of the mature clam and the artificially high survival rate of juvenile individuals in a culture environment ensure that sufficient seed for each year's production may be derived from only a small number of parents. When small numbers of breeder are used, or the contribution of each parent is unbalanced, or related individuals are mated, there may be a decrease in genetic variability of farmed stocks (Boudry et al., 2002; Li et al., 2004). The effects of inbreeding and genetic drift on a hatchery population with a limited number of captive stocks may result in a decrease in the quality of commercially important traits (e.g., growth rate, pathogen resistance, and maximum size). Therefore, for successful hatchery management, it is important to monitor the genetic profile of hatchery stocks during hatchery rearing.

Characterized as highly variable, neutral, and codominant systems amenable to genotyping by polymerase chain reaction (PCR), microsatellites emerged as genetic markers for precise identification of populations and individuals (Weber and May, 1989; Li et al., 2007a). Over the past decade, microsatellite markers have been extensively used to evaluate the genetic diversity and structure of farmed aquaculture species, such as Atlantic salmon (Norris et al., 1999), tiger shrimp (Xu et al., 2001), flat oyster (Launey et al., 2002), Pacific abalone (Hara and Sekino, 2007), and bay scallop (Wang et al., 2007). In the present study, we used ten microsatellite markers to estimate the level of genetic diversity within four wild populations and two hatchery stocks of Manila clam, and to compare the degree of genetic differentiation between them.

MATERIAL AND METHODS

Sampling

We surveyed four wild and two cultured populations of the Manila clam (Figure 1).

The wild population samples were collected from Dalian in Liaoning Province (DL, N = 52), Lianyungang in Jiangsu Province (LYG, N = 41), Ningbo in Zhejiang Province (NB, N = 44), and Pingtan in Fujian Province (PT, N = 48). Two cultured stocks were collected from Zhuanghe (ZH, N = 52) and Qingdao (QD, N = 56). The ZH hatchery population was founded in 2006 using hatchery-reared broodstocks, and no selected strains or lines have been established. No details as regards the founding and maintenance of the QD hatchery stock are available; however, the parents of the QD cultured stock have been hatchery-propagated over several generations in northern China.

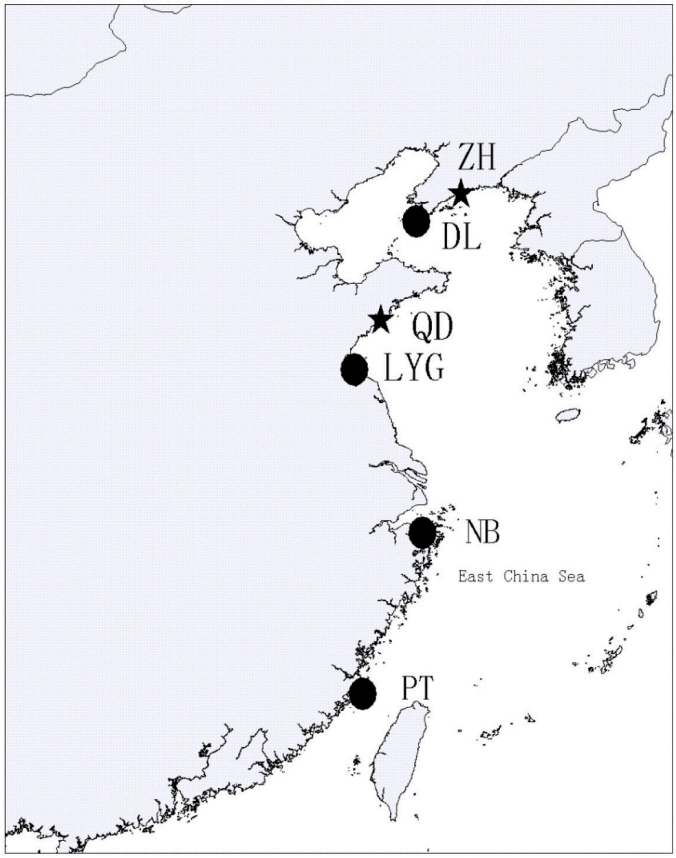

Figure 1. Map showing the locations and abbreviated names of four wild populations (circles) and two cultured populations (stars). DL = wild population of Dalian; LYG = wild population of Lianyungang; NB = wild population of Ningbo; PT = wild population of Pingtan; ZH = cultured population of Zhuanghe; QD = cultured population of Qingdao.

DNA extraction and microsatellite analysis

Genomic DNA was extracted from adductor muscles. Preserved tissue (about 100 mg) was digested with 1% SDS and 10 μg/mL proteinase K in 500 μL STE buffer (10 mM Tris-

HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) for 3 h at 55°C. The reaction mix was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol, washed with 70% ethanol, air-dried, and then suspended in distilled water. A total of 10 microsatellite markers (Table 1) were used in this study. PCRs were performed in a thermal cycler (PTC-100, Bio-Rad, USA) in a total volume of 20 μL containing 0.75 U *Taq* DNA polymerase (Takara), 1X PCR buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and about 50 ng template DNA. The PCR program was set at 5 min at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at the primer-specific annealing temperature listed in Table 1, and 30 s at 72°C, with a final extension for 10 min at 72°C. Amplification products were separated on a 12% non-denaturing polyacrylamide gel at 300 V for 1.5-2 h, stained with ethidium bromide, and visualized under ultraviolet light.

Table 1. Sequences of 10 microsatellite primers used in this study.

Locus	Primer sequence (5'-3')	Repeat motif	GenBank No.	No. of alleles	References
Asari16	F: GCTCGAGTCTGATTGGCTACTTGAA R: GGTATCTAGTCAGCTCTTGCAGTA	$(CT)_{12}$	AB257421	8	Yasuda et al., 2007
Asari24	F: CCTACGACATGTGGGTTATTT R: TGTTTTACCTGTCCATCATTCA	$(GT)_5CT(GT)_3$	AB257423	6	Yasuda et al., 2007
Asari55	F: TGGCTTTGTCAATTGTGTATTGTG R: GACAAATTGGCTTTATTTGGAGG	A_9…$(CTT)_8$	AB257427	14	Yasuda et al., 2007
Asari62	F: AGTTGTACAGCAGCTCAGCAT R: CAAATAACTAAACATTTTCAGACTGC	$(CT)_7C_3(CT)_5(GA)_3$	AB257428	10	Yasuda et al., 2007
Asari64	F: GTATCTGAATGGGTTGTTATTGTGAA R: GCAAAATCATTTCGTTTGATGC	$(CT)_2C_2(GA)_{12}$	AB257429	8	Yasuda et al., 2007
KTp5	F: ACAACTAGTATGTCAGGTCAA R: GTTTTTCACAAGAAGATTTT	$(TG)_5TT(TG)_6$	EF109755	10	An et al., 2009
KTp19	F: TTAACGCTAAACACAACACCT R: AAATGCAACAGCGTAATAAAG	$(TG)_3CG(TG)_{10}$	EF109760	9	An et al., 2009
KTp22	F: AAGTAACATGCGAATTGAAAA R: AACTGTGCTTGCCTGTAGATA	$(CAA)_7$	EF109761	16	An et al., 2009
KTp26	F: GCATATAGCGATTCACCTGT R: GCTGTTCCATACAAGAAGTCA	$(TG)_{10}$	EF109765	13	An et al., 2009
KTp30	F: GAAAAGATTTGGGACTTGTGT R: GCCGTTTAAATCGACCTG	$(TG)_{11}$	EF109766	11	An et al., 2009

Statistical analysis

For each locus in each population, the standard indices, including the number of alleles (N_A), allelic and genotypic frequencies, and expected (H_E) and observed (H_O) heterozygosities were calculated using GENEPOP 3.4 (Raymond and Rousset, 1995). We also estimated an overall inbreeding coefficient (*f*) (Weir and Cockerham, 1984) for each population and locus, by evaluating the significance through random allelic permutation procedures (minimum: 10,000 permutations) using FSTAT 2.9.3 (Goudet, 2001). A nonparametric analysis of variance (Mann-Whitney U-test) was performed to test for differences in the average values of N_A and H_E between the hatchery and wild populations using SPSS 10.0. Exact P value for testing conformity of genotypes with Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were estimated by the Markov chain method (1000 dememorization steps, 1000 batches, 1000 iterations per batch) employing GENEPOP. The significance levels were adjusted for sequential Bonferroni's correction (Rice, 1989). To determine the amount of genetic drift, the effective size of the Chinese hatchery stocks (N_E) was analyzed. Analysis of molecular vari-

ance (AMOVA) was calculated using the Arlequin 3.0 software (Excoffier et al., 2005). The extent of population differentiation was examined by calculating fixation indices based on an infinite allele model and a stepwise mutation model. For the former, the Microsatellite Analyzer (MSA) computer program (Dieringer and Schlötterer, 2003) was used to calculate conventional F_{ST} values. The values of F_{ST} were tested for significant departure from zero using random permutation procedures (1000 permutations). For the latter, R_{ST} values (Slatkin, 1995) were calculated using Arlequin. Departure of R_{ST} estimates from zero was tested with 1000 permutations. A neighbor-joining (NJ) tree was constructed based on F_{ST} using the MEGA 4.0 software (Tamura et al., 2007).

RESULTS

Genetic diversity in four wide populations and two cultured stocks

A total of 109 alleles were detected at the ten microsatellite loci across the six sets of samples. *KTp22* with 16 alleles was the most polymorphic microsatellite, while *Asari24* was the least variable with only 6 alleles. The allele number at each locus varied significantly in different sample sets (Table 2). The DL population displayed the highest allele number (9.10 ± 2.64), followed by LYG (8.5 ± 1.90), NB (8.50 ± 2.51) and PT (8.40 ± 2.07). The two cultured populations (ZH and QD) showed lower allele numbers (7.50 ± 1.51 and 7.40 ± 1.17, respectively). The wild populations (DL, LYG, NB, and PT) showed significantly higher allele richness than the cultured populations (ZH and QD) (Mann-Whitney U-test, $P < 0.05$). Compared to the N_A observed, the N_E at each locus was lower, indicating substantial unevenness in allele frequencies. The mean H_O was 0.68 and 0.70 in the hatchery populations, and ranged from 0.67 to 0.73 in the wide populations. However, the H_O difference between wild and hatchery populations is not statistically significant ($P > 0.05$).

The observed genotype frequencies were tested for agreement with HWE (Table 2). After sequential Bonferroni's correction for multiple tests, among the 60 population-locus cases (6 populations x 10 loci), 18 cases violated the equilibrium ($P < 0.005$), all resulting in heterozygote deficiencies. Generally, more markers departed from HWE in the cultured stocks than in the wild populations. In the PT wild population, only the *KTp22* marker did not conform to HWE, while in the QD-cultured stocks, five loci showed significant ($P < 0.005$) departure from HWE. Estimates of N_E using the mean F_k values are presented in Table 3. The N_E for the two cultured stocks (ZH and QD) was 59.9 and 48.2, respectively.

Population differentiation

F_{ST} and R_{ST} were used to estimate the population structure of the Manila clam (Table 4). Both showed that the tested populations of Manila clam were significantly ($P < 0.05$) differentiated. AMOVA analysis showed that 20.1% of total genetic variation originated from between-population variation, while the within-population variation explained 80.9% of total variation. Genetic differentiation between populations was analyzed using F_{ST} and corrected average pairwise differences. All estimates of pairwise F_{ST} were statistically significant ($P < 0.05$). An NJ phylogenetic tree was constructed using the F_{ST} genetic distance matrix. The 3 northern populations (DL, ZH, and QD) formed one group, while the 3 southern populations (LYG, NB and PT) clustered into another (Figure 2).

Table 2. Summary statistics for genetic variation at 10 microsatellite loci in six populations of Manila clam.

Population (N)		*Asari16*	*Asari24*	*Asari55*	*Asari62*	*Asari64*	*KTp5*	*KTp19*	*KTp22*	*KTp26*	*KTp30*	Average
DL (52)	N_A	7	6	12	10	6	8	7	12	13	10	9.10
	AR	5.2	4.8	9.2	7.2	4.5	6.1	4.9	9.6	9.2	7.5	6.82
	H_E	0.71	0.79	0.83	0.72	0.71	0.86	0.72	0.86	0.81	0.70	0.77
	H_O	0.64	0.65	0.69	0.60	0.72	0.70	0.68	0.71	0.68	0.68	0.67
	f	0.06	0.13*	0.03	0.02	-0.01	0.03	0.04	0.19*	0.15*	0.02	0.02
LYG (41)	N_A	6	6	11	9	7	9	7	11	9	10	8.50
	AR	4.8	4.6	9.6	7.8	5.8	7.1	5.2	8.4	6.5	7.8	6.76
	H_E	0.61	0.85	0.72	0.82	0.66	0.70	0.84	0.88	0.64	0.78	0.75
	H_O	0.57	0.83	0.69	0.72	0.56	0.64	0.85	0.75	0.66	0.76	0.70
	f	0.13*	0.01	0.10	0.12*	0.08	0.06	-0.01	0.06	-0.03	0.02	0.05
NB (44)	N_A	7	5	13	8	6	9	7	12	9	9	8.50
	AR	6.4	4.4	11.2	6.5	5.5	7.6	5.9	10.2	7.8	7.4	7.29
	H_E	0.71	0.81	0.77	0.81	0.72	0.78	0.88	0.86	0.57	0.77	0.77
	H_O	0.72	0.84	0.64	0.74	0.68	0.80	0.75	0.88	0.52	0.70	0.73
	f	-0.02	-0.03	0.17*	0.09	0.05	-0.03	0.15*	-0.02	0.09*	0.09	0.02
PT (48)	N_A	8	6	13	8	6	8	7	10	9	9	8.40
	AR	7.2	4.6	11.2	6.8	4.9	6.8	5.8	8.2	7.4	7.6	7.05
	H_E	0.72	0.81	0.75	0.81	0.68	0.71	0.89	0.87	0.59	0.80	0.76
	H_O	0.70	0.77	0.75	0.84	0.66	0.71	0.83	0.74	0.55	0.75	0.73
	f	0.02	0.04	0.00	-0.04	0.04	0.00	0.07	0.15*	0.06	0.06	0.03
ZH (52)	N_A	8	6	10	7	6	6	7	10	7	8	7.50
	AR	6.5	4.9	8.5	5.6	4.6	4.8	5.1	8.1	5.2	6.1	5.94
	H_E	0.60	0.80	0.85	0.62	0.73	0.76	0.90	0.84	0.71	0.65	0.75
	H_O	0.47	0.69	0.91	0.72	0.84	0.78	0.90	0.41	0.50	0.53	0.68
	f	0.23*	0.15	-0.06	-0.16	-0.17	-0.02	0.00	0.52*	0.29*	0.18*	0.00
QD (56)	N_A	6	6	9	8	6	8	7	8	7	9	7.40
	AR	4.6	4.5	7.5	6.2	4.6	6.5	5.4	6.1	5.3	7.5	5.82
	H_E	0.64	0.71	0.77	0.69	0.73	0.82	0.88	0.79	0.54	0.64	0.72
	H_O	0.60	0.55	0.79	0.63	0.79	0.74	0.94	0.69	0.44	0.58	0.70
	f	0.06	0.18*	-0.03	0.10*	-0.08	0.10*	-0.06	0.12	0.08*	0.09*	0.02

DL = wild population from Dalian; LYG = wild population from Lianyungang; NB = wild population from Ningbo; PT = wild population from Pingtan; ZH = cultured population from Zhuanghe; QD = cultured population from Qingdao. N = number of samples; N_A = allele number; AR = allele richness; H_E = expected heterozygosity; H_O = observed heterozygosity; f = fixation index. Table-wide significance levels were applied using the sequential Bonferroni's technique ($k = 9$). *Significant at $P < 0.005$.

Table 3. Effective population size (N_E) estimates for Chinese hatchery populations of *Ruditapes philippinarum*.

Population	Mean F_k	N_E	95%CI for N_E
ZH	0.102	59.9	25.6, 56.8
QD	1.189	48.2	38.7, 79.6

F_k = standard variance in allele frequency change; CI = confidence interval. ZH = Zhuanghe; QD = Qingdao.

Table 4. Analysis of genetic differentiation between pairs of samples based on estimates of F_{ST} (below diagonal) and R_{ST} (above diagonal).

	DL	LYG	NB	PT	ZH	QD
DL	-	0.3152*	0.4125*	0.5478*	0.0811*	0.1365*
LYG	0.0574*	-	0.1245*	0.1589*	0.1305*	0.0825*
NB	0.0692*	0.0342*	-	0.1421*	0.1345*	0.0896*
PT	0.0789*	0.0568*	0.0518*	-	0.4136*	0.3021*
ZH	0.0356*	0.0412*	0.0489*	0.0698*	-	0.1123*
QD	0.0468*	0.0345*	0.0369*	0.0561*	0.0321*	-

DL = Dalian; LYG = Lianyungang; NB = Ningbo; PT = Pingtan; ZH = Zhuanghe; QD = Qingdao. *Significant at $P < 0.05$.

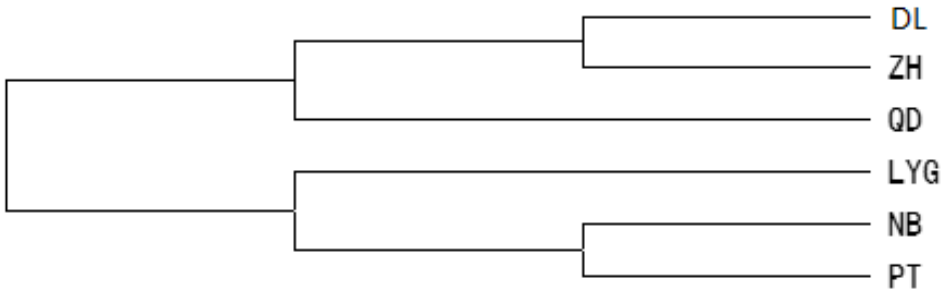

Figure 2. Neighbor-joining phylogenetic tree constructed with F_{ST} genetic distances. For abbreviations, see Table 2.

DISCUSSION

A high level of genetic diversity is essential for long-term survival of populations, since the extent of variation determines their ability to adapt to changing environments. In this study, substantial loss of allelic diversity was observed in two hatchery stocks compared to the natural populations. Loss of allelic variation at microsatellite loci in hatchery-produced animals has been reported in many aquaculture species, including fish (Sekino et al., 2002), shrimp (Xu et al., 2001) and shellfish (Wang et al., 2007; Li et al., 2004, 2007b). Manila clam possesses a thousand year aquaculture history in China, so there were concerns that genetic variability might have been lost during cumulative hatchery production. Bottleneck analysis showed that two cultured clam populations might have experienced a recent bottleneck, suggesting that these cultured stocks may have been set up by using only a few founder individuals, or an effective population size much lower than the real one due to lower and different contributions of founders to offspring, or due to different fitness of larvae/juveniles from different families. Clearly, the management practices used to produce these *R. philippinarum* commercial stocks would not have been sufficient to maintain genetic variability in the long run. Our results highlight the need to regularly expand hatchery stock genetic pools with additional wild collections to maintain genetically healthy *R. philippinarum* stocks in Chinese hatcheries.

In this study, we detected that 18 of the 60 population-locus tests deviated from HWE, all due to heterozygote deficiency. Generally, causes for such deviations include substructuring of the population sample, inbreeding, or the presence of null alleles. High frequency of null alleles has been reported in various bivalve mollusks (Zhan et al., 2007; Li et al., 2012a,b), and may complicate many types of population genetic analysis that rely on HWE, as false homozygotes would be common. Beyond the existence of null alleles, the heterozygote deficiency can increase due to other factors, such as nonrandom mating, admixture of independent populations, or artificial and natural selection during seed production and cultivation (Selkoe and Toonen, 2006). HWE deviation occurred more frequently in hatchery stocks than in the wild samples (Table 2). This could be the result of the long history of clam aquaculture and admixture of genetically differentiated populations. Since China has a long history of clam aquaculture, several batches of wild clams have been introduced to hatchery stocks to expand the gene pool. Repeated interbreeding between a natural population and hatchery stocks could result in a non-random mating system that could generate within-population substructuring (Wahlund effect), possibly leading to a characteristic heterozygote deficiency (Hartl and Clark, 1990).

Pairwise comparison of the allele frequencies (F_{ST} and R_{ST}) showed significant differentiation between all the populations tested. Possibly, the differentiation shown by the cultured strains is due to the low number of founding individuals in hatcheries, increasing the possibility of genetic drift. This has been well documented in other species such as Pacific oyster (Hedgecock and Sly, 1990), sea trout (Was and Wenne, 2002), Japanese flounder (Sekino et al., 2002), and Japanese scallop (Li et al., 2007b). However, some hatchery operations may also have led to changes in the genetic composition of cultured strains compared with source populations. An NJ phylogenetic tree based on F_{ST} showed a clear separation of the northern and southern populations. The large differentiation between wild populations may be related to the geographic separation of these populations. Therefore, we propose that future breeding programs should avoid farming in areas where local spawning occurs, and introgression between cultured and wild populations should be prohibited. Because of significant genetic differentiation between northern and southern populations, cultured populations should be managed separately, and any translocation between the two areas should be avoided.

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