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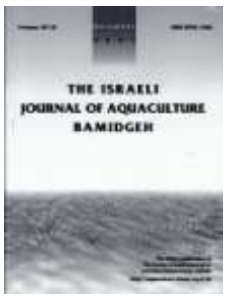

Using Microsatellites to Assess Genetic Variation in a Selective Breeding Program of Chinese Bay Scallop (*Argopecten irradians irradians*)

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Abstract

This study aimed to improve our understanding of the genetics of the Chinese bay scallop (*Argopecten irradians irradians*), one of the most important maricultured shellfish in China. Ten polymorphic microsatellite loci were examined to assess the allelic diversity, heterozygosity, and genetic variation between two domesticated populations selected for fast growth in breeding programs, and their base population. Forty-one alleles were found throughout the loci and the mean number of alleles per locus ranged 3.30-3.50. The average heterozygosity ranged 0.38-0.45, whereas the polymorphic information content ranged 0.1504-0.7518. Genetic differences between the three populations were detected based on the number of alleles per locus, effective number of alleles, Shannon index, inbreeding coefficient (Fis), *p* values, genetic distance, and pairwise Fst values. There was no significant loss of genetic variability in the breeding program but changes in gene frequencies were detectable over the populations, implying that the loci were affected by the pressures of selective culture.

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Introduction

The bay scallop, *Argopecten irradians irradians* Lamarck, is a marine bivalve of considerable economic importance, naturally distributed along the coasts of the Atlantic Ocean and the Gulf of Mexico (Wilbur and Gaffney, 1997). For about one century until the mid 1980s, bay scallops supported large commercial fisheries in Massachusetts, New York, and North Carolina and smaller fisheries in western Florida, USA. However, since then, bay scallop populations have dramatically declined due to large blooms of harmful toxic microscopic algae that cause high mortality (Belding, 1910; Goldberg and Tettelbach, 2008; Mackenzie, 2008). Since the introduction of 26 bay scallop specimens from the USA in 1982, a successful aquaculture industry was founded in China and the bay scallop became one of China's most important aquaculture species (Zhang et al., 1986; Guo et al., 1999). After that, serious problems affected scallop breeders in China, such as increased larvae and juvenile mortality and decreased growth rate (Zhang et al., 1997).

Genetic degradation of commercial Chinese bay scallop stocks was found by comparing mtDNA (Blake et al., 1997) and allozymes (Xue et al., 1999) to wild populations in America. To restore this important commercial species, a new broodstock of 401 individuals was introduced from Virginia and Massachusetts in 1998 and 1999 (Li et al., 2000). The new immigrants quickly replaced the original aging stocks and significantly boosted the industry. But genetic degradation in this hermaphroditic bivalve is a potential threat due to high inbreeding depression caused by self-fertilization (Stiles and Choromanski, 1995) and, in fact, genetic degradation has been identified by microsatellites (Wang et al., 2007).

Most genetically improved aquaculture strains are developed by traditional selective breeding, crossbreeding, and hybridization (Hulata, 2001). Selective breeding is the most effective way to obtain genetic improvement in cultured marine species (Zheng et al., 2006a). Selective breeding in oysters, hard calms, scallops, and other species can improve growth rates and disease resistance. Mass selection was used to increase production of *Saccostrea cucullata* (Jarayabhand and Thavornyutikarn, 1995), *Mercenaria mercenaria* (Hadley et al., 1991), and *Argopecten ventricosus* (Ibarra, 1999; Ibarra et al., 1999). Consequently, a program for selective breeding for fast growth of bay scallop was initiated in the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, in March 2002. The Chinese bay scallop was examined by establishing broodstocks for selection, estimating heritability for economically important traits, examining inbreeding depression and heterosis (Zheng et al., 2004, 2006a,b, 2007; Zhang et al., 2007a,b), and developing molecular markers for population genetics (Qin et al., 2007a,b,c; Li et al., 2008). Great genetic gains indicated that selection is a promising method for genetic improvement in the bay scallop (Zhang et al., 2008).

Microsatellites are the marker of choice for many types of genetic studies due to their high level of polymorphism, co-dominance, genomic distribution, and small locus size (Wang et al., 2007). Evidence of high genetic load was found in the Pacific oyster, *Crassostrea gigas*, by comparing the segregation ratios of microsatellite DNA markers at 6 h and 2-3 months post-fertilization in hybrid families (Launey and Hedgecock, 2001). Genetic variation in four successive mass selection lines of Tasmanian hatchery-produced stocks of Pacific oysters was characterized by allozymes and microsatellite loci (Appleyard and Ward, 2006). In this study, we examined genetic variations between two domesticated populations of Chinese bay scallop from our selective breeding program and their base population.

Materials and Methods

Sample collection. Fifty individuals were randomly collected from each of three populations: Zhongkehong stock R10, domesticated for 8 years; Zhongke No. 2 stock P10, domesticated for 4 years; and their base population from Jiaonan, China (120°02'E, 35°80'N). Genomic DNA from the three populations (total 300 individuals) was extracted from ethanol-preserved samples using a Fast200 DNA extraction kit (Fastagen, Shanghai, China) according to the manufacturer's instructions.

Microsatellite analysis. Ten polymorphic microsatellite loci were selected for polymerase chain reaction (PCR) analysis to detect genetic polymorphism (Zhan et al., 2006; Li et al., 2007). PCR amplifications were carried out in 15-µl reactions containing 1× PCR buffer, 0.34 µm of each primer, 200 µm of each dNTP, 1.5 mm $MgCl_2$, 0.5U Taq polymerase, and about 50 ng of template DNA on a TaKaRa TP600 PCR Thermal Cycler Dice (TaKaRa, Japan). The thermal profile for PCR amplification was 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at the primer-specific annealing temperature (Table 1), 30 s at 72°C, and a final step for 5 min at 72°C. PCR products were separated by electrophoresis on 12% polyacrylamide gel, sized with Marker DL 2000 (Takara, Dalian, China), and visualized by ethidium bromide stain under a UV transilluminator (BIORAD, USA). Gel-Pro Analyzer software (version 7.0, http://www.mediacy.com) was used to analyze the alleles detected by electrophoresis.

Table 1. Primer sequences, repeat types, and PCR conditions used to study ten microsatellite loci in Chinese bay scallop (*Argopecten irradians irradians*).

Locus	*Primer sequence (5′→3′)*	*Core sequence*	*Optimum annealing temperature (°C)*	*GenBank accession no.*
AIMS026[1]	F: CACTTCAGACACAAGTTACCGC R: TGAACCACCAAAGGTGACGGGG	(GAT)6	66	CB416920
AIMS027[1]	F: TGAACTTTTCCCTCATCGTGTC R: CACAACTATCAACCTGCCCATC	(CAG)5	65	CB414724
HWAD190[2]	F: CACTCGTGGGCGTAAATAG R: CAAATAAAGGAAGCAACCG	(TC)6TT(TC)21	50	DQ779953
Add026[3]	F: CGTTTGGGACTGATTTACC R: AACCTTTCTATTTCTTGTATTGAC	(TG)9…(TG)8	55	EU623919
Add050[3]	F: CATTGTTCGTGGTTCACTG R: AAAGCAACGGTTGTATTCC	(TG)13	54	EU623932
Aad171[3]	F: TATACGCTAAACCATCGCTG R: AAAAGTTACGAAACACTATCAAAAG	(CA)25CG(CA)11	58	EU623895
Add032[3]	F: CTTTGAATTGTATTTTTTCGC R: GTCCAAGGAGGCTTTATGC	(TC)19	56	EU623922
Aad071[3]	F: TACAATGTACTTTACCAGAATAGAC R: TGCTAATTTTTCGTTGACC	(AG)29	54	EU623847
Asd223[3]	F: ATTATTTTGTTTATACCATAGGAGC R: CGGTTTAGATTTGGAAGTTTG	(AG)19	58	EU623799
Aad092[3]	F: AATAGAGGATACTTTAGGAAGTTAC R: GTAAAAATCCTTCAGACATAATC	(AG)15	60	EU623859

[1] Zhan et al. (2006)
[2] Li et al. (2007)
[3] Li et al. (2012)

Statistical analysis. PopGen32 software (version 1.32, available at http:// www.ualberta.ca/~fyeh/popgene.html) was used to analyze the number of alleles per locus (A), the effective number of alleles (A_e), observed (H_o) and expected heterozygosity (H_e), Shannon Index (SI), inbreeding coefficient (Fis), statistical significance of Hardy-Weinberg equilibrium (HW), pairwise comparison of allele frequency among all the populations (Hartl and Clark, 1997) for each locus, genetic distance (D_C; Nei, 1978), and cluster analysis. Population pairwise Fst values based on the number of different alleles and allele frequencies were calculated using GenePop software version 3.4 (Raymond and Rousset, 1995). Polymorphism information content (PIC), a measure of locus polymorphism, was estimated using the formula of Botstein et al. (1980). Cluster analysis of the three populations was computed using the unweighted pair group method with arithmetic mean (UPGMA) in PopGene 32 (v. 1.32).

Results

Genetic diversity. Forty-one alleles were identified in the ten microsatellite loci from the 300 individuals (Table 2). The number of alleles per locus (A) ranged 2-6 (avg 3.4), whereas the effective number of alleles per locus (A_e) ranged 1.1959-4.6642 (avg 2.33).

The mean number of alleles per population ranged 3.3-3.5 and the allele size ranged 100-300 bp. The Shannon Index (SI) ranged 0.3025-1.5757, with an average for all loci in all populations of 0.8961±0.3427. The polymorphism information content (PIC) at each locus ranged 0.1504-0.7518, with an average of 0.4571±0.1622. There were significant differences (p<0.05) in average heterozygosis among the three populations. Eleven of the 30 population-locus cases (3 populations × 10 loci) significantly deviated from the Hardy-Weinberg (HW) equilibrium (p<0.01). While departure from the Hardy-Weinberg equilibrium was observed in every population and in most loci, the R10 population had the highest frequency of deviation.

Table 2. Number of alleles (A), effective number of alleles (A_e), observed heterozygosity (H_o), expected heterozygosity (H_e), Shannon Index (SI), inbreeding coefficient (Fis), probability value of Hardy-Weinberg equilibrium (HW), and polymorphism information content (PIC) at ten microsatellite loci surveyed in two populations from a selected breeding program (R10, P10) and their base population of the Chinese bay scallop, *Argopecten irradians irradians* (n = 50).

		Locus										
Population		*AIMS026*	*AIMS027*	*Add026*	*HWAD190*	*Add050*	*Aad171*	*Add032*	*Aad071*	*Asd223*	*Aad092*	*Mean of all loci*
R10	A	4	3	5	4	3	4	2	2	3	3	3.30±0.95
	A_e	2.8043	1.227	3.1368	3.0562	2.145	2.426	1.9992	1.7241	1.8362	1.9077	2.23±0.62
	H_o	0.4800	0.1800	0.4400	0.4000	0.3000	0.3600	0.6600	0.2400	0.3600	0.3800	0.38±0.13
	H_e	0.6499	0.1869	0.6881	0.6796	0.5392	0.5937	0.5048	0.4242	0.4600	0.4806	0.52±0.15
	SI	0.3944	1.2784	1.1641	0.8908	1.0113	0.6929	0.6109	0.6862	0.8338	0.8739	0.84±0.26
	Fis	0.2540	0.0270	0.3541	0.4055	0.4380	0.3875	-0.3205	0.4286	0.2095	0.2013	0.24±0.24
	HW*	**0.000025**	0.465823	**0.000136**	**0.000000**	**0.000000**	**0.001017**	0.028151	**0.001844**	0.358793	0.131811	-
	PIC	0.5861	0.1769	0.6267	0.6039	0.4621	0.5179	0.3749	0.3318	0.3604	0.4288	0.45±0.14
P10	A	4	2	5	6	4	2	3	3	2	4	3.50±1.35
	A_e	2.4594	1.4188	3.2852	4.1528	2.0912	1.1959	2.5316	1.9026	1.6756	2.1322	2.28±0.89
	H_o	0.4600	0.2800	0.7400	0.6200	0.3600	0.1800	0.4600	0.3600	0.4000	0.4400	0.43±0.16
	H_e	0.5994	0.2982	0.7026	0.7669	0.5271	0.1655	0.6111	0.4792	0.4073	0.5364	0.51±0.18
	SI	1.0971	0.4714	1.3706	1.5288	0.8691	0.3025	0.9986	0.7307	0.593	0.973	0.89±0.39
	Fis	0.2248	0.0515	-0.0638	0.1834	0.3101	-0.0989	0.2397	0.2411	0.0079	0.1714	0.13±0.14
	HW*	0.065992	0.658557	0.043627	0.031416	0.012018	0.511612	**0.006699**	0.070308	0.898001	**0.000002**	-
	PIC	0.5437	0.2516	0.6543	0.7199	0.4361	0.1504	0.5270	0.3793	0.3219	0.4810	0.45±0.18
Base	A	5	2	3	5	3	5	2	3	2	4	3.40±1.24
	A_e	2.3408	1.3931	1.6404	3.4130	2.8043	4.6642	1.9616	2.0517	1.3423	3.2446	2.49±1.05
	H_o	0.4200	0.2600	0.3600	0.6200	0.3400	0.4600	0.3800	0.5400	0.3000	0.7800	0.45±0.16
	H_e	0.5786	0.2851	0.3943	0.7141	0.6499	0.7935	0.4952	0.5178	0.2576	0.6988	0.54±0.18
	SI	1.1022	0.4559	0.7038	1.4063	1.0610	1.5757	0.6833	0.8521	0.4227	1.2495	0.95±0.39
	Fis	0.2668	0.0787	0.0779	0.1231	0.4716	0.4145	0.2248	-0.0535	-0.1765	-0.1765	0.13±0.22
	HW*	0.026437	0.523504	0.645819	0.210606	**0.000001**	**0.000000**	0.096550	**0.000077**	0.229949	0.040744	-
	PIC	0.5301	0.2424	0.3531	0.6682	0.5673	0.7518	0.3701	0.4397	0.2225	0.6324	0.48±0.18

* Significant p values for Hardy-Weinberg equilibrium are in bold

Genetic variation. Pairwise population comparisons of allele frequencies revealed that 23 of the 30 pairs significantly differed, even after Bonferroni correction for the significance level of three simultaneous tests (p<0.003). All pairs showed significant heterogeneity of allele frequencies for at least three loci (Table 3). Pairwise Fst values ranged 0.1664-0.2571 with significant differences among populations (p<0.003) after Bonferroni correction (Table 4). Further, six cases of observed heterozygosity excess (Fis<0) were observed in all populations (Table 2). The largest genetic distance (0.5048) was observed between R10 and the base population, while the smallest genetic distance (0.2560) was observed between P10 and the base population (Table 4). The neighbor-joining tree generated from the D_C values is shown in Fig. 1. The three populations fell into three clusters that corresponded to the period of domestication.

Table 3. Pairwise comparisons of allele frequencies between two selectively bred populations of Chinese bay scallop (R10 for 8 years and P10 for 4 years) and their base population at ten microsatellite loci.

Locus	*Population*	*R10*	*P10*	*Base*	*Locus*	*Population*	*R10*	*P10*	*Base*
	R10	-				R10	-		
AIMS026	P10	0.00000*	-		Aad171	P10	0.00000*	-	
	Base	0.00000*	0.76796	-		Base	0.00000*	0.00000*	-
	R10	-				R10	-		
AIMS027	P10	0.00000*	-		Add032	P10	0.00000*	-	
	Base	0.00000*	1.00000	-		Base	0.32417	0.00007*	-
	R10	-				R10	-		
Add026	P10	0.00000*	-		Aad071	P10	0.00000*	-	
	Base	0.00000*	0.00000*	-		Base	0.00000*	0.12857	-
	R10	-				R10	-		
HWAD190	P10	0.00000*	-		Asd223	P10	0.00000*	-	
	Base	0.00000*	0.00711	-		Base	0.00000*	0.03992	-
	R10	-				R10	-		
Add050	P10	0.00000*	-		Aad092	P10	0.15424	-	
	Base	0.00000*	0.00000*	-		Base	0.00000*	0.00012*	-

*Significant after Bonferroni correction (p<0.01-0.03). Probability values of homogeneity of allelic frequency distributions (P) estimated by a test analogous to the Fisher's exact test in the Markov-chain method are shown; wide significance levels were applied using the sequential Bonferroni technique (Rice, 1989; k = 3).

Table 4. Nei (1978) unbiased measures of genetic distance (below diagonal) and Fst values (above diagonal) among three populations of Chinese bay scallop.

Population	*R10*	*P10*	*Base*
R10		0.2368*	0.2571*
P10	0.4096		0.1664*
Base	0.5048	0.2560	

Wide significance levels were applied using the sequential Bonferroni technique (Rice, 1989; k=3). *Significant after Bonferroni correction (p<0.01/3)

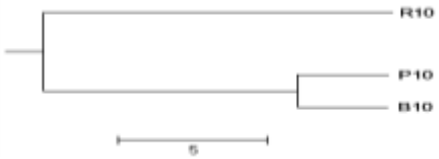

Fig. 1. Cluster analysis of two selectively bred populations of Chinese bay scallop (R10 for 8 years and P10 for 4 years) and their base population (B10) using the unweighted pair group method with arithmetic mean (UPGMA) in PopGene 32 (v. 1.32).

Discussion

The bay scallop is China's most successful industry built on an introduced species. However, high mortalities and slow growth rates eventually emerged in the small initial population that was maintained in artificial hatcheries and lacked a natural habitat. Bay scallop is simultaneously hermaphrodite and heterosexual. Thus, self-fertilization is common during seed production in the hatchery and this may have caused the high level of inbreeding depression detected in these stocks (Zheng et al., 2007). Selective breeding is widely used for bivalves. To avoid repeating past mistakes, we initiated a selective breeding program in new wild stock introduced from America in 1998 and 1999. In the program, F2 of the imported wild stock was used as a base population from which a strain named Zhongkehong (R10) was domesticated for fast growth for 8 years and a strain named Zhongke No. 2 (P10) was domesticated for fast growth for 4 years. The Zhongkehong strain is cultured on a large scale and accounted for over 60% of China's bay scallop production in 2012 (unpublished survey data).

As documented by mtDNA and DNA markers, a great proportion of the original genetic variation was lost due to generations of hatchery breeding (Blake et al., 1997; Qin et al., 2007a; Wang et al., 2007). With time, heterozygosity among the populations decreased, moreso in the R10 population than in the P10 and base populations (p<0.05). Although the loss of rare alleles is a meaningful measure of genetic change in hatchery stocks, heterozygosity is more sensitive (Hedgecock and Sly, 1990). There were no significant differences in mean allelic richness among the three populations. Long-term breeding programs aim to achieve a balance between continuous genetic gains and maintenance of adequate levels of genetic variation (Gaiotto et al., 1997). Compared to

the base population, populations R10 and P10 from the selective breeding program showed little reduction in genetic variation. Although a reduction in allele diversity in bay scallop strains was found in a similar microsatellite analysis between wild and hatchery populations of bay scallop, the trend was not significantly digressive (Wang et al., 2007). We believe that genetic variation of the Chinese bay scallop was maintained in some kind of balance, whereas commercial traits were considerably improved by our selective breeding program.

Significant deviations from Hardy-Weinberg (HW) expectations were observed in all populations (11 of the 30 locus tests), as is common (Zouros and Foltz, 1984; Estoup et al., 1995). This type of deviation is most likely caused by selection, admixture of genetically differentiated populations, non-random mating, and null-alleles (Callen et al., 1993; Rousset and Raymond, 1995; Li et al., 2003). Another likely factor is hybridization or mixture between two sub-species from the time of introduction (Zhang et al., 1997). The R10 population had a greater number of loci that deviated from the HW than the P10 or base population; the R10 population is the longer domesticated strain in the selective breeding program. Of concern is the high number of polyallelic loci in the Hardy-Weinberg disequilibrium for the R10 population, indicating that the population is undergoing great genetic change.

Among the three populations, the Fis value of the R10 population was highest. Inbreeding and the genetic substructure within sampling units can cause high Fis values. Inbreeding is most likely the major factor in our study, inferred from the remarkable increase seen with selection. As self-fertilization is very common in bay scallop, it would be difficult to minimize inbreeding and maintain genetic variability. Inbreeding significantly causes a decline of fitness and genetic variation and affects selective breeding efficiency (Zhang et al., 2005, 2007a; Qin et al., 2007a). More efficient methods are needed to reduce inbreeding during the breeding program. As hermaphroditic marine organisms, bay scallop disperse gametes into flowing sea water that travel during the planktonic larvae stage. But in a hatchery with a limited population size and space, the most effective way to prevent inbreeding is to increase the population size (Qin et al., 2007a).

Population differentiation was detected by traditional Fst (Weir and Cockerham, 1984) and pairwise comparisons of allele frequencies. Significant levels of differentiation were detected among all populations. The Fst between R10 and the base population (B10) was higher than the Fst between P10 and B10. Genetic distances and the neighbor-joining tree topology further describe the genetic variation of the domesticated populations. The R10 population was clearly separate from the other populations, while the P10 population was closer to the B10 population in genetic distance.

In conclusion, our study examined the genetic information of two domesticated strains in the Chinese bay scallop selective breeding program and found that the domesticated strains are undergoing genetic drift. Although no significant reduction in genetic diversity was detected, the domesticated strains showed notable genetic differentiation compared to the base population. Such changes may be the result of long-term artificial selection in the inbreeding population. This information allows us to monitor the genetic basis of Chinese bay scallop commercial strains and improve breeding programs. Inbreeding depression in bay scallop cannot be prevented by creating pedigrees or preventing consanguineous mating in commercial hatchery breeding. The only way to reduce inbreeding depression is to increase the effective population size. At least 340 individuals are necessary to maintain adequate genetic variation (Qin et al., 2007a). Further, as it is difficult to balance the contribution of each individual during fertilization, the safer choice is to spawn more seed than necessary.

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