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Full Length Research Paper

Low genetic diversity and genetic differentiation among domesticated stocks of the small abalone *Haliotis diversicolor* in China

Jianyong Liu[1], Bijian Zeng[2], Tao Du[1] and Yingzhu Rao[3]*

[1]Fisheries College, Guangdong Ocean University, 40 Jiefangdong Road, Zhanjiang, Guangdong 524025, China.
[2]Animal quarantine laboratory, Guangdong Entry-Exit Inspection and Quarantine Bureau, 66 Huacheng Road, Guangzhou Guangdong, 510623, China.
[3]Life Science and Technology School, Zhanjiang Normal University, 48 Cunjin Road, Zhanjiang, Guangdong, 524000, China.

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AFLP markers were used to investigate levels of genetic diversity within cultured populations of the small abalone *Haliotis diversicolor* in China and to compare them with a wild population. A total of 234 fragments, with a size range of 150-500 bp, were identified from 152 individuals by five AFLP primer combinations. High proportion of polymorphic loci (77.65%) and heterozygosity (0.2124) were found within the natural population, while low proportion of polymorphic loci (24.51 to 64.31%) and heterozygosity (0.0677 to 0.1665) were detected within the six cultured populations. The results show a significantly reduced genetic diversity in the hatchery stocks when compared with the wild population. AMOVA analysis, pairwise comparison of φ_{ST} values, and cluster analysis showed that both the wild population (DX) and the stock (TW) were significantly different from the other five domestic cultured populations. For the five hatchery stocks sampled from mainland China coast, however, genetic differences were not significant, except between SZ and ZJ, SZ and HN stocks. The genetic status detected in this study hints that mass mortality of *H. diversicolor* in recent years might have association with the reduction in genetic diversity. The results also indicate that all cultured populations, excluding TW and ZJ stocks, may not be suitable as source populations for selective breeding programs; some natural populations and foreign stocks introduced from outside China should be considered.

Key words: Amplified fragment length polymorphism, *Haliotis diversicolor*, domesticated stocks, genetic diversity.

NTRODUCTION

The small abalone *Haliotis diversicolor* naturally are distributed along the coastal waters of East Asia: from Japan to the Philippines (Lindberg et al., 1992). Due to habitat destruction and overexploitation, wild resource of this economic important species was depleted to unrecoverable status over the past several decades, especially along southern China and Taiwan coast (Nie, 1992; Roodt-Wilding, 2007). The reduction of wild-stocks and the increasing demand greatly promoted the abalone aquaculture industry. Culture of *H. diversiocolor* in Taiwan has expanded greatly since 1986 owing to successful artificial propagation and development of multiple-tier basket systems in grow-out farms (Yang and Ting, 1986). The species and farming methods were then introduced to mainland China in 1989, and the small abalone farming flourished after 1996 because of its short grow-out period and high profit (You et al., 2010). Hatchery cultured stock has been used as broodstock to produce the next cultured generation every year. Since

*Corresponding author. E-mail: raoyzh@Yahu.com.cn.

Abbreviations: AFLP, Amplified fragment length polymorphism; **AMOVA,** analysis of molecular variance.

2002, however, massive mortality of small abalone has occurred at post-larval (Cheng and Hsiao, 2004) and grow-out stages in southern China.

The high mortality and other problems such as slow growth and severely diseases have led to the striking reduction of small abalone production in recent years, and now, the aquaculture industry in southern China is close to collapse (You et al., 2009). The exact cause of this large-scale mortality remains unknown (Ma et al., 2008), though causations like the poor water quality, lack of food caused by diatoms, virus and bacterial infection, etc. were suspected (Xu et al., 2006); genetic degradation, resulted from inbreeding, may be the main reason for these problems (You et al., 2010).

Genetic diversity within a population increase its ability to withstand environmental perturbations and disease outbreaks (Gamfeldt and Kallstrom B, 2007), and the sufficient level of genetic variability is essential in order to maintain a sustained response from long-term selection for commercially important traits (Davis and Hetzel, 2000). Genetic structure and diversity of natural populations and culture stocks of abalone have been widely studied using molecular marks. A distinct reduction in genetic variability has been observed in the microsatellite analysis of hatchery produced *H. rubra* and *H. midae* (Evans et al., 2004) and cultured strains of *H. discus hannai*(Li et al., 2004; Li et al., 2007a). Genetic variation and population differentiation of the black abalone *H. cracherodii* along the California coastline was examined by mitochondrial cytochrome oxidase subunit I gene (COI), microsatellites, and AFLP markers (Gruenthal and Burton, 2008). Using three polymorphic enzyme-encoding loci and partial COI sequence, Hamm and Burton (2000) studied the genetic diversity and structure of seven black abalone *H. cracherodii* populations sampled along the central California coast. Klinbunga et al. (2003) examined genetic diversity of tropical abalone *H. asinina* originating from Thai waters by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of 18S and 16S rDNAs, and the lack of genetic heterogeneity were confirmed. Recently, significant genetic differentiation between the Gulf of Thailand and the Andaman Sea were observed using single-strand conformational polymorphism of AFLP-derived markers (Praipue et al., 2010).

Despite several decades of domestication in China, information about the genetic diversity in hatchery stocks of small abalone *H. diversicolor* is still limited and the conclusions from different research groups remain controversial. Jiang et al. (1995) reported fixed differences in mitochondrial DNA haplotypes between neighboring (35 km apart) Taiwanese *H. Diversicolor*. Using RAPD markers, Du et al. (2007) studied the genetic variation in five hatchery-produced small abalone stocks in South China, high level of genetic diversity and significant differentiation between each pair of the five populations were detected. In contrast, Li and Yan (2009) detected loss of genetic diversity in three hatchery produced stocks and low genetic differentiation among wild and hatchery populations by AFLP fingerprinting. The breeding program of small abalone *H. diversicolor* in China has been carried out for several decades. In recent years, the situation of its aquaculture industry was in chaos. No one knew the origin of parents and the seed stocks. As a result, levels of genetic diversity and patterns of gene pools of the present domesticated stocks should be comprehensively examined to reveal possible changes in variability caused by genetic drift, inbreeding, or selection.

AFLP analysis (Vos et al., 1995), which combines the strengths and overcomes the weaknesses of PCR-RFLP and random amplified polymorphic DNA (RAPD)-PCR (Williams et al., 1990), has been widely used for examination of levels of genetic diversity in abalone (Gruenthal et al., 2007; Gruenthal and Burton, 2008; Li and Yan, 2009; Praipue et al., 2010) and other marine organisms (Hsu et al., 2009; Lin et al., 2009; Liu et al., 2009). In this study, we used AFLP technique to more fully assess the genetic diversity and the patterns of gene pools of domesticated stocks of *H. diversicolor* in China. Our sampling range covers the main breeding areas, including five stocks along the mainland China coast, one at Taiwan coast. In addition, a natural population was used to detect the genetic degradation of cultured stocks. The results can provide necessary information for the ongoing breeding program, and benefit for the effective culturing and continued management for this species.

MATERIALS AND METHODS

Population sampling and DNA extraction

Small abalone samples were collected from six cultured populations of Dongshan in Fujian (FJ) Province, Shanwei (SW), Shenzhen (SZ) and Zhanjiang (ZJ) in Guangdong Province, Wenchang in Hainan (HN) province, and Tainan in Taiwan Province (TW), and a wild population from the coast of Dongxing Guangxi (DX). A total of 152 individuals were collected; the geographic locations and sample size are given in Figure 1. Muscle samples from each individual were cut and preserved in 95% ethanol. Genomic DNA was isolated using a DNA isolation Kit from Sangon (Shanghai, China) following the manufacturer's instructions. The concentration of DNA was measured by the spectrophotometer (Kodak, Biostep Germany).

AFLP analysis

AFLP was performed following the protocol described by Vos et al. (1995) with some modifications. Sequences of AFLP adapters and primers are listed in Table 1. Briefly, approximately 500 ng of genomic DNA was digested in a 20 µl of reaction volume including 3U EcoRI (MBI Fermentas), 3U MseI (TaKaRa Biotechnology Co., LTD.) and 2 µl 10× digestion buffer R at 37°C for 3 h, and then double-stranded adapters were ligated to the restriction fragments at 20°C overnight after addition of 2U T4 DNA ligase (TaKaRa Biotechnology Co., LTD.), 2 µl T4 DNA ligase buffer, 5 pmol EcoRI adapter (EcoRI-1/ EcoRI-2; Table 1) and 50 pmol MseI adapter

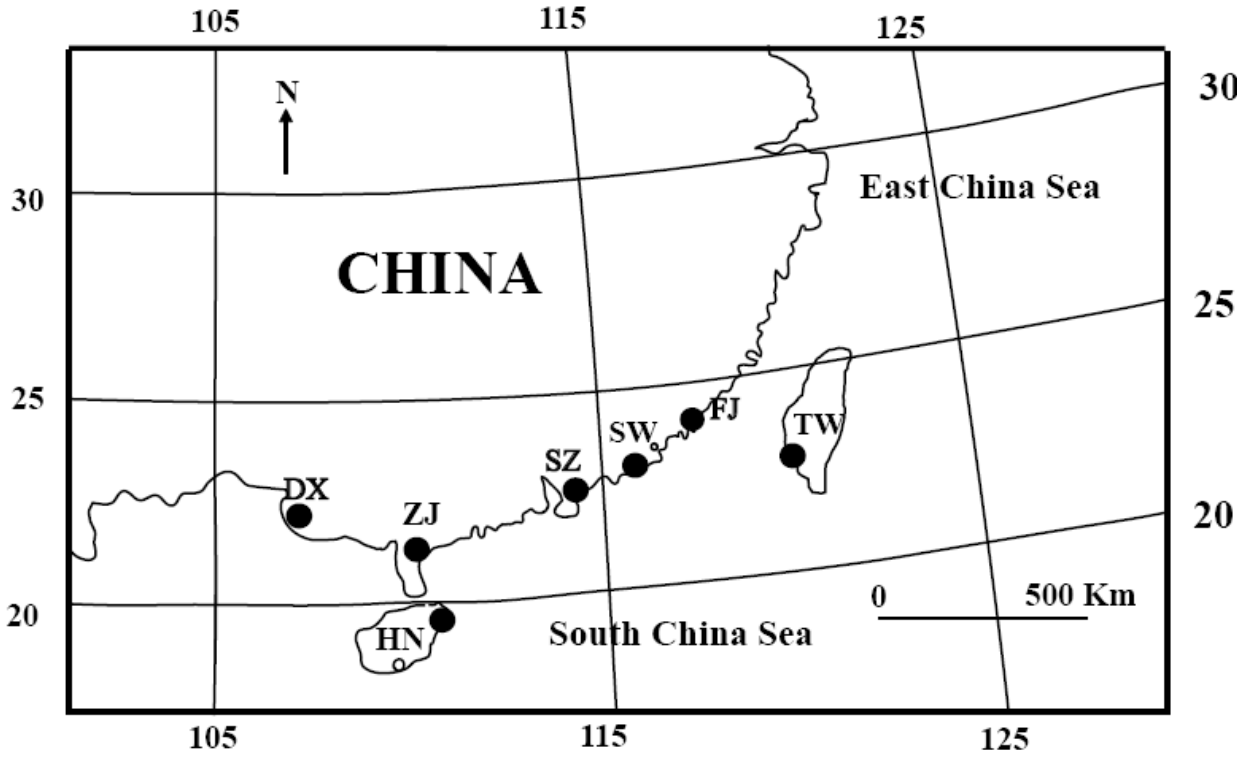

Figure 1. Locations for sample collection of small abalone populations with sample number followed in parentheses. FJ, Dongshan,Fujian Province (n = 22); SW, Shanwei, Guangdong Province (n = 22); SZ, Shenzhen, Guangdong Province (n = 22); ZJ, Zhanjiang, Guangdong Province (n = 22); HN, Wenchang, Hainan Province (n = 22); TW, Tainan, Taiwan Province; DX, Dongxing, Guangxi Province (n = 22).

Table 1. Oligonucleotide adaptors and primers used in AFLP analysis.

Adaptor or primer	Sequence(5'-3')
Adaptor	
EcoRI-1	CTC GTA GAC TGC GTA CC
EcoRI-2	AAT TGG TAC GCA GTC TAC
Mse I-1	GAC GAT GAG TCC TGA G
Mse I-2	TAC TCA GGA CTC AT
Primer of pre-amplification	
EcoRI+1	GAC TGC GTA CCA ATT CA
MseI+1	GAT GAG TCC TGA GTA AC
Primer for selective amplification	
E-AAG	GAC TGC GTA CCA ATT CAA G
E-ACT	GAC TGC GTA CCA ATT CAC T
M-CAA	GAT GAG TCC TGA GTA ACA A
M-CAT	GAT GAG TCC TGA GTA ACA T
M-CTA	GAT GAG TCC TGA GTA ACT A
M-CTG	GAT GAG TCC TGA GTA ACT G
M-CTC	GAT GAG TCC TGA GTA ACT C

(MseI-1/ MseI-2) with a final volume of 40 µl. A pre-amplification PCR reaction were conducted in a 20 µl volume containing 1 µl restriction-ligation mixture, 1U of Taq DNA polymerase (TaKaRa Biotechnology Co., LTD.), 10 × PCR reaction buffer, 1 µl of dNTP (25Mm), 50 ng of each pre-amplification primer (Table 1), with the following PCR program: 2 min at 72°C, 30 cycles with 30 s at 94°C, 30 s at 56°C, 2 min at 72°C; followed by 10 min at 60°C. The pre-amplification PCR product was diluted 50-fold in 10 mM Tris (pH

Table 2. Number of samples, average heterozygosity and percentage polymorphic loci for the seven populations of small abalone *Haliotis diversicolor* across 234 AFLP loci.

Population	Number of sample	Percentage polymorphic loci (95% criterion)	Average heterozygosity (unbiased)
At the population level			
ZJ	22	47.06	0.1270
HN	22	24.51	0.0677
SZ	22	37.25	0.0976
SW	22	36.27	0.0931
TW	20	64.31	0.1665
FJ	22	27.5	0.0811
Average of the six cultured stocks		39.48	0.1055
DX	22	77.65	0.2124
At the species level			
Total	152	68.63	0.1356

7.6) and 0.1 mM EDTA (TLE) buffer as the template of selective amplification. Selective amplifications were performed using five primer pairs; E-AAG/M-CTA, E-AAG/M-CTG, E-ACT/M-CAA, E-ACT/M-CAT and E-ACT/E-CTA, with the following temperature profile: an initial denaturation step of 2 min at 94°C; 12 cycles with 30 s at 94°C, 30 s at 65°C decreasing with 0.7°C per cycle, 2 min at 72°C; 23 cycles with 30 s at 94°C, 30 s at 56°C, 2 min at 72°C; followed by 10 min at 72°C. The method of silver staining followed that of Sanguinetti et al. (1994) with some modifications. Gels were fixed for 10 min with a mixed solution of 10% ethanol and 0.5% acetic acid, and then stained for 30 min with 0.2 g/ml AgNO3 solution. After being washed with distilled water the gels were developed in 1.75 g/ml NaOH solution with 0.4% formaldehyde until the bands noticeably appeared. Finally, the gels were washed with distilled water and air dried.

Clear and unambiguous bands between 150 and 500 bp were visually scored as “1” if present or “0” if absent.

Data analyses

The percentage of polymorphic loci (*P*) and average heterozygosity (*H*) were calculated using the TFPGA program (Miller, 1997). The polymorphic loci were estimated based on the percent of loci not fixed for one allele. Average heterozygosity for each locus was calculated following Nei’s (1978) unbiased heterozygosity formula.

The phenograms of the seven populations were constructed based on unweighted pair-group method UPGMA (Sokal and Michener, 1958) using the TFPGA program (Miller, 1997). The bootstrap values for the UPGMA tree were calculated by 1000 bootstrap re-sampling across loci. To further evaluate the relationships of the 152 individuals, principal coordinates analysis (PCA) based on Nei and Li (1979) distance coefficient was conducted using the software NTSYS-pc 2.02 (Rohlf, 1998).

The φ statistics, which are directly analogous to Wright’s F statistics (Excoffier et al., 1992), were adopted to analyze genetic variation within and between populations. The statistical significance of the total and pairwise φ values was estimated by comparing the observed distribution generated by 10000 permutations using the TFPGA program (Miller, 1997). Hierarchical molecular variance (AMOVA) was evaluated using the molecular variance software package in ARLEQUIN 2.0 (Excoffier et al., 1992). The φ statistics were adopted to analyze genetic variation at various hierarchical levels; φ_{ST} estimates the proportion of genetic variation within populations relative to the genetic variation of the whole sample, φ_{CT} evaluates the proportion of genetic variation among groups of populations relative to the whole species, and φ_{SC} estimates the variation among populations relative to a regional grouping of populations. We conducted hierarchical AMOVA analysis with three groups; samples from DX and TW were considered as the first two groups, while the five populations sample from mainland China were considered as the third group. The statistical significance of φ values was performed using Arlequin version 3.01 program (Excoffier et al., 1992) by comparing the observed distribution generated by 20000 permutations based on observed marker frequencies and assuming linkage equilibrium between loci.

RESULTS

AFLP polymorphism and genetic variation

A total of 234 fragments, with a size range of 150-500 bp, were identified by five AFLP primer combinations, with an average of 46.8 for each primer combination. The percentage of polymorphic loci and the average heterozygosity for each population are summarized in Table 2. Overall, the proportion of polymorphic loci and heterozygosity of all populations were 68.63% and 0.1356, respectively. The proportion of polymorphic loci and heterozygosity of the natural population (DX) were 77.65% and 0.2124, respectively. For the cultured stocks, the percentage polymorphic loci varied from 24.51 to 64.31% with an average of 39.48%, and the heterozygosity ranged from 0.0677 to 0.1665 with an average of 0.1055. TW stock had the greatest percentage of polymorphic loci (64.31%) and heterozygosity (0.1665), while HN stock exhibited the lowest percentage of polymorphic loci (24.51%) and heterozygosity (0.0677).

Population structure

The genetic differentiation coefficient among populations (φ_{ST}) was 0.388 ($P < 0.0010$), which indicated that there

Table 3. Hierarchical analysis of molecular variance (AMOVA) of small abalone *Haliotis diversicolor* based on AFLP phenotypes.

Source of variation	df	Sum of squares	Mean squares	Variance component (absolute)	Variance component (%)	φ statistic
Among groups	2	279.92	139.96	3.73	26.63	φ_{ct} =0.266**
Among populations within groups	4	64.31	16.08	0.28	1.97	φ_{st} =0.286**
Within populations	145	1449.30	10.00	10.00	71.39	φ_{sc}=0.027**
Total	151	1793.53				

df, Degree of freedom; $^{**}P < 0.01$.

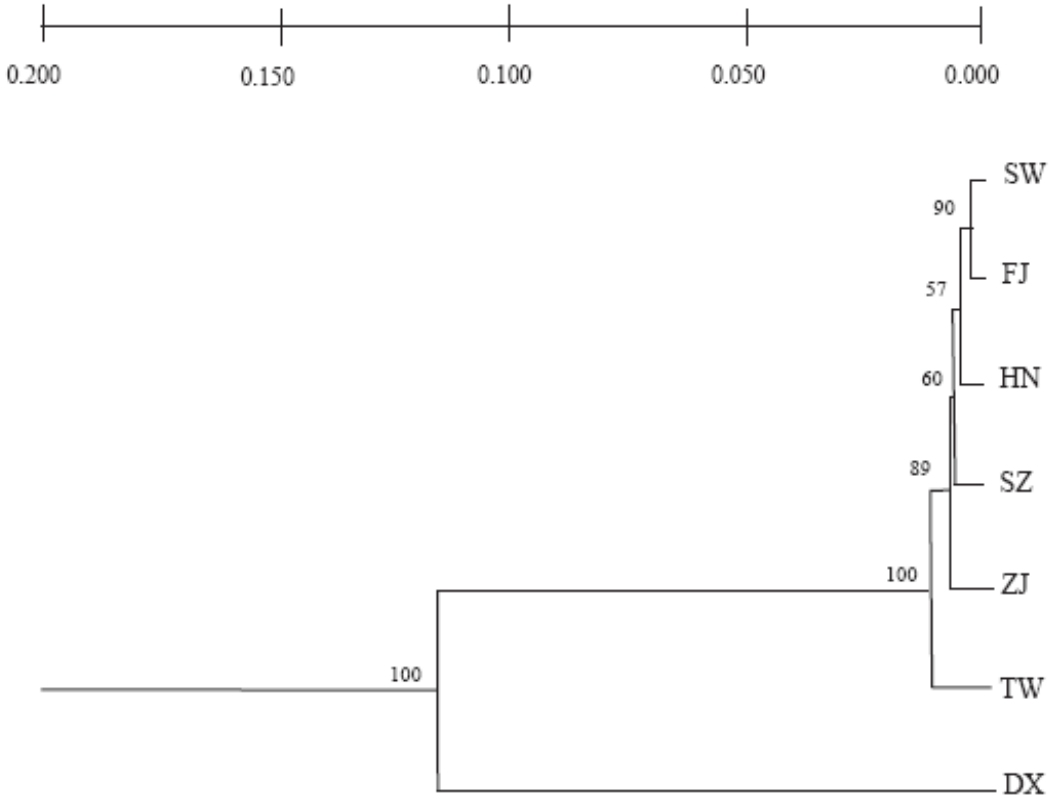

Figure 2. The UPGMA dendrogram showing the phylogenetic relationship among the seven populations of small abalone *Haliotis diversicolor*. The scale represents Nei's (1978) genetic distance. Significant bootstrapping value is indicated.

was highly significant genetic difference among the seven populations. Excluding the natural population (DX), the estimated φ_{ST} value was 0.056 (P <0.0010), indicating a significant population structuring among cultured populations. Significant genetic differentiation among the three groups was detected by hierarchical AMOVA tests, with 26.63% of genetic variation (P < 0.01). A small but significant (P < 0.01) value (1.97%) of genetic divergence was also found among populations within groups (Table 3).

The pairwise φ_{ST} values ranged from -0.0092 to 0.3905 (Table 4), the largest been between the DX and HN populations, and the lowest between the FJ and SW populations. Statistical tests indicated that both DX and TW populations were significantly genetically different from the other populations (P < 0.01). For the five hatchery stocks sampled from mainland China coast, however, genetic differences were not significant (P > 0.01), except between SZ and ZJ, SZ and HN stocks.

Cluster analysis

The UPGMA dendrogram based on Nei's (1978) genetic distance matrix is showed in Figure 2. In the dendrogram, the natural population appeared basal position, forming a

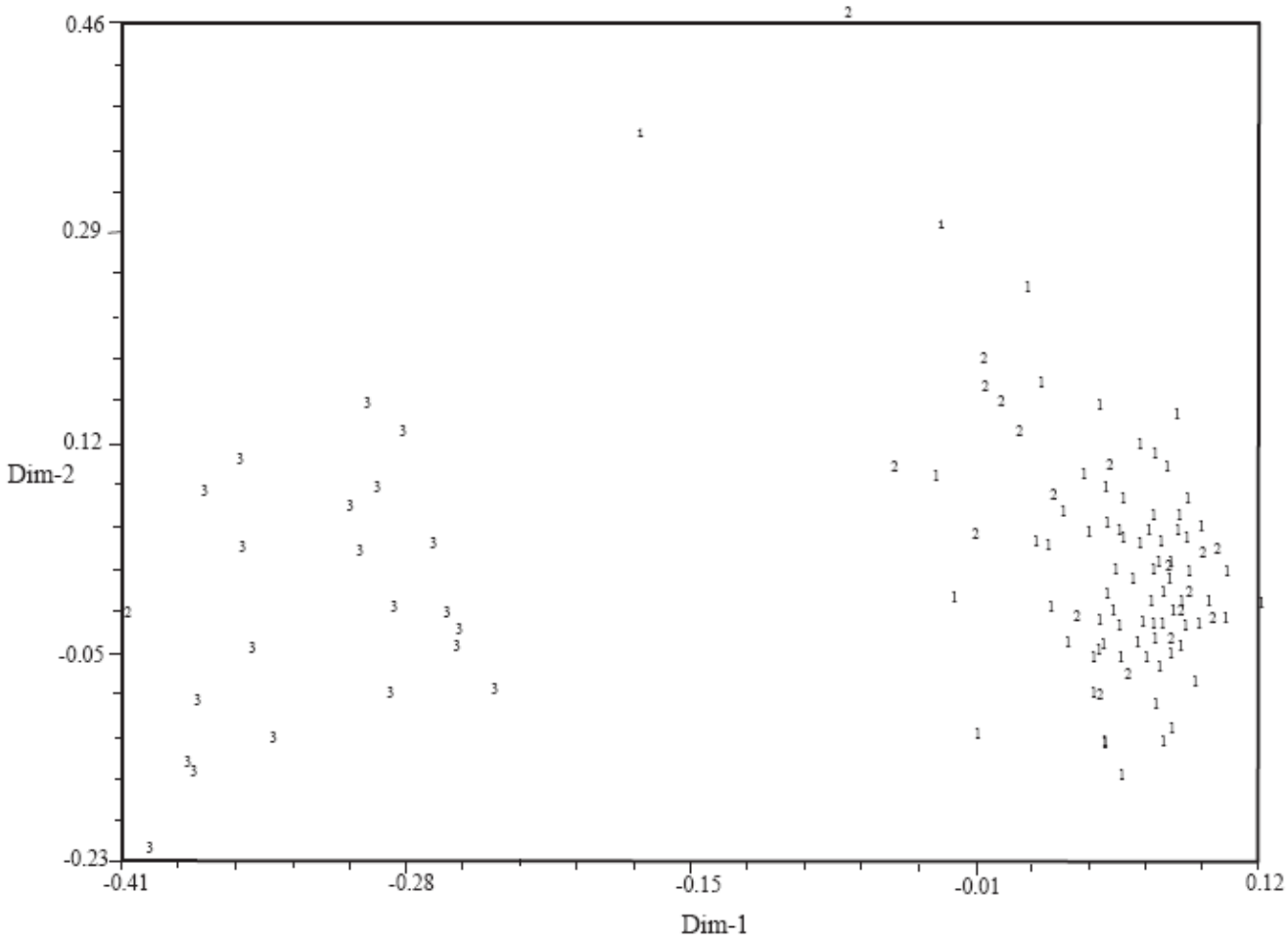

Figure 3. Principal coordinates analysis (PCA) using the Nei and Li (1979) distance coefficient computed from AFLP data collected from 152 individuals of small abalone *Haliotis diversicolor*. The proportion of total variance comprising each axis was 13.4% for axis 1 and 6.8% for axis 2. Numbers refer to populations as: 1, SW, FJ, HN, SZ, ZJ; 2, TW; 3, DX.

Table 4. Pairwise φ_{ST} values of small abalone *Haliotis diversicolor* based on AFLP data.

Population	ZJ	HN	SZ	SW	TW	FJ	DX
ZJ	*****						
HN	0.0567	*****					
SZ	0.0592**	0.0761**	*****				
SW	0.039	0.0379	0.0416	*****			
TW	0.0566**	0.1002**	0.0835**	0.0677**	*****		
FJ	0.0544	0.0444	0.0102	-0.0092	0.0863**	*****	
DX	0.3111**	0.3905**	0.3503**	0.3496**	0.2543**	0.3744**	*****

** $P < 0.01$.

separated cluster from the six cultured populations. The six cultured stocks clustered together with a bootstrapping confidence value of 100% and within this cluster, the five cultured stocks sampled from mainland China coast grouped together.

The result of principal coordinates analysis (PCA) using the Nei and Li (1979) distance coefficient is shown in Figure 3. The proportion of total variance comprising each axis was 13.4% for axis 1 and 6.8% for axis 2. The cluster status of individuals of each population was consistent with the UPGMA dendrogram. All individuals of natural population were concentrated in one large cluster. Most individuals of the cultured stocks clustered together and individuals showed similar genetic

distances, though some individuals from TW showed larger genetic distances from this main cluster.

DISCUSSION

In this study, genetic analysis of diversity among and within six hatchery and a natural populations of small abalone *H. diversicolor* in China was conducted using AFLP markers. The results show a significantly reduced genetic diversity in the hatchery stocks when compared with the wild population. For a long period, the problem of maintaining adequate levels of genetic variability within the hatchery stocks of small abalone was overlooked, or procedures were not undertaken to prevent its reduction, which may have led to the genetic diversity status of this species in China. The breeding program of *H. diversicolor* has been carried out in China for several decades, and hatchery cultured stock has been used as broodstock to produce the next cultured generation every year. The high fecundity of mature abalone and artificially high survival rate of juvenile abalone in a cultured environment ensured sufficient seed for each year's production. However, the culture practices such as mass spawning, use of uneven broodstock sex ratios, differential larval/juvenile survival during metamorphosis and size-based grading, all have the potential to drastically reduce the level of genetic variation remaining in the hatchery populations. Additionally, *H. diversicolor* is a highly fecund broadcast spawning species and the possibility exists that a few individuals produce the majority of offspring in a given generation.

Such closely related cohorts are more likely to be subsequently chosen as broodstock to produce the next generation, which can lead to inbreeding, and subsequently reduce the genetic diversity of the cultural populations. The accumulation of genetic diversity in natural populations is very slow. However, if appropriate precautions are not implemented throughout the culture process, this diversity can be lost in as little as a single generation (Porta et al., 2007). Loss of genetic diversity in three hatchery produced stocks of *H. diversicolor* in China has been reported (Li and Yan, 2009), which is consistent with our results. Similar results have been shown in many other aquaculture species including fish (Danzmann et al., 1988; Frost et al., 2006), crustaceans (Xu et al., 2001; De Lima et al., 2008) and other molluscs (Li et al., 2007b; Lind et al., 2009; Slabbert et al., 2009).

It is generally believed that declines in variation at neutral markers may be indicative of a loss of variation at coding regions. Minimizing this reduction is important to aquaculture, as this is the source of variation in important commercial traits such as growth rate and disease resistance (Vuorinen, 1984). Though selection cannot be ruled out in influencing our AFLP data, much evidence indicates that typically <5% of AFLP loci are under selective pressure (Campbell et al., 2003; Murray and Hare, 2006). The distinct reduction in genetic variation in the hatchery stocks detected in this study may hint that mass mortality of *H. diversicolor* in recent years might be related to the reduction in genetic diversity.

Reductions in genetic variation have been shown to be detrimental to commercially important traits such as growth rate (Koehn et al., 1988) and fitness (Danzmann et al., 1989) in other marine organisms. In addition, understanding the status of genetic diversity is critical to the long-term viability of abalone breeding programs, especially as breeders attempt to develop selected lines in hatcheries with small effective population sizes. The genetic status showed that, excluding TW and ZJ stocks, the other cultured populations may not be suitable as source populations for selective breeding programs.

AMOVA analysis, pairwise comparison of genetic distance and φ_{ST} values, and cluster analysis demonstrated significant genetic difference between natural and hatchery populations. These are consistent with the results that the hatchery stocks were characterized with lower percentage of polymorphic loci and heterozygosity, while the natural population showed much higher genetic diversity indexes. Owing to population bottleneck and genetic drift during the breeding practice, huge genetic divergence between hatchery stocks and natural populations of many other shellfish species had been observed (Arnaud-Haond et al., 2003; Yu and Chu, 2006; Li et al., 2007b; Praipue et al., 2010).

Conceptually, the formation of each hatchery group is similar to a mainland-island model of genetic structure, where genetic drift and migration between different hatchery groups (sink populations) will determine the degree of genetic divergence between different sinks (Frankham et al., 2002). If there is no migration between different sinks, it is likely that genetic drift will strengthen the differences in genetic structure between hatchery groups over several generations.

For the hatchery stocks studied, TW population was significantly genetically different from the other stocks, which was consistent with the mainland-island model of genetic structure. For the five hatchery stocks sampled from mainland China coast, genetic differences were not significant (P>0.01), except between SZ and ZJ and SZ and HN stocks. As an ecologically important species, *H. diversicolor* has been cultured in coastal areas of Southern China for several decades. The juveniles for aquaculture and broodstock for seedling production were released among different abalone aquaculture areas. The cross or gene flow between different hatchery stocks, similar to the migration between different sinks in mainland-island model, may contribute to low genetic difference of hatchery *H. diversicolor* stocks in mainland China. Our results were inconsistent with previous findings in RAPD survey (Du et al., 2007). Contrary to our results, low genetic differentiation between wild and hatchery populations of *H. diversicolor* in China were

detected by AFLP fingerprinting (Li and Yan, 2009).

The basic information on genetic population differentiation and levels of genetic diversity of *H. diversicolor* stocks is necessary to improve the stock selection program and establish an effective selective breeding program for this species.

According to our results, four of the five cultured stocks in mainland China (HN, SW, SZ, and FJ) may not be suitable as source populations for selective breeding programs; ZJ, TW stock, some natural populations and stocks introduction outside China should be considered. Unfortunately, sampling the wild population was very difficult during our experiment, and wild populations could hardly be found in most of its traditional habitats. In addition, there are many contradictions among the present results and previous findings (Du et al., 2007; Li and Yan, 2009) for the genetic survey of hatchery *H. diversicolor* stocks. This incongruence is simply ascribable to the different molecular markers and the sample sources. The situation of *H. diversicolor* aquaculture industry in mainland China was in chaos; no one knew the exact origin of parents and the seed stocks. Therefore, additional sampling and using more other molecular markers will be helpful to understanding the genetic diversity status of *H. diversicolor* in China.

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