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AFLP ANALYSIS OF POPULATIONS OF *HALIOTIS DISCUS HANNAI*, *HALIOTIS GIGANTEA*, AND THEIR HYBRIDS

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ABSTRACT Amplified fragment length polymorphism (AFLP) analysis of the population genetic structure and genetic diversity of *Haliotis discus hannai* (D), *Haliotis gigantea* (G), and their reciprocal hybrids D ♀ × G ♂ (DG) and G ♀ × D ♂ (GD) was carried out in this study. A total of 479 unambiguous and highly repeatable AFLP markers, 311 of which (64.93%) were polymorphic, were obtained using 7 primer combinations. The reciprocal hybrids inherited bands from both parents, indicating that the hybrids were truly heterogeneous. The Shannon diversity index for D, G, and their reciprocal hybrid populations DG and GD was 0.169 ± 0.188, 0.211 ± 0.227, 0.236 ± 0.267, and 0.231 ± 0.242, respectively. Analysis of molecular variance revealed that 29.58% of the variance was among populations, whereas 71.42% of variance was within populations. Genetic distance was maximum (0.681) between D and G, and was minimum (0.482) between GD and G. The 4 populations were clustered into 2 major clades using the unweighted pair group method with arithmetic mean. All genetic parameters indicated that there was plentiful genetic diversity in the reciprocal hybrids of D × G. Results of this study suggest that these AFLP markers can be used in the future to enhance current breeding practices in abalone culture because of the large numbers of polymorphic markers.

KEY WORDS: abalone, *Haliotis discus hannai*, *Haliotis gigantea*, hybridization, AFLP analysis

INTRODUCTION

Molecular markers, such as allozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat, intersimple sequence repeat, and DNA sequence, have proved to be very efficient means to investigate the population genetics of aquatic animals (Apte et al. 2003, Besse et al. 2004, Liu & Cordes 2004, Xia & Jiang 2006). In contrast to many types of molecular markers, AFLP is a recently developed system that combines the specificity of restriction enzyme analysis with the relative technical simplicity of the polymerase chain reaction, and combines the strengths and overcomes the weaknesses of the RFLP and RAPD methods (Vos et al. 1995). AFLPs have been used to fingerprint accessions, to determine genetic linkage mapping, and to analyze genetic diversity (Young et al. 1998, Liu et al. 1999, Liu et al. 2003, Liu et al. 2005, Wang et al. 2007). They have the advantages that they are easy to use, sample a large number of loci per reaction, and are reproducible between laboratories. As an ideal marker system for resolving genetic structure among individuals, populations, and species, they are frequently used in the field of molecular ecology and evolution (Bensch & Åkesson 2005).

The Pacific abalone *Haliotis discus hannai* Ino is an economically important gastropod species distributed in the coastal waters of China, Japan, and Korea. It has become a commercially important species because of its high economic value, as well as its large-scale cultivation since the late 1980s in China, where it has entered a stage of rapid development during the past 2 decades (Guo et al. 1999).

Haliotis gigantea, which is called Xishi abalone in China, is a valued commercial species along the coast of Japan. This species was introduced from Japan to China for mariculture in 2003 (Luo et al. 2006). The meat of *H. gigantea* is crisp and tender, and this species has excellent disease resistance (Gao et al. 2000). These traits make it an excellent potential species for abalone mariculture in China, and now it is intensively cultivated.

A genetic improvement program has been initiated in China with the goal of producing an improved hybrid abalone for commercial aquaculture and production. The hybrids have remarkable heterosis and have been cultured on a commercial scale. To initiate the improvement effort, a basic understanding of the genetic relationships among the hybrids and their parents was considered essential. In this article, we report the results of a genetic analysis of the interspecific hybrids produced by *H. discus hannai* and *H. gigantea* based on AFLP analysis.

MATERIALS AND METHODS

Samples

The broodstock of *H. discus hannai* (D) and *H. gigantea* (G) came from Nagasaki, Japan, and Dalian, China, respectively. Each sample was transported to the Jiefeng Abalone Farm in Fuzhou City, China.

In this study, a random sample of 40 mature males and 60 mature females from each of the 2 species was selected for spawning. A 2×2 factorial design was made between populations of D and G, resulting in 4 distinct groups: 2 reciprocal crosses *H. discus hannai* ♀ × *H. gigantean* ♂ (DG) and *H. gigantean* ♀ × *H. discus hannai* ♂ (GD) (crossing between 2 parental populations), and 2 parental groups D ♀ × D ♂ (DD) and G ♀ × G ♂ (GG) (crossing within each parental population). The reciprocal hybrids and the progeny of the purebred were reared in tanks separately to prevent intermixing. Live juvenile abalones ($n = 30$) of each cross were randomly sampled at 4 mo and used for AFLP analysis.

DNA Extraction

Total genomic DNA of each abalone was isolated from pedal muscle tissue by digesting it in 50 mM Tris-HCl, 100 mM

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ethylenediamene tetraacetic acid (pH, 8.0), 1% sodium dodecyl sulfate and 100 μg/mL of proteinase K at 55°C for 3 h, followed by 1 organic extraction with phenol and 1 with phenol:chloroform (v/v, 1:1). DNA was precipitated using ethanol, washed with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl and 1 mM ethylenediamene tetraacetic acid; pH, 8.0). The quality of extracted DNA was assessed using 1% agarose gel electrophoresis, the DNA concentration was measured with an ultraviolet spectrophotometer (Hitachi U-2001, Tokyo, Japan), and then the samples were stored at 4°C until use.

AFLP Analysis

AFLP analysis was performed using the protocol of Vos et al. (1995) with minor modifications as reported in Wang et al. (2004). Genomic DNA of each sample was double digested with restriction enzymes *Eco*R I and *Mse* I. The digested DNA fragments were ligated with *Eco*R and *Mse* adaptors (*Eco*R-F, 5′-CTCGTAGACTGCGTACC-3′; *Eco*R-R, 5′-AATTGGTACGCAGTCTAC-3′; *Mse*-F, 5′-GACGATGAGTCCTGAG-3′; *Mse*-R, 5′-TACTCAGGACTCAT-3′) using T4 ligase. The ligated DNA fragments were preamplified with an E-primer (5′-GACTGCGTACCAATTA-3′) and an M-primer (5′- GACTGCGTACCAATTA-3′). Seven primer combinations (E-AAG × M-CAG, E-AGG × M-CTG, E-AGC × M-CTT, E-ACG × M-CTC, E-ACG × M-CTG, E-AAG × M-CAA, E-AAC × M-CTA) were selected for selective amplification. The final polymerase chain reaction product was run on a 6% denaturing polyacrylamide gel in 1× TBE buffer. Analysis was carried out using silver staining of the gel (Sanguinetti et al. 1994) and overnight drying before samples were photographed.

The banding patterns of AFLP were compared between the conspecific groups and the hybrids with a consistent pattern in all the lines. AFLP band polymorphisms in reciprocal hybrids were also evaluated by scoring the presence or absence of fragments.

To test the reproducibility of AFLP fingerprints, all the protocol steps were performed twice independently on each sample, using 25 ng and 250 ng of genomic DNA.

Data Analysis

Unambiguous AFLP bands were scored for presence (1) or absence (0) of fragments between 50 bp and 500 bp, and then assembled in a 0/1 binary data matrix table. The data matrix was analyzed for population genetic diversity using the POPGENE software package (Yeh et al. 1999) in 3 ways: (1) the percentage of polymorphic loci (*P*), (2) Nei's gene diversity (*H*), and (3) Shannon's index of phenotypic diversity (Lewontin 1972).

Genetic distances between populations were calculated using Nei's (1978) unbiased distance and similarity measures. The percentages of polymorphic loci were estimated based on the percent of loci not fixed for 1 allele. Analysis of molecular variance (AMOVA) was conducted to calculate variance components and their significance levels for variation among hybrids and their parental populations using the AMOVA program version 1.55 (Excoffier 1993). Genetic population relationships were estimated by constructing an unweighted pair group method with arithmetic mean (UPGMA) tree based on Nei's standard genetic distance (Nei 1978).

RESULTS

AFLP Polymorphism

The 7 primer pair combinations used for AFLP analysis generated a total of 479 AFLP markers in the interval of 50–500 bp, of which 311 were polymorphic (64.93%). The number of markers generated varied from 58–78, and the percentage of polymorphic fragments ranged greatly from 35.06% (in DD cross) to 79.31% (in DG cross; Table 1). Figure 1 shows the AFLP fingerprint of conspecific and heterospecific crosses generated by using the primer combination E-AGC × M-CTT. The profiles of D and G indicated that they were relatively diverse, and each of them holds species-specific bands. Interspecific hybrid progenies showed in-between patterns to their parents in that species-specific bands were segregated. The specific bands from both D and G coexisted in hybrids of DG and GD crosses. Some of these bands are indicated in Figure 1.

Genetic Diversity Analysis

The observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's (1978) gene diversity (*H*), and Shannon's information index (*I*) of conspecific and heterospecific crosses are shown in Table 2. The values of *Na* ranged from 1.176–1.243 and *Ne* from 1.249–1.387. The population with the greatest percentage of polymorphic loci (63.91%), the highest gene diversity (0.131), and Shannon's information index (0.236) was DG. In parental populations, the lowest gene diversity and Shannon's information index were in DD, and they were 0.102 and 0.169, respectively. However, the percentage polymorphic loci of the D population (50.17%) was higher than the G population (49.18%).

The similarity indices and genetic distances among 4 crosses based on AFLP banding patterns are shown in Table 3. The similarity indices between DG and GD crosses were higher (0.587) than DD and GG groups (0.436). However, the genetic distances of reciprocal hybrids (0.482) were lower than conspecific groups (0.681). Genetic distance was maximum (0.681) between D and G, and was minimum (0.434) between GD and G. It was evident that these 2 parental species were genetically distinct, and it also suggested that the reciprocal hybrids, DG and GD crosses, were both close to G.

TABLE 1.

Number of AFLP loci and polymorphisms detected using 7 AFLP primer combinations in hybrids and parental crosses.

Primer Combinations	Total No. of Bands	Polymorphic Bands *n*				Polymorphic Bands %			
		DD	GG	DG	GD	DD	GG	DG	GD
E-AAG × M-CAG	71	43	46	52	54	60.56	64.79	73.24	76.06
E-AGG × M-CTG	78	46	47	57	62	58.97	60.26	73.08	79.49
E-AGC × M-CTT	58	38	35	46	42	65.52	60.34	79.31	72.41
E-ACG × M-CTC	63	31	28	36	37	49.21	44.44	57.14	58.73
E-ACG × M-CTG	63	26	23	36	31	41.27	36.51	57.14	49.21
E-AAG × M-CAA	77	27	31	37	33	35.06	40.26	48.05	42.86
E-AAC × M-CTA	69	28	26	41	39	40.58	37.68	59.42	56.52
Mean						50.17	49.18	63.91	62.18

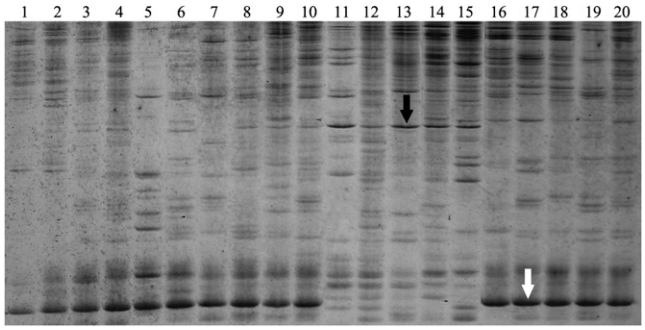

Figure 1. Representative AFLP profiles of *H. discus hannai*, *H. gigantea*, and interspecific hybrids generated by primer combination E-AGC × M-CTT. 1–5, F1 hybrids of GD cross; 6–10, F1 hybrids of DG cross; 11–15, F1 of DD cross; 16–20, F1 of GG cross. Black arrow indicates *H. discus hannai*-specific bands; white arrow indicates *H. gigantea*-specific bands.

The data from AMOVA were used to determine the variations of reciprocal hybrids and the differentiation between hybrids and their parental groups. AMOVA indicated that more variation (70.42%) was accounted for by differentiation within populations, with only 29.58% accounting for variation among populations, and there were significant differences ($P < 0.01$) under the significance tests with 1,000 permutations (Table 4).

Cluster Analysis

The dendrogram obtained from UPGMA cluster analysis of genetic distance based on 7 AFLP primer combinations is presented in Figure 2. The hybrid crosses and their parental groups were clustered into 2 major clades. Group 1 constituted only the DD cross and group 2 contained GG, DG, and GD crosses. The DG cross formed a separate cluster in group 2 and, therefore, group 2 could be further divided into 2 clusters.

DISCUSSION

DNA markers have proved to be a robust and cost-effective technology for assessment of genetic diversity in marine animals (Young et al. 1998, Barman et al. 2003, Zhu et al. 2006, Wang et al. 2007). Recently, the AFLP technique, which generates a large number of polymorphic markers, has been seen as one of the most powerful approaches for the assessment of genetic variation among populations, especially for species with a molecular genetic background that was not previously available (Lucchini 2003, Wang et al. 2004). In this study, genetic analysis of the reciprocal hybrid populations produced by D × G was conducted using AFLP markers. Our results, based on 7 AFLP primer sets, suggested that hybridization between D and G could occur in the same culture environment. We isolated some of the AFLP bands specific to D and to G, and diagnostic bands could be identified from both. The reciprocal hybrids induced by D and G resulted in the presence of parent-specific bands, which were inherited from both parents based on the AFLP profiles. These markers will be most useful for genetic identification and breeding studies in abalone aquaculture.

The average Nei genetic diversity of DD, GG, DG, and GD populations was 0.102, 0.116, 0.131, and 0.123, respectively, and the percentages of polymorphic loci were 50.17%, 49.18%, 63.91%, and 62.18%. The level of AFLP of hybrid populations was similar to that in catfish (Liu et al. 1998). The results also showed that the genetic diversity of hybrids was relatively higher than that of parental populations. This is important, because genetic diversity lays the foundation for aquatic animals to survive and adapt to changing environments, and is critical for adaptation to environmental changes and for the long-term survival of species. Understanding of genetic diversity and its causes can provide insight into ecological and evolutionary histories; and meanwhile, such information also may make for conservation and restoration (Hamrick & Godt 1996, Avise 2000). Increasing genetic variation also results in promoting the ability of creatures to adjust to changing living conditions. AFLP analysis on D and G showed that they were genetically distinct. It also revealed that these two parental species had a genetic basis of heterosis for their hybrids. Hybridization between D and G may cause the increasing heterozygosities of hybrids. Furthermore, the increase of genetic variation through hybridization was probably related to heterosis, because the hybrids showed fast growth and low mortality in aquaculture. It was also found that hybrids had high heterozygosity and showed heterosis (Ferdinandez & Coulman 2002).

The results of the UPGMA dendrogram indicated that the reciprocal hybrids were close to G. For instance, GD and GG clustered in the same group, whereas the DG cross formed a separate cluster. This was consistent with the results that more

TABLE 2.

Population genetics parameters for 4 populations of reciprocal hybrids and their parental groups.

Population	*Na*	*Ne*	*H*	*I*
DD	1.176	1.263	0.102	0.169
GG	1.183	1.249	0.116	0.211
DG	1.243	1.387	0.131	0.236
GD	1.231	1.384	0.123	0.231

H, Nei's gene diversity; *I,* Shannon's information index; *Na,* observed number of alleles; *Ne,* effective number of alleles.

TABLE 3.

Similarity indices (above diagonal) and genetic distances (below diagonal) between 4 crosses based on AFLP banding patterns.

Population	DD	GG	DG	GD
DD		0.436	0.521	0.534
GG	0.681		0.548	0.618
DG	0.638	0.568		0.587
GD	0.563	0.434	0.482	

TABLE 4.

Analysis of molecular variance within and among the populations of hybrids and parental groups.

Sources of Variation	df	Square Sum	Variance Components	Percentage	*P* Value
Among populations	3	463.342	17.79	29.58	<0.01
Within populations	119	1,728.218	42.35	70.42	<0.01

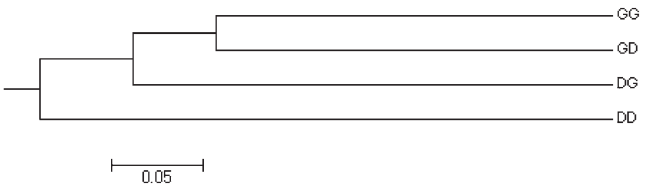

Figure 2. The UPGMA dendrogram based on AFLP data of *H. discus hannai*, *H. gigantea*, and their hybrids.

loci were shared by F1 hybrids and G, and indicated that the genetic differences between hybrids and their two parent species were not equal, but that they were more similar to G.

In conclusion, the AFLP markers clearly revealed the differences between the reciprocal hybrids and their parents. Our results also demonstrated the feasibility and practicality of using AFLP markers for genetic analysis, which will aid in future hybrid abalone breeding projects.

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