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ALLOGYOGENETIC PROGENY ARE PRODUCED FROM A HYBRID ABALONE CROSS OF FEMALE *HALIOTIS DIVERSICOLOR* AND MALE *HALIOTIS DISCUS DISCUS*

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ABSTRACT Interspecific hybrid families of female *Haliotis diversicolor* × male *H. discus discus* were produced and analyzed using amplified fragment length polymorphism (AFLP) technology to reveal the genetic makeup of F1 progenies. The survival rates of the hybrid F1 were very low, ranging from 0–0.13%. Twenty hybrid F1 from 3 families along with 3 different female parents and their common male parent were analyzed with 3 AFLP primer combinations. In total, 266 markers were detected. Genetic relationships among the progenies and the parents were evaluated by generating a similarity and genetic distance matrix. The genetic divergence between *Haliotis diversicolor* and *Haliotis discus* was at a high level, with genetic distance ranging from 1.471–1.492. The AFLP band patterns of hybrid F1 progeny were similar to those of the female parents, but were quite different from that of the male parent. The mean genetic distance between hybrid F1 and their female parents were 0.024–0.039, slightly less than that among the female parents, which indicates that the hybrid F1 shared high genetic similarity with their female parents, *Haliotis diversicolor*. However, 0–0.8% of total AFLP bands of each individual were found to be parental bands, and 0–3.3% were found to be nonparental bands. The possible reason for the presence of paternal-specific and nonparental bands is discussed.

KEY WORDS: abalone, interspecific hybridization, allogynogenesis, AFLP, *Haliotis diversicolor*, *Haliotis discus discus*

INTRODUCTION

Interspecific hybridization can be carried out to obtain broodstocks with combined traits of both parental species to support aquaculture. However, true hybrids are not always produced from interspecific hybridization. For example, in fish, gynogenesis, androgenesis, or karyogamy may result from heterospecific insemination (Chevassus 1983). A similar phenomenon has also been reported in molluscs. Jiang et al. (1983) and Li et al. (1983) proved that the karyotype and zygrams of the F1 individuals of *Pinctada fucata* × *P. chemnitzi* and *P. fucata* × *P. maxima* were similar to the female parent, which indicated that the F1 in both crosses may be gynogens (Jiang et al. 1983, Li et al. 1983).

Among the variety of molecular tools useful to detect outcross, amplified fragment length polymorphism (AFLP) (Vos et al. 1995) is popular for its convenience and informativeness. The application of AFLP markers is not restricted to species and does not require any prior genetic knowledge of the studied species. In addition, given the large number of polymorphic fragments that can be obtained with a relatively limited number of primer combinations, this method has been successfully used to survey the contribution of parental genomes and gene introgression in natural and artificial hybridization (O'Hanlon et al. 1999, Munoz et al. 2004, Hua et al. 2006).

The abalone *Haliotis diversicolor* and *H. discus discus* are 2 economically important species cultured in China. They spawn in different seasons, with an overlap when the water temperature is about 20–25°C. Ke et al. (2000) carried out interspecific hybridization between *H. diversicolor* and *H. discus discus* and produced viable F1 progeny. The preliminary results from observations showed that F1 in the *Haliotis diversicolor* × *H. discus discus* cross were not intermediate, but were more similar to the female parent. To verify the genetic makeup of F1 of the *H. diversicolor* × *H. discus discus* cross, we produced hybrid cross families between female *H. diversicolor* and male *H. discus discus*, and analyzed the genetic relationship among the families and their parents with AFLP.

MATERIALS AND METHODS

Broodstock and Crosses

Parent abalone used in the crosses were obtained from the Haitian Abalone Farm in Dongshan Island, Southeast China. *H. diversicolor* and *H. discus discus* were originated from coastal China and Japan, respectively, and have been reproduced at the farm for several generations since the 1990s. Paired matings were done at the Haitian Abalone Farm in August 2004. Each parent abalone was allotted to separated basins and was artificially induced to spawn with air exposure and ultraviolet-irradiated seawater. The spawning induction of *H. diversicolor* began 3–5 h earlier than that of *H. discus discus,* because *H. discus discus* is more sensitive to the induction. Semen of *H. discus discus* were collected and stored by refrigeration before insemination. As soon as the eggs were spawned, they were collected and immediately inseminated with semen of *H. discus discus*. A sample of unfertilized eggs was taken to ensure no independent cleavage or contamination of the sperm from the same species occurred. The fertilized eggs were rinsed with seawater every hour. After hatching, the larvae were collected and transferred to 200-L tanks equipped with diatom films, and cultured in seawater with 20 mg/L penicillin–streptomycin before larval settlement. Five families with different female parents and a common male were produced. Seawater was renewed every day after larval settlement. Adductor muscle samples were collected from each parent and fixed in 95% ethanol. The F1 progenies were sampled and fixed when the shell length was about 1 cm. The hybrid families were denoted as HF408091–HF4080095 according to the date and the

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sequence of larvae hatching-out, and the female parents were denoted as FP 408091–408095, respectively. The hybrid progenies in each family were observed and counted during development, including the early gastrula stage (6 h after insemination), before they were transferred into the rearing tank (22 h after insemination), and when they were sampled (2 mo after insemination). The fertilization rate and the viability at sampling time were calculated as follows:

$$\text{Fertilization rate} = \text{No. of developed embryos/No. of total eggs} \times 100\%$$

$$\text{Viability} = \text{No.of surviving individuals/No. of larvae put into the rearing tank} \times 100\%$$

DNA Extraction

Genomic DNA was extracted from 10–20 mg adductor muscle using the phenol–chloroform–isoamyl alcohol method (Sambrook et al. 1989).

Analysis of AFLP Markers

The standard AFLP procedure established by Vos et al. (1995) was essentially followed with minor modifications to generate the markers in the current study. One hundred nanograms of genomic DNA from each sample was double digested with restriction enzymes *Eco*R I and *Tru*1I (MBI Fermentas, Vilnius, Lithuania). The digested DNA fragments were ligated with the specific adaptors using T4 ligase (Promega, Madison, WI), and ligated DNA fragments were preamplified with an E-primer (5′-GACTGCGTACCAATTA-3′) and an M-primer 5′- GACTGCGTACCAATTA-3′. AFLP analysis (selective amplification) was then performed using primers with an additional dinucleotide extension. Three primer sets, E-AAC/M-CTA, E-AAG/M-CAA, and E-AGC/M-CTT, were used in this study. Amplified fragments were separated by 6% denaturing polyacrylamide gel electrophoresis in a Sequi-Gen GT/PowerPac 3000 System (BioRad, Hercules, CA) and visualized by silver staining (Wang et al. 2004).

Data Analysis

Each fragment was considered a dominant locus with 2 states: presence or absence. AFLP bands were scored manually, with 0 for absence and 1 for presence, and then transformed into a 0/1 binary matrix. Pairwise similarities (S_{AB}) and genetic distances (D_{AB}) were calculated based on the binary matrix with a Microsoft Excel-based Basic program (AFLP Data Analyser, version 1.3) (Wang et al. 2004). S_{AB} and D_{AB} for shared DNA fragments between 2 individuals A and B was calculated as follows (Lynch 1990):

$$S_{AB} = 2N_{AB}/(N_A + N_B) \quad (1)$$

$$D_{AB} = -lnS_{AB} \quad (2)$$

where N_{AB} is the number of DNA fragments in common between individuals A and B, and N_A and N_B are the total number of fragments possessed by individuals A and B.

TABLE 1.

Fertilization rates and viability at the age of 2 mo of F1 progenies from hybrid families of female *H. diversicolor* × male *H. discus discus*.

No. of Family	Fertilization Rate (%)	Viability (%)
408091	5.3	0.00
408092	14.6	0.11
408093	4.9	0.15
408094	5.5	0.09
408095	30.4	0.02

If the number of F1 equals n, the mean similarity within the families ($\overline{S}$) was computed as

$$\overline{S} = \frac{\sum S_{AB}}{n} \quad (3)$$

Cluster analysis based on UPGMA (unweighted pair group method with average means) and the dendrogram were conducted using Mega 3.1 (Kumar et al. 2004) based on the distance matrix.

RESULTS

Fertilization Rate and Viability

Five hybrid families between female *H. diversicolor* and male *H. discus discus* were produced in August 2004. The fertilization

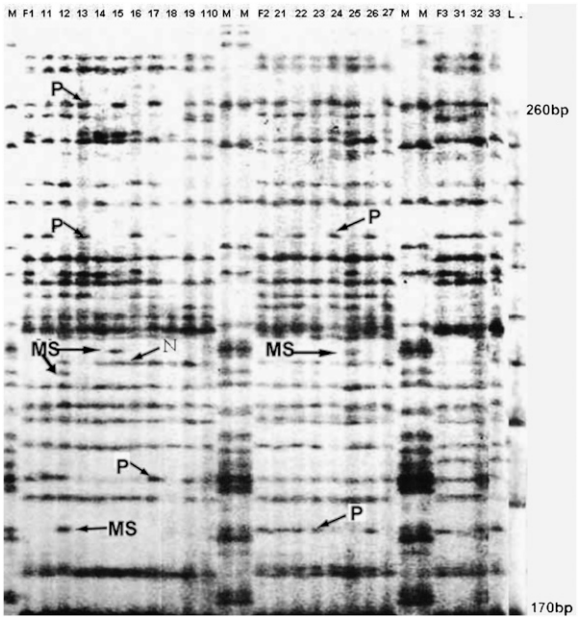

Figure 1. A portion of the AFLP fingerprint of 3 crossbred families of *Haliotis diversicolor* female × *H. discus discus* male using the E-AAC/M-CTA primer pair. Lane M, male parent; lanes F1, F2, and F3, female parent of family 408093, 408,094, and 408095, respectively; lanes 11–110, 21–27, and 31–33, F1 of family 408093, 408094, and 408095, respectively; lane L, DNA ladder. MS, male parent-specific band; N, nonparent locus band; P, polymorphic locus band.

TABLE 2.

The number of father-specific AFLP bands, mother-specific AFLP bands, and common AFLP bands of 2 parent abalone species in 3 hybrid families.

Family	Prime Pair	Father-Specific Band	Mother-Specific Band	Common Band
408093	AAC-CTA	49	32	7
	AAG-CAA	48	46	10
	AGC-CTT	30	35	10
408094	AAC-CTA	45	32	8
	AAG-CAA	43	46	9
	AGC-CTT	29	35	9
408095	AAC-CTA	46	32	8
	AAG-CAA	45	46	9
	AGC-CTT	31	35	10
Total		127	113	27

rates of these families varied from 4.9–30.4%. The viability of F1 was very low, only 0% to 0.15% at the age of 2 mo (Table 1).

AFLP Fingerprinting

Twenty hybrids from 3 families were analyzed with 3 AFLP primer combinations along with 3 different female parents and their common male parent. Figure 1 shows one part of the AFLP profile generated by using primer pair E-AAC/M-CTA. The primer combination revealed large differences between *H. diversicolor* and *H. discus discus* species based on their banding pattern. A total of 253 markers were detected from the 3 prime pairs in 2 parent species, out of which 127 were *H. discus discus* specific, 113 were *H. diversicolor* specific, only 27 were shared by both species (Table 2).

Most of the bands of F1 progenies were monomorphic, with 85.61%, 88.89%, and 95.97% of the scored bands in no. 408093, 408094, and 408095 families, respectively. Of the monomorphic bands in all 3 families, 51.38% were *H. diversicolor* specific, and 18.06% were common to both parents, but none was male parent specific. The polymorphic bands in F1 progenies were divided into 3 different subsets to study the band-sharing pattern between the F1 individuals and the parents:

1. The maternal segregated bands (MSB), which were bands found in some but not all F1 individuals, and were shared with the female parent but not the male parent, indicating that female parent-specific loci segregated among F1 progeny
2. Paternal-specific bands (PSB), which were bands found in F1 individuals with the same motility with male parent-specific bands
3. Nonparental bands (NPB), which were bands found in F1 individuals but in neither of the parents

The percentage of monomorphic bands, polymorphic bands, MSB, PSB, and NPB in 3 hybrid families is shown in Table 3. In the 3 families analyzed, 6.35–20.14% of bands were MSB, 0.81–3.17% of bands were PSB, and 1.61–5.76% of bands were NPB (Table 3). The frequency was 0.05–0.30 for PSB and 0.10–0.45 for NPB. Of the total bands for each individual, 0–3.3% were NPB and 0–0.8% were PSB.

Determination of Genetic Similarity and Cluster Analysis

Pairwise similarities and genetic distances were calculated based on the binary matrix with AFLP Date Analyser version 1.3 (Wang et al. 2004). Data obtained from 3 primer combinations were analyzed together. This showed that genetic divergence between *H. diversicolor* and *H. discus discus* was very large, with the genetic distance ranging from 1.471–1.492. The progenies of 3 families shared very low genetic similarity with their common male parent (range, 0.230–0.234). In contrast, similarities among the hybrid families and the female parents were all high (range, 0.919–0.962; Table 4).

Cluster analysis showed a clear separation of the 2 parental species. The male parent separated from the other as a distinct clade. All the hybrid F1 families clustered to their own female parent at first, and then 3 subclusters formed a clade distinct from the male clade (Fig. 2).

DISCUSSION

Hybrids obtained from *H. diversicolor* × *H. discus discus* crosses could potentially lead to positive qualities of both parents were expected, such as to expand ecological tolerance limits, so continuous efforts had been made to develop such hybrids in our laboratory. However, the appearance of the F1 individuals and the results from previous studies with AFLP technology indicated that the progenies from a hybrid cross of *H. diversicolor* female and *H. discus discus* male might be gynogens instead of true hybrids (Cai et al. 2009). To verify the genetic essence of *H. diversicolor* × *H. discus discus* hybrid F1 progeny, 3 hybrid families were analyzed using AFLP.

The detection of species-specific bands can be very useful for easy and early detection of any outcrossing event as well as assigning parentage. The results from our study showed that there was a high level of genetic divergence between *H.*

TABLE 3.

The percentage of monomorphic bands (MMB), polymorphic bands (PMB), maternal segregated bands (MSB), paternal-specific bands (PSB), and nonparental bands (NPB) in hybrid families of *H. diversicolor* female and *H. discus discus* male.

Family	No. of Individuals	No. of Total Bands	MMB (%)			PMB (%)			
			Total	MSB	Common Band	Total	MSB	PSB	NPB
408093	10	139	79.86	61.15	18.71	20.14	11.51	2.88	5.76
408094	7	126	86.51	65.50	20.63	13.49	6.35	3.17	3.97
408095	3	124	91.94	70.97	20.97	8.06	6.45	0.81	1.61
Total	20	144	69.44	51.38	18.06	30.56	17.36	3.47	9.03

TABLE 4.

The mean genetic distances and similarities (in parentheses) among progeny populations from hybrid families of *H. diversicolor* female and *H. discus discus* male and their parent.

Hybrid Families and Parents	MP	FP408093	HF408093	FP408094	HF408094	FP 408095	HF 408095
MP	0.000	1.492	1.466	1.487	1.452	1.471	1.470
FP408093	(0.225)	0.000	0.039	0.068	0.082	0.079	0.085
HF408093	(0.231)	(0.962)	0.043	0.069	0.081	0.080	0.082
FP408094	(0.226)	(0.934)	(0.933)	0.000	0.030	0.056	0.046
HF408094	(0.234)	(0.922)	(0.922)	(0.971)	0.035	0.061	0.053
FP408095	(0.230)	(0.924)	(0.923)	(0.946)	(0.941)	0.000	0.024
HF408095	(0.230)	(0.919)	(0.921)	(0.955)	(0.949)	(0.976)	0.028

FP, female parent; HF, hybrid family; MP, common male parent.

diversicolor and *H. discus discus,* and a lot of species-specific bands could be used to analyze the genome composition of hybrid progenies. The hybrid F1 *H. diversicolor* × *H. discus discus* showed high genetic similarity with *H. diversicolor* according to the band patterns. Most of the bands of the hybrid F1 were found in *H. diversicolor*, but not more than 0.08% of total bands in each individual were *H. discus discus* specific. These results indicate that the hybrid F1 might be allogynogens with little remaining of the male genome. It has been reported that gynogens or androgens might be produced in the interspecific hybridization in fish (Uyeno 1972, Stanley 1976, Chevassus 1983, Howell et al. 1995) and in molluscs (Jiang et al. 1983, Li et al. 1983). The viable resultant gynogens from hybridization may be the result of male chromosome elimination and spontaneous diploidization of the maternal chromosome set (SDM) (Ye et al. 1989). The viable hybrids described in the literature showed complete or nearly complete elimination of the chromosome complement from 1 parental species. That paternal chromosomes could be preferentially eliminated through mitotic abnormalities during early embryogenesis has been observed in the salmonid hybrids between *Oncorhynchus masou* and *O. mykiss* by using genomic *in situ* hybridization (Fujiwara et al. 1997). SDM has been reported to occur in some bisexual fish at low frequencies, ranging from 1–5% (Cherfas et al. 1995) or at a relative high frequency (about 30%) (Thorgaard & Gall 1979, Cherfas et al. 1991, Flajshans et al. 1993). Allotriploids were found at frequencies of 5.8–17.9% in hybrid larvae of *H. diversicolor* and *H. discus discus* based on karyotype analysis (Cai 2005), which suggested SDM also occurred in the hybrid F1.

NPB were observed in all 3 analyzed hybrid families, ranging from 5.04%, 3.97%, and 1.61% out of the total bands. NPB have been reported in other cases of interspecific hybridization. Allen and Gaffney (1993) detected NPB besides female PSB in hybrid adults between *Crassostrea gigas* and *C. rivularis* with allozyme electrophoresis. Wan et al. (2004) found that 16.7–18.7% bands in an ISSR profile of hybrid larvae between *Chlamys farreri* and *C. nobilis* were nonparental. Three possible reasons might attribute to the NPB: (1) artifact bands produced from shortcomings of the technique, (2) new bands produced from interaction between the 2 parental genomes, and (3) bands produced from contamination of exotic sperm and larvae in hatchery conditions. The current results show that the similarities observed intrafamily were all larger than those interfamily, and that hybrid individuals always clustered with their female parent first, which would happen infrequently if the families were contaminated with exotic sperm or larvae. Whether the NPB were produced because of interaction of heterogeneous genomes or shortcomings of AFLP is still unknown.

In addition, the viability of hybrid F1 was very low, with only 0–0.15% at the age of 2 mo. The low viability was consistent with the other hybrid cross that produced progenies

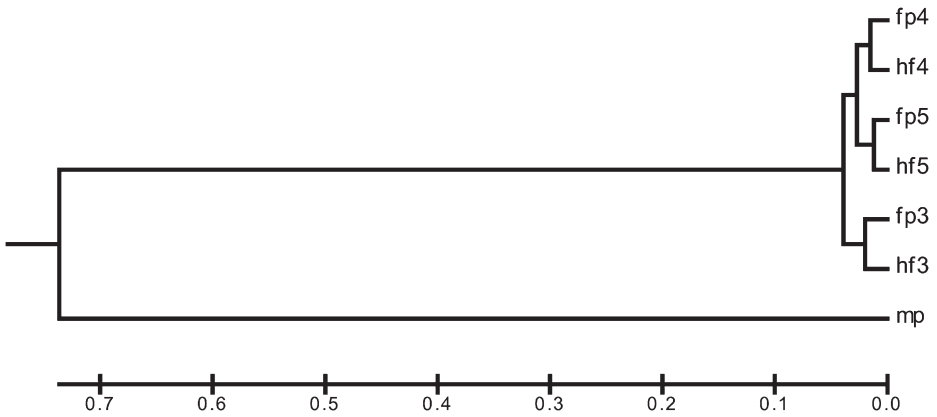

Figure 2. UPGMA dendrogram based on the distance matrix revealed the parentage relation among hybrid families and their parents. fp, female parent; hf, hybrid family; mp, male parent. The scale represents the genetic distance.

similar to 1 parent. Wei et al. (1983) gained viable juveniles in 5 of 12 interspecific crosses between *Pinctada martensii*, *P. chemnitzi,* and *P. chemnitzi*, with low viability ranging from 0.01–0.04%. Outcross incompatibility may be the reason for the low viability.

Thus, although the viable progenies from a hybrid cross between female *H. diversicolor* and male *H. discus discus* were not true hybrids but allogynogens, they still have a lot of potential applications in genetics and breeding in abalone. So far, the artificial induction of gynogenesis in abalone has not achieved complete success, because no one has declared that they have obtained sex-matured gynogenetic diploid in abalone. This article highlights a biological method to produce gynogenetic diploids in *H. diversicolor*, which would be helpful in rapid establishment of pure breeding.

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