AFLP Analysis of Seven Geographical Populations of *Epinephelus akaara*

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Abstract: The genetic diversity and genetic differentiation of *Epinephelus akaara* populations in seven geographical regions were analyzed by Aamplified Fragment Length Polymorphism (AFLP) technique. The analysis results revealed that the levels of genetic diversity among the 7 populations were different. It has been shown that the genetic diversity of *E. akaara* populations in Dayawan (DYW) and Zhoushan (ZS) were the lowest, and the genetic diversity of *E. akaara* population was the highest. Eighty-eight samples in 7 *E. akaara* populations could be apparently classified into three clades by UPGMA analysis, *E. akaara* population in Sanya (SY) was categorized into clade A. part of *E. akaara* population in Zhanjiang (ZJ) was categorized into the clade B. and other part of *E. akaara* population in Zhanjiang (ZJ), as well as rest of five *E. akaara* population were categorized into clade C. The six subclades were further clarified among clade C according to the geographical distribution. Taken together, our study would provide valuable information for the genetic conservation and improvement of *E. akaara*.

Key words: *Epinephelus akaara*; AFLP; genetic diversity and genetic differentiation
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赤点石斑鱼7个地理群体的AFLP分析

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摘 要：应用AFLP技术对赤点石斑鱼 *Epinephelus akaara* 7个地理群体进行了遗传多样性及遗传分化的分析。结果显示：不同群体的遗传多样性差异较大，大亚湾群体和舟山群体遗传多样性最低，湛江群体最高；通过UPGMA聚类，7个群体88个个体明显分成3支，三亚群体单独聚为一支（Clade A），湛江群体的部分个体聚类为一支（Clade B），湛江群体剩余个体和其他5个群体的个体聚为一支（Clade C）。其中在分支C中存6个小的分支，这6支中个体间基本以地理群体进行聚类。研究结果为赤点石斑鱼种质资源保护和遗传改良提供了遗传学依据。

关键词：赤点石斑鱼；AFLP；遗传多样性；遗传分化
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Epinephelus akaara is categorized into Perciformes, Serranidae, Epinephelinae, Epinephelus, there are around 100 species of *Epinephelus akaara* totally in the world. Most of *Epinephelus akaara* are living in the Indian Ocean and tropical and semitropical regions of the Pacific Ocean. Since its rapid growth and high market price, *Epinephelus akaara* , popularly cultured in the south of China, has become the main encaged culture fish in several provinces, such as Fujian, Guangdong, Zhejiang and Hainan

In recent years, genetic resource of *Epinephelus akaara* populations have rapidly decreased caused by

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capturing inordinately. Moreover, the cultural populations also encountered prevalent diseases and highly inbreedingon the reason of the development of fish breeding,. All of these would have effect on the conservation and exploiture of *Epinephelus akaara*. At present, the studies of *Epinephelus akaara* are mainly focusing on the regulation of reproduction, growth, and artificial breeding[1-3]. However, the study on the genetic diversity and differentiation of *Epinephelus akaara*. Has not been reported yet. Generally, genetic diversity of a species was determined by its adaptability, survivability and evolvability. Abundance genetic diversity means high survivability and great potential in application of genetic breeding [4].

Molecular markers have been widely applied in genetic diversity analysis of animals and plants, which is in comparsion to traditional marker techniques, according to the good reproducibility and high polymorphism. Among various molecular techniques, AFLP (amplified fragment length polymorphism) could be used to obtain mass markers without the genomic information by combination of the advantages of RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphic DNA). AFLP has been also applied in the study of genetic diversity and differentiation in many aquacultural fishes[5-9].

As one of the main species of Chinese aquiculture, *Epinephelus akaara* is exporting to many countries with high production. In order to keep the good seeds and avoid the negative effects from artificial breeding on wild resources, it is necessary to investigate the genetic diversity of wild populations. In present study, the AFLP technique was applied to analyze the genetic diversity and differentiation of *E. akaara* in 7 different wild populations from East China Sea to South China Sea. Our study would be useful for the conservation and genetic breeding of *E. akaara* in the future.

1 Materials and Methods

1.1 Fish samples

The Locations and times of harvesting *E. akaara* in 7 geographical populations were shown in Table 1. 5 ~ 16 individuals of each population, which have been conserved at −20 ℃, were used to study.

1.2 DNA extraction and AFLP analysis

Genomic DNA was extracted from blood with phenol/chloroform extraction as described by Sambrook et al[10].

AFLP analysis was conducted essentially as described by Vos et al[10]. Primers and adaptor were de signed according to the protocol of Qi and Lindhout[11]

Table 1 Sampling time of 7 geographical populations of *Epinephelus akaara*

Geographical population	Sampling time
Zhoushan population (ZS)	Sep. 2004
Pingtan population (PT)	Oct. 2004
Xiamen population (XM)	Jan. 2005
Zhelin population (ZL)	Nov. 2004
Dayawan population (DYW)	Dec. 2004
Zhanjiang population (ZJ)	Oct. 2004
Sanya population (SY)	Jan. 2005

were commercially synthesized by Sangon Co. (Shanghai). The DNA samples were digested with *Eco*R Ⅰ and *Mse* Ⅰ, and then ligated to restriction site-specific adaptors. Pre-amplification was carried out using adaptor-specific primers with one selective base at 3' end of each primer. The pre-amplification products were diluted 20-fold and used in selective amplification. The selective amplification used the primers with three selective bases at 3' end of each primer. Totally 13 primer combinations were selected for AFLP analysis.

Products of the selective amplification were separated by polyacrylamide gel electrophoresis at 60 V for 1.5 hours and were detected by silver staining. The electrophoretic images were scanned and then saved in computer for further analysis.

1.3 Data analysis

The data were scored as dominant markers. Bands present were scored as ''1'' and absent as ''0''. The AFLP marker names were referred to the primers used, for example E followed by numbers referred to the *Eco*R Ⅰ primer and M followed by numbers to the *Mse* Ⅰ primer. Bands were numbered serially in descending order of fragment length, thus the last numbers of the AFLP marker codes referred to the relative position of the band shown in the gel.

The main parameters used in this study were: Frequency of dominant genotype: $Pd = n_i/n$, n_i is the number of individuals which shows band in locus I; n is total number of individuals. Frequency of polymorphic loci: P = polymorphic loci/total loci. Index of genetic similarity: $S_{ij} = 2N_{ij}/(N_i + N_j)$, N_{ij} is the number of common bands between individual i and j; N_i and N_j are the number of specific bands in individual i and j, respectively. Genetic distance: $D = -\ln S$, S is the index of genetic similarity. Shannon's index (H), diversity within population: $H_0 = -\sum X_i \ln X_i/N$, X_i is the frequency of locus i in population; N is total number of markers in population. Average diversity among n different populations: $H_{pop} = -\sum H_0/n$, n is the number of populations. Diversity in population: H_{sp}

$= -\sum X \ln X/n$, X is total dominant frequency of locus i in n populations. Nei'rs index (h): $h_0 = 1 - \sum P_i^2$, P_i is the allele frequency of single locus. G_{st} can measure the genetic differentiation among populations: $G_{st} = 1 - h_s/h_t$, h_s is average heterozygosity in total population; h_t is average heterozygosity within population.

Genetic diversity index and Shannon's index were calculated with POPGEN1. 32 software, and AMOVA analysis was used to compute F_{st} with Arlequin software. UPGMA tree was constructed with MEGA 3.0 software.

2 Results

2.1 AFLP amplification

13 primer combinations were used to analysis 88 samples in 7 *E. akaara* populations. Totally, 941 bands ranging from 0.1 kb to 1.5 kb were obtained, and 672 of 941 (71.41%) were polymorphic bands. Great differences existed among different primer combinations. Total bands obtained with each primer combination ranged from 41 to 95, on average, 72. Polymorphic bands obtained with each primer combination ranged from 33 to 71 (58.00% ~80.49%), on average, 51.7 (Table 2).

Table 2 Amplification results of different primer combinations

Primer combinations	Number of loci amplified	Number of polymorphic loci amplified	Frequency of polymorphic loci
E3M3	69	57	68.67
E2M1	95	59	62.11
E4M1	93	71	76.34
E3M2	82	63	76.83
E4M4	83	57	68.67
E1M2	70	51	72.86
E1M4	48	33	68.75
E2M4	50	29	58.00
E3M4	64	51	80.00
E1M1	83	52	63.00
E4M2	95	69	72.63
E3M1	41	33	80.49
E1M3	68	47	69.12
Total	941	672	71.41

2.2 Genetic diversity analysis

Number of bands ranged from 614 to 807 among 7 populations. It showed the great difference existed among 7 populations. The least number of bands have been shown in DYW population, only 614, while that of ZJ population shown the largest number of bands, 807. Frequency of polymorphic loci ranged from 18.40% to 47.70%. Nei's index ranged from 0.044 7 to 0.142 9. Shannon's index ranged from 0.066 5 to 0.214 1 (Table 3, 4). Therefore, genetic diversity of DYW population was the lowest, while that of ZJ population was the highest. However, low number of individuals may cause underestimate genetic diversity of DYW population. Genetic similarity within populations ranged from 0.861 5 to 0.947 4. ZJ population was the lowest (genetic distance: 0.149 1), and ZS population was the highest (genetic distance: 0.054 1), indicating that the differentiation within population in ZS was lower than that in ZJ population.

Table 3 Number of loci amplified, ratio of polymorphic loci, index of genetic similarity and genetic distance of 7 *Epinephelus akaara* populations

Population	Total number of loci amplified	Number of polymorphic loci amplified	Frequency of polymorphic loci	Index of genetic similarity / Genetic distance
ZS	616	153	24.84	0.947 4/0.054 1
PT	666	198	29.73	0.943 6/0.058 1
XM	650	170	26.15	0.940 3/0.061 6
ZL	644	173	26.86	0.943 9/0.057 7
DYW	614	113	18.40	0.946 7/0.054 8
ZJ	807	385	47.70	0.861 5/0.149 1
SY	629	185	29.41	0.936 9/0.065 2

Table 4 Nei's index and Shannon's index of 7 *Epinephelus akaara* populations

Index	ZS	PT	XM	ZL	DYW	ZJ	SY
Nei's index	0.054 3	0.062 0	0.056 8	0.060 4	0.044 7	0.142 9	0.059 3
Shannon's index	0.081 9	0.094 3	0.086 0	0.090 9	0.066 5	0.214 1	0.091 0

2.3 Genetic structure and genetic differentiation

Based on AFLP data, a UPGMA tree was constructed for 88 samples from 7 populations (Fig. 2). The 88 samples could be classified into three clades. SY was categorized into single clade A. Part of ZJ population was categorized into single clade B, while the other part of ZJ population and other populations was categorized into clade C. Furthermore, Clade C could be subdivided into 6 subclades subsequently. All these subclades basically clustered according to their geographical location, which implied that the population structure is relative with their geographical location. We also used UPGMA method to construct a tree for 7 populations (Fig. 3), which has shown the same result to that abovementioned. Taken together, SY and ZJ populations were different from other 5 populations in genetic structure.

Based on AMOVA analysis, 56.63% variation obtained from among populations ($P < 0.01$) (Table 5), and 55.53% variation ontained from among populations based on Gst estimation. Both of the analysis results indicated great genetic variation and obvious genetic differentiation among populations. Also, Fst showed great genetic difference among 7 populations (Table 6). Genetic distance between populations varied from 0.076 9 to 0.346 7. Genetic distance between ZS and PT population is the lowest (0.076 9), while that SY and ZJ population are the highest (0.346 7) (Table 7). The genetic distance between SY population and other 6 populations ranged from 0.158 2 to 0.346 7, and much more exceeded among 5 populations in clade C, which implied that SY and ZJ population have significant genetic differentiation and might be relatively independent populations.

Fig. 1 UPGMA tree of 88 individuals of the 7 *Epinephelus akaara* populations
1 - 14: ZS; 15 - 29: PT;
30 - 39: XM; 40 - 52: ZL; 53 - 57: DYW;
58 - 73: ZJ; 74 - 88: SY

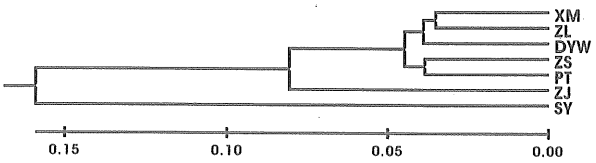

Fig. 2 UPGMA tree of the 7 *Epinephelus akaara* populations

Table 5 AMOVA analysis of the 7 *Epinephelus akaara* populations

Source of differentiation	freedom	Sum of squares	variation	frequency
Among population	6	4307.385	54.521	56.630
Within population	81	3382.024	41.753	43.370
Total	87	7689.409	96.274	100

Table 6 Fst value (above diagonal) and the corresponding P value (below diagonal) among 7 *Epinephelus akaara* populations

Population	ZS	PT	XM	ZL	DYW	ZJ	SY
ZS	—	0.000	0.000	0.000	0.000	0.000	0.000
PT	0.262	—	0.000	0.000	0.000	0.000	0.000
XM	0.313	0.269	—	0.000	0.000	0.001	0.000
ZL	0.384	0343	0.147	—	0.000	0.000	0.000
DYW	0.434	0.357	0.266	0.233	—	0.022	0.000
ZJ	0.369	0.346	0.302	0.321	0.263	—	0.00
SY	0.800	0.798	0.789	0.795	0.799	0.633	—

3 Discussion

3.1 Genetic diversity of 7 *E. akaara* populations

In order to evaluate the genetic diversity in differ

Table 7 Genetic similarity(above diagonal) and genetic distance(below diagonal) among the 7 *Epinephelus akaara* populations

Population	ZS	PT	XM	ZL	DYW	ZJ	SY
ZS		0.926 0	0.918 6	0.911 8	0.907 0	0.844 9	0.728 6
PT	0.076 9		0.920 7	0.914 3	0.913 9	0.848 8	0.727 0
XM	0.084 9	0.082 6		0.932 3	0.922 2	0.852 3	0.736 7
ZL	0.092 3	0.089 6	0.070 1		0.928 3	0.854 5	0.736
DYW	0.097 6	0.090 1	0.080 9	0.074 4		0.853 7	0.730 6
ZJ	0.168 5	0.163 8	0.159 8	0.157 2	0.158 2		0.707 0
SY	0.316 6	0.318 8	0.305 6	0.306 6	0.313 9	0.346 7	

ent populations, we sampled *E. akaara* from 7 representative locations. Using 13 primer combinations, 941 bands were obtained from 88 individuals of 7 populations. Among these bands, 672 of 941 (about 71.41%) were polymorphic. Therefore, AFLP could be used for obtaining more genetic information for a given species and could be a good technique to evaluate the genetic diversity.

Results achieved in this study revealed great difference among 7 populations. The frequency of polymorphic loci, the Nei's index, the Shannon's index and the genetic similarity within population varied from 18.40 to 47.70%, from 0.044 7 to 0.142 9, from 0.066 5 to 0.214 1, and from 0.861 5 to 0.947 4, respectively. The genetic variation was the lowest in DYW and ZS populations, while that was the highest in ZJ population. Compared with the current published data, frequency of polymorphic loci in *E. akaara* was lower than that in *Pseudosciaena crocea*, including wild (76.6%) and cultured (69.2%~70.6%) large yellow croaker[6], and Dai-chu race in Zhejiang province (55.80%)[9], it was also lower than that in 3 wild Red sea bream *Pagrus major* populations (64%~58.4%) and wild *Lutjanus argentimaculatus* (57.14%) in China[5,7]. But frequency of polymorphic loci in *E. akaara* was higher than that in wild (46.18%) and cultured (40.07%) flounder[8], partial cultured population of channel catfish *Lctalurus punctatus* (18.6%) and arowana *Scleropages formosus* (12.7%~15.6%)[12-13]. These results indicated that genetic diversity of *Epinephelus akaara* is below the middle level.

3.2 Genetic differentiation among 7 populaitons of *Epinephelus akaara*

The 7 populations could be classified into 3 clades. Clade A only contained SY population. But Clade B contained part of individuals of ZJ population and Clade C contained 6 subclades and these subclades basically clustered according to their geographical location. SY population contained 34 specific bands. Significant differentiation could be found among SY population and other populations. But there are no significant differentiation found within individuals of SY population. Individuals of ZJ population was classified into both clade B and C, which implied that significant differentiation was found within individuals of ZJ population. ZS, PT, XM, ZL, SY and part of ZJ population were classified into clade C. Most individuals of the 6 populations in clade C could be further clustered into several subclades. Result of AMOVA and Fst showed that great difference exists among populations. In the 7 populations, ZJ population contained two clades, while individuals from other populations basically were clustered into one clade., We also concluded that gene flow among different populations was weak by AFLP analysis, which may be limited by finite movement and other unknown geographical factors. Another possibility was individuals moving into a population may not be efficient to affect the genetic structure[14-16]. SY population was located in Hainan Island far away from the mainland land. This maight limit the gene flow between SY and other populations. Clade B contains only individuals of ZJ population. But clade C contained individuals of ZJ and other populations, which may indicated that gene flow occur between ZJ and other populations. This process which may be natural or artificial breeding, causes the total number of loci, frequency of polymorphic loci, Nei's index and Shannon's index are higher than other populations. In aquaculture, rich genetic diversity was the basic for seed selection. In this

study, we showed that there was significant genetic structure among different populations. Therefore, in order to maintain the development of *Epinephelus akaara* culture, we shall pay more attention to the conservation of wild resources when carrying out artificial breeding. Moreover, we found specific markers to clade A, B and C, respectively. These markers could be applied to population identification and might play important roles in the determination of seed source.

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