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## Analysis of genetic variation in selected stocks of hatchery flounder, *Paralichthys olivaceus*, using AFLP markers

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### Abstract

Amplified fragment length polymorphism (AFLP) analysis was performed in order to evaluate genetic characteristics of one common population and two selective hatchery populations of flounder *Paralichthys olivaceus*. A group of 60 genotypes belonging to three populations was screened using 10 different AFLP primer combinations. A total of 491 loci were produced in the three studied populations. The loci of 65.78%, 61.47% and 60.92% were polymorphic over all the genotypes tested in common, susceptible and resistant populations, respectively. The number of polymorphic loci detected by single primer combination ranged from 21 to 43. The average heterozygosity of common, susceptible and resistant populations was 0.1656, 0.1609 and 0.1586, respectively, which showed no significant difference. Compared with the common population, the two selective hatchery populations, susceptible and resistant, showed significant genetic differences including a smaller ( $P < 0.05$ ) number of total loci, a smaller ( $P < 0.05$ ) number of total polymorphic loci and a smaller ( $P < 0.05$ ) percentage of low frequency (0–0.2) polymorphic loci. AFLP banding pattern was transformed into

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binary data and matrices were processed with POPGENE and TFGPA software. Similarity relationships were described graphically by a dendrogram, which clustered the three populations. The AFLP fingerprinting technique was confirmed to be a reproducible and sensitive tool for the study of population genetics of flounder. The present study confirmed that it was important to detect the genetic variability of the selective hatchery populations for the conservation of natural flounder resources.

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*Keywords:* Flounder; *Paralichthys olivaceus*; AFLP marker; Genetic characteristics; Genetic variation

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## 1. Introduction

The flounder *Paralichthys olivaceus* is one of the most important aquaculture species in China. It is widely distributed throughout the coastal areas of North China. However, aquaculture production of this species has not increased greatly over the last decade in spite of extensive fishery management efforts. The major reason for this slow growth was viral and bacterial diseases. Juvenile flounder are susceptible to many bacterial and viral diseases, of which *Vibrio anguillarum* is of particular concern due to its ubiquitous presence and frequent occurrence in marine aquaculture, it can, pose a significant threat to commercial production of marine fish. An important approach to disease prevention is to culture strains of fish resistant to major diseases. Genetic variations in resistance to diseases do exist among or within reared strains of fishes infected with vibriosis, furunculosis and bacteria (Beacham and Evelyn, 1992). Such variations can be exploited to produce strains of fish that are more resistant to specific diseases for selective breeding by selecting the resistant individuals.

Initial selection for flounder with disease resistance has been conducted in China. However, assessment programs for selective breeding programs are still lacking. This would demand analysis of genetic structures of selected strains as compared to non-selected controls. In addition, while such selection programs may provide broodstocks with improved traits, their intentional and accidental release into wild environment could have major ecological consequences. If a large number of cultured flounders escape or are released from aquaculture facilities (Su et al., 1990), they could significantly alter the genetic composition of wild populations by either displacing them or interbreeding with them (Waples, 1999). Most hatchery stocks typically show a reduced genetic variability, which may possibly result in the reduction of the population's capability to adapt to new environments (Allendorf and Phelps, 1980). Since the size of cultured populations in breeding programs tends to be small and genetically less diverse than wild populations (Campton, 1995; Sunden and Davis, 1991; Wolfus et al., 1997; Sekino et al., 2002), significant reduction in genetic variability can occur even in the first hatchery generation (Verspoor, 1988). Upon release of such populations into natural environment, a decrease in genetic diversity and an overall decline in the fitness of the populations

may result (Waples, 1991; Lester, 1992; Ferguson et al., 1995; Alcivar-Warren et al., 1997). Therefore, it is important to establish a baseline information concerning genetic background of the aquaculture population both for genetic enhancement programs and for population genetic programs aiming at protection of the genetic integrity of natural populations.

Several types of molecular markers have been used for the assessment of genetic variations of hatchery and wild strains of flounder. For examples, using allozyme analysis, Liu et al. (1997) detected significant genetic differentiation among hatchery strains and wild populations, but detected no reductions in genetic variability in hatchery populations. However, this may have been due to the limited number of allozyme loci used in the study. In support for such a notion, significant reductions of genetic variability in several hatchery populations were detected by using mitochondrial DNA (mtDNA)-RFLP (Sugaya et al., 1999), mtDNA sequencing (Sekino et al., 2002), and microsatellites (Yoshida et al., 2000; Sekino and Hara, 2000, 2001; Sekino et al., 2002). These studies indicated that molecular marker systems with greater levels of capability for the detection of polymorphism might provide a better solution for the assessment of genetic variations. Amplified fragment length polymorphism (AFLP) is a PCR-based, multi-locus fingerprinting technique (Vos et al., 1995) that has tremendous power for revealing polymorphism (for a review, see Liu and Cordes, 2004). It has been successfully applied in many aquaculture species such as catfish (Mickett et al., 2003), rainbow trout (Young et al., 1998), kuruma prawn (Li et al., 2003; Moore et al., 1999) and black tiger (Wilson et al., 2002). In this project, our objectives were to determine if AFLP would be suitable for the analysis of genetic variations among aquaculture populations and to assess genetic variation of populations under selection for disease resistance traits. Here we report that AFLP provided high levels of polymorphism among aquaculture populations of flounder in China, and that genetic differences were observed among a resistant line, a susceptible line, and a common control line of flounder.

## 2. Materials and methods

### 2.1. Fish sampling

Three populations with a total of 60 individuals of flounder were used for the present study. The common population (C) was the second generation of the cultured flounder, which was founded using hundreds of wild populations without any artificial selection, that is, all of them were randomly selected. The populations were previously established from common population by injecting *V. anguillarum* for the selective breeding program. Approximately 200 individuals of susceptible and resistant flounder were obtained, respectively, to ensure the effective population size during the following successive generations (Bartley et al., 1995). We used the first generation of S and R populations for the present study. All of the three populations were obtained by spawning without an aid of stripping. These experimental fish were raised at Haiyang hatchery station, Yellow Sea Research Fisheries Institute, Chinese

Academy of Fishery Sciences. An overview of the flounder sample description is given in Table 1. Blood samples were collected from these fish to compare AFLP polymorphism of susceptible and resistant populations. Samples were stored frozen ( $-20\text{ }^{\circ}\text{C}$ ) until genetic analysis was performed.

## 2.2. Genomic DNA extraction

DNA extraction was performed as described by Strauss (1989) and Liu et al. (2005).

Blood samples (100  $\mu\text{l}$ ) were collected in 1 ml syringe and immediately expelled into a tube containing 500  $\mu\text{l}$  DNA extraction buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8, 25 mM EDTA, 0.5% SDS, and freshly added 0.1 mg/ml proteinase K). Blood was expelled into lysis buffer quickly to disperse the blood cells. The lysates were incubated at  $55\text{ }^{\circ}\text{C}$  overnight. DNA was extracted twice with phenol and once with chloroform. DNA was precipitated by adding half the original blood volume of 7.5 M ammonium acetate and two volumes of ethanol. DNA was collected by brief centrifugation and washed twice with 70% ethanol, air-dried, and redissolved in TE buffer. The concentration was measured with a GENEQUANT Pro (Pharmacia Biotech Ltd) RNA/DNA spectrophotometer for absorption at 260 nm.

## 2.3. AFLP reactions

Procedures of AFLP analysis were essentially based on Vos et al. (1995). Genomic DNA was processed using the ‘‘AFLP analysis System I’’ (Gibco BRL Life Technologies) according to the manufacturer’s instructions with some modifications. The *EcoRI* primers used were not radioactively labeled. Instead, a modified silver staining method detailed below was used. After partial digestion and ligation to adaptors, it was pre-amplified and amplified through two sequential steps. PCR pre-amplification, with primers carrying one selective nucleotide, was performed with 30 cycles at  $94\text{ }^{\circ}\text{C}$  for 30 s,  $56\text{ }^{\circ}\text{C}$  for 1 min and  $72\text{ }^{\circ}\text{C}$  for 1 min. Samples were processed immediately for the following step or stored at  $-20\text{ }^{\circ}\text{C}$ . After dilution up to 20 times in water, PCR products from the pre-amplification reaction were used as templates for selective amplification. The amplification step consisted of 12 cycles at  $94\text{ }^{\circ}\text{C}$  for 30 s,  $65\text{ }^{\circ}\text{C}$  for 30 s (with a decreasing ramp of  $0.7\text{ }^{\circ}\text{C}$  each cycle) and  $72\text{ }^{\circ}\text{C}$  for 1 min followed by 30 cycles at  $94\text{ }^{\circ}\text{C}$  for 30 s,  $56\text{ }^{\circ}\text{C}$  for 30 s and  $72\text{ }^{\circ}\text{C}$  for 1 min. Altogether 10 primer combinations were used in the analysis (Table 2).

Table 1  
Description of the three strains of flounder *Paralichthys olivaceus* used in the study

Strain name	Abbreviations	Source of material	Number of individuals	Dates sampled
Common	C	Qingdao, China	20	7/2003
Susceptible	S	Qingdao, China	20	8/2003
Resistant	R	Qingdao, China	20	8/2003

#### 2.4. Gel electrophoresis and silver staining

The PCR products were mixed with an equal volume of formamide dye (99% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were heated to 95 °C to denature for 5 min and immediately placed on ice. The gel was pre-electrophoresed at 60 W for 30 min, then 5.0 µl of the amplified DNA was loaded and run on a 5% denaturing polyacrylamide gel (4.75% acrylamide, 0.25% bisacrylamide, 7.5 M urea and 1 × TBE buffer) with 1 × TBE buffer on a DNA sequencing system (Liuyi corporation, China) at 80 W for 120 min.

Silver staining was conducted using the procedures of Merrill et al. (1979) and Liu et al. (2004) with modifications. After electrophoresis, the gel was fixed in 10% ethanoic acid for at least 30 min. The gel was rinsed in distilled water three times and stained with a mixture of 0.1% silver nitrate and 0.15% formaldehyde for 30 min. The stained gel was rinsed again with distilled water and immersed in a developing solution (3% sodium carbonate, 0.15% formaldehyde, 0.02% sodium thiosulphate). The development was subsequently stopped with 10% ethanoic acid when the bands became visualized and reached desirable intensity. Band sizes were estimated by a standard AFLP DNA ladder.

#### 2.5. AFLP reproducibility test

The reproducibility of AFLP fingerprints was performed to increase the consistency of the results by comparing the fingerprints of the same flounder individual. Two selected genotypes were independently processed from the beginning of the AFLP analysis for three replicates with two different primer combinations (No. 1 and 2 from Table 2). Reproducibility was calculated as the percentage of bands that showed consistent results over the three replicates analyzed per flounder sample.

Table 2  
AFLP primer combinations used in the study

Primer combinations	<i>EcoRI</i> primers	<i>MseI</i> primers
1	<i>EcoRI</i> primer + ACG	<i>MseI</i> primer + CAC
2	<i>EcoRI</i> primer + ACG	<i>MseI</i> primer + CTG
3	<i>EcoRI</i> primer + ACT	<i>MseI</i> primer + CTC
4	<i>EcoRI</i> primer + AGC	<i>MseI</i> primer + CTT
5	<i>EcoRI</i> primer + AAC	<i>MseI</i> primer + CTC
6	<i>EcoRI</i> primer + AGC	<i>MseI</i> primer + CTA
7	<i>EcoRI</i> primer + AAC	<i>MseI</i> primer + CAT
8	<i>EcoRI</i> primer + AAC	<i>MseI</i> primer + CAG
9	<i>EcoRI</i> primer + AGG	<i>MseI</i> primer + CTG
10	<i>EcoRI</i> primer + ACT	<i>MseI</i> primer + CAC

The sequences for *EcoRI* primer were 5'-GACTGCGTACCAATTC-3', and for *MseI* primer were 5'-GATGAGTCTGAGTAA-3'.

## 2.6. Data analysis

AFLP bands were scored for presence (1) or absence (0) by Crosscheck freeware (Buntjer, 1999), and transformed into 0/1 binary character matrix. Fragments that could not be scored unambiguously were not included in the analysis. The data matrix was analyzed for population genetic diversity using POPGENE software package (Yeh et al., 1999). Genetic distances between populations were calculated by Nei's (1978) unbiased distance and similarity measures. Genetic population relationships were estimated by constructing UPGMA tree based on Nei's standard genetic distance (Nei, 1978). The bootstrap values for the UPGMA tree were calculated by 1000 bootstrap re-sampling across loci. Average heterozygosities and percent polymorphic loci were estimated using the TFPGA program (Miller, 1997). Average heterozygosity estimates were calculated for each locus and then averaged over loci according to Nei's (1978) unbiased heterozygosity formula. The percentages of polymorphic loci were estimated based on the percent of loci not fixed for one allele. Confidence intervals were generated by bootstrapping analysis at the 99% confidence level with 1000 replications.

## 3. Results

### 3.1. AFLP polymorphism of the three populations of flounder

Reproducibility test was carried out with primer combinations 1 and 2 using two randomly selected DNA samples. A high degree of reproducibility (reproducibility score = 99%) was obtained for each of the primer combinations tested (data not shown). AFLP analysis of 60 flounder individuals using 10 primer combinations produced a total of 491 scoreable bands, of which 65.78%, 61.47% and 60.92% were polymorphic over all the genotypes tested in common, susceptible, and resistant populations, respectively (Table 3). The total number of polymorphic bands over all populations varied from 21 (for primer combination 5) to 43 (for primer combination 10) per primer combination. The average heterozygosity of common, susceptible, and resistant populations were 0.1656, 0.1609 and 0.1586, respectively.

### 3.2. Genetic differences between common and the two selective hatchery populations

A greater ( $P < 0.05$ ) number of total AFLP bands were observed from the common population than from the resistant or the susceptible populations. There were a total of 491 AFLP loci detected in common population, while they were 462 and 458 in susceptible and resistant populations, respectively (Table 3). The total number of polymorphic bands was also higher ( $P < 0.05$ ) in common population than in the other selected populations. The total polymorphic loci were 323 in common population (65.78%), 284 in susceptible population (61.47%), and 279 in

Table 3

A summary of AFLP analysis of genetic variations in flounder using the 10 primer combinations as listed in Table 2

Strain	Number of polymorphic loci											N	H	P
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Total			
C	31	29	30	31	26	33	35	38	27	43	323	491	0.1656	65.78
S	28	25	29	27	21	30	32	29	27	36	284	462	0.1609	61.47
R	23	27	25	30	24	32	29	30	27	32	279	458	0.1586	60.92

Three strains were investigated with C for common strain, S for susceptible strain, and R for resistant strain. P1 through P10 indicate primer combinations 1–10 as listed in Table 2. Total number of AFLP bands (N), average heterozygosity (H), and percentage of polymorphic loci (P) are given.

resistant population (60.92%). However, no significant difference was found in the proportion of polymorphic bands among the three populations.

Some differences in percentage of polymorphic loci among populations were found as shown in Fig. 1. The percentage of low frequency (0–0.2) polymorphic bands in the two selective hatchery populations was lower ( $P < 0.05$ ) than that in the common population, while the percentage of high frequency (0.6–1.0) polymorphic bands had a tendency to increase in the two selective hatchery populations as compared with the common population.

The UPGMA dendrogram constructed on the basis of the inter population genetic similarity is showed in Fig. 2. The susceptible and resistant populations were clearly separated from the common population. Fig. 3 showed the genetic similarity tree across all 60 individuals. Within the similarity tree, most of common, susceptible and resistant individuals formed distinct clusters. Genetic similarity among populations is summarized in Table 4. It appeared that the common population had the highest genetic similarity with the resistant population, while the resistant

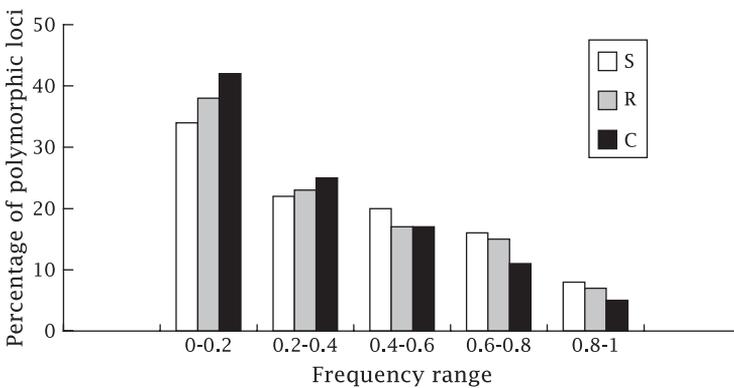


Fig. 1. Distribution of percentage of polymorphic loci at each frequency range in common, susceptible and resistant populations. Note: in the figure, percentage of polymorphic loci is the ratio of the number of polymorphic loci at each of the five frequency range and the total number of polymorphic loci in each population.

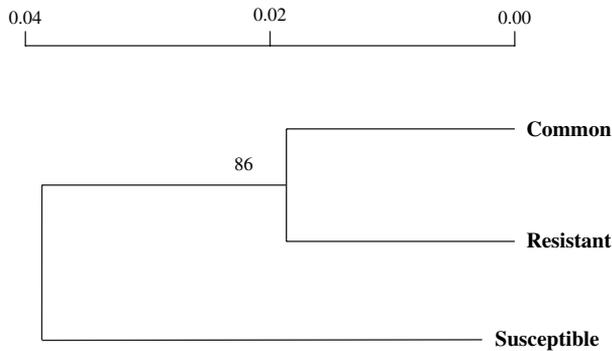


Fig. 2. UPGMA dendrogram showing the phylogenetic relationship among common and selective hatchery populations of flounder *Paralichthys olivaceus*. The scale represents Nei's (1978) genetic distance. Significant bootstrapping value is indicated.

population and the susceptible population had the lowest genetic similarity. These results suggested that genetic divergence between selective hatchery populations and common stock may have arisen. The susceptible and resistant populations had a much higher ( $P < 0.01$ ) value of genetic distance because they had undergone an opposed selective pressure (susceptible against resistant).

## 4. Discussion

### 4.1. AFLP polymorphism

Several PCR-based molecular methods of genotype analysis have been developed for the genetic analysis of fish (Grewe et al., 1993; reviewed by Liu and Cordes, 2004). Sequence analysis of specific mitochondrial or ribosomal DNA fragments and multiplex PCR of strain-conserved DNA fragments are efficient for fish strain identification (Bartlett and Davidson, 1991; Rocha-Olivares, 1998; Gharrett et al., 2001; Noell et al., 2001; Wasko et al., 2001). Compared with the DNA sequence methods, RAPD-based DNA fingerprinting is quicker and cheaper. In addition, the experimental requirements are lower (Cordes et al., 2001; Sebastio and Neri, 2001). However, RAPD provides low reproducibility (Hallden et al., 1994; Tommerup et al., 1995; Hampl et al., 2001), and its power for the detection of polymorphism is not very high (Cubeta et al., 1993; Cespedes et al., 1998). AFLP has the advantage over RAPD for its high reproducibility, as well as its great power for the detection of polymorphism. A large number of bands can be produced rapidly with a limited number of primer combinations. Clearly, AFLP is one of the most powerful approaches for the assessment of genetic variation among populations, especially for species in which no molecular genetic information was previously available. Here, we demonstrated that AFLP is highly efficient for the determination of genetic variations among selected hatchery populations of flounder. In the present study, AFLP

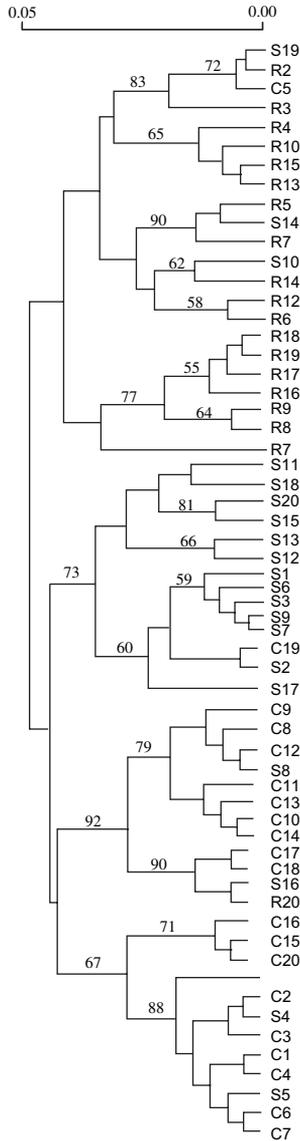


Fig. 3. UPGMA dendrogram showing the phylogenetic relationship among all 60 genotypes of flounder *Paralichthys olivaceus*. The scale represents Nei's (1978) genetic distance. Significant bootstrapping values are indicated.

analysis using 10 primer combinations generated nearly 500 fragments for all the three populations. Within populations, 60.92–65.78% of the fragments were polymorphic. Such a high level of polymorphism would make AFLP a powerful technique in elucidating genetic differentiation in flounder populations, and should be

Table 4

Genetic similarity of the common strain as compared with the two selective hatchery strains

Strain	<i>C</i>	<i>S</i>	<i>R</i>
<i>C</i>	***	0.9802	0.9831
<i>S</i>		***	0.9626
<i>R</i>			***

Genetic similarity was calculated as previously described by Nei (1978).

useful for the assessment of genetic structures of flounder in selective breeding programs.

Previously, genetic variation had been studied in cultured flounder in China utilizing allozyme (You et al., 2001). They reported percent polymorphic loci of 24.1%, and observed a mean heterozygosity value of 0.0788. These values are considerably lower ( $P < 0.01$ ) than those reported in the present study ( $P$ : 60.92–65.78%;  $H$ : 0.1586–0.1656). The differences are likely due to the ability of AFLP to resolve more loci and to detect greater levels of polymorphism than allozyme analysis.

#### 4.2. Genetic changes in selective hatchery populations

Results of AFLP analysis indicated that the two selective cultured populations were genetically different ( $P < 0.05$ ) from the common population. When channel fish were subjected to selection for growth, allozyme polymorphism was reduced compared to control strains, and analyses indicated that some of the reduced diversity was due to selection (Hallerman et al., 1986). In the current study, one flounder population selected for disease resistant and the other selected for disease susceptible also exhibited a reduction in AFLP polymorphism compared to the common population, implying that selection for disease resistance can reduce genetic diversity. Significant ( $P < 0.01$ ) genetic differences were also found between the two selective cultured populations and they might have resulted from different selection procedures. For the same reason, the UPGMA dendrogram showed that the susceptible population was clustered closer to the common population than to the resistant population.

In general, most selective cultured aquatic stocks represent genetically exogenous populations, thus, the intra-specific hybridization with wild stocks may result in a reduction of fitness in wild populations (Hindar et al., 1991; Lester, 1992; Ferguson et al., 1995). Even cultured populations that originated from the same local population may also threaten the fitness of the local population through the reduction of its effective population size (Ryman et al., 1995), displacement, behavior and other factors, especially when the absolute size of the wild population is small (Ryman and Laikre, 1991). Theoretically, genetic diversity is important because it can give populations the ability to adapt to environmental changes and artificial selection. Presently, one of the largest problems in the flounder industry is the prevalence of diseases. While selection for disease resistance is necessary and studies are being undertaken to map genetic markers that may be linked to disease resistance

in flounder in our laboratory, increased selection pressure without maintaining genetic variations could have long-term adverse effects on the future response of flounder stocks to selection. Genetic diversity data for the selective hatchery populations can be effectively used to mate individuals or populations having high performance, but having a varied genetic background, in order to maintain genetic diversity for future selective breeding while maintaining or improving performance for production traits. This work is the first assessment of genetic variation in selected stocks of hatchery flounder. Our findings indicated that such assessment of genetic structure should be conducted periodically along with selective breeding programs. In addition, caution should be exercised to avoid significant release of selected hatchery stocks into the wild, either intentionally, or accidentally.

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