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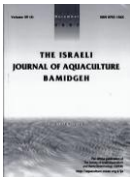

Genetics Features of Natural and Cultured Populations of Half-Smooth Tongue Sole (*Cynoglossus semilaevis*) Revealed by RAPD Markers

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Abstract

Randomly amplified polymorphism DNA (RAPD) was used to evaluate genetic diversity in natural and cultured populations of half-smooth tongue sole (*Cynoglossus semilaevis*). Two hundred genotypes from four natural populations (Laizhou, Weihai, Qingdao, Rizhao) and one cultured population (Mingbo) were screened with 18 RAPD primers. Of 101 loci in the five populations, 43.33%, 47.52%, 45.83%, 44.57%, and 42.86%, respectively, were polymorphic. The number of polymorphic loci detected by a single primer combination ranged 1-4. Average heterozygosity was 0.0735, 0.0893, 0.0871, 0.0847, and 0.0685, respectively. Weihai had greater genetic diversity than the other populations ($p<0.05$) including number of RAPD bands, number of polymorphic bands, average heterozygosity, and number of genotypes. Mingbo had the least genetic viability. Intentional or accidental release of cultured half-smooth tongue sole into natural sea areas may disrupt local gene pools and result in loss of genetic variability. Genetic variability of cultured populations should be monitored to conserve natural half-smooth tongue sole resources.

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Introduction

The half-smooth tongue sole (*Cynoglossus semilaevis*) is a marine fish species widely distributed throughout coastal areas of China. It is cultured in north China, especially in Shandong Peninsula, because of its good taste and high value. Long-term conservation of genetic diversity is important for any species. Half-smooth tongue sole resource management and enhancement is necessary to maintain long-term resource sustainability. Recognizing stock units in practical management is necessary for sustainable use of exploitable resources. A basic understanding of stock structure among geographical half-smooth tongue sole populations is thus required.

In general, the effective sizes of founding broodstock populations are restrained by farming conditions, resulting in the use of only a few individuals as broodstock. This practice may lead to erosion of the genetic diversity of the stocks, which compromises industrial performance. Intentional or accidental release of cultured half-smooth tongue soles into the wild environment could have major ecological consequences. If a large number of cultured half-smooth tongue soles escape or are released from aquaculture facilities, they could significantly alter the genetic composition of wild populations by either displacing them or interbreeding with them (Waples, 1999). Most cultured stocks show reduced genetic variability, which may result in the reduction of the population's capability to adapt to new environments. Therefore, it is important to establish baseline information concerning the genetic background of the cultured population, both for genetic enhancement programs and for genetic programs designed to protect the genetic integrity of natural populations.

Molecular markers provide a solution for the assessment of genetic variations. RAPD fingerprinting is quicker and cheaper in spite of lower polymorphism than microsatellite and some other molecular markers (Williams et al., 1990). It can be performed without knowledge of genomic sequence and be used to analyze genetic diversity, construct linkage maps, tag genes of interest, determine parentage, and detect genetic variation. RAPD has been used to study many aquaculture species including penaeid prawns (Garcia et al., 1995), red sea bream (Jiang et al., 2004), discus (Koh et al., 1999), phaeophyta (Wang et al., 2004), carp (Wang and Li, 2004), gilthead seabream (Bilgen et al., 2007), and flounder (Liu et al, 2007). However, genetic monitoring is limited because dominant markers are less efficiency than co-dominant marker. Fortunately, this limitation can be mitigated by the high number of loci and large size samples.

It is important to monitor genetic variability of cultured populations of half-smooth tongue sole for the conservation of natural resources. Here we report that RAPD revealed high levels of polymorphism among populations of half-smooth tongue sole in China, and that genetic differences were observed among four natural and one cultured populations.

Materials and Methods

Fish sampling. A total of 200 (40 individuals × 5 populations) half-smooth tongue sole (20 cm) were collected in 2005-2006 from four sites in the Yellow Sea (Laizhou Bay, Weihai, Qingdao, and Rizhao) and one hatchery station (Fig. 1). The hatchery (Mingbo Corp.) sample was obtained from a broodstock of approximately 30 individuals caught in Laizhou Bay. The 30 captives were mated in a spawning tank, by mesocosm spawning without stripping, and the first generation formed our hatchery sample. Blood samples were collected from the 200 fish and stored frozen (-20°C) until genetic analysis.

Genomic DNA extraction. DNA was extracted as described by Liu et al. (2005; 2006) with some modifications. Blood samples (100 µl) were collected with a 1-ml syringe and immediately expelled into a tube containing 500 µl 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 quickly expelled into a lysis buffer to disperse the blood cells. The lysates were incubated at 56°C overnight. DNA was extracted twice with phenol and once with chloroform. DNA was precipitated by adding half the original blood volume to 7.5 M ammonium acetate and two volumes of ethanol. DNA was collected by brief centrifugation and washed twice with 75% ethanol, air-dried, and dissolved in TE (Tris, a common pH buffer, and EDTA, a molecule chelating cations like Mg^{2+}) buffer.

RAPD PCR amplification. Random 10mer primers were purchased from Sangon (Shanghai) and screened on two randomly selected half-smooth tongue sole individuals. By comparing the effects of magnesium concentrations and annealing temperature during amplification, 18 primers that produced clear reproducible fragments were selected for further analysis (Table 1). RAPD reactions were carried out in a 20-µl reaction volume containing 1 µl template DNA solution (about 50 ng), 0.25 µM primer, 100 µM dATP, dGTP, dCTP, dTTP, each (Promega, USA), 2.5 mM Mg^{2+}, 1 × polymerase buffer (Promega), and 1.0 U Taq DNA polymerase (Promega). PCR amplification reaction was conducted with a Peltier Thermal Cycler (PTC-200). The cycling parameters were 94°C for 5 min; 45 cycles of 30 s at 94°C to denature; 1 min at 36°C to anneal; and 2 min at 72°C to extend. The amplification products were separated on 1.2%

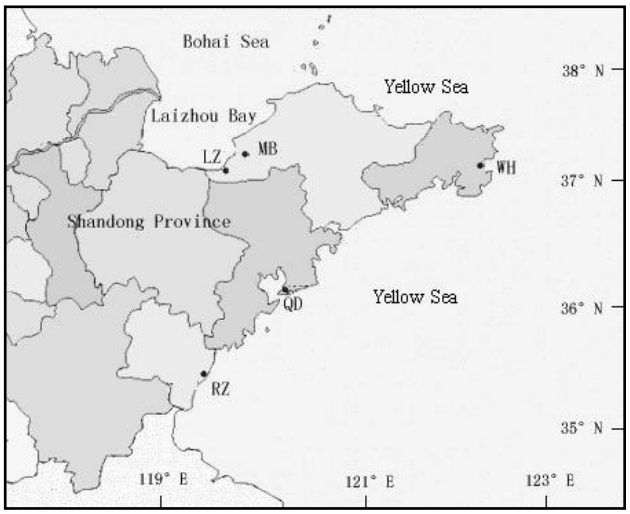

Fig. 1. Location of sampled half-smooth tongue sole (*Cynoglossus semilaevis*) populations examined by RAPD for genetic diversity. LZ = Laizhou Bay, MB = Mingbo hatchery; WH = Weihai; QD = Qingdao; RZ = Rizhao.

agarose gel for 1-2 h at 80 V, and recorded with a digital imager after staining with ethidium bromide (0.5 μg/ml).

Data analysis. RAPD bands were scored as present (1) or absent (0) for each DNA sample by Crosscheck software (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 sample genetic diversity using POPGENE software package (Yeh et al., 1999). Genetic distances between samples were calculated by unbiased distance and similarity measures (Nei, 1978). Analysis of Molecular Variance (AMOVA) was performed to analyze genetic distance among samples using the ARLEQUIN (Schneider et al., 2000). Average heterozygosities and percentages of polymorphic loci were estimated using the TFPGA program (Miller, 1997). Average heterozygosity estimates were calculated for each locus and then averaged over loci by the unbiased heterozygosity formula (Nei, 1978). Percentages of polymorphic loci were estimated based on the percent of loci not fixed for one allele. Significance were tested using *t* test ($p<0.05$ and $p<0.01$). Estimation of pairwise Fst values for sample combinations were performed using the ARLEQUIN program and evaluated by a test analogous to the Fisher's exact test using the Markov-Chain method. Significance was adjusted for multiple comparisons using the sequential Bonferroni correction (Rice, 1989).

Table 1. Primers used for RAPD amplification.

Primer	*Primer sequence*	*Annealing temp (°C)*
S15	GGAGGGTGTT	36
S271	CTGATGCGTG	36
S311	GGAGCCTCAG	36
S312	TCGCCAGCCA	37
S379	CACAGGCGGA	36
S381	GGCATGACCT	37
S407	CCGTGACTCA	37
S431	TCGCCGCAAA	37
S475	GGAAGCCAAC	37
S476	CCAAGCTGCC	37
S477	TGACCCGCCT	37
S479	GGGAAGGACA	35
S483	GGTCACCTCA	36
S486	GAGCGCCTTG	36
S495	GGGTAACGTG	37
S498	AGGCTGGGTG	36
S501	TGCGGGTCCT	37
S503	ACACAGAGGG	36

Results

RAPD polymorphism. RAPD analysis of 100 individuals using the 18 selected primers produced 101 scoreable bands ranging 250-2000 bp, corresponding to an average of 5.61 bands per primer. Every primer produced polymorphic bands. The highest number of bands was produced by S501, and the highest number of polymorphic bands by S271 and S498 (Table 2).

Structure and genetic differences between populations. The Weihai population had the greatest genetic diversity ($p<0.05$) including the most genotypes, as well as the highest number of RAPD bands, polymorphic bands, and average heterozygosity (Table 3). Among the natural populations, the Laizhou population had the lowest number of RAPD bands, polymorphic bands, average heterozygosity, and number of genotypes. All the natural

populations had more RAPD bands than the cultured population (p<0.05). The Shannon index was higher in the natural populations than in the cultured population, but there were no significant differences.

Table 2. Number of bands, polymorphic bands, and RAPD genotypes determined for each population by each primer.

Primer	*Bands (no.)*	*Polymorphic bands (no.)*	*RAPD genotypes (no.)*				
			Laizhou	*Weihai*	*Qingdao*	*Rizhao*	*Mingbo*
S15	5	3	2	3	3	3	2
S271	6	4	4	6	5	4	4
S311	4	2	3	3	3	3	2
S312	6	3	3	4	4	4	3
S379	5	3	2	3	2	2	2
S381	4	1	2	2	2	2	2
S407	6	2	2	3	3	2	2
S431	7	3	3	4	3	3	3
S475	6	3	3	3	3	3	3
S476	5	2	2	3	2	2	2
S477	5	1	2	2	2	2	2
S479	6	3	4	5	4	4	3
S483	5	3	3	3	3	3	3
S486	7	3	4	4	4	4	4
S495	5	3	3	4	3	3	2
S498	7	4	3	5	5	3	3
S501	8	3	5	5	5	5	4
S503	4	2	3	3	2	3	2
Total	101	48	53	65	58	55	48

Table 3. RAPD analysis of genetic variations in five half-smooth tongue sole (*Cynoglossus semilaevis*) populations.

Population	*No. bands*	*Polymorphic bands*		*Avg heterozygosity*	*Shannon index*
		No.	*%*		
Laizhou	90	39	43.33±0.05	0.0735±0.04	0.1127±0.02
Weihai	101	48	47.52±0.09	0.0893±0.02	0.1178±0.06
Qingdao	96	44	45.83±0.06	0.0871±0.03	0.1145±0.03
Rizhao	92	41	44.57±0.04	0.0847±0.04	0.1134±0.05
Mingbo	84	36	42.86±0.05	0.0685±0.01	0.1119±0.02

A UPGMA dendrogram was constructed on the basis of inter sample genetic similarity (Fig. 2). Genetic distances between the samples were determined by the POPGENE program (Table 4). Significant genetic differences were detected by AMOVA (p<0.05). Pairwise Fst values also indicated significant differences among the samples except between Laizhou and Mingbo, and between Qingdao and Rizhao (Table 5).

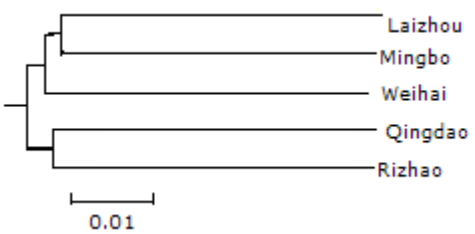

Fig. 2. UPGMA dendrogram showing phylogenetic relationships among five populations of half-smooth tongue sole (*Cynoglossus semilaevis*).

Table 4. Genetic distance (based on Nei, 1978) of five populations of half-smooth tongue sole (*Cynoglossus semilaevis*).

	Laizhou	Weihai	Qingdao	Rizhao	Mingbo
Laizhou	-	-	-	-	-
Weihai	0.0810*	-	-	-	-
Qingdao	0.0834*	0.0792*	-	-	-
Rizhao	0.0886*	0.0859*	0.0778	-	-
Mingbo	0.0765	0.0799*	0.0843*	0.0867*	-

* significantly different according to AMOVA (p<0.05).

Table 5. Fst values for pairwise comparison among five populations of *Cynoglossus semilaevis.*

	Laizhou	Weihai	Qingdao	Rizhao	Mingbo
Laizhou	-	-	-	-	-
Weihai	0.0116*	-	-	-	-
Qingdao	0.0124*	0.0114*	-	-	-
Rizhao	0.0134*	0.0128*	0.0112	-	-
Mingbo	0.0106	0.0118*	0.0126*	0.0130*	-

* significantly different, based on Fisher's technique after sequential Bonferroni correction (p<0.0125).

Discussion

RAPD polymorphism. Half-smooth tongue sole have been aquacultured for several years, but no attempts have been undertaken to assess the genetic status of natural and cultivated stocks. Sequence analysis of specific mitochondrial or ribosomal DNA fragments and multiplex PCR of strain-conserved DNA fragments are efficient for identifying fish strains (Bartlett and Davidson, 1991; Rocha-Olivares, 1998; Gharrett et al., 2001; Noell et al., 2001; Wasko et al., 2001). Compared with DNA sequence methods, however, RAPD fingerprinting is quicker and cheaper (Liu et al., 2004; Hallerman, 2006). In addition, the experimental requirements are lower (Cordes et al., 2001). Thus, RAPD is one of the better approaches for assessing genetic structure among populations, especially in species for which no molecular genetic information is available.

RAPD has the advantage over allozyme analysis for its high reproducibility as well as its power to detect polymorphism. A large number of bands can be produced rapidly with a limited number of primer combinations. A study of genetic variation in Japanese flounder in China using allozyme analysis reported mean heterozygosity values of 0.0788-0.0802 (You et al., 2001). These values were considerably lower (p<0.01) than those (0.2255–0.2739) reported using RAPD (You et al., 2002). The differences are likely due to the ability of RAPD to resolve more loci and detect greater levels of polymorphism than allozyme analysis.

In this study we demonstrated that RAPD is very efficient for determining genetic variations among natural and cultured populations of half-smooth tongue sole. RAPD using 18 primers generated more than 100 fragments for five populations. Within populations, 42.86-47.52% of the fragments were polymorphic. Such a high level of polymorphism shows that RAPD is a powerful technique for elucidating genetic differentiation in half-smooth tongue sole populations and should also be used to assess genetic structures in this species. In fact, we are planning to use RAPD to monitor genetic variability in half-smooth tongue sole selective breeding programs in hatcheries of China.

Genetic differences. Natural populations represent the primary source of genetic variability for aquacultured stocks. Genetic variability is an important attribute of domesticated species since those with greater variation are better able to develop productive traits. Genetic variability of cultured strains is likely characterized by substantial reductions caused by loss of low-frequency loci, probably due to the small number of effective parents in the founding strain. This suggests that the cultured strain is bottlenecked.

In the present study, the Weihai population had the highest diversity among the populations while the Mingbo population had the lowest. The Laizhou population had the lowest diversity among the natural populations. The Fst values of the Qingdao and Rizhao populations did not significantly differ since they are geographically near one another and had more chance for gene flow. The Weihai population was closer in genetic distance to the Qingdao population than the Laizhou population, probably due to the absence of physical barriers to migration between Weihai and Qingdao. The Mingbo population was closer in genetic distance to the Laizhou population, probably because the cultivated population was derived from the Laizhou population.

The Mingbo population was founded using about 30 individuals, but the number of effective parents may have been smaller, suggesting that genetic diversity in the natural population is not fully exploited in the cultured population. Diversity would be enhanced by frequently outcrossing cultivated half-smooth tongue sole with natural half-smooth tongue sole rather than selective breeding of cultivated varieties, followed by inbreeding. Enhancement of genetic variability among cultivated varieties would prevent the unplanned breeding that probably takes place in the present situation. For sustainable culture of half-smooth tongue sole, proper breeding programs must be implemented with careful management and monitoring such that there is frequent outcrossing with natural forms as well as maintenance of newly emerged traits by inbreeding.

Most cultured aquatic stocks represent genetically exogenous populations. Thus, intra-specific hybridization with natural stocks may result in a reduction of fitness in wild populations (Ferguson et al., 1995). Even cultured populations that originated from the same local population may threaten the fitness of the natural population by reducing its effective population size (Ryman et al., 1995), especially when the absolute size of the wild population is small. Significant release of cultured stocks of half-smooth tongue sole into the wild should be prevented.

Acknowledgements

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