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Genetic divergence and population structure of black sea bream *Acanthopagrus schlegeli* inferred from microsatellite analysis

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ABSTRACT: Four highly variable microsatellite loci, *Acs1**, *Acs3**, *Acs4** and *Acs9**, were isolated and then used to investigate genetic diversity and population structure in black sea bream, *Acanthopagrus schlegeli*, collected from wild populations of six locations (western Japan and southern Korea), and hatchery stock and post-stock populations from Hiroshima Bay. When allelic segregation of each microsatellite locus was examined using the single spawning pair and their progeny, all loci were well fitted to the Mendelian manner of inheritance. The genetic variations at these loci in the eight populations revealed high levels of variability. In the wild populations the mean number of alleles per locus was 10.8–13.5, and the mean observed hetrozygosity was 0.755–0.828. In contrast, these values in hatchery stock population were 10.0 and 0.776, respectively. Genetic distance indicates the existence of geographic divergence between western Japan and south Korea, and little genetic differentiation among populations in western Japan.

KEY WORDS: ***Acanthopagrus schlegeli*, black sea bream, genetic variability, Mendelian inheritance, microsatellite, population structure.**

INTRODUCTION

The understanding of genetic divergence and diversity is one of the most important steps in managing fishery resources. A large number of artificially produced offspring have been released into the natural environment for stock enhancement without considering the influence on genetic structure. The broodstocks for seed production have a small gene pool, so the stocked offspring will cause a fluctuation in gene frequencies as well as the decrease of genetic polymorphisms in the wild.[1,2] Tessier *et al.*[3] have reported an extreme case in which stocked offspring caused major genetic drift and a 50% reduction in effective population size of a wild Atlantic salmon population. Therefore the stocking projects should be done with better understanding of genetic variation and divergence.

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Microsatellite markers, tandem arrays of di-, tri- or tetra- nucleotide sequences flanked by regions of unique DNA sequences,[4,5] have proved to be a powerful tool for the assessment of genetic divergence and diversity of many fish.[6–12] Several studies have used microsatellite DNA markers to study the genetic variability of artificially stocked seedlings. For example, studies have been conducted in amberjack,[9] Japanese flounder[10] and red sea bream.[12]

In Japan, black sea bream, *Acanthopagrus schlegeli* are an important commercial fisheries resource and are popular in sport fishing. The total amount, which exceeded 6 million, was stocked in 2001. Several researchers have tried to determine the fish quality and survival rates of this stocking seed.[13–15] However, they could not determine the stock efficiency because of problems in tagging the stocked fish. Therefore highly sensitive genetic markers, such as microsatellite DNA, should be provided as a powerful tool.

The objective of the present paper is to report the isolation and characterization of microsatellite

loci in wild black sea bream from western Japan and Korea. Using these loci, levels of genetic divergence and variation were surveyed in the stocking seed.

MATERIALS AND METHODS

Hatchery stocked samples

Hatchery-stocked black sea bream were produced by the Hiroshima City Marine Products Promotion Association in 1997. They produced 51 broodstock, composed of 29 female and 22 male fish. When the fertilized egg production reached its peak in May, the eggs were collected for 1 day and transferred to a rearing tank. After 2 months' rearing the hatchery stock population was collected prior to release.

A total of 20 000 hatchery stock black sea bream with a mean total length of 4.0 cm, was released into Daio Bay of Nino-shima Island, Hiroshima Bay[13] where stock enhancement projects have been carried out since 1979. The samples of post-stock population were collected 10 days after stocking at the release point. According to the previous study, these samples might be composed of 50% hatchery stock and 50% indigenous recruiters.[13]

Wild samples

Before the stocking was carried out at Daio Bay of Nino-shima Island, indigenous recruiters were collected by a throwing net as a wild population at Hiroshima (Hiroshima pupulation). Other wild populations in western Japan, Kochi (Susaki city, Uchinoura Bay), Mie (Watarai gun, Gokasho Bay), Nagasaki (Konagai city, Isahaya Bay) and Tottori (Sakaiminato city, Miho Bay), were sampled by angling in 2000. These samples ranged from14.9 to 48.0 cm in body length. Wild population were also collected from Yosu city, in southern Korea, in 1999. Because Korea has not performed stock enhancement frequently, the Yosu samples will be a useful control when comparing frequently stocked populations in Japan. Sampling locations in the present study are shown in Fig. 1. Fifty fish were sampled from each location except Nagasaki ($n = 27$).

Microsatellite analysis

The DNA extraction from muscle tissue was carried out by the standard sodium dodecylsulfate (SDS)–phenol/chloroform procedure. The

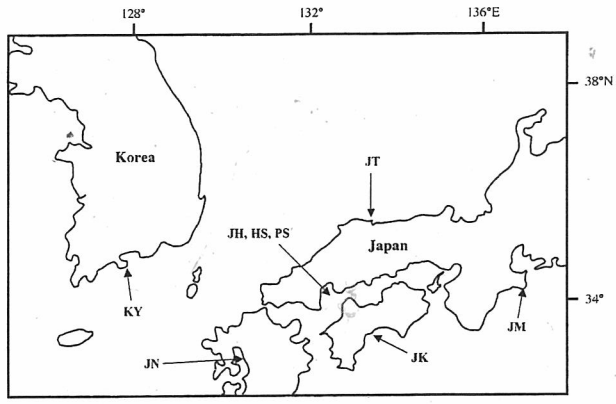


Fig. 1 Map showing sampling locations of black sea bream. KY, Yosu; JH, Hiroshima; HS, hatchery stock; PS, post stock; JK, Kochi; JM; Mie; JN, Nagasaki; JT; Tottori.

extracted DNA were digested with *Sau* 3AI, and fragments ranging between 400 and 600 bp were selected on agarose gel. The size selected fragments were ligated into the *Bam*HI site of pUC 18 plasmid vector. Positive transformations were screened by the chemiluminescence method with $(CA)_{10}$ synthetic biotinylated oligonucleotide. Positive clones were sequenced using ABI PRISM™ 373 A sequencer (PE Applied Biosystems, Japan). Primer sets of flanking microsatellites were designed using the DNASIS software version 3.2 (Hitachi Engineering, Japan).

In every set of primers, the 5′ end of the reverse primer was labeled with biotin. Polymerase chain reaction (PCR) was carried out in 30 cycles of 1 min at 94°C and 30 s at the annealing temperature (Table 1), and then extended by 30 s at 72°C. Each PCR product (2–3 μL) was electrophoresed in 8% polyacrylamide gel (7 M urea, 8% polyacrylamide). After the run, amplified DNA samples were transferred to the nylon membrane, and then detected by the chemiluminescence method (Sequencing high-Cycle; Toyobo, Japan). The size of the alleles was determined according to pUC18 ssDNA as the sequencer ladder.

Microsatellite loci were tested as to whether they inherit according to Mendelian manner or not. In the present study an inheritance experiment was conducted using a single spawning pair and their progeny, at the Hiroshima City Marine Products Promotion Association.

Data analyses

Genetic diversity for each location was evaluated by the number of alleles per locus, the observed heterozygosity (H_o), and the expected heterozygosity (H_e). Genotype frequencies at each locus in

Table 1 Core sequence of microsatellite loci and primers used in the present study

Locus	Core sequence	Primer (5′–3′)	Aneal temperature (C°)	GenBank number
*Acs1**	$(CA)_{13}$	F: TTGCAGCAGATGGGTTCAGA R: GGTCATCTGTATCGACGACT	58	AB102864
*Acs3**	$(CA)_{16}$	F: GGACCCATATGGCTGTCAAA R: TTTATGAGCGACCCTGAGTC	57	AB095008
*Acs6**	$(GT)_{12}$	F: GTCTGACATCATGCTCTGAG R: ACAACCGTCTTCTTCTTACG	56	AB095010
*Acs9**	$(CA)_{21}$	F: GAACTGACAGTAGTGACTGG R: GTGCTGCAGTTTATATCTGC	56	AB095011

F, forward; R, reverse.

Table 2 Results of allelic inheritance in the interbreeding experiment of black sea bream

Locus	Parental genotype		Offspring genotype				χ^2
	Male	Female	No. observed (expected)				
*Acs1**	aa	aa	aa 27 (27.0)				0.00
*Acs3**	ac	bb	ab 13 (13.5)	bc 14 (13.5)			0.04
*Acs6**	ac	bd	ab 7 (6.75)	bd 8 (6.75)	ac 7 (6.75)	cd 5 (6.75)	0.70
*Acs9**	ac	bd	ab 7 (6.75)	ad 8 (6.75)	bc 6 (6.75)	cd 6(6.75)	0.41

each population were tested for conformity to Hardy–Weinberg equilibrium following a Markov chain method. The significance of pairwise Wright's F_{ST} for population genetic difference was tested using AREQUIN version 1.1.[16] The PHYLIP version 3.5 software[17] was used for bootstrapping, genetic distance estimation and dendrogram construction. Allele frequencies were bootstrapped 1000 times by the PHYLIP program. An unweighted pair group method with arithmetic average (UPGMA) dendrogram was visualized with the TREEVIEW program.[18]

RESULTS

Four isolated microsatelitte loci and developed primers set for PCR amplification in black sea bream are shown in Table 1. Inheritance of each microsatellite locus was examined using the single spawning pair and genotypic segregations at the four loci are shown in Table 2. The *Acs1** loci was monomorphic because of only one genotype. The *Acs 3**, *Acs 4** and *Acs 9** loci were polymorphic. Isolated microsatellite in the present study exhibited Mendelian manner of inheritance.

Genetic variations at four microsatellite loci examined in the eight populations are summarized in Table 3. The number of alleles at each loci ranged from 6 to 21. *Acs9** was particularly diverse with 16–21 alleles. The mean number of alleles per locus in the wild populations ranged from 10.8 to 13.5 and was higher than that of the hatchery stock population at 10.0. The mean H_o and H_e ranged from 0.751 to 0.842 and 0.774–0.826, respectively. The H_o of the Tottori population (at 0.751) and Kochi populations (at 0.755) were low among the eight populations. For mean H_e, the Kochi populations had the lowest value. Low levels of H_o/H_e were detected at the *Acs1** loci in the Kochi and Tottori populations.

A deviation from Hardy–Weinberg equilibrium (HWE) assessed by the Markov chain procedure ($P < 0.05$) was observed in the Kochi, Tottori, Yosu, Hiroshima and the post-stock population at locus *Acs1**. The deviation was also observed in Hiroshima and Mie populations at locus *Acs3**, and in the Mie population at locus *Acs9** (Table 3).

Table 4 shows allele frequencies in eight populations. At locus *Acs1**, the allele *123* bp exhibited high frequencies ranging from 0.460 to 0.628 among the western Japan populations, while Yosu population was 0.277 only. Differences can be seen for the major allele *92* bp of locus *Acs3* between both Nagasaki and Kochi

Table 3 Genetic variability of four microsatellite loci in eight populations of black sea bream

Populations	*Acs1**	*Acs3**	*Acs4**	*Acs9**	Mean
Kochi					
No. samples	50	50	50	50	
No. alleles	6	14	7	16	10.8
Heterozygosity (H_o)	0.580	0.800	0.740	0.900	0.755
(H_e)	0.706	0.783	0.702	0.905	0.774
(H_o/H_e)	0.822**	1.022	1.054	0.994	0.975
Mie					
No. samples	50	47	50	50	
No. alleles	8	13	9	21	12.8
Heterozygosity (H_o)	0.660	0.808	0.820	0.880	0.792
(H_e)	0.689	0.802	0.795	0.929	0.804
(H_o/H_e)	0.958	1.007**	1.031	0.947	0.986**
Nagasaki					
No. samples	27	26	26	26	13.0
No. alleles	8	18	7	19	0.820
Heterozygosity (H_o)	0.704	0.846	0.846	0.885	0.826
(H_e)	0.680	0.905	0.791	0.930	0.993
(H_o/H_e)	1.035	0.935	1.070	0.952	
Tottori					
No. samples	47	46	49	49	13.5
No. alleles	10	18	8	18	0.751
Heterozygosity (H_o)	0.553	0.783	0.775	0.894	0.818
(H_e)	0.701	0.870	0.784	0.919	0.918
(H_o/H_e)	0.789**	0.900	0.989	0.973	
Yosu (Korea)					
No. samples	47	48	50	50	12.8
No. alleles	10	14	8	19	0.818
Heterozygosity (H_o)	0.681	0.792	0.800	1.000	0.816
(H_e)	0.748	0.807	0.791	0.919	1.002
(H_o/H_e)	0.910**	0.981	1.011	1.088	
Hiroshima					
No. samples	46	47	48	47	
No. alleles	6	15	6	20	11.8
Heterozygosity (H_o)	0.652	0.808	0.896	0.957	0.828
(H_e)	0.627	0.821	0.774	0.916	0.784
(H_o/H_e)	1.040**	0.984**	1.158	1.045	1.056
Hatchery stock in Hiroshima					
No. samples	47	42	47	49	
No. alleles	6	10	8	16	10.0
Heterozygosity (H_o)	0.574	0.881	0.915	1.000	0.842
(H_e)	0.547	0.812	0.838	0.909	0.776
(H_o/H_e)	1.049	1.085	1.092	1.100	1.085
Post-stock in Hiroshima					
No. samples	48	42	49	45	
No. alleles	8	13	7	17	11.2
Heterozygosity (H_o)	0.521	0.905	0.857	1.000	0.821
(H_e)	0.591	0.822	0.802	0.887	0.775
(H_o/H_e)	0.882**	1.101	1.069	1.127	1.045

H_o, observed; H_e, expected.
**Departure from Hardy–Weinberg equilibrium by Markov chain procedure ($P < 0.05$).

populations, and other populations. The major allele of the *Acs4** locus in Kochi population was 71 bp while that of other populations was 69 bp. The allele *85* bp and *97* bp at *Acs9** locus in Kochi population was different from other populations.

The pairwise F_{ST} test showed that the Kochi population was significantly different ($P < 0.05$) from all the other populations except for the Hiroshima population. Difference between the Nagasaki and Yosu population was also evident (Table 5).

Table 4 Allele frequencies in eight populations of black sea bream

Allele (bp)	Kochi	Mie	Nagasaki	Tottori	Yosu	Hiroshima	Hatchery stock	Post stock
*Acs1**								
109–119	0.100	0.080	0.000	0.022	0.032	0.000	0.011	0.031
121	0.080	0.290	0.148	0.128	0.074	0.130	0.223	0.167
123	0.480	0.460	0.498	0.467	0.277	0.566	0.628	0.604
125	0.180	0.040	0.074	0.234	0.382	0.087	0.053	0.104
127	0.050	0.100	0.204	0.085	0.096	0.163	0.074	0.063
129	0.000	0.000	0.019	0.021	0.117	0.000	0.000	0.021
131–137	0.110	0.030	0.057	0.043	0.022	0.054	0.011	0.010
*Acs3**								
74–88	0.010	0.000	0.096	0.076	0.021	0.011	0.000	0.000
90	0.090	0.074	0.193	0.130	0.156	0.160	0.107	0.107
92	0.420	0.340	0.058	0.283	0.344	0.351	0.345	0.357
94	0.060	0.191	0.019	0.043	0.198	0.096	0.155	0.143
96	0.110	0.149	0.135	0.098	0.052	0.085	0.083	0.036
98–128	0.200	0.235	0.346	0.218	0.155	0.244	0.227	0.204
*Acs4**								
59–65	0.040	0.120	0.058	0.154	0.060	0.042	0.191	0.133
67	0.070	0.100	0.096	0.214	0.080	0.104	0.191	0.133
69	0.220	0.340	0.135	0.326	0.280	0.219	0.160	0.214
71	0.470	0.190	0.308	0.214	0.220	0.312	0.245	0.133
73	0.140	0.190	0.269	0.082	0.260	0.250	0.149	0.316
75–77	0.060	0.060	0.134	0.010	0.100	0.073	0.064	0.071
*Acs9**								
71–81	0.180	0.270	0.212	0.347	0.230	0.180	0.101	0.145
83	0.130	0.100	0.058	0.071	0.140	0.074	0.082	0.033
85	0.170	0.050	0.038	0.071	0.080	0.117	0.133	0.067
87	0.060	0.080	0.077	0.153	0.120	0.021	0.071	0.056
89	0.000	0.080	0.135	0.092	0.080	0.160	0.133	0.256
91	0.010	0.040	0.077	0.092	0.070	0.117	0.133	0.133
93–119	0.450	0.380	0.403	0.174	0.280	0.331	0.347	0.310

Table 5 Pairwise comparison of F_{ST} in eight populations of black sea bream

Populations	Kochi	Mie	Nagasaki	Tottori	Yosu	Hiroshima	Hatchery stock
Kochi							
Mie	0.0325**						
Nagasaki	0.0259**	0.0158					
Tottori	0.0370**	−0.0006	0.0285				
Yosu	0.0274**	0.0001	0.0022**	0.0085			
Hiroshima	0.0227	0.0127	−0.0011	0.0242	0.0036		
Hatchery stock	0.0371**	0.0199	0. 0077	0.0136	0.0127	0.0034	
Post stock	0.0720**	0.0216	0.0085	0.0386	0.0139	0.0098	0.0148

**Significance level at $P < 0.05$.

A UPGMA dendrogram based on Nei's genetic distance is shown in Fig. 2. No geographic relationship and genetically differentiated populations were found in the western Japan populations. However, slight genetic divergence was found between the Yosu and the western Japan populations. The hatchery stock and post-stock population exhibited little genetic distance from the wild Hiroshima population.

DISCUSSION

Microsatellites DNA markers are more polymorphic than isozymes in the analysis of genetic diversity. For example, in analysis of wild and hatchery-reared black sea bream with 42 isozyme loci, the number of alleles per locus ranged from 1.095 to 1.35 and the H_o ranged from 0.048 to 0.066.[19] In contrast, the four microsatellite loci

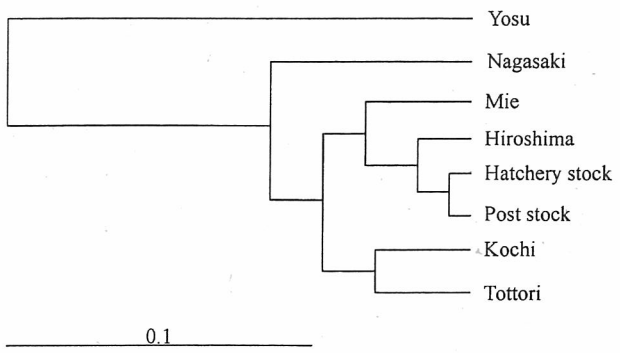


Fig. 2 UPGMA dendrogram of western Japan and southern Korea based on Nei's genetic distance.

used in the present study had a mean number of alleles per locus ranging from 10.0 to 13.5 and had a H_o between 0.751 and 0.842. On the microsatellite performance in black sea bream, the number of alleles per locus was fewer than that observed in red sea bream[2] and Japanese flounder[10] but the H_o was similar to those reported in the two species.

For the hatchery stock population in Hiroshima Bay, the mean H_o and H_e were 0.842 and 0.776, respectively. These values almost coincided with those estimated in wild populations. Based on the H_o and H_e, hatchery stock population seems to be conserved at a reasonable level of genetic variation. However, the lowest mean number of alleles per locus in hatchery stock population was noted, comparing with those in the wild populations. Therefore, to evaluate genetic diversity using microsatellite, the mean number of alleles per locus is a better indicator than H_o and H_e. The lowest value of the mean number of alleles per locus in hatchery stock population may be accounted for by the small number of broodstock ($n = 51$; composed of 29 female and 22 male fish).

A significant deviation from HWE was observed in some microsatellite loci of the western Japan populations with excess homozygosity. It is suggested that a large number of stocked fish might cause a mixing with indigenous populations and thus each population could include several separately spawning subpopulations. This situation was also suggested in red sea bream.[8] The explanation of mixing of stocked population with the indigenous population can be partly supported by the Kochi population. Here, 30% of them had a deformity in the internostril epidermis, which often arises under hatchery-reared conditions.[20]

For genetic divergence in the wild population in western Japan, the significantly different F_{ST} ($P < 0.05$) was detected only for the Kochi population, although the reason is not clear at present. As the mentioned here, it might be caused by a large number of stocking seed with genetic drift. Overall, according to the results of F_{ST} and genetic distance on the dendrogram, black sea bream in western Japan have little geographic divergence. This conclusion is the same as that reported by Taniguchi *et al.*, in which three natural populations were not different by the homogeneity test using 42 isozyme loci.[19] Kijima and Fujio also pointed out relatively little genetic differentiation in black sea bream among Japanese marine teleosts.[21] A question arises as to why black sea bream in western Japan could have little geographic divergence. This may be attributed to the random disperal of pelagic eggs and larvae, and extensive migration habit of the adult fish. The extensive migration of black sea bream was demonstrated by ultrasonic telemetry (Fukuda T, pers. comm., 2000).

The significantly different F_{ST} was seen in the Yosu and Nagasaki populations. Moreover, there was relatively large genetic distance on a dendrogram between Yosu population and the western Japan populations. These results partly support the existence of genetically differentiated populations between western Japan and southern Korea. Further research will be required for better understanding of the genetic structure of the wild population of black sea bream, using a larger number of samples from more sampling locations.

Focusing on the hatchery stock and post-stock populations, there was little increase in mean number of alleles per locus from the hatchery levels and a decrease from wild levels in the post-stock population. This can be partly explained by the mixing between the two populations. In fact, according to the mark-tracing experiment at Daio Bay of Nino-shima Island, the mixing rate of hatchery stock seed to the wild reached more than 50% at 10 days after stocking.[13] The negligible genetic distance between hatchery stock and post-stock population indicates that microsatellites developed in the present study have strong possibilities as a stock separation tool. From the viewpoint of conservation genetics, it is noteworthy that the little genetic distance between the hatchery stock and the wild Hiroshima population was marked.

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