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Genetic diversity of wild and farmed black sea bream populations in Jeju

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Black sea bream, *Acanthopagrus schlegelii*, is a commercially important fish in Korea. As a preliminary investigation into the effect of hatchery rearing for stock enhancement, we examined genetic diversity between wild and farmed black sea bream populations from Jeju using six microsatellite markers. High levels of polymorphism were observed between the two populations. A total of 87 different alleles were found at the loci, with some alleles being unique. Allelic variability ranged from 8 to 22 in the wild population and from 7 to 17 in the farmed one. Average observed and expected heterozygosities were estimated at 0.87 and 0.88 in the wild sample. The corresponding estimates were 0.83 and 0.86 in the farmed sample. Although a considerable loss of rare alleles was observed in the farmed sample, no statistically significant reductions were found in heterozygosity or allelic diversity in the farmed sample, compared with the wild one. Significant genetic heterogeneity was found between the wild and farmed populations. These results suggest that more intensive breeding practices for stock enhancement may have resulted in a further decrease of genetic diversity. Thus, it is necessary to monitor genetic variation in bloodstock, progeny, and target populations and control inbreeding in a commercial breeding program for conservation. This information may be useful for fisheries management and the aquaculture industry.

Keywords: black sea bream; *Acanthopagrus schlegelii*; microsatellite; genetic diversity; stock enhancement

Introduction

Black sea bream (*Acanthopagrus schlegelii*) is widely distributed, from the South China Sea to the coastal waters of Japan and Korea, and is a commercially valuable recreation and aquaculture fishery species in Pacific Asia. In Korea, artificial breeding methods have been developed to enhance black sea bream resources, and large quantities of cultured fry have been released into the Korean coastal sea at a number of prefecture government and private hatcheries since 2002. In Jeju, approximately 300,000 seeds have been released into the area around Jeju Island each year for the past two years; thus, it can be considered that the extent to which stocking impacts the wild population is small or little in the case of fish over 25 cm length (three years of age).

The genetic diversity of artificial seeds is generally lower than that of the wild population, due to unconscious selection and a limited number of parental broodstock in the hatchery. Thus, large-scale stocking may cause a reduction in genetic diversity in wild populations. This reduction invariably has the potential to result in the loss of genetic variation for disease resistance and reduce the capability of the population to adapt to new environments (Liu et al. 2005). The Food and Agriculture Organization of the United Nations (FAO) recommends genetic characterization of both the stocking population used in population enhancement programs and the target population, so any genetic change can be monitored for the sustainable management of exploited fish populations (FAO 1993). Thus, when conducting artificial stocking, the genetic variation between hatchery and wild populations and how this variation is maintained during hatchery rearing should be monitored from the initiation of stocking in each target species for successful management and sustainable use of the species in the future (Ståhl 1987; Primmer et al. 1999).

Many studies have reported the reduction in genetic variability, derived from limited numbers of breeders under hatchery conditions (Coughlan et al. 1998; Sekino et al. 2002; Hara and Sekino 2003; Jackson et al. 2003; Jeong et al. 2003; Nugrohoa and Taniguchi 2004; Skaala et al. 2004). To date, however, a complete genetic comparison of the wild black sea bream population to the hatchery one for stock enhancement in Korea based on high-resolution DNA markers has not been carried out.

Recent studies have demonstrated the strength of microsatellite DNA markers for monitoring changes in genetic variation of farmed stocks, parentage

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assignment, and fine-scale studies of population structure in marine species (Norris et al. 2000; Desvignes et al. 2001; Holland 2001; Alarcón et al. 2004; Li et al. 2004; An et al. 2008). Novel microsatellite markers have been characterized in wild black sea bream and have been found to be highly polymorphic (Jeong et al. 2003, 2007).

The aim of this preliminary study was to assess the genetic diversity in samples of wild black sea bream in Jeju, Korea, using microsatellite markers, and to compare the results with those observed in a hatchery undergoing stock enhancement. These data are necessary for founding and maintaining cultivated stocks in relation to stock enhancement with the aims of conserving diversity and minimizing inbreeding.

Materials and methods

Sample collection and DNA extraction

Samples of fin-clip tissue (approximately 1 cm^3) were obtained from wild caught black sea bream ($n = 50$; fish length, over 25–30 cm) from the coast of Jeju island, Korea, in 2008. This wild sample should represent the natural condition, uncontaminated by transplantation or stocking. Similarly, 50 black sea bream individuals were sampled from a hatchery-reared strain (fish length 5–6 cm), bought by the Jeju special self-governing province for a stock enhancement program in 2008. No details regarding the founding and maintenance of the farmed strain are available; however, their original parents were held at a farm on the southern coast of Korea. The tissues were stored in 2 mL 99% ethanol at 4°C until DNA extraction. Total DNA from each sample was extracted using a MagExtractor-genomic DNA purification kit (Toyobo). The DNA extractions were performed according to the manufacturer's recommendations for an automated DNA extraction system, MagExtractor MFX-2100 (Toyobo). The extracted genomic DNA was stored at −20°C until genotyping.

Microsatellite genotyping

Six highly variable previously characterized microsatellite loci (ACS4, ACS6, ACS9, ACS16, ACS17, ACS21) were selected for the present study (Jeong et al. 2003, 2007). A total of 100 black sea bream from the two populations were typed. Primer sequences, microsatellite repeat sequence, and the optimal annealing temperature for each locus are listed in Table 1. The forward primer from each primer set was 5′-fluorescent labeled with one of three dyes: 6-FAM, HEX, or NED (PE Applied Biosystems). PCR amplification of six microsatellite loci was carried out using an RTC 200 instrument (MJ Research) in 10 μL of solution containing 10–50 ng DNA, 1 × ExTaq buffer, 0.2 mM dNTPs, 10 pmol of each primer, and 0.25 U Taq DNA polymerase (Takara). The amplification protocol included an initial denaturation for 11 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at the optimal annealing temperature, and 1 min at 72°C, with a final extension step of 5 min at 72°C. For genotyping, 1 μL PCR product was added to each reaction containing formamide with a size standard GeneScan-400HD [ROX] (PE Applied Biosystems) and electrophoresed, using an ABI3130 DNA sequencer (PE Applied Biosystems). The fragment length of the PCR products was determined using the GeneMapper software (Version 4.0, PE Applied Biosystems).

Statistical analyses

To analyze the variation in microsatellite loci in the samples, the number of alleles per locus (N_A), allelic

Table 1. Six microsatellite marker sequences of black sea bream (*Acanthopagrus schlegelii*) used in this study, core repeats, and their specific annealing temperatures used in PCR amplification.

Locus	Repeat motif	Primer sequence (5′→3′)	T_a (°C)*	Genebank accession no.
ACS4	(GT)12	F: TTTACACACCGGGAGCTCAA R: GTAAAGATCCATGGAGGTGC	57	AB095009
ACS6	(GT)12	F: GTCTGACATCATGCTCTGAG R: ACAACCGTCTTCTTCTTACG	56	AB095010
ACS9	(CA)21	F: GAACTGACAGTAGTGACTGG R: GTGCTGCAGTTTATATCTGC	56	AB095011
ACS16	(CA)28	F: ACCAAGGACCCTTGTGAATG R: GCATGTCTGCAGCAACAGCA	53	AB095012
ACS17	(CA)24	F: ACAGGCGCATGTGTTTATGC R: ATCTCTCCGTATCTTCCTCC	64	AB095013
ACS21	(CA)12	F: CGGAAAGGAAGCAGCTCTTG R: AACTGTTAGCCAGGGTCAGC	64	AB095014

*T_a is the optimal annealing temperature.

richness (A_R), allele size range (S), frequency of the most common allele (F), number of unique alleles (U), and gene diversities (Nei 1987) were determined for each population at each locus, using the program FSTAT version 2.9.3.2 (Goudet 2002). The A_R measures the number of alleles independent of sample size. Effective number of alleles (a_e; Kimura and Crow 1964) was estimated based on the formula $a_e = 1/\sum x_i^2$, where x_i is the frequency of the ith allele for each locus. To estimate the genetic heterozygosity between the population samples, unbiased expected and observed heterozygosity values (H_e and H_o, respectively; Nei 1987) were calculated using the ARLEQUIN 2.0 (Schneider et al. 2000) software. Deviations from Hardy–Weinberg equilibrium (HWE) for each locus or globally across populations and loci were tested using probability tests or exact tests by the Markov-chain procedure.

The extent of population subdivision was examined by calculating fixation indices. Differentiation between populations was characterized using F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995; Michalakis and Excoffier 1996) estimates calculated with the computer program GENEPOP (version 3.4, over the internet; Rousset and Raymond 1995). R_{ST} incorporates the correlation of the weighted mean allele size expressed as the number of tandem repeats. Values of inbreeding coefficients (F_{IS}), F_{ST} and R_{ST} were tested for significant departure from zero, using 5000 permutations of alleles among individuals within samples and among samples, and of genotypes among samples, respectively. The sequential Bonferroni correction was applied to derive significance levels for the analysis involving multiple comparisons (Rice 1989).

Results

Genetic variation within samples

Samples of 50 wild black sea breams and 50 farmed ones were screened for variation at six microsatellite loci. Samples failing to amplify the first time were reamplified once. All six microsatellite loci were polymorphic in all samples of black sea bream, and levels of polymorphism varied depending on the locus. Genetic variability in each population is shown in Table 2. A total of 87 different alleles were observed at the six loci, and in each population some alleles were unique. The number of alleles per locus varied from seven at locus ACS6 to 22 at locus ACS9, with the average number of alleles being 12.9. Fewer alleles were found in the farmed sample, compared with the wild one. Overall allelic richness varied from 6.70 to 20.32, with the wild population showing higher allelic richness than the farmed one. Compared with the number of observed alleles, the effective number of alleles at each locus was lower, indicating that some alleles were abundant at one locus. Levels of observed (H_o) and expected (H_e) heterozygosity were high for two samples. The average of observed and expected heterozygosity ranged from 0.76 at ACS4 to 0.96 at ACS16, and from 0.81 at ACS4 to 0.92 at ACS16, respectively. No significant difference was found between samples at most loci in the observed allelic number and the average observed and expected heterozygosity.

The mean gene diversities (G_d) ranged from 0.81 for ACS4 to 0.92 for ACS9 and ACS16 in the two populations. The ranges of allele sizes (S) and number of unique alleles (U) are shown in Table 2. In total, 19 alleles were found to be unique to the two populations: 16 in wild samples and three in farmed. In most loci, except ACS4, the wild black sea bream had a higher number of unique alleles than the farmed fish.

Allele frequencies at all six selected loci in each sample are shown in Table 3 and Figure 1. These data reveal differences between samples. At ACS4, a total of 10 alleles were observed; the 60 allele was observed only in the wild samples, and the 62 and 76 alleles only in the farmed samples. At ACS6, in total, nine alleles were observed, and the major allele was 98. At ACS9, a total of 22 alleles were observed; the major allele was 78. The 64, 66, 96, 102, and 108 alleles were observed only in the wild samples, and no unique allele was seen in the farmed samples. At ACS16, in total, 18 alleles were observed. The 94, 114, and 118 alleles were observed only in the wild samples, and the 116 only in the farmed samples. At ACS17, in total, 12 alleles were observed. The 134 and 152 alleles were observed only in the wild samples, and no unique allele was seen in the farmed samples. At ACS21, in total, 16 alleles were observed. The 90, 104, and 118 alleles were observed only in the wild samples, and no unique allele was seen in the farmed samples.

Inbreeding coefficients (F_{IS}) varied between the markers, from -0.073 (ACS16) to 0.110 (ACS9) in the farmed sample and from -0.051 (ACS17) to 0.082 (ACS4) in the wild sample. Average F_{IS} including all markers was 0.039 in the farmed line and 0.012 in the wild sample. Significant departures from HWE ($P < 0.05$) in the direction of heterozygote deficiency were observed in two (at ACS6 and ACS21 for the farmed population) of 12 single-locus exact tests after sequential Bonferroni corrections. However, the inbreeding coefficient F_{IS} showed that ACS6 for the wild samples and ACS17 for the farmed samples also departed from zero ($P < 0.05$), which is a heterozygosity deficit condition (Table 2).

Genetic variation between samples

Genetic differentiation between two black sea bream populations in Jeju, Korea, was estimated using F_{ST}

Table 2. Allelic variability observed at six microsatellite loci in wild and hatchery *Acanthopagrus schlegeli* samples.

	Microsatellite loci						
Population (No)	ACS4	ACS6	ACS9	ACS16	ACS17	ACS21	Mean
Jeju captured ($n=50$)							
N_A (a_e)	8 (4.93)	9 (5.46)	22 (14.45)	17 (12.20)	12 (7.52)	16 (8.18)	14.00 (8.79)
A_R	7.95	8.59	20.32	15.95	11.2	14.67	13.11
G_d	0.81	0.83	0.94	0.93	0.88	0.89	0.88
S	60–78	90–118	64–110	86–120	132–158	86–126	
R	18	28	46	34	26	40	32.00
F	0.280	0.300	0.140	0.130	0.200	0.110	0.193
U	1	2	5	3	2	3	2.67
H_e	0.805	0.825	0.940	0.927	0.876	0.887	0.877
H_o	0.740	0.800	0.940	0.940	0.920	0.860	0.867
F_{IS}	0.082	0.031	0.000	−0.014	−0.051	0.030	0.012
	(0.430)	(0.010)	(0.422)	(0.900)	(0.381)	(0.185)	
P	0.000	0.427	1.000	0.000	0.000	0.313	
Jeju cultured ($n=50$)							
N_A (a_e)	9 (4.98)	7 (4.98)	17 (8.98)	15 (10.48)	10 (6.68)	13 (7.42)	11.83 (7.25)
A_R	8.67	6.7	15.69	14.32	9.39	11.98	11.13
G_d	0.81	0.81	0.90	0.91	0.86	0.88	0.86
S	62–78	94–108	66–110	86–120	132–158	86–126	
R	16	14	42	34	26	40	28.67
F	0.340	0.320	0.190	0.160	0.250	0.240	0.250
U	2	0	0	1	0	0	0.50
H_e	0.807	0.807	0.898	0.914	0.859	0.874	0.860
H_o	0.780	0.800	0.800	0.980	0.800	0.800	0.827
F_{IS}	0.034	0.009	0.110	−0.073	0.069	0.085	0.039
	(0.619)	(0.001)	(0.119)	(0.102)	(0.029)	(0.005)	
P	0.522	0.000	0.000	0.000	0.326	0.000	
Mean all populations							
N_A (a_e)	8.5 (4.95)	8 (5.22)	19.50 (11.71)	16.00 (11.34)	11.00 (7.10)	14.50 (7.80)	12.92 (8.02)
A_R	8.31	7.65	18.01	15.14	10.30	13.33	12.12
G_d	0.81	0.82	0.92	0.92	0.87	0.88	0.87
S	17.00	21.00	44.00	34.00	26.00	40.00	30.33
R	1.50	1.00	2.50	2.00	1.00	1.50	1.58
H_e	0.806	0.816	0.919	0.921	0.867	0.880	0.868
H_o	0.760	0.800	0.870	0.960	0.860	0.830	0.847

Note: Number of alleles per locus (N_A), effective number of alleles (a_e), allellic richness (A_R), gene diversity (Gd), size in bp of alleles (S), allelic size range (R), frequency (F) of the most common allele, number of unique alleles (U), expected heterozygosity (H_e), observed heterozygosity (H_o), inbreeding coefficient (F_{IS}), and probability of significant deviation from Hardy–Weinberg equibrium (P) are given for each population and locus.Calculations assume that individuals with one microsatellite band are homozygous for the allele. Number in parenthesis below F_{IS} indicates the probability of significant heterozygosity excess.

and R_{ST} estimates. The global multilocus F_{ST}, including all loci, was estimated to be 0.0105 ($P<0.01$), and the R_{ST} value was 0.0327 ($P<0.01$). The significant F_{ST} and R_{ST} estimates indicate genetic differentiation between these farmed and the wild black sea bream samples.

Discussion

All six microsatellite loci screened in this study were polymorphic in all samples analyzed. There was no indication that genotyping errors affected allele scoring (e.g., allele dropouts or stuttering) at any of the markers in any of the samples. Some observed peak patterns typically contained stuttering. However, as alleles were determined on the basis of relatively higher intensity, the stutter peak did not interfere with accurate scoring or genotype designation. Samples failing to amplify the first time were reamplified once. However, samples that failed to amplify were rare. Additionally, no sample failed to amplify at more than

Table 3. Frequency of each microsatellite allele in wild and hatchery *Acanthopagrus schlegeli* samples in Jeju of Korea.

Locus	Allele	Jeju captured	Jeju cultured	Locus	Allele	Jeju captured	Jeju cultured
ACS4	60	0.030	0.000	ACS16	86	0.010	0.010
	62	0.000	0.040		88	0.050	0.030
	64	0.090	0.080		90	0.120	0.050
	66	0.170	0.150		92	0.130	0.070
	68	0.280	0.340		94	0.030	0.000
	70	0.280	0.210		96	0.070	0.030
	72	0.030	0.070		98	0.090	0.090
	74	0.070	0.070		100	0.070	0.100
	76	0.000	0.010		102	0.090	0.160
	78	0.050	0.030		104	0.060	0.060
					106	0.010	0.030
ACS6	90	0.130	0.000		108	0.040	0.040
	94	0.030	0.060		110	0.080	0.070
	96	0.210	0.140		112	0.090	0.120
	98	0.300	0.230		114	0.010	0.000
	100	0.110	0.080		116	0.000	0.010
	102	0.080	0.320		118	0.030	0.000
	104	0.110	0.110		120	0.020	0.130
	108	0.020	0.060				
	118	0.010	0.000	ACS17	132	0.180	0.120
					134	0.020	0.000
ACS9	64	0.020	0.000		136	0.110	0.140
	66	0.010	0.000		138	0.030	0.060
	68	0.030	0.010		140	0.140	0.250
	70	0.090	0.110		142	0.110	0.100
	72	0.060	0.020		144	0.200	0.160
	74	0.080	0.110		146	0.100	0.110
	76	0.040	0.060		148	0.070	0.040
	78	0.140	0.180		150	0.010	0.010
	80	0.090	0.190		152	0.010	0.000
	82	0.050	0.080		158	0.020	0.010
	84	0.050	0.030				
	86	0.060	0.010	ACS21	86	0.010	0.010
	88	0.050	0.020		88	0.110	0.080
	90	0.020	0.050		90	0.110	0.000
	92	0.070	0.050		92	0.040	0.090
	94	0.010	0.030		94	0.030	0.110
	96	0.020	0.000		96	0.080	0.080
	100	0.050	0.020		98	0.090	0.110
	102	0.020	0.000		100	0.040	0.010
	104	0.020	0.020		102	0.260	0.240
	108	0.010	0.000		104	0.060	0.000
	118	0.010	0.010		114	0.010	0.010
					116	0.050	0.030
					118	0.020	0.000
					120	0.010	0.020
					124	0.070	0.170
					126	0.010	0.040

one locus (data not shown), and this makes it unlikely that poor DNA quality affected our results.

Molecular genetic diversity in fish has been found to be associated with life history traits reflecting habitat types (DeWoody and Avise 2000). In this study, high levels of polymorphism (mean heterozygosity = 0.87; mean allelic number = 12.9) were present in all six microsatellite loci examined. These values are considerably higher than those revealed by allozyme analysis (heterozygosity range = 0.048–0.066; allelic number range = 1.095–1.35) (Taniguchi et al. 1983). The levels of genetic diversity of *A. fasciatus* were similar to those

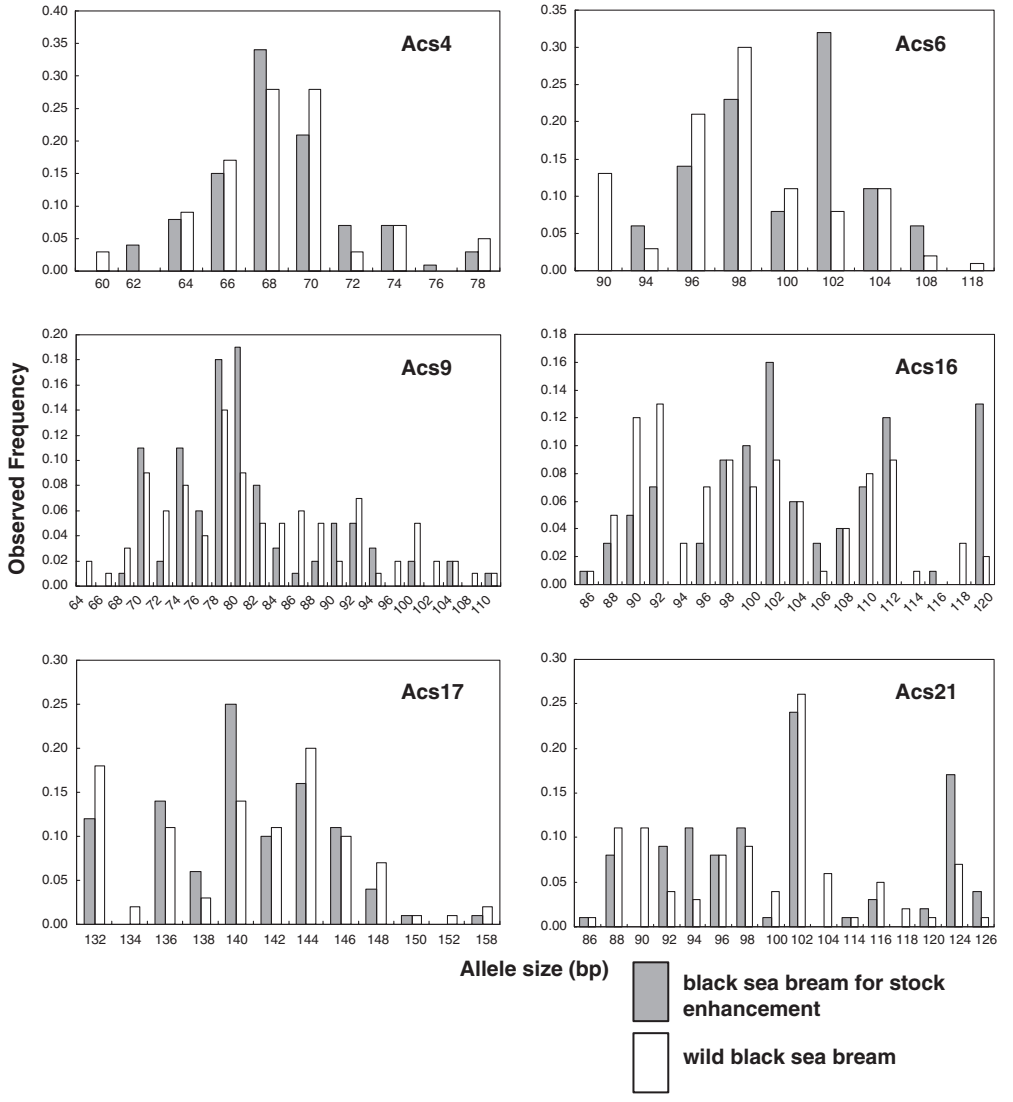

Figure 1. Allele size frequency distributions of the six microsatellite loci of *Acanthopagrus schlegelii* populations.

reported in the previous study (Jeong et al. 2003). They surveyed four microsatellite loci using about 400 *A. fasciatus* individuals in eight groups from coastal waters of Japan including Yeosu, Korea, and found that in the sample from Yeosu, Korea, the average number of alleles per locus was 12.8 and the average expected heterozygosity was 0.82. However, compared with those of other marine fisheries, they were slightly higher in genetic diversity and relatively lower in allele number per locus (mean heterozygosity = 0.79; mean allelic number = 20.6) (DeWoody and Avise 2000).

When the level of diversity in hatchery stocks was compared with that in the wild populations, on average 82.8% of the allelic richness observed within the wild samples was present in the hatchery samples, and the hatchery population displayed a slightly lower level of heterozygosity. However, no significant difference in the average number of alleles per locus or average expected heterozygosity was observed. High genetic variability in farmed stocks has also been reported for salmonids and rock bream (Elliott and Reilly 2003; An et al. 2008). Lower allele richness observed in the farmed samples may be caused by hatchery selection and inbreeding. Reduced variability in farmed strains has been described in other cultivated fish species (Porta et al. 2006). Tessier et al. (1997) 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. Continued intensive breeding practices for stock enhancement might lead to decreased genetic variability if the number of effective breeders is not maintained. To maintain levels of genetic variation within farmed strains comparable to wild source populations, good

broodstock management practices are required. Hence, continued genetic monitoring of the bloodstock is warranted in artificial seed production.

Significant deviations from Hardy–Weinberg equilibrium (HWE) in the direction of deficiencies of heterozygotes were detected in the hatchery collection at two of the six microsatellite loci, even after sequential Bonferroni correction for multiple tests. Additionally, significant inbreeding coefficient values were detected at two loci, ACS6 for the wild sample and ACS17 for the farmed sample, in a heterozygosity deficit condition. Relevant heterozygote deficiencies have been reported for many fish species (Waldman and McKinnon 1993; Hoarau et al. 2002). General causes for such deviations include substructuring of the population sample, inbreeding, or the presence of null alleles. From results of previous studies, null alleles seem to be a likely explanation (Ardren et al. 1999; Banks et al. 1999). However, admixture of more than two independent populations, nonrandom mating, or artificial selection forces during seed production and cultivation might account for the deviation from HWE in the hatchery population.

There was significant genetic differentiation detected by F_{ST} and R_{ST} values between the wild and hatchery black sea bream samples. This difference is likely to be a result of the reduction in the effective number of contributing parents and effects of artificial selection on the hatchery progeny.

A desirable characteristic of a stock enhancement program is to release genetically similar fish to the wild population. For black sea bream in Jeju of Korea, the progeny produced for release showed a different genetic composition, with considerable loss of rare alleles observed, but no statistically significant reduction was found in mean heterozygosity or diversity compared with the wild population. In fact, the loss of alleles is more important than the change in allele frequencies, because the latter can be changed again by random drift, but there is no way to recover a lost allele. For the proper management of stock enhancement programs, genetic monitoring for structure and diversity has to be considered in addition to biological, ecological, and fishery factors. Thus, samples from the wild population should be taken and analyzed with genetic markers before the fish are used as bloodstock. A sample of the hatchery-reared fish should then be taken for genetic analysis. This information will be useful to evaluate the feasibility of the enhancement program to maintain the genetic diversity of the wild population as well as to improve the management of the hatchery for the following season.

In summary, genetic diversity analysis revealed changes in the genetic composition between the hatchery and wild black sea bream populations in Jeju, Korea. The hatchery black sea bream produced for release in a stock enhancement program showed some level of genetic differentiation, compared with wild samples. Thus, for an adequate stocking strategy of black sea bream, periodic genetic evaluations of bloodstock, progeny, and target populations are necessary, not only in Jeju, but also in other provinces of Korea. Such genetic monitoring can be useful for the preservation and further genetic management of the Korean black sea bream.

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