J. Fish. Sci. Technol. 11(2), 82-87, 2008

JOURNAL OF
FISHERIES SCIENCE AND TECHNOLOGY
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Investigation of Genetic Diversity between Wild-caught and Hatchery-reared Rock Bream (*Oplegnathus fasciatus*) Using Microsatellite DNA Analysis

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Marine fisheries are important natural resources and must be maintained, especially fish species that are important sources of food. Despite the increase in stocking programs to maintain fisheries with artificially raised fish, the genetic impact stocking has on the wild fry population has not been addressed. Genetic variation in rock bream, *Oplegnathus fasciatus*, within and between wild-caught parents and the F_1 generation produced by them in 1 day was assayed using nine highly variable microsatellite markers. The nine microsatellite loci used in this study displayed diverse polymorphisms, and in total, 98 different alleles were observed over all loci. Differences in genetic variability of the F_1 offspring compared to their wild-caught parents (brood stock) were observed in terms of allele frequency, gene diversity, and heterozygosity. Although the F_1 generation of rock bream was missing 16% of the microsatellite alleles, no significant reduction was found in mean heterozygosity of the F_1 population compared to the brood stock. Eight of nine loci showed significant Hardy-Weinberg equilibrium (HWE) deviations in the F_1 population, while the brood stock deviated from HWE at three microsatellite loci (KOF85, KOF360 and KOF374). These deviations showed mostly a deficit of heterozygotes. Our results provide evidence for genetic differences in the F_1 hatchery offspring compared to their wild-caught parents and reinforce the need for a series of consecutive egg collections to avoid the loss of genetic variability. This also further underscores the importance of monitoring genetic variability of hatchery populations for the conservation of natural rock bream resources.

Key words: Rock bream, Genetic diversity, Brood stock, Offspring, Microsatellite marker

Introduction

Microsatellite markers are used in analyses of genetic diversity within species, population genetic structure, and genomic mapping of fishes. This technique has become a powerful tool and is very useful in many areas of fish genetics and breeding. Microsatellites have successfully been used to analyze genetic variation of several aquaculture fishes includeing Atlantic salmon (Verspoor, 1988; Norris et al., 1999), barramundi (Frost et al., 2006), carp (Hansen et al., 2006), red sea bream (Perez-Enriquez et al., 1999), and Japanese flounder (Sekino et al., 2002; Liu et al, 2005). As a consequence of increased artificial fry production, the potential genetic impact of the release of hatchery-reared fish on wild fish stocks is a growing concern. Previous studies have reported that hatchery practices can have a negative impact on the genetic diversity of hatchery populations when a small number of individuals are used for the brood stock, the contribution of each parent is unbalanced, or related individuals are mated (Coughlan et al., 1998; Jeong et al., 2006; Sekino et al., 2002). Most hatchery stocks typically show reduced genetic variability, which may result in the loss of disease resistance or in the reduction of the population's capability to adapt to new environments (Allendorf and Phelps, 1980). Thus, genetic monitoring of wild populations and hatchery stocks is recommended to preserve genetic variation in wild populations (FAO, 1993). The rock bream, *Oplegnathus fasciatus,* inhabits costal rocky reefs near southern Korea and is one of the most economically important fisheries

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resources in Korea. Because this species has a good prospect for culture as a sedentary high-class fish, artificial breeding has been employed to enhance resources. Although numerous cultured fry of rock bream have been released into Korean coastal waters, there is currently no information on how hatchery practices impact the levels of genetic diversity in this species. This study was conducted to assay the genetic differences within and between wild-caught rock bream and their F_1 offspring based on nine microsatellite DNA markers. Our results present the loss of genetic variability of hatchery strains and the potential uses of microsatellite DNA markers for the further genetic monitoring of hatchery strains.

Materials and Methods

Sample collection and DNA extraction

Wild rock bream were collected in Jeju coastal waters in early 2003 and reared in the hatchery for reproduction. The eggs produced by these fish were collected for 1 day and transferred to a rearing tank. We sampled part of the wild-caught brood stock (n=93) and their first-generation (F_1) offspring (n=500) prior to release. Total DNA from each fin tissue sample was extracted using an automated DNA extraction system (MagExtractor MFX-6100; Toyobo, Osaka, Japan) with a MagExtractor genomic DNA purification kit (Toyobo, Osaka, Japan). The extracted genomic DNA was stored at -20°C until genotyping.

Microsatellite genotyping

We performed genotyping analysis on 93 of the wild-caught brood stock and 500 of their F_1 offspring. Nine microsatellite loci were amplified using previously reported primers (An et al., 2006). Microsatellite repeat sequences and the optimal annealing template for each locus are shown in Table 1. The forward primer from each primer set was 5'-fluorescently labeled with one of three dyes (6-FAM, HEX, or NED; Applied Biosystems, Foster City, CA, USA). PCR amplification of nine microsatellite loci was carried out by the same method as described in a previous report (An et al., 2006). For genotyping, the PCR product was added to each reaction containing formamide with the size standard GENESCAN-400HD [ROX] (Applied Biosystems, Foster City, CA, USA) and electrophoresed using an ABI3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The fragment length of the PCR products was determined with GENEMAPPER software (Version 4.0, Applied Biosystems, Foster City, CA, USA).

Data analysis

Statistical analysis was performed by several programs to investigate determinants of genetic diversity within and between the brood stock and their F_1 offspring. FSTAT (version 2.9.3.2; Goudet, 1995) was used to calculate the number of alleles per locus (N_A),

Table 1. Sequences of 9 microsatellite markers and their specific annealing temperatures of PCR amplification. T_a is the optimal annealing temperature

Locus	Repeat motif	Primer sequences (5'-3')	T_a (°C)	Allele size range (bp)
KOF20	$(CA)_{14}$	F: AGCCAGCGCTTCTACCT R: CTTCAGAGTCTGGATGTCCC	58-60	236-266
KOF35	$(TG)_{11}$ … $(TG)_3$	F: ACTCTACTGTGCCTGGAGC R: ACACTAATGGGCAGATTCCT	58-60	152-176
KOF85	$(TG)_2(TC)_2(TG)_4TA(TG)_9$	F: TGGGAGAATTAGGCTTTCAT R: GAGGCCTGATGCTAAGTTTT	58-60	156-170
KOF175	$(TG)_{17}$	F: CCATGGAAAATGTGTTTCTG R: CAATGTAAAACCCTGCAAAA	58-60	158-176
KOF319	$(TG)_{13}$	F: GGATATCGCACGCTCTT R: CACATGTCATATCTGGGATT	58-60	104-112
KOF360	$(TG)_7CG(TG)_6(AG)_3$	F: GGTGATCTGAGTCTTTACCC R: TGCACAAACATACATACGC	58-60	102-144
KOF367	$(TG)_5$ … $(AG)_4$ … $(AG)_4$ …$(TG)_7(AG)_6$	F: CATTCTGTTACTGCCGTGTA R: CACTCCATCTTGCCTTGTT	58-60	254-258
KOF369	$(CA)_{11}$	F: GGATTCACTTACTCGCTTGC R: TTTTACACGCTGCTTCCTAA	58-60	126-130
KOF374	$(TG)_2AG(TG)_{10}TT(TG)_7TA(TG)_4$	F: GGGGACAGGAGTGTATTGTA R: GCTCAGTGCCATCTAATGA	58-60	148-184

gene diversity (G_d), and allelic richness (A_R), which allows for comparing numbers of alleles across samples of unequal sizes. Both observed and expected heterozygosity (H_O and H_E, respectively; Nei, 1987) of each locus were estimated using ARLEQUIN (version 2.0; Schneider et al., 2000). The frequency of the most common allele for each locus of each population was calculated using the software GENEPOP (version 3.4; Raymond and Rousset, 1995). An overall inbreeding coefficient (F_{IS}) of Weir and Cockerham (1984) and their significance test were calculated to measure the deviation from Hardy-Weinberg equilibrium (HWE) with GENEPOP (5,000 permutations).

Results

Allele sizes ranged from 96 to 272 bp across all microsatellite loci. All nine microsatellite loci were highly polymorphic, while the degree of variability was different at each locus. In total, 98 different alleles were found in the brood stock and the offspring population. The average number of alleles per locus ranged from 3.5 (KOF367 and KOF369) to 17.5 (KOF360). The average frequency of the most common alleles in the wild (brood stock) population (0.478) was lower than that of the F_1 population (0.538). The average A_R per locus varied from 3.21 (KOF369) to 16.21 (KOF360). The average A_R in the F_1 population (7.79) was lower than that of the brood stock (9.22). Consequently, the F_1 population showed a 16% reduction in the average A_R compared to the natural population. Both populations exhibited a high average G_d, ranging from 0.60 (F_1 offspring) to 0.66 (brood stock; Table 2).

The means of H_O and H_E (0.596 and 0.603, respectively) in the F_1 population were slightly lower

Table 2. Allelic variability observed at nine microsatellite loci in the rock bream broodstock and their offspring populations. Number of samples (No), number of alleles per locus (N_A), allelic richness (A_R), gene diversity (G_d), size in bp of alleles (S), 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 equilibrium (*P*) are given for each population and locus

Population (No)	Microsatellite loci									
	KOF20	KOF35	KOF85	KOF175	KOF319	KOF360	KOF367	KOF369	KOF374	Mean
Jeju parent (93)										
N_A	11	9	5	10	8	17	4	3	15	9.11
A_R	11.00	9.00	6.00	10.00	8.00	17.00	4.00	3.00	15.00	9.22
G_d	0.82	0.72	0.65	0.56	0.57	0.92	0.41	0.49	0.83	0.66
S	248-272	162-178	158-174	150-178	96-116	102-146	252-258	128-132	150-190	
F	0.301	0.478	0.452	0.645	0.613	0.177	0.726	0.661	0.253	0.478
U	1	1	1	4	3	1	1	0	2	1.556
H_E	0.819	0.716	0.648	0.556	0.569	0.915	0.414	0.488	0.835	0.662
H_O	0.849	0.731	0.731	0.495	0.505	0.914	0.387	0.398	0.849	0.651
F_{IS}	-0.037	-0.021	-0.130	0.111	0.112	0.001	0.065	0.186	-0.018	0.017
	(0.684)	(0.763)	(0.000)	(0.122)	(0.079)	(0.067)	(0.169)	(0.102)	(0.716)	
P	0.645	0.756	0.000	0.370	0.482	0.000	0.069	0.141	0.000	
Jeju offspring (500)										
N_A	12	8	7	9	9	18	3	4	15	9.44
A_R	10.56	7.52	5.19	6.31	5.54	15.41	2.99	3.41	13.20	7.79
G_d	0.81	0.69	0.59	0.36	0.55	0.89	0.35	0.39	0.80	0.60
S	248-270	160-178	160-174	156-182	98-120	102-146	254-258	126-132	156-186	
F	0.288	0.483	0.565	0.793	0.621	0.198	0.781	0.741	0.373	0.538
U	2	0	2	3	4	1	0	1	2	1.667
H_E	0.805	0.687	0.593	0.356	0.554	0.888	0.351	0.393	0.803	0.603
H_O	0.800	0.524	0.732	0.330	0.528	0.934	0.294	0.420	0.804	0.596
Fis	0.006	0.237	-0.235	0.074	0.047	-0.051	0.162	-0.070	-0.002	0.012
	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.273)	(0.000)	
P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.286	0.000	
Mean all populations										
N_A	11.50	8.50	6.00	9.50	8.50	17.50	3.50	3.50	15.00	9.28
A_R	10.78	8.26	5.60	8.16	6.77	16.21	3.50	3.21	14.10	8.51
G_d	0.81	0.70	0.62	0.46	0.56	0.90	0.38	0.44	0.82	0.63
U	1.50	0.50	1.50	3.50	3.50	1.00	0.50	0.50	2.00	1.611
H_E	0.812	0.702	0.621	0.456	0.562	0.902	0.383	0.441	0.819	0.633
H_O	0.825	0.628	0.732	0.412	0.517	0.924	0.341	0.409	0.827	0.624

than those of the brood stock (0.651 and 0.662, respectively), although no significant difference was detected ($p<0.05$; Table 2). The brood stock departed from HWE ($p<0.05$) at microsatellite loci KOF85, KOF360, and KOF374, while the F_1 population showed deviations from HWE at most microsatellite loci except locus KOF369. Departures from HWE were due to a deficit of heterozygotes at most loci. The F_{IS} value in the offspring population showed the same tendency toward HWE deviations across all loci.

Allele frequencies for the nine microsatellite loci in the F_1 and broodstock populations are shown in Table 3. Unique alleles (i.e., alleles observed in only one population) were found at eight microsatellite loci (all but KOF369) in the wild population and seven microsatellite loci (all but KOF35 and KOF367) in the F_1 population. The most common alleles of both the brood stock and the F_1 population were identical in most loci except KOF374 (allele 164 versus 174) and KOF20 (allele 256 versus 260; Table 3).

Discussion

A large number of artificially produced offspring have been released into the natural environment through stock enhancement programs without considering their influence on the genetic structure and genetic divergence and diversity is one of the most important steps for managing fishery resources. The nine highly polymorphic microsatellite loci examined in this study revealed the loss of genetic variability in the F_1 offspring population compared to their wild-caught parents. We found that eight microsatellite loci (all but KOF369) deviated from HWE in the F_1 population; in most cases, deviations from HWE were due to a deficit of heterozygotes except at loci

Table 3. Allele frequency of each microsatellite locus in the rock bream broodstock and their offspring populations

Locus	Allele	Parents	Offspring
KOF319	96	0.005	—
	98	—	0.001
	100	0.005	0.005
	102	—	0.001
	104	—	0.002
	106	0.086	0.090
	108	0.613	0.621
	110	0.220	0.223
	112	0.043	0.056
	114	0.022	—
	116	0.005	—
	120	—	0.001
KOF175	150	0.011	—
	152	0.011	—
	154	0.011	—
	156	0.005	0.001
	160	—	0.001
	166	—	0.021
	158	0.016	0.032
	170	0.091	0.031
	172	0.645	0.793
	174	0.134	0.113
	176	0.059	0.007
	178	0.016	—
	182	—	0.001

Table 3. (continued)

Locus	Allele	Parents	Offspring
KOF374	150	0.005	—
	156	0.005	0.002
	160	—	0.002
	162	0.220	0.077
	164	0.253	0.108
	166	0.005	0.088
	168	0.011	0.040
	170	0.022	0.023
	172	—	0.005
	174	0.204	0.373
	176	0.070	0.167
	178	0.065	0.018
	180	0.027	0.005
KOF20	182	0.065	0.037
	184	0.032	0.044
	186	0.005	0.011
	190	0.011	—
	248	0.102	0.006
	250	—	0.011
	252	0.065	0.032
	254	0.043	0.034
	256	0.301	0.254
	258	0.183	0.187
	260	0.204	0.288
	262	0.038	0.079
	264	0.027	0.059
	266	0.027	0.042
	268	0.005	0.007
	270	—	0.001
	272	0.005	—
KOF369	126	—	0.003
	128	0.661	0.741
	130	0.269	0.242
	132	0.070	0.014
KOF85	158	0.005	—
	160	—	0.001
	162	—	0.005
	164	0.183	0.241
	168	0.011	0.011
	170	0.344	0.174
	172	0.452	0.565
	174	0.005	0.003
KOF367	252	0.005	—
	254	0.726	0.781
	256	0.247	0.198
	258	0.022	0.021

KOF85, KOF360, and KOF374. These three loci showed an excess of heterozygotes. Although it is difficult to determine whether extensive heterozygosity deficiency in a study with microsatellite markers represents a real biological phenomenon or a technical artifact of PCR amplifications or misscoring of microsatellite loci (Jones et al., 1998; Li et al., 2002), null alleles are a possible explanation for the deficiencies in heterozygosity observed here (Jones et al., 1998; Li et al., 2002; Reece et al., 2004). Although the number of alleles per locus in the F_1 generation was reduced by 16% compared to the brood stock, we found no significant difference in mean heterozygosity between hatchery and wild populations. Similar results have been reported in several studies of farmed animals (Coughlan et al., 1998; Evans et al., 2004; Norris et al., 1999; Perez-Enriquez et al., 1999; Zhang et al., 2005). The loss of rare alleles from hatchery populations has been reported as a more meaningful measure of genetic variation than heterozygosity, because heterozygosity is insensitive to the substantial genetic changes that may occur in cultivated aquaculture stocks (Hedgecock and Sly, 1990). The loss of a number of alleles in the offspring population may have resulted from the eggs having been collected from only a few parents, if not all breeders released eggs during the sampling period. Jeong et al. (2006) reported that eggs collected for only 1 day after spawning of olive flounder showed lower genetic diversity than eggs collected for 2 days after spawning. This suggests that the egg-collection period is important in determining the genetic diversity of artificially-produced offspring. We found no differences in the frequencies of main alleles between the two populations. The F_1 population had unique alleles in most loci except KOF35 and KOF367, which may have been caused by unknown parents. The sample of 93 parents investigated did not represent the entire brood stock. We have no detailed records regarding the number of parents that actually contributed to the production of the offspring used in this study.

We found that the genetic diversity of the F_1 population was reduced compared to their wild-caught parents. When that variation is lost in first-generation hatchery stocks, it is lost to all subsequent generations within a closed breeding program, and may therefore limit the genetic improvement available within that stock. The ways to decrease these genetic impacts of hatchery stocks on natural stocks include improving artificial breeding methods and the genetic management for all hatchery strains by means of monitoring their genetic variability. In addition, we emphasize that all aspects of offspring production must be considered, including the egg-collection period and the number of effective parents.

Acknowledgments

This study was supported by the Internship Program of the Biotechnology Research Institute, National Fisheries Research and Development Institute (NFRDI), Republic of Korea (contribution no. RP-2008-BT-010) Views expressed herein are those of the authors and do not necessarily reflect the views of NFRDI.

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(Received May 2008, Accepted June 2008)