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Genetic characterization of hatchery populations of Korean spotted sea bass (*Lateolabrax maculatus*) using multiplex polymerase chain reaction assays

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ABSTRACT. The spotted sea bass, *Lateolabrax maculatus*, is an important commercial and recreational fishery resource in Korea. Aquacultural production of this species has increased because of recent resource declines, growing consumption, and ongoing government-operated stock release programs. Therefore, the genetic characterization of hatchery populations is necessary to maintain the genetic diversity of this species and to develop more effective aquaculture practices. In this study, the genetic diversity and structure of three cultured populations in Korea were assessed using multiplex assays with 12 highly polymorphic microsatellite loci; 144 alleles were identified. The number of alleles per locus ranged from 6 to 28, with an average of 13.1. The mean observed

and expected heterozygosities were 0.724 and 0.753, respectively. Low levels of inbreeding were detected according to the inbreeding coefficient (mean F_{IS} = 0.003-0.073). All hatchery populations were significantly differentiated from each other (overall fixation index (F_{ST}) = 0.027, P < 0.01), and no population formed a separate cluster. Pairwise multilocus F_{ST} tests, estimates of genetic distance, mantel test, and principal component analyses did not show a consistent relationship between geographic and genetic distances. These results could reflect the exchange of breeds and eggs between hatcheries and/or genetic drift due to intensive breeding practices. For optimal resource management, the genetic variation of hatchery stocks should be monitored and inbreeding controlled within the spotted sea bass stocks that are being released every year. This genetic information will be useful for the management of both *L. maculatus* fisheries and the aquaculture industry.

Key words: Genetic diversity; Genetic differentiation; Hatchery population of Korean spotted sea bass (*Lateolabrax maculatus*); Microsatellite loci; Multiplex polymerase chain reaction (PCR) assay

INTRODUCTION

The Korean spotted sea bass, *Lateolabrax maculatus* (Perciformes: Moronidae), is widely distributed along the Korean coast with the borders of Vietnam and China (Shao et al., 2009). *L. maculatus* has been designated as a congeneric species of *L. japonicus* based on morphological traits, but it was recently redescribed as a reef-associated fish species that is characterized by its distinctive characteristics including many clear black dots on the lateral body region (Yokogawa and Seki, 1995; Kim and Jun, 1997). In Pacific Asia, *L. maculatus* is one of the most important commercial fishery species. However, its natural population has been declining continuously for two decades in Korea (Ministry for Food, Agriculture, Forestry, and Fisheries, 2009). In response to decreased natural resources in the face of increasing demand, the aquacultural production of spotted sea bass has increased since the early 2000s, and it has rapidly developed in the past decade. Furthermore, the Korean government has sponsored artificial spotted sea bass seed release for coastal spotted sea bass resource enhancement, and more than one million spotted sea bass seeds reared in hatcheries were released into the Korean coastal sea areas in 2012.

Restocking and stock enhancement have been used as tools to recover stocks of commercially overexploited marine resources in several countries (Støttrup and Sparrevohn, 2007). However, the massive releases of hatchery-produced fish have raised concerns about their genetic effects on wild populations. Hatchery fish may have a reduced genetic variability, and this may eventually lower the genetic diversity and could potentially alter the genetic structure of natural populations (Taniguchi, 2003). Hence, hatchery production of spotted sea bass raises concerns regarding the maintenance of genetic diversity among cultured stocks. Therefore, an investigation of the genetic variation in cultured spotted sea bass stocks is urgently needed for successful hatchery management, the production of high-quality spotted sea bass, and to avoid reductions in the genetic variation in aquaculture stocks. Investigating the genetic diversity and population structure of species after overexploitation and/or introgression have occurred fails to maximize the potential for using this knowledge as a tool to aid in the development of policies that adequately protect biodiversity in the wild populations from anthropogenic influences.

The monitoring of genetic variation among marine resources can be achieved by molecular genetic analysis (Ward, 2006). Molecular methods that utilize polymerase chain reaction (PCR) and nucleotide sequence determination overcome many of the limitations of phenotypic methods (Choi and Kim, 2012; Lee and Hur, 2012). Most importantly, microsatellites emerged as molecular markers with the finest resolution to label populations and individuals because of their high variability, abundance, neutrality, codominant inheritance, and good reproducibility (Tautz, 1989; Kim et al., 2013). Microsatellite genotyping has proved to be a powerful tool in studies of genetic variation in many marine species (An et al., 2012; Han et al., 2012; Hong et al., 2012). However, despite the commercial importance and history of stocking practices of spotted sea bass in Korea, genetic studies on spotted sea bass using DNA markers have been relatively rare. The genetic structuring of Northwestern Pacific populations has been analyzed using mitochondrial DNA polymorphism (Liu et al., 2009), and microsatellite markers have been reported from *L. maculatus* in China (Shao et al., 2009). Quite recently, one wild population and one hatchery population in Korea were analyzed with eight polymorphic markers (An et al., 2013). No further data about the genetic diversity of cultured stocks in different regions are available. Furthermore, the increasing demand for routinely performing population genetic studies in *L. maculatus* to support optimum management and selection programs prompts the development of robust and low-cost genetic tagging tools. Microsatellite multiplex PCR by successive technical advances, in which several markers are simultaneously amplified in the same reaction, is used to increase the amount of information generated per assay and to reduce consumable and labor costs (Henegariu et al., 1997; Neff et al., 2000).

In this study, the genetic diversity and relationships within and between cultured populations of *L. maculatus* from different regions in Korea were investigated using multiplex PCR assays with 12 microsatellite markers. This study will provide useful data for effective monitoring and management of spotted sea bass populations as well as for the implementation of stock-enhancing programs.

MATERIAL AND METHODS

Sample collection and DNA extraction

For the analysis, 130 spotted sea basses (*L. maculatus*) were collected from three coastal locations in Korea between June and July 2013 (Figure 1). To cover the main aquaculture areas, three hatchery populations were sampled from three different areas in Yeosu (YS; 30 individuals) and Namhae (NH; 50 individuals) in the southern coastal areas and Hwaseong (HS; 50 individuals) in the western coastal areas (Table 1). The samples from hatchery-reared populations were acquired from a population of hatchery-reared offspring from small broodstocks composed of hatchery-produced adults. Although the original ancestors of the hatchery spotted sea bass were collected locally and the hatchery populations had been reared continuously, no detailed records of their founding and maintenance were available.

Caudal fin samples were collected from fresh specimens and immediately stored in 99.9% ethanol until DNA preparation. Genomic DNA for genotyping was extracted from fin clips of each sample using an automated DNA extraction system, MagExtractor MFX-2100 (TOYOBO, Osaka, Japan), with a MagExtractor-Genomic DNA Purification Kit (TOYOBO). The genomic DNA extracted was kept at -20°C until genotyping.

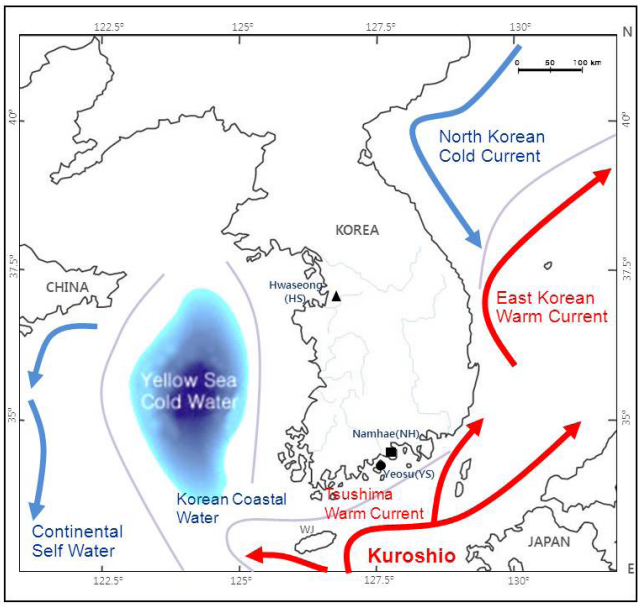

Figure 1. Approximate locations of the three hatchery population sample sites of *Lateolabrax maculatus* that were used in this study. The abbreviations are as follows: YS = Yeosu; NH = Namhae; and HS = Hwaseong.

Table 1. Collection details for three hatchery populations of Korean spotted sea bass, *Lateolabrax maculatus*.

Sampling area (abbreviation)	Sample locality	Sample size	Collection date
Yeosu (YS)	South Sea; 34°45'N, 127°39'E	30	June 2013
Namhae (NH)	South Sea; 34°48'N, 127°54'E	50	June 2013
Hwaseong (HS)	West Sea; 37°11'N, 126°50'E	50	July 2013

Multiplex PCR design and microsatellite genotyping

L. maculatus is closely related to a congeneric species, *Lateolabrax japonicus* based on morphological traits. Thus, 18 polymorphic microsatellite loci for *L. maculatus* (Shao et al., 2009) and 22 polymorphic cross-specific microsatellite loci for *L. japonicus* (Jiang et al., 2007) were tested to develop a multiplex PCR technique using eight wild spotted sea bass individuals. PCR was conducted in 25-µL reactions containing 12.5 µL 2X Multiplex PCR Pre-Mix (SolGent, Daejeon, Korea; Cat. No. SMP01-P096), 100 ng template DNA, and 10 pmol each primer, using an ABI 9700 Thermal Cycler System (Applied Biosystems, Foster City, CA, USA). PCRs were run for 15 min at 95°C followed by 30 cycles of 20 s at 95°C, 40 s at 54°C, and 1 min at 72°C before a 3-min final extension at 72°C. PCR products were separated on a 3% agarose gel to check their sizes. Initially we selected a set of 17 candidate microsatellite loci on the basis of their good amplification quality and the variation in the size of the resulting amplicons for primary development. Each forward primer was 5'-end-labeled with 6-FAM, NED, and HEX dyes (Applied Biosystems), and the eight samples were multiplexed for genotyping. The resulting mixture was analyzed using an ABI 3130 Prism Genetic

Analyzer (Applied Biosystems). The alleles from the raw data were analyzed using GeneMapper (ver. 4.0; Applied Biosystems). Finally, on the basis of the number of alleles per locus (N_A) in eight individuals, similar DNA amplification properties under multiplex PCR conditions, and allele range size, 12 microsatellite loci (Lama07, Lama36, Lama38, Lama40, Lama42, Lama44, Ljapo38, Ljapo44, Ljapo46, Ljapo48, Ljapo93, and Ljapo138) were selected for genotyping in PCR-based multiplex panels (Table 2). For this group of loci, optimal amplification conditions were established, mostly with respect to annealing temperatures (52, 54, 56, and 60°C). The results were evaluated, and this process was optimized until clear peaks for each locus were obtained. Four PCR-based multiplex systems were used for genotyping. The 12 microsatellite loci were placed into one of four three-plex PCR panels with an annealing temperature of 54°C on the basis of allelic size variation: 1) Lama07, Lama36, and Ljapo138; 2) Lama38, Lama40, and Lama42; 3) Lama44, Ljapo48, and Ljapo93; and 4) Ljapo38, Ljapo44, and Ljapo46.

Four three-plex PCR amplifications were performed with 130 spotted sea bass individuals using an ABI 9700 Thermal Cycler System (Applied Biosystems). The PCR conditions and thermocycling profiles described above were used. For genotyping, 1 μL PCR product was combined with formamide and a GeneScan-500 HD ROX size standard (Applied Biosystems) and subsequently electrophoresed using an ABI PRISM 3130 Automated DNA Sequencer (Applied Biosystems).

Table 2. Twelve microsatellite loci sequences for the hatchery spotted sea bass (*Lateolabrax maculatus*) populations used in this study. Core repeats and their specific annealing temperatures used in the multiplex polymerase chain reaction amplification assays are included.

Locus	Primer sequence (5'-3')	Ta (°C)	Repeats	Accession No.
Lama07	F: AAAGGGTTGAAAATCCGTGG ned R: CAGGAGGTAATAAAACAGGGCTA	54 (54)	$(CA)_{13}N(AC)_{12}$	EU090737
Lama36	F: CTAAAGGACCACAAGATACACG hex R: ACTCAGGCTCAAACCAGACA	54 (54)	$(AC)_{13}$	EU090766
Lama38	F: ACAAAACTCATCCATCAAGCAG ned R: AGTGTCCACGGAGACGGTAA	54 (54)	$(GT)_{11}$	EU090768
Lama40	F: TCTAAGTAGGCTTGGCTGTC fam R: TATCTGAAACCGTCTCCGTA	52 (54)	$(TG)_{12}$	EU090770
Lama42	F: GTATTTCACTATCCCATCCTG hex R: TATTGTTGCTCTTCCTCCTC	50 (54)	$(AC)_{15}$	EU090772
Lama44	F: GGGCAGTAATTGGTGAGGGA ned R: TCTTCAGGGCAAAAGGTGGT	54 (54)	$(GT)_{16}$	EU090774
Ljapo38	F: TATCGGTGAAAACCTTGAAA hex R: TAGCACTTGCCAGTATCCC	57 (54)	$(GT)_{22}$	EF010957
Ljapo44	F: GGGCTGGTGACATTGGAT ned R: TCTGTGTTGGCGTGGAAA	60 (54)	$(TG)_{11}AGAA(AG)_4$	EF010954
Ljapo46	F: TGAAATAAACTGATGGATAGATA fam R: CGATGGATTCAAGACCTC	51 (54)	$(GT)_{21}A(TG)_4(CA)_3$	EF010955
Ljapo48	F: AACGACTGTCTTCACTTGGT hex R: TGAGAGATTGTCATCACGG	55 (54)	$(TG)_9T(TG)_{11}CGCG(TG)_3$	EF010956
Ljapo93	F: CAGGTGTCATCTTCGTGC fam R: CTGCTGTGTATCCTTCGC	59 (54)	$(GA)_6(GT)_9$	EF010946
Ljapo138	F: CAGACGAAACTCTTCTTCTAACA fam R: TCATTTGGACAGCCTAACG	51 (54)	$(GT)_{20}$	EF010948

Ta is the multiplex annealing temperature and (Ta) is the original annealing temperature.

Data analysis

Possible null alleles and genotyping errors caused by stuttering and/or large-allele

dropout were tested using MICRO-CHECKER (1000 randomizations; van Oosterhout et al., 2004). Scoring and human error were estimated by duplicate analyses. Because the conclusion drawn from microsatellite loci strongly depends on their neutrality, the data were also tested to determine whether one or more microsatellites were under selection using the LOSITAN software (Antao et al., 2008), which is a selection-detection workbench based on a well-evaluated fixation index (F_{ST})-outlier detection method. The software calculated F_{ST} values and heterozygosity for each locus according to Weir and Cockerham (1984) and expected F_{ST} values for each locus weighted by its heterozygosity. Simulated distributions of F_{ST} values conditional on heterozygosity under a neutral model were obtained and compared with the observed F_{ST} values to identify potential outlier loci.

The genetic diversity of each sample was performed with the FSTAT ver. 2.9.3.2 software (http://www2.unil.ch/popgen/softwares/fstat.htm). The genetic parameters were the N_A, the allelic richness (A_R), which is N_A corrected for the sample size applying the rarefaction index (El Mousadik and Petit, 1996), the size range of an allele in bp, the number of observed unique alleles (U), the observed heterozygosity (H_O), the expected heterozygosity (H_E), and the polymorphic information content (PIC), which is an indicator of the utility of the marker for linkage or population genetic studies; these parameters were calculated using CERVUS version 3.0 (http://www.fieldgenetics.com/pages/aboutCervus_Overview.jsp). Differences in genetic diversity parameters were identified using a nonparametric analysis (Wilcoxon signed-rank test; Wilcoxon, 1945). The inbreeding coefficient (F_{IS}; Weir and Cockerham, 1984) for each population and locus was calculated to address deviations from the Hardy-Weinberg equilibrium (HWE), and the significance levels were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989). GENEPOP'007 was used for testing linkage disequilibrium to determine the extent of distortion from the independent segregation of loci. Because hatchery populations are often subjected to founder effects and bottlenecks that result in lower genetic diversity, the Bottleneck software program ver. 1.2.02 (Cornuet and Luikart, 1996) was used under the infinite allele model (IAM), stepwise-mutation model (SMM), and two-phased model of mutation with 1000 iterations to verify the existence of bottlenecks inferred by heterozygosity excess in the three samples. Significance was tested using the Wilcoxon signed-rank test (Wilcoxon, 1945).

The extent of population differentiation was examined by calculating the global multilocus F_{ST} values (Weir and Cockerham, 1984) and R_{ST} values (1000 permutations; Rousset, 1996). The indices of the pairwise F_{ST} values based on an IAM and R_{ST} values based on an SMM were calculated using ARLEQUIN. The R_{ST} index incorporates the correlation of the weighted mean allele size, which is expressed as the number of tandem repeats. Significance levels were evaluated after the sequential Bonferroni adjustment of critical probabilities (Rice, 1989).

Hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was used to partition genetic variance among subsamples within groups and among groups using ARLEQUIN ver. 3.0 (Excoffier et al., 2005), and the significances of the AMOVA components were tested using 1000 permutations. The strength of the relationships among the geographical populations was estimated from the genetic distances based on the chord distance, D_{CE}, between all pairs of populations (Cavalli-Sforza and Edwards, 1967). The correlation between the genetic and geographic distances was evaluated using the Mantel test, and genetic relationships between populations were evaluated using the principal component analysis (PCA) with

GenAlEx 6.5 (http://biology.anu.edu.au/GenAlEx/Welcome.html).

RESULTS

Multiplex PCR optimization

The development of the genotyping tool consisted of progressive selections to refine the PCR conditions of the 40 microsatellite loci in *L. maculatus* and *L. japonicus*, and a successful set of 12 loci was finally obtained. Most loci were amplified in eight individuals of *L. maculatus*. The 17 microsatellites preselected for multiplex PCR had good amplification quality and covered an optimal range of product lengths. The other 23 loci were discarded because of inappropriate product lengths. Crosschecking was performed on the 17 candidate primer pairs and was aimed at assessing primer suitability for multiplex PCR conditions. From the 17 preselected loci, the 12 primers with high polymorphism were selected for primary development, and the other 5 primers, which had less than 5 alleles in 8 individuals, were retained as potential substitutes. Finally, a set of 12 microsatellites was chosen to establish the multiplex genotyping tool, and four three-plex PCR combinations were selected. All 12 loci selected contained dinucleotide repeats (Table 2).

Genetic variability

The genetic variability in three natural populations of *L. maculatus* (total N = 130) were assessed using the multiplex method with the 12 polymorphic microsatellite loci presented here. The 12 primer sets yielded variable profiles.

The MICRO-CHECKER analysis did not detect any allele scoring errors caused by stuttering or large-allele dropout, but it indicated that 5 (Lama07, Ljapo38, Ljapo46, Ljapo48, and Ljapo138) of the 12 loci examined may include null alleles, and our data demonstrated that Ljapo46 was affected by null alleles in all three population samples. The global outlier test using three populations with LOSITAN under both the IAM and SMM determined that no locus had an excessively high or low F_{ST} value relative to neutral expectations, indicating that there was no candidate for selection pressure. To minimize the detection of false positives, we considered one locus (Ljapo46) to have null alleles. Therefore, this locus was eliminated from subsequent analyses. No evidence of highly significant linkage disequilibrium among loci was found.

All 11 microsatellite loci were polymorphic in the three population samples of spotted sea bass, and the level of polymorphism varied among the loci. The genetic diversity of each population is presented in Table 3. A total of 144 alleles were observed, and some alleles were unique in each population. The allelic diversity was 13.1, with the N_A ranging from 6 to 28; no population had a diagnostic allele. The mean A_R numbers (for comparing N_A between different sample sizes) varied from 4.29 for Lama40 to 17.78 for Ljapo138. The highest value was observed for Ljapo138 in the YS sample, with values up to 19 alleles. In the three populations, 49.1 to 60.5% of the alleles were rare alleles with a frequency <5%. Rare alleles were detected at most loci and were not associated with a particular locus in any population. A high degree of polymorphism with PIC values between 0.510 and 0.891 was detected per locus (Table 3). The average gene diversity (H_E) varied between 0.571 and 0.910, and the average H_O ranged between 0.529 and 0.918.

Table 3. Summary statistics for the 11 microsatellite loci in three hatchery populations of Korean spotted sea bass (*Lateolabrax maculatus*).

Population (No.)		Microsatellite loci											
		Lama07	Lama36	Lama38	Lama40	Lama42	Lama44	Ljapo38	Ljapo44	Ljapo48	Ljapo93	Ljapo138	Mean
	F_{ST}	0.026	0.035	0.014	0.019	0.041	0.042	0.003	0.024	0.022	0.031	0.027	0.027
YS (30)	N_A	8	6	4	4	7	13	13	16	9	10	19	9.91
	A_R	8.00	6.00	4.00	4.00	7.00	13.00	13.00	16.00	9.00	10.00	19.00	9.91
	U	0	2	0	1	1	2	2	1	1	1	0	1.00
	S	206-224	227-289	265-279	199-205	250-266	139-173	278-324	262-294	230-260	322-346	263-307	
	PIC	0.684	0.569	0.520	0.534	0.744	0.858	0.785	0.867	0.796	0.778	0.926	0.733
	H_E	0.734	0.647	0.581	0.608	0.790	0.885	0.813	0.892	0.831	0.813	0.946	0.776
	H_O	0.700	0.533	0.733	0.467	0.800	0.933	0.800	0.833	0.867	0.700	0.900	0.751
	F_{IS}	0.047	0.178	-0.267	0.236	-0.012	-0.056	0.016	0.067	-0.044	0.141	0.049	0.032
NH (50)	N_A	8	4	5	5	8	12	11	15	10	9	19	9.64
	A_R	7.32	3.60	4.95	4.44	7.44	11.72	9.95	12.73	8.42	8.57	16.28	7.77
	U	0	0	1	0	1	0	0	1	0	0	2	0.45
	S	192-218	279-285	265-279	195-207	248-264	143-183	278-324	262-294	230-260	326-344	261-311	
	PIC	0.705	0.569	0.576	0.434	0.763	0.863	0.695	0.790	0.661	0.826	0.893	0.707
	H_E	0.749	0.649	0.644	0.477	0.799	0.884	0.721	0.817	0.704	0.853	0.910	0.746
	H_O	0.800	0.700	0.580	0.520	0.680	0.920	0.640	0.860	0.760	0.760	0.940	0.742
	F_{IS}	-0.069	-0.079	0.101	-0.092	0.151	-0.041	0.114	-0.053	-0.081	0.110	-0.033*	0.003
HS (50)	N_A	14	5	5	5	7	16	10	12	10	12	23	10.82
	A_R	12.46	4.53	4.20	4.44	6.54	14.68	9.31	10.06	8.39	10.55	18.06	9.38
	U	3	0	1	0	0	3	2	0	0	2	4	1.36
	S	192-224	279-287	265-279	195-207	250-264	139-183	278-326	266-292	230-260	322-344	255-335	
	PIC	0.827	0.446	0.489	0.561	0.713	0.856	0.656	0.779	0.734	0.749	0.854	0.697
	H_E	0.852	0.512	0.546	0.627	0.754	0.875	0.683	0.813	0.771	0.779	0.874	0.735
	H_O	0.720	0.560	0.500	0.600	0.740	0.900	0.580	0.800	0.580	0.720	0.760	0.678
	F_{IS}	0.157*	-0.096	0.085	0.044	0.019	-0.029*	0.152	0.016	0.250*	0.077*	0.132*	0.073
Mean of all	N_A	10.00	5.00	4.67	4.67	7.33	13.67	11.33	14.33	9.67	10.33	20.33	10.12
populations	A_R	9.26	4.71	4.38	4.29	6.99	13.13	10.75	12.93	8.60	9.71	17.78	9.32
	PIC	0.739	0.528	0.528	0.510	0.740	0.859	0.712	0.812	0.730	0.784	0.891	0.712
	H_E	0.778	0.603	0.590	0.571	0.781	0.881	0.739	0.841	0.769	0.815	0.910	0.753
	H_O	0.740	0.598	0.604	0.529	0.740	0.918	0.673	0.831	0.736	0.727	0.867	0.724

YS = Yeosu, NH = Namhae, HS = Hwaseong, F_{ST} = single-locus fixation index, No. = number of samples, N_A = number of alleles per locus, S = size of alleles in bp, A_R = allelic richness, U = number of unique alleles, H_E = expected heterozygosity, H_O = observed heterozygosity, PIC = polymorphism information content, and F_{IS} = inbreeding coefficient are given for each population and locus. Calculations assume that individuals with one microsatellite band are homozygous for the allele. *Significant deviation from Hardy-Weinberg equilibrium after Bonferroni's correction (P, initial $\alpha = 0.05/11 = 0.005$).

Table 4. Frequencies of each microsatellite allele in three hatchery populations of Korean spotted sea bass (*Lateolabrax maculatus*).

Locus	Allele	YS	NH	HS	Locus	Allele	YS	NH	HS
Lama07	192	0.000	0.010	0.027		320	0.050	0.080	0.030
	194	0.000	0.000	0.008		322	0.050	0.010	0.100
	200	0.000	0.000	0.004		324	0.100	0.120	0.090
	202	0.000	0.000	0.008		326	0.000	0.000	0.010
	206	0.433	0.250	0.300					
	208	0.133	0.130	0.142	Ljapo44	262	0.017	0.020	0.000
	210	0.250	0.400	0.285		264	0.017	0.000	0.000
	212	0.000	0.020	0.015		266	0.017	0.000	0.010
	214	0.017	0.030	0.031		268	0.100	0.160	0.300
	216	0.000	0.030	0.054		270	0.083	0.050	0.020
	218	0.083	0.130	0.081		272	0.250	0.360	0.190
	220	0.050	0.000	0.019		274	0.133	0.150	0.230
	222	0.017	0.000	0.008		276	0.067	0.070	0.010
	224	0.017	0.000	0.019		278	0.033	0.020	0.010
						280	0.000	0.050	0.000
Lama36	277	0.017	0.000	0.000		282	0.050	0.020	0.060
	279	0.000	0.010	0.010		284	0.017	0.010	0.100
	281	0.467	0.420	0.650		286	0.117	0.040	0.040
	283	0.367	0.380	0.260		288	0.017	0.020	0.000
	285	0.100	0.190	0.050		290	0.033	0.010	0.020
	287	0.033	0.000	0.030		292	0.017	0.010	0.010
	289	0.017	0.000	0.000		294	0.033	0.010	0.000
Lama38	265	0.600	0.490	0.630	Ljapo48	230	0.017	0.030	0.010
	267	0.000	0.000	0.010		232	0.067	0.090	0.230
	273	0.000	0.040	0.000		234	0.117	0.010	0.110
	275	0.183	0.330	0.200		236	0.000	0.010	0.010
	277	0.183	0.100	0.150		242	0.033	0.000	0.000
	279	0.033	0.040	0.010		250	0.200	0.230	0.100
						252	0.117	0.100	0.050
Lama40	195	0.000	0.010	0.020		254	0.083	0.020	0.080
	199	0.017	0.000	0.000		256	0.000	0.010	0.010
	201	0.550	0.700	0.530		258	0.317	0.480	0.390
	203	0.267	0.110	0.200		260	0.050	0.020	0.010
	205	0.167	0.160	0.240					
	207	0.000	0.020	0.010	Ljapo93	322	0.017	0.000	0.100
						324	0.000	0.000	0.010
Lama42	248	0.000	0.140	0.000		326	0.100	0.070	0.060
	250	0.200	0.100	0.180		328	0.167	0.170	0.190
	252	0.067	0.010	0.120		330	0.000	0.000	0.040
	256	0.000	0.020	0.010		332	0.067	0.230	0.070
	258	0.050	0.250	0.070		334	0.367	0.190	0.410
	260	0.300	0.320	0.410		336	0.117	0.140	0.030
	262	0.283	0.060	0.180		338	0.050	0.070	0.050
	264	0.067	0.100	0.030		340	0.017	0.080	0.010
	266	0.033	0.000	0.000		342	0.000	0.040	0.020
						344	0.083	0.010	0.010
Lama44	139	0.017	0.000	0.070		346	0.017	0.000	0.000
	141	0.000	0.000	0.010					
	143	0.233	0.230	0.040	Ljapo138	255	0.000	0.000	0.010
	145	0.183	0.150	0.050		259	0.000	0.000	0.010
	147	0.067	0.000	0.000		261	0.000	0.100	0.090
	149	0.033	0.020	0.060		263	0.100	0.000	0.010
	151	0.033	0.120	0.290		265	0.017	0.010	0.020
	153	0.067	0.080	0.030		267	0.050	0.050	0.010
	155	0.033	0.040	0.140		269	0.100	0.190	0.040
	157	0.000	0.050	0.030		271	0.017	0.010	0.030
	159	0.050	0.030	0.070		273	0.050	0.080	0.020
	161	0.067	0.060	0.080		275	0.017	0.030	0.030
	163	0.067	0.040	0.020		277	0.100	0.080	0.260

Continued on next page

Table 4. Continued.

Locus	Allele	YS	NH	HS	Locus	Allele	YS	NH	HS
	165	0.133	0.120	0.040		279	0.100	0.100	0.210
	167	0.000	0.000	0.040		281	0.050	0.000	0.050
	173	0.017	0.000	0.000		283	0.067	0.000	0.030
	179	0.000	0.000	0.020		285	0.067	0.050	0.050
	183	0.000	0.060	0.010		287	0.017	0.020	0.010
						289	0.067	0.140	0.060
Ljapo38	278	0.067	0.030	0.040		291	0.000	0.050	0.010
	300	0.017	0.000	0.000		293	0.050	0.000	0.010
	302	0.000	0.000	0.020		295	0.050	0.020	0.000
	304	0.033	0.010	0.040		297	0.050	0.000	0.010
	306	0.017	0.000	0.000		299	0.017	0.010	0.000
	308	0.050	0.040	0.000		301	0.000	0.020	0.010
	310	0.033	0.060	0.000		303	0.000	0.000	0.010
	312	0.033	0.020	0.000		305	0.000	0.020	0.000
	314	0.033	0.060	0.040		307	0.017	0.010	0.000
	316	0.117	0.070	0.090		311	0.000	0.010	0.000
	318	0.400	0.050	0.540		335	0.000	0.000	0.010

For abrreviations, see legend to Table 3.

Differences in genetic diversity were also reflected in the three population samples. Allele frequencies of all 11 loci selected in each sample are presented (Table 4; Figure 2), revealing differences between the samples. However, despite these differences, no clear difference in the average genetic diversity statistics was found among the population samples (Wilcoxon signed-rank test, $P > 0.05$).

In total, 31 alleles were found to be unique to a single local population, and *U* ranged from 5 in the NH population to 15 in the HS population. Despite this, no population possessed a true diagnostic allele.

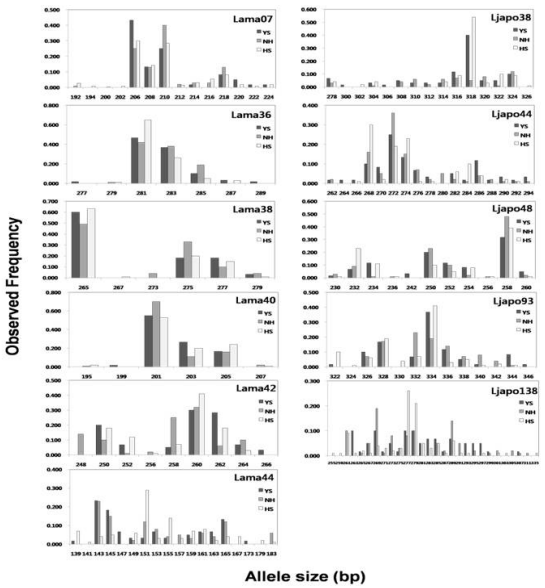

Figure 2. Allele size frequency distributions of the 11 microsatellite loci in the three hatchery populations of *Lateolabrax maculatus* used in this study.

The Hardy-Weinberg tests revealed that 27 (82%) of the 33 locus-population combinations were generally consistent with Hardy-Weinberg proportions; however, six cases exhibited significant deviations from HWE after adjustment of the P values across the 11 loci using the sequential Bonferroni method for multiple observations (Rice, 1989). These six cases resulted from four deficiencies of heterozygotes and two excesses of heterozygotes. Significant deviations were detected at five loci (Lama07, Lama44, Ljapo48, Ljapo93, and Ljapo138). Significant deviations were not evenly distributed among samples or loci, nor were they associated with a particular locus or sample. These deviations were observed at one locus in NH, but at five loci in HS.

The tests of mutation drift equilibrium to detect genetic bottlenecks showed that the YS and NH populations exhibited a heterozygosity excess of microsatellites under the IAM (Wilcoxon test: $P < 0.05$) through the Wilcoxon signed-rank test, suggesting a bottleneck in these two hatchery populations.

Population genetic differentiation

Low but significant genetic differentiation (overall $F_{ST} = 0.027$, $P < 0.001$; $R_{ST} = 0.012$, $P < 0.001$) was observed among three hatchery populations of Korean spotted sea bass. These two statistics with similar results suggested that the level of genetic heterogeneity among these populations was small but statistically significant. Table 5 shows the pattern of genetic differentiation among populations observed by comparing the D_{CE} value and the mean pairwise F_{ST} and R_{ST} values using composite allele frequency data. The pairwise F_{ST} values among populations were significantly different from zero in all pairwise comparisons (all $P < 0.01$ after sequential Bonferroni's correction). On the other hand, the pairwise R_{ST} values among populations were significantly different from zero in only one pairwise comparison between NH and HS. The D_{CE} values were similar to the F_{ST} and R_{ST} values, and they ranged from 0.271 to 0.296. The amount of variation attributable to differences between individuals within populations and within-individual differences were large (3.85 and 93.48, respectively; $P < 0.01$), while only a low but significant proportion of variation (2.67%; $P < 0.01$) was detected among populations (Table 6). The PCA scatter plot based on D_{CE} values showed that a separate cluster was not formed (Figure 3). In addition, the Mantel test was applied to all samples and revealed no significant relationship between the genetic and geographical distances, invalidating the isolation-by-distance model of genetic structure ($P > 0.05$). Together, pairwise F_{ST} and R_{ST} tests, the Mantel test, and the PCA on the three hatchery populations of *L. maculatus* did not show a consistent relationship between geographic and genetic distances.

Table 5. D_{CE} distance (below the diagonal) and mean F_{ST} and R_{ST} estimates (above the diagonal) between each pair of three hatchery populations of Korean spotted sea bass (*Lateolabrax maculatus*).

Population	YS	NH	HS
YS	-	0.018* (0.006)	0.019* (0.012)
NH	0.275	-	0.037* (0.015*)
HS	0.271	0.296	-

For abbreviations, see legend to Table 3.

Table 6. Analysis of molecular variance (AMOVA) of 11 microsatellite loci in three hatchery populations of Korean spotted sea bass (*Lateolabrax maculatus*).

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percent variation (%)	P value
Among populations	2	27.682	0.113	2.67	0.000
Among individuals within a population	127	544.030	0.163	3.85	0.000
Within individuals	130	514.500	3.958	93.48	0.000
Total	259	1086.212	4.234		

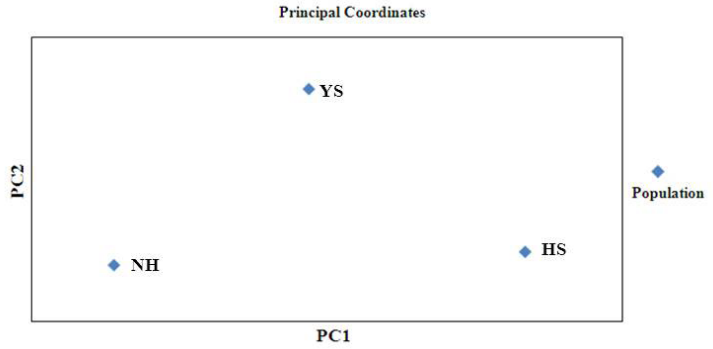

Figure 3. Principal component analysis (PCA) plot showing the phylogenetic relationships among three hatchery samples of the Korean spotted sea bass based on the D_{CE} (Cavalli-Sforza and Edwards, 1967) genetic distances. The abbreviations are as follows: YS = Yeosu; NH = Namhae; and HS = Hwaseong.

DISCUSSION

Microsatellite markers, because of their high polymorphism, are ideal for population studies (An et al., 2012). However, microsatellite genotyping is expensive and time consuming. Genotyping cost and time can be greatly reduced by multiplexing. Efficient multiplexing is especially important in situations in which a large number of samples need to be genotyped at multiple loci quickly and cost-effectively. In this study, we tested and developed four three-plex PCRs of 12 microsatellites for efficient genotyping in spotted sea bass.

Multiplex PCR is a demanding reaction, and optimization is needed to balance the amplification of different loci and avoid cross-amplification (Guichoux et al., 2011). We optimized the four three-plex assay by picking the best primer sets, adjusting primer concentrations, and optimizing annealing temperatures to accommodate 12 markers. The optimized protocol worked well with DNA samples. The combination of the simple DNA extraction protocol, which is fast and inexpensive, and microsatellite multiplexing would greatly reduce genotyping cost and turnaround time, allowing highly efficient genotyping of microsatellites. The multiplex PCR method developed in this study was shown to be capable of quantifying the genetic variability of three hatchery populations of Korean spotted sea bass. Our results indicate that it can be used as a fast, robust, and inexpensive tool with high discriminating power to evaluate the variability of *L. japonicus*.

The genetic variability of the 11 microsatellite loci proved to be extensive. The genetic variability in the three hatchery populations (average A_R = 9.32, average H_E = 0.753) was higher than previous measurements from Chinese natural populations (average N_A = 3 to

9, average H_e = 0.405 to 0.868 at 18 microsatellite loci) (Shao et al., 2009). Similar genetic variability in farmed spotted sea basses (N_A = 8.63, average H_E = 0.724 at 8 microsatellite loci) was also detected using microsatellite markers (An et al., 2013). An et al. (2013) examined one hatchery and one wild population of *L. japonicus* in Korea and found that the hatchery population showed very little reduction in genetic variation compared with the wild population, but the genetic heterogeneity between the populations was significant. Because most of the microsatellite markers used were the same, they can be compared on an equal basis. The microsatellite variation determined here was as high or similar to values in this species from other reports, indicating that a high level of genetic variation has been preserved during the domestication of these three cultured spotted sea bass populations in Korea. In Korea, spotted sea bass seeds are produced exclusively in hatcheries. The large number of effective breeders and/or mixing of genetically different lots produced separately might have a significant contribution to the high genetic variation in the populations studied. Not all loci were equally variable. Specifically, Lama44, Ljapo44, and Ljapo138 displayed greater allelic diversity and higher levels of heterozygosity than other loci.

Significant deviations from HWE were observed in 6 of the 33 population-locus cases. Four cases had lower heterozygosity than predicted by HWE. This deficit may be attributed to improper domestication processes in the hatchery populations, such as a limited number of founders, inbreeding, or both (Kohlmann et al., 2005). In addition, the null alleles observed at the microsatellite loci present a likely explanation for the deviation (Callen et al., 1993). In our study, null alleles were observed at 5 of the 12 loci, but the absence of a null allele at the Ljapo93 locus makes this explanation, alone, difficult to reconcile. In contrast, 2 of the 6 significant HWE deviations were caused by an excess of heterozygotes. A decrease in the effective population size can give rise to a reduction in homozygotes because the sampling bias of alleles can result from a small number of parents and differences in allele frequencies between the sexes (Spencer et al., 2000).

Genetic differentiation, detected by F_{ST} and R_{ST} values, was low but significant between most pairs of hatchery populations. Although we cannot trace the origins of the significant differences between the hatchery populations without records from the farms, this differentiation observed among cultured populations might have resulted from different founder populations. In addition, different selection procedures in hatchery practices also may have led to changes in the genetic composition of the hatchery populations (Li et al., 2007). Similar results were observed in the microsatellite analysis of other hatchery-reared species including fish (Was and Wenne, 2002; Alarcón et al., 2004). AMOVA of all 11 microsatellites revealed that 2.67% (F_{ST} = 0.027, $P < 0.001$) of the genetic variance occurred among the hatchery samples, with the remainder of the variance occurring between individuals within populations and within individuals. The Mantel test and the PCA scatter plot that was constructed using D_{CE} genetic distances did not indicate a regional structure, i.e., individuals from nearby regions were not grouped together (Figure 3). The relatively low F_{ST} value and the lack of a relationship between the geographic and genetic distances suggested that some gene flow occurred among populations during the extensive aquaculture of spotted sea bass, although the possibility of widespread exchanges of stocks and eggs between hatcheries by local farmers cannot be excluded.

In conclusion, an understanding of the genetic structure and diversity among marine resources, especially for species for which artificial stocks are produced by hatchery-produced

seed for natural resource restoration, is critical for the establishment of suitable guidelines for resource management and selective breeding. No detailed information about the genetic diversity of hatchery populations of spotted sea bass in Korea is available to date. In this study, we reported that relatively high genetic variability and significant but minor genetic differentiation were detected among the hatchery populations of spotted sea bass in Korea. Furthermore, the lack of a relationship between the geographic and genetic distances implied extensive spotted sea bass aquaculture. The allelic composition and diversity of spotted sea bass should be carefully considered regarding the seedling and stocking practices of hatcheries in order to conserve the genetic diversity of the natural population. Using the polymorphic microsatellites developed, especially the multiplex set consisting of 12 polymorphic markers, it would be easy to identify individuals to increase the genetic diversity of the existing stocks. Such information will be useful in the genetic management of fisheries and the successful implementation of stock-enhancement programs.

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