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Comparative genetic diversity of wild and released populations of Pacific abalone *Haliotis discus discus* in Jeju, Korea, based on cross-species microsatellite markers including two novel loci

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Pacific abalone *Haliotis discus discus* is an important fisheries resource in Jeju, Korea. For basic information about its current genetic status in relation to stock enhancement, the level and distribution of genetic variation between wild and released stocks of Pacific abalone in Jeju were examined at nine cross-species microsatellite markers including the use of two novel primers. High levels of polymorphism were observed between the two populations. A total of 146 different alleles were found at all loci, with some alleles being unique. The allelic variability ranged from five to 27 in the wild population and from four to 16 in the released sample. The average observed and expected heterozygosities were estimated to be 0.74 and 0.84 in the wild sample and 0.70 and 0.78 in the released sample, respectively. Although a considerable loss of rare alleles was observed in the released sample, no statistically significant reductions were found in heterozygosity or allelic diversity in the released sample compared to the wild population. Low but significant genetic differentiation was found between the wild and released populations. These results suggest that the intensive breeding practices for stock enhancement may have resulted in a further decrease in genetic diversity, and that the cross-species microsatellite markers used in this study represent a potentially efficient means for further genetic studies, providing beneficial information for the protection and management of *H. discus discus*.

Keywords: Pacific abalone; *Haliotis discus discus*; cross-species microsatellite; genetic diversity; stock enhancement

Introduction

Pacific abalone *Haliotis discus discus* is distributed mainly along the coastal waters of Jeju Island, Korea, and the warm south-western waters of Japan. On Jeju Island, this species has long been considered an important species in both fisheries and aquaculture. Recently, stock enhancement programs have been introduced to increase harvest yields, and many Pacific abalone seeds were released into coastal areas of Pyungdae in the island in 2007 by the Jeju province fisheries institute (JPFI). *H. discus discus* (Camac-jeonbok) is considered a sister subspecies of *H. discus hannai* (Cham-jeonbok), from which it is difficult to clearly discriminate based on morphologic characteristics (Ino 1952; An et al. 2005; Hara and Sekino 2005). Nevertheless, some fisheries scientists and fishermen from both the government and private hatcheries sectors in Jeju claim that Camac-jeonbok *H. discus discus* is quite different from the Cham-jeonbok *H. discus hannai*; thus, *H. discus discus* was used in this study.

The Food and Agriculture Organization of the United Nations (FAO) recommends the genetic characterization of both the stock population used in population enhancement programs and the target population, so that any genetic changes can be monitored for the sustainable management of exploited fish populations (FAO 1993). Thus, for the successful management and sustainable use of such species, 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 (Ståhl 1987; Primmer et al. 1999). Detailed information on the genetic status of exploited species can assist in the development of comprehensive long-term management and protection plans. Therefore, many studies have investigated the genetic variability of wild and farmed populations of marine species using several types of molecular markers (Hindar et al. 1991; Sekino et al. 2002; Hara and Sekino 2003; Alarcón et al. 2004; Skaala et al. 2004; Chen et al 2008; Gum et al. 2009). Currently, however, no information is available on the genetic diversity of wild and released populations of *H. discus discus* in relation to stock enhancement in Jeju, Korea.

Microsatellites, short tandem repeats of nuclear DNA, are highly polymorphic co-dominant markers. They are expected to be particularly useful for

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monitoring changes in genetic variation of farmed stocks, parentage assignment, and fine-scale studies of population structure in marine species (Jeong et al. 2003; Li et al. 2004; An et al. 2008). Novel microsatellite markers have been developed from Pacific abalone (Sekino and Hara 2001; Li et al. 2002; An and Han 2005; Sekino et al. 2005). Typically, the development of microsatellites for each new species demands considerable time, effort, and cost. Cross-species comparisons can identify polymorphic microsatellite loci, so that fewer species-specific markers are needed for studies. In an effort to increase the potential use of cross-species microsatellite markers for *H. discus discus* populations and pedigree analyses, we used previously developed primers (An et al. 2010) and two unpublished microsatellite loci from *H. discus hannai*. In the present study, we conducted a baseline survey of the genetic diversity of wild and released populations of *H. discus discus* in Jeju, Korea, using cross-species microsatellite markers.

Materials And Methods

Sample collection and DNA extraction

Two wild and released populations of *H. discus discus* (each $n = 50$; shell length, over 10 cm) were sampled from the coastal waters of Pyungdae in Jeju Island, Korea in April 2009 by some fisheries scientists of the JPFI. This coastal water was the place that many hatchery-raised seeds (shell length 4 cm, 18–20 months of age) had been released in 2007 as part of a stock enhancement program by the JPFI. Hatchery-raised abalone seeds were partially produced from the brood stock captured and reared for reproduction at the JPFI and bought partially by the Jeju special self-governing province. No details regarding the founding and maintenance of the hatchery-raised seeds bought are available; however, their original parents were held at a farm on the coast of Jeju, Korea. Samples were selected by very experienced researchers, based on the shell characteristics of wild animals; for released specimens, clear green shell coloration of 4 cm length on the top of shell, which is typically observed in hatchery-raised abalone, and/or metal tags on shells (which had been applied as part of the stocking practice) were also used for identification. It can be considered that the extent to which stocking impacts the wild population is small or little in the case of abalone with shell length over 10 cm (4 years of age). Tissue samples (approximately 1 cm^3) from two populations were taken and 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, Osaka, Japan). The DNA extractions were performed using an automated DNA extraction system (MagExtractor MFX-2100; Toyobo), according to the manufacturer's instructions. The extracted genomic DNA was stored at −20°C until needed.

Microsatellite genotyping

Seven highly variable microsatellite loci characterized recently (KHdh14, KHdh35, KHdh48, KHdh54, KHdh73, KHdh87 and KHdh88; An et al. 2010) and two unpublished loci (KHdh3 and KHdh4161) from *H. discus hannai* were used. A total of 100 abalones from the two populations were typed. Primer sequences, microsatellite repeat sequences, fluorescent labels, 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, Foster City, CA, USA). PCR amplification of nine microsatellite loci was performed using an RTC 200 instrument (MJ Research, Waltham, MA, USA) in 10 μL solution containing 10–50 ng DNA, 1 × ExTaq buffer, 0.2 mM dNTPs, 10 pmol each primer, and 0.25 U Taq DNA polymerase (TaKaRa Biomedical Inc., Shiga, Japan). 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 for 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

Table 1. Two novel cross-species primers of used for genotyping of *Haliotis discus discus*.

Locus	Repeat motif	Primer sequence (5′→3′) (F = forward, R = reverse)	*Ta*(°C)	Forward primer fluorescent label	Genebank accession no.
KHdh3	$(TG)_{10}$	F: GTCACATACCAAGCAAACTGT R: GTCAAACAAACCAGCCTCTA	59	HEX	AY948308
KHdh4161	$(TG)_4TA(TG)_{22}$	F: CGAGTCTCGTTTTCACCTC R: T C GGTGGAATCTACATC TATC	56	6-FAM	DQ225168

Ta is the octimal annealing temoerature

PCR products was determined using GeneMapper software (version 4.0, PE Applied Biosystems).

Statistical analyses

MICRO-CHECKER 2.2.3 software (van Oosterhout et al. 2004) was used to detect genotyping errors due to null alleles, stuttering or allele dropout (1000 randomizations). GENEPOP 3.1b software (Rousset and Raymond 1995) was used to identify deviations from Hardy-Weinberg equilibrium, HWE (exact tests, 1000 iterations), allelic size range, number of unique alleles, and observed and expected heterozygosities (indicating an excess or deficiency of heterozygotes). FSTAT 2.9.3.2 (Goudet 1995) was used to calculate number of alleles, gene diversity, and F_{IS} (Weir and Cockerham 1984) per locus and sample.

The extent of population subdivision was examined by calculating fixation indices. ARLEQUIN 3.0 software (Excoffier et al. 2005) was used to assess linkage disequilibrium for all pairs of loci (Slatkin and Excoffier 1996) and calculate single-locus F_{ST} and global multilocus F_{ST} values (1000 permutations) (Weir and Cockerham 1984). R_{ST} (Slatkin 1995; Michalakis and Excoffier 1996) estimates were calculated using the computer program GENEPOP version 3.4, available on the internet. R_{ST} incorporates the correlation of the weighted mean allele size expressed as the number of tandem repeats. Significance levels were adjusted for multiple tests using the sequential Bonferroni correction technique (Rice 1989).

Results

Cross-species amplification

The majority of cross-species amplifications from the closely related abalone species resulted in PCR fragments with the expected microsatellite morphology and correct allele sizes, indicating homologous loci. Homology was not tested through sequence comparison. More than 50% of all individuals were reanalysed to ensure the repeatability of allele scoring. Samples that failed to show amplification products were reamplified. There was no indication that genotyping errors affected allele scoring due to allele dropouts or stuttering at any of the markers in any of the samples. Samples that failed to amplify were not included and this makes it unlikely that poor DNA quality affected our results.

Genetic variability comparisons

All primers were polymorphic in all samples, and the level of polymorphism varied depending on the locus. The genetic variability in each population is presented in Table 2. In total, 146 different alleles were observed over nine loci, and some alleles were unique to each population. The number of alleles per locus varied from four at locus KHdh54 to 27 at locus KHdh14; fewer alleles were found in the released population than in the wild one, but the difference was not statistically significant ($P > 0.05$).

The MICRO-CHECKER analysis showed that some loci may have been influenced by one or more null alleles in both populations; loci KHdh3, KHdh54, and KHdh73 in the released samples and KHdh3 and KHdh73 in the wild population were affected. KHdh3 and KHdh73 appeared to have been influenced in both populations, which indicate that using these two loci for population genetic analyses that assume HWE may be problematic. Thus, a global multilocus F_{ST} value was estimated with and without this locus. However, locus KHdh54 was affected by null alleles in only one sample; thus, this was included in further analyses.

The levels of observed (*Ho*) and expected (*He*) heterozygosity varied between the two sample populations, ranging from 0.30 at KHdh3 to 1.00 at KHdh14, KHdh48, and KHdh87, and from 0.50 at KHdh3 to 0.97 at KHdh4161, respectively. No significant difference between *Ho* and *He* was found between samples at most loci (Kruskal-Wallis test, $P > 0.05$).

Wild samples had higher genetic diversity (G_d) and a wider range of allele sizes (S) at most loci (Table 2). In total, 89 alleles were unique to the two populations: 63 in wild samples and 26 in released ones. For most loci, wild abalone had more unique alleles than the released population. The allele frequencies at all nine selected loci in each sample are shown in Figure 1 and Table 3. These data reveal differences between the populations. The inbreeding coefficients (F_{IS}) varied among markers, from -0.318 (KHdh88) to 0.454 (KHdh54) in the released samples, and from -0.253 (KHdh87) to 0.524 (KHdh3) in the wild samples. The average F_{IS}, including all markers, was 0.17 in the released population and 0.15 in the wild one.

No significant linkage disequilibrium was found for all pairs of loci after sequential Bonferroni for each of 36 simultaneous tests in the wild and hatchery populations.

Significant departures from HWE ($P < 0.0055$) were found after Bonferroni correction at six loci (KHdh3, KHdh14, Khdh54, Khdh73, Khdh87, and KHdh88) in the released samples and at two loci (Khdh3 and KHdh73) in the wild ones, indicating that deviations from HWE were due to heterozygosity deficit or excess.

Genetic differentiation between the two populations was estimated using F_{ST} and R_{ST} estimates. The global multilocus F_{ST}, including all loci, was 0.021 ($P < 0.001$), and the R_{ST} value was 0.018

Table 2. Summary statistics for nine microsatellite loci between abalone collections.

Population (No)		Microsatellite loci									Average across loci
		Khdh3	Khdh4161	Khdh14	Khdh35	Khdh48	Khdh54	Khdh73	Khdh87	Khdh88	
	F_{ST} (R_{ST})	0.0142	0.0264	0.0169	0.0132	0.0620	0.0175	0.0163	0.0026	0.0142	0.0207 (0.0178)
HddR (50)	a	6	16	10	11	16	4	13	8	6	9.33
	Gd	0.50	0.64	0.91	0.84	0.89	0.61	0.90	0.75	0.66	0.74
	R	404–414	146–186	140–222	134–158	178–236	272–320	188–220	122–148	136–152	
	U	2	9	4	2	4	0	4	0	1	2.89
	He	0.497	0.867	0.816	0.840	0.888	0.606	0.890	0.912	0.691	0.779
	Ho	0.300	0.745	1.000	0.900	0.951	0.333	0.533	0.754	0.817	0.704
	*F*is	0.401	0.351	−0.094	−0.073	−0.129	0.454	0.405	0.333	−0.318	0.17
		(0.003)	(0.000)	(0.000)	(0.060)	(0.093)	(0.000)	(0.000)	(0.001)	(0.001)	
	P	0.004	0.010	0.000	0.047	0.132	0.000	0.000	0.001	0.003	
HddN (50)	a	6	27	16	11	22	5	12	14	13	12.33
	G_d	0.63	0.70	0.96	0.89	0.93	0.66	0.90	0.87	0.78	0.81
	R	392–410	136–188	144–240	134–166	170–246	272–320	176–224	122–174	118–162	
	U	2	20	9	2	10	1	5	6	8	7.00
	He	0.625	0.968	0.958	0.889	0.930	0.660	0.889	0.869	0.782	0.841
	Ho	0. 00	0.835	0.967	0.867	1.000	0.500	0.533	1.000	0.633	0.737
	*F*is	0.524	0.430	−0.010	0.025	−0.177	0.245	0.404	−0.253	0.193	0.15
		(0.000)	(0.000)	(0.218)	(0.556)	(0.480)	(0.040)	(0.000)	(0.358)	(0.053)	
	P	0.000	0.008	0.138	0.057	0.549	0.046	0.000	0.400	0.035	
Mean population	a	6.00	21.50	13.00	11.00	19.00	4.50	12.50	11.00	9.50	10.83
	G_d	0.57	0.67	0.94	0.87	0.91	0.64	0.90	0.81	0.72	0.77
	U	2.00	14.50	6.50	2.00	7.00	0.50	4.50	3.00	4.50	4.00
	He	0.561	0.918	0.887	0.865	0.909	0.633	0.890	0.891	0.737	0.815
	Ho	0.300	0.790	0.984	0.884	0.976	0.417	0.533	0.877	0.725	0.733

Single-locus F_{ST}, R_{ST}, number of samples (No), number of alleles per locus (a), gene dic = versity (G_d), allelic size range (R), number of unique alleles (U), expected heterozygosity (*He*), observed heterozygosity (*Ho*), inbreeding coefficient (*F*is), and probability of significant deviation from Hardy-Weinberg equibrium after Bonferroni correction (P, initial $\alpha = 0.05/9 = 0.0055$) are given for each population and locus. Calculation assume that individuals with one micosatellite with one microsatellite band are homozygous for the allele. Number in parentheses below Fis indicates the probability of significant heterozygosity excess or deficit.

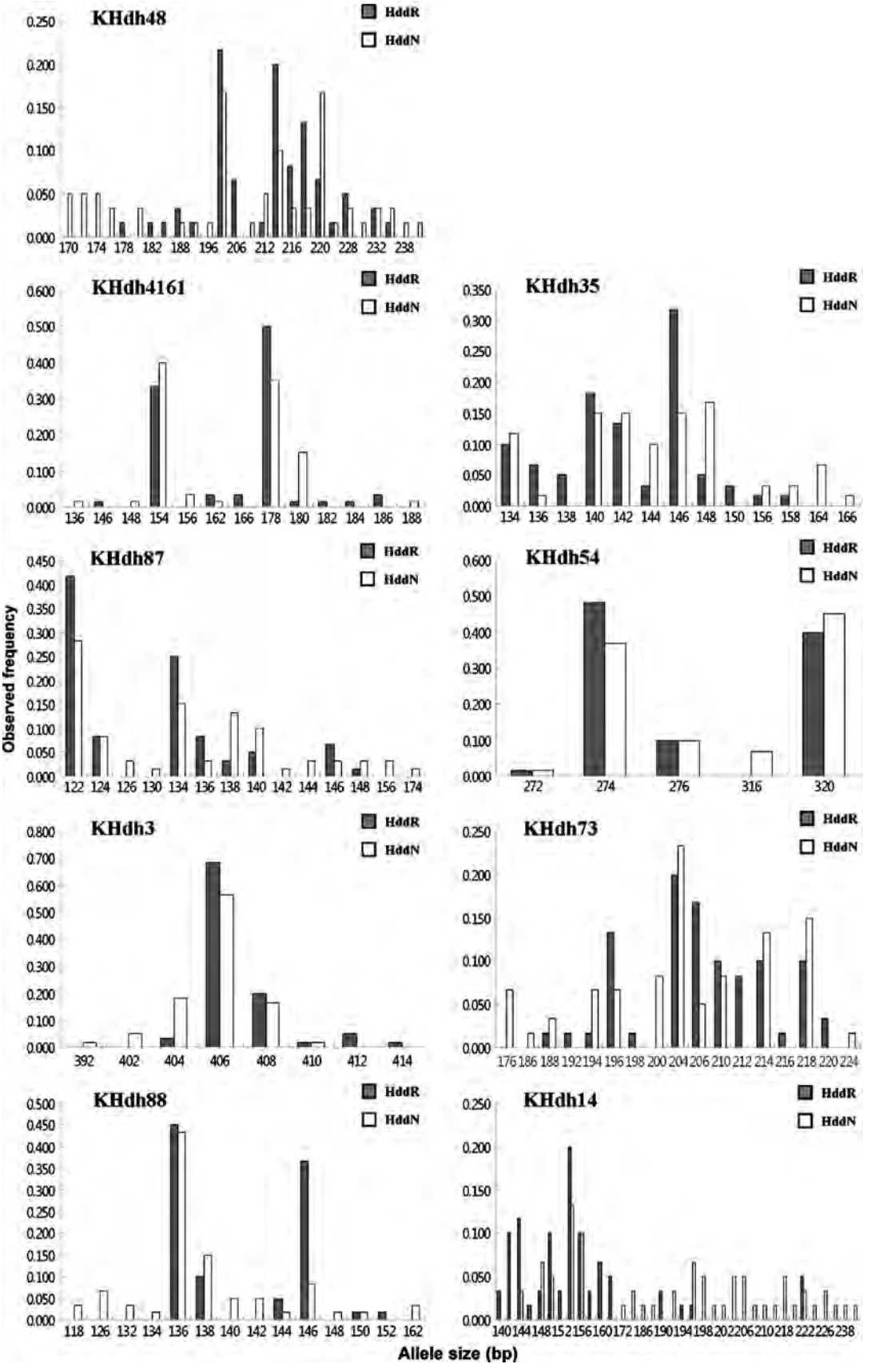

Figure 1. Allele size frequency distributions of the nine microsatellite loci in two *Haliotis discus discus* populations. HddR, released abalone; HddN, wild abalone.

Table 3. Frequency of each microsatellite allele in wild and hatchery *Haliotis discus discus* samples in Jeju, Korea.

Locus	allele	HddR	HddN	Locus	allele	HddR	HddN
khdh48	170	0.000	0.050	**khdh35**	134	0.100	0.117
	172	0.000	0.050		136	0.067	0.017
	174	0.000	0.050		138	0.050	0.000
	176	0.000	0.033		140	0.183	0.150
	178	0.017	0.000		142	0.133	0.150
	180	0.000	0.033		144	0.033	0.100
	182	0.017	0.000		146	0.317	0.150
	184	0.017	0.000		148	0.050	0.167
	188	0.033	0.017		150	0.033	0.000
	192	0.017	0.017		156	0.017	0.033
	196	0.000	0.017		158	0.017	0.033
	198	0.217	0.167		164	0.000	0.067
	206	0.067	0.000		166	0.000	0.017
	208	0.000	0.017				
	212	0.017	0.050	**khdh54**	272	0.017	0.017
	214	0.200	0.100		274	0.483	0.367
	216	0.083	0.033		276	0.100	0.100
	218	0.133	0.033		316	0.000	0.067
	220	0.067	0.167		320	0.400	0.450
	226	0.017	0.017				
	228	0.050	0.033	**khdh73-1**	176	0.000	0.067
	230	0.000	0.017		186	0.000	0.017
	232	0.033	0.033		188	0.017	0.033
	236	0.017	0.033		192	0.017	0.000
	238	0.000	0.017		194	0.017	0.067
	246	0.000	0.017		196	0.133	0.067
					198	0.017	0.000
khdd4-1/6-1	136	0.000	0.017		200	0.000	0.083
	146	0.017	0.000		204	0.200	0.233
	148	0.000	0.017		206	0.167	0.050
	154	0.333	0.400		210	0.100	0.083
	156	0.000	0.033		212	0.083	0.000
	162	0.033	0.017		214	0.100	0.133
	166	0.033	0.000		216	0.017	0.000
	178	0.500	0.350		218	0.100	0.150
	180	0.017	0.150		220	0.033	0.000
	182	0.017	0.000		224	0.000	0.017
	184	0.017	0.000				
	186	0.000	0.033	**khdh14**	140	0.033	0.000
	188	0.000	0.017		142	0.100	0.000
					144	0.117	0.033
khdd87	122	0.417	0.283		146	0.017	0.000
	124	0.083	0.083		148	0.033	0.067
	126	0.000	0.033		150	0.100	0.050
	130	0.000	0.017		152	0.033	0.000
	134	0.250	0.150		154	0.200	0.133
	136	0.083	0.033		156	0.100	0.100
	138	0.033	0.133		158	0.033	0.000
	140	0.050	0.100		160	0.067	0.000
	142	0.000	0.017		162	0.050	0.000
	144	0.000	0.033		172	0.000	0.017
	146	0.067	0.033		182	0.000	0.033
	148	0.017	0.033		186	0.000	0.017
	156	0.000	0.033		188	0.000	0.017
	174	0.000	0.017		190	0.033	0.000
					192	0.000	0.033
khdd3	392	0.000	0.017		194	0.017	0.000
	402	0.000	0.050		196	0.017	0.067
	404	0.033	0.183		198	0.000	0.050
	406	0.683	0.567		200	0.000	0.017
	408	0.200	0.167		202	0.000	0.017
	410	0.017	0.017		204	0.000	0.050
	412	0.050	0.000		206	0.000	0.050
	414	0.017	0.000		208	0.000	0.017
					210	0.000	0.017
khdd88	118	0.000	0.033		212	0.000	0.017
	126	0.000	0.067		218	0.000	0.050
	132	0.000	0.033		220	0.000	0.017
	134	0.000	0.017		222	0.050	0.033
	136	0.450	0.433		224	0.000	0.017
	138	0.100	0.150		226	0.000	0.033
	140	0.000	0.050		230	0.000	0.017
	142	0.000	0.050		238	0.000	0.017
	144	0.050	0.017		240	0.000	0.017
	146	0.367	0.083				
	148	0.000	0.017				
	150	0.017	0.017				
	152	0.017	0.000				
	162	0.000	0.033				

($P < 0.001$). When loci KHdh3 and Khdh73 were excluded, the global multilocus F_{ST} was 0.022 ($P < 0.001$). Low but significant F_{ST} and R_{ST} estimates indicate the presence of genetic differentiation between the released and wild populations.

Discussion

Despite the importance of *H. discus discus* as a highly valuable fishery resource in Jeju, Korea, there is little information regarding the genetic background of this species. The present study is the first report on the genetic status of this species using microsatellite markers.

We used previously developed microsatellite markers from *H. discus hannai* to evaluate the genetic variability of *H. discus discus*; these cross-species primers could serve as a valuable tool, sufficient to detect genetic differences between the wild and released populations. Although many more samples are needed to confirm these results, the cross-species primers might be valuable during the initial phase of genetic analyses, while taking into account the relatedness of species. Previously, Hara and Sekino (2005) reported that the microsatellite markers developed from *H. discus hannai* provided high resolution for studying the genetic

differences between *H. discus hannai* and *H. discus discus* populations.

We found that both the number of alleles and expected heterozygosity are reduced in the released hatchery-raised abalone population, showing only 76% of the average number of alleles and 90% of the expected heterozygosity expressed in the wild abalone population, with considerable loss of rare alleles, although no statistically significant reduction was found. A reduction in genetic diversity relative to wild populations seems to be a characteristic of hatchery-raised organisms. It may be caused by founder effects, as well as artificial and natural selection in the culture environment (Tessier et al. 1997; Coughlan et al. 1998; Was and Wenne 2002; Li et al. 2004, 2007; Porta et al. 2006; Marchant et al. 2008). Allelic frequency in wild populations is not always reflected in hatchery populations due to random changes in the production process of a new generation. In fact, allele loss has a greater impact on genetic diversity than changes in allele frequency, because the latter may change again by random genetic drift, but there is no way to recover a lost allele. High levels of polymorphism were detected in the released population (mean $He = 0.76$) over all nine microsatellite loci examined. Similar results have been reported for *H. discus hannai* and other cultivated fish species (Elliott and Reilly 2003; Li et al. 2007; An et al. 2008; Marchant et al. 2008).

Significant deviations from Hardy-Weinberg (H-W) expectations were observed in both populations. In general, the causes of such H-W disequilibrium, found frequently in microsatellite loci, probably include the substructuring of the population sample, inbreeding, homoplasy, or the presence of null alleles (Estoup et al. 1995; Banks et al. 1999; Li et al. 2003). In addition, a higher occurrence of H-W disequilibrium events occurred in the hatchery-raised stock than in the wild one (six versus two, respectively; Table 2). This may have resulted from admixture of more than two independent populations, non-random mating, or artificial and natural selection during seed production and cultivation in the hatchery stock. The occurrence of higher F_{IS} values at all loci in the released sample suggests that non-random mating might have occurred.

Based on F_{ST} and R_{ST} values, there is significant genetic differentiation between the populations. This difference is probably a result of reduced genetic variation due to a lower effective number of founding individuals in the hatchery, and the effects of artificial selection on the hatchery progeny (Table 2).

It has been suggested that reduced genetic variation can result in reduced performance in aquaculture, because this is the source of variation for important traits such as growth rate and disease resistance (Allendorf and Phelps 1980; Vuorinen 1984). There are several reports of reduced fitness in hatchery-bred samples when exposed to natural environments (Hansen 2002; Li et al. 2004). Release of genetically maladapted fishery reduces the effectiveness of enhancement, and is most detrimental overall if the fitness of hatchery raised fishery is only moderately lower than in the wild population. Thus, for an adequate stocking strategy for *H. discus discus* in Jeju, Korea, periodic genetic evaluations of broodstock, progeny, and target populations are necessary, on behalf of the health of the wild fishery resource.

For the proper management of stock enhancement programs, genetic monitoring for structure and diversity must be considered in addition to biological, ecological, and fishery factors. Before carrying out a large-scale release for stock enhancement, genetic differences between cultured and wild populations must be identified and quantified if effective monitoring of gene flow is to occur. It is therefore important that adequate amounts of this kind of genetic information be available both for hatchery-reared populations and for wild populations that may be affected by migration from them. This information will be useful for evaluating 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 population for the following season.

In summary, we investigated the genetic diversity of wild and released populations of *H. discus discus* in Jeju, Korea, for the first time, using cross-species microsatellite markers developed from *H. discus hannai*. Our results show that the markers used can detect changes in genetic composition between hatchery-raised and wild populations, and that hatchery-raised abalone has genetically differentiated from the wild population. This in turn indicates that genetic monitoring is a useful tool for the preservation and further genetic management of *H. discus discus* in Korea.

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