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Genetic Variation of Korean Masu Salmon (*Oncorhynchus masou*) Populations Inferred from Mitochondrial DNA Sequence Analysis

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We analyzed the nucleotide sequences of about 500 bp of the mitochondrial NADH dehydrogenase subunit 3 (ND3) gene to estimate the genetic variation of Korean masu salmon (*Oncorhynchus masou*) populations. DNA samples were collected from 104 river-only specimens and 52 anadromous specimens from three hatcheries and one river. There are no records of artificial release into the river. We amplified the ND3 gene by polymerase chain reaction, targeting areas that included parts of the cytochrome oxidase III gene and the NADH dehydrogenase subunit 4L gene, and defined 14 haplotypes based on 12 variable nucleotide sites in the examined region. Among the haplotypes, ten were specific to river-only specimens within hatchery populations. Haplotype diversity of river-only populations in hatcheries was higher than that of anadromous and wild populations. Pairwise population F_{ST} estimates and neighbor-joining tree analyses inferred that anadromous and river-only populations were distinct. These results suggest that sequence polymorphism in the ND3 region may be a useful marker for analyzing the genetic variation and population structure of masu salmon.

Key words: Genetic variation, Masu salmon, Mitochondrial DNA, *Oncorhynchus masou*

Introduction

Masu salmon, *Oncorhynchus masou*, are found only in the Far East, including Korea, Russia, and Japan, particularly in the East Sea, the Sea of Okhotsk, and the Northern Pacific Ocean (Kato, 1991). In Korea and central Japan, there are both anadromous and river-only populations. Genetic variation is important for the long-term survival of natural populations, because it confers the ability to adapt to changing environmental conditions, thereby increasing fitness (Frankel and Soulé, 1981). A lack of genetic variation or too much homozygosity caused by inbreeding can be detrimental to fitness. The degree of genetic variation within and among populations is determined by identifying populations (Avise, 1994). Among the limited number of studies that have applied genetic analyses to masu salmon in Korea, several have shown sequence divergence of mitochondrial (mt) DNA and allelic variation in protein-coding loci (Hong et al., 1994; Lee et al., 2000). However, recently developed molecular techniques have increased the accuracy and resolution of estimates of genetic variation compared to conventional allozyme analysis (Taniguchi et al., 2003). Maternally inherited mtDNA has more sequence variability than heterologous mtDNA molecules and most single-copy nuclear genes, which do not recombine with nuclear DNA (Brown et al., 1979). Therefore, analyzing maternally inherited mtDNA is a useful and important way to investigate the genetic variation of populations of aquatic organisms. Many fish species, including salmon, have been genetically analyzed based on mtDNA. However, both restriction fragment length polymorphism (RFLP) analyses (Park et al., 1993) and allozyme analyses (Seeb and Crane, 1999) have resulted in low levels of variation. Sato et al. (2001) found greater variation using nucleotide sequences. In particular, the NADH dehydrogenase subunit 3 (ND3) region of mtDNA may be a useful genetic marker, because it is conserved across all organisms and has a fast evolutionary rate (McKay et al., 1996; Verspoor et al., 1999).

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We conducted polymerase chain reaction (PCR) on the ND3 gene and adjacent genes in the mtDNA of Korean masu salmon to investigate its use as a competent DNA marker for population genetic analyses. Our results may help properly manage masu salmon and other fish species.

Materials and Methods

Fish samples and DNA extraction

Fin samples were collected from 156 masu salmon, including 29 wild, river-only specimens from the Mulchi River (MCR), and 127 specimens (75 river-only and 52 anadromous) of three different brood-stocks from the Cold Water Inland Fisheries Research Institute (CWIF), the Gyeongbuk Research Center for Freshwater Fish (GRCF), and the River Development Project Agency (RDPA; Table 1). The samples were preserved in 100% ethanol at room temperature until use. Total genomic DNA was extracted using a conventional phenol-chloroform method (Sambrook et al., 1989). An ultraviolet spectrophotometer (Shimadzu, Japan) was used to determine the quantity and quality of the isolated DNA. The DNA concentration was estimated by measuring absorbance at 260 nm. Protein contamination was estimated by calculating the ratio of absorbance at 260 nm and 280 nm.

PCR amplification and nucleotide sequence analysis

About 900 bp of the mtDNA ND3 region were amplified by PCR, targeting an area that included parts of the cytochrome oxidase III (COIII) gene and the NADH dehydrogenase subunit 4L (ND4L) gene. Primers and their sequences were designed based on the reported sequences of masu salmon, chum salmon (*O. keta*), and Atlantic salmon (*Salmo salar*; Oohara et al., 1997; Table 2 and Fig. 1). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) after examining size and quality with electrophoresis on a 1.5% agarose gel. Amplified products were cloned with the TOPO cloning® kit (Invitrogen) and sequenced with M13 forward and T7 reverse primers using the ABI3130xl capillary sequencer (Applied Biosystems). The ampli-fied PCR products were directly sequenced with the following reaction mixture: 50 μL reaction mixture with 0.5-1 μg template DNA, 1x PCR buffer, 1 unit *Taq* DNA polymerase (Qiagen), 0.2 mM dNTPs, and 25 pM of each forward and reverse primer. The cycling conditions included pre-cycling denaturation at 94°C for 10 min, following by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. One μL of PCR product was used for sequencing reactions with a BigDye® Terminator Cycle Sequencing kit version 1.1 (ABI) according to the manufacturer's instruction. The sequence data were aligned using DNASIS software (Hitachi) to determine the genotypes (haplo-types) of the ND3.

Population genetic analysis

We estimated haplotype and nucleotide diversity within populations, and nucleotide divergence between populations according to Nei (1987) and Nei and Tajima (1981), using the two parameter distance method of Kimura (1980) and the K and DA programs in REAP (MeElroy et al., 1993). Pairwise F_{ST} values were calculated to estimate the genetic distance between populations, following Slatkin and Hudson (1991) and using the Arlequin version 2.000 program package. A neighbor-joining tree was constructed for each replicate using Neighbor program, and a consensus tree was generated using Consensus program in Phylip version 3.5c (http://www.Evolution.genetics.washington.edu/phylip.html).

Results and Discussion

The primers OKM1F and OKM1R (Table 2 and Fig. 1) successfully amplified the ND3 region of samples from eight individuals. Sequencing of the cloned PCR products resulted in approximately 900 bp sequences with truncated 5' ends and complete 3' ends flanked by COIII and ND4L. A base homology analysis with reported chum salmon ND3 sequences (Oohara et al., 1997; GenBank accession number D84147) confirmed that the obtained sequences were indeed masu salmon mtDNA ND3. Direct sequencing of the PCR products revealed 548 bp sequences with

Table 1. Sampling information of masu salmon tissues used for mtDNA analysis

Sampling location	Code	Date of collection	Numbers of type sample
Gyeongbuk Research Center for Freshwater Fish	GRCF	Aug. 2006	River-only: 27 Anadromous: 24
River Development Project Agency	RDPA	Aug. 2006	River-only: 24 Anadromous: 28
Cold Water Inland Fisheries Research Institute	CWIF	Aug. 2006	River-only: 24
Mulchi River	MCR	Aug. 2006	River-only: 29

Table 2. Nucleotide sequences of oligonucleotide primers for PCR and sequencing

Name	Sequence (5'-3')
OKM1F	GCATCATAGAAGGTGAACGA
OKM1R	CATTTGAAGGGCTCATAGGG
OKM2F	TTACAATCGCTGACGGCG
OKM2R	GGTGCGGTGAAACGCGAGTC

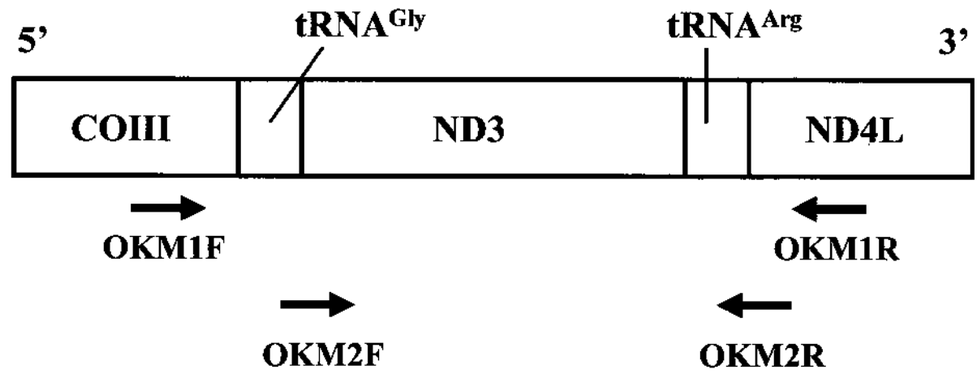

Fig. 1. Positions and directions of primers for PCR and sequencing.

the OKM2F and OKM2R primers (Table 2 and Fig. 1) in 156 specimens from all four localities, and 12 variable nucleotide sites defining a total of 14 haplotypes (Table 3). The nucleotide sequences of the 14 haplotypes were deposited in GenBank under accession numbers EU294012-EU294025. The 12 variable nucleotide sites were base substitutions with a transversion and transition of four and eight, respectively. Substitution at each site (except one of them) was biallelic (Table 3), suggesting a single base substitution among sequences. The distribution of the 14 haplotypes among the salmon populations is presented in Table 4. There were two to three haplotypes in each population, except for river-only salmon from RDPA and CWIF, which had five to nine haplotypes. OMM02, OMM04, OMM05, and OMM08-OMM14 were specific to river-only specimens, and OMM03 occurred in all populations (Table 4). These results suggest that most of the 14 haplotypes are randomly distributed throughout river-only and anadromous populations, but OMM01, OMM03, and OMM07 are shared.

Table 3. Variable nucleotide sites in 548 bp sequences of the ND3 region in the masu salmon mtDNA and defined haplotypes. Dots indicate the nucleotide identical to that in the OMM1 sequence

Haplotype	Variable nucleotide sites											
	1	10	37	38	287	383	431	446	482	500	521	531
OMM01	T	T	A	C	G	G	A	C	A	A	C	T
OMM02	.	.	.	.	A	.	.	.	.	C	.	.
OMM03	.	.	.	.	A	.	.	.	.	.	.	.
OMM04	.	.	.	.	A	.	.	.	G	.	.	.
OMM05	.	.	.	.	A	.	G	T	.	.	.	.
OMM06	.	.	.	.	A	A	.	.	.	.	.	.
OMM07	.	.	C	.	A	.	.	.	.	.	.	.
OMM08	.	.	C	.	A	.	.	.	G	C	.	.
OMM09	.	.	C	.	A	.	G	T	.	.	.	.
OMM10	.	A	.	.	A	.	.	.	G	.	T	A
OMM11	A	.	.	.	A	.	.	.	.	.	.	.
OMM12	A	.	.	T	A	A	.	.	.	.	.	.
OMM13	A	.	C	.	A	.	.	.	.	.	.	.
OMM14	G	.	.	T	A	.	.	.	.	.	.	.

Table 4. Distribution of ND3 haplotypes in 4 populations. Symbol "—" indicates no detection

Haplotype	GRCF		RDPA		CWIF	MCR
	River-only	Anadromous	River-only	Anadromous	River-only	River-only
OMM01	3	—	—	8	—	—
OMM02	—	—	—	—	1	—
OMM03	22	23	15	19	12	26
OMM04	—	—	—	—	5	—
OMM05	—	—	—	—	1	—
OMM06	—	—	5	—	—	—
OMM07	—	1	2	1	—	2
OMM08	—	—	—	—	1	—
OMM09	—	—	—	—	1	—
OMM10	—	—	—	—	1	—
OMM11	2	—	—	—	1	1
OMM12	—	—	1	—	—	—
OMM13	—	—	—	—	1	—
OMM14	—	—	1	—	—	—

Both haplotype and nucleotide diversity of river-only fish were higher than those of anadromous fish from GRCF and RDPA, suggesting more genetic variation in river-only populations (Table 5). However, the wild MCR river-only population had the lowest haplotype and nucleotide diversities. These results suggest that this wild population might have experienced a prolonged or severe demographic bottleneck in recent times (Nei et al., 1975). The CWIF population had the highest haplotype diversity (Table 5), possibly due to human-mediated introduction of stocks from foreign countries. Indeed, the CWIF population had a large number of haplotypes that were not found in other populations (Table 4).

Pairwise F_{ST} estimates revealed a significant difference between river-only and anadromous populations (Table 6), although there was no significant difference between anadromous populations from GRCF and MCR, or between the anadromous population from RDPA and the river-only population from GRCF. The unrooted consensus neighbor-joining tree showed a clearer difference between river-only and anadromous populations (Fig. 2). These results suggest low or restricted gene flow between river-only and anadromous populations in different geo-graphic locations of Korea. Further analyses using more populations from adjacent areas in Korea and other countries would help elucidate the gene flow pattern of masu salmon.

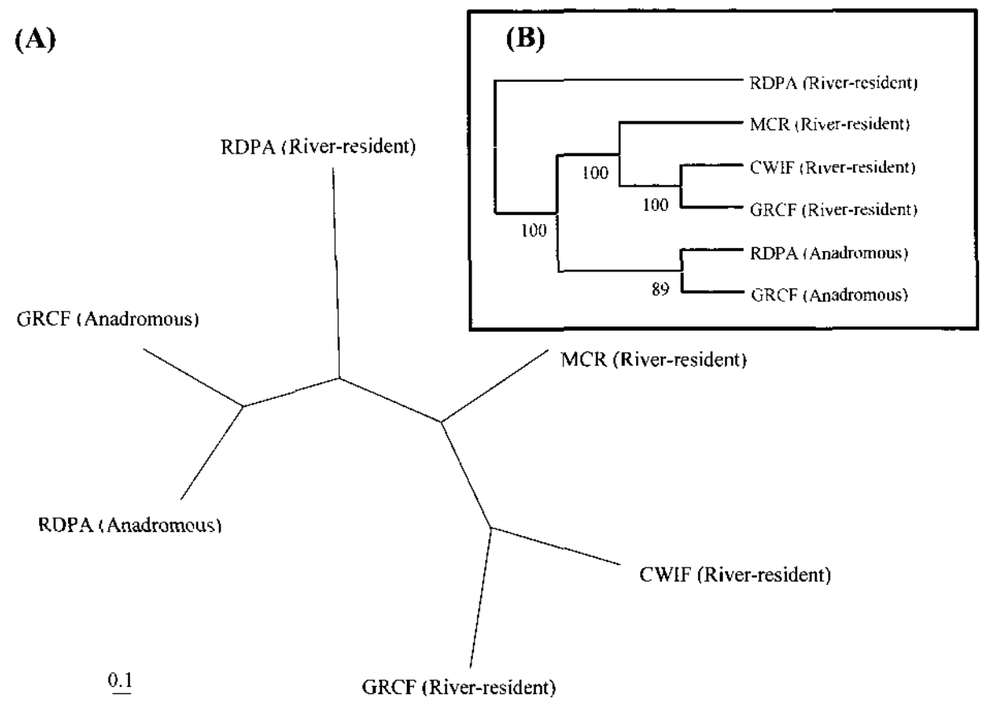

Fig. 2. Unrooted neighbor-joining tree showing genetic distance based on Kimura two-parameter model among 6 masu salmon populations (A). In the inset (B), the topology of the consensus tree (not scaled) is shown with nodal values for bootstrap support over 50% of the 1,000 replicated trees.

In conclusion, we found that the sequence polymorphism of the mtDNA ND3 region was a useful marker for analyzing the genetic variation of masu salmon in Korean populations. Our results may help obtain the baseline data necessary to improve stocking programs, conserve genetic variation, and maintain biodiversity.

Table 5. Number of total haplotypes, haplotype diversity (H) and nucleotide diversity ($\pi \pm SD$) in 4 populations of masu salmon

Population		Total haplotypes	H	π
GRCF	River-only	3	0.331	0.0006±0.0007
	Anadromous	2	0.083	0.0002±0.0003
RDPA	River-only	5	0.580	0.0016±0.0013
	Anadromous	3	0.473	0.0009±0.0009
CWIF	River-only	9	0.725	0.0027±0.0019
MCR	River-only	3	0.197	0.0004±0.0005

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Table 6. Population pairwise F_{ST} estimates of masu salmon based on the mtDNA ND3 sequence data. Symbol "*" indicates significant with significance level $P < 0.05$. Number of permutations was 1023.

Population		GRCF		RDPA		CWIF
		River-only	Anadromous	River-only	Anadromous	River-only
GRCF	River-only					
	Anadromous	0.0515				
RDPA	River-only	0.0749*	0.1661*			
	Anadromous	0.0318	0.1891*	0.0819*		
CWIF	River-only	0.1087*	0.2244*	0.0514	0.0958*	
MCR	River-only	0.0126	-0.0187	0.1127*	0.1397*	0.1736*

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