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Heteropolymorphism of mitochondrial NADH dehydrogenase subunit 3 gene for the population analysis of chum salmon, *Oncorhynchus keta*

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Abstract: *Mitochondrial DNAs (mtDNAs) has been frequently used as genetic markers for the population genetic studies. In this study, we used chum salmon (Oncorhynchus keta) from Korea, Japan and America, and compared their mitochondrial NADH dehydrogenase subunit 3 (ND3) genes by DNA sequence analysis. Sequence variation was studied in the ND3 among total 11 individuals from three populations. The ND3 gene was amplified by PCR targeting parts of cytochrome oxidase III gene (COIII) and NADH dehydrogenase subunit 4L gene (ND4L). ND3 gene sequence, encoded 752 bps, presented some genetic variation in the chum salmon populations. The observed nucleotide variations inferred the distinct genetic differentiation of American salmons from Korean and Japanese chum salmons. Six sites of single nucleotide polymorphism (SNP) were explored in the ND3 locus. Denaturing gradient gel electrophoresis analysis also showed a clear heterogenous band in American salmons compared to Asian salmons.*

Key words*: Chum salmon, Mitochondrial NADH dehydrogenase gene, SNP*
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Introduction

Chum salmon (*Oncorhynchus keta*) has received considerable attention due to its commercial importance in the Pacific area including Korea, Japan, Russia and North America. Among the *Oncorhynchus* species, the chum salmon has the longest migration towards feeding habitat (Salo, 1991). Such a behavior is common to Asian and North American stocks, and they present together sometimes during ocean migration stage in international waters. Thus, stocks identification of chum salmon has become a matter of management issue in the North Pacific area.

In the past few decades, genetic markers especially allozymes have been implemented for stock identification of chum salmons by management agencies in Pacific Rim countries (Urawa *et al*., 1997; Wilmot *et al*., 1998; Seeb and Crane 1999a,b). Allozyme analysis requires careful collection and handling of tissues (Brown *et al*., 1979), being often inadequate for discriminating causal factors of population divergence due to low frequency of polymorphic loci in chum salmon (Zhivotovsky *et al*., 1994). Recently, development molecular techniques, such as restriction fragment length polymorphism (RFLP) and sequence analysis using mitochondrial DNA (mtDNA), has shown higher resolution and accuracy than allozymes analyses (Park *et al*., 1993; Sato *et al*., 2001). Maternally inherited mtDNA has five times higher rates of nucleotide substitution than the nuclear DNA (Brown *et al*., 1979). Therefore, the analysis of mtDNA has become a method of choice for population genetics and evolutionary studies. So far, mtDNA studies using RFLP have been conducted in many fish species including salmon. However, Park *et al*. (1993) described low levels of mtDNA sequence variation in chum salmon. The variation level was similar to the divergence of allozymes (Seeb and Crane, 1999a). Sato *et al*., (2001) detected more variation in mtDNA by nucleotide sequence analysis than those obtained from RFLP. In particular, mtDNA ND3 region is useful as a genetic marker for stock identification and phylogeographic study because ND3 is conserved across all organisms and has a fast rate of sequence evolution (McKay *et al*., 1996; Verspoor *et al*., 1999).

In the present study, we cloned mtDNA ND3 genes from chum salmon compared variation of their nucleotide and amino acid sequences among and within Korean, Japanese and American salmons to develop molecular markers and accurate method to address stock identification problems.

Materials and Methods

Fish samples and DNA extraction: Fresh liver samples were excised from chum salmons collected from 3 locations for a total of 11 fish including 8 wild samples from Namdae river (n = 4) in Korea and Shokanbetu river in Japan (n = 4), and 3 cultured samples from Quilence hatchery in America from 1999 to 2000. Collected samples were stored at -80°C, or kept in 80% ethanol at room temperature until DNA extraction. DNA was extracted from approximately 70 mg of the stored specimens with a QIAGEN Blood and Cell Culture DNA Midi Kit (Qiagen, Germany) according to the manufacturer's instruction. A UV spectrophotometer (Shimadzu, Japan) was used to determine the quantity and quality of the isolated DNA. DNA concentration was estimated by measuring the absorbance at 260 nm wavelength. Protein contamination was estimated by the ratio of absorbance at 260 nm and 280 nm wavelength (Sambrook *et al*., 1989).

Table - 1: Sequence comparison in the variable sites of NADH dehydrogenase subunit 3 gene from 11 individuals

Strain	Single nucleotide position					
	+65	+311	+401	+541	+542	+599
Chum salmon*	C	C	C	G	G	G
Korean 1	T	C	C	T	A	A
Korean 2	T	C	C	T	A	A
Korean 3	C	C	C	T	G	A
Korean 4	C	C	C	T	G	A
Japanese 1	C	C	G	T	G	G
Japanese 2	T	C	G	T	A	A
Japanese 3	C	C	G	T	G	A
Japanese 4	T	C	C	T	A	A
American 1	C	T	C	T	G	G
American 2	C	T	C	T	G	G
American 3	C	T	C	T	G	G
Mutation type	transition	transition	transversion	transversion	transition	transition

* Nucleotide sequences of chum salmon in GenBank of NCBI (accession number D84147)

Table - 2: Comparison of NADH dehydrogenase subunit 3 amino acid sequences from 11 individuals

Strain	Amino acid substitution position					
	+12	+16	+18	+39	+53	+87
Chum salmon*	I	A	L	C	S	R
Korean 1	I	A	L	C	S	L
Korean 2	I	A	P	C	S	L
Korean 3	L	A	L	C	S	L
Korean 4	L	A	L	C	S	L
Japanese 1	I	A	L	W	P	L
Japanese 2	I	A	L	W	S	L
Japanese 3	I	A	L	W	S	L
Japanese 4	I	P	L	C	S	L
American 1	I	A	L	C	S	L
American 2	I	A	L	C	S	L
American 3	I	A	L	C	S	L

*Amino acid sequences of chum salmon in GenPept of NCBI (Accession Number BAAs99120155)

Table - 3: NADH dehydrogenase subunit 3 sequences data of Pacific salmons registered in Gen Bank

Species	Commom name	Accession No.
Oncorhynchus keta	Chum salmon	D84147
Oncorhynchus masou	Masu salmon	D63336
Oncorhynchus tshawytscha	Chinook salmon	AF392054
Oncorhynchus nerka	Sockeye salmon	D84148
Oncorhynchus rhodurs	Amago salmon	D63410
Oncorhynchus gorbuscha	Pink salmon	AF294831
Oncorhynchus kisutch	Coho salmon	AF294829

PCR amplification: Mitochondrial ND3 gene was amplified by polymerase chain reaction (PCR) in 50 µl of reaction mixture containing 0.5-1 µg of template DNA, 1X PCR buffer, 1 unit of Hotstar*Taq* DNA polymerase (Qiagen, Germany), 0.2mM dNTPs, and each 25 pM of forward and reverse primers. Cytochrome oxidate III gene (COIII: 5'-TTACAATCGCTGACGGCG-3') was used as a forward primer, and NADH dehydrogenase subunit 4L gene (ND4L; 5'-GGTGCGGTGAAACGCGAGTC-3') was used as a reverse primer (Domanico and Phillips, 1995). The condition of PCR amplification using a MiniCycler TM (MJ research, USA) was as follows; pre-cycling denaturation at 94ºC for 10 min, following by 35 cycles of 94ºC for 1 min, 50ºC for 1 min, and 72ºC for 1 min, with final-extension at 72ºC for 10 min. The amplified DNA fragments were determined for the size and quality with 0.8% agarose-gel electrophoresis and ethidium bromide staining, then purified by the QIAquick PCR Purification Kit (Qiagen, Germany).

Cloning and sequence analysis: The amplified DNA fragment was ligated to a pCR 2.1-Topo vector with the TOPO TA cloning ™ system (Invitrogen, Netherlands). The plasmid was cloned into *E. coli* Top 10 competent cell (Invitrogen, Netherands) for transformation. Sequencing of positive clones was carried out with an ABI PRISM 377 DNA sequencer (PE Biosystem, USA). Sequencing of positive clones was carried out with an ABI PRISM 337 DNA sequencer (PE Biosystem, USA). Sequence reaction was performed using an ABI PRISM dye terminator cycle sequencing kit (PE Biosystem, USA) according to the manufacturer's instruction. The obtained sequence

data were aligned by the GeneDoc software (version 2.4, Cris, USA) to determine the intraspecific single nucleotide polymorphisms (SNPs) among sequences of mtDNA ND3 region.

DGGE (Denaturing gradient gel electrophoresis) nalysis: PCR products (25 µl) were applied for the denaturing gradient gel electrophoresis (DGGE) separation using gradient denatured gel (10-30% acrylamide/bis acrylamide, 5×TAE, urea, deionized formamide). The PCR product were mixed with formamide-loading buffer (25 µl) and denatured at 100ºC for 5 min, and followed by electrophoresis in TAE buffer at 50ºC for 18 hr. The gel was stained with ethidium bromide and observed under UV light.

Results and Discussion

A primer pair of COIII and ND4L successfully amplified chum salmon mtDNA ND3 region from DNA samples of 11 individuals with occurrence of amplified fragments of about 750 bps. As a result, sequencing of the cloned PCR products disclosed 752 bps, flanked with 3' portion of COIII and 5' portion of ND4L. The base homology analysis with reported chum salmon mtDNA sequences (GenBank accession number D84147) revealed that the obtained PCR fragments from the Namdae population in Korea were due to the occurrence of +1 to +281 bps in the 3' portion of the COIII and +633 to +752 bps in the 5' portion of the ND4L.

As shown in Table 1, multiple alignments of the obtained sequences showed a polymorphic nucleotide site, which disclosed 5 variable sites within and among Korean, Japan and American samples. As compare with nucleotide sequence of chum salmon in GenBank of NCBI (Accession number D84147), the observed nucleotide sequence variations were base substitutions, *i.e.* G to T transversion at +541 nucleotide in all of the samples, C to T transition at +65 nucleotide, which was found only from American samples. On the other hand, the observed substitutions, C to G transversion at +401 nucleotide from three out of four Japanese samples were distinguished with Korean and American samples. G to A transition at +599 nucleotide was disclosed from only Korean and Japanese samples. However, in the SNPs of salmons the transition was more frequent than the transversion as reported Brown *et al*. (1979). Although the non-coding control region containing the D-loop has often higher sequence variability than the coding regions in the mtDNA genome, and has been recommended for investigating genetic variation at the intraspecific level in many fish species including salmon (Moritz *et al*., 1987; Park *et al*., 1993; Sato *et al*., 2001), other mtDNA regions such as ND3 and cytochrome *b* gene have been targeted for population analysis of marine organisms (Azuma *et al*., 2006; Kitanishi *et al*., 2007). The present preliminary sequence analysis of the mtDNA ND3 region of chum salmon showed clear genetic differentiation among Korean, Japanese and American salmons. These highly reproducible sequencing data, although more variations in this region would be expected as the number of populations and individuals increased, may be preferable for estimating the genetic diversity of chum salmon.

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                                  540                 560
Type I      TCAACTCAGTACCCCAACCCTAACACTTATTTGATCCACT
Type II     TCAACTCAGTACCCCAACCCTGACACTTATTTGATCCACT
McKay *     TCAACTCAGTACCCCAACCCTAACACTTATTTGATCCACT
Chum        TCAACTCAGTACCCCAACCCGGACACTTATTTGATCCACT
Masu        TCAACTCAACACCCCCGCCCTAACACTCGTCTGATCCACT
Chinook     TCAGCTCGACACCCCAACCCTAACACTTATTTGATCCACT
Sockeye     TCAACTCACCACCCCAGCCCTCACACTTGCCTGATCGACT
Amago       TCAACTCAACACCCCCGCCCTAACACTCGTCTGATCCACT
Pink        TCAACTCAGTACCCCAACCCTGACACTTATTTGATCCACT
Coho        TCAACTCAGTACCCCAACCCTGACACTTATTTGATCCACT
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Fig. 1: Variation of nucleotide sequences in the mitochondrial NADH dehydrogenase subunit 3 gene of Pacific salmons. *derived from McKay *et al*. (1996). Type I and Type II are from the present study. The others are from GenBank of NCBI

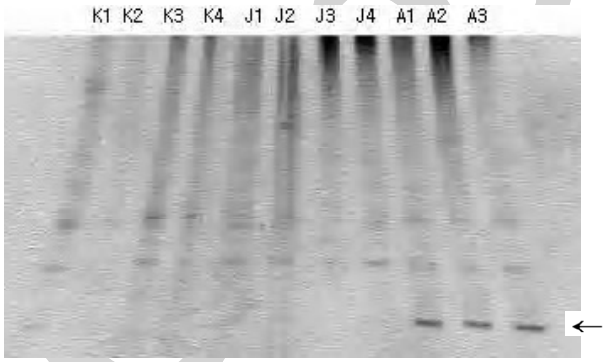

Fig. 2: Denaturing gradient gel electrophoresis analysis in the variable sites of NADH dehydrogenase subunit 3 gene from 11 individuals of Korean samples (K1, K2, K3, K4), Japanese samples (J1, J2, J3, J4), and American samples (A1,A2,A3). Arrow indicates heterogeneous bands in American samples

The deduced amino acid sequence of ND3 region is shown in Table 2. The methionine codon starts from +282 and the stop codon at +630-632 nucleotides. This sequence contains an open reading frame (ORF) encoding the putative 116 amino acid residues. The amino acid sequence variations were observed at 6 sites, which were substitutions, *i.e.* Isoleucine (Ile, I) to Leucine (Leu, L) at +12 amino acid from K3 and K4 samples from Korea, Cysteine (Cys, C) to Tryptophan (Trp, W) at +39 amino acid from all of Japanese samples. Multiple alignment of the nucleotide sequences of ND3 showed that the observed nucleotide variations in our data and the previous findings obtained by McKay *et al*. (1996) were distinguishable from those of NCBI data (Table 3). In addition, the sequence variations observed at +541 and +542 nucleotide were divided into Type I and Type II of *Oncorhynchus* species as shown in Fig. 1. The nucleotide A at +542 in Type I, masu salmon, chinook salmon and amago salmon was distinguished from the nucleotide G at +542 nucleotide in Type II, chum salmon, pink salmon, and coho salmon. Our sequence data from mtDNA ND3 region in salmonid is provided on the certain level of polymorphism among these two groups of species for use in conservative approach to stocking programs and genetic conservation.

On the other hand, the DGGE analysis using a primer set (5'-TTACAACAATCATTACTATT-3' and 5'-TACTGAGTTGA TCCCCCCAA-3') for PCR amplification, which was selected from ND3 region showed a clear heterogenous band in American salmons compared to Korean and Japan salmons analysed in this study (Fig. 2). In fact, mtDNA studies, mostly based on the RFLP, have been conducted so far in many fish species including salmon (Meyer, 1993). Low levels of mtDNA sequence variation shown by RFLP analysis were reported in chum salmon (Park *et al*., 1993) and other species of *Oncorhynchus* (Wilson *et al*., 1987). The sequence variation detected in the ND3 region of chum salmon by DGGE method is apparently greater than previous RFLP analyses on chum salmon. Therefore, our findings suggest that the DGGE analysis was proven to be useful for detection of SNPs of chum salmon.

In conclusion, the single nucleotide polymorphisms (SNPs) of ND3 region could be used as a genotyping tool for chum salmon, thereby providing a potential means for the estimation of stock identification.

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