

# Development of Salmon Identification DNA Chip based on Mitochondrial COIII-ND3-ND4L Variations

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## Abstract

Salmon are born in fresh water, migrate to the sea, and then return to their natal river for reproduction. This feature has been utilized for their enrichment as commercial resources because the artificial rearing and release of fry and the catching of returning salmon become easy tasks. For proper management of salmon resources and fair trade of salmon products, a reliable identification of salmon species is necessary. However, species identification based on morphological features has often been inaccurate and time consuming in many cases. In particular, for processed salmon products in the market, the application of morphological features toward species identification is impossible. To resolve this problem, we have developed a DNA chip that distinguishes 9 species of salmonid fishes. DNA sequence variations in the mitochondrial DNA COIII-ND3-ND4L region were utilized for designing species-specific oligonucleotide probes. The probes were synthesized and covalently linked onto a glass slide in duplicate. Hybridization of the probes with single-stranded DNA target molecules labeled with a fluorescent dye produced species-specific hybridization spot patterns. The whole process of species identification took only about 4 hours. This DNA chip system provides a sensitive, objective, and high-throughput method for salmonid species identification, being especially

useful for processed products in the market. This technique can be easily applied to other fish species.

**Keywords:** Salmon, Species identification, DNA chip, Microarray, COIII-ND3-ND4L

## Introduction

Salmonid fish include salmon and trout that inhabit the fresh waters as well as the Atlantic and Pacific Oceans. Most salmonid species are anadromous, but trout are restricted to fresh water throughout their lives. A typical salmon is born in fresh water, migrates to the sea, and then returns to its natal river for reproduction. This feature has been utilized for enrichment as a commercial resource because the artificial rearing and release of fry and the catching of returning salmon become easy tasks. Many countries in the Atlantic and Pacific have been operating salmon hatcheries for stock enhancement. For proper management of the resources and fair trade of salmon products, reliable identification of salmon species is necessary. However, species identification based on morphological features has often been inaccurate and time consuming in many cases. In particular, for processed salmon products in the market, the application of morphological features toward species identification is impossible. Recently, molecular methods have been introduced in fishery science<sup>1-9</sup>. Molecular techniques utilize genetic variations such as single nucleotide polymorphisms (SNP) and microsatellites<sup>3,4</sup>. These variations have already been applied to several cases of species identification<sup>10-12</sup>. Protein markers such as muscle soluble proteins were also successfully used in species identification, but they have a problem of heat lability<sup>5</sup>. A loss of structure and biological function of the protein markers was an obstacle for accurate species identification. Restriction fragment length polymorphism (RFLP) was proposed for species identification, but was of no use for salmon<sup>5</sup>. In addition, simple fragment patterns of artificial RFLP-PCR showed no congruent results among the experiments. On the other hand, mitochondrial DNA is known to have enough sequence variations to discriminate the species identity of fish<sup>13</sup>. DNA sequence variations in the mitochondrial DNA control region, ATP6, and ND3 gene sequences have shown consistent results for identification of salmon species and populations<sup>14,15</sup>.

|           |             |             |              |            |             |             |                 |
|-----------|-------------|-------------|--------------|------------|-------------|-------------|-----------------|
| chum_A1   | TCCACTTTCT  | TTGTCGCCAC  | AGGATTCCAC   | GGCCTACACG | TAATCATCGG  | CTCTACTTTT  | CTGGCTGTTT      |
| chum_B1   | .....       | .....       | .....        | .....      | .....       | .....C...   | .....           |
| chum_C1   | .....       | .....       | .....        | .....      | .....       | .....C...   | .....           |
| chum_D1   | .....       | .....       | .....        | .....      | .....       | .....C...   | .....           |
| chum_D2   | .....       | .....       | .....        | .....      | .....       | .....C...   | .....           |
| chum_D3   | .....       | .....       | .....        | .....      | .....       | .....C...   | .....           |
| chum_D4   | .....       | .....       | .....        | .....      | .....       | .....C...   | .....           |
| chum_D5   | .....       | .....       | .....        | .....      | .....       | .....C...   | .....           |
| pink      | .....       | .....A..... | .....T.....  | .....      | .....       | .....C...   | .....T....C..C. |
| sockeye   | ..T.....    | .....T..... | G.....T..... | ..T.....   | ...T..T..   | .....C..C   | .....C...       |
| chinook   | ..T.....    | .....       | C.....T..... | .....      | .G..T..T..  | ...A..C...  | ..A..C...       |
| coho      | ..T.....    | .....       | .....T.....  | .....      | ...T.....   | ...A..C...  | ..A..C...       |
| masu      | ..T.....    | .....T..    | .....T.....  | .....T..   | ...T..T..   | .....C...   | T..A..C...      |
| ishikawae | ..T.....    | .....T..    | .....T.....  | .....T..   | ...T.....   | .....C...   | T..A..C...      |
| rainbow   | ..T.....    | .....T..    | .....T.....  | .....      | ...T..T..   | .....C...   | .....C...       |
| cutthroat | ..T.....    | .....T..    | ..T.....T    | .....      | ...T..T..   | .....C...   | .....C...       |
| atlantic  | ..T.....    | .....A..... | .....        | .....T..   | .G..T.....  | ...C..C...  | T..A..CA..C.    |
| chum_A1   | GCCTTCTACG  | ACAAATTCAA  | TACCATTTCA   | CATCTGAACA | TCATTTTGGC  | TTTGAAGCCG  | CTGCTTGATA      |
| chum_B1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_C1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D2   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D3   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D4   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D5   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| pink      | .....       | .....       | .....C...    | .....G..   | .....       | .....       | .....           |
| sockeye   | .....       | .....       | .....C..T.   | .....      | .....       | .....       | .....           |
| chinook   | .....G..    | ..GG..C..   | .....C..T.   | .....C...  | .....       | .....T..    | .....           |
| coho      | .....       | ..G.....    | .....C..T.   | .....C...  | .....       | .....T..    | .....           |
| masu      | .....       | ..G.....    | ..T..C..T.   | .....      | ..C.....    | .....T..    | .....           |
| ishikawae | .....       | ..G.....    | ..T..C..T.   | .....      | ..C.....    | .....T..    | .....           |
| rainbow   | .....       | ..G.....    | ..T..C..T.   | .....      | .....       | .....T..    | .....C...       |
| cutthroat | .....       | ..G.....    | .....C..T.   | .....      | .....       | .....T..    | .....           |
| atlantic  | .....CT.... | .....       | ..T..C..T.   | .....C...  | .....C...   | .....T..    | ..C..C...       |
| chum_A1   | TTGACACTTT  | GTAGACGTTG  | TGTGACTCTT   | CCTATACGTT | TCTATTACT   | GATGAGGCTC  | ATAATCTTTC      |
| chum_B1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_C1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D2   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D3   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D4   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D5   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| pink      | .....       | .....       | .....        | .....C     | .....       | .....       | .....           |
| sockeye   | .....       | .....       | .....        | .....C     | .....       | .....       | .....C.         |
| chinook   | .....       | .....       | .....        | .....C     | .....       | .....       | .....           |
| coho      | .....       | .....       | ..A.....     | .....C     | .....       | .....       | .....           |
| masu      | .....       | .....       | ..A..G....   | .....C     | .....       | .....       | .....           |
| ishikawae | .....       | .....       | ..A..G....   | .....C     | .....       | .....       | .....           |
| rainbow   | .....       | .....       | ..A..G....   | .....C     | .....       | .....       | .....           |
| cutthroat | .....       | .....       | ..A.....     | .....C     | .....       | .....       | .....           |
| atlantic  | C.....      | .....C.     | ..A....A..   | .....C     | .....       | .....       | .....           |
| chum_A1   | TAGTATTAAT  | TAGTATAAGT  | GACTTCCAAT   | CACCCGGTCT | TGGTTAAAAAT | CCAAGGAAAAG | ATAATGAACT      |
| chum_B1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_C1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D1   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D2   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D3   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D4   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| chum_D5   | .....       | .....       | .....        | .....      | .....       | .....       | .....           |
| pink      | .....       | .....CG...  | .....T.....  | .....      | .....       | .....       | .....C          |
| sockeye   | .....C...   | G.....      | .....        | .....      | .....       | .....       | .....           |
| chinook   | .....C      | AC.....     | .....        | .....      | .....       | .....       | .....           |
| coho      | .....C      | GC.....     | .....        | .....      | .....       | .....       | .....           |
| masu      | .....C...   | A.....      | .....        | .....      | .....G.     | .....       | .....           |
| ishikawae | .....C...   | A.....      | .....        | .....      | .....G.     | .....       | .....           |
| rainbow   | .....       | AC.....     | .....        | .....      | .....       | .....       | .....T.         |
| cutthroat | .....C      | AC.....     | .....        | .....      | .....       | .....       | .....           |
| atlantic  | .....C      | AC.....     | .....        | .....      | .....G..    | .....       | .....           |

**Figure 1.** Alignment of nucleotide sequences of mtDNA of the COIII-ND3-ND4L region from 10 salmon species. chum A1, B1, C1, D1, D2, D3, D4 and D5 indicate subtypes of *Oncorhynchus keta* (chum salmon), pink, sockeye, Chinook, Coho, Masu, Ishikawae, rainbow, cutthroat and Atlantic corresponding to *O. gorbuscha*, *O. nerka*, *O. tshawytscha*, *O. kisutch*, *O. masou masou*, *O. masou ishikawae*, *O. mykiss*, *O. clarkia* and *Salmo salar*, respectively.

|           |             |             |             |              |             |             |            |
|-----------|-------------|-------------|-------------|--------------|-------------|-------------|------------|
| chum_A1   | TAATTACAAC  | AATCATTACT  | ATCACCATCA  | CATTGTCCGC   | AGTACTAGCC  | ACTATCTCTT  | TCTGATTACC |
| chum_B1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_C1   | .....       | .....       | ..T.....    | .....        | .....       | .....       | .....      |
| chum_D1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D2   | .....       | .....       | .....C..    | .....        | .....       | .....       | .....      |
| chum_D3   | .....       | .....       | .....C..    | .....        | .....       | .....       | .....      |
| chum_D4   | .....       | .....       | .....C..    | .....        | .....       | .....       | .....      |
| chum_D5   | .....       | .....       | .....C..    | .....        | .....       | .....       | .....      |
| pink      | .....       | G.....      | ..T.....    | ..C.....     | .....       | .....       | .....G..   |
| sockeye   | ..G.....    | .....CG..   | ..T.....    | ..C.....     | .....       | .....T...   | .....      |
| chinook   | .....       | .....C..    | ..T.....    | .....        | .....       | .....T...   | .....      |
| coho      | .....       | .....C..    | .....T..    | ..C...T..    | .....       | ..G.T...    | .....C...  |
| masu      | .....       | ..T.C...    | ..T.....    | ..C.A..T..   | .....       | .....T...   | .....      |
| ishikawae | .....       | ..T.C...    | ..T.....    | ..C.A..T..   | .....       | .....T...   | .....      |
| rainbow   | ...C.....   | .....C..    | ..T.....    | ...A.....    | .....       | .....T...   | .....      |
| cutthroat | .....       | .....C..    | .....       | .....        | .....       | .....T...   | .....      |
| atlantic  | .....       | ..T..G..    | ..T.....T.. | ..C.A..G..   | .....       | .....T..C.. | .....C...  |
|           |             |             |             |              |             |             |            |
| chum_A1   | ACAAATCTCC  | CCCGACGCAG  | AAAAGTTGTC  | TCCCTATGAG   | TGCGGATTTG  | ACCCACTAGG  | GTCCGCCCGC |
| chum_B1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_C1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D2   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D3   | .....       | .....       | .....       | .....        | .....       | .....T...   | .....      |
| chum_D4   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D5   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| pink      | .....T...   | .....       | .....A..    | C.....C..A.. | ..T.....C.. | ...CT.G...  | ...T.....T |
| sockeye   | .....T...   | .....       | .....A..    | C.....C..A.. | ..T.....    | ...CT.G...  | A.....T    |
| chinook   | T.....      | ..A.....    | ..G....A..  | C.....C..A.. | ..T.....    | ..T...T...  | .....      |
| coho      | .....       | .....       | ..G..AC..   | C.....C..A.. | ..T.....    | ..T...T...  | .....      |
| masu      | .....T..T   | ..A.....    | ..G..A....  | C.....C..A.. | ..T.....    | ...TT....   | A.....T    |
| ishikawae | .....T..T   | ..A.....    | ..G..A....  | C.....C..A.. | ..T.....    | ...TT....   | A.....T    |
| rainbow   | .....       | ..A.....    | ..G..A..A.. | C.....C..A.. | ..T.....    | ...CT....   | .....      |
| cutthroat | .....       | ..A.....    | ..G....A..  | C.....C..A.. | ..T.....    | ...C.....   | A.....     |
| atlantic  | .....AA.G   | .....       | .....AC.A.. | C.....C..A.. | ..T..C..C.. | ..T..C..... | A.....     |
|           |             |             |             |              |             |             |            |
| chum_A1   | CTCCCCCTCT  | CTTTACGCTT  | CTTTTAATT   | GCCATCCTCT   | TTCTCCTATT  | TGATCTAGAA  | ATTGCCCTCC |
| chum_B1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_C1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D1   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D2   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D3   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D4   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| chum_D5   | .....       | .....       | .....       | .....        | .....       | .....       | .....      |
| pink      | ..A.....    | .....       | .....C...C  | .....        | ..C.....    | C.....      | .....      |
| sockeye   | ..G.....    | ..C.....A.. | T...C...C   | .....G..     | ..C.....G.. | C..C.....   | ..C.....T. |
| chinook   | ..A.....    | ..C.....A.. | .....C...C  | .....A..     | ..C.....    | .....       | .....      |
| coho      | ..G.....    | ..C.....    | .....C...C  | .....A..     | ..C..A..    | .....       | .....      |
| masu      | ..A.....    | ..C.....    | .....CC...C | .....G..     | ..C..T....  | ...CT.G...  | ..C.....   |
| ishikawae | ..A.....    | ..C.....    | .....CC...C | .....G..     | ..C..T....  | ...CT.G...  | ..C.....   |
| rainbow   | ..G.....    | ..C.....    | .....C...C  | .....T.A..   | ..C.....    | .....       | ..C.....   |
| cutthroat | ..G..T...   | ..C.....    | .....C...C  | ..T...A..    | ..C.....G.. | .....       | .....      |
| atlantic  | ..A.....    | ..CC.G....  | .....C...   | .....T..A..  | .....       | .....       | ..C.....   |
|           |             |             |             |              |             |             |            |
| chum_A1   | TTCTCCCCCT  | ACCTTGGGGG  | GATCAACTCA  | GTACCCCAAC   | CCTAACACTT  | ATTTGATCCA  | CTGCCGTACT |
| chum_B1   | .....       | .....       | .....       | .....        | ..G.....    | .....       | .....      |
| chum_C1   | .....       | .....       | .....       | .....        | ..G.....    | .....       | .....      |
| chum_D1   | .....       | .....       | .....       | .....        | ..G.....    | .....       | .....      |
| chum_D2   | .....       | .....       | .....       | .....        | ..G.....    | .....       | .....      |
| chum_D3   | .....       | .....       | .....       | .....        | ..G.....    | .....       | .....      |
| chum_D4   | .....       | .....       | .....       | .....        | ..G.....    | .....       | .....      |
| chum_D5   | .....       | .....       | .....       | .....        | ..G.....    | .....       | .....      |
| pink      | ..C.....C.. | ...C..A...  | .....G...   | A.....       | T.....      | .....       | .....      |
| sockeye   | ...G...C..  | G.....      | .....G...   | A.G....G..   | .....C      | C..C.....   | .....      |
| chinook   | ...A..TC..  | ...A..A..A  | .....G..G   | AC.....      | ..T.....    | .....       | .....      |
| coho      | ...A..TC..  | G..A..A..A  | .....G...   | AC.....      | ..T.G....C  | G...G...    | .....      |
| masu      | ...A...C..  | G.....A..   | .....       | A.....CG.    | .....C      | G..C.....   | ...T....   |
| ishikawae | ...A...C..  | G.....A..A  | .....       | AC.....CG.   | .....C      | G..C.....   | ...T..G..  |
| rainbow   | ..T.G...C.. | ...A...     | .....C      | AC.....G..   | ..G.....C   | ..C.....    | ...T....   |
| cutthroat | ..T.A...C.. | G.....A...  | .....       | AC.....      | .....       | .....       | .....      |
| atlantic  | ..A.....C.. | T..C..A..A  | .....       | CC.....G..   | ..C.....    | GCC.....G   | .....      |

Figure 1. Continued.

|           |              |              |             |            |               |             |            |
|-----------|--------------|--------------|-------------|------------|---------------|-------------|------------|
| chum_A1   | CGCCCTCCTT   | ACTCTTGGCT   | TAATTTATGA  | ATGAACCCAA | GGAGGCTTGG    | AATGAGCCGA  | ATAGGCAGTT |
| chum_B1   | .....        | .....        | .....       | G.....     | .....         | .....       | .....      |
| chum_C1   | .....        | .....        | .....       | G.....     | .....         | .....       | .....      |
| chum_D1   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D2   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D3   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D4   | .....        | .....        | .....       | A.....     | .....         | .....       | .....      |
| chum_D5   | .....        | ...T.....    | .....       | .....      | .....         | .....       | .....      |
| pink      | .....        | .....        | .....       | G.....     | .....A.....   | .....       | .....      |
| sockeye   | .....        | .....AC..... | .....       | G.....     | .....A.....   | .....       | .....      |
| chinook   | .....        | .....        | .....       | .....      | .....A.....   | .....       | .....      |
| coho      | .....        | .....        | .....       | .....      | .....A.....   | .....       | .....      |
| masu      | T.....       | .....A.....  | .....C..... | .....      | .....A.....   | .....       | .....      |
| ishikawae | T.....       | .....A.....  | .....C..... | .....      | .....A.....   | .....       | .....      |
| rainbow   | A.....T..... | .....        | .....C..... | .....      | .....A.....   | .....G..... | .....      |
| cutthroat | A.....T..... | .....        | .....C..... | .....      | .....A.....   | .....G..... | .....      |
| atlantic  | T..T.....C   | .....C.....  | .....C..... | ...G.....G | .....C.T..... | .....       | .....      |
| chum_A1   | AGTCCAAAAC   | AAGACCCTTG   | ATTTCCGGCTC | AAAAGACCAT | GGTTTAAGTC    | CATGACCGCC  | TTATGACACC |
| chum_B1   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_C1   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D1   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D2   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D3   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D4   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D5   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| pink      | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| sockeye   | .....T.....  | .....        | .....       | .....      | .....         | .....T..... | .....      |
| chinook   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| coho      | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| masu      | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| ishikawae | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| rainbow   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| cutthroat | .....        | .....        | .....       | ...A.....  | .....         | .....       | .....      |
| atlantic  | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_A1   | AGTACACTTC   | AGCTTTACCT   | CAGCCTTTAT  | .....      | .....         | .....       | .....      |
| chum_B1   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_C1   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D1   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D2   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D3   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D4   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chum_D5   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| pink      | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| sockeye   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| chinook   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| coho      | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| masu      | .....T.....  | .....T.....  | .....       | .....      | .....         | .....       | .....      |
| ishikawae | .....T.....  | .....T.....  | .....       | .....      | .....         | .....       | .....      |
| rainbow   | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| cutthroat | .....        | .....        | .....       | .....      | .....         | .....       | .....      |
| atlantic  | .....        | .....        | .....       | .....      | .....         | .....       | .....      |

Figure 1. Continued.

In this study, we have developed a DNA chip for salmon species identification that can distinguish 9 species of salmon and trout based on mitochondrial DNA COIII-ND3-ND4L region variations. This region contains 203 base pairs of the 3' terminal region of COIII (1-203), 70 bp of tRNA-gly (204-273), 351 bp of ND3 (274-624), 68 bp of tRNA-Arg (625-692), and 52 bp of the 5' terminal region of ND4L (693-744). This region is known to harbor a high level of sequence variation and has been used for the phylogenetic analysis of Pacific salmon (*Oncorhynchus*)<sup>18</sup>. The 9 salmonid species in the DNA chip include chum sal-

mon (*Oncorhynchus keta*), pink salmon (*O. gorbuscha*), sockeye salmon (*O. nerka*), chinook salmon (*O. tshawytscha*) coho salmon (*O. kisutch*), cherry salmon (*O. masou masou*), Atlantic salmon (*Salmo salar*), ishikawae salmon (*O. masou ishikawae*), and rainbow trout (*O. mykiss*). Species-specific oligonucleotide probes were designed for each salmonid species and covalently linked onto a glass slide in duplicate. Using this DNA chip, we have successfully identified each species. This system will provide a sensitive, objective, and high-throughput method for salmonid species identification, being especially useful for proces-

sed products in the market.

## Results and Discussion

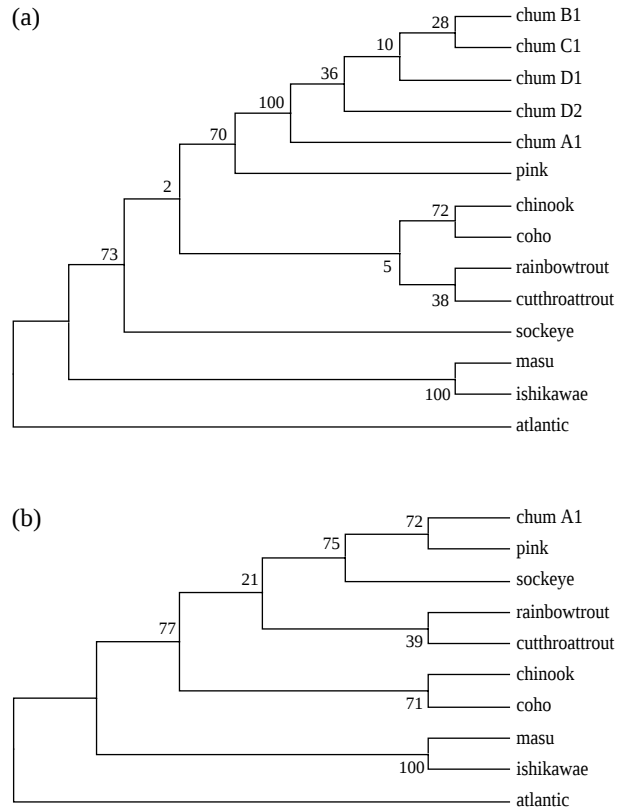
### Sequence Analysis

The mitochondrion is a cytoplasmic organelle having its own circular genome. The number of mitochondria present in a cell may vary, ranging between hundreds to thousands. Because of the high variable sequences among species, short standardized control sequences including COI and Cytochrome *b* (CYTB) genes are commonly used to distinguish species. However, the mitochondrial DNA control region of salmon is not suitable for species identification because the control region contains too little genetic variations to distinguish species. In order to solve this problem, we have selected the COIII-ND3-ND4L region of salmon mitochondrial DNA, which is oriented in the opposite direction of the control region. The region includes 203 bases (position 1-203) of the COIII (CO3) gene, 70 bases (204-273) of the tRNA-gly gene, 351 bases (274-624) of the ND3 gene, 68 bases (625-692) of the tRNA-Arg gene, and 52 bases (693-744) of the ND4L gene. In contrast with the control region, the sequence variation of the COIII, ND3, and ND4L areas showed polymorphisms enough to discriminate salmon species.

In this study, a sequence analysis result tells the possibility of identification by genetic marker (Figure 1). Nucleotide sequences of the mitochondrial DNA COIII-ND3-ND4L region were obtained from 9 salmon species, *Oncorhynchus keta*, *O. gorbuscha*, *O. nerka*, *O. tshawytscha*, *O. kisutch*, *O. masou masou*, *O. masou ishikawae*, *O. mykiss* and *Salmo salar*. A total of 730 bp sequences were aligned with an average composition of A (31.5%), T (28.1%), G (24.6%), and C (15.8%). Conserved sites were 566 (77.5%), variable

sites were 164 (22.5%), and singletons were 63 (8.6%).

For intra-specific identification, chum salmon sequences were obtained from 201 individuals from 6 populations (Korea, Japan, Russia, USA, Alaska and Canada) and represented by 5 types, named A1, B1, C1, D1, and D2, which were selected from 51 SNP



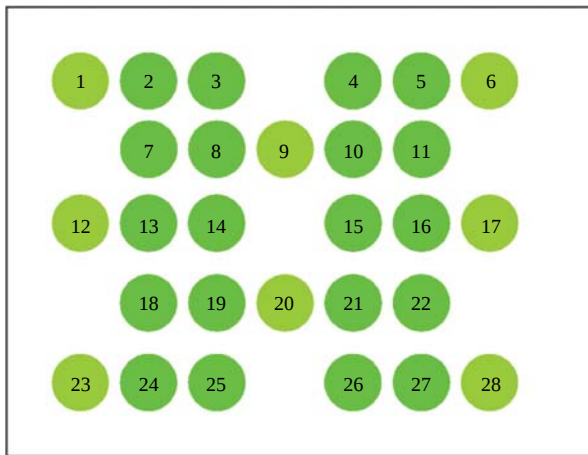
**Figure 2.** Phylogenetic analysis of 10 salmon species. (a) contains 5 types of chum salmon, while (b) contains only A1 type of chum salmon. Trees were made by NJ method (bootstrap 1000 repitations).

**Table 1.** Oligonucleotide probes for species identification of salmonid fish.

| Probe | Specific sequence                     | Common name                 | Genus               | Species                                  |
|-------|---------------------------------------|-----------------------------|---------------------|------------------------------------------|
| PM*   | 5'-NH2-T(10)GTTTCGCTCCTGATCCCTGG-3'   | —                           | —                   | —                                        |
| S01   | 5'-NH2-(10)ACGCAGAAAAGTTGTCTCCCT-3'   | Chum salmon                 | <i>Oncorhynchus</i> | <i>keta</i>                              |
| S02   | 5'-NH2-T(10)GATTTGATCCATTAGGGTCCGC-3' | Chinook salmon              | <i>Oncorhynchus</i> | <i>tshawytscha</i>                       |
| S03   | 5'-NH2-T(10)GACCCTTTAGGATCCGCCCGT-3'  | Cherry,<br>Ishikawae salmon | <i>Oncorhynchus</i> | <i>masou masou</i> ,<br><i>ishikawae</i> |
| S04   | 5'-NH2-T(10)ACTCTGGACTAATTTATGAGTG-3' | Sockeye salmon              | <i>Oncorhynchus</i> | <i>nerka</i>                             |
| S05   | 5'-NH2-T(10)CACTGCTGTACTTGCCCT-3'     | Cherry salmon               | <i>Oncorhynchus</i> | <i>masou masou</i>                       |
| S06   | 5'-NH2-T(10)ACTGCTGTGCTTGCCC-3'       | Ishikawae salmon            | <i>Oncorhynchus</i> | <i>ishikawae</i>                         |
| S07   | 5'-NH2-T(10)GACCCAGGGAGGCCT-3'        | Atlantic salmon             | <i>Salmo</i>        | <i>salar</i>                             |
| S08   | 5'-NH2-T(10)CCTCCCCCTACCCTGA-3'       | Pink salmon                 | <i>Oncorhynchus</i> | <i>gorbuscha</i>                         |
| S09   | 5'-NH2-T(10)CTTGACACTCGTTTGGTCCA-3'   | Coho slmon                  | <i>Oncorhynchus</i> | <i>kitutch</i>                           |
| S10   | 5'-NH2-T(10)TCAACTCCACACCCCGA-3'      | Rainbow trout               | <i>Oncorhynchus</i> | <i>mykiss</i>                            |

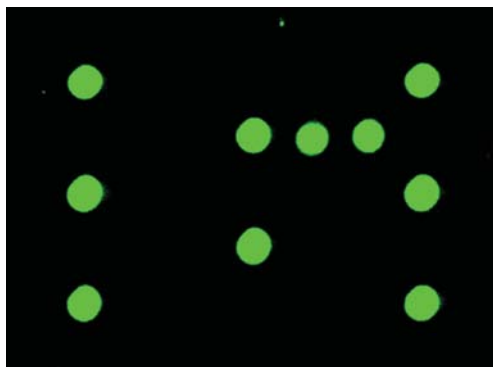
\*PM: Position Marker, non-homologous cy3 labeled probe

(a)

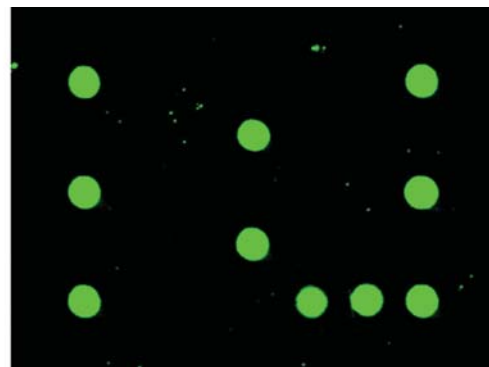


1, 6, 9, 12, 17, 20, 23, 28: PM  
 2, 3: S01  
 4, 5: S02  
 7, 8: S03  
 10, 11: S04  
 13, 14: S05  
 15, 16: S06  
 18, 19: S07  
 21, 22: S08  
 24, 25: S09  
 26, 27: S10

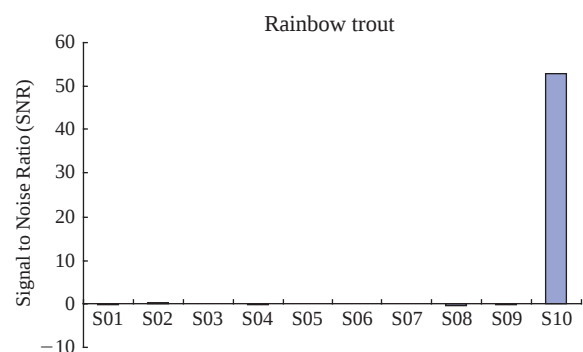
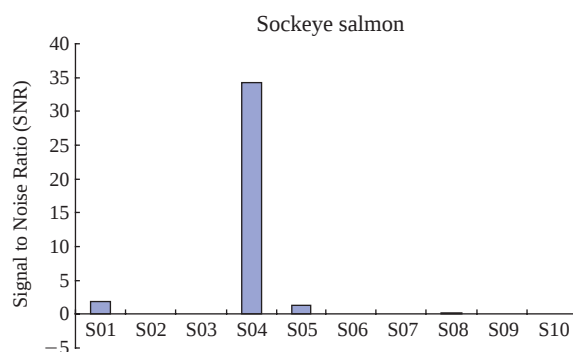
(b)



Sockeye salmon



Rainbow trout



**Figure 3.** (a) The layout of a DNA chip for species identification, and (b) hybridization results of sockeye salmon, and rainbow trout.

haplotypes<sup>20</sup>. Chum salmon type A1 is the most common sequence in Korean and Japanese populations, and chum salmon type B1 is the most common sequence in Russian, Alaskan, and Canadian populations. Chum salmon type C1 occurs almost exclusively in

the USA population, and chum salmon type D1 is another common sequence observed in Japanese, Canadian, and Alaskan populations, while type D2 occurs only in Korean populations. Types C1 and D1 are different from B1 by only a single nucleotide. The rela-

tionship of chum salmons from Korea, Japan, Canada, America, Alaska, and Russia can be grouped by Korea-Japan and Canada-Alaska-Russia and America. Identification of chum salmon populations between [Korea-Japan] and [Alaska-Canada-Russia-America] is possible.

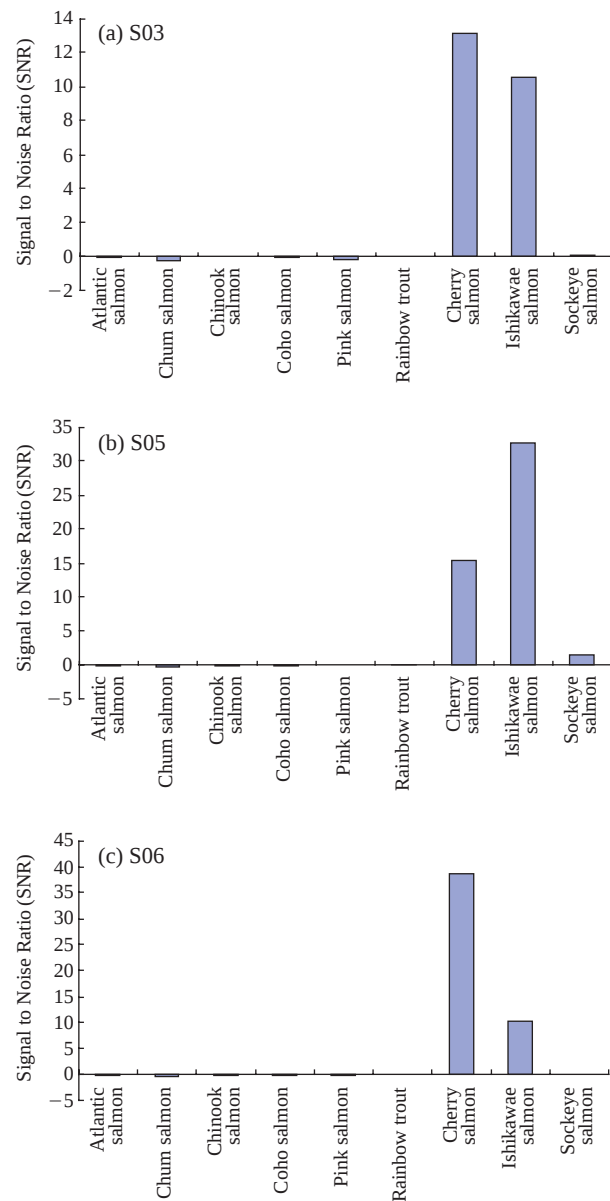
### Species Specific Probes

For salmon species, mtDNA COIII-ND3-ND4L sequences were analyzed and compared. Using species-specific sequences, DNA probes were designed and spotted on a DNA chip. To identify an unknown sample, the target DNA was amplified and hybridized with probes on the DNA chip. Salmon species are distinctive in their single nucleotide polymorphisms (SNPs) of mtDNA COIII-ND3-ND4L region sequences.

Specific probes for species identification were selected based on the alignment results of sequencing data (Figures 1, 2). In order to obtain optimal signal intensity, each probe contained a variation site within the central region of a probe sequence, and 10mers of oligo dTTP were added on the 5' direction of the probe to relieve steric hindrance<sup>16,17</sup>. A chum salmon specific probe was located in 365-385 of a COIII-ND3-ND4L fragment, and the melting temperature of the probe was 56.7 degrees. The melting temperatures of other salmon specific probes were around 50.1-56.2 degrees, and detailed information is as follows. Specific probes for chinook salmon, sockeye salmon, cherry salmon, ishikawae salmon, Atlantic salmon, coho salmon, and rainbow trout were located in 395-416, 400-420, 571-583, 549-567, 550-566, 594-609, 492-507, 531-550, and 513-529 of the COIII-ND3-ND4L fragment of the target site, respectively. Probe sequences spotted on a DNA chip and a sequence comparison between selected species are listed in Table 1 and Figure 3, respectively.

### Hybridization Patterns of Salmon Species Identification Using a DNA Chip

In order to label the target DNA, mitochondrial COIII-ND3-ND4L fragments were amplified with a cyanine 3-labeled primer. After asymmetric PCR, antisense single-stranded targets were hybridized with the sense probes on the DNA chip. The fluorescence hybridization patterns of selected probes on a DNA chip are shown in Figure 3. All of the DNA chip results show a perfect-match with the species sequence. In an experiment of sockeye salmon, the hybridization signal-to-noise ratio (SNR) was more than 30. Most of the SNR data obtained from other salmon experiments are more than 20. However, the hybridization signals from evolutionally near species such as ishikawae and cherry salmon show some weak ambi-



**Figure 4.** Analysis results of species specific probes. (a) S03, specific probe for cherry salmon and ishikawae salmon, (b) S05, specific probe for cherry salmon (c) S06, specific probe for ishikawae salmon.

guity due to genetic similarities (Figure 4). In this case, species identification can be easily measured by signal intensity thresholds between different species. Also, in order to distinguish ishikawae and cherry salmon from other salmons, 3 different probes were designed. An S03 probe can be hybridized with both cherry salmon and ishikawae salmon, but S05 and S06 probes are only specific for cherry and ishikawae, respectively (Figure 4).

## Conclusion

Recently, DNA microarray techniques have become attractive for species identification due to their simple, fast, and cost-effective way to discriminate large numbers of samples simultaneously. In this study, we have demonstrated that a DNA chip is a powerful tool for identification of salmon species. We were able to identify the salmon species using specific unique probes on a DNA chip. The hybridization pattern can easily distinguish between different salmon species. In this study, we have successfully developed a DNA chip that can distinguish 9 species of salmon and trout using the COIII-ND3-ND4L region of mitochondrial DNA. All results of hybridization were identical with that of the sequence information. This DNA chip system provides a sensitive and high-throughput method for identifying salmon species, and it can also be easily applied to other species identifications.

## Materials and Methods

### Samples and DNA Extraction

Twenty-eight salmon and trout DNA samples were collected using several ways. Chum salmon were obtained from both sides of the North Pacific and in the Bering Sea (Korean, Japanese, Russian (east Sakhalin), Canadian, American and Alaskan). The individual samples were collected when fish returned to their natal rivers. Liver or muscle tissues were taken from each individual and stored in ethanol until DNA extraction. Pink salmon were delivered from Russia, and other samples were from the National Fisheries Research and Development Institute of Korea. Genomic DNA was extracted from each samples using a DNeasy tissue kit (QIAGEN, Germany) following the manufacturer's protocol. DNA quality was measured using spectrophotometric analysis, and the size of the DNA was analyzed using gel electrophoresis.

### DNA Amplification, Cloning and Sequencing

To amplify the mitochondrial COIII-ND3-ND4L genes, the PCR reaction was carried out in a total volume of 50  $\mu$ L containing 5  $\mu$ L of a 10X reaction buffer (containing MgCl), 4  $\mu$ L of a 10 mM dNTPs mix, 3  $\mu$ L of COIII-ND3-ND4L universal primers each, 0.5–1  $\mu$ g DNA, and 0.5 units of Taq polymerase (TaKaRa, Japan). The COIII-ND3-ND4L universal primers were obtained from a published paper<sup>18</sup>: COIII forward primer 5'-TTA CAA TCG CTG ACG GCG-3', ND4L reverse primer 5'-GGT GCG GTG AAA CGC GAG TG-3'. PCR amplification was carried out

in a DNA-ENGINE (MJ Research, USA): preheating at 95°C for 5 min, followed by 35 cycles of 95°C for 15s, 50°C for 45s 72°C for 1 min, and a final extension of 72°C for 7 min. To confirm the correct size of the PCR products, the amplification products were electrophoresed on a 1% agarose gel. Amplified DNA products were purified using a QIAquick PCR purification kit (QIAGEN, Germany) and were used for PCR direct sequencing reactions. If the amplified DNA didn't have a clear band, they were cloned into the vector pCR2.1-TOPO with a TOPO-TA cloning system (Invitrogen, USA). Both samples were sequenced using an Automated DNA sequencer 377 or 3100 (Applied Biosystems, USA). The sequencing process was performed using fluorescent dye-termination methods (Big Dye, Applied Biosystems, CA), and sequence data were obtained using an automated DNA sequence system (Applied Biosystems, USA). The DNA sequence data obtained from a chromatogram that were read from the sequencer and assembled contigs were aligned using a ClustalW<sup>19</sup>.

### Fabrication of Microarray

To distinguish the species of salmon, specific probes were selected from sequence alignments of mitochondrial COIII-ND3-ND4L genes, and a specific probe for rainbow trout was also selected to divide the salmon and trout. The specific probes were positioned within the central region of mitochondrial COIII-ND3-ND4L genes, and were synthesized by Metabion (Germany). Species specific probes are described in Table 1. Salmon specific probes were printed onto silylated slides (Cell Associate, USA) using a robotic microarrayer (Cartesian Technology, USA). Each slide contained four sub-arrays, allowing the parallel testing of 4 different samples.

### DNA Amplification for Hybridization Experiment

The amplification of target DNA for hybridization was carried out using an asymmetric PCR strategy. A reverse primer was modified with CY3 fluorescent dye to detect DNA variation by the DNA chip, and the concentration of the reverse primer was ten times higher than the forward primer. Amplification condition was equal with process for sequencing.

### Hybridization and Washing Step

Hybridization of the amplified target sequence was performed in 100  $\mu$ L of 3  $\times$  SSC, 0.3% SDS for one hour at 55°C. In the four sub-arrays, the reaction solutions were separated from each other with a perfusion chamber (GraceBio, USA). After hybridization, the DNA chips were washed immediately in a 1X SSC

with 0.1% SDS for 3 min, 1X SSC for 3 min, and 0.1X SSC for one min.

### Image Analysis

Hybridization signals were detected using a Genepix 4000B (Axon instrument, USA) at a PMT gain of 600, and a laser power of 99%. After scanning, the fluorescence intensity was analyzed using Genepix 4.1 software. To verify a positive or negative result, the image pixel intensities of each spot were calculated, and the local background intensity was subtracted from the median value of each spot. Species determinations were based on the signal-to-noise ratio value.

### Acknowledgements

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