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Genetic Relationship among Three Subspecies of *Oncorhynchus masou* Determined by Mitochondrial DNA Sequence Analysis

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ABSTRACT—It is generally accepted that there are 3 subspecies of *Oncorhynchus masou* in Japan, namely, Masu salmon (*Oncorhynchus masou masou* (Brevoort)), Amago salmon (*O. masou ishikawae* Jordan & McGregor), and Biwa salmon (*O. masou rhodurus* Jordan & McGregor or *O. masou* subsp. Kimura). Since the genetic relationship of these three taxa is not well known, there has been considerable confusion over their nomenclature. We have clarified the genetic relationship among these three taxa by partially sequencing their mitochondrial DNA. Sequences of 948 base pairs from the 3' region of the ATPase subunit 6 gene to the 5' region of the cytochrome oxidase subunit 3 gene were obtained for 20 individuals including wild Biwa salmon, wild and farmed Amago and Masu salmon. Furthermore, 2,162 base pairs from the 3' region of ATPase subunit 6 gene to the 5' region of NADH dehydrogenase subunit 4L gene were determined in 4 individuals. In total, there were 26 sites of base substitutions. The haplotypes of Masu salmon and Amago salmon were similar. On the other hand, 17 of the 26 sites had substitutions characteristic of Biwa salmon. A matrix of genetic distances and maximum parsimony analysis among the haplotypes indicated that Biwa salmon is genetically more distant from Masu and Amago salmon, than Masu salmon is from Amago salmon. This means that Biwa salmon diverged from the common ancestor of the *Oncorhynchus masou* complex before the divergence between Masu salmon and Amago salmon.

INTRODUCTION

It is generally accepted that the *Oncorhynchus masou* complex in Japan comprises three subspecies: namely, (1) *Oncorhynchus masou masou* (Brevoort), the Masu salmon, in its anadromous form (also called “Yamame” in its non-anadromous form); (2) *O. masou ishikawae* Jordan & McGregor, the Amago salmon, in its non-anadromous form (also called “Satsuki salmon” in its anadromous form); and (3) *O. masou rhodurus* Jordan & McGregor or *O. masou* subsp. Kimura, the Biwa salmon (lacustrine form) (Araga and Ida, 1984; Hosoya, 1993; Kimura, 1990). Although the classification in this complex has been historically confused (for a review see Kimura, 1990), it is more widely accepted that there are three taxa, according to morphological characters (Kato 1973a; Kato, 1978a, b; Kimura, 1989) and life history (Honjo, 1976; Kato, 1973b; Kato, 1978b).

Opinion is divided as to their nomenclature (Araga and Ida, 1984; Kimura, 1990), partly because of the vagueness of the original descriptions of their type specimens (Jordan and McGregor, 1925). It is still questionable whether the rank of subspecies is totally adequate for classifying them, because the degree of genetic differences among the three taxa has not been precisely evaluated. Throughout this paper, we use the following common names for the three taxa: Masu salmon, Amago salmon and Biwa salmon.

Geographically, Masu salmon is distributed in and around the Sea of Japan and the adjoining rivers, southward

to Taiwan, and northward to the Sea of Okhotsk and the western slopes of the Kamchatkan Peninsula. Amago salmon inhabits only the southwestern part of Japan: the Seto Inland Sea of Japan and the adjoining rivers, the Pacific side of southern Honshu northward to Hakone, and the Pacific side of Shikoku. In the remainder of Japan only Masu salmon is found. The distribution of Amago salmon scarcely overlaps that of Masu salmon (Araga and Ida, 1984; Oshima, 1957). Biwa salmon is solely endemic to Lake Biwa and the rivers entering the lake (Araga and Ida, 1984; Kato, 1973a).

Morphologically, the sides of Amago salmon are marked with red spots, whereas those of Masu salmon are not (Oshima, 1957). Hence some researchers have called Amago salmon “Red-spotted Masu salmon” (Kuwahara and Iguchi, 1994). Biwa salmon has red spots on its sides, though the spots are found only in juveniles before their downstream migration into Lake Biwa from the rivers entering the lake (Kato, 1973a). It is therefore easy to distinguish Amago salmon from Biwa salmon in Lake Biwa.

We clarified the genetic relationship of these three subspecies by partially sequencing their mitochondrial DNA. This method is useful for genetic analyses of closely related subspecies, because mitochondrial DNA evolves considerably faster than nuclear DNA (Brown *et al.*, 1982). We amplified mitochondrial DNA by means of the polymerase chain reaction (PCR) (Saiki *et al.*, 1985).

MATERIALS AND METHODS

The specimens were obtained from the following localities in

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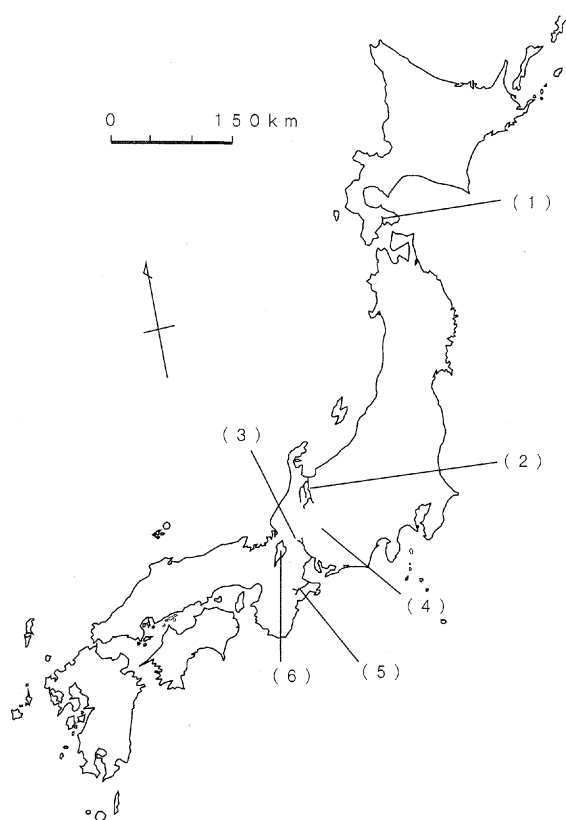


Fig. 1. The locations of sampling points in Japan. (1) Farmed Masu salmon from Hokkaido; (2) wild Masu salmon from the Jinzu River; (3) wild Amago salmon from the Nagara River; (4) farmed Amago salmon from Gifu; (5) wild Amago salmon from the Miya River; (6) wild Biwa salmon from Lake Biwa.

Japan (Fig. 1): from Hokkaido - farmed Masu salmon (4 specimens); from the Jinzu River - wild Masu salmon (5 specimens); from the Nagara River - wild Amago salmon (5 specimens); from Gifu - farmed Amago salmon (4 specimens); from the Miya River - wild Amago salmon (1 specimen); from Lake Biwa - wild Biwa salmon (5 specimens).

Each of the two wild populations of Amago salmon inhabits an upstream branch separated from the main river stem by a small dam, through which artificially released fish can not pass. According to fishermen, no fish lacking red spots have been observed for the past 60 years at these two locations. Therefore we conclude that the specimens in this study are from wild populations, in which hybridization of wild Amago salmon and Masu salmon is quite unlikely.

As for Masu salmon, individuals that had migrated up the Jinzu River for spawning were captured. In the Jinzu River, neither artificially reared Amago salmon nor cultured Masu salmon have yet been released. None of the 50 individuals observed had red spots. Therefore we conclude that the specimens are from a wild population of Masu salmon, in which hybridization of wild Masu salmon and Amago salmon is quite unlikely.

Mitochondrial DNA samples were prepared as described by Lansman *et al.* (1981). After CsCl ultra-centrifugation, only open circular fractions were used in this study.

Five pairs of PCR primer sequences were designed based on the reported sequences of mitochondrial DNA from Pacific salmonids (Thomas and Beckenbach, 1989), human (Anderson *et al.*, 1981) and *Xenopus laevis* (Roe *et al.*, 1985). The target region for each pair of PCR primers overlapped slightly with the adjacent target region(s) (Fig. 2). The amplified region spans from the 3' region of ATPase subunit 6 (ATPase 6) gene to the 5' region of NADH dehydrogenase subunit 4L (ND4L) gene (Fig. 2).

The sequences of primers were as follows: P01, 5'-AACTGACCATGAACCTAAGCTTCTTCGACCA-3'; P02, 5'-GCTGCTGTAGC-AATTAGTTG-3'; P11, 5'-ACTTACAGCCAATCTCACAG-3'; P12, 5'-TGGTAGAAGGCTCAGAAGAA-3'; P21, 5'-CGATATGGCATAATC-TTATT-3'; P22, 5'-AGACCGGGTGATTGGAAGTC-3'; P31, 5'-GTCT-CTATTTACTGATGAGG-3'; P32, 5'-ATGGTCTTTTGTAGCCGAAA-T-3'; P41, 5'-CGGAGTTAGTCCAAAAYAAG-3'; P42, 5'-CGTGCRG-

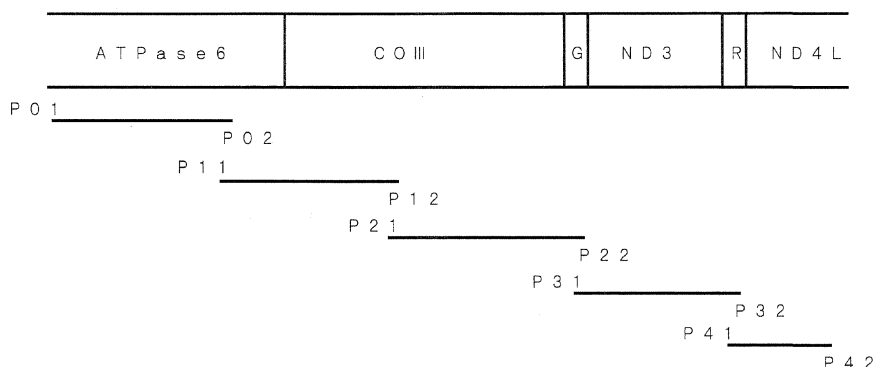


Fig. 2. The regions of PCR-amplification and sequencing in mitochondrial DNA. The 5 lines under the gene map correspond to the 5 amplification regions. Both strands were sequenced for each region. Names of primers for PCR and sequencing are also shown. The names of the genes: ATPase 6, ATPase subunit 6; COIII, cytochrome oxidase subunit 3; G, glycine tRNA; ND3, NADH dehydrogenase subunit 3; R, arginine tRNA; ND4L, NADH dehydrogenase subunit 4L.

TTGCTACTAGTAG-3', where Y is pyrimidine (T or C) and R is purine (A or G).

To sequence the amplified products using the universal primer, primers containing universal sequence (5'-TGTAACGACGGCCA-GT-3') in their 5' region were also synthesized. In total, 20 primers were prepared: 10 each with and without the universal sequence.

PCR proceeded for 35 cycles on a Perkin-Elmer Cetus thermal cycler, with denaturation at 96°C for 30 seconds, annealing at 55°C for 1 min and extension at 72°C for 2 min. The amplification was followed by a 7 minute final extension at 72°C and then the mixture was cooled at 4°C.

The amplified DNAs were resolved by electrophoresis on 2 % agarose gels, stained with ethidium bromide and purified with glass beads (Vogelstein and Gillespie, 1979). Sequence data were obtained using the dye-primer Taq cycle-sequencing reaction and an automated DNA sequencer (Applied Biosystems 370A). Sequences were determined for both DNA strands.

DNA sequence data were processed using Genetyx software programs (Software Development Co Ltd, Japan). Genetic distances were obtained using Kimura's two parameter method (Kimura, 1980) and the Jukes-Cantor method (Jukes and Cantor, 1969; Kimura and Ohta, 1972). The PAUP 3.1.1 computer program (Swofford, 1993) and the PHYLIP 3.5c computer package (Felsenstein, 1993) were used for maximum parsimony analysis and bootstrapping.

RESULTS

The sequences of 948 base pairs of DNA from the 3' region of ATPase 6 gene to the 5' region of cytochrome oxidase subunit 3 (CO III) gene were obtained using primer pairs P01/P02 and P11/P12 (Fig. 2), for 24 individuals (5 Biwa salmon, 5 Amago salmon from the Nagara River, 1 Amago salmon from the Miya River, 5 Masu salmon from the Jinzu River, 4 farmed Masu salmon that originated in Hokkaido and 4 farmed Amago salmon that originated in Gifu; see Fig. 1 for each location). In addition, 1,214 base-pair DNA sequences were determined and linked to the 948 base-pair sequences to obtain 2,162 base pairs from the 3' region of ATPase 6 gene to the 5' region of ND4L gene (Fig. 2), for 4 individuals (1 Biwa salmon, 1 Amago salmon from the Nagara River, 1 Amago salmon from the Miya River and 1 Masu salmon from the Jinzu River).

Figure 3 shows the 2,162 base pair sequence for Amago salmon from the Nagara River. This sequence has a 3 base pair insertion from base 440 to 442, and a 9 base pair insertion from 485 to 493, compared with the reported Pacific salmonid sequences (Thomas and Beckenbach, 1989). These correspond to 3 bases between 453 and 454, and 9 bases between 495 and 496 (Fig. 2A in Thomas and Beckenbach, 1989). On the other hand, taking the sequence in Figure 3 of our report as a standard, we found no insertions or deletions among the sequences of the Masu, Amago and Biwa salmon obtained in this study.

Table 1 summarizes the variable nucleotide positions among Masu, Amago and Biwa salmon, for 948 and 2,162 base pairs of data.

Among 948 bases, 15 sites were variable. Thirteen of them showed transitions, and 2 showed transversions. Thirteen synonymous and 2 non-synonymous substitutions

were found. Six haplotypes were designated as "a" to "f" (Table 1). The haplotypes of Masu salmon and Amago salmon were similar. Especially, farmed Masu salmon from Hokkaido and farmed Amago salmon from Gifu had the identical haplotype "c". On the other hand, 9 of the 15 substitutions were unique to Biwa salmon, indicating genetic peculiarities of Biwa salmon mitochondrial DNA among the *Oncorhynchus masou* complex.

Genetic distances among the 6 haplotypes were obtained with their standard deviations (Table 2A) using Kimura's two parameter method (Kimura, 1980). The intra-subspecies variations were from 0.21 % to 0.32 % for Masu salmon, from 0.11% to 0.42% for Amago salmon, and 0% for Biwa salmon. The range of the genetic distances between Masu and Amago salmon was from 0 to 0.42%, being comparable with intra-subspecies variations. On the other hand, the distances between Biwa salmon and the other two taxa were from 1.07% to 1.17%. These data showed that Biwa salmon is genetically more divergent from Masu and Amago salmon, than Masu salmon is from Amago salmon. The use of Jukes-Cantor's correction (Jukes and Cantor, 1969; Kimura and Ohta, 1972) instead of the Kimura's two parameter method, gave essentially the same results (not shown).

Among the four 2,162 base pair sequences (Table 1), 24 sites were variable: 20 of them showed transitions and 4 showed transversions. Eighteen of the 24 substitutions were synonymous, 5 were non-synonymous, and 1 was found in the glycine-tRNA gene. The haplotypes for Masu salmon and Amago salmon were again found to be similar, whereas Biwa salmon had 17 characteristic substitutions distinct from Amago and Masu salmon. Table 2B is the distance matrix among the four haplotypes, "a", "b", "d" and "f" for 2,162 base pairs of data. The distances between Biwa salmon and the other two taxa were from 0.89 to 0.93%, significantly larger than those between Masu and Amago salmon (from 0.14% to 0.23%).

Figure 4 shows the maximum parsimony networks for the haplotypes in Table 1, both for 948 (Fig. 4A) and 2,162 (Fig. 4B) base pairs of data. The two diagrams are quite similar. The haplotype of Biwa salmon "f" differs from those of Masu and Amago salmon more than Masu salmon does from Amago salmon. The haplotypes for Amago and Masu salmon gather into a cluster and intermingle with each other in Figure 4A. This indicates that the range of inter-subspecies difference between Amago and Masu salmon is nearly equal to the intra-subspecies variations within Amago and Masu salmon, being consistent with the genetic distance values in Table 2.

To draw a rooted maximum parsimony tree from 2,162 base pairs of data, the sequence for the rainbow trout *Oncorhynchus mykiss* (Thomas and Beckenbach, 1989) was included as an outgroup for the *Oncorhynchus masou* complex. Maximum parsimony analysis was carried out using the PAUP 3.1.1 computer program (Swofford, 1993). Two trees of equal maximum parsimony were obtained

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                                30                                60
ATTTATGAGC CCCACATACC TAGGTATTCC ACTTATCGCT GTAGCATTAA CCCTCCCATG

                                90                                120
AATTCTTTTC CCTACACCCT CTGCCCGATG ATTAAACAAC CGCCTAATTA CCCTGCAAGG

                                150                                180
ATGATTCATC AACCGATTTA CCCAACAACT CCTTTTACCA CTAAATTTAG GGGGTCACAA

                                210                                240
ATGAGCGGCT CTAATAACCT CCCTAATACT GTTTCTTATT ACCCTAAATA TACTAGGTCT

                                270                                300
ACTCCCTTAT ACATTTACCC CCACCACGCA ACTCTCCCTA AATATAGGCC TTGCAGTCCC

                                330                                360
ATTATGGCTC GCCACAGTAA TTATCGGTAT ACGAAATCAA CCCACTGCCG CCCTCGGCCA

                                390                                420
TCTTTTACCC GAAGGGACCC CCGTCCCACT AATCCCTGTA CTGATCATT TCGAAACAAT

                                450                                480
TAGCCTTTTT ATTGCCCCC TCGCCCTTGG CGTACGACTT ACAGCCAATC TCACAGCAGG

                                510                                540
CCACCTTCTC ATCCAATAA TTGCTACAGC AGCCTTTGTT CTCTTACCTC TAATACCCAC

                                570                                600
AGTGGAATC TTAACCTCTA TTGTTCTATT TCTACTTACC CTTCTTGAAA TTGCTGTAGC

                                630                                660
CATAATTCAA GCATACGTTT TTGTTTTACT CCTAAGCCTC TACCTACAAG AAAACGGTTA

                                690                                720
ATGGCACACC AAGCACACGC ATACCATATG GTTGACCAA GCCCCTGACC TCTAACGGC

                                750                                780
GCAATCGCCG CCCTTTTACT TACATCAGGC ACTGCAGTCT GATTCCATTT CCACTCACTC

                                810                                840
ACACTACTTA CCCTGGGTAA TGTTCTATTA CTTCTCACCA TGTATCAATG ATGACGGGAT

                                870                                900
ATTATTCGAG AGGGCACCTT TCAAGGACAT CACACACCCC CAGTCCAAAA AGGATTACGA

                                930                                960
TATGGTATAA TCTTGTTTAT TACCTCCGAA GTATTCTTTT TCCTGGGTTT CTTCTGAGCC

                                990                                1020
TTCTATCAGC CTAGCCTTGC CCCCACACCT GAATTAGGGG GTTGCTGACC CCCCACAGGC

                                1050                                1080
ATTACAACCTC TAGACCCCTT TGAGGTGCCC CTTCTTAATA CTGCAGTCCT TCTAGCATCT

                                1110                                1140
GGTGTTACCG TTACATGAGC CCATCATAGC ATCATAGAAG GTGAACGAAA ACAAAACATT

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1170                                1200
CAAGCTCTTA CTCTTACTAT TTTACTAGGA TTTTACTTCA CTTTCCTACA AGGTATAGAA

1230                                1260
TACTACGAAG CCCCATTTAC AATCGCTGAT GCGTATACG GCTCTACTTT CTTTGTGCGT

1290                                1320
ACAGGATTCC ATGGCCTACA CGTAATTATC GGCTCTACCT TTTTAGCCGT TTGCCTTCTA

1350                                1380
CGACAAGTTC AATATCACTT TACATCTGAA CATCACTTTG GCTTTGAAGC TGCTGCTTGA

1410                                1440
TATTGACACT TTGTAGACGT TGTATGGCTC TTCCTATACG TCTCTATTTA CTGATGAGGC

1470                                1500
TCATAATCTT TCTAGTACTA ATAAGTATAA GTGACTTCCA ATCACCCGGT CTTGGTTAA

1530                                1560
GTCCAAGGAA AGATAATGAA CTTAATTACA ACAATTATCA CTATTACCAT CACACTATCT

1590                                1620
GCAGTACTAG CCACTATTTT TTTCTGATTA CCACAAATTT CTCCAGACGC AGAGAAATTG

1650                                1680
TCCCCCTACG AATGTGGATT TGACCCTTTA GGATCCGCCG GTCTACCCTT CTCCTTACGC

1710                                1740
TTCTTCCTAA TCGCCATCCT GTTCCTTCTA TTGACTTGG AAATCGCCCT CCTTCTACCC

1770                                1800
CTGCCTTGAG GAGATCAACT CAACACCCCG GCCCTAACAC TCGTCTGATC CACTGCTGTA

1830                                1860
CTTGCCCTCC TTA CTCTCGG CTTAATCTAT GAATGAACCC AAGGAGGCTT AGAATGAGCC

1890                                1920
GAATAGGCAG TTAGTCCAAA ACAAGACCCT TGATTTCTGGC TCAAAGACC ATGGTTTAA

1950                                1980
TCCATGACCG CCTTATGACA CCAGTACATT TCAGCTTTAC TTCAGCCTTT ATTCTAGGGC

2010                                2040
TTATAGGACT CGCGTTTCAC CGCACCCATC TTCTTTTCA G CTTCTATGC CTAGAAGGAA

2070                                2100
TAATACTCTC TCTATTTATT GCCCTCTCCC TATGAGCCCT TCAAATGGAA GCAACCGGCT

2130                                2160
ATTCACTAGC TCCTATACTT CTCCTGGCAT TTTCAGCCTG TGAAGCAAGC GCAGGTTTAG

CC

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Fig. 3. Mitochondrial DNA sequence of 2,162 base pairs for Amago salmon from the Nagara River. Stop codons for protein coding regions are double-underlined, and start codons are darkened. From base 659 to 661, stop codon for ATPase 6; from 661 to 663, start codon for COIII; from 1444 to 1446, stop codon for COIII; from 1446 to 1515, glycine tRNA gene; from 1516 to 1518, start codon for ND3; from 1864 to 1866, stop codon for ND3; from 1865 to 1934, arginine tRNA gene; from 1935 to 1937, start codon for ND4L.

Table 1. Variable nucleotide positions in haplotypes among 3 subspecies of the *Oncorhynchus masou* complex. The same nucleotides as in the sample "Amago(N)1" are marked by hyphens.

| | | Relative nucleotide position in compared sequences | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--------------|---|--|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|--------|
| | | 1 1 1 1 1 1 1 1 1 1 2 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 1 1 1 3 3 4 6 8 8 8 9 9 2 4 4 4 6 6 7 7 7 8 0 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| sample | | 2 | 5 | 8 | 1 | 2 | 9 | 0 | 2 | 0 | 8 | 3 | 7 | 8 | 1 | 4 | 8 | 0 | 3 | 5 | 4 | 6 | 0 | 1 | 8 | 1 | 0 | haplo- |
| name(*) | | 2 | 0 | 8 | 2 | 7 | 4 | 1 | 8 | 7 | 7 | 7 | 9 | 2 | 5 | 5 | 1 | 9 | 9 | 8 | 1 | 2 | 1 | 9 | 3 | 8 | 9 | type |
| Amago(N) | 1 | A | A | A | C | C | C | A | T | A | T | G | C | A | G | G | C | T | G | C | T | T | G | G | G | C | T | a |
| Amago(M) | 1 | - | - | - | - | - | - | G | C | - | - | A | A | - | - | - | † | - | - | - | - | - | - | - | - | A | - | b |
| | 2 | - | - | - | - | - | - | G | C | - | - | A | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | b |
| | 3 | - | - | - | - | - | - | G | C | - | - | A | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | b |
| | 4 | - | - | - | - | - | - | G | C | - | - | A | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | b |
| | 5 | - | - | - | - | - | - | G | C | - | - | A | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | b |
| Amago(G) | 1 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| | 2 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| | 3 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| | 4 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| Masu(J) | 1 | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | A | - | d |
| | 2 | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | d |
| | 3 | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | d |
| | 4 | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | d |
| | 5 | - | - | - | A | - | T | - | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | e |
| Masu(H) | 1 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| | 2 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| | 3 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| | 4 | - | - | - | - | - | - | G | C | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | c |
| Biwa | 1 | G | G | G | - | T | - | - | C | G | C | A | - | G | A | A | - | A | C | T | - | C | A | A | A | A | C | f |
| | 2 | G | G | G | - | T | - | - | C | G | C | A | - | G | A | A | - | - | - | - | - | - | - | - | - | - | - | f |
| | 3 | G | G | G | - | T | - | - | C | G | C | A | - | G | A | A | - | - | - | - | - | - | - | - | - | - | - | f |
| | 4 | G | G | G | - | T | - | - | C | G | C | A | - | G | A | A | - | - | - | - | - | - | - | - | - | - | - | f |
| | 5 | G | G | G | - | T | - | - | C | G | C | A | - | G | A | A | - | - | - | - | - | - | - | - | - | - | - | f |
| codon | | 3 | 1 | 3 | 3 | 3 | 1 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | t | 3 | 3 | 3 | 3 | 1 | 3 | 3 | |
| position(**) | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Syn/Non- | | S | N | S | S | S | S | S | S | N | S | S | S | S | S | S | S | N | N | | S | S | S | S | N | S | S | |
| (***) | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

(*) Sample name: Amago(N), wild Amago salmon from the Nagara River; Amago(M), wild Amago salmon from the Miya River; Amago(G), farmed Amago salmon from Gifu; Masu(J), wild Masu salmon from the Jinzu River; Masu(H), farmed Masu salmon from Hokkaido; Biwa, wild Biwa salmon from Lake Biwa.

(**) Codon position: first(1), second(2) or third(3) codon position for protein coding regions; t, a site in glycine-tRNA coding region.

(***) Syn/Non-: Synonymous or non-synonymous substitution of protein coding sequences.

Table 2 Genetic distance matrix for the haplotypes. For each pair of haplotypes, the percentage (%) of genetic distance is given above the diagonal, and its standard deviation is given below the diagonal. These values were calculated using Kimura's two parameter method for correcting multiple-hits.

(A) Matrix based on 948 base pairs of data

| subspecies | haplotypes | Amago salmon | | | Masu salmon | | | Biwa salmon |
|--------------|------------|--------------|--------|--------|-------------|--------|--------|-------------|
| | | a | b | c | c | d | e | f |
| Amago salmon | a | — | 0.42 | 0.32 | 0.32 | 0.11 | 0.42 | 1.17 |
| | b | (0.21) | — | 0.11 | 0.11 | 0.32 | 0.42 | 1.17 |
| | c | (0.18) | (0.11) | — | 0 | 0.21 | 0.32 | 1.07 |
| Masu salmon | c | (0.18) | (0.11) | (0) | — | 0.21 | 0.32 | 1.07 |
| | d | (0.11) | (0.18) | (0.15) | (0.15) | — | 0.32 | 1.07 |
| | e | (0.21) | (0.21) | (0.18) | (0.18) | (0.18) | — | 1.17 |
| Biwa salmon | —f | (0.36) | (0.36) | (0.34) | (0.34) | (0.34) | (0.36) | — |

(B) Matrix based on 2,162 base pairs of data

| subspecies | haplotypes | Amago salmon | | Masu salmon | Biwa salmon |
|--------------|------------|--------------|--------|-------------|-------------|
| | | a | b | d | f |
| Amago salmon | a | — | 0.28 | 0.14 | 0.93 |
| | b | (0.11) | — | 0.23 | 0.93 |
| Masu salmon | —d | (0.08) | (0.10) | — | 0.89 |
| Biwa salmon | —f | (0.21) | (0.21) | (0.20) | — |

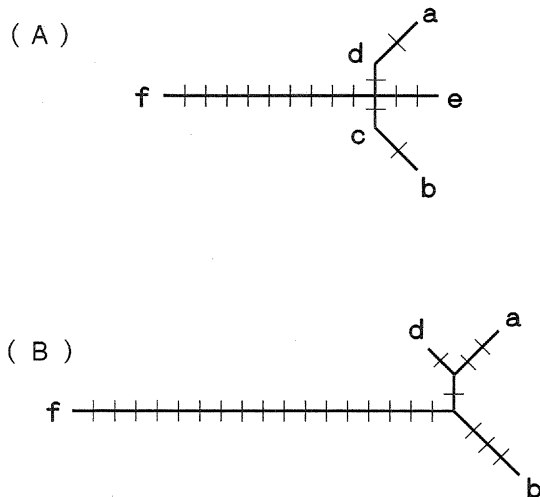


Fig. 4. Maximum parsimony networks for the haplotypes, (A) from 948 base pairs of data and (B) from 2,162 base pairs of data. Each of the small vertical bars on the lines connecting haplotypes represents a single substitution. The haplotypes "a" to "f" correspond to those shown in Table 1: briefly, Amago salmon corresponds to haplotypes "a", "b" and "c"; Masu salmon corresponds to haplotypes "c", "d" and "e"; and Biwa salmon corresponds to the haplotype "f".

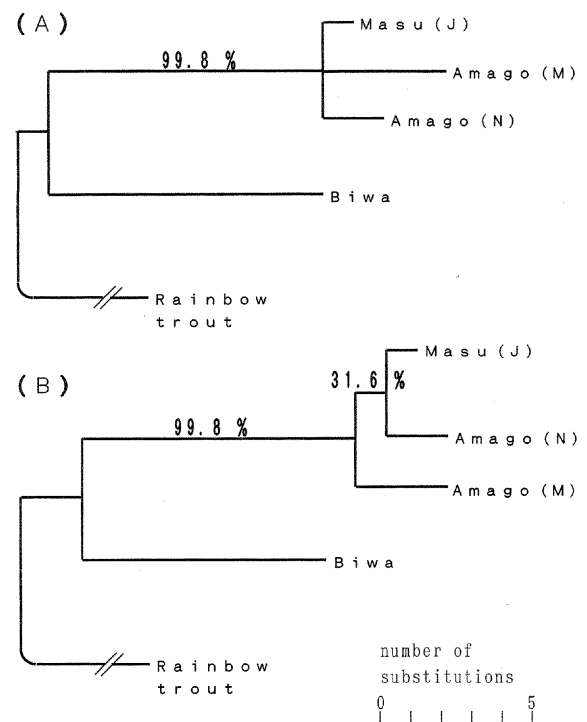


Fig. 5. The rooted maximum parsimony trees for Masu, Amago and Biwa salmon, based on 2,162 base pairs of data. Two trees of equal maximum parsimony are shown in (A) and (B). They were obtained by the parsimony analysis using the sequence for Rainbow trout as an outgroup for the *Oncorhynchus masou* complex. By bootstrapping, the topology of the 50% majority-rule consensus tree coincided with that of the tree in (A). The bootstrap probability is shown for each cluster.

(Fig. 5A and 5B). Bootstrap analysis generated a 50% majority-rule consensus tree, the topology of which coincided with that of the tree in Figure 5A. After 1,000 bootstrap replications, Masu salmon and Amago salmon were clustered together and separated from Biwa salmon with a probability of 99.8%. Essentially the same results were obtained using the PHYLIP 3.5c computer package (Felsenstein, 1993) instead of PAUP 3.1.1. These results indicate that Biwa salmon diverged from the common ancestor of the *Oncorhynchus masou* complex before Masu salmon and Amago salmon diverged.

DISCUSSION

Mitochondrial DNA sequencing revealed the genetic relationship among the three taxa of *Oncorhynchus masou* complex: Masu salmon, Amago salmon and Biwa salmon. Our results highlight the usefulness of mitochondrial DNA analysis for genetic studies among closely related organisms (Brown *et al.*, 1982; Lansman *et al.*, 1981; Wilson *et al.*, 1985). Other studies using isozymes have not led to conclusions regarding the relationship among the three taxa (Numachi, 1982; Numachi, 1984; Okazaki, 1974).

In general, for phylogenetic analyses by mitochondrial DNA sequencing, it is important to obtain DNA-sequences from several individuals for each taxon, because the range of sequence variations within each taxon can be similar to the range of sequence differences among taxa. Therefore, we determined 948 base pairs of mitochondrial DNA sequences for at least 5 individuals from each taxon (Table 1). We found that the genetic distance between Masu and Amago salmon is as small as the variations within Masu or Amago salmon, whereas the genetic distances between Biwa salmon and the other two taxa are significantly larger (Table 2A). We then used more sequence data (2,162 base pairs) from fewer individuals for bootstrap analysis of the parsimony trees, and obtained a majority-rule consensus tree supported with a high confidence level (Fig. 5A). This two-step approach was rationalized by the similarity between the two distance matrices (Table 2A and 2B) as well as between the two parsimony diagrams (Fig. 4A and 4B) obtained from 948 and 2,162 base pairs of data, respectively. The values for the genetic distances in Table 2B are a little smaller than those in Table 2A, partly because the two tRNA sequences included in the 2,162 base pairs of data evolves more slowly than protein coding sequences.

In this paper, we clarified that Biwa salmon is genetically more distant from Masu and Amago salmon, than Masu salmon is from Amago salmon. Other studies on morphology could not conclude this because the three taxa are very alike in morphology and meristic characters. Only a few morphological characters suggest the larger genetic divergence of Biwa salmon from the other two taxa: Biwa salmon have more pyloric caeca and fewer transverse scales than Masu and Amago salmon (Kato, 1973a; Kimura, 1989). Further, the common occurrence of red spots on the

sides of Amago salmon and juvenile Biwa salmon gave the misleading impression that Amago salmon and Biwa salmon might be genetically closer. Our results indicated that the common occurrence of red spots in this case is not a synapomorphy or evidence of closer kinship between the two taxa, though we do not deny the importance of the red spots as a key character to identifying the three taxa.

A generally used average rate of base substitutions for mitochondrial DNA between two vertebrate lineages is 2% per million years, including salmonids (Wilson *et al.*, 1985). A considerably lower substitution rate, however, was also reported for salmonids (0.5 - 0.9% per million years between two lineages), as well as for other poikilotherms (Martin and Palumbi, 1993). If we adopt the former substitution rate (Wilson *et al.*, 1985) for the *Oncorhynchus masou* complex, the values of genetic distance in Table 2 suggest that Biwa salmon diverged from the common ancestor of the *Oncorhynchus masou* complex about 500,000 years ago. According to paleolimnological studies, this agrees with when Lake Biwa became large and deep (450,000 years ago) (Yokoyama, 1984). Several taxa of fishes and snails endemic to Lake Biwa are considered to have differentiated concomitantly with the deepening of the lake (Tomoda, 1981, 1984). Biwa salmon may be one taxon that adapted itself to the deeper habitat of Lake Biwa. On the other hand, if we use the latter estimate of the evolutionary rate (Martin and Palumbi, 1993), the divergence time of Biwa salmon from the ancestor is calculated to be from 1.1 to 2 million years ago. Because of the conflict between the two estimates of the evolutionary rate (Martin and Palumbi, 1993; Wilson *et al.*, 1985), at present we cannot draw a conclusion on the time of divergence. Conversely, detailed studies on the fossil records of the endemic taxa of Lake Biwa, together with molecular evolutionary studies, will help us determine the absolute substitution rates of their mitochondrial DNA.

Although Oshima (1957) did not distinguish Biwa salmon from Amago salmon, our results support the notion that the Biwa salmon is genetically distinct from Amago salmon, and that the former should be regarded as a definite taxon. Therefore it is necessary to give Biwa salmon an appropriate scientific name. According to Kimura (1990), the name *Oncorhynchus masou rhodurus* given for Biwa salmon (Arago and Ida, 1984) is not adequate, because the type specimen of *Oncorhynchus masou rhodurus* is regarded as Masu or Amago salmon, judging from the morphological characters such as the number of pyloric caeca and transverse scales (Kato, 1973a; Kimura, 1989). On the other hand, the scale structure of the type specimen described by Jordan and McGregor (1925) resembles that for Biwa salmon, judging from the many ridges invading the exposed area (Kato, 1978a). Because of these ambiguities on the type specimen of *Oncorhynchus masou rhodurus*, it seems that Biwa salmon has no adequate scientific name and is tentatively called *Oncorhynchus masou* subsp. (Kimura, 1990).

The question of which level of taxonomy should be adopted, subspecies or species, for distinguishing Biwa salmon from Masu and Amago salmon, is intriguing. According to the definition of species by Mayr (1970), sympatric and morphologically distinct populations that are reproductively isolated from each other are regarded as different species. Therefore we raise two issues: (1) whether or not Biwa salmon and Amago salmon coexist at a single locality, and if so, (2) whether or not they are reproductively isolated from each other.

Although Biwa salmon and Amago salmon populate Lake Biwa and the rivers entering the lake today (Kato, 1978b; Kato, 1981; Kuwahara and Iguchi, 1994), this may be partly because the Amago salmon was artificially introduced, starting in 1970, to the rivers entering Lake Biwa (Kato, 1978b). It is not known whether wild Amago salmon already inhabited Lake Biwa and rivers entering the lake before the artificial introduction. The type specimen of Amago salmon *Oncorhynchus masou ishikawae* was captured from Lake Biwa (Jordan and McGregor, 1925), suggesting that wild Amago salmon were ubiquitous in Lake Biwa at that time.

Since Biwa salmon and Amago salmon coexist in Lake Biwa today, it is noteworthy that the former retains their mitochondrial DNAs distinct from those of Amago salmon. This suggests that Biwa salmon and Amago salmon are reproductively isolated in Lake Biwa and the rivers entering the lake. The idea of reproductive isolation is consistent with the observations that Biwa salmon differs from Amago salmon in ecological characters such as maturity age, growth rate and food habits (Kato, 1978b), as well as in morphological characters, such as the absence of red spots on individuals inhabiting the lake (Kato, 1973a). In summer, Biwa salmon inhabits cold layers of the lake which are deeper than those where Amago salmon is found (personal communication from fishermen). This also suggests two ecologically different populations.

Based on these data, it is suggested that Biwa salmon should be regarded as a new species distinct from *Oncorhynchus masou*, i.e. Amago salmon and Masu salmon. To obtain further evidence of reproductive isolation between Biwa salmon and Amago salmon, the mitochondrial as well as nuclear DNA sequences for Amago salmon captured in Lake Biwa should be determined and compared with those of Biwa salmon.

Another interesting finding of our study is that Amago salmon and Masu salmon are genetically quite closely related. The range of inter-subspecies difference (from 0 to 0.42 %) is nearly equal to the range of intra-subspecies variation (from 0.11 to 0.42 %). These data, however, do not deny the necessity of distinguishing the two as subspecies. A subspecies is defined as a population of a species inhabiting a geographic subdivision of the range of the species and differing morphologically from other populations of the species. Because Masu salmon and Amago salmon fulfill these criteria (geographic subdivision and presence or

absence of red spots), they should be regarded as two subspecies, even though they are genetically quite closely related.

Because the haplotypes of Amago salmon and Masu salmon are similar (Table 1) and they intermingle with each other in the parsimony diagram (Fig. 4A), it is not surprising that the haplotype "c" is commonly observed in domesticated Masu salmon and Amago salmon. No hybridization experiments have been conducted between either of these cultured populations and other subspecies. Therefore each individual of the domesticated population should have one of the haplotypes that the original wild population possessed.

Further, it should be noted that two wild individuals, namely, Amago(N)1 and Masu(J)1 in Table 1 also have very similar mitochondrial DNA sequences. Although the two were assigned to different haplotypes "a" and "d" by comparing 2,162 base pairs, the two sequences are identical from base 501 to base 1,500. This means that, if we had amplified the 1,000 base pairs in the sequence from 501 to 1,500, the two wild individuals would have been assigned the same haplotype name. In conclusion, the data in Table 1 indicate that mitochondrial DNA sequences can be identical over 1,000 base pairs between Masu salmon and Amago salmon, not only for domesticated populations, but also for wild populations.

It is notable that the two subspecies are genetically very closely related, despite the difference in the occurrence of the red spots. Presently, we intend to elucidate the population structure of Masu salmon and Amago salmon by applying molecular techniques to several other populations of both Amago salmon and Masu salmon.

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The 2,162 base pairs of DNA sequences reported in this paper have been submitted to the DDBJ/Genbank/EMBL Data bank, with the following accession numbers: D63335, for Biwa salmon; D63336, for Masu salmon; D63410, for Amago salmon.

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