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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 and Amago 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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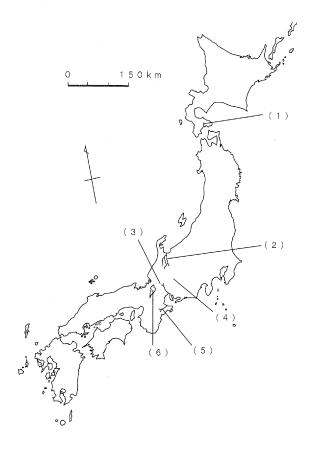


Fig. 1. The locations of sampling points in Japan. (1) Farmed Masu salmon from Hokkaido; (2) wild Masu salmon from the Jinzu River; (3) wild Amago salmon from the Nagara River; (4) farmed Amago salmon from Gifu; (5) wild Amago salmon from the Miya River; (6) wild Biwa salmon from Lake Biwa.

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'-AAACTGA-CCATGAACCTAAGCTTCTTCGACCA-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'-ATGGTCTTTTGAGCCGAAA-T-3'; P41, 5'-CGGAGTTAGTCCAAAAYAAG-3'; P42, 5'- CGTGCRG-

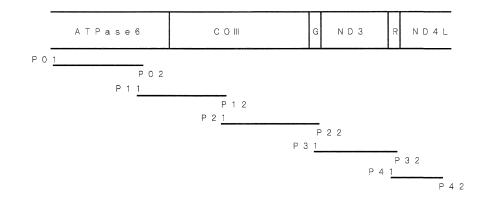


Fig. 2. The regions of PCR-amplification and sequencing in mitochondrial DNA. The 5 lines under the gene map correspond to the 5 amplification regions. Both strands were sequenced for each region. Names of primers for PCR and sequencing are also shown. The names of the genes: ATPase 6, ATPase subunit 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'-TGTAAAACGACGGCCA-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  $96^{\circ}$ C for 30 seconds, annealing at  $55^{\circ}$ C for 1 min and extension at  $72^{\circ}$ C for 2 min.The amplification was followed by a 7 minute final extension at  $72^{\circ}$ C and then the mixture was cooled at  $4^{\circ}$ 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 basepair 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 Figare 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 intrasubspecies 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 intersubspecies 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

ATTTATGAGC	CCCACATACC	30 TAGGTATTCC	ACTTATCGCT	GTAGCATTAA	60 CCCTCCCATG
AATTCTTTC	CCTACACCCT	90 CTGCCCGATG	ATTAAACAAC	CGCCTAATTA	120 CCCTGCAAGG
ATGATTCATC	AACCGATTTA	150 CCCAACAACT	CCTTTTACCA	CTAAATTTAG	180 GGGGTCACAA
ATGAGCGGCT	CTACTAACCT	210 CCCTAATACT	GTTTCTTATT	ACCCTAAATA	240 TACTAGGTCT
ACTCCCTTAT	ACATTTACCC	270 CCACCACGCA	ACTCTCCCTA	AATATAGGCC	300 TTGCAGTCCC
ATTATGGCTC	GCCACAGTAA	330 TTATCGGTAT	ACGAAATCAA	CCCACTGCCG	360 CCCTCGGCCA
TCTTTTACCC	GAAGGGACCC	390 CCGTCCCACT	AATCCCTGTA	CTGATCATTA	420 TCGAAACAAT
TAGCCTTTTT	ATTCGCCCCC	450 TCGCCCTTGG	CGTACGACTT	ACAGCCAATC	480 TCACAGCAGG
CCACCITCIC	АТССААСТАА	510 TIGCTACAGC	AGCCTTTGTT	CICTIACCIC	540 TAATACCCAC
	ТТААСТТСТА	570			600
	GCATACGTTT	630			660
		690			720
AIGGCACACC	AAGCACACGC		GTTGACCCAA	GCCCCTGACC	TCTAACCGGC
GCAATCGCCG	CCCTTTTACT	750 TACATCAGGC	ACTGCAGTCT	GATTCCATTT	780 CCACTCACTC
ACACTACTTA	CCCTGGGTAA	810 TGTTCTATTA	CTTCTCACCA	TGTATCAATG	840 Atgacgggat
ATTATTCGAG	AGGGCACCTT	870 TCAAGGACAT	CACACACCCC	CAGTCCAAAA	900 AGGATTACGA
TATGGTATAA	TCTTGTTTAT	930 TACCTCCGAA	GTATTCTTT	TCCTGGGTTT	960 CTTCTGAGCC
TTCTATCACG	CTAGCCTTGC	990 CCCCACACCT	GAATTAGGGG	GTTGCTGACC	1020 CCCCACAGGC
ATTACAACTC	TAGACCCCTT	1050 TGAGGTGCCC	СТТСТТААТА	CTGCAGTCCT	1080 TCTAGCATCT
GGTGTTACCG	TTACATGAGC	1110 CCATCATAGC	ATCATAGAAG	GTGAACGAAA	1140 ACAAACCATT

	CAAGCTCTTA	CTCTTACTAT	1170 TTTACTAGGA	TTTTACTTCA	CTTTCCTACA	1200 AGGTATAGAA	
	TACTACGAAG	CCCCATTTAC	1230 AATCGCTGAT	GGCGTATACG	GCTCTACTTT	1260 CTTTGTCGCT	
-	ACAGGATTCC	ATGGCCTACA	1290 CGTAATTATC	GGCTCTACCT	TTTTAGCCGT	1320 TTGCCTTCTA	
	CGACAAGTTC	AATATCACTT	1350 TACATCTGAA	CATCACTTTG	GCTTTGAAGC	1380 TGCTGCTTGA	
	TATTGACACT	TTGTAGACGT	1410 TGTATGGCTC	TTCCTATACG	ТСТСТАТТТА	1440 CTGATGAGGC	
	тса <u>таа</u> тстт	TCTAGTACTA	1470 ATAAGTATAA	GTGACTTCCA	ATCACCCGGT	1500 CTTGGTTAAA	
	GTCCAAGGAA	AGATA <mark>ATG</mark> AA	1530 CTTAATTACA	ACAATTATCA	CTATTACCAT	1560 CACACTATCT	
	GCAGTACTAG	CCACTATTTC	1590 TTTCTGATTA	CCACAAATTT	CTCCAGACGC	1620 Agagaaattg	
	TCCCCCTACG	AATGTGGATT	1650 TGACCCTTTA	GGATCCGCCC	GTCTACCCTT	1680 CTCCTTACGC	
	TTCTTCCTAA	TCGCCATCCT	1710 GTTCCTTCTA	TTTGACTTGG	AAATCGCCCT	1740 CCTTCTACCC	
	CTGCCTTGAG	GAGATCAACT	1770 CAACACCCCC	GCCCTAACAC	TCGTCTGATC	1800 Cactgctgta	
	CTTGCCCTCC	TTACTCTCGG	1830 CTTAATCTAT	GAATGAACCC	AAGGAGGCTT	1860 AGAATGAGCC	
	GAA <u>TAG</u> GCAG	TTAGTCCAAA	1890 ACAAGACCCT	TGATTTCGGC	TCAAAAGACC	1920 ATGGTTTAAG	
	TCCATGACCG	CCTTATGACA	1950 CCAGTACATT	TCAGCTTTAC	TTCAGCCTTT	1980 ATTCTAGGGC	
	TTATAGGACT	CGCGTTTCAC	2010 CGCACCCATC	TTCTTTCAGC	CCTTCTATGC	2040 CTAGAAGGAA	
	ТААТАСТСТС	TCTATTTATT	2070 GCCCTCTCCC	TATGAGCCCT	TCAAATGGAA	2100 GCAACCGGCT	
	ATTCAGTAGC	TCCTATACTT	2130 CTCCTGGCAT	TTTCAGCCTG	TGAAGCAAGC	2160 GCAGGTTTAG	

СС

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.

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

					Re	lati	ve r	nucl	eoti	ide	pos	itio	n in	col	mpa	arec	d se	que	ence	es								
																	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	А	А	А	С	С	С	А	Т	А	Т	G	С	А	G	G	С	Т	G	С	Т	т	G	G	G	С	Т	а
Amago(M)	1	-	-			-	-	G	С	-	-	А	А		-	-	Τ	-	-		-	-	-			А	-	b
	2				-		—	G	С	_	-	А	А															b
	3	—		-	-	-	-	G	С	-		А	А	-	_	-												b
	4							G	С			А	А		-													b
	5				—		-	G	С	_	-	А	А			-												b
Amago(G)	1		-	-		-		G	С	_		А				_ `												с
	2			-			-	G	С	-		А				· 												С
	3		-	-	-	-	-	G	С	-	-	А	-	-	-	-												С
	4				-		-	G	С	-		А			-	-												С
Masu(J)	1			-			-	_	С	-		-	_		_	-	_	_	_	_	С	_	-		_	А		d
	2				_	_	-	-	С		_	-		-	-	_												d
	3	_			-	_		-	С																			d
	4	_	-					_	С	-	·	-	-		-	-												d
	5		_		А		Т	-	С	-	_	А	-		-	-												е
Masu(H)	1				-			G	С	-	-	А	-	-	-	-												С
	2		-			-	_	G	С	-	-	А	-	-	_	-												С
	3		-	-	-		-	G	С	-	-	А	-	-	-													С
	4	—	-	-	-	-	_	G	С	-	-	А		-		-												с
Biwa	1	G	G	G	-	т	-		С	G	С	А		G	А	А		А	С	Т		С	А	А	А	А	С	f
	2	G	G	G		т	-	_								А												f
	3	G	G	G				_							А	А												f
	4	G	G	G								А		G	А	А												f
	5	G	G	G	_									G	А	А												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(**)		Ũ	•	2	2	5		2	-	•	2	2	2	2	2	5	2	-	_	•	5	Ŭ	J	5	•	5	5	
Syn/Non-		S	N	s	S	S	s	s	S	Ν	s	S	S	S	S	S	s	N	N		s	s	s	S	N	s	S	
(***)		0		5	5	5	5	5	5		5	5	5	5	5	5	0				5	0	0	5		5	5	

(\*) 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.

subspecies		An	nago salmor	י י	М	asu salmon		Biwa salmon
	haplotypes	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
	L_ 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 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)	-

(A) Matrix based on 948 base pairs of data

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

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

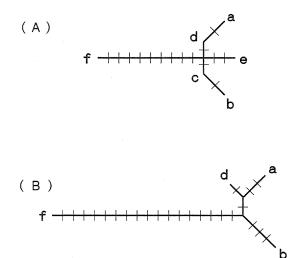


Fig. 4. Maximum parsimony networks for the haplotypes, (A) from 948 base pairs of data and (B) from 2,162 base pairs of data. Each of the small vertical bars on the lines connecting haplotypes represents a single substitution. The haplotypes "a" to "f" correspond to those shown in Table 1: briefly, Amago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

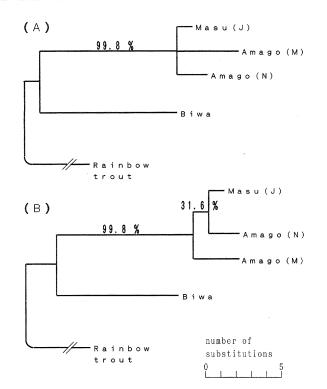


Fig. 5. The rooted maximum parsimony trees for Masu, Amago and Biwa salmon, based on 2,162 base pairs of data. Two trees of equal maximum parsimony are shown in (A) and (B). They were obtained by the parsimony analysis using the sequence for Rainbow trout as an outgroup for the *Oncorhynchus masou* complex. By bootstrapping, the topology of the 50% majorityrule 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 (Araga 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 masu 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/Genebank/EMBL Data bank, with the following accession numbers: D63335, for Biwa salmon; D63336, for Masu salmon; D63410, for Amago salmon.

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