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Phylogenetic relationship of the genus *Oncorhynchus* species inferred from nuclear and mitochondrial markers

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The phylogenetic relationship among the salmonid fishes of the genus *Oncorhynchus* has been analyzed using various kinds of markers for a long time. However, there are three major disagreements among those studies; (1) the authenticity of the Pacific salmon group as a monophyletic cluster, (2) the phylogenetic relationship among three Pacific salmons (pink salmon, sockeye salmon, and chum salmon), and (3) the phylogenetic position of masu salmon. We used allozyme electrophoresis to clarify the phylogenetic relationship between the Pacific salmon group and the Pacific trout group. Furthermore, we reanalysed published mitochondrial DNA D-loop sequences (Shedlock et al., 1992). Allozymic data and mtDNA data indicated the following consistent results; (1) all Pacific salmons formed a monophyletic cluster, (2) chum salmon and pink salmon were clustered within those Pacific salmons, (3) masu salmon formed a cluster with other Pacific salmons and diverged first in this group.

INTRODUCTION

The salmonid fish (the family Salmonidae) are distributed in a relatively cold water of northern hemisphere and normally have anadromous life histories, that is, naturally spawn only in some definite regions in the upper reaches of rivers or streams. These fish are also one of the most important resources as commercial or game fish in Japan as well as in Canada, North America, and eastern Siberia.

The genus *Oncorhynchus* belongs to the family Salmonidae and have been the subjects of numerous phylogenetic studies using morphology (Hikita, 1962; Smith and Stearley, 1989), allozyme electrophoresis (Utter et al., 1973), restriction fragment length polymorphism [RFLP] of ribosomal RNA genes [rDNA] (Phillips et al., 1992), RFLP of mitochondrial DNA [mtDNA] (Thomas et al., 1986), *Hin*dIII fragment (ATPase 6, CO III, $tRNA^{GLY}$, ND 3, $tRNA^{ARG}$, and ND 4L) sequences of mtDNA (Thomas and Beckenbach, 1989), D-loop sequences of mtDNA (Shedlock et al., 1992), ATPase 6 and ND 3 sequences of mtDNA (Domanico and Phillips, 1995), and short interspersed repetitive elements [SINEs] (Kido et al., 1991; Murata et al., 1993; Takasaki et al., 1996). These studies are in general agreements that *Oncorhynchus* had branched into four groups; (1) the Pacific trout group that consists of rainbow trout (*O. mykiss*; nijimasu) and cutthroat trout (*O. clarki*), (2) coho salmon (*O. kisutch*; ginzake) and chinook salmon (*O. tschawytscha*; masunosuke) of the Pacific salmon group [we call them A-lineage in this paper], (3) pink salmon (*O. gorbuscha*; karafutomasu), sockeye salmon (*O. nerka*; benizake), and chum salmon (*O. keta*; sake) of the Pacific salmon group [we call them B-lineage in this paper], and (4) masu salmon (*O. masou*; yamame) of the Pacific salmon group (Fig. 1). The Pacific trout group inhabits only western America, while the Pacific salmon group except masu salmon inhabits the whole area of the northern Pacific Ocean. Masu salmon has a restricted geographical distribution mainly in the Japanese archipelago. However, the phylogenetic relationship among above four groups differs depending on data.

In this study, we analyzed 19 allozyme loci by using protein electrophoresis for inferring the phylogenetic relationship among the four *Oncorhynchus* groups: the Pacific trout group, A- and B-lineages of the Pacific salmon group and masu salmon. It should be noted that Utter et al. (1973) used only 12 loci, and only 2 loci are overlapped among the 19 loci used in the present study. They simply computed similarities for interspecies comparisons, while we used the standard genetic distance (*D*) (Nei, 1972, 1978) to construct the phylogenetic tree.

Because mtDNA does not recombine, the same topology is expected to be obtained among different regions of an

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mtDNA genome. However, the result of D-loop sequences (Shedlock et al., 1992) is different from other mtDNA studies. We thus reexamined the mtDNA D-loop sequences data and compared with our allozyme data to help clarify the phylogenetic relationships among those *Oncorhynchus* species.

MATERIALS AND METHODS

Allozyme electrophoresis. Eight rainbow trout, four pink salmon, and two coho salmon individuals were bought from markets, while ten masu salmon were caught in Kamikasuo, Tochigi Prefecture. Two Japanese charr (*Salvelinus leucomaenis*; iwana) caught in Oorakumae river of Aomori Prefecture were also used as the outgroup.

Muscles, livers, and intestines of those fish were individually homogenized with the 20 mM phosphate buffer, pH 7.0, containing 0.1 M KCl and 20 mM EDTA under a cold temperature. The clear supernatants of homogenates obtained by centrifugation (15,000 rpm, 10 min, 4°C) were stored at -40°C before electrophoresis. Polyacrylamide gel (7.5%) was used for electrophoresis with the electrode buffer (0.38M Glycine-Tris, pH 8.3). The following thirteen enzymes were analyzed; α-glycerophosphate dehydrogenase (α-GPDH), glucose-6-phosphate dehydrogenase (G6PD), octanol dehydrogenase (ODH), nothing dehydrogenase (NDH), formaldehyde dehydrogenase (FDH), peroxidase (PO), superoxide dismutase (SOD), aspartate aminotransferase (AAT), phosphoglucomutase (PGM), esterase (EST), alkaline phosphatase (ALK), leucine aminopeptidase (LAP), and fumarase (FUM).

To estimate the genetic variability within and between species, average heterozygosity (H), genetic identity (I), and standard genetic distance (D) described by Nei (1972, 1978) were calculated from the allele frequency data. The phylogenetic trees were constructed by using the neighbor-joining method (Saitou and Nei, 1987) and bootstrap tests with 1000 replications (resampling loci) were performed. A series of programs developed by N. S. were used for assisting those phylogenetic analyses.

Nucleotide sequences of mtDNA. The D-loop sequence of rainbow trout was retrieved from the DDBJ/EMBL/GenBank international nucleotide sequence database under the accession number S68946 (Shedlock et al., 1992). D-loop sequences of the other salmonid species were obtained from published papers (Shedlock et al., 1992). CLUSTAL W version 1.6 (Thompson et al., 1994) was used for multiple alignment of the D-loop sequence data. The phylogenetic trees were constructed by using the neighbor-joining method (Saitou and Nei, 1987), the maximum likelihood method (Felsenstein, 1981), and the maximum parsimony method (Fitch, 1977). Split decomposition method (Bandelt and Dress, 1992) was also used. Bootstrap tests with 1000 replications were performed for the neighbor-joining analysis. Computer packages MEGA version 1.0 (Kumar et al., 1993), MOLPHY version 2.2 (Adachi and Hasegawa, 1994), PAUP version 3.1.1 (a commercial product distributed from Illinois Natural History Servey), and SplitsTree version 1.0 (developed by D. Huson and R. Wetzel) were used for assisting the neighbor-joining, the maximum likelihood, the maximum parsimony, and the split decomposition analyses, respectively.

RESULTS AND DISCUSSION

Allozyme electrophoresis. Table 1 shows allele frequencies for 19 genetic loci in five salmonid species. Monomorphism was observed at the *Po* locus for five salmonid species and at the *Aat* locus for four *Oncorhynchus* species. *Sod-2* and *Lap-1* were detected only in rainbow trout and masu salmon, respectively (data not shown). Only two loci (*Sod-1* and *Pgm*) overlapped with previous study (Utter et al., 1973).

Genetic identities (I) and standard genetic distances (D) among the five salmonid species are shown in Table 2. The I values range from 0.161 ± 0.083 (pink salmon - Japanese charr) to 0.504 ± 0.119 (pink salmon - coho salmon), and the D values range from 0.685 ± 0.236 (pink salmon - coho salmon) to 1.829 ± 0.515 (pink salmon - Japanese charr). The average heterozygosity (H) values are also shown in Table 2, and they range from 0.035 for coho salmon to 0.084 for pink salmon and masu salmon. These values are within the range of those for various fishes species previously reported (e.g., Gyllensten, 1985).

The phylogenetic tree for the four *Oncorhynchus* species was constructed from the matrix of D (Table 2) as shown in Fig. 2. The tree was rooted by applying the method described by Ishida et al. (1995), where constancy of evolutionary rate was assumed on the specified tree topology. In this tree, pink salmon and coho salmon formed a cluster and the phylogenetic position of rainbow trout indicated that the species diverged earlier among the *Oncorhynchus* species. The bootstrap values of both internal branches were 100%. The unrooted topology of this tree is the same as that of Utter et al. (1973), though the location of the root is different. This inconsistency is probably because they used UPGMA.

The origination time (t) of *Oncorhynchus* species was estimated by the equation described by Nei (1987) ($t = 5 \times 10^6 D$) from the tree (Fig. 2). The D value (1.02) of the first divergence among *Oncorhynchus* species was estimated under the assumption of molecular clock using the method described by Ishida et al. (1995). The time was thus estimated to be about 5 Ma (million years ago) within the Pliocene. The *Oncorhynchus* species is distributed only in the northern Pacific Ocean and they probably penetrated from the Atlantic Ocean through the Bering Seaway. Briggs (1970) suggested that the north Pacific

Table 1. Allele frequencies at 19 genetic loci in five salmonid species

Locus	A**	species* 1	2	3	4	5
α-gpdh	a	1.00	1.00	–	–	–
	b	–	–	0.88	1.00	–
	c	–	–	0.12	–	–
	d	–	–	–	–	1.00
		(16)	(20)	(8)	(4)	(4)
G6pd	a	1.00	–	–	–	1.00
	b	–	1.00	1.00	1.00	–
		(16)	(20)	(8)	(4)	(4)
Odh	a	–	–	1.00	–	–
	b	1.00	1.00	–	1.00	–
	c	–	–	–	–	1.00
		(16)	(20)	(8)	(4)	(4)
Ndh	a	1.00	–	–	–	–
	b	–	–	1.00	1.00	–
	c	–	1.00	–	–	1.00
		(16)	(20)	(8)	(4)	(4)
Fdh	a	–	1.00	1.00	–	–
	b	1.00	–	–	1.00	1.00
		(16)	(20)	(8)	(4)	(4)
Po	a	1.00	1.00	1.00	1.00	1.00
		(12)	(20)	(8)	(4)	(4)
Sod-1	a	–	–	0.37	–	–
	b	–	–	–	1.00	–
	c	–	–	0.63	–	0.50
	d	–	1.00	–	–	–
	e	0.94	–	–	–	–
	f	0.06	–	–	–	–
	g	–	–	–	–	0.50
		(16)	(20)	(8)	(4)	(4)
Aat	a	–	–	–	–	1.00
	b	1.00	1.00	1.00	1.00	–
		(16)	(20)	(8)	(4)	(4)
Pgm	a	–	1.00	–	–	–
	b	–	–	–	1.00	–
	c	1.00	–	–	–	1.00
	d	–	–	1.00	–	–
		(10)	(20)	(8)	(4)	(4)
Est-1	a	1.00	0.70	–	–	1.00
	b	–	0.30	1.00	1.00	–
		(10)	(20)	(6)	(4)	(4)

Locus	A**	species* 1	2	3	4	5
Est-2	a	–	1.00	–	–	–
	b	1.00	–	–	–	–
	c	–	–	–	1.00	–
	d	–	–	1.00	–	–
	e	–	–	–	–	1.00
		(12)	(2)	(8)	(2)	(4)
Est-3	a	–	1.00	–	1.00	–
	b	1.00	–	1.00	–	–
	c	–	–	–	–	1.00
		(16)	(20)	(8)	(4)	(4)
Est-4	a	1.00	1.00	1.00	–	–
	b	–	–	–	1.00	1.00
		(16)	(20)	(8)	(4)	(4)
Est-5	a	–	–	–	–	1.00
	b	–	0.80	–	–	–
	c	0.12	0.10	1.00	1.00	–
	d	0.44	–	–	–	–
	e	–	0.10	–	–	–
	f	0.44	–	–	–	–
		(16)	(20)	(8)	(4)	(4)
Alk	a	–	1.00	–	–	1.00
	b	–	–	0.88	1.00	–
	c	1.00	–	–	–	–
	d	–	–	0.12	–	–
		(16)	(12)	(8)	(4)	(2)
Lap-2	a	–	–	–	0.50	–
	b	–	1.00	–	–	1.00
	c	1.00	–	0.50	0.50	–
	d	–	–	0.50	–	–
		(16)	(20)	(8)	(4)	(4)
Lap-3	a	0.69	0.89	–	1.00	–
	b	–	0.11	1.00	–	0.50
	c	0.31	–	–	–	0.50
		(16)	(18)	(4)	(2)	(4)
Lap-4	a	1.00	–	1.00	1.00	1.00
	b	–	0.50	–	–	–
	c	–	0.50	–	–	–
		(6)	(4)	(8)	(4)	(4)
Fum	a	–	–	1.00	–	–
	b	1.00	1.00	–	1.00	1.00
		(16)	(20)	(8)	(4)	(4)

Numbers of genes sampled ($2N$) for each locus are in parentheses. A hyphen indicates zero frequency.

* 1: rainbow trout, 2: masu salmon, 3: pink salmon, 4: coho salmon, 5: Japanese charr.

** Allele names. They are alphabetically designated starting from "a" with increasing the electrophoretic mobility.

and Atlantic Oceans were isolated from each other from the late Mesozoic (200 Ma) until the late Cenozoic when two periods of trans-Arctic exchange occurred. The first period took place about 10-12 Ma during a brief Bering Seaway connection between the two ocean basins. The second period occurred during the most recent Bering Seaway opening some 3-5 Ma. The origination time of *Oncorhynchus* species estimated by allozyme data is con-

Table 2. Genetic identities, standard genetic distances, and average heterozygosities among five salmonid species based on 19 genetic loci

	rainbow	masu	pink	coho	charr
rainbow	0.061 ± 0.038	0.417 ± 0.116	0.319 ± 0.109	0.405 ± 0.113	0.404 ± 0.117
masu	0.874 ± 0.279	0.084 ± 0.042	0.317 ± 0.107	0.409 ± 0.114	0.329 ± 0.112
pink	1.141 ± 0.341	1.148 ± 0.339	0.084 ± 0.042	0.504 ± 0.119	0.161 ± 0.083
coho	0.904 ± 0.280	0.894 ± 0.279	0.685 ± 0.236	0.035 ± 0.035	0.278 ± 0.108
charr	0.907 ± 0.290	1.112 ± 0.339	1.829 ± 0.515	1.281 ± 0.388	0.070 ± 0.048

Note. Genetic identities are above the diagonal, standard genetic distances are below the diagonal, and average heterozygosities (H) are on the diagonal. Numbers with ± are standard errors.

Fig. 1. The scheme of the relationship for the *Oncorhynchus* species from various kind of data. The strictly consensus relationship of On*corhynchus* species from morphological, allozyme, rDNA, mtDNA, and SINEs data. Names in parentheses are English common names and Japanese scientific names.

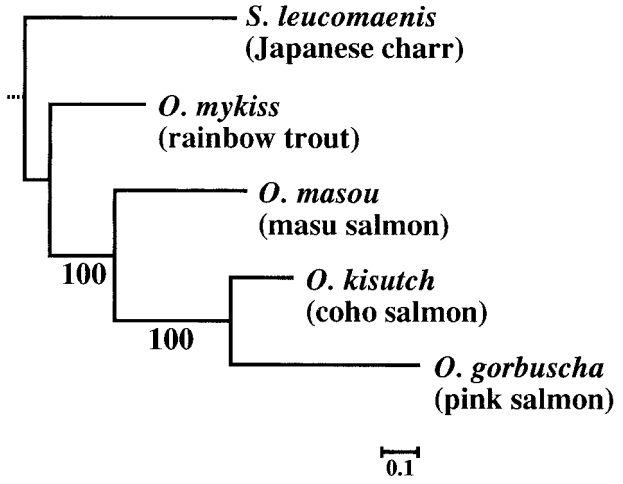

Fig. 2. The neighbor-joining tree for the five salmonid species constructed from Nei's standard genetic distances based on 19 genetic loci. The value given on each branch is the bootstrap probability (in %).

sistent with the second period. It is speculated that *Oncorhynchus* species penetrated and radiated in the northern Pacific Ocean during the second trans-Arctic connection. The fossil record of masu salmons also suggests that *Oncorhynchus* species existed in the late Cenozoic (Uyeno et al., 1975).

Nucleotide sequences of mtDNA. We first realigned the D-loop sequences for nine salmonid species. To obtain a more optimal alignment, profile alignment menu in CLUSTAL W program was used. In this procedure, the alignment of sequences for closely related three Pacific salmons (sockeye salmon, chum salmon, and pink salmon) was first performed. Next, five sequences of other *Oncorhynchus* species were joined to the aligned three sequences and finally the sequence of Atlantic salmon (*Salmo salar*) was joined. The resultant multiple alignment has the total length much shorter (about 80 sites) than that described by Shedlock et al. (1992). Shedlock et

al. (1992) treated gaps in the alignment as fifth character states for their maximum parsimony analysis, while we eliminated gaps. Unstable sites (the region immediately downstream of the tRNA[PHE] gene, that is after position 957) were eliminated following Shedlock et al. (1992). The remaining 906 nucleotide sites (913 sites when only eight *Oncorhynchus* species were analysed) were used for the following phylogenetic analyses. Variant sites are only shown (Table 3).

Because transitional nucleotide substitutions seem to be much more frequent than transversional substitutions in mtDNA (Brown et al., 1982), we first calculated the number of nucleotide substitutions among salmonid species by using the two-parameter method (Kimura, 1980) and constructed the phylogenetic tree by using the neighbor-joining tree. A method described by Ishida et al. (1995) was then applied to obtain integer-value branch lengths. However, the total number (255) of nucleotide substitutions inferred from this procedure was smaller than that of the smallest possible number (258). This underestimation was probably caused by the assumption of uniform evolutionary rate at every site. We thus used the two-parameter method described by Kimura (1980) with a gamma distribution model (Jin and Nei, 1990) to obtain a more reasonable distance matrix. The parameter a (0.723) in the gamma distribution was estimated by the method described by Tamura and Nei (1993). The mean (0.2859) and variance (0.3988) of the estimated number of nucleotide substitutions per site were estimated by parsimony analysis of first obtained 9 salmonid species tree. Resultant pairwise distances were shown in the lower diagonal of Table 4. Those values were considerably larger than proportions of nucleotide difference (upper diagonal of Table 4). This indicates that some parallel nucleotide changes, multiple substitutions, or back substitutions exist and needed to use a suitable corrected distance matrix method.

The resulting tree has the same topology as that obtained previously using the two-parameter method described by Kimura (1980) without gamma distribution. A total of 277 nucleotide substitutions was estimated by applying this procedure (Fig. 3). In this tree, the genus *Oncorhynchus* branched into the following four groups: the Pacific trout group (rainbow trout and cutthroat trout), the A-lineage of the Pacific salmon group (coho salmon and chinook salmon), the B-lineage of the Pacific salmon group (pink salmon, sockeye salmon, and chum salmon), and masu salmon. All Pacific salmon species formed a cluster. The tree indicated the same relationship with that estimated from the present allozyme data (Fig. 2). However, the bootstrap value of each branch was not so high.

We also used the maximum likelihood and the maximum parsimony methods. Because Atlantic salmon was much divergent from other *Oncorhynchus* species, we used only eight *Oncorhynchus* species for these analyses. All

Table 3. Sequence comparison of the Realigned D-loop region of nine salmonid mtDNAs

Species	Nucleotide position
	111111111111111111111111111112222222222222222222222222222223333333333333333
	1111111112222222334444556666667888999900011111111235555555677788889900122223333333444555555566677000011114444445555
	15678901234567803456749124519012345645703476790123456791101245692167023672504579234579123012458902835023924564567890 78
mykiss	AC----TACAGCTATTAACTGTATTTAAACTACATTATATTTCAT--CTGCAT-TATCGGGATTCCGCAATTTC-TTAGTTTTAATATTTATTCTAAAACCTCAACGGCTT-ATCGAT
clarki	..----C.T.A.....T.........TT.................--.....C-.........C-..T...A.AA.CTA.CC...........................-......
tschawytscha	..-CA-..-.C-.....TT...........G..C..........T-...TG.T.G.....G.C.........-..........GACC.C..C.C.G.......T....-...A..
kisutch	..-TAA..G.--.--..TT.................C.......--.....C-...A...G.C...T.....--ACGAG..........CCTC.C..GT...G.TA...-...A..
keta	G.-TA-C.-TC-C-CC.TT..C...........-..........--.....C-...TACT...G...T....-A.....C.GC.....CGC.....C........T..A.ATC-A..
nerka	..-GA-C.-.C-A-CC.TT.........................--.....C-.......G.C.T.......-A.....C.GC.....C.C..C...GG......TA.ACC..-A..
gorbuscha	..-GA-..-TT-.-CC.TT.......TT.ACTTCACG..CAC...--.....C-..CTAT.....TA.T.CA..A.....C.GC.....C.C..C..CG......A..T.CC..-ATA
masou	..GTA--.-.CA.--..TT....C..........C...G...T.C--..-..C---.............C...AA................C..CT.CG.....G.TA...-......
S. salar	.T----.T...........AA.T.ACT........C.......TATATC....-...-A..GA.....T...AAA.........CC...............ATT..TAT..A..-A..

Species	Nucleotide position
	3333333344444444444555555555555666666666666666666777777777777777777777777777788888888888888888888888888889999999999999999
	6788899900013334588000113333567001222333444455668111122233333344444555688990012333444445567777788889000111111223334555
	692453452490347190438902123489028645946724785606534580280457890123455674451223081473458945756789156733461245691504985 7
mykiss	TGGTAAGGCCATAGAGGA-TTTGCGCGT-CTAGACTTATTAACC-CGTACCGATTCGTCA--AGATCTCT-TC-AAGTTACA-GTAAT-AGGATGCCA--TAGGAAGCGCCCAATATG
clarki	..................-.........-.......C......-.........C.AC..--....T.TC-.A-.....GAG---...TGA.-.....TA..A.GG......T.-...
tschawytscha	...A..A...........-.........---..G......G.T-T..............--....T...-..-......T.-A.G..T.A.-.....TAC...........T.-.C.
kisutch	......A...........-.........---...........T-T......A....A..G--.TG.T.T.-..-.........-A.G..T.A.G....TTA........A...T.-...
keta	AA.A...-.A......C.-....T....---.A..C.........-.....A..........--.........-.AA........-.....-.A.......TA..A......TA.T.-...
nerka	..................-.........---...........TA-.T............T-.........-..-G.AC....A..T..-.A.......TA............T.-...
gorbuscha	..A.T.....T...G...-..........-..T..T........A-AT......C.......AG.TC.....C.AT........-.....-G........TA..........T.T.-...
masou	.....GA.T..C...T.C-.CGAT....C--......GCC.G..-...G.A..C...--.--......T.-.AA........-....A-......T..TT.....-A.....T.-...
S. salar	.A.........CTA..CTAC...TC--G---...............-..-.TGAT..TT...---AGA.C.C-Y.-.GA.C...-...C.T.ATGCCAT.TACTAA.-TG...TT--G.A

Data from the nucleic acid sequence data bases and Shedlock et al. (1992). Sequence identity is indicated by a dot, and a gap is indicated by a hyphen. At position 764 of *Salmo salar*, Y = C or T.

Table 4. Sequence divergences of D-loop sequences among nine salmonid species

	rainbow	cutthroat	chinook	coho	chum	sockeye	pink	masou	Atlantic salmon
rainbow		0.0298	0.0320	0.0408	0.0430	0.0320	0.0596	0.0430	0.0751
cutthroat	0.0315		0.0519	0.0475	0.0596	0.0508	0.0740	0.0640	0.0949
chinook	0.0342	0.0575		0.0397	0.0552	0.0342	0.0728	0.0574	0.0938
coho	0.0443	0.0518	0.0431		0.0662	0.0408	0.0817	0.0596	0.0960
chum	0.0463	0.0663	0.0612	0.0749		0.0430	0.0651	0.0640	0.0949
sockeye	0.0339	0.0558	0.0365	0.0441	0.0464		0.0563	0.0552	0.0916
pink	0.0661	0.0848	0.0831	0.0950	0.0730	0.0620		0.0806	0.1170
masu	0.0467	0.0727	0.0645	0.0670	0.0723	0.0613	0.0934		0.1015
Atlantic salmon	0.0858	0.1130	0.1119	0.1148	0.1126	0.1082	0.1448	0.1232	

Values above diagonal are proportions of nucleotide difference (*p*-distances) and those below diagonal are gamma distances with Kimura 2-parameter model

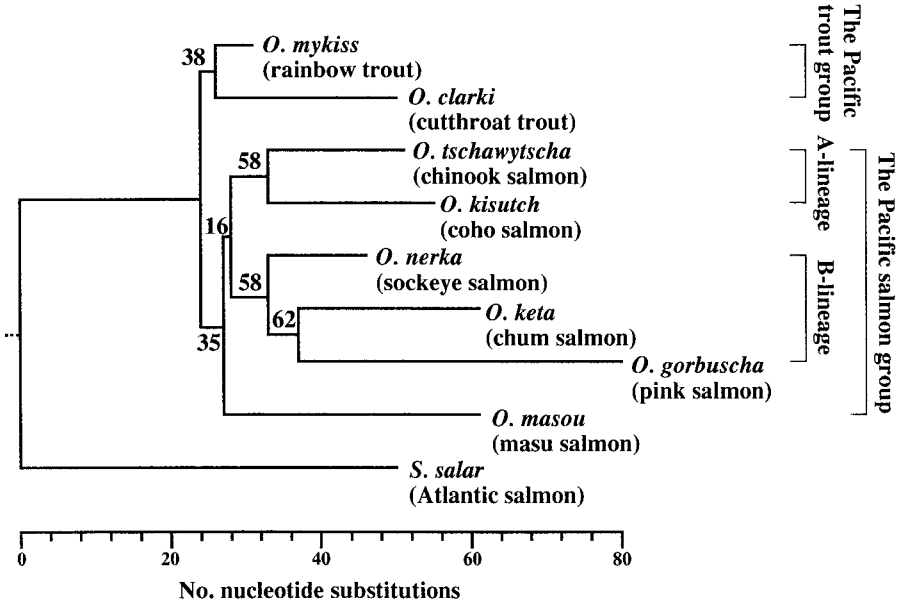

Fig. 3. The neighbor-joining tree for the nine salmonid species constructed from D-loop sequence data using gamma distances with Kimura's 2-parameter model. The value given to each branch is the bootstrap probability (in %).

the 10,395 possible tree topologies for 8 sequences were searched by using exhaustive options of MOLPHY and PAUP, and the results for the top 10 trees with highest likelihoods are presented in Table 5. The rainbow trout-cutthroat trout cluster and the chum salmon-pink salmon cluster were observed in all the 10 trees. The topology of the maximum likelihood tree (tree 1) was identical with the neighbor-joining tree of Fig. 3. Three equally maximum parsimonious trees (trees 1, 2 and 5) were found when transitions and transversions were not weighted (transitions/transversions ratio is 1.0). One of them had the same topology with the maximum likelihood tree. Because the estimated transitions/transversions ratio was 4.07 in the maximum likelihood analysis, we also used ratios 2.0 and 4.0 in the maximum parsimony analysis. Trees 1 and 2 were again equally parsimonious trees in both cases, while tree 5 was no longer parsimonious (see Table 5). The topology of the tree 2 was different only on the shortest branch (bootstrap value 16 %) from tree 1.

We also constructed the phylogenetic tree of six *Oncorhynchus* species (rainbow trout, cutthroat trout, coho salmon, chinook salmon, pink salmon, and sockeye salmon) by using the neighbor-joining and the maximum likelihood methods based on *Hin*dIII fragment sequences (Thomas and Beckenbach, 1989). The resultant trees had the identical topology with that of the tree in Fig. 3

Table 5. The maximum likelihood and the maximum parsimony analyses of various kind of topologies for eight *Oncorhynchus* species

Tree	Topology*	Δln L	RNM1.0	RNM2.0	RNM4.0
1	((my,cl),((ki,ts),((ke,go),ne)),ma)	0.0	**196**	**263**	**397**
2	(((my,cl),((ke,go),ne)),(ki,ts),ma)	–1.8	**196**	**263**	**397**
3	((my,cl),(ki,ts),(ma,((ke,go),ne)))	–2.6	197	264	398
4	((my,cl),((ki,ts),ne),(ma,(ke,go)))	–4.0	197	264	398
5	((my,cl),(((ki,ts),ne),(ke,go)),ma)	–4.1	**196**	264	400
6	((my,cl),((ki,ne),ts),(ma,(ke,go)))	–6.1	198	265	399
7	((my,cl),(((ki,ne),ts),(ke,go)),ma)	–6.5	197	265	401
8	(((my,cl),(ke,go)),((ki,ts),ne),ma)	–7.2	197	265	401
9	((my,cl),(ki,(ts,((ke,go),ne))),ma)	–7.5	197	265	401
10	((my,cl),((ki,((ke,go),ne)),ts),ma)	–8.5	199	267	403

Δln L : The difference in log-likelihood from the ML tree (–2334.6).
RNM : Required number of mutations when the maximum parsimony method was applied. The number followed RNM means the transition/transversion ratio used. Bold letters are minimal trees for each case.
*Species abbreviations are my : *O. mykiss* (rainbow trout), cl : *O. clarki* (cutthroat trout), ne : *O. nerka* (sockeye salmon), ts : *O. tschawytscha* (chinook salmon), ki : *O. kisutch* (coho salmon), ke : *O. keta* (chum salmon), go : *O. gorbuscha* (pink salmon), ma : *O. masou* (masu salmon).

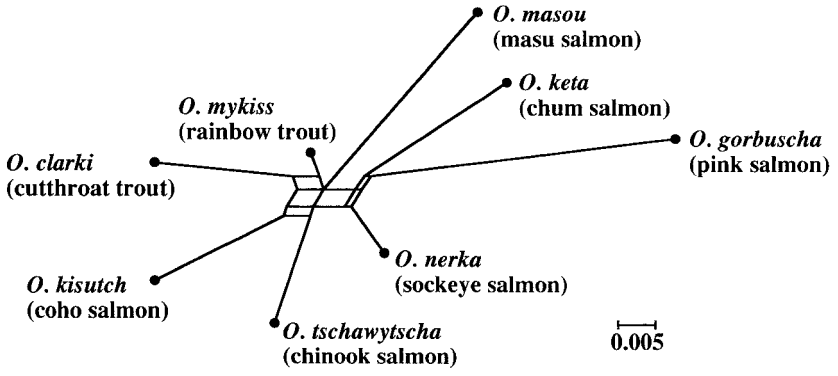

Fig. 4. A split decomposition network for the eight *Oncorhynchus* species based on proportions of nucleotide difference (Table 4). Edges were drawn to scale.

and bootstrap values were quite high at all branches (data not shown). However, two *Oncorhynchus* species (chum salmon and masu salmon) and an outgroup species (e.g., Atlantic salmon) were not included. Therefore *Hin*dIII fragment sequences were not so useful to resolve the phylogenetic problems in this study.

The split decomposition network of eight *Oncorhynchus* species was shown in Fig. 4. In this network, six parallelograms were observed and many tree topologies are embedded in this figure. There are three clusters different from the neighbor-joining tree (Fig. 3) in the network; (I) cutthroat trout and coho salmon, (II) coho salmon, chinook salmon, and sockeye salmon, and (III) pink salmon and sockeye salmon. However, these three clusters are supported only by a few nucleotide sites. Cluster I is supported by 4 sites (177, 224, 227, and 730), cluster II is supported by 1 site (155), and cluster III is supported by 3 sites (171, 345, and 660) (see Table 3). In this study sequences of mtDNA were used and the possibility of gene recombination is eliminated. Thus those sites probably appeared by some parallel nucleotide changes, multiple substitutions, or back substitutions. On the other hand, the position of masu salmon is not definite in this network. The bootstrap value of that branch in the neighbor-joining tree (Fig. 3) is also quite low.

We estimated the rate (λ) of nucleotide substitution in

the mtDNA D-loop region under the assumptions of the constant evolutionary rate. The equation $d = 2\lambda t$ was used, where d is the number of nucleotide substitutions per site between a pair of sequences, and t is the divergence time. As mentioned above, the last Bering Seaway opening occurred during some 3-5 Ma and it was suggested that *Oncorhynchus* species penetrated and radiated into the northern Pacific Ocean at around that period. Therefore, the time (t) of the root of the tree of Fig. 3 was assumed to be 3-5 Ma. The number of nucleotide substitutions per site of the first divergence among *Oncorhynchus* species ($d = 0.028$) was estimated by applying the method described by Ishida et al. (1995). Thus the rate ($\lambda = d/2t$) becomes $0.2 - 0.4 \times 10^{-8}$ per nucleotide site per year. This value is considerably smaller than that of human mtDNA ($2.5 - 15 \times 10^{-8}$ estimated by Tamura and Nei, 1993) and horse mtDNA ($2 - 4 \times 10^{-8}$ estimated by Ishida et al., 1995). Thomas and Beckenbach (1989) suggested that the evolutionary rate of mtDNA in salmonid fishes is considerably slower than that in primates. Martin et al. (1992) suggested that rates of evolution for mtDNA in sharks may be 5 - 7 times slower than that for mammals, because of the different metabolic physiology between poikilotherms and homoiotherms. The value estimated in this study is consistent with above two studies.

Comparison of various studies. The phylogenetic relationship among four groups (A-linage of the pacific salmon group, B-linage of the pacific salmon group, masu salmon, and the pacific trout group) differs depending on data (Table 6A). Allozyme (Utter et al., 1973) and RFLP of rDNA (Phillips et al., 1992) data suggest that A- and B-lineages of the Pacific salmon group form a cluster and masu salmon forms a cluster with the Pacific trout group (topology I). Morphology (Smith and Stearley, 1989) and SINEs (Takasaki et al., 1996) data suggest that masu salmon forms a cluster with A- and B- lineages of Pacific salmons and diverged first (topology II). Morphology (Hikita, 1962) data suggest that masu salmon forms a cluster with A-lineage of the Pacific salmon group (topology III). D-loop sequences of mtDNA (Shedlock et al., 1992) suggest that masu salmon forms a cluster with the B-lineage of the Pacific salmon group (topology IV). RFLP of mtDNA (Thomas et al., 1986), *Hin*dIII fragment sequences of mtDNA (Thomas and Beckenbach, 1989), and ATPase 6 and ND 3 sequences of mtDNA (Domanico and Phillips, 1995) do not contain masu salmon, therefore these results were not shown.

The phylogenetic relationship within the B-lineage of Pacific salmons (pink salmon, sockeye salmon, and chum salmon) is also not clear (Table 6B). Data based on morphology, allozyme, rDNA, and D-loop sequences of mtDNA suggest that pink salmon and sockeye salmon are sister species (topology I), while data on RFLP of mtDNA, ATPase 6 and ND 3 sequences of mtDNA (Domanico and Phillips, 1995), and SINEs suggest that pink salmon and chum salmon are sister species (topology II).

The phylogenetic tree constructed from the mtDNA reanalyses in this study (Fig. 3) indicated the same relation-

Table 6. Comparisons of various studies

A: The phylogenetic relationship among four groups

		Various studies*							
		1	2	3	4	6	6'	8	9
Topology I	((A,B),(M,P))			○	○				
Topology II	(((A,B),M),P)		○				○	○	○
Topology III	(((A,M),B),P)	○							
Topology IV	(((B,M),A),P)					○			

B: The phylogenetic relationship within the B-linage of the Pacific salmon group

		Various studies#								
		1	2	3	4	5	6	6'	7	8
Topology I	((pink,sockeye),chum)	○	○	○	○	○				
Topology II	((pink,chum),sockeye)						○	○	○	○
Topology III	((sockeye,chum),pink)									

A: A-linage of the Pacific salmon group, B: B-linage of the Pacific salmon group, M: masu salmon, P: The pacific trout group

* 1: morphology (Hikita, 1962), 2: morphology (Smith and Stearley, 1989), 3: allozyme electrophoresis (Utter et al., 1973), 4: RFLP of rDNA (Phillips et al., 1992), 5: RFLP of mtDNA (Thomas et al., 1986), 6: D-loop sequences of mtDNA (Shedlock et al., 1992), 6': D-loop sequences of mtDNA reanalysed (present study), 7: ATPase 6 and ND 3 sequences of mtDNA (Domanico and Phillips, 1995), 8: SINEs (Takasaki et al., 1996), 9: allozyme electrophoresis (present study)

ship with that estimated from the present allozyme data (Fig. 2). However, there are two differences between the previous results (Shedlock et al., 1992) and those of the present study. (1) Masu salmon is clustered with other Pacific salmons and is not clustered with only B-lineages of the Pacific salmon group (tree II). This result is supported by morphology (Smith and Stearley, 1989) and SINEs (Takasaki et al., 1996) data. (2) Pink salmon and chum salmon are clustered (topology II). This result is supported by two other mtDNA studies (Thomas et al., 1986; Domanico and Phillips, 1995), therefore the consistency of all mtDNA data was confirmed. This result is also supported by SINEs data. SINE insertions appear to be irreversibly therefore provide an informative markers of evolution (Murata et al., 1993).

In the previous study (Shedlock et al., 1992), they used all 9 salmonid fish (including Atlantic salmon) and Arctic grayling (*Thymallus arcticus*: this belongs to a different subfamily) to obtain the simultaneous alignment. They also considered only transitional and transversional substitution ratio to construct maximum parsimony and maximum likelihood trees. However we used the profile alignment procedure and excluded Arctic grayling to obtain a more optimal alignment. We also used a suitable corrected distance matrix method to consider transitional and transversional substitution ratio and the rate of substitution varies from site to site and some parallel nucleotide changes, multiple substitutions, or back substitutions. As a result, a more optimal relationship among salmonid fishes seemed to be obtained.

In conclusion, the following consistent results were obtained by our electrophoretic data and mtDNA analysis; (1) all the Pacific salmon species form a monophyletic cluster, (2) chum salmon and pink salmon are clustered within those Pacific salmons, and (3) masu salmon forms a cluster with other Pacific salmons and diverged first in this group. All of these results are consistent with SINEs data (Kido et al., 1991; Murata et al., 1993; Takasaki et al., 1996).

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