Phylogenetic analysis of Pacific salmon (genus *Oncorhynchus*) using nuclear and mitochondrial DNA sequences

M.J. Domanico, R.B. Phillips, and T.H. Oakley

Abstract: Recent phylogenetic analyses of Pacific salmon of genus *Oncorhynchus* based on sequences from mitochondrial DNA and one nuclear growth hormone intron supported two subgroups:one with chinook (*O. tshawytscha*) and coho (*O. kisutch*) salmon and the other with pink (*O. gorbuscha*), sockeye (*O. nerka*), and chum (*O. keta*) salmon. In the latter group, a sister relationship was indicated between pink and chum salmon. Previous studies based on morphological and allozyme data had suggested a closer relationship between pink and sockeye salmon. In this paper we present a combined analysis of 4365 base pairs aligned sequence from nuclear and mitochondrial genes including new sequence data from the internal transcribed spacers (*ITS1* and *ITS2*) of the nuclear ribosomal DNA, the sequences of three growth hormone introns (*GH1C*, *GH2C*, *GH2D*) and the sequences of the *ATPase6* and *ND3* genes of the mtDNA. Phylogenetic analysis of the combined data set gives strong support to a close relationship between pink and chum salmon and between coho and chinook salmon.

Résumé : Des analyses phylogénétiques récentes du saumon du Pacifique du genre *Oncorhynchus* fondées sur des séquences d'ADN mitochondrial et d'un intron d'une hormone de croissance nucléaire ont révélé l'existence de deux sous-groupes : un qui comprend le saumon quinnat (*O. tshawytscha*) et le saumon coho (*O. kisutch*) et l'autre comprenant le saumon rose (*O. gorbuscha*), le saumon sockeye (*O. nerka*) et le saumon kéta (*O. keta*). Dans ce dernier groupe, une relation soeur a été observée entre le saumon rose et le saumon kéta. Des études antérieures fondées sur des données morphologiques et des données sur les allozymes avaient laissé entendre une relation plus étroite entre le saumon rose et le saumon sockeye. Dans le présent article, nous présentons une analyse combinée d'une séquence de 4365 paires de bases alignées provenant de gènes nucléaires et mitochondriaux, y compris des données sur la nouvelle séquence provenant des espaceurs transcrits internes (*ITS1* et *ITS2*) de l'ADN ribosomique nucléaire, les séquences de trois introns d'hormone de croissance (*GH1C*, *GH2C*, *GH2D*) et les séquences des gènes *ATPase6* et *ND3* de l'ADNmt. L'analyse phylogénétique de l'ensemble de données combinées vient appuyer fortement l'existence d'une relation étroite entre le saumon rose et le saumon kéta et entre le saumon coho et le saumon quinnat.

[Traduit par la Rédaction]

Introduction

The salmonid genus *Oncorhynchus* includes chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), Asian masu salmon (*O. masou*), pink salmon (*O. gorbuscha*), chum salmon (*O. keta*), sockeye salmon (*O. nerka*), and trouts native to the North Pacific that were formerly included in the genus *Salmo* (Smith and Stearley 1989). Evolutionary relationships among *Oncorhynchus* species have been studied by comparing morphologies (Stearley 1992), allozymes (Utter et al. 1973), mitochondrial DNA (mtDNA) (Thomas et al. 1986;Thomas and Beckenbach 1989; Shedlock et al. 1992; Domanico and Phillips 1995), short interspersed repetitive elements (SINES) (Murata et al. 1993, 1996), ribosomal DNA (rDNA) restriction fragment length polymorphisms (RFLPs) (Phillips et al.1992), the nucleotide sequence of the D intron of growth hormone 2 (*GH2D*) (McKay et al. 1996) and mitochondrial DNA (mtDNA) sequence data (Domanico and Phillips 1995). Most of these studies found Asian masu salmon to be intermediate between the rainbow trout and other Pacific salmon species. Among Pacific salmon, two subgroups usually contained coho and chinook salmon, and pink, sockeye, and chum salmon, respectively.

Different data sets have supported different relationships among pink, sockeye, and chum salmon (reviewed in Smith 1992). Allozymes (Utter et al. 1973), rDNA RFLPs (Phillips et al. 1992), mtDNA D-loop sequences (Shedlock et al. 1992), and morphology (Stearley 1992) suggest that pink and sockeye are sister species. Life histories (Smith and Stearley 1989), mtDNA RFLPs (Thomas et al. 1986), mtDNA sequence data from two protein-coding genes (*ND3* and *ATPase6*) (Thomas and Beckenbach 1989; Domanico and Phillips 1995), nuclear repetitive elements or SINEs (Murata et al. 1993), and nuclear sequence data from the *GH2D* intron (McKay et al. 1996) suggest an alternative relationship in which pink and chum are sister species.

To resolve conflicting trees for Pacific salmon and to compare relationships suggested by nuclear DNA data with those indicated by mtDNA, we sequenced the internal transcribed spacers (*ITS1* and *ITS2*) of the nuclear rDNA. The *ITS1* and *ITS2* were chosen for this analysis because they are evolving rapidly compared with other nuclear regions (reviewed in Gerbi 1985). The spacers are located within the rDNA repeat

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Fig. 1. Aligned DNA sequence of the first internal transcribed spacer (*ITS1*) of ribosomal DNA for seven *Oncorhynchus* species. Sites in common with the first sequence are indicated by periods, gaps are indicated by minus signs, and phylogenetically informative sites are indicated by plus signs. (Informative sites in bases 547–586 were ignored in the analysis because alignment of this region was problematic.)

with *ITS1* between the 18S and 5.8S and *ITS2* between 5.8S and 28S RNA genes. Phylogenetic analysis of *ITS* sequences has clarified phylogenies of a number of different species including fishes of the genus *Salvelinus* (Pleyte et al. 1992; Phillips et al. 1994), primates (Gonzales et al. 1990), and several invertebrates (Vogler and DeSalle 1994; Fritz et al. 1994).

We have obtained additional nuclear sequence data from two growth hormone introns, *GH1C* (Oakley 1996) and *GH2C* (Domanico 1994) and combined these with sequences of the *GH2D* intron (McKay et al. 1996). In this paper we present results of a combined phylogenetic analysis of all of the DNA sequence data currently available (4365 bp) from Pacific salmon using rainbow trout as an outgroup.

Materials and methods

Materials and extractions

Fish samples used for determination of ITS sequences were obtained from the following sources: rainbow trout (Evergreen Hatchery, Pound, Wisconsin) chinook salmon (Warm Springs Hatchery, Washington), coho salmon (Juneau, Alaska), Asian masu salmon (Japan), chum salmon (Juneau, Alaska), sockeye salmon (Bristol Bay, Alaska), and pink salmon (Juneau and Prince William Sound, Alaska). DNA was extracted from the liver tissue of two fish from each sampling area, except for masu salmon where only one fish was used. Either the Stratagene kit or phenol was used to extract DNA (Sambrook et al. 1989).

Amplification and sequencing of the rDNA spacers

The region of rDNA spanning the *ITS1* and *ITS2* and including the 5.8S gene were amplified together as one fragment using the polymerase chain reaction (PCR) (Saiki et al. 1988). The 18S rDNA sequence from *Xenopus laevis* (Salim and Maden 1981) was used to design the forward primer (5′ CTTGACTATCTAGAGGAAGT 3′) and the 28S sequence from *Xenopus laevis* (Ware et al. 1983) was used to design the reverse primer (5′ ATATGCTTAAATTCAGCGGG 3′).

Amplifications of rDNA were carried out as described in Phillips et al. (1995*b*) using a 5′ biotinylated reverse 28S primer to facilitate production of single stranded DNA. Dynabeads M-280 streptavidin (Dynal, Inc.) were used according to the manufacturer's instructions for separating double-stranded PCR product into single-stranded DNA for use in the sequencing reactions.

Dideoxy sequencing reactions (Sanger et al. 1977) were done using Sequenase version 2.0 (U.S. Biochemical) according to the manufacturer's instructions. Sequencing primers used for sequencing the *ITS1* region were KP2 and 5.8S (Phillips et al. 1995*b*). The KP2 primer was used to sequence the 5′ portion (18S side) of the *ITS1* and the 5.8S primer was used to sequence the complimentary strand to obtain the sequence of the 3′ portion of the spacer. Nucleotide sequences of both strands were determined with an overlapping region of approximately 100 base pairs. The sequencing primer for the *ITS2* was 5′ CTACGCCTGTCTGAGTGTC 3′, which was designed from the 5.8S gene of rainbow trout (Nazar and Roy 1978) and was used to sequence the entire *ITS2*. Sequences were aligned using CLUSTALV (Higgins et al. 1992) and adjusted by eye. Percent sequence divergences were determined by calculating the absolute genetic distances with the PAUP program (Swofford 1993) and genetic distances calculated using the Jukes–Cantor method with the MEGA program (Kumar et al. 1993).

Phylogenetic analysis of rDNA sequences

The most parsimonious trees were determined using the exhaustive search option and majority rule consensus trees were constructed using the branch and bound method of PAUP (Swofford 1993) with 500 bootstrapped replications. Gaps were analyzed in two ways: (*i*) by treating gaps as missing data and (*ii*) by treating gaps as missing data and adding a separate binary character matrix based on presence or absence of each indel. Rainbow trout and Asian masu salmon were used as outgroups. For the final analyses a region of uncertain homology between bp 547 and 586 in the *ITS1* was excluded. This did not affect tree topology but increased character fitness statistics.

Combined analysis of nuclear and mitochondrial DNA sequences

For the combined analysis of all DNA sequences, gaps were treated as missing data. The most parsimonious (MP) tree was calculated using the exhaustive search method of PAUP. A majority rule consensus tree was also produced using the branch and bound method of PAUP with 500 bootstrapped replications. Jukes–Cantor distances were calculated and a phenogram constructed using the neighbor joining aligorithm of MEGA (Kumar et al. 1993) with 500 bootstrapped replications.

Results

Analysis of *ITS1* and *ITS2* sequences

Nucleotide sequences of the rDNA spacers (*ITS1* and *ITS2*) from seven *Oncorhynchus* species gave a final data set of 971 aligned base pairs, with *ITS1* containing 593 bp (Fig. 1) and *ITS2* containing 378 bp (Fig. 2).

When gaps were counted as missing data, there were 99 variable sites with 37 being phylogenetically informative. Percent sequence divergences ranged from 1.62 between pink and chum salmon to 8.29 between pink and chinook salmon (Table 1). The transition/transversion ratio averaged 1.06 (Table 2). Trees were rooted assuming masu salmon and rainbow trout as outgroups. One best cladogram of 49 steps was produced using PAUP (Fig. 3) with uninformative characters excluded. Chinook and coho cluster as sister species. Pink, sockeye, and chum form a clade and pink and chum show a sister relationship. A second best cladogram at 50 steps had the same topology except that chinook and coho were on separate adjacent branches

There were 79 indel characters (assuming contiguous gaps as single characters). Thirty five of the indel characters were phylogenetically informative. Combining these indel characters with the substitution matrix produced a single most parsimonious tree consistent with Fig. 3 only with higher bootstrap values.

A phylogenetic tree was also produced using the neighbor joining method of the MEGA program (Kumar et al.1993) with Jukes–Cantor distances (Table 3). This tree showed the same topology as those produced using the parsimony method of PAUP (Swofford 1993).

Combined nuclear and mitochondrial DNA sequence analysis

Data from three growth hormone introns (Devlin 1993; Du et al. 1993; Forbes et al.1994; Domanico, 1994; McKay et al.

```
                                          +++   +                       60
sockeye ACGGGTTGCC AGCCGCCGGC ATGGGGCTGA GGCT--CCAA AATCCAGCTA AGCTGCGGG-
chum    .......... .......... .......... .......... .......... T.........
pink    .......... .......... .......... .......... .......... T.........
coho    .......... .......... .......... .CGC...G.. .......... T.........
chinook .......... .......... .......... .CGCAC.A.. .......... T.........
masu    .......... .......... .......... .CTC...G.. .......... T.........
rainbow .......... ...T...... .......... .CAC...A.. .......... T........G

                                       +                               120
sockeye TTGGGTAGGG TA-GGGGGCT CACGCCTCCC GCCTCTCCCT TCTCCCGGCG CGGGTGTCAT
chum    .......... ..A....... .......... -......... .......... ...-......
pink    .......... .......... .......... .......... .......... ..........
coho    .......... .......... .......... .......... .......... ..........
chinook .......... .......... .....-...T .......T.. .......... ..........
masu    .......... .......... .......... .......... .......... ..........
rainbow .......... .......... .........T .......... .......... ..........

                                 +                                     180
sockeye CGGTCCTAGC CCGCTTCCCC GCAT-CCCCC -TTTGCCTGG GATGTGCC-G ACTGGCTCCA
chum    .......... .......... ....T..... -......... .......... ..........
pink    .......... .......... .......... -......... .......... ..........
coho    .......... .......... ....T..... C......... ........C. ..........
chinook .......... .......... ..T.T..... C......... ........C. ..........
masu    .......... .......... ....C..... C......... ........C. ..........
rainbow .......... .......... ....C..... C......... .......... ..........

                                 +                                     240
sockeye TCCCCTTTCC CCATTAGC-A CGGCTGCATG ACTCACCTAT GTGGCGGGTG GAGAGGCCGC
chum    ....--..-- --......C. .......... .......... .-........ ..........
pink    .......... ........C. .......... .......... .-........ .....CG...
coho    .......... ........C. ...T...... .......... .-........ ..........
chinook .......... ........C. ...T...... .......... .-........ ..........
masu    .......... ........C. .......... .......... .-........ ..........
rainbow .......... ........C. .......... .......... .-........ ..........
                                                                        300
                                +                                      ++++
sockeye TACCGAAGGA CTGGGGGTGT -CAGTGAACC GGGACTTTCC  AAAATGGTCT AACACTGACG
chum    .......... .....--... ........-. .......-..  .......... .T........
pink    .......... .......... .......... .......... ........... ...G......
coho    .......... .......... C.G....... .......C..  .......... ........T.
chinook .......... .......... C.G....... -......-..  .......... .......TT.
masu    .......... .......... ..G....... .......-..  .........C ....-.CTTA
rainbow .......... .......... ..G....... ..........  .......... ....-.CTTA

                                                                       360
sockeye TAAGCGGCTT TAGTATCGCC -AGTATCCTC GCGCGGCACT GG-A-CCCAG TCAACT-CTC
chum    ......-.. .......... .........T .......... .....A.... ......T.--
pink    ......... .......... .......... .......... .....A.... ......--..
coho    ......... .......... .......... .......... ...G.A..-.. ......T...
chinook ......... .......... .......... .......... ...G.A..-.. ......T...
masu    .....A... ..TT..C... .C........ .......... ...G.A.... ......T...
rainbow ......... .......... .C........ .......... ...G.A.... ......T...
                                                                       420
                    +             +                  +
sockeye TGCGCCCGGC GTAGGTGGGG GTT-AATGTC TGCGCGGCTT CACCGGCGCT TCGGCGACGA
chum    -----.... ..C....... .-..C..... ..-........ ......-... ..........
pink    .CT...... ..C....... .-..C..... ...-....... .......... ..........
coho    ......... .......... ....C..... ........... .CTT...... ..........
chinook ......... .......... .-..T..... .........A. AC........ ..........
masu    ......... .......... ....T..... ........... .......... ........--
rainbow ......... ..C....... ....T..... ..........C .......... ........--

        +    +                                           (1) +     480
sockeye ---CGCA-GC GCAGACTCCC GGAAGCCTCC CTATTCTTAA ACCTT----- --GTCTTTGA
chum    TGA..T.... ....-..... .......... .......... .....TGTCT TT....C...
pink    TGA..T.... ....-..... .......... .......... .......... ..........
coho    GGA....A.. ....-..... .......... .......... .......... ..........
chinook GGA....A. .....-..... .......... .......... .....T.... ..........
masu    --A...... .A...-..... .......... .......... ....-T.... .T....C...
rainbow --A...... .....-..... .......... .......... .....T.... ..........

                   +    +         +                          +           540
sockeye ACCATGGCCT -GCGTATTGC G---AAGGGC GGGTGGGGGA AA-GGAGGGT AACCTCCCAA
chum    ..-......  T...CTCT-- .GCC.AGT.. A......... .......... .........-
pink    ..-......  T...CAGGAG .GGC.GAT.. ...... ..T .........C .........-
coho    .-T......  G...CTC.-- .GCT...T.. ......A... .......... ........C.
chinook ..-......  G...CTC.-- .GC-...T.. .......... .......... ..........
masu    G.T-.....  G-..CTC.-- .GACT..T.. .......... ..A......C ..........
rainbow ..T......  G-..CTC.-- .GTGT..T.. .......... ..A....... C...-.....

                ++  +++   +      +               +               593
sockeye TCTCTGCCCA GCCACTGAGC CTCT-GCGTG C--AATGAAA AA---CAATA ATA
chum    ........TT .ATG..-G.. ...A...... .TT...C... ....C...G. G..
pink    ....... T- .ATG..T-..  .........  --....... ..A......  G..
coho    ....... TC ..TG-.-G.. ...A...... .GT ...... ..AAC....  G..
chinook ....... -G A.....-G.. .......... .G--....G. .-..-..... G..
masu    ....C..T.T .T.....A.. ....T..... .TT-..A.G. ..A..T.... G..
rainbow .......... ......-G.. .......... .G--...... .......... G..
```

Fig. 2. Aligned DNA sequence of the second internal transcribed spacer (*ITS2*) of ribosomal DNA for seven *Oncorhynchus* species. Sites in common with the first sequence are indicated by periods, gaps are indicated by minus signs, and phylogenetically informative sites are indicated by plus signs.

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                                                                       60
sockeye TGTCATCAAT CGGAACCTCT -GGTTTCCGC AGCTGGGGCA GT-GCAGGCG GCCACCGTGC
chum    .......... .......... G......... .......... ..C....... ..........
pink    .......... .......... G......... .......... ..C....... ..........
coho    .......... .......... G......... .......... ..C....... ..........
chinook .......... .......... G......... ..A....... ..C....... ..........
masu    .......... .......... .......... .......... ..C....... ..........
rainbow .......... .......... .......... .......... ..C....... ..........

                                                       +++            120
sockeye AGCCTTCGTC CCCCTAAGTT CAGACCAGGA CGGCTCGGTG GGGTT-GTTG AGGATGAGCT
chum    .......... .......... T......... .......... ..T....... ..........
pink    .......... .......... A......... .......... ..T....... ..........
coho    .......... .......... .......... .......... ..TAGC.... ..........
chinook .......... .......... .......... .......... .TTAGC.... ..........
masu    .......... ...-...... .......... .......... ..T....... ....G.....
rainbow .......... ...-...... .......... .......... ..A.G..... ..........

                                                                +     180
sockeye TCTG-CTCTA CTTCCG-A-T CCCCCGTGCG CTCTTCCTTT CCCTT-TCGC TTCG-CGAGA
chum    .......... .....GG..T .......... .......... .......... ....-.....
pink    .......... ......-..T .......... .......... .....C.... .C..-.....
coho    ....G..... .C....CT.T .......... .......... .....C.... .C..-.....
chinook .-..G..... .C....C-.T .......... .......... .....C.... .-..G.....
masu    .-..G....  ..-...C-.T .......... .......... .....C.... ....-.....
rainbow .-..G..... .C........ .......... .......... .....C.... ....G.....

                                                                 +    240
sockeye GGCGCCCACG TTCCCCGCAT GGTCTGGCGC GGCTGCCGGT GGACTCT-GT CTGTCCGTGC
chum    .......... .......... .......... .......... .......-.. ..C.......
pink    .......... .......... .......... .......... .......-.. ..C.......
coho    .......... .......... .......... .......... .....T.T.. ..C.......
chinook .......... .......... .......... .........- -AG....T.. ..T.......
masu    .......... .......... .......... .......... .......T.. ..T.......
rainbow .......... .......... .......... .......... ....A..T.. ..T.......

                 +        +                                 +         300
sockeye TGCCCGTGTT ACGCATGTGG TTCTCGGGGT AGCGCTCGGG GTTAGGTTAG GGCTGCGGAG
chum    .......... .......C.. .......... .......... ........T. ..........
pink    .......... .......C.. .......... .......... ........T. ..........
coho    .........A .......... .......... .......... .......... ..........
chinook .-.......A .........- .......... .......... .......... ..........
masu    .........A .......... .......... .......... .......... ...C......
rainbow .........A C......... .......... .......... .......... ...G......

                                                        +             360
sockeye CTCCGACCGC CGACCTGAAA CGTAATTGAG AAGGTGAGCC GGGCAACCGG CCACAATCCA
chum    .......... .......... ...-...... .......... .......... ..........
pink    .......... .......... ...-...... .......... .......... ..........
coho    ........A. .......... ...-...... .......... .......... ..........
chinook .....G-... ..T....... .......... .......... .......... ..........
masu    .......... .......... ...-...... .......... ....G..... ..........
rainbow .......... .......... .......... .......... ....G..... ..........

                        378
sockeye TTCACTTTGA CTACGACC
chum    .......... ........
pink    .......... ........
coho    .......... ........
chinook .......... ........
masu    .......... ........
rainbow .......... ........
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1995; Oakley 1996) are available for all North American Pacific salmon. Growth hormone intron sequences (*GH1C*, *GH2C*, *GH2D*), rDNA sequences (*ITS1* and *ITS2*), and mitochondrial protein coding sequences (*ATPase6* and *ND3*) were combined into one data set, which contained 4365 aligned base pairs. Sequence alignments will be presented elsewhere and are available from the authors. Aligned sequences were analyzed using maximum parsimony (PAUP; Swofford 1993) with the indels considered missing data. The most parsimonius tree was consistent with the most parsimonius trees obtained with the rDNA spacers and mtDNA data. Bootstrap values were higher for the combined analysis (Fig. 4). This tree, based on combined data, had 234 steps (ignoring uninformative characters), with the next most parsimonious tree having 251 steps. There were a total of 143 informative sites (Table 4), with 35 synapomorphies between pink and chum salmon; 43 between pink, chum, and sockeye; and 26 between chinook and coho salmon.

Table 1. Absolute distance of the *ITS1* and *ITS2* between seven *Oncorhynchus* species generated using PAUP.

	Sockeye	Chum	Pink	Coho	Chinook	Masu	Rainbow
Sockeye	—	0.0405	0.0324	0.0649	0.0600	0.0541	0.0514
Chum	15	—	0.0162	0.0610	0.0795	0.0725	0.0750
Pink	11	6	—	0.0649	0.0829	0.0750	0.0778
Coho	24	25	24	—	0.0231	0.0625	0.0575
Chinook	21	31	29	9	—	0.0500	0.0342
Masu	20	29	27	25	19	—	0.0300
Rainbow	19	30	28	23	13	12	—

Note: All alignment gaps were treated as missing data. Number of differences are given below the diagonal. Percent sequence divergences are given above diagonal.

Table 2. Transition to transversion ratios for the *ITS1* and *ITS2* DNA sequences for seven *Oncorhynchus* species.

	Sockeye	Chum	Pink	Coho	Chinook	Masu	Rainbow
Sockeye	—	1.3912	0.5847	0.9450	0.7060	1.2224	0.8326
Chum	0.6519	—	0.7531	1.5748	1.454	2.0519	1.1528
Pink	0.2824	0.4103	—	0.9547	0.7824	1.1391	0.6718
Coho	0.3569	0.6166	0.3387	—	1.3503	1.5300	1.3562
Chinook	0.2516	0.4004	0.2589	0.6011	—	1.2224	0.8073
Masu	0.4343	0.7668	0.3873	0.5776	0.4343	—	1.2147
Rainbow	0.3062	0.4150	0.2374	0.5246	0.3178	0.5256	—

Note: Transition/transversion ratios are given above the diagonal, and standard errors are given below the diagonal.

Fig. 3. Majority rule consensus tree based on 500 branch and bound bootstrapped replications of *ITS1* and *ITS2* DNA sequence data (971 bp). All gaps were counted as missing data. Informative sites in the section from bp 547 to 586 were ignored in the analysis, since this section had multiple plausible alignments. Numbers indicate the percentage that each branch was retained in 500 samplings of the data (parsimony bootstrap percentages above each node, Jukes–Cantor/neighbor joining percentages below each node). Rainbow trout and masu salmon were used as outgroups and the tree was rooted with a basal polytomy.

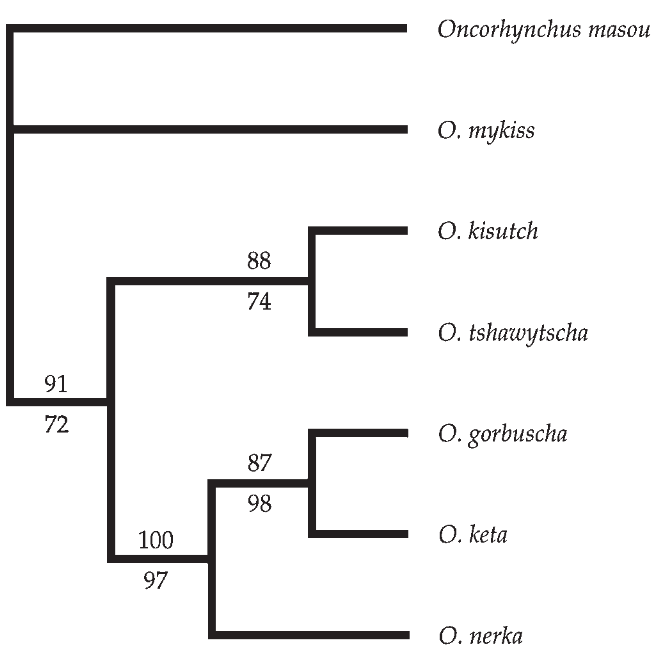

Fig. 4. Bootstrapped parsimony tree of the six *Oncorhynchus* species that represent the Pacific salmon using combined data set (4365 bp) of the sequences of the nuclear ITS1 and ITS2 rDNA, GH1C, GH2C, and GH2D and the mitochondrial *ND3* and *ATPase6* genes. *Oncorhynchus mykiss* (rainbow trout) was used as the outgroup. All alignment gaps were counted as missing data. Numbers indicate the percent that each node was retained in 500 bootstrap samplings of the data (parsimony bootstrap percentages above each node, Jukes–Cantor/neighbor-joining percentages below each node).

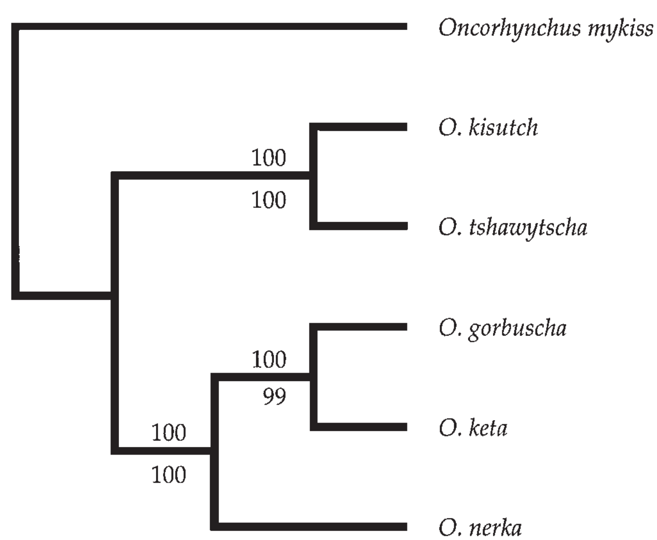

Table 3. Jukes–Cantor distances and associated standard errors among seven *Oncorhynchus* species based on combined data from *ITS1* and *ITS2* including 928 of 971 bp.

	Sockeye	Chum	Pink	Coho	Chinook	Masu	Rainbow
Sockeye	—	0.0231	0.0231	0.0356	0.0419	0.0406	0.0381
Chum	0.0053	—	0.0170	0.0343	0.0419	0.0406	0.0393
Pink	0.0053	0.0045	—	0.0406	0.0482	0.0444	0.0431
Coho	0.0066	0.0065	0.0071	—	0.0256	0.0386	0.0343
Chinook	0.0072	0.0072	0.0078	0.0056	—	0.0406	0.0331
Masu	0.0071	0.0071	0.0075	0.0068	0.0071	—	0.0268
Rainbow	0.0069	0.0070	0.0073	0.0065	0.0064	0.0057	—

Note: All alignment gaps were excluded in the distance measurement. Distances are given above the diagonal, and standard errors are given below the diagonal.

Table 4. Phylogenetically informative sites in the combined data set.

	Nuclear genes						Mitochondrial genes			
	ITS1	*ITS2*	*GH2C*	*GH2D*	*GH1C*	Total	*ND3*	*ATPase6*	Total	Grand total
Phylogenetically informative sites (PIS)	17	9	7	14	12	59	31	54	85	143
Total aligned sites (TAS)	555*	378	528	1327	714	3502	351	512	863	4365
PIS/TAS	0.031	0.024	0.013	0.011	0.017	0.017	0.088	0.105	0.098	0.033

*This is excluding a region of uncertain homology between bp 544 and 586 (see Fig. 1).

Table 5. Jukes–Cantor distances and associated standard errors among six *Oncorhynchus* species based on the combined data set of 4418 bp of nuclear and mitochondrial DNA sequence data.

	Sockeye	Chum	Pink	Coho	Chinook	Rainbow
Sockeye	—	0.0418	0.0379	0.0483	0.0449	0.0489
Chum	0.0034	—	0.0309	0.0540	0.0500	0.0546
Pink	0.0032	0.0029	—	0.0569	0.0506	0.0586
Coho	0.0037	0.0039	0.0040	—	0.0321	0.0503
Chinook	0.0035	0.0037	0.0038	0.0030	—	0.0427
Rainbow	0.0037	0.0039	0.0041	0.0038	0.0034	—

Note: All alignment gaps were excluded in the distance measurement. Distances are given above the diagonal, and standard errors are given below the diagonal.

Jukes–Cantor genetic distances were calculated (Table 5), and a tree was generated with the neighbor joining aligorithm (MEGA; Kumar et al. 1993) that had the same topology as the tree obtained using maximum parsimony.

Discussion

Molecular evolution of rDNA spacers and GH introns

The rDNA internal transcribed spacers provide excellent nuclear regions for investigating phylogenetic relationships among Pacific salmon. When alignment gaps were exluded from the analysis, the percentage sequence divergence varied from 1.62 to 8.29 between different taxa in the genus *Oncorhynchus*. Growth hormone introns appear to be evolving more slowly with maximum sequence divergence between introns of different species of 2.5%.

Intraindividual sequence variation has been shown to occur in the ribosomal internal transcribed spacers of tiger beetles (*Cincindela dorsalis*) (Vogler and DeSalle 1994) and lake trout (*Salvelinus namaycush*) (Zhuo et al. 1994) but not in the Pacific salmon. This difference may be due to the fact that the technique used in the present study was not as sensitive as cloning for finding polymorphisms, since sequencing was done directly from PCR products. However, if the polymorphisms were equally represented in the PCR product they would have been visible on the autoradiographs. Different sequence variants may be derived from rDNA repeats located on different chromosomes (Vogler and DeSalle 1994). The lack of intraindividual polymorphism in rDNA in Pacific salmon may be a consequence of the nucleolar organizer regions (NORs) in Pacific salmon being restricted to a single locus (Phillips et al. 1986), compared with lake trout, which has NORs at multiple chromosomal locations (reviewed in Phillips et al. 1989).

Phylogenetic analysis of DNA sequence data

The number of phylogenetically informative sites for the relationships among pink, chum, and sockeye for each part of the combined data set is shown in Table 4. There are fewer sites for the *ITS1* and *ITS2* than described earlier, because masu salmon was not included in the combined data. There were more informative sites in the data from mitochondrial genes than from nuclear genes, and the ratio of phylogenetically informative sites to total sites was twice as high for rDNA spacers as for growth hormone introns. Both nuclear data sets agree with those of mtDNA RFLPs (Thomas et al. 1986), mtDNA sequence (Domanico and Phillips 1995), SINEs (Murata et al. 1993), and life histories (Stearley and Smith 1993), which place pink and chum as sister species instead of pink and sockeye as shown by allozymes (Utter et al. 1973) and morphology

(Smith and Stearley 1989). With the nuclear sequence data and mitochondrial DNA data (Thomas et al. 1986; Domanico and Phillips 1995) in agreement, there is no support for introgression of mtDNA between pink and chum as suggested by Smith (1992).

The pink, sockeye, and chum lineages appear to have undergone rapid separation and evolved independently for some time, making the assignment of sister species difficult. Evidence that each of these species evolved independently for nearly equal times comes from sequence analysis of *GH2C* (Domanico 1994). Pink, sockeye, and chum salmon each had several unique polymorphisms, but there were very few phylogenetically informative sites that provided evidence for a sister relationship between any of these species. Rapid chromosomal evolution also occurred resulting in different karyoypes for each species ($2n = 52$ for pink, $2n = 57–58$ for sockeye, and $2n = 74$ for chum) (reviewed in Hartley 1987).

Reexamination of the data sets that produced trees supporting a sister relationship between pink salmon and sockeye salmon revealed that these trees were weakly supported. In the tree based on data from RFLPs of the of rDNA (Phillips et al. 1992), there was only one synapomorphy between pink and sockeye, and it was an *Apa*I site in the highly variable *IGS* region. *Apa*I recognizes CCCGGG, and sequencing of the spacer regions of the rDNA has revealed intraspecific variation in runs of Cs and Gs. In recent phylogenetic analyses utilizing RFLPs of rDNA, this enzyme has been eliminated (Phillips et al. 1995*a*). In the tree generated from allozyme data (Utter et al. 1973), 12 polymorphic loci were found among all species examined, and the only locus for which pink and sockeye shared a fixed allele was an undescribed muscle protein. In the cladogram based on morphology (Stearley 1992), four synapmorphies were listed for the pink and sockeye clade. Two of these are age-or size-specific, and two (number of gill rakers and tooth size) can be influenced by trophic adaptation.

In conclusion, analysis of the combined sequence data from mitochondrial DNA, nuclear rDNA spacers, and three nuclear growth hormone introns strongly supports a sister relationship of pink and chum salmon as well as between coho and chinook salmon.

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