Phylogeographic analysis of mitochondrial DNA variation in Alaskan coho salmon, *Oncorhynchus kisutch*

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Abstract—Mitochondrial DNA (mtDNA) haplotypes of coho salmon (*Oncorhynchus kisutch*) sampled from northern Pacific Ocean and Bering Sea drainages formed two monophyletic clades between which nucleotide divergences averaged 2.95 substitutions per 1000 nucleotides. These data were obtained from restriction endonuclease digestions of PCR products that included over 97% of the mtDNA genome and resolved 16 different haplotypes in 258 fish from 13 locations. Comparisons of haplotype compositions of populations indicated that the Bering Sea drainages and one Kodiak Island population clustered separately from nine other Gulf of Alaska populations, including one from Asia. Rates of gene flow among populations estimated from haplotype frequencies (assuming an equilibrium between gene flow and random drift) were low (about one female per generation between drainages within regions) in relation to allozyme-based estimates of gene flow for other Pacific salmon species. Much of the haplotype frequency variation was within-region variation. Haplotypes from both clades occur in many extant populations, suggesting that gene flow, population movements, or recolonization followed divergence of refugial isolates. Nested clade analysis of the geographic distribution of mtDNA haplotypes indicated that coho salmon demographic history has been influenced by recent isolation by distance and that historic population fragmentation was preceded by range expansion. These observations are consistent with effects expected from Pleistocene glacial advances and retreats.

Manuscript accepted 19 March 2001.
Fish. Bull. 99:528–544 (2001).

In drainages flowing into the Gulf of Alaska and Bering Sea, coho salmon (*Oncorhynchus kisutch*) are the least numerous and their population structure the least understood of Pacific salmon species (*Oncorhynchus* spp.). Many populations spawn in late fall or winter in remote drainages that are difficult to access. Spawning populations are often small and separated widely (Sandercock, 1991). In larger rivers spawning adults may return to small, often transient, headwater streams. After emergence, fry and juveniles may move to rearing areas, usually much lower in the river (Sandercock, 1991), forming complex admixtures from spawning populations in large drainages.

Studies of allozyme variation have provided insight into the population structure of Pacific salmon species (e.g. Zhivotovsky et al., 1994, and references therein). Patterns of genetic variability often provide evidence of relationships between populations resulting from coancestry or gene flow, and genetic divergence among populations may be used for stock identification (Shaklee et al., 1999). In coho salmon, the low level of allozyme variation resolves relatively little population genetic structure (Reisenbichler and Phelps, 1987; Wehrhahn and Powell, 1987; Bartley et al., 1992; Pustavoit, 1995). This low level of allozyme variation is consistent with numerous spawning populations that have small effective sizes and low levels of gene flow, such as that of coho salmon.

Analysis of DNA variation adds dimensions of interpretation not possible with allozyme data (Avise et al., 1988; Avise, 1989). "Gene trees" for mitochondrial DNA (mtDNA) haplotypes are especially informative. The mitochondrial genome is transmitted (primarily) maternally (Gyllensten et al., 1991), and mtDNA is haploid and clonally inherited with no recombination. Consequently, mutations accumulate over time within a clonal mtDNA line or haplotype. Comparison of different haplotypes provides a basis for reconstruction of matriarchal genealogies. There is also evidence that mtDNA sequences may diverge (evolve) faster than many nuclear sequences (Brown et al., 1982). The extent of nucleotide divergence provides a temporal basis for comparing haplotype lineages. The rates of divergence of mtDNA have been roughly estimated for a number of species pairs by comparing observed nucleotide sequence divergence with fossil records that document the emergence of those species (Brown et al., 1979; Shields and Wilson, 1987 and references therein). Although applications of these molecular "clocks" are questionable when extended to species for which the fossil record is poor or missing, deductions about relative (as opposed to absolute) divergence times can be made from the extent of nucleotide change within a species. Furthermore, the geographic distri-

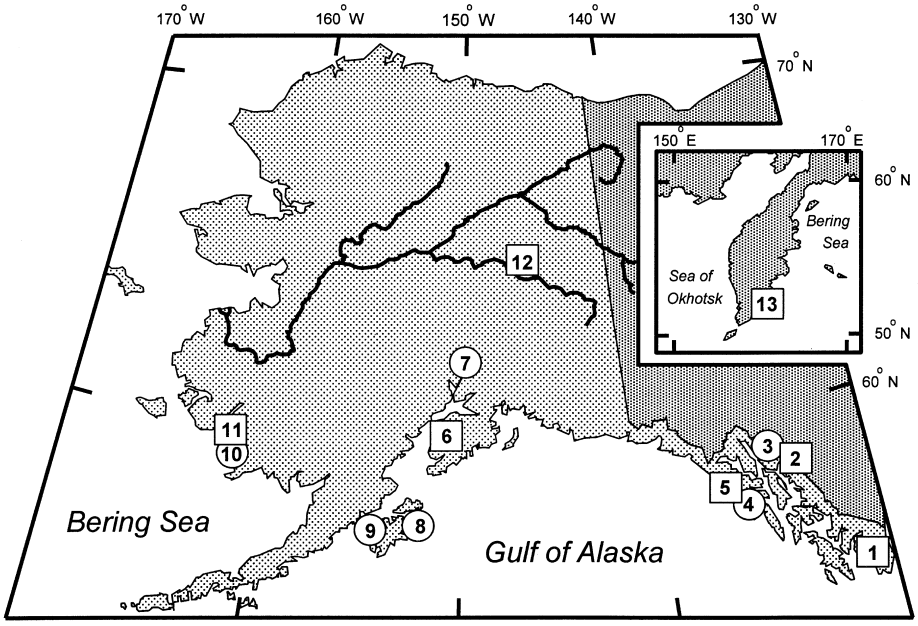

Figure 1

Sites from which coho salmon were sampled for mtDNA analysis. Squares denote samples used in both the preliminary and secondary analyses; circles denote collections used only for the secondary analysis. Locations are Hugh Smith River (1), Fish Creek, a Taku River tributary (2), Berner's River (3), Indian River (4), Ford Arm River (5), Crooked Creek (6), Little Susitna River (7), Buskin River (8), Karluk River (9), Eek River (10), Kanektok River (11), Delta Clearwater River, a Yukon River tributary (12), and Kamchatka River (13).

bution of haplotypes and their genealogical relationships can provide information about the historic demography and gene flow of a species. Templeton and colleagues (e.g. Templeton and Sing, 1993, Castelloe and Templeton, 1994; Templeton, 1998) have developed methods to examine both the shape of the "gene tree" and the geographic distribution of haplotypes, which they term "nested clade analysis of geographic distances."

The objectives of our study were to survey the geographic distribution of mtDNA variation in Alaskan coho salmon populations along the Gulf of Alaska and Bering Sea and to use that information and the mtDNA haplotype "gene tree" to deduce the nature of the historic demographic processes that influenced the contemporary geographic distribution of coho salmon.

Materials and methods

Coho salmon were sampled from 12 drainages in Alaska and one in Asia (Fig. 1). Samples of heart tissue from each specimen were preserved in 95% ethanol or a solution of 20% dimethyl sulfoxide (DMSO) and 0.25M ethylenediaminetetraacetic acid (EDTA) at pH 8, saturated with NaCl (Seutin et al., 1991).

Total genomic DNA was isolated by phenol-chloroform extraction (Wallace, 1987) or with Puregene DNA™ isolation kits (Gentra Systems Inc., Minneapolis, MN). Sequences were PCR-amplified using primers that targeted seven regions of the mtDNA genome in pieces that range from about 2115 to 2689 base pairs (bp) (Fig. 2, Table 1). The regions were designated ND3/ND4 (including genes for the NADH dehydrogenase-3 subunit and NADH dehydrogenase-4L and -4 subunit genes), ND5/ND6 (including genes for the NADH dehydrogenase-5 and -6 subunits), Cytb/D-loop (including the cytochrome *b* gene and the control region), 12S/16S (including 12S rRNA gene and most of the 16S rRNA gene), ND1/ND2 (including the NADH dehydrogenase-1 and NADH dehydrogenase-2 subunit genes), COI/COII (including most of the cytochrome oxidase I subunit gene and the cytochrome oxidase II subunit gene), and A8/COIII (including genes for the ATPase-8 and -6 subunits and the cytochrome oxidase III subunit gene). The seven mtDNA regions were amplified by denaturation at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C [0.2 mM of each dNTP, 0.2µ M of each primer, 2 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3 with 1 unit of Taq polymerase (Perkin Elmer, Norwalk, CT) in a 50-µL reaction], except that amplifications of regions A8/COIII and ND3/ND4 required 3 mM instead of 2 mM $MgCl_2$.

Subsamples of PCR products of each mtDNA region were digested with each of 12 restriction enzymes. The endonucleases recognized six bases (*Ase* I), multiple six-base sites (*Ava* I, *Hin*d II, *Sty* I), multiple 5 base sites (*Bst*N I), and four bases (*Bst*U I, *Cfo* I, *Dde* I, *Hin*f I, *Mbo* I, *Msp* I, *Rsa* I). Digestion reactions were carried out under conditions recommended by the manufacturers. The resulting frag-

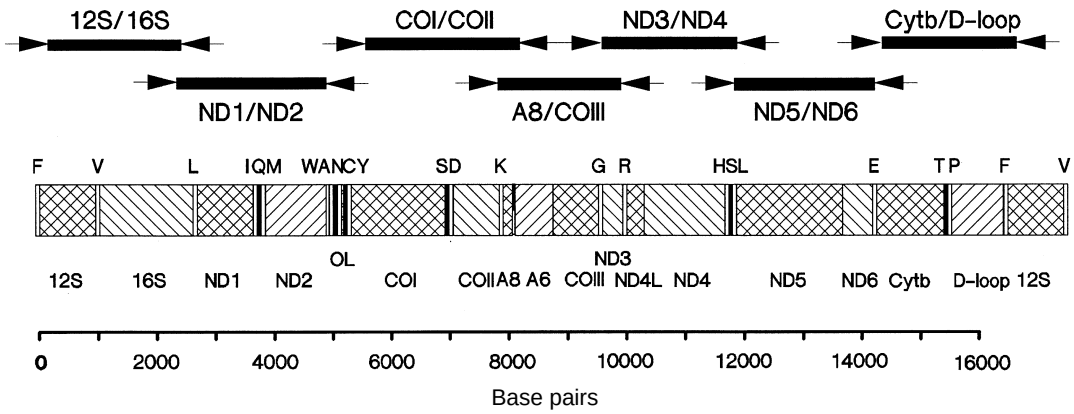

Figure 2

The gene map of linearized mtDNA and fragments that can be PCR amplified in coho salmon. The map is based on the sequence of *O. mykiss* mtDNA (Zardoya et al., 1995). More precise details of the range spanned by each primer pair are provided in Table 1.

Table 1

Primers for PCR amplification of coho salmon mitochondrial DNA (mtDNA), location in relation to *O. mykiss* (Zardoya et al., 1995), PCR fragment sizes, and fragment overlaps (bp).

Region	Sequence	*O. mykiss* locations	Fragment size	Overlap	Source
12S/16S	5′ AATTCAGCAGTGATAAACATT 3′	1234–1254			1
	5′ AGATAGAAACTGACCTGGATT 3′	3615–3635	2402		1
				121	
ND1/ND2	5′ ACCTCGATGTTGGATCAGG 3′	3515–3533			1
	5′ ATTAAAGTGNTTGA(T/G)TTGCATTC 3′	6181–6203	2689		1, 5
				–430	
COI/COII	5′TAATCGTCACAGCCCATGCCTTCGT 3′	6634–6658			2
	5′ GGTCAGTTTCAGGGTTCAGGTTTAGC 3′	9079–9104	2471		2
				166	
A8/COIII	5′ CTAGTGACATGCCCCAACTCAACC 3′	8939–8962			2, 3
	5′ TCATAAGGCGGTCATGGACTTAAACC 3′	11028–11053	2115		2, 3
				480	
ND3/ND4	5′ TTACGCGTATAAGTGACTTCCAA 3′	10574–10596			2, 3
	5′ TTTTGGTTCCTAAGACCAATGGAT 3′	12881–12904	2331		2, 3
				32	
ND5/ND6	5′ AACAGCTCATCCATTGGTCTTAGG 3′	12873–12896			2, 4, 5
	5′ TTACAACGATGGTTTTTCATGTCA 3′	15319–15342	2470		2, 4, 5
				19	
Cytb/D–loop	5′ TGAA(G/A)ACCACCGTTGTTATTCAA 3′	15324–15347			2, 4, 5
	5′ TAGGGCCTCTCGTATAACCG 3′	1321–1340	2659		2, 4, 5
				107	
12S/16S					

[1] Consensus from Anderson et al. (1981); Anderson et al. (1982); Bibb et al. (1981); Roe et al. (1985); Chang et al. (1994).
[2] Unpublished mtDNA sequence data from our lab.
[3] Thomas and Beckenbach (1989).
[4] Cronin et al. (1993).
[5] Carney et al. (1997).

ments were separated by electrophoresis through a 1.5% agarose gel (a mixture composed of one part Ultra Pure™ agarose [BRL Gibco, Grand, NY] and two parts Synergel™ [Diversified Biotech Inc., Boston, MA]) in 0.5×TBE buffer (TBE is 90 mM Tris-boric acid, and 2 mM EDTA, pH 7.5). DNA in the gel was stained with ethidium bromide and photographed on an ultraviolet light transilluminator. Digests that produced fragments too small for detection in agarose/Synergel™ gels were resolved in 12% polyacrylamide (29:1 acrylamide:bisacrylamide; 1×TBE) gels. DNA fragments separated in polyacrylamide gels were stained with SYBR Green 1 Nucleic Acid Stain™ (Molecular probes, Eugene, OR), which is more sensitive than ethidium bromide. Either a 1-kilobase (kb) ladder or *Hae* III digested $\phi\chi^{174}$ RF phage DNA (BRL Gibco, Grand, NY) was used as a molecular weight reference for estimating restriction fragment sizes.

Restriction sites were inferred from fragment patterns that could be related to each other by the gain or loss of a single site. Composite haplotypes were constructed from restriction fragment patterns of all restriction enzymes across all mtDNA PCR regions. Using the rules of Castelloe and Templeton (1994) to resolve ambiguities, we constructed the single most probable parsimonious tree depicting restriction site changes between haplotypes. Using REAP (McElroy et al., 1990), we estimated haplotype (nucleon) and nucleotide diversities within populations (Nei, 1987) as well as average nucleotide divergences between populations. Nucleotide divergence between populations takes into account both the haplotype frequency differences between populations and the nucleotide divergences between haplotypes (Nei and Tajima, 1983; Nei, 1987; Nei and Miller, 1990). Homogeneity of haplotype diversities among populations was tested by using the Monte-Carlo simulation in REAP (McElroy et al., 1990)(10,000 iterations; Hedges, 1992) to establish probability levels for goodness-of-fit statistics (Roff and Bentzen, 1989).

Populations were clustered from pair-wise nucleotide divergences by using the Fitch and Margoliash (1967) least-squares method (FITCH in PHYLIP; Felsenstein, 1995). For comparisons between populations, the precision of estimates of nucleotide divergence depends on sample size. Therefore, stability of the topology was examined by bootstrapping (2000 iterations; Hedges, 1992) over individuals within each collection. A consensus tree (CONSENSE in PHYLIP; Felsenstein, 1995) that shows the stability of the topology, but not the branch lengths, was generated from the set of bootstrapped trees.

The hierarchical structure of the expanded set of coho samples was analyzed by analysis of molecular variance (AMOVA; Excoffier et al., 1992) with Arlequin (Schneider et al., 1997). Collections were grouped geographically into four regions: Southeast Alaska, Southcentral Alaska, Bering Sea, and Asia. With appropriate choices of divergence matrices, the analysis can examine the structure from haplotype frequencies (e.g. Weir, 1996) or from nucleotide diversities based on paths between haplotypes traced through a haplotype tree (Excoffier et al., 1992). Significance (P_{MC}) of Φ-statistics (Excoffier et al., 1992) was estimated from distributions of the statistics generated by 17,000 permutations (Hedges, 1992) at the appropriate level of hierarchy.

Nested clade analysis of geographical distributions of haplotypes and subclades (e.g. Templeton and Sing, 1993; Castelloe and Templeton, 1994; Templeton, 1998) was conducted with GEODIS 2.0 (Posada et al., 2000).

Results

Our general approach was to conduct a broad preliminary survey to obtain genome-wide information for mtDNA restriction site variation. Subsequently, we focused on the variable restriction sites and examined larger sample sizes and additional populations. From those results we constructed a fine-scale mtDNA "gene tree" and analyzed the geographic distributions of mtDNA lineages to deduce the nature of the historical demographic changes that influenced present-day population structure. The approach also allowed us to determine the effects on estimates of molecular parameters that occur when analyses focus on variable restriction sites.

Diversities of coho salmon mtDNA

Ten coho salmon from each of seven drainages (Fig. 1) were analyzed to survey broadly the species' mtDNA variability using 12 restriction endonucleases (Appendix 1). The total number of restriction sites inferred from restriction fragment patterns was 298 (an average of 291.28 per haplotype), which corresponds to 1284 nucleotides (an average of 1254.80), or a maximum of 7.73% (an average of 7.56%) of the coho salmon mtDNA genome (Table 2). Sixteen sites (1.2% of the total) were variable. Individually, the regions averaged between 29 and 57 restriction sites, which correspond to a maximum of between 5.58% and 9.57% of the nucleotides in a region. Although the amplified regions had some overlaps (Table 1), no variable sites were observed in the areas of overlap; and no invariant sites were shared between regions, except possibly *Dde* I sites in the 408-bp overlap between A8/COIII and ND3/ND4 (Table 1). Because of the large total number of sites examined, a few overlapping *Dde* I sites would cause only a slight decrease in nucleotide diversity estimates and have little effect on nucleotide divergence estimates.

Restriction site variation was observed in five of the seven PCR-amplified mtDNA regions for the 12 restriction endonucleases used. Between zero and five variable sites were observed per region. No variation was detected in the A8/COIII and ND3/ND4 regions. The largest number of variable sites (5) and the highest level of nucleotide diversity (5.99 substitutions per 1000 base pairs) were observed in the ND5/ND6 region (Table 2).

Because there is no recombination between heterologous mtDNA molecules, the composite haplotype is the appropriate unit to consider in genetic analysis (e.g. Avise, 1989). Our preliminary survey discovered 11 haplotypes (Table 3). As a whole, the sample of 70 fish had a haplotype diversity of 0.803 and a nucleotide diversity of 1.70 substitutions per 1000 base pairs. Haplotype diversities within

Table 2

Number and variability of restriction sites observed in each of the seven mtDNA regions we examined using 12 restriction endonucleases in our preliminary survey.

Region	Fragment size	Mean number of sites	Mean number of nucleotides	% coverage	Number of variable sites	Number of haplotypes	Haplotype diversity (±SE)	Nucleotide diversity (per 1000)
12S/16S	2402	53.5	226.7	9.44	3	4	0.485 ±0.042	2.15
ND1/ND2	2689	41.5	184.7	6.87	3	4	0.485 ±0.042	2.65
COI/COII	2471	39.7	168	6.8	3	3	0.057 ±0.038	0.25
A8/COIII	2115	29	126.7	5.99	0	1	0	0
ND3/ND4	2331	31	130	5.58	0	1	0	0
ND5/ND6	2470	36.5	157.2	6.36	5	4	0.470 ±0.040	5.99
Cytb/D-loop	2659	57	248.7	9.35	2	3	0.670 ±0.014	1.87
Total	16,642	291.28	1254.8	7.54	16	11	0.803 ±0.024	1.70

Table 3

Observed numbers of each mtDNA haplotype, haplotype diversity, and nucleotide diversity (substitutions per 1000 bp) within collections of coho salmon examined in a preliminary survey of North Pacific Ocean coho salmon (Nei and Tajima, 1983; Nei ,1987). Standard errors are in parentheses. Homogeneity of haplotype frequencies ($P_{MC} < 10^{-4}$) was tested with using Monte-Carlo simulation based on 10,000 resampling iterations to estimate probability (Roff and Bentzen, 1989).

		A–D cluster						E–H cluster						
		Haplotype						Haplotype						
Collection	*n*	A	A′	B	C	C′	D	E	E′	F	G	H	Haplotype diversity	Nucleotide diversity
Hugh Smith River	10	3	0	0	4	1	0	2	0	0	0	0	0.78	1.31
Fish Creek (Taku River)	10	7	1	0	0	0	1	0	0	1	0	0	0.53	0.88
Ford Arm River	10	2	0	4	1	0	0	2	0	0	1	0	0.82	1.65
Crooked Creek	10	5	0	0	4	0	0	1	0	0	0	0	0.64	0.78
Eek River	10	0	0	0	4	0	0	4	0	0	0	2	0.71	1.87
Delta Clearwater River	10	0	0	0	0	0	0	0	0	0	0	10	0.00	0.00
Kamchatka River	10	5	0	0	4	0	0	0	1	0	0	0	0.06	0.94
Total	70	22	1	4	17	1	1	9	1	1	1	12	0.80	1.70
													(±0.024)	(±0.000)
Average													0.59	1.06
													(±0.11)	(±0.24)

collections from individual drainages ranged from 0.00 to 0.82 and nucleotide diversities ranged from 0.00 to 1.87 substitutions per 1000 bp. The distribution of haplotypes among collections was highly heterogeneous, which is noteworthy given the small sample sizes (n=10) (Table 3).

Phylogenetically, there were two clusters of haplotypes (haplotypes A–D and haplotypes E–H) (Fig. 3). Haplotypes of the two clusters differed by five or more restriction sites, and the average nucleotide divergence between individual haplotypes in the two clusters averaged 2.72 nucleotide substitutions per 1000 nucleotides, as compared to an average nucleotide diversity within each cluster of 0.87. The cluster of A–D accounted for the majority of fish, but haplotypes of both clusters were observed in collections from all drainages, except for fish from the Delta Clearwater River, which had only haplotype H. Bootstrap estimates of nucleotide divergence between clusters and average nucleotide diversity within clusters, estimated from the entire sample of 70 fish, were 2.98 ±0.06 and 0.35 ±0.05 (substitutions per 1000 nucleotides), respectively.

Expanded coho mtDNA survey based on variable sites

We increased the number of populations surveyed to 13 and increased sample sizes to 20, except for the Kam-

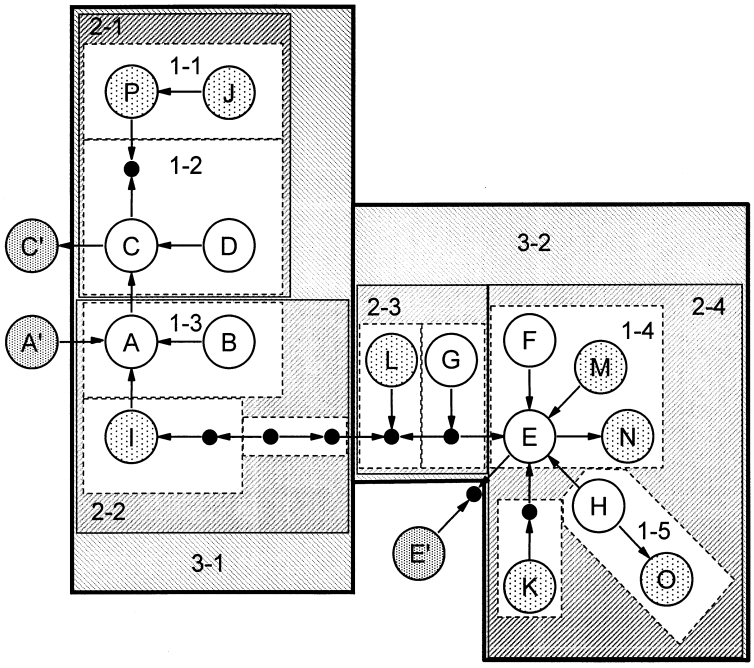

Figure 3

Haplotype tree for coho salmon mtDNA restriction site variation. Haplotypes A–H, including A′, C′, and E′, were observed in the preliminary survey (70 fish); haplotypes I–O were observed in the subsequent survey (188 additional fish); and haplotype P was detected in digests conducted to resolve positions on the tree of the latter haplotypes. Arrows indicate the direction of site gain. Ambiguities in the topology were resolved (Castelloe and Templeton, 1994) and the nested structure was constructed (Templeton and Sing, 1993). Haplotypes A′, C′, and E′ were pooled with A, C, and E, respectively, for the nested clade analysis. The results of the analysis are provided in Table 5.

chatka River (n=17) and Delta Clearwater River (n=21) populations. To make analysis of larger numbers of samples practical, we focused on restriction sites in five mtDNA regions that defined the most abundant eight (A–H) of the 11 haplotypes. Haplotypes A′, C′, and E′, each of which was represented by a single individual, were eliminated. In this survey, we analyzed site variation for the following PCR region by restriction endonuclease combinations:

12S/16S rRNA	Cyt b/D-loop	ND5/ND6	ND1/ND2	ND3/ND4
Cfo I	*Bst*N I	*Ava* I	*Dde* I	*Cfo* I
Dde I		*Dde* I	*Sty* I	

This survey detected 62 restriction sites (56.47 on average in each haplotype), which correspond to 262.67 nucleotides (238.22 on average). The proportion of the genome screened was a maximum of 1.58% (1.43% on average). To completely resolve the placement of haplotypes I, J, K, and L in the "gene tree," we digested their ND5/ND6 regions with *Mbo* I and their COI/COII regions with *Dde* I. An additional haplotype (P) was resolved. In total, the expanded survey resolved three additional haplotypes (I, J, and P) within the A–D clade and five additional haplotypes (K, L, M, N, and O) within the E–H clade (Table 4, Appendix 2, and Fig. 3). Haplotypes of both clusters appeared in most drainages. It is notable that four drainages included haplotypes of only a single clade: Delta Clearwater had 20 of haplotype H and one of the related haplotype O; Indian River had 19 of haplotype A and one of haplotype C; Berners River had five of hapotype A, 11 of haplotype C, one of haplotype I, two of haplotype J, and one of haplotype P; and the Little Susitna collection had four A haplotypes, 15 C haplotypes, and one I haplotype. Within drainages, haplotype diversities ranged from 0.10 to 0.73 and nucleotide diversities ranged from 0.22 to 9.07 substitutions per 1000 bp. The 13 collections exhibited strong heterogeneity ($P<10^{-4}$, Table 4).

In a Fitch-Margoliash phenogram that estimates relationships among drainages based on haplotype frequencies (Fig. 4), Delta Clearwater River differed strongly from the other collections, and the collections from systems that drain into the Bering Sea and from Karluk Lake on Kodiak Island, clustered separately. The remaining col-

Table 4

Observed numbers of each mtDNA haplotype, haplotype diversity, and nucleotide diversity (substitutions per 1000 bp) within collections of North Pacific coho salmon screened for variable sites detected in a preliminary survey (Table 3)(Nei and Tajima, 1983; Nei, 1987). Standard errors are in parentheses. Homogeneity of haplotype frequencies ($P_{MC} < 10^{-4}$) was tested by using Monte-Carlo simulation based on 10,000 resampling iterations to estimate probability (Roff and Bentzen, 1989).

		Haplotype																	
Collection	*n*	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Haplotype diversity	Nucleotide diversity
Hugh Smith River	20	6	0	12	0	2	0	0	0	0	0	0	0	0	0	0	0	0.57	3.64
Fish Creek (Taku River)	20	11	0	3	2	0	2	0	0	0	1	0	1	0	0	0	0	0.68	5.68
Berners River	20	5	0	11	0	0	0	0	0	1	2	0	0	0	0	0	1	0.64	2.40
Indian River	20	19	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0.10	0.22
Ford Arm River	20	10	6	1	0	2	0	1	0	0	0	0	0	0	0	0	0	0.68	4.51
Crooked Creek	20	9	0	10	0	1	0	0	0	0	0	0	0	0	0	0	0	0.57	2.50
Little Susitna River	20	4	0	15	0	0	0	0	0	1	0	0	0	0	0	0	0	0.42	1.13
Buskin River	20	5	0	12	0	3	0	0	0	0	0	0	0	0	0	0	0	0.58	4.69
Karluk River	20	1	0	9	0	5	0	0	0	0	0	1	0	0	4	0	0	0.73	9.07
Eek River	20	1	0	7	0	9	0	0	3	0	0	0	0	0	0	0	0	0.68	8.38
Kanektok River	20	5	0	8	0	5	0	0	1	0	0	0	0	1	0	0	0	0.75	7.91
Delta Clearwater River	21	0	0	0	0	0	0	0	20	0	0	0	0	0	0	1	0	0.1	0.2
Kamchatka River	17	9	0	7	0	1	0	0	0	0	0	0	0	0	0	0	0	0.58	2.7
Total	258	85	6	96	2	28	2	1	24	2	3	1	1	1	4	1	1	0.734	6.73
																		(±0.016)	(±0.00)
Average																		0.54	4.08
																		(±0.06)	(±0.83)

lections spanned the southern portion of the geographic range from southern Southeast Alaska to the Kamchatka Peninsula. Within that set of collections, the two coastal Southeast Alaskan collections (Ford Arm and Indian River) appeared to form a weak cluster, but there was no obvious structure among the remaining collections.

We conducted AMOVA analyses reflecting a geographical hierarchy: Southeast Alaska, Southcentral Alaska, western and interior Alaska (Bering Sea), and Asia to examine the geographic basis of variation. Although the Karluk River collection resembled Bering Sea collections more than other northern Gulf of Alaska collections, we included it with the Southcentral Alaska group to maintain the geographic basis of the analysis. Analyzing the data based on haplotype frequencies (analogous to allelic differences in analysis of variance described by Weir [1996]) revealed highly significant divergence among collections (Φ_{ST}=0.291, P_{MC}<0.0001), most of which is attributable to average divergence among drainages within a region (Φ_{SC}=0.227, P_{MC}≤0.0001), rather than differences between regions (Φ_{CT}=0.083, P_{MC}=0.094). Incorporating relationships between the haplotypes into the analysis increased the proportion of the total divergence observed among drainages (Φ_{ST}= 0.449, P_{MC}≤0.0001) and among drainages within regions (Φ_{SC}=0.273 , P_{MC}<0.0001). The estimate of the proportion of divergence among regions also increased (Φ_{CT}=0.242, P_{MC}=0.083). Estimates of long-term gene flow [$N_{e(f)}m=(\Phi_{XY}^{-1}-1)/2$] from Φ_{SC} and Φ_{CT} based on haplotype relationships were about 1 female per generation between collections within regions and about two females per generation between regions. Such estimates assume that an equilibrium between gene flow and random drift exists.

The nested clade analysis (Fig. 3, Table 5) collapses the "gene tree" from the periphery and analyzes each subclade for significantly small or large geographical distributions of the components as compared with the subclade as a whole. The first two levels of nesting are 1-step clades (nesting 0-step haplotypes) and 2-step clades (nesting 1-step clades). The significance in their geographic distributions were consistent with restricted gene flow with isolation by distance for haplotypes of the A–D (plus I, J, and P) subclade and past fragmentation for haplotypes of the E–H (less G plus M, N, and O) subclade. The 3-step clade (nested 2-step clades) that includes all of the E–H (plus L, M, N, and O) haplotypes also indicates past fragmentation. The most interior level of nesting, which contrasts the A group of haplotypes with the E group, assuming that the A group is interior (ancestral) based on the interpretations of Castelloe and Templeton (1994), is consistent with contiguous range expansion. We reanalyzed the data without the Delta Clearwater population, the Kamchatka population, and without either. These two geographically distinct populations did not alter the overall interpretation, but the Delta Clearwater population was responsible for significance of clade 2–4 and the Kamchatka population was responsible for the significance of clade 2–2.

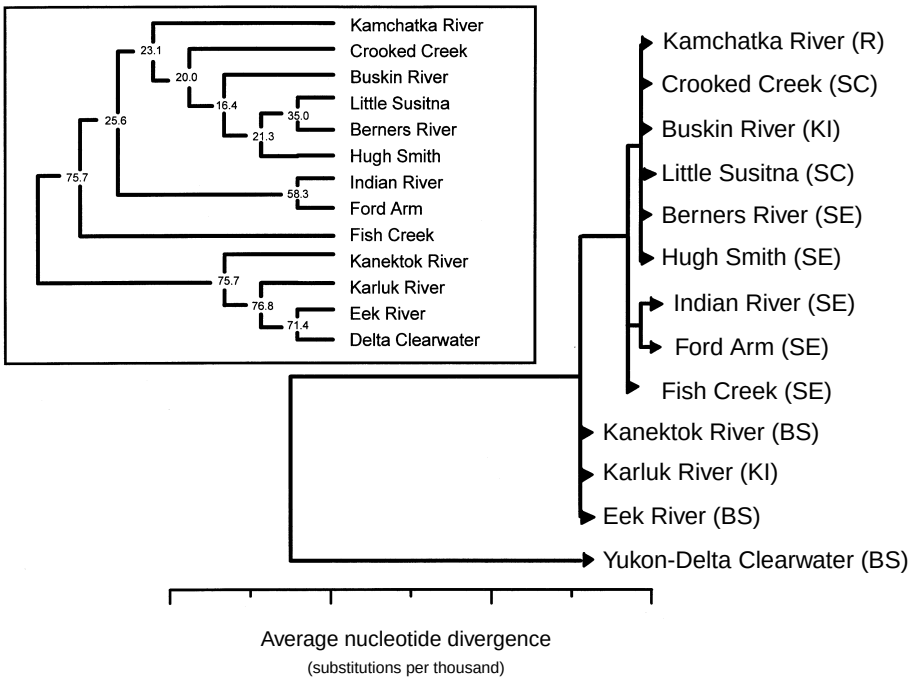

Figure 4

A Fitch-Margoliash least-squares phenogram constructed from nucleotide divergences among coho salmon collections and a consensus tree (inset) derived from 2000 iterations (Hedges, 1992) of bootstrap resampling from the individuals within each collection. Geographic regions for the samples are Russia (R), Southcentral Alaska (SC), Kodiak Island (KI), and Southeast Alaska (SE), and Bering Sea (BS), The consensus tree is unscaled and describes the confidence of the topology depicted in the phenogram.

Discussion

Coho salmon mtDNA variation

We examined the coho salmon mtDNA genome using restriction analysis of seven PCR products and detected fragments as small as 25 bp, which made it possible to sample nearly all (over 97%). of the mitochondrial genome. We are unaware of other fish species that have been as thoroughly surveyed. Our estimates of species-level parameters of molecular evolution should be quite robust in comparisons with those for other taxa, including fish.

Our results (Table 2) suggest that mtDNA variation is not evenly distributed throughout the genome and that focusing analysis on variable sites overestimates nucleotide diversity estimates (see Tables 3 and 4). Many previous studies of Pacific salmon species have limited the portion of the mtDNA genome surveyed (e.g. chum salmon [Cronin et al.; 1993; Park et al., 1993; Seeb and Crane, 1999]; sockeye salmon [Bickham et al., 1995; Taylor et al. 1996]; chinook [Cronin et al., 1993]). Other studies have included a limited geographic range of samples and have restricted the portion of the mtDNA genome studied (e.g. sockeye [Burger et al., 1997]; chinook [Adams et al., 1994]) or surveyed selected variable sites (e.g. pink salmon [Brykov et al., 1996; Seeb et al., 1999]; chum salmon [Scribner et al., 1998]; sockeye [Taylor et al., 1997]). Consequently, no previous studies have produced results that are appropriate for a broad comparison with our results.

We evaluated our results in the context of other fish species by plotting the effective number of haplotypes observed (the number of haplotypes which, if equally abundant, would result in the observed haplotype diversity, n_c, against the average nucleotide divergences between haplotypes for piscine species. For these comparisons, we used only data that were derived from 20 or more individuals and that surveyed the entire mtDNA genome (Fig. 5). Only two studies of Pacific salmon met those criteria; and our estimates of nucleotide diversity (0.4–4.5 per 1000 nucleotides) were generally lower than estimates for chinook salmon (6 haplotypes: 1.3–8.1; Wilson et al., 1987) and chum salmon (2 haplotypes: 2.4; Thomas et al., 1986).

Average nucleotide divergences between coho salmon haplotypes are quite low in relation to those of most other species studied; whereas, the effective number of haplotypes is near the median. The effective number of (selectively neutral) haplotypes (n_c) is monotonically related to the product of effective population number (of females

Table 5

Geographical nested clade analysis (Templeton, 1998) for Alaskan coho salmon mtDNA haplotpes based on the haplotype network in Figure 3 using GEODIS 2.0 (Posada et al., 2000). Only clades that exhibit significant results are shown. The analysis examines the geographical spread (D_C) of tip (T) and interior (I) subclades (Castelloe and Templeton, 1994), their relative spread within the clade (D_N), and contrasts their distrubutions (I–T) identifying distances that are significantly small (S), significantly large (L), or not significant (NS). Interpretations are based on Templeton's (1998) inference key.

1-step clades					2-step clades					3-step clades					4-step clades				
Clade	Sub-clades	Tip/ Interior	D_C	D_N	Clade	Sub-clades	Tip/ Interior	D_C	D_N	Clade	Sub-clades	Tip/ Interior	D_C	D_N	Clade	Sub-clades	Tip/ Interior	D_C	D_N
1-3	A	I	NS	NS	2-1	1-2	I	NS	NS	3-2	2-3	I	NS	L	4-1	3-1	I	S	S
	B	T	S	S		1-1	T	S	NS		2-4	T	S	S		3-2	T	NS	L
		I–T contrast	L	L			I–T contrast	L	NS			I–T contrast	NS	L			I–T contrast	NS	S
	Isolation by distance.					Isolation by distance.					Past fragmentation					Contiguous range expansion			
1-4	E	I	NS	NS	2-2	1-3	I	L	L										
	F	T	NS	L		I	T	NS	NS										
	M	T	NS	NS			I–T contrast	NS	NS										
	N	T	S	S		Islation by distance.													
		I–T contrast	L	NS															
	Past fragmentation				2-4	1-4	I	NS	L										
						1–5	T	S	NS										
						K	T	NS	NS										
							I–T contrast	L	NS										
						Past fragmentation													

in the case of mtDNA – $N_{e(f)}$) and mutation rate (μ) (Kimura and Crow, 1964; Ohta and Kimura, 1973). However, for μ that are relatively constant among species, n_c orders the relative size of historic effective sizes of the species (Avise et al., 1988). Estimates of n_c may be influenced by the number of individuals analyzed, the scope of sampling with respect to both geographic range, and the number of sites detected in the mtDNA genome.

The average nucleotide sequence divergence between haplotype pairs presents a more complex picture. Accumulation of nucleotide differences between haplotypes requires time. If the mutation rates (μ) are reasonably similar (although there is debate about the degree of similarity [e.g. Avise et al., 1988; Gibbons, 1997]), the extent of haplotype divergence should order the species according to the time that has elapsed since a major species bottleneck. Such ordering is a consequence of lineage sorting within isolates, which tends toward a set of related (monophyletic) haplotypes but fosters divergence among isolates. However, as a result of lineage sorting, species composed of isolates are likely to exhibit higher average divergence than species broadly connected by gene flow (Neigel and Avise, 1986). The small nucleotide divergence among haplotypes in coho salmon suggests that extant coho salmon share a relatively recent common female ancestor.

Two distinct clusters of haplotypes account for most of the divergence observed among coho salmon. The clusters are separated by an average of 2.72 substitutions per 1000 nucleotides, but divergence among haplotypes within each cluster average about 0.87 substitutions per thousand nucleotides.

Expanded coho mtDNA survey from variable sites

By concentrating our effort on variable sites, we evaluated the variation present in a larger number of fish and from additional populations. The two clusters of haplotypes observed in the preliminary survey persisted, each augmented by several new haplotypes. The average divergence between haplotypes within each cluster was 4.95 substitutions per 1000 nucleotides and the clusters averaged 10.28 substitutions per 1000 bp apart. By focussing on variable restriction sites,

estimates of nucleotide divergence were inflated more than threefold. However, the data remain useful for examining intraspecific structure, and strong heterogeneity among collections indicated structure among populations or at higher levels of population hierarchy.

The unrooted tree (Fig. 4) depicting relationships among collections shows that the Bering Sea and the Karluk River collections cluster separately from the other collections. The cluster-based structure of the "gene tree" accentuates the divergence between geographic regions because the highest abundance of E-cluster haplotypes occurs in the Bering Sea (and Karluk) collections. In fact, the Delta Clearwater collection is fixed for E-cluster haplotypes. The AMOVA analyses revealed a population structure that appears stronger within regions than among regions. Both estimates of gene flow (one to two females per generation) are low in relation to allozyme-based estimates of other Pacific salmon species (i.e. an exchange of 6.6 to 16.4 individuals per generation for pink salmon [McGregor, 1983; Beacham et al., 1985; Noll et al., 2001]; 6.3 to 9.0 for chum salmon [Kondzela et al., 1994; Phelps et al., 1994; Wilmot et al., 1994; Winans et al.. 1994]; 1.3 to 7.1 for sockeye salmon [Wood et al., 1994; Varnavskaya et al., 1994]; and 1.2 to 4.0 for chinook salmon [Gharrett et al., 1987; Utter et al., 1989; Bartley and Gall, 1990]) but consistent with estimates from coho salmon allozyme data (Wehrhan and Powell, 1987; Bartley et al., 1992).

It is essential to keep in mind that such gene-flow estimates assume an equilibrium between gene flow and random drift. The distribution of coho salmon haplotypes suggests that a broad equilibrium may not exist. For example, the Delta Clearwater River population was nearly fixed for haplotype H, an E-cluster haplotype found only in the Bering Sea populations. Its haplotype composition reflects its geographic isolation from other coho populations. Also, the haplotype distribution from the Kamchatka River population was surprising because it is so similar to Southeast Alaskan populations, but differed from the geographically closer Bering Sea populations. This makes sense if the genetic structure of extant coho salmon populations is strongly influenced by historic events, such as historic random drift, colonization from eastern populations, or survival in an different glacial refugia, and that an equilibrium between gene flow and random drift has not yet been reached.

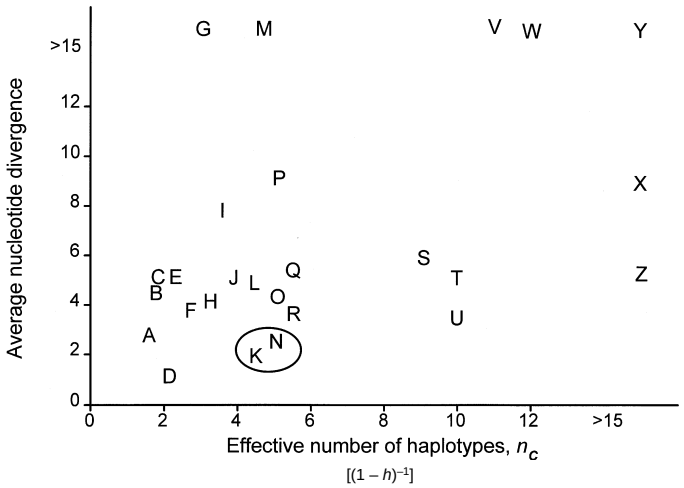

Figure 5

Comparison of the effective number of haplotypes (n_c) and the average nucleotide divergence between haplotypes within a species for 25 species of fish estimated from haplotype diversity (h). The number of effective haplotypes is monotonically related to $N_e\mu$ (μ is the mutation rate); larger average nucleotide divergences require longer times and reduced gene flow to develop. The following species were plotted: A = red grouper (Richardson and Gold, 1993, 1997); B = white marlin (Graves and McDowell, 1995); C = hardhead catfish (Avise et al., 1987, 1989); D = American eel (Avise et al., 1986, 1989); E = oyster toadfish (Avise et al., 1987, 1989); F = American shad (Bentzen et al., 1989); G = redear sunfish (Avise et al., 1987, 1988, 1989, 1992); H = *Sicyopterus stimpsoni* (Zink et al., 1996); I = lake whitefish (Bernatchez and Dodson, 1991); J = red snapper (Gold et al., 1994, 1997); K = coho salmon (Carney et al., 1997); L = black drum (Richardson and Gold, 1993; Gold et al., 1994); M = spotted sunfish (Avise et al., 1987, 1989, 1992; Avise, 1992); N = coho salmon (this study); O = NW Atlantic capelin (Dodson et al., 1991); P = bowfin (Avise et al., 1987, 1989, 1992; Avise, 1992); Q = striped marlin (Graves and McDowell, 1994; Graves and McDowell, 1995); R = *Stenogobius hawaiiensis* (Zink et al., 1996); S = *Lentipes concolor* (Zink et al., 1996); T = greater amberjack (Richardson and Gold, 1993); U = *Awaous guamensis* (Zink et al., 1996); V = Atlantic herring (Kornfield and Bogdanowicz, 1987; Richardson and Gold, 1993); W = largemouth bass (Nedbal and Phillipp, 1994); X = red drum (Gold and Richardson, 1991; Gold et al., 1993); Y = warmouth (Avise et al., 1987, 1989, 1992; Avise, 1992); Z = NE Atlantic capelin (Dodson et al., 1991). Estimates for coho salmon in this study (N) and our previous study (K) are circled.

Synthesis

The data obtained from restriction site analysis provide a present day "snapshot" of coho salmon mtDNA variation and have two levels of resolution. One level is the geographic distribution of haplotypes and the haplotype compositions of the populations sampled. The other level is the pattern and extent of divergence among the haplotypes. The mtDNA haplotype compositions of populations indicate that coho salmon, at least in their Alaskan range, generally have lower gene flow than other Pacific salmon species, although the comparisons assume equilibria between gene flow and random drift that may not yet have been reached. Coho salmon exhibit divergence among populations within regions, but generally not fixed differences. Many of the haplotypes were found in populations throughout the range examined, which suggests

that at some time the haplotypes were disseminated either through gene flow or colonization. Our data, are generally consistent with the known distributions and abundances of North Pacific coho salmon, that is, coho salmon is a species composed of many small spawning populations that have low levels of gene flow. The low geneflow rates estimated within several geographic ranges indicate that a finer scale study of coho salmon population structure is warranted.

Superimposed on this "snapshot" of population structure is the mtDNA "gene tree," which carries information about the demographic history of coho salmon. Nested clade analysis of the geographic distribution of haplotypes and clades of related haplotypes reveal recent isolation by distance, particularly for the geographical distribution of A-cluster haplotypes. There is also evidence of past population fragmentation in the distribution of E-cluster haplotypes, and the interior of the tree has evidence of range expansion. The topology of the tree also indicates recent demographic expansion preceded by stationary, or more likely, declining populations. Radiation of haplotypes to form a star-like pattern occurs when populations expand (Slatkin and Hudson, 1991), such as the haplotypes that surround haplotypes A and E. Whereas, in stationary or declining populations, the haplotype composition of a population eventually tends toward a single, evolutionarily related (monophyletic) set as a result of stochastic processes (Neigel and Avise, 1986), such as the A- or E-cluster.

It is likely that the demographic history of coho salmon is closely tied to Pleistocene events. During the Pleistocene Epoch, glaciers periodically advanced and retreated in regions bordering the northeastern Pacific Ocean. These advances were accompanied by a drop in sea level (exceeding 100 m), decreased sea surface temperatures, increased coverage of sea ice, and reduced marine surface water productivity (e.g. Porter, 1989; Bartlein et al., 1991; de Vernal and Pedersen, 1997). Much of the present day freshwater range was physically covered with ice (Hamilton, 1986). During the last glacial maximum, coho salmon must have been displaced from most of British Columbia and the Gulf of Alaska coast, now the center of their range. During each glacial advance, it is likely that marine and freshwater habitats of Pacific salmon populations were greatly reduced, especially for species that require freshwater rearing, such as coho salmon. Alteration and reduction of natural ranges during these advances probably resulted in the declines or extirpation of many populations. The patterns observed in the analysis of coho salmon mtDNA variation resulted from major demographic changes involving the range of drainages sampled and are consistent with the effects that would be expected as a result of glacial advances and retreats. For most of the last 500,000 years, the environment was much harsher than now, as indicated by the sea level (a measure of the amount of the world's water tied up in ice), which was more than 50 m lower than today during most of that period (Porter, 1989; Bartlein et al., 1991). Peaks of recent interglacial periods during which the environment probably approached modern conditions have occurred about every 100 thousand years, approximately 120 thousand years ago (120 ka) , 200 ka, 330 ka, and 400 ka (Porter, 1989; Petit et al., 1999).

A linkage between geologic record and molecular evolution requires application of a "molecular clock" to relate observed nucleotide divergences to a mutation rate. The calibration of mtDNA clocks is contentious. A rate of 2% nucleotide substitution per million years (Brown et al., 1982), referred to as the "standard clock," has been broadly applied. However, the rates for salmonids may be slower, and rates of 1% per million years or less (Smith, 1992) may be more appropriate.

An interpretation of our results based on the "standard clock" would be that divergence between clusters began 136 ka at the beginning of the last interglacial period and that divergence within the clusters began 43 ka, at the end of the last interglacial period. The small number of haplotypes within each cluster is consistent with a relatively small effective number of females ($N_{e(f)}$) within refugia followed by rapid expansion or the existence of several isolates within each refugium. Following the last glacial maximum, dispersion from one or more glacial refugia was followed by incomplete inter-refugial introgression. The objection to interpretations based on the "standard clock" is that the divergence rate is much faster than the rate generally accepted for salmonids. However, mtDNA clocks are calibrated from interspecies comparisons, which occur over times measured in millions of years (Thomas and Beckenbach, 1989; Martin et al., 1992; Bentzen et al., 1993; Phillips and Oakley, 1997). The mutation rate is certainly not homogeneous over the mtDNA genome, so it is possible that mutational hot spots dominate the rate in shorter time spans but are saturated and much less important in estimates over long time spans.

Although there are unquestionably strong demographic signals in the molecular evolutionary record of coho salmon, we can not match them unequivocally to specific Pleistocene events. To make such a match, several pieces of information are needed. First, the data available for calibrating a clock for the entire salmonid mtDNA genome are limited. Although there are numerous studies of particular mtDNA regions, rate differences among regions compromise their utility. Second, short-term, intraspecific clocks based on hot spots must be developed and compared with long-term, interspecific clocks. We are now examining data from other Pacific salmon species that share the northern Pacific Ocean range with coho salmon. Concordance among those results interpreted from the well-described Pleistocene environmental history may provide us with independent records of Pleistocene influences on the demography of Pacific salmon, which can be used to calibrate intraspecific mtDNA clocks.

The haplotype compositions of the populations that we studied leave us with questions about patterns of postglacial colonization and influences of the Cascadian and Beringian refugial stocks. Acquisition and analysis of samples from native populations in the Pacific Northwest should resolve questions about the composition of coho salmon in the Wisconsin Cascadian refugium. And, an intensive study of populations skirting the Alaska Peninsula and eastern Bering Sea should provide information about local colonization patterns and the composition of Beringian coho.

Acknowledgments

We appreciate the constructive and encouraging comments made by A.T. Beckenbach on an early draft. D. Churikov and B. Finney provided valuable discussions. We thank D. Churikov, J. Gharrett, and three anonymous reviewers for reviewing the manuscript. T. Chatto, S. Kelley, D. McBride, L. Schwartz, L. Shaul, C. Skaugstad, and S. Taylor kindly provided samples. This work is a result of research sponsored in part by the Saltonstall-Kennedy Fund NA36 FD0179 and in part by the Alaska Sea Grant College Program, which is cooperatively sponsored by NOAA, Office of Sea Grant and Extramural Programs, Department of Commerce, under grant NA90AA-D-SG066 (project R/02-16), and the University of Alaska with funds appropriated by the State. The manuscript was revised while AJG was at Hokkaido University and supported, in part, by the Japan Society for Promotion of Science.

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Appendix 1

Restriction fragment sizes for seven PCR-amplified coho salmon mtDNA regions for twelve restriction endonucleases. Composite haplotypes are shown in Appendix 2.

2S/165

Ase I	*Ava* I	*Bst*N I	*Bst*U1	*Cfo* I		*Dde* I			*Hin*d II	*Hin*f I	*Mbo* I		*Msp* I	*Rsa* I	*Sty* I
1	1	1	1	1	2	1	2	3	1	1	1	2	1	1	1
2400	1200	1000	1300	1210	1100	455	400	400	750	1800	1475	900	950	930	1500
	1200	410	475	380	380	400	280	280	720	600	375	575	650	600	500
		380	300	350	350	280	250	205	720		210	375	400	290	200
		310	275	150	150	155	205	205	210		170	210	190	200	200
		200		120	120	145	155	155			170	170	130	200	
		150			110	130	145	145				170	80	180	
						125	130	130							
						120	125	125							
						104	120	120							
						80	104	104							
						65	80	80							
						55	65	65							
						48	55	55							
						45	48	48							
						37	45	45							
						35	37	45							
						33	35	37							
						30	33	35							
						27	30	33							
							27	30							
								27							

ND1/ND2

Ase I	*Ava*I	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I			*Hin*d II	*Hin*f I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I			
1	1	1	1	1	1	2	3	1	1	1	1	1	1	2	3	4
1786	1596	1496	2026	1146	780	780	780	1846	1546	1026	1926	1176	650	650	550	550
520	600	1150	500	900	480	480	480	800	850	590	780	375	550	550	530	530
230	450		120	600	240	240	240		250	510		275	530	530	470	470
110					220	210	180			310		250	395	305	395	305
					180	180	148			210		220	305	305	305	305
					145	145	145					220	230	230	230	230
					123	123	123					130		90	180	180
					120	120	120									90
					115	115	115									
					95	95	95									
					78	78	78									
						(10)	62									
							(10)									

continued

Appendix 1 (continued)

A8/A6/COIII/ND3

Ase I	*Ava* I	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hin*f I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
1	1	1	1	1	1	1	1	1	1	1	1
2210	2210	1200	1700	1300	970	1300	500	1800	800	780	500
		700	500	900	970	900	498	210	600	680	450
		310			180		400	200	490	345	350
					80		300		320	240	340
							160			150	240
							150				220
							150				90

COI/COII

Ase I	*Ava* I	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I			*Hin*d II	*Hin*f I		*Mbo* I		*Msp* I	*Rsa* I	*Sty* I
1	1	1	1	1	1	2	3	1	1	2	1	2	1	1	1
1562	2432	1152	1422	1522	530	530	530	1682	575	500	650	550	2000	872	1282
650		890	500	1010	360	360	360	750	500	500	550	520	220	350	1000
220		390	410		330	330	330		410	410	480	480	212	350	150
					293	293	293		295	295	270	270		300	
					255	255	255		150	150	220	220		225	
					235	235	235		150	150	175	175		215	
					196	196	209		150	150	75	130		120	
					119	110	110		90	90		75			
					110	80	80			75					
					26	39	39								
					(13)	26	26								
						(13)									

ND3/ND4

Ase I	*Ava* I	*Bst*N I	*Bst*U I	*Cfo* I		*Dde* I	*Hin*d II	*Hin*f I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
1	1	1	1	1	2	1	1	1	1	1	1	1
1955	2105	1050	1800	1600	1100	525	2305	1730	525	900	875	1970
350	200	750	500	700	700	490		550	500	800	490	330
		500			500	400			400	300	365	
						290			350	250	230	
						150			190	115	140	
						150			190		110	
						90			150		90	
						90						

ND5/ND6

Ase I	*Ava* I		*Bst*N I		*Bst*U I	*Cfo* I	*Dde* I				*Hin*d II	*Hin*f I	*Mbo* I		*Msp* I	*Rsa* I	*Sty* I
1	1	2	1	2	1	1	1	2	3	4	1	1	1	2	1	1	1
1100	2530	2230	900	900	2025	1000	665	600	600	600	1950	550	1442	1442	1525	2530	1675
850		300	900	900	525	800	490	570	490	490	550	475	410	410	410		600
550			650	400		500	300	490	320	300		420	312	312	285		275
			100	250		200	270	320	300	270		320	286	281	200		
				100			200	160	270	200		275	100	100	130		
							160	150	160	160		210		(5)			
							150	145	150	150		175					
							145	65	145	145		125					
							120		65	120							
										65							

continued

Appendix 1 (continued)

CytB/D-Loop													
Ase I	*Ava* I	*Bst*N I			*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hin*f I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
1	1	1	2	3	1	1	1	1	1	1	1	1	1
1524	1534	900	900	900	2100	900	550	2094	690	810	700	1240	1050
600	520	850	540	540	444	694	430	450	650	610	330	400	580
420	315	415	415	400		510	310		470	400	240	390	450
	175	400	400	310		300	290		310	170	225	195	220
		130	310	220		140	280		250	165	210	140	140
			130	195			280		230	165	205	125	110
				130			175			150	205	105	70
							130			72	180		
							90				180		
											120		

Appendix 2

Key to haplotype composition inferred from variable fragment patterns in Appendix 1. No variation was observed in the A8/COIII region (Table 2). Numbers in parentheses are presumed haplotypes.

	ND1/ND2		12S/16S			ND3/ ND4	Cytb/ Dloop	ND5/ND6				COI/A8		
Haplotype	*Dde* I	*Sty* I	*Cfo* I	*Mbo* I	*Dde* I	*Cfo* I	*Bst*N I	*Ava* I	*Bst*N I	*Dde* I	*Mbo* I	*Dde* I	*Hin*F I	*Mbo* I
A	2	4	2	1	2	1	2	2	2	2	2	2	2	1
A′	2	4	2	1	2	1	2	2	1	2	2	2	2	1
B	2	4	1	1	2	1	2	2	2	2	2	2	2	1
C	2	4	2	1	2	1	3	2	2	2	2	2	2	1
C′	2	4	2	2	2	1	3	2	2	2	2	2	2	1
D	2	3	2	1	2	1	3	2	2	2	2	2	2	1
E	3	2	2	1	2	1	1	1	2	4	1	2	2	1
E′	3	2	2	1	2	1	1	1	2	4	1	2	1	2
F	3	1	2	1	2	1	1	1	2	4	1	2	2	1
G	3	2	2	1	2	1	1	1	2	3	1	1	2	1
H	3	2	2	1	1	1	1	1	2	4	1	2	2	1
I	2	4	2		2	1	1	2		2	2	2		
J	2	4	2		2	1	3	1		3	2	3		
K	1	2	2		2	1	1	1		4	2	2		
L	3	1	2		2	1	1	1		3	1	2		
M	3	2	2		2	1	1	1		1	(1)	(2)		
N	3	2	2		3	1	1	1		5	(1)	(2)		
O	3	2	2		1	2	1	1		5	(1)	(2)		
P	2	4	2		2	1	3	1		3	2	2		