Heredity (2009) 103, 299–309

www.nature.com/hdy

ORIGINAL ARTICLE

Long-term changes in the fine-scale population structure of coho salmon populations (*Oncorhynchus kisutch)* subject to extensive supportive breeding

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The long-term viability of a metapopulation depends partly on the gene flow among sub-populations. Management approaches such as translocations and supportive breeding between closely related populations may affect gene flow and overall structure, and therefore viability. Here, we examined temporal changes in the fine-scale population structure of coho salmon (*Oncorhynchus kisutch*) by comparing archived (1938) and modern (2001–2005) populations in six rivers within a single conservation unit (Puget Sound, Washington) sampled before and after an extended period of between-river transfers and releases of millions of cultured salmon. Genotype frequencies at eight microsatellite loci showed that current populations descended from historical Puget Sound populations, but populations in different rivers that exchanged fish for hatchery propagation share more of their ancestry recently than they did historically. Historically, populations in different rivers were isolated by geographic distance, but that relationship is no longer significant. Allelic richness among all populations declined significantly, suggesting that genetic drift has increased because of a population bottleneck. Populations in different rivers and within the same river have become more diverged, providing further evidence for a widespread bottleneck. Previously, we observed that genetic distance significantly decreased with the number of fish exchanged; however, some populations apparently resisted introgression. Altered gene flow and lost diversity may affect the complexity, and therefore resiliency of sub-populations within a conservation unit. Plans for artificial culture need to maintain existing genetic diversity and avoid disrupting the fine-scale structure by using local populations for parents whenever possible.
Heredity (2009) **103,** 299–309; doi:10.1038/hdy.2009.69; published online 15 July 2009

Keywords: hatchery; pacific salmon; microsatellite loci; genetic diversity; metapopulation; temporal

Introduction

Population structure has important consequences for the evolution of a species and for implications for conservation, because drift, selection, migration and mutation act to differentiate allelic diversity among semi-isolated populations (Whitlock, 2004). Phenotypic diversity can evolve over short genetic and geographic distances (McKay and Latta, 2002), and semi-isolated populations can diversify and become locally adapted (Taylor, 1991). Furthermore, the accumulation of deleterious mutations is dependent on the global effective population size, as well as on the levels of gene flow between populations (Waples, 2002; Whitlock, 2004). Semi-isolated populations represent the range of diversity within a species or conservation unit over a variety of environmental conditions, and afford resilience to perturbations (Allendorf and Luikart, 2007). To be most effective, conservation actions should consider the existing population structure and the processes that lead to structuring among populations.

The recovery of a species may require human intervention, but care is needed to maintain population structure. Activities such as supportive breeding and translocations are often used to increase population abundance, provide surplus fish for harvest and expand a species' range. However, these activities present risks after the escape or release of domesticated or nonindigenous fish (Ellstrand *et al*., 1999; Barilani *et al*., 2005; Halbert *et al*., 2005; Naish *et al*., 2007) that may culminate in introgression or a partial or complete displacement of native populations (Hansen, 2002; Haygood *et al*., 2003; Vasemagi *et al*., 2005). Ongoing supportive breeding could result in a loss of genetic diversity and reductions in effective population size (Ryman and Laikre, 1991; Gaffney *et al*., 1996) and could alter the sub-population structure (Utter, 2001; Birnbaum *et al*., 2003). Despite these risks, supportive breeding and translocations may provide an effective avenue for the recovery of a population's fitness or for the prevention of a loss of genetic diversity (Tallmon *et al*., 2004; Edmands, 2007). It thus becomes important to establish the relationship between evolutionary divergences and impacts on population structure that might be expected after human-mediated hybridization (McClelland and Naish, 2007).

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Received 12 August 2008; revised 3 February 2009; accepted 5 June 2009; published online 15 July 2009

When donor and recipient populations differ substantially at neutral genetic loci, assignment tests can identify descendants of introduced fish (Cornuet *et al*., 1999); however, this task is more difficult among closely related populations that experience high levels of gene flow wherein assignment tests are less reliable (Cornuet *et al*., 1999), and when baseline data are not available for all source populations. In the absence of complete baseline data, Bayesian assignment approaches have been used to identify fish with native, introduced or introgressed ancestry (Hansen *et al*., 2001). Alternatively, transfers may alter the fine-scale population structure. Archived tissues can provide an invaluable historical baseline for exploring changes to the allelic diversity within and among populations resulting from extensive human intervention (Nielsen *et al*., 1999).

Coho salmon populations (*Oncorhynchus kisutch*) on the West Coast of North America provide a framework for studying the effects of introgression due to supportive breeding and translocations. These populations have been classified within conservation units (here, evolutionarily significant units, ESUs; Waples, 1991) that represent divergent evolutionary components of species diversity. Several studies have shown that barriers to introgression tend be greater in anadromous salmonids than in freshwater or marine fish species, but introgression is frequent after transfers among closely related populations (reviewed in Utter, 2001). Translocations of coho salmon between two ESUs have been shown to be largely unsuccessful (Withler, 1982; Ford *et al*., 2004), but little is known about the effects of culture on population structure within an ESU. Coho salmon in the Puget Sound ESU of Washington State have been propagated and released into the wild for over 100 years to enhance harvest opportunities (Stickney, 1996), and the composite populations in this ESU have been designated a Species of Concern due to possible changes to genetic diversity caused by culture practices (NMFS, 2004). In an earlier study, we showed that the genetic distance among contemporary populations was the smallest between rivers that experienced the greatest numbers of transfers, and sub-population genetic divergence was reduced in rivers that had the highest number of hatchery releases (Eldridge and Naish, 2007). Population structure could not be explained by geographical distance. However, without a historical knowledge of gene diversity and gene flow before large-scale transfer and culture activities, it is impossible to eliminate the possibility that the observed patterns were due to natural causes.

Here, we examined the stability of the population structure and genetic diversity of coho salmon within an ESU that has experienced ongoing releases into the wild of cultured fish. We used microsatellite loci to compare genetic diversity, and the magnitude and patterns of gene flow among populations occupying six rivers around Puget Sound, Washington in 1938 and in the present, using archival samples, and we compared our results with those published earlier (Eldridge and Naish, 2007).

Materials and methods

Study area

This study was conducted in six rivers in the Washington State portion of the Puget Sound coho salmon ESU (Figure 1). Within each river basin, hatcheries have released into the wild millions of coho salmon from a variety of intra- and interbasin sources. Coho salmon releases in and around Puget Sound between 1952 and 2004 have been described previously (Eldridge and Naish, 2007). The goals of many coho salmon hatchery programs in Puget Sound were undefined until recently (Mobrand *et al*., 2005), and likely comprised a mixture of fishery enhancement and conservation activities. However, in all cases, broodstock was collected from adults during their spawning migrations in freshwater, and offspring were released into the wild as juveniles, often into multiple creeks in the same year.

Tissue samples and microsatellite analyses

Coho salmon tissues from the six Puget Sound rivers were available from two time periods (Table 1). Historical scales or skin patches were collected from adult coho salmon sampled from spawning grounds and from hatcheries in these rivers during the spawning cycle that began in the fall of 1938 ($N = 367$). More recently, scales, fin clips, operculum punches or clips from pectoral girdle were collected from adult and juvenile coho salmon from hatcheries and from the wild between 2001 and 2005 ($N = 643$); scales were stored dry on cards at an ambient temperature and other tissues were stored in ethanol. Additional scales were available for an intermediate period (1968) from adult coho salmon returning to the Voights Creek Hatchery on the Puyallup River ($N = 73$). A sample consisted of fish from a single creek from the same year where possible, except samples labeled as either North Fork, Middle Fork or South Fork in Table 1, which refer to small collections combined from nearby creeks, or samples labeled as Trap, which were collected from the mainstem of a river.

DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's directions, but the historical samples collected in 1938 were eluted in two centrifugation spins of 50 μl each. A total of 12 modern samples were previously screened (Eldridge and Naish, 2007). All historical, intermediate and four modern samples (NOnf05, NOmf01, NOsf01 and SGjk05) were unique to this study. We determined genotypes for all fish at eight microsatellite loci (*Olc8*, *Ogo1a*, *Ogo2aI*, *Ogo2aII*, *Omy1011*, *One13*, *Ots3* and *P53*). A total of 11 loci were originally screened (Eldridge and Naish, 2007), but genotypes in the historical samples could be reliably determined at only eight loci. Alleles at the locus with the shortest products (*Ots503*, 73–95 base pairs), and at two loci with large products (*Ots505* and *Oki23*, >230 base pairs), could not be distinguished from PCR artifacts and these loci were dropped from the analyses.

Amplification of microsatellite loci in the modern samples followed procedures described in Eldridge and Naish (2007). The PCR reaction reagents for the historical samples are described in Supplementary Table 1 and the PCR thermocycling conditions in Supplementary Table 2. PCR products were visualized on the MegaBACE automated sequencer (Amersham Biosciences, Piscataway, NJ, USA) using a ROX-labeled ET 550 internal size standard. Microsatellite allele size was determined with the Genetic Profiler v.2 software package (Amersham Biosciences). PCR thermocycling and allele scoring were repeated for 118 genotypes from 1938 to determine the

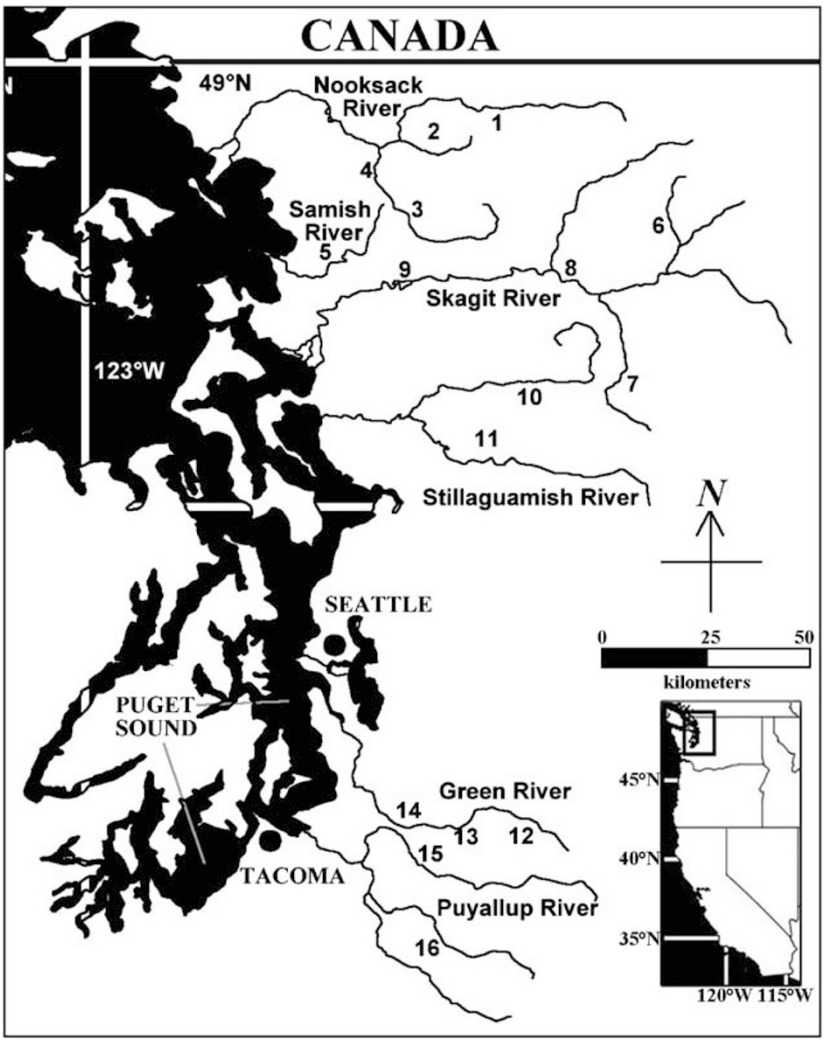

Figure 1 Map of coho salmon sampling locations around Puget Sound, Washington. Numbers correspond to sample labels in Table 1.

rates at which alleles were scored consistently among historical samples. Approximately, 94% of alleles (221 of 236) were scored consistently at all eight loci. Most discrepancies involved a fish being scored once as a heterozygote and a second time as a homozygote so that only one allele was consistent between repeat amplifications.

Statistical analyses

Each locus within each sample was tested for the presence of null alleles, and for scoring errors due to stuttering or allelic dropout (Micro-Checker v.2.2.1, Van Oosterhout *et al*., 2004). The genetic diversity within each sample was described by determining the number of unique allele classes at each locus, the observed and expected heterozygosities and the sample inbreeding coefficient (F_{IS}) (GenAlEx v.6.1, Peakall and Smouse, 2006). To test for panmixia within samples, the significance of F_{IS} over all loci in each sample was tested using the Hardy–Weinberg probability exact test (Genepop v.3.4, Raymond and Rousset, 1995) and tests for genotypic equilibrium were conducted between all pairs of loci (FSTAT v2.9.3.2, Goudet, 1995). 'Outlier' loci or samples were defined as those that consistently contributed to significant tests at the $P<0.05$ level. Outlier loci may arise because of null alleles, inconsistencies in amplification or linkage between loci, and outlier samples may suggest deviations from random mating. To determine whether any locus, locus pair or sample was involved in a disproportionately large number of significant deviations from Hardy–Weinberg or genotypic equilibrium, χ^2-tests were conducted comparing the observed number of significant tests with the expected number, if significant results were distributed randomly across loci, locus pairs or samples.

Changes in genetic diversity among and within rivers were assessed by testing whether the number of unique allelomorphs changed over time. We compared the allelic richness between samples (N_a, number of allelomorphs in samples of a common size) to test for a change in the number of allelomorphs. For each river and period, the allelic richness for a locus was determined by counting the number of allelomorphs in a draw of 2N alleles (as opposed to genotypes), taken without replacement, from a pool of all samples from a single river during the historical (1938), intermediate (Puyallup River in 1968) or modern (2001–2005) period. The draws were repeated

Table 1 Sample information and summary of genetic variation within samples for coho salmon from six rivers around Puget Sound

Sample label	*River*	*Location*	*Year*	*Age*[a]	N[b]	n[c]	*A*[d]	H_e[e]	H_o[f]	F_{IS}
1—Nonf38	Nooksack	North Fork	1938	A	21	15.1	6.5	0.71	0.52	0.26***
2—NOmf38	Nooksack	Middle Fork	1938	A	4	3.0	3.6	0.66	0.61	0.11
3—NOsf38	Nooksack	South Fork	1938	A	26	22.9	8.1	0.75	0.66	0.13*
5—SMtr38	Samish	Butler Creek & Trap	1938	A	13	11.4	6.4	0.72	0.64	0.10**
6—SGdi38	Skagit	Diobsud Creek	1938	A	35	30.6	9.3	0.73	0.71	0.04
7—SGsa38	Skagit	Sauk	1938	A	43	34.8	9.1	0.77	0.72	0.07***
8—SGjk38	Skagit	Jackman Creek	1938	A	33	28.3	8.8	0.74	0.70	0.05**
9—SGpo38	Skagit	Powell Creek	1938	A	28	23.8	8.0	0.76	0.66	0.12***
10—STnf38	Stillaguamish	North Fork	1938	A	20	14.6	7.6	0.76	0.69	0.09
11—STsf38	Stillaguamish	South Fork	1938	A	21	17.0	7.3	0.71	0.64	0.10**
12—GRdm38	Green	Headworks Dam	1938	A	15	11.5	6.9	0.68	0.62	0.07
14—GRh38	Green	Soos Hatchery	1938	A	66	56.6	9.6	0.74	0.75	−0.01
16—PUh38	Puyallup	Voights Hatchery	1938	A	42	30.9	9.3	0.73	0.69	0.06
16—PUh68	Puyallup	Voights Hatchery	1968	A	73	67.9	9.3	0.73	0.69	0.06***
1—NOnf01	Nooksack	North Fork	2001	A	94	88.8	8.0	0.73	0.73	−0.01***
1—NOnf05	Nooksack	North Fork	2005	A	17	16.6	6.3	0.73	0.70	0.04***
2—NOmf01	Nooksack	Middle Fork	2001	A	8	8.0	6.0	0.72	0.84	−0.15
3—NOsf01	Nooksack	South Fork	2001	A	36	34.4	8.5	0.74	0.75	−0.02
4—NOsf02	Nooksack	South Fork	2002	J	43	40.8	8.6	0.74	0.75	−0.01
5—SMen01	Samish	Ennis Creek	2001	A	46	43.6	7.9	0.73	0.75	−0.02
6—SGdi05	Skagit	Diobsud Creek	2005	J	40	39.6	8.6	0.73	0.72	0.02
7—SGsa05	Skagit	Sauk River	2005	J	29	28.8	8.1	0.74	0.73	0.01*
8—SGjk05	Skagit	Jackman Creek	2005	J	12	11.0	6.4	0.72	0.80	−0.11
9—SGcc05	Skagit	Childs Creek	2005	J	47	46.3	7.8	0.74	0.75	0.00**
10—STnf04	Stillaguamish	North Fork	2004	A	48	45.8	8.4	0.72	0.77	−0.07
11—STsf04	Stillaguamish	South Fork	2004	A	45	43.3	9.1	0.71	0.75	−0.07*
13—GRnw05	Green	Newakum Creek	2005	J	46	45.0	9.9	0.73	0.72	0.03
14—GRh02	Green	Soos Hatchery	2002	A	48	46.1	8.6	0.74	0.75	−0.01*
15—PUbo02	Puyallup	Boise Creek	2002	A	37	35.6	8.4	0.73	0.75	−0.03
16—PUh02	Puyallup	Voights Hatchery	2002	A	47	45.1	8.4	0.73	0.75	−0.04
	Mean 1938				28.2	23.1	7.7	0.73	0.66	0.09***
	Mean 2001–2005				40.2	38.7	8.1	0.73	0.75	−0.03***
	Mean 1938–2005				36.1	32.9	8.0	0.73	0.71	0.03***

[a]A, adult, J, age 0 except for NOsf02, which included some age 1+.
[b]Sample size.
[c]Average number of genotypes determined at eight microsatellite loci.
[d]Average number of alleles at eight microsatellite loci.
[e]Average expected heterozygosity at eight microsatellite loci.
[f]Average observed heterozygosity at eight microsatellite loci.
*, **, *** Significant deviation from panmixia at $*P<0.05$, $**P<0.01$ or $***P<0.001$.

1000 times and the average was recorded. The process was repeated after combining all the samples from a given time period to compare the overall historical and modern periods. Wilcoxon paired sample tests (Zar, 1999) were conducted to determine if the allelic richness among all eight loci was significantly different between historical samples (1938) and modern samples (2001–2005) from the same river, and between all rivers combined. The Samish River was excluded from the allelic richness tests because of small historical sample size.

The temporal and spatial scales at which allelic diversity was distributed within and between historical (1938) and modern samples (2001–2005) were evaluated by examining the genotype diversity within and among samples grouped by period or by river, using a series of hierarchical analysis of molecular variance (AMOVA) tests, interpolating for missing data with 999 random permutations to test for significance (GenAlEx v.6.1, Peakall and Smouse, 2006). We used genotype diversity (Φ-*statistics*) rather than allelic diversity (F-*statistics*) because of deviations from the Hardy–Weinberg equilibrium in some samples (Supplementary Table 3).

Isolation by distance among rivers historically (1938) and recently (2001–2005) was assessed by Mantel tests (Mantel and Valand, 1970), with 999 random permutations to determine significance (GenAlEx v.6.1). We compared rivers rather than individual samples because we were interested in the effects of transfers on the gene flow between rivers. In this analysis, the genetic distance between each pair of rivers (here, Φ_{RT}) was determined using an AMOVA that interpolated for missing data (GenAlEx v.6.1). When only one site was sampled in each river, the Φ_{ST} value estimated by AMOVA was used as the Φ_{RT} value. Geographic distances between rivers were determined to be the shortest path in fresh or saltwater between the respective river mouths using the distance calculator in the US Geological Survey National Map Viewer (http://nmviewogc.cr.usgs.gov/viewer.htm).

Pairwise tests of population differentiation based on randomizing multi-loci genotypes between samples were conducted (FSTAT v2.9.3.2, Goudet, 1995) to estimate the reproductive isolation between pairs of samples within and between rivers and between different years, and Φ_{ST} was calculated for each pair of sample (GenAlEx v.6.1). Population structure was visualized using a multi-dimensional scaling plot of Φ_{ST} (SPSS, version 10.0.5, SPSS, Chicago, IL, USA). To evaluate the changes in genetic distances over time, a matrix of the historical Φ_{ST} values was compared with a matrix of the modern values

between the same pairs of sampling locations using a Mantel test, with 999 random permutations to determine significance (GenAlEx v.6.1). Modern samples collected over 2 years were available for some locations, in which case only the earlier sample was used.

We also assigned fish to theoretical populations using a Bayesian algorithm that can account for an admixture of fish and partial baselines. Accounting for partial baselines was necessary because our historical samples did not include all Puget Sound sources of transferred fish (Eldridge and Naish, 2007). The Bayesian Analysis of Population Structure (BAPS v.4.14) computer program uses two steps to determine the mixture proportions in unknown samples (Corander and Marttinen, 2006). The algorithm first determines the optimum number of theoretical populations (up to a user-defined K_{MAX} that we set to 20 for 20 runs), which is used to determine the admixture proportions in all samples. Historical baselines were used to 'train' the algorithm, and during the admixture run, the minimum sample size was 5, the number of iterations was 100, the number of reference fish from each population was 200 and the number of iterations for reference fish was 20. Output is the proportion of a fish's genome derived from different sources, which we averaged across fish within the same sample to determine the assignment percentage. We used this approach rather than considering admixture proportions (Hansen *et al*., 2001), because most fish were admixed.

Results

Sample quality and tests for random mating

The success of PCR varied between markers and samples. More than 72% of genotypes in the historical and 92% in the modern samples were determined (estimates based on the average number of genotypes determined per fish in Table 1). No sample or locus was involved in more deviations from Hardy–Weinberg equilibrium than expected (historical samples: $\chi^2 = 9.3$, $df = 12$, $P = 0.67$; modern samples: $\chi^2 = 17.68$, $df = 16$, $P = 0.34$; all loci: $\chi^2 = 12.00$, $df = 7$, $P = 0.10$). The historical samples did not deviate from genotypic equilibrium more than expected ($\chi^2 = 4.36$, $df = 12$, $P = 0.98$), nor did any pair of the eight loci ($\chi^2 = 14.44$, $df = 27$, $P = 0.98$). Deviations from genotypic equilibrium were distributed nonrandomly among the modern samples ($\chi^2 = 50.71$, $df = 16$, $P < 0.001$). Five or more significant tests were observed in each of the Child Creek (SGcc05), Sauk River (SGsa05) and Samish River (SMen05) samples. Disequilibrium in the two Skagit River samples may have resulted from a nonrandom mating in these samples (as suggested by significant F_{IS}, Table 1, Supplementary Table 3).

Results generated using Micro-Checker indicated a significant homozygote excess in some of the historical samples at the same loci wherein deviations from Hardy–Weinberg equilibrium were observed. Failure to amplify large alleles or skipping base pairs during PCR replication (that is, stuttering) is unlikely to be the source of homozygote excess because the excess was distributed across allele classes. Furthermore, null alleles are probably not responsible, as no single locus was systematically affected across populations. To affirm that the problem was not caused by a tendency to incorrectly score a heterozygous fish as a homozygote, we compared the historical and modern samples for the number of loci at which fish were homozygous, and found no significant difference ($\chi^2 = 4.2$, $df = 6$, $P = 0.65$). Alternatively, homozygote excess could be explained by a Wahlund effect. Among the historical samples, fish from nearby creeks or from a trap were pooled and may have represented mixed populations; therefore, we separated the fish from different creeks in the older samples and repeated the Micro-Checker analyses. Only one creek in the 1938 NF Nooksack River continued to show homozygote excess after analyzing creeks separately, although the power to detect deviations among individual creeks was reduced because of small sample sizes (2–17 fish). The larger groupings were maintained for further analyses because they provided a greater sample representation, despite the possibility of the Wahlund effect. Inclusion or exclusion of the older NF Nooksack River sample (NOnf38) did not affect the results; therefore, only results including this sample are presented.

Changes in genetic diversity and population structure

Allelic diversity declined between historical and modern samples. Of the 120 unique allelomorphs observed at the eight loci, 16 allelomorphs appeared only in the historical samples, whereas four appeared only in the modern samples and another one was observed only in the intermediate sample. These allelomorphs were rare, occurring at frequencies below 0.10. Similarly, allelic richness declined over time for seven loci, and was unchanged for the eighth when all rivers were pooled, a statistically significant change (Table 2). However, in comparisons involving single rivers, allelic richness was significantly lower only in the Skagit River, possibly reflecting the smaller sample sizes when rivers were considered on their own.

Genotype distances among the historical and modern samples tended to be small ($\Phi_{ST} < 0.04$ historically and < 0.10 currently, Figure 4 and Supplementary Table 4), and a significant genotype differentiation occurred primarily between pairs of modern samples ($P < 0.05$; Supplementary Table 4). The historical samples often consisted of few fish, which limited the power to detect significant genotype differentiation between samples. Only the modern sample from the 2001 NF Nooksack River (NOnf01) was highly diverged from most other samples ($\Phi_{ST} > 0.06$), a result observed previously for this same sample location and year (Small *et al*., 2004; Eldridge and Naish, 2007). However, a second sample from the same location collected in 2005 (NOnf05) was less diverged from other modern Nooksack and Skagit River samples ($\Phi_{ST} < 0.06$), although this sample was smaller ($N = 17$). Bias in the analyses of modern samples might have been caused by the outlier modern NF Nooksack River sample (NOnf01); therefore, all further statistical analyses were conducted with and without this sample.

The Mantel tests indicated that there was a significant positive relationship between genetic distance and geographic distance between rivers historically (Figure 2, filled diamonds and solid line; $r = 0.595$, Mantel test $P = 0.007$). Recently, the isolation-by-distance pattern was still found to be positive, but the relationship was no

Table 2 Temporal comparisons of allelic richness at eight microsatellite loci for coho salmon from five rivers around Puget Sound, Washington

River and period	*2N*	Ocl8	Ogo1a	Ogo2aI	Ogo2aII	Omy1011	One13	Ots3	P53	P-*value*
Nooksack h	60	10.5	2.0	8.0	7.3	6.6	14.7	8.4	10.6	0.20
Nooksack m	60	11.6	2.0	6.8	6.6	7.4	12.9	7.5	8.9	
Skagit h	60	13.1	2.0	7.0	7.6	8.6	15.0	10.6	11.7	0.05*
Skagit m	60	13.6	2.0	6.7	6.5	7.7	11.6	8.6	10.7	
Stillaguamish h	52	12.4	2.0	6.9	7.8	10.4	14.0	8.7	8.9	0.20
Stillaguamish m	52	13.2	2.0	5.9	6.6	7.6	12.2	7.6	10.7	
Green h	60	13.1	2.0	8.3	7.4	8.2	13.4	7.8	10.3	0.44
Green m	60	12.0	2.0	9.1	5.7	8.1	13.0	7.4	11.2	
Puyallup h	30	11.7	2.0	5.8	5.8	4.9	10.0	7.9	11.6	0.11 h × i
Puyallup i	30	8.8	2.0	5.7	5.7	4.8	10.4	6.2	10.1	0.55 h × m
Puyallup m	30	10.2	2.0	5.7	6.3	5.5	11.9	5.9	8.3	0.55 i × m
All h	200	17.6	2.0	10.6	10.5	11.4	18.8	12.0	15.6	0.02*
All m	200	15.6	2.0	9.8	7.7	10.4	16.5	10.7	13.3	

Genotypes from the same river and period (historical (h, 1938), modern (m, 2001–2005) or intermediate (i, 1968) for Puyallup) were pooled and 2N alleles were drawn with replacement. Values presented are the average of 1000 draws. The Samish River was not considered because of small sample size.
*Significant non-parametric Wilcoxon paired sample test for difference in allelic richness ($P<0.05$).

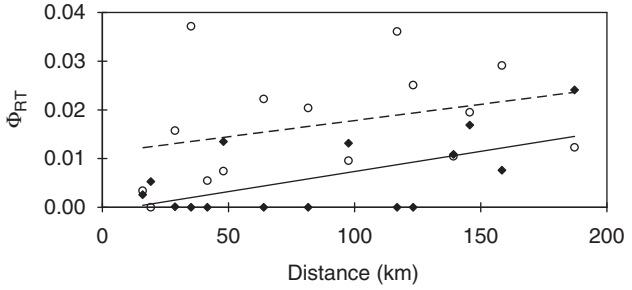

Figure 2 Geographic distance vs genetic divergence among pairs of rivers (Φ_{RT}) historically (1938, filled diamonds) or recently (2001–2005, empty circles) for coho salmon in six rivers around Puget Sound, Washington. The lines are regression historically (solid line) or recently (dashed line).

longer significant (Figure 2, empty circles and dashed line; $r=0.322$, Mantel test $P=0.127$) and on an average, distances were larger than they were historically. Removal of the modern NF Nooksack River sample (NOnf01) did not qualitatively affect these results.

In the hierarchical AMOVA, over 95% of genotype variation was between fish within samples, but the variance in genotype frequencies increased within and among rivers over time (Table 3). The structure was not significant between the historical and modern groups ($\Phi_{RT}=0.001$), but the structure among rivers was significant both historically ($\Phi_{RT}=0.008$) and during contemporary periods ($\Phi_{RT}=0.014$). Similarly, the differentiation between samples within rivers was significant in the historical ($\Phi_{SR}=0.015$) and modern samples ($\Phi_{SR}=0.039$). The increased divergence within and among rivers recently was largely due to a single population that has become highly diverged over time. The 2001 NF Nooksack River sample was previously identified as an outlier, and here, it accounted for a large proportion of the within-river variation among modern samples; removing this single sample from the combined samples across both time periods reduced the variation among sites within rivers ($\Phi_{SR}=0.040$ vs $\Phi_{SR}=0.031$), which was also observed among the modern samples ($\Phi_{SR}=0.039$ vs $\Phi_{SR}=0.028$).

A multidimensional scaling plot of the genotype distances revealed that the historical and modern samples overlapped in their distributions, except for the previously mentioned 2001 NF Nooksack River (Figure 3). The pairwise genotype distances between locations changed over time, such that there was no relationship between historical and modern comparisons between the same locations (Figure 4; $r=-0.099$, Mantel test $P=0.331$). One notable change was that the NF Nooksack River sample was consistently more diverged from other locations recently than it was historically (white diamonds, Figure 4).

The Bayesian analysis of individual admixture indicated that there were most likely 11 theoretical populations among the historical and current Puget Sound coho salmon samples (Figure 5). The theoretical populations were not equivalent to any real population; therefore, we interpreted assignments to the same theoretical population as indicating a shared common ancestry, that is, alleles identical by descent. Each historical and modern sample partially assigned to all 11 theoretical populations, and rarely did the assignment percentages exceed 50%; therefore, only the theoretical population to which the largest percentage of the genome assigned and the percent assignment are shown for each sample (Figure 5). The predominant assignment for the historic samples was different for each sample, but the modern samples were predominantly one of five different origins, and most current populations cluster on the basis of a history of mutual exchange. The Skagit River was the most common source for fish introduced into the Nooksack and Stillaguamish Rivers in the north (Eldridge and Naish, 2007), and the 2001 SF Nooksack sample, both Stillaguamish samples and three of the Skagit samples were predominantly one of two groups observed in two historic Skagit samples (either SGjk38 or SGdi38). The Green River was the most common source for fish introduced into the Puyallup River in the south (Eldridge and Naish, 2007), and the four Green and Puyallup River samples were predominantly of the group that dominated in the Green River historically. The two NF

Table 3 Hierarchical analysis of genotype frequency variation among groups (Φ_{RT}), among sample sites within groups (Φ_{SR}), and among all sample sites (Φ_{ST}) for coho salmon around Puget Sound, Washington

Samples	*Group*	Φ_{RT}	Φ_{SR}	Φ_{ST}	*Among groups (%)*	*Among sites within groups (%)*	*Within sites (%)*
Complete (29)	h/m (2)	0.001	0.040***	0.041***	0.10	3.98	95.92
Historical (13)	River (6)	0.008**	0.015***	0.023***	0.79	1.50	97.71
Modern (16)	River (6)	0.014***	0.039***	0.052***	1.37	3.84	94.79
Complete w/o Nonf01 (28)	h/m (2)	0.003**	0.031***	0.033***	0.26	3.06	96.68
Modern w/o NOnf01 (15)	River (6)	0.010***	0.028***	0.038***	1.04	2.76	96.20

Groups were defined by period (historic (h, 1938) or modern (m, 2001–2005)) or river. Modern samples were considered with and without the outlier 2001 NF Nooksack (NOnf01) sample. The number of samples and the number of groups are in parentheses.
, * Values significantly different from zero (**$P \leqslant 0.01$, ***$P \leqslant 0.001$).

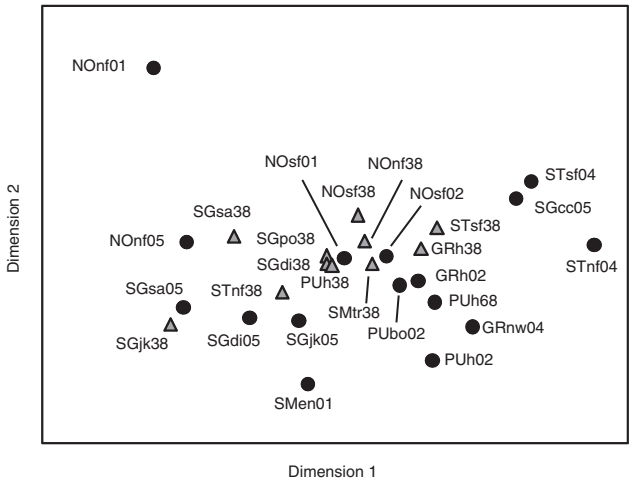

Figure 3 The first two dimensions of a multidimensional scaling plot based on pairwise Φ_{ST}, an analog of F_{ST} based on genotype diversity, of coho salmon around Puget Sound, Washington sampled in 1938 (gray triangles) or from 1968–2005 (black dots) and calculated from allele frequencies at eight microsatellite loci. Not included are the GRdm38, NOmf38 and NOmf01 samples, which were of small size (fewer than 15 fish).

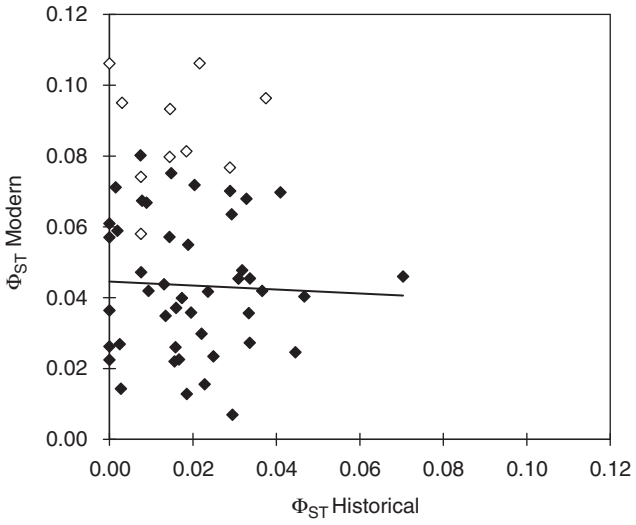

Figure 4 Historical (1938) vs modern (2001–2005) genotype divergence among pairs of locations (Φ_{ST}, an analog of F_{ST} based on genotype diversity) for coho salmon from six rivers around Puget Sound, Washington (filled symbols). Empty symbols indicate pairwise divergences with the outlier NF Nooksack population. The line is the regression among all samples.

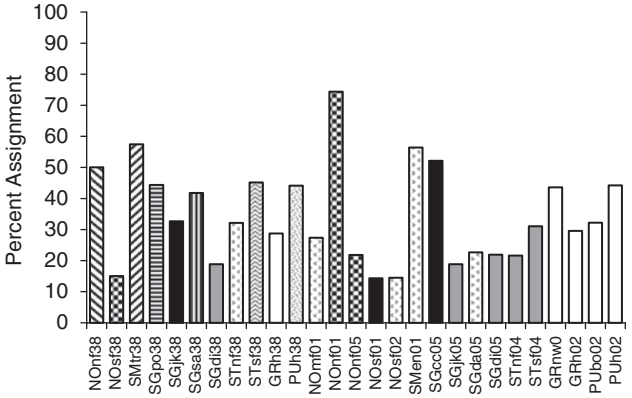

Figure 5 Theoretical population to which the largest percentage of the genome was assigned (indicated by pattern or shading of bar) and the percent assignment for historical and modern samples of Puget Sound coho salmon. A Bayesian assignment method (BAPS v.4.14) 'trained' using the samples from 1938 was used to create the 11 theoretical populations (each indicated by a unique pattern or shading of bar) and to perform assignments. Sample labels are as in Table 1. Each of the samples from 1938 predominantly assigned to a different theoretical population, but the 2001–2005 samples predominantly assigned to one of five theoretical populations.

Nooksak samples may be of native ancestry because they were predominantly of the same group that was dominant in the historic SF Nooksack. A grouping that is not easily explained by exchange history included the MF Nooksack sample, one SF Nooksack sample, the Samish sample, and the Skagit Sauk sample and the historical NF Stillaguamish sample, This anomalous grouping may reflect introgression after transfers from a river, in addition to the six rivers considered (Eldridge and Naish, 2007).

Discussion

Our results revealed changes in genotype diversity and fine-scale population structure among coho salmon within and between neighboring rivers around Puget Sound, Washington since 1938. The heirachical AMOVA, multidimensional scaling plot of genetic distances (Figure 3), assignment tests (Figure 5) as well as an earlier study (Eldridge and Naish, 2007), all indicated that the current populations of coho salmon in the Puget Sound ESU are descended from historical populations within the ESU. Historically, there was a pattern of reproductive isolation by geographic distance that

remains but is no longer statistically significant (Figure 2), and we found no relationship of genetic distances in the historical and modern comparisons between populations sampled at the same locations (Figure 4). Rather, there currently exists a significant relationship between genetic distance and transfer history (Eldridge and Naish, 2007). In addition, populations in different rivers share more of their ancestry recently than they did historically, and generally cluster on the basis of a history of mutual exchange (Figures 3 and 5). Coho salmon lost allelic diversity as well, suggesting that genetic drift increased due to a severe bottleneck in many populations. The hierarchical AMOVA also indicated that populations in different rivers are more diverged now than they were historically, as are different populations in the same river, which could result from an increased genetic drift within populations. There are two hatchery practices that may account for these changes in genetic diversity: transfers between hatcheries, which could alter patterns of gene flow and pooling the milt of multiple parents, which could reduce genetic diversity (that is, the Ryman–Laikre effect Ryman and Laikre, 1991). Similar changes in population structure have been observed in anadromous fish species subject to migration from genetically diverged populations after hatchery transfers (Nielsen *et al*., 1997; Hansen, 2002). At the same time, finding what seems to be a remnant population in the NF Nooksack River is additional evidence that habitat partitioning may allow some native populations to resist swamping by large releases of nonnative fish and heavy exploitation (Nielsen *et al*., 2001; Hansen, 2002).

Null alleles or allelic dropout due to poor DNA quantity and quality is a concern with historical samples (Nielsen *et al*., 1999). In addition, excess homozygote classes due to pooling diverged populations (a Wahlund effect) might have affected the interpretation of the results. However, we used tests that do not rely on an assumption of the Hardy–Weinberg equilibrium, namely, AMOVA based on genotype frequencies, allelic richness tests and genotype frequencies (Φ-*statistics*) as opposed to allele frequencies (F-*statistics*). Repeating the analyses with and without the poor quality 1938 NF Nooksack River sample did not detectably alter the results of the AMOVA and isolation by distance tests, suggesting that excess homozygote classes in the archived samples did not substantially bias the results. In any case, it is unlikely that null alleles or allelic dropout caused systematic errors in allele or genotype frequencies, leading to the results reported here.

The finding of isolation by distance in the historical but not the contemporary samples was likely not an artifact of outlier comparisons. There were seven Φ_{RT} values equal to zero among the historical samples (Figure 2). In total, four of these pairwise comparisons involved the Stillaguamish River, which is in the geographic middle of the rivers comprising the ESU, and therefore may have naturally exchanged a number of fish with rivers to the north and south (Figure 1). There was also a large genetic distance between the following two geographically close modern samples: the Stillaguamish and Samish rivers (Figure 2). Removing all pairwise comparisons associated with the modern Stillaguamish or Samish rivers did not change the results of the Mantel test for isolation by distance (results not shown), indicating that these samples had little effect on the conclusions. In addition, a simple comparison of the pairwise genetic distances between historical and modern samples (Figure 4) showed that population structure had likely changed over time.

Previously, it was reported that rivers that exchanged a large number of fish were the most similar, and it was hypothesized that the release of millions of coho salmon may have contributed to the homogenization of populations within rivers (Eldridge and Naish, 2007). However, coho salmon populations within rivers are more diverged recently (Φ_{SR} Table 3). There are two possible explanations for this outcome. First, the number of modern populations in the previous study was greater than that in this study and compared hatchery populations directly with wild populations. This study only included samples that were collected at the same locations as those of the historical populations, and may thus have a reduced resolution at this scale. Second, it seems that some populations were resistant to transfers and releases of cultured fish. The NF Nooksack River population became increasingly diverged from other populations, suggesting that cultured fish released into the wild may affect only some populations within a river. Previously, Small *et al.* (2004) postulated that behavioral and environmental forces might favor the reproductive isolation of certain wild coho salmon populations that do not experience direct hatchery activities, despite large numbers of cultured releases within 12 river kilometers. It is possible that culture practices can increase gene flow between donor and recipient populations, but nearby populations in the same river may remain isolated if they are not targeted by intentional cultured releases.

A fundamental question remains: Without the hatcheries, would historical and current populations of coho salmon from the same location be more closely related and would the current isolation-by-distance pattern be significant? A temporal stability of population structure has been observed in a number of taxa including marine, anadromous and freshwater fishes, (Nielsen *et al*., 1997; Tessier *et al*., 1997; Hansen *et al*., 2002; Bernal-Ramirez *et al*., 2003) and amphibians (Hoffman *et al*., 2004), often despite severe population bottlenecks or migration due to transferring genetically distinct populations. But stability of local population structure is by no means universal (Ruzzante *et al*., 2001), and depends on the spatial and temporal scales considered (Whitlock, 2004). It seems reasonable to assume that the population structure of long-standing populations of coho salmon should be stable over 60 years or 20 generations, in part because it takes many generations of reproductive isolation for the population differences in allele and genotype frequencies to evolve (Allendorf and Luikart, 2007). Hence, it is important to determine the natural factors and human actions that can disrupt population structure. Extinction and recolonization within a metapopulation is one natural factor (Whitlock, 2004), and differences may be observed between nuclear and mitochondrial DNA if there are sexual differences in philopatry (Bernal-Ramirez *et al*., 2003), but it is not clear whether bottlenecks or high levels of natural gene flow also lead to an instability of population structure (Ruzzante *et al*., 2001; Hoffman *et al*., 2004).

We believe that artificial culture practices including transfers and supportive breeding are the most likely mechanisms by which the pattern of gene flow and levels

of genotypic diversity among Puget Sound coho salmon might be altered. Clearly, human actions such as hatchery supplementation and transfers can have dramatic effects on the structure of wild populations (Nielsen *et al*., 2001; Hansen, 2002), but again, not always (Ford *et al*., 2004; Ruzzante *et al*., 2004). Studies that have detected adverse effects after transfers usually have been on systems in which many fish were transferred and released (hundreds of thousands to millions), in which transfers occurred for a long time (>10 years) or involved donor populations that were closely related to the recipient population (this study, Nielsen *et al*., 2001; Hansen, 2002). Systems in which negative effects after transfers were restricted to a small area or were absent usually involved fewer fish (tens of thousands), were over a shorter duration (<10 years) or involved source populations that were highly diverged from native populations (Ford *et al*., 2004; Ruzzante *et al*., 2004).

Harvest and habitat changes in the form of urbanization and logging have been extensive in the Puget Sound area (Pess *et al*., 2002), and population bottlenecks can affect genetic diversity (Hauser *et al*., 2002). Harvest and habitat changes may have contributed to population size decreases and reduced gene flow, and therefore reduced genotypic diversity within populations and increased the genetic distance between populations. However, neither harvest nor habitat changes seem solely sufficient to explain the changed population structure observed among rivers. We expect that fish dislocated from their natal sites by habitat changes would most likely seek out a suitable habitat nearby, and thus would likely blur but not disrupt an isolation by a distance pattern of gene flow (Leider, 1989). Moreover, separating harvest effects from the effects of hatcheries may be difficult, high harvest rates would not have been sustainable without the benefit of hatchery supplementation. For example, starting in the 1960s, escapement goals for some rivers were set to the amount needed to meet the broodstock goals for the local hatchery, about 10% of the total number of adults for some Puget Sound rivers (Nehlsen *et al*., 1991).

The available demographic data do not suggest a substantial decrease in the number of fish reaching maturity over time (Weitkamp *et al*., 1995); however, there is a reliance on hatcheries for a substantial portion of the production of adult coho salmon (over 50% of all adults in Puget Sound are born in hatcheries (Weitkamp *et al*., 1995)). We hypothesize that the dominant spawning population shifted from wild to hatchery and that this shift was coupled with poor hatchery practices such as pooling milt of multiple males, which may have led to a reduction of allelic diversity due to a decrease in the effective size within rivers (Ryman and Laikre, 1991; Eldridge and Killebrew, 2007).

Salmon hatchery operations started in Puget Sound in the 1890s, which may have altered the coho salmon population structure before the time the historical samples were collected in 1938. However, our study showed changes in population structure and genetic diversity between 1938 and the present, a finding that does not depend on the knowledge of the unaffected population structure. In addition, it was common hatchery practice to release coho salmon as presmolts (fry and fingerlings) before the 1940s. These releases were generally not successful, and it is unlikely that in the presence of healthy wild coho salmon populations, the hatchery-origin fish would have had much influence on the genetic variation (Kelez, 1937; Flagg *et al*., 1995).

The actual ancestries of the modern population could not be determined using the Bayesian assignment tests, although this is not surprising. Even in the absence of transfers, it may be very difficult to assign fish when populations are weakly diverged (Latch *et al*., 2006). In addition, population structure has changed from a relatively clean isolation-by-distance pattern of gene flow to one in which it is influenced by both natural migration and hatchery-mediated gene flow, which can make ancestry more difficult to resolve. Introductions have occurred from outside the Puget Sound coho salmon ESU, but despite these transfers, all contemporary Puget Sound coho salmon populations examined here are closely related to the historical populations we sampled within the ESU. Thus, there is no strong evidence that a highly differentiated exogenous population has substantially introgressed with, or replaced, the native populations, supporting observations in other systems (Utter, 2001).

Here, we showed that fine-scale population structure and gene flow have changed within a coho salmon ESU that experienced the release of hundreds of millions of fish over a long time period. This finding adds to a previous study, in which we showed that the numbers of fish transferred and released affected the coho salmon population structure (Eldridge and Naish, 2007). Fisheries propagation as a tool in restoration may become increasingly important (Leber *et al*., 2004), and interactions with wild populations may be inevitable (Naish *et al*., 2007). The evolutionary importance of metapopulation structure and gene flow has long been recognized for enabling adaptation through trial and error (Wright, 1932), for providing novel genetic diversity to counteract the effects of inbreeding by genetic drift (Mills and Allendorf, 1996) or for maintaining the long-term viability of the species in the face of environmental change (Hilborn *et al*., 2003). Conservation management plans should recognize the potential effects of culture on gene flow and the fine-scale structure of the target species, and recovery approaches should use fish from the target population as much as possible.

Acknowledgements

We thank S Karren and the staff at the National Archives, Pacific Alaska Region, for their assistance in locating and retrieving the historical samples from the Bureau of Fisheries Files, as well as M McClure, D Schindler, F Utter, L Hauser and four anonymous reviewers for their comments. This publication was partially funded by the Joint Institute for the Study of the Atmosphere and Ocean (JISAO) under NOAA Cooperative Agreement No. NA17RJ1232, Contribution 1429. WHE was supported by a grant from Washington Sea Grant, University of Washington, pursuant to National Oceanic and Atmospheric Administration Award No. NA05OAR41711742, Project No. E/I-11. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or of any of its subagencies.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)