

Genetic analysis of juvenile Chinook salmon collected in White Salmon River

Laboratory report for FONS 13210-2006-062

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Background

It is expected that Condit Dam will be removed starting in fall 2009, and that Chinook salmon (*Oncorhynchus tshawytscha*) populations that presently spawn below Condit Dam will be impacted. The present population structure, and the relationships between populations in White Salmon River and those in adjacent National Fish Hatcheries (NFH) is unclear.

This laboratory report describes methods used to genotype Chinook salmon samples collected in White Salmon River and to perform mixture analyses on those samples. A broader report that incorporates further analyses of the genetic data and integration of the genetic results with other data types is expected to follow. The purpose of the present report is to provide an update regarding the FONS-funded genetic analysis to the Condit Dam Removal Workgroup.

Work to be completed (as listed in FY07 statement of work)

- Process 13 microsatellite loci (Table 1) in ~1000 samples (depending on the number of 2007 smolts):
 - Smolts from rotary trap 2006 (USGS) 435
 - Smolts from rotary trap 2007 (USGS) ~400
 - Hood River 1992-2006 (ODFW) 150
 - Clackamas River 6

- Assemble Baseline
 - Add new pops (Hood River and Little White Salmon NFH)
 - Standardize new alleles (observed by CRITFC)
- Conduct power analysis of Baseline (simulations, blind assignment of 100 individuals)
- Assess genetic relationships between baseline populations
- Assign analyzed samples to reporting groups
- Write final genetics lab report

Methods and Materials

Samples processed at Abernathy Fish Technology Center (AFTC) to date include 427 juveniles collected from White Salmon River in 2006 and 612 juveniles collected in 2007. We also analyzed 150 adult samples collected from Hood River between 2001 and 2006.

Microsatellite Analysis

Genomic DNA was extracted from each individual using Chelex resin (Biorad) and the protocol described by Small et al. (1998). Thirteen microsatellite loci (Table 1) were amplified in each sample using the polymerase chain reaction (PCR). Reaction conditions, thermal cycling profiles and PCR product pooling protocols are listed in Appendix 1. Liquid handling was performed using a HydraII (ThermoFisher Scientific).

Raw microsatellite data (electropherograms) were analyzed using GENEMAPPER version 4.0 (Applied Biosystems). All genotypes were scored by two independent readers (double-scoring). Following completion of the data collection, 10% of all samples were re-analyzed as part of AFTC's QA/QC protocol.

It was noted during the analysis that some individual fish were yielding alleles that had not been previously described for Chinook salmon. For the present marker set and baseline, this made it highly likely that the individuals in question were not Chinook salmon. Results that led to this conclusion included out-of-range alleles, fixed alleles at loci that are usually highly polymorphic, and atypical peak morphologies for several loci. Six of these samples (614-085, 614-076, 614-088, 614-092, 660-010, and 660-019) plus four samples sent to AFTC by the Lower Columbia Fish Health Center (WS 1-4) and reference samples of Chinook salmon (*O. tshawytscha*; AFTC 07-80217 A) and steelhead (*O. mykiss*; AFTC 1039-004) were sent, without labels, to Dr. Linda Park at NOAA Fisheries Northwest Fisheries Science Center. Dr. Park performed sequencing analysis of the cytochrome oxidase III and NADH dehydrogenase 3 (COIII/ND3) region of the mitochondrial DNA. Based on the results she correctly identified the two reference samples, and identified nine of the ten other samples as coho salmon (*O. kisutch*). The tenth sample was not successfully analyzed. We assumed that all 128 fish in the dataset that exhibited the atypical alleles were coho salmon and excluded them from subsequent analyses.

Table 1. Thirteen microsatellite loci standardized by the Genetic Analysis of Pacific Salmonids (GAPS) consortium (Seeb et al. 2007).

Locus	Primer sequence (5' to 3')	Citation
<i>Ots201b</i>	F- CAGGGCGTGACAATTATGC R- TGGACATCTGTGCGTTGC	OSU unpublished
<i>Ots208b</i>	F- GGATGAACTGCAGCTTGTTATG R- GGCAATCACATACTTCAACTTCC	Greig et al. 2003
<i>Ots211</i>	F - TAGGTTACTGCTTCCGTCAATG R - GAGAGGTGGTAGGATTTGCAG	Greig et al. 2003
<i>Ots212</i>	F- TCTTCCCTGTTCTCGCTTC R- CCGATGAAGAGCAGAAGAGAC	Grieg et al. 2003
<i>Ogo4</i>	F- GTCGTCACTGGCATCAGCTA R- GAGTGGAGATGCAGCCAAAG	Olsen et al.1998
<i>Ogo2</i>	F- ACATCGCACACCATAAGCAT R- GTTCTTCGACTGTTTCTCTGTGTTGAG	Olsen et al. 1998
<i>Ots3M</i>	F- TGTCACTCACACTCTTTCAGGAG R- GAGAGTGCTGTCCAAAGGTGA	Banks et al. 1999
<i>Ots213</i>	F- CCCTACTCATGTCTCTATTTGGTG R- AGCCAAGGCATTTCTAAGTGAC	Greig et al. 2003
<i>Omm1080</i>	F- GAGACTGACACGGGTATTGA R- GTTATGTTGTCATGCCTAGGG	Rexroad et al. 2001
<i>Ssa408UOS</i>	F- AATGGATTACGGGTACGTTAGACA R- CTCTTGTGCAGGTTCTTCATCTGT	Cairney et al. 2000
<i>Ots9</i>	F- ATCAGGGAAAGCTTTGGAGA R- CCCTCTGTTACAGCTAGCA	Banks et al. 1999
<i>OtsG474</i>	F- TTAGCTTTGGACATTTTATCACAC R- CCAGAGCAGGGACCAGAAC	Williamson et al. 2002
<i>Oki100</i>	F- CCAGCACTCTCACTATTT R- CCAGAGTAGTCATCTCTG	unpublished

Columbia River genetic baseline

The Columbia River portion of the standardized multi-agency baseline (Seeb et al. 2007) was used for this work. Additionally, we used unpublished data for several Columbia River populations provided to us for this analysis by Columbia River Inter-Tribal Fish Commission (CRITFC) as well as data for Hood River (collected as part of the present work). In total, the baseline used here contained samples from 54 populations (Table 2).

Sub-division of White Salmon River samples

In order to evaluate the possibility of multiple populations within the White Salmon River samples, we used GENECLASS2 (Piry et al. 2004) to calculate the probability that the multi-locus genotype of each individual originated from each of the 54 baseline populations (Ranalla and Mountain 1997). Individuals with $\geq 90\%$ probability of originating from one of the baseline populations were assigned to the corresponding population. Assigned samples were sorted by collection date and we examined the data for discontinuities associated with the time during which few samples were collected in 2007 (the first half of May).

Genetic diversity observed in White Salmon River

We compared genetic diversity in White Salmon River to that in Spring Creek NFH, Little White Salmon NFH and other baseline samples within the Lower Columbia River. Number of alleles per locus were summed for each collection, and we examined the data for alleles observed in White Salmon River that were not present in Spring Creek NFH or Little White Salmon NFH. FSTAT (Goudet 2001) was used to calculate allelic richness (number of alleles per population, corrected for sample sizes) for each population.

Table 2. Populations and reporting groups in Columbia River genetic baseline. Mixture analysis accuracy is the proportion of simulated fish, in a mixture analyses of fish from that population, that were correctly assigned back to that population and the associated reporting group.

	Population	Reporting Unit	Mixture analysis accuracy	
			To population	To reporting group
1	Cowlitz Hat. (fall)	LowCol	0.9537	0.9953
2	Lewis R. (fall)	LowCol	0.8796	0.9938
3	Sandy R. (fall)	LowCol	0.9505	0.9922
4	Cowlitz Hat. (spring)	LowCol	0.9804	0.9928
5	Kalama Hat. (spring)	Willamette	0.9716	0.9729
6	Lewis Hat. (spring)	LewisHsp	0.9578	0.9578
7	McKenzie Hat. (spring)	Willamette	0.9586	0.9988
8	Hood River (fall)	LowCol	0.9574	0.9718
9	N. Santiam Hat. (spring)	Willamette	0.9605	0.9987
10	Little White Salmon NFH (fall)	MidupColOT	0.8268	0.9549
11	Little White Salmon NFH (spring)	upColST	0.8929	0.9453
12	Spring Cr. Hat. (fall tule)	LowCol	0.9857	0.9994
13	upDeschutes R. (summer)	DeschutesOT	0.9623	0.9882
14	lowDeschutes R. (fall)	DeschutesOT	0.8725	0.9048
15	Carson Hat. (spring)	upColST	0.9199	0.9570
16	Warm Springs Hat. (spring)	midColST	0.9952	0.9963
17	Klickitat R. (spring)	midColST	0.8791	0.8811
18	Klickitat R. (summer)	MidupColOT	0.5317	0.9165
19	Klickitat R. (fall)	MidupColOT	0.6327	0.9547
20	Shitike Cr. (spring)	midColST	0.9914	0.9921
21	John Day R. (spring)	midColST	0.9418	0.9425
22	Yakima Hat. (spring)	YakimaST	0.9942	0.9942
23	Wenatchee R. (spring)	upColST	0.9400	0.9721
24	Methow R. (spring)	upColST	0.8664	0.9296
25	Entiat R. (spring)	upColST	0.9835	0.9891
26	Hanford Reach (fall)	MidupColOT	0.8634	0.9495
27	Priest Rapids Hat. (fall)	MidupColOT	0.7655	0.9636
28	Wells Hat. (fall)	MidupColOT	0.8298	0.9816
29	Methow R. (summer)	MidupColOT	0.8170	0.9863
30	Tucannon R. (spring)	TucST	0.9955	0.9955
31	Imnaha (spring)	SFSalST	0.9610	0.9643
32	Minam R. (spring)	RapCWST	0.9068	0.9717
33	Lostine R. (spring)	LostST	0.9934	0.9934
34	Catherine Cr. (spring)	RapCWST	0.9055	0.9846
35	Lyons Ferry Hat. (fall)	SnakeOT	0.8051	0.9178
36	Clearwater R. (fall)	SnakeOT	0.7291	0.8910
37	Nez Perce Tribal Hat. (fall)	SnakeOT	0.7919	0.9205
38	Lolo Cr. (spring)	RapCWST	0.8023	0.9720
39	Newsome Cr. (spring)	RapCWST	0.8278	0.9946

		Mixture analysis accuracy		
		To	To reporting	
	Population	Reporting Unit	population	group
40	Dworshak Hat. (spring)	RapCWST	0.7876	0.9544
41	Red River (spring)	RapCWST	0.7980	0.9550
42	Powell Trap (spring)	RapCWST	0.8176	0.9817
43	S. Fork Clearwater R. (spring)	RapCWST	0.8842	0.9922
44	Rapid River Hat. (spring)	RapCWST	0.9256	0.9975
45	Big Creek a (spring)	MFSalST	0.9497	0.9712
46	Big Creek b (spring)	MFSalST	0.9641	0.9843
47	Johnson Cr. (spring)	SFSalST	0.9469	0.9915
48	Secesh R. (spring)	SFSalST	0.9660	0.9752
49	McCall Hat. (spring)	SFSalST	0.8917	0.9827
50	Sawtooth Hat. (spring)	upSalST	0.9652	0.9781
51	W. Fork Yankee Fork (spring)	upSalST	0.9726	0.9907
52	E. Fork Yankee Fork (spring)	upSalST	0.9583	0.9867
53	Pahsimeroi Hat. (spring)	upSalST	0.9603	0.9777
54	Marsh Cr. (spring)	MFSalST	0.8749	0.8811

Genetic divergence among populations

Component Analysis was performed using the program GENETIX (Belkhir et al. 2004) to reduce the genotype matrix to three dimensions and allow visual inspection of the baseline data.

Divergence among populations in the Lower Columbia River and between each of the stocks from Little White Salmon NFH and their nearest neighbors was examined using a test for allele frequency heterogeneity in GENEPOP (Raymond & Rousset 1997). The fixation index F_{ST} was calculated between each pair of populations using ARLEQUIN (Excoffier et al. 2005). Statistical significance of pairwise F_{ST} estimates were tested using a permutation procedure with 20,000 replicates.

Mixture analysis

The method of Ranalla and Mountain (1997) was used to assess the genotype probabilities in each population, as implemented in GMA (Kalinowski 2003). Prior to performing mixture analysis on the samples collected at White Salmon River, we tested the accuracy of the baseline using simulations and a blind sample. The simulations involved generating a mixture of 400 fish from

one population and then performing mixture analysis on those 400 fish and observing how many assigned back to the population used to generate them. If the baseline were powerful enough to allow perfect mixture analysis, then all 400 fish (100%) would assign back to the correct population. This was repeated 1,000 times for each population, and the mean proportions assigned back to the correct population are listed in Table 1.

Simulations may provide overly optimistic estimates of accuracy, so it is desirable to also test the baseline using blind samples, or, fish who are not in the baseline but whose true origin is known. To do this we randomly removed from the baseline 30 fish from Spring Creek NFH, 30 fish from Little White Salmon NFH fall run, 30 fish from Little White Salmon NFH spring run, and 10 fish from Cowlitz Hatchery. Mixture analysis was then performed on these 100 fish.

Finally, mixture analysis was performed on the juvenile samples collected from White Salmon River.

Results

Microsatellite Analysis

Following removal of failed samples, ambiguously labeled samples and coho salmon samples, the number of Chinook salmon genotyped at the time of this writing was 1,061: 313 White Salmon River samples from 2006, 608 White Salmon River samples from 2007, and 140 samples from Hood River. The PCR failure rate for this data set was ~1.5%, indicating that tissues were of high quality and the PCR protocols were robust. Ten conflicts were observed among 2,080 QC/QA genotypes, suggesting an error rate of ~0.5%.

Sub-division of White Salmon River samples

Of the 921 White Salmon River juveniles assigned to population using GENECLASS, 437 (47%) assigned to a baseline population with > 90% probability. In 2006, 165/170 (97%) of fish that were collected from March through April assigned to Spring Creek NFH and other populations in the LowCol reporting group, but only 1/8 (13%) of fish collected in May assigned back to these groups. Likewise in 2007, 222/226 (98%) of individuals collected from March through April assigned to Spring Creek NFH and other populations in the LowCol reporting group, but only 1/33 (3%) of fish collected in May and June assigned back to these groups. These results led us to

divide the 2007 White Salmon River sample into two populations (early and late). For population analysis, the division was done as close as possible to the time suggested by assignment of the 2007 samples (first week of May). For mixture analysis, we wanted to make the 2006 and 2007 as comparable as possible and thus divided the 2007 sample when sampling ended in 2006 (May 18).

Genetic diversity observed in White Salmon River

The number of alleles per locus in White Salmon River ranged from 4 at *Ots9* in the early 2007 collection to 43 at *Omm1080* in all three White Salmon River collections (2006, 2007 early and 2007 late). Allelic richness estimates for the White Salmon River 2006 and early 2007 collections were generally (12/13 loci) slightly higher than those for Spring Creek NFH, but were also generally (9/13 loci) slightly lower than average values for the LowCol reporting group. Allelic richness estimates for the White Salmon River 2007 late collection were very similar to those for Little White Salmon NFH and to the rest of the MidupColOT reporting group. For all loci, the three White Salmon River samples exhibited allelic richness estimates with 1.96 SD of the average for populations in the corresponding reporting groups.

Genetic divergence among populations

Component analysis clustered the Chinook salmon baseline samples into three broad groups, including 1) lower Columbia fall / hatchery, 2) mid-upper Columbia River fall – summer, and 3) spring run Chinook salmon (Figure 1). Five spring runs (Kalama Hatchery, McKenzie Hatchery, North Santiam Hatchery, and Klickitat River) did not fit inside these groups, illustrating a broad amount of variability among spring run Chinook salmon. This analysis revealed similarities between each of the White Salmon River samples and an adjacent hatchery stock. The 2006 and early 2007 samples clustered near the Spring Creek NFH sample, and the late 2007 sample clustered with the mid and upper Columbia River fall populations (which include the Little White Salmon NFH fall Chinook salmon).

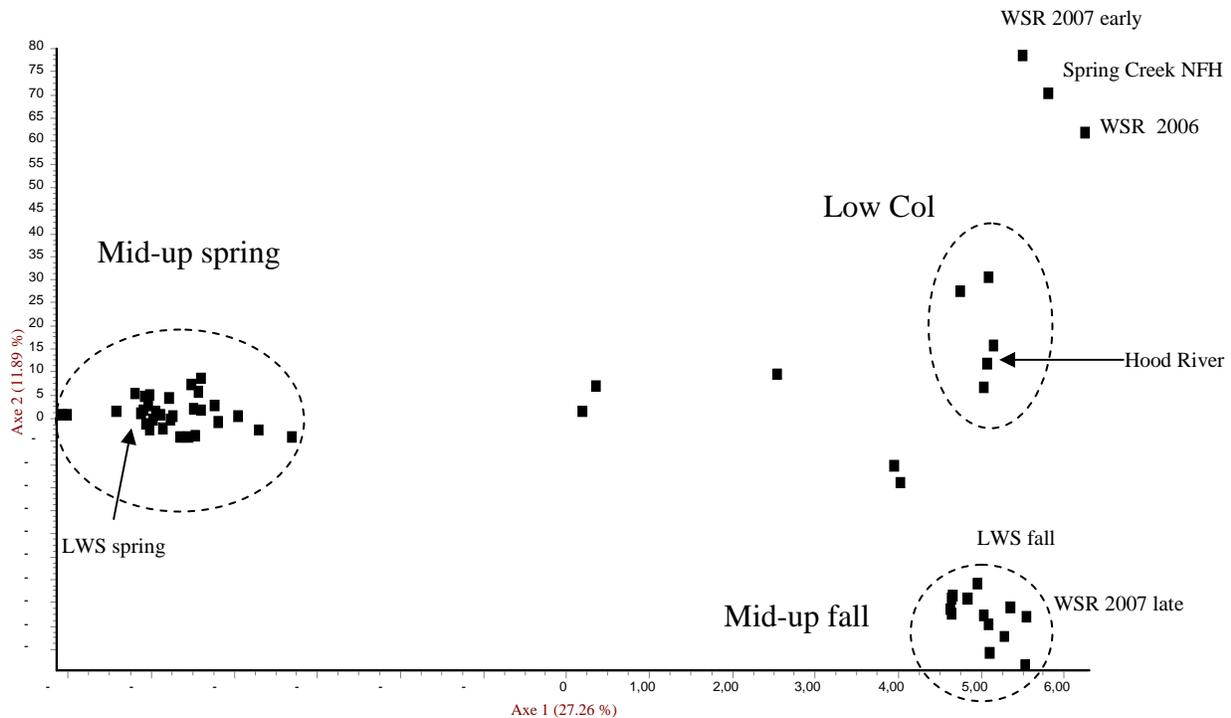


Figure 1. Correspondence analysis plot of 54 populations of Chinook salmon in the Columbia River based on 13 microsatellite DNA loci. Each square represents a population and the distance between each pair of squares is proportional to the genetic divergence between the corresponding populations. The early samples from White Salmon River (WSR 2006 & WSR 2007 early) are similar to the Spring Creek NFH sample, and the late sample from White Salmon River (WSR 2007 late) is similar several middle and upper fall populations, including the Little White Salmon NFH fall stock (LWS fall).

Heterogeneity tests revealed differences in allele frequencies between the 2006 and early 2007 White Salmon collections and between these and all baseline populations. The late 2007 sample was significantly different from all baseline populations except the Klickitat R. fall and summer Chinook. Pairwise F_{ST} between the 2006 and early 2007 was small (0.03) but significant ($P < 0.01$), but F_{ST} between each of these and the Spring Creek NFH were not significant. All other pairwise F_{ST} estimates involving these three collections were significant. Pairwise F_{ST} s between the late 2007 White Salmon River sample and several mid-upper Columbia fall populations (Klickitat R. (fall and summer), Hanford Reach, Priest Rapids Hatchery) were not significantly greater than zero.

Mixture analysis

Mixture analysis of simulated fish indicated that the mean accuracy to population was 89.7% (range 53.2% - 99.6%; Table 2). When populations were pooled based on genetic similarity into 15 reporting groups, mean accuracy rose to 96.8% (range 88.1% - 99.9%). An example of a group of populations for which mixture analysis accuracy was increased by pooling was the MidupColOT reporting group. In this case, accuracy to each population was below 90%, but simulated accuracy to the group was over 90% for each population. Assignment to the hatchery populations of primary interest to the present work suggested $>90\%$ accuracy to population for Spring Creek NFH and $<90\%$ accuracy for the two Little White Salmon NFH stocks. Accuracy to reporting group was $>90\%$ for all three stocks.

Mixture analysis of the blind samples suggested lower accuracies to reporting groups and populations than was suggested by the simulations (Table 3). Simulations are often overly optimistic as baseline samples are expected to be, on average, more divergent from one another than the populations that they represent are. Also an incomplete baseline will not impact simulation results but may well impact assignment of real fish. Another complication here is the baseline used to assign the blind samples was smaller than the true baseline (due to removal of the blind samples). The results of the blind sample analysis in the present study likely provide a conservative estimate of how accurate mixture analyses using the full baseline will be. We thus expect assignment to the LowCol and MidupColOT reporting groups to be accurate to within a couple of percent, but would be very cautious in interpretation of results regarding assignment to the upColST reporting group. Assignment to the specific hatcheries of interest was 6.2% off for

the relatively distinct Spring Creek NFH tules, but 16.5% off for the Little White Salmon fall stock (which appears more similar to other up river fall stocks; Fig 1).

Table 3. Mixture analysis results for samples of known origin, “blind samples”, removed from baseline. Reporting groups are defined in Table 2. Numbers in parentheses indicate the proportion assigned to NFH stocks of interest with each reporting group. For the LowCol reporting group this is the Spring Creek NFH tule stock, for MidupColOT this is the Little White Salmon NFH URB stock, and for upColST this is Little White Salmon NFH spring stock.

Reporting group	True proportion		Estimated proportion	
	To reporting group	To NFH Population	To reporting group	To NFH Population
LowCol	0.400	(0.300)	0.419	(0.238)
MidupColOT	0.300	(0.300)	0.258	(0.135)
SnakeOT	-		0.036	
midColST	-		0.025	
upColST	0.300	(0.300)	0.141	(0.125)
RapCWST	-		0.094	
MFSalST	-		0.013	
SFSalST	-		0.014	

Based on the simulations and blind tests we are confident in mixture analysis results that assign fish to reporting groups and are even somewhat confident in proportional assignments to Spring Creek NFH, but are less confident in this baseline to produce accurate proportional assignments to the Little White Salmon NFH.

As might have been predicted based on the individual assignments that led us to divide the 2007 sample from White Salmon River into two populations, early assignment in both years was predominantly to the LowCol reporting group and late assignment was predominantly to the MidupColOT reporting group (Table 4). The majority (68.5% – 72.1%) of early fish in both years

assigned to Spring Creek NFH, while a substantially smaller proportion (11.4%) of late fish in 2007 were assigned to Little White Salmon NFH.

Table 4. Mixture analysis results for juvenile Chinook salmon caught at White Salmon River. Reporting groups are defined in Table 2. Numbers in parentheses indicate the proportion assigned to NFH stocks of interest with each reporting group. For the LowCol reporting group this is the Spring Creek NFH tule stock, for MidupColOT this is the Little White Salmon NFH URB stock, and for upColST this is Little White Salmon NFH spring stock.

Reporting group	2006 early		2007 early		2007 late	
LowCol	0.894	(0.685)	0.937	(0.721)	0.027	
Willamette	0.003		0.003		-	
DeschutesOT	0.001		0.007		0.039	
upColST	-		0.011	(0.006)	-	
MidupColOT	0.088	(0.017)	0.013	(0.002)	0.769	(0.114)
SnakeOT	0.007		0.001		0.165	
RapCWST	0.003		0.025		-	
upSalST	-		0.003		-	

Conclusions

Simulations and analysis of blind samples indicated that the standardized Chinook salmon microsatellite baseline provides relatively accurate estimates of mixture composition to 15 reporting groups within the Columbia River. Of particular relevance to the present study, the Lower Columbia (LowCol) and middle up river bright (MidupColOT) groups were estimated to within a few percent of true values. Accuracy of mixture analysis to upper Columbia stream type (upColST) was lower, as was accuracy to individual populations within the 15 reporting groups.

The present data support the existence of two populations of Chinook salmon in the White Salmon River. One population, which we have designated “early” based on the relative out-migration time, appears genetically similar to fall tule runs and in particular to the tule stock at Spring Creek NFH. The second population, which we have called “late” here, appears genetically similar to fall stocks from the middle and upper Columbia River. Diversity within each of these populations is comparable to that in other tule and URB stocks, respectively. The numbers of alleles observed and allelic richness estimates do not support hypotheses that the number of successful spawners in the White Salmon River is smaller than in other populations. Divergence between these stocks is large relative to the total diversity of Chinook salmon within the Columbia River.

Little variation was observed between years in the early White Salmon River population. This could reflect stability of the population (i.e. effective population size large enough to prevent major allele frequency changes due to drift) and a substantial influence on this stock by Spring Creek NFH.

In the two years of samples examined here, transition between the two populations for out-migrating smolts took place in the first two weeks of May.

Acknowledgements

The results described here represent one piece of an ongoing collaborative study, and a broader account including contributions from several other groups will be described elsewhere. Samples from White Salmon River were collected by a United States Geological Survey team lead by Pat Connolly and Brady Allen. Hood River samples were collected by Eric Olsen and Rod French (Oregon Department of Fish and Wildlife). Don Campton, Larry Merchant and Speros Doulos provided insight and support throughout the study. We are grateful to Shawn Narum (Columbia River Inter-Tribal Fisheries Commission) for providing unpublished data that we included in the baseline used for this project. We are also grateful to Linda Park and Anna Elz (NOAA Fisheries) for performing genetic species identifications.

The views expressed here are those of the authors and do not necessarily represent those of the United States Fish and Wildlife Service.

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Appendix 1: Details for the amplification and pooling of thirteen microsatellite loci.

Reaction conditions (per 96-well plate):

Master Mix Components	2.0 mM MgCl ₂ ¹	1.75 mM MgCl ₂ ²
dH ₂ O	7.4	7.55
5X Buffer	3.0	3.0
25 mM MgCl ₂	1.2	1.05
10 mM dNTPs	0.3	0.3
10 μM Forward Primer	0.5	0.5
10 μM Reverse Primer	0.5	0.5
Go Taq	0.1	0.1
Total Volume	13	13

¹ *Ogo2, Ots208b, Ots212, Ots9, OtsG474, Ogo4, Ots213, Ots211*

² *Oki100, Omm1080, Ots3M, Ots201b, Ssa408*

Thermal cycling profiles:

Program ³	1	2	3	4	5	6	7	Loci
49C-36X	94°C, 3:00	94°C, 0:15	49°C, 0:30	72°C, 1:00	Go to 2, 36x	72°C, 30:00	25°C, for ever	Ots3M
54C	94°C, 3:00	94°C, 0:02	54°C, 0:30	72°C, 1:00	Go to 2, 37x	72°C, 7:00	25°C, for ever	Oki100, Ssa408
56C	94°C, 3:00	94°C, 0:02	56°C, 0:30	72°C, 1:00	Go to 2, 34x	72°C, 7:00	25°C, for ever	Omm1080, Ots201b
58C1-37X	94°C, 3:00	94°C, 0:02	58°C, 0:30	72°C, 1:00	Go to 2, 37x	72°C, 7:00	25°C, for ever	Ogo2, Ots212, OtsG474
58C2-34X	94°C, 3:00	94°C, 0:02	58°C, 0:30	72°C, 0:30	Go to 2, 34x	72°C, 7:00	25°C, for ever	Ots208b
60C2-30S	95°C, 5:00	94°C, 0:02	60°C, 0:30	72°C, 0:30	Go to 2, 37x	72°C, 10:00	25°C, for ever	Ots9
60C3-60S	94°C, 3:00	94°C, 0:02	60°C, 0:30	72°C, 1:00	Go to 2, 37x	72°C, 7:00	25°C, for ever	Ogo4, Ots213, Ots211

PCR product pooling:

Multiplex Set 1		Multiplex Set 2		Multiplex Set 3	
dH ₂ O	36.5 μL	dH ₂ O	36.5 μL	dH ₂ O	38 μL
Ots208b	5 μL	Omm1080	5 μL	Ssa408	5 μL
OtsG474	5 μL	Ots213	4 μL	Ots201b	2.5 μL
Ots212	1.5 μL	Ogo4	2.5 μL	Ots211	2.5 μL
Ogo2	1 μL	Oki100	2 μL	Ots3M	2 μL
Ots9	1 μL				
TOTAL	50 μL		50 μL		50 μL

