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ORIGINAL PAPER

Microsatellite variation reveals weak genetic structure and retention of genetic variability in threatened Chinook salmon (*Oncorhynchus tshawytscha*) within a Snake River watershed

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Abstract Pacific salmon (*Oncorhynchus* spp.) have been central to the development of management concepts associated with evolutionarily significant units (ESUs), yet there are still relatively few studies of genetic diversity within threatened and endangered ESUs for salmon or other species. We analyzed genetic variation at 10 microsatellite loci to evaluate spatial population structure and genetic variability in indigenous Chinook salmon (*Oncorhynchus tshawytscha*) across a large wilderness basin within a Snake River ESU. Despite dramatic 20th century declines in abundance, these populations retained robust levels of genetic variability. No significant genetic bottlenecks were found, although the bottleneck metric (M ratio) was significantly correlated with average population size and variability. Weak but significant genetic structure existed among tributaries despite evidence of high levels of gene flow, with the strongest genetic differentiation mirroring the physical segregation of fish from two sub-basins. Despite the more recent colonization of one sub-basin and differences between sub-basins in the natural level of fragmentation, gene diversity and genetic differentiation were similar between sub-basins. Various factors, such as the (unknown) genetic contribution of precocial males, genetic compensation, lack of hatchery influence, and high levels of current gene flow may have contributed to the persistence of genetic variability in this system in spite of historical declines. This unique study of indigenous Chinook salmon underscores the importance of maintaining natural populations in interconnected and complex habitats to minimize losses of genetic diversity within ESUs.

Keywords Chinook salmon · *Oncorhynchus tshawytscha* · Fine-scale genetic structure · Genetic diversity · Bottlenecks

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Introduction

Effective conservation requires the identification of biologically relevant units within species as the focus of management and legal protection efforts. The concept that has been adopted most generally for defining within-species diversity eligible for protection under the U.S. Endangered Species Act is that of the evolutionarily significant unit (ESU), originally proposed by Ryder (1986) and subsequently modified by many others (e.g., Waples 1991; Dizon et al. 1992; Moritz 1994; Crandall et al. 2000). Though determining the specific criteria for defining ESUs has been highly controversial, the common denominator of most definitions is the need to preserve units that are considered 'viable' and that maintain adequate diversity to ensure the future evolutionary potential of a species (see Fraser and Bernatchez 2001 for a thorough review).

Because of their 'conservation crisis' (Waples et al. 2004) and tremendous economic and cultural value, Pacific salmon (*Oncorhynchus* spp.) have been central to the development of management unit concepts in biology (Waples 1991, 1995; Fraser and Bernatchez 2001; Ford 2004). Over 50 ESUs have been defined for Pacific salmon in the lower 48 United States, based on information characterizing diversity in life history, morphology, behavior, habitat, and genetic variability among major stocks (see Ford 2004). Thus, ESUs often encompass wide geographic areas or regions that sustain relatively unique components of a species. However, the application of the ESU concept in Pacific salmon has not overshadowed the recognition that a great deal of diversity exists among local populations within ESUs that needs to be identified and managed at smaller scales (Waples et al. 2001). Local populations would not be expected to display as extensive reproductive isolation or as divergent morphological and ecological attributes as might be required for an ESU designation, but they are defined based on at least partial reproductive isolation from other groups and often exhibit some level of local-adaptation (Taylor 1991; Halupka et al. 2003). Thus, they are recognized as important components of diversity and practical units for management (Moritz 1994; Nielsen and Powers 1995; McElhany et al. 2000; Fraser and Bernatchez 2001; McClure et al. 2003a). At these smaller scales (i.e., 10's–100's kms), ecological processes (e.g., restricted dispersal) may take on increased importance in generating diversity, and information about habitat spatial structure and likely dispersal distances of salmonid species has been helpful in defining local population units (McElhany et al. 2000; McClure et al. 2003a). However, there is little genetic information to guide efforts to define local populations at the within-ESU scale in many salmonid species, particularly in indigenous populations unaffected by hatchery supplementation.

Pacific salmon are an especially interesting group for investigating population genetic diversity at small spatial scales because they possess several unique behaviors that may either promote or erode differences among local populations, and because of their conservation concern. Most Pacific salmon are anadromous, with the majority of individuals undertaking long-distance migrations to feed for one or several years in the ocean before returning to their natal stream to spawn through a behavior known as 'homing' (Quinn 1993). Migration in salmonids may facilitate 'straying' (dispersal from the natal site for breeding), which may act to homogenize populations genetically (Hansen and Mensberg 1998; Ford 2004; but see Utter 2004). In contrast, the characteristic strong homing behavior of these fish is expected to minimize dispersal and increase relatedness, potentially on a highly localized spatial scale (Taylor 1991; Bentzen et al. 2001). Exactly how these forces balance out in nature, and the degree to which this balance generates independent populations genetically differentiated at a local scale is not well known (Dittman and Quinn 1996; Quinn 2005).

Furthermore, the dramatic declines and/or fluctuations in population sizes suffered by many Pacific salmon populations would be expected to jeopardize their genetic integrity through losses of genetic diversity and significant genetic bottlenecks (Waples 1990a, c, 2002). Reduced genetic variability may impair the ability of populations to evolve with future environmental change and put them at greater risk of extinction (Saccheri et al. 1998; England et al. 2003; Frankham 2005). Losses of genetic diversity due to population declines are often further compounded by hatchery supplementation in many salmonid species (Ryman and Laikre 1991; Allendorf and Waples 1996). Genetic data are therefore useful not only for delineating relevant management units but also for understanding the extent to which local populations have maintained genetic diversity—a key component for the future evolutionary flexibility of species (Waples 1995)—in the face of such threats.

We analyzed genetic variation at 10 microsatellite loci to characterize population structure in wild Chinook salmon (*Oncorhynchus tshawytscha*) across a large wilderness basin in central Idaho (Fig. 1) that is one component of the Snake River ESU. The present study is part of a larger body of work on the distribution and abundance of Chinook salmon spawning in the study basin (see Isaak et al. 2003; Isaak and Thurow 2006). Here, we combine genetic information with insight from demographic data on population dynamics. First, we estimate the degree of genetic differentiation among spawning aggregations and determine the relationship between spatial genetic patterns and network structure. Second, we test for differences in gene diversity and genetic differentiation between areas with different geomorphic history and contemporary physical structure. Here, we predicted that the upper sub-basin of the system, which was colonized more recently following glacial retreats and has geographically closer and more continuous spawning gravels, should exhibit both decreased genetic diversity and less differentiation among sample sites than the lower sub-basin (see below, and Castric and Bernatchez 2003; Costello et al. 2003; Ramstad et al. 2004). Third, we evaluate whether populations that have undergone known demographic bottlenecks have

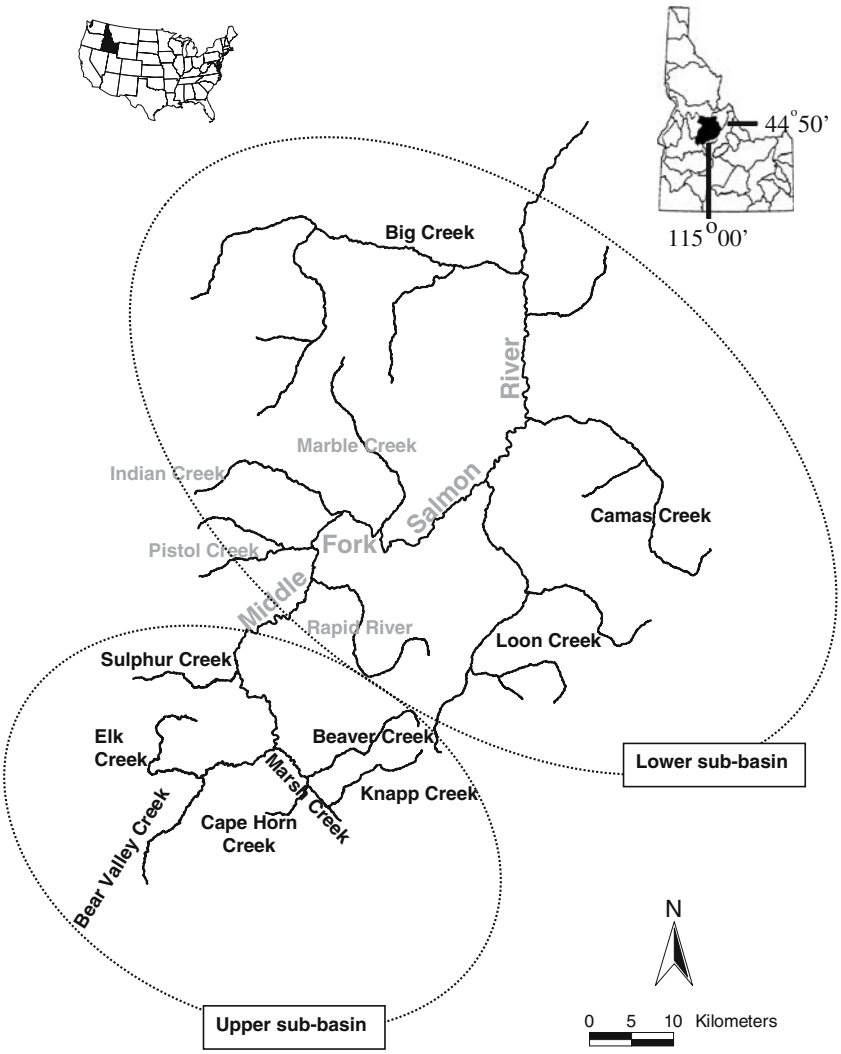

Fig. 1 Stream network in the Middle Fork Salmon River, Idaho used by Chinook salmon for spawning. Genetic samples were obtained from streams with names in black bold (tributaries with gray names were unsampled)

suffered significant genetic bottlenecks from recent demographic declines. This system provides a unique opportunity to examine various factors likely related to the maintenance of genetic diversity in an indigenous, naturally reproducing population within a threatened salmonid ESU.

Methods

Study site

Data were collected from Chinook salmon returning to spawn in the Middle Fork Salmon River (MFSR), which drains 7,330 km^2 of forested and mountainous terrain in central Idaho (Fig. 1). For most of its length, the MFSR flows through a wilderness area, and much of the habitat in the basin is in a relatively natural state. Valley morphologies in the upper MFSR sub-basin (Fig. 1) were heavily affected by late Pleistocene glaciation (Meyer and Leidecker 1999) and access to this area was blocked by a glacier at the mouth of Sulphur Creek until approximately 10,000 years ago (McPhail and Lindsey 1986; Utter et al. 1989); thus, the upper sub-basin is assumed to have been colonized more recently than the lower sub-basin. Additionally, this geologic history has created a diversity of habitats in the watershed. Deposits of glacial drift in the upper sub-basin have created large, open U-shaped valleys with extensive reaches of suitable spawning habitat, while in the lower sub-basin the river flows through narrow, V-shaped valleys with more limited spawning habitat (Isaak and Thurow 2006). Tributaries in the lower sub-basin are also generally larger and more spatially segregated than those in the upper sub-basin. Most spawning in the MFSR occurs in approximately 650 km of habitat distributed among major tributaries and the mainstem river (Fig. 1).

The MFSR is a tributary to the Snake River (part of the Columbia River system), which houses two

Chinook salmon ESUs listed as threatened in the early 1990s (57 Federal Register 14653). The two ESUs are the Snake River fall run Chinook salmon and the Snake River spring/summer run Chinook salmon, the latter of which encompasses MFSR populations. Chinook salmon in the MFSR are some of the few remaining indigenous populations (i.e., wild fish not influenced by hatchery fish) occurring in the lower 48 United States (Thurow et al. 2000). Like many other Chinook salmon stocks, populations within this basin have suffered large declines from their abundances in the 1950s (Fig. 2) (Brown 2002). Recently, salmon abundance in the MFSR and elsewhere in this ESU increased, probably stimulated by improved survival during portions of the life stage that occur outside the MFSR (Fig. 2; Fish Passage Center Annual report, BPA Contract Number 94-033 2003; Beamish 2004).

Redd counts

Annual censuses of redd (salmon nest) abundances were conducted using low-level helicopter flights from 1995 to 2002 within that portion of the stream network that historically supported Chinook salmon as detailed in Isaak and Thurow (2006). Censuses covered all occupied spawning habitat thought to support mostly 'spring' run fish, though the very lower reaches of lower sub-basin streams have been classified traditionally as 'summers' (Hassemer 1993). However, although genetic differences between runs can exist at larger scales (see, e.g., Waples et al. 2004, which considers all samples from the Salmon River as 'springs'), arrival times in the high-elevation MFSR are largely continuous and the classification of run times here has been murky at best (Lichatowich and Mobrand 1995). Furthermore, other recent evidence suggests that spring/summer run times in this system should be treated as a continuum rather than distinct 'races' (Brannon et al. 2004). Therefore, we did not consider run time explicitly in our analyses but cover this issue further in the Discussion. The cumulative number of redds at the end of the spawning season comprised each year's population estimates for each tributary.

Genetic sampling and laboratory methods

In August and September of 2001 and 2002, tissue was obtained from 908 carcasses sampled throughout census sites. Tissue was stored in 95% ethanol, and total genomic DNA was extracted using DNeasy extraction kits (Qiagen Inc, Valencia, CA, USA). We used fluorometry to quantify extracted DNA, which was then

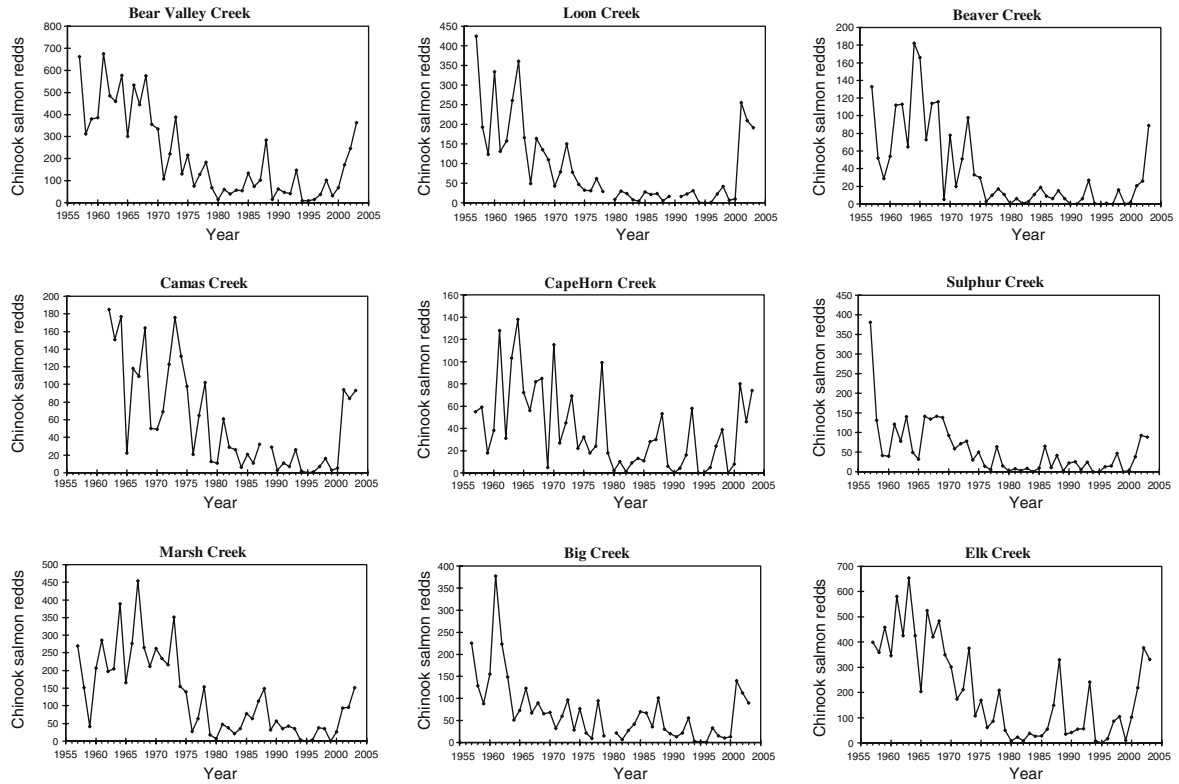

Fig. 2 Time series of Chinook salmon redd counts for nine streams (Knapp Creek excluded) in the Middle Fork Salmon River basin. Data are from annual Idaho Department of Fish and Game surveys of spawning index areas (Brown 2002)

diluted to 5 ng/μl. Polymerase chain reactions (PCRs) and fragment sizing using an Applied Biosystems (Foster City, CA, USA) Prism 3730 DNA Analyzer were performed by the Nevada Genomics Center (http://www.ag.unr.edu/genomics/, Reno, NV). Ten microsatellite loci were chosen in collaboration with NOAA Fisheries (Northwest Fisheries Science Center, Seattle, WA) and the Center for Salmonid and Freshwater Species at Risk (Hagerman, Idaho). These loci, references, Genbank accession numbers, and PCR conditions are given in Table 1. PCRs were performed in 10 μl reactions, each with 1 μl Titanium buffer, 0.2 μl Titanium taq, approximately 20 ng of DNA, dNTP and primer amounts given in Table 1, and the remainder made up with water. Individuals were genotyped manually by HN using Genemapper v3.0 (Applied Biosystems). In initial laboratory work, we found that the quality of DNA in many of our tissue samples was poor due to degradation of carcasses in the field. For quality control, each locus was amplified three times for each individual. Individuals were genotyped at a locus only if amplified successfully, consistently, and unambiguously at minimum twice for heterozygotes and three times for homozygotes.

Genetic analyses

Although temporal variation in genetic patterns can sometimes occur, spatial variation is generally more important in Pacific salmon (Waples et al. 2004) and we pooled samples across the two consecutive years to increase sample sizes and improve estimation of population allelic frequencies (see Waples 1990b; Beacham et al. 2003). For population-based analyses, i.e., those based on a priori designations of population boundaries (e.g., tests for null alleles, calculations of F_{ST}, tests for genetic bottlenecks, and population phenograms, see below) we included all individuals in the data set—even if they were not genotyped at some loci—to increase the accuracy of allelic frequency estimates within samples.

We used MICROCHECKER (Van Oosterhout et al. 2003) to test each sample at each locus for null alleles and allelic dropout before proceeding with further analyses. Adherence to Hardy–Weinberg equilibrium was assessed in FSTAT (Goudet 2001) by testing for excessive or deficient F_{IS} values for each population sample at each locus, with the program adjusting critical significance levels using Bonferroni corrections to account for simultaneous tests (Goudet 2001).

Estimates of allelic diversity such as allelic richness (R_S, a quantification of the number of alleles corrected for sample size) and an unbiased measure of gene

Table 1 Microsatellite markers used to genotype Chinook salmon sampled in the Middle Fork Salmon River, with references and Genbank accession number

Marker	Reference	Genbank #	Thermal protocol	dNTPS (μl)	Primer F/R (μl)
Otsg68	Williamson et al. (2002)	AF393187	95°C for 2 min; 94, 62, and 72°C each at 40 s, cycled 44 times; 72°C for 5 min	0.5	0.1
Otsg249	Williamson et al. (2002)	AF393192	95°C for 2 min; 94, 62, and 72°C each at 40 s, cycled 44 times; 72°C for 5 min	0.5	0.1
Otsg311	Williamson et al. (2002)	AF393194	95°C for 2 min; 94, 62, and 72°C each at 40 s, cycled 44 times; 72°C for 5 min	0.5	0.1
Omm1020	Rexroad et al. (2001)	AF346679	95°C for 2 min; 94, 62, and 72°C each at 40 s, cycled 44 times; 72°C for 5 min	0.5	0.1
Ots3	Banks et al. (1999)	AF107031	95°C for 2 min; 94, 53, and 72°C each at 40 s, cycled 44 times; 54°C for 40 min	0.54	0.2
Ots2M	Greig and Banks (1999)	*	95°C for 2 min; 94, 60, and 72°C each at 40 s, cycled 44 times; 60°C for 40 min	0.54	0.2
Ots10M	Greig and Banks (1999)	*	95°C for 2 min; 94, 60, and 72°C each at 40 s, cycled 44 times; 60°C for 40 min	0.54	0.2
Ogo4	Olsen et al. (1998)	AF009796	95°C for 2 min; 94, 60, and 72°C each at 40 s, cycled 44 times; 60°C for 40 min	0.8	0.2
OtsD9	Naish and Park (2002)	AY042709	95°C for 2 min; 94, 60, and 72°C each at 40 s, cycled 44 times; 60°C for 40 min	0.8	0.2
Ssa408	Cairney et al. (2000)	AJ402725	95°C for 2 min; 94, 60, and 72°C each at 40 s, cycled 44 times; 60°C for 40 min	0.8	0.2

Also given are the thermal protocols, and the amount of dNTPs and forward and reverse primer used in each 10 μl PCR reaction

*Modifications of original primers (Ots10 and Ots2): Ots2M F-gcc ttt taa aca cct cac act tag, Ots2M R-tta tct gcc ctc cgt caa g, Ots10M F-ggg cat gtg tgt gta gaa aga, Ots10M R-ggt ccc att gtc att act gct ac

diversity (H_E, Nei 1987) were calculated for each sample and averaged across loci using FSTAT. Because R_S can be biased downwards by small sample sizes, we excluded Knapp Creek (n=7) from this estimation. Based on historical colonization patterns in the upper sub-basin (see 'Methods', Study site), we tested for differences in allelic richness and gene diversity between the upper and lower sub-basins of the MFSR using randomization tests available in FSTAT. We predicted that populations in the upper sub-basin would have reduced genetic variation compared to those in the lower sub-basin due to post-glaciation colonization events and historical founder effects.

The extent of genetic differentiation among all samples was evaluated based on pair-wise F_{ST} values (Weir and Cockerham 1984), calculated in FSTAT. Five thousand permutations were used to assess significance, and alpha values were corrected for multiple comparisons as noted above. Again, we used randomization tests in FSTAT to test for differences between the two sub-basins in within sub-basin F_{ST} values. We predicted that populations in the upper sub-basin would have reduced differentiation due to the more recent colonization of this sub-basin and the more continual nature of its modern-day spawning habitat (see Methods, Study site), both of which we assumed would lead to greater genetic homogenization within the upper sub-basin.

To evaluate relationships among samples in an alternative manner, chord distances (D_{CE}, Cavalli-Sforza and Edwards 1967) were calculated and used to build neighbor-joining phenograms in POPULATIONS (Langella 2002). The chord distance was chosen over other distance estimators because of its greater accuracy in depicting correct topologies (Takezaki and Nei 1996) and because it does not assume any mutation model (Bernatchez and Martin 1996). The consistency of relationships was evaluated by bootstrapping over loci with 5,000 permutations. Phenograms were visualized using TREEVIEW (Page 1996).

Isolation by distance (IBD) was assessed based on a Mantel test implemented in GENEPOP version 3.3. (Raymond and Rousset 1995, 2004) using 5,000 permutations for statistical significance. F_{ST} was used as the genetic distance, and geographic distance was measured based on the stream distance separating each sample pair, with the mid-point of occupied spawning habitat serving as the location for each sample.

Given the population declines occurring in this system over the past 50 years, we tested for genetic bottlenecks using two different approaches. We first used the M ratio (Garza and Williamson 2001) because of its consistent performance in identifying populations with known bottlenecks and its theoretical ability to detect bottlenecks over a longer time-frame than other approaches (see Garza and Williamson 2001; Abdelkrim et al. 2005; Neville et al. in press-b). The M ratio capitalizes on changes that occur after a bottleneck in the distribution of allele sizes relative to the number of alleles in a population. Empirical data suggest that an M ratio less than 0.7 signifies a bottleneck (Garza and Williamson 2001, 2003). The significance of the observed M ratio can also be evaluated by comparison to M ratios of simulated populations at drift–migration equilibrium. These simulations require user input of theta ($4N_e\mu$, where N_e is the effective population size and μ is the microsatellite mutation rate; simplistically, N_e characterizes the ability of populations to retain genetic variation). Because mutation rates and values of N_e are unknown, two values of theta (0.1 and 20) bracketing those likely in systems such as ours were used (see Guinand and Scribner 2003). Theta does not change the M ratio itself, but affects the significance level indicated by simulations. Pearson's correlation coefficient was calculated to determine if M ratios varied with average population sizes and the coefficients of variation in population sizes for each creek from 1995 to 2002, the period following the most recent population declines and during which we have censused redds.

The program BOTTLENECK (Piry et al. 1999) was used as an alternative measure of genetic bottlenecks, to test for excess gene diversity relative to that expected under mutation–drift equilibrium. The program Bottleneck tests for excess heterozygosity using several mutation models, with the infinite allele model and the stepwise mutation model characterizing the two extremes of mutational processes (Cornuet and Luikart 1996; Balloux and Lugon-Moulin 2001). However, based on the fact that most microsatellites deviate from both of these models, Piry et al. (1997) suggest that a two-phase model (TPM, Di Rienzo et al. 1994) be used for microsatellite loci, where a certain proportion of mutations are not one-step mutations but constitute larger changes. We therefore assumed a TPM with the default value of 95% single-step mutations and 5% larger mutations. Statistical significance was based on 5,000 iterations and a Wilcoxin sign test.

The above analyses require one to define populations a priori (Manel et al. 2003), which can be a particularly difficult task for species such as salmon with mixtures of continuous and patchy habitats and migratory behavior (Neville et al. in press-a). We therefore used an individual-based clustering approach (STRUCTURE 2.1, Pritchard et al. 2000) to determine the most likely number of genetic clusters (k) in the MFSR. STRUCTURE iteratively sorts individual genotypes into clusters that maximize the fit of the data

to theoretical expectations derived from Hardy–Weinberg and linkage equilibrium. Once the most likely k is determined, individuals are assigned to the cluster in which they have the highest probability of membership (Q). This approach can reduce the need to define populations a priori, and also provides information about current movement as opposed to the long-term historical averages given by F_{ST}. However, in species with low differentiation there may be little statistical power for accurate assignment of individuals (see Cornuet et al. 1999) and it is unlikely that tangible clusters that can be used to define 'populations' in further analyses will be produced. Because we expected differentiation to be relatively low in this system, as is typical for anadromous fishes (Ward et al. 1994; Allendorf and Waples 1996), we used STRUCTURE as a complementary way to assess genetic structure but did not use it as a substitute for population-based analyses. For this individual-based analysis, which is likely to be more sensitive than frequency-based analyses to missing data, we included only individuals genotyped at 8 or more loci (n=593). Based on preliminary analyses, we evaluated the likelihood of k=2–12, with 5 runs performed for each k, and a burn-in length of 500,000 and 100,000 MCMC replicates for each run. We assumed an admixture model and correlated allele frequencies among populations (Pritchard et al. 2000).

Results

Eighty seven individuals could not be amplified consistently and were dropped from the data set, leaving a final number of 821 individuals used in population-based analyses. Two samples were identified by MICROCHECKER as having excess homozogytes at two loci (Elk Creek at Ots311 and Beaver Creek at Ots3). Homozygote excess can indicate the occurrence of null alleles or allelic dropout in affected loci. However, in each instance, the excess of homozygotes was restricted to one population for each of two different loci, and deviated from theoretical expectations by only one individual. With a true null allele or systematic dropout we would expect a consistent pattern of homozygote excess within affected loci. We believe, therefore, that neither null alleles nor allelic dropout are likely to have affected our results significantly.

None of the F_{IS} values were significantly different from zero when corrected for multiple tests, indicating our samples were in Hardy–Weinberg equilibrium (Table 2). The number of alleles per population averaged across loci ranged from 6.1 to 14.4, and allelic richness (R_S) ranged from 8.86 to 10.39 (Table 2). Average gene diversity (H_E) per population ranged from 0.70 to 0.74 (Table 2). Randomization tests did not show differences between the two sub-basins in allelic richness (within sub-basin R_S=9.78 vs. 9.76 in the upper and lower sub-basin, respectively, randomization P=0.95) or gene diversity (within sub-basin H_E=0.73 vs. 0.73, randomization P=0.78), contrary to our predictions.

An overall F_{ST} value of 0.016 (95% confidence interval = 0.014–0.018) suggested low, yet significant differentiation within the MFSR as a whole. Pair-wise F_{ST} comparisons ranged from −0.006 to 0.034 (negative values are occasional statistical artifacts of F_{ST} calculations; Table 3). All comparisons suggested statistically significant differentiation between locations except for the Cape Horn–Beaver Creek comparison and comparisons involving Knapp Creek, whose lack of significance may have been an artifact of small sample size (n=7). In contrast to our predictions about sub-basin differences in levels of differentiation, there was no difference in F_{ST} values among populations within the upper and lower sub-basins ($F_{STupper}$=0.010, $F_{STlower}$=0.014, randomization P=0.51).

Despite relatively low levels of differentiation in the system as indicated by F_{ST}, the neighbor-joining tree illustrated a well-supported geographic basis to population relationships (Fig. 3). The three streams in the lower sub-basin all grouped separately from upper sub-basin streams with relatively strong bootstrap support (80%), and the geographically proximate Loon and Camas Creeks paired consistently (81%) as more closely related to each other than to Big Creek. In the upper sub-basin, the neighboring Elk and Bear Valley streams paired with strong support (95%), as did Marsh and Sulphur Creeks (97%), which are separated by Elk and Bear Valley Creeks. Finally, Beaver, Knapp and Cape Horn Creeks (tributaries to Marsh) clustered with moderate support (60%) as distinct from other creeks in the upper sub-basin.

The Mantel test demonstrated significant isolation by distance (one-tailed P=0.01). However, inspection of the correlation graph (Fig. 4) indicates that the bulk of this relationship is due to differences in the impact of within- vs. among-sub-basin geographic distances on genetic distance (see Discussion).

M ratios ranged from 0.73 to 0.86 (Table 4). All were above the 0.7 value diagnostic of genetic bottlenecks, but all but one were below the 0.82 cut-off observed for populations known not to have suffered population reductions in Garza and Williamson's (2001) empirical review. None of these values were statistically significant when a theta of 20 was assumed,

Table 2 Genetic diversity statistics for each locus and population for Chinook salmon sampled from the MFSR

Locus		Beaver	Big	Bear Valley	Camas	Capehorn	Elk	Knapp	Loon	Marsh	Sulphur
Ogo4	H_E	0.80	0.80	0.83	0.79	0.82	0.80	0.88	0.79	0.83	0.80
	R_S	6.61	7.15	7.34	7.03	7.46	7.33	NA	5.85	7.40	7.32
	F_{IS}	−0.05	0.02	0.01	−0.09	0.04	−0.01	−0.14	−0.03	−0.04	0.00
Ots10M	H_E	0.47	0.45	0.50	0.52	0.42	0.50	0.26	0.54	0.50	0.48
	R_S	2.00	3.19	2.55	2.62	3.39	2.41	NA	3.63	2.00	2.00
	F_{IS}	0.03	−0.11	−0.04	−0.18	−0.06	−0.03	−0.09	−0.19	−0.27	0.09
Ots3	H_E	0.63	0.63	0.60	0.61	0.62	0.64	0.67	0.62	0.55	0.66
	R_S	3.94	3.67	4.67	4.81	3.93	4.84	NA	3.89	3.36	3.88
	F_{IS}	0.27	0.04	0.01	0.07	0.05	−0.10	−0.07	0.09	−0.11	−0.07
OtsD9	H_E	0.75	0.58	0.61	0.64	0.75	0.63	0.75	0.71	0.64	0.56
	R_S	4.81	4.28	4.44	4.83	5.47	4.57	NA	4.00	3.97	3.68
	F_{IS}	0.05	0.02	0.02	−0.15	−0.14	−0.03	−0.14	0.03	−0.07	0.01
Ssa408	H_E	0.80	0.73	0.83	0.87	0.84	0.82	0.90	0.87	0.83	0.73
	R_S	10.67	11.53	11.97	13.50	12.96	11.37	NA	14.95	12.16	10.29
	F_{IS}	−0.07	0.04	0.01	0.08	0.09	0.06	−0.11	0.03	−0.06	−0.04
Ots2M	H_E	0.60	0.58	0.51	0.53	0.51	0.55	0.48	0.53	0.53	0.50
	R_S	4.77	4.89	2.46	3.63	3.54	4.08	NA	3.34	3.70	2.97
	F_{IS}	−0.19	−0.17	−0.08	0.13	−0.20	−0.09	−0.50	−0.20	−0.16	−0.11
Ots311	H_E	0.95	0.95	0.96	0.95	0.96	0.95	0.96	0.96	0.95	0.95
	R_S	23.60	21.52	24.78	21.05	22.00	21.64	NA	20.99	21.13	21.14
	F_{IS}	0.09	−0.03	0.05	0.05	0.04	0.06	−0.04	0.06	0.08	0.02
Otsg68	H_E	0.89	0.95	0.94	0.95	0.92	0.94	0.94	0.94	0.94	0.92
	R_S	18.41	21.38	18.95	19.81	18.52	19.66	NA	19.39	17.99	17.68
	F_{IS}	−0.01	0.01	−0.03	−0.02	−0.02	0.02	−0.06	0.04	0.03	−0.03
Omm1020	H_E	0.45	0.43	0.57	0.49	0.61	0.57	0.50	0.37	0.35	0.62
	R_S	4.99	4.75	6.30	5.26	7.57	6.47	NA	5.01	4.58	5.11
	F_{IS}	−0.11	0.09	−0.01	0.08	0.09	−0.06	0.14	−0.12	−0.05	−0.08
Otsg249	H_E	0.93	0.92	0.94	0.93	0.94	0.93	0.96	0.92	0.94	0.89
	R_S	18.49	16.71	18.14	15.74	19.09	18.15	NA	14.48	17.50	14.55
	F_{IS}	0.07	−0.05	−0.02	0.00	0.00	−0.01	−0.04	−0.03	0.05	0.07
Average	H_E	0.73	0.70	0.73	0.73	0.74	0.73	0.73	0.73	0.71	0.71
	R_S	9.83	9.91	10.16	9.83	10.39	10.05	NA	9.55	9.38	8.86

H_E is Nei's gene diversity, R_S is allelic richness (Knapp creek not included due to low sample size; minimum n for other R_S estimates = 25), and F_{IS} is an inbreeding coefficient that measures deviation from H–W equilibrium

Table 3 Pair-wise F_{ST} values for Chinook salmon populations sampled across the Middle Fork Salmon River

Creek	Beaver	Cape Horn	Marsh	Bear Valley	Elk	Sulphur	Loon	Camas	Big
Knapp	−0.005	−0.006	0.016	0.002	0.008	0.023	−0.004	**0.008**	0.005
Beaver		0.009	**0.012**	**0.012**	**0.011**	**0.020**	**0.011**	**0.017**	**0.018**
Cape Horn			**0.019**	**0.011**	**0.014**	**0.020**	**0.016**	**0.029**	**0.018**
Marsh				**0.005**	**0.005**	**0.019**	**0.017**	**0.020**	**0.024**
Bear Valley					**0.003**	**0.017**	**0.015**	**0.016**	**0.017**
Elk						**0.012**	**0.020**	**0.020**	**0.021**
Sulphur							**0.034**	**0.032**	**0.023**
Loon								**0.009**	**0.018**
Camas									**0.022**

F_{ST} values in bold are significantly different from 0 at α=0.05

but the M ratios for several creeks (Beaver, Cape Horn, Marsh and Sulphur) were significantly smaller than the equilibrium M when assuming a theta of 0.1 (Table 4). M ratio values were significantly correlated to the arithmetic mean (r=0.81; P=0.01), the coefficient of variation (r=−0.73; P=0.03), and the harmonic mean (r=0.68; P=0.04) of population sizes from 1995 to 2002 (Table 4). No tests for excesses in heterozygosity were significant based on analyses using the program BOTTLENECK (P>0.05).

The individual-based clustering algorithm suggested the most likely number of clusters in the MFSR to be 7 (posterior probability of $k(7)$=1, compared to a posterior probability of $1.18 * e^{-25}$ for $k(6)$, the next most likely number of clusters). Probabilities of assignment of individuals to these clusters were rela-

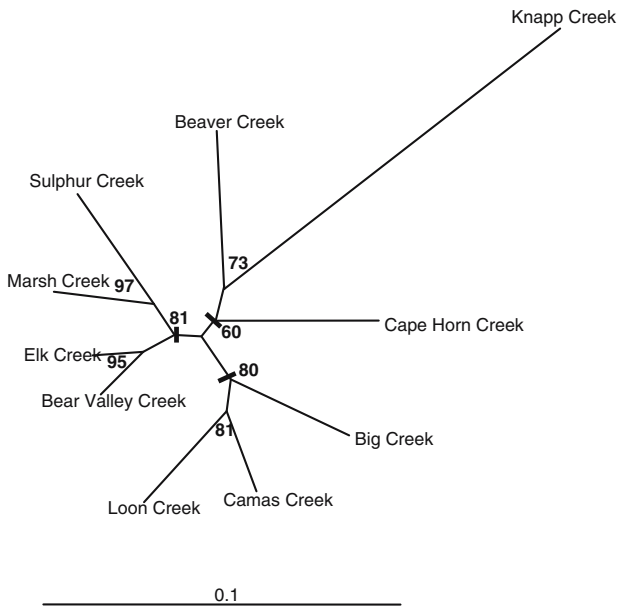

Fig. 3 Phenogram of Nei's D_A genetic distance relating populations of Chinook salmon in the Middle Fork Salmon River. Values at each fork indicate the percentage of 5,000 bootstrap iterations across loci that support the given relationship, with those above 50% shown

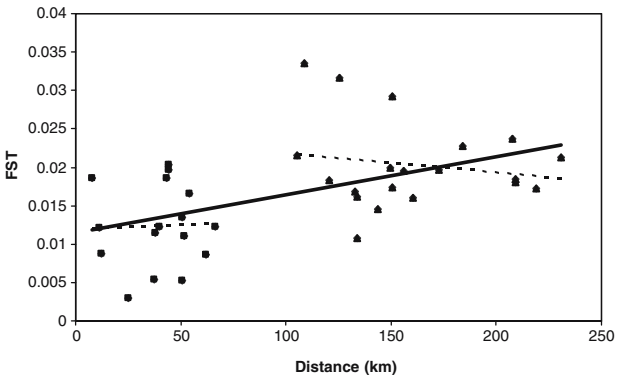

Fig. 4 Correlation of F_{ST} between pairs of Chinook salmon populations in the Middle Fork Salmon River and stream distance between the midpoints of spawning sites within each tributary. Trend lines drawn through entire figure (solid line) and for each cluster of points (dashed lines): the lower-left cluster represents comparisons within sub-basins while that in the upper right corner represents comparisons between sub-basins. See text for discussion

tively low: when taking the highest probability of membership in a cluster, Q, for each individual and averaging this across all individuals, average Q=0.58±0.20, indicating that weak differentiation in the system resulted in poor discriminatory power (Cornuet et al. 1999; Hansen et al. 2001). As a result, clusters defined by STRUCTURE had little clear geographic basis and were generally comprised of individuals from many sites. One exception was a moderate degree of clustering characterizing some fish from two of the three lower sub-basin streams (Big and Loon Creeks). Here, one autonomous cluster in each stream was comprised of fish from sampling areas throughout the stream. Drawing on results from the phenogram, we evaluated k=2 to mimic the split between the two sub-basins. This analysis had higher assignment probabilities (average highest Q=0.81±0.14) and showed clearer geographic membership of individuals in the two clusters.

Table 4 Arithmetic mean, harmonic mean, and coefficient of variation of yearly abundances for Chinook salmon sampled from the Middle Fork Salmon River from 1995 to 2002. Also presented are genetic sample sizes (n), and M ratios and their P values when assuming either a theta ($4N_e\mu$) of 0.1 or 20 (asterisks indicate significance at α=0.05). Knapp creek was omitted due to small sample size

Sample name	Arithmetic	Harmonic	C.V.	n	M ratio	M ratio P value	
						0.1	20
Knapp	NA	NA	NA	NA	NA	NA	NA
Beaver	29	5.06	1.40	33	0.74	0.02*	0.88
Cape Horn	31	16.20	1.07	36	0.74	0.03*	0.49
Marsh	56	26.95	1.12	69	0.73	0.02*	0.56
Bear Valley	128	38.22	0.95	170	0.81	0.14	0.79
Elk	144	48.43	0.87	173	0.86	0.31	0.98
Sulphur	40	5.48	1.02	59	0.77	0.05*	0.80
Loon	116	22.29	1.18	75	0.77	0.05*	0.80
Camas	61	16.45	1.08	116	0.79	0.08	0.74
Big	64	9.54	1.12	83	0.79	0.08	0.82

Discussion

System-wide genetic diversity and tests for bottlenecks

Local populations of Chinook salmon in the MFSR have retained a surprisingly robust level of genetic variability, as indicated by moderately high estimates of gene diversity and allelic richness. This was somewhat surprising given their recent demographic history, though Chinook salmon, in particular among the Pacific salmon species, are generally characterized by high heterozygosity levels (Waples et al. 2001). No M ratios were below the value 'diagnostic' of bottlenecks (though some were statistically significant if a very small theta value was assumed), suggesting these populations have not suffered severe or long-lasting genetic bottlenecks (Garza and Williamson 2001; see also Doerner et al. 2005, and see below for more detailed discussion). Genetic variation is often reduced follow-

ing demographic reductions in Chinook salmon. For example, several runs of Chinook salmon in the highly-altered Central Valley of California had relatively low gene diversity and showed other signs of bottlenecks, patterns that were consistent with both declines in the 20th century and known historical influences (Banks et al. 2000). However, the retention of diversity in the face of demographic instability is not unprecedented in this species: other Chinook salmon populations with recent demographic reductions have maintained high genetic variability and exhibited no evidence of genetic bottlenecks (Marshall et al. 2000; Teel et al. 2000). Fraser River (British Columbia) populations maintained relatively high levels of gene diversity but were significantly bottlenecked, and estimates of N_e were low and often declining even as population sizes rebounded (Shrimpton and Heath 2003).

Genetic responses to bottlenecks can be variable and context-dependent, depending on the generation time and life history of the species, the severity of the demographic decline, current levels of gene flow, and the nature of demographic rebound (Nei et al. 1975; Rundle et al. 1998; Garza and Williamson 2001). Several factors may have helped buffer the MFSR populations from dramatic changes in genetic variation despite severe demographic declines. First, while the majority of Chinook salmon populations in the lower 48 United States are hatchery supplemented (see e.g., Allendorf and Waples 1996), Chinook salmon in the MFSR comprise one of the few remaining indigenous wild stocks (Thurow et al. 2000). In most other populations, hatchery supplementation has been undertaken in an effort to reduce variability in reproductive success among spawners, but the ultimate effect of supplementation can actually be a dramatic reduction in effective population sizes and thus an increase in the rate of loss of genetic variability (Ryman and Laikre 1991; Tessier et al. 1997; Hansen et al. 2000). The genetic variability we see maintained in the MFSR may be partly due to the indigenous nature of these populations and the lack of hatchery influence in this region.

Secondly, although fish from various MFSR tributaries are genetically structured, differentiation in the system is relatively low and individual-based analyses suggest dispersal occurs among tributaries. Gene flow may mask the ability of diversity estimates, which may only detect long periods of severe bottlenecks and are very sensitive to low levels of migration, to characterize demographic declines. Additionally, dispersal among local populations may have served to 'rescue' them from loss of genetic variability (Hanski 1991; Stacey et al. 1997; Ingvarsson 2001; Consuegra et al. 2005). Under such a scenario, it may be the 'global' N_e (i.e., that of the lineage or species) that carries greater importance than the N_es of local populations. Given the distinctiveness of Interior Columbia stream-type (i.e., 'spring') fish in general (see Waples et al. 2004), current diversity levels in local populations might primarily reflect the long-term N_e of this lineage as a whole, which is not likely to be very small (R. Waples, personal communication). Similarly, demographic declines in the MFSR were followed by rapid recoveries in the last several years (Fig. 2) (Isaak and Thurow 2006). Theoretically, the impact of even severe bottlenecks can be small if the bottleneck is followed by a rapid flush of growth in which most genetic variability is maintained (Nei et al. 1975; Templeton 1980). Such an effect has recently been observed in rebounding white-tailed deer (*Odocoileus virginianus*) populations, which retained high levels of genetic variability despite dramatic historical reductions (Doerner et al. 2005). Additionally, overlapping generations in Pacific salmon can also help to buffer them from long-term losses of genetic variability in comparison to species with discrete generations (Waples 1990a). Furthermore, the large amount of relatively high-quality spawning habitat in the MFSR may have allowed for even representation in the breeding pool each year as populations have increased (see Shrimpton and Heath 2003). In steelhead (*Oncorhynchus mykiss*), reduced variance in reproductive success at low population numbers has been shown to have a 'genetic compensation' effect that increased N_e/N (Ardren and Kapuscinski 2003).

Finally, we cannot underestimate the genetic contribution of precocial males in this system. Precocial males have been found to contribute significantly to reproduction in other salmonid species (Garcia-Vazquez et al. 2001; Blanchfield et al. 2003). Though we have no information about the reproductive success of resident males in the MFSR, they are common in the system. Because these individuals do not migrate to the ocean, they are potentially less responsive to downstream factors thought to jeopardize anadromous Pacific salmon (e.g., poor ocean conditions, hydropower dams). Successful spawning by precocial males may increase effective population sizes, thus slowing losses of diversity to genetic drift (Consuegra et al. 2005).

Although we found little indication of the dramatic losses of diversity we expected based upon known demographic declines, we would like to emphasize that other patterns suggest that recent declines potentially *have* impacted genetic variation in these populations, even if only slightly. Effects of even diffuse genetic bottlenecks (England et al. 2003), such as decreased heterozygosity and the loss of rare alleles that contribute to genetic variation and perhaps future evolutionary

potential (Waples 1990a), may still be a concern for these populations. For instance, though tests for heterozygosity excess were insignificant and none of the M ratios were below the 0.7 diagnostic of bottlenecks, M ratios for several populations were below the 0.82 threshold found in populations of various species with known demographic stability (Garza and Williamson 2001). Thus, while M ratios here did not show evidence of significant bottlenecks, they also did not clearly demonstrate that MFSR populations have *not* been impacted genetically by their demographic declines. There are few other studies in salmon with which to compare M ratio results. In at least one instance, however, the continuous nature of the M ratio has been shown to be a good indicator of the relative demographic stability of Chinook populations, i.e., where impacted Chinook populations displayed $M < 0.68$ but a captive population originating from a large ancestral population generated an M ratio >0.9 (see Shrimpton and Heath 2003). Relic lake trout (*Salvelinus namaysuch*) populations all had M ratios < 0.70, though the statistical significance of these values depended somewhat on mutational models and theta values assumed (Guinand and Scribner 2003). One other system of sockeye salmon (*Oncorhychus nerka*) with documented periodic severe reductions in population sizes had M ratios similar to ours (M = 0.74–0.85, Habicht et al. 2004). The fact that M ratios for several populations in the MFSR were < 0.75, with some of these being significantly different from those expected for a population at demographic equilibrium when assuming a small theta, may indicate a degree of bottleneck 'signal' in allelic distributions. Finally, our M ratio values corresponded with demographic characteristics, suggesting the potential for weak impacts of population declines on genetic variation, and that the prevention of further losses should remain a focus of conservation efforts in the MFSR.

System-wide genetic structure

Salmonids are known for their ability to generate and maintain population structure, at least in stable environments (Utter 2004). Despite dramatic population fluctuations in recent history, we observed statistically significant differentiation among major spawning sites in the MFSR, with almost every tributary having a genetically distinct population even at distances of less than 5 km. F_{ST} values in the MFSR were relatively low but similar to those observed in other anadromous salmon and particularly other Chinook salmon populations (Ward et al. 1994; Allendorf and Waples 1996; see Waples et al. 2001). Still, the existence of even weak differentiation at this small within-watershed spatial scale, especially given the recent recovery dynamics of these populations, is an important result that stresses the need to identify and conserve within-ESU diversity.

Salmon and trout populations are often found to be structured by geographic distance and habitat structure (see Waples 1991; McConnell et al. 1997; Angers et al. 1999; Teel et al. 2000; Heath et al. 2001; King et al. 2001; Wenburg and Bentzen 2001), a helpful characteristic given that limited genetic information is often available for defining populations (McClure et al. 2003a). Here, despite the low levels of differentiation, genetic relationships based on the chord distance phenogram showed concordance with tributary branching patterns, with many of these relationships having high bootstrap support (>80%). Isolation by distance was significant, suggesting at first glance that equilibrium between dispersal and genetic drift has been reached system-wide. However, the significance of this relationship was primarily due to differences between sub-basins, as opposed to isolation by distance at all spatial scales. That is, sample pairs compared at the within sub-basin spatial scale (cluster of points at lower left of Fig. 4) did not demonstrate IBD, and there was similarly no correlation between genetic and geographic distance among pairs in different sub-basins (cluster of points at upper right of Fig. 4)—the statistical significance of the relationship comes from the fact that genetic distances between pairs in different sub-basins are higher than those between pairs within sub-basins (see Slatkin 1993; Hutchison and Templeton 1999). Similarly, although seven distinct clusters were identified by the individual-based clustering algorithm, they had little geographic basis save for some degree of genetic clustering within streams in the lower sub-basin and the clear segregation of individuals between sub-basins: individuals from the same tributary were often assigned to different clusters, and many individuals showed evidence of mixed ancestry (partial membership in several clusters).

The above analyses all suggested that the dominant factor contributing to differentiation in the MFSR is the organization of tributaries into two geographically separated sub-basins. We cannot rule out that this could arise as an artifact from a lack of samples from the middle of the MFSR (e.g., from Indian and Pistol creeks, and mainstem spawners). Another factor not explicitly considered here is the influence of life history type ('spring' vs. 'summer' run fish) on genetic differentiation. However, if divergent life history types existed in our samples (see Methods: Redd counts), we would have expected some departure from Hardy–Weinberg equilibrium (Wahlund effect: excess of

homozygotes) from pooling individuals from different runs, or distinctive clustering of individuals in streams where run-times may coexist (i.e., separating fish from the lower and upper portions of lower sub-basin streams). Overall, our results demonstrate that weak genetic structure among tributaries within sub-basins has been maintained despite a relatively high level of movement within these sub-basins, whereas the strongest genetic pattern correlates to the physical segregation of fish from the two sub-basins. Sub-basins (and perhaps even specific tributaries within the lower sub-basin in particular) may be important units for conservation focus within this watershed.

Within sub-basin differences in genetic diversity and structure

We observed no difference between the two sub-basins in within-sub-basin allelic richness, gene diversity, or genetic differentiation despite the more recent colonization of upper sub-basin streams following glacial retreats and differences in contemporary habitat structure. We had hypothesized that historical founder effects would have reduced the genetic diversity of the latter populations, as has been found in several other salmonid populations colonizing habitats after glacial retreats (e.g., King et al. 2001; Castric and Bernatchez 2003; Costello et al. 2003; Ramstad et al. 2004). We also expected the more recent colonization of the upper sub-basin would contribute to reduced levels of differentiation within this sub-basin, due to decreased time for among-tributary differences to accrue (this effect was also expected to be compounded by the geographically closer and more continuous nature of spawning habitat in this sub-basin). Although at a much larger spatial scale, a similar mechanism was proposed by Bartley and Gall (1990), who suggested that the greater structure among Chinook populations across California compared to those in more northern areas (Washington and Canada) corresponded with the lack of glaciation in California. Weakened differentiation among populations of Atlantic salmon (*Salmo salar*) has also been attributed to recent colonization following the retreat of glaciers in North America (King et al. 2001). The lack of differences in the distribution of genetic variation between the upper and lower sub-basins in the MFSR may result from several factors. The first may be the small sample size in the lower sub-basin (3 populations), which gives only low statistical power for this comparison. Secondly, though statistically significant, differentiation in this system as a whole is relatively low, and on-going gene flow both within and (less so) between sub-basins may have obscured any genetic patterns that may have been altered by these historical founding events.

Conclusions

Chinook salmon populations in the MFSR are vestiges of once-prolific wild runs of salmon and other anadromous fishes in the interior Columbia River basin (Nehlsen et al. 1991; Lee et al. 1997; Thurow et al. 1997). Despite extensive research at larger scales, how much diversity might exist in more localized regions within this species has been unclear. Our results revealed weak local population structuring that is loosely tied to the geography of spawning locations within the Middle Fork Salmon River, but also suggested that gene flow among spawning locations within sub-basins is common. Among other factors, gene flow may have provided an important buffer against expected losses of genetic variability in the face of both historical and recent population reductions. The natural landscape of the Middle Fork Salmon River is dynamic, with massive landslides (Meyer and Leidecker 1999), wildfires (Minshall et al. 2001), and climatic extremes influencing habitats and, undoubtedly, populations of Chinook salmon. In more recent decades, changes to migration corridor habitats outside the MFSR, combined with the influences of harvest and hatchery propagation, have likely contributed to population declines across the Columbia River basin (McClure et al. 2003b). Given these potential threats, Chinook salmon in the Middle Fork Salmon River appear to be surprisingly resilient and it is remarkable that populations have not exhibited some of the more 'diagnostic' or extreme genetic symptoms associated with extinction risk (see Dunham et al. 1999). Habitat connectivity that has allowed for re-expansion into extirpated habitats and on-going gene flow (Consuegra et al. 2005; Isaak and Thurow 2006), and perhaps even the natural genetic integrity of this indigenous stock, may have been key factors sustaining this resiliency. However, as we did uncover certain more moderate signs of genetic vulnerability, we caution that the future genetic integrity of Chinook salmon populations in the Middle Fork Salmon River may still be uncertain, particularly if human-caused threats outside the watershed persist. Our results emphasize the importance of maintaining indigenous populations within heterogeneous interconnected stream networks in order to minimize losses of genetic diversity in threatened and endangered ESUs.

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