North American Journal of Fisheries Management 24:672–685, 2004

Genetic Stock Identification of Steelhead in the Columbia River Basin: An Evaluation of Different Molecular Markers

GARY A. WINANS,* MELANIE M. PAQUIN, AND DONALD M. VAN DOORNIK

National Marine Fisheries Service, Northwest Fisheries Science Center, 2725 Montlake Boulevard East, Seattle, Washington 98112, USA

BRUCE M. BAKER

Washington Department of Fish and Wildlife, 600 Capital Way North, Olympia, Washington 98501, USA

PERRY THORNTON

National Marine Fisheries Service, Northwest Fisheries Science Center, 2725 Montlake Boulevard East, Seattle, Washington 98112, USA

DAN RAWDING AND ANNE MARSHALL

Washington Department of Fish and Wildlife, 600 Capital Way North, Olympia, Washington 98501, USA

PAUL MORAN

National Marine Fisheries Service, Northwest Fisheries Science Center, 2725 Montlake Boulevard East, Seattle, Washington 98112, USA

STEVEN KALINOWSKI

Montana State University, Department of Ecology, Bozeman, Montana 59717, USA

Abstract.—Protein genetic markers (allozymes) have been used during the last decade in a genetic stock identification (GSI) program by state and federal management agencies to monitor stocks of steelhead *Oncorhynchus mykiss* in the Columbia River basin. In this paper we report new data for five microsatellite and three intron loci from 32 steelhead populations in the three upriver evolutionarily significant units (ESUs) and compare the performance of allozyme, microsatellite, and intron markers for use in GSI mixture analyses. As expected, microsatellites and introns had high total heterozygosity (H_T) values; but there was little difference among marker classes in the magnitude of population differentiation as estimated by Wright's fixation index (F_{ST}), which ranged from 0.041 (microsatellite loci) to 0.047 (allozyme loci) and 0.050 (intron loci). For allozyme and microsatellite loci, the relationships among populations followed the patterns of geographic proximity. In computer-simulated mixture analyses, GSI estimates were more than 85% correct to the reporting group, the exact percentage depending on the marker data set and target group. Microsatellite loci provided the most accurate estimate (83%) in the 100% upper Columbia River ESU simulation, whereas simulation estimates for the 32-locus allozyme baseline were 93–94% for the 100% middle Columbia River ESU and two Snake River management groups. The simulations also showed that the estimates improved substantially up to a sample size of 50 fish per population. Technical advances will concomitantly increase the number of useful microsatellite loci and the rate of laboratory throughput, making this class of molecular marker more valuable for GSI mixture analyses in the near future. In the meantime, we recommend that steelhead management in the Columbia River rely on both allozyme and microsatellite data for GSI procedures.

Steelhead *Oncorhynchus mykiss* were once very abundant in the Columbia River, peak run sizes being put as high as 500,000 adults (TAC 1997). As with other species of Pacific salmon, steelhead population sizes were reduced to the tens of thousands in the last century by hydroelectric development, in-river and ocean fisheries, and stream habitat degradation. In the 1990s, concern about the reduction in the number and abundance of steelhead populations prompted an evaluation of population viability and abundance under the purview of the U.S. Endangered Species Act. Based

* Corresponding author: gary.winans@noaa.gov

Received March 13, 2003; accepted September 23, 2003

on available life history and genetic data, the National Marine Fisheries Service identified four evolutionarily significant units (ESUs) of steelhead in the basin (Busby et al. 1996). Three ESUs are found entirely above Bonneville Dam (river km 235 [measuring from the river's mouth]): the middle Columbia River ESU (roughly from Bonneville Dam to and including the Yakima River but excluding the Snake River), the upper Columbia River ESU (upstream of the Yakima River), and the Snake River ESU. The steelhead in these ESUs are considered inland-type steelhead *O. m. gairdneri* (as distinct from the coastal form *O. m. irideus*; Busby et al. 1996) and are almost exclusively stream-maturing populations.

Further biological diversity is recognized within these upriver populations of the Columbia River basin. Fishery managers have designated two ''run types,'' A and B, based on bimodal peaks in the run counts of adults at Bonneville Dam. By definition, adult A-run steelhead enter freshwater first and pass Bonneville Dam prior to August 25, whereas B-run steelhead pass after that date. A-run steelhead are predominantly age-1 ocean fish while B-run steelhead adults are age-2 ocean fish (Busby et al. 1996). The B-run stocks are thought to have higher growth rates and to be larger than A-run fish at a given age. B-run steelhead are thought to exist only in the Clearwater, Middle Fork Snake, and South Fork Snake rivers. Fishery agencies manage populations by ESU and, in some cases, by run group (TAC 1997).

Since 1997, protein genetic markers (allozymes) have been used in a genetic stock identification (GSI) program by state and federal management agencies to monitor steelhead stocks in the basin (Rawding et al. 1997). From April to September, fin clips and opercular punches have been taken nonlethally from returning adults at Bonneville Dam for GSI analysis to monitor passage dates by stock. Upriver, steelhead are incidentally caught in an in-river fishery (zone 6; see Figure 1) for Chinook salmon *O. tshawytscha* in August and September. To estimate and monitor stock composition by ESU, tissues (muscle, heart, liver, and retinal) are taken from incidentally killed steelhead for genetic analysis. For both GSI applications, allozyme data from mixture specimens are assessed against a baseline of 32 or more loci from each of 39 populations (Rawding et al. 1997).

While allozyme baselines are still being used in GSI applications (Seeb and Crane 1999; Wilmot et al. 2000; Winans et al. 2001), incipient baselines based on DNA markers are growing in number and geographic complexity (e.g., Beacham et al. 2001). The potential advantages of these new molecular markers (e.g., single-nucleotide polymorphisms of mitochondrial DNA and nuclear genes as well as nuclear microsatellites) are enhanced population discrimination and technical suitability for high laboratory output (e.g., number of loci per fish per working day; Park and Moran 1994). Small tissue requirements per specimen reduce fish-handling time and stress. Although heralded as the next boon for population genetic research (Avise 1994; Lewontin 1991), the accumulation of geographically complete baselines of DNA-based loci that match the preexisting allozyme baselines for Pacific salmon (Seeb and Crane 1999; Teel et al. 1999) has been slow (but see Beacham et al. 2001). There are also indications that some of the early predictions that DNA markers would provide more accurate population discrimination may not be fulfilled. For example, Allendorf and Seeb (2000) found concordance in the amount of genetic variation within and between populations of sockeye salmon *O. nerka* for allozyme, nuclear DNA, and mitochondrial DNA markers and Scribner et al. (1998) found no difference in the ability of allozymes, mitochondrial DNA, and microsatellite loci to discriminate between two groups comprised of eight populations of chum salmon *O. keta*.

In this paper, we report new data for five microsatellite and three intron loci from 32 steelhead populations in the three upriver ESUs (Figure 1). Our purposes are to (1) describe and compare the patterns and levels of variability among allozyme, microsatellite, and intron markers; (2) demonstrate the accuracy and precision of ESU estimates in simulated mixtures using various marker data sets; (3) identify subsets of ''important'' allozyme loci for use in GSI applications; and (4) recommend a set of loci for future GSI applications in steelhead management, monitoring, and conservation in the Columbia River basin.

Methods

Allozymes.—The allozyme baseline data set is composed of information collected over the last 10–15 years, primarily by the Washington Department of Fish and Wildlife. When the National Marine Fisheries Service evaluated the coastwide status of steelhead under the purview of the Endangered Species Act (Busby et al. 1996), fishery agencies constructed a standardized allozyme baseline with data from several sources (Waples et al. 1993; Phelps et al. 1994). These data have served as the in-river genetic baseline for GSI pro-

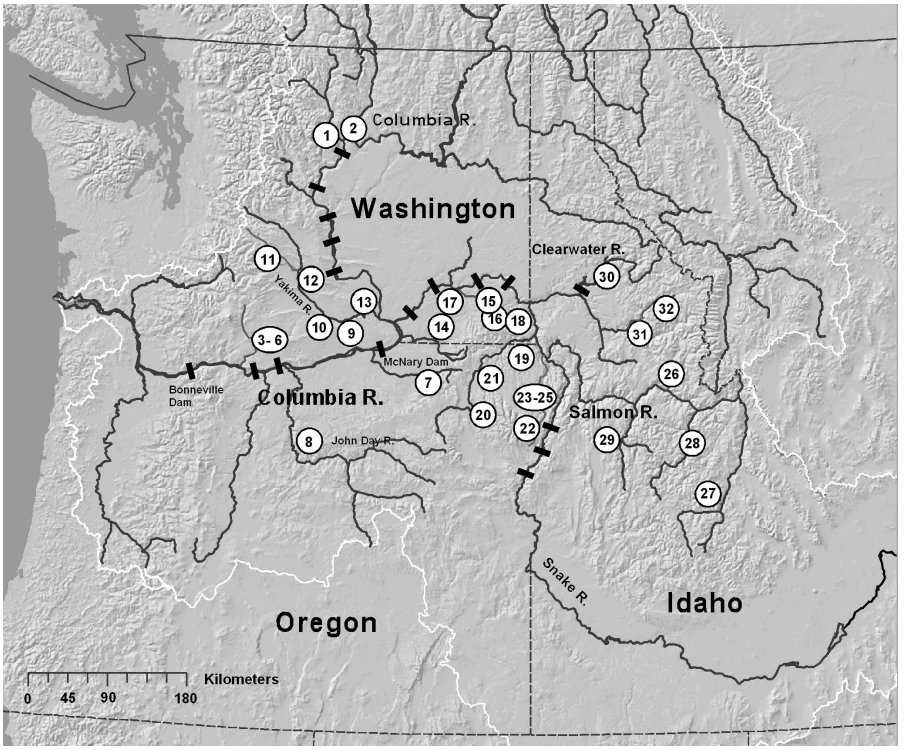

FIGURE 1.—Map showing the locations of 32 collections of steelhead in the Columbia River basin (delimited by the white line). Numbers correspond to the populations listed in Table 1. Main-stem dams are indicated by bars. The Zone 6 fishery is located upstream of Bonneville Dam (river kilometer 235, measuring from the river's mouth) and below McNary Dam (river kilometer 470).

cedures. Included in this data set are eight new populations, as indicated in Table 1.

All allozyme data were obtained by standard starch gel electrophoresis (Aebersold et al. 1987) under the laboratory conditions described in Waples et al. (1993). Population statistics have been described previously (Waples et al. 1993; Phelps et al. 1994). Distance metrics and genetic diversity (i.e., Wright's fixation index, F_{ST}) calculations were obtained with BIOSYS (Swofford and Selander 1981). Population differences were depicted by means of multidimensional scaling (MDS) of genetic distance metrics in NTSYS-pc (Rohlf 1994). A principal components analysis (PCA) was conducted on the correlation matrix among the frequencies of the most common allozyme alleles and, in some cases, those of secondary or tertiary alleles present at a frequency of at least 5% (e.g., $P_{0.95}$ alleles).

DNA loci.—DNA was extracted from tissues archived at −80°C using a Qiagen DNeasy DNA isolation kit. A 1-mm × 5-mm piece of fin clip or muscle tissue was added to lysis buffer and incubated at 55°C for 5.5–8 h (see Qiagen rodent tail and animal tissue protocol). We amplified five microsatellite loci by means of polymerase chain reaction (PCR): *One*μ*8* using primer sets 5′-AACATTCTGGGATGACAGGGTA-3′ and 5′-CTG TTCTGCTCC AGTGAAGTGGA-3′ (Scribner et al. 1996), *One*μ*14* using primer sets 5′-AGAAA CATGAGAACAGTCTAGGT-3′ and 5′- CCTTA TGAGTTTGGTCTCCATGT-3′ (Scribner et al. 1996).

Ots4 using primer sets 5′-GACCCAGAGCAC AGCACAA-3′ and 5′-GGAGGACACATTTCAG CAG-3′ (Banks et al. 1999), *Ssa289* using primer sets 5′-CTTTACAAATAGACAGACT-3′ and 5′-TCATACAGTCACTATCATC-3′ (McConnell et al. 1995), and a new microsatellite locus (*P53ms*) using primer sets (5′-TGACACATATCCTCGCT TTCTCC-3′ and 5′-CAACTCTCTTGGTGAGG C-3′. Polymerase chain reactions were performed in 10-μL reactions with 5–25 ng/μL of DNA, 0.2–0.4 μM of each primer, 1.75–2.5 mM $MgCl_2$, 0.2–0.4 mM deoxynucleotide triphosphates, and 0.5–2.5 units Promega *Taq* DNA polymerase. Initial

TABLE 1.—Sampling information for 32 populations of steelhead in the Columbia River basin. Population locations are shown in Figure 1. Data were previously described by the Washington Department of Fish and Wildlife (WDFW; Phelps et al. 1994, 1996, 2000) and the National Marine Fisheries Service, (NMFS; Waples et al. 1993) except for the 8 populations designated as ''new.''

Population code	Population name	Location[a]	Number of fish		Year(s) collected	Agency
			Allozyme data	DNA data		
		Upper Columbia River				
1	Wells Hatchery	Upper Columbia	90	48	1991	WDFW
2	Wells ''wild''	Upper Columbia	44	43	1995, 1998	WDFW (new)
		Middle Columbia River				
3	Upper Klickitat River	Klickitat	309	15	1991, 1994	WDFW
4	Bowman Creek	Klickitat	104	55	1991	WDFW
5	Little Klickitat River	Klickitat	110	42	1991	WDFW
6	Lower Klickitat River	Klickitat	75	21	1994	WDFW
7	Umatilla Hatchery	Umatilla	86	56	1996	NMFS (new)
8	Beech Creek	John Day Dam	105	47	1996	NMFS (new)
9	Satus River	Yakima	333	58	1989–1990	WDFW
10	Toppenish Creek	Yakima	175	56	1990	WDFW
11	Wapatox Trap	Yakima	370	44	1987	WDFW
12	Roza Trap	Yakima	175	55	1989	WDFW
13	Chandler Trap	Yakima	373	59	1987	WDFW
14	Touchet River	Walla Walla	99	53	1995	WDFW (new)
		A run, Snake River				
15	Lower Tucannon River	Tucannon	143	48	1989–1990	NMFS
16	Upper Tucannon River	Tucannon	184	38	1989–1990	NMFS
17	Lyons Ferry Hatchery	Tucannon	100	15	1991	NMFS
18	Asotin Creek	Snake	100	52	1995	WDFW (new)
19	Chesnimnus Creek	Grande Ronde	200	43	1989, 1990	NMFS
20	Deer Creek	Grande Ronde	200	60	1989, 1990	NMFS
21	Wallowa Hatchery	Grande Ronde	200	46	1990–1991	NMFS
22	Lick Creek	Imnaha	192	50	1989–1990	NMFS
23	Camp Creek	Imnaha	99	47	1990	NMFS
24	Little Sheep Creek	Imnaha	200	44	1989–1990	NMFS
25	Little Sheep Hatchery	Imnaha	200	47	1990–1991	NMFS
26	Bargamin Creek	Snake	46	45	1999	WDFW (new)
27	Pahsimeroi Hatchery	Snake	100	48	1990	NMFS
		B run, Snake River				
28	Loon Creek	Mid Fork Salmon	65	48	1999	NMFS (new)
29	Secesh River	South Fork Salmon	30	30	1999	WDFW (new)
30	Dworshak Hatchery	Clearwater	200	40	1989, 1991	NMFS
31	Selway River[b]	Clearwater	83	47	1990	NMFS
32	Lochsa River[c]	Clearwater	176	48	1989, 1990	NMFS

[a] River unless indicated otherwise.
[b] At Gedney Creek.
[c] At Fish Creek.

denaturation of 2–5 min at 95°C was followed by one of two thermalcycler profiles, depending on the locus. For *One*μ*8* (60°C annealing temperature), *P53ms* (61°C annealing temperature), and *Ots4* (54°C annealing temperature), initial denaturation was followed by 32 cycles of 40 s at 94°C, 40 s at the specified annealing temperature, and 40 s at 72°C. For *Ssa289* (54°C annealing temperature) and *One*μ*14* (58°C annealing temperature), initial denaturation was followed by 32 cycles of 70 s at 94°C, 70 s at the specified annealing temperature, and 70 s at 72°C. A final extension of 5 min at 72°C was added to the end of each thermalcycler profile. The forward primer from each pair was fluorescently labeled with one of five dyes (FAM, NED, HEX, VIC, or PET; Applied Biosystems), and electrophoresis was conducted on an Applied Biosystems Model 3100 automated sequencer. Fragment size was determined and genotyping analysis performed with Genescan 3.6 and Genotyper 3.6, respectively (Applied Biosystems). Restriction fragment length polymorphisms were analyzed to detect variation in the intron regions of three loci: *GnRH*, *IK*, and *RAG3′* (see Baker et al. 2002 for primer sequences, PCR conditions, and electrophoretic conditions). The PCR

products for each locus were digested with a specific restriction enzyme: *Mse*I for *GnRH, Dra*I for *IK,* and *Fok*I for *RAG3′* (see Baker et al. 2002). Fragment lengths were determined by visually comparing the electrophoresed fragments with a 100-base-pair DNA ladder from New England Bio Laboratories.

Computer simulations.—We analyzed computer-generated (i.e., simulated) population mixtures to evaluate the ability of different sets of loci to estimate the proportions of each ESU in a mixture. In each simulation, a mixture of 400 fish (i.e., 400 multilocus genotypes) was drawn from the baseline data and the stock composition of this mixture was estimated using a bootstrapped baseline data set. All sampling was done with replacement and assumed random mating and the independent assortment of loci. This sampling was repeated 1,000 times. Averages and standard deviations were recorded for each stock proportion estimate. In the first set of simulations, 100% of the fish in the mixture were from one ESU or management group. In the second set of simulations, we used three mixture scenarios that mimic the mixture compositions seen in past GSI work in the Columbia River. In the third set of simulations, we explored the effect of baseline sample size on the accuracy of the estimates of mixture proportions. The allozyme baseline data set is currently much larger than the microsatellite baseline data set (an average of 100 individuals per population for the allozyme data versus 48 individuals per population for the microsatellite data), and we wanted to estimate how much stock composition estimates might improve if the microsatellite data set were increased. In these simulations, we varied the baseline sample size from 10 to 300 individuals by randomly drawing alleles from the Rannala and Mountain probability distribution (which is very similar to the allele frequencies in the actual baseline data) for mixtures composed solely of individual ESUs.

The mixture analyses were conducted with Genetic Mixture Analysis (GMA; available at http://www.montana.edu/kalinowski/GMA/kalinowski_GMA.htm). In several instances, simulations were also conducted with the SPAM (Debevec et al. 2000) computer package (results not shown here). Both computer programs produce conditional maximum likelihood (Millar 1987) estimates of mixture proportions, but they use different methods for estimating the probability of sampling an allele from a baseline population. The important difference between these programs is that SPAM uses the maximum likelihood estimate of the probability of sampling the *i*th allele from a baseline population (n_i/n, where n_i is the number of copies of the *i*th allele that have been observed in a baseline sample of n genes) and GMA uses a Bayesian estimate of this probability ($\{n_i + [1/k]\}/[n + 1]$, where k is the number of alleles at the locus; Rannala and Mountain 1997). The distinction between these two estimates is that the maximum likelihood probability will equal zero for an allele not found in a baseline sample while the Bayesian probability will equal a small nonzero number. In our experience, the Bayesian approach completely eliminates the ''unclassified'' multilocus genotypes that are identified in mixture results based on SPAM.

Locus evaluation and selection criteria.—To evaluate whether a subset of allozyme loci would provide adequate population differentiation in a mixture analysis, we constructed three different subsets of 10 loci each. For each subset, we chose 10 loci as a baseline for simulation analyses based on the largest F_{ST} or total heterozygosity (H_T; i.e., the mean expected heterozygosity over populations) values or on the weighting coefficients along the first three components of a PCA. We then compared these results with the full data sets for each marker group.

Results

We collected data for all marker sets for 32 populations (Table 1). The average number of fish analyzed for allozymes was 158 per population. The average number of fish analyzed for DNA variability was 44 per population. Three populations from the lower Columbia River ESU (Wind, Panther, and Skamania rivers) used in the Washington Department of Fish and Wildlife allozyme baseline for GSI analyses (Kassler et al. 2002) were not included because of their absence or low occurrence (<1%) in mixture analyses to date (Winans, unpublished data). Allele frequency data are available for both allozyme (Washington Department of Fish and Wildlife, Genetics Section, 600 Capital Way North, Olympia, Washington 98501–1091) and DNA loci (http://www.nwfsc.noaa.gov/datasets/steelhead_allele_data.xls).

Allozymes

The allelic variability at 32 loci is summarized in Table 2. The allozyme loci were characterized by generally low levels of variation. Only 8 alleles (from 7 loci) were variable at the $P_{0.95}$ level in 50% or more populations (Table 2). Of the 32 al-

TABLE 2.—Summary of genetic variation at 32 loci in populations of steelhead. In the column headings, A is the number of alleles; F_{ST} is Wright's fixation index; PC loci are loci or alleles with large weights on principal component axes 1–3 (PC1–3) of 17 common alleles and 9 secondary or tertiary alleles; H_T is total heterozygosity; and a $P_{0.95}$ population is one with a secondary or tertiary (parentheses) allele with a frequency ≥ 0.05 (for microsatellite loci, the number of alleles at a locus with a frequency ≥ 0.05 in at least one population is given in brackets).

Locus	A	Mean allele frequency	F_{ST}	PC loci	H_T	No. of $P_{0.95}$ populations
			Allozymes			
*mAAT-1**	2	0.983	0.026		0.032	4
*sAAT-1,2**	3	0.970	0.010		0.058	7
*sAAT-3**	3	0.994	0.046		0.013	1
*ADA-1**	3	0.983	0.037		0.034	3
*ADA-2**	5	0.970	0.032		0.058	4
*ADH**	4	0.988	0.020		0.024	2
*sAH**	4	0.760	0.040		0.375	32 (5)
*ALAT**	3	0.948	0.044	PC2	0.098	13 (1)
*FH**	3	0.986	0.026		0.028	2
*GAPDH**	3	0.944	0.066	PC3	0.105	14 (1)
*G3PDH**	4	0.987	0.059	PC1	0.026	4
*GPI-A**	3	0.993	0.012	[a]	0.026	0
*GPI-B1**	4	0.993	0.043	[a]	0.014	1
*GPI-B2**	2	0.999	0.000	[a]	0.001	0
*mIDHP**	4	0.985	0.029		0.030	2
*sIDHP-1**	4	0.995	0.004		0.010	0
*sIDHP-2**	5	0.209	0.027		0.648	32 (32)
*LDH-B1**	2	0.999	0.015	[a]	0.002	0
*LDH-B2**	3	0.408	0.077	PC1	0.487	32
*LDH-C**	2	0.998	0.011	[a]	0.005	0
*sMDH-A1,2**	4	0.995	0.002	[a]	0.010	0
*sMDH-B1,2**	6	0.975	0.020	PC1	0.050	4 (1)
*sMEP-1**	2	0.988	0.107	PC1	0.023	3
*MPI**	3	0.943	0.065	PC2	0.109	13
*NTP**	4	0.721	0.054	PC1	0.410	32 (1)
*PEPA**	4	0.879	0.064		0.215	28 (3)
*PEPD-1**	4	0.980	0.042	PC1	0.039	4
*PEPLT**	2	0.991	0.026		0.017	2
*PGK-2**	4	0.583	0.036	PC3	0.511	32 (6)
*PGM-2**	3	0.990	0.022	PC1	0.019	2
*sSOD-1**	3	0.904	0.064	PC1	0.178	7 (16)
*TPI-3**	4	0.979	0.076		0.041	3
Average			0.047		0.116	
			Microsatellites			
*One*μ*8**	21	0.190[b]	0.044		0.842	32 [15]
*One*μ*14**	10	0.551[c]	0.027		0.593	32 [7]
*Ots4**	10	0.436[d]	0.036		0.701	32 [8]
*P53ms**	19	0.583[e]	0.054		0.587	32 [8]
*Ssa289**	8	0.648[f]	0.042		0.506	32 [5]
Average			0.041		0.646	
			Introns			
*IK**	2	0.568	0.072		0.455	32
*GnRH**	2	0.901	0.059		0.167	22
*RAG3**	2	0.896	0.036		0.179	29
Average			0.050		0.267	

[a] Dropped due to low variability.
[b] Allele **149*.
[c] Allele **154*.
[d] Allele **123*.
[e] Allele **160*.
[f] Allele **106*.

lozyme loci, 23 (72%) had 3–4 alleles per locus. The maximum number of alleles per locus was 6 (*sMDH-B1,2**). A total of 109 allozyme alleles were scored. Of the 172 tests for conformity to Hardy–Weinberg expectations in the 8 new populations (Table 1), only 2.5% were statistically significant. For the remaining 24 samples, previous test statistics were all approximately within the limits of what would be expected by chance alone (<5%; see Phelps et al. 1994, 1996, 2000; Waples et al. 1993), and the incidence of departure from Hardy–Weinberg expectations appeared to be randomly distributed among populations and gene loci. All chi-square comparisons of allele frequencies locus by locus (across populations) were statistically significant ($P < 0.001$), with the exceptions of *sMDH-A1,2** ($P = 0.017$) and *GPI-B2** ($P = 0.59$). The average F_{ST} for allozymes was 0.047, 10 loci having an F_{ST} greater than 0.05, indicating moderate levels of geographic differentiation (Hartl 1981). The H_T values ranged from 0.014 (*GPI-B1**) to 0.648 (*sIDH-P2**), averaging 0.116.

Microsatellites

Allelic variability at five microsatellite loci is summarized in Table 2. Sixty-eight alleles were scored over the five loci, ranging from 8 alleles at *Ssa289* to 21 alleles at *One*μ*8* (Table 2). From 5 to 15 alleles per locus had a frequency of at least 0.05 (i.e., $P_{0.95}$ level; Table 2). There were 21 (13%) statistically significant departures from Hardy–Weinberg proportions. Almost half (9) of these were due to heterozygote deficiencies in three Yakima River populations (Chandler, Rosa, and Wapatox traps) and may be due to downstream admixture of juveniles from semi-isolated populations. Allele frequency data from these collections will be used tentatively until further sampling is conducted. The remainder of the Hardy–Weinberg departures appeared to be randomly distributed across populations and loci. All chi-square contingency tests of allele frequencies over all populations were statistically significant ($P < 0.001$). The mean F_{ST} for microsatellite loci was 0.041; H_T ranged from 0.506 (*Ssa289*) to 0.842 (*One*μ*8*), averaging 0.646.

Introns

Two alleles were scored at each intron locus, for a total of 6 alleles (Table 2). Each locus was variable at the $P_{0.95}$ level in essentially all populations, and the allele frequencies were statistically significant among all populations ($P < 0.001$). The level of genotypic departure from Hardy–Weinberg expectations (6%) was close to that expected by chance alone (i.e., 5%). The mean F_{ST} for intron loci was 0.05; H_T ranged from 0.167 (*GnRH*) to 0.455 (*IK*), averaging 0.267.

Variation among Populations

Patterns of differentiation were generally congruent among marker classes (Figures 2–4). For example, MDS plots of allozyme and microsatellite data showed distinct clusterings of the middle Columbia River ESU and Snake River B-run populations (Figures 2, 3). Although the Snake River A-run populations clustered together, both data points for the upper Columbia River ESU fell within this group for the allozyme and microsatellite data sets on the first two axes. Only along the third MDS axis (which was based on microsatellite loci) was the upper Columbia River ESU distinct (Figure 3). A scatter plot of PCA scores based on allozyme frequencies and an MDS plot based on Nei's genetic distance (Nei 1978) (neither graph shown) were highly congruent with the ESU relationships seen in the allozyme plot. The distribution of populations in the MDS plot based on intron variation was similar to the patterns from the other markers but showed less of a geographic pattern with respect to population differentiation (Figure 4).

Simulations

100% ESU analyses.—The Snake River B-run group was the most readily identified over all simulations, and the upper Columbia River ESU had the lowest estimates (Table 3). Allozymes provided the most accurate estimates for the middle Columbia River ESU simulation (94%) and both Snake River reporting groups (93%), whereas microsatellite data had the highest estimate for the upper Columbia River ESU (83%). Over all scenarios, estimates based on intron data ranged from 47% to 77%. For each scenario, the all-loci data set provided the most accurate estimates (Table 3). It is worth noting that the estimates made by means of the standard algorithm procedure (i.e., SPAM, which does not accommodate rare alleles) were substantially lower (averaging only 72% for the all-loci analyses) and that 13–28% of the mixture genotypes were identified as unknowns.

Realistic-mixture analyses.—Past GSI work in the Bonneville Dam monitoring program and in the Zone 6 bycatch surveys has shown that the Snake River ESU (runs A and B together) is the numerically dominant ESU (Kassler et al. 2002). With these results in mind, we used values of 70,

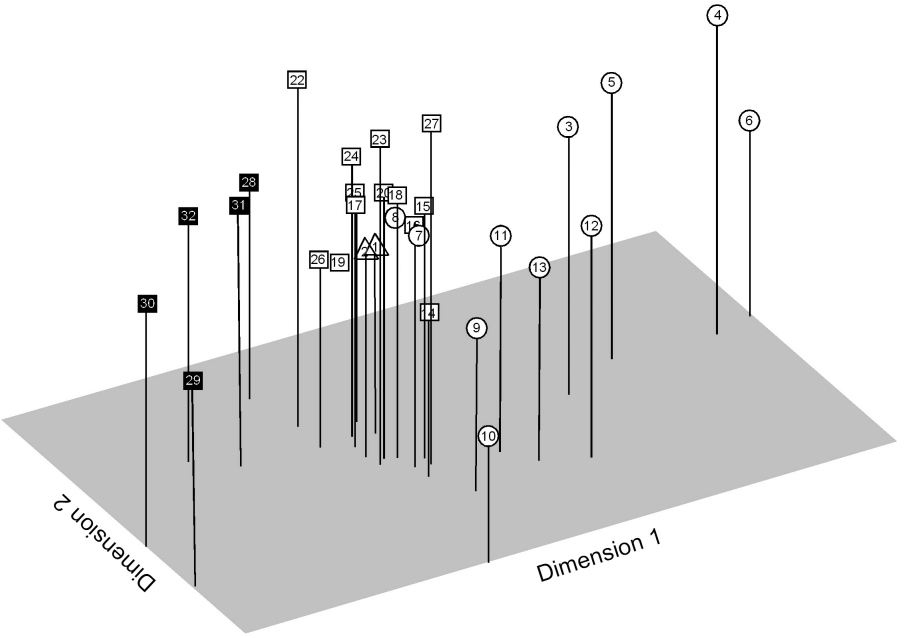

FIGURE 2.—Multidimensional scaling plot of steelhead populations based on chord distances (Cavalli-Sforza and Edwards 1967) derived from 32 allozyme markers. Triangles represent evolutionarily significant units (ESUs) from the upper Columbia River, circles ESUs from the middle Columbia River, and squares ESUs from the Snake River (open squares refer to run A and closed squares to run B).

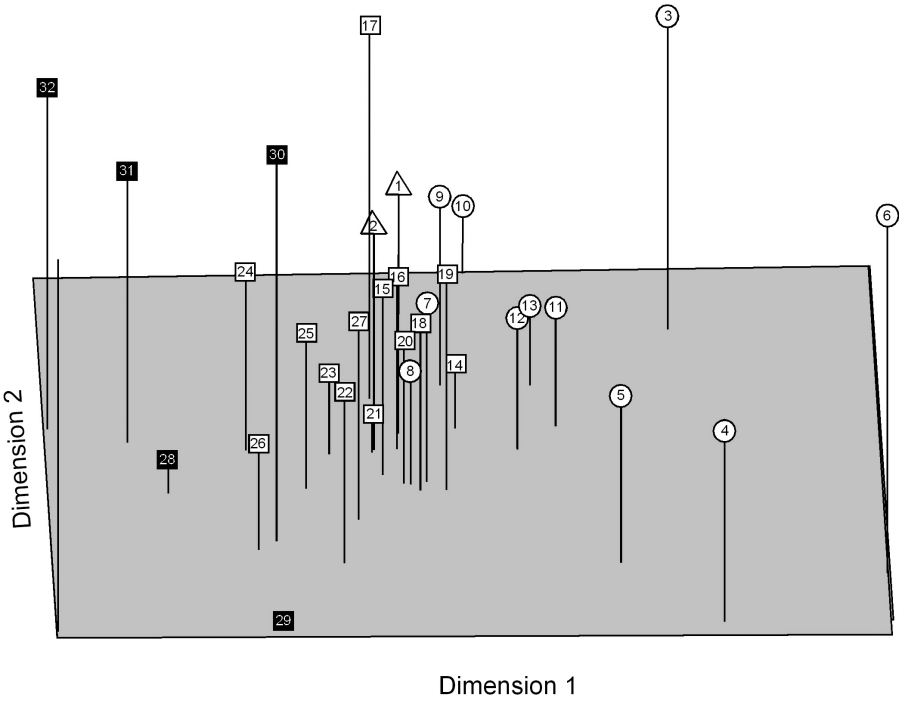

FIGURE 3.—Multidimensional scaling plot of steelhead populations based on chord distances (Cavalli-Sforza and Edwards 1967) derived from five microsatellite markers. See the caption to Figure 2 for an explanation of the symbols used.

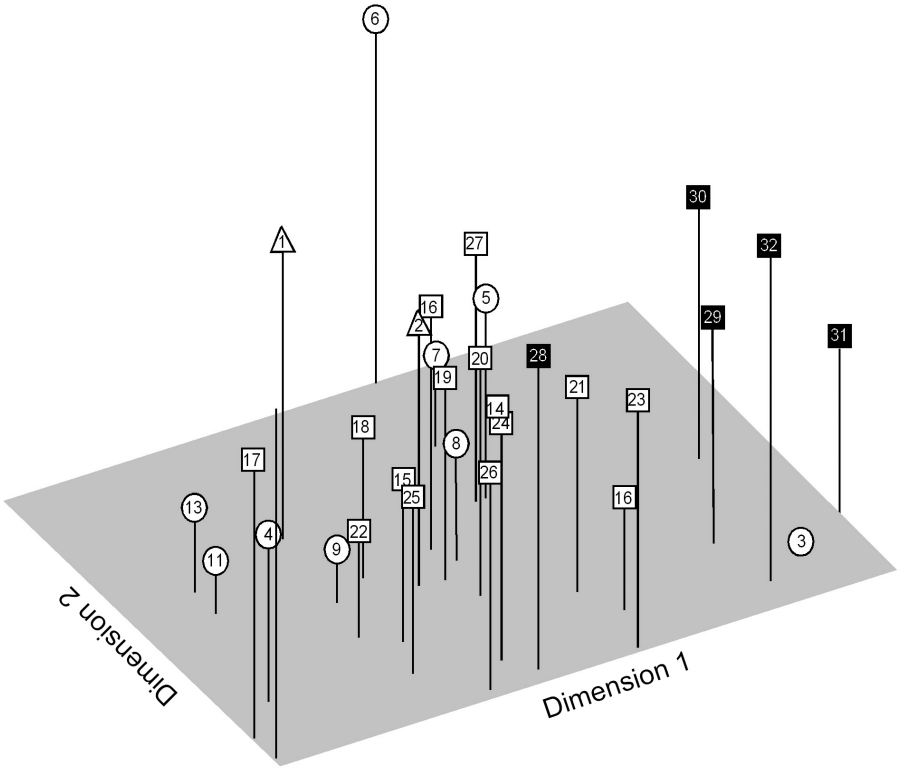

FIGURE 4.—Multidimensional scaling plot of steelhead populations based on chord distances (Cavalli-Sforza and Edwards 1967) derived from three intron markers. See the caption to Figure 2 for an explanation of the symbols used.

TABLE 3.—Results of four simulated mixture scenarios consisting entirely of one evolutionarily significant unit (ESU) or other management group. Analyses were based on four sets of loci (introns, microsatellites, allozymes, and all data). Mean (SDs in parentheses) estimates of percent compositions were calculated from 1,000 bootstrapped mixtures with 400 fish per mixture. Estimates were made for individual populations ($n = 32$) and pooled by ESU or other management group.

		Management group			
Genetic material	Population	Upper Columbia River	Middle Columbia River	A run, Snake River	B run, Snake River
Introns (3 loci, 6 alleles)	Upper Columbia River	47 (21)	25 (19)	23 (18)	5 (6)
	Middle Columbia River	4 (4)	73 (17)	18 (18)	5 (5)
	A run, Snake River	3 (6)	23 (16)	63 (20)	11 (12)
	B run, Snake River	1 (2)	5 (9)	17 (14)	77 (15)
Microsatellites (5 loci, 68 alleles)	Upper Columbia River	83 (5)	5 (3)	11 (4)	1 (1)
	Middle Columbia River	2 (2)	90 (4)	8 (3)	0 (1)
	A run, Snake River	4 (3)	9 (4)	85 (5)	2 (1)
	B run, Snake River	0 (0)	3 (2)	7 (3)	90 (3)
Allozymes (32 loci, 109 alleles)	Upper Columbia River	61 (7)	8 (4)	25 (7)	6 (3)
	Middle Columbia River	0 (0)	94 (3)	6 (3)	0 (1)
	A run, Snake River	1 (1)	4 (3)	93 (4)	2 (2)
	B run, Snake River	1 (1)	0 (1)	6 (3)	93 (3)
All data (40 loci, 183 alleles)	Upper Columbia River	84 (4)	4 (2)	11 (4)	1 (1)
	Middle Columbia River	0 (1)	95 (2)	5 (2)	0 (0)
	A run, Snake River	1 (1)	4 (2)	94 (2)	1 (1)
	B run, Snake River	1 (0)	0 (1)	3 (2)	96 (2)

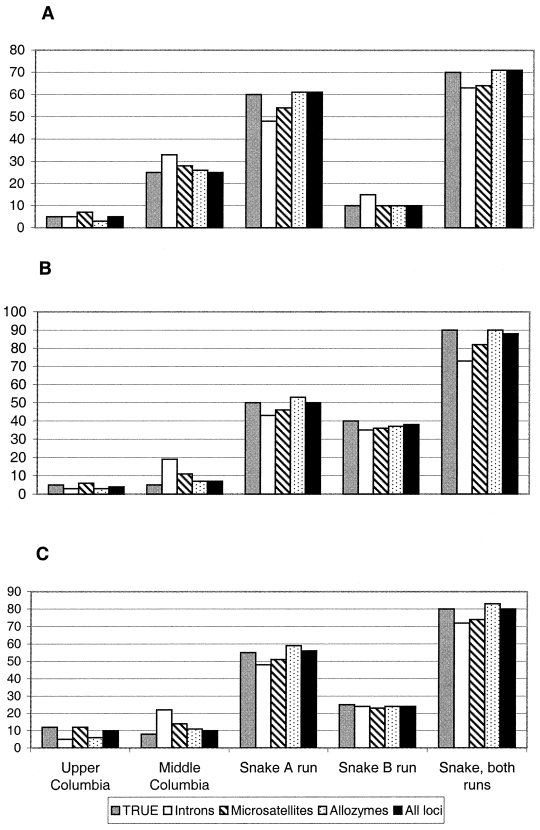

FIGURE 5.—Histograms of percent of mixture by steelhead population management group for three simulations **(A–C)** using four different marker sets (3 introns, 5 microsatellites, 32 allozymes, and all loci).

80, and 90% for this ESU to determine how the three marker classes would perform under more realistic scenarios (Figure 5). Based on the previously discussed simulations, it is not surprising that the microsatellite markers provided the best estimates for the upper Columbia River ESU when the true values were 5% or 12% (note that the intron markers also did well; Figure 5a). Allozyme data provided the most accurate estimates for the middle Columbia River ESU when it was present at proportions of 5, 8, or 25%. In general, the intron and microsatellite markers underestimated the Snake River groups, whereas the allozyme data were accurate or perhaps slightly positively biased for these two groups (Figure 5). Analyses using all-loci yielded the best results for the middle Columbia and Snake River groups.

Effect of Baseline Sample Size

Larger baseline samples produced better estimates of mixture proportions (Figure 6). However, increasing sample sizes produced diminishing returns, and in most cases sampling more than 50 individuals produced little improvement. Although the five microsatellite loci used in this analysis have many more alleles per locus than the allozyme loci, the microsatellite loci did not require or benefit from having larger sample sizes than the allozyme loci.

The mixtures that were composed entirely of fish from the upper Columbia River populations and that were analyzed with allozyme loci showed the greatest benefit from larger sample sizes. In this case, stock proportion estimates improved as baseline sample sizes increased beyond 200 individuals. It is not clear what aspects of the data effect different relationships between discriminatory power and sample size, but the relative indistinctiveness of the upper Columbia River collections from the Snake River collections with regard to allozyme variation is probably a factor.

Criteria for Selecting Subsets of Allozyme Loci

The three subsets of allozyme loci (plus the five microsatellite loci) identified by F_{ST}, PCA, and H_T yielded similar results in the 100% middle Columbia River ESU and 100% Snake River A-run scenarios; the estimates were 2–4% lower than the all-loci results (Table 4). However, the H_T loci results equaled the all-loci results for the 100% upper Columbia River ESU simulation and were only 1 percentage point less in the Snake River B-run simulation (Table 4). Previous simulations showed that a sample size of 50–100 individuals per population was sufficient for baseline data (Figure 6). We reran the simulations using the H_T allozymes plus the five microsatellite loci but assumed the allele frequencies were based on 100 fish per population for the DNA markers. The estimate for a simulated mixture consisting entirely of upper Columbia River ESU fish was 90%, 6 percentage points greater than the all-loci estimate. A simulation of the 100% Snake River B run led to a 98% estimate, 2 percentage points greater than the all-loci estimate.

Discussion

Relative to other species of Pacific salmon, steelhead have been shown to have high levels of heterozygosity and moderate levels of genetic differentiation, with a strong geographic component (Busby et al. 1996; Scribner et al. 1998; Beacham et al. 1999). Here, new microsatellite data from the Columbia River basin corroborate earlier findings.

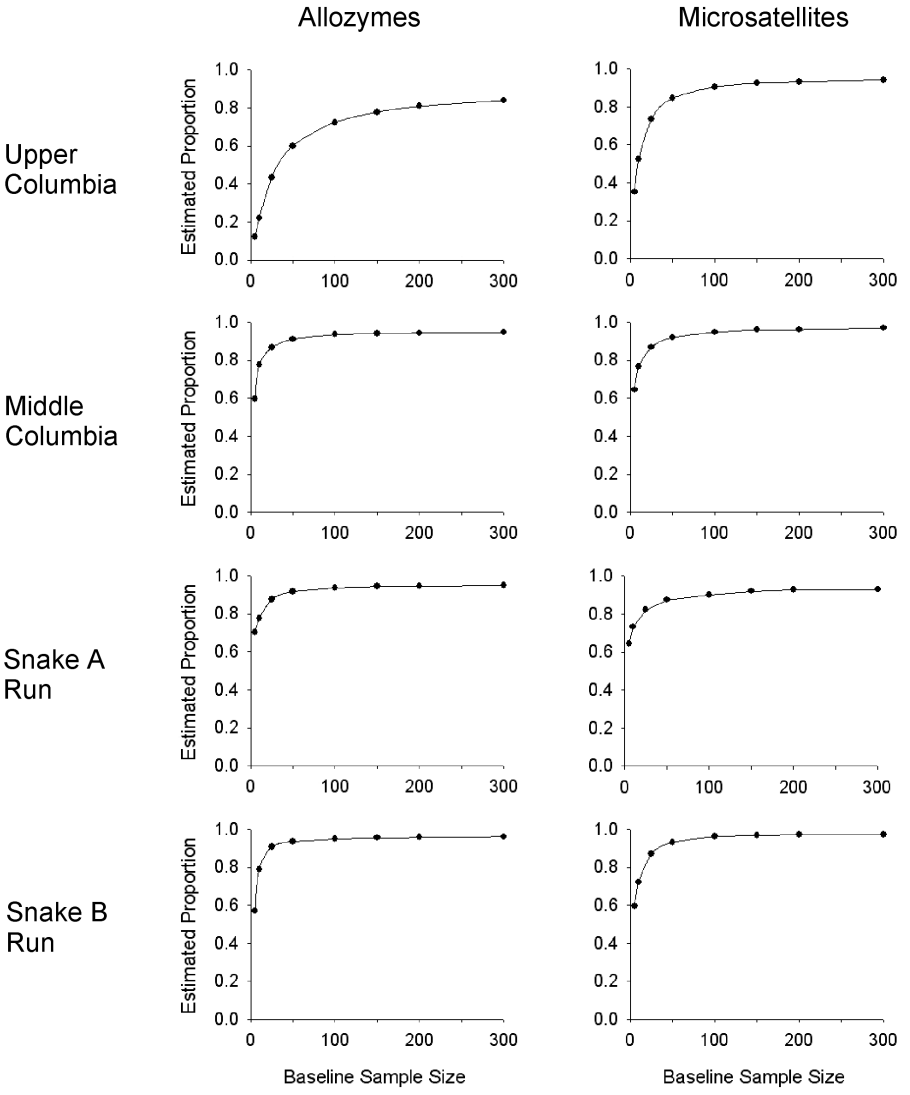

FIGURE 6.—Effects of eight sample sizes on estimates of mixture proportions in four simulated mixture scenarios. Mean estimates are based on 1,000 mixtures, each containing 400 fish.

As expected, microsatellite loci had a greater number of alleles per locus and larger H_T values than are typically observed at allozyme loci. Introns had intermediate H_T values and low allelic diversity. Despite these differences in total variation, there was little difference among marker classes in the magnitude of population differentiation as estimated by F_{ST}, which ranged from 0.041 (microsatellite loci) to 0.050 (intron loci). This result confirms findings of an earlier study reporting that different classes of markers have similar F_{ST} values (Allendorf and Seeb 2000). Regardless of marker type, statistically significant differences among populations were found for all loci. And with the exception of intron variability, genetic variability followed geographic patterns. The latter result has important practical consequences. It is unrealistic to expect to sample all contributing source populations in a mixture for GSI analysis. It is also statistically unwieldy to use baselines that contain multilocus data for a large number of populations (e.g., $N = 100–200$). But when populations are genetically related by geographic proximity, not all populations in an area need to be included in the genetic baseline and ''representative'' populations that include ma-

TABLE 4.—Estimates comparable to those reported in Table 3 but based on allozyme loci (identified in Table 2) together with five microsatellite loci. Allozyme loci were chosen based on the values of F_{ST} and H_T or because they had alleles with large weights on axes 1–3 in a principal components analysis (PCA; Table 2). See Table 3 for additional details.

Criterion	Upper Columbia River	Middle Columbia River	A run, Snake River	B run, Snake River
All data	84 (4)	95 (1)	94 (1)	96 (3)
F_{ST}[a]	83 (5)	93 (2)	90 (4)	93 (3)
PCA[a]	83 (5)	93 (3)	90 (4)	94 (2)
H_T[a]	84 (5)	93 (3)	90 (3)	95 (2)
H_T[b]	90 (5)			98 (2)

[a] $N \approx 48$ fish/locus/population.
[b] $N = 100$ fish/locus/population.

jor regional productivity will produce accurate regional GSI estimates (Beacham et al. 2001).

Genetic differences among steelhead populations at allozyme and microsatellite loci are sufficient for accurate GSI estimation. In general, allocation to ESU is more than 85% correct, although it can be more or less accurate than that depending on the particular data set and target ESU (Table 3). Empirically determined biases (e.g., estimates for the upper Columbia River ESU are likely to include individuals from the Snake River ESU; Table 3) can be partially explained by the overlapping genetic similarities seen in the MDS plots (Figures 2–4). It should be noted that we did not evaluate the effect of year-to-year variation in marker frequencies, which could be important in both GSI discrimination and locus selection. In this regard, (Waples et al. 1993) reported many cases in which between-year differences within populations were small compared with the differences between populations (although exceptions were noted).

Our initial results indicate that the greater the number of alleles used in a GSI analysis the better the resolution among ESUs. For example, the all-loci analyses yielded the most accurate estimates of the single-composition simulations (Table 3). However, the gain was minimal in most cases compared with estimates using locus subsets. For example, the estimate for the 100% upper Columbia River ESU was 83% with microsatellite loci (63 independent alleles) and 84% using all data (143 independent alleles). For the 100% Snake River B run, the results based on all data were only 3 percentage points greater than the allozyme estimate (67 independent alleles; 93%). The simulations based on subsets of allozyme loci provide an interesting perspective. We found that the results obtained with 10 ''H_T'' allozyme loci plus the 5 microsatellite loci (89 independent alleles) are comparable to those obtained with all genetic data (Table 4). We also found that better results were obtained using these same loci having assumed that the microsatellite sample size was 100 fish (Table 4). Recent theoretical work has shown that the greater the number of alleles used in simulated GSI analyses the better the estimates of stock proportions (Kalinowski, in press). This work is based on allele distributions with similar distribution patterns. Our results suggest that the empirical patterns and levels of allelic variation may strongly influence the resolution obtained for a particular allele set in a GSI application.

We evaluated whether the allozyme data set could be simplified by identifying ''important'' subsets of allozyme loci to use in mixture analyses. Of the three criteria, the loci selected by total heterozygosity (H_T) performed better in two of four scenarios. This finding translates into dropping 22 loci from the electrophoretic screening protocol with a loss of only 2–4% accuracy for the above scenarios. In contrast, Scribner et al. (1998) reported that the values of the likelihood ratio (G), F_{ST}, and H_T failed to isolate critical loci for GSI analyses of two management groups of chum salmon in the Yukon River. Evaluations of various methodologies and statistics for identifying subsets of discriminatory loci will continue as GSI applications increasingly use DNA-based loci with complex allele distributions and technical requirements.

Are DNA-based loci better than protein-genetic markers for GSI? Perhaps. Here we show that the variability in allozymes and microsatellites bears a similar genetic signature with respect to the microevolutionary events that produce spatial genetic relationships among populations (Scribner et al. 1998; Allendorf and Seeb 2000). From a practical perspective, either or both data sets can be used reliably for similar and accurate GSI estimations. From a technical perspective, DNA markers are becoming more cost effective, diverse, and automated. Importantly, extremely small tissue samples can be taken from juveniles as well as adult fish nonlethally—a vast advance over protein tissue requirements. Still, there are attendant problems with the technology. For example, standardization of allele scoring within and between laboratories may be more difficult for microsatellite markers than for allozymes due to the larger number of alleles per locus. The large number of alleles

at microsatellite loci may also lead to other problems. For example, it has been shown that loci with more than 30 alleles can lead to serious genotyping errors and inconsistencies (O'Reilly et al. 1998). We also note that the future of steelhead GSI may not lie totally with microsatellites: less developed molecular technologies may provide further easily automated procedures. For example, techniques to measure single nucleotide insert polymorphisms can provide data for biallelic loci—and these techniques are amenable to extremely high throughput for a large number of loci (Kalinowski, unpublished).

Which loci do we recommend? We suggest that mixture analysis of steelhead in the Columbia River include both allozyme and microsatellite data. The detection of fish from the upper Columbia River ESU is best accomplished with microsatellite loci; the two other upriver ESUs (three management units) are best resolved with a combination of allozyme and microsatellite data. Nonlethally sampling adult fish for allozymes in these two applications is not an issue, as an opercular punch or fin clip is sufficient (Van Doornik et al. 1999). The inconvenience of maintaining two field and laboratory protocols, however, will predictably end when a refined and expanded data set of DNA-based loci is completed.

Acknowledgments

Stevan Phelps (WDFW) helped initiate the original research project and manage earlier portions of the allozyme baseline. David Kuligowski (NMFS) performed a portion of the protein electrophoresis. Norm Switzler, Janine Rhone, Bill Ingram, Cherril Bowman, and Keith Sweeney participated in the data collection at WDFW. Bill Hopley (WDFW) and Paul Aebersold (NMFS) provided administrative and technical support; and Kathleen Neeley and Jennifer Burke (NMFS) helped with the illustrations. Reference to trade names does not imply endorsement by NMFS.

References

Aebersold, P. B., G. A. Winans, D. J. Teel, G. B. Milner, and F. M. Utter. 1987. Manual for starch gel electrophoresis: a method for the detection of genetic variation. NOAA Technical Report NMFS 61.

Allendorf, F. W., and L. W. Seeb. 2000. Concordance of genetic divergence among sockeye salmon populations at allozyme, nuclear DNA, and mitochondrial DNA markers. Evolution 54:640–651.

Avise, J. C. 1994. Molecular markers, natural history, and evolution. Chapman and Hall, New York.

Baker, J., P. Bentzen, and P. Moran. 2002. Molecular markers distinguish coastal cutthroat trout from coastal rainbow trout/steelhead and their hybrids. Transactions of the American Fisheries Society 131: 404–417.

Banks, M. A., M. S. Blouin, B. A. Baldwin, V. K. Rashbrook, H. A. Fitzgerald, S. M. Blankenship, and D. Hedgecock. 1999. Isolation and inheritance of novel microsatellites in Chinook salmon (*Oncorhynchus tschawytscha*) (correction). Journal of Heredity 90(3):U1–U1.

Beacham, T. D., J. R. Candy, K. J. Supernault, T. Ming, B. Deagle, A. Schulze, D. Tuck, K. H. Kaukinen, J. R. Irvine, K. M. Miller, and R. E. Withler. 2001. Evaluation and application of microsatellite and major histocompatibility complex variation for stock identification of coho salmon in British Columbia. Transactions of the American Fisheries Society 130: 1116–1149.

Beacham, T. D., S. Pollard, and K. D. Le. 1999. Population structure and stock identification of steelhead in southern British Columbia, Washington, and the Columbia River based on microsatellite DNA variation. Transactions of the American Fisheries Society 128:1068–1084.

Busby, P. J., T. C. Wainwright, G. J. Bryant, L. J. Lierheimer, R. S. Waples, F. W. Waknitz, and I. V. Lagomarsino. 1996. Status review of West Coast steelhead from Washington, Idaho, Oregon, and California. NOAA Technical Memorandum NMFS-NWFSC-27.

Cavalli-Sforza, L. L., and A. W. F. Edwards. 1967. Phylogenetic analysis: models and estimation procedures. Evolution 21:550–570.

Debevec, E. M., R. B. Gates, M. Masuda, J. Pella, J. J. Reynolds, and L. W. Seeb. 2000. SPAM (version 3.2): Statistics program for analyzing mixtures. Journal of Heredity 91:509–511.

Hartl, D. L. 1981. A primer of population genetics. Sinauer Associates, Sunderland, Massachusetts.

Kalinowski, S. T. In press. Genetic polymorphism and mixed stock fisheries analysis. Canadian Journal of Fisheries and Aquatic Sciences.

Kassler, T. W., D. Rawding, A. Marshall, B. Baker, and J. B. Shaklee. 2002. Genetic mixed-stock analysis of Columbia River steelhead at Bonneville Dam and in the Zone 6 fishery, 1997–2001. Washington Department of Fish and Wildlife, Report to Northwest Fisheries Science Center, Seattle.

Lewontin, R. C. 1991. Twenty-five years ago in genetics: electrophoresis in the development of evolutionary genetics—milestone or millstone? Genetics 128:657–662.

McConnell, S. K., P. O'Reilly, L. Hamilton, J. M. Wright, and P. Bentzen. 1995. Polymorphic microsatellite loci from Atlantic salmon (*Salmo salar*): genetic differentiation of North American and European populations. Canadian Journal of Fisheries and Aquatic Sciences 52:1863–1872.

Millar, R. B. 1987. Maximum likelihood estimation of mixed-stock fishery composition. Canadian Journal of Fisheries and Aquatic Sciences 44:583–590.

Nei, M. 1978. Estimation of average heterozygosity and

genetic distance from a small number of individuals. Genetics 89:583–590.

O'Reilly, P. T., C. Herbinger, and J. M. Wright. 1998. Analysis of parentage determination in Atlantic salmon (*Salmo salar*) using microsatellites. Animal Genetics 29:363–370.

Park, L. K., and P. Moran. 1994. Development in molecular genetic techniques in fisheries. Reviews in Fish Biology and Fisheries 4:272–299.

Phelps, S. R., B. M. Baker, and C. A. Busack. 2000. Genetic relationships and stock structure of Yakima River basin and Klickitat River basin steelhead populations. Washington Department of Fish and Wildlife, Fisheries Management Program, Progress Report, Olympia.

Phelps, S. R., B. M. Baker, P. L. Hulett, and S. A. Leider. 1994. Genetic analysis of Washington steelhead: initial electrophoretic analysis of wild and hatchery steelhead and rainbow trout. Washington Department of Fish and Wildlife, Fisheries Management Program Report 94-9, Olympia.

Phelps, S. R., S. A. Leider, P. L. Hulett, B. Baker, and T. Johnson. 1996. Genetic analyses of Washington steelhead: preliminary results incorporating 36 new collections from 1995 and 1996. Washington Department of Fish and Wildlife, Fisheries Management Program, Progress Report, Olympia.

Rannala, B., and J. L. Mountain. 1997. Detecting immigration by using multilocus genotypes. Proceedings of the National Academy of Sciences of the United States of America 94:9197–9201.

Rawding, D., S. Phelps, A. Marshall, and C. W. Hopley. 1997. Genetic stock identification of steelhead in Columbia River Zone 6 fishery and at Bonneville Dam. Washington Department of Fish and Wildlife, Annual Report of Research to the Northwest Fisheries Science Center, Seattle.

Rohlf, F. J. 1994. NTSYS-pc: numerical taxonomy and multivariate analysis system, version 1.80. Exeter Software, Setauket, New York.

Scribner, K. T., P. A. Crane, W. J. Spearman, and L. W. Seeb. 1998. DNA and allozyme markers provide concordant estimates of population differentiation: analyses of U.S. and Canadian populations of Yukon River fall-run chum salmon (*Oncorhynchus keta*). Canadian Journal of Fisheries and Aquatic Sciences 55:1748–1758.

Scribner, K. T., J. R. Gust, and R. L. Fields. 1996. Isolation and characterization of novel salmon microsatellite loci: cross-species amplification and population genetic applications. Canadian Journal of Fisheries and Aquatic Sciences 53:833–841.

Seeb, L. W., and P. A. Crane. 1999. Allozymes and mitochondrial DNA discriminate Asian and North American populations of chum salmon in mixed-stock fisheries along the south coast of the Alaska Peninsula. Transactions of American Fisheries Society 128:88–103.

Swofford, D. L., and R. B. Selander. 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. Journal of Heredity 72:281–283.

TAC (Technical Advisory Committee). 1997. Columbia River fish management plan: 1996 all-species review. Technical Advisory Committee, *U.S. v. Oregon*, Report 222, Portland, Oregon.

Teel, D. J., P. A. Crane, C. M. Guthrie III, A. R. Marshall, D. M. Van Doornik, W. D. Templin, N. V. Varnavskya, and L. W. Seeb. 1999. Comprehensive allozyme database discriminates Chinook salmon around the Pacific Rim. North Pacific Anadromous Fish Commission, Document 440, Anchorage, Alaska.

Van Doornik, D. M., G. A. Winans, and D. J. Teel. 1999. Allozyme studies of Pacific salmonids with nonlethal sampling of fin tissues. North American Journal of Fisheries Management 19:678–686.

Waples, R. S., O. W. Johnson, P. A. Aebersold, C. K. Shiflett, D. M. Van Doornik, D. J. Teel, and A. E. Cook. 1993. A genetic monitoring and evaluation program for supplemented populations of salmon and steelhead in the Snake River basin. Annual Repor of Research to the Bonneville Power Administration, Portland, Oregon.

Wilmot, R. L., C. M. Kondzela, C. M. Guthrie, III, A. Moles, J. J. Pella, and M. Masuda. 2000. Origins of salmon seized from the FV *Arctic Wind*. Auke Bay Laboratory, Alaska Fisheries Science Center, Juneau.

Winans, G. A., D. Viele, A. Grover, M. Palmer-Zwahlen, D. Teel, and D. M. Van Doornik. 2001. An update of genetic stock identification of Chinook salmon in the Pacific Northwest: test fisheries in California. Reviews in Fisheries Science 9:213–237.