Genetic Variation at Minisatellite DNA Loci Among North Pacific Populations of Steelhead and Rainbow Trout (*Oncorhynchus mykiss*)

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Genetic variation at minisatellite DNA, or variable number tandem repeat (VNTR), loci is widely studied in the context of animal breeding and pedigree analyses, but comparatively little information exists on the levels of variation at such loci in natural populations. I examined allelic variability at two VNTR loci (Ssa1 and T34) by Southern hybridization analyses within and between populations of steelhead and rainbow trout, the sea-run and freshwater resident life-history forms of *Oncorhynchus mykiss*, from eight populations tributary to the northeast Pacific Ocean. Single-locus expected heterozygosities ranged from an average of 61% (Ssa1) to 80% (T34) in the eight populations, and no significant departures from Hardy-Weinberg expected genotype frequencies were detected. Eighteen putative allelic fragments were resolved in the 267 steelhead and rainbow trout examined at Ssa1 [molecular weight range, 3.6–9.5 kilobase pairs (kbp)], and 26 alleles were resolved at T34 (1.7–9.4 kbp). At Ssa1, however, one allele accounted for 58% of all alleles scored and at T34 three alleles accounted for 72% of those scored. Allele frequencies at both loci were stable within two populations sampled over successive years, but varied significantly between populations within watersheds, and large frequency differences were detected between major geographic areas (e.g., Alaska versus British Columbia). Neighbor-joining analyses of genetic distances among populations accompanied by bootstrap analysis provided strong support (>70%) for clustering of populations by geographic region, as well as for a major genetic distinction (100% bootstrap support) between interior Fraser River populations of rainbow trout and coastal steelhead trout.

Assessing levels of genetic diversity within and between populations is an important aspect of conservation biology and management of fish resources (Lannan et al. 1989; Ryman and Utter 1987). Since its development in the mid-1960s, assays of biochemical genetic variation at protein-coding loci have been the workhorse genetic system in fisheries conservation and biology (Utter et al. 1987). Such assays have provided information on systematics and taxonomy (e.g., Utter et al. 1973), population genetic structure (e.g., Altukhov and Salmenkova 1991; Bartley and Gall 1990), population identification in mixed-fishery analyses (Beacham et al. 1987; Pella and Milner 1987), and evolutionary (Foote et al. 1989; Kornfield et al. 1982) and conservation genetics (Meffe 1990; Waples and Teel 1990). Over the past 10 years or so, a new generation of molecular genetic markers has become well established and widely applied in fishery biology. In particular, restriction site and sequence analyses of the mitochondrial DNA (mtDNA) genome have provided new insights and, at times, greater resolution to problems in phylogenetics, population structure, and resource management (e.g., Avise et al. 1987; Billington and Hebert 1991; Carr and Marshall 1991; Ferris and Berg 1987; Nolan et al. 1991; Ovenden 1990). Further, there are a vast number of relatively unexploited nuclear DNA genetic markers that may provide the ultimate resolving power for genetic differentiation in the form of DNA fingerprinting of individuals (Castelli et al. 1990; Hallerman and Beckmann 1988). Despite the great potential of nuclear DNA markers, there have been few studies of nuclear DNA variation in natural populations relative to more specialized pedigree-based applications in aquacultural genetics (Fields et al. 1989; Harris et al. 1991; Stevens et al. 1993; Taggart and Ferguson 1990b).

Oncorhynchus mykiss is a salmonid fish native to the North Pacific Ocean from California to Kamchatka and the Sea of Okhotsk (McPhail and Lindsey 1970). The

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species commonly occurs as two migratory life-history phenotypes: a sea-run or anadromous form, known as "steelhead," and a freshwater-resident form, known as "rainbow trout." Steelhead/rainbow trout are highly prized in freshwater recreational fisheries, and steelhead may comprise significant incidental bycatch in commercial net fisheries for Pacific salmon in localized areas (e.g., Jantz et al. 1990). Despite their importance to recreational fisheries and the potential impact on these resources from commercial fisheries, studies of genetic population structure and diversity in wild steelhead populations have been relatively few and intermittent (Parkinson 1984; Reisenbichler et al. 1992).

In this study, I employ two variable number tandem repeat (VNTR) nuclear DNA markers to survey genetic diversity within and between six populations of steelhead trout and two rainbow trout populations from five distinct watersheds tributary to the Northeast Pacific Ocean. These genetic loci are a class of repetitive DNA known as "minisatellite DNA," which consists of tandem arrays of nucleotides that share a "core" sequence typically between 10 and 65 base pairs in length (Jarman and Wells 1989; Wright 1993). My primary objectives in this paper were twofold: (i) to provide an illustration of the use of VNTR markers to survey genetic variation in natural populations, and (ii) to provide one of the first estimates of VNTR allelic frequencies in natural salmonid populations. My secondary goal was to conduct an assay of VNTR polymorphism to contribute to updating our understanding of genetic diversity and relationships in steelhead/rainbow trout populations from earlier surveys that employed protein electrophoresis or mitochondrial DNA (Parkinson 1984; Wilson et al. 1985).

Materials and Methods

Study Populations and Tissue Sampling

Blood samples (1–5 ml) were obtained from adult steelhead trout for DNA analysis by venipuncture using 21-gauge syringes and immediately mixed with 5–10 ml of 95% ethanol. These samples were stored at −20°C until DNA extraction (0.5–6 months). Although the focus of this study was on levels of interpopulation variation, an assessment of the relative level of allele frequency heterogeneity within populations (i.e., between years, between sexes, or among age classes) is necessary for meaningful evaluations of among population variation. The populations examined in this study, therefore, ranged from samples from the same sites over successive years, to multiple river populations from the same major drainage area, to populations from widely distant major drainage areas (Table 1 and Figure 1). In addition, blood sampling of one population (Keogh River) afforded the opportunity for sampling of scales for age determination, thus permitting tests for genetic differentiation among year-classes from a single population.

Table 1. Population names, drainage area, collection year, and sample sizes for steelhead and rainbow trout populations assayed for VNTR variation

Population	Drainage	Collection year	Sample size
Karluk River	Kodiak Island, Alaska	1992	27
Babine River	Skeena River, BC	1992	17
Babine River	Skeena River, BC	1993	28
Morice River	Skeena River, BC	1992	30
Morice River	Skeena River, BC	1993	23
Sustut River	Skeena River, BC	1993	19
Keogh River	Vancouver Island, BC	1993	47
Cowichan River	Vancouver Island, BC	1993	25
Blackwater River[a]	Fraser River, BC	1993	26
Badger Lake[b]	Fraser River, BC	1993	25

[a] Hatchery-raised progeny of 125 males × 175 females.
[b] Hatchery-raised progeny of 130 males × 89 females.

Previous studies of allozyme variation in *O. mykiss* have suggested that a major genetic distinction is present between "interior" populations of the Fraser and Columbia rivers and "coastal" rainbow and steelhead trout north and south of the Fraser-Columbia (Allendorf and Utter 1979). To determine whether this major grouping was detectable at minisatellite loci, liver samples (also stored in 95% ethanol) were also obtained from two interior Fraser River populations of rainbow trout (Table 1 and Figure 1).

DNA Extraction, Enzyme Restriction, and Southern Blotting

High molecular weight genomic DNA was obtained from ethanol-stored blood samples exactly as described by Taylor et al. (1994). DNA from liver samples was obtained using Pronase digestion and phenol/chloroform extraction as described by Taggart et al. (1992). The DNAs were resuspended in 50–200 μl of TE, quantified by spectrophotometry, and stored at −20°C. These extraction procedures typically yielded 30–200 μg of high molecular weight DNA.

Approximately 4 μg of genomic DNA from each individual was restricted with *Hae* III following the manufacturers' instructions (New England Biolabs or Bethesda Research Laboratories). The digested samples were size-fractionated by electrophoresis in 0.8% agarose gels in 0.5 × TBE buffer (45 mM Tris-Borate, 1 mM EDTA, pH 8.0). RNase was added to molten agarose (0.2 μg ml^{-1}) before gel casting to digest low molecular weight RNA in each sample during electrophoresis, and 50 ng of λ DNA restricted with *Hin*d III and *Eco*R I was included in four lanes of each gel to serve as a molecular weight size standard. After electrophoresis, the agarose gels were depurinated, alkali denatured, neutralized, and transferred under vacuum (Pharmacia Vacu-Gene) to nylon hybridization membranes (Amersham Hybond-N). After transfer, the membranes were washed briefly in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) and fixed to the membranes by crosslinking under ultraviolet illumination for 3 min.

Hybridization Studies

I assayed variation at two variable number tandem repeat (VNTR) loci in steelhead trout by hybridization with two minisatellite repeat sequence probes, Ssa1-rep (Ssa1) and 3.15.34 (T34). Both probes were derived from Atlantic salmon (*Salmo salar*) genomic libraries (Bentzen et al. 1993; Taggart and Ferguson 1990a) and have been shown to behave as single-locus, Mendelian markers in a variety of salmonids (Bentzen et al. 1991; Taggart and Ferguson 1990b; Taylor et al. 1994). Ssa1 hybridizes with homologous sequences in a number of salmonids (Bentzen et al. 1993) and detects variation in the number of tandemly arranged copies of a 16–base pair (bp) repeating sequence that occur between *Hae* III restriction enzyme cleavage sites. Approximately 100 ng of probe was labeled nonradioactively with the nucleotide analog digoxigenin-11-dUTP by random priming (Feinberg and Vogelstein 1983; Höltke et al. 1992). The membranes containing *Hae* III–digested steelhead trout genomic DNA were prehybridized in 1% BSA, 7% SDS, 0.26 M

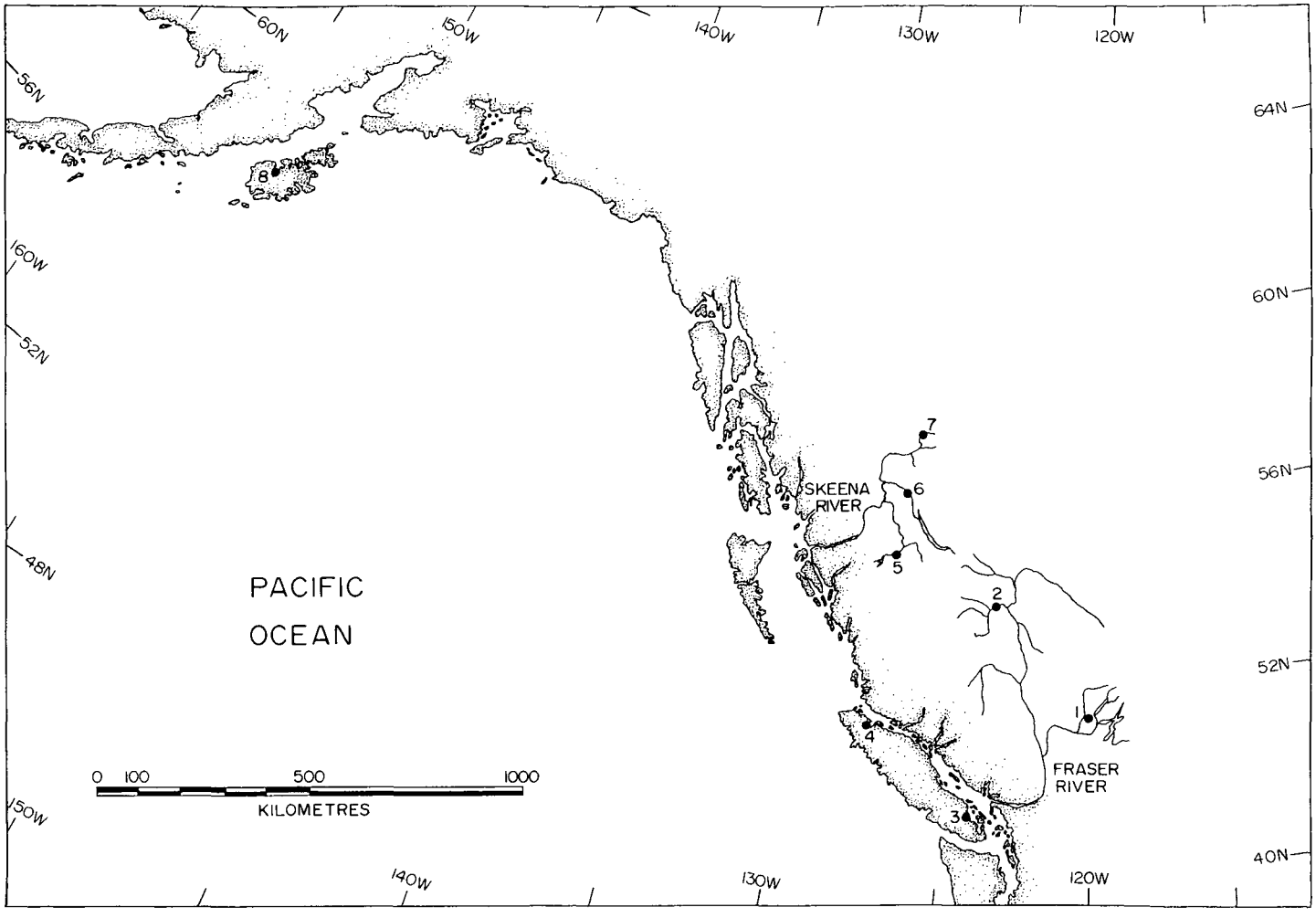

Figure 1. Geographic locations tributary to the eastern North Pacific Ocean of anadromous (steelhead trout) and nonanadromous (rainbow trout) *Oncorhynchus mykiss* populations sampled in the study. 1 = Badger Lake; 2 = Blackwater River; 3 = Cowichan River; 4 = Keogh River; 5 = Morice River; 6 = Babine River; 7 = Sustut River; 8 = Karluk River.

Na_2PO_4, 1 mM EDTA (Westneat et al. 1988) for at least 1 h at 65°C in a rotisserie style hybridization oven (Robbins Scientific), then hybridized by the addition of denatured probe, and incubated at 65°C for 16 h. Following hybridization, the membranes were washed in 2 × SSC/0.1% SDS at 65°C for 30 min and once in 1 × SSC/0.1% SDS under the same conditions. Hybridization experiments with probe T34 (12.5 ng) were completed under similar conditions, but also included Atlantic salmon competitor DNA (120 μg), to reduce background hybridization, and final stringency washes were performed with 0.2 × SSC/0.1% SDS. For both probes, the DNA hybrids were detected by chemiluminescence (Bronstein and McGrath 1989; Höltke et al. 1992) followed by exposure of the membranes to x-ray film (Kodak XOMAT-AR) at room temperature for 2–16 h.

Scoring of VNTR Variation

High levels of polymorphism and the limitations imposed by current techniques for resolving VNTR alleles coupled with the essentially continuous distribution of the molecular weights of such alleles make comparisons of restriction fragments within and between gels subject to measurement error (Galbraith et al. 1991). I minimized the potential for such errors in four ways. First, restricted DNAs of fish from each population were assayed across at least three gels and individuals from two or three populations were always electrophoresed on common gels. In this way, estimates of band molecular weights of individuals within each population would never be limited to a single gel. Second, a minimum of three λ size standards was included on each gel, and the mean migration distance of each fragment of known molecular weight was used to estimate the molecular weight of steelhead trout fragments resolved with the two probes. This procedure served to integrate mobility differences, owing to physical changes across single gels, in the size standards. To estimate the molecular weights of unknown steelhead trout restriction fragments, I calculated a least-squares regression between the known molecular weights of the λ size standards and the reciprocal of their migration distances visualized on the autoradiographs after Schaffer and Sederoff (1981). Third, and as recommended by Galbraith et al. (1991), "standard individuals" were included on each gel to serve as controls for running samples on different gels. The standard fish were three steelhead possessing alleles covering the entire range of allelic molecular weights observed between approximately 1 and 9.5 kilobase pairs (kbp). Within- and among-gel error in molecular weight estimation increased with increasing VNTR allele sizes (cf. Heath et al. 1994; Taylor et al. 1994). Estimated molecular weights of fragments electrophoresed in different lanes within the same gel varied <1.0% and the maximum among-gel variation was 1.5%. Final-

ly, when such errors resulted in any ambiguities regarding allelic distinctions, the individual steelhead or rainbow trout DNAs in question were restricted and hybridized with Ssa1 or T34 in side-by-side comparisons. The VNTR alleles resolved with Ssa1 and T34 were designated by single letter codes (e.g., A, B, etc.) in order of appearance.

Statistical Analysis of VNTR Allele Frequency Data

The analysis of VNTR allele variation in steelhead populations consisted of calculating allele frequencies and single locus heterozygosity estimates using the computer program BIOSYS (release 1.7; Swofford and Selander 1981). Tests of the fit of observed genotype frequencies within populations with Hardy-Weinberg equilibrium expectations were evaluated using χ^2 tests. There were a large number of alleles, and hence some of the expected frequencies were so low as to make the standard χ^2 test suspect. The goodness of fit tests, therefore, were conducted by pooling genotypes into three classes, with all alleles but the most common considered as one allele (Swofford and Selander 1981). The genotype classes so created were (i) homozygotes for the common allele, (ii) common and rare allele heterozygotes, and (iii) rare allele homozygotes and other heterozygotes (Swofford and Selander 1981). I then applied Yates' correction for continuity to improve performance of the χ^2 test for single degree of freedom tests. As multiple simultaneous statistical tests were performed, I used the sequential Bonferroni procedure (Rice 1989) to set significance levels for Type I errors. To conduct this procedure, each test is ranked by its attained χ^2 significance level, P, from lowest (P_1) to highest (P_k). Then the lowest P is considered, and if this P_1 is $\leq\alpha/k$ (where $\alpha = 0.05$), then the corresponding test denotes significance at the "table-wide" significance level of 0.05 (Rice 1989). If the inequality is not met, then the corresponding test (and all those with larger P) are not significant at $\alpha = 0.05$. If the first test is significant, then the next lowest P is considered and declared significant if its P_2 is $\leq\alpha/(k-1)$. The procedure is continued until the inequality $P_i \leq \alpha/(1+k-i)$ is not met (Rice 1989). For each of the χ^2 tests so performed, I cite the obtained probability level as well as the sequentially Bonferroni-adjusted α required for rejection of the null hypothesis. The extent of interpopulation genetic differentiation corrected for sampling error was expressed by calculating F_{ST}' following Workman and Niswander (1970) and Ruiz-Garcia (1993). The statistic F_{ST}' is related to Wright's (1978) F_{ST} by:

$$F_{ST}' = F_{ST} - 1/2\ Nt, \qquad (1)$$

where Nt is the total sample size. I also tested whether the calculated $F_{ST}' = 0$ utilizing the relationship between F_{ST}' and the χ^2 statistic following Workman and Niswander (1970) and Ruiz-Garcia (1993).

Tests of the significance of allele frequency differences within populations between years and among populations had to accommodate many cases of rare alleles. Consequently, I used the Monte Carlo χ^2 randomization procedure developed by Roff and Bentzen (1989) specifically to test for frequency differences when many classes (alleles) have low expected frequencies. For each such test I calculated probabilities after 1,000 randomizations of the frequency matrices. As multiple simultaneous tests were involved, significance levels were sequentially adjusted as outlined above. Estimates of relative similarity among the populations at the VNTR loci were summarized by calculating Nei's (1972) distance using GENDIST in the PHYLIP package of computer programs (version 3.4; Felsenstein 1990). Relationships among steelhead populations were inferred from the allele frequency data for both minisatellite loci by clustering the Nei's genetic distance estimates using the Neighbor-joining algorithm (Saitou and Nei 1987) with the NEIGHBOR program in PHYLIP. Neighbor-joining was selected over the more common UPGMA clustering technique because VNTR polymorphisms are generated by length mutations. The mechanisms generating such mutations, such as unequal crossing over or replication slippage, are fundamentally distinct from evolutionary change by point mutations which can more readily accommodate the assumption of equal rates of change across lineages in UPGMA analysis (Swofford and Olsen 1990). The robustness of the resulting topology was assessed by bootstrapping the allele frequency data matrix 100 times, calculating a matrix of genetic distances for each bootstrapped matrix, and then performing 100 Neighbor-joining analyses. Finally, a majority-rule consensus Neighbor-joining tree was constructed. The bootstrapping-consensus tree analysis was performed using the programs SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE, respectively—all of which are found in PHYLIP.

Results

Allelic Variation at VNTR Loci

Hybridization of *Hae* III–digested steelhead genomic DNA and Ssa1 resolved a total of 18 putative allelic fragments that ranged from approximately 3.6 to 9.4 kilobase pairs (kbp) in molecular weight (e.g., Figure 2 and Table 2). The most common allele in the total sample ($N = 267$) was a 6.7-kbp fragment (allele B) that accounted for ~58% of all alleles scored and ranged in frequency from 0.43 to 0.80 among the 10 population samples (Figure 3). One- or two-banded genotypes, consistent with homozygotic and heterozygotic conditions at a single locus, respectively, were observed in 90.3% of the total sample. Twenty-six individuals, however, were characterized by three-banded genotypes and were found in all populations except for Karluk and Sustut River steelhead. In each case, however, the third band observed was associated with two other more common bands, and the third band was not used in any of the subsequent allele frequency calculations. Expected heterozygosity among all samples averaged ($\pm$SE) 0.61 $\pm$ 0.04 and ranged from a low of 0.32 (Morice River 1992) to 0.74 (Sustut River). There was no evidence of reduced heterozygosity in the hatchery-raised trout where observed heterozygosities were 0.66 and 0.63 for Blackwater River and Badger Lake trout, respectively (Table 3). The χ^2 tests detected no significant deviations from genotypic frequency expectations for populations under Hardy-Weinberg equilibrium at the Ssa1 VNTR locus when adjusted for multiple tests (i.e., χ^2 range: 0.00–4.6, df = 1, minimum $P = .039$, Bonferroni α required for H_o rejection = 0.005).

The banding patterns of steelhead and rainbow trout *Hae* III–restricted DNA when resolved by hybridization with 3.15.34 (T34) were more complex than those observed with Ssa1 (Figure 2). Between one and four bands were observed per individual, and they ranged in molecular weight from 1.3 to 9.4 kbp. Such a banding pattern could result from detection of two related and disomically inherited loci or detection of a single tetrasomically inherited locus. Because of extensive overlap in the molecular weight distribution of the alleles, it was impossible to unambiguously assign alleles to specific loci for a two locus system. I assumed, therefore, that T34 detected a single tetrasomically inherited locus, assigned genotypes to each individual, and tested the observed distribution

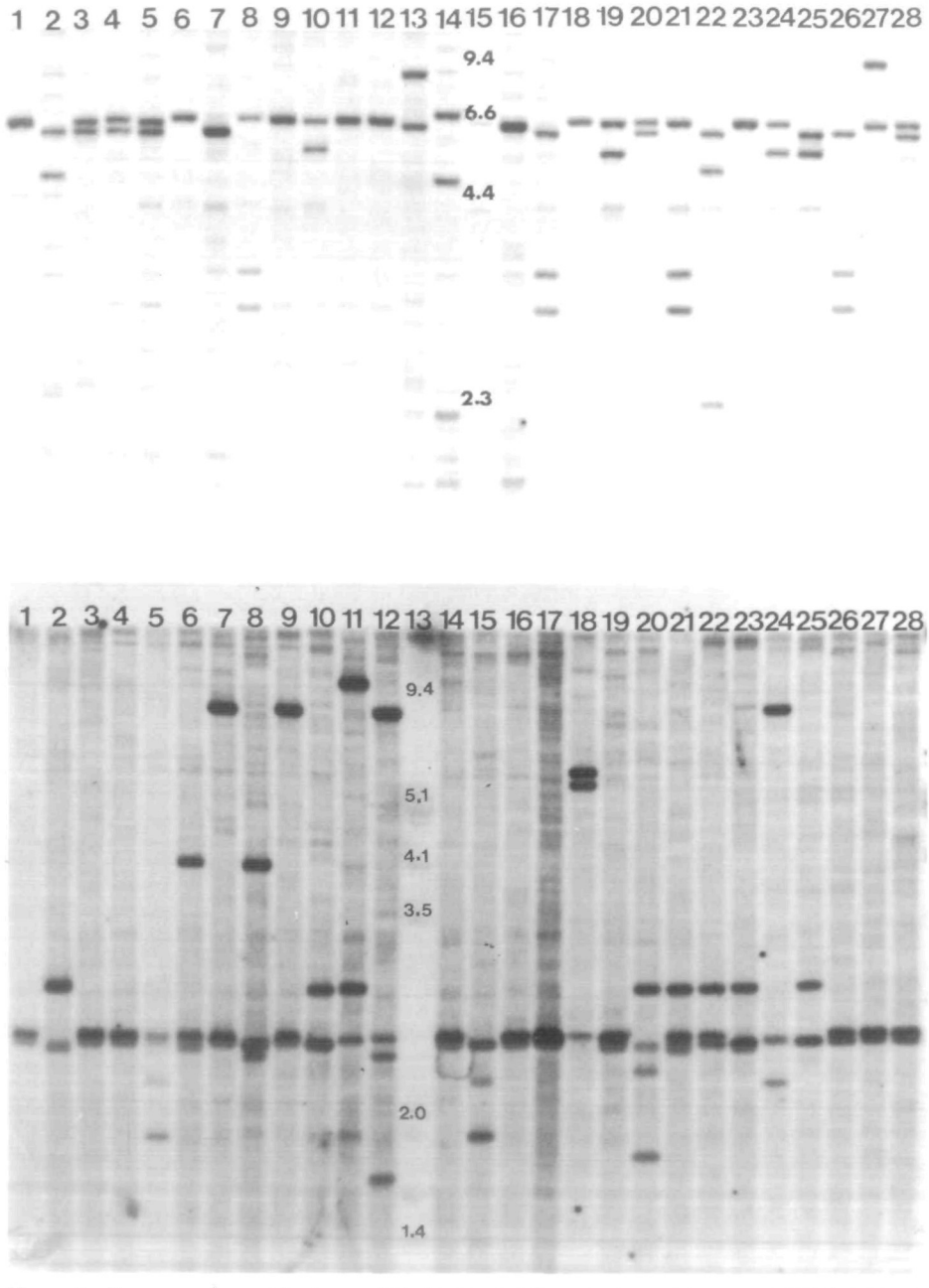

Figure 2. Representative autiolumigram of *Hae* III–digested *Oncorhynchus mykiss* genomic DNA hybridized with the variable number tandem repeat probe "Ssa1-rep" (Ssa1, upper) and "3.15.34" (T34, lower): **(Upper)** A total of 18 Ssa1 alleles was resolved varying from 3.6 to 9.4 kilobase pairs (kbp) in molecular weight; lanes 1 and 2 = Blackwater River; 3–12 = Badger Lake; 13 = Karluk River; 14 = Babine River; 15 = molecular weight marker (kpb); 16 = Morice River; 17–28 = Badger Lake; **(Lower)** A total of 26 T34 alleles was resolved varying from 1.6 to 9.4 kilobase pairs (kbp) in molecular weight; lanes 1 and 2 = Morice River; 3 = Babine River; 4–10 = Karluk River; 11 = Babine River; 12 = Morice River; 13 = molecular weight marker (kbp); 14–17 = Karluk River; 18–21 = Cowichan River; 22–26 = Karluk River; 27 and 28 = Keogh River. Hybridization was at 65°C, and final wash conditions were 1 × SSC/0.1% SDS for Ssa1 and 0.2 × SSC/0.1% SDS for T34.

of these genotypes against Hardy-Weinberg expectations by pooling the 26 observed alleles (Table 2) into two classes: the most common allele and all other alleles and using the equation:

$$p^4 + 4p^3q + 6p^2q^2 + 4pq^3 + q^4, \quad (2)$$

where p = the frequency of the most common allele and q = the frequency of all other alleles pooled (Allendorf and Thorgaard 1984). In none of the 10 population samples did the observed genotypic class distributions depart significantly from Hardy-Weinberg expectations under a single tetrasomically inherited locus model for the variation detected by T34 (χ^2 range: 0.00–6.3, df = 1, minimum $P = .025$, Bonferroni $\alpha = 0.005$). Average expected heterozygosity was 0.80 (±0.01) and ranged from a low of 0.72 in Blackwater River trout to 0.87 in Keogh River steelhead (Table 3).

Table 2. Approximate molecular weights (MW, in kilobase pairs) of designated alleles A–R (Ssa1) and A–Z (T34) at two minisatellite DNA loci in steelhead and rainbow trout (*Oncorhynchus mykiss*)

Ssa1		T34	
Allele	MW	Allele	MW
A	5.10	A	5.65
B	6.70	B	3.75
C	3.65	C	3.37
D	4.20	D	2.81
E	6.50	E	2.50
F	6.80	F	2.40
G	6.75	G	2.30
H	6.65	H	2.25
I	4.90	I	2.20
J	5.20	J	1.75
K	9.30	K	2.13
L	5.30	L	1.85
M	4.95	M	2.75
N	5.25	N	1.95
O	5.23	O	1.76
P	9.50	P	9.40
Q	5.60	Q	2.15
R	8.50	R	8.85
S	—	S	8.70
T	—	T	3.80
U	—	U	4.00
V	—	V	5.40
W	—	W	6.50
X	—	X	1.80
Y	—	Y	5.50
Z	—	Z	1.90

The distribution of alleles at T34 was broader than for Ssa1, and three alleles (D, E, and F, Figure 4) each had moderately high frequencies and together accounted for a mean (±SE) 0.72 ± 0.03 of the alleles scored in each population.

Allelic Variation Among Populations: Ssa1

Neither Babine or Morice river allele frequencies differed between sample years at Ssa1 (χ^2 randomization, $P = .73$ and .38, respectively) so these data were pooled within each population for further analyses. Allele frequency distributions were highly distinct between steelhead from the Babine and Morice rivers for these pooled data ($P = .0000$). Ssa1 allele frequency distributions were also highly distinct between Morice and Sustut rivers and between Babine and Sustut steelhead ($P = .0000$ and .0090, respectively; Bonferroni $\alpha = 0.0125$).

By contrast to the significant differences among populations within the Skeena River drainage, there was no significant difference in the frequency distribution of Ssa1 alleles between the two Vancouver Island populations (Keogh and Cowichan

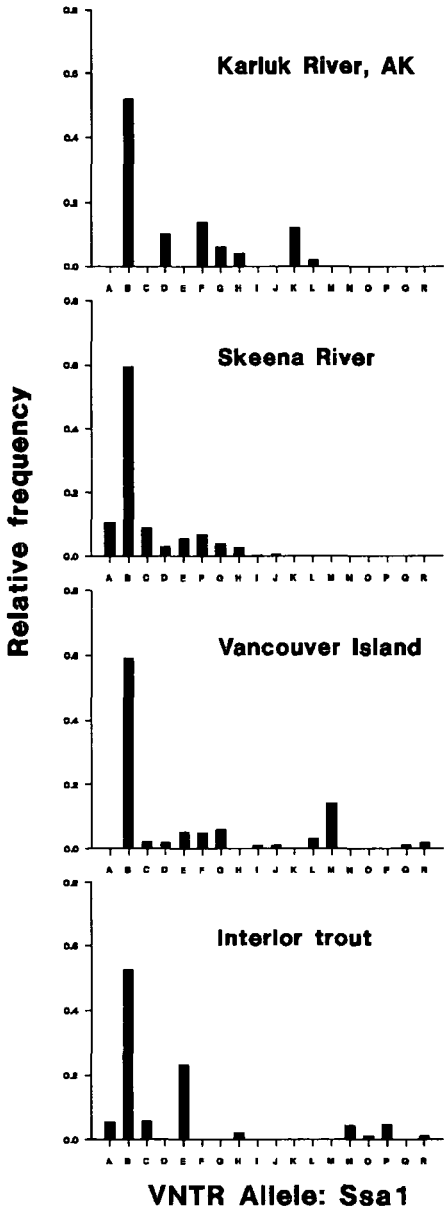

Figure 3. Relative frequency distributions of Ssa1 alleles in Karluk River (N = 25), Skeena River (N = 117, three populations), Vancouver Island steelhead (N = 72, two populations), and Interior rainbow trout (N = 51, 2 populations) regional groupings. Molecular weights of alleles are given in Table 2.

Rivers, P = .44), nor between the two populations of interior trout (P = .0180, Bonferroni α = 0.0100). When the allele frequencies were pooled by geographic region (Karluk River, Alaska; Skeena River, Vancouver Island, Interior), striking interregional differences were observed (P = .0000, Figure 3). Although the frequency of the most common allele, 6.7 kbp B, was fairly similar among regions, the relative frequencies of other alleles varied dramatically (Figure 3). For instance, the 4.9-kbp A allele was found in 5.6% of interior trout and in 9.5% of Skeena River steelhead, but

Table 3. Observed (Obs. H) and expected (Exp. H) heterozygosities under Hardy-Weinberg equilibrium at minisatellite VNTR loci Ssa1 and T34 in 10 population samples of steelhead and rainbow trout

	Locus			
	Ssa1		T34	
Population	Obs. H	Exp. H	Obs. H	Exp. H
Babine River 1992	0.88	0.66	0.81	0.82
Babine River 1993	0.68	0.71	0.82	0.78
Morice River 1992	0.36	0.44	0.80	0.83
Morice River 1993	0.32	0.32	0.86	0.84
Sustut River	0.68	0.74	0.84	0.79
Karluk River	0.72	0.68	0.60	0.79
Cowichan River	0.60	0.56	0.80	0.80
Keogh River	0.72	0.68	0.91	0.87
Blackwater River	0.74	0.66	0.61	0.72
Badger Lake	0.63	0.63	0.64	0.75
$\bar{x}$	0.633	0.608	0.769	0.798
SE	0.054	0.042	0.035	0.014

None of the observed versus expected heterozygosity comparisons were statistically significant after adjusting for multiple simultaneous comparisons (see text for details).

was absent both from Vancouver Island and Karluk River (Kodiak Island) steelhead (Figure 3). Also, allele E (6.5 kbp) was found in ~20% of interior rainbow trout, but was found in <5% of steelhead from the other geographic regions (Figure 3). Overall genetic differentiation at Ssa1 was moderately high and significantly different from zero (F_{ST}' = 0.058, χ^2 = 504, df = 153, P < .001).

Allelic Variation Among Populations: T34

For the Skeena River tributaries sampled both in 1992 and 1993 (Babine and Morice Rivers), there were no significant differences in allele frequency at the T34 locus between years (χ^2 randomizations, P = .28 and .09, respectively). When the two consecutive year samples were pooled within populations, genetic distinctions between Morice and Babine river steelhead were highly significant (P = .0000). Babine River steelhead allele frequency distribution was also highly distinct from that characterizing the Sustut River population (P = .006, Bonferroni α = 0.0100), but the latter and Morice steelhead were not significantly distinct at T34 (P = .0970).

As at the Ssa1 locus, the two Vancouver Island steelhead populations had similar T34 allele frequencies (P = .0440, Bonferroni α = 0.0125). The two interior rainbow trout populations were, however, highly distinct at T34 (P = .0000), with several of the less common alleles varying dramatically in frequency (Figure 4). When allele frequencies at T34 were pooled by geographic region (Kodiak Island, Skeena River, Vancouver Island, Interior), significant genetic differentiation among regions was resolved (P = .0000, Figure 4). One allele, the 3.0-kbp D allele was present in only 2.2% of interior trout, rose to 7–9% occurrence in the coastal islands steelhead, and increased to an average of 16.4% of Skeena River steelhead (Figure 4).

Overall genetic differentiation among populations was slightly lower at T34 than at Ssa1, but was still highly significant (F_{ST}' = 0.039, χ^2 = 500, df = 225, P < .001).

Allelic and Life-History Diversity: Keogh River

Total age (freshwater plus ocean ages) was determined for 28 of the 47 steelhead sampled from the Keogh River. The most common steelhead were 5- and 6-year-olds, which together accounted for 89% of those aged. Two ocean age classes were observed; ocean age 2 fish were more common (55%) than steelhead that had spent 3 years at sea before returning to freshwater to spawn (45%). A single freshwater age class predominated in the sample; 71% of adult Keogh River steelhead were 3 years of age at smolting.

I tested for allele frequency differences between two total age classes (total age 4 and 5 versus age 6 and 7, both N = 14), between the two classes of ocean age fish (ocean age 2 versus age 3, N = 19 and 9, respectively) and between two classes of freshwater age steelhead (freshwater age 3 versus ages 2 and 4, N = 20 and 8, respectively). In no case were any significant differences detected between Keogh River steelhead total, ocean, or freshwater age classes either at Ssa1 or T34 (all P > .4), and the same alleles were most common in all age groups.

Relationships Among Populations

The VNTR allele frequency matrix was used as input to Neighbor-joining clustering analysis to assess the genetic affinities among the 10 population samples (Figure 5). The analysis clearly separated interior Fraser River rainbow trout populations from coastal and Skeena River steelhead populations. The dendrogram also identified the Skeena River populations as distinct from Kodiak Island and Vancouver Island populations and linked annual samples within the Skeena River as genetically most similar to each other (Figure 5). These groupings of *O. mykiss* were well supported by bootstrap resampling of the VNTR allele frequency matrix (N = 100 replications). The bootstrapping analysis indicated 100% support for a distinction

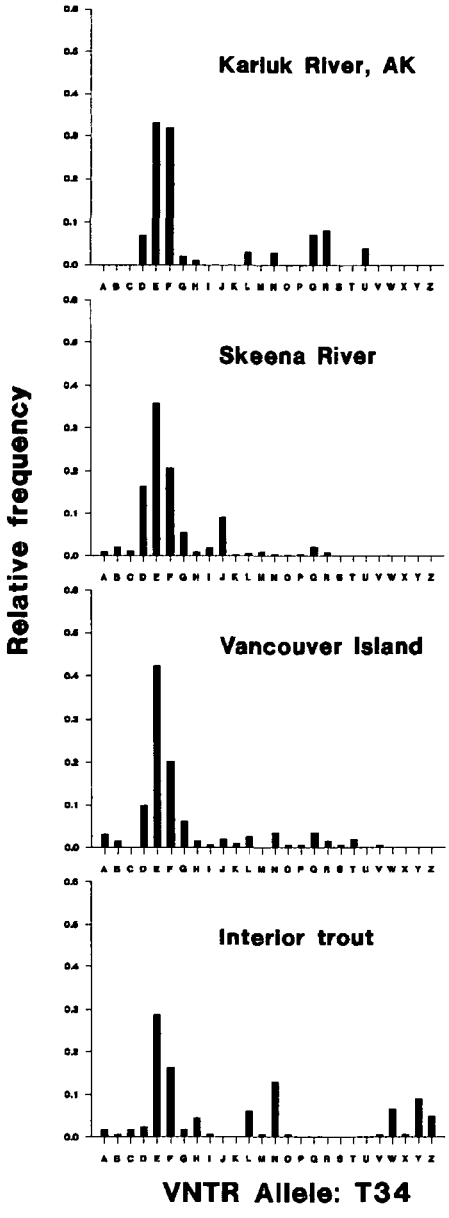

Figure 4. Relative frequency distributions of T34 alleles in Karluk River (N = 25), Skeena River (N = 117, three populations), Vancouver Island steelhead (N = 72, two populations), and Interior rainbow trout (N = 51, two populations) regional groupings. Molecular weights of alleles are given in Table 2.

between Fraser River interior *O. mykiss* (Blackwater and Badger) and anadromous populations from the north and south (Karluk, Skeena, Keogh, and Cowichan Rivers, Figure 5). This distinction was apparent no matter which population was used to arbitrarily root the Neighbor-joining analysis; the interior Fraser River populations always clustered distinct from the steelhead populations with 100% bootstrap support. As well, the analysis provided majority support for the distinctiveness of the Skeena River populations as a unit (71%) within the "non-Fraser" sub-

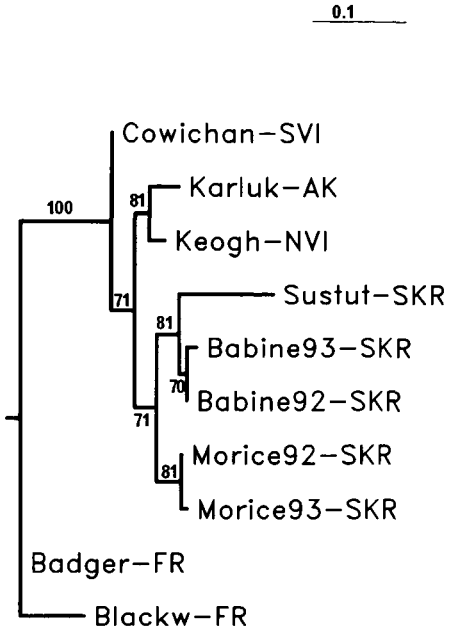

Figure 5. Neighbor-joining dendrogram of genetic affinities among 10 population samples of *Oncorhynchus mykiss*. Relationships were inferred from a matrix of Nei's (1972) genetic distances among populations calculated from allele frequencies at Ssa1 and T34 VNTR loci. Numbers at branch points indicate the number of times the group of populations below the branch was found out of 100 boot-strapped analyses. The branch length scale is 0.1 unit of Nei's (1972) genetic distance. NVI = Northern Vancouver Island; SVI = southern Vancouver Island; SKR = Skeena River; AK = Alaska; FR = Fraser River; 92 = 1992 collection year; 93 = 1993 collection year.

cluster as well as for an affinity between Keogh and Karluk rivers steelhead (81%) relative to the most southern coastal population, the Cowichan River (Figure 5).

Discussion

Inheritance at VNTR Loci

Both VNTR probes employed in this study provided informative, molecular markers for population genetic investigations in *Oncorhynchus mykiss*. Intrapopulation patterns of genotypic variation resolved with Ssa1 were consistent with disomic segregation at a single locus as has been documented experimentally in related species (Bentzen and Wright 1993; Taylor et al. 1994, in press). About 10% of fish assayed, however, displayed three-banded patterns also reported in chinook (*Oncorhynchus tshawytscha*; Heath et al. 1994) and Atlantic salmon (*Salmo salar*; Bentzen and Wright 1993). These three-banded phenotypes could result from (i) the occasional detection of variation at a second locus similar in sequence to Ssa1 (either by descent or parallel sequence evolution) or (ii) the detection of a "split allele" produced by the presence of an *Hae* III restriction site within the Ssa1 repeat of some individuals (cf. Saccheri and Bruford 1993). Arguing against an internal *Hae* III restriction site is the observation that cutting the DNAs from fish which displayed three-banded genotypes with enzymes other than *Hae* III (such as *Mbo* I, *Alu* I, and *Rsa* I, which recognize distinct sequences from *Hae* III) still produced three-banded genotypes in those fish (Taylor, unpublished data). In addition, assays of Ssa1 variability in Atlantic salmon (*Salmo salar*) with a probe based on unique sequence flanking the Ssa1 repeat, while detecting one or two bands in the vast majority of fish, also occasionally detects a third band (Bentzen and Wright 1993). Finally, in wild and pedigree populations of chum salmon (*Oncorhynchus keta*), Ssa1 reveals from two to four bands per individual, which Taylor et al. (1994) showed to represent variation at two linked loci. These observations suggest, therefore, that Ssa1 may represent a duplicated locus in Salmonidae, but that there have been different rates of divergence between the loci in the different species such that their co-detection by the probe is rare in some species (rainbow and steelhead trout, Atlantic salmon) and complete in others (chum salmon). Alternatively, a second locus may be present in all *O. mykiss*, but with most alleles in a molecular weight size range not included in my survey (i.e., <500 bp). The occasional detection of an allele at this locus within the detectable size range may simply reflect the relatively low frequency of such alleles in the study populations (i.e., approximately <2% per population). Also, infrequent detection of alleles at a putative second locus could stem from their being so-called "null alleles" (sensu Jeffreys et al. 1991) with which the probe will not hybridize.

Variation at the second VNTR locus, T34, was also consistent with Mendelian allelic variation and appeared to be tetrasomic in the wild and hatchery populations. Given that (i) Salmonidae have had a tetraploid origin and are still in the process of rediploidization (Allendorf and Thorgaard 1984; Ohno 1970), (ii) many salmonid genetic loci (particularly in *O. mykiss*) still exhibit tetrasomic inheritance (Allendorf and Thorgaard 1984), and (iii) tetraploid loci tend to be concentrated close to the telomeric regions of chromosomes (Allendorf and Thorgaard 1984) where minisatellite (at least in humans) loci tend also to be concentrated (Royle et al. 1988), it is not surprising that T34

might show tetrasomic inheritance in *O. mykiss*. There are also precedents for within species and even among family variation in tetraploid versus diploid inheritance at specific protein-coding loci in rainbow trout (Allendorf and Thorgaard 1984). Variation at T34, although disomic in Atlantic salmon, brown trout (*Salmo trutta*) and sockeye salmon (*Oncorhynchus nerka*) (Taggart and Ferguson 1990a; Taylor et al., in press), appears to be still in the process of rediploidization in rainbow and steelhead trout.

Allelic Variation Within Populations

Expected heterozygosities at both VNTR loci ranged from an average of 61% (Ssa1) to 80% (T34) among the study populations. For Ssa1, these values are higher than those reported for sockeye salmon and kokanee (average 47% for 22 populations) and Atlantic salmon (51% for three populations) (Bentzen and Wright 1993; Taylor et al., in press). For T34, the heterozygosity values are also higher than those reported for sockeye salmon and kokanee (34%) and in Atlantic salmon (64% for four populations) (Taggart and Ferguson 1990a; Taylor et al., in press). Both at Ssa1 and T34, therefore, *O. mykiss* displays higher levels of polymorphism than in at least two other salmonid species. Greater levels of genetic variation at VNTR loci in steelhead and rainbow trout are consistent with interspecies comparisons at protein-coding loci in these same species that averaged ~6% in rainbow and steelhead trout, but only 2.4% and 1.8% in Atlantic salmon and sockeye salmon and kokanee, respectively (Allendorf and Utter 1979). It is also suggested by these comparisons that variation at VNTR loci might average up to 10 times that at protein-coding loci, although reliable comparisons of average heterozygosity between genetic loci will have to await assays at more VNTR loci. Notwithstanding this limitation of intergenetic system comparisons based on different numbers of loci, the present analysis serves to demonstrate the potential for highly polymorphic genetic markers to reside at VNTR loci of salmonid fishes. In addition, the estimates of VNTR heterozygosity provided by the present study should probably be considered as conservative for three reasons. First, because of the continuous distribution of VNTR allele molecular weights and the limited resolving power of gel electrophoresis, alleles that differ by only a few repeat units would be undetected. Second, minisatellite alleles of the same molecular weight may, nonetheless, differ slightly in repeat motif sequences (e.g., Jeffreys et al. 1991). Finally, the aforementioned "null alleles" (sensu Jeffreys et al. 1991) or alleles of so small a molecular weight as to be undetectable under the present electrophoretic conditions may also exist (cf. Bentzen and Wright 1993).

Two of my study populations represented fish that were artificially propagated within hatcheries (Blackwater River and Badger Lake trout). A major concern of those responsible for hatchery production of salmonids is the possibility of reduced genetic variation that may accompany artificial propagation owing to low inbreeding effective population sizes (Allendorf and Ryman 1987; Allendorf and Utter 1979). Such declines in genetic variation, typically assayed at protein-coding loci, have been associated with reduced survival of affected hatchery populations (reviewed by Allendorf and Ryman 1987). Genetic variation resolved at VNTR loci in the present study provide a further and potentially more sensitive measure of genetic variation that can be monitored in hatchery populations. The expected heterozygosities of Blackwater River and Badger Lake rainbow trout averaged 69% across both VNTR loci compared to an average 71% in the wild steelhead populations. Although this comparison is only for two loci, and thus must be considered preliminary, it appears that these two hatchery populations of rainbow trout have levels of genetic variation equal to those of wild populations of steelhead at Ssa1 and T34 loci. Comparable levels of genetic variation between the wild and hatchery *O. mykiss* probably stems from the original derivation of Badger Lake trout from multiple interior British Columbia populations and from the use of relatively large numbers (>50) of wild fish as parents in the propagation of both populations in the hatchery (D. Larson, B.C. Fish and Wildlife Branch, Abbotsford, B.C., Canada, personal communication).

Zoogeography of VNTR Variation Among Populations

Polymorphism at protein-coding loci has been extensively surveyed in *O. mykiss*, and variation at such loci revealed evidence for two major genetic groups of North Pacific rainbow and steelhead trout: a "coastal" group (including Skeena River tributaries) extending from southern Kamchatka in the western Pacific to the Mad River system of northern California in the eastern Pacific and an "inland" group found exclusively in the upper Fraser and Columbia river systems east of the Cascade mountains (Allendorf and Utter 1979; Okazaki 1984; Reisenbichler et al. 1992; Utter et al. 1980). It is believed that these two genetic groups stem from isolation of *O. mykiss* in two refugia during the last (Wisconsinan) glaciation, which ended about 15,000 years ago (Lindsey and McPhail 1986; McPhail and Lindsey 1986). Coastal *O. mykiss* are thought to be derived from ancestral populations that survived glaciation in the south Kamchatka-western Alaska-Yukon River Valley (Beringian refuge) and coastal areas south of the ice sheet maximum (Cascadian refuge in the eastern North Pacific). By contrast, present-day inland populations were probably derived from *O. mykiss* that persisted within large proglacial lakes of the Pacific refuge formed by ice-dam impoundments of the upper Fraser and Columbia systems (McPhail and Lindsey 1986; Okazaki 1984). Variation at the VNTR loci Ssa1 and T34 assayed in the present study is broadly consistent with these genetic groupings in *O. mykiss*. Separation between Blackwater and Badger trout (found in the upper Fraser River east of the Cascade mountains, Figure 1) from coastal steelhead from southcentral Alaska to southern Vancouver Island and from Skeena River steelhead was supported in 100% of the bootstrapped Neighbor-joining analyses (Figure 5). The concordant association between genetic variation and zoogeographic separation at protein-coding and VNTR loci provides strong support for the idea that historical biogeographic events have played a prominent role in the organization of genetic structure of present-day *O. mykiss* populations.

The genetic distance/clustering analyses based on VNTR allele frequencies at the two loci also revealed structure on a finer geographic scale among the *O. mykiss* populations. For instance, annual samples within populations were most similar genetically, followed by populations within rivers systems (Babine, Morice, and Sustut Rivers of the Skeena River drainage, Figure 5). Notwithstanding the general clustering of *O. mykiss* populations by watershed and major geographic region, VNTR allele frequency distributions were significantly distinct among adjacent populations within the same watershed (e.g., among Babine, Sustut, and Morice rivers in the Skeena River system). By contrast, VNTR variation within populations was low; allele frequencies were temporally stable, and although sample sizes were

small, no differences among age classes within the Keogh River population were detected. Significant genetic differences among populations within drainages support the idea that individual steelhead trout populations are genetically distinct demes, isolated by geography and the tendency of fish to return to their natal streams to reproduce (cf. Parkinson 1984). The sensitivity of the VNTR assay presented here (as indicated by heterozygosity and F_{ST}' values) relative to variation at protein-coding loci suggests that VNTR polymorphisms should be useful in situations where protein electrophoresis has failed to detect significant genetic structuring among steelhead trout populations within drainages (e.g., Reisenbichler and Phelps 1989; Reisenbichler et al. 1992). The extent to which VNTR loci will improve resolution over more traditional genetic markers, however, must await surveys of variation at additional VNTR loci.

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