Isolation and characterization of novel salmon microsatellite loci: cross-species amplification and population genetic applications

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Abstract: Twenty-two variable number of tandem repeat microsatellite dinucleotide repeat ($[GA]_n$ and $[CA]_n$) loci were cloned from sockeye salmon (*Oncorhynchus nerka*) partial genomic libraries. Characteristics and optimal polymerase chain reaction (PCR) conditions were defined for each locus. The degree of conservation of sequences flanking microsatellite repeat motifs and the utility of heterologous PCR primers for analyses in closely related taxa was tested using 10 salmonid species from four genera. Nearly all microsatellite primers produce amplification products in multiple species, suggesting broad application in salmonid research. The utility of these loci for population genetic studies was tested using individuals (N = 83) from three spawning populations of chinook salmon (*Oncorhynchus tshawytscha*) from the Yukon River, Yukon Territories. Twelve of 16 loci screened were polymorphic (mean heterozygosity = 0.254). Genetic distance estimates between populations were concordant with results from a previous allozyme survey of these same populations. Discussions of the utility of microsatellite markers in salmonid population genetic research are presented in light of recently described statistical methodologies based on mutational properties and interallelic differences in repeat score.

Résumé : À partir de banques génomiques partielles de saumons sockeye (*Oncorhychus nerka*), on a cloné 22 loci de microsatellite séquences répétées en tandem dinucléotidiques ($[GA]_n$ et $[CA]_n$). Les caractéristiques et les conditions optimales pour la PCR ont été définies pour chaque locus. Le degré de conversion des séquences qui flanquent les motifs répétés des microsatellites et l'utilité des amorces hétérologues de la PCR pour des analyses dans des taxons étroitement apparentés ont été évalués avec 10 espèces de salmonidés appartenant à quatre genres. Presque toutes les amorces de microsatellites ont donné des produits d'amplification chez plusieurs espèces, ce qui laisse supposer qu'elles sont applicables à grande échelle dans les recherches sur les salmonidés. On a évalué l'utilité de ces loci dans les études génétiques des populations, en utilisant des individus (N = 83) provenant de trois populations de saumons quinnat (*Oncorhychus tshawytscha*) en cours de reproduction, vivant dans le fleuve Yukon (Territoires du Nord-ouest). Douze des 16 loci examinés étaient polymorphes (hétérozygotie moyenne = 0,254). Les estimations de distance génétique entre les populations concordaient avec les résultats obtenus au cours d'une étude allozymatique précédente des mêmes populations. On examine l'utilité de marqueurs à microsatellites dans les recherches génétiques de populations de salmonidés, à la lumière de méthodes statistiques récemment décrites, basées sur les propriétés mutationnelles et les différences inter-alléliques des résultats obtenus avec les séquences répétées.
[Traduit par la Rédaction]

Introduction

Characterization and quantification of genetic diversity has long been a major goal in both basic and applied fisheries research. For many years, protein allozyme electrophoresis has provided much of the empirical population-level data (Allendorf et al. 1987; Gharrett and Smoker 1994). However, recent advances in molecular technology have dramatically increased the number of genetic markers capable of resolving variation at the DNA level (see review by Park and Moran 1994). Many studies have utilized maternally inherited mitochondrial DNA (e.g., surveys summarized in Avise 1992), and an increasing number of nuclear genetic markers have been developed, including restriction fragment length polymorphism (RFLP) analysis of single-copy nuclear DNA (Wirgin and Maceda 1991), coding genes such as lactate dehydrogenase-B (Crawford et al. 1989), single locus minisatellites (Taggart and Ferguson 1990; Bentzen et al. 1991; Prodöhl et al. 1994), multi-locus DNA minisatellites (Prodöhl et al. 1992), and microsatellites (Estoup et al. 1993).

Despite the rapid development and growing interest in the use of molecular genetic markers for population-level analyses, few studies have addressed the utility of a single class of nuclear markers across a range of species, nor have results of molecular markers been compared with results using more widely employed techniques (i.e., allozymes). In addition, little is known of levels of genetic variability within and among populations at the DNA level.

One class of markers that is receiving increasing attention is that of variable number of tandem repeat (VNTR) microsatellite loci (Tautz 1989; Bruford and Wayne 1993).

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Microsatellites consist of tandem arrays of short (generally ≤5 bp) repeat motifs such as $[CA]_n$ or $[AAT]_n$. Many simple-sequence motifs occur in extremely high frequency in eukaryotic genomes (e.g., every 30 kb for $[GT]_n$ repeats in mammals; Stallings et al. 1991) relative to random motifs of equivalent length. High frequency of occurrence and uniformity of distribution within most eukaryotic genomes (Luty et al. 1990), and high levels of variation (e.g., Amos et al. 1993), have fostered a growing appreciation of their use in genome mapping (Dietrich et al. 1992), paternity (Morin et al. 1994), and forensics (Jeffreys et al. 1992). These markers have increasingly been used in population studies of both contemporary (e.g., Scribner et al. 1994; Taylor et al. 1994; Slatkin 1995) and historical (Ellegren 1991) samples. Microsatellites are particularly amenable for population-level analyses that necessitate the characterization of many individuals. Loci are assayed using polymerase chain reaction (PCR)-based techniques, in which PCR primers are designed from sequences flanking the repeat motif, and amplification products are run on denaturing polyacrylamide gels. Further, microsatellites that have been characterized in specific taxa have proved to be homologous and polymorphic in a diverse number of species of varying degrees of relatedness (Moore et al. 1991; Schlotterer et al. 1991).

In this study we present data from a series of novel dinucleotide ($[GA]_n$ and $[CA]_n$) microsatellite loci that were developed from sockeye salmon (*Oncorhynchus nerka*). Optimal PCR conditions are described and results of cross-species amplifications are presented in the hope of stimulating interest in the use of these markers for future salmonid research. The utility of these markers for population genetic studies was tested using a *Oncorhynchus tshawytscha* data set from tributaries of the Yukon River. Levels of interpopulation heterogeneity in allele frequency are compared with a previous allozyme study of these same populations.

Methods and materials

Sampling locations

Samples of adult chinook salmon were collected from specific spawning grounds on the North Fork of the Klondike River (N = 29), McQuesten River (N = 27), and Stoney Creek of the Takhini River (N = 27) during August 23–30, 1990. Tissues were taken in the field and placed immediately into liquid N_2. Samples were subsequently stored at –70°C in the laboratory until DNAs were extracted. Samples of the remaining nine species used in cross-species amplifications were as follows: *Oncorhynchus kisutch*, Kenai River, Alaska; *Oncorhynchus gorbuscha*, Kenai River, Alaska; *Oncorhynchus keta*, Fishing Branch of the Porcupine River, Yukon Territory; *O. nerka*, Kenai River, Alaska; *Oncorhynchus mykiss*, Kenai River, Alaska; *Oncorhynchus clarki*, brood stock from Montana State University; *Thymallus arcticus*, Coleville River, Alaska; *Salvelinus malma*, Kenai River, Alaska; and *Salmo salar*, Dee River, Scotland, U.K.

DNA extractions

DNA was extracted using standard proteinase K, phenol–chloroform techniques (Sambrook et al. 1989; Bruford et al. 1992) and resuspended in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). DNA concentrations were determined using fluorometry, and working stocks of 50 ng/mL were made for each sample. One hundred nanograms of DNA were used for each PCR reaction (see below).

Cloning and characterization of microsatellite loci

Two procedures were used for library construction and screening of microsatellites. Initial libraries were made using a modification of procedures described in Rassmann et al. (1991). In brief, *O. nerka* genomic DNA was isolated as above and was completely digested using three restriction enzymes (*Alu*I, *Rsa*I, and *Hae*III). The 300- to 700-bp size fraction was isolated using an electroelution procedure (Bruford et al. 1992), and was ligated into a *Sma*I digested, dephosphorylated pUC18 (Stratogene) plasmid (50 ng genomic DNA to 200 ng vector). The ligation reaction was transformed using electroporation into *E. coli* JS5 (BioRad, Richmond, Calif.), which were plated at low density on agar containing IPTG/X-gal and ampicillin (100 mg/mL). Filter replicates of each plate were made using Hybond-Nfp (Amersham Corp., Arlington Heights, Ill.) nylon membranes. Membranes were probed using [γ-^{32}P]ATP end-labeled simple-sequence polymers ($[GA]_{20}$ and $[CA]_{20}$). Positive clones were identified by autoradiography. Putative positive recombinant plasmid DNAs were purified using miniprep columns (Promega, Madison, Wis.) and transferred to Nytran (Schleicher and Schuell, Keene, N.H.) membranes using a 96-well dot-blot apparatus (BioRad). A second screening was performed that included oligo $[GA/CT]_n$ and $[CA/GT]_n$ standards (Pharmacia, Alameda, Calif.) as positive controls. Positives from the second screening were sequenced using modifications of standard di-deoxy double-stranded Sequenase (USB) protocols, including DMSO (Winship 1989).

Greater efficiency, in terms of the number of positives identified per unit effort, was obtained using a modification of library enrichment procedures described in Ostrander et al. (1992). Genomic DNA was completely digested using the restriction enzyme *Sau*3AI, size-selected as described above, and ligated into a dephosphorylated pBluescript KS+ (Strategene) phagemid vector. Portions of the ligation mixture were transformed using electroporation into *E. coli* CJ236 (BioRad) deficient in dUTPase (*dut* gene product) and uracil-*N*-glycosylase (*ung* gene product). Colonies were plated at high density on large (150 × 15 mm) LB plates containing ampicillin (100 mg/mL). After overnight incubation, colonies were washed with 10 mL of 2× YT media (Sambrook et al. 1989), after which cell suspensions were collected. One hundred millilitres of washed cells were used to inoculate an overnight culture, which was grown to saturation. Each of 24 tubes (2 mL of 2× YT media) was inoculated with 100 µL of overnight culture and infected with M13KO7 helper phage. Single-stranded DNA was prepared using PEG precipitation (Sambrook et al. 1989), and primer extension reactions were carried out by combining 3 µg of single-stranded DNA, 20 pmol of $[CA]_{20}$ or $[GA]_{20}$ primer, 10 µL of 10× PCR buffer (Perkin Elmer, Branchburg, N.J.), 200 µM each dNTP in a final volume of 100 µL using conditions specified in Ostrander et al. (1992). Primer extension reactions were terminated by extracting the mixture using one volume of phenol – chloroform – isoamyl alcohol (25:24:1) followed by extraction using chloroform –isoamyl alcohol (24:1). DNA was precipitated in the presence of salt (0.1 volume 3 M sodium acetate, pH 5.5) and glycogen (2 µL of 1 mg/mL) using 100% ethanol, followed by washing in 70% ethanol. Precipitates were resuspended in 1× TE and incubated overnight at 4°C with T4 ligase under conditions specified by the manufacturer (Pharmacia). The ligation mix was heat-inactivated for 10 min at 65°C, cleaned using phenol–chloroform, precipitated as above, and resuspended in 15 µL H_2O. One to three microlitres of the resuspended double-stranded DNAs were transformed using electroporation into *E. coli* MV1109 (BioRad) and plated at low density. Colony screening was preformed as described in Ostrander et al. (1992), and a second screening was conducted as above. Positive colonies were isolated and DNA was prepared as above. Double-stranded sequencing was performed for all positive colonies using standard Sequenase (USB) procedures, including DMSO as above.

Development of PCR primers and primer optimization

Primer sequences within flanking regions adjacent to microsatellite

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loci were developed using the software OLIGO (Rychlik and Rhoads 1989). PCR conditions for each primer pair were optimized using sockeye and chinook genomic DNAs using a 30-µL reaction mix consisting of 10 pmol of each primer, dNTPs at 200 µmol each, 0.25 units *Taq* DNA polymerase (Cetus), and PCR buffer (10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.01% NP–40, 0.01% Triton X–100) for 30–35 cycles. Products were visualized on 1.5% agarose gels using ethidium bromide staining. Primer sequences and annealing temperatures are provided in Table 1.

Chinook population samples were screened for variation using [γ-^{32}P]ATP end-labeled primers. One primer from each pair was end-labeled using T4 polynucleotide kinase according to manufacturer's specifications (Pharmacia). PCR reactions were as described above (using locus-specific annealing temperatures; Table 1) except that concentrations of both primers and dNTPs were reduced to one-tenth. The entire PCR reaction was mixed with 12 µL of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and heated for 5 min at 95°C before loading onto a 6% denaturing sequencing gel. An M13 control sequencing reaction (USB sequence kit) was run adjacent to the samples to provide an unambiguous size marker for the microsatellite alleles. Gels were dried and autoradiographed overnight at –70°C using intensifying screens.

Analysis of microsatellite polymorphisms

Levels of genetic variation for each of three chinook salmon populations was assessed as the mean number of alleles per locus (A), observed heterozygosity (H_{DC}), and expected (under Hardy–Weinberg) heterozygosity (H_E). Deviations from Hardy–Weinberg equilibrium were tested for each variable locus using χ^2 tests with pooling (Hartl and Clarke 1988) to account for the presence of many rare alleles. The variance in allele frequency among populations was tested using θ as described by Weir and Cockerham (1984), and significance of θ was tested by jackknifing across samples (Weir 1990). Nei's genetic distance (Nei 1972) and recently defined distance measures (Δ, Goldstein et al. 1995; D_{AS}, Bowcock et al. 1994) based on population allele frequencies and allele repeat scores were calculated for all pairwise population comparisons. Measures of interpopulation genetic distance were compared with estimates for these sample populations based on an allozyme survey (22 loci) conducted previously (Wilmot et al. 1992). Interpopulation distances were further summarized in the form of UPGMA phenograms. Estimates of allele frequency, Nei's genetic distance, and heterozygosity were calculated using the BIOSYS–1 program (Swofford and Selander 1981).

Results

One hundred and sixty-one positive clones were identified from screenings based on colony or plaque hybridizations and were sequenced. Sixty-two (39%) contained repetitive $[GA]_n$ (N = 35) and $[CA]_n$ (N = 27) regions. A large number of microsatellite-containing clones (N = 36) were unusable because of the close proximity of repeat regions to the cloning site or were found to contain small repeat regions (repeats) or incomplete repeats (i.e., presence of single or multiple base pair substitutions within the repeat region). The high number of clones with *Sau*3AI restriction sites near repeat regions suggests that alternative restriction enzymes may be more appropriate for future libraries. Twenty-six positive clones containing microsatellite repeats had adequate flanking sequence to develop PCR primers (Table 1).

PCR amplification products of the expected size (based on the cloned sequence) were obtained for 22 of 26 microsatellite primer pairs tested, for at least one species (Table 2). Four primer pairs failed to produce unambiguous PCR products and were not used in subsequent analyses. While attempts were not made to optimize conditions for each species, most primer pairs amplified regions of the appropriate size in the majority of species tested using PCR conditions developed for chinook (loci *One*µ1–18) or sockeye (loci *One*µ19–22). Two primer pairs (*One*µ15 and *One*µ16) amplify what appears to be duplicated loci based on the presence of multiple alleles (3) of the appropriate size in sockeye and chinook salmon (data not shown). Locus *One*µ13 exhibited null alleles in high frequency in chinook salmon (see beyond).

Analyses of chinook salmon collected from each of three spawning aggregations from the Yukon River confirm the utility of these loci for population genetic analyses. Twelve of 16 loci were variable (mean H_{DC} = 0.254) with 2–11 alleles segregating at each variable locus within a population (Table 3). Many variable loci were allozyme-like in that a common allele was typically present in high frequency accompanied by one or few rare alleles. Several loci (e.g., *One*µ2, *One*µ8, and *One*µ14; Table 3) were highly polymorphic. Genotypic frequencies for each of l2 variable loci surveyed for the three populations conformed to Hardy–Weinberg expectations (P > 0.05) with the exception of locus *One*µ13, which was characterized by high frequencies of a null allele(s) (Table 3).

Populations differed greatly in allele frequency (Table 3). Heterogeneity in allele frequency among populations was documented for 7 of 12 polymorphic loci (mean θ = 0.036, P < 0.01; Table 3).

The degree of interpopulation variation was further quantified using genetic distance measures (Fig. 1). Using a standard distance measure (Nei 1972), a measure based on the proportion of shared alleles (D_{AS}, Bowcock et al. 1994), and one based on population allele frequency and repeat copy scores (Δ, Goldstein et al. 1995), the Klondike and McQuesten populations were genetically most similar, while the Klondike and Stoney Creek populations were genetically most divergent (Fig. 1). Microsatellite results, in terms of the rank ordering of interpopulation estimates of divergence, and thus the topologies of resulting UPGMA phenograms, agreed with results from an earlier allozyme survey of these same populations (Wilmot et al. 1992; Fig. 1).

Discussion

Analyses using novel salmonid microsatellite loci revealed several major results. First, the potential of using heterologous PCR primers was explored and a majority of the microsatellites appear to be conserved across a diverse number of salmonid species. Second, the utility of these markers for population genetic analyses was confirmed. The majority of microsatellite loci surveyed were polymorphic, and many showed significant spatial heterogeneity in allele frequency among chinook salmon populations within one major river drainage. Finally, results of microsatellite population surveys agreed with those from an earlier allozyme study of the same populations, suggesting wide utility for a variety of basic and applied research questions.

Use of heterologous PCR primers and conservation of microsatellite loci

Presence of amplification products of expected size (based on sequences of sockeye clones) for each of 22 microsatellite

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Table 1. Characteristics of 22 sockeye salmon (*Oncorhynchus nerka*) microsatellite PCR primers, amplification conditions, repeat motifs, and expected product sizes.

Locus	Length (bp) of PCR product[a]	Repeat motif[b]	Annealing temp. (°C)[c]	Primer sequences (5′–3′)[d]
*One*μ1	113	$[GA]_{14}$	60–50	F: GTCTTACCAAATGTCTTCCTCCT R: GCCATTTAGCATACGATTTTATC
*One*μ2	188	$[GA]_{11}$	60–50	F: GGTGCCAAGGTTCAGTTTATGTT R: CAGGAATTTACAGGACCCAGGTT
*One*μ3	186	$[GA]_{18}$	52	F: TCTCCTTGGTCTCTCTGTCCCTT R: CTATCAGCCAATCGCATCAGGAC
*One*μ4	124	$[GA]_{23}$	60	F: TAATTTACATATCAGGTTCTGCC R: TATGCTAGTCATGGCTCTTACAT
*One*μ5[e]	178	$[CA]_{12}$	60	F: AACACACCAGCTGTGAAAACAAA R: TGTCTATCGCCAATCTCTCTGCT
*One*μ6	222	$[GA]_{18}$	60	F: CAGAGTGGCCTAGATGCTTTAAT R: CCACACACCAAATCCTACCCTTA
*One*μ7	190	$[GA]_{11}$ $N_2[GA]_4$	52	F: ACACTGCAAACACTCTGCTTACT R: CAAGAAGAAACCCTGTCCTCAAG
*One*μ8	208	$[CA]_{24}$	60	F: AACATTCTGGGATGACAGGGGTA R: CTGTTCTGCTCCAGTGAAGTGGA
*One*μ9	230	$[CA]_{54}$	60	F: CTCTCTTTGGCTCGGGGAATGTT R: GCATGTTCTGACAGCCTACAGCT
*One*μ10	133	$[CA]_{25}$	60–50	F: ATGGGGAACAGAAGAGGAAT R: CTGTAGGTGTGAAATGTATTTAAA
*One*μ11	152	$[CA]_{13}$	60	F: GTTTGGATGACTCAGATGGGACT R: TCTATCTTTCCTGTCAACTTCCA
*One*μ12	163	$[GA]_{24}$	60	F: ACTTATGCTAGTCATGGCTCTT R: TCGGTCATCGAAAGATACTTTT
*One*μ13	164	$[GA]_{20}$	52	F: TCATACCCCATGCCTCTTCTGTT R: GATGAGTGAAAGAGAGGGAGCGA
*One*μ14	143	$[CA]_{26}$	52	F: AGAAACATGAGAACAGTCTAGGT R: CCTTATGAGTTTGGTCTCCATGT
*One*μ15[f]	109	$[CA]_7$ $N_2[CA]_{13}$	52	F: AGCTTGACATCATAAAATGCGTC R: TTTCTTCTCTCATTCTCACACGA
*One*μ16[f]	142	$[CA]_{33}$	60	F: ATGCTGTAACCAGTGAATCCCTT R: TATCAACAGAATGCCAACCTTTT
*One*μ17	121	$[GA]_{20}$	60	F: ATGGCAGGATTGTTTTAGGTTGT R: GCCATGAGGAAGACACATCAATA
*One*μ18[e]	179	$[CA]_{16}$	54	F: ATGGCTGCATCTAATGGAGAGTAA R: AAACCACACACACTGTACGCCAA
*One*μ19	149	$[CA]_{33}$	56	F: CTGGAAAGCACAGAGAGAGCCTT R: TCCAACAGTCTAACAGTCTAACCA
*One*μ20	163	$[CA]_{16}$	60–50	F: TCTGTGGACAAAACATGAGATTA R: CTCCCATTTTCCCATTTATTGTT
*One*μ21	145	$[GA]_{35}$	56	F: GGTTACAGTGGGTTCACTCTACA R: GTTATGACAACAGTCTCTGTCGC
*One*μ22	168	$[GT]_{22}$	58	F: TTCTCTACAGGCGATGAACTGAT R: TTCTTACCTCCACGATGACACAA

[a]Expected PCR product size based on cloned sockeye salmon allele of specified repeat number. Product sizes may vary in other species.
[b]Core repeat motif from cloned sockeye salmon sequence.
[c]PCR annealing temperatures were optimized either for chinook (loci *One*μ1–18) or sockeye salmon (loci *One*μ19–22). Optimal conditions may vary for additional species (see Table 2). Ranges in temperature refer to "touch-down" conditions (annealing temperature are decreased by 2°C at 2-cycle intervals and 30 cycles are run at the final temperature).
[d]F and R refer to forward and reverse primer sequences, respectively.
[e]Two distinct genomic regions amplified. The lower molecular weight fragment is of the expected size.
[f]Multiple allelic banding patterns observed for sockeye and chinook salmon suggest gene duplication.

primer pairs suggests that dinucleotide repeat regions and sequences flanking these motifs are highly conserved across species differing greatly in degree of relatedness (Table 2). The degree of homology has some degree of phylogenetic basis in that a greater number of primer pairs produce amplification products within the genus *Oncorhynchus* compared with those of more distantly related (Stearley and Smith 1994) taxa. Findings of high interspecific homology are consistent with results

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Table 2. Cross-species amplification using 22 pairs of sockeye salmon (*Oncorhynchus nerka*) microsatellite primers.

	Oncorhynchus							*Salmo*	*Thymallus*	*Salvelinus*
Locus	*kisutch*	*gorbuscha*	*keta*	*nerka*	*tshawytscha*	*mykiss*	*clarki*	*salar*	*arcticus*	*malma*
*One*μ1	*	+	+[a]	+	+	+	+	+	*	+
*One*μ2	+	*	+[a]	+	+	+	+	+	+	*
*One*μ3	*	+	*	*	+	*	+	+	*	*
*One*μ4	+	+	*	+	+	+	*	+[a]	*	*
*One*μ5	*	*	+[a]	+	+	+	+	+	*	*
*One*μ6	+	+	+	+	+	+	+	+	+	*
*One*μ7	+	*	+	+	+	+	+	*	+	+
*One*μ8	–	+[a]	*	+	+	+	+	–	+	*
*One*μ9	*	+	+	+	+	*	*	+	+	*
*One*μ10	*	+	+	+	+	+	+	+	+	+
*One*μ11	*	*	+	+	+	+	+	+	*	*
*One*μ12	*	*	–	+	+	*	*	–	*	*
*One*μ13	*	+	+	+	+	+	*	+	*	*
*One*μ14	*	*	+[a]	+	+	*	+	+	*	*
*One*μ15[b]	*	*	*[a]	+	+	+	+	+	*	+
*One*μ16[b]	*	*	–	+	*	–	*	–	*	–
*One*μ17	+	+	+	+	+	+	+	+	+	+
*One*μ18	+	*[a]	+	+	+[c]	+	+	+[c]	*	*
*One*μ19	+[a]	+[a]	+	+	+	+	+	–	*	–
*One*μ20	*	+	*	+	*	+	*	*	*	*
*One*μ21	*	*	*	+	–	*	*	–	*	*
*One*μ22	*	*	*	+	+	–	*	–	+	–

Note: Symbols associated with each locus-species category refer to the presence or absence and quality of PCR products based on conditions optimized for chinook salmon: +, a single clear PCR product was obtained; *, a PCR product of the expected size was obtained but conditions were suboptimal; –, no PCR product was observed.

[a]PCR product was not of the size range (>100 bp difference) documented for the cloned sockeye salmon sequence.

[b]Multiple PCR products.

[c]Duplicated locus.

from other vertebrates. Schlotterer et al. (1991) found that homologous loci could be amplified from a diverse range of toothed (Odontoceti) and baleen (Mysticeti) whales with estimated divergence times of 35–40 Myr. Moore et al. (1991) found microsatellites were conserved across species as diverse as primates, artiodactyls, and rodents. Microsatellites isolated in domestic dogs were used in studies of a variety of canid species (Gotelli et al. 1994; Roy et al. 1994). Use of heterologous PCR primers should significantly reduce the cost of developing similar markers for other salmonid species.

Spatial heterogeneity in allele frequency among populations

Allelic variation within populations was high, both in terms of the number of alleles per locus and single- and multi-locus heterozygosity (Table 3). Heterozygosities were substantially greater than earlier estimates based on allozyme analyses (H_E = 0.201–0.226 versus 0.080–0.098, respectively). Relatively low sample sizes preclude any definitive interpretation of the utility of the presence of rare alleles within specific populations (i.e., in the context of estimating gene flow; Slatkin 1985). However, various criteria were proposed for determining appropriate sample size requirements for various statistical analyses (Chakraborty 1992).

The majority of polymorphic microsatellite loci surveyed exhibited significant spatial variation in allele frequency across the three sampled chinook populations (Table 3). The topologies of each UPGMA phenogram, in terms of identifying the rank-order of degree of between-population variation, were the same for each genetic distance measure used (Fig. 1). Estimates of Nei's (1972) interpopulation genetic distances were concordant between microsatellite and earlier allozyme analyses for these populations. Similar findings were reported for comparisons of minisatellite, microsatellite, and allozyme data sets collected for *Bufo bufo* (Scribner et al. 1994), suggesting that different loci classes are able to capture the same signature of microevolutionary events. Differences in branch length were observed, presumably because of the unique properties of each of the three distance measures employed (see below).

Comparison of the utility of microsatellites and other nuclear genetic markers

The utility of any genetic marker for ecological or evolutionary genetic studies depends principally on the research question of interest and the inherent properties of markers themselves. Studies at the intraspecific level routinely rely on protein electrophoresis and mtDNA. Indeed, for years allozyme electrophoresis has been the workhorse of empirical fisheries population genetics. However, for various reasons (e.g., low levels of detectable polymorphism, sample storage requirements, desirability of nondestructive sampling, and possible confounding effects of selection), allozymes may not always be appropriate for population-level analyses. Microsatellites are assayed using PCR-based techniques and thus are amenable to nondestructive sampling (i.e., using fin clips,

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Table 3. Allele frequencies and estimates of genetic variability for 16 microsatellite loci surveyed for three spawning populations of Chinook salmon (*Oncorhynchus tshawytscha*) from the Yukon River, Yukon Territories, Canada.

Locus	Allele (bp)	McQuesten ($N = 29$)	Klondike ($N = 27$)	Stoney Creek ($N = 27$)	θ^a
*One*μ1	142	1.000	1.000	1.000	
*One*μ2	194	0.707	0.685	0.796	0.002
	196	0.103	0.148	0.037	
	200	0.138	0.111	0.074	
	204	0.017	0.000	0.000	
	208	0.000	0.037	0.056	
	210	0.000	0.000	0.037	
	216	0.034	0.019	0.000	
*One*μ3	162	0.034	0.037	0.185	0.078*
	166	0.966	0.963	0.815	
*One*μ4	95	0.948	0.778	0.963	0.089**
	97	0.000	0.037	0.000	
	109	0.017	0.185	0.019	
	111	0.034	0.000	0.019	
*One*μ5	183	1.000	1.000	0.923	0.063
	185	0.000	0.000	0.077	
*One*μ6	206	1.000	1.000	1.000	
*One*μ7	191	0.121	0.074	0.130	0.001
	193	0.879	0.926	0.870	
*One*μ8	156	0.241	0.241	0.167	0.013
	158	0.000	0.019	0.000	
	160	0.000	0.074	0.037	
	170	0.537	0.370	0.593	
	174	0.056	0.167	0.056	
	178	0.148	0.130	0.148	
	184	0.019	0.000	0.000	
*One*μ9	165	0.000	0.000	0.148	0.137**
	169	1.000	1.000	0.852	
*One*μ10	135	0.103	0.222	0.019	0.074**
	137	0.621	0.611	0.463	
	141	0.000	0.019	0.000	
	143	0.276	0.148	0.519	
*One*μ11	139	1.000	1.000	1.000	
*One*μ12	131	0.865	0.759	0.963	0.057**
	133	0.000	0.037	0.000	
	145	0.135	0.204	0.037	
*One*μ13[b]	148	0.048	0.000	0.000	Not tested
	150	0.167	0.000	0.000	
	152	0.143	0.438	0.022	
	154	0.524	0.250	0.522	
	174	0.119	0.313	0.457	
	null	0.525	0.678	0.385	
*One*μ14	187	0.000	0.019	0.019	0.017*
	189	0.034	0.000	0.000	
	191	0.017	0.000	0.000	
	193	0.103	0.074	0.093	
	195	0.000	0.000	0.019	
	197	0.121	0.130	0.074	
	199	0.000	0.000	0.093	
	201	0.069	0.222	0.204	
	203	0.397	0.296	0.296	
	209	0.155	0.111	0.000	
	213	0.000	0.019	0.000	
	215	0.069	0.111	0.000	
	237	0.017	0.000	0.000	
	239	0.000	0.000	0.037	
	241	0.000	0.000	0.056	
	243	0.017	0.000	0.056	
	245	0.000	0.000	0.037	
	247	0.000	0.019	0.000	
	253	0.000	0.000	0.019	
*One*μ17	96	1.000	1.000	1.000	
*One*μ18	167	0.792	0.827	0.600	0.051*
	177	0.146	0.077	0.175	
	179	0.063	0.096	0.225	
Mean±SE					0.036±0.012
Observed heterozygosity (direct count)		0.201	0.226	0.221	
Expected heterozygosity (under Hardy–Weinberg)		0.227	0.267	0.239	
Pc[c]		0.615	0.615	0.769	
A[d]		2.5	2.5	2.5	

[a] Significance of θ: *, $P < 0.05$; **, $P < 0.01$.
[b] Locus not used in calculations of θ and genetic distance (Fig. 1) because of the presence of null alleles.
[c] Percentage of loci polymorphic.
[d] Mean number of alleles per locus.

scales, blood) because of the lack of constraints on amounts of DNA needed. Microgram quantities of DNA are routinely obtained from adapose or dorsal fin clips, and all loci reported herein were successfully assayed using these material sources. This is of particular concern when sampling from protected parks and preserves or when sampling threatened and endangered species. Because of the tissue-specific expression of many proteins, large-scale allozyme analyses frequently require destructive sampling. Field collections of samples for DNA-based analyses does not require cryopreservation. In addition, microsatellites occur in noncoding regions and are thought to be free from selective constraints, an assumption often accepted but rarely tested for allozyme variants (see Verspoor and Jordon 1989; Karl and Avise 1992).

Microsatellites also have distinct advantages over other classes of highly polymorphic VNTR loci such as minisatellites. Minisatellite variation is routinely assessed using Southern-blotting techniques. Measurement errors within and across gels may exceed repeat unit length. Thus, estimates of fragment mobilities may not correspond directly to repeat copy number. The continuous distribution of fragment sizes frequently necessitates allelic classifications based on "floating" (Weir 1992) or "fixed" (Budowle et al. 1991) bins. Both the precision of allelic classification (based on bin assignment) and the abundance of allelic and genotypic classes raises a challenge for testing classical population genetics models (Scribner et al. 1994). Conversely, microsatellite allelic designation can be assigned unambiguously, as PCR products can

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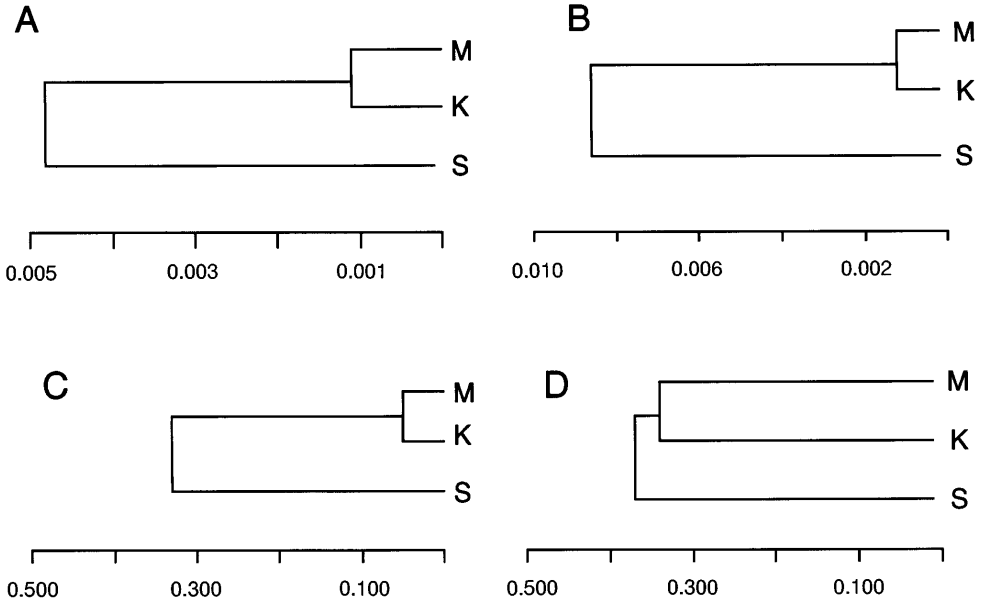
Fig. 1. UPGMA phenograms derived using three measures of genetic distance, summarizing relationships among three populations of chinook salmon (*O. tshawytscha*) from the Yukon River. M, McQuesten; K, Klondike; S, Stoney Creek. (A) *D* (Nei 1972) for allozymes. (B) *D* (Nei 1972) for microsatellites. (C) Δ (Goldstein et al. 1995) for microsatellites. (D) D_{AS} (Bowcock et al. 1994) for microsatellites.



be run on high resolution polyacrylamide sequencing gels and scored using a reference sequence standard (e.g., M13 or the cloned allele). The ability to precisely score alleles greatly enhances the potential for comparisons across studies if locus nomenclature (see Tautz 1993) and allelic standards are established with the rigor pursued by researchers employing protein allozymes.

Unique properties of microsatellite loci

The high information content relative to more standard genetic markers suggests great utility to a variety of fisheries research and management issues. The question is how to make the best use of the unique properties (i.e., rate and mode of mutation) of these loci. What assumptions must be satisfied when conducting statistical analyses?

The role of mutation has traditionally been downplayed as an evolutionary force impacting the genetic divergence among populations. If mutation rates are much less than migration rates, than for neutral alleles, divergence (as estimated by F_{st}) is shown to be nearly independent of mutation rate (Crow and Aoki 1984). If the mutation rate is high relative to the rate of interpopulation gene flow, then for neutral and highly mutable loci, novel mutations might contribute significantly to estimates of interpopulation variation (Scribner et al. 1994).

High mutation rates and the reported stepwise mutation process by which alleles mutate to smaller or larger size (i.e., by small number of repeat units; Schlotterer and Tautz 1993; Weber and Wong 1993) does not erase information about ancestral states. Evidence from direct studies of mutations in human families (e.g., Weber and Wong 1993) revealed that the size of a new mutant allele depends on the size of the allele that mutated (i.e., nearly all mutations differ from their ancestors by one or two repeat units). Regardless of whether all mutations are of only small incremental changes as assumed under the stepwise mutation models of Valdes et al. (1993), Shriver et al. (1993), and Goldstein et al. (1995), or are occasionally of larger magnitude as suggested under the two-step mutation model of Di Rienzo et al. (1994), assumptions made when using numerous standard measures of divergence (i.e., measures based on divergence in allele frequency such as F_{st}; Wright 1951) to estimate N_m or other demographic parameters may not be satisfied (Slatkin 1995). High mutation rates could also result in convergence in allele size (i.e., identify in state) in different geographic locales independent of common ancestry. Additional complications may arise because of the presence of nonamplifying or null alleles that are due to mutations in one of the priming sites (Callen et al. 1993; Pemberton et al. 1995).

Determining the relative importance of various evolutionary forces that cumulatively affect the degree of interpopulation divergence in microsatellite allele frequencies (i.e., drift, migration, mutation) will require further empirical and theoretical work. Likewise, the assumptions and caveats underlying the use of various measures of interpopulation differentiation will certainly be refined with the accumulation of additional empirical data. Recent attempts were made to provide population statistics appropriate for microsatellite analyses (Bowcock et al. 1994; Goldstein et al. 1995; Slatkin 1995). Goldstein et al. (1995) derived a distance measure (Δ) that is reported to be linear with time when used for loci undergoing a strict stepwise mutation process. Unlike other distance measures (e.g., Nei's distance; Nei 1972) that measure interpopulation differentiation caused by sampling, Δ is a function of mutation rate and not population size. Using simulation studies, these authors show that for populations separated for 100 generations, divergence (Δ) is linear over time.

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Bowcock et al. (1994) defined a distance measure between pairs of individuals ($1–P_S$), where P_S is the number of shared alleles, summed over loci, divided by twice the number of loci assayed. This measure can be extended to populations in a manner similar to that used for band-sharing coefficients in multilocus minisatellite analyses.

Goldstein et al. (1995) showed that the estimator Δ and distance measures based on the proportion of shared alleles (Bowcock et al. 1994) may be useful in identification of group membership of individuals within admixed groups composed of a set of previously distinct populations. If sufficient differences exit across subpopulations, it is possible in principle to assign individuals within an admixed group to their population of origin using clustering programs.

Interpopulation divergence, as quantified using various distance measures, increases monotonically (though in a nonlinear fashion) as a function of the time since separation. Over relatively short periods of time, drift would be expected to be the most pervasive evolutionary force causing divergence in allele frequencies. Therefore, genetic distance measures that are based on differences in allele frequency (e.g., Nei's distance) would be expected to track microevolutionary changes more adequately than measures that incorporate mutational processes (e.g., Δ). Mutation-based distance measures may prove more appropriate for resolving deeper branches of a population phylogeny, extending over greater periods of post-separation time.

Differences among distance measures are exemplified in Fig. 1 by variation in interpopulation branch lengths. While all measures show the Klondike and McQuesten populations to be more closely related than either are to Stoney Creek (a result consistent with population geographic proximities), the branch lengths based on Δ (Fig. 1C) are a order of magnitude larger than those based on Nei's genetic distance (Figs. 1A and 1B). This is presumably a function of the wide range of allele sizes (and frequency heterogeneity) at some loci (i.e., *One*μ14). Resolution of the Klondike–McQuesten versus Stoney Creek branches was less clear based on the proportion of shared alleles (D_{AS}; Fig. 1D), though the results, in part due to high number of private alleles, were consistent with topologies derived from other measures.

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