Isolation and Inheritance of Novel Microsatellites in Chinook Salmon (*Oncorhynchus tschawytscha*)

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We describe the isolation, PCR amplification, and characterization of 10 new microsatellite loci (*Ots-1–Ots-10*) for the federally protected chinook salmon (*Oncorhynchus tschawytscha*). We investigate the inheritance and linkage of these loci as well as a previously published locus, *Oneμ13,* in families obtained from artificial crosses. Mendelian transmission is confirmed for 76 of 80 segregations observed. Of the four deviations, two appear to have resulted from gametic segregation distortion. The other two provide evidence for the existence of at least one null allele. We also identify "drop out" of large alleles in these two families owing to competitive PCR amplification of smaller alleles. There is no evidence for linkage between any pair of loci. One mutation observed at *Ots-2* is reported and confirmed by DNA sequencing. We estimate the mutation rate at this locus to be 6.5×10^{-4} (95% confidence interval 3.6×10^{-3} to 1.6×10^{-4}, respectively). Characterizing a mutant allele at *Ots-2* offers the first step toward understanding mutation rates for chinook microsatellites. Owing to their Mendelian inheritance, these new loci provide reliable markers for high-resolution population genetics studies of this species.

Recent studies in population, ecological, and conservation genetics have focused on molecular markers known as microsatellites. These markers are extremely polymorphic within and among populations (Goldstein and Pollock 1997; Jarne and Lagoda 1996) and therefore offer a source of detailed information for studies of closely related populations (Andersen et al. 1997; Bowcock et al. 1994; De Knijff et al. 1997; Ellegren et al. 1996; Jorde et al. 1997; Lehmann et al. 1996; O'Reilly et al. 1996; Roewer et al. 1996; Tessier et al. 1997). Population genetics inferences for studies using microsatellite data require that transmission of these markers across generations conform to Mendelian expectation, yet relatively few empirical studies formally document their mode of inheritance (but see Chistyakov et al. 1997; Morral et al. 1993; Naciri et al. 1995; Traut et al. 1992).

Our research uses microsatellites to characterize and manage winter-run chinook salmon (*Oncorhynchus tschawytscha*) in an artificial propagation and captive breeding program. This program was initiated as a means of enhancing the survival of the wild winter-run population in the Sacramento River, which was placed on the Federal Endangered Species List in 1994 (NMFS 1994). Artificial mating in this program required molecular determination of parentage to avoid inbreeding. Effective population size estimates for wild and captive populations were necessary to ensure that the supplementation program was indeed enhancing and not reducing the genetic integrity of the wild population (Hedrick et al. 1995). The importance of run identification of potential spawning candidates was emphasized by the discovery of hybridization between winter and nonwinter chinook in the captive-rearing program (Hedgecock et al., unpublished data). The use of microsatellite data for these purposes relies on the verification that microsatellites are indeed inherited in Mendelian fashion.

An added complication for inheritance in the family Salmonidae is residual tetrasomic inheritance owing to their divergence from a single tetraploid ancestor within the last 25–100 million years (Allendorf and Danzmann 1997). While there is a general tendency for tetrasomic segregation to diploidize, evidence in tree frogs and salmonids indicates that certain loci segregate disomically in some families and tetrasomically in others (Allendorf and Danzmann 1997; Danzmann and Bogart 1982,1983; May 1982; Marsden et al. 1987). Duplicated loci that have alleles in the same size range yield phenotypes that make it challenging to discriminate be-

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Table 1. PCR primer sequence and reaction conditions for 10 microsatellites developed in chinook salmon (*Oncorhynchus tschawytscha*)

Locus	Primer sequence	Repeat motif	Annealing temperature	DNA (ng)	$MgCl_2$ (mM)	dNTPs (mM)	Primer (μM)	Allele size range (bp)
Ots-1	5′-GGAAAGAGCAGATGTTGTTAA-3′ 5′-TGCTTTATCTGCTGCTTCA-3′	$TG_3N_8TG_3N_4TG_3N_2TG_7N_{16}TG_9$	59	50	1.5	0.1	0.8	180–192
Ots-2	5′-ACACCTCACACTTAGA-3′ 5′-CAGTGTGAAGGATATTAAA-3′	CA_{17}	45	100	1.0	0.05	1.0	64–106
Ots-3	5′-CACACTCTTTCAGGAG-3′ 5′-CTTCCATTGTGATTCT-3′	TC_{18}	50	50	1.5	0.2	1.0	83–103
Ots-4	5′-GACCCAGAGCACAGCACAA-3′ 5′-CTGCTGAAATGTGTCCTCC-3′	$GA_2N_2GA_{17}N_9GA_5$	60	50	1.5	0.2	0.5	146–156
Ots-5	5′-ACAGCAGTCTACATTGACC-3′ 5′-TTTTTGGTTTTAATGAACA-3′	$AT_5N_2AT_3$	47	50	1.5	0.2	1.0	122–126
Ots-6	5′-TCTCTTCCAGCACCACACA-3′ 5′-GGATGTGGAAAAACTGTCT-3′	$CA_{13}N_3CA_3$	60	100	1.0	0.2	1.0	196–202
Ots-7	5′-TACTGGCCCAATGCT-3′[a] 5′-CTAGGCTACCTTCCGCTACCG-3′ 5′-TGTTGGCATCAGAGACATGTA-3′	AC_{34}	60	50	1.0	0.2	0.6	152–204
Ots-8	5′-TTAATATGGTCCGAAGAGGAT-3′ 5′-AACCTTTAATTTGCATTCATT-3′	$AC_{39}N_6AC_3N_2AC_4$	54	100	1.5	0.1	1.0	166–240
Ots-9	5′-CAGGGAAAGCTTTGGAGA-3′ 5′-GAACAGAGGGTCAATGAAAGA-3′	GT_{12}	59	100	1.0	0.1	0.5	114–122
Ots-10	5′-TCTGTCTACTGTATGTGCTGT-3′ 5′-AGGGTAGCAGTAATGACAA-3′	$GA_3N_4GA_{19}$	57	100	1.5	0.1	0.5	180–204

Optimizations were performed in 10 μl reactions using 1× PCR buffer and 0.25 U *Taq* DNA polymerase.
[a] Second version upstream primer.

tween disomic and tetrasomic as most likely inheritance models (Allendorf and Danzmann 1997; Marsden et al. 1987).

Besides the need to demonstrate stable inheritance for new marker types, an understanding of the mechanisms and modes by which microsatellites accumulate variance within populations is critically important, particularly given that microsatellites are thought to have exceptionally high rates of mutation (10^{-2}–10^{-5}; Weber and Wong 1993). Polymerase slippage during DNA replication is emerging as the favored mechanism for microsatellite mutation (Levinson and Gutman 1987). It is also proposed that larger mutational changes result from unequal crossover between alternate alleles (Mornet et al. 1996). This is affirmed by studies of $(CA)_n$ fragments inserted into defined positions of the yeast genome which demonstrate that microsatellites significantly enhance recombination (Treco and Arnheim 1986). Studies in fish genetics are just beginning to explore some of these issues; Brooker et al. (1994) note that microsatellite repeats in Atlantic cod, rainbow trout, and Atlantic salmon are generally larger than microsatellites of mammals, while Angers and Bernatchez (1997) reveal evidence for multiple mutational events and emphasize the impreciseness of drawing inferences on mutational rates on the basis of microsatellite allele size information alone.

This article describes how 10 new microsatellite loci were isolated and characterized in winter-run chinook salmon by means of the polymerase chain reaction (PCR) and how these loci, together with an additional locus from the literature, were inherited in controlled crosses. Our objective is to establish whether these microsatellites are indeed a reliable source of data for use in population genetics studies for the management and protection of endangered salmon populations.

Methods

Library Construction

Genomic DNA was sampled from liver tissue in 14 winter-run fish sacrificed for spawning at the Coleman National Fish Hatchery in June 1991. Standard proteinase-K digestion was followed by phenol: choloroform extraction (Sambrook et al. 1989). This genomic DNA was digested with the restriction enzyme *Dpn*II (New England Biolabs) and size fractionated using agarose (1.5%, BRL) gel electrophoresis. Fragments approximately 250–550 bp in size were eluted from the gel onto DEAE paper (Schleicher & Schuell #23430) and recovered according to manufacturers protocol. Phagemid vector (pBluescript SK^-, Stratagene) was digested with *Bam*HI (New England Biolabs) and treated with calf-intestine alkaline phosphatase (Stratagene). The above size-selected salmon DNA fragments were ligated into the *Bam*HI site and this vector was transformed into *E. coli* (XL1-blue cells, Stratagene). Competent cells were prepared using standard protocol (Sambrook et al. 1989). Recombinant clones were selected by plating on LB Amp^{50} plates containing IPTG and X-Gal.

Library Screening, Allele Cloning, and Sequencing

The phagemid library was screened with $(CA)_{15}$ and $(CT)_{15}$ oligonucleotide probes synthesized with 5′ biotinylation by the San Francisco State University DNA laboratory. Positive hybridizations were visualized using a colony images kit [United States Biochemical (USB)]. Positive clones were confirmed by Southern blotting. Plasmid DNA was purified by alkali lysis (Sambrook et al. 1989) and sequenced using a sequenase kit (USB).

PCR Primer Design and PCR Optimization

PCR primers for 10 loci, *Ots-1* through *Ots-10,* were designed using the OLIGO software package (NBI), synthesized using a Pharmacia Gene Assembler II, and purified on NAP-10 columns (Pharmacia). General PCR components were 50 ng/μl template; 1, 2, or 3 mM $MgCl_2$; 0.2 mM dNTPs; 1 μM each PCR primer; 1.25 μCi α^{33}P-dATP and 0.25 U *Taq* DNA polymerase (Promega, Madison, Wisconsin); 50 mM KCl; 10 mM Tris-Cl (pH 9.0); and 0.1% Triton X-100. Primers were initially tested using α^{33}P but were later labeled with fluorescent phosphoamidites (HEX and fluorescein) and visualized on an FMBIO fluorescent imaging system (Hitachi). Primer sequences and reaction conditions for each optimized primer pair are given in Table 1.

Genomic DNA for characterization of

Table 2. Family crosses grouped together in each of four lots

Lot 1	Lot 2	Lot 3	Lot 4
D×4	C×8	L×9	D×10
E×4		L×10	J×10
G×4			
H×4			
B×6			
C×6			
C×7			

Letters indicate males and numbers represent females.

the progeny of the 1991, 1993, and 1994 year classes was extracted from 1 mm^2 caudal fin-clips using 5% Chelex (Biorad protocols). Parents and offspring were genotyped at loci *Ots-1* through *Ots-10* and *Oneμ13* (Schribner et al. 1996) for the 1991 year class, and *Ots-1, -3, -9, -10,* and *Oneμ13* in year classes 1993 and 1994. PCR products were run on an 8% denaturing polyacrylamide gel. The microsatellites were originally sized using an M13 sequencing ladder (USB), but known-size-allele standards were used for calibration on later gels.

Crosses

In 1991, 14 adult winter chinook salmon were crossed to produce 12 families. Eleven of these families were subsequently grouped into three separate lots (1, 3, and 4) in order to maintain adequate rearing densities among juveniles (Table 2). A fourth lot (lot 2) consisted of a single family, from male C × female 8, in which inheritance of *Ots-2, -3, -4, -6, -8, -9, -10,* and *Oneμ13* was verified (*Ots-1* and *Ots-5* were not tested because both parents in this family were homozygous for the same allele). *Ots-1* through *Ots-6* were then used to provide family determination for juveniles in the mixed lots (1, 3, and 4) using a computer program (McGoldrick and Hedgecock 1997; SAS Institute 1994).

The broods of 1993 and 1994 were individually tagged with unique passive integrated transponders (PIT; Prentice et al. 1990) that were injected into the abdominal cavity of juveniles. When these fish reached maturity their parentage was verified using microsatellite analysis. Family sizes in 1991 are notably larger than in 1993 and 1994 as these fish were genotyped at an earlier life-stage before much mortality had occurred. We only focus on five loci (*Ots-2, -3, -9, -10,* and *Oneμ13*) in the 1993 and 1994 year classes because of their importance in discriminating winter-run from the other Central Valley chinook populations (Banks et al. 1996). We present segregation data for seven, two, and nine families for brood years 1991, 1993, and 1994, respectively (Table 3). Sample sizes in 5 families from 1991 and 11 families from 1993 were too small to allow segregation analysis.

Inheritance

A minimum family sample size of 12 was adopted so that the lowest expected phenotypic frequency in a 1:1:1:1 segregation would be three. Initial evaluation involved verification of consistency between offspring and parental phenotypes. Standard *G* tests (Sokal and Rohlf 1995) were used to check for compliance of phenotypic frequencies among families assuming the model of codominant Mendelian inheritance (Table 3). Corrections for multiple testing within a locus (Cooper 1968) were applied. Fisher's exact test was applied to verify the significance of *G* test results when the expected number of phenotypes within a class was less than five. Cases for which significant deviation from Mendelian expectation was observed were investigated using chi-square tests to determine whether or not the exceptions could be explained by distorted gametic segregation.

Linkage Analysis

Exact tests for linkage disequilibrium between the 11 loci considered in this study (*Ots-1* through *Ots-10* and *Oneμ13*) were performed according to Weir et al. (1996) and Zaykin et al. (1995) using genetic data analysis software.

Mutant Verification

Verification of family origins for the 1993 and 1994 broods revealed a unique allele (72 bp) at *Ots-2* not observed in any of the parents in either year. This allele appeared in one F_1 individual from the 1993 broodstock. Cloned *Ots-2* alleles from this individual, her parents, and two of her progeny (F_2) were obtained by ligating *Ots-2* PCR products into a simple T-ended cloning vector (Promega pGem-T). The recombinant vectors were transformed into competent *E. coli* (JM109, Promega) and selected by growth on LB Amp^{50} plates top-spread with IPTG and X-Gal. Plasmid DNA was extracted using a standard alkali lysis protocol. This DNA was then amplified by PCR to confirm the presence of the cloned *Ots-2* alleles. At least two clones of each allele of interest were then sequenced using a cycle sequenase kit (Amersham), in conjunction with Hex-labeled M13 forward primer (USB).

Confidence intervals for estimated rates of mutation were calculated assuming that mutations follow a Poisson distribution for which the expected number of mutations is λ. The probability of observing at most one mutation under such a model was assumed to equal $(1 + \lambda)e^{-\lambda}$ resulting in 95% upper and lower confidence limits of 5.57 and 0.24, respectively (Schug et al. 1997).

Results

Microsatellite Isolation, PCR Primer Design, and PCR Optimization

Library screening resulted in 49 positive clones. Sequencing these clones revealed 22 microsatellites that appeared promising for further development, 14 that did not have sufficient quality sequence on both sides of the repeat to allow design of PCR primers, and 13 that were false positives. Experimentation with 47 PCR primers resulted in the selection of 10 loci for continued research; four perfect repeat microsatellites (*Ots-2, -3, -7,* and *-9*) and six interrupted microsatellites (*Ots-1, -4, -5, -6, -8,* and *-10*). Optimized PCR reaction conditions (Table 1) yielded repeatable amplification. Parental phenotypes were consistent among gels and used as controls for all offspring characterization (Table 4). Neither homology for microsatellite flanking sequences nor open reading frames were detected in GenBank.

Inheritance

None of the PCR primer pairs *Ots-1* through *Ots-10* demonstrated evidence for duplicated loci, although we have observed evidence for this phenomenon in characterizing other loci in winter-run chinook families (Scribner KT, personal communication). Twenty crosses in this study produced identical genotypes in the offspring, thus verifying consistency between parental and offspring phenotypes only. In general, phenotypic classes observed in the F_1 generation were in agreement with parental phenotypes (98 of 100 crosses, Table 3). However, there were two exceptions in brood year 1991: family L×9 at *Ots-8* and family C×8 at *Ots-7.* Offspring in family L×9 were expected to have equal frequencies of phenotypes 190/190, 190/210, 190/245, and 210/245, yet the 245 bp allele of parent L appeared missing in all offspring. The observed phenotypes form only three classes that have frequencies consistent with a 1:2:1 expectation. Hypothetical dropout of the 245 bp allele in the last two expected classes would result in the observed elevated frequencies of the

Table 3. (a) *G* test results for 100 transmission studies for microsatellite loci *Ots-1* through *Ots-6*, and (b) *G* test results for 100 transmission studies for microsatellite loci *Ots-7* through *Ots-10* and *Oneμ13*

(a)	*Ots-1*			*Ots-2*			*Ots-3*			*Ots-4*			*Ots-5*			*Ots-6*		
Family	P	O	G	P	O	G/E	P	O	G	P	O	G	P	O	G	P	O	G
1991 Brood																		
C×6	184/192	25		66/66	12	0.04		—		154/156	15	1.01	124/124	25		196/198	7	8.43
				68/86	13					152/158	10					196/200	12	
																200/202	3	
																198/202	3	
C×7	192/192	47		66/66	24	0.02	85/95	26	20.91**	154/154	16	4.87	124/124	28	2.19	196/196	16	9.92
				66/86	23		95/95	3		152/154	31		124/126	18		196/200	18	
																196/202	6	
																200/202	7	
C×8	192/192	80	70/86	35	0.01	95/97	74		150/154	14	1.83	124/124	71		196/196	15	2.66	
			66/70	36					150/152	19					200/202	22		
				71					148/154	18					196/202	15		
									148/152	22					196/200	22		
L×9	184/192	10	2.84	66/66	19	2.84	91/95	5	1.92	154/154	29		124/124	15	0.03	198/198	18	1.71
	192/192	19		66/74	10		91/97	10					124/126	14		196/198	11	
							93/95	6										
							93/97	7										
M×9	184/192	14		74/86	4	/0.06	85/95	9	7.84	154/156	7	0.07	124/124	15		198/198	5	0.72
				66/86	5		85/93	4		150/154	8					196/198	6	
				68/74	6		91/91	10								196/196	4	
				66/66	0		91/93	2										
D×10	192/192	6	5.09	66/66	20		85/91	7	0.25	154/156	1	16.76**	124/124	20		198/198	5	0.29
	184/192	4					85/85	9		154/154	6					196/198	9	
	184/184	7								152/156	1					196/196	6	
										152/154	12							
J×10	192/192	6	0.44	66/66		27	85/85	13	0.04	154/156	12	0.33	124/126	15	0.33	198/202	8	0.74
	184/192	12					85/91	12		152/156	15		124/124	12		198/198	5	
	184/184	8														196/202	7	
																196/198	7	
1993 Brood																		
C×6		—		66/66	6	4.30	85/91	1	3.59		—			—			—	
				66/86	5		85/95	5										
				86/86	1		91/97	2										
							95/97	4										
C×10		—		66/86	9	3.14	91/91	4	3.13		—			—			—	
				86/86	3		91/95	1										
							95/99	3										
							91/99	5										
1994 Brood																		
J×7		—		66/66	12	2.97	91/95	9	0.06		—			—			—	
				66/86	5		95/95	8										
H×11		—		66/66	2	1.86	85/95	2	2.09		—			—			—	
				66/70	10		85/97	4										
				70/70	5		95/95	4										
							95/97	6										
I×11		—		66/66	6	2.60	93/95	2	2.38		—			—			—	
				66/70	3		93/97	3										
				66/96	5		95/97	6										
				70/96	2		97/97	3										
E×12		—		66/70	16		95/95	7	0.07		—			—			—	
							95/97	8										
I×18		—		66/66	9	1.16	93/93	1	3.20		—			—			—	
				66/96	5		93/97	8										
							97/97	5										
G×7		—		66/70	6	0.6	85/91	3	1.62		—			—			—	
				70/86	9		85/95	6										
							91/95	3										
							95/95	3										
E×19		—		66/70	13		93/95	4	1.97		—			—			—	
							95/95	9										
H×9		—		66/66	9	0.25	95/95	4	1.40		—			—			—	
				68/70	7		85/95	4										
							85/97	5										
							95/97	2										
B×15		—		66/70	13		93/97	2	6.86		—			—			—	
							97/97	11										

(b)	*Ots-7*			*Ots-8*			*Ots-9*			*Ots-10*			*Oneu13*		
Family	P	O	G	P	O	G	P	O	G	P	O	G/E	P	O	G/E
1991 Brood															
C×8	158/160	21	6.65	194/198	32	0.62	114/114	24	0.47	194/194	14	0.31	150/160	29	0.00
	158/204&n	25		190/194	26		114/122	29		194/198	24		160/168	29	
	n/160	15								198/198	14				
	n/204&n/n	32													
L×9		—		190/210	10	2.17	118/118	13	1.13	194/198	31		150/160	8	4.47
				190/190	19		114/118	19					160/160	5	
				210/210	5								150/172	13	
													160/172	6	
M×9		—		190/190	10	1.90		—		194/194	16	0.31	160/172	15	0.33
				190/230	3					194/198	13		160/160	12	
				190/210	6										
				210/230	3										

Table 3. Continued

(b)	*Ots-7*			*Ots-8*			*Ots-9*			*Ots-10*			*Oneu13*		
Family	P	O	G	P	O	G	P	O	G	P	O	G/E	P	O	G/E
J×10		—			—			—		194/194	14	0.67		—	
										194/198	10				
											24				
1993 Brood															
C×6		—			—			—		194/194	3	/0.002	150/150	4	/0.005
										194/198	9		150/160	8	
										198/198	0		160/160	0	
C×10		—			—			—		194/194	4	0.70	150/150	3	0.68
										194/198	5		150/160	2	
										198/198	4		150/164	3	
													160/164	4	
1994 Brood															
J×7		—			—		114/114	3	4.66	194/194	2	2.66	150/160	12	2.97
							114/122	5		194/198	10		160/160	5	
							122/122	8		198/198	2				
H×11		—			—		114/122	3	0.00	194/198	6	0.08	150/160	6	1.49
							118/122	6		198/198	7		160/160	11	
							122/122	3							
I×11		—			—			—		194/194	9	1.97	150/160	16	
										194/198	4				
E×12		—			—			—		194/194	2	2.16	150/160	9	0.60
										194/198	4		160/160	6	
										198/198	5				
I×18		—			—			—		194/198	14		150/150	10	2.66
													150/160	4	
G×7		—			—			—		194/198	4	0.11	160/160	14	
										198/198	5				
E×19		—			—			—		194/198	7	1.65	150/160	7	0.33
										198/198	3		160/160	5	
H×9		—			—			—		194/198	6	1.02	160/160	6	0.00
										198/198	3		150/160	6	
B×15		—			—			—		198/198	10		150/160	6	0.00
													160/160	6	

Family indicates parent IDs; P indicates offspring phenotypes; O reports number of cases an individual phenotype is observed; G reports *G*-test results; E reports Fisher's exact probability. Significance ($P < .001$) is indicated by **. There were no observations in the significance range $.05 > P > .001$. Symbols:—represents crosses that were not studied, n represents hypothetical "null" alleles.

190/190 class and new 210/210 class. A similarly perplexing inconsistency between parent and expected offspring phenotypes was observed in family C×8 at *Ots-7*. Expected offspring phenotypes were 158/160 or 158/204. The following six phenotypes were observed: 158/160 (21), 158/204 (4), 158 (21), 160 (15), 204 (19), and no apparent PCR product (13). Assuming parents to have genotypes 158/null and 160/204 (sometimes null), offspring genotypes could be grouped as follows: 158/160 (21), 158/204 + 158/null (25), null/160 (15), and null/204 + null/null (32). *G* tests for these assumed frequencies do not demonstrate significant deviation from expectation (Table 3b). To resolve these unusual phenotypes and explore the hypothesis that they could result from mutations at PCR priming sites (null alleles), DNA from this family was amplified using a different upstream primer (Table 1). Results from using this new primer were similar to those described above.

Family classification of F_1 juveniles was inconsistent with parental phenotypes in 10 of 3936 parent/offspring comparisons. DNA was reextracted from fin-clip samples of these fish and three replicate PCR amplifications were performed to verify the data for these 10 exceptions. Nine of the inconsistent phenotypes proved to be a result of scoring errors, which when corrected allowed these individuals to be assigned correctly to their expected families. One F_1 juvenile female remained inconsistent and is discussed later.

Despite confirmation of Mendelian inheritance in 96 of the 100 crosses considered in this study (Table 3), 2 of 80 segregations show significant non-Mendelian phenotypic ratios (family D×10 in the 1991 year class at *Ots-4* and family C×7 in the 1991 year class at *Ots-3*). This may provide evidence for gametic distortion. Male D (154/156) in family D×10 transmits 18 alleles of 154 bp but only 2 alleles of 156 bp ($P = 1.9 \times 10^{-5}$), while female 7 in family C×7 transmits 26 alleles of 85 bp but only three alleles of 95 bp ($P = .004$). These two cases occur among a total of 89 families where genotype details allow observation of gamete contribution from each parent. Their small overall frequency (0.02) indicates that this phenomenon will not substantially affect the accuracy of these microsatellites as molecular markers.

Linkage Analysis

Pairwise exact tests for linkage disequilibrium in a total of 192 two-locus combinations across seven families revealed 16 significant deviations from expectation. Considering the two families with large sample size (greater than 40), no two-locus combination was significant in both families. This indicates that the disequilibrium observed in the 16 significant observations was a function of factors unique to a few combinations within particular families and not evidence of linkage between loci.

Mutant Verification

The *Ots-2* phenotype (66/72) of one 1993 year class fish was incompatible with the phenotypes of its parents (female 8 and male I). DNA sequencing confirmed parent 8 (66/70) is a $(CA)_7/(CA)_9$ heterozygote and parent I (66/66) is a $(CA)_7/(CA)_7$ homozygote, while their mutant offspring (66/72) was a $(CA)_7/(CA)_{10}$ heterozygote. The phenotypes of this fish for *Ots-3, -4, -6, -9, -10,* and *Oneμ13* were consistent with family I×8 and inconsistent with every other family. Furthermore, we have not observed a 72 bp allele in any other broodstock spawned at the Coleman National Fish Hatchery in 1993 or any of the other years we have characterized (1991–1995). Phenotypic data for 12 full sibs from family I×8 and 9 half sibs (family A×8) did not

Table 4. Microsatellite genotypes for winter-run chinook captive broodstock used in crosses for inheritance studies

ID number	Sex	*Ots-1*	*Ots-2*	*Ots-3*	*Ots-4*	*Ots-5*	*Ots-6*	*Ots-7*	*Ots-8*	*Ots-9*	*Ots-10*	*Oneu13*
BY1991												
6	F	184/184	66/66	95/97	156/156	124/124	198/200	152/152	190/190	114/118	198/198	160/160
7	F	192/192	66/66	85/95	154/154	124/126	196/200	—	194/198	114/122	198/198	150/160
8	F	192/192	70/70	97/97	148/150	124/124	196/200	158/158	194/194	114/114	194/198	150/168
9	F	192/192	66/74	91/93	154/154	124/124	196/198	152/152	190/210	118/118	194/194	160/172
10	F	184/192	66/66	85/85	152/154	124/124	196/198	152/160	190/210	118/122	194/198	160/160
C	M	192/192	66/86	95/95	152/154	124/124	196/202	160/204	190/198	114/122	194/198	160/160
D	M	184/192	66/66	85/91	154/156	124/124	196/198	160/160	190/190	114/118	194/198	160/160
J	M	184/192	66/66	85/95	156/156	124/126	198/202	—	190/210	114/122	194/194	160/160
L	M	184/192	66/66	95/97	154/154	124/126	198/198	152/152	190/245	114/118	198/198	150/160
M	M	184/184	66/86	85/91	150/156	124/124	196/198	152/152	190/230	122/122	194/198	160/160
BY1993												
6	F	—	66/86	85/97	—	—	—	—	—	114/114	194/198	150/160
10	F	—	86/86	91/99	—	—	—	—	—	114/114	194/198	150/164
C	M	—	66/86	91/95	—	—	—	—	—	114/122	194/198	150/160
BY1994												
7	F	—	66/86	91/95	—	—	—	—	—	114/122	194/198	160/160
9	F	—	66/66	95/97	—	—	—	—	—	122/122	194/198	160/160
10	F	—	84/86	91/91	—	—	—	—	—	122/122	194/194	150/168
11	F	—	66/70	95/97	—	—	—	—	—	118/122	194/198	160/160
12	F	—	66/66	95/97	—	—	—	—	—	114/118	194/198	150/160
15	F	—	66/66	93/97	—	—	—	—	—	114/122	198/198	150/160
18	F	—	66/66	93/97	—	—	—	—	—	122/122	198/198	150/160
19	F	—	66/66	93/95	—	—	—	—	—	118/122	198/198	150/160
G	M	—	70/70	85/95	—	—	—	—	—	114/122	198/198	160/160
H	M	—	66/70	85/95	—	—	—	—	—	114/122	198/198	150/160
I	M	—	66/96	93/97	—	—	—	—	—	122/122	194/194	150/150
E	M	—	70/70	95/95	—	—	—	—	—	114/114	194/198	160/160
B	M	—	70/70	97/97	—	—	—	—	—	114/122	198/198	160/160
J	M	—	66/66	95/95	—	—	—	—	—	114/122	194/198	150/160

show this 72 bp allele. The fish determined to have the 72 bp allele was reared until maturity and crossed with two unrelated males having 66/66 *Ots-2* phenotypes. PCR amplification of 49 F_2 juveniles from the resulting families revealed totals of 30 heterozygotes (66/72) and 19 homozygotes (66/66), not significantly different from the Mendelian 1:1 expectation. DNA sequences generated from these F_2 juveniles revealed typical allele sequences consistent with those observed in the F_1 parents.

This new allele at the locus *Ots-2* was observed in a total of 1530 parent/offspring comparisons at this locus which corresponds to a mutation rate of 6.5×10^{-4}, with upper and lower confidence limits of 3.6×10^{-3} and 1.6×10^{-4}, respectively.

Discussion

Statistical analyses of phenotypic ratios for the 11 loci tested in a total of 100 chinook salmon crosses reveal convincing evidence for normal Mendelian inheritance. Unlike the extremely unstable inheritance and mutational patterns now well known for trinucleotide microsatellites associated with human disease (Longshore and Tarleton 1996), our findings suggest that the majority of loci we consider provide a reliable source of molecular genetic data for population genetic studies of chinook salmon.

Our findings reveal some limitations in characterizing microsatellite variation, as well as two cases of segregation distortion. First, let us focus on two exceptions with regard to agreement between parental and offspring phenotypes. Phenotypes observed among offspring in families L×9 at *Ots-8* and C×8 at *Ots-7* were clearly not consistent with expectations. In both cases large parental alleles (245 bp in L×9 and 204 bp in C×8) were not transmitted to offspring as expected. In heterozygous individuals, preferential amplification of a smaller allele over a much larger allele would falsely indicate non-Mendelian inheritance even though larger alleles may indeed have been transmitted. This problem of large allele "drop out" is well documented in the minisatellite literature (Van Pijlen et al. 1995). In contrast to large size differences between alleles in the minisatellite study, however, the increased size of alleles for which the "dropout" artifact was observed in our study was only about 50 bp.

An alternative explanation for the inconsistent parent/offspring phenotypes observed in these two families may be the loss of alleles among offspring. Irrespective of the sizes of hypothetical alternate alleles, nonamplification may result from variation in the PCR priming site. This phenomenon is well documented in other microsatellite studies (Brand and Ron 1997; Brookfield 1996; Callen et al. 1993; Gullberg et al. 1997; Koorey 1993; Pemberton et al. 1995). It is difficult to favor either null allele or large allele "dropout" artifacts as a cause of these observations as both may play a role. Null alleles due to primer site variation may cause inconsistent amplification in different populations. For example, the locus *Oneμ13* demonstrates perfect transmission frequencies in winter-run chinook families of our study. Scribner et al. (1996) describes evidence for a null allele at this locus in chinook populations from the Yukon River, Alaska.

Evidence for significant gametic segregation distortion in two families is our second anomalous result. Disproportional transmission of alleles by either male D or female 7 in families D×10 and C×7 is inconsistent with Mendel's law of independent assortment. We explored "secondary tetrasomy" (Allendorf and Danzmann 1997) as a likely explanation for these results. This phenomenon has not been observed in female salmonids (Allendorf and Danzmann 1997) and is therefore unlikely

to apply to family C×7. Furthermore, we found that the observed ratios in family D×10 remained significantly different to expected ratios assuming tetrasomy (Allendorf and Thorgaard 1984; Burnham 1962). Other prezygotic events, however, such as gene conversion or meiotic drive (Lyttle 1991, 1993; Silver 1993), or even virus-mediated non-Mendelian transmission, such as identified in the fruit fly (Fleuriet and Periquet 1993; Lopez Ferber et al. 1997), are possible explanations for these results. Postzygotic events, such as linkage to a dominant lethal locus, are also feasible. Mendelian transmission of the same alleles in other families and loss of significance when pooling data indicates that these rare phenomena may not significantly bias microsatellite data in population studies. Nevertheless, further studies of these families may prove interesting, particularly given the uncertainty of how rare meiotic drive elements are maintained in populations (Leeflang et al. 1996).

Mutations that change the number of repeat units within a microsatellite are more easily characterized in routine PCR-product electrophoresis than the phenomena described above. DNA sequencing confirms that the 72 bp allele detected in one of the offspring differs from either of its parental alleles by an insertion of either one or three dinucleotide repeat units. Regardless, this observed mutation is consistent with the emerging view that most microsatellite mutations result from DNA polymerase strand slippage (Levinson and Gutman 1987), as well as the apparent bias toward an increased number of repeat units observed in mutational events (Weber and Wong 1993). It is at odds, however, with the expectation that larger microsatellites mutate at higher rates (Jin et al. 1996; Schug et al. 1997); when compared with the other loci developed in this study, winter-run alleles at *Ots-2* have the smallest number of repeat units. Mendelian transmission of the 72 bp allele affirms that this was indeed a germline mutation. We have a unique opportunity to document the fate of this allele during forthcoming years, given that a 72 bp allele has not been observed in any winter-run fish studied from 1991 through 1998 and also appears scarce in other chinook populations from California's Central Valley.

Our 6.5×10^{-4} estimate for the rate of mutation at *Ots-2* is within the generally accepted range of 10^{-2} to 10^{-5} (Weber and Wong 1993). Studies in 40 CEPH human reference families and confirmation of rates using DNA from untransformed cells resulted in an estimated mutation rate of 1.2×10^{-3} per locus per gamete per generation (Weber and Wong 1993). For mice, estimated rates of 1.2×10^{-4} and 4.7×10^{-4} resulted from five observed events in different inbred and recombinant strains (Dallas 1992) and the low rate estimated for *Drosophila* is 6.3×10^{-6} (Schug et al. 1997). Within species, factors such as chromosomal location, length and type of repeat motif, as well as the presence or absence of interruptions explains why the pattern and rate of mutations at microsatellites vary among loci (Edwards et al. 1992; Jin et al. 1996). Invoking the assumption that such mutations are well approximated by the Poisson distribution, we estimate an upper 95% limit of 1.3×10^{-4} for the other loci studied in our investigation. These rates are two to five orders of magnitude higher than estimated rates of mtDNA and allozyme mutation. Salmon microsatellites are therefore highly likely to resolve parentage and to discriminate between closely related populations.

In this study we report the isolation and development of 10 original microsatellite loci for use in chinook salmon population discrimination. Capitalizing on our captive broodstock resource, we used known crosses to examine the transmission of these loci. Eight of the 10 loci conformed to Mendelian inheritance expectations and show no evidence for linkage. Null allele and/or large allele "dropout" artifacts reduced the reliability of the other two loci. This underscores the necessity of empirical transmission studies for new molecular markers used in population genetics.

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