

Incidence of Hybridization between Naturally Sympatric Westslope Cutthroat Trout and Rainbow Trout in the Middle Fork Salmon River Drainage, Idaho

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Abstract.—Introgressive hybridization has been widely reported for westslope cutthroat trout (WCT) *Oncorhynchus clarkii lewisi* and rainbow trout (RBT) *O. mykiss* and is often a result of introductions of nonnative RBT into previously allopatric populations of westslope cutthroat trout. The WCT evolved in sympatry with RBT in a portion of its native range. Few studies have evaluated natural hybrid zone structure in sympatric populations or the effects of nonnative introductions within sympatric populations. We used one mitochondrial DNA marker and three co-dominant nuclear DNA markers to examine 17 populations of WCT that were sympatric with native RBT—steelhead (anadromous RBT). As 5 of the 11 sample locations were situated downstream of stocked headwater mountain lakes, we wanted to determine the effects of headwater lake introductions on naturally sympatric populations of WCT and RBT—steelhead below the lakes. Hybrids were found in streams below stocked and unstocked headwater lakes. Our results indicated that the majority of the populations displayed a bimodal hybrid structure, linkage disequilibrium, and Hardy–Weinberg disequilibrium. This suggests recent, ongoing hybridization but also strong assortative mating, which may be indicative of minimal impacts from stocking or independent events of natural hybridization. Natural hybrid zones should be addressed in current political deliberations about the inclusion of hybridized populations of WCT in Endangered Species Act considerations. A decision to discount protective actions for such stocks may lead to a loss of potentially adaptive genetic diversity and the fragmentation of populations.

Hybridization has been widely reported in fishes (Scribner et al. 2001), yet the mechanisms of hybridization and the role of hybridized populations in conservation and management are still uncertain (Allendorf et al. 2001). While hybridization was once thought of as rare (Mayr 1963), it is now a threat for many native fishes and especially poses challenges to conservation and management of native cutthroat trout *Oncorhynchus clarkii* throughout western North America (Allendorf et al. 2001; Peacock and Kirchoff 2004). Many cutthroat trout subspecies are currently designated as sensitive species by state agencies or are protected under the U.S. Endangered Species Act (ESA), and hybridization with nonnative rainbow trout

(RBT) *O. mykiss* is considered a primary reason for those designations (Utter 2003).

One of the most difficult challenges in the conservation and management of hybridized populations is the categorization of hybrid zones (Allendorf et al. 2001). Hybrid zones are areas of contact between two genetically divergent populations where interbreeding occurs (Allendorf et al. 2001). Little is known about the factors that influence the establishment and perpetuation of hybrid zones. Hybridization can result in four different outcomes: (1) reinforcement of reproductive isolating mechanisms, (2) speciation, (3) introgression, where the genes of one species becomes integrated into another species, and (4) formation of hybrid swarms (Ostberg and Rodriguez 2006). Many evolutionary factors interact to affect the outcome of hybridization events, and no single factor is common to all hybridization events (Scribner et al. 2001). The size

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and structure of the hybrid zone are affected by development of (or lack of) reproductive isolating mechanisms, role of selection, dispersal potential of hybrids, abundance and rate of gene flow among parental types, genetic compatibility, and environment, as well as the interaction of these factors (Scribner et al. 2001). As a result, a continuum of hybrid zones has been documented within salmonids, ranging from occasional hybridization (Redenbach and Taylor 2003) to complete breakdown of reproductive isolation and the formation of hybrid swarms (Docker et al. 2003). Occasional hybridization has been regarded as both a historical and contemporary process that has shaped the evolution of many sympatric species (Taylor 2004). In contrast, risk of extinction from introgressive hybridization (namely, the formation of hybrid swarms) has been recognized as a major consequence of nonnative introductions into formerly allopatric populations. As these hybrid zones reflect different evolutionary trajectories, it is important to recognize the different mechanisms that may exist for species that evolved in sympatry as opposed to allopatry.

Evaluations of hybrid zone structure can reveal important underlying processes and help differentiate the type of hybridization. Hybrid zone structure can be classified as bimodal, intermediate, or unimodal (Harrison and Bogdanowicz 1997). Bimodal hybrid zones consist mainly of genotypes resembling the parental forms with few intermediate hybrid genotypes and are strongly associated with well-developed (but incomplete) prezygotic isolation (Jiggins and Mallet 2000). The occurrence of natural hybridization among sympatric species indicates incomplete isolation coupled with reinforcement of isolating mechanisms that maintains species integrity over evolutionary time. Intermediate hybrid zones consist of a more even mixture of both parental and hybrid genotypes, while unimodal hybrid zones, most often referred to as hybrid swarms, predominantly consist of hybrid genotypes (Jiggins and Mallet 2000). Unimodal hybrid zones generally reflect a complete breakdown of isolating mechanisms or lack of selection against hybrids and are often the result of secondary contact among allopatric species.

The westslope cutthroat trout (WCT) *O. clarkii lewisi* is 1 of 14 subspecies of cutthroat trout for which introgressive hybridization is of concern. Westslope cutthroat trout were historically distributed throughout central and northern Idaho, western Montana, and portions of northwestern Wyoming, eastern Washington, and north-central Oregon in the United States, and southwestern Saskatchewan, southern Alberta, and southeastern British Columbia in Canada (Behnke

1992). Westslope cutthroat trout and RBT–steelhead (anadromous RBT) evolved in allopatry throughout most of their historic ranges (Behnke 1992). The areas where they evolved in sympatry include the Kootenai, Salmon, and Clearwater River drainages in Idaho; the John Day River drainage in Oregon; and the middle Columbia River basin in Washington. Historic introductions of nonnative RBT have also occurred throughout the range of WCT, resulting in introgressive hybridization (Allendorf and Leary 1988). Recent concerns regarding the decline of WCT through introgressive hybridization have led to a petition to list the subspecies as threatened under the ESA and a new policy regarding hybridized populations (USFWS 2003).

Introgressive hybridization has been cited as the greatest threat facing WCT, yet the establishment and maintenance of hybridized populations is not understood (Hitt et al. 2003). Hybridization has been documented in allopatric WCT populations which have been stocked with nonnative RBT (Forbes and Allendorf 1991; Rubidge et al. 2001; Hitt et al. 2003; Rubidge and Taylor 2004, 2005; Ostberg and Rodriguez 2006), but few studies have documented ongoing hybridization among native WCT and native RBT–steelhead (Howell and Spreull 2003; Weigel et al. 2003; Peterson et al. 2004). In two of these studies (Howell and Spreull 2003; Weigel et al. 2003), stocking of nonnative fish may have directly impacted the WCT populations (Allendorf et al. 2005); therefore, the documentation of the natural hybrid zone within the range of sympatric RBT and WCT is unclear. To date, there is little information regarding the structure of hybrid zones in areas of natural sympatry and the processes that influence that structure.

In this study, our primary objective was to document the extent of hybridization within sympatric populations of WCT and RBT–steelhead in the Middle Fork Salmon River (MFSR) drainage in Idaho. As 5 of the 11 sample locations were situated downstream of headwater mountain lakes (historically stocked with nonnative trout), a second objective was to determine the effects of headwater lake introductions on naturally sympatric populations of WCT and RBT–steelhead below the lakes. We predicted that populations downstream of headwater lake introductions would have the highest levels of hybridization and that recent hybridization would also be evident at these locations due to invasion by nonnative trout. We assess levels of assortative mating, directionality of hybridization, and spatial patterns of hybridization throughout the MFSR drainage to determine the mechanisms that may be important to the observed levels and patterns of hybridization in sympatry. We also interpret results

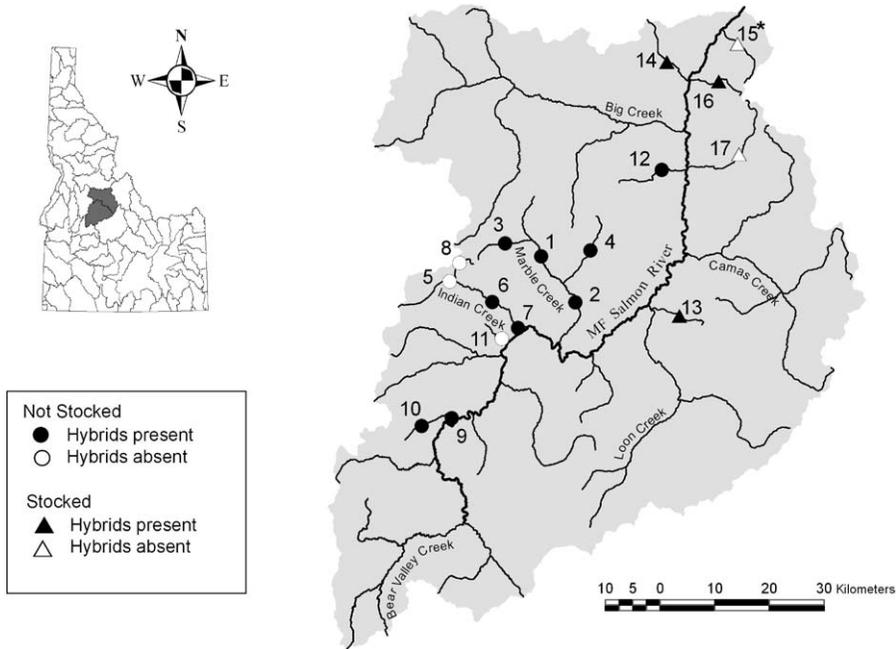


FIGURE 1.—Stocking history and distribution of hybridization for westslope cutthroat trout populations sampled in 17 creeks of the Middle Fork Salmon River drainage, Idaho: (1) upper main-stem Marble, (2) middle main-stem Marble, (3) Big Cottonwood, (4) Trail, (5) upper main-stem Indian, (6) middle main-stem Indian, (7) lower main-stem Indian, (8) Little Indian, (9) lower Elkhorn, (10) upper Elkhorn, (11) Garden, (12) Soldier, (13) Cache, (14) Papoose, (15) Roaring, (16) Ship Island, and (17) Wilson. In Roaring Creek, westslope cutthroat trout–Yellowstone cutthroat trout hybrids were detected.

within the context of recently proposed guidelines by the U.S. Fish and Wildlife Service (USFWS) for conserving hybridized populations under the ESA.

Methods

Study area.—Sampling was conducted within the MFSR drainage in central Idaho (Figure 1). The MFSR supports naturally sympatric populations of WCT, resident RBT, and anadromous steelhead as well as other native salmonids. Road and trail densities are low, and little stocking has occurred within the drainage; however, intermittent stocking of hatchery-reared WCT, RBT, cutthroat–RBT hybrids, golden trout *O. mykiss aguabonita*, and Yellowstone cutthroat trout *O. clarkii bouvieri* has occurred in the headwater lakes of some MFSR tributaries from the early 1930s through 2001. The MFSR drains 7,330 km² through mountainous terrain, and elevation ranges from 1,550 to 3,150 m. Land in the study area is primarily managed as wilderness, and the MFSR main stem has been protected as a wild and scenic river by the U.S. Forest Service since 1969.

Stocking of nonnative trout was assessed through the Idaho Department of Fish and Game (IDFG) electronic database, which contains information from 1967 to the

present (available at <http://fishandgame.idaho.gov/apps/stocking/>), and handwritten IDFG records, which date back to 1920. All stocking occurred within the high mountain lakes at the headwaters of the tributaries, and no stocking occurred within the main-stem MFSR or its tributaries (Table A.1; Figure 1).

Population samples.—Tissue (fin clip) samples were nonlethally obtained from a total of 839 fish from 17 locations in 2002. Fish were collected by angling; sample sizes ranged from 25 to 62 fish per sample location (Table 1). Samples were collected from all *Oncorhynchus* spp. regardless of phenotype. Sampling was conducted uniformly from the headwaters to the mouths of the main stems, where possible. All fin clips were stored at ambient temperature in 100% non-denatured ethanol until DNA extraction.

Eighty-one golden trout from the Mt. Whitney State Hatchery (Cottonwood Lakes strain) were sampled for use as a reference population, since stocking records indicated that this strain was used for golden trout stocking in Idaho. Currently, there are no diagnostic markers available to differentiate hatchery strains of WCT and RBT from native populations (Table A.1).

Genetic analysis.—Total genomic DNA was extracted from samples based on methods described by

TABLE 1.—Number of samples collected (N), and number of westslope cutthroat trout (WCT) like genotypes, rainbow trout (RBT) like genotypes, F_1 hybrid genotypes, and F_n hybrid genotypes detected among the 17 sample locations in the Middle Fork Salmon River drainage, Idaho. Hardy–Weinberg and linkage equilibrium results are listed along with the introgression levels (with 95% lower and upper confidence bounds).

Drainage	Stream	N	H–W equilibrium	Linkage equilibrium	WCT-like	F_1 hybrids	F_n hybrids	RBT-like	Introgression level (%)
Marble Creek	(1) Upper main-stem Marble Creek	46	Yes	No	41	2	3		1 (1–4)
	(2) Middle main-stem Marble Creek	59	No	No	31	2	2	24	2 (1–5)
	(3) Big Cottonwood Creek	57	Yes	No	54	2	1		<1 (0–2)
	(4) Trail Creek	44	No	Yes	8		1	35	3 (1–11)
Indian Creek	(5) Upper main-stem Indian Creek	56	Yes	Yes	56				0
	(6) Middle main-stem Indian Creek	58	No	Yes	35		1	22	2 (1–5)
	(7) Lower main-stem Indian Creek	57	No	Yes	26		1	30	2 (1–5)
	(8) Little Indian Creek	51	Yes	Yes	51				0
Middle Fork Salmon River	(9) Lower Elkhorn Creek	20	No	No	6	1	2	11	6 (2–17)
	(10) Upper Elkhorn Creek	25	Yes	No	23	1		1	3 (1–7) ^a
	(11) Garden Creek	50	Yes	Yes	50				0
	(12) Soldier Creek	54	No	No	3	1	2	48	8 (3–21)
	(13) Cache Creek ^b	45	Yes	No	43	1		1	2 (1–5) ^a
	(14) Papoose Creek ^b	62	No	No	7	1	2	52	10 (3–25)
	(15) Roaring Creek ^b	39	No	Yes	33		2 ^c	4	0 ^c
	(16) Ship Island Creek ^b	57	Yes	Yes	2	3	29	23	48 (42–55)
	(17) Wilson Creek ^b	59	No	Yes	13			46	0

^a Hardy Weinberg equilibrium was not rejected, so introgression was calculated according to the methods.

^b Locations downstream of stocked headwater lakes.

^c Two samples had mtDNA of Yellowstone cutthroat trout.

Paragamian et al. (1999). Four co-dominant nuclear loci were used to differentiate between RBT and WCT (Table 2): two intron restriction fragment length polymorphisms (RFLPs) and two species-specific simple-sequence repeats (SSRs). The two RFLP nuclear markers, *Ikaros* (*IK*) and recombination action gene (*RAG3'*), were previously reported to show diagnostic RFLPs between RBT and cutthroat trout when cut with the appropriate restriction enzyme (Rubidge et al. 2001; Baker et al. 2002; Campbell et

al. 2002). The other two nuclear markers (*OCC-16*, *OM-13*) reveal size-based differences in the polymerase chain reaction (PCR) products (Table 2). Amplification procedures and restriction digests (if applicable) followed the methods of Campbell et al. (2002) and Ostberg and Rodriguez (2002, 2004). Because mitochondrial DNA (mtDNA) is maternally inherited, it can provide information regarding the directionality of hybridization when coupled with nuclear DNA analyses. Therefore, the cytochrome *b*

TABLE 2.—Marker type, restriction enzyme, total size of amplified product (number of base pairs), and species-specific diagnostic fragment allele sizes for molecular markers used to differentiate westslope cutthroat trout (WCT) and rainbow trout (RBT); n/a = not applicable.

Locus	Enzyme	Type of marker	Expression	Number of base pairs	Diagnostic allele sizes	Reference
Cytb	<i>Hae-III</i>	mtDNA RFLP	Haploid	1,300	WCT: 910, 165, 110, 75, 50, 40 WCT: 600, 310, 165, 110, 75, 50 RBT: 310, 300, 265, 165, 130, 110 GT: 300, 290, 265, 195, 120, 110	Baker et al. 2002
IK	<i>Hinf-I</i>	Intron RFLP	Co-dominant	813	WCT: 519, 294 RBT: 813	Baker et al. 2002
Rag3'	<i>Dde-I</i>	Intron RFLP	Co-dominant	1,013	WCT: 544, 286, 183 WCT: 324, 286, 220, 183 RBT: 544, 469	Baker et al. 2002
OCC-16	n/a	SSR	Co-dominant	280–380	WCT: 380 RBT: 280	Ostberg and Rodriguez 2002
OM-13	n/a	SSR	Co-dominant	175–190	WCT: 190 RBT: 175	Ostberg and Rodriguez 2002
OCC-38 ^a	n/a	SSR	Co-dominant	150–175	WCT: 175 RBT: 150	Osterberg and Rodriguez 2004
OCC-42 ^a	n/a	SSR	Co-dominant	160–190	WCT: 190 RBT: 160	Osterberg and Rodriguez 2004

^a OCC-38 and OCC-42 were only amplified for the genotypes indicative of F_1 hybrids.

gene (*Cytb*) was amplified and digested with the *HaeIII* enzyme (Table 2; Baker et al. 2002). All RFLP and PCR products were separated by horizontal gel electrophoreses through a 3% agarose-synergel (Diversified Biotech) gel and were stained with ethidium bromide to reveal diagnostic banding patterns.

One of the nuclear markers identified as diagnostic between WCT and RBT did not appear to be species specific in our study. The *IK* locus apparently had a higher percentage of hybrid genotypes than the other loci examined. For example, no hybrid genotypes were detected in Roaring and Garden creeks at three of the nuclear loci, yet 50% and 7% of the samples, respectively, had hybrid genotypes at the *IK* locus. We decided to exclude this locus from further analyses because it did not appear to be fixed in this study and others (M. R. Campbell, unpublished data) and because its inclusion had the potential to bias our introgression estimates upward.

We calculated our probability of detecting introgression in each of the populations with three nuclear markers using the following equation from Kanda et al. (2002):

$$A^{2N \cdot X} = B,$$

where $A = 1$ minus the percent introgression to be detected, $B = 1$ minus the percent chance of detecting that level of introgression, $N =$ the number of fish sampled per site, and $X =$ the number of diagnostic nuclear markers. Based upon our sample sizes in Table 1, we had a 97% chance of detecting RBT introgression as low as 3% at all of the sites.

A hybrid index (Jiggins and Mallet 2000) was used to generate a frequency distribution of nuclear genotypes differentiating individuals with WCT parental genotypes, RBT parental genotypes, and mixed (hybrid) genotypes. For the hybrid index, each allele was scored 0 for a WCT allele and 1 for a RBT allele. The sum across all loci was used to generate the hybrid index. A cumulative score of 0 indicates that only WCT-like genotypes were sampled, and a cumulative score of 6 indicates that only RBT-like genotypes were sampled. Intermediate scores (ranging from 2 to 5) indicate mixed (hybrid) genotypes.

While the hybrid index is useful for characterizing hybridized populations, it cannot distinguish individuals that are heterozygous at all loci from individuals that are homozygous for alternative alleles. For example, an individual with a hybrid score of 3 could either be heterozygous at all loci or homozygous for WCT at one locus, homozygous for RBT at another locus, and heterozygous for both species at the third locus. Individual classification is commonly

used to characterize hybridized populations (Ostberg and Rodriguez 2006). Therefore, individual classification was also performed based upon composite nuclear and mtDNA genotypes. Genotypes were classified as WCT-like if they were homozygous for WCT at all loci, RBT-like if they were homozygous for RBT at all loci, and hybrid if they possessed a mixture of alleles from the two parental species. Hybrid genotypes were further classified into first-generation (F_1) hybrids and later-generation (F_n) hybrids. The F_1 hybrid genotypes were heterozygous for both RBT and westslope trout at all loci, and F_n hybrid genotypes were those that possessed a mix of heterozygous and homozygous loci or mtDNA of one species and nuclear loci representing the other species. Since we were interested in distinguishing recent versus historic hybridization events, we chose to amplify two additional co-dominant loci for the genotypes identified as F_1 hybrids to reduce the probability of mistaking an F_n genotype for an F_1 genotype (Table 2). The *OCC-42* and *OCC-38* markers were amplified by following the methodology of Ostberg and Rodriguez (2004).

Tests for Hardy–Weinberg equilibrium and linkage equilibrium were performed to determine whether each sample location consisted of a single randomly mating population, indicating either a hybrid swarm, the presence of only parental genotypes from one species, or recent mating between RBT and WCT. Fisher's exact tests for Hardy–Weinberg equilibrium were performed using GENEPOP (Raymond and Rousset 1995), and genotypic disequilibrium between all pairs of loci was estimated using GENEPOP. A sequential Bonferroni adjustment ($\alpha = 0.05$) was used to account for multiple simultaneous tests.

The level of hybridization at each sample location was reported in two ways: (1) the number and type of hybrid genotypes (F_1 , F_n) and parental (WCT-like, RBT-like) genotypes and (2) the percentage of RBT introgression, calculated as the number of RBT alleles observed out of the total number of *Oncorhynchus* spp. alleles examined in either a randomly mating population (excluding F_1 genotypes) or a nonrandomly mating population (excluding F_1 genotypes and RBT-like genotypes). Hardy–Weinberg results were used to determine whether a sample location contained a single randomly mating population. Upper and lower confidence bounds were calculated for introgression levels using the equation for proportions (Newcombe 1998).

Allelic cytonuclear disequilibrium (cD) and genotypic cD (cD_1 , cD_2 , cD_3) were calculated for all locations in which hybrid genotypes were identified; for these calculations, we used the formulas of

Asmussen et al. (1987):

$$\begin{aligned} cD_1 &= \text{freq}(R/r) - [\text{freq}(R) \times \text{freq}(r)], \\ cD_2 &= \text{freq}(H/r) - [\text{freq}(H) \times \text{freq}(r)], \\ cD_3 &= \text{freq}(W/r) - [\text{freq}(W) \times \text{freq}(r)], \end{aligned}$$

and

$$cD = cD_1 + 0.5cD_2,$$

where R = the RBT nuclear genotype, r = the RBT mtDNA haplotype, H = the hybrid nuclear genotype, and W = the WCT nuclear genotype.

This parameter was used to test the association between nuclear genotypes and mtDNA haplotypes and infer directionality of mating. A positive cD_1 value indicates that RBT mtDNA was associated with RBT nuclear alleles more often than random expectations, a positive cD_2 value indicates that RBT mtDNA was associated with nuclear heterozygotes more often than expected, a negative cD_2 value indicates that WCT mtDNA was associated with nuclear heterozygotes more often than expected, and a negative cD_3 value indicates that WCT mtDNA was associated with WCT nuclear alleles more often than expected (Asmussen et al. 1987). Averages across loci were reported in instances where cytonuclear values were consistent across loci.

We also examined spatial patterns in the distribution of hybrids to determine whether hybrids were spreading throughout the drainage from locations downstream of stocked headwater lakes. A distance matrix of fluvial distance between all pairs of sample locations was constructed along with a hybrid matrix based upon the presence or absence of hybrids in each pair of sample locations. Fluvial distance was measured in kilometers following stream networks for each pair of sampling locations using a program written for ArcView 3.2. The presence of hybrids was coded 1 if both sample locations contained hybrids and 0 if neither or only one sample location within the pair contained hybrids. Autocorrelation between fluvial distance and the presence of hybrids was investigated with a Mantel test (Mantel 1967) using the add-in STATXL for Microsoft Excel. We also used a contingency table to test for differential introgression (selection) across the three loci by comparing genotypic frequencies of WCT (BB), hybrids (AB), and RBT (AA).

Results

The mtDNA marker, *Cytb* digested with *HaeIII*, yielded a polymorphism within 78 of the 81 golden trout samples analyzed, which had not been identified previously in RBT (Mays 2001; Table 2). While this marker was only partially fixed (polymorphism

appeared in 96% of the samples), the presence of the polymorphism was used to assess hybridization with golden trout. None of the fish displayed the golden trout mtDNA haplotype in this study.

Hybrid genotypes were detected in four of the five sample locations with a prior headwater lake stocking history (Table 1), and introgression levels ranged from 0% to 55%. Yellowstone cutthroat trout mtDNA was detected in three fish from Roaring Creek, but no WCT–RBT hybrid genotypes were detected in this stream. No hybrid genotypes were detected in Wilson Creek. All of the sample locations below stocked headwater lakes, except that of Ship Island Creek, had less than 20% introgression, although the upper confidence bounds for Papoose Creek exceeded 20%. Fisher's exact tests of Hardy–Weinberg equilibrium rejected the null hypothesis of random mating at all sites examined except Cache and Ship Island creeks (Table 1). Linkage equilibrium was rejected for Papoose and Cache creeks (Table 1), and F_1 hybrid genotypes were detected in both of these sample locations.

Fish with hybrid genotypes were also detected within 9 of the 12 sample locations with no prior history of headwater lake stocking (Table 1). Fish with only WCT-like genotypes were detected in three sample locations (upper Indian, Little Indian, and Garden creeks; Figure 2). The hybrid index illustrated that both WCT-like and RBT-like genotypes were present in seven of the sample locations where hybrid genotypes were detected (Figure 2). However, one parental type was dominant in the majority of the streams. Rainbow trout-like genotypes were not detected in two of the sample locations at which hybrid genotypes were identified (upper Marble and Big Cottonwood creeks). All of the unstocked sample locations had less than 10% introgression, although the upper confidence bounds for Soldier Creek exceeded 20% (Table 1).

Fisher's exact tests of Hardy–Weinberg equilibrium rejected the null hypothesis of random mating at all of the unstocked headwater lake sample locations except upper Indian, Little Indian, upper Elkhorn, and Garden creeks (Table 1). Linkage equilibrium was rejected for upper Elkhorn, lower Elkhorn, Soldier, upper Marble, middle Marble, and Big Cottonwood creeks (Table 1). Genotypes indicative of F_1 hybrids were detected in all of the sample locations with significant linkage disequilibrium.

Cytonuclear associations are useful in determining directionality and randomness of mating (Table 3). In upper Elkhorn and Cache creeks, hybridization without introgression was observed because only genotypes indicative of F_1 hybrids were detected. The cytonuclear signatures indicated nonrandom mating in all locations

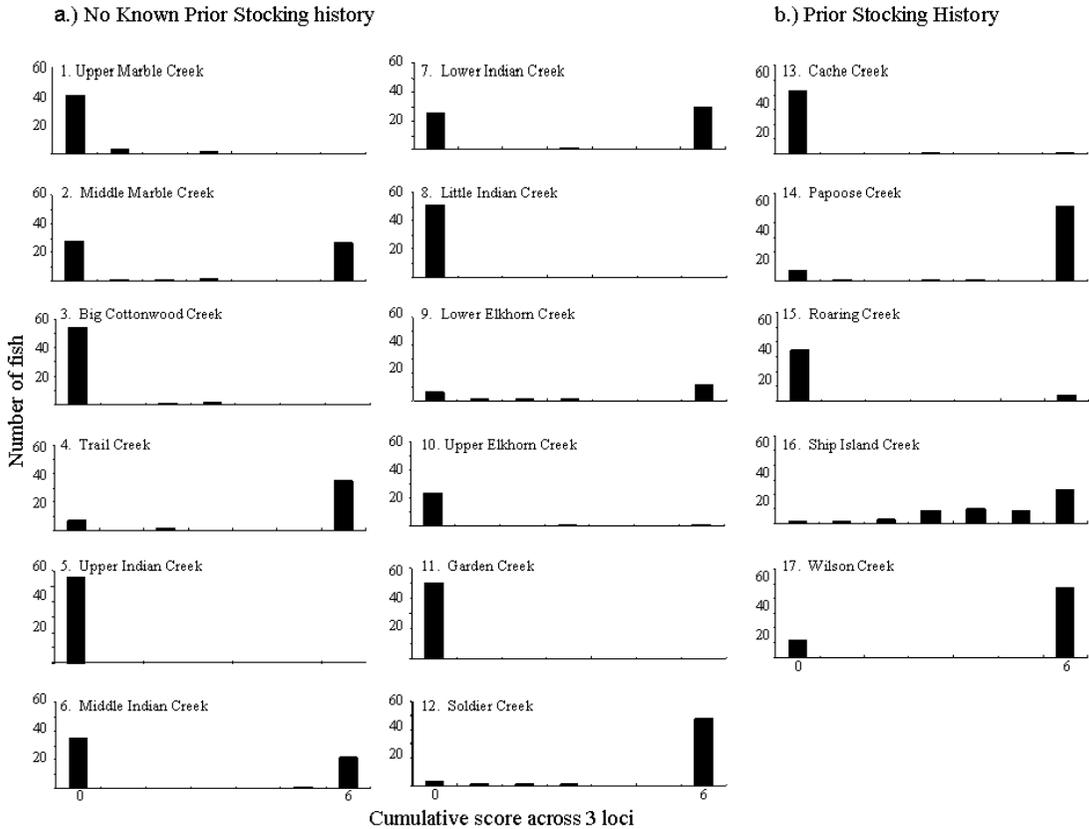


FIGURE 2.—Hybrid zone structure of Middle Fork Salmon River (Idaho) westslope cutthroat trout (WCT) and rainbow trout (RBT) in (a) 12 populations with no known history of stocking (1–12) and (b) five populations with a history of stocking (13–17). Values on the x-axis represent the total number of RBT alleles, ranging from 0 (pure WCT) to 6 (pure RBT), as cumulatively scored across three loci.

except upper Marble and Big Cottonwood creeks. In the other sample locations, introgression was detected, as were strong assortative mating and sex-based directionality to mating. All of the locations had positive cD_1 values, indicating a strong association between RBT mtDNA and RBT nuclear DNA and a tendency for RBT to mate with one another. All of the locations, with two exceptions, had a 0 or negative cD_2 value, which suggests an association between WCT mtDNA and nuclear hybrid genotypes. This indicates asymmetric mating and a tendency for hybrids to mate with WCT in preference to RBT. The two exceptions were Ship Island and middle Indian creeks. Both of these sample locations had positive cD_2 values, suggesting a tendency for hybrids to mate with RBT. The majority of the sample locations also displayed a 0 or negative cD_3 value, indicating a strong association between WCT mtDNA and WCT nuclear DNA and tendency for WCT to mate with one another. The

TABLE 3.—Allelic (cD) and genotypic cytonuclear disequilibria (cD_1 , cD_2 , cD_3) results averaged over all three loci for 12 hybridized populations within the Middle Fork Salmon River drainage, Idaho. The abbreviation cD_1 refers to the association between rainbow trout mitochondrial haplotype and rainbow trout nuclear alleles, cD_2 refers to the association between each parental type and heterozygous loci, and cD_3 refers to the association between the cutthroat trout haplotype and cutthroat trout nuclear alleles.

Population	cD_a	cD_2	cD_3	cD
Big Cottonwood Creek	0.00	0.00	0.00	0.00
Cache Creek	0.02	0.00	-0.02	0.02
Lower Elkhorn Creek	0.22	-0.01	-0.21	0.22
Upper Elkhorn Creek	0.04	0.00	-0.04	0.04
Middle main-stem Indian Creek	0.23	0.01	-0.24	0.24
Lower main-stem Indian Creek	0.25	0.00	-0.24	0.25
Middle main-stem Indian Creek	0.23	-0.003	-0.22	0.24
Upper main-stem Marble Creek	0.00	0.00	0.00	0.00
Papoose Creek	0.13	-0.03	-0.10	0.12
Soldier Creek	0.08	-0.02	-0.07	0.07
Ship Island Creek	0.23	0.04	0.05	0.10
Trail Creek	0.15	0.00	-0.15	0.15

TABLE 4.—Pooled genotypic frequencies across the 17 westslope cutthroat trout populations examined in the Middle Fork Salmon River drainage, Idaho; AA = rainbow trout genotypes, AB = hybrid genotypes, BB = westslope cutthroat trout genotypes.

Locus	Genotype		
	AA	AB	BB
Rag3	266	25	538
Occ16	262	29	537
Omm13	260	29	538

exception was Ship Island Creek, where introgression into both parental species was detected.

Directionality to mating was also inferred based on mtDNA. The hybrid genotypes had a greater proportion of WCT mtDNA than RBT mtDNA. Of the F_1 hybrids identified, 79% had WCT mtDNA. The distribution of hybrid genotypes also indicated that later-generation hybrids with WCT mtDNA were more common (detected in eight populations) than later-generation hybrids with RBT mtDNA (detected in two populations).

No significant autocorrelation was detected between fluvial distance and the presence of hybrids ($r = -0.112$; $P = 0.09$) within the drainage, although low levels of hybridization were found in the majority of sample locations (Figure 2). There was also no difference among genotypic frequencies at each locus (Table 4; $\chi^2 = 0.46$, $P = 0.97$).

Discussion

Our results provided evidence for hybridization within populations of WCT and RBT–steelhead in the MFSR drainage. The management of hybridized populations depends on the causes of hybridization (natural or anthropogenic) and the trajectory of the hybridization event. Therefore, the objectives of this study were not only to document the extent of hybridization within the MFSR drainage but to attempt to categorize hybridization. The sample locations could be divided into four different groups: (1) hybridized locations below stocked headwater lakes, (2) non-hybridized locations below stocked headwater lakes, (3) hybridized locations below unstocked headwater lakes, and (4) nonhybridized locations below unstocked headwater lakes. Of the 17 sample locations analyzed in this study, four (24%) were categorized into the first group, one (6%) was categorized into the second group, nine (53%) were categorized into the third group, and three (17%) were categorized into the fourth group. We formulated the following predictions that should be fulfilled if stocking of nonnative fish is the primary cause for hybridization. First, the majority

of the sample locations would fall into the first and last groups, as stocking would primarily influence locations directly downstream of the headwater lakes unless hybrids are spreading throughout the drainage. Second, the highest levels of hybridization would be found in locations below stocked headwater lakes. Third, spatial patterns of linkage disequilibrium would indicate that recent hybridization is occurring directly downstream of the headwater lake introductions due to the influx of nonnative trout at these locations. These predictions were based upon the assumption that spatial and temporal differences in spawning behavior have evolved for naturally sympatric RBT and WCT and that hatchery fish diminish these differences. While some of our predictions were met, we found that the patterns of hybridization in this drainage were complex and could arise from stocking, natural causes, or both.

Individual classifications were used to describe the genotype of individuals but may not necessarily reflect the true ancestry of each individual. Since only three nuclear markers were used, this type of classification could result in an overestimate of pure parental types and an underestimate of F_n hybrids, as some of the genotypes classified as F_1 hybrids or parental types could actually be F_n hybrids. The probability of mistaking an F_2 hybrid genotype for an F_1 hybrid genotype is 12% when three co-dominant nuclear markers are used (Boecklen and Howard 1997). Since we were interested in distinguishing between recent and historic hybridization events, we chose to amplify two additional loci for the genotypes identified as F_1 hybrids to reduce the potential misclassification of these hybrid genotypes. The addition of two more nuclear markers reduces the probability of mistaking an F_2 hybrid genotype for an F_1 hybrid genotype to less than 5%. If the individuals are more advanced backcrosses ($>F_2$), the probability of mistaking them for an F_1 hybrid with five loci is less than 1%. Since we did not amplify additional loci for the other genotypes, we may have missed some hybrid genotypes. Therefore, caution must be taken regarding the ancestry of individual fish.

Hybrid genotypes were found in four of the five streams below stocked headwater lakes. However, contrary to our second prediction, only Ship Island Creek had higher levels of hybridization than the sample locations below unstocked headwater lakes. In Roaring Creek, Yellowstone cutthroat trout were stocked in the high mountain lakes at the headwaters of the stream, and Yellowstone cutthroat trout mtDNA was detected in three of the fish. Since our nuclear markers were not diagnostic between WCT and Yellowstone cutthroat trout, we were unable to determine the extent of Yellowstone cutthroat trout

introgression in Roaring Creek. Rainbow trout–WCT hybrids were detected in Cache and Papoose creeks. However, only one F_1 hybrid was detected within Cache Creek, and few hybrids ($n = 3$) were detected within Papoose Creek despite a prior stocking history. Following our prediction, significant linkage disequilibrium was evident in Cache and Papoose creeks, indicating recent hybridization at these two locations.

Ship Island Creek had the highest reported levels of introgressive hybridization (48%) in this study. Although tests for Hardy–Weinberg and linkage equilibrium failed to reject random mating within this population, the cytonuclear signature (Avisé 2001) did not indicate that it was a hybrid swarm due to the presence of RBT-like genotypes. However, the architecture of the hybrid zone suggests that there has been a complete breakdown of reproductive isolating mechanisms in this system. This could be due to the stocking of nonnative fish in its headwater lakes or natural habitat disturbances, as both factors may act similarly in disrupting reproductive isolating mechanisms. Additional samples are needed to determine whether this breakdown will result in a stable hybrid zone, reemergence of reproductive isolating mechanisms, or complete genetic introgression (Betts et al. 2005).

The presence of low levels of hybridization in the majority of the streams appeared to be independent of whether the sample locations were directly downstream of headwater lake introductions. No hybrids were detected in Wilson Creek and only hybrids with Yellowstone cutthroat trout mtDNA were found in Roaring Creek despite prior stocking of nonnative RBT in their headwater lakes. Furthermore, hybrids were found in nine locations below headwater lakes with no known history of stocking. Significant linkage disequilibrium was also evident in six of these nine locations where hybrids were detected. This could be the result of recent hybridization among WCT and RBT parental types or straying of F_1 hybrids (or nonnative trout) into these locations. Currently, no known markers are available to discriminate between hatchery and native trout; such markers are needed to determine conclusively whether the hybrids in these streams are the progeny of native or introduced trout. In the absence of diagnostic markers, we evaluated stocking data and spatial distribution of hybrids to determine the extent to which nonnative trout or hybrids could be impacting the streams below unstocked headwater lakes.

The consequence of hybridization after nonnative introductions is dependent upon the invasion success of the nonnative taxa below the headwater lakes, which largely depends upon the magnitude, timing, and frequency of the introductions (Moyle and Light 1996). In the MFSR drainage, the magnitude and

frequency of introductions have been low due to the drainage's inaccessibility. On average, 750 fry were stocked in unequal intervals ranging from about 2 to 4 years (Table A.1). The most recent incidence of RBT stocking occurred in 1986, and the most recent incidence of WCT stocking occurred in 2000 (Table A.1). The detection of significant linkage disequilibrium and F_1 hybrids in the majority of the sample locations indicated a recent hybridization event. The effects of nonnative RBT on these recent hybridizing events are probably minimal unless there are self-reproducing populations of hatchery RBT (which invaded the drainage in the late 1980s) or straying of hatchery adult steelhead (McMichael and Pearsons 2001). Hatchery origin WCT would be more likely to act as invaders, as they were stocked more recently. The ability of these fish to invade locations downstream of the introductions and successfully reproduce is dependent upon survival to sexual maturity and available rearing and spawning habitat.

The degree to which nonnative trout have impacted the unstocked headwater lake locations is further dependent upon the dispersal capabilities of the nonnative trout or hybrid progeny. Previous studies investigating hybridization between WCT and nonnative RBT have indicated that straying of hybrids can expand hybridization and impact unstocked locations (Hitt et al. 2003; Rubidge and Taylor 2005). This observation was based upon the following results: (1) hybridized populations were found in closer proximity to one another than pure localities, (2) low levels of F_1 individuals relative to F_n individuals, and (3) decreasing levels of hybridization with increased distance from the current source of nonnative RBT introductions (Hitt et al. 2003; Rubidge and Taylor 2005). We did not detect a pattern of hybrid straying in our study. Hybridized localities were not found in closer proximity to one another than pure localities. Rather, hybrids were randomly distributed throughout the drainage. We also observed a relatively equal distribution of F_1 and F_n fish in all of the sample locations except Ship Island Creek. This suggested that F_1 hybrids have recently appeared in multiple locations throughout the drainage. Lastly, if Ship Island Creek was the source for straying hybrids, then we would expect introgression to be highest in close proximity to, and decrease with distance from, Ship Island Creek. A regression analysis was performed, and no relationship was detected between the level of introgression and distance from this location ($R^2 = 0.01$). Therefore, hybrid straying was not correlated with the spatial distribution of hybrids within the MFSR drainage. This possibility cannot be ruled out entirely, as we did not sample all of the MFSR drainage and there could be long-distance

migrations of hybrid fish. Additional information regarding population genetic structure among the sampled locations may provide insights into the degree of gene flow among populations and potential for migration. However, it seems less likely that all of the F_1 hybrids or nonnative trout moved into these streams from nearby hybridized populations, and this is evidence of independent events of ongoing hybridization.

In all but one creek, mating with hybrids and RBT has not caused a loss of genetic identity in WCT, despite ongoing hybridization. This is in contrast to the hybrid swarms that have been reported for native WCT and introduced RBT that evolved in allopatry (Rubidge et al. 2001; Hitt et al. 2003). All populations, except Ship Island Creek, displayed a bimodal hybrid index that was characterized by a high frequency of parental types and few hybrids. Hardy–Weinberg equilibrium proportions also indicated that the majority of the streams consisted of nonrandomly mating populations of RBT and WCT. This suggests strong assortative mating is occurring within these streams to allow the species to remain discrete after contact (Jiggins and Mallet 2000). The bimodal structure of the hybrid zones suggests either strong prezygotic isolation and weak selection or postzygotic selection against hybrid genotypes (Jiggins and Mallet 2000). Our observation of few advanced generation hybrids could be reflective of selection against certain hybrid crosses (Arnold 1997). However, there were instances when advanced generation hybrids were detected in our study as well as others (Rubidge and Taylor 2004, 2005). The superior performance of hybrids under certain laboratory conditions (Ferguson et al. 1985; Leary et al. 1995; Allendorf et al. 2004) indicates that postzygotic selection is probably weak and that prezygotic isolation may be more important in areas of sympatry. Jiggins and Mallett (2000) indicated that prezygotic isolation probably plays an important role in structuring bimodal hybrid zones.

Several factors may be involved in prezygotic isolation of WCT and RBT. Strong spatial and temporal reproductive segregation has been reported for RBT and cutthroat trout, which probably limits contact and subsequent hybridization events. Rainbow trout generally spawn in lower stream reaches, while WCT are restricted to the headwaters (Hanson 1977). Steelhead also prefer larger substrate for spawning. Segregation by elevation may also be prominent, where spawning WCT utilize higher-elevation tributaries (Bozek and Hubert 1992; Magee et al. 1996). Rainbow trout generally spawn earlier than cutthroat trout as well (Likens and Graham 1988; Henderson et al. 2000). Our data suggest that in sympatry, these prezygotic

mechanisms likely play an important role in limiting introgression between WCT and RBT–steelhead. The demonstration of low levels of hybridization in many streams, however, indicates that prezygotic reproductive isolation is somewhat incomplete.

The patterns of cytonuclear disequilibrium indicate that the initial hybridization events (F_1 hybrids) were more often the result of a female cutthroat trout mating with a male RBT–steelhead, although it occurred in both directions. Size differences between sexes can constrain hybridization events and lead to unidirectional hybridization or promote sneaking behavior (Taylor 2004). The higher-order hybrids also displayed directionality or strong asymmetric mating. Westslope cutthroat trout mtDNA was strongly associated with heterozygous alleles in all of the streams, suggesting that mating was more often the result of an F_1 hybrid mating with a WCT. This could be due to selection against some hybrid crosses or asymmetric abundance of species within the streams. The hybrid index indicates that a predominant parental type was present in the majority of the streams. In the exceptions where later-generation hybrids had RBT mtDNA (middle Indian, Papoose, and Ship Island creeks), RBT were the predominant parental type. Asymmetric mating is commonly observed when naturally sympatric salmonids hybridize (Redenbach and Taylor 2003; Ostberg and Rodriguez 2004; Baumsteiger et al. 2005), while symmetric mating is often observed as a result of secondary contact between formerly allopatric salmonids (Ostberg and Rodriguez 2006).

While stocking may have had some impact within the MFSR drainage, the patterns of hybridization in the majority of the sample locations (especially those below unstocked lakes) were also consistent with recent, natural hybridization events. This is based upon the following observations commonly reported for naturally hybridizing taxa: (1) low levels of introgression and F_n individuals where the two taxa co-occur, (2) asymmetric mating, (3) bimodal hybrid zone structure, and (4) strong assortative mating (Redenbach and Taylor 2003; Ostberg et al. 2004). Natural hybridization has also been documented in the Big Creek drainage, Idaho (Peterson et al. 2004). Natural hybridization has historically occurred within other portions of the range of WCT (Leary et al. 1987; Brown et al. 2004) and has shaped the evolution of many sympatric salmonids (see Taylor 2004). Brown et al. (2004) and Leary et al. (1987) observed mtDNA of WCT in a steelhead population in the Tuccannon River, where there has been no documented stocking of WCT. Redenbach and Taylor (2002) documented natural hybridization within Dolly Varden *Salvelinus malma* and bull trout *S. confluentus*, and Bernatchez et

al. (1995) detected mtDNA of Arctic char *S. alpinus* in allopatric populations of brook trout *S. fontinalis*. Natural hybridization has also been extensively reported among sympatric populations of coastal cutthroat trout and RBT–steelhead (Campton 1987; Wenburg and Bentzen 2001; Young et al. 2001; Baumsteiger et al. 2005; Bettles et al. 2005). Our results indicate that natural hybridization may also be occurring between naturally sympatric WCT and RBT–steelhead. We believe that this hypothesis should be accorded equal consideration given its historical documentation in WCT and recent documentation in other sympatric salmonids.

Conservation and Management Implications

Hybridization with nonnative trout, such as RBT and Yellowstone cutthroat trout, was considered a genetic threat to some WCT populations during the subspecies' most recent status review (Costello and Rubidge 2004; Shepard et al. 2005), initiating an ongoing scientific and policy dispute regarding the appropriateness of including hybrids within ESA listing deliberations for WCT (USFWS 2003; Allendorf et al. 2004; Allendorf et al. 2005; Campton and Kaeding 2005). Currently, any WCT population that maintains the phenotypic characteristics of the species and has less than 20% of its genes derived from other taxa is eligible for ESA listing consideration (USFWS 2003). However, concerns over genomic extinctions have led to an alternate endorsement of a 0% policy (Allendorf et al. 2004, 2005) and new litigation. Currently, the USFWS does not make a distinction between natural or anthropogenically caused introgression in classifying populations under the ESA. Under the current 20% policy, all of our sample locations, except Ship Island Creek, would be eligible for listing consideration. If confidence bounds are required in hybridization assessments, then Papoose and Soldier creeks would also be excluded. A 0% policy would mean that the majority of populations within the MFSR drainage would be excluded from protection under the ESA if listing occurred (including those reported in Peterson et al. 2004).

An effective conservation and management policy must consider risks from alternative strategies (type A and type B errors). Type A error may result in the protection of a hybridized population that is of little conservation value (e.g., hybrid swarm), while type B error may result in failure to protect a hybridized population that is of conservation value (e.g., natural introgression). Each of these errors has significant consequences. Type A error could result in wasted valuable resources and protection of populations that threaten other populations, while type B errors could

result in an eradication of potentially unique genetic variation or increased fragmentation due to the protection of small, isolated populations. The 0% policy (Allendorf et al. 2004) is based upon the dangers of type A error, while the 20% policy (Campton and Kaeding 2005) is based upon the dangers of type B error. We recognize that both of these risks are of importance, and we suggest that a flexible hybrid policy is needed to minimize both errors. A policy that recognizes naturally hybridizing populations is needed when evaluating species status, since natural hybridization may be part of the evolution of the species in areas of sympatry. This may result in separate policies for hybridized populations existing in sympatric versus allopatric zones.

Our study highlights the difficulty in distinguishing between natural and anthropogenic causes when some stocking has occurred throughout the sympatric range of WCT and RBT. In the absence of diagnostic markers, conservation and management decisions could be made using information gained from this study and others (Rubidge and Taylor 2004; Ostberg and Rodriguez 2006), such as hybrid zone structure or genetic monitoring for increasing introgression levels (Rubidge and Taylor 2005). Eligibility for protection within a more flexible policy would not rely on an acceptable level of introgression (e.g., 0% or 20%) but would require genetic monitoring to determine the architecture of the hybrid zone and determine whether the observed event will result in (1) a stable hybrid zone, (2) reemergence of reproductive isolating mechanisms, (3) complete genetic introgression, and (4) invasion of nonhybridized populations. Maintenance of the maximum amount of genetic variation is central to this policy and would result in the protection of hybridized populations if there are no perceived risks. While we were not able to conclusively determine the causes of hybridization in this drainage, our results indicated that reproductive isolating mechanisms were disrupted in only one location and that all of the populations except that of Ship Island Creek still maintain conservation value. We recommend genetic monitoring in the next generation to confirm the stability of the hybrid zones prior to protective management actions.

Allendorf et al. (2001) indicated that the lack of a hybrid policy under the ESA probably results from the difficulty in writing one policy that would apply to all types of species and situations. Whereas Allendorf et al. (2001) referred to the application of one hybrid policy to many species, we suggest that one policy may not even apply for all types of hybrid zones within a single species, such as the WCT. Managers and policy makers should carefully consider the risks of applying

one static policy to a multifaceted and dynamic phenomenon like introgressive hybridization and should acknowledge the complexity of hybridization.

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Appendix: Stocking of trout species in the Middle Fork Salmon River drainage, Idaho

TABLE A.1.—Headwater lakes where stocking has occurred, sampled streams that they flow into, species, and dates of the stocking events.

Sample location	Lake	Species ^a	Dates
Roaring Creek	Roaring Creek Lake 1	ct	1955, 1965
	Roaring Creek Lake 2	ct	1955, 1965
	McGuire Lake	ct	1968, 1971, 1974, 1977
Ship Island Creek	Ship Island Lake	ct	1937
		rbt	1937
	Ship Island Lake 2	wct	1992, 1998
		gt	1969, 1970
	Ship Island Lake 4	yct	1986
	Airplane Lake	wct	1989, 1992, 1996, 1998, 2001
		rbt	1963
		ct	1937
	Shoban Lake	wct	1963, 1977, 1983, 1986, 1989, 1992, 1996, 1998
	Sheapeater Lake	wct	1989, 1992, 1996, 1998, 2001
Wilson Creek	Buck Lake	yct	1963, 1977
		wct	1986, 1989, 1992, 1998
		yct	1983
	Harbor Lake	rxc	1980
		wct	1986, 1989, 1992, 1998
		rxc	1980
		rbt	1951, 1959, 1964, 1974, 1977, 1983
		ct	1968, 1971
	Heart Lake	wct	1989, 1992, 1996, 1998, 2001
		rbt	1959, 1974, 1977, 1983, 1986
		rxc	1980
	Parragon Lake	ct	1968, 1971
		wct	1989, 1992, 1996, 1998
		yct	1983, 1986
	Ramshorn Lake	rxc	1980
		wct	1989, 1992, 1996, 1998, 2001
		yct	1983, 1986
Welcome Lake	rxc	1980	
	wct	1989, 1992, 1996, 1998, 2001	
	rbt	1964, 1974, 1977, 1983	
	rxc	1980	
	ct	1968, 1971	
Wilson Lake	rbt	1964	
	wct	1989, 1992, 1996, 1998, 2001	
	yct	1983, 1986	
	rxc	1980	
	ct	1968, 1971, 1974, 1977	
Papoose Creek	Papoose Creek Lake	wct	1985, 1998, 1991, 1994, 2000
		yct	1978
		ct	1975, 1982
		gt	1977
	Papoose Creek Lake 2	ct	1975, 1978

TABLE A.1.—Continued.

Sample location	Lake	Species ^a	Dates
Cache Creek	Cache Creek Lake 1	wct	1990, 1996, 1999
		yct	1984
		rxc	1981
	Cache Creek Lake 2	ct	1968, 1971, 1974, 1977
		rxc	1981
		ct	1968, 1971, 1974, 1977
	Cache Creek Lake 3	wct	1987, 1990, 1996, 1999
		yct	1984
		rxc	1981
	Cache Creek Lake 4	ct	1968, 1954, 1971, 1974, 1977
		ct	1954, 1959, 1968, 1971, 1974
		wct	1987
	Cache Creek Lake 5	yct	1984
		rxc	1981
	Cache Creek Lake 6	ct	1971, 1974, 1977
		rbr	1968
		ct	1968, 1971, 1974
	Cache Creek Lake 7	ct	1968, 1971, 1974

^a ct = unspecified cutthroat trout, wct = westslope cutthroat trout, rbr = rainbow trout, yct = Yellowstone cutthroat trout, gt = golden trout, rxc = rainbow × cutthroat trout.