

Identifying Introgressive Hybridization in Native Populations of California Golden Trout Based on Molecular Markers

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Abstract.—The California golden trout *Oncorhynchus mykiss aguabonita* is one of three subspecies within the rainbow trout–redband trout complex endemic to the Kern River basin and historically restricted to Golden Trout Creek and the South Fork Kern River. Past allozyme studies have indicated that native populations of California golden trout in the Golden Trout Creek drainage may have become introgressed with rainbow trout alleles through interaction with hybrids of rainbow trout and California golden trout stocked into nearby headwater lakes that are connected to tributaries in the drainage. We used six microsatellites and a minisatellite marker to estimate the genetic diversity and levels of introgression in approximately 700 California golden trout taken from 23 locations in Golden Trout Creek, its tributaries, and surrounding lakes. Indications of introgression were found in all but one of the sampled Golden Trout Creek drainage locations, the lowest average levels (0–8%) occurring in the lower reaches of Golden Trout Creek. The highest levels (12–17%) were in the Cottonwood Lakes populations that have been used as California golden trout broodstock by the California Department of Fish and Game. Evidence of introgression was also found in fish sampled from the upper reaches of the South Fork Kern River. This suggests that past and present stocking policies and hybridization with introduced rainbow trout currently threaten the genetic integrity of California golden trout populations across all of their native range.

The California golden trout *Oncorhynchus mykiss aguabonita* is one of three native subspecies of rainbow trout found in the Kern River basin of California's southern Sierra Nevada range (Moyle 2002), the other two being the Little Kern golden trout *O. mykiss whitei* and the Kern River rainbow trout *O. mykiss gilberti*. Currently classified as an inland subspecies of rainbow trout, the California golden trout and Little Kern Golden trout share distinction as California's state fish. A species of special concern according to the California Department of Fish and Game (CDFG), California golden trout recently underwent a status review for listing as an endangered species (USFWS 2002).

Prehistoric distributions of California golden trout (CGT) were probably limited to the South Fork Kern River and Golden Trout Creek (Figure 1), a tributary of the Kern River that, before geologic activity within the last 5,000–10,000 years,

emptied into the South Fork Kern River (Moore and Lamphere 1983). Since then, isolation between populations of CGT in Golden Trout Creek and Kern River rainbow trout has been maintained by a natural barrier: a 57.1-m waterfall where Golden Trout Creek empties into the Kern River.

Transplantation of CGT outside of their native waters began at least as early as the latter half of the 19th century. Establishment of populations in the originally fishless Cottonwood Lakes (Figure 1) has been traced back to a pre-1876 introduction of fish from Golden Trout Creek (E. Ober, California Department of Fish and Game, field correspondence, 1935; but see Evermann 1906 for another possible source) to fishless Mulkey Creek, a tributary of the South Fork Kern River. Twelve fish from Mulkey Creek were subsequently transplanted into Cottonwood Creek in 1876, and 21 fish from Cottonwood Creek were used to establish the Cottonwood Lakes populations in 1891 (Evermann 1906).

Beginning in 1918, the Cottonwood Lakes fish served as the source population for fingerling CGT planted in waters throughout California and a number of other states. Adult CGT were trapped and artificially spawned at Cottonwood Lakes, and the fertilized eggs were packed to nearby Mt. Whitney Hatchery, where they were hatched and grown out

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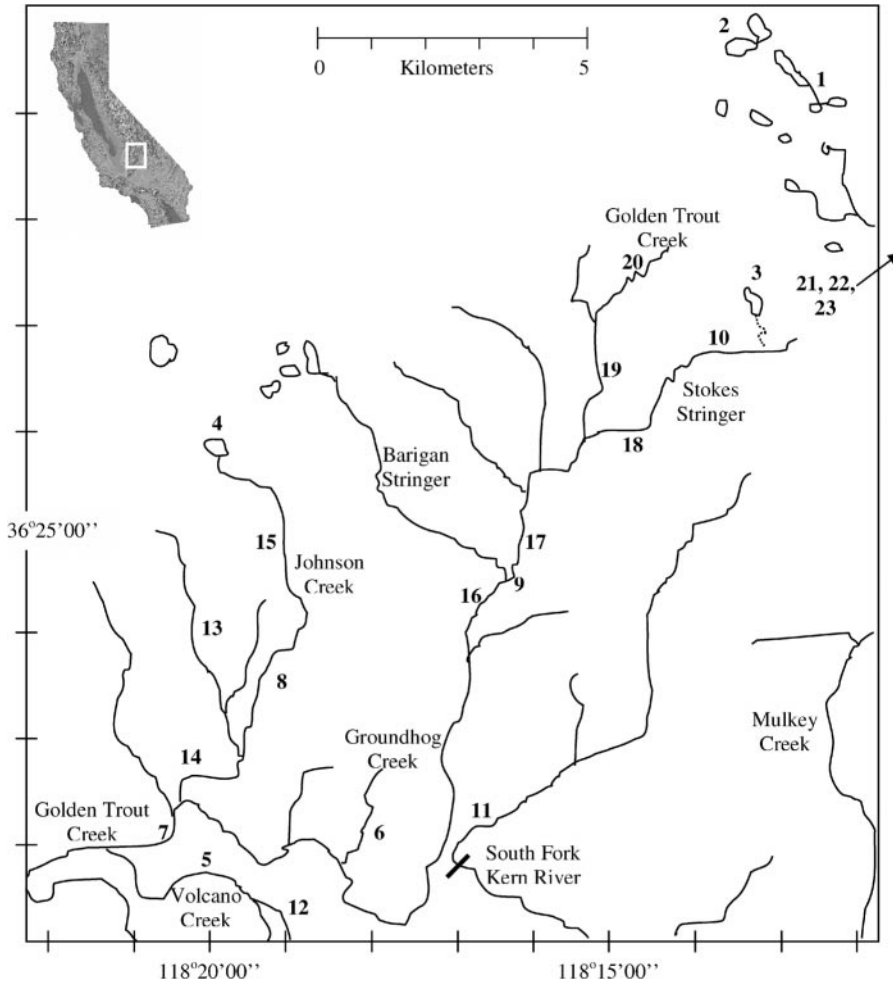


FIGURE 1.—Location of sample sites used to examine introgressive hybridization in native populations of California golden trout. Numbers correspond to the location numbers given in Table 1. Sample sites 21, 22, and 23 are part of the Cottonwood Creek system that drains from the Cottonwood Lakes (not pictured).

to fingerling size (Stan Stephens, CDFG, unpublished report). In the early 1930s, hatchery workers began to notice changes in the appearance of Cottonwood Lakes golden trout, raising concern about the purity of the broodstock. Because returning unused CGT fingerlings to Cottonwood Lakes was routinely practiced starting as early as 1923, it is believed that rainbow trout fingerlings also raised at the hatchery may have inadvertently been stocked into the lakes as well (Stan Stephens, CDFG, personal communication). Although rainbow trout have never been planted by the CDFG in Golden Trout Creek, it was feared that its headwater lakes had become introgressed through planting with hybridized CGT from the Cottonwood Lakes broodstock.

Unpublished evidence based on protein allozyme analyses (R. F. Leary, Division of Biological Sciences, University of Montana, Missoula, Montana, USA, in a letter to CDFG) concluded that the Cottonwood Creek and Cottonwood Lakes populations of CGT had become introgressed with alleles from rainbow trout. Due to the hybridization of the Cottonwood Lakes populations, most, if not all, of the CGT populations transplanted in California, Wyoming, Idaho, and elsewhere over the past 75 years are most likely introgressed with rainbow trout DNA. In addition, evidence suggests large portions of the CGT populations in the South Fork Kern River are also introgressed (Gall and May 1997; Bagley et al. 1998, 1999; Cordes et al. 2000). Consequently, one of the most widely trans-

planted western trout has become a candidate for listing as endangered, because the distribution of nonhybridized fish may now be restricted to the Golden Trout Creek watershed and the extreme headwaters of the South Fork Kern River, an area representing only 36% of its former range (Stephens, unpublished). Unfortunately, evidence of introgression based on allozymes (R.F. Leary, unpublished data) was also found in a number of headwater lakes in the Golden Trout Creek watershed that had been planted with fish from Cottonwood Lakes throughout most of the 20th century. Currently there is concern that native CGT populations in Golden Trout Creek are becoming introgressed with rainbow trout alleles infiltrating from these headwater lakes via their outlet streams.

Molecular markers have been used extensively in recent years to investigate hybridization and introgression between introduced and native western American salmonids (Carmichael et al. 1993; Rubidge et al. 2001; Spruell et al. 2001; Baker et al. 2002; Campbell et al. 2002; Ostberg and Rodriguez 2004), including members of the rainbow trout-redband trout complex (Nielsen et al. 1999). The goal of the present study was to use nuclear microsatellite and minisatellite markers to investigate the genetic status of CGT populations in Golden Trout Creek, its tributaries, and the surrounding lakes thought to contain hybridized fish. More specifically, the objectives were to (1) compare presumably nonhybridized populations of CGT from lower Golden Trout Creek with hybridized fish from the Cottonwood Lakes to identify possible rainbow trout alleles in the drainage, (2) survey other lakes in the region with connections to Golden Trout Creek for evidence of rainbow trout hybridization, and (3) survey fish in the upper reaches of Golden Trout Creek and its tributaries for evidence that rainbow trout may alleles have infiltrated these populations.

Methods

Sample collections.—Tissue samples were collected in the summers of 1999 and 2000 by California Department of Fish and Game (CDFG) and the U.S. Forest Service (USFS) personnel. Samples were collected from various locations on Golden Trout Creek, its tributaries, a number of surrounding lakes (including Cottonwood Lakes), the South Fork Kern River, and the Cottonwood Creek drainage (Table 1; Figure 1). Samples from the various populations used in the microsatellite analysis were grouped into four categories: (1) hybridized lake populations (Johnson Lake, Chicken

Springs Lake, Cottonwood Lakes Number 2, and Cottonwood Lakes Number 4), (2) presumably nonhybridized CGT populations (Volcano Creek, Little Whitney Meadow, and Groundhog Creek), (3) creek populations threatened by introgression (Middle Johnson Creek, Mouth of Barigan Stringer, and Upper Stokes Stringer), and (4) a presumably nonhybridized CGT population from above the Ramshaw barrier on the South Fork Kern River. Built in 1973 to eliminate predation of CGT by introduced brown trout *Salmo trutta*, the barrier is believed to have protected CGT in the headwaters of the South Fork Kern River from introgression with rainbow trout stocked further downstream (Stephens, unpublished). Additional samples from creek populations threatened by introgression used in the minisatellite analysis are listed in Table 1 and shown in Figure 1.

Samples consisted of fin clips from adult CGT either dried or preserved in DMSO storage buffer (20% DMSO, 0.25 M EDTA, NaCl to saturation, pH 7.8) and stored at room temperature. For development of the minisatellite locus, we also used wild rainbow trout fin clip samples stored in DMSO buffer collected from the North Fork American River, dried fin clip samples collected from Putah Creek by the CDFG, and farmed rainbow trout fin clip samples provided by Clear Springs Food, Inc. (Buhl, Idaho) and preserved in ethanol.

Genetic analyses.—Whole genomic DNA was extracted from samples using the Qiagen DNeasy Tissue Kit. Microsatellite loci (Table 2) originally developed in Chinook salmon *Oncorhynchus tshawytscha* (Williamson et al. 2001) and previously shown in this laboratory to be polymorphic in various lineages of rainbow trout were amplified from the isolated DNA samples via the polymerase chain reaction (PCR). Amplifications of all microsatellite loci were carried out in 10- μ L reactions containing 5.15 μ L sterile dH₂O, 1.0 μ L 10 \times PCR buffer, 0.40 μ L of 50-mM MgCl₂, 0.80 μ L of 2.5-mM deoxynucleotide triphosphate (dNTP) mixture, 0.2 μ L of 1- μ M forward primer labeled with one of three fluorescent dyes (Table 2), 0.40 μ L of 10- μ M unlabeled reverse primer, 0.05 μ L of *Taq* I polymerase (0.25 U total), and 2.0 μ L of DNA (approximately 50 ng DNA total). Samples were first denatured for 5 min at 95°C, followed by 30–35 cycles of PCR amplification performed under the following conditions: 1 min at 95°C, 1 min at 52°C, and 1 min at 72°C. The PCR product alleles were separated electrophoretically on a 5.5% polyacrylamide gel using the MJ Research

TABLE 1.—Sample locations and codes, sample sizes (*N*), years, and collecting agencies for California golden trout and rainbow trout samples. Location numbers correspond to those in Figure 1. Abbreviations include GTC for Golden Trout Creek, SFK for South Fork Kern River, CDFG for California Department of Fish and Game, and USFS for U.S. Forest Service.

Sample groups	Location (code)	<i>N</i>	Year	Agency
Microsatellite and minisatellite analyses				
Hybridized lake	Cottonwood Lakes 1, 2, 3 (CL2)	32	2000	CDFG
	Cottonwood Lakes 4, 5 (CL4)	30	2000	CDFG
	Chicken Springs Lake (CSL)	31	2000	CDFG
	Johnson Lake (JL)	30	2000	CDFG
Presumably nonhybridized	Volcano Creek (VC)	32	2000	USFS
	Groundhog Creek (GC)	32	2000	USFS
	GTC at Little Whitney Meadow (LWM)	31	2000	USFS
Threatened by introgression	Middle Johnson Creek (MJC)	30	1999	CDFG
	Mouth of Barigan Stringer (MBS)	30	1999	CDFG
	Upper Stokes Stringer (USS)	30	1999	CDFG
South Fork Kern River	SFK above Ramshaw barrier (SFK)	28	1999	CDFG
Additional minisatellite analyses				
Additional locations threatened by introgression	Lower Volcano Creek (GTLV)	13	1996	CDFG
	Salt Lick Creek (SLC)	40	2000	USFS
	Lower Johnson Creek (LJC)	32	1999	CDFG
	Johnson Creek (JC)	24	2000	USFS
	GTC below Barigan Stringer (BBS)	29	1999	CDFG
	GTC below Stokes Stringer (BSS)	31	1999	CDFG
	Middle Stokes Stringer (MSS)	29	1999	CDFG
	GTC at Big Whitney Meadows (BWM)	40	2000	USFS
	GTC headwaters (HW)	30	1999	CDFG
	Horseshoe Creek (HC)	30	1999	CDFG
	Little Cottonwood Creek 1 (LCC1)	25	2000	CDFG
	Little Cottonwood Creek 2 (LCC2)	25	2000	CDFG
	Rainbow trout	Putah Creek (PC)	5	1999
Cold Springs Food, Inc. (CSF)		3	1998	
North Fork American River (NFAR)		22	2000	CDFG

TABLE 2.—Primer information for six microsatellite and one (*ACCCTG-6*) minisatellite locus used to investigate the introgression of rainbow trout alleles into California golden trout populations in the Golden Trout Creek drainage.

Locus	Primers (5'-3') ^a	Fluorophore ^b	Size (base pairs)	Repeat sequence	Reference
<i>Ots-G3</i>	F: GGACAGGAGCGTCTGCTAAATGACTG	FAM	146–242	(GAAT) ₈ N ₁₂ (GATA) ₁₁	Williamson et al. (2001)
	R: GGATGGATTGATGAATGGGTGGG				
<i>OtsG-85</i>	F: CCATGTCAGCACTGACTTAAT	FAM	151–287	N ₁₂ (GATA) ₂₆ (GATA) ₁₉	Williamson et al. (2001)
	R: GGATGTTGTTCTTAATGTTTT				
<i>Ots-G249b</i>	F: ATGGCAGTTAAGAGAACAAAAGTT	HEX	147–187	(TAGA) ₁₉	Williamson et al., unpublished ^c
<i>Ots-G423</i>	R: CCTACCCTTCTCATTCGAAGACTAA	TET	104–204	(GATA) ₄₁	Williamson et al. (2001)
	F: AGGCCTGCCAGGCACTAAAGGTAT				
<i>OMM-1082</i>	R: GCAAGCAAACATGTAGCTTCATGG	FAM	177–237	(GATA) ₁₇	Rexroad et al. (2002)
	F: CAAGAGCACTAACGACCATGT				
<i>OMM-1083</i>	R: CGCAAGCAAGCTAACACA	TET	137–469	(GATA) ₂₆	Rexroad et al. (2002)
	F: GCCCTGACCAACCTAACACA				
<i>ACCCTG-6</i>	R: TGTCTGACATTCGGTTAGTAGTGG		254–676	Table A.2	This study
	<i>MseC</i> : ATGCGGTGATCACGTTTC				
	<i>EcoA</i> : GTCTGTTGATACAGGAC				

^a F = forward, R = reverse.

^b Column entries are trademarked names used by Invitrogen Corp., Carlsbad, California.

^c K. S. Williamson, Department of Animal Science, University of California, Davis, unpublished data.

BaseStation gel analysis system (MJ Research, San Francisco, California). The Genescan 500 internal size standard (Applied Biosystems) labeled with a fourth fluorescent dye (ROX) was run in each lane. Alleles were scored using Cartographer software from MJ Research.

A minisatellite locus was developed by generating potential species-specific markers through amplified fragment length polymorphism (AFLP) analysis (Tranah et al. 2003). Briefly, DNA from rainbow trout and CGT samples was digested with the restriction enzymes *EcoRI* and *Mse I*, and adapters to the *Mse I* and *EcoRI* restriction sites were then ligated to the ends of the DNA fragments. The adapters were designed to have a final 3' nucleotide that is different from the original sequence, so the restriction site was destroyed by the ligation of the adapter sequences. The fragments were then preamplified via the polymerase chain reaction using primers complementary to the adapter sequences and designed with an additional selective nucleotide overhanging the 3' end of the adapters (e.g., *Eco ANN + Mse CNN*). Fragments were then amplified using various combinations of more specific primers with two or three selective nucleotides (e.g., *Eco ACC + Mse CGT*). After amplification, the products were run out on a 5% acrylamide denaturing gel. Amplified bands that appeared fixed and unique to a single subgroup (either golden trout or rainbow trout) were cut out of the denaturing gels, reamplified using non-labeled primers under preamplification PCR conditions, purified using the Qiagen Qiaquick PCR purification kit, and sequenced using BigDye Dye Terminator chemistry from Perkin Elmer/ABI. The sequences generated from these fragments were used to design new primers to specifically amplify the potentially informative fragments.

Primers developed from the sequenced AFLP fragments described above were used to amplify DNA from a panel of individuals of both the rainbow trout and CGT subgroups. The PCR conditions for these amplifications were 94°C for 2 min followed by steps of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s for 30 cycles, and a final extension step of 72°C for 5 min after cycling. The PCR products were then run on a 5% polyacrylamide denaturing gel for 1.5 h at 35 W and scanned with a Molecular Dynamics 595 FluorImager. A single locus (*ACCCTG-6*; Table 2) exhibited a definitive allele size difference between the subgroups and was used to investigate the genetic character of various CGT populations sampled in the Golden Trout Creek watershed and surrounding lakes (Ta-

ble 1; Figure 1). Amplifications of the *ACCCTG-6* locus in CGT and rainbow trout individuals were performed under the conditions previously described. Fragments were run on a 5% polyacrylamide gel, and individual alleles were excised from the gel. We eluted DNA from the acrylamide fragments with 200 μ L TLE (1M Tris-HCL, 0.5M EDTA, pH 8.0), reamplified using the same PCR protocol and sequenced as described above. Allele sequences were obtained for CGT from Golden Trout Creek, the headwater lakes, and the South Fork Kern River above Ramshaw barrier; sequences were obtained for rainbow trout from the North Fork American River.

Statistical analyses.—California golden trout population genetic characteristics for all loci, including number of alleles, allele frequencies, observed and expected heterozygosities, and deviations from Hardy-Weinberg equilibrium, were estimated using Genes in Populations 2.2 (Krueger and May 1995). Tests of gametic disequilibria involving the microsatellite loci were performed using GENEPOP (Raymond & Rousset 1995; available at http://wbiomed.curtin.edu.au/genepop/genepop_op1.html). The Arlequin 1.1 software program of Schneider et al. (1997) was used to evaluate patterns of genetic diversity and divergence within and between populations based on the analysis of molecular variance (AMOVA) of Excoffier et al. (1992), which generates *F*-statistics analogous to the θ -values of Weir and Cockerham (1984). Significance of *F*-statistics was evaluated using exact *F* permutation procedures (Excoffier et al. 1992). Type I error was controlled for all multiple testing using the sequential Bonferroni method of Rice (1989).

Genetic relationships among samples based on the microsatellite data were examined using the software package PHYLIP 3.5c (Felsenstein 1995). Cavalli-Sforza and Edwards's (1967) chord distances (D_{CE}) were calculated among samples using the GENDIST program and plotted as a neighbor-joining (NJ) phenogram (Saitou and Nei 1987) using NEIGHBOR. The original allele frequency matrix was then resampled 1,000 times using BOOTSTRAP and the chord distances among samples were estimated for each resulting matrix. A consensus NJ phenogram was generated using CONSENSE, and all bootstrap values were plotted onto the phenogram from the original sample matrix to indicate stability of the nodes. Principal components analysis (PCA) using PCA-GEN (Goudet 1999) was also used to examine genetic relationships among samples. This involves the linear transformation of observed allele frequen-

TABLE 3.—Observed heterozygosities at six microsatellite loci for 11 populations of California golden trout. Asterisks denote significant deviations from Hardy–Weinberg equilibrium after Bonferroni correction for multiple tests (initial $\alpha = 0.001$). Water bodies included in the analysis are Volcano Creek (VC), Groundhog Creek (GC), Golden Trout Creek at Little Whitney Meadow (LWM), Johnson Lake (JL), Chicken Springs Lake (CSL), Cottonwood Lake 2 (CL2), Cottonwood Lake 4 (CL4), Middle Johnson Creek (MJC), Golden Trout Creek at Mouth of Barigan Stringer (MBS), Upper Stokes Stringer (USS), and South Fork Kern River (SFK).

Population	Loci						Average
	<i>Ots-G3</i>	<i>Ots-G85</i>	<i>Ots-G249b</i>	<i>Ots-G423</i>	<i>OMM-1082</i>	<i>OMM-1083</i>	
VC	0	0.90	0.61	0.56*	0.57	0.81	0.58
GC	0	0.65	0.50	0.44*	0.44	0.74	0.46
LWM	0.03	0.88	0.50	0.35*	0.40	0.61	0.46
JL	0.07	0.83	0.81	0.80	0.22	0.79	0.59
CSL	0.36	0	0.77	0.74	0.10	0.52	0.42
CL2	0.03	0.68	0.78	0.71	0.26	0.77	0.54
CL4	0.13	0.60	0.67	0.72	0.40	0.86	0.66
MJC	0	0.81	0.46	0.18*	0.53	0.89	0.48
MBS	0	0.73	0.55	0.32*	0.61	0.76	0.50
USS	0	0.59	0.52	0.45*	0.57	0.93	0.51
SFK	0	0.45*	0.91	0.40*	0.80	0.77	0.56

cies such that differences among groups of samples are plotted in a two-dimensional space by maximizing the variation of the transformed data measured along each axis (component).

Results

Microsatellite Loci

Six microsatellite loci were amplified in the 11 population samples listed in Table 1. All microsatellite loci were polymorphic in all samples except *Ots-G3*, which was polymorphic in the four lake samples and monomorphic in all of the Golden Trout Creek and South Fork Kern River samples (except Golden Trout Creek at Little Whitney Meadow). Allele frequency distributions for each population and locus are given in the appendix (Table A.1); observed heterozygosities (H_o) and test results for Hardy–Weinberg equilibrium are provided in Table 3. The number of alleles per locus ranged from three (*Ots-G3*) to 35 (*OMM-1083*), and H_o averaged for each location over all loci ranged from 0.42 (Chicken Springs Lake) to 0.66 (Cottonwood Lakes Number 4). A cursory comparison of microsatellite allele distributions revealed a total of 38 alleles across six loci (average 5.2 alleles/locus) found in the presumably nonhybridized creek samples that were not present in any of the lake samples (Table A.1).

Genotype distributions for five of the six microsatellite loci did not differ significantly from Hardy–Weinberg expectations at any of the collection locations after correcting for multiple tests (the exception was *Ots-G85* for the South Fork Kern River sample). In contrast, the *Ots-G423* locus exhibited significant deviations from Hardy–

Weinberg expectations due to an excess of homozygotes for all of the Golden Trout Creek and South Fork Kern River samples but not for any of the lake samples. Calculation of genetic disequilibrium for the six loci and 11 samples (66 pairwise comparisons total) revealed a single departure from equilibrium in the Johnson Lake sample after correction for multiple testing ($k = 15$ tests per population, $\alpha = 0.00333$), as expected due solely to chance.

To test for population structuring, microsatellite loci were analyzed as a combined data set. The AMOVA tests were conducted by first grouping populations into the four categories outlined in the Methods section; a second test was then run by combining into a single group creek samples of nonhybridized fish and fish threatened by introgression (Table 4). Test results for both grouping scenarios revealed significant differences in genetic variation, both among groups (V_a) and among populations, within groups (V_b). Among group percent variation was larger and within group percent variation was smaller when all of the Golden trout creek samples were treated as a single group. Pairwise F_{ST} values for the combined-loci population ranged from less than 0.001 for many population pairs to 0.41 between Middle Johnson Creek and Chicken Springs Lake (Table 5). Exact F permutation tests revealed statistically significant pairwise F_{ST} values between Volcano Creek and all other populations except Little Whitney Meadow. Pairwise F_{ST} values were also statistically significant between all lake and presumably nonhybridized creek populations. Likewise, all F_{ST} values between the lake and creek populations threatened

TABLE 4.—Analysis of molecular variance (AMOVA) results for six microsatellite loci used to test population heterogeneity within and among groups of 11 California golden trout samples. In the first analysis, groups consisted of (1) presumably nonhybridized creek populations (Volcano Creek, Groundhog Creek, and Golden Trout Creek at Little Whitney Meadow) and creek populations threatened by introgression (Middle Johnson Creek, Mouth of Barigan Stringer, and Upper Stokes Stringer), (2) lake populations (Cottonwood Lake 2, Cottonwood Lake 4, Chicken Springs Lake, and Johnson Lake), and (3) South Fork Kern River above the Ramshaw barrier. In the second analysis, the first group was broken down into two presumed components, nonhybridized and threatened by introgression.

Source of variation	df	Sum of squares	Variance	% Variation	P-value
Based on three groups					
Among groups	2	73.918	0.15952	10.21	0.008
Among populations, groups	8	59.340	0.09927	6.35	<0.001
Within populations	661	861.629	1.30352	83.44	<0.001
Total	671	994.887	1.56231		
Based on four groups					
Among groups	3	73.628	0.10237	6.72	0.044
Among populations, groups	7	59.630	0.11715	7.69	<0.001
Within populations	661	861.629	1.30352	85.59	<0.001
Total	671	994.887	1.52305		

by introgression were statistically significant. Excluding Volcano Creek, all values between the presumably nonhybridized and creek populations threatened by introgression were nonsignificant. Pairwise F_{ST} values were significant between the South Fork Kern River population and all of the lakes, Volcano Creek in the nonhybridized group, and Middle Johnson Creek from the group of creek sites threatened by introgression. Within the lake group, Chicken Springs Lake proved significantly different from all the other samples, which did not differ significantly from each other. The only significant pairwise F_{ST} in the creek samples threatened by introgression was between Middle Johnson Creek and Golden Trout Creek at the Mouth of Barigan Stringer.

To estimate the degree and geographic extent of

rainbow trout introgression in the Golden Trout Creek drainage, we first identified those alleles present in the lake samples that were absent from the presumably nonhybridized Golden Trout Creek and South Fork Kern River populations. This yielded a total of 14 presumed rainbow trout alleles spread over five of the six microsatellite loci (Table A.1). Twelve of these alleles occurred in Cottonwood Lakes Number 2, nine in Cottonwood Lakes Number 4, six in Johnson Lake, and three in Chicken Springs Lake. Nine of these alleles have been found to occur in wild steelhead (anadromous rainbow trout) and various strains of hatchery rainbow trout (authors, unpublished data). To date, the remaining five alleles have not been found outside of the lake populations tested here. Frequencies (f) of the presumed rainbow trout alleles found in the

TABLE 5.—Population pairwise F_{ST} values for 11 populations of California golden trout based on six microsatellite loci. Asterisks denote significant values after Bonferroni correction for multiple tests (initial $\alpha = 0.001$).

Population ^a	Population										
	Nonhybridized			Hybridized lakes				Threatened creeks			
	VC	GC	LWM	JL	CSL	CL2	CL4	MJC	MBS	USS	SFK
VC											
GC	0.05*										
LWM	0.01	<0.01									
JL	0.20*	0.15*	0.15*								
CSL	0.36*	0.37*	0.38*	0.28*							
CL2	0.18*	0.12*	0.09*	0.01	0.24*						
CL4	0.19*	0.12*	0.09*	<0.01	0.25*	0.01					
MJC	0.08*	0.02	-0.02	0.20*	0.41*	0.19*	0.20*				
MBS	0.08*	-0.01	-0.04	0.12*	0.32*	0.11*	0.13*	0.05*			
USS	0.06*	-0.05	-0.07	0.14*	0.33*	0.12*	0.15*	0.03	0.02		
SFK	0.04*	0.01	-0.02	0.05*	0.27*	0.02*	0.04*	0.10*	0.02	0.03	

^a Population codes for sample locations are presented in Table 3.

TABLE 6.—Frequency estimates of putative rainbow trout alleles in samples of California golden trout from the four lake and three creek populations threatened by introgression (Table 1), based on six microsatellite loci. Frequencies of rainbow trout alleles at the *ACCCTG-6* minisatellite locus are given for comparison.

Population ^a	<i>Ots-G3</i>	<i>Ots-G85</i>	<i>Ots-G249b</i>	<i>Ots-G423</i>	<i>OMM-1082</i>	<i>OMM-1083</i>	Average	<i>ACCCTG-6</i>
JL				0.38		0.16	0.10	
CSL	0.18			0.44	0.02	0.11	0.12	
CL2		0.10		0.38	0.02	0.22	0.12	0.11
CL4	0.03	0.13		0.54		0.33	0.17	0.11
MJC				0.02			<0.01	
MBS		0.10		0.29	0.02	0.08	0.08	
USS				0.07		0.02	0.02	
Lake samples							0.13	0.06
Threatened creek samples							0.03	0

^a Population codes for sample locations are presented in Table 3.

lake samples ranged from 0.02 to 0.54. Frequency estimates of introgressed alleles per locus in the lakes (Table 6) ranged from an average of 0.10 (Johnson Lake) to 0.17 (Cottonwood Lake No. 4). For the six loci analyzed, 175 of the 1,382 scored alleles (13%) in the four lake samples were presumed to be rainbow trout alleles. Of the 31 fish from Cottonwood Lake Number 4 (the sample with the highest level of introgression), 26 carried at least one presumed rainbow trout allele; they averaged 1.9 out of 12 alleles per fish surveyed.

Six of the 14 presumed rainbow trout alleles were found in one or more of the three creek sites threatened by introgression (Table A.1): one in Middle Johnson Creek, three in Upper Stokes Stringer, and five at the Mouth of Barigan Stringer. Frequencies of each of these presumably introgressed alleles were generally low (Table A.1) and usually represented only one or two instances in a geographic sample, except for the *Ots-G423* 136-base-pair (bp) allele ($f = 0.29$) and the *Ots-G85* 207-bp allele ($f = 0.10$) found in the Mouth of Barigan Stringer sample. Frequencies of individual alleles of presumed rainbow trout found in the creek samples threatened by introgression ranged from 0.02 to 0.29. Frequency estimates of introgressed alleles per locus in the creek samples threatened by introgression (Table 6) ranged from an average of less than 0.01 (Middle Johnson Creek) to 0.08 (Mouth of Barigan Stringer). For the six loci analyzed, 34 of the 999 scored alleles (3%) in the three creek samples threatened by introgression were presumed rainbow trout alleles; 28 of these were found in the sample taken from the Mouth of Barigan Stringer, and 17 of those were the *Ots-G423* 136-bp allele. Of the 30 fish from the Mouth of Barigan Stringer (the creek sample with the highest level of introgression), 11 carried at least one presumed rainbow trout allele,

and they averaged 0.8 out of 12 alleles per fish surveyed. Levels of presumed rainbow trout alleles were much lower in the other two creek samples threatened by introgression. In the upper Stokes Stringer sample, three fish carried a single introgressed allele, and a single fish carried two. In the sample from middle Johnson Creek, only a single introgressed allele was found in the 30 fish analyzed.

The NJ tree based on D_{CE} illustrates the relationships among the four groupings of samples (Figure 2). The lake samples formed a distinct cluster, and the Golden Trout Creek samples another; the South Fork Kern River sample was intermediate. The Mouth of Barigan Stringer also appeared intermediate between other GTC samples and the South Fork Kern sample. Within the lake cluster, Chicken Springs Lake was the most divergent of the samples. Bootstrap support of the branchings was moderate to high (44–100%), all but the lowest value being greater than 50%.

The results of the PCA closely mirrored the topology of the NJ tree (Figure 3). The first and second component accounted for 51.4% ($P = 0.001$) and 21.8% ($P = 0.018$) of the total variance, respectively. Two main groups were revealed, one composed of the lake samples and one of the Golden Trout Creek samples, the South Fork Kern River sample being intermediate between them. The introgressed Mouth of Barigan Stringer sample occupied an intermediate position between the Golden Trout Creek and South Fork Kern River samples. Chicken Springs Lake was highly divergent from the remaining cluster of lake samples.

Minisatellite Locus

To provide an independent estimate of hybridization levels that could be compared with the results from the microsatellite analysis, we devel-

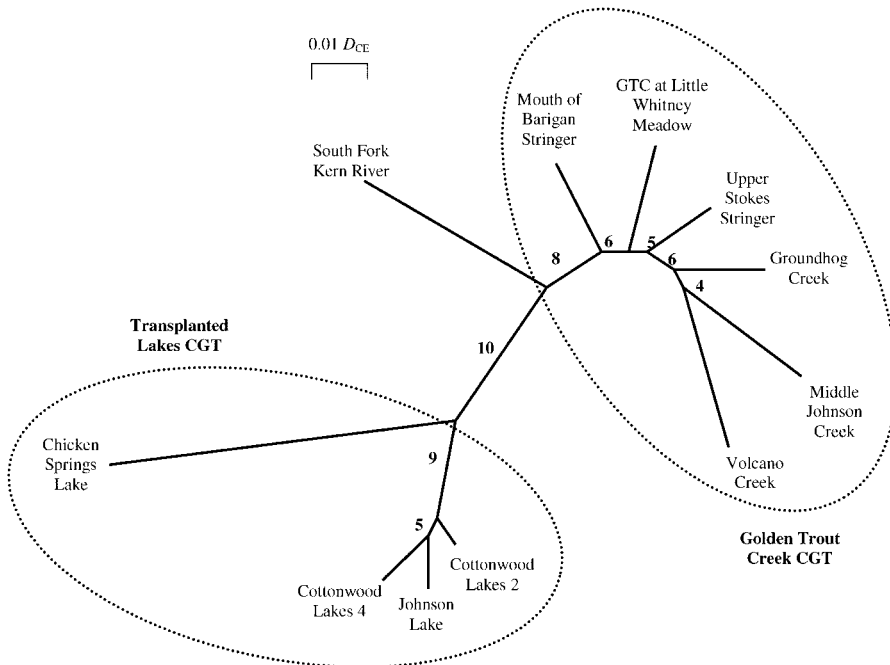


FIGURE 2.—Neighbor-joining tree based on Cavalli-Sforza and Edward's chord distance (D_{CE}) for 11 California golden trout samples. Presumably genetically nonhybridized creek samples include Volcano Creek, Groundhog Creek, and Golden Trout Creek at Little Whitney Meadow. Lake samples include Johnson Lake, Chicken Springs Lake, Cottonwood Lake 2, and Cottonwood Lake 4. Creek populations threatened by introgression with rainbow trout allele introgression include Middle Johnson Creek, Golden Trout Creek, Golden Trout Creek at the Mouth of Barigan Stringer, and Upper Stokes Stringer. Bootstrap values at the nodes indicate the percentage of times that populations beyond the node grouped together based on 1,000 bootstrap iterations.

oped an anonymous single-copy nuclear marker diagnostic between CGT and rainbow trout. We used 64 AFLP primer combinations to examine variation between rainbow trout and CGT subgroups. From these 64 combinations, 87 potentially informative fragments were cut out and sequenced. From these sequences, primers to amplify 23 novel loci were developed. A single locus (*ACCCTG-6*) revealed disjunct allele size distributions between CGT and rainbow trout. Rainbow trout screened for this locus had alleles ranging in size from 252 to 399 bp ($N = 7$ alleles), whereas CGT exhibited a range of 636–676 bp ($N = 3$ alleles).

Seven of the 10 allele samples from rainbow trout in North Fork American River and California golden trout in Golden Trout Creek and South Fork Kern River were sequenced to better identify the genetic characters of these subgroups. Sequencing of representative alleles revealed that the locus contained a complex minisatellite made up of variable repeat units that ranged in size from 15 to 18 bp, some repeat units exhibiting point muta-

tions and insertion–deletion events in their sequence. Alleles could thus be characterized by the number and type of repeat units contained within their minisatellite region (Table A.2). The CGT type alleles were all found to be closely related but distinct from the rainbow-type alleles. The rainbow-type alleles 399 and 297 found in the Golden Trout Creek samples (Golden Trout Creek below Stokes Stringer, Golden Trout Creek below Barigan Stringer, Groundhog Creek, and Golden Trout Creek at Little Whitney Meadow), and the alleles 297 and 267 found in the Cottonwood Lakes (Cottonwood Lakes 2, Cottonwood Lakes 4), were identical in sequence to the same-sized alleles in the rainbow trout reference sample. Variation among alleles was due almost exclusively to the insertion or deletion of repeat units, rather than changes in flanking region sequences. The locus was subsequently amplified in 23 population samples of CGT, including the 11 from the microsatellite analysis (Table 1).

Allele frequencies for the entire sample set are listed in Table A.3. Allele 651, the most common

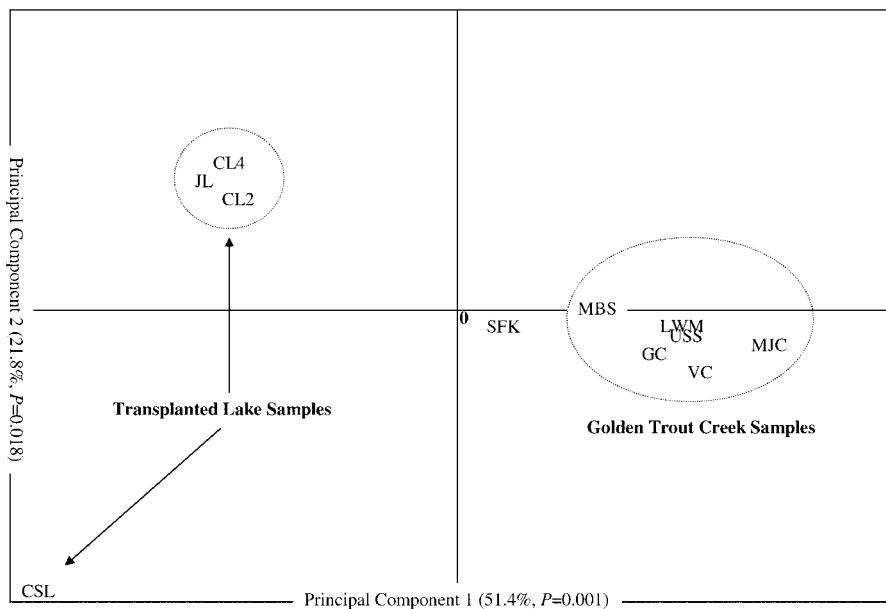


FIGURE 3.—Principal components analysis of six California golden trout and steelhead/rainbow trout samples. Creeks included in the analysis are Volcano Creek (VC), Groundhog Creek (GC), Golden Trout Creek at Little Whitney Meadow (LWM), Johnson Lake (JL), Chicken Springs Lake (CSL), Cottonwood Lake 2 (CL2), Cottonwood Lake 4 (CL4), Middle Johnson Creek (MJC), Golden Trout Creek at Mouth of Barigan Stringer (MBS), Upper Stokes Stringer (USS), and South Fork Kern River (SFK). Percentages indicate the amount of the total variance explained by the first and second principal components.

allele in the Golden Trout Creek drainage (average $f = 0.50$), was absent from all of the lake samples. All of the lake samples showed marked shifts in allele frequencies compared with Golden Trout Creek populations (Table A.3). A total of four different rainbow-type alleles were found in Golden Trout Creek, South Fork Kern River, and lake samples. Neither the Johnson Lake nor Chicken Springs Lake samples contained rainbow-type alleles, whereas the two Cottonwood Lakes samples (Cottonwood Lakes Nos. 2 and 4) exhibited relatively high frequencies of these alleles (average $f = 0.11$), which was similar to that estimated by the microsatellite data ($f = 0.15$). Six fish in the Cottonwood Lake 2 sample and 10 in the Cottonwood Lake 4 sample carried a single rainbow-type allele. A single fish in the Cottonwood Lake 4 sample contained two rainbow-type alleles.

Five fish from the Golden Trout Creek drainage carried a single allele that fell within the rainbow trout size range. Single instances of rainbow-type alleles occurred in Groundhog Creek and Golden Trout Creek at Little Whitney Meadow (samples that had been presumed to be taken from nonhybridized CGT populations in the microsatellite analysis). One fish with a single rainbow-type al-

lele was also found at Golden Trout Creek below Stokes Stringer, and two were found in the sample from below Barigan Stringer (but none at the Mouth of Barigan Stringer or from Upper Stokes Stringer, contrary to the microsatellite data). A single fish with two rainbow trout type alleles was also found below Barigan Stringer. No rainbow trout alleles were found in Volcano Creek or in the Little Cottonwood Creek and Horseshoe Meadow Creek samples. Three rainbow type alleles in three fish were found in the South Fork Kern River sample. Overall, the frequency of rainbow trout alleles in the 15 Golden Trout Creek samples included in the *ACCTG-6* locus analysis was 0.01 (including all of the presumably nonhybridized and creek samples threatened by introgression from the microsatellite analysis). The frequency of rainbow trout alleles in the South Fork Kern River sample was 0.071.

Discussion

Microsatellite Data

Given the small number of fish used to establish Cottonwood Lakes, one would expect reduced genetic variation in these, as well as the Johnson

Lake and Chicken Springs Lake populations, which were founded from the Cottonwood Lakes stocks. (Other introductions of California golden trout into the headwater lakes may have occurred. Between 1929 and 1943, approximately 300 adult California golden trout were collected annually from Golden Trout Creek in the vicinity of Big Whitney Meadows and transported to Rocky Basin Lakes and possibly Johnson Lake, where they were stocked by Mt. Whitney District Ranger, Everest Shellenbarger, in 1952. Volunteers working with U.S. Department of Agriculture personnel captured 581 California golden trout from Golden Trout Creek and stocked them in Rocky Basin Lake, according to Stan Stephens, CDFG fishery management files, Bishop, California). Such reduction is evident in the large number of alleles found in the presumably nonhybridized CGT populations but not found in any of the lakes. However, reduction in CGT polymorphism is not reflected in average observed heterozygosities (H_o). The Cottonwood Lake 2 sample had the highest H_o in the study, and values for the other lake samples (excluding Chicken Springs Lake) fell within the range exhibited by the South Fork Kern River, nonhybridized creek, and creek samples threatened by introgression. These results indicate that the drop in genetic variation expected in populations founded from a small number of fish has been masked by the introduction of rainbow trout alleles. Similarly, Carmichael et al. (1993) showed elevated allozyme polymorphism in hybridized populations of Apache trout *O. apache* compared with nonhybridized sites believed to have undergone repeated bottlenecks. Chicken Springs Lake, in contrast, although showing evidence of introgressed rainbow trout alleles, still had the lowest H_o in the study.

The source of the five alleles found only in the lake populations remains elusive. Because they were not found in any of the Golden Trout Creek samples (whether presumably nonhybridized or threatened by introgression), it seems unlikely they are CGT alleles, although we cannot discount the possibility of rare alleles, undetected in Golden Trout Creek, making their way into the Cottonwood Lakes populations. To date the alleles have not been found in samples of steelhead from the Navarro River, rainbow trout from four hatcheries, or a wild population of rainbow trout from the North Fork American River (authors' unpublished data), but given the high levels of genetic diversity in both wild and domestic stocks of rainbow trout (Berg and Gall 1988; Bagley 1997; Bagley and

Gall 1998), this sampling may not be adequately exhaustive. Because these alleles did not occur at any of the creek sites threatened by introgression, they did not influence our estimates of nonnative alleles at these sites.

Hardy–Weinberg equilibrium is expected to be regained within one to a few generations in a relatively large, randomly mating population once outside input has ceased (Hartl and Clark 1997). Test results showed that samples did not differ significantly from Hardy–Weinberg expectations for five of the six loci tested. This indicates that the Cottonwood and Golden Trout Creek headwater lake populations have regained equilibrium since the presumed introduction of rainbow trout alleles. This is consistent with the fact that the initial hybridization event occurred sometime before the 1930s and no recent input of rainbow trout is suspected (Stephens, personal communication). It is difficult to ascertain whether Hardy–Weinberg equilibrium in the creek populations threatened by introgression is due to the regaining of equilibrium, as outlined above, or occurred because the input of rainbow trout alleles remains too small to have disrupted equilibrium. The *Ots-G423* locus differed significantly from Hardy–Weinberg equilibrium due to an excess of homozygotes in all of the South Fork Kern River, nonhybridized creek, and threatened by introgression creek samples but not in any of the lake samples. The simplest explanation for these results is the presence of a naturally occurring null allele in the South Fork Kern River and Golden Trout Creek populations that did not make it through the population bottleneck and into the Golden Trout Creek headwater lakes populations. Lu et al. (2001) also attributed heterozygote deficiency centered on a single locus to a probable null allele in a study of lake whitefish *Coregonus clupeaformis*. Berrebi et al. (2000) found departures from Hardy–Weinberg equilibrium due to hybridization in populations of marble trout *Salmo marmoratus*, but this seems unlikely in our case because only a single locus was involved. Another explanation that cannot be ruled out is that this locus is subject to selective pressure or is closely linked to one that is; however, this seems unlikely given that departures from equilibrium were exhibited only in a subset of geographically and environmentally proximate populations.

The AMOVA indicated significant differences in genetic variation both within and between the lake, nonhybridized creek, creek threatened by introgression, and South Fork Kern River groups.

Differences among groups were stronger and differences within groups were weaker when the non-hybridized fish and creek fish threatened by introgression were treated as a single group, suggesting that introgression has not yet led to significant genetic differentiation among populations within Golden Trout Creek and its tributaries. This is reflected in the lack of significant pairwise F_{ST} values between most of these samples (excluding Volcano Creek, as discussed below).

Significant pairwise F_{ST} values derived from selectively neutral markers can result from mutation, random drift of allele frequencies in isolated populations, or the introduction of new alleles (gene flow) into some populations but not others (Avice 2004). As expected for hybridized populations, each of the lake samples was significantly different from all other Golden Trout Creek and South Fork Kern River samples. In addition, Chicken Springs Lake was significantly different from the other three lake samples, a result consistent with a founder effect associated with establishing a population from relatively few individuals. With regard to the presumably nonhybridized creek samples, Volcano Creek differed significantly from all other samples in the study except Golden Trout Creek at Little Whitney Meadow. Why Volcano Creek differed from Groundhog Creek (the other presumably nonhybridized site) but not Little Whitney Meadow may be due to a number of factors, including the closer proximity of Volcano Creek to the Little Whitney Meadow site and the evidence for slightly more introgression of the Groundhog Creek sample suggested by the *ACCCGT-6* marker results. Leary (R.F. Leary, unpublished data, in a letter to the CDFG) also found Volcano Creek to be genetically distinct, based on allozyme markers, and did not recommend it as a source population for future restocking of CGT populations because of reduced genetic variability and shifts in allele frequencies compared with other Golden Trout Creek populations.

Two of the samples from creeks threatened by introgression (Middle Johnson Creek and Mouth of Barigan Stringer) did exhibit a significant pairwise F_{ST} ; this may be due to the differential introgression of various rainbow trout alleles into these populations. The relatively high frequency of two presumed rainbow trout alleles at the Mouth of Barigan Stringer suggests a relatively high gene flow rate of introgressed alleles out of Rocky Basin Lakes 1 (the largest of the lakes) into Barigan Stringer. Samples from the Rocky Basin lakes were not available for our analysis (the populations were

recently removed by gill netting; Stephens, personal communication), but based on allozyme electrophoresis data, Leary (R. F. Leary, unpublished data) found it to be hybridized.

The close relationship of all the presumably nonhybridized site and creek sites threatened by introgression compared with the lake samples is well illustrated in the graphic results from the NJ and PCA analyses (Figures 2, 3). The Mouth of Barigan Stringer sample, despite having the highest level of suspected introgression for any of the creek locations, remained widely separated from the heavily introgressed lakes, although its position could be seen as slightly intermediate between the two. This intermediacy was not reflected in the significance of population pairwise F_{ST} values discussed above—at least after use of the sequential Bonferroni correction. The lack of significant population pairwise F_{ST} values between the South Fork Kern River and Golden Trout Creek samples (excluding Volcano Creek and Middle Johnson Creek) contradicts the more clearly intermediate position the South Fork Kern River sample holds in the NJ and PCA analyses. The relatively high level of rainbow trout alleles in the South Fork Kern River sample indicated by the *ACCCTG-6* locus (discussed below) supports this intermediate position and suggests that CGT populations in the South Fork Kern River above the constructed Ramshaw Barrier are hybridized and should not be used as source stocks in restoration efforts of CGT populations. Similar examples of intermediacy in graphical ordination analyses have been documented in both interspecific (Young et al. 2001; Roques et al. 2001) and intraspecific (Berrebi et al. 2000; Hansen 2002) hybrid populations. To answer whether the intermediate position of the South Fork Kern River sample is due to introgression or genetic differentiation produced by historical isolation will require a more thorough examination of populations within the South Fork Kern drainage.

The levels of rainbow trout introgression reported here should be considered conservative. One difficulty in estimating the number of rainbow trout alleles in the Golden Trout Creek drainage and surrounding lakes is the impossibility of sampling the rainbow trout responsible for the original introduction of foreign alleles into the Cottonwood Lakes because the initial source of the Mt. Whitney hatchery stock is uncertain and the current stock has been modified since the 1930s (Stephens, personal communication). Instead, we had to assume that any alleles found in the lakes that were not

found in the presumably nonhybridized creek or South Fork Kern River samples had been introduced through introgression with rainbow trout. This assumption is not unreasonable because all the suspected nonnative alleles found in the Golden Trout Creek samples occur in rainbow trout populations. Furthermore, it is unlikely that rare CGT alleles not found in our nonhybridized CGT populations survived the repeated bottlenecks that led to the establishment of the Cottonwood Lakes populations. Nevertheless, consideration of the above would lead us to assume that the number of presumed rainbow trout alleles that we found in the lake sites and creek sites threatened by introgression is an upper estimate. Conversely, evidence from the *ACCCTG-6* locus (discussed below) indicates that a small number of rainbow trout alleles are present in some of the presumably nonhybridized sample locations (Groundhog Creek, Golden Trout Creek at Little Whitney Meadow, and South Fork Kern River above Ramshaw Barrier). This would lead to the misidentification of a small number of rainbow trout alleles as naturally occurring CGT alleles and would depress the number of introgressed alleles that we could identify. Both of these effects should be small; more important is the overlap in microsatellite allele size distributions between CGT and rainbow trout. This overlap means that we cannot identify all of the alleles in the Golden Trout Creek system that were derived from rainbow trout, which results in an underestimation of introduced nonnative alleles.

Minisatellite Locus

The results based on the *ACCCTG-6* locus highlight the importance of using multiple markers when trying to assess low levels of introgression in a natural system because, if only a single locus is used, random sampling error could easily result in missed introgressed alleles in a particular sample. The need for multiple markers becomes particularly important when trying to identify pure (anthropogenically uncorrupted) populations of threatened or endangered species (e.g., Cordes et al. 2004; Peacock and Kirchoff 2004; Wares et al. 2004), employ various individual assignment and admixture tests (e.g., Lu et al. 2001; Roques et al. 2001; Hansen 2002), assess introgression beyond the F_1 generation (e.g., Carmichael et al. 1993; Young et al. 2001, Campbell et al. 2002), or determine the direction of backcrossing and introgression (e.g., Dowling and Childs 1992; Rubidge et al. 2001). The complex minisatellite utilized in this study provides another nuclear marker, with

the added benefit of having diagnostic allele size ranges for determining subspecies identity.

The absence of the most common CGT minisatellite allele in the Golden Trout Creek samples suggests the lake populations went through a rather severe population bottleneck before the introduction of rainbow trout alleles into the system, a scenario supported by the microsatellite data. No evidence of introgression was seen in any of the three Johnson Creek samples, although one instance of a presumed rainbow trout allele did show up (microsatellite analysis) in the Middle Johnson Creek sample.

A single instance of a rainbow trout type allele was found in samples from Golden Trout Creek below Whitney Meadow and Groundhog Creek, whereas three such instances were found in the sample from the South Fork Kern River. This indicates, when analyzing the microsatellite data, that some introgression has occurred even in the samples that we had assumed to be nonhybridized. Sequencing of the *ACCCTG-6* locus showed that the golden trout and rainbow-type alleles are highly divergent, separated by both size polymorphisms and sequence variation in the minisatellite region (Table A.3). Due to the complexity of the repeat it is difficult to estimate the rate of change of the locus; however, the large size and sequence differences between the CGT and rainbow trout alleles indicate a long isolation between the two classes. Thus, it seems unlikely that the rainbow-type alleles found in the Golden Trout Creek and South Fork Kern River samples are ancestral alleles shared by the CGT and rainbow trout subgroups. We found no evidence of hybridization in our Little Cottonwood Creek or Horseshoe Creek samples, suggesting that introgression has not yet spread significantly through the rest of the Cottonwood Creek subbasin. These results are preliminary, however, because analyses of these additional samples (Table 1, Group B) are based solely on the *ACCGT-6* locus. A more thorough survey using multiple markers is needed to accurately map introgression in the Cottonwood Creek drainage.

Conclusions.—Results of this study indicate that nonhybridized populations of CGT within their historical range may be much rarer than previously believed. Nonnative genetic infiltration due to hybridization has been documented in a number of threatened or endangered native western trout, including Apache trout *O. apache* (Carmichael et al. 1993), Gila trout *O. gilae* (Wares et al. 2004), west-slope cutthroat trout *O. clarkii lewisii* (Rubidge et al. 2001), Yellowstone cutthroat trout *O. clarkii*

bouvierii (Campbell et al. 2002), and Lahontan cutthroat trout *O. clarkii henshawii* (Peacock and Kirchoff 2004). In general, our microsatellite results indicate that all of the CGT lake populations tested suffered reductions in genetic variability due to one or more population bottlenecks before being extensively introgressed with rainbow trout alleles introduced into the system. Based on the combination of loci employed here, it appears that subsequent movement and reproduction of introgressed fish from these lakes into the headwaters of the Golden Trout Creek drainage has resulted in the presence of rainbow trout alleles at low frequencies in all but one (Volcano Creek) of the Golden Trout Creek samples tested.

Rhymer and Simberloff (1996) drew attention to the issue of species extinction through hybridization, and more recently Allendorf et al. (2001) proposed a set of criteria for categorizing different levels of hybridization in native populations. Under their system, the CGT populations studied here display type-5 hybridization: widespread hybridization and introgression (in our case at relatively low frequencies) and an introduced species, though some nonhybridized populations still remain. Type-6 hybridization is characterized by admixture with no remaining nonhybridized populations. Given that only Volcano Creek has shown a complete absence of rainbow trout alleles to date, CGT may slip into type-6 hybridization in the near future, depending on management in the Golden Trout Creek watershed and the results of ongoing genetic assessments in the South Fork Kern River.

Currently no federally recognized policy on the role of hybridized populations in the conservation of threatened or endangered species exists (Allendorf et al. 2004), leading to controversy over management of native fish like the westslope cutthroat trout (Allendorf et al. 2004; Campton and Kaeding 2005). Restoration efforts in many native inland trout have focused on isolation of nonhybridized populations above constructed barriers (e.g., USFWS 1998), replication of all known nonhybridized lineages in suitable habitats (e.g., USFWS 2003), and restoration to native ranges via chemical treatment and subsequent restocking from nonhybridized stocks (e.g., USFWS 2004). In the past, chemical treatment and restocking used to restore populations of Little Kern golden trout within their native range have produced mixed results (Bagley et al. 1998, 1999). Although the relative isolation of Golden Trout Creek behind natural barriers would lend itself to such a strategy, until nonhybridized, genetically representative

CGT source populations can be identified, other options should be considered. Volcano Creek has been suggested as a source population, and results from this study indicate it is the only nonintrogressed Golden Trout Creek tributary tested to date. However, its reduced genetic variability and genetic distinctiveness from other Golden Trout Creek populations would not support its use as a sole founder stock. Whether this genetic distinctiveness is due to the introgression of rainbow trout alleles in the other populations or the result of some degree of isolation from Golden Trout Creek and its other tributaries is an open question. Nielsen et al. (1999) reported similar results in a microsatellite-based study of relic McCloud River redband trout populations. In that study Sheephaven Creek, the type-locality and only population not believed to be introgressed with nonnative coastal rainbow trout alleles, exhibited reduced genetic variability and distinctiveness compared with other McCloud river populations. South Fork Kern River populations located above the Ramshaw Barrier have also been suggested as a source population for restocking, but our minisatellite marker data show the presence of rainbow trout alleles in a sample from above the barrier. A more thorough investigation into the genetic status of South Fork Kern River populations and the probability of identifying nonhybridized sources of CGT is currently being concluded (J.F.C., M.R.S., and B.M., unpublished data).

Until suitable source populations can be identified, an attempt to eradicate rainbow trout alleles through chemical treatment and subsequent restocking would probably result in CGT populations that are more genetically dissimilar to historical populations than the current ones. Cordes et al. (2004) showed that the genetic affinities of populations of Paiute cutthroat trout *O. c. seleniris* subjected to multiple cycles of chemical treatment and restocking to eliminate the effects of rainbow trout introgression more closely reflected stocking history than geographic proximity or stream connectivity. In the meantime, other efforts to halt further introgression of rainbow trout alleles into the Golden Trout Creek system have already been implemented (Stephens, personal communication). These include gill-netting to remove all fish from Johnson Lake, Chicken Springs Lake, and the Rocky Basin Lakes; electrofishing around the Mouth of Barigan Stringer to reduce the number of introgressed fish; and the discontinuation of all fish transplants into any of the lakes and creeks associated with the Golden Trout Creek drainage.

However, given the levels of introgression observed in this study, it may become necessary at some point to consider incorporating introgressed populations into the overall recovery plan for this species.

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TABLE A.1.—Continued.

Alleles (base pair)	Populations										
	VC	GC	LWM	CL2	CL4	CSL	JL	MJC	MBS	USS	SFK
<i>Ots-G3</i>											
146	1.00	1.00	1.00	1.00	0.93	0.82	0.97	1.00	1.00	1.00	1.00
200 ^b	0.00	0.00	0.00	0.00	0.03	0.18	0.00	0.00	0.00	0.00	0.00
242	0.00	0.00	0.00	0.00	0.03	0.00	0.03	0.00	0.00	0.00	0.00
<i>OMM-1082</i>											
177 ^a	0.64	0.53	0.33	0.00	0.00	0.00	0.00	0.57	0.43	0.53	0.21
181	0.27	0.44	0.29	0.85	0.78	0.95	0.89	0.43	0.50	0.45	0.53
185	0.09	0.00	0.00	0.06	0.10	0.00	0.04	0.00	0.04	0.02	0.04
189 ^a	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04
197	0.00	0.00	0.00	0.03	0.05	0.02	0.07	0.00	0.00	0.00	0.02
199	0.00	0.00	0.02	0.02	0.05	0.02	0.07	0.00	0.00	0.00	0.08
201 ^c	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.02	0.00	0.00
205	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04
209	0.00	0.03	0.00	0.02	0.02	0.00	0.00	0.00	0.02	0.00	0.04
237	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
<i>OMM-1083</i>											
137 ^c	0.00	0.00	0.00	0.06	0.31	0.00	0.04	0.00	0.04	0.00	0.00
149 ^a	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
157 ^b	0.00	0.00	0.00	0.05	0.02	0.00	0.00	0.00	0.00	0.00	0.00
161 ^b	0.00	0.00	0.00	0.02	0.00	0.00	0.06	0.00	0.00	0.00	0.00
173	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
177 ^a	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
181 ^a	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.09	0.02	0.00	0.02
185	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.06	0.00	0.02	0.08
189 ^a	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.07	0.04	0.04	0.02
193	0.19	0.09	0.13	0.00	0.02	0.00	0.00	0.02	0.11	0.04	0.33
197	0.17	0.13	0.11	0.03	0.00	0.00	0.00	0.04	0.19	0.07	0.08
201	0.28	0.31	0.20	0.26	0.26	0.29	0.50	0.24	0.25	0.29	0.06
205	0.02	0.27	0.07	0.32	0.19	0.71	0.13	0.00	0.18	0.33	0.27
209 ^a	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.02
211 ^a	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
213 ^a	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
217 ^a	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
221 ^a	0.04	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.11	0.04	0.02
225	0.06	0.07	0.04	0.08	0.09	0.00	0.21	0.20	0.02	0.07	0.00
229	0.08	0.04	0.09	0.02	0.02	0.00	0.00	0.26	0.02	0.04	0.00
233 ^a	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
237 ^c	0.00	0.00	0.00	0.11	0.05	0.00	0.06	0.00	0.04	0.02	0.00
241 ^b	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
245 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
413 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
417 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
425 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
429 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
437 ^a	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
469 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00

^a Alleles found in presumably nonhybridized and threatened-by-introgression Golden Trout Creek samples but not in any of the lake samples.

^b Alleles found only in lake samples.

^c Alleles found in lake and threatened-by-introgression creek samples but not in presumably nonhybridized samples.

TABLE A.2.—Size and sequence differences in California golden trout (CGT) and rainbow trout alleles for the *ACCCTG-6* locus. Included are the relative positions of repeat units in aligned alleles. Location codes are given in Table 1 in the text.

Allele size (bp)	Location(s) found	Repeat units ^a
676	All CGT samples including SFK	CAECACAFCDACAFCCAFAFAFCD-AGHCH-HHI-IJ
651	All CGT samples including SFK	CAECACAFCDACAFCCAFAFAFCD-AGHC--HHI-IJ
636	All CGT samples including SFK	CAECACAFCDACAFCCAFAFAFCD-AG-C--HHI-IJ
	NFAR, LWM, BSS, BBS,	-----CAFCD-----FCAF--CDB-----FHIIHIIJ
399	SFK	
369	NFAR	-----CAFCD--- --FCAF--CD-A----FHIIHIIJ
297	NFAR, CL2, CL4, GC	-----CAFCD- -----FHIIHIIJ
267	NFAR, CL2, CL4	-- --CAFCD- -----FHII--IJ

^a Unit sequences are as follows (nucleotide substitutions are given in bold italics):

Repeat units	Size (bp)	Sequence
A	18	TACA-GCACCTGTTGTTGA
B	18	TACA-GCACCTGTTG GT GA
C	18	TACA-GCACCTGTTGTTG G
D	18	TACA-GCAC T GTTGTTGA
E	16	TACATGCACCT---GTT GG
F	15	TACA-GCACCT---GTT GG
G	15	TACA-GCACCT---GTTGA
H	15	TACA- GA ACCT---GTTGA
I	15	TACA- GA ACCT---GTT GG
J	15	TACA-GC GC CT---GT AGG

TABLE A.3.—Allele frequencies and observed (H_o) and expected (H_s) heterozygosities for the *ACCCTG-6* locus in 24 samples of California golden trout and rainbow trout. Population codes are given in Table 1.

Popu- lation	N	Alleles										H_o	H_s
		676	651	636	399	369	354	297	282	267	252		
CL-2	32	0.44	0.00	0.45	0.00	0.00	0.00	0.08 ^a	0.00	0.01 ^a	0.02 ^a	0.80	0.60
CL-4	30	0.54	0.00	0.35	0.00	0.00	0.00	0.08 ^a	0.00	0.02 ^a	0.01 ^a	0.57	0.58
CSL	28	0.43	0.00	0.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.49
JL	31	0.71	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.41
VC	32	0.30	0.46	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.58	0.64
LWM	22	0.30	0.46	0.23	0.02 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.77	0.65
MJC	29	0.31	0.62	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.51
GC	32	0.26	0.56	0.17	0.00	0.00	0.00	0.01 ^a	0.00	0.00	0.00	0.56	0.59
MBS	27	0.39	0.37	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.65
USS	27	0.35	0.44	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.63	0.64
SFK	21	0.21	0.57	0.14	0.02 ^a	0.00	0.00	0.05 ^a	0.00	0.00	0.00	0.33	0.60
GTLV	13	0.23	0.33	0.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.65
SLC	40	0.20	0.60	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.58	0.56
LJC	32	0.23	0.50	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.62
JC	23	0.26	0.65	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.50
BBS	28	0.14	0.52	0.29	0.02 ^a	0.00	0.00	0.04 ^a	0.00	0.00	0.00	0.57	0.63
BSS	27	0.26	0.44	0.28	0.02 ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.59	0.66
MSS	24	0.25	0.52	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.58	0.61
BWM	26	0.29	0.40	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.73	0.66
HW	27	0.24	0.48	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.63
HC	27	0.32	0.04	0.65	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.48
LCC-1	20	0.28	0.70	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.43
LCC-2	25	0.30	0.68	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.45
NFAR	8	0.00	0.00	0.00	0.11 ^a	0.17 ^a	0.06 ^a	0.64 ^a	0.03 ^a	0.00	0.00		

^a Alleles confirmed by DNA sequencing.