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## CONSERVATION OF PAIUTE CUTTHROAT TROUT: THE GENETIC LEGACY OF POPULATION TRANSPLANTS IN AN ENDEMIC CALIFORNIA SALMONID

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**Paiute cutthroat trout, *Oncorhynchus clarki seleniris*, are threatened by loss of genetic diversity, restricted distributions, and historical hybridization with other introduced trout species. In this study, we used a single copy nuclear (scnDNA) marker and several nuclear microsatellites to assess levels of rainbow trout (RT) hybridization, estimate existing amounts of genetic variation, and characterize relationships among nine populations of Paiute cutthroat trout (PCT). No evidence of RT introgression was found in any of the PCT populations based on an scnDNA marker and five microsatellite loci. The two polymorphic microsatellite markers revealed population heterozygosities ranging from 0.138-0.657 (average=0.469) and 0.336-0.722 (average=0.544), respectively. Allele frequency distributions differed significantly from Hardy-Weinberg equilibrium in a single sample at one locus. Log-likelihood  $G$  tests and population pairwise  $F_{ST}$  estimates indicated significant differentiation among most of the samples, and a neighbor-joining phenogram of  $D_{CE}$  genetic distances revealed genetic relationships among populations that closely reflected PCT stocking history. Results support that efforts to eradicate hybridized PCT have been successful. However, the remaining populations are fragmented with limited genetic variation.**

### INTRODUCTION

The Paiute cutthroat trout, *Oncorhynchus clarki seleniris*, is a narrowly distributed form of cutthroat trout most closely related to the more widespread Lahontan cutthroat, *O. c. henshawi*, of eastern California, Nevada, and southern Oregon (Nielsen and Sage 2002). Historically, Paiute cutthroat trout (PCT) were probably restricted to an approximately 10-km stretch of Silver King Creek below Llewellyn Falls and above Silver King Canyon gorge (Fig. 1), which effectively isolated them from Lahontan cutthroat trout (LCT) inhabiting the adjacent East Fork Carson River. Over time, PCT evolved distinct phenotypic characteristics, including an almost complete lack of body spotting and an iridescent hue, which distinguished them from other cutthroat and rainbow trout (RT) populations. Snyder (1933, 1934) first recognized PCT as a subspecies based on

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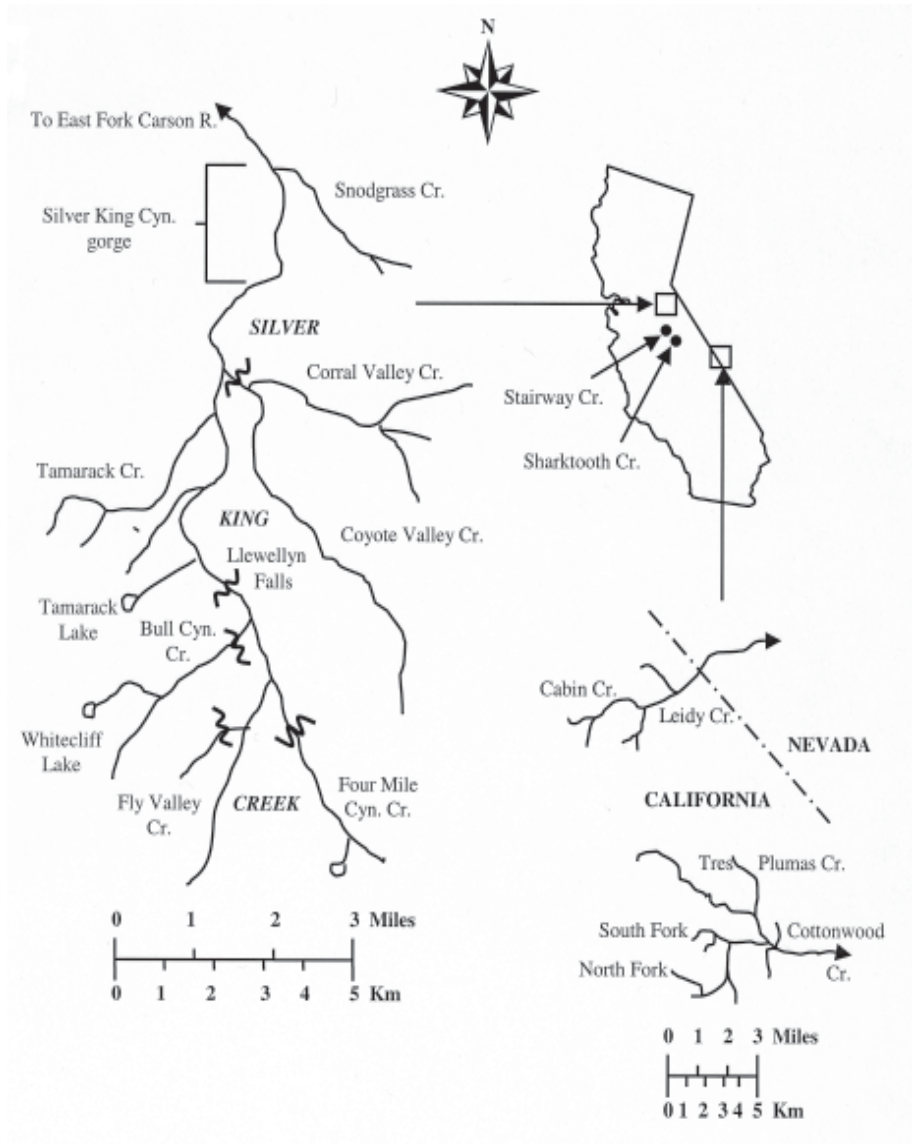


Figure 1. Map depicting the current distribution of Paiute cutthroat trout in California. Populations in Stairway Creek, Sharktooth Creek, and the White Mountains represent out-of-basin transfers from various locations in the Silver King Creek basin. Natural barriers to fish migration occurring in various streams are indicated with a wavy line.

the meristics of PCT populations that had been planted above Llewellyn Falls early in the last century.

The first documented transfer of PCT occurred in 1912, when a Basque sheepherder transplanted a small number of PCT from Silver King Creek below Llewellyn Falls (Fig. 2) into the fishless waters above (V. S. Connell; in Ryan and Nicola 1976<sup>1</sup>). As early as 1924, putative hybrid LCT/PCT and RT/PCT were found in Silver King Creek below Llewellyn Falls (Ryan and Nicola 1976<sup>1</sup>). By the time PCT were described as a subspecies (Snyder 1933, 1934), it is probable that no natural, non-hybridized populations existed in its native historic range. Early in the last century, poorly documented, unauthorized transfers of PCT into fishless tributaries of Silver King Creek above various barriers (Fig. 1) included Four Mile Canyon Creek, Corral Valley Creek, and Coyote Valley Creek (Ryan and Nicola 1976<sup>1</sup>). These populations served as critical early refugia from introgression once unauthorized transfers of rainbow and LCT created hybrids in Silver King Creek below Llewellyn Falls. In 1947, the California Department of Fish and Game (CDFG) made additional intra-basin transfers from Silver King Creek above Llewellyn Falls into Fly Valley and Bull Canyon Creeks (Vestal 1964<sup>2</sup>). However, only the Fly Valley Creek transfer appeared successful (Ryan and Nicola 1976<sup>1</sup>). PCT have also been successfully transplanted out of their native range to other drainages in California (Fig. 1) including North Fork Cottonwood Creek and Cabin Creek in the White Mountains (Mono County), Stairway Creek (Madera County), and Sharktooth Creek (Fresno County). In 1949, an unauthorized introduction of RT resulted in hybridization of PCT above Llewellyn Falls. In addition, introductions of LCT into Silver King Creek may have occurred prior to 1949, and an accidental aerial planting into Whitecliff Lake is suspected to have occurred in 1955 (Ryan and Nicola 1976<sup>1</sup>). In 1964, PCT collected from Corral Valley and Coyote Valley Creeks were described as slightly hybridized with RT based on meristics (Ryan and Nicola 1976<sup>1</sup>).

The California Fish and Game Commission recognized the threats of hybridization and over-fishing to the endemic PCT soon after its discovery (Ryan and Nicola 1976<sup>1</sup>). The threat of extinction from hybridization and introgression with introduced rainbow and LCT resulted in the federal listing of PCT as endangered in 1970 (Federal Register 35:16047<sup>3</sup>), though it was downlisted to threatened in 1975 (Federal Register 40: 29863<sup>4</sup>) to allow for aggressive management. In 1974, after reviewing Snyder's original meristic description of *O. c. seleniris*, the CDFG realized that body spotting was an unreliable diagnostic tool for evaluating hybridization (Ryan and Nicola 1976<sup>1</sup>). The CDFG turned to genetic analysis of certain populations to detect hybridization and determine if

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<sup>1</sup>Ryan, J.H. and S.J. Nicola. 1976. Status of the Paiute cutthroat trout, *Salmo clarki seleniris* Snyder, in California. California Department of Fish and Game Inland Fisheries Administrative Report #76-3. 56 pp.

<sup>2</sup>Vestal, E.H. 1964. Memo to Dick Beland. California Department of Fish and Game Memorandum. March 17, 1964. 1pp.

<sup>3</sup>Federal Register. 1970. Conservation of endangered species and other fish or wildlife. 35: 197(October 12, 1970) p. 16047-16048.

<sup>4</sup>Federal Register. 1975. Threatened status for three species of trout. 40: 137 (July 16, 1975) p. 29863-29864.

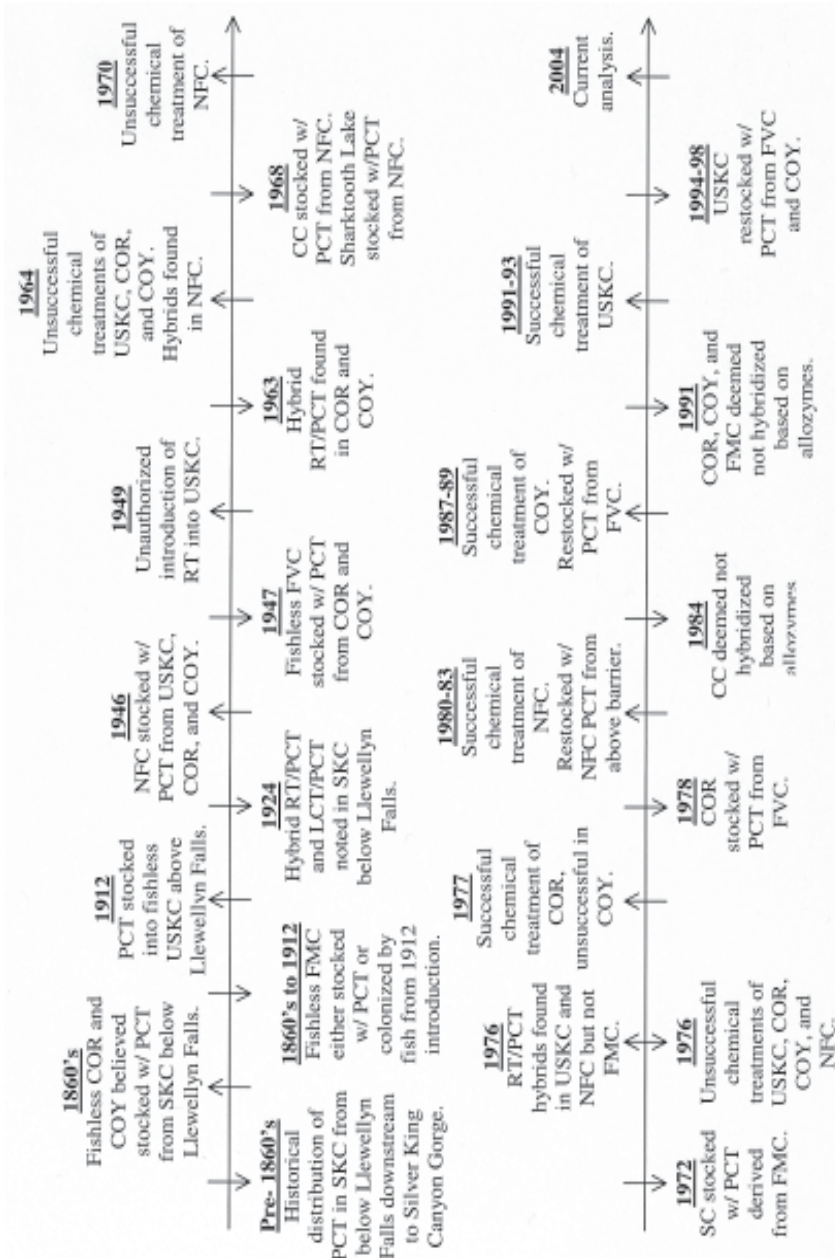


Figure 2. A timeline of important stocking and chemical treatment events involving Paiute cutthroat trout in the Silver King Creek basin and various out-of-basin transplant populations. Population codes follow those given in Table 1. Information taken from Vestal (1964), Ryan and Nicola (1976), Flint (1980), and Busack and Gall (1981) as cited in text.

various chemical treatments were successfully removing all introgressed fish. Allozyme analysis of PCT collected in 1976 found introgression by RT in upper Silver King Creek and North Fork Cottonwood Creek, but not in Four Mile Canyon Creek (Busack and Gall 1981). A 1976 chemical treatment of upper Silver King Creek was unsuccessful. In 1977, Corral Valley and Coyote Valley Creeks were treated, but only the Corral Valley treatment was deemed successful. Coyote Valley Creek was again treated during 1987-88 to remove hybrids. The North Fork of Cottonwood Creek was treated in 1980-81. Between 1991 and 1993, DFG chemically treated Silver King Creek in reaches where hybrids (based on body spotting and allozyme analysis) continued to be found. The repeated cycles of chemical treatment and restocking with presumably pure trout has been the main influence linking extant populations of PCT, so that current genetic relationships probably reflect stocking history rather than any pattern of natural affinities. Limitations of allozyme analyses (including limited variability and destructive sampling) and the continued uncertainties surrounding the genetic integrity of various PCT populations required non-invasive, highly polymorphic genetic tools to identify current threats of hybridization and introgression, and evaluate the success of restocking efforts.

The goals of this study were to use nuclear microsatellite and single copy nuclear (scn) DNA markers to investigate the genetic status of PCT in Silver King Creek, its tributaries, and out-of-basin populations. More specifically, the objectives were to: (1) evaluate potential markers to differentiate RT, LCT, and PCT, (2) survey PCT populations in the Silver King Creek drainage and out-of-basin transplants for evidence of RT introgression, and (3) characterize the genetic diversity within and among extant PCT populations.

## MATERIALS AND METHODS

### Sample Collection

Fin clip samples of PCT, LCT, and RT were collected in the summer months between 1996 and 2000 (Table 1). In 1996, California Department of Fish and Game (CDFG) personnel collected PCT samples from the North Fork of Cottonwood Creek. Also in 1996, U.S. Geological Survey/Biological Research Division (USGS/BRD) personnel collected LCT samples from the East Carson River, Murray Canyon Creek, and Poison Flat Creek. U.S. Forest Service (USFS) biologists collected samples of PCT from Sharktooth Creek in 1999 and Stairway Creek in 2000. All of the above samples were dried and stored in scale envelopes. Also in 2000, CDFG biologists collected fin clip samples from PCT at five locations in the Silver King Creek basin and Cabin Creek. They also collected two suspected hybridized PCT from upper Fish Valley (upper Silver King Creek above Llewellyn Falls) and one from Bull Canyon Creek (Table 1). Finally, CDFG personnel collected RT samples from the North Fork American River in 2000. Samples from the Silver King Creek basin, Cabin Creek, and the North Fork American River were placed in DMSO storage buffer (20% DMSO, 0.25 MEDTA, NaCl to saturation, pH 7.8), and stored at room temperature.

Table 1. Collection names, locations, sizes, and collection dates of Paiute cutthroat trout samples collected between 1996 and 2000. Location codes are given in parentheses.

Species	Sample name	County	Sample size (n)	Collection date
Paiute cutthroat	Cabin Cr. (CC)	Mono	26	08/23-24/2000
	N. Fork Cottonwood Cr. (NFC)	Mono	31	07/17/2000
	Stairway Cr. (SC)	Madera	30	08/04/2000
	Sharktooth Cr. (STC)	Fresno	25	08/22/2000
	Corral Valley Cr. (COR)	Alpine	33	08/11/2000
	Four Mile Canyon Cr. (FMC)	Alpine	31	08/14/2000
	Coyote Valley Cr. (COY)	Alpine	30	08/11/2000
	Silver King Cr. (SKC)	Alpine	28	08/12/2000
Suspected hybrids <sup>a</sup>	Fly Valley Cr. (FVC)	Alpine	35	08/14/2000
	Upper Silver King Cr. (USKC)	Alpine	2	08/27/2000
Lahontan cutthroat	Bull Canyon Cr. (BCC)	Alpine	1	08/27/2000
	East Carson R. (ECR)	Alpine	1	08/29/1996
	Murray Canyon Cr. (MCC)	Alpine	2	08/29/1996
Rainbow trout	Poison Flat Cr. (PFC)	Alpine	3	08/30/1996
	N. Fork American R. (NFAR)	Placer	6	08/03/2000

<sup>a</sup>Suspected Paiute cutthroat/rainbow trout hybrids based on body spotting (B. Somers, California Dept. of Fish and Game, personal communication)

## Genetic Analyses

Whole genomic DNA was extracted from fin clip samples using the Qiagen DNeasy™ Tissue Kit. To screen for polymorphisms, we assessed 11 microsatellite loci via the polymerase chain reaction (PCR) in six individuals each of PCT, LCT, and RT. Initially, 10  $\mu$ L amplification reactions were carried out containing 4.85  $\mu$ L sterile dH<sub>2</sub>O, 1.0  $\mu$ L 10X PCR buffer, 0.3  $\mu$ L 50 mM MgCl<sub>2</sub>, 0.8  $\mu$ L 2.5 mM dNTP mixture, 1  $\mu$ L 10 mM forward primer, 1  $\mu$ L 10 mM reverse primer, 0.05  $\mu$ L Taq I polymerase (0.25 U total), and 1  $\mu$ L sample DNA (approximately 5 ng DNA total). PCR reactions were first denatured for 1 min 30 sec at 95°C, followed by steps of 1 min at 95°C, 30 sec at 52°C, and 1 min at 72°C for 30 cycles. PCR products were then electrophoresed on a 5% polyacrylamide denaturing gel for 1.5 hours at 35 watts, and alleles were visualized using a Molecular Dynamics 595 FluorImager. Allele sizes were estimated using the Molecular Dynamic's FragneNT Analysis application (Version 1.1, 1993-4). Seven of the 11 microsatellite loci (Table 2) consistently amplified in PCT, LCT, and RT, and only 2 (OMM1058 and OMM1088) proved polymorphic in PCT.

PCR conditions for amplifying PCT DNA at the two polymorphic loci were optimized for use with fluorescent-labeled primers. The OMM1058 and OMM1088 forward primers were labeled with HEX and FAM fluorophores, respectively. PCR was carried out with 5.15  $\mu$ L of sterile H<sub>2</sub>O, 1.0  $\mu$ L 10X PCR buffer, 0.8  $\mu$ L 2.5 mM dNTP mixture, 0.4  $\mu$ L

Table 2. Characteristics of 7 microsatellite loci evaluated for use in this study. Size ranges are given in base pairs (bp). All microsatellite primer sets are from Rexroad et al. (2001).

Microsatellite Loci	Paiute Cutthroat Trout		Lahontan Cutthroat Trout		Rainbow Trout	
	#alleles	Size range	#alleles	Size range	#alleles	Size range
OMM 1050	1	220	4	224-236	8	240-280
OMM 1051	1	428	5	400-496	7	232-276
OMM 1058	4	216-228	3	208-216	7	184-240
OMM 1086	1	194	3	194-202	5	194-242
OMM 1088	6	204-224	5	144-240	5	120-140
OMM 1104	1	224	1	240	5	168-216
OMM 1108	1	156	6	144-176	4	148-188

50 mM MgCl<sub>2</sub>, 0.05  $\mu$ l Taq I polymerase (0.25 U total), 0.2  $\mu$ l of 1 mM labeled forward primer, 0.4  $\mu$ l of 10 mM reverse primer, and 2  $\mu$ l PCT sample DNA (approximately 10 ng total). PCR amplification conditions were the same as described above. Alleles were separated electrophoretically on a 5.5% polyacrylamide gel using the MJ Research BaseStation gel analysis system (MJ Research, Inc., Boston, Ma, USA) and analyzed using MJ Research's Cartographer software. The Genescan 500 size standard (MJ Research) labeled with ROX fluorescent dye was run in each lane.

An anonymous single copy nuclear (ascnDNA) marker was developed following protocols established in the Genomic Variation Laboratory at the University of California, Davis (Tranah et al. 2003). Briefly, 200 ng of DNA from RT, LCT, and PCT samples were digested for 1 hour at 37 °C with the restriction enzymes EcoRI and Mse I using 0.1  $\mu$ l of each enzyme, 2.0  $\mu$ l 10X buffer, 0.16  $\mu$ l 400X BSA, and ddH<sub>2</sub>O to 16  $\mu$ l. Adapters to Mse I and EcoRI restriction sites were then ligated to the ends of the DNA fragments by adding 0.4  $\mu$ l Eco Adapter (5 pmoles), 0.4  $\mu$ l Mse Adapter (50 pmoles), 0.2  $\mu$ l 100mM ATP, 0.2  $\mu$ l T4 DNA Ligase, 0.4  $\mu$ l 10x buffer, 0.1  $\mu$ l 400X BSA, and 2.3 ml H<sub>2</sub>O to the 16 ml reaction. The ligation reaction was performed overnight at room temperature, after which 180  $\mu$ l TLE was added. The adapters were designed to have a final 3' nucleotide that was different from the original sequence, thus destroying the original restriction site. The fragments were then amplified via PCR using primers complimentary to the adapter sequences. The primers used were designed with one to three varying nucleotides overhanging the 3' end of the adapters. Fragments were selectively amplified by varying the specific primers that were used. Fragments were first amplified using shorter, less specific preamp primers. The PCR conditions were: 94°C for 1.5 minutes, followed by 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute for 23 cycles; 180  $\mu$ l of TLE were added to the preamp PCR product for each sample. Fragments were then amplified with different combinations of the specific primers, using a touchdown PCR program with a denaturing step of 94°C for 30 seconds, an annealing step beginning at 65°C for the first cycle and decreasing 0.7°C each cycle until 56°C for 30 seconds, and an elongation step of 72°C for 1 minute. Thirty-five cycles



were performed for the entire reaction. After amplification, the products were electrophoresed on a 5% acrylamide denaturing gel for 1.5 hours at 35 W, and scanned with a Molecular Dynamics 595 FluorImager. Bands of PCR-amplified product that appeared fixed and unique to a single subgroup (RT, LCT, or PCT) were cut out of the denaturing gels, and re-suspended in 200  $\mu$ l TLE. These fragments were re-amplified using non-labeled primers under preamp PCR conditions and sequenced using Perkin Elmer/ABI BigDye Dye Terminator chemistry. New primers were designed that specifically amplified the potentially informative fragments using the software Primer Select (DNASar, Inc.).

The sequences generated from these fragments were used to design new primers. From the sequenced alleles described above, we developed primers for the single locus AGGCCG-3 (F: 5'-GGGGCAAAGGGGGTCTGAC-3'; R: 5'-AACCGGGGGTTGACGTACATTATA-3'), which exhibited a definitive allele size difference between RT and LCT/PCT. No fragments exhibiting size differences between LCT and PCT were found. The AGGCCG-3 locus was used to investigate the possibility of RT introgression in the PCT populations sampled in this study. The PCR conditions for amplification of this locus were: 94°C for 2 minutes, followed by steps of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds for 30 cycles, and a final extension step of 72°C for 5 minutes after cycling. PCR products were then electrophoresed on a 5% polyacrylamide denaturing gel for 1.5 hours at 35 W, and imaged with a Molecular Dynamics 595 FluorImager. Allele sizes were estimated using Molecular Dynamic's Fragment Analysis application.

### Statistical Analyses

Standard molecular indices including allele frequencies, expected heterozygosities ( $H_e$ ), and observed heterozygosities ( $H_o$ ) were calculated using the program Genes in Populations<sup>5</sup>. Conformance to Hardy-Weinberg expectations for each locus/population combination was tested by the Markov chain method to obtain unbiased estimates of Fisher's exact test based on 1000 iterations (Rousset and Raymond 1995) using the web-based software program Genepop on the Web (available at [http://wbio.med.curtin.edu.au/genepop/genepop\\_op1.html](http://wbio.med.curtin.edu.au/genepop/genepop_op1.html)). Log-likelihood  $G$  tests using the Markov chain method for population differentiation at each locus and for all loci combined, as well as calculations of Weir and Cockerham's (1984) estimates of  $F_{ST}$  were also performed using Genepop on the Web. Statistical significance of population pairwise  $F_{ST}$  values was determined using the software program FSTAT 2.9.3 (updated from Goudet 1995; available at <http://www.unil.ch/izea/software/fstat.html>). All significance values resulting from multiple comparisons were corrected for Type I error using the Bonferroni correction of Rice (1989)

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<sup>5</sup>Genes in Populations 2.2. Designed by B. May and C. Krueger and written in C by W. Eng and E. Paul; program available for download at <http://animalscience.ucdavis.edu/extension/Gene.htm>

Cavalli-Sforza and Edward's (1967) chord distances ( $D_{CE}$ ) were calculated among samples and plotted as a neighbor-joining (NJ) phenogram (Saitou and Nei 1987) using Phylip 3.5c (Felsenstein 1995). The original allele frequency matrix was then resampled 1000 times by bootstrapping and the genetic distances between samples were estimated for each resulting matrix. A consensus UPGMA diagram was generated and all bootstrap values above 50% were plotted on to the dendrogram from the original sample matrix to indicate stability of the nodes.

## RESULTS

### Hybridization Assessment

Sixty-four AFLP primer combinations were used to screen for genetic differences among PCT, LCT, and RT. From these 64 combinations, 87 potentially informative fragments were cut out and sequenced. From these sequences, primers to amplify 23 novel loci were developed. Only one (AGGCCG-3) of the 23 novel loci screened showed fixed differences between PCT and RT, while none showed fixed differences between Paiute and LCT. All RT screened for the AGGCCG-3 locus shared a single band 219 base pairs (bp) in length, while all PCT and LCT samples exhibited a single band of 189 bp. All individuals from each PCT collection (Table 1) were amplified at the AGGCCG-3 locus and no rainbow-type bands were found, indicating that none of the tested samples were a product of hybridization with RT.

Of the seven microsatellite loci screened (Table 2), one (1088) was diagnostic, exhibiting disjunct distributions of alleles between PCT and RT. As in the AGGCCG-3 marker above, no rainbow-type bands were found in any of the PCT collections tested. This is consistent with the findings from the five microsatellite loci (OMM1050, OMM1051, OMM1086, OMM1104, and OMM1108) that were monomorphic in PCT. Five of these loci were polymorphic in RT (Table 2), and none of the RT alleles at any locus were found in 75 tested individuals composed of subsets of eight samples from each PCT population and the suspected hybrids.

Four of the microsatellite loci (Table 2) monomorphic in PCT were polymorphic in LCT and some may be useful as diagnostic markers for identifying instances of hybridization between the two subspecies. However, since the present study focused on identifying diagnostic markers between PCT and RT, we limited our survey of LCT to fish derived from the East Carson River. A more extensive survey of LCT populations in California and Nevada would be necessary to assess the usefulness of the AGGCCG-3 or microsatellite markers in distinguishing PCT from LCT. We chose the North Fork American River population as our reference RT samples because it is a wild population shown to contain nearly all of the diversity found in three different strains of hatchery RT, including the Hot Creek strain that was the source of RT introduction into Silver King Creek above Llewellyn Falls in 1949 (J.F. Cordes, unpublished data based on a survey of six microsatellite loci.).

## Genetic Variation and Distances

For the seven microsatellite loci listed in Table 2, all but two were monomorphic in PCT. The number of alleles per locus for polymorphic OMM1058 and OMM1088 was 4 and 6, respectively. Since a goal of the microsatellite analysis was to evaluate variability between PCT populations, we did not use the numerous monomorphic loci for the rest of the analyses. Microsatellite variation was therefore evaluated at two loci, OMM1058 and OMM1088.

Allele frequencies, observed heterozygosity ( $H_o$ ), and expected heterozygosity ( $H_e$ ) are listed in Table 3. Allele sizes for the OMM1058 locus ranged from 216-228 bp. All four of the OMM1058 alleles were found in the North Fork Cottonwood Creek sample; all other populations had three alleles present. Observed heterozygosities ( $H_o$ ) for OMM1058 ranged from 0.138 (Stairway Creek) to 0.657 (Fly Valley Creek), with an average of 0.469. Allele sizes for the OMM1088 locus ranged from 205-225 bp. Although all of the populations contained a majority of the six OMM1088 alleles, none contained all of them (Table 3). The observed heterozygosities ( $H_o$ ) at this locus ranged from 0.336 (Stairway Creek) to 0.722 (Cabin Creek), with an average of 0.544. The only significant deviation ( $P < 0.05$ ) from Hardy-Weinberg expectations after correction for multiple tests occurred at the OMM1088 locus in the Sharktooth Creek collection.

Table 3. Allele frequencies, sample sizes (n), observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosities, and  $P$  values for tests of Hardy-Weinberg equilibrium for nine populations of Paiute cutthroat trout. Location codes are given in Table 1. An (\*) indicates a significant deviation from Hardy-Weinberg equilibrium ( $P < 0.01$ ) after correction for multiple tests (initial  $\alpha = 0.001$ ).

Locus	Allele	CC	NFC	SC	STC	COY	COR	FMC	SKC	FVC
OMM1058	216	-	0.067	-	-	-	-	-	-	-
	220	0.389	0.117	-	0.200	0.121	0.387	0.050	0.143	0.186
	224	0.361	0.800	0.931	0.680	0.586	0.323	0.800	0.571	0.414
	228	0.250	0.017	0.069	0.120	0.293	0.290	0.150	0.286	0.400
	n	18	30	29	25	31	30	29	28	35
	$H_o$	0.611	0.333	0.138	0.480	0.643	0.581	0.333	0.657	0.657
	$H_e$	0.656	0.342	0.128	0.483	0.571	0.662	0.335	0.634	0.634
	$P$	0.098	0.174	1.000	0.365	0.138	0.587	1.000	0.680	0.966
OMM1088	209	0.417	0.536	-	0.405	-	-	-	-	-
	213	0.083	0.018	0.150	0.095	0.033	-	0.100	0.259	0.156
	217	0.306	0.411	0.633	0.357	0.650	0.470	0.400	0.667	0.641
	221	0.111	0.036	-	0.024	0.167	0.061	-	0.074	0.125
	225	0.083	-	0.217	0.119	0.150	0.470	0.483	-	0.078
	229	-	-	-	-	-	-	0.017	-	-
	n	18	28	30	21	33	30	30	27	32
	$H_o$	0.722	0.417	0.336	0.383	0.525	0.609	0.483	0.61	0.563
$H_e$	0.681	0.442	0.329	0.584	0.527	0.608	0.466	0.589	0.543	
$P$	0.075	0.444	0.142	<b>0.000*</b>	0.434	0.224	1.000	0.537	0.800	

Log-likelihood *G* tests revealed significant genetic differentiation among populations over all for both loci individually and combined. Population pairwise  $F_{st}$  values indicated significant differentiation among all samples with the following exceptions: Coyote Creek and Fly Valley Creek; Silver King Creek and Fly Valley Creek; Stairway Creek and Four Mile Canyon Creek; Sharktooth Creek and Cabin Creek; and Sharktooth Creek and North Fork Cottonwood Creek (Table 4).

Table 4. Population pairwise estimates (above diagonal) and log likelihood *G* tests of allele frequency differences (below diagonal) for two microsatellite loci. An (\*) above the diagonal indicates a statistically significant population pairwise  $F_{ST}$  value; numbers below the diagonal indicate loci (1=OMM1058, 2=OMM1088) exhibiting a significant difference in allele frequencies. Results based on  $P < 0.05$  after correction for multiple tests (initial  $\alpha = 0.013$ ).

Population	COR	COY	SKC	FVC	FMC	SC	STC	CC	NFC
COR	-	<b>0.089*</b>	<b>0.146*</b>	<b>0.076*</b>	<b>0.144*</b>	<b>0.246*</b>	<b>0.137*</b>	<b>0.093*</b>	<b>0.271*</b>
COY	2	-	<b>0.029*</b>	0.008	<b>0.114*</b>	<b>0.093*</b>	<b>0.090*</b>	<b>0.138*</b>	<b>0.175*</b>
SKC2	2	-	0.017	<b>0.149*</b>	<b>0.121*</b>	<b>0.102*</b>	<b>0.153</b>	<b>0.193*</b>	
FVC2	0	0	-	<b>0.150*</b>	<b>0.165*</b>	<b>0.108*</b>	<b>0.109*</b>	<b>0.212*</b>	
FMC	1,2	2	2	1,2	-	0.076	<b>0.104*</b>	<b>0.213*</b>	<b>0.208*</b>
SC 1,2	1	1,2	1	0	-	<b>0.137*</b>	<b>0.297*</b>	<b>0.186*</b>	
STC1,2	2	2	2	2	1,2	-	0.040	0.016	
CC 2	2	2	2	1,2	1,2	0	-	<b>0.142*</b>	
NFC1,2	1,2	1,2	1,2	1,2	1,2	0	1	-	

In order to examine the relatedness between populations, a NJ phenogram was constructed based on  $D_{CE}$  genetic distances. This phenogram and a graphical depiction of stock transfers are detailed in Figure 3. All of the nodes were supported with bootstrap values greater than 50% (range 51-76%), and genetic relationships among populations closely followed their stocking histories. This concordance in the data between genetic relationships and stocking history lent confidence to our results despite the relatively few number of microsatellite markers employed; however, a more extensive analysis of additional samples using a larger suite of markers would help to verify our initial findings.

## DISCUSSION

### Hybridization Assessment

The AGGCCG-3 locus was developed to distinguish RT from PCT. The fixed allele for PCT was the only allele observed in all samples from every PCT population as well as the suspected hybrids (Table 1) from Bull Canyon Creek and Silver King Creek. No rainbow-type alleles were found in any of the PCT samples. Although it has been

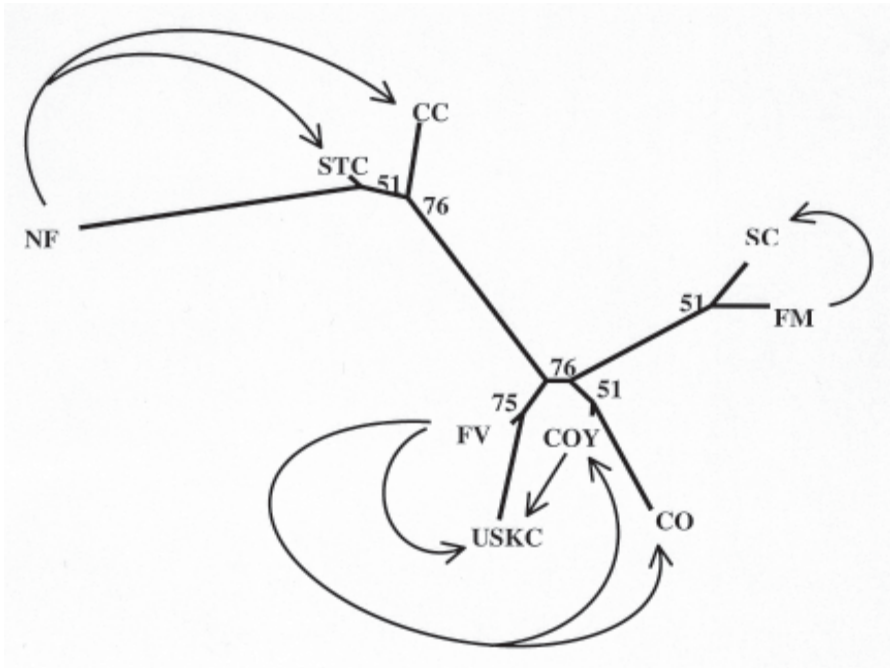


Figure 3. Neighbor-joining tree depicting relationships among nine creek populations of Paiute cutthroat trout based on Cavalli-Sforza and Edwards  $D_{CE}$  genetic distances. Bootstrap values at the nodes indicate the percentage of times populations beyond the node grouped together based on 1,000 iterations. Arrows correspond to direction of most recent stocking from source to current populations sampled in this study (discussed in text).

suggested that the Cabin Creek population may be introgressed (USFWS 1985<sup>6</sup>, p.13), no rainbow-type band was found in any individuals from Cabin Creek. These results suggest that none of the tested PCT populations have undergone recent hybridization with RT. Introgression from past hybridization events may be difficult to detect when relying on a single genetic marker, but such introgression seems unlikely since the absence of RT alleles in any of the PCT populations for the polymorphic 1088 microsatellite locus or the five monomorphic loci supports the conclusion that hybridization has not occurred. Thus it appears that the combination of chemical and electro-shocking treatments that have taken place since hybridization was last genetically assessed (Busack and Gall 1981) has been successful at eradicating PCT/RT hybrids to below detectable levels from all of the tested populations in Silver King Creek basin and the out-of-basin transfers.

### Genetic Variation and Stocking History

This study detected limited genetic variability in PCT. Of seven microsatellite loci screened, only two were polymorphic in PCT (average number of alleles/locus= 2.14),

in contrast to the six loci found to be polymorphic in a small sample of LCT from the neighboring East Fork Carson River (average number alleles/locus = 3.86). Higher levels of microsatellite variability in larger sample sizes of LCT have been reported by Nielsen and Sage (2002) who screened 16 amplifiable loci derived from various trout and salmon species and found 13 to be polymorphic (average number of alleles/locus of 15.0). Higher levels of microsatellite variability based on average number of alleles/locus and observed heterozygosities have also been shown in other native California trout including California golden trout (Cordes et al. 2001<sup>7</sup>) and redband trout (Nielsen et al. 1999), arguably making PCT the most narrowly distributed and genetically limited native trout in the state. This limited genetic variability may be due to a bottleneck associated with the original isolation of PCT from a common ancestor with the LCT, bottlenecks resulting from the small number of fish typically used as transplant stocks, or a combination of the two.

Log-likelihood  $G$  tests and population pairwise  $F_{st}$  values revealed significant genetic differentiation among a majority of the samples. Given the stocking history of the basin, this probably represents the result of founder effects and genetic drift on populations started with small numbers of stocked fish, rather than naturally occurring differentiation of reproductively isolated native populations. All non-significant estimates of population differentiation (Coyote Creek and Fly Valley Creek; Silver King Creek and Fly Valley Creek; Stairway Creek and Four Mile Canyon Creek; Sharktooth Creek and Cabin Creek; and Sharktooth Creek and North Fork Cottonwood Creek) occurred between samples that shared common recent stocking histories (see discussion on stocking groups below).

Genetic distances suggest that three relatively discrete stocking groups of PCT currently exist, and that relationships among populations have been largely determined by stocking history (Fig. 3). The first group included fish from North Fork Cottonwood, Cabin, and Sharktooth Creeks. A second group was comprised of samples from upper Silver King, Fly Valley, Corral Valley, and Coyote Creeks. The third group consisted of samples from Stairway and Four Mile Canyon Creeks.

The first group of samples from North Fork Cottonwood Creek, Sharktooth Creek, and Cabin Creek were relatively distinct from all remaining samples on the NJ tree. The North Fork Cottonwood Creek samples may represent the oldest extant PCT population (but see discussion on Four Mile Canyon Creek fish). Established in 1946, this was the first out-of-basin transfer of PCT from upper Silver King, Coyote Valley, and Corral Valley Creeks (Vestal 1947). This transfer occurred prior to the introduction of RT above Llewellyn Falls from Hot Creek Hatchery in 1949; the fish collected from Corral Valley and Coyote Valley Creeks are believed derived from PCT planted by loggers in the late 1860's (Vestal 1964<sup>2</sup>). All three of these donor populations have since been extirpated by hybridization and subsequent chemical treatment.

<sup>6</sup>U.S. Fish and Wildlife Service. 1985. Paiute Cutthroat Trout Recovery Plan. U.S. Fish and Wildlife Service, Portland, OR. 68 pp.

<sup>7</sup>Cordes, J.F., M.A. Blumberg, G.A.E. Gall, and B. May. 2001. Genetic status of California golden trout populations in the headwaters of Golden Trout Creek. Report to the California Department of Fish and Game Threatened Trout Committee. September 2001. 34 pp.

The North Fork Cottonwood Creek population was the only sample to have all four alleles at the OMM1058 locus, suggesting that the slightly higher level of variation within this sample may reflect its history as the oldest known remaining population. At the OMM1088 locus, we observed four out of six alleles. Interestingly, the 225 base pair allele at the OMM1088 locus was not found in this population, but was found in Cabin and Sharktooth Creeks, two out-of-basin transfers made with PCT from North Fork Cottonwood Creek. Each of these translocated populations had five alleles at this locus. Comparison of allele frequencies at this locus between the North Fork Cottonwood Creek sample and both of these populations reveals that the same two alleles (209 and 217 bp) account for more than 70% of the variation in all three samples. One of these alleles (209 bp) is not found in any of the other sample populations, and may be a relict of translocation from the now-extirpated upper Fish Valley populations. The Sharktooth Creek population was established in 1968 from 29 fish (23 from N. Fork Cottonwood Creek and six from Delaney Creek) placed into a headwater lake (Sharktooth Lake; Ryan and Nicola 1976<sup>1</sup>). By 1975, a survey of the lake found no fish, though PCT had apparently become established in the creek<sup>8</sup>.

Apparently, an unauthorized transfer of RT led to putative hybrids becoming prevalent in North Fork Cottonwood Creek as far as a natural barrier in the Granite Meadow area by 1964. In 1970, the creek was electrofished and chemically treated; 75% of the fish (those with fewer than five spots) were returned to the stream and a survey in 1973 found no evidence of heavily spotted fish (putative hybrids). However, Busack and Gall (1981) described genetic evidence of introgression between PCT and RT in a 1976 collection taken from this stream below the Granite Meadow barrier. North Fork Cottonwood Creek was chemically treated below Granite Meadow in 1980-81, and fish were relocated downstream from above the barrier in 1982-83. In the present study, no evidence of hybridization was observed in the North Fork Cottonwood Creek sample taken from above the Granite Meadow barrier.

The second group on the NJ tree consisted of four loosely associated samples from upper Silver King, Fly Valley, Coyote, and Corral Valley Creeks. As mentioned previously, Silver King Creek above Llewellyn Falls was first stocked with presumably pure PCT in 1912. PCT in Fly Valley Creek were established in 1947 (Vestal 1964<sup>1</sup>) from a transfer of 54 fish; Ryan and Nicola (1976: p. 30) “assumed that it [the source] was either Coyote or Corral Valley Creeks or both.” It is believed that Coyote and Corral Valley Creeks were originally fishless, but stocked by loggers in the late 1860’s (Vestal 1964<sup>1</sup>), also with presumably pure PCT from below Llewellyn Falls. Upper Silver King Creek above Llewellyn Falls received its first transfer of RT in 1949, and a selective sportfishery and repeated electrofishing were ineffective at removing rainbow and hybrid trout after 13 years (Flint et al. 1998). Single chemical treatments were not

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<sup>8</sup>It is unclear why the only significant departure from Hardy-Weinberg equilibrium occurred at the OMM1088 locus in this sample ( $H_o=0.383$ ,  $H_e=0.584$ ,  $P<0.001$ ). The presence of a null allele (one that doesn’t amplify via PCR due to sequence changes in the flanking regions where the primers bind) is possible, although it seems unlikely that the allele would be rare or absent in all other populations.

successful at removing putative hybrids in 1964 and 1976, and a chemical treatment project took place between 1990-1993 to remove any remaining hybrids in this reach of stream. In 1994-1998, pure PCT were relocated back in to upper Silver King Creek from Coyote Valley and Fly Valley Creeks. The location of the upper Silver King population in this group reflects this shared ancestry. However, these translocations were not genetically complete, since the OMM1088/225 bp allele present in both the Coyote Valley Creek and Fly Valley Creek samples is missing in the upper Silver King Creek sample. Also, allele frequencies differed between these three populations, although they continued to share the most common allele at both the OMM1058 and OMM1088 loci.

The Coyote Valley Creek population was re-established from the Fly Valley Creek population after successive chemical treatments in 1987 and again in 1988. This followed unsuccessful treatments in 1964 and 1977. Fifty-four PCT were transferred from Fly Valley Creek into Coyote Valley Creek in 1989. The decreased observed heterozygosity seen in the Coyote Valley Creek population suggests inbreeding as a result of the small initial number of transferred fish, although there was no loss of alleles at either loci when compared to the Fly Valley source population. Although the majority of the fish (87%) used to establish the upper Silver King Creek population came from Coyote Valley Creek, the upper Silver King Creek sample aligned more closely with the Fly Valley Creek sample in the dendrogram, suggesting differential reproduction, random drift, or both have affected the relationships among these three populations.

The most recent chemical treatment of Corral Valley Creek was in 1977 with a subsequent restocking of twenty fish from Fly Valley Creek. An initial decrease in population is believed to have occurred, as few fish were observed the year following the initial transfer (Bacon 1978<sup>9</sup>). Since the 1977 treatment, possible putative hybrids were identified and removed. In a comparison of allele frequencies between the samples from Fly Valley and Corral Valley Creeks, we observed the loss of the OMM1058 213 bp allele in the Corral Valley Creek sample. This and the fixation of the 225 bp allele at the OMM1088 locus in the Corral Valley Creek sample suggests a bottleneck likely occurred when this population was re-established.

The third cluster consists of Stairway and Four Mile Canyon creeks. The out-of-basin Stairway Creek population was established in 1972 from 77 Delaney Creek fish. The source populations for Delaney Creek were Four Mile Canyon Creek (n=43) and Fly Valley Creek (n=3). This translocation explains the grouping of Stairway Creek with Four Mile Canyon Creek. Both of these populations have four alleles at the OMM1088 locus, although the Four Mile Canyon Creek population contains an allele not represented in any other population. The Stairway Creek population has lost a single allele at the OMM1058 locus relative to its source populations, and only has two alleles. This population has the lowest overall heterozygosity of any population at both the OMM1088 ( $H_o=0.336$ ) and OMM1058 ( $H_o=0.138$ ) loci.

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<sup>9</sup>Bacon, M. 1978. Coyote Valley and Corral Valley Creeks, Alpine County, California Department of Fish and Game Memorandum. October 20, 1978. 1 pp.



PCT in Four Mile Canyon Creek were either established by stockmen prior to 1912 (letter from C.S. Kabel to Elden Vestal, September 18, 1956; in Ryan and Nicola 1976) or ascended into this reach after the 1912 introduction into upper Silver King Creek above Llewellyn Falls. If these fish naturally colonized Four Mile Canyon Creek from upper Silver King Creek (before beaver dams presented a series of barriers to migration) they may represent the oldest continuous population of PCT. Electrofishing and removal of spotted trout was done between 1972 and 1992 and allozyme analysis in 1976 and 1991 did not produce evidence of RT introgression. About 1 mile of upstream habitat known to contain fish was never electrofished (William Somer, CDFG, personal communication).

PCT probably diverged from a more widely distributed PCT/LCT ancestor after a presumably small subpopulation of fish was isolated from the rest of the East Fork Carson River drainage by natural barriers in a gorge in lower Silver King Creek Canyon. Although the limited genetic diversity in PCT revealed in this study may have resulted from this initial separation, it has almost certainly been exacerbated by the repeated chemical treatment and restocking of virtually all of the extant populations. Fragmentation of an already narrowly distributed subspecies such as PCT into smaller, isolated populations can lead to accelerated genetic drift, resulting in random shifts in allele frequencies among populations, loss of genetic diversity, fixation of different alleles in different populations, and genetic diversification (fragmentation) of replicate populations originally from a single source (Frankham et al. 2002). Spruell et al. (1999) argued that local populations of Bull trout faced a high risk of extinction based on a significant degree of genetic differentiation and low numbers of spawners. Results of this study show that loss of genetic diversity, shifts in allele frequency, and genetic differentiation (fragmentation) is already apparent in PCT populations. In light of these findings, extant populations of PCT should be considered part of a single management unit with regard to planned restoration. The translocation of hundreds of fish from Coyote Valley and Fly Valley Creeks into upper Silver King Creek, and the apparent loss of an allele in the upper Silver King sample, displays the difficulty of estimating what contribution is necessary from donor populations when restocking fishless habitat. Future translocation efforts should ideally consist of large numbers of fish from multiple donor populations containing as much genetic variation as possible in order to minimize loss of diversity and the effects of inbreeding. Ultimately, management goals should include reconnection of as many of the extant populations in the Silver King Creek basin as possible. Efforts to reconnect PCT populations above Llewellyn Falls and re-establish uncompromised populations from below the falls downstream to the gorge in Silver King Creek Canyon are already in the planning stage (Will Somer, CDFG, personal communication). Out-of-basin PCT populations such as North Fork Cottonwood Creek and Four Mile Canyon Creek may constitute an important part of any restoration efforts, since unique alleles were found in both of these samples. Additionally, the development of molecular markers that can distinguish between LCT and PCT would be important for determining their genetic relationship and investigating the possibility of introgressive hybridization between the two groups prior to any restorations.

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