

# Across the great divide: genetic forensics reveals misidentification of endangered cutthroat trout populations

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## Abstract

**Accurate assessment of species identity is fundamental for conservation biology. Using molecular markers from the mitochondrial and nuclear genomes, we discovered that many putatively native populations of greenback cutthroat trout (*Oncorhynchus clarkii stomias*) comprised another subspecies of cutthroat trout, Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus*). The error can be explained by the introduction of Colorado River cutthroat trout throughout the native range of greenback cutthroat trout in the late 19th and early 20th centuries by fish stocking activities. Our results suggest greenback cutthroat trout within its native range is at a higher risk of extinction than ever before despite conservation activities spanning more than two decades.**

*Keywords:* AFLP, conservation genetics, cutthroat trout, endangered species, forensics, microsatellite

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## Introduction

Cutthroat trout (*Oncorhynchus clarkii*) are widely distributed throughout the drainage basins of inland western North America. Since the first description of the species in 1541 from the upper Pecos River in New Mexico, 14 subspecies have been described based on morphology, genetics, and geography (Behnke 2002). Of these, two have gone extinct within the last century, several are listed under the Endangered Species Act as Threatened or Endangered, and many are candidates for federal protection. One of the federally protected subspecies is the greenback cutthroat trout (*Oncorhynchus clarkii stomias*). The historic native range of greenback cutthroat trout included streams and lakes of Colorado's South Platte and Arkansas River drainages on the east side of the Continental Divide. Over the last 150 years, the species' range declined from over 13 000 km of lakes, rivers and streams to about 70 km (Young & Harig 2001). This decline occurred as a consequence of mining

pollution, fishing pressure, and displacement by non-native salmonids introduced by state and federal hatcheries and private enterprises for recreational and sustenance fisheries (Young & Harig 2001). By 1919, greenback cutthroat trout persisted in only a handful of tributaries of the upper Arkansas River and in 1937, the subspecies was declared extinct.

In the 1950s, the subspecies was resurrected when a putatively pure population was discovered in a headwater stream of the South Platte River. Subsequently, additional relict greenback cutthroat trout populations were found in high-elevation streams (above 2100 m) of the South Platte and Arkansas River drainages. These discoveries prompted federal protection for the taxon under the US Endangered Species Act (USFWS 1978) and a management and restoration plan was implemented. Over more than two decades, greenback cutthroat trout were propagated in hatcheries, appropriate habitats were identified and cleared of non-native salmonids, and large numbers of cutthroat trout were introduced in an attempt to reach the conservation goal of 20 self-sustaining greenback cutthroat trout populations (Young & Harig 2001). In many places, populations were subject to regular monitoring and habitats received

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**Table 1** Study populations are listed with population abbreviations, major and minor drainage, latitude, longitude, and the number of individuals in each population that were included in the mitochondrial, microsatellite and AFLP data sets

Pop. name	Pop. abbr.	Major drainage	Sub-drainage	Lat.	Long.	COI ND2 mtDNA tree	ND2 AMOVA	Microsat	AFLPs
South Prong Hayden Creek	HAY	Arkansas River	Arkansas River	38.31	-105.82	9	15	30	19
Severy Creek	SEV	Arkansas River	Cascade Creek	38.88	-105.03	8	10	10	10
Bear Creek	BER	Arkansas River		38.80	-104.95	8	17	20	20
South Apache Creek	APA	Arkansas River	Huerfano River	37.86	-105.01	16	21	30	20
Graneros Creek	GRA	Arkansas River	St. Charles River	37.89	-105.00	13	20	30	20
Hunters Creek	HUN	South Platte	St. Vrain Creek	40.21	-105.59	14	16	30	20
Como Creek	COM	South Platte	Boulder Creek	40.03	-105.53	14	20	30	19
Dry Gulch	DRY	South Platte		39.70	-105.87	8	17	30	20
Little South Fork Poudre	PDR	South Platte	Cache la Poudre River	40.60	-105.54	5	17	26	18
South Fork Slater	SLT	Colorado River	Little Snake River	40.83	-107.30	9	14	14	14
South Fork Parachute Creek	PAR	Colorado River	Colorado River	39.63	-107.99	5	10	10	10
Lake Nanita	NAN	Colorado River	Colorado River	40.48	-104.77	10	19	24	22
East Fork Piedra Creek	PIE	Colorado River	San Juan River	37.53	-107.05	12	19	29	20
Big Beaver Creek	BVR	Colorado River	White River	40.05	-107.62	4	20	22	20
West Antelope Creek	ANT	Colorado River	Gunnison River	38.66	-107.04	10	21	30	24

multiple introductions of fish over the course of several years. The intensive recovery effort was recently declared a success and the taxon is poised for removal from the list of federally protected species (USGS 2006).

As part of a study on the population genetics of greenback cutthroat trout, we surveyed all putatively pure, native populations of greenback cutthroat trout from the Arkansas and South Platte River drainages on the east slope of the Continental Divide in Colorado and several reportedly pure populations of the Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus*) native to the Colorado River system west of the continental divide. Colorado River cutthroat trout is thought to be the sister taxon of greenback cutthroat trout (Behnke 2002). Included in our survey were populations used as sources of brood stock for propagating greenback cutthroat trout used in the recovery effort. We discovered an unexpected and complicated distribution of genotypes best explained by extensive propagation and movement of cutthroat trout that began in the 19th century. Furthermore, fewer relict populations of pure greenback cutthroat trout exist than previously thought and restoration populations may not be greenback cutthroat trout because non-native trout contaminated brood stocks. Our results imply that more than two decades of work towards bringing the species back from extinction have failed to improve the species' status.

## Methods

### Samples

All populations surveyed from the South Platte and Arkansas river drainages were previously identified as

pure greenback cutthroat trout based on morphological and genetic data (Behnke 1976; Young *et al.* 2002). With the exception of Lake Nanita, all populations surveyed from the Colorado River drainage were putatively pure native Colorado River cutthroat trout and included representative populations from five major subdrainages: Yampa River, White River, Colorado River, Gunnison River, and the San Juan River. Lake Nanita was included because it is considered pure Colorado River cutthroat trout and has been used widely for brood stock in the state of Colorado, although the population probably originated from fish propagation activities before 1950. We extracted DNA from adipose fin clips stored in 70% ethanol using QIAGEN DNeasy extraction kits according to manufacturer's instructions. Detailed information on samples can be found in Table 1.

### Sequencing and phylogenetic analysis of mitochondrial DNA

We targeted two regions of the mitochondrial genome for polymerase chain reaction (PCR) amplification and DNA sequencing: a 641-bp region of the cytochrome oxidase I (COI) gene flanked by the primers COI F (5'-ATCTCTCA-GTACCAAACCCC-3') and COI- aH redo (5'-CACAGTG-TRTAGGCGTCTGG-3') and an 889-bp region of the NADH dehydrogenase 2 (ND2) mitochondrial gene flanked by primers NdintF4 (GGGCAGTGGCACAAACTATT) and NDVarR (GCTTTGAAGGCTCTTGGTCT) (Novak *et al.* 2005). COI was amplified using 1× buffer (New England Biolabs), 1 mM dNTPs, 0.25 U *Taq* polymerase (NEB) at 94 °C for 2 min, 94 °C 30 s, 55 °C 45 s, and 72 °C 75 s for 35 cycles and a 72-°C extension for 5 min. ND2 was amplified

using 1× buffer, 1 mM dNTPs, 0.25 U *Taq* polymerase (NEB) at 94 °C for 2 min, 94 °C 30 s, 58 °C 45 s, and 72 °C 75 s for 35 cycles and a 72-°C extension for 7 min. Sequences were edited and aligned in SEQUENCHER 4.6. A total of 10 unique haplotypes were defined within the range of greenback cutthroat trout and Colorado River cutthroat trout by the concatenated COI and ND2 sequences from analysis of 146 individuals (GenBank Accession nos EF673223–EF73232 and EF673250–EF673259). We also sequenced the COI and ND2 genes for individuals from several other subspecies of cutthroat trout, including representative populations from the Rio Grande drainage (*Oncorhynchus clarkii virginialis*), the Lahontan basin (*Oncorhynchus clarkii henshawi*), the Bonneville Basin of Utah (*Oncorhynchus clarkii utah*), and the Snake River drainage (*Oncorhynchus clarkii bouvieri*) (GenBank Accession nos EF673233–EF673249 and EF673260–EF673276).

We inferred phylogenetic relationships among distinct haplotypes using Bayesian methods, implemented in MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001). Using MRMODELTEST (Nylander 2004), we found the best evolutionary models for COI was HKY + I and for ND2 was GTR + I based on Akaike information criterion (AIC). Using MRBAYES 3.1.2, we combined the results from two separate runs of 1 000 000 generations using an empirically determined burn-in of 28 000 generations.

### Genotyping

We used two sources of data to examine nuclear genetic variation: microsatellite allele length polymorphism and amplified fragment length polymorphism (AFLP). Genetic identification services (GIS, www.genetic-id-services.com) genotyped 367 individuals for 10 microsatellite loci using protocols previously described (Pritchard *et al.* 2007). AFLP reactions were completed for 276 individuals (Table 1) using the Applied Biosystems AFLP Plant Mapping Kit. The simultaneous restriction and ligation (R–L) was completed in a PerkinElmer 9700 thermal cycler at 37 °C for 2 h. Instead of the suggested 1 µL for both the *MseI* and *EcoI* adaptor pairs, 1.5 µL and 2 µL were used, respectively. All dilutions were made using 0.2× Buffer AE from QIAGEN (2 mM Tris-Cl, 0.1 mM EDTA, pH 9.0). The R–L product was diluted to 0.2× for use in the preselective amplification, and the preselective product was diluted to 0.05× for use in the selective amplification. The following parameters were used for the selective amplification: 2-min denaturation at 94 °C, 10 touchdown cycles with the annealing temperature changing from 66 to 56 °C by 1 °C per cycle (20 s at 94 °C, 30 s at 66–56 °C, 2 min at 72 °C), 20 cycles of 20 s at 94 °C, 2 min at 56 °C, 2 min at 72 °C, followed by a 30-min extension step at 60 °C. All amplifications were completed in a PerkinElmer 9700 thermal cycler using the 9600 ramp speed. Two selective primer combinations were used to

amplify and fluorescently tag the fragments: *Eco*-act and *Eco*-agg, *Mse*-cag and *Mse*-caa. Samples were denatured in Hi-Di formamide and sized on an ABI PRISM 3130 Genetic Analyser at a 5-kV injection voltage with an internal ROX-500 dye (ABI). GENEMAPPER 4.0 software was used to analyse and score the markers for two alleles (present/absent), producing a binary matrix.

### STRUCTURE analysis

A Bayesian clustering analysis implemented in STRUCTURE (Pritchard *et al.* 2000) provided the basis to assess the degree of concordance across multiple assessments of genetic variation. We used the program to cluster individuals into two groups ( $K = 2$ ) to test whether the nuclear data were congruent with the two mitochondrial DNA (mtDNA) lineages present within the geographical range of greenback cutthroat trout and Colorado River cutthroat trout (see results). STRUCTURE simulations assumed a model of admixed ancestry and independent allele frequencies between groups. For microsatellite data, 20 separate simulations were run, each with a burn-in of 100 000 generations and a subsequent Markov chain of 1 000 000 generations. For AFLPs, 20 separate simulations were run, each with a burn-in of 100 000 generations and a subsequent Markov chain of 500 000 generations.

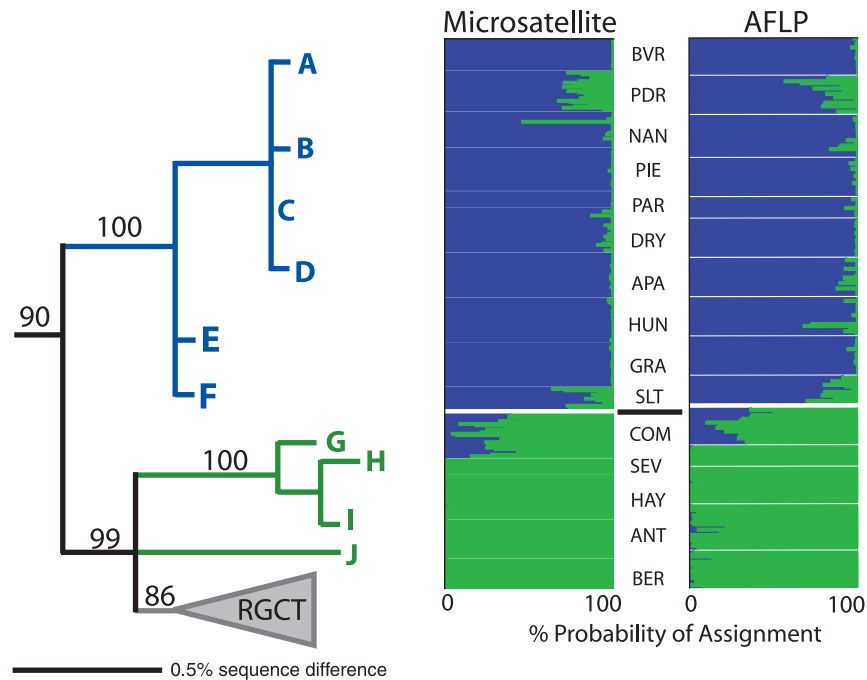
### Estimates of population differentiation

We assessed the distribution of molecular variation within and among populations for the three data types using an analysis of molecular variance (AMOVA) implemented in ARLEQUIN version 2.0 (Schneider *et al.* 2000). We defined two groups based on the current subspecies designations; notably, the two groups are separated by the Continental Divide.

To confirm whether migration had occurred recently among populations, we employed a semi-Bayesian multi-locus assignment test available in GENECLASS 2.0 to assign each individual to its most likely population of origin based on microsatellite data (Piry *et al.* 2004). A probability of assignment was calculated for each individual based on 10 000 random genotypes that were simulated for each reference population (Paetkau *et al.* 2004). Individuals with assignment probabilities of less than 0.05 to any population were excluded from consideration.

### Results

Phylogenetic analysis of the combined COI and ND2 mitochondrial gene sequences (1530 bp) revealed two divergent lineages within the ranges of greenback cutthroat trout and Colorado River cutthroat trout consisting of 10 unique haplotypes (Fig. 1). The average uncorrected



**Fig. 1** A phylogram of mtDNA sequence data and *STRUCTURE* bar plots from microsatellite and AFLP data. Ten unique greenback cutthroat trout and Colorado River cutthroat trout haplotypes were evident from 1530 bp of COI and ND2 mitochondrial sequence data. Rio Grande cutthroat trout haplotypes from the Rio Grande drainage in Colorado and New Mexico are summarized by a grey triangle. Yellowstone cutthroat trout and Lahontan cutthroat trout were used to root the phylogenetic tree. Posterior probabilities from a Bayesian analysis of phylogenetic hypotheses are shown for relevant nodes. Bayesian clustering of genetic variation for 10 microsatellite loci and 401 AFLP loci in *STRUCTURE* provided corroborating support for two distinct evolutionary clades within Colorado. Probabilities of assignment of each individual to each cluster (green or blue) are shown along the x-axis. A horizontal line represents an individual and white lines designate populations. Population abbreviations are defined in Table 1. The branches and haplotype letters in the phylogram were coloured to represent the major split among lineages discovered in the nuclear and mtDNA data.

sequence divergence between the two lineages was 1.7%; the corresponding value assuming the Hasegawa–Kishino–Yano (HKY) model of sequence evolution was 1.8%. These divergent lineages differ as much from each other as they do from other recognized subspecies. The average divergence among cutthroat trout haplotypes from the Lahontan Basin in Nevada, the Bonneville Basin of Utah, the Snake River system in Wyoming, and the Rio Grande River system of New Mexico was 1.6% (1.7% assuming the HKY model). For the same gene regions, the degree of divergence between rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout was 6.6% (8.7% assuming the HKY model). Times of divergence for rainbow and cutthroat trout range from 3.5 to 8 million years ago (McKay *et al.* 1996; Smith *et al.* 2002), suggesting the cutthroat trout lineages within the geographical range of greenback cutthroat trout and Colorado River cutthroat trout diverged about 0.7–2.0 million years ago.

Bayesian cluster analysis assuming two groups ( $K = 2$ ) for 10 microsatellite loci and 401 AFLP loci showed strong concordance with the genetic break defined by the mtDNA

(Fig. 1): 12 of the 15 populations sampled grouped with high probability ( $q > 0.95$ ) into one or the other genotype cluster. Values for Como Creek were the lowest:  $q$  was approximately 0.67 for both the microsatellite and AFLP data. The average  $q$  values from the microsatellite and AFLP data for South Fork Poudre and South Fork Slater populations were 0.84 and 0.87, respectively.

Unexpectedly, the divergent evolutionary lineages defined by mitochondrial and nuclear DNA markers did not separate geographically on either side of the Continental Divide (Fig. 2). One lineage was common on the west slope, comprising five of the six populations surveyed. We designated this lineage Colorado River cutthroat trout. Colorado River cutthroat trout also was common in the Arkansas and South Platte rivers east of the divide. The other lineage, likely corresponding to greenback cutthroat trout, was restricted to four populations on the east slope, and one population on the west slope.

AMOVA on the mtDNA, microsatellite and AFLP data revealed a lack of significant partitioning of genetic variation between the west and east slopes of the Rocky Mountains,

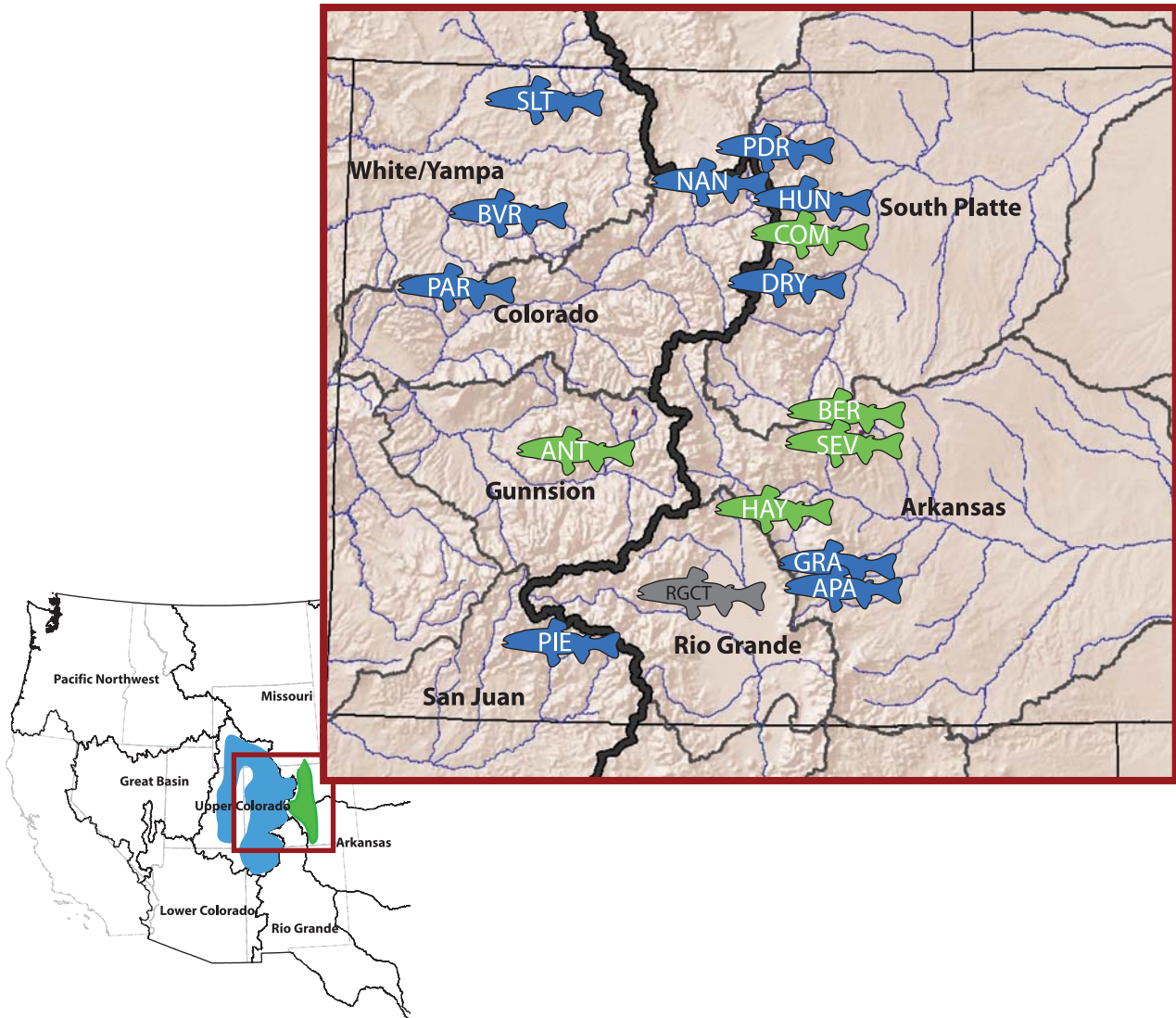
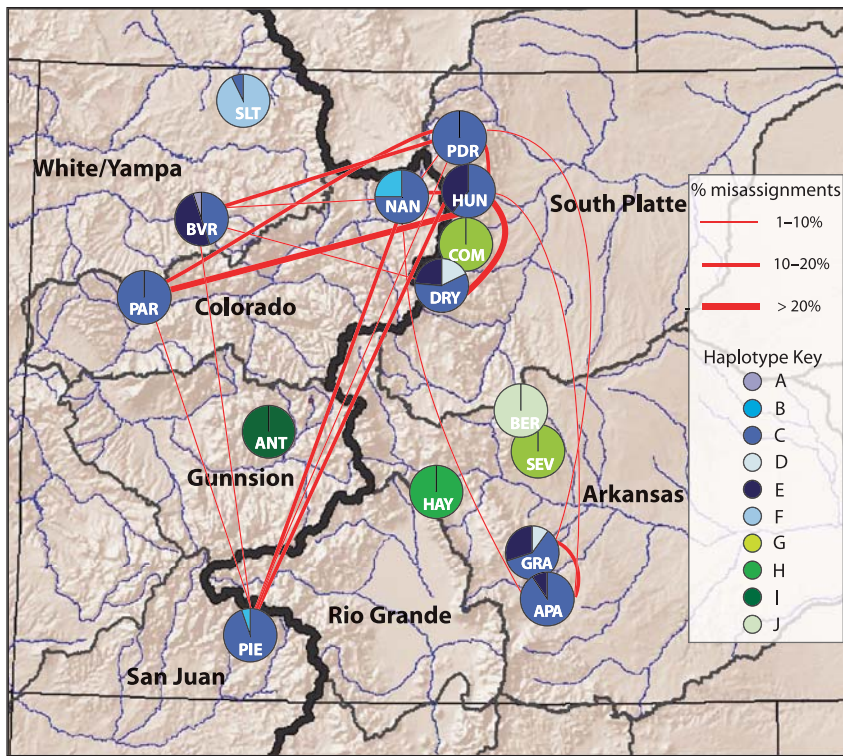


Fig. 2 Left, map showing a portion of inland western United States. Blue and green depict the ancestral range maps of Colorado River cutthroat trout and greenback cutthroat trout, respectively. Right, the portion within the rectangle is enlarged (right) showing the approximate locations of populations surveyed (abbreviations correspond to Table 1) and subspecies designations determined in this study, where blue is Colorado River cutthroat trout and green is greenback cutthroat trout. The current locations of the two subspecies are out-of-place with respect to the putative ancestral ranges. The thick black line running down the centre of the figure indicates the Continental Divide and the labelled sub-basins on each slope are delineated with thinner black lines. A grey fish represents the native Rio Grande cutthroat trout, which is found in the Rio Grande drainage in southern Colorado and New Mexico.

with less than 1% of the genetic variation distributed across the divide for all three molecular markers. AMOVA showed similar patterns across the two sets of nuclear markers, with about 40% and 60% of the genetic variation partitioned among and within populations, respectively ( $P < 0.0001$ ). The vast majority of the variation for mtDNA was partitioned among populations ( $\approx 95\%$ ;  $P < 0.0001$ ) with only a small amount of variation ( $\approx 5\%$ ) within populations. Low within-population mtDNA diversity was particularly evident for genetically defined greenback

cutthroat trout populations sampled: all five populations lacked variation (Fig. 3). Notably, two populations (Severy and Como) shared the same haplotype. Assignment tests for the microsatellite data mirrored patterns suggested by the AMOVA results; namely, the data suggested recent gene exchange across the Continental Divide for populations identified as Colorado River cutthroat trout, even between geographically remote populations (e.g. San Juan to South Platte) (Fig. 3). By contrast, all of the individuals from greenback cutthroat trout populations assigned to their



**Fig. 3** Proportions of ND2 mtDNA haplotypes and misassignments based on microsatellite data in each population. Haplotype pie chart colours correspond to haplotypes (A–J) depicted in Fig. 1. Population abbreviations shown on haplotype pie charts correspond to Table 1. The percentage of misassignment between populations using 10 microsatellite loci are illustrated with thin red lines (1–10%), medium red lines (10–20%), and thick red lines (greater than 20%). Note that lines connect only the Colorado River cutthroat populations (blue fish) and the largest inferences of recent gene flow are mostly confined to comparisons across the continental divide.

population of origin. Overall, these results suggested gene exchange within the Colorado River cutthroat trout subspecies was more frequent across the Continental Divide than between sampled localities within a drainage basin.

### Discussion

Two divergent lineages were discovered from this set of populations using a combination of mtDNA and nuclear gene analyses. These two lineages most likely correspond with two described subspecies: greenback cutthroat trout (*Oncorhynchus clarkii stomias*) and Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus*). Before our assessment using molecular markers, nine greenback cutthroat trout populations were recognized: Hunters Creek, Como Creek, South Fork of the Poudre River, and Dry Gulch in the South Platte river system; and South Prong Hayden Creek, Severy Creek, South Apache Creek, Graneros Creek, and Bear Creek within the Arkansas River basin. Our molecular assessment revealed that most (five) of the putatively relict greenback cutthroat trout populations were Colorado River cutthroat trout and more similar to populations west of the Continental Divide in the Colorado River basin than to greenback cutthroat trout populations in the Arkansas or South Platte rivers.

The observed similarity of populations across the Continental Divide and between geographically remote

sub-basins can be explained by recent natural migrations (Behnke 1976). Several lines of evidence suggest that this hypothesis is unlikely, however. Although geologically recent interbasin transfers of fish have been hypothesized for the intermountain west (Smith *et al.* 2002), such events have not been recorded for the Rockies of Colorado. Moreover, repeated advances and retreats of mountain glaciers that may have caused a shifting allegiance of high-elevation streams likely ended 10 000 years ago (Menounos & Reasoner 1997). If the interbasin movement of fish occurred 10 000 years ago or longer, extraordinarily large long-term effective population sizes —  $N_e = t / \{2 \ln[1 / (1 - F_{ST})]\} = N_e \sim 7000$  — would be required to explain the observed low levels of differentiation between Colorado River cutthroat trout populations on either side of the Continental Divide (Nei *et al.* 1977; Waples *et al.* 2004). Yet surveys of contemporary populations of cutthroat trout suggest  $N_e$  is one to three orders of magnitude smaller (Neville *et al.* 2006) and estimates of effective population size for Colorado River cutthroat trout populations in this study using methods relying on linkage disequilibrium (Hill 1981) and software developed by R.S. Waples ranged from 3 to 518, with an average of 73. Finally, the Continental Divide defines a clear biogeographical separation of North American freshwater fish faunas, implying long-term isolation for the two regions (Simons & Mayden 1998).

As a means of establishing the expected degree of differentiation for populations within and between drainages in

**Table 2** Partial summary from the State Archives and Fisheries Commission reports of greenback cutthroat trout (GCT) and Colorado River cutthroat trout (CRCT) movement across the Continental Divide and between the Arkansas and South Platte rivers that involved localities near to sample sites. The taxon stocked is identified as greenback cutthroat trout or Colorado River cutthroat trout based on geographical source. If the source is unknown and the historic records listed 'native trout,' we use the code 'CUT'; these fish were most likely Colorado River cutthroat trout. Note that this list is incomplete because of missing records and the failure to record sufficient information from which to determine species, source, or destination of stocking efforts

Taxon	Year	Source	Destinations	Closest study site (subspecies identified by this study)
GCT	1894	Leadville Area, Arkansas R.	Tomichi Creek, near Sargents, Gunnison R.	ANT (GCT)
GCT	1894	Twin Lakes, Leadville area, Arkansas R.	Gunnison R.	ANT (GCT)
CRCT	1893	Hetzer's Lake, Grand County, Colorado R.	Twin Lakes, Leadville area, Arkansas R.	HAY (GCT)
CRCT	1899	Emerald Lake, San Juan R.	Streams near Salida, Arkansas R.	HAY (GCT)
CRCT	1899	Emerald Lake, San Juan R.	Lakes near Leadville, Arkansas R.	HAY (GCT)
CRCT	1900	Grand Mesa Lakes, Colorado R.	Streams in Lake County, Arkansas R.	HAY (GCT)
CRCT	1901	Emerald Lake, San Juan R.	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
CRCT	1901	Grand Mesa Lakes, Colorado R.	Pueblo Co., Arkansas R.	GRA, APA (CRCT)
CRCT	1901	Grand Mesa Lakes, Colorado R.	St. Vrain R., S. Platte R.	HUN (CRCT)
CRCT	1901	Grand Mesa Lakes, Colorado R.	Boulder Creek, S. Platte R.	COM (GCT/CRCT)
CRCT	1914	Grand Mesa Lakes, Colorado R.	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
CRCT	1915	Grand Mesa Lakes, Colorado R.	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
CRCT	1925	Grand Mesa Lakes, Trappers Lake, or Marvine Lake	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
CRCT	1927	Grand Mesa Lakes, Trappers Lake, or Marvine Lake	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
CUT	1931	Grand Mesa Lakes, Trappers Lake, or Marvine Lake	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
CUT	1932	Grand Mesa Lakes, Trappers Lake, or Marvine Lake	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
CUT	1932	Grand Mesa Lakes, Trappers Lake, or Marvine Lake	Graneros Creek, Pueblo Co., Arkansas R.	GRA, APA (CRCT)
CUT	1934	Grand Mesa Lakes, Trappers Lake, or Marvine Lake	Graneros Creek, Pueblo Co., Arkansas R.	GRA, APA (CRCT)
CUT	1935	Grand Mesa Lakes, Trappers Lake, or Marvine Lake	Upper Poudre River, Larimer Co., S. Platte R.	PDR (CRCT)
GCT	1893	Arkansas River	Mammoth Cr, S. Platte R.	COM (GCT)
GCT	1893	Arkansas River	Michigan Cr, S. Platte R.	COM (GCT)
GCT	1894	Arkansas River	Upper Boulder Cr, S. Platte R.	COM (GCT)
GCT	1894	Arkansas River	Mammoth Cr, S. Platte R.	COM (GCT)
GCT	1897	Arkansas River	Mammoth Cr, S. Platte R.	COM (GCT)

the range of Colorado River cutthroat trout, we estimated the genetic differentiation between populations based on microsatellite loci for a closely related subspecies, the Rio Grande cutthroat (*Oncorhynchus clarkii virginalis*), in three river drainages in New Mexico. Average observed within-population heterozygosity was similar for Rio Grande cutthroat trout and Colorado River cutthroat trout (0.42 and 0.44, respectively) as was allelic richness (2.62 and 3.07, respectively). The average  $F_{ST}$  within and between drainages for Rio Grande cutthroat trout was 0.37 and 0.49, respectively (Fig. S1, Supplementary material), whereas the average  $F_{ST}$  between Colorado River cutthroat trout populations separated by the Continental Divide was 0.17 (Table S1, Supplementary material). Furthermore, *trans-divide*  $F_{ST}$  values for Colorado River cutthroat trout populations were smaller than 80% of the  $F_{ST}$  values for pairs of Rio Grande cutthroat trout populations from nearby locations *within the same drainage* and smaller than 100% of the  $F_{ST}$  values for pairs of Rio Grande cutthroat trout populations sampled from *different drainages*. Put differently, the degree of differentiation for Colorado River

cutthroat trout populations separated by the Continental Divide was less than the differentiation between samples of Rio Grande cutthroat trout from nearby localities within the same river in New Mexico.

A long history of propagation and movement of trout throughout Colorado (Wiltzius 1985) prompted us to survey historical records for evidence of fish movement to explain the checkerboard pattern of greenback cutthroat trout and Colorado River cutthroat trout lineages in the Arkansas and South Platte drainages. Records in the State Archives and Fisheries Commission reports beginning in the late 1800s revealed abundant evidence for repeated movement of (i) Colorado River cutthroat trout from west slope sources to sites across the entire historical range of greenback cutthroat trout; (ii) greenback cutthroat trout from the east slope to selected west slope localities; and (iii) fish between the Arkansas and South Platte and among sub-basins within the Colorado River basin (Table 2). In fact, before the rediscovery of greenback cutthroat trout in 1953, more than 300 million cutthroat trout had been distributed across Colorado through the state and federal

hatchery programmes (Wiltzius 1985). Many private trout production facilities also propagated and distributed cutthroat trout throughout the state, and scattered records indicate anglers actively moved fish from stream to stream, sometimes over the continental divide (Wiltzius 1985). Stocking records provide explanations for similarity of populations despite wide geographical separation. For example, the southernmost population sampled (East Fork Piedra) from the San Juan drainage of the west slope and the northernmost population sampled from the South Platte drainage on the east slope (South Fork Poudre River) shared identical mtDNA haplotypes and had individuals misassigned between populations. Early records indicate Colorado River cutthroat trout derived from Emerald Lakes in the San Juan drainage of southern Colorado were actively propagated and distributed across the divide to the rivers of the Front Range, including the Poudre River (Table 2). Additionally, greenback cutthroat trout from the Arkansas River were propagated and stocked in west slope waters of the Gunnison and Colorado Rivers (Table 2) providing an explanation for the similarity of fish from the Gunnison and Arkansas rivers despite being separated by the Continental Divide.

Unfortunately, the movement of fish across basins within Colorado has compromised the intensive greenback cutthroat trout recovery efforts implemented over the last two decades. The brood stocks developed for the Arkansas River and South Platte River were derived from populations of Colorado River cutthroat trout, or from a combination of greenback cutthroat trout and Colorado River cutthroat trout. For instance, the Arkansas River brood-stock included fish from South Apache Creek and Graneros Creek, both populations identified as Colorado River cutthroat trout based on our genetic analysis. The main South Platte River brood-stock included South Fork Poudre River, Hunters Creek, and Como Creek fish. Our genetic data identified South Fork Poudre and Hunters Creek as Colorado River cutthroat trout. Como Creek appears to be a greenback cutthroat trout, although the population may not be native to the South Platte because all individuals possessed an mtDNA haplotype sampled from Severy Creek in the Arkansas River drainage. Identical mitochondrial haplotypes in headwater streams from two widely separated drainages is highly unlikely, especially for trout that typically show strong genetic differentiation between different drainage basins (MacHordom *et al.* 2000; McCusker *et al.* 2000). Furthermore, early stocking records indicated movement of greenback cutthroat trout from sources in the Arkansas River to Boulder Creek watershed (Como Creek is within the Boulder Creek watershed) on three separate occasions (Table 2).

Before our work, there were nine putatively pure relict populations of greenback cutthroat trout spanning most of the ancestral range of the species; furthermore, the recovery

goal of 20 self-sustaining populations had been established by the restoration activities. Our genetic data suggest that within the native range of greenback cutthroat trout, only four pure greenback cutthroat trout populations exist that collectively inhabit about a dozen kilometres of stream habitat. An additional discovery was that within each of these populations, we failed to detect mtDNA diversity and levels of nuclear gene diversity were low [average  $H_{OBS} (\pm 1 SD) = 0.23 \pm 0.11$ ] relative to other cutthroat trout suggesting the remaining greenback cutthroat trout populations may suffer from inbreeding depression or a high genetic load.

Our discovery of a greenback cutthroat trout population on the west slope (West Antelope Creek) in the Gunnison River Basin (Fig. 2) and perusal of genetic information for many west slope trout available in agency reports suggest there may be populations of greenback cutthroat trout on the west slope that retain some of the genetic variation that was present before greenback cutthroat trout were nearly exterminated from the drainages of the Front Range. Greenback cutthroat trout were actively propagated in hatcheries and stocked in several locations from 1885 to 1896 (Wiltzius 1985), including high elevation streams in the Gunnison River and Colorado River basins (Table 2). Ironically, trout stocking activities that contributed to the decline of greenback cutthroat trout and compromised recovery efforts preserved portions of the ancestral gene pool that ultimately may prove useful for managing the genetic diversity of greenback cutthroat trout. A thorough genetic investigation of cutthroat trout populations on the west slope of Colorado's Continental Divide is critical to the recovery of greenback cutthroat trout.

Errors in taxonomy can compromise our understanding of nature (Knowlton *et al.* 1992) and can lead to the irreversible loss of biodiversity (Avice & Nelson 1989). Effective implementation of the Endangered Species Act depends on accurate identification of species and elucidation of their geographical distribution. This seemingly simple task is complicated because in any one place, existing biological diversity reflects contributions from both *in situ* diversification of native species and recent additions of species associated with human activity (Rahel 2000). In some cases, introduced species may be mistakenly considered native because invasions or introductions happened before the distribution and biological characteristics of native species were documented by biologists (Willis & Birks 2006). These problems have certainly confounded status assessments of the greenback cutthroat trout, *O. c. stomias*. By the time David Starr Jordan discovered this native trout in the clear waters of the Rocky Mountains east of the Continental Divide, non-native salmonids had been introduced and many populations of greenback cutthroat trout had probably already suffered localized extirpations. Yet, as we



demonstrate, it is possible to sort out what is native from diversity introduced directly or indirectly by the industrious environmental engineering of humans. The challenge now is to use our knowledge and continue engineering the landscape towards the goal of restoring native species across their ancestral ranges.

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Jessica Metcalf recently completed her Ph.D. work on greenback cutthroat trout conservation genetics at the University of Colorado. She is currently working on cutthroat trout phylogeography and sequencing DNA from cutthroat trout museum samples. She is also looking for a postdoc position. Andrew Martin is an associate professor at the University of Colorado researching evolutionary questions about trout, sharks, prairie dogs, and microbes. The other authors work on various cutthroat trout research projects.

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## Supplementary material

The following supplemental material is available for this article:

**Fig. S1** A histogram showing Rio Grande cutthroat trout pairwise  $F_{ST}$  values.

**Table S1** Pairwise  $F_{ST}$  values for 10 microsatellite loci for greenback cutthroat trout and Colorado River cutthroat trout

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