

Historical stocking data and 19th century DNA reveal human-induced changes to native diversity and distribution of cutthroat trout

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Abstract

Many species are threatened with extinction and efforts are underway worldwide to restore imperilled species to their native ranges. Restoration requires knowledge of species' historical diversity and distribution. For some species, many populations were extirpated or individuals moved beyond their native range before native diversity and distribution were documented, resulting in a lack of accurate information for establishing restoration goals. Moreover, traditional taxonomic assessments often failed to accurately capture phylogenetic diversity. We illustrate a general approach for estimating regional native diversity and distribution for cutthroat trout in the Southern Rocky Mountains. We assembled a large archive of historical records documenting human-mediated change in the distribution of cutthroat trout (*Oncorhynchus clarkii*) and combined these data with phylogenetic analysis of 19th century samples from museums collected prior to trout stocking activities and contemporary DNA samples. Our study of the trout in the Southern Rocky Mountains uncovered six divergent lineages, two of which went extinct, probably in the early 20th century. A third lineage, previously declared extinct, was discovered surviving in a single stream outside of its native range. Comparison of the historical and modern distributions with stocking records revealed that the current distribution of trout largely reflects intensive stocking early in the late 19th and early 20th century from two phylogenetically and geographically distinct sources. Our documentation of recent extinctions, undescribed lineages, errors in taxonomy and dramatic range changes induced by human movement of fish underscores the importance of the historical record when developing and implementing conservation plans for threatened and endangered species.

Keywords: ancient DNA, conservation genetics, greenback cutthroat trout, historical records, *Oncorhynchus clarkii*, phylogeography

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Introduction

The diversity and distribution of many taxa have changed dramatically over the last couple of centuries largely in response to human activities. Extirpation of populations has resulted in cases in which the contemporary range underestimates the historical range

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(France & Collins 1993; Mingozi & Esteve 1997; Beaver *et al.* 2003; Sousa *et al.* 2010). In other cases, movement of individuals has expanded the distribution of species beyond their native range (Carlton 1996; McKinney & Lockwood 1999; Kowarik 2003). Sorting past and present patterns of diversity are further complicated by traditional taxonomic treatments that may not accurately reflect phylogenetic diversity (Graham *et al.* 2004; Pfenninger & Schwenk 2007). Historical records documenting the actions that ultimately altered diversity and distribution provide one means of assessing the cause and magnitude of change (Westley & Fleming 2011) and reconstructing historical distributions of species (Franco & Morgan 2007; Gil-Sanchez & McCain 2011). Another way to estimate regional diversity and historical distributions, particularly for taxa plagued with taxonomic uncertainties, is to analyse genetic data from samples collected prior to human activities (e.g. Valentine *et al.* 2008; Hansen *et al.* 2009; Paplinska *et al.* 2011; Iwamoto *et al.* 2012). In concert, these two approaches can lead to an understanding of how actions by humans have changed the diversity and distribution of species. This information is critical for establishing a baseline of historical conditions to guide restoration goals for species in decline or threatened with extinction.

Our study focuses on a biological icon of the western United States, the cutthroat trout (*Oncorhynchus clarkii*). We know from historical records that native trout suffered widespread extirpations (Allendorf & Waples 1996; Dunham *et al.* 1997; Behnke 2002; Harig & Fausch 2002; Novinger & Rahel 2003; Young 2009; USFWS 2010), were propagated and moved across the landscape (Wiltzius 1985; Pister 2001; Dunham *et al.* 2004), and that the taxonomic record is rife with errors (Behnke 2002; Metcalf *et al.* 2007). Here, we evaluate hypotheses about the distribution and diversity of cutthroat trout across seven major drainages encompassing the Pacific and Atlantic slopes of the Continental Divide in the Southern Rocky Mountains, North America.

The prevailing view of native diversity and distribution

Historically, four distinct subspecies of cutthroat were described from Colorado (Fig. 1). The Colorado River cutthroat trout (*O. c. pleuriticus*) was described as native to all major drainages of the western slope of the Continental Divide, including the San Juan, Gunnison, Colorado and Yampa River basins (Behnke 1992). The greenback cutthroat trout (*O. c. stomias*) was described from the Arkansas and South Platte basins east of the Continental Divide (Jordan 1891; Behnke 2002). The Rio Grande cutthroat trout (*O. c. virginialis*) was documented

from the Pecos, Canadian and Rio Grande Rivers on the east slope of the Continental Divide (Behnke 1992). The fourth taxon, the Yellowfin cutthroat trout (*O. c. macdonaldi*), was restricted to Twin Lakes in the headwaters of the Arkansas River (Jordan 1891; Behnke 1992). Both *O. c. macdonaldi* and *O. c. stomias* were declared extinct at one time (Behnke 1992), although the greenback cutthroat trout was purportedly rediscovered in the 1950s—an event that initiated a large-scale restoration effort aimed at re-establishing the subspecies to a large number of tributaries in both the South Platte and Arkansas river basins (Behnke 1969; Young & Harig 2001).

A published phylogenetic inference based on mitochondrial DNA (mtDNA) revealed four divergent lineages, which were additionally supported by clustering methods using both microsatellite and AFLP nuclear markers (Fig. 2) (Metcalf *et al.* 2007; Pritchard *et al.* 2009). While the number of taxa aligned with the prevailing view of cutthroat trout diversity in the Southern Rockies, the geographical distribution of subspecies did not. In fact, trout putatively identified as *O. c. stomias* and *O. c. pleuriticus* were found in streams and lakes on both slopes of the Continental Divide, a pattern interpreted as an effect of fish stocking (Metcalf *et al.* 2007). In addition, a fourth, divergent lineage was discovered, but it was only found in a single stream (Bear Creek) in the Arkansas River drainage. Whether this lineage represented a named subspecies, such as *O. c. stomias* or the extinct *O. c. macdonaldi*, could not be determined without understanding past diversity and the influence of stocking on the distribution of subspecies. These findings called into question the current taxonomy of cutthroat trout and prompted a thorough investigation into the potential effects of past stocking and propagation on the current diversity and distribution of cutthroat trout.

Extirpation and propagation effects

Review of historical records indicated that native trout populations in Colorado suffered dramatic declines beginning in the middle 1800s. Initially, trout populations were decimated by overfishing, mining pollution and agricultural practices (Supporting information; Young & Harig 2001). Coincidentally, a number of private individuals, and later, state and federal agencies, began propagating trout for commercial and recreational purposes (Wiltzius 1985). The first documented movement of native trout within the state occurred in 1873 (Miner July 8th, 1873). Although our research through the public records does not provide a complete account of the propagation and stocking activities by private citizens, what is clear from the newspaper

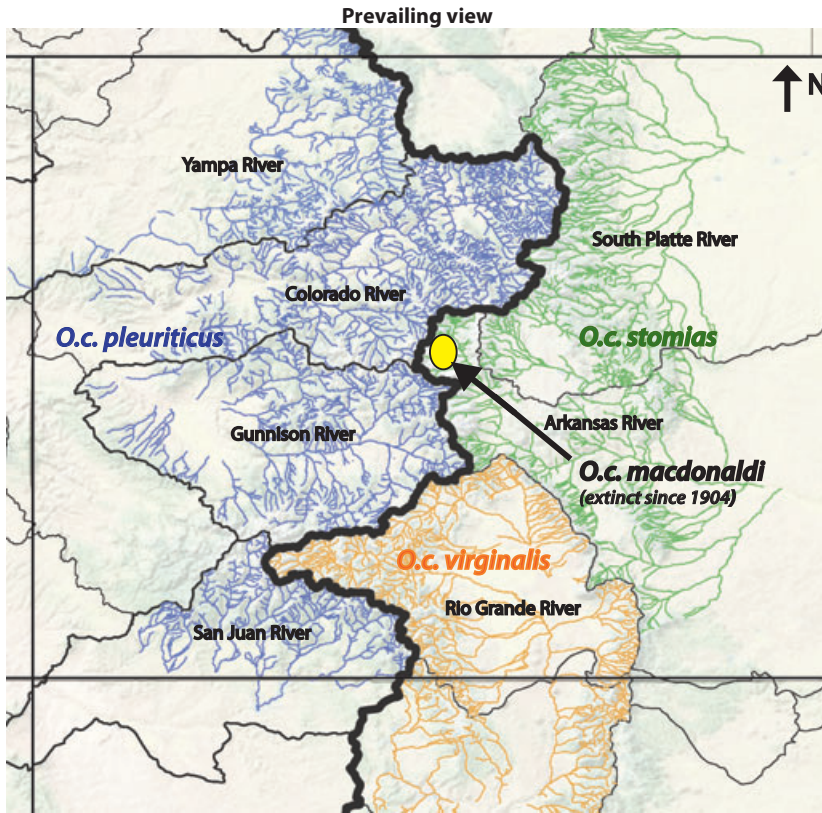


Fig. 1 The prevailing view of cutthroat diversity and distribution in Colorado and northern New Mexico. Four subspecies were described for Colorado. Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus*) was described for drainages west of the Continental Divide (blue). Greenback cutthroat trout (*O. c. stomias*) were described for drainages east of the Divide (green) in the South Platte and Arkansas River drainages. Yellowfin cutthroat trout (*O. c. macdonaldi*) were described for the Arkansas River drainage basin as well, albeit restricted to a small lake system (yellow circle). Finally, Rio Grande cutthroat trout (*O. c. virginalls*) were described for waters in the Rio Grande River basin east of the Divide in southern Colorado and New Mexico (orange).

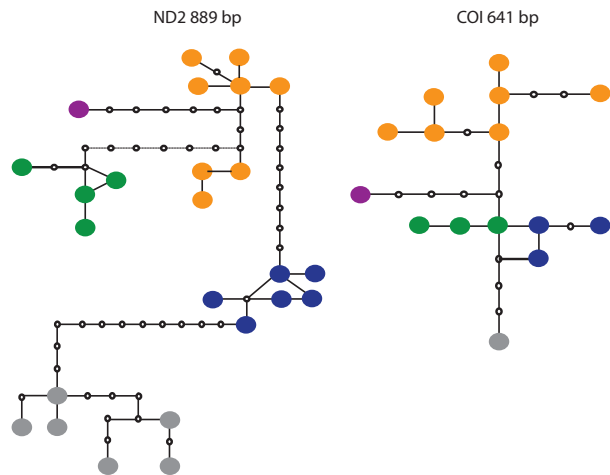


Fig. 2 Statistical parsimony haplotype network based on mitochondrial ND2 (889 basepairs) and COI (641 basepairs) sequence data sampled from modern populations. Four distinct mitochondrial lineages were sampled in Colorado (lineages shown in blue, green, purple and orange). The current distribution of blue, green and purple lineages does not correspond to the prevailing view in Fig. 1 (see Metcalf *et al.* 2007), and thus, their taxonomy is unclear. The lineage composed of haplotypes shown in orange corresponds to Rio Grande cutthroat trout, which is still found in its native range today (Rio Grande River drainage). Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*), are also included as an outgroup, shown in grey.

accounts is that trout propagation of both native and introduced species was occurring in Colorado in the early 1870s and continued until the present (Supporting information). State and federal hatcheries began propagation and stocking of trout for the perceived public good in the early 1880s. The number of trout propagated and stocked into the waters of Colorado was substantial. Between 1885 and 1953 there were 41 014 documented fish stocking events in Colorado by state or federal agencies. The vast majority of these involved brook trout (*Salvelinus fontinalis*), rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*O. clarkii*) (Fig. 3, supporting information). Remarkably, over 750 million fish of these three species were stocked from hatcheries into streams and lakes in Colorado over this period of time. Introductions of brook trout and rainbow trout probably had devastating effects on native cutthroat trout populations because brook trout are superior competitors and rainbow trout hybridize with cutthroat trout (Young & Harig 2001). The combined effects of pollution, over-fishing and the large-scale stocking of non-native trout taxa largely explained the widespread decline of native trout populations (Young & Harig 2001).

Although trout were imported into Colorado from across the globe, large numbers of trout native to the Southern Rocky Mountains were propagated and

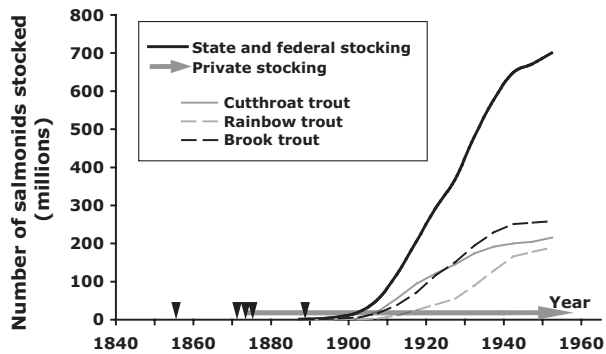


Fig. 3 Cumulative numbers (in millions of fish) of cutthroat trout, rainbow trout and brook trout stocked in Colorado waters from 1885 to 1953 by state and federal agencies. Private interests also stocked fish around the state, but their efforts were generally smaller in scale, and much less well documented. Dates of museum collections used in this study are indicated by arrowheads.

stocked throughout the state. Production of native cutthroat trout by federal fish biologists began in 1899 at the Grand Mesa Lakes region of the Gunnison River basin in Colorado. By 1909, that effort alone produced 29 000 000 trout in 798 lots that were stocked into lakes and streams of all major drainages on both the Pacific and Atlantic slopes in the state of Colorado. Stocking of Gunnison River basin trout across the state continued until 1931. In 1903, a second source of native cutthroat trout, also from the western slope of the Continental Divide (Trappers Lake), was brought into the hatchery system and propagated until 1938. For the period 1914–1925 when the stocking data were most complete, over 26 000 000 fish were stocked in 989 different lots to tributaries of all major drainages across the state. By contrast, cutthroat trout native to the east slope of the Continental Divide (*O. c. stomias*) were rarely used for large-scale hatchery broodstock. Importantly, many cutthroat trout were stocked into habitats that were originally fishless, usually above waterfalls that served as barriers to upstream movement of fish (Fausch *et al.* 2009). Therefore, the signal of the native phylogeography may have been erased by extirpation of native populations coupled with widespread stocking of western slope cutthroat trout in high alpine lakes and streams on both slopes of the Continental Divide. The stocking records suggest that the cutthroat trout lineages that are widespread today may be descendants of ancestors derived from two major drainages—the Gunnison and the Yampa—of Colorado’s western slope. The absence of commensurate large-scale hatchery production of native cutthroat trout (*O. c. stomias* and *O. c. macdonaldi*) east of the Continental Divide in Colorado meant that similar reservoirs of genetic

diversity may not have been available to buffer native trout declines in the Arkansas and South Platte basins.

In this study, we use DNA recovered from 19th century museum specimens of cutthroat trout to test three hypotheses about the native diversity and distribution of cutthroat trout in the Southern Rocky Mountains and how it has changed in recent times. First, we test the prevailing view that cutthroat trout diversity includes four divergent and distinct lineages within Colorado corresponding to the four named subspecies (H1). Also in line with prevailing view, we test that divergent lineages were largely confined to major drainage basins: *O. c. pleuriticus* to the San Juan, Gunnison, Colorado, Yampa and Green River drainages west of the Continental Divide; *O. c. stomias* to the Arkansas and South Platte east of the Continental Divide; *O. c. macdonaldi* was restricted to a pair of lakes in the upper Arkansas Basin; and *O. c. virginialis* to waters in the Rio Grande River drainage basin east of the Continental Divide (H2). Finally, based on the historical stocking data, we test that the current distribution of cutthroat trout subspecies differs markedly from the historical distribution, with trout native to the western slope of the Continental Divide becoming widespread beyond their native range (H3).

Methods

Museum specimen tissue collection, DNA extraction and sequence generation

Skin, gill, muscle and bone were sampled from cutthroat trout specimens stored in ethanol and housed at the California Academy of Sciences, Smithsonian Museum of Natural History, the Academy of Natural Sciences in Philadelphia and the Museum of Comparative Zoology at Harvard University (Table S1). Because intensive propagation and stocking of native trout began in earnest with state and federally operated hatcheries as early as 1885, with efforts expanding considerably in the very early years in the first decade of twentieth century, we focused our efforts on securing samples collected prior to the first decade of twentieth century. We note that a low-level of propagation and movement of trout by private interests probably began by the 1870s. We sampled specimens collected between the years 1857 and 1889 across seven drainage basins in Colorado and New Mexico: South Platte, Arkansas, Rio Grande, San Juan, Gunnison, Colorado and Yampa River drainage basins (Table 1). Collection locality details and notes were recorded (Table S1). As some museum specimens were collected after fish stocking activities initiated, albeit at a scale much lower than in the 20th century, some uncertainty in their native status

Table 1 For museum fish, the number of localities sampled within each drainage, year range of collection and number of individual successfully sequenced are listed

Drainage	Localities	Years	Individuals sequenced
South Platte	5	1871–1889	9
Arkansas	4	1889	8
Rio Grande	2	1873–1889	2
San Juan	1	1874	2
Gunnison	1	1889	2
Colorado	3	1889	4
White/Yampa	1	1889	1
Unknown	1	1857	2
Total	18	1857–1889	30

Thirty museum fish were sampled and successfully sequenced from seven drainages across a total of 18 different streams and lakes. Details on each museum fish, including an additional 14 samples that did not produce sufficient DNA for sequencing, can be found in Table S1.

exists. In the case of two samples with uncertain locality information (Behnke 2002)—the type specimens of *Oncorhynchus clarkii stomias* [Academy of Natural Sciences in Philadelphia (ANSP) 7825 and 7826]—additional historical research was conducted (results in Supporting Information). Tissue and bone material from these samples were collected using disposable, sterile metal blades and placed into sterile glass or plastic vials in 70% ethanol and transported to the University of Colorado, Boulder (CU).

To ensure endogenous DNA was recovered from each historical sample and to minimize risk of contamination, all pre-polymerase chain reaction (PCR) steps were performed in a building that never housed modern cutthroat trout samples, DNA or PCR product (Knight lab, Porter Sciences, CU). Clean clothes and shoes were designated solely for use in the laboratory. Furthermore, all pre-PCR steps were performed while wearing a disposable lab coat, facemask and two pairs of gloves. Before DNA extraction, samples were repeatedly rinsed with 70% ethanol and allowed to dry in a PCR workstation equipped with UV lights and HEPA-filtered airflow (Fisher Scientific). Once samples were dried, total genomic DNA was extracted using a Qiagen animal blood and tissue extraction kit, for which we modified two steps of the protocol. We added two times the recommended amount of buffer ATL and eluted DNA from the silica column with three 30- μ L aliquots of Qiagen buffer AE. One mock extraction control was included for every four or five samples. Both extraction controls and no-template PCR controls were amplified with every four or five samples. Replicate DNA extractions, PCR and sequencing were carried out

for a subset of samples (listed in Table S1) at CU and in a specialized historical DNA laboratory at the Australian Centre for Ancient DNA (ACAD), University of Adelaide, Australia.

Owing to the degraded nature of the DNA in the museum fish samples, multiple short, overlapping mitochondrial DNA fragments were amplified from each successful DNA extraction and subjected to Sanger sequencing. We generated data for five ND2 gene fragments and one COI gene fragment, ranging in size from 91 to 135 nucleotides including primers (primer sequences are listed in Table S2). These gene fragments included several diagnostic mitochondrial single nucleotide polymorphisms (SNPs) characterized in extant cutthroat trout subspecies. At CU, PCR amplicons were cloned and 5–8 clones were sequenced per successful amplification, whereas at ACAD, amplicons were sequenced directly. Sequence data were imported and edited in Sequencher 4.6 (Gene Codes Corporation). Primer sequences were removed and ND2 sequence fragments were grouped by sample. Disagreements between base calls were resolved by majority rule (best two out of three) or left ambiguous (e.g. Y = C or T).

Modern tissue collection, DNA extraction and sequence generation

For comparison to the historical samples, we sequenced a 648- or 889-basepair region of the ND2 gene for at least 10 individuals from 53 contemporary populations (Table S3). Fish were sampled from across the species' range in the Southern Rockies as part of previous studies (Metcalf *et al.* 2007; Rogers 2008), and ND2 sequencing methods were performed as described in Metcalf *et al.* (2007).

Genetic analysis

For the four extant lineages of cutthroat trout, we identified unambiguously diagnostic SNPs in the 430-bp 'museum subset' of ND2 and COI gene sequence data. Given the difficulty referring to lineages that have different geographical locations in different time periods and the confusion of pre-existing taxonomy in relation to those lineages, we refer to three of these lineages by colour (as shown in Fig. 2) and resolve these lineages to taxonomy later in this study. As Rio Grande cutthroat trout (shown in orange in Fig. 2) do not appear to have been propagated and stocked extensively (Pritchard *et al.* 2009), we refer to this lineage by name. We assigned museum samples to one of the extant lineages based on the presence of lineage-defining SNPs. Second, we generated a statistical parsimony network of haplotypes that were present in both museum and modern

samples based on the 430-bp mitochondrial DNA data set using TCS (Clement *et al.* 2000). Finally, we inferred a phylogeny of extant and historical haplotypes based on the same 430-bp sequence data set. In the phylogenetic analysis, we included sequence data for all major subspecies of cutthroat trout (*O. c. bouvieri*, *O. c. utah*, *O. c. henshawi*, *O. c. lewisi* and *O. c. clarki*) as well as rainbow trout (*Oncorhynchus mykiss*) to clarify whether museum samples represent a trout stocked from another region in North America. These additional sequence data were originally published in Metcalf *et al.* (2007) or Loxterman & Keeley (2012) (GenBank No's EF673223–EF73232; EF673250–EF673259; EF673233–EF673249; EF673260–EF673276; JQ747557–JQ747623). We used a nucleotide substitution model of GTR+I (Metcalf *et al.* 2007). We inferred a phylogeny using maximum-likelihood methods with the software PhyML 3.0 (Guindon *et al.* 2010). We used NNI for tree improvement and computed branch support using the approximate likelihood-ratio test for branches described in Anisimova *et al.* (2011). We also constructed phylogenies using BEAST v1.6.1 (Drummond & Rambaut 2007). As our analysis spans the boundary between populations and species, we used two different tree priors (i) a coalescent model assuming a constant population size and (ii) a Yule speciation process model. Each analysis was run for 100 000 000 MCMC generations, sampling every 1000 trees with a burn-in of 10 000 000 generations. Stationarity of the posterior probabilities distribution and the ESS values for the priors was examined in Tracer v1.4 (Rambaut & Drummond 2007) to confirm the burn-in was appropriate. Maximum clade credibility consensus trees were generated from posterior distributions of 90 000 trees using TreeAnnotator v1.6.1 (Drummond & Rambaut 2007).

Additionally, we investigated the geographical distribution of ND2 molecular variation for both museum samples and a broad survey of modern populations. We performed an analysis of molecular variance among drainages of historical (1857–1889 A.D.) and modern (2000 A.D. to present) populations separately to understand how the distribution of variance has changed over time. For the modern data, we included only the portion of ND2 sequenced for museum samples (378 bp) in the analysis. We generated distance matrices and performed AMOVA analyses using tools included in the ape and pegas packages in the R statistical environment (Paradis *et al.* 2004; Paradis 2010). Distance matrices were generated using default parameters, except loci with missing data were not excluded from the analysis. Drainage delineations included South Platte, Arkansas, Rio Grande, San Juan, Gunnison and Colorado River basins. Samples from the Yampa River drainage were not included in the analysis because the

museum data set had only one sample from this drainage. To account for the difference in sample size between the museum and modern data set, we randomly subsampled the modern data set 1000 times using the same number of samples per drainage as the museum data set. Subsampled data were generated using a custom Python v2.7.2 script (www.python.org) in conjunction with the NumPy v1.6.1 (www.numpy.org) numerical Python library. Using R 2.14.2 with pegas v0.4-1, we performed the AMOVA analysis on each subsampled data set to generate a range of possible modern values to compare with the historical estimate.

Results

Museum fish DNA

Skin, gill, muscle and bone were sampled from 44 cutthroat trout specimens stored in ethanol. Several samples that yielded DNA were extracted multiple times (up to four times) to confirm authenticity of endogenous DNA (Table S1). Sixty-one museum sample extractions were performed (not including mock extraction controls). Extracted DNA was highly degraded. PCR success declined with increasing target fragment size (Fig. S1) and 14 of the 44 individuals (32%) failed to yield DNA that could be amplified using PCR. Nonetheless, 30 individuals (Tables 1 and S1) yielded a total of 430 bps of mtDNA (two ND2 gene fragments and one COI gene fragment) that permitted robust inference of the phylogeny and regional biogeography of native cutthroat trout (GenBank No's JX195398–JX195487). Furthermore, sequence data were reproducible—data generated from independent extractions of museum samples were identical. In particular, the consensus sequence from cloned amplicons (at CU) matched amplicons directly sequenced (at ACAD) at >99% of nucleotides. Most mismatches were probably due to deamination (postmortem chemical damage). In a few cases in which mismatches were not resolved, bases were left ambiguous.

Phylogenetic analyses reveal historical diversity of Colorado's cutthroat trout

Individual museum samples were assigned to one of the four extant subspecies depending on the clade-defining mutations (new modern haplotypes include Genbank No's JX195488–JX195497) (Table S4). Twenty-one museum samples assigned to modern clades and nine did not (Supporting information). A statistical parsimony haplotype network including museum sequences and contemporary haplotypes revealed six distinct clusters, each of which was separated by at

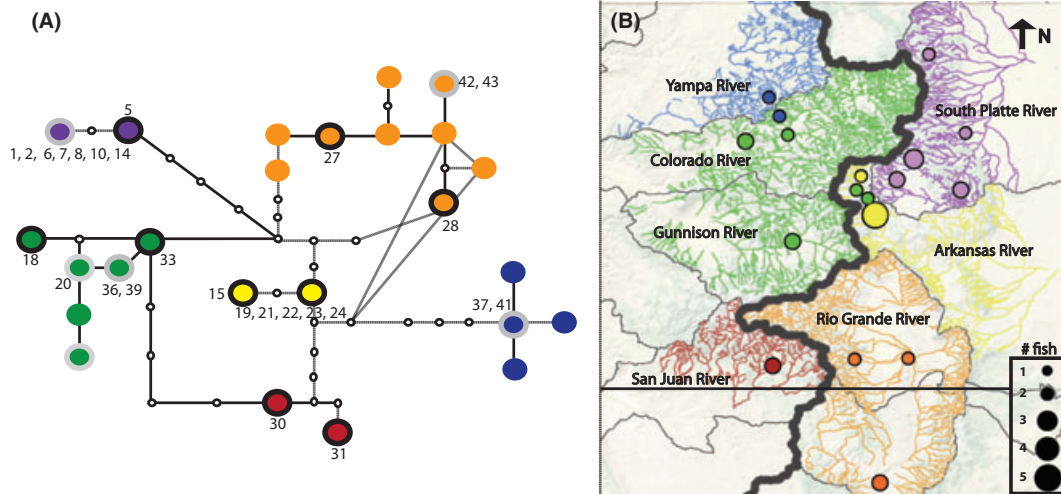


Fig. 4 (A) Statistical parsimony haplotype network using a subset of 430 basepairs of combined ND2 and COI mitochondrial sequence data. Haplotype colours match those shown in Fig. 2. Haplotypes present in (i) museum samples only have a black outline (ii) museum and modern samples have a grey outline and (iii) modern populations only have no outline. Putative unsampled ancestors are represented by small, open circles. Numbers next to museum haplotypes refer to fish sample numbers in Table S1. (B) Geographical distribution of museum samples, with the colour of each circle corresponding to the lineage colour in the haplotype network. The size of each circle is proportional to the number of museum individuals sampled with a particular lineage. Colours for drainages correspond with our hypothesis of the historical distribution of clades.

least two and as many as 12 mutations (~ 0.5 to 3% sequence difference) (Fig. 4A). Six of the 15 haplotypes represented in the museum samples were identical to modern haplotypes, and nine were unique (Fig. 4A). Importantly, museum samples from the Arkansas and San Juan basins did not group with any of the contemporary lineages, and the two sets of haplotypes were separated by a minimum of four mutations from any other clade (Fig. 4A).

Our phylogenetic inference using maximum-likelihood and Bayesian analyses included samples representative of contemporary and historical subspecies in Colorado as well as cutthroat trout subspecies and rainbow trout native to drainages outside of the state. The combination of modern and museum samples revealed high maximum-likelihood ratios and posterior probabilities (using both tree priors—a coalescent model assuming constant population size and a Yule speciation process model) for eleven North American cutthroat trout clades. Of the six clades represented by fish collected from Colorado, two were only detected in museum samples from either the San Juan or Arkansas drainages (Fig. 5). Furthermore, twenty-one museum samples grouped within four contemporary clades with high posterior probabilities in a phylogenetic tree (Fig. 5). Importantly, none of the branches subtending two or more clades had consistently high support across phylogenetic analyses, underscoring that the relationships among clades remain uncertain. If branching

order disagreed between tree inference methods, a polytomy was enforced (Fig. 5). Bayesian posterior probabilities and approximate likelihood test ratios were reported for all other branches. For the Bayesian analyses, posterior probabilities were highly similar ($<1\%$ in most cases) regardless of tree prior, and therefore, only the values from the coalescent analysis are shown in Fig. 5.

Phylogenetic analyses reveal historical distribution of Colorado's cutthroat trout

There were six divergent mtDNA clades discovered from sequencing of the museum samples—of these, four were restricted to single drainage basins (Fig. 4B). All historical samples from the Rio Grande River drainage grouped with the lineage designated Rio Grande cutthroat trout (orange lineage). All samples that grouped with the purple lineage were restricted to the South Platte drainage. The two distinct clades that did not cluster with any of the modern clades (shown in red and yellow) were restricted to either the San Juan basin or the Arkansas basin, respectively (Fig. 4B). The blue and green lineages, however, were discovered in multiple drainages. It is noteworthy that the museum samples comprising these two lineages were sampled in 1889, after the initiation of stocking activities in the state. Therefore, their native status is somewhat uncertain. The two museum samples that grouped with

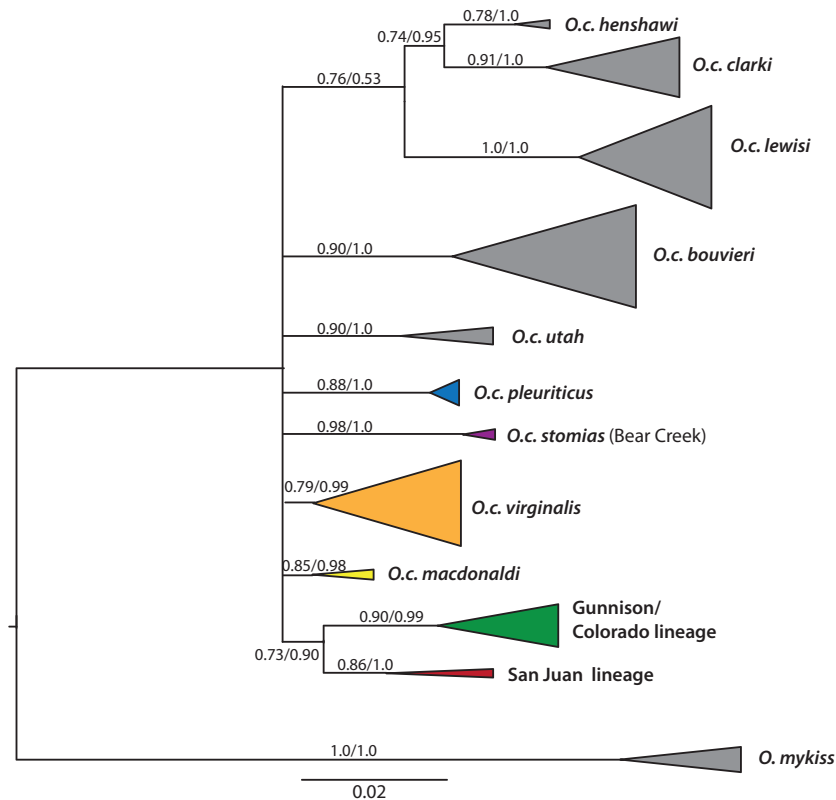


Fig. 5 Phylogenetic inference for the extant lineages of cutthroat trout in the Southern Rocky Mountains based on 430 bp of mitochondrial DNA sequence data. The maximum-likelihood tree shown was generated using PhyML. Branches were collapsed to a polytomy in cases where branching order conflicted with results from analyses using Bayesian approaches in BEAST. Approximate likelihood-ratio test for branch support and Bayesian posterior probabilities are shown for all branches (only BPPs for the analysis incorporating a coalescent tree prior are shown). The sizes of triangles are proportional to the diversity present in each clade.

contemporary blue lineage were sampled from the Yampa and Colorado River drainages (one individual in each drainage). Of the seven museum samples that grouped with green lineage, five were sampled from the Colorado and Gunnison basins west of the Continental Divide and two were sampled from Twin Lakes in the Arkansas River basin east of the Continental Divide.

Comparison of historical and modern distribution of distinct lineages

An analysis of molecular variance (AMOVA) revealed the amount of genetic variation distributed among drainages declined significantly since historical times (Fig. 6), highlighting the change in distribution of trout over the last century. Historically, the per cent of sequence variation distributed by drainage was 64%; by contrast, 95% (one-tailed) of values from modern data (subsamped to an equally small sample size as the museum data) ranged from 6 to 61% with a mean of 30%. These results suggest several long-standing biogeographical barriers to gene flow were breached towards the end of the 19th century.

The modern distribution of lineages shows some similarities and some important differences with the historical phylogeography. The historical and modern ranges

are similar for the Rio Grande cutthroat trout (shown in orange in Figs 2 and 4). For the purple lineage, the only modern representative is currently restricted to a single stream within the Arkansas River basin (Bear Creek); by contrast, all historical samples were restricted to streams in the South Platte drainage. Because the museum samples from the Arkansas River basin were clearly divergent from the purple lineage, these results suggest that the lineage currently restricted to Bear Creek was originally native to the South Platte basin. The other two extant mtDNA lineages (green and blue) are both widely distributed today across multiple basins on both slopes of the Continental Divide. The blue lineage fish were historically restricted to one or two drainage basins on the western slope of the Continental Divide. Today, they have been sampled from multiple localities in the Arkansas, South Platte, San Juan, Gunnison, Colorado and Yampa River basins (Table S5). The green lineage trout were sampled from three drainages in 1889, the Colorado, Gunnison and Arkansas River. Today, they are present in the Colorado, Gunnison, Arkansas and in the South Platte basin as well (Table S5).

Discussion

The diversity and distribution of cutthroat trout have changed dramatically over the last 150 years in the

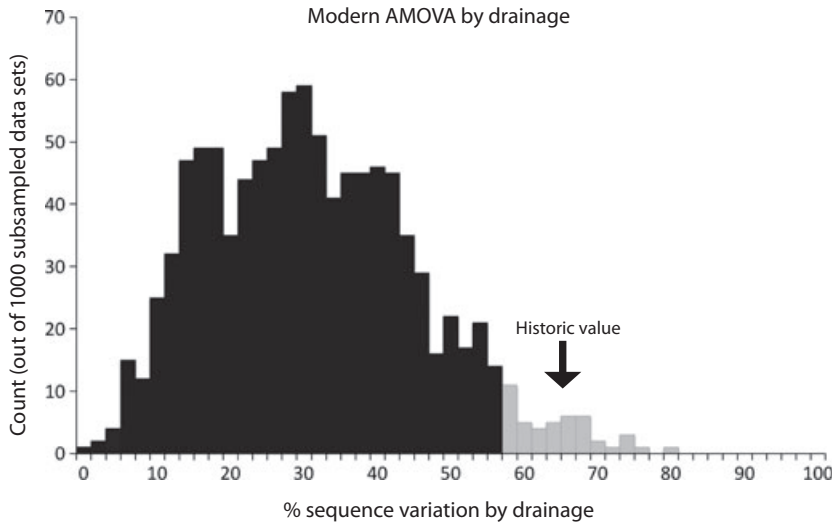


Fig. 6 AMOVA analysis by drainage. The modern sequence data set was subsampled so that the number of samples from each drainage basin matches the sample size of the museum data set. The per cent sequence variation explained by drainage for 1000 subsampled modern data sets is shown as a histogram in black (95% of values) and grey (5% tail of values). The per cent variation explained by drainage for museum samples is indicated with an arrow.

Southern Rocky Mountains. Our phylogenetic survey of fish collected prior to the onset of extensive propagation and stocking of fish revealed six divergent lineages, not four as originally described by 19 and 20th century fish taxonomists (H1). Each cutthroat lineage was probably endemic to either a single drainage basin or two adjacent drainage basins, but the native distribution of subspecies probably differs from the prevailing view in several ways (H2). Notably, instead of the expected one, there were three distinct lineages historically native to the drainage basins on the western slope of the Continental Divide. Furthermore, the subspecies native to the South Platte was probably endemic to the drainage basin, and not a native of the Arkansas River drainage as originally described. Additionally, museum specimens identified as *Oncorhynchus clarkii macdonaldi* were found in the Arkansas drainage as expected, but not just to a pair of headwater lakes as originally described (Jordan 1891). The distribution of *O. c. virginalis* aligned with the prevailing view and was only detected in the Rio Grande River drainage basin. Our third hypothesis could not be refuted. We discovered that the current and historical distribution of cutthroat trout lineages were significantly different. The difference probably reflects the success of past fish propagation and stocking activities, which broadly distributed two lineages of cutthroat trout that were historically native to waters west of the Continental Divide. The decline from six lineages in the museum samples to four today suggests there were two extinction events: one in the Arkansas River basin and another in the San Juan River system. Importantly, we discovered that the cutthroat lineage historically native to the South Platte, that at one time was declared extinct, persists in a single stream outside its native range.

The role of stocking in the contemporary distribution of Colorado's cutthroat trout

AMOVA results support the hypothesis that stocking has radically changed the geographical distribution of cutthroat trout lineages in Colorado. Discordance between the historical and modern distribution of diversity is best explained by the widespread stocking of cutthroat trout across the state of Colorado. More specifically, stocking activities spanning 1899–1931 from the Grand Mesa Lakes within the Gunnison Basin and from 1903–1938 from Trappers and Marvine Lakes in the headwaters of the White River within the Yampa basin may explain why the two lineages native to these drainages are abundant in high elevation streams across major drainage basins on both slopes of the Continental Divide (Fig. 7). Importantly, many trout were stocked into historically fishless waters above barriers. Following the founding stocking events, those same barriers have protected these populations from non-native salmonids such as brook, brown (*Salmo trutta*) and rainbow trout that tend to replace or hybridize with native cutthroat trout (McGrath & Lewis 2007; Metcalf *et al.* 2008; Peterson *et al.* 2008; Bennett *et al.* 2010; Benjamin *et al.* 2011). The end result is a patchwork of cutthroat trout lineages that persist in small, high elevation populations across the state of Colorado.

In addition to stocking by federal and state agencies, stocking activities by private fish culturists that began in the late 19th century also appears to have played a role in the distribution of cutthroat trout in Colorado. Exhaustive surveys of contemporary populations within the South Platte basin have failed to find the clade characterized in museum specimens as native to the South Platte River. Instead, we discovered the trout native to the South Platte persists in an approximately four-mile

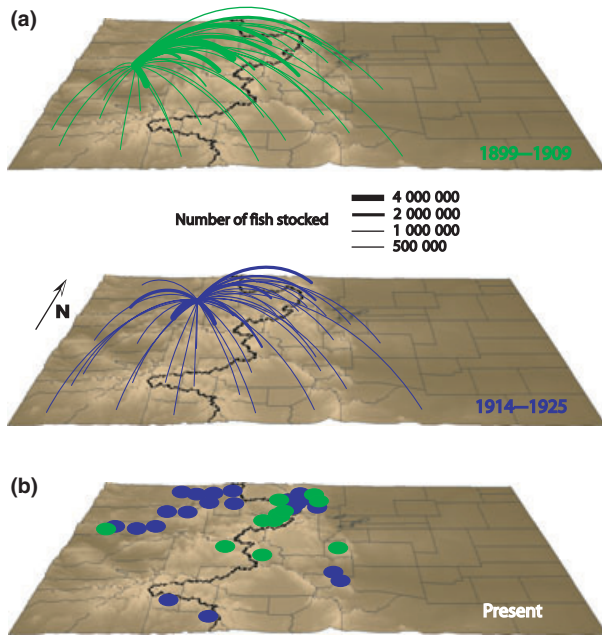


Fig. 7 The effect of fish stocking on modern cutthroat trout distribution. (A) Numbers of cutthroat trout stocked using two divergent lineages from different drainage basins (indicated by different colours) between 1899 and 1925. Colour schemes reflect new hypothesized historical distributions of native cutthroat trout lineages (see Figs 2 and 3). (B) Coloured circles represent genetic identification of cutthroat trout lineages in contemporary populations examined in this study (Table S4). Only populations without evidence of introgression between distinct clades of cutthroat trout are included. Colours correspond with those in the haplotype networks (Figs 2 and 4). Contemporary distribution of native Rio Grande trout can be found in Pritchard *et al.* (2009).

stretch of a small stream (Bear Creek) within the Arkansas River basin above several natural barriers to upstream movement of fish. Historical records indicate that Bear Creek was originally fishless, but in 1882, an early homesteader had built a trout pond in the headwaters. With no state or federal hatcheries propagating native cutthroat trout at that time, fish would probably have been obtained from one of two private hatcheries both of which obtained their fish from a tributary of the South Platte drainage (Kennedy 2010). Ironically, while stocking contributed to the decline of native cutthroat trout throughout their range (Behnke 1992; Young 2009), it appears to have also inadvertently prevented the extinction of this unique lineage.

Implications for taxonomy of Colorado's cutthroat trout

Mitochondrial DNA sequence data support recognition of six divergent, native lineages of cutthroat trout Colorado. Although we acknowledge limitations inherent in

using single gene trees to describe phylogeny, we did previously use clustering methods (Metcalf *et al.* 2007; Pritchard *et al.* 2009) to demonstrate nuclear support for extant lineages. Therefore, based on the historical and modern mitochondrial sequence data, we suggest a new working hypothesis for taxonomy of cutthroat trout in the Southern Rocky Mountains.

In some cases, taxonomic inference is fairly straightforward. The two haplotypes comprising the yellow lineage included the type specimen of *O. c. macdonaldi*, and thus most probably represents the Yellowfin cutthroat trout *O. c. macdonaldi*. Moreover, the lack of modern samples representing the *O. c. macdonaldi* clade confirmed reports from the early twentieth century documenting its extinction (Wiltzius 1985). Additionally, the phylogeography of the modern (see Pritchard *et al.* 2009) and historical data provide little doubt that *O. c. virginalis*, first described from the Rio Grande basin in 1853, is an evolutionarily distinct lineage. Finally, the native to the San Juan drainage does not fall into one of the four named lineages (Fig. 4A) and appears to have also gone extinct since historical times.

This leaves three lineages for which taxonomy is less clear. The purple lineage was once restricted to the South Platte. Early taxonomists were clear that the designation *O. c. stomias* belonged to cutthroat trout native to the east side of the Continental Divide (Cope 1871; Jordan 1891; Cockerell 1908). With *O. c. macdonaldi* occupying the Arkansas River drainage, the name *stomias* reasonably falls to those fish of the South Platte drainage. Complicating the issue, however, is a mislabelling of locality information on *O. c. stomias* type specimens (suggested in Behnke 1976), which appear to have been collected near Santa Fe, New Mexico in 1855—a belief confirmed by genetic data in this study (see results for ANSP 7825 and 7826)—and not in the Platte River. The error stems from mislabelled specimens many years later when the samples were designated *O. c. stomias* (Supporting Information). Therefore, it could also be argued that the name *O. c. stomias* is a diminutive, second name for Rio Grande cutthroat trout (*O. c. virginalis*), for which type specimens were collected in 1853 (Behnke 2002). We argue that the greenback cutthroat trout has been held as the native to the South Platte and Front Range of Colorado for over a century, and thus, the lineage native to the South Platte should keep the designation *O. c. stomias*. Indeed, as the first person to use the name greenback cutthroat trout to describe *O. c. stomias*, David Starr Jordan wrote that he 'adopted the name *stomias* for trout of the Platte' (Jordan 1891).

This leaves two lineages (blue and green) for which there is a need for taxonomic resolution and perhaps

revision. Because these lineages are represented by museum samples collected after the onset of intensive fish stocking activities (~1885) and they are the only lineages not restricted to single drainages, their native distribution and taxonomy remain uncertain. However, we argue that the blue lineage is probably *O. c. pleuriticus*. In both the historical and modern samples, the Yampa River basin appears inhabited by cutthroat trout of the blue lineage (Fig. 4B and Table S5). Importantly, no green lineage cutthroat trout were detected in modern Yampa River samples, a pattern also supported by nuclear data previously generated for 59 Yampa River populations (Rogers 2010). Thus, we infer that the geographical range of the blue lineage was probably restricted to the Yampa and Green River basins and was not widely distributed historically across all major drainages of the west slope. The type specimens of *O. c. pleuriticus* were collected in the Green River drainage basin, which encompasses the Yampa River. Therefore, these specimens probably harbour blue lineage haplotypes, although this needs to be confirmed with additional sampling of museum specimens. Because *O. c. pleuriticus* was the only described subspecies from the western slope, the green lineage may represent an undescribed distinct taxon. This lineage appears native to the Colorado and Gunnison rivers, and potentially the Arkansas River basin. Although considered separate basins, the Colorado and Gunnison rivers converge well upstream of the warm desert confluence with the Green River and its major tributary the Yampa River. Cold water temperatures would have allowed cutthroat trout to move between the Colorado and Gunnison Rivers, giving rise to a monophyletic group in those two basins. Given the significant barrier between the east and western slopes of the Continental Divide, we believe it is unlikely that the green lineage was native to the Arkansas River drainage. Instead, we believe that by the time the samples were collected in 1889, early stocking activities may have established this lineage in the headwaters of the Arkansas River drainage. The Twin Lakes fishery, from which the samples were collected, had already seen considerable fish stocking activity by 1889 (Wiltzius 1985). Twin Lakes lie near the first federal fish hatchery in Colorado, and just a dozen miles south of the bustling city of Leadville, the second most populous city in the state at the time.

Bringing back O. c. stomias

We believe the descendants of fish that were stocked into Bear Creek are the last remaining representatives of *O. c. stomias*, the greenback cutthroat trout currently recognized as a threatened under the Endangered Species Act. This fish was declared extinct in the 1930s

(Green 1937); however, in 1953, a collection of fish from a small stream in the South Platte drainage were identified as *O. c. stomias* (although not without uncertainty), marking the 'rediscovery' of the greenback cutthroat trout that would eventually launch an intensive recovery effort. Conservation efforts aimed at restoring *O. c. stomias* in the South Platte and Arkansas River basins were based on taxonomic assessments that were compromised by the extensive mixing of trout among drainages that began in the late 1870s, however. Of the streams and lakes in the South Platte and Arkansas River basins that were subjected to trout removal and restocking with what the best available science suggested was *O. c. stomias*, all appear to harbour lineages native to the western slope of the Continental Divide.

Currently, *O. c. stomias* appears to persist as a single self-sustaining population in a locality outside the native range of the subspecies. The population harbours little genetic variation for loci that are typically variable in cutthroat trout populations (Metcalf *et al.* 2007) probably the result of few founding fish used in the initial stocking effort or a subsequent population bottleneck. Low genetic diversity may compromise rehabilitation of the taxon, making it essential that planned propagation efforts attempt to maximize genetic diversity and assess subsequent fitness. It is encouraging, nonetheless, that the taxon has persisted within a relatively short stretch of a small stream for more than a century, suggesting it is likely to survive and perhaps thrive in other places.

Conclusion

Regional diversity and distribution of many taxa in North America have changed, sometimes dramatically over the last 150 years (Rahel 2000). For trout native to the Southern Rocky Mountains, anthropogenically driven changes resulted in the extinction of some lineages, and significant declines, range expansions or range shifts of others. Our work comparing historical records of trout propagation and movement with the phylogenetic diversity of historical and modern samples increased our understanding of the status of described taxa and resulted in the rediscovery of a taxon, *O. c. stomias*, that was at one time declared extinct.

Other threatened and endangered species may be subject to similar uncertainty regarding their native diversity and distribution (e.g. Boessenkool *et al.* 2009; Athrey *et al.* 2011; Hekkala *et al.* 2011). In many cases, historical specimens exist in museums that are available for inferring native diversity and taxonomy. With specimens collected up to 150 years ago, our study pushes back the age for recovering DNA from ethanol-preserved specimens for population-level studies by

over two-fold and demonstrates the feasibility of molecular mining of archived samples. Establishing the timing of human disturbance, coupled with the use of ancient DNA techniques for retrieving phylogenetic information from predisturbance historical samples, paves the way forward for developing conservation goals aimed at restoring threatened and endangered species to their native ranges.

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An interdisciplinary team of scientists conducting research on all aspects of conservation for Colorado's native salmonid resources assembled the research presented here. J.L.M. is a

postdoctoral researcher who uses ancient DNA methods to answer questions in ecology, evolution, conservation, forensics and history (<https://sites.google.com/site/jessicametcalf/>). A. P.M. is a Professor at CU working on a wide range of evolutionary and conservation-focused research projects of native species (http://stripe.colorado.edu/~am/Site/Martin_Lab). To find out more about cutthroat trout conservation in Colorado, please see [http://wildlife.state.co.us/Research/Aquatic/Cutthroat Trout](http://wildlife.state.co.us/Research/Aquatic/Cutthroat_Trout).

Data accessibility

Historical and modern sequence data sets are available on NCBI GenBank under accession numbers JX195488–JX195497.

Alignments of sequence data and haplotype frequency data can be found at doi: 10.5061/dryad.b4783.

A haplotype frequency table for 53 modern populations as well as fish stocking data can be found at <http://stripe.colorado.edu/~am/Site/Trout.html>.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 (a) Plot showing a decrease in average PCR success across all samples as target amplicon size increased. Mito-

chondrial amplicons ranged from 91 to 135 basepairs. (B) Logistic regression of PCR amplification success (1) or failure (0) as a function of fragment size for 322 PCR reactions. Chi square = 39.0657; df = 1; $P = 0.0000$.

Table S1 List of museum samples used in the study, including information on the drainage, museum, specimen accession number, collector, year collected, collection locality, the number of DNA extractions completed at the University of Colorado (CU) and the Australian Center for Ancient DNA (ACAD), and the number of DNA samples that yielded successful amplification of DNA.

Table S2 Mitochondrial DNA primers sequences used in the study.

Table S3 Modern cutthroat trout populations sampled across seven drainage basins in Colorado and New Mexico.

Table S4 The extant lineage, the diagnostic SNPs for each lineage (based on 430 bps), and the number of historic samples that have all of the diagnostic SNPs of each extant lineage.

Table S5 Number of ND2 mitochondrial haplotypes assigned to extant major lineages from fish that were sampled from 53 populations across major drainage systems.

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