Distinct Phenotypes of Native Cutthroat Trout Emerge under a Molecular Model of Lineage Distributions

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Abstract

Recent molecular investigations using contemporary and century-old museum specimens questioned the traditional four-subspecies taxonomic arrangement of Cutthroat Trout Oncorhynchus clarkii in the southern Rocky Mountains and revealed six lineages, including two that are likely extinct. We examined extant lineage specimens to determine whether morpho-meristic taxonomic approaches better classified Cutthroat Trout under (1) the traditional Geographic Model, which recognizes different subspecies east and west of the Continental Divide and in the Rio Grande basin; or (2) the Molecular Model, which uses genotypes to assign populations to four lineages. Classification success of the Molecular Model was higher than that of the Geographic Model, whether comparisons involved single-trait, principal components, or discriminant function analyses. Native east slope South Platte River trout (putative Greenback Cutthroat Trout O. clarkii stomias) were distinct and correctly classified, as were 83% of Rio Grande Cutthroat Trout O. clarkii virginalis populations. In all, 100% of the Blue Lineage populations of Colorado River Cutthroat Trout O. clarkii pleuriticus (putative west slope native of the White, Yampa, Green, and downstream Colorado River drainages) and 71% of the Green Lineage populations of Colorado River Cutthroat Trout (native to west slope Gunnison and Dolores River drainages and Colorado River headwaters) were correctly classified (89% overall) under the Molecular Model. Green Lineage misclassifications were mainly from morphologically and genetically distinct populations located east of the Continental Divide, whose native status remains unknown. In contrast, only 63% of those east slope and west slope Cutthroat Trout populations were correctly classified under the Geographic Model. Cohesion of distinct phenotypes and genotypes of present-day native Cutthroat Trout lineages was remarkable given widespread and massive early stocking of various lineages outside of their native ranges. Strong congruence of morphological and molecular patterns demonstrated the power of joint morphological and molecular analyses. We encourage management that preserves diversity of these rare Cutthroat Trout lineages that evolved in concert with their environment.

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Morphological similarities of organisms have been used to define taxonomic and systematic relationships for centuries (Linnaeus 1758), a process recently aided by development of molecular-based investigations (Moritz 1994; Tautz et al. 2003). Molecular studies have been particularly useful for defining taxa and cryptic forms that have broad morphological variation or when morphologically conserved forms have few or indistinct physical structures (Shaffer et al. 2004; Egge and Simons 2006; Kon et al. 2007; Berendzen et al. 2009; Pinzon et al. 2013). Molecular approaches have also been useful in a “biological forensics” framework, to reconstruct poorly documented historical distribution patterns of organisms altered by human activities, and to verify identity of morphologically determined pure populations (Metcalf et al. 2007, 2012; Peacock et al. 2017). Thus, paired morphological and molecular analyses can be a powerful tool to understand lineage distributions and evolutionary relationships of organisms and to guide conservation actions (Vredenburg et al. 2007). Morphological analyses are also important because phenotypic traits are sometimes the legal foundation upon which to define taxa eligible for protected status (Campton and Kaeding 2005), findings that may be strengthened by the inclusion of molecular data.

Morphologically variable subspecies and lineages of widespread and iconic Cutthroat Trout *Oncorhynchus clarkii* existed nearly exclusively as allopatric populations in drainages across western North America, but human activities have muddled their native distribution patterns and created confusion regarding the conservation status of rare forms (Metcalf et al. 2007; Stowell et al. 2015; Young et al. 2016). Ease of Cutthroat Trout culture and their popularity with anglers resulted in early and widespread stocking in and outside of subspecies’ native ranges (Wiltzius 1985; Dunham et al. 2004; Metcalf et al. 2012), which, when coupled with incomplete stocking records, has resulted in taxonomically mixed and enigmatic salmonid communities across the landscape. In addition, widespread stocking of Rainbow Trout *O. mykiss* throughout the 20th century has confounded identification of native stocks, as Rainbow Trout hybridize readily with Cutthroat Trout (Behnke 1970; Leary et al. 1987; Bartley and Gall 1991; Weigel et al. 2003; Allendorf et al. 2005). Stacking of other non-native salmonids that replace native trout (Peterson et al. 2004; Fausch et al. 2009), coupled with large-scale alteration or destruction of habitat, including mining, timber harvest, cattle grazing, and interbasin exchange and development of water resources, further altered distributions of native Cutthroat Trout in the intermountain western USA (Penaluna et al. 2016).

Indiscriminate stocking across altered habitats created a challenging backdrop for scientists and managers who were subsequently interested in the conservation of Cutthroat Trout. Robert J. Behnke dedicated his life work to identifying native diversity of Cutthroat Trout subspecies and the relationships among them, thus facilitating their conservation (Behnke 1992, 2002). This task was difficult because early scientists described many taxa based on minor morphological differences (Jordan 1891, 1920; Schreck and Behnke 1971), and when combined with inadequate or mistaken locality descriptions and collection of few and often poorly preserved museum specimens, resulted in a confusing and tortuous taxonomic history for many lineages of Cutthroat Trout. Based on comparisons with specimens collected early in history and a drainage-defined approach, Behnke hypothesized which forms best represented legitimate taxonomic units and which populations among them were likely pure. His work was challenging because few morphological and meristic traits could reliably discriminate taxa, particularly in the southern Rocky Mountains (SRM), where a legacy of robust stocking resulted in mixed lineages across the landscape and confused the natural distributions of Cutthroat Trout by the late 1800s, when taxonomic studies were just beginning (Cope 1871; Jordan 1891; Evermann and Rutter 1895; Wiltzius 1985; Metcalf et al. 2012).

Increasingly sophisticated molecular techniques facilitated evaluations of Cutthroat Trout distributions, diversity, and systematic relationships (Pritchard et al. 2009; Wilson and Turner 2009; Houston et al. 2012; Loxterman and Keeley 2012; Brunelli et al. 2013). Ancient DNA techniques using museum specimens collected before the extensive mixing of various Cutthroat Trout stocks have verified the identity of some pure lineages (Peacock et al. 2017) and have generated hypotheses about native distributions of Cutthroat Trout different than those based on earlier morphological analyses, especially in the SRM (Behnke 1992; Metcalf et al. 2007, 2012). Resolution of differences regarding those hypothesized distributions is important because many native SRM Cutthroat Trout are rare, including the federally listed Greenback Cutthroat Trout (GBCT) *O. clarkii stomias* (threatened; USFWS 1998). Understanding the discriminatory power of morphological traits to identify subspecies or lineages of Cutthroat Trout and their distributions is especially critical because the U.S. Fish and Wildlife Service (USFWS) concluded that introgressed Cutthroat Trout populations warrant Endangered Species Act (ESA) protection if they “conform phenotypically” to the scientific description of the subspecies (USFWS 2003). This position was affirmed in listing deliberations for Westslope Cutthroat Trout *O. clarkii lewisi* (American Wildlands v. Kempthorne 2008). Whether to include molecular information to define taxonomy and populations that are eligible for protection is controversial—supported by some (Tautz et al. 2003; Allendorf et al. 2004, 2005) and not by others (O’Brien and Mayr 1991; Dowling and Childs 1992; Campton and Kaeding 2005). Nevertheless, because phenotypic traits are sometimes the
foundation upon which taxonomic decisions are based, the morphometric as well as molecular information presented herein will assist in determining whether extant Cutthroat Trout lineages in the SRM represent discrete taxa or are simply variants of a more broadly distributed taxon.

There are presently four recognized subspecies of Cutthroat Trout in the SRM (Leary et al. 1987; Behnke 1992; Utter and Allendorf 1994; Loxterman and Keeley 2012; Penaluna et al. 2016), among the 14 subspecies recognized for this wide-ranging western North American species (Behnke 1992, 2002; Trotter 2008; Trotter et al. 2018). The SRM subspecies historically included (1) the Colorado River Cutthroat Trout (CRCT) *O. clarkii pleuriticus*, which was thought native and restricted to the Colorado River basin in streams west of the Continental Divide; (2) the Rio Grande Cutthroat Trout (RGCT) *O. clarkii virginalis*, the southernmost subspecies and native to the upper Rio Grande basin in southern Colorado, New Mexico, and west Texas, in the Pecos and Canadian River drainages, and streams in the Rio Grande proper; (3) the GBCT, which was thought native to streams on the east slope of the Continental Divide in the Arkansas and South Platte River basins, Colorado and Wyoming; and (4) the endemic Yellowfin Cutthroat Trout *O. clarkii macdonaldi*, which was assumed sympatric with GBCT and restricted only to the headwaters of the Arkansas River in Twin Lakes (Jordan 1891; Behnke 2002; but see Wiltzius 1985). Apparent co-occurrence of Yellowfin Cutthroat Trout and GBCT represents the only instance of apparent sympathy for native Cutthroat Trout subspecies in North America (Behnke 1992, 2002; Trotter 2008), which is relevant to later discussions regarding the native status of Cutthroat Trout in that basin.

More recently, Metcalf et al. (2007, 2012) used a combination of mitochondrial DNA (mtDNA) and nuclear DNA analyses to refine distribution patterns reported by Behnke (1992) and demonstrated that six lineages (four extant) of Cutthroat Trout historically occupied the SRM rather than just the four subspecies described above. Metcalf et al. (2007, 2012) showed that two distinct Colorado River basin forms exist and were widely scattered across the SRM landscape through stocking, thereby obscuring the native distributions of these fish. The complicated taxonomic history of Cutthroat Trout in the SRM, along with further justification for this study, can be found in the Appendix and Bestgen et al. (2013).

Our main goal was to evaluate alternative distribution hypotheses for native Cutthroat Trout in the SRM. First, we used a large number of populations and specimens from a broad geographic range to determine whether patterns of molecular divergence supported those described by Metcalf et al. (2007, 2012). Molecular techniques were also used to screen presumptive conservation populations to confirm genetic lineage and to exclude introgressed specimens from populations that may otherwise confuse morphological findings. Second, we used the same samples to quantify morphological variation and to determine whether a particular distributional hypothesis—Behnke (1992) or Metcalf et al. (2012)—was better supported. Specifically, we determined whether distributions of extant SRM Cutthroat Trout were better classified as GBCT, CRCT, and RGCT subspecies per Behnke (1992; hereafter, “Geographic Model”); whether taxonomic distributions better corresponded to those proposed by Metcalf et al. (2012; hereafter, “Molecular Model”) wherein CRCT were separated into Blue and Green lineages, GBCT were present only as Bear Creek fish, and RGCT remained the same; or whether some combination of the two hypotheses was warranted. Concurrent use of molecular and especially morphological traits, combined with broad-based, comprehensive sampling efforts, clarifies taxonomic relationships of Cutthroat Trout in the SRM, informs possible ESA listing decisions and conservation actions (USFWS 2003; Allendorf et al. 2005; Campton and Kaeding 2005), and should guide future conservation efforts.

METHODS

Population selection protocol.—A fundamental principle of this study was to ensure comprehensive representation of the range of variation for molecular and morphological—meristic (hereafter, “morphological”) characteristics present in Cutthroat Trout among the various lineages investigated. Random and comprehensive selection of sampling sites was not necessarily a feature of historical studies, which instead used many samples from convenience localities or were from streams that supported specimens with unusual traits, such that general range-wide patterns in morphology were not revealed. Geographic bounds of each lineage suggested by Metcalf et al. (2007, 2012), Rogers (2010), and Rogers et al. (2018) were fully evaluated with data from this study. We surmised that variation within lineages would be spatially organized based on potential for isolation and differentiation in or across drainage basins, as it is for most Cutthroat Trout lineages (Penaluna et al. 2016). Thus, populations were grouped within U.S. Geological Survey four-digit hydrologic unit code (HUC) areas that also serve as geographic management units (GMUs) for the various conservation teams responsible for native trout management (Figure 1; Shepard et al. 2005; Hirsch et al. 2006; Alves et al. 2008; Muhlfeld et al. 2015). The assumed native ranges of the described subspecies in the SRM span 14 GMUs. Eight GMUs were thought to be occupied by CRCT (four by the Blue Lineage and four by the Green Lineage), two by GBCT, and four by RGCT.

Cutthroat Trout databases maintained by the CRCT Conservation Team (Hirsch et al. 2006), RGCT...
Conservation Team (Alves et al. 2008), and GBCT Recovery Team (unpublished) were used to identify candidate populations for taxonomic investigation. Only core conservation populations (unaltered genetic status, variously determined with molecular studies by recovery teams) from streams were considered for inclusion in this study. Three candidate populations from each GMU were selected at random (drawn from a list of numbers) to ensure that morphological and genetic diversity was well represented and not influenced by personal knowledge of...
phenotypes or the perceived need to include a particular stream because the population had unusual characteristics. If both Blue and Green lineages of CRCT were present in a GMU, up to three populations of each were selected. In some drainages, limited numbers of lineage populations restricted the number of study streams.

Inclusion of a stream population in the study was allowed only for those meeting three additional criteria: (1) a population from the same eight-digit HUC was not already selected, (2) molecular data were available to make a determination on the lineage present (Rogers 2008), and (3) estimated population size exceeded 150 adult Cutthroat Trout per 1.6 km (1 mi) of stream to minimize the negative consequences of removing 12 or 24 fish from the population. Thus, the stream selection protocol generated a relatively unbiased sample of study populations, with minimal influence on relatively small populations of trout.

Twenty-four fish were collected from the first population selected from each GMU to characterize within-population variability of morphological characteristics. If that stream could not support the removal of 24 fish because of small population size, only 12 were collected, and another population was substituted for the 24-fish sample. In a few instances after sampling began, sufficient numbers of fish were not obtained from a stream, so a substitute was identified, again based on a random draw from the remaining populations in that GMU. A small number of wild specimens and a larger number of their progeny raised in a hatchery (similarities of those groups are described below and by Bestgen et al. 2013) were also available from the limited Bear Creek population in the Arkansas River drainage, Colorado, which was noteworthy for its distinct genetic profile (Proebstel et al. 1996; Evans and Shiozawa 2002; Metcalf et al. 2007, 2012). We investigated the sufficiency of 12 specimens to capture trait variation compared to 24 specimens and found that smaller samples represented most (90%) of the variation present in large samples (Bestgen et al. 2013).

Sample collection.—We restricted specimens to a comparatively narrow length range of 178 to 229 mm TL so that any variation in traits due to specimen size differences was minimized (Bestgen et al. 2013) per the recommendations of Mottley (1936). We assumed no temperature-induced differences of meristic or morphological traits, as most streams (lake populations were excluded; e.g., Keeley et al. 2005; Seiler and Keeley 2009) were similar coldwater systems at high elevations. We also assumed that any changes in characteristics related to fish size or age would be uniform across subspecies and lineages—an assumption that deserves further research. In practice, molecular techniques are also usually used to confirm lineage designations, given widespread historical stocking of other lineages and the potential for introgression.

Specimens were captured by electrofishing or by hook and line. Fin clips (upper caudal or right pelvic) were retained for subsequent genetic analysis, and care was taken to ensure that tissue collection did not compromise morphological examination. After tissue collection, specimens were anaesthetized in tricaine methanesulfonate (MS-222), were placed in 10% formalin for a minimum of 3 weeks, and were transferred to a final preservative of 70% ethanol. Individual fish were tagged with a coded label, and jars were similarly labeled, all by a third party, to ensure that the collection locality of samples was unknown to investigators conducting both molecular and morphological assessments. This strict blind protocol ensured that investigators were not influenced by knowledge of the geographic locality of the stream or specimens. All whole-fish specimens are housed at the Larval Fish Laboratory, Colorado State University.

Morphological data collection.—Traits that were selected to measure or count were based, in part, on those historically used in Cutthroat Trout taxonomic studies so that literature comparisons could be made. Because historical studies of trout (e.g., Behnke 1992) excluded strictly mensural traits (e.g., head length and body depth), presumably because of variation induced by environmental or other effects (Mottley 1934, 1936; Keeley et al. 2005), we also chose to exclude those traits from this study. Morphological trait data (counts of lateral series scales, anterior gill rakers [upper and lower limbs], basibranchial teeth, pyloric caeca, and pelvic fin rays) were generated according to Hubbs and Lagler (1947) or Behnke (1992), with modifications as described by Bestgen et al. (2013). Counts of scales above the lateral line, a historically used trait (Behnke 1992), were deemed unreliable because scales were often deeply embedded and counts were not replicable. We quantified the minimal variation within and among investigators to ensure that our trait data collection was accurate and precise; more detailed explanations of the techniques used to obtain morphological data are provided by Bestgen et al. (2013).

Spotting patterns of Cutthroat Trout, including the size, distribution, and number of spots, have been used to describe various taxa but usually only in a qualitative manner (Behnke 1992; but see Qadri 1959 and Diefenbach 1966). We wanted to better quantify spotting patterns of Cutthroat Trout and counted spots (a pigment concentration at the surface of the skin, visible to the naked eye regardless of size, but not including deeper pigmentation concentrations such as Parr marks) in seven areas: lateral surface of the head and six regions of the trunk, excluding those on fins (Figure 2). Head spots included those on the top of the head and opercle. The trunk of the fish in lateral view was divided into anterior, middle, and posterior thirds, and each of those were further divided into two sections by the lateral line (n = 6 regions). A spot was counted in a section if more than
FIGURE 2. Zone demarcation used to count Cutthroat Trout spots on the trunk of the body. Dashed lines separate anterior, middle, and posterior thirds of the trunk; spots posterior to the dashed line on the caudal peduncle were not counted. Head spots (those on the operculum and bony skull) were counted separately from the trunk. The thirds of the trunk were separated into upper and lower zones by the lateral line.

half of it was located inside the section boundary. Presence (yes/no) of spots on the top of the head, a separate trait, was determined by examination of the top of the head on either side of the occipital division.

We summed the number of spots in the three upper and three lower trunk regions and divided the former by the latter. Because Cutthroat Trout spots are typically concentrated dorsally (and posteriorly; Behnke 1992), the resulting ratio was nearly always greater than 1; a ratio of about 1 suggested a relatively even spot distribution laterally, and a number less than 1 suggested a greater concentration of spots ventrally. We also calculated ratios describing spot distribution by dividing the total number of spots in the anterior-most two sections (one above and one below the lateral line) and the two middle-body sections by the total spot counts in the upper and lower posterior-most sections that included the caudal peduncle area (fore-trunk spot and mid-trunk spot ratios, respectively). Each of those ratios was typically less than 1 because spots on Cutthroat Trout are usually concentrated posteriorly; a ratio of approximately 1 suggested a more even spot distribution from anterior to posterior, while a number greater than 1 suggested that spots were concentrated anteriorly. Largest mean spot size estimation was accomplished by measuring several spots determined as candidates for the largest, and the three with the greatest diameter (nearest 0.1 mm) were used to calculate the mean.

Molecular data collection.—Sample DNA was isolated from each fin tissue by using a proteinase K (enzyme code 3.4.21.64) tissue lysis and spin-column DNA purification protocol following the manufacturer’s specifications (DNeasy Kit; Qiagen, Hilden, Germany). Population assignment to lineages was confirmed by sequencing a 648-base-pair (bp) fragment of the NADH dehydrogenase (enzyme code 1.6.99.3) subunit 2 (ND2) mitochondrial gene (described by Bestgen et al. 2013). This represents a slightly shorter subset of the 889-bp fragment used in other studies (Metcalf et al. 2007; Loxterman and Keeley 2012), imposed by the sequencing equipment available. Sequences were aligned in ClustalW (Thompson et al. 1994), and the evolutionary history was inferred using the maximum likelihood method (Tamura and Nei 1993) in MEGA7 (Kumar et al. 2016). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was calculated (Felsenstein 1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004). Aligned sequence data were exported from MEGA7 to Arlequin using PGDSpider (Lischer and Excoffier 2012), where pairwise distances between haplotypes were calculated (Excoffier et al. 2005). The resulting table was then imported into HapStar (Teacher and Griffiths 2011) to generate minimum spanning networks.

Although all conservation populations were assumed pure, introgressive hybridization with other Oncorhynchus taxa was possible and could affect morphological results. Thus, admixture in the nuclear genome was explored with amplified fragment length polymorphisms (AFLPs). Fragment size was evaluated on an ABI 3130 sequencer (Applied Biosystems, Foster City, California). A molecular signature for each individual was produced in GeneMapper version 4.0 (Applied Biosystems) by scoring for the presence or absence of a standardized set of 119 markers between 50 and 450 bp in size generated from reference Cutthroat Trout populations. The genetic profiles of individuals in the test population were compared to those found in reference populations (Rogers 2008) by using a Bayesian approach for identifying population clusters (Pritchard et al. 2000). The program STRUCTURE version 2.2 (Falush et al. 2007; Pritchard et al. 2007) was used to determine similarity between test individuals and reference populations. Reference populations were selected and grouped by their mtDNA lineage (Metcalf et al. 2007) and not by geographic or historic subspecies classifications. Genetic similarity or dissimilarity was scored as the admixture proportion, or the probability that each test individual shared a genetic background with each of the Cutthroat Trout subspecies reference population groups. Proportions were expressed as $q$-values for each subspecies. These $q$-values were obtained by running STRUCTURE 10 times for each population of interest using a burn-in of 50,000 steps followed by 50,000 Markov chain–Monte Carlo replicates. Average $q$-values from the run with the highest log-likelihood (Pritchard et al. 2007) were used to generate the admixture proportions for the unknown population. Confidence intervals around admixture proportions were generated with the software application QSTRAP version 3.1 (available at https://cpw.state.co.us/learn/Pages/ResearchAquaticSoftware.aspx). Although only core conservation populations identified in databases were considered during the selection process, our AFLP
screening detected several possible instances of introgressive hybridization in native Cutthroat Trout specimens with Rainbow Trout or Yellowstone Cutthroat Trout *O. clarkii bouvieri*. If AFLP data suggested that native Cutthroat Trout individuals displayed more than 0.5% admixture with either alien taxon, those individuals were eliminated from further analysis. Elsewhere (Bestgen et al. 2013), we report the details for specimens screened with molecular methods to confirm that they fit within their anticipated clades using mitochondrial sequence data and for AFLP markers that were used to assess evidence of introgressive hybridization in the nuclear genome (Table 1).

**Data analysis.**—We explored how different the morphotypes of various Cutthroat Trout populations were, assuming the traditional Geographic Model distributions for subspecies were correct (i.e., Behnke 1992), and compared those distributions to morphotypes corresponding to the lineage and subspecies (hereafter, “lineage”) distributions described by the Molecular Model. The Geographic Model had groups that were consistent with recognized subspecies from the east slope of the Continental Divide, Colorado (GBCT); the west slope (CRCT) in Wyoming, Utah, and Colorado; and the Rio Grande basin (RGCT) in Colorado and New Mexico. We also included in this classification a fourth group, GBCT from Bear Creek (GBCT-Bear Creek), to provide consistency with the alignment suggested by the Molecular Model (Metcalf et al. 2007, 2012). Thus, the Molecular Model recognized the Bear Creek and Rio Grande basin groups, in addition to the CRCT-Blue Lineage and CRCT-Green Lineage previously described. This analysis provided a multi-trait view of historical Cutthroat Trout taxonomy, which has not been conducted for those groups. We then compared findings for Cutthroat Trout distributions under the Geographic Model with the distributions consistent with the Molecular Model, which assumes that lineages outlined by Metcalf et al. (2007, 2012) represent the historical diversity of Cutthroat Trout. Cutthroat Trout morphological characteristics are known to have considerable variation, so classifications were for mean trait values for populations in individual streams. Use of means for traits by stream is consistent with historical analyses (e.g., Wernsman 1973; Behnke 1992) and also allowed for comparisons with those in the literature. Although the use of means could mask the presence of an individual admixed with alien alleles in a sample, all fish were screened with AFLPs, and when introgressive hybridization was detected, that individual was eliminated from further analyses.

We first compared summary data for morphological traits (e.g., mean, 95% confidence interval, and range) for subspecies (Geographic Model) and lineages (Molecular Model), understanding that GBCT-Bear Creek were included in each. We focused on four traditional measures (lateral series scale counts, total number of anterior gill rakers, number of basibranchial teeth, and number of pyloric caeca; Behnke 1992) and four nontraditional metrics (total trunk spots, fore-trunk spot ratio, mid-trunk spot ratio, and mean largest spot size). Trait selection was based, in part, on the largest $F$-values for traits obtained from linear discriminant function analyses (DFAs; PROC DISCRIM in SAS; SAS Institute 1988) using all specimen data.

We then conducted a principal components analysis (PCA; SAS PROC PRINCOMP, with correlation matrix) of trait data to assign populations to the Geographic Model or Molecular Model. This allowed groups to cluster in the principal component (PC) space (three axes plotted, only the first two showed relevant patterns) without a priori assignment of streams to specific taxa or lineages. This also allowed assessment of the overlap of morphotypes previously reported and, more importantly, allowed us to examine whether any subspecies or lineages were distinct from each other in the PCA for the Geographic Model and Molecular Model, respectively. Mean component scores in PC space for subspecies and lineage populations (not individuals) were enclosed in 99% confidence ellipses to ensure a robust test of mean differences among populations. We plotted but do not show wider 90% prediction ellipses, as those demonstrated essentially the same patterns as confidence ellipses. Examination of overlap among groups is clearly a subjective technique but importantly does not make assumptions regarding where populations should be assigned a priori. We conducted the PCAs using traditional meristic data (counts of gill rakers, lateral series scales, pyloric caeca, and basibranchial teeth) and spot traits (total trunk spots, fore-trunk spot and mid-trunk spot ratios, and mean largest spot size), and all trait values except ratios were log-transformed to meet normality assumptions.

The DFAs assessed group differences and classification rates for populations, again using either the Geographic Model or the Molecular Model. We first tested for differences among subspecies (Geographic Model) or lineages (Molecular Model) with multivariate ANOVA (MANOVA option; SAS PROC DISCRIM) using the same eight morphological traits used in PCA. We then used DFA (pooled covariance matrix; SAS Institute 1988) with the same eight variables to determine classification rates for populations from different subspecies or lineages. Constructing discriminant functions from a data set and then obtaining classification rates using the same data can lead to inflated classification rates (Lance et al. 2000). Therefore, the CROSSVALIDATE option (a jackknife resubstitution procedure), which is nearly unbiased (SAS Institute 1988), was used because observations are individually removed and the discriminant function is then rerun to reassess classification rates. We used the same procedure to determine classification rates of populations under the assumptions of the Geographic and Molecular models,
TABLE 1. Sample location and associated lineage designations based on mitochondrial haplotypes and amplified fragment length polymorphisms (AFLPs) for the 49 populations of Cutthroat Trout used in this study. Geographic management units (GMUs) and stream numbers are shown in Figure 1. Lineage designations are per Metcalf et al. (2012): Blue is the Colorado River Cutthroat Trout (CRCT) lineage (CRCT-Blue Lineage) thought native to the Yampa, Green, and lower Colorado River GMUs; Green is the CRCT-Green Lineage thought native to the upper Colorado, Gunnison, and Dolores River GMUs; S. Platte is the Greenback Cutthroat Trout (GBCT) thought native to the South Platte River basin GMU (GBCT-Bear Creek); Yellowstone is Yellowstone Cutthroat Trout; Rio Grande is Rio Grande Cutthroat Trout (RGCT); and Rainbow is Rainbow Trout.

| Drainage  | GMU       | Stream          | Stream number | Lineage | AFLP (% purity) |
|-----------|-----------|-----------------|---------------|---------|-----------------|
| Arkansas  | Arkansas  | South Apache Creek | 6             | Blue    | 100             |
| River     | River     | North Taylor Creek | 26            | Blue    | 100             |
|           |           | Graneros Creek   | 43            | Blue    | 100             |
|           |           | Hayden Creek, South Prong | 3 | Green    | 96   4 |
|           |           | Severy Creek, Bear Creek | 19 | Green    | 100             |
| Colorado  | Upper     | Little Green Creek | 29            | Blue    | 100             |
| River     | Colorado  | Mitchell Creek   | 42            | Blue    | 100             |
|           | River     | Abrams Creek     | 25            | Green   | 100             |
|           |           | Cunningham Creek | 31            | Green   | 100             |
|           |           | Henderson        | 34            | Green   | 100             |
|           |           | Bears Creek, Horseshoe Pond | 49 | S. Platte |               |
| Colorado  | Dolores   | Tabeguache Creek | 12            | Blue    | 99  1 |
| River     | River     | Little Taylor Creek | 18           | Green   | 1  95  3 |
|           |           | Big Red Creek    | 21            | Green   | 7  88  3  3 |
|           |           | Canyon Creek     |               |         |                 |
|           |           | Deep Creek, East Fork | 24 | Green    | 11  89             |
| Colorado  | Gunnison  | Nate Creek       | 8             | Green   | 1  98  1 |
| River     | River     | Deep Creek       | 11            | Green   | 100             |
|           |           | Doug Creek       | 47            | Green   | 100             |
| Colorado  | Upper     | Steel Creek      | 7             | Blue    | 98  2 |
| River     | Green     | South Beaver Creek | 41         | Blue    | 100             |
|           | River     | Irish Canyon Creek | 2            | Yellowstone | 2  98             |
| Colorado  | Lower     | Little West Fork | 16            | Blue    | 100             |
| River     | Green     | South Brownie Creek | 38       | Blue    | 96  2  2     |
|           | River     | Johnson Fork     | 44            | Blue    | 99  1 |
| Colorado  | Yampa     | Milk Creek       | 23            | Blue    | 100             |
| River     | River     | Snell Creek      | 30            | Blue    | 100             |
|           |           | Deep Creek       | 35            | Blue    | 100             |
| Colorado  | Lower     | Pine Creek       | 5             | Blue    | 100             |
| River     | Colorado  | Right Fork       | 40            | Blue    | 100             |
|           | River     | U M Creek        |               |         |                 |
|           |           | West Fork Boulder Creek | 45 | Blue    | 100             |
using mean trait values for each population both as the training data set and as the cross-validation data (A. Hess, Department of Statistics, Colorado State University, personal communication). Although this is a slight departure from the analysis by Bestgen et al. (2013), who used individual fish traits as the training data set and population means as the cross-validation data, the classification results were largely unchanged.

We conducted an additional discriminant classification analysis to characterize similarity and variation among populations within lineages. Because of the limited numbers of populations, all individuals within a lineage were used to establish the training data set, and then cross-validation was used to determine classification rates of populations to the correct GMU within the lineage.

**RESULTS**

The stream selection protocol resulted in a relatively even representation of populations from throughout the ranges and GMUs of recognized subspecies or lineages (Table 1; Figure 1). Out of 837 specimens available for study, 744 remained after censoring introgressed individuals and removing what was deemed a feral population of Yellowstone Cutthroat Trout (stream 2 [Irish Canyon Creek]; Bestgen et al. 2013). Thus, populations included 10 GBCT (east slope, Geographic Model) and 25 CRCT (west slope) populations. Those same 35 stream populations were distributed as 21 CRCT-Blue Lineage and 14 CRCT-Green Lineage populations under the Molecular Model. Twelve populations of RGCT (all Rio Grande drainage) were included, as well as two GBCT-Bear Creek samples (49 total population samples; Table 1). Bear Creek was retained as distinct from other GBCT populations because it would not have historically qualified as a pure population based on morphological characteristics (Proebstel et al. 1996), but Bear Creek samples are used in both the Geographic and Molecular Model comparisons.

Phylogenetic relationships inferred from 648 bp of the mitochondrial ND2 gene from our samples showed the
presence of four divergent SRM Cutthroat Trout clades in addition to a handful of Rainbow Trout and Yellowstone Cutthroat Trout haplotypes (Figure 3). These four clades were aligned with those described in the Molecular Model, with CRCT-Blue Lineage and CRCT-Green Lineage fish being discrete along with RGCT and GBCT-Bear Creek. This arrangement is not consistent with the Geographic Model, which would have grouped the six CRCT-Blue Lineage populations and the four CRCT-Green Lineage populations from east of the Continental Divide into GBCT, while combining the 16 CRCT-Blue Lineage populations and 9 CRCT-Green Lineage populations from west of the Continental Divide into CRCT.

In all, we recovered 34 different ND2 mitochondrial haplotypes that were distributed among six distinct clades (Figure 3). Eight haplotypes were unique to single fish while the remaining 26 occurred in more than one individual, and 15 were shared among two or more populations. In addition to four haplotypes commonly found in Yellowstone Cutthroat Trout and two haplotypes found in Rainbow Trout, we recovered 12 RGCT haplotypes, 9 CRCT-Green Lineage haplotypes, and 6 CRCT-Blue Lineage haplotypes. The GBCT-Bear Creek population had a single distinct haplotype. The ND2 sequence data suggested that only two populations were incorrectly assigned to their anticipated lineages (Table 1; Figure 3). One putative CRCT-Blue Lineage population was identified as Yellowstone Cutthroat Trout (Irish Canyon; morphological description provided by Bestgen et al. 2013) and was excluded from further analysis. The other (Abrams Creek, Colorado) aligned with the CRCT-Green Lineage rather than the CRCT-Blue Lineage as anticipated, so this population was placed into its correct lineage.

Number and geographic distribution of haplotypes in networks offered insights into the relationships of Cutthroat Trout populations in various subspecies and lineages. For example, with its high number of haplotypes recovered, the RGCT showed clear separation of streams from the Rio Grande drainage proper (Figures 1 and 3B, lower cluster of streams) compared to streams in the Pecos and Canadian River drainages (upper cluster). Streams in the Pecos River (populations 9, 10, and 14) and Canadian River (populations 22, 36, and 39) drainages were also distinguished by a unique haplotype. Geographic distribution of haplotypes in the Rio Grande drainage proper was more widespread, although most individuals from streams in the lower Rio Grande (populations 4, 20, and 33) shared a distinct haplotype.

The CRCT-Green Lineage specimens contained nine haplotypes and also showed consistency in geographic groupings. For example, east slope streams (populations 3, 17, 19, and 37; Figure 3C) had two distinct haplotypes that were not shared by their counterparts in the Colorado, Gunnison, or Dolores River drainages. In contrast to

FIGURE 3. Phylogenetic relationships inferred from 648 base pairs of the mitochondrial NADH dehydrogenase subunit 2 gene for Cutthroat Trout from the southern Rocky Mountains. Four Yellowstone Cutthroat Trout haplotypes were detected in two populations (streams 2 and 44; stream numbers are defined in Table 1 and depicted in Figure 1) and were included in the analysis, as was a Rainbow Trout haplotype detected in a single fish from stream 21 and another in five fish from stream 18. (A) The evolutionary history was developed with the maximum likelihood method, and the tree with the highest log-likelihood is shown. Percent branching support was evaluated with 1,000 bootstrap replicates. Branches with more than 60% support were retained, while those with less support were collapsed into a polytomy. Phylogenetic analyses were conducted in MEGA7, with evolutionary distance units representing the number of base substitutions per site. Major clades relevant for this study are displayed as minimum spanning networks, with line segments representing single mutation, for (B) Rio Grande Cutthroat Trout, (C) Colorado River Cutthroat Trout (CRCT) Green Lineage, and (D) CRCT-Blue Lineage. Stream numbers are listed next to open circles that represent sampled haplotypes (GenBank accession numbers MK473752-MK473783), whereas black dots represent unsampled inferred haplotypes.
of unique haplotypes in some drainages and wide dispersal of a single more common haplotype in other populations. For example, native populations in three western-most drainages—the lower Colorado River (populations 5, 40, and 45), lower Green River (populations 16, 38, and 44), and upper Green River (populations 7 and 41)—each had a unique haplotype (Figure 3D). Most other populations, whether in the presumptive native Yampa/White River drainage or in widespread populations transplanted outside the putative native range, shared a haplotype from Trappers Lake (located just south of population 23) in the White River drainage, Colorado, suggesting a common heritage for those fish. From a predominantly Blue Lineage population of CRCT in the Dolores River drainage, Tabeguache Creek (population 12), 2 of 24 specimens had a CRCT-Green Lineage haplotype, while the remaining 22 fish had the common Trappers Lake Blue Lineage haplotype. This was the only instance where fish in one stream were assigned to two different clades.

**Individual Meristic Character Comparisons**

Under the Geographic Model, traditionally used morphological characteristics indicated few differences between subspecies, especially GBCT and CRCT (Figure 4). In contrast, morphological traits defined by the Molecular Model pointed to the distinctness of RGCT as well as CRCT-Blue Lineage and CRCT-Green Lineage populations. The GBCT-Bear Creek samples were distinct from other taxonomic groups in both models. Lateral series scale counts were lowest for GBCT-Bear Creek and RGCT specimens and were substantially different for the CRCT-Blue Lineage and CRCT-Green Lineage under the Molecular Model but were similar for GBCT and CRCT under the Geographic Model (population details given by Bestgen et al. 2013). Anterior gill raker counts were lowest for GBCT-Bear Creek specimens and similar for all other subspecies or lineages (Molecular and Geographic models). The GBCT-Bear Creek specimens had the lowest basibranchial tooth counts and the highest incidence of specimens without basibranchial teeth (61%). Although mean basibranchial tooth count of RGCT specimens was comparatively low (5.9), the range for populations was broad (0 to 11) and reflected geographic structuring, with higher counts being more common in the Rio Grande drainage proper than elsewhere. Basibranchial tooth counts were slightly lower for presumptive GBCT and CRCT, while counts were similar for CRCT-Blue Lineage and CRCT-Green Lineage populations. Mean pyloric caecum counts differed by about 2 for GBCT and CRCT populations. Mean number of pyloric caeca was similar among groups, with RGCT specimens having a comparatively high number (Figure 4).

Spotting patterns, similar to the traditionally used morphological characteristics discussed above, indicated distinctness of GBCT-Bear Creek and RGCT as well as CRCT-Blue and CRCT-Green lineages, while fewer differences were noted between GBCT and CRCT populations. For example, spot counts were relatively high for CRCT-Blue Lineage populations compared to CRCT-Green Lineage populations. The GBCT-Bear Creek population had the highest overall count, and only CRCT-Green Lineage and RGCT counts were similar. Conversely, trunk spot counts were similar among CRCT and GBCT (Geographic Model, west slope and east slope groups, respectively). Like total spot counts, CRCT and GBCT groups had similar fore-trunk spot ratios, but those varied substantially between the CRCT-Blue Lineage (higher) and CRCT-Green Lineage (lower). The GBCT-Bear Creek specimens had the highest ratio, and RGCT had the lowest. Mid-trunk spot ratios were typically higher than fore-trunk spot ratios for specimens in all subspecies or lineages, indicating increased spot density posteriorly. Mid-trunk spot ratios were similar for CRCT and GBCT (Geographic Model) but were substantially different (non-overlapping 95% confidence limits) between the CRCT-Blue Lineage and CRCT-Green Lineage under the Molecular Model hypothesis. Mean largest spot size varied little among groups, including those for CRCT and GBCT subspecies, although GBCT-Bear Creek fish had smaller spots.

Within-lineage comparisons were presented elsewhere (Bestgen et al. 2013), so they are not discussed here except to note the geographic trait structuring in CRCT-Green Lineage populations located on the east and west slopes of the Continental Divide. For example, lateral series scale counts were higher in their presumed native west slope range (mean for GMU means = 208) than in east slope populations of indeterminate native status (mean = 194). Mean trunk spot counts were lowest for presumed west slope CRCT-Green Lineage fish (mean = 96) and higher in east slope populations (mean = 136). Similarly, fore-trunk and mid-trunk spot ratios were lowest for west slope CRCT-Green Lineage fish (0.35 and 0.50, respectively) and higher for east slope CRCT-Green Lineage fish (0.54 and 0.68, respectively).

**Multivariate Analyses: Principal Components Analysis**

The first two PCs using stream population mean values for four morphological traits and four spot traits accounted for 53% of the total variation in the data (Table 2; Figure 5). The two samples from Bear Creek were well separated from all other populations along PC2 in the Geographic Model due to relatively low lateral series scale, basibranchial tooth, and gill raker counts as well as relatively small mean spot size. Morphological data for GBCT, CRCT, and RGCT populations in the Geographic Model overlapped broadly with each other in PC space, and 99% confidence ellipses about centroids for GBCT.
and CRCT broadly overlapped each other, indicating similarity, especially for spot counts and fore-trunk and mid-trunk spot ratios. The RGCT populations were moderately distinctive from GBCT and CRCT along PC1 due to lower scale and trunk spot counts and lower fore-trunk and mid-trunk spot ratios. The RGCT from Macho and McCrystal creeks were in the extreme negative region of PC1 due to very low mean trunk spot counts (69 in both populations), and Carnero Creek was in the extreme positive region due to high spot counts and high spot ratios.

Under the Molecular Model, GBCT-Bear Creek samples were distinct from other lineages, similar to the Geographic Model. Unlike the Geographic Model, separation of confidence ellipses for CRCT-Green Lineage and CRCT-Blue Lineage populations was complete along PC1. The CRCT-Blue Lineage was separated in space along PC1 by relatively high lateral series scale counts, high trunk spot counts, and high fore-trunk and mid-trunk spot ratios relative to CRCT-Green Lineage and RGCT populations. The CRCT-Blue Lineage populations showed low variation, as indicated by the relatively small size of the confidence ellipse. Most CRCT-Green Lineage populations were closely aligned with the RGCT along PC1 and PC2 in the Molecular Model, which was mainly a
Consequence of mean trunk spot count similarities as well as CRCT-Green Lineage fore-trunk and mid-trunk spot ratios that were intermediate between those of RGCT and the CRCT-Blue Lineage. Morphological distinctness of the four east slope CRCT-Green Lineage populations was also evident. The RGCT centroid and confidence ellipse were well separated from those of CRCT-Blue Lineage populations on PC1, with one exception: Carnero Creek was located in the CRCT-Blue Lineage confidence ellipse.

Multivariate Analyses: Discriminant Function Analysis

The F-statistics and P-values produced in the DFA supported use of the eight trait variables for describing and classifying populations of Cutthroat Trout in the SRM (Table 3), and F-values generally corresponded to the magnitude of standardized coefficient loadings for the first and second discriminant function axes (DF I and DF II). Highest magnitude loadings for traits in the Geographic Model on DF I were for lateral series scales, mid-trunk spot ratio, and pyloric caecum counts, while gill raker number, fore-trunk spot ratio, and spot size were highest for DF II.

Overall, the DFA supported the individual trait analysis and PCA findings that various Cutthroat Trout populations aligned more closely with the lineages in the Molecular Model than with subspecies in the Geographic Model. For example, although the Wilks’ λ statistic indicated differences among the groups (F = 6.42, P < 0.0001) and pairwise comparisons indicated significant mean differences among most subspecies pair combinations (P < 0.0001), the pair GBCT and CRCT were not significantly different (P = 0.38).

Under the Geographic Model, GBCT-Bear Creek samples were correctly classified (Table 4). However, only 68% of CRCT populations were correctly classified, and 32% (8 of 25 streams) were misclassified. Additionally, 50% (5 of 10) of GBCT populations were correctly classified, with 63% overall classification success for those populations and CRCT populations. Misclassified CRCT and GBCT populations were most often misclassified as the other subspecies, indicating their similarity under the Geographic Model. The DFA correctly classified 10 of 12 (83%) RGCT populations, with both misclassifications (McCryystal and Columbine creeks) as CRCT. Overall classification success for populations in the Geographic Model was 69%.

| Trait                  | PC1     | PC2     |
|------------------------|---------|---------|
| Lateral series scales  | 0.136364| 0.383937|
| Anterior gill rakers   | 0.266916| 0.486462|
| Basibranchial teeth    | 0.246829| 0.517502|
| Pyloric caeca          | −0.015329| 0.089507|
| Trunk spots            | 0.448089| −0.219062|
| Fore-trunk spot ratio  | 0.553238| −0.235753|
| Mid-trunk spot ratio   | 0.584709| −0.149556|
| Mean spot size         | 0.016143| 0.462809|
| Percentage of variation explained | 53      |
The Wilks’ λ statistic for the Molecular Model MANOVA indicated significant differences among the lineages ($F = 10.17, P < 0.0001$). Pairwise comparisons indicated significant mean differences among all lineage pair combinations ($P < 0.0001$), including CRCT-Blue Lineage and CRCT-Green Lineage populations. Highest magnitude loadings for traits in the Molecular Model were similar to those for the Geographic Model, with variation only in the order of importance.

Classification success for populations to their correct lineage under the Molecular Model was relatively high and 100% for GBCT-Bear Creek samples ($n = 2$) and CRCT-Blue Lineage ($n = 21$) populations. Seventy-one percent (10 of 14 populations) of CRCT-Green Lineage populations were correctly classified, with three misclassified as CRCT-Blue Lineage populations and one misclassified as RGCT. Classification success for RGCT (83%) was as described in the Geographic Model. Overall classification success for populations by using morphological traits under the Molecular Model was 88%; 89% of CRCT-Blue Lineage and CRCT-Green Lineage populations were correctly classified. The CRCT-Green Lineage population misclassified as RGCT was from East Fork Deep Creek (in the Dolores River drainage). Importantly, the remaining three misclassified CRCT-Green Lineage populations were all from east slope streams (Como Creek in the South Platte River drainage; Severy Creek and South Prong Hayden Creek in the Arkansas River drainage), all of which showed distinctive morphological characteristics and an ND2 haplotype that was not found in west slope CRCT-Green Lineage populations.

**DISCUSSION**

Cutthroat Trout of the SRM showed substantial population structuring and high classification rates to correct lineages using morphological data. This was unexpected, based on the historical literature that indicated broad morphological overlap among CRCT and GBCT (Behnke 1992, 2002). Population structuring based on morphological data also corroborated our molecular analyses and those of others (Pritchard et al. 2009; Metcalf et al. 2012;
Rogers et al. 2018; Thorgaard et al. 2018; Trotter et al. 2018). This highlighted the power of side-by-side morphological and molecular analyses, especially when data are obtained from the same specimens.

Patterns of lineage differentiation and diversity that remain across SRM drainage basins also supported an emerging thesis in the literature: that phenotypic, genetic, and life history differences of native salmonids are cohesive and often persist (Pritchard et al. 2009; Jones et al. 2018; Trotter et al. 2018) despite high potential for gene flow and admixture with stocked hatchery fish. This is especially remarkable for SRM Cutthroat Trout, given the millions of fish of different species—or different lineages of the same species—that were stocked across the landscape over long periods of time (Metcalf et al. 2012).

Although the mechanisms responsible for persistence of those traits are not well understood, managers should protect and replicate those stocks as adaptive storehouses of genetic material (sensu Behnke 1972), especially given a future of changing environmental conditions (Isaak et al. 2015; Udall and Overpeck 2017).

We also showed that aspects of Cutthroat Trout distribution patterns supported by Behnke (1992) under the Geographic Model remained valid, but more frequently other aspects of distributions were more similar to patterns proposed by the Molecular Model. For example, distribution and distinctness of RGCT were unchanged under either model (Behnke 1992; Pritchard et al. 2009). Consistent with both models and with our larger data set, GBCT were found east of the Continental Divide, but only in a single stream, Bear Creek. Molecular and morphological data indicated GBCT-Bear Creek representatives of that subspecies were distinctive, and that stream supported the only remaining population, even though it is found in a stream outside of its presumed South Platte River basin native range.

Our analysis also indicated the CRCT-Blue and CRCT-Green lineages better represented native CRCT, in support of the Molecular Model. Similar to Metcalf et al. (2012), we found that populations of the CRCT-Blue Lineage, native to the Green, Yampa, and White River drainages, were established in many other streams throughout the Colorado River basin as well as in streams east of the Continental Divide. The CRCT-Blue Lineage populations established outside of their native range were likely founded from the stocking of hatchery fish, based on molecular and morphological similarities across populations (Bestgen et al. 2013), which is reasonable given that all populations retained a haplotype consistent with the one known from the widely disseminated Trappers Lake, Colorado, source stock.

Similarly, Colorado River basin CRCT-Green Lineage fish, native to the upper Colorado, Gunnison, and Dolores River basins, were also found on the east slope of the

### Table 4

Discriminant function analysis results using the jackknife resubstitution procedure (one sample removed at a time for reclassification) that describes percent correct classification of southern Rocky Mountain Cutthroat Trout populations under the Geographic Model or the Molecular Model. Number of populations used in each classification group is shown in parentheses (GBCT-BC = Greenback Cutthroat Trout from Bear Creek [under both models]; GBCT = Greenback Cutthroat Trout [Geographic Model]; CRCT = Colorado River Cutthroat Trout [Geographic Model]; CRCT-Blue = Colorado River Cutthroat Trout Blue Lineage [Molecular Model]; CRCT-Green = Colorado River Cutthroat Trout Green Lineage [Molecular Model]; RGCT = Rio Grande Cutthroat Trout [both models]). The numbers on the diagonal of each matrix depict the percentage of populations that were correctly classified, while off-diagonal numbers depict the percentage of populations that were misclassified to other subspecies or lineages. The total percent correct figures are the percentages of all populations that were correctly classified in all subspecies or lineages.

| Model and taxonomic group | Geographic Model subspecies | Molecular Model lineage |
|--------------------------|-----------------------------|-------------------------|
|                         | GBCT-BC GBCT CRCT RGCT | GBCT-BC CRCT-Blue CRCT-Green RGCT |
| GBCT-BC (2)              | 100 0 0 0                | 100 0 0 0               |
| GBCT (10)                | 0 50 40 10               | 0 100 0 0               |
| CRCT (25)                | 4 28 68 0                | 0 21 71 7               |
| RGCT (12)                | 0 0 17 83                | 0 0 17 83               |
| Total percent correct    |                           |                         |
| (all subspecies)         |                           |                         |
| GBCT-BC (2)              | 100 0 0 0                | 100 0 0 0               |
| CRCT-Blue (21)           | 0 100 0 0                | 0 100 0 0               |
| CRCT-Green (14)          | 0 21 71 7                | 0 21 71 7               |
| RGCT (12)                | 0 0 17 83                | 0 0 17 83               |
| Total percent correct    |                           |                         |
| (all lineages)           |                           |                         |

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Continental Divide. However, unlike east slope CRCT-Blue Lineage populations, east slope CRCT-Green Lineage populations were morphologically distinct and possessed haplotypes not found in west slope populations. An additional finding was that individual traits and DFA demonstrated substantial morphological structuring in all Molecular Model lineages, organized by drainage (GMUs), and those differences were often supported by the presence of distinctive haplotypes as well. Nuances of these findings are discussed below.

**Phylogeny and Haplotype Networks**

Invasions of Cutthroat Trout into the SRM region are hypothesized to have originated from the upper Snake River drainage (Behnke 2002), although there is evidence to suggest other routes of colonization (Trotter et al. 2018). Presence of the CRCT-Blue Lineage in the lower Colorado River basin GMU was unexpected, given the presence of presumably native CRCT-Green Lineage fish in the proximal Dolores River and upper Colorado River basin GMUs. Headwater dispersal of CRCT-Blue Lineage stocks from the GMU in the lower Green River basin may explain the presence of CRCT-Blue Lineage fish in the lower Colorado River. Since they share a unique mitochondrial haplotype not found elsewhere across the range of the CRCT-Blue Lineage, we hypothesize that these are indigenous populations rather than founded by stocking.

Invasion of west slope Colorado River basin streams south of the presumed native distribution of CRCT-Blue Lineage fish may have occurred along several fronts and resulted in a CRCT-Green Lineage fish with fewer and larger spots and more scales. The CRCT-Green Lineage populations are more morphologically similar to RGCT than CRCT-Blue Lineage populations and have fewer and larger spots and lower numbers of basibranchial teeth. Behnke (1992, 2002) and Pritchard et al. (2009) discussed the distribution of RGCT among various drainages and, as our data support, they suggested wide differentiation among Rio Grande proper populations from those residing in the Pecos and Cimarron River drainages.

Origin of east slope populations of Cutthroat Trout in the South Platte and Arkansas River drainages is uncertain. If native South Platte River drainage GBCT are represented well by Bear Creek fish, they have strongest affinities with CRCT-Blue Lineage fish in terms of spotting patterns. However, mtDNA analyses (Figure 3) do not support such a relationship and instead suggest closer affinity to CRCT-Green Lineage and RGCT populations. Thus, the dispersal pathways and evolutionary relationships of SRM taxa are uncertain.

**Distinctiveness of Subspecies and Lineages**

Morphological and molecular differences we noted for GBCT-Bear Creek compared to other subspecies or lineages of Cutthroat Trout examined in this study were noted by earlier investigators (Proebstel et al. 1996; Behnke 2002). However, it was difficult to compare traits of contemporary GBCT-Bear Creek fish to historical GBCT samples for several reasons, including issues of historical specimen purity (see Appendix). Furthermore, morphological analysis of museum specimens, whose DNA was used by Metcalf et al. (2012) to define South Platte River GBCT, has not been completed. Inspection of those specimens will be helpful in determining morphological trait variation of GBCT and whether the Bear Creek population (with low gill raker counts and a high proportion of fish absent basibranchial teeth), which may have undergone a genetic bottleneck by population founder effects or extreme environmental events, represents that variation. A rare strain of Lahontan Cutthroat Trout *O. clarkii henshawi*, which was also discovered as a single population in a small stream outside of its native range (https://www.fs.fed.us/lahontanfbcw), was first identified with morphological traits (Hickman and Behnke 1979) and then was verified with molecular techniques nearly 40 years later (Peacock et al. 2017). That example demonstrated the value of joint morphological and molecular approaches, which may ultimately be useful for understanding whether the traits of GBCT-Bear Creek specimens are similar to those historically found in the South Platte River drainage.

Our morphological analysis of CRCT populations supported the proposition of two extant lineages in the upper Colorado River, per Metcalf et al. (2012). This conclusion was based in part on misclassification of one-third of the CRCT populations and half of the GBCT populations (only 63% total correct) under the Geographic Model, compared to correct classification of 89% of CRCT-Blue Lineage and CRCT-Green Lineage populations with the Molecular Model. Under the Molecular Model, all 21 populations of CRCT-Blue Lineage fish grouped together in the DFA, mainly on the basis of scale counts and spotting patterns. Limited morphological variation in CRCT-Blue Lineage fish (Bestgen et al. 2013) was consistent with low mitochondrial haplotype diversity (six Blue Lineage haplotypes across 21 streams; Rogers et al. 2014; this study), which, when combined with the wide establishment of morphologically similar Trappers Lake fish, likely contributed to their high classification rates.

Classification rates for CRCT-Green Lineage populations were lower, which may be due to the morphological traits intermediate between CRCT-Blue Lineage populations and RGCT. Lower classification rates for CRCT-Green Lineage populations may also be a function of geographic clustering of populations with different morphological characteristics, as three of the four misclassified Green Lineage populations were from streams east of the
Continental Divide (the other east slope population, Fern Creek, was correctly classified). Similarities among east slope CRCT-Green Lineage populations may be a result of (1) stocking of morphologically uniform hatchery fish that were different from extant native west slope CRCT-Green Lineage populations or (2) founder effects that gave rise to distinct phenotypes and mitochondrial haplotypes, as has been postulated for other Cutthroat Trout introductions (Hickman and Behnke 1979).

Alternatively, distinctive east slope CRCT-Green Lineage fish may represent archetypal diversity from native Cutthroat Trout that invaded from the west slope and persisted despite extensive stocking of Cutthroat Trout from other sources (Metcalf et al. 2012). This explanation is supported, in part, by the presence of a relatively rare but dominant haplotype in CRCT-Green Lineage fish from three east slope streams (populations 17, 19, and 37) as well as a unique haplotype recovered from South Prong Hayden Creek fish (population 3) in the Arkansas River drainage. The latter haplotype was otherwise known only from two specimens collected in 1889 from Twin Lakes (headwaters of the Arkansas River; Metcalf et al. 2012); together, they lend support to the natural establishment of CRCT-Green Lineage fish on the east slope via headwater capture. This scenario is possible given that both Westslope Cutthroat Trout and Yellowstone Cutthroat Trout occur on both sides of the Continental Divide (Behnke 1992). Contrary to this idea, we do not find other coolwater or coldwater west slope fish in Colorado drainages (e.g., Speckled Dace *Rhinichthys osculus*, Mountain Sucker *Catostomus platyrhynchos*, and Mottled Sculpin *Cottus bairdii*) in east slope streams, nor are subspecies of Cutthroat Trout known to overlap in any other portion of their extensive range (Behnke 1992). Although our study results cannot demonstrate whether CRCT-Green Lineage fish are native to streams east of the Continental Divide in the SRM, the presence of rare haplotypes, the morphological consistencies of east slope CRCT-Green Lineage fish, and their differences from west slope Green Lineage populations justify continued conservation efforts.

We found distinct traits for RGCT in this study, as was the case for other investigators using morphological and molecular techniques (Jordan 1891; Behnke 1992, 2002; Pritchard et al. 2009). Compared to GBCT and CRCT, fewer hatchery-produced fish were apparently stocked across the native range of RGCT, which undoubtedly aided the preservation of native populations. Pritchard et al. (2009) also discussed the apparent resiliency of Pecos and Canadian River populations, which retained their genetic integrity despite the stocking of some hatchery-derived Cutthroat Trout from a main basin Rio Grande stock. A possible exception is the RGCT from Middle Carnero Creek, Colorado. We confidently classified those fish as RGCT by using molecular techniques. However, morphological data placed that population in the middle of CRCT-Blue Lineage populations in the PCA on the basis of relatively high spot counts and high spot count ratios, and it also had a high and distinctive pyloric caecum count (mean = 48.5). Historical stocking records from the early 1900s showed that downstream portions of Carnero Creek were stocked with Trappers Lake (CRCT-Blue Lineage) Cutthroat Trout. If, after additional sampling, CRCT-Blue Lineage haplotypes are recovered there, a more parsimonious explanation for the presence of divergent morphological characteristics may be available.

High classification rates of CRCT-Blue Lineage, CRCT-Green Lineage, and RGCT populations to GMUs indicated strong morphological similarities and population structuring at the scale of relatively small drainage basins. Morphological consistencies at those scales were supported by microsatellite data (Pritchard et al. 2009) as well as the presence of unique haplotypes in SRM Cutthroat Trout in many drainages. Although the number of populations and specimens from all of these groups imposes some limitations on interpretations of our results, including the use of individual fish as the training data set in classifications, structuring by GMU and major river basin seems a feature of all Cutthroat Trout lineages considered (Trotter et al. 2018). Those consistent patterns indicate that the management of SRM Cutthroat Trout should proceed at the level of the GMU (fourth-level HUC) unless new information suggests otherwise.

We found a dearth of published studies with which to compare our geographically detailed and range-wide morphological analysis of Cutthroat Trout taxa from the SRM, whereas recent molecular analyses were common (Pritchard et al. 2009; Wilson and Turner 2009; Loxterman and Keeley 2012; Metcalf et al. 2012). An exception was Williams (2004), who studied range-wide morphological and allozyme variation of Coastal Cutthroat Trout *O. clarkii clarkii* and found high intra- and interpopulation diversity across a diverse and dynamic landscape. We do not include studies of morphology related to environmental conditions or life history attributes here (Keeley et al. 2005; Seiler and Keeley 2009), as those included few or no meristic characters in common with our study, and most were not range-wide in nature. Data to describe more nuanced geographic variation in Cutthroat Trout may exist from previous works (e.g., Behnke 1992) but have not yet been discovered. The combination of morphological variation and molecular studies we used uncovered variation in SRM Cutthroat Trout that was not available from molecular studies alone, including morphological differences of CRCT-Green Lineage populations east of the Continental Divide and distinctiveness of Cutthroat Trout by drainage basin (here, four-digit HUCs). Combined morphological and molecular studies of
other Cutthroat Trout subspecies or lineages beyond the SRM (e.g., Busack and Gall 1981) may likewise be useful to better describe geographic variation and distinctive population segments, which will aid their conservation.

**Management**

It is not yet clear how the results presented here and those from other molecular studies on Cutthroat Trout of the SRM (Metcalf et al. 2007, 2012; Rogers 2010; Loxterman and Keeley 2012) will shape future management. A logical first step is to determine whether the four lineages studied here constitute recognized groups at some level of taxonomic organization. This would lead to better-informed listing decisions for rare taxa wherein phenotypic characteristics are used as the basis to distinguish taxa (e.g., Campton and Kaeding 2005). Metcalf et al. (2012) suggested that Bear Creek fish likely represent GBCT native to the South Platte River basin, which seems reasonable. They also reasoned that CRCT-Blue Lineage fish are best represented by the subspecies *O. clarkii pleuriticus* but with a natural distribution restricted to the Green, Yampa, and White River drainages of Wyoming, Utah, and Colorado. Taxonomic status of RGCT is largely unchanged by recent genetic and morphological studies, although differences among the Pecos River, Canadian River, and Rio Grande drainages support possible recognition of distinct population segments or evolutionarily significant units (Moritz 1994; Fraser and Bernatchez 2001), as proposed by Pritchard et al. (2009).

For the SRM Cutthroat Trout considered here, only CRCT-Green Lineage populations are unaccounted for in terms of assignment to a recognized taxonomic entity. Regardless of whether formal designation as a taxonomic entity is warranted, description of morphological variation is appropriate and needed for all lineages in the SRM and perhaps other areas as well. Minimally, this would assist managers with understanding historical and taxonomic origin of yet-undiscovered or incompletely studied populations of Cutthroat Trout and would help to focus conservation and recovery actions. Population structuring at the drainage basin level, as recognized with our morphological and molecular data, supports the long-held notion that population management and restoration activities should emphasize preservation of the unique phenotypes and genotypes in populations that evolved in concert with the environment (Behnke 1972, 2002; Allendorf and Leary 1988). Preservation of that diversity, regardless of where it resides on the landscape, should be a guiding principle for future management.

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The four recognized subspecies of Cutthroat Trout in the southern Rocky Mountains (SRM) have a tortuous and interesting taxonomic history, which merits additional description and study justification for readers not familiar with those details. The taxonomic arrangement of the Greenback Cutthroat Trout (GBCT), Colorado River Cutthroat Trout (CRCT), and Rio Grande Cutthroat Trout (RGCT) subspecies, as well as the Yellowfin Cutthroat Trout, was relatively stable for several decades into the early 21st century, based primarily on the work of Behnke (1992; Fausch et al., in press). Following that period of stasis, and using contemporary Cutthroat Trout specimens from east and west of the Continental Divide in Colorado (east slope and west slope, respectively), Metcalf et al. (2007) found CRCT and GBCT subspecies in their presumed historical drainages, but they also found populations of the former on the east slope and populations of the latter on the west slope. Metcalf et al. (2007) presumed that the presence of CRCT in east slope streams was due to widespread stocking with fish from several sources on the west slope (Metcalf et al. 2012). Their investigations revealed that CRCT were still found in their west slope range, but native populations were presumably restricted to northwest Colorado in the Green, Yampa, and White River drainages (herein, CRCT-Blue Lineage; sensu Metcalf et al. 2012). These included a large population in the headwaters of the White River, Colorado, at Trappers Lake, from which millions of fish were historically obtained for stocking in west slope as well as east slope locations (Metcalf et al. 2012; Rogers 2012). A second CRCT group was identified in the upper Colorado, Gunnison, and Dolores River drainages south of the distribution of Blue Lineage fish and was designated the “green lineage” (herein, CRCT-Green Lineage; Metcalf et al. 2012). The CRCT-Green Lineage included Cutthroat Trout from a hatchery on the Grand Mesi in the Colorado–Gunnison River basin, which was also a historical source of fish stocked in streams east and west of the Continental Divide (Metcalf et al. 2012; Rogers 2012).

Molecular examination of museum specimens also revealed that putative GBCT from several South Platte River basin locations had a distinct genetic profile—one not found in historical specimens from the Arkansas River basin, where the GBCT was also considered native (Jordan 1891). Contemporary specimens from all but one sampled population in the South Platte and Arkansas River drainages had genetic material consistent only with presumptive west slope fish of the CRCT-Blue Lineage or CRCT-Green Lineage. The only extant east slope population examined

Appendix: Taxonomic History of Cutthroat Trout in the Southern Rocky Mountains

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that had genetic traits consistent with historical South Platte River basin GBCT museum specimens was found in Bear Creek (hereafter, GBCT-Bear Creek), a small stream near Colorado Springs, Colorado, in the Arkansas River basin, outside of its presumed native range. The GBCT-Bear Creek population may derive from a late-19th-century stocking event into the historically fishless stream with trout from a South Platte River source (Kennedy 2010; Metcalf et al. 2012; Rogers 2012).

Molecular analysis of museum specimens also verified morphological distinctness of a fine-spotted native Arkansas River salmonid, the Yellowfin Cutthroat Trout (Jordan and Evermann 1890; Jordan 1891; Behnke 1992; Metcalf et al. 2012). In addition, some fish from the same Twin Lakes collection, including some labeled as GBCT, also shared a distinct Yellowfin Cutthroat Trout mitochondrial haplotype, suggesting that some of those specimens may have been misidentified. Metcalf et al. (2012) found no molecular evidence of South Platte River basin native GBCT in the Arkansas River basin other than in Bear Creek, and the remaining “Greenback Cutthroats” in 1889 collections from Twin Lakes (Jordan 1891) were genetically consistent with CRCT-Green Lineage fish rather than the South Platte River native form. Whether Twin Lakes CRCT-Green Lineage fish were native or transplanted, even in 1889, is unknown, but we acknowledge that many other taxa were already introduced into the lakes by that time (Jordan 1891; Juday 1906). Only the distribution of RGCT still mirrors previous descriptions (Behnke 2002), with molecular analysis demonstrating that they remain extant across their native range in Colorado and New Mexico (Pritchard et al. 2009; Metcalf et al. 2012). Thus, of the six presumptive lineages detected by molecular analyses of historical museum specimens, including an extinct mitochondrial clade from the San Juan River drainage of southwestern Colorado and New Mexico (Metcalf et al. 2012), only CRCT-Blue Lineage and CRCT-Green Lineage fish, the putative native trout of the South Platte River basin (sensu Metcalf et al. 2012; GBCT-Bear Creek), and RGCT are believed extant.

A reasonable question, then, was how well the distribution patterns of SRM Cutthroat Trout used in historical morphological studies corresponded to patterns proposed by molecular analyses, a task that proved difficult for several reasons. First, original descriptions may be inaccurate, because Jordan (1891) likely described GBCT from RGCT specimens (Metcalf et al. 2012; Rogers 2012). Second, and more general to all SRM Cutthroat Trout taxa, past studies typically defined morphological traits and variation only as means and ranges for specimens from broadly defined geographic locations (Wernsman 1973; Behnke and Zarn 1976; Behnke 1992), information that is not suitable for more fine-scale geographic comparisons proposed by molecular analyses. Because original descriptive data from individual samples are apparently missing (R. J. Behnke, Colorado State University, personal communication, 2012), future comparisons with historical data may also be limited. A final difficulty is that historical taxonomic data used to describe Cutthroat Trout morphological variation were sometimes based on populations that are now known to be, or were postulated to be, of mixed or unknown genetic heritage. For example, Dieffenbach (1966) used specimens from Black Hollow Creek, Colorado, to describe variation of GBCT from the South Platte River drainage, but it was later discovered that they likely hybridized with Rainbow Trout (Wernsman 1973). Hybridization status of those specimens is now impossible to determine, as the Black Hollow Creek population was extirpated after Dieffenbach’s (1966) study was completed (Wernsman 1973). Other putative GBCT specimens used in descriptive studies were from historically fishless sections of Roaring and Como creeks (isolated downstream by natural waterfall barriers), so those populations, by necessity, were introduced from sources of unknown provenance (Dieffenbach 1966; Wernsman 1973). For example, our data indicated that those populations represent CRCT-Blue Lineage and CRCT-Green Lineage fish, respectively, further eroding confidence in the original morphological descriptions of GBCT. Lack of finer-scale geographic definition of samples, small sample sizes, and use of populations with unknown genetic heritage necessitated that we gather new specimens to better understand the morphological variation of Cutthroat Trout lineages across the SRM (Bestgen et al. 2013).