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ARTICLE

Morphological and genetic concordance of cutthroat trout (*Oncorhynchus clarkii*) diversification from western North America

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Abstract: The cutthroat trout (*Oncorhynchus clarkii* (Richardson, 1836)) is one of the most widely distributed species of freshwater fish in western North America. Occupying a diverse range of habitats, they exhibit significant phenotypic variability that is often recognized by intraspecific taxonomy. Recent molecular phylogenies have described phylogenetic diversification across cutthroat trout populations, but no study has provided a range-wide morphological comparison of taxonomic divisions. In this study, we used linear- and geometric-based morphometrics to determine if phylogenetic and subspecies divisions correspond to morphological variation in cutthroat trout, using replicate populations from throughout the geographic range of the species. Our data indicate significant morphological divergence of intraspecific categories in some, but not all, cutthroat trout subspecies. We also compare morphological distance measures with distance measures of mtDNA sequence divergence. DNA sequence divergence was positively correlated with morphological distance measures, indicating that morphologically more similar subspecies have lower sequence divergence in comparison to morphologically distant subspecies. Given these results, integrating both approaches to describing intraspecific variation may be necessary for developing a comprehensive conservation plan in wide-ranging species.

Key words: phenotype, diversity, morphology, native distribution, intraspecific variation, cutthroat trout, *Oncorhynchus clarkii*.

Résumé : La truite fardée (*Oncorhynchus clarkii* (Richardson, 1836)) est une des espèces de poissons d'eau douce les plus largement réparties dans l'ouest de l'Amérique du Nord. L'espèce, qui occupe une grande variété d'habitats, présente une variabilité phénotypique considérable souvent soulignée par une taxonomie intraspécifique. Des phylogénies moléculaires récentes ont décrit une diversification phylogénétique parmi les populations de truites fardées, mais aucune étude n'a présenté une comparaison morphologique des divisions taxonomiques pour l'ensemble de l'aire de répartition. Nous avons utilisé des paramètres morphométriques linéaires et basés sur la géométrie pour déterminer si des divisions phylogénétiques et de sous-espèces correspondent à des variations morphologiques parmi les truites fardées, en utilisant des populations répétées provenant de l'ensemble de l'aire de répartition géographique de l'espèce. Nos données indiquent une divergence morphologique significative de catégories intraspécifiques au sein de certaines sous-espèces de truite fardée, mais pas toutes. Nous comparons également des mesures de distance morphologique à des mesures de distance de la divergence de séquences d'ADNmt. La divergence de séquences d'ADN est positivement corrélée aux mesures de distance morphologique, ce qui indique que des sous-espèces morphologiquement semblables présentent une moins grande divergence séquentielle que des sous-espèces morphologiquement distantes. À la lumière de ces résultats, l'intégration des deux approches de description des variations intraspécifiques pourrait être nécessaire à l'élaboration d'un plan de conservation exhaustif pour les espèces à grande aire de répartition. [Traduit par la Rédaction]

Mots-clés : phénotype, diversité, morphologie, aire de répartition indigène, variation intraspécifique, truite fardée, *Oncorhynchus clarkii*.

Introduction

Widely distributed species often exhibit significant phenotypic variation across their geographic range (Mayr 1963). Intraspecific variability can represent substantial levels of diversity, allowing different populations to occupy a variety of habitats or ecological conditions (Kolbe et al. 2012; Nosil 2012; Wellborn and Langerhans 2015; Des Roches et al. 2018). Biologists have long recognized the importance of within-species measures of biodiversity by assigning subspecies names to populations from different geographic locations that exhibit phenotypic differences (Wilting et al. 2015). More recent approaches have attempted to recognize within-species diversity by assigning populations to "evolutionarily significant units" or "distinct population segments" based on phenotypic or genetic differences (Waples 1991; Haig and D'Elia 2010). The need to use such approaches has been reinforced by examples of locally adapted populations with unique morphological, behavioral, or life-history characteristics (Smith and Skulason 1996; Schluter 2000; Gillespie 2012; Blanquart et al. 2013). When dramatic reductions in widely distributed species occur, how to implement recovery programs can be complicated by a lack of understanding, or uncertainty, in identifying intraspecific diversity (Bálint et al. 2011). In the

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face of a rapidly changing planet, maintaining the range of diversity found within a species may increase the probability of species persistence and function within ecosystems (Bellard et al. 2012; Des Roches et al. 2018; Raffard et al. 2019).

Historically, efforts to understand and describe intraspecific diversity have often occurred when biologists document significant phenotypic variation among populations of a species, and as a result, they describe different subspecies (Haig and D'Elia 2010; Braby et al. 2012). As many subspecies names originated in the 19th and early 20th century, before the application of quantitative statistical techniques, subspecies descriptions are frequently only qualitative in form and may be based on limited geographic sampling (Remsen 2010; Winker 2010). The widespread occurrence of habitat alteration and fragmentation has raised numerous concerns for many taxa, often resulting in the need to reevaluate their distribution and conservation status (Haig et al. 2006; Hanski 2011). The development of modern DNA technologies and molecular markers has facilitated the ability of biologists to identify and to establish the extent of focal taxa; however, the use of such molecular data has at times created conflicting views with past qualitative descriptions of intraspecific variation (Apostolidis et al. 1997; Fitzpatrick 2010; Huang and Knowles 2016). Although DNA divergence data may provide an enticingly straightforward method of defining intraspecific groups, phylogenetic grouping based on a single genetic locus or a few genetic loci may not capture recently evolved adaptive differentiation (Zink 2004; Taylor and McPhail 1999; Lamichhaney et al. 2012). Multivariate morphometric techniques provide an additional tool for quantifying a wide range of morphological variation that can incorporate a suite of phenotypic characteristics, including measures of adaptive variation (Rohlf and Marcus 1993; McGuigan et al. 2003; Komiya et al. 2011). Digital imaging technology has further simplified and standardized the collection of morphometric data and improved the ability of researchers to increase the geographic extent of samples included in analyses (Mojekwu and Anumudu 2015; Cardini 2020). Despite the improvements made in quantifying intraspecific variation using molecular and morphometric methods, the two approaches have often been pursued in isolation of each other and sometimes leading to arguments for one method over the other (Hillis 1987; Phillimore et al. 2008; Losos et al. 2012). Given the need for evaluating the status of subspecies of conservation concern (Haig et al. 2006; Venter et al. 2006; IUCN 2020), a better understanding of the concordance between morphometric and molecular methods in quantifying intraspecific variation is greatly needed.

The cutthroat trout (*Oncorhynchus clarkii* (Richardson, 1836)) is a widely distributed freshwater fish species native to western North America (Behnke 1980, 1992). Since first being described by Richardson (1836), numerous common and scientific names have been used to describe different species, subspecies, and populations of this polytypic group (Jordan et al. 1930; La Rivers 1962; Behnke 1965, 1992; Trotter 2008). Current taxonomic classifications list 14 subspecies of cutthroat trout, two of which are thought to be extinct (Behnke 1992; Trotter 2008; Trotter et al. 2018). Cutthroat trout are distributed along a north–south axis from Alaska to New Mexico (Fig. 1), commonly occupying stream and lake habitats in contrasting areas ranging from temperate rainforests to arid desert ecosystems (Behnke 1992; Trotter 2008). Numerous populations of cutthroat trout have declined in abundance or have been extirpated as a result of human-induced factors, including habitat loss and alteration, and by competition, predation, disease, and hybridization through the introduction of non-native species (Young 1995; Trotter 2008). Phylogenetic analyses of DNA sequence variation of cutthroat trout have revealed concordance with some subspecies categories, as well as more recently recognized evolutionary lineages (Wilson and Turner 2009; Houston et al. 2012; Loxterman and Keeley 2012; Brunelli et al. 2013; Shiozawa et al. 2018); however, little comparative data exist for range-wide morphological comparisons of subspecies categories or its concordance with molecular data (for some pairwise comparisons see Qadri 1959; Dieffenbach 1966; Bestgen et al. 2019).

In this study, we compare populations of cutthroat trout from across their geographic range to (*i*) determine whether a significant component of morphological variation is accounted for by subspecies classifications and (*ii*) determine if phylogenetic lineages identified by genetic variation are correlated with morphological variation. In doing so, we determine the utility, importance, and possible divergence of both approaches in describing intraspecific variation in a wide-ranging fish species of conservation concern.

Materials and methods

Sampling locations

To quantify morphological variation in cutthroat trout, we sampled populations from across the geographic range of this polytypic species (Fig. 1). We identified and designed our sampling to capture as wide a geographic range as possible, given the restricted range of some subspecies of cutthroat trout. For all subspecies compared, we were able to collect replicate populations of each subspecies or evolutionary lineage of cutthroat trout (Supplementary Table S1).[1] To identify candidate populations, we searched published papers, technical reports, and government databases for records of cutthroat trout populations and consulted with regional biologists for suggested populations to sample. For each population, we attempted to collect between 30 and 50 individuals as a representative sample of the morphological variation within a population, based on past studies that were able to detect significant intraspecific morphological variation among salmonid populations with similar numbers of fish per population (Swain and Holtby 1989; Keeley et al. 2005, 2007; Seiler et al. 2009). We collected as wide a range of fish sizes as possible from each sampling location to ensure overlapping sizes across all populations. Fish that were less than 50 mm in length were generally not included in samples due to the difficulty in positioning small fins for measurements from photographs (see below). We assumed populations were free of introgression with rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) based on our observations that rainbow trout were absent and that the fish sampled had no intermediate characteristics indicative of introgression between the two species. Discussions with regional biologists also alerted us to which candidate populations were thought to be introgressed and previously published studies also informed or confirmed which populations were free of introgression with rainbow trout (Cegelski et al. 2006; Eaton et al. 2018). Finally, a subset of fish used in phylogenetic comparisons (Loxterman and Keeley 2012) were identified by genetic haplotype to be cutthroat trout, and a larger proportion of fish used in Gunnell et al. (2008), Loxterman et al. (2014), and Eaton et al. (2018) were tested for introgression and removed from morphometric samples if any rainbow trout alleles were detected. These past studies indicate introgression was very low or absent in the areas that we selected for sampling. We included replicate populations of cutthroat trout typically classified as Bonneville cutthroat trout (*Oncorhynchus clarkii utah* (Suckley, 1874)), coastal cutthroat trout (*Oncorhynchus clarkii clarkii* (Richardson, 1836)), Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus* (Cope, 1872)), Lahontan cutthroat trout (*Oncorhynchus clarkii henshawi* (Gill and Jordan, 1878)), Rio Grande cutthroat trout (*Oncorhynchus clarkii virginalis* (Girard, 1856)), westslope cutthroat trout (*Oncorhynchus clarkii lewisi* (Girard, 1856)), and Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri* (Jordan and Gilbert, 1883)). We

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[1] Supplementary table is available with the article at https://doi.org/10.1139/cjz-2020-0106.

Fig. 1. Sampling locations of cutthroat trout (red circles) used for morphometric analyses within the estimated historical distribution of cutthroat trout (*Oncorhynchus clarkii*) subspecies (colored polygons) in western North America (Behnke 1980, 1992, 2002; McPhail 2007). Subspecies names label the polygon(s) estimating the extent of each major subspecies of cutthroat trout. Location data was collected from sampling sites using a hand-held GPS device. Figure was created using ArcMap version 10.7 (esri, Inc., Redlands, California, USA) and assembled from the following data sources: USDA/NRCS – National Cartography and Geospatial Center, 8-Digit Watershed Boundary Data 1:24000; ArcMap base map data layers.

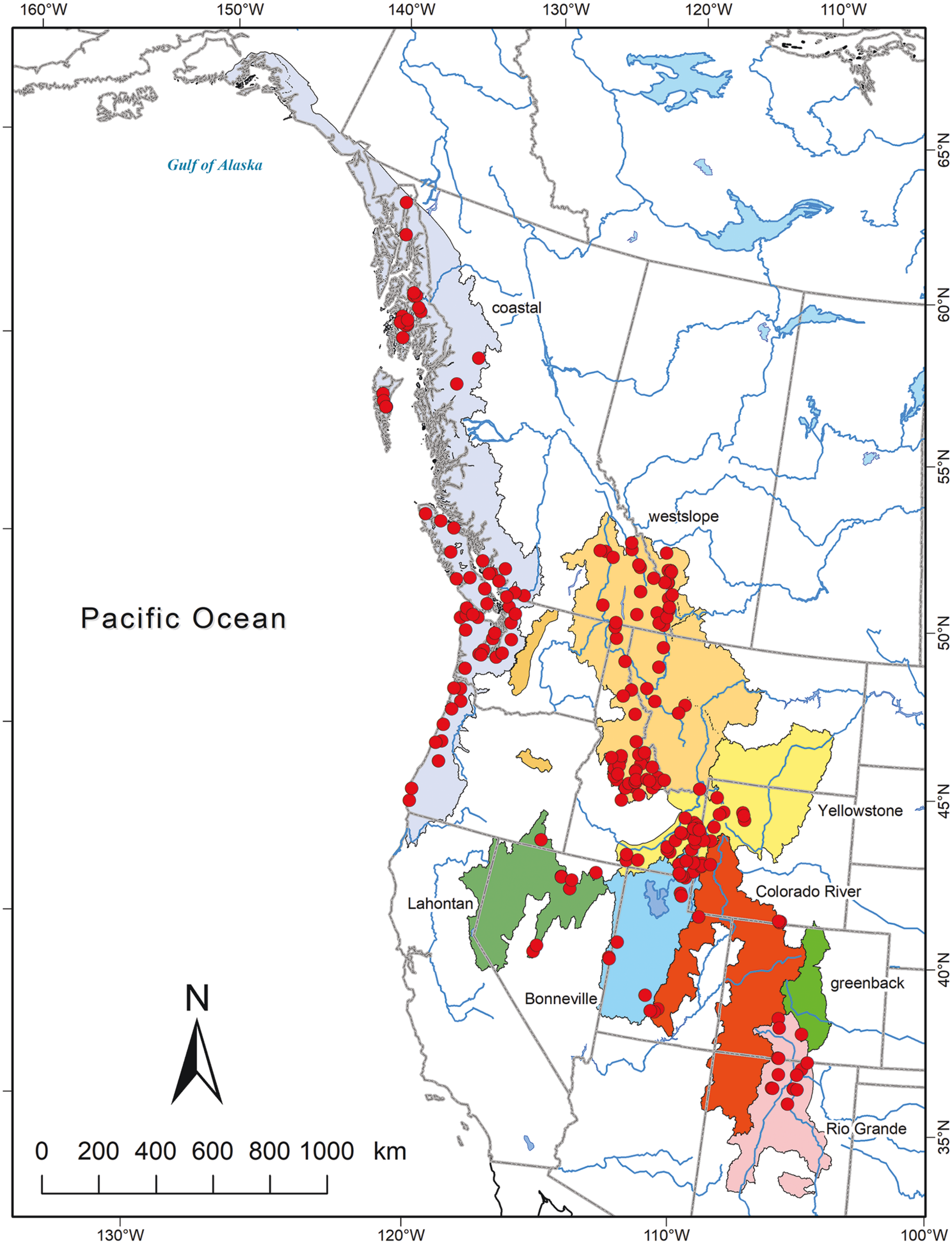
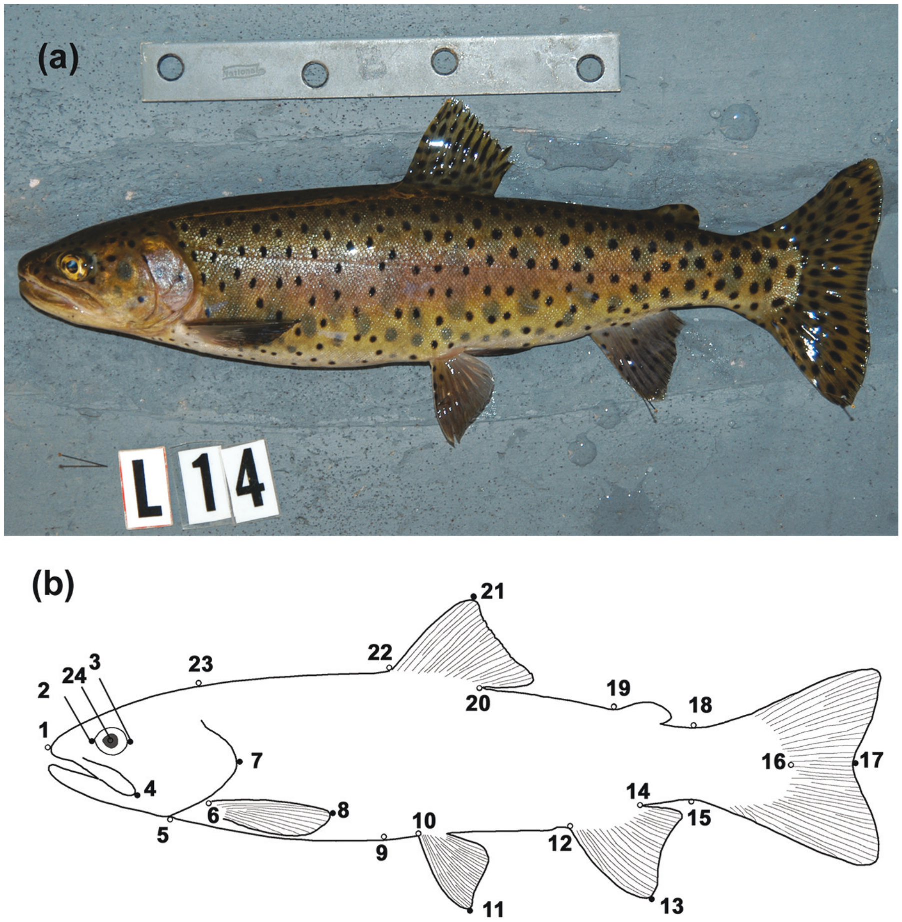


Fig. 2. (*a*) Example of a digital photograph of a cutthroat trout (*Oncorhynchus clarkii*) used for morphometric measurements. The scale bar is used to convert distances in pixels into distances in millimetres. (*b*) Location of 24 landmark positions used for digitizing morphometric measurements from cutthroat trout photographs. All landmarks were used in calculating linear morphometric measurements, whereas only landmarks with open circles were used for geometric morphometric comparisons (i.e., landmarks 1, 5, 6, 9, 10, 12, 14, 15, 16, 18, 19, 20, 22, 23, and 24). Color version online.



categorized some populations as a Great Basin lineage of cutthroat trout based on the phylogenetic relationships described by Loxterman and Keeley (2012), using mtDNA sequence divergence of the *ND2* gene, with the same divergence noted in earlier phylogenetic studies (Smith et al. 2002; Campbell et al. 2011*a*; Houston et al. 2012). We also included samples of cutthroat trout from populations considered by some to be separate subspecies of cutthroat trout, including Willow/Whitehorse (*Oncorhynchus clarkii* ssp.), Humboldt cutthroat trout (*Oncorhynchus clarkii humboldtensis* Trotter and Behnke, 2008), and Snake River finespotted cutthroat trout (*Oncorhynchus clarkii behnkei* Montgomery, 1995) (Behnke 2002; Trotter and Behnke 2008; Trotter 2008). We grouped the Willow/Whitehorse and Humboldt populations with Lahontan cutthroat trout, and we grouped fish from locations considered to be Snake River finespotted cutthroat trout to be Yellowstone cutthroat trout, given little genetic differentiation from each of these two major subspecies based on mtDNA sequence data (Loxterman and Keeley 2012). We could not include samples of greenback cutthroat trout (*Oncorhynchus clarkii stomias* (Cope, 1871)) due to conservation concerns in sampling representative populations of that subspecies. In total, we sampled 215 populations representing eight subspecies or primary evolutionary lineages of cutthroat trout (Supplementary Table S1).[1] Given the geographic extent of cutthroat trout populations, it took multiple field seasons spanning about 10 years to collect all the samples used in this study. Sampling was conducted over the years 2003 to 2012 during summer to fall months after spring spawning and snowmelt periods had passed.

Collection techniques

Cutthroat trout were collected from each location using one or more sampling techniques. In stream and river habitats, we sampled fish using a backpack or boat-mounted electroshocker or by angling. For lake populations, we sampled fish by angling, or by using multipanel monofilament gillnets, minnow traps, or hoop nets (Memphis Net and Twine, Memphis, Tennessee, USA). In almost all cases, fish were captured alive, placed in a holding container supplied with fresh water, and then anesthetized one-at-a-time by placing individuals in a 5 L anesthetic water bath. Depending on which state or province the fish were collected in, we used one of three different anesthetics: a solution of buffered tricaine methanesulfonate (MS-222; Argent Chemicals, Redmond, Washington, USA) at a concentration of 30 mg/L, carbon dioxide gas bubbled into the water bath, or a mixture of Eugenol (clove oil) and ethanol (at a ratio of 1:10) diluted into the water bath. Handling and anesthetizing fish one-at-a-time gave us more control in processing each fish and minimized the risk of mortality. After being placed in the anesthetic bath, processing a single fish typically took 3 to 4 min. Once anesthetized, we measured each fish for fork length (±1 mm) and wet mass (±0.01 g), and a digital photograph was taken of the specimen's left side by placing it on a board covered with polystyrene sheeting (Nikon D100 camera, 50 mm focal length; Fig. 2*a*). To ensure that we could measure the length of fins from the photographs, we used insect mounting pins to extend and hold dorsal, pelvic, caudal, and anal fins in an erect position (Fig. 2*a*). We standardized how photographs were taken across all populations by using a camera position perpendicular to the specimen, a consistent focal length of 50 mm, and an electronic flash. We also included a unique identification number, as well as a scale bar, in each photograph to allow for the conversion of distances in pixels into distances in millimetres. After photographing each fish, it was allowed to recover from the anesthesia in a holding container supplied with fresh water then released near the point of capture.

Morphological comparisons

To quantify morphological variation across cutthroat trout populations, we assigned landmarks to the digital photograph collected for all specimens in this study. Twenty-four landmarks were digitized onto each photograph using the computer software tpsDig (Fig. 2*b*; Rohlf 2004). Landmark positions from each photograph were then used to calculate linear morphological distances by extracting measurements between landmarks using a computer algorithm programmed in BASIC (Kemeny and Kurtz 1964).

We compiled measurements of body length, jaw length, head length, eye diameter, caudal peduncle depth, and fin lengths, as well as several measures of body depth, from the landmark locations (Fig. 2*b*; Table 1). After extraction of linear distances, we regressed each measurement against fish length and then used the within-group slope (group = population) to adjust each trait to the mean body size of all trout using the method of Thorpe (1976). Given the large number of possible pairwise comparison of slopes, we could not assume that the slope describing the relationship between each morphological trait and body size was equal across all populations; hence, we used the population-specific slope in the size-correction equations for morphological variables. All linear measurements used in regressions were $\log_{10}$ transformed to meet the assumption of homogeneity of variance, and we visually examined plots of residuals regressed against fork length to ensure that we met this assumption.

In addition to linear morphometric techniques, we also used geometric morphometric methods to assess differences in overall body shape between subspecies (Bookstein 1991; Zelditch et al. 2012), using a subset of the digitized landmarks (Fig. 2*b*, open circles). As we were interested in an overall measure of shape variation, the variation related to the size of the fish was removed through a generalized least-squares Procrustes analysis, creating a consensus configuration from landmark locations across all specimens. This procedure uses tpsRelw software (Rohlf 2010) to scale all specimens to a common body size by superimposing each specimen's landmark configuration over all others. A reference configuration was then produced, representing the mean shape of all fish. Shape parameters, including partial warp scores and uniform shape components, were then generated for each individual using a thin-plate spline function, allowing us to compare each specimen's landmark locations to the reference configuration (Zelditch et al. 2012). For visual interpretation of the overall body shape change in figures, we produced deformation grids from tps software.

Genetic and morphological distances

To calculate genetic distance between subspecies of cutthroat trout, we used mtDNA sequence variation of the *ND2* gene to estimate genetic distance between cutthroat trout subspecies, based on the data reported in Loxterman and Keeley (2012). Pairwise estimates of genetic distance were based on mean sequence divergence as calculated by the Tamura and Nei (1993) method of nucleotide substitution. Morphological distances between cutthroat trout categories were Euclidean distances calculated from principal components (PC) axes of linear morphological variables and relative warp (RW) axes for geometric morphological variables using PROC DISTANCE in SAS version 9.3 (SAS Institute, Inc. 2011).

Table 1. Loading coefficients from a principal components (PC) analysis based on 40 size-adjusted linear measurements of cutthroat trout (*Oncorhynchus clarkii*) morphology.

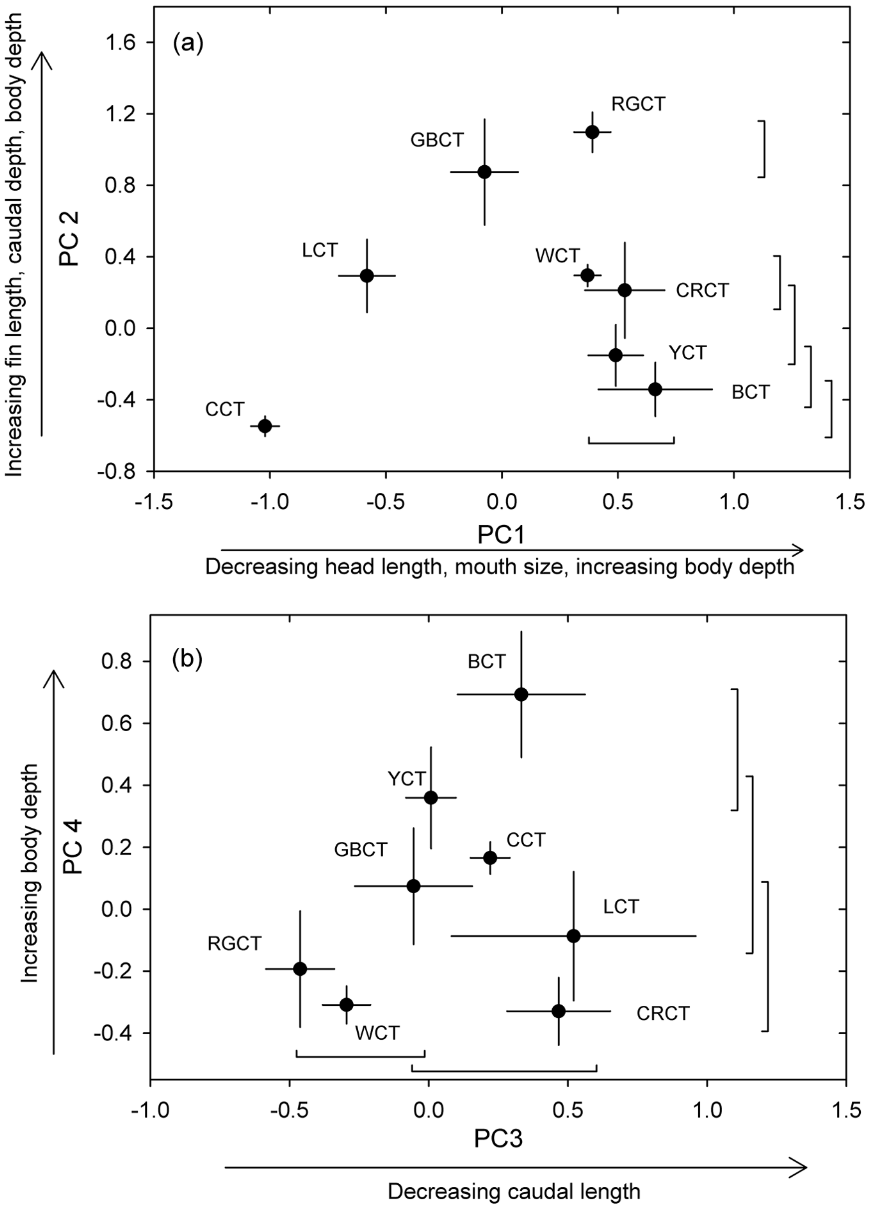
	Landmarks	PC1	PC2	PC3	PC4
Morphological character					
Head length 1	1–7	−0.86	0.23	0.19	0.18
Head length 2	1–23	−0.79	0.28	0.05	0.23
Head length 3	1–5	−0.67	0.01	0.31	0.20
Head length 4	5–23	−0.40	0.70	0.13	0.25
Premaxilla length	1–4	−0.86	0.18	0.13	0.12
Eye diameter	2–3	−0.59	0.18	−0.07	0.17
Snout length	1–24	−0.88	0.12	0.12	0.13
Pectoral fin length	6–8	−0.27	0.60	−0.11	0.06
Pelvic fin length	10–11	−0.33	0.35	−0.09	0.00
Anal fin length	12–13	−0.28	0.37	−0.22	0.15
Anal fin width	12–14	−0.11	0.40	−0.40	0.29
Dorsal fin length	21–22	−0.31	0.28	−0.25	0.07
Dorsal fin width	19–20	0.19	0.56	−0.38	−0.03
Caudal fin length	16–17	−0.40	0.44	−0.18	−0.15
Caudal length 1	14–17	0.25	0.08	−0.30	−0.71
Caudal length 2	13–16	0.49	−0.21	−0.12	−0.51
Caudal length 3	17–19	0.07	0.42	−0.60	−0.45
Caudal length 4	16–19	0.37	0.14	−0.47	−0.34
Caudal peduncle depth	15–18	0.07	0.85	−0.06	0.04
Inner body length	1–16	0.38	−0.44	0.18	0.15
Anterior body length	6–10	0.58	0.17	0.44	−0.13
Anterior to mid-body length 1	6–12	0.73	−0.28	0.26	0.15
Anterior to mid-body length 2	6–20	0.36	0.54	0.26	−0.15
Anterior to mid-body length 3	6–22	0.27	0.46	0.62	−0.13
Anterior body depth 1	6–23	−0.44	0.59	0.27	0.20
Anterior body depth 2	9–22	0.43	0.83	0.12	0.06
Mid-body length 1	10–12	0.38	−0.56	−0.16	0.37
Mid-body length 2	10–14	0.31	−0.30	−0.44	0.56
Mid-body length 3	10–19	0.61	−0.10	−0.08	0.38
Mid-body length 4	19–20	0.45	−0.60	0.10	0.33
Mid-body depth 1	10–20	0.38	0.78	0.04	0.04
Mid-body depth 2	10–22	0.46	0.82	0.11	0.05
Mid-body depth 3	10–23	0.53	0.33	0.56	−0.10
Posterior body length	22–23	0.25	−0.15	0.68	−0.24
Posterior body depth 1	12–19	0.38	0.77	0.04	0.08
Posterior body depth 2	12–20	0.68	0.32	0.16	0.23
Posterior body depth 3	12–22	0.75	0.44	−0.10	0.26
Posterior body depth 4	14–19	0.42	0.78	−0.02	−0.02
Posterior body depth 5	14–20	0.66	−0.11	−0.08	0.45
Posterior body depth 6	14–22	0.70	0.27	−0.38	0.41
Eigenvalue		10.22	8.65	3.39	2.87
Percentage of total variation		26	22	9	7

Note: Landmark numbers refer to body locations depicted in Fig. 2*b*.

Statistical analyses

To determine if differences occurred among subspecies when integrated across all linear morphological variables, we used a multivariate analysis of variance (MANOVA) to test for differences in size-adjusted linear measurements among subspecies while accounting for intercorrelation among response variables (Tabachnick and Fidell 1996; Zelditch et al. 2012). As we were primarily interested in comparing between subspecies differences, we considered each population to be a unit of replication; hence, we calculated a mean value for each size-adjusted morphological trait for each population and then used these values as observations in the MANOVA. To summarize patterns of morphological

Fig. 3. Mean (±1 SE) principal components (PC) scores from a principal components analysis of 40 size-adjusted measurements of cutthroat trout (*Oncorhynchus clarkii*) morphology for (*a*) PC 1 versus PC 2 and (*b*) PC 3 versus PC 4. On each axis, means that share brackets do not differ significantly (least significant difference (LSD) test, α = 0.05). Abbreviations beside symbols identify cutthroat trout groups: Bonneville cutthroat trout (BCT), coastal cutthroat trout (CCT), Colorado River cutthroat trout (CRCT), Great Basin lineage of cutthroat trout (GBCT), Lahontan cutthroat trout (LCT), Rio Grande cutthroat trout (RGCT), westslope cutthroat trout (WCT), and Yellowstone cutthroat trout (YCT). On each axis, means that share a line do not differ from each other (LSD test, $P > 0.05$).

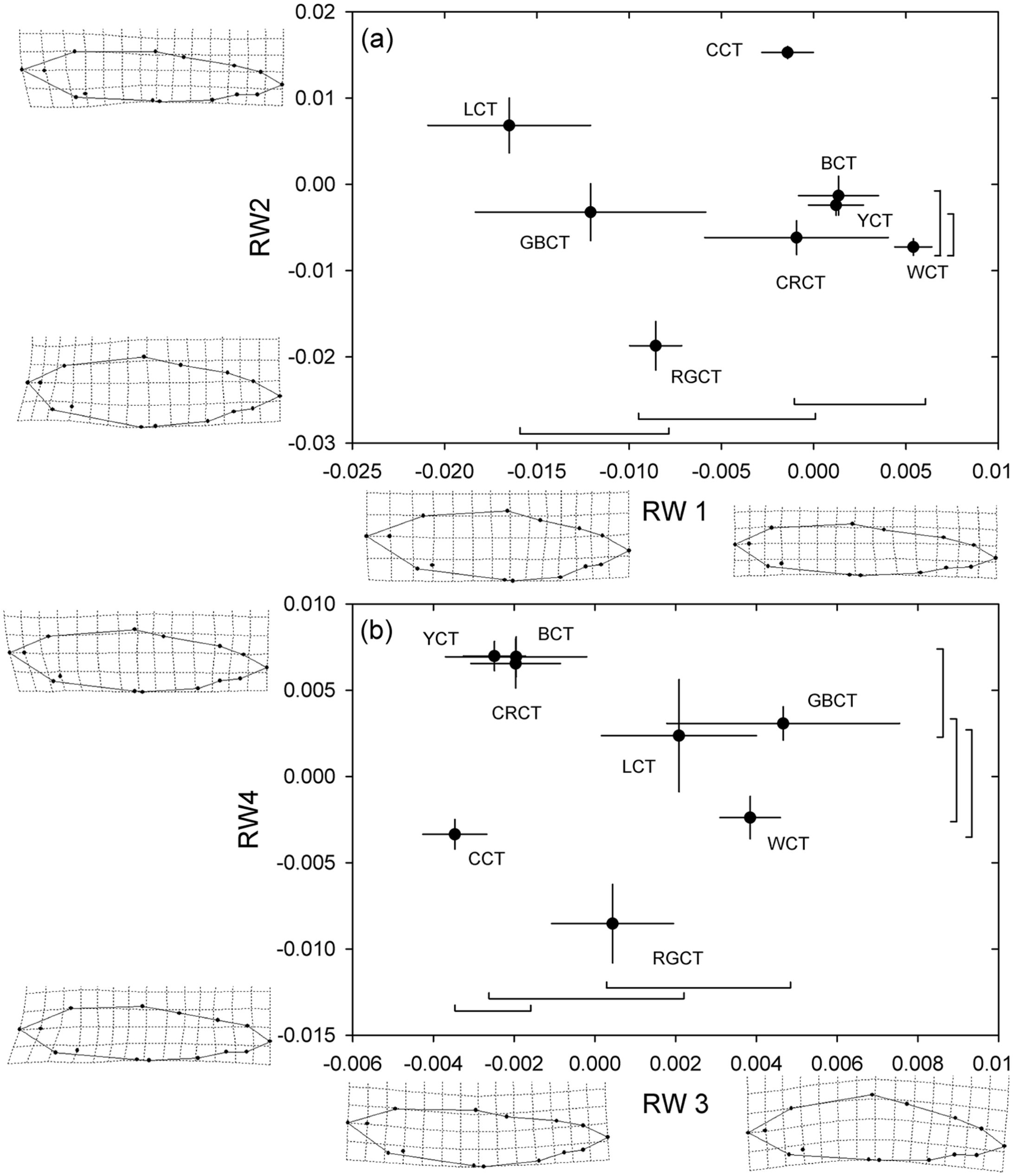


variation, we performed a principal components analysis (PCA) on correlation matrices of the size-adjusted linear measurements to describe the pattern of relative size differences in morphology among subspecies. We assessed differences across subspecies using PC axes that accounted for a significant proportion of the total variation using eigenvalues > 1 (Tabachnick and Fidell 1996), but we limited our comparisons to the first four PC axes as a description of main morphometric differences. Mean PC scores were calculated for each population along each axis and then used to test for differences in morphology across subspecies with a single-factor ANOVA (Fleming et al. 1994; Zelditch et al. 2012).

Geometric body shape variation was compared across subspecies using a PCA on partial warp scores with uniform shape components, which is often referred to as a relative warps analysis (Rohlf 1993). As with linear measures, we calculated mean PC scores for each cutthroat trout group based on population as the unit of replication. We compared mean PC scores using a single-factor ANOVA to determine if a significant proportion of morphological variation was accounted for by subspecies groupings. Subsequent figures represent mean (±1 SE) differences according to subspecies or evolutionary lineage. All tests of significance were based on type III sum of squares using the GLM procedure in SAS version 9.3, with pairwise differences between means identified by the least significant difference (LSD) test (SAS Institute, Inc. 2011).

To compare the degree of concordance between measures of morphological and genetic distance and according to subspecies or evolutionary lineages of cutthroat trout, we used the

Fig. 4. Mean (±1 SE) relative warp (RW) scores from a relative warps analysis of 15 landmarks of cutthroat trout (*Oncorhynchus clarkii*) morphology for (*a*) RW 1 versus RW 2 and (*b*) RW 3 versus RW 4. On each axis, means that share brackets do not differ significantly (least significant difference (LSD) test, α = 0.05). Abbreviations beside symbols identify cutthroat trout groups: Bonneville cutthroat trout (BCT), coastal cutthroat trout (CCT), Colorado River cutthroat trout (CRCT), Great Basin lineage of cutthroat trout (GBCT), Lahontan cutthroat trout (LCT), Rio Grande cutthroat trout (RGCT), westslope cutthroat trout (WCT), and Yellowstone cutthroat trout (YCT).



congruence among distance matrices (CADM) function (Campbell et al. 2011*b*) from the APE package (version 3.3) implemented in R statistical software (Paradis et al. 2004). Probability of concordance between matrices occurring by chance alone was based on 10 000 permutations.

Results

We collected cutthroat trout for morphometric analyses from 215 locations representing populations over much of the geographic range for this species (Fig. 1; Supplementary Table S1[1]). On average, we collected photographs from 42 individuals (range: 8–152) for each population. After compiling and digitizing landmarks on the photographs, 8503 individual cutthroat trout were included for morphometric analyses.

Linear morphological comparisons

Our analysis of morphological variation in cutthroat trout revealed that a significant proportion of variation was accounted for by differences among subspecies categories. When all size-adjusted linear measures were combined in a MANOVA, significant differences in morphology were accounted for by differences among categories (Wilks' λ = 0.00092, $F_{[280,1124]}$ = 3.90, P < 0.0001). When we summarized the pattern of linear measures of morphological variation by PCA, four main axes explained 64% of the total variation (Table 1). Among the categories compared, the most distinct separation occurred along the first axis, primarily separating coastal, Great Basin, and Lahontan groups from the five remaining categories (Fig. 3*a*). ANOVA on PCA scores from the first axis revealed that 62% of the variation was accounted for by subspecies differences ($F_{[7,208]} = 49.37$, $P < 0.0001$; Fig. 3*a*). The morphological features most strongly correlated along the first axis indicated that the coastal, Great Basin, and Lahontan groups had the largest head and mouth sizes, whereas fish from the opposite end of the axis, such as Bonneville, Colorado River, and Yellowstone groups, had the deepest bodies. Scores from the second PCA axis accounted for 41% of the differences among categories ($F_{[7,208]} = 20.50$, $P < 0.0001$; Fig. 3*a*). The second axis was primarily correlated with measures of fin length, mid-body depth, and caudal peduncle depth, and separated the Rio Grande and Great Basin groups from all others (Fig. 3*a*; Table 1). Scores from the third PCA axis explained 16% of the variation among subspecies groups compared ($F_{[7,208]} = 5.82$, $P < 0.0001$), but was only able to separate groups from the extreme range of the axis (Fig. 3*b*), as did scores from the fourth PCA axis, which accounted for 22% of the variation in PCA scores ($F_{[7,208]} = 8.71$, $P < 0.0001$).

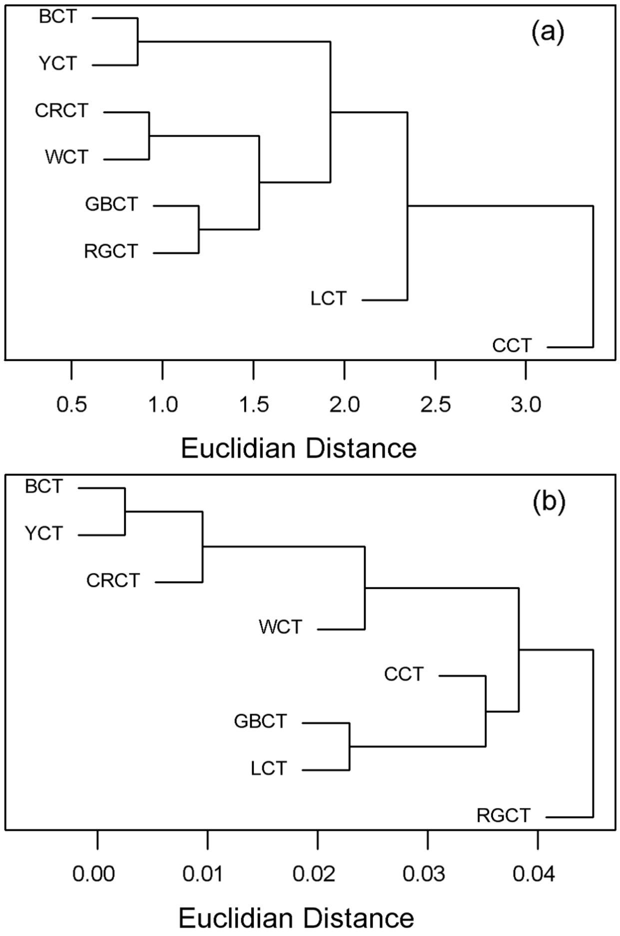
Geometric morphological comparisons

Body shape differences summarized by geometric analyses also revealed significant differences among cutthroat trout subspecies. When RW scores were combined in a MANOVA, significant differences in morphology were accounted for by differences among subspecies (Wilks' $\lambda = 0.14$, $F_{[28,740]} = 19.61$, $P < 0.0001$). When we summarized the pattern of body shape variation by a relative warp analysis, four main axes explained about 53% of the total variation. Among subspecies, some separation occurred along the first RW axis, separating subspecies groups along an axis of decreasing head and body depth (Fig. 4*a*). ANOVA on RW scores from the first axis revealed that 24% of the variation was accounted for by subspecies differences ($F_{[7,208]} = 9.37$, $P < 0.0001$). The morphological features that differentiated groups most strongly along the first axis indicated that fish from the coastal, Great Basin, and Lahontan groups had the largest head and mouth sizes (Fig. 4*a*). Based on scores from the second RW axis, 68% of the variation was accounted for by differences among subspecies ($F_{[7,214]} = 63.65$, $P < 0.0001$). The second RW axis detected differences related to body depth and head length, separating coastal cutthroat trout and Lahontan cutthroat trout from most other subspecies categories (Fig. 4*a*). The Rio Grande group had the lowest scores of cutthroat trout subspecies along the second axis, indicating the smallest head length and deepest body shape. Based on scores from the third RW axis, 24% of the variation was accounted for by subspecies differences ($F_{[7,208]} = 9.35$, $P < 0.0001$). Scores from the fourth RW axis explained 26% of the variation among subspecies categories ($F_{[7,208]} = 10.47$, $P < 0.0001$; Fig. 4*b*).

Genetic and morphological distances

Morphological distance measures between cutthroat trout subspecies indicate that some subspecies are much more similar to each other than other groups. Euclidian distance measures of morphology indicate that Bonneville and Yellowstone cutthroat trout were most similar to each other, regardless of whether linear methods (Fig. 5*a*) or geometric methods (Fig. 5*b*) were used. Similarly, westslope and Colorado River cutthroat trout were ranked as the next most similar to Bonneville and Yellowstone cutthroat trout using both morphometric methods; however, geometric methods placed westslope cutthroat trout as more distant to the first three groups than linear-based morphometrics (Fig. 5*a* versus Fig. 5*b*). Coastal and Lahontan cutthroat trout were ranked as most distant to all other groups using linear measurements, with the Great Basin and Rio Grande groups as intermediate (Fig. 5*a*). Geometric methods of body shape indicated that the Rio Grande group was the most divergent morphologically, followed by a Great Basin – Lahontan – coastal grouping (Fig. 5*b*).

Fig. 5. Dendrogram of morphological distance measures based on (*a*) linear measurements or (*b*) geometric measurements of cutthroat trout (*Oncorhynchus clarkii*) morphology. Abbreviations identify cutthroat trout groups: Bonneville cutthroat trout (BCT), coastal cutthroat trout (CCT), Colorado River cutthroat trout (CRCT), Great Basin lineage of cutthroat trout (GBCT), Lahontan cutthroat trout (LCT), Rio Grande cutthroat trout (RGCT), westslope cutthroat trout (WCT), and Yellowstone cutthroat trout (YCT).

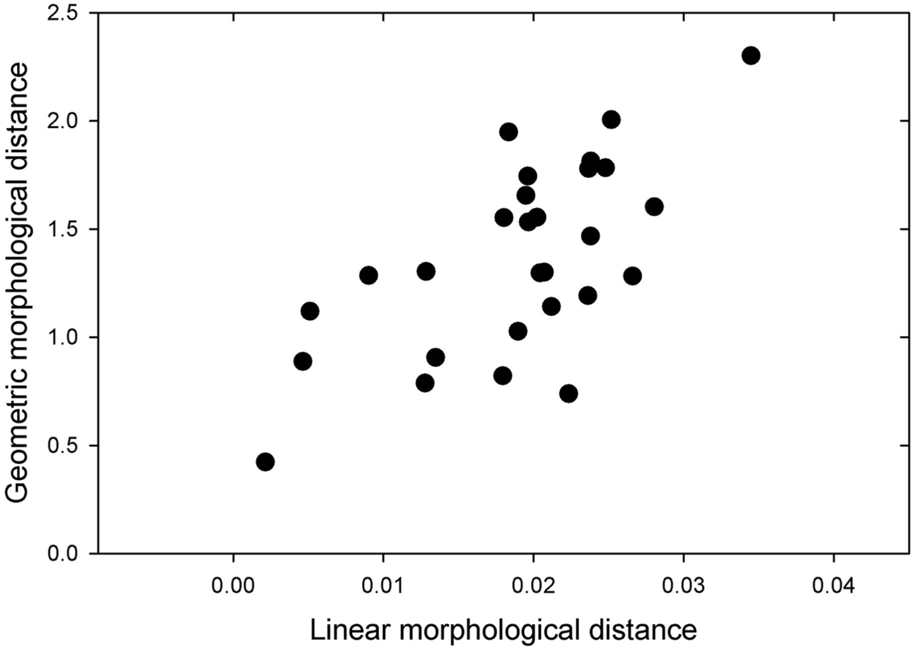


A comparison of morphological methods between cutthroat trout groups indicated that linear and geometrically based methods were positively correlated with each other (Mantel $r = 0.56$, $P = 0.0050$; Fig. 6). However, the concordance between morphological distance and genetic distance differed between the two distance measures. Morphological distance of cutthroat trout groups using linear measurements was positively correlated with Tamura–Nei genetic distance (Mantel $r = 0.38$, $P = 0.035$; Fig. 7*a*). Morphological distance of cutthroat trout groups based on geometric measurements was not significantly related to genetic distance (Mantel $r = -0.15$, $P = 0.73$; Fig. 7*b*).

Discussion

In this study, we compared cutthroat trout from populations distributed across its geographic range to determine if intraspecific groupings explain a significant proportion of phenotypic variation in this species. We found that morphological variation in cutthroat trout is related to the classification used to define intraspecific diversity in cutthroat trout, but some subspecies

Fig. 6. The relationship between pairwise Euclidian distance of cutthroat trout (*Oncorhynchus clarkii*) morphology based on linear morphological variables versus geometric morphological variables for each of the eight subspecies or lineages compared.

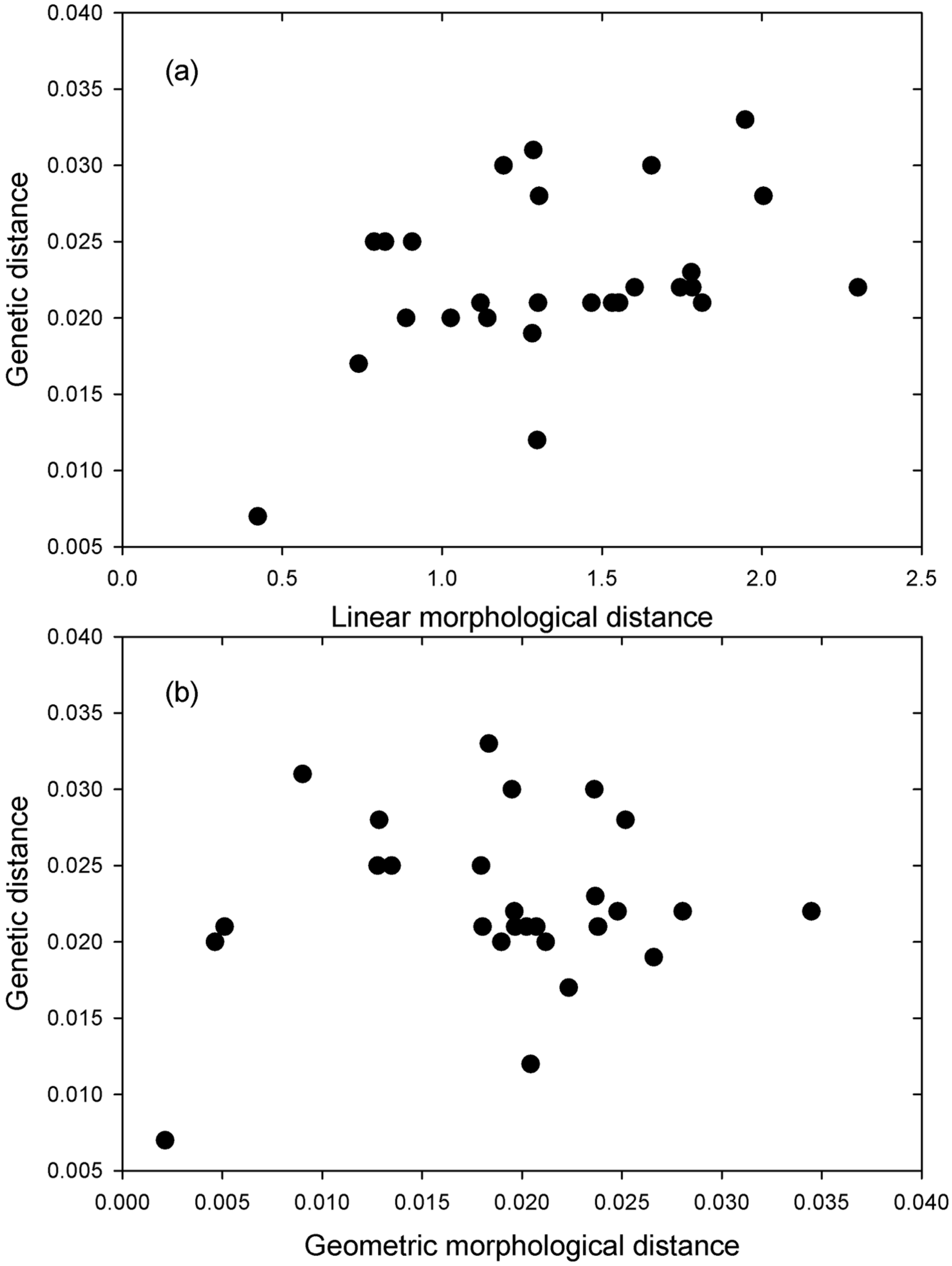


are much more similar to each other than others. We also examined the concordance between genetic and morphological measures of intraspecific variation and found a positive correlation between the two approaches when morphological measurements included linear-based characteristics. Our data indicate that early qualitative descriptions of intraspecific variation in cutthroat trout do capture morphological differences observed between populations from geographically distant locations; however, molecular methods have found important differences not originally identified by subspecies categories (Smith et al. 2002; Campbell et al. 2011*a*; Loxterman and Keeley 2012). Combining both morphometric and molecular methods in assessing intraspecific variation provides a comprehensive assessment and understanding of intraspecific diversity.

Across all subspecies of cutthroat trout that we examined, those that exhibited the most divergent morphology, such as coastal cutthroat trout and Lahontan cutthroat trout, also tended to be from watersheds that were more distant or isolated from other subspecies. Therefore, our data indicate some concordance between morphometric and genetic methods and suggest significant divergence between geographically isolated subspecies. However, for some of the more geographically proximate subspecies, the pattern of divergence is more complicated and should be approached cautiously. Bonneville cutthroat trout and Yellowstone cutthroat trout are geographically adjacent to each other and morphologically similar. They also share some of the same genetic haplotypes, possibly because of historical connections between the upper Snake River and Bear River watersheds (Campbell et al. 2011*a*, 2018; Loxterman and Keeley 2012; Eaton et al. 2018). Some additional populations of cutthroat trout found in the Bonneville Basin and upper Snake River exhibit mtDNA haplotypes that are much more divergent than geographic proximity would indicate, such as the Great Basin lineage (Loxterman and Keeley 2012), and whose distribution overlaps the range previously understood to be Bonneville or Yellowstone cutthroat trout (Behnke 1992, 2002). In cases of adjacent watersheds, detailed geographic sampling of populations may be required to identify boundaries or contact zones for different subspecies of cutthroat trout and should be used to inform any future conservation programs that are designed to re-establish populations (Bestgen et al. 2019).

Diagnostic meristic or morphological characters are often used to differentiate species of fishes and are important reference tools for establishing the presence and range of a species (Scott and Crossman 1973; McPhail 2007; Sigler and Zaroban 2018). Diagnostic characters capable of distinguishing phenotypically similar species may represent, in part, the genetic distance that has accumulated between two species diverging from the most recent common ancestor. Intraspecific variation is sometimes difficult to diagnose morphologically because of the similarity of forms and that early qualitative descriptions may represent extreme values of more continuous or clinal variation from populations at distant locations, or because of plasticity in characters induced by contrasting environmental conditions (Fitzpatrick 2010; Winker 2010; Kinnison et al. 2015; Campbell et al. 2017). The importance of such factors may only become evident with more geographic sampling and study. Cutthroat trout were first described in the scientific literature more than 175 years ago, but its entire distribution and indications of its phenotypic variability were not fully appreciated until decades later (Jordan et al. 1930; La Rivers 1962; Behnke 1992). Descriptions of different subspecies of cutthroat trout began appearing in scientific literature as morphologically variant populations were discovered in newly examined locations (Jordan et al. 1930; Thorgaard et al. 2018). It was not until the late 20th century that a more organized and consolidated grouping of cutthroat trout populations, principally by major watershed boundaries, occurred (Behnke 1979, 1992, 2002). While descriptions of different subspecies of cutthroat trout are often based on spotting patterns or meristic counts (Behnke 1992, 2002), overlapping ranges of some characters have made it difficult to unambiguously define diagnostic features in some subspecies (Qadri 1959; Dieffenbach 1966; Allendorf et al. 2005; Bestgen et al. 2019). In our study, we demonstrate morphological differences between cutthroat trout categories using continuously distributed features that typically are not simple enough to be used as diagnostic features in

Fig. 7. (*a*) The relationship between pairwise Euclidian distance of cutthroat trout (*Oncorhynchus clarkii*) morphology based on linear morphological variables versus Tamura–Nei genetic distance based on mtDNA sequence divergence of the *ND2* gene. (*b*) The relationship between Euclidian distance of cutthroat trout morphology based on geometric morphological variables versus Tamura–Nei genetic distance based on mtDNA sequence divergence of the *ND2* gene.



dichotomous keys. Nevertheless, our study does provide quantitative morphological evidence indicating that different subspecies categories do account for a significant component of phenotypic and genetic variation.

Despite the difficulties in identifying diagnostic characters among closely related groups, differences among populations of the same species are often thought to be a result of local adaptive differentiation that can represent important intraspecific variation (Taylor 1991; Smith and Skulason 1996). Salmonid fishes are well known for exhibiting polymorphisms associated with morphological or life-history characteristics (Sandlund et al. 1992; Chavarie et al. 2015; Kazyak et al. 2015; Koene et al. 2020). Intraspecific diversity requires quantitative measures from a variety of data sources, both phenotypic and genetic. Descriptions using only one approach may overlook important considerations at other levels. For example, cutthroat trout from Bear Lake (Idaho–Utah, USA) represent a lacustrine form of Bonneville cutthroat trout that achieves a large size at maturity by feeding on four different species of fish that are endemic to the lake (Ruzycki et al. 2001). Although this population has the same mtDNA haplotype as other stream-dwelling Bonneville cutthroat trout populations, it has distinct morphological, life-history, and ecological relationships to other species that merits special conservation concern because it represents unique intraspecific variation (Seiler and Keeley 2009; Loxterman and Keeley 2012). A similar life-history form of Lahontan cutthroat trout was also described from

Pyramid Lake, Nevada, USA (Hickman and Behnke 1979), and for rainbow trout from Kootenay Lake, British Columbia, Canada (Irvine 1978; Keeley et al. 2005). Such important variation may not be captured by studies of genetic or morphological data without an understanding of the ecological variation present across populations.

Past studies of morphological variation in fish taxa indicate that some features are related to differences in trophic ecology or habitat conditions occupied by different populations (Wainwright 1996; Berchtold et al. 2015). In our study, coastal cutthroat trout, Lahontan cutthroat trout, and the Great Basin lineage of cutthroat trout had the largest head and mouth sizes across the subspecies that we compared. Large head size in salmonid fishes has been associated with populations that feed on relatively large prey items, such as other species of fish, often in large, deep lakes (Irvine 1978; Campbell 1979; Chavarie et al. 2015). The historical association of pluvial Lake Lahontan and Lake Bonneville with the range of Lahontan cutthroat trout and the Great Basin lineage of cutthroat trout, as well as the current association of coastal cutthroat trout with marine habitats, may explain the relatively large head size of those populations with similar standing water habitat where piscivory in more commonly observed. In addition, most cutthroat trout populations occupy headwater streams for spawning purposes and often remain in those streams for rearing, and may remain in stream habitat throughout their entire life cycle (Northcote 1997). While strong selective pressure probably constrains the general body form of salmonids for flowing water habitat, significant variability and plasticity in salmonid morphology has been observed across populations that is related to differences in water velocity or migration distance (McLaughlin and Grant 1994; Pakkasmaa and Piironen 2000; Pavey et al. 2010; Bowen and Marchetti 2016). Even relatively small differences in body morphology can have important effects on critical physiological or behavioral characteristics such as swimming ability or predator avoidance (Taylor and McPhail 1985; Hawkins and Quinn 1996; Seiler and Keeley 2007). In contrast to differences in morphology that may occur as a result of environmentally induced phenotypic plasticity (Imre et al. 2002; Peres-Neto and Magnan 2004), the significant differences that we detected across replicate populations of different subspecies or evolutionary lineages of cutthroat trout indicate genetically based differences between the groups compared. Such differences may simply reflect accumulated differences between subspecies; however, they may also reflect locally adapted variation that can be important in maintaining viable populations over large geographic areas.

Widespread changes to natural ecosystems have created numerous conservation concerns for many species occupying threatened habitats and a need to understand the distribution and status of these species. Cutthroat trout populations were historically found in significant portions of major watersheds in western North America, but have declined in many areas due to negative interactions associated with non-native species through competition, predation, disease, and hybridization (Gresswell 2011; Penaluna et al. 2016). Similarly, habitat alteration from stream flow reduction, over-grazing of riparian areas by livestock, and resource extraction activities have also negatively affected cutthroat trout populations (Young 1995; Trotter 2008). Determining the conservation status of cutthroat trout populations and developing recovery plans requires an understanding of the distribution of cutthroat trout diversity. Regional cutthroat trout management plans (Thompson 2002; Teuscher and Capurso 2007) often operate on the basis of past taxonomic descriptions that have been complicated by more recent re-evaluations of genetic diversity. The advent and refinement of molecular techniques in conservation genetics has facilitated relatively rapid assessments of species distributions and connectivity of populations across an increasingly fragmented landscape (Cegelski et al. 2006; Neville et al. 2006). However, molecular assessments have also uncovered conflicting patterns with existing taxonomic categories by revealing cryptic divisions not evident in past taxonomic descriptions (Bowen et al. 2007; Trigo et al. 2013; Feinberg et al. 2014). For example, the leatherside chub (*Gila copei* (Jordan and Gilbert, 1881)), a minnow native to localized drainages of the western United States, was recently assigned to a different genus and divided into two species, the northern leatherside chub (*Lepidomeda copei* (Jordan and Gilbert, 1881)) and the southern leatherside chub (*Lepidomeda aliciae* (Jouy, 1881)), based largely on mtDNA sequence data that revealed two distinct phylogenetic lineages and a closer relationship to the spinedaces (genus *Lepidomeda* Cope, 1874) (Johnson et al. 2004; Page et al. 2013). For cutthroat trout, phylogenetic analyses have also identified subdivisions of populations that do not always correspond to previous subspecies descriptions (Smith et al. 2002; Campbell et al. 2007; Houston et al. 2012; Loxterman and Keeley 2012). Our comparisons, which included a Great Basin lineage of cutthroat trout identified by mtDNA sequence divergence (Loxterman and Keeley 2012), indicated significant morphological divergence. Despite more recent molecular evidence and other widely used classification schemes of cutthroat trout that recognize multiple subspecies (Behnke 1992, 2002), only one species of cutthroat trout is currently recognized in the list of fishes for North America by the joint committee of the American Fisheries Society and the American Society of Ichthyologists and Herpetologists (Page et al. 2013). How to incorporate new sources of information such as from this study and how to decide whether species or subspecies classifications should be revised or used as a basis for conservation decisions will be one of the challenges that arise as different taxa are evaluated to determine their conservation status (for an example see U.S. Fish and Wildlife Service 2015).

Conclusions

Widespread threats to aquatic habitats have created a need for conservation plans for cutthroat trout, but uncertainty about the distribution of cutthroat trout subspecies has sometimes hindered progress. Genetic and morphological distances indicate strong support for some past groupings, while others are more recent and need to be better appreciated and integrated into conservation plans. In addition to genetic and morphological data, comprehensive conservation plans should also include life-history data or measures of ecologically based variation. By doing so, biologists will have the best opportunity to maintain all remaining levels of intraspecific variation found across cutthroat trout populations.

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