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Molecular Systematics of Redband Trout from Genome-Wide DNA Sequencing Substantiates the Description of a New Taxon (Salmonidae: *Oncorhynchus mykiss calisulat*) from the McCloud River

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Abstract

Rainbow Trout, *Oncorhynchus mykiss*, exhibit high levels of phenotypic diversity leading to the recognition of numerous subspecies. A major distinction among Rainbow Trout subspecies exists between Coastal Rainbow Trout (*O. m. irideus*), which occurs west of the Cascade and Sierra Nevada mountain ranges, and interior Redband Trout (*O. mykiss* sspp.), largely distributed to the east. Interior Redband Trout are composed of three primary lineages and can share various outward, anatomical or physiological characteristics that are often symplesiomorphies or examples of convergence. We examine high-throughput DNA sequence data from Sacramento Redband Trout *O. m. stonei* from the Upper Pit and Upper McCloud Rivers along with representatives of Rainbow Trout and Golden Trout lineages to clarify the composition and relationships of the Sacramento Redband Trout. We find *O. m. stonei* to be polyphyletic, divided between populations in the Pit River and the Upper McCloud River. Redband Trout obtained from the Pit River are most-closely related to Great Basin Redband Trout *O. m. newberrii* and to fish of the Warner Lakes Basin and Surprise Valley within the *O. m. newberrii* lineage. The type specimen of *O. m. stonei,* collected from the Lower McCloud River, is phenotypically similar to Great Basin Redband Trout. We find as well that the isolated populations of trout in the Upper McCloud River Basin represent a lineage of Rainbow Trout now restricted to that region, are monophyletic and are not most closely related to the interior Redband Trout lineages of *O. m. newberrii* or *O. m. gairdnerii*. Furthermore, they are not represented by the type specimens of *O. m. stonei* or *O. m. shasta*. Consequently, we formally describe the McCloud River Redband Trout *O. mykiss calisulat,* new subspecies.

Key words: Molecular Phylogenetics, Native Trout, Pacific Trouts, Rainbow Trout, Sheepheaven Creek, Taxonomy

Introduction

For most of the time since description, Rainbow Trout, *Oncorhynchus mykiss* (Walbaum 1792), in North America were classified as *Salmo gairdnerii* Richardson 1836 based on a Columbia River Basin fish and termed ‘Redband Trout’ (Richardson 1836), or as *S. irideus* (originally *S. iridea* Gibbons 1855) based on a specimen from San Leandro Creek, California, representative of a coastal form (Gibbons 1855). Recognition that the North American *S. gairdnerii* and *S. irideus* were conspecific to Asian *O. mykiss* (originally *S. mykiss* Walbaum 1792), was followed by the application of *gairdnerii* and *irideus* as subspecies of *mykiss* and the movement of Pacific trouts into the genus

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TABLE 1 Origins of samples in the phylogenetic data set of this study. The common name and abbreviation used in text along with subspecies nomenclature for Rainbow Trout (*Oncorhynchus mykiss*) is provided.

Name Used in Text	Abbreviation Used in Text	Major Group	Group	Subspecies Nomenclature	Additional Strain or Type Info	Watershed	General Location
Ingroup							
Coastal Rainbow Trout	Coastal RT	CRT	CRT	*O. m. irideus*	Coleman Hatchery Strain		Crystal Hatchery
					Resident Rainbow Trout	American River	N. Fork American River
					Anadromous Rainbow Trout	Yuba River	Lower Yuba River
Eagle Lake Rainbow Trout	Eagle Lake RT	EGLK	EGLK	*O. m. aquilarum*	Eagle Lake	Eagle Lake	Eagle Lake
Great Basin and Upper Klamath Lake Redband Trout	Great Basin RB	REDB	REDB	*O. m. newberrii*	Goose Lake	Goose Lake	Cottonwood Creek
				O. m. newberrii	Warner Lakes	Warner Lakes	Dismal Creek
Columbia River Redband Trout	Columbia River RB	REDB	REDB	*O. m. gairdnerii*		Owyhee River	Little Jacks Creek
						Payette River	Fawn Creek
California Golden Trout	California GT	CGTC	CAGT	*O. a. aguabonita*	California Golden Trout	Golden Trout Creek	Volcano Creek Left Stringer
							Salt Lick Creek
Little Kern Golden Trout	Little Kern GT	CGTC	LKGT	*O. a. whitei*	Little Kern River Golden Trout	Little Kern River	Silver Lake
						Little Kern River	Upper Willow Creek
Kern River Rainbow Trout	Kern River RT	CGTC	KRRT	*O. m. gilberti*	Kern River Rainbow Trout	Kern River	Chagoopa Creek
							Kern-Kaweah Creek
Sacramento River Redband Trout	Sacramento RB	REDB	REDB	*O. m. stonei*		North Fork Pit River	Shields Creek
						South Fork Pit River	Fitzhugh Creek
McCloud River Redband Trout	McCloud River RB	MRRB	MRRB	*O. m. calisulat,* **ssp. nov.**		McCloud River	Sheepheaven Creek
							Dry Creek
							Edson Creek
							Moosehead Creek
Outgroup							
Lahontan Cuttroat Trout	Lahontan CT			*O. clarkii henshawi*	Eastern Management Area	Humboldt River	Gance Creek
					Pilot Peak Strain	Truckee River	Pilot Peak Hatchery

TABLE 1. (Continued)

Name Used in Text	Latitude	Longitude	Year	Location Code	Abbreviation Used in Figures	Individual Code
Ingroup						
Coastal Rainbow Trout	40.93185	-121.54696	2011	COLE	HRNB_COLE	HRNB_COLE_01
						HRNB_COLE_02
	39.17881	-120.72978	2006	NFAR	RAIN_NFAR	RAIN_NFAR_02
	39.20253	-120.61774				RAIN_NFAR_05
	39.23429	-121.31727	2009	LYBA	RAIN_LYBA	STLH_LYBA_01
						STLH_LYBA_02
Eagle Lake Rainbow Trout	40.58906	-120.77413	2009	EGLK	RAIN_EGLK	RAIN_EGLK_01
						RAIN_EGLK_02
						RAIN_EGLK_04
Great Basin and Upper Klamath Lake Redband Trout	41.94813	-120.29138	2004	CTWD	REDB_CTWD	REDB_CTWD_01
						REDB_CTWD_02
	41.98884	-120.17188	2002	DISM	REDB_DISM	REDB_DISM_01
						REDB_DISM_03
Columbia River Redband Trout	42.75043	−116.091990	?	JCKS	CRRB_JCKS	CRRB_JCKS_01
						CRRB_JCKS_02
	44.38714	−116.0615	?	FWN	CRRB_FAWN	CRRB_FWN_01
						CRRB_FWN_01
California Golden Trout	36.34926	-118.32317	2005	VCLS	CAGT_VCLS	CAGT_VCLS_01
						CAGT_VCLS_02
	36.38941	-118.33517	2005	STLC	CAGT_STLC	CAGT_STLC_01
						CAGT_STLC_02
Little Kern Golden Trout	36.38226	-118.53203	2007	SILV	LKGT_SILV	LKGT_SILV_01
						LKGT_SILV_02
	36.28728	-118.50044	2011	UWLC	LKGT_UWLC	LKGT_UWLC_01

......*continued on the next page*

TABLE 1. (Continued)

Name Used in Text	Latitude	Longitude	Year	Location Code	Abbreviation Used in Figures	Individual Code
						LKGT_UWLC_02
Kern River Rainbow Trout	36.47844	-118.44470	2007	CHGC	KRNB_CHGC	KRNB_CHGC_01
	36.59522	-118.47470	2007	KKWC	KRNB_KKWC	KRNB_KKWC_01
Sacramento River Redband Trout	41.43737	-120.34846	2009	SHLD	REDB_SHLD	REDB_SHLD_01
						REDB_SHLD_02
	41.36312	-120.30054	2003	FTZH	REDB_FITZ	REDB_FTZH_02
						REDB_FTZH_05
McCloud River Redband Trout	41.32840	-121.82547	2002	SHPN	REDB_SHPN	REDB_SHPN_01
						REDB_SHPN_02
	41.19658	-121.84220	2008	DRYC	REDB_DRYC	REDB_DRYC_01
						REDB_DRYC_03
	41.29322	-121.94003	2007	EDSN	REDB_EDSN	REDB_EDSN_01
						REDB_EDSN_04
	41.18441	-121.80454	2016	MOHD	REDB_MOHD	REDB_MOHD_01
						REDB_MOHD_02
Outgroup						
Lahontan Cuttroat Trout			2001	LCT	LCT_GACR	LCT_GACR_10
			2008	LCT	LCT_PPP1	PCT_PPP1_01

Oncorhynchus (Behnke 1966; Smith and Stearley 1989; Behnke 2002). Various subspecies of *O. mykiss* have been recognized, and the split between Coastal Rainbow Trout, *O. m. irideus* (Coastal RT), and the three main lineages of interior Redband Trout remains a major phylogenetic division. The naming of main lineages of Redband Trout follows their distribution: the Columbia River Redband Trout, *O. m. gairdnerii*, (Columbia River RB), the northern Great Basin and Upper Klamath Lake Redband Trout *O. m. newberrii* (originally *S. newberrii* Girard 1859, Great Basin RB) and, the Sacramento Redband Trout, *O. m. stonei* (originally *S. gairdneri stonei* Jordan 1894, Sacramento RB) (see Table 1 for full list of subspecies and abbreviations used).

The McCloud and Pit River Basins

The historically defined range of the Sacramento RB was largely the McCloud and Pit Rivers (Behnke 2002). These rivers joined under present day Shasta Lake (Figure 1). The McCloud River and the Pit River are distinctive in character though geographically close (Rutter 1908). The McCloud River originates from spring-fed streams near Mount Shasta and then passes over McCloud Falls, consisting of three waterfalls that are impassable to fishes migrating upstream (Figure 1). The watershed may be divided into an Upper McCloud above the falls and a Lower McCloud below these falls. The spring-fed nature of the McCloud River provided a thermal profile that was favorable to now-extirpated Bull Trout, *Salvelinus confluentus* (Suckley 1859), for its entire distribution from the confluence with the Sacramento River up to Lower Falls (Rode 1990). Volcanic activity around Mount Shasta has acted on the tributaries of the Upper McCloud leading to the isolation of spring-origin streams, and, in the case of several large basaltic flows, the creation of the McCloud Falls and the subsequent isolation of the Upper McCloud to upstream movement of fishes such as Chinook Salmon, *Oncorhynchus tshawytscha* (Walbaum 1792) (Yoshiyama *et al.* 2001). The Sacramento RB historically was the only known species of fish from the Upper McCloud (Rutter 1908).

The Pit River originates as a precipitation fed river in northeastern California that exhibits intermittent connections with Goose Lake to the north (Figure 1). The Goose Lake Basin is typically endorheic but may at times flow into the Pit River, observed as recently as 1868 (Grayson 2011). Goose Lake then for various reasons may be classified as part of the Sacramento River Basin as opposed to part of the Great Basin e.g. (Rutter 1908; Goose Lake Fishes Working Group 1996). Spring-derived flows make a large contribution in the middle section of the Pit River, below the confluence of the Fall River in the Pit River Basin. Historically, the Fall River was the farthest upstream tributary to be known to host spawning Chinook Salmon in the Pit River drainage with major waterfalls of the Pit River obstructing anadromous fish movements (Yoshiyama et al. 2001). In addition to physical barriers to fish movement, the contribution of the Fall River changes the character of the Pit River at their confluence, limiting consistent cold-water fish movement into the upper Pit River. From a desert stream generally inhospitable to trout except for headwaters, the Pit River becomes broadly more hospitable to trout due to the cold spring-fed influence of the Fall River. The upper reaches of the Pit River are known to contain various fishes. Rainbow Trout in tributaries of the Upper Pit River, appear similar to Great Basin RB (Behnke 2002) and may be frequently found along with Pit Sculpin, *Cottus pitensis* Bailey & Bond 1963, and hybrid Sacramento Sucker *Catostomus occidentalis* Ayres 1854 x Modoc Sucker *Ca. microps* Rutter 1908 (Cooper 1983). A species of Tui Chub likely of Great Basin origin—*Siphateles thalassinus* (Cope 1883)—is found in the Pit River and is also found in the Goose Lake Basin, Warner Lakes Basin and Cow Head Lake Basin (Harris 2000; Remple 2013). The mix of species in Pit River with different biogeographic origins may be attributed to pre-Pleistocene connection of the Pit River Basin to the upper Klamath River and also to a large river exiting the Great Basin (Moyle 2002). The stream community of Great Basin RB, Speckled Dace, *Rhinicthys osculus* (Girard 1856), Modoc Sucker, Marbled Sculpin, *Co. klamathensis* Gilbert 1898, and Pit-Klamath Brook Lamprey, *Entosphenus lethophagus* (Hubbs 1971), subsequently was subject to invasion of Sacramento River fishes, largely eliminating the Great Basin stream community and resulting in the hybridization of Sacramento Sucker and Modoc Sucker, the Pit Sculpin and Inland Riffle Sculpin, *Co. gulosus* (Girard 1854), creating a hybrid taxon, the Sacramento Riffle Sculpin, *Co. g. wintu* Moyle & Campbell 2022, and other taxa (Baumsteiger *et al.* 2017; Moyle and Campbell 2022).

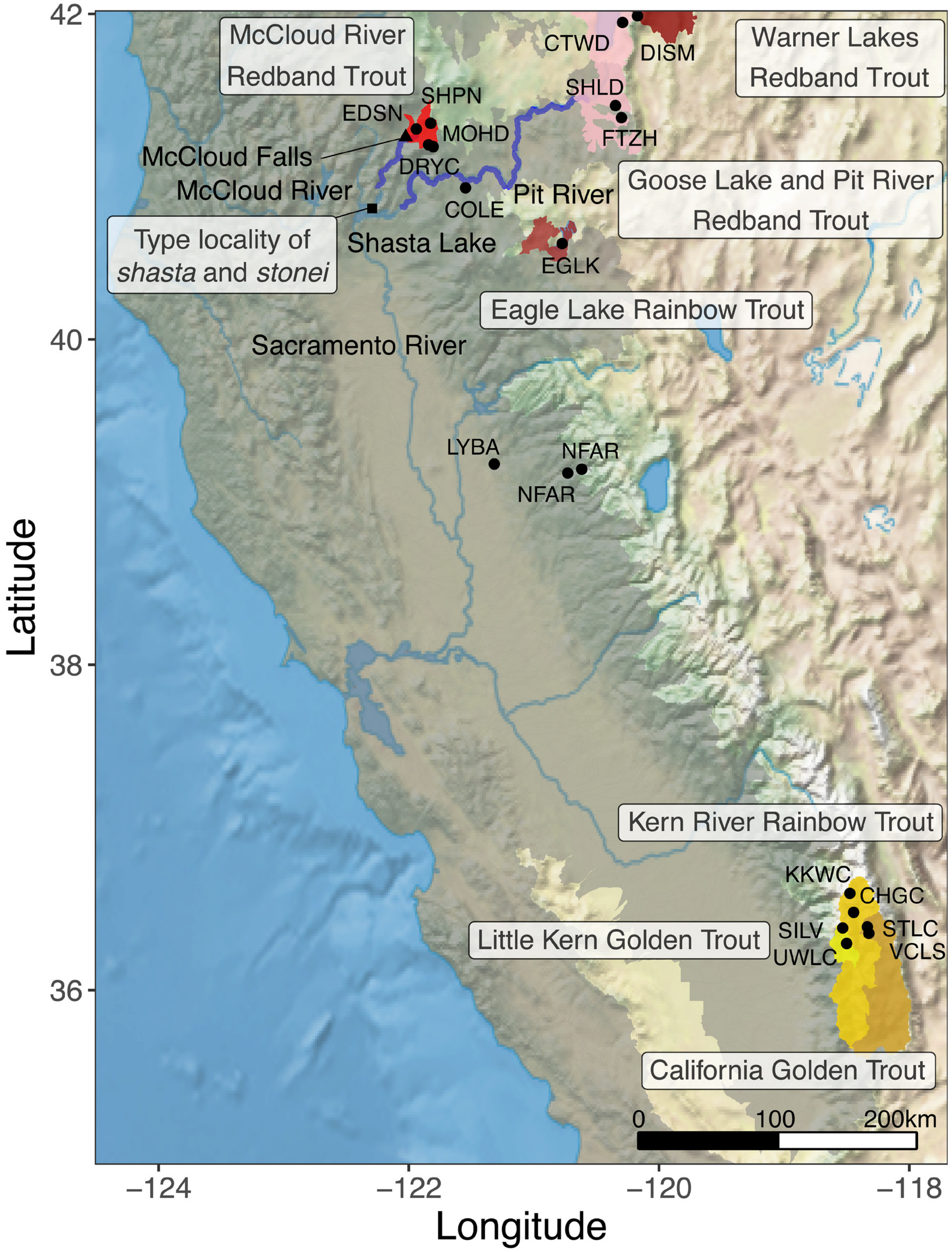

FIGURE 1. Map of key features and distributions of key lineages in this study. The distribution of Coastal Rainbow Trout (*Oncorhynchus mykiss irideus*) is shown in grey shading. Other lineages are labeled in different colors. Distributions were retrieved from the PISCES database (pisces.ucdavis.edu, "Historic Range—Expert Opinion") except for Warner Lakes Redband Trout (*O. m.* ssp.), which is represented by a polygon of hydrologic unit code (HUC) 17120007. Sampling locations used in phylogenetic analyses are indicated with a four-letter code that corresponds to Table 1 and Supplemental Table S1. Samples of *O. m. gairdnerii* from Idaho are not shown.

Redband Trout of the Northern Sacramento River Basin

Livingston Stone, director of the former Baird Station Fish Hatchery, observed and collected unique trout in the late 1800's from the McCloud River near Baird Station (40.793709, -122.298594 WGS84; Figure 1) and sent them to David Starr Jordan, an eminent ichthyologist and then president of Stanford University. The Sacramento RB was initially described as a subspecies of Columbia River RB from these samples as *Salmo gairdneri stonei* (CAS-SU 10900, holotype), honoring Livingston Stone with the subspecific epithet (Jordan 1894a). A second subspecies of Columbia River RB was also described by Jordan also from the McCloud River at Baird Station, *Salmo gairdneri* var. *shasta* (Jordan 1894b) (CAS-SU 10903, lectotype). Subsequent authorities chose the name *stonei* over *shasta* for Redband Trout found below the McCloud Falls in the McCloud River e.g. (Snyder 1933; Wales 1939). At a later date, *S. g. shasta* was synonymized with *S. g. stonei* (Needham and Behnke 1962), *S. gairdnerii* was synonymized with *S. mykiss* (though still recognized as a valid subspecies; Behnke 1992), and the western North American members of *Salmo* were transferred to *Oncorhynchus* (Smith and Stearley 1989) resulting in the current designation of *O. m. stonei* for the Sacramento RB.

Above the impassable McCloud Falls in the McCloud River, a distinctive trout native to the Upper McCloud River is present (Figure 1). These distinctive trout were termed 'southern Sierra golden trout' by Wales (1939, pg. 290) because he considered them to be different than *stonei*. The fish observed by Wales (1939) are not Golden Trout (*O. aguabonita*) which has two subspecies, California Golden Trout *O. a. aguabonita* (Jordan 1892) (California GT) and Little Kern Golden Trout *O. a. whitei* (Evermann 1906) (Little Kern GT). The distributions of both of these subspecies are in the southern Sierra Nevada and geographically distant from the McCloud River (Figure 1). The observation of Wales (1939) of so-called golden trout in the McCloud River drainage does indicate phenotypic similarities between Redband Trout of the Upper McCloud and fish in the Golden Trout Complex consisting of California GT, Little Kern GT and Kern River Rainbow Trout *O. m. gilberti* (Jordan 1894) (Kern River RT) (Behnke 2002). Behnke (1979; 1992; 2002) and Stephens (2007) advocated that trout in Sheepheaven Creek in the McCloud River drainage be described taxonomically as a new subspecies of *O. mykiss* distinctive from Sacramento RB. Sheepheaven Creek, while located in the McCloud River drainage basin, is an isolated spring-fed creek that flows ~1.6 km before all surface water returns underground. Genetic investigation has revealed that Sheepheaven Creek trout are one of a few largely non-admixed populations of a lineage occurring in the Upper McCloud River (Simmons *et al.* 2010; Habibi *et al.* 2022).

The reason why Upper McCloud River Redband Trout, McCloud River RB hereafter, appear so different from those below the falls may be attributable to a combination of ancient and ongoing isolation. McCloud River RB and the Golden Trout Complex are present at opposite ends of the California Central Valley, and this distribution along with some similarities has been interpreted as evidence of common ancestry of the two lineages (Schreck and Behnke 1971). In the vicinity of Mount Shasta in the northern Sierra Nevada and the Kern River in the southern Sierra Nevada, several plant and animal species exhibit disjunct distributions attributable to survival in refugia during Plesitocence glaciations. The Foxtail Pine (*Pinus balfouriana*) is a notable species mentioned by Schreck and Behnke (1971) that has an approximately 500 km gap in distribution between subspecies. Within this species, divergence between subspecies dating to the Middle or Early Pleistocene is attributable to a Pleistocene glacial event (Bailey 1970; Hickman 1993; Eckert *et al.* 2008). These lines of evidence point to the isolation of the McCloud River RB and survival during Pleistocene glacial extent in a refugium. This isolation continues as trout in the Lower McCloud River are unable to move above the McCloud Falls. Coastal RT for example, are hypothesized to have entered the Lower McCloud River much later than McCloud River RB, after the falls had become impassable to fish moving upstream (Behnke 1979).

As a result there was admixture of different evolutionary lineages below the falls in the Lower McCloud, probably including more than one Redband Trout lineage with Coastal RT. Behnke (1979, pg. 134) states, "there is such variability in numbers of vertebrae, scales, and pyloric caeca in the populations in various tributaries of the McCloud and Pit rivers that the name [*O. m.*] *stonei* would be useful only as name for the trout of a particular geographical region (upper Sacramento River Basin), not as a natural evolutionary unit." Behnke (2002) extended *O. m. stonei* to include Redband Trout with a historical distribution in the McCloud River, the Pit River, and, a potential distribution reaching south in the Sacramento River Basin to the Feather River. The application of *O. m. stonei* to a geographic region, as opposed to fish of a particular descent, is taxonomically problematic, and what constitutes *O. m. stonei* continues to vary in current literature and accepted use.

At times, the name *O. m. stonei* has been broadly applied not only to Redband Trout of the McCloud River and Pit River but it is also expanded to include Goose Lake, Warner Lakes and the Chewaucan Basin e.g. (Currens *et al.* 2009). Such a broad application of *O. m. stonei* relies on the monophyly of the subspecies, which as Behnke (1979) has already indicated, is likely not true. At other times, the name *O. m. stonei* is applied narrowly, and only to distinctive fish in the Upper McCloud River, that is McCloud River RB (Moyle 2002). The application of a restricted use of *O. m. stonei* only to fish above McCloud Falls excludes the type locality and type specimen of *O. m. stonei* from the historic range of *O. m. stonei* and omits Redband Trout occurring within the Pit River (Figure 1).

Phylogenetic Relationships of Sacramento River Redband Trout

Anatomical evidence indicates that McCloud River RB from Sheepheaven Creek are unlike Rainbow Trout as well as other Pacific trouts, and that the McCloud River RB are clearly distinguishable from the type specimen of *S. g. stonei* (Gold 1977; Behnke 1979). A comprehensive anatomical investigation has not been undertaken that included trout from various tributaries of the Upper McCloud, and anatomical characteristics across the range of McCloud River RB are not well characterized. Pit River RB on the other hand, are anatomically similar to the type specimen of *S. g. stonei*, the type specimen of *S. g.* var. *shasta* and Goose Lake Redband Trout (Goose Lake RB), a member of the Great Basin RB lineage *O. m. newberrii* (Behnke 1979). Behnke (1979) examined specimens collected from headwaters of the Pit River (n=10, South Fork Parker Creek) as well as museum specimens collected in 1904 (n=6, Joseph Creek) in support of this conclusion. Biogeographically, there exists a simple explanation, as Goose Lake drains southward at times into the Pit River (Behnke 1992; Goose Lake Fishes Working Group 1996). Furthermore, the Pit River exhibited ancient connectivity to the Great Basin (Moyle 2002). As a result, the distribution of Goose Lake RB trout may be extended into the Pit River, e.g. Figure 1.

While anatomical evidence is straightforward in indicating polyphyly of Sacramento RB, it lacks resolution in placing McCloud River RB in relation to Rainbow Trout lineages. Molecular phylogenetic studies have the potential to better resolve the relationships of McCloud River Trout and Pit River RB; however, molecular phylogenetic studies have produced conflicting results and have generated a great deal of uncertainty regarding Sacramento RB e.g. (Berg 1987; Nielsen *et al.* 1997; Nielsen *et al.* 1999; Pearse and Garza 2015; Abadía-Cardoso *et al.* 2019; Pearse and Campbell 2018). In part, the conflict among molecular phylogenetic studies comes from sampling strategies. Sampling for molecular investigations that included Redband Trout from the McCloud River, Pit River, Goose Lake and elsewhere to provide proper context has been limited e.g. (Berg 1987). Studies that include diverse Pacific trout lineages in general along with McCloud River RB, are notably absent as well. Another contribution to the uncertainty around a molecular phylogenetic consensus regarding the composition and relationships of Sacramento RB is the nature of genetic data collection. Genetic data collection methods have advanced rapidly, leading to various studies with different data types, resolution, and ability to detect admixture. The inclusion of mitochondrial and nuclear markers in a study of McCloud River RB can be self-contradictory e.g. (Bagley and Gall 1998). How genetic data are modeled, analytical complexity, is an additional important consideration. Not all studies modeled or addressed biological processes such as admixture with out of basin lineages in McCloud River RB and Pit River RB or incomplete lineage sorting (ILS).

We address many of the limitations of previous studies by examining genome-wide DNA sequence data in several analytical frameworks with appropriate sampling. We investigate Sacramento RB trout from the McCloud River and Pit River, potential near relatives, and major Rainbow Trout lineages to clarify questions about the composition, distribution and phylogenetic relationships of Sacramento RB.

Methods

In this study we analyzed two high-throughput sequencing data sets to investigate the population genetic and phylogenetic relationships of Sacramento River RB from the McCloud River and Pit River. First we sample broadly for population genetics including both subspecies of Golden Trout, then use a subset of those samples that exhibit minimal admixture as a result of human mediated translocations for phylogenetic analysis. Cutthroat Trout, *O. clarkii* (Richardson 1836), is included in phylogenetic analyses to provide rooted phylogenetic hypotheses and examine if the sister species has contributed to ingroup gene pools. We align our data to the Rainbow Trout genome and exclude residually tetrasomic genomic regions as well as known inversions to increase phylogenetic accuracy. The concordance and differences observed between analyses undertaken permits conclusions about the evolutionary relationships and origins of Redbands Trout of the Northern Sacramento River Basin to be drawn while accommodating for some of the complexities of biological evolution that have hindered clear differentiation among named groups to date.

Sampling and Sequencing

We sampled a taxonomically comprehensive set of lineages focusing on Redband Trout of the Sacramento River Basin, but also divergent lineages of Rainbow Trout. We obtained representatives of McCloud River RB from across the Upper McCloud drainage including four sampling locations that have been previously determined to have the least admixture with hatchery rainbow trout strains and are not descendants of known translocations: Dry Creek, Edson Creek, Moosehead Creek, and Sheepheaven Creek (Simmons *et al.* 2010; Stephens *et al.* 2013; Habibi *et al.* 2022). We sampled broadly from the Pit River Basin, including two geographically distant sampling locations from the Pit River, Shields Creek in the North Fork Pit River and Fitzhugh Creek in the South Fork Pit River without detectable introgression with Coastal RT (Habibi *et al.* 2022). We included Eagle Lake Rainbow Trout *O. m. aquilarum* (Snyder 1917) (Eagle Lake RT) individuals to represent this distinctive lineage at times considered a Redband Trout that likely originated from the Sacramento River Basin and has a long history of hatchery propagation and transfer.

To represent the remaining two main Redband Trout lineages, we included Great Basin RB from the Great Basin and the Goose Lake Basin with two locations that exhibit low admixture with Coastal RT (Habibi *et al.* 2022). The low admixture individuals were identified from the Warner Lakes Basin (Warner Lakes Redband Trout, Warner Lakes RB, DISM sampling location) as well as Goose Lake RB (CTWD sampling location, Figure 1). We obtained sequence data from Columbia River RB to represent the *O. m. gairdnerii* lineage in molecular phylogenetic analyses from different locations in Idaho (Chen *et al.* 2018: SRR5933416.1, SRR5933417.1, SRR5933432.1, SRR5933433.1).

Native Rainbow Trout of the Sacramento River Basin are represented by Coastal RT from the Yuba River and North Fork American River and are used in both population genetic and phylogenetic analyses. We also obtained sequence data from representative Coastal RT from California (Campbell *et al.* 2021) and Washington (Elwha River: Fraik *et al.* 2021). To identify potential contributions of hatchery RT strains, we obtained sequences from several widely used hatchery strains: Hot Creek, Coleman, Mt. Shasta, and Pit. We included sequence data from Coleman Hatchery Strain individuals in phylogenetic analyses. All three subspecies of the Golden Trout Complex (Kern River RT, Little Kern GT, California GT) were obtained, focusing on localities with high genetic integrity (Cordes *et al.* 2006; Stephens 2007; Erickson 2013; Habibi *et al.* 2022). To orient phylogenetic trees and to be able to identify any contribution from the sister species of Rainbow Trout, we included two divergent samples of Lahontan Cutthroat Trout, *O. c. henshawi* (Gill & Jordan 1978) (LCT), previously reported as individuals LCT_GACR_10 and PCT_PPP1_01 (Saglam *et al.* 2017).

Alignment to the Rainbow Trout genome was conducted as previously described (Habibi *et al.* 2022). Briefly, library preparation of Restriction-site Associated DNA sequencing (RADseq) libraries for data sets was conducted using *SbfI* as a restriction enzyme e.g. (Ali *et al.* 2016). However, the samples from Chen *et al.* (2018) were sequenced with single-end sequencing compared to paired-end for other samples. The mem algorithm of BWA version 0.7.17-r1188 (Li and Durbin 2009) was used for alignment and reads were filtered to concordantly mapped pairs and deduplicated with SAMtools version 1.7 (Li *et al.* 2009).

Genotype Likelihoods, Principal Component and Admixture Analyses

We conducted population genetic analyses in a genotype likelihood (GL) framework by first generating a GL file with ANGSD version 0.930 (Korneliussen *et al.* 2014). Genotype likelihood files were generated for all samples and for all Redband Trout separately. For both data sets we specified a minimum presence of the genotypes in 90% of individuals (-minInd) with the following additional options relating to quality and creation of the GL file: -GL 1 -doGLF 2 -doMajorMinor 1 -doMaf 2 -SNP_pval 1e-6 -minMapQ 20 -minQ 20. We generated covariance matrices for Principal Components (PC) analysis with PCAngsd (Meisner and Albrechtsen 2018). The covariance matrix for both all samples and Redband Trout was imported into R (R Development Core Team 2020) for conversion to PCs and visualization with ggplot (Wickham 2009).

Admixture analyses of the full population genetics GL file and the subset of Redband Trout GL file were conducted with NGSAdmix subprogram of ANGSD version 0.930 (Skotte *et al.* 2013). We used default parameters and assessed convergence so that the maximum iterations may be increased if necessary. The optimal number of *K* clusters was determined by conducting 10 runs at each *K* value (1 to 6 for population genetics data set and 1 to 5 for the subset of Redband Trout). The resulting likelihood values were supplied to the CluMPAK server (http://clumpak.tau.ac.il/bestK.html) and the best *K* identified as described in Evanno *et al.* (2005).

Multiple Sequence Alignment Generation

Samples exhibiting minimal to no admixture were selected to represent key lineages for phylogenetics based on the results of Habibi *et al.* (2022) and admixture results in this study (Table 1; Figure 1). In addition to the data from Habibi *et al.* (2022), Columbia River RB samples were included in this alignment (FWN and JCKS sampling locations). For the phylogenetic data set we generated an alignment of SNPs from BAM files by first creating a Variant Call File (VCF) with ANGSD version 0.930 (Korneliussen *et al.* 2014). For quality control thresholds on genotypes with ANGSD we required a site to be present in > 95 % of individuals (-minInd 39), a minimum minor allele frequency of 5% (-minMaf 0.05), quality score of read alignment (-minMapQ 30), quality score of base (-minQ 20), significance (-SNP_pval 1e-6) and a posterior cutoff of 0.95 (-postCutoff 0.95). The resulting VCF file was sorted with Awk version 4.1.4 (Aho *et al.* 1988) and linked SNPs removed using the *+prune* algorithm of bcftools version 1.10.1 (available at https://github.com/samtools/bcftools) with the following command line specifications: -l 0.9 -w 30000. Conversion of VCF to a phylip formatted file used the *vcf2phylip.py* script version 2.0 (available at https://github.com/edgardomortiz/vcf2phylip) and sites filtered for analysis under an ascertainment bias correction with *ascbias.py* (available at https://github.com/btmartin721/raxml_ascbias/blob/master/ascbias.py).

The recently produced chromosomal-level genome assembly of Rainbow Trout (Pearse *et al.* 2019) provides significant advantages when analyzing high-throughput sequence data for molecular phylogenetic analysis. Of particular note is that with increased representation of genome sequences from Salmonidae, residual tetrasomy across 10-15% of the genome and 25% of chromosomes is typical e.g. (Allendorf *et al.* 2015). Residual tetrasomy in salmonids is broadly conserved and well characterized in Rainbow Trout (Campbell *et al.* 2019; Pearse *et al.* 2019; Blumstein *et al.* 2020). In terms of phylogenetic analysis, two problems are created by the analysis of residually tetrasomic genomic regions: (1) Residual tetrasomy increases the effective population size of loci, exacerbating ILS e.g. (Campbell *et al.* 2020) and, (2), these residually tetrasomic regions are independently reverting to diploidy across lineages, creating difficulties in assigning orthology and potentially creating conflict between gene trees and species trees (Robertson *et al.* 2017; Campbell *et al.* 2020). Thus, the exclusion of residually tetrasomic regions of the Rainbow Trout genome should decrease gene-tree and species-tree conflict and increase phylogenetic accuracy.

Beyond the ancient genome duplication event, recently identified chromosomal inversions in Rainbow Trout have been located on two chromosomes: Omy05 and Omy20. These inversions do not recombine with the corresponding non-inverted genome regions, contain many linked variants and appear to be adaptive. The Omy05 inversion is well-characterized, contains > 1200 genes and is associated with anadromy and residency or warm and cold temperatures e.g. (Miller *et al.* 2012; Pearse *et al.* 2014; Pearse *et al.* 2019). The Omy05 inverted haplotypes (Resident-type) are strongly selected for in populations without access to either the ocean or large lakes and rivers, and notably above barriers and waterfalls e.g. (Martínez *et al.* 2011; Leitwein *et al.* 2017). Clear phylogeographic signal is seen in the ancestral (non-inverted, Anadromous-type) variants, but not in the inverted haplotypes (Pearse *et al.* 2019). Given the selection on this region, high frequency of inverted haplotypes in focal populations, and potential influence in concatenated analyses we excluded the Omy05 inversion region from our phylogenetic analyses. The Omy20 inversion forms have been found to vary in frequency between small, high gradient streams without ocean, lake or large river access when compared to a Rainbow Trout population with ocean access (Campbell *et al.* 2021). Given the properties of these inversions, the genomic regions containing them should be excluded from phylogenetic analyses to increase accuracy. Therefore, we excluded tetrasomically pairing regions of the Rainbow Trout genome and chromosomal inversions located on Omy05 and Omy20 when generating genotype calls (available in GitHub repository https://github.com/MacCampbell/o-m-calisulat).

Maximum Likelihood Phylogenetic Inference & Hypothesis Testing

We first conducted an unconstrained Maximum Likelihood (ML) tree inference with IQ-TREE multicore version 2.0-rc1 to produce both a best tree (ML best tree) and a consensus tree (ML consensus tree) (Nguyen *et al.* 2014; Trifinopoulos *et al.* 2016). We selected nucleotide evolution models based on the Bayesian information criterion (BIC) and corrected for ascertainment bias (-m MFP+ASC). Support for the ML best tree nodes was assessed with the Shimodaira-Hasegawa approximate Likelihood Ratio Test (SH-alRT) (Guindon *et al.* 2010) (-alrt 1000) and 1,000 rapid bootstraps (Hoang *et al.* 2017) (-bb 1000). The ML consensus tree was generated as a consensus from the 1,000 bootstrap trees generated by the rapid bootstrap algorithm and support for nodes also assessed by bootstrapping. To test support for previously hypothesized relationships of McCloud River RB, we evaluated the following scenarios against the ML best tree with IQ-TREE: (1) The consensus tree, (2) McCloud River RB and the Golden Trout Complex are monophyletic, (3) McCloud River RB, Great Basin RB and Columbia River RB are monophyletic, and (4) McCloud River RB and Coastal RT are monophyletic. To conduct the topology tests, we specified the best-fit model of sequence evolution identified in the ML tree search, and estimated model parameters from both the consensus tree and an initial parsimony tree. The constrained trees (e.g. McCloud River RB and Golden Trout Complex are monophyletic) were then passed to IQ-TREE to compute all available topology tests within the IQ-TREE program. An parameter of 10,000 replicates was specified with the RELL method (Kishino *et al.* 1990) (-zb 10000 -zw -au) and model parameters estimated from the consensus ML tree topology with the -te option.

Species Tree Analysis

A concatenated phylogenetic inference enforces the same genealogy on all loci, and while unrealistic, is often a useful model to use. As it is clear gene-tree and species-tree discordance exists as a result of ILS, it is sensible to model it explicitly if possible (Maddison 1997; Degnan and Rosenberg 2009). We applied a multispecies coalescent model to phylogenetic analyses in this study. This approach specifically addresses ILS (deep coalescence) (Maddison 1997), which can lead to incorrect phylogenetic estimation e.g. (Knowles and Kubatko 2010). The multispecies coalescent was applied and a species tree generated by analyzing the SNP alignment with SVDQuartets distributed as part of PAUP* version 4.0a (Swofford 2003; Chifman and Kubatko 2014; Chifman and Kubatko 2015). To use SVDQuartets, we pooled samples from the same sampling locations for analysis, except LCT, which were combined despite originating from two different sampling locations. Keeping recognized subspecies or Rainbow Trout separated across sampling locations allowed for evaluation of the phylogenetic independence of McCloud River RB sampling locations. Additionally, Great Basin RB in the concatenated analyses indicated that Pit River RB may be more closely related to Warner Lakes RB, and this intra-clade relationship would be able to be evaluated under the multispecies coalescent model by not combining sampling locations across pre-defined subspecies. Within SVDQuartets, we evaluated all possible quartets and evaluated topological support with 100 bootstrap replicates.

Phylogenetic Network Analysis

Any model of evolution that assumes a single bifurcating tree poorly describes complex evolutionary scenarios. Furthermore, the separation of species before or after hybridization can create identical gene tree conflict as observed from ILS (Yu *et al.* 2011). To address these limitations, we created a phylogenetic network. Phylogenetic networks allow the visualization of reticulation events such as hybridization and provide a more complete picture of conflicting signals in a data set e.g. (Huson and Bryant 2005; Solís-Lemus and Ané 2016). We constructed an implicit phylogenetic network to display alternative evolutionary scenarios present in our data set (Huson *et al.* 2010). We generated the phylogenetic network with SplitsTree version 4.15.1 (Huson and Bryant 2005) from a neighbor-net algorithm using the SNP alignment previously examined.

Taxonomic Methods

We examined specimens of McCloud River RB and material of *Oncorhynchus mykiss* housed in the Museum of Wildlife and Fish Biology, University of California, Davis (WFB), and the Biodiversity Research and Teaching Collections, Texas A&M University, College Station (TCWC). We report only those counts and measurements reported by Gold (1977: Table 1), though counts of pyloric caecae and gill rakers could not be obtained from WFB material. Counts and measurements were generally obtained following Hubbs and Lagler (1949). Counts of vertebrae include the two ural centra and were obtained from radiographs or from cleared and double stained material (Taylor and Van Dyke 1985). Counts of dorsal, anal, and caudal-fin rays were obtained from radiographs or from cleared and double stained material. The two posteriormost rays of the dorsal and anal fin (which articulate with a single pterygiophore) were counted as one ray.

Results

Population Genetics Sampling

We obtained sequence data from 318 Rainbow Trout that we analyzed in a population genetics framework (Supplemental Figure S1, Supplemental Table S1). These 318 trout make up the *population genetics data set* and contain 72 McCloud River RB samples and 132 additional trout from the Sacramento River drainage and Great Basin sampling locations identified as Redband Trout when collected. Additionally, we obtained 10 representatives of Eagle Lake RT as a putative Redband Trout of the Sacramento River drainage. None of the paired end data was composed of Columbia River RB and it is not present in the population genetics dataset.

Coastal RT is represented by 38 samples, from the Sacramento River drainage (three sampling locations, n=8), Coastal California (four sampling locations, n=25), and the Elwha Drainage of western Washington State (n=5). The four hatchery rainbow strains are represented by 17 individuals. From the Golden Trout Complex, 49 samples are divided between California GT (n=22), Kern River RT (n=9) and Little Kern GT (n=18).

Genotype Likelihoods, Principal Component and Admixture Analyses

The GL file of the 318 individuals in the population genetics data set contains 119,325 SNPs with a minimum MAF of 0.05. Three main groupings are apparent in the PCA of the population genetics data set corresponding to McCloud River RB, the California Golden Trout Complex, and other Redband Trout + Coastal RT + Eagle Lake RT + hatchery strains (Figure 2A). The first PC of the population genetics data set separates McCloud River RB from other Rainbow Trout (13.04% of variance). The second PC separates the Golden Trout Complex from Rainbow Trout lineages most broadly (11.04% of variance). Within large groupings, the separation of California GT from Kern River RT + Little Kern GT is apparent as well as other Redband Trout from Coastal RT + Eagle Lake RT + hatchery strains.

Admixture results of the population genetics data set indicate a best K of 3. At $K = 2$, McCloud River RB separate from all other trouts. At $K = 3$, the Golden Trout Complex and other Redband Trouts form additional clear clusters. Coastal RT, Eagle Lake RT, and hatchery strains consist of varying degrees of contributions from the three main ancestry components. Increasing K creates a cluster of Coastal RT + Eagle Lake RT + hatchery strains that shows substantial contributions to some individuals identified as Redband Trout. Additional Ks identify a unique Eagle Lake RT ancestry component and indicate the distinctiveness of California GT within the California Golden Trout Complex.

The McCloud River RB and other Redband Trout subset of the population genetics analysis consists of 204 individuals and a GL file with 96,926 sites with a minimum MAF of 0.05. From this PC analysis, the first PC (21.25% of variance) separates McCloud River RB from other Redband Trout (Figure 2B). Redband Trout in this data set are further separated along the second PC (3.11% of variance) between Goose Lake drainages and other sampling locations in the Sacramento River Basin as well as the Warner Valley and Surprise Valley. Admixture results indicated a best $K = 2$, separating McCloud River Trout from all other Redband Trout (Figure 4). Increasing Ks separate a likely Coastal RT or hatchery strain contribution to the ancestry of individuals. At $K = 4$ a separate ancestry component within Great Basin RB from the Goose Lake Basin is present.

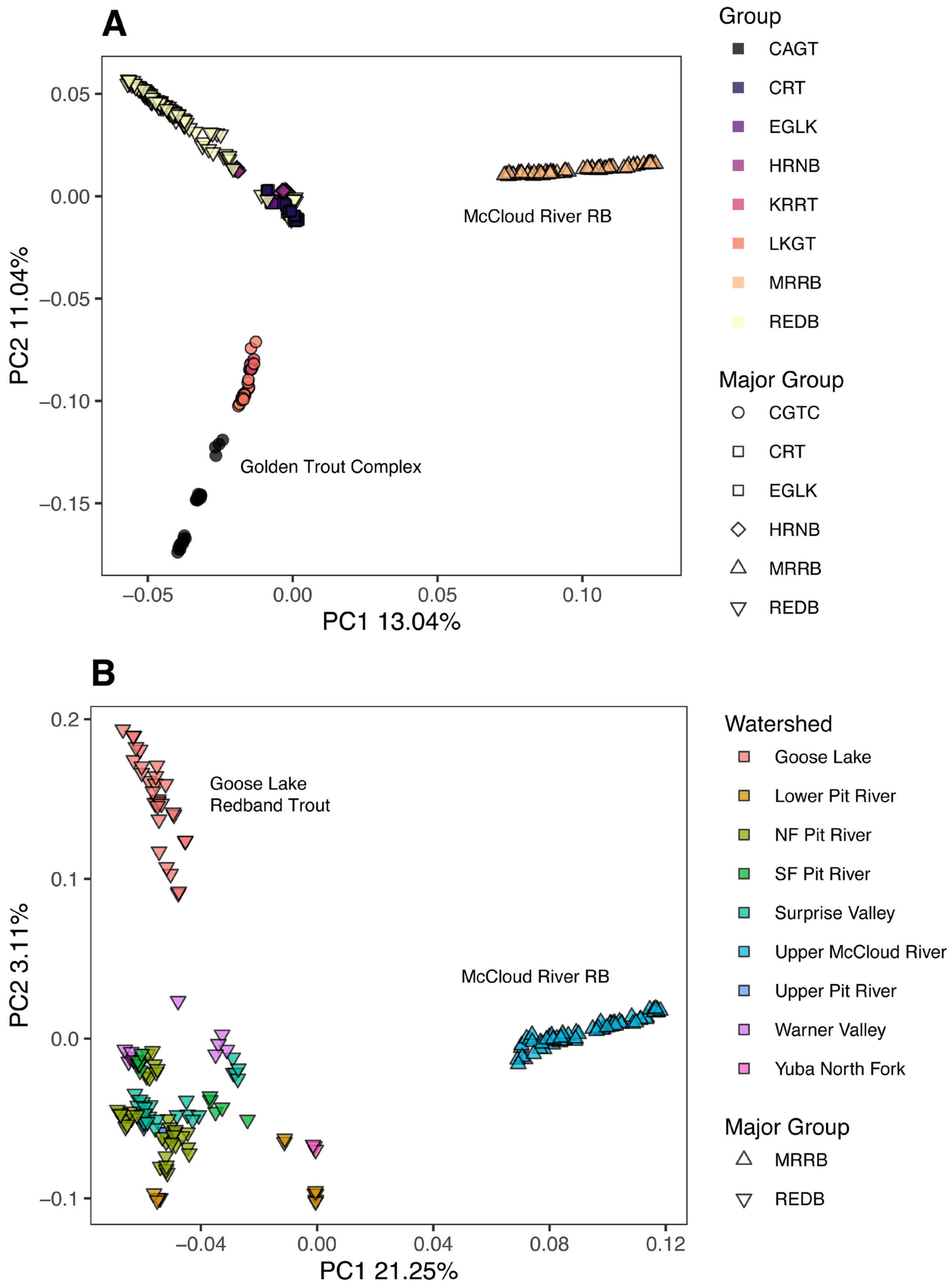

FIGURE 2. Principal Component Analyses. The first two Principal Components (PCs) are presented for all samples (n = 318) in the population genetics analysis in A and Redband Trout samples (n = 204) in B. Genotype likelihoods were generated separately for the PCs presented in each panel. In A points are color coded by Group corresponding broadly to lineage, and further condensed into a Major Group by consolidating the California Golden Trout Complex and represented by shape (Table 1). In B, points are colored by watershed and the same shape applied to the Major Group (MRRB and REDB). Abbreviations for Major Group are explained in the text.

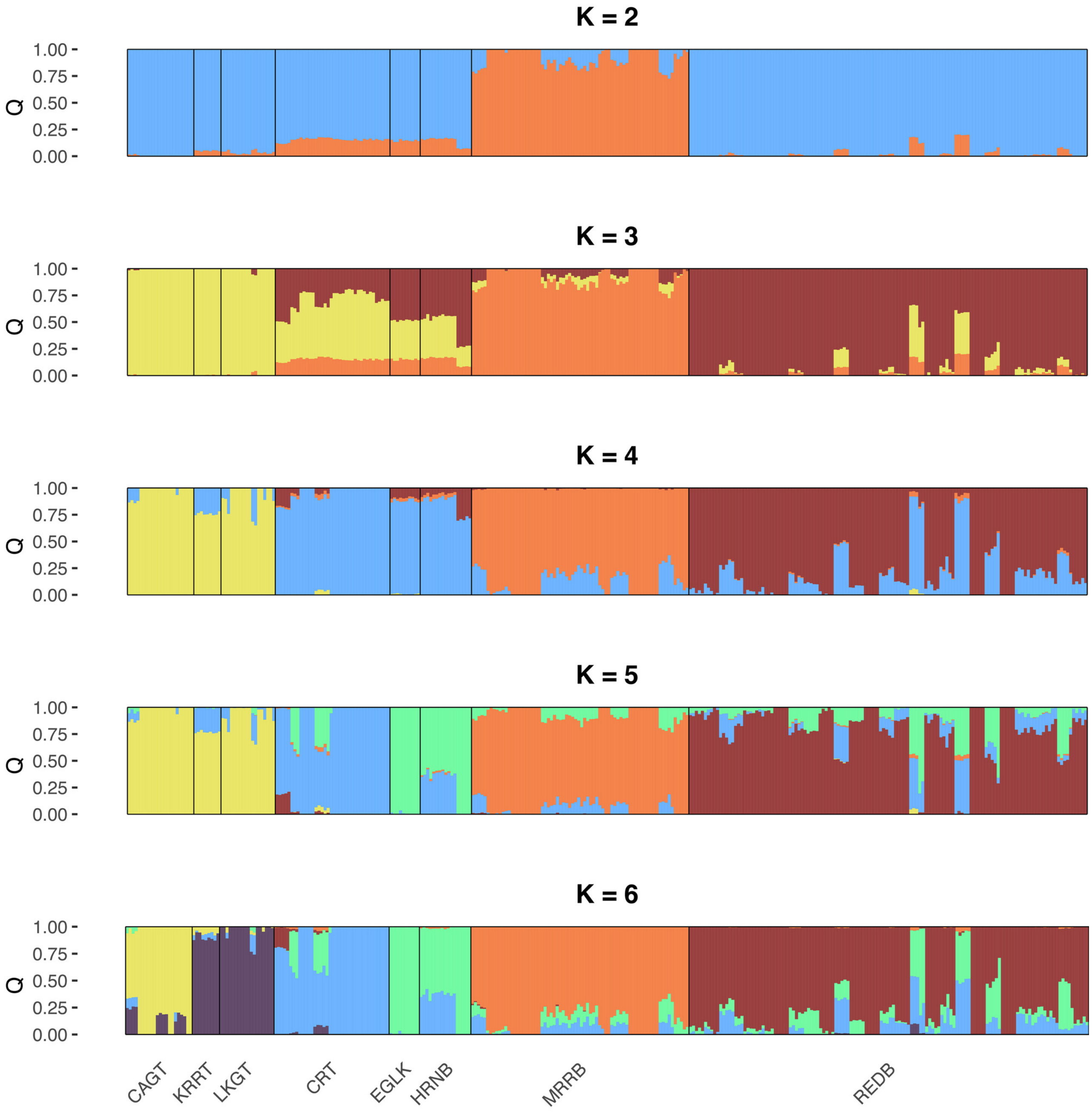

FIGURE 3. Admixture plots from the population genetics data set. Number of genetic clusters (*K*) presented for *K* = 2–6 from all samples (n = 318) analyzed in a population genetics framework. Admixture analysis was conducted in NGSAdmix with an optimal *K* = 3. Labeling of *x*-axis is according to Group as in Table 1: CAGT, California Golden Trout; KRRT, Kern River Rainbow Trout; LKGT, Little Kern Golden Trout; CRT, Coastal Rainbow Trout; EGLK, Eagle Lake Rainbow Trout; HRNB, Hatchery Rainbow Trout; MRRB, McCloud River Redband Trout; REDB, all other Redband Trout.

Phylogenetic Alignment Characteristics

After excluding residually tetrasomic regions and chromosomal inversions, SNPs were called from approximately two-thirds of the bases in assembled Rainbow Trout chromosomes. From the 41 individuals in the alignment, a total of 9,787 SNPs were called and pruning linked SNPs reduced the number of SNPs to 4,266. The final SNP data set after ascertainment bias correction is 2,999 SNPs, with 2,990 distinct site patterns and 1,775 parsimony informative sites. The alignment file is provided in the Data Supplement (available at https://github.com/MacCampbell/o-m-calisulat).

Maximum Likelihood Phylogenetic Inference & Hypothesis Testing

The best-fit model of nucleotide evolution as determined by BIC is TVM+F+ASC+R2 with the ML tree presented in Figure 5A and the consensus tree in Figure 5B. Output files from IQ-TREE are provided in the Data Supplement. In all analyses, the placement of McCloud River RB and Pit River RB indicates non-monophyly of Sacramento RB. Pit River RB samples are consistently placed within the Great Basin RB (*O. m. newberrii*) lineage. The ML tree (Figure 5A) indicates the Eagle Lake RT is the earliest branching lineage of *O. mykiss* and receives maximal SH-aLRT support (100) as well as maximal bootstrap support (BS, 100%). The Golden Trout Complex is the next successive branching within the *O. mykiss* lineage and its monophyly is supported by SH-aLRT (97) and bootstrap support (95%). Within the Golden Trout Complex there is support for the Kern River RT and Little Kern GT as sister taxa (SH-aLRT 87, BS=91%). Generally, taxonomic units are well-supported, except for *O. m. irideus* (SH-aLRT=67 and BS=28%) and the monophyly of Sacramento RB is not present. All McCloud River samples had strong support for monophyly (maximal SH-aLRT and BS), with a sister relationship to *O. m. gairdnerii* + *O. m. newberrii* not well-supported with a SH-aLRT of 94 and BS of 37%. Sheepheaven Creek samples of McCloud River RB are nested within other Upper McCloud River sampling locations.

The consensus ML tree (Figure 5B) identified McCloud River RB as the earliest branching *O. mykiss* lineage with maximal bootstrap support for monophyly with the Sheepheaven Creek population nested within other sampling locations of this subspecies with the same branching pattern as the ML tree (Figure 5A). The monophyly of *O. m. gairdnerii* and *O. m. newberrii* (including Pit River RB) as well as the Eagle Lake RT and the Golden Trout Complex are well-supported (BS = 90%, 100% and 95%, respectively). Within the Golden Trout Complex, Kern River RT and Little Kern GT are well-supported as sister taxa (BS = 91%). However, the support for the branching pattern among the Golden Trout Complex, Coastal RT and Eagle Lake RT is not strong. Eagle Lake RT and Coastal RT are sister taxa in this analysis, with very low BS (20%).

TABLE 2. Phylogenetic hypothesis testing results. For alternative hypothesis to the ML tree shown in Figure 5A, the log Likelihood score is shown as well as the difference between the ML tree and the alternative hypotheses. Statistics evaluating support for alternative hypotheses as shown, with a significant score indicated in bold. The test scores are: bp-RELL, bootstrap proportion using RELL method; p-KH, *p* - value of one sided Kishino-Hasegawa test; p-SH, *p* - value of Shimodaira-Hasegawa test; p-WKH, *p* - value of weighted Kishino-Hasegawa test; *p* - value of weighted Shimodaira-Hasegawa test; c-ELW, Expected Likelihood Weight; p-AU, *p* - value of approximately unbiased test.

Hypothesis	**log Likelihood**	**log L. Difference**	**bp-RELL**	**p-KH**	**p-SH**
ML Consensus Tree	-26541.62	33.42	0.08	0.11	0.18
McCloud River RB and Golden Trout Complex are Monophyletic	-26528.18	19.98	**0.02**	0.11	0.32
Columbia River RB, Great Basin RB and McCloud River RB are Monophyletic	-26516.46	8.26	0.24	0.27	0.63
McCloud River RB and Coastal RT are Monophyletic	-26523.39	15.19	0.04	0.08	0.43

Continued.

Hypothesis	**p-WKH**	**p-WSH**	**c-ELW**	**p-AU**
ML Consensus Tree	0.11	0.18	0.08	0.13
McCloud River RB and Golden Trout Complex are Monophyletic	0.06	0.17	**0.02**	**0.04**
Columbia River RB, Great Basin RB and McCloud River RB are Monophyletic	0.27	0.64	0.24	0.42
McCloud River RB and Coastal RT are Monophyletic	0.08	0.21	0.04	0.12

Hypothesis testing results are provided in the Data Supplement and in Table 2. The use of either the ML best tree or an initial parsimony tree did not change which tests were significant, and only the ML consensus tree for model parameter estimation results are presented. The ML best tree indicated Columbia River RB, Great Basin RB and McCloud River RB to be monophyletic with weak support; however, only three results reject an alternative hypothesis to the ML best tree. All three of these are from the constraint of McCloud River RB and the Golden Trout Complex as monophyletic. Monophyly of McCloud River RB and the Golden Trout Complex was rejected by the bootstrap proportion using the RELL method (0.02), the Expected Likelihood Weight test (*p*-value = 0.02) and the Approximately Unbiased test (*p*-value = 0.04) (Kishino *et al.* 1990; Shimodaira 2002; Strimmer and Rambaut 2002).

Species Tree Analysis and Phylogenetic Network

The species tree has maximal support for each node (BS = 100%) and indicates the Golden Trout Complex is the earliest-branching lineage of *O. mykiss* (Figure 6). Kern River RT and Little Kern GT are sister taxa within the Golden Trout Complex. McCloud River RB branch successively following the Golden Trout Complex with Sheepheaven Creek again nested within other McCloud River RB populations. A clade of Coastal RT and Eagle Lake RT is present, with Eagle Lake RT sister to Coleman Hatchery fish. The Columbia River RB trout are sister to Warner Lakes RB plus Pit River RB.

The neighbor net phylogenetic network exhibits several reticulations (Figure 7). Reticulations are present among species of the Golden Trout Complex, Lahontan CT and California GT, and, Coleman Hatchery and Eagle Lake RT lineages; however, McCloud River Trout do not have any reticulations between other taxonomic units.

Discussion

Origins and Evolutionary Relationships of Northern Sacramento River Basin Redband Trouts

Problematically, implied monophyly is associated with the use of 'redband' as part of the common name of various Rainbow Trout subspecies. If Redband Trout are monophyletic, a parsimonious explanation would be that McCloud River RB of the Upper McCloud River are most closely related to another geographically proximate Redband Trout lineage, such as the Pit River RB, that also occurs in the Sacramento River Basin. Prior to this study it was already apparent that McCloud River RB and Pit River RB may be separate lineages e.g. (Behnke 1979) and Redband Trout as a whole are not monophyletic e.g. (Wishard *et al.* 1984). However, we can expand on these concepts with a finer resolution of the higher-level relationships of Redband Trout. Our study finds evidence that McCloud River RB persisted in a glacial refugium in the Upper McCloud River during the Pleistocene, thus have persisted over time, and may have become isolated by the formation of waterfalls on the McCloud River. The Pit River RB samples in this study are most closely related to Redband Trout of the Warner Lakes and the Surprise Valley. Pit River RB most likely entered the Sacramento River Basin from the Great Basin; however, Goose Lake RB do not provide the majority of the ancestry of Pit River RB although it is considered part of the Sacramento River drainage. We find that none of the Sacramento River Basin Redband lineages—McCloud River RB, Pit River RB and Eagle Lake RT—are closest relatives of each other.

To understand the evolutionary relationships of Northern Sacramento River Basin Redband Trouts, the examination of material from the type locality of *O. m. stonei* would undoubtedly be informative in a genetic study. However, the type locality, Baird Station, is now underwater due to the construction of the Shasta Dam, and as such material from this locality could not be resampled for analyses in the present study. Furthermore, numerous human-mediated transfers of Rainbow Trout have occurred around what was the type locality of *O. m. stonei* e.g. (Pearse and Garza 2015) such that physically proximate sampling locations undoubtedly have some human-mediated influence. The type specimens of *S. g.* var. *shasta* and *S. g. stonei* from Baird Station are anatomically similar to Goose Lake RB and historic samples from the Pit River (Behnke 1992). Without being able to obtain genetic sequence data from the type locality of *S. g. stonei*, we can still advance understanding of the origins and evolutionary relationships of extant populations of McCloud River RB, Pit River RB, and the Eagle Lake RT.

What is a McCloud River Trout?

McCloud River RB exhibit taxonomic uncertainty across several levels. Are they a single phylogenetic entity? Are McCloud River RB nested within another subspecies or did they originate from hybridization or some other complex evolutionary scenario? What are the nearest relatives of McCloud River RB? Previously, Sheepheaven Creek trout have been reported to be anatomically distinctive and separate taxonomic recognition has been proposed e.g. (Behnke 2002). The population genetic analyses in this study demonstrate the uniqueness of McCloud River RB as a gene pool and that it is distributed in a restricted geographic area above McCloud River Falls (Figures 2, 3 and 4) (Habibi *et al.* 2022). There exist several genetically intact populations of McCloud River RB not subjected to out of basin transfers, including the Sheepheaven Creek population (Simmons *et al.* 2010; Habibi *et al.* 2022). The presence of genetic variants that are found in McCloud River RB and Coastal RT may represent ancient hybridization of Coastal RT with archaic trouts or migration from the McCloud River RB downstream into Coastal RT gene pools, or a combination of both. The molecular phylogenetic analyses conducted in this study find the four sampled populations representing genetically pure McCloud River RB to be monophyletic and that Sheepheaven Creek trout are nested within the other populations (Figures 5 and 6). Based on the data, assumptions, and analyses presented here, the recognition of Sheepheaven Creek trout alone as a separate taxonomic entity is not warranted e.g. (Simmons *et al.* 2010). Instead, there are several populations of McCloud River RB that are most-closely related to each other and restricted in distribution to the Upper McCloud.

Another level of taxonomic uncertainty with McCloud River RB is the independence of this lineage. In all analyses we conducted with population genetics techniques, we identified a coherent McCloud River RB lineage that is not composed of mixed lineages (Figures 2, 3 and 4). With three distinct phylogenetic analytical frameworks, we find concordant support for the independence of the McCloud River RB lineage and it is not nested within another subspecies (Figures 5, 6 and 7). The sampled populations representing McCloud River RB received maximal support for monophyly and the phylogenetic network does not demonstrate reticulation events between McCloud River RB and other taxa that would suggest major hybridization events.

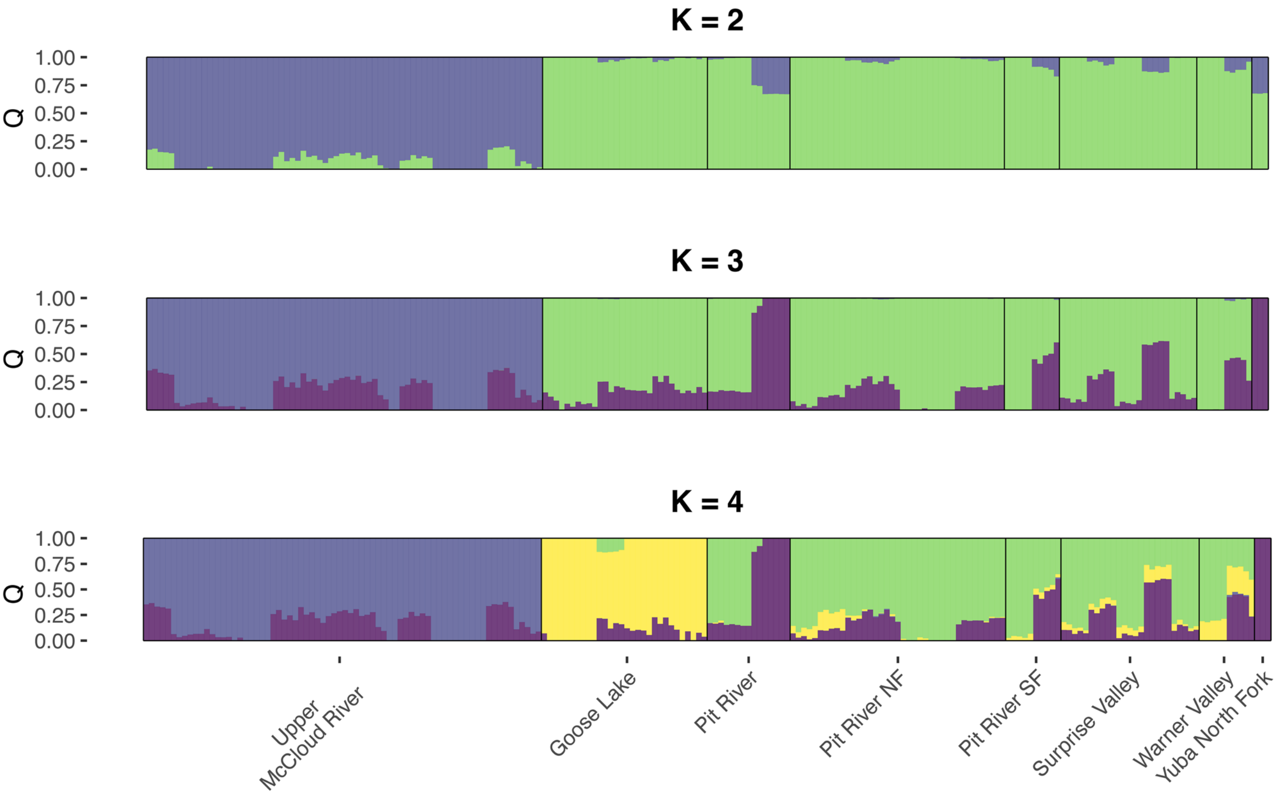

FIGURE 4. Admixture plots from McCloud river trout and other Redband Trout in population genetics dataset. Admixture results from NGSAdmix for genetic clusters (*K*) from 2-4 with the subset of samples collected as Redband Trout. Sample size of 204, optimal *K* = 2. The *x*-axis labels are labeled according to watershed.

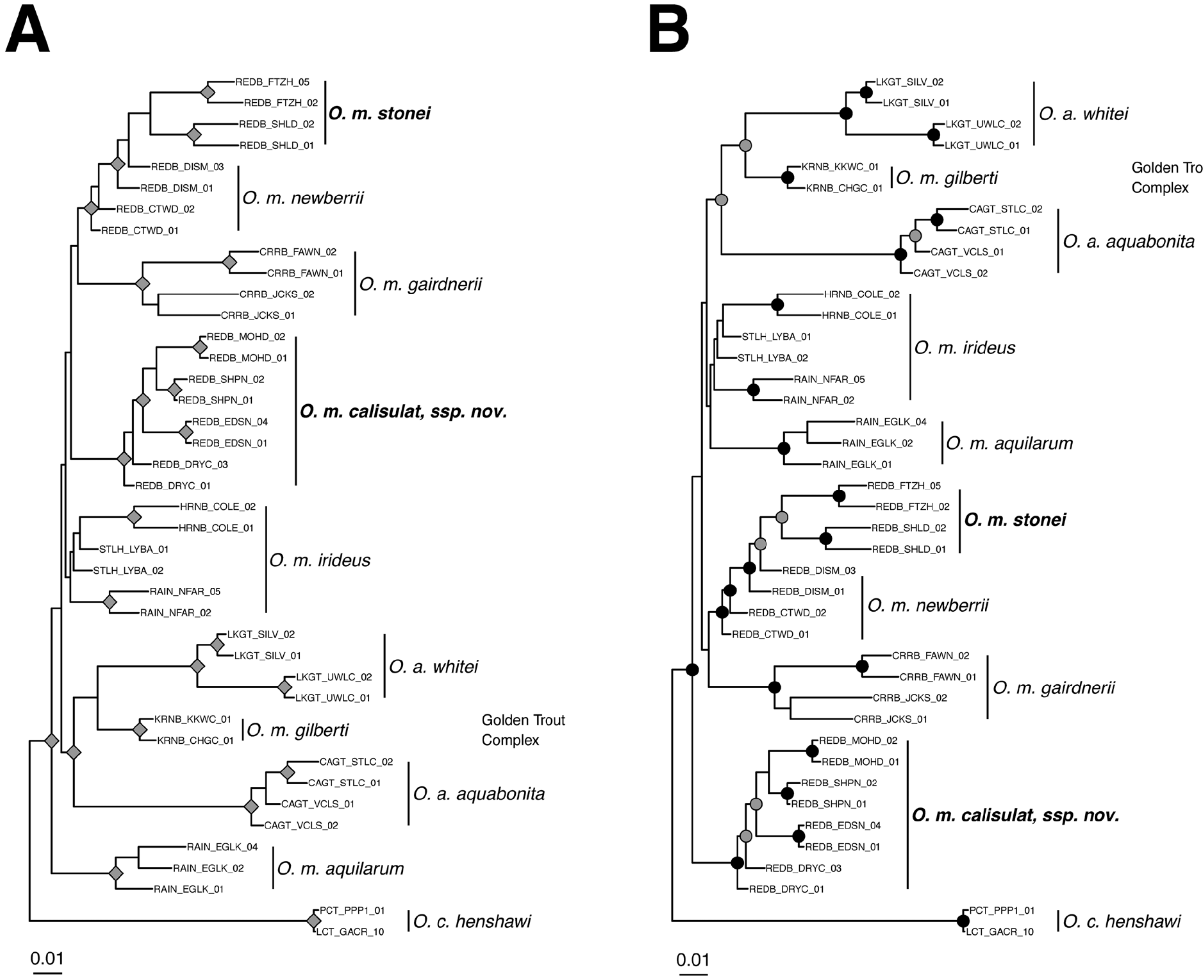

FIGURE 5. Maximum Likelihood (ML) tree (A) and ML consensus tree (B). In both panels subspecies of Rainbow Trout (*Oncorhynchus mykiss*) are indicated along with members of the Golden Trout Complex. McCloud River Redband Trout (*O. m. calisulat*, **ssp. nov.**) and Sacramento Redband Trout (*O. m. stonei*) are indicated with bold text. Individual sample names are provided at tips and further described in Table 1. In 5A, nodes receiving Shimodaira-Hasegawa approximate Likelihood Ratio Test scores ≥ 80 and bootstrap support (BS) ≥ 95% are indicated with a diamond. In 5B, two spans of bootstrap support are presented, with 100% ≥ BS > 95% as solid black circles and 95% ≥ BS > 90% as grey circles at nodes.

The placement of McCloud River RB among other lineages of Rainbow Trout is much less clear. Population genetics techniques indicate that most genetic variance would separate McCloud River RB from all other Rainbow Trout subspecies as well as the Golden Trout Complex, rather than being most closely related to a particular lineage (Figure 2A and 2B). Admixture results consistently indicate the first separation is of McCloud River RB from all other sampled lineages ($K = 2$, Figures 3 and 4). Testing of previous hypotheses in a phylogenetic framework finds evidence to reject the idea that McCloud River RB are most-closely related to any member of the Golden Trout complex e.g. (Stephens 2007) (Schreck and Behnke 1971; Miller 1972; Behnke 1992). Hypothesis testing did not find any of the other hypotheses examined to be significantly better or worse than the ML tree. Under the concatenated ML framework in this paper, it is unclear if the three major Redband Trout lineages identified here taken together are monophyletic. By modeling the multispecies coalescent, strong support of McCloud River RB as a lineage most closely related to ((Coastal RT + Eagle Lake RT), (Columbia River RB + Great Basin RB)) is indicated. The phylogenetic network does not clearly indicate a near relative of McCloud River RB and shows the difficulty in resolving branching patterns phylogenetically due to a complex evolutionary history.

Phylogenetic Relationships of Pit River and Eagle Lake Redband Trouts

We examined Redband Trout of the Pit River in addition to the Upper McCloud River to test the composition of Sacramento RB and what constitutes *O. m. stonei*. The population genetics analyses indicate that Pit River RB are most similar in composition to Great Basin RB, and to the Warner Valley and Surprise Valley sampling locations within the Great Basin RB lineage. This is counter to the expectation that Goose Lake, as a result of intermittent connections would be the primary source of Pit River RB ancestry, though it is detectable in the North Fork Pit River ($K = 4$, Figure 4). From a phylogenetic perspective, Pit River RB samples are clearly nested within *O. m. newberrii,* itself the sister lineage of *O. m. gairdnerii* (Figures 5, 6 and 7). Our results indicate that that Surprise Valley and Warner Lakes Basin trout are very closely related (Figures 2B and 4), with a Northern Great Basin origin of Surprise Valley trout, as was also found by Stephens (2007). While Goose Lake has clear hydrological connections to the Pit River, it is possible that headwater transfer events have occurred between the Upper Pit River and the Surprise Valley across the Warner Mountains. To determine the validity and geographic extent of *O. m. stonei*, comprehensive sampling of Redband Trout of the Northern Great Basin is needed in addition to Sacramento RB.

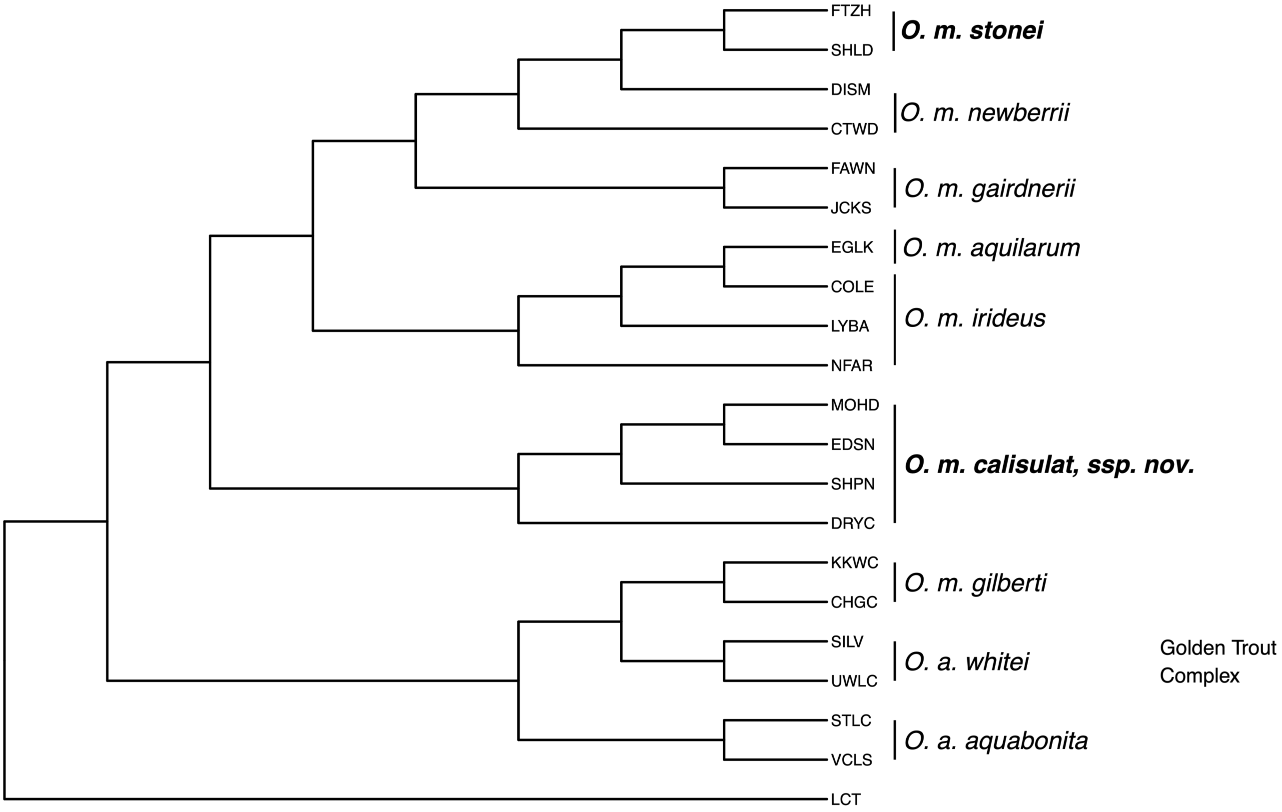

FIGURE 6. Species tree produced by SVDQuartets. The species tree branch lengths are equal and bootstrap support was maximal for all nodes and not shown. Each subspecies of Rainbow Trout (*Oncorhynchus mykiss*) is indicated with McCloud River Redband Trout (*O. m. calisulat*, **ssp. nov.**) and Sacramento Redband Trout (*O. m. stonei*) in bold text. For Rainbow Trout subspecies, sampling locations are labeled with a four-letter code corresponding to Figure 1 and Table 1. The two samples of Lahontan Cutthroat Trout (*O. clarkii henshawi*) are labeled as LCT.

Eagle Lake RT likely originated from the Sacramento River Basin and are considered a subspecies (*O. m. aquilarum*) and at times a Redband Trout. Our population genetic analyses indicate a close relationship of Eagle Lake RT and Coastal RT (Figures 2 and 3). There is an Eagle Lake RT ancestry component present in admixture results of $K = 5$ and $K = 6$ (Figure 3). Eagle Lake RT is known to be widely propagated and incorporated into hatchery stocks, thus detecting Eagle Lake ancestry widely as a result of human activities is not surprising. Concatenated phylogenetic analyses indicate the independence of Eagle Lake RT (Figure 5), while the species-tree indicated a sister relationship to Coleman Hatchery strain fish (Figure 6). Reticulations present in the phylogenetic network (Figure 7) lend support to the idea that more recent hybridization has led to the violations of the assumptions of the multispecies coalescent model and the placement of Eagle Lake RT as sister to Coleman Hatchery fish. A signature of hybridization of Eagle Lake RT and other Rainbow Trout has been demonstrated in some individuals of this subspecies (Rodzen and Ahrens 2019), and transfers of hatchery Rainbow Trout have occurred into Eagle Lake though they are thought to be unsuccessful (Busack *et al.* 1980). As we do not examine the timing of the hybridization between Eagle Lake RT and Coastal RT, if the hybridization is ancient or recent is unknown, as is the direction. That is, as Eagle Lake RT was so widely propagated, gene flow may have occurred into the Coleman Hatchery strain, e.g. Figure 3. The placement of Eagle Lake RT is uncertain in this study. However, Eagle Lake RT is not a close relative of McCloud River Trout or Pit River RB.

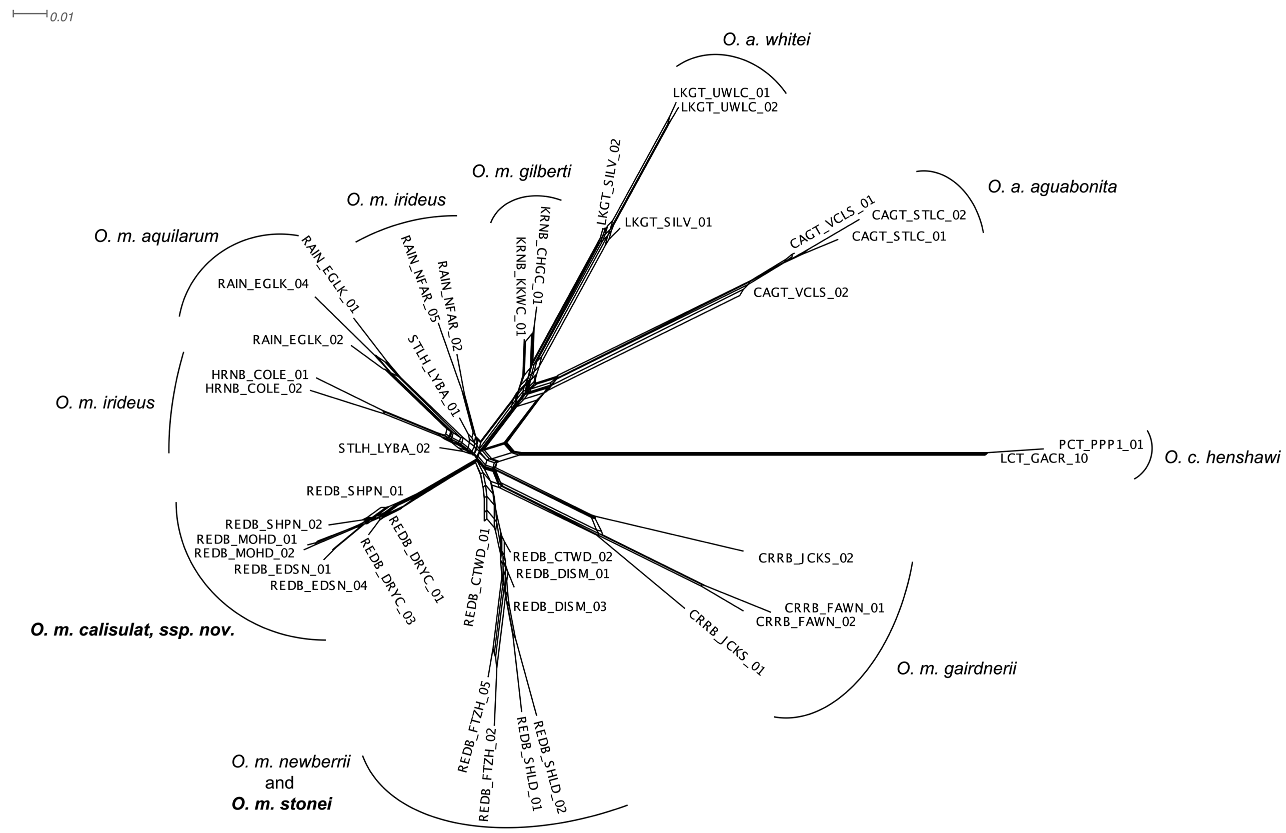

FIGURE 7. Phylogenetic network of individuals examined in this study produced by the neighbor net algorithm. Tips are labeled by individual codes described in Table 1. Each subspecies of Rainbow Trout (*Oncorhynchus mykiss*) is indicated. McCloud River Redband Trout (*O. m. calisulat*, **ssp. nov.**) and Sacramento River Redband Trout (*O. m. stonei*) are show in bold. The outgroup species, Lahontan Cutthroat Trout (*O. clarkii henshawi*) is indicated.

The Golden Trout Complex and Kern Basin Lineages

Oncorhynchus aguabonita is considered valid with two subspecies in this manuscript following Eschmeyer's Catalog of Fishes, the American Fisheries Society, and other sources e.g. (Page and Burr 2011; Page *et al.* 2013; Penaluna *et al.* 2016; Fricke *et al.* 2022). We consistently find a third taxon placed with California GT and Little Kern GT, the Kern River RT, and refer to all three as the Golden Trout Complex. These three lineages originate from the same geographic area (Figure 1) and are placed together in both population genetic (Figure 2A, Figure 3) and phylogenetic frameworks (Figures 5-7). Admixture results are particularly informative and show that the Kern River RT and Little Kern GT are mostly composed of the same genetic cluster, with high ancestry proportions (i.e. Figure 3, $K = 6$). Admixture of California GT in Kern River RT is present, and may be a result of natural gene flow within the Kern River Basin and/or originate from more recent human-mediated movements of California GT. Evidence of admixture within Kern River RT with Coastal RT may be a result of human-mediated movements of hatchery fish or a result of the greater diversity of genetic variants within Coastal RT that may in part reflect shared ancestral variation or gene flow from Kern River RT into Coastal RT.

If *Oncorhynchus aguabonita* is continued to be considered a valid species, the recognition of Kern River RT as a third subspecies (*O. a. gilberti*) is sensible based on the genetic data we have investigated. Anatomical data also supports this assertion. Shreck and Behnke (1971) indicate that museum specimens of *O. m. gilberti* and *O. a. whitei,* collected in 1904 and earlier (and thus predating human-mediated movement of nonnative lineages into the Kern River Basin), have no differences in meristic characters. Furthermore, both these species descriptions are "based on virtually identical specimens" (Behnke 1992, pg. 189). Placing the three subspecies of *aguabonita*, *gilberti* and *whitei* into a single species grouping would unite the distinctive native trouts of the Kern Basin and resolve the paraphyly of *O. aguabonita* demonstrated in this study (e.g. Figures 6 & 7).

Conclusions

We find evidence that the Sacramento RB contains at least two different lineages, with samples in this study not exhibiting admixture between lineages. The Pit River RB is most closely related to Great Basin RB (*O. m. newberrii*). Resolution of relationships among Great Basin RB lineage fish requires broad sampling beyond the scope of this study, but should include Pit River RB. McCloud River RB are geographically restricted to the Upper McCloud River and represent a distinct lineage not most closely related to a single Rainbow Trout lineage. The description of *O. m. stonei* (Jordan 1894) is based on trout phenotypically similar to Pit River RB and Redband Trout of Great Basin origin, collected from a location downstream of a barrier impassable to upstream movement on the McCloud River, and outside the historic range of McCloud River RB (Figure 1). Consequently, the subspecific epithet *stonei* is not appropriately applied to McCloud River RB and we agree with previous researchers' conclusions that a new taxon should be described, e.g. (Gold 1977). We find McCloud River RB is a subspecies of Rainbow Trout not most closely related to other Redband Trout, and, based on the species tree, represents the sister taxon of a combined lineage of Coastal RT, Eagle Lake RT, Columbia River RB and Great Basin RB. The resulting non-monopyhly of Redband Trout is in agreement with previous research that the three main redband subspecies of Rainbow Trout are not monophyletic e.g. (Wishard *et al.* 1984), though the results of some analyses provided strong support for the monophyly of Columbia River RB and Great Basin RB, e.g. Figure 6.

Herein, we describe the McCloud River Redband Trout *O. m. calisulat,* new subspecies.

***Oncorhynchus mykiss calisulat* ssp. nov., Campbell and Conway**

urn:lsid:zoobank.org:act:60E62FE9-D41A-46E7-8A5F-ADF021D1CBFB
McCloud River Redband Trout, Cali Sulat (Figure 8 and Table 3)

Holotype. WFB 5020, formerly WFB 76-05-23, 120 mm SL; USA, California, Shasta County, McCloud River, Sheepheaven Creek (no latitude or longitude provided), 8 October 1974. Sturgess, Hoopaugh and Staley, collectors.

Paratypes. USA: California: WFB 5021, 1, 118 mm SL; same as holotype—WFB 5022, 1, 118 mm SL; same as holotype—WFB 5023, 1, 104 mm SL; same as holotype—WFB 5024, 1, 75 mml SL; same as holotype — TCWC 2872.01, 23 (2 c&s), 79–144 mm SL; McCloud River, Sheepheaven Creek, 41.32735, -121.8276, 1974 — LACM 38568-1, 2, 96–101 mm SL; McCloud River, Sheepheaven Creek, 41.32278, -121.82389, 1 June 1974

Diagnosis. *Oncorhynchus mykiss calisulat* is distinguished from *O. m. stonei*, by a lower number of gill rakers on the first arch (14–18, mode 15 vs. 17–22, 20), a lower number of branchiostegal rays (8–11, 9 vs. 10–13, 12) and a greater number of scales in the lateral series (151–174, 162 vs. 139–160, 146). The numbers of gill rakers on first gill arch, branchiostegal rays and pectoral fin rays (13–14, 13) are distinctive in comparison to other Pacific Trouts, with the number of gill rakers on first gill arch (14–18, mode 15) the lowest of any Pacific Trouts. *Oncorhynchus m. calisulat* is further distinguished from *O. mykiss* and *O. aguabonita* by 44 SNPs with fixed allele differences described by Habibi *et al.* (2022).

Description. See Figure 8 for general appearance and Table 3 for morphometric and meristic characters obtained from holotype and paratypes. Chromosomes 2N = 58, NF = 104. Characterized by small overall adult size (largest specimen examined 143 mm SL) with well-developed parr marks present on adults. Body laterally compressed, greatest depth midway between occiput and dorsal fin origin. Head short, 25–30% of SL, laterally compressed.

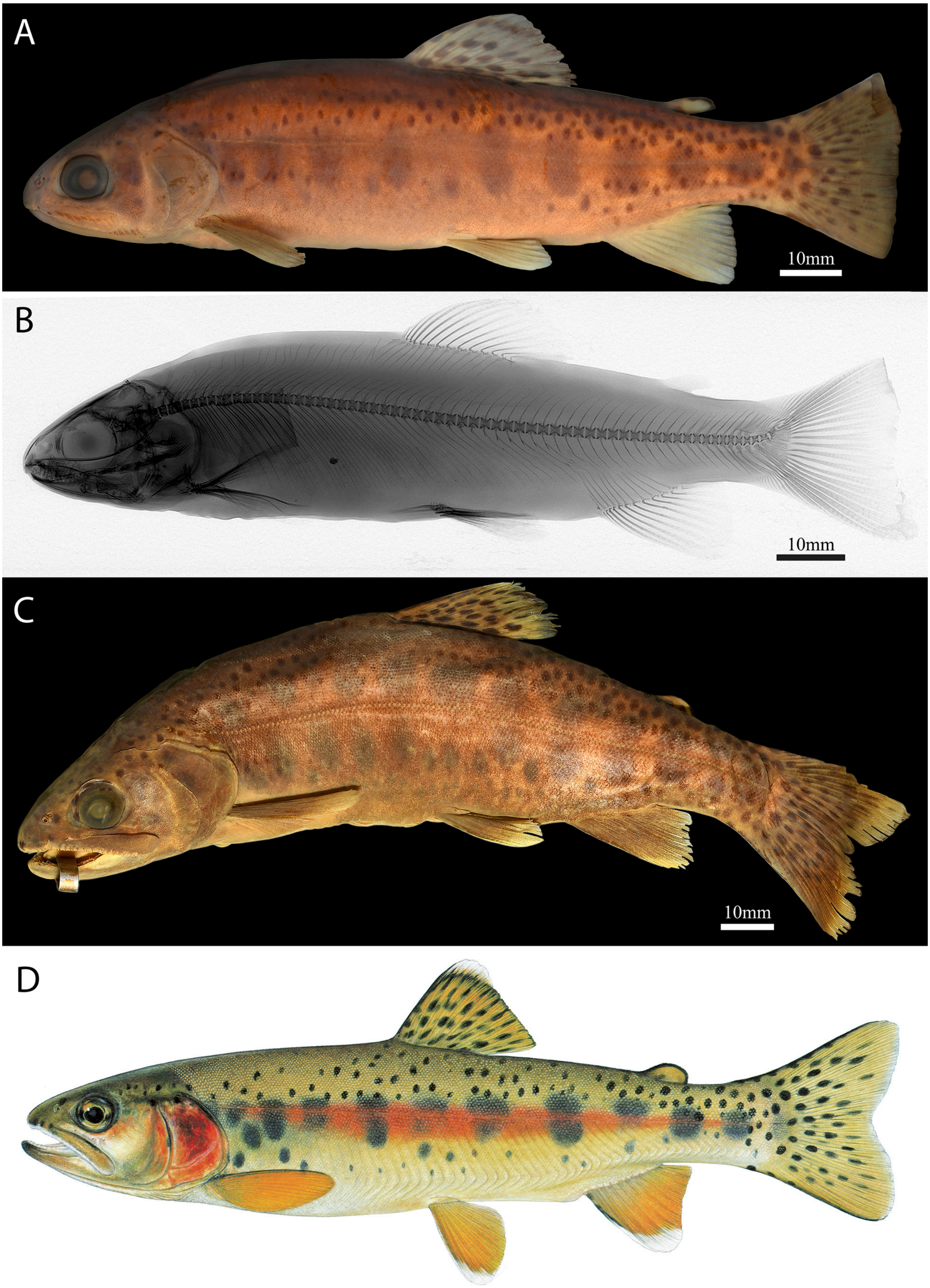

FIGURE 8. McCloud River Redband Trout, *Onchorhynchus mykiss calisulat*, **ssp. nov.**, Sheepheaven Creek. A. WFB 5020, holotype, 120 mm SL. B. same specimen as in A, radiograph. C. TCWC 28772.01, paratype, 144 mm SL. D. Illustration of *O. m. calisulat*, **ssp. nov.**, showing life colors, © J. Tomelleri, used with permission.

TABLE 3. Morphometric and meristic characters of holotype (WFB 5020) and 7 paratypes of *Oncorhynchus mykiss calisulat* **ssp. nov.** Values placed in parentheses represent means and standard deviation for values obtained from paratypes. Ranges for meristic characters marked with an asterisk (*) are from Gold (1977).

	Holotype	**Paratypes**	
	WFB 5020	WFB 5021, 5022 (N=2)	TCWC 2872.01 (N=5)
Standard Length (SL)	120	118, 118	111.4–144
In % SL			
Head length	26.4	26.7–27.4 (27.0, 0.5)	25.8–29.3 (27.0, 1.4)
Body depth	24	21.9–24.3 (23.1, 1.7)	22.4–27.6 (24.3, 2.4)
Predorsal length	53	52.2–54.5 (53.3, 1.6)	52.4–55.5 (53.8, 1.2)
Preanal length	77.6	75.3–76.7 (76.0, 1.0)	74.3–78.1 (75.5, 1.5)
Prepectoral length	22.9	23.1–23.2 (23.2, 0.1)	23.7–26.4 (25.0, 1.0)
Prepelvic length	55.6	54.8–54.9 (54.9, 0.1)	56.2–57.1 (56.7, 0.4)
Preadipose length	85.6	84.7–84.9 84.8, 0.1)	84.3–87.5 (85.1, 1.3)
Length of caudal peduncle	15.2	15.2–16.9 (16.0, 1.2)	13.0–14.8 (13.8, 0.8)
Depth of caudal peduncle	10.3	9.7–10.9 (10.1, 0.6)	9.7–11.0 (10.4, 0.5)
Length of dorsal-fin base	14.9	13.2–13.7 (13.5, 0.4)	12.4–15.0 (13.6, 1.3)
Length of anal-fin base	11.6	10.4–11.9 (11.2, 1.1)	11.0–12.5 (11.6, 0.5)
Length of pectoral fin	16.8	16.6–16.9 (16.8, 0.2)	15.2–18.7 (16.9, 1.3)
Length of pelvic fin	14.3	12.9–13.3 (13.1, 0.3)	11.6–14.9 (13.0, 1.3)
In % HL			
Head width	37.9	37.2–37.8 (37.5, 0.5)	35.7–38.6 (37.4, 1.1)
Interorbital width	29	26.0–29.5 (27.8, 2.5)	26.2–31.3 (28.4, 1.9)
Occiput to snout tip	70.3	66.7–67.8 (67.2, 0.8)	65.7–71.4 (69.3, 2.2)
Length of maxilla	43.5	42.9–44.0 (43.4, 0.8)	41.9–47.4 (44.1, 2.0)
Width of gape	39.1	35.0–36.2 (35.9, 0.4)	37.1–38.6 (37.7, 0.6)
Eye diameter	24	22.3–24.8 (23.5, 1.7)	22.8–24.5 (23.6, 0.7)
Dorsal-fin rays	15	13–14	10–14*
Anal-fin rays	12	13	10–12*
Pectoral-fin rays	13	13–14	13–14*
Pelvic-fin rays	9	9	9–10*
Principal caudal-fin rays	10+8	10+9	10+9
Dorsal procurrent rays	12	11–13	12
Ventral procurrent rays	12	12	10–12
Branchiostegal rays (left/right)	9/9	9–10/9–10	8–10/8–11*
Gill rakers on first arch	-	-	14–18*
Scales in lateral line row	116	115–121	113–123*
Scales in lateral series	151	153–158	153–174*
Scales above lateral line	32	32–34	32–36*
Scales below lateral line	26	27	23–27*
Total vertebrae	61	61	60–63*
Pyloric cecae	-	-	29–42*
Parr marks	8	9–10.	7–10.

Eye large, diameter greater than snout length. Snout rounded in lateral aspect. Mouth large, posteriormost tip of upper jaw (maxilla plus small supramaxilla) located posterior to imaginary vertical line through posterior margin of orbit. Scales in lateral line row, 113–123 (mean of 117 reported by Gold 1977; holotype 116). Scales in lateral series, 151–174 (mean of 162 reported by Gold 1977; holotype 151). Scales above lateral line 32–36 (mean of 33 reported by Gold 1977; holotype 32). Scales below lateral line 23–27 (mean of 25 reported by Gold 1977; holotype 26). Pyloric caeca 29–42 (mean of 36 reported by Gold 1977). Dorsal-fin rays 10–15 (holotype 15), comprising iv,9, v,10 (holotype) or v,11 in x-rayed (n=3) and cleared and stained material (n =2). Anal-fin rays 10–13 (holotype 12), comprising iii,9 (holotype) or iiii,9 in x-rayed and cleared and stained material. Pectoral-fin rays 13–14 (holotype 13). Pelvic-fin rays 9–10 (holotype 9). Caudal-fin rays 10+8 (holotype) or 10+9. Dorsal procurrent ray 10–13 (holotype 12). Ventral procurrent rays 10–12 (holotype 12). Branchiostegal rays 8–11 (holotype 9). Gill rakers on first arch 14–18. Basibranchial dentition vestigial, with 0–5 teeth on basibranchal 1 (Schreck and Behnke 1971; Gold 1977; 0 or 1 tooth only on basibranchal 1 in cleared and stained material examined herein). Basihyal with two rows of large, slightly recurved teeth. Total vertebrae 60–63 (holotype 61).

Coloration. In preservative (Fig. 8A, C), background color olive brown dorsally, fading to lighter cream brown ventrally. Circular to dorsoventrally elongate dark brown parr marks on body side; 7-10 (holotype 9). Small dark brown spots, each covering surface of 3–5 scales, scattered over dorsolateral and lateral surface of body; densest above lateral line below and posterior to dorsal fin and on caudal peduncle; few above lateral line anterior to dorsal fin or below lateral line anterior to anal fin. Dorsal surface of head with few small dark brown spots, similar in size to small spots on body. Lateral surface of head dusted with tiny dark brown melanophores, most prominent on cheek and operculum. Dorsal and caudal fin with small dark brown spots similar in size to small dark brown spots on side of body. Pectoral, pelvic and anal fin light creamy brown; with weak (holotype) or dense scattered dark brown melanophores. Adipose fin whitish; without (holotype) or with few small dark brown spots; posterior margin outlined dark brown. In life (Fig. 8D), body background color light olive brown dorsally, fading to pale yellow ventrally. Brick red lateral stripe extends along body side, deepest below dorsal fin. Parr marks dark blue-grey. Small spots on body, dorsal fin, and caudal fins black. Dorsal, adipose and caudal fins yellow. Pectoral, pelvic and anal fin orange. Tip of dorsal, pelvic and anal fins white. Posterior margin of adipose fin outlined black. Side of operculum orange red. A weak yellow cutthroat mark on ventrolateral surface of head.

Etymology. From the Winnemem Wintu name for the subspecies *cali sulat*, beautiful trout.

Distribution. Known from the McCloud River drainage basin upstream of McCloud Falls. Human mediated movements of other Rainbow Trout into the range of this subspecies has resulted in admixture of some McCloud River Redband Trout populations with other Rainbow Trout. Given that McCloud River Redband Trout appeared to occupy the McCloud River, and, likely the Upper Sacramento River Basin prior to Coastal Rainbow Trout (*O. m. irideus*) and Great Basin Redband Trout (*O. m. newberrii*) moving into the Upper Sacramento River Basin, there is a slight possibility that other populations closely related to McCloud River Redband Trout may have persisted above natural barriers to fish movement outside the core distribution of McCloud River Redband Trout known today. Rutter (1908) provides a detailed description of trout from a tributary of the Upper Sacramento River, South Fork Battle Creek, and comments on their similarity to McCloud River Redband Trout. The description of 11 South Fork Battle Creek specimens collected upstream of Battle Creek Falls, a barrier to fish movement, indicates similarity to key anatomical features of McCloud River Redband Trout. An average is only provided for the number of scales in the lateral series (163 vs. 162). Other characteristics are presented as a range (gill rakers 15–19 vs. 14–18; branchiostegal rays 10–12 vs. 8–11), with no pectoral-fin ray counts provided. Combined with additional evidence presented by Rutter (1908), it could be interpreted that Redband Trout historically were present as far south as the Feather River (Behnke 1992). Future investigations of isolated populations can provide insight into the number of Redband Trout lineages that historically occupied the Upper Sacramento River Basin and their natural contribution to Coastal Rainbow Trout populations.

Associated Species. The McCloud River Redband Trout is the only known native fish from the McCloud River upstream of McCloud Falls.

Data Availability

Code for analyses, figure generation and files associated with phylogenetic analyses are available at https://github.com/MacCampbell/o-m-calisulat. Raw sequencing data is available from the NCBI Sequence Read Archive under BioProject PRJNA804589.

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SUPPLEMENTAL FIGURE S1—Distribution of samples examined in this study

Samples examined in this study are enumerated based on Hydrographic Unit Code 8 (HUC8) and colored according to lineage. Abbreviations follow Table 1. In two HUC8 basins, Kern River and Yuba River, more than one lineage is sampled and the HUC8 polygon is split to reflect this. The Sacramento River Basin is shaded blue. Samples of Coastal Rainbow Trout from the Elwha River and Columbia River Redband are not shown.