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Cryptic Species of Freshwater Sculpin (Cottidae: *Cottus*) in California, USA

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Abstract

The Riffle Sculpin (*Cottus gulosus*) is a small, bottom-dwelling fish regarded as widespread in the cool-water streams that flow into California's Central Valley and into streams of the central California coast. Using population genomics, supported by other genetic, distributional, and meristic studies, we demonstrate that *C. gulosus* consists of three cryptic species with four subspecies (five lineages), all but one entirely endemic to California:

Cottus pitensis, Pit Sculpin Bailey and Bond 1963
Cottus gulosus, Inland Riffle Sculpin (Girard 1854)
    C. g. gulosus: San Joaquin Riffle Sculpin (Girard 1854), nominate subspecies
    C. g. wintu: Sacramento Riffle Sculpin, Moyle and Campbell 2022, new subspecies
Cottus ohlone, Coastal Riffle Sculpin Moyle and Campbell 2022, new species
    C. o. ohlone, Ohlone Riffle Sculpin Moyle and Campbell 2022, nominate subspecies
    C. o. pomo, Pomo Riffle Sculpin Moyle and Campbell 2022, new subspecies.

The three species are endemic to California watersheds although the range of *C. pitensis* extends into southeastern Oregon. All are confined to cool headwater streams or to rivers with cold water releases below dams. Their populations are increasingly isolated from one another because of anthropogenic changes to California's river systems and some are threatened with extinction. Providing taxonomic recognition of the distinct forms will improve conservation efforts on their behalf. This study also demonstrates how genomics can be used to resolve situations where signals from mitochondrial and nuclear DNA are in conflict.

Key words: endemism, genomics, taxonomy, Scorpaeniformes, riffle sculpin, Pit sculpin, mitochondrial introgression, cytonuclear discordance

Introduction

Many more species exist than can be described with traditional taxonomic methods that rely on morphology, morphometrics, behavior and other traits observable by humans. Many of these undescribed species are cryptic species, defined as "two or more distinct species that are erroneously classified (and hidden) under one species name (Bickford *et al*. 2007, p.148)." Protecting cryptic species can be challenging if they are soley regarded as populations of widespread common species. Fortunately, improved genetic techniques have provided a means to identify cryptic fish species. For example, DNA barcoding indicates that a *Gambusia* complex in the fresh waters of Cuba contains at least four cryptic species (Lara *et al*. 2010). Using similar techniques, Winterbottom *et al*. (2014) found 52−94 new species in their samples of the marine goby genus *Trimma*; if DNA barcoding is accepted for species designation, their predictions are that the current 73 recognized species would increase to nearly 200. Likewise, Kon *et al*. (2007) found that three morphologically defined species of the tiny gobiid fish *Schindleria* were made up of 21 distinct lineages, presumably cryptic species.

The literature on fishes is replete with examples, such as those above, that show the existence of many cryptic species based largely on mitochondrial and nuclear DNA analyses. The general conclusion is that fish diversity is

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considerably higher than indicated if just morphological species are counted. But formal recognition of fish species based on differences in DNA, as reflected in phylogenies, is rare, presumably because describing species using data of any kind is a tedious process. While describing species based mostly on genetics is not yet widely used it is increasingly accepted. For example, Egge and Simons (2006) described a new species of catfish (Ictaluridae) based on nuclear and mitochondrial DNA even though morphometric and other features did not distinguish it from a related species, with which it had been combined. In addition, federal agencies such as the National Marine Fisheries Service use genetically based Evolutionary Significant Units and Distinct Population Segments to manage endangered fishes as defined under the Endangered Species Act of 1973 (Moyle *et al.* 2017). However, Hudson and Coyne (2002) and Krishnamurthy and Francis (2012) have cautioned against using genetic techniques/markers for identification based on inherent limitations of the methods, although genetic data are commonly used to lump multiple recognized species into single taxa (e.g., Copus *et al.* 2018). Thus, whenever possible, cryptic species recognized through mitochondrial DNA (mtDNA) or barcoding should also be defined using more comprehensive genomic approaches (Yang and Rannala 2017) and additional sources of information such as zoogeography.

The conservation of cryptic species makes species delimitation through DNA-based methods appropriate and necessary (Tautz *et al.* 2003, Hebert and Gregory 2005, Barnes and Turner 2016). For example, in California, USA, most recognized native fish species are endemic to the region and are in decline, some to the point of extinction (Moyle 2002, Moyle *et al.* 2013 Leidy and Moyle 2021). A number of species, however, are cryptic, so do not have formal recognition. Baumsteiger and Moyle (2019), using the genomics approach described in Baumsteiger *et al.* (2017), found that one widespread fish species (California Roach, *Hesperoleucus symmetricus*) was actually five species. Four of these cryptic species had previously been described but their names were synonymized under *H. symmetricus* by later taxonomists who could not find morphometric or other characters to make the species readily identifiable (Moyle 2002). The genomics study of Baumsteiger *et al.* (2017) resurrected some of the original species names, important for conservation. Yet other cryptic species are not so fortunate as to have previous species descriptions that may be applied to them. Such cryptic species may therefore require DNA sequences or other molecular characters as the *de facto* characters for species designation/identification.

In this paper, we apply methods previously used with California Roach to objectively identify cryptic species of sculpins (*Cottus*) endemic to California (Fig. 1). The genus *Cottus* is no stranger to the difficulties of species identification due to limited morphological and meristic differences among species (Kinziger *et al.* 2005, Yokoyama and Goto 2005, Young *et al.* 2013). In particular, *Cottus gulosus,* the Riffle Sculpin, is composed of many disjunct populations; these populations share a basic life history and narrow environmental tolerances that make them prone to isolation and extirpation (Moyle 2002). Thus, their possession of benthic (rather than pelagic) larvae limits their ability to disperse in response to rapid environmental change as does their requirement for cool (<25 °C), clear, well-oxygenated water that is most typical of headwaters (which are increasingly isolated from one another).

Baumsteiger *et al.* (2012) conducted a comprehensive phylogenetic study of California *Cottus* using nine nuclear and two mitochondrial DNA markers. All recognized species were included in their study, which showed that (a) "*C. gulosus*" in Washington and Oregon is an undescribed species closely related to *C. marginatus* but not to California *C. gulosus,* (b) *C. pitensis* is a valid species closely related to *C. gulosus,* and (c) *C. gulosus* in California is made up of several distinct lineages that suggest multiple taxa. The third point was further supported by Baumsteiger *et al.* (2014) who noted that the geographic locations of these lineages were related to known phylogeographic breaks in the landscape. Despite this strong evidence, no formal taxonomic recognition of the lineages has yet been proposed. Consequently, here we address the question: are evolutionary lineages within *Cottus gulosus* cryptic species that can be formally recognized even when distinct meristic and morphological differences are lacking? Given the sensitivity of members of this complex to poor water quality (especially warm temperatures) and stream alteration, formal recognition of lineages by assigning them names is important for their conservation.

Taxonomic history of *Cottus Gulosus*

Sculpins of the genus *Cottus* are small benthic fishes that inhabit cool to cold water streams, lakes, and estuaries in Eurasia and North America (Kinziger *et al.* 2005). They are readily recognizable by their large, flattened heads, large pectoral fins, scale-less bodies, and mottled coloration that blends in with the rocky substrates where they live. Kinziger *et al.* (2005) found that the genus consisted of five primary lineages (clades) and that all *Cottus* on the west coast of North America belonged to just one of these clades (*Cottopsis*).
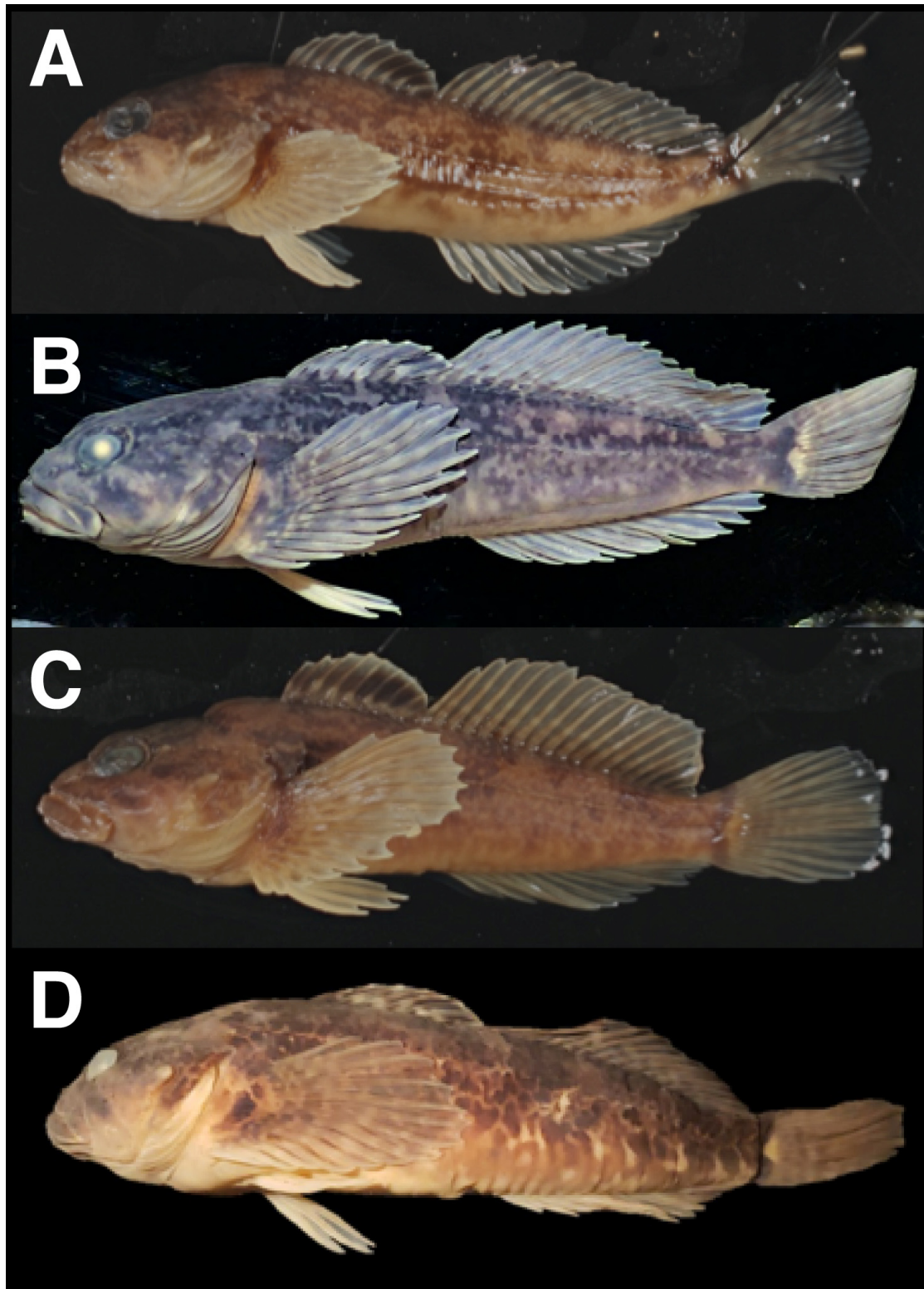
FIGURE 1. Inland (A, B) and Coastal (C, D) Riffle Sculpins from the UC Davis Museum of Wildlife and Fish Biology. (A) San Joaquin Riffle Sculpin, *Cottus gulosus gulosus*. WFB-277-08-09. Kaweah River, Tulare County, California (68 mm SL, 82 mm TL). Coll. Larry Brown, September 10, 1985. Holotype is same as for *C. gulosus* (USNM 291). (B) Sacramento Riffle Sculpin, *Cottus gulosus wintu*. Holotype WFB-3464. North Fork Feather River, Butte County, California (78 mm SL, 96 mm TL). Coll. Jason Baumsteiger, October 5, 2017. (C) Coastal Riffle Sculpin, *Cottus ohlone*. Holotype. WFB-3402. Also, holotype for Ohlone Sculpin, *C. o. ohlone*. Guadalupe River, Santa Clara County, California (65 mm SL, 79 mm TL). Coll: J. J. Smith, November 18, 1986. (D) Pomo Riffle Sculpin, *Cottus ohlone pomo*. Holotype. WFB-3396. Pieta Creek near mouth on Russian River, Mendocino County, California (90 mm SL, 105 mm TL). Coll. P Moyle, J. Baumsteiger, August 2, 2017.

Cottus gulosus was first described by Charles Girard (1854) as *Cottopsis gulosus* from the San Joaquin River, California. In 1857, Girard also identified the same species from the upper Pit River (Evermann and Clark 1931). Eigenmann and Eigenmann (1889) described *Uranidea semiscabra centropleura*, collected from a single spring in Lake County, which Jordan and Evermann (1898) synonymized with *C. gulosus,* a move that Robins and Miller (1957) confirmed. Jordan (1896) described *Cottus shasta* from the McCloud River, a species that Snyder (1905) found to be indistinguishable from *C. gulosus*. Robins and Miller (1957) examined the types of both *C. shasta* and *C. gulosus* and also found no differences. To confuse things further, Shapovalov and Dill (1950) considered *C. gulosus* to be a subspecies of *C. bairdi* (a widespread species in eastern North America) but listed it as *C. b. shasta*. Robins and Miller (1957) demonstrated there was no justification for this designation.

Jordan and Evermann (1898) cataloged all known information on the taxonomy and distribution of *C. gulosus*. Unfortunately, they mistakenly listed San Mateo Creek (San Mateo County) as the type locality, rather than the San Joaquin River, but their re-description of the species was based on fish from nearby San Francisquito Creek, Santa Clara County. Jordan and Evermann (1898) noted, however, that coastal and interior types were "not distinguishable by any permanent character (p. 1945)". They considered the distribution of *C. gulosus* to be streams of the Coast Range south to Point Conception and, vaguely, "streams of the interior" of California. This description indicates already considerable confusion in identifying sculpin species, given that Riffle Sculpin do not occur south of Monterey Bay watersheds (Snyder 1913, Moyle 2002, Baumsteiger *et al.* 2014). Otherwise, the known distribution seemed to be in streams tributary to San Francisco Bay and Monterey Bay, the Russian River watershed, and the Sacramento-San Joaquin drainage from the Pit River in the north to the San Joaquin River in the south. Bailey and Bond (1963) later recognized that *C. pitensis* was a species separate from *C. gulosus*. *Cottus pitensis* is the common stream sculpin of the Pit River watershed, which drains much of northeastern California before flowing into the Sacramento River. The differences between the two species were the absence of palatine teeth in *C. pitensis*, preopercular spines usually two rather than three (in *C. gulosus*), and lateral line usually complete (87%) in *C. pitensis* and usually incomplete (77%) in *C. gulosus*. These differences are easiest to see in specimens over 50 mm SL (Bailey and Bond 1963).

To determine phylogenetic breaks in the distribution patterns of both species, Baumsteiger *et al.* (2014), used a comprehensive array of genetic techniques, including nuclear and mitochondrial sequence markers and microsatellites on samples of both species over their entire ranges (total n = 872). Analyses were also conducted of phylogeny, gene flow, and fine-scale population structure. This work supported the conclusions from more limited numerical and genetic sampling in Baumsteiger *et al.* (2012) of relevant sculpins that *C. gulosus* is paraphyletic, showing that (a) one lineage is more closely related to *C. pitensis* than to other *C. gulosus*; (b) *C. gulosus* populations from coastal watersheds and from the San Joaquin basin are two divergent lineages that most likely are cryptic species and (c) the genetics of *C. gulosus* in the Sacramento Valley had been modified slightly by an ancient hybridization event with *C. pitensis,* without evidence of ongoing hybridization. This suggested that the Sacramento Riffle Sculpin should also be treated as a distinct taxon.

Confusion on the identification of *C. gulosus* has been enhanced by acceptance of the species as widespread in coastal streams of Washington and Oregon (Wydoski and Whitney 2003, Markle 2016). Robins and Miller (1957) concluded the identity of these sculpins was based on misidentification of individuals of *Cottus perplexus*. Likewise, Baumsteiger *et al.* (2012, 2014) concluded that Washington and Oregon riffle sculpins were also distinct from the *C. gulosus* originally described by Charles Girard, making *C. gulosus* endemic to central California.

Methods

Meristics

To identify meristic and morphometric differences among individuals at locations within the range of *Cottus gulosus*, we compiled data for 17 traits that Page and Burr (2011) and Moyle (2002) used to identify *Cottus* species: (1) number of anal fin rays, (2) number of spines in the first dorsal fin, (3) number of rays in the second dorsal fin, (4) dorsal fins joined/not joined, (5) large spot on the dorsal fin (yes, no), (6) palatine teeth (yes, no), (7) maxilla reaches to back, middle or front of eye, (8) chin pores 0,1, or 2, (9) pelvic fin with 1 spine and 3–4 rays, (10) depressed pelvic fins touch vent (yes/no), (11) pectoral fin rays 15–16, (12) preopercular spines 2–3, (13) lateral line complete (yes/no), (14) lateral line pores 21–38, (15) prickling (none, axillary, widespread), (16) caudal peduncle (narrow rounded vs deep compressed), (17) saddles of pigmentation on the back (0–6) in fresh specimens (Table 1). Pigmentation is otherwise variable and faded in preserved specimens, so did not prove to be a useful characteristic.

TABLE 1. Meristic values for Riffle Sculpin samples from the UC Davis Museum of Wildlife and Fish and the California Academy of Sciences, San Francisco. *Cottus asper* and *C.pitensis* data is presented for comparison. ? means 'could not be determined'. Locations include the year collected. See Table 2 for more precise location information. Sculpins at locations with * were *C. asper* originally identified as *C. gulosus*

	#	Anal Fin Rays	Dorsal Fin 1st Spine	Dorsal Fin 2nd Ray	Joined	Spot	Palatine Teeth	Mouth (to eye)	Chin pore
C. asper	24	16–19	7–10	19–23	yes	yes	yes	variable	1 [2]
C. gulosus	72	12–17	7–9	16–19	yes	yes	yes	back	1
C. pitensis	8	12–15	7–10	16–19	no	yes	no	back	absent
COASTAL LOCATIONS									
Russian R—2017	10	13–14	7–8	15–19	no	yes	yes	back	2
Salmon Cr—2000	1	14	9	18	no	yes	yes	middle	2
Lagunitas Crk—1894	1	13	6	18	no	yes	yes	back	?
Moore Crk-Napa-1945	9	12–14	7–8	16–18	mixed	yes	yes	back	1–2
Alameda Crk—1938	1	14	8	17	no	?	yes	middle	2
Bird Crk—2004	1	14	6	18	barely	yes	yes	mid eye	none
Carnadero Crk—1945	1	14	8	19	no	yes	?	front	2
Llagas Crk—1938	1	14	7	18	barely	yes	yes	middle	2
Llagas Crk—1896	10	13–14	7–8	18–20	mixed	yes	yes	middle	1–2
Guadalupe Crk—2013	10	13–14	7–8	16–18	barely	yes	yes	mid/front	0–2
INLAND LOCATIONS									
NF Feather R—1981	10	12–15	8–10	14–19	no	yes	yes	mid	0–2
Deer Creek-Sac-1980	5	13–14	7–8	17–19	barely	yes	yes/no	front/back	1
NF Kings—1985	3	12–13	8–9	17–18	yes/no	yes	yes	mid	1–2
MF Kaweah 1985	5	12–15	8–9	17–18	yes/no	yes	yes	front/mid	1
Cache Creek*—1985	1	14	7	18	no	yes	yes	front	2
NF Cache Crk*- 1963	1	13–14	8	17	yes	yes	yes	back	2
Putah Creek*—1987	3	13–14S	6	17–18	yes	yes	yes	front/mid	1

TABLE 1. (Continued)

	#	Pelvic Fin			Pect Fins	Preoperc Spines	Lateral Line	Prickling
		Spine	Rays	Touch vent	Rays		Complete/Pores	
C. asper	24	1	4	-	15–18	2–3	yes/28–43	yes
C. gulosus	72	1	3–4	no	14–15	2–3	both/ 21–38	behind pect
C. pitensis	8	1	3	-	12–16	2[3]	yes/31–39	behind pect
COASTAL LOCATIONS								
Russian R—2017	10	1	3	no	14–15	2	yes/27	axillary
Salmon Cr—2000	1	1	3	no	15	2	yes/?	axillary
Lagunitas Crk–1894	1	1	3	no	15	2	?	axillary
Moore Crk-Napa-1945	9	1	3	no	14–16	2	yes/30–35	axillary
Alameda Crk—1938	1	1	3	no	15	2	yes/28	axillary
Bird Crk—2004	1	1	3	no	14	2	no/?	axillary
Carnadero Crk—1945	1	1	3	no	?	2	yes/?	axillary
Llagas Crk—1938	1	1	3	no	15	2	yes/?	axillary
Llagas Crk—1896	10	1	3	no	14–15	2	yes/27–33	axillary
Guadalupe Crk—2013	10	1	3	no	12–15	2	both/25–29	none/axillary
INLAND LOCATIONS								
NF Feather R—1981	10	1	3	yes/no	14–15	1–2	yes/?	none/axillary
Deer Creek-Sac-1980	5	1	3	no	14–16	2	both/26–34	axillary
NF Kings—1985	3	1	3	no	15–16	1–2	yes/28–34	none/axillary
MF Kaweah 1985	5	1	3	no	15–16	1	yes/32–38	axillary
Cache Creek*—1985	1	1	2	no	14	2	yes/28	heavy
NF Cache Crk*- 1963	1	1	3	no	14	2	yes/28	axillary
Putah Creek*—1987	3	1	3	both	13–14	2	yes/28	none-heavy

Individuals (n = 72 for *C. gulosus,* 24 for *C. asper,* 8 for *C. pitensis*), were selected from museum collections at UC Davis and the California Academy of Sciences based on availability and condition.

Sampling

We sampled individuals from 13 locations identified as containing *Cottus gulosus* (Table 2). Clips from fins were collected within three years of the analysis and stored in non-denatured ethanol or as dried clips on Whatman paper. We included additional samples for comparison: Prickly Sculpin *C. asper* (7 locations), Pit Sculpin *C. pitensis* (2), and Paiute Sculpin *C. beldingii* (1). All samples were collected with a Smith Root backpack electrofisher using standard protocols except for the *C. beldingii*, which required hand netting. All specimens are deposited in the Museum of Wildlife and Fish Biology, University of California, Davis.

TABLE 2. Sampling locations, showing number of samples collected and used in the genomic analyses. Locations are sorted by species based on the original taxonomic designation under which they were collected. Codes identify locations in the figures. Cr = creek, Res= reservoir, R = River. RL=Robert Leidy, PM=Peter Moyle, JS = Jerry J. Smith, RQ= Rebecca Quiñones, JB=Jason Baumsteiger.

No.	No. used	Code	Location	Region	Latitude	Longitude	Collector
'Riffle' sculpin							
4	4	COY	Coyote Cr	Santa Clara	37.11325	-121.47717	RL
8	8	HSS	Hot Springs	Lassen	40.435258	-121.36544	PM
8	8	GUAD	Guadalupe Cr	San Jose	37.2250	-121.9050	JS
4	0	McC FR	Fish Rock	McCloud	41.045	-122.18356	RQ
8	8	McC HB	Hamilton Bend	McCloud	41.0444	-122.195	RQ
4	0	McC SQ	Squaw Cr	McCloud	41.04	-122.207	RQ
8	3	MER	Merced R	Central Valley	37.6532	-119.7825	JB
8	8	MOK	Mokelumne R	Central Valley	38.3159	-120.7104	JB
8	8	PEN	Penitencia Cr	Santa Clara	37.3980	-121.8000	JS
8	8	RUS	Russian R	Sonoma	38.9082	-123.05793	JB
8	8	RUSEF	EF Russian R	Sonoma	39.249561	123.12017	JB
4	2	SAC	Sacramento R	Cantara	41.2661	-122.3078	JB
8	8	UVAS	Uvas Cr	Santa Clara	37.0120	-121.6270	JS
Pit Sculpin							
4	4	PITMC	Mill Cr	Pit	41.26669	-120.30001	RQ
4	3	PITRC	Rush Cr	Pit	41.283491	-120.86686	RQ
Prickly sculpin							
4	1	CLS	Clear Lake	Clear Lake	38.995	-122.705	JB
8	8	McC, M	Shasta Res	McCloud	40.932808	-122.24928	JB
4	3	NAP	Napa R	Napa	38.268	-122.284	JB
4	4	PUT BC	Big Canyon Cr	Putah	38.833417	-122.65006	RQ
4	4	PUT RR	Russell Ranch	Putah	38.533401	-121.85026	RQ
4	2	SUS	Suisun Marsh	Sac Delta	38.07	-122.07	JB
4	4	WAD	Waddell Cr	Santa Cruz	37.099517	-122.27549	RQ
Paiute Sculpin							
4	4	SAG	Sagehen Cr	Truckee	39.4322	-120.25026	RQ
132	118						

RADseq and de novo assembly

DNA was extracted from fin clips using a Qiagen DNeasy Blood & Tissue Kit. DNA was quantified using a Qubit fluorometer and kit (Invitrogen) and normalized to 5 ng/µl for library preparation. DNA was digested with the restriction enzyme *SbfI* and paired-end 100 bp read libraries built using the protocol of Ali *et al*. (2016) and sequenced

on an Illumina HiSeq 2500 All RAD sequencing data required a perfect barcode and partial restriction site match. The genome assembler PRICE (Ruby *et al.* 2013) was used to create a *de novo* partial genome of RAD sequences from eight Pit Sculpin (*C. pitensis*) individuals. To remove potential paralogous loci and chimeric reference sequences, loci with five or more SNPs were removed from the reference assembly. Sequences from all individuals in this study were then aligned to this reference assembly using the Burrows-Wheeler aligner (Li and Durbin 2009) under default parameters. SAMTOOLS was used to eliminate PCR duplicates and create Binary Alignment Map (BAM) files (Li *et al.* 2009). A minimum threshold of approximately 10x coverage (i.e. reads) was set per locus for all individuals included in our analyses. For a more thorough explanation of the *de novo* assembly and RADseq construction, see Baumsteiger *et al.* (2017).

Population genomics

Population genomic analyses were performed within a probabilistic framework. We conducted Principal Component (PC) analyses, admixture, and F_{st} analyses to differentiate genomic variation among currently recognized species and to validate these lineages. These analyses made use of the entire suite of loci available from the *de novo* assembly. BAM files for each individual were used with angsd (Korneliussen *et al.* 2014) to identify polymorphic sites, infer major/minor alleles (doMajorMinor 1), estimate allele frequencies (doMaf 2), and retain single nucleotide polymorphisms (SNPs) with a minor allele frequency of at least 0.05 (minMaf 0.05). Quality control options used with ANGSD included a SNP p – value of 1.0 x 10^{-6} (SNP_pval 1e-6), a minimum base quality of 20 (minQ 20) and a minimum alignment score of 10 for reads (-minMapQ 10). Principal Component analyses were conducted by using covariance matrices from genotypes called by ANGSD using the NGSCOVAR program within NGSTOOLS and plotting results in R (R Core Team 2021; Fumagalli *et al.* 2014). Admixture analyses were completed by generating BEAGLE-formatted Genotype Likelihood input files in ANGSD (with the same parameters as the PCA) and running those input files in NGSADMIX. Analyses were conducted from K = 2 to K = 7, with minimum 10 iterations. The optimal number of clusters, K, was assessed using delta-K, following Evanno *et al.* (2005). Results were processed and visualized using POPHELPER (Francis 2017). Uncorrected F_{st} analyses were conducted using the same locations/groupings as above, generating simple allele frequency files and then performing pairwise comparisons between each group or location with the realSFS subprogram of angsd. Again, more thorough explanations of these analyses can be found in Baumsteiger *et al.* (2017).

Molecular phylogenetic analysis

We generated phylogenetic hypotheses by generating genotype calls and analyzing those with SVDQuartets distributed with PAUP* version 4.0a (Chifman and Kubatko 2014, 2015, Swofford 2003) as well as TREEMIX (Pickrell and Pritchard, 2012). Genotype calls were generated from the in-group Riffle Sculpin samples which had been analyzed for population genomics. Outgroup samples of *C. asper* and *C. beldingii* samples were reduced to increase the number of variable sites for analysis. We selected two individuals of *C. asper* and two individuals of *C. beldingii* with the greatest number of read counts to use as outgroups. Genotypes were called with ANGSD (doGeno 4) under the SAMTOOLS model (GL 1) specifying a fixed major and minor frequency (doMaf 1) with frequency as a prior (doPost 1). For quality control we applied minimum mapping and base quality cutoffs (minMapQ 20, minQ 20), a minimum minor allele frequency (minMaf 0.05), a SNP p – val (SNP_pval 1e-6) and a posterior probability cutoff (postCutoff 0.95). Futhermore, we required a SNP to be present in 95% (minInd 80) of individuals.

The genotype calls were prepared for analysis by pruning linked SNPs with BCFTOOLS (+prune -l 0.9 -w 10000), converted to a phylip formatted file with *vcf2phylip.py* (https://github.com/edgardomortiz/vcf2phylip). Invariant sites for the purposes of ascertainment bias correction were removed with *asbias*.py (https://github.com/btmartin721/raxml_ascbias). The resulting SNP alignment was supplied to SVDQUARTETS with Riffle Sculpins collected from the same sampling locations grouped together and the outgroup representatives consolidated into two species-representing groups. We evaluated all possible quartets and assessed confidence with 100 standard bootstrap replicates.

We then used the resulting pruned VCF-formatted file to generate input files for TreeMix by pooling individuals in the same manner as SVDQUARTETS. We ran TREEMIX for a number of migration edges allowed of zero, one, or two (-m option), specified *C. beldingii* as the outgroup (-root), and for covariance matrix estimation set the number of SNPs per block to 10 (-k 10). Due to the small sample size of the SAC sampling location and for *C. beldingii* and *C. asper* (n = 2 for all), we disabled sample size corrections (-noss).

Mitochondrial data

We compiled an alignment of mitochondrial cytochrome b (cytb) sequences from publicly available data as mitochondrial introgression of Pit Sculpin into Sacramento region Riffle Sculpin was previously documented. From Baumsteiger *et al*. (2014) we obtained sequences from accessioned *C. gulosus* and *C. pitensis* (NCBI PopSet 655167758 and 655167638) and renamed these according to the proposed taxonomy. We aligned sequences with MAFFT with default parameters (Katoh and Toh, 2008). The mtDNA alignment was imported into R (R Core Team 2021) with the read.dna function of *ape* (Paradis and Schliep 2019). A haplotype network was created with the haplotype function of *pegas* (Paradis 2010). We used the default settings of the haplotype function which uses uncorrected distances and pairwise deletion of missing data. The network was visualized as a minimum spanning tree with the size of circles proportional to the frequency of those haplotypes.

Distribution

Distributions were initially mapped using the *Pisces* data base (https://pisces.ucdavis.edu/fish) for California fishes, which maps species presence or absence in HUC12 watersheds (Santos *et al*. 2013). HUC 12 watersheds are the smallest units currently mapped under the USGS Hydrologic Unit Code (https://water.usgs.gov/GIS/huc.html). After initial mapping, distributions were refined by checking museum records (Museum of Wildlife and Fish, University of California, Davis, and California Academy of Sciences). Maps were refined using field notes and collection records of Moyle and J. Baumsteiger, including those listed in Baumsteiger *et al*. (2014, 2017) and by consulting with regional experts on fishes.

Species designations

Based on the discussion in Freudenstein *et al*. (2017), we define the sculpin species in this project as biological species because (a) each lineage is also a metapopulation with a common ancestor, (b) each lineage is confined to a geographically defined area in which the geologic history can explain isolation and evolution of a distinct entity, (c) each lineage is broadly separable from other known species of *Cottus* as determined by F_{st}, PC, and admixture analyses of genomic data, (d) some sculpin lineages can be separated from other lineages by minor, if overlapping, differences in meristics and morphometrics, and (e) each lineage has a distinctive role in the cool-to-cold headwater stream ecosystem of which it is part. The role (niche) of the members of the riffle sculpin complex is that of a benthic predator on benthic, rock-dwelling aquatic invertebrates and small fish. Sculpins serve as an important prey for larger fish and for aquatic birds and snakes. This role varies somewhat from stream to stream, depending on environmental and biological features. This role of sculpins in cool-water stream ecosystems is characteristic of *Cottus* species throughout the northern hemisphere; they typically coexist with one or more trout species (Salmonidae) and one or more species of ostariophysan fishes such as suckers (Catostomidae) and dace (*Rhinichth*ys) (Moyle and Cech 2004).

While Freundenstein *et al*. (2017) prefer not to recognize cryptic species as legitimate species, we think all California *Cottus* species, including those described in this paper, fit the definition of biological species (above) well. Our subspecies designations, however, are more problematic, because their recognition is based on geographic isolation from other subspecies within a species and relatively small genomic differences, indicating recent or incomplete reproductive isolation. Because naming a taxon is important for conservation of biological diversity, the basic choice becomes designating all significant branches within a lineage as species, subspecies, or Distinct Population Segments (DPS). Subspecies and DPS designations allow for naming populations below the species level and to have them formally protected under the USA and California Endangered Species Acts. In this paper we prefer to use subspecies designations because the subspecies have a geographic foundation and show genetic evidence of isolation from other subspecies.

Results

The sculpins historically identified as *Cottus gulosus* are now assigned to three species with four subspecies that have largely non-overlapping distributions (Figs. 1, 2).
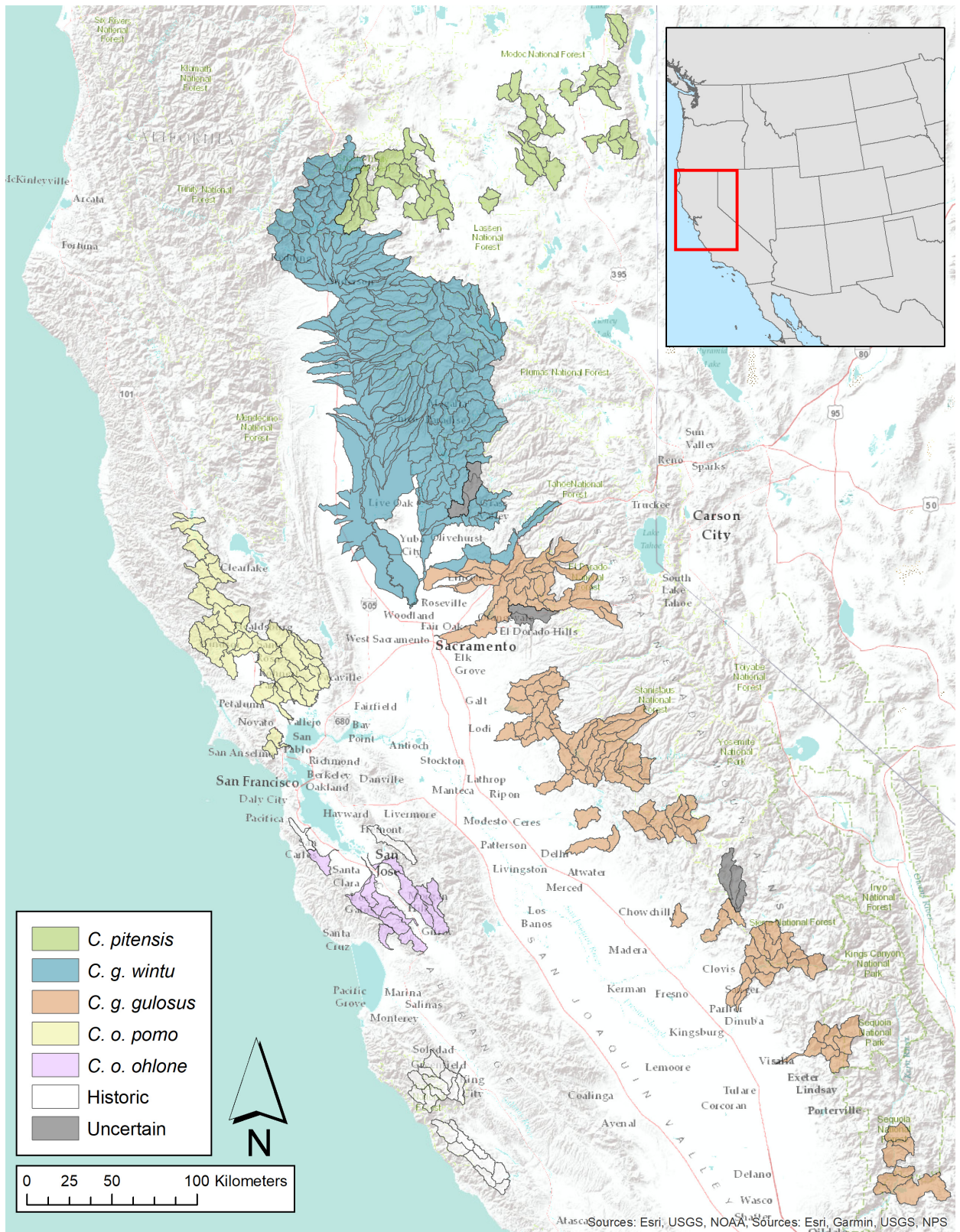
FIGURE 2. Distribution of Riffle Sculpin taxa in California, described in the text. Pit Sculpin (*Cottus pitensis*) is found in the northeast (light green). Inland Riffle Sculpin (*Cottus gulosus*) is found mainly in streams of the Central Valley (blue/orange), while Coastal Riffle Sculpin (*Cottus ohlone*) is confined to coastal and South San Francisco Bay watersheds (yellow/pink).
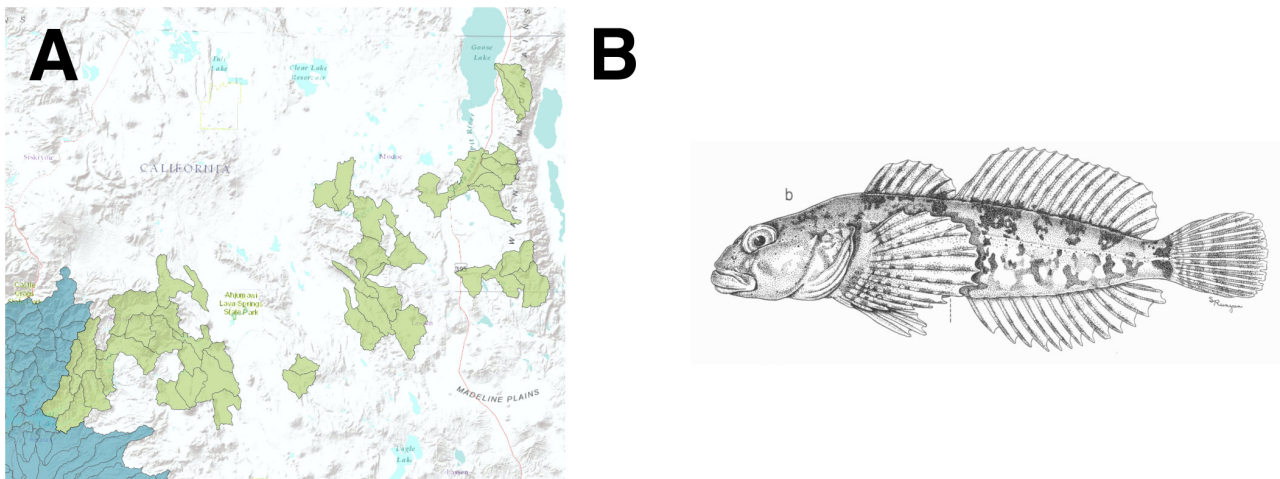
FIGURE 3. (A) Distribution of *Cottus pitensis* in northeastern California (light green). This distribution is mostly in streams of the Pit River basin in California but extends into Oregon tributaries of Goose Lake (upper right corner of map). (B) Drawing of *C. pitensis* holotype from Bailey and Bond (1963), collected from the North Fork Pit River, Modoc County, CA.

Meristics

The analysis of these factors proved to be inconclusive, failing to reveal any specific characteristics that differentiate individuals from coastal and inland locations (Table 1), although *C. pitensis* was confirmed as a separate species. For *C. gulosus*, 45 coastal individuals from nine locations and 28 inland individuals from seven locations had meristic measurements that are overlapping. No specific meristic characters are apparent to separate these fish. Lateral line pore counts were not useful because most of the fish available were less than 50 mm total length.

DNA sample collection, sequencing and SNP generation

Raw sequence data, alignments and code associated with analyses are available at https://github.com/MacCampbell/ca-cryptic-sculpins and in a Dryad repository: doi:10.25338/B86Q0K. A total of 132 individuals were collected initially for the analysis but only 110 were analyzed for population genomics analyses (Table 2). Only two sampling locations were lost from the McCloud River with reduced representation of some other locations. The *de novo* assembly consisted of 41,764 contigs greater than 300bp in length and 175,913 SNPs were identified from the 110 samples (as Genotype Likelihoods). These SNPs were used for population genomic analyses where all samples were included. Secondary analyses, where some samples were removed, used slightly fewer SNPs by necessity because some of the variation found in the removed individuals was no longer present. For the analyses of Riffle Sculpins, 81 fish produced 121,816 SNPs (as Genotype Likelihoods).

Population genomics

Principal Component analyses on all 110 samples separated Riffle Sculpin individuals collected from coastal locations from all other sampling locations along PC 1 (16.81% of variance (Fig. 4A).This distinguishes Coastal Riffle Sculpin from five currently recognized species of *Cottus* (Riffle, Pit, Prickly, Coastrange, and Paiute Sculpin) found in California. The second PC (12.34%) differentiates Paiute Sculpin (central group) from Prickly Sculpin (lower group) and Pit/Riffle Sculpin (upper group) samples.

A subsequent PC analysis was performed on fish identified as Riffle or Pit Sculpin. Pit Sculpin were included due to their apparent overlap with Inland Riffle Sculpin locations in the initial PC analysis. Once again PC 1 (24.75% of the variation) differentiated Coastal Riffle Sculpin individuals from Pit Sculpin and Inland Riffle Sculpin individuals (Fig. 4B). Principal Component 2, although explaining substantially less variation (5.45%), separates several groups. Within Coastal Riffle Sculpin, there are two groups separated along PC 2, a northern Russian River/north San Francisco Bay group and a southern Santa Clara Valley region group. The natural geographic division of Russian River and Santa Clara Valley Coastal Riffle Sculpin is San Francisco Bay. The Inland Riffle/Pit Sculpin samples are split into three groups: Pit Sculpin (upper group), Sacramento region Riffle Sculpin (central group) and finally a more southern Central Valley group (lower).
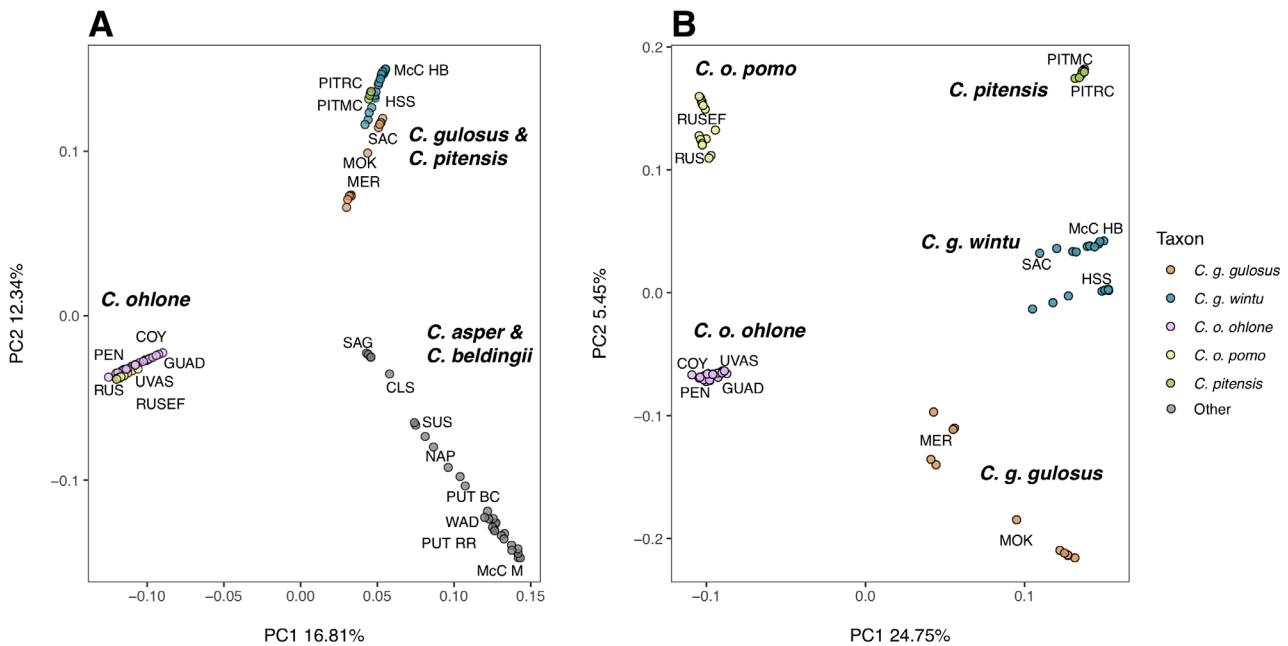
FIGURE 4. Principal Components analyses of genome-wide SNP data from all sampling locations in study (A) and from locations where the fish were only identified as Riffle or Pit Sculpin when collected (B). Points are color coded by taxon as indicated in the legend with sampling locations indicated corresponding to Table 2. The total variance explained by each Principal Component is indicated in the axis labels.

Admixture analyses on all samples is presented as $K = 2 − 6$ genetic clusters (Fig. 5). Similar to the PC analysis, $K = 2$ produces two clusters that separate Coastal Riffle Sculpin from all other species. The next cluster to emerge ($K = 3$) is Prickly Sculpin, followed by the Pit/Inland Riffle Sculpin ($K = 4$), Paiute Sculpin ($K = 5$), and Russian River samples from the remaining Coastal Riffle Sculpin cluster. Attempts to determine the optimal K using the method of Evanno *et al.* (2005) were inconclusive and attempts to identify clusters above $K = 6$ subdivided previous clusters and were inconsistent, indicating over-parameterization. Admixture results of the Riffle Sculpin complex individuals are presented for $K = 2$ through $K = 6$ in Supplementary Figure S1. Similar to the results of PC analysis of the same samples, Coastal Riffle Sculpin individuals are separated from Pit Sculpin and Inland Riffle Sculpin individuals at $K = 2$. Subsequent values of K separate the two Russian River sampling locations of Coastal Riffle Sculpin ($K = 3$), the Sacramento region Riffle Sculpin with evidence of admixture with a combined Pit Sculpin and Central Valley Inland Riffle Sculpin ancestry component ($K = 4$), and the separation of Central Valley Inland Riffle Sculpin from Pit Sculpin ($K = 5$). The highest K examined ($K = 6$) combines Central Valley Inland Riffle Sculpin and Sacramento Riffle Sculpin again, and shows additional admixture of Pit Sculpin with Sacramento region Riffle Sculpin individuals. At this level, Coastal Riffle Sculpin is highly subdivided (four clusters).

An uncorrected pairwise F_{st} estimate derived between each of the four known species, along with the separation of coastal and inland populations of Riffle Sculpin, is presented in Table 3. The highest pairwise values are between Paiute Sculpin and any other species (0.87–0.96). The second highest values are between Coastal Riffle Sculpin and any of the remaining species (0.71–0.76). Values separating Coastal and Inland Riffle Sculpin (0.71) are higher than between Inland Riffle and either Pit (0.51) or Prickly (0.67) Sculpin (Table 3). The lowest values observed were between inland Riffle and Pit Sculpin, consistent with PC and admixture analyses. The second set of uncorrected pairwise F_{st} estimates between sampling locations revealed similar patterns to the larger groupings above (Supplementary Fig. S2). Values were highest for comparisons between Paiute Sculpin and any other species. Otherwise, values were highest between Coastal Riffle Sculpin and other species. Examining sculpin samples from *within* the species identified above produced lower F_{st} estimates than any comparison between species. The lowest values observed were between locations identified as Prickly Sculpin (0.21–0.59). Locations within Coastal Riffle Sculpin showed higher F_{st} estimates between fish from the more northern Russian River and fish from the Santa Clara Valley than any fish within those locations (0.59–0.70 for between the two regions; 0.21–0.49 for within regions, Supplementary Fig. S1).
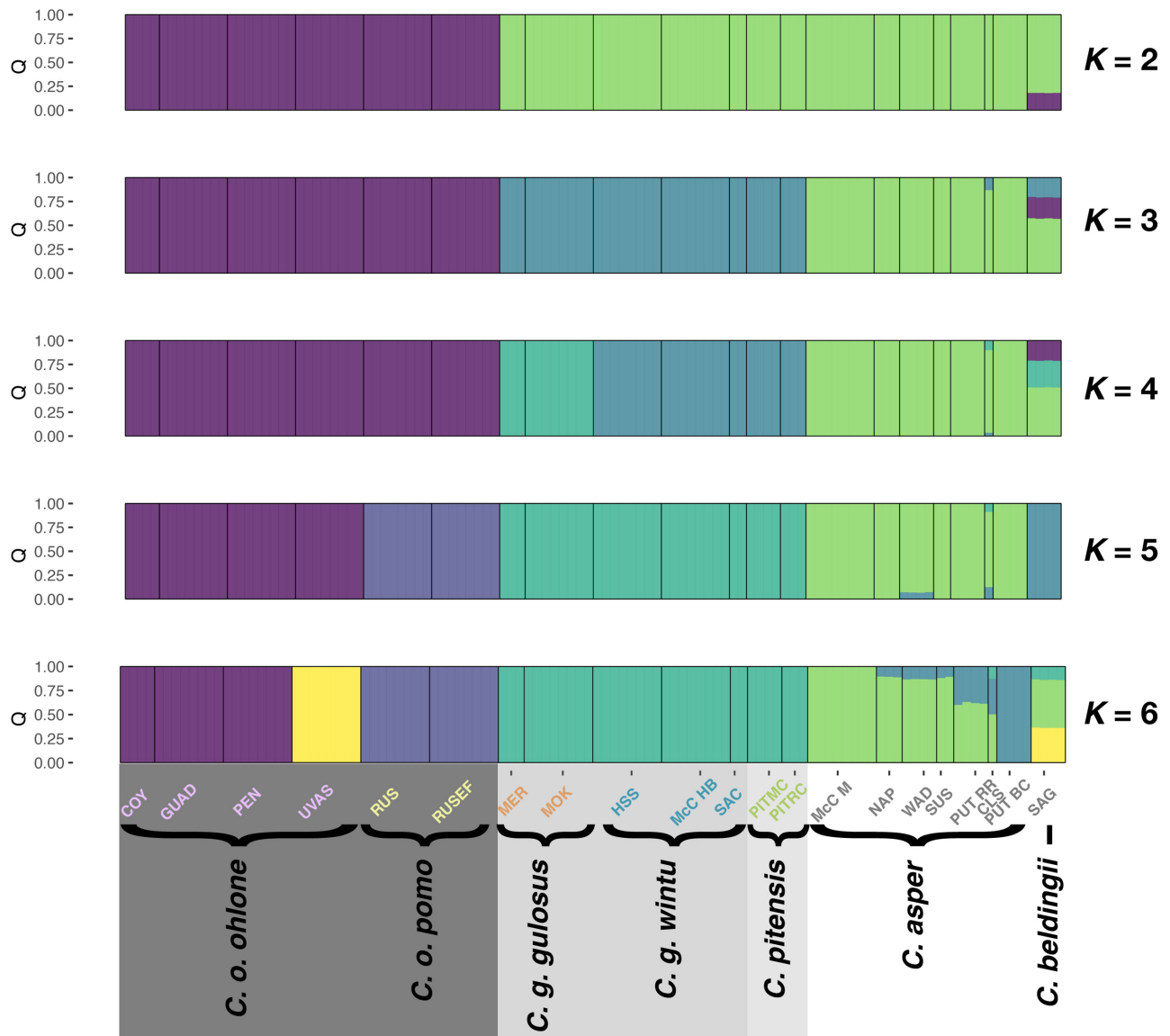
FIGURE 5. Admixture analysis of all sampling locations from $K = 2$ to 6 genetic clusters. Sampling locations along the *x*-axis are color coded by sampling location as in Figure 4 and correspond to Table 2. *Cottus* taxa are designated on the *x*-axis.

TABLE 3. Uncorrected pairwise Fst estimates for sculpin lineages discussed in this paper. The higher the estimate, the more distantly related the species in each pair.

	Inland R.	Coastal R.	Pit	Prickly	Paiute
Inland R.	—	0.71	0.51	0.67	0.91
Coastal R.		—	0.76	0.73	0.92
Pit			—	0.71	0.96
Prickly				—	0.87
Paiute					—

Molecular phylogenetic analysis

Our initial sculpin data set (n =110) was reduced to 84 fish decreasing Paiute Sculpin and Prickly Sculpin to two individuals each. An initial 1,682 SNPs was ultimately reduced to 749 after preparing for input into SVDQuartets. The exhaustive sampling of quartets (1,215,196) produced a topology supported with maximal bootstrap support (100%) at each node and shown in Fig. 6. The input file for PAUP* and output treefiles are available at https://github.com/MacCampbell/ca-cryptic-sculpins/tree/main/treefiles. We found Coastal Riffle Sculpin and Inland Rif-

fle Sculpin to be sister lineages in the topology produced by SVDQUARTETS. Within Coastal Riffle Sculpin, the sampling locations representing Russian River sampling locations and all other sampling locations formed the first division within this clade. The Inland Riffle Sculpin was clearly divided between Sacramento region and Central Valley sampling locations. Based on the species-tree topology, the Pit Sculpin was not most closely related to, or nested within, any other species in this data set.

We created the input data files for TREEMIX following guidelines and scripts available with the source program code (https://bitbucket.org/nygcresearch/treemix/src/master/) and 1,407 SNPs present after pruning of the data set. The TREEMIX outputs were plotted using the plotting_funcs.R script distributed with the program (Fig. 7A, 7B and 7C). With zero migration edges, a topology of Coastal Riffle Sculpin branching first with a sister Inland Riffle Sculpin and Pit Sculpin relationship (Fig. 7A). The inclusion of a first migration edge shows a relatively large amount of migration from Pit Sculpin into Sacramento region Inland Riffle Sculpin but does not result in a change of relationships among sampling locations (Fig. 7B). The addition of a second migration edge results in agreement with the previous migration edge and the addition of a migration edge of much lower weight from MER sampling location of Inland Riffle Sculpin to the UVAS sampling location of Coastal Riffle Sculpin (Fig. 7C) and reduces the magnitude of the drift parameter overall (x – axis). This two-migration edge hypothesis has a minor change in the relationships among PEN, GUAD and COY sampling locations compared to the lower migration edge hypotheses.
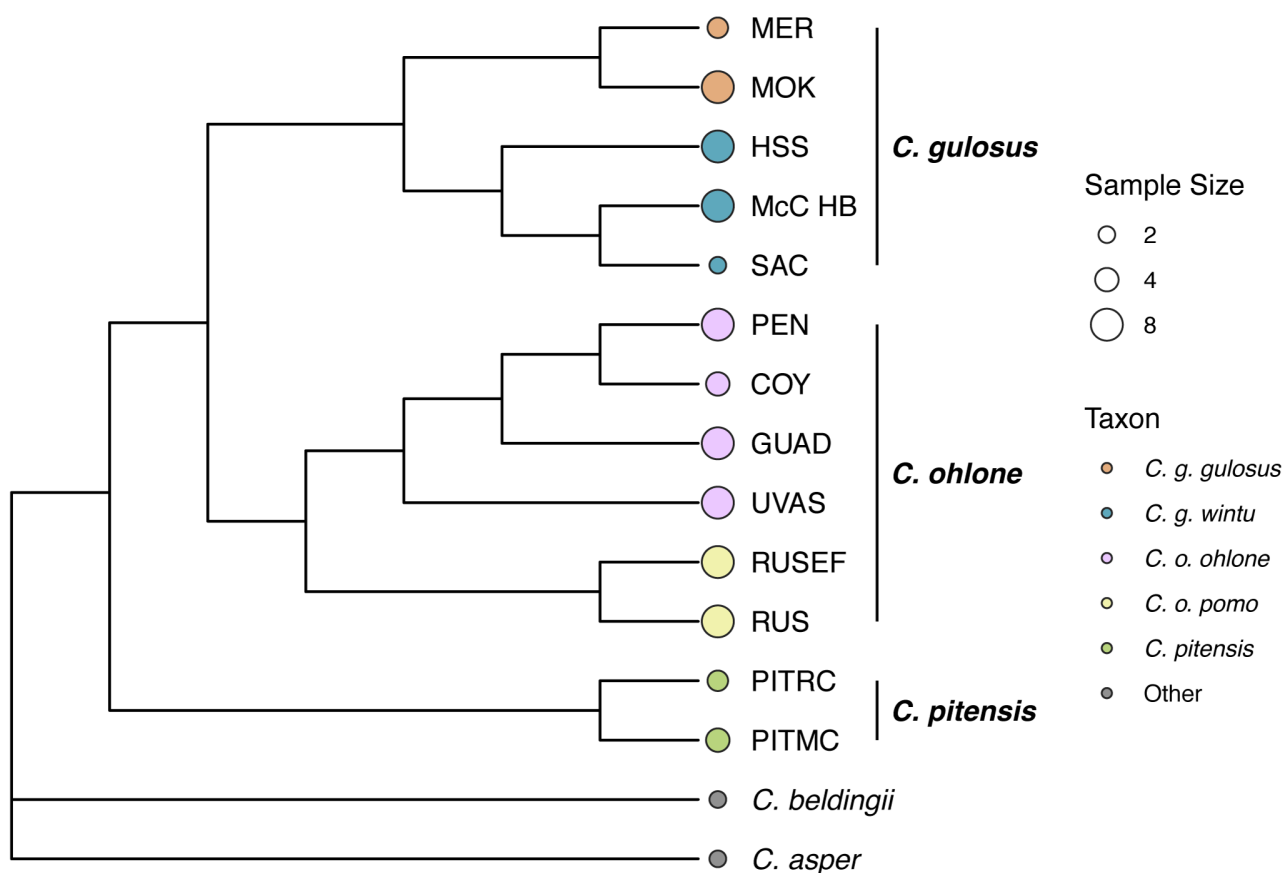

FIGURE 6. Species tree generated from genotype calls with SVDQuartets. The tree is rooted by *Cottus asper* and *C. beldingii*. Circles at tips are proportional to sample size and colored by taxon. Nodal support was maximal (bootstrap support = 100%) for all nodes, and is not indicated.

Mitochondrial Data

We created an alignment 1,081 base pairs in length with 192 mtDNA sequences total from Central Valley Riffle Sculpin (n = 56), Sacramento Riffle Sculpin (n = 76) and Pit Sculpin (n=60). There are a total of 42 unique haplotypes, with the minimum spanning tree shown in Fig. 7D. Sacramento Riffle Sculpin sampling locations may have individuals from either of the two divergent mtDNA lineages separated by 23 steps. Pit Sculpin and Sacramento Riffle Sculpin individuals, however, have mitochondrial haplotypes restricted to alternative mtDNA lineages. The most common haplotype occurs in 30 individuals from Sacramento Riffle Sculpin and 33 Pit Sculpin individuals.
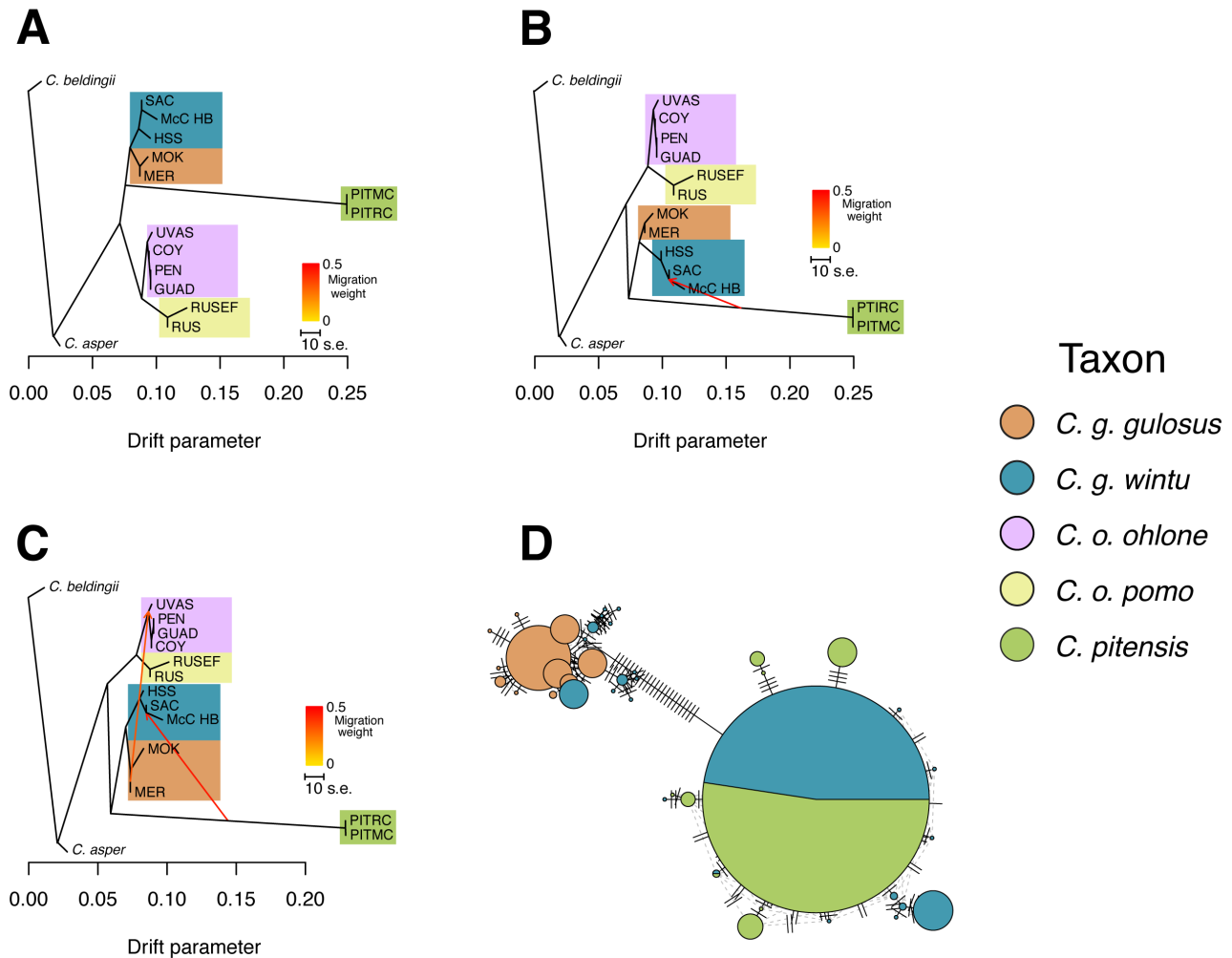
FIGURE 7. Phylogenetic hypotheses generated by TreeMix from genotype calls with *Cottus beldingii* designated as the root, permitting no migration (A), one migration edge (B) and two migration edges (C). A minimum-spanning network is shown in panel D generated from cytochrome b data from Inland Riffle Sculpin individuals. Color-coding of taxa is shown in the figure key.

Distribution

The five lineages within the Riffle Sculpin complex occupy distinct geographic regions (Fig. 2). Only *C. gulosus wintu* (described below) has a range that meets, and perhaps overlaps at times, with two other lineages, but the only consistent evidence of hybridization between species is an apparently ancient hybridization event between *C. pitensis* and the ancestor of *C. g. wintu* as identified by Baumsteiger *et al.* (2012). See Supplementary Fig. S1 and Fig. 7 of this study for corroborating evidence of hybridization between these two lineages.

As cool-water specialists, members of all five lineages are largely confined to headwater regions or to regulated rivers below dams where low water temperatures are typically maintained to support salmonid fishes. This isolation is enhanced by their benthic larvae, in contrast to *C. asper* and *C. aleuticus,* which have pelagic larvae with high mobility (Smith 1982, Moyle 2002). The current distribution of four of the lineages is small or highly fragmented, indicating human-caused changes to their native river systems, which isolate most sculpin populations within lineages from one another. Figures 2 and 3 reflect their distribution in 2019, showing how distributions have been fragmented. The mainstem San Joaquin and Pit rivers, for example, are now too warm for sculpins for long reaches and are dominated by non-native fishes, largely because of human modifications such as dams and diversions (Moyle and Daniels 1982, Moyle 2002). An exception is *C. g. wintu,* which appears to be widespread in the Sacramento River drainage. This is partly an artifact of using HUC 12 watersheds to map distribution. If a watershed flows into the sculpin-rich Sacramento River or a major tributary, it is classified as having sculpins, even if the habitat is unsuitable (e.g., the stream is intermittent). In fact, while year-around cool water is abundant in the main Sacramento

River, thanks to dam releases, the distribution of sculpins in the river system is poorly documented. Riffle Sculpins are apparently absent from watersheds marked as 'historic' (Fig. 2) although the southernmost patch of these watersheds is in the Salinas River drainage which has not been adequately sampled.

Determination of taxa

Our analysis revealed two species and four subspecies within the range of the species originally designated as *C. gulosus* and confirmed the species status of closely related *Cottus pitensis*. Four lineages were found to be new subspecies with largely non-overlapping ranges (Fig. 3).

Cottus gulosus **(Girard 1854), Inland Riffle Sculpin**
(Fig. 1)

Originally described by Charles Girard as *Cottopsis gulosus* from the San Joaquin River, as follows: "Largest specimens a little over three inches {76 cm} in total length; of which the head forms the third, the caudal fin excepted. Preopercle provided with two small spines, such as may be observed in several species of *Cottus* proper, the head otherwise is smooth; mouth proportionately large; posterior extremity of upper maxillary reaching a vertical line, which would pass behind the pupil. A space of five twentieths of an inch exists between the origin of the first dorsal and the occiput. Second dorsal connected with the first by a low membrane proceeding from the last spiny ray. Tips of posterior soft rays not quite reaching the base of caudal. Anal, about as high as second dorsal, but shorter. Caudal, well developed and posteriorly rounded. Origin of ventrals midway between the tip of lower jaw and the anus. Pectorals broad and large, its longest rays extending posteriorly as far as the fifth ray of second dorsal. D IX. 18. A 13. C 3. 1. 5. 4. 1. 2. V I. 4. P 15. Lateral line uninterrupted from thoracic region to base of caudal. Abdomen beset with minute prickles; skin elsewhere smooth. Ground color reddish brown; head and dorsal region spotted with black. Dorsal, caudal and pectorals barred with black; first dorsal provided posteriorly with an elongated black spot. Anal and ventrals unicolor. (Girard 1854; p.130). This description still fits the species complex today, although larger samples show more variation in fin ray counts and characters (Table 1).

Characteristics of Riffle Sculpins used in this study (n = 23) are as follows: anal-fin rays (12–15), dorsal spines (6–10), dorsal rays (14–19), dorsal fins usually joined, black spot on dorsal (yes), palatine teeth present (variable), mouth vs eye (variable but maxilla mostly reaches mid-eye), chin pores (0–2), pelvic fins spines + rays (1+2–3), pelvics touch vent when depressed (variable but mostly not), pectoral fin rays (13–16), preopercular spines (1–2), lateral line completeness variable, and lateral line pores (26–38).

Holotype. USNM 291, *Cottopsis gulosus* from San Joaquin River, California (two specimens), Collected by A. L. Heermann and described by Charles Girard (1854).

Diagnosis. It is distinguished from other members of the *C. gulosus* complex as a distinct lineage determined by genomic studies and its limited geographic distribution (Figs. 1, 3). In streams, it can co-occur with *C. asper* from which it can be most easily separated by its short anal fin (12–15 rays vs. 16–19 for *C. asper*) and short pelvic fins, which do not touch the vent when depressed.

Distribution. *C. gulosus* is the name historically assigned to all populations in the species complex as described in this paper, including *C. pitensis*. Our study shows that the name should be restricted to (a) populations found in streams in the western Sierra Nevada, on the east side of the southern Central Valley, from the American River watershed in the north to the Kern River watershed in the south (Fig. 2), including the San Joaquin River and its tributaries and (b) populations in the Sacramento River and streams tributary to it, except the Pit River. Baumsteiger *et al*. (2014) used mitochondrial and nuclear DNA to examine a large sample (n = 872) of sculpins assigned to the *C. gulosus* species complex. They found a distinct, genetically-based separation of *C. gulosus* from streams flowing into the southern Central Valley, from similar sculpins in streams flowing into the northern Central Valley (Sacramento Valley). These populations here are treated here as new subspecies. The two river systems join in the tidal Sacramento-San Joaquin Delta. A similar break was found in the distribution of the Sacramento Riffle Sculpin lineage at the mouth of the Pit River (now under Shasta Reservoir); sculpins above this break are genetically Pit Sculpins while those below the break are a subspecies that shows evidence of ancient hybridization with *C. pitensis* (Baumsteiger *et al*. 2014).

Etymology. *Cottus* was the Roman name for European sculpin, while *gulosus* roughly translates as 'big mouth' or 'gluttonous' (Moyle 2002).

MOYLE & CAMPBELL

Cottus gulosus gulosus **(Girard 1854), San Joaquin Riffle Sculpin, nominate subspecies**

Description is the same as for the *C. gulosus* (Fig. 1)

    Holotype: USNM 291. Same as for *C. gulosus*, above.

    Paratype: None designated

    Diagnosis: Distinguished from other members of the *C. gulosus* complex as a distinct lineage as determined by genomic studies (Baumsteiger *et al*. 2014, this study) and by its limited geographic distribution (Fig. 3). See species description for distinguishing it from co-occurring *C. asper*.

    Distribution. These California endemic sculpins are found in cold-water riffles, in headwaters or below dams with cold water releases into rivers in the southern Sierra Nevada on the eastern side of the Central Valley, except for the American River (which flows directly into the Sacramento River), the rivers that contain now-isolated populations all flow into the southern Central Valley, especially the San Joaquin River. Rivers with populations include the Mokelumne, Stanislaus, Tuolumne, Merced, Fresno, San Joaquin, Kings, and Kern rivers.

    Etymology. See *C. gulosus*

Cottus gulosus wintu **Moyle and Campbell 2022. Sacramento Riffle Sculpin, new subspecies.**

Description as for *C. gulosus* (Table 1, Fig. 1). Characteristics of *C. g. wintu* used in this study (n=15) are as follows: anal fin rays (12–15), dorsal spines (7–10), dorsal rays (14–19), dorsal fins joined? (variable), black spot on dorsal (yes), palatine teeth present (variable), mouth vs eye (variable but maxilla mostly reaches mid-eye), chin pores (0–2), pelvic fins spines + rays (1+3), pelvics fins mostly do not touch vent, pectoral fin rays (14–16), preopercular spines (1–2), lateral line variable, lateral line pores (26–38). Other characters are listed in Table 1.

    Holotype. WFB 3424. 78 mm SL, 96 mm TL. Fig. 1. North Fork Feather River, Butte County, California. Coll. Jason Baumsteiger, Oct. 5, 2017. Anal-fin rays 13; dorsal-fin spines 8; dorsal-fin rays 17; dorsal fins joined; dorsal fin spot present; palatine teeth absent; maxilla reaches middle of eye; 1 chin pore; pelvic fins 1 spine, 3 rays; pelvics do not touch vent; pectora1 fin rays 14; preopercular spines 1; lateral line complete; lateral line pores, 35; axillary prickles present.

    Paratypes (4). WFB 3425–3428. 78–89 mm SL, 96–108 TL. Anal-fin rays 13; dorsal spines 8; dorsal-fin rays 17–18; dorsal fins joined; dorsal fin spot present on 3; palatine teeth absent; maxilla reaches middle of eye; 1–2 chin pores; pelvic fins 1 spine, 3 rays; pelvics do not touch vent; pectora1 fin rays 15; preopercular spines 1; lateral line complete; lateral line pores, 36–41; axillary prickles present.

    Distribution. Found in the Sacramento River watershed (northern Central Valley), from the American River (and tributaries) north to Shasta Dam. Above Shasta Reservoir, it occurs primarily in the upper Sacramento River and its tributaries (Baumsteiger *et al*. 2014). Other tributaries to Shasta Reservoir (e.g. McCloud River, Squaw Valley Creek) support *C. pitensis*, while the reservoir itself supports *C. asper* (Prickly Sculpin). It inhabits cool-water permanent tributaries and rivers and rivers below dams, where reservoir releases provide cool water (generally less than 24°C).

    Etymology. The species name honors the Wintu people who were the region's original inhabitants, with their lands encompassing many of the streams and rivers where this subspecies lives. The Winnemem Wintu (The Middle Water People) still live in the area and are working to restore salmon runs and to protect sacred rivers and sites in the region.

Cottus pitensis **Bailey and Bond 1963. Pit Sculpin (Fig. 3).**

Cottus pitensis is described in detail by Bailey and Bond (1963), a description that largely overlaps that of *C. gulosus*. Diagnostic features are given below.

    Holotype. UMMZ 130558, adult male 83 mm SL, from North Fork, Pit River, 1934.

    Paratypes. UMMZ 130559, six specimens 55–76 mm SL.

    Diagnosis. "Distinguished from *gulosus* by consistent absence of palatine teeth, the usually better-developed lateral line, and the typical complement of two preopercular spines (Bailey and Bond 1963, p. 21). According to Bai-

ley and Bond (1963), *C. gulosus* has 2–4 preopercular spines but usually 3, while *C. pitensis* has 1–3, but usually 2. In the original description, Girard (1854) noted that *C. gulosus* has two preopercular spines. All other characteristics show broad overlap (Bailey and Bond 1963). Genomic studies (Baumsteiger *et al*. 2014, this study) confirm its species status, as does the distinct distribution. See Table 1 for meristic counts on specimens used in this study (n=8).

Distribution. Endemic to the Pit River watershed in northeastern California and southeastern Oregon. Oregon populations are found in tributaries to Goose Lake, including Drews, Cottonwood, and Thomas creeks (Lake County, Oregon). In California, the Pit Sculpin is found in tributaries to Goose Lake (Lassen and Willow Creeks) and then south in streams throughout the Pit River watershed (Modoc and Shasta counties) to Squaw Valley Creek, now a tributary to Shasta Reservoir. Kinziger *et al*. (2016) note that the complex geologic history of the region has resulted in some population structure in the Pit Sculpin, suggesting further investigation of its taxonomic relationships is needed.

Etymology. Named after the Pit River, which encompasses its distribution. The Pit River was so named because early Euro-American visitors were impressed by the deep pits dug by the native peoples to trap wildlife (Gudde and Bright 1998).

Cottus ohlone Moyle and Campbell 2022. Coastal Riffle Sculpin, new species
urn:lsid:zoobank.org:act:8F6E51D6-5317-4F4E-9246-B2F30FBE7207

Description as for *C. gulosus*. Meristics of sculpins used in this study (Table 1, n = 45): anal-fin rays (12–14), dorsal spines (6–9), dorsal-fin rays (15–19), dorsal fins joined? (variable), black spot present on dorsal, palatine teeth usually present (variable), mouth vs eye (variable but maxilla mostly reaches mid-eye), chin pores (0–2), pelvic fin spines + rays (1+3), pelvics do not touch vent, pectoral-fin rays (14–16), preopercular spines (2), lateral line (complete/incomplete, variable), lateral line pores (25–35). Other characters are listed in Table 1.

Holotype. WFB-3402. 65 m SL, 79 mm TL from Guadalupe Creek, Santa Clara County, California. November 18, 1986. Collected by Jerry J. Smith (Fig. 1). 65 mm SL, 79 mm TL. Anal fin rays 13; dorsal fin spines 8; dorsal fin rays 19; dorsal fins joined; dorsal fin spot present; palatine teeth absent; maxilla reaches middle of eye; 1 chin pore; pelvic fins 1 spine, 3 rays; pelvics do not touch vent; pectora1-fin rays 15; preopercular spines 1; lateral line incomplete; lateral line pores, 27; axillary prickles present but small.

Paratypes. WFB-3403, 3404, 3405, 3406 from same location. 58–60 mm SL, 74–81 TL. Anal-fin rays 13; dorsal fin spines 8; dorsal fin rays 17–19; dorsal fins variable in joining; dorsal fin spot present; palatine teeth absent; maxilla reaches middle of eye; 1–2 chin pores; pelvic fins 1 spine, 3 rays; pelvic fins do not touch vent in 3 of 4; pectora1 fin rays 13–15; preopercular spines 1; lateral line incomplete in most; lateral line pores, 26–33; axillary prickles present but small.

Diagnosis. Distinguished from other members of the *C. gulosus* complex as a distinct lineage as determined by molecular phylogenetic and population genomic evidence (Baumsteiger *et al*. 2014, this study) and by its limited distribution (Fig. 2).

Distribution. Restricted to drainages flowing west or southwest along the Coast Range Mountains of California, with two distinct lineages, described as subspecies here. The watersheds include (a) the Russian River and Redwood Creek (which flow directly into the Pacific Ocean, (b) the Napa River, Sonoma Creek and nearby watersheds that flow into north San Francisco Bay, (c) streams draining the Diablo and Coastal ranges through the Santa Clara Valley and nearby areas and flowing into south San Francisco Bay, and (d) tributaries to the Pajaro River, in the Salinas River watershed (Fig. 2). Populations may also exist in the headwaters of the Salinas River (Snyder 1913) although there are no recent records. Habitats are restricted to cool, clear permanent streams with rocky riffles, mostly in headwaters.

Etymology. *Ohlone* honors the Ohlone peoples, who were the original human inhabitants of the Santa Clara Valley region and much of the southern San Francisco Bay region (see http://www.muwekma.org/). The name Ohlone refers to the more than 50 peoples that spoke similar languages and interacted with one another in the region. Their descendants are largely encompassed in the present-day Muwekma Ohlone Tribe.

Cottus ohlone ohlone Moyle and Campbell 2022. Nominate subspecies, Ohlone Riffle Sculpin.

Holotype and paratypes. Same as for the species, *C. ohlone*.

    Diagnosis. Distinguished from other members of the *C. gulosus* complex as a distinct lineage as determined by genetic and genomic studies (Baumsteiger *et al*. 2014, this study) and by its distinctive distribution (Fig. 3).

    Description. Same as for *C. ohlone* above (Fig. 1).

    Distribution. Largely restricted to upper portions of streams in and around the highly urbanized (e.g., San Jose) Santa Clara Valley, including the upper Guadalupe River and upper Penitencia Creek, which is tributary to Coyote Creek. All known locations occur west of the Coast Range (Diablo Range), in the hills around the Santa Clara Valley, or in streams flowing into San Francisco Bay from the Coast Range. The southernmost sample collected was from Bird Creek, near Hollister Hills (see Baumsteiger *et al*. 2014). The northernmost location known was San Mateo Creek (Jordan and Everman 1896) from which they have been extirpated (Leidy 2007).

Cottus ohlone pomo Moyle and Campbell 2022. Pomo Riffle Sculpin, new subspecies

Description as for *C. gulosus*. Meristics of sculpins used in this study (Table 1, n = 21): anal-fin rays (12–14), dorsal spines (6–8), dorsal rays (15–19), dorsal fins joined? (variable), black spot on dorsal (yes), palatine teeth present (no), maxilla reaches eye (variable), chin pores (1–2), pelvic fins spines + rays (1+3), pelvic fins do not touch vent, pectoral -in rays (14–16), preopercular spines (2), lateral line complete, lateral line pores 27–35. Other characters are listed in Table 1.

    Holotype. WFB 3396 90 mm SL, 105 mm TL from Pieta Creek, upstream of mouth on Russian River, Mendocino County, California, N 38.925278 W 123.054500. Collected by J. Baumsteiger and P. Moyle, August 2, 2017. 90 mm SL, 105 mm TL. Anal-fin rays 13; dorsal-fin spines 8; dorsal-fin rays 17; dorsal fins not joined; dorsal fin spot present; palatine teeth absent; maxilla reaches middle of eye; chin pores 2; pelvic fins 1 spine, 3 rays; pelvic fins do not touch vent; pectoral1-fin rays 14; preopercular spines 1; lateral line incomplete; lateral line pores 31; axillary prickles present.

    Paratypes. WFB 3397, 3398, 3399, 3400 from same location. 71–81 mm SL, 91–105 mm TL. Anal fin rays 13; dorsal fin spines 8; dorsal fin rays 17; dorsal fins not joined; dorsal fin spot variable in presence; palatine teeth absent; maxilla reaches middle of eye; 2 chin pores; pelvic fins 1 spine, 3 rays; pelvic fins do not touch vent; pectoral1 fin rays 14–15; preopercular spines 1; lateral line completeness variable; lateral line pores, 25–30; axillary prickles present.

    Diagnosis. Distinguished from other members of the *C. gulosus* complex as a distinct lineage as determined by genomics and by its distinctive distribution (Fig. 1).

    Distribution. Present in the upper portions of the Russian River, above the mouth of Mark West Creek, including the East Fork, as well as in tributaries to northern San Francisco Bay, including the Napa River, Petaluma River, Sonoma Creek, and smaller tributaries (Fig. 3). These streams had connections in the past to the Russian River, via the shifting headwaters of Sonoma Creek.

    Etymology: *Ohlone* honors the Ohlone people, as discussed in the names section for *C. o. ohlone* (see http://www.muwekma.org/). *Pomo* honors the diverse native peoples who were once the principal human inhabitants of the Russian River region, which contains streams that are important habitat for Pomo Riffle Sculpin.

Discussion

Cryptic species of *Cottus*

Our study shows that fishes currently identified as *C. gulosus* in California belong to three species and four subspecies, with no or only minor observable external differences, as described below. The lack of detectable phenotypic differences among the taxa, except those minor characteristics distinguishing *C. pitensis* (Bailey and Bond 1963), is no real surprise. Throughout more than 100 years of sculpin descriptions in California (Jordan 1896, Jordan and Everman 1898, Rutter 1908, Krejsa 1967, Daniels and Moyle 1984, Moyle 2002, Rowsey and Egge 2017), mistaken or incomplete identifications are common, so biologists and others sampling fishes frequently identify sculpins as just 'Cottus species.' Rowsey and Egge (2017) after an extensive morphological and meristic study of *C. gulosus* and *C. perplexus* (a widespread species in Oregon and Washington) failed to find useful characters for separation

of the species. Contributing to the confusion was the fact that sculpins identified as *C. gulosus* in Oregon belong to a different evolutionary lineage than *C. gulosus* in California (Baumsteiger *et al*. 2012, 2014). Indeed, freshwater sculpin species are notoriously difficult to differentiate (Kinziger *et al*. 2005, Yokoyama and Goto 2005, Young *et al*. 2013). The genus *Cottus* likely contains numerous additional cryptic species, consistent with our study. More and more studies of other groups of organisms acknowledge that species structure can be present even when phenotypic differences are lacking (Belyaeva and Taylor 2009, Victor 2015, Liu *et al*. 2018). However, novel approaches may yet discover differences that are currently undetectable (Zúniga-Reinoso and Benítez 2015).

Our genomic analyses make use of an extensive set of loci taken from throughout the genome of these fishes. Such analyses greatly diminish errors associated with previous genetic analyses that used limited nuclear loci or mitochondrial DNA (Luikart *et al*. 2003, Allendorf 2017). We found that mitochondrial DNA sequence data cannot reliably separate Inland Riffle Sculpin species and subspecies (Fig. 7D). In this study, the mitochondrial-nuclear conflict with regard to *C. gulosus* and *C. pitensis* highlights the utility of examining mitochondrial and nuclear DNA data sources together. Incomplete lineage sorting and admixture are difficult to distinguish and treating gene tree discordance as incomplete lineage sorting through the multispecies coalescent would lead to the conclusion that *C. gulosus* and *C. ohlone* are sister taxa (Fig. 6). However, when treating this discordance as hybridization, *C. gulosus* and *C. pitensis* are shown to be sister lineages (Figs. 7A, 7B and 7C). Combined, mitochondrial and nuclear data support historical hybridization followed by mitochondrial introgression leading to replacement of *C. g. wintu* mitochondrial haplotypes by *C. pitensis* haplotypes in regions of the range of *C. g. wintu* (Fig. 7D). Evolutionary investigations, beyond the scope of this study, are needed to determine the root cause of this process, e.g. thermal adaptation (Wilson and Bernatchez, 1998). This and other efforts have found that genomic techniques can reliably differentiate other species of fish in California (Baumsteiger *et al*. 2017, Baumsteiger and Moyle 2019, Baumsteiger *et al*. 2019).

The ability to identify cryptic species through DNA is important for protecting aquatic diversity. We can no longer assume phenotypic characteristics are the only metrics useful for differentiating lineages and their divergence over time. Our study, along with previous studies (Baumsteiger *et al*. 2012, 2014), clearly differentiate Coastal Riffle Sculpin from Inland Riffle Sculpin in California. This pattern is consistent over multiple types of genetic markers and analyses, ranging from mitochondrial and nuclear DNA (Baumsteiger *et al*. 2012, 2014) to thousands of nuclear SNPs (this study) taken from throughout the genome. Additionally, no study, including this one, has successfully identified morphological or meristic differences among the five lineages, except for minor differences separating *C. pitensis* from the other species (Bailey and Bond 1963).

A significant finding of all three genomic analyses used in this study is that Coastal Riffle Sculpin are highly differentiated from any currently identified *Cottus* species in California, confirming the results of Baumsteiger *et al*. (2014). Principal Component, admixture and TREEMIX phylogenetic analyses all demonstrate the independence of the Coastal Riffle Sculpin lineage from other sculpin lineages in the data set and shows substantial genetic differentiation (e.g. PC1 of Fig. 4A, and *K* = 2 of Fig. 5). We conclude, therefore, that the Coastal Riffle Sculpin is a distinct species. Phylogeographic evidence supports such a strong differentiation, coinciding with the rise of the Coast Range Mountains millions of years ago (Baumsteiger *et al*. 2014). The creation of this geographic barrier to dispersal (especially given the sensitive ecological conditions needed for these species—see Moyle 2002) is a textbook example of allopatric speciation (Hoskin *et al*. 2005). Ancestral Riffle Sculpins became isolated in two regions. They then underwent extensive and independent evolutionary changes throughout their genomes resulting in two modern species, with little, if any phenotypic change: Coastal Riffle Sculpin and Inland Riffle Sculpin.

Pit and Inland Riffle Sculpin

Bailey and Bond (1963) hypothesized that Pit Sculpin (*C. pitensis*) diverged from the ancestral *Cottus gulosus* lineage following the rise of a barrier (Pit Falls) on the lower Pit River, which flows into the Sacramento River. Baumsteiger *et al*. (2014), using limited nuclear loci and mitochondrial DNA, confirmed the close relationship between Inland Riffle Sculpin from the Sacramento watershed and Pit Sculpin and concluded that they are nevertheless separate species. Baumsteiger *et al*. (2014) also found that presumed *C. gulosus* within the Sacramento River drainage had experienced limited, if ancient, hybridization with *C. pitensis*. Our current genomic study supports this finding. Non-introgressed *C. pitensis* exist within the Pit River drainage, above the mouth of the Pit River and below Pit Falls. In contrast non-introgressed *C. gulosus* exist south of the American River (the last major tributary Sacramento River) in tributaries to the southern Central Valley (primarily the San Joaquin watershed) (Fig. 3).

Historic hydrological conditions provided limited connectivity among all three groups but there is no evidence of recent/ongoing hybridization (Baumsteiger *et al*. 2014). The hybridization appears to have been a unique event, creating a genotype that became established in the Sacramento watershed after the original split of *C. pitensis* from *C. gulosus* and after the spread of *C. pitensis* to streams throughout the Pit River watershed. Presumably, the hybrid form could not invade San Joaquin watershed streams because much drier conditions isolated populations from one another in the upper parts of tributary watersheds, as reflected in contemporary distribution patterns (Fig. 3). Given that C. *gulosus* in the Sacramento River watershed can be geographically and genomically defined, we consider it to be a cryptic subspecies of *C. gulosus*. Evidence of past hybridization with Pit Sculpin is found in the nuclear genome of Sacramento Riffle Sculpin. It is shown in all sampling locations in that geographic region including the American and Feather Rivers (Baumsteiger *et al*. 2014). Admixture with Pit Sculpin increases to the north, closer to populations of pure Pit Sculpin. In this study, we detected hybridization with Pit Sculpin in the Mc HB and SAC sampling locations (Figs. 7B and 7C, Supplementary Fig. S1). Mitochondrial haplotypes sampled from *C. gulosus* in the Sacramento River drainage north of the American and Feather Rivers are completely derived from a Pit Sculpin mitochondrial lineage (Baumsteiger *et al*. 2014, but see also Fig. 7D). A substantial mitochondrial-nuclear discordance thus exists. This means that using mtDNA to identify sculpins in the Sacramento River basin will not correctly separate Pit Sculpin and Sacramento Riffle Sculpin, while a nuclear DNA-based assay is able to do so.

Coastal Riffle Sculpin

Genomic results not only show that Coastal Riffle Sculpin is a distinct species and that there are two separate lineages within the species, one from the Santa Clara Valley region and one from the Russian-Napa rivers region. The two lineages are separated by San Francisco Bay-Estuary, which is of relatively recent origin (<10,000 years). However, the complex, active geology of the region would have made earlier separation possible (Sloan 2006). Genomic differentiation of populations between these two regions is similar to that seen between Pit (*C. pitensis*) and Inland Riffle (*C. gulosus*) sculpins, suggesting that, from a sculpin perspective, separation of the two regions has a longer history. The genetic differences are significant enough so that an argument could be made they represent two separate species. However, the genomic support is somewhat mixed between analyses and is not as strong as the support for separation of *C. ohlone* from *C. gulosus*. Therefore, we conclude that the two forms are best designated as subspecies (*C. o. pomo* and *C. o. ohlone*).

There are considerable conservation implications of recognizing the species and its two subspecies. Climate change has the potential to reduce rainfall in the Coast Range mountains, increasing variability of stream flows for this region and the propensity for wildfires (Westerling and Bryant 2008). All the sculpin taxa are dependent on cool, clean, highly oxygenated water (Moyle 2002), which is increasingly scarce in regions so close to major human populations. While headwaters may be protected to some degree, alteration of lower reaches of most streams by urban development, agriculture, and water diversions means that if sculpins have been extirpated from headwater streams by natural causes (e.g., predicted megadroughts), natural recolonization from nearby populations is unlikely. The addition of non-native predatory species into these systems increases the threat to sculpins. For example, *C. o. ohlone* is common in headwater streams which are largely on public lands such as Henry Coe State Park (R. Leidy, pers. comm. 2020). However, the lower reaches of these streams flow through urbanized areas in the Santa Clara Valley (a.k.a. 'Silicon Valley') and southern San Francisco Bay, where appropriate habitat and the Ohlone riffle sculpin are entirely lacking. Thus, they are at risk of extinction through the combined effect of many local extirpations over the years, especially from the effects of climate change and megadroughts (Williams *et al*. 2020).

Conclusions

The taxon long identified as just *C. gulosus* consists of three species (including *C. pitensis*) and four subspecies (five lineages total). These taxa are endemic to limited regions and need special consideration to avoid extirpation of isolated populations when their streams or watersheds are altered. Members of this species complex are physiologically adapted to cool headwater streams, so they are prone to isolation as downstream areas are dammed, diverted, polluted, and otherwise altered. This isolation is also enhanced by the sculpin's limited dispersal abilities. Their larvae and juveniles are benthic and tend to stay in the parental region, unlike species such as *C. asper* which have pelagic larvae and can disperse rapidly (Smith 1982, Moyle 2002). Local isolation means that individual popula-

tions can disappear during severe drought but have no way to re-colonize the vacated area. Populations in greatest danger of extinction in the near future are those of the Ohlone Riffle Sculpin followed by most populations of Riffle Sculpin in the San Joaquin River drainage. Fortunately, high-quality waters that provide good habitat for salmon and trout (fish important to people) can also provide good habitat for the five endemic lineages of Riffle Sculpin. This increases their probability of survival in the coming decades of variable conditions created by human activity and enhanced by climate change.

Acknowledgements

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Supplementary Figure S1
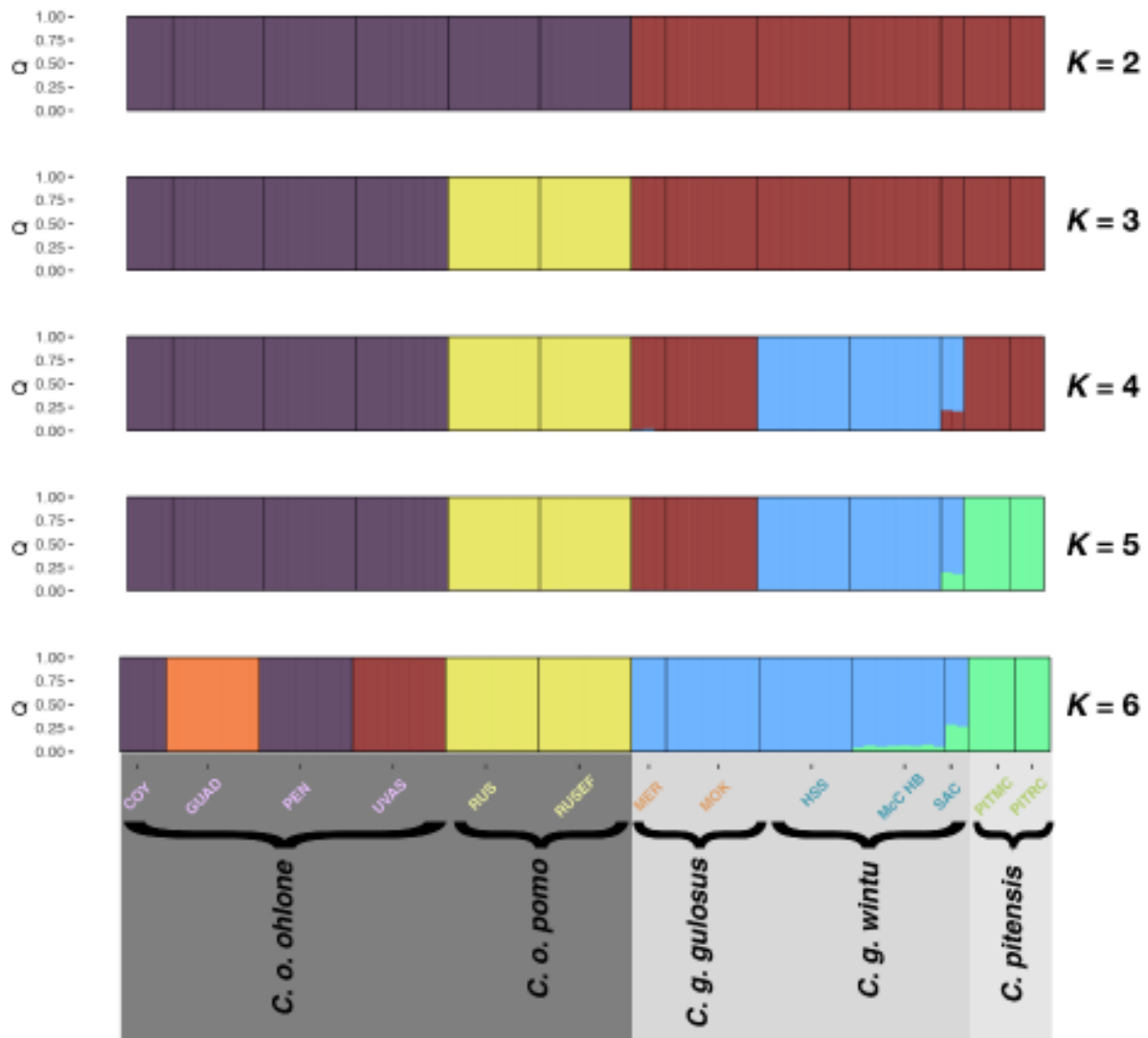
Supplementary Figure S1. Admixture analysis of Riffle Sculpin sampling locations from $K = 2$ to 6 genetic clusters. Sampling locations along the x - axis are color coded by sampling location as in Figure 4 and correspond to Table 2. *Cottus* taxa are designated on the x - axis.

Supplementary Figure S2
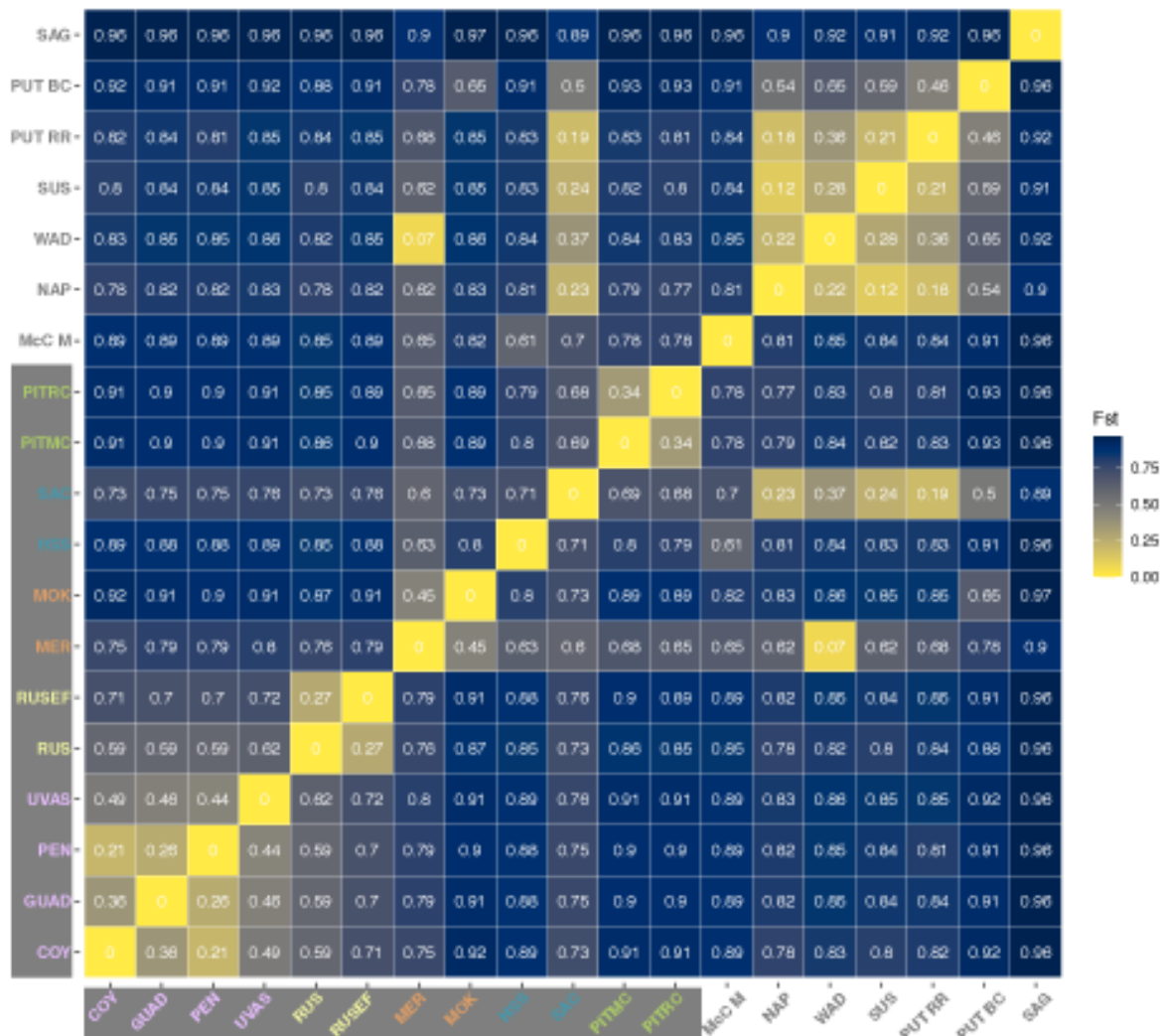
Supplementary Figure S2. Uncorrected Pairwise Fst estimates between sampling locations. Abbreviations are from Table 2. The higher the estimate, the more distantly related the species in each pair are to each other. Note that COY, GUAD, PEN, UVAS, RUS and RUSEF are *C. ohlone*; MER, MOK, HSS and SAC are *C. gulosus*; PITMC and PITRC are *C. pitensis*; McC M, NAP, WAD, SUS, PUT RR and PUT BC are *C. asper*; SAG is *C. beldingii*.