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2019 Report

Genetic Evaluation of Upper Basin Colorado River *Gila cypha*

Photo Credit T. Kennedy

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LIST OF ABBREVIATIONS

A_R = Allelic richness

ARRC = Aquatic Resource and Recovery Center

BP = Base-pairs

Cyt b = Mitochondrial gene *cytochrome b*

DAPC = Discriminant Analysis of Principal Components

ddRAD = Double digest Restriction-site Associated DNA

F_{IS} = Inbreeding Coefficient

F_{ST} = Fixation Index

GIS = Geographic Information Systems

H_E = Expected Heterozygosity

H_O = Observed Heterozygosity

mtDNA = Mitochondrial DNA

ND2 = Mitochondrial gene *NADH dehydrogenase subunit two*

N_E = Effective population size

PCA = Principal Components Analysis

PCR = Polymerase Chain Reaction

QA/QC = Quality Control/Quality Assurance

SAP = Shrimp alkaline phosphatase

SNARRC = Southwestern Native Aquatic Resource and Recovery Center

SSCP = Single-stranded conformational polymorphism

LIST OF KEYWORDS

humpback chub, *Gila cypha*, roundtail chub, *Gila robusta*, bonytail, *Gila elegans*, conservation genetics, population structure, hybridization, introgression, genetic diversity, microsatellite, *ND2*, Upper Colorado River Basin

EXECUTIVE SUMMARY

Conservation of the federally endangered humpback chub (*Gila cypha*) in the Upper Colorado River Basin (Upper Basin) has been complicated by challenges in distinguishing *G. cypha* from roundtail chub (*G. robusta*). This is further convoluted through contemporary hybridization with *G. robusta* and bonytail (*G. elegans*). Proper management of *G. cypha* necessitates identification of management units, design and establishment of broodstock, assessment of *G. robusta* by *G. cypha* hybridization, and the calculation of effective population size.

These goals were accomplished through analysis of 512 *G. cypha* from five Upper Basin sites (Cataract Canyon, Black Rocks, Desolation/Gray Canyon, Westwater Canyon, and Yampa Canyon) as well as one site (the Little Colorado River) in the Lower Basin. Included in the analysis were 179 *G. robusta*, 22 *G. elegans*, and 13 suspected hybrids. Samples were genotyped for 18 microsatellite loci and sequenced for the ND2 mitochondrial (mtDNA) locus.

Genetic diversity levels were slightly higher for *G. cypha* in the Upper Basin compared to the Lower Basin. Tests for population structure revealed three discrete populations for *G. cypha*: 1) Black Rocks/Westwater Canyon and Cataract Canyon, 2) Desolation/Gray Canyon, and 3) the Lower Basin. Yampa Canyon "*G. cypha"* were genetically identified as *G. robusta*, which supports field reports of *G. cypha* being extirpated from Yampa Canyon. Private microsatellite alleles and mtDNA haplotypes were found in all *G. cypha* populations. Two distinctly separate mtDNA haplotype groups were found: 1) *G. elegans* and 2) *G. cypha*/*G. robusta*. The most common haplotypes were shared by *G. cypha* and *G. robusta*. Hybridization between *G. cypha* and *G. robusta* was prevalent at Black Rocks and Westwater Canyon, but rare in Desolation Canyon. *Gila cypha* diverged from *G. robusta* as stream distance from Black Rocks increased. Estimates of effective population size were similar to census estimates in the Upper Basin.

Overall these results support three management units for *G. cypha*: 1) Black Rocks/Westwater Canyon and Cataract Canyon, 2) Desolation/Gray Canyon, and 3) the Lower Basin. Any future development of broodstock for the Upper Basin should separate Desolation Canyon from Black Rocks/Westwater Canyon due to the variable incidence of hybridization observed at these two sites. Lower Basin fish should not be used to supplement Upper Basin diversity because this study revealed greater genetic diversity estimates in the Upper Basin.

RECOMMENDATIONS

We recommend that three management units be designated for *G. cypha*: 1) Black Rocks/Westwater Canyon and Cataract Canyon, 2) Desolation/Gray Canyon, and 3) the Lower Basin. These three units contain distinct genetic diversity that should be maintained. Both Upper Basin units had slightly higher levels of genetic diversity than the Lower Basin unit.

Upper Basin broodstocks should be maintained separately from the Lower Basin broodstock to preserve the genetic differences found in this study. Upper Basin stocks should not be supplemented with Lower Basin diversity since the Upper Basin has greater genetic diversity compared to the Lower Basin. Black Rocks/Westwater Canyon and Desolation Canyon broodstocks should be maintained separately because they represent distinct populations with differing levels of interspecific hybridization.

Effective population size estimates ranged from 492 (Desolation Canyon) to 1548 (Black Rocks) in the Upper Basin. However, multiple factors may influence these estimates. Age structuring may be deflating estimates, whereas the prevalence of hybridization in Black Rocks and Westwater Canyon may be inflating effective population sizes. Desolation Canyon had both the fewest individuals with hybrid ancestry, and the lowest effective population size estimates. Maintaining higher effective population sizes will prevent genetic diversity from decreasing in the future.

A study should be conducted to determine the number of effective breeders (N_b) for *G. cypha* in the Upper Basin. This would be a multi-year study to account for annual variation in reproduction resulting from skip spawning or environmental fluctuations. Young-of-year fish would be sampled from multiple sites throughout the Upper Basin to allow for improved N_b calculations.

A multi-decadal population genetic study should be conducted to observe changes in hybridization rates over time. Such a study could be feasible by sourcing tissue samples and extracted DNA from museum archives and genetics labs that have studied Upper Basin *Gila* populations in the past. These could be combined with new collections (i.e., 2019 and onwards) from sites analyzed in this report. Results could be analyzed in the context of water usage derived from Geographic Information Systems (GIS) databases to better understand the effects of habitat modification on genetic diversity of *Gila* populations.

All populations had private alleles. Those occurring at relatively high frequencies (i.e., >0.1) should be considered when developing future broodstocks. Broodstock should be collected from multiple locations within each canyon and at multiple time points to increase the probability of sampling the entire range of Upper Basin genetic diversity. Genetic screening of broodstock would ensure that the current genetic diversity is represented.

A study should be undertaken to monitor *G. cypha* spawning in the Upper Basin to determine when and where spawning occurs. Tissue samples should be taken from adults as they leave spawning grounds and/or from eggs or larvae sampled from different spawning events.

INTRODUCTION

The humpack chub (*Gila cypha*) is endemic to the Upper Colorado River Basin (Upper Basin, above Glen Canyon Dam) and northern reaches of the Lower Colorado River Basin (Figure 1). It is listed as federally endangered (32 FR 4001), but a downlisting to threatened status has recently been recommended (U.S. Fish and Wildlife Service 2018). *Gila cypha* inhabit canyons within fast-moving, turbulent rivers. Their young are most likely to occupy slow, shallow shoreline areas or areas under cliffs (Gerig et al. 2014). Adults often occupy eddies, which may result in reduced swimming effort and provide greater food availability. Spawning occurs when flows are at their highest or receding, and *G. cypha* exhibit more site fidelity during spawning than their sympatric congener, roundtail chub *Gila robusta* (Kaeding et al. 1990).

Gila cypha is a long-lived species with an iteroparous reproductive strategy, however lifespan and generation time differs between the Upper and Lower Colorado River Basins. Lower Basin fish are capable of living up to 40 years and have a generation time of approximately 16 years (Coggins & Walters 2009; Yackulic et al. 2014), whereas this is reduced in the Upper Basin to approximately 8 years (Francis et al. 2016; Hines et al. 2016; Howard & Caldwell 2017). Female *G. cypha* produce approximately 2,500 eggs and participate in communal spawning over gravel substrate (U.S. Fish & Wildlife Service 2018). Evidence for skipped spawning has also been observed in the Lower Basin (Little Colorado River: Pearson et al. 2015). Sex ratios for *G. cypha* are assumed to be 1:1 in the Lower Basin (Valdez and Ryel 1997), however surveys in April and May of 2018 revealed male:female sex ratios of 1.6:1 and 1.3:1, respectively (Pillow 2018).

Over the past 20 years, *G. cypha* census size estimates have undergone a decline followed by apparent stabilization in the Upper Basin (U.S. Fish and Wildlife Service 2017). One population (Yampa Canyon) was extirpated during this time. Individual *G. cypha* populations remain vulnerable to several threats despite the apparent stabilization (U.S. Fish and Wildlife Service 2018). For example, anthropogenic changes in stream flow and water quality are thought to threaten population levels. Predation by nonnative rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* on juvenile *G. cypha* threatens *G. cypha* recruitment, and has been exacerbated by an anthropogenic decrease in turbidity in parts of their range (Ward et al. 2016). Predation by nonnative fishes (primarily smallmouth bass, *Micropterus dolomieu*) also occurs in the Upper Basin (U.S. Fish and Wildlife Service 2017). Little Colorado River *G. cypha* have been infected with Asian tapeworm (*Bothriocephalus acheilognathi*) and that infection is expected to spread, decreasing growth and survival in infected fish (Clarkson et al. 1997). In addition, hybridization has been documented among several *Gila* species (DeMarais et al. 1992, Dowling and DeMarais 1993, Minckley and DeMarais 2000, Gerber et al. 2001, Chafin et al. 2019), including hybridization of *G. cypha* with *G. robusta* (not listed) and *G. elegans* (bonytail, endangered).

Conservation efforts have been undertaken to aid in recovery of *G. cypha*, including the maintenance of a refuge population at the Southwestern Native Aquatic Resources and Recovery Center (Southwestern ARRC) that was created using the Little Colorado River as the source population. Spurgeon et al. (2015) found evidence of growth and survival of juvenile *G. cypha* translocated into Shinumo Creek (Colorado River tributary, Grand Canyon National Park). They concluded that translocation of *G. cypha* is an effective strategy for establishing refuge populations in the Lower Basin, and would likely be successful in other tributaries of the Colorado River. Nonnative predator removal has also been undertaken, although the effects on *G. cypha* recruitment are uncertain (Coggins et al. 2011), and stocking agreements are in place to prevent the introduction of nonnative fishes into *G. cypha* habitat (U.S. Fish and Wildlife Service 2011). The Lower Basin population has shown increasing

census size estimates (approximately 12,250 individuals) and recruitment numbers after declining for several years (U.S. Fish and Wildlife Service 2018).

Six population units were designated for *G. cypha* (U.S. Fish and Wildlife Service 2011): Black Rocks (Colorado); Yampa Canyon (Dinosaur National Monument, Colorado); Westwater Canyon (Utah); Desolation/Gray Canyons (Utah, here referred to as Desolation Canyon); Cataract Canyon (Utah); and the Lower Basin population (Arizona). However, the designation of the six *G. cypha* population units was based on geographic separation and may not reflect actual population dynamics (e.g. migration and gene flow). It is also important to document contemporary genetic diversity throughout the Upper Basin, and evaluate changes in diversity patterns that may have occurred since the Douglas and Douglas (2007) report due to population declines that occurred during the early 2000s (i.e., Black Rocks, Westwater Canyon, and Yampa Canyon: U.S. Fish and Wildlife Service 2017). A population's probability of persistence is linked to within-population genetic diversity in areas experiencing envionmental fluctuations, with greater diversity allowing for adaptation (Lande and Shannon 1996). The maintenance of genetic diversity within a population is in turn related to factors such as effective population size, genetic drift, migration, and selection (Lande and Barrowclough 1987); therefore, assessing and monitoring population genetic diversity and effective population size are important when determining both the status of *G. cypha* and its likelihood of persistence.

Gila cypha is a member of the western chubs (*Gila* spp.) and in order to assess genetic diversity of *G. cypha* populations, it is important to consider the historical relationship of *Gila* spp. within the Colorado River Basin due to complex speciation events, many involving hybridization. Virgin River chub (*G. seminuda*) may be the result of speciation by hybridization of *G. robusta* and *G. elegans,* resulting in intermediate morphology and allozyme frequencies, and the presence of *G. elegans* mitochondrial (mtDNA) haplotypes (DeMarais et al. 1992). Dowling and DeMarais (1993) analyzed allozymes and mtDNA restriction digest patterns from *G. robusta*, *G. cypha*, and *G. elegans* to investigate hybridization among these species. Based on haplotype and allozyme frequencies in now allopatric populations, they concluded that Upper Basin *G. robusta* had *G. cypha* mtDNA haplotypes and intermediate allozyme frequencies. They also concluded that Chevelon Creek (upper Little Colorado River tributary) and Upper Basin *G. robusta* had similar haplotypes to *G. cypha* (lower Little Colorado River) while *G. seminuda* had similar haplotypes to *G. elegans*. Overall, Dowling and DeMarais (1993) concluded that allozyme frequencies were more consistent with sampling locations and morphological identifications than mtDNA haplotypes and that haplotype similarity of *G. robusta* and *G. cypha* was caused by recent introgression.

Gerber et al. (2001) compared mtDNA (*cytochrome b* [*cyt b*] and *NADH dehydrogenase subunit two* [*ND2*]) restriction endonuclease and single-stranded conformational polymorphisms (SSCP) to identify species-specific haplotypes based on Lower Basin allopatric populations. Their results indicated that Lower Basin *G. cypha* and *G. robusta* had discrete haplotypes, although some *G. cypha* had *G. elegans* haplotypes, and that isolated Upper Basin *G. cypha* and *G. robusta* contained *G. elegans* haplotypes. They then concluded that all of the remaining Upper Basin *G. robusta* contained *G. cypha* haplotypes, indicating widespread introgression where *G. robusta* haplotypes had been replaced with *G. cypha* haplotypes, and that this pattern of introgression began before human alteration of the area. Finally, they concluded that Pahranagat roundtail chub (*Gila robusta jordani*), which contained *G. cypha* haplotypes, was a separate taxon derived from hybridization events between *G. robusta* and *G. cypha*.

In view of the observed hybridization among these species, it is not surprising that morphological identification can be problematic, especially in the field; however, several studies have

used morphological and meristic characters that can differentiate among *G. cypha*, *G. robusta*, and *G. elegans* using statistical analyses, many using characters described by Minckley (1973) and Smith et al. (1979). Using seven qualitative morphological characters (mouth, jaw length, nuchal [predorsal] hump, skull depression, caudal peduncle, nuchal and breast scale development) of *G. cypha* and *G. robusta* samples from the Yampa River, Douglas et al. (1989) used Principal Components Analysis (PCA) to successfully discriminate among the two species (i.e., successfully sorting the species into their proper groups). Morphological measurements (e.g. nuchal depression and caudal peduncle depth) and meristic characters (number of dorsal and anal fin rays) analyzed by PCA discriminated between spawning *G. cypha* and *G. robusta* in Black Rocks (Kaeding et al. 1990). Canonical variates analysis of morphometric landmarks effectively discriminated between Upper Basin *G. cypha* and *G. robusta* and found population differences within each species (McElroy and Douglas 1995). Meristic and morphometric characters of museum specimens from all three species analyzed in a Discriminant Function Analysis successfully discriminated among all species (Douglas et al. 1998). Most recently, geometric morphometrics was shown to be successful in distinguishing between *G. cypha* and *G. robusta* and showed population differences within *G. cypha* (Douglas et al. 2001). Despite the clear differences found through statistical analysis, however, quick identification in the field without the benefit of laboratory methods and statistics was found to be difficult in all of the above studies.

Widespread hybridization of *G. cypha* and *G. robusta* may pose a threat to maintaining the genetic integrity of *G. cypha*. Therefore Douglas and Douglas (2007) analyzed genetic diversity and hybridization of *G. cypha* and *G. robusta* in the Upper Basin using microsatellite markers and mtDNA sequence data. They found that *G. robusta* and *G. cypha* shared mtDNA haplotypes while both were easily separated from *G. elegans*. Based on this evidence, they concluded that *G. cypha* and *G. robusta* underwent population reductions and a wide-scale hybridization event at the end of the Pleistocene, causing a mixture of *G. robusta* and *G. cypha* mtDNA haplotypes. Like Gerber et al. (2001), Douglas and Douglas (2007) found *G. elegans* haplotypes in *G. cypha* (Marble, Grand and Desolation canyons) and a single *G. robusta* (Desolation Canyon). Analysis of nuclear microsatellite markers was also unable to distinguish among Upper Basin *G. cypha* and *G. robusta* and was insufficient for the identification of Upper Basin *G. cypha* population structure; nevertheless, Douglas and Douglas (2007) defined six management units among the three species: *G. elegans*; Grand Canyon *G. cypha*; Desolation Canyon (including *G. cypha* and *G. robusta*); Upper Basin *G. cypha* outside of Desolation Canyon; Yampa River *G. robusta*; and Upper Basin *G. robusta* outside of Desolation Canyon and Yampa River.

The management units identified by Douglas and Douglas (2007) are problematic in some respects. The inability to distinguish *G. cypha* from *G. robusta* in Desolation Canyon prevents genetic monitoring of each species in that area. The lumping of Upper Basin *G. robusta* outside of Desolation Canyon and Yampa River is problematic when this species is found upstream of these sites in the Green River drainage and in the Upper Colorado River drainage, where individuals within the same management unit would have to pass through Desolation Canyon to connect these areas. *Gila cypha* has also been found upstream of Desolation Canyon and in the Upper Colorado River at Black Rocks, suggesting that more population structure may exist within the species. Therefore, further work is needed to determine how best to effectively manage *G. cypha* in the Upper Basin.

The Southwestern ARRC considered the following questions concerning Upper Basin *G. cypha*:

a) What is the current state of genetic diversity of Upper Basin populations (primarily Black Rocks and Desolation Canyon) that needs to be maintained?

b) Should Upper and Lower Basin stocks be kept separate or merged?

c) Should Black Rocks and Desolation Canyon stocks be kept separate or merged?

d) If Upper and Lower Basin stocks are kept separate, should the Upper Basin stock be supplemented with Lower Basin diversity?

e) Estimate the effective population size for Upper Basin populations.

f) Using our common loci, has there been a loss of allelic diversity within particular populations or in the Upper Basin as a whole since the Douglas and Douglas (2007) study?

g) Are there private (unique) alleles in populations/locations that should be considered when developing broodstock which the Program should attempt to collect from the wild? Would these private alleles suggest keeping individual populations separate or would we incorporate those into a smaller number of broodstocks?

MATERIALS AND METHODS

Tissues

Tissue samples were collected by several partners and submitted to Southwestern ARRC for genotyping (Table 1). Collection locations are marked on the map in Figure 1. Tissues were preserved in 95% ethanol and archived at -80°C.

Extraction, PCR, and Genotyping

Genomic DNA was extracted using DNeasy® Blood and Tissue Kits (Qiagen®) following the manufacturer's protocol, after which samples were archived at -80 °C. Eighteen microsatellite loci were amplified via polymerase chain reaction (PCR) using forward primers (Applied Biosystems®) labeled with one of four fluorescent dyes (Appendix I). Amplification was carried out in 10 µL reactions consisting of 1 µL template DNA, 0.8X Multiplex PCR Kit (Qiagen®), and up to 200 nM each of forward and reverse primers using a GeneAmp® 9700 or ProFlex™ PCR System (Applied Biosystems®). Touchdown cycling (annealing temperature decreased by 0.2°C per cycle) consisted of an initial denaturing step at 95°C for 15 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 60 s, and extension at 72°C for 60 s, with a final extension at 72°C for 30 min.

Amplified microsatellite loci were visualized on an automated 3130xl genetic analyzer (Applied Biosystems®) with GeneScan™ LIZ 500 size standard, and loci were genotyped with GeneMapper® Software 5 (Applied Biosystems®). Accuracy in genotyping was ensured by a Quality Control/Quality Assurance (QA/QC) process consisting of secondary scoring of all genotypes by a second researcher, and independently re-extracting, amplifying, and scoring 10% of the samples. Approximately 0.5% of allele calls differed between the initial and re-evaluated samples.

Haplotype Sequencing

One mtDNA locus, *NADH dehydrogenase 2* (*ND2*), was amplified using the AA_{Gila}-L (5'-TATCCCCCTAATGGCACA G-3') and E_{Gila}-H (5'-TTCTGCTTAGAGCTTTGAAGGC-3') primers from Gerber et al. (2001). A 954 base-pair (bp) fragment (from which 888bp were used) was amplified in a 20 µL reaction

containing 0.6X Multiplex PCR Kit (Qiagen®), 50 nM each primer, and 2 µL of template DNA. Thermal cycling conditions included an initial 15 min denaturing step at 95°C followed by 35 cycles of 15 s at 95°C, a 15 s annealing phase with an initial annealing temperature of 50°C that increased by 0.4°C each cycle, and 30 s at 72°C, followed by a final extension phase of 7 min at 72°C. Amplified PCR product was cleaned for sequencing with 0.42 $u \cdot \mu L^{-1}$ exonuclease I (Thermo Fisher Scientific) and 0.04 $u \cdot \mu L^{-1}$ shrimp-alkaline-phosphatase (SAP) (Affymetrix) in 1X exonuclease I reaction buffer (Thermo Fisher Scientific). The reaction was incubated at 37°C for 30 min followed by denaturation at 65°C for 15 min. Each 10 µL sequencing reaction consisted of 5 µL cleaned PCR product, 1X BigDye® Terminator Sequencing Buffer (Applied Biosystems®), 0.1X BigDye® v3.1 Terminator Reaction Mix (Applied Biosystems®), and 100 nM primer. Sequencing thermal cycler conditions consisted of an initial 1 min 95°C denaturing step followed by 25 cycles of 30 s at 95°C, 20 s at 50°C, and 4 min at 60°C. Sequencing product was ethanol precipitated in 95% ethanol (Sigma-Aldrich) with 30 mM Ethylenediaminetetraacetic acid (EDTA) and 5 mM sodium acetate and eluted in 10 µL highly deionized (Hi-Di™) formamide (Applied Biosystems®). After 3 min incubation at 95°C, the product was sequenced on a 3130xl genetic analyzer (Applied Biosystems®) and sequences were edited in Sequencher v5.1 (Gene Codes).

Microsatellite Analysis

Micro-Checker 2.2.3 (van Oosterhout et al. 2004) was used to check allele scoring for potential genotyping errors, null alleles, and large allele drop-out. Micro-Checker tests for null alleles by testing for homozygote excess. Genepop v4.7 (Raymond and Rousset 1995, Rousset 2008) was used to test for departures from Hardy-Weinberg (HW) equilibrium and for linkage disequilibrium in each population. Alpha (0.05) was adjusted for multiple comparisons in all Genepop tests using a Bonferroni correction (Rice 1989).

The frequency function in GenAlEx (Peakall and Smouse 2006) was used to calculate expected heterozygosity (H_E), observed heterozygosity (H_O), and inbreeding coefficients (F_{IS}) and to identify private alleles in each sample, for each site, and for each species. Arlequin v3.5 (Excoffier and Lischer 2010) was used to calculate Weir and Cockerham's (1984) pairwise F_{ST} estimates of divergence among populations. Rarefaction (HP-Rare: Kalinowski 2004; 2005) was utilized to control for disparate sample sizes across groups when calculating allelic richness (A_R). Due to the difficulty in obtaining large sample sizes for some site-year combinations, A_R values were standardized based upon 16 alleles. This number was selected to allow inclusion of Upper Basin sites with low sample sizes (N=8 samples; Desolation Canyon 2012). Genetic diversity measures were compared across species, sites, and years for all markers. Estimates from this study were compared to those from Douglas and Douglas (2007) for five loci common to both studies.

The effective population size (N_E) was estimated for each sampling location (single sample estimate) and year in NeEstimator v2.1 (Do et al. 2014) using the linkage disequilibrium method (Waples and Do 2008) with a modified correction for missing data. The frequency at which rare alleles were excluded from the analysis (P_{CRIT}) followed recommendations made by Waples and Do (2010). For sample groups with N>25, P_{CRIT}=0.02 was used. For all other sites, P_{CRIT} was based on 1/2N where N was the number of individuals genotyped for that sample. The 95% jackknife confidence intervals (Jones et al. 2016) were reported.

The Bayesian clustering method of Structure v2.3.4 (Pritchard et al. 2000, Hubisz et al. 2009) was used to investigate the number of genetic clusters (K). The admixture model that assumes gene flow among populations and allows for correlated allele frequencies across populations was applied. Twenty

replicates were performed for each K with K ranging from 1 to 20. All runs had a burn-in of 250,000 iterations followed by 500,000 iterations of data collection. StructureHarvester v0.6.94 (Earl & vonHoldt 2012) facilitated determination of optimal K values through ΔK calculations. The run with the highest log-likelihood for each K was also identified. Structure results were visualized using Distruct (Rosenberg 2004).

Another Bayesian clustering program, NewHybrids v1.1 beta 3 (Anderson and Thompson 2002), was used to assess the hybrid status of *G. cypha* and *G. robusta* samples. The "z" option was utilized to assign *G. cypha* from the Little Colorado River and *G. robusta* from Muddy Creek samples as "pure" populations. These decisions were made from Structure results, following methods used for sturgeon species in the Missouri River basin (Schrey et al. 2011; Eichelberger et al. 2014). Muddy Creek (Little Snake River tributary) was also selected to represent *G. robusta* because it is not considered a "population center" of *G. cypha* despite its occasional presence in this system (Wick et al. 1991; U.S. Fish & Wildlife Service 2017). NewHybrids tested each sample for classification in six categories: Pure *G. cypha*, Pure *G. robusta*, first generation (F1) *G. cypha* x *G. robusta* hybrid, second generation (F2) *G. cypha* x *G. robusta* hybrid, an F1 backcross with pure *G. cypha*, or an F1 backcross with pure *G. robusta*. The program was run for 100,000 generations of burn-in followed by 1,000,000 generations of data collection.

A Discriminant Analysis of Principal Components (DAPC) (Jombart et al. 2010) was performed using the R (R Core Team 2017) package adegenet (Jombart and Ahmed 2011) to visualize the genetic differences among locations and years. The number of principal component axes to include was determined through cross-validation, and all discriminant axes were included in the analysis.

Mitochondrial Haplotype Analysis

Sequences were aligned in Sequencher® v5.1 (GeneCodes). The number of segregating sites (S), nucleotide diversity (π), Tajima's D (Tajima 1989), Fu's F_S test (Fu 1997), and pair-wise comparisons of sequence divergence (F_{ST}) were calculated in Arlequin (Excoffier and Lischer 2010) using an 888 bp region of *ND2*. Statistical parsimony haplotype networks (Templeton et al. 1992; Clement et al. 2000) were produced in Popart using the TCS network option (Leigh and Bryant 2015) to show the haplotype relationships and composition of each population. Sites with >5% missing data were excluded and only the sequences without missing data for the remaining polymorphic sites were included in the haplotype network. *Gila cypha* and *G. robusta* samples from the entire range of the study (N=660), *G. elegans* (N=21), *G. seminuda* (N=16), and *G. robusta jordani* (N=14) were included. A second haplotype network was also included to determine if haplotype differences were present among the Yampa Canyon individuals that were visually identified as either *G. cypha* or *G. robusta*. Haplotype diversity was compared across species and populations. Haplotype richness was calculated using rarefaction in PAST v3 (Hammer et al. 2001). Estimates were standardized to a sample size of 14 to control for disparate sample sizes and allow for comparison of site-year combinations with low sample numbers.

RESULTS AND DISCUSSION

Microsatellite Analysis

Micro-Checker found evidence of null alleles in six loci, but only *Gbig34* showed consistent evidence of null alleles in multiple populations and across multiple years (Black Rocks, Cataract Canyon, Desolation Canyon, and Westwater Canyon). Therefore *Gbig34* was excluded from the Structure analysis, genetic divergence (F_{ST}) comparisons, and N_E estimates. *Gbig87* showed evidence of null alleles

in Little Colorado, Muddy Creek, and two of the five Desolation Canyon samples (2006 and 2015). *Ca12* showed evidence of null alleles in Chevelon Creek and the 2010 Desolation Canyon sample. Three loci showed evidence of null alleles in only one sample (*Cypg26* in the Yampa Canyon individuals identified as *G. cypha*, *Gel227* in the 2010 Desolation Canyon sample, and *Pluc41* in Little Colorado). These findings may be the result of running multiple comparisons in Micro-Checker without being able to adjust for the number of comparisons; therefore only *Gbig34* was excluded from analyses.

None of the microsatellite loci consistently deviated from Hardy-Weinberg equilibrium across sample years and localities in Genepop following Bonferroni correction. Appendix II contains p-values for the test of heterozygote excess, and Appendix III exhibits p-values for the heterozygote deficiency test. *Pluc11* significantly deviated from Hardy-Weinberg equilibrium in the 2012 Black Rocks sample, but not in the 2013 or 2014 samples. Given potential mixture among *G. cypha* and *G. robusta*, and the field misidentifications that can occur as a result, it is possible that this results from a Wahlund effect (i.e., pooling of multiple distinct populations: Waples 2015). Some loci significantly deviated from linkage equilibrium in some samples: *Ca12* with *Pluc22* in Little Colorado and with *Gbig294* in Desolation Canyon in 2015; *Gbig294* with *Pluc22* in Desolation Canyon (2006, 2014, and 2015), Westwater Canyon, and in *G. elegans*; *Gel228* with *Pluc22* in Westwater Canyon; *Pluc11* with *Pluc19* in Desolation Canyon in 2014; *Cypg3* with *Pluc11* in Desolation Canyon in 2006; *Cypg26* with *Pluc41* in Muddy Creek; *Gbig34* with *Gel226* in *G. elegans*; and *Gel227* with *Gbig294* and *Pluc22* in *G. elegans*. However, no loci consistently showed linkage across high numbers of sample years and localities.

Genetic diversity estimates for all sample locations and years are reported in Table 2 for *G. cypha* and in Table 3 for *G. robusta* and *G. elegans*. Locus-specific results are available in Appendix IV (*G. cypha*) and Appendix V (*G. robusta*; *G. elegans*). For *G. cypha*, Little Colorado had the lowest allelic richness. The 2015 Cataract Canyon sample had the lowest heterozygosity, but 2013 sample heterozygosity was comparable to that of *G. cypha* at other sites. Lower Basin *G. robusta* had much lower allelic richness and heterozygosity than the Upper Basin sites. Overall, *G. cypha* (A_R = 8.31, H_O = 0.82, H_E = 0.81) had higher genetic diversity than *G. robusta* (A_R = 7.19, H_O = 0.76, H_E = 0.74). The difference in diversity estimates narrowed when Lower Basin sites were excluded from estimates (*G. cypha*: A_R = 8.44, H_O = 0.82, H_E = 0.81; *G. robusta*: A_R = 7.79, H_O = 0.79, H_E = 0.77). None of the fixation indices showed signs of inbreeding (*G. cypha* F_{IS}=-0.01; *G. robusta* F_{IS}=-0.04).

Five microsatellite markers used in this study were also used in the Douglas and Douglas (2007) report (Table 4). Expected heterozygosity (H_E) was used to compare genetic diversity across studies since sample sizes were variable. Heterozygosity estimates remained the same or increased slightly for all sampling locations relative to Douglas and Douglas (2007), with the exception of Black Rocks which saw a small decrease (2007 H_E=0.82; 2014 H_E=0.77). Inbreeding coefficient estimates were comparable for the two studies (Douglas & Douglas F_{IS}=-0.09 to 0.05; Bohn et al. F_{IS}=-0.15 to 0.08).

Gila cypha had over three times as many private alleles as *G. robusta* (Table 5). However, only a portion of the total *G. robusta* range was sampled, and approximately one-third as many *G. robusta* were collected for this study relative to *G. cypha*. Many of the alleles occurring at low frequency (<0.1) may only appear to be private due to disparate sample sizes. For example, Desolation Canyon had the most private alleles for *G. cypha* (N=29), which is unsurprising given it had the largest sample size of all localities, meaning rare alleles that occur at low frequency in all populations were more likely to be detected. Yampa Canyon tied with Black Rocks for the most private alleles in *G. robusta* (N=9). Chevelon Creek had private alleles occurring at high frequency (≤ 0.56), which is unsurprising given the natural (Grand Falls) and anthropogenic (Glen Canyon Dam) barriers that prevent gene flow between this Little

Colorado tributary and the Upper Basin. Yampa Canyon chubs visually identified as *G. cypha* did not have any private alleles compared to Yampa Canyon *G. robusta*. Only the Whirlpool Canyon *G. robusta* and the Yampa Canyon *G. cypha* lacked private alleles, however sample sizes for these localities were low (N=6 and N=24, respectively) so there was reduced potential to capture rare, low frequency alleles.

Population Structure

All within-site pairwise comparisons in Arlequin were non-significant and showed no indication of temporal variation during the ten-year sampling period of this study ($F_{ST} \leq 0.005$). Pairwise comparisons of each site are shown in Table 6. All of the *G. cypha* sites were significantly different from each other with the exception of Black Rocks and Westwater Canyon. The Yampa Canyon samples that had been visually identified as *G. cypha* showed no significant differentiation from those visually identified as *G. robusta*. The *G. robusta* from Yampa Canyon and Whirlpool Canyon were not significantly different and could be considered a single population. The Westwater Canyon *G. robusta* were not significantly different from either Black Rocks *G. robusta* or *G. cypha*. The remaining *G. robusta* sites were all significantly different from each other. Chevelon Creek *G. robusta* are as divergent from Upper Basin *G. robusta* as the Upper Basin *G. robusta* are from *G. cypha* and *G. elegans*. This difference is consistent with the hypothesis that Lower Basin *G. robusta* may be a separate subspecies from Upper Basin *G. robusta*. However, a broader range of samples and phylogenetic analysis would be required to determine if the differences are basin-wide or if Chevelon Creek is a small population that has undergone a large amount of genetic drift.

StructureHarvester revealed that K=2 (ΔK=21.37) was the favored K value according to the ΔK method (Evanno et al. 2005), however this value fails to account for the three species evaluated in this study. This result matches the trend of studies utilizing ΔK which more frequently identify K=2 as the preferred K value relative to those employing other methods (Janes et al. 2017). The next most highly favored value was K=3 (ΔK=10.69) but this result also failed to distinguish *G. elegans* from Desolation Canyon *G. cypha*. This failure to identify *G. elegans* at low K values may be attributed to imbalanced sampling (Wang 2017) since *G. elegans* accounts for just 3% of the samples used in this study. The third most favored value was K=5 (ΔK=8.46), which was the lowest K value that differentiated *G. elegans* from the other *Gila* species. Therefore, K=5 was considered the preferred result determined via ΔK, and other means of assessing K were explored to investigate the potential for hierarchical population structure. Results for K<5 are presented in Appendix VI.

The most probable Structure run for each K was selected and the log-likelihood for each run was plotted in Figure 2. This indicated structure at K=10; a result which showed population substructure within Black Rocks, Westwater Canyon, and Desolation Canyon. An increasing number of clusters were split among the *G. cypha* populations for K>10 in Black Rocks, Westwater Canyon, and Desolation Canyon, but the overall clustering of the sampled sites did not change. Given these results it is probable that further clustering exists within *G. cypha* in Black Rocks, Westwater Canyon, and Desolation Canyon, but for this analysis we report K≤10 in order to establish geographic population units for management purposes.

The outputs from K=5, K=6, and K=10 are shown in Figure 3. The output for K=5 (Figure 3a) is included to show that there are clear species differences between Upper Basin *G. cypha* and *G. robusta*, although Yampa Canyon fish visually identified as *G. cypha* clustered with *G. robusta*. The output for K=6 (Figure 3b) shows three geographic regions within *G. cypha* and two within *G. robusta*. This

demonstrates both species can be divided into three clusters: 1) Lower Basin, 2) Black Rocks/Westwater Canyon, and 3) the remainder of the Upper Basin.

The output for K=10 (Figure 3c) is presented to demonstrate potential for further clustering within *G. cypha* sample sites. Desolation Canyon *G. cypha* contains a mix of two genetic clusters, one of which is also prominent in Cataract Canyon. Black Rocks and Westwater Canyon are subdivided into three genetic clusters. Mark-recapture studies have shown strong site-fidelity in Desolation Canyon (Badame 2012) and Westwater Canyon (Jackson 2010), suggesting that spatial separation of spawning sites could be responsible for the multiple clusters. Results presented here suggest similarity of Cataract Canyon to both Desolation Canyon and Black Rocks, despite strong site-fidelity. Cataract Canyon is below the confluence of the Green and Colorado Rivers, meaning this could be a contact zone between different populations. The Upper Basin *G. robusta* clustered into two geographic units when K=10: Black Rocks and Whirlpool/Yampa Canyon/Muddy Creek. The Black Rocks *G. robusta* show evidence of mixed ancestry with both the other Upper Basin *G. robusta* cluster and Black Rocks/Westwater Canyon *G. cypha*.

Population structure was further explored through DAPC. The first 200 principal components (PCs) and 12 discriminant axes (DAs) retained in the DAPC analysis explained 92.8% of the genetic variation in the dataset (Figure 4). The first DA separated *G. elegans* from *G. cypha* and *G. robusta*. The second DA separated *G. cypha* from *G. robusta*, although these two species are not as clearly separated from each other as either is from *G. elegans*.

Gila elegans was excluded from a second DAPC analysis to better visualize differences between *G. cypha* and *G. robusta*. The first 200 PCs and 11 DAs explained 93.1% of the genetic variation (Figure 5). The first DA separated the Lower Basin from the Upper Basin, while the second DA separated *G. cypha* from *G. robusta*. The Black Rocks and Westwater Canyon populations are closest together, with the possible hybrids from Black Rocks (Gsp_BR) spreading across *G. cypha* and *G. robusta*. Possible hybrids from Westwater Canyon (Gsp_WWC) are located further from the *G. robusta* samples in the plot. Moving through the Upper Basin and away from Black Rocks/Westwater Canyon, the species diverge with the populations most geographically distant from Black Rocks/Westwater Canyon being farthest apart on the horizontal axis. The *G. cypha* from Yampa Canyon overlap the *G. robusta* from Whirlpool, Muddy Creek, and Yampa Canyon.

Hybridization

NewHybrids revealed a high degree of introgression into *G. cypha* by *G. robusta*, and vice versa (Figure 6). NewHybrids tended to categorize fish in groups that were similar to their field identifications (e.g., fish collected as *G. cypha* tended to be identified as pure *G. cypha* or *G. cypha* backcrosses), with Yampa Canyon *G. cypha* being a notable exception (Table 7). Detection of hyrbids was most prevalent for both species at Black Rocks and Westwater Canyon. Desolation Canyon exhibited very low levels of among-species mixing in comparison. For *G. cypha*, just 42/113 samples (37%) from Black Rocks were classified as pure *G. cypha*. Only 25 (22%) of these were assigned with high confidence (probability > 0.8). For Westwater Canyon, 40/135 samples (29.6%) were recovered as pure with 12 (9%) being assigned at high confidence. Desolation Canyon provides a contrasting image of these sites with 155/176 samples (88%) identified as pure, 120 (68%) of which were assigned with high confidence. An absence of *G. cypha* in Yampa Canyon was again noted, with all samples collected in the field as *G. cypha* being detected as pure *G. robusta* (11/24), F2 hybrids (1/24), or *G. robusta* backcrosses (12/24: 50%).

Gila robusta samples exhibited similar trends, with Black Rocks and Westwater Canyon again showing the greatest degree of hybridization. Nearly all *G. robusta* samples from this locality were multi-generational hybrids, with 52/81 samples (64%) being classified as *G. robusta* backcrosses. Thirty-one (38%) of these were assigned with high confidence. All seven samples collected as *G. robusta* in Westwater Canyon were assigned as hybrids, being almost evenly split among F2 hybrids (N=3), *G. cypha* backcrosses (N=2), and *G. robusta* backcrosses (N=2).

Interestingly, no fish included in this study were identified as F1 hybrids. However, the presence of F2 hybrids and backcrossed individuals implies their existence despite a lack of detection. This trend matches the results of Chafin et al. (2019) who also noted a lack of F1 hybrids. Their study utilized double digest Restriction-site Associated DNA (ddRAD) sequencing to evaluate hybridization in the same samples that were analyzed for the Douglas and Douglas (2007) report (Tyler Chafin – personal communication). The Chafin et al. study also discovered a high prevalence of hybridization at Black Rocks and Westwater Canyon. However, their results for Yampa Canyon and Desolation Canyon differed. Whereas this study found Desolation Canyon to be comprised mostly of pure *G. cypha*, the Chafin study found most of the samples from this locality to be F2 hybrids and *G. cypha* backcrosses. Additionally, their *G. cypha* Yampa Canyon samples were recovered as F2 hybrids whereas the equivalent samples from this study were mostly a mix of pure *G. robusta* and *G. robusta* backcrosses.

Years of collection for the Chafin et al. study were 2000 for Black Rocks and Westwater Canyon, 2001 for Desolation Canyon, and 1999-2001 for Yampa Canyon (Tyler Chafin – personal communication). This likely accounts for the reduced detection of *G. cypha* ancestry in our 2007 Yampa Canyon samples relative to the Chafin et al. (2019) study. The other major discrepancy concerns Desolation Canyon – samples collected there in 2001 show a much greater incidence of hybridization relative to those collected from 2006 to 2015. This may be a matter of sample size (N=23, Chafin et al. 2019 vs. N=176 for this report).

Effective Population Size

For *G. cypha*, Black Rocks had the highest effective population size (N_E=1,548) overall and Desolation Canyon (N_E=492) had the lowest (Table 8). An infinite population size with a wide confidence interval (N_E=44 to ∞) was calculated for Cataract Canyon, with this estimate based upon just 10 samples. For *G. robusta*, Yampa Canyon had a higher estimated N_E than the other sites (N_E=1,467) and Chevelon Creek had the lowest (N_E=110). An infinite population size was calculated for Black Rocks, however the low end of its 95% confidence interval was N_E=630. The sum of Upper basin *G. cypha* census size estimates (3,691) is slightly larger, but similar to the sum of harmonic mean values for our study (2,842). However, census sizes for individual sample sites (Desolation Canyon = 1,672; Westwater Canyon = 1,315; Black Rocks = 404; Cataract Canyon = 300; Yampa Canyon = assumed extirpated: U.S. Fish and Wildlife Service 2018) differ substantially from these N_E estimates.

Age classes of *G. cypha* samples (Yackulic et al. 2014) are presented in Table 9. Nearly all *G. cypha* captured for this study were adults, with only a few sub-adults or juveniles recorded across all sites (Age class ≤3; N=10). Size data were not available for two sites (Black Rocks 2012; Desolation canyon 2010) however these fish were known to have been PIT tagged and are assumed to have similar size distributions to the other collection years at their respective sites. Size data for *G. robusta* was only available for Yampa Canyon (2010, 2012) and Muddy Creek (2006). All fish from Yampa Canyon were adults (>200mm), ranging from 204mm to 410mm in length (Mean = 343mm). However the size range of fish at Muddy Creek varied between 82 and 296mm (Mean = 153). Since these fish are known to

mature in small tributaries at 150-200mm in length, this collection site likely contained the greatest diversity in age classes among both species investigated (Joseph et al. 1977).

Based on N_E estimates, most populations superficially appear unlikely to experience genetic diversity loss in the short-term. This is based upon assumptions of the 50/500 rule (Franklin 1980). This rule is frequently invoked to provide context for N_E estimates by providing a guideline to assess short- and long-term viability of a population. In other words, a minimum population size of 50 is required to minimize inbreeding, and 500 to combat loss of alleles due to genetic drift over longer timescales. Inbreeding should be minimized under these assumptions at all sites, but potential for loss of alleles due to drift remains (Desolation Canyon *G. cypha*; Chevelon Creek *G. robusta*; Muddy Creek *G. robusta*).

However, it is important to consider additional factors in the interpretation of N_E estimates and invocation of the 50/500 rule. For example, recent discussion has criticized applicability of this rule to complex systems, and suggested the need for revised rules with greater minimum N_E thresholds (Jamieson & Allendorf 2012; 2013; Frankham et al. 2013). Multiple factors can also bias N_E estimates. For example, age structure can deflate N_E estimates (i.e., actual effective size may be larger than reported here). Age class data (Table 9) indicate samples are comprised of adults of unknown age. Given that skipped spawning has been observed in the Lower Basin (Pearson et al. 2015), we cannot rule out the possibility that N_E estimates are biased as a result of combining different cohorts that rarely interbreed with one another (Waples et al. 2014). In contrast, hybridization may have an opposing influence on N_E estimates. For example, Peters et al. (2014) found higher than expected N_E for mottled ducks, which was attributed to hybridization with mallards. Populations examined here with the highest N_E are coincidentally those with the greatest incidence of observed hybridization for both *G. robusta* and *G. cypha* (Black Rocks, Westwater Canyon), thus raising the issue that hybridization between the two species may be inflating N_E estimates at these sites.

Mitochondrial Haplotype Analysis

Sequence diversity was calculated for each species and sampling site from 711 sequences of an 888 bp region of *ND2* (Table 10). For *G. cypha*, Cataract Canyon had the highest nucleotide diversity and the Yampa Canyon individuals visually identified as *G. cypha* had the lowest nucleotide diversity. *Gila robusta* had consistently lower nucleotide diversity than *G. cypha* and had the lowest nucleotide diversity of the four species. A single haplotype was found in *G. robusta jordani*, but only 14 individuals were sequenced.

Both *G. cypha* and *G. robusta* had signficantly negative values for Tajima's D, which would be expected when multiple populations have been combined (Hartl and Clark 2007). The Little Colorado, Westwater Canyon, and Desolation Canyon populations of *G. cypha* and the Black Rocks population of *G. robusta* also had a signficantly negative Tajima's D, which could indicate recent bottleneck events (Tajima 1989), admixture from multiple populations, or selection againt deleterious haplotypes (Hartl and Clark 2007). Only the combined *G. robusta* and Black Rocks *G. robusta* samples had significantly negative Fu's (1997) F_S, which could indicate population expansion. While this test may be confounded by the combination of multiple populations into one sample, it is worth noting that Fu's F_S was negative or close to zero in most *G. robusta* populations but positive in the *G. cypha* populations (except for Yampa Canyon, which genotypes as *G. robusta*). Chevelon Creek and Whirlpool Canyon , for which only 2 and 8 samples were successfully sequenced, are the exceptions to this pattern. This trend could indicate that Upper Basin *G. robusta* is either expanding, stable, or showing less severe declines than *G. cypha*.

The haplotype pairwise F_{ST} comparisons (Table 11) were more variable than the microsatellite pairwise F_{ST} comparisons (Table 6). While interspecies comparisons (except for Yampa Canyon) were signficantly different with the nuclear markers, less than half of the *G. cypha*/*G. robusta* populations were significantly different for the *ND2* haplotypes. Conversely, all populations of *G. cypha* and *G. robusta* were highly differentiated from *G. elegans* (F_{ST} > 0.768). Chevelon Creek showed high differentiation from all other *G. robusta* populations (F_{ST} > 0.510), but not from the *G. cypha* populations and showed no differentiation from Little Colorado *G. cypha* (F_{ST} = 0.007).

The discrepancies between microsatellite genetic distances and mtDNA genetic distances are likely due to the inheritance mode of each DNA type, differing mutation rates, and selective pressures acting on *ND2*. *Gila robusta* and *G. cypha* populations with high mtDNA genetic distances have likely undergone long periods of isolation and/or genetic bottlenecks that have decreased the number of haplotypes persisting in those populations. Rapid mutation of microsatellites produces large numbers of alleles, allowing for the detection of population structure when alleles are shared among populations. The similarity of haplotypes in the Little Colorado River and Chevelon Creek suggests that selective pressures acting on *ND2* or other mtDNA genes differ between the Lower and Upper Basins. Although this unusual pattern could be the result of mtDNA introgression, after the complete replacement of Chevelon Creek *G. robusta* haplotypes with *G. cypha* haplotypes we would expect to see greater microsatellite similarity between Little Colorado and Chevelon Creek. Instead, Chevelon Creek is more distant from Little Colorado than it is from any of the other *G. robusta* or *G. cypha* populations (Table 6).

A statistical parsimony haplotype network of 711 sequences with no missing data for 95 polymorphic sites is shown in Figure 7. Nodes are colored to match the ten Structure clusters identified in Figure 3c. All individuals were assigned to the cluster representing the greatest proportion of their assigned ancestry. There are two groups of haplotypes in the network that are separated by at least 49 mutations. The top group consists of primarily *G. elegans* and *G. seminuda* with some *G. cypha*. The bottom group consists of only *G. robusta* (*including G. robusta jordani*) and *G. cypha*. For simplicity, we will refer to the top group as *G. elegans* haplotypes and the bottom group as *G. robusta* haplotypes. None of the *G. robusta* had *G. elegans* haplotypes while 25 *G. cypha* had *G. elegans* haplotypes. The majority of these were collected from Black Rocks (N=8) and Westwater Canyon (N=7), with a minority coming from Desolation Canyon (N=5), Cataract Canyon (N=3), and the Little Colorado River (N=2). Structure attributed a very low proportion of ancestry for each individual to *G. elegans*. The maximum proportion of ancestry (Q) attributed to *G elegans* was 0.349 with the majority (22/25 individuals) being less than 0.01. One of the *G. elegans* haplotypes was found among all *G. cypha* populations, with another being found in all Black Rocks / Westwater Canyon clusters and Desolation Canyon. None of the *G. seminuda* had *G. robusta* haplotypes.

In the *G. robusta* haplotype group, the most common haplotypes are found throughout *G. robusta* and *G. cypha*. The most common haplotype is found in all clusters of *G. cypha*, all populations of *G. robusta* except for Chevelon Creek, and differs from *G. robusta jordani* by a single mutation. Some haplotypes were only found in *G. robusta* or *G. cypha*, but all single-species haplotypes are within four mutations of one found in both species.

Based on the haplotypes identified in Popart, haplotype frequencies were calculated for each species and sampling location and compared to the Douglas and Douglas (2007) report (Table 12). Haplotype frequencies were higher in the current study for all species-sample site pairs except *G. robusta* from Westwater Canyon. These differences can be attributed to sample size. *Gila cypha* had more haplotypes and private haplotypes than *G. robusta*. Cataract Canyon had the highest haplotype

richness for *G. cypha*, followed by Westwater Canyon and Black Rocks. *Gila robusta* exhibited the greatest haplotype richness at Black Rocks. Increased hybridization in Black Rocks and Westwater Canyon (Figure 6) may be increasing haplotype richness. All *G. cypha* populations had private haplotypes that would be lost if a population became extirpated. Chevelon Creek did not have private haplotypes, which is unexpected given this site is at the perimeter of the area sampled for this study. However, the Chevelon Creek haplotype was absent from the Upper Basin.

The 2007 Yampa Canyon samples were identified in the field as *G. cypha* or *G. robusta* and submitted to the Southwestern ARRC for genetic confirmation. All individuals genotyped as *G. robusta* in the Structure analyses, but it is possible *G. cypha* introgression led to intermediate morphology. To determine if there were any differences in the mtDNA haplotypes of these individuals, a haplotype network was produced in Popart that included *G. cypha* from the nearest population, Desolation Canyon, and the 2007 sample from Yampa Canyon (Figure 8). The sharing of haplotypes between *G. cypha* and *G. robusta* makes the identification of *G. cypha* haplotypes problematic, and none of the Yampa samples identified as *G. cypha* had haplotypes private to *G. cypha*. However, two haplotypes were found in the Yampa Canyon *G. cypha* that were not present in the Yampa Canyon *G. robusta*. Conversely, all of the Yampa Canyon *G. robusta* haplotypes were shared with the Yampa Canyon *G. cypha*.

Hybridization, Conservation, and Broodstock Considerations

Based upon the microsatellite data, we can define three management units to preserve genetic diversity within *G. cypha*: 1) Black Rocks, Westwater, and Cataract Canyons, 2) Desolation Canyon, and 3) the Lower Basin. Contrary to the management units defined by Douglas and Douglas (2007), we recommend that *G. cypha* be managed separately from *G. robusta* in Desolation Canyon. Cataract Canyon is a potential contact zone between Desolation Canyon and Black Rocks/Westwater Canyon which complicates its placement. Based upon incidence of interspecific hybridization it is more appropriately placed with Black Rocks and Westwater Canyon rather than Desolation Canyon. However, it is worth noting that occasional upstream movement of *G. cypha* from Cataract Canyon to Desolation Canyon has been observed (U.S. Fish and Wildlife Service 2017).

The Structure analysis demonstrates that Black Rocks and Westwater Canyon are composed of the same three genetic clusters (K=10; Figure 3c) and grouping of these populations is corroborated by observed movement of *G. cypha* between Black Rocks and Westwater Canyon (Francis and McAda 2011; Elverud 2012). Until we can determine the mechanism creating multiple genetic clusters at these sites it is difficult to determine how those clusters should affect management decisions. If the clusters result from natal spawning site fidelity when adults move upstream to spawn, then the relevant spawning sites should be identified and steps may need to be taken to preserve spawning habitat. Spatial segregation of spawning groups is a likely explanation for these clusters as *G. cypha* show less movement during spawning than *G. robusta* (Kaeding et al. 1990) and these clusters were not observed in *G. robusta*. If the clusters are the result of temporal spawning variation, flow regimes may need to be managed to ensure a diverse range of spawners within a given spawning year. If possible, a study should be undertaken to monitor *G. cypha* spawning in the Upper Basin to determine when and where spawning occurs and tissue samples should be taken from adults as they leave spawning grounds and/or from eggs or larvae sampled from different spawning events.

We found no genetic evidence of *G. cypha* in the Yampa Canyon samples. This is consistent with observations of the decline and functional extirpation of the Yampa Canyon population (Tyus 1998; U.S.

Fish and Wildlife Service 2017). It is important to note that Douglas et al. (1989) found clear morphological differences among two groups in Yampa Canyon which they identified as *G. cypha* and *G. robusta*. However, without genetic analysis of these samples it is difficult to determine if those groupings are reflective of the morphologically disparate samples with identical genetics that were evaluated in this study.

We saw increased hybridization in Black Rocks/Westwater Canyon and found that *G. cypha* and *G. robusta* were least divergent from one another in these locations. Species divergence also increased as geographic distance from Black Rocks/Westwater Canyon increased. These results contradict the findings of Douglas and Douglas (2007) and Chafin et al. (2019) which found *G. cypha* and *G. robusta* were genetically indistinguishable in Desolation Canyon and formed separate genetic clusters in Black Rocks. McElroy and Douglas (1995) also found that *G. cypha* and *G. robusta* showed the greatest morphological similarity in Desolation Canyon and Cataract Canyon. Although *G. robusta* from Desolation and Cataract Canyons were not included in this study, we still would expect to identify contemporary hybridization with *G. cypha* in these sites as we did in Black Rocks if it is occurring. We also found Desolation Canyon to be more divergent from *G. robusta* than other localities, likely as a result of different rates of hybridization in various *G. cypha* populations. One possible explanation for the discrepancy among studies is the misclassification of Desolation Canyon *G. cypha* as *G. robusta* in Douglas and Douglas (2007) and McElroy and Douglas (1995). It is possible that the samples of *G. cypha* and *G. robusta* being analyzed truly were *G. cypha*. As in Yampa Canyon, concurrent morphological and genetic analysis of a random sample of *Gila* spp. may help clarify this issue.

Several hypotheses have been proposed to explain the shared mtDNA haplotypes of *G. cypha* and *G. robusta*. Dowling and DeMarais (1993) proposed that hybridization occurred when available habitat decreased during the Pleistocene and Holocene, causing allopatric species to occupy the same habitat. Gerber et al. (2001) hypothesized that widespread introgression was the result of *G. robusta* and *G. cypha* hybridizing as they moved into the warming Upper Basin in the Pleistocene. Douglas and Douglas (2007) proposed that hybridization occurred during late-Pleistocene bottlenecks. All hypotheses posit that hybridization occurred long after speciation and long before human intervention.

The labeling of these shared haplotypes as introgression is based on the assumption that Lower Basin populations of *G. robusta* and *G. cypha* are "pure" and provide a good source for determining which haplotypes are attributed to *G. cypha* and which are attributed to *G. robusta*. Gerber et al. (2001) based their haplotype categories on Lower Basin samples, concluding that all Upper Basin *G. robusta* had *G. cypha* haplotypes. However, we found *G. robusta* haplotypes in the Little Colorado River that were shared with *G. cypha*, indicating that the Lower Basin as a whole does not follow this trend. All Lower Basin *G. robusta* samples from Gerber et al. (2001) were collected only from the Gila River and its tributaries. Furthermore, *G robusta* has been recovered as polyphyletic using nuclear markers (Schönhuth et al. 2013). The assumption that Lower Basin *G. robusta* haplotypes, or those from Gila River fish specifically, can be used to inform timing or direction of introgression is therefore complicated by potential polyphyly.

Regardless of historic patterns of gene flow and hybridization, current Upper Basin *G. cypha* hybridize with and share haplotypes with *G. robusta* and to a lesser extent *G. elegans*. Despite varying hypotheses of how hybridization occurred, the consensus is that not all hybridization is a result of human intervention (Dowling and DeMarais 1993; Douglas and Douglas 2007; Gerber et al. 2001). It is therefore important to consider whether contemporary hybridization rates are a response to habitat modifications or other anthropogenic influences. Chafin et al. (2019) attempt to address this question,

and note an increase in hybridization in *G. robusta* samples from the San Rafael River that were collected eight years apart. They also noted a weak yet significant relationship between hybridization and water consumption, which implicates anthropogenic habitat modification in the breakdown of species barriers.

Similar changes in hybridization rates were not observed over the time encompassed by this study, but many sites analyzed herein are represented by a single point in time. The only site represented by an equivalent timeframe to San Rafael *G. robusta* collections in the Chafin et al. (2019) study is Desolation Canyon (9 years: 2006-2015). The span of time represented by other collections is too short to reasonably expect observation of accelerated hybridization rates for a fish with a generation time estimated around 8 years (U.S. Fish and Wildlife Service 2017). A multi-decadal study would instead be necessary to reliably observe changes in hybridization rates over time. Such a study could be feasible by sourcing tissue samples and extracted DNA from museum archives and genetics labs that have studied Upper Basin *Gila* populations in the past. These could be combined with new collections (i.e., 2019 and onwards) from sites analyzed in this report.

Multiple researchers have argued that natural hybridization should not prevent members of an endangered species from being protected, and those individuals with greater than 50% ancestry attributable to a protected species merit protection (Allendorf et al. 2001; Wayne et al. 2016). We do not recommend the removal of hybrids or prevention of hybridization in these species. We also see no reason to fully exclude hybrid ancestry from *G. cypha* broodstock as long as *G. robusta* and *G. elegans* alleles do not exceed their naturally occurring frequencies. However, the results of this study indicate stark differences in prevalence of hybridization among populations that should be taken into account for the design and maintenance of broodstock. Hybrid ancestry in hatchery stocks should not exceed observed frequencies for corresponding wild populations.

Despite its apparent natural occurrence, hybridization still poses a threat to these species if climate change or anthropomorphic effects reduce available spawning grounds to the point where hybridization increases above its natural rate. Monitoring available spawning habitat and managing flows could aid in mitigation of this problem.

CONCLUSIONS

a) What is the current state of genetic diversity of Upper Basin populations (primarily Black Rocks and Desolation Canyon) that needs to be maintained?

We recommend defining three management units to preserve genetic diversity within *G. cypha*: 1) Black Rocks, Westwater Canyon, and Cataract Canyon; 2) Desolation and Gray Canyons; and 3) the Lower Basin. We recommend investigating the source of the genetic clustering found within these management units. In case these clusters are the result of spawning site fidelity we recommend identifying and maintaining disparate spawning grounds within each management unit to preserve all of the available genetic diversity within the unit. Upper Basin *G. cypha* (A_R=8.44; H_O=0.82) had higher genetic diversity than Lower Basin *G. cypha* (A_R=6.78; H_O=0.78) and levels of genetic diversity were similar across both Upper Basin management units.

b) Should Upper and Lower Basin stocks be kept separate or merged?

Given the nuclear and mtDNA genetic differences between Lower Basin and Upper Basin *G. cypha*, separate Upper and Lower Basin stocks should be maintained. Structure and DAPC analyses indicated separation of the Upper and Lower Basins.

c) Should Black Rocks and Desolation Canyon stocks be kept separate or merged?

Desolation Canyon and Black Rocks represent separate population units, and differ drastically in observed incidence of hybridization between *G. cypha* and *G. robusta*. Fewer individuals from Desolation Canyon were found to have hybrid ancestry relative to Black Rocks. We therefore recommend separation of these stocks to prevent future potential introduction of *G. robusta* ancestry into the *G. cypha* population at Desolation Canyon. We also recommend that the disparate genetic clusters identified within these sites be represented in broodstock design to prevent genetic diversity loss. Sampling from multiple locations within each site and at multiple times should foster representation of these groups, and the Southwestern ARRC can provide confirmation through genetic analysis of collected broodstock.

d) If Upper and Lower Basin stocks are kept separate, should the Upper Basin stock be supplemented with Lower Basin diversity?

We find no reason to supplement Upper Basin stocks with Lower Basin genetic diversity. Genetic diversity was higher in Upper Basin *G. cypha* (A_R=8.44; H_O=0.82) than in Lower Basin *G. cypha* (A_R=6.78; H_O=0.78).

e) Estimate the effective population size for Upper Basin populations.

Effective population size estimates ranged from 492 (Desolation Canyon) to 1548 (Black Rocks) in the Upper Basin. However, the prevalence of hybridization in Black Rocks and Westwater Canyon may be inflating effective population sizes. Desolation Canyon had both the fewest individuals with hybrid ancestry, and the lowest effective population size estimates.

f) Using our common loci, has there been a loss of allelic diversity within particular populations or in the Upper Basin as a whole since the Douglas and Douglas (2007) study?

Genetic diversity has not measurably changed in *G. cypha* since Douglas and Douglas (2007) or during the sampling period of this study. Heterozygosity estimates remained the same or increased slightly for all sampling locations relative to Douglas and Douglas (2007), with the exception of Black Rocks which saw a small decrease (2007 H_E=0.82; 2014 H_E=0.77). Maintaining higher effective population sizes will prevent genetic diversity from decreasing in the future.

g) Are there private (unique) alleles in populations/locations that should be considered when developing broodstock which the Program should attempt to collect from the wild? Would these private alleles suggest keeping individual populations separate or would we incorporate those into a smaller number of broodstocks?

All of the *G. cypha* sampling locations contained private alleles and private haplotypes that should be preserved in future broodstocks. Separate populations should be maintained (i.e., Desolation Canyon vs. Black Rocks/Westwater Canyon) due to differences in hybridization rates at these sites which may influence allelic contributions from *G. robusta*.

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Table 1. Summary of collection locations and times of tissue samples used in this study. See Figure 1 for a map of sampling locations. N = number of individuals genotyped.

Species	MEL IDs	N	Sampling Location	Drainage	Year
humpback chub (*G. cypha*)		**512**			
	GcypW13LCR_001-050	50	Little Colorado River	Lower Colorado River	2013
	GcypW13CTC_001-010	10	Cataract Canyon	Upper Colorado River	2013
	GcypW15CTC_001-004	4	Cataract Canyon	Upper Colorado River	2015
	GcypW12BR_001-089	87	Black Rocks	Upper Colorado River	2012
	GcypW13BR_001-013	13	Black Rocks	Upper Colorado River	2013
	GcypOU14BR_001-013	13	Black Rocks	Upper Colorado River	2014
	GcypW16WWCa_001-63	63	Westwater Canyon	Upper Colorado River	2016
	GcypW16WWCb_001-84	72	Westwater Canyon	Upper Colorado River	2016
	GcypW09GR_001-050	48	Desolation Canyon	Green River	2006
	GcypW10DC_001-018	18	Desolation Canyon	Green River	2010
	GcypW12DC_001-008	8	Desolation Canyon	Green River	2012
	GcypW14DC_001-052	52	Desolation Canyon	Green River	2014
	GcypW15DC_001-050	50	Desolation Canyon	Green River	2015
	GcypW09YC_001-025*	24	Yampa Canyon	Yampa River	2007
roundtail chub (*G. robusta robusta*)		**179**			
	GrobW14CHEV_001-009	9	Chevelon Creek	Lower Colorado River	2014
	GrobW12BR_001-080	80	Black Rocks	Upper Colorado River	2012
	GcypW16WWCb_002-005,7,49,59	7	Westwater Canyon	Upper Colorado River	2016
	GrobW12WP_001-006	6	Whirlpool	Green River	2012
	GrobW09YC_001-018*	16	Yampa Canyon	Yampa River	2007
	GrobW10YC_001-012	12	Yampa Canyon	Yampa River	2010
	GrobW12YC_001-018	18	Yampa Canyon	Yampa River	2012
	GrobW06MC_001-048	31	Muddy Creek	Little Snake River	2006
putative hybrid chubs		**14**			
	GcypHW12BR_001-006	6	Black Rocks	Upper Colorado River	2012
	GcypHW13BR_001-002	2	Black Rocks	Upper Colorado River	2013
	GcypW16WWCa_064	1	Westwater Canyon	Upper Colorado River	2016
	GcypW16WWCb_009,010,012,022	4	Westwater Canyon	Upper Colorado River	2016
bonytail (*G. elegans*)		**22**			
	GeleDX12_001-030	22	Southwestern ARRC	Lower Colorado River	2012
	GcypW16WWCb_066	1	Westwater Canyon	Upper Colorado River	2016
Pahranagat roundtail chub (*G. robusta jordani*)		**14**			
	GrobjDXR_001-014**	14	Southwestern ARRC	Pahranagat River	2016
Virgin chub (*G. seminuda*)		**16**			
	GsemDX15R_001-016**	16	Southwestern ARRC	Virgin River	2015

*The 2007 Yampa Canyon samples were morphologically identified as either *G. cypha* or *G. robusta*, but due to ambiguous morphology tissue samples were submitted to Southwestern ARRC for confirmation.
**The *G. robusta jordani* and *G. seminuda* were sequenced for haplotype comparisons only.

Table 2. *Gila cypha* summary statistics of the eighteen microsatellite loci. N_A = number of alleles, A_R = allelic richness rarified for 16 alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, and F_{IS} = inbreeding coefficients. LCR = Little Colorado River, WWC = Westwater Canyon, and YC = Yampa Canyon.

	LCR	Cataract		Black Rocks			WWC	Desolation Canyon					YC
	2013	2013	2015	2012	2013	2014	2016	2006	2010	2012	2014	2015	2007
N_A	12.4	9.2	5.3	20.7	10.6	11.6	22.0	17.2	12.9	8.9	16.8	16.3	12.7
A_R	6.78	8.27	-	8.83	8.39	8.96	9.02	8.25	8.54	8.89	8.09	7.79	7.86
H_O	0.78	0.84	0.75	0.84	0.81	0.79	0.84	0.82	0.83	0.91	0.83	0.82	0.78
H_E	0.78	0.81	0.73	0.85	0.81	0.81	0.84	0.82	0.82	0.81	0.82	0.80	0.78
F_{IS}	0.01	-0.03	0.01	0.01	0.01	0.01	0.00	0.01	-0.02	-0.13	0.00	-0.02	0.00

Table 3. *Gila robusta* and *G. elegans* summary statistics of the eighteen microsatellite loci. N_A = number of alleles, A_R = allelic richness rarified for 16 alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, and F_{IS} = inbreeding coefficients. LCR = Chevelon Creek, BR = Black Rocks, WWC = Westwater Canyon, WP = Whirlpool Canyon, and MC = Muddy Creek.

	Gila robusta								*Gila elegans*
	LCR	BR	WWC	WP	Yampa Canyon			MC	2012
	2014	2012	2016	2012	2007	2010	2012	2006	
N_A	3.6	18.5	8.06	6.3	11.7	9.6	10.7	11.9	7.0
A_R	3.56	8.01	8.06	-	8.19	7.85	7.51	7.13	5.63
H_O	0.54	0.80	0.84	0.78	0.81	0.77	0.77	0.79	0.77
H_E	0.49	0.81	0.78	0.73	0.79	0.76	0.76	0.77	0.74
F_{IS}	-0.10	0.01	-0.09	-	-0.03	-0.02	0.00	-0.03	-0.02

Table 4. Comparison of genetic diversity measures for the five microsatellite loci used in Douglas and Douglas (2007) and in this study. Samples marked "DD07" are from the Douglas and Douglas report. All other years are from the current study. N = sample size, H_E = expected heterozygosity, and F_{IS} = inbreeding coefficients.

		Gbig34		*Gbig294*		*Ca12*		*Gbig87*		*Gel228*		Mean	
	N	H_E	F_{IS}	H_E	F_{IS}	H_E	F_{IS}	H_E	F_{IS}	H_E	F_{IS}	H_E	F_{IS}
G. cypha													
Little Colorado													
DD07	11	0.90	-0.01	0.37	-0.24	0.79	-0.04	0.78	-0.17	0.86	0.05	0.74	-0.08
2013	49	0.91	0.06	0.33	0.08	0.83	-0.03	0.83	0.13	0.87	-0.05	0.76	0.04
Black Rocks													
DD07	51	0.94	0.08	0.46	-0.03	0.87	0.00	0.93	0.16	0.92	0.04	0.82	0.05
2012	87	0.94	0.18	0.33	0.05	0.90	0.00	0.94	0.00	0.92	0.01	0.81	0.05
2013	13	0.88	0.22	0.45	0.15	0.83	-0.20	0.90	-0.02	0.90	-0.11	0.79	0.01
2014	13	0.93	0.25	0.21	-0.08	0.88	0.03	0.92	-0.08	0.91	-0.09	0.77	0.01
Desolation Canyon													
DD07	51	0.93	0.07	0.25	0.06	0.87	0.00	0.88	0.04	0.80	-0.08	0.75	0.02
2006	48	0.94	0.16	0.36	0.08	0.87	-0.03	0.88	0.16	0.84	0.01	0.78	0.08
2010	18	0.93	0.16	0.38	-0.16	0.82	0.26	0.90	0.14	0.85	-0.18	0.78	0.04
2012	8	0.90	-0.11	0.33	-0.14	0.84	-0.19	0.88	-0.13	0.85	-0.17	0.76	-0.15
2014	52	0.93	0.12	0.31	0.13	0.86	-0.03	0.86	0.08	0.82	-0.03	0.76	0.05
2015	50	0.93	0.08	0.38	-0.16	0.88	-0.07	0.81	0.14	0.82	-0.06	0.76	-0.01
Westwater Canyon													
DD07	77	0.94	0.09	0.39	-0.03	0.89	-0.04	0.94	0.08	0.90	-0.01	0.81	0.02
2016	135	0.95	0.15	0.37	0.00	0.91	0.00	0.94	0.01	0.91	0.04	0.82	0.04
Yampa River													
DD07	7	0.86	-0.17	0.25	-0.17	0.84	-0.20	0.76	0.24	0.86	-0.17	0.71	-0.09
2007	24	0.82	-0.07	0.08	-0.04	0.88	-0.08	0.93	0.01	0.91	-0.06	0.72	-0.05
G. robusta													
Black Rocks													
DD07	43	0.90	-0.01	0.18	-0.07	0.87	-0.04	0.92	0.02	0.91	0.01	0.76	-0.02
2012	80	0.91	0.15	0.21	-0.01	0.88	-0.02	0.93	-0.01	0.91	-0.05	0.77	0.01
Westwater Canyon													
DD07	21	0.90	0.02	0.16	-0.06	0.89	0.03	0.92	0.01	0.92	-0.01	0.76	0.00
2016	7	0.92	0.17	0.14	-0.08	0.90	-0.20	0.95	-0.14	0.96	-0.13	0.78	-0.08
Yampa River													
DD07	44	0.91	0.08	0.02	-0.01	0.90	-0.04	0.91	0.10	0.86	0.00	0.72	0.03
2007	16	0.89	-0.12	0.06	-0.03	0.89	-0.12	0.91	-0.03	0.88	-0.07	0.73	-0.08
2010	12	0.86	-0.06	0.00	-	0.88	0.05	0.90	-0.11	0.78	0.10	0.68	0.00
2012	18	0.85	0.03	0.05	-0.03	0.89	-0.06	0.91	-0.04	0.89	-0.13	0.72	-0.05
G. elegans													
DD07	15	0.00	-	0.72	0.26	0.68	-0.09	0.77	-0.04	0.72	-0.01	0.58	0.02
2012	22	0.79	0.13	0.70	-0.04	0.76	0.16	0.81	0.10	0.82	-0.05	0.77	0.06

Table 5. Number of private alleles observed in each species, site, and year. The maximum frequency at which private alleles were observed is reported along with the sample size for each sample. Private allele frequencies were calculated independently for each level (species, site, and year). Species totals include alleles private to the species but found in multiple samples and are not equal to the sum of private alleles from each site.

Sample	Private Alleles	Maximum Frequency	Sample Size
G. cypha	**105**	**0.05**	**377**
Little Colorado	**3**	**0.01**	**50**
Cataract Canyon	**2**	**0.14**	**14**
2013	1	0.05	10
2015	0	-	4
Black Rocks	**13**	**0.03**	**113**
2012	7	0.01	87
2013	1	0.04	13
2014	2	0.04	13
Westwater Canyon	**14**	**0.01**	**135**
Desolation Canyon	**29**	**0.06**	**176**
2006	2	0.01	48
2010	4	0.03	18
2012	0	-	8
2014	3	0.01	52
2015	6	0.01	50
Yampa Canyon	**0**	**-**	**24**
G. robusta	**31**	**0.03**	**172**
Chevelon Creek	**3**	**0.56**	**9**
Black Rocks	**9**	**0.03**	**80**
Westwater Canyon	**1**	**0.08**	**7**
Whirlpool Canyon	**0**	**-**	**6**
Yampa Canyon	**9**	**0.02**	**46**
2007	2	0.03	16
2010	3	0.04	12
2012	2	0.03	18
Muddy Creek	**4**	**0.02**	**31**
G. elegans	**10**	**0.52**	**23**

Table 6. Pairwise estimates of genetic divergence (F_{ST}) among populations. Pairwise F_{ST} values that were significant after adjusting for nominal comparisons ($p \leq 0.000641$) are in bold-faced type. Negative F_{ST} estimates were converted to zero. Pairwise comparisons involving the Whirlpool *G. robusta* population should be interpreted cautiously due to low sample size for this population (N=6). Medium gray = within *G. cypha*, light gray = between *G. cypha* and *G. robusta,* white = within *G. robusta*, and purple = with *G. elegans*.

		G. cypha					*G. robusta*						
		Cataract Canyon N=14	Black Rocks N=113	Westwater Canyon N=148	Desolation Canyon N=158	Yampa Canyon N=24	Chevelon Creek N=9	Black Rocks N=80	Westwater Canyon N=7	Whirlpool Canyon N=6	Yampa Canyon N=46	Muddy Creek N=31	*G. elegans* N=22
G. cypha	Little Colorado	**0.063**	**0.039**	**0.039**	**0.062**	**0.079**	**0.213**	**0.074**	**0.078**	**0.098**	**0.085**	**0.101**	**0.155**
	Cataract Canyon		**0.025**	**0.023**	**0.022**	**0.043**	**0.187**	**0.041**	**0.038**	**0.053**	**0.049**	**0.068**	**0.140**
	Black Rocks			0.002	**0.026**	**0.041**	**0.172**	**0.026**	0.016	**0.053**	**0.044**	**0.054**	**0.136**
	Westwater Canyon				**0.028**	**0.041**	**0.173**	**0.025**	**0.017**	**0.055**	**0.046**	**0.056**	**0.135**
	Desolation Canyon					**0.059**	**0.176**	**0.048**	**0.040**	**0.070**	**0.060**	**0.074**	**0.138**
	Yampa Canyon						**0.156**	**0.030**	**0.050**	0.006	0.000	**0.030**	**0.183**
G. robusta	Chevelon Creek							**0.168**	**0.222**	**0.152**	**0.149**	**0.180**	**0.305**
	Black Rocks								0.009	**0.035**	**0.030**	**0.041**	**0.169**
	Westwater Canyon									0.053	**0.050**	**0.064**	**0.156**
	Whirlpool Canyon										0.003	**0.037**	**0.194**
	Yampa Canyon											**0.027**	**0.178**
	Muddy Creek												**0.188**

Table 7. Classification of individuals in NewHybrids. Each individual analyzed by NewHybrids was tested to determine if it belongs to one of six categories: Pure *Gila cypha* (HBC), pure *Gila robusta* (RTC), First generation *G. cypha* by *G. robusta* hybrid (F1), Second generation *G. cypha* by *G. robusta* hybrid (F2), F1 hybrid by *G. cypha* backcross (HBC Bx), or F1 hybrid by *G. robusta* backcross (RTC Bx). Each classification for each locality is represented by two numbers. The first is the number of individuals classified at high confidence (probability > 0.8). The second is the total number of individuals for which that category was the most probable assignment.

Species	**Locality**	**HBC**	**RTC**	**F1**	**F2**	**HBC Bx**	**RTC Bx**
G. cypha	Little Colorado River	35/47	-	-	-	0/3	-
	Cataract Canyon	2/4	-	-	0/2	4/8	-
	Black Rocks	25/42	-	-	0/11	19/55	1/5
	Westwater Canyon	12/40	-	-	3/16	16/75	2/5
	Desolation Canyon	120/155	-	-	0/1	4/19	0/1
	Yampa Canyon	-	5/11	-	0/1	-	3/12
G. robusta	Chevelon Creek	-	0/2	-	1/1	-	4/6
	Black Rocks	-	2/5	-	5/19	0/5	31/52
	Westwater Canyon	-	-	-	1/3	0/2	1/2
	Whirlpool Canyon	-	0/2	-	-	-	3/5
	Yampa Canyon	-	10/19	-	-	-	15/28
	Muddy Creek	-	20/28	-	-	-	0/4
Gila sp.	Black Rocks	-	-	-	1/5	0/2	0/2
	Westwater Canyon	-	-	-	0/2	1/2	-

Table 8. Effective population size estimates for each sampling location by year. Ninety-five percent jackknife confidence intervals are reported below point estimates, which are reported in bold-faced type. Harmonic means are reported for all sites.

	Gila cypha				*Gila robusta*			
	Cataract Canyon	Black Rocks	Westwater Canyon	Desolation Canyon	Chevelon Creek	Black Rocks	Yampa Canyon	Muddy Creek
2006				**292** 151-2149	**110** 10-∞			**271** 96-∞
2007							**489** 65-∞	
2010				**∞** 261-∞			**∞** 38-∞	
2012		**516** 253-12616				**∞** 630-∞	**∞** 225-∞	
2013	**∞** 44-∞	**∞** 105-∞						
2014		**∞** ∞-∞		**458** 186-∞				
2015				**396** 184-∞				
2016			**774** 441-2660					
Mean	**∞**	**1548**	**774**	**492**	**110**	**∞**	**1467**	**271**

Table 9. Size class data for *Gila cypha* samples used in N_E calculations. Size Class 1 = Juvenile (40-100mm). Size Class 2 = Small sub-adult (100-150mm). Size Class 3 = Large sub-adult (150-200mm). Size Class 4 = Small adult (200-250mm). Size class 5 = Large adult (250mm+). No size data were available for Black Rocks (2012) or Desolation Canyon (2010), however it is known that these fish were large enough to have been PIT tagged.

		Size Class				
Site	**Year**	**1**	**2**	**3**	**4**	**5**
Cataract Canyon	2013	0	0	1	5	4
Black Rocks	2012	-	-	-	-	-
	2013	0	1	1	4	6
	2014	0	0	0	4	9
Westwater Canyon	2016	0	2	1	33	96
Deslation Canyon	2006	0	0	0	17	33
	2010	-	-	-	-	-
	2014	0	0	2	22	28
	2015	0	0	2	13	31

Table 10. Sequence diversity statistics for 888 bp of *ND2*. N = sample size, *S* = number of segregating sites, π = nucleotide diversity, D = Tajima's D, *p*(D) = p-value for Tajima's D, F_S = Fu's F_S test, *p*(F_S) = p-value for Fu's F_S.

Sample	**N**	***S***	**π**	**D**	***p*(D)**	**Fs**	***p*(Fs)**
G. cypha	491	104	0.0088	-1.42	0.038	-2.19	0.373
Little Colorado	50	82	0.0081	-2.15	0.002	6.21	0.963
Cataract Canyon	14	82	0.0320	0.46	0.728	5.58	0.984
Black Rocks	108	80	0.0114	-1.09	0.128	8.38	0.962
Westwater Canyon	129	82	0.0090	-1.50	0.034	2.07	0.768
Desolation Canyon	168	85	0.0058	-2.04	0.002	1.97	0.777
Yampa Canyon	22	4	0.0010	-0.44	0.366	-0.09	0.455
G. robusta	169	20	0.0012	-1.90	0.005	-11.28	0.000
Chevelon Creek	8	0	0.0000	0.00	1.000	-	-
Black Rocks	76	13	0.0012	-1.66	0.024	-6.49	0.004
Westwater Canyon	7	5	0.0019	-0.79	0.281	-0.43	0.287
Whirlpool Canyon	2	0	0.0000	0.00	1.000	-	-
Yampa Canyon	47	5	0.0005	-1.41	0.064	0.26	0.493
Muddy Creek	29	5	0.0015	0.19	0.611	0.15	0.556
G. elegans	21	7	0.0037	2.26	0.990	2.38	0.875
G. robusta jordani	14	0	0.0000	0.00	1.000	-	-
G. seminuda	16	12	0.0039	-0.20	0.457	-1.07	0.274

Table 11. Pairwise F_{ST} comparisons of *ND2* sequences across sites. Pairwise F_{ST} values that were significant after adjusting for nominal comparisons ($p \leq 0.00055$) are in bold-faced type. Negative F_{ST} estimates were converted to zero. Sample sizes are reported in Table 9. Pairwise comparisons involving the Whirlpool *G. robusta* population should be interpreted cautiously due to low sample size for this population (N=2). LCR = Little Colorado River. Medium gray = within *G. cypha*, light gray = between *G. cypha* and *G. robusta,* white = within *G. robusta*, and purple = with *G. elegans*.

		G. cypha						*G. robusta*							
		Cataract	Black Rocks	Westwater	Desolation	Yampa	Chevelon	Black Rocks	Westwater	Whirlpool	Yampa	Muddy	*G. elegans*	*G. r. jordani*	*G. seminuda*
	LCR	0.181	0.018	0.030	0.025	0.034	0.007	**0.075**	0.000	0.000	**0.056**	0.059	**0.918**	**0.388**	**0.915**
G. cypha	Cataract		0.116	0.166	0.288	**0.223**	0.158	**0.417**	0.074	0.000	**0.363**	**0.246**	**0.768**	**0.254**	**0.742**
G. cypha	Black Rocks			0.000	0.012	0.016	0.081	0.043	0.000	0.000	0.038	0.023	**0.876**	0.258	**0.872**
G. cypha	Westwater				0.014	0.016	0.113	0.038	0.000	0.000	0.043	0.014	**0.899**	0.289	**0.897**
G. cypha	Desolation					0.000	0.141	0.012	0.000	0.000	0.008	0.018	**0.933**	**0.408**	**0.932**
G. cypha	Yampa						**0.600**	0.012	0.027	0.000	0.056	0.035	**0.972**	**0.860**	**0.974**
G. robusta	Chevelon							**0.510**	**0.594**	1.000	**0.703**	**0.522**	**0.968**	**1.000**	**0.970**
G. robusta	Black Rocks								0.000	0.000	0.046	0.062	**0.979**	**0.788**	**0.980**
G. robusta	Westwater									0.000	0.186	0.000	**0.961**	**0.850**	**0.961**
G. robusta	Whirlpool										0.000	0.000	0.959	1.000	0.958
G. robusta	Yampa											0.148	**0.982**	**0.910**	**0.984**
G. robusta	Muddy												**0.971**	**0.782**	**0.972**
	G. elegans													**0.973**	0.105
	G. r. jordani														**0.975**

Table 12. Haplotype frequencies for each species and site. Douglas and Douglas (2007) *ND2* haplotype frequencies are also reported for comparison purposes. N = number of individuals sequenced, N_H = number of haplotypes, H_d = haplotype diversity, H_R = haplotype richness rarified for N=14, and P_H = number of private haplotypes.

	Current Study					2007 Report	
Sample	N	N_H	H_d	H_R	P_H	N	N_H
G. cypha	491	40	0.76	-	26		
Little Colorado	50	8	0.70	4.63	2	9	3
Cataract Canyon	14	8	0.89	8.00	2		
Black Rocks	108	13	0.60	5.23	2	16	4
Westwater Canyon	129	19	0.71	5.92	6	20	8
Desolation Canyon	168	15	0.41	3.83	6	21	2
Yampa Canyon	22	4	0.72	3.48	1	5	3
G. robusta	169	17	0.76	-	6		
Chevelon Creek	8	1	0.00	-	0		
Black Rocks	76	12	0.53	4.69	5	19	3
Westwater Canyon	7	4	0.71	-	0	21	9
Whirlpool Canyon	2	1	0.00	-	0		
Yampa Canyon	47	3	0.20	2.07	0	20	3
Muddy Creek	29	5	0.66	4.28	1		
G. elegans	21	5	0.73	4.33	1		
G. robusta jordani	14	1	0.00	1.00	1		
G. seminuda	16	8	0.84	7.37	6		

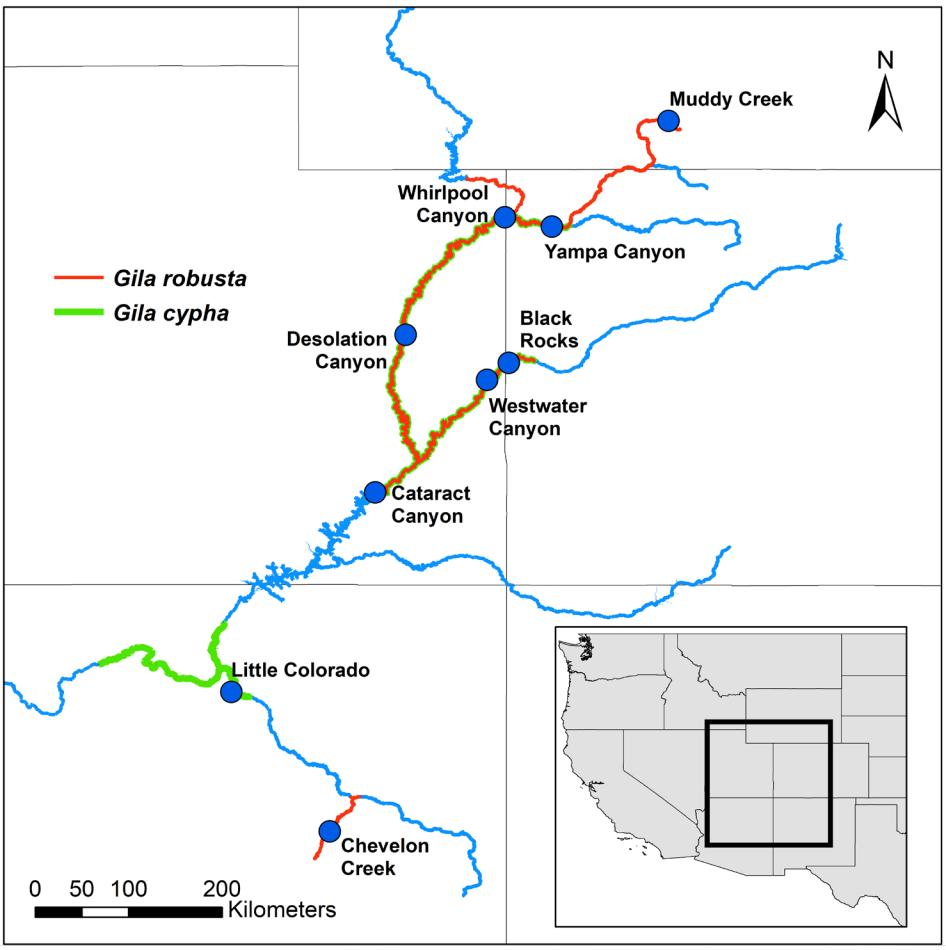

Figure 1. Extant range of humpback chub *G. cypha* (green) alongside the range of roundtail chub *G. robusta* (red) within the study area. Rivers highlighted in both shades represent overlapping species ranges where the potential for hybridization is increased. Sites where samples were collected for this study are also marked.

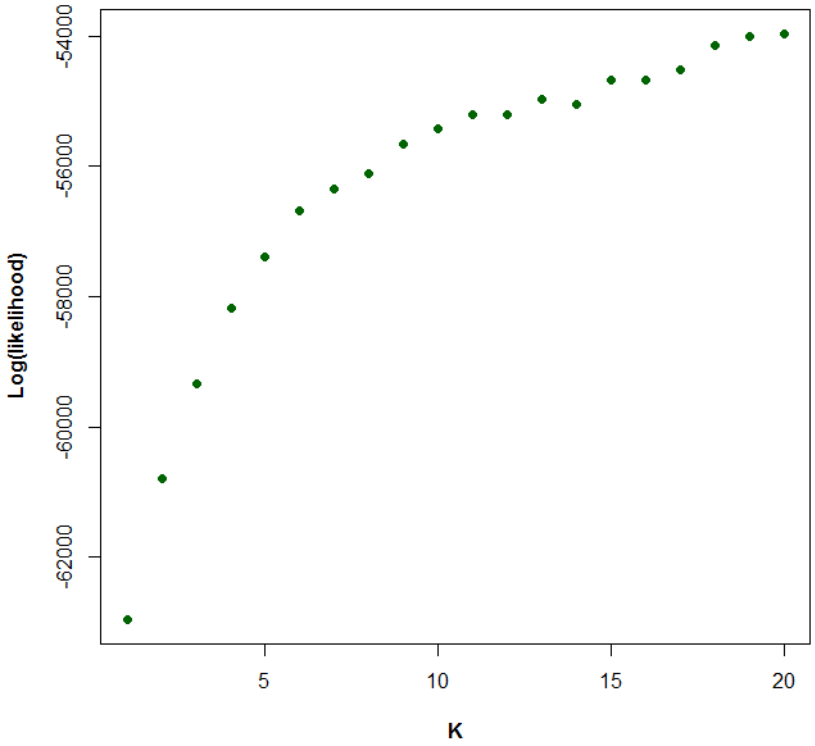

Figure 2. Graphical representation of the STRUCTURE analysis showing the log-likelihood for the highest probability run from each K.

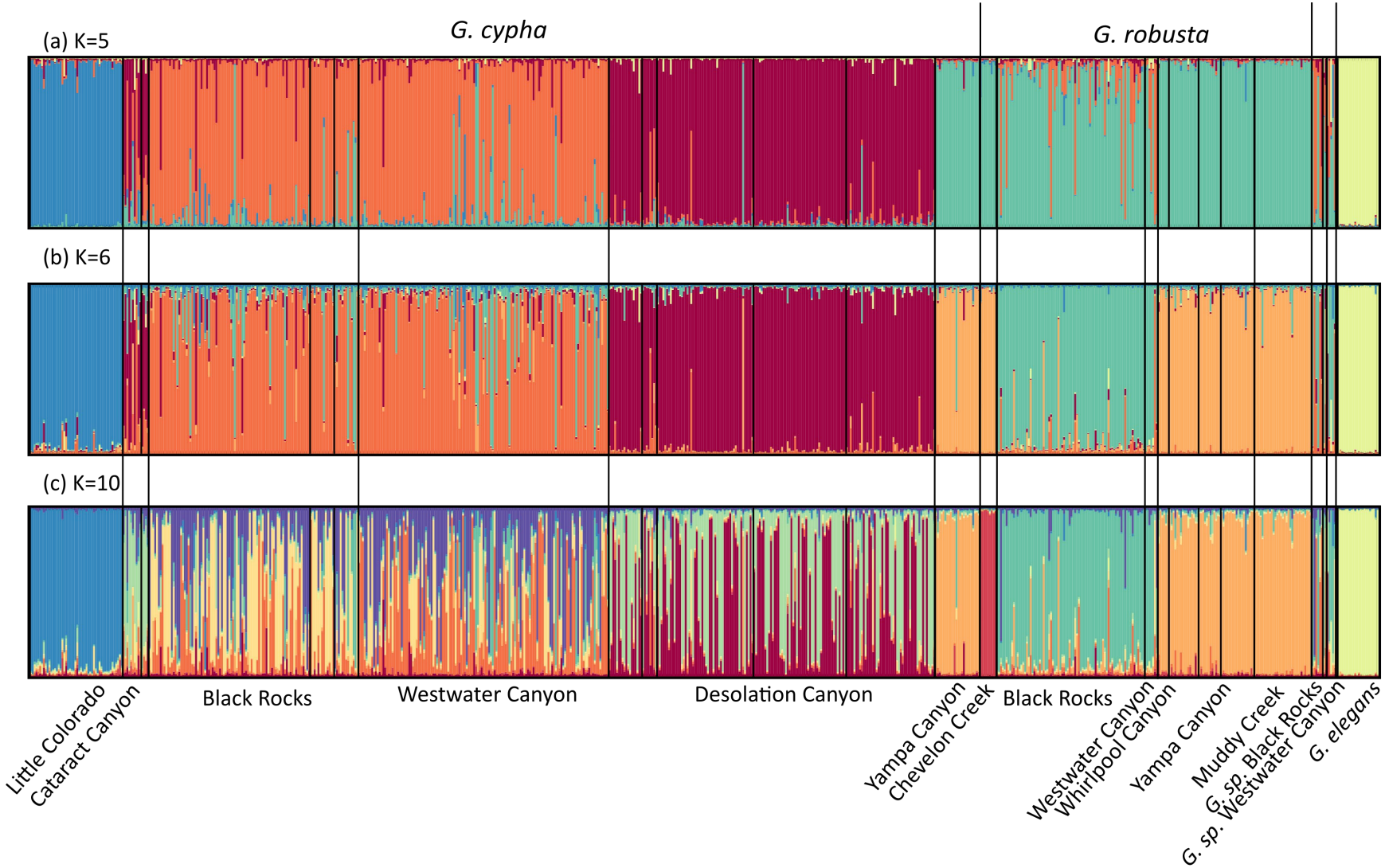

Figure 3. Graphical representation of the STRUCTURE analysis created in Distruct. Each individual fish is represented by a single vertical bar, with the proportion of color in each bar representing the estimated proportion of ancestry attributed to each cluster. Black lines within each output separate samples by year while lines that extend across outputs separate sample sites.

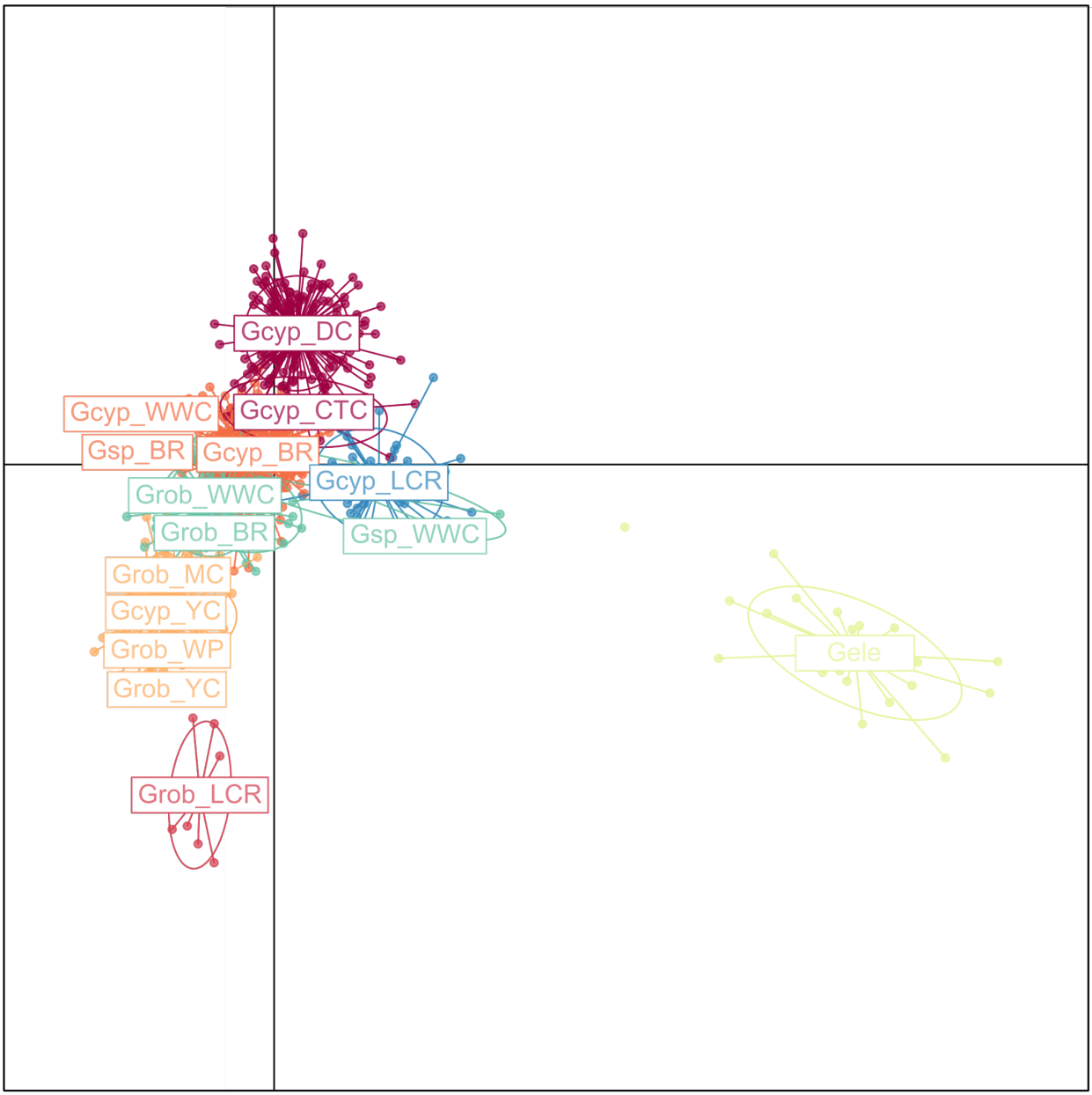

Figure 4. Discriminant Analysis of Principal Components (DAPC) scatter plot constructed using the adegenet package in R. Ellipses are the 67% inertia ellipses for each group and all lines radiate from the centroids for each group. Gcyp_LCR = Little Colorado *G. cypha*, Gcyp_CTC = Cataract Canyon *G. cypha*, Gcyp_BR = Black Rocks *G. cypha*, Gcyp_WWC = Westwater Canyon *G. cypha*, Gcyp_DC = Desolation Canyon *G. cypha*, Gcyp_YC = Yampa Canyon *G. cypha*, Grob_LCR = Chevelon Creek *G. robusta*, Grob_BR = Black Rocks *G. robusta*, Grob_WWC = Westwater Canyon *G. robusta*, Grob_WP = Whirlpool Canyon *G. robusta*, Grob_YC = Yampa Canyon *G. robusta*, Grob_MC = Muddy Creek *G. robusta*, Gsp_BR = possible hybrids from Black Rocks, Gsp_WWC = possible hybrids from Westwater Canyon, Gele = *G. elegans*.

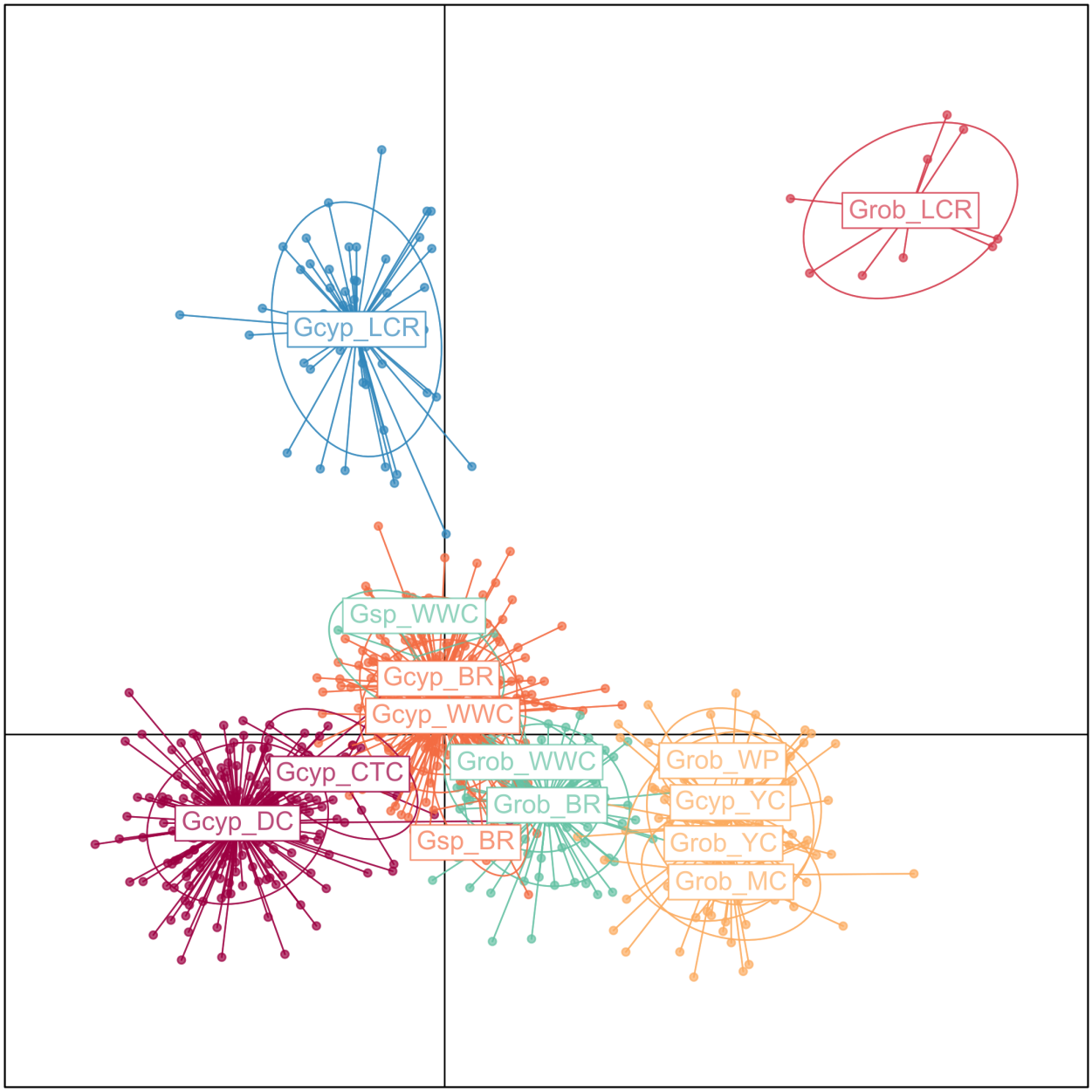

Figure 5. Discriminant Analysis of Principal Components (DAPC) results with *G. elegans* excluded from the analysis. Scatter plot constructed using the adegenet package in R. Labeled rectangles represent the centroids for each sample and ellipses are the 67% inertia ellipses for each group. Gcyp_LCR = Little Colorado *G. cypha*, Gcyp_CTC = Cataract Canyon *G. cypha*, Gcyp_BR = Black Rocks *G. cypha*, Gcyp_WWC = Westwater Canyon *G. cypha*, Gcyp_DC = Desolation Canyon *G. cypha*, Gcyp_YC = Yampa Canyon *G. cypha*, Grob_LCR = Chevelon Creek *G. robusta*, Grob_BR = Black Rocks *G. robusta*, Grob_WWC = Westwater Canyon *G. robusta*, Grob_WP = Whirlpool Canyon *G. robusta*, Grob_YC = Yampa Canyon *G. robusta*, Grob_MC = Muddy Creek *G. robusta*, Gsp_BR = possible hybrids from Black Rocks, Gsp_WWC = possible hybrids from Westwater Canyon.

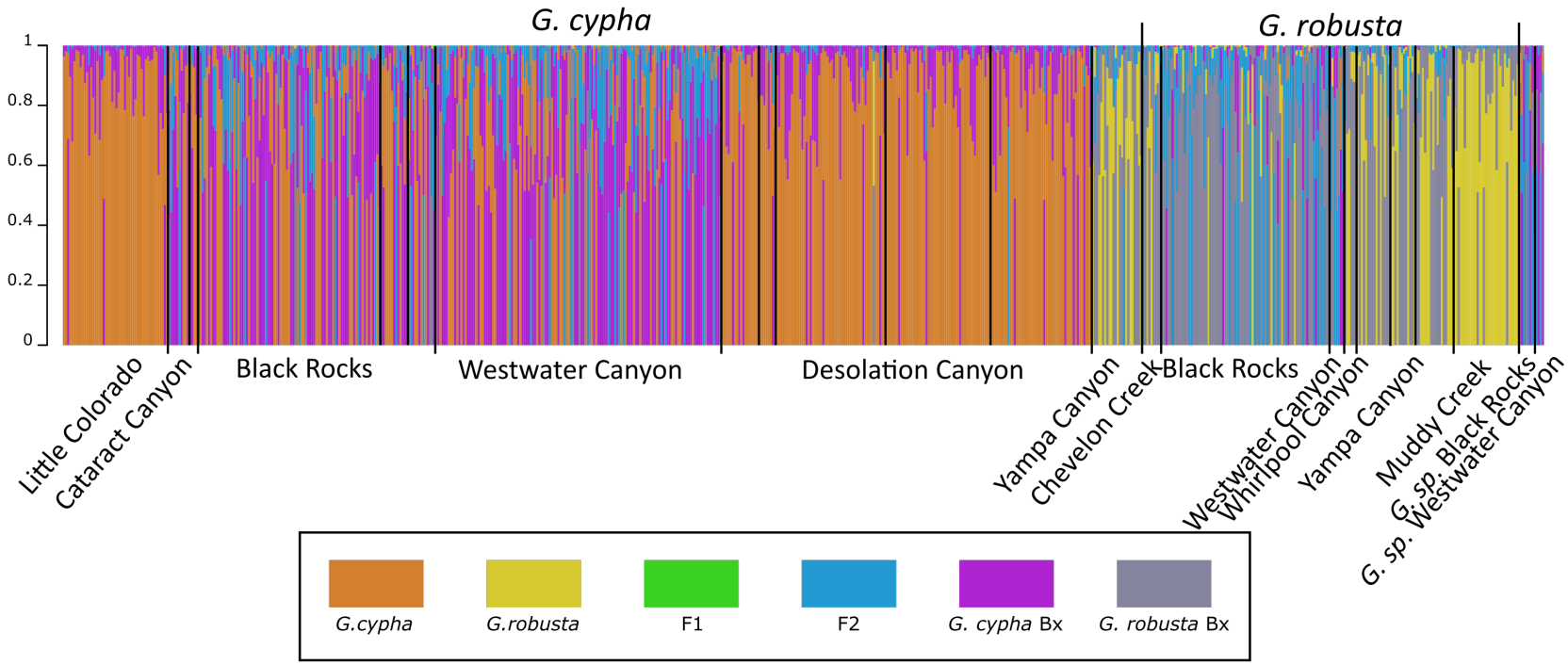

Figure 6. A barplot representing the results of NewHybrids. Each individual fish is represented by a vertical bar. The proportion of color in each bar represents the probability of a fish being assigned to one of six categories: Pure *Gila cypha* (*G. cypha*), pure *Gila robusta* (*G. robusta*), First generation *G. cypha* by *G. robusta* hybrid (F1), Second generation *G. cypha* by *G. robusta* hybrid (F2), F1 hybrid by *G. cypha* backcross (*G. cypha* Bx), or F1 hybrid by *G. robusta* backcross (*G. robusta* Bx).

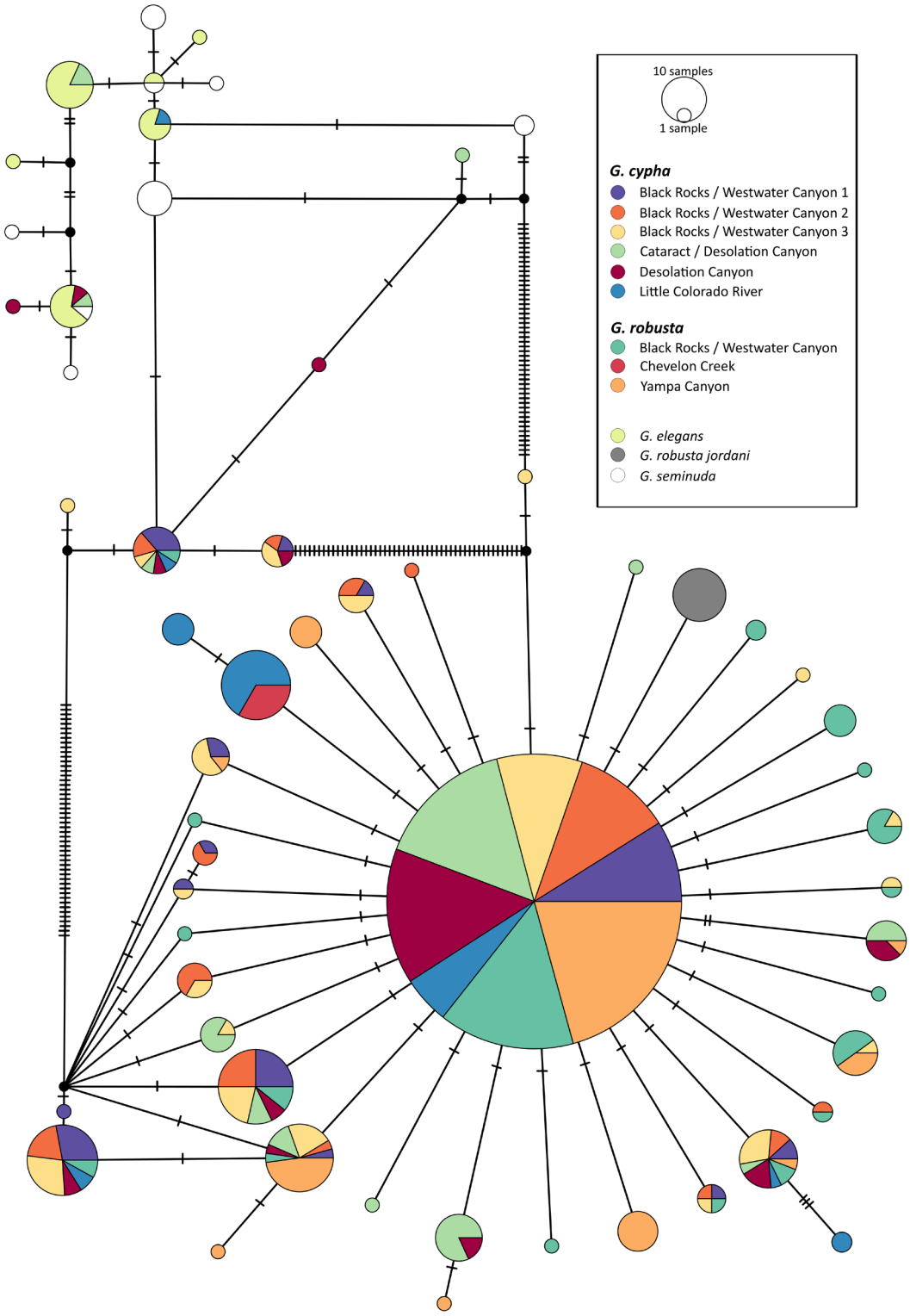

Figure 7. Statistical parsimony haplotype network of an 888 bp region of *ND2* from Popart. Colors match the individual assignments in the K=10 Structure output, shown in Figure 3c. *Gila robusta jordani* and *G. seminuda* are represented by grey and white, respectively. Segregating sites (S) = 95, parsimony informative sites = 83.

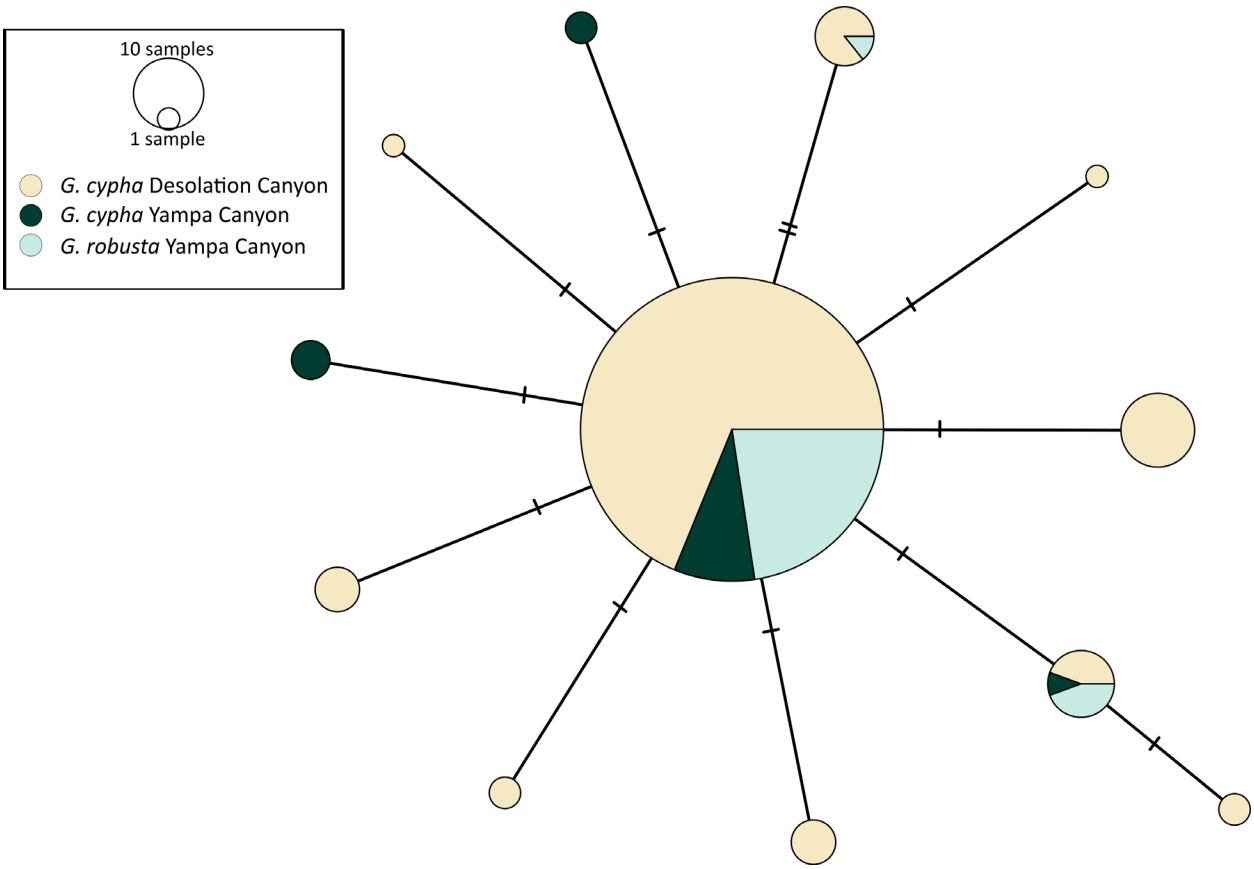

Figure 8. Haplotype network of the 2007 Yampa Canyon samples and the 2006 Desolation Canyon sample. Yampa Canyon samples are shaded to match the visual identification of each fish. Segregating sites (S) = 12, parsimony informative sites = 10.

94 Appendix I. Details of the 18 microsatellite loci used to screen *Gila* from the Upper Basin.

Panel Set	Locus	Primer Sequences	Repeat Motif	Reference
A	*Gbig34*	F: NED-GTCTCCGGGTCTCCAACTCC R: GCTCGCCCCTGTCACCA	$(GATA)_{14}$	Meredith and May (2002)
	Gel226	F: VIC-TTGCACATGAACTTACATAGAGG R: ACCGTAGATAAAAACAATACAACG	$(TAGA)_3TT(GATA)_7GATA(GACA)_6$ GATA	Keeler-Foster et al. (2004)
B	*Ca12*	F: VIC-GTGAAGCATGGCATAGCACA R: CAGGAAAGTGCCAGCATACAC	$(CA)_{16}$	Dimsoski et al. (2000)
	Gbig87	F: NED-TGTGGCTTTAAGTAAATGATGACC R: TCGGGTGTTATAGAAAATGTTCC	$(GATA)_{10}$	Meredith and May (2002)
	Gel227	F: 6FAM-TGTGAGATGGTTGTGCAAAG R: TTTTAATTGGCCACGACACA	$(CTAT)_6(CTGT)_{14}(CTTGT)_4$	Keeler-Foster et al. (2004)
C	*Cypg3*	F: 6FAM-AGTAGGTTTCCCAGCATCATTGT R: GACTGGACGCCTCTACTTTCATA	$(CAGA)_2(TAGA)_{11}$	Baerwald and May (2004)
	Gbig294	F: PET-TGTTCCCCTCATCATCATAG R: AGAACAATAGAACAATACACAGA	$(GATA)_7$	Meredith and May (2002)
	Pluc13	F: NED-GGGTGGGTGGCTAAGGTAGG R: TTCACATAAATGTGTAATCTAGAGAAGTGC	ATCT	Martin et al. (2015)
	Pluc22	F: VIC-TCATGTGCTCCTATCTGAGTGC R: TCATTCTTGAAATCTCATTGGTCC	AAAG	Martin et al. (2015)
D	*Cypg26*	F: NED-TTGGTTAGACACGATTGATGT R: TCCAAGTCTAAAGTATACAACAGTC	$(CAGA)_2(TAGA)_2TGA(CAGA)_2TAG$ $A(CAGA)_2CAAA(CAGA)_5(TAGA)_3$	Baerwald and May (2004)
	Pluc03	F: 6FAM-AGGTTCTTTCTTCTTCATCATGG R: TGAGTAGCATCATGTCTTGCG	AATAG	Martin et al. (2015)
	Pluc11	F: PET-CTTTGCGACGTCGATTTCC R: AACAATGCGGTTCTTCATGC	ATCT	Martin et al. (2015)
E	*Gel228*	F: PET-CCAGAAAATAATGCAACTCTTG R: CTGTCACAGGATCTCCAGAAG	$(TCTA)_{21}$	Keeler-Foster et al. (2004)
	Pluc19	F: 6FAM-GAGTTGTTTCATACAGGTATGCCC R: TTAGTTTCTTGCTGATGGATGG	ATCT	Martin et al. (2015)
	Pluc42	F: NED-TTGACCATTGTTACAAGCAACC R: CACTATTATACTGTGATAGACGGACGG	ATCT	Martin et al. (2015)
F	*Pluc30*	F: PET-AAATGCTGAATGTTGTAAATGAGC R: TCTGCACGGAATCCATTAGC	AAAG	Martin et al. (2015)
	Pluc41	F: 6FAM-GAATGCATTGATTCAGCTCACC R: TGCACATACTGTTGGTTTGGC	AAAG	Martin et al. (2015)
	Pluc44	F: NED-AATACAATTACGCAACAGAAGCC R: CCATTTAAAGATAAACAGCAAGTGG	AAAG	Martin et al. (2015)

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Appendix II. Heterozygote Excess. The p-value for each test of heterozygote excess is provided for each locus used in this study. Results are organized by species, sampling locality (Site) and year of collection. The null hypothesis for the heterozygote excess test assumes that no excess of heterozygous individuals exists. Dashes represent cases in which an inadequate sample was available to perform the test.

Species	Site	Year	Ca12	Cypg26	Cypg3	Gbig294	Gbig34	Gbig87	Gel226	Gel227	Gel228	Pluc03	Pluc11	Pluc13	Pluc19	Pluc22	Pluc30	Pluc41	Pluc42	Pluc44
G. cypha	Little Colorado River	2013	0.4092	0.2632	0.2878	0.7736	0.9355	0.9996	0.7781	0.1737	0.2809	0.9568	0.5627	0.7075	0.8737	0.6496	0.6061	0.9189	0.6048	0.3737
	Cataract Canyon	2013	0.5887	0.3315	0.3725	0.6227	1.0000	0.7725	0.5770	0.6635	0.9859	0.8016	0.7134	0.6132	0.9464	0.8216	0.6060	0.3280	0.5627	0.5453
	Cataract Canyon	2015	0.9117	1.0000	0.9719	1.0000	1.0000	0.8439	0.7461	0.8611	0.9328	0.8571	0.8571	0.7429	1.0000	1.0000	1.0000	0.8650	0.7394	0.8613
	Black Rocks	2012	0.6399	0.1776	0.3032	0.5138	1.0000	0.5779	0.5321	0.4705	0.6803	0.5237	1.0000	0.8019	0.0291	0.8896	0.7910	0.6768	0.4790	0.7951
	Black Rocks	2013	0.1462	0.7300	0.8624	0.9162	0.9974	0.8048	0.7413	0.7436	0.3907	0.5841	0.9581	0.7434	0.8175	0.3722	0.4614	0.9210	0.5338	0.5549
	Black Rocks	2014	0.9291	0.8621	0.9865	0.8800	1.0000	0.5911	0.9103	0.8348	0.5279	0.9531	0.9644	0.7746	0.4987	0.2205	0.9180	0.5595	0.8916	0.9730
	Westwater Canyon	2016	0.6064	0.5600	0.5353	0.8190	1.0000	0.9274	0.6287	0.1068	0.9274	0.5161	0.6471	0.8655	0.8757	0.2340	0.2684	0.5253	0.5347	0.1997
	Desolation Canyon	2006	0.8357	0.9405	0.4454	0.8978	1.0000	1.0000	0.2470	0.9969	0.5343	0.3148	0.8454	0.6950	0.0902	0.0811	0.1791	0.5867	0.2446	0.3053
	Desolation Canyon	2010	0.9755	0.6713	0.8034	0.3656	0.9952	0.9425	0.6015	0.9864	0.0778	0.5602	0.1663	0.3476	0.1797	0.3252	0.2804	0.7615	0.2280	0.5362
	Desolation Canyon	2012	0.4147	0.9519	0.6458	0.8000	0.7157	0.5918	0.6711	0.4772	0.4260	0.9289	0.3663	0.2183	0.4818	0.3862	0.1540	0.6115	0.9529	0.6515
	Desolation Canyon	2014	0.5162	0.0351	0.4827	0.9001	1.0000	0.8824	0.2417	0.6047	0.4290	0.5346	0.8281	0.7388	0.4693	0.0275	0.3967	0.7312	0.3229	0.3759
	Desolation Canyon	2015	0.0854	0.8105	0.3997	0.0484	0.9991	0.9986	0.9527	0.0243	0.1682	0.2110	0.4276	0.5624	0.7982	0.7129	0.1045	0.3225	0.9914	0.1768
	Yampa Canyon	2007	0.2372	0.9842	0.8547	0.9787	0.3126	0.8595	0.7308	0.6035	0.3243	0.5814	0.9762	0.8445	0.4572	0.8918	0.1460	0.4759	0.8475	0.8025
G. robusta	Chevelon Creek	2014	0.9741	0.3861	0.7332	-	0.7769	-	0.4018	0.7995	0.5309	0.2896	-	0.2574	0.4739	-	0.2896	0.4933	-	0.1673
	Black Rocks	2012	0.2381	0.1207	0.4701	0.6630	1.0000	0.7305	0.9544	0.8530	0.1439	0.7841	0.8179	0.5877	0.8015	0.3086	0.4922	0.7611	0.5736	0.9370
	Westwater Canyon	2016	0.4913	0.7270	0.5812	-	0.9981	0.6781	0.9992	0.9984	0.7030	0.3608	0.7883	0.5308	0.6783	0.5594	0.6684	0.4057	0.5682	0.7086
	Whirlpool	2012	0.7635	0.7549	0.6002	-	0.9106	0.4246	0.8240	0.9215	0.8330	0.8734	0.7913	0.8913	0.6513	0.6195	0.7273	0.9721	0.7273	0.5140
	Yampa Canyon	2007	0.2753	0.4364	0.9519	-	0.2523	0.5730	0.5200	0.6850	0.3526	0.9240	0.9419	0.7381	0.9327	0.4748	0.8654	0.5932	0.5566	0.6264
	Yampa Canyon	2010	0.9201	0.3853	0.7486	-	0.5973	0.4870	0.9577	0.8479	0.8674	0.4855	0.4264	0.6579	0.5885	0.7902	0.1808	0.9607	0.9046	0.9176
	Yampa Canyon	2012	0.4907	0.3966	0.9709	-	0.5630	0.6077	0.4410	0.8917	0.1932	0.5722	0.9979	0.5672	0.4127	0.7801	0.6100	0.9995	0.3454	0.4202
	Muddy Creek	2006	0.0681	0.4390	0.2268	-	0.9140	0.9992	0.4710	0.3510	0.3071	0.0499	0.7396	0.5266	0.9698	0.1787	0.7229	0.3540	0.4406	0.1428
G. sp.	Black Rocks	2012	0.9985	0.6061	0.8173	0.6646	0.9065	0.9227	0.9853	0.9712	0.4848	0.9895	0.3654	-	0.7543	0.8379	0.8325	0.8447	1.0000	0.6915
	Black Rocks	2013	-	-	1.0000	-	-	1.0000	-	1.0000	-	-	-	-	1.0000	1.0000	1.0000	-	1.0000	1.0000
	Westwater Canyon	2016	0.7063	0.9967	1.0000	0.6667	1.0000	0.9261	0.7928	0.9884	0.9782	0.6471	1.0000	0.5882	0.7877	0.3937	1.0000	0.5625	0.3019	0.5356
G. elegans	Westwater Canyon	2016	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Southwestern ARRC	2012	0.9149	0.4152	0.7522	0.5019	0.9761	0.9063	0.1421	0.0111	0.4129	0.4126	0.9154	0.6838	0.4037	0.7165	0.3988	0.0510	0.1994	0.1982

Appendix III. Heterozygote Deficiency. The p-value for each test of heterozygote deficiency is provided for each locus used in this study. Results are organized by species, sampling locality (Site) and year of collection. The null hypothesis for the heterozygote deficiency test assumes that no deficit of heterozygous individuals exists. Dashes represent cases in which an inadequate sample was available to perform the test.

Species	Site	Year	Ca12	Cypg26	Cypg3	Gbig294	Gbig34	Gbig87	Gel226	Gel227	Gel228	Pluc03	Pluc11	Pluc13	Pluc19	Pluc22	Pluc30	Pluc41	Pluc42	Pluc44
G. cypha	Little Colorado River	2013	0.6138	0.7470	0.7193	0.2991	0.0597	0.0042	0.2223	0.8424	0.7185	0.0525	0.4661	0.2978	0.1068	0.3701	0.4058	0.0877	0.3929	0.6302
	Cataract Canyon	2013	0.4945	1.0000	1.0000	0.8093	0.0004	0.4275	1.0000	0.7303	0.0324	0.2376	0.5576	0.5826	0.2210	0.2852	0.6483	0.8962	0.6212	1.0000
	Cataract Canyon	2015	0.7683	0.4348	0.2534	0.4286	1.0000	1.0000	1.0000	1.0000	0.3458	0.4286	0.4286	1.0000	0.1493	0.4286	0.0874	1.0000	1.0000	0.4890
	Black Rocks	2012	0.3668	0.8333	0.6660	0.5188	0.0000	0.4279	0.4521	0.5106	0.3226	0.4897	0.0026	0.2347	0.9844	0.1203	0.2045	0.2892	0.5431	0.2046
	Black Rocks	2013	1.0000	0.3683	0.1638	0.2213	0.0084	0.6165	0.3181	0.3216	1.0000	0.5251	0.2026	0.3572	0.2683	0.6554	0.5575	0.1098	1.0000	0.6489
	Black Rocks	2014	0.1053	0.1760	0.0312	1.0000	0.0000	1.0000	0.1568	0.2760	1.0000	0.0634	0.0425	0.3804	1.0000	0.9449	0.0989	0.5200	0.1453	0.0485
	Westwater Canyon	2016	0.3771	0.4918	0.4706	0.1900	0.0000	0.0691	0.3285	0.8782	0.0887	0.4980	0.3862	0.1593	0.2125	0.7989	0.7241	0.4779	0.4972	0.7936
	Desolation Canyon	2006	0.1928	0.0448	0.5456	0.1315	0.0002	0.0012	0.8756	0.0013	0.4647	0.6680	0.1627	0.2814	1.0000	0.9264	0.8454	0.4253	0.7665	0.6613
	Desolation Canyon	2010	0.0242	0.4183	0.2012	1.0000	0.0120	0.0771	0.6899	0.0247	1.0000	0.5048	0.9371	1.0000	1.0000	0.7607	0.8235	0.2561	0.9407	0.7487
	Desolation Canyon	2012	1.0000	0.1807	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.1605	0.9298	1.0000	1.0000	0.8020	1.0000	1.0000	0.1091	1.0000
	Desolation Canyon	2014	0.4716	0.9715	0.5287	0.1170	0.0000	0.1437	0.9208	0.3841	0.5756	0.4787	0.1668	0.3087	0.5891	0.9748	0.6122	0.2266	0.6679	0.6716
	Desolation Canyon	2015	0.9295	0.1956	0.6086	1.0000	0.0000	0.0059	0.0453	0.9870	0.8446	0.7933	0.6976	0.4089	0.2400	0.3073	0.9223	0.6854	0.0094	0.7968
	Yampa Canyon	2007	0.9280	0.0166	0.1494	1.0000	0.8666	0.2060	0.3714	0.4315	0.8738	0.4388	0.0400	0.1667	1.0000	0.1272	0.8873	0.5344	0.1557	0.2136
G. robusta	Chevelon Creek	2014	0.0317	0.8552	0.3337	-	0.3252	-	0.8933	0.3697	0.5349	1.0000	-	0.8099	1.0000	-	1.0000	0.8117	-	0.9672
	Black Rocks	2012	0.8184	0.8789	0.5207	0.6700	0.0000	0.2664	0.0513	0.1438	0.8366	0.2115	0.1871	0.4154	0.2662	0.6913	0.5304	0.2241	0.4283	0.0788
	Westwater Canyon	2016	1.0000	1.0000	1.0000	-	0.0436	1.0000	0.0200	0.0202	1.0000	0.9637	0.5539	1.0000	1.0000	1.0000	0.6430	1.0000	0.8780	1.0000
	Whirlpool	2012	0.5354	0.4353	1.0000	-	0.3313	1.0000	1.0000	0.2065	0.4936	0.2503	0.7583	0.5502	1.0000	0.7326	0.7576	0.0927	1.0000	1.0000
	Yampa Canyon	2007	1.0000	0.6901	0.0448	-	1.0000	0.6924	1.0000	0.4330	0.8316	0.0726	0.1338	0.3821	0.1846	0.5998	0.1579	0.4231	0.4686	0.5233
	Yampa Canyon	2010	0.1024	1.0000	0.4180	-	0.7322	1.0000	0.2252	0.1946	0.2402	0.5797	1.0000	0.6645	1.0000	0.3910	0.9585	0.0640	0.1316	0.2710
	Yampa Canyon	2012	0.6260	0.6418	0.0300	-	0.5049	0.7141	1.0000	0.1562	1.0000	0.4854	0.0038	0.4487	1.0000	0.2369	0.6275	0.0007	0.8074	0.6393
	Muddy Creek	2006	0.9856	0.5885	0.8898	-	0.0644	0.0021	0.6446	0.6535	0.7384	0.9591	0.2761	0.4658	0.0248	0.8229	0.2899	0.6787	0.6026	0.9643
G. sp.	Black Rocks	2012	0.0128	0.7576	1.0000	1.0000	1.0000	0.3377	0.1622	0.2409	0.5844	0.0640	1.0000	-	1.0000	1.0000	0.3819	0.2670	0.0915	1.0000
	Black Rocks	2013	-	-	1.0000	-	-	0.3333	-	1.0000	-	-	-	-	0.3333	1.0000	1.0000	-	1.0000	0.3333
	Westwater Canyon	2016	1.0000	0.0425	0.0175	1.0000	0.0179	0.3560	1.0000	0.2022	0.2104	0.6983	0.2384	1.0000	1.0000	1.0000	0.0281	1.0000	1.0000	1.0000
G. elegans	Westwater Canyon	2016	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Southwestern ARRC	2012	0.0975	0.8544	0.2632	0.5144	0.0364	0.1374	0.9427	1.0000	0.6197	0.7051	0.3438	0.3745	0.6847	0.3498	0.6149	1.0000	0.8867	0.8456

Appendix IV. *Gila cypha* summary statistics of the eighteen microsatellite loci. N = sample size, N_A = number of alleles, A_R = allelic richness rarified for 16 alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, and F_{IS} = inbreeding coefficients. LCR = Little Colorado River, WWC = Westwater Canyon, and YC = Yampa Canyon.

	LCR	Cataract		Black Rocks			WWC	Desolation Canyon					YC
	2013	2013	2015	2012	2013	2014	2016	2006	2010	2012	2014	2015	2007
Gbig34	N=49	N=9	N=4	N=87	N=13	N=13	N=131	N=47	N=18	N=8	N=51	N=49	N=24
N_A	19	12	8	31	12	18	31	27	19	12	24	24	16
A_R	9.87	11.20	-	11.25	9.52	12.52	11.62	11.40	11.67	12.00	10.81	11.01	8.29
H_O	0.86	0.67	1.00	0.77	0.69	0.69	0.81	0.79	0.78	1.00	0.82	0.86	0.88
H_E	0.91	0.90	0.88	0.94	0.88	0.93	0.95	0.94	0.93	0.90	0.93	0.93	0.82
F_{IS}	0.06	0.26	-0.14	0.18	0.22	0.25	0.15	0.16	0.16	-0.11	0.12	0.08	-0.07
Gel226	N=47	N=10	N=4	N=87	N=13	N=13	N=135	N=47	N=18	N=8	N=52	N=50	N=23
N_A	16	12	6	33	14	15	35	29	23	11	28	30	20
A_R	7.56	10.67	-	11.31	10.44	11.11	11.46	11.00	11.93	11.00	11.09	11.38	11.44
H_O	0.81	1.00	1.00	0.93	0.92	0.85	0.93	0.96	0.94	1.00	0.96	0.88	0.91
H_E	0.85	0.90	0.81	0.94	0.90	0.91	0.95	0.92	0.91	0.89	0.92	0.93	0.93
F_{IS}	0.05	-0.11	-0.23	0.01	-0.03	0.07	0.02	-0.04	-0.04	-0.12	-0.04	0.06	0.02
Ca12	N=48	N=10	N=4	N=86	N=13	N=13	N=134	N=46	N=18	N=8	N=52	N=50	N=24
N_A	13	9	5	18	9	10	21	13	9	9	11	15	14
A_R	6.68	7.97	-	9.29	7.35	8.60	9.19	7.63	6.67	9.00	7.29	7.96	8.83
H_O	0.85	0.90	0.75	0.91	1.00	0.85	0.90	0.89	0.61	1.00	0.89	0.94	0.96
H_E	0.83	0.83	0.69	0.90	0.83	0.88	0.90	0.87	0.82	0.84	0.86	0.88	0.88
F_{IS}	-0.03	-0.09	-0.09	0.00	-0.20	0.03	0.00	-0.03	0.26	-0.19	-0.03	-0.07	-0.08
Gbig87	N=48	N=10	N=4	N=86	N=13	N=13	N=133	N=46	N=18	N=8	N=52	N=50	N=24
N_A	20	12	7	31	15	15	31	22	15	10	23	19	20
A_R	7.99	10.67	-	11.13	10.84	11.63	10.79	9.10	10.02	10.00	8.81	7.79	11.18
H_O	0.73	0.90	1.00	0.94	0.92	1.00	0.93	0.74	0.78	1.00	0.79	0.70	0.92
H_E	0.83	0.90	0.84	0.94	0.90	0.92	0.94	0.88	0.90	0.88	0.86	0.81	0.93
F_{IS}	0.13	0.00	-0.19	0.00	-0.02	-0.08	0.01	0.16	0.14	-0.13	0.08	0.14	0.01
Gel227	N=48	N=10	N=4	N=87	N=13	N=13	N=147	N=47	N=18	N=8	N=52	N=50	N=24
N_A	13	11	7	20	11	12	24	15	14	8	19	14	16
A_R	7.52	9.53	-	9.89	9.03	9.79	10.38	9.51	9.62	8.00	9.29	9.02	9.81
H_O	0.90	0.90	1.00	0.93	0.85	0.85	0.95	0.85	0.72	1.00	0.90	0.98	0.92
H_E	0.85	0.85	0.84	0.92	0.88	0.90	0.93	0.92	0.90	0.85	0.90	0.91	0.90
F_{IS}	-0.06	-0.06	-0.19	-0.01	0.04	0.06	-0.02	0.07	0.19	-0.17	0.00	-0.08	-0.02
Cypg3	N=48	N=10	N=4	N=87	N=13	N=11	N=134	N=47	N=18	N=8	N=52	N=50	N=24
N_A	14	11	6	22	12	12	30	21	11	11	22	18	12
A_R	7.34	9.70	-	10.14	9.71	10.25	10.26	9.91	8.24	11.00	10.07	9.59	8.84
H_O	0.90	1.00	0.75	0.95	0.85	0.82	0.90	0.92	0.83	1.00	0.94	0.92	0.83
H_E	0.85	0.87	0.81	0.92	0.89	0.89	0.92	0.90	0.87	0.89	0.90	0.90	0.89
F_{IS}	-0.06	-0.15	0.08	-0.04	0.05	0.08	0.02	-0.02	0.04	-0.12	-0.04	-0.03	0.07
Gbig294	N=49	N=10	N=4	N=87	N=13	N=13	N=135	N=48	N=18	N=8	N=52	N=50	N=24
N_A	4	5	2	6	6	4	10	7	6	4	9	8	2
A_R	2.83	4.57	-	3.23	4.57	2.85	3.72	3.38	3.98	4.00	3.25	3.59	1.56

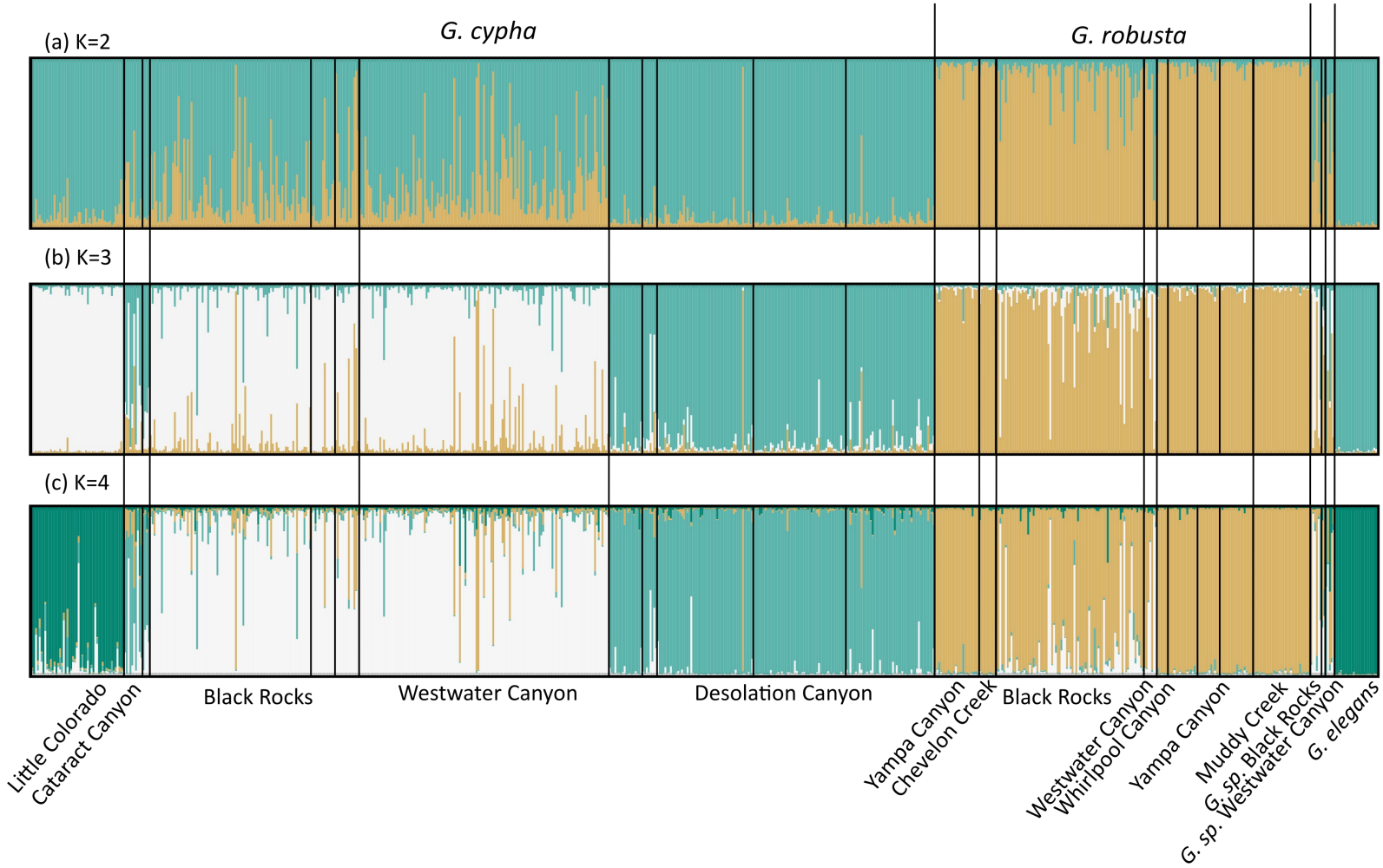
	LCR	Cataract		Black Rocks			WWC	Desolation Canyon					YC
	2013	2013	2015	2012	2013	2014	2016	2006	2010	2012	2014	2015	2007
H_O	0.31	0.60	0.25	0.31	0.39	0.23	0.37	0.33	0.44	0.38	0.27	0.44	0.08
H_E	0.33	0.54	0.47	0.33	0.45	0.21	0.37	0.36	0.38	0.33	0.31	0.38	0.08
F_{IS}	0.08	-0.11	0.47	0.05	0.15	-0.08	0.00	0.08	-0.16	-0.14	0.13	-0.16	-0.04
Pluc13	N=49	N=9	N=4	N=87	N=13	N=13	N=133	N=48	N=18	N=8	N=51	N=50	N=24
N_A	12	9	6	24	13	13	25	21	16	10	21	20	14
A_R	6.25	8.54	-	9.78	10.16	10.00	10.04	10.07	10.66	10.00	9.52	9.31	9.42
H_O	0.84	0.89	1.00	0.90	0.92	0.92	0.89	0.90	1.00	1.00	0.86	0.92	0.88
H_E	0.80	0.86	0.81	0.92	0.90	0.90	0.93	0.92	0.92	0.81	0.90	0.90	0.90
F_{IS}	-0.05	-0.04	-0.23	0.02	-0.03	-0.03	0.04	0.03	-0.09	-0.23	0.04	-0.03	0.03
Pluc22	N=49	N=10	N=4	N=87	N=13	N=13	N=133	N=48	N=18	N=8	N=52	N=50	N=24
N_A	5	5	2	6	3	3	7	6	7	5	6	7	5
A_R	3.78	4.60	-	3.09	2.99	2.99	3.32	4.08	4.98	5.00	3.92	4.21	3.66
H_O	0.55	0.60	0.25	0.49	0.54	0.69	0.47	0.73	0.67	0.75	0.79	0.60	0.58
H_E	0.58	0.69	0.47	0.53	0.54	0.54	0.46	0.64	0.63	0.64	0.69	0.61	0.68
F_{IS}	0.05	0.13	0.47	0.06	0.01	-0.27	-0.01	-0.14	-0.06	-0.17	-0.15	0.02	0.14
Cypg26	N=47	N=10	N=4	N=85	N=12	N=13	N=135	N=48	N=18	N=8	N=51	N=50	N=23
N_A	14	9	6	25	13	12	24	18	14	11	16	14	16
A_R	7.53	8.30	-	10.43	10.31	10.00	9.93	8.60	9.53	11.00	7.97	6.49	9.43
H_O	0.89	1.00	0.75	0.95	0.92	0.85	0.92	0.85	0.89	0.88	0.94	0.86	0.74
H_E	0.85	0.86	0.78	0.93	0.89	0.90	0.92	0.88	0.88	0.89	0.86	0.79	0.89
F_{IS}	-0.05	-0.17	0.04	-0.02	-0.03	0.06	0.00	0.03	-0.01	0.02	-0.09	-0.09	0.17
Pluc03	N=50	N=10	N=4	N=87	N=13	N=13	N=135	N=47	N=18	N=8	N=52	N=50	N=24
N_A	10	7	3	11	8	9	10	10	9	7	8	8	6
A_R	6.00	6.40	-	7.00	7.10	7.14	6.80	6.52	7.18	7.00	6.11	5.64	5.31
H_O	0.82	0.80	0.50	0.90	0.85	0.77	0.84	0.85	0.83	0.75	0.85	0.88	0.71
H_E	0.79	0.81	0.53	0.85	0.82	0.80	0.85	0.84	0.84	0.81	0.83	0.80	0.75
F_{IS}	-0.04	0.01	0.06	-0.05	-0.03	0.04	0.01	-0.02	0.01	0.08	-0.02	-0.10	0.06
Pluc11	N=50	N=10	N=4	N=87	N=13	N=13	N=135	N=48	N=18	N=8	N=52	N=50	N=24
N_A	8	5	3	18	5	7	22	14	13	6	14	15	10
A_R	4.03	4.73	-	5.45	3.84	5.31	5.71	5.10	7.40	6.00	5.72	4.85	4.45
H_O	0.56	0.60	0.50	0.66	0.39	0.62	0.69	0.60	0.89	0.88	0.65	0.58	0.38
H_E	0.54	0.60	0.53	0.70	0.48	0.59	0.69	0.66	0.77	0.72	0.70	0.58	0.40
F_{IS}	-0.03	-0.01	0.06	0.06	0.20	-0.05	0.00	0.09	-0.15	-0.22	0.06	-0.01	0.06
Gel228	N=48	N=10	N=4	N=85	N=13	N=13	N=135	N=47	N=18	N=8	N=52	N=50	N=24
N_A	12	11	5	20	13	15	21	16	11	9	16	16	17
A_R	7.62	9.56	-	9.79	10.29	11.26	9.32	7.90	7.82	9.00	7.45	7.37	10.28
H_O	0.92	0.70	0.75	0.91	1.00	1.00	0.87	0.83	1.00	1.00	0.85	0.86	0.96
H_E	0.87	0.87	0.78	0.92	0.90	0.91	0.91	0.84	0.85	0.85	0.82	0.82	0.91
F_{IS}	-0.05	0.19	0.04	0.01	-0.11	-0.09	0.04	0.01	-0.18	-0.17	-0.03	-0.06	-0.06
Pluc19	N=44	N=10	N=4	N=84	N=13	N=13	N=135	N=47	N=16	N=8	N=50	N=49	N=22
N_A	17	13	7	31	11	15	35	25	16	10	22	24	23
A_R	8.61	11.44	-	10.82	8.94	11.32	11.81	11.67	10.14	10.00	11.41	10.80	12.46

	LCR	Cataract		Black Rocks			WWC	Desolation Canyon					YC
	2013	2013	2015	2012	2013	2014	2016	2006	2010	2012	2014	2015	2007
H_O	0.84	0.90	0.75	0.99	0.85	1.00	0.93	1.00	1.00	1.00	0.96	0.88	1.00
H_E	0.89	0.91	0.84	0.93	0.87	0.92	0.95	0.94	0.88	0.86	0.94	0.92	0.94
F_{IS}	0.05	0.01	0.11	-0.06	0.03	-0.09	0.03	-0.06	-0.13	-0.16	-0.02	0.05	-0.06
Pluc42	N=33	N=8	N=4	N=84	N=13	N=13	N=135	N=48	N=18	N=8	N=51	N=50	N=24
N_A	10	7	6	18	14	13	19	14	11	10	14	14	7
A_R	6.93	7.00	-	9.42	11.11	9.60	9.71	7.43	8.35	10.00	7.92	7.99	4.89
H_O	0.85	0.88	1.00	0.92	1.00	0.85	0.92	0.85	0.89	0.75	0.86	0.82	0.75
H_E	0.83	0.83	0.81	0.90	0.92	0.87	0.91	0.82	0.83	0.86	0.85	0.86	0.70
F_{IS}	-0.03	-0.06	-0.23	-0.02	-0.09	0.03	-0.01	-0.04	-0.07	0.13	-0.02	0.05	-0.07
Pluc30	N=50	N=10	N=4	N=87	N=13	N=13	N=135	N=48	N=18	N=8	N=52	N=50	N=24
N_A	7	7	5	10	6	5	11	12	9	6	11	11	7
A_R	5.32	6.53	-	5.80	5.05	4.46	6.17	6.58	6.01	6.00	5.53	5.93	5.54
H_O	0.72	0.80	0.50	0.83	0.69	0.54	0.84	0.83	0.83	1.00	0.73	0.82	0.83
H_E	0.75	0.77	0.69	0.81	0.69	0.68	0.81	0.78	0.73	0.76	0.73	0.74	0.76
F_{IS}	0.04	-0.04	0.27	-0.03	0.00	0.21	-0.03	-0.06	-0.14	-0.32	0.00	-0.11	-0.10
Pluc41	N=49	N=10	N=4	N=87	N=13	N=13	N=132	N=48	N=18	N=8	N=52	N=50	N=24
N_A	15	7	7	25	13	13	23	18	14	10	21	16	10
A_R	7.96	6.37	-	10.01	10.07	10.37	9.63	8.50	8.93	10.00	9.63	7.28	7.12
H_O	0.78	0.90	1.00	0.92	0.85	0.92	0.91	0.81	0.89	1.00	0.90	0.80	0.83
H_E	0.88	0.78	0.84	0.92	0.89	0.91	0.91	0.83	0.87	0.88	0.88	0.76	0.83
F_{IS}	0.11	-0.16	-0.19	0.00	0.05	-0.02	0.01	0.02	-0.02	-0.13	-0.02	-0.06	-0.01
Pluc44	N=50	N=10	N=4	N=86	N=13	N=13	N=132	N=48	N=17	N=8	N=51	N=50	N=24
N_A	14	13	5	24	12	17	27	21	16	11	18	21	13
A_R	8.16	11.13	-	11.05	9.71	12.16	10.96	10.18	10.59	11.00	9.92	10.08	8.97
H_O	0.90	1.00	0.75	0.93	0.92	0.85	0.97	0.96	0.94	1.00	0.94	0.94	0.88
H_E	0.88	0.90	0.75	0.94	0.89	0.92	0.94	0.91	0.91	0.89	0.92	0.92	0.90
F_{IS}	-0.02	-0.12	0.00	0.01	-0.04	0.08	-0.03	-0.05	-0.04	-0.12	-0.03	-0.02	0.02

Appendix V. *Gila robusta* and *G. elegans* summary statistics of the eighteen microsatellite loci. N = sample size, N_A = number of alleles, A_R = allelic richness rarified for 16 alleles , H_O = observed heterozygosity, H_E = expected heterozygosity, and F_{IS} = inbreeding coefficients. LCR = Chevelon Creek, BR = Black Rocks, WP = Whirlpool Canyon, and MC = Muddy Creek.

		Gila robusta								*Gila elegans*
		LCR	BR	WWC	WP	Yampa Canyon			MC	
		2014	2012	2016	2012	2007	2010	2012	2006	2012
Gbig34		N=9	N=78	N=7	N=6	N=16	N=11	N=17	N=30	N=16
	N_A	5	27	10	9	17	12	13	21	9
	A_R	4.99	10.12	10.00	-	10.44	9.69	8.80	9.99	6.72
	H_O	0.78	0.77	0.71	0.83	1.00	0.91	0.82	0.83	0.69
	H_E	0.78	0.91	0.86	0.86	0.89	0.86	0.85	0.89	0.79
	F_{IS}	0.00	0.15	0.17	0.03	-0.12	-0.06	0.03	0.07	0.13
Gel226		N=9	N=75	N=7	N=6	N=16	N=11	N=17	N=31	N=22
	N_A	7	24	11	10	18	15	17	21	5
	A_R	6.65	10.45	11.00	-	12.10	12.21	11.46	10.51	4.61
	H_O	0.89	0.88	0.71	1.00	1.00	0.91	1.00	0.94	0.77
	H_E	0.77	0.93	0.86	0.89	0.93	0.92	0.93	0.92	0.67
	F_{IS}	-0.16	0.05	0.17	-	-0.07	0.01	-0.08	-0.02	-0.16
Ca12		N=9	N=78	N=7	N=6	N=16	N=12	N=18	N=31	N=22
	N_A	3	17	7	6	12	11	12	12	8
	A_R	2.99	8.52	7.00	-	9.15	9.07	8.98	8.02	5.57
	H_O	0.22	0.90	1.0	0.83	1.00	0.83	0.94	0.97	0.64
	H_E	0.59	0.88	0.84	0.81	0.89	0.88	0.89	0.87	0.76
	F_{IS}	0.62	-0.02	-0.2	-	-0.12	0.05	-0.06	-0.12	0.16
Gbig87		N=9	N=79	N=7	N=6	N=16	N=12	N=18	N=30	N=22
	N_A	1	24	10	7	17	13	17	15	9
	A_R	1.00	10.51	10.00	-	10.89	10.37	10.59	9.87	6.42
	H_O	0.00	0.94	1.0	1.00	0.94	1.00	0.94	0.80	0.73
	H_E	0.00	0.93	0.88	0.81	0.91	0.90	0.91	0.91	0.81
	F_{IS}	–	-0.01	-0.14	-	-0.03	-0.11	-0.04	0.13	0.10
Gel227		N=9	N=77	N=7	N=6	N=16	N=12	N=18	N=31	N=22
	N_A	4	23	11	6	14	10	11	16	8
	A_R	3.89	9.29	11.00	-	10.13	8.16	7.11	8.62	6.42
	H_O	0.67	0.84	0.71	0.67	0.94	0.83	0.72	0.84	1.00
	H_E	0.70	0.89	0.90	0.78	0.90	0.79	0.78	0.86	0.81
	F_{IS}	0.04	0.05	0.21	0.14	-0.04	-0.06	0.08	0.02	-0.24
Cypg3		N=9	N=80	N=7	N=6	N=16	N=10	N=18	N=30	N=22
	N_A	5	21	8	8	11	8	12	13	10
	A_R	4.88	9.62	8.00	-	8.09	7.19	8.72	6.54	6.99
	H_O	0.56	0.93	1.0	1.00	0.81	0.80	0.83	0.77	0.82
	H_E	0.64	0.91	0.86	0.85	0.84	0.82	0.88	0.71	0.83
	F_{IS}	0.13	-0.01	-0.17	-	0.03	0.02	0.05	-0.08	0.01

		Gila robusta								*Gila elegans* 2012
		LCR	BR	WWC	WP	Yampa Canyon			MC	
		2014	2012	2016	2012	2007	2010	2012	2006	
Gbig294		N=9	N=80	N=7	N=6	N=16	N=12	N=18	N=31	N=22
	N_A	1	6	2	2	2	1	2	1	5
	A_R	1.00	2.56	2.00	-	1.50	1.00	1.44	1.00	4.20
	H_O	0.00	0.21	0.14	0.17	0.06	0.00	0.06	0.00	0.73
	H_E	0.00	0.21	0.13	0.15	0.06	0.00	0.05	0.00	0.70
	F_{IS}	–	-0.01	-0.08	-	-0.03	–	-0.03	–	-0.04
Pluc13		N=9	N=80	N=7	N=6	N=16	N=12	N=18	N=31	N=22
	N_A	4	25	8	8	16	11	11	14	6
	A_R	3.89	9.31	8.00	-	10.75	9.08	8.12	8.38	4.82
	H_O	0.89	0.89	1.0	0.83	0.94	0.92	0.83	0.87	0.73
	H_E	0.69	0.90	0.85	0.82	0.91	0.88	0.86	0.88	0.71
	F_{IS}	-0.29	0.02	-0.18	-	-0.03	-0.04	0.03	0.01	-0.03
Pluc22		N=9	N=79	N=7	N=6	N=16	N=12	N=18	N=31	N=22
	N_A	1	6	4	5	4	4	4	4	7
	A_R	1.00	3.46	4.00	-	3.88	3.80	3.98	3.88	5.42
	H_O	0.00	0.67	0.57	0.83	0.69	0.58	0.72	0.84	0.59
	H_E	0.00	0.63	0.46	0.74	0.67	0.64	0.74	0.71	0.66
	F_{IS}	–	-0.07	-0.24	-	-0.02	0.09	0.02	-0.18	0.11
Cypg26		N=9	N=78	N=7	N=6	N=16	N=12	N=18	N=30	N=11
	N_A	3	18	10	7	10	13	12	12	8
	A_R	2.99	7.77	10.00	-	6.96	9.94	8.37	7.47	7.09
	H_O	0.67	0.95	1.0	0.83	0.88	1.00	0.94	0.87	0.91
	H_E	0.54	0.87	0.89	0.83	0.82	0.88	0.87	0.85	0.81
	F_{IS}	-0.24	-0.09	-0.13	0.00	-0.07	-0.13	-0.09	-0.02	-0.12
Pluc03		N=9	N=79	N=7	N=6	N=16	N=12	N=18	N=30	N=22
	N_A	2	9	5	5	6	7	7	6	8
	A_R	2.00	5.48	5.00	-	5.87	6.03	5.78	4.64	7.10
	H_O	0.67	0.76	0.86	0.67	0.69	0.75	0.78	0.83	0.91
	H_E	0.44	0.78	0.67	0.76	0.83	0.74	0.72	0.73	0.86
	F_{IS}	-0.50	0.02	-0.27	0.13	0.17	-0.01	-0.08	-0.15	-0.06
Pluc11		N=9	N=79	N=7	N=6	N=16	N=12	N=18	N=30	N=22
	N_A	2	18	7	5	8	7	8	8	2
	A_R	1.89	6.52	7.00	-	5.53	5.00	4.76	5.23	2.00
	H_O	0.11	0.70	0.86	0.67	0.50	0.50	0.33	0.60	0.36
	H_E	0.11	0.69	0.83	0.61	0.58	0.43	0.47	0.61	0.43
	F_{IS}	-0.06	-0.01	-0.04	-	0.14	-0.17	0.28	0.02	0.16
Gel228		N=9	N=80	N=7	N=6	N=16	N=10	N=18	N=30	N=21
	N_A	3	20	11	7	13	8	13	11	8
	A_R	3.00	9.41	11.00	-	9.10	7.00	9.28	6.99	6.60
	H_O	0.67	0.95	1.0	0.83	0.94	0.70	1.00	0.87	0.86
	H_E	0.66	0.91	0.89	0.82	0.88	0.78	0.89	0.80	0.82
	F_{IS}	-0.01	-0.05	-0.13	-	-0.07	0.10	-0.13	-0.08	-0.05

		Gila robusta								*Gila elegans* 2012
		LCR	BR	WWC	WP	Yampa Canyon			MC	
		2014	2012	2016	2012	2007	2010	2012	2006	
Pluc19		N=8	N=78	N=7	N=6	N=16	N=11	N=17	N=31	N=22
	N_A	9	34	10	9	21	15	16	20	6
	A_R	9.00	11.49	10.00	-	12.76	12.01	11.06	10.23	4.90
	H_O	1.00	0.94	1.0	1.00	0.94	1.00	1.00	0.84	0.73
	H_E	0.86	0.94	0.88	0.86	0.94	0.91	0.92	0.90	0.70
	F_{IS}	-0.16	0.01	-0.14	-	0.00	-0.10	-0.08	0.06	-0.04
Pluc42		N=8	N=79	N=7	N=6	N=16	N=12	N=17	N=31	N=22
	N_A	2	12	7	3	6	7	7	7	9
	A_R	2.00	5.97	7.00	-	5.01	5.97	6.04	4.86	7.22
	H_O	0.13	0.76	0.86	0.50	0.75	0.67	0.82	0.77	0.91
	H_E	0.12	0.79	0.75	0.40	0.73	0.78	0.77	0.74	0.84
	F_{IS}	-0.07	0.04	-0.15	-	-0.03	0.14	-0.08	-0.04	-0.08
Pluc30		N=9	N=80	N=7	N=6	N=16	N=12	N=18	N=31	N=22
	N_A	3	9	5	4	8	7	8	8	3
	A_R	2.99	4.79	5.00	-	5.62	5.89	5.38	5.79	2.85
	H_O	0.67	0.54	0.71	0.67	0.69	0.92	0.61	0.81	0.59
	H_E	0.49	0.55	0.67	0.60	0.64	0.76	0.60	0.75	0.58
	F_{IS}	-0.35	0.021	-0.06	-	-0.07	-0.21	-0.02	-0.07	-0.02
Pluc41		N=9	N=80	N=7	N=6	N=16	N=12	N=18	N=31	N=22
	N_A	6	17	8	6	13	12	9	11	9
	A_R	5.98	8.86	8.00	-	9.19	9.57	6.34	7.72	7.40
	H_O	0.89	0.86	1.0	0.67	0.94	0.75	0.61	0.90	1.00
	H_E	0.80	0.90	0.83	0.81	0.88	0.86	0.74	0.87	0.86
	F_{IS}	-0.12	0.04	-0.21	0.17	-0.07	0.13	0.18	-0.04	-0.17
Pluc44		N=9	N=78	N=7	N=6	N=16	N=12	N=18	N=30	N=21
	N_A	4	23	11	7	15	12	13	15	6
	A_R	3.99	10.09	11.00	-	10.56	9.31	8.96	8.67	5.11
	H_O	0.89	0.89	1.0	1.00	0.94	0.83	0.94	0.93	0.86
	H_E	0.67	0.92	0.89	0.82	0.91	0.88	0.89	0.85	0.77
	F_{IS}	-0.33	0.03	-0.13	-	-0.03	0.05	-0.07	-0.10	-0.11

Appendix VI. Structure Plot for K=2, 3, and 4. Each individual fish is represented by a single vertical bar, with the proportion of color representing the estimated proportion of ancestry attributed to each cluster. Black lines within each output separate samples by year while lines that extend across outputs separate sample sites.