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**Project Title**
Genetic Evaluation of Hybridization among Three Native Sucker Species of the San Juan River

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**Lead Agency:**
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**Principal Investigator:**
Steven M. Mussmann, Geneticist
U.S. Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
7116 Hatchery Road
Dexter, NM 88230

Present address:
Abernathy Fish Technology Center
1440 Abernathy Creek Rd
Longview, WA 98632
(360) 442-7981
steven_mussmann@fws.gov

Melody J. Saltzgiver, Geneticist
U.S. Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
7116 Hatchery Road
Dexter, NM 88230
(575) 734-5910 x154
melody_saltzgiver@fws.gov

Tracy A. Diver, Fish Biologist
U.S. Fish and Wildlife Service
New Mexico Fish and Wildlife Conservation Office
3800 Commons Ave. N.E.
Albuquerque, New Mexico 87109
(505) 342-9900 x115
tracy_diver@fws.gov

Benjamin J. Schleicher, Fish Biologist
U. S. Fish and Wildlife Service
Grand Junction Fish and Wildlife Conservation Office
445 West Gunnison Ave, Suite 140
Grand Junction, Colorado 81501
(970) 628-7205
benjamin_schleicher@fws.gov

**Abstract:**

Interspecific hybridization is common in fishes, particularly in the family Catostomidae. Following the reported recruitment of Razorback Sucker (*Xyrauchen texanus*) in the San Juan River in 2018, a decision was made to investigate juvenile Razorback Sucker for evidence of hybridization with Flannelmouth Sucker (*Catostomus latipinnis*) due to the physical similarities of some juvenile fish to age-1 Flannelmouth Sucker. Genetic assessment focused on five sample groups collected from the San Juan River in 2019-2020: adult Razorback Sucker ($N$ = 123), juvenile (i.e., < 300 mm TL) Razorback Sucker ($N$ = 61), Flannelmouth Sucker *x* Razorback Sucker hybrids ($N$ = 41), Razorback Sucker larvae ($N$ = 156), and Catostomidae larvae that could not be identified to species via existing morphological keys ($N$ = 42). We utilized double digest Restriction-site Associated DNA sequencing (ddRAD) to evaluate hybridization among native sucker species at 23,186 loci. All sample groups were compared against wild adult Bluehead Sucker (*C. discobolus;* $N$ = 24), Flannelmouth Sucker ($N$ = 24), and Razorback Sucker ($N$ = 24) collected from the San Juan River during 2019-2020. Analyses were performed to quantify the proportion of hybrids in each group, determine the sex of parental species of hybrids using maternally inherited mitochondrial DNA (mtDNA), and evaluate signatures of selection acting upon hybrids using genomic cline analyses. Hybridization with Bluehead Sucker was rarely observed. We found that just 23.7% of Razorback Sucker larvae resulted from hybrid crosses among native sucker species. However, 100% of juvenile Razorback Sucker identified in the field were determined to have some level of hybridization with most being first-generation Razorback Sucker *x* Flannelmouth Sucker hybrids. Hybrid status was also confirmed for most field-identified Razorback Sucker *x* Flannelmouth Sucker hybrids. Genomic cline analysis provided little indication of strong selection acting upon alleles from either Razorback Sucker or Flannelmouth Sucker; however, mtDNA analysis indicated that hybrid offspring were almost exclusively descended from male Flannelmouth Sucker and female Razorback Sucker. While the mechanism causing this pattern is currently unknown, these trends could be explained by genetic incompatibilities or behavior (e.g., female mate choice). Regardless, species boundaries for Razorback Sucker have thus far been maintained, as evidenced by a lack of significant introgression from Flannelmouth Sucker into naturally recruiting Razorback Sucker. However, genetic monitoring should occur to verify that this trend continues. Our results further confirm the lack of recruitment from larval to juvenile and adult life stages for Razorback Sucker in the San Juan River despite successful annual reproduction.

**Introduction:**

Interspecific hybridization among animal species has long been a controversial subject in biology. It was once considered an exceptionally rare occurrence by prominent biologists (Dobzhansky 1937; Mayr 1942), although their contemporaries recognized that hybridization occurred more commonly in certain animal taxa, such as fishes (Hubbs 1955). Biologists have since concluded that species boundaries are more permeable than once believed, with interspecific hybridization and

introgression events now documented for at least 10% of animal species (Mallet 2005). Consequently, perspectives among evolutionary biologists on hybridization have shifted to recognize it as a natural process that contributes to adaptation and speciation (Arnold 1992).

Although natural, interspecific hybridization remains a contentious subject in conservation. Its outcomes often conflict with conservation goals (Genovart 2008), yet some controversial perspectives maintain that it could aid imperiled species in adaptation to changing environments (Quilodrán et al. 2020; Ottenburghs 2021). Regardless of perspective, it is an issue that we are forced to confront while anthropogenic modifications of habitats persist (Buggs 2007; Chunco 2014). Hybridization can pose several threats to population recovery, including wasted reproductive effort through the production of sterile first-generation (F1) hybrids (Wolf et al. 2001), a competitive advantage conferred to hybrid offspring through hybrid vigor (Whitlock et al. 2000; Hedrick and Fredrickson 2010), loss of fitness due to outbreeding depression (Lynch 1991), and extinction (Allendorf et al. 2010; Fitzpatrick et al. 2015). Conservation policy and legal complications also arise, due to a lack of explicit protections for hybrids in the Endangered Species Act of 1973 (Allendorf et al. 2001).

The above concepts intersect in endangered fish conservation. Many fishes readily hybridize with both congeneric and confamilial species (Scribner et al. 2000). For example, native Catostomidae in western North America have long been known to hybridize with one another (Hubbs 1955). Presence of hybrids in a system has traditionally been determined using morphological data (Hubbs et al. 1943; Dauble and Buschbom 1981; Cole et al. 2008; Quist et al. 2009). However, hybrid analysis has shifted primarily to genetic and genomic methods which allow for more precise quantification of hybrid prevalence (Twyford and Ennos 2012). These advancements, especially in laboratory methods that rapidly identify genetic markers in non-model organisms (Campbell et al. 2018), are useful for attaining unprecedented resolution of interspecific hybridization and introgression in natural systems (Pardo-Diaz et al. 2012). These methods can illuminate cryptic or multigenerational hybridization when they are partnered with sensitive algorithms capable of detecting admixture among species (Pritchard et al. 2016; Blischak et al. 2018; Beugin et al. 2018). This has created opportunities to better understand hybridization in response to anthropogenic activity (Bangs et al. 2017) and its importance in speciation (Dowling and DeMarais 1993; Barton 2001; Bangs et al. 2018).

Hybridization has frequently been studied in a management context to evaluate admixture with non-native species (McDonald et al. 2008; Bangs et al. 2020a). This phenomenon has been less frequently evaluated when it exclusively involves native catostomids (Dowling et al. 2016; Bangs et al. 2020b). Existing studies indicate hybridization is common among sympatric Catostomidae in their native habitats; although its frequency may vary from one system to the next, even when the same species assemblages are present in different locations (Mandeville et al. 2015, 2017).

Hybridization recently became a management concern in the San Juan River, which is home to three native catostomids: Bluehead Sucker (*Catostomus discobolus*), Flannelmouth Sucker (*C. latipinnis*), and Razorback Sucker (*Xyrauchen texanus*). Two of these species, Bluehead Sucker (excluding Zuni Bluehead Sucker *C. d. yarrowi*) and Flannelmouth Sucker, are not listed as federally threatened or endangered. However, Razorback Sucker is listed under the Endangered Species Act of 1973, as amended (16 U.S.C. 1531-1544, 87 Stat. 884), and was historically found in the San Juan River until its extirpation in the early 1990s (Ryden 2005). The San Juan River Basin Recovery Implementation Program (SJRRIP) has since led efforts to recover endangered Razorback Sucker populations. Tens of thousands of Razorback Sucker have been stocked in the San Juan River since the 2000s, with many of these individuals known to persist and reproduce (Franssen et al. 2016; Diver et al. 2021).

The SJRRIP's conservation actions were briefly thought to have spurred successful recruitment of Razorback Sucker. Such an event would be notable, due to the rarity of recruitment for the species throughout its range (Carson et al. 2016). Record recruitment of Razorback Sucker to juvenile life stages was reported in 2018, but uncertainty in field identifications lingered due to the physical similarities of some fish to juvenile Flannelmouth Sucker. Questions remained concerning the proportions of Razorback Sucker *vs*. the number of hybrids present among those observations. This is because wild-spawned Razorback Sucker are rarely encountered beyond larval life stages (Albrecht et al. 2010; Schleicher et al. 2020), suspected hybrids with Flannelmouth Sucker are frequently observed (Schleicher et al. 2020), and simultaneous occupation of spawning habitat by both Razorback Sucker and Flannelmouth Sucker has also been noted (Ryden 2000). Furthermore, hybridization of Razorback Sucker with Flannelmouth Sucker is known to occur through prior genetic studies and historical documentation (Buth et al. 1987; Douglas and Marsh 1998; Dowling et al. 2012). Consequently, these factors prompted genetic evaluation of purported juvenile Razorback Sucker from the San Juan River.

Despite being a natural process, hybridization is a potential impediment to Razorback Sucker recovery when conservation goals aim to recover non-admixed individuals in their former habitat. Uncertainty surrounding hybridization outcomes therefore necessitates careful evaluation of its impacts when endangered species are involved. The San Juan River provides a unique opportunity to evaluate hybridization patterns among native sucker species by exploring selection acting upon genomic loci in hybrids and backcrossed individuals. In this study we take the initial steps in addressing hybridization among native San Juan River suckers by quantifying the number of hybrids detected at multiple life stages during the 2019 and 2020 field seasons. Additionally, we categorize the fish according to generational hybrid classes and work to identify potential selection that favors alleles from one species over the other. These analyses collectively provide initial assessments of hybridization among native suckers in the San Juan River and early evaluations of threats posed.

**Methods:**

*Sample Collection*

Samples representing adult, juvenile (< 300 mm TL), and larval catostomids were collected from the San Juan River in 2019 and 2020 (Table 1). Larval samples (i.e., protolarval through metalarval developmental stages) were collected as a part of annual larval fish surveys conducted by American Southwest Ichthyological Researchers (ASIR). Two groups of larvae were sequenced: those morphologically identified as Razorback Sucker, and a group identified to familial level (hereafter referred to as 'Catostomidae' larvae) that could not be confidently assigned to species through morphological keys (Farrington et al. 2022). Juvenile fish in 2019 were collected during various monitoring efforts (i.e., demographic monitoring and non-native removal); however, 2020 fish were collected as part of the fall 2020 Razorback Sucker monitoring that occurred in lieu of demographic monitoring. Adult Razorback Sucker were collected by Kansas State University as a part of their efforts to translocate Razorback Sucker above the Piute Farms waterfall. Adult Bluehead Sucker and Flannelmouth Sucker were collected during routine monitoring in 2019.

*Library Preparation*

Double digest restriction-site associated DNA (ddRAD) library preparation followed the methods of Peterson et al. (2012). Samples were digested using restriction enzymes PstI and MspI for 24 hours before 8 base pair (bp) barcodes were ligated to the PstI end of each restriction fragment. Barcoded samples (25 to 100 ng DNA each; standardized per library) were pooled in sets of 48 following Illumina adapter ligation, then size selected using the Pippin Prep System (Sage Science) to

retrieve DNA fragments 353 to 403 bp in length. This range was identified using the methods of Chafin et al. (2018). Size-selected DNA was subjected to 12 cycles of polymerase chain reaction (PCR) amplification using Phusion high-fidelity DNA polymerase (New England BioLabs; Ipswich, MA, USA) according to manufacturer protocols. Subsequent quality checks were performed via fragment analysis (Agilent 2100 Bioanalyzer) and quantitative PCR (ABI QuantStudio 5) to confirm successful library amplification. Four to six indexed libraries (N = 48 samples per library) were pooled at an equimolar concentration of 4 nM and sequenced through three sequencing runs on the Illumina NextSeq 500 at Whitney Genetics Laboratory (Midwest Fisheries Center; Onalaska, WI). Sequencing was performed using Illumina High Output v2.5 150 cycle kits configured for 150 bp single-end reads.

*Data Assembly*

Resulting sequence files were first evaluated in FASTQC (Andrews 2010) to evaluate data quality. A low-quality sequence region corresponding to the PstI restriction cut site was identified in six libraries. We compensated for this by first running the *process_radtags* module of STACKS v2.60 (Rochette et al. 2019) to demultiplex all libraries without performing a restriction cut site quality check. The demultiplexed reads were then trimmed in FASTP (Chen et al. 2018) to remove sequencing artifacts (poly-G tails: De-Kayne et al. 2021), discard the first 8 bp from the 5' end of each sequence, and trim bases from the 3' end of each sequence until a maximum of 100 bp remained. Sequences < 100 bp in length were discarded from the dataset. Finally, sequences were subjected to quality filtering in *process_radtags* to remove sequences with uncalled bases and discard reads containing low quality scores (average phred quality score < 10 within a sliding window of 15 bp).

A *de novo* assembly was then conducted in STACKS v2.60. Due to the large number of samples (N = 495), the STACKS pipeline was operated through a custom script to control the number of samples used to build the locus catalog. Large numbers of samples (N > 500) negatively impact *de novo* assembly of RAD data through propagation of sequencing error in the locus catalog (Rochette and Catchen 2017). Twenty-four representatives of each species (Bluehead Sucker, Flannelmouth Sucker, Razorback Sucker) were selected from adult samples for locus catalog construction. Assembly parameters were optimized according to the recommendations and methods of Paris et al. (2017). This yielded $M = 3$ (the number of mismatches allowed between putative alleles), $n = 3$ (the number of mismatches allowed between putative loci), and $N = 5$ (the number of mismatches allowed to align secondary reads). All other assembly parameters were left as default values. Single nucleotide polymorphisms (SNPs) were genotyped using the *populations* module of STACKS and filtered according to the requirements of downstream analyses.

*Admixture Analysis*

Samples were first processed to quantify species ancestry proportions ($Q$) per individual. This was accomplished through a maximum likelihood approach in ADMIXTURE to assess the contributions of Bluehead, Flannelmouth, and Razorback Sucker to the genomic composition of each sample (Alexander et al. 2009; Alexander and Lange 2011). Data were filtered in *populations* to retain loci present in at least two species, a minimum of 50% of individuals from each species, and having a minor allele frequency (MAF) > 0.05. Loci with extremely high observed heterozygosity (global $H_O > 0.9$) were discarded under the assumption they represented paralogs (McKinney et al. 2017). One SNP per ddRAD locus was retained to minimize linkage disequilibrium. The ADMIXTURE analysis was carried out in ADMIXPIPE v3.0 (Mussmann et al. 2020) using a clustering ($K$) value of 3 with 40 replicates performed. Replicates were summarized in CLUMPAK (Kopelman et al. 2015).

*Hybrid Analysis*

Results from ADMIXTURE were used to identify non-admixed individuals of Bluehead, Flannelmouth, and Razorback Sucker. Up to 24 individuals per species were used as non-admixed references for all downstream hybrid analyses. Reference individuals were required to exhibit ancestry from a single species and be an adult individual with genetic ancestry matching its species identification in the field. More than 24 Razorback Sucker fit these criteria, so the 24 samples with the highest overall sequencing output were selected.

Hybrid classifications were assessed in a Bayesian framework using NEWHYBRIDS v2.0 (Anderson and Thompson 2002). This program explicitly classifies individuals into hybrid or non-hybrid categories. The program evaluates six possible categories by default: 1) Species A, 2) Species B, 3) first generation (F1) hybrid of Species A and B, 4) second generation (F2) hybrid (i.e., offspring of two F1 hybrids), 5) Species A backcross (offspring resulting from an F1 *x* Species A cross), and 6) Species B backcross (offspring from an F1 *x* Species B cross). NEWHYBRIDS cannot evaluate hybrids involving three or more species simultaneously, so we evaluated all pairwise interspecific crosses separately (i.e., Bluehead Sucker *x* Flannelmouth Sucker, Bluehead Sucker *x* Razorback Sucker, and Flannelmouth Sucker *x* Razorback Sucker). Each data file was comprised of individuals containing full or partial ancestry for the species pair under evaluation. To prepare data for NEWHYBRIDS, the *populations* module of STACKS was rerun three times to prepare files of SNPs common to each pair of species. The resulting SNP files were then reduced to 200 loci showing the greatest among-population differentiation ($F_{ST}$) and lowest linkage disequilibrium ($r^2 < 0.2$) as determined through GENEPOPEDIT (Stanley et al. 2017). NEWHYBRIDS was run for 100,000 Markov chain Monte Carlo (MCMC) burn-in generations followed by 1,000,000 MCMC generations of data collection. The 'z' option was employed in NEWHYBRIDS to specify reference genotypes for non-admixed individuals during each run of NEWHYBRIDS.

Finally, we used the *est.h* function in the INTROGRESS R package (Gompert and Buerkle 2010) to calculate a hybrid index for each species pair (Buerkle 2005). Data were filtered to acquire only SNPs representing fixed differences among each pair of species. Interspecific heterozygosity (i.e., the proportion of loci at which an individual is heterozygous for the two parental alleles) was calculated for each individual using the *calc.intersp.het* function in INTROGRESS, and results were visualized using the *triangle.plot* function.

*Genomic Cline*

A genomic cline analysis was conducted for Flannelmouth Sucker *x* Razorback Sucker hybrids. This analysis could not be performed for other species pairs due to the paucity of interspecific hybrids involving Bluehead Sucker. The program BGC v1.03 was used to identify species-specific SNPs that exhibited patterns of introgression deviating significantly from neutral expectations (Gompert and Buerkle 2012). The *populations* module of STACKS was run again to further reduce missing data and exclude larvae from the analysis. To this end, SNPs were required to be present in at least 50% of individuals from each Razorback Sucker, Flannelmouth Sucker, or hybrid sample group. Larvae were excluded because it was assumed that any environmental factors corresponding to selective pressures may not have had adequate time to act upon these individuals. A MAF filter > 0.05 was applied, and maximum heterozygosity was set at 0.9. The resulting SNP matrix was input into BGC and evaluated for 50,000 generations of burn-in followed by 75,000 generations of data collection. Four independent MCMC runs were performed. The R package CLINEHELPR v1.1 (Martin et al. 2021) was used to combine independent outputs, assess MCMC convergence, detect outlier SNPs under selection, and prepare summary plots. Two outlier detection methods were utilized (Gompert and Buerkle 2011, 2012),

the first of which tests if cline parameter confidence intervals (α = cline center; β = cline rate) exclude the neutral expectations (α = 0; β = 0). The second method typically provides a more conservative estimate of outlier loci by testing if per-locus cline parameters are statistically unlikely given the overall distribution of observed parameter values.

*mtDNA genotyping*

A 648 bp region of cytochrome c oxidase subunit I (COI) was sequenced for 153 samples that were determined to be hybrids. COI is a mitochondrial (mtDNA) gene, meaning it is maternally inherited and therefore facilitates species identification of the maternal lineage in hybrid crosses. PCR was conducted using the FishF1 and FishR1 COI barcoding primers (Ward et al. 2005) in 20 µL reaction volumes containing 8 µL 2x Qiagen® Multiplex Master Mix; 1 µL each of 10 µM forward and reverse primers, 1 µL DNA template, and 9 µL nuclease-free water. Conditions for amplification consisted of an initial denaturation step at 95°C for 15 minutes followed by 35 cycles of denaturation at 95°C (45 s), annealing at 56°C (60 s), and extension at 72°C (60 s) with a final extension at 72°C for 30 minutes. Reactions were purified by combining Exonuclease I and Shrimp Alkaline Phosphatase using manufacturer protocols (New England BioLabs; Ipswich, MA, USA). Sequencing reactions were performed bidirectionally using BigDye v3.1 Terminator chemistry (Applied Biosystems®) according to manufacturer protocols. Sequence products were concentrated via ethanol precipitation, dried, and eluted in 10 µL HiDi-Formamide solution prior to capillary electrophoresis (ABI 3500xL Genetic Analyzer). Forward and reverse sequences for each sample were aligned and edited to verify base calls using Geneious® R11 (Biomatters; Auckland, New Zealand).

**Results:**

*Data Assembly*

After completing all quality filtering steps for raw Illumina data, 597,839,646 reads remained ($\bar{x}$ = 1,207,757 reads per sample; $\sigma$ = 615,239; minimum = 227,260 reads per sample; maximum = 4,109,864 reads per sample). Mean per-sample sequencing depth was 17.0x ($\sigma$ = 7.8x, minimum = 5.2x, maximum = 47.8x). The initial STACKS assembly yielded 23,186 unlinked SNPs (20.9% missing data), which were used for determinations of admixture among the three species. The results of more stringent filtering schemes used for other analyses are provided below.

*Admixture Analysis*

The ADMIXTURE analysis (Figure 1) indicated a high prevalence of hybridization among Flannelmouth Sucker and Razorback Sucker, but hybridization involving Bluehead Sucker was rare. No three-species hybrids were detected. Excluding fish that were field-identified as Bluehead Sucker, just seven samples were determined to have any level of Bluehead Sucker ancestry (Bluehead Sucker *Q* = 0.140 to 1.000). Fish collected as Flannelmouth Sucker tended to have Flannelmouth Sucker ancestry (Flannelmouth Sucker *Q* = 0.944 to 1.000). Field-identified Flannelmouth Sucker *x* Razorback Sucker hybrids tended to indicate hybrid ancestry in the ADMIXTURE analysis. With the exceptions of three misidentified Flannelmouth Sucker, all field-identified hybrids indicated mixed ancestry (Razorback Sucker *Q* = 0.186 to 0.501; mean *Q* = 0.442).

All field-identified juvenile Razorback Sucker exhibited similar patterns of mixed ancestry. Most had ancestry proportions consistent with those expected for Razorback Sucker *x* Flannelmouth Sucker hybrids (Razorback Sucker *Q* = 0.145 to 0.516; mean *Q* = 0.463). In contrast, most Razorback Sucker larvae were correctly identified, with just 35 exhibiting Razorback Sucker ancestry levels consistent

with F1 hybrids (Razorback Sucker $Q = 0.473$ to 0.545). Adult Razorback Sucker had very little Flannelmouth Sucker ancestry (Razorback Sucker $Q = 0.891$ to 1.000; mean $Q = 0.993$).

The 'Catostomidae' larval group included several non-admixed representatives of all three native species (i.e., $Q = 1.000$) [Flannelmouth Sucker = 12 (28.6%); Razorback Sucker = 4 (9.5%); Bluehead Sucker = 2 (4.8%)], whereas the remainder had varying proportions of ancestry [N = 24 (57.1%)]. The majority of these [N = 22 (52.4%)] had mixtures of Razorback Sucker and Flannelmouth Sucker ancestry.

*Hybrid Analysis*

NEWHYBRIDS results were congruent with the ADMIXTURE outputs. However, NEWHYBRIDS classifies all individuals into discrete categories, thus some individuals that exhibited minor amounts of introgression from another species in the ADMIXTURE analysis (e.g., $Q < 0.1$) were classified as non-admixed by NEWHYBRIDS (Table 2). All NEWHYBRIDS classifications were assigned with high confidence (Bayesian posterior probability; BPP > 0.99), except for one adult Razorback Sucker assigned with BPP = 0.69). All adult Bluehead Sucker and Flannelmouth Sucker were classified as non-admixed. Most adult Razorback Sucker were also non-admixed, except for two individuals that were classified as Razorback Sucker backcrosses involving Flannelmouth Sucker. Nearly all juvenile fish collected in the field as Flannelmouth Sucker *x* Razorback Sucker hybrids were confirmed as hybrids. Three exceptions were determined to be misidentified Flannelmouth Sucker. Most other field-identified hybrids (30/38) were classified as F1 hybrids of Razorback Sucker and Flannelmouth Sucker, with the remainder being Flannelmouth Sucker backcrosses (8/38). Juvenile fish collected as Razorback Sucker in 2019-2020 were almost exclusively F1 hybrids with Flannelmouth Sucker (53/61), with the exceptions being an F2 hybrid (1/61), and several Flannelmouth Sucker backcrosses (7/61). Razorback Sucker larvae were mostly non-admixed Razorback Sucker (119/156). However, 32 F1 hybrids with Flannelmouth Sucker were observed, as were three F2 hybrids, and a single Razorback Sucker backcross. One larva was determined to be a Bluehead Sucker *x* Razorback Sucker F2 hybrid. 'Catostomidae' larvae that could not be identified to species via morphology represented a mixture of non-admixed species and varying classes of hybrids. The majority were non-admixed representatives of Bluehead Sucker (N = 2), Flannelmouth Sucker (N = 12), and Razorback Sucker (N = 8). Seventeen individuals were Flannelmouth Sucker *x* Razorback Sucker F1 hybrids. The remaining three individuals were F2 hybrids or backcrosses (Table 2).

Hybrid larvae were unevenly distributed among sampling localities, with 'locality' defined as the river mile (RM) at which a group of larvae were collected. Hybrids were found at 15 of the 37 sites from which the 2020 Razorback Sucker larvae were collected. However, these were not evenly distributed among the sites. Just three of these 15 sites represented 54% of the total hybrids detected. These three collections occurred at RM 9.6, 57.9, and 73.5. Larvae from multiple hybrid classes (e.g., a mixture of F1 and F2 hybrids) were also detected at three of 15 sites, indicating parental contribution from multiple adults of each species. These three sites were located at RM 9.6, 52.4, and 73.5. The 'Catostomidae' larval group was collected from 20 different sites, with hybrids being found at 15 of these sites. Just two of these sites exhibited larvae from multiple hybrid classes (RM 170.9 and 180.6).

Hybrid index results from INTROGRESS were mostly congruent with the results from ADMIXTURE and NEWHYBRIDS. Data filtering procedures for this analysis yielded 6,732 SNPs representing fixed differences between Bluehead Sucker and Flannelmouth Sucker, 5,862 fixed differences between Bluehead Sucker and Razorback Sucker, and 5,676 fixed differences between Flannelmouth Sucker and Razorback Sucker. Unlike NEWHYBRIDS, which places individuals into discrete categories, a hybrid index offers a continuous scale of hybrid ancestry. However, approximate hybrid categories can be

visualized when the hybrid index is plotted against interspecific heterozygosity (Figure 2A). In general, hybrids involving Bluehead Sucker were rare, and tended to be F1 hybrids when observed (Figure 2B and 2C). This was the only instance in which the hybrid index conflicted with NEWHYBRIDS, which had determined all hybrids involving Bluehead Sucker to be either backcrosses or F2 hybrids. Backcrosses involving Flannelmouth Sucker were observed for Bluehead Sucker (Figure 2B) but not Razorback Sucker (Figure 2C). However, hybrids of Bluehead Sucker and Razorback Sucker were rarely observed overall. Plots representing Flannelmouth Sucker and Razorback Sucker hybrids indicated again that most hybrids are F1 hybrids, with F2 hybrids and Razorback Sucker backcrosses being infrequent (Figure 2D). Rare instances of backcrossing among these two species tended to yield Flannelmouth Sucker backcrosses (Figure 2D).

*Genomic Cline*

The additional filtering in *populations* to meet the stringent missing data requirements of BGC recovered 2,786 SNPs representing fixed differences between Razorback Sucker and Flannelmouth Sucker. Using the first significance test in CLINEHELPR (Figure 3), 107 SNPs were α-outliers significantly favoring Razorback Sucker, while an additional 192 SNPs were α-outliers significantly favoring Flannelmouth Sucker. Thirteen β-outlier SNPs were also detected, which generally represented a slight heterozygote advantage. Four of the β-outlier loci were also detected as α-outliers, with two of these having an additional slight advantage for the Razorback Sucker allele vs. the Flannelmouth Sucker allele at their respective loci. When applying the second, more stringent significance test, only α-outliers favoring Flannelmouth Sucker (N = 64) were detected (Figure 4).

*mtDNA genotyping*

Nearly all instances of hybridization involved male Flannelmouth Sucker and female Razorback Sucker (Table 3). This was especially true for F1 hybrids, in which 128 of 129 F1 hybrids had a Razorback Sucker mother. This trend also held for other hybrid classes involving these two species. Among Flannelmouth Sucker backcrosses, just two of 16 observations involved a female Flannelmouth Sucker parent. All F2 hybrids and Razorback Sucker backcrosses exhibited a Razorback Sucker maternal lineage. All hybrids involving Bluehead Sucker had Bluehead Sucker mtDNA; however, very few examples of these crosses were documented overall (N = 3).

**Discussion:**

Although hybridization is a common, natural phenomenon among fishes and within Catostomidae in particular, our results highlight surprising trends that will have implications for recovery of Razorback Sucker in the San Juan River. An unquantified amount of Razorback Sucker reproductive effort is wasted through hybridization with Flannelmouth Sucker; however, the annual presence of Razorback Sucker larvae suggests the wasted effort is a lesser impediment to recovery (Diver et al. 2021). Most concerningly, we found a lack of Razorback Sucker recruitment to juvenile life stages. Rather, juvenile individuals that had been field-identified as Razorback Sucker were genetically determined to be misidentified Flannelmouth Sucker *x* Razorback Sucker hybrids. This indicates a bottleneck to recruitment that may impact only Razorback Sucker at a particular life stage. The complete absence of juvenile Razorback Sucker recruits therefore makes it impossible to quantify recruitment as a response to any variable. However, observable Flannelmouth Sucker backcrossing trends and selection acting upon loci shared among Razorback Sucker and Flannelmouth Sucker can provide insights about the threats posed to Razorback Sucker recovery in the San Juan River. Therefore, we addressed the

degree to which hybridization poses an immediate threat to Razorback Sucker conservation, the caveats associated with these conclusions, and necessary future directions for this research.

*Conservation Threats and Observed Patterns of Hybridization*

The greatest immediate conservation threat is the lack of recruitment for Razorback Sucker beyond larval life stages. Most Razorback Sucker larvae collected in 2020 were correctly identified as Razorback Sucker. However, the percentage of accurate larval identifications (76.3%) was lower than the typical 87.7% to 95.2% accuracy observed from 2009 to 2019 (Diver et al. 2021; Saltzgiver and Mussmann 2022). This could be explained by the limited sampling that occurred in 2020 due to the COVID-19 pandemic (Farrington et al. 2022); therefore, we do not attribute the increased proportion of detected hybrids to an actual increase of hybrids in the system. The presence of Razorback Sucker larvae indicates that adult Razorback Sucker are successfully reproducing in the San Juan River, but the classification of all field-identified Razorback Sucker juveniles collected in 2019-2020 as hybrids indicates that these larvae are not recruiting to the next life stage.

In contrast, Flannelmouth Sucker *x* Razorback Sucker F1 hybrids are recruiting to sexual maturity. Juvenile Flannelmouth Sucker backcrosses collected in 2019 and 2020 demonstrate the successful reproduction of F1 hybrids with non-admixed Flannelmouth Sucker. However, the opposite phenomenon was rarely observed, with just two adults and one larva detected as Razorback Sucker backcrosses. F2 hybrids were also rare (N = 3 larvae; 1 juvenile), indicating that either F1 hybrids rarely reproduce with one another or Razorback Sucker, or these crosses infrequently yield viable offspring. Consequently, Razorback Sucker alleles are being incorporated into the Flannelmouth Sucker population, but at present there is little integration of Flannelmouth Sucker alleles into the Razorback Sucker population, with uncertainty as to whether this trend will hold. Hatchery propagation and supplementation of Razorback Sucker also provides a constant influx of non-admixed fish. Therefore, the formation of a hybrid swarm (i.e., the blurring of distinction among species as a consequence of repeated hybridization and backcrossing: Harrison, 1993) is unlikely to occur for Razorback Sucker in the San Juan River at this time.

Interestingly, the survival of hybrids with Flannelmouth Sucker ancestry suggests that Flannelmouth Sucker alleles confer an advantage in the San Juan River. However, this pattern exists despite little evidence of selection for alleles of one species over the other in the genomic cline results. The application of stringent methods for determining significance only recovered SNP loci that favor Flannelmouth Sucker, albeit weakly (Figure 4). This may indicate weak favoritism for Flannelmouth Sucker alleles, but could also result from a lack of sampling for Razorback Sucker backcrosses (if they are more prominent in the San Juan River than we have thus far detected). There is also scant evidence of selection favoring hybrids. However, genomic cline models operate on individual genetic loci rather than groups of genes (Gompert and Buerkle 2011). Thus, we cannot discount the possibility that novel combinations of Flannelmouth Sucker and Razorback Sucker alleles could confer a selective advantage due to environmental factors in the modern San Juan River (Fitzpatrick 2013).

Evaluation of mitochondrial DNA data provides an additional layer of complexity to the above results and helps elucidate possible explanations for the observed trends. Nearly all Flannelmouth Sucker *x* Razorback Sucker hybrids, regardless of hybrid classification, have Razorback Sucker mtDNA. This indicates that either female Razorback Sucker are choosing to mate with Flannelmouth Sucker, or that primarily hybrid offspring with a Razorback Sucker maternal lineage survive to maturity. This pattern could be explained by multiple scenarios, including: 1) genetic incompatibilities, 2) population size differences, or 3) behavior.

Offspring viability due to genetic incompatibility could explain the differential rates of introgression into each species. Currently, knowledge of F1 hybrid viability is limited, but reciprocal crosses of Razorback Sucker and Flannelmouth Sucker yielded offspring that survived to at least 36 days post-hatch; therefore, it is known that at least a portion of F1 hybrid larvae are able to survive under controlled conditions (Wolters et al. 2019). Unfortunately, survival of multigenerational hybrids has not been studied in a controlled system, and hatchery environments are subject to very different selective pressures compared to the wild (Frankham 2008; Christie et al. 2016), thus it is unknown if survival rates from a controlled environment would directly translate to a natural environment. However, the mtDNA inheritance patterns observed in this study indicate potential incompatibilities exist and are perhaps influenced by environmental factors. An environmental interaction seems probable, given drainage-specific patterns in hybridization observed across the Colorado River Basin for other catostomid assemblages (Mandeville et al. 2015, 2017).

Genetic incompatibilities can arise when coadapted gene complexes are disrupted as a consequence of hybridization (Lynch 1991; Frankham et al. 2011). A common incompatibility can arise from necessary interactions between mitochondrial and nuclear genes (i.e., mitonuclear incompatibility). Mitochondrial genes necessary for ATP synthesis must be compatible with complementary genes in the nuclear genome. Unlike mitochondrial DNA, which is inherited as a single unit, the nuclear genes in question are distributed among separate chromosomes or portions of the same chromosome that are not always inherited as a single unit due to recombination during meiosis. The disruption of these gene complexes through hybridization can reduce fitness of hybrid offspring (Wolff et al. 2014; Tobler et al. 2019). These problems may manifest in second-generation hybrids when co-evolved gene complexes are more greatly disrupted (Burton et al. 2006). Such a phenomenon could explain the lack of observed F2 hybrids and Razorback Sucker backcrosses.

Population size differences for Razorback Sucker and Flannelmouth Sucker populations could also contribute to hybridization. Although census size estimates are unavailable for Flannelmouth Sucker, the number of breeders estimated to contribute to annual cohorts is an order of magnitude greater than estimated for Razorback Sucker (Diver et al. 2021), and Flannelmouth Sucker exhibits the greatest catch per unit effort for any large-bodied fish in the San Juan River (Schleicher 2018). Furthermore, native Colorado River Basin catostomids are known to share spawning habitat (Tyus and Karp 1990). Therefore, F1 hybrids in the San Juan River may encounter Flannelmouth Sucker spawning partners more frequently than they encounter Razorback Sucker.

Lastly, behavior such as female mate choice could also be driving the observed patterns of F1 hybrids backcrossing with Flannelmouth Sucker. Spawning behavior in Catostomidae is relatively understudied, but little variability is typically noted among species (Díaz-Muñoz et al. 2014). Typical spawning accounts for Catostomidae describe males as occupying territory above spawning beds, while females enter their territory to spawn with multiple males simultaneously (Page and Johnston 1990). Similar behavior has been observed for both Flannelmouth Sucker and Razorback Sucker (Mueller 1989; Weiss et al. 1998). It is therefore possible that female F1 hybrids are entering spawning beds and choosing to mate with Flannelmouth Sucker males. Female mate choice favoring or disfavoring hybrids has been observed in other fish species; sometimes maintaining species boundaries or leading to speciation (van der Sluijs et al. 2008; Svensson et al. 2017), and other times allowing hybrid species complexes to persist (Morgado-Santos et al. 2015; Aubier et al. 2019). Therefore, we cannot discount the possibility that similar phenomena are driving the observed frequency of Flannelmouth Sucker backcrosses in the San Juan River.

*Future Recommendations*

Our study provides a window into the current patterns of hybridization among native Catostomidae in the San Juan River. However, the extent to which this hybridization has been driven by environmental alterations remains unknown, and patterns could change as anthropogenic activity continues to impact the environment (Comte et al. 2022). Therefore, continued monitoring of hybridization in the San Juan River is paramount to establishing a more complete picture of interspecific interactions among Razorback Sucker and Flannelmouth Sucker, and tracking the potential threats posed by hybridization over time. Although some results described herein are encouraging for Razorback Sucker conservation (e.g., a general lack of Flannelmouth Sucker introgression into the Razorback Sucker population), it is uncertain if these trends will continue. Our analysis would also benefit from sampling of additional groups, such as collection of juvenile Flannelmouth Sucker from the San Juan River. It has been suggested that hybrid offspring may be more similar morphologically to the species of their maternal parent because of egg morphology; however, it is unlikely these minor body shape differences are detectable in the field (Wolters-Rinker et al. 2022). Therefore, we find it unlikely that a reservoir of hybrid individuals with Flannelmouth Sucker mtDNA exists among the San Juan River Flannelmouth Sucker population, but we cannot completely exclude this possibility.

Continued analysis of existing data will provide further insights into the timespan over which hybridization has occurred. For example, NEWHYBRIDS classifies individuals into discrete hybrid categories, whereas INTROGRESS calculates a hybrid index to quantify species ancestry as a continuous value between 0 and 1. In Figure 2D, the samples corresponding to the 'Flannelmouth Sucker Backcross' group are distributed along the left side of the triangle, indicating a wide range of hybrid index and interspecific heterozygosity values. Some of these individuals could represent 3$^{rd}$-generation (or later) hybrid crosses. Accurate classification of individuals into 3$^{rd}$-generation hybrid categories is possible with NEWHYBRIDS, albeit with some caveats. For example, computation is more time consuming due to the exponential increase in the number of possible 3$^{rd}$-generation hybrid categories, and some of these classes (such as F2 *vs*. F3) are indistinguishable from one another (Pritchard et al. 2016).

Forthcoming genomic resources will also aid in hybrid analysis. A reference genome will soon be published for Razorback Sucker (Trevor Krabbenhoft, University of Buffalo, personal communication), which will open additional analytical opportunities for detecting gene associations with the loci we have identified to be under selection (Ahrens et al. 2018).

*Conclusion*

In summary, the lack of Razorback Sucker recruitment remains highly concerning. However, some encouraging trends remain, such as a lack of Flannelmouth Sucker introgression into the Razorback Sucker population. There is also a lack of evidence for strong selection in favor of hybrids, or for species-specific selection acting upon the nuclear loci evaluated in this study. Other patterns are mildly concerning, such as the discovery of statistically significant outlier loci favoring only Flannelmouth Sucker when a certain significance test is employed, but this trend could also result from a potential sampling bias (i.e., a scarcity of Razorback Sucker backcrosses to include in the genomic cline analysis). These results combined with observed mtDNA trends indicate several possible explanations for the observed patterns, including behavior, population size, and mitonuclear incompatibility. Although we cannot determine a definitive cause for the observed mtDNA patterns, it is likely that one or more of these factors is contributing to the maintenance of species boundaries among suckers in the San Juan River. Continued genetic monitoring of the San Juan River population will be necessary to

determine whether current trends will hold over time, and to further evaluate selection acting upon the Razorback Sucker and Flannelmouth Sucker populations.

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**Signed:**
Steve Mussmann, Melody Saltzgiver, Tracy Diver, Ben Schleicher
Principal Investigators
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**Table 1.**
Samples collected (N) for Bluehead Sucker (BHS), Flannelmouth Sucker (FMS), and Razorback Sucker (RBS) as well as FMS *x* RBS hybrids. Year and life stage at collection are also provided. All samples are categorized according to their morphological identifications. Larvae that could not be reliably identified to species via morphology are listed as 'Catostomidae.' The number of sampling localities is provided per larval group, with 'locality' defined as the river mile at which a group of samples were collected. River miles representing collection locations are also provided. However, river mile data were not collected for three hybrid samples and 25 juvenile RBS samples in 2020. Adult RBS from 2020 were collected below the Piute Farms Waterfall and translocated above it.

| Species | Year | Life Stage | N | Localities | River Mile |
|---|---|---|---|---|---|
| **BHS** | 2019 | Adult | 24 | - | 143-147.9 |
| **FMS** | 2019 | Adult | 24 | - | 145-147.9 |
| **FMS *x* RBS Hybrid** | 2019 | Juvenile | 16 | - | 76-133 |
| **FMS *x* RBS Hybrid** | 2020 | Juvenile | 25 | - | 25-91 |
| **RBS** | 2019 | Juvenile | 6 | - | 80-106 |
| **RBS** | 2020 | Larvae | 156 | 37 | 3.2-76.2; 161.2-184.3 |
| **RBS** | 2020 | Juvenile | 55 | - | 34-133 |
| **RBS** | 2020 | Adult | 147 | - | 0 |
| **Catostomidae** | 2020 | Larvae | 42 | 20 | 11.4-76.1; 165.2-184.3 |

**Table 2.**
NEWHYBRIDS classification results for all genotyped individuals. All species pairs of Bluehead Sucker (BHS), Flannelmouth Sucker (FMS), and Razorback Sucker (RBS) were evaluated to detect interspecific hybridization. The 'Field ID' column indicates morphologically-based species identifications performed in the wild (adult and juvenile life stages) or in a laboratory (larval life stage). Genetic determinations in NEWHYBRIDS are indicated in columns with shaded headings (blue = non-admixed; red = F1 hybrids; green = 2nd generation hybrid categories). Hybrid categories in column headings also indicate the species involved. Backcross categories are listed according to the species comprising the majority of the hybrid ancestry. For example, fish counted under the column heading 'FMS Bx (BHS)' had parents that were 1) a first-generation (F1) Bluehead Sucker *x* Flannelmouth Sucker hybrid, and 2) a non-admixed Flannelmouth Sucker.

| Field ID | Year | Life Stage | BHS | FMS | RBS | F1 Hybrid (FMS x RBS) | F2 Hybrid (BHS x FMS) | F2 Hybrid (BHS x RBS) | F2 Hybrid (FMS x RBS) | FMS Bx (BHS) | FMS Bx (RBS) | RBS Bx (FMS) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **BHS** | 2019 | Adult | 24 | - | - | - | - | - | - | - | - | - |
| **FMS** | 2019 | Adult | - | 24 | - | - | - | - | - | - | - | - |
| **FMS x RBS Hybrid** | 2019 | Juvenile | - | 1 | - | 14 | - | - | - | - | 1 | - |
| **FMS x RBS Hybrid** | 2020 | Juvenile | - | 2 | - | 16 | - | - | - | - | 7 | - |
| **RBS** | 2019 | Juvenile | - | - | - | 5 | - | - | - | - | 1 | - |
| **RBS** | 2020 | Larvae | - | - | 119 | 32 | - | 1 | 3 | - | - | 1 |
| **RBS** | 2020 | Juvenile | - | - | - | 48 | - | - | 1 | - | 6 | - |
| **RBS** | 2020 | Adult | - | - | 145 | - | - | - | - | - | - | 2 |
| **Catostomidae** | 2020 | Larvae | 2 | 12 | 8 | 17 | 1 | - | - | 1 | 1 | - |
| **Total** | | | **26** | **39** | **272** | **132** | **1** | **1** | **4** | **1** | **16** | **3** |

**Table 3.**
Mitochondrial DNA (mtDNA) haplotypes for larvae and juveniles determined to be hybrids through genetic analysis. The number of hybrid samples corresponding to each mtDNA haplotype (BHS = Bluehead Sucker, FMS = Flannelmouth Sucker, and RBS = Razorback Sucker) are provided according to their NEWHYBRIDS classifications. Hybrid categories in column headings also indicate the species involved. Backcross categories are listed according to the species comprising the majority of the hybrid ancestry. For example, fish counted under the column heading 'FMS Bx (BHS)' had parents that were 1) a first-generation (F1) Bluehead Sucker *x* Flannelmouth Sucker hybrid, and 2) a non-admixed Flannelmouth Sucker.

| Life Stage | mtDNA Haplotype | F1 Hybrid (FMS x RBS) | F2 Hybrid (BHS x FMS) | F2 Hybrid (BHS x RBS) | F2 Hybrid (FMS x RBS) | FMS Bx (BHS) | FMS Bx (RBS) | RBS Bx (FMS) |
|---|---|---|---|---|---|---|---|---|
| **2019-2020 Juveniles** | BHS | - | - | - | - | - | - | - |
| | FMS | 1 | - | - | - | - | 2 | - |
| | RBS | 79 | - | - | 1 | - | 13 | - |
| **2020 Larvae** | BHS | - | 1 | 1 | - | 1 | - | - |
| | FMS | - | - | - | - | - | - | - |
| | RBS | 49 | - | - | 3 | - | 1 | 1 |

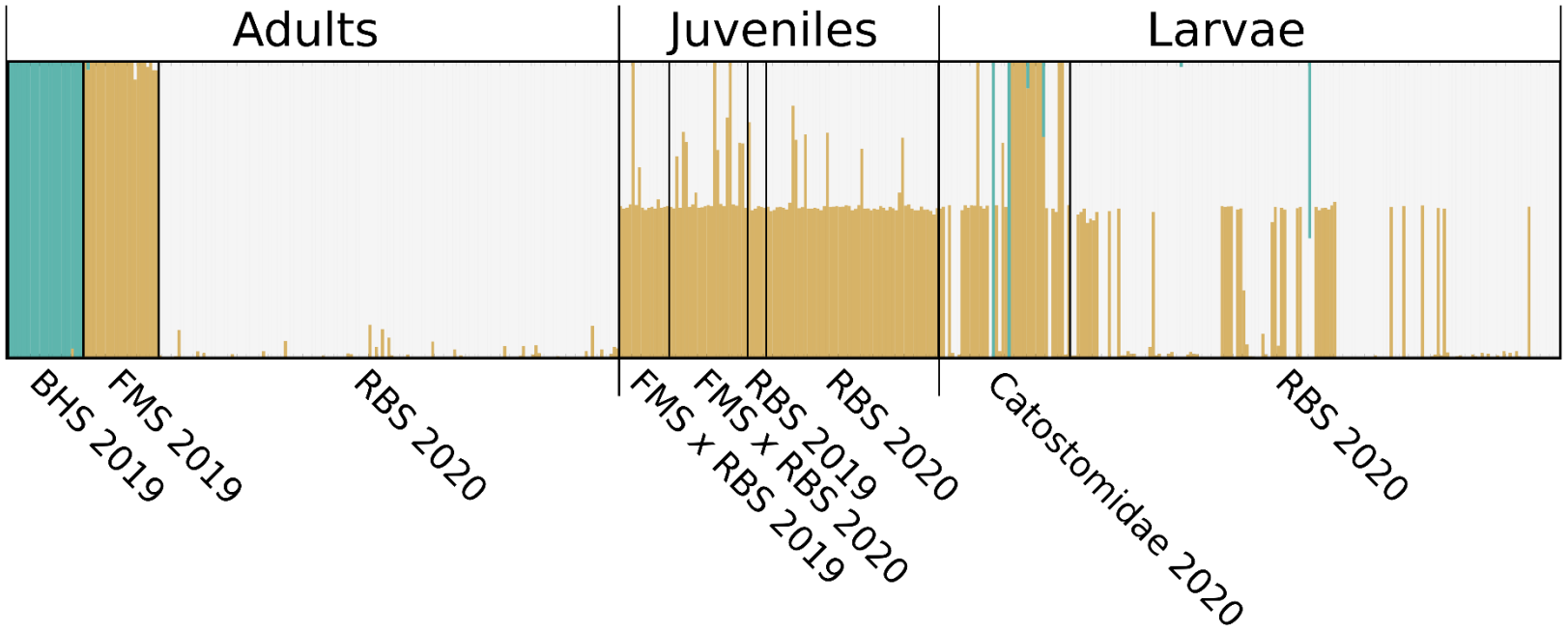


**Figure 1.**
Results of ADMIXTURE analysis on 23,186 unlinked single nucleotide polymorphisms (SNPs) in 495 samples. Each sample is represented by a vertical bar, with the proportions of color in each bar representing proportions of ancestry assigned to each of three different species. Green corresponds to Bluehead Sucker ancestry (BHS), brown represents Flannelmouth Sucker ancestry (FMS), and white indicates Razorback Sucker ancestry (RBS). Labels across the top of the plot represent the life stage at which sample groups were collected (adult, juvenile, or larval). Labels on the bottom of the plot indicate the morphological species identification of each sample and year of collection.

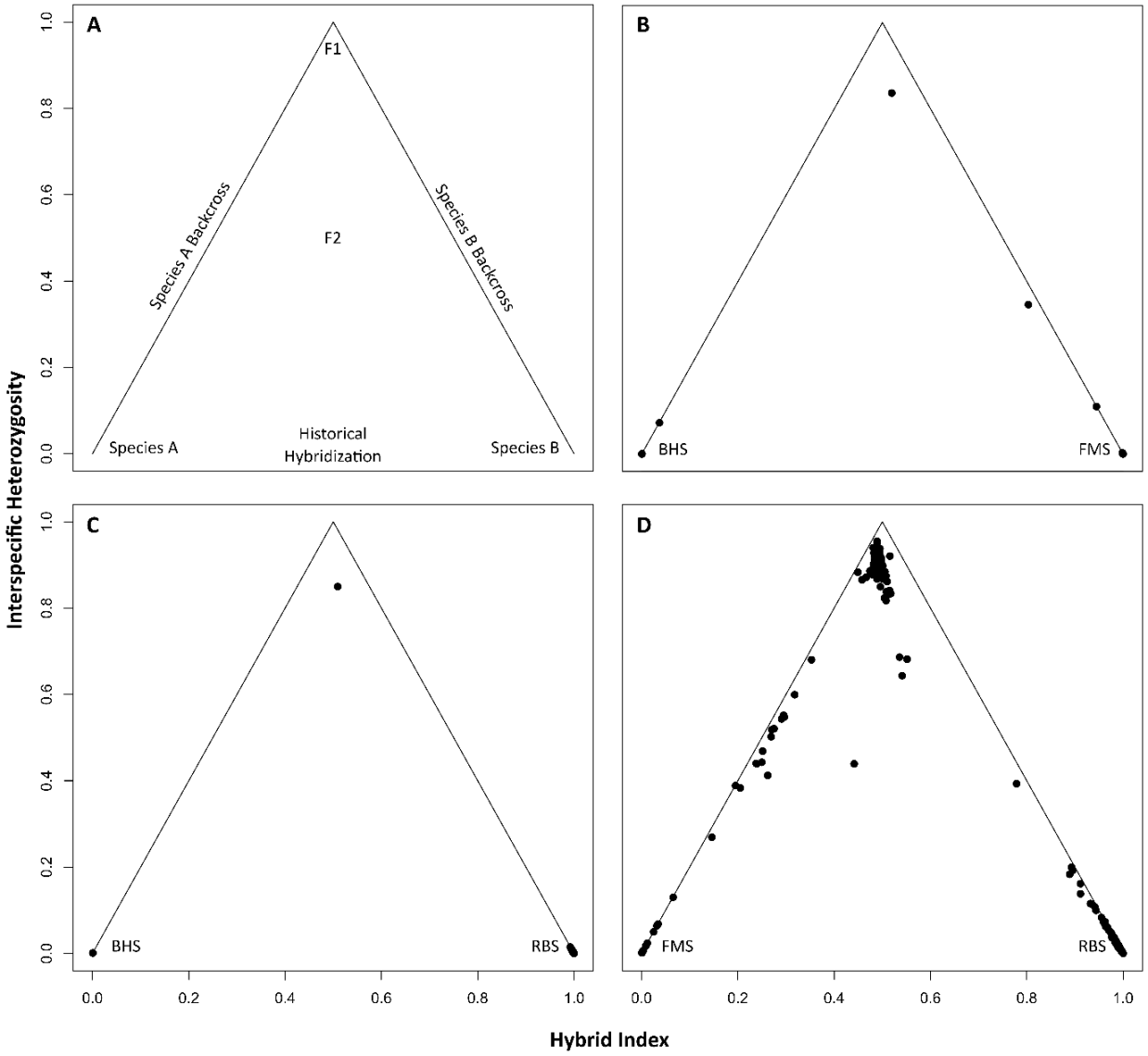


**Figure 2.**
Hybrid index results for each species pair. Each point represents a single sample. The x-axis represents the hybrid index of each sample as calculated in the R package INTROGRESS. The y-axis represents interspecific heterozygosity per sample. Plot A = an example of where different classifications of hybrids are expected to be observed in each triangle plot; B = Bluehead Sucker (BHS) *x* Flannelmouth Sucker (FMS); C = Bluehead Sucker *x* Razorback Sucker (RBS); and D = Flannelmouth Sucker *x* Razorback Sucker.

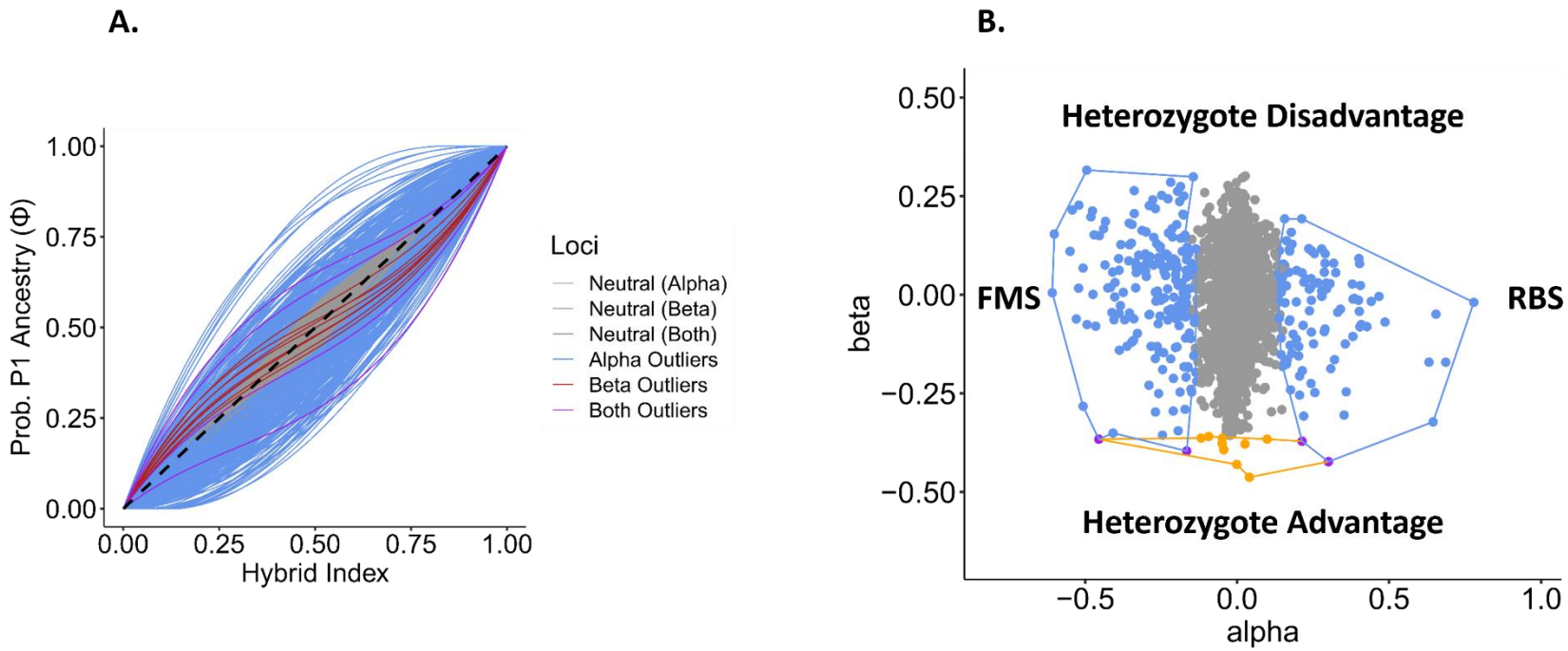


**Figure 3.**
The results of a Bayesian genomic cline analysis in BGC v1.03, which analyzed 2,786 single nucleotide polymorphisms (SNPs) representing fixed differences between Flannelmouth Sucker (FMS) and Razorback Sucker (RBS). Panel A shows the cline plot, in which the hybrid index (x-axis) reflects species ancestry (0.0 = Flannelmouth Sucker, 1.0 = Razorback Sucker) and the y-axis reflects the probability of an individual having a Razorback Sucker allele. Panel B plots α and β cline parameters for each locus (α = cline center; β = cline rate), with significant α-outliers in blue, β-outliers colored in yellow, and outliers for both cline parameters in purple. Using the first significance test in CLINEHELPR, which tests if cline parameter confidence intervals exclude the neutral expectations (α = 0; β = 0), 107 SNPs were determined to be α-outliers significantly favoring Razorback Sucker. An additional 192 SNPs were α-outliers significantly favoring Flannelmouth Sucker. Thirteen β-outlier SNPs were also detected, which generally represented a slight heterozygote advantage. Four of the β-outlier loci were also detected as α-outliers, with two having an additional slight advantage for the Razorback Sucker allele vs. the Flannelmouth Sucker allele at their respective loci.

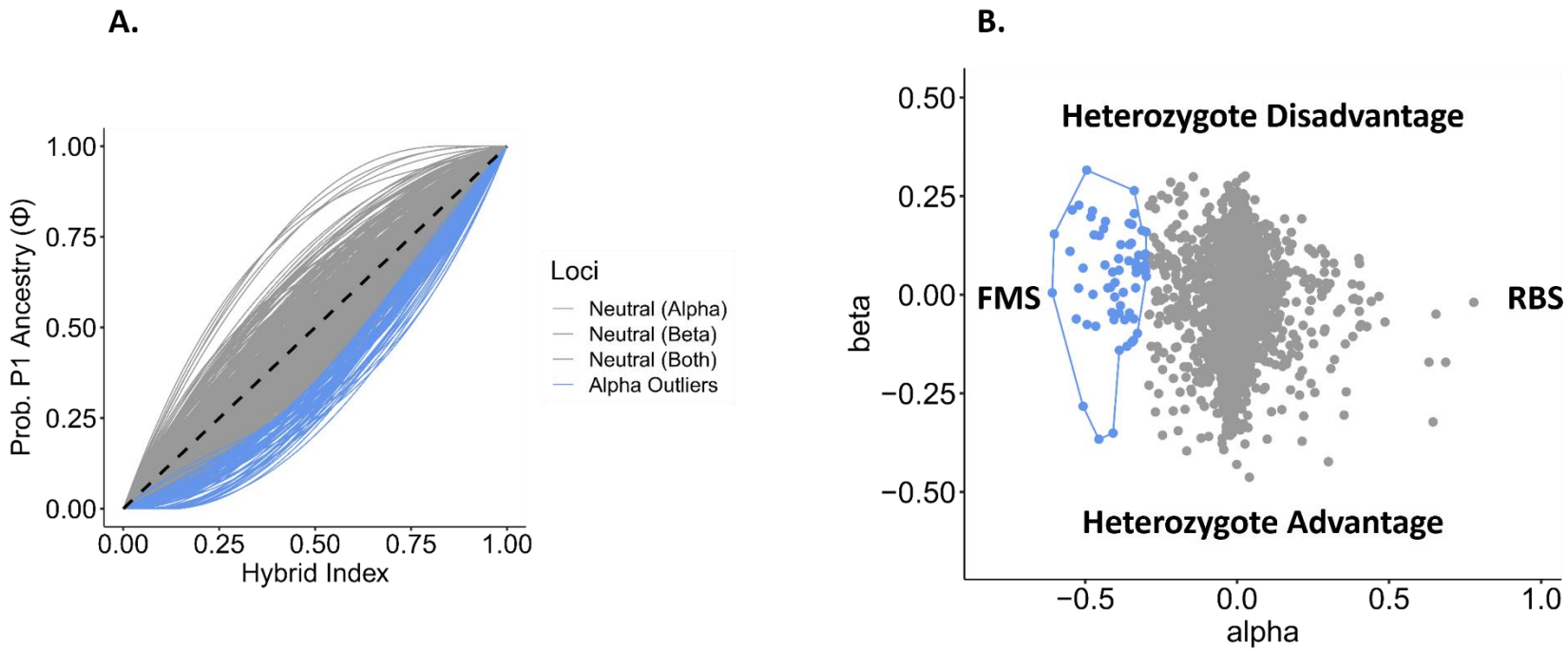


**Figure 4.**
The results of a Bayesian genomic cline analysis in BGC v1.03, which analyzed 2,786 single nucleotide polymorphisms (SNPs) representing fixed differences between Flannelmouth Sucker (FMS) and Razorback Sucker (RBS). Panel A shows the cline plot, in which the hybrid index (x-axis) reflects species ancestry (0.0 = Flannelmouth Sucker, 1.0 = Razorback Sucker) and the y-axis reflects the probability of an individual having a Razorback Sucker allele. Panel B plots α and β cline parameters for each locus (α = cline center; β = cline rate), with significant α-outliers in blue. Only α-outliers favoring Flannelmouth Sucker (N = 64) were detected when using the second significance test in CLINEHELPR, which typically provides a more conservative estimate of outlier loci by testing if per-locus cline parameters are statistically unlikely given the overall distribution of observed parameter values.