12-2016

Fishes as a Template for Reticulate Evolution: A Case Study Involving Catostomus in the Colorado River Basin of Western North America

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Fishes as a Template for Reticulate Evolution: A Case Study Involving *Catostomus* in the Colorado River Basin of Western North America

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Biology

by

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December 2016
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Abstract

Hybridization is neither simplistic nor phylogenetically constrained, and *post hoc* introgression can have profound evolutionary effects. Most studies have focused on tractable model systems, rather than organisms with complicated phylogenetic histories. Finescale Sucker (genus *Catostomus*) in western North America is recognized as a paradigm of fish hybridization. Yet, its extent of historic and contemporary introgression is largely unstudied, an aspect that impedes the resolution of its phylogeny as a baseline for conservation. To explore reticulation in this group, I assayed variation of 20 *Catostomus* species across temporal and geographic scales by analyzing hundreds of samples and employing a combination of molecular and bioinformatic approaches.

Chapter-1 examined hybridization among native suckers in an anthropogenically-fragmented environment using sequence analysis of mitochondrial and nuclear markers. Introgression was not detected, but hybridization with Utah Sucker likely lowers recruitment in the rarer Bluehead Sucker in the Bonneville Basin.

Chapter-2 tested discordant mitochondrial and morphological hypotheses by evaluating historical introgression in *Catostomus* using 14,007 ddRAD loci comprising 179,811 SNPs. A well-supported phylogeny offered insights into the effects of admixture on different phylogenetic methods, but tests for introgression allowed resolving previous taxonomic discords.

Chapter-3 dissected phylogenomic patterns and tested species-delimitations for taxa with admixed ancestry. Comparative population genetic and phylogenetic analyses supported taxonomic revisions in two species of conservation concern, and highlight that response to vicariant events is modulated by species-specific life history variation.

Chapter-4 assessed historic and contemporary admixture across 10 co-occurring endemic and invasive species using ~90k SNPs with hundreds of unlinked, fixed species-specific markers.

This genomic approach allowed to discern complex hybridization patterns across an entire basin and revealed elevated reproductive isolation at greater phylogenetic distance.

In combination, these analyses examined evolutionary reticulation among freshwater fishes of conservation concern in a large, geographically diverse, but heavily altered watershed, the Colorado River Basin, and highlighted both the complexity and constraints of introgressive hybridization. Insights from this study will aid in conservation of aquatic ecosystems in the arid Southwest further jeopardized by anthropogenic threats and an uncertain climatic future.

Acknowledgements

Numerous state, federal, and tribal agencies contributed field expertise, specimens, technical assistance, collecting permits, funding or comments for completion of this projects, Particular thanks goes to Paul Thompson and Krissy Wilson from Utah Division of Wildlife Resources who provided samples and support for Chapter 1. Samples were also provided by Brian Sidlauskas from the Oregon State Museum, Thomas Turner and Lex Snyder from the Museum of Southwestern Biology at University of New Mexico, Mary Peacock at the University of Nevada, and Jonathan Richmond at USGS. I would also like to acknowledge my fellow lab mates especially Steve Mussman and Tyler Chafin for their assistance and comments. I also acknowledge my advisors Michael Douglas and Marlis Douglas as well as my committee members Thomas Turner and Andrew Alverson for providing guidance and support. The Arkansas High Performance Computing Center also provided support and hardware accent. This research would not have been possible without funding provided by various state and federal agencies, and last but not least by the University of Arkansas through scholarships and generous endowments: The Bruker Professorship in Life Sciences (M.R.D.), 21st Century Chair in Global Change Biology (M.E.D.), Professor Delbert Swartz Endowed Graduate Fellowship, and James and Carol Hendren Fellowship.

Table of Contents

I. Introduction

Hybridization has traditionally been viewed as an evolutionary aberration, in that it contradicted the early modern synthesis with its focus on geographic isolation and selection in the development of reproductive isolation (Dobzhansky 1973). Sir Ronald Fisher went as far as to state that hybridization was "… the grossest blunder in sexual preference which we can conceive of an animal making" (Fisher 1930:130). However, even Carolus Linnaeus, who focused his life's work on describing species, abandoned his fixation on their discreteness after recognizing the importance of hybridization in *Linaria* plants (Larson 1968). Today, many examples of naturally occurring or artificially induced hybridization have been revealed, with causation often attributed to anthropogenic introductions and/or habitat modifications (Kane et al. 2009).

Rather than hybridization being viewed as a contradiction to the Biological Species Concept (Mayr 1942), it instead offers an opportunity to explore how a lack of reproductive isolation can actually facilitate the evolutionary process (Good et al. 2003). Hybridization, especially when coupled with introgression, has been suggested to play a diversifying role in both plants (Arnold 1992) and animals (Dowling and Secor 1997). Introgression, the incorporation of alleles from one species into the gene pool of another, can promote evolutionary change by i) generating new genetic variations, ii) transferring adaptive traits, and iii) producing new lineages that can exploit a novel niche in which neither parental taxa could succeed (Dowling and Secor 1997, Seehausen et al. 2014). At the same time, introgressive hybridization can have detrimental consequences for a species, especially if induced because of anthropogenic introductions of non-native species, by either i) disrupting local adaptation, or ii) genetically swamping endemics leading to the effective extinction of a species (Rhymer and Simberloff 1996).

Studying introgressive hybridization has been a near-Sisyphean task due to the inability to assay enough independent genetic markers that can discern introgression from incomplete lineage sorting (Eaton and Ree 2013), where the latter is defined as a situation in which alleles within one species share a more recent common ancestor with another species due to random assortment of ancestral polymorphisms, thus mimicking gene transfer due to introgression (Seehausen et al. 2014). Recent advancements in sequence technologies, however, allow the evolutionary effects of hybridization to be more formally evaluated by generating vast amounts of data across the entire genome, thus promoting more expansive research (Kane et al. 2009, Dasmahapatra et al. 2012, Eaton and Ree 2013).

These approaches have, in turn, underscored the 'semipermeable nature of species boundaries,' i.e. the ability of some regions of the genome to be introgressed more readily than others, a concept that has long been suggested (Key 1968), but only recently examined at a genomic level (Harrison and Larson 2014). While semipermablity itself is not surprising, particularly given its acceptance by early proponents of the strict reproductive isolation hypothesis (Mayr 1982), it has often been considered unimportant in the evolutionary process (Harrison and Larson 2014). This viewpoint was driven by the assumption that hybridization is rare, and that introgression usually leads to a 'genetic swamping' of one or both parental species due to a loss of reproductive barriers. The first argument has long since been rejected (Arnold 1992, Dowling and Secor 1997), whereas the second has only recently come under scrutiny. For example, introgression is now known to occur without dismantling species boundaries (Fontaine et al. 2015), and likewise, with a rather precise transmission of adaptive traits (Dasmahapatra et al. 2012, Nadeau et al. 2012). Consequently, these results have led to a more accepting view of introgressive hybridization as an evolutionary process, one that instead promotes gene flow

across semipermeable species boundaries, and has a precise effect on the genome (Nosil et al. 2009, Michel et al. 2010, Harrison 2012).

Large amounts of sequence data allow for simultaneous resolution of introgression at two levels: an historical context that relates to the semipermeable transfers of traits and the hybrid origin of species, as well as a more contemporary scale that reflects its variability across genomic and geographic clines (Fitzpatrick et al. 2010). Examination of historical introgression and its impacts on the genome, but also improved identification of backcrossed hybrids, are tasks highly relevant for management and conservation, which has often been hampered by the conflicting views of introgressive hybridization (Allendorf 2001).

In this dissertation, hybridization and introgression are examined at various temporal, geographic and taxonomic scales in Finescale Suckers (genus *Catostomus*), a group of freshwater fishes widely distributed throughout western North America, with several taxa of conservation concern. Species in the genus are known to readily hybridize, especially when invasive congeners have been introduced and/or habitats modified (Holden 1975, Douglas and Douglas 2010). Beyond initial admixture of two species, hybridization may have more far-reaching, albeit subtle effects such as potentially providing a bridge for introgression among native species that would not naturally hybridize (McDonald et al. 2008).

On top of contemporary admixture, hybridization in suckers (Catostomidae) has a long phylogenetic history driven by the tumultuous geologic and climatic events of western North America over the last 50mya, including volcanism, glaciation, extreme flooding and drought. These processes have driven catostomid diversification by providing long periods of isolation, augmented by episodes of secondary contact due to stream capture (Smith et al. 2010). Both, the

evolutionary history of the lineage and the ecological theatre that shaped its diversity, are aspects of interest and were explored in this dissertation.

In Chapter 1, hybridization was examined on a localized scale between two native catostomids (Utah Sucker *C. ardens* and Bluehead Sucker *C. Pantosteus discobolus*) in the Bonneville Basin. Anecdotal evidence suggested hybridization was of recent origin, likely driven by anthropogenic habitat modification. Sequence analysis of three nuclear loci and two mitochondrial genes was employed to test for (1) hybridization, and (2) introgression between the two species. In addition, morphological characters were used to test if (3) admixed individuals were morphological intermediate. The predictions were that hybridization was indeed occurring, but not introgression, and admixed individuals could indeed be identified based on phenotypic intermediacy.

For this study, using a small number of legacy markers (single gene sequencing) was appropriate, given the presumed recentness of hybridization, and sufficient to test for shallow introgression. However, the other three studies examined systems with a longer history of admixture, extended over larger geographic areas and involved additional taxa, requiring a more powerful approach. Hence, a next generation sequencing method (double digest restriction-site associated DNA, or ddRAD) was employed, that facilitated the generation of tens of thousands of nuclear loci to examine historic introgression and high levels of admixture.

In Chapter 2, discordance between morphology- versus mtDNA-based phylogenies in *Catostomus* was examined. Two valid hypotheses have been previously proposed for these discords: introgressive hybridization (Smith et al. 2013) and convergent evolution of morphologies (Chen and Mayden 2012). The former (i.e., 'Introgression Hypothesis') offers an explanation for admixed genotypes in morphologically distinct lineages, whereas the latter (i.e.,

'Convergent Evolution Hypothesis') posits that mtDNA genealogies accurately reflect the species tree, with distinct morphology arising multiple times through convergent evolution. To test several taxonomic hypotheses that related to (a) monophyly of the subgenera *Catostomus* and *Pantosteus,* and (b) position of unusual taxa such as *Xyrauchen*, a robust molecular phylogeny was first established using ddRAD data and a variety of phylogenic methods. Next, to detect historical introgression, a series of tests was conducted using Patterson's D-statistic. By mapping historical introgression events onto the phylogeny, the discords amongst competing hypotheses could be explored.

In Chapter 3, taxonomic hypotheses were tested at a finer geographic scale by examining concordance of population and phylogenetic patterns across two co-distributed species of the Upper Colorado River Basin (Flannelmouth Sucker *C. latipinnis* and Bluehead Sucker *C. P. discobolus*). Both are considered 'species of concern' and include a subspecies recently listed under the Endangered Species Act (Zuni Bluehead Sucker *C. P. d. yarrowi*). Analyses of ddRAD data was used to delineate species and identify evolutionary significant units. Congruent and discordant patterns were then interpreted within the context of drainage evolution and its interplay with unique life history attributes of each taxon.

In Chapter 4, contemporary hybridization and introgression was further explored across a broad geographic range, the Upper Colorado River Basin, and including all catostomids native and introduced into the system, again using ddRAD data. The objective of this large-scale analysis was to gain a nuanced perspective of the extent of admixture and relate it to covariates such as phylogenetic relatedness, geologic history, and anthropogenic disturbances prevalent in the region.

Combined, the four studies in this dissertation explored the complexity of introgressive hybridization in a system with a long history of admixture. Recent advancements in sequencing methods and new analytical protocols were employed to disentangle contemporary from historic signals and to resolve phylogeographic patterns, previously untested, but now interpretable within the complex geologic history of the region. This in turn allowed testing of alternate taxonomic hypotheses and to explore discordance amongst different phylogenies. In addition, unraveling patterns of introgressive hybridization at different temporal scales provides insights into a fundamental evolutionary process highly relevant for conservation efforts, especially with regard to fishes of southwestern North America and the numerous anthropogenic threats they face.

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II. Anthropogenic Impacts Facilitate Native Fish Hybridization in the Bonneville Basin of Western North America

Abstract

Drought, habitat modification, and invasive species are serious issues for native fishes that will indeed elevate in western North America concomitant with anthropogenic demands for water. The Bluehead Sucker (*Catostomus* (*Pantosteus*) *discobolus*) is both a "species of concern" and a benchmark for these impacts throughout its range, with sharp declines in recruitment a result of reduced flows, degraded habitat, elevated water temperatures, and introduced Brown Trout (*Salmo trutta*). However, an additional (and emerging) threat to the already low recruitment of Bluehead Sucker in the Bonneville Basin of Utah is hybridization with native Utah Sucker (*C. ardens*). Hybridization often stems from the forced co-mingling of species due to environmental degradation, with diminished recruitment of impacted species a result. Here we utilized three diagnostic nuclear loci and two mitochondrial genes to evaluate potential hybrids found in a recent assessment of Bluehead Sucker in the Bonneville Basin. Individuals putatively identified as hybrids in a targeted morphological evaluation were indeed verified as such, underscoring the utility of a combined morphological and molecular approach to hybrid verification. We also identified hybridization in both the Bear (one site) and Weber (three sites) rivers, but surprisingly with no evidence of introgression, suggesting in turn a reproductive barrier that circumvents backcrossing. Furthermore, a molecular approach also allows the managerial implications of hybridization to be more properly gauged, in that its impacts on reproductive output can be more appropriately calculated.

Introduction

Recent droughts in southwestern North American are predicted to extend in both prevalence (Seager and Vecchi 2010) and duration (Ault et al. 2014) as the climate continues to fluctuate. This issue, coupled with an increasingly anthropogenic demand for water, will prolong and extend already manifested impacts, and further imperil the depauperate biota of southwestern North America (Ficke et al. 2007). Unfortunately, this situation is greatly magnified in the Bonneville and Colorado River basins (Hinck et al. 2007).

In addition to anthropogenic and natural dewatering, endemic fishes are also impacted by contemporary fragmentation of an already reduced aquatic habitat (Fagan et al. 2002; Gober and Kirkwood 2010), and from introductions of non-native species (Fuller et al. 1999; Sabo et al. 2010). The former situation has drastically altered the physical and chemical properties of the riverine system, which has in turn diminished reproduction, slowed growth, and lowered survival rates of endemic fishes (Douglas and Douglas 2000). Furthermore, habitat alterations reduce the effectiveness of local adaptations and also promote successful invasions by creating habitats that juxtapose with the ecological affinities of the invader (Martinez et al. 1994). These factors, in combination, have reduced abundance and distribution of native fishes in the Bonneville and Colorado River basins (Minckley et al. 2003).

The Bluehead Sucker (*Catostomus* (*Pantosteus*) *discobolus*) is native to the Upper Snake River, Bonneville, and Colorado River basins (Page and Burr 2011), yet now occurs in less than 50% of its historic range, a situation that has led to its designation as a ‘species of concern’ in multiple states (Bezzerides and Bestgen 2002). Recent surveys of the Bonneville Basin (2004-2009) by Utah Division of Wildlife Resources (UDWR) did not detect Bluehead Sucker in the Utah portion of the Bear River, and only in several numerically reduced and heavily fragmented

populations in the Weber River (Webber et al. 2012). This reduction is especially disconcerting in that Bluehead Sucker represents not only an evolutionary significant unit (ESU) in these areas (Hopken et al. 2013), but potentially a distinct species as well [*C.* (*P.*) *virescens*; Smith et al. 2013; Unmack et al. 2014]. Regardless of its uniqueness, it is now confronted with considerable conservation and management issues.

In addition, one (of two) populations studied by Webber et al. (2012) in the Weber River now has limited recruitment, an aspect attributed to (1) delayed/ reduced spawning that stems from a reduction in both flows and summer water temperatures, and/or (2) predation on juveniles by the introduced and numerically superior (at 50:1) Brown Trout (*Salmo truuta*). A reduction in the recruitment of juvenile Bluehead Sucker may also be exacerbated by hybridization with native Utah Sucker (*C. ardens*). For example, Hopken et al. (2013) identified several potential hybrids in both the Bear and Weber rivers (the latter representing the largest populations in the drainage). However, the potential for introgression and its extent have not been quantified, and potential impacts on Bluehead Sucker remain but a supposition.

Here we used both mitochondrial and nuclear DNA loci to evaluate hybridization and the potential for introgression between these native species. We also assessed the targeted resampling of hybrids in the Weber River by UDWR so as to ascertain the efficacy of field-based identifications.

Methods

Samples were collected from the largest extant populations in the Bonneville Basin: one site on Smith's Fork near the confluence with the Bear River (Lincoln, WY) and three on the Weber River (Morgan, Weber and Summit counties, UT) (Figure 1). Sampling of adult Bluehead Sucker was done near Ogden on the Weber River in 2004 (N=14) and 2006 (N=26), and near

Coalville in 2007 (N=30). Sampling was also done on Smith's Fork in 2007 near the confluence with the Bear River (N=4). Subsequent targeted sampling of hybrids was done near Morgan on the Weber River in 2009 (N=5), and near Ogden in 2011 (N=2). Fin clips (total N=81) were stored in 95% ethanol for subsequent genetic analyses.

Samples were captured via electrofishing and putatively diagnosed was performed using morphological characteristics. Utah Sucker in the Weber River is predominant over Bluehead Sucker by at least 50:1, consequently initial identification consisted of looking for morphological characteristics that would distinguish a Bluehead Sucker or hybrid from a Utah Sucker while the fish was still immobilized in the water. Characteristics employed to do so were: 1) yellow pigment in the eye, and 2) a reduction in the size of scales posterior to the head. Additionally, lateral line scales of Bluehead Sucker and hybrids lack or had minimal black borders found in Utah Sucker. Earlier sampling (2004-2007) stopped here, thus all Bluehead Sucker and hybrids were sampled. Later sampling (2009-2011) went a step further by targeting only morphological hybrids using criteria listed in Table 1 once the samples were culled.

Genomic DNA was extracted from fin clips using the Qiagen DNAeasy Tissue Kit (QIAGEN Corporation, Maryland, USA), and used as template in polymerase chain reactions (PCR) to amplify mitochondrial (mt) DNA ATPase 6 and 8 genes, following Douglas and Douglas (2010). Three nuclear loci (CKA7, S7, and RP40) were also amplified following Quattro and Jones (1999), Palumbi and Baker (1994), and Friesen et al. (1999), respectively. Amplicons were sequenced using BigDye v. 3.1 (Applied Biosystem Inc., Forest City CA) and analyzed on an ABI Prism 3100 Genetic Analyzer.

Sequences were manually edited using Sequencher (v 5.4, Gene Codes, Ann Arbor MI) and aligned with BioEdit (v. 7.2.5, Ibis Biosciences, Carlsbad CA) against a reference database

of *Catostomus* species endemic or invasive to the Bonneville and Colorado River drainages, including Flannelmouth Sucker (*C. latipinnis*), Sonora Sucker (*C. insignis*), White Sucker (*C. commersonii*), Mountain Sucker (*C. platyrhynchus*), and Desert Sucker (*C. clarkii*). Individuals were then classified as either pure Bluehead Sucker, pure Utah Sucker, F1 hybrid, or backcrossed hybrid, following Bangs (2011).

Results

A total of 949 base pair (bp) was obtained across the three nuclear loci: CK7 (415 bp), RP40 (323 bp), and S7 (211 bp). Across all three loci 66 polymorphic sites were detected between Bluehead and Utah sucker, of which 46 where diagnostic for either Bluehead or Utah sucker (Figure 2). Across all catostomids found in the Colorado River and Bonneville basins, 122 polymorphic sites (12.86%) were detected across the nuclear loci, of which 94 were parsimony informative (Table 2).

During targeted sampling of Bluehead Sucker in both the Weber and Bear rivers some hybrids were collected, as confirmed by the molecular analyses. Of the four samples in the Bear River, one (25%) was a F1 (first-filial) cross between Bluehead and Utah sucker, while the remainder was pure Bluehead Sucker. Six of 30 sucker (20%) collected in the Weber River near Coalville (2007) were also F1 hybrids, with the remaining 24 (80%) again diagnosed as pure Bluehead Sucker. No hybrids were detected on the Weber River near Ogden in 2004 or 2006.

Putative hybrids captured during targeted sampling of morphological hybrids on the Weber River in 2009 (N=5) and 2011 (N=2) all were substantiated using molecular markers. No introgression was detected at any location. Of the 14 total hybrid samples, six had mtDNA of Bluehead Sucker while the remaining eight had mtDNA of Utah Sucker.

Discussion

Translocation of species and habitat modifications are important conservation issues in that they accelerate the rates of hybridization globally (Allendorf et al. 2001). Habitat modifications not only depress recruitment (and for a plethora of reasons; Didham et al. 2007) but also eliminate reproductive barriers that maintain the integrity of species (Holden 1975; Maan et al. 2010). For example, Bluehead Sucker in the Bonneville and Upper Snake River basins exhibits a reduced juvenile recruitment due to extensive habitat alterations and the introduction of invasive Brown Trout (Webber et al. 2012). It may additionally be impacted by hybridization with its native congener, Utah Sucker. Therefore, any reduction in its recruitment would have potentially serious results.

Hopken et al. (2013) found several potential hybrids during a range wide molecular assessment of Bluehead Sucker. We re-evaluated these samples so as to determine extent of hybridization and presence of introgression. In addition, putative hybrids were also morphologically determined in the field by UDWR personnel, and a molecular verification of these calls would be an important contribution to the management of the system.

In total, 14 hybrids between Bluehead and Utah sucker were detected across three locations on the Weber River and one location on the Bear River. This included three of the largest populations of Bluehead Sucker in the Weber River (i.e., Coalville, Morgan and Ogden).

Seven hybrids were found in both the Weber and Bear rivers in 2007 during targeted sampling of Bluehead Sucker, with no evidence of introgression. The lack of introgression may represent postzygotic reproductive isolation between these species, with sterile F1 hybrids the result. Similar reproductive isolation has been suggested in Bluehead Sucker, in that it will hybridize without introgression with both invasive White Sucker (*C. commersonii*) and

Longnose Sucker (*C. catostomus*) (Mandeville et al. 2015). A lack of introgression between Bluehead Sucker and other catostomids outside of the *Pantosteus* subgenus is also not surprising, in that postzygotic reproductive isolation (Stelkens et al. 2009) should be promoted by the antiquity of the separation (>15mya; Unmack et al. 2014).

A lack of introgression between Bluehead and Utah sucker also may be explained by its contemporary nature, in that it has not been documented prior to this study. Hence, our data may represent the first evidence within the system. Genetic monitoring should be continued so as to confirm a lack of introgression. Furthermore, sampling should be done using a non-targeted strategy so as to avoid bias with regard to hybridization rates in target populations.

Although a lack of introgression essentially avoids the issue of admixture and potential genetic swamping of the Bluehead Sucker gene pool, it nevertheless promotes a loss of reproductive output. For example, European Mink (*Mustela lutreola*) does not produce viable hybrids when hybridizing with introduced North American Mink (*M. vision*), yet is still in decline due to a loss of reproductive output (Rozhnov 1993). Similar results have also been noted with regards to hybridization in Salmonidae (Leary et al. 1993) and Cyprinidae (Konishi and Takata 2004). This aspect is more deleterious for the numerically diminished species, in this case Bluehead Sucker, since proportionally more of its total reproductive effort is lost as a result.

An additional exacerbation could be the potential presence of asymmetrical mating, e.g. preferential crossing between females of one species and males of another (Leary et al. 1993). For example, the endangered freshwater minnow *Pseudorasbora pumila* is in decline due to the introduction of its congener *P. parva*, with which it asymmetrically hybridizes to produce sterile offspring. The vast majority of these involve the female of the native species crossing with males of the introduced species, and as such, the loss of reproductive effort is considerably more

apparent in the native species (Konishi and Takata 2004). However, in our study, no evidence of asymmetrical mating was apparent, in that six of 14 hybrids (43%) exhibited mtDNA haplotypes diagnostic for Bluehead Sucker, whereas the other eight (57%) were characteristic of Utah Sucker. The lack of asymmetric mating could also be due to reduced sampling, yet it also fits with previous studies documenting hybridization among Bluehead, Flannelmouth (*C. latipinnis*), and White sucker (Douglas and Douglas 2010).

Nevertheless, a loss of reproductive output is an important issue for the ongoing conservation and management of Bluehead Sucker in the Bonneville Basin, and its elucidation requires an accurate identification of both species and hybrids (Godbout et al. 2009). Here we provide a genetic tool for identification of hybrids and assessment of introgression. In addition, we also evaluated field-based targeted resampling of hybrids, a process that successfully identified only hybrids (N=7), all of whom were adult F1s. Hybridization was found in the three largest Bluehead Sucker populations in the Weber River (Coalville, Morgan and Ogden), again indicating the broad extent of hybridization.

Conclusion

Hybridization without introgression has occurred between Bluehead and Utah sucker in both the Bear and Weber rivers of the Bonneville Basin, and represents a loss of reproductive output for Bluehead Sucker already hampered by low recruitment and small population sizes (Webber et al. 2012). Further genetic monitoring should be performed so as to confirm a lack of introgression, and herein we provide a simple and affordable tool for this purpose. If hybridization without introgression is indeed occurring, then its accurate morphological identification sustained by molecular analyses will be important for ongoing assessment.

Acknowledgements

Numerous agencies contributed field expertise, specimens, technical assistance, collecting permits, funding or comments for the completion of this study. First and foremost, we acknowledge Aaron Webber, Cassie Melon, and Krissy Wilson, who initiated this study as part of Utah Department of Wildlife Resources native fish monitoring. Thanks also to other UDWR personnel, in particular P. Martin, S. Jones, K. Beidinger, M. Breen, T. Hedrick, S. McKay, and C. Walker. We acknowledge natural resource agencies in Arizona, Colorado, New Mexico, Utah and Wyoming as well as the National Park Service, U.S. Bureau of Reclamation, and U.S. Bureau of Land Management. We are also in debt to students, postdoctorals and faculty who contributed to the development of our research: A. Albores, W. Anthonysamy, P. Brunner, T. Dowling, R. Cooper, J. Cotter, M. Davis, E. Fetherman, M. Hopken, M. Kwiatkowski, S. Mussmann, A. Reynolds, J. Reynolds, C. Secor, and P. Unmack. Sampling procedures were approved under IACUC permit 98-456R (Arizona State University) and 01-036A-01 (Colorado State University). Funding was provided by the Utah Division of Wildlife Resources through Endangered Species Mitigation Funds and State Wildlife Grants.

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Tables

Table 1: Morphological characteristics used to distinguish Bluehead Sucker (*Catostomus* (*Pantosteus*) *discobolus*) from Bluehead Sucker x Utah Sucker (*Catostomus ardens*) hybrids in the Weber River, Utah.

Morphological Characteristic	**Bluehead Sucker**	**Bluehead Sucker/Utah Sucker hybrid**
lateral line scale size	small (anterior) to large (posterior)	moderate (anterior) to large (posterior)
head length	short	moderate
cartilaginous ridge	strongly defined	moderately defined
lateral notches	strongly defined	moderately defined
papillae rows	7-8 on lower lip	5-6 on lower lip
papillae size	small	moderate

Table 2: Molecular characteristics of the three nuclear loci used to test for hybridity between Bluehead Sucker (*Catostomus* (*Pantosteus*) *discobolus*) and Utah Sucker (*Catostomus ardens*) in the Bonneville and Colorado River basins.

	Locus			
	CK7	**RB40**	**S7**	**Total**
Length (bp)	415	323	211	949
Parsimony informative	42	39	13	94
Variable sites	49	50	23	122
% variable sites	11.8%	15.5%	10.9%	12.9%

Figure Headings

Figure 1: Map of the Bonneville Basin (Utah). Dots represent cities and stars represent sample sites.

Figure 2: Polymorphic sites found in three nuclear DNA loci that distinguish Utah Sucker (UTS) and Bluehead Sucker (BHS). Rows are individual alleles whereas columns represent location of the nucleotide base within the sequence. A base matching the reference allele (UTS1) is represented by (.), whereas (-) represents a deletion.

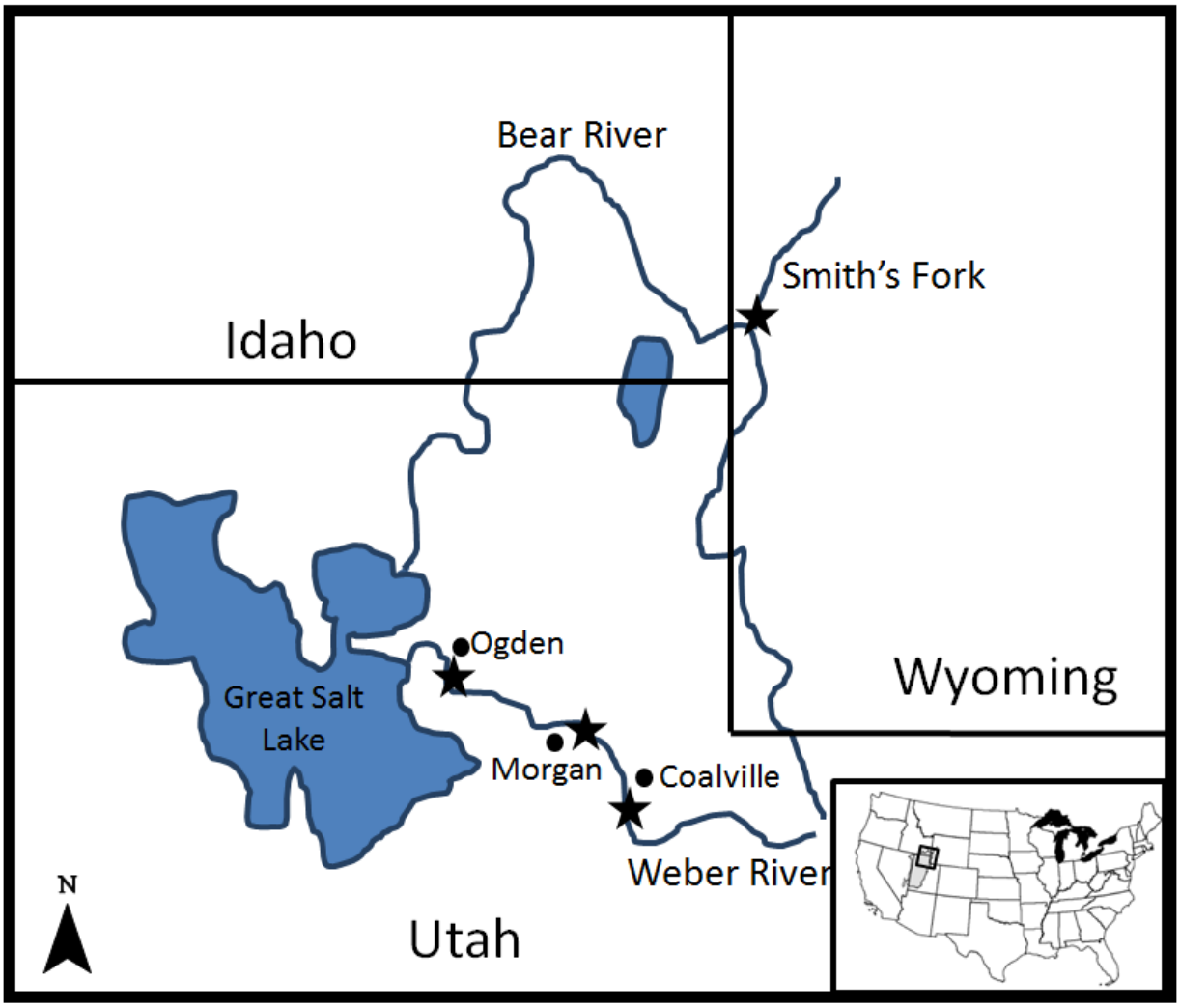
Bear River
Smith's Fork
Idaho
Wyoming
Ogden
Great Salt Lake
Morgan
Coalville
Weber River
N
Utah

Figure 1.

CK7

```
     0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 3 3 3
     6 9 0 3 4 5 5 6 6 6 6 6 6 6 6 6 6 7 8 2 3 0 1 9
     5 2 3 9 0 8 9 0 1 2 3 4 5 6 7 8 9 9 4 5 0 1 2 2
UTS1 G A A T G G T T G G T T A G C T G C T G T A A T
UTS2 . . C . . . . . . . . . . . . . . . . . . . . .
BHS1 A C . C A - - - - - - - - - - - - - . A . G T C C
BHS2 A C . C A - - - - - - - - - - - - - A A . G T C C
BHS3 A C . C A - - - - - - - - - - - - - . . A G T C C
```

S7

```
     0 0 0 0 0 0 1 1 1 1 1
     2 6 9 9 9 9 0 1 3 4 6
     5 6 1 3 4 5 0 6 1 2 5
UTS1 A A G G T G G T C G T
UTS2 . . . . . . . A . . .
UTS3 . . . . . . . . . A .
UTS4 G . . . . . . . . . .
BHS1 . G A T G A . . . . A
BHS2 . G A T G A T . . . A
BHS3 . G A T G A . . A . A
```

RB40

```
     1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
     4 5 6 7 8 8 9 9 9 0 0 0 0 0 0 1 1 1 1 2 4 4 4 5 5 5 5 5 6 9
     9 1 1 6 0 4 7 8 9 0 1 2 3 5 6 4 5 6 8 1 7 8 9 0 1 2 3 4 9 1 7
UTS1 G T T G T C C A G T G C C A C T C T G G T T T G T T T G T - G
UTS2 . . . . . . . . . . . . . . . . . . T . - - - - - - - - . . .
UTS3 . . . . . . . . . . . . . . G . . . . T . - - - - - - - - . . .
UTS4 . . . . . . . . . . . . . . . . . . . . . . . . - - - - . . .
UTS5 . . . . . . . . . . . . . . . . . . . . . . . - - - - - - - . . .
BHS1 T A A A C T - - - - - - - - . T - - - - . T . . . . - - - - - G C A
BHS2 T A A A C T - - - - - - - - . T - - - - . T . . . . - - - - - . C A
```

Figure 2.

III. The Effects of Introgression in Resolving Phylogenetic Discord in *Catostomus* (Pisces: Catostomidae)

Abstract

Phylogenomics now permits porous species boundaries to be more adequately explored, which has resulted in a greater understanding of, and appreciation for, reticulation as an evolutionary process. Incongruence among hypotheses derived from different genes still remains as a challenge in systematics, but with opportunities to examine the complexity that resides within these spatio-temporal patterns. This is especially true for non-model organism with potentially complex admixture histories, such as the genus *Catostomus* (Pisces, Catostomidae). We contrasted alternative mitochondrial and morphological hypotheses suggested for *Catostomus* by utilizing 14,007 loci generated from ddRAD sequencing. We first derived a phylogeny, then applied Patterson's D-statistic to resolve potential discords and tested the putative hybrid origin of two species. Our phylogenomic results juxtaposed well with a morphologically derived hypothesis, and revealed introgression within the genus that, in turn, limits the veracity of phylogenies based solely on mitochondrial data. Introgression also impacted the topologies produced by concatenation *versus* multispecies coalescent methods, which was explored through use of naïve binning. These results verified earlier simulation studies by providing an unambiguous and empirical example of the process. Fine-grained phylogenomic patterns underscored considerable diversity within *Catostomus* that extended across southwestern North America, and highlighted in particular two drainages (Virgin and Little Colorado rivers) that contain unique diversity. The complex introgressive histories uncovered herein should become particular foci for conservation and management.

Introduction

A principle goal of evolutionary biology is to resolve relationships among living organisms, and has relied traditionally on the assumption that gene trees represent the true evolutionary history of species (Rokas et al. 2003). However, recent phylogenomic studies have recovered considerable incongruence among gene trees and this has, in turn, promoted a deeper and more synthetic search for complex evolutionary patterns (Posada 2016). In addition, phylogenomic approaches yield large, multi-gene datasets that now permit incongruence and its causation to be more thoroughly parsed, to include the extent of ancestral introgression (Green et al. 2010). The more complex evolutionary histories that are recovered can then be juxtaposed against phylogeographic patterns derived from legacy approaches, and as a means of sharpening our evolutionary focus (Eaton and Ree 2013, Som 2015).

Generally, incongruence among genealogies, as implicated by introgression, is perceived as inconsequential to phylogeographic patterns. This perspective reflects the assumption that hybridization and introgression are not only rare but will also produce an inevitable 'genetic swamping' of parental taxa. Although the 'rarity' argument has long been rejected (Arnold 1992, Dowling and Secor 1997), that of 'genetic swamping' has only recently come under scrutiny. For example, introgression not only occurs without the subsequent dismantling of species boundaries (Fontaine et al. 2015), but also with adaptive traits rather precisely transmitted (Dasmahapatra et al. 2012, Nadeau et al. 2012). Consequently, a less myopic view of introgressive hybridization has now emerged, one that promotes species boundaries as semipermeable and with rather precise effects on genome evolution (Nosil et al. 2009, Michel et al. 2010, Harrison 2012).

However, traditional phylogeographic studies have relied on individual mitochondrial DNA genes, as excerpted from a single molecule, and thus may not reflect the complex

evolutionary history of study species (Avise 2009). This can be especially problematic in cases involving introgressive hybridization, which can often lead to mito-nuclear incongruence, particularly since mitochondrial genes are prone to purifying selection (Bermingham and Moritz 1998). Dobzhansky-Muller incompatibilities (i.e., the accumulation of incompatible epistatic interactions between diverging species), can also lead to asymmetric introgression, and with a rapid fixation of conspecific haplotypes when incompatibilities arise between the mitochondrial genes of one species and nuclear genes of a second (Burton and Barreto 2012). Consequently, mitochondrial genes can reflect a phylogenetic history quite different from that of the nuclear genome, and may thus conflict with the true species tree. Such mito-nuclear incongruence has been noted in numerous taxa: fruit flies (Bachtrog et al. 2006), lizards (Renoult et al. 2009), birds (Humphries and Winker 2011, Peters et al. 2014), frogs (Chen et al. 2009, Bryson et al. 2014), mammals (Galbreath et al 2010, Phillips et al. 2013), and fishes (Bossu and Near 2009, Willis et al. 2014, Akishinonomiya et al. 2016).

Mito-nuclear incongruence is especially problematic in fishes, due to the prevalence of hybridization, as facilitated by a natural history that involves external fertilization, weak reproductive isolation, and a relatively linear mode of stream dispersal (Hubbs, 1955; Campton, 1987). In the genus *Catostomus*, commonly known as Finescale Suckers, mito-nuclear incongruence has been proposed, because these freshwater fishes readily hybridize when invasive congeners are introduced and/or habitats modified (Holden and Stalnaker 1975, Douglas and Douglas 2010). Historically, periods of introgression among diverging lineages may have been promoted by the tumultuous geologic history of western North America, as species' ranges and abundances fluctuated in tandem with volcanism, glaciation, extreme flooding, and extended drought (Smith et al. 2013). These synergistic occurrences have propelled diversification by

providing long periods of vicariant-derived isolation, sporadically augmented by periods of secondary contact due to stream capture (Douglas et al. 1999, Smith et al. 2010).

The evolutionary history of *Catostomus* has proven contentious, due largely to conflicts between mitochondrial and morphological phylogenies. Two valid hypotheses have been proposed to explain these discrepancies: Introgressive hybridization (Smith et al. 2013), and the convergent evolution of morphologies (Chen and Mayden 2012). The former (i.e., the 'Introgression Hypothesis') offers an explanation for admixed genotypes in morphologically distinct lineages, with support provided by several well-documented and contemporary hybridization events. The second (i.e., the 'Convergent Evolution Hypothesis') posits that mtDNA genealogies accurately reflect the species tree, but with distinct morphologies arising multiple times through convergent evolution, thus promoting an argument that "…the long-thought idea of widespread genetic exchange across taxa represents a series of declarations that are either less parsimonious or cannot be tested" (Chen and Mayden 2012:207).

Indeed, an examination of historic introgression can be a near-Sisyphean task, in that separating introgression from incomplete lineage sorting is difficult, where 'lineage sorting' is defined as a situation in which alleles in one species share a more recent common ancestor with another due to random assortment of ancestral polymorphisms (Castillo-Ramírez and González 2008). However, recent research has clearly deciphered patterns of historical introgression through the use of Patterson's D-statistic (Durand et al. 2011), first employed to test for hybridization among early hominid lineages (Green et al. 2010), then successfully applied across a variety of subsequent taxa: *Heliconius* butterflies (Dasmahapatra et al. 2012), *Sceloporus* lizards (Leaché et al. 2013), and *Xiphophorus* fishes (Cui et al. 2013). This test relies on thousands of loci that can be generated by various methods, including restriction-associated

DNA (RAD) sequencing methods, that not only yield thousands of loci, but at a reduced cost (Baird et al. 2008, Kane et al. 2009, Dasmahapatra et al. 2012). Here we apply one such method (double digest restriction-site associated DNA, or ddRAD; Peterson et al. 2012) to resolve discord between mitochondrial and morphological phylogenies by testing for the presence of introgression among species. Different phylogenetic methods were also applied (concatenated SNPs *versus* multispecies coalescent) so as to understand the impacts of introgression on the capacity of various algorithms to resolve this complex evolutionary history. We also examined the phylogeography of *Catostomus*, with a special focus on the deserts of southwestern North America, and juxtaposed these results against the geologic history and drainage evolution of the region in an attempt to topographically define patterns of congruence amongst clades. This, in turn, can promote the conservation and management of species now in decline throughout the region.

Methods

Sampling

The genus *Catostomus* comprises at least 26 species, distributed primarily throughout western North America (Warren and Burr 2014). Recent phylogenetic evaluations (Smith et al. 2013, Unmack et al. 2014) suggest the necessity of taxonomic revisions, to include: Potential recognition of four new species in the subgenus *Pantosteus* (*C. P. virescens*, *C. P. bondi*, *C. P. lahontan*, and *C. P. jordani*); Confirmation of hybrid origin for two species (*C. P. columbianus*, *C. P. discobolus yarrowi*); And the clarification of alternative and conflicting phylogenetic hypotheses (Chen and Mayden 2012, Smith et al. 2013).

Our sampling included 20 species of *Catostomus*, plus *Xyrauchen texanus*, given its questionable phylogenic placement within the family. Two species of *Moxostoma* were added as outgroup (Table 1). The genus' status as MRCA (most recent common ancestor) with an estimated divergence time of <50mya (Ferris 1984) places it within a temporal frame appropriate for ddRAD analyses (Rubin et al. 2012, DaCosta and Sorenson 2015, Leaché et al. 2015). Fin clips and tissue plugs were collected between 1995-2011, and spanned the range of the focal taxa in western North America. This diversity of species and locations permitted a rather fine-grained examination of phylogeographic patterns (Table 1). Additional samples were obtained from museums: Ichthyology Collection, Oregon State University/ Corvallis; and Museum of Southwestern Biology, University of New Mexico/ Albuquerque), and were extracted as above (Table 1, see Acknowledgements for accession numbers).

Data Collection

Genomic DNA was extracted from tissue samples using the PureGene® Purification Kit or DNeasy® Tissue Kit (Qiagen Inc.) and stored in DNA hydrating solution. The quantity and quality of high molecular weight DNA were visualized on 2% agarose gels and quantified using a Qubit fluorometer (Thermo Fisher Scientific, Inc.). Library development followed previously published protocols (Peterson et al. 2012). Digest were performed using 1µg of genomic DNA with 10 units each of PstI (5'-CTGCAG-3') and MspI (5'-CCGG-3') in CutSmart buffer (New England Biosciences) for 20 hours at 37°C. Digests were visualized on 2% agarose gels, cleaned using AMPure XP beads, and quantified using a Qubit fluorometer. Approximately 0.1 µg of DNA was then ligated with barcoded Illumina adaptors, using custom oligos (Peterson et al. 2012). All barcodes differed by at least two bases so as to avoid fragment mis-assignments.

Ligations were pooled in sets of 48, cleaned and concentrated using AMPure XP beads, then size selected at 350-400 bps using the Pippin Prep automated size fractionator (Sage Sciences). Size-selected DNA served as template for Phusion high-fidelity DNA polymerase reactions using indexed primers and 10 cycles, following the manufacture's protocol (New England Biosciences). Reactions were cleaned with AMPure XP beads, and visualized on the Agilent 2200 TapeStation to confirm successful amplification. A final quality check of libraries was performed via qPCR at the University of Wisconsin Biotechnology Center (Madison), and two index libraries (96 samples) were pooled per lane for Illumina HiSeq 2000 100-bp single-end sequencing.

Filtering and Alignment

All analyses were conducted on the Arkansas High Performance Computing Cluster (AHPCC) at the University of Arkansas. Illumina reads were filtered and aligned using the pipeline PyRAD v.3.0.5 (Eaton and Ree 2013). Restriction site sequences and barcodes were removed, resulting in 87bp-fragments. Loci were discarded if they exhibited: 1) <5 reads within an individual; 2) >10 heterozygous sites per individual consensus, 3) >2 haplotypes per individual; 4) >75% heterozygosity per site among individuals; and 5) <50% of individuals within a given locus (per Leaché et al. 2015). Individuals with more than 80% missing data were also discarded.

Clustering thresholds were tested from 60%-95% and all yielded the same concatenated topologies, with differences found only in support values (i.e., similar clustering thresholds of 70%-85%). Previously derived uncorrected sequence variation in catostomid fishes (Chen and Mayden 2012) provided a mechanism to fix upon the 80% value (per Leaché et al. 2015).

Phylogenetic Methods

Loci produced in PyRAD were used to generate phylogenies based on concatenated SNPs: A maximum likelihood (ML) phylogeny (RAxML v. 7.3.2; Stamatakis 2006), using GTRCAT with 1,000 bootstraps; and a Bayesian (BA) phylogeny (MrBayes v. 3.2.3; Ronquist et al. 2012) using GTR with 10 million generations sampled every 1,000, with the first 25% discarded as burn-in.

Methods employing concatenated SNPs can inflate support values for poorly supported or erroneous nodes (Liu et al. 2015, Edwards et al. 2016). This is especially problematic if introgression has occurred between groups, potentially resulting in a topology unsupported by the majority of loci (Twyford and Ennos 2012, Leaché et al. 2014). Since introgression has potentially occurred between several species of *Catostomus*, two multispecies coalescent analyses (MSC) suitable for RAD loci were also employed (Leaché et al. 2015).

One MSC method (SVDquartets, Chifman and Kubatko 2015, as implemented in Paup* v. 4.0, Swofford 2003), utilizes one SNP per RAD-locus, with frequencies of SNPs for each species used to test support for quartets. To do so, individuals are *a priori* partitioned into species (or populations) based upon concordance between taxonomic hypotheses, geographic distributions, and high support values from phylogenetic analyses based on concatenated SNPs. All possible quartets were sampled and bootstrapped (N=1000).

The second MSC method (implemented in Astral v.4.7.8, Mirarb et al. 2014) constructs RAxML phylogenies using whole RAD loci, and then assesses support within these phylogenies using quartets. However, the small size of the RAD loci (87bps) may in turn promote poor support and, to correct for low resolution, a naïve binning method (Bayzid and Warnow 2013) was used such that RAD loci are randomly grouped into larger "supergenes" that then served as

input. The binning was varied, to include analyses with 1, 2, 3, 5, 10, 20, 50, and 100 combined RAD loci, so as to assess any potential bias similar to that found when SNPs are concatenated. Nevertheless, tradeoffs are still apparent in that lower binning levels yield less bias, but also provide less potential resolution, whereas greater binning levels may contain higher resolution albeit with greater bias (similar to methods based on concatenated SNPs). All ASTRAL runs were bootstrapped (n=128) with results reported as percentages.

Patterson's D-statistic

Once phylogenetic hypotheses were established with the above four methods, proposed introgression events based on incongruence between morphological and mitochondrial phylogenies were then tested, using Patterson's D-statistic to gauge reproductive isolation with subsequent gene flow (Durand et al. 2011, as implemented in PYRAD).

Based on phylogenetic conflicts between morphological (Smith et al. 2013) and mitochondrial (Chen and Mayden 2012) analyses, the following pairs of species were examined: Bluehead Sucker (*C. P. discobolus*) x Mountain Sucker (*C. P. platyrhynchus*); Bluehead Sucker x Desert Sucker (*C. P. clarkii*); Bluehead Sucker x Rio Grande Sucker (*C. P. plebeius*); Flannelmouth Sucker (*C. latipinnis*) x Sonora Sucker (*C. insignis*); Sonora Sucker x Razorback Sucker (*X. texanus*); and Flannelmouth Sucker x Razorback Sucker. The putative hybrid origin of the Bridgelip Sucker (*C. P. columbianus*) was also examined.

All members of a given species were used in each D-statistic test, and all combinations were permuted so as to provide multiple tests per introgression event. Z-scores for individual permutations were derived from 1,000 bootstrapped calculations (per Eaton and Ree 2013). Each permutation within a given introgression test was also used to calculate an overall Z-score and its

range. Thresholds for significance were adjusted for multiple tests using the Holm-Bonferroni correction, with α=0.01 (per Eaton et al. 2015). In cases where variance was high within a species, populations were split according to geographic regions so as to more appropriately gauge fine-grained patterns of introgression that may have impacted some populations more so than others.

Results

After filtering, 14,007 loci containing 179,811 SNPs were obtained, of which 67.9% (N=122,128) were parsimoniously informative and contained 32.68% missing data. These data also produced 13,989 unlinked SNPs. Average post-filtering coverage was 17.3x and all individuals (N=184) had >8.6x coverage, with <80% missing data.

Phylogenetic Analyses

Both ML and BA concatenated SNP methods produced the same topology, with support among-clades at 100% (Figure 1). However, support within clades varied somewhat, reflecting fine-grained phylogeographic patterns that are less distinct and which indicate potential ongoing gene flow among populations. These were subsequently collapsed (results not shown).

With the exception of Longnose Sucker (*C. catostomus*) falling outside of all in-group species (Figure 1), the remainder of *Catostomus* (clade A) divided into two large clades, one of which consisted of what Smith et al. (2013) termed subgenus *Catostomus* (clade C), but with Razorback Sucker (*X. texanus*) as sister to both Sonora Sucker (*C. insignis*) and Flannelmouth Sucker (*C. latipinnis*) (clade M). The second represented the subgenus *Pantosteus* (clade V),

containing all *Pantosteus* with the exception of the Bridgelip Sucker (*C. P. columbianus*) that fell as sister to Tahoe Sucker (*C. tahoensis*) (clade J) within the subgenus *Catostomus* (clade C).

Within *Pantosteus* (Figure 1: clade V), two distinct monophyletic sister clades were identified, one corresponding to what is referred to as '*platyrhynchus*' (clade W), and containing five monophyletic groups that corresponded to species described by Smith et al. (2013) as: *C. P. jordani* (Missouri River Basin), *C. P. bondi* (Columbia River Basin), *C. P. lahontan* (Lahontan Basin), and two groups of *C. P. platyrhynchus* (Upper Snake River/ Bonneville/ Colorado River basins) (clades DD and EE). The remainder of *Pantosteus* (i.e., '*discobolus clade*'; clade FF) clustered into six monophyletic groups, three of which corresponded to the previously-described Colorado River Basin Bluehead Sucker (*C. P. discobolus*; clade PP), and the Upper Snake River/Bonneville Basin Bluehead Sucker (*C. P. virescens*; clade MM), as well as an undescribed clade (OO) that included Zuni Bluehead Sucker (*C. P. d. yarrowi*) and Bluehead Sucker from the Little Colorado River. The remaining three clades corresponded to Rio Grande Sucker (*C. P. plebeius*; clade HH), Santa Ana Sucker (*C. P. santaanae*; clade JJ), and Desert Sucker (*C. P. clarkii*; clade KK).

The MSC method SVDQUARTETS produced a topology similar to those from the concatenated SNP methods, but with differences in placement of the root within the '*discobolus*' clade (FF; Figure 1). The concatenated methods placed Bluehead Sucker (*C. P. discobolus* and *C. P. virescens*) outside the remaining species (GG; Figure 2A), whereas Rio Grande Sucker (*C. P. plebeius*) was placed outside by SVDQUARTETS (GG'; Figure 2B).

Binning of <5 RAD loci in the ASTRAL analysis resulted in little or no nodal support, and values were thus not reported. When binning included 5-10 RAD loci, the topology matched that of other MSC methods, with Rio Grande Sucker at the root of the '*discobolus*' clade (GG';

Figure 2B). With binning of >10 RAD loci, nodal support was generally higher and the topology reflected that from the concatenation SNPs method, with Bluehead Sucker at the root of the '*discobolus*' clade (GG; Figure 2A, Table 2).

Phylogenetic Discordance and Introgression

Tests for introgression in the Bridgelip Sucker, a species of putative hybrid origin, were not significant despite employing several potentially co-occurring *Pantosteus* species, to include Bluehead Sucker from the Upper Snake River/ Bonneville Basin, and Mountain Sucker from the Columbia River/ Lahontan/ Upper Snake River/ Bonneville basins (Table 3A).

Mountain, Desert, Bluehead, and Flannelmouth suckers were each split for the remainder of the tests, due to their high within-species variance. Mountain Sucker partitioned into five clades representing the four revised species (above), as well as the split of *C. P. platyrhynchus* between Bonneville/Snake and Colorado rivers. Desert Sucker was split into three clades (i.e., Virgin, Bill Williams, and Gila rivers), all of which were supported at 100% in both concatenated SNPs phylogenetic methods. Flannelmouth Sucker was split into the same three clades previously derived by phylogenetic methods (i.e., Virgin, Little Colorado, and Colorado rivers), then further split between Grand Canyon and the remainder of the Upper Colorado River. Samples from Wenima Wildlife Area (AZ) were also split from the rest of the Little Colorado River, due to their substantially different D-statistic values. Bluehead Sucker was divided into Bonneville Basin, Grand Canyon, Little Colorado, and Upper Colorado rivers, much as was found in Flannelmouth Sucker, and (again) with a further split in the Little Colorado River due to the presence of conspecific alleles (as noted by Turner and Wilson 2009).

Introgression between Flannelmouth and Sonora suckers (Table 3B) was also noted at two sites (Virgin River and Wenima Wildlife Area), with but two individuals (67%) significant in the latter. Evidence was also detected for introgression between Razorback and Sonora suckers (Table 3C), but not between Flannelmouth and Razorback suckers (Table 3D).

Introgression was also detected between Bluehead and Desert suckers, but with considerable variance in the D-statistic that exhibited a geographic pattern among sites. In Bluehead Sucker, all groups in the Colorado River basin were significantly introgressed, save for two sites in the Little Colorado River drainage (i.e., Willow and Silver creeks). The D-statistic was higher for sites in the Upper Colorado River Basin above Lake Powell (AZ/UT border) than for Grand Canyon and the Little Colorado River, its major tributary in the Lower Basin. No introgression was detected in Desert Sucker, save for a single sample from the Virgin River (Table 3E).

Significant introgression was also detected between Bluehead and Mountain suckers in both the Colorado River and the Upper Snake/ Bonneville basins. However, there were no significant geographic patterns of introgression for these two species when compared within and between basins (Table 3F).

Interestingly, introgression of Rio Grande Sucker into Bluehead Sucker was not detected, save for a single population in the Rio Nutria of the Zuni River, NM (a tributary of the Little Colorado River). However, other Zuni River populations (i.e., Agua Remora and Tampico Springs) and the remainder of the Little Colorado River showed no significant introgression (Table 3G). Similarly, there was a lack of significant introgression among Desert, Santa Anna, and Rio Grande suckers (Table 3H), despite their unusual arrangement in the concatenated phylogenies (Figure 1).

Discussion

Incongruence among phylogenies produced by different genes and alternative methods is an unmitigated difficulty for modern systematics (Rokas et al. 2003), so much so that it recently appeared as a symposium topic in the annual meeting of the *Society for Systematic Biology* (Posada 2016). However, an opportunity to resolve these complex evolutionary histories is provided by phylogenomics, even in the face of reticulated evolution and the phylogenetic incongruence it fosters (Som 2015). Yet, the question of how precisely can these large multi-locus datasets be evaluated in the face of incongruence (i.e. concatenation *versus* multi-species coalescent methods) has sparked several recent debates (Springer and Gatesy 2016, Edwards et al. 2016).

In this study, phylogenomic analyses of *Catostomus* revealed a clade considerably impacted by historical introgression. Several different analytical approaches were employed as a means of evaluation: A) Effects of gene incongruence on concatenated and multi-species coalescent methods; and B) Naïve binning as a novel method to test for potential effects of concatenation. The reticulate evolutionary history of this group was then resolved by using D-statistic tests to successfully unravel phylogenetic discord, followed by comparative phylogenomics that established congruent patterns for the evolutionary history of the two major clades.

Effects of Introgression on Concatenated and MSC Phylogenetic Analyses

While all phylogenetic methods used in this study produced largely congruent topologies, one source of contention was the node produced by concatenated SNP methods (ML and BA) *versus* one resulting from multi-species coalescent methods (quartet assembly). This particular

conflict fixed on the root-placement of the '*discobolus*' clade in *Pantosteus* (per Smith et al. 2013) that comprised Bluehead, Desert, Rio Grande, and Santa Anna suckers. The concatenated method approach strongly supported Bluehead Sucker as the basal branch of this clade (Figure 2A), whereas the MSC method (SVDQUARTETS) supported instead Rio Grande Sucker (Figure 2B); the latter also represented as such in previous morphological (Smith et al. 2013) and mitochondrial phylogenies (Unmack et al. 2014).

One potential explanation for this could be an erroneous phylogenetic grouping caused by introgression, a situation that may also impact results from our concatenated methods in that only a small percentage of introgressed alleles are required to contravene the relationship expressed by the majority of loci. Thus, our multi-species coalescent (MSC) phylogeny may provide a more appropriate resolution in that it utilizes only unlinked/ independent SNPs, and thus reflects relationships found in the majority of loci, whereas our concatenated method results could be biased and driven instead by potential introgression between Mountain and Bluehead suckers, as was detected in the D-statistic (Table 3F).

To further investigate this argument, we applied a naïve binning approach in which varying amounts of RAD-loci were randomly binned then subsequently treated as "supergenes" for analysis using another MSC method (i.e., ASTRAL). Here, the assumption was that fewer binned RAD-loci should yield results similar to the MSC phylogeny, and if concatenation itself is the cause of discordance, then binning with a greater number of RAD-loci should shift support to the topology identified by the concatenated SNPs methods. And in fact, this is exactly what we found. Lower levels of binning (≤10 loci) yielded a topology congruent with that of the MSC phylogeny, whereas greater levels (≥15 loci) produced instead a topology that juxtaposed with concatenated methods (Table 2). It should be noted that D-statistic results showed no significant

introgression between Desert and Rio Grande sucker, thus eliminating another potential explanation for the erroneous grouping (Table 3H).

These results also parallel the recent debate between concatenated and MSC methods. Springer and Gatesy (2016) argued that MSC methods relied on unrealistic models that failed to account for gene incongruence, other than from incomplete lineage sorting, and are thus inappropriate for resolving introgressed phylogenies. Concatenation was favored instead, since introgression should be masked and the resulting phylogeny will then represent the major of the loci. However, recent studies employing simulated data (Leaché et al. 2014, Solís-Lemus et al. 2016) showed that even with low levels of introgression, the concatenated methods consistently failed to capture the true species tree, whereas MSC methods not only did so, but also amid low levels of introgression. Support for the latter is reflected in our results, and thus provides an empirical proof of concept for a theoretical idea that was fostered via simulations.

Tests for Introgression that Resolve Phylogenetic Discord

We found several statistically significant introgression events using the D-statistic test, (Table 3), and these resolved the discords observed between our phylogeny and previous mitochondrial phylogenies. This included the following erroneous placements in mitochondrial phylogenies: 1) Razorback Sucker as sister to Sonora Sucker, 2) Mountain Sucker from Colorado River/ Bonneville Basin as sister to Bluehead Sucker, 3) some Flannelmouth Sucker populations placed within Sonora Sucker, and 4) some Bluehead Sucker populations that fell within Rio Grande and Desert suckers (see mitochondrial phylogenies in Doosey et al. 2010, Chen and Mayden 2012, Unmack et al. 2014). These data confirm the ‘Introgression Hypothesis’ (Smith et

al. 2013), and reflect the importance of phylogenomic analyses in resolving cases of reticulated evolution, as found in *Catostomus*.

Our results also underscore potential dangers inherent in the reliance upon single-gene phylogenies, such as those based on markers from the mitochondrion, in that Dobzhansky-Muller incompatibilities coupled with purifying selection can lead to a rapid fixation of invasive mitochondria, thus yielding phylogenies discordant with species histories (Burton and Barreto 2012). Despite this admonition, studies that resolve species and develop conservation plans have largely relied upon single mitochondrial or nuclear gene phylogenies (Allendorf et al. 2010, Frankham 2010, Carstens et al. 2012). Our results underscore the importance of genomic approaches in these situations, and provide tacit support for previous admonitions (i.e. Carstens et al. 2013, McCormack et al. 2013, Steiner et al. 2013).

Species of Hybrid Origin

Two species in the subgenus *Pantosteus* have been proposed as species of hybrid origin, yet these projections are not supported by our analyses. The first is the Bridgelip Sucker (*C. P. columbianus*), originally described as a *Pantosteus* (i.e., *P. columbianus*, Snake River; Eigenmann and Eigenmann 1893), then subsequently re-described as a *Catostomus* (i.e., *C. syncheilus*, Hubbs and Schultz 1932), and finally as a hybrid lineage based on morphological characteristics shared with Tahoe Sucker (*C. tahoensis*) and an unidentified *Pantosteus* (Smith et al. 2013). Our results instead place Bridgelip Sucker as sister to Tahoe Sucker, a situation congruent with the mitochondrial phylogeny. We also found no molecular evidence of potential introgression from any *Pantosteus* that may have been potentially sympatric with Bridgelip Sucker (Table 3A). However, our diagnosis is based on but two samples of Bridgelip Sucker

(Donner und Blitzen River; Oregon State Museum) and thus further sampling should be performed.

The Zuni Bluehead Sucker (*C. P. discobolus yarrowi*) was also postulated as being of hybrid origin between Bluehead Sucker (*C. P. discobolus*) and Rio Grande Sucker (*C. P. plebeius*) (Smith et al. 1983). However, our results refute the argument of hybrid origin by identifying but a single population (Rio Nutria) that reflects alleles introgressed from Rio Grande Sucker (Table 3G). Our results are also congruent with other allozyme (Crabtree and Buth 1987) and single-gene sequencing studies (Turner and Wilson 2009).

Phylogeography of the Desert Southwest

Fossil data indicate that *Catostomus* originated in the Pacific Northwest and subsequently diversified south and east through the Great Basin during the Miocene (Smith et al. 2013). Based on our phylogeny, the separation between *Catostomus* and *Pantosteus* subgenera occurred before their dispersal south and east, due to the presence of early splits in *Catostomus* between northern taxa that spread throughout the proto-Columbia Basin (*C. rimiculus*, *C. microps*) and drainages east of the continental divide (*C. commersonii*), and southern taxa (Figure 1: clade G) that radiated throughout the Great Basin and neighboring Colorado River Basin. Given this, the phylogeography of the two subgenera (i.e., *Catostomus* and *Pantosteus*) then represents a comparative analysis of this radiation, and can be compared and contrasted throughout southwestern North America, the region from which much of our sampling is based (Figure 3).

Diversification within *Pantosteus* occurred after its spread into the Great Basin, with the first split occurring in Mid-Miocene between the '*discobolus*' (clade FF) and '*platyrhynchus'* (clade W) species-groups (Smith et al. 2013). All extant members of the '*discobolus*' group

(clade FF) occur in southwestern North America, with three (of six) species found in the Colorado River Basin. Our MSC phylogeny concurred with both previous morphological and mitochondrial data, as well as the fossil record, with Rio Grande Sucker representing the basal separation. This presumably occurred during a period of elevated tectonism and climatic oscillations that facilitated a connection not only between the Rio Grande and the Colorado River basins during Mid-to-Late Miocene, but also rivers in northern Mexico (Chamberlain et al. 2012).

Colorado River Basin

Subsequent to the split between the Rio Grande and Colorado River basins, there was a separation between Lower and Upper Colorado River basins, with Desert Sucker sequestered in the former and Bluehead Sucker in the latter (Figure 4G). This presumably occurred during the formation of Grand Wash and following formation of the Grand Canyon at 4.8mya (Kimmel 1975, Spencer et al. 2013). This same split is also found in the *Catostomus* subgenera, with Sonora Sucker in the Lower Colorado River Basin as sister to Flannelmouth Sucker in the Upper Basin. It has also been noted in snails (Hershler and Sada 2002) and other fishes in the region (DeMarais et al 1992).

Subsequent to this separation, there were additional reconnections that promoted introgression between Bluehead and Desert suckers, as well as Flannelmouth and Sonora suckers. However, introgression is seemingly limited to the Upper Colorado River Basin and Virgin River, and was not detected in the Lower Colorado River Basin. This may relate to Late Pleistocene drought (i.e., the Hypsithermal; Pielou 1974) that encouraged southward movement of Upper Colorado River species (Flannelmouth and Bluehead suckers) into the Grand Canyon,

thus promoting hybridization with Lower Colorado River species. Subsequent movement back into the Upper Colorado River occurred once pluvial conditions returned (Douglas et al 2003).

Within the '*discobolus*' group, Desert and Santa Anna suckers are sister taxa (clade II), a seemingly incongruent geographic occurrence in that they are separated by the Mojave Desert. However, a connection has long been hypothesized between the Lower Colorado River and the coastal drainages of Southern California, due to the apparent taxonomic similarities found in each (Hubbs and Miller 1948, Oakley et al. 2004). A reasonable window for such an inter-basin transfer would be Mid-to-Late-Pleistocene, particularly given that pluvial lakes formed in the Mojave Desert during this period, a result of flooding by the Colorado River (Enzel et al. 2003, Roskowski et al. 2010). Within the Lower Colorado River Basin, two well-supported clades (i.e. Bill Williams and Gila rivers) separated by the mainstem Colorado River, were detected in both Sonora and Desert suckers (Figure 4A, F).

Potential Endemism in the Little Colorado and Virgin rivers

In the Upper Colorado River Basin, both Bluehead and Flannelmouth suckers in the Little Colorado River consistently split from the rest of the conspecific populations in the basin (Figure 4C, D). The Upper Little Colorado River became isolated from the Colorado River by the formation of Grand Falls some 20kya (Duffield et al. 2006). However, and despite the late occurrence of this vicariant event, the Little Colorado River also harbors other unique species to include the Little Colorado River Spinedace, *Lepidomeda vittata* (Minckley and Carufel 1967), a potentially unique form of Flannelmouth Sucker (Miller 1972, Minckley 1973), as well as a unique subspecies of Bluehead Sucker (Zuni Bluehead Sucker; Cope and Yarrow 1875).

The Zuni Bluehead Sucker, currently listed under the Endangered Species Act (Federal Register 2014) is presumed to occur on the Defiance Plateau and in the Zuni River, both of which drain into the Little Colorado River. The Defiance Plateau and the Zuni River also separate as discrete, monophyletic populations within a larger paraphyletic group. This paraphyly can be resolved by grouping the remainder of the Little Colorado River with the Defiance Plateau and Zuni River, as potentially supported by the larger caudal fins and more terete body found throughout the Little Colorado River, and which represent distinguishing characteristics for the Zuni Bluehead Sucker (Minckley 1973).

The Little Colorado River Flannelmouth Sucker was also mentioned as a potential new species distinct from the rest of Flannelmouth Sucker (Minckley 1973), replete with a 'manuscript name' (*C. sp. "crassicauda,"* Miller 1972). While the Little Colorado River Sucker does emerge from our data as a distinct group, it falls within a paraphyletic Flannelmouth Sucker. Its putative recognition as distinct would necessitate a separation of the Virgin River Flannelmouth Sucker from Flannelmouth Sucker *sensu lato*, thus yielding three separate taxa. Morphological support for the Virgin River Flannelmouth Sucker has also been suggested (Miller 1952) and may result from hybridization with Sonora or Razorback suckers, as suggested by its morphological variation (Minckley 1980). D-statistic test also showed that Virgin River Flannelmouth Sucker had significantly more introgression from Sonora Sucker than any other Flannelmouth population further supporting this idea.

Pantosteus from the Virgin River grouped with Desert Sucker from the Lower Colorado River, but showed introgression with Bluehead Sucker from the Upper Colorado River, whereas Flannelmouth Sucker from the Virgin River group together with conspecifics from the Upper Colorado River in the *Catostomus* subclade, yet reflect introgression with Sonora Sucker from

the Lower Colorado River (Figure 4G, H). Even though the phylogeographic pattern is incongruent, introgression between distinct clades from the Lower and Upper Colorado River basins is still apparent in the Virgin River (Figure 4G, H). The extent of this introgression is a worthwhile topic to pursue, in that our samples represent but a single site for each species.

Great Basin

The Bonneville Basin (a component of the Great Basin) abuts to the Colorado River Basin in Utah and shares with it *Pantosteus* species, to include both Bluehead and Mountain suckers. Bluehead Sucker, once listed as a single species, has recently been split again into *C. P. virescens* (Bonneville Basin/ Upper Snake River), and *C. P. discobolus* (Colorado River Basin). Mountain Sucker, on the other hand, currently represents but a single species (*C. P. platyrhynchus*) but can be further separated into Bonneville and Colorado River basin components, with the added complication that individuals in the Price and San Rafael rivers (i.e., Colorado River Basin) represent introductions from the Bonneville Basin (per Sigler and Miller 1963). They thus fall within the Bonneville clade. This, in turn, suggests the potential for additional delineations in Mountain Sucker between the two basins, as reflected by morphological divergence between these groups. This has also been attributed to introgression by different Bluehead Sucker species (Smith et al 2013).

However, the mitochondrial phylogeny suggests that all Mountain Sucker have haplotypes more closely related to *C. P. discobolus* of the Upper Colorado River Basin (Hopken et al. 2013), subsequently calibrated at 2-3mya, a time when both the Green River of the Upper Colorado River Basin and the Bonneville Basin flooded into the Upper Snake River (Unmack et al. 2014). This, in turn, may have facilitated a transfer of individuals among basins. Our D-

statistic test does indeed reflect introgression between Bluehead and Mountain suckers, but values are non-significant when basins are compared. This suggests that introgression was not merely within each basin, but instead among basins and species.

The placement of Utah Sucker within *Catostomus* contrasts with the evolution of *Pantosteus*, in that Utah Sucker from the Bonneville Basin shares ancestry with species from the Lahontan (i.e., Tahoe Sucker) and Columbia River basins (i.e., Bridgelip Sucker; Figure 1), as opposed to *Pantosteus* in the Colorado River Basin. One potential explanation for this discrepancy is that flooding of the Bonneville Basin during the Late-Pliocene/ Early-Pleistocene allowed an exchange between the Upper Colorado River Basin to the east and the Lahontan River Basin to the west (Reheis et al. 2002). In this sense, *Pantosteus* of the Bonneville Basin represents a response to drainage connectivity with the Colorado River Basin to the east, whereas Utah Sucker is a response to such temporary drainage rearrangements with the Lahontan Basin to the west.

In the same sense, flooding of the Lahontan Basin during Late-Pliocene/Early-Pleistocene could also have transferred lineages to the Columbia Basin via the Lower Snake River. This connection is also seen for *Pantosteus* with regard to the Lahontan Mountain Sucker (*C. P. lahontan*) and the Columbia Mountain Sucker (*C. P. bondi*), and, between Tahoe Sucker and Bridgelip Sucker in *Catostomus* as well (Figure 1). This then allowed the initial radiation through the Great Basin to again return to the Columbia Basin from which it originated.

Conclusion

Phylogeographic and systematic analyses of many non-model organisms, particularly those that possess a history replete with reticulated evolution, have often been hampered by the

discordance between mitochondrial and morphological analyses. However, recent advances in molecular sequencing technology have opened avenues for deciphering the phylogenomics of non-model organisms, and that can permit the resolution of incongruences as well as promote unambiguous tests of historical introgression. In this regard, our phylogenomic analyses highlight both benefits and deficiencies with regard to different phylogenetic methods, particularly regarding concatenation and multi-species coalescent methods. Our data also serve as a empirical confirmation of conclusions that stem from simulated data (Leaché et al. 2014, Solís-Lemus et al. 2016), in that an 'real-world' example is provided of the bias effect that can occur when concatenation is employed, due largely to the introgression between distantly related taxa.

The taxonomic veracity of the subgenus *Pantosteus* was supported herein (Smith et al. 2013, Unmack et al. 2014), as was the proposed taxonomic revisions, but with the removal of Bridgelip Sucker as a component. Our data also argue that additional morphological and molecular data are needed to substantiate the subgenus *Catostomus* (Smith et al. 2013), and that such analyses must involve the remainder of *Catostomus,* and well as Lake Suckers, *Chasmistes* and *Deltistes*.

Comparative phylogeography of the southern species of *Catostomus* revealed both similarities and differences with respect to diversification patterns. Fine-grained phylogeographic patterns in the Colorado River Basin also warrant additional study, especially with regard to Virgin and Little Colorado rivers, in that populations of conservation concern are harbored therein and may demonstrate complex histories blurred by recent and historic admixture. These resolutions promote *Catostomus* as a model system from which the effects of reticulate evolution can be more fully interpreted, and to promote the management and conservation of desert fishes.

The aquatic biodiversity inherent to the rivers of southwestern North America has great contemporary importance, for this region is subjected to ongoing drought and excessive anthropogenic water use that act in synergy to threaten the longevity and biological integrity of these components.

Acknowledgements

Steven M. Mussman provided considerable support with the development of the naïve binning in ASTRAL, and deserves special acknowledgement for his contribution. Numerous agencies contributed field expertise, specimens, technical assistance, collecting permits, funding, or commentary, all of which have facilitated the completion of this study. Additional samples were kindly provided by: B. Sidlauskas (Oregon State Museum), T. Turner and A. Snyder (Museum of Southwestern Biology, University of New Mexico), M. Peacock (University of Nevada), and J. Richmond (U.S. Geological Survey). We are also indebted to those students, postdoctorals, and faculty who have promoted our research: A. Albores, P. Brunner, M. Davis, T. Dowling, R. Cooper, J. Cotter, E. Fetherman, M. Hopken, M. Kwiatkowski, A. Reynolds, J. Reynolds, C. Secor, and P. Unmack. Sampling procedures were approved under IACUC permit 98-456R (Arizona State University) and 01-036A-01 (Colorado State University). Accession numbers for samples from the Museum of Southwestern Biology included; MSB 44046, 44047, 44052, 44059, 44067, 49715, 49962, 53500, 63249, and 88362. Accession numbers for samples from the Oregon State Museum included; OS 15636, 17152, 17158, 17548, 17553, and FW316-3. This research was supported by generous endowments to the University of Arkansas: The Bruker Professorship in Life Sciences (M.R.D.) and 21st Century Chair in Global Change Biology (M.E.D.).

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Tables

Table 1: Sample sizes by drainage for *Catostomus, the subgenus Pantosteus*, *Xyrauchen*, and the outgroup *Moxostoma*. Also included are number of sample sites (Sites) and number of samples (N) for each species.

Species	Major Drainage	State	Sites	N
C. ardens	Bonneville Basin	WY, UT	2	4
C. latipinnis	Upper Colorado River	WY, UT, CO, AZ, NM	11	11
	Grand Canyon	AZ	3	5
	Virgin River	UT	1	8
C. "crassicada"	Little Colorado River	AZ	3	8
C. insignis	Lower Colorado River	AZ, NM	5	7
C. (P.) jordani	Missouri River	MT	1	2
C. (P.) lahontan	Lahontan Basin	NV	2	5
C. (P.) bondi	Columbia River	OR	1	2
C. (P.) platyrhynchus	Bonneville	WY, UT	4	6
	Upper Colorado River	WY, UT, CO	16	20
C. (P.) virescens	Bonneville Basin	WY, UT	5	5
C. (P.) discobolus	Upper Colorado River	WY, UT, CO, AZ, NM	29	31
	Grand Canyon	AZ	5	6
	Little Colorado River	AZ	8	13
C. (P.) d. yarrowi	Zuni River	NM	3	12
C. (P.) clarkii	Virgin River	NV	1	1
	Lower Colorado River	AZ, NM	7	8
C. (P.) santaanae	Los Angeles River	CA	1	3
C. (P.) plebeius	Rio Grande	CO, NM	6	6
C. commersonii	Mississippi River	ND, IL	3	3
	Upper Colorado River	WY, CO	2	2
C. tahoensis	Lahontan Basin	NV	1	3
C. rimiculus	Rogue River	OR	1	1
C. microps	Goose Lake	OR	1	1
C. (P.) columbianus	Donner und Blitzen River	OR	2	2
C. catostomus	Upper Colorado River	WY	1	3
X. texanus	Upper Colorado River	UT, NM	2	4
M. macrolepidotum	Mississippi River	ND	1	1
M. valenciennesi	Mississippi River	MN	1	1
		Total	**129**	**184**

Table 2: Node support values for all phylogenetic methods. Numbers next to ASTRAL represent number of loci binned for each run. Letters correspond to nodes in Figures 1 and 2. Numbers beneath each node represent bootstrap support. Blue boxes with no values represent 100% bootstrap support (=1.0 posterior probability). Colors are arranged from blue to red, with blue representing greater support and red representing least support; (-) represents a lack of support.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
RAXML																						
MR.BAYES																						
SVDQUARTETS																						
ASTRAL-5	67	95	68	99	55	72	63	65	95	68	32	23	94	51	40	61	26	36	-	-	-	88
ASTRAL-10	93				97		96					83			98		99	94	12	19	58	
ASTRAL-15							98					95			97				37	44	55	
ASTRAL-20																			53	60	67	
ASTRAL-50																			97	90		
ASTRAL-100																			98	92	95	

	V	W	X	Y	Z	AA	BB	CC	DD	EE	FF	GG	GG'	HH	II	JJ	KK	LL	MM	NN	OO	PP
RAXML													-									
MR.BAYES													-									
SVDQUARTETS				99	97			90				-	83					98				
ASTRAL-5	88	80	97	16	28	67	95	11	29	45	86	-	30	84	61	24	27	11	28	24	16	41
ASTRAL-10				62	88	89		45	75	82		-	63		98	69	59	32		66	57	81
ASTRAL-15				83	99	98		54	82			55	-				84	34		91	91	
ASTRAL-20				96				60				67	-				91	49		93	98	
ASTRAL-50				99				76				90	-				89	67			99	
ASTRAL-100				95				79				98	-				95	75			98	

100 >90 >80 >70 >50 <50

Table 3: Patterson's D-statistic results (per format in Eaton et al. 2015). Tests with significant Z-scores are in bold with the species involved with introgression also in bold. Range of Z-scores for each set of tests (RangeZ) and the number of significant tests out of the total number of tests (nSig/ntest) are also reported, as it the overall Z-score. Abbreviations are as follows: THS=Tahoe Sucker, BLS=Bridgelip Sucker, BBS=Bonneville Bluehead Sucker, BHS=Bluehead Sucker (split into UC=Upper Colorado River Basin, GC=Grand Canyon, def=Defiance Plateau, tam=Tampico Springs, agr=Agra Remora, rnu=Rio Nutria, LC=Upper Little Colorado, wil=Willow Creek, and sil=Silver Creek), MTS=Mountain Sucker (split into MR=Missouri River, LB=Lahontan, CB=Columbia River Basin, BB=Bonneville, CR=Colorado River), LNS=Longnose Sucker, SOS=Sonora Sucker, FMS=Flannelmouth Sucker (split into GC=Grand Canyon, UC=Upper Colorado River Basin, LC=Little Colorado without Wenima Wildlife Area, wen=Wenima Wildlife Area, VR=Virgin River), DES=Desert Sucker (split into VR=Virgin River, BW=Bill Williams River, GI=Gila River Basin), WTS=White Sucker, RBS=Razorback Sucker, SAS=Santa Anna Sucker, RGS=Rio Grande Sucker.

A

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
THS	BLS	BBS	LNS	0.20	0.48	0.40	6	10	1198	0 , 2.88	0/90
THS	BLS	MTS (CB)	LNS	0.20	0.65	0.31	4	6	945	0 , 2.73	0/36
THS	BLS	MTS (LB)	LNS	0.19	0.56	0.34	7	10	1206	0 , 2.97	0/90
THS	BLS	MTS (BB)	LNS	0.19	0.53	0.36	7	10	1273	0 , 2.97	0/108

B

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
FMS (UC)	FMS (GC)	SOS	WTS	0.08	0.27	0.30	13	15	4586	0 , 2.96	0/360
FMS (UC)	**FMS (VR)**	**SOS**	WTS	**0.64**	0.09	**7.43**	21	98	4706	**4.76 , 12.69**	**420/420**
FMS (GC)	**FMS (VR)**	**SOS**	WTS	**0.64**	0.07	**9.01**	18	84	4604	**4.74 , 11.02**	**420/420**
FMS (LC)	**FMS (VR)**	**SOS**	WTS	**0.63**	0.11	**5.80**	18	77	3340	3.51 , **11.68**	**338/350**
FMS (wen)	FMS (VR)	SOS	WTS	0.14	0.21	0.68	43	57	3280	0.09 , **4.51**	**35/210**
FMS (LC)	FMS (UC)	SOS	WTS	0.02	0.15	0.12	20	21	3295	0 , 1.54	0/300
FMS (LC)	FMS (GC)	SOS	WTS	0.08	0.18	0.42	20	24	3208	0 , 2.2	0/300
FMS (UC)	**FMS (wen)**	**SOS**	WTS	**0.48**	0.10	**4.56**	17	49	3213	0.37 , **7.19**	**129/180**
FMS (GC)	**FMS (wen)**	**SOS**	WTS	**0.49**	0.12	**4.08**	17	50	3126	0.21 , **7.43**	**126/180**

C

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
FMS (UC)	**SOS**	**RBS**	WTS	**0.54**	0.05	**10.16**	44	149	3823	**4.38 , 10.81**	**240/240**
FMS (GC)	**SOS**	**RBS**	WTS	**0.56**	0.06	**9.10**	40	141	3745	**4.10 . 11.00**	**240/240**
FMS (VR)	**SOS**	**RBS**	WTS	**0.49**	0.05	**9.80**	40	116	3871	3.76 , **8.31**	**276/280**
FMS (LC)	**SOS**	**RBS**	WTS	**0.53**	0.08	**6.41**	32	105	2711	2.92 , **10.47**	**184/200**
FMS (wen)	**SOS**	**RBS**	WTS	**0.54**	0.10	**5.33**	20	66	2672	2.73 , **8.43**	**99/120**

D

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
FMS (GC)	FMS (UC)	RBS	WTS	0.00	0.31	0.01	12	12	4259	0 , 2.69	0/288
FMS (UC)	FMS (LC)	RBS	WTS	0.06	0.24	0.27	12	14	3019	0 , 2.00	0/240
FMS (GC)	FMS (LC)	RBS	WTS	0.10	0.26	0.38	13	16	2944	0 , 2.48	0/240
FMS (UC)	FMS (wen)	RBS	WTS	0.18	0.23	0.76	13	18	2891	0 , 2.45	0/144
FMS (GC)	FMS (wen)	RBS	WTS	0.13	0.25	0.51	14	18	2817	0.13 , 2.39	0/144
FMS (UC)	FMS (VR)	RBS	WTS	0.44	0.14	3.10	18	46	4337	1.04, **6.01**	**112/336**
FMS (GC)	FMS (VR)	RBS	WTS	0.43	0.15	2.90	16	39	4249	0.98 , **5.57**	**81/336**
FMS (LC)	FMS (VR)	RBS	WTS	0.42	0.12	3.33	15	37	3013	0.91 , **5.05**	**63/280**
FMS (wen)	FMS (VR)	RBS	WTS	0.25	0.17	1.45	17	28	2911	0.18 , 3.26	0/168

E

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
BBS	**BHS (UC)**	**DES (GI)**	MTS (MR)	**0.69**	0.07	**9.95**	34	186	3761	**8.01 , 18.35**	**250/250**
BBS	**BHS (GC)**	**DES (GI)**	MTS (MR)	**0.45**	0.09	**5.00**	43	116	3189	**4.56 , 17.96**	**300/300**
BBS	**BHS (def)**	**DES (GI)**	MTS (MR)	**0.40**	0.09	**4.72**	52	123	3598	2.53 , **7.53**	**245/300**
BBS	**BHS (tam)**	**DES (GI)**	MTS (MR)	**0.36**	0.08	**4.52**	50	106	3599	3.13 , **6.05**	**122/200**
BBS	**BHS (agr)**	**DES (GI)**	MTS (MR)	**0.37**	0.08	**4.80**	49	109	3315	3.14 , **6.15**	**148/200**
BBS	**BHS (rnu)**	**DES (GI)**	MTS (MR)	**0.43**	0.08	**5.64**	43	108	3325	3.61 , **6.90**	**184/200**
BBS	**BHS (LC)**	**DES (GI)**	MTS (MR)	**0.44**	0.10	**4.58**	41	104	3213	2.72 , **5.77**	**68/100**
BBS	BHS (wil)	DES (GI)	MTS (MR)	0.13	0.11	1.21	38	49	2361	0.12 , 2.66	0/100
BBS	BHS (sic)	DES (GI)	MTS (MR)	0.13	0.12	1.13	56	72	2877	0.14 , 2.96	0/100
SAS	**DES (VR)**	**BHS (UC)**	MTS (MR)	**0.43**	0.09	**4.63**	41	104	3853	**3.99 , 7.08**	**30/30**

SAS	DES (BW)	BHS (UC)	MTS (MR)	0.18	0.15	1.20	25	37	2317	0.21 , 2.66	0/60
SAS	DES (GI)	BHS (UC)	MTS (MR)	0.28	0.10	2.70	25	44	3188	0.89 , 3.13	0/150

F

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
RGS	**BBS**	**MTS (BB)**	LNS	**0.36**	0.09	**4.12**	24	51	2397	2.22 , **5.21**	**384/540**
RGS	**BHS (UC)**	**MTS (BB)**	LNS	**0.44**	0.08	**5.32**	22	57	2790	2.58 , **6.60**	**443/540**
RGS	**BHS (GC)**	**MTS (BB)**	LNS	**0.43**	0.09	**4.98**	19	49	2285	2.17 , **6.16**	**475/648**
RGS	**BBS**	**MTS (CR)**	LNS	**0.40**	0.09	**4.54**	21	49	2454	2.24 , **5.63**	**434/540**
RGS	**BHS (UC)**	**MTS (CR)**	LNS	**0.49**	0.09	**5.56**	21	60	2863	2.87 , **9.28**	**497/540**
RGS	**BHS (GC)**	**MTS (CR)**	LNS	**0.48**	0.10	**5.02**	18	51	2347	2.32 , **7.57**	**507/648**
MTS (MR)	**MTS (BB)**	**BBS**	LNS	**0.38**	0.09	**4.00**	25	55	2222	2.71 , **6.30**	**156/180**
MTS (MR)	**MTS (BB)**	**BHS (UC)**	LNS	**0.32**	0.08	**4.07**	30	58	2565	2.26 , **5.58**	**140/180**
MTS (MR)	**MTS (BB)**	**BHS (GC)**	LNS	**0.35**	0.09	**4.05**	22	47	2105	2.21 , **5.63**	**163/216**
MTS (MR)	**MTS (CR)**	**BBS**	LNS	**0.43**	0.09	**4.68**	23	58	2250	3.00 , **9.65**	**171/180**
MTS (MR)	**MTS (CR)**	**BHS (UC)**	LNS	**0.45**	0.09	**4.83**	25	67	2608	2.83 , **7.66**	**164/180**
MTS (MR)	**MTS (CR)**	**BHS (GC)**	LNS	**0.49**	0.10	**4.98**	19	55	2139	2.88 , **11.72**	**202/216**
BHS (UC)	BBS	MTS (BB)	LNS	0.02	0.15	0.15	26	27	3182	0 , 1.78	0/450
BHS (GC)	BBS	MTS (BB)	LNS	0.01	0.21	0.03	23	22	2593	0 , 2.06	0/540
BBS	BHS (UC)	MTS (CR)	LNS	0.04	0.17	0.21	25	27	3263	0 , 2.01	0/450
BBS	BHS (GC)	MTS (CR)	LNS	0.07	0.21	0.34	21	24	2664	0 , 2.41	0/540

G

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
BHS (agr)	**BHS (rnu)**	**RGS**	LNS	**0.95**	0.05	**20.04**	3	114	3127	**7.27 , 62.25**	**192/192**
BHS (tam)	**BHS (rnu)**	**RGS**	LNS	**0.94**	0.04	**21.56**	4	132	3567	**10.51 , 63.19**	**192/192**
BHS (LC)	**BHS (rnu)**	**RGS**	LNS	**0.84**	0.10	**8.27**	12	133	2907	**4.35 , 36.52**	**96/96**
BHS (wil)	**BHS (rnu)**	**RGS**	LNS	**0.83**	0.09	**9.31**	9	96	2141	**4.64 , 26.64**	**96/96**
BHS (sic)	**BHS (rnu)**	**RGS**	LNS	**0.85**	0.06	**14.65**	10	125	2657	**6.24 , 31.60**	**96/96**
BHS (def)	**BHS (rnu)**	**RGS**	LNS	**0.78**	0.06	**13.44**	21	174	3325	**8.97 , 23.64**	**288/288**
BHS (GC)	**BHS (rnu)**	**RGS**	LNS	**0.73**	0.08	**8.93**	26	164	2904	**4.72 , 18.77**	**288/288**
BHS (agr)	BHS (tam)	RGS	MTS (MR)	0.10	0.52	0.19	5	6	2511	0.06 , 0.11	0/64

BHS (LC)	BHS (agr)	RGS	MTS (MR)	0.11	0.29	0.37	14	17	1772	0.14 , 1.17	0/128
BHS (LC)	BHS (tam)	RGS	MTS (MR)	0.09	0.31	0.29	17	21	2012	0.01 , 0.91	0/128

H

P1	P2	P3	O	D	std(D)	Z	BABA	ABBA	nloci	RangeZ	nSig/ntest
DES (GI)	SAS	RGS	MTS (MR)	0.15	0.20	0.75	17	23	2381	0.18 , 2.51	0/180
DES (VR)	SAS	RGS	MTS (MR)	0.22	0.20	1.10	24	38	2845	0.23 , 3.52	0/36
DES (BW)	SAS	RGS	MTS (MR)	0.06	0.24	0.23	17	19	1745	0 , 2.32	0/72

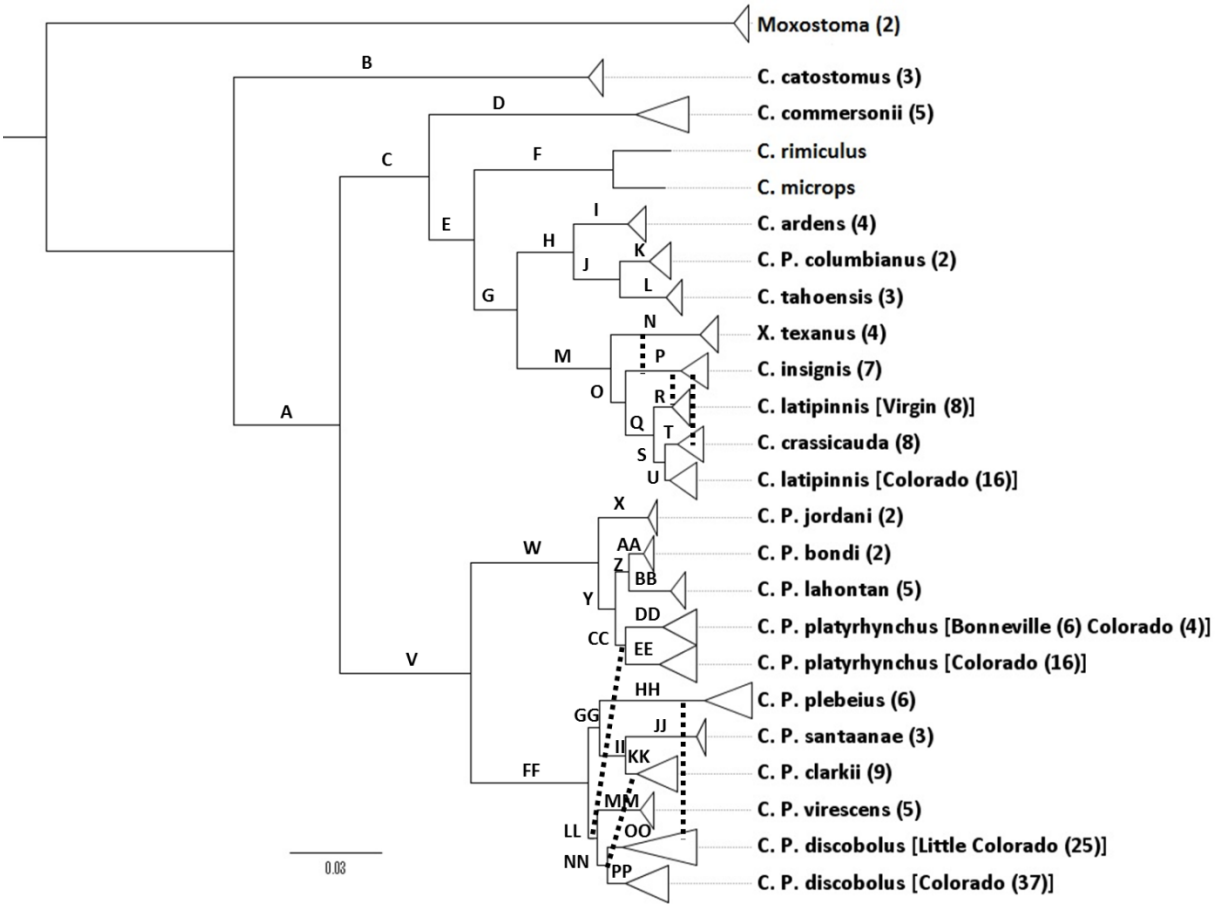
Figure Headings

Figure 1: Phylogeny of *Catostomus* with branch lengths from RAxML. Letters at nodes correspond with columns in Table 2 containing support values for all analyses. Collapse of nodes is based on species and level of support. Dotted lines represent significant introgression events per D-statistic tests. Numbers in parentheses represent number of individuals for each collapsed node.

Figure 2: Alternative phylogenetic hypotheses for taxa in the subgenus *Pantosteus*, as derived by (A) concatenated SNP approaches (RAxML and MrBayes) and (B) multispecies coalescent approach (SVDquartets). Letters at nodes correspond with columns in Table 2 that contain the support values for all analyzes.

Figure 3: Map of Colorado River Basin and Bonneville Basin.

Figure 4: Phylogeographic patterns for (A) Desert Sucker, (B) Rio Grande Sucker, (C) Bluehead Sucker, (D) Flannelmouth Sucker, (E) Mountain Sucker, (F) Sonora Sucker, (G) combined *Pantosteus*, and (H) combined *Catostomus* not in *Pantosteus*. Dotted lines represent significant introgression events.


Moxostoma (2)
C. catostomus (3)
C. commersonii (5)
C. rimiculus
C. microps
C. ardens (4)
C. P. columbianus (2)
C. tahoensis (3)
X. texanus (4)
C. insignis (7)
C. latipinnis [Virgin (8)]
C. crassicauda (8)
C. latipinnis [Colorado (16)]
C. P. jordani (2)
C. P. bondi (2)
C. P. lahontan (5)
C. P. platyrhynchus [Bonneville (6) Colorado (4)]
C. P. platyrhynchus [Colorado (16)]
C. P. plebeius (6)
C. P. santaanae (3)
C. P. clarkii (9)
C. P. virescens (5)
C. P. discobolus [Little Colorado (25)]
C. P. discobolus [Colorado (37)]
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
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PP
0.03

Figure 1.

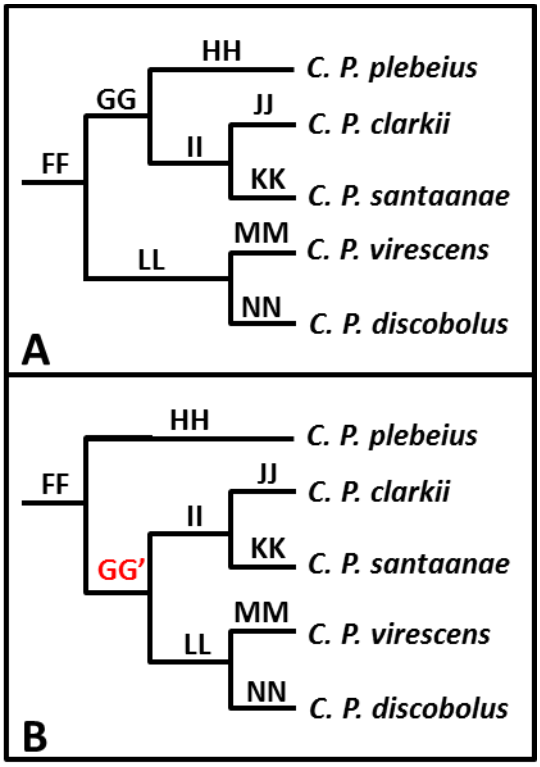
HH
C. P. plebeius
GG
JJ
C. P. clarkii
II
FF
KK
C. P. santaanae
MM
C. P. virescens
LL
NN
C. P. discobolus
A
HH
C. P. plebeius
FF
JJ
C. P. clarkii
II
KK
GG'
C. P. santaanae
MM
C. P. virescens
LL
NN
C. P. discobolus
B

Figure 2.

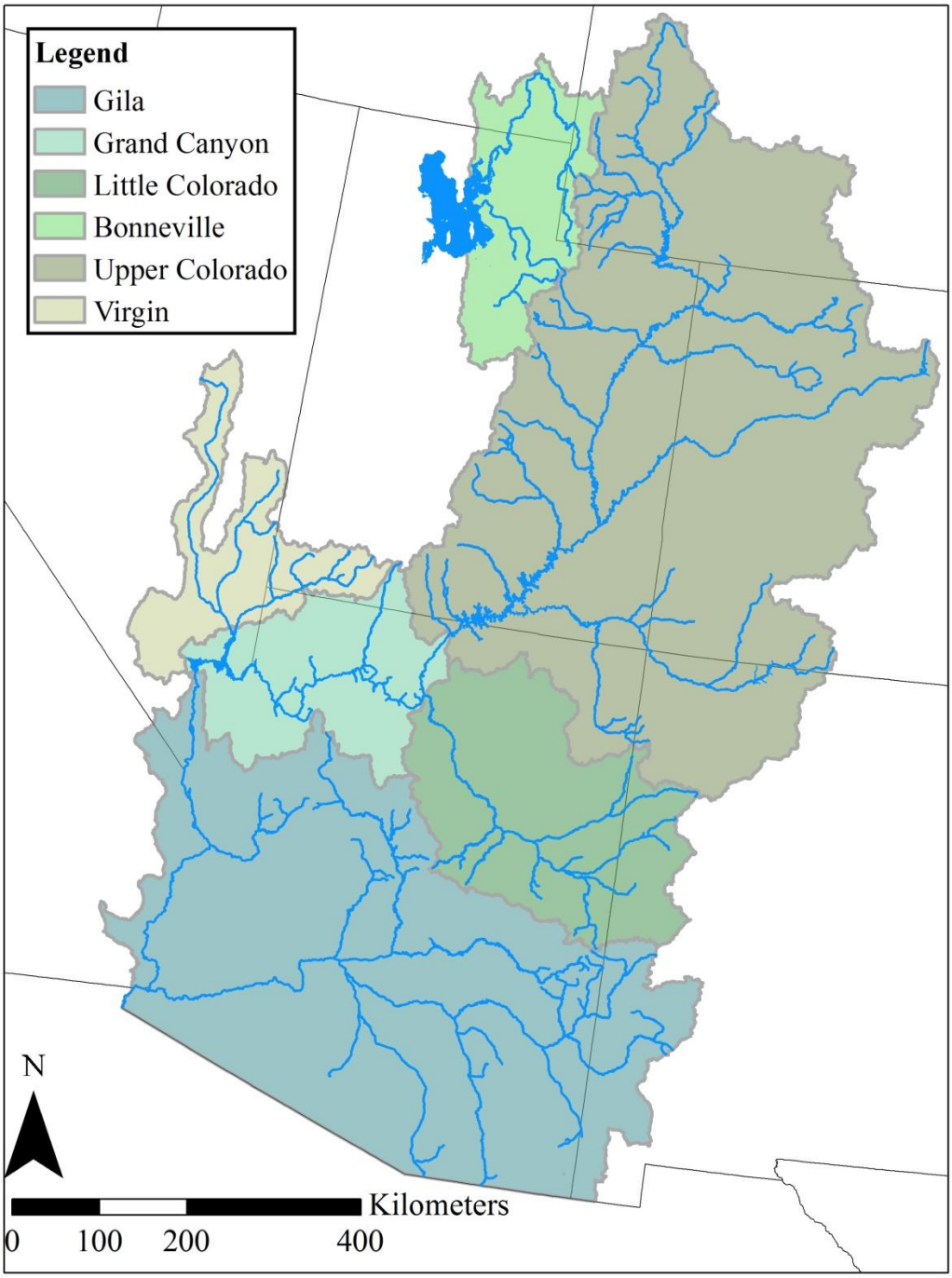
Legend
Gila
Grand Canyon
Little Colorado
Bonneville
Upper Colorado
Virgin
N
Kilometers
0
100
200
400

Figure 3.

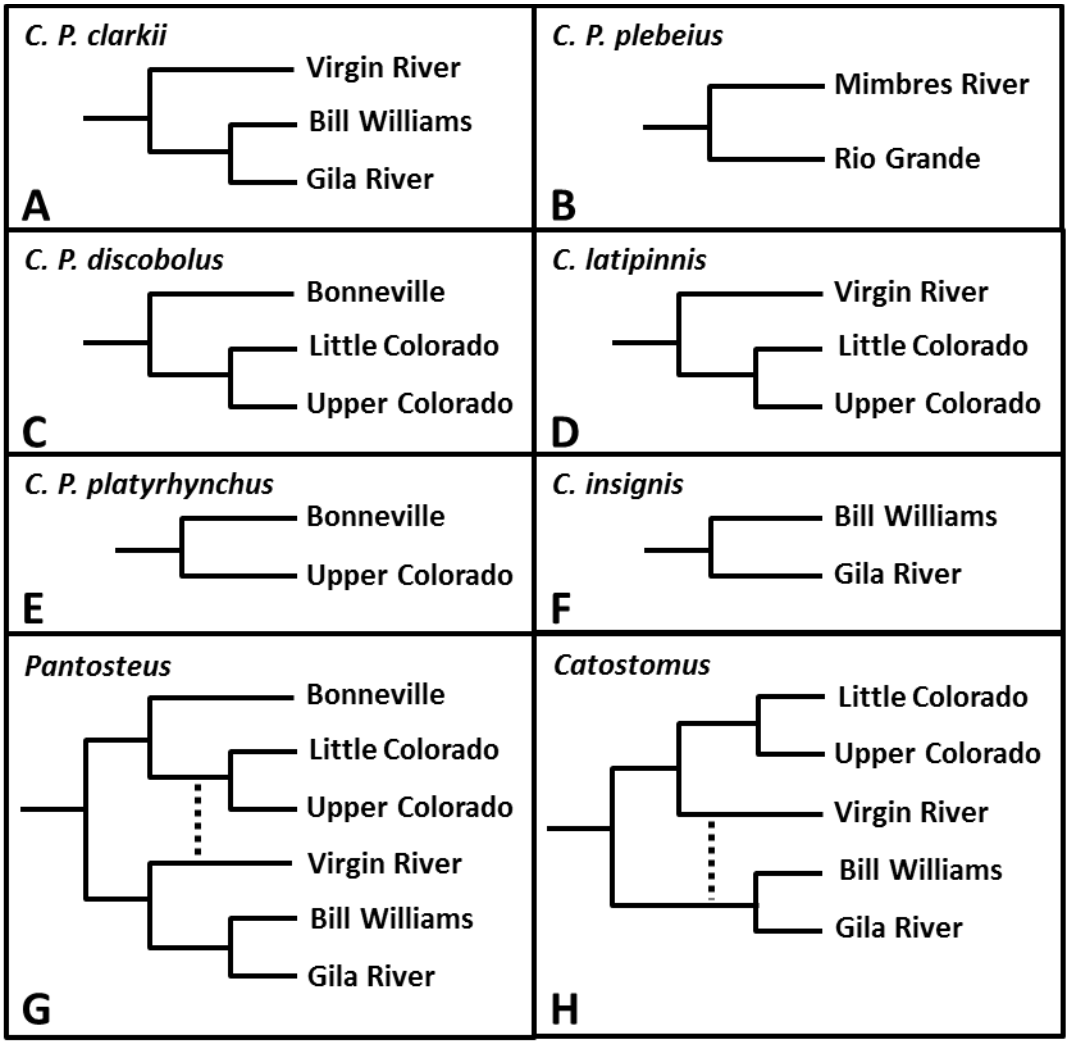
C. P. clarkii
Virgin River
Bill Williams
Gila River
A
C. P. plebeius
Mimbres River
Rio Grande
B
C. P. discobolus
Bonneville
Little Colorado
Upper Colorado
C
C. latipinnis
Virgin River
Little Colorado
Upper Colorado
D
C. P. platyrhynchus
Bonneville
Upper Colorado
E
C. insignis
Bill Williams
Gila River
F
Pantosteus
Bonneville
Little Colorado
Upper Colorado
Virgin River
Bill Williams
Gila River
G
Catostomus
Little Colorado
Upper Colorado
Virgin River
Bill Williams
Gila River
H

Figure 4.

IV. Comparative Species Delimitations in the Presence of Interspecific Gene Flow: Flannelmouth (*Catostomus latipinnis*) and Bluehead Sucker (*C. Pantosteus discobolus*) as Case Studies

Abstract

Delimitation of species is an essential step for the assessment of biodiversity and its conservation. Recent advancements in Next-Generation-Sequencing (NGS), combined with new analytical approaches, have promoted our capacity to accomplish these tasks, particularly with species whose boundaries are obscured by reticulation. A comparative approach, particularly with regard to the incorporation of divergent life histories, allows for active integration of the ecology of the species within their riverscapes. Here we utilized double digest restriction associated DNA sequencing (ddRAD) to examine phylogeographic patterns and proposed taxonomic revisions in two co-distributed catostomids (Flannelmouth Sucker, *Catostomus latipinnis* and Bluehead Sucker, *C. Pantosteus discobolus*) with dissimilar life-histories in the Colorado River Basin. Three phylogenetic methods and a Bayesian assignment test highlighted similar phylogeographic patterns in the two species, but also underscored divergence times and evolutionary histories that differed. Three lineages of Bluehead Sucker were detected in all methods, supporting the elevation of *C. P. virescens* in the Bonneville Basin and Upper Snake River as a species separate from *C. P. discobolus* in the Colorado River, as well as support the Zuni Bluehead Sucker as a unique form. However, admixture detected here highlights the complex history of the Zuni Bluehead Sucker and warrants the need for reevaluation of its range. While three lineages were detected in Flannelmouth Sucker, they more accurately represent evolutionary significant units (ESUs), due to the slight phenotypic and genetic differentiation, current geographic isolation, and lack of concordance required for recognition under the

genealogical concordance component of the phylogenetic species concept. Two hybrid detection methods also indicated several instances of introgressive hybridization that have impacted both species, especially populations in the Little Colorado and Virgin rivers. Through the incorporation of these methods, as well as relating to previous morphological, enzymatic, and mitochondrial work, allowed for examining species delimitation and disentanglement of complex histories of isolation and secondary contact, exemplified in Southwestern fishes.

Introduction

One of the most fundamental issues in biology is the concept of species delimitation, an area vital to evolutionary biology, as well as conservation and management (Carstens et al. 2013). This is now most commonly done using DNA-based approaches, but with frequent reliance upon a single marker (i.e. DNA barcoding) (Ahrens et al. 2016), a questionable approach given that many taxa are now recognized as having semipermeable species boundaries (Dasmahapatra et al. 2012, Nadeau et al. 2012, Fontaine et al. 2015). This highlights the importance of invoking multi-locus data for such tasks, especially when study species are known to possess complex histories that involve admixture (Petit and Excoffier 2009).

Many methods for species delimitation using multi-locus datasets have been proposed, but all seemingly apply the genealogical concordance concept for phylogenetic species recognition (Avise and Ball 1990), which defines a species as the smallest diagnosable unit that reflects concordance across multiple gene genealogies (Taylor et al. 2000). This concept has become more amenable with the application of next generation sequencing, but the potential over-splitting of taxa still remains problematic (Agapow et al. 2004, Hedin et al. 2015). Furthermore, these methods may actually be less accurate when study species have a history of introgression (Camargo et al. 2012, Stewart et al. 2014).

As a means to avoid these issues, a newer framework (Leaché and Fujita 2010) starts with the genetic clustering of species with admixed histories, so as to detect potentially erroneous species delimitation based on inter-specific gene flow (Camargo et al. 2012, Stewart et al. 2014). This approach gains additional power when multiple lines of evidence are integrated, such as life history, geographical distributions, and morphology (Knowles and Carstens 2007, Schlick-Steiner et al. 2010, Fujita et al. 2012). As a result, complex histories can now be more

clearly discerned and species then delineated despite introgression. This is a particularly appealing aspect when problematic species are of conservation concern (Pyron et al. 2016).

For example, Flannelmouth Sucker (*Catostomus latipinnis*) and Bluehead Sucker (*C. Pantosteus discobolus*) have complex histories that reflect historical introgression (Smith et al. 2013), as well as contemporary hybridization with various congeners (Douglas and Douglas 2010, Mandeville et al. 2015). Until recently, both species have remained understudied, but conservation concerns have accelerated due to a prolonged natural drought and an ever-increasing anthropogenic demand for water (Seager and Vecchi 2010). A federal and multi-state effort has now coalesced to focus on the basin-wide mitigation and recovery of both species (Carmen 2007). Thus, the question of species delimitation as well as the potential for conservation units, are issues of great importance, especially given the fact that historically both have been the most abundant species with the greatest biomass in the Upper Colorado River Basin (Hubbs et al. 1948).

Each species also presents a different life history, with Flannelmouth Sucker primarily found in the mainstem and Bluehead Sucker in higher elevation streams that have unfortunately become more fragmented over time (Douglas et al. 2003, Hopken et al. 2013). These ecological differences seemingly underpin the manner by which both species have responded to the tumultuous geologic history of western North America. In this sense, vicariant processes (i.e., volcanism and glaciation) coupled with episodic drought, have induced long periods of isolation sporadically augmented by more pluvial periods that promoted secondary contact due to stream capture (Smith et al. 2010). Thus, a comparative study of both species can provide not only insights into how admixture has influenced their evolution, but also clarify our understanding of

the basin itself, and how its phylogeography can provide additional insights into those diversities displayed by other species in the system.

Both study species are primarily endemic to the Upper Colorado River Basin, but with Flannelmouth Sucker also in the Virgin River of the Lower Colorado River Basin and Bluehead Sucker in the neighboring Bonneville Basin. The latter may potentially represent a different species (*C. P. virescens*), as judged by morphological (Smith et al. 2013), mitochondrial (Hopken et al. 2013, Unmack et al. 2014), and nuclear phylogenies (Chapter 2). Taxonomic uncertainties within Flannelmouth and Bluehead suckers are additional management complications, especially with regard to the presence for each of potentially unique lineages in the Little Colorado River. One of these may represent a unique species (i.e., Little Colorado Sucker), currently grouped with Flannelmouth Sucker, and a second may be a unique subspecies (i.e., Zuni Bluehead Sucker, *C. P. d. yarrowi*) now found only in the Zuni River (NM) and Kin Lee Chee Creek (AZ) but with an historic distribution that potentially included the entire Little Colorado River (Minckley 1973).

The quantification of molecular variability in both of these catostomids is a key element in delimiting their species/ management units and defining their patterns of reticulation. Both aspects are important with regards to the management and conservation of each species, and the basin as a whole. Here we build upon the results of Chapter 2 by applying phylogenomic (i.e., concatenated and multispecies coalescence) and population genomic (i.e., clustering) methods to delimit potential species throughout their respective ranges, but with special focus on the Little Colorado River. In this regard, the impacts of divergent life histories as well as the role of stream capture and hybridization are particularly germane with regard to rates of differentiation.

Methods

Sample Acquisition

Samples were collected between 1995 and 2011 and consisted of either fin clips or tissue plugs. Genomic DNA was extracted using the PureGene® Purification Kit or DNeasy® Tissue Kit (Qiagen Inc., Valencia CA) following manufacturer's protocols, and stored in DNA hydrating solution. Additional samples were obtained from the Museum of Southwestern Biology (University of New Mexico) (accession numbers provided in Acknowledgements).

A total of 139 samples were evaluated (Table 1). These included 81 samples from the subgenus *Pantosteus* and 57 samples in the subgenus *Catostomus* (per Smith et al. 2013). Bluehead Sucker (*C. P. discobolus*) (N=65) were obtained from throughout its range, including the Bonneville Basin (N=5), Grand Canyon AZ (N=10), Chinle Wash NM (N=10), Little Colorado River (N=29), and various sites in the Upper Colorado River Basin above Grand Canyon (N=11). Rio Grande Sucker (*C. P. plebeius*; N=6) and Desert Sucker (*C. P. clarkii*; N=8) were also sampled so as to evaluate their potential for hybridization with other *Pantosteus*. Mountain Sucker (*C. P. jordani*) from the Missouri River Basin (N=2) was included as outgroup for all analyses involving subgenus *Pantosteus* (Figure 1, Table 1).

Flannelmouth Sucker (N=35) was collected from throughout its range, to include the Virgin River UT (N=8), Little Colorado River (N=14), Grand Canyon AZ (N=5), and various sites in the Upper Colorado River Basin above Grand Canyon (N=8). White Sucker (*C. commersonii*) from locations in its native range (N=3) and from the introduced population in the Colorado River (N=2) were included as outgroup for all analyses of the *Catostomus* subgenus. (Figure1, Table 1). Sonora Sucker (*C. insignis*; N=10), Utah Sucker (*C. ardens*; N=4), and Razorback Sucker (*Xyrauchen texanus*; N=4) were also included in analyses of Flannelmouth

Sucker, due to their geographic proximity, close phylogenetic relationships, and potential for hybridization.

Data Collection

DNA was extracted with PureGene® Purification Kit or DNeasy® Tissue Kit (Qiagen Inc., Valencia CA) and stored in DNA hydrating solution (same kits). Libraries for double digest restriction-site associated DNA (ddRAD) were generated following the protocol outlined in Chapter 2. This included digesting with PstI (5'-CTGCAG-3') and MspI (5'-CCGG-3'), pooling 48 individuals prior to a size selection of 350-400bps, PCR amplification, and combining two libraries per lane of Illumina HiSeq 2000 single end 100bp sequencing. Samples for each reference species, region, and hybrid type were randomly distributed across several libraries and lanes so as to reduce the potential for library preparation bias. Sequencing was performed at the University of Wisconsin Biotechnology Center in Madison.

Filtering and Alignment

Illumina reads were filtered and aligned (protocol, Chapter 2) using PyRAD v.3.0.5 (Eaton and Ree 2013). This included: a clustering threshold of 80% based the uncorrected sequence variation in catostomid fishes (Chen and Mayden 2012, Chapter 1), and removal of restriction site sequence and barcode. In addition, loci were removed if they displayed: 1) <5 reads per individual), 2) >10 heterozygous sites within a consensus, 3) >2 haplotypes for an individual, 4) >75% heterozygosity for a site among individuals, and 5) <50% of individuals at a given locus.

Clustering Algorithm

All analyses utilized unlinked SNPs generated from PyRAD. Bayesian clustering (STRUCTURE v. 2.3.4; Pritchard et al. 2000) employed the admixture model with correlated allele frequencies and a burn-in of 100,000 generation, followed by 500,000 generations post-burn-in. No population priors were used. Genetic clusters (k) were each run with 15 iterations (with k=1 to k=16), then averaged across iterations to determine final values. The most likely genetic cluster was resolved by using the estimated log probability of data Pr(x|k) and the Δk statistic (per Evanno et al. 2005). Bayesian clustering also allowed for the conformation that all contemporary hybrids with invasive White Sucker had been removed.

Phylogenetic Methods

Concatenated SNPs were used to generate both maximum likelihood (ML) and Bayesian phylogenies without *a priori* assumption, with the ML analysis conducted in RAxML (v. 7.3.2; Stamatakis 2006) using GTRCAT with 1,000 bootstraps. The Bayesian analysis was performed in MrBayes (v. 3.2.3; Ronquist et al. 2012) using GTR (10,000,000 generations), sampling every 1,000 generations, and a 25% burn-in that was discarded.

However, concatenated SNPs methods can potentially overestimate support values for erroneous or poorly supported nodes (Liu et al. 2015, Edwards et al. 2016). This is especially problematic if introgression has occurred because the majority of loci may not support the resulting topology (Twyford and Ennos 2012, Leaché et al. 2014). However, multispecies coalescent methods perform well in situations with limited introgression, and are thus important for the delimitation of species with admixed ancestry (Edwards et al. 2016).

A multispecies coalescent species tree was generated in SVDQUARTETS (Chifman and Kubatko 2015) as implemented in PAUP* v. 4.0 (Swofford 2003) using unlinked SNPs. SVDQUARTETS uses a coalescent model to calculate frequencies of SNPs for each species to test support for quartets. This does not require the concatenation but does necessitate that individuals be *a priori* partitioned into species or populations. Species were subdivided into populations based on high support under both concatenated SNP methods. All possible quartets were exhaustively sampled using 1000 bootstraps.

Hybrid Detection

A hybrid index was calculated as a second means of assessing admixture, and to assess contemporary hybrid events by mapping against interspecific heterozygosity. The est.h function in the R-package INTROGRESS (Gompert and Buerkle 2010) was used to estimate the hybrid index (Gompert and Buerkle 2009) for samples in locations with potential admixed ancestry. This included hybridization among: 1) Rio Grande and Bluehead sucker in the Zuni River (NM), 2) Sonora and Flannelmouth sucker in the Little Colorado and Virgin rivers, and 3) admixture between linegages of Bluehead Sucker in the Little Colorado River. The calc.intersp.het and triangle.plot functions in INTROGRESS were also used to assess recentness of hybrid events by calculating interspecific heterozygosity and generating triangle plots for each admixture test, with a recent hybrid identified via its high interspecific heterozygosity.

NEWHYBRIDS (Anderson and Thompson 2002) was used to test the probability of hybrid assignment, to include first-filial (F1), second-filial (F2), and first and second generation backcross (Bx), as well as more ancestral crossings that are based on Hardy-Weinberg expectations for random mating over several generations. Unlinked SNPs were used in both

INTROGRESS and NEWHYBRIDS analyses, with additional filtering that included removal of: (a) loci found only in a single species, (b) loci found in <80% of individuals, and (c) loci with a minimum allele frequency >10%

Results

After filtering, a total of 20,928 loci and 98,230 SNPs were recovered in *Pantosteus,* with 60.8% of the SNPs (N=59,729) being parsimony-informative and contained 29.28% missing data. For the subgenus *Catostomus*, 21,306 loci and 104,372 SNPs were recovered, with 66.4% of SNPs (N=69,306) being parsimony-informative and contained 28.16% missing data. Unlinked SNPs (N=19,717: *Catostomus*; N=20,038: *Pantosteus*) were used to generate Bayesian clustering and multispecies coalescent phylogenies for each subgenus. Average coverage post-filtering was 17.8x, with all individuals >8.9x coverage and with <80% missing data.

Phylogeny

Both concatenated SNP methods produced the same topology (Figures 2A and 3A), with posterior probabilities of one and a bootstrap support of 100% for all nodes at the species level as well as for some populations within species. The multispecies coalescent phylogenies returned the same general topology as that produced by concatenated methods, but with variance in placement of the root of the Rio Grande Sucker (*C. P. plebeius*) / Desert Sucker (*C. P. clarkii*) / Bluehead Sucker (*C. P. discobolus* and *C. P. virescens*) clade in the *Pantosteus* subgenus (Figure 2B and 3B). For the concatenated methods, Bluehead Sucker was placed outside the remaining species (Figure 2A), whereas Rio Grande Sucker was placed outside for the multispecies coalescent method (Figure 2B). The latter reflects previous research, to include morphological

phylogenies and the fossil record (Smith et al. 2013), as well as mitochondrial phylogenies (Chen and Mayden 2012, Unmack et al. 2014), and our results from Chapter 2.

For *Pantosteus*, isolated drainages were identified with high support in all phylogenetic analyses, to include: The 1) Mimbres and Rio Grande rivers for Rio Grande Sucker, 2) Bill Williams and Gila rivers for Desert Sucker, and 3) Bonneville Basin, Upper Colorado and Little Colorado rivers for Bluehead Sucker (Figure 2A and B). There was scant resolution among populations in the Upper Colorado River, but with some highly-supported nodes for management units that are consistent with previous microsatellite and mitochondrial analyses (Hopken et al. 2013). Several highly supported groups were found within the Little Colorado River, to include: 1) Defiance Plateau (AZ), 2) Willow Creek (AZ), 3) Silver Creek (AZ), 4) Upper Little Colorado River (AZ), and 5) Zuni River (NM) (Figure 2A and B).

For *Catostomus*, highly supported splits were found between species as well as within Flannelmouth Sucker (i.e., Virgin River, Upper Colorado River, and Little Colorado River; Figure 3A). The Little Colorado River clade was sister to the Upper Colorado River samples, with the Virgin River samples outside of this grouping and consistent with the results from Chapter 2 (Figure 3A and 3B). Within the Little Colorado River, ML analyses indicated three moderately-supported groups (80-90% bootstrap support): 1) Chevelon Canyon Lake (AZ), 2) Silver Creek (AZ), and 3) Wenima Wildlife Area (AZ) in the Upper Little Colorado River (AZ) (Figure 3A). These groups were supported by 1.0 Bayesian posterior probability in MRBAYES, but less so by SVDQUARTETS (<70% bootstrap support). Also, the split between Upper Colorado and Little Colorado rivers showed only moderately support (at 86%). It should be noted that the Wenima population was left out of the SVDQUARTETS phylogeny due to spurious results

produced by hybridization with Sonora Sucker. Removing the Wenima population had no effect on topology or supports.

Structure

The optimum number of supported clusters for *Pantosteus* was k=6, corresponding to: 1) Mountain Sucker (*C. P. jordani*), 2) Desert Sucker (*C. P. clarkii*), 3) Rio Grande Sucker (*C. P. plebeius*), and three clusters within Bluehead Sucker that corresponded to 4) Bonneville Basin (*C. P. virescens*), 5) Colorado River, and 6) Little Colorado River. Rio Nutria (NM) was the only population in the Zuni River to assign with Rio Grande Sucker (Figure 2C).

The only other mixing among clusters in *Pantosteus* was between Bluehead Sucker from the Colorado and Little Colorado rivers. This occurred in: 1) two out of ten samples from Chinle Wash (AZ), a tributary of the San Juan River, and 2) all Little Colorado River samples with the exception of the Zuni River populations (the only group fully assigned to the Little Colorado River cluster). The proportion of assignments to cluster varied between regions in the Little Colorado River, but was largely consistent within each, with the Defiance Plateau (AZ) having the greatest assignment to the Colorado River cluster (32.7-38.6%), followed by populations from the Upper Little Colorado River (12.9-22.4%), and Willow and Silver creeks (AZ) (0.5-1.6%) (Figure 2C).

For *Catostomus*, the optimum number of supported clusters was k=5, corresponding to the five currently recognized species, including: 1) White Sucker (*C. commersonii*), 2) Utah Sucker (*C. ardens*), 3) Razorback Sucker (*Xyrauchen texanus*), 4) Sonora Sucker (*C. insignis*), and 5) Flannelmouth Sucker (*C. latipinnis*). No structure was detected within Flannelmouth Sucker, even at higher k-values. The Wenima Wildlife Area in the Little Colorado River was the

only population to have mixed assignment with allocation to both Flannelmouth and Sonora sucker gene pools, but with some variation, in that four samples had lower assignments to Sonora Sucker (10.3-13.9%) when compared to the other three (26.9-28.3%). This, in turn, may represent different classes of hybrid (Figure 3C).

Hybridization

Individuals (N=4) from the Rio Nutria were tested for hybridization by using the Rio Grande Sucker (N=6) and other Zuni Bluehead Sucker (N=8) as parentals, with no missing data in the 302 unlinked SNPs employed [59.2% of which (N=179) were fixed between species]. All four samples assigned with perfect support in NEWHYBRIDS to the "random mating over several generations" category. An evaluation of the Rio Nutria individuals by INTROGRESS yielded hybrid index values somewhat larger for Rio Grande Sucker (0.228-0.347) than the q-scores from STRUCTURE (0.170-0.252). However, the 95% confidence intervals in INTROGRESS overlapped with the q-scores from STRUCTURE (Figure 4A), suggesting their agreement.

Hybridization between Sonora Sucker and Flannelmouth Sucker was also tested in the Little Colorado (N=14) and Virgin rivers (N=8), using Sonora Sucker (N=10) and the remaining Flannelmouth Sucker (N=13) as parentals. This analysis had 12.8% missing data, with 625 unlinked SNPs [38.9% of which (N=243) were fixed between species]. Wenima Wildlife Area (AZ) was the only Little Colorado River population to reflect statistically significant hybridization with Sonora Sucker. Here, the evaluation of Wenima individuals by INTROGRESS yielded hybrid index values for Sonora Sucker (0.170-0.320) that were slightly larger than q-scores from STRUCTURE (0.103-0.283). Again, the 95% confidence interval generated by INTROGRESS overlapped with q-scores from STRUCTURE, indicating agreement. NEWHYBRIDS

assigned four Wenima samples with greater than 95% probably as second generation (Bx) backcrosses into Flannelmouth Sucker. It also failed to assign the other three samples to any hybrid class, but instead assigned each to different classes: F2, second generation backcrosses (Bx) into Flannelmouth Sucker, and "the random mating over several generations" category. All Flannelmouth Sucker samples from the Virgin River also had low, but significant, hybrid index values for Sonora Sucker (0.079-0.096). This was not delineated in STRUCTURE, but it is consistent with the significant Patterson's D-statistic in Chapter 2 that points to potential historic introgression (Figure 4B).

Chinle Wash (N=10) and the Little Colorado River (N=17) with exclusion of Zuni River were evaluated for mixing between the two clusters of Bluehead Sucker found in the Colorado River Basin, with parentals being 1) Bluehead Sucker from throughout the Upper Colorado River (N=21), and 2) those from the Agua Remora and Tampico Springs of the Zuni River (N=8). The latter were used as a parentals since they assigned completely to the Little Colorado River cluster in STRUCTURE (Figure 2C). A total of 546 unlinked SNPs were input to INTROGRESS [17.9% fixed differences (N= 98) with 11.3% missing data]. Results essentially mirrored those of STRUCTURE, with the highest hybrid index values for the Colorado River Bluehead cluster found in the Defiance Plateau (0.601-0.628), followed by Upper Little Colorado River populations (0.355-0.403), then Silver and Willow creeks (AZ) (0.266-0.333). However hybrid index values for all admixed individuals were significantly higher than were q-scores, based on 95% confidence intervals in INTROGRESS. Two Chinle Wash samples also showed significant admixture, with hybrid index values for the Colorado River cluster being 0.654 and 0.945. However, the former value had a high interspecific heterozygosity value, indicating a potential recent admixture (Figure 4C).

Discussion

In 2004, six states that encompass the Colorado River Basin signed a 'Range-wide Conservation Agreement Plan' with the goal of managing three fish species (Roundtail Chub *Gila robusta*, Flannelmouth Sucker *Catostomus latipinnis*, and Bluehead Sucker *C. Pantosteus discobolus*) basin-wide in order to avoid potential listing under the Endangered Species Act (Carmen 2007). All three exhibit distinct life histories and habitat preferences that may have differentially driven their divergences within the basin. Here we examined two of these species (Flannelmouth Sucker and Bluehead Sucker), the former a large- river omnivore whereas the latter is an algavore largely restricted to higher elevations. Our purpose was to potentially distinguish similarities and differences in their patterns of divergence, and to suggest taxonomic revisions, if appropriate. In doing so, we also examined the impacts of introgression within and among species, as a means to disentangle their complex evolutionary histories, as driven by the deep and tumultuous history of the basin.

Life History and its Effects on Differentiation

In Chapter 2, comparative phylogeography of the *Catostomus* and *Pantosteus* subgenera (per Smith et al. 2013) revealed patterns of parallel phylogeography throughout much of the Colorado River and neighboring basins (Figure 5). However, the scale of divergence differed greatly between these groups, as highlighted by the focus of this study, the Upper Colorado River Basin.

Although three distinct clades were identified in Flannelmouth Sucker, the split appears to be relatively recently (i.e., within tens of thousands of years, corresponding to Late Pleistocene). The divergences were likely climate-driven, augmented by volcanic barriers that

appeared during this period, such as Grand Falls on the Little Colorado River (i.e., ~20kya). Lineages of Bluehead Sucker, on the other hand, reflect temporally deeper origins per our branch lengths and clustering, as well as previous mitochondrial dating (4.5-3.5mya; Unmack et al. 2014). However secondary contact between these lineages, as well as their hybridization with other species, has further complicated the disentanglement of their phylogenetic histories, and consequently delayed their conservation and management.

The contrasting timescales seen above for these clades may have been driven by difference in habitat preference between subgenera. The subgenus *Pantosteus* is commonly referred to as 'mountain sucker' due to its preference for higher elevation streams with cooler habitats, whereas the subgenus *Catostomus* contains physically larger omnivorous fish restricted to lower-elevations in larger rivers of the basins (Sigler and Miller 1962, Smith 1966). Thus, although Bluehead and Flannelmouth suckers largely co-occur, their preferences in habitat differ with profound effects on their diversification rates. For example, Douglas et al. (2003) suggested that Flannelmouth Sucker in the Upper Colorado River Basin were driven into the Lower Basin by the intense warming and drying induced by the Hypsithermal in Late Pleistocene (Pielou 1974), and later recolonized the Upper Basin from the Grand Canyon. This same pattern was observed in mainstem Bluehead Sucker. However, populations likely persisted in the upper mountain reaches of various tributaries of the Upper Colorado River Basin, the results of which are reflected by shallow, but discernable genetic divergence among populations, consistent with recognition as several management units (MUs) (Hopken et al. 2013).

Bonneville Basin

Although both species are sympatric in the Colorado River Basin, the Bluehead Sucker also occurs in the Bonneville and Upper Snake River basins. The Bonneville Basin/ Snake River form may represent a unique species, originally described as such (*C. P. virescens*; Cope and Yarrow 1875, Snyder 1924) but subsequently collapsed into *C. P. discobolus* (Smith 1966). The split between *C. P. virescens* in the Bonneville Basin/ Snake River and *C. P. discobolus* in the Colorado River Basin is supported in all analyses herein, to include population clustering and three different phylogenetic methods (Figure 2). The convergence of all methods, along with recent morphological (Smith et al. 2013) and mitochondrial phylogenies (Hopken et al. 2013, Unmack et al. 2014), supports the re-elevation of the Bonneville Bluehead Sucker. Furthermore, the timing of the split between these two species (i.e., ~4.8mya) exceeds the timing of diversification found in other catostomid species (Unmack et al. 2014), highlighting the deep divergences between the subgenera.

Little Colorado River Basin

The results of all phylogenetic analyses presented herein separate Flannelmouth and Bluehead suckers in the Little Colorado River from the Upper Colorado River Basin, to include the Grand Canyon (Figures 2 and 3). These Little Colorado River lineages represent 1) Zuni Bluehead Sucker (*C. P. discobolus yarrowi*), which has a drastically reduced range that promoted its recent listing under the Endangered Species Act (Federal Register 2014), and 2) Little Colorado River Sucker, which is currently recognized by Arizona Game and Fish as an undescribed species distinct from Flannelmouth Sucker, largely due to morphological differences (Miller 1972, Minckley 1980).

Zuni Bluehead Sucker

Zuni Bluehead Sucker was originally allocated as a separate species when *Pantosteus* was first described (Cope and Yarrow 1875). It was subsequently defined instead as a subspecies, based on allozymes and morphologically data (Smith et al. 1983). The latter work also suggested it was of hybrid origin between Bluehead and Rio Grande suckers. However several evaluations now refute this hypothesis: A) Only one population (Rio Nutria) contains alleles from Rio Grande Sucker (Figure 2C, Figure 4A; D-statistics, Chapter 2); B) Allozyme analyses (Crabtree and Buth 1987) lack support; and C) Data from single-gene sequencing likewise lack congruence (Turner and Wilson 2009, Hopken et al. 2013).

Zuni Bluehead Sucker is also believed to have originated in the mountains of northeast Arizona and northwest New Mexico, to include Zuni River and Kin Lee Chee Creek of the Defiance Plateau (Smith et al. 1983). However, phylogenetic analyses place populations from Kin Lee Chee Creek and the entire Defiance Plateau as paraphyletic to the Zuni River samples, due to the sister relationship between the Zuni River and the remainder of the Little Colorado River populations (Figure 2A, 2B). In addition, the entire Little Colorado River Basin clade forms a monophyletic group that is sister to the rest of the Colorado River Bluehead Sucker populations (Figure 2A, 2B). This suggests that the Zuni Bluehead Sucker spread into the Little Colorado River when mountain streams integrated with the Little Colorado River (as suggested by Minckley 1973, Smith et al. 1983). The current hypothesis (Smith et al. 1983) suggests that it was subsequent replaced by Bluehead Sucker in all drainages of the Little Colorado River drainage, save the Zuni River and Kin Lee Chee Creek.

However, population-clustering analyses yielded a clade unique to the Little Colorado River within which only populations from the Zuni River were assigned (Figure 2C). All other

populations were assigned to a composite that represented this cluster and the remainder of the Colorado River Basin, with proportions for the latter ranging from 0.5-38.6%. This admixture was also detected in hybrid index analyses, indicating that the remainder of the Little Colorado River Basin may be of admixed ancestry between these two lineages (Figure 4C). Thus, instead of Bluehead Sucker replacing Zuni Bluehead Sucker in the Little Colorado River, it may have instead hybridized with it, with admixed populations now found in all but the Zuni River. Also, the Defiance Plateau may represent the origin for this Bluehead Sucker invasion, based on the greatest proportion of assignments to the Colorado River cluster. This may presumably be the result of a stream capture with Chinle Wash, due to its close location and the presence of two Chinle Wash individuals partially assigned to Zuni Bluehead Sucker (Figure 2C and 4C).

Further investigations employing a diversity of techniques (e.g., morphology, stable isotopes, and transcriptomes) are needed to understand how admixture has affected the breadth of lineages in the Little Colorado River. Our results support the presence of the Zuni Bluehead Sucker, and highlight the necessity of including the entire Little Colorado River clade in further assessments of its status. This also mandates a reassessment of the Zuni Bluehead Sucker distribution, so as to either remove from it the Kin Lee Chee Creek population, or include it within the Little Colorado River Basin as a whole.

Little Colorado River Sucker

In contrast to the Zuni Bluehead Sucker, the Little Colorado River Sucker did not cluster as a separate lineage in population clustering analysis, despite its representation as a monophyletic group in all phylogenetic analyses (Figure 3). This may reflect the recent origin of the Little Colorado River Sucker, presumed to have occurred concomitant with formation of

Grand Falls some 20kya. This vicariant break separates the Upper Little Colorado River from the rest of the Colorado River and prevents contemporary upstream gene flow (Duffield et al. 2006). Thus, despite similar contemporary phylogeographic patterns found in Zuni Bluehead Sucker and Little Colorado River Sucker, different evolutionary histories were driven by habitat preference that ultimately resulted in different levels of divergence but similar contemporary ranges. This underscores the chaotic history of the Desert Southwest and the need for comparative studies that disentangle the organismal histories that coexist there.

Hybridization was also detected between Sonora and Flannelmouth suckers in one population in the Little Colorado River (Wenima Wildlife Area; Figure 3C). These admixed individuals are presumably due to a recent hybrid event, as gauged by the variation found in Sonora Sucker with regards to q-scores (Figure 3C), hybrid index values (Figure 4B), high interspecies heterozygosity (Figure 4B), and the presence of four second-generation hybrids. Regardless, further sampling is needed to confirm this assumption.

Virgin River

Despite forming a monophyletic group, the Little Colorado River Sucker fell within a paraphyletic Flannelmouth Sucker. This was due largely to the placement of the Virgin River population outside of the rest of Flannelmouth Sucker (Figure 3). The Virgin River form has also been suggested as being potentially unique due to its elevated morphological variation, previously attributed to hybridization with Sonora Sucker (*C. insignis*) and Razorback Sucker (*Xyrauchen texanus*) (Minckley 1980). Historic introgression with Sonora Sucker was detected in all Virgin River samples, a reflection of the elevated hybrid index values and low interspecies heterozygosity (Figure 4B). While the proportion of Sonora Sucker is reduced in this population,

it is nevertheless significant based on previous D-statistic tests (Chapter 2) and significant hybrid index values for all samples (Figure 4B).

However, the three Flannelmouth Sucker groups (i.e., Upper Colorado, Little Colorado, and Virgin River) grouped as a single cluster (Figure 3C) and the split of the groups as seen in the phylogeny could not be replicated in cluster analyses, even at higher k-values. This, in turn, may reflect the recent origins of these groups, a supposition further supported by the short branch lengths separating the groups in the phylogeny (Figure 3A). There is also a lack of fixed differences between these lineages in a previous mitochondrial analysis (Douglas et al. 2003). These aspects fit with the previous assumption that the Virgin River population may have separated only recently, i.e., Late Pleistocene, most likely due to climatic oscillations that may have alternately connected and separated the Grand Canyon and Virgin River populations as recently as 7.5kya (Douglas et al. 2003).

Conclusion

Flannelmouth Sucker and Bluehead Sucker are two species of concern in the Colorado River Basin. Several proposed taxonomic revisions could affect not only the management of these species but also the basin as a whole. Comparative phylogeographic patterns appear very similar for both species, yet contrasting levels of divergence reflect very different evolutionary histories that, in turn, impact species delimitation. Three lineages of Bluehead Sucker were detected in all phylogenetic and population genetic methods applied. This supports the elevation of *C. P. virescens* in the Bonneville and Upper Snake River as a species separate from *C. P. discobolus* in the Colorado River (as suggested by Smith et al. 2013, and Unmack et al. 2014). Results also support the Zuni Bluehead Sucker as a unique form. However, the current

designation of Kin Lee Chee Creek individuals as congruent with the Zuni River populations, is an erroneous decision based on paraphyletic grouping. This can be resolved by including all Little Colorado River Bluehead Sucker populations in this grouping, or the removal of Kin Lee Chee Creek from the listing of the Zuni Bluehead Sucker. The situation is further complicated by hybridization with Rio Grande Sucker, and Bluehead Sucker from the Colorado River.

The Little Colorado River Sucker was placed within a paraphyletic Flannelmouth Sucker that could only be resolved by designating the Virgin River population as a unique lineage. However, the recent origin of these three clades is sustained by a failure to detect them in all population genetic analyses, and consistent with the lack of resolution seen in mitochondrial analyses (Douglas et al. 2003). Thus, the three lineages of Flannelmouth Sucker more accurately represent evolutionary significant units (ESUs), due to their slight phenotypic and genetic differentiation, their current geographic isolation, and the lack of concordance required for recognition under the genealogical concordance component of the phylogenetic species concept.

Acknowledgements

Numerous agencies contributed field expertise, specimens, technical assistance, collecting permits, funding or comments for the completion of this study. We are in debt to students, postdoctorals and faculty who contributed to the development of our research: A. Albores, P. Brunner, M. Davis, T. Dowling, R. Cooper, J. Cotter, E. Fetherman, M. Hopken, M. Kwiatkowski, A. Reynolds, J. Reynolds, C. Secor, and P. Unmack. Sampling procedures were approved under IACUC permit 98-456R (Arizona State University) and 01-036A-01 (Colorado State University). Samples were also provided by the Museum of Southwestern Biology, University of New Mexico (T. Turner, L. Snyder). Accession numbers for samples from the Museum of Southwestern Biology are: MSB 44046, 44047, 44052, 44059, 44067, 49715, 49962, 53500, 63249, and 88362. This research was supported by generous endowments to the University of Arkansas: The Bruker Professorship in Life Sciences (M.R.D.) and 21st Century Chair in Global Change Biology (M.E.D.).

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Tables

Table 1: Sample sizes from each species by drainage. Included are number of sample sites (Sites) and samples (N) for each species.

Species	Major Drainage	Location	State	Sites	N
C. (P.) jordani	Missouri	Beaver Creek	MT	1	2
C. (P.) virescens	Bonneville	Various	WY, UT	5	5
C. (P.) discobolus	Upper Colorado	Green River	WY, UT, CO	4	4
		Colorado River	UT, CO	4	4
		San Juan River	UT, NM	3	3
		Chinle Wash	AZ	5	10
	Grand Canyon	Grand Canyon	AZ	5	10
	Little Colorado	Defiance Plateau	AZ	3	6
		Upper Little Colorado	AZ	3	6
		Silver Creek	AZ	2	3
		Willow Creek	AZ	1	2
C. (P.) d. yarrowi	Little Colorado	Zuni River	NM	3	12
C. (P.) clarkii	Bill Williams	Bill Williams River	AZ	1	2
	Gila	Verde River	AZ	2	2
		Gila River	NM	2	2
		San Francisco River	NM	2	2
C. (P.) plebeius	Mimbres	Mimbres River	NM	2	2
	Rio Grande	Rio Grande	CO, NM	4	4
X. texanus	Upper Colorado	San Juan River	UT, NM	2	4
C. ardens	Bonneville	Various	WY,UT	2	4
C. latipinnis	Upper Colorado	Green River	WY, UT, CO	4	4
		Colorado River	UT, AZ	2	2
		San Juan River	UT, NM	2	2
	Grand Canyon	Grand Canyon	AZ	5	5
	Virgin River	Beaver Dam Wash	UT	1	8
C.* sp. cf *latipinnis	Little Colorado	Chevelon Canyon	AZ	1	4
		Silver Creek	AZ	1	3
		Wenima	AZ	1	7
C. insignis	Bill Williams	Bill Williams River	AZ	1	2
	Gila	Verde River	AZ	2	2
		Gila River	NM	2	2
		San Francisco River	NM	2	4
C. commersonii	Mississippi	Various	ND, IL	3	3
	Upper Colorado	Green River	WY, CO	2	2
			Total	**85**	**139**

Figures

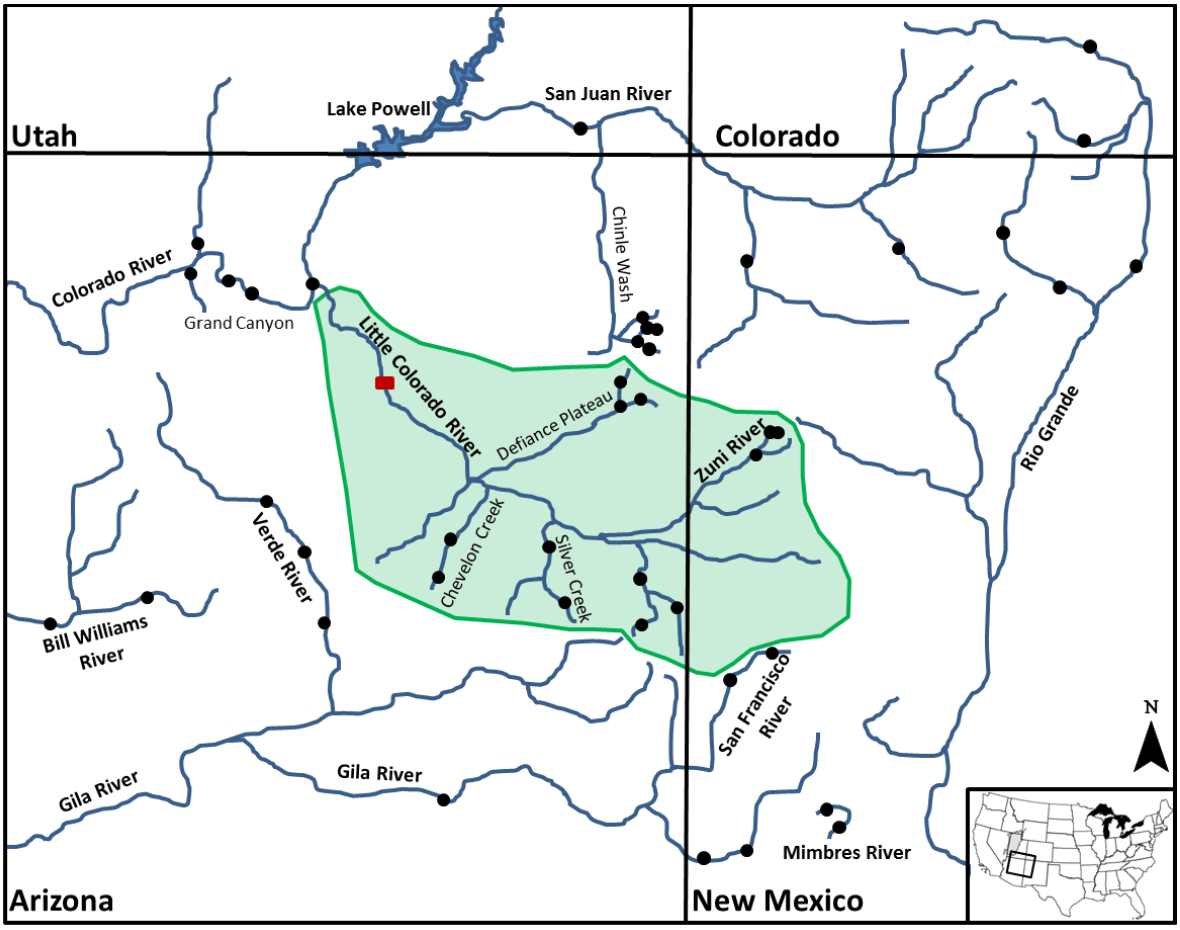
Utah
Colorado
Arizona
New Mexico
Lake Powell
San Juan River
Colorado River
Grand Canyon
Chinle Wash
Little Colorado River
Defiance Plateau
Zuni River
Rio Grande
Verde River
Chevelon Creek
Silver Creek
Bill Williams River
San Francisco River
Gila River
Gila River
Mimbres River
N

Figure 1.

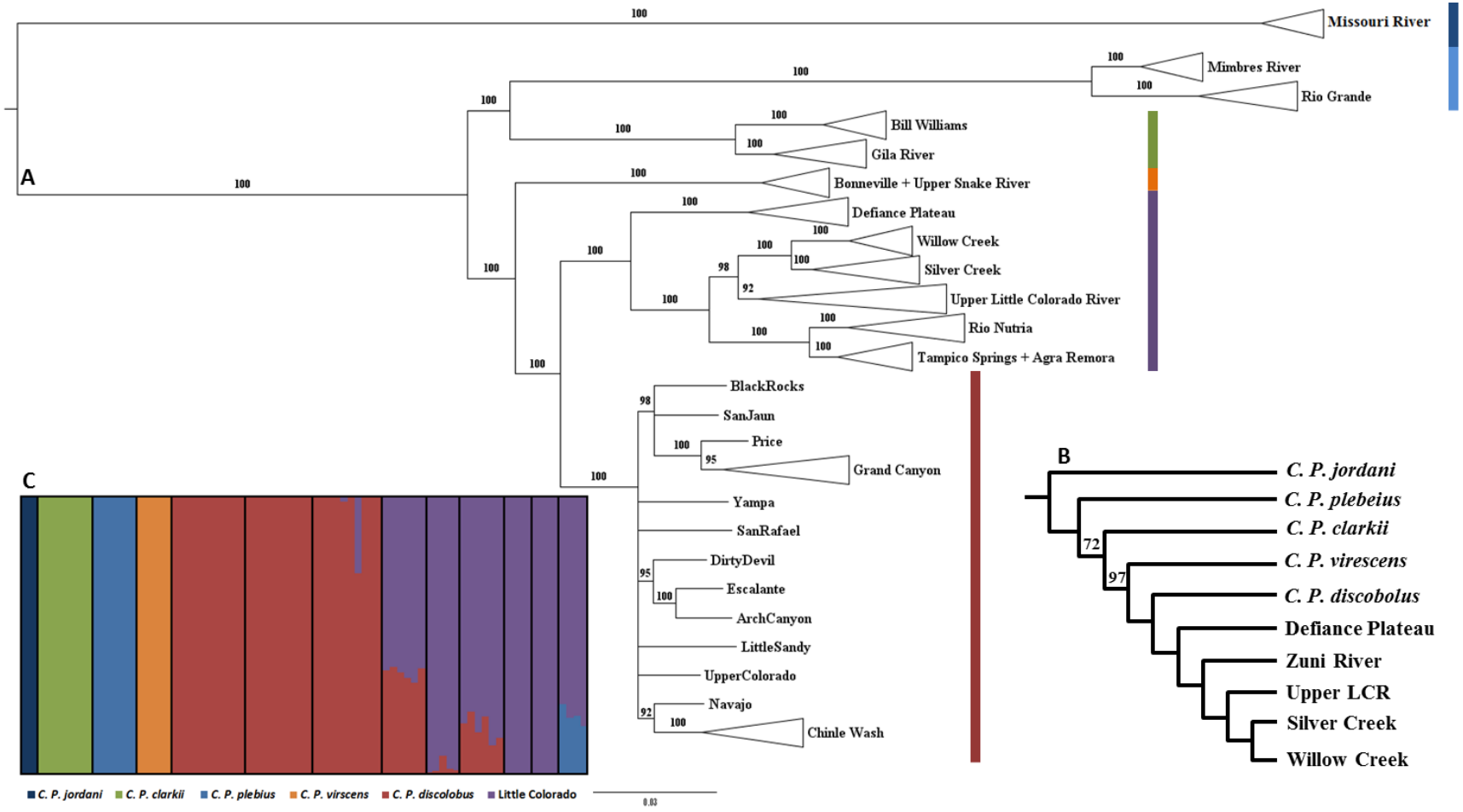
A
Missouri River
Mimbres River
Rio Grande
Bill Williams
Gila River
Bonneville + Upper Snake River
Defiance Plateau
Willow Creek
Silver Creek
Upper Little Colorado River
Rio Nutria
Tampico Springs + Agra Remora
BlackRocks
SanJaun
Price
Grand Canyon
Yampa
SanRafael
DirtyDevil
Escalante
ArchCanyon
LittleSandy
UpperColorado
Navajo
Chinle Wash
B
C. P. jordani
C. P. plebeius
C. P. clarkii
C. P. virescens
C. P. discobolus
Defiance Plateau
Zuni River
Upper LCR
Silver Creek
Willow Creek
C
C. P. jordani
C. P. clarkii
C. P. plebius
C. P. virscens
C. P. discolobus
Little Colorado
0.03

Figure 2.

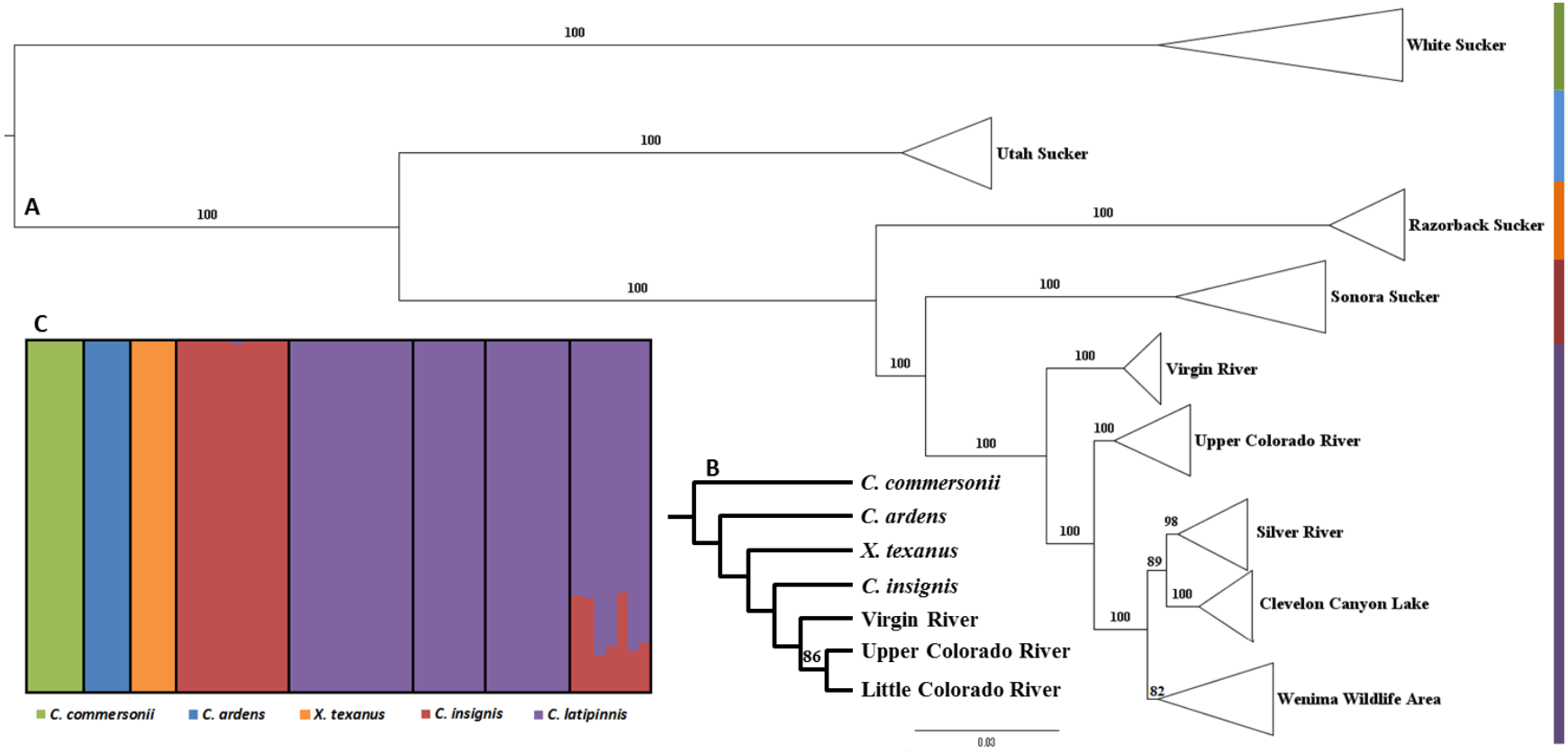
A
100
White Sucker
100
Utah Sucker
100
Razorback Sucker
100
Sonora Sucker
100
Virgin River
100
Upper Colorado River
100
Silver River
98
89
Clevelon Canyon Lake
100
Wenima Wildlife Area
82
100
100
100
100
100
100
B
C. commersonii
C. ardens
X. texanus
C. insignis
Virgin River
Upper Colorado River
86
Little Colorado River
0.03
C
C. commersonii
C. ardens
X. texanus
C. insignis
C. latipinnis

Figure 3.

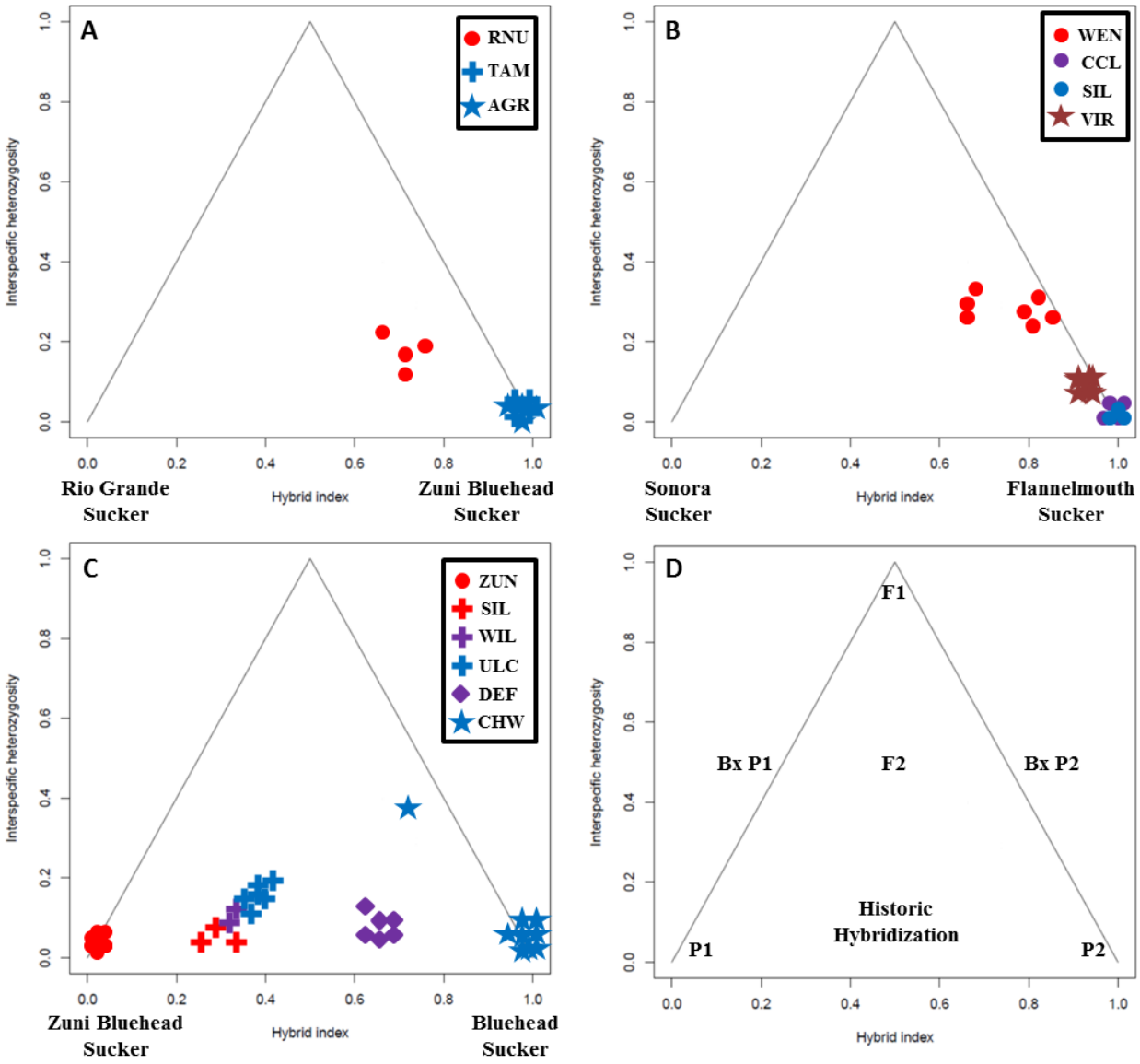
A
RNU
TAM
AGR
Interspecific heterozygosity
Hybrid index
Rio Grande Sucker
Zuni Bluehead Sucker
B
WEN
CCL
SIL
VIR
Sonora Sucker
Flannelmouth Sucker
C
ZUN
SIL
WIL
ULC
DEF
CHW
Zuni Bluehead Sucker
Bluehead Sucker
D
F1
Bx P1
F2
Bx P2
Historic Hybridization
P1
P2

Figure 4.

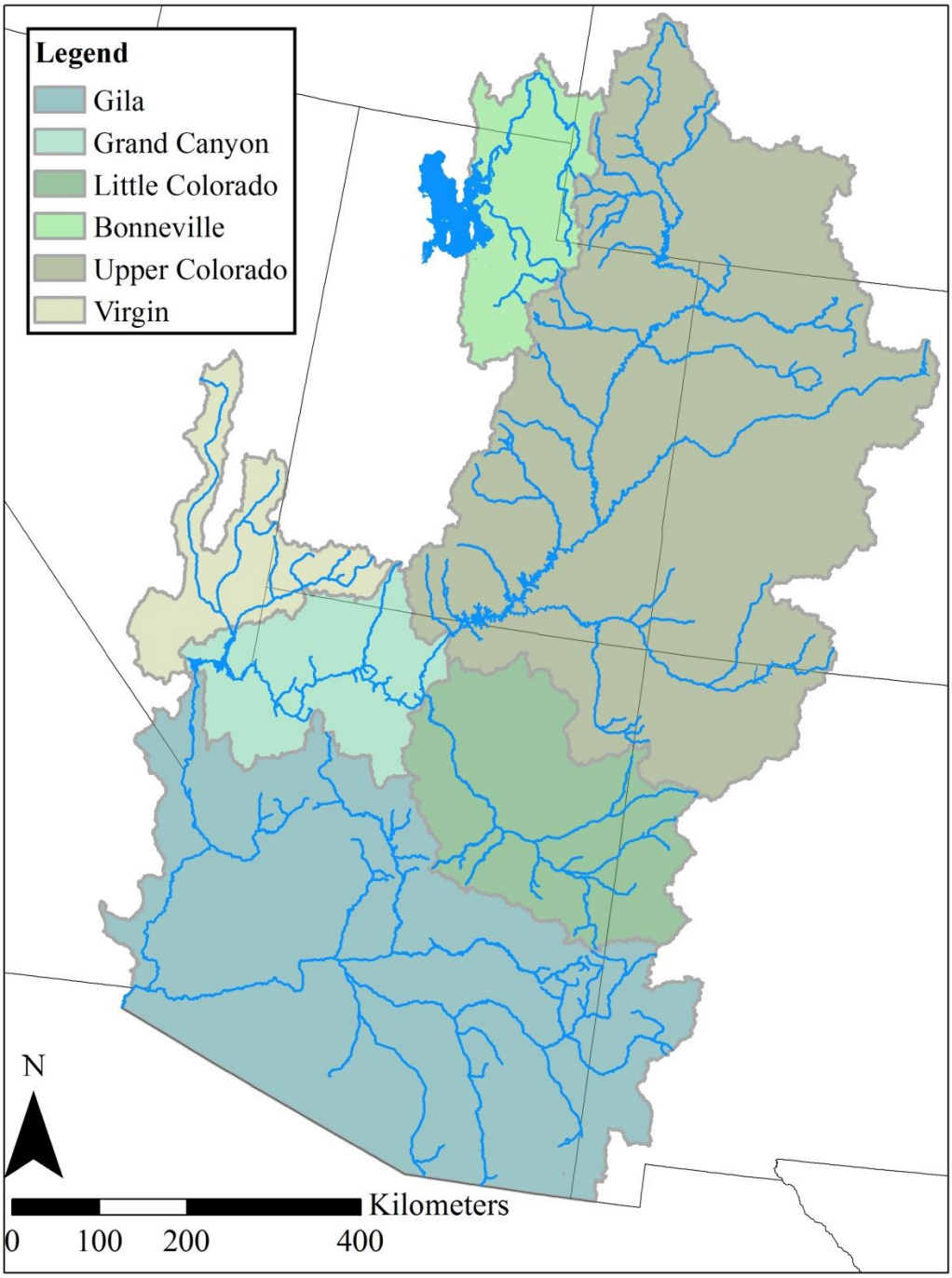
Legend
Gila
Grand Canyon
Little Colorado
Bonneville
Upper Colorado
Virgin
N
Kilometers
0
100
200
400

Figure 5.

V. Phylogenetic Divergence and Reproductive Compatibility Bookmark the Reticulated Evolution of Endemic Suckers (Pisces: Catostomidae) in the Colorado River Ecosystem of Western North America

Abstract

Reticulation has driven the evolution of catostomid fishes in the Colorado River ecosystem of western North America and constrained our understanding of their phylogenomic relationships and distributions. The resulting complexity has been confounded of late by admixture with introduced congeners, and the deciphering of these reticulations has been limited by the weak resolution of morphological and legacy molecular data. Here, we employ double digest restriction-site associated DNA (ddRAD) to (a) clarify the introgression and hybridization of catostomid fishes in the Colorado River Basin, (b) assay its breadth by placing it within the context of phylogenetic distance and 'ecological specialization' among dyads, and (c) examine potential drivers of introgression over different hybrid crosses. Admixture was detected involving ten different pairs of species with rates of introgression and breakdown of reproductive isolation increasing with habitat alteration, a situation that may suppress behavioral isolating mechanisms and/or promote hybrid survival. Although hybridization occurs throughout the genus despite phylogenetic distance, introgression is only found within subgenera, implicating distance and/or ecological specialization as drivers of reproductive isolation. Our study examines hybridization and introgression across an entire freshwater basin, to include all catostomids native or introduced into the system. Understanding patterns of hybridization and reproductive isolation across the range of species provides a baseline for disentangling the long history of hybridization that in turn will help with adaptive management and conservation.

Introduction

Reticulated evolution is a product of several (often interacting) phenomena, including horizontal gene transfer, polyploidization, and hybridization/introgression (Wendel and Doyle 1998). All have been traditionally viewed as examples of 'aberrant evolution,' in that their occurrence was not only peripheral to, but also disruptive of diversifying processes, with outcomes translated as an anastomosing network rather than a more traditional, bifurcating tree. This connotation of aberrancy is best reflected by legacy perspectives regarding hybridization (i.e., "… the grossest blunder in sexual preference which we can conceive of an animal making;" Fisher 1930:130), and (…the "infection" of one species with the genes from a second; DuRietz 1930:376, 380, 386, 411).

Rather than a contradiction to evolution, hybridization offers instead the opportunity to grasp how the evolutionary process can be facilitated despite reproductive isolation (Good et al. 2003). Hybridization, especially when coupled with introgression, has long been thought to play a beneficial evolutionary role in both plants (Arnold 1992) and animals (Dowling and Secor 1997). Thus, introgression, the incorporation of alleles from one species into the gene pool of another, can spur evolution by i) generating new genetic variation, ii) transferring adaptive traits, and iii) producing new lineages that can exploit a novel niche within which neither parental taxa could succeed (Darras et al. 2014, Seehausen et al. 2014). At the same time, introgressive hybridization can have negative consequences, especially with regard to anthropogenic introductions, by either i) disrupting local adaptations, or ii) genetically swamping endemics, leading to the effective extinction of the species (Rhymer and Simberloff 1996). These conflicting views of introgressive hybridization have often complicated conservation management (Allendorf et al. 2001).

Hybridization is relatively common in fishes, as facilitated by a natural history that involves external fertilization, weak reproductive isolation, and a relatively linear mode of dispersal in streams (Hubbs 1955, Campton 1987). Introgressive hybridization has also been documented in a myriad of fish taxa throughout North America, including trout (Leary et al. 1984), pupfish (Echelle and Connor 1989), mosquito fish (Quattro et al. 1991), bass (Barwick et al. 2006), and carp (Lamer et al. 2010). Habitat alterations and introduction of invasive congeners most often promote admixture, as frequently noted in taxa of southwestern North America: chubs (Hamman 1981, Douglas et al. 1989), pupfish (Echelle and Echelle 1994) and suckers (Douglas and Marsh 1998, Clarkson and Minckley 1988, McDonald et al. 2008, Douglas and Douglas 2010).

Species in the genus *Catostomus,* like many cypriniforms, readily hybridize, especially when invasive congeners have been introduced and/or habitats disturbed (Holden and Stalnaker1975, Douglas and Douglas 2010). This phenomenon may also yield more subtle effects, such as potentially providing a 'hybrid bridge' for introgression among species that would not naturally hybridize (McDonald et al. 2008). Hybridization has also been noted among native sympatric species without the influence of introduced congeners (Hubbs et al. 1943, Nelson 1968), and has occurred between genera within families (McAda and Wydoski 1980, Buth et al. 1992, Tranah and May 2006). However, the taxonomic placement of these genera has been a subject of debate (Chen and Mayden 2012, Chapter 2).

Here we focus on three native species that have potentially hybridized in the Upper Colorado River Basin: Flannelmouth Sucker (*Catostomus latipinnis*), Bluehead Sucker (*C. Pantosteus discobolus*), and Mountain Sucker (*C. P. platyrhynchus*). These species show admixture 1) amongst themselves, 2) with sympatric Razorback Sucker (*Xyrauchen texanus*), 3)

with Lower Colorado River Basin Sonora Sucker (*C. insignis*) and Desert Sucker (*C. P. clarkii*), and 4) contemporaneously with introduced White Sucker (*C. commersonii*) and Longnose Sucker (*C. catostomus*). Our study examines range-wide molecular evidence of hybridization and introgression that employs all members of the clade, both native and introduced, and as such, it provides a blueprint for disentangling historical events from those more contemporary. This, in turn, promotes the adaptive management and conservation of these species in western North America, and sheds light on the evolution of reproductive isolation with increasing phylogenetic divergence.

Methods

Sample acquisition

Fin clips and tissue plugs were collected throughout the Upper Colorado River Basin during 1995-2011 (Douglas and Marsh 1998, Douglas et al. 2003, Douglas and Douglas 2010, Hopken et al. 2013). Additional samples were obtained from the Museum of Southwestern Biology (University of New Mexico). Nine potential areas of hybridization were partitioned from these samples: 1) Big Sandy River (WY: N=45), 2) Blacks Fork (WY: N=50), 3) Upper Green River (WY: N=27), 4) Middle Green River (UT: N=11), 5) Yampa River (CO/UT: N=60), 6) Price River (UT: N=25), 7) San Juan River (NM/UT: N=47), 8) Grand Canyon (AZ: N=67), and 9) Virgin River (UT/NV: N=11) (Figure 1, Table 1). Details with regard to species, samples, and regions are provided in Appendix 2.

Data Collection

DNA was extracted with PureGene® Purification Kit or DNeasy® Tissue Kit (Qiagen Inc., Valencia CA) and stored in DNA hydrating solution (same kits). Libraries for double digest restriction-site associated DNA (ddRAD) were generated following the protocol outlined in Chapter 2. This included digesting with PstI (5'-CTGCAG-3') and MspI (5'-CCGG-3'), pooling 48 individuals prior to a size selection of 350-400bps, PCR amplification, and combining two libraries per lane of Illumina HiSeq 2000 single end 100bp sequencing. Samples for each reference species, region, and hybrid type were randomly distributed across several libraries and lanes so as to reduce the potential for library preparation bias or lane-effects. Sequencing was performed at the University of Wisconsin Biotechnology Center in Madison.

Filtering and Alignment

Illumina reads were filtered and aligned (protocol, Chapter 2) using PYRAD v.3.0.5 (Eaton and Ree 2013). This included: a clustering threshold of 80% based the uncorrected sequence variation in catostomid fishes (Chen and Mayden 2012, Chapter 1), and removal of restriction site sequence and barcode. In addition, loci were removed if they displayed: 1) <5 reads per individual), 2) >10 heterozygous sites within a consensus, 3) >2 haplotypes for an individual, 4) >75% heterozygosity for a site among individuals, and 5) <50% of individuals at a given locus.

Clustering Algorithm

All analyses utilized unlinked SNPs generated from PYRAD. Bayesian clustering (STRUCTURE v. 2.3.4; Pritchard et al. 2000) employed the admixture model with correlated allele frequencies and a burn-in of 100,000 generation, followed by 500,000 generations post-burn-in.

No population priors were used. Tests for genetic clusters (k) were each run with 15 iterations (with k=1 to k=16), then averaged across iterations to determine final values. The most likely number of genetic cluster was resolved by using the estimated log probability of data Pr(x|k) and the Δk statistic (per Evanno et al. 2005).

Hybrid Detection

For hybrid analyses, unlinked SNPs were used after additional filtering to include only fixed differences between the two parental species and the removal of loci that contained <80% individuals. Only fixed differences between species were used to ensure accurate interspecific heterozygosity. Both hybrid analyses required the designation of parental populations, with only two per test. Thus, the programs could only test hybrid ancestry from two parental species.

The R-package INTROGRESS (Gompert and Buerkle 2010) was used to develop a hybrid index (Gompert and Buerkle 2009) for each cross. This involved a test of hybridization between: 1) Flannelmouth Sucker x White Sucker, 2) Bluehead Sucker x White Sucker, 3) Flannelmouth Sucker x Bluehead Sucker, 4) Bluehead Sucker x Longnose Sucker, 5) White Sucker x Longnose Sucker, 6) Bluehead Sucker x Mountain Sucker, 7) Bluehead Sucker x Desert Sucker, 8) Bluehead Sucker x Razorback Sucker, 9) Flannelmouth Sucker x Razorback Sucker, and 10) Flannelmouth Sucker x Sonora Sucker. The same package (above) was used to create a triangle plot of hybrid index by interspecific heterozygosity for each admixture test and (occasionally) by populations as well.

NEWHYBRIDS (Anderson and Thompson 2002) was used to test the probability of assignment to a hybrid class, including first-filial (F1), second-filial (F2), and first- and second-generation backcross (Bx). Additional crossings, while of interest, would fail to assign

individuals to any of the designed hybrid or parental categories. Only first- and second-generation backcrosses were designated, since the potential exists for ancestral crosses to be spuriously assigned to later generation backcross categories (i.e., third, forth, etc.). Individuals would thus be designated as more contemporaneous then they actually were.

Results

Post-filtering 11,669 loci where obtained. These contained 89,868 SNPs of which 66,151were parsimoniously informative, with 32.39% missing data. Average coverage was 19x, with all individuals >11.5x coverage and with missing data <80%. A total of 11,501 unlinked SNPs were used in Bayesian clustering runs. The total number of fixed SNPs, number of individuals, and amount of missing data used for each hybrid cross are presented in Table 2.

Bayesian Clustering

The most likely number of genetic clusters was k=10, corresponding to the 10 species in this study. Each reference sample (Chapter 2) assigned to a single cluster, with the exception of one Virgin River sample that displayed Desert Sucker x Bluehead Sucker ancestry. It was excluded from the Desert Sucker reference sample in subsequent analyses. Flannelmouth Sucker from the Virgin River did assign to the Flannelmouth Sucker cluster, but was excluded as a reference sample in subsequent hybrid analyses as it reflected historical introgression with Sonora Sucker (per Chapters 2 and 3).

One Navajo River sample assigned to Bluehead Sucker (q=0.50), White Sucker (q=0.37), and Flannelmouth Sucker (q=0.13). A high interspecific heterozygosity value suggested it was most likely a back-cross between a first generation White Sucker x Flannelmouth Sucker hybrid

and a Bluehead Sucker. All other samples were, at most, assigned to two clusters, and were thus utilized for hybrid analyses (Figure 2).

Hybridization with Invasive Species

White Sucker x Flannelmouth Sucker hybrids occurred in all regions where both parentals are common. These included: All three regions above Flaming Gorge Dam, the Yampa River, and the Navajo River in the San Juan River region. White Sucker x Bluehead sucker were also found in the same locations, with the exception of the Upper Green River region where Bluehead Sucker is not common (Figure 2).

All White x Bluehead sucker hybrids (N=29) reflected hybrid indexes of ~0.50 and high interspecific heterozygosity (Figure 3B), and were assigned as F1 by NEWHYBRIDS. White x Flannelmouth sucker (N=68) were identified by q-scores (STRUCTURE: Figure 2) and hybrid indices (INTROGRESS: Figure 3A). Of these, NEWHYBRIDS assigned 46 as F1, three as F2, six as first generation Bx to White Sucker, eight as first generation Bx to Flannelmouth Sucker, two as second generation Bx to Flannelmouth Sucker, and three as undetermined. However, most study regions had only F1 hybrids and first-generation backcrosses to Flannelmouth Sucker. F2-hybrids were only found in the Upper Green (N=2) and Muddy Creek of the Yampa River (N=1) (Figure 4D, E). First generation White Sucker backcrosses were found only in the Upper Green (N=4) and Ham's Fork of Blacks Fork (N=2) (Figure 4B, D). Second generation backcrosses with Flannelmouth Sucker (N=2) as well as undetermined hybrid classes (N=3) were only in the Upper Green (Figure 4D).

Longnose Sucker was only found in the Big Sandy River, where it hybridized with both invasive White Sucker (N=1) and native Bluehead Sucker (N=2). All three were assigned a q-

score and hybrid index of 0.50-Longnose Sucker and 0.50-Bluehead or White sucker. High interspecific heterozygosity values (Figures 3C and D) and output from NEWHYBRIDS also pointed to an F1 hybrid status.

Hybridization between Native Species

Hybrids between Flannelmouth and Bluehead sucker were found in the Yampa River near Lily Park (N=3), as well as throughout the Middle Green region, to include the White River (N=1) and the mainstem Green River between its confluence with the White River and Flaming Gorge Dam (N=3). All seven had hybrid indices of ~0.50 with high interspecific heterozygosity (Figure 5A), and were assigned as F1 by NEWHYBRIDS. These assignments were consistent with q-scores that approximated 0.50 (Figure 2)

F1-hybrids involving Razorback Sucker were only found in the mainstem San Juan River near the confluence with the Colorado River. These included one F1-hybrid with Bluehead Sucker (Figure 5B) and one with Flannelmouth Sucker (Figure 6F). The F1-classification was consistent across all three analyses. Introgressed hybrids between Razorback Sucker and Flannelmouth Sucker were found in the Grand Canyon (N=4) and the Virgin River (N=1). All appeared to be high level backcrosses to Flannelmouth Sucker, since NEWHYBRIDS failed to assign them to any hybrid category, and all contained q-scores and hybrid indexes >0.75 for Flannelmouth Sucker (Figures 2 and 6F).

One Bluehead x Desert sucker hybrid and one Flannelmouth x Sonora sucker hybrid were found in Grand Canyon. The only sample of Desert Sucker from the Virgin River was also a Bluehead x Desert sucker hybrid. These assignments were consistent across both Bayesian clustering (Figure 2) and hybrid index (Figure 6D, E), but had low interspecific heterozygosity as

expected from historical hybridization, and thus were not assigned to any hybrid category. Flannelmouth Sucker from the Virgin River assigned completely to the Flannelmouth Sucker cluster (Figure 2), but with significant hybrid indexes based on a 95% confidence interval that indicated some level of historic introgression (Figure 6E).

Bluehead x Mountain sucker hybrids were only found in the Blacks Fork region (N=9) and the Price River (N=8) (Figure 6A). Interestingly, all Price River individuals were field-identified as Mountain Sucker, whereas one (of nine) from Blacks Fork was field-identified as Bluehead Sucker. Of the nine hybrids in Blacks Fork, two were classified as a first-generation backcross to Mountain Sucker, with the remaining seven as later-generation backcrosses, based on their high assignments to Mountain Sucker in both Bayesian clustering (Figure 2) and hybrid index (Figure 6B). Of the eight hybrids in the Price River region, five were caught in the Price River and three in the White River (Figure 6C). NEWHYBRIDS classified two from the White River as F1-hybrids, one from the Price River as first-generation backcross to Bluehead Sucker, while the remaining five were undetermined and presumably higher-level backcrosses into either Bluehead Sucker (N=2) or Mountain Sucker (N=3). Unlike Blacks Fork, the Price River also had several (N=10) field-identified Mountain Sucker that were assigned as such by Bayesian clustering (Figure 2).

Discussion

Recent advancements in sequence technologies have allow the evolutionary effects of hybridization, and reticulate evolution in general, to be more formally evaluated, thus promoting more expansive research (Kane et al., 2009; Dasmahapatra et al., 2012; Eaton and Ree, 2013). Consequently, a less awkward view of introgressive hybridization has developed, one that

promotes instead the maintenance of semipermeable species boundaries, and its effects on the evolution of the genome (Nosil et al., 2009; Michel et al., 2010; Harrison, 2012). For example, introgression is now known to occur without dismantling species boundaries (Fontaine et al., 2015), and likewise, with a rather precise transmission of adaptive traits (Dasmahapatra et al., 2012; Nadeau et al., 2012). This has reshaped both our view of speciation as well as the formation of reproductive isolation in the face of hybridization (Edmands et al. 2002).

Catostomid fishes provide a good system for the manner by which reproductive isolation has evolved. This is due to several reasons: They have long been recognized to hybridize (Hubbs et al. 1943, Nelson 1968, Buth et al. 1992, Tranah and May 2006), have a deep and chaotic history of isolation and secondary contact driven by the geology of Western North America (Smith et al. 2013), and are of conservation concern as a result of hybridization with invasive congeners (McDonald et al. 2008, Douglas and Douglas 2010). However, and despite these caveats, their introgression remains relatively enigmatic, particularly across geographical and temporal scales.

In this study, we document patterns of introgression across an array of hybrid crosses, to include all possibilities across ten species in the Colorado River Basin (Figure 7). Results highlight a level of reproductive isolation that increases with phylogenetic distance, as well as variability across the entire basin in the various outcomes of hybridization. These data provide insights into the evolution of reproductive isolation, a consequence that can both inform conservation and aid in predicting patterns of introgression and hybridization as rivers dwindle due to drought and anthropogenic water use.

Reproductive Isolation Increases with Phylogenetic Distance

Reproductive isolation is expected to increase with phylogenetic divergence, especially if phenotypic differences promote ecological specialization among taxa (Coyne and Orr 2004). Ecological divergence is an important driver of reproductive isolation (Funk et al. 2006) and has been suggested as such in *Catostomus,* despite repeated instances of hybridization and introgression (Mandeville et al. 2015). In this study, we found that while hybridization transected all phylogenetic levels within the genus, barriers to introgression increased with phylogenetic distance, particularly between those subgenera that display different life histories and habitat preferences.

The phylogeny of *Catostomus* (Chapter 2) reflects two subgenera (*Catostomus* and *Pantosteus*), as suggested by Smith et al. (2013), with Longnose Sucker as sister. Crosses between the subgenera (i.e., Flannelmouth x Bluehead sucker, White x Bluehead sucker, Razorback x Bluehead sucker) appeared not conducive to introgression, as was found with crosses between Longnose Sucker and either White or Bluehead sucker (Figure 5). In comparison, all five crosses within subgenera reflected introgression (Figure 6). This includeed Flannelmouth x Razorback sucker, each currently within a different genus, but with nuclear (Chapter 2) and mitochondrial (Chen and Mayden 2012) markers suggesting their placement within the subgenus *Catostomus.* This is also congruent with recent fossil evidence that reflects both have seemingly diverged within the last 6my (Smith 2015).

This pattern of introgression within- and a lack between-subgenera remained consistent, even when expanded to include other hybrid crosses and drainages: the Bonneville Basin (Utah x Bluehead sucker, Chapter 1), the Lower Colorado River Basin (Desert x Sonora sucker, Clarkson and Minckley 1988; Sonora x Razorback sucker, Chapter 2), and the Little Colorado River (Bluehead x Rio Grande sucker, and Flannelmouth x Sonora sucker, Chapter 3). All seven

crosses among-subgenera reflected introgression, either contemporaneously or historically, whereas it was absent in all seven crosses between-subgenera (Figure 7). The stark differences observed in patterns of introgression within *versus* between subgenera relate to a breadth and depth of reproductive isolation. Increased isolation may be driven simply by the degree of phylogenetic divergence, such as ~24mya between subgenera and ~14mya within (Unmack et al. 2014).

This pattern may also be ecologically driven. For example, the subgenus *Pantosteus* prefers higher elevations and cooler habitats compared to the subgenus *Catostomus* (Sigler and Miller 1962). *Pantosteus* also reflects a series of specialized morphological adaptions that facilitate the scraping of diatoms and biofilm from the substrate of high-velocity streams (Smith 1966). Thus, ecologically specializations may drive reproductive isolation, such that the fitness of hybrids is depressed in either parental environment.

Hybrids between Flannelmouth and Bluehead sucker without the White Sucker Bridge

White Sucker has been introduced throughout the Upper Green River above Flaming Gorge Dam, the Yampa River, and the San Juan River (Sublette et al. 1990, Holden 1991). Several studies noted that White Sucker in these areas hybridizes with both Flannelmouth and Bluehead suckers (Holden and Stalnaker 1975, McDonald et al. 2008, Quist et al. 2008, Douglas and Douglas 2010). It has been suggested (McDonald et al. 2008) that introduced White Sucker threaten reproductive isolation of native Flannelmouth and Bluehead suckers by acting as a 'hybrid bridge' between the two. However, results herein underscore the fact White Sucker is not required for the breakdown of reproductive isolation between Flannelmouth and Bluehead sucker. Seven F1 hybrids between Flannelmouth x Bluehead sucker were found, four of which

occurred in the Middle Green River region where White Sucker is absent or uncommon. All were found below Flaming Gorge Dam, to include the White and Yampa rivers, and the Green River between Flaming Gorge Dam and the White River. The closing of Flaming Gorge Dam had profound effects on distribution and abundance of suckers, to include the promotion of White Sucker and hybrids (Holden and Stalnaker 1975). Thus, habitat modifications may be more disruptive to the reproductive isolation of these species in the Middle Green, White, and Yampa rivers, if not other drainages as well.

If White Sucker does act as a hybrid bridge, one would also expect to find hybrids that contained ancestry to all three species. One such hybrid was found in the Navajo River (San Juan River region) but no other locations, despite the presence of Flannelmouth x White sucker and Bluehead x White sucker hybrids. Thus, three-way crosses appear rare and limited to certain geographic regions where introgression between Flannelmouth and White sucker is more common, and Bluehead Sucker are abundant. This is also reflected in a recent study that confirmed the presence of admixed individuals between the three species in Muddy Creek (Yampa River region), but not in the Big Sandy, even though Flannelmouth x White sucker and Bluehead x White sucker hybrids were present (Mandeville et al. 2015). These researchers also noted that three-way hybrids were 50% ancestral to Bluehead Sucker, and thus may represent a first generation cross between Bluehead sucker and Flannelmouth x White sucker hybrids (as noted herein). This fits well with the previously-posed argument for a lack of introgression across subgenera.

In either case, introgression was not detected for any Bluehead Sucker hybrids, except within the subgenus *Pantosteus*. Thus, admixture with Flannelmouth Sucker, White Sucker, and with Flannelmouth x White sucker hybrids is not considered as a threat to the genetic integrity of

Bluehead Sucker, and is clearly not contributory to a hybrid swarm, or even a new 'mutt sucker' (McDonald et al. 2008). It represents instead a loss of reproductive effort and should therefore be managed as such.

Flannelmouth Sucker x White Sucker Introgression varies with Habitat

Introgression between native Flannelmouth Sucker and introduced White Sucker can, however, be construed as a threat to the genetic integrity of the native species, already listed as a 'species of concern' throughout its range. However, our data demonstrate that such a threat varies by drainage. Some sites (i.e., Upper Green River and Blacks Fork regions) reflected greater levels of introgression than did others (Figure 4). The Yampa River region contained F2 and Bx hybrids, but solely in the Muddy Creek, a drainage previously impacted by extensive introgression (McDonald et al. 2008, Mandeville et al. 2015). Despite the presence of several F1 hybrids, no evidence for introgression was found in the mainstem Yampa and Little Snake rivers, a result consistent with that of Douglas and Douglas (2010). The Big Sandy River contained only one Bx and several F1 hybrids, again juxtaposing with the limited introgression found in this region (Mandeville et al. 2015).

The extent of introgression between these species can be attributed to habitat alterations. All sites with obvious evidence for introgression were found in Wyoming, an area with a history of anthropogenic impacts, to include dumping of industrial pollutants and raw effluent in the 1940s (Bosley 1960), development of Flaming Gorge and Fontanelle dams in the early 1960s, extensive rotenone treatment in 1962 to remove 'trash' fish, and introduction of numerous invasive fishes (Holden 1991). Collectively, these activities have reduced native fish densities, particularly among suckers, as well as greatly modified the habitat of the region

(Quartarone 1995, Wiley 2008). Homogenization of habitat may also induce change of behaviors that historically maintained reproductive isolation, and combined these effects might promote both the initiation and survival of hybrids. This is especially apparent in the Upper Green River region, which has receive the brunt of these impacts, and consequently reflects considerable declines in Bluehead and Razorback sucker, now listed as rare or absent (Wiley 2008). This region contained also the locales where the greatest levels of introgression were found between Flannelmouth and White sucker (Figure 4D).

No Evidence for Introgression in Longnose Sucker Hybrids

In comparison to White Sucker, the impact of introduced Longnose Sucker on native suckers has been minimal. Its presence was recorded only in the Big Sandy River, where it had hybridized (N=3) with both native Bluehead and introduced White sucker. However, no introgression was found, a result not surprising given the deep phylogenetic divergence between these species (~27.9mya; Unmack et al. 2014). This is also supported by other studies in the Big Sandy River that found only a few Bluehead x Longnose sucker hybrids, all of which were presumably F1s (Mandeville et al. 2015).

Contemporaneous Bluehead x Mountain Sucker Hybrids

Bluehead and Mountain sucker share a long history of introgression in the Colorado and Bonneville river basins (Chapter 2) and were also found to hybridize in the Little Sandy River (Mandeville et al. 2015). However their range-wide and contemporaneous hybridization has yet to be examined. We found introgressive hybridization between these species in the Blacks Fork and Price River, including the presence of two F1 hybrids in the White River (Price River

region). These data emphasize how recent the hybridization between these species has been, and in turn, reflects not only habitat alterations in the Upper Green River (WY) but also the introduction of Mountain Sucker from the Bonneville Basin into the Price River (Sigler and Miller 1962, Chapter 2).

Hybrids with Razorback Sucker

Razorback Sucker has experienced drastic declines throughout the Colorado River Basin (Minckley 1983) and is the only catostomid in the basin to be placed on the endangered species list (Federal Register 1991). Its decline has been attributed to habitat alterations, to include development of dams, that not only disrupted recruitment, but increased opportunities for hybridization with Flannelmouth Sucker (Buth et al. 1987, Douglas and Marsh 1998). Several of these hybrids in the Grand Canyon and Virgin River were high-level backcrosses with Flannelmouth Sucker, as would be expected from an initial hybridization followed by several generations of backcrossing with Flannelmouth Sucker (Figure 2 and 6F). While introgression between these species may seem odd given their placement in different genera, our data indicate both fall within the subgenus *Catostomus,* and fossil evidence suggests a recent divergence at 6mya (Smith 2015).

In addition to finding hybrids in the Grand Canyon and Virgin river, two F1 Razorback hybrids were also found in the San Juan River, and unsurprisingly, one was with Flannelmouth Sucker (which co-occurs throughout its post-impoundment range), whereas the second was a F1 cross with Bluehead Sucker from the mainstem San Juan River that, to our knowledge, has yet to be documented. However, if reproductive isolation does increase with phylogenetic distance, as proposed herein, the above example may then be but a minor threat to the genetic purity of the

Razorback Sucker Yet, it still represents a loss of reproductive output and lowers natural recruitment, both of which are drastically reduced in the endangered Razorback Sucker (Minckley 1995). This may be important given that stocking programs were started in 1991 to rehabilitate Razorback Sucker, and have subsequently augment several populations, to include the San Juan River (Minckley 1995, Dowling et al. 1996).

Historical Hybridization between Lower and Upper Basin Species

Sonora and Desert sucker in the Lower Colorado River Basin are ecologically equivalent and sister to Upper Basin Flannelmouth and Bluehead Sucker respectfully. One Flannelmouth x Sonora sucker hybrid and one between Bluehead x Desert sucker were found in Grand Canyon, and were assumed to be residuals of historic hybridization between these species. The Grand Canyon is the conduit between Upper and Lower Colorado River basins, and hybridization between these species has been suggested due to elevated morphological variation in both Grand Canyon and Virgin River (Minckley 1980), as well as the presence of conspecific mitochondrial haplotypes in the Upper Basin and Grand Canyon (Douglas et al. 2003, Douglas and Douglas 2010, Hopken et al. 2013). One explanation for this historical contact could be Late Pleistocene climate change, to include a reduction in flow of the Colorado River during the Hypsithermal, which could have forced a retreat of Upper Basin species down into the Lower Basin, thus facilitating contact between sister-species (per Douglas et al. 2003). Such a drought-induced range shift, in turn, would promote historical admixture.

Introgression was also found between Bluehead x Desert sucker and Flannelmouth x Sonora sucker in the Virgin River. The Virgin River comprises a unique assemblage of native suckers, due to the presence of both Flannelmouth Sucker (native to the Upper Basin), and

Desert Sucker (native to the Lower Basin). However, the Virgin River does not fall within the ranges of the other two species, Sonora Sucker (Lower Basin) and Bluehead Sucker (Upper Basin). However, highly significant introgression was found between species-pairs in all catostomids from the Virgin River, suggesting the species that occur there may be of possible hybrid origin. This same pattern has also been suggested for the origin of other fishes in the Virgin River (i.e. *Gila seminuda*; DeMarais et al. 1992). Additional samples of Flannelmouth and Desert suckers are needed from the Virgin River before the effects of introgression can be fully elucidated in this region, since only one site was sampled for each species and in the case of Desert Sucker only one sample was available.

Conclusions

While hybridization is a common phenomenon in catostomid fishes, introgression seemingly decreases with phylogenetic distance and may be driven by the ecological specialization between subgenera. Introgression between native Flannelmouth Sucker and introduced White Sucker has also increased concomitant with habitat disturbance, as previously suggested (Mandeville et al. 2015). However, there is limited (if any) capacity for White Sucker to serve as a 'hybrid bridge' between native species (McDonald et al. 2008), particularly given the extreme influence of habitat alterations in promoting breakdown of reproductive isolation among native species (per Middle Green Yampa, and White rivers). Based on our comprehensive analyses, the implication that multiple species will potentially collapse into a 'mutt sucker' (McDonald et al. 2008) is improbable at best, due to minimal rates of introgression found in most locations coupled with the increased level of reproductive isolation that is concomitant with increased phylogenetic divergence. The presence of historical admixture between native species

also provides an example of how species boundaries can be maintained, even in the presence of introgression induced by anthropogenic disturbances. Previous studies provided a limited perspective with regards to hybridization and introgression, using small sample sizes and narrow geographic locales. Our study instead examined hybridization and introgression across an entire freshwater basin, and included all catostomids native or introduced into the system. Understanding extant patterns of hybridization and reproductive isolation across the diverse range of these species provides a baseline necessary to disentangle the long history of hybridization in this region. These data, in turn, will promote adaptive management and conservation of this clade region-wide.

Acknowledgements

Numerous agencies contributed field expertise, specimens, technical assistance, collecting permits, funding, or comments. Rio Grande and Razorback suckers were provided by the Museum of Southwestern Biology, University of New Mexico (T. Turner and L. Snyder). Accession numbers for samples from the Museum of Southwestern Biology are: MSB 44046, 44047, 44052, 44059, 44067, 49715, 49962, 53500, 63249, and 88362. Numerous students, postdoctorals and faculty contributed to the development of our research: A. Albores, P. Brunner, M. Davis, T. Dowling, R. Cooper, J. Cotter, E. Fetherman, M. Hopken, M. Kwiatkowski, A. Reynolds, J. Reynolds, C. Secor, and P. Unmack. Sampling procedures were approved under IACUC permit 98-456R (Arizona State University) and 01-036A-01 (Colorado State University). This research was supported by generous endowments to the University of Arkansas: The Bruker Professorship in Life Sciences (M.R.D.) and 21st Century Chair in Global Change Biology (M.E.D.).

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Tables

Table 1: Sample sites and number of samples based on field identification. FMS=Flannelmouth Sucker, BHS=Bluehead Sucker, WHS=White Sucker, MTS=Mountain Sucker, LNS=Longnose Sucker, RBS=Razorback Sucker, DES=Desert Sucker, SOS=Sonora Sucker, RGS=Rio Grande Sucker, UTS= Utah Sucker, HYB=Hybrid.

Region	**Location**	**State**	N	FMS	BHS	WHS	MTS	LNS	RBS	DES	SOS	RGS	UTS	HYB
Upper Green River	Horse Creek	WY	4											4
	Cottonwood Creek	WY	10	4										6
	Bitter Creek	WY	7	3										4
	Flaming Gorge Dam	WY	6	2		2								2
Big Sandy River	Big Sandy River	WY	25	4	6		1	5						9
	Little Sandy River	WY	20	1	6		1							12
Blacks Fork	Hams Fork	WY	13	4										9
	Muddy Creek	WY	9	3										6
	Blacks Fork	WY	20	2	3		7							8
	Henrys Fork	WY	8	3	2		1							2
Yampa River	Little Snake River	WY	21	2	4	3								12
	Yampa River	CO	39	5	5	5	1							23
Middle Green River	Green River	UT	7		4									3
	White River	UT	4	1	2									1
Price River	Price River	UT	15		8		7							
	White River	UT	10				10							
San Juan River	Navajo River	NM	17	2	3									12
	Arch Canyon	UT	20		20									
	San Juan River	UT	10	2	2				4					2
Grand Canyon	Havasu Creek	AZ	10		10									
	Matkatamiba Canyon	AZ	15		15									
	Kanab Creek	AZ	12	10	2									
	Shinumo Creek	AZ	12	10	2									
	Conf. Little Colorado	AZ	18	3	15									
Virgin River	Beaver Dam Wash	UT	10	10										
	Meadow Valley Wash	NV	1							1				
Other Sites			66	5	11	3	14			8	15	6	4	
Total			**409**	**76**	**120**	**13**	**42**	**5**	**4**	**9**	**15**	**6**	**4**	**115**

Table 2: Number of fixed SNPs used for hybrid analysis between each cross. Abbreviations for crosses are as in Table 1. Also included are percent missing data (% missing), number of individuals (# indiv), and number of samples identified as admixed by STRUCTURE (# hybrid).

Cross	SNPs	% missing	# indiv	# hybrid
FMS x WHS	260	13.3	108	68
FMS x SOS	403	11	51	1
FMS x RBS	399	10.1	44	6
BHS x FMS	302	12.6	58	7
BHS x WHS	253	14.1	73	29
BHS x LNS	251	12.7	34	2
BHS x RBS	232	10.4	7	1
BHS x DES	99	10	100	2
BHS x MTS	274	11.6	144	17
WHS x LNS	477	9.5	8	1

Figures Headings

Figure 1: Map of the Upper Colorado River and neighboring basins. The nine regions in this study are highlighted in blue.

Figure 2: Bayesian clustering plots by region for populations that had admixed ancestry. All reference sites from Chapter 2 (not depicted) were assigned to one cluster.

Figure 3: Triangle plots depicting hybrid indices versus interspecific heterozygosity for species of *Catostomus*, to include introduced White and Longnose suckers. Crosses include A) White x Flannelmouth sucker, B) White x Bluehead sucker, C) White x Longnose sucker and D) Longnose x Bluehead sucker.

Figure 4: Triangle plots depicting hybrid indices versus interspecific heterozygosity for White x Flannelmouth sucker by location.

Figure 5: Triangle plots depicting hybrid indices versus interspecific heterozygosity for Bluehead Sucker and other *Catostomus* species external to the subgenus *Pantosteus*. Crosses include A) Bluehead x Flannelmouth sucker, B) Razorback x Bluehead sucker, C) White x Bluehead sucker, and D) Longnose x Bluehead sucker.

Figure 6: Triangle plots depicting hybrid indices versus interspecific heterozygosity between species within the same subgenus (A-D *Pantosteus*, E-F *Catostomus*). Crosses include A-C) Mountain x Bluehead sucker, D) Desert x Bluehead sucker, E) Sonora x Flannelmouth sucker and F) Razorback x Sonora sucker.

Figure 7: Network depicting crosses among study species. Solid lines represent those recorded in this study, whereas dashed lines represent those recorded in previous studies (Chapters 2, 3, Clarkson and Minckley 1988). Red lines represent introgression whereas black lines represent hybridization without introgression. Species abbreviations are as in Table 1 and are colored by subgenus or species (green = *Catostomus*, blue = *Pantosteus*, orange = *Xyrauchen*, purple = Longnose Sucker). A phylogeny in Chapter 2 reflects species-relationships.

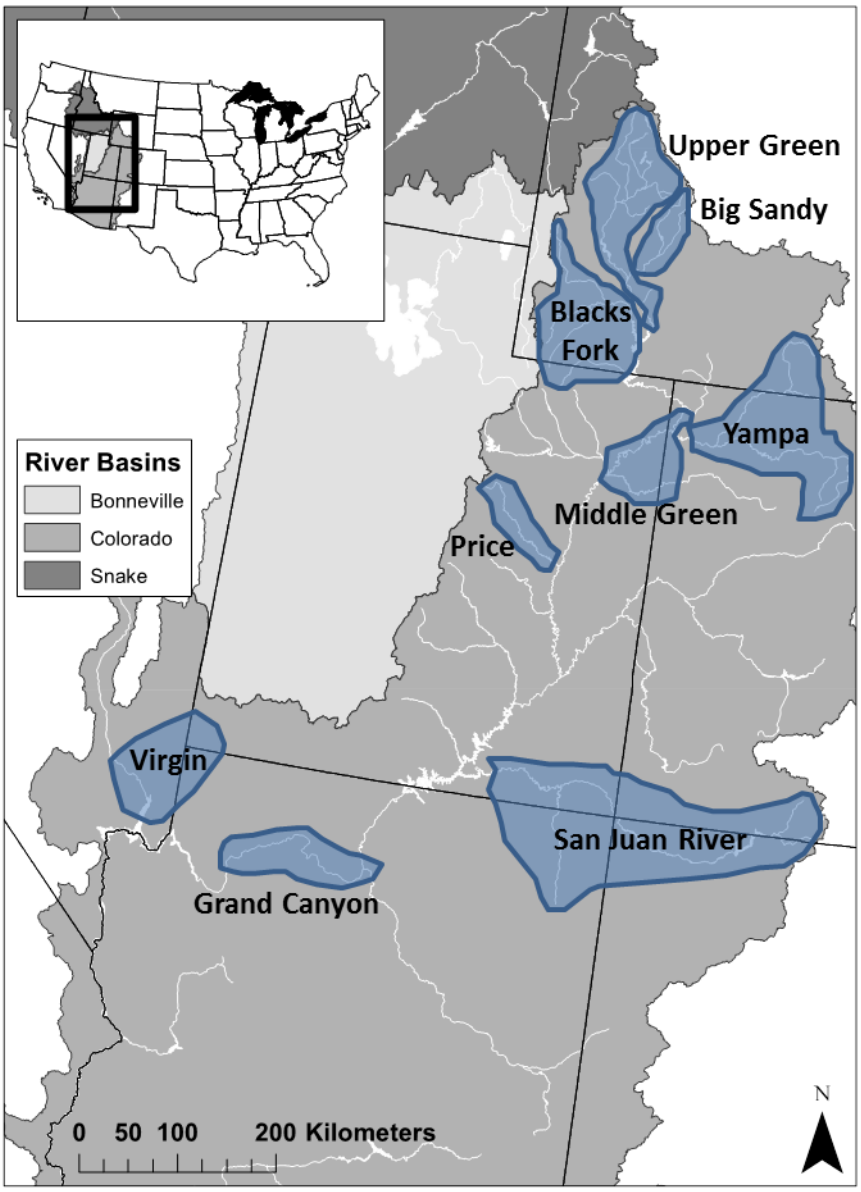
Upper Green
Big Sandy
Blacks Fork
Yampa
Middle Green
Price
Virgin
San Juan River
Grand Canyon
River Basins
Bonneville
Colorado
Snake
0 50 100 200 Kilometers
N

Figure 1.

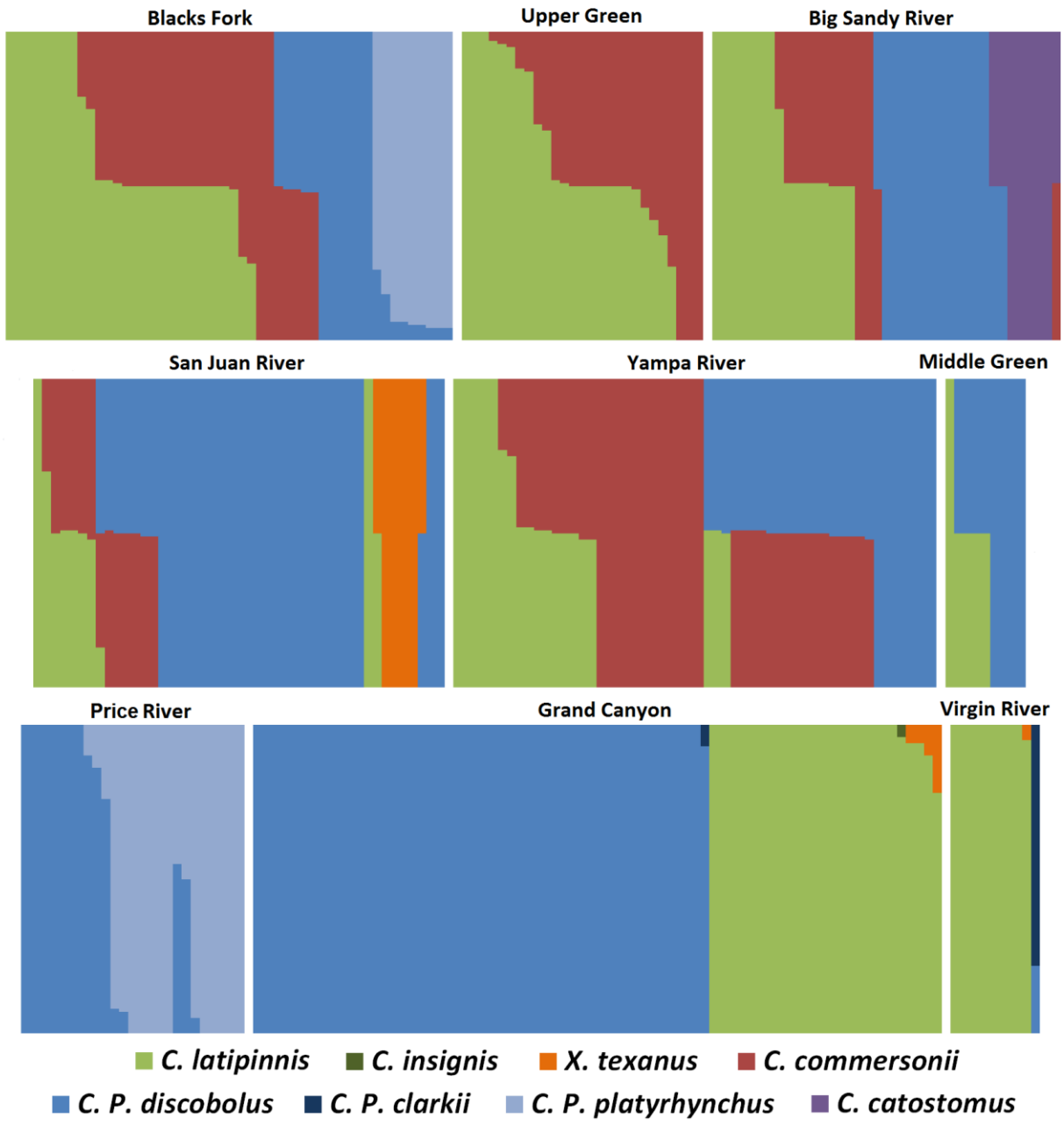
Blacks Fork
Upper Green
Big Sandy River
San Juan River
Yampa River
Middle Green
Price River
Grand Canyon
Virgin River
C. latipinnis
C. insignis
X. texanus
C. commersonii
C. P. discobolus
C. P. clarkii
C. P. platyrhynchus
C. catostomus

Figure 2.

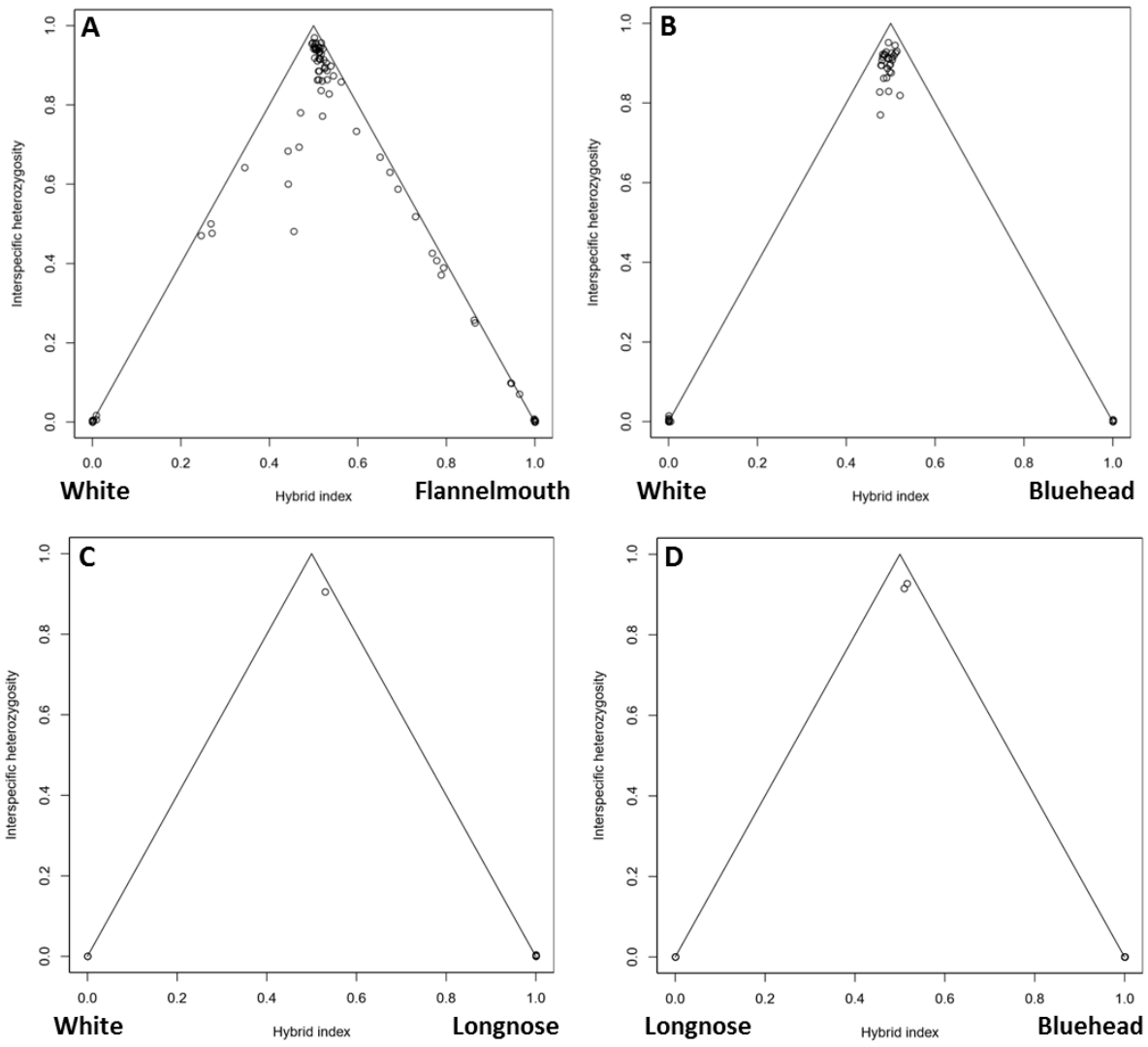
A
B
C
D
Interspecific heterozygosity
Hybrid index
White
Flannelmouth
White
Bluehead
White
Longnose
Longnose
Bluehead

Figure 3.

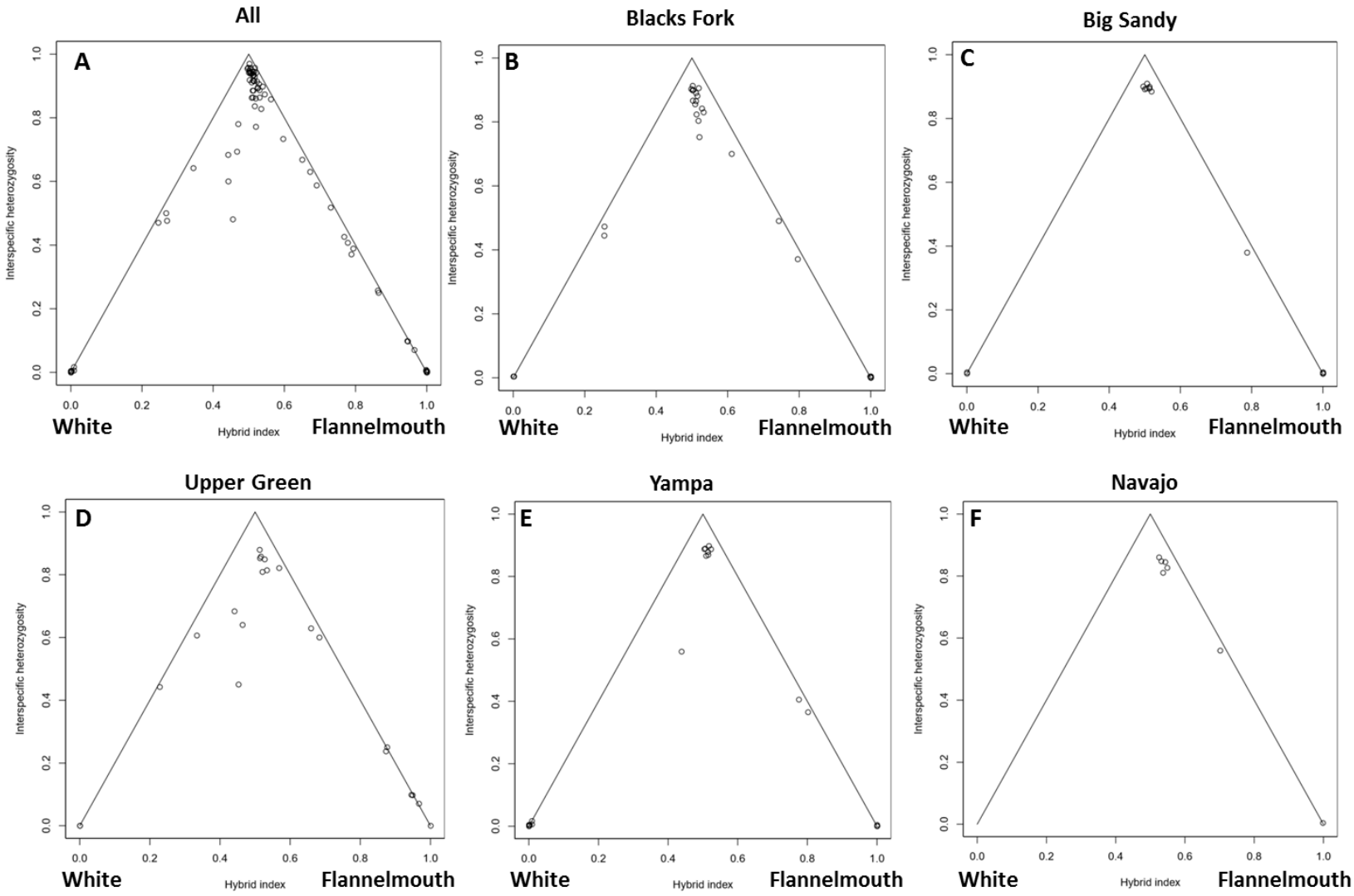
All
A
Blacks Fork
B
Big Sandy
C
Upper Green
D
Yampa
E
Navajo
F
Interspecific heterozygosity
Hybrid index
White
Flannelmouth
0.0
0.2
0.4
0.6
0.8
1.0

Figure 4.

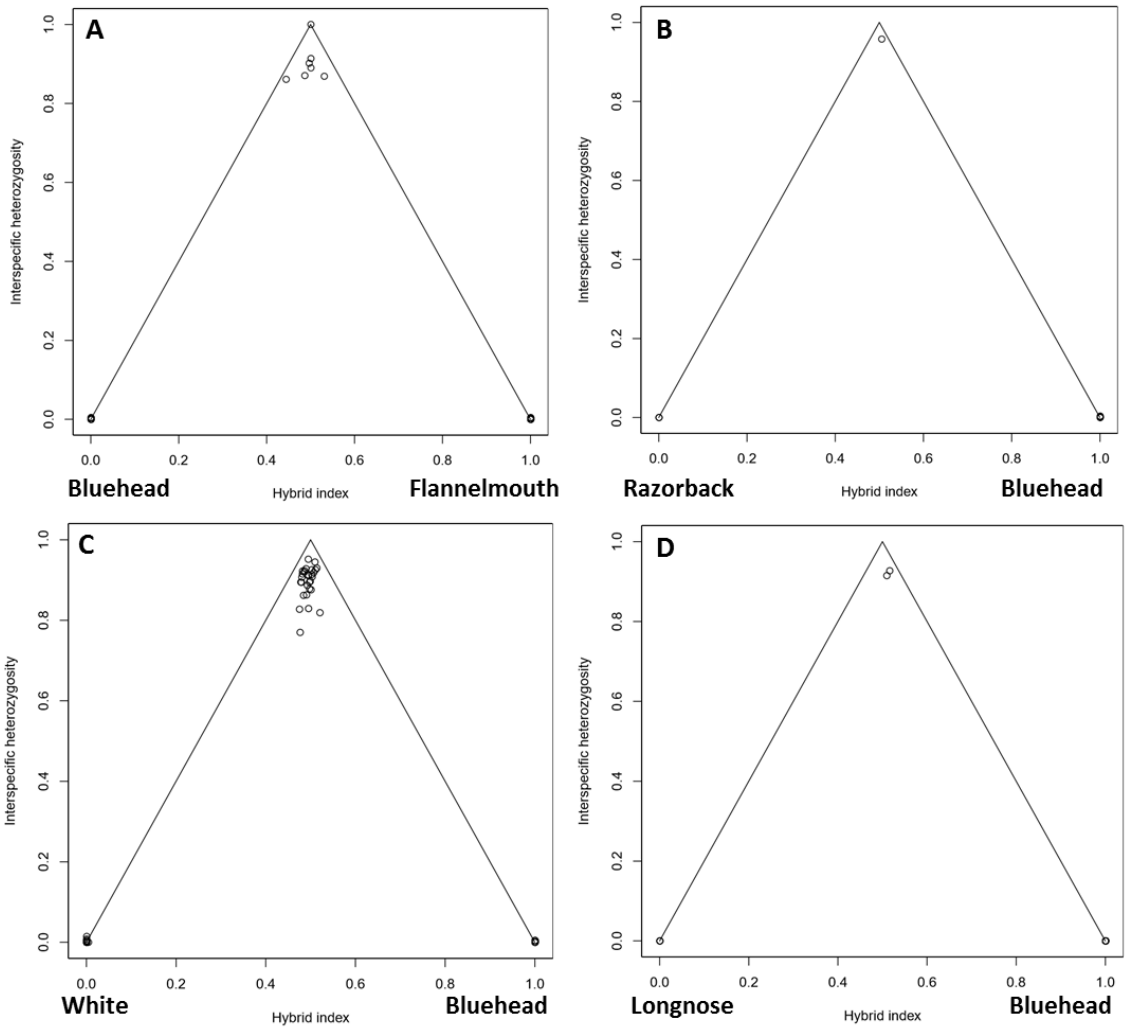
A
Interspecific heterozygosity
0.0
0.2
0.4
0.6
0.8
1.0
Bluehead
Hybrid index
Flannelmouth
B
Razorback
Hybrid index
Bluehead
C
White
Hybrid index
Bluehead
D
Longnose
Hybrid index
Bluehead

Figure 5.

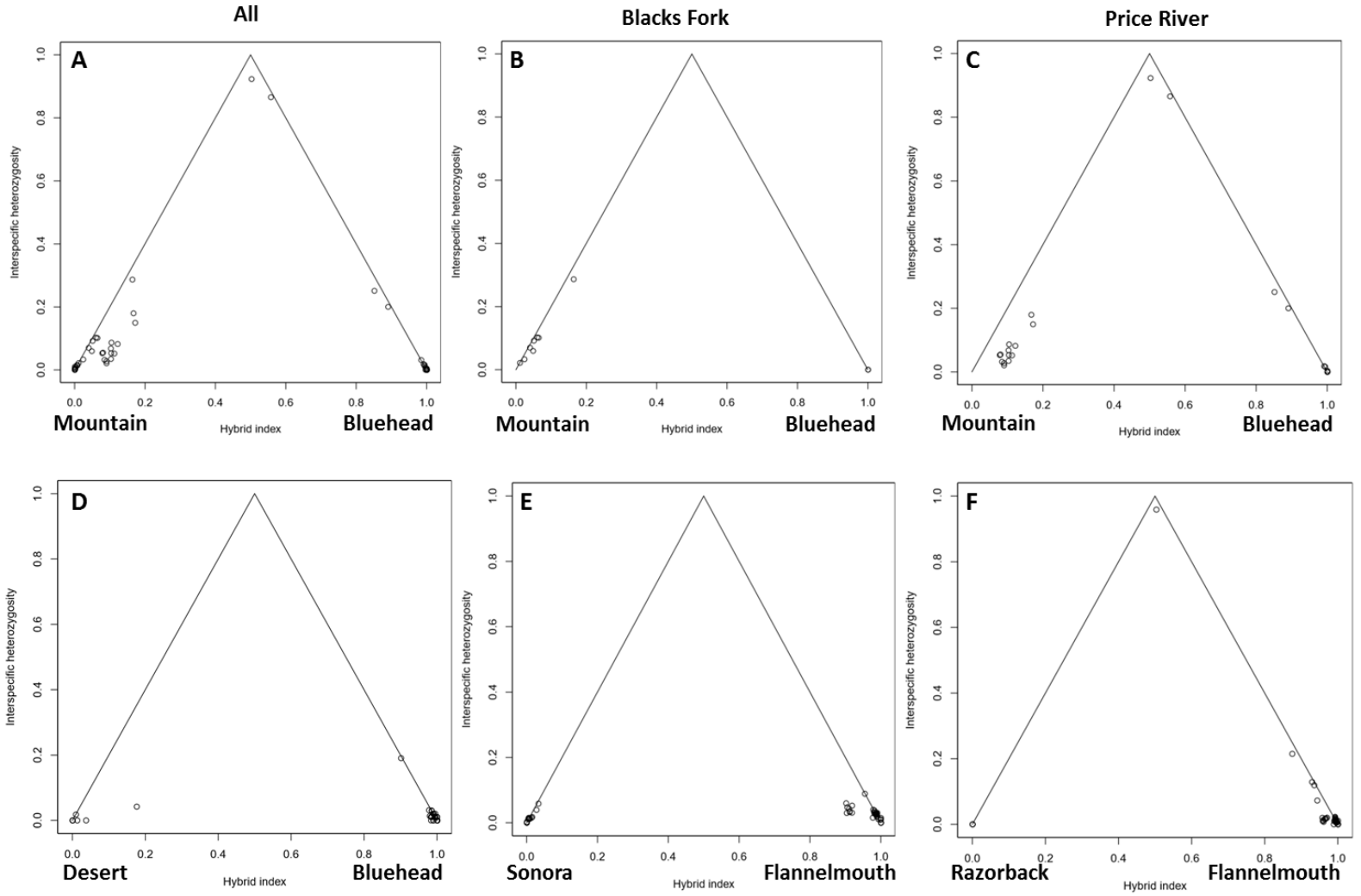
All
Blacks Fork
Price River
A
B
C
D
E
F
Interspecific heterozygosity
Hybrid index
Mountain
Bluehead
Desert
Sonora
Flannelmouth
Razorback

Figure 6.

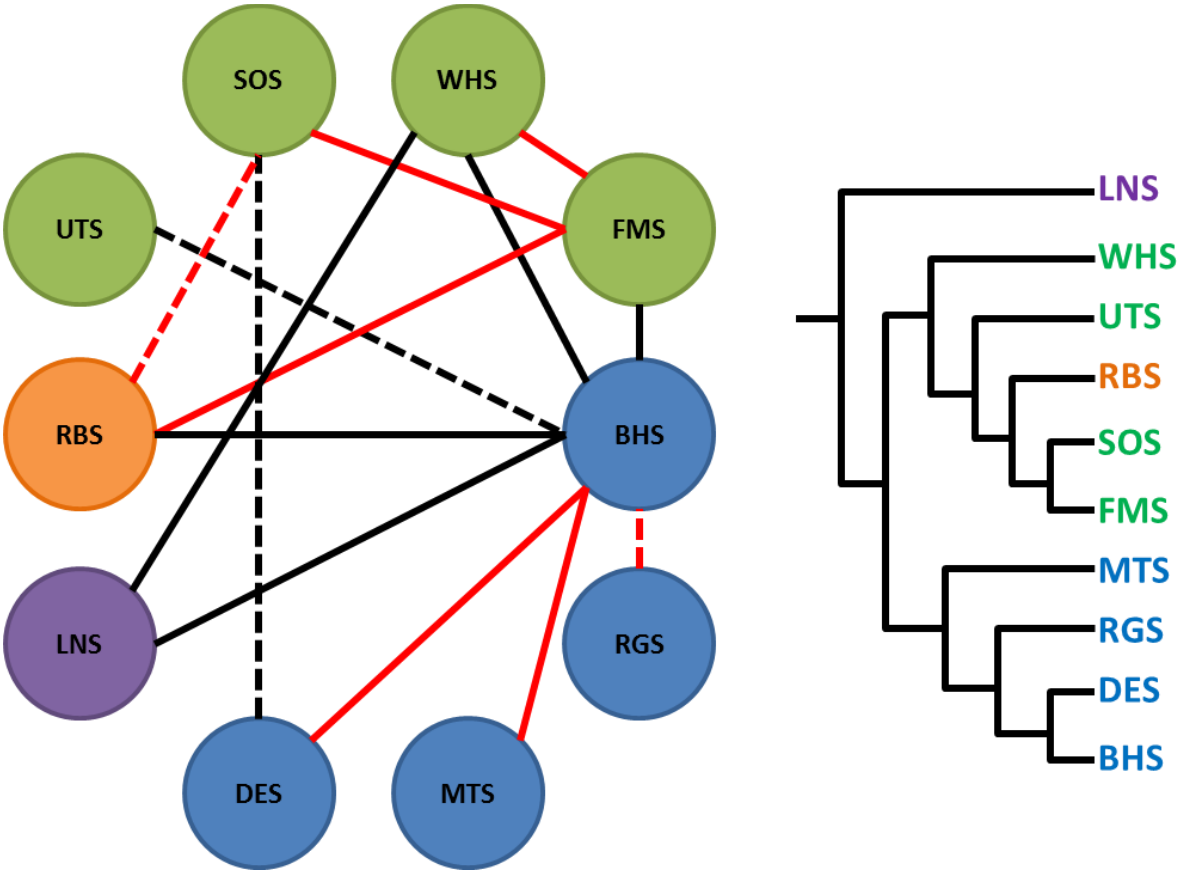
SOS
WHS
UTS
FMS
RBS
BHS
LNS
RGS
DES
MTS
LNS
WHS
UTS
RBS
SOS
FMS
MTS
RGS
DES
BHS

Figure 7.

VI. Conclusion

In the last 15my, Western North America has experienced a chaotic geological history, driven by tectonics and volcanism as well as climate change (Spencer et al. 2008, Chamberlin et al. 2012). These processes shaped a topology defined by isolation and reconnection of basins, that in turn lead to diversification and secondary contact of species endemic to this region (Hubbs and Miller 1948, Hershler and Sada 2002). *Catostomus*, known as Finescale Suckers, exemplifies this through a history of speciation, punctuated by reticulation, resulting in discordance between morphological (Smith et al. 2013) and mitochondrial phylogenies (Chen and Mayden 2012, Unmack et al. 2014). In this dissertation, phylogeographic and phylogenetic patterns were examined in *Catostomus* at various temporal, geographic and taxonomic scales to resolve this discordance, by (a) deriving a robust phylogeny based on thousands of nuclear loci and hundreds of samples, (b) documenting and statistically testing for historic and contemporary introgression, (c) interpreting results within the context of the geologic history of the region and natural life history of focal species, and (d) translating inferences to clarify proposed and suggest additional taxonomic revisions.

In Chapter 1, contemporary hybridization was examined on a localized scale (the Bonneville Basin) between two native species, Utah Sucker (*C. ardens*) and Bluehead Sucker (*C. Pantosteus discobolus*). Sequence analysis of three nuclear loci and two mitochondrial genes revealed hybridization, but no back-crossing, suggesting F1-hybrids are not reproducing in the system. Sterile hybrids may represent a loss of recruitment especially problematic for the numerically reduced Bluehead Sucker. Individuals putatively identified as hybrids in a targeted morphological evaluation were all verified as such, underscoring the utility of a combined morphological and molecular approach to hybrid verification.

In Chapter 2, discordance between morphological- and mitochondrial-based taxonomic hypotheses was resolved by deriving a phylogeny that formed a framework for statistically testing and mapping historic introgression. These were requisites for a more complete examination of proposed taxonomic revisions (see Appendix 3 for details), detailed examination of two subgenera to define intra-specific diversity (Chapter 3), and a large-scale analysis of contemporary introgression involving endemic and introduced species (Chapter 4). Results also highlighted the ability, or lack thereof, to resolve the true species tree in the face of introgression and lent itself as an empirical example in the debate between concatenation and multispecies coalescent methodology.

Many introgression events detected in Chapter 2 pertained to the species of the Colorado River Basin. In Chapter 3, a comparative fine-scale evaluation between two of these species, Flannelmouth Sucker (*C. latipinnis*) and Bluehead Sucker (*C. P. discobolus*), revealed that phylogeographic patterns, while similar, were not molded by the same vicariant events. Divergence among intra-specific lineages differed greatly between species, indicating diversification occurred over different time scales – a prime example of the ecological theater defining the evolutionary play (Hutchinson 1965). Marked differences in habitat preferences were likely driving the process of diversification, with isolation in mountain streams primarily defining Bluehead Sucker populations, whereas recent climate oscillations and formation of late Pleistocene barriers were impacting Flannelmouth Sucker. Hybridization and introgression driven by stream captures have also further complicated delimitation of lineages in both species, especially in the recently listed endangered Zuni Bluehead Sucker (Federal Registry 2014).

Contemporary hybridization has also been widely noted in catostomid fishes (Hubbs et al. 1943, Buth et al. 1992, Tranah and May 2006), possibly driven by introduction of invasive

species (McDonald et al. 2008, Douglas and Douglas 2010) and modification of habitats (Holden and Stalnaker 1975). However, hybridization across large geographical and temporal scales remains more enigmatic, especially with regards to introgression. In Chapter 4, hybridization was examined in all species across the Colorado River Basin as a whole. While hybridization was detected throughout the basin across all levels of phylogenetic distance, introgression appears to only occur within subgenera, suggesting increased reproductive isolation with phylogenetic distance or ecological specialization. Similar findings were reflected in the neighboring Bonneville Basin (Chapter 1), and in patterns of introgression at increasing phylogenetic scales (Chapters 2 and 3). Furthermore, hybridization and introgression appeared more prevalent in areas impacted by habitat modification, a pattern that held true both within native species, as well as between native and introduced species, suggesting that anthropogenic disturbances have the capacity to break down reproductive barriers.

While hybridization and introgression has a long been recognized in *Catostomus*, the analysis of large, multi-loci datasets and availability of hundreds of samples collected over two decades, allowed for the disentanglement of complex, reticulate evolutionary histories. This dissertation also contributes to the recognition that introgression can occur without dismantling species boundaries (Fontaine et al. 2015), and often with a rather precise transmission of adaptive traits (Dasmahapatra et al. 2012, Nadeau et al. 2012), and specific effects on genome evolution (Nosil et al. 2009, Michel et al. 2010, Harrison 2012). Consequently, introgressive hybridization is now recognized as a fundamental evolutionary process, rather than a rare aberration. Reticulation can promote diversification, while simultaneously maintaining semipermeable species boundaries, and this dissertation contributes empirical evidence to this

debate exploring large-scale patterns of introgressive hybridization within the context of the evolutionary history of a lineage and the ecological theatre that shaped its diversity.

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Appendix 1: Feasibility of Examining Gene Duplication using ddRAD Sequencing

Gene duplication is believed to play an important role in the creation of genetic novelty (Bridges 1935, Stephens 1951, Koonin 2005). Ohno (1970) championed the adaptive significance of gene duplication by citing the growing body of examples of gene and whole genome duplications, as well as providing models for maintenance of gene duplicates. These outcomes included 1) *gene conservation*, where both duplicates maintain full ancestral function, 2) *subfunctionalization*, division of ancestral function, and 3) *neofunctionalization* (later coined by Force 1999), where one duplicate develops a new function.

Some of the examples for the evolutionary importance of maintained duplicates include the duplication and neofunctionalization of *vtgaa* gene in Acanthopterygii, which played an important role in the radiation of ray-finned fishes into the ocean (Finn and Kristoffsen 2007), the duplication and gene conservation of the salivary amylase gene *amy1* among human with starch-rich diets (Perry et al. 2007), and duplication and subfunctionalization of acetylcholinesterase in the common mosquito that provides resistance to organophosphate pesticides (Labbe et al. 2007). However with the growing body of examples the relative importance of each of the outcomes has remained subject of debate and appears to vary greatly across taxonomic groups (Hahn 2009).

To add to the complication, gene duplication can occur at varying levels from a single gene or segment of a chromosome (Finn and Kristoffsen 2007), to a whole chromosome (Tunca et al. 2000), or even whole genomes (Tang et al. 2008). Single genes and segments of a chromosome can be duplicated through a variety of different mechanisms including unequal crossing-over, duplicative transposition, and retrotransposition, while whole genome duplications are a result of polyploidization (Hahn 2009).

Single segment verse whole genome duplications differ greatly in their maintenance of duplication. For example, the size of duplication can greatly dictate the maintenance of duplicates, especially those with a high number of interactions, which are over-retained after whole genome duplications through the "gene balance hypothesis" (Conant and Wolfe 2007) and under-retained after small scale duplication (Blomme et al. 2006, Hufton et al. 2009). However, no matter the form of duplication most duplicates are not maintained for very long especially after polyploidization (Maere et al. 2005, Paterson et al 2006).

Whole genome duplications (WGD) are due to polyploidization and are often viewed an evolutionary dead end that results from either non-reduction in meiosis or somatic doubling in mitosis of parental germline or early embryos (Glasauer and Neuhauss 2014). Following polyploidization duplicated chromosomes accumulate mutation and eventually fail to form quadrivalents resulting it re-diploidization of the genome (Zheng et al. 2008). The resulting lineage while no longer polyploid still retains the duplicates, termed ohnologs, which in return may provide an adaptive advantage (Glasauer and Neuhauss 2014). In fact polypoid species are often more robust to environmental change and have reduce extinction risks compared to their diploid relatives (Fawcett et al. 2009).

WGD can also increase speciation due to differential gene loss (Scannell et al. 2006) and many radiation events have occurred after WGD including *Paramecium* (Aury et al. 2006), yeast (Scannell et al. 2006), flowering plants (Jaillion et al. 2007, Tang et al. 2008), vertebrates (Dehal and Boore 2005), teleost (Glasauer and Neuhauss 2014), and salmonids (Macquee and Johnston 2014). However the importance of WGD in these radiation events still remain debated due to the delay in time after WGD and the presence in only some of the lineages while others remain species-poor (Soltis et al. 2009, Near et al. 2012).

In the evolutionary history of *Catostomus* there have been at least four WGD events including two at the base of the all vertebrates (Dehal and Boore 2005), one at the base of teleost estimated at between 350mya-320mya (Christoffels 2004, Vandepoele et al. 2004), and one at the base of the family Catostomidae (Uyeno and Smith 1972) that occurred at least 50mya (Ferris 1984). Similar WGD have been found in various teleost including some cyprinids (Wolf et al. 1969), cobitids (Ohno et al. 1967, Ferris and Whitt 1977), poecilids (Schultz 1969), and salmonids (Ohno 1970).

In comparison to the salmonid and teleost WGD little work has been done on the catostomid WGD. Uyeno and Smith (1972) first suggested genome duplication followed by re-diploidization at the root of Catostomidae based on the increase in chromosome number (2n=98-100) as compared to other sister lineages (2n=48-50) with the exception of some cobitids (2n=96-100) which may have also had a WGD. Using allozymes Ferris and Whitt (1977) found gene silencing of 40-50% of ohnologs in catostomids, a result recently matched with the sequencing of several catostomid transcriptomes that found 44.2-50% gene silencing (Krabbenhoft et al. 2015). Neofunctionalization was also suggested to be uncommon in catostomids due to equal branch lengths between ohnologs (Krabbenhoft et al. 2015).

The date of the catostomid WGD has also remained relatively unknown due to the conflicted age of Catostomidae. Ferris (1984) estimated the WGD at ~50mya at the root of Catostomidae. However, recent fossil calibrations using several mitochondrial genes suggest an older origin of Catostomidae (Unmack et al. 2014) and as a result this WGD event may indeed be older in origin. Thus, all that is known at this point is that the polyploidization occurred at least 50mya.

Being able to examine the catostomid WGD using genomic data may provide new insight to this limitedly studied WGD. However, I doubt the feasibility of being able to do this with the ddRAD single end 100 base pair sequencing since the ability to cluster ohnologs from the catostomid WGD may be difficult due to the long divergence time (>50mya). RAD sequencing methods (RAD-seq, GBS, ddRAD, etc.) are often highly variable and as such are great for resolving recent divergence events, but are limited in their ability to resolve more historical splits.

For example, Rubin *et al.* (2012) examined the limitations of RAD-seq to resolve nodes of different ages in *Drosophlia*, mammals, and fungi and had difficulty reliably recovering nodes >60mya do to the lack of recovery of homologous RAD loci. The lack of recovery of homologous RAD loci was attributed to the gain and loss of enzyme recognition sites. DaCosta and Sorenson (2015) further explored recovery of homologous RAD loci over varying divergence times in finches and found that the gain and loss of enzyme recognition sites was so predictable that they could reliability construct phylogenies based simply on the number of shared homologous loci.

It should also note that both of these studies utilized pair-end sequencing of randomly sheared RAD fragments and were able to obtain loci ~1kb. In contrast, the data presented in this dissertation consist on size selected single-end sequencing of ddRAD loci which produces loci that are on average 87bps and relies on the presence of two enzyme cut site, which further exacerbates these limitations. Thus, the feasibility of being able to detect a duplication event that occurred more than 50mya is highly unlikely.

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Appendix 2: The Grouping of Regional Sites with Hybridization

Six of the nine regions of potential hybridization are located within the Green River Basin, the largest tributary of the Colorado River Basin. Three of these are above Flaming Gorge Dam and contain native Flannelmouth Sucker (*Catostomus latipinnis*) and introduced White Sucker (*C. commersonii*). Big Sandy River provides habitat for native Bluehead Sucker (*C. Pantosteus discobolus*) and Mountain Sucker (*C. P. platyrhynchus*), as well as introduced Longnose Sucker (*C. catostomus*). Blacks Fork is represented by native Bluehead Sucker, Mountain Sucker, and their potential hybrids. The remaining sites above Flaming Gorge Dam (Upper Green River region) drain into the mainstem Green River and contain very few native Bluehead Sucker and Mountain Sucker, a residual of ill-fated attempts in 1962 to remove native fish for the purposes of establishing a Rainbow Trout fishery (Holden 1991, Hilton and Smith 2014).

The remaining sites in the Green River Basin include: 1) Yampa River/Little Snake River, a source of previous hybrid studies between White Sucker, Flannelmouth Sucker, and Bluehead Sucker (McDonald et al. 2008, Douglas and Douglas 2010), 2) Middle Green River, which does not contain White Sucker, but potential hybrids between Flannelmouth Sucker and Bluehead Sucker, and 3) Price River, with a population of Mountain Sucker (Sigler and Miller 1963) introduced from the Bonneville Basin (Chapter 2), and which may be hybridizing with native Bluehead Sucker.

Samples from the San Juan River were collected from the Navajo River, Arch Canyon, and the mainstem San Juan River. White Sucker is rare in the San Juan, with exception of perennial tributaries such as the Animas and Navajo rivers (Carmen 2007). In a range-wide analysis of Bluehead Sucker, Hopken et al. (2013) found haplotypes of Desert Sucker (*C. P.*

clarkii) throughout Upper Colorado River Basin, but especially so in Arch Canyon, an isolated population within the San Juan. The mainstem San Juan River supports native Flannelmouth and Bluehead suckers, and is a recovery site for the endangered Razorback Sucker (*Xyrauchen texanus*), a species that will hybridize with Flannelmouth Sucker (Douglas and Marsh 1998, Carmen 2007).

The Grand Canyon and Virgin River link the Lower and the Upper Colorado River basins, and as such may represent the potential mixing of sister species between them. This would include: Sonora Sucker (*C. insignis*) x Flannelmouth Sucker, and Desert Sucker x Bluehead Sucker. Hybridization between Razorback Sucker and Flannelmouth Sucker has also been detected in this region (Douglas and Marsh 1998).

The Little Colorado River was separated from the Colorado River Basin for 20kya by Grand Falls (Duffield et al. 2006), and was thus not included in this study. However, hybridization has been noted in the Little Colorado River, and those analyses are found in Chapter 3.

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Appendix 3: Evaluation of Taxonomic Revisions in *Catostomus*

Subgenus *Pantosteus*

Results from Chapter 2 support the subgeneric placement of *Pantosteus* (per Smith et al. 2013) with the exception of the Bridgelip Sucker (*C. P. columbianus*). This taxonomic grouping was originally at the generic level (Cope and Yarrow 1875), and consisted of the former *Minomus* (*M. yarrowi* and *M. platyrhynchus*) and *C. discobolus* (Cope 1872), as well as a new species, *P. virescens*. Several other species were subsequently described and allocated to *Pantosteus*, and include: *P. jordani* (Evermann 1893), *P. columbianus*, *P. lahontan* (Rutter 1903), and *P. santaanae* (Snyder 1908).

Pantosteus was later reevaluated and collapsed it to six species, and reallocated it as a subgenus of *Catostomu*s (Smith 1966). Its components were: 1) Desert Sucker, *P. clarkii* (formerly *Notolepidomyzon utahensis* and *N. intermedius*); 2) Bluehead Sucker, *P. discobolus* (that also included *P. virescens* and *P. yarrowi*); 3) Mountain Sucker, *P. platyrhynchus* (with *P. lahontan* and *P. jordani*); 4) Rio Grande, *P. plebeius* (formerly *C. guzmaniensis*); 5) Santa Anna Sucker, *P. santaanae*; and 6) Bridgelip Sucker, *P. columbianus*.

Our results support the placement of the first five of these species but not the Bridgelip Sucker, which placed sister to the Tahoe Sucker (*C. tahoensis*). The removal of Bridgelip Sucker from *Pantosteus* is not surprising since previous mitochondrial work has also placed Bridgelip Sucker sister to Tahoe Sucker (Chen and Mayden 2012, Unmack et al. 2014) and recent morphological has suggested a hybrid origin of the species (Smith et al. 2013). However, no introgression was detected from any *Pantosteus*, suggesting instead a non-hybridogenic origin for Bridgelip Sucker that would potentially involve its separation from Tahoe Sucker following the Lahontan Basin spillover during the Late-Pliocene to Early-Pleistocene (Reheis et al. 2002).

Several splits with two species within *Pantosteus*, Mountain Sucker and Bluehead Sucker, have recently been suggested based on morphological (Smith et al. 2013) and mitochondrial phylogenies (Unmack et al. 2014). Each of these species are discussed below.

C. P. jordani (Evermann 1893); Mountain Sucker (Missouri River Basin)

Collapsed into *C. P. platyrhynchus* based on lack of morphologically differentiation (Smith 1966) and later elevated back to species based of more expansive morphologically work (Smith et al. 2013) and mitochondrial phylogeny (Unmack et al. 2014). Results from Chapter 2 support this elevation based on monophyly in all methods.

C. P. lahanton (Rutter 1903); Mountain Sucker (Lahanton Basin)

Collapsed into *C. P. platyrhynchus* based on lack of morphologically differentiation (Smith 1966) and later elevated back to species based of more expansive morphologically work (Smith et al. 2013) and mitochondrial phylogeny (Unmack et al. 2014). Results from Chapter 2 support this elevation based on monophyly in all methods.

C. P. bondi (Smith et al. 2013); Mountain Sucker (Columbia River Basin)

Newly described species elevated from *C. P. platyrhynchus* based on morphologically (Smith et al. 2013) and mitochondrial phylogenies (Unmack et al. 2014). Results from Chapter 2 support this elevation based on monophyly in all methods. *C. P. bondi* also placed sister to *C. P. lahanton* suggesting that it may have diverged from Lahontan Basin spillover during the Late-Pliocene to Early-Pleistocene (Reheis et al. 2002), consistent with fossil calibrated mitochondrial dating (~4.5mya; Unmack et al. 2014)

C. P. platyrhynchus (Cope 1874); Mountain Sucker (Colorado River, Upper Snake River, and Bonneville basins)

Originally described as *Minomus platyrhynchus* and later placed in *Pantosteus* (Cope and Yarrow 1875). *C. P. lahanton* and *C. P. jordani* were collapsed into *C. P. platyrhynchus* based on lack of morphologically differentiation (Smith 1966) but later elevated back to species along with the description of a new species *C. P. bondi* based of more expansive morphologically work (Smith et al. 2013). Mitochondrial work however place *C. P. platyrhynchus* within sympatric Bluehead Sucker (Chen and Mayden 2012, Unmack et al. 2014). Results from Chapter 2 support this elevation based on monophyly in all methods and Patterson's D-statistic supports introgression with Bluehead Sucker resulting in mitochondrial swamping.

Results also support two clades; one representing the Colorado River Basin and the other representing that Bonneville and Upper Snake River basins including two introduced populations in the Price and San Rafael rivers (Sigler and Miller, 1963) that are physiographically within the Colorado River Basin but group phylogenetically with the Bonneville Basin.

C. P. virescens (Cope 1875 in Cope and Yarrow 1875); Bluehead Sucker (Bonneville and Upper Snake River basins)

Originally described from a specimen labeled from the San Juan River of the Colorado River Basin (Cope and Yarrow 1875) but later determined that this specimen was from the Weber River on the Bonneville Basin (Snyder 1924). Collapsed into *C. P. discobolus* based on lack of morphologically differentiation (Smith 1966) and later elevated back to species based of more expansive morphologically work (Smith et al. 2013) and mitochondrial phylogeny (Unmack et al. 2014). Results from Chapter 2 support this elevation based on monophyly in all methods, as well as support from Chapter 3 based on phylogenetic and bayesian clustering methods.

C. P. discobolus (Cope 1872); Bluehead Sucker (Colorado River Basin)

Originally described as *C. discobolus* (Cope 1872) before being placed in *Pantosteus* (Cope and Yarrow 1875). *C. P. virescens* was collapsed into *C. P. discobolus* based on lack of morphologically differentiation (Smith 1966) and later elevated back to species based of more expansive morphologically work (Smith et al. 2013) and mitochondrial phylogeny (Unmack et al. 2014). Results from Chapter 2 support this elevation based on monophyly in all methods, as well as support from Chapter 3 based on phylogenetic and bayesian clustering methods.

Results also support a split of two clusters within *C. P. discobolus*, one representing the Little Colorado River and the other representing the rest of the Upper Colorado River Basin (Chapter 2 and 3). The Little Colorado River cluster may represent the Zuni Bluehead Sucker discussed below.

C. P. discobolus yarrowi (Cope 1874); Zuni Bluehead Sucker (Little Colorado River Basin)

Originally described as *Minomus yarrowi* (Cope 1874) before being placed in *Pantosteus* (Cope and Yarrow 1875) and then later collapsed into *C. P. discobolus* based on lack of morphologically differentiation (Smith 1966). Later described as a subspecies of hybrid origin between Bluehead Sucker and Rio Grande Sucker originating in the Zuni and Chuska Mountains of northeast Arizona and northwest New Mexico (Smith et al. 1983). However, this hypothesis is refuted given that only one population (Rio Nutria) contained Rio Grande Sucker alleles in population clustering, hybrid analyses, and D-statistics presented here (Chapter 2 and 3), as well as previous allozyme analysis (Crabtree and Buth 1987) and single gene sequencing (Turner and Wilson 2009).

The current listing under the Endangered Species Act (Federal Register 2014) lists two populations in the Little Colorado River (Zuni River and Kin Lee Chee Creek). However, results indicate that the entire Little Colorado River forms a clade and the two populations currently

listed are paraphyletic (Chapter 2 and 3). Thus, reevaluation of the Zuni Bluehead Suckers is needed and should include all populations in the Little Colorado River. It should also be noted that secondary contact between Bluehead Sucker and Zuni Bluehead Sucker may have also occurred in all populations of the Little Colorado River with the exception of the Zuni River based on bayesian clustering (Chapter 3), further complicating the evolutionary history of the Zuni Bluehead Sucker.

Subgenus *Catostomus*

Smith et al. (2013) argued for the recognition of a subgenus *Catostomus* that would include all members of the genus not already allocated to subgenera (in this sense, subgenera would represent *Pantosteus*, *Xyrauchen*, *Chasmistes*, and *Deltistes*, with the latter three being external to *Catostomus*). Morphological work recognized the potential paraphyly inherent to the subgenus *Catostomus*, in that some of its species would split basal to *Pantosteus*, thus splitting the group (Smith et al. 2013). However, two of these species that previously grouped external to *Pantosteus* (i.e., *C. tahoensis and C. rimiculus*) fall in a clade with the other *Catostomus* species. In fact, all *Catostomus* not within *Pantosteus* fall into a well support clade (but with the exception of the Longnose Sucker, *Catostomus catostomus*). This may in turn represent the subgenus argued for by Smith et al. (2013). However, it also includes Razorback Sucker (*Xyrauchen texanus*) and possibly all other lake suckers, *Chasmistes* and *Deltistes*. It should also include the Bridgelip Sucker that fell within this group instead of the subgenus *Pantosteus*, which it is currently listed in. More work is also needed to include all species of *Catostomus* with increase sampling to figure out the validity of all subgenera.

C. latipinnis (Baird and Girard 1853); Flannelmouth Sucker (Colorado River Basin)

Three clades were detected in Flannelmouth Sucker to include 1) Virgin River, 2) Little Colorado River, and 3) Upper Colorado River. However, based on lack of differentiation in bayesian clustering and short branch lengths these may better represent evolutionary significant units separated in the last 20kya and not different species. This is important since the Little Colorado River populations are currently recognized as a distinct species ("Little Colorado River Sucker"; *C. sp. "crassicauda"*) by Arizona Game and Fish.

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