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Article

A Complicated History of Mitogenome Introgression Among *Luxilus* Species (Teleostei, Family Leuciscidae) in the Ozark Highlands

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Abstract

Hybridization is common among minnows and shiners in the family Leuciscidae, and mitonuclear discordance can reveal evidence of historical hybridization and introgression events that have shaped extant species and populations. We have identified a notable case of serial mitogenome replacement in populations of two shiner species, *Luxilus zonatus* and *L. chrysocephalus*, which are syntopic in drainages throughout the northern and eastern Ozark Interior Highlands of North America. These mitogenome replacement events involved *L. zonatus* acquiring the mitogenome of *L. chrysocephalus*, and populations of *L. chrysocephalus* acquiring the mitogenome of an allopatric congeneric species, *L. cornutus*. The latter species has a more northern distribution that was likely shifted southward by glacial advances during the Pleistocene. The geographic extent of mitogenome replacements in both species span multiple separate drainages encompassing most of the major river systems that comprise the northern and eastern Ozark Highlands. We attribute these patterns to shifting species distributions which were impacted by multiple glacial advances and coincident geomorphological changes to Ozark Highland drainages throughout the Pleistocene. The serial nature of mitogenome replacements in *L. zonatus* and *L. chrysocephalus* may exclude a role for natural selection in these introgression events, but the dynamic shifts in species distributions and gene flow connections throughout the Pleistocene may have favored an invasion-with-hybridization model that predicts massive asymmetric introgression between invading and resident species.

Keywords: hybrid introgression; mitonuclear discordance; Ozark Highlands; *Luxilus*; Leuciscidae; Pleistocene

Key Contribution: Pleistocene hybrid introgression has led to two congeneric minnow species experiencing mitogenome replacement across largely congruent Ozark Highland drainages

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1. Introduction

Hybridization and introgression occur frequently in nature and can be an important source of genetic variation in species (Arnold, 1997; Dowling & Secor, 1997; Edelman & Mallet, 2025; Hedrick, 2013; Mallet, 2005). Discordant patterns of mitonuclear introgression, which in the most extreme cases can result in mitogenome replacement even in the absence of measurable nuclear introgression, are well documented (Toews & Brelsford, 2012). Furthermore, the evidence of historical hybrid zones that have moved or disappeared is often apparent from genetic traces of introgression in the extant populations of impacted species (Wielstra, 2019).

Introgression may be driven by deterministic factors. Natural selection may favor introgression of locally adapted mitogenomes when species encounter novel or extreme environments or drive genetic rescue when mitogenomes have accumulated mutation load (Bonnet et al., 2017; Hill, 2019; Sloan et al., 2017). Mitochondrial replacement also may be nonadaptive and driven by sex-biased fitness of hybrid offspring (Dowling & Hoeh, 1991; Gerber et al., 2001; MacPherson et al., 2023). Alternatively, introgression may be driven as an outcome of range shifts and species invasion. When range shifts lead to invading species encountering congeneric species and engaging in hybridization, the outcome can lead to massive introgression in the genome of the invading species as a purely neutral demographic process (Currat et al., 2008; Wielstra & Arntzen, 2012).

Hybridization is common in fishes (Hubbs, 1955), and particularly prevalent in minnows and shiners of the family Leuciscidae (Scribner et al., 2001; Zbinden et al., 2023). Multi-species unguarded nesting behaviors are common among minnows and shiners and associated with high rates of hybridization (Corush et al., 2021). Discordant mitonuclear introgression patterns have been extensively reported among fish species, with examples of complete mitogenome replacement at the population level reported in charrs (Doiron et al., 2002; Wilson & Bernatchez, 1998), cichlids (Nevado et al., 2009), darters (Bossu & Near, 2009), pupfish (Carson & Dowling, 2006), topminnows (Duvernell & Schaefer, 2014), and shiners in the genus *Luxilus* (Duvernell & Aspinwall, 1995).

The genus *Luxilus* is comprised of nine North American shiner species (Dowling & Naylor, 1997). Studies have demonstrated the frequent occurrence of hybridization among sympatric *Luxilus* species (Dowling et al., 1989; Dowling & Moore, 1984; Meagher & Dowling, 1991), and members of the genus commonly spawn in heterospecific groups that can include multiple congeners (Pflieger, 1997). Within the zone of extant sympatry between *L. chrysocephalus* and *L. cornutus*, mechanisms of reproductive isolation and the dynamics of hybridization vary among populations isolated in separate drainages (Dowling et al., 1997; Dowling & Hoeh, 1991; Gleason & Berra, 1993). In the Ozark Interior Highlands, outside the zone of extant species sympatry, historical hybrid introgression has resulted in *L. chrysocephalus* populations in drainages throughout the region appearing monomorphic for *L. cornutus* mitogenomes (Duvernell & Aspinwall, 1995).

Recently, it was discovered that *L. zonatus* populations in some Ozark drainages exhibit *L. chrysocephalus* mitogenomes (Halas, 2011). What makes this particularly noteworthy is that in the river drainages where *L. zonatus* exhibit *L. chrysocephalus* mitogenomes, the *L. chrysocephalus* populations themselves are monomorphic for *L. cornutus* mitogenomes, suggesting a complex history of hybridization, introgression, and serial mitogenome replacement among *Luxilus* species in the Ozarks (Halas, 2011). The objective of our study was to determine the geographic extent of mitogenome introgression among *L. zonatus* populations, determine which drainages were impacted, and interpret these introgression events in a historical phylogeographic context.

2. Materials and Methods

The genus *Luxilus* is a relatively common minnow found throughout the eastern United States, including the *cornutus* and *zonatus* species groups. The *cornutus* group is comprised of four species, *L. chrysocephalus*, *L. cornutus*, *L. cerasinus*, and *L. albeolus*. *Luxilus chrysocephalus* is one of the most widely distributed members of this group with a range that spans the southern Great Lakes and the eastern two-thirds of the Mississippi River basin, as well as south into Gulf Coast drainages. *Luxilus cornutus*, has a similarly large range but is more northern in its distribution, including the northwestern portion of the Mississippi River basin, through the Great Lakes basin, and extending east to Atlantic drainages from Nova Scotia to the mid-Atlantic coast. A zone of extant sympatry between the species spans drainages of the Great Lakes, upper Illinois River, and portions of the upper Ohio River basin. The other two species of the group are more narrowly distributed in mid-Atlantic drainages with *L. albeolus* ranging from the Roanoke River of Virginia, southward to the Cape Fear River of North Carolina while *L. cerasinus* is found primarily in the Roanoke and New rivers.

There are three allopatric species in the *L. zonatus* species group, restricted mostly to the Ozark Highlands. They include *L. cardinalis* in the Arkansas River drainage, *L. pilsbryi* in the White River drainage, and *L. zonatus* in Missouri and Mississippi River tributaries that drain the Ozarks, including the Osage, Gasconade, and Meramec River drainages in the northern Ozark Highlands, and the Black and St. Francis River drainages in the southeastern Ozark Highlands (Mayden, 1988). All three species in the *L. zonatus* complex are sympatric with *L. chrysocephalus*.

We sampled *L. zonatus* from eleven sites in each of five major Ozark river drainages where they are found, along with *L. pilsbryi* from three sites in the White River drainage, and *L. cardinalis* from two sites in the Arkansas River drainage (Table 1). Specimens were collected by seining moderate to strong flowing current in the main channels of creeks and rivers. We also collected *L. chrysocephalus* from ten sites within the Ozark Highland study area (Table 1) and combined current with previously reported results (Duvernell & Aspinwall, 1995) for the species in this region. Additional reference sequences of *Luxilus* species outside the Ozark Highlands study area were included from previous published and unpublished sources (Dowling et al., 1992; Dowling & Naylor, 1997).

Table 1. Ozark Highlands sample locations, drainages, and species collected.

Site #	River	Drainage	Species	Lat/Long
1	Charrette Creek	Missouri River	*L. chrysocephalus*	38.686 N 91.104 W
2	Big River	Meramec River	*L. zonatus*	38.169 N 90.730 W
3	Crooked Creek	Meramec River	*L. zonatus*	37.800 N 91.349 W
4	Dry Fork	Meramec River	*L. zonatus*	37.991 N 91.555 W
5	Little Piney Creek	Gasconade River	*L. zonatus, L. chrysocephalus*	37.910 N 91.903 W
6	Osage Fork	Gasconade River	*L. zonatus*	37.633 N 92.452 W
7	Niangua River	Osage River	*L. zonatus, L. chrysocephalus*	37.642 N 93.044 W
8	Pomme de Terre	Osage River	*L. zonatus*	37.556 N 93.307 W
9	Sac River	Osage River	*L. zonatus*	37.878 N 93.720 W
10	Little St. Francis River	St. Francis River	*L. zonatus, L. chrysocephalus*	37.547 N 90.388 W
11	St. Francis River	St. Francis River	*L. chrysocephalus*	37.595 N 90.498 W
12	Whitewater River	St. Francis River	*L. chrysocephalus*	37.579 N 90.001 W
13	Black River	Black River	*L. zonatus*	37.328 N 90.768 W
14	Current River	Black River	*L. zonatus*	37.287 N 91.410 W
15	North Fork	White River	*L. pilsbryi, L. chrysocephalus*	36.851 N 92.187 W
16	Little Beaver Creek	White River	*L. pilsbryi*	36.801 N 92.909 W
17	James River	White River	*L. pilsbryi, L. chrysocephalus*	37.156 N 93.199 W
18	Spring River	White River	*L. chrysocephalus*	36.317 N 91.493 W
19	Kings River	White River	*L. chrysocephalus*	36.137 N 93.582 W
20	Spring River	Arkansas River	*L. cardinalis*	37.150 N 94.062 W
21	Arkansas River	Arkansas River	*L. cardinalis*	

NA - Sample location coordinates not available, but recorded as Arkansas River drainage, Crawford Co, AR.

Fin clips were preserved in 95% ethanol, and voucher specimens were preserved in 10% formalin. All specimens were identified to species based on diagnostic morphological features (Pflieger, 1997). Ozark Highlands samples collected for this study were extracted using Blood and Tissue extraction columns (Qiagen). Subunit 2 of the NADH dehydrogenase gene (ND2) of mitochondrial DNA (mtDNA) was isolated by polymerase chain reaction (PCR) using primers designed from an *L. chrysocephalus* reference sequence. The primers were LuxND2_4010F 5'-GCCCATACCCCGAACATGAC-3' and LuxND2_5145R 5'-TCTGCTTAGAGCTTTGAAGGCT-3'. PCR was conducted using Thermo Scientific PCR Master Mix (2X) (Thermo Fischer), and cycling conditions of an initial denaturing step at 95°C for 4 min, 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec, followed by 72°C for 4 min. PCR products were prepared for Sanger Sequencing using HighPrep PCR magnetic beads (Magbio Genomics). Sanger Sequencing of forward and reverse strands was conducted at the MU Genomic Technology Core (mugenomicscore.missouri.edu/) using the PCR primers. Chromatograms were assembled and aligned using Geneious Prime (Dotmatics). Additional reference samples of *Luxilus* species were prepared using similar PCR and Sanger sequencing methods as previously described (Dowling & Naylor, 1997).

A phylogeny was generated using the Maximum Likelihood (ML) method employed in MEGA11 (Tamura et al., 2021) with the GTR+I nucleotide substitution model and 1000 bootstrap replicates. *Ericymba dorsalis*, *Notropis atherinoides*, and *N. buccata* were included as outgroups.

Nucleotide diversity (π), and nucleotide divergence (D_{xy}) parameters were estimated from ND2 sequence alignments using DnaSP 6.12.03 (Rozas et al., 2017).

Molecular divergence times of mitochondrial lineages were estimated using the suite of programs from BEAST 2.7.3 (Bouckaert et al., 2019) and reported as mean divergence time +/- 95% HPDs. Fossil-based calibration is confounded in Leuciscids by a poor fossil record. Therefore, we conservatively employed a uniform prior distribution of divergence rates as recommended by Smith and Larson (pers. comm.) to generate estimates of divergence time. Divergence rates for minnows and suckers correlate with size. *Luxilus* minnows (3g – 100g) lie at the low end of the size range for the family, with a corresponding expected rate of 2.5 – 3.5 times the pairwise divergence rate per million years. Input files were generated using BEAUti 2 and *BEAST was run with a species tree relaxed clock with a log normal rate distribution spanning the rate range reported by Smith and Larson. Analyses were run for 20 million generations with parameters logged every 1,000 generations. Multiple runs were conducted, and outputs were examined in Tracer 1.7.2 (Drummond & Rambaut, 2007) to verify that key parameters had effective sample sizes of 200 or greater. Log and tree files from four runs were combined using LogCombiner 2.7.3 (Drummond & Rambaut, 2007). with a 10% burnin. Mean age estimates and topology were based on the maximum clade credibility using common ancestor heights from our combined trees using TreeAnnotator 2.7.3 and the tree was visualized in FigTree 1.4.4.

3. Results

A mtDNA ML phylogeny (Figure 1) was consistent with previously reported species relationships in the genus *Luxilus* (Dowling & Naylor, 1997). Samples of the *L. zonatus* group (*L. pilsbryi*, *L. cardinalis*, and *L. zonatus*) formed a monophyletic clade (98% of bootstrap replicates). Within the *L. zonatus* species group, *L. cardinalis* and *L. pilsbryi* were most closely related (86% bootstrap replicates), followed by *L. zonatus* as expected (Dowling & Naylor, 1997; Mayden, 1988). However, among morphologically identified *L. zonatus* populations, the *L. zonatus* mtDNA haplogroup was only found in specimens from a portion of its range, in the Black/Current River drainage (Table 2, Figure 2a).

Table 2. Summary of numbers of individuals of each species exhibiting each mtDNA haplogroup (clade according to Figure 1) at each sampled site.

Species	River	Introgressed *L. zonatus*	*L. chry*	Introgressed *L. chry*	*L. zon.*	*L. pil.*	*L. car.*
L. chrysocephalis							
	Charrette Creek			1			
	Little Piney Creek			1			
	Niangua River			1			
	Little St. Francis River	9	1				
	St. Francis River		1				
	Whitewater River		1				
	North Fork			1			
	James River			1			
	Kings River			1			
L. zonatus							
	Big River	14					
	Crooked Creek	1					
	Dry Fork	3					
	Little Piney Creek	4		1			
	Osage Fork	3					
	Niangua River	5					
	Pomme de Terre	6					

Sac River	4				
Little St. Francis River	3	1			
Black River			17		
Current River			4		
L. pilsbryi					
North Fork				2	
Little Beaver Creek				1	
James River				2	
L. cardinalis					
Spring River					2
Arkansas River					1

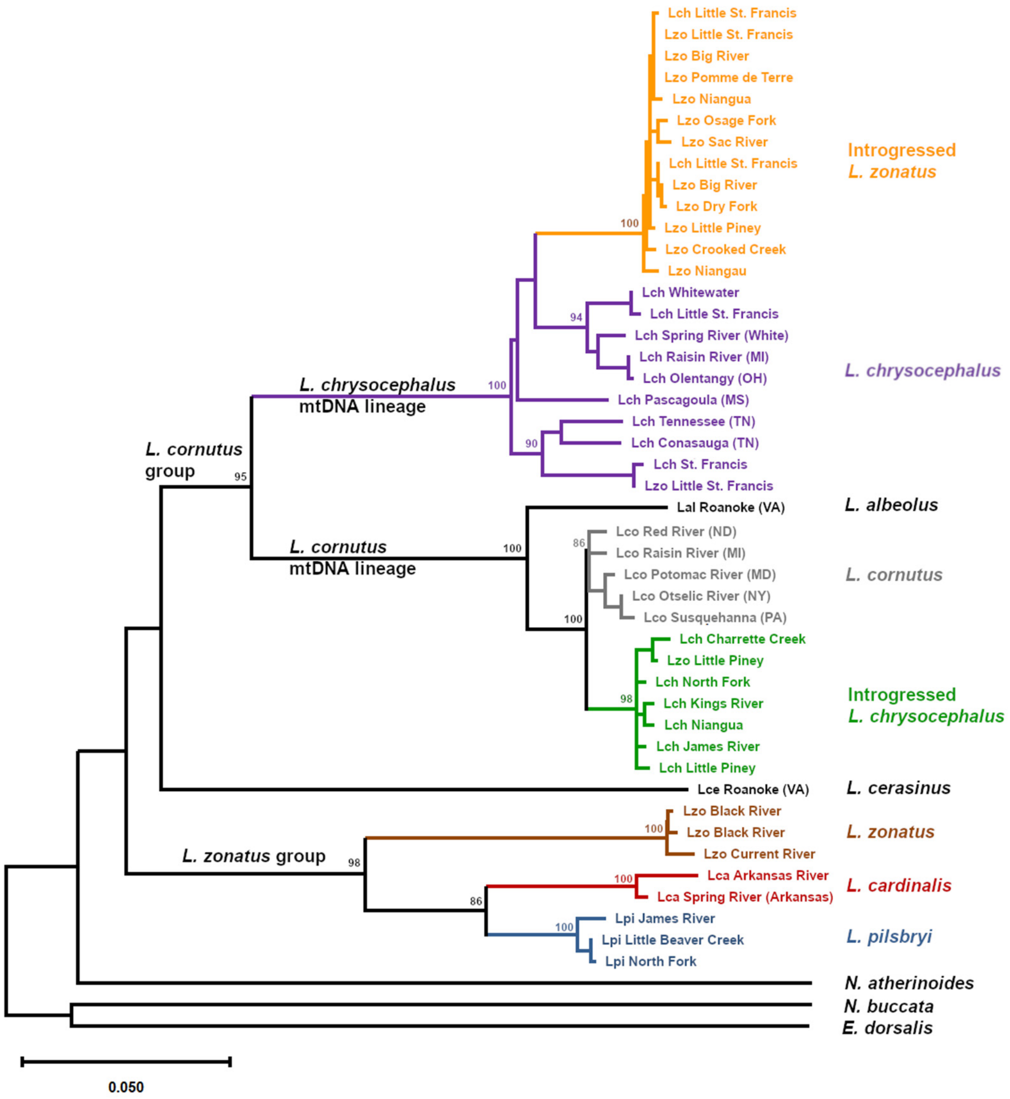

Figure 1. Maximum Likelihood phylogeny of mitochondrial ND2 mtDNA sequences of *Luxilus* spp. identified by sample location (Table 1). Morphological species abbreviations: *L. chrysocephalus* Lch, *L. cornutus* Lco, *L. zonatus* Lzo, *L. cardinalis* Lca, *L. pilsbryi* Lpi, *L. albeolus* Lal, *L. cerasinus* Lce. Clades are labeled according to predominant species and highlighted by separate colors. Reference specimens from outside the Ozark Highlands are identified by drainage and state, and duplicate river names are clarified by corresponding drainage. The *L. cornutus* and *L. zonatus* groups, and nested *L. chrysocephalus* and *L. cornutus* mtDNA lineages are labeled at interior nodes. Nodes with bootstrap support ≥80% are indicated. Outgroups included *Notropis atherinoides*, *Notropis* buccata, and *Ericymba dorsalis*.

The *cornutus* group excluding *L. cerasinus* (ie. *L. cornutus*, *L albeolus*, and *L. chrysocephalus*) formed a second monophyletic clade (95% of bootstrap replicates). *Luxilus cerasinus* was sister to the rest of the *cornutus* group, though without bootstrap support. There were two mtDNA lineages within the *cornutus* group, one contained the *L. cornutus* mtDNA lineage including *L. albeolus* (100% of bootstrap replicates) while the other contained the *L. chrysocephalus* mtDNA lineage (100% of bootstrap replicates).

Comparison of mtDNA results with morphological species assignments identified considerable introgression among these taxa. A well-defined mtDNA lineage of morphologically identified *L. zonatus* (100% of bootstrap replicates) was nested within the range-wide *L. chrysocephalus* mtDNA lineage, indicating that this distinct lineage (hereafter 'introgressed *L. zonatus*') resulted from mitochondrial introgression from *L. chrysocephalus* into *L. zonatus*. These morphologically identified introgressed *L. zonatus* were from the St. Francis, Meramec, Gasconade, and Osage river drainages. Most individuals sampled from these populations exhibited the introgressed *L. zonatus* mtDNA haplogroup (Table 2). The paraphyletic *L. chrysocephalus* range-wide mtDNA clade, exclusive of introgressed *L. zonatus*, exhibited nucleotide diversity (π) of 0.033. Nucleotide diversity for the monophyletic introgressed *L. zonatus* lineage was nearly an order of magnitude lower at 0.004.

The *L. cornutus* mtDNA haplogroup also exhibited evidence of introgression as previously reported (Duvernell & Aspinwall, 1995). Sampled populations of morphologically identified *L. chrysocephalus* from drainages throughout the northern Ozark Highlands, as well as the White River drainage in the southern Ozark Highlands all exhibited a monophyletic clade of introgressed *L. cornutus* mtDNA (98% support, known hereafter as 'introgressed *L. chrysocephalus*') (Table 2, Figure 2b) and were the sister lineage to a range-wide *L. cornutus* clade. The range-wide *L. cornutus* and introgressed *L. chrysocephalus* clades appeared reciprocally monophyletic, though with lower bootstrap support (86%) and small sample size for the *L. cornutus* clade exclusive of the introgressed *L. chrysocephalus* clade. The *L. cornutus* clade exhibited nucleotide diversity of 0.010 compared to 0.006 for the introgressed *L. chrysocephalus* clade. Samples of morphologically identified *L. chrysocephalus* taken from the St. Francis River and Whitewater River, exhibited range-wide *L. chrysocephalus* haplotypes while the Little St. Francis River sample included both range-wide *L. chrysocephalus* and introgressed *L. zonatus* haplotypes (Table 2, Figure 2b).

Among all sampled *L. zonatus* individuals, the only ones that did not exhibit the *L. zonatus* haplotype or the here-defined introgressed *L. zonatus* haplotype were one individual in the Little Piney, a tributary of the Gasconade River, which exhibited an introgressed *L. chrysocephalus* haplotype, and one *L. zonatus* individual from the Little St. Francis River exhibiting an *L. chrysocephalus* haplotype (Table 2, Figure 2a).

A calibrated phylogeny (Figure 3) exhibited very large HPD confidence intervals in part due to calibration uncertainty built into the analysis. Nevertheless, confidence intervals placed the coalescent ages of most clades of interest within the Pleistocene (2.6 to 0.01 Ma). Our analysis placed the most likely age of the most recent shared ancestor between *L. cornutus* and *L. chrysocephalus* as pre-Pleistocene, at 5.05 Ma (0.61 - 11.77 Ma), and the *L. zonatus* group (*L. zonatus*, *L. pilsbryi*, *L. cardinalis*) at 4.75 Ma (0.59-11.07) (Figure 3). The coalescent age of all samples in the *L. chrysocephalus* mtDNA lineage was 1.98 Ma (0.24 – 4.62 Ma), and the introgressed *L. zonatus* clade within the *L. chrysocephalus* mtDNA lineage was 0.30 Ma (0.03 – 0.72 Ma). Nucleotide divergence (D_{xy}) between the introgressed *L. zonatus* clade and the next most closely related *L. chrysocephalus* haplotype was 0.049 with an estimated age of 1.52 Ma (0.16-3.57 Ma). The age of the ancestor of the *L. cornutus* and introgressed *L. chrysocephalus* mtDNA lineage (i.e. excluding *L. albeolus*) was 0.73 Ma (0.07 – 1.72 Ma), with an age for the nested range-wide *L. cornutus* clade estimated at 0.42 Ma (0.04 –1.02 Ma), and the introgressed *L. chrysocephalus* clade estimated at 0.31 Ma (0.03 – 0.74 Ma), respectively.

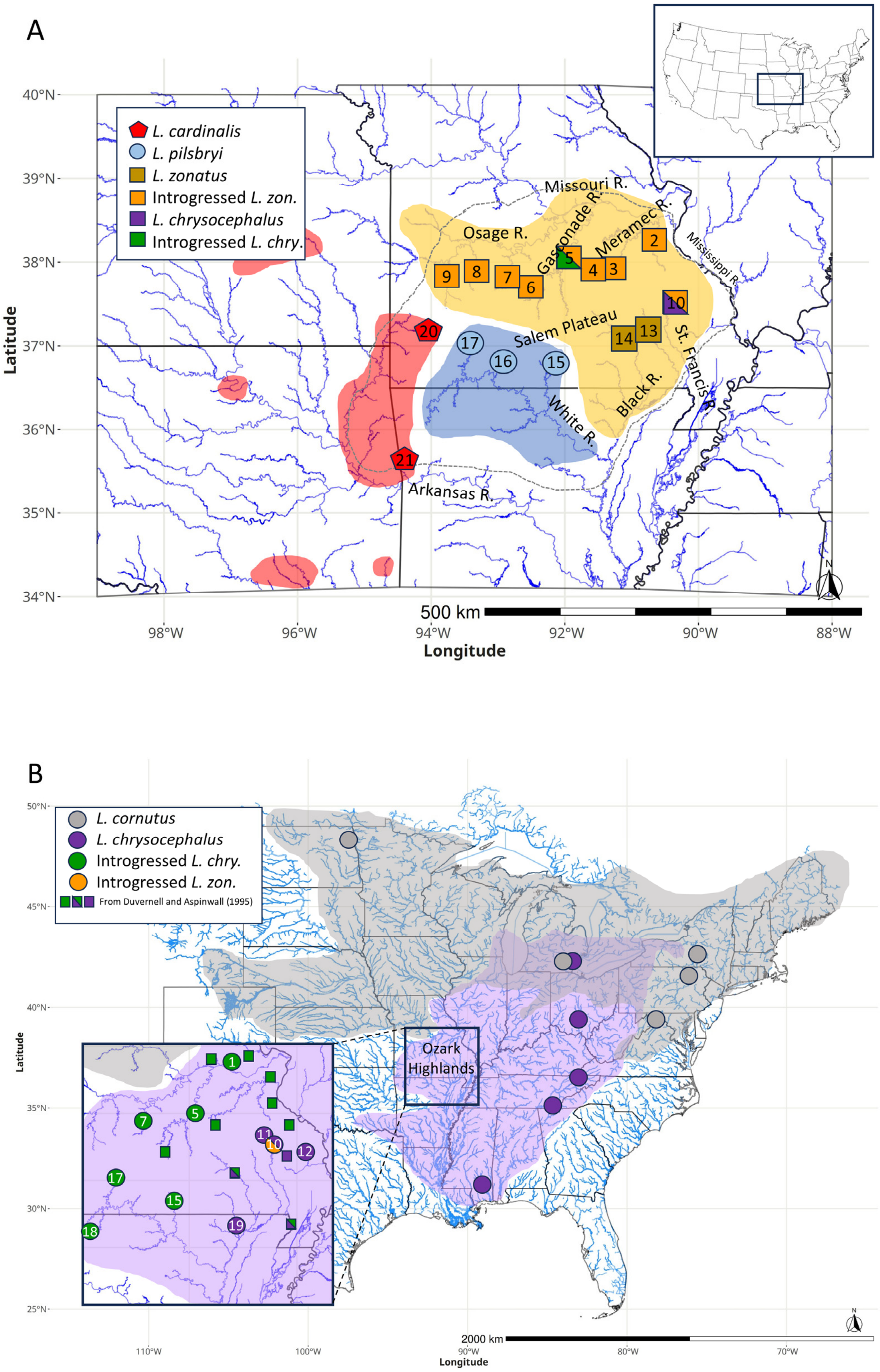

Figure 2. Distribution of species and mitochondrial linages for morphologically assigned members of the A) *L. zonatus* group and B) *L. cornutus* group. Shaded areas delineate species distributions, and symbols with colors indicate mitochondrial lineages. Split symbols indicate sample mitochondrial polymorphism. Numbered sample locations are identified according to Table 1. Mitochondrial lineage colors match clades in Figure 1.

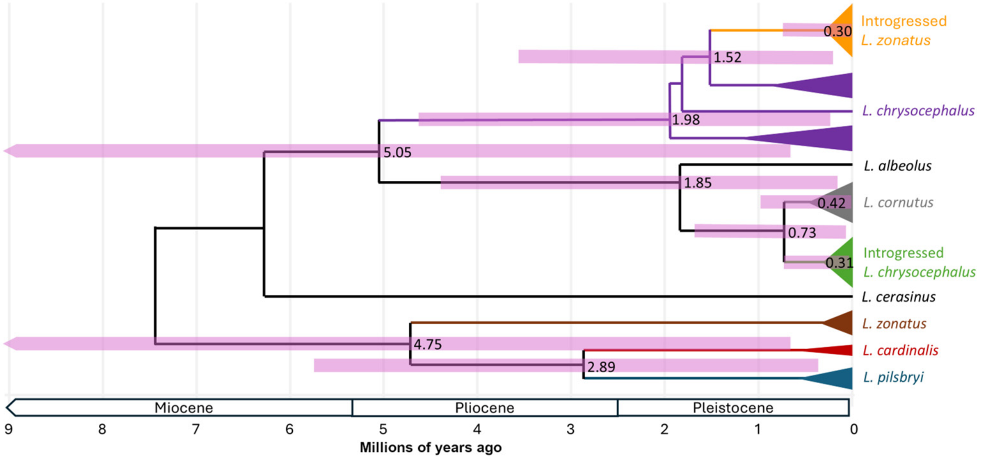

Figure 3. Time calibrated phylogeny resulting from BEAST analysis. Mean ages are shown at each node, and horizontal bars present the 95% Highest Posterior Density (HPD) intervals for the estimated divergence times.

4. Discussion

Discordant mitonuclear introgression has been widely documented in many organisms (Bonnet et al., 2017; Hill, 2019; Sloan et al., 2017; Toews & Brelsford, 2012). The features that make our study exceptional are first that mitogenome replacement appears to have occurred twice in succession involving three congeneric shiner species. Second, the ranges of impacted populations in the two affected species are largely congruent and cover multiple isolated drainages that span the northern and eastern Ozark Highlands. The phylogeographic patterns of mitogenome introgression of both *L. chrysocephalus* and *L. zonatus* require at least two separate incidences of mitonuclear introgression that are Pleistocene in age, leading to mitogenome replacement in ancestral populations of both species. The broad, congruent geographic overlap of impacted populations, involving numerous Ozark Highland drainages, may reflect the impact of shifting distributions of both species associated with Pleistocene glacial cycles, and coincident with Ozark Highlands geomorphological processes.

Glaciation in North America west of the present day Mississippi River valley was marked by the Laurentide Ice Sheet advancing as far south as the vicinity of the Missouri River Valley at the northern edge of the unglaciated Ozark Highlands. Five glacial advances occurred in this region prior to the Illinoian stage at approximately 2.4, 1.3, 0.8, 0.4, and 0.2 Ma (Rovey & Balco, 2011). These events modified the course of rivers in the region, pushing the range of affected species southward (Robison, 1986). Periglacial lakes along the glacial front would have increased in size as glaciers melted. This would have also increased connection of periglacial lakes along the glacial front, and overflow of meltwater may have allowed for east-west dispersal of fishes along the northern border region of the Ozark Highlands (Robison, 1986).

The major drainages of the Ozark Highlands radiate out of the Salem Plateau. Biogeographically, drainages north of the Salem Plateau (e.g. Osage, Gasconade, Meramec) have similar ichthyofaunal species assemblages that differ in notable respects from drainages south of the Salem Plateau (e.g. St. Francis, Black, White). These biogeographic differences are evident in some Leuciscids (*Campostoma* spp., *Cyprinella* spp., *Notropis* spp.), Centrarchids (*Ambloplites* spp., *Lepomis* spp.), Ictalurids (*Noturus* spp.) (Pflieger, 1997), and intraspecific phylogeography of the *Etheostoma caeruleum* complex (Ray et al., 2006). There is also evidence that connections occurred occasionally between drainages that were otherwise isolated by the Salem Plateau. A population genetic study of the topminnow, *Fundulus olivaceus*, identified gene flow between the Big and St. Francis rivers, the Meramec and Black rivers, the Big Piney and North Fork rivers, and the Meramec and Gasconade rivers (Duvernell et al., 2019). Study of fluvial geomorphology in the Ozark Highlands offers evidence that headwaters on opposite sides of the Salem Plateau have commingled historically through numerous stream capture events (Beeson et al., 2017) creating opportunities for faunal exchanges among drainages.

The *L. zonatus* species group (*L. cardinalis*, *L. pilsbryi*, and *L. zonatus*) diversified in isolated drainages, likely beginning in the Pliocene (common ancestor estimated here at 4.7 Ma), and the phylogeographic distribution of these three species is consistent with their origin in the southern Ozark Highlands followed by a range expansion of *L. zonatus* into the more northern drainages within its range. Those northern *L. zonatus* populations appear monophyletic for the introgressed mitogenome acquired from *L. chrysocephalus*. Among *L. zonatus* populations, mitogenome introgression has impacted at least four of the five major Ozark drainages encompassed by the extant species distribution such that, range-wide, far more *L. zonatus* individuals exhibit introgressed than non-introgressed mitogenomes. The distribution of mitogenome replacement in northern *L. zonatus* populations is consistent with massive introgression predicted by an invasion-with-hybridization model driven by demographic inequality at a northward invasion front (Currat et al., 2008). According to this prediction, as *L. zonatus* expanded its range into eastern and northern portions of the Ozark Highlands where *L. chrysocephalus* was already present, the demographic inequality alone between resident and invading populations could have been sufficient to drive asymmetric mtDNA introgression (Currat et al., 2008; Wielstra & Arntzen, 2012). The reduced nucleotide diversity, and monophyly of the introgressed *L. zonatus* clade relative to other range-wide *L. chrysocephalus* haplotypes may indicate that this mitogenome replacement event coincided with a founder event initially within a small geographic region (e.g. within one drainage) and then expanded into other drainages through continued *L. zonatus* range expansion. The broad extant syntopy of *L. zonatus* and *L. chrysocephalus* throughout the Ozark Highlands suggests a northward expansion of *L. zonatus* without competition or species replacement.

The age of the split between *L. cornutus* and *L. chrysocephalus* is similarly dated to the Pliocene (5.05 Ma). With a northern *L. cornutus* range that was almost completely formerly glaciated, the presence of introgressed *L. cornutus* mitogenomes in Ozark *L. chrysocephalus* populations provides evidence for range overlap within or in the vicinity of the Ozark Highlands at least sometime during the Pleistocene (Duvernell & Aspinwall, 1995). The divergence between introgressed haplotypes found in Ozark *L. chrysocephalus* and the range-wide *L. cornutus* species haplogroup is dated to approximately mid-late Pleistocene (0.73 Ma). Evidence that hybrid introgression resulted in *L. chrysocephalus* mitogenome replacement from an *L. cornutus* donor spans all drainages originating in the Ozark Highlands, with most drainages appearing monophyletic for the introgressed haplogroup, and only drainages in the southeastern Ozark Highlands (Black, St. Francis, and Whitewater Rivers) exhibiting predominantly non-introgressed *L. chrysocephalus* mitogenomes (Duvernell & Aspinwall, 1995).

Our phylogenetic analysis shows that, after introgression, range-wide *L. cornutus* and introgressed Ozark *L. chrysocephalus* populations achieved reciprocal monophyly. The similar levels of nucleotide diversity between range-wide *L. cornutus* and introgressed Ozark *L. chrysocephalus* suggests that an *L. cornutus* range-wide expansion was similarly Pleistocene in age and occurred on a similar time frame to when the Ozark *L. chrysocephalus* introgression event occurred. The relatively limited range-wide nucleotide diversity in the northerly distributed *L. cornutus* compared to the more southerly distributed *L. chrysocephalus* is consistent with contrasting and disproportionate impacts of Pleistocene glaciation on these species in their respective extant latitudinal distributions.

The notable feature of these overlapping patterns of introgression is that in multiple drainages where *L. zonatus* appears monomorphic for an introgressed haplogroup from an *L. chrysocephalus* donor, sympatric *L. chrysocephalus* populations appear monomorphic for a different introgressed haplogroup from an *L. cornutus* donor. This dual mitogenome replacement pattern was observed in all drainages north of the Salem Plateau, including all samples in the Osage, Gasconade and Meramec River drainages. The coalescent ages of the introgressed *L. chrysocephalus* and *L. zonatus* haplogroup clades are both pre-Illinoian late Pleistocene, and with essentially the same age estimates (310 and 297 kya, respectively), suggesting that introgressed populations of both species may have established nearly concurrently via either range expansions or gene flow. However, *L. zonatus* introgression would have had to preceded *L. chrysocephalus* introgression or else introgressed *L. zonatus* populations would exhibit *L. cornutus*-lineage haplotypes instead of *L. chrysocephalus*-lineage haplotypes.

The phylogeographic distribution of mitogenome replacement events across multiple isolated drainages may suggest a deterministic driver operating similarly in separate drainages throughout the Ozark Highlands. However, the concurrent prevalence of introgressed mitogenomes in populations of both *L. chrysocephalus* and *L. zonatus* presents challenges to the invocation of natural selection as a driving factor. If mitogenome replacement was prevalent across multiple Ozark drainages because natural selection favored introgression of locally adapted mitogenomes in both species, then this does not account for why natural selection would favor different non-native mitogenomes in sympatric populations of each species. Similarly, if the driver of selection favoring introgressed mitogenomes was to alleviate some form of accumulated mutation load, as might have been caused by founder effects resulting from shifting ranges driven by glacial cycles, then that would not address the multi-drainage pattern of introgression or explain why introgressed mitogenomes were more fit than native mitogenomes in both species. The alternative explanation for the broad distribution of introgressed mitogenomes in both *Luxilus* species across very similar geographic areas that encompass most of the same Ozark drainages is range expansions and drainage invasions that could have occurred repeatedly for both species. The shared history of mitogenome haplogroups points to a complicated history of hybridization and introgression between these species. This calls for a better understanding of the phylogeographic histories of both species that will only be possible with further sampling and nuclear genome data.

Distributions of mitochondrial haplogroups between *L. zonatus* and *L. chrysocephalus* are further complicated in the St. Francis River drainage in the southeastern Ozark Highlands. The *L. chrysocephalus* sampled in the St. Francis River drainage in this study exhibited exceptional mitochondrial sequence diversity that reflected the entire range-wide mitogenome diversity of *L. chrysocephalus* (Figure 1). Results reported here are consistent with those reported by Duvernell and Aspinwall (Duvernell & Aspinwall, 1995) except that the earlier study relied on a PCR-RFLP assay that only resolved *L. cornutus* and *L. chrysocephalus* mtDNA lineages, and did not delineate differences between the introgressed *L. zonatus* haplogroup and all other range-wide *L. chrysocephalus* haplotypes.

In the Little St. Francis River, both *L. zonatus* and *L. chrysocephalus* were polymorphic for *L. chrysocephalus* haplotypes, including a predominance of the introgressed *L. zonatus* haplogroup in both species. The prevalence of the introgressed *L. zonatus* haplogroup could be indicative of an ancestral polymorphism in the Little St. Francis *L. chrysocephalus* population that has been shared with *L. zonatus* through introgressive hybridization. The St. Francis River *L. chrysocephalus* could be the source of the introgressed *L. zonatus* haplogroup that subsequently expanded into other Ozark Highland drainages. Alternatively, the shared mitochondrial polymorphisms between *L. zonatus* and *L. chrysocephalus* in the Little St. Francis River could indicate more recent, ongoing hybridization between the species. Further sampling within this drainage, with the inclusion of nuclear genetic data, is needed before conclusions can be drawn. Either way, this level of interspecies haplogroup sharing is distinctive within this system and was not encountered to the same extent in any other drainages. In the Osage, Gasconade and Meramec drainages where *L. chrysocephalus* and *L. zonatus* are syntopic and both essentially monomorphic for introgressed haplogroups, there is little evidence of recent or ongoing hybridization between the species except for one *L. zonatus* individual from Little Piney Creek in the Gasconade River drainage that exhibited the introgressed *L. chrysocephalus* mitogenome.

5. Conclusions

The concurrent patterns of mitogenome introgression among *Luxilus* species within the Ozark Highlands offer an exceptional example of mitonuclear discordance impacting multiple species and provide unique insights into Pleistocene-era impacts of gene flow and range shifts driven or facilitated by glacial advances and geomorphological processes occurring in the Ozark Highlands during this period.

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Abbreviations

The following abbreviations are used in this manuscript:

mtDNA	Mitochondrial DNA
ML	Maximum Likelihood
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
HPD	Highest Posterior Density

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