

Pleistocene speciation and glacial refugia in the Gilt Darter (*Percidae: Percina evides*) species complex

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Abstract

Among temperate regions of the world, freshwater fish species richness is highest in the Central Highlands of eastern North America. Historical biogeographic and phylogeographic researchers have investigated mechanisms driving this exceptional diversity, yet the role of major climatic events, like Pleistocene glaciation, is incompletely characterized. In this study, we analyze genomic DNA sequence data sampled from populations of the widely distributed Gilt Darter, *Percina evides*, to reconstruct pre-glacial drainage patterns and assess the impact of Pleistocene glaciation on generating the high species diversity of eastern North American freshwater fishes. Phylogenomics, population genomic analyses, and evaluation of morphology delimit 5 species currently classified as *P. evides*. These species likely diverged via allopatric speciation among the disjunct regions of the Central Highlands driven by the onset of Pleistocene glaciation. Divergence times among newly delimited species of the Gilt Darter complex are congruent with the onset of glaciation, periods of river incision and aggradation, and river network rearrangement. The discovery of new species in the *P. evides* complex and reconstruction of the timing of their diversification provide insight into the role that Pleistocene glaciation and glacial refugia contributed to the remarkable temperate freshwater biodiversity hotspot of the Central Highlands.

Keywords: phylogeography, speciation, darters, species delimitation

Introduction

Eastern North America hosts the most species-rich freshwater fish fauna among temperate regions of the world (García-Andrade et al., 2023; Lundberg et al., 2000). This exceptional biodiversity is concentrated in the Central Highlands, a geographic region comprising two distinct areas: the Eastern Highlands (encompassing southern Appalachia and the Cumberland Plateau) and the Interior Highlands (containing the Ozark and Ouachita Highlands) (Jenkins et al., 2015; Mayden, 1985, 1987a, 1987b, 1988). Within the Central Highlands, distantly related species of freshwater fishes exhibit a similar pattern of geographic distributions; present in the disjunct Eastern and Interior Highlands but absent in the intervening lowland areas (Mayden, 1987b; Pflieger, 1971; Wiley & Mayden, 1985). Level II Ecoregions, which are regions of similar ecosystems, serve as a useful proxy for mapping these highland regions and are illustrated in Figure 1 (Level II Ecoregion data sourced from the United States Environmental Protection Agency). This distinctive distribution pattern has motivated considerable scientific interest, leading to numerous studies examining the paleogeographic events and speciation mechanisms that have shaped this biodiversity hotspot (Berendzen et al., 2003, 2008a; Bossu et al., 2013; Halas & Simons, 2014; Hundt et al., 2016; Mayden, 1988; Near et al., 2001).

During the Pleistocene epoch, glacial advances dramatically reshaped river drainages across eastern North America, significantly impacting the Central Highlands both within

and south of the maximum glacial extent (Goldthwait, 1991; Mayden, 1987b, 1988). The Teays-Mahomet River was a pre-Pleistocene river system hypothesized to have originated along the Blue Ridge Escarpment (Figure 1), flowing north to northwest across Ohio, Indiana, and Illinois, USA, before converging with the Old Mississippi River (Goldthwait, 1991; Ray, 1974; Thornbury, 1965; Tight, 1903; ver Steeg, 1946). This ancient river system included drainages that are now tributaries of the middle and upper Ohio River system, including the Great Miami, Kentucky, and New Rivers. As glaciers advanced southward, the course of the Teays-Mahomet River was disrupted and dammed, creating several episodes of proglacial lakes such as Lake Tight (Erjavec, 2018; Wolfe, 1942) and Lake Monongahela (Lessig, 1963) along the margin of the Laurentide Ice Sheet. During these glacial stages, existing drainage divides were overtopped, and upon ice sheet retreat, rivers continued to flow through the paleo-divides, ultimately forming the modern Ohio River system (Erjavec, 2018; Fowke, 1925; Granger et al., 2001; Ray, 1974; Tight, 1903). Because of their hypothesized pre-Pleistocene connection via the Teays-Mahomet River system, lineages distributed throughout the upper Ohio River are expected to be more closely related to lineages in the Interior Highlands compared to other Eastern lineages distributed outside of the original Teays-Mahomet River system (Mayden, 1988). Others have questioned the age and existence of a discrete Teays River, proposing instead three “Teays rivers,” two existed during the late

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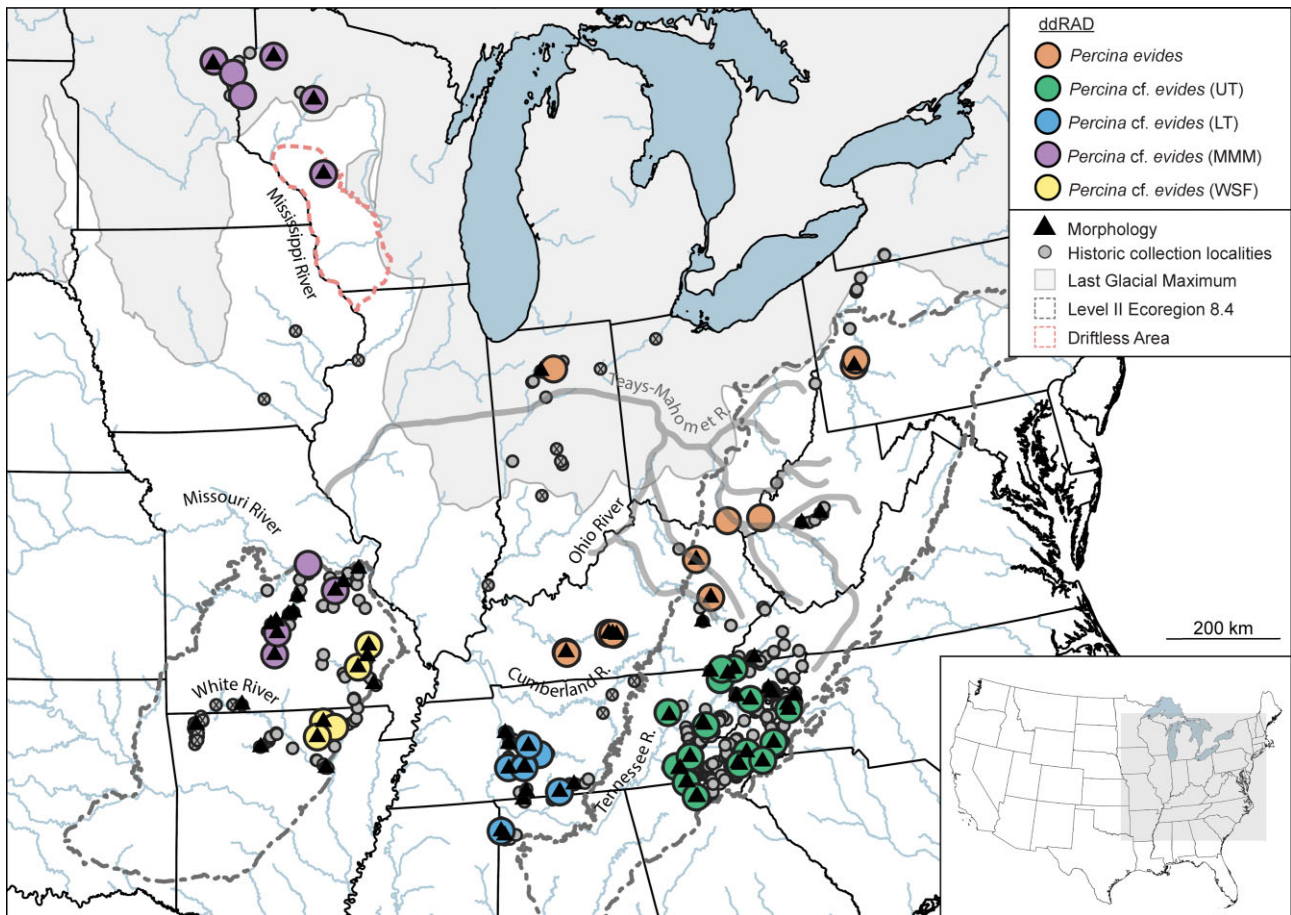


Figure 1. Map of the eastern United States with *Percina evides* sampling locations, data type collected, and the extent of the Last Glacial Maximum. Teays-Mahomet River (translucent gray line) adapted from [Mayden \(1988\)](#). Historical localities were curated from Fishnet2 and GBIF; records with erroneous or mismatched georeferenced data were corrected to the best of our ability based on the given locality or removed; historical localities with a grey “x” indicate the population is extirpated ([Fisher, 2008](#); [Metzke et al., 2022](#); [Rice & Zimmerman, 2019](#); [Robison & Buchanan, 2020](#); [Simon, 2006](#); [Trautman, 1981](#)). Level II Ecoregion 8.4 represents the Ozark, Ouachita-Appalachian Forests and corresponds to the Interior and Eastern Highlands.

Tertiary period and one dating to a more recent glacial age, which give the illusion of a continuous buried river valley ([Gray, 1991](#); [Melhorn & Kempton, 1991](#)). Pleistocene climatic shifts also influenced rivers south of the maximum glacial extent, such as the Tennessee River, by triggering cycles of bedrock incision and sediment aggradation that may have altered fluvial habitat. Phylogeographic studies of aquatic lineages offer insight into how these dramatic river drainage reconfigurations and climatic changes impacted speciation and biogeography in the Eastern North American biodiversity hotspot ([Berendzen et al., 2003, 2008a, 2008b](#); [Bossu et al., 2013](#); [Hundt et al., 2016](#); [Near et al., 2001](#); [Simons, 2004](#)).

The Gilt Darter, *Percina evides* ([Jordan & Copeland, 1877](#)), is distributed throughout the Central Highlands and upper Mississippi River system with disjunct populations in the Interior Highlands, Eastern Highlands, and the upper Mississippi River system in Wisconsin and Minnesota, USA ([Figure 1](#)). The distribution of *P. evides* includes areas in the Ohio River system heavily impacted by glacial fluctuation during the Pleistocene as well as the Driftless Area ([Figure 1](#)) that exhibits no evidence of past glaciation during the Quaternary ([Carson et al., 2023](#); [Hobbs, 1999](#); [Knox, 2019](#)). The Driftless Area is hypothesized to have served as glacial refugia for several plant and animal species ([Beatty](#)

& [Provan, 2010, 2011](#); [Lee-Yaw et al., 2008](#); [Rowe et al., 2004](#)). Following isolation in glacial refugia like the Driftless Area, post-glacial colonization following the leading edge of the ice sheet could lead to rapid geographic expansion of populations or could involve gene flow between previously isolated populations, leading to decreased or increased genetic diversity, respectively ([Shafer et al., 2010](#)). In addition, species could persist in glacial refugia with little contact between other populations following glacial retreat, facilitating increased genetic differentiation.

Divergence in morphological characters and variation of mitochondrial DNA haplotypes within the *P. evides* complex have been well documented over the past half century ([Denoncourt, 1969](#); [Near et al., 2001](#)). Variation in meristic traits and patterns of pigmentation led to the delimitation of three taxa that were never formally named: the lineages in the Missouri and White Rivers, the upper Tennessee River, and all other populations ([Denoncourt, 1969](#)). Phylogenetic analysis of mitochondrial DNA resolved three lineages of *P. evides* distributed throughout drainages east of the Mississippi River, the Missouri and upper Mississippi River systems, and the White River system ([Near et al., 2001](#)). Traditionally, species discovery and delimitation of fishes have relied exclusively on morphological trait differences; however, contemporary approaches now integrate

molecular and morphological data to provide a more comprehensive approach to demarcating species boundaries (Artaev et al., 2024; Egge & Simons, 2006; Ghezelayagh et al., 2025; Harrington & Near, 2012; Kim et al., 2022; Layman & Mayden, 2012; MacGuigan et al., 2021; Near et al., 2021; Wood et al., 2023). In the genomics era, this integrated protocol offers a more robust method for identifying independently evolving lineages, distinguishing true species boundaries from population-level variation (Sukumaran & Knowles, 2017). Developing an accurate account of species diversity within the *P. evides* complex through this integrative approach is necessary for conducting comprehensive historical biogeographic analyses and investigating the mechanisms responsible for the remarkable temperate freshwater biodiversity hotspot of the Central Highlands.

In this study, we use double-digest restriction-site associated DNA (ddRAD) sequence data and traditional morphological traits to infer phylogenetic relationships, deploy an integrated approach to species delimitation, and address the role of Pleistocene glaciation on diversification in the *P. evides* complex. We hypothesize *P. evides* from the Ohio River drainage will be more closely related to *P. evides* present in the Interior Highlands than to other lineages in the Eastern Highlands, reflective of historical connectivity via the Teays-Mahomet River.

Materials and methods

Specimen collection and ddRAD library preparation

Specimens of the *P. evides* complex were obtained through field collections and research museum collections (Table S1). Fin clips were taken from the right pectoral and pelvic fins and were preserved in 95% ethanol; voucher specimens were preserved in 10% formalin and transferred to 70% ethanol for long-term preservation. Tissues were deposited in the Yale Fish Tissue Collection, and voucher specimens were deposited in the Vertebrate Zoology Division Ichthyological Collection, Yale Peabody Museum. All handling of animals was conducted accordingly under IACUC No. 2018-10681 and 2023-10681. Genomic DNA was isolated using the Qiagen DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA). ddRAD sequencing libraries were prepared following Peterson et al. (2012). Approximately 400 ng of DNA per sample was digested for 16 hr using MspI/PstI restriction enzymes. Following digestion, DNA samples were ligated with 96 unique barcodes. Samples were amplified using PCR and purified using Qiagen QIAquick PCR Purification Kit with a modified protocol. Purified PCR samples were pooled into a single 96-sample library and standardized to approximately 2,000–4,000 ng of DNA. Size selection and sequencing of the library were performed by the University of Oregon GC3F facility. DNA fragments of 300–500 bp were selected using a BluePippin and sequenced on an Illumina NovaSeq 6000 using single-end sequencing.

Reads (BioProject ID: PRJNA1214323) were aligned to a *P. caprodes* reference genome (genome assembly GCA_013375575.1) available at the National Center for Biotechnology Information (NCBI) using ipyrad v. 0.9.92 (Eaton & Overcast, 2020). For read filtering, “TGCAG, CCG” set for restriction overhang, and the default was used for all other parameters as follows: *Q* score of 33, maximum of five low-quality base calls per read, minimum of six

for depth for statistical and majority-rule base calling, strict filtering for adapters/primers, zero allowable mismatches to barcodes, a minimum length of 35 reads after adapter trim, a maximum of 0.05 for maximum number of uncalled bases and heterozygotes in a consensus, a maximum of 0.2 SNPs per locus, a maximum of eight indels per locus, and a maximum of 0.5 heterozygous sites per locus.

Phylogenetic inference

A maximum-likelihood phylogeny of the *P. evides* complex was inferred using IQ-TREE v. 2.1.2 (Minh et al., 2020) with 1,000 ultrafast bootstrap replicates (Hoang et al., 2017), using concatenated ddRAD loci. The dataset used for maximum-likelihood phylogenetic inference included 164 specimens with a minimum of 70 specimens per locus. Three *P. copelandi* and five *P. vigil* specimens were included as outgroups following the results of molecular phylogenetic analyses using Sanger-sequenced nuclear genes (Near & Keck, 2013). The maximum-likelihood phylogeny was inferred using the TVM + F + I + G4 molecular evolutionary model, which was identified as the best-fitting model using ModelFinder (Kalyaanamoorthy et al., 2017).

Population structure and admixture

To inform population genomic structure, we employed sparse non-negative matrix factorization (sNMF) to estimate admixture coefficients for each genotyped specimen, and calculated the fixation index, F_{ST} . For sNMF, an unlinked SNP dataset, which included one random SNP per locus, was generated using ipyrad to remove as much missing data as possible, so as not to confound the analyses. The dataset included 140 individuals of the *P. evides* complex with a minimum of 138 individuals per locus. We used the R package LEA v. 3.14.0 (Gain & Francois, 2021) for cross-entropy validation analysis and sNMF estimation of ancestry coefficients. Cross-entropy validation was performed for populations (K) 1–10 with 100 replicates and an alpha value of 100 to determine the appropriate number of populations to represent the data. We plotted the cross-entropy values and chose the optimum K value based on the point where the cross-entropy curve reaches a plateau (Francois, 2016). Pairwise F_{ST} values among the major lineages of the *P. evides* complex were calculated using Weir and Cockerham (1984) methodology with the R package hierfstat v. 0.5.11 (Goudet, 2005) and a dataset that included 147 individuals with a minimum of 120 specimens per locus.

Genomic species delimitation, species tree inference, and divergence time estimation

We inferred species trees using two methods: SVDquartets (Chifman & Kubatko, 2014, 2015) implemented in PAUP (v. 4.0a) (Swofford, 2003) and the SNP and AFLP Package for Phylogenetic analysis (SNAPP v. 1.6.1) (Bouckaert et al., 2019; Bryant et al., 2012). In both species tree analyses, we treated *P. evides* as five lineages or species following the ddRAD maximum likelihood inferred phylogeny. SVDquartets is a method that infers phylogenetic relationships among quartets of taxa under the coalescent model and produces unrooted phylogenies (Chifman & Kubatko, 2014). For this analysis, we used unlinked SNPs from 161 individuals, including the outgroup *P. vigil*, with a minimum of 140 individuals per locus, and performed 100 bootstrap

replicates. Unlinked SNP files were generated using ipyrad, where one random SNP per locus was sampled. SNAPP uses independent biallelic markers to infer species trees and demography through a Bayesian Markov chain Monte Carlo method. Given the computational intensity of SNAPP, the dataset was down-sampled to include 25 individuals sampled across the geographic range of *P. evides* and *P. vigil* (Table S1), with a minimum of 23 individuals per locus. The dataset was pruned to remove non-binary SNPs and to code missing data using the R package phrynomics (<https://github.com/bbanbury/phrynomics>). The timing of divergence between the most recent common ancestor (MRCA) of *P. vigil* and the *P. evides* complex was calibrated with divergence time estimates provided in Near and Keck (2013), with an MRCA of 11.49 Ma (95% HPD: 14.06–8.87 Ma). We performed one run of 3,669,000 replicates, logging every 500 replicates. Using Tracer v. 1.7.2 (Rambaut et al., 2018), we verified that effective sample sizes (ESS) exceeded 200. The mean height of the maximum clade credibility tree was annotated after applying a 10% burn-in.

To delimit species using genomic data, we calculated the genealogical divergence index for the *P. evides* complex under a five-species model (*gdi*; Jackson et al., 2017). We used the species tree inferred from the SVDquartets analysis as the guide tree. Genetic species delimitation analysis BPP v. 4.6.2 (Flouri et al., 2018; Yang, 2015) was conducted using 8–10 geographically spaced individuals per lineage of the *P. evides* complex. The dataset was pared down due to the computational intensity of the analysis, and geographically spaced individuals were selected to reduce sampling bias. The theta and tau parameters—a proxy for effective population size and divergence time, respectively—estimated from the A00 algorithm of BPP were utilized for *gdi* calculation. Nucleotide diversity (Nei, 1987) within and between lineages was estimated using PopGenome (Pfeifer et al., 2014) as a basis for theta and tau priors. We performed 800,000 generations, sampled every 20 generations, with a burn-in of 100,000 generations. Tracer was used to confirm that ESS values were above 200.

Demographic and phylogeographic history of Interior Highlands and upper Mississippi River watershed

To address whether populations of the *P. evides* complex located in and upstream of the Driftless Area are the result of geographic isolation during glacial periods or of post-glacial range expansion, the demographic history of these populations was reconstructed. We used *fastsimcoal2*, which utilizes the site frequency spectrum (SFS) to fit model parameters under a coalescent model (Excoffier et al., 2021). We used a dataset of 32 individuals, 8–10 per lineage in concordance with phylogenetic analysis, from geographically spaced sampling locations (Table S1) with a minimum of 27 specimens per locus. Individuals from the White, St. Francis, Missouri, Meramec, Ohio, and upper Mississippi River systems were used for *fastsimcoal2* analysis. The SFS was converted from Variant Call Format and projected down to include 762 unlinked SNPs using easySFS v. 0.0.1 (<https://github.com/isaacovercast/easySFS>) (Gutenkunst et al., 2009). The crown age was constrained using the mean age of the MRCA of *P. evides* in the Ohio River system and populations in the Interior Highlands-upper Mississippi

River system extracted from SNAPP analysis; 1,000 parametric bootstrapping replicates were performed.

Morphological analyses

Comparative meristic trait data were collected from 460 specimens of *P. evides*. Using a standard dissecting microscope, numbers of scale rows and fin elements were counted following Hubbs et al. (2004) and Page (1981); transverse scale row count follows Page (1983: 16, Figure 2). Meristic traits included in our study are the number of scales along the lateral line, the number of scales above and below the lateral line, the number of transverse scale rows, the number of scale rows around the caudal peduncle, the number of dorsal fin spines and rays, and the number of anal and pectoral fin rays. Frequency tables are reported in Tables S2–S10. A principal component analysis (PCA) was performed through the *prcomp* function in the stats R package (scaled and centered) and visualized using the R package factoextra (v. 1.0.7) (Kassambara & Mundt, 2016). The R package ggplot2 was used to plot the PCA coordinates for final visualization (v. 3.4.4) (Wickham, 2016). A linear discriminant analysis (LDA) was performed to assess the ability to properly assign individuals in a given population using the R package MASS (v. 7.3.60) (Venables & Ripley, 1997). The data were first scaled, then 134 individuals were randomly selected to train the linear discriminant model (~30% of the total data). *T*-tests accounting for unequal variance were performed on the means of meristic counts, followed by Bonferroni *p*-value correction, excluding above and below lateral line scale rows because transverse scale rows encompass these counts. These tests were performed using the *t.test* and *p.adjust* functions in the stats R package (v. 4.4.1).

Results

Phylogenomic inference, population genetic analyses, divergence time estimates, and genomic species delimitation

The maximum-likelihood phylogeny inferred from the concatenated ddRAD-loci resolves a monophyletic *P. evides* species complex and five reciprocally monophyletic groups in the complex (bootstrap support = 100, Figure 2A): *P. cf. evides* from the upper Tennessee River (UT), *P. cf. evides* from the lower Tennessee River (LT), *P. evides sensu stricto* (*s.s.*) from the Ohio River system, *P. cf. evides* from the Missouri, Meramec, and upper Mississippi River (MMM) systems, and *P. cf. evides* from the White and St. Francis Rivers (WSF). The phylogeny resolves a sister relationship between *P. evides* (*s.s.*) and the species present in the Interior Highlands and upper Mississippi River system, rendering the populations distributed in the Eastern Highlands paraphyletic (Figure 2A). Lineages from the upper and lower Tennessee River resolve as successive sister lineages to all other populations of the *P. evides* complex; therefore, populations distributed in the Tennessee River system do not resolve as a clade (Figure 2A).

Cross-entropy validation analysis prior to the sNMF analysis identified five genomic groups as the optimal number of distinct genotypic clusters ($K = 5$). Under the $K = 5$ scenario, genomic clusters are consistent with the five lineages resolved in the maximum-likelihood analysis of the

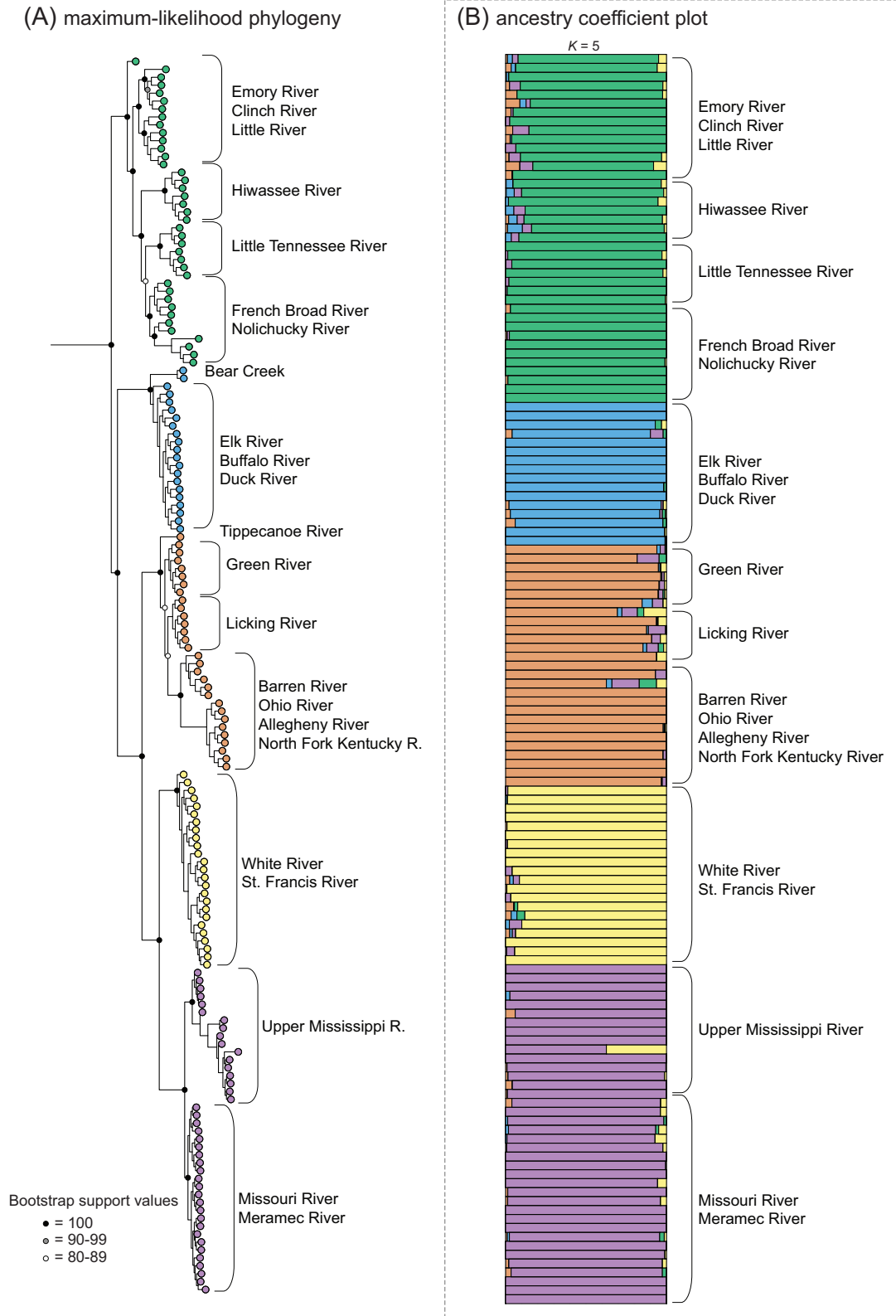


Figure 2. (A) Maximum-likelihood inferred phylogeny of the *Percina evides* species complex, outgroups *Percina vigil*, and *P. copelandi* omitted from figure. (B) sNMF bar plot of the *Percina evides* complex for five lineages.

concatenated ddRAD dataset and display little to no admixture (Figure 2A, B).

The fixation index (F_{ST}) was highest (0.52) between *P. cf. evides* (LT) and *P. cf. evides* (MMM) and lowest (0.34) be-

tween the lineages in the Interior Highlands, *P. cf. evides* (WSF) and *P. cf. evides* (MMM) (Table 1). The F_{ST} values indicate that gene flow is rare among species of the *P. evides* complex. The magnitude of F_{ST} values among species of the

Table 1. Fixation index (F_{ST}) pairwise calculations for five lineages in the *Percina evides* complex.

	<i>Percina cf. evides</i> Lower Tennessee River	<i>Percina cf. evides</i> Missouri, Meramec, and Upper Mississippi Rivers	<i>Percina evides</i>	<i>Percina cf. evides</i> Upper Tennessee River
<i>Percina cf. evides</i> Missouri, Meramec, and Upper Mississippi Rivers	0.52			
<i>Percina evides</i>	0.44	0.42		
<i>Percina cf. evides</i> Upper Tennessee River	0.35	0.45	0.36	
<i>Percina cf. evides</i> White and St. Francis Rivers	0.49	0.34	0.38	0.42

Note. F_{ST} was calculated using Weir and Cockerham (1984) methodology.

P. evides complex is within the range calculated for 12 pairs of darter sister species (Ghezelayagh et al., 2025).

Phylogenetic relationships within the *P. evides* complex inferred in the SVDquartets species tree are congruent with those in the concatenated ddRAD-loci maximum-likelihood phylogeny (Figure 3A). The SNAPP species tree, alternatively, resolves the two species in the Tennessee River as a clade. The SNAPP species tree and strict clock analysis estimate the age of the MRCA of the *P. evides* complex as 2.51 Ma (95% HPD: 1.76–3.25 Ma; Figure 3C). The age of the MRCA of upper and lower Tennessee River lineages is 1.98 Ma (95% HPD: 1.41–2.59 Ma; Figure 3C). The age of the MRCA of the *P. evides* and the Interior Highlands and upper Mississippi system species is 1.85 Ma (95% HPD: 2.39–1.28 Ma; Figure 3C). The age of the MRCA of *P. cf. evides* (WSF) and *P. cf. evides* (MMM) is 0.98 Ma (95% HPD: 0.68–1.27 Ma; Figure 3C). The age of the MRCA of *P. cf. evides* (MMM) is estimated as 28 Ka (95% HPD: 15–39 Ka) (Figure 3B).

We estimated *gdi*, which provides an estimate of the degree of genetic differentiation due to isolation and gene flow (Jackson et al., 2017), under a five-species model. Values of *gdi* below 0.2 are consistent with populations within a species, whereas values above 0.7 are expected for distinct species (Jackson et al., 2017). Values of *gdi* between 0.2 and 0.7 may still represent distinct species that are supported by additional lines of evidence, such as morphology, behavior, or ecology (Jackson et al., 2017). Resulting *gdi* values of the *P. evides* complex are moderate to high for all five lineages (between 0.5 and 0.8) (Figure 3D).

Meristic analyses

Variation in meristic traits was visualized through PCA. The first two principal components (PCs) account for 61.7% of the variation (Figure 4A). *Percina cf. evides* (WSF) overlaps almost entirely in PC-space with *P. cf. evides* (MMM) (Figure 4A), and LDA correctly identified 47% of *P. cf. evides* (WSF) from all other lineages (Table 2). LDA correctly identifies 74% of *P. evides*, 75% of *P. cf. evides* (UT), and 84% of *P. cf. evides* (LT) and *P. cf. evides* (MMM). Overlap in PC-space and incorrect assignment by LDA is observed in lineages that all exhibit low or high meristic counts. Significant differences between the means for lateral line scale rows, transverse scale rows, caudal peduncle scale rows, dorsal fin spines, dorsal fin rays, anal fin rays, and pectoral fin rays were significant ($p < .05$) in 57 out of 70 comparisons following Bonferroni correction (Tables S11–S17).

Discussion

Glaciation as a driver of speciation

The diversification of the *P. evides* complex has been profoundly impacted both directly by Pleistocene glaciation and indirectly by associated environmental changes. The relationship between glaciation and the origin of the species-rich fish fauna of the Central Highlands in eastern North America has been debated. One hypothesis attributes the species' disjunct distributional patterns to advancing glaciers that created environmental conditions that facilitated dispersal east to west, or vice versa, across the Mississippi River (Mayden, 1987b, 1988). Lineage diversification, in this case, would have occurred following dispersal and isolation during the Pleistocene. Conversely, based on congruent and replicated patterns of speciation, the Central Highlands Vicariance Hypothesis (CHVH) proposes that patterns of diversity existed prior to the Pleistocene throughout a continuous highland province (Mayden, 1988). The CHVH also predicts that lineages or species in the Ohio River system and the Interior Highlands will share a more recent common ancestor due to pre-Pleistocene connectedness via the Teays-Mahomet River (Figure 1; Mayden, 1988). The age of the MRCA of the *P. evides* complex is 2.51 Ma (95% HPD: 1.76–3.25 Ma), temporally congruent with the Pliocene–Pleistocene transition at 2.58 Ma. Our phylogenetic analysis resolves *P. evides* from tributaries and the mainstem of the Ohio River as sister to *P. cf. evides* in the Interior Highlands–upper Mississippi River system (Figure 2A), matching the predictions based on the connectedness of pre-Pleistocene river systems proposed by Mayden (1988). The age of the MRCA of *P. evides* and *P. cf. evides* in the Interior Highlands–upper Mississippi River system is estimated as 1.85 Ma (95% HPD: 2.39–1.28 Ma; Figure 3C), roughly corresponding to the rearrangement of the Teays River system approximately 2 Ma (Granger et al., 2001). The phylogenetic relationships of the *P. evides* complex are thus consistent with a geographically widespread common ancestor prior to the onset of Pleistocene glaciation, followed by allopatric speciation resulting from glacial advance that fragmented river networks, isolating ancestral populations (Figures 1 and 2A).

The Tennessee River system, unlike the Teays-Mahomet River, was not directly disrupted by glacial processes; however, it was likely indirectly affected by climate changes associated with glacial cycles, which may have influenced habitat connectivity. Throughout the Pleistocene, water and sediment fluxes in rivers throughout southeastern North

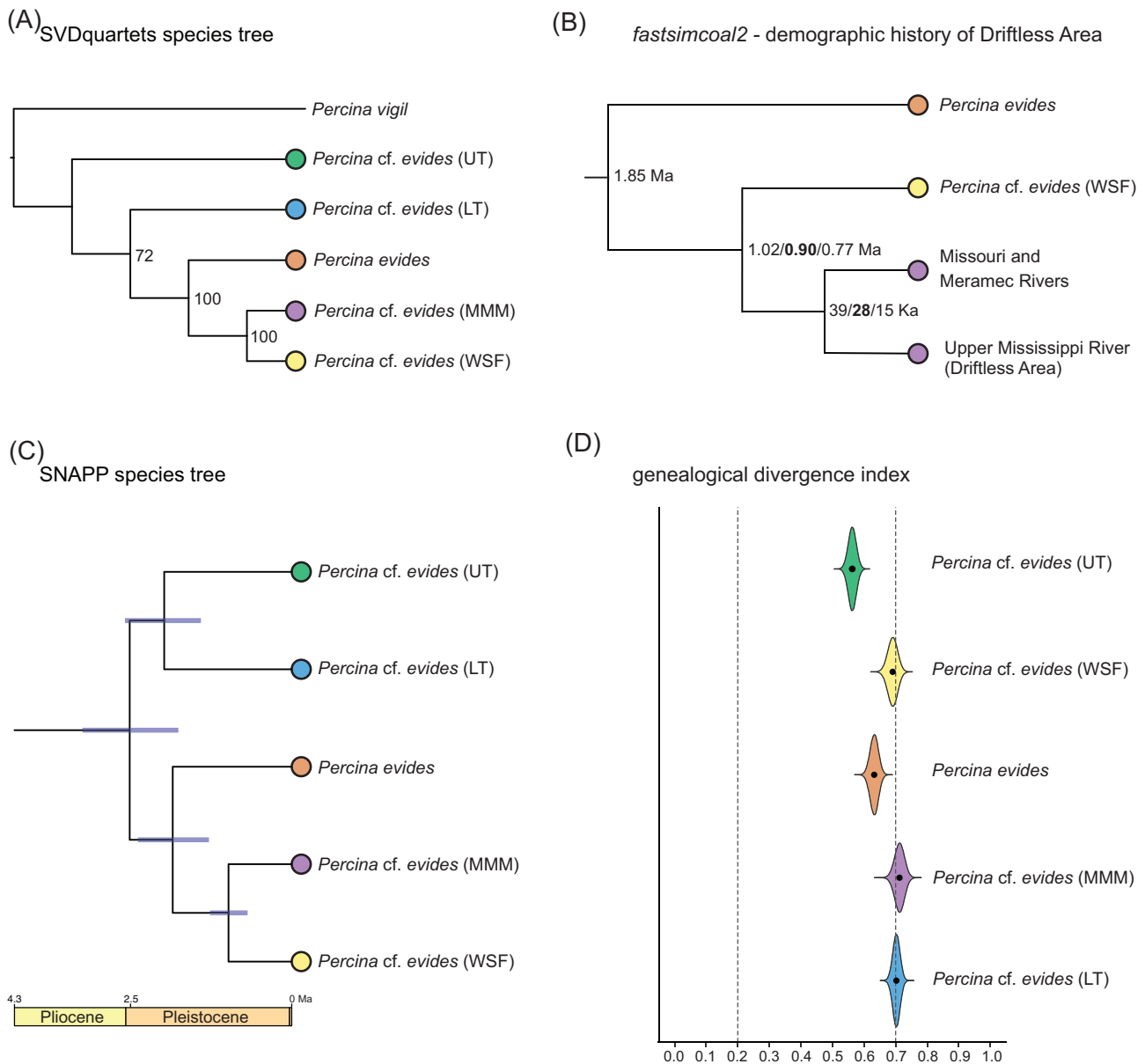


Figure 3. (A) SVDquartets species tree. (B) Demographic history reconstruction of *Percina cf. evides* in the Driftless Area. Crown age constrained to 1.85 Ma. Upper bound of 95% HPD, mean age estimate in bold, and lower bound of 95% HPD, respectively, are reported for each node. (C) SNAPP species tree calibrated with divergence time estimates provided by Near and Keck (2013). Horizontal bars represent the 95% HPD for each node. Outgroup *Percina vigil* omitted from figure. (D) Plot of genealogical divergence index values for the *Percina evides* species complex for five lineages.

America fluctuated as evidenced by river terraces and fluvial sediments stranded in caves (Anthony & Granger, 2007; Granger et al., 2001; Rittenour et al., 2007). During glacial periods, periglacial weathering processes may have increased sediment supply to the Tennessee River (Marshall et al., 2021), leading to sediment aggradation and an increase in turbidity. During interglacial periods, reduced sediment supply and increased precipitation rates may have led to less sediment transport, clearer water, and the exposure and incision of the channel bed (Rittenour et al., 2007; Wickert et al., 2019). These fluctuations between sediment aggradation and river incision may have influenced habitat associated with the *P. evides* complex, which is characterized by clear water with permanent flow and substrates composed of gravel or rubble (Etnier & Starnes, 1993; Hatch,

1985; Robison & Buchanan, 2020; Trautman, 1981). For example, fluvial sediment stranded in caves in the Green River suggests a notable period of river aggradation 2.3–2.4 Ma followed by episodes of river incision approximately 2.0, 1.5, and 1.2 Ma, and aggradation 0.7–0.8 Ma (Granger et al., 2001). Additionally, cave sediments stranded above the Cumberland River system indicate a period of incision 2.4–2.5 Ma (Anthony & Granger, 2007). While less is known about the geologic history of river incision and aggradation in the lower Tennessee River, it may be similar to that of the Green and Cumberland Rivers (Odom & Granger, 2022). The divergence between the two *P. evides* lineages in the Tennessee River is estimated at 1.98 Ma, coinciding with a hypothesized episode of river incision at 2 Ma (Odom & Granger, 2022). Waves of bedrock erosion in

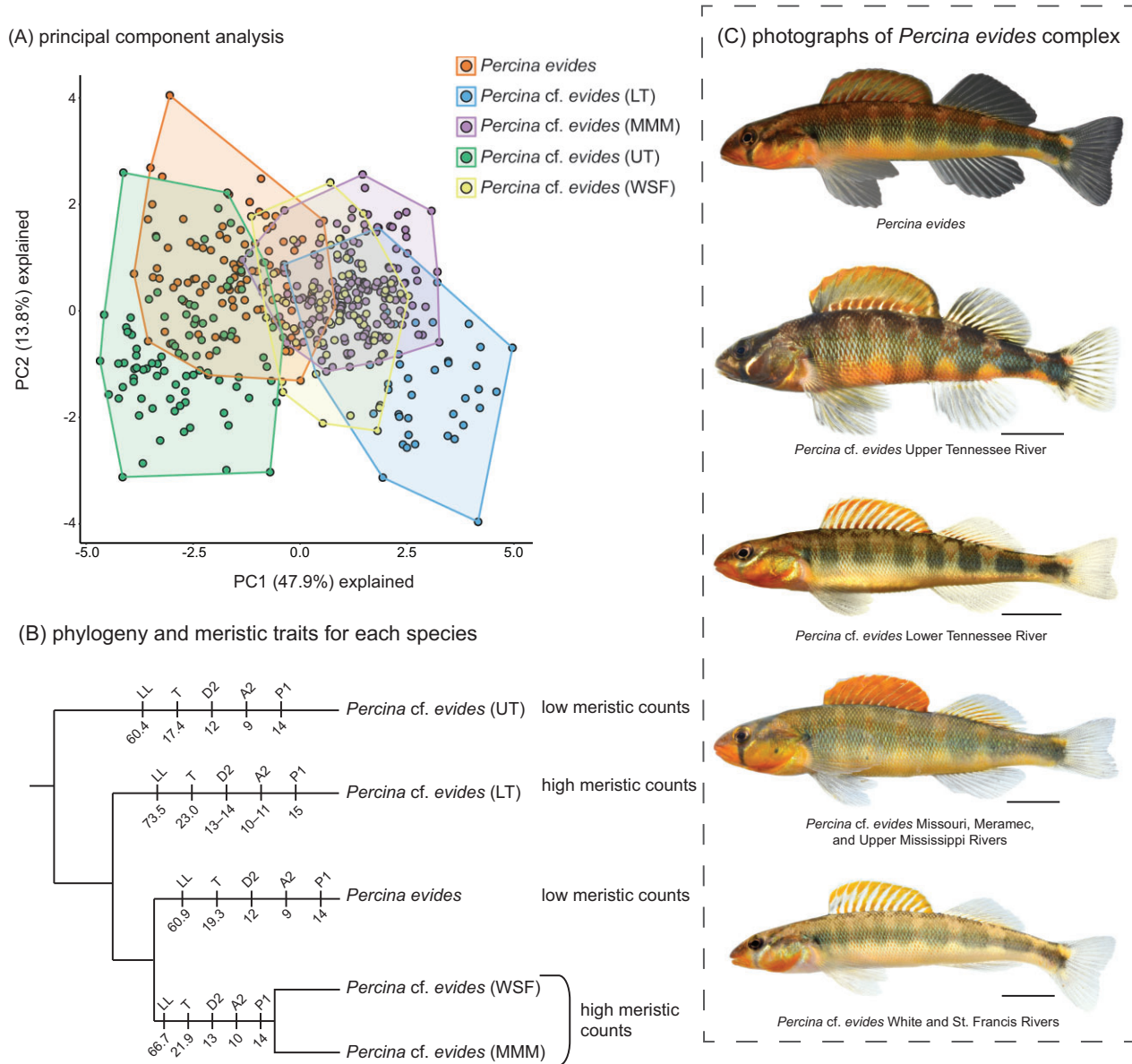


Figure 4. (A) Principal component analysis of meristic trait data of the *Percina evides* species complex. (B) Phylogeny of the *Percina evides* species complex with meristic trait data plotted for the corresponding lineage. Lateral line and transverse scales are the mean value; the mode is reported for all other traits. (C) Species of the *Percina evides* complex. *Percina evides* photograph courtesy Brian Zimmerman. Each black horizontal bar is 1 cm; the standard length of *Percina evides* is unknown.

the Tennessee River likely did not propagate upstream of the resistant Muscle Shoals, Alabama, knickpoint (Odom & Granger, 2022). Thus, the Muscle Shoals knickpoint may have been a more formidable barrier during periods of river incision when the gradient of the river was steeper. The 36-mile Muscle Shoals section of the Tennessee River (Burgess, 1916) corresponds to the geographic break between *P. cf. evides* in the upper and lower Tennessee River. Alternatively, periods of sediment aggradation may have made the main-stem Tennessee River less preferable to species of the *P. evides* complex. A lack of detailed information on the history of local changes in fluvial habitat in the Tennessee River precludes distinguishing between these scenarios, but both possibilities illustrate how glacial cycles may have contributed to a lack of gene flow between populations and eventual al-

lopatric speciation in river systems south of the glacial maximum.

Results of phylogenetic and demographic analysis of *P. cf. evides* in the upper Mississippi system provide supporting evidence for the hypothesis of occupation in glacial refugia followed by post-glacial range expansion. The boundaries of the Driftless Area have been debated, but are currently considered to include western Wisconsin and the northwestern tip of Illinois, where there is no evidence of glaciation during the Quaternary (Carson et al., 2023; Hobbs, 1999; Knox, 2019). Populations of *P. cf. evides* in the upper Mississippi River system form a reciprocally monophyletic group (Figure 2A), suggesting the Driftless Area as a potential Pleistocene glacial refugium. *Fastsimcoal2* demographic coalescent analysis estimates the age of the MRCA of the lineages

Table 2. Linear discriminant analysis of meristic trait data of the *Percina evides* complex when treating the complex as five lineages.

	<i>Percina cf. evides</i> Lower Tennessee River	<i>Percina cf. evides</i> issouri, Meramec, and Upper Mississippi Rivers	<i>Percina evides</i>	<i>Percina cf. evides</i> Upper Tennessee River	<i>Percina cf. evides</i> White and St. Francis Rivers
<i>Percina cf. evides</i> Lower Tennessee River	38	2	0	0	7
<i>Percina cf. evides</i> Missouri, Meramec, and Upper Mississippi Rivers	3	138	4	0	28
<i>Percina evides</i>	1	3	66	21	0
<i>Percina cf. evides</i> Upper Tennessee River	0	0	17	71	0
<i>Percina cf. evides</i> White and St. Francis Rivers	3	22	2	3	31
% predicted correctly	84	84	74	75	47

Note. Values are the number of individuals for each predicted assignment.

distributed in the upper Mississippi River system and the Missouri River as 28 Ka (95% HPD: 15–39 Ka, Figure 3B), roughly corresponding to the earliest estimates of the Local Last Glacial Maximum (Clark et al., 2009) and predating Wisconsin Glacial retreat (Carson et al., 2023). Populations upstream of the Driftless Area, such as in the St. Croix and Chippewa Rivers, are likely a result of post-glacial range expansion of the ancestral population that was isolated in the Driftless Area. Single specimens of *P. cf. evides* were collected in the late 19th century in the Des Moines and Cedar Rivers in Iowa and in the early 20th century from the lower Rock River in Illinois (Denoncourt, 1969; Metzke et al., 2022; Smith, 1979), which are located between the Missouri River and the upper Mississippi River system (Figure 1).

Comparative phylogeography of the Central Highlands

Researchers have proposed both vicariant and dispersal events as drivers for the current distributions of fish species with disjunct ranges across the Central Highlands (Berendzen et al., 2008a; Near et al., 2001; Simons, 2004). However, the level of support for each mechanism varies among species, rendering a single explanation or event insufficient to describe biogeographic patterns. For instance, the slender chubs (*Erimystax*), a clade of minnows native to eastern North America, diversified far prior to the Pleistocene in the Miocene, and their geographic distribution is explained both by vicariance among widely distributed species and dispersal events across the Mississippi River and into previously glaciated regions (Simons, 2004). Similarly, the Bigeye Chub (*Hybopsis amblops*) species complex initially diversified prior to the Pleistocene and shows evidence of post-glacial range expansion (Berendzen et al., 2008a). Additionally, species complexes like the Studfish (*Fundulus catenatus*) exhibit divergence dating to the Miocene, with current distributions shaped by both vicariant and more recent dispersal processes (Fast et al., 2025; Hundt et al., 2016). In contrast, the Banded Darter (*Etheostoma zonale*) has a history of significant divergence that commences around the Plio-Pleistocene transition (Halas & Simons, 2014), in discordance with expectations of the CHVH that predicts lineage divergence pre-dates the Pleistocene (Mayden, 1987b, 1988). The Hellbender (*Cryptobranchus alleganiensis*) has a disjunct geographic distribution in the Central Highlands potentially resulting from his-

torical connectivity via the Teays-Mahomet River (Sabatino & Routman, 2009). Akin to the *P. evides* species complex, several of these complexes—*H. amblops*, *E. zonale*, and *C. alleganiensis*—exhibit lineage diversification approximately 2 Ma, during the Pleistocene epoch (Berendzen et al., 2008a; Halas & Simons, 2014; Hime et al., 2021), suggesting that direct and indirect impacts of glaciation had a common impact across different taxa. Similar to species of the *P. evides* complex, both *H. amblops* and *C. alleganiensis* prefer clear, cool, highly oxygenated streams, which were likely disrupted by Pleistocene climate changes, contributing to their contemporary distribution (Berendzen et al., 2008a; Hime et al., 2021; Sabatino & Routman, 2009).

A tenet of the CHVH is that Pleistocene glaciation was not instrumental in driving speciation in Central Highlands fishes, except for between Interior Highland regions that were dissected by the Arkansas River, and that lineage diversification occurred prior to the Pleistocene (Mayden, 1987b, 1988). In contrast, the results of previous phylogeographic studies incorporating molecular clock-derived age estimations (Berendzen et al., 2008a; Halas & Simons, 2014; Hime et al., 2021) and those presented here suggest glacial cycles and associated effects on fluvial environments during the Pleistocene had a significant impact on the speciation and diversification of freshwater organisms present in the Central Highlands. The contrasting timing of speciation across different freshwater taxa demonstrates that a single dispersal or vicariant event cannot explain the shared biogeographic pattern and underscores the importance of studies focused on individual species complexes that can illuminate differences in drivers of allopatric speciation. Our results highlight how using temporal data for testing hypotheses about the origin of shared biogeographic patterns can help test for pseudo-congruence (Donoghue & Moore, 2003; Near & Keck, 2005), where similar patterns are a result of processes occurring at different times.

Discovery of biodiversity

The advancement of molecular data collection methods and the multispecies coalescent model has revolutionized species delimitation (Fujita et al., 2012; Ghezelayagh et al., 2025; Harrington & Near, 2012; Leaché et al., 2019; Rannala, 2015; Sites & Marshall, 2003). Because species are the fundamental unit of most biodiversity studies and conservation practices, it is imperative that species delimitation and

description use an integrative approach combining morphological and molecular data to most accurately describe species-level biodiversity (Taylor et al., 2021; Yan et al., 2018).

Our phylogenetic and population structure analyses support five species within the *P. evides* complex. Analyses of traditional meristic data are fairly robust for delineating species of the *P. evides* complex; however, LDA does not discern specimens of *P. cf. evides* (WSF) from other lineages (Table 2B). Species in the upper Tennessee and Ohio rivers show less disparity in meristic traits, as reflected by overlap in PC-space (Figure 4A; Tables S2–S10) but do not share common ancestry relative to other species in the complex (Figures 2A and 4B). Thus, the overlap in morphological traits among the species in the *P. evides* complex does not reflect their phylogenetic relationships (Figures 2A and 4B). There is minimal overlap between the sister species pair of *P. evides s.s.* distributed in the Ohio River system and *P. cf. evides* distributed in the Interior Highlands-upper Mississippi River system (Figure 4A). The overall pattern of overlap in meristic PC-space for the species complex is similar to several other closely related species of darters (Kozal et al., 2017; Layman & Mayden, 2009, 2012; Near et al., 2017, 2021; Wood et al., 2023). Variation in coloration of nuptial males is often used to diagnose closely related species of darters (e.g., Bauer et al., 1995; Layman & Mayden, 2012; Page et al., 1992). Variation in the *P. evides* complex has been previously noted (Denoncourt, 1969), where the form in the Upper Tennessee (excluding Clinch and Holston River systems) is characterized by bars on the second dorsal and caudal fin, the western form is characterized by red-orange pigment restricted to the lower half of the head, and all other populations are characterized by orange or orange-copper pigment present on lower half of entire body. There is variation between the species delimited here, which may serve as diagnostic features, but more thorough characterization is required. *Percina evides (s.s.)* and *P. cf. evides (UT)* breeding males are similar and display turquoise-teal lateral blotches, and a tricolor first dorsal fin. *P. cf. evides (UT)* breeding males often exhibit dark brown heads. Although *P. cf. evides (MMM)* and *P. cf. evides (WSF)* exhibit low disparity in meristic traits, they can be distinguished by the presence of a fully orange first dorsal fin in *P. cf. evides (MMM)* compared to clear panes along the first dorsal fin spines in *P. cf. evides (WSF)*.

Here, we delimit five distinct species in the *P. evides* complex that are characterized morphologically with traditional meristic traits, a class of data ichthyologists have used to delimit and describe species for more than 100 years (Moenkhaus, 1894). Our delimitation of five species in the *P. evides* complex deploys a robust analysis of genomic data coupled with an examination of traditional morphological characters, instead of relying solely on delimiting methods that are prone to species over splitting (Sukumaran & Knowles, 2017). The *P. evides* complex includes (1) *P. evides* distributed throughout the mainstem of the Ohio River and its tributaries upstream of the confluence with the Cumberland River, (2) *P. cf. evides* distributed in tributaries of the Tennessee River system upstream of the Tennessee River Gorge around Chattanooga, Tennessee, (3) *P. cf. evides* distributed in the lower Tennessee River system in the Elk River, Shoal Creek, Bear Creek, and the Duck River systems, (4) *P. cf. evides* in the Missouri, Meramec and upper Missis-

sippi River system, and (5) *P. cf. evides* in the White and St. Francis Rivers (Figures 1 and 2). We define these lineages as species under the unified general lineage species concept, and treat results from genomic species delimitation as a hypothesis with additional validation provided by morphological analysis (de Queiroz, 2007; Sukumaran & Knowles, 2017).

Despite nearly two centuries of scientific exploration of the diversity of North American freshwater fishes, a significant portion of species diversity—an estimated 10%—remains undescribed (April et al., 2011). The integration of morphological and molecular data offers a powerful approach for the discovery and delimitation of species diversity. Additionally, many widespread species contain unrecognized or undescribed diversity, and comprehensive geographic sampling is paramount for accurate delimitation (Berendzen et al., 2008a; Halas & Simons, 2014; Harrington & Near, 2012; Hundt et al., 2016; Kim & Conway, 2014; Kim et al., 2022; Layman & Mayden, 2012; MacGuigan et al., 2023; Nagle & Simons, 2012; Piller et al., 2008; Schönhuth et al., 2012; Wood et al., 2023). The ongoing process of discovery and description of North American freshwater fish diversity represents a dynamic field of research, underscoring that our understanding of aquatic biodiversity continues to evolve. Each new study has the potential to uncover previously unrecognized species, challenge existing taxonomic classifications, and provide deeper insights into the richness and complexity of evolutionary mechanisms responsible for the high species richness that characterizes the eastern North American aquatic biodiversity hotspot.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

Data are available on Dryad (<https://doi.org/10.5061/dryad.8gtht7701>) and NCBI SRA (BioProject ID: PR-JNA1214323).

Author contributions

J.E.W. collected ddRAD data and performed phylogenetic and morphological analyses. A.T. collected morphological data. D.K. performed genetic analyses of species delimitation and population demography. M.F.S. collected ddRAD data. J.E.W. and T.J.N. wrote the first draft with revisions from A.T., D.K., and M.F.S.

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Conflict of interest

The authors have no known conflicts of interest to report.

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