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Intraspecific phylogeography of *Percina evides* (Percidae: Etheostomatinae): an additional test of the Central Highlands pre-Pleistocene vicariance hypothesis

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Abstract

North America exhibits the most diverse freshwater fish fauna among temperate regions of the world. Species diversity is concentrated in the Central Highlands, drained by the Mississippi, Gulf Slope and Atlantic Slope river systems. Previous investigations of Central Highlands biogeography have led to conflicting hypotheses involving dispersal and vicariance to explain the diversity and distribution of the freshwater fish fauna. In this investigation predictions of the Central Highlands pre-Pleistocene vicariance hypothesis are tested with a phylogeographic analysis of the percid species *Percina evides*, which is widely distributed in several disjunct areas of the Central Highlands. Phylogenetic analysis of complete gene sequences of mitochondrially encoded cytochrome *b* recover three phylogroups, with very low levels of sequence polymorphism within groups. The two western phylogroups are monophyletic with respect to the eastern phylogroup. The recovery of two monophyletic lineages with an eastern and western distribution in the disjunct highland areas is a pattern expected from vicariance, but is not predicted by the Central Highlands pre-Pleistocene vicariance hypothesis. The recovery of very limited mitochondrial DNA polymorphism and lack of phylogeographic structuring across the entire range of the eastern clade, very shallow polymorphism between the disjunct Missouri River and upper Mississippi River populations, and lack of sequence polymorphism in the upper Mississippi River populations, support a hypothesis of dispersal during or following the Pleistocene. The present distribution of *P. evides* is best explained by both vicariant and dispersal events.

Keywords: cytochrome *b*, darters, historical biogeography, mitochondrial DNA, Percidae, phylogeography

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Introduction

Among temperate regions of the world, North America contains the greatest diversity of freshwater fishes. Much of this diversity is concentrated in the eastern half of the continent in the Central Highlands Region. The Central Highlands contain two disjunct upland areas, the Eastern Highlands (Appalachian Mountains) and the Interior Highlands (Ozark and Ouachita mountains), which are interrupted by the Central Lowlands (Mayden 1988). Three major drainage systems are contained in these areas, the Mississippi (including the Ohio, Tennessee and Missouri river drainages), the Atlantic Slope, and the Mobile Basin and Gulf of Mexico drainages. Distributional data for freshwater fish species in the Central Highlands are robust, making the region a prime candidate for intensive comparative phylogeographic investigations.

Initially, the origin and mode of speciation of the fishes in these highland areas involved hypotheses concerning centres of origin and dispersal. It was assumed that most species evolved in the Eastern Highlands and dispersed, from east to west, across the Central Lowlands to the Interior Highlands during the Pleistocene. This is referred to as the Central Highlands-Pleistocene dispersal hypothesis. Alternatively, Mayden (1985, 1987a, 1987b, 1988) and Wiley & Mayden (1985) proposed that pre-Pleistocene vicariance

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was largely responsible for the speciation and extant distribution of Central Highland fishes. The Central Highlands pre-Pleistocene vicariance hypothesis assumes a previously vast highland region that was, prior to the Quaternary, drained by the ancient Old Mississippi and Teays-Mahomet River systems (Mayden 1988). This region contained a diverse fauna that was fragmented by Pleistocene glaciation (Mayden 1985, 1988; Wiley & Mayden 1985). Thus, several species and clades which are found collectively in the Eastern Highlands, Interior Highlands and the upper Mississippi are hypothesized to represent remnants of pre-Pleistocene distributions (Mayden 1985, 1988). There are several phylogeographic predictions of the Central Highlands pre-Pleistocene vicariance hypothesis (Mayden 1988). For example, populations distributed in tributaries of the former Teays River drainage will form a monophyletic lineage. Therefore, populations of species widely distributed in the modern Ohio River drainage should not form a monophyletic group, as populations from former Teays River tributaries (upper Ohio) will be more closely related to populations from the Interior Highlands and upper Mississippi River drainage than to populations from tributaries that were not a part of the Teays River (lower Ohio). Populations in the upper Mississippi River drainage are expected to be most closely related to populations in the Interior Highlands (Mayden 1988).

Strange & Burr (1997) critically examined the pre-Pleistocene vicariance hypothesis using mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) variation. They examined the phylogeography of five clades of Central Highland fishes and attempted to place a temporal dimension to cladogenic events in the study species. Of the five clades examined, three (*Erimystax dissimilis*, *Percina* subgenus *Odontopholis* and *Etheostoma* subgenus *Litocara*) were phylogeographically and temporally consistent with the Central Highlands pre-Pleistocene vicariance hypothesis. The two remaining clades (*Cottus carolinae* and *Fundulus catenatus*) were characterized by weak phylogeographic structuring and low levels of inferred mtDNA sequence polymorphism, a pattern consistent with recent dispersal between the Eastern and Interior Highlands (Strange & Burr 1997).

In this investigation we attempt to provide an additional test of the Central Highlands pre-Pleistocene vicariance hypothesis with a phylogeographic analysis of the gilt darter, *Percina evides*, which is widely distributed in disjunct areas among the upper Mississippi, Interior Highlands and Eastern Highlands (Becker 1983; Page 1983). *P. evides* is found in the Missouri, Meramec, St. Francis and White river drainages in the Ozark Mountains of the Interior Highlands and in the St Croix, Chippewa and Black rivers in the upper Mississippi River drainage. East of the Mississippi River, *P. evides* is distributed throughout the Eastern Highlands in several tributaries of the Ohio River and in the lower and upper Tennessee River. Populations of *P. evides* in Illinois, Iowa and Ohio are thought to be extirpated (Denoncourt 1969; Smith 1979; Trautman 1981; Harlan *et al*. 1987).

This analysis utilizes complete gene sequences of the mitochondrially encoded cytochrome *b* to test two predictions of the Central Highlands pre-Pleistocene vicariance hypothesis. The predictions are: (i) disjunct populations of *P. evides* form distinct phylogroups; and (ii) populations of *P. evides* in the rivers of the Central Lowlands (e.g. upper Mississippi and Tippecannoe rivers) and former Teays tributaries (e.g. Kentucky River) are more closely related to populations from the Interior Highlands than to populations in the lower Ohio River drainage. Comparing the estimated phylogeography with these predictions permits an assessment of the Central Highlands pre-Pleistocene vicariance hypothesis in explaining the present distribution of *P. evides* (Mayden 1985, 1988; Wiley & Mayden 1985).

Materials and methods

Fishes were collected from several localities throughout the range of *Percina evides* using a minnow seine and backpack electroshocker. When possible, voucher specimens were deposited in the Illinois Natural History Survey (INHS) Fish Collection or the University of Alabama Ichthyology Collection (UAIC). Drainage groups, collection localities and catalogue number for vouchered specimens are as follows: (**Tennessee Drainage**) Little Bear Creek Franklin Co. AL, INHS 45577; Clinch River Grainger Co. TN, UAIC 10530.17; North Fork French Broad River Transylvania Co. NC, UAIC 7954.16; Nolichucky River Green Co. TN, UAIC 9826.20; Little River Blount Co. TN, UAIC 8444.07; (**Ohio Drainage**) Licking River Bath Co. KY, no voucher; Tippecanoe River Fulton Co. IN (2), UAIC 10314.14; Green River Green Co. KY, INHS 6410; Middle Fork Kentucky River Lee Co KY, no voucher; (**White Drainage**) Current River Ripley Co. MO, UAIC 10786.10; White River Independence Co. AR, UAIC 11362.18; Spring River Sharp Co. AR, UAIC 10064.22; (**Meramec Drainage**) Big River Jefferson Co. MO, UAIC 10356.17; (**Upper Mississippi Drainage**) St Croix River Chisago Co. MN, INHS 40661; Black River Jackson Co. WI, INHS 47466; Snake River Kanabec Co. MN, INHS 40719; (**Missouri Drainage**) Gasconade River Gasconade Co. MO, no voucher. Several specimens designated for molecular analysis were frozen whole in liquid nitrogen and were kept at −80 °C for long-term storage. Genomic DNA was isolated from 0.01–0.05 g of frozen muscle tissue using standard proteinase K digestion and phenol–chloroform extraction. Nucleic acids were precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. Precipitated DNA extractions were resuspended in 100 μL of 1× Tris–ethylenediaminetetraacetic acid solution.

The complete coding region of the mitochondrially encoded cytochrome *b* gene was amplified by polymerase chain reaction (PCR) using primers and conditions given in Near *et al*. (2000). PCR products were purified using the Qiagen QIAquick kit and used as template for Big Dye (Perkin Elmer) terminator cycle sequencing reactions following the manufacturer's recommendations. Sequencing products were cleaned of excess nucleotides via centrifugation on Sephadex columns. Sequences were read with an ABI 377 automated sequencer at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois Urbana-Champaign. Individual sequence files were edited using EDITVIEW ver. 1.0.1, and complete cytochrome *b* sequences were assembled from individual sequencing reactions using the program SEQUENCHER ver. 3.0 (Gene Codes, Ann Arbor, MI). All sequences used in this study were submitted to GenBank (AF375938–AF375955).

Sequences were aligned by hand to the percid cytochrome *b* data set of Song *et al*. (1998). Pairwise genetic distances, pairwise transition : transversion ratios, and base composition values were calculated using PAUP* 4.0 (Swofford 2000). The occurrence of multiple substitutions at a given nucleotide position (saturation) was assessed by plotting observed numbers of transitions (C ↔ T; A ↔ G) vs. observed transversions (C ↔ G; C ↔ A; T ↔ A; T ↔ G). Sequence divergences were corrected for within-group variation using the following formula: $\delta = \delta_{xy} - 0.5(\delta_x + \delta_y)$ where δ_x and δ_y are the mean sequence polymorphism between phylogroups *x* and *y* (Edwards 1997).

Phylogenetic relationships were estimated using both maximum-parsimony and maximum-likelihood optimality criteria using PAUP* 4.0. Initially, all of the *P. evides* sequences were analysed along with a data set that included complete cytochrome *b* sequences from all species of *Percina* (Near in press) and representative species from nine of 10 percid genera (Song *et al*. 1998). After identifying *P. aurantiaca* as the sister species of *P. evides*, it was included with two basal species of *Percina* (*P. roanoka* and *Percina* species, undescribed) that were designated as outgroup species for all subsequent analyses.

Branch-and-bound tree searches were used for maximum-parsimony analysis. Bootstrap (2000 pseudoreplications) analysis was used to examine levels of relative support for clades. Maximum-likelihood analysis utilized the HKY85 model of nucleotide substitution, with a correction for among-site rate variation (HKY85 + Γ). This model was chosen using Akaike information criterion, as implemented in the MODELTEST ver. 2.0 computer program (Posada & Crandall 1998). Heuristic searches were used to find the topology with the best likelihood score using tree–bisection–reconnection (TBR) with steepest descent option and 20 random addition sequences. Maximum-likelihood bootstrap analysis utilized 100 pseudoreplications.

Results and Discussion

Uncorrected *p*-distances from 18 individual *Percina evides* from 17 populations ranged from 0.0000 to 0.0404 substitutions per site. Plotting numbers of transitions vs. transversions did not detect saturation of nucleotide substitutions at any codon position (plots not shown). Among the 1140 nucleotides sequenced for 18 *P. evides* specimens, 70 sites are variable and 60 of these polymorphic sites are third codon position changes. There are only two polymorphic second codon nucleotide sites. One of the second codon nucleotide polymorphic sites is found in one of two individuals sampled from the Tippecanoe River (Wabash-Ohio drainage). It is an A ↔ G transition and results in a conservative amino acid change. Four additional amino acid substitutions involve first codon position nucleotide changes. Three of these four polymorphic amino acids involve a change in a single individual; however, at amino acid 327 all individuals from the Tennessee and Ohio populations code for alanine (Ala) and all individuals from the Missouri River drainage–upper Mississippi River drainage and White River drainage populations code for threonine (Thr). This is a nonconservative change involving a shift between a hydrophobic (Ala) and hydrophilic (Thr) amino acid.

Two patterns of sequence divergence are apparent. First, mitochondrial polymorphism is shallow within three regions: (i) Ohio and Tennessee drainages (mean 0.0003 substitutions per site); (ii) Missouri River drainage–upper Mississippi River drainage (mean 0.0001 substitutions per site); and (iii) White River drainage (mean 0.0004 substitutions per site). Second, comparatively large sequence polymorphism exists between populations east and west of the Mississippi River. Divergence between the Ohio River drainage–Tennessee River drainage and White River drainage populations (mean 0.0367 substitutions per site) exceeds divergences exhibited between the Ohio River drainage–Tennessee River drainage and Missouri River drainage–upper Mississippi River drainage (mean 0.0298 substitutions per site). The individual sampled from the Meramec exhibits moderate divergence from the Missouri River drainage–upper Mississippi River drainage populations (mean 0.0077 substitutions per site). There is comparatively large sequence polymorphism between the Missouri River drainage–upper Mississippi River drainage and White River populations (mean 0.0230 substitutions per site). The large level of sequence polymorphism among *P. evides* haplotypes from different geographical areas is illustrated in a frequency histogram of pairwise sequence divergences (Fig. 1). The bimodal distribution reflects the shallow sequence polymorphism within drainage areas and the deep divergences among drainage areas.

In both maximum-parsimony (Fig. 2) and maximum-likelihood (tree not shown) analyses, the eastern and western

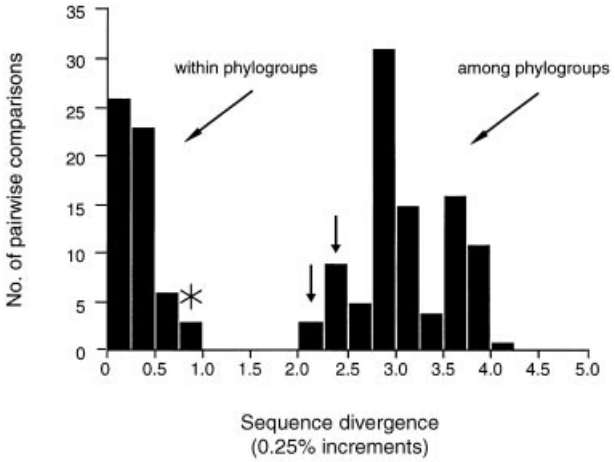

Fig. 1 Histogram of uncorrected cytochrome *b* sequence divergence among all 18 *Percina evides* sampled. Comparisons between Meramec River and Missouri river drainage–upper Mississippi River drainage populations indicated with an asterisk. Comparisons between White River drainage and Missouri River drainage–upper Mississippi River drainage populations indicated with an arrow.

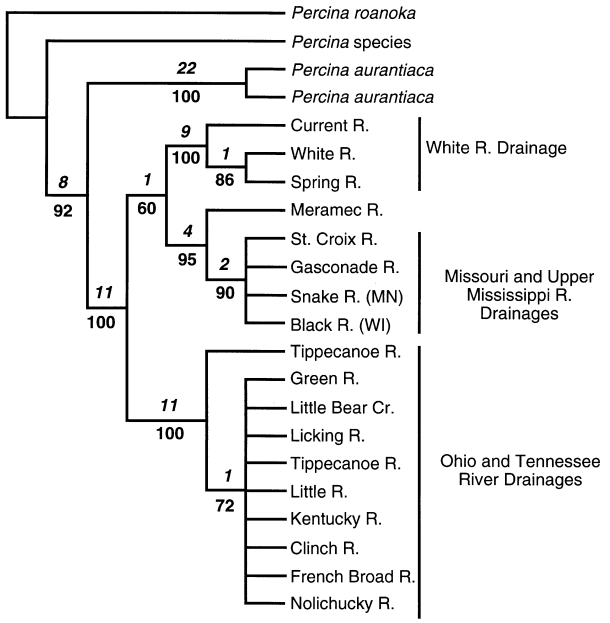

Fig. 2 Strict consensus of three most-parsimonious trees. Tree length is 363 steps and the consistency index (excluding uninformative characters) is 0.722. Numbers below nodes represent per cent recovery in bootstrap analysis (2000 pseudoreplicates). Italic numbers above nodes represent decay values. Drainage areas for *Percina evides* haplotypes are indicated to the right.

populations of *P. evides* were recovered as monophyletic lineages with moderate (60–69%) to strong (> 70%) bootstrap support. Within the eastern populations, mitochondrial haplotypes are so similar that there is no phylogenetic structuring among these populations (Fig. 3). In the western populations, the sequence polymorphism observed between the Missouri River drainage–upper Mississippi River drainage and the White River drainage populations is reflected in the recovery of each as a monophyletic group (Figs 2 and 3). The sister taxon relationship of these two lineages is supported with only 60% bootstrap recovery and a decay score of 1 (Fig. 2). The recovery of each of these two monophyletic lineages and the pairwise sequence distances between Missouri River drainage–upper Mississippi River drainage and White River drainage populations (Fig. 1) support a conclusion that each lineage is a distinct phylogroup (Fig. 3). Relationships within each of these two phylogroups are unresolved due to the lack of significant mitochondrial haplotype variation.

The observed sequence polymorphism among the disjunct populations of *P. evides* (Fig. 1) and estimated phylogeography (Figs 2 and 3) indicate both congruence and incongruence between predictions of the pre-Pleistocene vicariance hypothesis and the present distribution of *P. evides*. The recovery of three monophyletic phylogroups with substantial sequence divergence and a deep phylogeographic split between populations distributed in the eastern and western clades supports a Central Highlands pre-Pleistocene origin for this species (Fig. 3). The onset of Pleistocene glaciation may have been the vicariant event isolating the eastern and western clades, as evidenced by the observed phylogeographic structure (Figs 2 and 3). The failure to recover Ohio River populations of *P. evides* as a polyphyletic group, the lack of phylogeographic structure among the sampled upper Mississippi River populations, and the remarkable lack of sequence polymorphism between the Missouri River and upper Mississippi River populations does not support the pre-Pleistocene vicariance hypothesis (Mayden 1988) of a widespread historical distribution that is similar to the present distribution in this species. Based on the very shallow genetic distances observed and reflected in a lack of phylogenetic structure (Figs 1 and 2), post-Pleistocene dispersal of *P. evides* is hypothesized to account for much of the distribution across the eastern portion of the range (Fig. 3). The lack of haplotype polymorphism between populations of *P. evides* in the Missouri River drainage and upper Mississippi River drainage is explained by either dispersal during the Sangamonian Interglacial (Pflieger 1971), the period between the Illinoian and Wisconsinan glaciations (approximately 75 000 years b.p.), or post-Pleistocene dispersal (Denoncourt 1969) from the Missouri River Drainage in the northern Ozarks (Fig. 3). Several other species of freshwater fishes exhibit a similar disjunct distribution between the Ozark Mountains and upper Mississippi River and are hypothesized to have re-invaded glaciated regions after the Pleistocene from Ozarkian refugia (Burr & Page 1986).

This phylogeographic analysis demonstrates that mitochondrial gene sequence data provide sufficient phylogenetic resolution to discriminate among competing

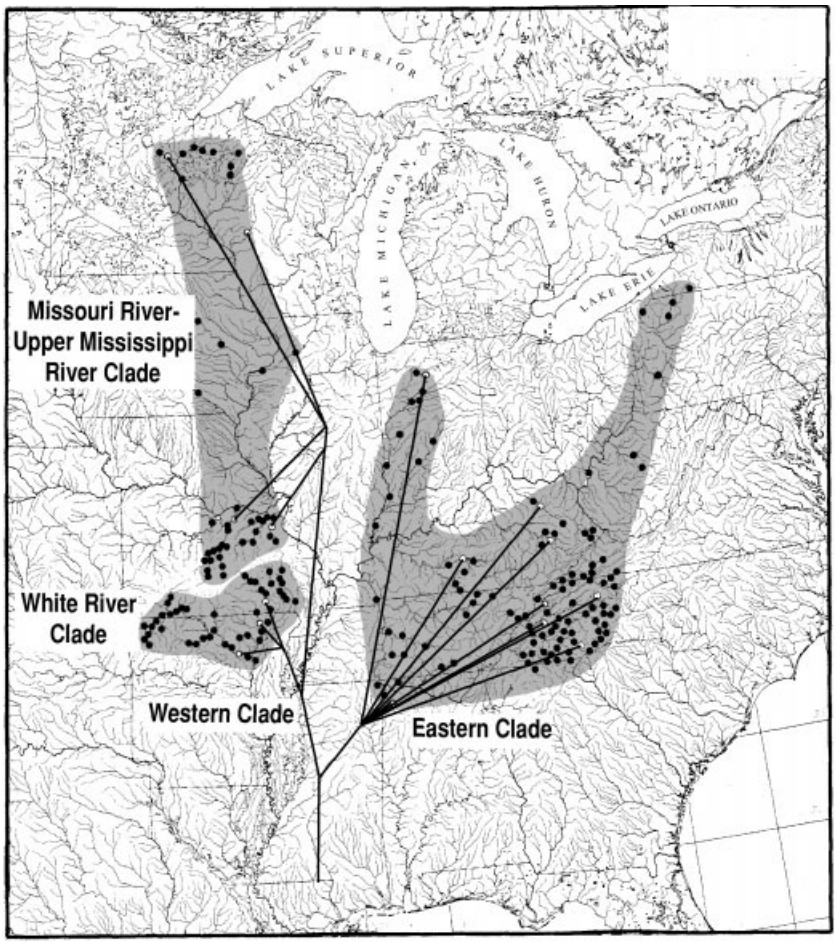

Fig. 3 Mitochondrial DNA inferred phylogeny (Fig. 2) superimposed on the *Percina evides* distribution map and collection localities. Open circles designate collection localities sampled for this study. Geographic areas containing monophyletic phylogroups are shaded.

hypotheses concerning the origin and diversification of *P. evides*. The conclusions of this investigation in relation to the Central Highlands pre-Pleistocene vicariance hypothesis are similar to conclusions presented by Strange & Burr (1997). The North American freshwater fish fauna is the product of several historical events that are not necessarily shared among all species in the Central Highlands. For example, the pre-Pleistocene vicariance hypothesis provides a robust explanation for the distribution of *P. evides* and observed mtDNA sequence polymorphism between the Interior and Eastern Highlands, but fails to explain the distribution of this species in the upper Mississippi River and within the Eastern Highlands (Fig. 3). The mtDNA polymorphism (Fig. 1) and phylogeographic relationships (Fig. 2) suggest that both vicariance and dispersal are necessary to explain the present distribution of *P. evides*. As a result of the mtDNA inferred phylogeography, a novel hypothesis to explain the origin of *P. evides* involves a synthesis of the vicariance model from the Central Highlands pre-Pleistocene hypothesis and dispersal from areas of refugia during Pleistocene glaciation. The generality of this conclusion in explaining the roles of vicariance and dispersal in the evolution of the North American Central Highlands freshwater fish fauna will be realized through the culmination of intensive phylogeographic investigations that adequately represent the extant diversity of species in this biogeographically unique region of the world.

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T.J. Near investigated the phylogenetic relationships of percid fishes for his PhD, and is currently exploring the utility of mitochondrial and nuclear gene sequences in resolving species relationships among darters in the genus *Percina*. L.M. Page has published 65 papers on the systematics and ecology of darters. He is currently revising the *Handbook of Darters* and investigating the evolution of reproductive behaviours of darters. R.L. Mayden continues to examine the systematic and biogeographic histories of North American freshwater fishes.
