

Dispersal, vicariance, and timing of diversification in *Nothonotus* darters

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Abstract

The species diversity of North American freshwater fishes is unparalleled among temperate regions of the planet. This diversity is concentrated in the Central Highlands of eastern North America and this distribution pattern has inspired different models involving either dispersal or vicariance to explain the high species diversity of North American fishes. The most popular of these models is the Central Highlands vicariance hypothesis (CHVH), which proposes an ancient and diverse widespread fauna that existed across a previously continuous highland landscape that is much different from today. The mechanisms of isolation in the CHVH involve specific instances of vicariance that affected several diverse lineages of Central Highlands fishes. We tested predictions of the CHVH and alternative models using a cytochrome *b*-inferred phylogeny of the darter clade *Nothonotus*. A Bayesian mixed-model method was used for phylogenetic analysis. The phylogenetic data set included all 20 recognized *Nothonotus* species, and most species were represented with multiple sequences. We were able to convert genetic branch lengths to absolute age using external fossil calibrations in the freshwater perciform fish clade Centrarchidae. Using a well-resolved *Nothonotus* phylogeny and divergence time estimates, we identify equal numbers of instances of both vicariance and dispersal among disjunct regions of the Central Highlands, biogeographic pseudocongruence, rather recent speciation in *Nothonotus*, and a surprisingly large amount of speciation within highland areas. With regard to *Nothonotus*, previous Central Highlands biogeographic models offer little in the way of providing possible mechanisms responsible for diversification in the clade. Patterns of speciation in *Nothonotus* are similar to those discovered in recent efforts that have included speciation as a parameter into classic models of island biogeography.

Keywords: biogeography, centrarchidae, divergence times, molecular clocks

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Introduction

The most diverse temperate freshwater fish fauna on Earth is found in North America (Briggs 1986; Lundberg *et al.* 2000). Hypotheses concerning formative events and mechanisms responsible for this exceptional diversity have focused primarily on geographical processes of speciation of fishes distributed in the disjunct regions of the Central Highlands (Wiley & Mayden 1985; Mayden 1988). A common pattern among Central Highland fishes is the presence of closely related species distributed among multiple disjunct highland regions (Pflieger 1971; Wiley & Mayden 1985; Mayden 1987;

Strange & Burr 1997). The Central Highlands include the Eastern Highlands, Interior Highlands (Ozark and Ouachita Mountains), and the upper portions of the Mobile Basin (Fig. 1), and are characterized by clear high-gradient rivers and streams with silt-free gravel or rubble substrates. Dispersal of aquatic organisms between these highland areas is thought to be restricted by intervening low land areas with larger typically silt bottomed low-gradient streams (Fig. 1).

One hypothesis explaining the pattern of geographical distribution of Central Highland fishes is the Pleistocene dispersal model, where the Eastern Highlands served as a centre of origin for many lineages that subsequently dispersed to unoccupied regions of the Interior Highlands during several different Pleistocene glaciations. Facilitating

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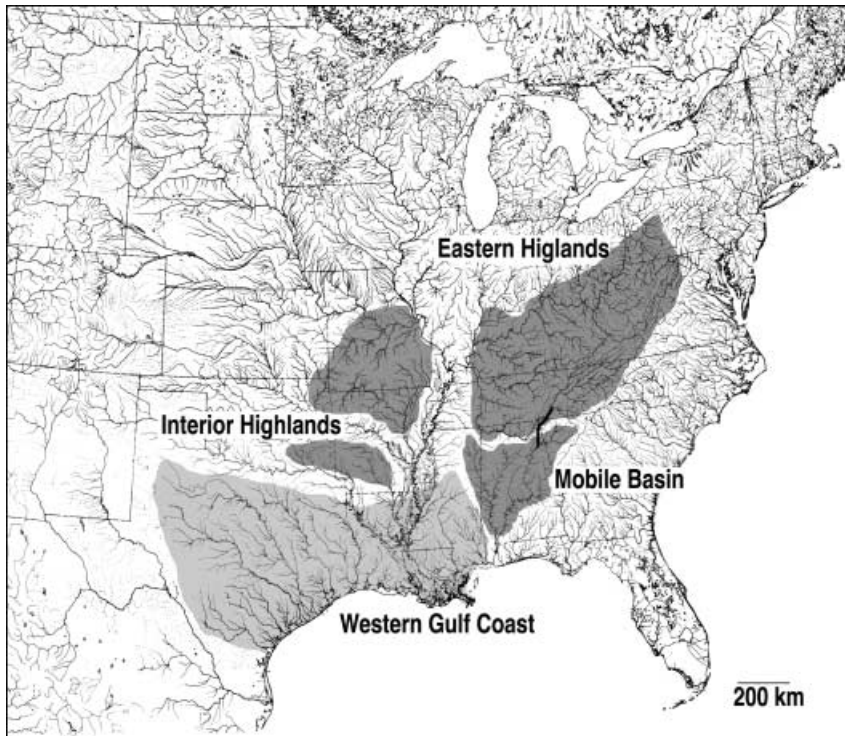


Fig. 1 Map of eastern North America showing regions discussed in the text. Disjunct areas of the Central Highlands are shaded with dark grey. A solid black line approximates the location of the hypothesized Appalachian river, connecting the Eastern Highlands with the Mobile Basin.

this dispersal were interconnecting waterways along ice sheets, or water level reductions creating continuous high-gradient stream conditions between disjunct highland regions. After the recession of Pleistocene glacial fronts, populations of species that dispersed from the Eastern Highlands were subsequently isolated in the Interior Highlands, resulting in eventual speciation (reviewed in Mayden 1987).

An alternative idea is the Central Highlands vicariance hypothesis (CHVH), which proposes vicariance as opposed to dispersal from centres of origin was the mechanism responsible for the geographical pattern of speciation exhibited among Central Highland fishes. In the model proposed by the CHVH, the present-day disjunct distribution of highland endemic fishes represents a vestige of a fauna that occupied a once widespread continuous highland region that was drained by an interconnected river system that is much different from today (Pflieger 1971; Wiley & Mayden 1985; Mayden 1987; Strange & Burr 1997). This extensive highland region and its diverse fish fauna was fragmented by Pleistocene glaciation through the destruction of highland geomorphic features and the drastic alteration of aquatic habitats (Thornbury 1965). The glacial destruction of the highland habitats in the present-day Central Lowlands, and the diversion of ancient river drainage courses resulted in the pattern of disjunct distribution of fishes and other aquatic organisms in the Interior and Eastern Highlands, and Mobile Basin (Wiley & Mayden 1985; Mayden 1987; Crandall & Templeton 1999).

These two alternative hypotheses make explicit predictions regarding the timing of speciation and biogeographic patterns in Central Highland fishes. The Pleistocene dispersal hypothesis proposes that the Eastern Highlands represent the ancestral area and the Interior Highlands were colonized from the Eastern Highlands, and the most recent common ancestor of sister species distributed in the Eastern and Interior Highlands should be no older than the initiation of glacial cycles at the Pliocene–Pleistocene interval, approximately 2.5 million years ago (Ma). In contrast, the CHVH proposes that the upper Gulf of Mexico Coastal Plain (Mobile Basin) forms the sister group to all other Central Highland-distributed species, and species or clades in the Eastern Highlands and Interior Highlands are sister groups (Fig. 2). In addition, since vicariance is the mechanism of geographical isolation in the CHVH, the age of speciation events associated with particular area relationships should correlate among groups and correspond to geomorphic Earth history events. For example, clades that exhibit a sister taxon relationship between species distributed in the Mobile Basin and the remaining highland areas are hypothesized to have a fairly ancient age, extending to the mid-Miocene, 16.4–11.2 Ma (Fig. 2). This timing is thought to correlate with the break-up of the hypothesized Appalachian river that connected the Tennessee River drainage in the Eastern Highlands with the Coosa River in the upper Mobile Basin (Mayden 1988); however, recent studies of the geomorphology of the Tennessee River indicate this connection may be much older than the mid-Miocene

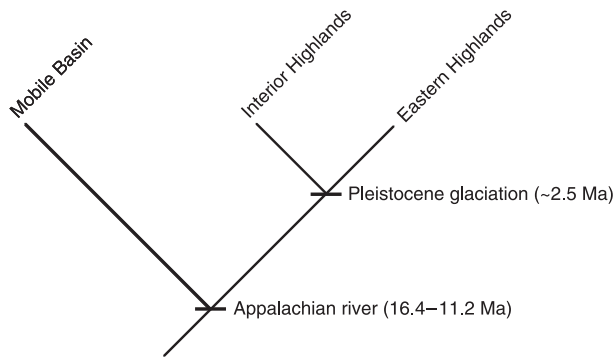


Fig. 2 Area relationships among disjunct highland areas of the Central Highlands predicted from the Central Highlands vicariance hypothesis (CHVH). Timing of speciation events proposed in the CHVH is given at the internal nodes.

(Mills & Kaye 2001). In addition, sister species, or lineages, distributed in the Interior and Eastern Highlands are hypothesized to be approximately 2.5 Ma, the age of the onset of Pleistocene glaciation that fragmented a once continuous highland area containing the Eastern and Interior Highlands (Fig. 2) (Mayden 1987). Since the CHVH proposes a pre-Pleistocene widespread and diverse fauna, most speciation events are hypothesized to have occurred much earlier than the onset of Pleistocene glaciation (greater than 2.5 Ma), regardless of the particular biogeographic relationships among species and lineages.

Previous uses of molecular phylogenies to investigate the history of Central Highland fishes and aquatic organisms have found some consistency with biogeographic patterns and predictions of the CHVH (Crandall & Templeton 1999; Near *et al.* 2001; Berendzen *et al.* 2003; Simons 2004). However, the temporal predictions of the Pleistocene dispersal model and the CHVH have not been as extensively tested, as few studies have used molecular data to estimate absolute ages of species and lineages (Strange & Burr 1997; Near *et al.* 2001; Berendzen *et al.* 2003; Simons 2004).

To date the only methods used to investigate the historical biogeography of Central Highland fishes have been pattern based (Mayden 1988), which explicitly avoid making assumptions regarding evolutionary processes such as dispersal and extinction (Rosen 1978). Relatively recent developments have led to alternative methods known as event-based analyses that do not reject natural processes such as dispersal a priori, and attempt biogeographic reconstruction using explicit process models (Ronquist & Nylin 1990; Ronquist 1997). Such considerations may be important to the study of Central Highland fishes since in addition to patterns suggestive of vicariance, several studies have uncovered genetic and phylogenetic signatures of recent and widespread dispersal (Kinziger *et al.* 2001; Near *et al.* 2001; Berendzen *et al.* 2003; Simons 2004).

Dispersal–vicariance analysis uses a phylogenetic tree to reconstruct ancestral ranges using a parsimony-based method. It differs from classic cladistic biogeographic methods in that it characterizes the history of geographical speciation by minimizing the number of implied dispersal and extinction events, where there is a cost for dispersal and extinction and no cost for vicariance (Ronquist 1997). One strategy to test predictions of the Pleistocene dispersal model and the CHVH is to use phylogenetic hypotheses of Central Highland fishes and dispersal–vicariance analysis to reconstruct ancestral distributions, and use molecular data to estimate species divergence times. Time-calibrated phylogenetic trees (chronograms) can test both the hypothesized area relationships among the disjunct areas of the Central Highlands and the hypothesized timing of dispersal and vicariant events (Fig. 2). Historical biogeographic analyses that do not incorporate information on the absolute timing of diversification of lineages risk being misled by pseudocongruence (Donoghue & Moore 2003), where similar biogeographic patterns are the result of multiple events affecting a single geographical area that happened at distinctly different times (Cunningham & Collins 1994).

The darter clade *Nothonotus* contains 20 recognized species and has been classified as a subgenus of *Etheostoma* (Kuehne & Barbour 1983; Page 1983); however, recent phylogenetic analyses of mtDNA sequences (Song *et al.* 1998; Sloss *et al.* 2004) and lack of morphological synapomorphies to diagnose *Etheostoma* (Bailey *et al.* 1954), justify the recognition of *Nothonotus* as a distinct darter genus. Aspects of the geographical distribution and natural history of *Nothonotus* make the group an attractive candidate to investigate hypotheses regarding the historical biogeography of eastern North America (Wood 1996). Species of *Nothonotus* are distributed among all the disjunct areas of the Central Highlands, as well as the Western Gulf Coast (Fig. 1) (Lee *et al.* 1980; Wood 1996). Phylogenetic hypotheses of *Nothonotus* based on allozyme frequency data and external morphology have been consistent with the CHVH with regard to the ancient divergence of the Mobile Basin endemic species, and the relationship of the Mobile Basin species to other Central Highlands species (Etnier & Williams 1989; Wood 1996). Based on the hypothesized age of the Mobile Basin–Eastern Highlands vicariance inferred from the allozyme phylogenies, approximately half of the speciation events in *Nothonotus* are thought to be fairly ancient, dating to the Miocene (Wood 1996).

In this study we present a phylogenetic analysis of all 20 recognized *Nothonotus* species using DNA sequences from the mitochondrial encoded cytochrome *b* (*cyt b*) gene. We test biogeographic predictions of the Central Highlands by assessing area relationships and using dispersal–vicariance analysis to reconstruct ancestral areas in the clade. In addition, we estimate divergence times in *Nothonotus* with an external fossil calibration of the molecular phylogeny and

methods to compensate for molecular evolutionary rate heterogeneity among lineages. Our analyses indicate two potential instances of pseudocongruence in the biogeographic history of *Nothonotus*, patterns of speciation that involve both vicariance and dispersal between disjunct highland regions, relatively recent divergence times of many species in the clade, and appreciable degrees of diversification within, as opposed to among, disjunct components of the Central Highlands. We discuss these results with regard to understanding the causes for high species diversity in the Central Highlands, and offer the possibility of introducing ideas and methods of island biogeography to the study of mechanisms that have shaped the unique fish fauna of the Central Highlands.

Materials and methods

Specimen sampling and DNA sequencing

The taxon sampling we used in this study was designed to provide a complete species sampled phylogeny of *Nothonotus*, as well as provide adequate external fossil calibration points. Reflecting the uncertainty of relationships among darter genera (Song *et al.* 1998; Sloss *et al.* 2004), we chose to root the *Nothonotus* phylogeny using three non-darter percid species (Table 1). External fossil calibrations were included by sampling 21 species from Centrarchidae (Table 1).

For many species in our analysis, complete coding sequences of the mitochondrial gene *cyt b* were downloaded directly from GenBank (Table 1). All other *cyt b* sequences were collected using DNA isolation, polymerase chain reaction (PCR), and sequencing protocols used in other studies of darter molecular phylogenetics (Near *et al.* 2000; Near 2002). Since the *cyt b* genes of all the species examined were of the same length, alignment of DNA sequences was performed by eye.

Phylogenetic analysis and divergence time estimation

Calibration of *Nothonotus* molecular phylogenies relied on previous use of fossil information in centrarchid fishes to convert relative molecular divergence to absolute age estimates (Near *et al.* 2003). Centrarchid fishes are classified in the same taxonomic order as darters, have a rich fossil record, and there are extensive comparative molecular data sets for this clade (Near *et al.* 2003, 2004). Since darters have a poor fossil record (Smith 1981; Cavender 1986), the opportunity to use such information to calibrate molecular phylogenies is essentially nonexistent. Fossil-calibrated centrarchid molecular phylogenies offer a set of external calibrations to estimate divergence times in darters (Near & Benard 2004).

Phylogenetic relationships of *Nothonotus* were investigated with a partitioned mixed-model Bayesian (Ronquist & Huelsenbeck 2003) analysis (pMM Bayesian) with posterior

probabilities estimated using Metropolis-coupled Markov chain Monte Carlo (MC3) (Larget & Simon 1999; Huelsenbeck *et al.* 2001). Each of the three codon positions of *cyt b* were treated as separate data partitions, and the optimal maximum-likelihood model of sequence evolution for each codon position was determined with hierarchical likelihood-ratio tests using the computer program MODELTEST 3.0 (Posada & Crandall 1998). The different models of sequence evolution that were selected for each particular data partition were assigned in the computer program MRBAYES 3.0 (Ronquist & Huelsenbeck 2003) with the APPLYTO command, and model parameter values were estimated for each data partition using UNLINK commands. The models used in the pMM Bayesian analyses differed by no more than three parameters. Each model had a substitution matrix (one, two, or six substitution rates) corresponding to JC or F81, HKY85 or K80, and GTR models of sequence evolution, among-site rate variation (equal vs. gamma distributed rates), and whether or not the presence of invariant sites was modelled (Swofford *et al.* 1996). MRBAYES 3.0 was run with 5×10^6 generations to ensure convergence of the MC3 algorithm in the estimation of tree topology and branch lengths. The burn-in period of the MC3 analysis was determined by graphically tracking the maximum-likelihood scores at each generation to determine the point where generations and maximum-likelihood scores reach a plateau. Trees and parameter values resulting from generations prior to the burn-in were discarded. The frequency that a particular clade occurs within the collection of post burn-in trees was interpreted as a measure of node support.

To determine if there was significant molecular evolutionary rate heterogeneity among lineages in the molecular phylogeny, a likelihood-ratio test was used to compare rate-variable and rate-constant models of sequence evolution. The likelihood-ratio test statistic was compared to a chi-squared distribution with $s-2$ degrees of freedom, where s was equal to the number of taxa included in the analysis (Felsenstein 1981).

Two centrarchid fossils were used to calibrate the *Nothonotus* molecular phylogeny. These were the oldest fossils that could be assigned as sister lineages to the extant species *Lepomis gulosus* (6.6 Ma) and *Archoplites interruptus* (15.5 Ma). Cross-validation of fossil dates in centrarchids revealed that both of these fossils were among the most consistent among a total of 10 individual fossil calibrations (Near *et al.* submitted). In these analyses, fossil dates were assigned to stem group nodes, so the node immediately preceding the most recent common ancestor of the fossil and extant species were dated with the calibrations (Doyle & Donoghue 1993; Magallon & Sanderson 2001). Details regarding the centrarchid fossils, their phylogenetic placement, and ages are given in Near *et al.* (2003) and Near & Benard (2004). Fossil dates assigned to the phylogeny were treated as fixed minimal ages in the computer program r8s

Table 1 Collection locations, voucher information, and GenBank Accession nos for specimens used in this study. All collections, except *Zingel zingel*, were made in the United States. Tissue vouchers are from the University of Tennessee Tissue Collection (UTTC)

Species	Locality	Tissue voucher	GenBank Accession no.
<i>Nothonotus acuticeps</i>	Nolichucky River at TN 340, Green County, Tennessee	UTTC 2205	AY742655
<i>Nothonotus aquali</i>	Buffalo River, Lewis County, Tennessee	UTTC 68	AF386537
<i>Nothonotus aquali</i>	Duck River at Halls Mill Road, Bedford County, Tennessee	UTTC 2562	DQ025497
<i>Nothonotus bellum</i>	Goose Creek, 1.6 km south of Crossroads, Russell County, Kentucky	UTTC 754	AY742656
<i>Nothonotus bellum</i>	Long Creek at Dotson Road, 10 km northeast of Laffayette, Macon County, Tennessee	UTTC 4865	DQ025498
<i>Nothonotus camurum</i>	Middle Fork of the Vermillion River, 3.2 km east-northeast of Collison, Vermilion County, Illinois	UTTC 1203	AF386536
<i>Nothonotus camurum</i>	Little River at TN 411 bridge, Blount County, Tennessee	UTTC 2578	DQ025499
<i>Nothonotus camurum</i>	Clinch River at Grissom Island, Claiborne County, Tennessee	UTTC 2582	DQ025400
<i>Nothonotus chlorobranchium</i>	Burningtown Creek, 11.5 km northwest of Franklin, Macon County, Georgia	UTTC 2120	AY742657
<i>Nothonotus chlorobranchium</i>	Little Pigeon River below US 321 bridge, Sevier County, Tennessee	UTTC 3094	DQ025401
<i>Nothonotus chuckwachatte</i>	Tallapoosa River at US Highway 27, 7.2 km north-northwest of Buchanan, Haralson County, Georgia	UTTC 2275	AY742658
<i>Nothonotus denoncourti</i>	Buffalo R at TN 13, north of Lobelville, Perry County, Tennessee	GenBank	AF274479
<i>Nothonotus douglasi</i>	Sipsey Fork at County Road 60, 15.2 km north Double Springs, Winston County, Alabama	UTTC 2116	AY742659
<i>Nothonotus etowahae</i>	Etowah River at State Highway 52, 7.41 km north-northwest Dahlonega, Lumpkin County, Georgia	UTTC 2272	AY742660
<i>Nothonotus etowahae</i>	Shoal Creek at Shoal Creek Road, Dawson County, Georgia	UTTC 4091	DQ025402
<i>Nothonotus jordani</i>	Conasauga River, 1.6 km north of Conasauga, Polk County, Tennessee	UTTC 985	AY742661
<i>Nothonotus jordani</i>	Conasauga River at TN 74 bridge, 19 km southeast of Cleveland, Bradley County, Tennessee	UTTC 2363	DQ025403
<i>Nothonotus juliae</i>	Buffalo River at US Highway 65, 3.2 km west of Gilbert, Searcy County, Arkansas	UTTC 897	AY742662
<i>Nothonotus juliae</i>	Buffalo River at US Highway 65, 3.2 km west of Gilbert, Searcy County, Arkansas	UTTC 2957	DQ025404
<i>Nothonotus maculatum</i>	Green River at Greensburg at US Highway 68, Green County, Kentucky	UTTC 446	AY742663
<i>Nothonotus microlepidum</i>	Harpeth River at US Highway 70, Cheetam County, Tennessee	UTTC 2249	AY742664
<i>Nothonotus moorei</i>	Middle Fork Little Red River at Shirley, Van Buren County, Arkansas	GenBank	AF274445
<i>Nothonotus moorei</i>	South Fork Little Red River upstream of US Highway 65 bridge, south side of Clinton, Van Buren County, Arkansas	UTTC 2884	DQ025405
<i>Nothonotus rubrum</i>	Foster Creek, Copiah County, Mississippi	GenBank	AF274446
<i>Nothonotus rubrum</i>	Bayou Pierre, 9.7 km west of Hazlehurst and Smyrna bridge, Copiah County, Mississippi	UTTC 4912	DQ025406
<i>Nothonotus rubrum</i>	Bayou Pierre at MS Highway 18, Copiah County, Mississippi	UTTC 4913	DQ025407
<i>Nothonotus rufilineatum</i>	Buffalo River at mouth of Grinders Creek, Lewis County, Tennessee	GenBank	AF274447
<i>Nothonotus sanguifluum</i>	Rockcastle River, Rockcastle County, Kentucky	UTTC 940	AY742665
<i>Nothonotus tippecanoe</i>	Harpeth River at Narrows of the Harpeth State Park, Cheatham County, Tennessee	GenBank	AF274471
<i>Nothonotus vulneratum</i>	Toccoa River, Fannin County, Georgia	UTTC 2203	AY742666
<i>Nothonotus vulneratum</i>	Oconaluftee River at US Highway 441, Jackson County, North Carolina	UTTC 2204	AY742667
<i>Nothonotus wapiti</i>	Elk River at TN Highway 273, Lincoln County, Tennessee	UTTC 2134	AY742668
<i>Nothonotus wapiti</i>	Elk River at TN Highway 273, Lincoln County, Tennessee	UTTC 4096	DQ025408
Non-darter percids			
<i>Perca flavescens</i>	Lake Andrusia, Beltrami County, Minnesota	UTTC 261	AY20099
<i>Sander vitreus</i>	Mississippi River, Rock Island County, Illinois	UTTC 312	AF386602
<i>Zingel zingel</i>	Bulgaria	UTTC 951	AY742669
Centrarchidae			
<i>Acantharchus pomotis</i>	Mullica River, Camden County, New Jersey	GenBank	AY115994
<i>Ambloplites cavifrons</i>	Tar River, Franklin County, North Carolina	GenBank	AY115980
<i>Ambloplites rupestris</i>	Indian Creek, Hardin County, Tennessee	GenBank	AY115978
<i>Ambloplites rupestris</i>	Lake Andrusia, Beltrami County, Minnesota	UTTC 284	AY225663
<i>Archoplites interruptus</i>	Hume Lake, Fresno County, California	UTTC 1077	AY225665
<i>Centrarchus macropterus</i>	Mud Creek, Hardin County, Tennessee	UTTC 384	AY225666

Table 1 Continued

Species	Locality	Tissue voucher	GenBank Accession no.
<i>Enneacanthus chaetodon</i>	Mullica River, Camden County, New Jersey	GenBank	AY115984
<i>Enneacanthus gloriosus</i>	Cedar Creek, Mobile County, Alabama	GenBank	AY115985
<i>Enneacanthus obesus</i>	Mullica River, Camden County, New Jersey	GenBank	AY115988
<i>Pomoxis annularis</i>	Big Muddy River, Union County, Illinois	GenBank	AY115990
<i>Pomoxis nigromaculatus</i>	Little River, McCurtain County, Oklahoma	GenBank	AY115992
<i>Lepomis cyanellus</i>	Whiteside Creek, LaFayette County, Wisconsin	GenBank	AY115974
<i>Lepomis gulosus</i>	Easley Creek, Hot Springs County, Arkansas	GenBank	AY115972
<i>Lepomis macrochirus</i>	Blue River, Crawford County, Indiana	UTTC 424	AY225667
<i>Lepomis miniatus</i>	Conasauga River, Bradley County, Tennessee	UTTC 444	AY225668
<i>Micropterus salmoides</i>	Lipset Lake, Burnett County, Wisconsin	GenBank	AY225675
<i>Micropterus dolomieu</i>	Fox River, Kenosha County, Wisconsin	GenBank	AY225687
Rerciform outgroup			
<i>Aplodinotus grunniens</i>	Mississippi River, Jo Daviess County, Illinois	UTTC 515	AY225662

using the FIXAGE command (Sanderson 2003). The optimal smoothing parameter value for penalized likelihood analysis in *r8s* was determined by cross-validation (Sanderson 2002). Error in divergence time estimates due to data sampling was determined by a bootstrap analysis. One thousand bootstrap replicate data sets were generated using CodonBootstrap, and for each replicate the optimal model and parameter values were used to estimate maximum-likelihood branch lengths on the maximum-parsimony tree. Trees were imported into *r8s* and the PROFILE command was used to determine the estimated divergence time for a given node across all trees. The confidence interval was calculated from the central 95% of the age estimates for a given node across all trees (Baldwin & Sanderson 1998).

Dispersal–vicariance analysis in *Nothonotus*

We gathered distributional data for all *Nothonotus* species from sources that were based on documented collections in museums (Lee *et al.* 1980; Page 1983; Etnier & Williams 1989; Etnier & Starnes 1993; Mettee *et al.* 1996; Wood 1996; Skelton & Etnier 2000). Each species was assigned to one of four areas in eastern North America, Eastern Highlands, Interior Highlands, Mobile Basin, and Western Gulf Coast (Fig. 1). No species of *Nothonotus* is distributed among multiple areas. We used dispersal–vicariance analysis (Ronquist 1997) to estimate ancestral areas in the *Nothonotus* phylogeny, and possible instances of dispersal between disjunct areas of the Central Highlands. The ancestral areas for all internal nodes were reconstructed using the computer program DIVA 1.1 (Ronquist 1996). The appearance of new areas in the optimizations was interpreted as dispersal events and separation of areas were interpreted as resulting from vicariance. The three-dimensional cost matrix used in the DIVA search algorithm searches for the reconstruction

minimizes dispersal using two rules: the ancestral area must be occupied by at least one descendent species and an ancestral area must contain at least one from each of the two descendent species or lineages (Ronquist 1997). In DIVA, we did not restrict the number of ancestral areas optimized for a given node, allowing for the possibility of widespread ancestral species.

Testing biogeographic predictions

The CHVH makes predictions regarding the phylogenetic relationships of species, based on the hypothesized area relationship of the disjunct regions of the Central Highlands (Fig. 2). To compare the mtDNA phylogeny with the topology predicted by the area relationships, we compared the best phylogenetic hypothesis of *Nothonotus* species that represented the CHVH, with the best tree resulting from maximum-parsimony analysis. We used maximum-parsimony constraints to find the best tree of *Nothonotus* species that had all Mobile Basin species as the sister lineage to all other *Nothonotus* species, and all Eastern and Interior Highland species were each reciprocally monophyletic. Since the CHVH does not make predictions regarding the relationship of Western Gulf Coast species, the placement of *Nothonotus rubrum* in these alternative hypotheses was determined by using backbone constraint heuristic tree searches in PAUP*. The significance of tree topology differences between the phylogeny predicted from the CHVH and the best maximum-parsimony trees was determined by using a maximum-likelihood Shimodaira–Hasegawa test (Shimodaira & Hasegawa 1999; Goldman *et al.* 2000), as implemented in PAUP*.

The hypothesized ages of speciation events in the Pleistocene dispersal model and the CHVH (Fig. 2) were compared to the molecular divergence time estimates

within *Nothonotus*. In order to identify possible instances of pseudocongruence in the historical biogeography of *Nothonotus*, we also compared divergence times of different nodes identified as vicariance involving the same areas of the Central Highlands. In particular, we were interested in testing the timing of vicariance between the Mobile Basin and other areas of the Central Highlands and between the Eastern and Interior Highlands. Divergence times at nodes with nonoverlapping error estimates were considered significantly different.

Results and discussion

Cytochrome *b* variation, phylogeny and model selection

The coding region of the *cyt b* gene was 1140 base pairs in all species examined. The alignment contained no insertions or deletions. Among interspecific comparisons in *Nothonotus*, pairwise differences at *cyt b* ranged from 1.9% (*Nothonotus sanguifluum* vs. *Nothonotus microlepidum*) to 17.3% (*Nothonotus juliae* vs. *Nothonotus denoncourti*). Likelihood-ratio tests using MODELTEST 3.0 identified a different maximum-likelihood model of DNA sequence evolution for each codon position, two substitution types, invariant sites, and gamma distributed rates for the first codon position; two substitution types and gamma distributed rates for the second codon position; and six substitution types, invariant sites, and gamma distributed rates for the third codon position.

Bayesian analysis was run four times for 5×10^6 generations with nearly identical results from each run. The last of these four runs was used to assess relationships and estimate divergence times of *Nothonotus*. After 1 million generations, the MC3 algorithm converged on a stable likelihood score, and the first 10 000 trees were discarded as the burn-in. The mean maximum-likelihood score of the 40 000 post burn-in trees was -14008.16 ± 9.10 , and the 50% majority rule consensus tree was well resolved with most nodes supported with a significant (> 0.94) Bayesian posterior probability (Fig. 3).

The phylogeny inferred from the *cyt b* sequence data is very similar to previous phylogenetic hypotheses resulting from analyses from allozyme data and external morphology (Etnier & Williams 1989; Wood 1996). In particular, our new phylogeny and the previous trees all agree that *N. juliae* is the sister species of all other *Nothonotus* species, and the six species comprising the *Nothonotus maculatum* species group (*N. maculatum*, *N. wapiti*, *N. vulneratum*, *N. aquali*, *N. sanguifluum*, and *N. microlepidum*) form a strongly supported monophyletic group.

A likelihood-ratio test was able to reject a rate-constant model, over a rate-variable model of nucleotide evolution ($\chi^2 = 116.82$, d.f. = 55, $P < 0.01$), thus invalidating the use of a simple maximum-likelihood molecular clock model for divergence time estimation.

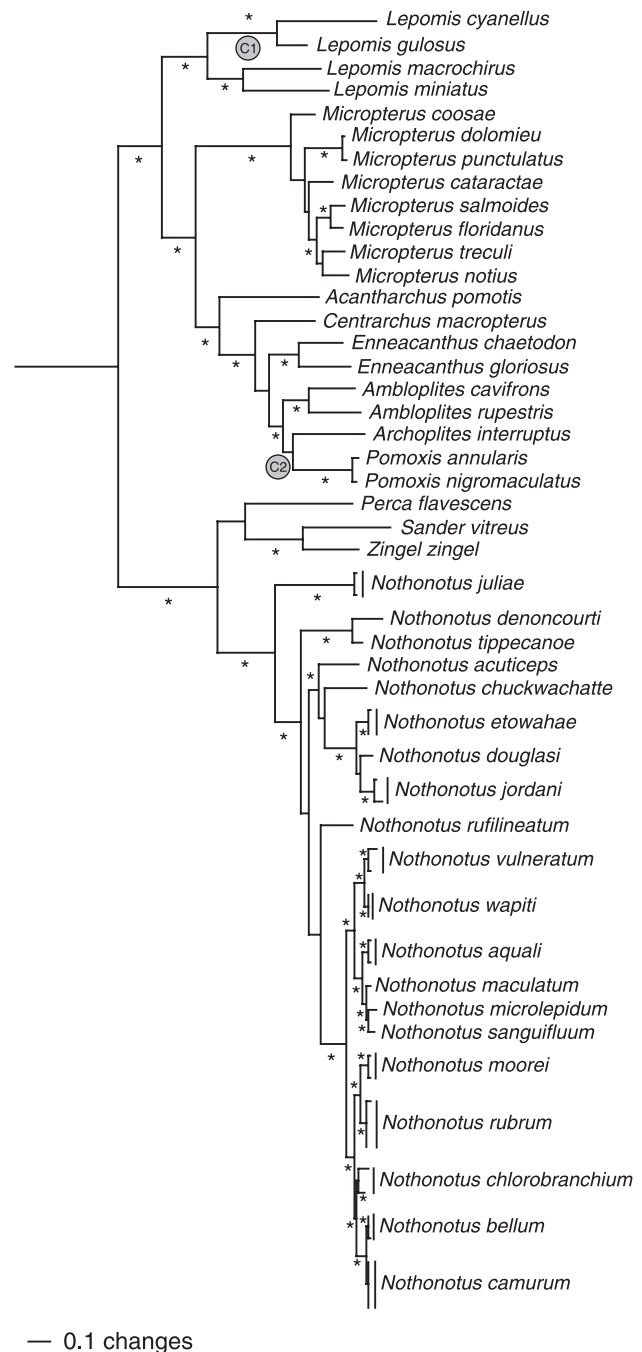


Fig. 3 Bayesian-inferred phylogeny using a mixed-model strategy. Nodes supported with significant Bayesian posterior probabilities are marked with an asterisk. Circled 'C' numbers designate nodes in Centrarchidae dated with fossil information.

Divergence time estimates, dispersal–vicariance analysis, and biogeographic predictions

Cross-validation in penalized likelihood analysis identified 316.23 as the optimal smoothing parameter value. Interspecific divergence time estimates from penalized

Node	Age (Ma)	Bootstrap estimate of error on age (Ma)	Ancestral area	Inferred biogeographic mechanism
1	18.46	± 3.53	EI	Vicariance
2	13.67	± 2.86	E	Speciation within area
3	3.80	± 0.78	E	Speciation within area
4	11.85	± 2.35	E	Speciation within area
5	10.05	± 2.00	EM	Dispersal
6	9.00	± 1.73	M	Vicariance
7	3.24	± 0.57	M	Speciation within area
8	2.73	± 0.64	M	Speciation within area
9	9.41	± 1.88	E	Speciation within area
10	4.90	± 1.12	E	Speciation within area
11	3.44	± 0.59	E	Speciation within area
12	1.42	± 0.38	E	Speciation within area
13	1.93	± 0.46	E	Speciation within area
14	1.41	± 0.32	E	Speciation within area
15	1.00	± 0.55	E	Speciation within area
16	2.91	± 0.71	EI, EW, EIW	Dispersal
17	1.89	± 0.51	IW	Dispersal*
18	2.38	± 0.64	E	Speciation within area
19	0.62	± 0.27	E	Speciation within area

*Dispersal if node 16 is optimized as EI, or EW.

Table 2 Divergence time estimates, bootstrap estimated confidence intervals, ancestral area reconstruction (E, Eastern Highlands; I, Interior Highlands; M, Mobile Basin; and W, Western Gulf Coast), biogeographic mechanism. Ma, million years ago

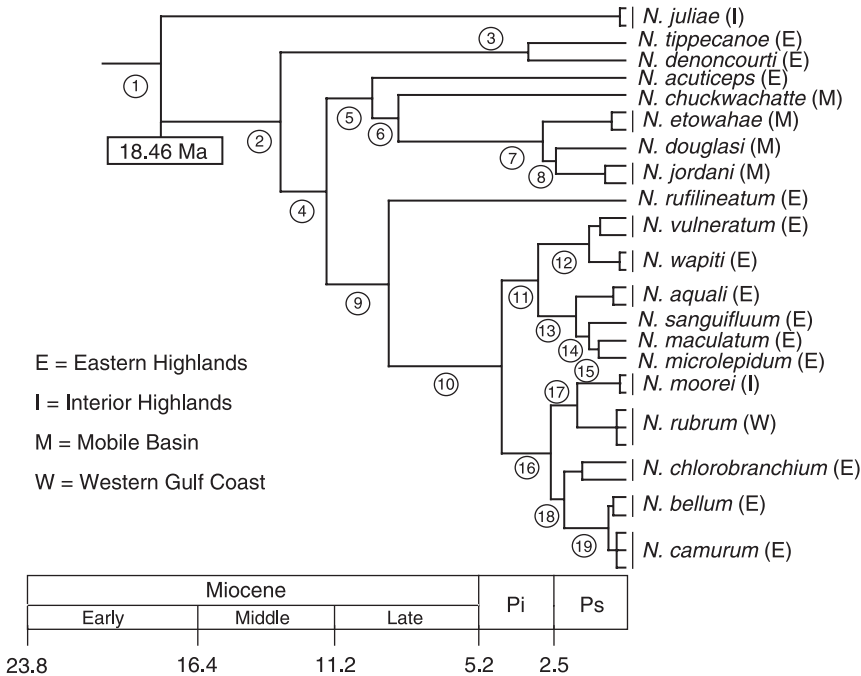


Fig. 4 Time-calibrated phylogeny (chronogram) of *Nothonotus*. The chronogram is calibrated against the geological time scale (Berggren *et al.* 1995). Exact age estimates, error on divergence times are given in Table 2. Biogeographic scoring of individual species is indicated in parentheses after species name. Dispersal–vicariance reconstruction of ancestral nodes is given in Table 2. Pi, Pliocene; Ps, Pleistocene.

likelihood are presented in Table 2, and a time-calibrated phylogeny (chronogram) is shown in Fig. 4. The age of the most recent common ancestor of all *Nothonotus* species was 18.46 ± 3.33 Ma, and the youngest sister species pair was *Nothonotus camurum* and *Nothonotus bellum* (0.62 ± 0.27 Ma). Unlike predictions based on allozyme-inferred phylogenies of *Nothonotus* (Wood 1996), the mtDNA penalized likelihood divergence time estimates indicate that few of the

diversification events in the clade occurred during the Miocene, with most speciation occurring in the Pliocene and Pleistocene (Table 2, Fig. 4).

Dispersal–vicariance analysis suggests that the ancestral area for *Nothonotus* is the Eastern and Interior Highlands (Table 2). Only one node was characterized by an ambiguous ancestral area reconstruction (node 16; Table 2). Movement of *Nothonotus* lineages between disjunct areas of the

Central Highlands is indicated by the reconstruction of at least two, and possibly three dispersal events in the history of the clade (Table 2, Fig. 4). These dispersal events are from the Eastern Highlands to the Mobile Basin (at internode between nodes 5 and 6; Table 2, Fig. 4), from the Eastern Highlands to the Interior Highlands, or Western Gulf Coast, or both the Interior Highlands and Western Gulf Coast (at internode between nodes 10 and 16). The third reconstructed dispersal event is dependent on the ambiguous optimization at node 16. If this node is either the Eastern Highlands–Interior Highlands or Eastern Highlands–Western Gulf Coast, then a dispersal event to either the Western Gulf Coast or Interior Highlands is inferred at the internode between nodes 16 and 17 (Table 2, Fig. 4). If dispersal from the Eastern Highlands to the Interior Highlands was optimized at this node, the timing of this speciation event (2.91 ± 0.71 Ma) and the fact that the Eastern Highlands is the ancestral area for nodes 9 and 10 (Table 2, Fig. 4) would fit the predictions of the Pleistocene dispersal model to explain the origins of *Nothonotus moorei* and *Nothonotus rubrum* (Mayden 1987).

The history of speciation in *Nothonotus*, as inferred from the molecular phylogeny (Fig. 4), is different from phylogenetic relationships predicted by the CHVH (Fig. 2). We found the best *cyt b* phylogeny that fit the tree topology predicted by the CHVH was significantly worse than the optimal maximum-parsimony tree in a Shimodaira–Hasegawa test (difference in likelihood score = 108.7, $P < 0.01$). The CHVH predicts that the basal split in a given clade distributed among the disjunct areas of the Central Highlands should be between the Mobile Basin and the Eastern and Interior Highlands (Fig. 2). However, the basal split in *Nothonotus* is a vicariance between the Eastern and Interior Highlands (Table 2, Fig. 4). There is a near-basal relationship with regard to the Mobile Basin endemic species; however, the phylogeny and ancestral area reconstruction using *DIVA* indicates that occurrence of *Nothonotus* species in the Mobile Basin can ultimately be attributed to an ancestral instance of dispersal from the Eastern Highlands (Table 2, Fig. 4).

In addition to predictions of phylogeny, the CHVH predicts that vicariance involving the Mobile Basin and the Eastern and Interior Highlands should occur sometime in the Middle Miocene (Mayden 1988; Wood 1996). The *DIVA*-inferred vicariance between these areas at nodes 5 and 6 is slightly later than this time, but the implications of this close agreement with the CHVH are unclear. The CHVH predictions regarding the biogeographic relationship of the Central Highlands with the Mobile Basin are based on the hypothesized Appalachian river that would have connected upper Tennessee River tributaries in the Eastern Highlands with the Coosa River in the Mobile Basin (Mayden 1988; Clabaugh *et al.* 1996; Wood 1996). The connection between the Tennessee River and Mobile Basin, via

the Appalachian river has been debated in the literature, with the observation that there was little geological evidence to support the timing of such a connection (Starnes & Etnier 1986). Recent geological analysis indicates that if such a connection between the upper Tennessee River and Mobile Basin did occur, it was formed sometime during the Palaeozoic and was severed before the Early Cenozoic (Mills & Kaye 2001). The divergence time estimates in *Nothonotus* involving the Eastern Highlands and the Mobile Basin are far too young to be attributed to such a connection between these rivers (Table 2, Fig. 4). However, the dates of connections between the lower Tennessee River and other eastern Mobile Basin rivers (e.g. Tombigbee River and Sipsey Fork) are within the range of the divergence times observed at the node where there is an inferred vicariance between these areas (Mills & Kaye 2001).

There are two separate reconstructed instances of vicariance between the Eastern and Interior Highlands in the history of speciation in *Nothonotus* (Table 2, Fig. 4). These two events involving the same areas of the Central Highlands occur at significantly different times, indicating pseudocongruence. The Eastern–Interior Highlands vicariance at node 1 occurred at 18.46 ± 3.53 Ma, and a similar vicariance reconstructed at node 16 occurred 2.91 ± 0.71 Ma, with the latter fitting the predicted timing of dispersal between the Eastern and Interior Highlands in the Pleistocene dispersal model.

Conclusions and new directions for understanding the biogeography of speciation in Central Highlands fishes

At first pass it appears that neither the Pleistocene dispersal model nor the CHVH explain most of the instances of speciation observed in *Nothonotus*. Does this mean that these hypotheses have outlived their utility to provide possible mechanisms to explain the incredible species richness observed in aquatic habitats of the Central Highlands? We believe that such overt dismissal of either of these hypotheses would be premature, since our study of *Nothonotus* includes a small fraction of Central Highland fish species diversity, and is the first attempt to test these ideas with a combination of phylogenetic hypotheses, ancestral area reconstructions, and divergence time estimates. We propose that future studies of Central Highlands biogeography minimally include these types of data, and recognize that the presence and reality of pseudocongruence in biogeographic studies necessitates the inclusion of absolute divergence time estimates (Donoghue & Moore 2003). Also, one must consider the effects of lineage extinction that results in a greater likelihood of recovering recent rather than ancient speciation events. With respect to present-day species diversity, extinction could have eliminated other lineages within *Nothonotus* that exhibit patterns predicted by the CHVH.

With respect to the present-day species diversity, the most general result from our analyses of speciation in *Nothonotus* is the limited instances of both vicariance and dispersal between disjunct highland areas in the history of the clade (Table 2), as well as the large amount of speciation within disjunct areas of the Central Highlands (Fig. 4). Even if the phylogeny, ancestral area reconstructions, and divergence time estimates in *Nothonotus* were found to fit either the Pleistocene dispersal model or the CHVH, only 31% of all speciation events in the clade would be explained by dispersal or vicariance between the disjunct highland areas. In other words, even if the patterns of speciation fit a priori predictions, either disjunct highland model would fail to explain 69% of the speciation events in *Nothonotus*.

Hypothesized mechanisms resulting in the high diversity of freshwater fishes in the Central Highlands have focused primarily on the geological and climatic stability of the region, as well as processes of vicariance and dispersal leading to speciation between disjunct highland regions (Briggs 1986; Mayden 1988). Absent from discussions of the evolution of this fauna have been observed patterns and theoretical insights from the study of species–area relationships and island biogeography (MacArthur & Wilson 1967; Rosenzweig 1995). Specifically, in these models the effect of area size on the numbers of species is determined by rates of immigration and extinction, where larger islands have more species than comparable smaller islands (MacArthur & Wilson 1967). Recently, the role of speciation has been introduced to the study of the area–species relationship (Losos & Schluter 2000; Hubbell 2001). If we consider the disjunct regions of the Central Highlands as islands separated by barriers to dispersal, an interesting pattern of within-highland-area speciation is observed in *Nothonotus*. The Eastern Highlands is the largest area and at least 12 allopatric speciation events in *Nothonotus* have occurred without dispersal or vicariance involving the other highland areas. The Interior Highlands and Mobile Basin are comparable in size, but there are no *in situ* speciation events in *Nothonotus* recovered in the Interior Highlands and three speciation events in *Nothonotus* are observed in the Mobile Basin (Table 2). We note that speciation within disjunct highland areas is similar to observed patterns of speciation in *Anolis* lizards on Caribbean islands, where the number of speciation events and the speciation rate is positively correlated with island area (Losos & Schluter 2000).

Ultimately the search for mechanisms that have resulted in the incredible temperate diversity of North American freshwater fishes will be greatly facilitated with the availability of phylogenetic hypotheses, ancestral area reconstructions, and divergence time estimates for multiple clades distributed in the Central Highlands. Previous hypotheses explaining the diversity of North American freshwater fishes have provided a valuable set of expectations

to test; however, there is much room to incorporate ideas from theoretical and empirical studies of island biogeography in unravelling the evolutionary mysteries of this exceptional continental fauna.

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