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TEMPORAL PATTERNS OF DIVERSIFICATION AND MICROENDEMISM IN EASTERN HIGHLAND ENDEMIC BARCHEEK DARTERS (PERCIDAE: ETHEOSTOMATINAE)

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Eastern North America is the location of the world's most species-rich temperate freshwater fish fauna. Hypotheses regarding the geographic and temporal scale of teleost diversification in this region have not been broadly investigated using absolute divergence time estimates among the constituent lineages. This study used time-calibrated molecular phylogenies estimated from mitochondrial and nuclear genes to investigate the temporal and geographic signatures of diversification within barcheek darters, a clade of allopatrically distributed species endemic to the Eastern Highlands. Results from divergence time estimates using an uncorrelated lognormal model suggest that the barcheek darters are an ancient group with a crown node estimate of 16.3 mya, 95% highest posterior density (HPD): [12.4, 20.5], and the clade is characterized by substantial intraspecific divergence times within several species. In particular, the Caney Fork endemic *Etheostoma basilare* comprises five strongly supported and deeply divergent clades with a most recent common ancestor estimated at 8.0 mya, 95% HPD: [5.6, 10.7]. These results are concordant with the hypothesis that geologically stable areas of eastern North America have facilitated both the generation and preservation of lineages across a substantial breadth of evolutionary time, and that allopatric speciation in darters has occurred at much smaller spatial scales than previously realized.

KEY WORDS: Allopatric, Catonotus, divergence times, Etheostoma, fossil calibration, speciation.

Eastern North America contains impressive evolutionary radiations of a number of aquatic animal lineages, including plethodontid salamanders (Petranka 1998; Wiens et al. 2006), crayfish (Crandall and Templeton 1999; Taylor and Schuster 2004), unionid mussels (Parmalee and Bogan 1998), and several clades of freshwater teleost fishes (Briggs 1986; Lundberg et al. 2000). In fact, this region contains the greatest number of freshwater fish species of any temperate region in the world (Lundberg et al. 2000). Most of the species diversity in these teleost fish lineages is restricted to three areas of disjunct highland topography, the Appalachians (Eastern Highlands) in the east and the Ozark and Ouachita Mountains (Interior Highlands) in the west, collectively known as the Central Highlands (Thornbury 1965; Pflieger 1971; Wiley and Mayden 1985; Mayden 1987, 1988; Soltis et al. 2006). Taxonomic affinities of organisms endemic to these highland regions have long intrigued students of biogeography, in particular those studying the evolution of eastern North America's exceptionally diverse freshwater fish fauna (Cope 1868; Jordan 1905; Metcalf 1966; Pflieger 1971; Wiley and Mayden 1985; Starnes and Etnier 1986; Mayden 1987, 1988; Near et al. 2001; Near and

Keck 2005; Berendzen et al. 2008; Piller et al. 2008). However, there has been limited utilization of molecular phylogenies to investigate the age of diversification and the geographic scale of speciation among species comprising the exceptionally diverse freshwater fish fauna of the Central Highlands.

TEMPORAL PATTERNS OF DIVERSIFICATION OF CENTRAL HIGHLANDS FISHES

The Central Highlands Vicariance Hypothesis (CHVH), a paradigm in the study of North American freshwater biota, proposes that prior to the Pleistocene the Central Highlands were contiguous and were subsequently fragmented into their contemporary configuration as a result of glacial-related geomorphologic alterations during the Pleistocene (Wiley and Mayden 1985; Mayden 1987, 1988; Strange and Burr 1997). According to the CHVH, fragmentation of the Central Highlands and the associated alterations of freshwater drainage configurations resulted in the isolation of widespread lineages and ultimately drove allopatric speciation in a number of freshwater fish clades. Predictions of this hypothesis have been tested using molecular phylogenies of teleost fishes (Strange and Burr 1997; Near et al. 2001; Hardy et al. 2002; Berendzen et al. 2003; Simons 2004; Berendzen et al. 2008; Piller et al. 2008), plethodontid salamanders (Kozak et al. 2006a), unionid mussels (Lieberman 2001), and crayfish (Crandall and Templeton 1999). However, these studies have suffered by the lack of absolute divergence time estimates. Without temporal information these analyses could be prone to the problem of temporal pseudocongruence, where a similar phylogenetic and distributional relationship across multiple lineages is misinterpreted as resulting from a single vicariant event when the pattern is actually driven by separate events occurring at different points in time (Donoghue and Moore 2003).

Near and Keck (2005) and Berendzen et al. (2008) investigated the temporal predictions of the CHVH with molecular divergence time estimates in *Nothonotus* darters (Percidae: Etheostomatinae) and the *Notropis rubellus* clade (Cyprinidae), respectively. Both studies determined that much of the diversity in the two example clades pre-dates the Pleistocene. This result is consistent with the CHVH that proposes a widespread and diverse pre-Pleistocene fish fauna (Mayden 1988). Both of these studies also suggest that a substantial amount of pre-Pleistocene lineage diversification has taken place within contemporarily disjunct highland areas, as opposed to between them (Near and Keck 2005; Berendzen et al. 2008). This pattern of within-highland area diversification is not explained in the CHVH, but may be more common than previously proposed.

The timing of diversification events presented in the CHVH are based on the timing of paleogeographic events that resulted in the fragmentation of the Central Highlands and the alteration of pre-Pleistocene river drainages (Mayden 1987). However, the rivers in the unglaciated areas of the Eastern Highlands, the largest and most species-rich of the three disjunct regions, are almost without exception entrenched into bedrock channels, have exhibited little diversion during the Pleistocene (Thornbury 1965; Teller and Goldthwait 1991; Anthony and Granger 2004), and exhibit a pre-Pleistocene configuration that is similar to the present day (Thornbury 1965; Metcalf 1966; Pflieger 1971; Mayden 1988). Therefore, there is little paleogeographic evidence to provide age estimates for lineages endemic to the geologically stable Eastern Highlands.

GEOGRAPHIC SCALE OF SPECIATION IN CENTRAL HIGHLAND FISH

For nearly 150 years, biologists have postulated that speciation in eastern North American fish clades is predominantly driven by allopatric processes (Cope 1868; Jordan 1905, 1908; Mayden 1988). Phylogenetic studies of Central Highland endemic teleost fish clades have supported this hypothesis (Kinziger et al. 2001; Near and Benard 2004; Simons 2004; Near and Keck 2005; George et al. 2006), and several synthetic works have used Central Highland distributed fish clades as examples of allopatric speciation (Wiley and Mayden 1985; Brooks and McLennan 1991; Berlocher 1998). Given the postulate that diversification of fish lineages in the Central Highlands is driven primarily by allopatric speciation, it remains unclear if allopatric diversification occurs at large versus small geographic scales, and between versus within major drainage systems. A common pattern observed in eastern North American freshwater fish is that of sister species occupying adjacent drainages (Wood 1996; Wiley and Hagen 1997; Near et al. 2000; Kinziger et al. 2001; Switzer and Wood 2002; Wood et al. 2002; Near and Benard 2004; Near and Keck 2005; Near and Hardman 2006). Vicariance resulting in such distributions has been invoked as working on small geographic scales through stream capture (Kuehne and Bailey 1961; Branson and Batch 1971) or on a much larger geographic scale involving major reconfigurations of drainage systems due to large scale paleogeographic events, such as Pleistocene glacial cycles (Pflieger 1971; Mayden 1988; Strange and Burr 1997; Near et al. 2001; Berendzen et al. 2003, 2008). These patterns suggest that diversification via allopatric speciation resulting from vicariance between drainage basins may be common. However, it remains unclear if allopatric diversification can take place within drainage systems in the absence of obvious geographic barriers to gene flow, and on what geographic scale such within drainage diversification has taken place.

STUDY SYSTEM: BARCHEEK DARTERS

The barcheek darter species group consists of seven described species that are distributed in a mosaic of adjacent and allopatric ranges along the Cumberland, Tennessee, and Green River

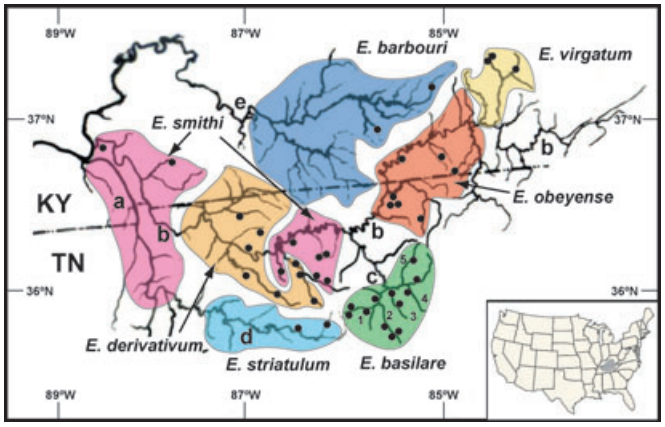

Figure 1. Drainage map illustrating the geographic distributions of barcheek darter species. Dots mark approximate collection localities of specimens used in this study (Table 1). Numbers identify tributaries of the upper Caney Fork river system (shaded in green): (1) Barren Fork, (2) Collins River, (3) Rocky River, (4) Cane Creek and Caney Fork River proper, and (5) Calfkiller River. Lower case letters identify major river systems: (a) Tennessee River, (b) Cumberland River, (c) Caney Fork River, (d) Duck River, and (e) Green River.

systems in Tennessee and Kentucky (Fig. 1) (Page and Schemske 1978; Page 1983; Etnier and Starnes 1993; Page et al. 2003). The upper, or eastern, portions of these three river systems are thought to have remained relatively unaltered in their courses through the Pleistocene glacial cycles (Thornbury 1965; Starnes and Etnier 1986; Sasowsky et al. 1995; Anthony and Granger 2006). However, the lower Tennessee, lower Duck, and lower Cumberland rivers are thought to have experienced course reconfigurations during the Pleistocene (Thornbury 1965; Braasch and Mayden 1985; Starnes and Etnier 1986). Given that the barcheek species *E. smithi* is found in the lower Tennessee, lower Duck, and lower Cumberland rivers (Fig. 1) there is the potential for differential impacts of Pleistocene glacial cycles across the clade.

All barcheek darter species exhibit male parental care, where males construct and defend nest sites under "slab" rocks and court females who deposit eggs on the rock ceiling (Page 1983). Barcheek darter species are restricted to rocky headwater streams containing suitable "slab-rock" pool spawning habitats and both niche conservatism and sexual selection have been hypothesized to be important in the history of diversification in the clade (Page and Schemske 1978; Porter et al. 2002). Barcheek darters are also noted for the presence of cryptic species, where morphological stasis has resulted in an underestimation of species diversity. Using phylogenetic trees estimated from nuclear and mtDNA gene sequences, and analyses of external morphology, Page et al. (2003) discovered two new species (*E. derivativum* and *E. basilare*) that were masquerading as disjunct populations of *E. virgatum* (Fig. 1).

Page et al. (2003) presented a phylogeny of the barcheek darter clade based on combined data partitioned Bayesian analysis, and recent phylogenetic analysis of AFLP markers resulted in a similar phylogeny of the barcheek darters (Mendelson and Simons 2006). An interesting result presented by Mendelson and Simons (2006) was the presence of significant intraspecific genetic structure in several barcheek darter species. We initiated a preliminary investigation focusing on the Caney Fork River endemic *E. basilare*. Our preliminary analyses revealed extensive genetic differentiation of mtDNA sequence data among populations of *E. basilare* distributed in tributaries of the Caney Fork River system (Fig. 1).

Using a multigene (mtDNA + nDNA) phylogeny and fossil calibration methods similar to those used in other studies of darters (Near and Benard 2004; Near and Keck 2005) we attempt to answer three general questions regarding the evolutionary history of the barcheek species group: (1) What are estimated divergence times between species and populations within the clade? (2) Is there evidence for differential impacts of Pleistocene glacial cycles on the evolution of the clade? (3) Are cryptic species present within *E. basilare* that would suggest that within-drainage allopatric speciation has occurred on a much smaller spatial scale than previously realized in the species-rich North American freshwater fish fauna? Our ultimate goal is to determine if patterns observed in barcheek darters can be extrapolated to develop a mechanistic explanation for the abundant species diversity of freshwater fish in eastern North America that incorporates patterns of diversification at a more regional scale.

Materials and Methods

SPECIMEN COLLECTION AND DNA SEQUENCING

Specimens were collected using a minnow seine, or were obtained from museum collections. Sampling localities and specimen voucher information are given in Table 1, and mapped in Figure 1. In our field collections, tissue samples were obtained either by preserving a whole fish in absolute ethanol or removing an individual's right pectoral fin and preserving it in a 2 mL tube containing absolute ethanol. If a fin sample was taken from an individual the remaining specimen was fixed in formalin and deposited as a voucher in the University of Tennessee Research Collection of Fishes.

DNA was isolated from muscle or fin tissue using standard phenol-chloroform extraction and ethanol precipitation procedures or using Qiagen DNAeasy tissue kits (Qiagen, Valencia, CA). Complete coding regions of the mitochondrial NADH dehydrogenase 2 (ND2) and cytochrome *b* (cyt*b*) genes were PCR amplified using primers and conditions given in Kocher et al. (1995) and Near et al. (2000), respectively. In addition to the ND2 gene, the primers in Kocher et al. (1995) also amplified three complete tRNA gene regions (Met, Trp, and Ala). The nuclear encoded first intron of the S7 ribosomal protein (S7) was amplified using

Table 1. Specimen collection data. Absence of specific latitude and longitude data is denoted with n/a. Museum codes include University of Tennessee Research Collection of Fishes (UT), Illinois Natural History Survey (INHS), and North Carolina State Museum (NCSM).

Species	Locality (number of individuals)	Latitude	Longitude	Museum Voucher
Etheostoma barbouri	Price Creek, Casey Co., Kentucky (2)	37°10′50"N	84°56′34"W	INHS 27864
	East Fork Little Barren River, Metcalfe Co., Kentucky (2)	n/a	n/a	no voucher
Etheostoma basilare	Tributary to Duke Creek, Cannon Co., Tennessee (2)	35°40′23"N	86°05′03"W	INHS 27838
	Calfkiller River at Mill Creek, Putnam Co., Tennessee (10)	36°05′02"N	85°19′40"W	no voucher
	Scott Creek, Warren Co., Tennessee (3)	35°34′17"N	85°42′42"W	no voucher
	Scott Creek, Warren Co., Tennessee (5)	35°34′17"N	85°42′42"W	UT 91.6704
	Charles Creek, Warren Co., Tennessee (5)	35°43′27"N	85°47′04"W	UT 91.6589
	Cane Creek, Van Buren Co., Tennessee (10)	35°46′57"N	85°24′17"W	UT 91.6624
	Rocky River, Van Buren Co., Tennessee (9)	35°41′03"N	85°34′42"W	UT 91.6582
	Caney Fork River, White Co., Tennessee (3)	35°50′01"N	85°19′33"W	UT 91.6592
	Laurel Creek, Van Buren Co., Tennessee (9)	35°45′03"N	85°33′54"W	UT 91.6939
	Collins River, Grundy Co., Tennessee (10)	35°31′05"N	85°40′26"W	UT 91.6948
	Collins River, Warren Co., Tennessee (10)	35°40′32"N	85°42′36"W	UT 91.6940
	Duke Creek, Cannon Co., Tennessee (9)	35°39′59"N	86°03′51"W	UT 91.6944
	McMahan Creek, Cannon Co., Tennessee (9)	35°43′13"N	86°03′26"W	UT 91.6946
	Garner Branch, Warren Co., Tennessee (6)	35°38′39"N	85°54′01"W	UT 91.7106
Etheostoma derivativum	Arrington Creek, Williamson Co., Tennessee (2)	35°51′56"N	86°42′27"W	no voucher
	Little Marrowbone Creek, Davidson Co., Tennessee (1)	36°16′26"N	86°53′44"W	INHS 88930
	Sycamore Creek, Rutherford/Davidson Co., Tennessee (2)	36°23′23"N	86°53′56"W	no voucher
	West Fork Stones River, Rutherford Co., Tennessee (3)	35°47′11"N	86°25′19"W	no voucher
	Hurricane Creek, Rutherford Co., Tennessee (2)	35°42′40"N	86°16′20"W	no voucher
	Carr Creek, Robertson Co., Tennessee (3)	n/a	n/a	no voucher
	South Harpeth River, Williamson Co., Tennessee (3)	35°59′32"N	87°02′55"W	UT 91.7001
	North Fork Suggs Creek, Wilson Co., Tennessee (3)	n/a	n/a	no voucher
Etheostoma obeyense	Dutch Creek, Cumberland Co., Kentucky (1)	36°49′06"N	85°26′55"W	no voucher
	Duncan Branch, Wayne Co., KY (1)	36°45′32"N	84°52′38"W	no voucher
	West Fork Obey River, Overton Co., Tennessee (2)	36°19′46"N	85°11′18"W	no voucher
	Barn Branch of Mill Creek, Overton Co., Tennessee (1)	36°28′13"N	85°26′40"W	UT 91.6690
	Bryan's Fork of Mill Creek, Overton Co., Tennessee (2)	36°27′05"N	85°25′29"W	UT 91.6692
	Morgan Creek, Overton Co., Tennessee (2)	36°27′35"N	85°24′23"W	UT 91.6710
	Little South Fork River, Wayne Co., Kentucky (5)	36°39′05"N	84°47′50"W	UT 91.7523
Etheostoma smithi	Spencer Creek, Wilson Co., Tennessee (1)	36°14′31"N	86°25′56"W	no voucher
	Spencer Creek, Wilson Co., Tennessee (2)	36°14′31"N	86°25′56"W	UT 91.7084
	Ferguson Creek, Livingston Co., Kentucky (1)	37°08′28"N	88°21′35"W	INHS 28316
	Spring Creek, Wilson Co., Tennessee (2)	36°05′18"N	86°13′39"W	no voucher
	West Fork Spring Creek, Wilson Co., Tennessee (2)	36°06′33"N	86°15′28"W	no voucher
	East Fork Stones River, Rutherford Co., Tennessee (2)	35°53′26"N	86°17′08"W	no voucher
	Florida Creek, Wilson Co., Tennessee (2)	36°00′10"N	86°14′35"W	no voucher
	Mill Creek, Davidson Co., Tennessee (3)	35°59′41"N	86°41′33"W	UT 91.7100
	Muddy Fork, Christian Co., Kentucky (1)	36°59′06"N	87°38′29"W	UT 91.7290
Etheostoma striatulum	Hurricane Creek, Bedford Co., Tennessee (1)	35°33′25"N	86°29′56"W	no voucher
	Hurricane Creek, Bedford Co., Tennessee (1)	35°33′25"N	86°29′56"W	NCSM 29833
	Wartrace Creek, Bedford Co., Tennessee (1)	35°35′14"N	86°20′24"W	no voucher
Etheostoma virgatum	tributary to Roundstone Creek, Rockcastle Co., Kentucky (1)	37°26′17"N	84°18′55"W	INHS 27832
	Clear Creek, Rockcastle Co., Kentucky (1)	37°28′39"N	84°15′37"W	INHS 37939
	Middle Fork Rockcastle River, Jackson Co., Kentucky (1)	37°20′36"N	84°04′44"W	no voucher
Etheostoma ditrema	Camp Branch, Shelby Co., Alabama	n/a	n/a	no voucher
Etheostoma squamiceps	Big Creek, Hardin Co., Illinois	n/a	n/a	INHS 48199
Etheostoma oophylax	McMullough Fork, Calloway Co., Kentucky	n/a	n/a	INHS 44536
Etheostoma percnurum	Copper Creek, Scott Co., Virginia	n/a	n/a	no voucher
Etheostoma flabellare	Middle Fork Vermillion River, Vermillion Co., Illinois	n/a	n/a	no voucher
Etheostoma kennicotti	Poor Fork, Letcher Co., Kentucky	n/a	n/a	no voucher
Percina caprodes	Big Piney Fork, Sharp Co., Arkansas	n/a	n/a	INHS 41160
Percina roanoka	Blackwater River Franklin Co., Virginia	n/a	n/a	INHS 64359

primers and conditions presented in Chow and Hazama (1998). Amplified PCR products were prepared for DNA sequencing with enzymatic degradation using shrimp alkaline phosphatase and exonuclease I, incubated at 37°C for 15 minutes and 80°C for 15 minutes. Cycle sequencing reactions using PCR templates were performed at the Molecular Systematics and Conservation Genetics Laboratory, Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, USA. Gene sequences were constructed from individual sequencing reactions using Sequencher version 4.5 (Gene Codes, Ann Arbor, MI). No individual was observed to possess S7 intron 1 alleles that differed in size, but all heterozygous sites were scored using the ambiguity codes and treated as missing data in all subsequent analyses.

Based on previous phylogenetic studies of the dater clade *Catonotus* (Braasch and Mayden 1985; Porterfield et al. 1999; Page et al. 2003), the barcheek darter phylogenies were rooted using a set of outgroups that contained sequence data from five species sampled from the two other species groups in the clade: Spottail (*E. squamiceps* group) and fantail darters (*E. flabellare* group) (Page 1975; Braasch and Mayden 1985). Gene sequences of ND2, cyt*b*, and S7 for the outgroup taxa were downloaded from GenBank. Data used for external fossil calibration were downloaded from GenBank and consisted of ND2 and S7 DNA sequence data for 46 individuals representing 32 species sampled from the freshwater fish clade Centrarchidae (Near et al. 2004, 2005).

DNA SEQUENCE ALIGNMENT, MODEL SELECTION, AND PHYLOGENETIC ANALYSIS

We performed two sets of DNA alignments for our phylogenetic analyses and estimation of divergence times in barcheek darters. The first set of DNA alignments included the mtDNA genes (cyt*b*, ND2, and tRNAs) and the S7 intron sequences collected from 159 individuals sampled from all seven barcheek darter species and the five sampled species from other subclades of *Catonotus*. Because cyt*b* sequences were not available for the centrarchid species, the second set of DNA alignments included the ND2 and S7 intron sequences from all individuals included in the first set of alignments plus three darter species not classified as *Catonotus* (*Etheostoma ditrema*, *Percina roanoka*, and *P. caprodes*), and 47 individuals sampled from Centrarchidae. Alignments of the cyt*b*, ND2 and tRNA genes were done by eye. The computer program MUSCLE (Edgar 2004) was used to align the S7 intron DNA sequences. Genbank accession numbers for the centrarchid ND2 and S7 intron DNA sequences are given in Near et al. (2005), and those for the cyt*b*, ND2, and S7 intron DNA sequences sampled from the darter species are given in the Supporting Appendix S1.

Earlier phylogenetic analyses suggested incongruence among gene trees for barcheek darters generated from mitochondrial and nuclear genes, with mitochondrial phylogenies resulting in a paraphyletic barcheek darter clade. However, model based maximum likelihood analysis of combined mitochondrial and nuclear gene DNA sequences resulted in the monophyly of the barcheek darters (Page et al. 2003). Our preliminary analyses reflected these results and we used Bayesian analyses of the combined DNA sequences from the mitochondrial and nuclear genes. The same phylogenetic analyses were performed on both sets of our DNA sequence alignments.

Phylogenetic hypotheses were generated with a partitioned Bayesian analysis (Ronquist and Huelsenbeck 2003) with posterior probabilities estimated using metropolis-coupled Markov chain Monte Carlo (Larget and Simon 1999; Huelsenbeck et al. 2001). Eight data partitions were identified based on previous studies (Near et al. 2005; Near and Keck 2005; Keck and Near 2008): three codon positions from each of the two protein coding genes (ND2 and cyt*b*), the pooled tRNA genes, and the S7 intron. The optimal maximum likelihood model for each partition was determined with AIC as executed in the computer program Modeltest 3.0 (Posada and Crandall 1998). The models identified for each individual data partition were used in a partitioned Bayesian analysis using the computer program MrBayes 3.1 (Ronquist and Huelsenbeck 2003), which was run three separate times for 6.0×10^6 generations each to ensure convergence of the MC3 algorithm. Model parameter values that included the nucleotide substitution rate matrix, the shape parameter for the gamma distribution that models among site rate variation, and nucleotide frequencies were unlinked among the partitions in the MrBayes runs. The number of generations discarded from each MrBayes run and treated as the burn-in was determined by assessing convergence using the computer program Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer/) to plot the maximum likelihood score versus the number of generations. The posterior probabilities of nodes in the phylogeny were calculated from the set of post burn-in trees.

We tested alternative phylogenetic hypotheses using a maximum likelihood Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa 1999; Goldman et al. 2000) as implemented in the computer program PAUP* 4.0b (Swofford 2003). The six alternative phylogenetic hypothesis were developed to reflect the expected relationships if sister species, or lineages, are found in adjacent river systems. These alternative phylogenies based on the expected biogeographic pattern include *E. barbouri* as the sister species to all other barcheek darters, *E. derivativum* and *E. smithi* as sister species, *E. derivativum* and *E. striatulum* as sister species, *E. basilare* and *E. derivativum* as sister species, *E. obeyense* and *E. virgatum* as sister species, and *E. basilare* and *E. obeyense* as sister species. The best trees representing these alternative hypotheses were generated with maximum parsimony constraint tree searches in PAUP* 4.0.

MOLECULAR DIVERGENCE TIME ESTIMATES

Because of substantial heterogeneity in nucleotide substitution rates among lineages, a relaxed clock method was used to estimate divergence times in the barcheek darter clade. Most relaxed clock methods assume that rate variation between ancestral and descendent branches is autocorrelated, but methods that use an uncorrelated lognormal model (UCLN) assume that substitution rates on adjacent branches are independently sampled from a lognormal distribution (Drummond et al. 2006). The UCLN model was implemented in our study by using the computer program BEAST version 1.4.6 (Drummond and Rambaut 2007).

The darter fossil record is very poor (Smith 1981; Cavender 1986), and previous attempts to estimate divergence times using darter molecular phylogenies have used external fossil calibrations from the Centrarchidae (Near and Benard 2004; Near and Keck 2005). Centrarchid fish are classified in the same taxonomic order as darters, have a rich fossil record that has been used to calibrate molecular phylogenies, and there are extensive comparative molecular data available for this clade (Near et al. 2003, 2004, 2005). The ND2 and S7 gene sequences sampled from barcheek darter species were aligned with those previously sampled from all other centrarchid species (specimen information given in Near et al. 2005). Five centrarchid fossils identified as producing internally consistent age estimates among a set of 10 fossil calibrations using a fossil cross-validation analysis were used to calibrate the barcheek darter phylogeny (Near et al. 2005). The phylogenetic placement of the fossils within Centrarchidae was identical to that reported in Near et al. (2005).

The UCLN model executed in BEAST allows uncertainty in the absolute ages of the fossil calibrations that is represented as prior distributions as opposed to using point age estimates, or fixed ages. The centrarchid fossil age estimates were treated as probability distribution-based calibrations, using a lognormal distribution with a zero point minimum bound reflecting the geologic age estimate of the fossil (Ho 2007). The uncertainty of the zero point minimum bound age estimate of the fossil was determined through the results of previous fossil cross-validation analyses (Near et al. 2005) and the temporal bounds of geological chrons or Land Mammal Age intervals of which particular centrarchid fossil bearing formations are dated. The mean and standard deviation of the lognormal prior distribution of the fossil age was modified to allow the 95% of the distribution to contain the estimated lower bound. The age, taxonomic identification, and specifics of the lower and upper bound ages used for the fossil calibrations are given in Table 2.

The posterior probability density of divergence times was estimated using the optimal molecular evolutionary models determined from likelihood ratio tests and an assumption that the UCLN model best explains the evolution of nucleotide substitution rates in barcheek darters. A Yule process speciation prior was used for the branching rates in the phylogeny. A total of six independent runs were executed in BEAST and each run consisted of 2.0×10^7 generations and the resulting trees and log files from each run were combined using the computer program LogCombiner version 1.4.6 (http://beast.bio.ed.ac.uk/LogCombiner). Convergence of parameter values and estimated ages of nodes to optimal distributions was assessed by plotting the marginal probabilities using Tracer version 1.4 (Drummond and Rambaut 2007). The posterior probability density of the combined tree and log files was summarized using TreeAnnotator version 1.4.6 (Drummond and Rambaut 2007). The mean and 95% highest posterior density (HPD) estimates of divergence times were visualized on the chronogram using the computer program FigTree version 1.1 (Drummond and Rambaut 2007). BEAST was also run using an empty alignment, or without data, to determine the effect of the calibration priors on the divergence time estimates. The .xml format file used for the BEAST analyses is available in the Supporting Appendix S2.

Table 2. Centrarchid fossil calibrations, minimal fossil ages, lognormal mean and SD used to shape and scale the lognormal prior distribution on node ages, and the 95% lognormal prior on the calibration age. A dagger identifies extinct species.

Fossil	Minimal fossil age (mya)	Lognormal mean	Lognormal SD	Age at 95% lognormal prior distribution (mya)
Lepomis megalotis	2.4	0.5	0.5	6.1
Lepomis humilis	3.4	0.5	0.5	7.1
Lepomis †*kansasensis*	6.6	0.4	0.5	10.0
Pomoxis sp.	12.0	0.5	0.5	15.7
Archoplites †*clarki*	15.5	0.1	0.5	18.0

Results

The combined cyt*b*, ND2, tRNA, and S7 dataset contained 2926 aligned sites. The only missing data were approximately 30 base pairs from the 5′ end of cyt*b* for eight individuals, six *E. basilare* and two *E. striatulum.* Partitioned Bayesian analyses on this alignment and the less inclusive alignment used in divergence time analysis resulted in identical tree topologies with regard to relationships among the barcheek darter species (Fig. 2). Therefore, inclusion of the more distantly related centrarchid species to provide external fossil calibrations seemed to have no effect on the resulting barcheek topologies. Most of the interspecific nodes in the barcheek phylogeny, including the most recent common ancestor (MRCA) of the entire barcheek darter clade, were supported with significant Bayesian posterior probabilities (Fig. 2). However, the MRCA of *E. barbouri* and *E. basilare* as well as the MRCA of

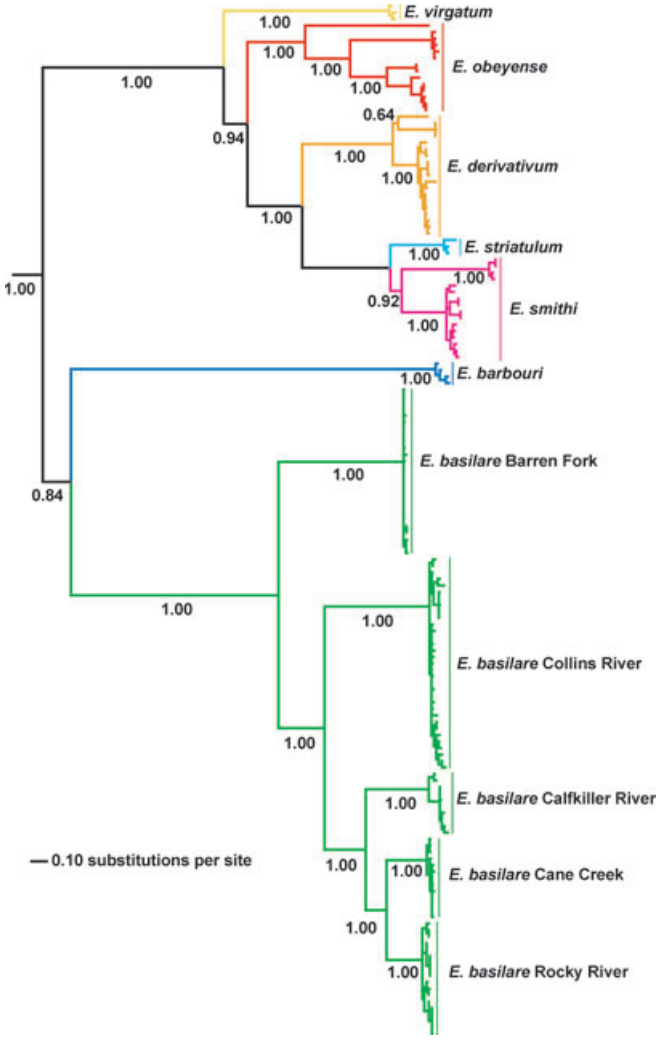

Figure 2. Phylogeny of barcheek darter species resulting from partitioned Bayesian analysis of combined mitochondrial (cytochrome b and NADH subunit 2) and nuclear encoded S7 ribosomal protein intron 1 gene sequences. Numbers below nodes correspond to Bayesian posterior probabilities. Colored branches correspond to species as in Figure 1.

E. obeyense and the clade containing *E. derivativum*, *E. smithi*, and *E. striatulum* were not supported with significant Bayesian posterior probabilities (Fig. 2). All seven barcheek species were reciprocally monophyletic (Fig. 2).

Shimodaira–Hasegawa maximum likelihood tree topology tests were able to reject four of the six alternative hypotheses of relationships within the barcheek darter clade that were constructed to depict species being found in adjacent areas as sister lineages (Fig. 3). The alternative phylogeny that has *E. barbouri* as the sister species to all other barcheek darter species could not be rejected. The optimal Bayesian phylogeny includes *E. barbouri* and *E. basilare* as sister species (Fig. 2). Additionally, the Bayesian phylogenetic analysis results in phylogenies that place *E. virgatum* as sister to a clade containing *E. obeyense*, *E. derivativum*, *E. smithi*, and *E. striatulum* (Fig. 2); however, the Shimodaira–Hasegawa test cannot reject the hypothesis that *E. obeyense* and *E. virgatum* are sister species (Fig. 3).

Extensive intraspecific genetic structure was observed in the four barcheek darter species represented in our study by more than three sampled populations. Maximum pairwise sequence divergence at both the mtDNA genes and the nuclear encoded S7 intron within species was high for *E. basilare*, *E. obeyense*, *E. smithi*, and *E. derivativum*, with maximum pairwise differences at the mtDNA genes ranging between 2.74% and 9.72% and those at the nuclear encoded S7 intron ranging between 1.34% and 2.30% (Table 3). Thorough sampling across the geographic range of *E. basilare* (Fig. 1) revealed a striking pattern of population structure. Five reciprocally monophyletic and deeply divergence clades were discovered within this species, with each clade restricted to a tributary system of the relatively small Caney Fork River (Figs. 1 and 2).

The distribution of observed S7 ribosomal protein intron 1 alleles among populations of *E. basilare* is presented in Table 4. Unique S7 intron 1 alleles were present in three (Barren Fork, Collins River, and Cane Creek) of the five clades identified in the phylogenetic analysis of combined mitochondrial and nuclear gene sequence (Fig. 2). Alleles shared between clades reflected the topology of the phylogenetic tree, as two alleles were shared between the Barren Fork and Collins River clades. These lineages comprise the earliest splits in the *E. basilare* intraspecific phylogeny and each of these two clades contains a high number of private alleles relative to the other three clades (Table 4). One rare allele was shared between the Calfkiller River and Cane Creek clades, and a common allele was shared among most sampled individuals in the Calfkiller River, Cane Creek, and Rocky River clades that form a monophyletic group in the phylogeny (Fig. 2; Table 4).

The BEAST UCLN analyses identified substantial nucleotide substitution rate heterogeneity in the combined ND2-S7 intron dataset, as measured by the coefficient of variation statistic logged in BEAST ($\sigma = 0.316$, 95% HPD: [0.225, 0.411]). Rate variation across ancestor and descendent lineages were uncorrelated (covariance = 0.025, 95% HPD: [−0.122, 0.172]). Running BEAST with the empty alignment resulted in divergence time estimates that were considerably older than analyses using the data. This result indicated that the priors used in the model are not having a strong effect on the estimated divergence times. The chronogram that includes Centrarchidae and all sampled darter species is shown in Figure 4. The estimated age of the Centrarchidae MRCA (32.6 mya, 95% HPD: [25.3, 39.9]) is very similar to a previous estimate (33.6 ± 3.6 mya) using fixed fossil calibrations in a penalized likelihood analysis that included a larger sampling of both mitochondrial and nuclear genes (Near et al. 2005).

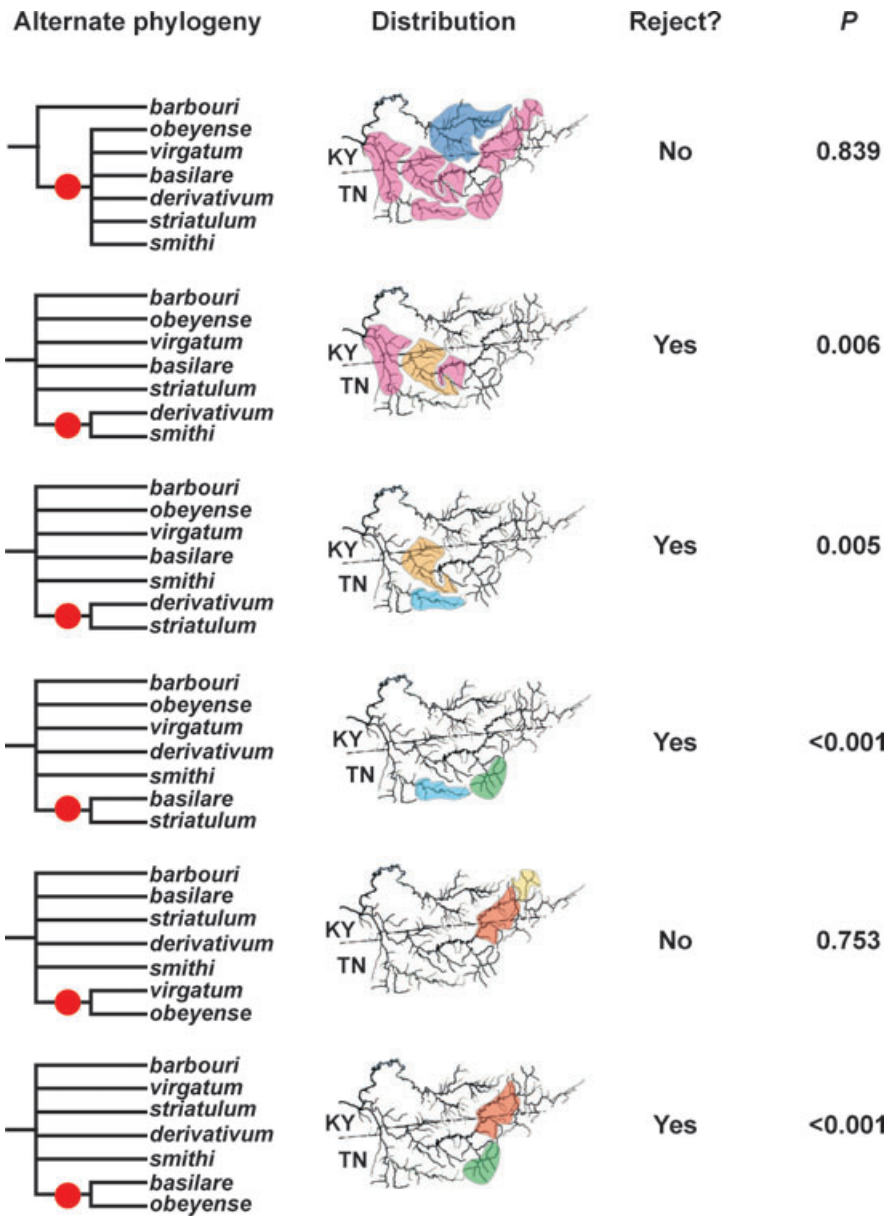

Figure 3. **Alternative phylogenies and results of Shimodaira–Hasegawa tree topology tests. Maps show the geographic distribution of the alternative sister lineage relationships compared to the optimal Bayesian tree shown in Figure 2.**

The UCLN age estimate of the MRCA of the barcheek darter clade was 16.3 mya, 95% HPD: [12.4, 20.5], and the estimated ages of all other interspecific nodes in the barcheek darter phylogeny ranged between 14.9 mya, 95% HPD: [11.0, 18.8] and 4.0 mya, 95% HPD: [2.6, 5.5] (Fig. 5, Table 5). As expected, most of the intraspecific nodes were younger than the interspecific nodes many dating to less than 1.0 mya. However, several of the intraspecific nodes where characterized by very old age estimates. For example, three major clades were observed in *E. obeyense* and the age estimate for the MRCA of all sampled *E. obeyense* populations was 6.0 mya, 95% HPD: [4.0, 8.1] (Fig. 5, Table 5). The first split in the *E. obeyense* clade involved a population sampled from Dutch Branch (Beaver Creek system) in Kentucky, and the other two clades with appreciable divergence times include a population sampled from the Little South Fork River in Kentucky, and all populations sampled west of the Beaver Creek population. Among the barcheek darters, the oldest estimate for an intraspecific node was the MRCA of all sampled *E. basilare* populations [8.0 mya, 95% HPD: (5.6, 10.7)] (Fig. 5, Table 5). This node represented the MRCA of the Barren Fork River population and the rest of the clade (Fig. 5). The four other clades present within *E. basilare* also exhibited relatively high divergence time estimates ranging from 5.4 mya, 95% HPD: [3.6, 7.2], between the populations sampled from the Collins

Table 3. Uncorrected pairwise intraspecific genetic distances observed at mitochondrial and nuclear genes for barcheek darter species.

Species	No. examined	Maximal mtDNA divergence	Maximal nDNA divergence
E. barbouri	4	0.50%	0.19%
E. basilare	100	9.72%	1.52%
E. derivativum	19	2.74%	1.34%
E. obeyense	14	8.15%	2.30%
E. smithi	16	4.80%	1.52%
E. striatulum	3	0.46%	0.19%
E. virgatum	3	0.27%	0.19%

River system and the rest of the clade, to 2.0 mya, 95% HPD: [1.2, 2.9], between the geographically adjacent Rocky River and Cane Creek–upper Caney Fork River proper populations (Figs. 1 and 5).

Discussion

The phylogenetic analyses of combined mitochondrial and nuclear gene DNA sequences sampled from the barcheek darter species clade resulted in a resolved and strongly supported phylogenetic tree (Fig. 2). Our results and those presented in Mendelson and Simons (2006) both revealed substantial intraspecific genetic divergence at both mtDNA and nuclear genes for every recognized barcheek species sampled broadly throughout its geographic distribution (Fig. 2; Table 3). The relationships among the barcheek darter species in our phylogenies are very similar to previous studies (Page et al. 2003; Mendelson and Simons 2006) and provide an important historical framework to examine the timing and pattern of diversification of the barcheek darter clade.

Table 4. Distribution of S7 ribosomal protein intron 1 alleles among 100 sampled *Etheostoma basilare* specimens.

Allele	Frequency	Barren Fork	Collins River	Calfkiller River	Cane Creek	Rocky River
1	0.37	16	22	0	0	0
2	0.02	0	0	1	1	0
3	0.01	0	0	0	1	0
4	0.38	0	0	9	11	18
5	0.01	1	0	0	0	0
6	0.02	2	0	0	0	0
7	0.11	3	8	0	0	0
8	0.02	0	2	0	0	0
9	0.01	0	1	0	0	0
10	0.01	0	1	0	0	0
11	0.03	3	0	0	0	0
12	0.01	1	0	0	0	0

TIMING OF DIVERSIFICATION IN BARCHEEK DARTERS AND INSIGHTS TO THE RICH BIODIVERSITY OF EASTERN NORTH AMERICA

Given the fact darters are essentially absent from the known fossil record of North American freshwater teleosts (Smith 1981; Cavender 1986), inferences on the timing of diversification in darters has been based on patterns observed in other freshwater teleost clades and the correlation of paleogeographic events with instances of lineage diversification. The initial estimates of the timing of diversification in darters and other clades of North American freshwater teleosts hypothesized a very recent origin (less than 2.5 mya) based on comparisons of these clades (i.e., darters, minnows, suckers) with examples of "explosive" speciation in African Rift lake cichlids and Lake Lanao cyprinids (Jenkins et al. 1972). Older age estimates were based on the timing of paleogeographic events associated with the Central Highlands. For example, sister clades distributed in the disjunct Interior and Eastern Highlands were dated minimally at the onset of Pleistocene glaciation approximately 2.5 mya (Pflieger 1971; Mayden 1987, 1988); however, because it was hypothesized that clades were present prior to the Pleistocene, many were expected to have origins prior to 2.5 mya (Wiley and Mayden 1985). Phylogenetic studies of Central Highlands freshwater teleost clades that have used genetic data to infer divergence times indicate that the timing of diversification for some clades is consistent with previous hypotheses, but many other clades do not fit specific temporal predictions and exhibit pseudocongruence with regard to biogeographic patterns (Strange and Burr 1997; Near et al. 2003; Near and Keck 2005).

Because barcheek darters are endemic to a single highland region, the Central Highlands Vicariance Hypothesis cannot provide a prediction regarding the timing of diversification in this clade. The lack of appreciable morphological disparity among the barcheek darter species and the fact that the species in the clade exhibit very little variation in habitat and resource utilization are important factors when considering their strict allopatric distribution (Page and Burr 1976; Flynn and Hoyt 1979; Page 1980; Braasch and Mayden 1985; Kopp 1985; Page et al. 2003). The absence of disparity among the barcheek darter species may be interpreted as evidence for a recent evolutionary origin of the clade. However, inferences from this study based on lineage age estimates resulting from molecular analyses indicate that barcheek darters are a relatively ancient darter clade with an origin close to 16 mya (Figs. 4 and 5; Table 5).

Both the ancient inter- and intraspecific age estimates indicate that diversification in barcheek darters has been influenced by ancient fragmentation among populations, and there appears to have be little movement of populations between tributaries of the Cumberland River Basin, particularly in the eastern portion of the system. Pleistocene glacial cycles, however, may have had more

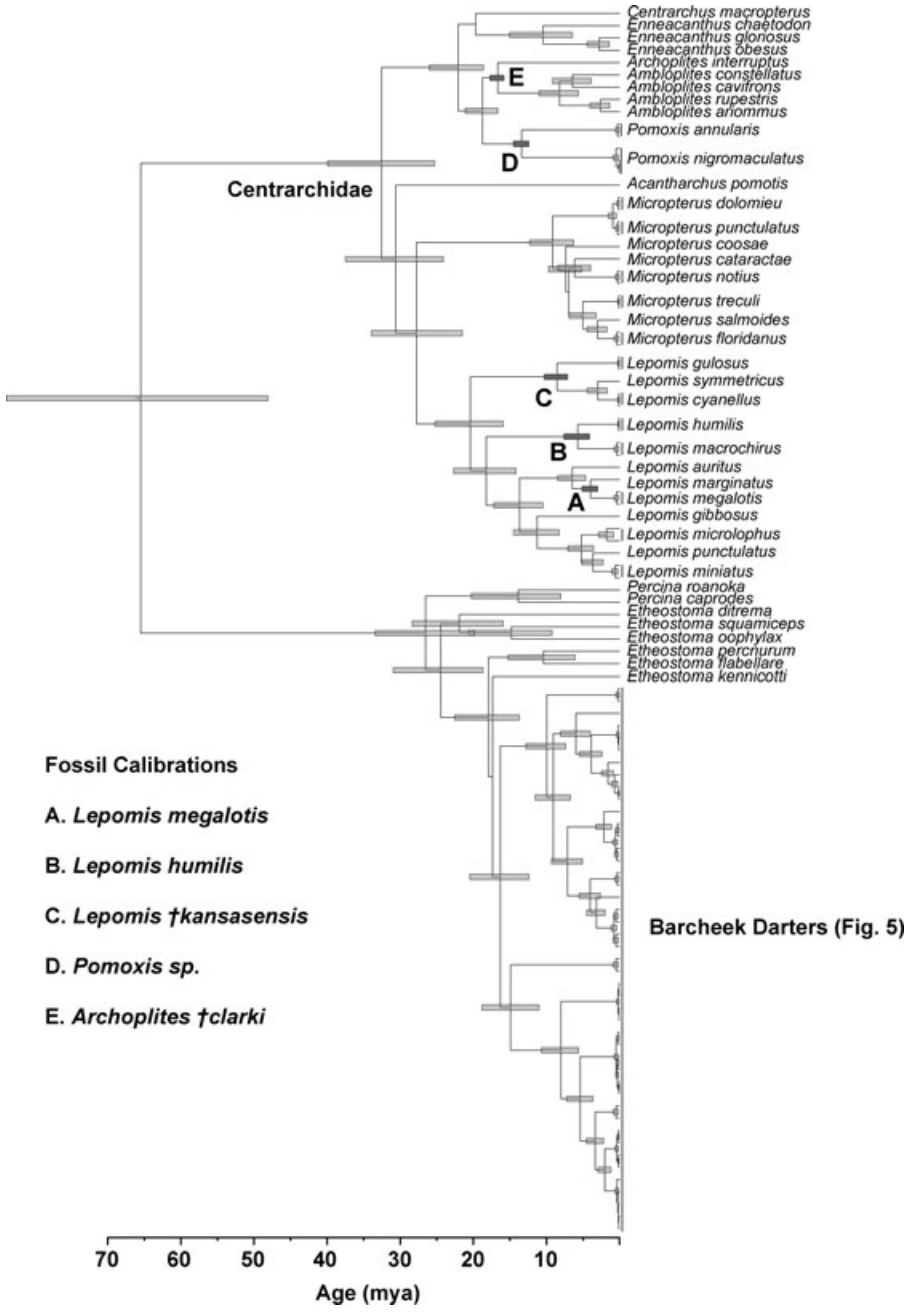

Figure 4. **Chronogram for centrarchid and darter species estimated from a set of combined BEAST analyses using the combined mitochondrial (NADH subunit 2) and nuclear (S7 ribosomal protein intron 1) encoded gene sequences. The phylogenetic placement of the individual calibrations listed in Table 2 are marked with capital letters (A–E). Uncertainty in the divergence times are shown by bars on nodes with the length corresponding to the 95% highest posterior density (HPD) of the node ages. HPD bars for calibration nodes are shaded with dark gray.**

influence on the evolution of the clade in the western portion of its range. There is a temporal signature of diversification in barcheek darters that is consistent with this hypothesis, as the youngest diversification events involve barcheek darter species distributed in the western Cumberland River Basin, Duck River, and lower Tennessee River Basin, and the older diversification events involve lineages distributed in the eastern Cumberland River Basin (Figs. 1 and 5). This may reflect the paleogeographic disturbance of these areas at the onset of the Pleistocene (Thornbury 1965; Braasch and Mayden 1985; Starnes and Etnier 1986).

The mean posterior age estimates for the MRCA nodes in *E. basilare* (8.0 mya), *E. obeyense* (6.0 mya), and *E. smithi* (3.2) mya are surprisingly ancient, and exceed many of the interspecific age estimates in other darter clades (Fig. 5) (Near and Benard

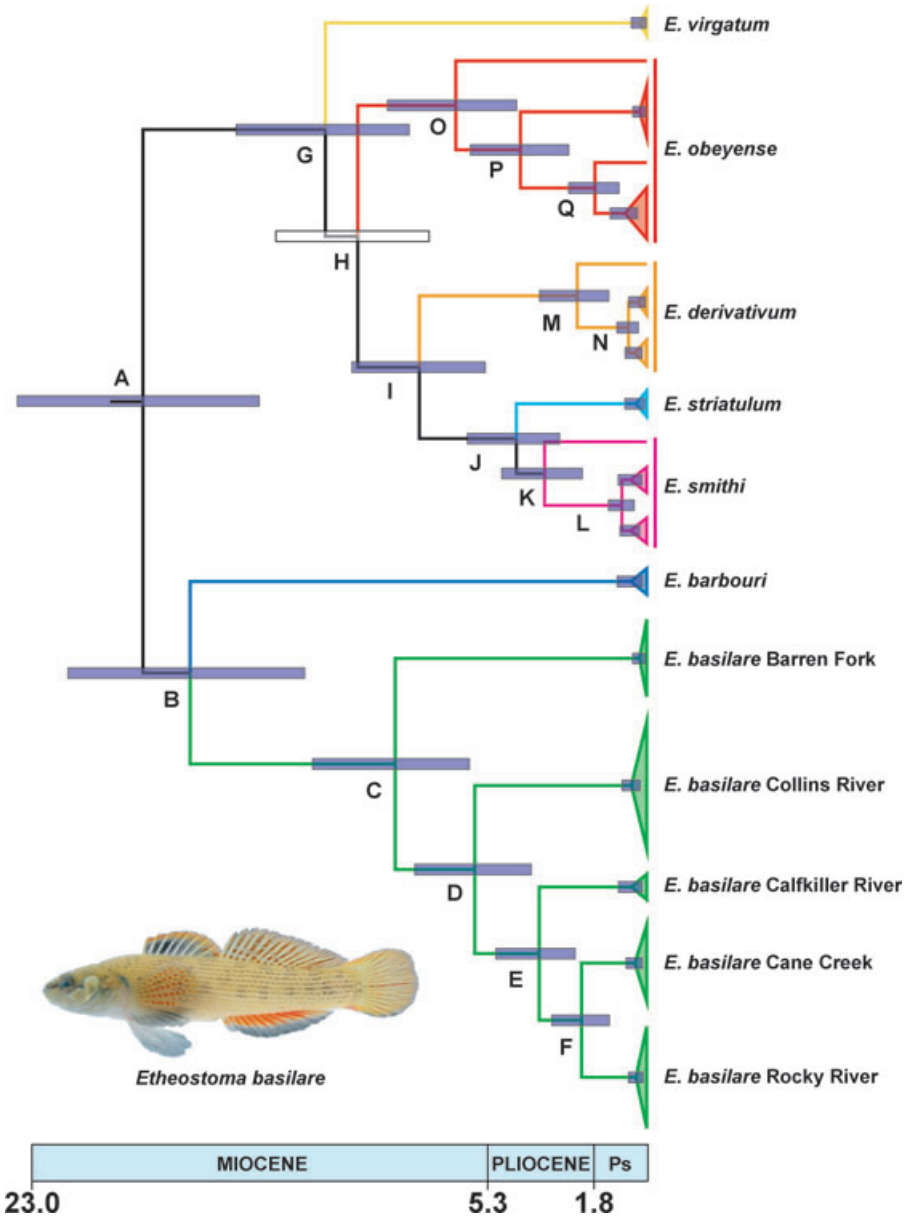

Figure 5. Chronogram for barcheek darter species estimated from a set of combined BEAST analyses using the combined mitochondrial (NADH subunit 2) and nuclear (S7 ribosomal protein intron 1) encoded gene sequences. The chronogram is calibrated against the Neogene geologic time scale (Lourens et al. 2004). Uncertainty in the tree topology and divergence times are shown by bars on nodes with the length corresponding to the 95% highest posterior density (HPD) of the node ages. The shading communicates the posterior probabilities of nodes with shaded bars greater or equal to 0.95 and open bars less than 0.95. Age estimates and 95% HPD for labeled nodes is given in Table 5. Colored branches correspond to species as in Figure 1.

2004; Near and Keck 2005). Given these deep splits between and within species, a reasonable exercise would involve investigating any possible geographic barriers to dispersal that could have resulted in long-term isolation of barcheek darter populations. The origin of *E. barbouri* and *E. striatulum* is straightforward, because both of these species exist outside of the Cumberland Basin (Fig. 1). However, all of the other barcheek darter species are distributed in the Cumberland Basin. It is possible that the main stem of the Cumberland River serves as a barrier to dispersal, because such large river habitat may not be suitable for barcheek darters. However, this hypothesis fails to explain the substantial divergence observed between populations within *E. basilare*, *E. obeyense*, and *E. smithi* that are distributed among interconnected smaller streams and tributaries of the Cumberland River. In addition, the complex and interdigitated geographic distributions exhibited by *E. smithi* and *E. derivativum* indicate that historical instances of dispersal through the main stem of the Cumberland River have occurred multiple times (Fig. 1).

Table 5. Divergence times of barcheek darters reported as posterior mean ages in millions of years (mya) estimated using an uncorrelated lognormal relaxed clock method. The credibility intervals for age estimates are reported as the 95% highest posterior density (HPD). Nodes correspond to the chronogram shown in Figure 5.

Node	Posterior mean age (mya)	95% HPD
A	16.3	12.4, 20.5
B	14.9	11.0, 18.8
C	8.0	5.6, 10.7
D	5.4	3.6, 7.2
E	3.3	2.2, 4.6
F	2.0	1.2, 2.9
G	10.0	7.4, 12.8
H	9.1	6.7, 11.6
I	7.1	5.1, 9.3
J	4.0	2.6, 5.5
K	3.2	2.0, 4.5
L	0.8	0.4, 1.2
M	2.1	1.1, 3.2
N	0.6	0.3, 0.9
O	6.0	4.0, 8.1
P	3.9	2.4, 5.5
Q	2.0	0.9, 2.4

As more divergence time estimates for aquatic vertebrate clades distributed in the Central Highlands become available, it will be possible to investigate if the temporal history of barcheek darters has revealed a pattern of diversification generally observed in other lineages that comprise the species-rich Central Highlands freshwater fauna. Specifically, it should be assessed if other clades and species lineages exhibit the ancient divergence times and substantial microendemic population genetic divergence observed among barcheek darter species. Investigations of eastern plethodontid salamanders have revealed extensive genetic divergence among populations of *Eurycea cirrigera* distributed in the Cumberland River Basin (Kozak et al. 2006a). Estimation of divergence times among species of *Plethodon* distributed in eastern portion of the Central Highlands reveals that the clade exhibits a divergence time predating the age estimate for the MRCA of barcheek darters by less than 10 million years (Kozak et al. 2006b; Wiens et al. 2006).

In contrast to these ancient intra- and interspecific divergence time estimates, several examples of relatively shallow genetic differentiation and recent divergence times between and within species are present in Central Highland endemic clades. *Etheostoma cinereum* and *Percina burtoni* are two darter species endemic and distributed throughout the Tennessee, Cumberland, and Duck River basins (Lee et al. 1980; Page 1983; Burr and Warren 1986; Etnier and Starnes 1993; Boschung and Mayden 2004). Phylogeographic analyses of mtDNA sequences resulted in reciprocal monophyly of *E. cinereum* and *P. burtoni* populations sampled from each of these major river basins (Powers et al. 2004; George et al. 2006). We examined the intraspecific phylogenies and sequence data from these two studies in an unpublished analysis and estimated the age of the intraspecific MRCA of *E. cinereum* close to 1.5 mya and that of *P. burtoni* approximately 2.0 mya. The *Nothonotus maculatus* clade contains five species with strict allopatric distributions in the Tennessee and Cumberland River basins (*N. maculatus* does extend to the Ohio River Basin), and is another example of a Central Highlands clade exhibiting recent divergence times among species with many species in the clade as young as 1.0 ± 0.6 mya (Near and Keck 2005).

The emergent pattern from divergence time estimates among endemic clades of the Central Highlands is a wide range of intraspecific coalescent times and divergence times between closely related species. Some of the most biodiversity-rich areas of the world are large geographic areas characterized by long periods of climatic and geologic stability (Rosenzweig 1995), such as the disjunct elements of the Central Highlands. These areas may act as "museums" by preserving ancient clades through reduced extinction (Stebbins 1974). However, the presence of younger clades endemic to the Central Highlands indicates that in addition to accumulating biodiversity as a "museum," this region may also be acting as a "cradle" of diversification (Stebbins 1974). Combinations of the museum and cradle models formulated by Stebbins (1974) have recently been proposed to explain high biodiversity in the tropics (Jablonski et al. 2006; McKenna and Farrell 2006). We propose that in addition to serving as the area where aquatic biodiversity is generated, the geologic and climatic stability of the Central Highlands facilitates the preservation of processes of population differentiation and speciation that have occurred across substantial time scales.

PATTERN AND SCALE OF SPECIATION IN BARCHEEK DARTERS

The complete lack of sympatry among barcheek darter species is consistent with the long-standing observation that the history of diversification among North American freshwater fishes is characterized by allopatric speciation (Cope 1868; Jordan 1905, 1908; Wiley and Mayden 1985; Near et al. 2003; Near and Benard 2004; Near and Keck 2005). However, most studies have investigated allopatric speciation among North American fishes on a scale between major river drainages or between disjunct highland areas. Recently, investigation into the phylogenetics of *Nothonotus* darters (Near and Keck 2005; Keck and Near 2008) and minnows in the *Notropis rubellus* species clade (Berendzen et al. 2008) has revealed that extensive diversification in these clades has occurred within major river drainages as opposed to between them. The barcheek darters seem to fit this pattern of within-drainage

speciation as the majority of the clade is restricted to the Cumberland River basin (Fig. 1). The time-calibrated phylogeny and distribution of barcheek darters indicates that allopatric speciation has occurred continually throughout the history of the clade, and at much smaller geographic scales than has been typically investigated among North American freshwater teleost fishes. This diversification could have been facilitated through geographic isolation of populations in rocky headwater tributaries coupled with sexual selection (Porter et al. 2002), eventually leading to speciation.

A study on the goby clade *Elacatinus* highlights an example of speciation at small geographic scales that is accompanied by morphological and ecological divergence among closely related species (Taylor and Hellberg 2005). The lack of apparent morphological and ecological diversification among barcheek darter species, despite their hypothesized allopatry through a significant portion of geological time, is a striking contrast to the pattern observed in *Elacatinus* gobies and suggests stabilizing selection and/or niche conservatism have been important factors in the history of diversification in barcheek darters. Explorations of niche modeling for barcheek darter species could potentially identify fine scale differences in habitats occupied by different barcheek species and discriminate between hypotheses of diversification based upon niche conservatism (Peterson et al. 1999; Wiens and Graham 2005; Kozak and Wiens 2006; Martínez-Meyer and Peterson 2006) versus ecological divergence among closely related species (Graham et al. 2004; Rissler and Apodaca 2007).

The relatively small geographic scale of diversification among barcheek darter species in the Cumberland River system appears expansive when considering the extreme pattern of microendemism exhibited among several cryptic lineages discovered in *E. basilare*. This study intensively sampled *E. basilare* populations and the phylogeny indicates that *E. basilare* is comprised of five clades distributed allopatrically among specific tributaries of the Caney Fork River (Figs. 1, 2, and 5). Each of these lineages exhibits ancient divergence times with a mean interval of 2.0 mya between diversification events among lineages. The monophyletic and divergent nature of these populations suggests *E. basilare* is comprised of five cryptic species that are phylogenetically diagnosable and reproductively isolated. Slight morphological differences in populations have been noted (Page et al. 2003), and formal species recognition may be warranted.

An alternative hypothesis to the presence of five species classified as *E. basilare* is that female philopatry and male-biased dispersal has resulted in a strong geographic structuring of mtDNA haplotypes among Caney Fork tributaries, leading to apparent differentiation of populations that are not necessarily reproductively isolated (Hoelzer 1997). Despite sampling a nuclear gene in our study, much of the phylogenetic signal is driven by variation in the sampled mtDNA genes. However, Mendelson and Simons (2006) found similar population structure within *E. basilare* based on AFLP markers that provide a screen of variation in the nuclear genome, and should be resistant to difficulties in interpretation due to sex-biased gene flow. Distance analyses of the AFLP markers resulted in three clusters that correspond to three clades present in the mtDNA and nDNA phylogeny (Fig. 2), a Collins River cluster, a Calfkiller River cluster, and a Barren Fork River cluster (Mendelson and Simons 2006). Our analysis of the S7 ribosomal intron 1 locus revealed limited sharing of alleles among the five clades comprising *E. basilare* (Table 4). Unique alleles were detected in three of these clades (Table 4). The presence of a larger number of unique alleles in the phylogenetically basal lineages (Barren Fork River and Collins River) is indicative of a fairly high historical effective population size and is consistent with a long period of genetic isolation. The congruence between the inferred clades in our Bayesian analyses and the clustering of populations in Mendelson and Simons' (2006) AFLP study coupled with the presence of unique nDNA alleles in three of the five populations is strong support for our hypothesis of cryptic species diversity within *E. basilare*.

The evolutionary processes that resulted in, and are currently maintaining, this fine-scale allopatric diversity in *E. basilare* could be similar to the mechanisms of diversification in other clades of eastern North American teleost fishes, particularly those restricted to the climatically and geologically stable regions of the Central Highlands. Our results suggest that allopatric divergence can take place within river systems in the absence of traditionally recognized barriers to gene flow and at much smaller spatial scales than previously realized. This is exemplified by the *E. basilare* clade that during the past eight million years has diversified into five distinct evolutionary lineages within the approximately 2000 km^2 drainage area that comprises the Caney Fork River and its tributaries.

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Supporting Information

The following supporting information is available for this article:

Appendix S1. GenBank accession information for gene sequences of cytochrome b (cyt*b*), NADH subunit 2 (ND2), and S7 ribosomal protein intron 1 (S7), and sampling locations for the darter specimens used in this study.

Appendix S2. Xml formatted file used in BEAST analyses.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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