# A Mitochondrial DNA Analysis of the Species Status of the Endemic Waccamaw Darter, *Etheostoma perlongum*

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**The Waccamaw Darter *Etheostoma perlongum* is one of three teleost fish species and several molluscs endemic to Lake Waccamaw, a large Carolina Bay in southeastern North Carolina. We assessed species status by sequencing most of the mitochondrial cytochrome *b (cyt b)* gene in *E. perlongum* and its putative sister species, *E. olmstedi*, from the adjoining Waccamaw River and streams in the nearby Pee Dee and Cape Fear Drainages. Phylogeographic analysis shows strongly supported river- and drainage-specific clades with divergence times dating to the Pleistocene. Haplotypes of *Etheostoma perlongum* are very similar and not phylogenetically distinct from sequences from the Waccamaw River population of *E. olmstedi*. However, strong haplotype frequency differentiation shows that free genetic exchange does not occur between the river and lake, and along with life history distinctiveness, suggests reproductive isolation despite contact between these populations. Coalescent simulations showed that incomplete lineage sorting between reproductively isolated populations is a plausible explanation for the failure of *cyt b* sequences to have achieved reciprocal monophyly. Gene tree/species tree discord equal to that seen in our *cyt b* data was produced in ≥ 5% of coalescent trees, using lake-river divergence times of T = 5–10 thousand years ago (kya), no gene flow and $N_e = 10^5$; simulations with more realistic values of $N_e = 10^4$ and T = 10–20 kya required recurrent lake-river migration to generate comparable levels of incomplete sorting of haplotypes. Studies of behavioral reproductive isolation and multilocus studies of nuclear DNA should be used to distinguish these scenarios and to further evaluate the species status of the endemic Waccamaw Darter.**

CAROLINA Bays are shallow, elliptical depressions in the landscape of the southeastern Atlantic coastal plain, with their axes oriented in a characteristic northwest-southeasterly direction. Earlier theories for their origin involving meteorite impact (Prouty, 1952) were replaced by geomorphological theories (Kaczorowski, 1977; Carver and Brook, 1989), but evidence favoring extraterrestrial impact origin has recently re-emerged (Firestone et al., 2007). Concentrated in North and South Carolina, Bays have a characteristic associated flora and are often filled with water. One of the largest is Lake Waccamaw in Columbus County, North Carolina. Pollen records (Frey, 1953, 1955), stratigraphic analysis, and radiocarbon dating of Waccamaw Lake sediments (Riggs et al., 2000) yield a maximum age of about 30,000 years, and suggest that lake formation postdated the last glacial maximum (20,000 to 14,000 years ago). Nevertheless, the lake contains several endemic species that include three fishes, each from a separate family (Hubbs and Raney, 1946), as well as gastropods and unionid bivalves (LeGrand et al., 2006).

One of these endemics is the Waccamaw Darter, *Etheostoma perlongum*, unique among the darters in being confined exclusively to a lentic environment. The Waccamaw Darter shows a number of morphological traits (e.g., a more fusiform body with more vertebrae) typical of lacustrine derivatives of stream-dwelling fishes (Hubbs and Raney, 1946). Distinctiveness of these characters was sufficient to lead Hubbs and Raney (1946) to describe *E. perlongum* as a separate species, most likely derived from the Tesselated Darter, *E. olmstedi*, which occurs in the adjacent Waccamaw River and in streams of the Atlantic coastal plain from northern New England to North Florida (Cole, 1967). Waccamaw Darters were also shown to grow more rapidly and to a larger terminal size, then to die after one breeding season (Shute et al., 1982), while Tesselated Darters reach a smaller terminal size and live from two to four years (Raney and Lachner, 1943). A subsequent morphological and allozyme analysis (Shute, 1984), nevertheless, questioned the species status of *E. perlongum*, suggesting it instead to be an ecomorph of *E. olmstedi*, and proposed that the two morphs freely interbreed where they meet in the upper Waccamaw River. In particular, Shute (1984) reports clines extending over 40 km (from 6 km below the lake to the South Carolina border) in the characters used in Hubbs and Raney's (1946) original species description, including the number of lateral-line scales and cheek and nape squamation. A cline in frequencies of one allele at the isocitrate dehydrogenase allozyme locus is also evident (Shute, 1984).

In this report, we use mitochondrial DNA (mtDNA) sequences to re-examine the species status of the Waccamaw Darter. To do so, we studied *E. perlongum* and several regional populations of *E. olmstedi*. Our goals were to determine whether *E. perlongum* shows phylogenetic distinctiveness of mtDNA sequences, and to infer its affinities to geographic populations of its putative sister species. While there are exceptions that depend on the genetic structure of the ancestral population, the demographic history of speciation, and the effective population sizes of the emergent forms, the time to reciprocal monophyly of nuclear DNA clades is expected to be approximately four times that of mtDNA clades (Neigel and Avise, 1986; Birky, 1991; Edwards and Beerli, 2000). Therefore, while Shute (1984) found shared allozyme polymorphisms between lake and river forms, it is reasonable to propose reciprocal monophyly of these populations for mtDNA. Following Shute's (1984) study, we focus on populations within the Waccamaw and Pee Dee drainages, because these most likely share common ancestry with *E. perlongum*.

## MATERIALS AND METHODS

***Fish collection and tissue sampling.***—Darters were collected from the following six sites from June 2002 through

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**Fig. 1.** Sampling locations for *Etheostoma perlongum* (Lake Waccamaw) and *E. olmstedi* (other sites). (A) Map of the sampled regions of the three watersheds. Site in the Cape Fear watershed: BR = Black River. (B) Sites in the Pee Dee watershed: PD = Pee Dee River, LPD = Little Pee Dee River, LR = Lumber River. (C) Sites in the Waccamaw watershed: WR = Waccamaw River (1: Old Dock bridge, 2: Seven Creeks, 3: Norris Lake Rd. bridge on Camp Swamp Creek, 4: Buck Creek); LW = Lake Waccamaw (1: NCWRC access, 2: North Shore, 3: near dam on Waccamaw River outlet).

November 2003 (Fig. 1). Locations are followed by site abbreviations and sample sizes ($n$) denoting the number of fishes characterized from each: Lake Waccamaw (LW; $n = 15$), Waccamaw River (WR; $n = 15$), Lumber River (LR; $n = 5$), Little Pee Dee River (LPD; $n = 3$), Pee Dee River (PD; $n = 5$), and Black River (BR; $n = 9$). Stream sampling for *E. olmstedi* was by kick-seining, and lake sampling for *E. perlongum* was by snorkeling with hand nets. Gill tissue was removed and immediately placed into salt-saturated DMSO preservative (Seutin et al., 1993). Specimens were returned to the laboratory and fixed in 10% formalin, washed extensively in water, then transferred into 70% ethanol, and are stored at the University of North Carolina at Wilmington.

***DNA extraction, PCR, and DNA sequencing.***—DNA was extracted from gill tissue using a modification of the ''Rapid Isolation of Mammalian DNA'' protocol in Sambrook and Russell (2000). The full-length mitochondrial cytochrome *b* (*cyt b*) gene was amplified using primers designed by Schmidt and Gold (1993). PCR reactions contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM each dNTP, 0.5 μM each primer, and 0.75 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA). Thermal cycling conditions included an initial denaturation step of 1 minute at 94°C, followed by 35 cycles of (1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C), then by 5 minutes at 72°C. Amplicons were purified over QIAquick columns (QIAGEN, Valencia, CA), and sequenced using Big Dye Terminator Cycle Sequencing Kits (ABI) and internal sequencing primers (available upon request). Sequenced products were analyzed on an ABI 3100 Genetic Analyzer.

***Sequence analysis.***—Sequences were edited using Sequencher (Gene Codes Corp., Ann Arbor, MI) and have been deposited on GenBank. Two *cyt b* sequences of the Johnny Darter, *E. nigrum*, and one sequence of the Riverweed Darter, *E. podostemone*, from GenBank were included in the alignment as outgroups. The alignment was checked for errors and stop codons, and identical haplotypes were merged using MacClade 4.0 (Maddison and Maddison, 2000).

Modeltest 3.7 (Posada and Crandall, 1998) was used to select the best-fitting model of molecular evolution (using the Akaike Information Criterion): a Tamura-Nei (1993)

model with rates γ-distributed across sites (α = 1.637; hereafter termed TrN+G). This model was applied to analyses of molecular variance (AMOVA) and phylogenetic analyses. MEGA 3.0 (Kumar et al., 2001) was used to calculate genetic distances. Arlequin 2.0 (Schneider et al., 2000) was used to perform AMOVA (Excoffier et al., 1992) and to compute $\Phi_{ST}$ between populations. For AMOVA, we treated the Waccamaw and Pee Dee as separate drainages, as in Jenkins et al. (1971). $\Phi_{ST}$ was based on the coalescent method of Slatkin (1991), and its significance was determined using 1000 randomizations.

Maximum likelihood (ML) and neighbor-joining (NJ: Saitou and Nei, 1987) phylogenetic trees were built using PAUP 4.0b10 (Swofford, 2003). The ML heuristic search employed ten replicate random-sequence stepwise additions for starting trees and TBR branch swapping. Support for nodes in the trees was assessed using 500 fast-stepwise replicates. As an alternative means for viewing the gene genealogy, a haplotype network was constructed using TCS 1.2.1 (Clement et al., 2001) with connection limits relaxed so that all populations could be joined in a single network.

Coalescent simulations were used to evaluate the hypothesis that incomplete lineage sorting of ancestral polymorphism was responsible for the failure of Waccamaw river and lake sequences to form reciprocally monophyletic groups. Simulations employed Mesquite 2.5 (Maddison and Maddison, 2008). An alignment of sequences of all individuals from the Waccamaw river and lake was used (identical haplotypes were not merged). A single individual from the Pee Dee River was supplied as an outgroup sequence, and a neighbor joining tree of this alignment was provided as the empirical gene tree. For the species tree, divergence time (T) between lake and river clades was varied between 2,500 and 100,000 generations, with the total depth of the species tree (back to the Pee Dee/Waccamaw common ancestor) maintained at 500,000 generations. Discordance between gene trees (both true and simulated) and species trees was measured using the number of deep coalescents (Maddison, 1997). Simulations assuming no migration were used to evaluate the hypothesis that discordance was due entirely to incomplete lineage sorting between recently separated populations showing no gene exchange. These no-migration simulations used a base effective population size ($N_e$) = 100,000. Simulations with migration were used to test the hypothesis of divergence with limited gene flow. These used $N_e$ = 10,000 and T = 10,000, with migration rates varied between 0 and $5 \times 10^{-4}$ per individual per generation.

We estimated divergence times between populations using a relaxed-clock model (Drummond et al., 2006) implemented in the software BEAST 1.4.7 (Drummond and Rambaut, 2007). The program employs a Bayesian Markov chain Monte Carlo (MCMC) method that can co-estimate the tree topology and ages of target nodes, while allowing for molecular rate heterogeneity among lineages. Darters have a poor fossil record (Smith, 1981), so we used information from centrarchid fossils as external calibrations for our darter dataset. In addition to being in the same taxonomic order as the percids, centrarchids have several fossils that have been cross-validated (Near et al., 2005) and reliably used to date other darter groups (Near and Benard, 2004; Near and Keck, 2005).

The calibration points used were the fossils that have been assigned as the most recent common ancestor (MRCA) of *Archoplites interruptus* and *Ambloplites* (15.5 million years ago [mya]) and the MRCA of *Lepomis gulosus* and *L. cyanellus* (6.6 mya: Near et al., 2005; Near and Keck, 2005). Cytochrome *b* sequences for these and other centrarchid and percid outgroup species were obtained from GenBank. To avoid biases associated with the divergence time-dependent nature of molecular evolutionary rates (Wayne et al., 1991; Penny, 2005; Ho et al., 2007), we separated our dataset into two: one at the intraspecific phylogeographic level, and the other at a deeper, interspecific level. Prior to analysis in BEAST, the best-fitting model of molecular evolution for each dataset was selected using Modeltest as above.

In the dataset containing the outgroup species, we included only the most common darter haplotype from each of our sampled drainages (Waccamaw, Pee Dee, and Cape Fear). A relaxed clock was used, with rates among lineages assumed to be uncorrelated and sampled from a lognormal distribution. A TrN+G+I model of substitution was used, and a Yule model of speciation employed as branching prior. The nodes for the two fossil calibrations were given priors that followed a lognormal distribution with the dated ages used as the means. This yielded a skewed distribution with a hard minimum bound and a soft maximum bound (i.e., long right tail) for the fossil ages, which better represents the uncertainty and bias associated with fossil dating (Yang and Rannala, 2006; Sanders and Lee, 2007). The MCMC was run twice, each for $10^7$ generations and sampled every five-hundredth generation, with a burn-in of $10^6$. Upon checking that the runs converged on the same parameters, their outputs were combined using LogCombiner v1.4.7 and viewed in Tracer v1.4 (Drummond and Rambaut, 2007).

The time for the MRCA (tMRCA) of the three *E. olmstedi* haplotypes estimated from the analysis above was then used as the calibration for a second analysis with BEAST, which included all of our *E. olmstedi* and *E. perlongum* samples but no other species. The calibration time estimate and its associated 95% highest posterior density (HPD), following a lognormal distribution, were assigned to the root of the tree of this second dataset, since only fish from the three sampled drainages were included. We employed a TrN+G substitution model and a coalescent prior of constant population size. MCMC runs were performed as described above.

## RESULTS

The edited alignment included 906 nucleotide positions and 301 of the 380 codons in the full-length cytochrome *b* gene. Fifty-four nucleotide positions were variable (Table 1), of which six were singleton substitutions and 48 were parsimony-informative. Of the total 35 substitutions that were variable between at least two but fixed within all populations, three substitutions were at first codon positions and 32 were at third positions. Of the 13 sites that were polymorphic in at least one population, two were at first codon positions and 11 were at third positions. There were nine amino acid replacement polymorphisms, of which five were singletons; the latter were confirmed by repeat PCRs. As expected from previous reports of high functional constraint on *cyt b* (Irwin et al., 1991), all of the polymorphic and fixed amino acid substitutions were conservative. Hence, *cyt b* evolution in these darters, showing high polymorphism but relatively strong amino acid conservation, is typical of studies of conspecific vertebrate populations. Averaged over all pairwise compar-

**Table 1.** Condensed Alignment of Variable Nucleotide Positions for *Etheostoma olmstedi* and *E. perlongum* Cytochrome *b* Haplotypes. Haplotype codes refer to source locations (Fig. 1). Dots indicate sites that are identical to the reference haplotype (BR-1: GenBank Acc. GQ184438). ? = missing data.

| | | Nucleotide position | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | Location (frequency) | 39 | 46 | 67 | 136 | 148 | 171 | 180 | 207 | 234 | 235 | 237 | 240 | 241 | 243 |
| BR-1 | Black R. (2) | G | G | A | A | G | C | G | G | T | A | T | A | G | A |
| BR-2 | Black R. (4) | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| BR-3 | Black R. (1) | . | T | . | . | . | . | . | . | . | . | . | . | . | . |
| PD-4 | Pee Dee R. (2) | C | . | . | G | . | . | . | . | . | . | . | . | . | G |
| PD-5 | Pee Dee R. (2) | C | . | . | G | . | . | C | . | . | . | . | . | . | G |
| PD-6 | Pee Dee R. (1) | C | . | . | G | . | . | C | . | . | . | . | . | . | G |
| LPD-7 | L Pee Dee R. (3) | C | . | . | G | . | T | C | . | C | . | . | . | . | G |
| LR-8 | Lumber R. (3) | C | . | . | G | . | . | C | A | . | G | . | . | . | G |
| LR-9 | Lumber R. (1) | ? | ? | . | G | . | . | C | A | . | G | . | . | . | G |
| LR-10 | Lumber R. (1) | ? | T | . | G | . | . | C | A | . | G | . | . | . | G |
| WR-11 | Waccamaw R. (11), Lake Waccamaw (3) | C | . | . | . | . | . | . | A | . | . | . | G | . | G |
| WR-12 | Waccamaw R. (1) | C | . | . | . | A | . | . | A | . | . | . | G | . | G |
| WR-13 | Waccamaw R. (1) | ? | ? | ? | . | . | . | . | A | . | . | . | G | . | G |
| LW-14 | Lake Waccamaw (8), Waccamaw R. (2) | C | . | . | . | . | . | . | A | . | . | . | G | . | G |
| LW-15 | Lake Waccamaw (1) | C | . | C | . | . | . | . | A | . | . | . | G | . | G |
| LW-16 | Lake Waccamaw (1) | C | . | . | . | . | . | . | A | . | . | C | G | . | G |
| LW-17 | Lake Waccamaw (1) | C | . | . | . | . | . | . | A | . | . | . | G | A | G |
| LW-18 | Lake Waccamaw (1) | ? | ? | ? | . | . | . | . | A | . | . | . | G | . | G |

| | | Nucleotide position | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | Location (frequency) | 246 | 252 | 285 | 297 | 339 | 358 | 360 | 363 | 426 | 432 | 442 | 465 | 507 | 582 |
| BR-1 | Black R. (2) | C | C | T | A | T | G | G | C | C | T | C | G | A | G |
| BR-2 | Black R. (4) | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| BR-3 | Black R. (1) | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| PD-4 | Pee Dee R. (2) | T | . | C | G | C | . | A | T | . | . | . | A | . | A |
| PD-5 | Pee Dee R. (2) | T | . | C | G | C | . | A | T | . | . | . | A | . | A |
| PD-6 | Pee Dee R. (1) | T | . | C | G | C | . | . | T | . | . | . | A | . | A |
| LPD-7 | L Pee Dee R. (3) | T | . | C | G | C | . | . | T | . | . | . | A | . | A |
| LR-8 | Lumber R. (3) | T | . | C | G | C | . | . | T | . | . | . | A | . | A |
| LR-9 | Lumber R. (1) | T | . | C | G | C | . | . | T | G | . | . | A | . | A |
| LR-10 | Lumber R. (1) | T | . | C | G | C | . | . | T | . | . | . | A | . | A |
| WR-11 | Waccamaw R. (11), Lake Waccamaw (3) | T | . | . | G | . | . | . | T | . | C | T | A | G | . |
| WR-12 | Waccamaw R. (1) | T | . | . | G | . | . | . | T | . | C | T | A | G | . |
| WR-13 | Waccamaw R. (1) | T | T | . | G | . | A | . | T | . | C | T | A | G | . |
| LW-14 | Lake Waccamaw (8), Waccamaw R. (2) | T | T | . | G | . | . | . | T | . | C | T | A | G | . |
| LW-15 | Lake Waccamaw (1) | T | T | . | G | . | . | . | T | . | C | T | A | G | . |
| LW-16 | Lake Waccamaw (1) | T | T | . | G | . | . | . | T | . | C | T | A | G | . |
| LW-17 | Lake Waccamaw (1) | T | . | . | G | . | . | . | T | . | C | T | A | G | . |
| LW-18 | Lake Waccamaw (1) | T | T | . | G | . | . | . | T | . | C | T | A | G | . |

| | | Nucleotide position | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | Location (frequency) | 594 | 600 | 621 | 633 | 639 | 681 | 687 | 702 | 711 | 732 | 744 | 753 | 759 | 762 |
| BR-1 | Black R. (2) | A | G | T | A | C | G | A | T | C | C | T | G | G | A |
| BR-2 | Black R. (4) | . | . | . | . | . | . | . | . | T | . | . | . | A | . |
| BR-3 | Black R. (1) | . | . | . | . | . | . | . | . | T | . | . | . | A | . |
| PD-4 | Pee Dee R. (2) | G | T | C | . | . | . | . | . | T | T | C | . | . | T |
| PD-5 | Pee Dee R. (2) | G | T | C | . | T | A | G | . | T | T | C | A | . | T |
| PD-6 | Pee Dee R. (1) | G | T | C | . | . | . | G | . | T | T | C | . | A | T |
| LPD-7 | L Pee Dee R. (3) | G | T | C | . | . | . | G | . | T | T | C | . | . | T |
| LR-8 | Lumber R. (3) | G | T | C | . | . | . | G | . | T | T | C | . | . | T |
| LR-9 | Lumber R. (1) | G | T | C | . | . | . | G | . | T | T | C | . | . | T |

**Table 1.** Continued.

| Haplotype | Location (frequency) | Nucleotide position 594 | 600 | 621 | 633 | 639 | 681 | 687 | 702 | 711 | 732 | 744 | 753 | 759 | 762 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LR-10 | Lumber R. (1) | G | T | C | . | . | . | G | . | T | T | C | . | . | T |
| WR-11 | Waccamaw R. (11), Lake Waccamaw (3) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |
| WR-12 | Waccamaw R. (1) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |
| WR-13 | Waccamaw R. (1) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |
| LW-14 | Lake Waccamaw (8), Waccamaw R. (2) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |
| LW-15 | Lake Waccamaw (1) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |
| LW-16 | Lake Waccamaw (1) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |
| LW-17 | Lake Waccamaw (1) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |
| LW-18 | Lake Waccamaw (1) | G | T | C | G | T | . | . | C | T | T | C | . | . | . |

| Haplotype | Location (frequency) | Nucleotide position 789 | 802 | 804 | 807 | 828 | 834 | 840 | 849 | 870 | 876 | 882 | 888 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BR-1 | Black R. (2) | A | G | C | A | A | A | G | C | A | A | A | T |
| BR-2 | Black R. (4) | . | . | . | . | . | . | . | . | . | . | . | . |
| BR-3 | Black R. (1) | . | . | . | . | . | . | . | . | . | . | . | . |
| PD-4 | Pee Dee R. (2) | . | . | . | . | G | . | . | T | . | G | G | C |
| PD-5 | Pee Dee R. (2) | . | A | . | G | G | . | C | T | . | G | G | C |
| PD-6 | Pee Dee R. (1) | . | . | . | . | G | . | C | T | . | G | G | C |
| LPD-7 | L Pee Dee R. (3) | G | . | . | . | G | . | C | T | . | G | G | G |
| LR-8 | Lumber R. (3) | . | . | . | . | G | G | C | T | . | G | G | G |
| LR-9 | Lumber R. (1) | . | . | . | . | G | G | C | T | . | G | G | G |
| LR-10 | Lumber R. (1) | . | . | . | . | G | G | C | T | . | G | G | G |
| WR-11 | Waccamaw R. (11), Lake Waccamaw (3) | . | . | T | . | G | . | . | T | G | . | G | C |
| WR-12 | Waccamaw R. (1) | . | . | T | . | G | . | . | T | G | . | G | C |
| WR-13 | Waccamaw R. (1) | . | . | T | . | G | . | . | T | G | . | G | C |
| LW-14 | Lake Waccamaw (8), Waccamaw R. (2) | . | . | T | . | G | . | . | T | G | . | G | C |
| LW-15 | Lake Waccamaw (1) | . | . | T | . | G | . | . | T | G | C | G | C |
| LW-16 | Lake Waccamaw (1) | . | . | T | . | G | . | . | T | G | . | G | C |
| LW-17 | Lake Waccamaw (1) | . | . | T | . | G | . | . | T | G | . | G | C |
| LW-18 | Lake Waccamaw (1) | . | . | T | . | G | . | . | T | G | . | . | C |

isons, substitutions showed a strong transitional bias that pervades mtDNA in fishes (Meyer, 1993), but were greater in magnitude (10.1:1) than in other studies of darters (Near et al., 2000; Switzer and Wood, 2002).

The ML heuristic search produced two trees with identical scores (*ln* L = 2432.15). These trees and the NJ tree showed very slight differences in relationships near their tips, but the same major clades. All trees (one ML tree shown in Fig. 2) showed three drainage-specific clades (Cape Fear, Pee Dee, and Waccamaw) with > 95% bootstrap support. The first split on the trees separated the Cape Fear from the Pee Dee and Waccamaw clades, which grouped together with strong support. Within the Pee Dee clade, the Lumber River population formed a monophyletic group. The three Little Pee Dee sequences were identical, and distinct from those from the Lumber River. Sequences from the Pee Dee River population showed greater than six times the next-highest nucleotide diversity of any other water body (Table 2) and did not form a clade, but were polyphyletic and basal within the clade containing all individuals from the Pee Dee Drainage (Fig. 2).

The 49 specimens displayed a total of 18 unique haplotypes, most of which were confined to single sampling sites (Table 1). In addition, the Lake Waccamaw population showed one common haplotype, shared between eight Lake Waccamaw and two Waccamaw River fish, and the Waccamaw River population showed one common haplotype, shared between nine Waccamaw River and three Lake Waccamaw fish (Table 1, Fig. 2). Sequences from the Waccamaw drainage formed two shallow paraphyletic groups receiving moderate support (60 and 62%, respectively) in the NJ and ML trees. One group contained 12 WR and four LW fish, and the other group contained 11 LW and three WR fish, a strong frequency difference. These groups differed at only a single nucleotide site, position 252 in the alignment (Table 1), a derived ''T'' substitution that was present in no other specimens than the 11 *E. perlongum* and three Waccamaw River *E. olmstedi*. Two of the three *E. olmstedi* were collected 16 km and one was collected 32 km downstream from the lake near the South Carolina border, so these fish are not likely to result from dispersal from the lake. Inadequate sampling at present, however, prevents us

**Table 2.** Mean Percent Genetic Distances between Geographic Populations of *E. perlongum* and *E. olmstedi*. Within-population nucleotide variation is shown on the diagonal. All values were corrected with the TrN+G model of sequence evolution.

| | Black R. | Pee Dee R. | Little Pee Dee R. | Lumber R. | Waccamaw R. | Lake Waccamaw |
|---|---|---|---|---|---|---|
| Black R. | 0.12 | | | | | |
| Pee Dee R. | 3.07 | 0.74 | | | | |
| Little Pee Dee R. | 3.26 | 0.95 | 0 | | | |
| Lumber R. | 3.31 | 0.99 | 0.82 | 0.05 | | |
| Waccamaw R. | 3.04 | 2.48 | 2.74 | 2.49 | 0.08 | |
| Lake Waccamaw | 3.11 | 2.55 | 2.82 | 2.57 | 0.12 | 0.09 |

from determining whether the frequency of the nucleotide substitution shows clinal variation in the river.

The difference in the frequency of this mtDNA substitution was reflected in a highly significant $\Phi_{ST}$ of 0.241 between LW and WR (Table 3). This value was the smallest of all pairwise population comparisons, however. Pairwise comparisons of *E. olmstedi* populations between drainages showed very high $\Phi_{ST}$ values that approached fixation (from 0.89 to 0.98). Barriers to gene flow between populations within the Pee Dee drainage were strong as well, despite interconnection of these populations through the main stem of the Pee Dee River. The Little Pee Dee and Lumber River populations inhabit adjacent tributaries, which meet to form a common waterway about 75 km upstream from where they join the main stem (Fig. 1). Yet, they showed a $\Phi_{ST}$ value of 0.938 (Table 3) and were distinguished by six fixed substitutions (Table 2). AMOVA analysis showed that 85.4% of the total genetic variation was among, and 9.3% was within drainages (fixation indices for differentiation among drainages $\Phi_{CT}$ = 0.854, for among waterbodies within drainages $\Phi_{SC}$ = 0.636, and for both among and within drainages $\Phi_{ST}$ = 0.947, $P < 0.001$ for each). These calculations are based on a small number of populations and should be treated with caution, but they do indicate deeper separations between than within river drainages.

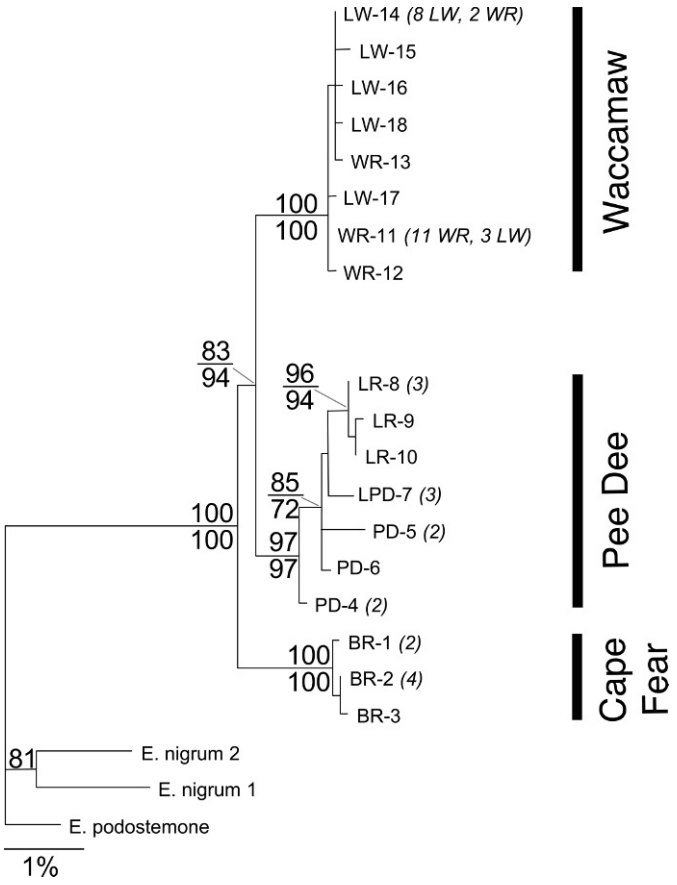


**Fig. 2.** Maximum likelihood phylogeny for cytochrome *b* haplotypes of *Etheostoma perlongum* and *E. olmstedi* cytochrome *b* haplotypes. The tree is one of two ML trees with equal likelihood scores (*ln* L = 2432.15). Numbers above branches are bootstrap support values > 70% (500 replications); upper values from fast-stepwise and lower values from NJ bootstrap. Distances are based upon the TrN+G model. Haplotype codes (Table 1) on branch termini are followed with sample size in italics when shared by multiple individuals and by site codes when present in > 1 population.

Conflict between the c*yt b* gene tree and the species tree of *E. perlongum* and *E. olmstedi* from the Waccamaw River was considerable; 23 deep coalescents (Maddison, 1997) were required to reconcile the two trees. Coalescent simulations without migratixon modeled a scenario of incomplete lineage sorting as the sole factor responsible for discordance (Fig. 3A). At T = 10,000, 5,000, and 2,500 generations, 0, 4.7, and 54.4% of simulated trees showed ≥ 23 deep coalescents, respectively. Smaller effective population sizes required recurrent gene flow to produce comparable results. At $N_e$ = 10,000 and T = 10,000 generations (10,000 years for *E. perlongum* and approximately 20,000 years for *E. olmstedi*: Shute et al., 1982), a migration rate of $5 \times 10^{-4}$ (equivalent to five individuals exchanged each generation) was sufficient to produce ≥ 23 deep coalescent events in 25.6% of simulated trees; migration rates of $1 \times 10^{-4}$ or less generated this number of deep coalescents in < 1% of trees (Fig. 3B). Similar results (not shown) were obtained in migration models using $N_e$ = 100,000 and T = 100,000 generations; again, a recurrent migration rate of five individuals per generation was required to produce ≥ 23 deep coalescents.

Calculations of genetic distances (Table 2) showed that sequence variation within the Lake Waccamaw population was similar to that of the Black, Lumber, and Waccamaw rivers. With the caveat that sample sizes are small, this supports no major reduction in polymorphism associated with colonization of the lake. Genetic distance between Waccamaw and Pee Dee Drainage populations was considerably larger (range 2.48–2.82%) than between populations within the Pee Dee drainage (range 0.74–0.99%).

For divergence time estimates, posterior distributions of tMRCAs were unimodal and right-skewed, so median values were more appropriate. Based upon centrarchid fossil calibrations, the common ancestor between *E. olmstedi* populations in the Cape Fear drainage and those in the Waccamaw and Pee Dee drainages was dated at 1.57 (95% HPD: 0.761–2.82) mya. Darters from the Pee Dee drainage

**Table 3.** $\Phi_{ST}$ between Populations of *E. perlongum* and *E. olmstedi*. Estimates were based on coalescent times as defined by Slatkin (1991) and were calculated using Arlequin. The values for *P* (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$) are based on the proportion of 1000 randomizations that generated a $F_{ST}$ greater than the value estimated from the real data.

| | Black R. | Pee Dee R. | Little Pee Dee R. | Lumber R. | Waccamaw R. |
|---|---|---|---|---|---|
| Pee Dee R. | 0.890*** | | | | |
| Little Pee Dee R. | 0.970*** | 0.569* | | | |
| Lumber R. | 0.967*** | 0.634** | 0.938* | | |
| Waccamaw R. | 0.971*** | 0.915*** | 0.978** | 0.972*** | |
| Lake Waccamaw | 0.961*** | 0.902*** | 0.963*** | 0.958*** | 0.241** |

split from those in the Waccamaw drainage more recently, 1.15 mya (0.574–1.69), while populations within the Pee Dee drainage shared a common ancestor 520 (188–860) thousand years ago (kya). Finally, divergence of *E. perlongum* in Lake Waccamaw from *E. olmstedi* in the Waccamaw River was dated at 190 (42–400) kya.

## DISCUSSION

The original description of *E. perlongum* (Hubbs and Raney, 1946) suggested a sister relationship to *E. olmstedi* (*Boleosoma nigrum olmstedi* at their writing). The present study leaves little doubt that *E. perlongum* originated from an ancestral population shared with *E. olmstedi* in the Waccamaw River. It is therefore appropriate to evaluate the endemic status of *E. perlongum* within a phylogenetic framework of nearby geographic populations. Our study reveals strong drainage and river-specific differentiation in *E. olmstedi*. Earlier work uncovered comparable genetic separations between populations of darters in streams of the Missouri River (Switzer and Wood, 2002), in the Ozark and Appalachian mountains (Strange and Burr, 1997), and in Eastern Kentucky (Strange, 1998), but in these cases, Pleistocene glaciations were responsible. Populations of *Etheostoma olmstedi* in southeastern Atlantic slope rivers and streams are far south of the line of glacial advance, but show historical separations of similar age. Tesselated Darters, therefore, join a growing list of examples from madtoms (Bennetts et al., 1999), pygmy sunfishes (Quattro et al., 2001), and *Lepomis* sunfishes (Bermingham and Avise, 1986) in which strong phylogeographic pattern has been recorded from coastal streams in this region.

While details of the history of the physical environment of the Southeastern U.S. coastal plain since the Pliocene are not well understood, reasonable hypotheses can be posed that are in accord with our estimated divergence dates (Bermingham and Avise, 1986; Avise, 1992). The most ancient separations are likely to have coincided with the highest sea level stands of the early Pleistocene, when seawater would have flooded coastal streams and displaced populations to the headwaters of the Cape Fear and Pee Dee. Adjacent tributaries of the Pee Dee appear as sister populations, and these closer relationships cannot be due to current-day gene flow connecting them through the main stem of the Pee Dee River, which our analysis indicates is absent or nearly so. Perhaps lateral stream capture events during high sea level stands formed interconnections between upper reaches of these neighboring streams that were severed as sea levels dropped. Other darter populations in adjacent streams have shown even greater genetic similarities that have been attributed to stream capture events (Strange, 1998; Switzer and Wood, 2002).

Separation of the Waccamaw populations about 1 mya is surprising. The Waccamaw runs parallel to, and only 20–40 km inland from, the present shoreline, and it has no headwater that would have been protected from seawater that would have flooded the entire river much more recently. Shute et al. (1981) cite evidence from Zullo and Harris (1979) that both the Waccamaw and Little Pee Dee rivers (including the Lumber as a tributary of the latter) previously had extensive watersheds throughout the inner Coastal Plain and into the Piedmont. These could have served as refuges, but evidence also suggests that some 75,000 years ago in the Wisconsin glacial period, uplift of the Cape Fear Fault caused diversion of stream flow into the Cape Fear River (Zullo and Harris, 1979). This left both the Little Pee Dee and Waccamaw with their present-day shrunken watersheds, restricted only to the outer Coastal Plain, but would also predict recent exchange between Cape Fear, Waccamaw, and Little Pee Dee populations, which we have not detected. At present, our findings are only suggestive, and a more detailed survey of the region is required to adequately address the historical biogeography of populations of *E. olmstedi* here.

Divergence between Waccamaw lake and river populations is less than between any two geographic populations of *E. olmstedi* in this study. But do our results support the conclusion that *E. perlongum* and *E. olmstedi* are reproductively isolated? Coalescent simulations confirm that lineage sorting between historically isolated populations is a plausible explanation for the *cyt b* gene tree, and suggest two rather different scenarios. The first describes complete reproductive isolation between lake and river populations originating just 5–10,000 years ago in relatively large ancestral populations. This timing would correspond with reinvasion of the lake about 20,000 years after the last major sea level regression. An effective size of 100,000 is fairly large but not inconceivable, as present day census number in Lake Waccamaw alone (Lindquist and Yarbrough, 1982) has been estimated at 125,000 individuals (which the authors note is probably an underestimate). However, demographic estimates of $N_e$ that take into account population size fluctuation, variance in offspring number, and sex ratio variation show that long-term values for $N_e/N$ average just 0.11 across a number of vertebrate species (Frankham, 1995). With a $N_e$ about one-tenth the current census number of the Lake Waccamaw population, and divergence 10–20,000 years ago, moderate gene flow (a rate of $5 \times 10^{-4}$ per individual) between populations each generation was required to generate the same level of gene tree-species tree discord. This would suggest that reproductive isolation between *E. perlongum* and *E. olmstedi* is not complete, but that gene flow is restricted. Either scenario (migration

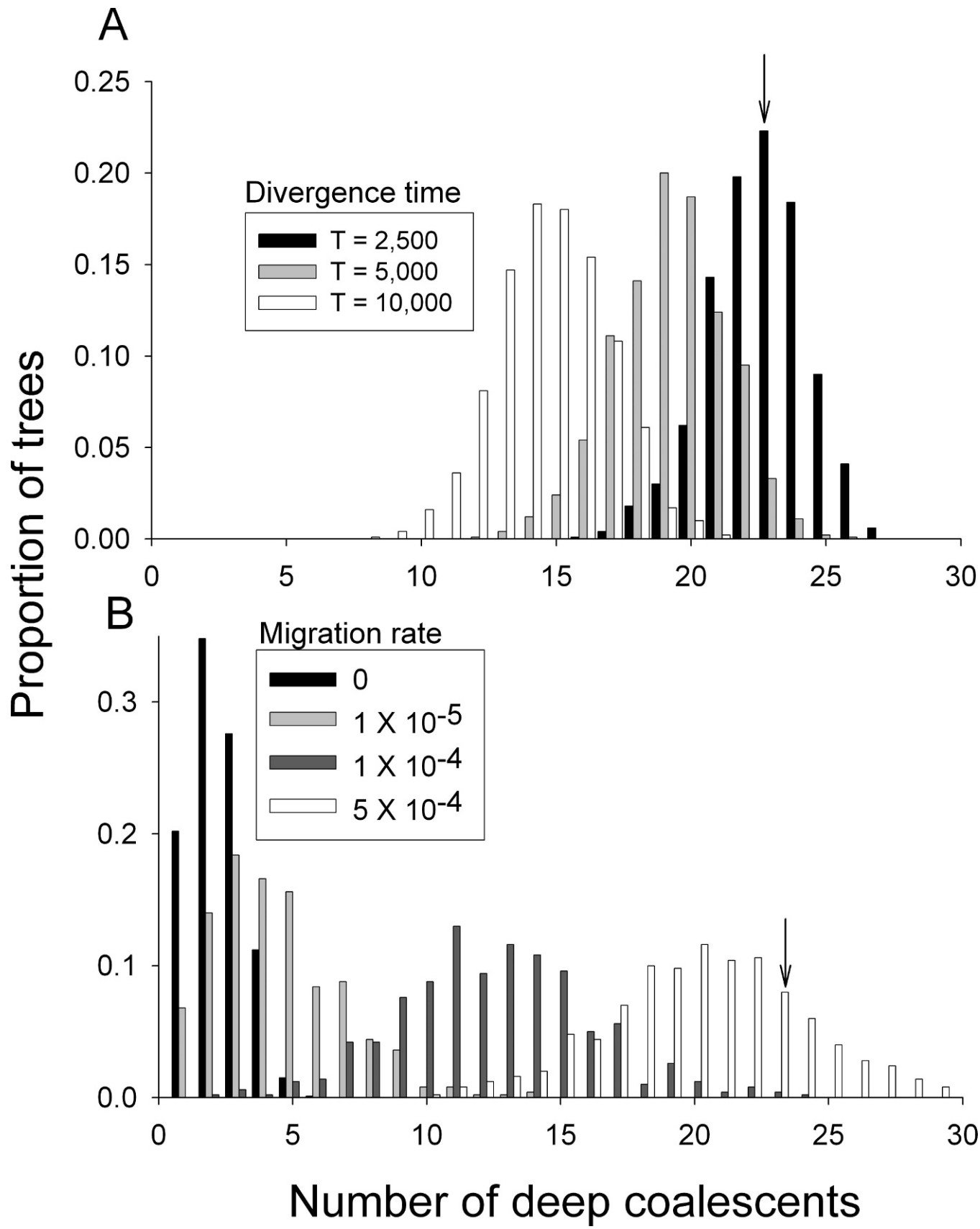


**Fig. 3.** Coalescent simulations. Each figure plots the distribution of the number of deep coalescents, a measure of discord between a reciprocally monophyletic species tree and 1000 gene trees simulated under a neutral coalescent model. (A) Simulations performed over a range of divergence times between river and lake populations (T in generations), with base effective population size $N_e$ = 100,000, and no migration. (B) Simulations performed over a range of migration rates (per individual per generation) at $N_e$ = 10,000 and T = 10,000 generations. Arrows show observed number of deep coalescents between the species tree and the empirical *cyt b* tree.

absent or present) could be consistent with observed levels of haplotype frequency and sequence differentiation.

A result with gene flow between the endemic and sister as restricted as this is not biologically trivial. All other populations of *E. olmstedi* we studied are allopatric, but *E. perlongum* and *E. olmstedi* meet along a common boundary. During high water periods, such as occurred several times during the present study (pers. obs.), the Waccamaw River floods over the small dam and is in complete contact with the lake at the head of the river, and opportunities for migration must have been even less restricted prior to the dam's construction. This suggests that other isolating factors, ecological and/or reproductive in nature, must restrict genetic exchange between *E. perlongum* and *E. olmstedi*. Lindquist et al. (1981) used field observations and laboratory cinematography to describe unique behaviors during the elaborate courtship and mating of *E. perlongum*. Constanz (1985) used *in situ* observations of *E. olmstedi* in a creek in West Virginia to detail complex spawning behavior, including male alloparental care of egg clusters. Together these studies indicate highly complex mating and courtship behaviors that should be further studied to determine the degree to which Waccamaw lake and river populations show reproductive isolation.

Nevertheless, despite its designation as a morphological species, and its ecological and life history distinctiveness, *E. perlongum* does not qualify as a phylogenetic species. A strict adherence to the criteria proposed by Vogler and DeSalle (1994) and Moritz (1995)—namely, unique clusters of haplotypes and monophyly of mitochondrial DNA sequences—would lead us to reject Evolutionary Significant Unit (ESU) status for *E. perlongum*. Instead, based on differences in mtDNA haplotype frequencies and following the recommendations of Moritz (1995), we would be led to designate *E. perlongum* as a management unit of *E. olmstedi*, appropriate for shorter-term demographic monitoring, but not for long-term preservation as a threatened species.

Other analyses are also needed to further interpret patterns of clinal variation in morphology and allozymes reported previously (Shute, 1984). Expanded sampling of sites at varying distances downstream of the lake, morphological analysis, and multilocus analysis of mitochondrial and nuclear DNA markers are called for. A challenge for these studies would be to distinguish a hypothesis of introgression between recently separated allopatric forms, from one of intergradation between ecomorphs of the same species, the interpretation preferred by Shute (1984). These two scenarios would have very different conservation implications.

The present results indicate that derived life history and morphological traits of the Waccamaw Darter have evolved rapidly due to adaptation to the novel lake environment. They point to further studies of mating behavior and population genetics to more fully address whether this unique lake-dwelling population is reproductively isolated. Only with these studies can we fully evaluate both its vulnerability to extinction and strategies for recovery.

## MATERIAL EXAMINED

Listed is species binomial, collection site (for specimens in this study), voucher catalog numbers (NA = not available); in parentheses are number of individuals and GenBank accession numbers. Sequences from GenBank each correspond to a single individual.

***Specimens collected for this study.***—*Etheostoma olmstedi:* Black River: UNCW09.01.06 (7, GQ184438–GQ184440); Little Pee Dee River: UNCW09.01.04 (3, GQ184444); Lumber River: UNCW09.01.03 (5, GQ184445–GQ184447); Pee Dee River: UNCW09.01.05 (5, GQ184441–GQ184443); Waccamaw River: UNCW09.01.02 (15, GQ184448–GQ184450). *Etheostoma perlongum:* Lake Waccamaw: UNCW09.01.01 (15, GQ184451–GQ184455).

***Sequences from GenBank.***—*Ambloplites rupestris:* TJN 284 (AY225663); *Archoplites interruptus:* TJN 1077 (AY225665); *Etheostoma nigrum:* INHS 39507 (AF183945), NA (AY374268); *Etheostoma podostemone:* NA (AF045346); *Lepomis cyanellus:* UAIC12528.01 (AY115974); *Lepomis gulosus:* UAIC12420.01 (AY115972); *Micropterus dolomieu:* NA (AY225687); *Micropterus salmoides:* NA (AY225675); *Nothonotus acuticeps:* UTTC 2205 (AY742655); *Nothonotus moorei:* NA (AF274445); *Perca flavescens:* NA (AY374280).

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## LITERATURE CITED

**Avise, J. C.** 1992. Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. Oikos 63:62–76.

**Bennetts, R. Q., J. M. Grady, F. C. Rhode, and J. M. Quattro**. 1999. Discordant patterns of morphological and molecular change in broadtail madtoms (genus *Noturus*). Molecular Ecology 8:1563–1569.

**Bermingham, E., and J. C. Avise**. 1986. Molecular zoogeography of freshwater fishes in the southeastern United States. Genetics 113:939–965.

**Birky, C. W., Jr.** 1991. Evolution and population genetics of organelle genes: Mechanisms and models, p. 112–134. *In*: Evolution at the Molecular Level. R. K. Selander, A. G. Clark, and T. S. Whittam (eds.). Sinauer and Associates, Sunderland, Massachusetts.

**Carver, R. E., and G. A. Brook**. 1989. Late Pleistocene paleowind directions, Atlantic Coastal Plain, USA. Palaeogeography, Palaeoclimatology, Palaeoecology 74:205–216.

**Clement, M., D. Posada, and K. A. Crandall**. 2001. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657–1659.

**Cole, C. F.** 1967. A study of the eastern Johnny darter, *Etheostoma olmstedi* Storer (Teleostei, Percidae). Chesapeake Science 8:28–51.

**Constanz, G. D.** 1985. Alloparental care in the Tesselated Darter, *Etheostoma olmstedi* (Pisces: Percidae). Environmental Biology of Fishes 14:175–183.

**Drummond, A. J., S. Y. W. Ho, M. J. Phillips, and A. Rambaut**. 2006. Relaxed phylogenetics and dating with confidence. PLoS Biology 4:699–710.

**Drummond, A. J., and A. Rambaut**. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7:214–221.

**Edwards, S. V., and P. Beerli**. 2000. Perspective: gene divergence, population divergence, and the variance in coalescence time in phylogeographic studies. Evolution 54:1839–1854.

**Excoffier, L., P. Smouse, and J. Quattro**. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

**Firestone, R. B., A. West, J. P. Kennett, L. Becker, T. E. Bunch, Z. S. Revay, P. H. Schultz, T. Belgya, D. J. Kennett, J. M. Erlandson, O. J. Dickenson, A. C. Goodyear, R. S. Harris, G. A. Howard, J. B. Kloosterman, P. Lechler, P. A. Mayewski, J. Montgomery, R. Poreda, T. Darrah, S. S. Que Hee, A. R. Smith, A. Stich, W. Topping, J. H. Wittke, and W. S. Wolbach**. 2007. Evidence for an extraterrestrial impact 12,900 years ago that contributed to the megafaunal extinctions and the Younger Dryas cooling. Proceedings of the National Academy of Sciences of the United States of America 104:16016–16021.

**Frankham, R.** 1995. Effective population size/adult population size ratios in wildlife: a review. Genetical Research 66:95–107.

**Frey, D. G.** 1953. Regional aspects of the late-glacial and post-glacial pollen succession of south-eastern North Carolina. Ecological Monographs 23:289–313.

**Frey, D. G.** 1955. A time revision of the Pleistocene pollen chronology of southeastern North Carolina. Ecology 36:762–763.

**Ho, S. Y. W., B. Shapiro, M. J. Phillips, A. Cooper, and A. J. Drummond**. 2007. Evidence for time dependency of molecular rates estimates. Systematic Biology 56:515–522.

**Hubbs, C. L., and E. C. Raney**. 1946. Endemic fish fauna of Lake Waccamaw, North Carolina. Miscellaneous Publications of the University of Michigan Museum of Zoology 65:1–30.

**Irwin, D. M., T. D. Kocher, and A. C. Wilson**. 1991. Evolution of the cytochrome *b* gene of mammals. Journal of Molecular Evolution 32:128–144.

**Jenkins, R. E., E. A. Lachner, and F. J. Schwartz**. 1971. Fishes of the Central Appalachian Drainages: their distribution and dispersal, p. 43–117. *In*: The Distributional History of the Biota of the Southern Appalachians. Part III: Vertebrates. P. C. Holt (ed.). Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

**Kaczorowski, R. T.** 1977. The Carolina Bays and their relationship to modern oriented lakes. Unpubl. Ph.D. diss., University of South Carolina, Columbia, South Carolina.

**Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei**. 2001. MEGA2: molecular evolutionary genetics analysis. Arizona State University, Tempe, Arizona.

**LeGrand, H. E., Jr., S. P. Hall, S. E. Mc Rae, and J. T. Finnegan**. 2006. Natural Heritage Program List of the Rare Animal Species of North Carolina, p. 112. North Carolina Natural Heritage Program, North Carolina Department of Environment and Natural Resources, Raleigh, North Carolina.

**Lindquist, D. G., J. R. Shute, and P. W. Shute**. 1981. Spawning and nesting behavior of the Waccamaw darter, *Etheostoma perlongum*. Environmental Biology of Fishes 6:177–191.

**Lindquist, D. G., and C. G. Yarbrough**. 1982. Status of the endemic ichthyofauna of Lake Waccamaw, North Carolina. Technical Report E-1-VI, North Carolina Wildlife Resources Commission.

**Maddison, W. P.** 1997. Gene trees in species trees. Systematic Biology 46:523–536.

**Maddison, W. P., and D. R. Maddison**. 2000. MacClade. Sinauer Associates, Inc., Sunderland, Massachusetts.

**Maddison, W. P., and D. R. Maddison**. 2008. Mesquite: a modular system for evolutionary analysis. http://mesquiteproject.org

**Meyer, A.** 1993. Evolution of mitochondrial DNA in fishes, p. 1–38. *In*: Molecular Biology Frontiers, Biochemistry and Molecular Biology of Fishes. P. W. Hochacka and T. P. Mommsen (eds.). Elsevier, Amsterdam.

**Moritz, C.** 1995. Defining ''evolutionarily significant units'' for conservation. Trends in Ecology and Evolution 9:373–375.

**Near, T. J., and M. F. Benard**. 2004. Rapid allopatric speciation in logperch darters (Percidae: *Percina*). Evolution 58:2798–2808.

**Near, T. J., D. I. Bolnick, and P. C. Wainwright**. 2005. Fossil calibrations and molecular divergence time estimates in centrarchid fishes (Teleostei: Centrarchidae). Evolution 59:1768–1782.

**Near, T. J., and B. P. Keck**. 2005. Dispersal, vicariance, and timing of diversification in *Nothonotus* darters. Molecular Ecology 14:3485–3496.

**Near, T. J., J. C. Porterfield, and L. M. Page**. 2000. Evolution of cytochrome *b* and the molecular systematics of *Ammocrypta* (Percidae: Etheostomatinae). Copeia 2000:701–711.

**Neigel, J. E., and J. C. Avise**. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation, p. 515–534. *In*: Evolutionary Processes and Theory. E. Nevo and S. Karlin (eds.). Academic Press, New York.

**Penny, D.** 2005. Relativity of molecular clocks. Nature 426:183–184.

**Posada, D., and K. A. Crandall**. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

**Prouty, W. F.** 1952. Carolina bays and their origin. Bulletin of the Geological Society of America 63:167–224.

**Quattro, J. M., W. J. Jones, and F. C. Rohde**. 2001. Evolutionarily significant units of rare pygmy sunfishes (genus *Elassoma*). Copeia 2001:514–520.

**Raney, E. C., and E. A. Lachner**. 1943. Age and growth of johnny darters, *Boleosoma nigrum olmstedi* (Storer) and *Boleosoma longimanum* (Jordan). American Midland Naturalist 29:229–238.

**Riggs, S. R., D. V. Ames, D. R. Brant, and E. D. Sager**. 2000. The Waccamaw Drainage System: Geology and Dynamics of a Coastal Wetland, Southeastern North Carolina. Division of Water Resources, North Carolina Department of Environment and Natural Resources, Raleigh, North Carolina.

**Saitou, N., and M. Nei**. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

**Sambrook, J., and D. W. Russell**. 2000. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

**Sanders, K. L., and M. S. Y. Lee**. 2007. Evaluating molecular clock calibrations using Bayesian analyses with soft and hard bounds. Biology Letters 3:275–279.

**Schmidt, T. R., and J. R. Gold**. 1993. Complete sequence of the mitochondrial cytochrome *b* gene in the cherryfin shiner, *Lythurus roseipinnis* (Teleostei: Cyprinidae). Copeia 1993:880–883.

**Schneider, S., D. Roessli, and L. Excoffier**. 2000. ARLEQUIN Version 2.000. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

**Seutin, G., J. Brawn, R. E. Ricklefs, and E. Bermingham**. 1993. Genetic divergence among populations of a tropical passerine, the Streaked Saltator (*Saltator albicollis*). Auk 110:117–126.

**Shute, J. R.** 1984. A systematic evaluation of the Waccamaw darter, *Etheostoma perlongum* (Hubbs and Raney), with comments on relationships within the subgenus *Boleosoma* (Percidae: Etheostomatini). Unpubl. M.S. thesis, University of Tennessee, Knoxville.

**Shute, J. R., P. W. Shute, and D. G. Lindquist**. 1981. Fishes of the Waccamaw River drainage. Brimleyana 6:1–24.

**Shute, P. W., J. R. Shute, and D. G. Lindquist**. 1982. Age, growth, and early life history of the Waccamaw darter, *Etheostoma perlongum*. Copeia 1982:561–567.

**Slatkin, M.** 1991. Inbreeding coefficients and coalescence times. Genetical Research 58:167–175.

**Smith, G. R.** 1981. Late Cenozoic freshwater fishes of North America. Annual Reviews of Ecology and Systematics 12:163–193.

**Strange, R. M.** 1998. Mitochondrial DNA variation in johnny darters (Pisces: Percidae) from eastern Kentucky supports stream capture for the origin of upper Cumberland River fishes. American Midland Naturalist 140:96–102.

**Strange, R. M., and B. M. Burr**. 1997. Intraspecific phylogeography of North American highland fishes: a test of the Pleistocene vicariance hypothesis. Evolution 51:885–897.

**Switzer, J. F., and R. M. Wood**. 2002. Molecular systematics and historical biogeography of the Missouri saddled darter *Etheostoma tetrazonum* (Actinopterygii: Percidae). Copeia 2002:450–455.

**Swofford, D. L.** 2003. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, Massachusetts.

**Tamura, K., and M. Nei**. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512–526.

**Vogler, A. P., and R. DeSalle**. 1994. Diagnosing units of conservation management. Conservation Biology 8:354–363.

**Wayne, R. K., B. van Valkenburgh, and S. J. O'Brien**. 1991. Molecular distance and divergence time in carnivores and primates. Molecular Biology and Evolution 8:297–319.

**Yang, Z., and B. Rannala**. 2006. Bayesian estimation of species divergence times under a molecular clock using multiple fossil calibrations with soft bounds. Molecular Biology and Evolution 23:212–226.

**Zullo, V. A., and W. B. Harris**. 1979. Plio-pleistocene crustal warping in the outer Coastal Plain of North Carolina, p. 31–40. *In*: Structural and Stratigraphic Framework for the Coastal Plain of North Carolina. G. R. Baum, W. B. Harris, and V. A. Zullo (eds.). Carolina Geological Society.