

Concordant Mitochondrial and Nuclear DNA Partitions Define Evolutionarily Significant Units in the Imperiled Pinewoods Darter, *Etheostoma mariae* (Pisces: Percidae)

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A major challenge faced by conservation officials is determining which habitats are most vulnerable to anthropogenic perturbation and thus are in most critical need of protection. We utilized a fish with life history attributes presumably conducive to low rates of gene flow and small effective population size to gain insight into the appropriate conservation units in the Carolina Sandhills, a threatened ecosystem in the southeastern United States. We assessed variation in nuclear and mitochondrial DNA sequence data throughout the range of the Pinewoods Darter, *Etheostoma mariae* (Percidae), a species endemic to headwater streams of the Lumber and Little Pee Dee rivers (Pee Dee Drainage) in the Carolina Sandhills. Concordant partitions in nuclear and mitochondrial DNA loci support the designation of two Evolutionarily Significant Units (ESUs): one each from Lumber and Little Pee Dee rivers. The data suggest these populations have been historically isolated and are on distinct evolutionary trajectories. Additionally, this research underscores the potential importance of fine-scale sampling in conservation genetics studies of organisms predisposed to genetic differentiation and demonstrates that significant population structure can occur even within a single drainage.

FRESHWATER fishes of the southeastern United States exhibit varying propensities for population differentiation and speciation. This is reflected in the number of species within a lineage and the relative size of the geographical ranges of these species. The precise reasons why certain taxa speciate more frequently and exhibit greater population sundering than others are not known. However, these taxa often share life history characteristics conducive to low rates of dispersal, and therefore low gene flow (Avice, 2000). For example, many such species guard nests, have limited dispersal capabilities, or have noticeably small census (and presumably effective) population sizes. One taxon with a propensity for rapid population differentiation is the darters (Percidae: Etheostomatini), a speciose group of North American freshwater fishes whose members often exhibit extreme microendemism, particularly in the southeastern United States (Powers and Mayden, 2003). Several ecological characteristics of darters may play a role in their apparent high rate of divergence (Turner and Trexler, 1998). Darters often exhibit preference for small headwater streams where available habitat might be limited. They are often intolerant of lower reaches of rivers and consequently dispersal might be restricted even within drainages. Habitat preference, coupled with the male nest-guarding behaviors that characterize many species, are presumably responsible for small effective population sizes and reduced within-population relative to among-population genetic variation in several species (Turner et al., 1996; Turner and Trexler, 1998).

Many darter species are faced with immediate risk of extinction (Warren et al., 2000). The fact that most darters have a high propensity for isolation renders them valuable

to conservation genetics studies. Due to the life history characteristics described above, darters are presumably among the first fishes to exhibit population differentiation in sundered habitats. Conversely, their habitat preferences and life history attributes also predispose them to be among the first taxa to go extinct in disturbed areas. Thus, darters may provide insight into the appropriate units of habitat conservation in threatened aquatic environments of the southeastern United States.

In recent years, freshwater ecosystems of the southeastern United States have begun garnering attention for their diverse and highly endangered faunas (Lydeard and Mayden, 1995). Fishes in this region frequently have extremely small geographic distributions, with many restricted to a single drainage (Swift et al., 1986). This fact, coupled with recent anthropogenic habitat degradation, has led to the highly imperiled status of a large portion of this fauna. For example, 187 of approximately 662 fish taxa (28.2%) in the southeastern United States are recognized as extinct, endangered, threatened, or vulnerable (Warren et al., 2000). Additionally, systematic relationships of fishes of the southeastern United States are poorly resolved, which has hindered effective management and conservation of these species. However, recent population genetic and phylogenetic analyses have revealed deep gene-genealogies among adjacent drainage basins in several taxa (Bennetts et al., 1999; Quattro et al., 2001, 2002; Wirgin et al., 2001), a pattern that is not surprising given the old age of these drainages. This region was not glaciated during the Pleistocene and consequently, these drainage systems are much older, for example, than drainages in the northern U.S. and Canada (Jenkins et al., 1971; Swift et al., 1986). The old age

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of these drainages, when coupled with low levels of inter-drainage gene flow, promotes population differentiation (Bernatchez and Wilson, 1998). An emerging theme arising from the frequent observation of deep gene-genealogies among populations of freshwater fishes of the southeastern United States is the need for drainage-specific management plans (Bennetts et al., 1999; Quattro et al., 2001, 2002; Wirgin et al., 2001). In this paper we ask whether there are cases where even finer scale resolution might be necessary.

One distinctive region within the southeastern United States that remains particularly poorly understood in the context of conservation is the Carolina Sandhills. Formed in the Miocene (approximately 9–12 mya; Murphy, 1995), the Carolina Sandhills is one of the oldest extant ecosystems in the southeastern United States. Not surprisingly, the Carolina Sandhills is home to numerous rare and/or endemic species including the Red-cockaded Woodpecker (*Picoides borealis*; Carter et al., 1983), Pine Barrens Treefrog (*Hyla andersonii*; Martof et al., 1980), chubs (*Cyprinella* sp.; Menhinick and Braswell, 1997), Sandhills Chub (*Semotilus lumbee*; Rohde and Arndt, 1991), and the Pinewoods Darter (*Etheostoma mariae*; Rohde and Arndt, 1991). Currently, the Sandhills ecosystem is threatened by residential expansion, as well as commercial (golf courses) and military activities (Rohde and Arndt, 1991). Creation of an efficacious management plan for the Sandhills is precluded by having a sound understanding of geographic variation in the genetic composition of its biota. Presently, there is very little known about genetic variation in these species, except for the Red-cockaded Woodpecker which exhibited a relatively large among-population component of genetic variation (Stangel et al., 1992). We assessed nuclear and mitochondrial DNA variation in the Pinewoods Darter (*Etheostoma mariae*), a species endemic to headwater streams of the Lumber and Little Pee Dee (Pee Dee Drainage) in the Carolina Sandhills for use as a proxy for conservation units of this unique ecosystem.

Etheostoma mariae prefers cool, clear, fast-flowing primary and secondary streams and appears to be susceptible to habitat destruction (Rohde and Ross, 1987; Rohde and Arndt, 1991). We predicted Lumber and Little Pee Dee individuals would be genetically distinct from one another, as the confluence of these rivers lies outside of the Sandhills, and that the majority of variation would occur between rather than within these rivers. We employed nuclear and mitochondrial DNA markers to test these hypotheses and to assess whether this species can be managed as a single unit or whether smaller-scale management is warranted using Moritz's (1994, 1995) criteria for designating Evolutionarily Significant Units (ESUs). We discuss these findings in light of conservation implications for the species as well as for this unique ecosystem in general.

MATERIALS AND METHODS

DNA extraction, amplification, and sequencing.—Pinewoods Darters were collected with seines and dipnets from 17 localities representing 11 streams throughout their range (Fig. 1). Upon capture, specimens were immediately preserved in 95% alcohol. Total DNA was obtained from caudal-fin clippings using DNeasy Tissue Kits (Qiagen, Valencia, CA) following the manufacturer's protocol. A portion of the mitochondrially encoded cytochrome *b* (*cytb*) locus and a nuclear intron, S7 intron 1, were amplified from extracted

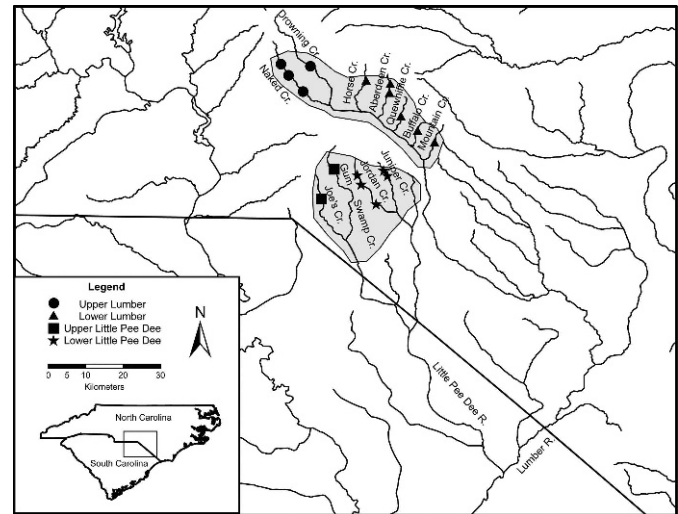


Fig. 1. Geographic distribution and sample localities of *Etheostoma mariae* in North Carolina, USA. Shaded areas represent the approximate distribution of this species. Note that the Lumber and Little Pee Dee rivers coalesce south of the Carolina Sandhills and are part of the Pee Dee Drainage.

DNA using the polymerase chain reaction (PCR). S7 was amplified with forward S7RPEX1F and reverse S7RPEX2R primers (Chow and Hazama, 1998), while *cytb* was amplified with modified H15149 (modified from Kocher et al., 1989) and GLUDG-L (Palumbi et al., 1991) oligonucleotides. PCR conditions for S7 and *cytb* included an initial denaturation for 4 minutes at 94°C, followed by 40 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 7 min. Amplicons (5.0 µl) were purified with 0.75 µl of 1 u/µl shrimp alkaline phosphatase (Promega, Madison, WI) and 0.25 µl of 20 u/µl Exonuclease I (New England Biolabs, Ipswich, MA) and subjected to the following thermal regime: 37°C for 15 min followed by 80°C for 15 min. Purified PCR products (1 µl) were used as template for Big Dye terminator cycle sequencing reactions (Applied Biosystems, Foster City, CA). Amplicons were sequenced with both forward and reverse primers and precipitated with ethanol/sodium acetate. Sequences were run out on an ABI 3700. Base pair calls were verified with Sequencher (version 4.1; Gene Codes Corp., Ann Arbor, MI), and sequences were aligned by eye in BioEdit (Hall, 1999). Heterozygotes for the nuclear intron were diagnosed as two overlapping, equally intense bands at single base positions. The original specimens used for genetic analysis were lost during shipping. We subsequently recollected specimens from the same eleven streams. Genetic analyses were conducted on the combined dataset comprising vouchered and non-vouchered specimens.

Genetic analyses.—Maximum parsimony and likelihood (ML) trees were generated in PAUP* (version 4.0b10, D. L. Swofford, PAUP*: phylogenetic analysis using parsimony [*and other methods], Sinauer Associates, Sunderland, MA, 2002) and used to assess phylogenetic relationships among *cytb* haplotypes. Hierarchical likelihood ratio tests (hLRT) implemented in MODELTEST (version 3.7; Posada and Crandall, 1998) were used to select a model of DNA substitution for ML. *Etheostoma fricksium*, the putative sister species to *E. mariae* (Richards, 1963), was used as the outgroup taxon. Support for internal nodes of ML and

parsimony trees was calculated using 1,000 bootstrap replicates. Additionally, an analysis of molecular variance (AMOVA; Excoffier et al., 1992) was used to partition genetic variation into within- and between-population components for *cytb* and S7. AMOVA analyses conducted on mitochondrial haplotypes and nuclear alleles utilized Φ_{ST} (uncorrected pairwise differences were used as a between-haplotype metric) and 'traditional' F_{ST} values (using observed allele frequency differences), respectively. More complex models of sequence evolution for inter-haplotype distance comparisons had negligible effects on estimates of Φ -statistics; consequently, only results using uncorrected pairwise distances are presented here. Genotypic data were tested for departures from Hardy-Weinberg equilibrium (HWE) within Lumber and Little Pee Dee rivers with Guo and Thompson's (1992) analogue of Fisher's exact test. All population genetic analyses were implemented in ARLEQUIN (version 3.0; Excoffier et al., 2005).

RESULTS

Mitochondrial DNA.—A total of 401 base pairs of the mitochondrially encoded cytochrome *b* gene (including 27 bp of tRNA upstream of *cytb*) was sequenced from 70 individuals representing 17 sampling locations. Eleven variable sites defined seven haplotypes within *Etheostoma mariae* (Table 1). Hierarchical LRT selected the HKY model (Hasegawa et al., 1985) + Γ with a shape parameter of 0.0026. Phylogenetic reconstruction revealed relatively deep splits between the Lumber and Little Pee Dee rivers (Fig. 2). Maximum parsimony and likelihood recovered the same topology, with reciprocal monophyly between the populations in the Lumber and Little Pee Dee Rivers. Seven fixed differences separated the Lumber clade of two haplotypes from the Little Pee Dee clade of five haplotypes. Percent sequence divergence between haplotypes from the two clades ranged from 1.8% to 2.3% (avg. = 2.0%). The AMOVA confirmed that the majority of genetic variation in *E. mariae* was between rather than within these rivers ($\Phi_{ST} = 0.920$, $P < 0.001$). Additional structure was found within the Little Pee Dee, with one fixed difference between streams of the upper and lower portions. Because of the presence of distinct population structure within the Little Pee Dee, we conducted a hierarchical AMOVA with 'populations' defined as streams in the upper and lower Lumber and upper and lower Little Pee Dee and 'groups' defined as the Lumber and Little Pee Dee. Our decision to divide the Lumber into upper and lower portions stemmed from a lack of HWE in that river for the S7 data (see below). Hierarchical AMOVA revealed significant population structure not only between the Lumber and Little Pee Dee ($\Phi_{CT} = 0.858$, $P < 0.001$), but also between the upper and lower portions of these rivers ($\Phi_{SC} = 0.814$, $P < 0.001$). Note that these models are nested; hence, the total variance sums to greater than one.

Nuclear introns.—Of 528 base pairs of S7 intron 1 sequenced in 56 individuals, eight polymorphic sites resulted in eight alleles in *Etheostoma mariae* (Table 2), with six in the Lumber River and three in the Little Pee Dee. Allele frequency differences between the two populations accounted for 39.2% of the total S7 diversity ($F_{ST} = 0.392$, $P < 0.001$). However, exact tests of HWE were significant for the Lumber (obs. $H = 0.519$; exp. $H = 0.767$; $P < 0.01$) and nearly

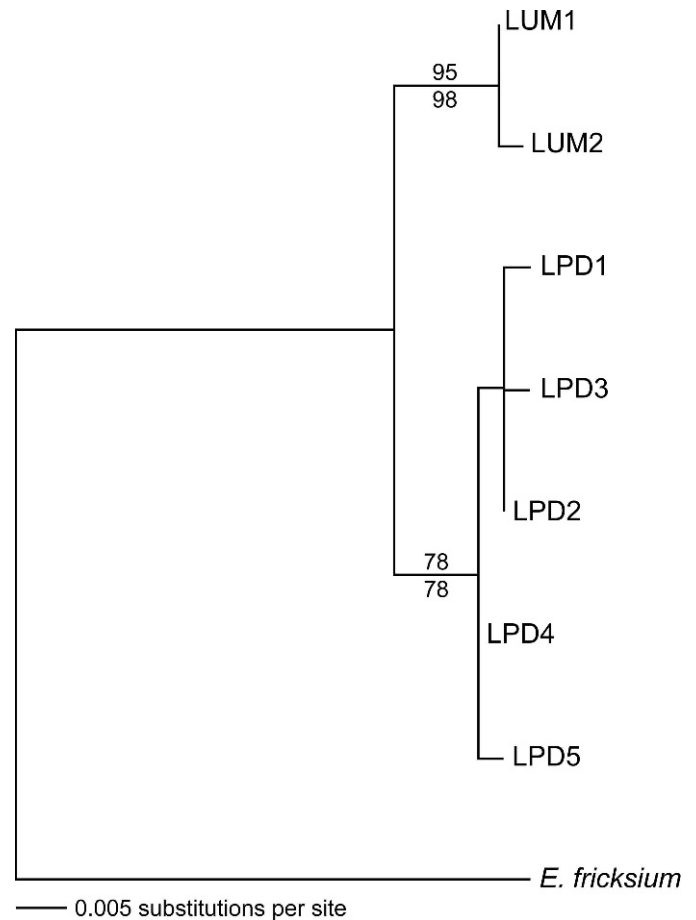


Fig. 2. Maximum-likelihood phenogram based on cytochrome *b* data. The maximum parsimony tree did not differ in topology. Terminal branches correspond to haplotypes and may represent multiple individuals. Bootstrap values for the ML and parsimony trees are listed above and below branches, respectively. For clarity, only bootstrap values greater than 70% are presented. Scale bar represents number of pairwise nucleotide differences per site.

significant (at $\alpha = 0.01$) in the Little Pee Dee (obs. $H = 0.103$; exp. $H = 0.220$; $P = 0.015$).

Significant divergence from Hardy-Weinberg expectations arising from a deficiency of heterozygotes can result from numerous factors including non-random mating, selection, and the Wahlund Effect (Wahlund, 1928; Hartl and Clark, 1997). The presence of a fixed difference in mitochondrial DNA within the Little Pee Dee (Table 1) suggests that it is not unreasonable to expect population structure within the Lumber as well. To assess the possibility of the Wahlund Effect arising from latent population structure within the Lumber River, we conducted a hierarchical AMOVA and HWE exact tests with populations defined as streams in the upper and lower Lumber and upper and lower Little Pee Dee. Lower Lumber samples did not deviate from HW expectation (obs. $H = 0.611$; exp. $H = 0.735$; $P = 0.088$), while upper Lumber did (obs. $H = 0.333$; exp. $H = 0.758$; $P < 0.01$). However, in the upper Lumber, five alleles are represented in a sample of just nine individuals. The lumping of a small number of individuals from nearby streams likely accounts for the deviation from HWE. In this case the deviation from HWE is also due to a true deficiency of heterozygotes. Because of the small sample size and number of locations sampled in the upper Lumber, a finer scale analysis was not possible. The upper Little Pee Dee was

Pee Dee ESUs should be managed independently; (2) the paucity of genetic diversity (S7) in the Little Pee Dee samples indicates a need for particularly close monitoring of this ESU; and (3) if captive-breeding or reintroduction programs are implemented in the future, care should be taken to prevent genetic exchange between ESUs.

Several researchers have pointed out the importance of utilizing additional data sources other than genetic data for determining ESUs (reviewed in Fraser and Bernatchez, 2001). Specifically, others have suggested the need to consider phenotypic and ecological differences (Taylor, 1999). In light of the controversy over criteria for designating ESUs, Fraser and Bernatchez (2001) proposed a dynamic, context-based framework based on available data termed 'adaptive evolutionary conservation'. Aside from the genetic data presented in this paper, other lines of evidence regarding the status of *E. mariae* are lacking. For example, very little morphological work has been done on *E. mariae*. In the only explicit study of Pinewoods Darter morphology, Richards (1963) noted no differences between Little Pee Dee and Lumber populations. While Richards examined specimens from both rivers, he was primarily concerned with interspecific relationships rather than intraspecific variation. Regardless, the lack of obvious morphological differences between Lumber and Little Pee Dee darters is somewhat surprising given the relatively deep genetic split between the two. The combination of high levels of endemism and overall depauperate nature of the Carolina Sandhills fauna (in terms of species diversity) suggests that there may be strong selective pressures associated with this environment, a hypothesis that requires testing.

Additionally, there is a need for similar studies in co-distributed Sandhills species. Further phylogeographic and population genetic studies of other imperiled fishes of the Carolina Sandhills are needed to assess whether patterns of genetic variation in *Etheostoma mariae* are concordant with co-distributed species. For example, *Semotilus lumbee* is a second Carolina Sandhills endemic that also occurs in headwater streams of the Pee Dee Drainage (but is less restricted in its habitat preferences; Rohde and Arndt, 1991), and thus might exhibit similar patterns of genetic variation. Preliminary data suggests that this species exhibits a similar, albeit shallower split between the Lumber and Little Pee Dee rivers (Quattro et al., unpubl.). Presently, genetic data for *E. mariae* represent a conservative estimate for appropriate management units in the Carolina Sandhills: namely, maintaining the biotic integrity of the Lumber and Little Pee Dee portions independently.

This study underscores the importance of fine-scale sampling in conservation genetics studies (Turner and Robison, 2006) and suggests that drainage-by-drainage assessments may not always provide sufficient resolution for this vein of research (Quattro et al., 2002). Data presented herein suggest that even geographically restricted endemic species can exhibit significant population structure on an extremely fine scale. Pinewoods Darters are an extreme example, with among the smallest spatial scales for reciprocally monophyletic populations known of any North American freshwater fish.

MATERIAL EXAMINED

Vouchered specimens.—North Carolina: Little Pee Dee tributaries: 4 specimens, NCSM 47646, Scotland Co., Juniper Cr.

at US HWY 15-501, 9 February 2008, F. C. Rohde; 6, NCSM 47647, Scotland Co., Jordan Cr. at SR 1324, 9 February 2008, F. C. Rohde; 7, NCSM 47648, Scotland Co., Gum Swamp Cr. at SR 1342, 9 February 2008, F. C. Rohde; 8, NCSM 47649, Scotland Co., Joe's Cr. at SR 1152, 9 February 2008, F. C. Rohde. Lumber tributaries: 7, NCSM 47650, Moore Co., Aberdeen City Limits, Ray's Mill Cr., a tributary of Aberdeen Cr., at Sandhills Blvd. and Maple St., 9 February 2008, F. C. Rohde; 8, NCSM 47651, Moore Co., Horse Cr. at SR 1112, 9 February 2008, F. C. Rohde; 1, NCSM 47652, Moore Co., Drowning Cr. at SR 1531, 9 February 2008, F. C. Rohde; 1, NCSM 47653, Richmond Co., Naked Cr. at SR 1321, 9 February 2008, F. C. Rohde; 1, NCSM 47654, Hoke Co., Mountain Cr. at SR 1215, 9 February 2008, F. C. Rohde; 6, NCSM 47655, Hoke Co., Quewhiffle Cr. at SR 1225, 9 February 2008, F. C. Rohde; 6, NCSM 47656, Hoke Co., Buffalo Cr. at SR 1203, 9 February 2008, F. C. Rohde.

Non-vouchered specimens.—North Carolina: Little Pee Dee tributaries: 1 specimen, Scotland Co., Joe's Cr. at SR 1152, 10 October 2000, F. C. Rohde; 3, Scotland Co., Joe's Cr. at SR 1152, 12 June 2001, F. C. Rohde and J. M. Quattro; 6, Scotland Co., Gum Swamp Cr. at SR 1342, 12 June 2001, F. C. Rohde and J. M. Quattro; 1, Scotland Co., Jordan Cr. at Sneadtown Road, 34.870432°, -79.485131°, 10 April 2005, T. J. Krabbenhof; 5, Scotland Co., Jordan Cr. at Sneadtown Road, 34.870432°, -79.485131°, 23 October 2005, T. J. Krabbenhof; 3, Scotland Co., Jordan Cr. at US HWY 15-501, 34.833437°, -79.45924°, 22 October 2005, T. J. Krabbenhof; 4, Scotland Co., Juniper Cr. at US HWY 15-501, 34.883859°, -79.451967°, 22 October 2005, T. J. Krabbenhof; 1, Scotland Co., Juniper Cr. at Nashville Creek Road, 34.899755°, -79.471459°, 23 October 2005, T. J. Krabbenhof. Lumber tributaries: 3 specimens, Montgomery/Richmond Co. line, Naked Cr. at SR 1527, approximately 3 km east of Norman, NC, 35.178148°, -79.698181°, 23 October 2005, T. J. Krabbenhof; 3, Richmond Co., Naked Cr. at SR 1003, 12 June 2001, F. C. Rohde and J. M. Quattro; 2, Montgomery/Moore Co. line, Drowning Cr. at Derby Road bridge, 35.175128°, -79.632285°, 10 April 2005, T. J. Krabbenhof; 5, Moore Co., Horse Cr. at Roseland Road, 35.132254°, -79.492261°, 10 April 2005, T. J. Krabbenhof; 2, Moore Co., Rays Mill Cr., a tributary to Aberdeen Cr., on corner of Maple St. and Sandhills Blvd., Aberdeen city limits, 35.134987°, -79.426690°, 23 October 2005, T. J. Krabbenhof; 3, Moore Co., unnamed tributary to Aberdeen Cr. at US HWY 1, 1 km north of Pinebluff, NC, 12 June 2001, F. C. Rohde and J. M. Quattro; 6, Hoke Co., Quewhiffle Cr. at Ashemont Road a.k.a. Chicken Road, 35.048991°, -79.416582°, 9 April 2005, T. J. Krabbenhof; 1, Hoke Co., Mountain Cr. at Winecuff Road a.k.a. Montrose Road, near McFarland, NC, 35.014386°, -79.390103°, 10 April 2005, T. J. Krabbenhof; 1, Hoke Co., Buffalo Cr. at Turnpike Road a.k.a. Horace Walter Road, 34.975322°, -79.358248°, 10 April 2005, T. J. Krabbenhof; 1, Hoke Co., Buffalo Cr. at Turnpike Road a.k.a. Horace Walter Road, 34.975322°, -79.358248°, 23 October 2005, T. J. Krabbenhof.

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of all haplotypes and alleles were submitted to GenBank (accession numbers: EU350091–EU350105); an alignment is available from the corresponding author. Specimens were collected under North Carolina Wildlife Resources Commission collecting licenses #0751 and #1038.

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