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MITOCHONDRIAL DNA PHYLOGEOGRAPHY OF THE POLYTYPIC NORTH AMERICAN RAT SNAKE (*ELAPHE OBSOLETA*): A CRITIQUE OF THE SUBSPECIES CONCEPT

FRANK T. BURBRINK,[1] ROBIN LAWSON,[2] AND JOSEPH B. SLOWINSKI[3]

[1]*Museum of Natural Science and Department of Biology, 119 Foster Hall, Louisiana State University, Baton Rouge, Louisiana 70803*
E-mail: fburbri@unix1.sncc.lsu.edu

[2]*Osher Foundation Laboratory for Molecular Systematics and Department of Herpetology, California Academy of Sciences, Golden Gate Park, San Francisco, California 94118*
E-mail: rlawson@mail.calacademy.org

[3]*Department of Herpetology, California Academy of Sciences, Golden Gate Park, San Francisco, California 94118*
E-mail: jslowins@calacademy.org

Abstract.—Subspecies have been considered artificial subdivisions of species, pattern classes, or incipient species. However, with more data and modern phylogenetic techniques, some subspecies may be found to represent true species. Mitochondrial DNA analysis of the polytypic snake, *Elaphe obsoleta,* yields well-supported clades that do not conform to any of the currently accepted subspecies. Complete nucleotide sequences of the cytochrome *b* gene and the mitochondrial control region produced robust maximum-parsimony and maximum-likelihood trees that do not differ statistically. Both trees were significantly shorter than a most parsimonious tree in which each subspecies was constrained to be monophyletic. Thus, the subspecies of *E. obsoleta* do not represent distinct genetic lineages. Instead, the evidence points to three well-supported mitochondrial DNA clades confined to particular geographic areas in the eastern United States. This research underscores the potential problems of recognizing subspecies based on one or a few characters.

Key words.—Control region 1, cytochrome *b*, *Elaphe obsoleta*, evolutionary biology, maximum likelihood, maximum parsimony, mitochondrial DNA, species, subspecies.

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The validity of the subspecies rank has received criticism for over 50 years (Mayr 1942, 1982; Wilson and Brown 1953; Cracraft 1983; McKitrick and Zink 1988; Frost et al. 1992; Frost and Kluge 1994). Commenting on the use of subspecies, Mayr (1982) stated that "the subspecies was not a concept of evolutionary biology but simply a handle of convenience for the clerical work of the museum curator." Moreover, subspecies that represent only arbitrary sections of a cline should not be considered as evolutionarily distinct entities (Wilson and Brown 1953; Mayr 1982; Cracraft 1983; Frost and Hillis 1990). Alternatively, subspecies may be used to designate isolated sublineages that may evolve into full species (incipient species; Mayr 1942). The suggestion that some subspecies may evolve into full species requires unobtainable knowledge of the future. Furthermore, incipient species cannot be differentiated from real species phylogenetically (Frost et al. 1992). Therefore, susbspecies have no real taxonomic meaning if they are used to represent arbitrary pattern classes or incipient species.

It is likely that there are species masquerading as subspecies and only additional studies can reveal their true status. Frost et al. (1992) suggested that in museum curatorial operations, it is prudent to retain trinomials for taxa with diagnosable characters having parapatric or allopatric ranges in case future work may find them to be distinct species. In this paper, we examine the phylogeography of the North American rat snake (*Elaphe obsoleta*) to determine if any of the subspecies represent distinct evolutionary lineages.

The North American rat snake is a common reptile in most forested areas of the central and eastern United States. Over the past 176 years, the following eight subspecies of *E. obsoleta* have been described based on adult color pattern: *E. o. obsoleta, E. o. quadrivittata, E. o. lindheimeri, E. o. spiloides, E. o. bairdi, E. o. deckerti, E. o. williamsi,* and *E. o. rossalleni.* Juveniles from all of these subspecies appear very similar in that they all have a light ground color with dark blotches.

The subspecies are defined by the following key characters: *E. o. obsoleta* (Say 1823) has a dark brown or black dorsum with little evidence of any pattern (Wright and Wright 1957; Conant and Collins 1991); *E. o. quadrivittata* (Holbrook 1842) has four dark dorsal stripes on a ground color of yellow or tan in Florida to gray in North Carolina (Conant and Collins 1991; Schultz 1996); *E. o. lindheimeri* (Baird and Girard 1853) has 25–35 large dorsal blotches on a brown, yellow, or orange ground color and is subject to considerable variation in color pattern (Conant and Collins 1991; Schultz 1996); *E. o. spiloides* (Duméril, Bibron, and Duméril 1854) resembles *E. o. lindheimeri,* where both retain the blotches evident in the juvenile color pattern in all of *E. obsoleta,* however, *E. o. spiloides* is distinguished from *E. o. lindheimeri* by having a gray or grayish white ground color (Schultz 1996); *E. o. deckerti* (Brady 1932) has four brown longitudinal stripes on an orange, yellow, or tan ground color, while still retaining the dorsal blotches found in the juveniles (Neill 1949; Wright and Wright 1957); *E. o. williamsi* (Barbour and Carr 1940) exhibits both blotches and stripes on a ground color of white or gray (Wright and Wright 1957; Schultz

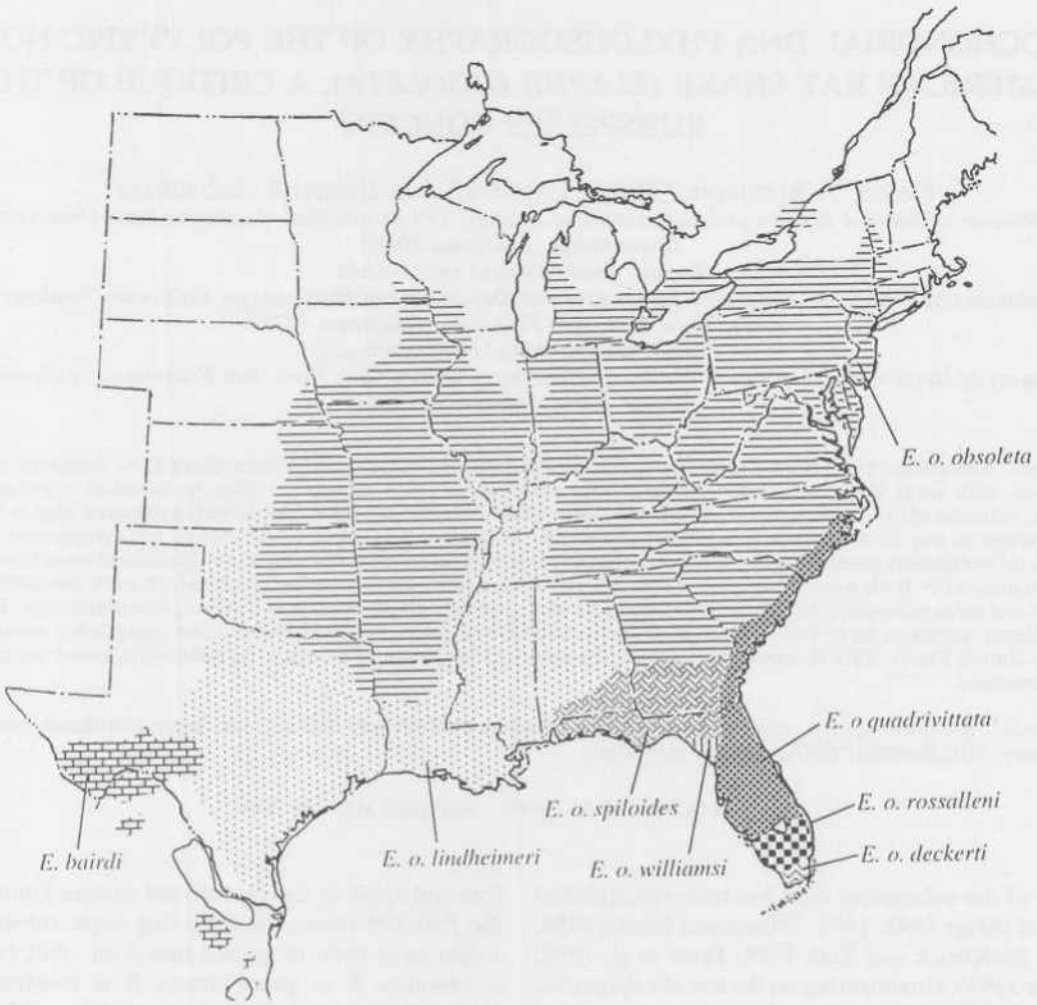


FIG. 1. Map of the eastern United States showing the geographic range of the subspecies of *Elaphe obsoleta* and *E. bairdi*. Note that the range of *E. o. spiloides* and *E. o. lindheimeri* differ from the maps in Conant and Collins (1991). During detailed morphological studies, F. T. Burbrink found that specimens displaying the gray ground color described for *E. o. spiloides* can only be found in southeastern Alabama, western Florida, and southern Georgia.

1996); *E. o. rossalleni* (Neill 1949) has an orange, orange-yellow, or orange-brown ground color with four poorly defined longitudinal stripes (Wright and Wright 1957); *E. o. bairdi* (Yarrow 1880 in Cope 1888) often has four poorly defined longitudinal stripes and numerous dorsal blotches on a ground color of brown to gray with a wash of yellow or orange at the edge of each scale (Conant and Collins 1991). This last subspecies was elevated to species status by Olson (1977). Ranges of these subspecies are illustrated in Figure 1.

Intergrades are assumed to occur where the ranges of these subspecies overlap (Neill 1949; Schultz 1996). However, there are no intergrades between *E. o. lindheimeri* and *E. bairdi* (Lawson and Lieb 1990). Neill (1949) and Dowling (1951) examined the geographic color variation in *E. obsoleta*. Each relied on color pattern to describe distinct geographic subspecies. Neill (1949), with no mention of actual specimens examined, believed that *E. o. obsoleta, E. o. spliloides* (referred to in his paper as *E. o. confinis*), *E. o. williamsi, E. o quadrivittata, E. o. rossalleni, E. o. deckerti,* and *E. o. lindheimeri* were all valid subspecies. However, Dowling (1951, 1952) recognized only four subspecies: *E. o. obsoleta, E. o. spiloides, E. o. quadrivittata,* and *E. o. bairdi. Elaphe o. rossalleni, E. o. deckerti,* and *E. o. williamsi* were placed in synonomy with *E. o. quadrivittata. Elaphe bairdi* was considered a subspecies of *E. obsoleta* because it was assumed that it intergrades with *E. o. lindheimeri.* Schultz (1996) reviewed the taxonomic history of the group.

To examine the relationships and phylogeography of *E. obsoleta,* we sequenced the complete cytochrome *b* and control region 1 mitochondrial genes from specimens of *E. obsoleta* and *E. bairdi* sampled from throughout its range (Fig. 2). We documented patterns of variation in the nucleotide sequences of these two genes within and among subspecies and reconstructed the phylogeny of these mitochondrial (mtDNA) genes. We used this gene tree to assess whether the currently recognized subspecies form natural groups. We maintain that if these eight subspecies form independently evolving lineages (species), then their distinct histories should be apparent in the mtDNA phylogeny, barring incomplete lineage sorting. In addition, we present a hypothesis

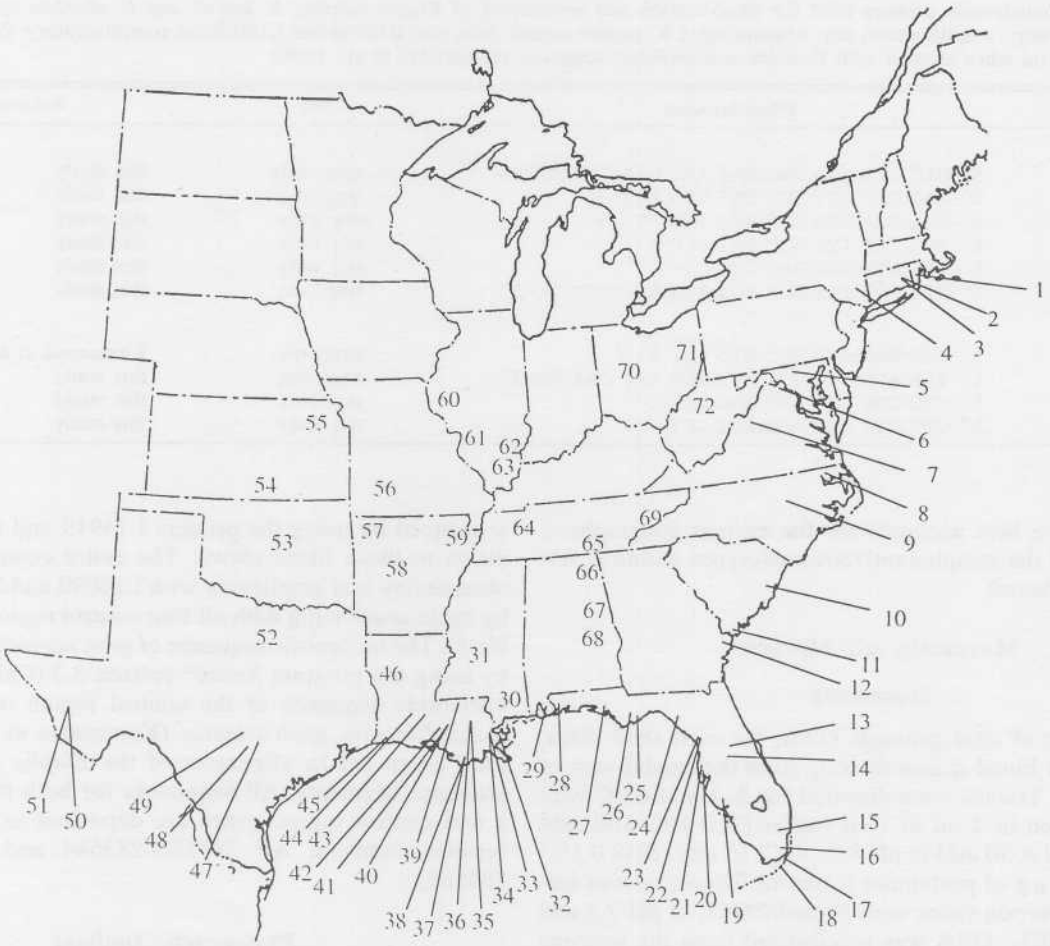


FIG. 2. Map of the eastern United States showing the localities of specimens used for the molecular analysis. The numbers in parentheses represent the locality numbers on the map. The following abbreviations in parentheses after each locality refer to tissues obtained from the following: B, Robin Lawson collection deposited at the Louisiana State University Museum of Natural Science; H, Louisiana State University Museum of Natural Science Herpetological Tissue Collection, RLB, blood from living snake taken by R. L. Lawson; CAS, California Academy of Sciences Tissue Collection; FB, shed skins.

(1)RI: Washington Co. (FB-9); (2)CT: Middlesex Co., Split Rock State Park (H-15889); (3) CT: Middlesex Co., Haddam (H-15890); (4)NY: Orange Co., Port Jervis (H-8455); 5 MD: Frederick Co., Brunswick (H-9349); (6) VA; Loudon Co., Middleburg (H-3191); (7) VA: Rockingham Co. (FB-14); (8) NC: Perquimans Co., Hartford (H-8825); (9) NC: Wake Co., Raleigh (H-8826); (10) SC: Williamsburg Co. (FB 9); (11) SC: Jasper Co., Hardeeville (H-3384); (12) GA: Chatham Co., Basin Road (old Hwy 17; H-9475); (13) FL: Putnam Co., Rodman Resevoir, State Rd 310 at Deep Creek (H-15884); (14) FL: Alachua Co., Gainesville (H-3377); (15) FL: Broward Co., Fort Lauderdale (H-3189); (16) FL: Dade Co., SW of Miami, Krone Ave (H-2229); (17) FL: Monroe Co., Key Largo, Tavernier (B13p35a); (18) FL: Monroe Co., Key Largo, Tavernier (B13p35b); (19) FL: Sarasota Co., Sarosota City (FB-20): (20) FL: Pinnelas Co., St. Petersburg, Tampa Bay, Placido Bayou (H-7783); (21) FL: Hernando Co., Brooksville, Hancock Lake (H-15028); (22) FL: Levy Co. (B13p35c); (23) FL: Levy Co., Hwy 24 and 345 (H-8775); (24) FL: Taylor Co., Perry (H-3212); (25) FL: Wakulla Co., US 98 and 319 (CAS 203079); (26) FL: Liberty Co., FL Hwy 13 on the west side of Ochlokonee River (CAS-203083); (27) FL: Walton Co., SE of Freeport (H-3276); (28) FL: Santa Rosa Co., Blackwater River State Forest (H-3309); (29) AL: Baldwin Co., Bay Minnette (H-3190); (30) MS: Forrest Co., Brooklyn (H-3186); (31) MS: Hinds Co. (FB-3); (32) LA: Orleans Par., New Orleans (H-8838); (33) LA: St. Tammany Par. 1.; Southern part of Parish at Pearl River (H-3379); (34) LA: St. Tammany Par. 2.: Abita Creek Preserve (H-15888); (35) LA: Tanghipahoa Par. 1, Natlbany River and Tickfaw River (H-3188); (36) LA: Tangipahoa Par. 2, Hammond (H-3246); (37) LA: Terrebonne Par. 1, Houma (H-8473); (38) LA: Terrebonne Par. 2, Houma (H-9338); (39) LA: East Feleciana Par., Clinton (H-3209); (40) LA: East Baton Rouge Par. 1, Baton Rouge (H-3169); (41) LA: East Baton Rouge Par 2., Baton Rouge (H-3306); (42) LA: Iberville Par., Plaquemine (H-8824); (43) LA: St. Landry Par., Hwy 71 (H-3280); (44) LA: Evangeline, Hwy 106 at Bayou Chico (H-15891); (45) LA: Cameron Par., Johnson's Bayou (H-8925); (46) LA: Natchitoches Par., Derry (H-15892); (47) TX: Comal Co., New Braunfels (H-8678); (48) TX: Medina Co., south of Medina Lake; (49) TX: Kerr Co., 16 mi South of Kerrville (H-8911); (50) TX: Jefferson Davis Co., 1 mi east of McDonald Observatory (H-3381); (51) TX: Jefferson Davis Co., 1 mi east of McDonald Observatory (H-3382); (52) TX: Palo Pinto Co., Brazos River (H-7572); (53) OK: Cleveland Co., vicinity of Norman (H-9337); (54) KS: Sumner Co., T33S, R1W, Sec. 6 (H-3394); (55) KS: Geary Co., T13S, R5E, Sec. 29 (H-3388); (56) MO: Greene Co., Springfield (CAS-162004); (57) AR: Madison Co., 0.5 mi east of Beaver Lake (H-15896); (58) AR: Garland Co., Lake Little Switzerland (H-14782); (59) AR: Craighead Co., Jonesboro (H-14781); (60) IL: Pike Co., 39°42′53′N, 90°39′16″W (H-9251); (61) IL: St. Claire Co., 8.5 km southeast of Mascoutah (H-14724); (62) IL: Gallatin Co., 7 km north of Old Shawneetown (H-15031); (63) IL: Johnson Co., 2.5 mi. northeast of Belknap (H-15030); (64) TN: Decatur Co., 1.5 mi. northwest of Decaturville (H-3206); (65) TN: Grundy Co., Savage Rd west of Co. Rd 399 (H-2286); (66) AL: Madison Co., Owana Cross Roads (H-8827); (67) AL: Talladega Co., 1.1 mi south of Rendalia (H-3385); (68) AL: Lee Co., Hwy 280 at Hwy 147 (H-3345); (69) TN: Knox Co., Knoxville (H-3376); (70) OH: Delaware Co., Delaware (FB-6); (71) OH: Stark Co., Canton (CAS 208631, RLB13P27F); (72) WV: Wood Co. (H-7572).

TABLE 1. Oligonucleotide primers used for amplification and sequencing of *Elaphe vulpina, E. bairdi,* and *E. obsoleta* cytochrome *b* and control regions. amp., amplification; seq., sequencing; CR, control region. Note that H16064 and L16090 are complimentary. Primer numbering refers to the 3′ end when aligned with *Dinodon semicarinatus* sequence (Kumazawa et al., 1998).

Primer	Primer sequence	Use	Reference
Cytochrome *b*			
L14910	5′-GAC CTG TGA TMT GAA AAC CAY CGT TGT-3′	amp. only	this study
L14919	5′-AAC CAC CGT TGT TAT TCA ACT-3′	amp. seq.	this study
L15324	5′-CCA TGA GGA CAA ATA TCA TTC-3′	seq. only	this study
L15584	5′-TCC CAT TYC ACC CAT ACC A-3′	seq. only	this study
L15399	5′-TTA ATT GAG AAT CCG CC-3′	seq. only	this study
H16064	5′-CTT TGG TTT ACA AGA ACA ATG CTT TA-3′	amp. seq.	this study
Control region			
H690	5′-GTT GAG GCT TGC ATG TAT A-3′	amp. seq.	Kumazawa et al. 1996
L16090	5′-TAA AGC ATT GTT CTT GTA AAC CAA AG-3′	amp. seq.	this study
H16602	5′-TTC CGG GCC ATT AAG ATG-3′	seq. only	this study
L16577	5′-GTT CTT TCC AAG ACC GCT-3′	seq. only	this study

that we believe best accounts for the current geographical distribution of the sampled mtDNA haplotypes within *E. obsoleta* and *E. bairdi.*

MATERIALS AND METHODS

Sequencing

As a source of total genomic DNA, we used shed skins, liver tissue, or blood drawn directly from the caudal vein of living snakes. Tissues were digested for 3–4 h at 65°C with constant motion in 2 ml of lysis buffer (Tris HCl 100 mM at pH 8.0, EDTA 50 mM at pH 8.0, NaCl 10 mM, SDS 0.5%) containing 60 μg of proteinase K per ml. Digestion was followed by extraction twice with phenol/$CHCl_3$ at pH 7.3 and once with $CHCl_3$. DNA was precipitated from the aqueous layer with 2.5 volumes of pure ethanol and the usually spooled DNA was then washed in 80% ethanol, dried, and redissolved in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0). Template DNA for the polymerase chain reaction (PCR) was prepared by diluting stock DNA with TE buffer to give spectrophotometric readings at A260 between 0.2 A and 0.7 A. Mitochondrial DNA was amplified from template DNA in 100 μl reactions using a hot-start method in a thermal cycler with a 7-min denaturing step at 94°C followed by 40 cycles

sequenced by using the primers L14919 and H16064 in addition to those listed above. The entire control region was obtained by first amplifying with L16090 and H690 followed by cycle sequencing with all four control region primers (Table 1). The nucleotide sequence of gene segments was aligned by using the program Xesee© version 3.2 (Cabot 1998). The nucleotide sequence of the control region of the colubrid snake *Dinodon semicarinatus* (Kumazawa et al. 1998) was used as an aid in alignment of the *Elaphe* control region sequence segments. All sequences for both the cytochrome *b* and control region genes are deposited in Genbank (accession numbers: AF 283576–283644 and AF 284138–284182).

Phylogenetic Analyses

The aligned sequences resulted in 2151 nucleotides (1117 cytochrome *b* positions and 1034 control region positions). The 70 *E. obsoleta*, two *E. bairdi,* and one *E. vulpina* sequences were analyzed by using maximum-parsimony and maximum-likelihood optimality criteria implemented with PAUP* 4.0 (Swofford 1999). The maximum-parsimony (MP) analysis was performed with all sites weighted equally. For MP, 100 heuristic searches

Tavaré 1986; Rodriguez et al. 1990) models. Because the three codon sites in protein-coding genes experience different substitution rates, rate heterogeneity was assumed and rate variation was modeled as a discrete gamma distribution (Yang 1993, 1996) with four rate categories. The three models were tested by using the MP tree. The gamma HKY85 model with a −ln value of 5688.85 was shown to perform significantly better than the gamma F81 model with a −ln value of 5937.68 (X^2 = 497.65, df = 1, $P < 0.001$). The gamma GTR model with a −ln value of 5685.07 did not perform significantly better than the HKY85 (X^2 = 5.56, df = 4, P = 0.4). Thus, the gamma HKY85 is considered the appropriate model for these data.

Different tree topologies were examined statistically using Templeton (Templeton 1983), winning sites (Prager and Wilson 1988), and Kishino-Hasegawa (Kishino and Hasegawa 1989) tests. With these tests, it is possible to determine if different tree topologies produced by MP or ML are significantly different. In addition, trees that constrain subspecies into monophyletic groups can be statistically compared to the shortest MP and ML trees without topological constraints.

Pairwise Genetic Comparisons

Using the sequence data, we examined the relationship between genetic distance and geographic distance (Wright 1943; Kimura 1953; Kimura and Weiss 1964; Hutchison and Templeton 1999). All pairwise genetic distances of individuals with both complete gene sequences corrected for multiple substitutions by the gamma HKY85 model with invariable sites estimated from the data were regressed against estimated pairwise geographic distance between collecting sites. Straight-line distances between pairs of samples were used in this study.

Results

Sequence Characteristics

Cytochrome b

Because the amplification primers are located within highly conserved transfer RNA genes that flank the cytochrome *b* gene and because our sequences contain an open reading frame without stop codons, it may be inferred that the sequences represent the functional cytochrome *b* gene rather than nuclear pseudogenes. Additionally, the translated amino acid sequence is sufficiently similar to that of other snakes to support this contention.

Characteristics of the complete snake cytochrome *b* nucleotide sequences were discussed by Campbell (1997) and Slowinski and Keogh (2000). In the rat snakes, as in other advanced snakes, the cytochrome *b* sequence commences with the start codon ATG coding for the amino acid methionine. In the *Elaphe* examined in this study, the cytochrome *b* gene is always 1116 bp long (372 amino acids). Instead of a stop codon, the gene ends with a thymine that is posttranscriptionally polyadenylated to form a functional stop codon (UAA), a common mechanism in snakes (Campbell 1997; Kumazawa et al. 1998; Slowinski and Keogh 2000). Additionally, as in other advanced snakes, 10 amino acid positions are deleted relative to the cytochrome *b* molecule of most other vertebrates; nine codon deletions occur near the 5′ end and one near the 3′ end of the gene (Campbell 1997; Slowinski and Keogh 2000). These deletions are in the matrix domain of the cytochrome *b* molecule (Degli Esposti 1993), which, because of functional differences between domains, has been shown to be the least conserved of the three domains comprising the molecule (Griffiths 1997). Of the 372 amino acids coded in the snake cytochrome *b* gene, there are two fixed differences between the ingroup and outgroup taxa and 12 positions that show variability among the ingroup taxa.

Nucleotide compositional bias exists mainly among the three codon sites, rather than as bias among the sampled sequences (all cytochrome *b* sites: X^2 = 14.35, df = 216, P = 1.00). The frequencies of A, C, G, and T at the first position were 0.350, 0.230, 0.178, and 0.241, respectively. For the second positions, they were 0.198, 0.280, 0.111, and 0.410. For the third positions, they were 0.451, 0.269, 0.036, and 0.245. This pattern of bias, with As dominating at first sites, Ts at second sites, and As at third sites, is similar to the pattern found by Campbell (1997) and Slowinski and Keogh (2000) in other snakes and is generally similar to the pattern in other vertebrates (e.g., mammals: Irwin et al. 1991).

Control Region

There are few descriptions of the control region in snakes. Here we compare the control region sequence for the rat snakes used in this study with that published for *D. semicarinatus,* an Asian colubrine snake (Kumazawa et al. 1998). The control region in vertebrates is composed of three domains, a 5′ domain that contains one or more termination-associated sequences (TAS), a central conserved domain, and a 3′ domain that contains the site of initiation for heavy-strand replication, as well as two to three conserved sequence blocks (CSB; Brown et al. 1986; Saccone et al. 1991; Taberlet 1996). The displacement loop is part of the control region from the termination-associated sequences and spans the site of initiation of heavy-strand replication (Clayton 1982, 1991; Taberlet 1996). In vertebrates, sites with increased variability are different among species, but generally are found in the 5′ and 3′ domains where insertions, deletions, and base substitutions occur more commonly (Taberlet 1996).

In *D. semicarinatus,* the entire control region has been duplicated, with the duplicate sequence inserted within the isoleucine-glutamine-asparagine (IQM) tRNA gene cluster (Kumazawa et al. 1996, 1998). Because this duplication has been found in colubrids, boids, and viperids, but not in *Leptotyphlops,* its presence in all advanced snakes seems probable (Kumazawa et al. 1996). These two control regions have been designated control region 1 for the original, located between tRNA-proline and tRNA-phenylalanine, and control region 2 for the duplicate (Kumazawa et al. 1998). To ensure that only control region 1 was amplified, primers located in tRNA-Thr and a highly conserved segment of 12S ribosomal DNA flanking this region were used.

The control region 1 of the three species of *Elaphe* sampled show a high level of sequence similarity with that of *Dinodon.* Near the 5′ end of the left domain is a region known as the C-rich region, which in *Dinodon* consists of nine Cs followed

by TA and then an additional nine Cs. In all three *Elaphe* species sampled, the C-region is similar to that of *Dinodon*, with nine Cs in the 5′ block but only eight in the 3′ block. Unique to the *E. vulpina* examined was a sequence of 10 nucleotides inserted within the 5′ C block. Of three left domain and one right domain hairpin-like secondary structures in the *Dinodon* control region, hairpins 1 and 2 are nearly identical in *Elaphe*, hairpin 3 is identical, and hairpin 4 is variously identical or nearly so among different *E. obsoleta* specimens, *E. bairdi*, and the single *E. vulpina*. Likewise, TAS 1 and TAS 2 are identical between *Dinodon* and the three *Elaphe* species. CSB 1, 2, and 3 are very similar in sequence between *Dinodon* and *Elaphe*, but do show some variability among *E. obsoleta* individuals. Finally, a singly repeated 20-bp segment and a doubly repeated 49-bp segment found in *Dinodon* also occur in the three species of *Elaphe*, where they have a high degree of sequence similarity to *Dinodon*.

Relative to *Dinodon*, there are six nucleotide sites that are absent from *E. vulpina* and eight that are absent from *E. obsoleta* and *E. bairdi*. In *E. vulpina*, there are, in addition to the 10-nucleotide segment inserted within the C-rich region mentioned above, four nucleotide sites not found in *Dinodon*. Among *E. bairdi* and *E. obsoleta* individuals, there are two to six sites absent from *Dinodon*. With the present data, it is not possible to tell whether these indels are insertions or deletions.

The *Dinodon* control region is 1018 bp (Kumazawa et al. 1996), *E. vulpina* control region is 1032 bp, and the *E. obsoleta* control region is variably between 1020 bp and 1022 bp.

Phylogenetic Analyses

The parsimony search found 18,479 shortest trees of length 424 steps (uninformative sites excluded; CI = 0.644, RI = 0.950). The g1 statistic for these data was −0.607, indicating highly structured data (Hillis 1991; Hillis and Huelsenbeck 1992). Results of the nonparametric bootstrap analysis are presented in Figure 3. Very high support for three major mtDNA clades of *E. obsoleta* was obtained from the bootstrap analysis: (1) an eastern clade comprised of rat snakes east of the Apalachicola River and the Appalachian Mountains (98% bootstrap support); (2) a central clade located west of the Apalachicola River and the Appalachian Mountains and east of the Mississippi River (99% bootstrap support); and (3) a clade west of the Mississippi River (79% bootstrap support). A monophyletic *E. bairdi* mtDNA clade (100% bootstrap support) is placed as the sister taxon to the western clade.

The ML analysis provided a tree with −lnL of 5676.90. This value was compared to the MP tree's −lnL value of 5679.52 using the Kishino-Hasegawa test, which did not show a significant difference ($P = 0.375$). The parsimony tree length for the ML topology was 428 steps (CI = 0.638, RI = 0.949), which was not significantly different from the length of the MP tree according to the Templeton ($P = 0.157$), winning sites ($P = 0.375$), and Kishino-Hasegawa ($P = 0.158$) tests.

To test the hypothesis that the subspecies form natural groups, a parsimony analysis was undertaken in which subspecies were constrained to be monophyletic. This generated 10,811 equally parsimonious unconstrained trees of 695 steps (CI = 0.393, RI = 0.861). This tree is 271 steps greater than the most parsimonious trees (424), which is significant according to Kishino-Hasegawa ($P < 0.0001$; Kishino and Hasegawa 1989), Templeton ($P < 0.0001$; Templeton 1983), and winning sites ($P < 0.001$; Prager and Wilson 1988 tests). Constraining subspecies monophyly also produced a ML tree with a significantly lower likelihood score than the unconstrained ML estimate to the Kishino-Hasegawa test (difference in −lnL = 1083.51, $P < 0.0001$).

Haplotypes and Pairwise Genetic Comparisons

In the 72 ingroup specimens examined in this study, a total of 59 distinct haplotypes were discovered. The cytochrome *b* gene alone produced 56 distinct haplotypes for 72 ingroup specimens, whereas the control region yielded 26 distinct haplotypes for 68 ingroup specimens. Members of the following groups of taxa share the same haplotypes: (1) Johnson County, Illinois, Gallatin County, Illinois, Craighead County, Arkansas, Decatur County, Tennessee; (2) Delaware County, Ohio, Stark County, Ohio, Pike County, Illinois; (3) Madison County, Arkansas, Garland County, Arkansas; (4) Orleans Parish, Louisiana, East Baton Rouge Parish, Louisiana 2; (5) Pinellas County, Florida, Broward, County, Florida; (6) Monroe County, Florida 1, Monroe County, Florida 2. Both the control region and the cytochrome *b* gene were sequenced in all 73 specimens except for the following: Sumner County, Kansas; Greene County, Missouri; Cleveland County, Oklahoma; and St. Landry Parish, Louisiana. Only the control region sequences were determined for those snakes. These four specimens shared the same control region haplotypes with several other specimens found within the western clade: (1) Sumner County, Kansas, Greene County, Missouri, Cleveland County, Oklahoma, Geary County, Kansas; (2) Cameron County, Louisiana, St. Landry Parish, Louisiana; (3) Natchitoches Parish, Louisiana, St. Landry Parish, Louisiana; (4) Terrebone Parish, Louisiana 1, St. Landry Parish, Louisiana; (5) Terrebone Parish, Louisiana 2, St. Landry Parish, Louisiana.

Pairwise geographic and genetic distances are categorized as intraclade comparisons if all of the comparisons were made between members found only within the eastern clade, central clade, or western clade. They are categorized as interclade comparisons if all the comparisons were made between individuals from different clades (i.e., eastern and central clades, eastern and western clades, central and western clades, eastern and *E. bairdi* clades, central and *E. bairdi* clades, and western and *E. bairdi* clades). The range of values for inter- and intraclade genetic distances for each gene and combined genes are shown in Table 2. For the combined genes and the cytochrome *b* genes alone, all interclade distances are higher than all intraclade distances. This evidence, along with the phylogenies, indicates that these mtDNA clades represent independent evolutionary lineages and that members within each clade are more closely related to each other than they are to members of other clades (Avise 1994).

Regressing pairwise genetic distance against pairwise geographic distance for all intraclade comparisons produced

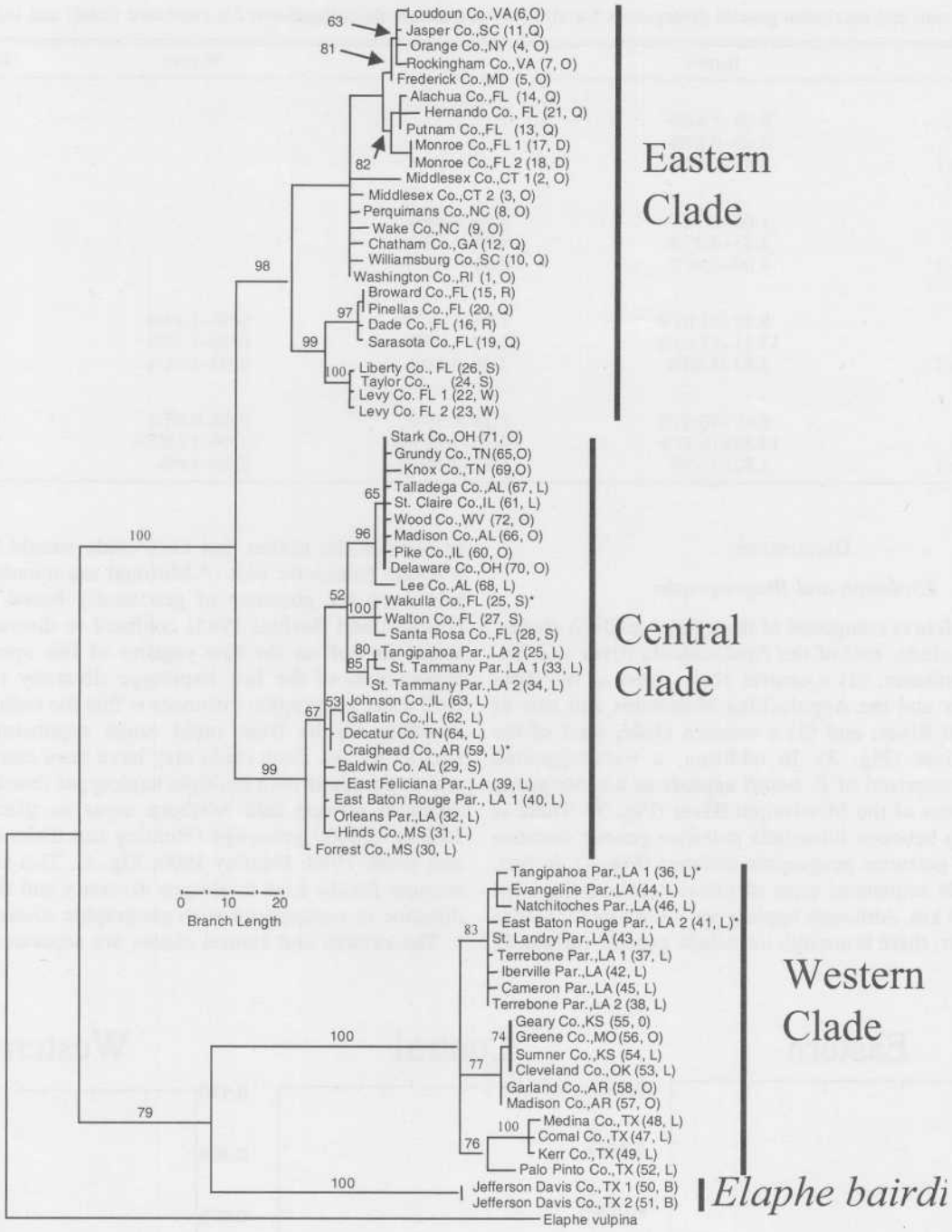


FIG. 3. Bootstrap, strict consensus tree for 18,479 shortest trees of length 424 steps. Numbers in parentheses correspond to localities listed in Figure 2. Letters in parentheses correspond to subspecies designation where: O, *Elaphe obsoleta obsoleta*; Q, *E. o. quadrivittata*; D, *E. o. deckerti*; S, *E. o. spiloides*; R, *E. o. rossalleni*; W, *E. o. williamsi*; L, *E. o. lindheimeri*; and B, *E. bairdi*. Specimens found outside of the geographic area represented by their clade are marked with an asterick.

graphs that were inconsistent with isolation by distance (a slope close to zero with low r^2-values; no points on the regression are nonindependent; Fig. 4). The intraclade comparisons failed to demonstrate that pairwise genetic distance increases with pairwise geographic distance. This may indicate that the populations within each clade have recently expanded outward from a smaller area and there has not been enough time for these genes to have diverged significantly (Fig. 5). Although less likely, the results could also suggest that each of the three major clades represents a panmictic population (see Discussion). Because the interclade genetic distances are much greater than the intraclade distances, a correlation between genetic distance and geographic distance between members of different clades is not expected.

TABLE 2. Minimum and maximum genetic divergences for all inter- and intraclade comparisons for combined (total) and individual genes.

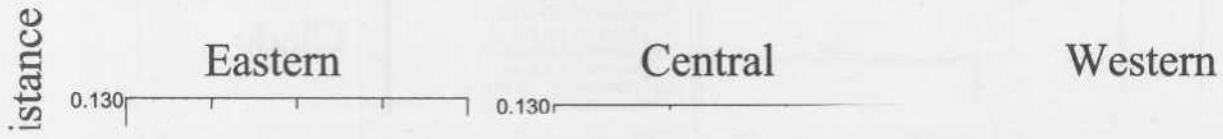
Clade	Eastern	Central	Western	*Elaphe bairdi*
Eastern				
Total	0.00–1.44%			
Cytochrome *b*	0.00–2.07%			
Control region 1	0.00–1.13%			
Central				
Total	1.60–2.76%	0.00–0.84%		
Cytochrome *b*	2.83–4.37%	0.00–1.53%		
Control region 1	0.00–1.41%	0.00–1.41%		
Western				
Total	8.59–11.01%	8.96–11.45%	0.00–1.16%	
Cytochrome *b*	13.11–15.65%	13.89–16.9%	0.00–1.55%	
Control region 1	3.64–5.80%	3.46–4.99%	0.00–1.11%	
E. bairdi				
Total	8.49–10.49%	8.28–9.53%	7.65–9.06%	0.09%
Cytochrome *b*	14.82–16.77%	14.60–16.19%	11.64–13.02%	0.001%
Control region 1	1.82–2.97%	1.82–2.769%	2.66–3.6%	0.001%

DISCUSSION

Evolution and Biogeography

Elaphe obsoleta is composed of three large mtDNA clades: (1) an eastern clade, east of the Apalachicola River and Appalachian Mountains; (2) a central clade, west of the Apalachicola River and the Appalachian Mountains and east of the Mississippi River; and (3) a western clade, west of the Mississippi River (Fig. 3). In addition, a well-supported fourth clade comprised of *E. bairdi* appears as a sister group to the clade west of the Mississippi River (Fig. 3). There is no relationship between intraclade pairwise genetic distance and intraclade pairwise geographic distance (Fig. 4). In fact, some intraclade sequences were identical despite being separated by 1000 km. Although haplotypes within major clades are very similar, there is enough intraclade genetic variability to exclude the notion that each clade should be considered a single panmictic unit. Additional arguments against panmixis are the presence of genetically based color morphs (Bechtel and Bechtel 1985) confined to discrete geographic areas, as well as the low vagility of this species. A better explanation of the low haplotype diversity in comparison with great geographic distances is that the reduced intraclade diversity results from rapid range expansion from much smaller nuclei. Each clade may have been composed of several populations with multiple haplotypes that dispersed from southern refugia into northern areas as glaciers retreated 18,000 to 6000 years ago (Huntley and Birks 1983; Huntley and Webb 1988; Huntley 1990; Fig. 5). This scenario would account for the high haplotype diversity and the low genetic distance in comparison with geographic distance.

The eastern and central clades are separated by the Apa-



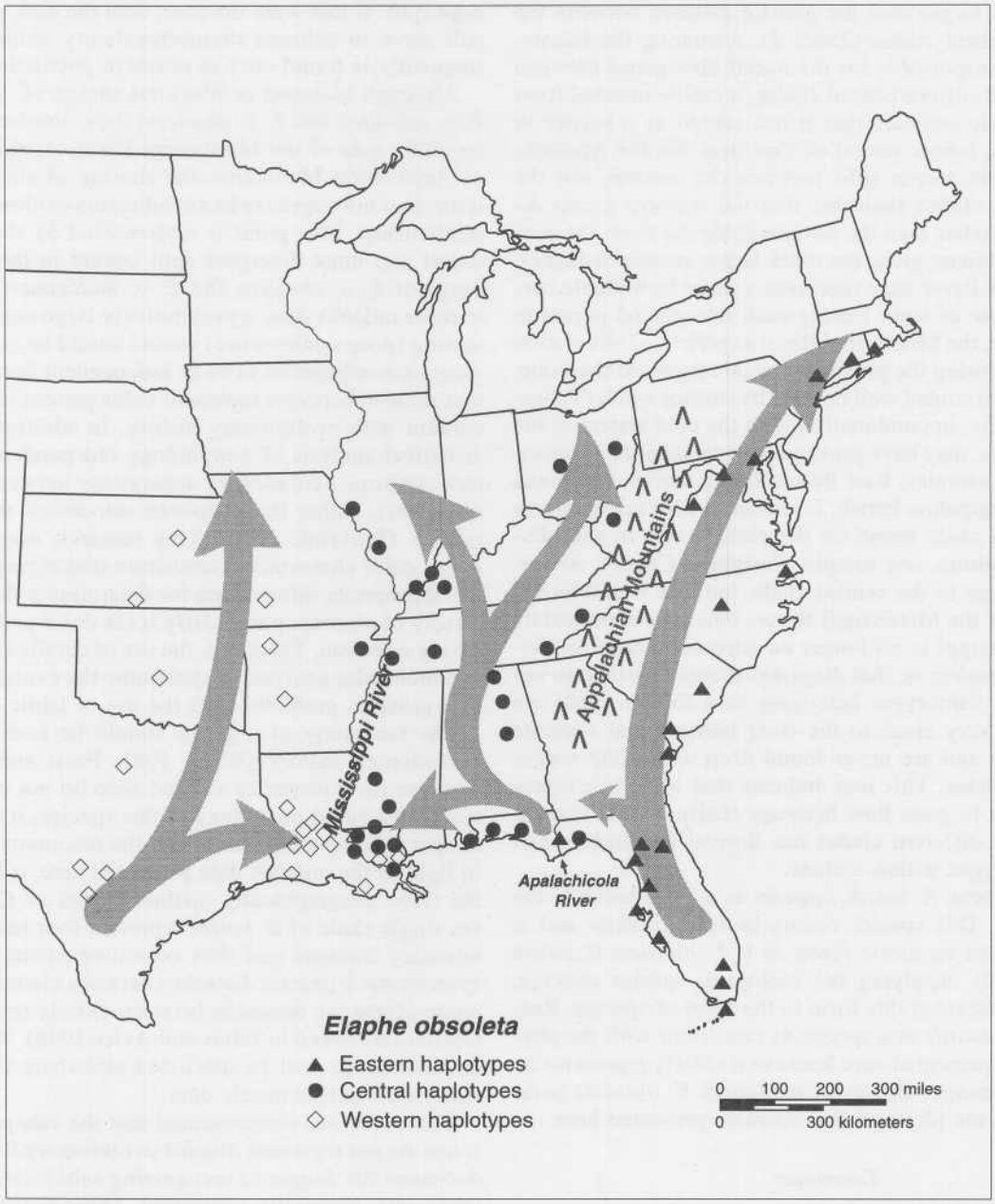


FIG. 5. Map showing northern dispersal patterns of *Elaphe obsoleta* mitochondrial clades from southern refugia following glacial retreat.

lachicola River. This river is the site of morphological and molecular discontinuities within fish, amphibian, and reptile species or clades (see reviews in Neill 1957; Blainey 1971; Swift et al. 1985; Bermingham and Avise 1986; Lawson 1987; Avise 1992, 1996). At the maximum rise in sea level during interglacial periods in the Pliocene and Pleistocene, the Apalachicola River was embayed as a large saltwater channel well into Georgia and Alabama, possibly up to the Fall Line in northern Georgia (Cooke 1945; Neill 1957; Blainey 1971). It was then that the Apalachicola River, in conjunction with the Appalachian Mountains to the north, apparently served as a barrier separating the eastern and central clades of *E. obsoleta*. However, the Wakulla County, Florida (26) sample is anomalous, in that it is a member of the central clade found east of the Apalachicola River. This may indicate that the Apalachicola River is no longer a barrier to east-west dispersal of *E. obsoleta* or that limited amounts of dispersal have always occurred. It has not yet been determined if the members of the eastern and central clade are hybridizing.

The central clade is separated from the western clade by the Mississippi River, a role the Mississippi has played in dividing the ranges of many species of fishes, amphibians, and reptiles in the central United States (Blair 1958, 1965; Wiley and Mayden 1985; Mayden 1988; Walker et al. 1998). The genetic distance between the western and central/eastern

clades is much larger than the genetic distance between the eastern and central clades (Table 2). Assuming the Mississippi River is responsible for the initial divergence between the western and eastern/central clades, it can be inferred from the phylogenetic estimate that it has served as a barrier to gene flow for a longer period of time than has the Apalachicola River. The deeper split between the western and the eastern/central clades indicates that the western group diverged much earlier than the eastern clade did from the central clade. Moreover, given the much larger genetic distances, the Mississippi River may represent a more formidable barrier to gene flow as well. During each interglacial period in the Pleistocene, the Mississippi River experienced alluviation of valleys cut during the previous glacial stages. At that time, the floodplain extended well beyond its current width (Thornbury 1965). This, in combination with the cold waters of the melting glaciers, may have prevented dispersal of *E. obsoleta.* However, two samples, East Baton Rouge Parish, Louisiana 2 (41) and Tangipahoa Parish, Louisiana 1 (36) are members of the western clade found on the eastern side of the Mississippi. In addition, one sample, Craighead County, Arkansas (59), belongs to the central clade, but was found on the western side of the Mississippi River. This may demonstrate that the Mississippi is no longer an adequate barrier to dispersal of *E. obsoleta* or that dispersal events continue to occur. However, haplotypes belonging to a foreign clade are always found very close to the river barriers that separate the two clades and are never found deep within the ranges of the other clades. This may indicate that there is a reproductive barrier to gene flow between clades or that competition between different clades has limited the dispersal of foreign haplotypes within a clade.

The desert form, *E. bairdi,* appears as a sister taxon to the western clade. This species occurs in xeric habitats and is not as dependent on mesic forest as is *E. obsoleta* (Lawson and Lieb 1990). Applying the biological species concept, Olson (1977) elevated this form to the level of species. Recognition of *E. bairdi* as a species is consistent with the phylogenetic data presented here because it clearly represents an independent lineage. However, it renders *E. obsoleta* paraphyletic given the phylogenetic estimate presented here.

Taxonomy

The well-supported clades demonstrate that none of the currently accepted subspecies represents a distinct evolutionary lineage (Fig. 3). As described in the introduction, many of these subspecies are diagnosed by color pattern. Apparently, these color characters are labile and bear few clues to the phylogenetic history of *E. obsoleta.* In fact, if the primitive color pattern of *E. obsoleta* is blotched (this is assumed because the outgroup, *E. vulpina,* is blotched and blotches are present in *E. obsoleta* very early in ontogeny), then the northern black coloration has evolved independently several times. This dark color pattern may have evolved multiple times in response to selection for enhanced thermoregulatory capabilities in cooler northern environments (Braswell 1977). Alternatively, these dark color patterns may have initially been found in the ancestor of the eastern, central, and western clades and subsequently persisted after each lineage split. If that were the case, then the dark coloration may still serve to enhance thermoregulatory abilities because it frequently is found only in northern populations.

Although blotched or black rat snakes (*E. o. lindheimeri, E. o. spiloides,* and *E. o. obsoleta*) show similar color patterns on either side of the Mississippi River, Apalachicola River, or Appalachin Mountains, the sharing of similar color patterns does not appear to be an indication of close phylogenetic relationship. This point is underscored by the fact that the oldest and most divergent split occurs in the center of the range of *E. o. obsoleta* and *E. o. lindheimeri.* With respect to these mtDNA data, a prohibitively large number of lineage sorting (deep coalescence) events would be required to consider each subspecies to be an independent lineage. It appears that these subspecies represent color pattern classes not concordant with evolutionary history. In addition, multivariate statistical analysis of morphology independent of color pattern confirms evolutionary separations between clades identified here, rather than between subspecies defined by coloration (Burbrink 2000). This research may indicate that vague color characters in temperate snakes might not provide the appropriate information for determining the evolutionary history of a group, particularly if the color patterns are under strong selection. Therefore, the use of detailed morphological and molecular analyses to determine the evolutionary history of a group is preferred over the use of labile color patterns.

The taxonomy of a group should be consistent with its evolutionary history (Wiley 1981; Frost and Hillis 1990). Because the subspecies studied here do not conform to the molecular-based phylogeny of this species, it is recommended that they be eliminated from the taxonomy of this group. In light of the mtDNA data presented here, it is possible that the three geographically distinct clades of *E. obsoleta* and the single clade of *E. bairdi* represent four independent evolutionary lineages and thus constitute distinct species. The cytochrome *b* genetic distances between clades are within the range of genetic distances between closely related species of reptiles (reviewed in Johns and Avise 1998). Taxonomic recommendations will be discussed elsewhere following evaluations of morphometric data.

This study has demonstrated that the subspecies of *E. obsoleta* do not represent distinct evolutionary lineages and underscores the danger of recognizing subspecies based on few characters, especially coloration. These poorly defined subspecies actually mask the evolutionary history of the group. Therefore, describing or recognizing subspecies from a few characters may not simply be a harmless handle of convenience for museum curators, but may be detrimental to understanding evolutionary history.

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