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Refugia and Speciation in North American Scarlet Snakes (*Cemophora*)

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Abstract.—**Scarlet Snakes (*Cemophora coccinea*) are monotypic, poorly studied, semifossorial habitat specialists from southeastern United States that traditionally include three subspecies based on color pattern and morphology. We sequenced two mitochondrial and two nuclear loci for 62 individuals from across the species range, and analyzed data with the use of parsimony and Bayesian phylogenetic methods to test a previously proposed phylogenetic hypothesis for *C. coccinea*. Our results suggest two Pliocene or Pleistocene refugia for *Cemophora*, one in southern Texas, and the other in the region extending from southeastern Louisiana through Florida. In light of our results we elevate *Cemophora coccinea lineri* to a full species, *C. lineri*, that differs from *C. coccinea* sensu stricto genetically and phenotypically.**

The Pleistocene Epoch, 2.58 million (Ma) to 11.7 thousand (Ka) years ago, was characterized by a series of cold, mesic glacial periods separated by warmer, drier interglacial periods, causing many species ranges in the northern hemisphere to shift south during glacial maxima and north during interglacials (Auffenberg and Milstead, 1965; Soltis et al., 2006). Whether Pleistocene climate changes were important for speciation of North American reptiles has been controversial. Historically, it was thought that virtually all extant reptile species in North America originated during the Miocene or earlier (Auffenberg and Milstead, 1965); however, genetic data suggest that Pleistocene speciation was more common than previously thought (Soltis et al., 2006). Nevertheless, many species have been neglected from genetic studies and whether speciation during the Pleistocene was common still is unclear.

Scarlet Snakes (*Cemophora coccinea*) are small, fossorial colubrid snakes, distributed widely across southeastern Unites States, and are monotypic with three geographic populations traditionally referred to as subspecies. *Cemophora coccinea coccinea* (Blumenbach, 1788) is endemic to peninsular Florida; *Cemophora coccinea copei* Jan, 1863 (Jan, 1863), ranges from eastern Oklahoma and Texas to southern New Jersey, and to northern Florida where it meets *C. c. coccinea*; and *Cemophora coccinea lineri* Williams et al. (1966), has an allopatric distribution and is endemic to southeastern Texas (Fig. 1A). The broad distribution of *C. coccinea* makes it an interesting species to investigate phylogeographically, but little has been published on *Cemophora* since Williams and Wilson (1967) reviewed the genus nearly 50 yr ago.

We tested the hypothesis that cold Pleistocene temperatures forced *C. coccinea* into two isolated refugia, one in southern Texas, and the other in Florida and adjacent areas. For this test, we sequenced two mitochondrial and two nuclear loci, inferred gene trees and a time-calibrated phylogeny, and compared color pattern and lepidosis for all three subspecies of *C. coccinea*. We predicted that divergence within *Cemophora* began during the Pleistocene, that *C. c. lineri* is the sister group to the remaining groups within *C. coccinea*, and that *C. c. lineri* is phenotypically distinguishable from other *C. coccinea*. Additionally, we compared genetic variation across the species range to test if *C. coccinea* expanded north out of the hypothesized Florida refugium during the Pleistocene, predicting that *C. c. copei* and *C. c. coccinea* are closely related genetically and that genetic variation is highest in Florida and adjacent areas.

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Materials and Methods

DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing.—We extracted genomic DNA from 62 *C. coccinea* and from 10 individuals used as outgroup taxa (two *Arizona elegans*, two *Lampropeltis gentilis*, one *Lampropeltis holbrooki*, one *Lampropeltis nigra*, one *Pantherophis obsoletus*, one *Pantherophis obsoletus spiloides*, and two *Rhinocheilus lecontei*) with the use of a standard salt DNA extraction protocol (Austin et al., 2010). We performed PCR to amplify two mitochondrial and two nuclear loci, including cytochrome oxidase 1 (COI), NADH dehydrogenase 4 (ND4) plus Histidine and Serine tRNAs, intron 1 of Spectrin Beta Non-Erythrocytic 1 (SPTBN1 intron 1), and intron 15 of the Z-chromosome gene Glutamate Decarboxylase 2 (GAD2 intron 15). PCR and sequencing primers are shown in Table 1. Our PCR products were purified and sequenced by Beckman Coulter Genomics, Inc. (Danvers, Massachusetts, USA). We supplemented new sequence data with sequences obtained from GenBank for nine outgroup individuals (Appendix 1). We edited sequence chromatograms in Sequencher v4.7 (Gene Codes Corporation; Ann Arbor, MI), aligned sequences with the use of ClustalX v2.0 (Larkin et al., 2007), and independently phased GAD2 intron 15 and SPTBN1 intron 1 data with the use of PHASE v2.1 (Stephens et al., 2001) to determine haplotypes when multiple heterozygous sites existed.

Phylogenetic and Phylogeographic Analyses.—Sex chromosomes can be highly variable among species, and the characteristics of sex chromosomes are poorly understood for most snake species (Vicoso et al., 2013; Mezzasalma et al., 2014). Therefore, prior to phylogenetic inference, we assessed whether females were not all heterozygous at GAD2 intron 15, in order to verify that gametologs (loci on chromosomes that do not recombine and can have different phylogenetic histories) do not exist. Subsequently, we partitioned DNA sequence data according to two different partitioning schemes. Under the first scheme (Scheme I) we inferred gene trees for each locus with the use of the best-fit substitution model inferred via the *modelTest* function in the R (R Core Team, 2014) package 'phangorn' (Schliep and Paradis, 2015). We used 'PartitionFinder' (Lanfear et al., 2012) to identify the optimal partitioning scheme (Scheme II), and the best-fit substitution model (the model with the lowest AIC score) for

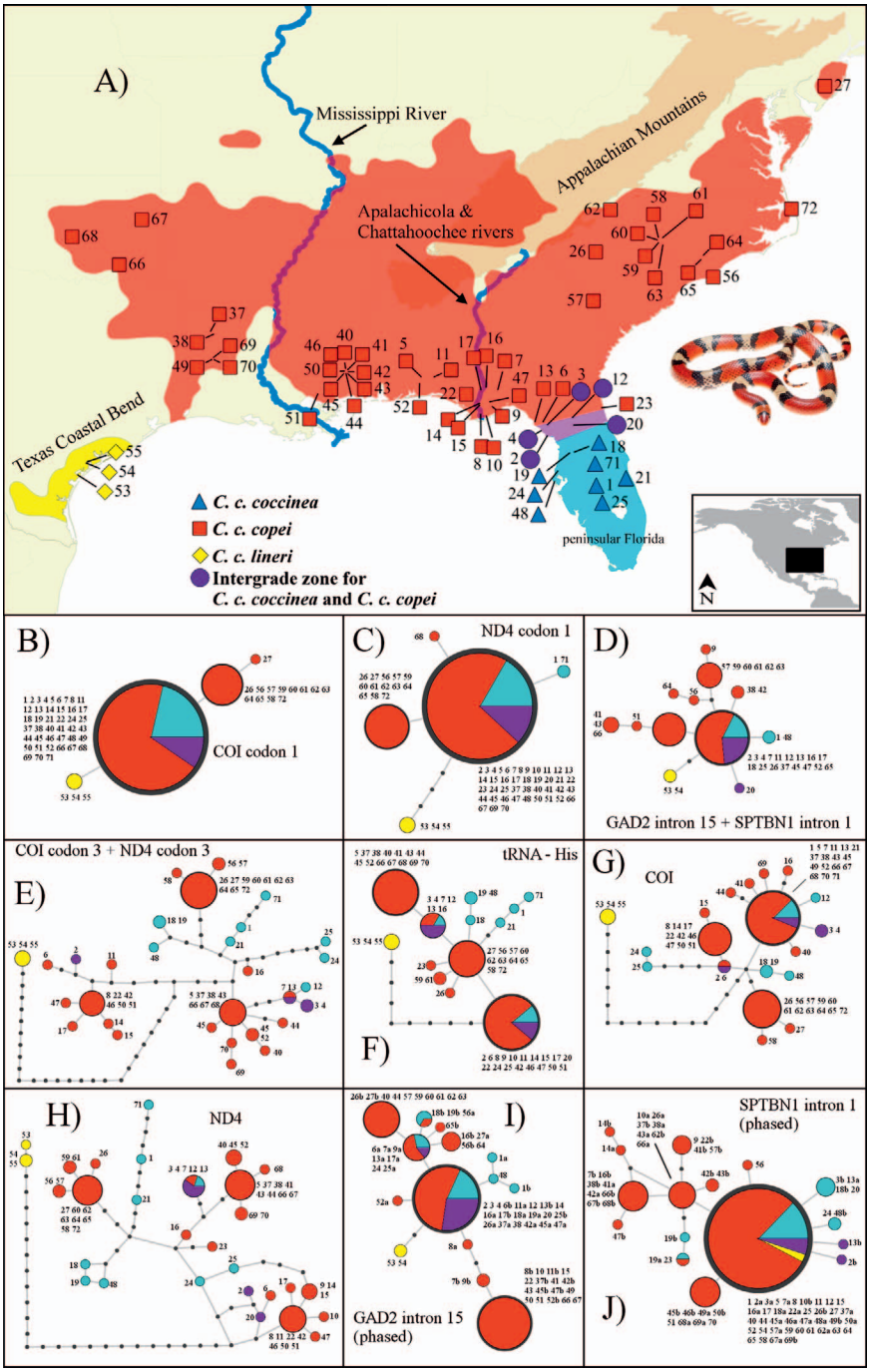

FIG. 1. Distribution, sampling and haplotype networks for *Cemophora coccinea*. Colors indicate *C. c. coccinea* (blue), *Cemophora c. copei* (red), *Cemophora c. lineri* (yellow), and the intergrade zone of *C. c. coccinea* and *C. c. copei* (purple). (A) Geographic ranges and sampling localities for *C. c. coccinea* (triangles), *C. c. copei* (squares), *C. c. lineri* (diamonds), and the intergrade zone of *C. c. coccinea* and *C. c. copei* (circles). Haplotype networks: (B) COI codon 1, (C) ND4 codon 1, (D) GAD2 intron 15 + SPTBN1 intron 1 (concatenated), (E) COI codon 3 + ND4 codon 3 (concatenated), (F) tRNA-Histidine, (G) COI, (H) ND4, (I) GAD2 intron 15 (phased), and (J) SPTBN1 intron 1 (phased); numbers are the individual ID numbers (Appendix 1); letters "a" and "b", when following individual ID numbers, indicate the two alleles carried by individuals heterozygous at GAD2 intron 15 or SPTBN1 intron 1. The range map is adapted from Conant and Collins (1998) and the photograph of *C. coccinea* is used with permission from Paul Marcellini.

each partition subset of Scheme II. We analyzed each partition subset of each partition scheme separately for downstream analyses and calculated nucleotide diversity, π (Nei, 1987), and its variance with the use of the R package 'pegas' (Paradis et al., 2015). We used TCS v1.21 (Clement et al., 2000) to generate haplotype networks. We used two criteria to resolve haplotype network ambiguity loops: 1) rare haplotypes are most likely to occur at tip nodes, and common haplotypes are most likely to occur at internal nodes; and 2) singleton haplotypes are more likely to be connected to haplotype nodes from the same locality than to haplotypes from different localities (Crandall and Templeton, 1993). For each partition subset, we conducted a Mantel's test with the use of the R package 'ade4' (Dray et al., 2015) to test for isolation by distance.

We used BEAST v2.1.1 (Bouckaert et al., 2014) for Bayesian phylogenetic inference of gene trees, with the use of uncorre-

TABLE 1. Primers used for PCR and DNA sequencing.

Locus	Primer name	Primer sequence	Source
COI	COI(+)b	TAAATAATATAAGCTTCTGACTGCTACCACC	Utiger et al. (2002)
COI	COI(−)bdeg1	ATTATTGTTGCYGCTGTRAARTAGGCTCG	Utiger et al. (2002)
ND4	ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	Arevalo et al. (1994)
ND4	Leu	CATTACTTTTACTTGGATTTGCACCA	Arevalo et al. (1994)
GAD2 intron 5	EST-GAD2-15F	CACACAAATGTYTGCTTCTGG	Ruane et al. (2014)
GAD2 intron 5	EST-GAD2-16R	ATGCGGAARAAATTGACCTTGTC	Ruane et al. (2014)
GAD2 intron 5	CCAG766R	TTGCATAATCCTGGACACCA	This study
SPTBN1 intron 1	SPTBN1F-APR-2010	TTGGCTGATGCCAGTTGTA	Ruane et al. (2014)
SPTBN1 intron 1	SPTBN1R-APR-2010	CAGGGTTTGTAACCTKTCCA	Ruane et al. (2014)

lated relaxed lognormal clock models, coalescent tree models, and substitution models with the lowest AIC score determined by modelTest (Scheme I partitions) or PartitionFinder (Scheme II partitions). *Cemophora coccinea*, as well as outgroup species, were each constrained to be monophyletic, but relationships among species were left unconstrained. We ran MCMC analyses for 200 million generations, sampling from the posterior distribution every 100,000 generations, with the first 20 million samples discarded as burn-in.

We used BEAST v1.8.2 (Drummond et al., 2012) to infer a time-calibrated phylogeny for *Cemophora* from ND4 + COI DNA sequence data. We calibrated the phylogeny with a normal distribution prior for the time since the most recent common ancestor of *Cemophora* and *Lampropeltis* ($X = 13.7$ Ma; SD = 2), which was previously estimated (Pyron and Burbrink, 2009). We used independent substitution models (GTR + gamma + invariant sites) and uncorrelated relaxed lognormal clocks for COI and ND4 partitions. We ran the analysis for 100 million generations, sampling from the posterior every 100,000 generations, and we discarded the first 10 million samples as burn in.

After running BEAST, we used Tracer v1.5 (Rambaut et al., 2014a) to check for convergence of any posteriors that occurred when the estimated sample size (ESS) of each parameter was $\geq$250. In cases where chain convergence was not reached we ran the analysis for a greater number of generations (i.e., until ESS $\geq$ 250). We used TreeAnnotator v1.8.0 (Rambaut et al., 2014b) to generate Maximum Clade Credibility trees with mean divergence times, and FigTree v1.4.0 (Rambaut, 2012) to visualize trees.

Genetic Variation.—To test for population expansion, we compared nucleotide diversity (π) across the range of *C. coccinea* (Nei, 1987). We used the R package 'pegas' (Paradis et al., 2015) to estimate π for 1) *C. c. coccinea*, 2) *C. c. copei*, 3) *C. c. coccinea* + *C. c. copei*, 4) *C. coccinea* from peninsular Florida, 5) *C. coccinea* from the region extending from southeastern Louisiana through the Florida panhandle, 6) *C. coccinea* from the region extending from South Carolina through New Jersey, and 7) *C. c. copei* west of the Mississippi River. A high π value in the region extending from southeastern Louisiana through peninsular Florida, and low π values north of Florida or west of the Mississippi River would support the hypothesis that *C. coccinea* expanded north out of Florida and surrounding regions.

Phenotypic Data.—We collected body size, lepidosis, and color pattern data from the holotype and five additional specimens of *C. c. lineri* to verify Williams and Wilsons (1967) assertion that *C. c. lineri* is phenotypically distinct from other *C. coccinea* and to test the hypothesis that *C. c. lineri* has been evolving in isolation from other *C. coccinea* populations, that predicts morphological divergence. We measured snout–vent length (SVL) and tail length (TAL) with a flexible rule to the nearest 1 mm and head length (HL) with dial calipers to the nearest 0.02 mm, examined the arrangement and number of cephalic scales, and counted the number of ventral, subcaudal, and midbody dorsal scale rows. For consistency with previous literature describing *Cemophora*, we included the gular scales, but not the anal scale, within ventral scale counts. We recorded the position of the black head band and the first black dorsal body band relative to cephalic scales, the number of red dorsal blotches before the tail, whether black dorsal bands connect laterally below red dorsal blotches, and the number of scale rows separating black dorsal bands from ventral scales (between midbody and tail). Additionally, we recorded the sex of specimens that were preserved with hemipenes everted (as male), or if sex was indicated on a specimen's field tag or in previous literature. We compared our data for *C. c. lineri* to previously reported data for *C. c. coccinea*, *C. c. copei*, and *C. c. lineri* (Williams et al., 1966; Williams and Wilson, 1967).

RESULTS

DNA Sequences, Haplotype Diversity, and Genetic Divergence.—We obtained DNA sequences at COI (475 bp), ND4 (753 bp), SPTBN1 intron 1 (704 bp), and GAD2 intron 15 (625 bp) loci for 53–61 *C. coccinea* plus 10 outgroup individuals (Appendix 1), verified that gametologs do not exist at GAD2 intron 15, determined optimal partitioning schemes, and calculated summary statistics for genetic data, and inferred haplotype networks. Our DNA sequences were given GenBank accession numbers (Appendix 1). Females, the heterogametic sex, were never heterozygous at GAD2 intron 15, indicating that gametologs did not occur, allowing us to use GAD2 intron 15 for phylogenetic inference. The optimal partitioning scheme inferred with PartitionFinder included seven partition subsets: COI codon 1, ND4 codon 1, COI codon 2 + ND4 codon 2, COI codon 3 + ND4 codon 3, tRNA-Histidine, tRNA-Serine (partial sequence), and GAD2 intron 15 + SPTBN1 intron 1. The tRNA-Serine and COI codon 2 + ND4 codon 2 partition subsets did not contain any parsimony informative sites in the ingroup, and therefore, these two partition subsets were not analyzed in downstream analyses. Summary statistics (sequence length, number of sequences, nucleotide diversity (π), number of parsimony informative sites, and % missing data) are reported for each partition subset, and show that nucleotide diversity is greatest for the tRNA-Histidine subset, but the ND4 partition contains the greatest number of parsimony informative sites (Table 2). Although we used criteria described by Crandall and Templeton (1993) to resolve ambiguities in haplotype networks, some ambiguities could not be resolved (Fig. 1B). Some COI, ND4, and GAD2 intron 15 haplotypes were unique to *C. c. copei* or to *C. c. coccinea*, and other haplotypes were shared between these two populations (Fig. 1G–I). Haplotypes of COI, ND4, and GAD2 intron 15 of *C. c. lineri* were never shared with *C. c.*

TABLE 2. Summary statistics for DNA sequence partition subsets. Each partition scheme consists of multiple partition subsets; partition subsets of the same partition scheme do not contain any of the same data. In Scheme I, each partition subset includes contiguous DNA sequences (i.e., from the same locus); Scheme II is the partitioning scheme inferred from PartitionFinder, and DNA sequences in a particular partition subset evolve in a similar way, despite not being contiguous on a chromosome; bp = base pairs, π = nucleotide diversity, P.I. sites = number of parsimony informative sites, % M.D. = % missing data.

DNA sequence partition subset	Partitioning scheme	Length (bp)	*n*	Mean $\pi \pm$ SE	P.I. sites	% M.D.
COI	I	475	67	3.48E-2 ± 3.02E-4	28	0.219
ND4	I	753	71	3.86E-2 ± 3.59E-4	64	0
GAD2 intron 15 (phased)	I	625	89	2.82E-3 ± 3.34E-6	11	3.68
SPTBN1 intron 1 (phased)	I	703	92	3.28E-3 ± 4.02E-6	13	0.240
COI codon 1	II	159	67	1.00E-3 ± 1.55E-3	2	0.225
ND4 codon 1	II	148	71	5.52E-3 ± 4.46E-3	6	0
COI codon 3 + ND4 codon 3	II	306	69	3.66E-2 ± 1.87E-2	52	3.85
tRNA-Histidine	II	270	71	1.02E-2 ± 6.09E-3	17	0
GAD2 intron 15 + SPTBN1 intron 1	II	1329	53	4.16E-4 ± 3.86E-4	44	1.03

coccinea or *C. c. copei*. Some SPTBN1 intron 1 haplotypes were shared among all populations, but other haplotypes were unique to either *C. c. copei* or *C. c. coccinea* (Fig. 1J). A Mantel's test supported isolation by distance for GAD2 intron 15 + SPTBN1 intron 1 and phased GAD2 intron 15 partition subsets, but not for other partition subsets.

Bayesian Phylogenetic Inference.—Phylogenetic analyses generally support *C. c. lineri* as monophyletic and *C. c. coccinea* and *C. c. copei* as paraphyletic, with divergence between *C. c. lineri* and other *C. coccinea* occurring during the Pliocene or early Pleistocene. Posterior effective sample sizes (ESSs) were high (ESS > 250) for all BEAST runs, indicating that analyses reached stationarity. *Cemophora c. lineri* is strongly supported as monophyletic (PP > 0.95) in all trees except the SPTBN1 intron 1 tree (Figs. 2, 3); the SPTBN1 intron 1 tree does not provide strong support for, or against, monophyly of *C. c. lineri*, because

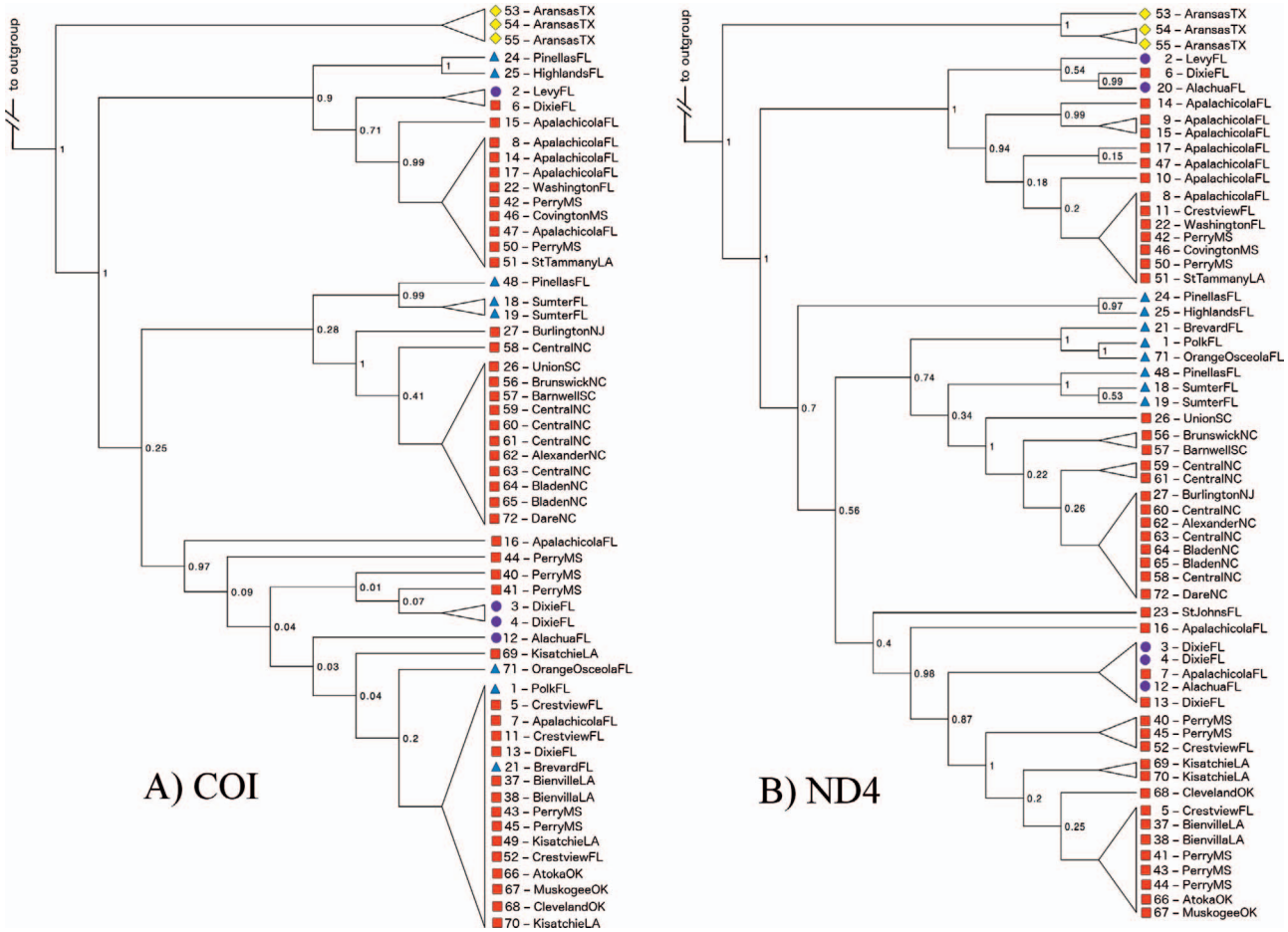

FIG. 2. BEAST phylogenetic trees for COI and ND4 loci. BEAST trees for (A) COI and (B) ND4 loci; numbers at internal nodes indicate posterior probabilities. Colored shapes at tips indicate subspecies: *Cemophora c. coccinea* (blue triangles), *Cemophora c. copei* (red squares), *Cemophora c. lineri* (yellow triangles), or *C. c. coccinea* and *C. c. copei* intergrade zone (purple circles). Individual ID numbers (Appendix 1) and locality information are also indicated at tree tips. Clades collapsed as triangles indicate a set of individuals that share the same haplotype; phylogenies are represented as simple cladograms (branch lengths are not proportional to divergence) so that posterior probabilities could be displayed.

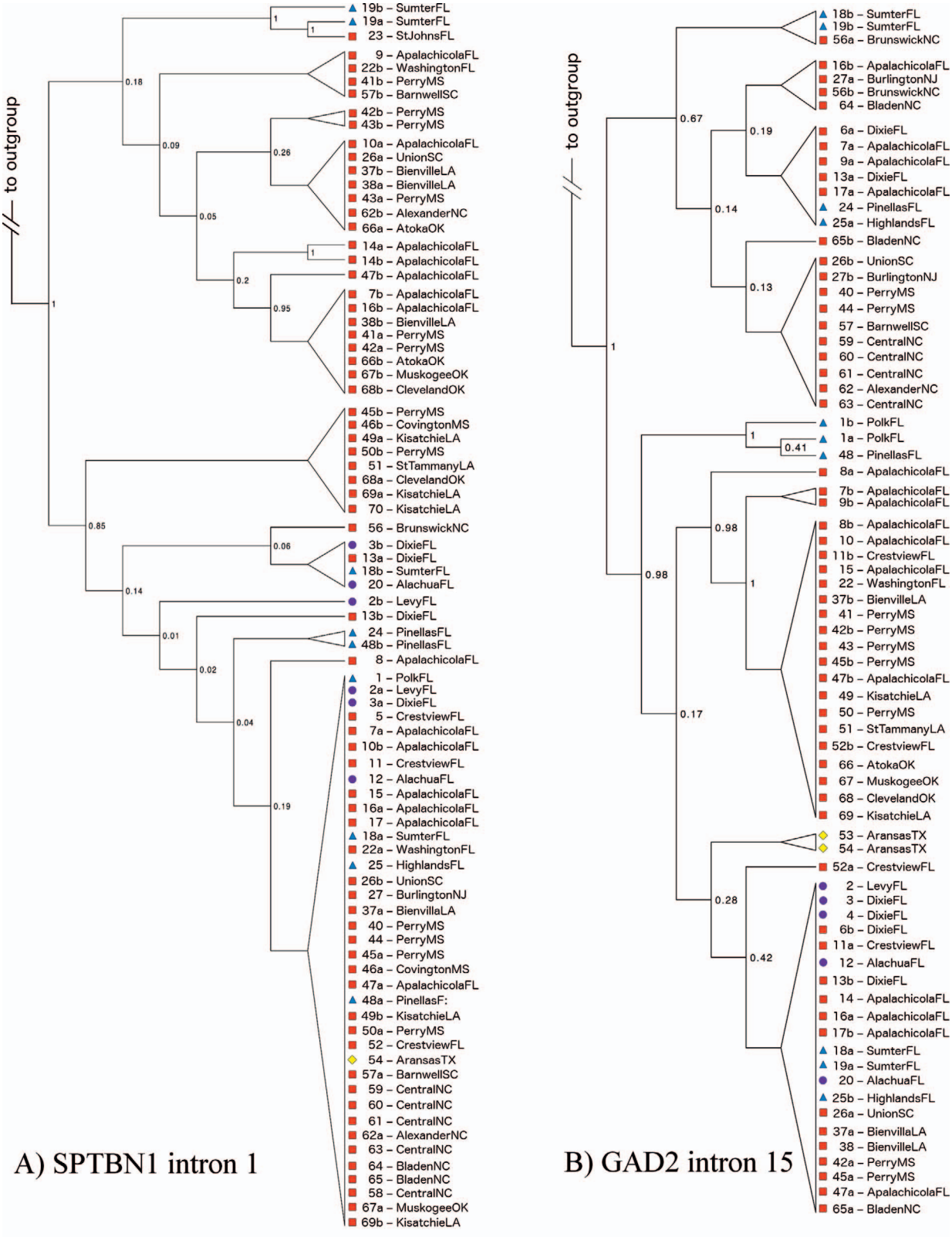

FIG. 3. BEAST phylogenetic trees for phased nuclear loci. BEAST trees for (A) SPTBN1 intron 1 (phased) and (B) GAD2 intron 15 (phased); numbers at internal nodes indicate posterior probabilities. Colored shapes at tips indicate subspecies: *Cemophora c. coccinea* (blue triangles), *Cemophora c. copei* (red squares), *Cemophora c. lineri* (yellow triangles), or *C. c. coccinea* and *C. c. copei* intergrade zone (purple circles). Individual ID numbers (Appendix 1) and locality information are also indicated at tree tips. Clades collapsed as triangles indicate a set of individuals that share the same haplotype. For individuals heterozygous at GAD2 intron 15 or SPTBN1 intron 1, the letters "a" and "b" are added to the end of the individual's ID number to indicate the two alleles carried by that individual; phylogenies are represented as simple cladograms (branch lengths are not proportional to divergence) so that posterior probabilities could be displayed.

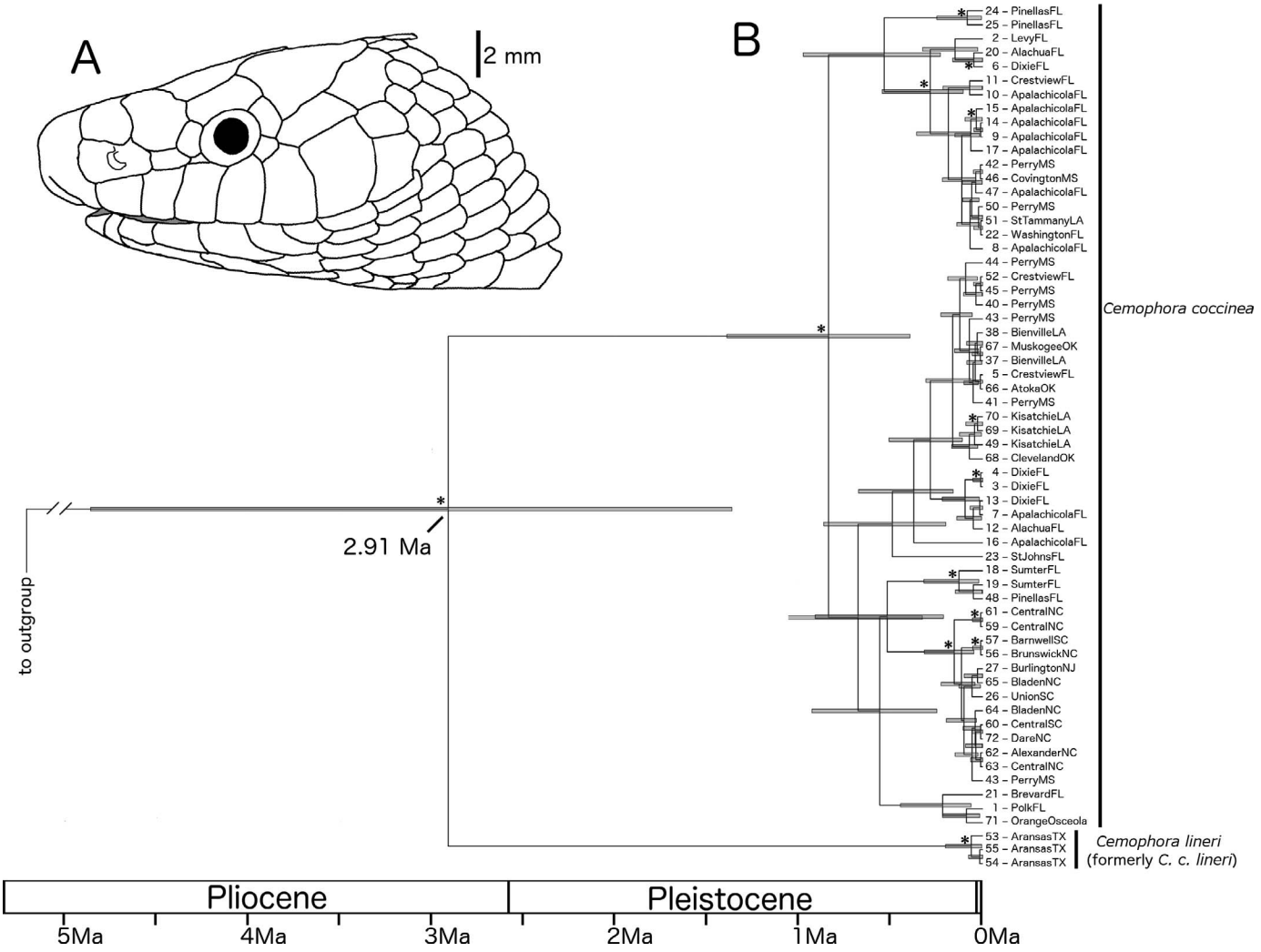

FIG. 4. (A) Cephalic scalation of *Cemophora lineri* (formerly *C. c. lineri*), specimen AMNHR 108916, Jim Hogg County, Texas; (B) time-calibrated mitochondrial (ND4 + COI) phylogeny of *Cemophora*; asterisks indicate clades with posterior probability >0.95; horizontal gray bars at internal nodes indicate clade age (95% HPD). Individual ID numbers (Appendix 1) and locality information are indicated at tree tips.

we obtained only SPTBN1 sequences for one *C. c. lineri* individual (Fig. 3A). Additionally, COI (Fig. 2A), ND4 (Fig. 2B), ND4 codon 1 (not shown), COI codon 3 + ND4 codon 3 (not shown), tRNA-Histidine trees (not shown), and the time-calibrated ND4 + COI phylogeny (Fig. 4B) strongly supported the hypothesis that *C. c. lineri* is sister to the remaining groups of *C. coccinea*. In contrast, SPTBN1 intron 1 (Fig. 3A) and GAD2 intron 15 (Fig. 3B) trees strongly support *C. c. lineri* as embedded in clades containing some *C. c. coccinea* and *C. c. copei* individuals. The GAD2 intron 15 + SPTBN1 intron 1 (not shown) and COI codon 1 (not shown) trees do not provide strong support for, or against, a sister relationship between *C. c. lineri* and the rest of *C. coccinea*, because posterior probabilities (PP) were low (<0.95). Most trees provide strong support against the monophyly of *C. c. coccinea* or *C. c. copei*, although ND4 (Fig. 2B) and COI codon 3 + ND4 codon 3 (not shown) trees and the time-calibrated ND4 + COI phylogeny do not provide support for, or against, monophyly of *C. c. coccinea* or *C. c. copei*, because posterior probabilities were low (PP < 0.95); none of the inferred trees strongly support *C. c. coccinea* or *C. c. copei* as monophyletic. All trees provide strong support against a sister relationship between either *C. c. coccinea* and other *C. coccinea*, or *C. c. copei* and other *C. coccinea*. The time-calibrated ND4 + COI phylogeny supports a Pliocene or early Pleistocene divergence between *C. c. lineri* and remaining *C. coccinea* (median divergence time [DT] estimate = 2.91 Ma; DT 95% highest posterior density (HPD) = 4.86–1.37 Ma; Fig. 4B).

Genetic Variation.—The range of nucleotide diversity across loci (π_{range}) was highest in peninsular Florida (π_{range} = 0.000480–0.0101) and relatively high in the region spanning southeastern Louisiana through the Florida panhandle (π_{range} = 0.000281–0.00703), suggesting that *C. coccinea* was restricted to a region extending from southeastern Louisiana though peninsular Florida. Additionally, π was low for *C. c. copei* west of the Mississippi River (π_{range} = 0–0.00102) or east of the Appalachian Mountains from South Carolina through New Jersey (π_{range} = 0–0.000962), suggesting that *C. coccinea* colonized these regions relatively recently. Values of π at each locus of *C. coccinea* subspecies or geographic groups are shown in Table 3.

Phenotypic Data.—*Cemophora c. lineri* are distinct from other *C. coccinea* in color pattern and lepidosis, supporting the hypothesis that *C. c. lineri* remained isolated after becoming separated from other *C. coccinea* (see Systematic Account section). *Cemophora c. lineri* has a greater average number of ventral scales (178–195, X = 186.1, n = 7) than other *C. coccinea* (150–185, X = 167.5, n = 239) and black dorsal bands extend laterally

TABLE 3. Nucleotide diversity (π) at COI, ND4, GAD2, and SPTBN1 loci for *Cemophora coccinea* subspecies and geographic groups. Low sample sizes ($n \leq 3$ sequenced for each locus) of *Cemophora c. lineri* prevented estimation of π for this taxon.

C. coccinea subspecies or geographic group	Mean π (COI)	Mean π (ND4)	Mean π (GAD2)	Mean π (SPTBN1)
C. c. coccinea	0.00865	0.00780	0.000859	0.00143
Cemophora c. copei	0.00552	0.00830	<0.000001	0.000359
C. c. coccinea + *C. c. copei*	0.00608	0.00907	0.000128	0.000285
Peninsular Florida	0.00689	0.0101	0.000488	0.00131
SE Louisiana through Florida Panhandle	0.00480	0.00703	0.000307	0.000281
South Carolina through New Jersey	0.000648	0.000962	<0.000001	0.000238
C. c. copei west of Mississippi River	0.000526	0.00102	<0.000001	<0.000001

to dorsal scale rows 3–5 in *C. c. lineri* versus dorsal scale rows 1–2 for other *C. coccinea*. Within *C. c. lineri*, the number of midbody scale rows is 19 ($n = 3$), mean relative head length (HL/SVL) = 0.031 ($n = 2$, range = 0.0223–0.0397), mean relative tail length (TAL/SVL) = 0.155 ($n = 5$, range = 0.142–0.183), mean number of subcaudal scales = 43.6 ($n = 5$, range = 41–48), mean number of scales separating the first black band on the body from the parietals = 1.67 ($n = 6$, range = 0–4), mean number of red dorsal blotches before the tail = 15.5 ($n = 4$, range = 13–18), and 50% of individuals ($n = 6$) have black dorsal bands that meet below at least one red dorsal blotch.

DISCUSSION

Genetic analyses suggest that speciation within *Cemophora* occurred when populations were separated during the Pliocene or early Pleistocene. Williams and Wilson (1967) hypothesized that the common ancestor of extant *Cemophora* occupied an area similar to the current *C. coccinea* species range, and that cold temperatures during the Pleistocene forced *Cemophora* into two isolated thermal refugia: one in Texas, where *C. c. lineri* evolved, and another in Florida and surrounding areas. Previous studies have suggested Pleistocene refugia in Florida (e.g., *Anolis carolinensis*), east of the southern Appalachian Mountains (e.g., *Blarina brevicauda*), along the northern coast of the Gulf of Mexico (e.g., *Plestiodon fasciatus*), and in southern Texas and Mexico (e.g., *Crotalus atrox*) (Brant and Ortí, 2003; Howes et al., 2006; Castoe et al., 2007; Campbell-Staton et al., 2012). Our results suggest that divergence in *Cemophora* began 4.86–1.37 Ma and that *C. c. lineri* is sister to the remaining groups in *C. coccinea*, supporting historical separation of *Cemophora* populations into two Pliocene or Pleistocene refugia, one in southern Texas (and perhaps northern Mexico) and the other further east, extending from southeastern Louisiana to southern Florida. Furthermore, our results suggest the most recent common ancestor of individuals from the eastern refugium existed during the Pleistocene 1.39–0.4 Ma, supporting northward expansion out of the eastern refugium during the Pleistocene.

We propose the elevation of *C. c. lineri* from subspecies to full species status, *C. lineri*. Collins (1991) applied the Evolutionary Species Concept, and proposed the elevation of *C. c. lineri* and 54 other subspecies to full species, because of their allopatric ranges (Frost and Hillis, 1990). Although taxonomists initially rejected Collins' (1991) taxonomic changes, subsequent studies corroborate nearly half of these changes (Van Devender, 1992; Crother, 2014); however, new data in support of, or against, recognition of *C. lineri* was not available until the current study. Like *C. c. coccinea* and *C. c. copei*, *C. c. lineri* is small, fossorial, and exhibits facultative oophagy, but *C. c. lineri* differs from other *C. coccinea* in color pattern and lepidosis: *C. c. lineri* has black dorsal bands that extend laterally to the third, fourth, or fifth scale row (between midbody and tail), whereas other *C. coccinea* have black dorsal bands that extend laterally to the first or second scale row, and *C. c. lineri* usually have more ventral scales than other *C. coccinea*. Additionally, mitochondrial divergence between *C. c. lineri* and other *C. coccinea* (~4%) is similar to levels of divergence observed between other snake species (Burbrink et al., 2000; Crother et al., 2011). We choose not to recognize any *Cemophora* subspecies, elevating *C. c. lineri* to a full species (see Systematic Account) according to the general lineage species concept (de Queiroz, 2007), while restricting *C. coccinea* to include populations formerly assigned to *C. c. coccinea* and *C. c. copei*. Our description of *C. lineri* is similar to the original description of *C. c. lineri* (Williams et al., 1966), but is expanded to account for greater phenotypic variation than previously described.

SYSTEMATIC ACCOUNT

CEMOPHORA LINERI

TEXAS SCARLET SNAKES

Cemophora coccinea lineri Williams, Brown, and Wilson, 1966

Holotype.—AMNH 75307, adult female, from 55.5 km S of Riviera, Kenedy County, Texas, United States, approximately 26 °47′40.74"N, 97°48′50.4714"W, 5 m elevation, collected by Ernest A. Liner and Richard Whitten on 29 June 1963 (Williams et al., 1966).

Paratype.—BCB 10993, adult female, from King Ranch, Kenedy County, Texas, precise coordinates unknown, collected by Brian P. Glass in 1951 (Williams et al., 1966).

Diagnosis.—A medium-sized species of the genus *Cemophora*, tribe Lampropeltini, family Colubridae. Distinguished from other lampropeltine species by the following combination of characters: 7 supralabial scales; enlarged posterior maxillary teeth; dorsal scales smooth in 19 rows at midbody; anal scale entire; 178–195 ventral scales; 13–18 red dorsal blotches before tail, bordered anteriorly and posteriorly by black bands; black dorsal bands extend laterally to the third, fourth, or fifth dorsal scale row (between midbody and tail); background dorsal color yellow, white, or gray; ventral color white.

Comparisons.—*Cemophora lineri* differs from *C. coccinea* in having a greater average number of ventral scales (*C. lineri*: 178–195 [$X = 186.1$]; *C. coccinea*: 150–185 [$X = 167.5$]) and black dorsal bands that extend to the third, fourth, or fifth dorsal scale row (vs. extending laterally to dorsal scale row one or two in *C. coccinea*).

Description of the Holotype.—An adult female, 538 mm SVL, 12 mm HL (2.23% of SVL), tail incomplete. Body cylindrical. Rostral scale large, wider than long in dorsal view, rounded and slightly convex in lateral view, triangular in anterior view, and extending between the internasal scales for a distance twice the length of the internasal suture; rostral contacts nasal and first

supralabial scales. Nasal scale longer than tall, roughly hourglass shaped and constricted dorsoventrally, surrounding a hook-shaped naris; nasal contacts rostral, internasal, prefrontal, loreal, and first two supralabial scales. Internasal scales wider than long, pentagonal, paired with medial suture; internasals contact nasal, prefrontal, and rostral scales. Prefrontal scales wider than long, paired with medial suture; length of suture between prefrontal scales less than half length of suture between internasal scales; prefrontals contact frontal, internasal, loreal, nasal, preocular, and supraocular scales. Frontal scale large, slightly longer than wide; frontal contacts prefrontal, parietal, and supraocular scales. Parietal scales large, longer than wide in dorsal view and nearly as long as frontal scale, and paired with medial suture; parietals contact nuchal, anterior temporal (left side only), sixth supralabial (right side only), suprapostocular, supraocular, frontal, and dorsal body scales. Loreal scale wider than tall, contacting nasal, prefrontal, preocular, and second and third supralabial scales. Preocular scale taller than wide, larger than loreal scale; preocular scale contacts loreal, prefrontal, supraocular, and third supralabial scales. Two postocular scales: suprapostocular and infrapostocular; suprapostocular larger than infrapostocular, and contacts infrapostocular, anterior temporal (left side only), sixth supralabial scale (right side only), parietal, and supraocular scales; infrapostocular contacts anterior temporal (left side only), fourth and fifth supralabial, sixth supralabial (right side only), and suprapostocular scales. Supraocular scales large, longer than wide, and contact preocular, prefrontal, frontal, parietal, and suprapostocular scales. Posterior temporal scale smaller than seventh supralabial scale; posterior temporal contacts nuchal, anterior temporal (left side only), and sixth and seventh supralabial scales. Seven supralabial scales, increasing in size in the following order: 2–1–3–4–5–7–6, with third and fourth entering the orbit. Eight infralabial scales, with fourth much larger than others; first infralabial pair with medial suture, before chin shields. Anterior chin shields longer than wide, rectangular, paired with medial suture; anterior chin shields contact posterior chin shields, first four infralabials, and intergenial scales separating posterior chin shields. Posterior chin shields much smaller than anterior chin shields, paired, separated by intergenial scales; posterior chin shields contact anterior chin shields, fourth infralabial, dorsal scale rows that extend ventrally. Dorsal scales smooth, in 19 rows; 195 ventrals, smooth; anal scale single; subcaudal scales paired, in >24 rows (tail incomplete).

Color Pattern of the (Liquid-Preserved) Holotype.—Background head color gray, dorsally; supralabials white; black head band extends from postorbital region, across anterior one-third of parietals, posterior edge of supraoculars, and posterior tip of frontal; first black dorsal band of body is separated from parietal scales by 1–2 gray dorsal scale rows; 17 dorsal blotches before tail, each dorsal blotch 7.5–10 scale rows long, red in life (Williams et al., 1966), white with scattered gray flecking in liquid preserved specimen, and bordered anteriorly and posteriorly by black dorsal bands (1.5–2.5 scale rows long middorsally); black dorsal bands extend laterally to third, fourth, or fifth scale row, not meeting below red dorsal blotches; black-bordered dorsal blotches separated by gray interspace regions 3.5–4.5 scale rows long; entire ventral region white.

Variation.—Five *C. lineri* specimens vary in size and scale counts from the holotype. AMNHR 108916 (Fig. 4A): male, 498 mm SVL, 71 mm TAL (14.3% of SVL), 186 ventrals, 44 subcaudals, 2–3 scales separating first black band on body from parietals, 14 red dorsal blotches before tail, two preocular scales. AMNHR 169158: sex unknown, 250 mm SVL, 37 mm TAL (14.8% of SVL), 185 ventrals, 41 subcaudals, 2–3 scales separating first black band on body from parietals. AMNHR 169159: male, 301 mm SVL, 55 mm TAL (18.3% of SVL), 11.96 mm HL (3.97% of SVL), 186 ventrals, 48 subcaudals, 3–4 scales separating first black body band from parietals, 13 red dorsal blotches before tail, black bands meeting below left side of first dorsal blotch. AMNHR 169160: sex unknown, 452 mm SVL, 64 mm TAL (14.2% of SVL), 189 ventrals, 42 subcaudals, <1 scale separating first black band on body from parietals, 18 red dorsal blotches before tail, black dorsal bands meeting below first red dorsal blotch. AMNHR 169161: sex unknown, 408 mm SVL, 65 mm TAL (15.9% of SVL), 184 ventrals, 43 subcaudals, first black band on body touches parietals, black bands meeting below right side of first dorsal blotch, two preoculars.

Distribution and Ecology.—*Cemophora lineri* is known from Aransas, Brooks, Calhoun, Jim Hogg, Kenedy, Matagorda, Nueces, and San Patricio counties, Texas, USA (Jackson et al., 2005; Werner and Dixon, 2010), including North Padre Island, a long barrier island 1–2 km off the Texas coast in the Gulf of Mexico (Mike Duran, pers. comm.). *Cemophora lineri* may also occur in Duval, Jackson, Jim Wells, Kleberg, Refugio, and Victoria counties, Texas, and perhaps in northern Tamaulipas, Mexico, because these areas contain habitats presumably suitable for *C. lineri* (Werler and Dixon, 2010). *Cemophora lineri* is fossorial and, like *C. coccinea*, probably nocturnal (e.g., field notes indicate *C. lineri* specimen AMNHR 108916 was found alive on road near midnight) (Nelson and Gibbons, 1972). *Cemophora lineri* has been found in association with a variety of plants, including oak (*Quercus*), mesquite, and prickly pear (*Opuntia*), and is usually found near loose, sandy soil (Werler and Dixon, 2010). *Cemophora lineri* probably has a diet similar to that of *C. coccinea*, which feeds primarily on reptile eggs; a captive *C. lineri* consumed nine eggs of a Texas Spiny Lizard *Sceloporus occellatus* (Werler and Dixon, 2010).

Etymology.—The specific name *lineri* is a patronym, named for Ernest A. Liner, who collected the holotype in 1963 with Richard Whitten (Williams et al., 1966).

Conservation.—The Texas Parks and Wildlife Department's list of rare, threatened, and endangered species (TPWD list) includes *C. c. lineri* as a threatened subspecies (Campbell, 2003). In light of elevating *C. c. lineri* to *C. lineri*, we recommend that the TPWD list include *C. lineri* as a threatened species.

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Jackman (Villanova University). We did not use live animals for any portion of this study. All tissue samples or sequences were obtained from museum tissue collections or from previously published GenBank sequences. This research was supported by a National Science Foundation grant (DEB 1146033) to CCA.

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Appendix 1

Institutional Abbreviations.—Institutional abbreviations used in this paper for specimen, tissue, and extracted DNA catalogue numbers: AMNH, American Museum of Natural History; CAS, California Academy of Sciences; FLMNH, Florida Museum of Natural History; FMNH, Field Museum of Natural History; FTB, Frank Burbrink tissue collection; LSUMZ, Louisiana Museum of Natural Science; MVZ, Museum of Vertebrate Zoology; NCSM, North Carolina Museum of Natural Sciences; OCGR, Oklahoma Collection of Genomic Resources; SLU, Southeastern Louisiana University; USNM, Smithsonian National Museum of Natural History.

Specimens Sequenced.—ID numbers of individuals included in this study are shown below, organized by subspecies and the state the individual is from. Each ID number is followed by a colon and then, in parentheses, the museum catalogue number (for specimen, tissue, or DNA extraction), GenBank accession numbers, and geographic coordinates (datum = WGS84). *Cemophora coccinea coccinea*: Florida: 1:(LSUMZ H22755, KM655135, KM655205, KM655027, KM655088, N27.698/W81.533), 18:(CAS207261, KM655115, KM655182, KM654992, KM655052, N28.686/W82.251), 19:(CAS208619, KM655157, KM655227, KM655008, KM655067, N28.686/W82.251), 21:(FLMNH151464, KM655143, KM655213, N27.916/W80.642), 24:(FLMNH164358, KM655136, KM655206, KM655028, KM655089, N28.130/W82.684), 25:(FMNH266587, KM655164, KM655234, KM655021, KM655082, N27.183/W81.352), 48:(LSUMZ42158, KM655118, KM655186, KM654997, KM655056, N27.834/W82.776), 71:(USNM541732, KM655130, KM655185, N28.350/W81.567). *Cemophora coccinea copei*: Florida: 5:(LSUMZ H22759, KM655173, KM655244, KM655103, N30.930/W86.754), 6:(LSUMZ H22760, KM655110, KM655177, KM654984, N29.610/W83.136), 7:(LSUMZ H22761, KM655112, KM655179, KM654987, KM655047, N30.430/W84.531), 8:(LSUMZ H22762, KM655126, KM655196, KM655012, KM655073, N29.870/W84.982), 9:(LSUMZ H22763, KM655188, KM655000, KM655059, N30.073/W84.657), 10:(LSUMZ H22764, KM655202, KM655024, KM655085, N29.829/W84.867), 11:(LSUMZ H22765, KM655174, KM655245, KM655042, KM655106, N30.967/W86.395), 13:(CAS196038, KM655166, KM655236, KM655022, KM655083, N29.609/W83.367), 14:(CAS203080, KM655137, KM655207, KM655029, KM655091, N30.176/W84.985), 15:(CAS203081, KM655162, KM655232, KM655017, KM655079, N30.176/W84.985), 16:(CAS203086, KM655127, KM655197, KM655014, KM655075, N30.590/W84.844), 17:(CAS203087, KM655133, KM655203, KM655025, KM655086, N30.525/W84.970), 22:(FLMNH153024, KM655123, KM655192, KM655007, KM655066, N30.436/W85.441), 23:(FLMNH157353, KM655195, KM655072, N30.021/W81.336), 52:(LSUMZ82144, KM655121, KM655190, KM655002, KM655061, N30.789/W86.841); Louisiana: 37:(LSUMZ 90393, KM655150, KM655220, KM654990, KM655050, N32.339/W93.109), 38:(LSUMZ 93381, KM655163, KM655233, KM655016, KM655078, N32.245/W93.030), 47:(LSUMZ 40484, KM654989, KM655225, KM654982, KM655160, N30.146/W84.854), 49:(LSUMZ 56140, KM655147, KM655041, KM655105, N31.493/W93.033), 51:(LSUMZ 80995, KM655238, KM655030, KM655093, N30.445/W89.897), 69:(SLU06087, KM655156, KM655224, KM655003, KM655062, N31.493/W93.033), 70:(SLU06088, KM655138, KM655208, KM655092, N31.493/W93.033); Mississippi: 40:(LSUMZ H22768, KM655139, KM655209, KM655031, KM655094, N31.187/W89.091), 41:(LSUMZ H22769, KM655140, KM655210, KM655033, KM655096, N31.132/W89.061), 42:(LSUMZ H22770, KM655116, KM655183, KM654993, KM655053, N31.088/W89.007), 43:(LSUMZ H22771, KM655134, KM655204, KM655026, KM655087, N31.090/W89.000), 44:(LSUMZ H22772, KM655119, KM655187, KM654999, KM655058, N31.088/W89.007), 45:(LSUMZ H22773, KM655109, KM655176, KM654983, KM655045, N31.087/W89.007), 46:(LSUMZ H22774, KM655159, KM655230, KM655071, N31.628/W89.511), 50:(LSUMZ56151, KM655122, KM655191, KM655006, KM655065, N31.163/W89.037); North Carolina: 56:(MVZ150181, KM655131, KM655200, KM655020, KM655081, N33.951/W78.019), 58:(LSUMZ H22777, KM655167, KM655237, KM655090, N35.086/W79.565), 59:(NCSM75916, KM655141, KM655211, KM655034, KM655097, N35.080/W79.561), 60:(NCSM75917, KM655154, KM655226, KM655005, KM655064, N35.058/W79.547), 61:(NCSM76183, KM655161, KM655231, KM655013, KM655074, N35.139/W79.536), 62:(NCSM77237, KM655144, KM655214, KM655037, KM655100, N35.972/W81.109), 63:(NCSM77275, KM655165, KM655235, KM655019, KM655080, N34.931/W79.521), 64:(NCSM81015, KM655145, KM655215, KM655039, KM655102, N34.601/W78.310), 65:(NCSM81017, KM655169, KM655239, KM655032, KM655095, N34.483/W78.376), 72:(USNM550133, KM655170, KM655240, N36.000/W75.667); New Jersey: 27:(LSUMZ H22767, KM655146, KM655216, KM655040, KM655104, N39.686/W74.666); Oklahoma: 66:(OCGR6520, KM655155, KM655225, KM655004, KM655063, N34.325/W95.870), 67:(OCGR6691, KM655151, KM655219, KM654989, KM655049, N35.675/W95.189), 68:(OCGR7191, KM655160, KM655229, KM655011, KM655070, N35.160/W97.283); South Carolina: 26:(LSUMZ H22766, KM655114, KM655181, KM654991, KM655051, N34.707/W81.553), 57:(MVZ233286, KM655153, KM655223, KM654998, KM655057, N33.238/W81.622). *Cemophora coccinea lineri*: Texas: 53:(TCWC90245, KM655117, KM655184, KM654994, N28.012/W97.096), 54:(LSUMZ H22776, KM655152, KM655222, KM654996, KM655055, N28.395/W96.867), 55:(TCWC87305, KM655124, KM655193, N28.395/W96.867). *Cemophora coccinea* (intergrade zone): Florida: 2:(LSUMZ H22756, KM655113, KM655180, KM654988, KM655048, N29.184/W83.015), 3:(LSUMZ H22757, KM655172, KM655243, KM655038, KM655101, N29.510/W82.998), 4:(LSUMZ H22758, KM655132, KM655201, KM655023, KM655084, N29.385/W83.083), 12:(AMNH153377, KM655158, KM655228, KM655010, KM655069, N29.616/W82.335), 20:(FLMNH123869, KM655221, KM654995, KM655054, N29.519/W82.216). Outgroup individuals: *Arizona elegans*: 30:(LSUMZ84809, KM655128, KM655198, KM655015, KM655076), 39:(LSUMZ40347, KM655199, KM655018, KM655077), 73:(FTB1937, KF215766, KF215113), 74:(FTB1854, KF215765, FJ627922); *Rhinocheilus lecontei*: 28:(LSUMZ84796, KM655148, KM655217, KM655043, KM655107), 29:(LSUMZ84799, KM655142, KM655212, KM655035, KM655098); *Lampropeltis holbrooki*: 33:(LSUMZ86457, KM655120, KM655189, KM655001, KM655060, N30.540/W91.742), 77:(DBS1570, KF215753,

JX648607), 78:(TNHC61234, ABC88070); *Lampropeltis gentilis*: 31:(LSUMZ86104, KM655242, KM655099, N30.074/W90.749), 35:(LSUMZ86475, KM655111, KM655178, KM654986, N30.303/W91.004), 79:(FTB1817, KF215812, KF215168), 80:(FTB1805, KF215811, KF215167); *Lampropeltis nigra*: 34:(LSUMZ86472, KM655125, KM655194, KM655009, KM655068, N30.324/W91.015), 81:(FTB1505, KF215755, JX648608); *Pantherophis obsoletus*: 36:(LSUMZ86672, KM655149, KM655218, KM654985, KM655046, N31.744/W92.546), 75:(LJV11562, FJ627930); *Pantherophis spiloides*: 32:(LSUMZ86381, KM655171, KM655241, KM655036, N30.074/W90.749), 76:(FTB622, FJ627806, FJ627849, FJ627925).