EVOLUTION, PHYLOGEOGRAPHY AND SPECIES BOUNDARIES OF THE RINGNECK SNAKE GENUS *DIADOPHIS*

BY

FRANK MATTHEW FONTANELLA

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2009

This manuscript has been read and accepted for the

Graduate Faculty in Biology in satisfaction of the

dissertation requirement for the degree of Doctor of Philosophy.

Mark E. Siddall

Date:

Chair of Examining Committee

Richard L. Chappell

Date:

Executive Officer

Frank T. Burbrink

Robert F. Rockwell

John J. Wiens

Richard L. Glor

Supervisory Committee

The City University of New York

ABSTRACT

EVOLUTION, PHYLOGEOGRAPHY AND SPECIES BOUNDARIES OF THE RINGNECK SNAKE GENUS *DIADOPHIS*

ADVISER: Dr. Mark E. Siddall

The monotypic snake genus *Diadophis* Baird and Girard, 1853 is one of the most widely distributed and morphologically variable snakes throughout North America. This snake (*Diadophis punctatus)* exploits a variety of habitats and ecological niches ranging from the mixed hardwood forests of southern Canada to the desert of the Southwestern US and Central Mexico (excluding the northern Great Plains states. Ringneck snakes have traditionally been divided into 13 subspecies based on a combination of morphology and geography. This study has provided the first range-wide molecular study and provides a framework for the origin, evolution and biodiversity in the genus *Diadophis*. Analysis of mtDNA sequence data inferred 16 mtDNA lineages that replace each other geographically across the United States and into central Mexico. In contrast to previous hypothesis, ancestral area reconstructions inferred an origin for *D. punctatus* in the southeastern United States during the Miocene, followed by a southeast to northeast then westward directionality of historical migration. Demographic analyses indicate that independent lineages currently occupying previously glaciated or unsuitable areas in eastern, central and western U.S. underwent post-glacial population expansion likely from southern refugia during the late Pleistocene/early Holocene. Conversely, southern lineages display patterns consistent with long-term population stability. For the purposes of species delineation, ecological niche modeling does not show isolation due to unsuitable habitat. Ten novel microsatellite loci developed for this study suggest that

most species boundaries are maintained through a balance of selection and dispersal in hybrid zones. Based on the geographic distribution of the mtDNA lineages and the microsatellite frequency data, we hypothesize that there are eight species within the genus *Diadophis* throughout the Eastern United States. This body of work provides a rich framework for further study including, examining species boundaries in the western lineages, determining population structure within species, and detailed examination of hybrid zones.

ACKNOWLEDGEMENTS

I would like to thank the following institutions and persons for providing help with obtaining tissue samples: Jens Vindum and Robin Lawson (California Academy of Sciences), Chris Austin and Donna Dittmann (Louisiana State University Museum of Natural Sciences), Chris Phillips and John Petzing (Illinois Natural History Survey, Robert Espinoza (California State University, Northridge Herpetology Collection, Laurie Vitt and Don Shepard (Oklahoma Museum of Natural History), Jimmie McGuire and Carla Cicero (Museum of Vertebrate Zoology), Greg Watkins-Colwell (Yale Peadbody Museum of Natural History), John Wilson (Savannah River Ecology Lab), C. S. Leib (University of Texas El Paso), Leslie Rissler (University of Alabama), John Campbell, Eric Smith, Carl. J. Franklin and Robert. Jadin (University of Texas at Arlington), Wayne Van Devender (Appalachian State University), Joe Collins, Travis Taggart and Carl Smith (Sternberg Museum Collection), Matt Brandley, Dan G. Mulchahy, Bob L. Christman, Michael R. Cummer, Richard F. Hoyer, Richard E. Weaver, Nick J. Anderson, John F. Parham, Mike Hazel, William Bosworth, Jack Meik, Kevin Setser, Kelly Irwin, Phil Frank, John Gilhen, Jace Robinson, Bill Peterman, Corey Roelky and Tony Gamble.

I owe special thanks to those who accompanied my on field trips and the collection tissues, Frank Burbrink, Tim J. Guiher, Alex Pyron, Cynthia Jimenez, Liz Borda, Eric Waltari, Ken McDonald, Susan Perkins, Anna Rothschild, Mike Percival, Jeff Pollock, and Kathryn Perez.

I am grateful to Amy Hunter, Jeff Pollock, Liz Borda and Kathryn Perez for their generous hospitality and allowing me to crash at their homes during field trips.

I am indebted to the following individuals who have sculpted and help lay for foundation for my career:

Dr. Thomas S. Hopkins (University of Alabama) for his inspiration and never ending amazement with the natural world. I would like to thank him for making field trips mandatory for his undergraduate courses and teaching me that fieldwork continues regardless of weather conditions.

Dr. Mark Siddall (American Museum of Natural History), my mentor, friend, confidant and fellow connoisseur of fine whiskies. Without Mark's exceptional generosity, this work would not have been possible. Not only has he provided me the opportunity to explore my own interests, but has helped shape those interests. He has provided countless opportunities for career advancement, collaboration, travel, and exploration as well as a serving as a model for the type of mentor I one-day hope to become. I am forever indebted to you.

I thank my family, especially my parents, Frances and Joseph Fontanella, and my brothers, Joe and Mark, for their support, encouragement, and never-ending love. I'd also like to thank them for understanding the missed birthdays, family outings, holidays and especially the golf trips.

Two American Museum of Natural History Theodore Roosevelt Memorial Fund awards, a City University of New York student travel award and Mark Siddall's unmatched generosity supported this dissertation research. I'd also thank the following agencies that granted scientific collecting permits: Alabama Department of Conservation and Natural Resources, Arkansas Game and Fish Commission, Georgia Department of Wildlife Resources, West Virginia Division of Natural Resources, Kentucky Department

of Fish and Wildlife Resources, South Carolina Division of Game, Tennessee Game and Fish Commission, Virginia Commission of Game and Inland Fisheries, Mississippi Game and Fish Commission and the New York State Department of Environmental Conservation.

TABLE OF CONTENTS

LISTS OF TABLES

PAGE

LISTS OF FIGURES

FIGURE PAGE

CHAPTER 1

Historical Overview of *Diadophis punctatus*

INTRODUCTION

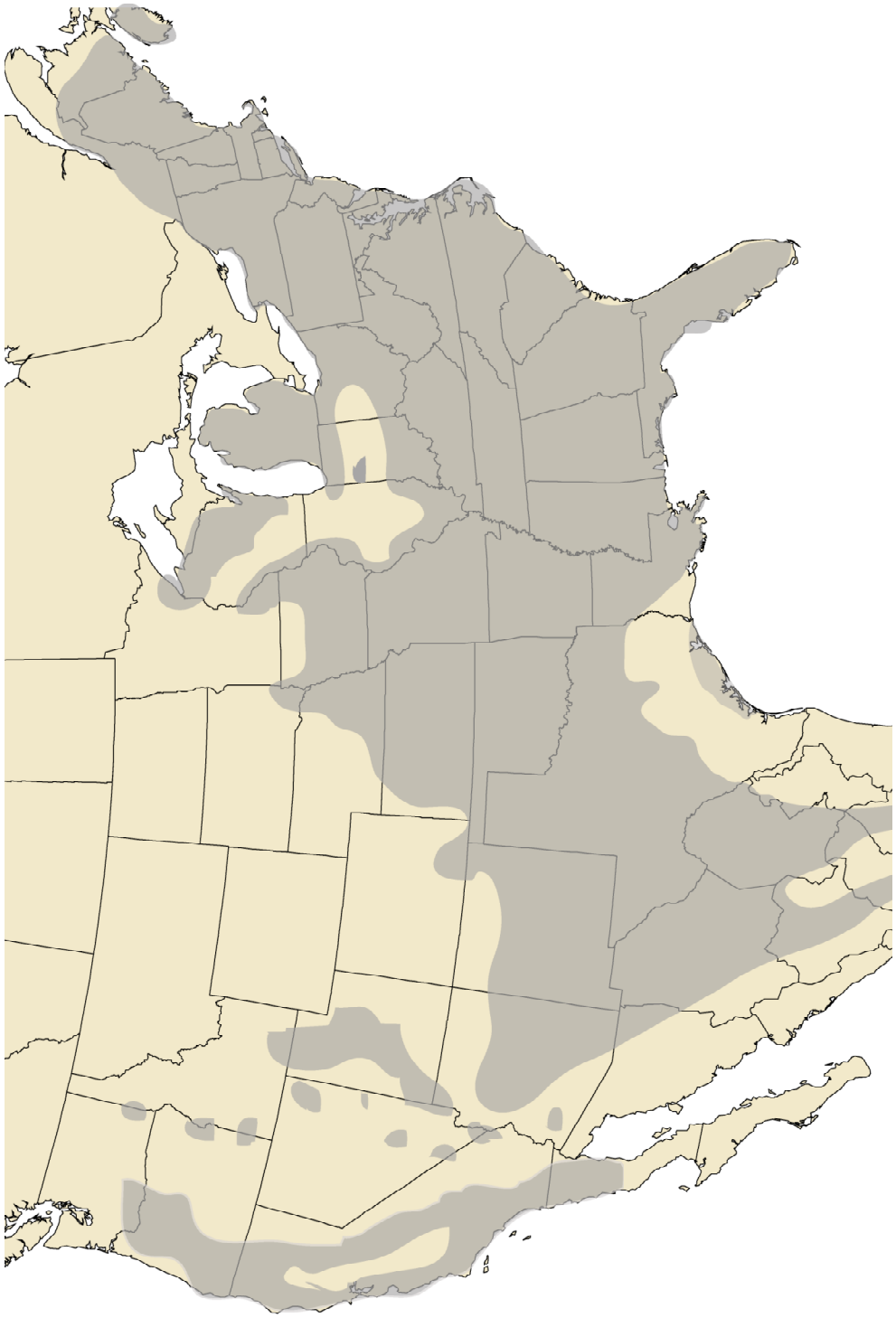
The monotypic snake genus *Diadophis* Baird and Girard, 1853 is one of the most widely distributed and morphologically variable snakes throughout North America. This snake (*Diadophis punctatus)* exploits a variety of habitats and ecological niches ranging from the mixed hardwood forests of southern Canada to the desert of the Southwestern US and Central Mexico (excluding the northern Great Plains states) (Fig. 1). Commonly known as the ringneck snake, this species ranges from altitudes of 0 to 2647 meters and is usually associated with canyon bottoms, riparian habitats, forests, and marshlands but is not aquatic (Hammerson 1982; Rosen et al 1996). Though often considered rare throughout much of its range due to its secretive or retiring habits, they can be both abundant and ecologically prominent.

ECOLOGICAL REVIEW

With a transcontinental distribution, it is not surprising that habitat preferences, nesting sites, behavior, and life history traits differ between geographic localities. Snakes from the northeastern and northwestern regions prefer coverage under rocks or in damp rotting stumps with loose bark or in open woodlands near rocky hillsides. Ringnecks throughout the Southerneastern United States occur in conspicuously wet locales such as damp forests, riparian woodlands and swamps, whereas southwestern and Mexican populations inhabit desert grasslands (Dundee and Miller III, 1968; Fitch, 1975, Stebbins, 1985). With the exception of southwestern populations, which feed primarily on other snakes and lizards (Gehlbach, 1974), the genus *Diadophis* is considered euryphagous, preying on small amphibians, reptiles, worms, grubs, and insects (Blanchard, 1942; Fitch, 1975; Stebbins, 1985; Connant and Collins, 1991).

FIGURE 1.

The geographic range of *Diadophis punctatus* across North America.



Seasonal activity varies in accordance with changing latitude. The small body size and slender habitus permit rapid adjustment of temperature with little effort, allowing ringnecks to maintain an optimum body temperature compared to the ambient temperature. This thermoregulation allows for a relatively long season of activity when compared with other reptiles. At more northerly latitudes, ringnecks typically become active in March and retire to hibernation in late October or early November. In warmer climates, ringnecks can remain active throughout the year, although their observable abundance drastically decreases from December through February and July and August (Anderson, 1942; Myers, 1965).

Life expectancy for ringneck snakes ranges from 10-15 years, reaching sexual maturity at 3-4 years with reproduction typically occurring during April/May and September/October (Fitch 1975). Clutch size averages 3-4 eggs per year but can range from 1-10 per year. Fitch (1975) noted that adult females can store sperm in vascularized pouches of the oviducts for relatively long periods and the deposition of eggs is affected by varying temperature cycles during the spring. Though detailed data on nesting is scarce, den sites vary depending on locality and consist of damp soil clumps or rotting logs for southeastern populations (Myers, 1965), limestone outcrops or under large flattened rocks in the central US (Fitch 1975), or sagebrush (*Artemisia tridentate)* and cheatgrass (*Bromus tectorum*) for ringnecks in Utah (Parker and Brown 1974). Both Fitch (1975) and Parker and Brown (1974) found evidence of communal over-wintering and the ability to return to specific hibernacula indicating that ringnecks can orient within familiar areas.

Dispersal exerts an important influence on population genetics and demography, as well as our ability to predict population level responses to environmental disturbance (Greenwood 1980; Dobson 1982). While there are only a few studies that examined dispersal capabilities in ringnecks, the works were extensive. Of the 433 measurements made over 26 years, nearly one fourth of the snakes traveled less than 10 meters and one third were between 10 and 70 m. The larger number of records less than 10 m indicates an affinity to smaller home areas. The relevance of these short movements may be due to the slow rate of travel and habitual return to a favored spot within the home range. Given the sharp drop in recorded movements after 140 m, Fitch (1975) suggested a home range with a diameter of approximately 70 m.

While many genera of snakes, both venomous and non-venomous, are known to have tail displays to ward off natural enemies (Greene, 1973), tail coiling in the genus *Diadophis* is peculiar. Myers (1965) showed that the response varies geographically, and that it is correlated with the intensity of the ventral coloring. Populations that have little to no variation between the coloration of the tail and the ventral body surface do not display coiling behaviors. In contrast, populations that have yellow pigmentation along the body that grade into bright red tails, have a well-developed coiling response. Fitch (1975) noticed that although the ventral color patterns of ringnecks throughout northeastern Kansas showed ventral color grading, the behavioral response varied. In his study, only 12% of the ringnecks captured showed the typical tail spiraling without further molestation, where as 26% did not show any response regardless of the level of stimulation.

SYSTEMATIC REVIEW

Historically this genus is known from an extinct species, *D. elimorae* Auffenber 1963(a), from the Late Miocene with fossils of the extant species *D. punctatus* (Linnaeus, 1766) from the Late Pleistocene (Holman, 2000). The genus is diagnosed morphologically as having 9-21 (9-14 in western forms and 12-21 in eastern forms) solid maxillary teeth with the last two enlarged and separated from proceeding teeth. The cephalic plates consist of a single frontal scale and paired parietal, prefrontal, internasal and supraocular scales. There are two preocular and postocular scales situated around the moderately sized eye. Typically there is one posterior temporal plate, except in specimens from Mexico, which have two. The upper and lower labials number seven to eight, with the upper labials meeting at the median line behind a triangular mental plate. The two pair of chin shields are equal in length, however the posterior shields are narrower than the anterior sheilds. The head is darker in color than the body and distinguished by a yellow to red band with narrow black borders around the neck. Ground color ranges from light gray to dark brown on the dorsum and yellow to red ventrally. The ventral scales that are often patterned with black dots extending from the throat to the tail. Dorsal scutellation ranges from 13-19 longitudinal rows of smooth scales that become wider ventro-laterally. The venter consists of a single row 126-239 transverse plates that are terminated by a divided oblique anal plate. The tail is short and tapers to a point with the paired caudal plates ranging from 30 to 76.

Previously, most examinations of the morphological variation within this species have been used to diagnose subspecies. Blanchard (1942) conducted the first range wide study recognizing four species complexes based on a combination of morphology and geography. The *punctatus* groups from the Eastern United States consisted of 1) *D.*

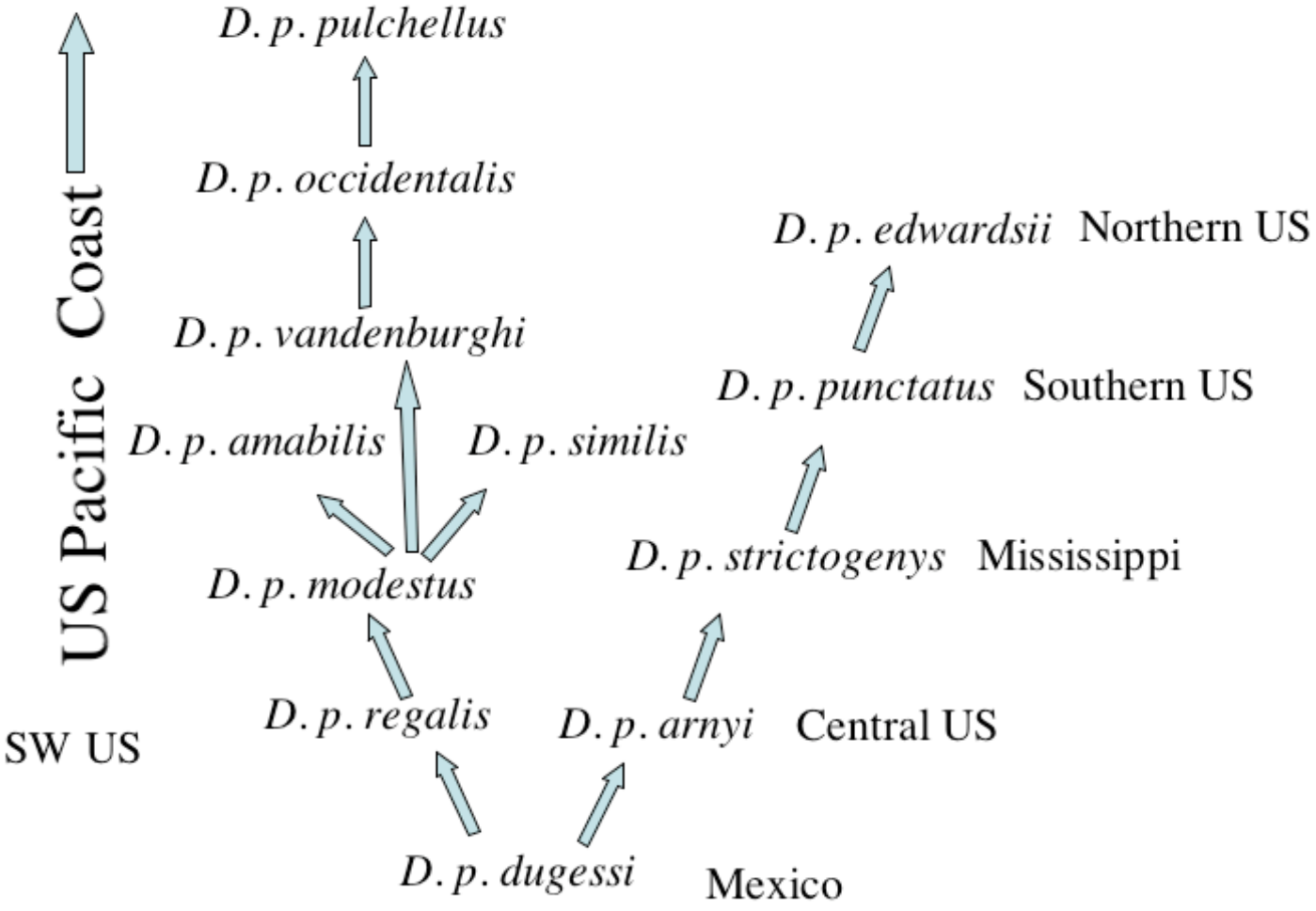
punctatus (punctatus, edwardsii, arnyi, stictyogens, docilis), 2) the *amabilis* group from California, *D. amabilis (modestus, vandenburghii, occidentalis, pulchellus, anthonyi, amabilis, similis*, 3) the *regalis* group from the Southwestern US, *D. regalis (laetus* and *regalis)* and 4) *D. dugesii* from Mexico. These four species groups were recognized based on the shape and length of the body, average number of ventral scales, pigmentation, as well as geographic ranges that did not overlap. Blanchard (1942) hypothesized that the genus *Diadophis* originated on the Mexican plateau with *D. dugesii,* then radiated north, diversifying into the western and eastern groups. The formation of each subspecies was thought to have been a result of the expansion of the species into new habitats (Fig 2). Blanchard (1942) did not explicitly state his criteria for designating species or subspecies of *Diadophis*. His views on their formation reflect ideas of phyletic evolution from the 1940's, and lack consideration of vicariant events, treating species as lineages or the influence that environmental factors may have played in shaping the formation and distribution of the groups.

In later studies, increased sampling across the full range suggested that the morphological characters used by Blanchard (1942) represent either clinal variation, or possible gene flow and that all of *Diadophis* should be considered conspecific (Mecham, 1956; McCoy, 1964; Croulet, 1965; Gehlbach, 1965, 1974).

Mecham (1956) examined four samples from an area within the Guadalupe Mountains between the known ranges of *D. regalis* and *D. punctatus*. In three of these specimens the neck ring was absent and was broadly interrupted in the fourth specimen. The absence or reduction of the neck ring was thought to be a diagnostic character for *D. regalis*. However, the ventral scale counts for the Guadalupe specimens were in the

FIGURE 2.

Evolutionary hypothesis of the currently recognized subspecies and the corresponding geographic ranges of *Diadophis punctatus* modified from Blanchard 1942. Arrows depict Blanchard's views on the phyletic evolution and dispersal of each subspecies.


US Pacific Coast
D. p. pulchellus
D. p. occidentalis
D. p. vandenburghi
D. p. amabilis
D. p. similis
D. p. modestus
D. p. regalis
SW US
D. p. edwardsii Northern US
D. p. punctatus Southern US
D. p. strictogenys Mississippi
D. p. arnyi Central US
D. p. dugessi Mexico

range described for *D. p. arnyi*. These samples led Mecham (1956) to conclude that *D. regalis* and *D. punctatus* represented a single geographically variable species. McCoy (1964) supported the findings of Mecham (1956) and suggested that *D. regalis* should be conspecific with *D. dugesi* based on specimens collected from southern Chihuahua. Similarly, Croulet (1965) considered *D. amabilis* to be conspecific with *D. regalis,* concluding that all *Diadophis* are conspecific under *D. punctatus*.

While examining samples from across Texas, New Mexico, Arizona and Chihuahua, Gehlbach (1974) found five statistically different groups based on ventral scale counts. Total body length and the number of ventral scales increased from east to west and north to south. Similar clinal trends were observed for dorsal scale row counts and coloration of the dorsum. However, the condition of the neck ring did not show any evidence of clinal variation.

The variation in morphological characters combined with the lack of any additional diagnostic characters led to the synonymy of all taxa into 13 subspecies of *D. punctatus* (Stebbins, 1985; Connant and Collins 1991). After first separating all samples into the respective subspecies groups based on morphology and geographic location, Hibbitts (1994) examined scutellation, size variation and color patterns to examine the phylogenetic relationships of the subspecies and the variation within *Diadophis*. Based on the characters measured, all subspecies of *Diadophis* were significantly different with the exception of the Northern California subspecies and *D. p. stictogenys* from *D. p. punctatus*. However, when compared, the range of the diagnostic characters used by Hibbitts (1994) shows considerable overlap between subspecies suggesting that

characters (e.g. ventral scale counts, tail length, ventral color pattern) are unreliable for species designation (Fig. 3).

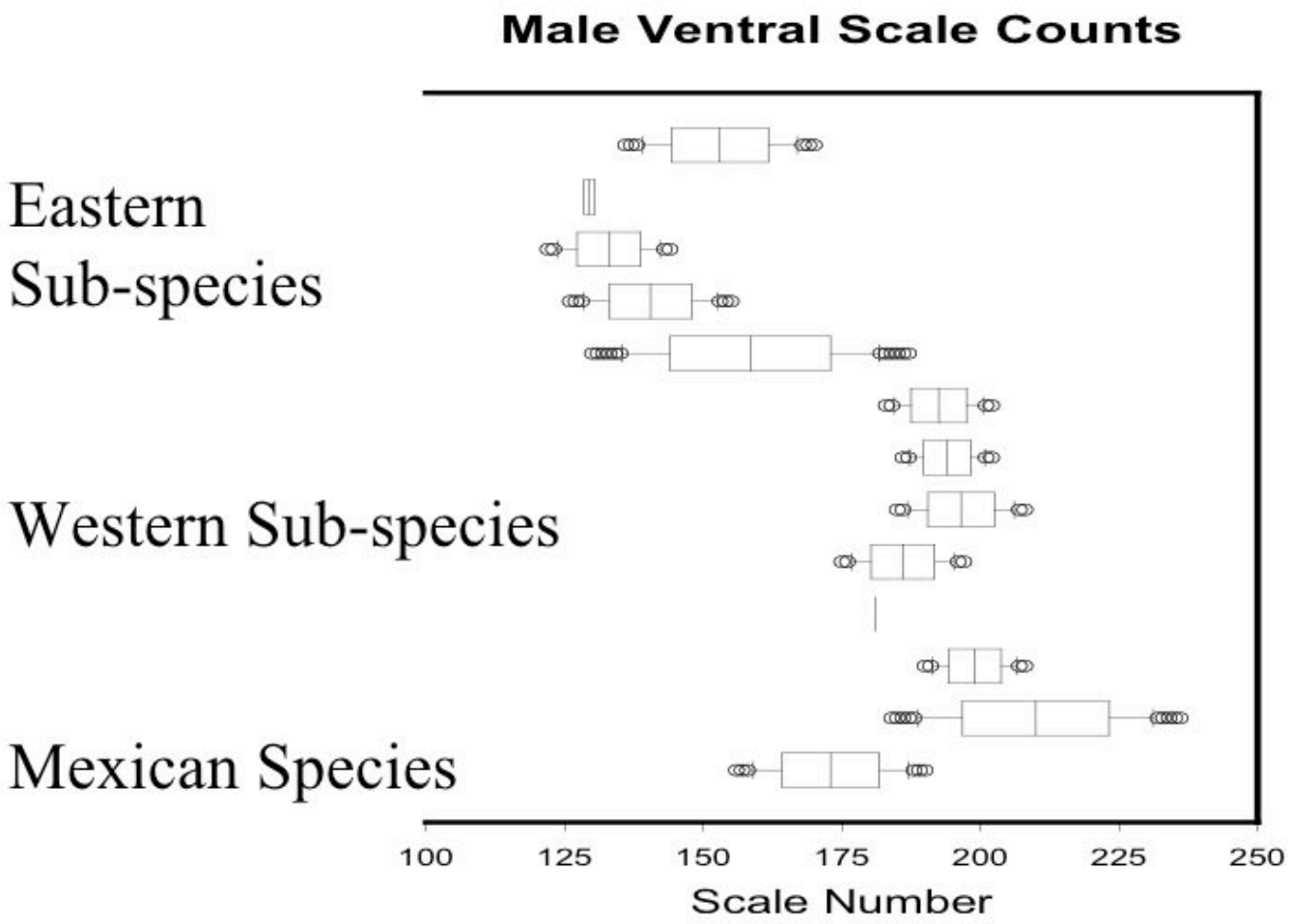
The paucity of fixed diagnostic morphological characters for the species within *Diadophis* lead to the use of molecular data in the more recent studies. Estimates of amino acid differences between population samples of *D. punctatus edwardsii* and samples from California showed degrees of divergence similar to those found between species of other vertebrate groups (Pinou *et al*. 1995). The range of amino acid divergences within *Diadophis* dates back approximately 15 mya to the middle of the Miocene epoch, the same time period as the *D. elinorae* fossil from Florida. These results suggest that *Diadophis* may not be monotypic and may contain at least two genetically distinct species.

Feldman (2000) examined phylogeographic patterns of *Diadophis* throughout California using the mitochondrial gene ND-4. While some phylogeographic structure was apparent, there was neither geographic division nor morphological characters (Hibbitts, 1994) to support the recognition of subspecies from California as described by Blanchard (1942). Based on these findings, Feldman (2000) proposed that the Pacific ring-necks be synonymized under *D. amabilis*.

The results from Feldman (2000) and Pinou (1995) both suggest that *Diadophis* does not consist of a single species. However because these were not range wide studies, questions as to the number of lineages, the processes that shape their ranges, where the lineages originated and how much morphological variation is present within each genealogical lineage could not be answered.

FIGURE 3

Range of ventral scale counts for each subspecies of *Diadophis punctatus*. Vertical bar in box plots represent mean scale count value. (Data from Hibbits 1994).


Male Ventral Scale Counts
Eastern
Sub-species
Western Sub-species
Mexican Species
100
125
150
175
200
225
250
Scale Number

SUB-SPECIES DISCRIPTIONS

Diadophis p. arnyi Kennicott 1859 (Fig. 4)

Diadophis p. arnyi ranges from southern New Mexico through most of the central Plains states and into South Dakota and western Wisconsin. This species displays sexual dimorphism in the number of ventral and caudal scales, as well as tail and head length. Males possess a lower number of ventral scales (158 males; 170 females) but have a greater number of caudals (47 males; 41 females) (Hibbitts, 1994). The collar is usually complete, however there are a few samples (<17) from throughout the range that have been found either lacking a ring or with one that is interrupted. The dorsal scale rows are either 17-15 or 15-15 with the ventral pigment rarely encroaching on to the dorsal scales. The neck ring is usually 1-3 scales wide and the posterior edge lined in black. The head is darker than the dorsum and the chin and lower labials show the same spotting pattern as the belly (Blanchard, 1942).

Localized studies suggest that the variability within the characters used to describe this widely distributed subspecies are a result of clinal variation, morphological plasticity or potential gene flow (Trauth 1996). Within Arkansas *D. p. arnyi* have dorsal scale rows of 17-17, which was considered to be the most useful diagnostic character in distinguishing *D. p. arnyi* from *D. p. stictogenys* (15-15) (Trauth, 1996). In areas of proposed intergradations with *D. p. stictogenys,* dorsal scale counts are either 15-16 or 16-17. In areas where *D. p. arnyi* is sympatric with *D. p. regalis,* specimens have ventral scale counts consistent with those of *arnyi* but either lack a neck ring or the ring is interrupted. Hibbitts (1994) proposed that this was indicative of gene flow between the two sub-species but failed to explain the occurrence of interrupted rings found in the

populations from Minnesota, Missouri, Oklahoma, Arkansas or Kansas, which are not sympatric with *D. p. regalis*. The Arkansas and Kansas populations also possess variable spotting patterns displaying as many as six different patterns. Within Kansas alone, Fitch (1975) found variation in body size, dorsal color, ventral color, size, shape and distribution of the ventral spotting patterns, presence and width of the neck ring, markings on the facial region, number of dorsal scale rows, number of ventrals, subcaudals and supralabials.

FIGURE 4.

Diadophis punctatus arnyi. Photo and identification by Scott Waters. Reprinted with permission.


© SCOTT WATERS

Diadophis p. edwardsii (Merrem 1820) (Fig. 5)

Commonly known as the Northern ringneck, this subspecies ranges from the northeast portion of Minnesota east to Nova Scotia, Canada and south along the Appalachian Mountains to northern Alabama. Sexual dimorphism is evident in the ventral scale numbers, (males average 153; females average 157) and subcaudal scales (males 56; females average 49) (Hibbitts, 1994). Dorsal scale rows are 15-15 with *D. p. edwardsii* being the only form of *Diadophis* with 8 supralabials with all other forms having 7. The head is darker than the slate-gray body and the neck ring is either complete or interrupted mid-dorsally. The ventral pigment typically does not encroach onto the dorsum with the majority of specimens lacking pigment on the chin, throat or labial scales (Blanchard, 1942; Hibbitts, 1994), while a few have heavy spotting on the infralabials (Gilhen, 1971). The ventral color patterns are variable ranging from no spotting pattern to a linear row of solid to half moon spots (Gilhen, 1971).

FIGURE 5.

Diadophis punctatus edwardsii. Photo and identification by Frank M. Fontanella

Diadophis p. punctatus (Linnaeus 1766*)* (Fig. 6)

A small ringneck from the southern coastal plain ranging from Chesapeake Bay southeast along the spine of the Appalachian Mountains to the eastern side of Mobile Bay. Sexual dimorphism is evident in tail length (females 18% of total length; males 21.5%), ventral scale count, (females average 144; males average 136) and subcaudals (females average 41; males average 47) (Hibbitts, 1994). From the material examined by Blanchard (1942) and Hibbitts (1994), the highest number of ventral scales are in specimens from northern Georgia, while those with the lowest ventral counts were from peninsular Florida. The head is black and the neck ring is often partially or completely interrupted and bordered with black. The chin and labials are often heavily pigmented and the ventral spots are crescent shaped and aligned mid-ventrally.

FIGURE 6.

Diadophis punctatus punctatus. Photo and identification by John Sullivan. Reprinted with permission.

Diadophis. p. acricus Paulson 1966 (Fig. 7)

This subspecies occurs only on Big Pine Key, Florida. The diagnostic character for this subspecies is the lack of a neck ring. Due to a lack of preserved specimens the morphological information is limited to two male specimens (Hibbitts, 1994). The length of the tail is approximately 22% of the total body length, 130 ventral scales and 49 subcaudal scales. The dorsal scale rows are 15-15 however no color description is available. Typical of the eastern *Diadophis,* the ventral pigment does not encroach on to the dorsum. Spotting patterns on the chin and labials are present but faint, and the ventral spots are similar arranged into a single midventral row similar to the *punctatus* subspecies.

FIGURE 7.

Diadophis punctatus acricus. Photo and identification by Nathan Shepard. Reprinted with permission.


Nathan Shepard

Diadophis. p. stictogenys Cope 1860 (Fig. 8)

D. p. stictogenys occupies mainly the Mississippi River Valley and adjacent areas. The range extends into eastern Texas, southeastern Oklahoma and southeastern Missouri. Blanchard (1942) described the distribution of *D. p. arnyi* in Arkansas as occurring in the "higher parts of Arkansas" and *D. p. stictogenys* as "south of the Ozark uplands". Range maps have generally followed Blanchard (1942) by separating *D. p. arnyi* and *D. p. stictogenys* along a boundary that splits the two physiographic regions of Arkansas into the lowland Mississippi Delta and Gulf Coastal Plain and the upper Interior Highlands (Ozark Mountains and Ouachita Mountains) (Wright and Wright, 1957; Conant, 1958, 1975; Connant and Collins, 1991). Trauth (1996) used characters similar to Blanchard and Hibbitts (1994) to identify a broad region of sympatry and possible integradation that does not coincide with the physiographic regions described by Blanchrd (1942). The most recent distribution depicts *D. p. stictogenys* extending well into the southern regions of the Ouachita Mountains (Trauth, 1996). Similar to other Eastern subspecies, *D. p. stictogenys* are also sexually dimorphic. Males possess fewer ventrals than females but have a greater number of subcaudals (Ernst and Barbour, 1989). The average number of ventrals in males and females is 149 and 151 respectively and subcaudal scale counts were 49 for males and 42 for females. Hibbitts (1994) recorded higher counts for both ventral and subcaudal scales but did not examine any *D. p. stictogenys* from Arkansas. The dorsal scale counts are 15-15 and are considered the most useful diagnostic character for distinguishing between *stictogenys* and *D. p. arnyi* (Blanchard, 1942; Smith, 1961; Gehlbach, 1974; Conant and Collins, 1991; Hibbitts, 1994). The number of ventral scales tends to increase from the east (where the range overlaps with *D. p. punctatus)* to

west (range overlaps with *D. p. arnyi)*. Some authors have used this variation in the scale counts as evidence of hybridization between the two subspecies (Blanchard, 1942; Gelbach, 1974; Hibbitts, 1994; Trauth, 1996). The ventral spotting is less variable than other forms and usually has a double row of ventral spots extending the length of the body. The head is typically darker than the body; the neck ring is 1/2 to 1.5 scales wide and bordered by black bands (Blanchard 1942; Hibbitts, 1994).

FIGURE 8.

Diadophis punctatus stictogenys. Photo and identification by Kory Roberts. Reprinted with permission.

Diadophis. p. regalis Baird and Girard 1853 (Fig. 9)

D. p. regalis is the largest of the *Diadophis* subspecies reaching lengths of up to 772 mm. There are several disjunct populations of this subspecies from the grasslands, mountains and deserts of the southwestern US and northern Mexico. The largest population ranges from eastern Texas westward to Tuscon, Arizona and north to Flagstaff, AZ. A large disjunct population exists in the Wasatch Mountains of central Utah ranging extending southward to the Utah/Nevada/Arizona border and north into southeastern Idaho. Several smaller populations exist through out western Arizona, Utah and southeastern Nevada. In Mexico, this form extends along the eastern slopes of the Sierra Madre Occidental south to northeastern Durango. In the Sierra Madre Oriental, the range extends south to San Luis Potosi and is largely absent from the Mexican Central Plateau (Blanchard, 1942; Hibbitts, 1994).

Ventral scale counts, tail length and subcaudal counts all suggest sexual dimorphism with males possessing less ventrals but longer tails and more subcaudals. Males average 216 ventrals, with a tail length 18.5% of the total length and 65 subcaudal scales. Females have tail lengths 16% of the total body length with an average of 59 subcaudals and 216 ventral scales. The absence of a neck ring varies in frequency from population to population. All 14 specimens from Utah examined by Hibbitts (1994) lacked a neck ring while the ring was absent in only 3 of the 35 specimens from Arizona, while 22 out of 50 specimens from Texas had a collar. All specimens examined by both Blanchard (1942) and Hibbitts (1994) had heavily spotted chins and throats with randomly arranged ventral spots. The head is slightly darker than the bluish to gray body. When present, the neck ring is three scales wide and is not bordered by a black

band. The yellow to brick red color of the abdomen frequently encroaches onto the first row of dorsal scales (Blanchard, 1942).

FIGURE 9.

Diadophis punctatus regalis. Photo and identification by Michael Price. Reprinted with permission.

Diadophis. p. amabilis Baird and Girard 1853 (Fig. 10)

D. p. amabilis is a relatively small snake occurring chiefly in the San Francisco Bay area in Central California. The number of ventral scales, tail length and head length all show sexual dimorphism. In females the ventral scales average 204 with a tail length that is 17% of the total body length and 56 subcaudal scales. Males average 191 ventral scales with a tail length 20% of the total body length and 63 subcaudals. The head is typically darker than the light gray to black body. The ventral color is yellow becoming reddish towards the posterior, with the color extending over the first half to 1.5 dorsal scale rows. The ventral spots are often round but also show transversely elongated spots that are arranged randomly. The chin, throat and labials have no fewer than 24 spots. The neck ring is narrow, typically one to one and one half scales wide and is interrupted in 25% of the specimens examined (Blanchard, 942; Hibbitts, 1994).

FIGURE 10.

Diadophis puncatus amabilis. Photo and identification by John Sullivan. Reprinted with permission.

Diadophis. p. dugesi Villada 1875 (Fig. 11)

This is the only subspecies of *Diadophis* restricted to the Mexican plateau. The range extends from southwestern Chihuahua, Mexico, along both slopes of the Sierra Madre Occidental and western slopes of the Sierra Madre Oriental, south to Michoacan and east to Vera Cruz. Information about this subspecies is limited to less then 20 specimens (Blanchard, 1942; Hibbitts, 1994). The dorsal scale rows are 15-15 in most samples but can be 15-17-15. Dorsal coloration is described as "dark" and extending over all dorsal scales down to the ventrals. The neck ring completely encircles the neck and is typically two scales wide. The head is slightly darker than the body and the color extends around the angle of the jaw onto the chin scales. The ventral scales have small, scattered black spots and average 180 scales in both males and females. Both male and female tail length is approximately 18% of the total body length with each sex having 54 subcaudal scales (Blanchard, 1942; and Hibbitts, 1994).

FIGURE 11.

Diadophis punctatus dugesi. Photo and identification by Ron Burkhardt. Reprinted with permission.

Diadophis p. modestus Bobourt 1866 (Fig. 12)

Commonly known as the Los Angeles ringneck, this form is restricted to the vicinity of Los Angeles, California ranging from San Bernardino west to Santa Barbara. The dorsal scales are bluish to black in color and the rows number 17-15 (Blanchard, 1942; Hibbitts, 1994). The head is typically darker than the body with a bright red neck ring one half to two scales wide and bordered behind by black spots. Tail length is 21% of the total body length in males with an average of 65 subcaudal scales. In females the tail length is 18% of the total body length and possess 60 subcaudal scales. Ventral scale counts average 188 and 202 in males and females respectively. The ventral color is yellowish and extends onto the first of scales. The ventral spots are generally round and aligned along the midline of the belly. The chin and labial scales have numerous (> 40) black spots (Hibbitts, 1994).

FIGURE 12.

Diadophis punctatus modestus. Photo and identification by Gary Nats. Reprinted with permission.


© Gary Nafis

Diadophis p. occidentalis Blanchrad 1923 (Fig. 13)

The northern most form of the Pacific ringnecks, the range of *D. p. occidentalis* extends from north of San Francisco Bay to southern Washington. The uninterrupted bright red neck ring is 1.5 to 2 scales in width and bordered by black bands. The head is darker than the dorsal surface. The dorsal scales number 15-15 or 15-13. Similar to other forms of *Diadophis* tail length, subcaudal scale counts and ventral scale counts all display sexual dimorphism. In females the tail length comprises 17% of the total length with an average of 57 subcaudal scales. Males average 65 subcaudals with 21% of the total length due to the length of the tail (Hibbitts, 1994). The ventral scales are 188 and 204 in males and females respectively. The ventral color is yellow to reddish and extends onto the first two to rows of dorsal scales. The chin and lower labials are prominently flecked with black spots that diminish in size and number on the ventral surface becoming random along the length of the body.

FIGURE 13.

Diadophis punctatus occidentalis. Photo and identification by Alan Barron. Reprinted with permission.


© Alan Barron

Diadophis p. pulchellus Baird and Girard 1853 (Fig. 14)

D. p. pulchellus occurs primarily along the western slopes of the Sierra Nevada's of California south to Tejon Pass north of Los Angeles. The broad, uninterrupted orange neck ring is two to three scales wide and bordered with black bands (Blanchard, 1942). The dorsal scales are 15-15 or 15-13 and are a light blue to slate gray in color. The ventral scales average 197 in males and 208 in females. Males typically have longer tails and more subcaudal scales than females. The tails in males typically make up 20% of the total body length and have 64 subcaudals where the tails of females are 18% of the total body length and 60 subcaudal scales (Hibbitts, 1994). The ventral color is bright orange or red and extends onto the first two rows of dorsal scales. The chin and labials have few small black spots that either continue down the ventral scales or stop after the first 10 or so scales (Blanchard, 1942).

FIGURE 14.

Diadophis punctatus pulchellus. Photo and identification by John Sullivan. Reprinted with permission.


© G. Nafis

Diadophis p. similis Blanchard 1923 (Fig. 15)

D. p. similis occupies the range described by Blanchard (1942) for both *D. p. similis* and *D. p. anthonyi*. This subspecies ranges from San Diego south to northern Baja California. The dorsal scale rows are 15-13 and are olive in color with a darker head. The neck ring is one-half to one and one-half scales wide, dull red and bordered with a few black spots (Blanchard, 192). The ventral scales, averaging 194 in males and 201 in females, are orange-yellow changing to scarlet towards the tail. The spotting pattern on the belly is sparse and unorganized but each ventral scute end has a black dash forming a ventro-lateral row of spots throughout the length of the body and tail. The tail length is 19% and 17% of the total body in males and females respectively. The labials, chin and throat are spotted with black (Hibbitts, 1994).

FIGURE 15.

Diadophis punctatus similis. Photo and identification by John Sullivan. Reprinted with permission.

Diadophis p. vandenburghii Blanchard 1923 (Fig. 16)

D. p. vandenburghii ranges from Monterey, California south along the coast to just north of Ventura, California. The dorsal scale rows are 17-15 and olive in color (Blanchard, 192). The uninterrupted neck ring is one to two and one half scales wide, yellow to red in color. The ventral scales average 190 in males and 200 in females (Blanchard, 1942; Hibbitts, 1994). The light ventral color extends over one and one-half to two of the lower most dorsal rows. There are few small black spots on the ventral scales that extend up to the chin shields and onto the labials. Males have longer tails than females, 21% and 18% of the body length respectively and higher subcaudal counts, 64 and 51 respectively (Hibbitts, 1994).

FIGURE 16.

Diadophis punctatus vandenburghii. Photo and identification by John Sullivan. Reprinted with permission.

CHAPTER 2

Phylogeography of *Diadophis punctatus*: extensive lineage diversity and repeated patterns of historical demography in a trans-continental snake.

(From: Fontanella *et al.* Phylogeography of *Diadophis punctatus*: extensive lineage diversity and repeated patterns of historical demography in a trans-continental snake. *Molecular Phlogenetics and Evolution* 46: 1049-1070)

INTRODUCTION

Historical processes such as population division due to isolation, long-distance dispersal or range expansion are expected to leave characteristic signals on the distribution and frequency of alleles (Hewitt 1996). When analyzed using phylogenetic methods, the tree structure can be superimposed over the range of the population, revealing whether the population history has been one of isolation, panmixia or a combination of the two. Predictions based on population genetic theory can then be incorporated to reconstruct the demographic histories of these populations and to examine the genetic variation within and among populations. Combining these methods has allowed biologists to investigate how the distribution of taxa may have changed in response to climatic shifts in geologic history (Avise and Walker 1998; Douglas et al. 2003; Zamudio and Savage 2003; Mahoney 2004).

During the Pleistocene, at least six glacial advances affected the physical and biological environments of the Northern Hemisphere (Cox and Moore 2000). The expansion and contraction of glacial ice sheets are thought to have played an important role in shaping the distribution and biodiversity throughout the northern temperate regions (Hewitt 1996). At the time of the last glacial maximum in North America, ~22-18 kya, ice sheets extended from southeastern Alaska throughout most of Canada and into the northeastern and northwestern United States (Mann and Hamilton 1995). The presence of these glaciers combined with the climatic changes during the Pleistocene are hypothesized to have stimulated intra-specific diversification by separating populations through the formation of glacial barriers and by shifting the location of suitable habitat further south (Durand et al. 1999; Hewitt 2000; Brunsfeld et al. 2001; Waltari et al.

2007). While phylogeographic patterns can vary across taxa, the concerted retreat into southern refugia has been proposed as an historical factor underlying the formation of major genetic lineages (Swenson and Howard 2005). Thus, concordant patterns of population expansion from southern refugia into previously glaciated areas have been inferred across a variety of taxa with a broad range of life history strategies, indicating the overriding impact of glacial vicariant events on structuring genetic diversity at northern latitudes. For many amphibian and reptile species, southern refugia have been proposed from across the conterminous United States including the Appalachian Mountains and Interior Highlands (Burbrink et al. 2000; Austin et al. 2002, 2004; Zamudio and Savage 2003), the Atlantic and Gulf Coast coastal plains (Austin et al. 2004; Burbrink et al. 2000; Church et al. 2003; Zamudio and Savage 2003), Northern Texas (Howes et al. 2006), the Clearwater drainage in Idaho (Carstens et al. 2004), the Sonoran and Chihauhuan deserts of the southwest (Castoe et al. 2007), the Columbia River Valley in Oregon and the Klamanth/Siskyou mountains of Northern California (Steele and Storfer 2006). Although the role of glaciation events on speciation has been debated (Klicka and Zink 1997, 1998, 1999; Avise and Walker 1998; Avise et al. 1998), the formation of different geographic refugia allowed existing lineages to persist. Additionally, many of these species have retained geographic ranges separated by distinct geographic boundaries. However, in some cases phylogeographic breaks can occur that do not coincide with known geographic boundaries (Irwin 2002). Examination of species distributions has revealed that non-climatic ecological factors such as breeding sites, host availability and especially species interactions can lead to genetic differentiation of local

populations in unglaciated areas (Ehrlich 1961; Hairston 1987; Crespi et al. 2003; Kozak et al. 2006).

Widespread taxa occupying both historically glaciated and unglaciated regions provide an excellent opportunity to study the effects of climatic cycles on population fragmentation and historical demography. Such taxa also serve as excellent models because their geographic ranges likely span many previously identified phylogeographic barriers and provide an opportunity to uncover genetic breaks that are not associated with geographic boundaries. In addition to a broad geographic range, several features make certain taxa more amenable to phylogeographic study for the purposes of examining the influence of climatic shifts. Limited or restricted dispersal of a species facilitates the successful inference of historical patterns of migration by maintaining the genetic patterns created during the establishment of the current distribution (Crandall and Templeton 1996; Templeton 1998). If a species becomes restricted to a particular area or colonizes an unoccupied region, the stability and maintenance of phylogeographic patterns will depend on longevity and vagility (Hewitt 1996). Thus, taxa with poor dispersal capabilities and exhibiting strong philopatry should retain the genetic patterns that developed while occupying these regions. Because mutations occurring in cytoplasmic genomes are transmitted maternally, patterns of variation inferred from mtDNA should reflect the demographic history and historical processes responsible for the contemporary distribution (Avise 2000).

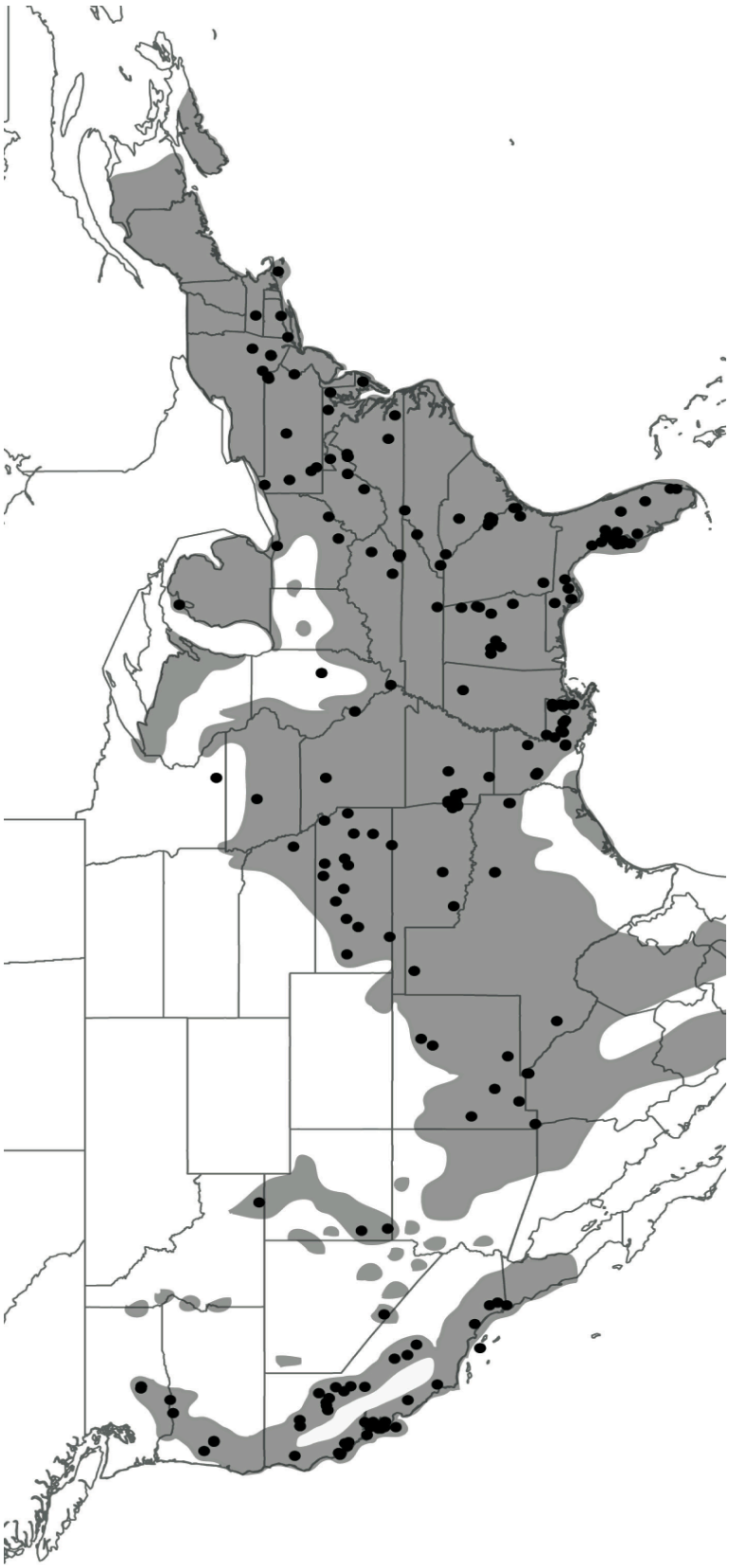
The ringneck snake *Diadophis punctatus* possesses several of the aforementioned features making it an excellent organism to study phylogeography and to examine the effects of climatic cycles on demographic history. As one of only seven North American

squamates with a transcontinental distribution, this snake exploits a variety of habitats and ecological niches ranging from the mixed hardwood forests of southern Canada to the deserts of the Southwestern US and Northern Mexico (Stebbins 2003) (Fig. 17). This species is also characterized by morphological and behavioral variation (Blanchard 1942; Gehlbach 1965; Fitch 1975; Blanchard et al. 1979; Connant and Collins 1991; Stebbins 2003) suggestive of extensive genetic variation. With the exception of the southwestern populations that feed exclusively on reptiles (Gehlbach 1965), ringnecks have generalist diets, preying on salamanders, earthworms, reptiles and insects (Blanchard 1942; Fitch 1975; Blanchard et al. 1979; Stebbins 2003; Connant and Collins 1991). This species is relatively long lived among small snakes, surviving to an average age of over ten years. Home ranges are estimated to have a diameter of a mere 70 meters (Fitch 1975; Blanchard et al. 1979) and Parker and Brown (1973) found that ringnecks in Utah returned to specific hibernacula over multiple years. The longevity, small home range and limited dispersal abilities of this species fulfill a number of the conditions beneficial to the inference of historical patterns of migration and fragmentation that have resulted in the current distribution of genetic diversity.

Despite the extensive variation in ecology, morphology, and life history of ringneck snakes, only two molecular studies have addressed lineage diversity within *D. punctatus*. However, neither was comprehensive; Pinou et al. (1995) examined immunological distances for a small sample of ringnecks while Feldman and Spicer (2006) restricted their phylogeographic analysis of mtDNA sequences to populations in California. Here we examine the historical patterns of *D. punctatus* with comprehensive transcontinental sampling using both phylogenetic and population genetic methods. Our

FIGURE 17.

Map of the United States showing the proposed range of *Diadophis punctatus* and location of samples used in this study. Locality and label designations are given in Appendix 1.



goals are to: 1) describe the lineage diversity and phylogeographic patterns of the ringneck snake *Diadophis punctatus* and 2) to evaluate the effects of Pleistocene glaciation on population demography in glaciated and unglaciated regions.

MATERIALS AND METHODS

Molecular analyses

Sampling and geographic distribution. Our geographic sampling (n = 286) covers most of the known the range of this species across the US (Fig 17; Appendix 1). Due to uncertainty of the sister group and to assess the monophly of *D. punctatus*, outgroup taxa included the other North American xenodontine snakes *Contia tenuis*, *Hypsiglena torquata, Rhadinaea flavilata, Francia abacura, Heterodon simus* and *Heterodon platyrhinos* (Pinou et al. 2004; Lawson et al. 2005).

DNA extraction, amplification and sequencing. We extracted DNA from liver, muscle, blood or shed skin preserved in ethanol or frozen in liquid nitrogen using phenol chloroform and CTAB modified from Saghi-Maroof et al. (1984). We amplified the mitochondrial genes *cytochrome b* (1117 bp) and a portion of the *NADH* 4 subunit (659 bp) via PCR using previously published primers and protocols (Burbrink et al. 2000) and (Arevalo et al. 1994), respectively.

Each of these genes has been used successfully to examine intraspecific variation in snakes (Burbrink et al. 2000; Feldman and Spicer 2002; Nagy et al. 2004). Amplifications were purified using either 2ul of ExoSap-it (USB Corp.) per 10 ul of PCR

product or AMPURE (Agentcourt) following the manufacturer's instructions. Purified PCR products were sequenced in both directions for both genes using BigDye v.1.1 (Applied Biosystems, Perkin-Elmer, California USA). For the cytochrome *b* gene, sequencing reactions were performed using internal sequencing primers designed specifically for *D. punctatus* (Dp-F CCTTCTGAGCAGCAACAGTAA) and (Dp-R GAAGAATCGTGTGAGGGTTGG). Occasionally, extension of the Dp-R primer failed to cover the forward section of cytochrome *b*. When this occurred, samples were re-sequenced using the L14910 (de Queiroz et al. 2002) primer. Sequencing reactions for ND-4 were carried out using the amplification primers. Reactions were purified with the CleanSeq Dye-terminator removal kit (Agentcourt) and analyzed on an ABI Prism 3730 sequencer (Applied Biosystems, Perkin-Elmer, California USA). Sequences were assembled, edited, and aligned using SEQUENCHER 4.7 (Gene Codes, Corp.) and an open reading frame was verified for each gene. The alignments were unambiguous with no gaps present in either of the mitochondrial genes for any of the *D. punctatus* sequenced in this study. Although the ND 4 primers amplify portions of the downstream Serine, Histidine, and Leucine trna genes, only the protein coding region was used in the analyses. The tRNA gene regions were deleted due to problems of alignment resulting from secondary structure and incomplete sequencing. Sequences were deposited in GenBank under the accession numbers (EU193950-194234 and EU193663-193948).

Phylogenetic analyses

Phylogenetic relationships of *Diadophis punctatus* samples were estimated for each gene separately and from the combined cytochrome *b* and ND 4 data sets with

Maximum Parsimony (MP) in TNT Goloboff et al. 2003) and Bayesian inference (BI) using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

Maximum Parsimony analyses were conducted using a heuristic search method with equally weighted characters, 1000 random addition-sequence replicates and the tree-bisection-reconnection (TBR) branch-swapping algorithm. Support for internal nodes was assessed using non-parametric bootstrapping (BS) (Felsenstein 1985) with 1000 pseudo-replicates and 100 random sequence-addition replicates.

ModelTest v3.7 (Posada and Crandall 1998) was used to select the best-fit model of nucleotide change based on the Akaike Information Criterion (AIC) independently for each gene (Akaike 1973). The GTR+Γ+I (general time reversible model with gamma-distributed among-site rate variation and with a proportion of invariant sites) model was selected for each gene and implemented in MrBayes. In the separate analyses, two methods of model partitioning were explored for each gene. The first method implemented the GTR+Γ+I model without considering differences in codon position. The second method partitioned the model across the first, second and third codon positions 3(GTR+Γ+I). Additionally, a mixed model analysis was performed for the concatenated data to infer trees using the evolutionary information from both genes. Two differently partitioned analyses were conducted for the concatenated data set because how a data set is partitioned can have a greater influence on the mean –lnL and estimated posterior probabilities than the overall number of partitions (Brandley et al. 2005). In the first analyses, the appropriate model was partitioned across each gene 2(GTR+Γ+I). In the second, models were partitioned across the first, second and third codon positions of

each gene 6(GTR+Γ+I). Each of the four analyses (implemented with two simultaneous runs) was conducted using default priors for parameters of the four markov chains with the model parameters unlinked among partitions for each run. Each run used a random starting tree and was run for 1 $x10^7$ generations. Trees and their parameters were sampled once every 1000 generations. Stationarity of the likelihood scores was determined by examining the convergence in posterior probabilities between the simultaneous runs using the standard deviation of split frequencies based on Rubin and Gelmans's "r" statistic (Gelman et al. 1995). The partitioned models were evaluated using Bayes factors calculated from the harmonic mean of the posterior probability distribution for the models (Nylander et al. 2004). When comparing models using Bayes factors, a value greater than 10 is considered strong evidence for the selection of the more complex model (Kass and Raferty 1995).

In order to conservatively delimit boundaries for each lineage, we followed the tree-based method of Wiens and Penkrot (2002), which incorporates geographic patterns of coalescence among DNA haplotypes to test for gene exchange between closely related lineages. This method assumes that discordance between haplotype clades and the geographic areas from which they are found or the failure of haplotypes from a given area to form a clade is evidence of potential gene flow with other populations (Slatkin and Maddison 1989).

Population genetic analyses

Diversity indices and population structure. We examined genetic structure and demographic patterns using both traditional population genetic and coalescent methods.

All lineages were treated as separate units for tests of demographic history and genetic structure. The majority of the major clades inferred from the phylogenetic analyses occupied non-overlapping geographical areas. In regions where lineages overlapped there was no evidence of introgression of the mitochondria (see Results). To summarize the genetic variation among and within populations, we first conducted an analysis of molecular variation (AMOVA, Excoffier et al. 1992) in Arlequin v2.0 (Schneider et al. 2000).

Historical demography. Haplotype (Hd) and nucleotide (θ) diversity were calculated to measure DNA polymorphisms using DNAsp v3.0 (Roza and Roza 1999). These measures are appropriate for this type of study because they do not depend on sequence length or sample size (Nei and Li 1979; Nei 1987). Nucleotide diversity can be calculated using the average number of pairwise nucleotide differences (π) or by calculating the number of segregating sites (S). Under the null hypothesis of population stability, the difference between these two values, Tajima's *D* (Tajima 1989) and Fu's F_s (Fu 1997), can be used to infer demographic history of a population. If populations have been stable over time both statistics are expected to be close to zero (Tajima 1989, Fu 1997). Significant deviation from zero (positive or negative) permits for the rejection of the null hypothesis of population stability. Under the assumption of neutrality, negative values are expected in populations that have undergone recent increases because rare alleles are more numerous than expected. Positive values occur if rare alleles are eliminated from populations following genetic bottlenecks (Tajima 1989). The validity of the assumed stepwise expansion model and the significance of *D* and F_s were calculated by constructing 10,000 coalescent simulations in DnaSP.

Mismatch distributions were also calculated for each lineage to infer changes in population size. Assuming an infinite sites model, population expansion would be depicted as a unimodal distribution whereas the distribution expected for population stability is ragged and multimodal (Harpending et al. 1998). The fit of the observed data was compared using the sum of squares deviations between observed and expected data estimated from 10,000 coalescent simulations in DnaSP. Although the R_2 raggedness index (Ramos-Onsins and Rozas 2002) is often used to assess significance of mismatch plots, non-unimodel models that fit sudden expansion models indicate structuring within populations or populations that are stable or shrinking (Excoffier and Schneider 1999; Rogers and Harpending 1992; Rogers et al. 1996). Therefore we do not consider the good fit of sudden expansion models to multimodal mismatch distributions strong evidence of population expansion. However, multimodal distributions can be inferred for populations that have undergone recent expansion but were recently sub-divided, subjected to substantial migration and/or have undergone historical contractions (Bertorelle and Slatkin 1995; Marjoram and Donnelly 1994; Ray et al. 2003; Castoe et al. 2007).

Although D, F_s and mismatch distributions are able to provide insights into whether or not population growth has been expansive, they are not able to provide information about the shape of population growth over time. Non-significant negative values of D and F_s are indications that populations have not undergone expansive growth relative to a null hypothesis of population stability. However, such values are agnostic as to whether populations are expanding slowly, are contracting or remaining relatively constant in size. Therefore, to estimate the shape of population growth through time we

constructed Bayesian skyline plots implemented in BEAST v1.4 (Drummond and Rambaut 2003; Drummond et al. 2005). This method uses MCMC sampling procedures to estimate the posterior distribution for the effective population size given a model of nucleotide substitution and a set of aligned DNA sequences (Drummond et al. 2005). As above, the appropriate model of nucleotide substitution for each lineage was determined using ModelTest. Geneologies and model parameters for each lineage were sampled every 1000th iteration for 1 $x10^7$ generations under a relaxed lognormal molecular clock with uniformly distributed priors and a pre-burnin of 100. Coalescent intervals (*m)* ranged from 3 to 15 depending on the total number of samples per lineage. We noticed that varying values of *m* had little effect on the analyses and that the demographic function was highly consistent across a wide range of values for the *m* hypervariable (Drummond et al. 2005). Demographic plots for each analysis were visualized using Tracer v1.0.1 (Rambout and Drummond 2003). If population sizes are constant through time then the slope of the skyline plot should not be significantly different than zero. We tested the slope of the skyline plot for each lineage against the null hypothesis of population stability (slope of zero) using GraphPAD Prism v.5.0 (GraphPad Software, Inc.)

Divergence Dating and Dating demographic change.

To infer the date of origin for each lineage without relying on a molecular clock and considering uncertainty in tree topology and branch length (i.e., 'the relaxed phylogenetics' method) we used BEAST v1.4.4 (Drummond and Rambaut, 2003). Phylogenetic estimates were constructed under the GTR+Γ+I model with an uncorrelated

lognormal tree prior with a constant population size prior. Derived from fossil data (Holman 1979; 2000), a mean calibration point of 7.5 my was placed at the root of *Diadophis* with a lognormal standard deviation of 0.29 producing a 95% credible sampling interval (CI) from 4.55 to 12.1 my. This time frame spans the North American Land Mammal Age (Hemphillian) into the Clarendonian Age; the latter age has not produced fossils for this species and is a reasonable cutoff point for the upper CI. Analyses were run for 20 million generations and sampled every 1000^{th} iteration following a pre-burnin of 2000.

We estimated the date of expansion for each lineage using the formula $T= \tau /2u$, where τ is the relative time (in generations) since expansion obtained from 1000 replicates from Arlequin and u is the product of the mutation rate, the generation time, and the sequence length. Females usually reach sexual maturity at approximately 3 years (Fitch 1975; Blanchard et al. 1979) and the total sequence length was 1776 bp. To obtain mutation rates for each lineage we used the semiparametric approach utilizing penalized likelihood (PL) with the truncated Newton (TN) algorithm implemented in r8s v1.70 (Sanderson 2003). Since large data sets with low sequence variation (such as population-level data) or phylogenies with very short branch lengths (phylogeographic studies) can lead to spurious results due to zero branch lengths, we followed the suggestions of Sanderson (2003) and constructed phylogeny using one exemplar from each lineage. This reduced data set was analyzed with the 6 (GTR+Γ+I) partitioned model. Outgroup taxa were pruned prior to rate smoothing analyses with the root of *D. punctatus* set to 7.5 million years ago. An optimal smoothing value of 1000 was obtained using the cross-

validation procedure implemented in r8s.

RESULTS

Phylogenetic reconstruction

Two hundred and ninety five sequences for 1776 aligned base pairs were obtained for *D. punctatus* and nine outgroup taxa. The absence of any internal stop codons in either protein coding gene and a bias against guanine on the light strand indicate that the sequences were from the mitochondrial genome and are not nuclear-integrated copies or pseudogenes (Zhang and Hewitt 1996).

Of the total 1776 characters analyzed, 832 were constant and 758 variable characters were parsimony informative (considering both ingroup and outgroup taxa). Maximum Parsimony analyses produced 14 trees of 3801 steps. Because both analyses produced highly congruent estimates of phylogenetic relationship for the major clades only the Bayesian consensus phylogram is presented with posterior probabilities and nonparametric bootstrap values from the MP analyses for the shared branches.

When genes were analyzed separately for the Bayesian analyses, 'burn in' occurred prior to one million generations both for the partitioned and un-partitioned analyses. As such, results hereafter were based on harmonic means calculated from the remaining nine million generations. The data were combined and analyzed under a mixed model since each of the separate analyses inferred trees with similar topologies. Burnin for the mixed model analyses occurred between one and 2.5 million generations depending on how the data were partitioned. For each of the four analyses, Bayes factors always chose the most complex model with a value greater than 1000. Therefore, tree topology and posterior probabilities (PP) were inferred from the 6 (GTR+Γ+I) model.

The combined mixed-model analyses produced a 50% majority-rule consensus tree with a mean lnL of –24033.25 following a 'burn in' of 2500 generations.

The *Diadophis punctatus* complex formed a well-supported monophyletic group exclusive of the other North American xenodontines. This species complex was composed of four major clades consisting of 14 phylogenetically and geographically distinct, putatively independent, evolutionary lineages.

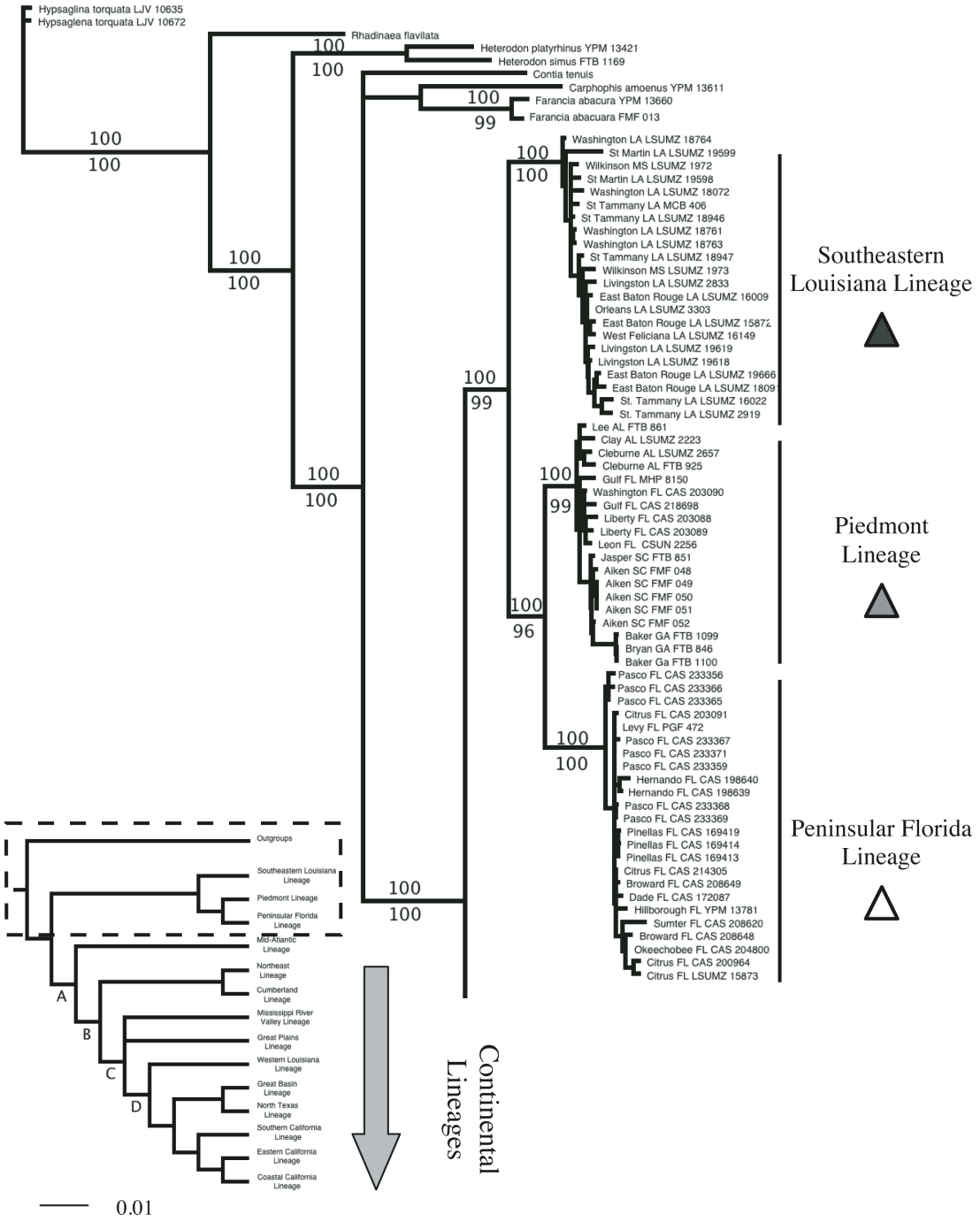
The basal node in the complex subtends a Gulf Coast clade (A) from the Mid-Atlantic and remaining clades of the continental United States (Fig. 18, 19). The Gulf Coast clade extends from southeastern Louisiana to southern Florida and comprises three lineages. The first-diverging lineage is restricted to southeastern Louisiana and is the sister taxon to a group containing the Piedmont lineage ranging from southeastern South Carolina to eastern Alabama and a peninsular Florida lineage distributed from the Florida Keys north to the Suwannee River.

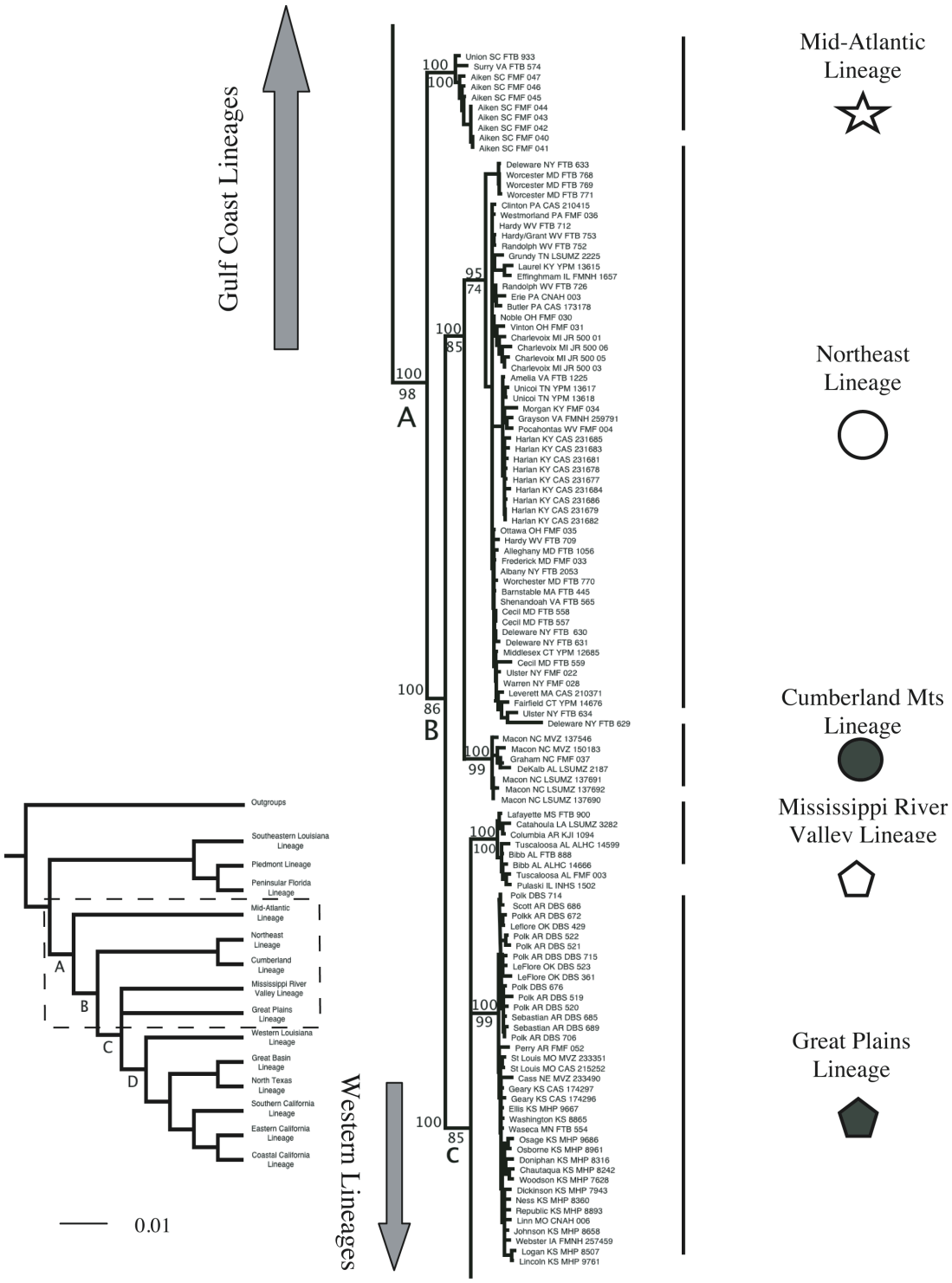
The second major division occurs between the Mid-Atlantic clade (B) and the more diverse continental clades west of the Appalachians. This clade consists of a single lineage that is confined to the eastern side of the Appalachian Mountains from costal southern Virginia to southeastern South Carolina.

The third major split occurs between the Appalachian (C) and Western clades (D), both of which comprise multiple lineages west and north of the Atlantic and Gulf Coast coastal plains. Within the Appalachian clade, the Northeast lineage ranges from central Tennessee north to Massachusetts and west to central Illinois above the Mississippi River Embayment. The Cumberland lineage includes populations confined to the mountains of northeastern Alabama and western North Carolina.

FIGURE 18.

Bayesian 50% majority-rule consensus tree for the 286 *Diadophis punctatus* samples and nine outgroup taxa. Posterior probabilities based on 7500 post burn-in trees are shown above the branches; nonparametric bootstrap proportions based on 1000 pseudo-replicates are listed below. Designations of the major population lineages follow the text. Symbols correspond to the geographic distribution of each lineage in Fig. 19. The dashed rectangle on the simplified tree depicts the position of the lineages in relation to entire phylogeny.


Hypsaglina torquata LJV 10635
Hypsaglena torquata LJV 10672
Rhadinaea flavilata
Heterodon platyrhinus YPM 13421
Heterodon simus FTB 1169
Contia tenuis
Carphophis amoenus YPM 13611
Farancia abacura YPM 13660
Farancia abacuara FMF 013
Washington LA LSUMZ 18764
St Martin LA LSUMZ 19599
Wilkinson MS LSUMZ 1972
St Martin LA LSUMZ 19598
Washington LA LSUMZ 18072
St Tammany LA MCB 406
St Tammany LA LSUMZ 18946
Washington LA LSUMZ 18761
Washington LA LSUMZ 18763
St Tammany LA LSUMZ 18947
Wilkinson MS LSUMZ 1973
Livingston LA LSUMZ 2833
East Baton Rouge LA LSUMZ 16009
Orleans LA LSUMZ 3303
East Baton Rouge LA LSUMZ 15872
West Feliciana LA LSUMZ 16149
Livingston LA LSUMZ 19619
Livingston LA LSUMZ 19618
East Baton Rouge LA LSUMZ 19666
East Baton Rouge LA LSUMZ 1809
St. Tammany LA LSUMZ 16022
St. Tammany LA LSUMZ 2919
Lee AL FTB 861
Clay AL LSUMZ 2223
Cleburne AL LSUMZ 2657
Cleburne AL FTB 925
Gulf FL MHP 8150
Washington FL CAS 203090
Gulf FL CAS 218698
Liberty FL CAS 203088
Liberty FL CAS 203089
Leon FL CSUN 2256
Jasper SC FTB 851
Aiken SC FMF 048
Aiken SC FMF 049
Aiken SC FMF 050
Aiken SC FMF 051
Aiken SC FMF 052
Baker GA FTB 1099
Bryan GA FTB 846
Baker Ga FTB 1100
Pasco FL CAS 233356
Pasco FL CAS 233366
Pasco FL CAS 233365
Citrus FL CAS 203091
Levy FL PGF 472
Pasco FL CAS 233367
Pasco FL CAS 233371
Pasco FL CAS 233359
Hernando FL CAS 198640
Hernando FL CAS 198639
Pasco FL CAS 233368
Pasco FL CAS 233369
Pinellas FL CAS 169419
Pinellas FL CAS 169414
Pinellas FL CAS 169413
Citrus FL CAS 214305
Broward FL CAS 208649
Dade FL CAS 172087
Hillborough FL YPM 13781
Sumter FL CAS 208620
Broward FL CAS 208648
Okeechobee FL CAS 204800
Citrus FL CAS 200964
Citrus FL LSUMZ 15873
Southeastern Louisiana Lineage
Piedmont Lineage
Peninsular Florida Lineage
Continental Lineages
Outgroups
Southeastern Louisiana Lineage
Piedmont Lineage
Peninsular Florida Lineage
Mid-Atlantic Lineage
Northeast Lineage
Cumberland Lineage
Mississippi River Valley Lineage
Great Plains Lineage
Western Louisiana Lineage
Great Basin Lineage
North Texas Lineage
Southern California Lineage
Eastern California Lineage
Coastal California Lineage
A
B
C
D
0.01


Gulf Coast Lineages
Western Lineages
Union SC FTB 933
Surry VA FTB 574
Aiken SC FMF 047
Aiken SC FMF 046
Aiken SC FMF 045
Aiken SC FMF 044
Aiken SC FMF 043
Aiken SC FMF 042
Aiken SC FMF 040
Aiken SC FMF 041
Deleware NY FTB 633
Worcester MD FTB 768
Worcester MD FTB 769
Worcester MD FTB 771
Clinton PA CAS 210415
Westmorland PA FMF 036
Hardy WV FTB 712
Hardy/Grant WV FTB 753
Randolph WV FTB 752
Grundy TN LSUMZ 2225
Laurel KY YPM 13615
Effinghmam IL FMNH 1657
Randolph WV FTB 726
Erie PA CNAH 003
Butler PA CAS 173178
Noble OH FMF 030
Vinton OH FMF 031
Charlevoix MI JR 500 01
Charlevoix MI JR 500 06
Charlevoix MI JR 500 05
Charlevoix MI JR 500 03
Amelia VA FTB 1225
Unicoi TN YPM 13617
Unicoi TN YPM 13618
Morgan KY FMF 034
Grayson VA FMNH 259791
Pocahontas WV FMF 004
Harlan KY CAS 231685
Harlan KY CAS 231683
Harlan KY CAS 231681
Harlan KY CAS 231678
Harlan KY CAS 231677
Harlan KY CAS 231684
Harlan KY CAS 231686
Harlan KY CAS 231679
Harlan KY CAS 231682
Ottawa OH FMF 035
Hardy WV FTB 709
Alleghany MD FTB 1056
Frederick MD FMF 033
Albany NY FTB 2053
Worchester MD FTB 770
Barnstable MA FTB 445
Shenandoah VA FTB 565
Cecil MD FTB 558
Cecil MD FTB 557
Deleware NY FTB 630
Deleware NY FTB 631
Middlesex CT YPM 12685
Cecil MD FTB 559
Ulster NY FMF 022
Warren NY FMF 028
Leverett MA CAS 210371
Fairfield CT YPM 14676
Ulster NY FTB 634
Deleware NY FTB 629
Macon NC MVZ 137546
Macon NC MVZ 150183
Graham NC FMF 037
DeKalb AL LSUMZ 2187
Macon NC LSUMZ 137691
Macon NC LSUMZ 137692
Macon NC LSUMZ 137690
Lafayette MS FTB 900
Catahoula LA LSUMZ 3282
Columbia AR KJI 1094
Tuscaloosa AL ALHC 14599
Bibb AL FTB 888
Bibb AL ALHC 14666
Tuscaloosa AL FMF 003
Pulaski IL INHS 1502
Polk DBS 714
Scott AR DBS 686
Polk AR DBS 672
Leflore OK DBS 429
Polk AR DBS 522
Polk AR DBS 521
Polk AR DBS DBS 715
LeFlore OK DBS 523
LeFlore OK DBS 361
Polk DBS 676
Polk AR DBS 519
Polk AR DBS 520
Sebastian AR DBS 685
Sebastian AR DBS 689
Polk AR DBS 706
Perry AR FMF 052
St Louis MO MVZ 233351
St Louis MO CAS 215252
Cass NE MVZ 233490
Geary KS CAS 174297
Geary KS CAS 174296
Ellis KS MHP 9667
Washington KS 8865
Waseca MN FTB 554
Osage KS MHP 9686
Osborne KS MHP 8961
Doniphan KS MHP 8316
Chautaqua KS MHP 8242
Woodson KS MHP 7628
Dickinson KS MHP 7943
Ness KS MHP 8360
Republic KS MHP 8893
Linn MO CNAH 006
Johnson KS MHP 8658
Webster IA FMNH 257459
Logan KS MHP 8507
Lincoln KS MHP 9761
A
B
C
Outgroups
Southeastern Louisiana Lineage
Piedmont Lineage
Peninsular Florida Lineage
Mid-Atlantic Lineage
Northeast Lineage
Cumberland Lineage
Mississippi River Valley Lineage
Great Plains Lineage
Western Louisiana Lineage
Great Basin Lineage
North Texas Lineage
Southern California Lineage
Eastern California Lineage
Coastal California Lineage
A
B
C
D
0.01
Mid-Atlantic Lineage
Northeast Lineage
Cumberland Mts Lineage
Mississippi River Valley Lineage
Great Plains Lineage

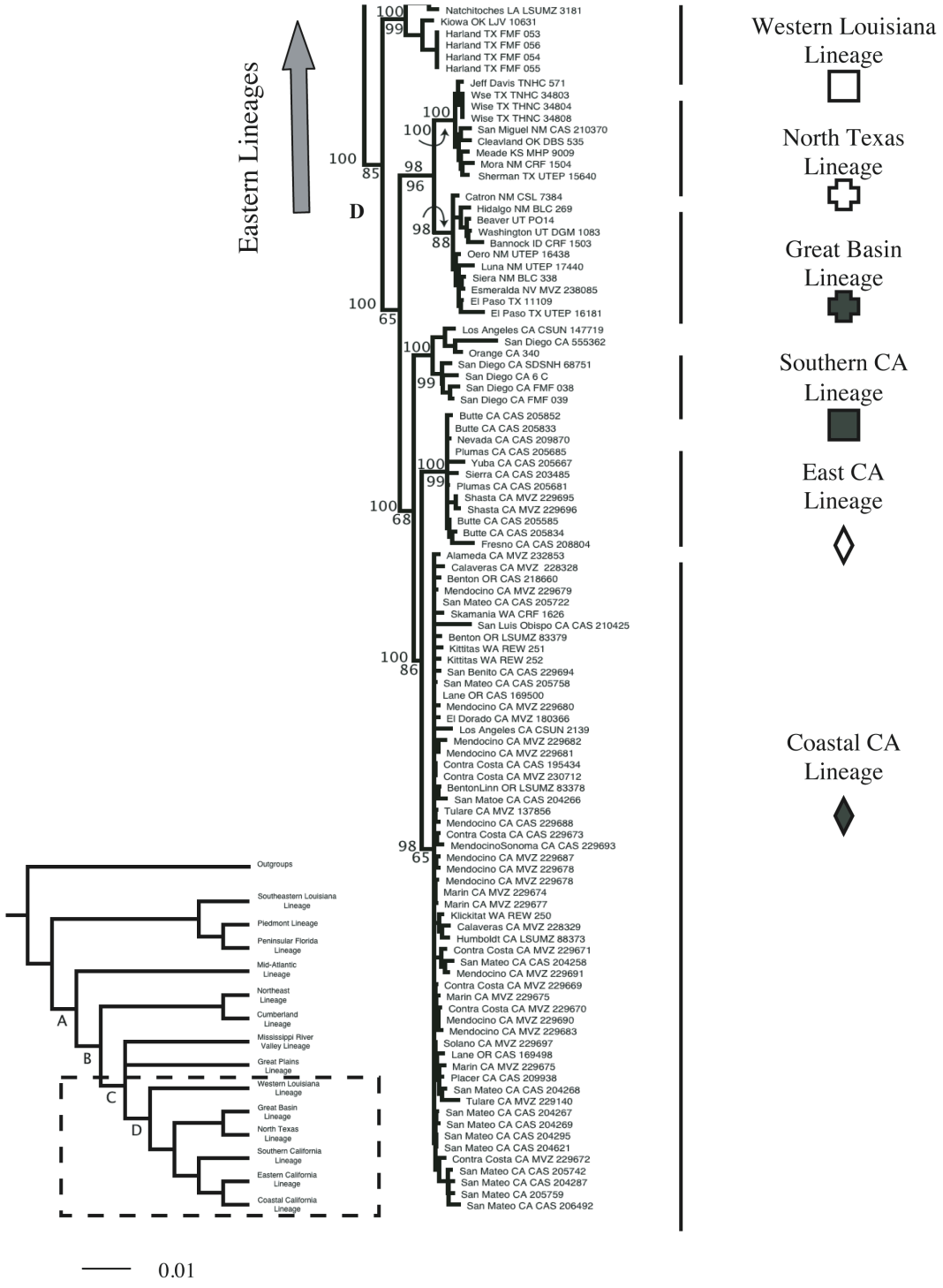
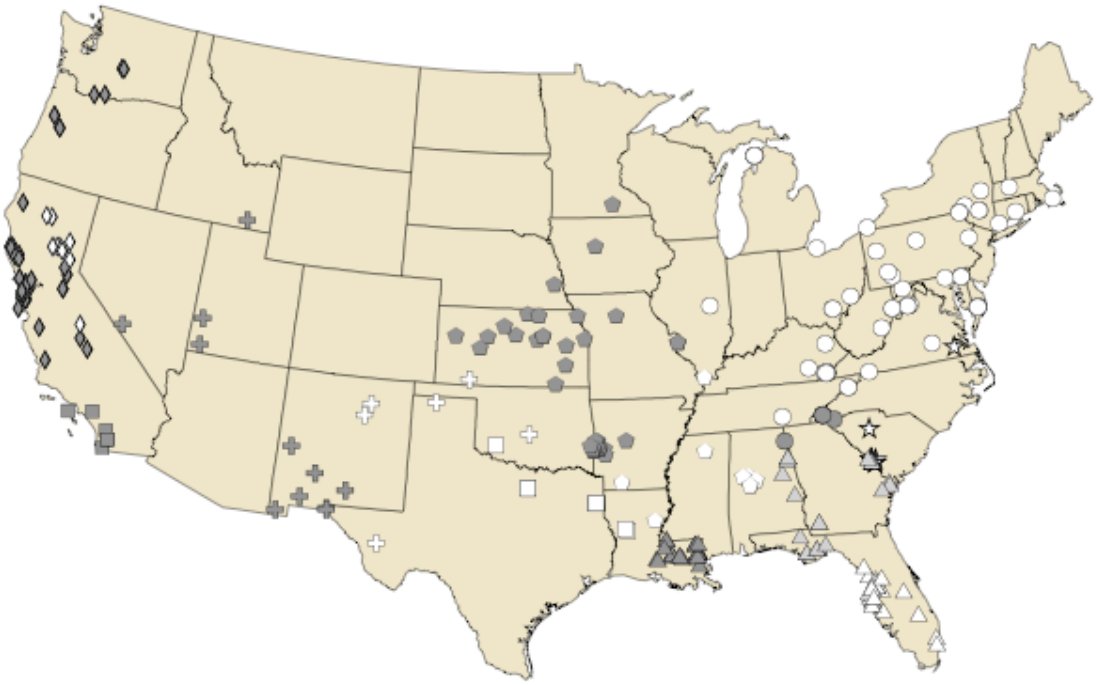

Eastern Lineages
D
Natchitoches LA LSUMZ 3181
Kiowa OK LJV 10631
Harland TX FMF 053
Harland TX FMF 056
Harland TX FMF 054
Harland TX FMF 055
Jeff Davis TNHC 571
Wse TX TNHC 34803
Wise TX THNC 34804
Wise TX THNC 34808
San Miguel NM CAS 210370
Cleavland OK DBS 535
Meade KS MHP 9009
Mora NM CRF 1504
Sherman TX UTEP 15640
Catron NM CSL 7384
Hidalgo NM BLC 269
Beaver UT PO14
Washington UT DGM 1083
Bannock ID CRF 1503
Oero NM UTEP 16438
Luna NM UTEP 17440
Siera NM BLC 338
Esmeralda NV MVZ 238085
El Paso TX 11109
El Paso TX UTEP 16181
Los Angeles CA CSUN 147719
San Diego CA 555362
Orange CA 340
San Diego CA SDSNH 68751
San Diego CA 6 C
San Diego CA FMF 038
San Diego CA FMF 039
Butte CA CAS 205852
Butte CA CAS 205833
Nevada CA CAS 209870
Plumas CA CAS 205685
Yuba CA CAS 205667
Sierra CA CAS 203485
Plumas CA CAS 205681
Shasta CA MVZ 229695
Shasta CA MVZ 229696
Butte CA CAS 205585
Butte CA CAS 205834
Fresno CA CAS 208804
Alameda CA MVZ 232853
Calaveras CA MVZ 228328
Benton OR CAS 218660
Mendocino CA MVZ 229679
San Mateo CA CAS 205722
Skamania WA CRF 1626
San Luis Obispo CA CAS 210425
Benton OR LSUMZ 83379
Kittitas WA REW 251
Kittitas WA REW 252
San Benito CA CAS 229694
San Mateo CA CAS 205758
Lane OR CAS 169500
Mendocino CA MVZ 229680
El Dorado CA MVZ 180366
Los Angeles CA CSUN 2139
Mendocino CA MVZ 229682
Mendocino CA MVZ 229681
Contra Costa CA CAS 195434
Contra Costa CA MVZ 230712
BentonLinn OR LSUMZ 83378
San Matoe CA CAS 204266
Tulare CA MVZ 137856
Mendocino CA CAS 229688
Contra Costa CA CAS 229673
MendocinoSonoma CA CAS 229693
Mendocino CA MVZ 229687
Mendocino CA MVZ 229678
Mendocino CA MVZ 229678
Marin CA MVZ 229674
Marin CA MVZ 229677
Klickitat WA REW 250
Calaveras CA MVZ 228329
Humboldt CA LSUMZ 88373
Contra Costa CA MVZ 229671
San Mateo CA CAS 204258
Mendocino CA MVZ 229691
Contra Costa CA MVZ 229669
Marin CA MVZ 229675
Contra Costa CA MVZ 229670
Mendocino CA MVZ 229690
Mendocino CA MVZ 229683
Solano CA MVZ 229697
Lane OR CAS 169498
Marin CA MVZ 229675
Placer CA CAS 209938
San Mateo CA CAS 204268
Tulare CA MVZ 229140
San Mateo CA CAS 204267
San Mateo CA CAS 204269
San Mateo CA CAS 204295
San Mateo CA CAS 204621
Contra Costa CA MVZ 229672
San Mateo CA CAS 205742
San Mateo CA CAS 204287
San Mateo CA 205759
San Mateo CA CAS 206492
Western Louisiana Lineage
North Texas Lineage
Great Basin Lineage
Southern CA Lineage
East CA Lineage
Coastal CA Lineage
Outgroups
Southeastern Louisiana Lineage
Piedmont Lineage
Peninsular Florida Lineage
Mid-Atlantic Lineage
Northeast Lineage
Cumberland Lineage
Mississippi River Valley Lineage
Great Plains Lineage
Western Louisiana Lineage
Great Basin Lineage
North Texas Lineage
Southern California Lineage
Eastern California Lineage
Coastal California Lineage
A
B
C
D
0.01

FIGURE 19.

Map showing the distribution of each *D. punctatus* population lineage diagnosed by mtDNA variation. Numbers above branches on the simplified tree are the mean dates of origin for each of the major lineages and haplogroups. Numbers below branches refer to Table 1.



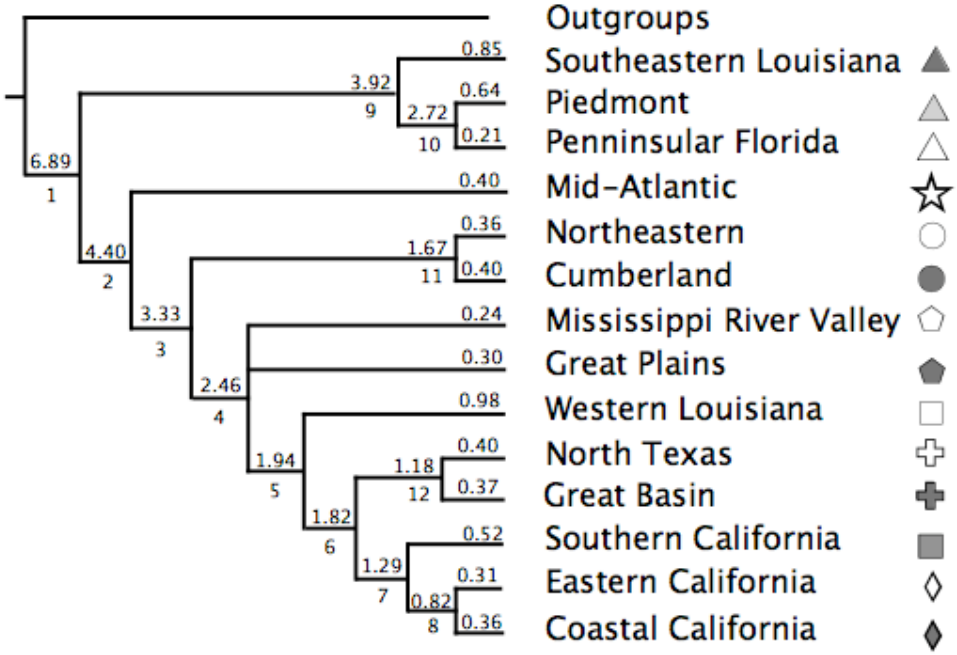

0.85
3.92
0.64
9
2.72
0.21
10
6.89
1
0.40
0.36
4.40
1.67
2
11
0.40
3.33
0.24
3
0.30
2.46
4
0.98
1.94
0.40
1.18
5
12
0.37
1.82
0.52
6
1.29
0.31
7
0.82
8
0.36
Outgroups
Southeastern Louisiana
Piedmont
Penninsular Florida
Mid-Atlantic
Northeastern
Cumberland
Mississippi River Valley
Great Plains
Western Louisiana
North Texas
Great Basin
Southern California
Eastern California
Coastal California

Table 1. The mean date of origin for each lineage of *Diadophis punctatus* and the time to the most recent common ancestor (tmrca) of each haplogroup. Values in parentheses represent standard deviation (SD) and the 95% confidence interval (CI) estimated using the uncorrelated lognormal Bayesian relaxed molecular clock in BEAST v. 1.4.4. Numbers for nodes follow Figure 3.

Node	Origin (MYA)	MtDNA Lineage	tmrca of Haplogroups (MYA)
1	6.689 (SD=0.0207; 95% CI=3.32-10.63)	SE LA	0.845 (SD=0.0074; 95% CI=0.237-1.63)
2	4.396 (SD=0.0213; 95% CI=1.84-7.388)	Piedmont	0.638 (SD=0.0056; 95% CI=1.59-1.291)
3	3.328 (SD=0.0195; 95% CI=1.353-5.638)	Penn. FL	0.211 (SD=0.00189; 95% CI=0.0354-0.455)
4	2.461 (SD=0.0155; 95% CI=0.998-4.172)	Mid-Atlantic	0.402 (SD=0.00398; 95% CI=0.0075-0.830)
5	1.943 (SD=0.0130; 95% CI=0.769-3.297)	Northeast	0.364 (SD=0.00349; 95% CI=0.081-0.768)
6	1.829 (SD=0.0121; 95% CI=0.738-3.315)	Cumberland	0.399 (SD=0.00366; 95% CI=0.0835-0.834)
7	1.288 (SD=0.0148; 95% CI=0.562-2.014)	MRV	0.238 (SD=0.00220; 95% CI=0.0385-0.515)
8	0.819 (SD=0.0056; 95% CI=0.312-1.483)	Great Plains	0.304 (SD=0.00279; 95% CI=0.0548-0.657
9	3.920 (SD=0.0225; 95% CI=1.477-6.683)	Western LA	0.984 (SD=0.00817; 95% CI=0.32-1.855)
10	2.721 (SD=0.0198; 95% CI=0.953-4.88)	Great Basin	0.377 (SD=0.0030; 95% CI=0.0877-0.761)
11	1.674 (SD=0.0144; 95% CI=0.511-3.178)	North Texas	0.396 (SD=0.0033; 95% CI=0.0799-0.801)
12	1.178 (SD=0.00813; 95% CI=0.411-2.071)	South CA	0.523 (SD=0.0032; 95% CI=0.122-0.940)
		Eastern CA	0.310 (SD=0.0023; 95% CI=0.0758-0.611)
		Coastal CA	0.361 (SD=0.0028; 95% CI=0.0841-0.706)

The Western clade is distributed from western Alabama to the coast of California and is divided further into two sub-clades. The Central sub-clade consists of two lineages, a Mississippi River Valley lineage ranging from southern Illinois south to central Arkansas and western Alabama and a Great Plains lineage extending throughout the prairies and grasslands of the central US from southern Minnesota to central Arkansas. Within the second sub-clade, a Western Louisiana lineage is distributed from west central Louisiana through eastern Texas and into southwestern Oklahoma. Further to the west, the North Texas lineage extends from east of the Guadalupe Mountains in New Mexico through north central Texas and into southern Kansas. Bound by the Guadalupe Mountains in the east and the Sierra Nevada Mountains in the West, the Great Basin lineage ranges from southern New Mexico north to southern Idaho. West of the Sierra Nevada Mountains three lineages were inferred ranging from southern California to central Washington. The Southern California lineage is composed entirely of populations south of the Transverse Mountains. North of the Transverse Mountains, the Coastal California lineage extends along the west coast of California into central Oregon. Haplotypes from this lineage were also present east of the Central Valley as far north as San Francisco Bay where they overlap with the southern range of the Eastern California lineage.

Divergence Dating

Although the origin of *Diadophis* remains unknown due to the lack of sampling in Mexico, the initial divergence within the US occurred between the Gulf Coast clade and the continental clades during the late-Miocene (~6.7 mya Fig. 19; Table 1). The remaining major divisions (B, C, D; Fig. 18) and the divergences within the Gulf Coast

clade and between the southwestern and California lineages occurred during the Pliocene. Each of the extant lineages shared a most recent common ancestor during the Pleistocene ranging from 0.238 to 0.985 mya, suggesting that the Laurentide ice sheet was not directly related to the formation of these lineages.

Population genetic analyses

Diversity indices and population structure.

Nucleotide diversity (π) across all *D. punctatus* haplotypes was 0.065 and the mean number of pairwise differences (κ) was 95.202. Fu's F_s statistic was significantly negative for the entire species (-33.467) suggesting a recent range-wide expansion; however, Tajima's *D* was not significant (-0.32475). The discrepancy between *D* and *F* is likely due to the decreased statistical power of *D* in detecting significant changes in population size (Ramos-Onsins and Rozas 2002). The molecular analysis of variance (AMOVA) suggests that genetic variation in the *D. punctatus* complex is apportioned non-randomly ($p < 0.01$) with 86% of the variance between lineages, 4% between populations within lineages, and only 10% within populations (Table 2).

Historical demography and dating population growth.

Tajima's *D* and Fu's F_s statistic were significant ($p=0.05$) for the Northeast, Great Plains, Coastal California and Eastern California lineages. The Eastern Louisiana lineage

Table 2. Results of the hierarchical analysis of molecular variance (AMOVA) testing the geographic structure for the 14 inferred lineages.

Source of Variation	d.f.	Sum of Squares	Percentage of Variation
Among Groups	13	14130.29	85.72
Among Populations within Groups	29	581.28	4.20
Wihin Populations	248	16285.32	10.07

was significant for Fu's F_s and approached significance for Tajima's D (p=0.057). The remaining lineages, though not significant, were consistently negative for Tajima's D (Table 3) indicating an excess of low frequency haplotypes and putatively gradual population growth. The mismatch distributions of pairwise nucleotide differences were multimodal for most lineages with the exception of the Great Plains, Coastal California, Eastern California and Northeast lineages.

The effective sample size (ESS) for each of the Bayesian skyline analyses was greater than 200, suggesting that the 10 million generations were sufficient to determine the demographic history for each lineage (Fig. 20). None of the plots show any evidence of genetic bottlenecks, recent sub-divisions or historical population contractions. Furthermore, the Mid-Atlantic and West Louisiana lineages had slopes that were not significantly different than zero, suggesting that these populations have maintained a relatively stable size.

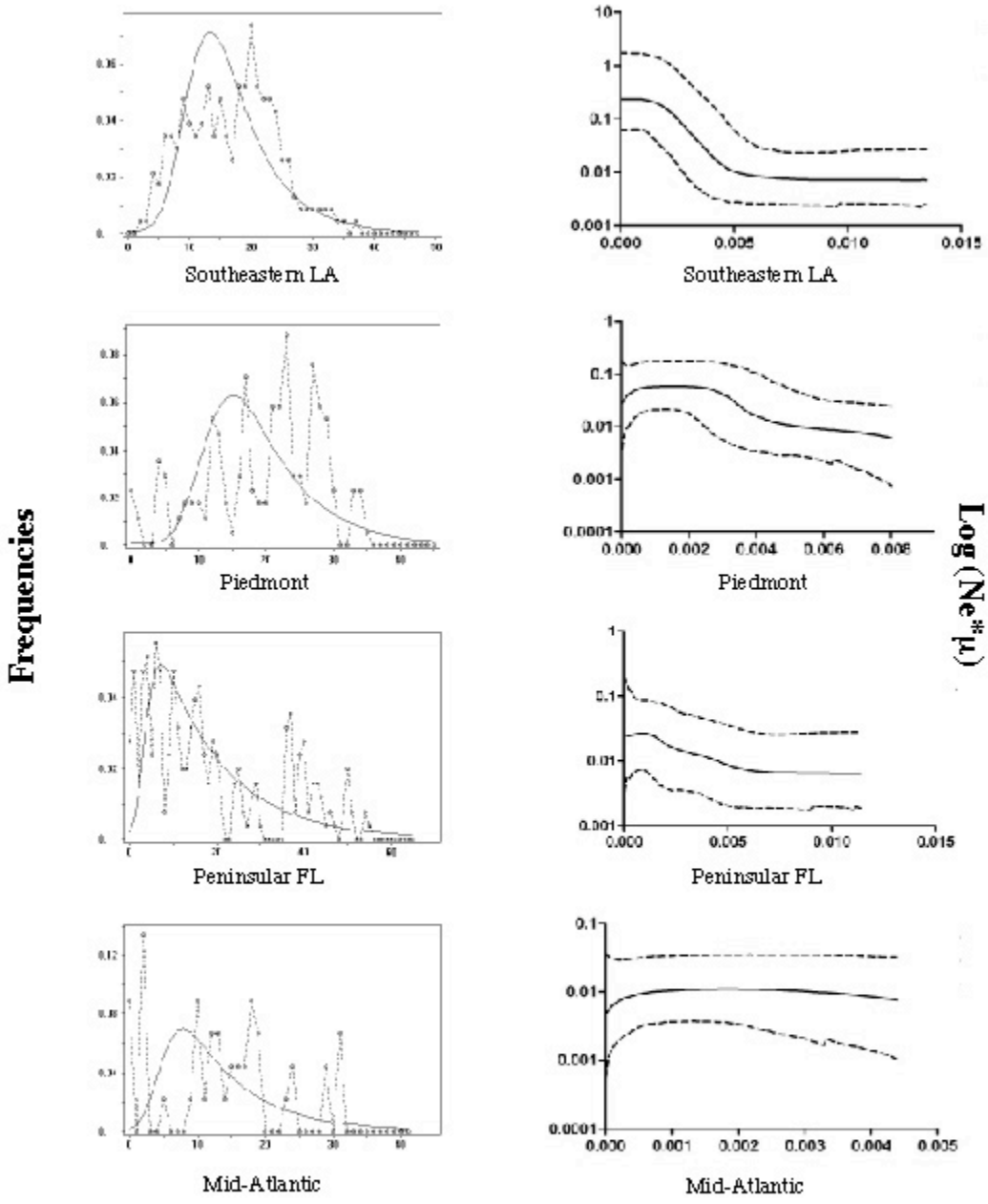
Unimodal distributions with low raggedness values, significantly negative D and F_s values and the Bayesian skyline plots depicting growth provide strong support that the Coastal California, Eastern California, Great Plains and Northeast lineages have undergone sudden expansion. However, the lack of evidence for population declines and significantly positive slopes from skyline plot suggests that the other lineages have also undergone population growth, albeit very slowly. Therefore, for each lineage we estimated the years since expansion based on the estimate of τ from the mismatch distribution. Results suggest that expansion ages of the lineages range from 5 kya

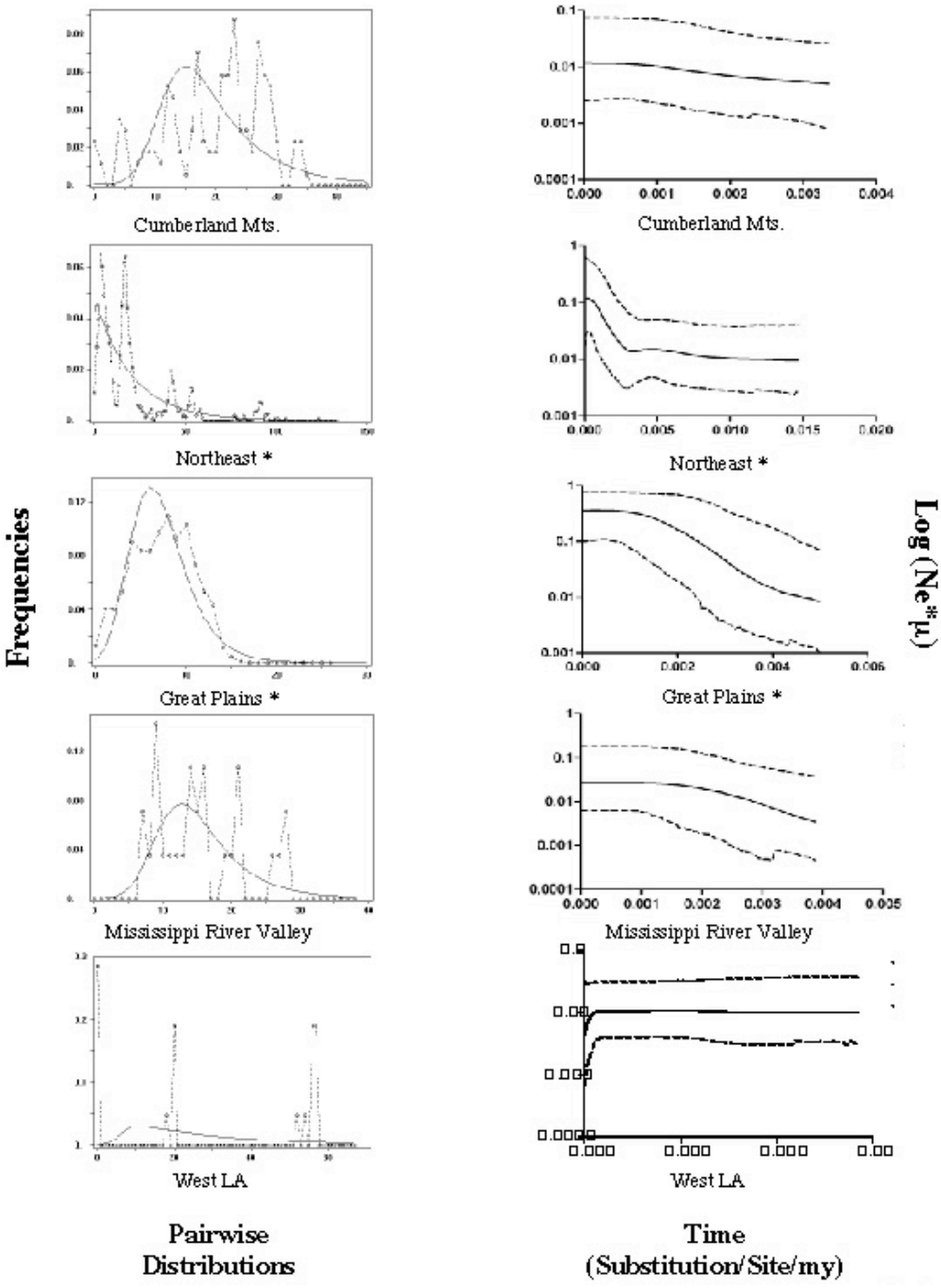
Table 3. Hapotype diversity (Hd), nucleotide diversity (π), average number of pairwise differences (k) and results of Tajima's D and Fu's F_s statistic for each lineage of *Diadophis punctatus* calculated for all sites of the concatenated data set. Tests that were significantly different at a p=0.05 are labeled with an *. The raggedness statistic, r_g, and the Ramos-Orsins and Rozas R_2 statistic are reported for the mismatch distributions. Substitution rates/million years were obtained from r8's (Sanderson 2003) using a calibration point of 7.5 my. The average tau (τ) and expansion time are listed with the upper and lower bounds in parentheses.

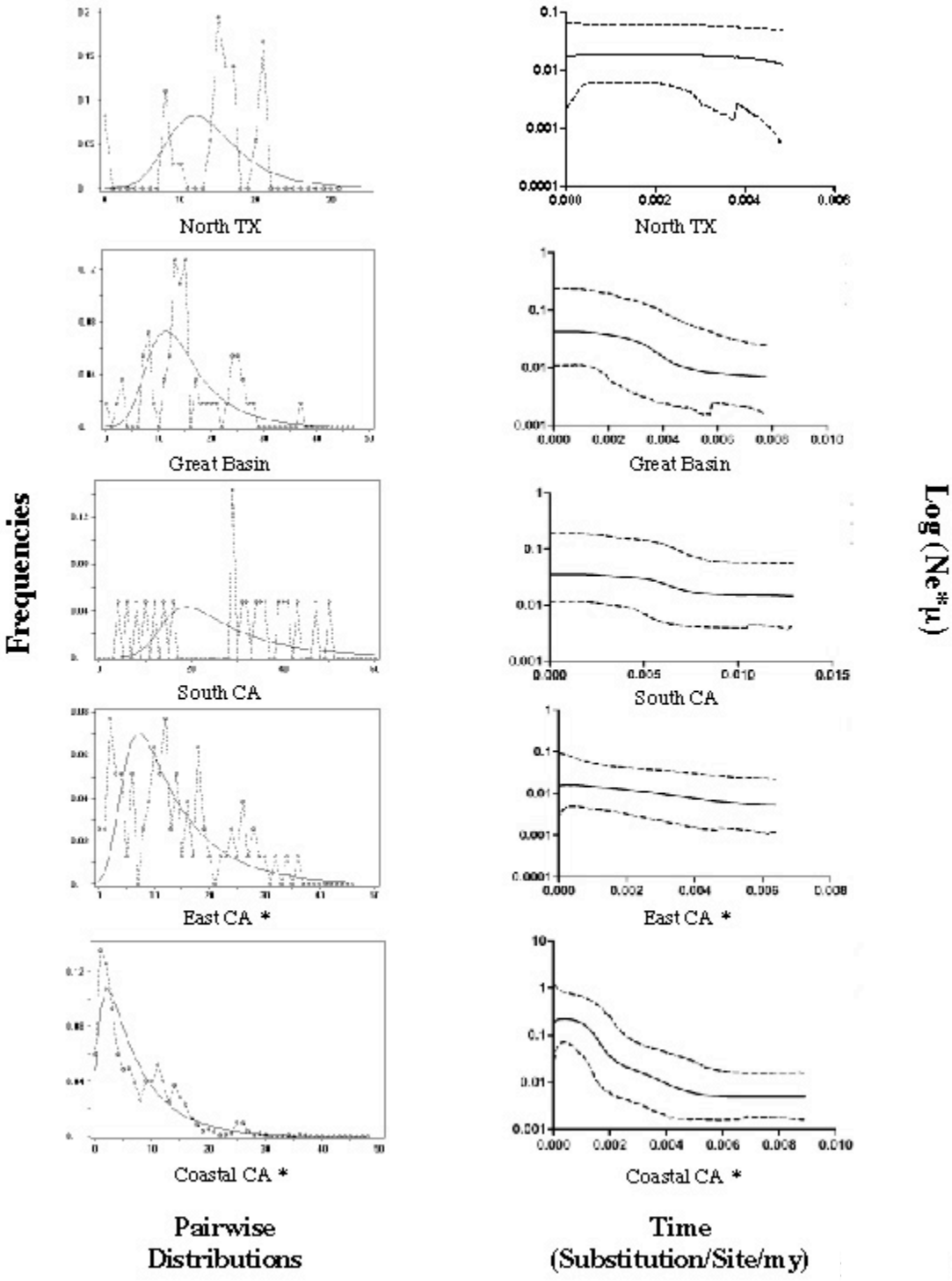
Lineage	Tajima's D	Fu's F_s	Hd	π	K	r	R_2	τ	Rate of Sub/my	Expansion Time
SE LA	-1.405	-10.05	1.0	0.0099	16.81	0.0042	0.074	14.23 (4.60-39.44)	6.58E-08	10,137 (3,274-28,108)
Piedmont	-1.118	-1.800	0.977	0.0126	19.43	0.0219	0.085	23.33 (8.36-37.76)	6.51E-08	15,365 (6,021-27,201)
Penn. FL	-1.447	-2.736	0.972	0.0109	18.68	0.0124	0.072	8.32 (0-31.319)	6.46E-08	6,043 (0-22,753)
Mid-Atlantic	-0.292	-1.612	0.911	0.0077	13.622	0.081	0.151	16.61 (2.51-38.61)	6.32E-08	12,326 (1,861-28,657)
Northeast	-2.073*	-15.76*	0.989	0.0133	19.966	0.0036	0.054	22.04 (8.07-42.47)	6.41E-08	16,145 (5,911-31,104)
Cumberland	-0.873	-1.280	1.0	0.0067	11.762	0.118	0.113	12.36 (7.18-18.48)	6.48E-08	8,943 (5,194-13,374)
MRV	-0.768	-1.333	1.0	0.0098	15.536	0.0740	0.103	10.8 (4.50-20.41)	6.95E-08	7,293 (3,032-13,783)
Great Plains	-1.690*	-21.996*	0.986	0.0044	7.287	0.0054	0.056	9.79 (4.41-17.17)	7.27E-08	6,318 (2,846-11,080)
West LA	0.119	6.968	0.714	0.0181	31.238	0.2404	0.174	25.01 (4.33-64.40)	7.86E-08	14,927 (2,586-38,445)
North TX	-0.879	1.027	0.917	0.0084	14.306	0.1150	0.104	16.98 (11.80-22.08)	7.87E-08	10,114 (7,026-13,154)
Great Basin	-1.111	-1.20	0.982	0.0090	15.164	0.0380	0.109	14.20 (6.48-25.08)	7.83E-08	8,500 (3,879-15,020)
South CA	-0.378	-0.016	1.0	0.0170	27.857	0.0988	0.150	35.27 (21.11-51.73)	7.74E-08	15,320 (6,741-25,299)
East CA	-1.754*	-1.249*	0.974	0.0078	13.205	0.0250	0.095	15.69 (1.22-38.15)	7.66E-08	9,609 (748-23,368)
Coastal CA	-2.410*	-25.75*	0.940	0.0044	7.112	0.0095	0.039	11.86 (2.40-26.31)	7.59E-08	7,326 (1,482-16,250)

FIGURE 20.

Mismatch distributions (left) and Bayesian skyline plots (right) depicting the demographic history for each lineage of *D. punctatus*. For the mismatch distributions, open circles represent the observed distribution of pairwise differences and the solid line represents the expected distribution assuming population expansion. For the skyline plots, data were analyzed for 10 million generations in the program BEAST v1.4 (Drummond and Rambaut 2003; Drummond et al. 2005) using the appropriate model of evolution determined from ModelTest 3.7 (Posada and Crandall 1998). The solid line represents the median value for the log of the population size (log N_e) and the dashed lines represent the upper and lower 95% credible intervals. Graphs labeled with an asterisk represent expanding populations.


Frequencies
Southeastern LA
Piedmont
Peninsular FL
Mid-Atlantic
Pairwise Distributions
Log (Ne*μ)
Southeastern LA
Piedmont
Peninsular FL
Mid-Atlantic
Time (Expected/Substitution/ Site/my)


Frequencies
Cumberland Mts.
Northeast *
Great Plains *
Mississippi River Valley
West LA
Pairwise Distributions
Log (Ne*μ)
Cumberland Mts.
Northeast *
Great Plains *
Mississippi River Valley
West LA
Time (Substitution/Site/my)


Frequencies
North TX
Great Basin
South CA
East CA *
Coastal CA *
Pairwise Distributions
North TX
Great Basin
South CA
East CA *
Coastal CA *
Time (Substitution/Site/my)
Log (Ne*μ)

(Peninsular FL) to 16 kya (Northeast US) (Table 3), all of which follow the recession of the Laurentide ice sheets ~18 kya.

DISCUSSION

Phylogeographic patterns across North America

Our analyses revealed four major clades composed of 14 geographical lineages within *D. punctatus* (Fig. 18). Within the US, the pattern of genetics breaks originate in the southeastern US and spread along the eastern coastal plain into the northeast, then east across the central US and into the west throughout California. The phylogeographic patterns inferred from west of the Mississippi River are broadly concordant with those of previous studies of North American taxa (Calsbeek et al. 2003; Pook et al. 2000; Castoe *et al*. 2007). However, the lineage diversity and phylogeographic patterns east of the Mississippi River are not concordant with most other vertebrates studied (Hayes and Harrison 1992; Burbrink et al. 2000; Burbrink 2002; Church et al. 2003).

The basal split within *D. punctatus* took placed during the late Miocene and subtends the Gulf Coast clade from the three continental clades, which originated during the Pliocene. Within the Gulf Coast clade, the Southeastern Louisiana lineage is limited to lowland areas of southern Louisiana and southwestern Mississippi, confined by the Red River to the north. During the Pleistocene, the mouth of the Mississippi River was shifted to the west towards the present day Atchafalaya River (Mayden 1988). Glacial melt waters carried increased sediment loads causing an eastward shift of the river basin (Brown and Kennett 1998). The occurrence of Southeastern Louisiana haplotypes west of

the current Mississippi River could be a result of these recent shifts. The Piedmont lineage is confined to the southeastern coastal plain where the Southeastern Fall Line separates the eastern coastal plain from the Appalachian Mountains. Throughout the southeastern United States, the east-west genetic discontinuities observed in freshwater and terrestrial species have largely been explained by two topographic features, the Tombigbee River in eastern Alabama and the Apalachicola River that transects the panhandle of Florida (Avise 2000; 2004; Burbrink et al. 2000; Near et al. 2001; Hoffman and Blouin 2004; Soltis et al. 2006; Pauly et al. 2007). This lineage represents one of the few squamate distributions that does not have an east-west genetic break associated with the Apalachicola/Tombigbee Rivers, raising questions as to why and how this low-vagility, coastal-plain endemic has been able to disperse across barriers that have been important in shaping phylogeographic patterns of this region. A second factor thought to have influenced the genetic patterns of Florida taxa has been fluctuations in sea level during the late Miocene/early Pliocene. As Florida became inundated, the north-south central ridges persisted as a series of offshore islands separating populations from the mainland (Webb 1990; Clark et al. 1999). Although further sampling is needed to determine the northern extent of the Peninsular Florida lineage, several taxa are endemic to this region of Florida including squamates (Conant and Collins 1991), birds (Peterson 1980), mammals (Burt and Grossenheider 1976), turtles (Walker and Avise 1998), butterflies (Kimball 1965) and plants (Elias 1987).

The second major division (B) corresponds to a southern Appalachian Mountain discontinuity, separating the Mid-Atlantic clade from the Appalachian and Western clades. This lineage is restricted to mesic forests and floodplains of the Atlantic Coastal

Plain from southeastern Virginia to western South Carolina. Similar east-west genetic breaks have been found in the unglaciated regions of the Appalachian Mountains for salamanders (Donovan et al. 2000; Zamudio and Savage 2003; Church et al. 2003), turtles (Walker and Avise 1998), Atlantic white cedar (Mylecraine et al. 2004) and the groundnut (Joly and Bruneau 2004). In western South Carolina, a secondary contact zone between the Mid-Atlantic and Piedmont lineages is present along the Fall Line separating the upland Piedmont of the Appalachians from the lowland Coastal Plain.

North of the Coastal Plain, the Northeast lineage of the Appalachian clade spans the Appalachian Mountains and is confined to the east by the Mississippi River. A genetic discontinuity associated with the Mississippi River has been well documented both for plants and animals (Near et al. 2001; Al-Rabab' ah and Williams 2002). However, several animal species show additional sub-structuring associated with the Apalachicola and Tombigbee Rivers (Burbrink et al. 2000; Brant and Orti 2003; Soltis et al. 2006). These rivers appear to have had little effect on the genetic structure of the Northeast lineage, which occupies mostly previously glaciated regions. Similar patterns observed in the leopard frog (*Rana pipiens*) are thought to be due to expansion into previously glaciated areas from northern refugia (Hoffman and Blouin 2004). At the southern extent of the Appalachian Mountains, the Cumberland lineage is confined to the montane environments of Western North Carolina and the Cumberland Plateau of northeastern Alabama. Elevational changes across the range of a species can influence dispersal patterns, causing genetic divergence between populations that have become adapted to different elevations (Slatkin 1987; Manel et al. 2003; Palo et al. 2003). The preference for high-elevation habitat may be a factor in limiting the dispersal of these

snakes into the lowland areas currently occupied by other lineages of *D. punctatus*. Although uncommon in North American squamates, genetic differentiation has been shown between low-altitude and high-altitude populations in the Pacific jumping mouse (Vignieri 2005), the tiger salamander (Spear et al. 2005) and the long-toed salamander (Giordano et al. 2007).

Within the Western clade, the Central sub-clade consists of the Mississippi River Valley (MRV) and the Great Plains lineages. Although the relationship between these two groups remains unresolved, each lineage was well supported (100 PP and 95 BS) in both analyses. The distribution of the MRV lineage is restricted to low elevation (< 200 meters) regions associated with the Mississippi Alluvial Plain (MAP). This flood plain extends through seven states (southern Illinois, southeastern Missouri, western Kentucky, eastern Arkansas, western Mississippi and Louisiana) and formed as a result of an influx of water into the Mississippi River due to the melting of the North American ice sheets (Perlmutter 1985). Although outside what is normally considered the MAP, western Alabama consists of lowland areas (< 200 m) associated with the western extent of the Gulf Coast coastal plain. The apparent association with flood plain and lowland forest habitat may be a factor inhibiting dispersal into the surrounding higher-elevation areas while expansion into southern Louisiana appears to be restricted by the Red River. For several species of plants and animals, the Mississippi River represents a major biogeographical barrier with distinct mtDNA haplotype groups occurring east and west of the river (Brown et al. 1996; Burbrink et al. 2000; Near et al. 2001; Berendzen et al. 2003; Brant and Orti 2003). Our study indicates that *D. punctatus* may be the only squamate that does not correspond to the east-west genetic break typical of other taxa.

The distribution spanning the Mississippi River below the Embayment of southern Illinois again raises questions as to how this low-vagility snake remains unimpeded by barriers that have shaped the phylogeographic patterns of other taxa within this region. North of the embayment, the influence of the Mississippi River becomes evident separating the Northeast and Great Plains lineages. West of the Mississippi River, the Great Plains lineage extends throughout the grasslands and prairies of the central US, from southern Minnesota to the Central Highlands of western Arkansas. These snakes occupy areas with elevations above 200 m, which may restrict dispersal into the lower-elevation eastern flood plain occupied by the MRV lineage.

Restricted in the east by the Mississippi River and the Red River to the north, the Western Louisiana lineage extends through the Piney Woods forests of western Louisiana, eastern Texas and southern Oklahoma. A preference for these high-density coniferous forests may be a factor limiting dispersal into the adjacent hardwood forests of the East Central Texas Plains. Throughout the North American Deserts, *D. punctatus* becomes less abundant occurring in several small disjunct populations (Fig. 17). Two lineages reciprocally monophyletic for mtDNA haplotypes were inferred within the southwest, corresponding to a Southern Rocky Mountain genetic discontinuity. East of the Guadalupe Mountains, the North Texas lineage occupies the semi-arid highland regions associated with the eastern Chihuahuan Desert and the Southern Plains of the southwestern and central US. Haplotypes from both the Western Louisiana and North Texas lineages occur in central Oklahoma, where the habitat shifts from the semi-arid steppe of the southwest to the grassland prairies of the central US. West of the Rocky Mountains, the Great Basin lineage is confined to the cooler, high elevations of the

Sonoran and Mojave Deserts extending north throughout the Great Basin. The Colorado Plateau may be a genetic barrier isolating the Great Basin and North Texas lineages. Ringneck snakes are largely absent from the high elevation xeric scrublands typical of this region and are limited to small disjunct populations across the Great Basin and Mojave Deserts (Fig. 19). Their limited dispersal abilities combined with the unfavorable habitat and smaller population sizes have likely contributed to maintaining a stable or non-expanding demographic signal. The east-west split inferred across the North American Deserts is broadly concordant with the vicariant phylogeographic patterns inferred for several other desert species, including fence lizards (Leaché and Reeder 2002), western rattlesnakes (Pook et al. 2000; Ashton and de Queiroz 2001), diamondback rattlesnakes (Castoe et al. 2007), horned lizards (Reeder and Montanucci 2001; Leaché and McGuire 2006) and scaled quails (Zink and Blackwell 1998). However, the distribution of the genetic break points is not shared across taxa, suggesting that the patterns represent different underlying causal factors. It would be of interest to expand upon the sampling within these studies and to evaluate the geographical structure and timing of molecular diversification across this region.

The phylogeographic patterns of *D. punctatus* in California and along the West Coast have been detailed elsewhere (Feldman and Spicer 2006). However, our increased mitochondrial sampling has delimited a third haplo-group restricted to the Sierra Nevada and Cascade Ranges. This Eastern California lineage may come into contact with the Coastal California lineage at several points along the central Sierra Nevada and again in the Cascade Range. Whether the presence of Coastal haplotypes in the interior ranges of California represents dispersal or simply the retention of ancestral haplotypes that have

otherwise gone extinct in eastern California is unknown. Dispersal events across the Central Valley or through the Transverse Ranges into the Sierra Nevada have been proposed for a number of species (Moritz et al. 1992; Tan and Wake 1995; Rodriguez-Robles et al. 1999; Feldman and Spicer 2002; Janzen et al. 2002; Spinks and Shaffer 2005). The apparent absence of a geographical barrier between the Coastal and Eastern California lineages provides an additional opportunity to test the genetic exclusivity of these minimally diverged mtDNA lineages.

Demographic History

During the peak of the last continental glaciation, approximately 18,000 years ago, the Laurentide Ice Sheet extended south to about 39° N in eastern North America (Delcourt and Delcourt 1987). Glaciers and associated climatic changes drove high latitude populations into more southern habitats (Hewitt 1999). The leading edge model of population expansion predicts that lineages at the glacial margins would have undergone rapid population expansion as previously unsuitable habitat became colonized. Such rapid or step-wise colonizations would be characterized by low levels of genetic diversity as each new founding population represented only a fraction of the ancestral population's genetic diversity (Nichols and Hewitt 1994; Hewitt 2000).

Eastern US.

For the six lineages inferred east of the Mississippi River, only the Northeast had significantly negative values for Tajima's D (-2.07) and Fu's F_s (-15.76), unimodal mismatch distributions and Bayesian skyline plots consistent with rapid population

expansion (Fig. 20). The average estimates of τ suggest that expansion began during late Pleistocene (~ 16 kya) coinciding with retreat of the ice sheets. Additionally, the distribution of haplotypes throughout shows extensive panmixia between northern and southern populations with little to no sub-structuring across great distances. These results, when combined with the high haplotype and low nucleotide diversity, are consistent with patterns of population expansion out of more southern refugia. Given the complex lineage diversity inferred throughout the southeast, populations of the Northeast lineage likely survived glacial cooling periods further north in the Appalachian Mountains or interior refugia near the edge of the glacial ice sheets. Several lines of evidence have emerged suggesting that re-colonization of glaciated landscapes in the eastern US may have occurred from refugia located further north than previously proposed (McLachlan et al. 2005).

In contrast to the Northeast lineage, demographic analyses for the three Gulf Coast lineages as well as the Mid-Atlantic, Cumberland and Mississippi River Valley lineages revealed multimodal mismatch distributions and non-significant D and F_s statistics (Fig. 20, Table 3). Bayesian skyline plots for each group also showed little to no growth over time, with the exception of the Southeastern Louisiana lineage (Fig. 20 part 1). Further sampling across southern Mississippi and western Alabama may help to clarify the demographic history of this lineage. Thus, the other eastern lineages do not appear to have undergone a significant population expansion or contraction but have remained relatively stable through time.

The maintenance of population stability across the complex ecosystems characterizing the southeastern US is likely due to a combination of extrinsic and

intrinsic barriers to dispersal. Even in the absence of geological barriers, habitat requirements alone can limit the ability of individuals to disperse across inhospitable landscapes or into novel communities (Crespi et al. 2003). Furthermore, the precedence of established lineages, competitive interactions between lineages or the presence of narrow hybrid zones may restrict gene flow and limit dispersal (Hewitt 1996). These factors should have had little effect on the expansion of temperate biota from long-term southern refugia into previously unsuitable unoccupied areas. As populations confined in northern refugia expanded following retreat of the glaciers, competitive effects between neighboring southern lineages may have restricted dispersal, maintaining the genetic structure of each lineage (Hewitt 1996). Inter-lineage interactions affecting population expansion have been proposed for the distribution of other Southeastern taxa with limited dispersal capabilities (Zamudio and Savage, 2003; Crespi et al. 2003; Kozak et al. 2006).

Central US.

Even though the Laurentide Ice Sheets did not extend as far south in the center of the continent as they did in the East, the effects on regional biota were similar (Brant and Orti 2003). Genetic variation in the Great Plains lineage conforms to predictions of population expansion models with unimodal mismatch distributions, significantly negative values of D (-1.690) and F_s (-21.99), and Bayesian skyline plot depicting rapid growth. Although the average time since expansion was relatively recent (< 7 kya), the high haplotype diversity and low nucleotide diversity are consistent with population expansion following colonization of previously glaciated regions. The relatively homogenous topography and climatic conditions of the Midwest region likely have

contributed to a lack of local differentiation among Great Plains ringnecks as well as other species (Stein et al. 2000). Tree topologies with shallow branches, little internal resolution (Fig. 18 part 2) and low sequence divergence further suggest recent population expansion from refugial areas. Throughout the Midwest, glacial refugia have been proposed in Oklahoma, northern Texas and the Interior Highlands, the latter of which includes the Ozark and Ouachita Uplands of Missouri and Arkansas (Wiley and Mayden 1985; Mayden 1988; Brant and Orti 2003; Zamudio and Savage 2003). The lack of Great Plains haplotypes within western Oklahoma and northern Texas suggests that the Interior Highlands acted as a southern refugium for ringnecks during times of glacial expansion (Fig. 19).

The historical demographic patterns inferred between northern and southern lineages of the central U.S. were similar to those in the eastern U. S. Neither the Western Louisiana nor the North Texas lineage showed signatures of population expansion in the demographic analyses, suggesting long-term population stability (Table 3 Fig. 20). These results are consistent with the prediction that northern lineages nearest the receding glacial edge underwent exponential population growth, while ecosystem stability or competitive interactions between southern lineages restricted geographic expansion (Hewitt 1996). Additional factors affecting expansion of the North Texas lineage may be the unsuitable habitat of the Rocky Mountains and Intermontane Plateaus to the north and the Guadalupe Mountains to the west.

Western US.

In the arid West, *Diadophis* appears restricted to patches of suitable mesic environments surrounded by less hospitable xeric habitats. Thus widespread movements or colonization events seem unlikely. Indeed, genetic variation in the Great Basin lineage this group conforms to predictions regarding long-term population stability (Table 3 Fig. 20). The specific habitat requirements of these snakes may be inhibiting population expansion into the surrounding areas. Increased sampling and the incorporation of microsatellite loci would be appropriate for testing the localized effects of altitude, geography and habitat shifts on the genetic diversity and connectivity between fragmented southwestern populations.

With respect to the California clade, Feldman and Spicer (2006) have recently provided a detailed explanation of the evolutionary and demographic history of California woodland reptiles. Our analyses of the combined data set inferred an additional northern California lineage confined to the Sierra Nevada Mountains (Fig 19). The separate demographic analyses of the two northern California lineages are consistent with the findings of Feldman and Spicer (2006), regarding Coastal and East California populations experiencing rapid population expansion during the Holocene approximately 7 (kya) and 10 (kya) respectively (Table 3 Fig. 20). This expansion would have been exacerbated by Holocene warming explaining a spread of suitable woodland habitat in the central and northern regions of California (Van Devender and Spaulding 1979; Smith et al. 2000)). This warming trend simultaneously decreased the extent of suitable habitat south of the Transverse Mountains. The demographic results for the Southern California lineage (Table 3 Fig. 20) lend further support to the conclusions of Feldman and Spicer

(2006) that the loss of suitable habitat throughout southern California had a stabilizing effect on the populations.

5. CONCLUSIONS

This study is the first comprehensive phylogeographic analysis of the trans-continental snake *Diadophis punctatus*. Separate and combined analyses of cytochrome *b* and ND 4 data sets inferred 14 well-supported mtDNA lineages occupying specific habitats separated by putative physical, biotic or ecological barriers. Previous studies of such broad ranging species have revealed considerable cryptic diversity and a more complex evolutionary history than postulated by previous workers, however the distribution and diversity of lineages in *D. punctatus* is unlike any other North American squamate. Whereas the patterns inferred throughout the western U. S. were broadly concordant with previously identified genetic barriers, several lineages in the eastern U. S. exhibited distinct patterns not shared by any other species. We attribute these patterns to the presence of Pleistocene glaciers and the associated climatic shifts. With the exception of the fragmented populations throughout the southwest, coalescent and non-coalescent measures of demographic history suggest post-glacial expansion into previously unsuitable habitats for each of the four northernmost lineages. However, the estimates of time since expansion suggest that factors leading to population growth differed between regions. In the Eastern and Central U. S., advancing Pleistocene glaciers drove northern populations into southern refugia promoting intra-specific lineage diversity. As glaciers receded, lineages in relatively northern refugia expanded into previously glaciated areas, while poor dispersal abilities and possibly increased inter-

lineage competition restricted southern lineages resulting in the current phylogeographic patterns (Fig. 21). The general warming trends that led to the recession of glaciers caused fundamental ecosystem-wide changes across North America. In accordance with these changes, demographic patterns suggest that the northern California lineages expanded as suitable habitat spread further north as temperatures increased during the Holocene.

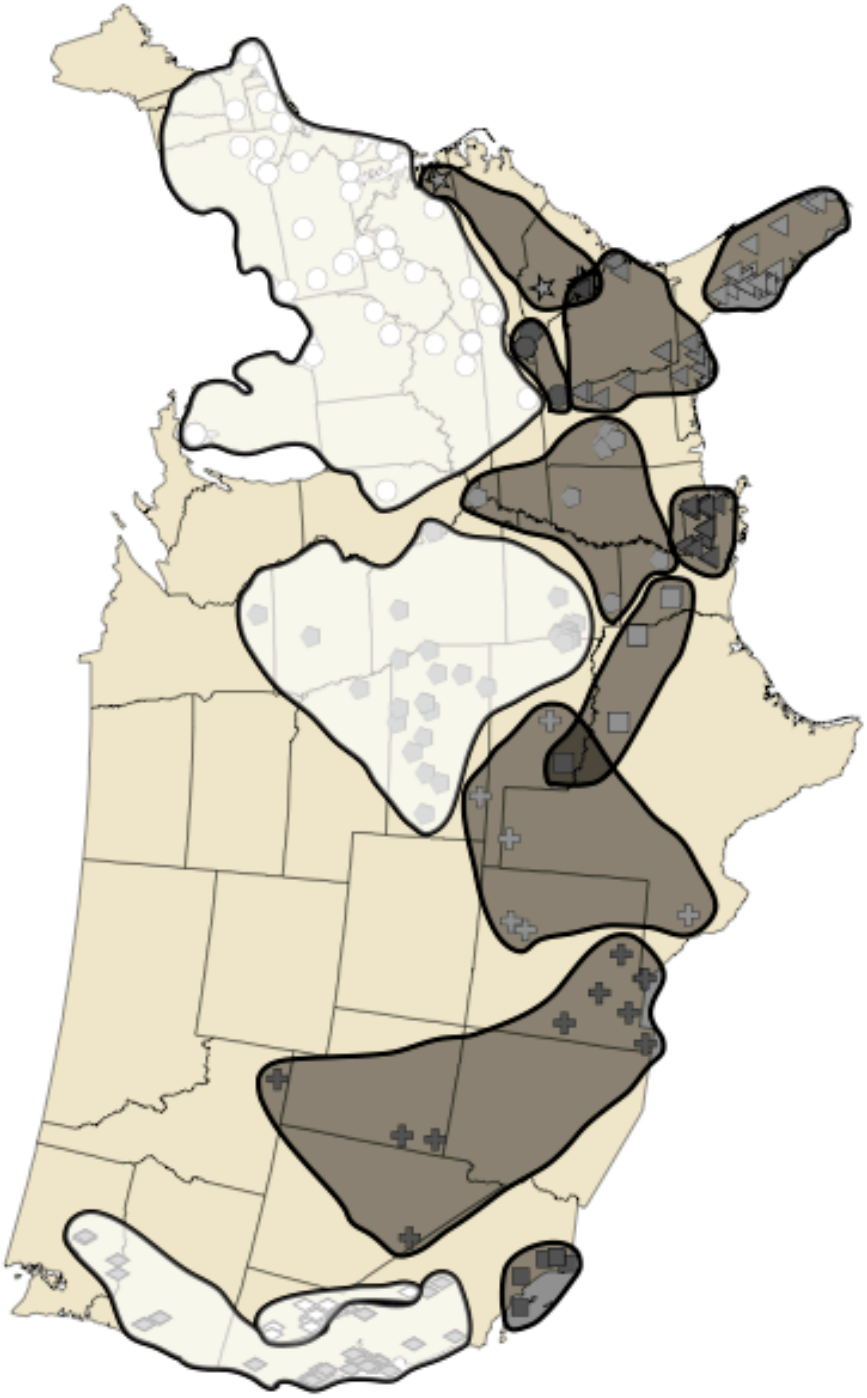
An additional striking outcome of our study is the identification of three putative areas of secondary contact. Such zones are of particular interest because they can provide important information about the interaction between lineages, how populations merge or diverge, and even how adaptive or maladaptive variation can be transferred through introgressive hybridization (Wake 1997). The contact zones within Oklahoma and South Carolina are especially intriguing because they appear to be associated with ecological transition zones. Secondary contact zones that are situated along ecotones provide an opportunity to test the Bounded Hybrid Superiority model (Moore 1977). Under this model ecological requirements are viewed as the causal factors in determining variation in fitness among individuals, making hybrids that are more fit in transitional habitats than either of the parental lineages and less fit in the parental habitats (Anderson 1949; Moore 1977). Finer scale sampling incorporating nuclear loci may reveal details of the genetic properties (cline shape, width, fitness) within these putative contact zones.

Finally, it seems apparent from these results that the species level diversity is currently underestimated and that a full taxonomic review is warranted. However given the extensive lineage diversity and multiple secondary contact zones, a taxonomic revision should not be undertaken without further sampling, particularly throughout

Mexico, and the addition of nuclear data to examine gene flow across the putative barriers.

FIGURE 21

Map showing the distribution of the lineages depicting rapid expansion of the northern most lineages (light gray) and long-term population stability of the southern lineages (dark gray). Dark lines represent the proposed geographic range for each group. Symbols correspond to Fig. 18



CHAPTER 3

Evaluating Hypotheses on the Origin and Diversification of the Ringneck Snake, *Diadophis punctatus* (Colubridae: Dipsadinae).

(Adapted from Fontanella, F.M and Siddall, M. E. 2009. Evaluating hypotheses on the origin and diversification of the ringneck snake, *Diadophis punctatus* (Colubridae: Dipsadinae)

Zoological Journal of the Linnean Society XX: XX-XXXX

Text extracts reproduced by permission from the Publisher

INTRODUCTION

Determining the origin, assembly and evolution of North American biota has long been of interest to biogeographers. Central Mexico has been of particular interest due to its rich biodiversity and having been recognized as a transition zone between the Nearctic and Neotropical biogeographic regions. However, any line demarcating these two regions has been blurred by the variety of ranges for the Mexican fauna (Wallace, 1876; Halffter, 1976) leading several authors to suggest a large transition zone between strictly northern and southern biota (Heilprin, 1887; Halffter, 1976). Although comparative biogeographic studies have shown the existence of a definitive transition zone between northern and southern taxa, there is no consensus region applicable for all taxa (Escalante, Rodriguez, & Morrone, 2004; Huidobro *et al*., 2006; Morrone, 2006). Despite this inability to precisely demarcate a boundary between the Nearctic and Neotropics, many species inhabiting the tropics reach their northern most limits in central or southern Mexico near where Nearctic species reach their southern limits. This transition between these regions combined with the extensive biodiversity and complex geology has led authors to consider Mexico an ancestral area for several species extending throughout Mexico and into the conterminous United States (Gloyd, 1940; Blanchard, 1942; Santacruz–Varela *et al*., 2004).

The close affinities recognized between taxa in Mexico and the contiguous United States has led to a variety of biogeographic scenarios (Klauber, 1972; Rosen, 1978; Webb & Wilkins, 1984; Zink, 2002). An "Out of Mexico" hypothesis proposes that certain species originated within Mexico and dispersed into the United States followed by isolation and allopatric speciation. The majority of phylogeographic studies involving

species inhabiting Mexico and the conterminous United States have focused on taxa distributed throughout the warm deserts of northern Mexico and the southwestern United States (Riddle & Honeycutt, 1990; Lee, Riddle & Lee, 1996; Upton & Murphy, 1997; Zink & Blackwell, 1998; Orange, Riddle & Nickle, 1999; Nason, Hamrick & Fleming, 2002; Haffner & Riddle, 2005; Jaeger, Riddle & Bradford, 2005). These studies have revealed genetic discontinuities, hybrid zones and species boundaries consistent with a model of allopatric speciation for a variety of taxa. A comparative phylogeographic study of birds, mammals and reptiles inhabiting the arid–lands of North America revealed a concordant split for 13 of the 16 species, each associated with the formation of the mid–peninsular seaway in Baja California (Zink, 2002) pointing to an ancestral configuration for the Baja biota.

Although few biogeographic studies have been conducted across the central portions of Mexico, a second ancestral area has been proposed for the southern portion of the Mexican Plateau near the foothills of the Trans–Mexican Volcanic Belt (Blanchard, 1942; Klauber, 1972; Wikler & Gordon, 2000; Place & Abramson, 2004). This large highland occupies much of northern and central Mexico and is defined by the Sierra Madre Occidental to the west, the Sierra Madre Oriental to the east and the Trans–Mexican Volcanic Belt to the south. Although once a semiarid savanna that extended into the southwestern United States, the combined rainshadows of the Sierra Madre Occidental and the Sierra Madre Oriental have resulted in a contemporary desert–scrub–steppe woodland habitat (Alexander & Riddle, 2005). The combination of climate change, mountain ranges and high elevation has facilitated the isolation of the central plateau resulting in high species diversity and multiple areas of endemism

(Escalante, Sanches–Cordero & Morrone, 2007) that could have served as a center of origin for species radiating northward. Recent efforts using biogeographical mapping or molecular phylogeography have revealed Mexican origins for a various Nearctic taxa including the Bicknell's thrush (*Catharus bicknelli*) (Outlaw *et al*., 2003), rattlesnakes *(*genu*s Crotalus*) (Gloyd, 1940; Klauber, 1972; Greene, 1997; Place & Abramson, 2004), thamnophine snakes (Ruthven, 1908; Alfaro & Arnold, 2001), corn (*Zea mays)* (Santacruz–Varela *et al*., 2004) and a fungus (*Fusarium circinatum)* (Wikler & Gordon, 2000). The ringneck snake *Diadophis punctatus* (L.) provides an opportunity to evaluate the "Out of Mexico" hypothesis because it has a remarkably large and continuous range extending from the southern portion of the Mexican Plateau and Trans–Mexican Volcanic Belt northward throughout the conterminous United States and into eastern Canada. Ringneck snakes have generalist diets, preying on salamanders, earthworms, reptiles and insects (Blanchard, 1942; Blanchard, Gilreath & Blanchard, 1979; Stebbins, 1985; Conant & Collins, 1991) allowing them to exploit a variety of habitats ranging from the mixed hardwood forests of southern Canada to the deserts of the Southwestern US and into the desert–scrub–steppe woodland habitat of central Mexico (Stebbins, 1985). This species is proposed to have low vagility, as well as extensive morphological and behavioral variation (Blanchard, 1942; Gehlbach, 1965; Blanchard *et al*., 1979; Connant & Collins, 1991; Stebbins, 2003). Based on the morphological variation, Blanchard (1942) considered the southern portion of the Mexican Plateau to be the center of origin for *D. punctatus*. As support for this notion, he cited the moderate number of ventral scales and larger body size for Mexican ringnecks relative to the 13 currently recognized sub–species distributed throughout the conterminous United States.

In this study, we expand on preexisting (Fontanella *et al*., 2008) phylogeographic data for *D. punctatus,* incorporating new data from previously unsampled areas appropriate to critically assessing hypotheses regarding a Mexican origin for the species.

MATERIALS AND METHODS

Specimens examined

Due to uncertainty of the sister group and to assess the monophyly of *D. punctatus,* outgroup taxa included xenodontine snakes from North America (*Contia tenuis* (Baird & Girard, 1852), *Hypsiglena torquata* (Gunther, 1860), *Rhadinaea flavilata* (Cope, 1871), *Farancia abacura* (Holbrook, 1836), *Heterodon simus* (L., 1766) and *Heterodon platyrhinos* (Latreille, 1801), the Central American *Alsophis portoricensis* (Reinhardt & Lutken, 1863) and *Arrhyton exiguum* (Cope, 1863), and the South American *Helicops angulatus* (L., 1758) (Pinou *et al*., 2004; Lawson *et al*., 2005). Ingroup sampling included new data for more than 100 individuals from previously unsampled areas throughout North America. Samples from southern Mexico were collected within the proposed range of the Mexican ringneck snake *D. p. dugesii* (Blanchard, 1942), allowing us to determine the origin of the genus *Diadophis*. Additional population level sampling was included to refine the boundaries of and test the exclusivity of the mtDNA lineages in addition the 286 individuals previously sampled (Fontanella *et al*., 2008) from across the contiguous United States (Supplementary Material 1).

Molecular methods

Genomic DNA was obtained from liver, muscle or shed skins using the Qiagen DNA extraction kit. We amplified the complete cytochrome *b* (1117 bp) mitochondrial gene for each of the new samples via PCR using published primers and protocols (Burbrink, Lawson & Slowinski, 2000). In order to compare divergence date estimates to Fontanella *et al*. (2008), we also amplified a 659 bp portion of the *ND 4* gene for those individuals used in the dating analyses (see below). This gene fragment was amplified using published primers and protocols (Arevalo, Davis & Sites, 1994). Amplifications were purified using AMPURE (Agencourt) following the manufacturer's instructions. Purified PCR products were sequenced, purified and aligned following the protocols of Fontanella *et al*. (2008). The alignments were unambiguous with no gaps present for each *D. punctatus* sequenced in this study. Sequences were deposited in GenBank under the accession numbers (FJ358734-FJ358829).

Phylogenetic analyses

Phylogenetic trees were constructed using both maximum parsimony (MP) and maximum likelihood (ML) criteria. Maximum parsimony analyses were conducted in TNT (Goloboff, Farris & Nixon, 2003) using a heuristic search method with equally weighted characters, 1000 random addition–sequence replicates and the tree–bisection–reconnection (TBR) branch–swapping algorithm. Support for internal nodes was assessed using non–parametric bootstrapping (BS) (Felsenstein, 1985) with 1000 pseudo–replicates and 100 random addition–sequence replicates.

ModelTest v3.7 (Posada & Crandall, 1998) was used to select the best–fit model of nucleotide change based on the Akaike Information Criterion (AIC) (Akaike, 1973). The

GTR + Γ + I (general time reversible model with Γ–distributed among–site rate variation and with a proportion of invariant sites) model was selected as the most appropriate model of nucleotide substitution. We conducted ML analyses using RAXML–VI–HPC version 7.0.4 (Stamatakis, 2006). Our RAXML analyses implemented the GTRGAMMA nucleotide substitution model with an among–site rate heterogeneity parameter partitioned across each codon position with a proportion of invariant sites. The implemented model is equivalent to the general time–reversible model with an among–site rate heterogeneity parameter determined by ModelTest. To assess support values for the inferred relationships, we implemented 10000 nonparametric bootstrap replicates. Analyses were run on the Cyberinfrastructure for Phylogenetic Research (CIPRES) cluster at the San Diego supercomputer center (Stamatakis, Hoover & Rougemont, 2008).

Hypothesis Testing

To discriminate specific topological scenarios under likelihood based criteria we implemented the Shimodaira–Hasegawa (S–H) test (Shimodaira & Hasegawa, 1999) in PAUP* VERSION 4.0b (Swofford, 2002) and the Approximately Unbiased (AU) test (Shimodaira, 2002) in CONSEL (Shimodaira & Hasegawa, 2001). Because likelihood searches for alternative topologies would be time consuming, all constraint tests were conducted on topologies constructed from a reduced data set. These tests allowed us to examine whether the best tree possible under the proposed constraint is a significantly worse fit to the data than the best tree without the constraint. These tests are useful for assessing the confidence of conclusions when data cannot resolve individual nodes of a

tree or when nodes have low support. The reduced data sets were analyzed using the same model parameters listed above.

We first tested Blanchard's (1942) hypothesis that the genus *Diadophis* originated in central Mexico and served as the source population for the conterminous United States. Blanchard's (1942) hypothesis was based on an increased number of dorsal scales and the possession of two posterior temporal scutes compared to the decreased number of dorsal scales and single posterior temporal scutes found in ringnecks throughout the conterminous United States. This scenario predicts that the populations from central Mexico are phylogenetically more closely related to the US lineages as a whole than to any single US lineage.

The second prediction was that central Mexico served as the source population for the lineages confined to the eastern United States. Close affinities have been proposed between multiple species in the southeastern United States and Central Mexico with the Neotropics (Hubbs, 1936; Martin & Harrell, 1957; Wiley, 1976). During glacial intervals, sea levels were nearly140 m lower in comparison to present levels opening corridors along the Gulf Coast allowing dispersal of species into the Southeastern US (Webb & Wilkins, 1984; Ellsworth *et al*., 1994). Fontanella *et al*. (2008) inferred the initial divergence of *D. punctatus* as a southeastern origin, however their analyses did not contain representatives from Mexico. Under this scenario, the Mexican populations would be phylogenetically more closely related to the populations in the southeastern United States than to other lineages.

Finally, we expand upon the hypothesis that *D. punctatus* originated in the southeastern United Sates by testing against a west to east dispersal pattern. The

southeastern United States is a geologically and topographically complex area that has long been recognized as a center for origin and dispersal for eastern biota (Adams, 1902). The region has remained largely undisturbed throughout oscillating climatic cycles, which allowed for existence of multiple refugial areas during times of glacial advancement (Soltis *et al*., 2006; Waltari *et al*., 2007; Fontanella *et al*., 2008). This combination of long–term stability and complex topography has contributed to the region's high endemicity and extensive biodiversity.

Divergence Dating

In order to compare divergence dates between lineages for the new topologies to those of Fontanella *et al*. (2008), we included the 659 bp portion of the mitochondrial gene *ND 4*. BEAST v1.4.4 (Drummond & Rambaut, 2003) was used to infer the date of origin for each lineage without relying on a molecular clock and considering uncertainty in tree topology and branch length (i.e., 'the relaxed phylogenetics' method). Phylogenetic estimates were constructed under the GTR+Γ model with an uncorrelated lognormal tree prior and a constant population size prior. Derived from fossil data (Holman, 1979, 2000), a mean calibration point of 7.5 Mya was placed at the root of *D. punctatus* with a lognormal standard deviation of 0.29 producing a 95% credible sampling interval (CI) from 4.55 to 12.1 Mya. This time frame spans the North American Land Mammal Age (Hemphillian) into the Clarendonian Age; the latter age has not produced fossils for this species and is a reasonable cutoff point for the upper CI. Analyses were run for 30 million generations and sampled every 1000th iteration following a burn–in of 3000.

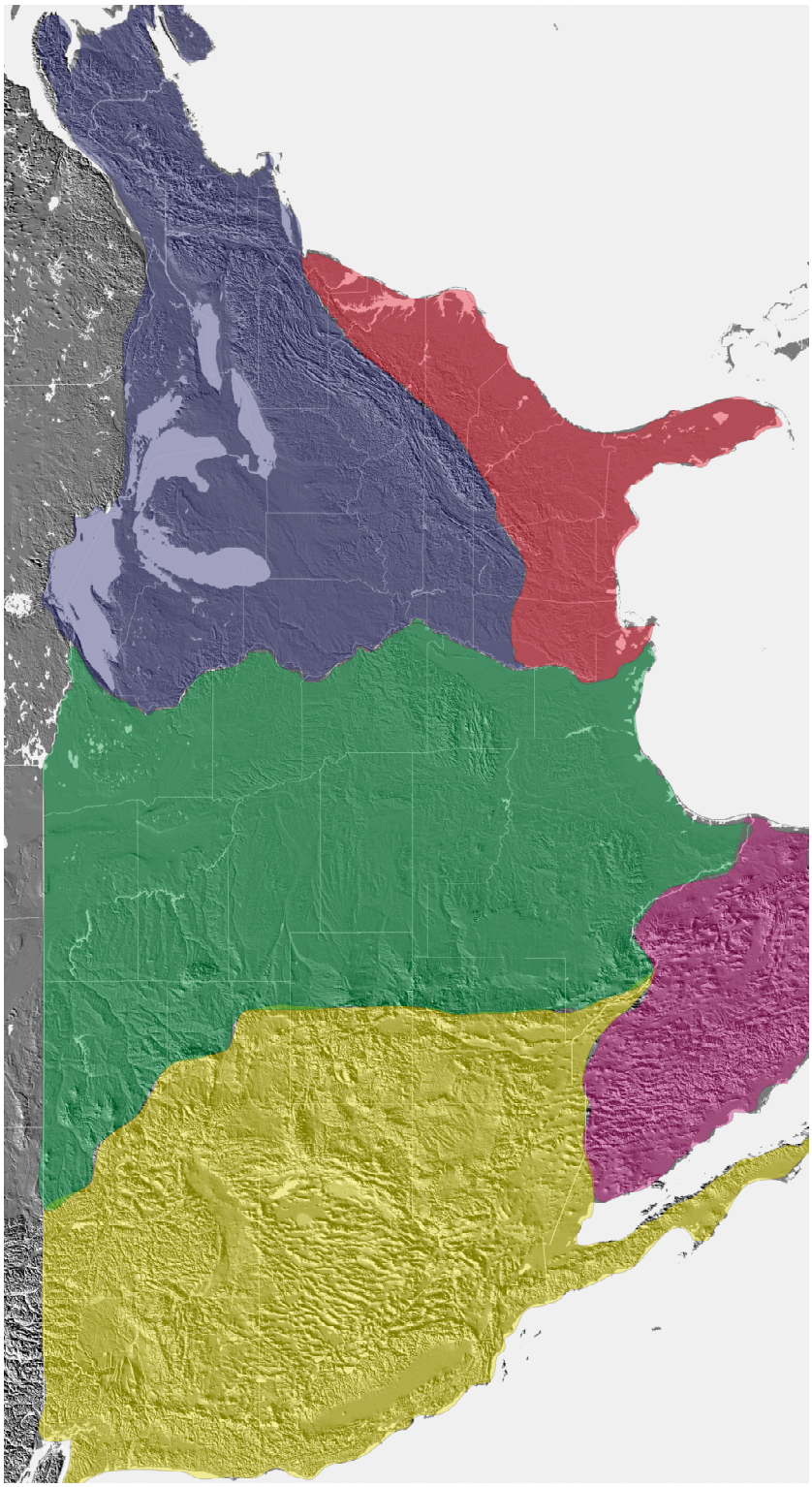
Ancestral Area Reconstruction

To reconstruct the ancestral distributions on the phylogeny of *D. punctatus*, we implemented the dispersal–vicariance analysis in DIVA v.1.1 (Ronquist, 1996). This method attempts to optimize the reconstruction of ancestral distributions by assuming a vicariant model while also incorporating the potential for dispersal and extinction in shaping the current distributional pattern. Five areas were defined by geographical boundaries: A) Coast Plain south of the Appalachian Mountains and east of the Mississippi, B) Northeastern United States north of the Appalachian Mountains and east of the Mississippi River C) West of the Mississippi River to the Rocky Mountains, D) West of the Rocky Mountains, and E) Mexico (Fig. 22).

Analyses were run using the exact search without further restriction of the number of areas in which the ancestor occurred (i.e. admitting the possibility of a widespread ancestor). This assumes that the dispersal ability of the ancestor was not greater than any of the descendants and asks the question: if the group had a center of origin in the past, what would be the most likely ancestral area for the group?

FIGURE 22.

Map of the United States and Mexico showing the five geographic regions used in the ancestral area reconstruction analysis.



RESULTS

Phylogenetic reconstruction

Our aligned mtDNA data set of 1117 sites comprises 286 previously published (Fontanella *et al*., 2008) and 95 sequences from previously unsampled regions throughout the United States and Mexico. The absence of premature stop codons and a strong bias against guanine in the light strand suggest that these sequences are from the mitochondrial genome and not nuclear integrated copies or pseudogenes (Zhang & Hewitt, 1996).

The Maximum Likelihood analyses produced a well resolved phylogeny (Fig. 23) with a mean ln–likelihood score of –18077.783742 and mean parameter values of alpha= 0.84563 , proportion of invariant=0.317964, G ↔ T = 1.000, C ↔ T=4.359, C ↔ G = 1.485, A ↔ T = 0.759, A ↔ G = 6.671, A ↔ C = 1.101 and a tree length of 2.662. Maximum parsimony analysis of the 442 parsimony informative characters produced 53 equally parsimonious trees of 2351 steps. Because Maximum Likelihood and Maximum–Parsimony topologies were highly congruent, only the maximum likelihood tree is presented. Support values from both methods are indicated where the two trees are concordant. The inclusion of the additional 100 samples resulted in the inference of an additional two well–supported mtDNA lineages and provides a better understanding of the geographic boundaries for mtDNA lineages.

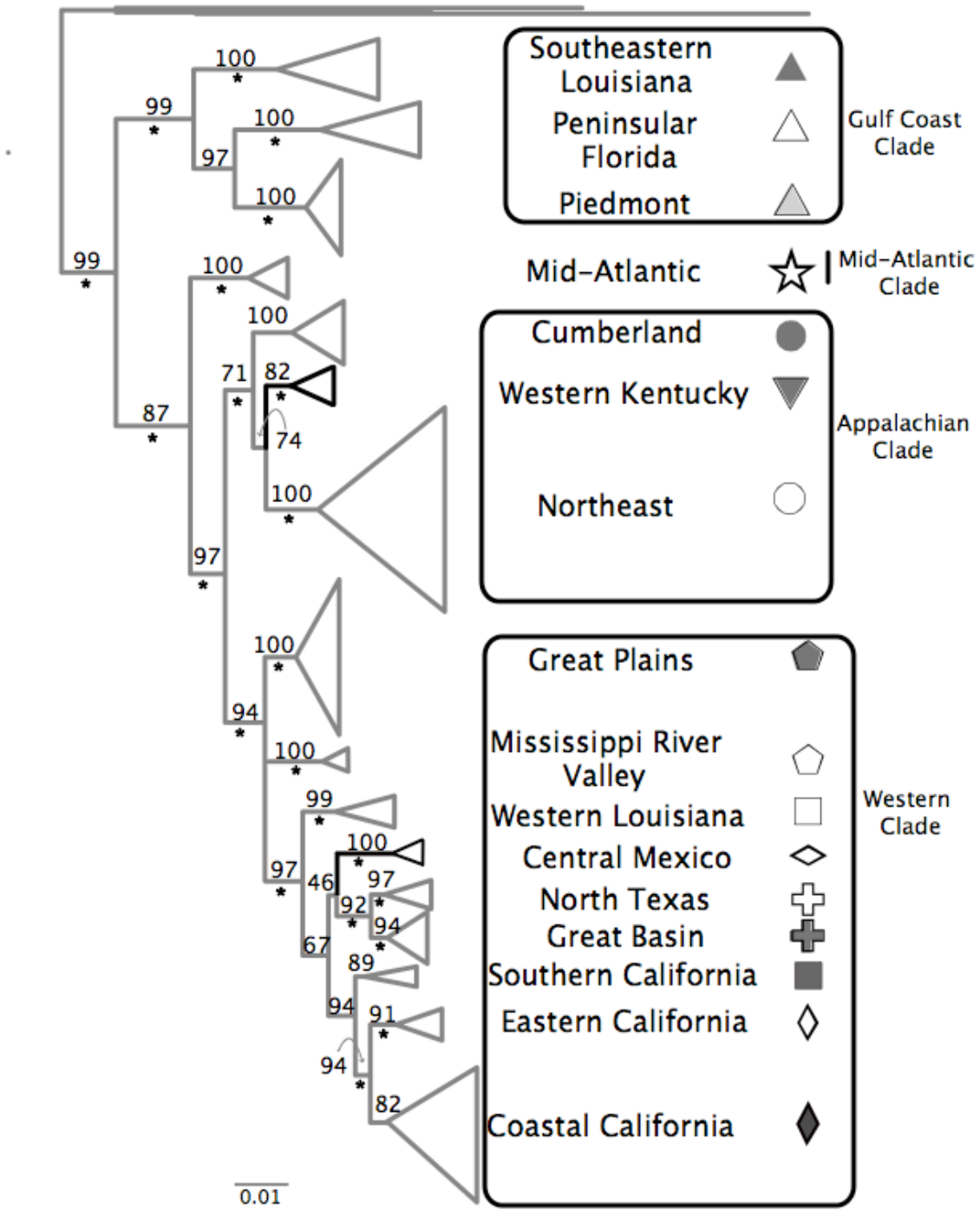
The *Diadophis punctatus* complex formed a well–supported monophyletic group exclusive of the other xenodontines. Similar to Fontanella *et al*. (2008), the resulting hypothesis illustrates four major clades across North America; Gulf Coast, Mid–Atlantic, Appalachian and Western (Fig. 23). Within the Appalachian clade we inferred an

additional mtDNA lineage that extends from eastern Illinois above the Mississippi River Embayment southeast to south central Tennessee. This Western Kentucky lineage is sister to the Northeast lineage, re–positioning the Cumberland lineage as sister to the Western Kentucky+Northeast clade.

The samples included from central Mexico formed a well–supported lineage extending from eastern Michoacan east to the boarder of Guanajuato and Queretaro. Within the Western clade, the Mexican lineage is sister to the North Texas+Great Basin clade. However, the placement of this lineage had little support in both analyses. Because the populations from southern Mexico were nested within the Western clade and analyses indicated that the position within the tree was somewhat ambiguous, we evaluated support for the alternative phylogenetic scenarios. Results from both the S–H and AU test rejected each of the proposed biogeographic hypotheses (Table 4).

FIGURE 23.

Maximum Likelihood tree for the 386 *Diadophis punctatus* samples and nine outgroup taxa. Nonparametric bootstrap proportions based on 1000 pseudo–replicates are listed above branches. Asterisk below represent nodes with MP bootstrap values greater than 70. Symbols correspond to the geographic distribution of each lineage in Fig. 25. New mtDNA lineages are indicated with dark branches


Southeastern Louisiana
Peninsular Florida
Piedmont
Gulf Coast Clade
Mid-Atlantic
Mid-Atlantic Clade
Cumberland
Western Kentucky
Northeast
Appalachian Clade
Great Plains
Mississippi River Valley
Western Louisiana
Central Mexico
North Texas
Great Basin
Southern California
Eastern California
Coastal California
Western Clade
0.01

Divergence Dating

Based on the combined analyses of cytochrome *b* and ND 4, the initial divergence within *Diadophis* occurred between the Gulf Coast clade and the conterminous clades during the late Miocene (~6.5 Mya Fig. 24; Table 5). The four major clades (Gulf Coast, Appalachian, Mid–Atlantic and Western) and the divergences between the lineages of the Gulf Coast clade occurred during the Pliocene. Regional divergences within the Appalachian and Western clades as well as the origins of the extant lineages occurred during the Pleistocene.

Ancestral Area Reconstruction

The DIVA results indicated that the optimal area distribution required 9 dispersal events (Fig. 24). For the primary radiation of *D. punctatus,* DIVA unequivocally inferred an optimal ancestral area in the southeastern region indicating a restricted ancestral distribution followed by dispersal throughout the northeastern states. For the lineages strictly west of the Mississippi River, DIVA inferred an ancestral area in the central states west of the Mississippi River. The ancestral areas for the lineages occupying Mexico, the western deserts and California were largely ambiguous.

Table 4. Results of hypothesis testing using the SH method of Shimodaira & Hasegawa (1999) and the AU method of Shimodaira & Hasegawa (2001).

Alternate Hypothesis	S–H Test	AU Test
Mexico as ancestral species	< 0.005	< 0.005
Gulf Coast corridor Hypothesis	< 0.03	< 0.005
West–East Dispersal Hypothesis	< 0.02	< 0.01

FIGURE 24.

Simplified tree with the mean dates of origin (in millions of years) for each of the major lineages and haplogroups. Numbers below nodes refer to Table 2. Letters above nodes refer to the optimal reconstructions of ancestral areas; A=Coast Plain south of the Appalachian Mountains and east of the Mississippi, B=Northeastern United States north of the Appalachian Mountains and east of the Mississippi River, C=West of the Mississippi River to the Rocky Mountains, D=West of the Rocky Mountains, and E=Mexico.

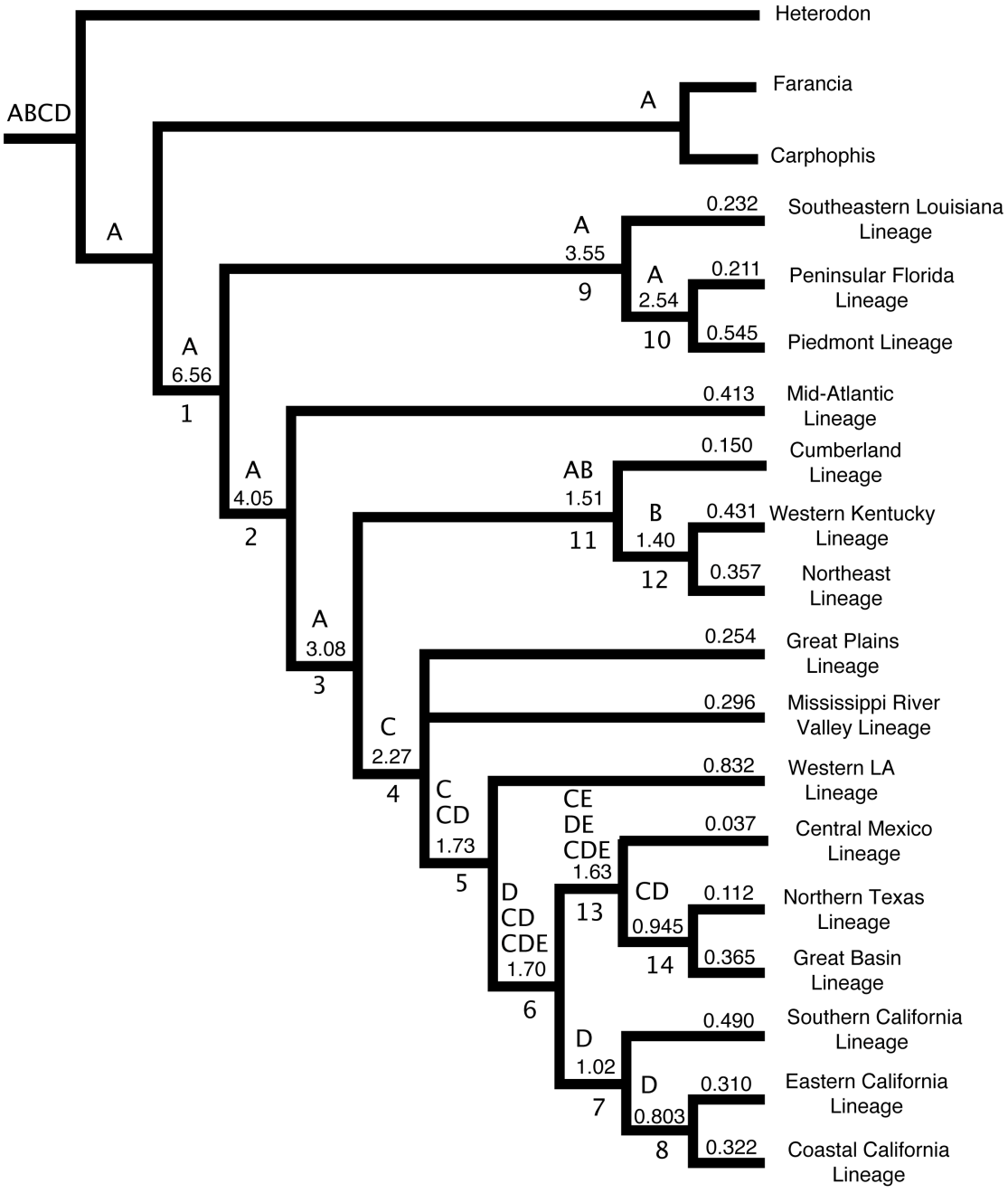

Heterodon
Farancia
Carphophis
ABCD
A
A
0.232
Southeastern Louisiana Lineage
A
3.55
9
A
2.54
0.211
Peninsular Florida Lineage
10
0.545
Piedmont Lineage
A
6.56
1
0.413
Mid-Atlantic Lineage
A
4.05
2
AB
1.51
0.150
Cumberland Lineage
B
1.40
11
0.431
Western Kentucky Lineage
12
0.357
Northeast Lineage
A
3.08
3
0.254
Great Plains Lineage
0.296
Mississippi River Valley Lineage
C
2.27
4
C
CD
1.73
5
0.832
Western LA Lineage
CE
DE
CDE
1.63
13
0.037
Central Mexico Lineage
CD
0.945
14
0.112
Northern Texas Lineage
0.365
Great Basin Lineage
D
CD
CDE
1.70
6
D
1.02
7
0.490
Southern California Lineage
D
0.803
8
0.310
Eastern California Lineage
0.322
Coastal California Lineage

Table 5. The mean date of origin for each lineage of *Diadophis punctatus* and the time to the most recent common ancestor (TMRCA) of each haplogroup. Values in parentheses represent standard deviation (SD) and the 95% confidence interval (CI) estimated using the uncorrelated lognormal Bayesian relaxed molecular clock in BEAST v. 1.4.4. Numbers for nodes follow Figure 3.

Node	Origin (MYA)	MtDNA Lineage	TMRCA of Haplogroups (MYA)
1	6.56 (SD=0.031; CI=3.15–10.46)	SE LA	0.23 (SD=0.003; CI=0.037–0.47)
2	4.05 (SD=0.031; CI=1.67–6.87)	Piedmont	0.55 (SD=0.007; CI=0.134–1.11)
3	3.08 (SD=0.026; CI=1.26–5.28)	Penn. FL	0.21 (SD=0.003; CI=0.036–0.46)
4	2.27 (SD=0.021; CI=0.88–3.89)	Mid–Atlantic	0.41 (SD=0.005; CI=0.009–0.86)
5	1.73 (SD=0.019; CI=0.66–2.95)	Cumberland	0.15 (SD=0.002; CI=0.022–0.32)
6	1.70 (SD=0.018; CI=0.63–2.93)	Western Kentucky	0.43 (SD=0.005; CI=0.103–0.88)
7	1.02 (SD=0.096; CI=0.40–1.81)	Northeast	0.75 (SD=0.009; CI=0.207–1.45)
8	0.80 (SD=0.008; CI=0.28–1.46)	MRV	0.29 (SD=0.004; CI=0.060–0.61)
9	3.55 (SD=0.033; CI=1.29–6.24)	Great Plains	0.25 (SD=0.003; CI=0.005–0.54)
10	2.54 (SD=0.035; CI=0.81–4.65)	Western LA	0.83 (SD=0.001; CI=0.260–1.53)
11	1.51 (SD=0.017; CI=0.53–2.75)	Southern Mexico	0.04 (SD=0.001; CI=0.002–0.105)
12	1.40 (SD=0.016; CI=0.40–2.59)	Great Basin	0.37 (SD=0.004; CI=0.009–0.71)
13	1.63 (SD=0.017; CI=0.59–2.87)	North Texas	0.10 (SD=0.001; CI=0.009–0.23)
14	0.95 (SD=0.010; CI=0.29–1.70)	South CA	0.49 (SD=0.006; CI=0.139–0.94)
		Eastern CA	0.33 (SD=0.004; CI=0.070–0.64)
		Coastal CA	0.26 (SD=0.003; CI=0.050–0.54)

DISCUSSION

Origin and diversification of Diadophis punctatus

Our analyses agree neither with the hypothesis of Blanchard (1942), who suggested that the ancestral area of *D. punctatus* was in central Mexico and that *D. p. dugesii* arose first, nor with the hypothesis that the lineages throughout the eastern United States are derived from a Mexican ancestor. Under a phylogenetic framework, Blanchard's (1942) hypothesis implies that the southern Mexico populations should be the earliest diverging lineage for any phylogenetic analyses. Likewise, the Gulf Coast corridor hypothesis suggests the drop in sea level during glacial periods of the Miocene/Pliocene exposed Gulf Coast shelf, allowing dispersal from southern Mexico into the southeastern United States. Yet the topologies that we estimated indicate that the populations from Mexico are nested well within *D. punctatus,* in a derived position more closely related to the lineages occupying the western United States rather than basal to *Diadophis* as a whole or to any of the eastern lineages. The results of all the topological constraint tests indicate a significantly worse topology when the Mexican populations are forced to be the first lineage of the genus *Diadophis* or sister to the lineages occupying the eastern United States (Table 4). The placement of Mexican populations nested within the tree suggests that the possession of two posterior temporal scales and moderate number of scale counts, characters considered by Blanchard (1942) to be primitive, are actually derived and apomorphic for the Mexican lineages. That said, the results of the constraint tests indicate that the precise placement of the Mexican lineage is not statistically well supported with respect to the western lineages. The central Mexico lineage can be shifted as sister to all of the lineages of the Western clade without a

significant change in likelihood score. Although the likelihood value of this topology is not significantly worse, it presents a convoluted biogeographic pattern.

Regardless of optimality criterion, the origin of the genus *Diadophis* appears to be in southeastern States. We further tested this hypothesis by constraining lineages in the eastern United States to be most closely related to lineages in the western United States, each of which resulted in significantly worse topologies (Table 4). These conclusions are supported by the ancestral area reconstructions, which unequivocally inferred a southeastern origin for *D. punctatus*. Additionally, the oldest fossil of the genus *Diadophis* was found in southern Florida lending additional support for a southeastern origin (Holman, 2000). Species origins in the southeastern United States have been attributed to a combination of geological and zoogeographic factors (Adams, 1902). The Southeast is an ancient and highly diverse landscape over half a billion years in age and composed of numerous physiographic provinces. Differentiation of species also has been fostered by the Southeast's stable geologic history. This region was neither significantly disturbed during ice ages nor completely inundated by rising sea levels during interglacial periods. These upland areas have served as the primary "spawning sites" for the evolution of new species (Adams, 1902; Butler & Mayden, 2003).

The combined data inferred an initial divergence within *D. punctatus* during the late Miocene approximately 6.5 Mya with the separation of the Gulf Coast lineages from the remaining conterminous lineages (Fig. 24, Table 5). This time period was marked by a major cooling trend with the progression of glaciers across the northern and Pacific regions (Dorf, 1959). The remaining major divergences occurred during the warming trend of the Pliocene, between 5–3 million years ago, following the retreat of the

Miocene glaciers. From the southeastern United States, the major biogeographic pattern follows a southeast to northeast then westward directionality of historical migration, with the divergence of the Mid–Atlantic lineage followed by a split across the northeastern US separating the Appalachian clade of Fontanella *et al*. (2008). The new biogeographic scenario within the Appalachian clade suggests a southern Appalachian origin followed by the divergence of the Northeast and Western Kentucky lineages respectively.

Across the southwest, the results suggest an historical migration from the central United States into Mexico and across the southwestern deserts. The position within the phylogeny, the date estimate and the ancestral area reconstructions for the southwestern + Mexico clade combined suggests a relatively recent invasion into central Mexico. Typically, reptiles occupying the Western United States are confined to the xeric habitats of the Chihuahuan and Sonoran deserts in northern Mexico (Riddle & Hafner, 2006) and do not extend into the grasslands of central Mexico or as far south as the Trans–Volcanic Belt. Interestingly, the divergence date estimate for the central Mexican lineage indicates a recent origin, approximately 34 kya. Based on palynological records, the Pleistocene conditions of Mexico were very different from present. Northern Mexico had a more temperate forested climate with extensive lakes and wetland areas while in central Mexico, glaciers expanded and there were extensive pine forests mixed with alpine grasslands (Metcalf *et al*., 2000). Since that time, the reorganization of atmospheric circulation caused major changes to seasonality and precipitation throughout Mexico leading to the drying out of Northern Mexico and resulting in significant changes to species distributions (Metcalf, 2006). Unfortunately, the sparse sampling throughout Mexico still limits our understanding of the southern extent of the North Texas and Great

Basin lineages and genetic barriers between these lineages and the one sampled Mexican lineage. Further sampling throughout these areas is needed to more accurately define the range limits and potential barriers to these lineages.

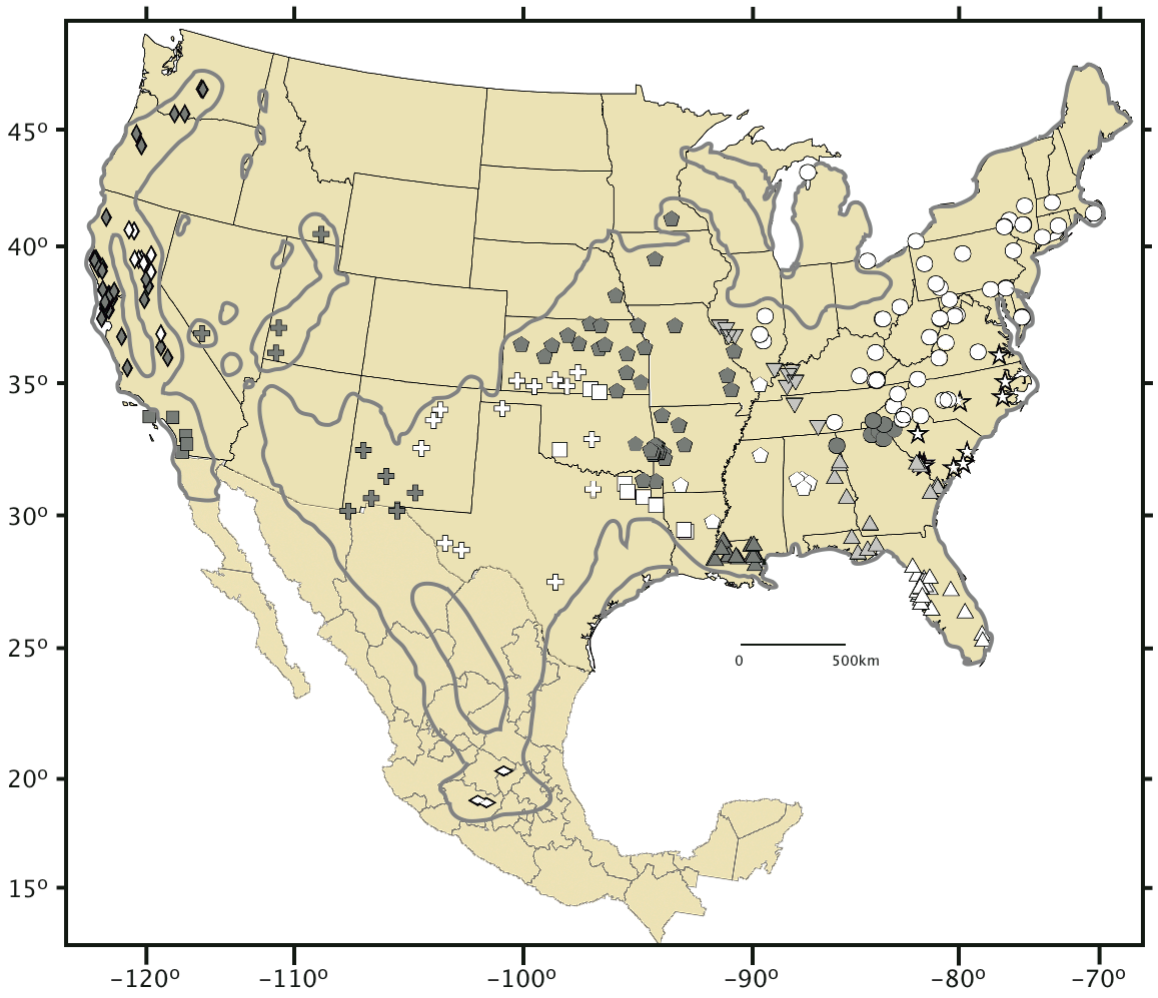
Phylogeographic Patterns

Analyses of our expanded sampling revealed four major clades inferred in Fontanella *et al*. (2008) plus an additional two lineages from previously unsampled areas, resulting in a total of 16 mtDNA lineages throughout the range of *D. punctatus* (Fig. 25). The addition of these lineages provides better estimates of the divergence dates for the origin of the genus *Diadophis* as well as dates for origin of the contemporary lineages. Rather than reiterating all of the previously published details (Fontanella *et al*., 2008), we limit our discussion to the distribution of newly inferred mtDNA lineages, their relationships to other *D. punctatus* lineages and changes to the geographic range of certain mtDNA lineages.

The newly sampled Western Kentucky lineage extends from south central Tennessee west along the Tennessee River and into to Lincoln Hills of southeastern Illinois. This lineage is associated with the mixed upland oak stands of the Tennessee Western Highland Rim and the hardwood forests of southern Illinois. Dispersal of these snakes into the flood plains of the Mississippi Alluvial Plain, which is occupied by the Mississippi River Valley lineage, appears to be inhibited by the Tennessee River to the west and the Shawnee Hills in southern Illinois. In western Illinois the Mississippi River

FIGURE 25.

Map showing the distribution of each *D. punctatus* lineage diagnosed by mtDNA variation. Symbols correspond to Fig. 23. Dark line corresponds to distribution of *Diadophis punctatus* throughout the United States and Mexico.


45°
40°
35°
30°
25°
20°
15°
−120°
−110°
−100°
−90°
−80°
−70°
0
500km

appears to be a barrier to western dispersal, isolating Western Kentucky populations from those of the Great Plains lineage. The lack of an apparent geographic barrier and the physiographic homogeneity of the Illinois Basin suggest the likely existence of a secondary contact zone between the Western Kentucky and Northeastern lineage in central Illinois.

Throughout the eastern United States *D. punctatus* is considered widely abundant, however they are sparsely distributed throughout much of the southwestern deserts and Northern Mexico (Conant & Collins, 1991; Mendelson & Jennings, 1992). Two lineages were inferred throughout this region: the North Texas lineage that extends into the northern regions of the Chihuahuan Desert and the Great Basin lineage confined to the Great Basin and portions of the Sonoran Desert (Fontanella *et al*., 2008). Although the sampling is sparse, the Mexican lineage appears to be associated with the grassland habitats of central Mexico extending from Michoacan to Guanjuato. Although the make–up of the biogeographic provinces throughout Mexico differs between studies (Aranda, Escobedo & Pozo, 1997; Morrone *et al*., 2002; Zink, 2002), this region of central Mexico is considered an endemic area associated with the northern region of the Transmexican Volcanic Belt zone (Marshall *et al*., 2000; Morrone *et al*., 2002).

Expanded lineage distributions across the US

Thorough sampling of mtDNA lineages that do not to conform to major geographic barriers is important for determining not only the extent of these lineages but also the location and breadth of contact zones. Along the eastern coast of the United States, the boundary between the low–lying mixed forests of the Atlantic Coastal Plain

and the more geologically complex Piedmont Plateau is demarcated by the Fall Line. This transition zone serves as the boundary between species in the alluvial valleys associated with the Coastal Plain and those in the Appalachian Highlands (Shankman & Hart, 2007; Beamer & Lamb, 2008). The addition of samples throughout these regions further defines the range of the Northeast, Mid–Atlantic and Cumberland lineages and identifies additional regions of secondary contact. Individuals collected from the Blue Ridge Mountains north of the French Broad River and within the western portion of the Piedmont share mtDNA haplotypes with individuals from the Northeast lineage. However, sampling along the Fall Line in central North Carolina and the transition zone between the foothills of the Blue Ridge Mountains and the Piedmont Plateau in western North Carolina suggest regions of parapatry or narrow contact zones between the Northeast and Mid–Atlantic lineages and the Cumberland and Northeast lineages, respectively (Fig. 25). Similarly, the additional sampling throughout the central United States suggests broad areas of contact between the Western Louisiana, North Texas and Great Plains lineages throughout Oklahoma. Contact zones across Oklahoma have been found for several vertebrates including pocket gophers (*Geomys bursarius*) (Cothran & Zimmerman, 1985) the white–footed mouse (*Peromyscus leocopus)* (Stangl, 1986) and the northern leopard frog (*Lithobates pipiens)* complex (Hillis, 1981). The haplotypes distributed throughout central Kansas south of the Arkansas River are nested within the Western Louisiana lineage, suggesting a range throughout the savannah and mixed grass plains of Oklahoma. The habitat shift from tall to short grass prairie may limit dispersal of the North Texas and Western Louisiana lineages across Kansas. The Arkansas River appears to isolate these two lineages from the Great Plains lineage further north. The

distribution of Great Plains haplotypes throughout eastern Oklahoma and Arkansas may be due to repeated shifts in the Arkansas River basin followed by range expansion.

Conservation and Taxonomic Implications

In determining conservational units, subspecies are often treated as evolutionarily independent units. However, molecular studies rarely infer phylogeographic patterns that are consistent with subspecies designations (Burbrink, Lawson & Slowinski, 2000; Fontanella *et al*. 2008). Moreover, lineages that are consistently disjunct under one data source (e.g., mtDNA) may prove to be less so when other (e.g., nuclear DNA) data are brought to bear. Yet, it is these unnamed disjunct units or lineages that should be considered when guiding conservation efforts and identifying genetic diversity, not the named subspecies. If the purpose of defining evolutionarily significant units (ESU's) is to recognize the evolutionary heritage and maintain the evolutionary potential of populations (Moritz, 1994), then the results of this study provide vital information about the historical population structure of *D. punctatus*.

Throughout most of its range *D. punctatus* is considered common and listed as secure or apparently secure for most states by NatureServe. Classifications that misrepresent the abundance and genetic diversity within species will have adverse effects on the assessments of species diversity, endemic species and the risk levels to those species. Examples include lineages in California and Louisiana. California ranks first for species diversity and endemic species and is second in species at–risk. The recovery of three genetically distinct lineages suggests that the six recognized subspecies over–represent the diversity of this snake throughout California. Conversely, the one

recognized subspecies throughout Louisiana under–represents the lineage diversity inferred from these data. Based on these findings the status of *D. punctatus* should be re–evaluated on both the national and regional levels.

CHAPTER 4

Development and characterization of 14 polymorphic microsatellite loci in the ringneck snake *Diadophis punctatus* (Colubridae: Dipsadinae)

(Adapted from Fontanella, F.M and Siddall, M. E. 2009b.

Development and characterization of 14 polymorphic microsatellite loci in the ringneck snake *Diadophis punctatus* (Colubridae: Dipsadinae)

*Conservation Genetics (*in press)

INTRODUCTION

The ringneck snake, *Diadophis punctatus,* has one of the largest geographic distributions of any species of snake in North America. Ranging from central Mexico to southern Canada (excluding the northern Great Plains states), this snake exploits a variety of habitats and ecological niches ranging from northeastern mixed hardwood forests to the desert grasslands of the southwestern US and central Mexico (Stebbins 1985). In certain portions of their range, populations of *D. punctatus* have experienced a severe decline in recent decades due to anthropogenic pressure and habitat fragmentation (Fitch 2008; USDA Forest Service). Analyses of mtDNA sequence data (cytochrome *b* and a portion of NADH *4*) inferred 16 lineages across the range, several of which were not isolated by geographic barriers resulting in multiple areas of secondary contact (Fontanella et al. 2008; Fontanella and Siddall 2009). The use of mtDNA sequence data routinely has been used to determine species boundaries (Hewitt 2001), however the usefulness of mitochondrial data in determining species boundaries is not universally agreed upon due to its maternal and non-recombining mode of inheritance (Davis 1996).

Microsatellites are abundant bi-parentally inherited loci that show high levels of polymorphisms (Jarne and Lagoda 1996). These markers facilitate rapid screening of multiple nuclear loci to determine species boundaries and examine gene flow between species (Fu and Zeng 2008).

We developed the first microsatellite library for *D. punctatus* by following and adapting the protocol written by Dr Travis Glenn at the University of Georgia. Because of the broad range and extensive lineage diversity within *D. punctatus,* DNA was isolated from one individual for each of the four major mtDNA clades (Fontanella et al 2008)

using the DNeasy Animal Tissue Kit (QIAGEN). Muscle tissue from the caudal region or shed skins was used to prevent contamination from potential liver parasites. Pooled genomic DNA was digested with *Rsa*I, ligated to an SNX linker and hybridized to a mixture of di- and tri- nucleotide biotinylated probes (i.e. GT, CAC, CTC and CAG). After hybridization to the repeat probes, the linker-ligated DNA was bound to streptavidin-coated (biotin-attracting) Dynabeads® (Dynal Biotech) and collected using a magnet. Repeat-enriched DNA was rendered double stranded and amplified in a polymerase chain reaction (PCR) using the forward SNX linker as a primer. Amplification was verified on a 2.5% agarose electrophoresis gel. Microsatellite rich fragments then were cloned using the Topo TA cloning kit (Invitrogen), transformed into *Escherichia coli OneShot* cells and grown on ampicillin selective plates. Individual colonies were sequenced directly using 0.5 μl of M13 and T7 primers (10X) and 3 μl of BigDye on an ABI 3700 XL automated sequencer.

Eighty-four clones with microsatellite regions of sufficient length (> 5 repeats) and with substantial flanking regions were identified with SEQUENCHER 4.7 (Gene Codes, Corp.). Di-, tri-, and tetranucleotide repeats were the most common but larger repeats, including a heptamer, were observed. TROLL (Castelo et al. 2002) was used to design PCR primers in the flanking regions with priming sites approximately 30-150 bp away from each microsatellite repeat. A total of 14 primer pairs (Table 1) were selected from 30 primer pairs. Rather than use each forward primer with a fluorescent label, we adapted the M13-tailed microsatellite protocol of Boutin-Ganache et al. (2001) where each forward primer is 5' augmented with an M13 forward sequence (CACGACGTTGTAAAACGAC). This tailed primer was then used in combination with

a 6-FAM fluorescently labeled M13 primer. Thus, amplification reactions contained three primers: a forward M13 fluorescent primer, 5'-augmented microsatellite forward primer and an unmodified microsatellite reverse primer. Reactions consisted of 1 μL of DNA, 0.025 μL of the 10μM forward M13 tailed primer, 0.25 μL of the 10μM reverse primer, 0.45 μL of 10μM reverse primer, 1.25 μL of $MgCl_2$, 1.25 μL of PCR Buffer II (Perkin Elmer), 0.2 μL of a 10 μM dNTP mixture, 0.1 μL of taq and 8 μL of water for a total volume of 12.5 μL. The thermal profile was 94 °C for 2 min followed by 25 cycles of 94 °C for 30s, the primer specific annealing temperature (Table 1) for 30s and 68 °C for 30s, followed by a 3 min extension at 68°C.

Depending on the overall strength of the amplification reaction, PCR products were diluted 1:10 or 1:30 with water into a single plate for genotyping. One microliter of the bulk PCR dilution was added to a plate containing 0.09 μL of the GeneScan 500 LIZ genotyping standard and 9.91 μL of HiDi Formamide (Applied Biosystems). Reactions were genotyped on an ABI 3730XL automated sequencer and preliminarily scored using GENMAPPER 3.7 (Applied Biosystems). Final scoring of microsatellite alleles was verified by eye for each sample.

Variability of these 14 loci was assessed using 34 individuals from the Peninsular Florida and Southeastern Louisiana populations. Conditions and characteristics of the 14 polymorphic loci are given in Table 6. Population genetic parameters were estimated in Genepop version 3.4 (Raymond and Rousset 1995). The number of alleles per locus ranged from 5 to 29 (Table 1). The observed heterozygosity ranged from 0.452 to 0.889 and the expected heterozygosity ranged from 0.816 to 0.962. Seven loci (Dp 29, E5, C4 Dp 26, Dp19, H1 and C9) significantly deviated from Hardy-Weinberg equilibrium after

Bonferroni correction ($P < 0.0004$). This is likely due to a Wahlund effect and may represent additional population structure. No significant linkage association was found among the loci. The microsatellite loci developed here provide a powerful tool for determining species boundaries in the ringneck snake, analyzing intraspecific phenomena related to genetic sub-structuring of populations and determining the conservation status of imperiled populations.

Table 6 Characteristics of 14 polymorphic microsatellite loci in the ringneck snake (*Diadophis punctatus*).

Locus	Motif	Primer Sequence	Annealing Temp (°C)	Size Range (bp)	N_A	H_O	H_E
Dp 1	$(AC)_{18}$	F-CATCTTGTTCCAAGTTGGTGCC R-TGACAATGGACTAAGTTCAGCAGA	58	276-320	12	0.734	0.878
Dp 6	$(TG)_{23}$	F-AGGAGAACTGTGTTTGGAGC R-CATAAGACTGGATGAGATTTGGC	55	311-365	22	0.801	0.903
Dp 11	$(GCT)_{23}$	F-CAGCTCAGGACTGCGTTGAC R-ACCTCGACAGACTCGAACAC	52	254-262	5	0.773	0.884
Dp 29*	$(TG)_{29}$	F-GCCACTGGAACTGTTGGTGTG R-CATTCGTCCACTTGCTTGGTCC	50	168-190	13	0.452	0.867
E 5*	$(GT)_{24}$	F- ATCCAGTGATATTTGTTCCCAG R- AGATTTCAGGTTTGTCATCAGC	55	244-330	10	0.589	0.923
C 4*	$(AGGA)_{121}$	F- TTCTCATCCCACTCAAATCACA R- ACCTCCATCCTCTTTTCCTTTT	52	362-400	15	0.698	0.816
E 4*	$(GT)_{29}$	F- CACTGCTTGTGTTTGCTGCT R- TTAAGACATTCGTCCACTTGCT	58	313-376	17	0.812	0.836
H 2	$(AC)_{24}$	F- TTTCCCTTTGCTACTTTGCCTA R- CCTTTAATTGAGACAAGGCGAT	58	246-302	17	0.889	0.932
Dp 17	$(AGTAG)_5$	F- CCTGATCCAATTTGCCTTCATTC R- TCCTTGTGTGTCCAATCACAC	56	220-267	29	0.841	0.962
Dp 26*	$(AAGG)_{20}$	F- ATTGCCCAAACTCCCCAGCC R- ACCAGACCAGTTGCATGTAGAC	56	202-226	11	0.597	0.873
Dp 25	$(GAGAA)_{15}$	F- TGGCTCTCACATTGAGCTGAGG R- CCTCATCCCTATACATCCTGTTC	52	250-320	21	0.801	0.837
Dp 19*	$(AC)_{27}$	F- GACCTCAGATCATTTCCAGGA R- ATTAAGGCAGGATTGAGTGGC	57	310-375	21	0.658	0.934
H 1*	$(TGC)_{24}$	F- CTCTGTTTATGCAGCTCAGGAC R- AATTTCCAGATGATAGCAAGCC	58	362-404	24	0.773	0.919
C 9*	$(TG)_{29}$	F- CGAAGCAGAACATCCAGA R- GCACACTTACATTGCACACA	58	208-224	14	0.612	0.904

N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity. Asterisks denote loci showing significant deviation from HWE.

CHAPTER 5

Delimiting species boundaries in the genus *Diadophis* across Eastern North America

INTRODUCTION

Mechanisms that prevent populations from merging or that act to further isolate populations will undoubtedly contribute to the maintenance of biological diversity. These mechanisms can include geographical, environmental and genetic factors that result in the cessation of gene flow through some level of isolation eventually resulting in the accumulation of biodiversity. These processes often are tested in phylogeographic studies, which seek to uncover the historical process responsible for the contemporary geographical distribution of genealogical lineages. Usually, the patterns of gene flow from gene genealogies seems straightforward where there is a sharp geographic boundary between two widely distributed clades (Upton and Murphy 1997; Patton and de Silva 1998; Riddle et al 2000). In these instances, researchers assume that the genetic breaks result from geographic barriers to gene flow between species or cryptic species boundaries. These patterns are prominent in the eastern United States, where several recurrent phylogeographic breaks have emerged associated with the Appalachian Mountains, the Apalachicola River and the Mississippi River (Soltis et al 2006). These patterns occur both in plant and animal species and provides support for the phylogeographic generalizations attributable to isolation and differentiation.

In the absence of obvious geographic barriers, vicariance can be caused by rapid changes to the environmental conditions in a species range. When these changes occur faster than individuals' ability to adapt, the species ancestral range becomes fragmented (Weins 2004). As populations track their ancestral conditions, dispersal becomes limited between populations by the intervening unsuitable habitat resulting in a cessation of gene flow (Kozak and Weins 2006; Rissler and Apodaca 2007).

In the absence of either physical or ecological barriers, genetic barriers can arise due to hybridization and introgression. Hybridization typically refers to the interbreeding of two closely related species, suggesting that there is some type of inter-specific barrier that keep the taxa on diverging, independent evolutionary pathways (Harrison 1990; Arnold 1997). When hybridization continues over several generations it can result in the formation of a hybrid zone, a geographic region where steep character clines between otherwise homogenous populations form (Harrison 1993). In this context, the dynamic aspect of hybridization events is termed introgression, which refers to the movement of genes mediated by backcrossing between two species (Avise 2004).

Hybridization historically has been viewed as rare among animals, however we now know that hybridization is relatively wide-spread and in some instances has resulted in the origin of new species (Dowling and Secor 1997; Seehausen 2004; Mavarez et al. 2006). Hybrids are usually rare on an individual basis, but species undergoing occasional inter-specific hybridization are common. Existing surveys suggest that approximately 10% of animal species are known to hybridize with at least one other species (Mallet 2005). However, hybridization between species does not guarantee that genes will pass between species because hybrids are typically selected against. While there are numerous descriptions on the development and maintenance of hybrid zones, they can be classified under two broad based models. The geographical-selection-gradient model assumes that two closely related species are adapted to a different environment in allopatry and produce a zone of secondary contact following range expansion. This contact results in a hybrid zone formed through exogenous selection, involving adaptations to distinct environmental conditions by the two taxa (Endler 1977; Moore and

Price 1993). These zones are not necessarily stable, with the position, width, and maintenance being determined by the environmental conditions. In the second model, hybrid zones can form in parapatry or as the result of secondary contact, however the zone is not associated with an environmental gradient instead being maintained by endogenous selection (internal genetic incompatibilities). Barton and Hewitt (1985) termed this a "tension zone" because of the two opposing forces, dispersal of parental species towards the hybrid zones and selection against hybrids. Under both models, if the fitness of the hybrids is lower than both parental species, selection will work to either strengthen or maintain the reproductive isolating mechanisms.

Species boundaries and patterns of speciation have routinely been determined using mitochondrial data due largely to the reduced effective population size and short coalescence time compared to most nuclear genes. Held (2003) argued that species could be identified solely on mtDNA data if the population displayed certain characteristics such as a bimodal distribution of pairwise differences and high levels of genetic differentiation in areas of sympatry. However, the usefulness of mtDNA data for determining species boundaries is not universally accepted. Several have argued against the use of mtDNA haplotypes data because a non-recombining mode of inheritance always results in a bifurcating tree of ancestor-descendent relationships (e.g. Davis 1996). In some cases, these patterns have been found below the species level when there is strong geographical population structure, resulting in what appears to be several species throughout a range when in fact they are part of one species with extensive male-mediated gene flow (Irwin 2001; Roberts et al 2004). This phenomenon is likely intensified in species with limited dispersal capabilities. Additionally, the maternal

inheritance and lack of recombination complicate the determination of hybridizing taxa and the amount of gene flow between species.

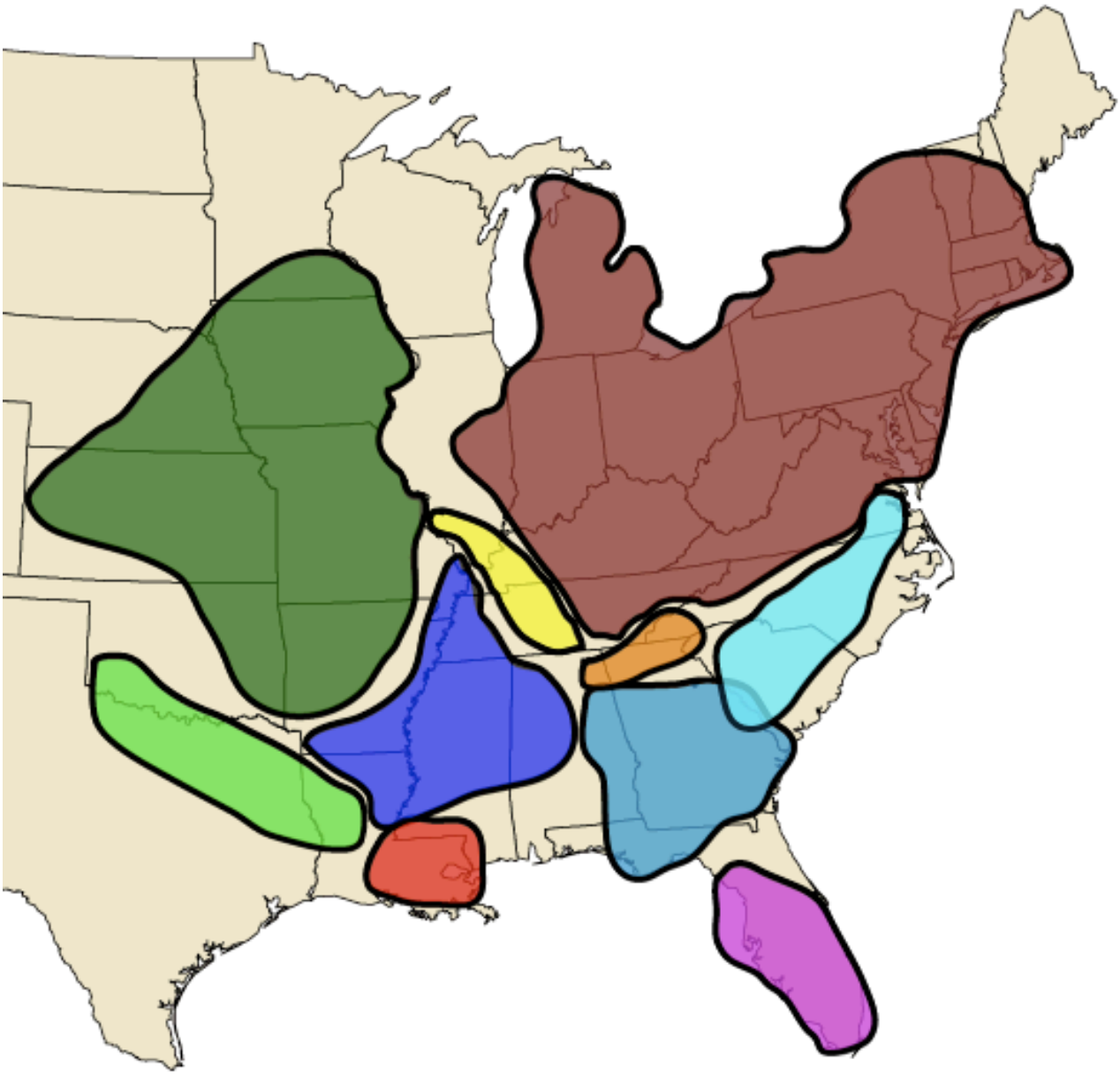
Microsatellites are popular markers for the study of gene flow and species delimitation because of their biparental inheritance and, unlike nuclear genes microsatellites have high levels of polymorphism (Jarne and Lagoda 1996). Microsatellites provide an easy way to screen multiple nuclear loci for variation between and within species. However, analysis of microsatellite data can be challenged by their complex mutation process (Palsboll et al 1999; Rubinsztein et al 1999), by the difficulty to distinguish identical-by-descent from identical-by-state alleles (Estoup et al 2002) and by their typically high mutation rates that may lead to problems of among-population differentiation (Hedrick 1999). Despite these concerns, the importance of assessing the congruence among genetic markers has been widely discussed (e.g. Avise 2000). New data sets can lead to independent corroboration (or rejection) of previously established hypothesis, and combined analysis of multiple loci can reduce the variance in the estimated parameters that is due to random effects of sampling and lineage sorting (e.g Takahata 1989; Hudson 1990). Also, contrasting results across markers, especially when those markers have distinct modes of inheritance, such as microsatellites and mtDNA, may provide new insights that could not be obtained with either type of data alone (Prugnolle and Meeus 2002).

Species that display phylogeographic breaks in the absence of obvious physical barriers or that display multiple secondary contact zones provide a unique opportunity to test the effects of the ecological factors and hybrid zones on speciation. The ringneck snake, *Diadophis punctatus,* has one of the largest geographic distributions of any species

of snake in North America. Ranging from central Mexico to southern Canada (excluding the northern Great Plains states), this snake exploits a variety of habitats and ecological niches ranging from northeastern mixed hardwood forests to the desert grasslands of the Southwestern US and Central Mexico (Stebbins 1985). Previous molecular studies using mtDNA inferred 16 well-defined lineages, several of which traverse previously identified genetic barriers for terrestrial vertebrates including the Appalachian Mountains, and the Mississippi and Apalachicola Rivers. These lineages appear to be confined to specific habitats (floodplains, grasslands, montane environments) resulting in several instances of secondary contact some of which appear to be associated with ecologic and physiographic transition zones. The vast majority of the genetic diversity and evidence of contact zones for these snakes occurs in the eastern United States. The southeastern United States is a geologically and topographically complex area characterized by extensive biodiversity.

Given the patterns of genetic diversity, we use 10 well-defined mtDNA lineages in the eastern United States (Fig. 26) to examine species boundaries in the ringneck snake *Diadophis punctatus* using species distribution modeling and novel microsatellite loci. We first determine the fundamental niche of each mtDNA lineage and assess whether these lineages are currently isolated by environmental factors. Using these methods, if the species distribution model closely matches the geographic range of a lineage, then the range is considered to a result of environmental factors. Conversely, if the predicted model extends well beyond the geographic range of the lineage, then factors other than environmental tolerances (such as competition) could be setting the range limit (Smith et al. 2005). We then use 10 novel microsatellite loci (Fontanella and Siddall 2009b) to

FIGURE. 26 Distribution of the 10-mtDNA lineages used in this study from Fontanella and Siddall (2009).



examine gene flow across putative genetic barriers. If lineages are not isolated by niche, we examine if areas of niche parapatry are acting as corridors for gene flow between lineages or if these areas coincide with hybrid zones.

MATERIALS AND METHODS

Ecological Niche Modeling

Ecological niche models for the eastern mtDNA lineages of *D. punctatus* were created using MAXENT version 3.0 (Phillips et al 2006). MAXENT creates species distributional models by combining presence only data with ecological layers under a statistical framework known as maximum entropy. This approach estimates a species' potential distribution by finding a probability distribution that is based on a distribution of maximum entropy. This method is equivalent to finding the maximum-likelihood distribution of a species based on the available information (Phillips et al 2004). MAXENT has been shown to perform better than other established methods (Phillips et al. 2004; 2006).

MAXENT analyses were run using point locality information from contemporary specimens, from natural history museum collections and the climatic layers downloaded from the WorldClim database with a 1 km resolution (Hijmans et al 2005). These variables represent summaries of means and variation in temperature and precipitation, and characterize the dimensions of climate considered particularly relevant in determining species distributions. Museum samples were assigned to a particular lineage if they placed inside the known range of an mtDNA lineage distribution. Samples that fell outside the currently know ranges were omitted. We recognize that the omission of these samples may bias the range estimation for lineages. We used the default

convergence threshold (10^{-5}) and maximum number of iterations (3000) values, using 10% of localities for model testing. Because phylogeographic breaks can form within a continuously distributed species even when there are no barriers to gene flow (Irwin 2002), background sampling points (100,000) were drawn from across the range of the genus *Diadophis* in the conterminous United States. We let the program select both suitable regularization values and functions of environmental variables automatically, which it achieves based on considerations of sample size. MAXENT outputs a continuous probability value, ranging from 0 to 1, an indicator of relative suitability for the species, based on the principle of maximum entropy, as constrained by the occurrence data. The predicted potential distribution for each lineage was displayed as a single category using the minimum training presence. We re-classed the MAXENT output files into binary partitions based on the LPT and used the overlay function in DIVA-GIS (Hijmans et al. 2001) to determine the size and shape of overlapping areas of suitable habitat between neighboring mtDNA lineages.

Molecular Methods

Sampling

We expanded our sampling from the previous studies, here examining a 325 specimens of *D. punctatus* collected from 10 previously described mtDNA lineages (Fontanella and Siddall 2009). Sampling within lineages ranged from 12 to 117 individuals. Sampling strategies focused on obtaining individuals near proposed secondary contact zones, however in several regions natural rarity limited our sample size. Tissues, consisting either of tail clips, liver or muscle tissue or shed skins were

stored in either 95% EtOH or a DNA storage buffer (4 M NaCl, 0.25 M EDTA ph 8.0, DMSO 20%).

Microsatellite Genotyping

Genomic DNA was isolated using the Dneasy Tissue Kit (QIAGEN). We selected ten microsatellite primers specifically designed for *D. punctatus* from Fontanella and Siddall 2009b). Each forward primer was augmented on the 5' end with an M13 forward sequence (CACGACGTTGTAAACGAC). This modified primer was then used in combination with the 5' FAM fluorescently labeled forward M13 primer in subsequent amplification reactions. Thus final PCR reactions consisted of 1 μL of DNA, 0.25 μL of unmodified reverse primer (10X), 0.025 μL of M13 tailed forward primer (10X), 0.45 μL of 6-FAM (Geneworks) fluorescently labeled M13 primer (10X), 1.25 μL of PCR Buffer, 1.25 μL of 25 mM $MgCl_2$, 0.2 μL of 10mM dNTP mixture, 0.1 μL of Taq and 8.0 μL of water for a total volume of 12.5 μL. The thermal profile was 95 ºC for 2 min followed by 30 cycles of 94 ºC for 30 s, the primer-specific annealing temperature and time (Table X), and 68 ºC for 30 s followed by a 4 min extension.

Depending on the overall strength of the amplification reaction, PCR reactions were diluted 1:10 or 1:30 with water into a single plate for genotyping. One microliter of the bulk PCR dilution was added to a plate containing 0.09 μL of the GeneScan 500 LIZ genotyping standard and 9.91 μL of HiDi Formamide (Applied Biosystems). Reactions were genotyped on an ABI 3730XL automated sequencer and automatically scored using GENOTYPER 3.7 (Applied Biosystems). Scoring of microsatellite alleles was verified by eye for each sample. MICRO-CHECKER software (Oosterbout *et al*. 2004) was used

to assess the presence of genotyping errors, such as non-amplified alleles, short allele dominance, and scoring of stutter peaks.

Microsatellite Loci Analysis

For each microsatellite locus, we calculated the total number of alleles, the range of allele sizes, total genetic diversity, and the number of private alleles to assess overall polymorphisms. Tests for departure from Hardy-Weinberg equilibrium and linkage disequilibrium were conducted using exact tests (Guo and Thompson 1992) with sequential Bonferroni corrections for multiple tests as implemented in FSTAT version 2.9.3.2 (Goudet 2001).

Geographical trends in the distribution of genetic diversity were investigated with Bayesian methods to infer population structure (Pritchard et al. 2000). Under these methods, the multilocus genotype data X are considered to be random draws from a parametric model, which describes their organization into clusters. The joint probability distribution $P(X,\theta)$ is determined over the data X, the model parameters and the missing data θ. The JPD consists of the prior distribution $P(\theta)$, which allows for prior information such as the geographical location of samples to be incorporated and the likelihood $P(X \mid \theta)$. The posterior distribution $P(\theta \mid X)$ of the model, conditional on the data, is then determined and used to infer model parameters such as the joint estimation of cluster membership and allele frequencies for the different clusters. Because θ consists of several multidimensional variables, computing $P(\theta \mid X)$ is usually not possible Therefore we generated an approximate sample of $P(\theta \mid X)$ using a Markov chain Monte Carlo simulation approach. The methods of Prichard et al. (2000) require multilocus genotype from unlinked genetic markers. In order to verify independence of our

microsatellite loci, we tested for linkage disequilibrium for all pairs of loci in each sampled using exact tests (Guo and Thompson 1992) with sequential Bonferroni corrections for multiple tests as implemented in GENEPOP version 4.0.7 (Raymond & Rousset 1995).

The model based clustering method in the program STRUCTURE (Pritchard et al 2000) probabilistically assigns individual multilocus genotypes to a user defined number of *K* clusters while maximizing linkage equilibrium within clusters. Because we were interested in assessing potential gene flow between lineages and not population structuring within lineages, we fixed *K*=10 based on the number of inferred mtDNA lineages (Fontanella and Siddall 2009). Three separate analyses were run for 3^6 iterations after burin in period of 1.5^6 without using any prior information on the population of origin for each individual. Because we were interested in examining potential gene flow between lineages, we used the admixture model with correlated allele frequencies. These parameters allow for the determination of hybrids by estimating the fraction of ancestry for each individual from each cluster.

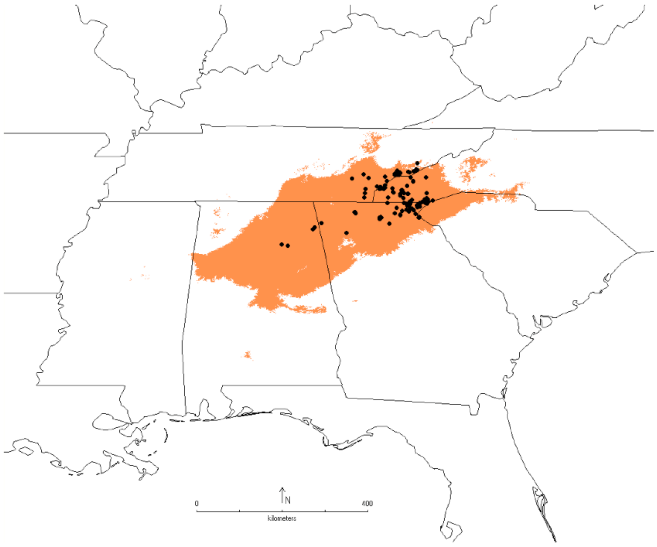
RESULTS

Ecological Niche Modeling

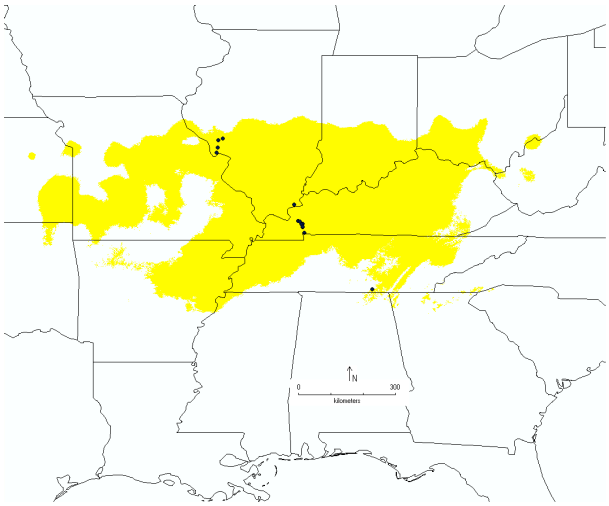
Most of the niche models (7 out of 10) showed predictions that extended well beyond the known range of the mtDNA distributions, with exceptions for the Southeastern Louisiana (SELA), Peninsular Florida and Mid-Atlantic lineages (Fig. 27). The latter predictions suggest that these distributions may be maintained by climatic gradients. However in all cases, the climatic niche models show that borders of the predicted ranges for neighboring lineages appear to be broadly suitable for both lineages. These predictions

suggest additional contact zones not inferred based on the mtDNA data due to limited sampling. Additionally, climatic models show suitable habitat along multiple borders for lineages that are not isolated by a physical barrier. These overlapping patterns suggest that the range limits of these lineages are set by factors other than climatic tolerances, such as inter-lineages interactions or possible hybrid zones. Overlaying the predictions of all ten lineages depicts that the central portion of the eastern United States as a large area of suitable habitat for several mtDNA lineages (Fig. 27), which may facilitate gene flow between lineages. Individuals moving into the range of the other species are more likely to mate with the other species than their own, simply because heterospecifics are much more common. If the offspring of hybrid matings are viable and fertile, they can breed successfully and thereby generate gene flow between the two species.

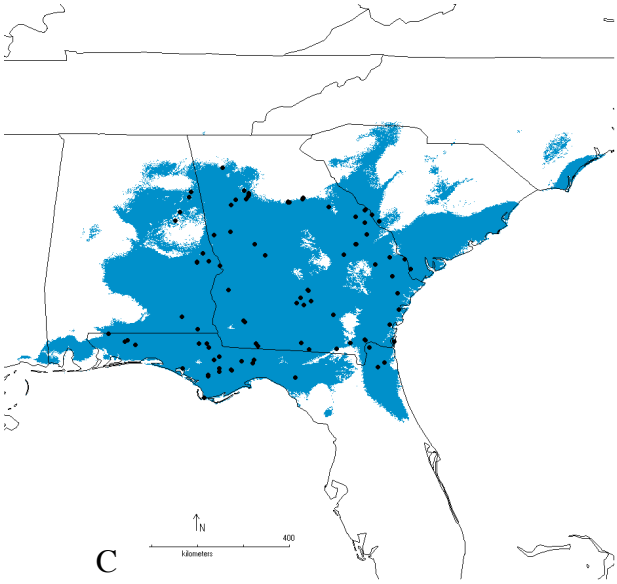
FIGURE. 27. Predicted geographic distribution lineage based on ecological niche modeling for the A) Cumberland lineage, B) Western Kentucky lineage, C) Piedmont lineage, D) Peninsular Florida


N
0
400
kilometers

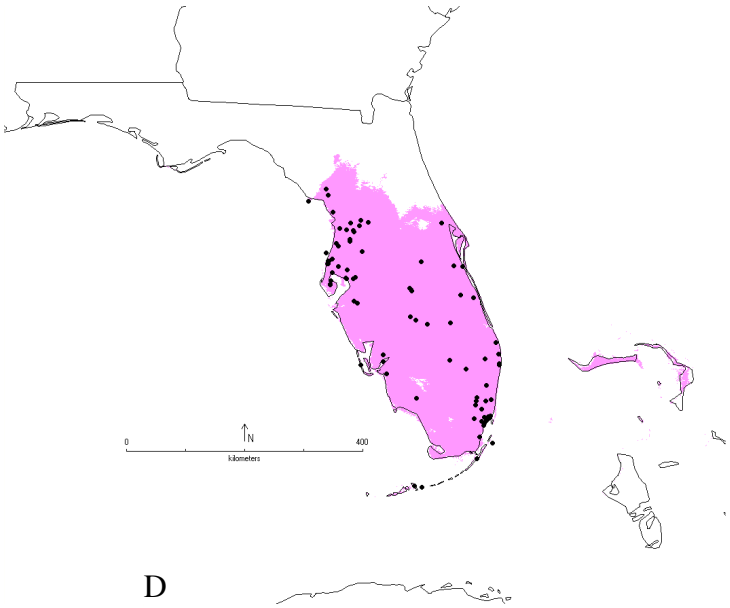
A


N
0
300
kilometers

B

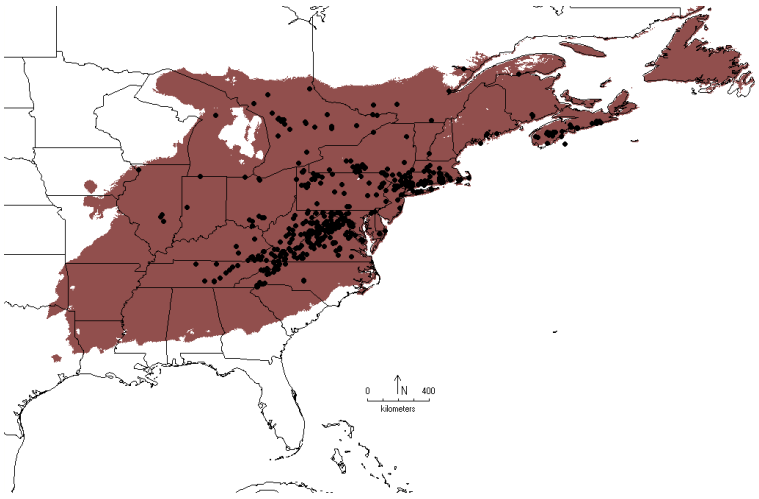

N
400
kilometers

C

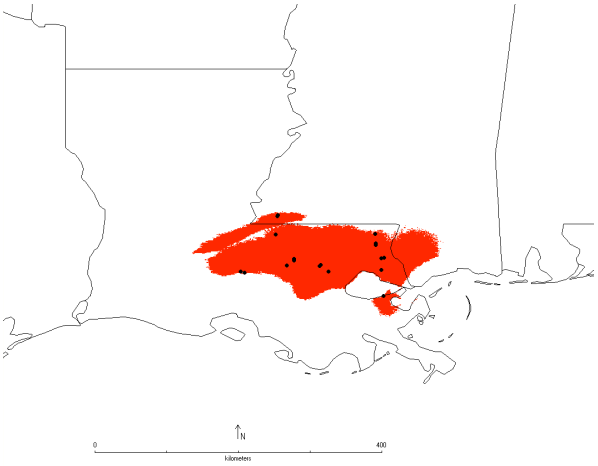

N
0
400
kilometers

D

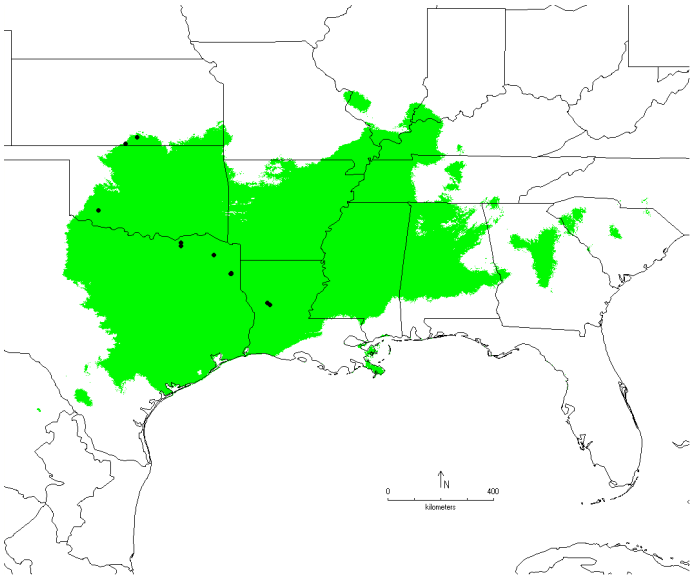
FIGURE. 27 continued. Predicted geographic distribution lineage based on ecological niche modeling for the E) Northeast lineage, F) Southeast Louisiana lineage, G) Western Louisiana lineage, H) Mid-Atlantic lineage.


0
N
400
kilometers

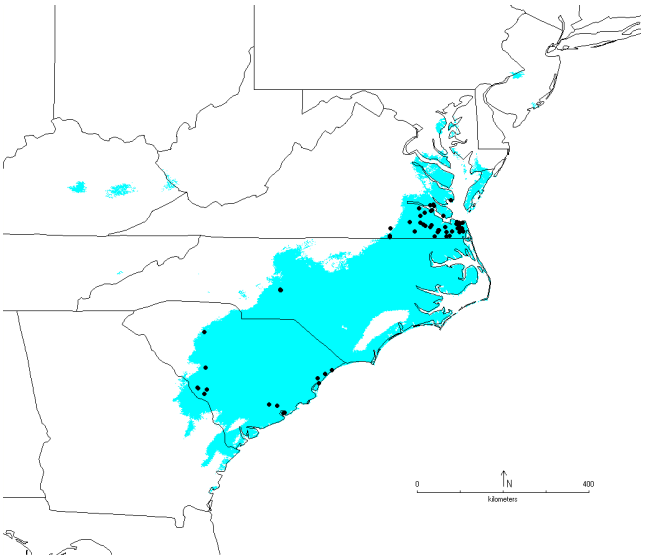
E


N
0
400
kilometers

F

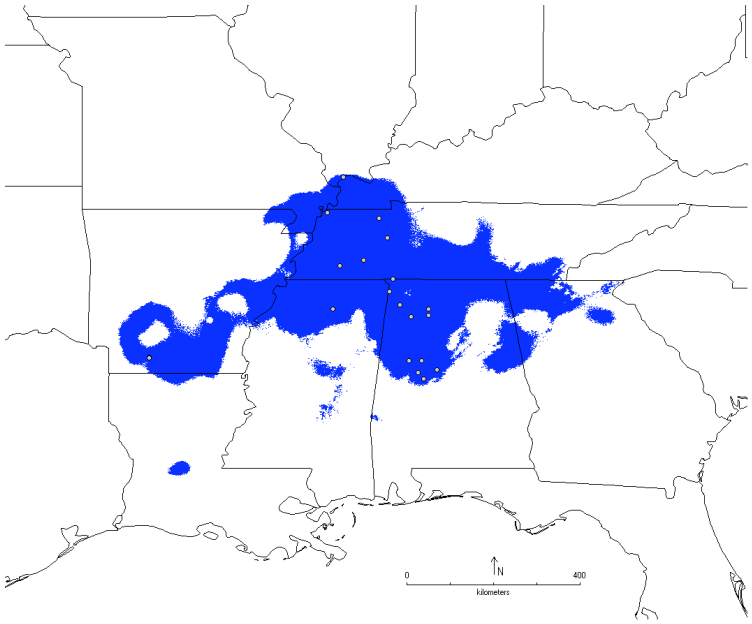

N
0
400
kilometers

G

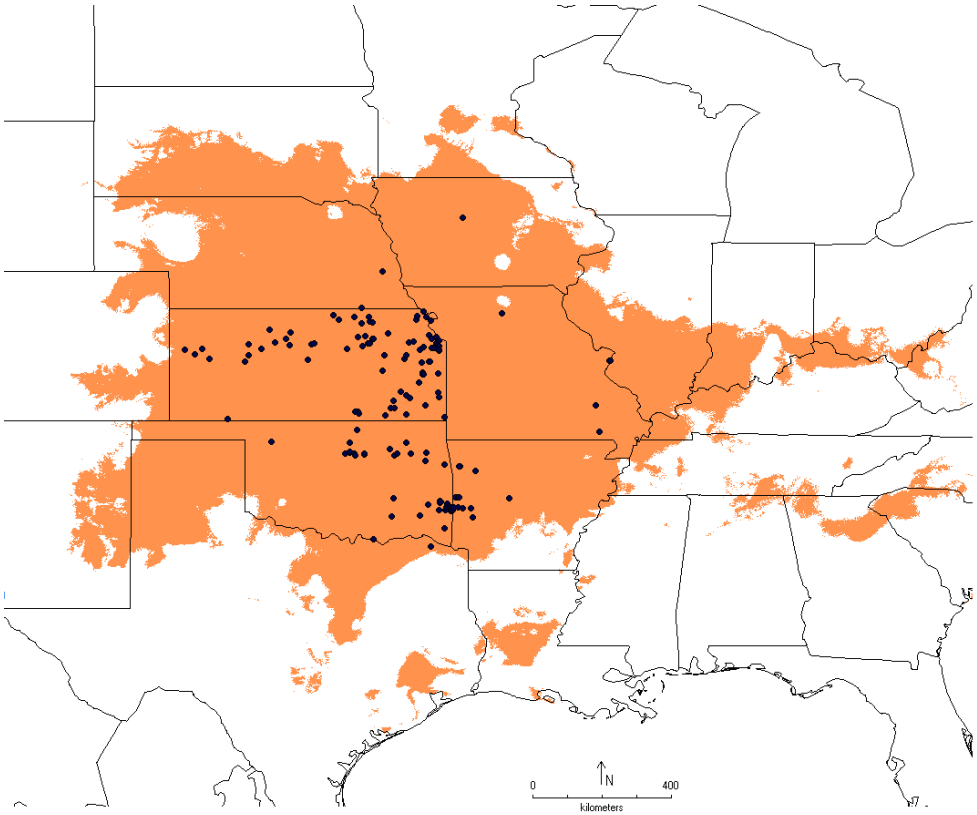

N
0
400
kilometers

H

FIGURE. 27 continued. Predicted geographic distribution based on ecological niche modeling for the I) Mississippi River Valley lineage and J) Great Plains lineage.

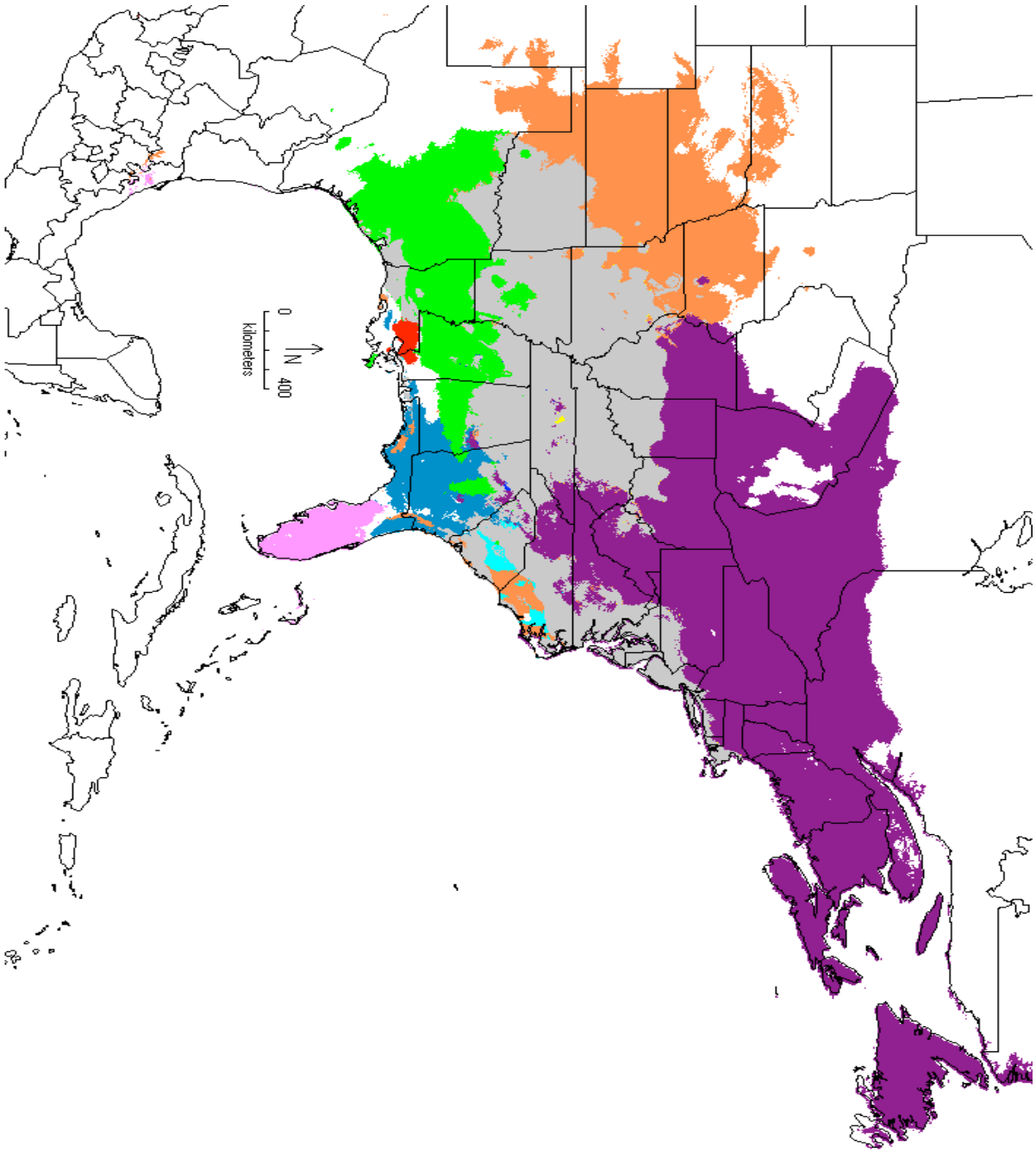


I



J

FIGURE. 27. Map of all ten species distribution models from MAXENT. Colors correspond to lineages in Fig. 26. Areas of overlapping niche space between lineages are shaded in grey.


0
kilometers
N
400

Microsatellite Analyses

Null alleles were detected for loci C4, H2 and Dp 26 in the Piedmont lineage. In each case, re-amplification under less stringent reaction conditions did not produce any readable genotype. One locus (E5), had fixed allele sizes within the Peninsular Florida and Great Plains lineages. Significant deviations from Hardy-Weinberg Equilibrium (HWE) were detected for all loci following a sequential Bonferroni correction. Deviation from HWE can be caused by several factors including null alleles (Dankin and Avise 2004), which were present in the Piedmont lineage, inbreeding or population sub-structuring (Wahlund effect). Analysis of population level structure within each lineage using STRUCTURE suggested that each lineage is comprised of at least four sub-populations (data not shown).

Levels of expected heterozygosity for ten microsatellite loci ranged from 0.42 to 1.0 in the ten lineages. The total number of alleles at each locus was high (64-120) and each lineage contained 0-24 alleles/locus (Table 7). Lineage level and global tests of linkage disequilibrium based on genotype frequencies failed to detect any instances of significant linkage among the ten microsatellite markers, supporting locus independence.

Mean expected heterozygosity computed over all lineages was considerably high (0.766). Exact tests of allele frequencies showed significant differences between all lineage pairs (Fisher's exact test, $P < 0.05$) both at individual loci and over all loci combined. The global F_{ST} value of 0.147 ($P < 0.05$) depicts significant differentiation between lineages as a whole. Pairwise F_{ST} comparisons ranged from 0.007 to 0.29. Comparisons between lineages were significantly different following Bonferroni corrections ($P < 0.05$) with the exception of the Mississippi River Valley, which was not

significantly different from any lineage and comparisons between Southeastern Louisiana and West Louisiana (Table 8).

The STRUCTURE analyses of the 10-microsatellite loci depicted varying levels of gene flow and hybridization between the mtDNA lineages (Fig 28). The Peninsular Florida, Great Plains, and Piedmont lineages did not show evidence of introgression with any of the other mtDNA lineages. The individuals in each of these lineages had values of $Q > 90$. In the SELA lineage 66% of the individuals had values of $Q > 90$. The remaining 34% showed shared alleles with the Mississippi River Valley and Western Louisiana lineages. In the Mid-Atlantic lineage 38% of the individuals had values of $Q>90$, while the remaining 62% showed shared ancestry with either the Piedmont lineage or the Peninsular Florida lineage. With the exception of one individual, which showed shared ancestry with the Mid-Atlantic lineage, all individuals from the Cumberland lineage showed $Q > 90$. In the Northeast lineage 87% of all individuals had $Q > 90$, with the remaining 13% having shared ancestry with the Cumberland, Western Kentucky or Mid-Atlantic lineages. The Western Louisiana lineage had two individuals that showed putative hybridization with the SELA lineage. The Mississippi River Valley lineage did not have any individuals that had values of $Q > 90$, instead every individual showed some level of hybridization with either the Piedmont, Western Louisiana, or SELA lineage. Pie charts showing the proportion of ancestry for all individuals with $Q < 90$ were mapped over the geographic range

Table 7. Expected (H_E) and observed (H_O) heterozygosities, total number of alleles (A_N), average number of private alleles (A_P), for ten mtDNA lineages of *D. punctatus* at ten microsatellite loci.

Lineage		19	H1	25	E4	26	H2	17	E5	C6	C4	Ave
SELA	H_O	0.75	0.69	0.85	0.79	0.73	0.77	0.71	0.84	0.81	0.88	0.78
	H_E	0.91	0.92	0.94	0.91	0.88	0.89	0.96	0.90	0.94	0.96	0.92
	N_A	16	18	21	14	13	17	23	13	20	20	21.6
	A_P											2.66
Piedmont	H_O	0.56	0.71	0.48	0.81	0	0	0.77	0.89	0.81	0	0.72
	H_E	0.97	0.93	0.94	0.96	0	0	0.99	0.93	0.90	0	0.95
	N_A	16	12	17	14	0	0	19	10	12	0	14.2
	A_P											NA
Pen FL	H_O	0.66	0.81	0.72	0.55	0.63	0.59	0.66	1	0.84	0.51	0.70
	H_E	0.78	0.91	0.82	0.81	0.70	0.82	0.97	1	0.90	0.72	0.84
	N_A	9	13	10	11	5	9	15	1	12	4	8.9
	A_P											2.20
Mid-Atl	H_O	0.67	0.55	0.48	0.66	0.45	0.49	0.72	0.68	0.61	0.47	0.58
	H_E	0.82	0.76	0.76	0.88	0.71	0.85	0.83	0.75	0.65	0.78	0.78
	N_A	9	10	5	9	5	7	11	6	9	5	7.6
	A_P											1.92
Cumberland	H_O	0.77	0.88	0.56	0.66	0.47	0.71	0.70	0.74	0.62	0.71	0.68
	H_E	0.92	0.95	0.67	0.78	0.87	0.76	0.83	0.82	0.87	0.79	0.83
	N_A	11	10	6	6	7	6	8	7	7	8	7.6
	A_P											2.02
Northeast	H_O	0.45	0.34	0.56	0.44	0.48	0.45	0.55	0.62	0.68	0.81	0.54
	H_E	0.95	0.92	0.93	0.90	0.91	0.66	0.91	0.81	0.92	0.90	0.88
	N_A	21	24	22	18	17	12	13	8	15	12	16.2
	A_P											2.72
WKY	H_O	0.71	0.79	0.67	0.61	0.59	0.77	0.69	0.78	0.72	0.33	0.66
	H_E	0.94	0.89	0.85	0.78	0.79	0.81	0.84	0.92	0.82	0.42	0.81
	N_A	11	9	9	7	7	6	7	8	7	4	7.5
	A_P											1.88
MRV	H_O	0.91	0.80	0.83	0.90	0.89	0.79	0.76	0.77	0.67	0.55	0.79
	H_E	0.97	0.99	0.92	0.92	0.99	0.89	0.94	0.95	0.98	0.96	0.95
	N_A	9	10	5	9	5	7	11	6	9	5	7.6
	A_P											2.00

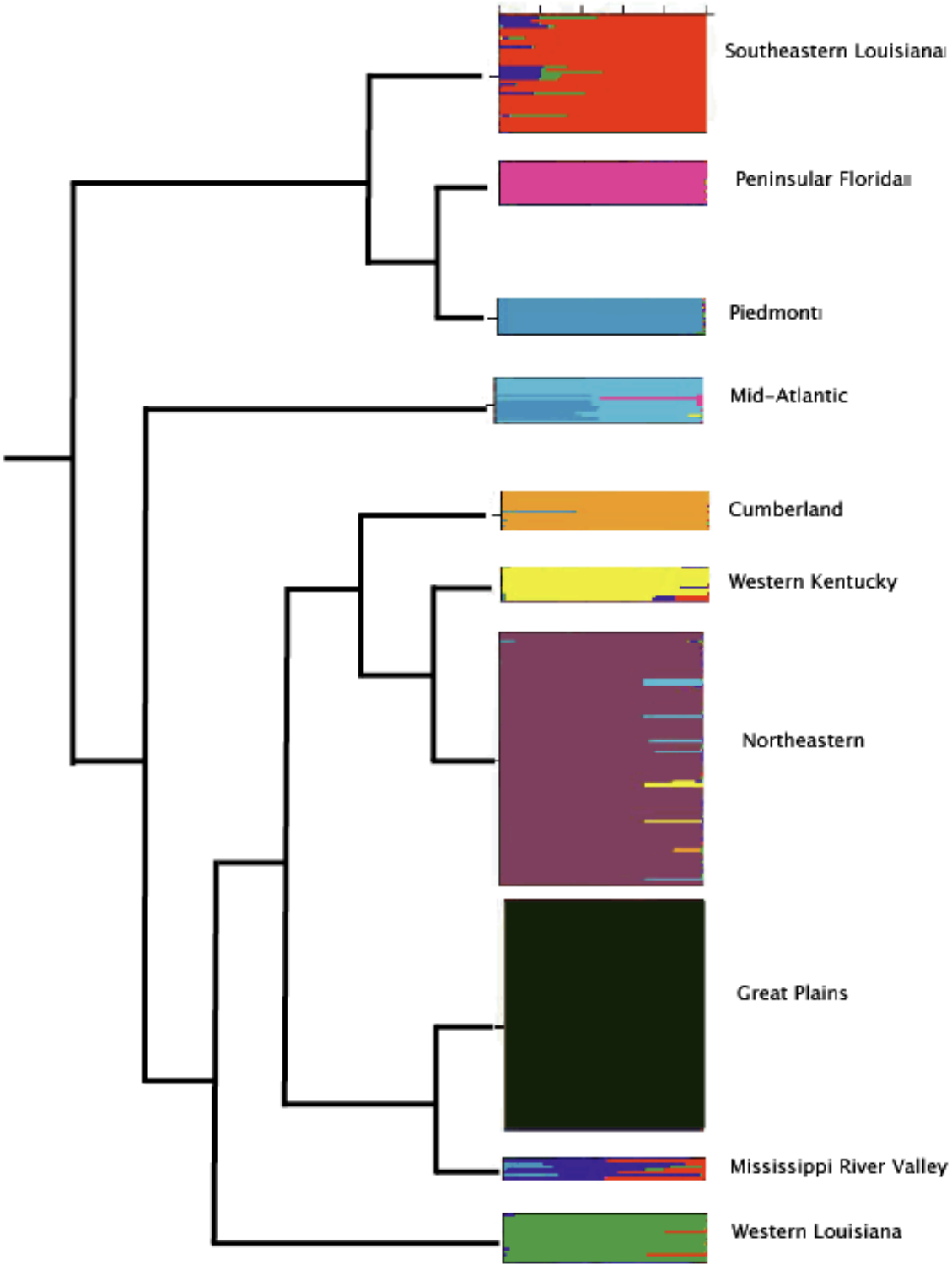
Table 7 continued

Lineage		19	H1	25	E4	26	H2	17	E5	C6	C4	Ave
Great Plains	H_O	0.65	0.53	0.60	0.59	0.67	0.67	0.78	1	0.78	0.71	0.70
	H_E	0.79	0.71	0.91	0.85	0.73	0.72	0.89	1	0.84	0.81	0.83
	N_A	8	5	10	6	4	4	8	1	7	7	6.0
	A_P											2.81
W LA	H_O	0.66	0.59	0.61	0.71	0.52	0.67	0.44	0.63	0.59	0.61	0.60
	H_E	0.93	0.96	0.82	0.84	0.80	0.85	0.69	0.92	0.87	0.71	0.84
	N_A	9	9	5	6	3	5	4	4	5	4	5.4
	A_P											1.45

Table 8. Results for Pairwise F_{ST} values for lineage differentiation between 10 mtDNA lineages. Asterisks indicate significance following a Bonferroni correction (P > 0.05)

	SELA	WLA	MRV	WKY	Pen FL	Mid Atl	Cumb	NE	GP	Pied
SELA	0	0.074	0.007	0.095*	0.128*	0.114*	0.095*	0.079*	0.154*	0.185*
WLA		0	0.072	0.713*	0.115*	0.108*	0.165*	0.129*	0.132*	0.170*
MRV			0	0.108	0.146	0.126	0.101	0.064	0.160	0.207
WKY				0	0.179*	0.171*	0.155*	0.111*	0.231*	0.260*
Pen FL					0	0.190*	0.188*	0.154*	0.252*	0.290*
Mid-Atl						0	0.146*	0.130*	0.241*	0.274*
Cumb							0	0.098*	0.210*	0.249*
NE								0	0.172*	0.195*
GP									0	0.291*
Pied										0

FIGURE. 28. Phylogenetic tree based on mtDNA sequence data with STRUCTURE plot as terminal taxa. Colors in STRUCTURE plot correspond to Fig. 26.


Southeastern Louisiana
Peninsular Florida
Piedmont
Mid-Atlantic
Cumberland
Western Kentucky
Northeastern
Great Plains
Mississippi River Valley
Western Louisiana

Discussion

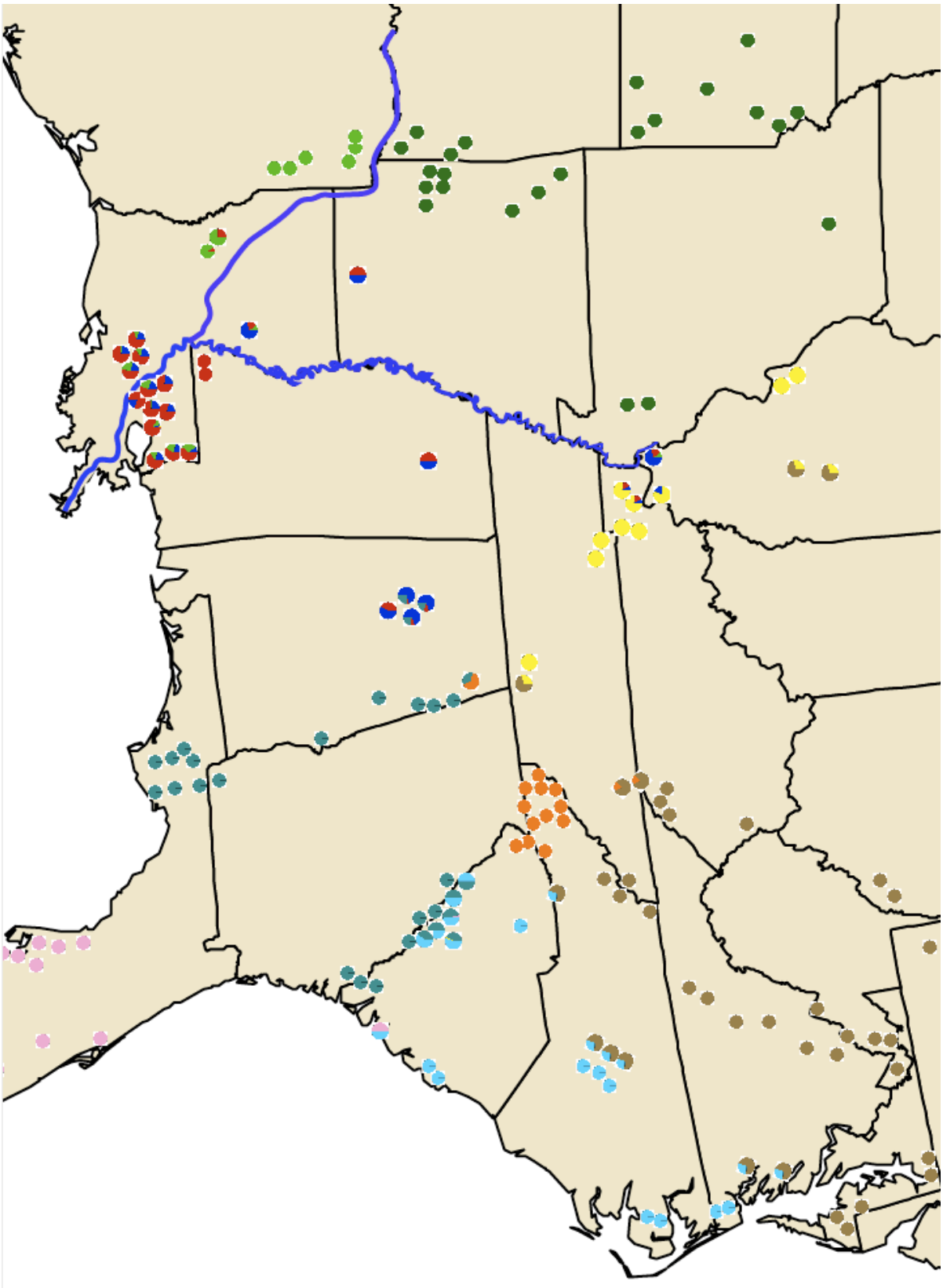
Hybrid Zones and Contemporary Admixture

While the microsatellite data show that these species are not reproductively isolated, for the most part, the hybrid individuals appear to be confined to narrow regions along the secondary contact zones between lineages (Fig. 29). The absence any putative hybrids beyond the contact zones indicate that areas of niche parapatry are not acting as corridors for gene flow or dispersal between species. In most instances, the width of the hybrid zone is narrower than the area of niche parapatry, implying that these represent tension zones maintained by a balance between dispersal and selection against hybrids and not an adaptation to environment (Barton and Hewitt 1985). In tension zones, hybrids are typically less fit because their new combinations of alleles are untested by selection (Barton 2001). Hybridization therefore imposes a genetic load on populations in the zone through gene-gene interactions in addition to the gene-environment migration loads associated with environmental margins. Both types of load will lead to a reduced fitness and population density, thereby reducing the chance that locally beneficial alleles will arise within the zone. The decreased fitness, often in the form of hybrid sterility, forms a barrier to gene flow by forming a hybrid sink, stopping the movement of alleles across the zone and into the parental populations. While the patterns inferred by the current data are consistent with the predictions of isolation by tension zones, extensive sampling will be needed in order to further test these hypotheses.

The inference of contemporary gene flow between the Southeastern Louisiana and Mississippi River Valley lineage is disconcerting. Contrary to the patterns described above for other species of *Diadophis*, hybrid individuals are distributed throughout both

geographic ranges, evidence of gene flow or dispersal beyond a tension zone or hybrid barrier (Fig. 29). While evidence of contemporary gene flow between sister lineages is becoming more evident (Shaw 2002; Kembert 2008), the appearance of shared alleles between distantly related lineages is rare and may have several explanations. The simplest explanation for the appearance of gene flow between distantly related lineages could be attributed to homoplasy. Because of the rapid evolutionary pace of microsatellites and the underlying nature of their mutational processes, alleles that are identical in size (state) are not necessarily identical by descent (Estoup et al 2002). Therefore, the presence of similar allele sizes in distantly related species could be due to convergence and not contemporary gene flow. In this the case however, homoplasy could then be used to explain every instance of putative gene flow between lineages, regardless of their time since divergence. If the mtDNA lineages are "valid" species, these data suggest that the process of divergence that ultimately results in reproductively isolated species can be prolonged. The fact that genomes can remain open to gene flow long after the speciation process is initiated greatly expands the novelty that can be generated from introgression. Based on these data, some portion of the genetic diversity in *Diadophis* has likely arisen from introgressive hybridization. If hybridization is maintained between distantly related species, introgression has the potential to provide the plethora of genetic variation required to drive species radiations (Arias et al 2008). For many organisms, introgression may allow for the continued exchange of genetic material necessary for adaptive change.

FIGURE. 29. Map with pie charts showing estimated proportion of ancestry from STRUCTURE plots showing individuals with values of $Q < 90$. Pie chart colors correspond to Fig. 26.



CONCLUSIONS

For the purposes of species delineation, we demonstrate that DNA sequence data, particularly those derived from the mitochondria, can provide initial indications of species, but are insufficient if used alone. Evidence for the restricted gene flow between the lineages based on morphology, ecology, behavior or independent molecular data are necessary. Although species distribution modeling does not show isolation due to unsuitable habitat, the microsatellite data suggest that most species boundaries are maintained through hybrid zones. Despite limited sampling, these zones appear to be contained to contact zones and maintained by a balance of dispersal and selection as opposed to ecological factors. In most cases, "pure" individuals were present in the area of niche parapatry but beyond the putative hybrid zone. The microsatellite data also suggest extensive gene flow and dispersal between two distantly related, geographically neighboring mtDNA lineages. While further study is needed in this region, given the amount of contemporary gene flow, these mtDNA lineages would not be considered "species" under any current species concept. The use of mtDNA alone has been cautioned against because the evolutionary rates of various genes are vastly different; some genes may reach coalescence within populations while others may remain non-coalescent even at the species level. The relative short coalescent times of mitochondrial genes provide an excellent starting point for species delimitation. However, maternal inheritance, potential male biased dispersal and the bifurcating nature of trees can obfuscate patterns of continued gene flow between mtDNA lineages, as well as the existence and position of hybrid zones.

Frequency data from multiple nuclear gene loci, particularly microsatellites or single nucleotide polymorphisms, provide a better source of data for inferring gene flow between proposed species groups and across proposed genetic barriers. If it is generally true that closely related species are often permeable to introgression, the nature of such species, their ecology, causes of speciation (Arnold 1997; Machado 2002), phylogeny reconstruction (Kliman 2000) as well as conservation issues concerning hybridizing populations (Allendorf 2001) all require re-evaluation. In particular, phylogenetic reconstruction and diagnostic tests for species status employing single genes must be used with great caution (Schluter 2000; Seehausen 2003; Verheyen et al. 2003).

CHAPTER 6

Summary

This study has provided the first range-wide molecular study and provides a framework for the origin, evolution and biodiversity in the genus *Diadophis*. Before the initiation of this study, our knowledge and understanding of the evolutionary history and biodiversity within the genus *Diadophis* was limited to two range-wide morphological studies (Blanchard 1942; Hibbitts 1994). These studies suggested that *Diadophis* originated in Mexico and diversified north along the Mexican Plateau into North America. While the two studies differ on the number of species, localized works (Trauth 1996) focusing on morphology found that several of the morphological characters used by Blanchard (1942) represent clinal variation. Due to the lack of fixed diagnostic morphological characters, all taxa were relegated to 13 subspecies of *D. punctatus* (Stebbins, 1985; Connant and Collins 1991).

The extensive variation in morphological characters led to the use of molecular data in the more recent studies (Pinou et al 1995; Feldman 2000refs). Estimates of amino acid differences between population samples in the Maryland versus California suggest that *Diadophis* may not be monotypic and may contain at least two genetically distinct species (Pinou et al 1995). Feldman (2000) examined phylogeographic patterns of *Diadophis* throughout California using the mitochondrial gene ND-4. While some phylogeographic structure was apparent, there was no geographic division or morphological characters (Hibbitts, 1994) to support the recognition of subspecies from California as described by Blanchard (1942). The results from Feldman (2000) and Pinou (1995) both suggest that *Diadophis* does not merely comprise a single species.

In this dissertation, analysis of mtDNA and ancestral area reconstructions (Chapter 3) inferred an origin for *D. punctatus* in the southeastern United States followed

by a southeast to northeast then westward directionality of historical migration. Whereas morphological studies may have previously (ref) suggested an origin on the Mexican plateau, the position within the phylogeny and date estimate for the southwestern + Mexico clade suggests a recent invasion into central Mexico with expansion into the Nearctic/Neotropic transition zone (Chapter 3).

Molecular dating suggests that the genus *Diadophis* originated during the late-Miocene (~6.7 mya), with the extant lineages diverging during the Pleistocene (Chapter 3). These data suggest each lineage arose prior to the last glacial maximum (~18 kya) and were therefore affected by changing climatic cycles. With the exception of the fragmented populations throughout the southwest, demographic analysis suggest post-glacial expansion for each of the four northernmost lineages. However, the estimates of time since expansion suggest that factors leading to population growth differed between regions. In the Eastern and Central U. S., advancing Pleistocene glaciers drove northern populations into southern refugia promoting intra-specific lineage diversity. As glaciers receded, lineages in relatively northern refugia appear to have expanded into previously glaciated areas, while poor dispersal abilities and possibly competition restricted the expansion of southern lineages. The general warming trends that led to the recession of glaciers caused fundamental ecosystem-wide changes across North America . In accordance with these changes, demographic patterns suggest that the northern California lineages expanded with the spread of suitable habitat spread during the Holocene (Chapter 2).

With respect to the current estimates of diversity, if subspecies described from morphological or geographic descriptions are to be considered as valid taxonomic

entities, then the genetic differentiation that underlies this variation should exhibit similar if not concordant patterns of evolutionary history (Burbrink *et. al.* 2000; Janzen *et. al.* 2004). None of the morphologically based subspecies designations of *D. punctatus* evaluated in this study are valid based on the molecular data. With the exception of California, the geographic range for each subspecies is composed of multiple well-supported mtDNA lineages. The phylogeographic patterns uncovered within *D. punctatus* and their concordance with putative genetic barriers suggests that the current single-species taxonomy underestimates the species level diversity and obscures the biogeographic history of this group. While many of these lineages appear to be isolated into different physiographic regions, others span previously identified genetic barriers or have distributions that result in secondary contact zones (Chapter 2). Such zones are of particular interest because they can provide important information about the interaction between lineages, how populations merge or diverge the factors that maintain species boundaries.

The combination of species distribution modeling and microsatellite loci suggests that while not reproductively isolated, putative hybrid zones maintain species in the eastern United States (Chapter 5). The position and width of these zones within broad areas of niche parapatry suggests they are likely controlled by a combination of selection and dispersal. The presence of shared alleles between the divergent South Eastern Louisiana and Mississippi River Valley lineages provides a unique opportunity to examine the effects of historical introgression on species and species boundaries (Chapter 5).

Although the results from this study have provided of the most extensive information to date regarding the origin, diversification and genetic diversity of *Diadophis*, several questions remain unanswered. This body of work provides a rich framework for further study including, examining species boundaries in the western lineages, determining population structure within species, and detailed examination of hybrid zones.

LITERATURE CITED

Adams CC. 1902. Southeastern United States as a center for geographical distribution of flora and fauna. *The Biological Bulletin* **3**: 115–131.

Akaike H. 1973. Information theory and an extension of the maximum–likelihood principle. In: Petrov BN, Csaki F, eds. *Second International Symposium on Information. Theory, Tsahkadzor, Armenia, USSR*. Budapest: Akademiai Kiado.

Alexander LF, Riddle BR. 2005. Phylogenetics of the new world rodent family Heteromyidae. *Journal of Mammalogy* **86**: 366–379.

Alfaro ME, Arnold SJ. 2001. Molecular systematics and evolution of *Regina* and Thamnophine snakes. *Molecular Phylogenetics and Evolution* **21:** 408–423.

Allendorf FW, Leary RF, Spruell P, Wengurg JK. 2001. The problems with hybrids: setting conservation guidelines. *Trends in Ecology and Evolution,* **16:** 613-622.

Al-Rabab'as MA, Williams CG. 2002. Population dynamics of *Pinus taeda* L. based on nuclear microsatellites. *Forest Ecology and Management,* **163:** 1109-1123.

Anderson E. 1949. *Introgressive hybridization*. John Wiley and Sons, New York, NY, USA.

Aranda M, Escobedo JE, Pozo C. 1997. Registros recientes de *Otonychtomys hatti (*Rodentia: Muridae) en Quintana Roo, Mexico. *Acta Zoologica Mexicana (nueva serie)* **72:** 63–65.

Arevalo E, Davis SK, Sites JW. 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. *Systematic Biology* **43**: 387–418.

Arnold ML. 1997. *Natural Hybridization and Evolution*. Oxford University Press, New York

Ashton KG, de Queiroz A. 2001. Molecular systematics of the western rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-loop in phylogenetic studies of snakes. *Molecular Phylogenetics and Evolution* **21:** 176-189.

Austin JD, Lougheed SC, Boag PT. 2004. Discordant temporal and geographic patterns in maternal lineages of eastern north American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae). *Molecular Phylogenetics and Evolution* **32:** 799-816.

Avise JC. 2000. *Phylogeography. The history and formation of species*. Harvard University Press, Cambridge, Massachusetts.

Avise JC. 2004. *Molecular Markers, Natural History, and Evolution,* 2nd edn. Sinauer & Associates, Sunderland, Massachusetts

Avise JC, Walker D. 1998. Pleistocene phylogeographic effects on avian populations and the speciation process. *Proceedings of the Royal Society of London Series B* **265:** 457-463.

Avise JC, Walker D, Johns GC. 1998. Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proceedings of the Royal Society of London Series B* **265:** 1707-1712.

Axelrod DI. 1980. Holocene climatic changes in relation to vegetation disjunction and speciation. *American Naturalist* **117:** 847-870.

Barton NH. 2001. Adaptation at the edge of a species' range. In *Integrating Ecology and Evolution in a Spatial Context*. (Silverton J and Antonovics J. eds). 365-392, Blackwell.

Barton NH, Hewitt GM. 1985. Analysis of hybrid zones. *Annual Review of Ecology and Systematics,* **6**, 113-148.

Beamer DA, Lamb T. 2008. Dusky salamanders (*Desmognathus,* Plethodontidae) from the Coastal Plain: Multiple independent lineages and their bearing on the molecular phylogeny of the genus. *Molecular Phylogenetics and Evolution* **47**: 143–153

Berendzen PB, Simons AM, Wood RM. 2003. Phylogeography of the northern hogsucker, *Hypentelium nigricans* (Teleostei: Cypriniformes). Genetic evidence for the existence of the ancient Teays River. *Journal of Biogeography* **30:** 1139-1152.

Bertorelle G, Slatkin M. 1995. The number of segregating sites in expanding human populations, with implications for estimates of demographic parameters. *Molecular Biology and Evolution* **12:** 887-892.

Blanchard FN, Gilreath M, Blanchard F. 1979. The eastern ring–neck snake (*Diadophis punctatus edwardsii*) in northern Michigan. *Journal of Herpetology* **13:** 377–402.

Blanchard FN. 1942. The Ringneck Snakes, Genus *Diadophis*. *Bulletin of the Chicago Acadademy of Science*. **7**: 1-144.

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF. 2001. M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *Biotechniques*, **31:** 53-54.

Brandley M, Schmitz A, Reeder T. 2005. Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid Lizards. *Systematic Biology* **54:** 373-390.

Brant SV, Orti G. 2003. Phylogeography of the Northern short-tailed shrew, *Blarina brevicauda* (Insectivora: Soricidae): past fragmentation and postglacial recolonization. *Molecular Ecology*, **12:** 1435-1449.

Brown JM, Abrahamson WG, Way PA. 1996. Mitochondrial DNA phylogeography of host races of the Goldenrod ball gallmaker, *Eurosia solidaginis* (Diptera: Tephritidae). *Evolution* **50:** 777-786.

Brown PA, Kennett JP. 1998. Megaflood erosion and meltwater plumbing changes during last North American deglaciation recorded in Gulf of Mexico sediments. *Geology* **26:**599-602.

Brunsfeld SJ, Sullivan J, Soltis DE, Soltis PS. 2001. Comparative phylogeography of northwestern North America: a synthesis. In: *Integrating Ecology and Evolution in a Spatial Context* (eds Silvertown J, Antonovics F), pp. 319-339. Blackwell Science, Oxford.

Burbrink FT, Lawson R, Slowinski JB. 2000. Mitochondrial DNA phylogeography of the polytypic North American ratsnake (*Elaphe obsolete*): a critique of the subspecies concept. *Evolution* **54:** 2107–2118.

Burt WH, Grossenheider RP. 1976. *Field Guide to the Mammals: North America North of Mexico*. Third edition. Houghton Mifflin Company, Boston MA, USA.

Butler RS, Mayden RL. 2003. Cryptic biodiversity–rich biodiversity in the southeastern waters of the United States. *Endangered Species Bulletin* **28:** 24–26.

Calsbeek R, Thompson JN, Richardson JE. 2003. Patterns of molecular evolution and diversification in a biodiversity hotspot: the California Floristic Province. *Molecular Ecology*, **12:** 1021-1029.

Carstens BC, Stevenson AL, Degenhardt J, Sullivan J. 2004. Testing nested phylogenetic and phylogeographic hypotheses in the *Plethodon vandykei* species group. *Systematic Biology* **53:** 781-792.

Castoe TA, Spencer CL, Parkinson CL. 2007. Phylogeographic structure and historical demography of the western diamondback rattlesnake (*Crotalus atrox*): A perspective on North American desert biogeography. *Molecular Phylogenetics and Evolution*, **42:** 193-212.

Church SA, Kraus JM, Mitchell JC, Church DR, Taylor DR. 2003. Evidence for multiple Pleistocene refugia in the postglacial expansion of the eastern tiger salamander, *Ambystoma tigrinum tigrinum*. *Evolution* **57:** 372-383.

Clark AM, Bowen BW, Branch LC. 1999. Effects of habitat fragmentation on an endemic scrub lizard (*Sceloporus woodi):* an historical perspective based on a mitochondrial gene geneology. *Molecular Ecology* **8:** 1093-1104.

Connant R, Collins JT. 1991. *A Field Guide to the Reptiles and Amphibians: Eastern and Central North America*. Houghton Mifflin Company, Boston

Cothran EG, Zimmerman EG. 1985. Electrophoretic analysis of the contact zone between *Geomys breviceps* and *Geomys bursarius*. *Journal of Mammalogy* **66:** 489–497.

Cox CB, Moore PD. 2000. *Biogeography: an ecological and evolutionary approach*. Blackwell Science, London, UK.

Crandall KA, Templeton AR. 1996. Applications of intraspecific phylogenies. In *New Uses for New Phylogenies* (eds. Harvey, P. H. et al.) pp. 81-102 Oxford University Press.

Crespi EJ, Rissler LJ, Browne RA. 2003. Testing Pleistocene refugia theory: phylogeographical analysis of *Desmognathus wrighti,* a high-elevation salamander in the southern Appalachians. *Molecular Ecology* **12:** 969-984.

Dankin EE, Avise JC. 2004. Microsatellite null alleles in parentage analysis. *Heredity,* **93,** 504-509.

Davis JI. 1996 Phylogenetics, molecular variation and species concepts. *Bioscience*, **46**, 502-511.

de Queiroz A, Lawson R, Lemos-Espinal JA. 2002. Phylogenetic relationships of North American garter snakes (*Thamnophis*) based on four mitochondrial genes: how much DNA sequence is enough? *Molecular Phylogenetics and Evolution* **22:** 315-329.

de Quirioz K. 1998. The general lineage concept of species, species criteria, and the process of speciation. In: *Endless forms: Species and speciation*. (Howard DJ, Berlocher, SH, eds). 57-75 Oxford University Press, Oxford, UK

Delcourt PA, Delcourt HR. 1987. *Long term forest dynamics of the Temperate Zone*, Springer-Verlag, N.Y., USA.

Donovan MF, Semlitsch RD, Routman EJ. 2000. Biogeography of the southeastern United States: a comparison of salamander phylogeoraphic studies. *Evolution* **54:** 1449-1456.

Dorf E. 1959. Climatic changes of the past and present. *Contributions from the Museum of Paleontology,* University of Michigan, **13:** 181–210.

Douglas MR, Brunner PC, Douglas ME. 2003. Drought in an evolutionary context: molecular variability in Flannelmouth Sucker (*Catostomus latipinnis)* from the Colorodo River basin of western North America. *Freshwater Biology* **48:** 1254-1273.

Dowling TE, Secor CL. 1997. The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics,* **28**, 593-619.

Drummond AJ, Rambaut A. 2006. BEAST v 1.4, Available from http://beast.bio.ed.ac.uk/

Drummond AJ, Rambaut A, Shapiro B, Pybus OG. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution* **22:** 1185-1192.

Durand JD, Persat H, Bouvet Y. 1999. Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. *Molecular Ecology* **8,** 989-997.

Edmands S. 2001. Phylogeography of the intertidal copepod *Tigriopus californicus* reveals substantially reduced population differentiation at northern latitudes. *Molecular Ecology* **10:** 1743-1750.

Ehrlich PR. 1961. Intrinsic barriers to dispersal in the checkerspot butterfly. *Science,* **134:** 108-109.

Elias TS. 1987. *The Complete Trees of North America; A Field Guide and Natural History*. N.Y. Gramercy Publishing Company.

Ellsworth DL, Honeycutt RL, Silvy NJ, Bickham JW, Klimstra WD. 1994. Historical biogeography and contemporary patterns of mitochondrial DNA variation in white–tailed deer from the Southeastern United States. *Evolution* **48:** 122–136.

Endler JA. 1977. *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton, New Jersey.

Escalante T, Rodriguez G, Morrone JJ. 2004. The diversification of Nearctic mammals in the Mexican transition zone. *Biological Journal of the Linnean Society* **83:** 327–339.

Escalante T, Sanches–Cordero V, Morrone JJ. 2007. Areas of endemism of Mexican terrestrial mammals: a case study using species' ecological niche modeling, parsimony analysis of endemicity and Goloboff fit. *Interciencia* 32:151–159.

Estoup A, Jarne P, Cornuet J-M. 2002. Homoplasy and mutation model at microsatellite loci and their consequences for population genetic analysis. *Molecular Ecology,* **11**: 1591-1604

Excoffier L, Schneider S. 1999. Why hunter-gatherer populations do not show signs of Pleistocene demographic expansions. *Proceedings of the National Academy of Science* USA **96:**10597-10602.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA restriction data. *Genetics* **131:** 479-491.

Feldman CR, Spicer GS. 2002. Mitochondrial variation in sharp-tailed snakes (*Contia tenuis):* evidence of cryptic species. *Journal of Herpetology* **36:** 648-655.

Feldman CR, Spicer GS. 2007. Comparative phylogeography of woodland reptiles in California. *Molecular Ecology* **15:** 2201-2222.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783-791.

Fitch HS. 1975. A demographic study of the ringneck snake (*Diadophis punctatus*) in Kansas. *University of Kansas, Museum of Natural History Miscellaneous Publications* **62:** 1-53.

Fitch HS. 2006. Ecological succession on a natural area in northeastern Kansas from 1948 to 2006. Herp. Conserv. Biol 1:1-5.

Fontanella FM, Feldman CR, Siddall ME, Burbrink FT. 2008. Phylogeography of *Diadophis punctatus*: extensive lineage diversity and repeated patterns of historical demography in a trans–conterminous snake. *Molecular Phylogenetics and Evolution* **46:** 1049–1070.

Fontanella FM, Siddall ME. 2009. Evaluating hypothesis on the origin and diversification of the ringneck snake, *Diadophis punctatus* (Colubridae: Dipsadinae). **Zoological Journal of the Linnean Society** (in press)

Fu JZ, Zeng XM. 2008. How many species are in the genus *Batrachuperus*? A phylogeographical analysis of the stream salamanders (family Hynobiidae) from southwestern China. *Molecular Ecology* **17:** 1469-1488. doi:10.1111/j.1365-294X.2007.03681.x

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking, and background selection. *Genetics* **147:** 915-925.

Gehlbach FR. 1965. Herpetology of the Zuni Mountains region, northwestern New Mexico. *Proceedings of the Natural History Museum* **116:** 243–332.

Gelman A, Carlin JB, Stern HS, Rubin DB. 1995. *Bayesian Data Analysis*. Chapman and Hall, London, UK

Giordano AR, Ridenhour BJ, Storfer A. 2007. The influence of altitude and topography on genetic structure in the long-toed salamander (*Ambystoma macrodactulym*). *Molecular Ecology* **16:** 1625-1637.

Gloyd HK.1940. *The Rattlesnakes, Genera Sistrurus and Crotalus: A Study in Zoogeography and Evolution*. Special Publication No. 4. Chicago Academy of Sciences, Chicago.

Goudet J. 2001. FSTAT. *A program to estimate and test gene diversities and fixation indicies (version 2.9.3)*. Available from http://www.unil.ch/izea/softwares/fstat.heml. Updated from Goudet (1995).

Goloboff PJ, Farris F, Nixon K. 2003. T.N.T.: Tree Analysis Using New Technology. Available at http://www.zmuc.dk/public/phylogeny/tnt.

Greene HW. 1997. *Snakes: The Evolution of Mystery in Nature*. University of California Press, Berkeley.

Guo SW, Thompson EA. **1992.** Performing the exact test of Hardy–Weingberg proportion for multiple alleles. *Biometrics,* **48**, 361–372.

Haffner DJ, Riddle BR. 2005. Mammalian phylogeography and evolutionary history of northern Mexico's deserts. In: Cartron, J–L, Ceballos, G, Felger, RS, eds., *Biodiversity, Ecosystems and Conservation in Northern Mexico*. Oxford University Press, New York, 225–245.

Hairston NG. 1987. *Community Ecology and Salamander Guilds*. Cambridge University Press, Cambridge

Halffter G. 1976. Distribucion de los insectos en la zona de transicion Meicana. Relaciones con la entomofauana de Norteamericana. *Folia Entomologica Mexicana* **35:** 1–64.

Harpending HC, Batzer MA, Gurven M, Jorde L, Rogers AR, Sherry ST. 1998. Genetic traces of ancient demography. *Proceedings of the National Academy of Science* USA, **95***:* 1961-1967.

Harrison RG. 1990. Hybrid zones: windows on evolutionary process. In: *Oxford Surveys in Evolutionary Biology* (eds Futuyma D, Antonovics J), Oxford University Press, Oxford UK.

Harrison RG. 1993. *Hybrid Zones and the Evolutionary Process*. Oxford University Press, New York and Oxford.

Hayes JP, Harrison RG. 1992. Variation in mitochondrial DNA and the biogeographic history of woodrats *(Neotoma)* of the eastern United States. *Systematic Biology* **41:** 331-344.

Heilprin A. 1887. *The Geographical and Geological Distribution of Animals*. D. Appleton, New York.

Held C. 2003. Molecular evidence for cryptic speciation within the widespread Antarctic crustacean *Ceratoserolis trilobitoides* (Crustacea, Isopoda). In: *Antarctic Biology in a Global Context (*eds Huiskes AHL, Gieskes WWC, Rozema J, Schorno RML, Van der Vies SM, Wolff WJ), Backhuys Publishers, Leiden, The Netherlands.

Hendrick PW. 1999. Perspective: highly variable loci and their interpretation in evolution and conservation. *Evolution* **53**: 313-318.

Heulsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17:** 754-755

Hewitt GM. 2001. Speciation, hybrid zones and phylogeography–or seeing genes in space and time. *Molecular Ecology* **10:** 537-549. doi:10.1046/j.1365-294x.2001.01202.x

Hewitt GM. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58:** 347-256.

Hewitt GM. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405:** 907-913.

Hijmans RJ, Guarino L, Cruz M, Rojas E. 2001. Computer tools for spatial analysis of plant genetic resourses data: 1. DIVA-GIS. *Plant Genetic Resources Newsletter*, **127:**15-19.

Hijmans RJS, Cameron S, Parra J, Jones P, Jarvis A. 2005. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology,* **25:** 1965-1978.

Hillis DM. 1981. Premating isolating mechanisms and adaptation of the larvae of *Rana pipiens* complex in Texas and southern Oklahoma. *Copeia* **1981:** 312–319.

Hoffman EA. Blouin MS. 2004. Evolutionary history of the northern leopard frog: reconstruction of phylogeny, phylogeography and historical changes in population demography from mitochondrial DNA. *Evolution*, **58:** 145-159.

Holman JA. 1979. A review of North American Pleistocene snakes. *Publication of the Museum, Michigan State Univiversity, (Paleontological Series)* **1:** 203–260.

Holman JA. 2000. *Fossil Snakes of North America. Origin, Evolution, Distribution, Paleoecology.* Indiana University Press, Indianapolis.

Howes BJ, Lindsay B, Lougheed SC. 2006. Range-wide phylogeography of a temperate lizard, the five-lined skink (*Eumeces fasciatus*). *Molecular Phylogenetics and Evolution*, **40:** 183-194.

Hubbs CL. 1936. *Fishes of the Yucatan peninsula*. Carnegie Institution of Washington Publication.

Hudson RR. 1990. Gene genealogies and the coalescent process. In: *Oxford Surveys in Evolutionary Biology* (eds Futuyma D, Antonovics J), Oxford University Press, Oxford UK.

Huidobro L, Morrone JJ, Villalobos JL, Alvarez F. 2006. Distributional patterns of freshwater taxa (fishes, crustaceans and plants) from the Mexican Transition Zone *Journal of Biogeography* **33:** 731–741

Irwin DE. 2002. Phylogeographic breaks without geographic barriers to gene flow. *Evolution,* **56:** 2383-2394.

Jaeger JR, Riddle BR, Bradford DF. 2005. Cryptic Neogene vicariance and Quaternary dispersal of the red–spotted toad (*Bufo punctatus*) insights on the evolution of North American warm desert biotas. *Molecular Ecology* **14:** 3033–3048.

Jarne P, Lagoda PJL. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution,* **11:** 424-429.

Joly S, Bruneau A. 2004. Evolution of triploidy in *Apios americana* (Leguminosae) revealed by genealogical analysis of the histone H3-D gene. *Evolution* **58:** 284-295.

Kass RE, Raferty AE. 1995. Bayes factors and model uncertainty. *Journal of the American Statistical Association* **90:** 773-795.

Kembert K. 2008. Good species despite massive hybridization: genetic research on the contact zone between the watersnakes *Nerodia sipedon* and *N. fasciata* in the Carolinas, USA. *Molecular Ecology* **17:** 1918-1929.

Kimball B. 1965. *The Lepidoptera of Florida. An Annotated Checklist. Arthropods of Florida and Neighboring Land Areas*, 1, 1-163, Florida Department of Agriculture, Gainesville, Florida

Klauber LM. 1972. *Rattlesnakes: Their Life History, Habits and Influence on Mankind*. 2 volumes, 2nd ed. University of California Press, Berkeley.

Klicka J, Zink RM. 1997. The importance of recent ice ages in speciation, a failed paradigm. *Science* **277:** 1666-1669.

Klicka J, Zink RM. 1998. "Response" to Pleistocene speciation and the mitochondrial DNA clock. *Science* **282:** 1995.

Klicka J, Zink RM. 1999. Pleistocene effects on North American songbird evolution. *Proceedings of the Royal Society of London series B* **266:** 695-700.

Kliman RM, Andolfatto P, Coyne JA, Depaulis F, Kreitman M, Berry AJ, McCarter J, Wakeley J, Hey J. 2000. The population genetics of the origin and divergence of the *Drosophila simulans* complex species. *Genetics*, **156:** 1913-193**1.**

Kozak KH, Wiens JJ. 2006. Does niche conservatism promote speciation? A case study in North American salamanders. *Evolution,* **60**: 2604-2621.

Kozak KH, Blaine RA, Russell AB, Larson A. 2006. Gene lineages and eastern North American palaeodrainage basins: phylogeography and speciation in salamanders of the *Eurycea bislineata* species complex. *Molecular Ecology*, **15:** 191-207.

Lancaster DL, Wise SE, 1996. Differential response by the ringneck snake (*Diadophis punctatus*) to odors of tail-autotomizing prey. *Herpetologica*, **52:** 98-108.

Lawson R, Slowinski JB, Crother BI, Burbrink FT. 2005. Phylogeny of the Colubroidea (Serpentes): New evidence from mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution* **37:** 581–601.

Leaché AD, McGuire JA. 2006. Phylogenetic relationships of horned lizards (*Phrynosoma*) based on nuclear and mitochondrial data: Evidence for a misleading mitochondrial gene tree. *Molecular Phylogenetics and Evolution*, **39:** 628-644.

Leaché AD, Reeder TW. 2002. Molecular systematics of the eastern fence lizard (*Sceloporus undulates*): a comparison of parsimony, likelihood, and Bayesian approaches. *Systematic Biology,* **51:** 44-68.

Lee TE, Riddle BR, Lee PL. 1996. Speciation in the desert pocket mouse (*Chaetodipus penicillatus* Woodhouse). *Journal of Mammalogy* **77:** 58–68.

Machado CA, Kliman RM, Markert JA, Hey J. 2002. Inferring the history of speciation from multilocus DNA sequence data: the case of *Drosophila pseudoobscura* and its close relatives. *Molecular Biology and Evolution,* **19:** 472-488.

Mahoney MJ. 2004. Molecular systematics and phylogeography of the *Plethodon elongatus* species group: combining phylogenetic and population genetic methods to investigate species history. *Molecular Ecology*, **13:** 149-166.

Mallet J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology and Evolution,* **20:** 229-237.

Manel S, Schwartz MK, Luikart G, Taberlet P. 2003. Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology and Evolution,* **18:** 189-197.

Mann DH, Hamilton TD. 1995. Late Pleistocene and Holocene paleoenvironments of the North Pacific coast. *Quarterly Science Review*, **14:** 449-471.

Marjoram P, Donnelly P. 1994. Pairwise comparisons of mitochondrial DNA sequences in subdivided populations and implications for early human evolution. *Genetics,* **136:** 673-683.

Marshall CJ, Liebherr JK. 2000. Cladistic biogeography of the Mexican transition zone. *Journal of Biogeography* **27:** 203–216.

Martin PS, Harrell BE. 1957. The Pleistocene history of temperate biotas in Mexico and Eastern United States. *Ecology* **3:** 468–480.

Mavarez J, Salazar CA, Bermingham E *et al.* 2006. Speciation by hybridization in *Heliconius* butterflies. *Nature,* **441**: 868-871.

Mayden RL. 1988. Vicariance biogeography, parsimony, and evolution in North American freshwater fishes. *Systematic Zoology,* **37:** 329-355.

McLachlan JS, Clark JS, Manos PS. 2005. Molecular indicators of tree migration capacity under rapid climate change. *Ecology,* **86:** 2088-2098.

Mendelson JR, Jennings, WB. 1992. Shifts in the relative abundance of snakes in a desert grassland. *Journal of Herpetology* **2:** 38–45.

Metcalf SE, O'Hara SL, Caballero M, Davies SJ. 2000. Records of Late Pleistocene–Holocene climatic change in Mexico–A review. *Quaternary Scientific Review* **19:** 699–721.

Metcalf SE. 2006. Late quaternary environments of the northern deserts and central transvolcanic belt of Mexcio. *Annals of the Missouri Botanical Garden* **93:** 258–273.

Moore WS, Price JT. 1993. Nature of selection in the Northern Flicker hybrid zone and its implications for speciation theory. In: *Hybrid zones and the Evolutionary Process* (eds. Harrison RG), Oxford University Press, New York and Oxford.

Moore WS. 1977. An evaluation of narrow hybrid zones in vertebrates. *Quarterly Review of Biology*, **52:** 263-277.

Moritz C. 1994. Defining 'evolutionary significant units' for conservation. *Trends in Ecology and Evolution* **9:** 373–375.

Moritz C, Schneider CJ, Wake DB. 1992. Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Systematic Biology,* **41:** 273-291.

Morrone JJ, Espinosa–Organista D, Llorente Bousquets J. 2002. Mexican biogeographic provinces: preliminary scheme, general characterizations, and synomyms. *Acta Zoologica Mexicana (nueva serie)* **85:** 83–108.

Morrone JJ. 2006. Biogeographic areas and transition zones of Latin America and the Caribbean Islands based on panbiogeographic and cladistic analyses of the entomofauna. *Annual Review of Entomology* **51:** 467–494.

Myers C. 1965. Biology of the ringneck snake *Diadophis punctatus* in Florida. *Bulletin of the Florida State Museum, Biological Sciences*, **10:** 43-90.

Mylecraine KA, Kuser JE, Smouse PE, Zimmermann GL. 2004. Geographic allozyme variation in Atlantic white-ceder, *Chamaecyparis thyoides* (Cupressaceae). *Canadian Journal of Forest Research,* **34:** 2443-2454.

Nagy ZT, Lawson R, Joger U, Wink M. 2004. Molecular systematics of racers, whipsnakes and relatives (Reptilia: Colubridae) using mitochondrial and nuclear markers. J. Zoo. Syst. Evol. Res., **42:** 223-233.

Nason JD, Hamrick JL, Fleming TH. 2002. Historical vicariance and postglacial colonization effects on the evolution of genetic structure in *Lophocereus*, a Sonoran Desert columnar cactus. ***Evolution* 56:** 2214–2226.

Near TJ, Page LM, Mayden RL. 2001. Intraspecific phylogeography of *Percina evides* (Percidae: Etheostomatinae): an additional test of the Central highlands pre-Pleistocene vicariance hypothesis. *Molecular Ecology*, **10:** 2235-2240.

Nei M. 1987. *Molecular evolutionary genetics*. Columbia University Press. New York.

Nei M, Li W-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science,* USA, **76:** 5269-5273.

Nichols RA, Hewitt GM. 1994. The genetic consequences of long distance dispersal during colonization. *Heredity,* **72:** 312-317.

Nylander JAA, Ronquist F, Huelsenbeck JP, Aldrey JL. 2004. Bayesian phylogenetic analysis of combined data. *Systematic Biology,* **53:** 47-67.

Oosterhout CV, Hutchinson WF, Willis DPM, Shipley P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes,* **4:** 535-538.

Orange DI, Riddle BR, Nickle DC. 1999. Phylogeography of a wide–ranging desert lizard, *Bambelia wislizenii* (Crotaphytidae). *Copeia* **2:** 267–273.

Outlaw DC, Voelker G, Mila B, Girman DJ. 2003. Evolution of long–distance migration in and historical biogeography of *Catharus* thrushes: a molecular phylogenetic approach. *The Auk* **120:** 299–310.

Palo JU, O'Hara RB, Laugen AT, Laurila A, Primmer CR, Merila J. 2003. Latitudinal divergence of common frog (*Rana temporaria*) by natural selection: evidence from a comparison of molecular and quantitative genetic data. *Molecular Ecology*, **12:** 1963-1978.

Palsboll PJ, Berube M, Jortensen H. 1999. Multiple levels of single-strand slippage at cetacean tri- and tetranucleotide repeat microsatellite loci. *Genetics*, **151:** 285-296.

Parker WS, Brown WS. 1974. Notes on the ecology of regal ringneck snakes (*Diadophis punctatus regalis*) in northern Utah. *Journal of Herpetology*, **8:** 262-263.

Patton JL, de Silva MNF. 1998. Rivers, refuges, and ridges: the geography of speciation of Amazonian mammals. In: *Endless forms: Species and speciation*. (Howard DJ, Berlocher, SH, eds). 57-75 Oxford University Press, Oxford, UK

Pauly GB, Piskurek O, Shaffer HB. 2007. Phylogeographic concordance in the southeastern United States: the flatwoods salamander, *Ambystoma cingulatum*, as a test case. *Molecular Ecology*, **16:** 415-429.

Perlmutter MA. 1985. Deep water clastic reservoirs in the Gulf of Mexio A depositional model. Geo-Marine Letters, **5:** 105-112.

Peterson RT. 1980. *A field guide to the birds*. Houghton Mifflin Company, Boston

Phillips SJ, Anderson RP, Schapire RE. 2006. Maximum entropy modeling of species geographic distribution. *Ecological Modeling*, **190:** 231-259.

Phillips SJ, Dudik M, Schapire RE. 2004. A maximum entropy approach to species distribution modeling. *Proceedings of the Twenty-First International Conference on Machine Learning*, **69:** 83-89.

Pinou T, Vicario S, Marschner M, Caccone A. 2004. Relict snakes of North America and their relationships within Caenophidia, using likelihood–based Bayesian methods on mitochondrial sequences. *Molecular Phylogenetics and Evolution* **32:** 563–574.

Pinou T, Hass CA, Maxson LR. 1995. Geographic variation of serum albumin in the monotypic snake genus *Diadophis* (Colubridae: Xenodontinae). *Journal of Herpetology,* **29:** 105-110.

Place AJ, Abramson CI. 2004. A quantitative analysis of the ancestral area of rattlesnakes. *Journal of Herpetology 38:* 151–156.

Pook CE, Wuster W, Thorpe RS. 2000. Historical biogeography of the western rattlesnake (Serpenetes: Viperidae: *Crotalus viridis*), inferred from mitochondrial DNA sequence information. *Molecular Phylogenetics and Evolution*, **15:** 269-282.

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

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics,* **155:** 945-959.

Prugnolle F, Meeus TD. 2002. Inferring sex biased dispersal from population genetic tools: a review. *Heridity*, **88:** 161-165.

Rambaut, A., Drummond, A.J., 2003. Tracer. Version 1.0.1. Available at http://wvolve.zoo.ox.ac.uk/

Ramos-Onsins SE, Rozas J. 2002. Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution,* **19:** 2092-2100.

Ray N, Currat M, Excoffier L. 2003. Intra-deme molecular diversity in spatially expanding populations. *Molecular Biology and Evolution* **20:** 76-86.

Raymond M, Rousset F, 1995. GENEPOP version 1.2: population genetics software for exact tests and ecumenicism. *Journal of Heredity,* **86:** 248-249.

Reeder TW, Montanucci RR. 2001. A phylogenetic analysis of the horned lizards (Phrynosomatidae: *Phyrnosoma*): Evidence from mitochondrial DNA and morphology. *Copeia,* **2:** 309-323.

Riddle BR, Hafner DJ. 2006. A step–wise approach to integrating phylogeographic and phylogenetic biogeographic perspectives on the history of a core North American warm deserts biota. *Journal of Arid Environments* **66:** 435–461.

Riddle BR, Hafner DJ, Alexander LF, Jaeger JR. 2000. Cryptic vicariance in the historical assembly of a Baja California Peninsular Desert biota. *Proceedings of the National Academy of Science,* USA, **97:** 14438-14443.

Riddle BR, Honeycutt RL. 1990. Historical biogeography in North American arid regions: an approach using mitochondrial DNA phylogeny in grasshopper mice (genus *Onychomys*). *Evolution* **44:** 1–15.

Rissler LF, Apodaca JJ. 2007. Adding more ecology into species delimitation: ecological niche models and phylogeography help define cryptic species in the black salamander *(Aneides flavipunctatus)*, *Systematic Biology,* **56:** 924-942.

Roberts MA, Schwartz TS, Karl SA. 2004. Global population genetic structure and male-mediated gene flow in the green sea turtle (*Chelonia mydas*): analysis of microsatellite loci. *Genetics,* **166:**1857-1870.

Rodriguez-Robles JA, DeNardo DF, Staub RE. 1999. Phylogeography of the California mountain kingsnake, *Lampropeltis zonata* (Colubridae). *Molecular Ecology*, **8:** 1923-1934.

Rogers AR, Fraley AE, Bamshad MJ, Watkins WS, Jorde LB. 1996. Mitochondrial mismatch analysis is insensitive to the mutational process. *Molecular Biology and Evolution*, **13:** 895-902.

Rogers AR, Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9:** 552-569.

Ronquist F. 1996. DIVA version 1.1 Computer program and manual. Available from Uppsala University at: http://www..ebc.uu.se/systzoo/research/diva/diva.html.

Ronquist F, Heulsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics,* **19:** 1572-1574.

Rosen DE. 1978. Vicariant patterns and historical explanation in biogeography. *Systematic Zoology* **27:** 159–188.

Roza J, Roza R. 1999. DnaSP, Version 3: An integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15:** 174-175.

Rubinsztein DC, Amos B, Cooper G. 1999. Microsatellite and trinucleotide-repeat evolution: evidence for mutational bias and different rates of evolution in different lineages. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences,* **354:** 1095-1099.

Ruthven AG. 1908. Variations and genetic relationships of the gartersnakes. *Bulletin of the U. S. National Museum* **61:** 1–201.

Saghai-Maroof MA, Solliman KM, Jorgensen, RA, Allard RW. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceeding of the National Academy of Science* USA, **81:** 8014-8018.

Sanderson MJ. 2003. r8s; inferring absolute rates of evolution and divergence times in the absence of a molecular clock. *Bioinformatics,* **19:** 301-302.

Santacruz–Varela A, Widrlechner P, Ziegler KE, Salvador RJ, Millard MJ, Bretting PK. 2004. Phylogenetic relationships among North American popcorns and their evolutionary links to Mexican and South American popcorns. *Crop Science* **44:** 1456–1467.

Schluter D. 2000. *The Ecology of Adaptive Radiation,* Oxford University Press, New York, NY

Schneider S, Foessli D, Excoffier L. 2000. Arlequin. Version 2.0. A software for population genetics analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Seehausen O. 2004. Hybridization and adaptive radiation. *Trends in Ecology and Systematics* **19:** 198-207.

Seymore R. 1987. Scaling of cardiovascular physiology in snakes. *Integrative Comparative Biology*, **27:** 97-109.

Shankman D, Hart JL. 2007. The Fall Line: A physiographic–forest vegetation boundary. *Geographical Review* **97:** 502–519.

Shaw KL. 2002. Conflict between nuclear and mitochondrial DNA phylogenies of a recent species radiation: What mtDNA reveals and coneals about modes of speciation in Hawiian crickets. *Proceedings of the National Academy of Science* **10:** 16122-16127.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log–likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* **16:** 1114–1116.

Shimodaira H, Hasegawa M. 2001. *CONSEL:* for assessing the confidence of phylogenetic tree selection. *Bioinformatics* **17:** 1246–1247.

Shimodaira H. 2002. An approximately unbiased test of phylogenetic tree selection. *Systematic Biology* **51:** 492–508.

Sites JW, Marshall JCM. 2004. Operational criteria for delimiting species. *Annual Review of Ecology and Systematics*, **35:** 199-227.

Slatkin M. 1987. Gene flow and the geographic structure of natural populations. *Science*, **236:** 787-792.

Slatkin M, Maddison WP. 1989. A cladistic measure of gene flow inferred from the phylogenies of alleles. *Genetics*, **123:** 603-613.

Smith SA, Stephens PR, Wiens JJ. 2005. Replicate patterns of species richness, historical biogeography and phylogeny in holarctic treefrogs. *Evolution,* **59:** 2433-2450.

Soltis DE, Morris AB, McLachlan JS, Manos PS, Soltis PS. 2006. Comparative phylogeography of unglaciated eastern North America. *Molecular Ecology* **15:** 4261–4293.

Spear SF, Peterson CR, Matocq MD, Storfer A. 2005. Landscape genetics of the blotched tiger salamander (*Ambystoma tigrinum melanostictum*). *Molecular Ecology*, **14:** 2553-2564.

Spinks, P. Q., Shaffer, H. B. 2005. Rangewide molecular analyses of the western pond turtle (*Emys marmorta*) cryptic variation, isolation by disatnace, and their conservation implications. *Molecular Ecology*, **14:** 2047-2064.

Stamatakis A, Hoover P, Rougemont J. 2008. A Rapid Bootstrap Algorithm for the RAxML Web–Servers, *Systematic Biology* in press.

Stamatakis A. 2006. RaxML–VI–HPC: maximum likelihood–based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22:** 2688–2690.

Stangl FB. 1986. Aspects of a contact zone between two chromosomal races of *Peromyscus leucopus* (Rodentia: Cricetidae). *Journal of Mammalogy* **67:** 465–473.

Stebbins RC. 1985. *A Field Guide to Western Reptiles and Amphibians*. Houghton Mifflin, Boston, MA.

Steele CA, Storfer A. 2006. Coalescent-based hypothesis testing supports multiple Pleistocene refugia in the Pacific Northwest for the Pacific Giant Salamander *(Dicamptodon tenebrosus*). *Molecular Ecology*, **15:** 2477-2487.

Stein BA, Kutner LS, Hammerson GA et al. 2000. State of the States: Geographic patterns of diversity, rarity, and endemism. In: *Precious Heritage: the Status of Biodiversity in the United States* (eds Stein BA, Kutner LS, Adams JS), pp. 119-157, Oxford University Press, Oxford

Swenson NG, Howard DJ. 2005. Clustering of contact zones, hybrid zones, and phylogeographic breaks in North America. American Naturalist **166:** 581-591.

Swofford DL. 2001. *PAUP*; Phylogenetic Analyses Using Parsimony (*and other Methods)*. Version 4. Sineauer Associates, Sunderland, Massachusetts.

Tajima F. 1989. Statisitcal method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics,* **123:** 597-601.

Takahata N. 1989. Gene geneology in three related populations: consistency probability between gene and population trees. *Genetics*, **122:** 957-966.

Tan AM, Wake DB. 1995. MtDNA phylogeography of the California newt, *Taricha torosa* (Caudata, Salamandridae. *Molecular Phylogenetics and Evolution* **4:** 383-394.

Templeton AR. 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7:** 381-397.

Upton DE. Murphy RW. 1997. Phylogeny of the side–blotched lizards (Phrynosomatideae: *Uta*) based on mtDNA sequences: support for a mid–peninsular seaway in Baja California. *Molecular Phylogenetics and Evolution* **8:** 104–113.

Verheyen E, Salzburger W, Snoeks J, Meyer A. 2003. Origin of the super-flock of cichlid fishes from Lake Victoria, East Africa. *Science,* **300:** 325-329.

Vignieri SN. 2005. Streams over mountains: influence of riparian connectivity on gene flow in the Pacific jumping mouse *(Zapus trinotatus*). *Molecular Ecology*, **14:** 1925-1937.

Wake DB. 2006. Problems with species: Patterns and processes of species formation in salamanders. *Annuals of Missouri Botanical Garden*, **93**: 8-23.

Walker D, Avise JC. 1998. Principles of phylogeography as illustrated by freshwater and terrestrial turtles in the southeastern United States. *Annual Review of Ecology and Systematics*, **29:** 23-58.

Wallace AR. 1876. *The Geographical Distribution of Animals, with a Study of the Relations of Living and Extinct Faunas as Elucidating the Past Changes of the Earth's Surface*. MacMillan, London.

Waltari E, Hijmans RJ, Peterson AT, Nyari AS, Perkins SL, Guralnick RP. 2007. Locating Pleistocene refugia: comparing phylogeographic and ecological niche model predictions. *PloS ONE* **7:** e563.

Webb SD, Wilkins KT. 1984. Historical biogeography of Florida Pleistocene mammals in HH, Genoways & MR, Dawson, eds. *Contributions in Quaternary Vertebrate Paleontology: A Volume in Memorial to John E. Guilday*. Special Publication No. 8, Carnegie Museum of Natural History 370–383.

Webb SD. 1990. Historical Biogeography. In Ecosystems of Florida (eds, Myers RL and Ewel JJ), pp. 70-102, University of Central Florida Press, Orlando, Florida

Wiens JJ. 2004. Speciation and ecology revisited: phylogenetic niche conservatism and the origin of species. *Evolution,* **58:** 193-197.

Wiens JJ, Penkrot TA. 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology*, **51:** 69-91.

Wiley EO. 1978. The evolutionary species concept reconsidered. *Systematic Zoology*, **27:** 17-26.

Wiley EO. 1976. The phylogeny and biogeography of fossil and recent gars (Actinopterygii: Lepisosteidae). *Miscellaneous Publications of the Museum of Natural History University of Kansas* **64:** 1–111.

Wiley EO, Mayden RL. 1985. Species and speciation in phylogenetic systematics, with examples from the North American fish fauna. *Annals of the Missouri Botanical Garden,* **72:** 596-635.

Winkler K, Gordon TR. 2000. An initial assessment of genetic relationships among populations of *Fusarium circinatum* in different parts of the world. *Canadian Journal of Botony* **78:** 709–717.

Zamudio KR, Savage WK. 2003. Historical isolation, range expansion, and secondary contact of two highly divergent mitochondrial lineages in spotted salamanders (*Ambystoma maculatum*). *Evolution*, **57:** 1631-1652.

Zhang DX, Hewitt GM. 1996. Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution* **11:** 247–251.

Zink RM, Blackwell RC. 1998. Molecular systematics and biogeography of arid–land gnatcatchers (Genus *Polioptila*) and evidences supporting species status of the California gnatcatcher (*Polioptila californica*). *Molecular Phylogenetics and Evolution*, **9:** 26–32.

Zink RM. 2002. Methods in comparative phylogeography, and their application to studying evolution in the North American aridlands. *Integrative and Comparative Biology* **42:** 953–959.

Zink RM, Blackwell RC. 1998. Molecular systematics of the scaled quail complex (Genus *Callipepla*). *Auk* **115:** 349-403.