SYSTEMATICS OF THE POLYTYPIC SNAKE ELAPHE GUTTATA

(SERPENTES: COLUBRIDAE)

THESIS

Presented to the Graduate Council of Southwest Texas State University
in Partial Fulfillment of the Requirements

for the Degree of Master of Science

By

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San Marcos, Texas

August 2002

ACKOWLEDGEMENTS

I would like to thank my committee members. Dr. Richard W. Manning, who can always think of questions that I don't know the answers to, and make me worry that he is saving the best ones for my oral exam. Dr. James R. Dixon, whose expertise in herpetology is bewildering. The opportunity to collaborate and learn from someone so knowledgeable is an experience I will not soon forget. I would like to extend a special thanks to my committee chair, Dr. Michael R.J. Forstner, who refused to let me take the easy way out. I'm not sure how he made the idea of completing a thesis in such a short period of time sound appealing, but thanks to his ability to persuade, I've learned a great deal.

I would like to thank my family, especially my parents Stephen and Elvia. They could not have been more supportive of my academic endeavors. Thanks to the rest of my family for all the help and support they have given me.

I would like to thank my fellow graduate students and everyone in the Forstner lab, especially Sue Morris, Diana McHenry, Aaron Marquardt, Jamie Dixson, and David Rodriguez.

Finally I would like to thank my girlfriend, Tiffany, who somehow managed to put up with me while I was writing this thesis.

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ABSTRACT

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Supervising Professor: Michael R. J. Forstner

The separation of species into subspecies has been debated and its usefulness is unclear. Subspecific classifications may be an aid to identification by taxonomists, or misapplied to incorrectly recognize organisms that may be more properly designated as species. While arguments of the species concept continue, the role of subspecies will continue to be uncertain. Newer methods for determining the relationships between taxa can be employed to examine polytypic species in which subspecies separations have been used. Mitochondrial DNA analyses were used to determine the evolutionary relationship

among subspecies of *Elaphe guttata* within Texas and relationships among subspecies of *E. guttata* in Texas, Colorado, New Mexico, Louisiana, Georgia, and Florida. Molecularsystematic analysis of the corn snake *E. guttata* revealed the presence of three distinct clades; a clade composed of snakes from Florida and Georgia to the Mississippi River, a clade composed of snakes from the Mississippi River to the Austroriparian Region of Texas, and a clade composed of snakes in central and south Texas extending into New Mexico and Colorado. The separations in these clades supported the current subspecific taxonomy, which is based on morphological characters. Distance measures among the clades indicate that the snakes from south and central Texas should be classified as a distinct species as recognized in 1951, and the two remaining clades should remain subspecies of *E. guttata*. The presence of distinct clades that agree with current morphological characters suggests, that in the case of *E. guttata*, subspecific taxonomic separations are useful systematic tools that describe distinct evolutionary lineages.

INTRODUCTION

Although the use of subspecies in the identification and classification of organisms is widely encountered, their application in taxonomy has remained unclear (Mayr, 1942; Simpson, 1961; Wilson and Brown, 1953; Frost et al., 1992). The separation of a species into subspecies may be an aid to identification by taxonomists, or misapplied to incorrectly recognize organisms more properly designated as species. Such confusion at the subspecies level may stem from debate over the species concept (Burma and Mayr, 1949). The classic biological species concept proposed by Dobzahansky (1937) and Mayr (1942) has been criticized in the light of newer ideas and techniques that allow insight into the evolutionary history of current taxa (Simpson, 1961; Wiley, 1978, 1980; Frost and Hillis, 1990; Cole, 1990; Smith, 1990; Highton, 1990). The evolutionary species concept proposed by Simpson (1961) and Wiley (1978, 1980) and the phylogenetic species concept proposed by Cracraft (1983) both seek to incorporate evolutionary history of the taxa. Most current taxonomy has been established by comparative anatomists who employed morphological characters exclusively to classify organisms into distinct taxa. While there are advantages to using morphological characters, the limited ability to infer phylogenetic history (Hillis, 1987) restrict their utility in describing species under the evolutionary criteria of phylogenetic species concepts. In light of molecular systematic techniques, polytypic species that have been divided into subspecies based on morphological characters should be reevaluated to

determine if the current taxonomic separations consist of evolutionarily distinct lineages, or simply groups of individuals with different phenotypic traits.

In many organisms, the organization of related taxa into groups could benefit from the use of molecular systematic techniques. Classifications of reptiles and amphibians have frequently resulted in incorrect or incomplete classifications. One consequence of this has been a continuously changing taxonomic system. A good example would be the classification of snakes which has always presented problems for zoologists due to limited morphological characters consequent of their reduced body plan (Cope, 1894). All modern or extant snakes are assigned to the superfamily Colubroidea (Lopez and Maxson, 1995), although no single set of morphological synapomorphies exists for all members of this taxon (Cadle, 1987; Dowling et al., 1983). The family Colubridae contains approximately 70% of all known snake species (Pough et al., 1998). Attempts to derive an accurate phylogeny of the colubrids, using both molecular and morphological analyses, have been unsuccessful (Cope, 1894; Dowling and Duellman, 1978; Smith et al., 1977; Dowling et al., 1983). Examination of extant colubrine snake species shows that the genus *Elaphe* may itself be polyphyletic (Lopez and Maxson, 1995, Dowling et al., 1983; Dowling et al., 1996; Dowling and Price, 1988). Distinct clades delineate new world and old world *Elaphe* with some North American *Elaphe* more closely related to snakes in the genera *Pituophis* and *Cemophora* than to rat snakes in the Asian or Mediterranean clades (Lopez and Maxson, 1995; Dowling et al., 1983).

A possible explanation for such problematic taxa is the changing methods that have been used in classification. Historically organisms were named and classified by biologists who were considered experts in their field without using a methodical approach

to taxonomy. The earliest of these classifications were established before any species concepts were developed (Dowling and Price, 1988). It is these early classifications that form the basis of our present day taxonomy. More recently scientists have used precise measurements of morphological characters and statistical analyses to group organisms together. In the classification of snakes, morphological characters such as scale counts (ventral, subcaudal, labial), number of blotches, color variation, degree of pigmentation, morphology of the hemipenes, have been used. Classical univariate and multivariate statistical analyses have been used to demonstrate the presence of statistically significant groupings based on these morphological characters (Vaughan et al., 1996; Smith et al., 1994). Analysis of these groupings along with geographic data showing where each sample was taken allowed biologists to infer ranges for these taxonomic groups (Vaughan et al., 1996; Smith et al., 1994). However, the use of these techniques did not show the evolutionary history between groups (Hillis, 1987).

In recent years molecular systematics has allowed biologists to use information contained within the DNA of organisms as a new set of characters available to assist in making more accurate taxonomic inferences (Hillis, 1987). Quick and efficient methods for extracting, amplifying, and sequencing DNA have provided biologists with large datasets which can be used to infer phylogenetic relationships. Although any DNA sequence has potential use in phylogenetic analysis, the use of mitochondrial DNA, or mtDNA, in phylogenetics has been especially helpful in reconstructing phylogenies between related taxa (Hillis, 1987; Avise et al., 1987; Moritz et al., 1987; Avise, 1991; Graur and Li, 2000). Unlike genomic nuclear DNA, mtDNA has many characteristics that favor its use in phylogenetic analysis. Mitochondrial DNA is much smaller, simpler

in structure, uniparentally inherited, and has a highly conserved gene order throughout the animal kingdom (Hillis et al., 1996; Graur and Li, 2001). Another important characteristic of mtDNA is its high rate of mutation compared to nuclear genes. Different regions of the mtDNA genome evolve at different rates but always at ten times or greater than nuclear counterparts. The mtDNA of every organism is maternally inherited avoiding any mechanism designed to increase genetic diversity as a result of sexual reproduction. This aspect further increases the relative evolutionary rate by eliminating recombination and subsequent corrections. Each gene sequence is composed of two types of nucleotide bases purines (adenine and guanine) and pyrimidines (cytosine and thymine). Changes in the arrangement of these bases, or mutations, are categorized as transitions and transversions. The divergence of one sequence from another is a measure of the amount of substitutions that have occurred in the genes being investigated. The frequency of transitions and transversions is important in phylogenetic reconstruction. Due to the changes in DNA secondary structure caused by the different substitutions as well as effects on gene function transitions have been shown to occur at higher frequencies than transversions. In the examination of a dataset, the occurrence of a transversion therefore may be more significant than transitions.

The use of DNA sequences to construct evolutionary relationships has increased dramatically in recent years. An increased capability in computing power has allowed programs such as PAUP* 4.0 (Phylogenetic Analysis Using Parsimony) (Swofford, 1999), Phylip 3.0 (Felsenstein, 1993), and MacClade 3.05 (Maddison and Maddison, 1992) to be used to resolve the evolutionary history recorded in the DNA sequences. These programs use parsimony, distance, and maximum likelihood analyses to produce

phylogenetic trees from the DNA dataset. Maximum parsimony analysis attempts to construct a phylogeny based on the minimum number of events needed to explain the dataset. The optimal tree under maximum parsimony is the tree that requires the fewest number of character state changes. Pairwise distance analyses measures the number of differences between two sequences. Maximum likelihood analyses attempt to find the tree that has the highest probability of depicting the evolution of the sequence dataset based on the observed data and the model of evolution.

Characteristics of the dataset such as base frequencies and substitution ratios must be taken into account during the analysis. This is accomplished by using substitution stepmatricies in parsimony analysis, DNA correction models in distance analysis, or estimation of the values from the dataset for use in maximum likelihood. Like all experimental results, the reliability of any phylogenetic tree must be tested. However, unlike most experimental results, replication and statistical analysis of phylogenetic trees is not straightforward. The use of bootstrap and jackknife analyses allow pseudo-replication of the dataset (Felsenstein, 1985; Hillis et al., 1996; Graur and Li., 2001). Bootstrap analysis consists of random sampling of the dataset to produce a new sample of the same length. Jackknife analysis consists of randomly deleting a set number of characters. In the process of randomly deleting or rearranging the characters some of the original observations will be reproduced while others will not. By repeating these techniques several thousand times, the observations that are seen at high frequencies can be assumed to be strongly supported by the data, while those that occur at lower frequencies are not. Bootstrap and Jackknife consensus trees are produced with a value at each node in the phylogeny indicating the percentage of replications in which the given

node was seen. Using these molecular systematic techniques groupings of closely related taxa, or clades, can be seen, and unlike morphological analysis the historical relationships among the clades is revealed through the relationships established in the phylogeny.

Many comparisons of molecular and morphological characters and their ability to infer taxonomic relationships have been attempted. Unfortunately as most evolutionary lineages are unique events, seldom can a phylogeny be used to predict results in another. Thus each phylogenetic hypothesis is the result of forces that may or may not have also shaped the evolutionary history of other organisms. For example, many taxa in the southeastern United States reflect the historical climatic changes in the region. The genus *Elaphe* is unique in that it allows this type of comparison between two sister clades (the North American rat snake, *Elaphe obsoleta*, and the Corn snake, *Elaphe guttata*). Both snakes are polytypic species that occur in sympatry throughout much of their range in the United States. Each species has been separated into several subspecies based on morphological characters, and both are currently under examination by molecular techniques.

The results completed thus far are intriguing. Recent work (Burbrink et al., 2000) using phylogenetic analysis at the subspecies level suggested that numerous distinct evolutionary lineages exist in *Elaphe obsoleta*. However, these lineages did not conform to current taxonomic subspecies that were based on morphological characters (Burbrink et al., 2000). As summarized in Burbrink et al. (2000), the North American rat snake, *Elaphe obsoleta*, is divided into seven subspecies based on color morphology (Table 1). Phylogenetic analysis of these subspecies suggested that four evolutionarily distinct clades were present: an eastern, central, western, and a clade from extreme western

Table 1. Descriptions of the current subspecies of *Elaphe obsoleta* drawn from Burbrink *et al.*, 2000; Wright and Wright, 1957; Conant and Collins, 1991.

Subspecies	Description
Elaphe obsoleta obsoleta	Dark brown or black dorsum, little evidence of pattern
Elaphe obsoleta quadrivittata	Four dark dorsal stripes Body color ranges from yellow- tan to gray
Elaphe obsoleta lindheimeri	25-35 dorsal blotches, brown, yellow, or orange ground color
Elaphe obsoleta spiloides	25-35 dorsal blotches, gray to grayish white body color
Elaphe obsoleta deckerti	Four longitudinal stripes, orange- yellow or tan body
Elaphe obsoleta williamsi	Blotches and stripes, gray or white color body
Elaphe obsoleta rossalleni	Body color orange-yellow to orange-brown, poorly defined longitudinal stripes
Elaphe bairdi (formerly *Elaphe obsoleta bairdi)*	Ground color of brown to gray, four poorly defined longitudinal stripes

Texas composed of Baird s rat snake, *Elaphe bairdi*. The eastern clade was composed of snakes east of the Appalachian Mountains. The central clade was composed of snakes between the Appalachian Mountains and the Mississippi River, and the Western clade was composed of snakes west of the Mississippi River. The *E. bairdi* clade was composed of two samples from Jeff Davis County, Texas. Results from this analysis show that the phylogenetic groupings do not conform to current taxonomy based on the color morphology. Representatives from certain subspecies (*E. o. obsoleta, E. o. lindheimeri*, and *E. o. spiloides*) were found in more than one clade suggesting that the subspecies based on color morphology are not evolutionarily distinct lineages (Burbrink et al., 2000).

The sister taxon to *Elaphe obsoleta*, *Elaphe guttata,* is a polytypic species that also exhibits color pattern variation throughout its range. The taxonomic history of *E. guttata* has been complicated due to repeated changes in names assigned to the taxa. Specimens of *E. guttata* from Texas differ from specimens found in the eastern part of the range from Louisiana to Florida. Individuals from Texas lack the bright colors seen in the snakes from the eastern portions of the range (Werler and Dixon 2000). Historically the corn snakes in Texas that are now the Great Plains Rat snake, *E. g. emoryi,* were classified as Emory s rat snake, *Elaphe emoryi emoryi,* and the range was limited to the central and western portions of the state (Figure 1, A) (Wright and Wright 1957). Later all corn snakes in Texas were classified as the Great Plains rat snake, *Elaphe guttata emoryi,* (Thomas, 1974). After further analysis of the morphological characters this taxon was divided into the corn snake, *Elaphe guttata guttata*, and the Southwestern rat snake, *Elaphe guttata meahllmorum,* (Smith et al., 1994). As reported

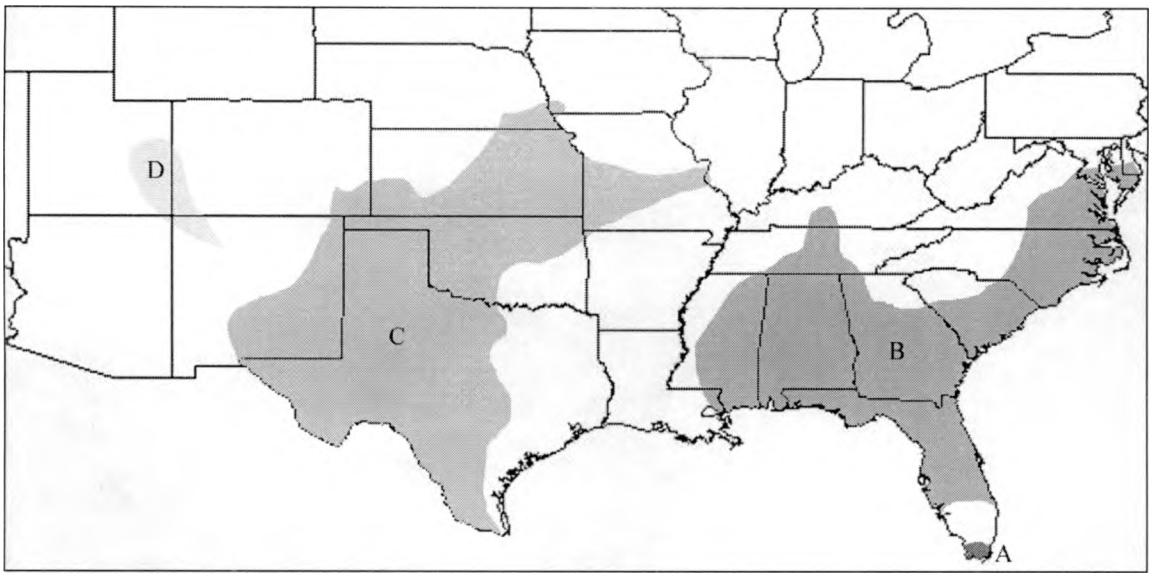

Figure 1. Historical map of the southern United States showing the taxonomy and range of *Elaphe guttata* as of 1957. Four subspecies represented on the map include: A) the Pink rat snake, *Elaphe guttata rosacea* B) the corn snake, *Elaphe guttata guttata* C) the Great Plains rat snake, *Elaphe guttata emoryi* (formerly *Elaphe emoryi emoryi*) D) the Intermountain rat snake, *Elaphe emoryi intermontana*. (Adapted from Wright and Wright 1957).

by Vaughan et al. (1996), in Texas the species has been divided into the three subspecies the corn snake, *Elaphe guttata guttata*, the Great Plains rat snake, *Elaphe guttata emoryi*, and the Southwestern rat snake, *Elaphe guttata meahllmorum* (Figure 2, A-D). These separations were based on the number of dorsal body and tail blotches, the number of ventral and subcaudal scales, the degree of ventral pigmentation, and presence or absence of subcaudal stripes (Table 2). Texas corn snakes range from tan to orange tan with a row of large brown to reddish brown blotches along the dorsal midline. *Elaphe guttata guttata* has 50.7 dorsal and tail blotches, 282.3 ventral and subcaudal scales, a high degree of ventral pigmentation, and subcaudal stripes are present in 90.9% of individuals (Vaughan et al., 1996; Werler and Dixon 2000; Dixon, 2000). The Great Plains rat snake, *E. g. emoryi*, is pale gray to grayish brown with a dorsal row of dark gray to grayish brown blotches outlined in dark brown or black. *Elaphe guttata emoryi* has 67.2 dorsal and tail blotches, 282.6 ventral and subcaudal scales, an intermediate to heavy degree of ventral pigmentation, and subcaudal stripes are present in 82.1% of individuals (Vaughan et al., 1996; Werler and Dixon, 2000; Dixon, 2000). The Southwestern rat snake, *Elaphe guttata meahllmorum*, is pale gray with a dorsal row of darker gray to grayish brown blotches. *Elaphe guttata meahllmorum* has 55.1 dorsal and tail blotches, 295.2 ventral and subcaudal scales, a small degree of ventral pigmentation, and subcaudal stripes are present in 27.5% of individuals (Vaughan et al., 1996, Werler and Dixon 2000; Dixon, 2000).

Mitochondrial DNA analysis of the *Elaphe guttata* complex seeks to evaluate the validity of the currently recognized taxonomy. It is possible that the topologies produced

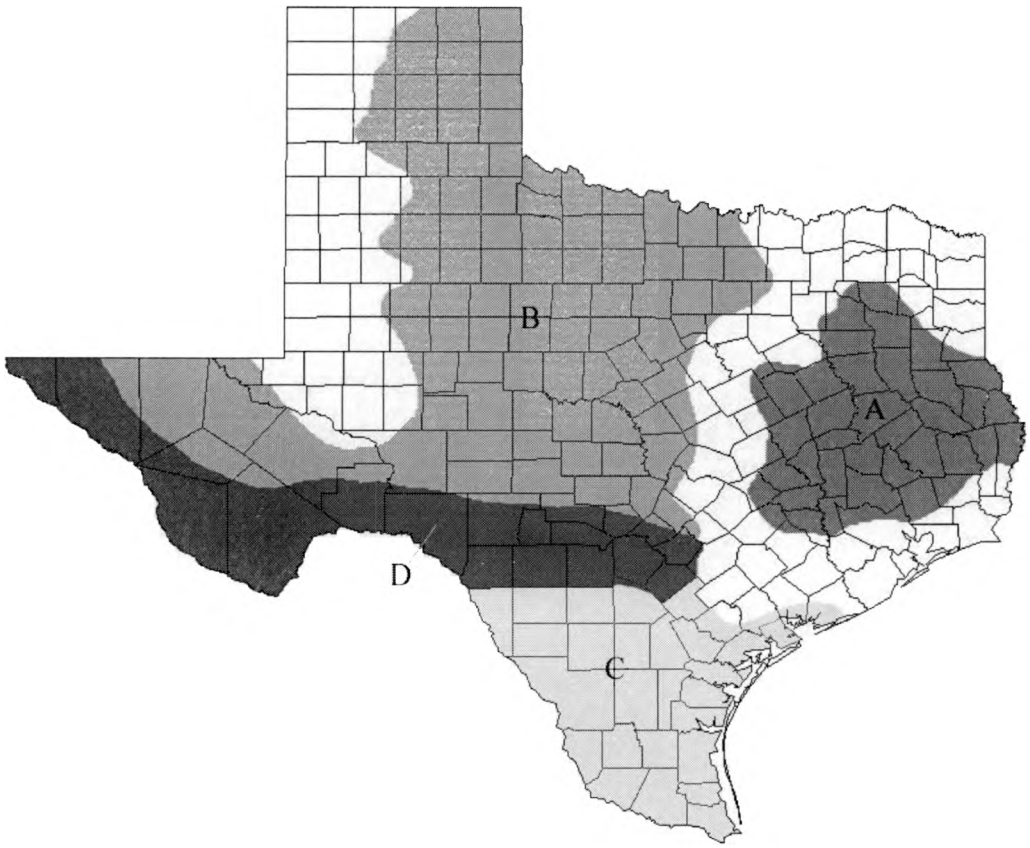

Figure 2. Map of Texas showing the geographic distribution of *Elaphe guttata* according to Vaughan *et al.*, 1996. Shaded regions denote: (A) Range of the corn snake, *E. g. guttata*; (B) range of the Great Plains rat snake, *E. g. emoryi*; (C) range of the Southwestern rat snake, *E. g. meahllmorum*; and (D) intergradation zone between *E. g. emoryi* and *E. g. meahllmorum.*

Table 2. Morphological characters used in the separation of *Elaphe guttata* into *E. g. guttata*, *E. g. emoryi*, and *E. g. meahllmorum*. Modified after Vaughan *et al.*, 1996.

Subspecies	Dorsal and tail blotches mean (min -max)	Ventral and subcaudal scales mean (min -max)	Degree of ventral pigmentation	Percent of individuals with subcaudal stripes
E. g. guttata	50.7 (44-59)	282.3 (272-299)	High	90.9%
E. g. emoryi	67.2 (57-81)	282.6 (271-293)	Intermediate to heavy	82.2%
E. g. meahllmorum	55.1 (39-67)	295.2 (275-312)	Small	27.5%

will support the current subspecific classification. An alternate outcome is that the topology produced will not support the current taxonomy which is based on morphological characters. Another possibility is an intermediate of the first two in that topologies produced by mtDNA analysis and the morphological classification will agree in some individuals but not in others. Given the results in *obsoleta* several distinct clades in *guttata* would underscore the importance in understanding the biogeography of related organisms, and illustrate the predictive nature of systematics. A more complete understanding of the evolutionary lineages and decisive agreement on taxonomy and classification may help put an end to repeated reclassification of *E. guttata.*

The objectives of this investigation include: 1) Determine the phylogenetic relationships among the three subspecies of corn snake in Texas *Elaphe guttata guttata*, *Elaphe guttata emoryi*, and *Elaphe guttata meahllmorum* through mtDNA analysis. 2) Determine the phylogenetic relationships between the Texas corn snakes and other subspecies of *E. guttata* from the eastern part of their range in Louisiana, Georgia, and Florida. 3) Determine intergradation zones between subspecies throughout the range of *E. guttata* if distinct lineages are shown in the mtDNA analysis.

MATERIALS AND METHODS

Samples were obtained from several collections, assigned an MF number, and combined into the Forstner tissue collection, Southwest Texas State University. Although only two samples are voucher specimens at the Texas Cooperative Wildlife Collection (TCWC), several other samples were taken from live specimens that are still in the possession of their owners.

DNA Analysis

DNA was extracted from frozen tissue using the Quigen DNeasy extraction kit and protocol. Extracted samples were amplified in 100µl reactions using 20µl Taq buffer (0.3M TRIS, 0.0075M $MgCl_2$, 0.075M $(NH_4)_2SO_4$, pH 8.5) 0.5µl Taq polymerase, 1.0µl dNTP's (2.5mM dATP, dCTP. dGTP, and dTTP) 1.0µl (10pM) forward (ND4) and reverse (Leu) primers, 1.0µl DMSO, and 74.5µl ddH_2O. GeneAmp ® PCR System 9700 was used to perform 40 cycles of denaturation, annealing, and extension using 95°C for thirty seconds, 50°C for 1 minute, and 72°C for 1 minute followed by a final extension period of five minutes at 72°C. Successfully amplified samples were cleaned using Concert ™ Rapid PCR Purification System and protocol. Clean PCR products were cycle sequenced using Big Dye v 2.0 in 9.0µl reactions using 3.0µl Big Dye, 1.0-2.0µl clean PCR product (depending on concentration), 0.5µl primer (Table 3), 3.5-4.5µl ddH_2O

Table 3. Primer sequence used to generate mtDNA sequence spanning a portion of the ND4 (NADH dehydrogenase subunit 4), $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ genes.

Primer Name	Primer Sequence 5' to 3'
ND4	CACCTATGACTACCAAAAGCTCATGTAGAAC
Leu	ACCACTGGGAGGTTCATTTTCATTAC
Corn F3	CTACAY*ATATTY*CTATCAACACA
Corn R2	CAACCTGR**TGAY*TACTAATTAAC

* Y indicates the presence of the nucleotides Cytosine or Thymine

** R indicates the presence of the nucleotides Adenine or Guanine

(depending on concentration of PCR product). GeneAmp ® PCR System 9700 were used to perform 35 cycles of denaturation, annealing, and extension using 95°C for thirty seconds, 55°C for 1 minute, and 60°C for 1 minute. Cycle sequencing products were cleaned using 700µl Sephadex solution (0.0625g/ml) in a Centri-sep column (Princeton Separations). Cycle sequencing products will be placed in the column and centrifuged for three minutes at 3,000 rpm. Clean cycle sequencing reaction products were assayed on a 6.0% polyacrylamide gel using the ABI prism 377 DNA sequencer. Sequencher™ 4.1 were used to assemble a contig (multiple sequence assembly) and produce a consensus sequence for each individual. Consensus sequences were assembled into a final alignment. The final alignment will be compared to previously aligned sequences, the nucleotide substitutions compared, and the tRNA structural integrity verified before phylogenetic analysis.

Phylogenetic Analysis

A Nexus format file was exported from the verified sequence assembly. PAUP* 4.0 (Swofford, 1999) will be used for all phylogenetic analyses. A thorough examination of the dataset was prepared to determine the optimal analyses to perform. Modeltest 3.06 (Posada and Crandall, 1998) was used to analyze the dataset for base frequencies, transition/ transversion ratio, proportion of invariable sites, gamma distribution, and correct DNA correction model. Using an uncorrected pairwise distance algorithm as well as the DNA correction model determined from Modeltest, the characters in question were graphed to determine which characters, if any, were saturated in the dataset. Parsimony, neighbor joining, and maximum likelihood analyses were performed to determine the

optimal topology representing the evolutionary history of the taxa. Starting with parsimony a heuristic search will be performed with 1,000 random additions excluding uninformative and ambiguous characters. To determine support of the nodes produced in this analysis a bootstrap and jackknife searches with 10,000 replications was performed using a full heuristic search. For analysis using distance criteria a neighbor joining analysis was performed using the correction algorithm determined from analysis by Modeltest. Bootstrap and jackknife analyses with 10,000 replicates were performed using a neighbor joining search to determine support for the distance topology. Maximum likelihood analysis was performed using base frequencies, substitution ratio, proportion of invariable sites, and gamma distribution calculated by Modeltest. Topologies produced were examined to determine if distinct clades were present, and to determine if any clades present represent distinct lineages represented by current subspecific taxonomy.

RESULTS

Dataset characteristics

NADH dehydrogenase subunit 4, $tRNA^{Leu}$, $tRNA^{His}$, and $tRNA^{Ser}$ genes were sequenced from twenty-nine samples from Texas, Colorado, Louisiana, Florida, and Georgia (Figure 3). Analysis of the dataset using Modeltest determined that the correct DNA correction model was HKY (Hasegawa et al., 1985) + I (proportion of invariant sites) + G (gamma distribution). Base frequencies of A= 0.3539, C= 0.2637, G=0.1171, and T= 0.2653, transition/transversion ratio of 6.3095, proportion of invariable sites= 0.5314, and a gamma distribution of 0.4890 were also determined by Modeltest. Examination of the transitions and transversions in the dataset show that characters in the dataset were not saturated (Figures 4-8).

Phylogenetic analysis

Parsimony analysis produced twelve equally parsimonious solutions with a tree length of 185 steps (CI=0.654, RI=0.893, RC=0.584). A g1 statistic of –0.539374 for the parsimony analysis indicates that data is structured (Hillis and Huelsenbeck, 1992). Bootstrap and jackknife values showed support for three distinct clades (Figure 9); A clade composed of samples east of the Mississippi River (100/100 bootstrap (BP) /jackknife (JK) support) with the exception of sample 2 (see Figure 3), a clade composed of snakes from the Mississippi River west into the Eastern parts of Texas defined as the

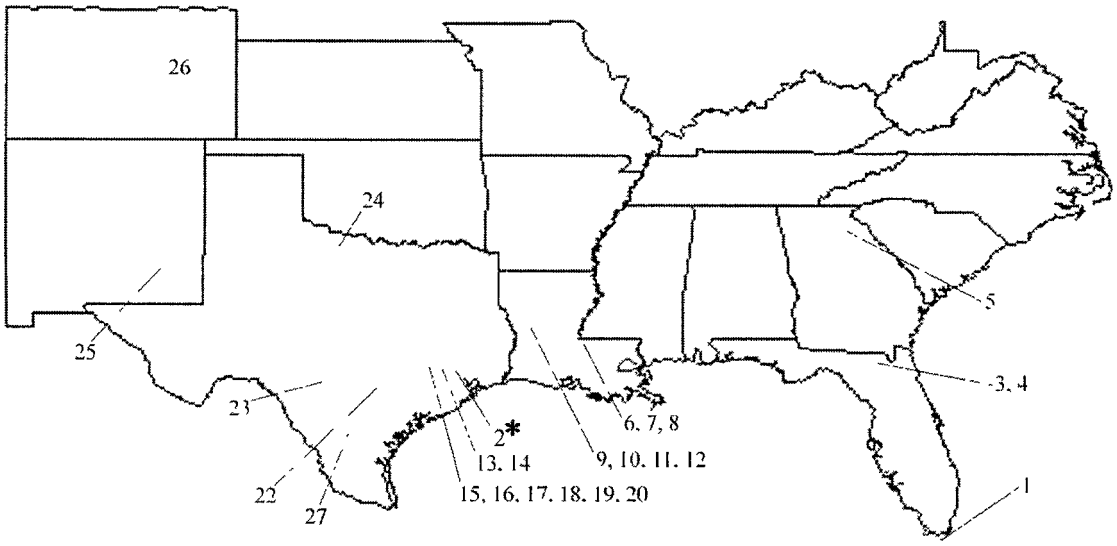

Figure 3. Map of the southern United States Showing the localities of specimens used for phylogenetic analysis. All tissues are cataloged using MF numbers and stored in the Michael Forstner tissue collection, Southwest Texas State University. More complete sample information can be found in Appendix II.

* Sample believed to be an escaped pet due to unusual coloration for the area in which it was found

(1) MF 5309· Monroe Co , FL., **(2)** MF 5383· Montgomery Co , TX, **(3)** MF 2935 Hamilton Co , FL,, **(4)** MF 3104 Hamilton Co , FL, **(5)** MF 5114 Barrow Co., GA, **(6)** MF 5290 W Feliciana Par , LA, **(7)** MF 5300 W Feliciana Par , **(8)** MF 5299 W Feliciana Par , LA, **(9)** MF 5289 Natchitoches Par , LA, **(10)** MF 5297 Natchitoches Par , LA, **(11)** MF 5298 Natchitoches Par , LA, **(12)** MF 5308. Natchitoches Par., LA, **(13)** MF 5779. Grime Co , TX, **(14)** MF 4995. Grimes Co , TX, **(15)** MF 4988 Grimes Co , TX, **(16)** MF 4994 Brazos Co , TX, **(17)** MF 4993 Brazos Co , TX, **(18)** MF 3801 Brazos Co , TX, **(19)** MF 130· Brazos Co , TX, **(20)** MF 4991· Brazos Co , TX **(21)** MF 4989 Brazos Co , Tx, **(22)** MF 3799 Guadalupe Co , TX, **(23)** MF 2491 Bandera Co , TX, **(24)** MF 3800 Archer Co , TX, **(25)** MF 5117 Chavez Co, NM, **(26)** MF 5305. Colorado (East of Denver), **(27)** MF 1402 McMullen Co , TX

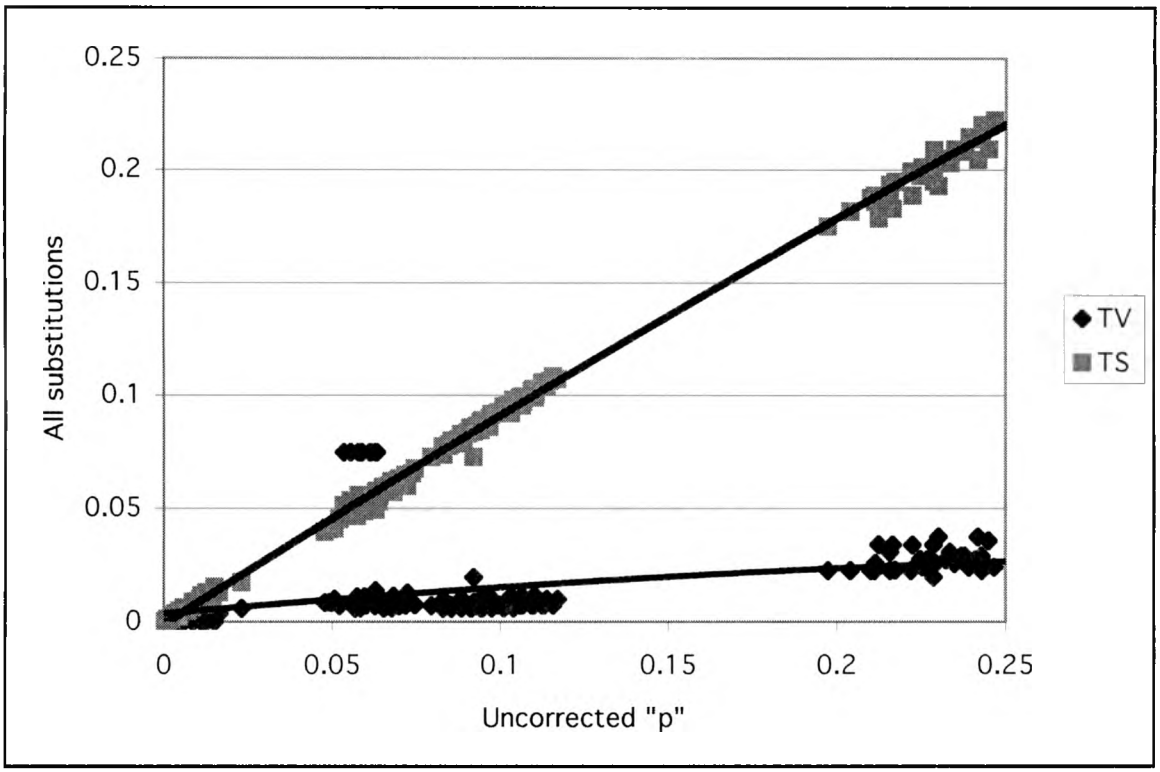

Figure 4. Examination of transitions and transversions in the *E. guttata* dataset composed of ND4, $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ mitochondrial genes. As shown by the figure neither transversions nor transversions are not saturated. Graph shows uncorrected distances. Note the presence of abberant transversions in the uncorrected data.

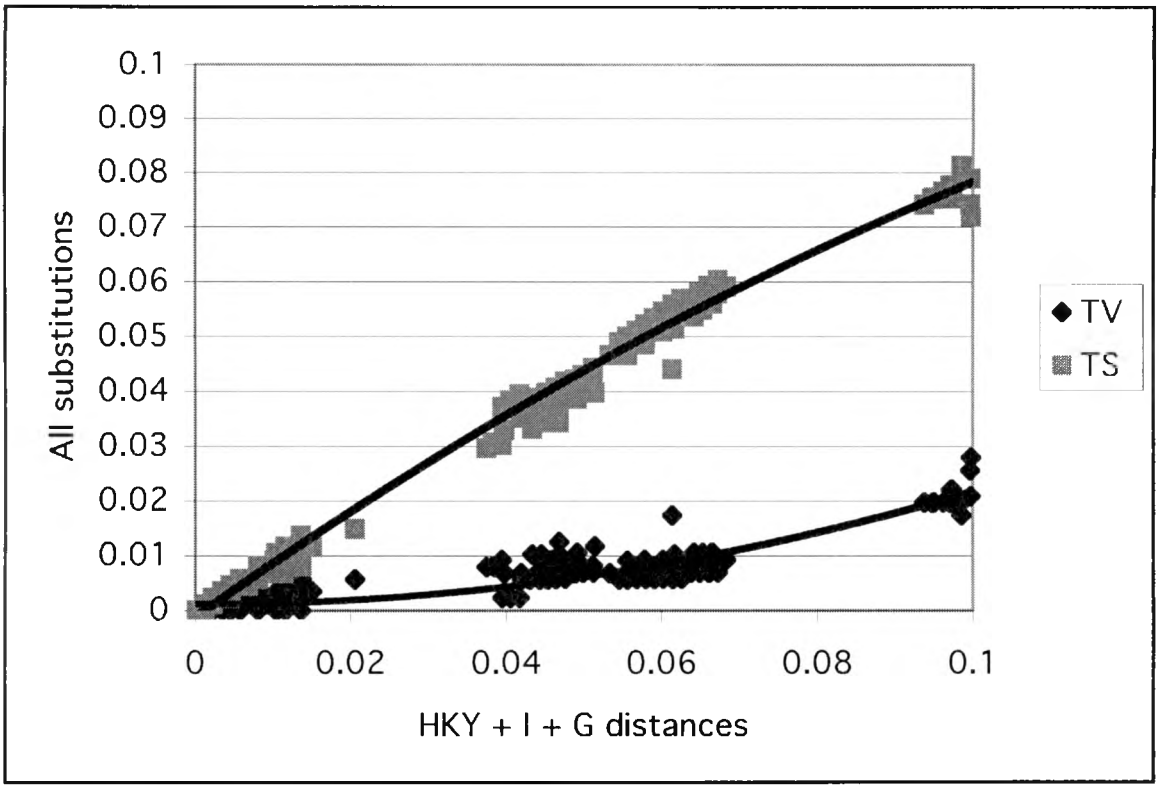

Figure 5. Examination of transitions and transversions in the *E. guttata* dataset composed of ND4, $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ mitochondrial genes. Graph shows the effects of the HKY + I + G DNA correction algorithm on the dataset. As shown by the figure transitions and transversions are not saturated with the DNA correction algorithm. Note the abberant transversions are now in line.

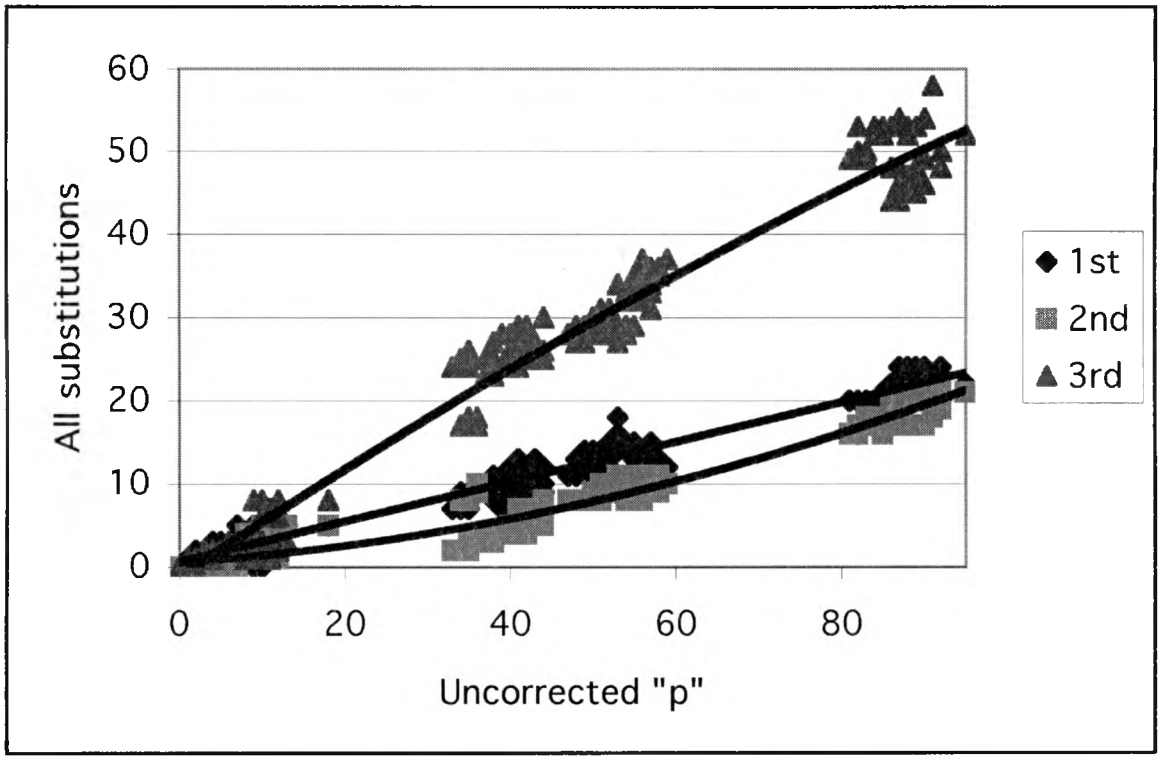

Figure 6. Examination of 1st, 2nd, and 3rd codon positions in the *E. guttata* dataset composed of 650 codons in the 3' end of the ND4 mitochondrial gene. Graphs shown are for uncorrected distances. As shown by the figure 1st, 2nd, and 3rd codon positions are not saturated.

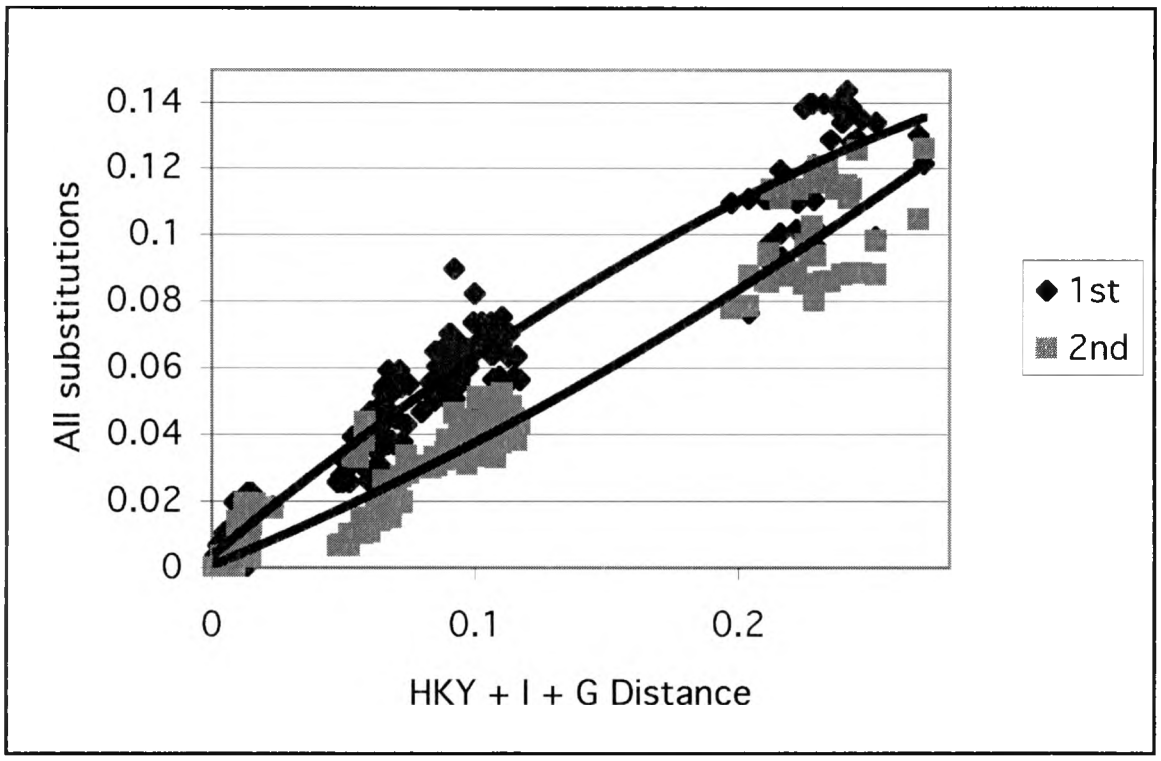

Figure 7. Examination of 1st and 2nd codon positions in the *E. guttata* dataset composed of 650 codons in the 3' end of the ND4 mitochondrial gene. Graphs show the effects of the HKY + I + G DNA correction algorithm on the dataset. As shown by the figure, 1st and 2nd codon positions are not saturated with the DNA correction algorithm.

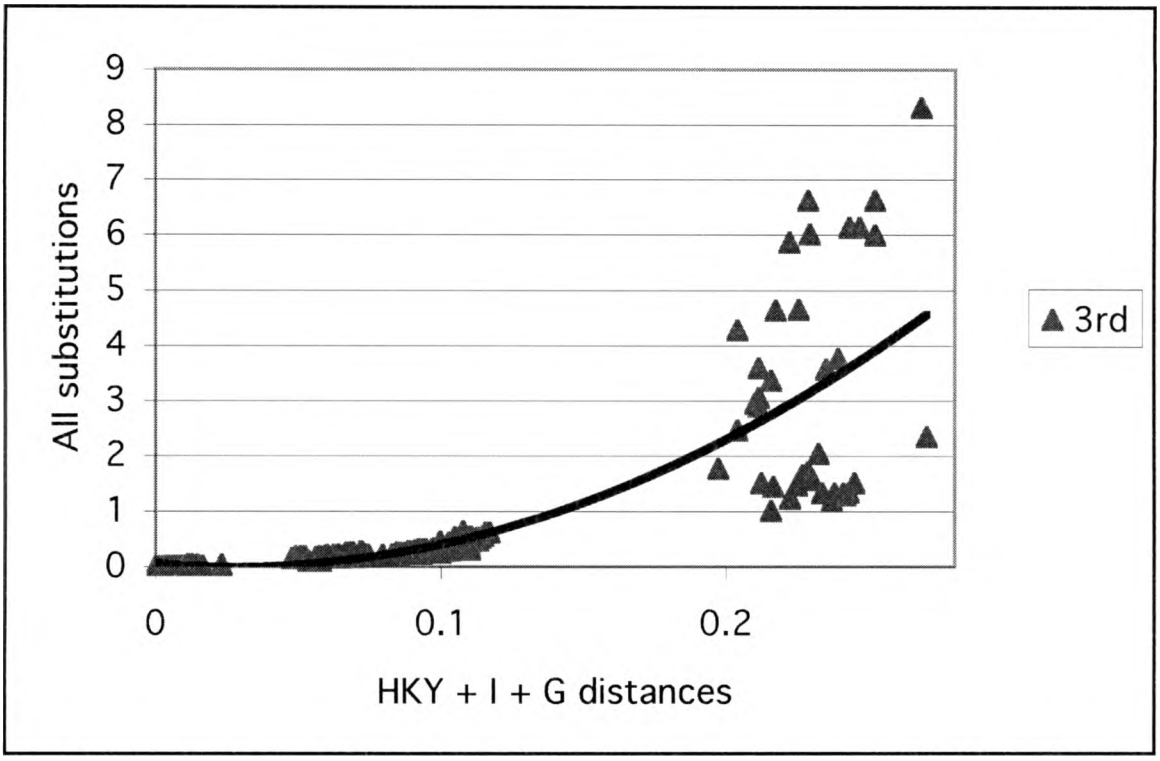

Figure 8. Examination of 3rd codon positions in the *E. guttata* dataset composed of 650 codons in the 3' end of the ND4 mitochondrial gene. Graphs show the effects of the HKY + I + G DNA correction algorithm on the dataset. As shown by the figure 3rd codon positions are not saturated with the DNA correction algorithm.

Austroriparian Region (Vaughan et al., 1996; Blair, 1949) (95/95 BP/ JK support), and a clade composed of snakes from central and south Texas extending into New Mexico and Colorado (87/86 BP/ JK support). Distance analysis using the HKY + I + G correction algorithm produced 15,121 trees with a tree score of 0.43448 (ME-score 350.399, g1= -0.6968) (Figure 10). Maximum likelihood analysis produced one tree with a –ln L of 894.69249 (Figure 11). Comparison of the parsimony, distance, and maximum likelihood topologies using the Kishino- Hasegawa, Templeton, and winning sites tests showed that the topologies did not differ significantly under maximum parsimony. Distance topologies had a tree score of 189 steps under parsimony (CI= 0.640, RI= 0.886, RC= 0.584, Kishino- Hasegawa P=0.3195, Templeton P=0.3173, winning sites P= 0.6250). The maximum likelihood topology had a tree score of 187 steps under parsimony (CI= 0.647, RI= 0.890, RC= 0.584, Kishino- Hasegawa P=0.1583, Templeton P=0.1573, winning sites P= 0.2891).

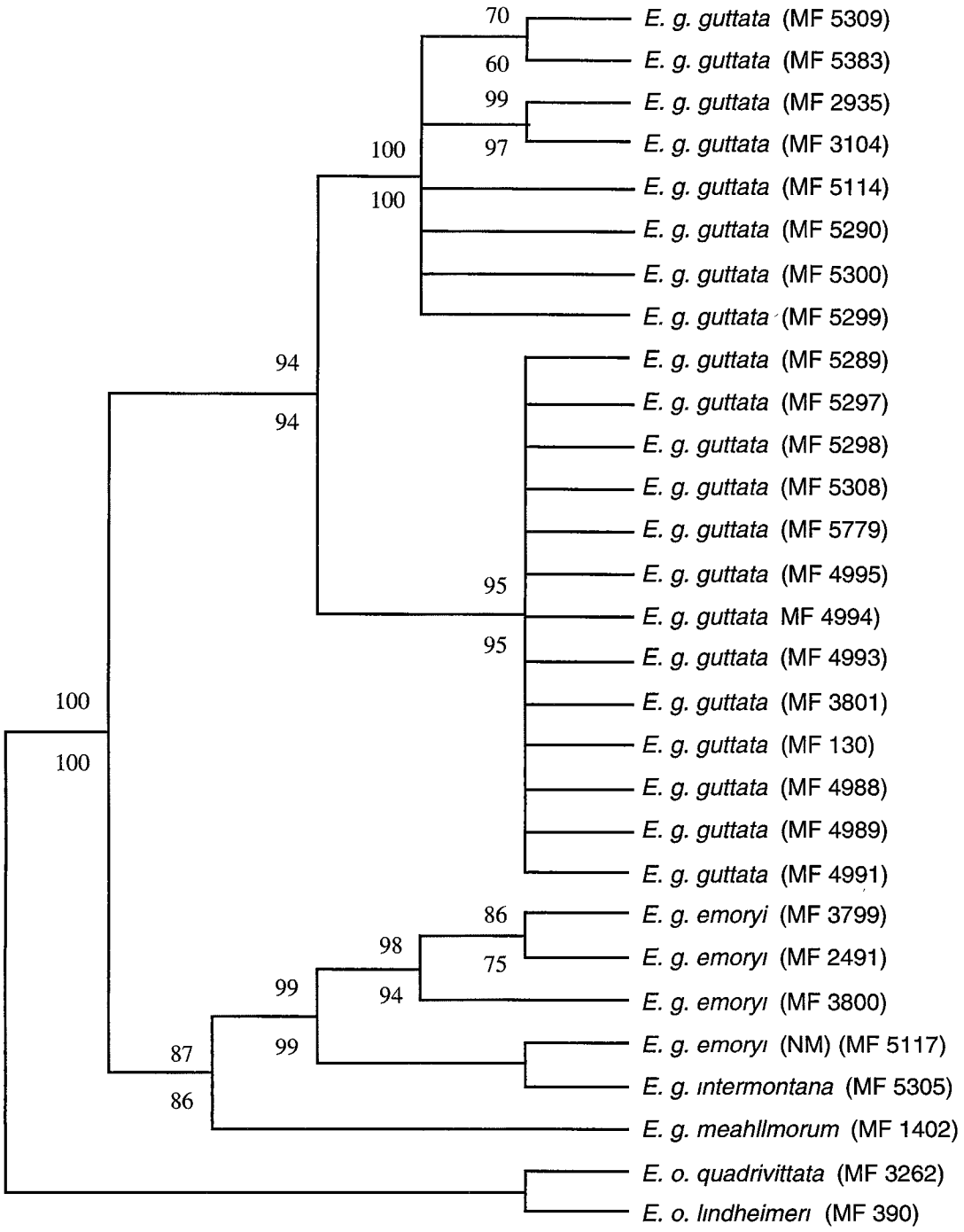

Figure 9. Strict consensus of twelve equally parsimonious solutions from a full heuristic search under maximum parsimony using tree-bisection-reconnection branch swapping algorithm. Random addition sequence was used with 10,000 replicates All trees had a tree length of 185 steps. Bootstrap and jackknife values are shown with bootstrap values above and jackknife values below the nodes for which they support. Analysis included 875 mt DNA nucleotides.

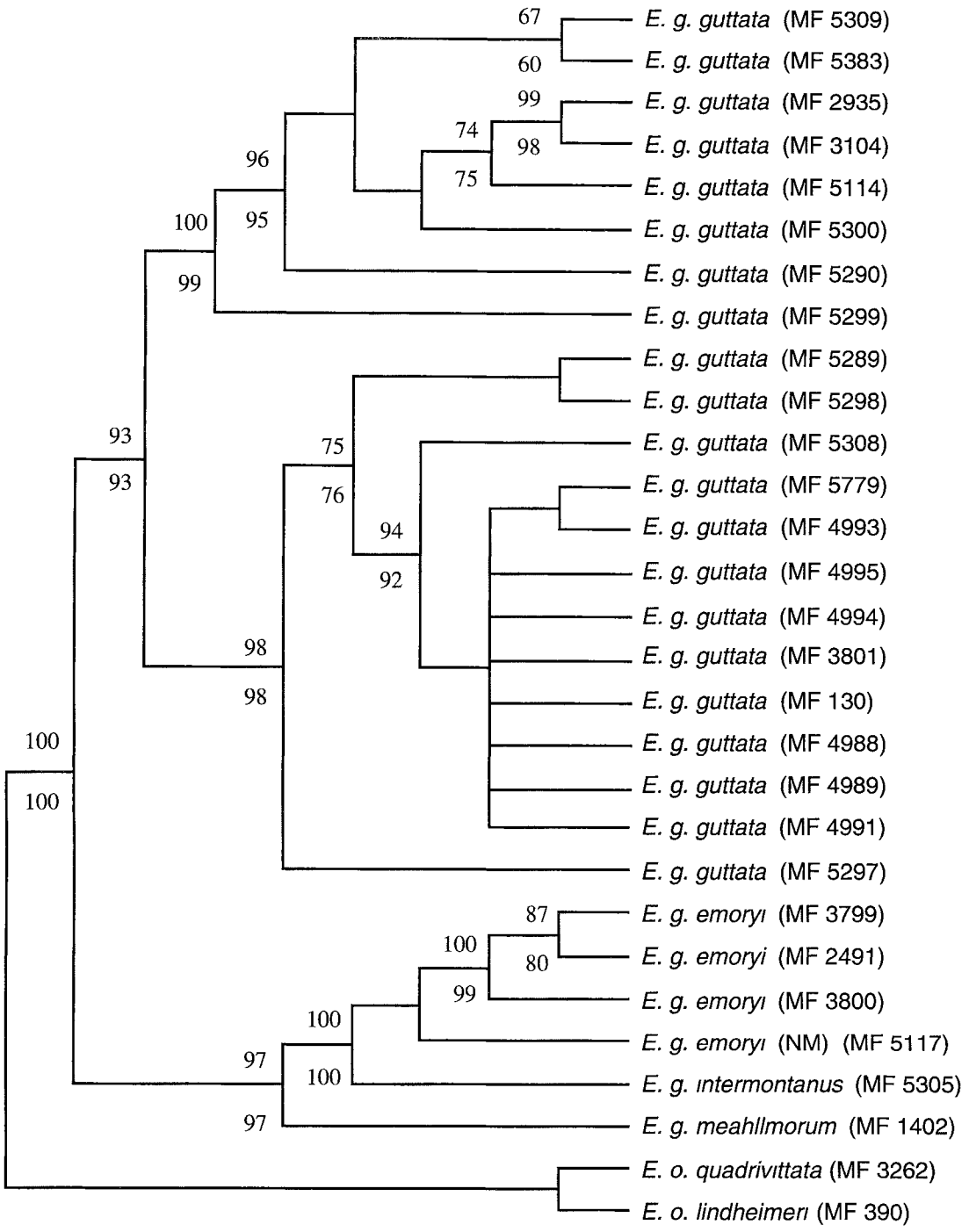

Figure 10. Strict consensus of 15, 212 trees with a tree score of 0.43448 following a neighbor joining heuristic search of 139,375,127 rearrangements using the HKY+ I+ G DNA correction algorithm (I=0.5314, G=0.4890). Bootstrap and jackknife values are shown with bootstrap values above and jackknife values below the nodes for which they support. Analysis included 875 mt DNA nucleotides.

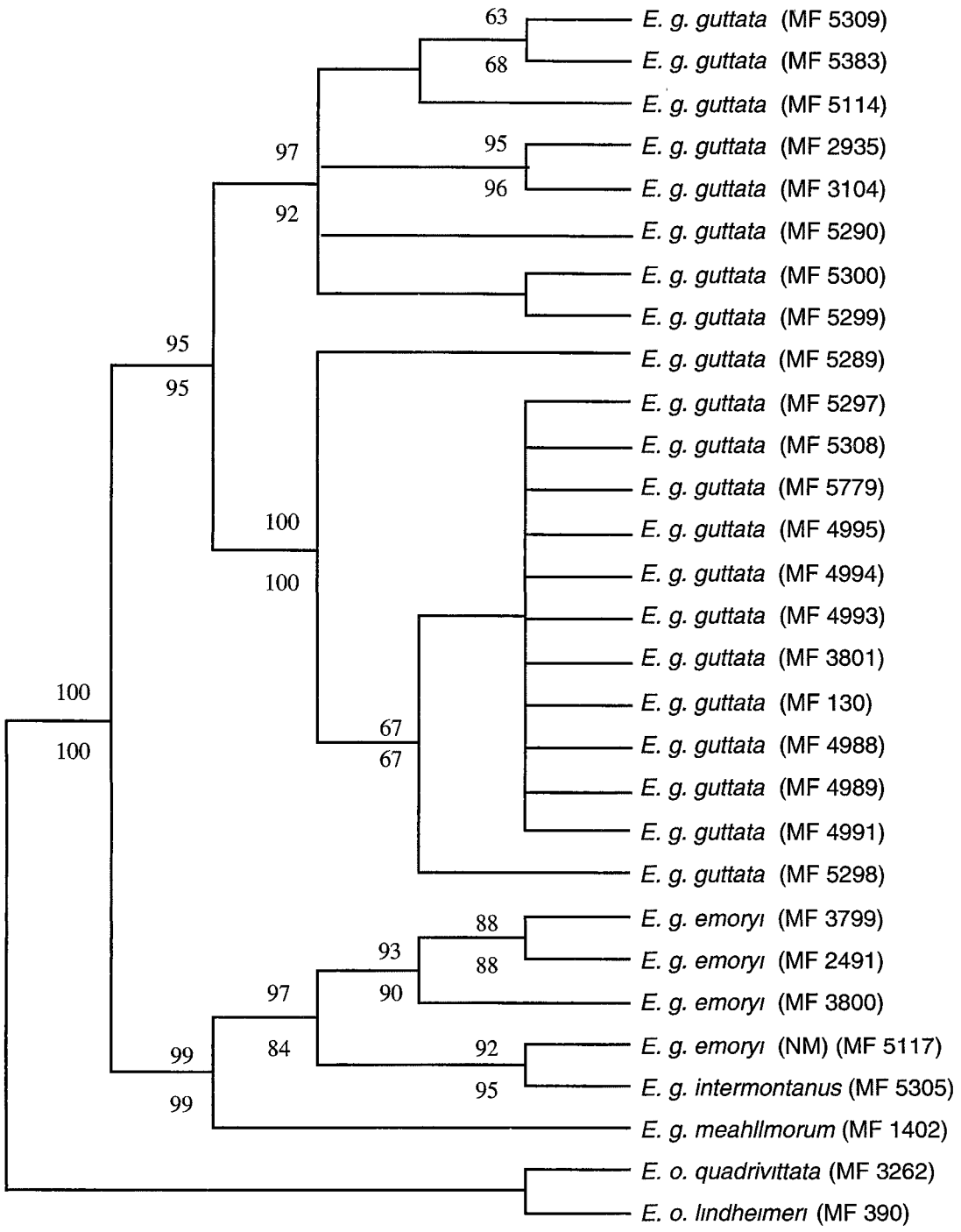

Figure 11. Maximum likelihood analysis produced one tree with a –ln L of 894.69249 following a heuristic search with 1,000 replicates. Bootstrap and jackknife values are shown with bootstrap values above and jackknife values below the nodes for which they support. Analysis included 875 mt DNA nucleotides.

DISCUSSION

Summary of Results

Molecular systematic analysis of the corn snake *Elaphe guttata* revealed the presence of three distinct clades. A clade composed of snakes from Florida and Georgia to the Mississippi River, a clade composed of snakes from the Mississippi River to the Austroriparian Region of Texas, and a clade composed of snakes in central and south Texas extending into New Mexico and Colorado (Figure 12). The easternmost clade, and the clade ranging from the Mississippi River are both composed of snakes that have been classified as *Elaphe guttata guttata* (Vaughan et al., 1996; Wright and Wright 1957). The western most clade is composed of snakes classified as *Elaphe guttata emoryi*, *E. g. meahllmorum*, and *E. g. intermontana* (Vaughan et al., 1996; Smith et al., 1994; Wright and Wright, 1957). Unlike the *E. g. guttata* clades (G1 and G2), the third clade is much more structured and there is much more variation among individuals within the clade (Figure 13). In all analyses *E. g. meahllmorum*, and *E. g. intermontana* remain distinct units within the *E. g. emoryi* clade. In addition, a sample from southeastern New Mexico is clearly shown to be *E. g. intermontana.* This result was unexpected as this geographic region is believed to be within the range of *E. g. emoryi* (Wright and Wright, 1957; Thomas, 1974). The variation shown within the *emoryi* clade is consistent with morphological separations proposed by Vaughan et al., (1996), and Smith et al., (1994).

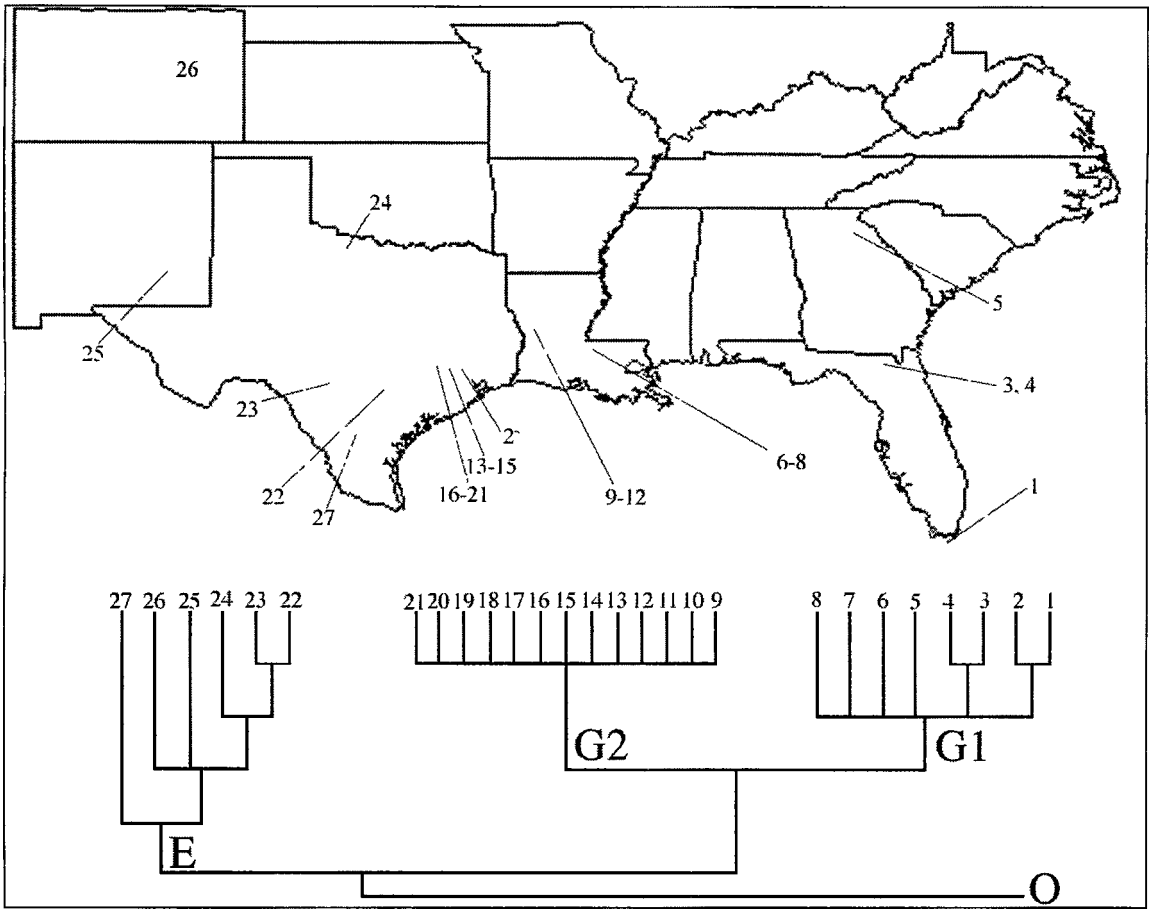

Figure 12. Map of the Southern United States showing sample localities for *E. guttata* with a strict consensus of all topologies produced by parsimony (12 trees), distance (15,121 trees), and maximum likelihood (1 tree). ND4, $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ genes were used in all analyses. Three distinct clades are present: G1, All samples east of the Mississippi River; G2, Samples west of the Mississippi River to the Austroriparian Region of east Texas; and E, samples from west and south Texas, New Mexico and Colorado. O represents the outgroup (*E. o. quadrivittata* and *E. o. lindheimeri*).

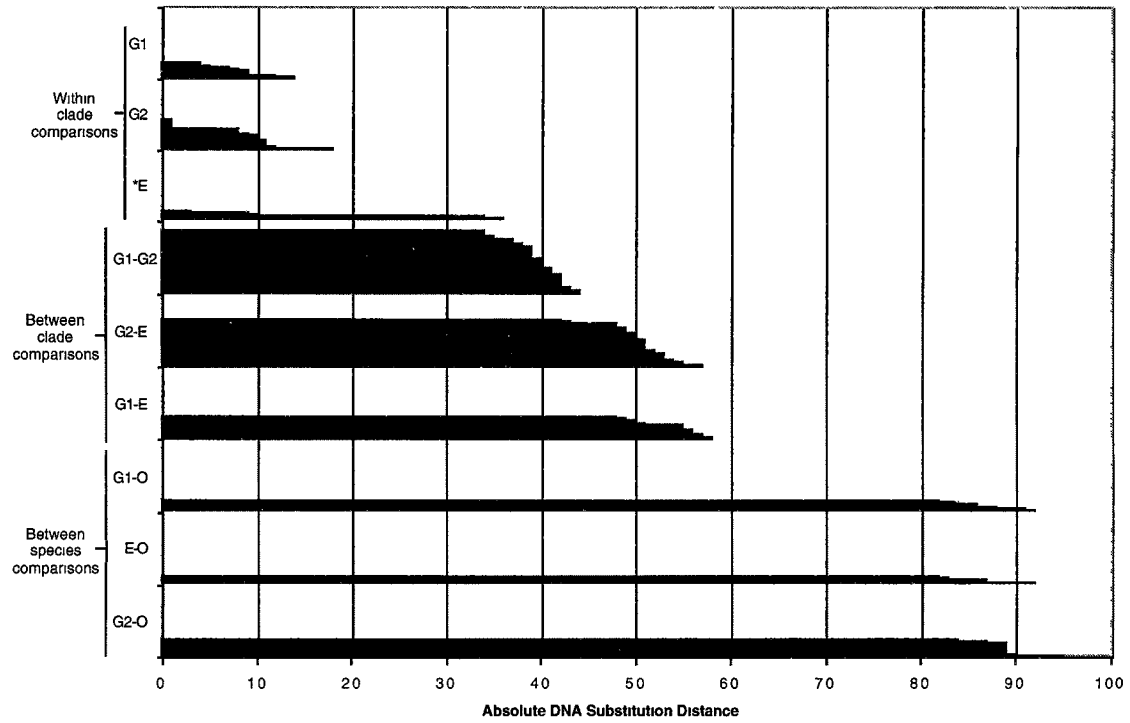

Figure 13. Absolute difference within and between clades over two taxonomic levels. The easternmost clade composed of snakes east of the Mississippi is represented by G1, the central clade composed of samples west of the Mississippi to eastern Texas is represented by G2, and the westernmost clade is represented by E. Between species comparisons are made with the outgroup *E. obsoleta* (O).

* Large distances within the westernmost clade are a result of pairwise comparisons of *E. g. emoryi* and *E. g. meahllmorum.*

Molecular Phylogeny of Elaphe obsoleta

Examination of the results found in the sister clade also showed that *Elaphe obsoleta* also was composed of three distinct clades: An eastern clade located east of the Appalachian Mountains, a central clade located between the Mississippi River and the Appalachian Mountains, and a western clade located west of the Mississippi River (Burbrink et al., 2000). The authors hypothesized that each clade originated from populations isolated in southern refugia, which migrated north as the glacial ice retreated 6,000-18,000 years ago. Unlike the results from the examination of *Elaphe guttata* presented here, the clades did not conform to current subspecific separations. In a separate analysis, Burbrink (2001) suggested that the classification of *E. obsoleta* be changed to reflect the results found in the molecular systematic analysis. The eastern clade composed of *E. o. obsoleta, E. o. quadrivittata, E. o. deckerti, E. o. rossalleni*, and *E. o. williamsi* would comprise the single species *Elaphe alleghaniensis.* The central clade composed of *E. o. obsoleta, E. o. lindheimeri*, and *E. o. spiloides* would comprise the species *Elaphe spiloides,* and the western clade composed of *E. o. obsoleta* and *E. o. lindheimeri* would comprise the species *Elaphe obsoleta.* Examination of the distances among the clades of *E. obsoleta* shows that all between clade comparisons are higher than those found in *E. guttata* except for the distance between the eastern and central clades (Figure 14), which may question the assignment of a specific names for the eastern and central clades.

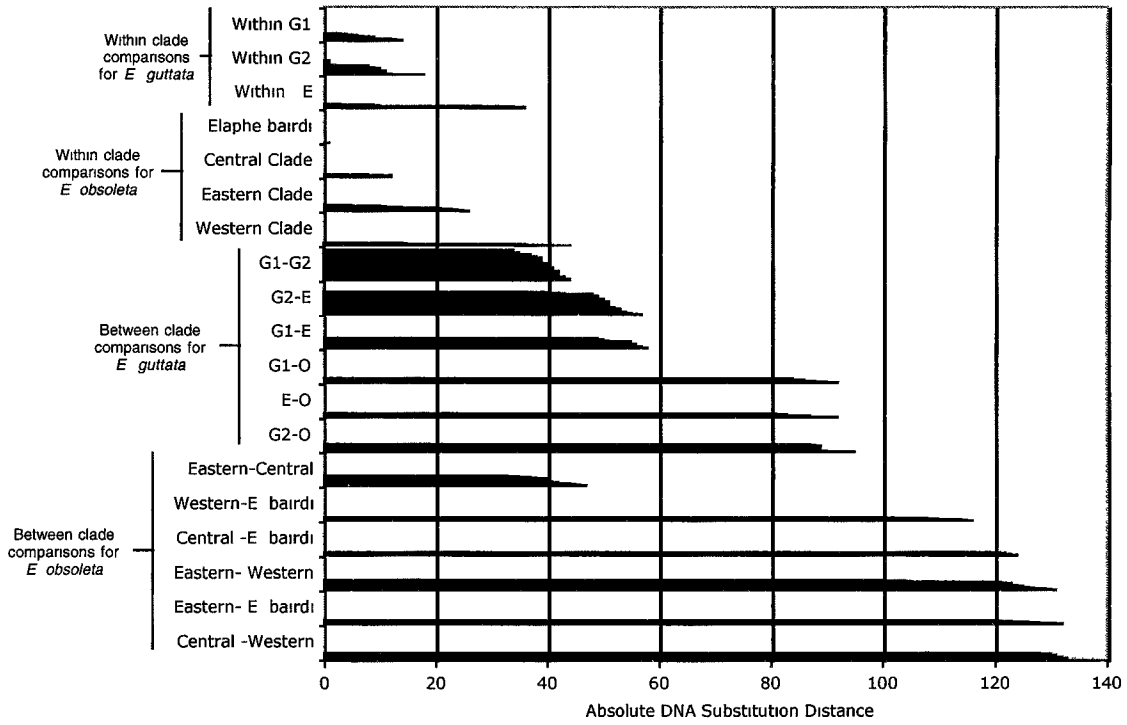

Figure 14. Comparison of within clade and between clade differences for *E. obsoleta* and *E. guttata* following mtDNA analysis. As shown in the diagram the distances between clades is greater for the comparisons of *E. obsoleta* with the exception of the comparison of the eastern and central clades.

Historical Biogeography of Elaphe guttata

The presence of distinct clades in *Elaphe obsoleta* and *E. guttata* and their sympatric ranges indicates that these taxa may have been exposed to similar evolutionary forces. However the clade boundaries for the sister taxa are not the same. In Burbrink et al., 2000, the authors suggested that clade boundaries occurred at the Appalachian Mountains and at the Mississippi River as the result of environmental phenomena during the Pleistocene. In *E. guttata* the clade boundaries occur at the Mississippi River and along the border of the Austroriparian Region of Texas. These boundaries are also believed to be a result of Pleistocene effects. As proposed by Deevey (1949), climate changes in the Pleistocene drove many species of plants and animals into two major refugia, peninsular Florida and Mexico. Under this hypothesis, the group that now makes up the *E. g. emoryi* clade would have occurred in a northern Mexican refugia. These individuals migrated north as the glacial sheet receded into their present ranges. The presence of a refugia in southern Florida might indicate that the eastern most *guttata* clade may have occurred in south Florida and thus expanded northward at the end of the Pleistocene towards the Mississippi River. This migration was stopped at the Mississippi River due to the marked increase in size that the Mississippi experienced as the glacial sheets melted (Thornbury, 1965). The western clade likely remained in sympatry with *E. g. emoryi* in South Texas. As the two groups expanded, individuals in the *E. g. guttata* clade were unable to tolerate the drier environments of west Texas and their range was eventually limited to eastern Texas and Louisiana. Migration once again was stopped at the Mississippi River (Figure 15, Hypothesis 1). Other studies suggest that areas in the southern United States retained suitable habitats during the Pleistocene glaciations

(Dillon, 1956; Flint, 1957; Flint 1971). Under this hypothesis *E. guttata* may have existed in southern Texas during the Pleistocene as indicated by Hill (1971). These individuals were most likely members of the *E. g. emoryi* clade. Decreases in sea levels during glaciations created refugia along portions of the Gulf Coast along the continental shelf that were once above sea level (Britton and Morton, 1989; Brown and Lomolino, 1998; Thomas, 1974). The presence of suitable habitat along the Gulf Coast may have provided adequate habitat for *E. g. guttata* in areas south of present day Louisiana. The expansion of the Mississippi River separated individuals in this area causing the separation of *E. g. guttata* into two clades. The western clade would have then expanded eastward into part of Texas, and the eastern clade expanded to the east into Florida and northward into Georgia (Figure 15, Hypothesis 2).

Support for the historic migratory patterns of *E. guttata* and *E. emoryi* can be extrapolated from the phylogenetic topologies. The *emoryi* clade's origination from a South Texas refugia is supported by the position of *E. g. meahllmorum* at the basal position within the clade. This subspecies' range is limited to the southern part of Texas into northern Mexico (Figure 2). Examination of the *guttata* clades shows that the basal members of each clade are from localities in Louisiana in the distance and maximum likelihood analyses. This would support the separation of a single refugia in southern portions of Louisiana by the increased width of the Mississippi River and subsequent migrations to the east and west by respective clades.

Conclusions

Mitochondrial DNA analysis poses several possible options for stable taxonomy and systematics in *E. guttata*. Since each clade was seemingly shown to be a distinct

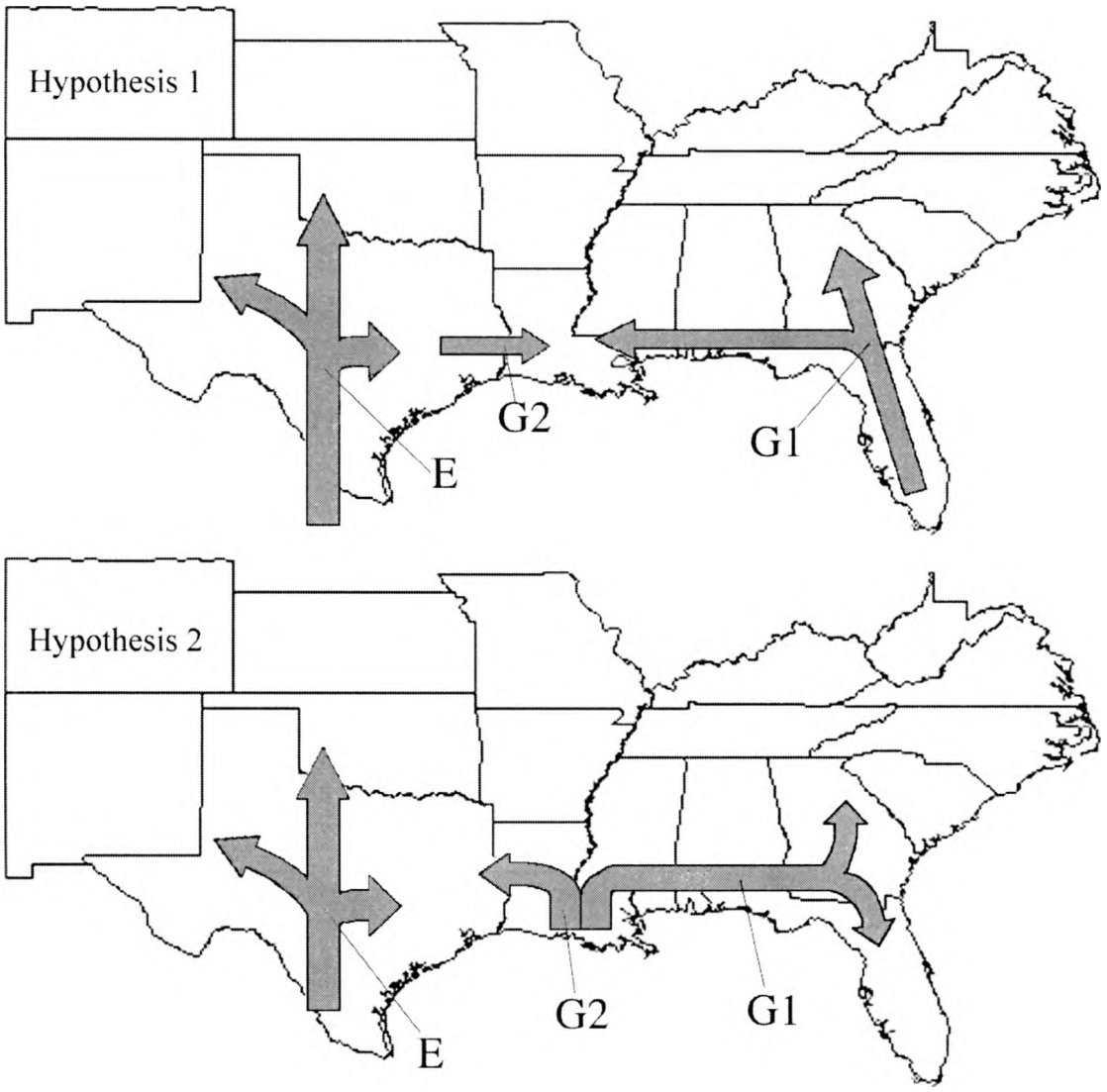

Figure 15. Maps of the southern United States showing possible responses to post Pleistocene environmental changes resulting in distinct evolutionary lineages for *E. guttata*. Hypothesis 1 shows the possible migratory history resulting from refugia limited to northern Mexico and peninsular Florida. Hypothesis 2 shows the presence of *E. guttata* in southern Louisiana which was separated due to changes in the Mississippi River as ice sheets melted.

evolutionary lineage, under the definitions of Wiley (1978, 1980) and Cracraft (1983), one or all of these subspecies may be designated as separate species. This is the logic followed by Burbrink, (2001) for *E. obsoleta.* Due to high variation among all taxa discrete genetic distances that distinguish subspecific from specific classification do not exist. However, comparison of genetic distances for closely related taxa can provide some indication of the genetic divergence between species at this level. Examination of the distances between the two clades composed of *E. g. guttata* shows that the distance between theses groups is less than the distance between either *E. g. guttata* clade and the western most clade composed of *E. g. emoryi* (Figure 13). Examination of the distances between other closely related snakes also shows that the distance between the two *E. guttata* clades is less than all other between species comparisons (Figure 16). The differences between the two *E. guttata* clades and the westernmost clade composed of *E. g. emoryi, E. g. meahllmorum,* and *E. g. intermontana* questions the validity of subspecies designation for *E. g. emoryi.* Comparison of the distance between either *E. g. guttata* clade (G1 or G2) and the western clade (E) shows that these distances are greater than the distance between *E. o. lindheimeri* and *E. bairdi* (Figure 16) which have been shown to be separate species by both morphological analysis and molecular analysis (Olson, 1977; Burbrink et al., 2000).

The presence of three distinct clades in the mitochondrial analysis suggests that each clade is a distinct evolutionary lineage that may have been separated 10,000- 15,000 years ago during the Pleistocene. In light of this finding some would argue that each lineage is a species as determined for *E. obsoleta* (Burbrink, 2001). However the distance measures show that the divergence between the *E. g. guttata* clades is less that

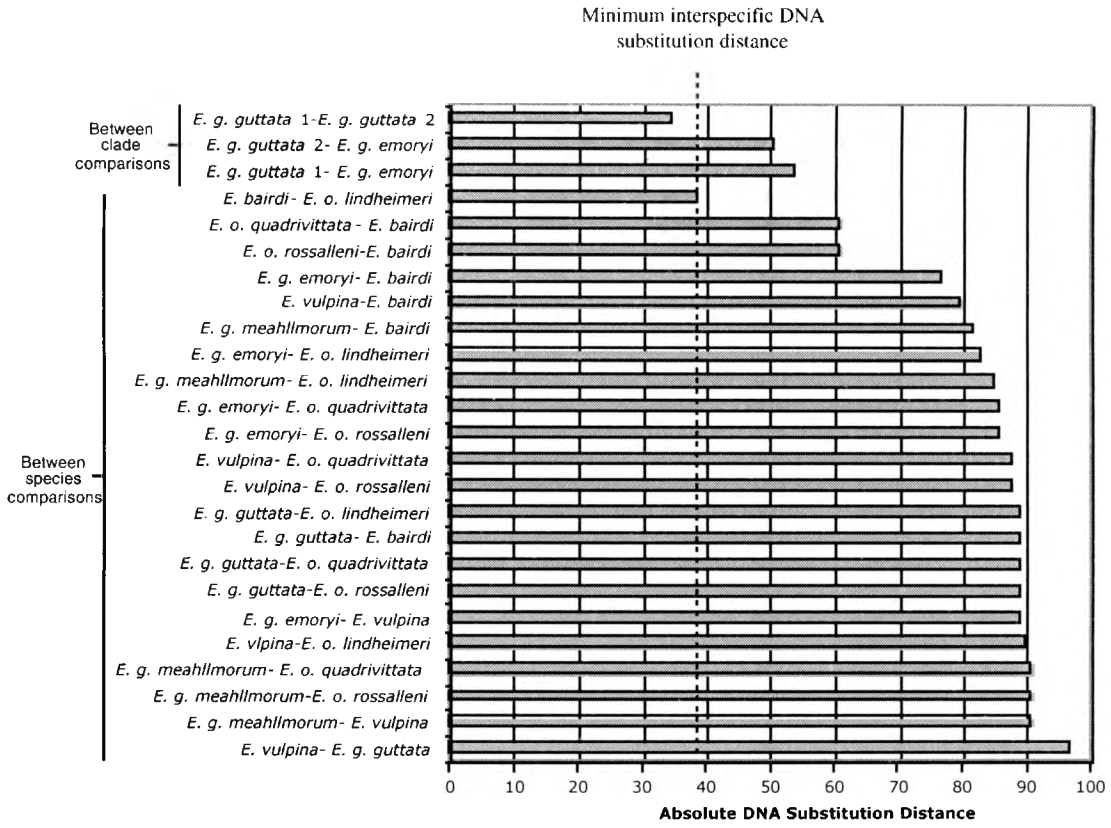

Figure 16. Comparison of absolute distances between several species of the genus *Elaphe*. Between clade distances for the analysis within *E. guttata* are also shown. Minimum interspecific DNA substitution distance seen between *E. o. lindheimeri* and *E. bairdi* is represented throughout the diagram for reference.

any between species comparison of closely related taxa (Figure 16). Investigation of the two *E. guttata* clades suggests that two distinct mitochondrial DNA lineages of this subspecies exist, although the designation of species status to these two groups is not appropriate. Therefore the clade east of the Mississippi River should retain the name *E. g. guttata* as named in Wright and Wright (1957) and Dowling (1951). The clade ranging from the Mississippi River west into eastern Texas not included in Wright and Wright (1957) should be described with a new subspecific name. Evaluation of the westernmost clade suggests that the subspecies *E. g. emoryi* may be more properly named *Elaphe emoryi emoryi* as previously named by Dowling (1951) and Wright and Wright (1957). This would appropriately recognize both the level of phylogenetic structure and monophyly associated with *emoryi*. The closely related snakes within the *emoryi* clade would be transferred to *emoryi* from subspecies of *guttata*. *Elaphe emoryi meahllmorum*, and *Elaphe emoryi intermontana* would thus become valid taxa.

These analyses were used to examine a polytypic species in order to better understand the relationships between several closely related organisms that have a long and confusing taxonomic history. Results of these analyses showed that several evolutionary distinct mitochondrial lineages are present in *E. guttata*. While these results may be interpreted as distinct specific lineages, care must be taken in order to avoid unnecessary reclassification and renaming of organisms based on a single mitochondrial analysis. These single mitochondrial analysis, while informative, exclude male genetic contributions through the populations. Complete evolutionary histories must be examined using multiple gene analyses that include nuclear genetic material. The presence of distinct mitochondrial lineages does however merit the designation at a

subspecific level. The use of subspecies in this manner will include some evolutionary history of the organisms that can be useful in resolving taxonomy of closely related taxa. Therefore, designating each of the clades of *E. g. guttata* as new species from a single mitochondrial analysis may exacerbate the confusion surrounding their taxonomy. Although the premature recognition of the *guttata* clades as distinct species is avoided, the use of these analyses to recognize *Elaphe emoryi* as a distinct species is warranted. In addition to the analyses presented here, which show a distinct separation between the guttata clades and the adjacent emoyi clade, this species was previously distinguished from other species of *Elaphe* dating back to 1853 (Baird and Girard, 1853), and verified by Dowling (1951). Naming the *guttata* cades as subspecies recognizes their monophyly and would direct future studies to examine nuclear gene data to describe the nature of that variation. Should the monophyly found here in mtDNA be supported by nDNA then species level recognition would be warranted.

APPENDIX I- GLOSSARY

Definitions for terms used in this thesis are taken from Hillis et al., 1996.

Alignment- The juxtaposition of amino acids or nucleotides in homologous molecules to maximize similarity or minimize the number of inferred changes among the sequences. Alignment is used to infer positional homology (qv) prior to or concurrent with phylogenetic analysis.

Bootstrap- A statistical method based on repeated random sampling with replacement from an original sample to provide a collection of new pseudoreplicate samples, from which sampling variance can be estimated.

Heuristic method- Any analysis procedure that does not guarantee finding the optimal solution to a problem (usually used to obtain a large increase in speed over exact models).

Jackknifing- A statistical method of numerical resampling based on deleting a portion of the original observations in subsequent samples.

Maximum likelihood- A criterion for estimating a parameter from observed data under an explicit model. In phylogenetic analysis, the optimal tree under the maximum likelihood criterion in the tree that id most likely to have occurred given the observed data and the assumed model of evolution.

Maximum parsimony- A criterion for estimating a parameter from observed data based on the principle of minimizing the number of events needed to explain the data. In phylogenetic analysis, the optimal tree under maximum parsimony criterion is the tree that requires the fewest number of character-state changes (which may be differentially weighted across characters and/ or character states). Often simply called parsimony.

Molecular Systematics- The detection, description, and explanation of molecular biological diversity, both within and among species.

Monophyletic- A group of taxa that contains an ancestor and al its descendants.

Neighbor joining- A heuristic method for obtaining a point estimate of minimum evolution tree.

Outgroup- One or more taxa assumed to be outside the ingroup.

OTU- Operational taxonomic unit. Synonymous with terminal taxon.

Phylogeny- The historical relationships among lineages of organisms or their parts (e.g., genes).

Synapomorphy- A shared derived character state that is indicative of a phylogenetic relationship among two or more OTUs.

APPENDIX II- SAMPLE INFORMATION

Sample	Collector	Date	Specific locality
MF 5309	GC		Marathon Key, Florida
MF 5383	JRD		Woodlands High School. Spring, Texas
MF 2935	MJF 1413	5-29-1999	Hwy 129 at I-75
MF 3104	MJF 1424		Hwy 129 N of I-75 2 miles on 129
MF 5114	JRD 34700	7-14-2001	Brazelton, GA
MF 5290	RKV 71	5-2000	8 mi NW Jackson, LA
MF 5300	RKV 66	5-2000	6 mi. NW Jackson, LA
MF 5299	RKV 64	5-2000	5 mi. NE Star Hill, LA, State Hwy 965
MF 5289	RKV 74	4-07-2001	Forest Ser. Rd. 309, 1/2 mi. E Hwy 119
MF 5297	RKV 69	9-25-01	Kisatchie Nat For , Longleaf Vista Trail
MF 5298	RKV 80	6-2000	W Vista Trail, 2 mi from Jct with Hwy119
MF 5308	SC	6-05-2001	Long Leaf Vista Road
MF 5779	JRD		
MF 4991			TCWC # 84709
MF 4989			TCWC # 84708
MF 4995	JRD 34474	6-15-2001	
MF 4993	JRD 34476	6-15-2001	
MF 3801	JRD 34135		
MF 130		4-30-1993	1.2 mi S of Rockprairie Rd intersection with Greensprairie Rd.
MF 4988	JRD 34788		
MF 3799	MJF		
MF 2491	JHM 397	6-14-2002	Lost Maples Natural Area, 4 mi N Vanderpool, TX
MF 3800	JRD 34217	1-02-2000	9 5 mi N Olney, TX
MF 5117	JHM 1126	6-23-2001	Bitter Lake National Wildlife refuge 7 0 mi. NE Roswell, NM
MF 5305	JRD	1-02-2001	
MF 1402	JRD 33450	5-01-1997	2.7 mi S Hwy 16, crossing of Nueces River

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