MORPHOLOGY, MOLECULES, AND DELINEATION OF THE GULF COAST BOX TURTLE

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2008

To Sara

ACKNOWLEDGMENTS

I thank my supervisory committee (C. Kenneth Dodd, James Austin and James P. Ross) for their insight and support. I thank the members of the UF Conservation Genetics lab for inspiration and camaraderie. I thank the staffs at the Florida Museum of Natural History, the National Museum of Natural History, the Sternberg Museum of Natural History, the University of Kansas Natural History Museum and the Florida Integrated Science Center for their insight and assistance. I thank multiple individuals who provided guidance and support, whether they realize it or not, including Ben Atkinson, Matt Aresco, Frank Fontanella, Phil Spinks, Brad Shaffer, Joe Collins, Albert Meier, Larry Wilson and several employees of the Florida Fish and Wildlife Conservation Commission. I thank the American Museum of Natural History, James P. Ross and James Austin of the University of Florida and the Society for the Study of Amphibians and Reptiles for financial assistance. This endeavor could not have been accomplished without the unconditional support of my wife, Sara Moore Butler.

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December 2008

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Major: Wildlife Ecology and Conservation

The eastern box turtle (*Terrapene carolina*) is a widespread and often abundant component of regional faunas. Despite the familiarity of *T. carolina*, little is known regarding the relationships among recognized subspecies. In the Florida panhandle, an area containing known genetic discontinuities in other turtles, four subspecies of *T. carolina* are distributed in close proximity. I implemented morphological and molecular analysis to understand the relationships among subspecific *T. carolina* taxa and explore how lineages are distributed across the Florida panhandle. I performed discriminate function analysis on 31 morphological characters from 723 individual *T. carolina* to develop canonical functions descriptive of *T. carolina* taxa. I validated these taxa as distinct evolutionary lineages by comparing mitochondrial DNA sequence fragments. I tested for intergradation between these taxa through analysis of a multilocus microsatellite dataset. I used this dataset to test for genetic structure among box turtles in the Florida panhandle. Morphological functions and mitochondrial DNA sequences may be used to discriminate between *T. carolina* subspecies. However, specimens from the Florida panhandle exhibit several distinct phenotypes and mitochondrial haplotypes. Despite this apparent geographic overlap of lineages, microsatellite analysis did not reveal genetic structure related to

mitochondrial haplotypes, geographic distance or physiographic features. Spatial autocorrelation analysis suggests genetic structuring among box turtles may occur at relatively large geographic scales. The lack of fine-scale genetic structure may be attributed to the presence of transient males, juvenile dispersal or human relocation of *T. carolina.*

CHAPTER 1
INTRODUCTION

The eastern box turtle (*Terrapene carolina*) is a charismatic and important component of eastern North America fauna. Despite the familiarity of this turtle, relatively little is known regarding the evolutionary history and relationships of the many phenotypically variable *Terrapene* taxa. I investigated the relationships among *Terrapene* in the Florida panhandle, where the distributions of four subspecies are in proximity. Morphological and molecular techniques possess unique benefits and limitations for understanding intra-specific relationships. I analyzed mitochondrial and nuclear DNA as well as morphology to understand the spatial genetic structure of *Terrapene* across the Florida panhandle.

Regional Phylogeography

Dozens of studies have examined concordance in distribution patterns, explored physiographic barriers to gene flow or attempted to elucidated mechanisms of speciation across a broad array of eastern North American resident taxa (reviewed by Soltis et al. 2006). Southeastern North America is a particularly interesting biogeographic area due to its physiographic complexity and habitat heterogeneity. The Coastal Plain has functioned as a refugium for taxa during Pleistocene glaciations (Braun 1947, Church et al. 2003, Austin et al. 2004) and xerothermic periods (Smith 1957). These periodic rufugial events allowed for allopatric diversification and produced an ecologically important area. The Florida panhandle, in particular, contains many rare, protected and endemic organisms (Wolfe et al. 1988). Expansion following these refugial events facilitated contact of related organisms - the Coastal Plain is recognized as a major area of secondary contact for a broad array of taxa (Swenson and Howard 2005). The Apalachicola River, which bisects the Florida panhandle into eastern and western segments, is one of the most commonly recognized genetic discontinuities in various

southeastern North American taxa (Soltis et al. 2006). The area adjacent to the river is a hotspot for diversity, endemism and phylogeographic breaks (Stein et al. 2000, Swenson and Howard 2005). Genetic structuring, often associated with the Apalachicola River, is evident among many taxa including mammals (Ellsworth et al. 1994), fishes (Bermingham and Avise 1986) and insects (Maskas et al. 2000) within the Florida panhandle. This region also contains a diverse herpetofaunal assemblage punctuated by a high degree of endemism and intraspecific genetic diversity (Walker and Avise 1998). The distributions of many intra-specific lineages reflect associations with current and historic physiographic features in the region. Numerous reptile taxa exhibit a genetic break across the Apalachicola River (Burbrink 2001, Means and Krysko 2001), including several turtles (Walker and Avise 1998). These examples illustrate the biogeographic and conservation importance of the southeastern coastal plain of North America and, more specifically, the Apalachicola region of the Florida panhandle.

Phylogeography of the Box Turtle

The eastern box turtle (Testudines: Emydidae: *Terrapene carolina*) is a functional component of the regional fauna. Aside from aiding in seed dispersal in certain habitats (Liu et al. 2004), box turtles are known vectors for invertebrate parasites and prey items for many vertebrates (reviewed by Dodd 2001). Despite this ecological importance, little is known about the evolutionary histories, relationships or distributions of *Terrapene* lineages. Evolutionary studies often delineate unique lineages which may be important when considering management decisions. Several biological characteristics advocate the use of *Terrapene* as an exemplar species for a phylogeographic study. These attributes are outlined below.

Terrapene is a moderately-sized organism with apparently low dispersal capability. Adult box turtles maintain relatively small home-ranges (Dodd 2001) and typically do not venture vast distances (Iglay et al. 2007, Schwartz 1974). Unlike birds or large mammals, box

turtles do not autonomously disperse across landscapes. Additionally, box turtles are too large to be dispersed by wind like many invertebrates and plants. The lack of potential for widespread dispersal suggests *Terrapene* should demonstrate genetic structuring at both landscape and range-wide scales.

Unlike most members of the family Emydidae which are dependent on permanent water for survival, *Terrapene carolina* is terrestrially adapted and maintains an existence loosely tied to aquatic habitats (Carr 1952). Box turtles can disperse independent of aquatic connections and their potential ranges are not greatly affected by stream capture or flooding (for biogeographic studies of aquatic taxa in southeastern North America, see Bermingham et al. 1986, Gilbert 1987).

Terrapene possesses a dense and unique shell that permits preservation and recognition in the fossil record. Pleistocene deposits in Florida commonly yield partial and complete *Terrapene* specimens (Hulbert 2001). Consideration and analysis of the fossil record contributes to our understanding of primary mechanisms of evolutionary processes (Gandolfo 2008). Appreciating when and where box turtles historically resided provides insight beyond biogeographic snapshots generated from modern range and habitat delineations.

Box turtles exhibit high phenotypic variability. There are currently six recognized subspecies of eastern box turtle; four of these (*T. c. carolina, T. c. bauri, T. c. major, T. c. triungus*) occur in the vicinity of the Florida Panhandle and exhibit unique phenotypic affinities. The geographic variation in morphological characters provides quantitative characters for developing phylogeographic hypotheses and comparing genotypic and phenotypic variation.

Relationships of the Box Turtle

The intergrade zone dynamics and subspecies status of box turtles has been addressed and debated in multiple studies since the 19th century. Ditmars (1934) provided a comprehensive

review of studies from 1820 to 1933 that described *Terrapene* taxa and addressed the relationships among them. Milstead (1969), Ward (1980) and Minx (1996) conducted intensive morphology-based investigations but reached no conclusive consensus of the inter-generic relationships of *Terrapene*. In southeastern North America, intergrade zones among subspecies of *T. carolina* have been proposed exclusively on phenotype (Carr 1952; Conant and Collins 1991; Milstead 1969). Carr (1952) and Milstead (1969) described the contact zones between these taxa as geographically expansive areas containing phenotypic intermediates (Figure 1-1a). Ward (1980) disagreed and diagnosed the intermediates described by Milstead within the variation of specific taxa (Figure 1-1b). Recently published distribution maps do not reflect either of these descriptions (see Conant and Collins 1991, Minx 1996, Dodd 2001), but do exhibit the distributional proximity of four subspecies to the Florida panhandle. The lack of concordance in results from phenotypic investigations of *Terrapene* relationships (Ward 1980, Milstead 1969, Minx 1996) shows the need for a study using molecular techniques.

Molecular methods provide powerful techniques to determine the nature and extent of contact zones (Harrison 1990), particularly in taxa where phenotypic characterizations are highly variable. Analysis of mitochondrial deoxyribonucleic acid (mtDNA) sequence data can reveal relationships between, and the distribution of, lineages of organisms. Southeastern North American reptiles exhibit divergent mtDNA sequences in lizards (Clark et al. 1999, Leache and Reeder 2002), snakes (Burbrink et al. 2001, Burbrink et al. 2002) and turtles (Starkey et al. 2003, Walker and Avise 1998). A recent chelonian checklist includes four species of *Terrapene* (*T. carolina*, *T. cohuila*, *T. nelsoni*, *T. ornata*) with six subspecies (*T. carolina bauri*, *T. c. carolina*, *T. c. major*, *T. c. mexicana*, *T. c. triungus*, *T. c. yucatana*) within the *T. carolina* complex (Fritz and Havas 2007), each of which may possess diagnostic mtDNA haplotypes. Previous

descriptions of *T. carolina* suggest intergrade zones occur near the panhandle region of Florida (Carr 1952, Dodd 2001, Milstead 1969). These observations imply mtDNA haplotypes representative of subspecific *T.carolina* lineages may occur in the Florida panhandle and clines, or gradation among characters (Huxley 1938), exist in the landscape between them (Figure 1-1a). The investigation by Ward (1980) concluded *Terrapene* lineages do not exhibit extensive intergradation and implies representative genotypic and phenotypic suites co-occur in narrow geographic ranges (Figure 1-1b). Both of these patterns may result from secondary contact of related lineages (Endler 1977). The conclusions of Milstead (1969) suggested gradual clines of characters in large geographic contact zones, whereas Ward's (1980) proposal suggested steep clines in characters in narrow contact zones.

Due to the recombinant nature of nuclear DNA and rapid mutational rates of microsatellites (Goldstein and Scholotterer 1999), these markers are ideal tools for investigating intra-specific relationships. Genetic variation, flow and structure between closely related lineages of reptiles have been explored with frequency data of nuclear microsatellite alleles (Davis et al. 2002, Hoehn et al. 2007, MacAvoy et al. 2007).

An sample of box turtles from the Florida panhandle should exhibit genetic structuring reflective of the intensity of intergradation. An expansive intergradation zone would exhibit limited genetic structure whereas structuring should be obvious across a narrow geographic area of intergradation

Study Objectives

The prevalence of genetic discontinuities in a variety of organisms has encouraged *a priori* hypothesis testing of herpetofaunal studies conducted in the Apalachicola region of Florida (Pauley et al. 2007). My study attempts to elucidate the genetic structure of a widespread, putatively low-dispersing organism in a complex biogeographic region. First, I test

for the presence of multiple lineages associated with described *T.carolina* supspecies across the Florida panhandle. I examined mtDNA sequences and a suite of morphological characters to diagnose sub-specific *T. carolina* taxa. I then examine population structuring and gene flow between lineages in the Florida panhandle through analysis of a multi-locus microsatellite dataset. I attempt to address the following questions: Are subspecies diagnosable using mtDNA haplotypes and statistical assignment of morphological characters? Is there evidence of a clinal pattern of intergradation among lineages based on microsatellite loci and phenotypic variation? Finally, based on previous phylogeographic investigations of chelonians (Walker and Avise 1998), I ask whether the Apalachicola River has genetically structured *Terrapene* across the Florida panhandle?

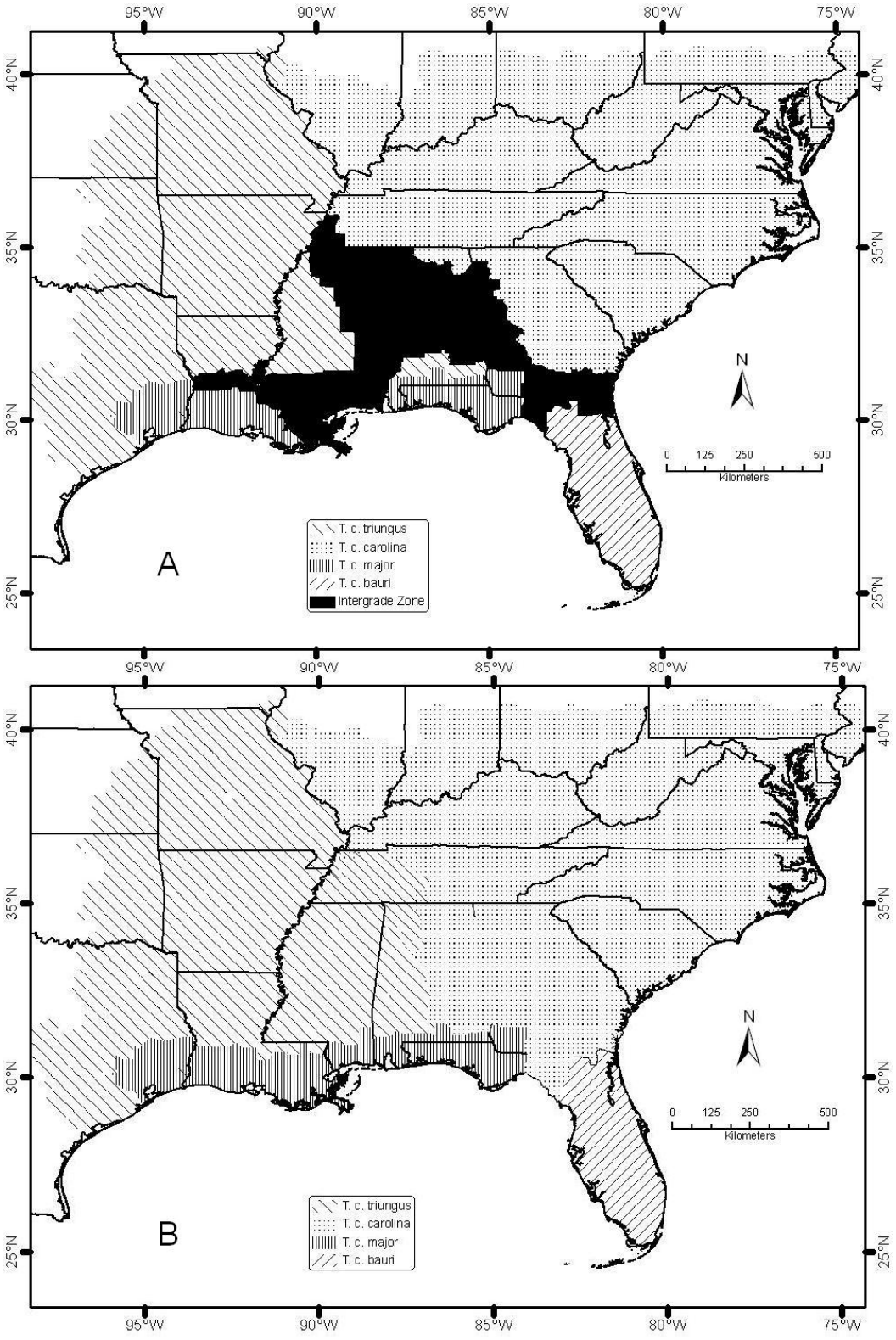


Figure 1-1. Hypothesized distributions of *Terrapene* taxa in southeastern North America. A) adapted from Carr (1952) and B) compiled from the range descriptions of Ward (1980).

CHAPTER 2
MATERIALS AND METHODS

Sampling Strategy

I examined 723 specimens of *Terrapene carolina* from four museum collections for a suite of discrete, meristic and color pattern characters (Appendix B). I selected specimens to maximize sampling coverage of southeastern North America (Figure 2-1) with emphasis on the Florida panhandle on either side of the Apalachicola River (Figure 2-2). I focused on specimens preserved in fluid as opposed to skeletal specimens which lose morphological characters during maceration. When coordinates were not available, I used DELORME TOPO 6.0 to georeference each specimen based on collection locality data. Specimens whose locality could not be determined within 10 kilometers were omitted from analysis. Many museum specimens are geographically clustered around collection hotspots. These hotspots typically contained several dozen specimens from an area less than 5,000 hectares. Specimens of *Terrapene carolina* in each hotspot were grouped together to permit in-depth multivariate analysis. I grouped between 20 and 30 individuals in each of the eleven areas (Figure 2-4).

Tissue samples were collected from museum and personal collections. I received the majority of Florida panhandle samples from M. Aresco, who began collecting Gulf Coast box turtle tissue nearly a decade ago. I also acquired tissue from specimens deep within the ranges of each currently recognized subspecies (Fig 2-1). Most *Terrapene* tissue samples obtained from M. Aresco and other sources were collected from dead on the road (DOR) specimens. Tissue was collected from 125 *Terrapene* specimens (Appendix C).

Fixation with formalin preserves museum specimens and denatures DNA. Most collectors have only recently begun archiving tissue prior to preserving specimens. Therefore, tissue samples were not available from the majority of museum specimens I examined.

Conversely, although an excellent source of DNA, DOR turtles are usually too damaged to permit accurate morphological examination. Due to the nature of sampling and limited tissue collections associated with fixed specimens, few morphological and tissue specimens originate from the same animal, although there is broad geographic overlap.

Morphological Data Collection

I examined several commonly used morphological characters and several novel characters. Twenty-three discrete character observations, including three sexually dichromatic characters, were made for each turtle. I also measured eight straight-line carapace and plastron lengths with vernier calipers (Figure 2-3). Each measurement was taken three times and the mean and variance of repeated measurements were calculated (Yezerinac et al. 1992). I scrutinized any average measurement with a variation greater than 1.0 mm. Many of these highly variable means were associated with erroneous data entry (i.e. 102.5 mm versus 1002.5 mm) and corrected respectively. Specimens with means whose high variation could not be accounted for were omitted from analysis. For a full description of each character, including its potential for bias, see Appendix 1.

Molecular Data Collection

I extracted DNA from chelonian tissue using standard phenol-chloroform techniques (Sambrook and Russel 2001) following a proteinase-K digestion. Although the time for completing an extraction may be longer, phenol-chloroform techniques cost less and provide higher DNA concentrations than kit extractions. The concentration of template DNA was measured using a Nanodrop spectrophotometer (Nanodrop ND-100, Wilmington, Deleware). However, DNA recovered from DOR samples can result in unreliable measures of template quality with spectrophotometry due to the high concentration of short, degraded DNA fragments.

Rosenbaum et al. (2007) found varying phylogeographic information in three mtDNA genes applied to an Emydid turtle. I examined the most variable of these genes, a fragment of the control region displacement loop (d-loop), in 117 *Terrapene carolina* specimens. King and Julian (2004) identified 22 polymorphic microsatellite loci in box turtles. I screened each of these loci for variability across a subset of box turtle samples. I selected six loci for amplification across all Florida panhandle samples based on ease of amplification and variability assayed from visualization on an Elchrom SEA-2000 (Cham, Switzerland) electrophoresis apparatus.

I performed polymerase chain reaction (PCR) amplifications of double-stranded product on a Eppendorf thermocycler. PCR reactions for mtDNA and microsatellite amplification contained 5 µl of 1 X PCR buffer (Promega, Madison, Wisconsin), a final concentration of 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 units of Taq polymerase and varying concentrations of primer and template DNA in a total volume of 20 µl. Conditions for PCR differed between loci but all included an initial denaturation at 94°C for 3 min, 35 amplification cycles of 45 s denaturation at 94°C, 45 s annealing at varying temperatures, 45 s extension at 72°C and a final extension of 5 min at 72°C. Primer sequences, primer and template concentrations and annealing conditions for PCR of individual genes are found in Table 2-1.

Mitochondrial sequences were cleaned of unincorporated dNTPs through application of ExoSAP-IT (USB, Cleveland, Ohio) before following standard sequencing protocol on an ABI 3130xl automated sequencer (Applied Biosystems, Foster City, California). I aligned d-loop sequences in the CLC DNA Workbench program (CLC bio, Katrinebjerg, Denmark) under default protocols.

Standard genotyping protocols were followed for fragment analysis of fluorescently labeled microsatellite product on an ABI 3130xl sequencer. I used GENEMARKER (Softgenetics, State College, Pennsylvania) to score microsatellite alleles based on ROX GS500 size standard (Applied Biosystems, Foster City, California). Several loci from the microsatellite dataset were amplified and genotyped twice to address possible allelic dropout and assure consistent allelic scoring. Since degraded DNA was included in this study I tested for null alleles using the software MICRO-CHECKER (Oosterhout et al. 2004).

Lineage Validation and Assignment

Morphology

I assessed the diagnostic utility of morphological characters in order to validate and detect differences between box turtle lineages. Diagnostic morphological characters were developed through analysis of carapace and plastron measurements corrected for body size. Milstead (1969) and Ward (1980) used measurement ratios to distinguish *Terrapene* taxa; however, the statistical behavior of ratios may invalidate analytical results (Atchley 1976). I corrected for size by regressing all characters against a size standard and reserving the residuals free from allometry (Reist 1986). I used shell volume calculated from the product of depth, width and length measurements as a size standard.

Step-wise discriminate function analyses (DFAs, JMP 7.0) were performed on combined and gender specific datasets to account for sexual dimorphism. I chose analysis models by excluding all characters which did not meet a discriminating significance probability (p) greater than 0.05. Several assumptions underlie the application of DFA, although large sample datasets may be robust to some violations (McGarigal et al. 2000). I investigated the violation of two of the assumptions that have the potential to invalidate results. The canonical plots of groups that do not possess equal variance-covariance matrices may be distorted (Williams 1983). I used a

logarithm transformation to standardize variables so variances were equal between groups. Initial analysis of transformed and raw datasets did not produce differing results, so I used raw datasets for all analysis. Outliers may also influence results from DFAs (McGarigal et al. 2000). When I observed outliers in canonical plots, I removed them and reanalyzed the data.

I evaluated sexually dimorphic characters to filter datasets by gender. I produced a male dataset by removing all specimens that did not have an enlarged tail. The female dataset consisted of individuals without a concave posterior plastron lobe, enlarged tail or enlarged rear claws. Many specimens could not be sexed based on these characters. I classified the sex of these individuals a posteriori by treating them as unknowns in an DFA of the specimens of known sex (Iverson and McCord 1991). I omitted unknown specimens that could not be assigned to a gender with at least 95% assignment probability.

I used DFA to explore among versus within group morphological variation of box turtles from collection hotspots. I expected groups within the same geographic distribution of a subspecies to overlap in canonical distributions. I formed higher-level groups based on the overlap in canonical distributions and developed group-specific formulas from their canonical functions through DFA. The suitability of higher and lower level group models was evaluated through comparisons of the amount of explained variation from the first two canonical functions and the percent of misidentified specimens. I chose the model with the most explanatory power to diagnose intraspecific box turtle lineages. Using this model, I assigned all morphological specimens to their most appropriate lineage. Specimens with a classification probability lower than 95% were omitted from further analysis. I visualized the distribution of morpho-lineages by mapping combined and gender-specific results. Finally, I examined the assignments of specimens from the Florida panhandle to determine which lineages were present in the region.

MtDNA Sequences

The d-loop sequence data was also used to examine the divergence and monophyly of putative subspecies and to compare distributions of mtDNA with morphological lineages assigned to box turtle specimens from the Florida panhandle. Using sequences from each subspecies' range and a subset of those from the panhandle, I developed a haplotype network in TCS 1.13 under default settings. Exploring intraspecific relationships with a network is often more appropriate than a phylogenetic tree due to the bifurcating assumptions of tree building and the limited resolution of intra-specific data (Posada and Crandall 2001). I recognized lineages based on the number of mutational steps between haplotypes and haplotype clusters. I compared corrected and uncorrected pairwise genetic distances within and among these lineages using MEGA 4.0. Standard error of these distance were calculated through a 1000-replicate bootstrap.

I assigned all specimens to a lineage and mapped these lineages over the specimen collection locality. A map of the panhandle specimens was assessed to determine which lineages contribute to the region's genetic structure.

Gene Flow

I looked for overlap in microsatellite allele frequencies between box turtle lineages in the Florida panhandle to confirm or refute intergradation. I implemented a Fisher's exact test with GENEPOP 4.0 to test the null hypothesis that all alleles in all lineages are from the same genetic population. Populations composed of hybrids may exhibit excess heterozygosity. I tested for excess heterozygosity and deviation from Hardy-Wienberg equilibrium (HWE) in box turtle lineages alone and pooled with GENEPOP 4.0 (Raymond and Rousset 1995).

I explored the suitability of the microsatellite dataset to detect natural clusters of related box turtles through a Bayesian approach implemented in STRUCTURE 2.1 (Pritchard et al. 2000).

STRUCTURE attempts to detect natural population clusters by simultaneously estimating cluster allele frequencies and assigning individuals to a cluster or a combination of clusters. These natural clusters may represent genetic patches or groups of individuals with more potential gene flow between each other than with individuals from other groups. The user inputs a genotypic dataset and a prediction for the number of clusters (k). STRUCTURE models the likelihood of the observed dataset being produced by the predicted number of clusters through an admixture model using a set number of generations. To ensure equilibrium of the proposed populations, I chose a model that incorporated 500,000 generations after a discarded burn-in period of 30,000 generations to calculate the likelihood of K = 1 to 10 populations. I calculated the average likelihood for each potential number of populations from five iterations of each model.

To explore the potential relationship between gene flow and geography, I used microsatellite genotypes to test for isolation by distance (IBD) across the Florida panhandle. I examined the regression of pairwise genetic differences against log transformed geographic distances (Rousett 2000) with a Mantel test in GenePop 4.0. I also looked for isolation by distance at a larger geographic scale through analysis of the discrete morphological characters. I implemented a spatial autocorrelation analysis using Alleles in Space (AIS, Miller 2005) to explore phenotypic similarity between individuals at various distance classes across the landscape. I plotted the slope of the autocorrelation statistic (A_y) across 10, 20 and 30 distance classes and tested for its significance against a 1000 replicate randomization. Using AIS, I interpolated a genetic surface between morphological box turtle samples across eastern North America to visualize potential areas of genetic discontinuity. I used a 100 X 100 grid and a distance weighting value of $a = 0.5$ in AIS to develop the surface.

Table 2-1. Primers and conditions used in this study

	Locus	Primer Sequences	Flourescence/ Repeat Motif	Primer Concentration	Template (ng)	Anealing Temperature (˚C)	Size Range (bp)
Starkey et al. 2003	mtDNA						
	DES1 (5'-3')	GCA TTC ATC TAT TTT CCG TTA GCA	-	0.5	20	52	725
	DES2 (3'-5')	GGA TTT AGG GGT TTG ACG AGA AT	-	0.5			
King and Julian 2004	Microsatellites						
	GmuB08 (5'-3')	CTC TGA GAC CCT TAT TCA CGT C	HEX (green) /	0.15	20	60	189 - 208
	R: (3'-5')	AGC CTT TGT CTG TAA GCT GTT C	TAC	0.15			
	GmuB12 (5'-3')	TCA ATC TTC CAG CCT AAC TGT G	FAM (blue)	0.3	20	60	177-190
	R: (3'-5')	AGG GAT GTG TTT TGC AAC TGG	TAC	0.3			
	GmuD21 (5'-3')	GCA GTT AGG CAT TAC TCA ACA TC	TAMN (yellow)	0.75	40	55	149 - 190
	R: (3'-5')	AGG GTA TGA ATA CAG GGG TGT C	ATCT	0.75			
	GmuD55 (5'-3')	GTG ATA CTC TGC AAC CCA TCC	FAM (blue)	0.45	20	55	154 - 212
	R: (3'-5')	TTG CAT TCA GAA TAT CCA TCAG	ATCT	0.45			
	GmuD90 (5'-3')	ATA GCA GGA CAA TTA CCA CCA G	TAMN (yellow)	0.75	20	60	130 - 157
	R: (3'-5')	CCT AGT TGC TGC TGA CTC CAC	ATCT	0.75			
	GmuD121 (5'-3')	GGC AAA TAT CCA ATA GAA ATC C	HEX (green)	0.9	20	55	123 - 163
	R: (3'-5')	CAA CTT CCT CGT GGG TTC AG	ATCT	0.9			

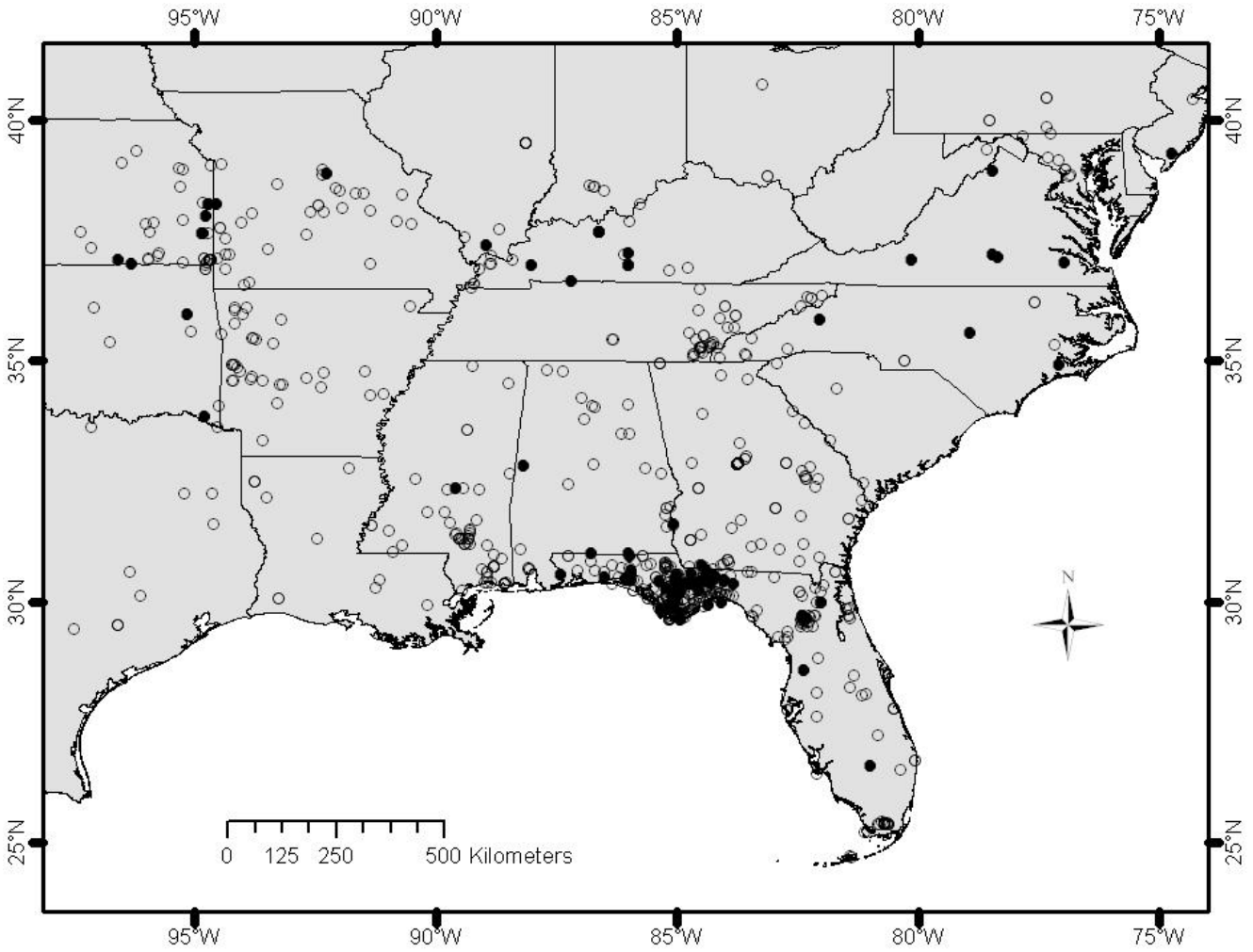


Figure 2-1. Sampling localities for this study. Hollow circles (○) represent museum samples used in morphological analysis; solid circles (●) represent tissue sample localities. One circle may represent more than one specimen.

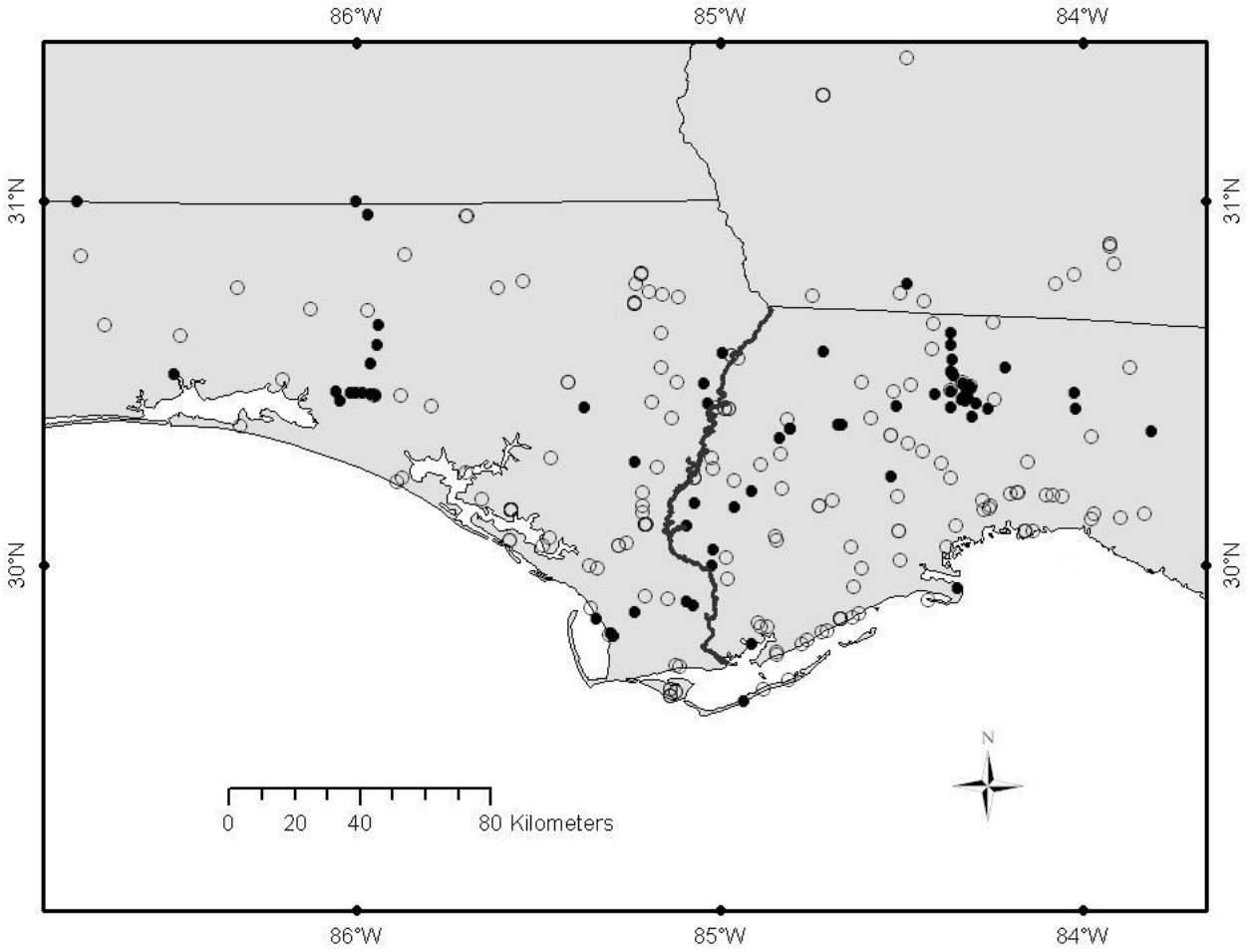


Figure 2-2. Sampling localities in the Florida Panhandle. Hollow circles (○) represent museum samples used in morphological analysis, solid circles (●) represent tissue sample localities. One circle may represent more than one individual. The course of the Apalachicola river through the Florida Panhandle is outlined in dark grey.

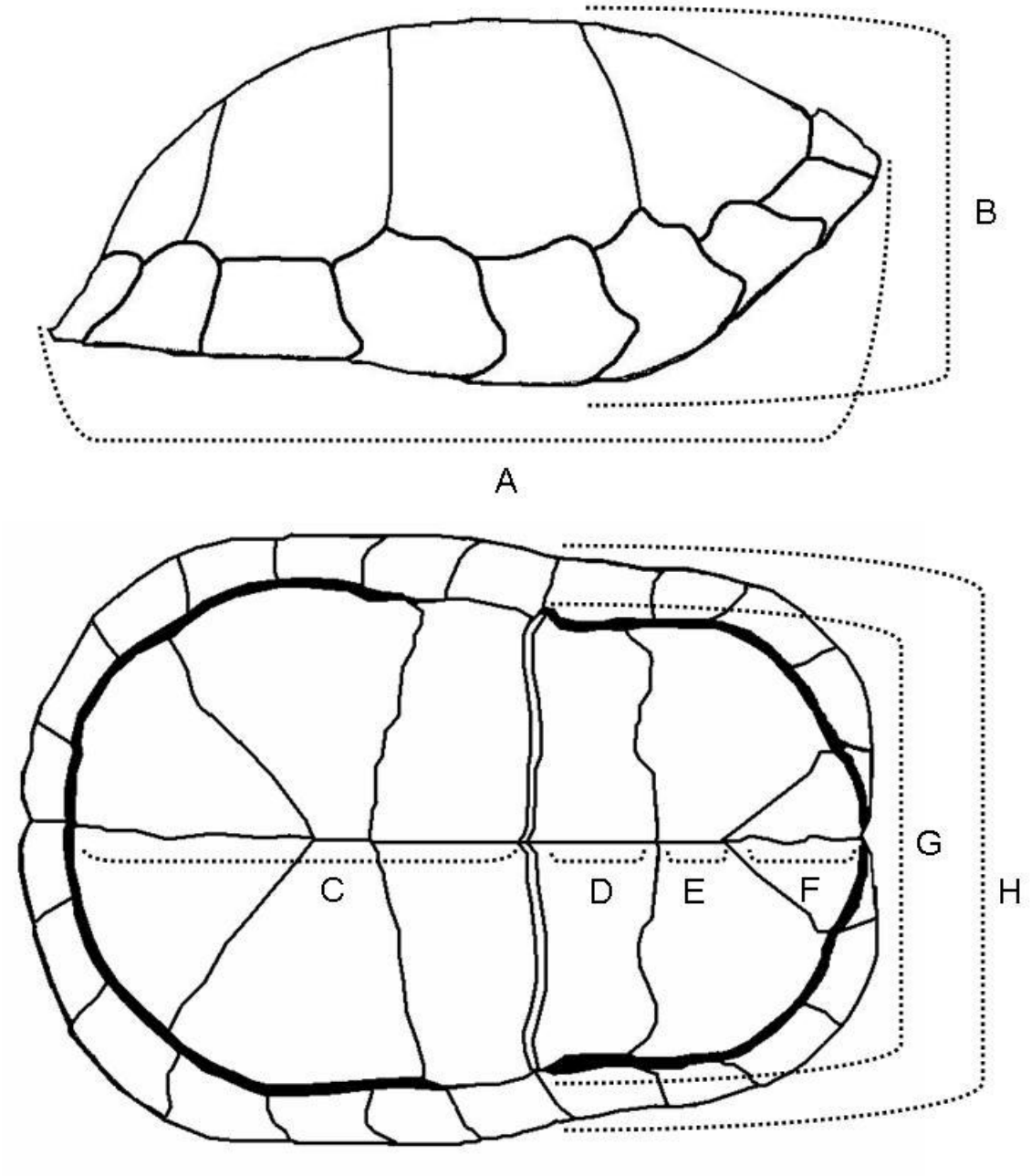


Figure 2-3. Shell distances measured in this study. A) Straight-line carapace. B) Shell height. C) Posterior plastral lobe. D) Interpectoral seam. E) Interhumeral seam. F) Interregular seam. G) Hinge. H) Shell width.

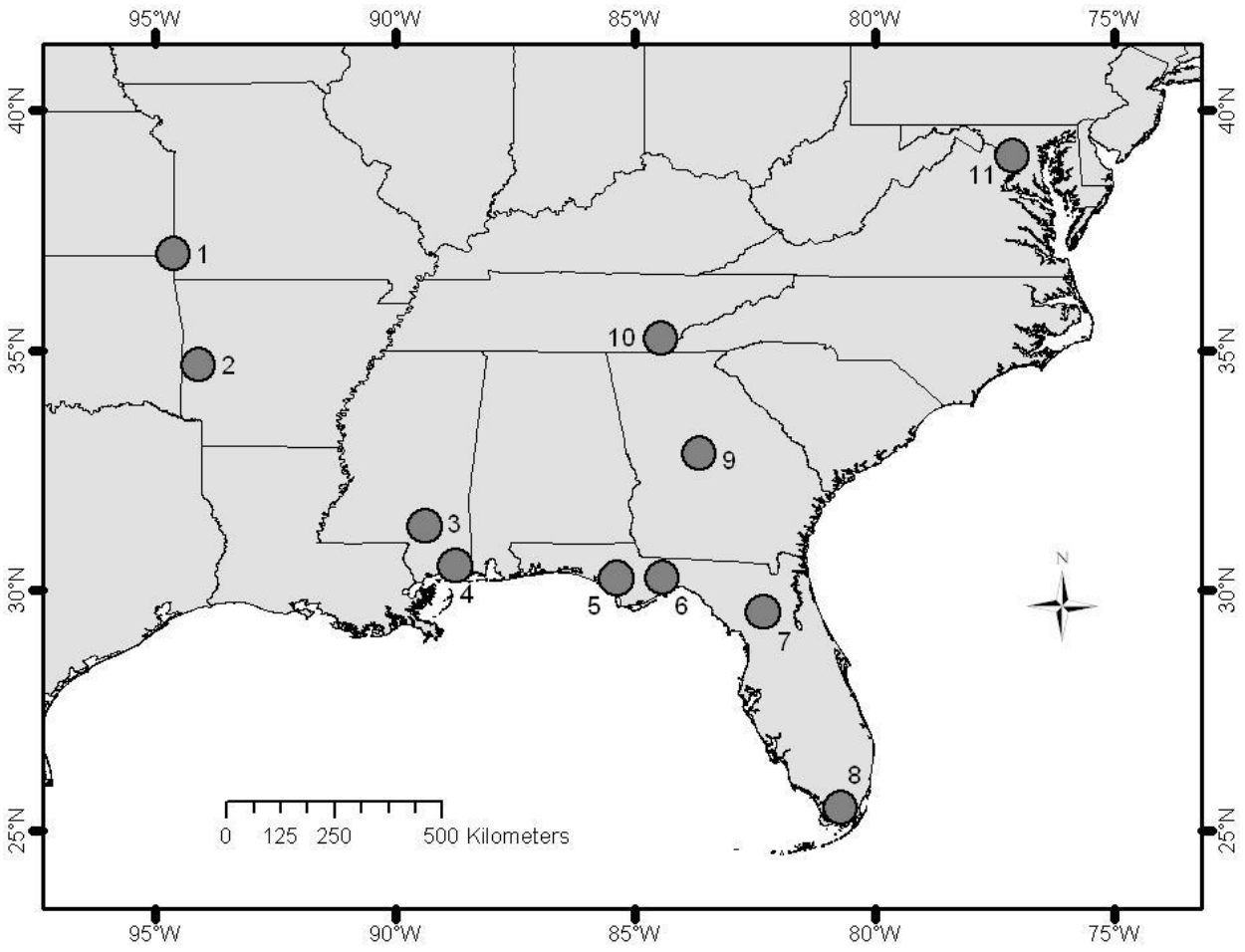


Figure 2-4. Sampling hotspots. Circles represent hotspots of *Terrapene carolina* specimen localities from several museum collections. The locality centers of these hotspots are as follow: 1 – Cherokee Co., Kansas, 2 – Scott Co., Arkansas, 3 – Forest Co., Mississippi, 4 – Jackson Co., Mississippi, Calhoun Co., Florida, Liberty Co., Florida, 7 – Alachua Co., Florida, 8 – Dade Co., Florida, 9 – Bibb Co., Georgia, 10 – McMinn Co., Tennessee and 11 – Prince Georges Co., Maryland. These specimens were considered groups in multivariate analyses.

CHAPTER 3
RESULTS

Lineage Validation and Assignment

Morphology

I removed 65 specimens whose sex could not be determined with at least 95% accuracy through DFA. The resulting dataset contained 340 males and 318 females. DFAs did not exhibit obvious differences between sexes so results are presented for all specimens.

The first lineage-based DFA identified a suite of 17 characters with a significant discriminating probability ($p > 0.05$). The mean confidence limit ellipses (MCLE), or the area with a 95% probability of containing a group's average specimen, of many collection hotspots overlap considerably in canonical space (Figure 3-1A). Many of the groups exhibiting overlap were located within the distribution of a common subspecies. Specimens from hotspots one through three fall within the distribution of *T. c. triungus* and have overlapping MCLEs. Similarly, the two groups within the range of *T. c. bauri* overlap in canonical space. All DFA defined groups within the ranges of *T. c. major* and *T. c. carolina* overlap. The first two canonical functions of this model account for 71% of the discriminating power of the characters (Table 3-1). Under this model, 23.6% of the specimens are misclassified (i.e. a specimen from group 1 should belong in group 2).

Based on the observed overlap in the first lineage DFA, I developed two higher level models (Figure 3-1B and C) and compared their discriminating power. Both models were composed of clusters of groups which overlapped in MCLEs. I omitted groups within the Florida panhandle from one of the models (Figure 3-1C) to account for potential function bias introduced by intergradating specimens (phenotypic intermediates). The model with all eleven groups clustered into three higher levels did not exhibit overlapping MCLEs between groups

(Figure 3-1A). Under this model, 1.7% of specimens are misclassified and the first two canonical functions account for 99% of the discriminating power (Table 3-1). The model that did not include panhandle specimens also did not exhibit overlap between group MCLEs (Figure 3-1C). This model did not reveal any misclassification of specimens and accounted for 100% of the discriminating power in the first two canonical functions. A suite of 12 characters with significant discriminating power (Table 3-2) was used to develop this model.

The final DFA assigned all but 84 specimens to one of the three clusters with 95% probability. The maps of the localities of each specimen assignment across the sampling range (Figure 3-2) roughly reflect recognized subspecific box turtle distributions (Figure 1-2). The map of assigned specimens in the Florida panhandle (Figure 3-3) exhibits members from each cluster as well as many specimens which could not be reliably assigned.

MtDNA Sequences

The d-loop haplotype network revealed four distinct clusters separated by at least seven mutational steps (Figure 3-4). The geographic localities of these clusters roughly reflect recognized subspecific distributions with the exception of the largest, more geographically extensive cluster (Figure 3-5). Specimens from two of the four clusters were collected from the Florida panhandle (Figure 3-6). One of these clusters is not represented by specimens outside the panhandle. The uncorrected genetic divergence within these clusters ranges between 0.6 and 1.0% while differentiation between lineages is between 2.1% and 6% (Table 3-3).

Gene Flow

The software MICROCHECKER recognized the potential for null alleles in one microsatellite locus (GmuD121). This locus was not included in analysis. The two distinct d-loop lineages (Figure 3-4 B, C) in the Florida panhandle share microsatellite alleles. The lineage restricted to the panhandle (lineage C) does not possess any unique microsatellite alleles (Table

3-4). Fisher's exact tests of individual allele frequencies did not reject the null hypothesis that the specimens in the Florida panhandle are from the same population ($p > 0.94$). HWE tests in GENEPOP did not support heterozygotic deficit or excess across pooled sample. Global tests did not rejected HWE across pooled samples and loci.

Structure analysis strongly supported one cluster ($k = 1$) in the Florida panhandle. Posterior probabilities of the average likelihoods from each simulated k were higher than 99% for $k = 1$ and less than 1% for all other values. I did not find evidence for IBD in the panhandle; the regression slope of genetic distance against geographic distance did not significantly differ from zero.

Morphological analysis in AIS revealed significant spatial autocorrelation ($p > 0.95$) in each set of distances (Table 3-5, Figure 3-7). The last distance class to exhibit significant autocorrelation was between 581 and 664 kilometers in the 30-class analysis. The interpolation of the genetic surface from AIS depicts one region of extremely high variability (Figure 3-8). This region corresponds with the Florida panhandle.

Table 3-1. Discriminate Function Analysis (DFA) models

Model		Canonical Discriminating Power			Misclassified
Clusters	Groups	Function 1	Function 2	Total	
11	11	38%	33%	71%	23.6%
3	11	55%	44%	99%	1.7%
3	9	52%	48%	100%	0%

These percentages represent model summaries from DFA of morphological specimens. The first model represents each of the sampling groups analyzed separately while the second and third models represent combined sampling groups that overlap in canonical space.

Table 3-2. Discriminate Function Analysis characters

	Character Frequency								Mean Residuals			
Group Cluster	Head/Leg Pattern	Notched Beak	Dark Carapace Background	No. of rear toes	Vertebral Ridge	Streaks	Spots	Blotches	Posterior Lobe	Interregular Seam	Interhumeral Seam	Depth
1,2,3	0.67	0.74	0.23	3.03	0.32	0.24	0.44	0.18	-3.51	0.98	-1.72	-0.87
7,8	0.84	0.29	1.00	3.43	0.84	0.94	0.68	0.01	3.11	-1.80	2.69	4.49
9,10,11	0.64	0.28	0.98	3.97	0.49	0.21	0.51	0.88	-1.26	-0.05	-0.75	-3.06

These discriminating characters from DFA were significant between clustered groups.

Table 3-3. Genetic distances

		Pairwise				
Lineage	Within	A	B	C	D	E
A	0.010 ±0.003					
B	0.008 ±0.002	0.038 ±0.007				
C	0.004 ±0.002	0.033 ±0.007	0.029 ±0.007			
D	0.006 ±0.003	0.054 ±0.009	0.057 ±0.009	0.059 ±0.010		
E	-	0.029 ±0.007	0.028 ±0.007	0.029 ±0.007	0.060 ±0.010	
F	-	0.033 ±0.007	0.041 ±0.008	0.036 ±0.008	0.051 ±0.009	0.021 ±0.006

Within and between group uncorrected genetic distances with bootstrap values from 1000 replicates of similar clusters of d-loop haplotypes

Table 3-4. Allele summaries

		gmuB12		gmuB08		gmuD21		gmuD55	
d-loop lineage	n	No. of Alleles	Unique	No. of Alleles	Unique	No. of Alleles	Unique	No. of Alleles	Unique
B	56	8	2	8	2	12	6	23	11
C	15	6	0	6	0	6	0	12	0

Microsatellite allele summaries for the mitochondrial lineages in the Florida panhandle

Table 3-5. Autocorrelation statistics

# of classes	Distance per class (km)	1	2	3	4	5	6	7
5	500	0.39*	0.47	0.53	0.53	0.57	-	-
10	250	0.35*	0.42*	0.46	0.49	0.52	0.53	0.53
20	125	0.31*	0.39*	0.42*	0.42*	0.45*	0.48	0.47
30	83	0.29*	0.35*	0.41*	0.41*	0.43*	0.42*	0.44*

These values are the spatial autocorrelation statistics for four sets of distance classes. Asterisks indicate statistics significantly different from a random distribution.

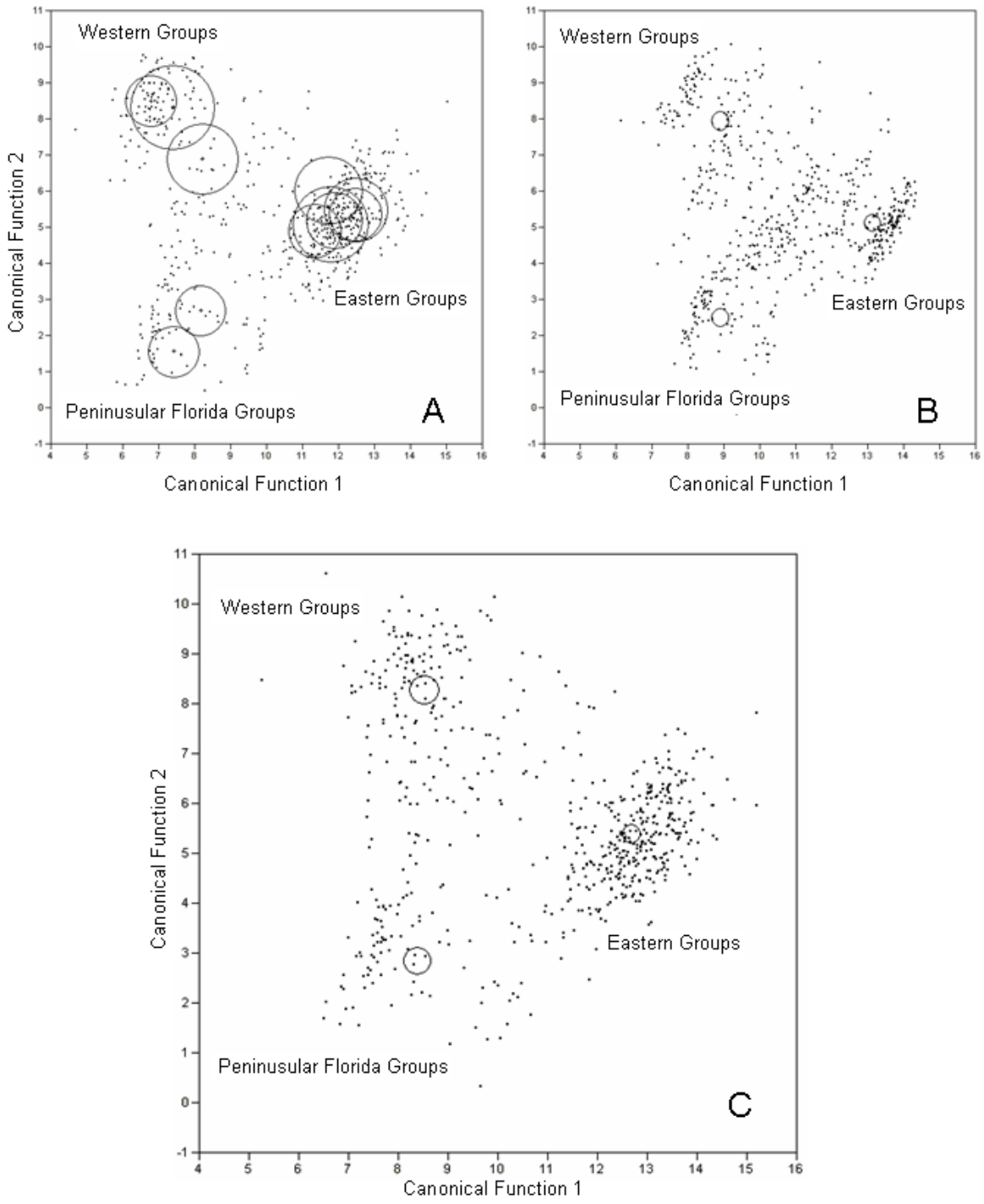


Figure 3-1. Canonical plots. These canonical plots are from three discriminate function analysis models. A) the plot from the model of all groups separate, B) and C) plots from the models of clustered groups.

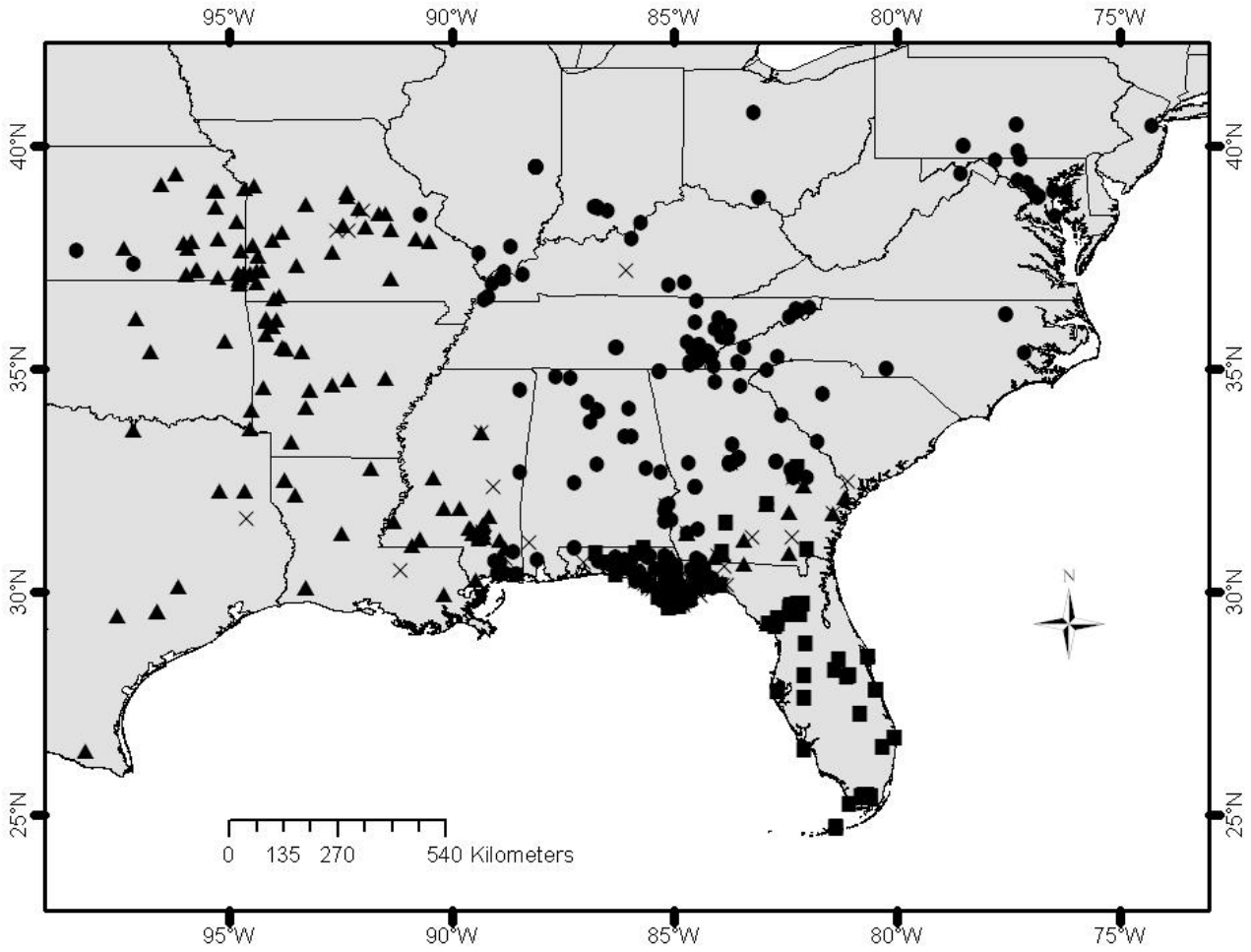


Figure 3-2. Morphological specimen assignments. Specimens that could not be assigned through discriminate function analysis with 95% probability are represented by Xs.

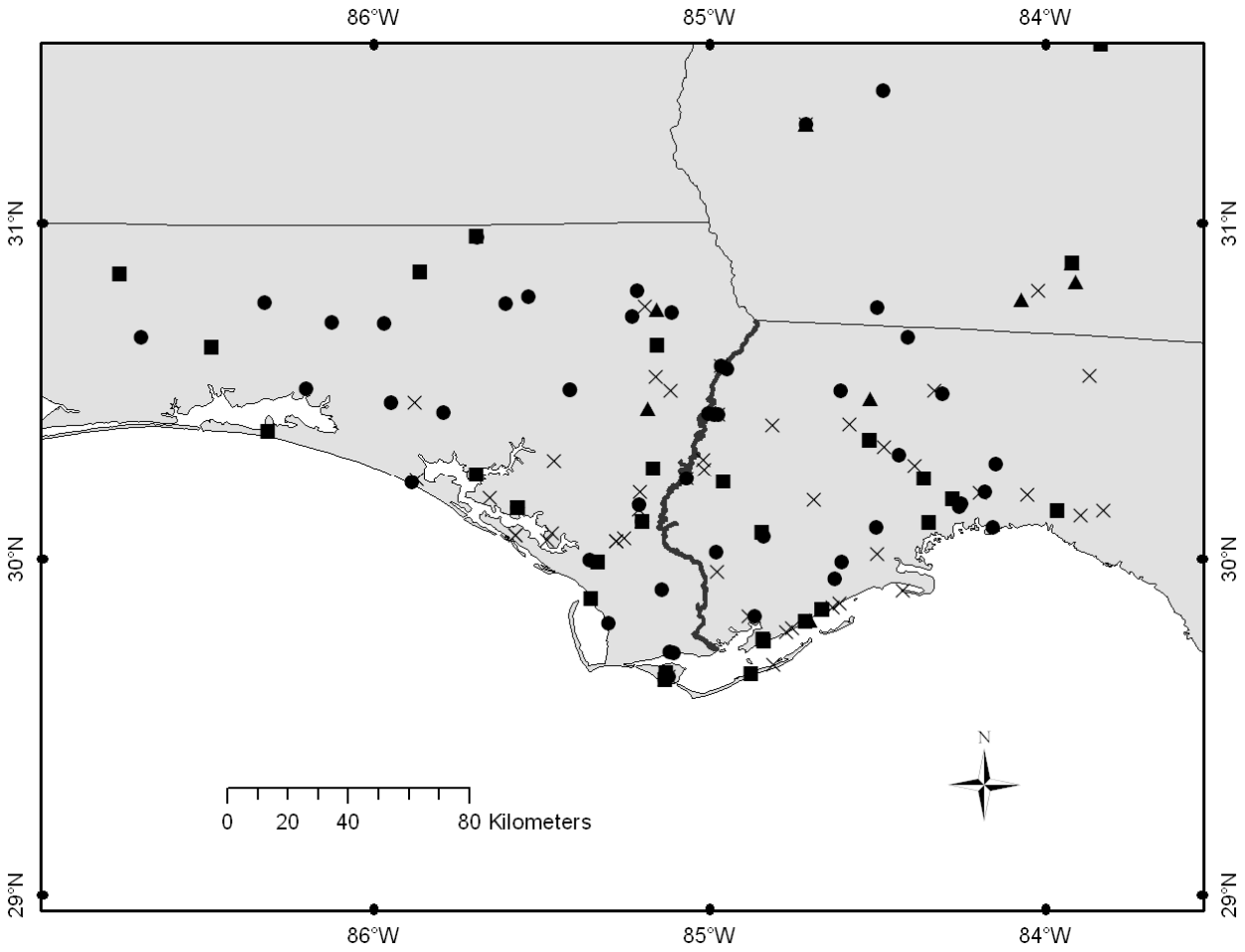


Figure 3-3. Florida panhandle morphological specimen assignments. Specimens that could not be assigned with 95% probability are represented by Xs.

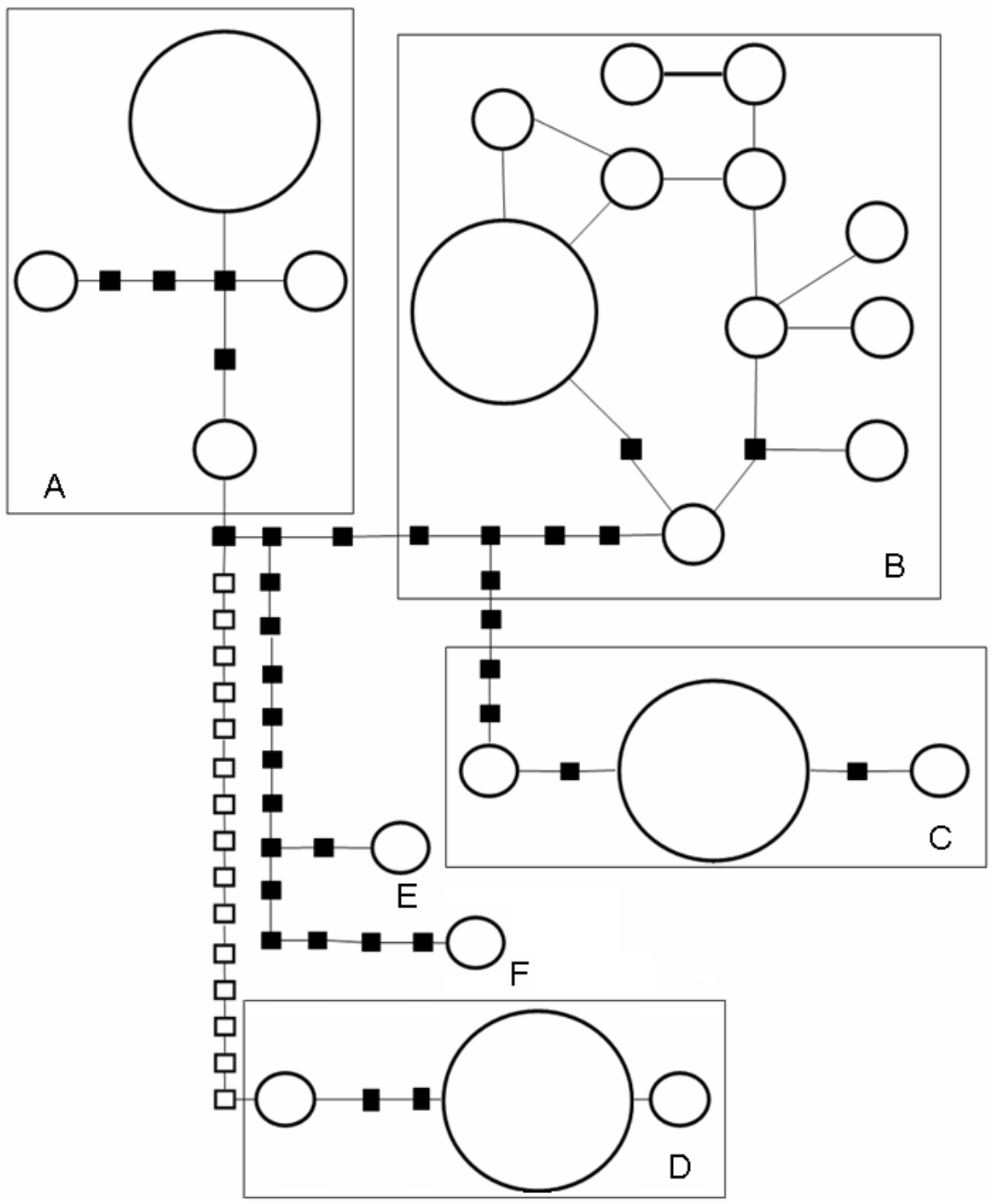


Figure 3-4. Mitochondrial haplotype network. This haplotype network was derived from d-loop sequences across the sampling range. Each square represents a base pair mutational step. Hollow squares indicate a network connection with less than 95% probability. Lineages A, B, C and D represent western, eastern, panhandle and peninsular clades respectively. Lineages E and F represent one specimen each from Escambia County, Florida and *Terrapene coahuila* respectively.

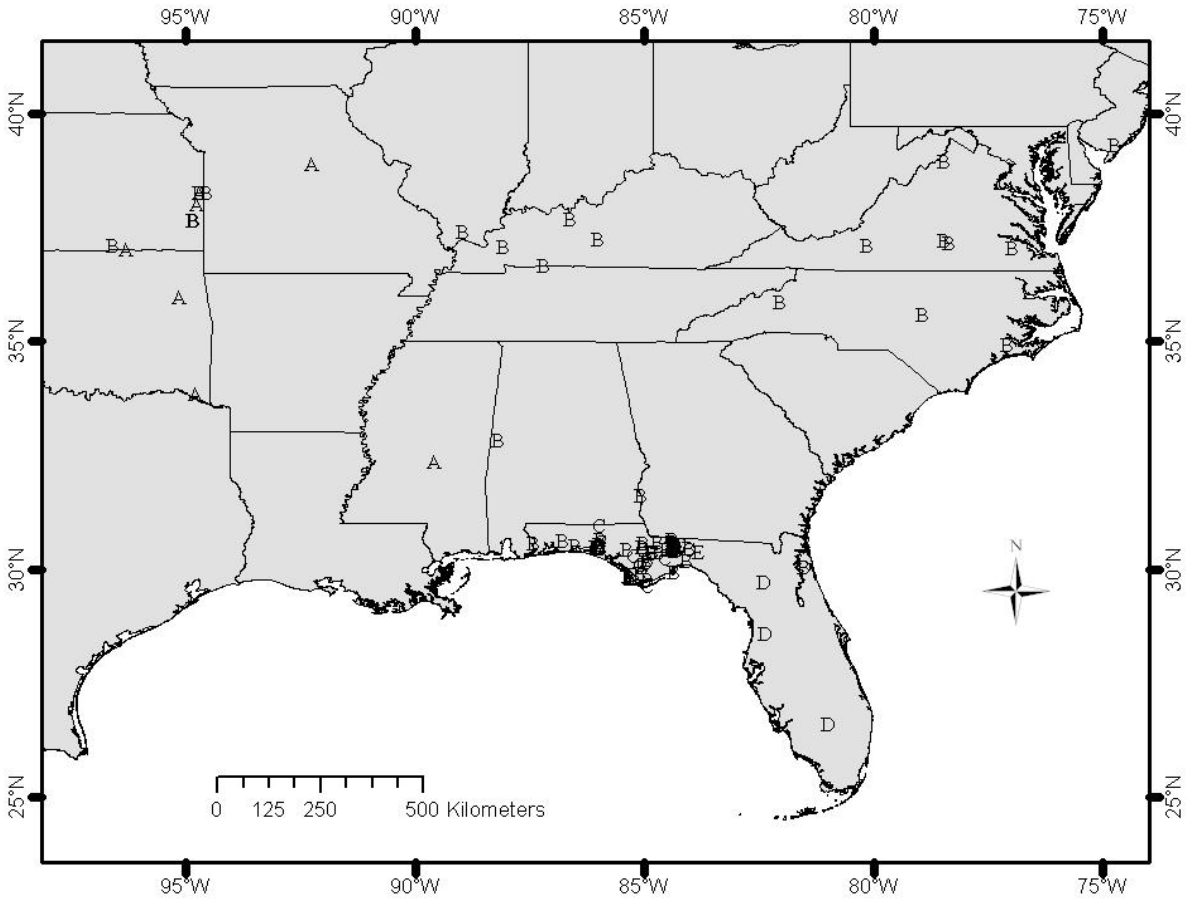


Figure 3-5. Haplotype distributions. Specimen localities are labeled based on clades from d-loop sequences. Lineage A is found primarily within the distribution of *T. c. triungus*, Lineage B within the distribution of *T. carolina carolina*, Lineage C within the Florida Panhandle and Lineage D within the range of *T. c. bauri*. Several lineage B specimens are found within the range of *T. c. triungus* and in the Florida panhandle.

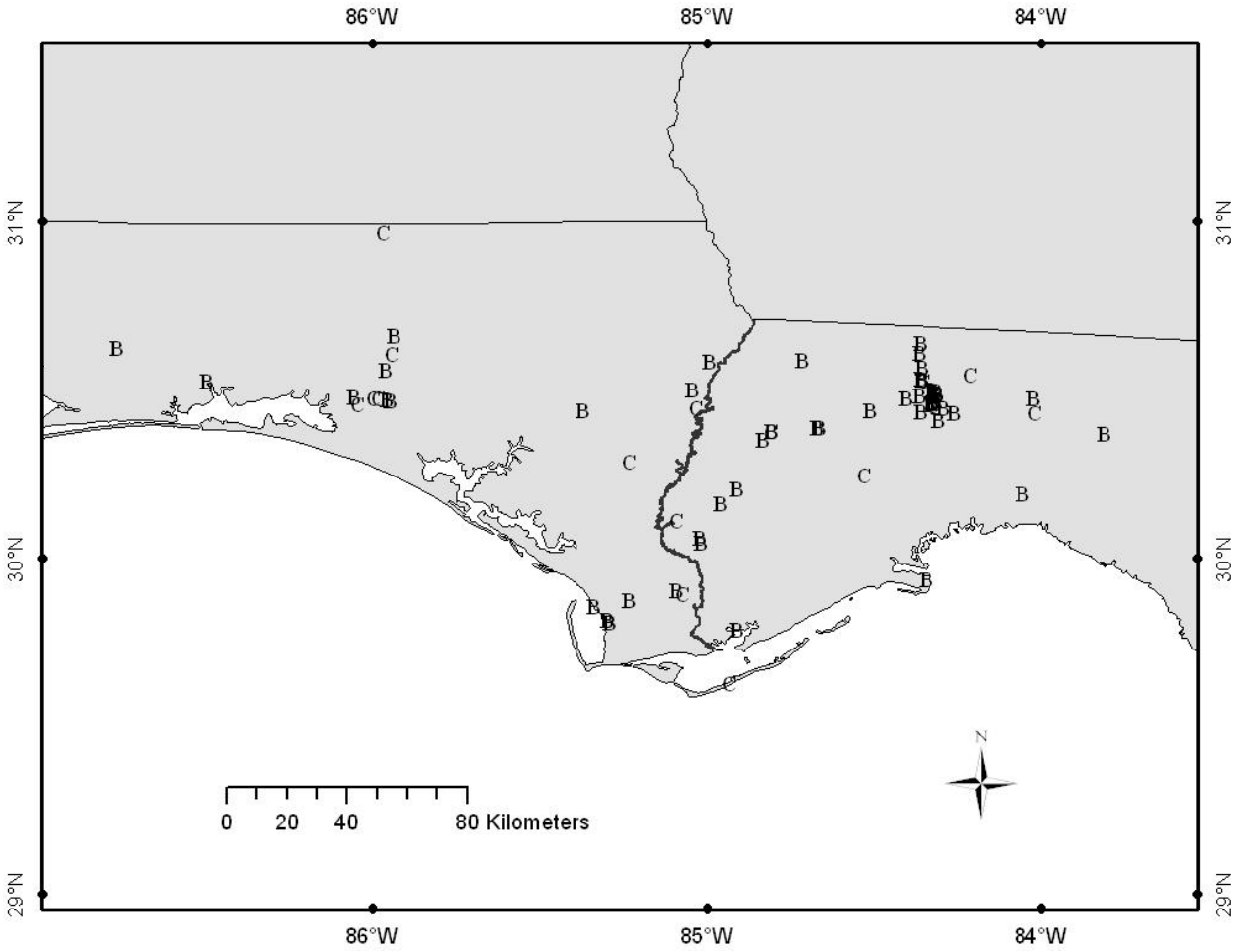


Figure 3-6. Panhandle detail of haplotype distributions. Specimen localities are labeled based on clades from d-loop sequences. Lineages B and C co-occur in this area and do not appear to possess geographic affinities. The Apalachicola River as it flows through Florida is outlined in grey.

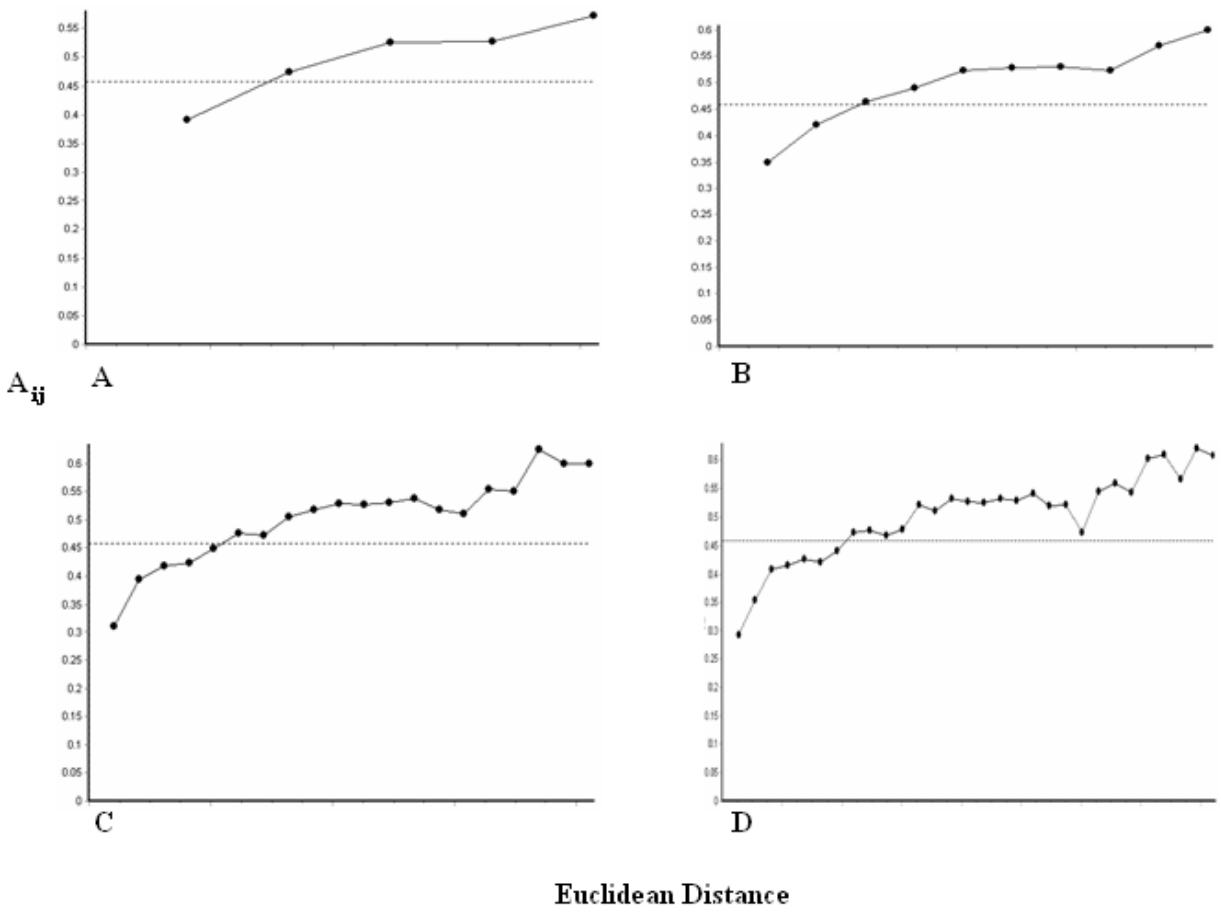


Figure 3-7. Autocorrelation plots. These plots show the spatial autocorrelation of morphological characters across the sampling range. The x axis depicts pairwise geographic distances and the y axis depicts the autocorrelation statistics for A) 5, B) 10, C) 20 and D) 30 distance classes. The dashed line is the average spatial autocorrelation statistic and its intercept with the observed autocorrelation line indicates the maximum distance of significant spatial autocorrelation.

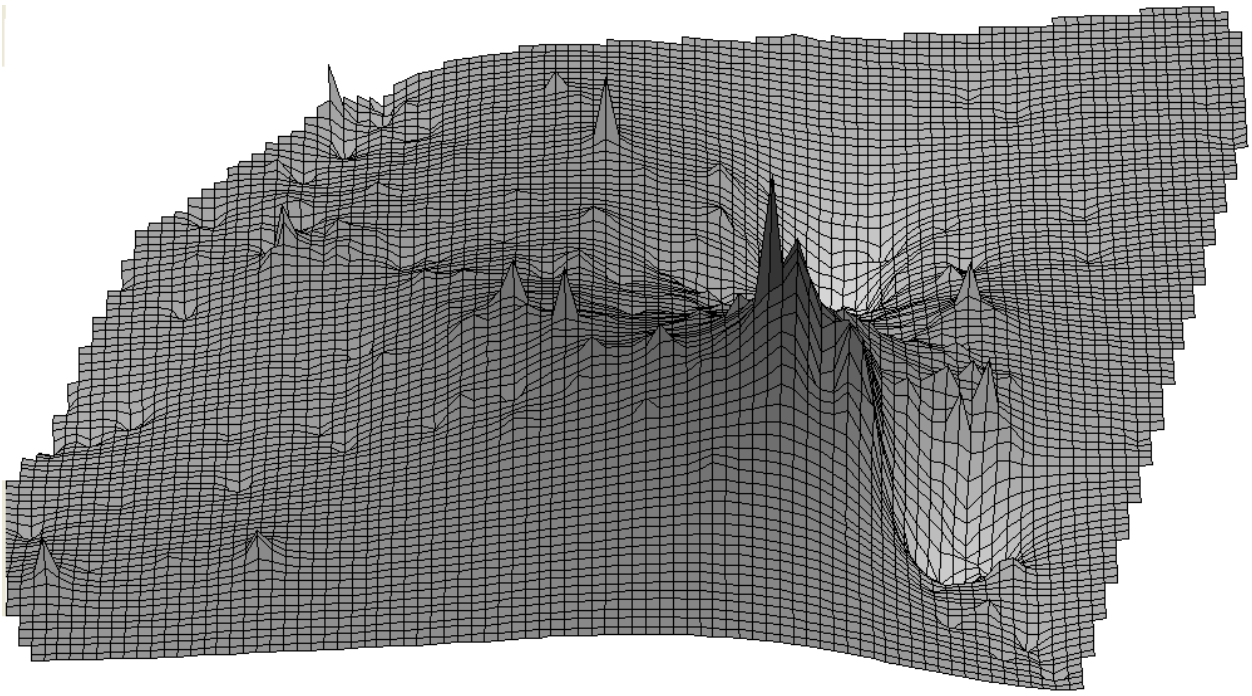


Figure 3-8. Genetic surface. This interpolation shows the range-wide genetic surface from the phenotypic dataset. The bottom right corner and the upper left corner are equivalent to the specimen localities from the southeast and northwest respectively.

CHAPTER 4
DISCUSSION

Lineage Validation and Assignment

In eastern North America, three of the four currently recognized subspecies of *Terrapene carolina* possess unique morphological and mitochondrial characters. The DFAs revealed differences in the morphology of a western, an eastern and a Florida lineage representative of the currently recognized *T. c. triungus*, *T. c. carolina* and *T. c. bauri* respectively. These lineages also possess unique mitochondrial haplotypes (Figure 3-5, clade A, clade B and clade D respectively). The distributions of specimens assigned to these lineages are nearly concordant with current subspecies distributional maps.

I do not recognize the validity of the Gulf Coast box turtle as a distinct evolutionary lineage. I was unable to distinguish specimens within the recognized range of *T. c. major* from the eastern lineage. Many of the phenotypic characters used to describe gulf coast box turtles are highly variable and also occur among eastern box turtles. Additionally, d-loop sequences from most specimens within the range of *T. c. major* are the same as those of the eastern lineage. However, another unique mtDNA haplotype (lineage C, Figure 3-6) occurs in this area, and many male box turtles from the gulf coast region seem to possess unique characteristics including a white head (Milstead 1969), a deeply cusped beak and a thick zygomatic arch (Minx 1996). These morphological characters and the lineage C mtDNA haplotype may have been inherited from the extinct giant box turtle *T. c. putnami* (Milstead 1969). Interestingly, specimens exhibiting classic *T. c. carolina* phenotypes possess lineage C d-loop haplotypes and specimens exhibiting *T. c. major* phenotypes possess eastern d-loop haplotypes (M. Aresco, upublished data). These results corroborate Milstead's (1969) hypothesis that *T. c. major* was not a distinct lineage, but instead a mixture of extant subspecies plus the extinct *T. c. putnami.*

The distinct geographic distributions, character suites and divergent mitochondrial haplotypes suggest the western, eastern and Florida lineages of box turtles underwent allopatry and divergence. The divergence of clade C may also be a product of historic vicariance due to physiographic features.

Avise (1992) estimated mtDNA divergence rates of turtles to be between 0.2-0.4% per million years. Under this estimate, the eastern, western and Florida clades diverged between 9.5 and 28.5 mya. The earliest known box turtle fossils allocated to *T. carolina* are from the late Miocene (~5 mya, Holman unpublished data–see Dodd 2001). Either much older *T. carolina* fossils remain undiscovered or the mtDNA divergence estimates of Avise (1992) are not applicable to the *T. carolina* mitochondrial control region.

Rates in mtDNA variation may differ severalfold among turtle taxa (Walker and Avise 1998), so I considered fossil evidence and historic geologic events to calibrate divergence rates in *T. carolina.* Eustatic estimates indicate sea levels of the coast of South Carolina were approximately 35 meters higher than current levels during the middle Pliocene (3.5 – 2.5 mya, Dowsett and Cronin 1990). Sea levels of this height would have inundated much of Florida, leaving an island disjunct from continental North America (Figure 4-1). Box turtle specimens are known from peninsular Florida prior to this event (Hulbert 2001). If box turtles persisted on the Pliocene island they would have diverged from those of the mainland, perhaps forming the Florida clade observed in this study. Using this event as a calibration point, the divergence rate of the control region of *T.carolina* mtDNA is between 1.54 to 2.36% per million years. This estimate overlaps the conventional 2.0% divergence rate estimated for non-turtle vertebrates (Wilson et al. 1985).

Under this estimate, eastern and western box turtles diverged between 1.61 – 2.47 mya while lineage C diverged from eastern box turtles between 1.23 and 1.88 mya.. These dates correspond with glaciations associated with the divergence of *Chrysemys* (Starkey et al. 2003). During interglacial periods, the cool waters and large floodplain of the Mississippi River may have prevented dispersal of rat snakes (Burbrink et al. 2000). Similarly, divergence of eastern and western box turtle clades may have resulted from vicariance due to river valley inundations. The haplotype and phenotype distributions appear to be associated more with the Mobile basin than the Mississippi basin. The ancestral Mobile River basin drained a larger area than the present day basin, including the Appalachian drainages that currently feed the Tennessee River (Mayden 1988). During glaciations, the Mobile River basin may have produced vicariance in box turtle populations.

Islands created during high sea levels in the early Pleistocene may have provided refugia for box turtles. A unique lineage of kingsnakes is thought to have diverged while isolated on Pleistocene islands in the panhandle of Florida (Means and Krysko 2001). The unique mitochondrial haplotypes of clade C may represent a box turtle lineage which diverged from the eastern clade by persisting on islands in the Coastal Plain during times of high sea level.

The divergence of clade B and C lineages could also be the result of landscape-level habitat affinities. During high sea levels the Coastal Plain may be reduced to a series of islands, however during glaciations and low sea levels the Coastal Plain would extend seaward beyond its current distribution. *T. c. putnami* may have flourished in these low, coastal areas. As sea levels advanced to modern levels, the habitat of *T. c. putnami* would have been reduced. The higher elevation areas of the Coastal Plain would provide refugia for *T. c. putnami* but also promote mixing with lineages adapted to the Piedmont.

A study incorporating carefully-structured sampling across the Coastal Plain of North American could test these potential scenarios. An island-refugia hypothesis would suggests several distinct but closely related lineages exist across the Coastal Plain, with each of these distinct lineages representing a refugial island available during high sea-levels. Alternatively, a Coastal Plain habitat hypothesis would suggests one major lineage across the entire landscape.

Intergradation and Gene Flow

The most phenotypically diverse area across the sampling range, as depicted by the genetic surface interpolation from AIS, is the Florida panhandle (Figure 3-8). Phenotypic assignment through DFA showed that box turtles from the Florida panhandle are composed of western, eastern and Florida lineages (Figure 3-3), as well as many specimens which could not be assigned with confidence. However, of these three lineages, only eastern mtDNA haplotypes occur in the Florida panhandle samples. Based on the mtDNA evidence from the samples in this study, *Terrapene* in the Florida panhandle are apparently not composed of intergrades between western, eastern and Florida lineages as proposed by Milstead (1969). However, this conclusion is not definitive since the evidence is based solely on one mtDNA gene. This pattern could also occur through biological selection; the maternally inherited mtDNA from the eastern lineage may be more prevalent if female eastern *Terrapene* are more reproductively fit. Corroboration of this conclusion can be confirmed through congruence with an unlinked gene (i.e., nuclear intron).

Although western and Florida lineages are not represented by panhandle specimens, lineage C haplotypes occur sympatrically with eastern haplotypes in the Florida panhandle. Recolonization and secondary contact of these lineages could produce the observed pattern of mtDNA haplotypes in the Florida panhandle. Analysis of the microsatellite dataset suggests these two lineages are not reproductively isolated. Although lineages B and C may have

diverged through allopatry, these lineages have since reticulated. The prevalence of eastern lineage specimens and the lack of unique microsatellite alleles in lineage C suggest complete swamping, at least in the Florida panhandle. One potential scenario involves eustatic allopatry – the ancestral stock to lineages B and C were separated by high sea levels. The eastern lineage maintained a geographically expansive distribution while lineage C was confined to Coastal Plain islands. The lineages diverged; lineage B into *T. c. carolina* and lineage C into *T. c. putnami*. As sea levels receded, box turtles colonized new, dry areas and the lineages made secondary contact.

Another possible scenario involves landscape-level allopatry through habitat affinities and segregation; lineage B representing a taxon inhabiting the Piedmont and lineage C inhabiting the Coastal Plain. Under this scenario, divergence would occur during glaciations and low sea level and mixing would be promoted as sea levels advanced.

Regardless of the mechanism, it is apparent that the eastern lineage swamped the now extinct *T. c. putnam*i, at least in the Florida panhandle. Swamping may have occured by *T. c. carolina* outnumbering *T. c. putnami*, but selection likely contributed to the extinction of the giant box turtle. The larger *T. c. putnami* would have been selected against by predators (including man) and fire.

Genetic Structure

Tests for IBD and cluster assignment did not reveal structuring among box turtles within the Florida panhandle. These results, in addition to the distribution of d-loop lineages B and C, imply the Apalachicola River does not and may have never acted as a barrier to gene flow in box turtles. The lack of discontinuity associated with the Apalachicola River separates the phylogeographic pattern of box turtles from the pattern of many other members of the family Emydid, and aligns it closer to the mtDNA pattern exhibited by the gopher tortoise (*Gopherus*

polyphemus, Walker and Avise 1998). Interestingly, the several Emydid taxa that do exhibit genetic discontinuities associated with the Apalachicola River inhabit aquatic environments, while the gopher tortoise is fully terrestrial. The Apalachicola River genetic discontinuity does not seem to apply to terrestrial chelonians.

Analysis of microsatellite loci did not reveal structuring within the Florida panhandle. Although the lack of apparent structure may be due to size homoplasy in informative microsatellite markers, limited structure could also result from high gene flow at the observed geographic scale. I examined sample-wide spatial autocorrelation of phenotypic characters to estimate larger geographic scales of genetic structure. Significant spatial autocorrelation occurred between pairs of individuals at least 581 kilometers apart. Autocorrelation at this scale suggest the area of genetic patches may greatly exceed levels suggested by the sedentary nature of many box turtles. The home ranges of most box turtles remain constant over several years (Nichols 1939). The box turtles that do not maintain a stable home range may be responsible for the lack of small-scale genetic structuring apparent from this study. My results corroborate the suggestions of Kiester (1982) that proposed enhanced gene flow by transient box turtles would result in a lack of subdivided populations of box turtles except at large scales. Additionally, no studies have investigated the dispersal of hatchling and juvenile box turtles from their nest site. Young box turtles dispersing large distances before settling into home ranges may also produce a lack of small-scale genetic structure.

Aside from natural movements, the lack of genetic structuring in box turtles may be due to human relocation. Box turtles are often removed from the wild and kept as pets (Dodd 2001). These turtles may later escape or be released. During their captivity, box turtles can be transported great distances. A three-toed box turtle collected in Guam serves as an extreme

example of this process (McCoid 1992). Human relocations of box turtles have likely disrupted the genetic integrity of the Florida panhandle.

Conclusion

Distinct evolutionary lineages of *Terrapene* can be diagnosed through statistical analysis of morphological suites and identification of mtDNA haplotypes. At least four divergent mtDNA lineages are present in southeastern North America, although morphological analysis recognized three distinct clades. Western, eastern and peninsular Florida lineages are represented by both unique mtDNA haplotypes and morphological suites. The fourth mtDNA haplotype occurs sympatrically with eastern haplotypes within the described range of the Gulf coast box turtle, *Terrapene carolina major*. Most specimens from this region are morphologically assignable to the eastern box turtle, although several individuals cannot be confidently assigned.

A microsatellite dataset was compiled from *Terrapene* specimens in the Florida panhandle. Analysis of this dataset did not show genetic structure associated with unique mtDNA haplotypes or the Apalachicola River. The lack of structure may be a result of microsatellite size homoplasy, although the spatial autocorrelation of morphological traits at a large geographic scale suggests a biological explanation. Gene flow across large geographic distance may be facilitated by transient males or extensive dispersal of juvenile box turtles. Additionally, genetic structure has likely been disrupted by human relocations of *Terrapene*.

Future phylogeographic studies could elucidate the contact zone dynamics between other *Terrapene* taxa. These studies should incorporate mitochondrial and nuclear markers from samples across a large geographic scale. Additional studies are needed to better understand the dispersal capability of *Terrapene.* These studies should focus on hatchling and transient male

turtles. The influence of human relocations on natural *Terrapene* populations should also be examined.

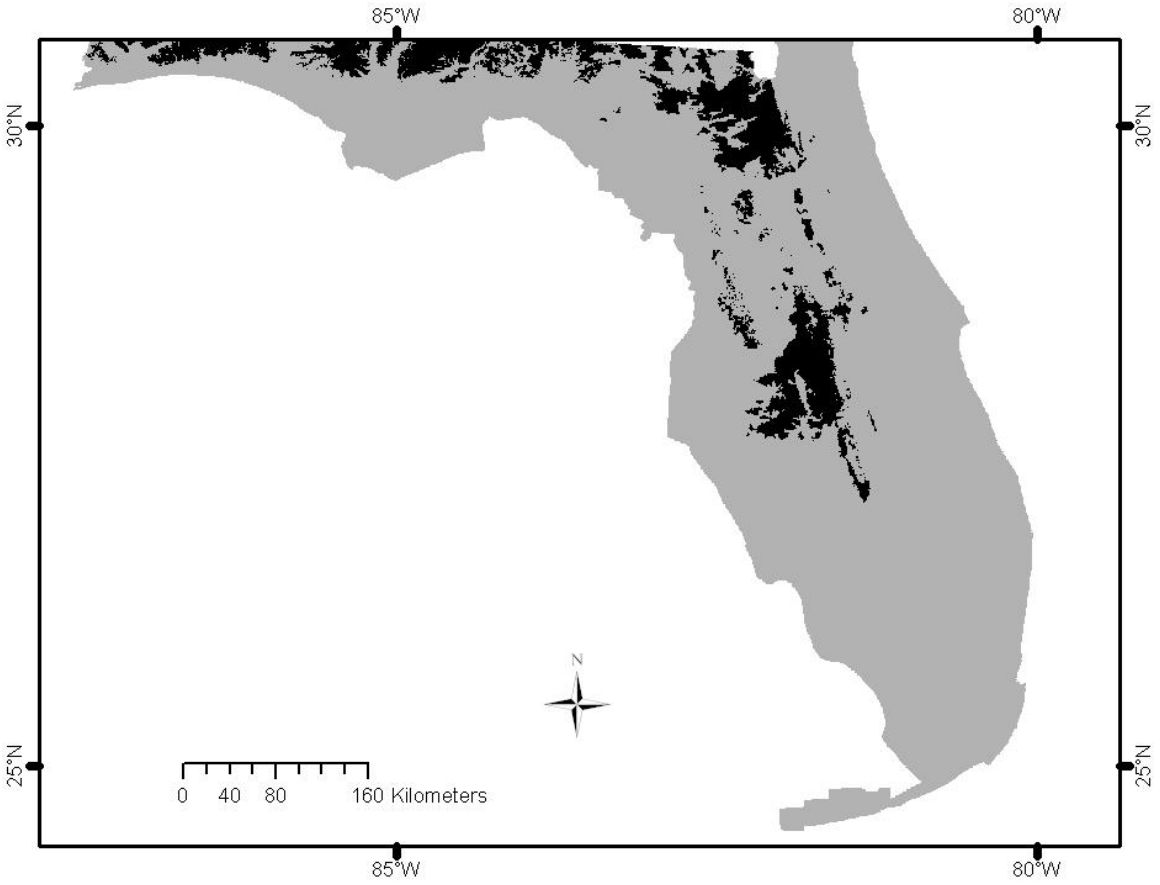


Figure 4-1. Florida coastlines. Black areas indicate the sections of present day Florida which would remain above the sea if levels rose to those estimated during the Pliocene (+ 35m, Dowsett and Cronin, 1990)

APPENDIX A
DISCRETE MORPHOLOGICAL CHARACTERS

The following descriptions detail each character or measurement and include the source and potential diagnostic problems when relative. Figures 2-3 and A-1 through A-3 depict character states and delineate landmarks of osteological measurements.

Head coloration: The coloration of fixed specimens fades with time and preservation. The presence of dark or light coloration is typically still obvious. I recorded whether the head of a specimen exhibited dark, light or both colorations.

Head and leg pattern: The head of Florida box turtles are usually patterned with two light stripes on either side, where those of gulf coast box turtles may be uniformly black (Carr 1952). I recorded whether specimens' heads and front limbs were patterned or plain. This character may also fade with time in preserved specimens.

Notched beak: Many box turtles exhibit a notch or cleft in the premaxilla. The notch in the beak of some specimens may be up to a centimeter deep while the beaks of others may be completely flat. Ditmars (1934) considered the presence of a notched maxillary beak a variable character and Minx (1996) found differences in the frequency of notched beaks between subspecies of *Terrapene carolina*. In life, the beak of a turtle may wear with time, potentially obscuring the presence of this character. I observed this character across all age groups, indicating its usefulness for most specimens.

Flared anterior carapace margin: Carr (1952) reported flaring of the rear carapace marginals in *T. c. carolina* and *T. c. major*. The rear marginals of box turtles are extremely variable and range from perpendicular to the plastron to upwardly curved like a gutter (see Figure 6E in Milstead 1969). I considered the marginal of specimens to be flared if its outer periphery was parallel or upturned to the plastron (Figure A-1).

Carapace background color: The background carapace color of *Terrapene carolina* is typically dark, although *T.carolina triungus* may possess a lighter horn-colored carapace background (Ditmars 1934). I recorded whether the carapace background was dark or light.

Rear toe count: The number of rear toes or claws varies between the subspecies of *T. carolina* (Minx 1996). I recorded whether specimens possessed three or four toes on each rear foot. Some specimens possessed three toes on one rear foot and four toes on the other. I recorded when this phenotype was observed, but unfortunately did not record which foot possessed the observed number of toes. Specimens missing rear feet were noted.

Imbricate anterior carapace margin: While examining specimens, I noticed that some exhibit an imbricate or serrated anterior margin of the carapace (Figure A-2). I recorded the presence or absence of this character.

Vertebral Ridge: Many specimens exhibit a prominent vertebral ridge, or middorsal keel – a thin (< 1.0 cm), raised section of carapace bisecting the animal dorsally (Minx 1996). I

recorded the presence of a ridge when apparent across at least one-half of the carapace. Shell wear may reduce the prominence of a vertebral ridge, although I typically found ridges observable in very old specimens.

Vertebral Stripe: Taylor (1895) originally described the Florida box turtle as possessing a yellow keel. I recorded the presence of a light vertebral strip when it was apparent over at least one half of the shell. Shell wear and scarring can obscure the presence of this character.

Carapace pattern: Box turtles exhibit remarkable variation in carapace pattern. Differences in pattern between subspecies have been noted (Carr 1952). I recorded the presence of streaks, spots or blotches on the carapace of specimens. I defined streaks as numerous straight, light lines at least 3 cm in length; spots as numerous round, light areas of pigmentation less than 1 cm in diameter; and blotches as numerous irregular light markings greater than 1 square centimeter in area. Many specimens exhibited combinations of these patterns and were noted accordingly. This character could not be accurately determined for several badly scarred or poorly preserved specimens.

Plastron pattern: Eastern and Florida box turtles may exhibit a patterned plastron while the plastrons of gulf coast and three-toed box turtles may be uniformly colored (Dodd 2001). I recorded whether the plastron of each specimen was uniform or patterned. With age, the plastron of a turtle may wear smooth and obscure any obvious patterns. I addressed this potential bias by comparing the frequency of patterned plastrons between specimens with a low number of annuli and specimens with annuli completely worn over.

Plastron coloration: The plastrons of gulf coast box turtles are dark brown or black while three toed box turtles usually possess a uniformly yellow plastron (Dodd 2001). Although plastron coloration may fade in preserved specimens, I easily recorded whether specimens possessed a light or dark plastron.

Urn-shaped nucal: Auffenberg (1967) described the presence of an urn-shaped anterior vertebral bone (or nucal) as a distinguishing characteristic between *T.carolina* major and *T.carolina* subspecies. I recorded the presence of an urn-shaped nucal. A diagram of this character along with other commonly observed nucal shapes is provided (Figure A-3).

Sexually Dimorphic Characters

Adult box turtles exhibit sexual dimorphism. The tail of male turtles is typically larger and longer than those of similarly sized females. Several studies report variation in sexually dimorphic characters between subspecies of box turtles. I recorded the presence of an enlarged tail along with several other sexually dimorphic characters described below.

Concave plastron: The concave posterior plastron of male *Terrapene carolina* facilitates fitting of the shell of females during reproduction (Dodd 2001). Male three-toed box turtles do not usually have a plastron concavity (Milstead 1969). I recorded the plastron state for each examined specimen.

Depth of plastron concavity: The relative depth of the plastron concavity differs between subspecies of *T. carolina* with gulf coast box turtle's concavities being the deepest (Dodd 2001). I considered the depth of a plaston to be deep if it was more than 20% of the length of the concave region.

Shape of concave region: The plastron concavity of male box turtles is typically round, although gulf coast box turtles often possess an elongate concavity (Milstead 1969). I recorded concavities as elongate if their length was at least 110% of their width.

Enlarged rear toes: The claws on the rear feet of male adult turtles often grow much larger than the rear claws of females of comparable size. Carr (1952) reported this character across many box turtle taxa. I recorded rear toes of a specimen as enlarged if their size appeared at least twice that of toes on the front feet. I noted when I could not determine the state of this character due to specimens missing their rear feet.

Continuous Characters

I measured straight-line distances three times per character for most specimens. Measurements were made to the nearest $1/100^{th}$ of a millimeter with a 20 cm digital vernier caliper. Accurate measurements of some specimens were inhibited due to scarring of the animal or awkward position of the limbs. I recorded when these difficulties occurred so their potential for influencing analytical abnormalities could be examined.

Posterior lobe of plastron: This distance is the length of the posterior half of the plastron, measured from the center of the hinge to the posterior end of the interanal seam.

Interregular seam: I recorded this measure as the distance between the pair of the most anterior scutes on the plastron.

Interhumeral seam: This measurement is the length of the seam between the pair of scutes in the center of the frontal lobe of the plastron.

Interpectoral seam: The most posterior pair of scutes on the frontal lobe of the plastron connect to form the interpectoral seam. I measured the length of this seam.

Hinge: I took this measurement across the posterior portion of the frontal lobe along the hinge in the center of the plastron. As mentioned earlier, many museum specimens have a detached plastron. Taking this measurement along the posterior end of the frontal lobe allowed for consistent measurements across all specimens.

Carapace width: I measured the width of the shell in line with the plastron hinge.

Shell depth: I measured the depth of the shell between the hinge and the top of the carapace directly above the hinge. The plastrons of some specimens were detached, likely for diet or reproduction studies. I recorded when plastrons were detached and fitted the plastron of these specimens snuggly against the carapace before taking depth measurements. The position

of greatest shell depth differs between some box turtle subspecies (Carr 1952). Unfortunately, I did not record where the greatest shell depth occurred on each specimen.

Carapace Length: I measured the straight-lined distance from the notch between the pair of anterior marginals and the notch between the two most posterior marginals.

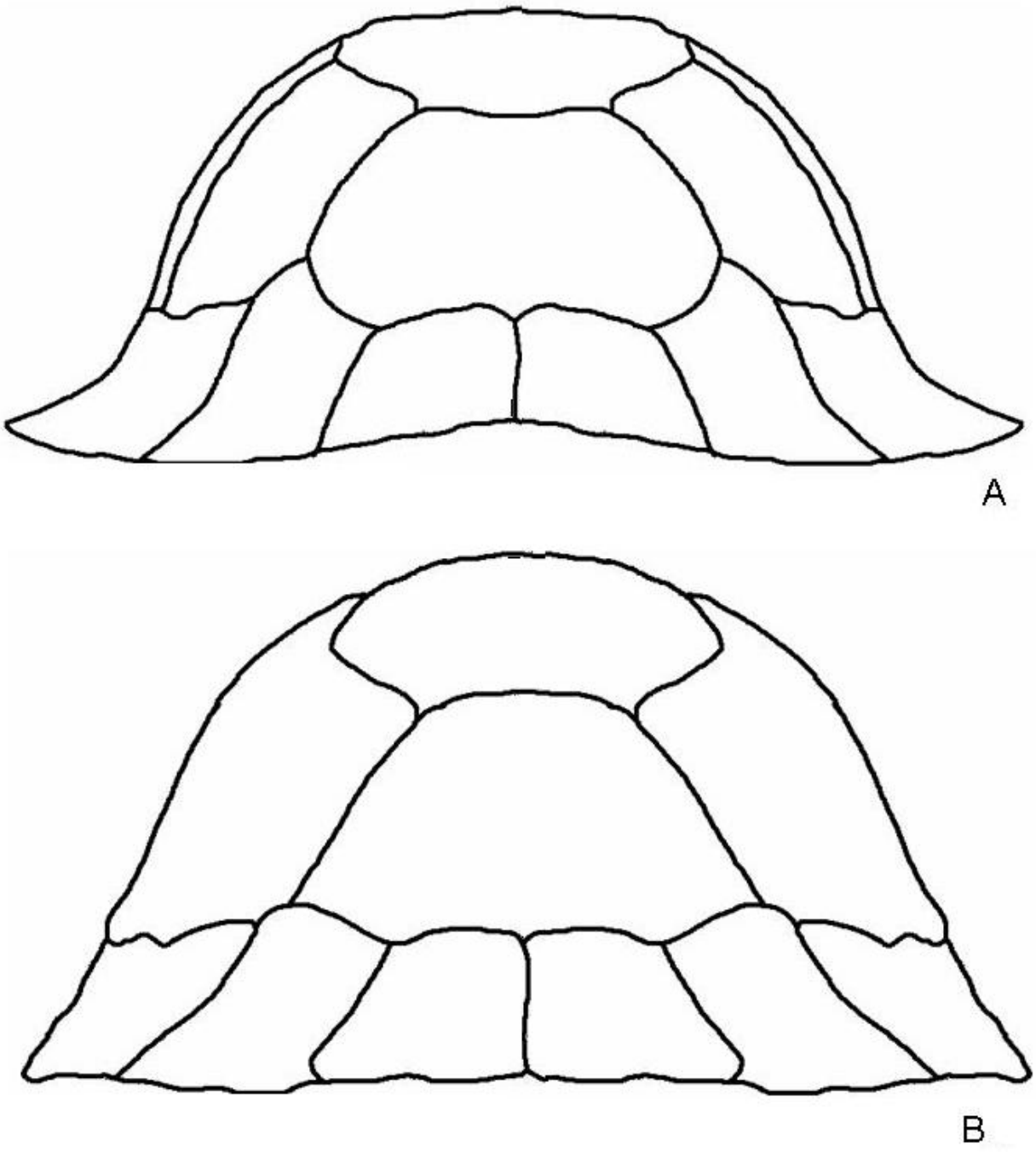


Figure A-1. Marginal flare. Medial view of the posterior carapace of two *Terrapene*. A) flared rear marginal, B) rear marginal not flared.

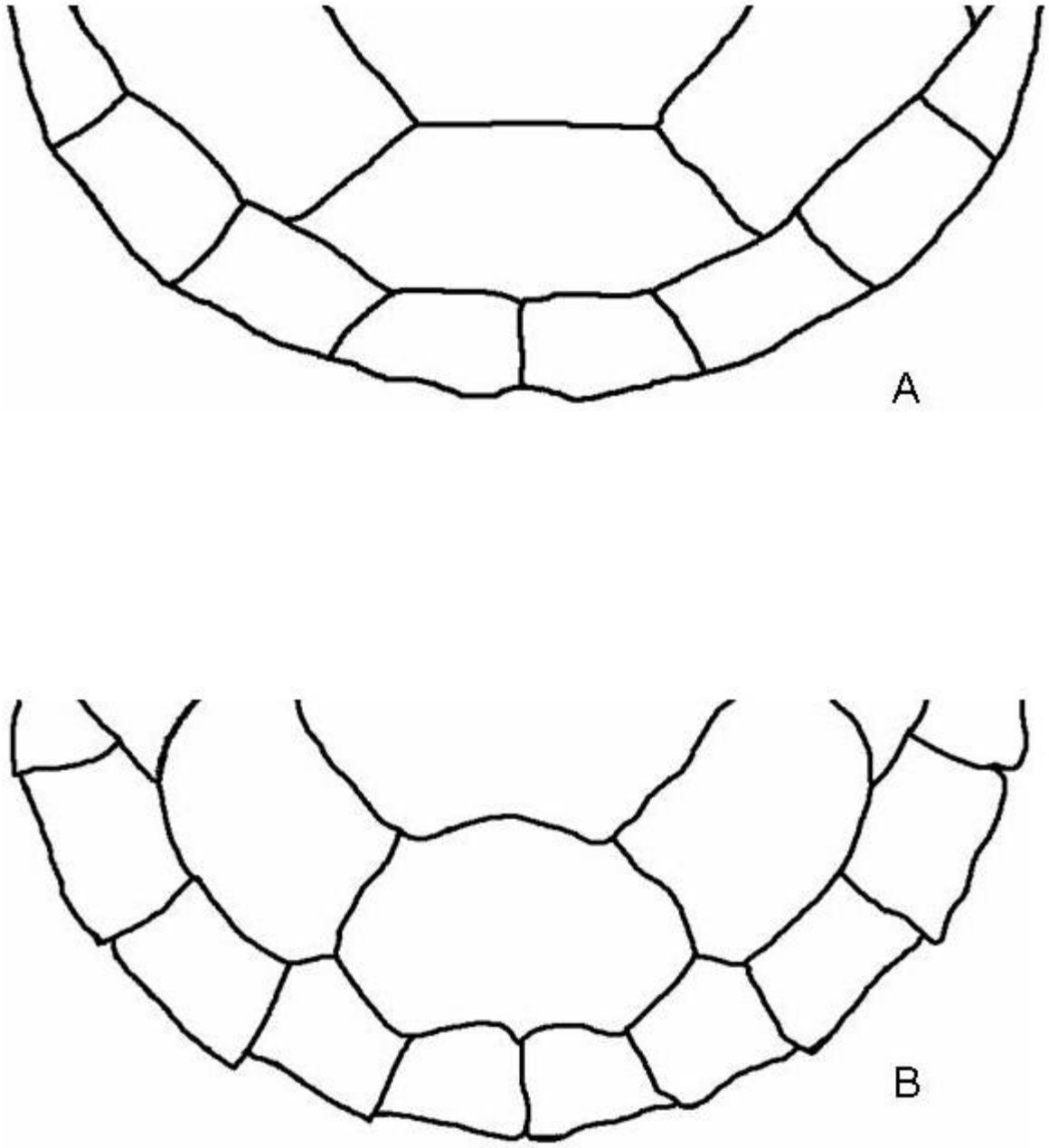


Figure A-2. Posterior marginals. A dorsal view of the posterior carapace of *Terrapene* shows the differences in posterior marginals. A) exhibits smooth rear marginals, B) represents serrated rear marginals.

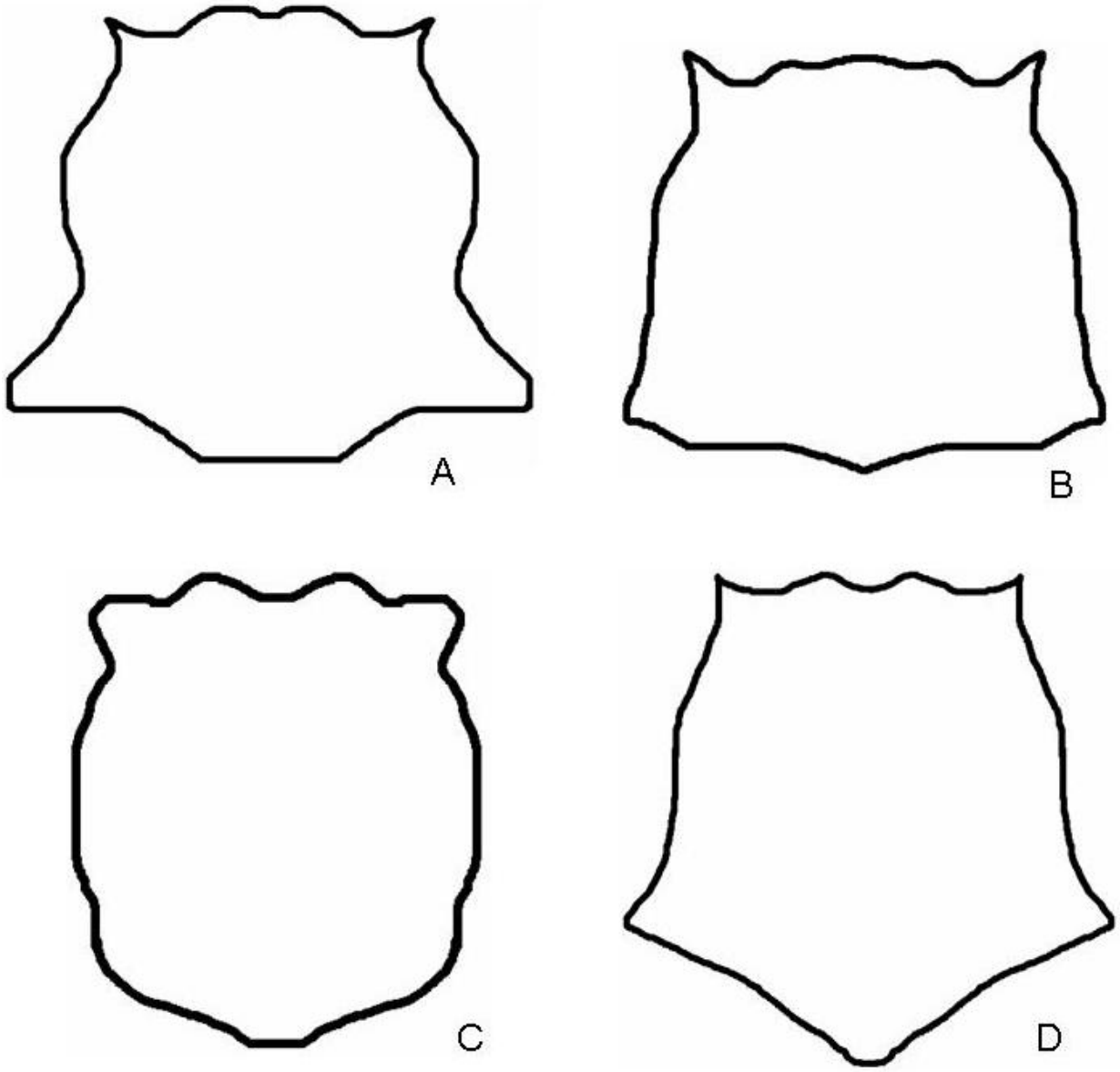


Figure A-3. Nucal shapes. These nucal shapes are common in *Terrapene carolina* including the urn-shape described by Auffenberg (A, 1952).

APPENDIX B
MUSEUM AND TISSUE DATA OF *TERRAPENE* USED IN THIS STUDY

Table B-1. Museum specimens of *Terrapene* used in this study.

KU	2540	KU	46722	KU	46805	KU	47350	KU	51452	KU	217160
KU	3013	KU	46752	KU	46807	KU	47352	KU	51453	KU	218795
KU	3014	KU	46753	KU	46808	KU	47353	KU	51454	KU	289712
KU	3063	KU	46758	KU	46810	KU	47354	KU	51455	UF	19
KU	3068	KU	46759	KU	46811	KU	47355	KU	51456	UF	659
KU	3093	KU	46760	KU	46812	KU	47357	KU	51457	UF	1411
KU	3142	KU	46761	KU	46813	KU	47358	KU	51458	UF	2349
KU	3143	KU	46762	KU	46814	KU	47360	KU	51460	UF	2377
KU	3144	KU	46763	KU	46815	KU	47361	KU	51461	UF	2378
KU	3396	KU	46765	KU	46816	KU	47362	KU	61854	UF	2379
KU	3832	KU	46766	KU	46817	KU	47363	KU	70969	UF	2380
KU	15828	KU	46767	KU	46818	KU	47364	KU	75135	UF	3328
KU	15829	KU	46768	KU	46819	KU	47365	KU	75137	UF	4019
KU	15886	KU	46769	KU	46820	KU	47366	KU	88838	UF	4020
KU	15890	KU	46770	KU	46821	KU	47368	KU	88841	UF	4225
KU	17367	KU	46771	KU	46822	KU	47369	KU	91357	UF	4226
KU	18387	KU	46773	KU	46823	KU	47370	KU	143854	UF	4227
KU	19343	KU	46774	KU	46824	KU	47371	KU	143855	UF	4228
KU	19344	KU	46775	KU	46825	KU	47372	KU	144569	UF	4230
KU	19348	KU	46776	KU	46826	KU	47373	KU	151894	UF	4231
KU	19478	KU	46778	KU	46827	KU	47374	KU	151895	UF	4232
KU	19738	KU	46780	KU	46828	KU	47478	KU	153637	UF	4233
KU	19739	KU	46781	KU	46829	KU	47479	KU	177148	UF	4234
KU	19741	KU	46784	KU	46830	KU	47482	KU	177149	UF	4235
KU	20936	KU	46786	KU	46831	KU	47483	KU	177217	UF	4236
KU	20937	KU	46787	KU	46851	KU	48240	KU	192259	UF	4237
KU	22818	KU	46788	KU	46853	KU	48241	KU	192404	UF	4239
KU	23039	KU	46789	KU	46854	KU	48242	KU	197234	UF	4241
KU	23337	KU	46790	KU	46893	KU	48243	KU	203625	UF	4245
KU	23338	KU	46791	KU	46894	KU	48244	KU	207136	UF	4247
KU	23340	KU	46793	KU	46917	KU	48245	KU	208133	UF	4406
KU	23341	KU	46794	KU	46918	KU	48246	KU	214312	UF	4407
KU	23342	KU	46797	KU	46919	KU	48247	KU	214313	UF	4408
KU	23343	KU	46798	KU	47341	KU	48249	KU	214317	UF	4409
KU	23344	KU	46799	KU	47342	KU	48250	KU	214318	UF	4410
KU	23345	KU	46800	KU	47343	KU	48258	KU	214320	UF	4412
KU	23346	KU	46801	KU	47344	KU	50505	KU	214321	UF	4413
KU	23348	KU	46802	KU	47346	KU	50507	KU	214322	UF	4414
KU	23349	KU	46803	KU	47347	KU	50752	KU	214323	UF	4415
KU	41570	KU	46804	KU	47349	KU	51433	KU	214324	UF	4416

Table B-1. Continued

UF	4418	UF	7865	UF	10127	UF	21400	UF	47872	UF	48161
UF	4419	UF	7874	UF	10128	UF	21476	UF	47874	UF	48162
UF	4420	UF	7907	UF	10147	UF	21477	UF	47879	UF	50789
UF	4422	UF	8464	UF	10181	UF	21478	UF	47883	UF	50802
UF	4423	UF	8465	UF	11120	UF	21479	UF	47887	UF	50813
UF	4423	UF	8473	UF	11121	UF	27612	UF	47888	UF	53406
UF	4424	UF	8590	UF	12012	UF	27613	UF	47890	UF	54660
UF	4425	UF	8591	UF	12348	UF	27614	UF	47896	UF	54661
UF	4426	UF	8592	UF	12447	UF	30177	UF	47898	UF	54662
UF	4427	UF	8619	UF	14199	UF	30178	UF	47900	UF	63785
UF	4428	UF	8739	UF	14666	UF	30179	UF	47901	UF	63786
UF	4429	UF	8905	UF	14668	UF	30180	UF	47902	UF	63788
UF	4430	UF	8906	UF	14669	UF	30181	UF	47903	UF	63790
UF	4431	UF	8937	UF	14670	UF	30182	UF	47904	UF	63791
UF	4432	UF	9253	UF	14671	UF	30183	UF	47905	UF	63792
UF	4433	UF	9350	UF	14672	UF	30185	UF	47906	UF	63793
UF	4434	UF	9390	UF	16224	UF	30186	UF	47907	UF	63793
UF	4437	UF	9391	UF	19736	UF	30187	UF	47908	UF	63795
UF	4438	UF	9392	UF	19737	UF	30188	UF	47910	UF	65954
UF	4439	UF	9409	UF	20544	UF	30189	UF	47960	UF	66321
UF	4440	UF	9493	UF	21171	UF	30256	UF	47961	UF	66322
UF	4441	UF	9497	UF	21172	UF	30257	UF	48121	UF	66324
UF	4442	UF	9498	UF	21173	UF	30259	UF	48122	UF	66352
UF	4443	UF	9499	UF	21174	UF	30275	UF	48123	UF	66353
UF	4444	UF	9500	UF	21175	UF	30276	UF	48124	UF	66354
UF	4445	UF	9501	UF	21176	UF	47779	UF	48125	UF	66423
UF	4446	UF	9502	UF	21181	UF	47780	UF	48127	UF	66426
UF	4447	UF	9704	UF	21182	UF	47781	UF	48128	UF	66591
UF	4448	UF	9708	UF	21183	UF	47782	UF	48131	UF	66593
UF	4450	UF	9710	UF	21185	UF	47784	UF	48133	UF	67752
UF	4452	UF	9711	UF	21186	UF	47785	UF	48134	UF	67756
UF	4453	UF	9731	UF	21190	UF	47786	UF	48135	UF	73785
UF	6510	UF	9732	UF	21191	UF	47787	UF	48138	UF	73786
UF	6511	UF	9734	UF	21192	UF	47788	UF	48140	UF	73787
UF	6512	UF	9735	UF	21194	UF	47789	UF	48145	UF	74704
UF	6513	UF	9736	UF	21195	UF	47790	UF	48146	UF	87651
UF	6514	UF	9738	UF	21196	UF	47791	UF	48147	UF	89498
UF	6515	UF	9739	UF	21198	UF	47792	UF	48150	UF	91092
UF	6610	UF	9740	UF	21200	UF	47793	UF	48151	UF	123198
UF	6611	UF	9742	UF	21201	UF	47794	UF	48153	UF	137227
UF	7443	UF	9757	UF	21202	UF	47795	UF	48154	UF	141802
UF	7444	UF	9787	UF	21204	UF	47796	UF	48155	UF	141876
UF	7446	UF	9815	UF	21396	UF	47797	UF	48156	UF	141880

Table B-1. Continued

UF	149147	UF	10126-2	USNM	45774	USNM	288236	USNM	326347
UF	149148	UF	10126-3	USNM	55589	USNM	288237	USNM	326348
UF	149150	UF	9492-1	USNM	55590	USNM	288238	USNM	326349
UF	149152	UF	9492-2	USNM	55648	USNM	292080	USNM	326350
UF	149153	UF	9689-1	USNM	60898	USNM	292081	USNM	326351
UF	149154	UF	9756-1	USNM	60899	USNM	304355	USNM	326352
UF	149156	UF	9756-2	USNM	60900	USNM	323057	USNM	326353
UF	149157	UF	7535	USNM	60901	USNM	323058	USNM	326354
UF	149158	UF	7537	USNM	64600	USNM	323073	USNM	326355
UF	149159	UF	7804	USNM	64989	USNM	323074	USNM	326356
UF	149160	UF	9832	USNM	69547	USNM	326204	USNM	326357
UF	149161	UF	9872	USNM	79370	USNM	326285	USNM	326358
UF	149162	UF	9881	USNM	81032	USNM	326294	USNM	326359
UF	149163	UF	21397	USNM	94375	USNM	326295	USNM	326360
UF	149164	UF	21398	USNM	95329	USNM	326297	USNM	326361
UF	149165	UF	21399	USNM	95331	USNM	326298	USNM	326362
UF	149166	UF	47798	USNM	95332	USNM	326299	USNM	326363
UF	149167	UF	47799	USNM	95333	USNM	326301	USNM	326364
UF	149168	UF	47866	USNM	95335	USNM	326319	USNM	326365
UF	149169	UF	48157	USNM	95337	USNM	326320	USNM	326366
UF	149170	UF	48158	USNM	95338	USNM	326321	USNM	326367
UF	149171	UF	48160	USNM	95346	USNM	326322	USNM	326368
UF	149173	UF	141890	USNM	95348	USNM	326324	USNM	326369
UF	149174	UF	141912	USNM	95349	USNM	326325	USNM	326370
UF	149175	UF	149146	USNM	95353	USNM	326327	USNM	326371
UF	149176	USNM	53	USNM	95357	USNM	326328	USNM	326388
UF	149177	USNM	11613	USNM	95358	USNM	326330	USNM	327978
UF	149178	USNM	19481	USNM	99841	USNM	326331	USNM	328081
UF	149179	USNM	22340	USNM	100359	USNM	326332	USNM	336217
UF	149180	USNM	22502	USNM	100519	USNM	326333	USNM	497306
UF	149181	USNM	22681	USNM	101060	USNM	326334	USNM	83992
UF	149182	USNM	28436	USNM	118169	USNM	326335	USNM	84447
UF	149183	USNM	29211	USNM	142098	USNM	326336	USNM	84448
UF	149184	USNM	45302	USNM	142099	USNM	326337	USNM	84450
UF	149197	USNM	45303	USNM	142100	USNM	326339	USNM	84451
UF	151190	USNM	45308	USNM	197487	USNM	326340	USNM	84452
UF	151365	USNM	45317	USNM	197490	USNM	326341	USNM	84457
UF	151368	USNM	45322	USNM	218797	USNM	326342	USNM	84882
UF	151372	USNM	45338	USNM	218798	USNM	326343	USNM	86443
UF	151373	USNM	45342	USNM	220293	USNM	326344	USNM	91226
UF	151375	USNM	45343	USNM	288178	USNM	326345	USNM	92019
UF	10126-1	USNM	45772	USNM	288235	USNM	326346	USNM	94372

KU=University of Kansas Natural History Museum, UF=Florida Museum of Natural History, USNM=Smithsonian Institution National Museum of Natural History.

Table B-2. Localities of tissue specimens used in this study

id	St	County	Lat	Long
tcar001	FL	Liberty	30.3877	-84.6714
tcar002	FL	Leon	30.4371	-84.5119
tcar003	FL	Liberty	30.1702	-85.0660
tcar004	FL	Liberty	30.3740	-84.8058
tcar005	FL	Liberty	30.3740	-84.8058
tcar006	FL	Liberty	30.1101	-85.0905
tcar007	FL	Liberty	30.3492	-84.8324
tcar008	FL	Leon	30.4308	-84.2580
tcar009	FL	Leon	30.4445	-84.2929
tcar010	FL	Leon	30.4631	-84.3253
tcar011	FL	Liberty	30.3739	-84.8059
tcar012	FL	Liberty	30.2020	-84.9104
tcar013	FL	Liberty	30.3693	-83.8118
tcar014	FL	Liberty	30.3872	-84.6621
tcar015	FL	Leon	30.4584	-84.3235
tcar016	FL	Leon	30.5275	-84.3572
tcar017	FL	Leon	30.5275	-84.3572
tcar018	FL	Leon	30.4853	-84.3098
tcar019	FL	Leon	30.4324	-84.3605
tcar020	FL	Liberty	30.2451	-84.5272
tcar021	FL	Leon	30.4584	-84.3244
tcar022	FL	Jefferson	30.4730	-84.0215
tcar023	FL	Leon	30.5275	-84.3572
tcar024	FL	Leon	30.4929	-84.3207
tcar025	FL	Leon	30.5275	-84.3572
tcar026	FL	Leon	30.4584	-84.3235
tcar027	FL	Leon	30.4937	-84.3121
tcar028	FL	Leon	30.4584	-84.3235
tcar029	FL	Leon	30.4955	-84.3256
tcar030	FL	Leon	30.5007	-84.3283
tcar031	FL	Jefferson	30.4306	-84.0197
tcar032	FL	Leon	30.4554	-84.3331
tcar033	FL	Gadsden	30.6384	-84.3619
tcar034	FL	Gadsden	30.6066	-84.3639
tcar035	GA	Decatur	30.7725	-84.4833
tcar036	GA	Clay	31.6151	-85.0479
tcar037	FL	Leon	30.4959	-84.3257
tcar038	FL	Liberty	30.0417	-85.0175
tcar039	FL	Liberty	30.1609	-84.9589
tcar040	FL	Leon	30.4793	-84.3633
tcar041	FL	Franklin	29.6261	-84.9327
tcar042	FL	Leon	30.4092	-84.3053
tcar043	FL	Leon	30.4720	-84.4051
tcar044	FL	Leon	30.4603	-84.3137

Table B-2. Continued

id	St	County	Lat	Long
tcar045	FL	Leon	30.5653	-84.3586
tcar046	FL	Leon	30.4841	-84.3085
tcar047	FL	Leon	30.5421	-84.2109
tcar048	FL	Leon	30.5233	-84.3534
tcar049	FL	Leon	30.4513	-84.3229
tcar050	FL	Leon	30.4880	-84.3089
tcar051	FL	Leon	30.4622	-84.3239
tcar052	FL	Leon	30.4926	-84.3199
tcar053	FL	Walton	30.4736	-85.9809
tcar054	FL	Leon	30.5310	-84.3609
tcar055	FL	Walton	30.4703	-85.9582
tcar056	FL	Walton	30.4747	-85.9988
tcar057	FL	Walton	30.6062	-85.9417
tcar059	FL	Walton	30.4537	-86.0426
tcar060	FL	Gadsden	30.5863	-84.7140
tcar061	FL	calhoun	30.4354	-85.3718
tcar062	FL	Walton	30.6601	-85.9366
tcar063	FL	Walton	30.4770	-86.0553
tcar064	FL	Walton	30.5555	-85.9599
tcar065	FL	Walton	30.4755	-86.0131
tcar066	FL	calhoun	30.4432	-85.0314
tcar067	FL	Walton	30.4665	-85.9449
tcar100	FL	Alachua	29.7060	-82.3990
tcar101	FL	Alachua	29.7060	-82.3990
tcar102	FL	"panhandle"	-	-
tcar103	KY	Breckenridge	37.6614	-86.5994
tcar104	KY	Breckenridge	37.6614	-86.5994
tcar105	KY	Breckenridge	37.6614	-86.5994
tcar106	FL	St. Johns	30.0541	-81.5153
tcar107	FL	St. Johns	30.0541	-81.5153
tcar108	FL	Okaloosae	30.6246	-86.7667
tcar109	FL	Okaloosae	30.5263	-86.4976
tcar110	FL	Gulf	29.8524	-85.3357
tcar111	FL	Holmes	30.9643	-85.9666
tcar112	FL	Hernando	28.5930	-82.3708
tcar113	FL	Escambia	30.5696	-87.3996
tcar114	FL	Hendry	26.5963	-80.9826
tcar115	FL	Gulf	29.8140	-85.2957
tcar116	FL	Gulf	29.8140	-85.2957
tcar117	KY	Todd	36.6467	-87.1726
tcar118	NC	Chatham	35.5907	-78.9109
tcar120	KY	Hart	37.2403	-86.0087
tcar121	KY	Hart	37.2464	-85.9961
tcar122	KY	Lyon	37.0806	-88.0737

Table B-2. Continued

id	St	County	Lat	Long
tcar123	NC	Avery	35.8589	-82.0385
tcar124	MS	Scott	32.3637	-89.5646
tcar125	VA	Shenandoah	38.9376	-78.4523
tcar126	VA	Surry	37.0333	-76.9574
tcar127	NC	Craven	34.9324	-77.0738
tcar128	AL	Sumter	32.8160	-88.1660
tcar129	NJ	Cumberland	39.2891	-74.7408
tcar130	VA	Prince Edward	37.2131	-78.4438
tcar131	VA	Prince Edward	37.1471	-78.3327
tcar132	IL	Johnson	37.4056	-88.9516
tcar133	KS	Linn	38.2626	-94.7029
tcar134	KS	Linn	38.2626	-94.7029
tcar135	MO	Bates	38.2560	-94.5330
tcar136	MO	Boone	38.8763	-92.2544
tcar137	KS	Cowley	37.1071	-96.5719
tcar138	KS	Chatauqua	37.0018	-96.3030
tcar139	OK	Cherokee	35.9785	-95.1369
tcar140	OK	McCurtain	33.8449	-94.7916
tcar141	FL	Franklin	29.9358	-84.3422
tcar142	KS	Crawford	37.6463	-94.8250
tcar143	KS	Crawford	37.6446	-94.8134
tcar144	KS	Bourbon	38.0084	-94.7670
tcar145	FL	Gulf	29.9016	-85.0887
tcar146	FL	Gulf	29.8714	-85.2319
tcar147	FL	Franklin	29.7840	-84.9106
tcar148	FL	Gulf	29.8064	-85.2918
tcar149	FL	Gulf	29.8912	-85.0720
tcar150	FL	Calhoun	30.5825	-84.9913
tcar151	FL	Calhoun	30.4982	-85.0420
tcar152	FL	Calhoun	30.2848	-85.2303
tcar153	FL	Walton	30.6969	-85.9935
tcar154	FL	Alachua	29.6813	-82.3533
tcar155	VA	Floyd	37.1046	-80.1298
tcar156	FL	Liberty	30.0588	-85.0200
tcar157	FL	Jefferson	30.1901	-84.0548

APPENDIX C
MTDNA HAPOTYPES AND MICROSATELLITE ALLELES

Table C-1. Variable base pair localities in mtDNA haplotypes

	Base Pair Location																			
HAP	3	6	22	46	67	77	80	84	87	92	96	104	120	131	132	138	169	193	215	254
1	G		C	T	T	A	A		A	C	C	A	T	G	T	G	A	A	C	A
2	.	C	T	.	C	.	.	.	G	.	T	T	.	A	C	T	G	.	T	G
3	.	C	T	.	.	.	.	.	G	T	T	T	.	A	C	T	G	.	T	G
4	.	C	T	.	.	.	.	.	G	.	T	T	.	A	C	T	G	.	T	G
5	T	.	.	.	.	.	.	.	.	.	.	T	C	.	.	.	G	G	T	.
6	T	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	G	G	T	.
7	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
8	T	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	G	G	T	.
9	T	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	G	.	T	.
10	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
11	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
12	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	.	T	.
13	T	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	G	G	T	.
14	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
15	T	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	G	G	T	.
16	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
17	T	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	G	G	T	.
18	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
19	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
20	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
21	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
22	T	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	G	G	T	.
23	T	.	.	.	.	.	.	.	G	.	.	T	C	.	.	.	G	G	T	.
24	T	.	.	.	C	.	.	G	G	.	.	.	.	.	.	.	G	.	.	.
25	T	.	.	.	.	.	G	.	.	T	.	T	.	.	.	.	G	.	.	.
26	T	.	.	.	.	.	G	.	.	T	.	T	.	.	.	.	G	.	.	.
27	T	.	.	.	.	.	G	.	.	T	.	T	.	.	.	.	G	.	.	.
28	T	.	.	.	.	.	G	.	G	T	.	T	.	.	.	.	G	.	.	.

Table C-1. Continued

	Base Pair Location																			
HAP	3	6	22	46	67	77	80	84	87	92	96	104	120	131	132	138	169	193	215	254
29	T	.	.	.	.	.	G	.	.	T	.	T	.	.	.	.	G	.	.	.
30	T	.	.	.	.	.	G	.	.	T	.	T	.	.	.	.	G	.	.	.
31	A	.	.	.	.	.	.	.	G	T	.	T	.	.	.	.	G	.	T	.
32	A	.	.	.	.	G	.	.	G	T	T	T	.	.	.	.	G	.	.	.
33	A	.	.	.	.	.	.	.	G	T	.	T	.	.	.	.	G	.	.	.
34	A	.	.	.	.	.	.	.	G	T	.	T	.	.	.	.	G	.	.	.
35	A	.	.	.	.	G	.	.	G	T	T	T	.	.	.	.	G	.	.	.

Table C-1. Continued

	Base Pair Location																		
HAP	274	277	286	331	332	333	334	336	337	345	372	396	397	400	412	417	419	420	421
1	A	A	C	C	T	T	A	T	A	T	A	G	C	G	G	T	T	G	A
2	.	.	T	T	C	.	.	C	.	.	.	.	.	A	.	C	C	A	.
3	.	.	.	T	C	.	.	.	.	C	.	.	.	A	.	C	C	A	.
4	.	.	T	T	C	.	.	C	.	.	.	.	.	A	.	C	C	A	.
5	G	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
6	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
7	G	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	.	.	G
8	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
9	.	G	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
10	G	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
11	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
12	G	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
13	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
14	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
15	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
16	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
17	G	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
18	G	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
19	.	.	.	.	.	.	.	.	.	C	.	A	T	A	A	C	C	A	G
20	G	.	.	.	.	.	.	C	.	C	.	A	T	A	A	C	C	A	G
21	G	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
22	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
23	.	.	.	.	.	.	G	.	.	C	.	A	T	A	A	C	C	A	G
24	.	.	.	.	.	.	.	.	.	C	.	A	T	.	.	.	.	A	G
25	.	.	.	.	.	.	.	C	.	.	.	A	T	.	A	C	.	A	G
26	.	.	.	.	.	.	.	C	.	.	.	A	T	.	A	C	.	A	G
27	.	.	.	.	.	.	.	C	.	.	.	A	T	.	A	C	C	A	G
28	.	.	.	.	.	.	.	C	.	.	.	A	T	A	A	C	.	A	G
29	.	G	.	.	.	.	.	C	.	.	.	A	T	.	A	C	.	A	G

Table C-1. Continued

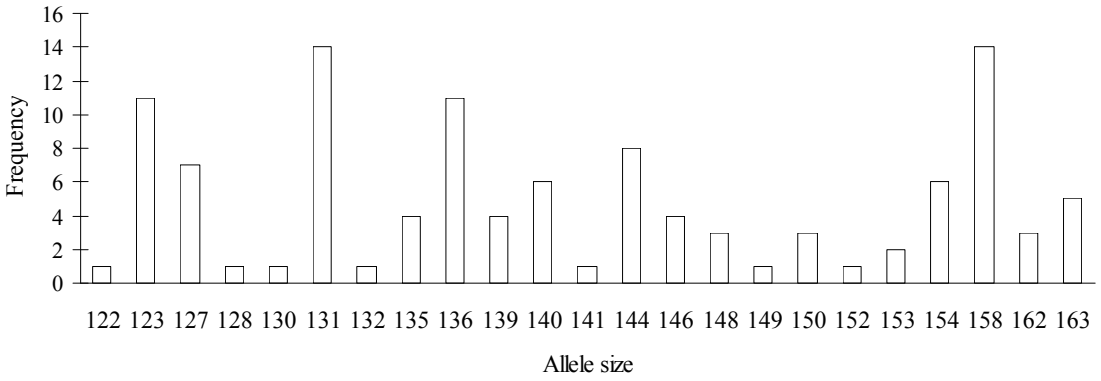
	Base Pair Location																		
HAP	274	277	286	331	332	333	334	336	337	345	372	396	397	400	412	417	419	420	421
30	.	.	.	.	.	.	.	C	.	.	.	A	T	.	A	C	.	A	G
31	.	.	.	.	.	.	.	.	.	C	G	A	T	.	.	C	.	A	.
32	.	.	.	.	.	A	.	.	G	C	G	A	T	.	.	.	.	A	.
33	.	.	.	.	.	.	.	.	G	C	G	A	T	.	A	C	.	A	.
34	.	.	.	.	.	.	.	.	G	C	G	A	T	.	.	C	.	A	.
35	.	.	.	.	.	.	.	.	G	C	G	A	T	.	.	.	.	A	.

Table C-1. Continued

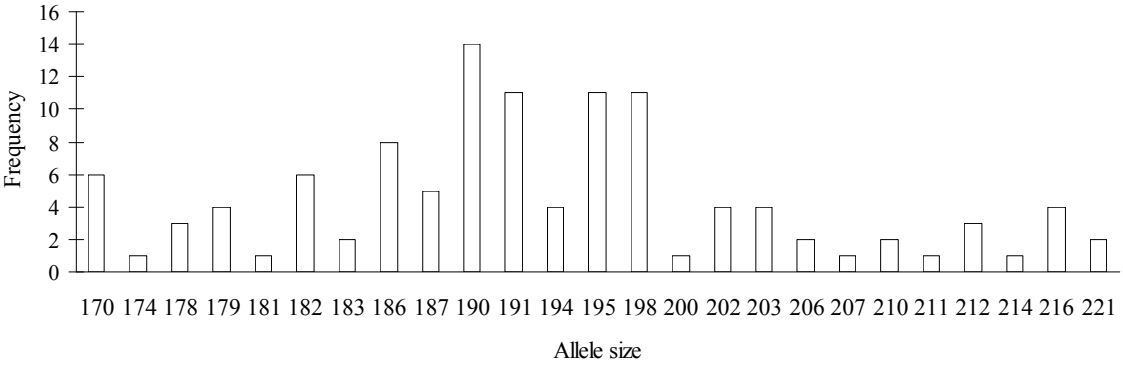
	Base Pair Location																	
HAP	431	454	460	462	463	470	472	482	491	509	510	511	519	520	521	522	526	527
1	G	G	T	T	T	T	G	T	C	T	G	A	A	T	G	T	T	A
2	A	A	.	.	C	.	.	.	T	.	.	.	.	.	.	A	C	.
3	A	A	.	.	C	.	.	.	T	.	.	.	.	.	.	A	C	.
4	A	A	.	.	C	.	.	.	T	.	.	.	.	.	.	A	C	.
5	A	.	.	C	.	.	.	.	.	C	A	.	G	.	.	.	.	.
6	A	.	.	C	.	C	.	.	.	C	A	.	.	.	.	.	.	.
7	A	.	.	C	.	.	A	.	.	C	A	.	.	.	.	.	.	.
8	A	.	.	C	.	C	.	.	.	.	A	.	.	.	.	.	.	.
9	A	.	.	C	.	C	.	.	.	.	A	.	.	.	.	.	.	.
10	A	.	.	C	.	C	.	.	.	C	A	.	.	.	.	.	.	.
11	A	.	.	C	.	C	.	.	.	C	A	.	.	.	.	.	.	.
12	A	.	.	C	.	.	A	.	.	C	A	C	.	.	.	.	.	.
13	A	.	.	.	.	C	.	.	.	C	A	.	.	.	.	.	.	.
14	A	.	.	.	.	C	.	.	.	C	A	.	.	.	.	.	.	.
15	A	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.
16	A	.	.	.	.	.	.	.	.	C	A	G	.	C	.	.	.	.
17	A	.	.	C	.	C	.	.	.	C	A	.	.	.	.	.	.	.
18	A	.	.	C	.	.	.	C	.	C	A	.	.	.	.	.	.	.
19	A	.	.	C	.	C	.	.	.	C	A	.	.	.	.	.	.	.
20	A	.	.	C	.	.	.	.	.	C	A	.	.	.	.	.	.	.
21	A	.	.	C	.	.	.	.	.	C	A	.	.	.	.	.	.	.
22	A	.	.	C	.	C	.	.	.	.	A	.	.	.	.	.	.	.
23	A	.	.	C	.	C	.	.	.	C	A	.	.	.	.	.	.	.
24	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
25	A	.	C	.	.	.	.	.	.	C	A	C	.	.	.	.	.	.
26	A	.	C	.	.	.	.	.	.	C	A	C	.	.	.	.	.	.
27	A	.	C	.	.	.	.	.	.	C	A	C	.	.	.	.	.	.
28	A	.	C	.	.	.	.	.	.	C	A	C	.	.	.	.	.	.
29	A	.	C	.	.	.	.	.	.	C	A	C	.	.	.	.	.	.

Table C-1. Continued

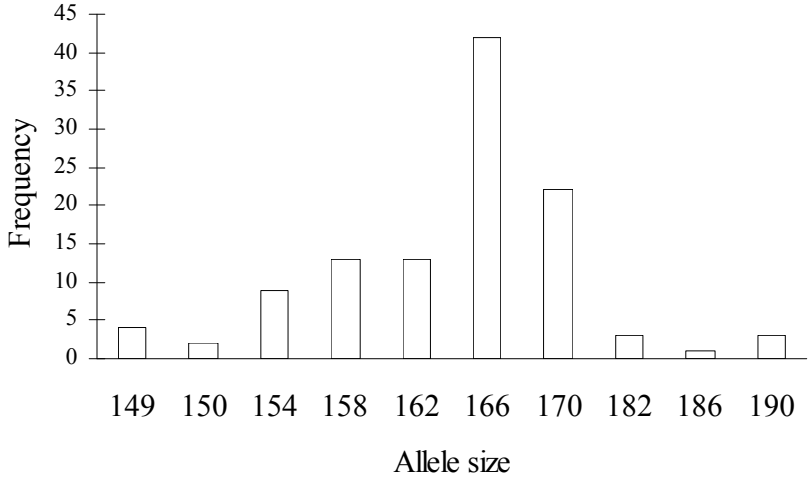
	Base Pair Location																
HAP	431	454	460	462	463	470	472	482	491	509	510	511	519	520	521	522	526	527
30	A	.	C	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.
31	A	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	.
32	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G
33	A	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	.
34	A	.	.	.	.	.	A	.	.	.	.	.	G	.	A	.	.	.
35	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.



A

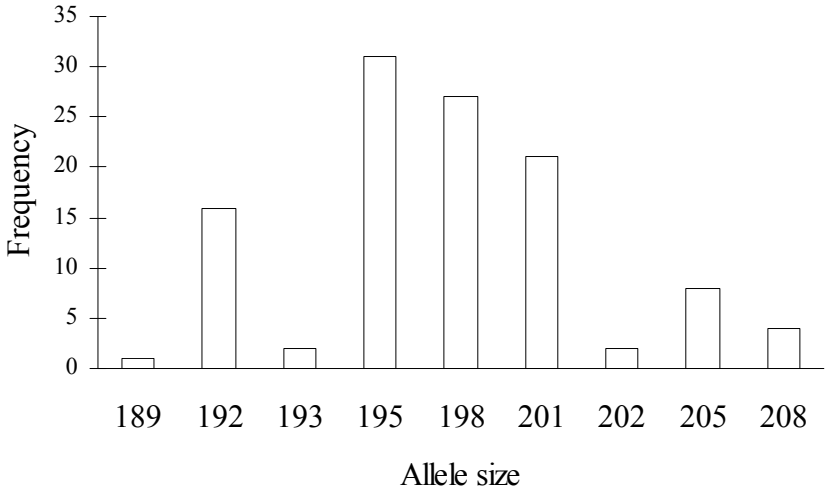


B

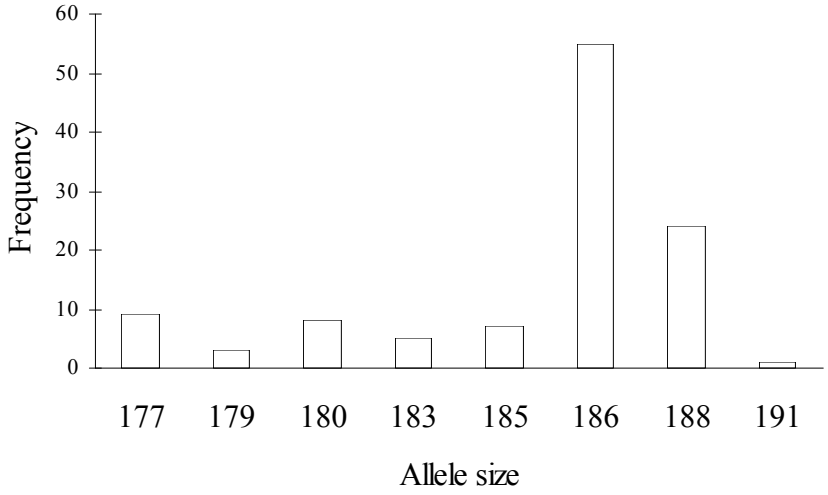


C

Figure C-1. Microsatellite allele frequencies. A) GmuD121, B) GmuD55, C) GmuD21, D) GmuB08 and E) GmuB12.



D



E

Figure C-1. Continued

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BIOGRAPHICAL SKETCH

Jason Michael Butler was born in 1981, in Harford Kentucky. The oldest of four children, he grew up mostly in Philpot, Kentucky. He earned his B.S. in biology at Western Kentucky University in 2005. He began working toward a M.S. in wildlife ecology and conservation in 2006.

After completing his master's program, Jason will return to Kentucky to pursue a career in conservation and ecological research. Jason has been married to Sara N. Moore Butler for two years.