

## ABSTRACT

Matthew F. Osentoski. mtDNA VARIATION IN THE GOPHER TORTOISE, Gopherus polyphemus. (Under the direction of Dr. Trip Lamb) Department of Biology, December, 1993.

The slow rate of mtDNA evolution in turtles poses a limitation on the levels of intraspecific variation detectable by conventional restriction fragment surveys. Here I explore an alternative assay for turtle evolutionary genetics, in which PCR-amplified regions of the mitochondrial genome are restricted using endonucleases with tetranucleotide recognition sites. The PCR-restriction approach was used to examine genetic variation in the gopher tortoise, *Gopherus polyphemus*. Restriction fragment polymorphisms, representing four amplified regions of the mitochondrial genome, were used to assess population genetic structure of the tortoise range-wide. Eight restriction endonucleases were used to survey 112 tortoise (55 localities), distinguishing a total of 36 mtDNA genotypes. Both UPGMA and parsimony analyses identified three major geographic assemblages: Eastern, Western, and Mid-Florida. The Eastern and Western assemblages bisect the species' range in the vicinity of the Apalachicola-Chattahoochee drainages, whereas the smaller Mid-Florida assemblage is associated largely with the Brooksville Ridge. The observed east-west break is roughly concordant with other distributional and genetic (nuclear and mitochondrial) studies that have been conducted in the Southeast. Both the Eastern/Western and Mid-Florida patterns may have been shaped

by historic eustatic sea level fluctuation. Overall, compared to conventional methodology, the PCR-restriction approach offers promise for revealing intraspecific variation in turtles.



mtDNA VARIATION  
IN THE  
GOPHER TORTOISE, Gopherus polyphemus

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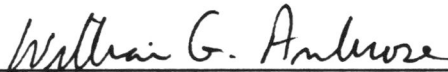
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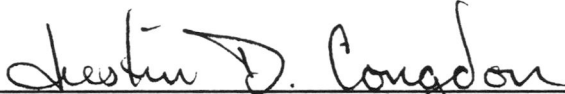
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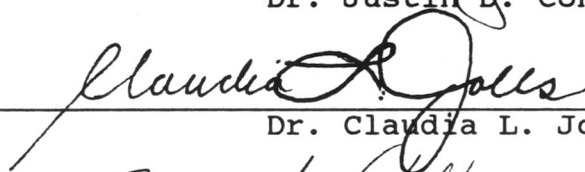
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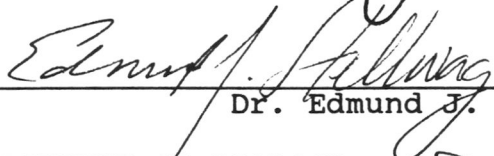
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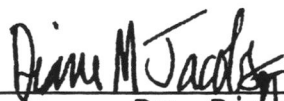
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## TABLE OF CONTENTS

|                                                                                              |    |
|----------------------------------------------------------------------------------------------|----|
| List of Figures and Tables.....                                                              | iv |
| Introduction .....                                                                           | 1  |
| Mitochondrial DNA and Its Role in Population and<br>Evolutionary Genetics.....               | 1  |
| MtDNA and Intraspecific Phylogeography.....                                                  | 1  |
| Methods of mtDNA Analysis.....                                                               | 9  |
| Assessing Intraspecific mtDNA Variation in Turtles:<br>Limitations and Future Prospects..... | 13 |
| The Gopher Tortoise, <i>Gopherus polyphemus</i> .....                                        | 14 |
| Research Objectives.....                                                                     | 20 |
| Materials and Methods.....                                                                   | 21 |
| Tortoise Collection.....                                                                     | 21 |
| MtDNA Amplification and Restriction Assay.....                                               | 21 |
| Data Analysis.....                                                                           | 23 |
| Results.....                                                                                 | 25 |
| Identification and Diversity of Haplotypes.....                                              | 25 |
| Geographic Variation.....                                                                    | 26 |
| Discussion.....                                                                              | 29 |
| The PCR-Restriction Approach.....                                                            | 29 |
| Southeastern Regional Biogeography.....                                                      | 31 |
| Geologic Background of the Southeastern United<br>States.....                                | 31 |
| Evidence for an East-West Break and Its Bearing<br>on the Tortoise mtDNA Data.....           | 32 |
| Biogeography of the Florida and Its Bearing<br>on the Tortoise.....                          | 36 |
| Summary.....                                                                                 | 39 |
| References.....                                                                              | 41 |

|                                                           |    |
|-----------------------------------------------------------|----|
| Figures.....                                              | 49 |
| Tables.....                                               | 59 |
| Appendix A: List of Collection Localities.....            | 64 |
| Appendix B: Methylene Chloride DNA Extraction Protocol... | 66 |
| Appendix C: Amplification Conditions.....                 | 68 |
| Appendix D: Restriction Profiles.....                     | 69 |

## LIST OF FIGURE AND TABLES

### FIGURES

|                                                                                                                      |    |
|----------------------------------------------------------------------------------------------------------------------|----|
| 1. Map of Sampling Localities.....                                                                                   | 49 |
| 2a. Photograph of Wire Box Trap Placement.....                                                                       | 50 |
| 2b. Photograph of Blood Extraction Technique.....                                                                    | 50 |
| 3. The Mitochondrial Genome.....                                                                                     | 51 |
| 4. UPGMA Phenogram.....                                                                                              | 52 |
| 5. Map Depicting Geographic Distribution of mtDNA<br>Clones.....                                                     | 53 |
| 6a. Parsimony Network Exclusive of Alabama Individuals..                                                             | 54 |
| 6b. Parsimony Network Including Alabama Individuals.....                                                             | 55 |
| 7a. UPGMA Phenograms of Data Exclusive of Cyt. <i>b</i> /D-loop<br>and NADH5/NADH6 Data.....                         | 56 |
| 7a. UPGMA Phenograms of Data Exclusive of NADH2/CO I<br>and 12S/16S rRNA Data.....                                   | 57 |
| 8. Maps Depicting the Geographic Arrangement of mtDNA<br>Clones in the Pocket Gopher and the Gopher<br>Tortoise..... | 58 |

### TABLES

|                                                                                 |    |
|---------------------------------------------------------------------------------|----|
| 1. Estimates of Base Substitutions Per Nucleotide Among<br>mtDNA Genotypes..... | 59 |
| 2. Composite mtDNA Genotypes.....                                               | 61 |
| 3. Summary of Turtle Phylogeographic Studies.....                               | 63 |

## INTRODUCTION

### *Mitochondrial DNA and Its Role in Population and Evolutionary Genetics*

*mtDNA and intraspecific phylogeography.* --- Traditionally, genetic studies of microevolution (e.g., intraspecific phylogenies) have relied on variability of nuclear encoded proteins, typically in the form of allozymes. Unfortunately, allozyme loci represent qualitative, multi-state traits from which phylogenetic order (at intraspecific or higher levels) can be difficult to infer. When additional complications posed by segregation and recombination are considered, the allozyme approach favors a population genetic perspective (allelic frequencies, heterozygosities) over an evolutionary (phylogenetic) one. Alternatively, a genetic system ideally suited for intraspecific phylogenetic studies should: 1) exhibit a mode of inheritance without recombination or genetic rearrangements; 2) lack introns, pseudogenes, and repetitive or transposable elements; and 3) evolve rapidly such that extensive genetic polymorphism arises within the lifespan of a species (Avice et al., 1987a). In many respects, mitochondrial DNA (mtDNA) meets these criteria.

The animal mitochondrial genome is a small (16,000 to 22,000 bp) covalently closed, circular molecule. The genome is highly conserved with regard to gene content and gene arrangement, encoding 22 tRNAs, two rRNAs, and 13 protein

subunits that function in the electron transport chain. Additionally, the molecule contains a non-coding control region called the D-loop; some 800 base pairs in length, the D-loop contains the origin of heavy strand replication. Pseudogenes, introns, and sizeable spacer sequences commonly found in nuclear DNA are altogether absent in mtDNA. Several reviews outlining the general structure and function of the mitochondrial genome have been published (Awise and Lansman, 1983; Brown, 1983; Brown, 1985).

At the nucleotide level, animal mtDNA evolves 1-10 times more rapidly than single copy nuclear DNA (scnDNA; Awise et al., 1987a) averaging an estimated 2% sequence divergence per million years (Brown et al., 1979). Awise (1986) proposed two hypotheses to account for the rapid rate of mtDNA evolution: 1) an enhanced mutation rate; and 2) relaxed selective constraints on the molecule's functional products. Neither hypothesis has been strongly supported, but circumstantial evidence suggests that mtDNA has a less stringent repair mechanism than nuclear DNA. Inefficient proofreading is implicated by the large number of simple base substitutions (with transitions greatly outnumbering transversions), the molecule exhibits a relatively small number of additions/deletions and even fewer large length variants (Moritz et al., 1987). Contrary to Awise's (1986) second hypothesis, mtDNA gene products do appear to be under strong selective pressure, as they are involved in ATP



production. Evidence for such functional constraint is apparent in the patterns of codon mutation. The frequency of base substitutions by codon position varies as follows: third > first > second. Third position changes are the least likely to cause amino acid replacement than are first position changes. Similarly, first position changes are less likely to cause amino acid replacement than are second position changes, thus implicating selection against amino acid replacement. Overall, the mtDNA molecule exhibits an accelerated, yet predictable, rate of sequence evolution.

Several studies have indicated that different regions within the mtDNA genome evolve at different rates (Fauron and Wolstenholme, 1980; Aquadro and Greenberg, 1983; Greenberg et al., 1983; Kocher et al., 1989). The most rapidly evolving regions include the control region (D-loop) and small intergenic spacers. Ribosomal RNAs exhibit an intermediate rate of evolution, whereas the tRNAs show a high degree of conservation; the protein coding regions exhibit varying degrees of conservation (Harrison, 1989). As noted previously, the high level of conservation observed among coding regions is likely attributable to strong selective pressure on their functional products.

Initially, geneticists expressed some concern that the large number of mitochondria per cell would yield multiple mtDNA genotypes within a given individual, a condition known as heteroplasmy. Mutations within a cell line generate the

majority of heteroplasmic cases (as opposed to "paternal leakage," see below), though the heteroplasmic condition is typically quite transitory due to rapid sorting of mtDNA molecules in germ cell lineages. Heteroplasmy has been documented in several major groups (Awise et al., 1987a), and is often observed at relatively low frequencies in lower vertebrates (Bermingham et al., 1986) and invertebrates (Boyce et al., 1989). Most individuals appear homoplasmic, however, while population surveys detecting heteroplasmic individuals have rarely encountered problems analyzing or interpreting their data. Heteroplasmy resulting from "paternal leakage" (paternal mtDNA invading the egg during fertilization) has been documented (Gyllenstein et al., 1991) but does not seem to be very common (detected paternal mtDNA at a frequency of  $10^{-4}$ ).

Indeed, one of the principal features that makes mtDNA such a powerful phylogenetic tool is its maternally mediated mode of inheritance. Cytoplasmic organelles, and hence mtDNA, are contributed to the offspring via the egg; neither recombination with paternal DNA nor rearrangement of the mitochondrial genome occurs. As a consequence, non-deleterious mutations are maintained within the maternal lineage. Each lineage can be considered a maternal "pedigree" or genealogy that contains a record of the mtDNA's mutational history. Mitochondrial DNA lineages can be traced back hundreds if not thousands of generations,

allowing mtDNA genotypes to be viewed (and analyzed) in a phylogenetic context. Often, the terminal groups of mtDNA phylogenies display a distinct geographic arrangement, a phenomenon termed *intraspecific phylogeography* (Avice et al., 1987a).

One significant aspect of maternal inheritance is that mtDNA genotypic frequencies are necessarily influenced by a specific set of population parameters, e.g., female survivorship, the number of female offspring, etc. As such, rare mtDNA genotypes can be lost much more quickly under this uniparental mode of inheritance than would be rare nuclear alleles under a biparental inheritance mode. Through the stochastic loss of rare mtDNA lineages, populations are often characterized by one or a few mtDNA genotypes. Neigel and Avice (1986) examined this phenomenon using computer simulations in an attempt to estimate the time required for various-sized populations to reach mtDNA monophyly. They concluded that for stable populations with initial sizes of  $N$  females or a carrying capacity near or equal to  $N$ , there was a high probability that within  $4N$  generations all descendants could be traced to a single founding female. Additionally, this same phenomenon can be applied to geographic isolation, where the number of generations required for the isolated populations to become genetically distinct is 2-4 times their effective population size (Neigel and Avice, 1986). As a whole, stochastic

lineage sorting simplifies the assessment of phylogeographic patterns by limiting genotypic diversity within populations while enhancing the effects of significant isolation events.

Avise et al. (1987a) identified five phylogeographic outcomes that could arise, in theory: I- phylogenetic discontinuities with spatial separation; II- phylogenetic discontinuities without spatial separation; III- phylogenetic continuity with spatial separation; IV- phylogenetic continuity without spatial separation; and V- phylogenetic continuity with partial spatial separation. These phylogenetic outcomes are outlined below.

Empirically, category I, phylogenetic discontinuities with spatial separation, is the most common pattern encountered. It is exemplified by arrays of distantly related mtDNA genotypes separated from each other by long-term zoogeographic barriers. Long-term barriers create periods of reduced gene flow sufficient to allow two isolated populations to attain reciprocal monophyly (loss of shared ancestral genotypes). Species exhibiting this pattern include the southeastern pocket gopher, *Geomys pinetis*, (Avise et al., 1979), three freshwater fish (Bermingham and Avise, 1986), and the desert tortoise, *Gopherus agassizii* (Lamb et al., 1989). In each case, genotypic assemblages are clearly allopatric and exhibit pronounced levels of mtDNA sequence divergence.

Category II, phylogenetic discontinuities without

spatial separation, has no empirical documentation to date. This anomalous genetic pattern requires the evolution and maintenance of two distinct, sympatric groups of genotypes. Although unlikely, genetically distinct groups of haplotypes in sympatry could be realized during a transitory period of admixture between two recently reunited groups, or during the spontaneous occurrence of intrinsic reproductive barriers within a species.

The third category, phylogenetic continuity with spatial separation, is characterized by little to no genotypic variation among geographically disjunct populations. Genetic panmixis in geographically disjunct populations is best exemplified by the oyster toadfish, *Opsanus tau*, which maintains a series of slightly variant (though not significantly different with estimated sequence divergence ( $p$ ) < 0.008) mtDNA clones geographically dispersed along the Atlantic coast from Massachusetts to Georgia (Avice et al., 1987b). It is presumed that genetic continuity is maintained by frequent disruption and reestablishment of gene flow among toadfish populations. Similar patterns have been documented for the diamondback terrapin, *Malaclemys terrapin* (Lamb and Avice, 1992) and old field mouse, *Peromyscus polionotus* (Avice et al., 1983).

Phylogenetic continuity with no spatial separation, or genotypic panmixis, is the fourth category. This panmictic pattern is most evident in species exhibiting high levels of

gene flow. Genotypic panmixis was detected in the Red-winged Blackbird, *Agelaius phoeniceus* (Ball et al., 1988) and certain coastal marine species (Avice et al., 1987b), whose free-ranging habit permit a high level of mixing throughout the range.

The final phylogeographic category, phylogenetic continuity and partial spatial separation, is typified by the co-occurrence of both localized and widespread genotypes. Although it is difficult to determine which situation predominates, this pattern could result from either the recent reuniting of two distinct populations or from intermediate levels of gene flow within a species. Examples of this pattern are rare, but a convincing case can be made for the bowfin, *Amia calva* (Bermingham and Avice, 1986). The bowfin exhibits a widespread "common" genotype and a set of more localized genotypes nested within the range of the common genotype. Bermingham and Avice (1986) suggested that the common genotype is a variant derived from one or more of the more confined genotypes but were unable to confirm this evolutionary scenario.

Patterns of phylogenetic outcome provide some insight to the importance of gene flow and its effect on intraspecific mtDNA phylogenies. As indicated by the strong empirical support, many intraspecific phylogenies exhibit pronounced geographic structure shaped by physiographic or ecological barriers to gene flow. This geographic structure

of genotypes is especially apparent in those species with low vagility. By extension, concordant phylogeographic patterns across several different species can provide researchers with important baseline information concerning regional biogeography (Avice, 1992).

In summary, the advantages of mtDNA analysis in assessing intraspecific phylogeographic patterns are several fold. The molecule's rapid evolutionary rate generally provides extensive genetic polymorphism that can be readily assayed by several molecular techniques. The maintenance of mutational records through maternal inheritance (in combination with stochastic lineage sorting) often generates straight forward patterns for assessing intraspecific phylogeography.

*Methods of mtDNA analysis.* --- Variation in the mitochondrial genome is routinely surveyed by restriction endonuclease assay or, increasingly, by DNA sequencing. The former approach generates restriction fragment length polymorphisms (RFLPs) or restriction site data, whereas the latter approach generates actual sequence data from which base substitutions (transitions and transversions) as well as base additions and deletions can be identified. My study involves restriction site data, which is typically analyzed by one of two fundamentally different approaches -- one quantitative, the other qualitative.

In the quantitative approach, restriction sites are converted to estimates of nucleotide sequence divergence,  $p$ , using the algorithm of Nei and Miller (1990). The  $p$ -value represents a pair-wise genetic distance between two distinct mtDNA genotypes. A  $p$ -value is calculated for all possible genotypic pairings, and these estimates are assembled in a matrix from which the haplotypes are clustered using UPGMA (unweighted pair-group method using arithmetic averages) analysis. UPGMA analysis constructs a tree or "phenogram" by linking the least distant pairs of genotypes (smallest  $p$ -values) first, followed successively by more and more distantly related genotypes. UPGMA maintains only one assumption: all distance values are ultrametric. This is a constrictive assumption since ultrametricity implies that every common ancestor is equidistant from all descendants. In other words, there is a constant rate of sequence evolution across lineages. However, genes seldom evolve at a uniform rate, and as a result, errors can be generated by UPGMA because the algorithm attempts to fit non-ultrametric data to an ultrametric tree. Although violation of this assumption may distort comparisons, especially those addressing higher level systematics, the analysis can be useful in assessing relationships among less divergent lineages (such as intraspecific studies). However, complications may arise even when the data are ultrametric because finite sampling of the genome may yield statistical



fluctuations in detected variability. The primary drawback for UPGMA analysis is not ultrametricity per se; rather it is that the algorithm lacks objective criteria for an optimal tree should the data fail to meet ultrametricity. As such, it is impossible to evaluate distortion attributable to non-ultrametric data sets. UPGMA is best used in a comparative context with other independent methods of analysis.

In the qualitative analysis, restriction sites are scored as either present (1) or absent (0) for each genotype. A presence/absence matrix generated for all genotypes is then used as input data and analyzed using the method of maximum parsimony. An "outgroup" is generally used, which provides tree direction by polarizing restriction sites as either ancestral or derived. The resulting tree, rooted by the outgroup, allows one to infer relationships among haplotypes examined relative to the ancestral outgroup. In the absence of an outgroup, the tree remains unrooted and is simply termed a "network." Nonetheless, a network is often informative regarding relative relationships among haplotypes.

Unlike a UPGMA phenogram, the integrity or strength of branching patterns in a parsimony network can be evaluated using a bootstrap analysis. For each bootstrap replicate, a new data set is randomly created by sampling with replacement from the original set of genotypes. Thus, the

new data set may be identical to the original or may contain multiple representatives of a given genotype. Typically, the data are subjected to 100 bootstrap iterations, for which each bootstrap replication data set is analyzed, and the resultant trees are compared. The percent occurrence of a given branching pattern can be calculated, where the higher the percentage, the stronger the support for a branch identifying an assemblage of genotypes.

*Assessing Intraspecific mtDNA Variation in Turtles: Current  
Limitations and Future Perspectives*

Surveys of intraspecific mtDNA variation have been conducted for several turtle species (desert tortoise, Lamb et al., 1989; Kemp's and olive ridleys, Bowen et al., 1991; green turtle, Bowen et al., 1992; diamondback terrapin, Lamb and Avise, 1992; pond slider and map turtle, Avise et al., 1992). A notable feature shared among these restriction-site studies is the limited number of mtDNA haplotypes uncovered and the unusually low genetic variability detected within each species. For example, Atlantic and Pacific populations of olive ridley (*Lepidochelys olivacea*), separated by 25,000 km of ocean, displayed no sequence variability among some 80 assayed restriction sites (Bowen et al., 1991). Similarly, only one mtDNA haplotype was detected in a range-wide survey of the map turtle, *Graptemys geographica* (Avise et al., 1992). Even studies, such as the desert tortoise, which did reveal substantive variation between major geographic regions found negligible variation within geographic regions (Lamb et al., 1989). Comparing intraspecific variation of turtle species with that of non-turtle species, Avise et al. (1992) compiled evidence suggesting an mtDNA evolutionary rate 2-14 times slower in turtles relative to other higher animal lineages. The reduced microevolutionary rate observed in turtle mtDNA could account for the limited intraspecific variability

detected by conventional restriction assays (6-base) of the entire mtDNA genome.

A primary aim of my research was to develop an alternative restriction assay for turtle mtDNA in an attempt to disclose population genetic variation in an otherwise slow-evolving genome. I used the polymerase chain reaction (PCR) to amplify specific regions of the mitochondrial genome of the gopher tortoise (*Gopherus polyphemus*). I surveyed variation within each region using 4-base restriction endonucleases to enhance fragment generation and, ostensibly, detectable genetic variability. The effectiveness of the PCR-restriction approach was assessed by comparing the intraspecific variation resolved for the gopher tortoise relative to conventional restriction surveys of other turtles.

### ***The Gopher Tortoise, Gopherus polyphemus***

The gopher tortoises (genus *Gopherus*) are a distinctly North American lineage of testudinid turtles characterized by structural specialization for digging and burrowing. With four living species and nine fossil taxa, the group has a remarkably complete fossil record, which reveals an early Oligocene origin for these tortoises, ostensibly derived from the Eocene genus *Stylemys* (Auffenberg, 1976; Bramble, 1971). Though the sister taxon status of *Stylemys* is generally accepted, considerably less consensus exists

regarding evolutionary relationships among the gopher tortoises themselves (Auffenberg, 1976; Bramble, 1982; Crumly, 1985; 1993). Traditionally, all recent and fossil taxa were assembled under a single generic heading, *Gopherus*, though two complexes were apparent osteologically (Auffenberg, 1976). Compiling additional morphological and paleontological evidence, Bramble (1982) argued for generic separation, differentiating the more specialized, fossorial species (*Gopherus*: *G. polyphemus*, gopher tortoise; *G. flavomarginatus*, Bolson tortoise) from the generalized lineage *Scaptochelys* (= *Xerobates*; Bour and Dubois, 1984: *X. agassizii*, desert tortoise; *X. berlandieri*, Texas tortoise). Both Bramble (1982) and Crumly (1993) point to *G. brevisterna* from the early Middle Miocene as the probable inceptive lineage of *Gopherus sensu stricto*. UPGMA and Wagner parsimony dendrograms generated from the mtDNA restriction fragment polymorphisms (RFLPs) were consistent with Bramble's classification for extant species (Lamb et al., 1989). Additional sequence data has been compared in a cladistic study fully supporting this relationship (Lamb and Lydeard, submitted).

The only extant representative of *Gopherus* east of the Mississippi River is the gopher tortoise, *G. polyphemus*. Gopher tortoise range from northeastern Louisiana east across southern Mississippi, Alabama, and Georgia, and southward into Florida (excluding the Everglades) (Auffenberg

and Franz, 1982). Two extremely localized populations occur in South Carolina (Jasper county) just north of the Savannah River; nonetheless they are considered natural, not introduced, populations and are probably relicts of a historical range that extended to the coast (Auffenberg and Franz, 1982).

The gopher tortoise is a habitat specialist restricted largely to upland sandhill communities. These open habitats provide many of the tortoise's requirements including sandy soils (>1 m in depth) that facilitate burrowing, low-growing herbaceous forage, open nest sites, and partial shade. Historically, the upland communities were dominated by longleaf pine stands, but tortoises will opportunistically occupy any sandy habitat sufficiently open to support low growing forbs. Suitable alternative habitats include cultivated forest stands, xeric hammocks, pine flatwoods, dry prairies, sand pine scrub, and mixed hardwood-pine communities (Auffenberg and Franz, 1982; Deimer, 1986).

The gopher tortoise exhibits a "classic" turtle life history: late sexual maturity, low fecundity, and long life span. Age at sexual maturity can range from 10-15 years in the southern portion of the range to 16-21 years in more northern locales (Georgia; Iverson, 1980; Landers and Garner, 1981). The mating season extends from April to early June, and nest construction generally occurs from mid-May to mid-June (Iverson, 1980). Clutch size is small

rangewide; tortoises produce a single clutch averaging five to seven eggs annually that incubate in the nest for 80-110 days (Iverson, 1980). During this period, predation is high; nest predation rates up to 87% have been recorded in southern Georgia (Landers et al., 1982). Even after hatching, hatchlings and juveniles remain susceptible to avian, reptilian, and mammalian predators until their shells become ossified (5-7 years). Once shells become ossified, individuals may live more than 40-60 years (Deimer, 1986). The low reproductive potential that results from this suite of life history characters is susceptible to heavy or sustained environmental perturbations, which can lead to rapid population declines and, consequently, slow recovery rates.

Though formerly quite common, the gopher tortoise has been extirpated from portions of its range, and many of the remaining populations are in a state of decline. Auffenberg and Franz (1982) estimated that gopher tortoise numbers have been reduced by 80% in the last century. The extreme eastern and western portions of the range contain only relictual populations. Some contiguous populations remain in Georgia, Alabama, and Mississippi though the tortoise is still fairly widespread in Florida (Auffenberg and Franz, 1982).

The most profound threat to the tortoise is irreversible habitat loss, resulting from intensive land

alteration for housing, industrial centers, broad-scale agriculture, intensive forest management, phosphate mining, and sand extraction (Deimer, 1989). These practices often kill some tortoises directly, but more importantly, they permanently destroy tortoise habitat. Even when habitat is preserved, its suitability will be slowly degraded without proper fire management. Periodic burning of natural longleaf/pine-scrub oak communities facilitates canopy opening and reduces ground cover, factors that promote tortoise food production and nest site availability. Landers and Buckner (1981) demonstrated that when burning was excluded from these habitats for eight or more years, resident tortoise populations were reduced by 60-80%.

An additional impact on tortoise numbers has been consumption by humans. The tortoise has been exploited for over 4,000 years, and, in fact, during the Great Depression provided a major food source for many rural southerners (Taylor, 1982). Although this practice has steadily declined, some states such as Florida allowed legal harvests as recently as 1988. Tortoise harvests for food continues in some portions of the range.

The notable decline of gopher tortoises has prompted recent protection efforts. Louisiana and South Carolina populations have received federal endangered status. The tortoise has federal threatened status in western Alabama and Mississippi (coupled with state endangered status), and



proposed state threatened status in Georgia.

### *Research Objectives*

In this study, I used the PCR-restriction assay approach to analyze mtDNA variation throughout the tortoise's range. I attempted to assess the feasibility of the PCR-restriction approach relative to conventional restriction surveys of other turtle species to determine whether the technique improved genetic resolution. To assess possible historical influences on the tortoise, I also compared levels and patterns of genetic variability in the tortoise to those of other species surveyed in the southeastern United States.

## MATERIALS AND METHODS

*Tortoise collection.* --- One hundred twelve *G. polyphemus* representing 55 localities across the species' range were examined (Fig. 1; specific localities in Appendix A). Tortoises were either captured by hand or in unbaited wire box traps (28" x 15" x 10", Tomahawk Live Trap Company, Tomahawk, WI) set at burrow entrances (Fig. 2a). Animals were processed in the field, where a 1-1.5 ml blood sample was drawn from the scapular vein/brachial artery using a diabetic syringe rinsed with 0.2 M EDTA (as an anticoagulant) (Fig. 2b). Post-puncture bleeding was minimized by applying direct pressure to the forelimb; tortoises were monitored 5-10 min to insure that bleeding had stopped and subsequently released on site. Blood samples were stabilized in 0.5 ml of sodium citrate buffer (ACD solution B; 0.02 M citric acid, 0.04 M sodium citrate, 0.08 M glucose), placed on wet ice, and then stored at -70° C upon return to the laboratory.

*mtDNA amplification and restriction assay.* --- Total genomic DNA was isolated from 40 µl of whole blood using the NaCl/methylene chloride extraction protocol of Hillis and Moritz (1990; Appendix B). Four 1.6-2.2 kb segments, representing slow (12S/16S ribosomal genes), moderate (NADH5/NADH6 genes; NADH2/cytochrome oxidase I), and rapidly (cytochrome *b*/D-loop) evolving regions of the mitochondrial

genome (Brown, 1985), were amplified using PCR (Fig. 3; Appendix C). Amplifications were performed in 85  $\mu$ l of reaction volume containing 1-10 ng of genomic DNA, each dNTP at 0.2 mM, each primer at 1  $\mu$ M, 4 mM  $MgCl_2$ , 8.5  $\mu$ l Cetus 10X PCR buffer II, and 2.15 units of AmpliTaq polymerase. Reactions were amplified for 30-35 cycles, each involving denaturation at 92° C for 1 min, hybridization at 50-60° C for 2 min, and extension at 72° C for 3 min. Primer sequences and optimal cycling conditions for each amplified region are listed in Appendix C.

Amplified products were precipitated in 2x ethanol, rehydrated in 60  $\mu$ l of distilled deionized water, and stored at 4° C for DNA assay. Restriction digests of each amplified region were generated with the following eight restriction endonucleases: *Bst*UI, *Dpn*II, *Hae*III, *Hinf*I, *Hin*PI, *Msp*I, *Rsa*I, and *Taq*<sup>a</sup>I. Given the relatively small size of each region (1.6-2.2 kb), I selected endonucleases with tetranucleotide recognition sequences (exclusive of the pentanucleotide *Hinf*I), thereby increasing the likelihood of fragment generation. Restriction digests were conducted according to the conditions suggested by the supplier (New England Biolabs, Beverly, MA); I used 7  $\mu$ l of rehydrated PCR product and approximately 2 units of enzyme in a total reaction volume of 15  $\mu$ l. Digestion fragments were separated according to molecular weight by electrophoresis through either 9% polyacrylamide (34:1 acrylamide:bis-

acrylamide) or 2% agarose (3:1 Nuseive:agarose; Thevierre, 1992) gels. These two electrophoretic techniques were used to enhance the resolution of smaller fragments (<100 bps) generally produced by tetranucleotide enzymes. Gels were stained in 1  $\mu\text{g/ml}$  ethidium bromide for 10-20 min to allow fragments to be visualized and photographed under UV light. Photography required 8-15 min exposures using Polaroid type 55 pos./neg. film (ASA 50; Chapman and Brown, 1989). Fragment lengths were determined by comparison to a 1-kb molecular weight standard (Bethesda Research Labs) and/or the GelMarker ladder (Research Genetics).

*Data analysis.* --- Each unique restriction profile produced by a given enzyme/region combination was assigned an upper-case letter code. Following convention, the most common profile was designated "C,"; profiles that differed by a single restriction site loss or gain were encoded "B" or "D," respectively. Non-adjacent letter designations represent a more than one site gain/loss relative to the "C" profile. Letter codes were compiled across all regions to create a composite mtDNA genotype or "haplotype" for each individual. In turn, each haplotype was designated by numerical code (e.g., haplotype "1," etc.).

MtDNA clonal diversity was estimated using the approach of Nei and Tajima (1981):

$$G = n(1 - \sum f_i^2) / (n - 1)$$

where  $f_i$  is the frequency of the  $i$ th genotype and  $n$  is the number of individuals surveyed. Genotypic diversity values can range from 0, when all individuals have the same haplotype, to 1.0 when all individuals have unique haplotypes.

The Restriction Enzyme Analysis Package (REAP) computer program (McElroy et al., 1992) was used to estimate nucleotide sequence divergence ( $p$ ) between pairs of haplotypes via the "site" approach of Nei and Miller (1990; eq. 4). The resulting matrix of  $p$ -values (Table 1) was used to construct a UPGMA phenogram (Sneath and Sokal, 1973) using the computer program NTSYS (version 1.70; Rohlf, 1991).

Additionally, parsimony analysis of the mtDNA haplotypes was conducted using the PAUP (Phylogenetic Analysis Using Parsimony) computer program (Swofford, 1993). A data matrix encoding the presence/absence status of each fragment for all haplotypes was generated using the MacClade software package (Maddison and Maddison, 1992) and analyzed using PAUP's heuristic search option, which generates the most parsimonious trees. A 50% majority rule tree was constructed from all equally parsimonious trees identified by the heuristic search. A bootstrap analysis involving 100 iterations (using the Bootstrap option in PAUP) was conducted to estimate confidence limits for the topological patterns revealed by parsimony analysis.

## RESULTS

*Identification and diversity of haplotypes.* --- The four amplified regions summed to 7.8 kb, accounting for roughly half of the tortoise mitochondrial genome (15.8 kb; Lamb et al., 1989). Of the 32 possible enzyme-region combinations, four such pairings failed to produce any restriction fragments (*Bst*UI: 12S/16S rRNA; *Msp*I: cyt. *b*/D-loop; *Bst*UI:NADH2/CO I; *Msp*I:NADH2/CO I) and will not be considered further. The remaining 28 enzyme-region combinations produced 126 restriction fragments and generated 47 unique digestion profiles (Appendix D). Overall, 36 haplotypes were detected among the 112 tortoises surveyed (Table 2). I was unable to amplify the NADH2/CO I region for any of the six Alabama samples. Two of the six Alabama individuals were assigned a haplotype (haplotype 36) with the understanding that it represents an incomplete composite genotype. Though excluded from the UPGMA analysis, haplotype 36 was included in the parsimony analysis, an inclusion justified in part by the highly conserved nature of the NADH2/CO I region (only *Taq*<sup>a</sup>I generated more than a single digestion profile for this region).

Only two of the 36 observed haplotypes (2 and 5) were common, representing 18 and 19 tortoises, respectively. The remaining haplotypes were relatively rare, with 17 (46%) being restricted to single individuals (Appendix A). In

light of these findings, mtDNA genotypic diversity was quite high,  $G = 0.932$ . Even though most haplotypes differed by only single site changes, the level of clonal diversity was surprising given the reduced mtDNA evolutionary rate observed in turtles (Avice et al., 1992).

*Geographic variation.* --- Although 21 haplotypes were restricted to single collection sites, those present at two or more locales were geographically localized. Even the most widespread haplotypes, 2 and 5, displayed a remarkable degree of geographic continuity.

The UPGMA analysis of  $p$ -values clustered the haplotypes into three major genetic assemblages, each with a distinct geographic orientation: Western, Eastern, and Mid-Florida (Fig 4). The Eastern/Mid-Florida assemblages, differed from the Western assemblage by 3.2% sequence divergence. The seven haplotypes that compose the Western assemblage encompass an area from Louisiana eastward to Taylor county, Florida, and north to Talbot county, Georgia, along the Chattahoochee River drainage (Fig. 5). The larger Eastern assemblage contains the most common haplotypes (2 and 5) and extends over the remainder of the range from South Carolina south through peninsular Florida. Eastern versus Western haplotypes are not completely parapatric, through overlap appeared limited to three locales. One incidence of overlap involved a single tortoise displaying an eastern haplotype



(haplotype 2) present in Florida's panhandle (Okaloosa county). The remaining two overlap localities - Emanuel county (central Georgia) contained one western to three eastern haplotypes, whereas Marion county (western Georgia) contained two western to six eastern haplotypes. Although, every possible effort was made to avoid translocated individuals, extensive relocation practices across the tortoise's range make certain locales and samples suspect, e.g. the Okaloosa county tortoise is probably a translocation (pers. comm. Paul Moler) and is so designated by a question mark (Fig. 5).

The third assemblage, Mid-Florida, was distinguished from the Eastern assemblage by an unexpected level of genetic divergence (2.7%). The assemblage is composed of seven haplotypes ( $p = 0.004 - 0.010$ ) that delineate a triangular shaped region from southern Levy county to Pinellas county along the Gulf coast, eastward to north of the Hillsborough River, and northeast to Orange/Osceola counties (Fig. 5). A limited overlap of haplotypes between assemblages was detected in the Mid-Florida region, where two individuals from Citrus and Hernando counties possessed Eastern haplotypes (1, 25). As with the overlap observed between Eastern and Western assemblages, it is difficult to determine whether these locales represent areas of secondary contact between assemblages or simply involve translocated individuals.

Parsimony analysis of the presence/absence matrix provided results similar to that of the UPGMA analysis. The parsimony network identifies and groups the same haplotypes for both Western and Mid-Florida assemblages (Fig. 6a). Moreover, bootstrap support for these assemblages is strong, at 92% for the Western group and 89% for the Mid-Florida group (Fig. 6a). Addition of the incomplete Alabama haplotype supported its inclusion in the Western group, though bootstrap values dropped slightly for both assemblages (77% Western and 84% Mid-Florida; Fig. 6b). In the absence of an outgroup, the parsimony network does not represent a true phylogeny but nonetheless provides a different qualitative perspective of general relationships among the tortoise haplotypes. It offers additional, independent support for the recognition of the three major mtDNA assemblages.

## DISCUSSION

*The PCR-Restriction Enzyme Approach*

Increasingly, studies of sequence variation stress the degree of rate heterogeneity observed among mitochondrial genes (Brown and Simpson, 1982; Wilson et al., 1987; Kocher et al., 1989). A fundamental component of this study involved determining which regions of the mitochondrial genome might be best suited to resolve population genetic relationships in the gopher tortoise. I examined genetic variation in four regions of the mtDNA molecule that exhibit a wide range of evolutionary rates. To determine the relative contribution of each region to overall genetic variation, I examined the number of bases surveyed and unique digestion profiles generated for each amplified segment. The cyt. *b*/D-loop segment exhibited a slight bias with respect to the relative number of bases surveyed accounting for 140 of the 394 bases surveyed (36%). The remaining base contributions were distributed as follows: 12S/16S for 96 bases (24%); NADH5/NADH6 for 72 bases (18%); and NADH2/CO I for 66 bases (17%). Evidence for regional bias was more apparent for the relative number of unique digestion profiles: cyt *b*/D-loop accounted for 18 (38%); 12S/16S for 12 (26%); NADH5/NADH6 for 9 (19%); and NADH2/CO I for 8 (17%).

The high level of genetic variation observed in the cyt. *b*/D-loop region was not unexpected. High rates of

substitution, increased levels of insertion and deletion, and numerous short repeated sequences appear to characterize the control region of various species (Shedlock et al., 1992; Wenick et al., 1993). Comparative studies of mammals (Brown et al., 1986; Saccone et al., 1987; Foran et al., 1988) and salmon (Shedlock et al., 1992) have demonstrated that the majority of the variation is attributable to the first 500 bps of the D-loop (corresponding to the region amplified in this survey). Recent sequencing studies have identified the utility of this portion of the D-loop in addressing phylogeographic and lower-level systematic questions (Wenick et al., 1993; Allard et al., in press; Lamb et al., submitted).

The contribution of the *cyt. b*/D-loop region notwithstanding, unique, geographically informative restriction sites were also present in each of the other three mitochondrial regions. Each amplified region's contribution to delineation of the three major assemblages was examined by systematically excluding the restriction data of each region. Four additional UPGMA analyses were conducted, successively eliminating the restriction site data provided by one of the four amplified regions. As illustrated in the resulting phenograms (Fig. 7a and 7b), the integrity of the three major assemblages was lost, indicating that each region provided important geographic information.

Compared to previous turtle surveys using conventional restriction assays the PCR-restriction approach revealed a marked increase in the number of haplotypes and estimates of genotypic diversity (Table 3). Such increases are likely attributable to tetranucleotide endonuclease assay and to the examination of a sizable portion of the D-loop. For example, when the cyt. *b*/D-loop region is excluded, the number of haplotypes drops from 36 to 19. The tortoise data (even without cyt. *b*/D-loop region) illustrate the potential utility of the PCR-restriction approach for detecting intraspecific genetic variation in species with slow mtDNA evolutionary rates.

### *Southeastern Regional Biogeography*

*Geologic background of the southeastern U.S.* --- The Coastal Plain topography of the southeastern United States has been altered repeatedly by eustatic sea level fluctuation (Swift et al., 1985). Throughout the Pliocene and Pleistocene, sea level has varied some 80 m above and below present levels (Vail and Mitchum, 1979). The Southeast's twelve major drainages, as well as numerous smaller drainages, were variously affected by these fluctuations. As sea level rose, several smaller drainages were completely inundated while transgressions of the larger rivers created extensive estuaries and isolated upland terrestrial habitats. In addition, the North American ice

sheet significantly altered the region's climate and ecology (Awise, 1992). As the ice sheet advanced, temperate biota were forced southward and, conversely, returned northward during periods of glacial recession. Either singly or in tandem, sea level fluctuation and climatic alterations likely impacted the aquatic and terrestrial biota of the Southeast. General accounts outlining historical influences on the region's biota are provided by Swift et al. (1985), Webb (1990), and Awise (1992). Details of specific regions and events that figure prominently in the biogeography of the Southeast are summarized in the following section.

*Evidence for an east-west break and its bearing on the tortoise mtDNA data.* --- Efforts to determine the biogeographic significance of geologic events in the southeastern U.S. have focused primarily on the region's freshwater fish fauna. In a phenetic analysis of distribution patterns for 240 freshwater fish, Swift et al. (1985) revealed a major east-west break centered along the Apalachicola-Ochlochnee divide. On a finer scale, ten zoogeographic provinces were delineated, each linked to a major southeastern drainage (including both Apalachicola and Ochlochnee-Satilla provinces). Overall, the study indicated substantial biogeographic structuring that Swift et al. (1985) attributed to regional geomorphic features as well as vicariant events mediated by eustatic sea level fluctuation.

Population genetic analysis has revealed similar east-west subdivisions for several southeastern freshwater fishes. Allozymic surveys of largemouth bass, *Micropterus salmoides* (Philipp et al., 1981), bluegill sunfish, *Lepomis macrochirus* (A vise and Smith, 1974), and mosquitofish, *Gambusia* spp. (Wooten et al. 1988), all detected east vs. west genetic assemblages defined by various drainages, e.g., the Suwannee River, Ochlochnee River, and Mobile Bay. Bermingham and A vise (1986) surveyed mtDNA variation in four additional species: the bowfin (*Amia calva*), spotted sunfish (*Lepomis punctatus*), redear sunfish (*Lepomis microlophus*), and warmouth (*Lepomis gulosus*). They also detected east-west genetic breaks associated with: 1) the Apalachicola-Suwannee drainages for the bowfin, redear, and spotted sunfish; and 2) the adjacent Mobile Bay drainage for the warmouth. Although geographic patterns vary slightly in these studies, the distributional, allozymic, and mitochondrial data are, overall, reasonably concordant and consistent with the hypothesized influences of eustatic sea level fluctuation (Swift et al., 1985; Bermingham and A vise, 1986; Gilbert, 1987).

In contrast to the numerous biogeographic studies of southeastern freshwater fish, the region's terrestrial fauna has received little attention regarding the effects of historical vicariance events. In one of the few terrestrial species surveys in the Southeast, A vise et al. (1979)

conducted a mtDNA RFLP analysis of the pocket gopher, *Geomys pinetis*. Their study is of particular interest because the pocket gopher's geographic range and habitat requirements are largely coincident with that of the gopher tortoise. Both species are confined to sandy upland situations with friable soils that facilitate burrowing. The mtDNA phylogenetic network for the pocket gopher exhibits a distinct east-west break in the vicinity of the Apalachicola drainage (Avice et al., 1979) that is, overall, remarkably similar to the geographic pattern observed among tortoise haplotypes (Fig. 8). In turn, the concordant features of both tortoise and pocket gopher phylogenies extend to the general phylogeographic patterns observed for certain freshwater fishes (Bermingham and Avice, 1986).

The appearance of concordant geographic patterns among independently evolving lineages often indicates a shared geologic history (Croizat et al., 1974; Platnik and Nelson, 1978; Rosen, 1978). Such concordance involves a temporal as well as spatial component. However, geologic estimates for southeastern vicariance events vary widely and thus likely represent a series of episodic changes. Swift et al. (1985) attributed different distributional patterns in fish species to geologic events ranging from the Miocene to the Pleistocene. Using estimates of genetic divergence, Bermingham and Avice (1986) attributed the genetic structure in several sunfish species (*Lepomis*) to a period of elevated



sea level (50-80 m) that occurred during the Pliocene interglacial. However, the concordant genetic break displayed by the bowfin (*Amia*) exhibits considerably less genetic divergence, thus indicating a more recent separation date of  $< 0.5$  Mya (Bermingham and Avise, 1986).

As with the fish species, it is possible that one or more episodic events influenced the southeastern terrestrial biota, although it is difficult to ascertain precisely which events were significant with respect to the gopher tortoise. Little is known about historic habitats of the Southeast, a factor that would have influenced a habitat specialist such as the tortoise. It can be hypothesized that periods of elevated sea level were accompanied by climatological fluctuations, causing habitats to shift in latitude and thus forcing terrestrial species northward away from coastal areas. When the sea level receded, the habitat would once again become suitable for recolonization. When these colonization/recolonization events are considered in light of the older (late Cenozoic) more prominent drainages in the region, substantial periods of isolation and thereby an east-west structure may have resulted. These drainages, such as the Chattahoochee-Apalachicola, could have maintained an east-west separation during all but extreme northern latitudinal shifts. Although translocation cannot be ruled out, the current overlap of eastern and western haplotypes in Marion and Emanuel counties may be relicts of

a recent contact during a northern shift event.

*Biogeography of peninsular Florida and its bearing on the tortoise.* --- Several accounts of the biogeography of peninsular Florida have stressed the historical roles played by the region's sand ridges (Webb 1990; Jackson, 1973; Burgess and Franz, 1978). In general, Florida's peninsular topography can be divided into five highland areas or ridges ranging from 80-100 m in elevation (White, 1958). The peninsula's overall low elevation has made it susceptible to marine transgression during periods of elevated sea level. However, evidence suggests that most ridges were not completely inundated; rather they persisted as a series of islands even during periods of maximal sea level rise (Cooke, 1939; MacNiel, 1949; White 1970; Webb, 1990, Neill 1957). Support for historic ridge-island effects is underscored by the presence of several endemic species on the Lake Wales Ridge. Endemic vertebrates include the scrub lizard, *Sceloporus woodi* (Jackson, 1973); sand skink, *Neoseps reynoldsi*; and the Florida scrub jay, *Aphelocoma caerulescens*. In addition, some 189 plant species have been identified as Lake Wales endemics (Neill, 1957).

The region delimited by the Mid-Florida tortoise assemblage corresponds roughly with a second major ridge, the Brooksville Ridge. The Mid-Florida assemblage is bordered on the north and east by the Withlacoochee River,

and on the south by Tampa Bay. It follows the Hillsborough River to the east and extends across the Lake Wales Ridge into northern Osceola county (Fig. 5).

The Withlacoochee and Hillsborough drainages, which occupy the lowland areas between ridges, were subject to incursion during periods of elevated sea level. Test wells along the upper Withlacoochee valley (east of the Brooksville Ridge) reveal periodic sedimentation layers corresponding to historic Pleistocene high sea levels (White, 1958). Historic marine transgression is further evidenced by relict Pleistocene beaches that run continuously along the eastern edge of the Brooksville Ridge from the northern Withlacoochee to the Hillsborough drainage (White, 1958; Vernon, 1951). The inundation of these low lying areas would have effectively isolated the Brooksville Ridge from the adjacent Lake Wales Ridge, forcing lowland terrestrial inhabitants upland.

It is relatively easy to infer isolation events resulting from transgression of lowland areas; an additional consideration involves how isolation may have been maintained during alternate periods of reduced sea level. Geological evidence suggests that even during reduced sea level the Withlacoochee and Hillsborough drainages may have served as barriers to terrestrial dispersal (Vernon, 1951; White, 1958). Several lines of evidence suggest the age and stability of the Hillsborough River's current drainage

pattern: 1) an extensive present day estuary, 2) a broad swampy flood plain (evidence of a formerly more extensive estuary), and 3) the degree of dissolution through the Pamlico terrace (White, 1958). The Hillsborough's age implies its potential influence as a barrier to terrestrial dispersal. Unfortunately, peiziometric data suggests that the Hillsborough may have been seriously reduced during extensive water table reduction that accompanied interglacial periods (Vernon, 1951); it is unclear if the Hillsborough served as a terrestrial barrier to dispersal during sea level reductions.

Geologic data indicates that the Withlacoochee River has experienced a very different regional history, though evidence suggests that it may have served as a barrier to terrestrial dispersal as well. The Withlacoochee, together with a series of lakes (Tsala Apoka), occupy low lying area east of the Brooksville Ridge. White (1958) suggested this shallow basin was once completely filled, creating a single large lake along the eastern edge of the Brooksville Ridge. The ancient lake, in combination with the Hillsborough River, would have limited dispersal along the eastern and southern edges of the Brooksville ridge. However, the northern portion of the ridge remained open to terrestrial dispersal. Thus, the presence of several tortoises with eastern haplotypes within the Mid-Florida assemblage may be the result of this connection, although translocation is

again a tenable alternative explanation.

Additional evidence for historic isolation of the Brooksville Ridge is provided by presence of endemic species. Several plants, including a bellflower (*Rotanthea robinsiae*) and a clematis (*Clematis micrantha*), are only found in Hernando County (Neill, 1957). A water strider (*Metrobates spp.*) is also endemic to the region (Neill, 1957).

Evidence for historic transgression coupled with the area's drainage history suggests sustained isolation of the Brooksville Ridge; nonetheless, a portion of the Mid-Florida assemblage extends east of the ridge. Although translocation cannot be ruled out, the Osceola and Polk county animals may provide evidence for recent migration possibly during the late Pleistocene sea level reductions.

#### *Summary*

Compared to conventional methodology, the PCR-restriction approach offers much promise for detecting intraspecific variation in turtles. The ability to examine specific regions using PCR, coupled with the use of primarily tetranucleotide restriction endonucleases, appears to have circumvented the difficulties detecting significant genetic variability within turtle species. Even in the absence of the cyt. *b*/D-loop region, this approach revealed marked increase in mtDNA genotypes relative to turtle studied using conventional methodologies.

Phylogeographic structure observed among tortoise mtDNA haplotypes was largely congruent with the genetic breaks observed in other southeastern fauna. Although I was unable to determine the specific historical events that may have shaped the tortoise's phylogeography, its concordance with other intraspecific phylogenies supports the influence of regional sea level fluctuation.

Overall, the geographic boundaries defined by the mtDNA assemblages should be useful in future tortoise management and relocation practices. With increased population declines and relocation practices encountered rangewide, the phylogenetic perspective provided by tortoise mtDNA holds promise for guiding future conservation policies.

## REFERENCES

- Allard, M.W., M.M. Miyamoto, K.A. Bjorndal, A.B. Bolten, and B.B. Bowen. 1993. Support for natal homing in green turtles from mitochondrial DNA sequences. *Copeia in press*.
- Aquadro, C.F. and B.D. Greenberg. 1983. Human mitochondrial DNA variation and evolution: Analysis of nucleotide sequences from seven individuals. *Genetics* 103: 287-312.
- Auffenberg, W. 1976. The genus *Gopherus* Testudinidae Part 1: osteology and relationships of extant species. *Bull. Fla. State Mus. Biol. Sci.* 20: 1-110.
- Auffenberg, W. and R. Franz. 1982. The status and distribution of the gopher tortoise (*Gopherus polyphemus*), p. 95-126. In *North American tortoises: conservation and ecology*, R.B. Bury (ed). Wildlife Research Report No. 12, Washington, D.C.: United States Dept. of Interior, Fish and Wildlife Service.
- Avise, J.C. 1992. Molecular population structure and biogeographic history of a regional fauna: A case history with lessons for conservation biology. *Oikos* 63: 62-76.
- Avise, J.C. 1986. Mitochondrial DNA and the evolutionary genetics of higher animals. *Phil. Trans. R. Soc. Lond. B.* 312: 325-342.
- Avise, J.C., B.W. Bowen, T. Lamb, A.B. Meylan, and E. Bermingham. 1992. Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Mol. Biol. Evol.* 9: 457-473.
- Avise, J.C., G. Giblin-Davidson, J. Laerm, J.C. Patton, and R.A. Lansman. 1979. Mitochondrial DNA clones and matriarchical phylogeny within and among geographic populations of the pocket gopher, *Geomys pinetis*. *Proc. Natl. Acad. Sci. USA* 76: 6694-6698.
- Avise, J.C. and R.A. Lansman. 1983. Polymorphism of mitochondrial DNA in populations of higher animals, pp. 147-164. In Evolution of Genes and Proteins, M. Nei and R.K. Koehn (eds.). Sinauer, Sunderland, MA.

- Avise, J.C., J. Arnold, M.B. Ball, E. Bermingham, T. Lamb, J.E. Neigel, C.A. Reeb, and N.C. Saunders. 1987a. Intraspecific phylogeography: The bridge between population genetics and systematics. *Ann. Rev. Syst.* 18: 489-522.
- Avise, J.C., C.A. Reeb, and N.C. Saunders. 1987b. Geographic population structure and species differences in mitochondrial DNA of marine mouthbrooding catfish (Ariidae) and demersal spawning toadfish (Brachoididae). *Evolution* 41: 991-1002.
- Avise, J.C. and M.H. Smith. 1974. Biochemical genetics of sunfish. I. Geographic variation and subspecific intergradation in the bluegill, *Lepomis macrochirus*. *Evolution* 28: 42-56.
- Avise, J.C., J.F. Shapira, S.W. Daniel, C.F. Aquadro, and R.A. Lansman. 1983. Mitochondrial DNA differentiation during the speciation process in *Peromyscus*. *Mol. Biol. Evol.* 1: 38-56.
- Ball, R.M., Jr., S. Freeman, F.C. James, E. Bermingham, and J.C. Avise. 1988. Phylogeographic population structure of Red-winged Blackbirds assessed by mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 85: 1558-1562.
- Bermingham, E. and J.C. Avise. 1986. Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* 113: 939-965.
- Bermingham, E., T. Lamb, and J.C. Avise. 1986. Size polymorphism and heteroplasmy in mitochondrial DNA of lower vertebrates. *J. Heredity* 77: 249-252.
- Bour, R. and A. Dubois. 1984. *Xerobates* Agassiz, 1857, synonyme plus ancien de *Scaptochelys* Bramble, 1982. (Reptilia, Chelonii, Testudinidae) *Bull. Soc. Linneenne Lyon* 53: 30-32.
- Bowen, B.W., A.B. Meylan, and J.C. Avise. 1991. Evolutionary distinctiveness of the endangered Kemp's ridley sea turtle. *Nature* 352: 709-711.
- Bowen, B.W., A.B. Meylan, J.P. Ross, C.J. Limpus, G.H. Balazs, and J.C. Avise. 1992. Global population structure and natural history of the green turtle (*Chelonia mydas*) in terms of matriarchal phylogeny. *Evolution* 46: 865-881.



- Boyce, T.M., M.E. Zwick, and C.F. Aquadro. 1989. Mitochondrial DNA in the bark weevils: Size, structure and heteroplasmy. *Genetics* 123: 825-836.
- Bramble, D.M. 1971. Functional morphology, evolution and paleoecology of gopher tortoises. Ph.D. Diss., Univ. California, Berkeley.
- Bramble, D.M. 1982. *Scaptochelys*: Generic revision and evolution of gopher tortoises. *Copeia* 1982: 852-867.
- Brown, G.G., G. Gadaleta, G. Pepe, C. Saccone, and E. Sbisa. 1986. Structural conservation and variation in the D-loop containing region of vertebrate mitochondrial DNA. *J. Mol. Biol.* 192: 503-511.
- Brown, G.G. and M.V. Simpson. 1982. Novel features of animal mtDNA as shown by sequence of two cytochrome oxidase subunit II genes. *Proc. Natl. Acad. Sci. USA* 77: 3605-3609.
- Brown, W.M. 1983. Evolution of animal mitochondrial DNA, pp. 62-88. In Evolution of Genes and Proteins, M. Nei and R.K. Koehn (eds.). Sinauer Associates Inc., Sunderland, MA.
- Brown, W.M. 1985. The mitochondrial genome of animals, pp. 95-130. In Molecular Evolutionary Genetics, R.J. MacIntyre (ed.). Plenum, New York, NY.
- Brown, W.M., M. George Jr., and A.C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 76: 1967-1971.
- Burgess, G.H. and R. Franz. 1978. Zoogeography of the aquatic fauna of the St. Johns river system with comments on adjacent peninsular faunas. *Amer. Midl. Nat.* 100: 160-170.
- Chapman, B.W. and B.L. Brown. 1989. Two methods to detect DNA fragments produced by restriction enzymes. *Anal. Biochem.* 177: 199-202.
- Cooke, C.W. 1939. Geology of Florida. Florida Geol. Survey Bull. No. 17.
- Croizat, L.G., G. Nelson and D.E. Rosen. 1974. Centers of origins and related concepts. *Syst. Zool.* 23: 265-287.

- Crumly, C.R. 1985. A hypothesis for the relationships of land tortoise genera (family Testudinidae), pp. 115-124. In Studia Paleocheloniologica, F. DeBroin and E. Jimenez-Fuentes (eds.). Stud. Geol. Univ., Salamanca.
- Crumly, C.R. 1993. The phylogenetic systematics of North American tortoises (genus *Gopherus*): Evidence for their classification. In Biology of North American Tortoises, R.B. Bury and D.J. Germano (eds.). U.S. Fish Wildl. Serv. Fish Wildl. Res. Washington, D.C., in press.
- Deimer, J.E. 1986. The ecology and management of the gopher tortoise in the southeastern United States. *Herpetologica* 42: 125-133.
- Deimer, J.E. 1989. An overview of gopher tortoise relocation. Florida Game and Freshwater Fish Commission, Nongame Wildlife Program Technical Report No. 5.
- Fauron, C. and D.R. Wolstenholme. 1980. Extensive diversity among *Drosophila* species with respect to nucleotide sequences with adenine + thymine rich region of mitochondrial DNA molecules. *Nucleic Acids Res.* 11: 2439-2453.
- Foran, D.R., J.E. Hixon, and W.M. Brown. 1988. Comparisons of ape and human sequences that regulate mitochondrial DNA transcription and D-loop synthesis. *Nucleic Acids Res.* 16: 5841-5861.
- Gilbert, C.R. 1987. Zoogeography of the freshwater fish fauna of southern Georgia and peninsular Florida. *Brimleyana* 13: 25-54.
- Greenberg, B.D., Newbold, J.E., and A. Sugino. 1983. Intraspecific nucleotide sequence variability surrounding the origin of replication in human mitochondrial DNA. *Gene* 21:33-49.
- Gyllensten, U., D. Wharton, A. Josefsson, and A.C. Wilson. 1991. Paternal inheritance of mitochondrial DNA in mice. *Nature* 352: 255-257.
- Harrison, R.G. 1989. Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *TREE* 4: 6-11.
- Hillis, D.M. and C. Moritz. 1990. Molecular Systematics. Sinauer Associates Inc., Sunderland, MA.

- Iverson, J.B. 1980. The reproductive biology of the *Gopherus polyphemus* Chelonia Testudinidae. Am. Midl. Nat. 103: 353-359.
- Jackson, J.F. 1973. Distribution and population phenetics of the Florida scrub lizard, *Sceloporus woodi*. Copeia 4: 746-761.
- Kocher, T.D., W.K. Thomas, A. Meyer, S.V. Edwards, A. Paabo, F.X. Villablanca, and A.C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86: 6196-6200.
- Lamb, T., Avise, J.C., and J.W. Gibbons. 1989. Phylogeographic patterns in mitochondrial DNA of the desert tortoise (*Xerobates agassizii*), and evolutionary relationships among the North American tortoises. Evolution 43: 76-87.
- Lamb, T. and J.C. Avise. 1992. Molecular and population genetic aspects of mitochondrial DNA variability in the diamondback terrapin, *Malaclemys terrapin*. J. Heredity 83: 262-269.
- Lamb, T. and C. Lydeard. 1993. A molecular phylogeny of the gopher tortoises, with comments on the systematic status of the Testudinidae. Mol. Phyl. Ecol., submitted.
- Landers, J.L. and J.L. Buckner. 1981. The gopher tortoise: effects of forest management and critical aspects of its ecology. Southlands Exp. For. Tech. Note 56: 1-7.
- Landers, J.L. and J.A. Garner. 1981. Status and distribution of the gopher tortoise in Georgia. In , Proceedings of the Nongame and Endangered Wildlife Symposium, R. Odum and J. Guthrie (eds.).
- Landers, J.L., W.A. McRae and J.A. Garner. 1982. Growth and maturity of the gopher tortoise, *Gopherus polyphemus*, in southwestern Georgia, USA. Bull Fla. State Mus. Biol. Sci. 27: 81-110.
- Maddison, W.P. and D.R. Maddison. 1992. MacClade Version 3.0. Sinauer Associates Inc., Sunderland, MA.
- MacNeil, F.S. 1949. Pleistocene shorelines in Florida and Georgia. U.S. Geol. Survey Prof. Paper 221-F: 91-107.

- McElroy, D., P. Morgan, E. Bermingham, and I. Kornfield. 1992. REAP: An integrated environment for the manipulation and phylogenetic analysis of restriction data. *J. Heredity* 83: 157-158.
- Moritz, C., T.E. Dowling, and W.M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Ann. Rev. Ecol. Syst.* 18: 269-292.
- Nei, M. and J.C. Miller. 1990. A simple method for estimating average number of nucleotide substitutions within and between populations from restriction data. *Genetics* 125: 873-879.
- Nei, M. and F. Tajima. 1981. DNA polymorphism detectable by restriction endonucleases. *Genetics* 97: 145-163.
- Neigel, J.E. and J.C. Avise. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation, pp. 513-534. In Evolutionary Process and Theory, E. Nevo and S. Karlin (eds.). Academic Press, New York, NY.
- Neill, W.T. 1957. Historical biogeography of present-day Florida. *Bull. Fla. Mus.* 2: 175-220.
- Philipp, D.P., W.F. Childers, and G.S. Whitt. 1981. Management implications for different stocks of largemouth bass (*Micropterus salmoides*) in the United States. *Can. J. Fish. Aquat. Sci. USA* 83: 9090-9093.
- Platnick, N.I. and G. Nelson. 1978. A model of analysis for historical biogeography. *Syst. Zool.* 27: 1-16.
- Riggs, S.M. 1984. Paleooceanographic model of Neogene phosphorite deposition, U.S. Atlantic continental margin. *Science* 223: 123-131.
- Rohlf, F.J. 1991. NTSYS-pc, Version 1.70. Stony Brook, New York.
- Rosen, D.E. 1978. Vicariant patterns and historical explanation in biogeography. *Syst. Zool.* 27: 159-188.
- Saccone, C., G. Pesole, and E. Sbisà. 1991. The main regulatory region of mammalian mitochondrial DNA: Structure-function model and evolutionary pattern. *J. Mol. Evol.* 33: 83-91.

- Shedlock, A.M., J.D. Parker, D.A. Crispin, T.W. Pietsch, and G.C. Burmer. 1992. Evolution of the salmonid mitochondrial control region. *Mol. Phyl. Evol.* 1: 179-192.
- Sneath, P.H.A. and R.R. Sokal. 1973. Numerical Taxonomy. Freeman, San Francisco, CA.
- Swift, C.C., C.R. Gilbert, S.A. Boretone, G.H. Burgess and R.W. Yerger. 1985. Zoogeography of the freshwater fishes of the southeastern United States: Savannah River to Lake Ponchartrain, pp. 213-265. In Zoogeography of North American Freshwater Fishes, C.H. Hocutt and E.O. Wiley (eds.). John Wiley & Sons, New York, NY.
- Swofford, D.L. 1993. Phylogenetic analysis using parsimony (PAUP), Version 3.1. Illinois Natural History Survey, Champaign, IL.
- Taylor, R.W., Jr. 1982. Human predation on the gopher tortoise, *Gopherus polyphemus* in north central Florida, USA. *Bull. Fla. State Mus. Biol. Sci.* 28: 79-102.
- Thevierge, B. 1992. Choosing an appropriate agarose for separation and purification of PCR products. *Biotechnology Laboratory*, February: 12.
- Vail, P.R. and R.M. Mitchum, Jr. 1979. Global cycles of relative changes of sea levels from seismic stratigraphy, pp. 469-472. In Geological and Geophysical Investigation of Continental Margins No. 26, J.S. Watkins (ed.). American Association of Petroleum Geologists Memoir.
- Vernon, R.O. 1951. Geology of Citrus and Levy counties, Florida. *Florida Geol. Survey Bull.* No. 33.
- Webb, S.D. 1990. Historical Biogeography, pp. 70-100. In Ecosystems of Florida, R.L. Meyers and J.J. Ewel (eds.). University of Central Florida Press, Orlando, FL.
- Wenink, P.W., A.J. Baker, and M.G.J. Tilanus. 1993. Hypervariable-control-region sequences reveal global population structuring in a long-distance migrant shorebird, the Dunlin (*Calidris alpina*). *Proc. Natl. Acad. Sci. USA* 90: 94-98.
- White, W.A. 1958. Some geomorphic features of central peninsular Florida. *Fla. Geol. Survey Bull.* No. 41.

- White, W.A. 1970 The Geomorphology of the Florida Peninsula. Geological Bulletin No. 51, Bureau of Geology, Div. of Interior Resc., Florida Dept. of Nat. Resc.
- Wilson, A.C., M. Stoneking, R.L. Cann, E.M. Prager, S.D. Ferris, L.A. Wrischnik, and R.G. Higuchi. 1987. Mitochondrial clans and the age of our common mother, pp. 158-164. In Human Genetics, Proc. Intl. Cong., Berlin 1986, F. Vogel and K. Sperling (eds.). Springer-Verlag, Berlin, Germany.
- Wooten, M.C., K.T. Scribner, and M.H. Smith. 1988. Genetic variability and systematics of *Gambusia* in the southeastern United States. *Copeia* 1988: 283-289.

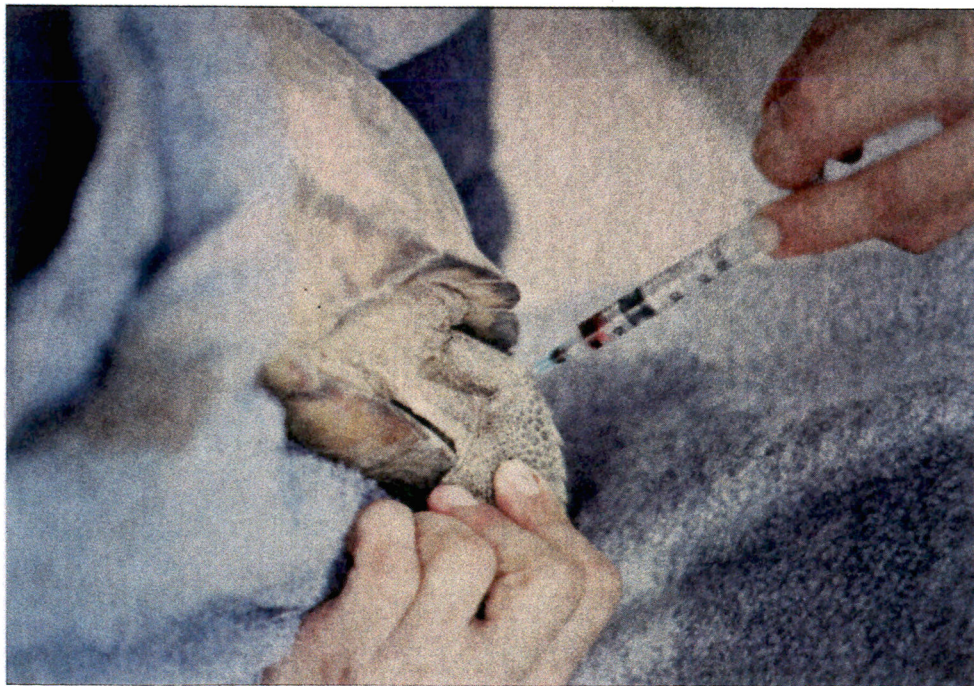


**Figure 1.** Map of the southeastern United States depicting specific sampling localities. A complete list of localities appears in Appendix A.



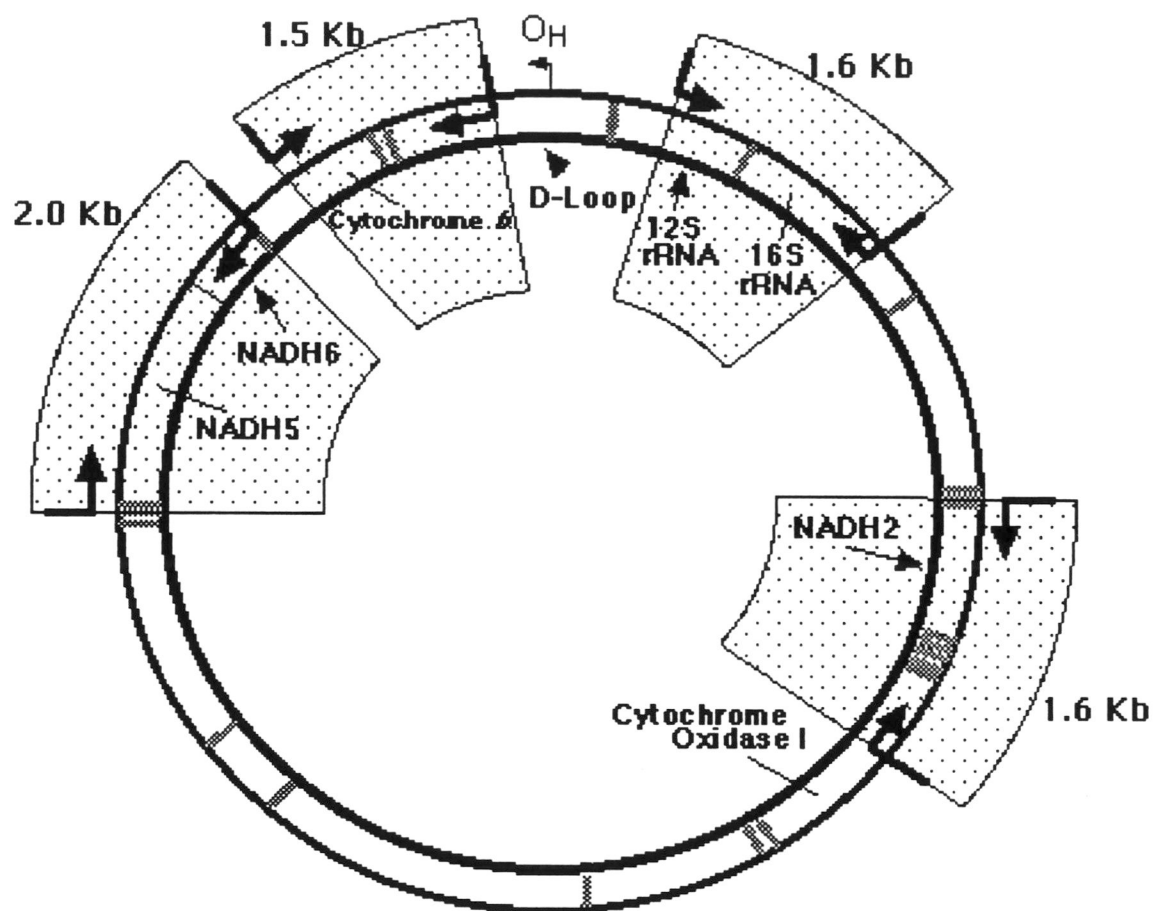


**Figure 2a.** Placement of wire box trap at entrance of tortoise burrow.

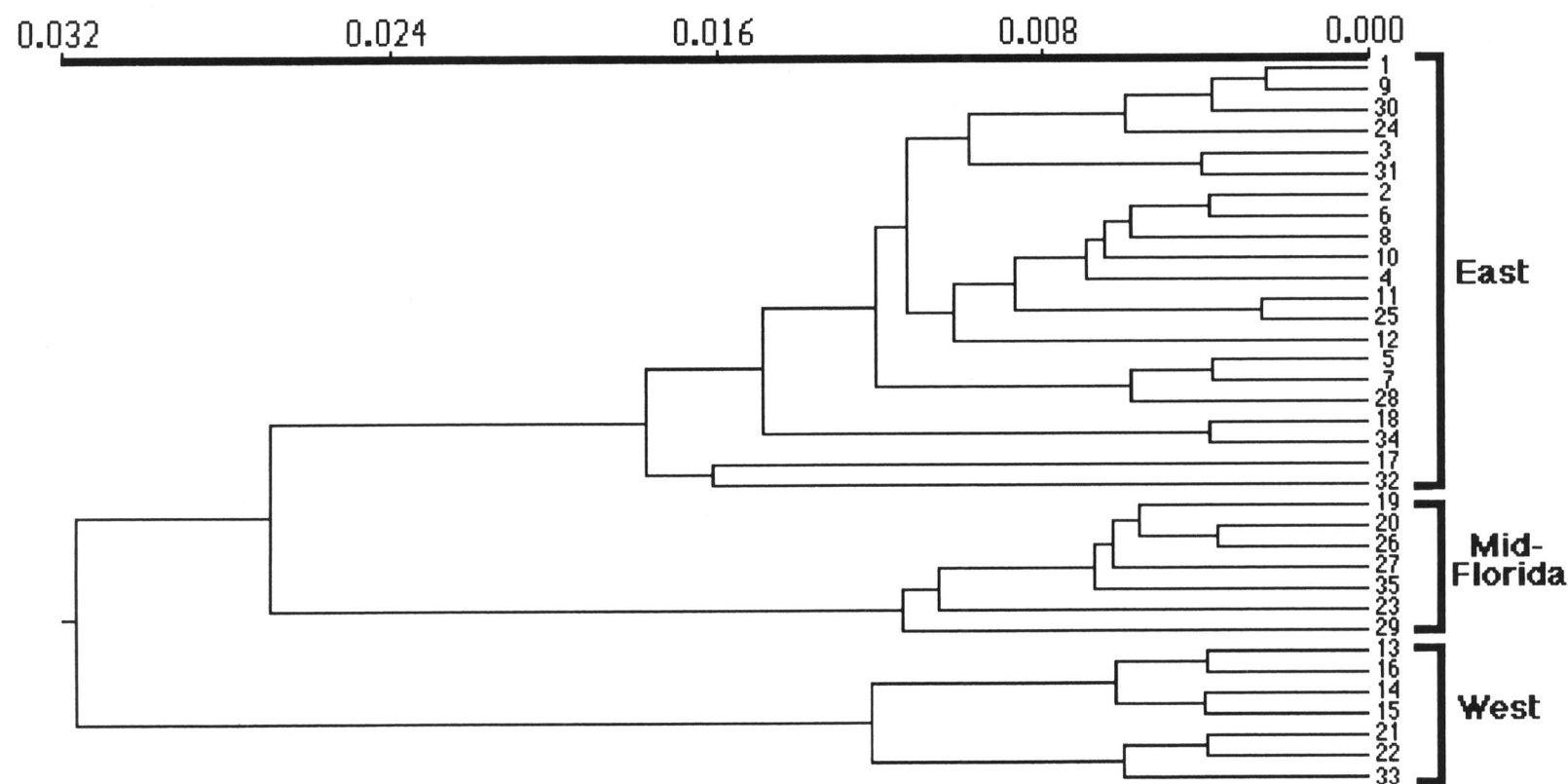


**Figure 2b.** Blood extraction technique.

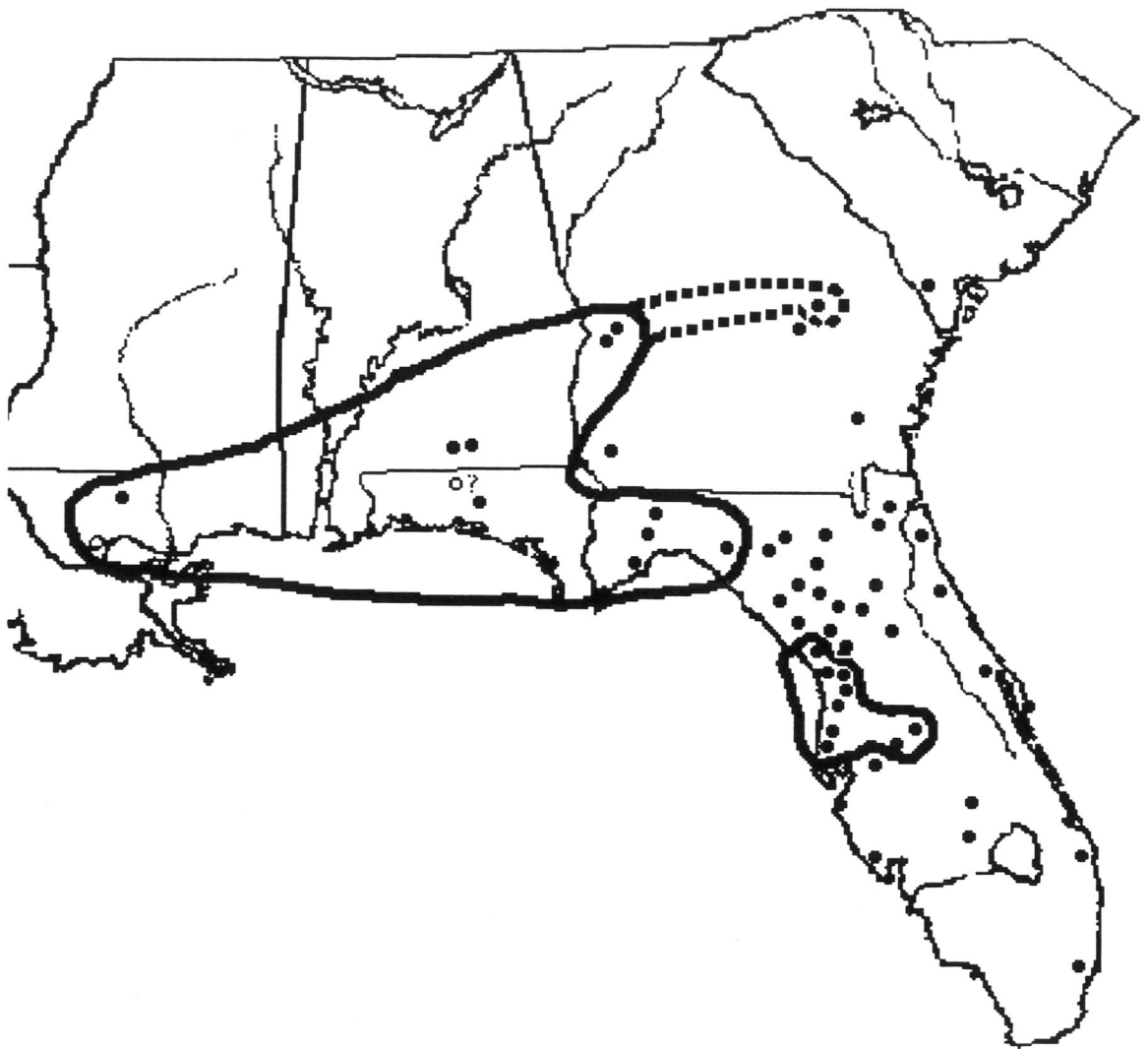




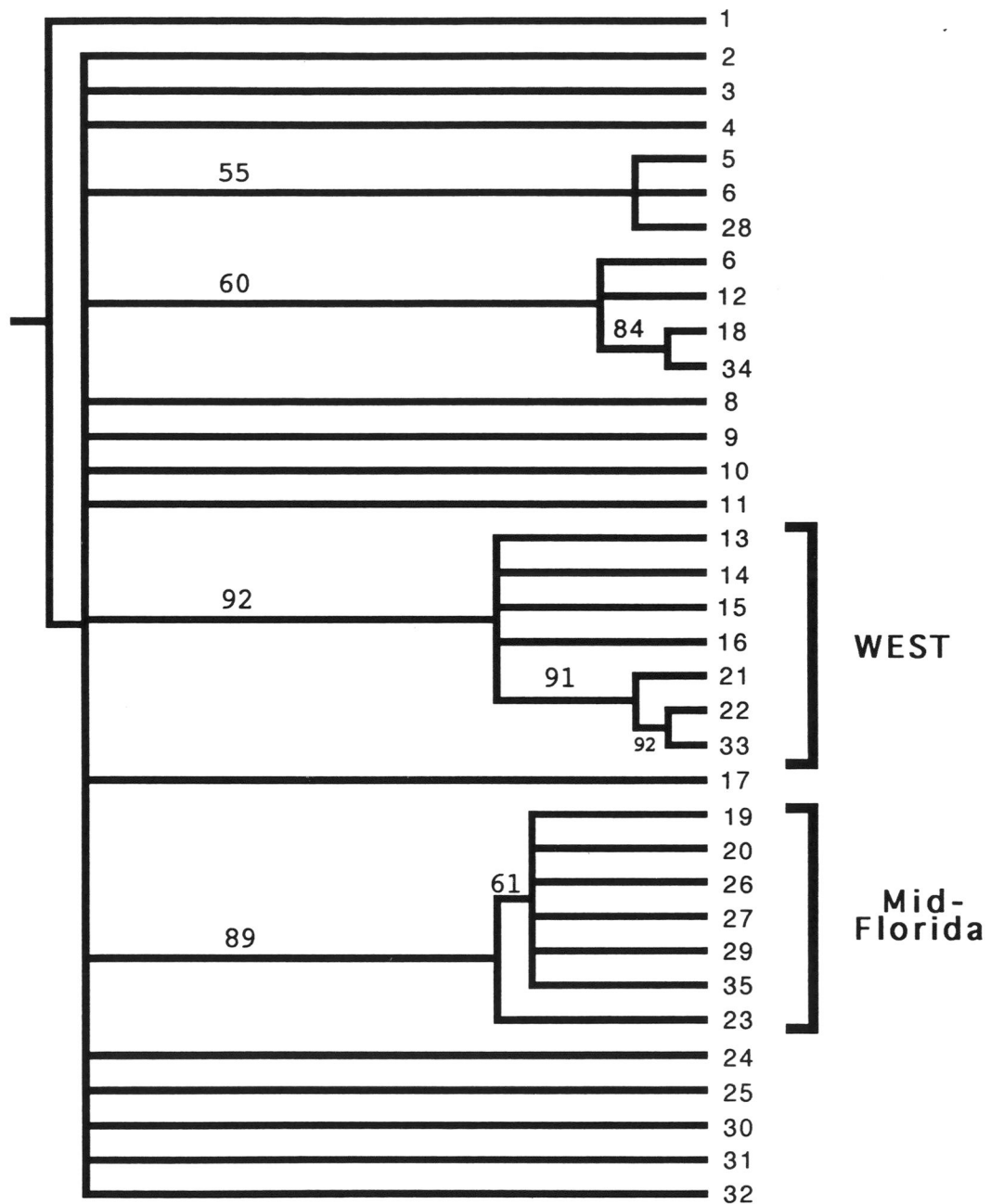
**Figure 3.** The mitochondrial genome. Highlighted areas represent amplified regions of the molecule.



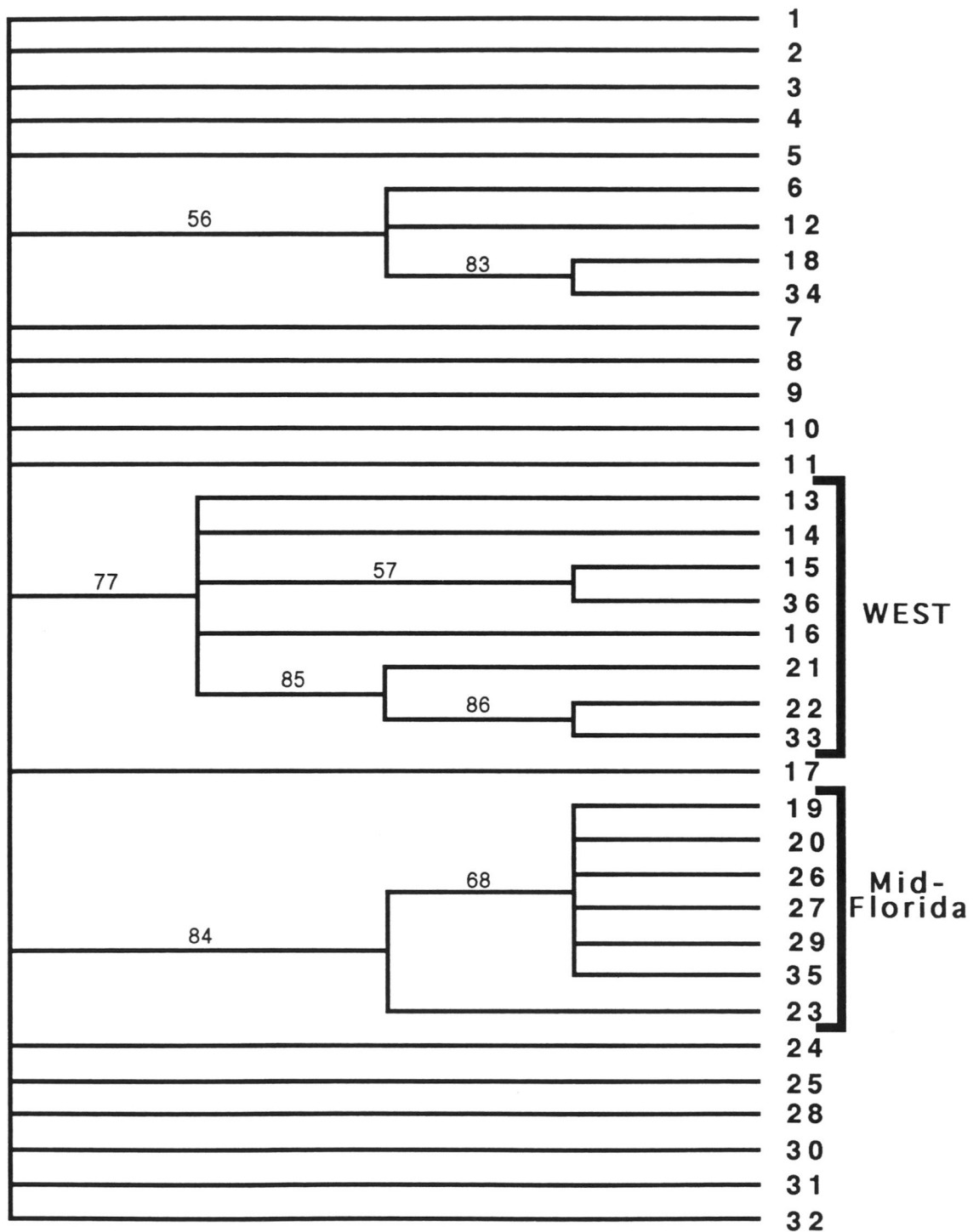
**Figure 4.** A phenogram depicting relationships among tortoise mtDNA genotypes generated by UPGMA analysis of genetic distance ( $p$ ) estimates. Highlighted areas correspond to geographic assemblages.



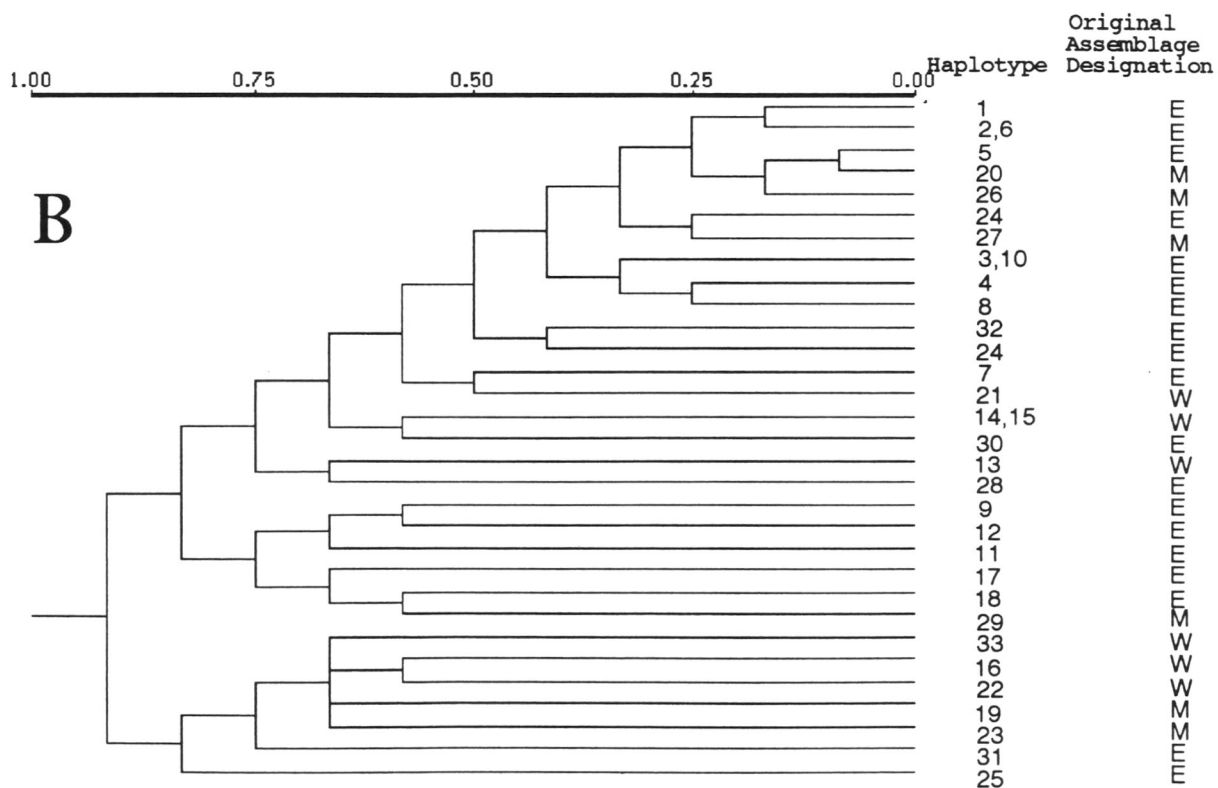
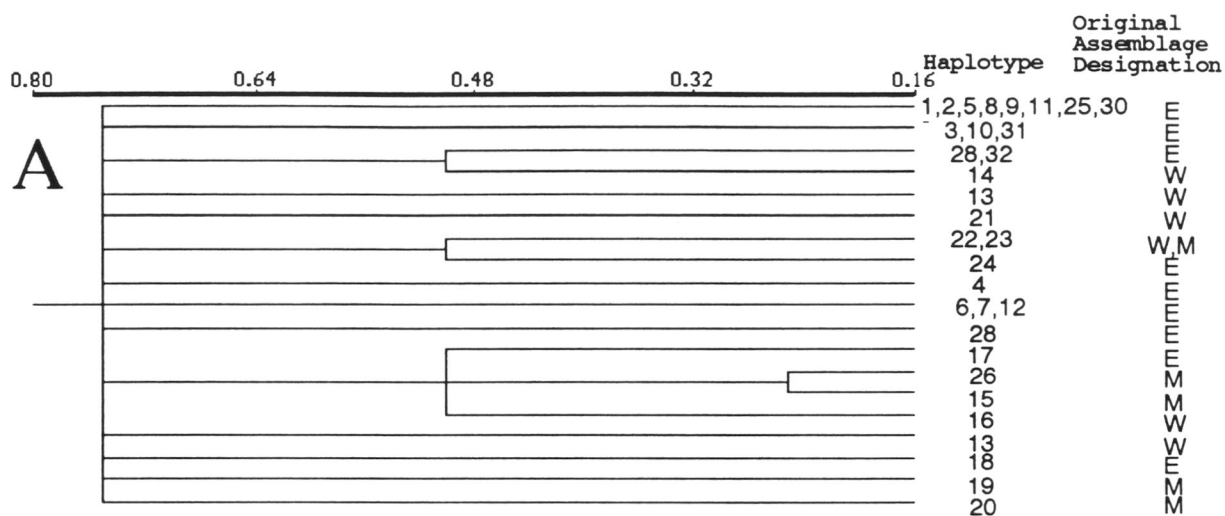
**Figure 5.** Geographic distribution of mtDNA clones as delineated by UPGMA and parsimony analyses. Each dot represents a collection site while solid lines encompass both the Western and Mid-Florida assemblages. Dashed line denotes mix of eastern and western genotypes in Emanuel county, Georgia. "?" denotes likely translocated individual from Okaloosa county, Florida.



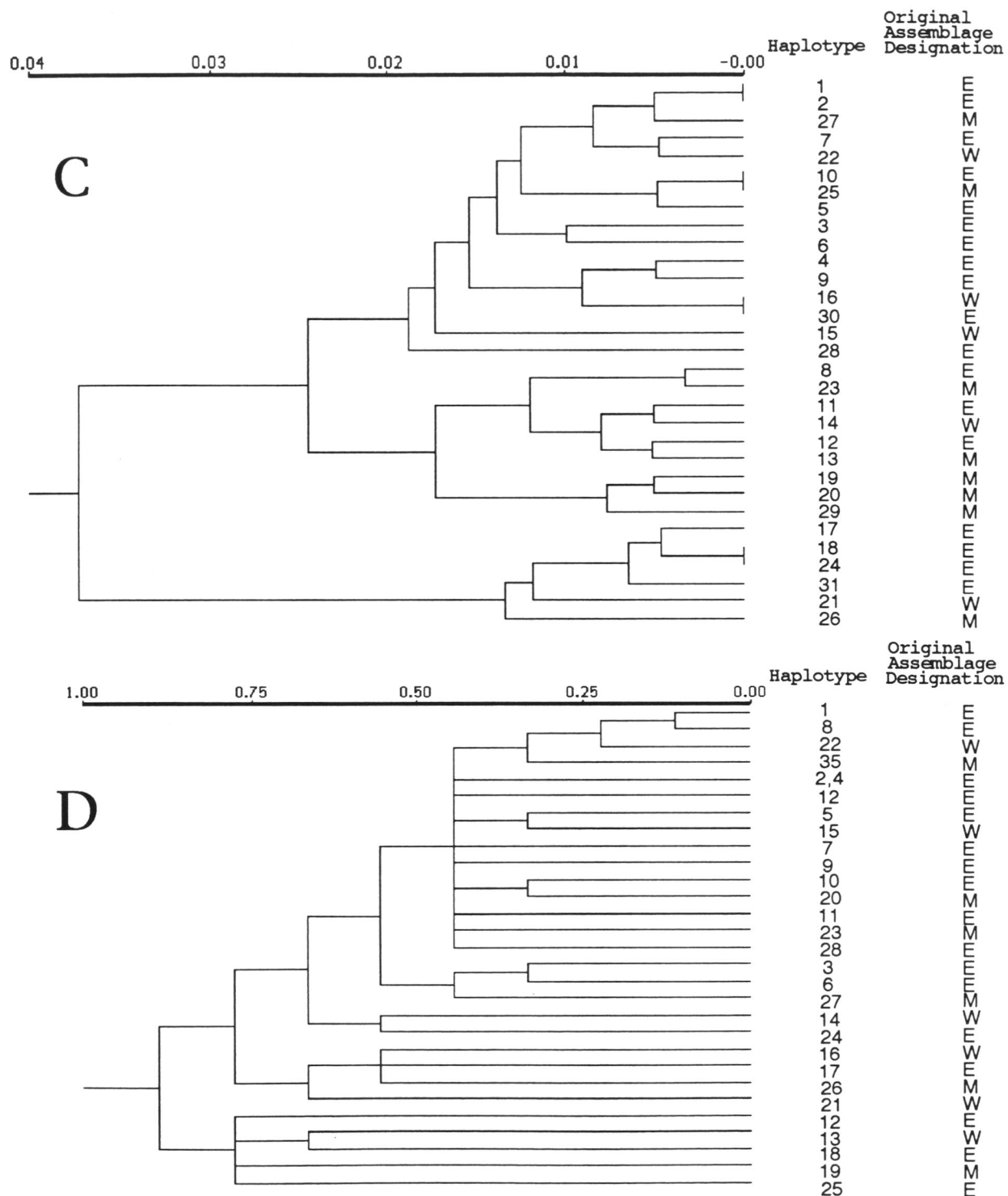
**Figure 6a.** Strict consensus tree of tortoise mtDNA genotypes produced by HUERISTIC option of PAUP exclusive of Alabama individuals (haplotype 36). Bootstrap values are listed on tree.



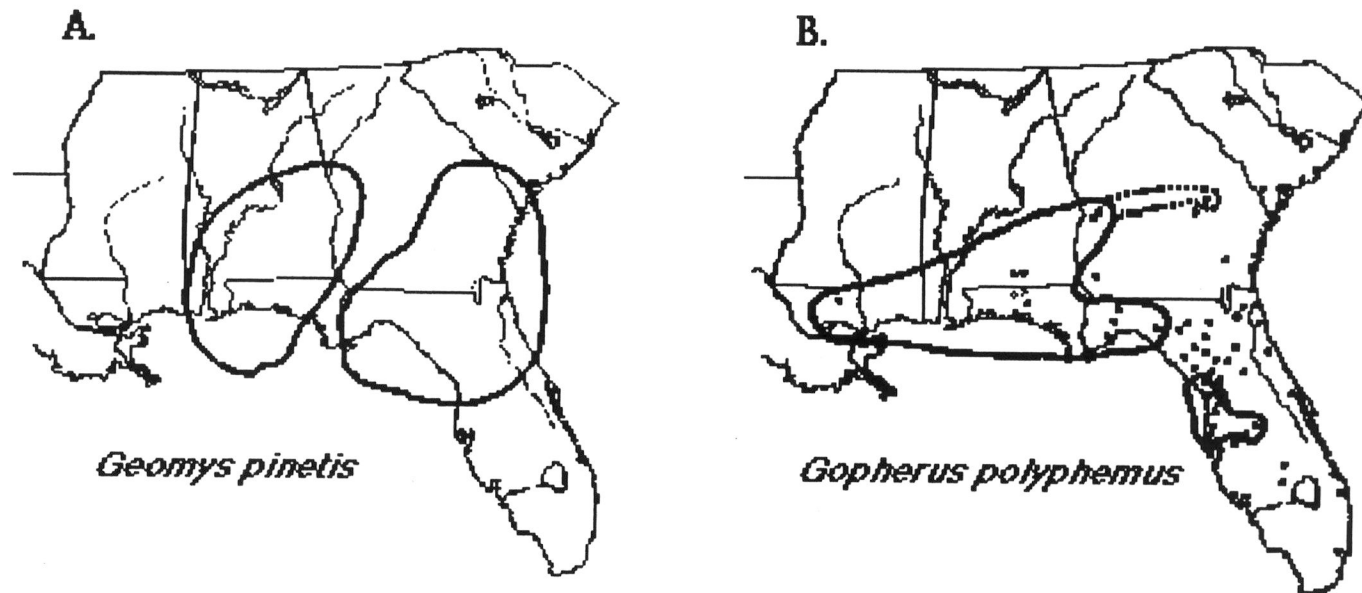
**Figure 6b.** Strict consensus tree of tortoise mtDNA genotypes produced by HUERISTIC option of PAUP including the Alabama individuals (haplotype 36). Bootstrap values are listed on tree.



**Figure 7a.** Phenograms depicting the relationship among mtDNA genotypes generated by UPGMA analysis of genetic distance ( $p$ ) estimates using three of the four amplified regions. Phenogram A: Cytochrome *b*/D-loop region excluded, Phenogram B: NADH5/NADH6 region excluded. Genotypes are classified by assemblage they appeared in UPGMA analysis of entire data set: E = Eastern assemblage, W = Western assemblage, and M = Mid-Florida assemblage.



**Figure 7b.** Phenograms depicting the relationship among mtDNA genotypes generated by UPGMA analysis of genetic distance ( $p$ ) estimates using three of the four amplified regions. Phenogram C: NADH2/CO I region excluded, Phenogram D: 12S/16S rRNA region excluded. Genotypes are classified by assemblage they appeared in UPGMA analysis of entire data set: E = Eastern assemblage, W = Western assemblage, and M = Mid-Florida assemblage.



**Figure 8.** Geographic arrangement of mtDNA genotypes of A) the pocket gopher (*Geomys pinetis*, Avise et al., 1979) and B) the gopher tortoise (*Gopherus polyphemus*).



**Table 1.** Estimates of base substitutions per nucleotide ( $p$ ) among mtDNA genotypes of the gopher tortoise, *Gopherus polyphemus*. Specific genotype localities appear in Appendix A.

| <u>Genotype</u> | <u>Genotype</u> |        |        |        |        |        |        |        |        |        |        |        |
|-----------------|-----------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                 | 1               | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| 1               | 0.0000          |        |        |        |        |        |        |        |        |        |        |        |
| 2               | 0.0078          | 0.0000 |        |        |        |        |        |        |        |        |        |        |
| 3               | 0.0118          | 0.0039 | 0.0000 |        |        |        |        |        |        |        |        |        |
| 4               | 0.0122          | 0.0040 | 0.0079 | 0.0000 |        |        |        |        |        |        |        |        |
| 5               | 0.0065          | 0.0064 | 0.0105 | 0.0104 | 0.0000 |        |        |        |        |        |        |        |
| 6               | 0.0117          | 0.0038 | 0.0078 | 0.0078 | 0.0104 | 0.0000 |        |        |        |        |        |        |
| 7               | 0.0103          | 0.0104 | 0.0145 | 0.0143 | 0.0038 | 0.0064 | 0.0000 |        |        |        |        |        |
| 8               | 0.0117          | 0.0038 | 0.0078 | 0.0079 | 0.0104 | 0.0078 | 0.0144 | 0.0000 |        |        |        |        |
| 9               | 0.0025          | 0.0079 | 0.0118 | 0.0122 | 0.0091 | 0.0117 | 0.0130 | 0.0117 | 0.0000 |        |        |        |
| 10              | 0.0117          | 0.0039 | 0.0078 | 0.0079 | 0.0104 | 0.0078 | 0.0143 | 0.0078 | 0.0117 | 0.0000 |        |        |
| 11              | 0.0151          | 0.0067 | 0.0107 | 0.0112 | 0.0080 | 0.0106 | 0.0119 | 0.0106 | 0.0151 | 0.0106 | 0.0000 |        |
| 12              | 0.0158          | 0.0078 | 0.0119 | 0.0118 | 0.0145 | 0.0039 | 0.0104 | 0.0091 | 0.0158 | 0.0118 | 0.0147 | 0.0000 |
| 13              | 0.0274          | 0.0189 | 0.0149 | 0.0234 | 0.0202 | 0.0230 | 0.0243 | 0.0202 | 0.0274 | 0.0147 | 0.0121 | 0.0189 |
| 14              | 0.0330          | 0.0238 | 0.0197 | 0.0253 | 0.0251 | 0.0279 | 0.0292 | 0.0251 | 0.0330 | 0.0195 | 0.0164 | 0.0238 |
| 15              | 0.0371          | 0.0279 | 0.0238 | 0.0294 | 0.0292 | 0.0235 | 0.0248 | 0.0292 | 0.0371 | 0.0235 | 0.0205 | 0.0195 |
| 16              | 0.0315          | 0.0230 | 0.0189 | 0.0274 | 0.0243 | 0.0187 | 0.0200 | 0.0243 | 0.0315 | 0.0187 | 0.0162 | 0.0147 |
| 17              | 0.0117          | 0.0200 | 0.0160 | 0.0243 | 0.0185 | 0.0156 | 0.0143 | 0.0240 | 0.0143 | 0.0240 | 0.0274 | 0.0199 |
| 18              | 0.0170          | 0.0091 | 0.0132 | 0.0131 | 0.0158 | 0.0051 | 0.0117 | 0.0131 | 0.0170 | 0.0131 | 0.0160 | 0.0091 |
| 19              | 0.0224          | 0.0264 | 0.0306 | 0.0212 | 0.0303 | 0.0303 | 0.0342 | 0.0303 | 0.0198 | 0.0303 | 0.0312 | 0.0346 |
| 20              | 0.0222          | 0.0261 | 0.0303 | 0.0210 | 0.0300 | 0.0300 | 0.0340 | 0.0300 | 0.0196 | 0.0220 | 0.0309 | 0.0342 |
| 21              | 0.0373          | 0.0287 | 0.0246 | 0.0331 | 0.0300 | 0.0243 | 0.0256 | 0.0300 | 0.0373 | 0.0243 | 0.0219 | 0.0202 |
| 22              | 0.0420          | 0.0333 | 0.0291 | 0.0377 | 0.0346 | 0.0287 | 0.0300 | 0.0346 | 0.0420 | 0.0287 | 0.0264 | 0.0246 |
| 23              | 0.0183          | 0.0223 | 0.0264 | 0.0172 | 0.0261 | 0.0183 | 0.0220 | 0.0261 | 0.0158 | 0.0261 | 0.0270 | 0.0223 |
| 24              | 0.0063          | 0.0117 | 0.0158 | 0.0161 | 0.0130 | 0.0078 | 0.0090 | 0.0156 | 0.0038 | 0.0156 | 0.0190 | 0.0117 |
| 25              | 0.0125          | 0.0041 | 0.0081 | 0.0087 | 0.0106 | 0.0080 | 0.0145 | 0.0080 | 0.0125 | 0.0080 | 0.0026 | 0.0120 |
| 26              | 0.0184          | 0.0223 | 0.0264 | 0.0172 | 0.0261 | 0.0261 | 0.0300 | 0.0261 | 0.0158 | 0.0261 | 0.0270 | 0.0303 |
| 27              | 0.0224          | 0.0264 | 0.0225 | 0.0212 | 0.0303 | 0.0303 | 0.0342 | 0.0303 | 0.0198 | 0.0303 | 0.0312 | 0.0346 |
| 28              | 0.0105          | 0.0105 | 0.0066 | 0.0145 | 0.0039 | 0.0145 | 0.0078 | 0.0145 | 0.0131 | 0.0145 | 0.0120 | 0.0188 |
| 29              | 0.0266          | 0.0306 | 0.0349 | 0.0254 | 0.0346 | 0.0346 | 0.0386 | 0.0346 | 0.0239 | 0.0346 | 0.0355 | 0.0390 |
| 30              | 0.0038          | 0.0040 | 0.0079 | 0.0085 | 0.0105 | 0.0079 | 0.0143 | 0.0079 | 0.0038 | 0.0079 | 0.0112 | 0.0118 |
| 31              | 0.0077          | 0.0079 | 0.0041 | 0.0124 | 0.0145 | 0.0118 | 0.0185 | 0.0118 | 0.0077 | 0.0118 | 0.0153 | 0.0159 |
| 32              | 0.0201          | 0.0121 | 0.0080 | 0.0161 | 0.0190 | 0.0162 | 0.0232 | 0.0162 | 0.0201 | 0.0162 | 0.0191 | 0.0205 |
| 33              | 0.0377          | 0.0291 | 0.0248 | 0.0335 | 0.0304 | 0.0333 | 0.0346 | 0.0304 | 0.0377 | 0.0246 | 0.0221 | 0.0291 |
| 34              | 0.0212          | 0.0132 | 0.0092 | 0.0172 | 0.0201 | 0.0091 | 0.0158 | 0.0173 | 0.0212 | 0.0173 | 0.0202 | 0.0132 |
| 35              | 0.0142          | 0.0174 | 0.0216 | 0.0223 | 0.0213 | 0.0213 | 0.0252 | 0.0213 | 0.0115 | 0.0213 | 0.0219 | 0.0255 |

**Table 1 (con't).** Estimates of base substitutions per nucleotide ( $p$ ) among mtDNA genotypes of the gopher tortoise, *Gopherus polyphemus*. Specific genotype localities appear in Appendix A.

| <u>Genotype</u> | 13     | 14     | 15     | 16     | 17     | <u>Genotype</u> |        | 20     | 21     | 22     | 23     | 24     |
|-----------------|--------|--------|--------|--------|--------|-----------------|--------|--------|--------|--------|--------|--------|
| 13              | 0.0000 |        |        |        |        | 18              | 19     |        |        |        |        |        |
| 14              | 0.0042 | 0.0000 |        |        |        |                 |        |        |        |        |        |        |
| 15              | 0.0082 | 0.0040 | 0.0000 |        |        |                 |        |        |        |        |        |        |
| 16              | 0.0040 | 0.0082 | 0.0042 | 0.0000 |        |                 |        |        |        |        |        |        |
| 17              | 0.0319 | 0.0375 | 0.0330 | 0.0274 | 0.0000 |                 |        |        |        |        |        |        |
| 18              | 0.0287 | 0.0337 | 0.0292 | 0.0243 | 0.0212 | 0.0000          |        |        |        |        |        |        |
| 19              | 0.0441 | 0.0465 | 0.0507 | 0.0483 | 0.0350 | 0.0358          | 0.0000 |        |        |        |        |        |
| 20              | 0.0352 | 0.0375 | 0.0415 | 0.0392 | 0.0346 | 0.0354          | 0.0075 | 0.0000 |        |        |        |        |
| 21              | 0.0093 | 0.0136 | 0.0095 | 0.0053 | 0.0331 | 0.0187          | 0.0543 | 0.0449 | 0.0000 |        |        |        |
| 22              | 0.0136 | 0.0179 | 0.0136 | 0.0093 | 0.0290 | 0.0230          | 0.0593 | 0.0495 | 0.0040 | 0.0000 |        |        |
| 23              | 0.0396 | 0.0419 | 0.0375 | 0.0352 | 0.0224 | 0.0236          | 0.0114 | 0.0113 | 0.0408 | 0.0454 | 0.0000 |        |
| 24              | 0.0315 | 0.0371 | 0.0327 | 0.0272 | 0.0103 | 0.0130          | 0.0237 | 0.0234 | 0.0328 | 0.0373 | 0.0119 | 0.0000 |
| 25              | 0.0149 | 0.0192 | 0.0233 | 0.0190 | 0.0247 | 0.0133          | 0.0285 | 0.0282 | 0.0248 | 0.0294 | 0.0243 | 0.0163 |
| 26              | 0.0396 | 0.0420 | 0.0461 | 0.0437 | 0.0306 | 0.0315          | 0.0037 | 0.0037 | 0.0495 | 0.0544 | 0.0075 | 0.0196 |
| 27              | 0.0355 | 0.0379 | 0.0419 | 0.0396 | 0.0266 | 0.0358          | 0.0076 | 0.0075 | 0.0454 | 0.0501 | 0.0114 | 0.0237 |
| 28              | 0.0161 | 0.0210 | 0.0251 | 0.0202 | 0.0145 | 0.0201          | 0.0346 | 0.0342 | 0.0259 | 0.0304 | 0.0303 | 0.0170 |
| 29              | 0.0488 | 0.0513 | 0.0556 | 0.0531 | 0.0309 | 0.0402          | 0.0115 | 0.0114 | 0.0593 | 0.0550 | 0.0154 | 0.0278 |
| 30              | 0.0234 | 0.0290 | 0.0330 | 0.0274 | 0.0158 | 0.0131          | 0.0212 | 0.0210 | 0.0331 | 0.0377 | 0.0172 | 0.0077 |
| 31              | 0.0194 | 0.0250 | 0.0290 | 0.0234 | 0.0118 | 0.0172          | 0.0254 | 0.0251 | 0.0290 | 0.0335 | 0.0212 | 0.0117 |
| 32              | 0.0235 | 0.0285 | 0.0327 | 0.0278 | 0.0161 | 0.0219          | 0.0394 | 0.0390 | 0.0337 | 0.0294 | 0.0349 | 0.0242 |
| 33              | 0.0095 | 0.0138 | 0.0179 | 0.0136 | 0.0335 | 0.0274          | 0.0550 | 0.0454 | 0.0080 | 0.0040 | 0.0501 | 0.0420 |
| 34              | 0.0246 | 0.0295 | 0.0251 | 0.0202 | 0.0172 | 0.0039          | 0.0403 | 0.0398 | 0.0147 | 0.0189 | 0.0276 | 0.0170 |
| 35              | 0.0345 | 0.0403 | 0.0445 | 0.0387 | 0.0264 | 0.0267          | 0.0076 | 0.0076 | 0.0446 | 0.0494 | 0.0114 | 0.0154 |

| <u>Genotype</u> | 25     | 26     | 27     | 28     | 29     | <u>Genotype</u> |        | 32     | 33     | 34     | 35     |
|-----------------|--------|--------|--------|--------|--------|-----------------|--------|--------|--------|--------|--------|
| 25              | 0.0000 |        |        |        |        | 30              | 31     |        |        |        |        |
| 26              | 0.0243 | 0.0000 |        |        |        |                 |        |        |        |        |        |
| 27              | 0.0285 | 0.0037 | 0.0000 |        |        |                 |        |        |        |        |        |
| 28              | 0.0147 | 0.0303 | 0.0264 | 0.0000 |        |                 |        |        |        |        |        |
| 29              | 0.0327 | 0.0075 | 0.0115 | 0.0390 | 0.0000 |                 |        |        |        |        |        |
| 30              | 0.0087 | 0.0172 | 0.0212 | 0.0145 | 0.0254 | 0.0000          |        |        |        |        |        |
| 31              | 0.0126 | 0.0212 | 0.0173 | 0.0106 | 0.0297 | 0.0039          | 0.0000 |        |        |        |        |
| 32              | 0.0163 | 0.0349 | 0.0309 | 0.0149 | 0.0269 | 0.0161          | 0.0121 | 0.0000 |        |        |        |
| 33              | 0.0251 | 0.0501 | 0.0459 | 0.0261 | 0.0506 | 0.0335          | 0.0293 | 0.0251 | 0.0000 |        |        |
| 34              | 0.0174 | 0.0358 | 0.0318 | 0.0160 | 0.0449 | 0.0172          | 0.0132 | 0.0177 | 0.0233 | 0.0000 |        |
| 35              | 0.0192 | 0.0039 | 0.0076 | 0.0255 | 0.0115 | 0.0129          | 0.0170 | 0.0301 | 0.0451 | 0.0311 | 0.0000 |

**Table 2.** MtDNA haplotypes observed in *Gopherus polyphemus*. Letters refer to digestion profiles produced by restriction endonucleases; their numerical superscripts distinguish the four amplified regions as follows:

| Haplotype | Composite Code by region: |                           |                          |                        |
|-----------|---------------------------|---------------------------|--------------------------|------------------------|
|           | 12S/16S rRNA <sup>a</sup> | cyt b/D-Loop <sup>b</sup> | NADH5/NADH6 <sup>c</sup> | NADH2/COI <sup>d</sup> |
| 1         | CCCCCCC                   | CCCCCCC                   | CCCCCCCC                 | CCCCC                  |
| 2         | CCCCCCC                   | CDCBCCC                   | CCCCCCCC                 | CCCCC                  |
| 3         | CCCCCCC                   | CDCBCCC                   | CCCCCCCC                 | CCCBCC                 |
| 4         | CCDCCCC                   | CDCBCCC                   | CCCCCCCC                 | CCCCC                  |
| 5         | CCCCCCC                   | CCCB CDC                  | CCCCCCCC                 | CCCCC                  |
| 6         | CCCCCCC                   | CDCBCCC                   | CCCCCCCCB                | CCCCC                  |
| 7         | CCCCCCC                   | CBCDCCC                   | CCCCCCCCB                | CCCCC                  |
| 8         | CCCCCCC                   | CDDBCCC                   | CCCCCCCC                 | CCCCC                  |
| 9         | CCCCCCC                   | CACCCCC                   | CCCCCCCC                 | CCCCC                  |
| 10        | CCCCCCC                   | CDCBCCC                   | CCCCCCCC                 | CCCCBC                 |
| 11        | CCCCCCC                   | CDCACDC                   | CCCCCCCC                 | CCCCC                  |
| 12        | CCCCCCC                   | CDBBCCC                   | CCCCCCCCB                | CCCCC                  |
| 13        | CCCCCCC                   | CDBACDC                   | CCCCCCCC                 | CCCBBC                 |
| 14        | CCBCCCC                   | CDBACDC                   | CCCCCCCC                 | CCCBBC                 |
| 15        | CCBCCCC                   | CDBACDC                   | CCCCCCCCB                | CCCBBC                 |
| 16        | CCCCCCC                   | CDBACDC                   | CCCCCCCCB                | CCCBBC                 |
| 17        | CCCCCCC                   | BCCCCC                    | CCCCCCCCB                | CCCBCC                 |
| 18        | DCCCCC                    | CDCBCCC                   | CCCCCCCCB                | CCCCC                  |
| 19        | CCDCBDC                   | CBCDCCC                   | CCCCCCCC                 | CCCCC                  |
| 20        | CCDCCDC                   | CBCDCCC                   | CCCCCCCC                 | CCCCBC                 |
| 21        | DCCCCC                    | CDBACDC                   | CCCCCCCCB                | CCCBBC                 |
| 22        | DCCCCC                    | BDBACDC                   | CCCCCCCCB                | CCCBBC                 |
| 23        | CCDCCCC                   | CBCDCCC                   | CCCCCCCCB                | CCCCC                  |
| 24        | CCCCCCC                   | CACCCCC                   | CCCCCCCCB                | CCCCC                  |

**Table 2 (con't).** MtDNA haplotypes observed in *Gopherus polyphemus*. Letters refer to digestion profiles produced by restriction endonucleases; their numerical superscripts distinguish the four amplified regions as follows:

|    |         |          |           |        |
|----|---------|----------|-----------|--------|
| 25 | CCCCCCC | CDCACCC  | CCCCCCCC  | CCCCCC |
| 26 | CCDCCDC | CBCDCCC  | CCCCCCCC  | CCCCCC |
| 27 | CCDCCDC | CBCDCCC  | CCCCCCCC  | CCCBCC |
| 28 | CCCCCCC | CCCB CDC | CCCCCCCC  | CCCBCC |
| 29 | CCDCCDC | BBCDBCC  | CCCCCCCC  | CCCCCC |
| 30 | CCCCCCC | CDCCCCC  | CCCCCCCC  | CCCCCC |
| 31 | CCCCCCC | CDCCCCC  | CCCCCCCC  | CCCBCC |
| 32 | CCCCCCC | BDCBBCC  | CCCCCCCC  | CCCBCC |
| 33 | DCCCCCC | BDBACDC  | CCCCCCCC  | CCCBBC |
| 34 | DCCCCCC | CDCBCCC  | CCCCCCCCB | CCCBCC |
| 35 | CCCCCDC | CBCDCCC  | CCCCCCCC  | CCCCCC |
| 36 | DCBCCCB | CABACCC  | CCCCCCCCB |        |

a 12S/16S rRNA letter codes depict profiles produced by the following seven enzymes (in order from left to right): *DpnII*, *HaeIII*, *HinfI*, *HinPI*, *MspI*, *RsaI*, *Taq<sup>a</sup>I*.

b cyt b/D-loop letter codes depict profiles produced by the following seven enzymes (in order from left to right): *BstUI*, *DpnII*, *HaeIII*, *HinfI*, *HinPI*, *RsaI*, *Taq<sup>a</sup>I*.

c NADH5/NADH6 letter codes depict profiles produced by the following eight enzymes (in order from left to right): *BstUI*, *DpnII*, *HaeIII*, *HinfI*, *HinPI*, *MspI*, *RsaI*, *Taq<sup>a</sup>I*.

d NADH2/CO I letter codes depict profiles produced by the following six enzymes (in order from left to right): *DpnII*, *HaeIII*, *HinfI*, *HinPI*, *RsaI*, *Taq<sup>a</sup>I*.

**Table 3.** Summary of turtle phylogeographic studies.

| Species                      | N   | Haplotypes | Assay approach                            | Reference                          |
|------------------------------|-----|------------|-------------------------------------------|------------------------------------|
| <i>Chelonia mydas</i>        | 226 | 4          | Restriction analysis with 6 base enzymes  | Bowen et al. (1989)                |
| <i>Lepidochelys kempi</i>    | 6   | 2          | Restriction analysis with 6 base enzymes  | Bowen et al. (1991)                |
| <i>Lepidochelys olivacea</i> | 21  | 1          | Restriction analysis using 6 base enzymes | Bowen et al. (1991)                |
| <i>Malaclemys terrapin</i>   | 53  | 6          | Restriction analysis using 6 base enzymes | Lamb and Avise (1992)              |
| <i>Trachemys scripta</i>     | 55  | 2          | Restriction analysis using 6 base enzymes | Birmingham and Avise (unpub. data) |
| <i>Xerobates agassizii</i>   | 56  | 5          | Restriction analysis using 6 base enzymes | Lamb et al. (1989)                 |
| <i>Graptemys geographica</i> | 12  | 1          | Restriction analysis using 6 base enzymes | Lamb et al. (submit'd)             |
| <i>Gopherus polyphemus</i>   | 112 | 36         | PCR-restriction approach                  | Osentoski and Lamb                 |

**APPENDIX A.** List of specific tortoise collection localities with corresponding composite geonotype designation(s).

| County              | Genotype | N | Locality                                                        |
|---------------------|----------|---|-----------------------------------------------------------------|
| Covington Co. AL    | 36       | 1 | Nellie's Pond, Conecuh Nat'l Forest                             |
| Escambia Co. AL     | 36       | 1 | Dixon Forestry Research Center                                  |
| Alachua Co. FL      | 2        | 1 | 2 mi. S of CR236, 1/4 mi E. Gilchrist Co. line. T8S-R17E-Sec 18 |
| Alachua Co. FL      | 2        | 1 | I-10 at State Hwy 121                                           |
| Alachua Co. FL      | 6        | 1 | Williston Rd. near GFC                                          |
| Alachua Co. FL      | 34       | 2 | Lochloosa Wildlife Mgmt Area, SR325 1 mi. S jct SR346           |
| Brevard Co. FL      | 9        | 3 | Merritt Island, Kennedy Space Center, Cape Canaveral            |
| Citrus Co. FL       | 1        | 1 | Citrus Wildlife Management Area, CR581 ca. 5 mi. S jct SR44     |
| Citrus Co. FL       | 27       | 1 | Homosassa                                                       |
| Citrus Co. FL       | 29       | 1 | Timberlane Dr. S of Beverly Hills                               |
| Columbia Co. FL     | 2        | 1 | Lake City Middle School                                         |
| Columbia Co. FL     | 2,3      | 2 | 1 mi. W CR131, 3.5 mi. N jct. SR18                              |
| Dade Co. FL         | 32       | 1 | Rehabilitation tortoise                                         |
| Dixie Co. FL        | 4        | 1 | CR349 1 mi. N CR351                                             |
| Duval Co. FL        | 2        | 1 | Dunn Ave. between Lem Turner Rd. and I-95 (Columbia 3)          |
| Duval Co. FL        | 2        | 1 | Plummers Rd. near Nassau Co. line                               |
| Duval Co. FL        | 30       | 1 | Dee Dot Ranch                                                   |
| Flagler Co. FL      | 5,25     | 4 | N side of St. Joe Rd, 1 mi E I-95 T11S-R31E-Sec.41              |
| Gilchrist Co. FL    | 5        | 1 | SR47, 2 mi. N of jct. of US 129                                 |
| Hernando Co. FL     | 23,24,26 | 3 | T21S-R18E-Sec.2                                                 |
| Highlands Co. FL    | 17       | 1 | Archbold Biological Station                                     |
| Highlands Co. FL    | 9        | 1 | Avon Park Bombing Range, T33S-R30E-Sec.28                       |
| Hillsborough Co. FL | 1        | 1 | 7.9 air mi. W Plant City, T28S-R20E-Sec.25, SE 1/4              |
| Lafayette Co. FL    | 2,25     | 2 | US 27 between Perry and Mayo                                    |
| Lee Co. FL          | 9        | 1 | I-75 N of Corkscrew Rd.                                         |

**APPENDIX A (con't).** List of specific tortoise collection localities with corresponding composite geonotype designation(s).

|                 |                |   |                                                             |
|-----------------|----------------|---|-------------------------------------------------------------|
| Leon Co. FL     | 14,15,34       | 3 | Nat'l Forest Rd 305, Approx. 2 mi W of State Hwy 319a       |
| Levy Co. FL     | 2              | 1 | SR121, 2.2 mi. N CR326                                      |
| Levy Co. FL     | 18             | 1 | CR326, 0.9 mi. E CR343, 3 mi. ENE of Gulf Hammock Community |
| Levy Co. FL     | 2              | 1 | Wacasassa Hunt Club N of Bronson                            |
| Marion Co. FL   | 35             | 1 | Rainbow Lake Estates, T15S-R18E-Sec. 19                     |
| Marion Co. FL   | 2              | 1 | US41, T15S-R18E-Sec.36                                      |
| Marion Co. FL   | 2              | 1 | Ocala Nat'l Forest, FR97 (Pipeline Rd) 2.9 mi. N SR 40      |
| Martin Co. FL   | 35             | 1 | US 1 approx. 2 mi. N of Tequesta                            |
| Pasco Co. FL    | 26             | 2 | Autumn Oaks Development, T24S-R17E-Sec2                     |
| Pinellas Co. FL | 26             | 1 | Vicinity of Tarpon Springs                                  |
| Polk Co. FL     | 26             | 1 | Teneroc State Park, T27S-R24E-ca.Sec25                      |
| Putnam Co. FL   | 2,3            | 3 | Ordway Preserve                                             |
| Okaloosa Co. FL | 2              | 1 | Eglin AFB                                                   |
| Osceola Co. FL  | 31             | 1 | Disney Property                                             |
| Orange Co. FL   | 19,20,26       | 3 | Disney Property N of US192 ca. 3-5 mi. W I-4                |
| Sarasota Co. FL | 1              | 3 | Venice, Caspersen County Park                               |
| Suwanee Co. FL  | 2              | 1 | CR349, 1.2 mi. S jct. SR51                                  |
| Suwanee Co. FL  | 2              | 1 | Dowling Park                                                |
| Volusia Co. FL  | 9              | 1 | US1 approx. 1 mi. N of Brevard Co. line                     |
| Wakulla Co. FL  | 16             | 1 | Ochlockonee State Park                                      |
| Wakulla Co. FL  | 13             | 1 | St Hwy 319, 0.8 mi. S of CR373A                             |
| Emanuel Co. GA  | 5,15           | 4 | Near I-16 just E of Hwy 1                                   |
| Marion Co. GA   | 10,11,12<br>14 | 8 | SR352, 0.35 mi. S of SR35                                   |
| Marion Co. GA   | 15             | 3 | St Hwy 352 0.32 mi. E of jct w/ St Hwy 355                  |
| Marion Co. GA   | 16             | 1 | CR64 0.8 mi. N of St Hwy 352                                |
| Miller Co. GA   | 2,8            | 7 | 7.3 mi. E of Colquitt on Belview Rd.                        |

**APPENDIX A (con't).** List of specific tortoise collection localities with corresponding composite geonotype designation(s).

|                     |          |    |                                        |
|---------------------|----------|----|----------------------------------------|
| Talbot Co. GA       | 14       | 1  | Hwy 96, 3.2 mi. W of W jct with Hwy 90 |
| Wayne Co. GA        | 5,6,7    | 5  | 3.9 mi. N of old Hwy 341 on Hwy 169    |
| Washington Prsh. LA | 21,22,33 | 7  | Ben's Creek Wildlife Management Area   |
| Jasper Co. SC       | 5,7,28   | 10 | Tillman Sandridge                      |



**Appendix B**  
**NaCl and Methylene Chloride DNA Extraction**

**Reagents**

Proteinase K (10 mg/ml)

20% SDS

STE (100 mM NaCl, 50 mM Tris pH 8.0, 10 mM EDTA pH 8.0)

5M NaCl

Methylene Chloride/Isoamyl Alcohol (24:1) solution

100% Ethanol

70% Ethanol

T<sub>10</sub>E<sub>0.1</sub>

**For each sample:**

1. In a sterile 1.5 ml microfuge tube, combine 40  $\mu$ l of whole blood (in sodium citrate buffer), 25  $\mu$ l Proteinase K (10 mg/ml), 35  $\mu$ l 20% SDS, and 450  $\mu$ l STE. Vortex the sample until homogeneous. The sample should appear milky white or slightly pink in color.
2. Digest sample at 55°C for one hour.
3. Add 150  $\mu$ l of 5M NaCl and shake vigorously. Add 45  $\mu$ l of 24:1 methylene chloride/isoamyl alcohol and shake until solution becomes homogeneous.
4. Place sample on ice for one-half to one hour.
5. Centrifuge in Eppendorf microcentrifuge at 2° C for 5 min at 5000 rpm, then increase to 9000 rpm for an additional 10 min.
6. Transfer the supernate to a new microfuge tube. Add 500  $\mu$ l 24:1 methylene chloride/isoamyl alcohol and shake until homogeneous. Centrifuge at 2° C for 5 min at 9000 rpm.
7. Carefully transfer the supernate to another new microfuge tube. Double the volume with 100% EtOH and add 150  $\mu$ l of 3M sodium acetate. Gently invert tube until fully mixed.
8. Place at -70° C for 2 hours to overnight.
9. Centrifuge at 2° C for 10-15 minutes at 14000 rpm to pellet the DNA.
9. Carefully decant the solution. Gently dislodge the pellet from the wall of the tube with 500  $\mu$ l of 70% EtOH and centrifuge at 2° C for 10 min at 14000 rpm.
10. Again, carefully decant the solution. Let DNA pellets dry (either air dry or speed vac).

## Appendix B (con't)

11. Resuspend the samples in 100  $\mu$ l ddH<sub>2</sub>O or 100  $\mu$ l of T<sub>10</sub>E<sub>0.1</sub>. Allow at least 4 hours for resuspension before using for amplification.

## Appendix C

### Specific Amplification Conditions

Region: **12S rRNA/16S rRNA**

Primers: **284/384**

Conditions: 5 minute at 94° C initial denaturation

Initial 5 cycles:

1 minute at 92° C  
2 minutes at 51° C  
3 minutes at 72° C

25 cycles at:

1 minute at 92° C  
2 minutes at 53° C  
3 minutes at 72° C

Region: **Cytochrome b/D-loop**

Primers: **H14724/L16464**

Conditions: 5 minute at 94° C initial denaturation

Initial 5 cycles:

1 minute at 92° C  
2 minutes at 51° C  
3 minutes at 72° C

25 cycles at:

1 minute at 92° C  
2 minutes at 53° C  
3 minutes at 72° C

Region: **NADH5/NADH6**

Primers: **763/764**

Conditions: 5 minute at 94° C initial denaturation

Initial 5 cycles:

1 minute at 92° C  
2 minutes at 50° C  
3 minutes at 72° C

25 cycles at:

1 minute at 92° C  
2 minutes at 52° C  
3 minutes at 72° C

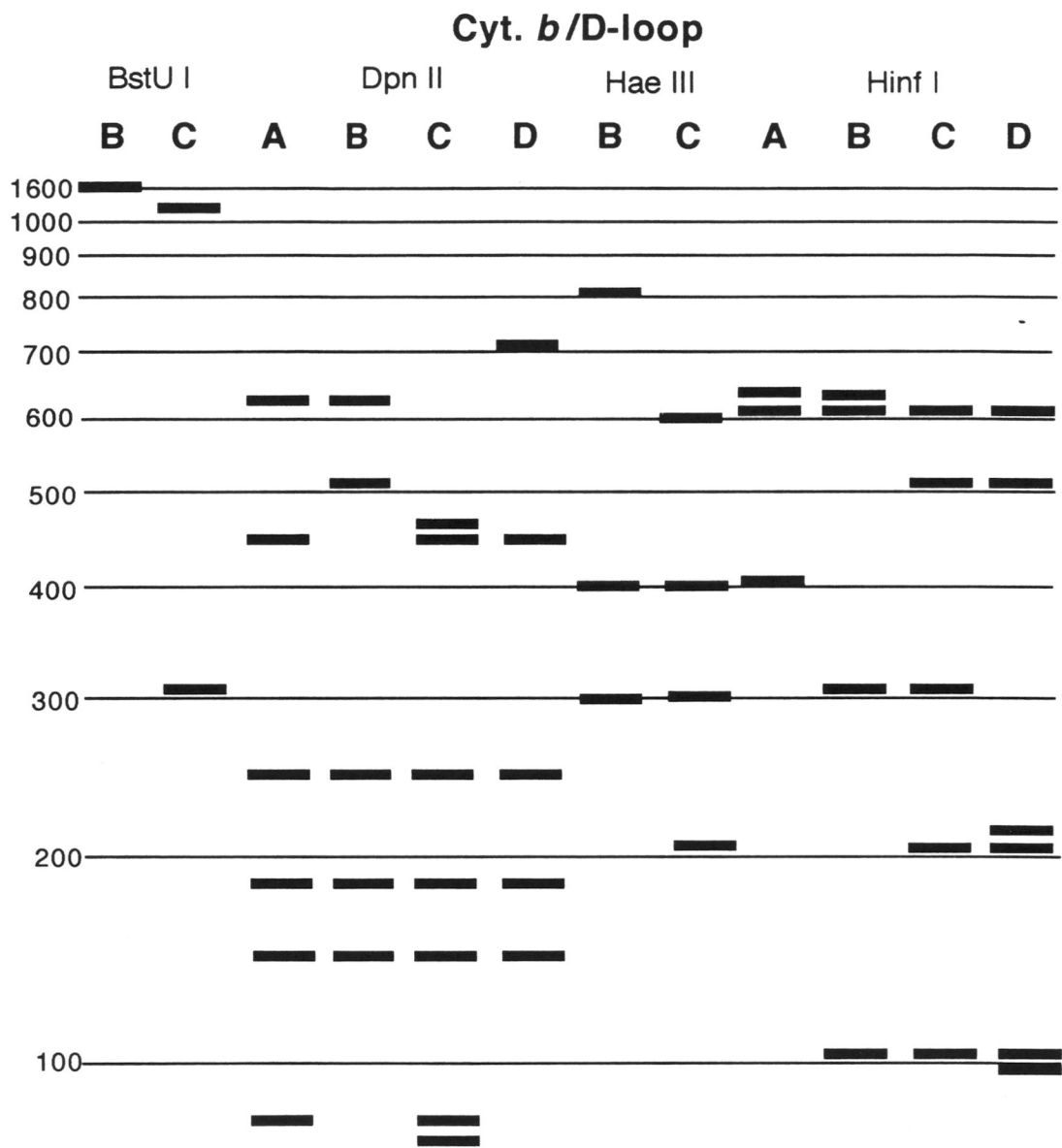
Region: **NADH2/Cytochrome Oxidase I**

Primers: **452/562**

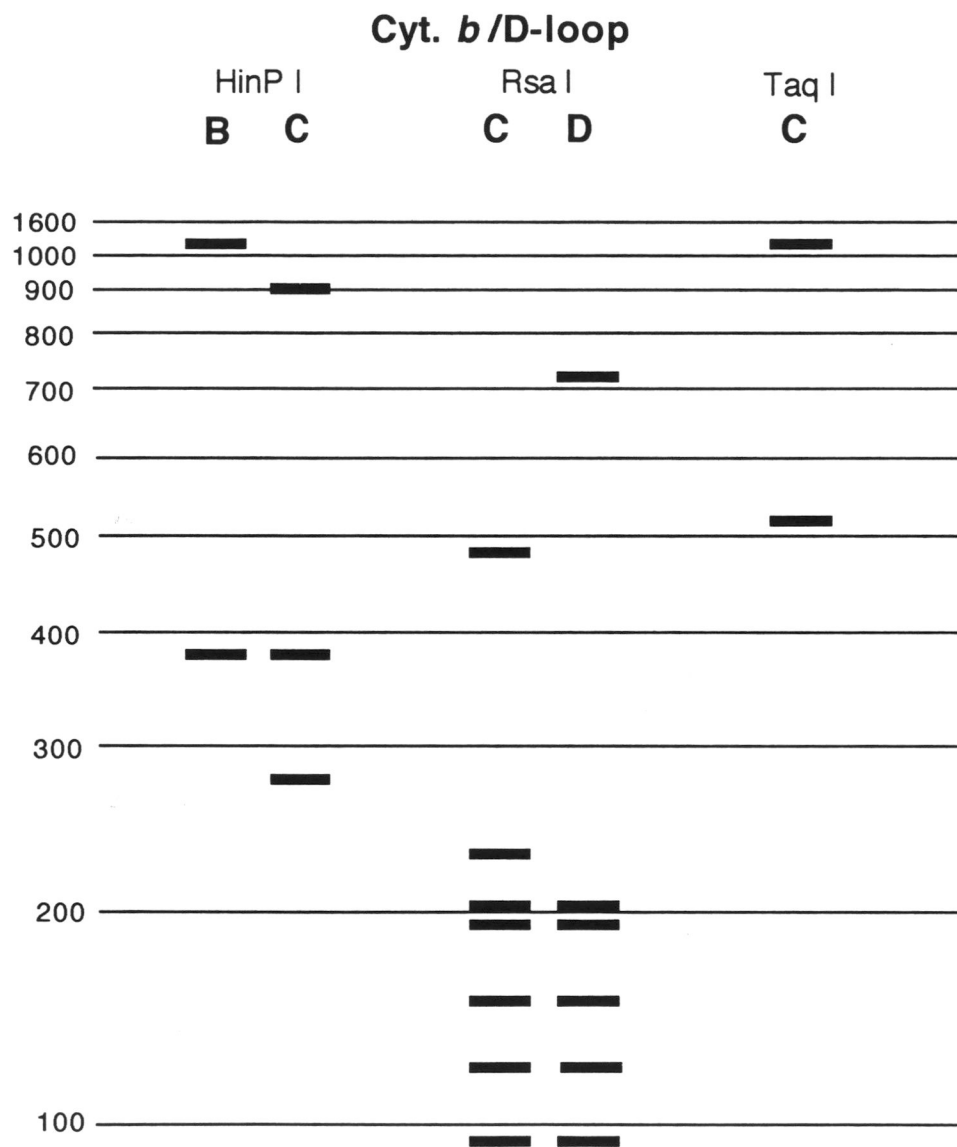
Conditions: 5 minute at 94° C initial denaturation

35 cycles:

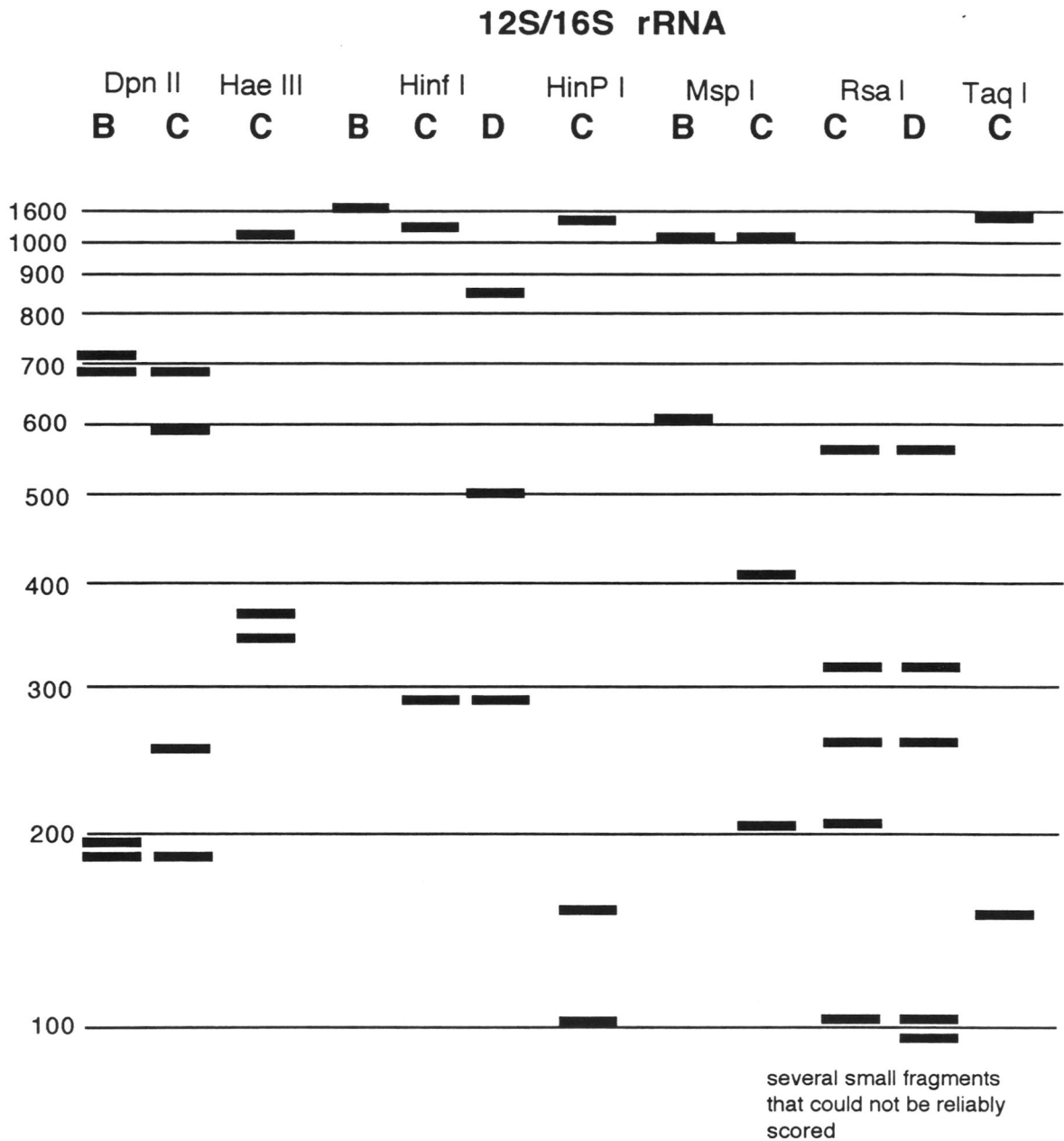
1 minute at 92° C  
2 minutes at 60° C  
3 minutes at 72° C



**Appendix D:** Restriction profiles generated from Cytochrome *b* /D-loop region.

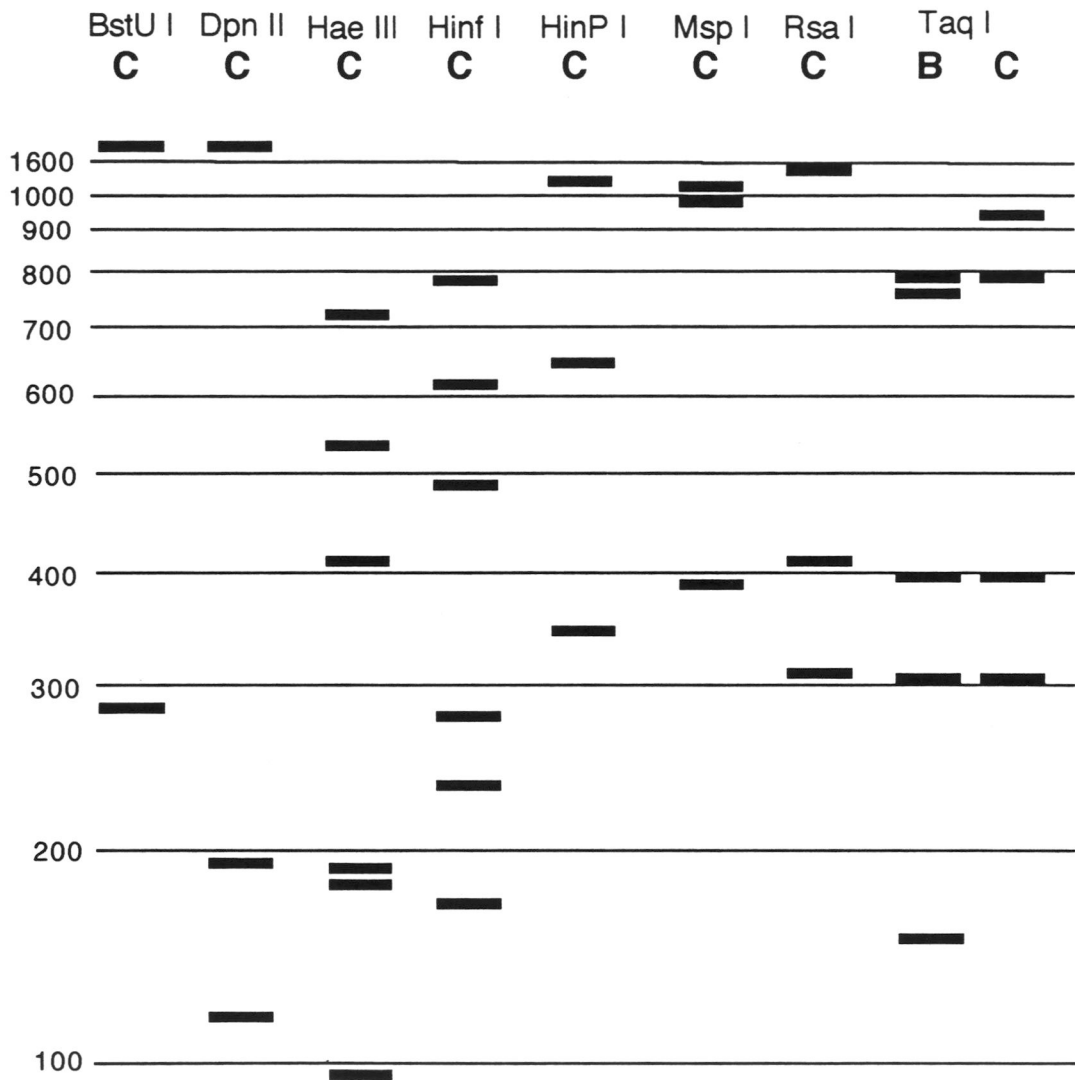


**Appendix D (con't):** Restriction profiles generated from Cytochrome *b* /D-loop region.



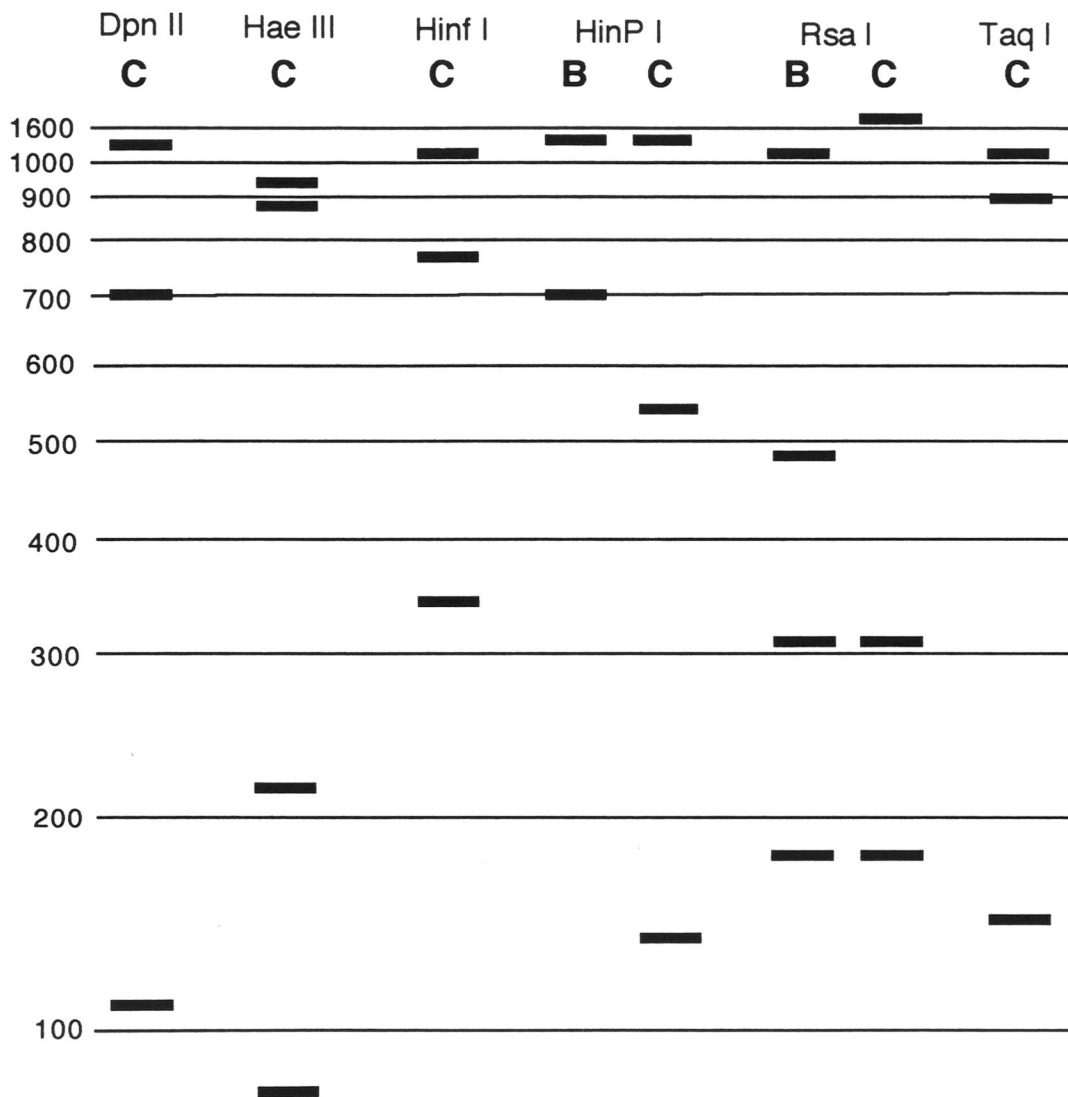
**Appendix D (con't):** Restriction profiles generated from 12S/16S rRNA region.

## NADH5/NADH6



**Appendix D (con 't):** Restriction profiles generated from NADH5/NADH6 region.

## NADH2/CO I



**Appendix D (con't):** Restriction profiles generated from NADH2/CO I region.