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Conservation Genetics and Systematics of Several Turtles Species in the Southeastern United States

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CONSERVATION GENETICS AND SYSTEMATICS OF SEVERAL TURTLES SPECIES IN THE SOUTHEASTERN UNITED STATES

by

Joshua Robert Ennen

A Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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ABSTRACT

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Chelonians (i.e., turtles) are an imperiled group of reptiles with about 66% of the recognized species listed as threatened by the IUCN. Most chelonian species have a unique set of life history traits (i.e., longevity, delayed sexual maturity, and low juvenile survivorship), which makes their populations exceedingly sensitive to increases in adult and juvenile moralities. With numerous anthropogenic effects (e.g., habitat alteration, exploitation, and over harvesting) negatively influencing mortality rates, chelonians have experienced global precipitous declines and extinctions.

This dissertation focuses on species within two chelonian genera, *Gopherus* and *Graptemys*. Although these two genera are vastly different ecologically, they are exposed to similar threats and possess similar conservation requirements. Within the genus *Graptemys* this dissertation focuses on the conservation genetics and systematics of three species (i.e., *G. gibbonsi*, *G. oculifera*, and *G. flavimaculata*) with distributions restricted to the Pearl River of Louisiana and the Pascagoula River of Mississippi. Since the taxonomic status of these species is still unresolved, I used molecular (i.e., mitochondrial) and morphological (only for the *G. oculifera* and *G. flavimaculata* comparison) data to assess the degree of differentiation and divergence between these species. The last two chapters focus on the conservation genetics of *Gopherus polyphemus*. In particular, I examined the level of genetic diversity within several western populations experiencing

aberrantly low hatching success using microsatellites, and conducted a range-wide phylogeographical study in an attempt to relate patterns of genetic structure with current management units for the species.

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I would like to thank my committee members for their assistance and patience during the completion of this dissertation. I like to thank B. Kreiser and C. Qualls for allowing me the flexibility to pursue many of my research interests. For his proclivity to find funding, I am forever indebted to C. Qualls. For his patience in the laboratory, I am greatly appreciative of B. Kreiser's assistance in the molecular side of many of my

projects. I thank J. Schaefer for comments and assistance in the multivariate statistical analyses, and M. Davis for his input and editorial efforts.

Finally, I would like to thank my family and friends for their unwavering support through my graduate career. In particular, I thank my parents S. and G. Ennen, my brother M. Ennen, and my sister S. Potter. Also, a special thanks to B. Fulmer. I would like to dedicate this dissertation to my late grandfather, R. G. German, Jr.

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CHAPTER I

GENETIC AND MORPHOLOGICAL VARIATION BETWEEN POPULATIONS OF THE PASCAGOULA MAP TURTLE (*GRAPTEMYS GIBBONSI*) IN THE PEARL AND PASCAGOULA RIVERS

Abstract

Cryptic species pose a major concern in conservation biology. Managing multiple species collectively as a single group could precipitate the loss of genetic variation and unique populations, and could even lead to extinction of an undiscovered species. An example of cryptic species phenomenon, *Graptemys pulchra* (*sensu lato*) was originally described as inhabiting Gulf coastal rivers from the Pearl River drainage in Louisiana to the Yellow River in Florida and south Alabama. Based on mostly colorimetric data, *G. pulchra* was split through the description of two new species, *G. gibbonsi* and *G. ernsti*. Each species, except for *G. gibbonsi*, possesses a drainage-specific distribution. Molecular data (mitochondrial DNA) later supported the recognition of each species in the "*pulchra* clade" (*G. pulchra*, *G. barbouri*, *G. ernsti*, and *G. gibbonsi*), but failed to include samples of *G. gibbonsi* from the Pascagoula River. Recently, *G. gibbonsi* was found to be less abundant than the two federally threatened species, *G. oculifera* and *G. flavimaculata*, that shares its range. My goal was to include *G. gibbonsi* samples from both rivers in a molecular assessment of the taxonomic status of this species. I compared the extent of genetic differentiation between *G. gibbonsi* populations with members within the "*pulchra* clade" and with *G. oculifera* and *G. flavimaculata*. My mtDNA sequence data showed greater genetic differentiation between the two *G. gibbonsi* populations than between the two recognized species, *G. oculifera* and *G. flavimaculata*,

but revealed only a modest degree of differentiation when compared to other members of the "*pulchra* clade."

Key Words.– *Graptemys*, Conservation, mtDNA, *G. gibbonsi*

Introduction

The phenomenon of cryptic species has been identified as a major concern in conservation biology (Lovich and Gibbons, 1997). Managing multiple species collectively as a single species could precipitate loss of genetic variation and unique populations within a species, and could even lead to extinction of an undiscovered species. Even after recognition, usually there is a lack of basic ecological knowledge for newly described species since earlier work on a group within a broad-ranging taxa was assumed to be applicable to the rest of its range. These scenarios are especially relevant to the Southeastern United States where researchers are still describing new species from previously believed wide-spread taxa (e.g., *Percina* – Williams et al., 2007; *Pseudacris* – Lemmon et al., 2008). The genus *Graptemys* is another example where new species have been described but with limited ecological study following the description.

Graptemys pulchra (Alabama map turtle) was split by Lovich and McCoy (1992) into three species (*G. ernsti*, *G. gibbonsi*, and *G. pulchra*) based on morphological data, which along with *G. barbouri* make up the "*pulchra* clade" (Lamb et al., 1994). All members of the "*pulchra* clade" possess restricted distributions, limited to only one of a few coastal basins along the eastern Gulf of Mexico in the southeastern United States (Figure 1.1). *Graptemys barbouri* and *G. pulchra* are each confined to a single drainage with *G. barbouri* in the Apalachicola River in Florida and southern Georgia (Sanderson and Lovich, 1998) and *G. pulchra* inhabiting the Mobile River drainage in Alabama

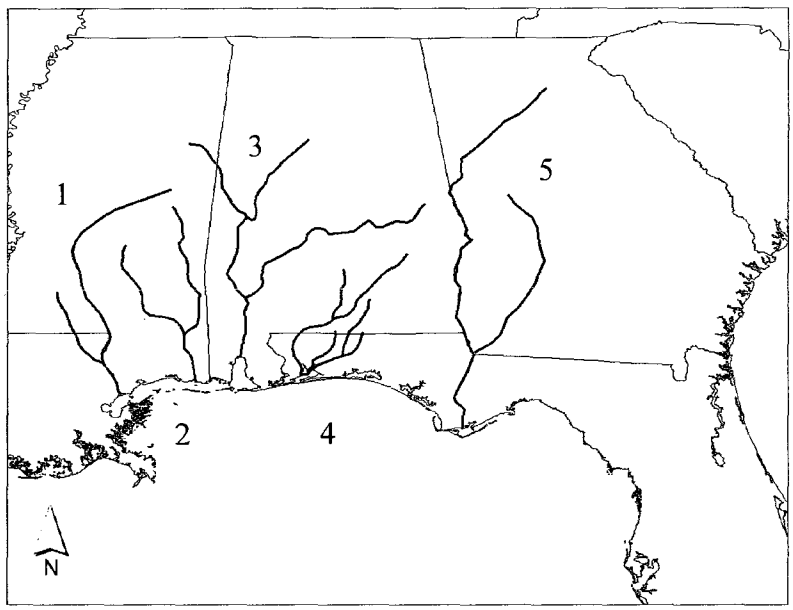

Figure 1.1. The five coastal drainages inhabited by six *Graptemys* species, *G. oculifera*, *G. flavimaculata*, *G. gibbonsi*, *G. pulchra*, *G. ernsti*, and *G. barbouri*. 1) Pearl River, 2) Pascagoula River, 3) Mobile Bay Basin, 4) Escambian Bay, and 5) Apalachicola River.

(Lovich and McCoy, 1992). Populations of *Graptemys ernsti* are known from several rivers (Conecuh, Escambia, Yellow, and Shoal) associated with the Escambia Bay drainage in the panhandle of Florida. *Graptemys gibbonsi* inhabits the Pearl and Pascagoula river systems in Louisiana and Mississippi and represents the only species in the "*pulchra* clade" not restricted to a single drainage system (Lovich and McCoy, 1992; Lovich and McCoy, 1994).

Interestingly, the distribution of *G. gibbonsi* overlaps that of two congeneric sister species, *G. oculifera* and *G. flavimaculata*, in the Pearl and Pascagoula rivers, respectively. Both mitochondrial DNA (Lamb et al., 1994) and morphological (Cagle, 1954) data have confirmed the sister status of these species. Sea level fluctuations associated with glacial cycles are likely the main mechanism behind speciation within the *Graptemys* genus (Lamb et al., 1994; Wood, 1977). The geologic history that led to the isolation and divergence of *G. oculifera* and *G. flavimaculata* would have also influenced the evolution of *G. gibbonsi* as well, but to what extent? Lovich and McCoy (1992) presented evidence that both *G. gibbonsi* populations in the Pearl and Pascagoula river drainages have limited divergence in morphological characters relevant to other members of the "*pulchra* clade" and suggested that they have been isolated for a relatively short period of time. This raises the question of how the geological history of the Pearl and Pascagoula rivers could result in speciation of the *G. oculifera/flavimaculata* ancestor, yet not produce a similar degree of genetic divergence between the *G. gibbonsi* populations in these same rivers. One possibility is that the Pearl and Pascagoula populations of *G. gibbonsi*, although morphologically similar, could represent "cryptic species" or "covert species." This phenomenon is taxonomically widespread (e.g.,

Lovich and Gibbons, 1997; salamanders- Tilley and Mahoney, 1996, Larson, 1984, Larson 1989; fishes- Kreiser et al., 2001), especially in species with a broad distribution.

"Cryptic species" are of interest to more than just systematists since they also pose challenges to conservation efforts. *Graptemys pulchra* (*sensu lato*) was only recently divided into multiple species by Lovich and McCoy (1992), and thus most of the relevant ecological literature pertains to *G. pulchra* (reviewed in Lovich and McCoy, in press a; Shealy, 1976) and *G. ernsti* (Shealy, 1976; reviewed in Lovich and McCoy, in press b). For *G. gibbonsi* there is a paucity of literature focusing on this species' basic ecology and life history making it one of the most poorly studied turtle species in the United States (J. E. Lovich, unpublished data.). Similarly, this is not an isolated instance within the genus *Graptemys*. Both *G. ernsti* and *G. versa* have poorly understood life histories (J. E. Lovich, unpublished data; Lindeman, 2005). This gap in our knowledge is unfortunate since, although, *G. gibbonsi* is listed as G3G4 and S3 in Mississippi and Louisiana, their populations have recently been reported as less abundant than the two federally threatened species *G. oculifera* (G2 and S2) and *G. flavimaculata* (G2 and S2) (Selman and Qualls, 2007). Because Lamb et al. (1994) failed to include both populations of G. gibbonsi, clarifying the taxonomic status of the two *G. gibbonsi* populations is also critical to identifying research needs and for effective management planning.

My goal was to use molecular (mtDNA) data to assess the taxonomic and conservation status of *G. gibbonsi* populations in the Pearl and Pascagoula Rivers through comparison with other *Graptemys* species in the same drainage and members of the "*pulchra*" clade. First, I used mtDNA sequences to compare the extent of genetic differentiation between *G. gibbonsi* populations with that found between *G.*

flavimaculata and *G. oculifera,* the sister species inhabiting the same drainages. Second, mitochondrial sequence data were to be used to compare the extent of genetic differentiation between *G. gibbonsi* populations with that found in recognized species within the "*pulchra* clade". These molecular comparisons provide an important extension to the work of Lamb et al. (1994), since they did not include individuals from both populations of *G. gibbonsi.*

Materials and Methods

Collections

I acquired samples of *G. gibbonsi*, *G. flavimaculata*, and *G. oculifera* from W. Selman from several localities (Figure 1.2). *Graptemys gibbonsi* was collected in the Chickasawhay River at Leakesville (31° 08.999 'N, 088° 32.853 'W), Leaf River north of Hattiesburg (31°22.610 'N, 089°16.641'W), Lower Pascagoula (30° 30.938 'N, 088° 36.197 'W), and the Pearl River at Columbia (31° 17.177 'N, 089° 52.479 'W). *Graptemys flavimaculata* was collected from both the Leaf and Chickasawhay river sites within the Pascagoula drainage, and *G. oculifera* was collected from the Pearl River at Columbia, Mississippi. For other species in the "*pulchra* clade", I acquired two tail tip samples (preserved in ethanol) from each of the three "*pulchra* clade" species, which were provided by B. Thompson. Both samples of *G. ernsti* were collected in the Conecuh River at River Falls, Alabama (31° 20.936.'N, 086° 31.772 'W). Likewise, both *G. barbouri* samples were collected from the Chipola River at a boat ramp near Mariena, Florida (30° 00.588'N, 085° 02.377'W). One *G. pulchra* sample was collected from the Tombigbee River at Tuscahoma Landing, Alabama (32° 03.672'N, 088° 06.646'W), while the other was collected from the Tallapoosa River in Elmore County, Alabama (32° 29.66.'N, 086° 14.23'W). Total genomic DNA was extracted with the

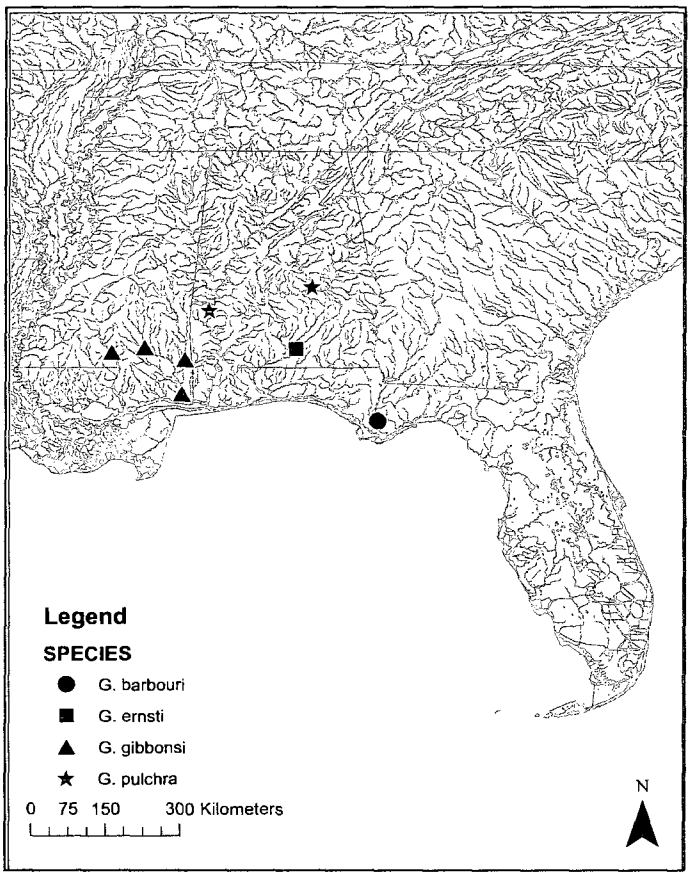

Figure 1.2. Distribution of samples across the southeastern United States including five coastal drainages (e.g., Pearl, Pascagoula, Mobile Bay, Escambia Bay, and Apalachicola) and the four species in the "*pulchra* clade."

DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA) and gel checked on agarose to assess DNA quality. Sequence data for *Chrysemys picta* obtained from GenBank (AF069423) was used as an outgroup in all the phylogenetic analyses.

mtDNA

Since Lamb et al., (1994) showed that the control region of mitochondrial genome had the most phylogenetic signal and cyt *b* provided poor phylogenetic resolution within *Graptemys*, I amplified a larger and separate portion of the control region and a different portion of the genome (i.e., ND4) using the primers reported by Spinks and Shaffer (2005). Amplifications were conducted in a total volume of either 25 µl or 50 µl using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 200 µM dNTPs, 2 mM $MgCl_2$, 0.5 units of *Taq* polymerase (Promega Co.), 0.3 µM of each primer, 20-150 ng of template DNA, and water to the final volume. PCR products were cleaned using the ExoSAP-IT system (USB Co., Cleveland, OH, USA), and then used as the template in a cycle sequencing reaction with an ABI BigDye Terminator cycle sequencing kit (Foster City, CA, USA) using the primers described above. All sequencing reactions were sephadex cleaned (Princeton Separations, Adelphia, NJ, USA) prior to gel runs at the Iowa State University DNA Sequencing and Synthesis Facility. Sequence data were edited and aligned using Sequencher v. 4.1 (GeneCodes Co., Ann Arbor, MI, USA).

PAUP* 4.0b10 (Swofford, 2002) was used to calculate pairwise uncorrected *p* distances between all haplotypes within the ingroup taxa. The degree of congruence in the phylogenetic signal of the control region and ND4 datasets was examined using the incongruence length difference test as implemented by PAUP* (Farris et al., 1994). Phylogenetic were inferred using maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. The maximum parsimony analysis was performed by PAUP*

with a branch-and-bound search, and the initial upper bound was calculated by stepwise addition. The most appropriate model of sequence evolution for the ML analysis was selected by ModelTest v. 3.5 (Posada and Crandall, 1998) as a HKY+G model with a Gamma distribution shape parameter of 0.0137. A Bayesian inference of the phylogeny was performed using MrBayes v. 3.1 (Ronquist and Huelsenbeck, 2003). Tree space was explored starting with a random tree and employing two independent runs of four Markov chains of 1,000,000 generations, each sampled every 100 generations. Plots of log-likelihood scores versus generation time were examined to ensure that each run had reached stationarity, and the first 2,500 trees were then discarded as burn-in. Phylogenetic support was assessed through bootstrapping (Felsenstein, 1985) with 1,000 rounds of resampling for the MP and ML analyses. The majority-rule consensus of the 7,500 trees saved by the Bayesian analysis was used to obtain the posterior probabilities of each clade.

Results

For the 6 species, I obtained sequences for 36 individuals for the control region (666 bp) and sequences of 40 individuals for ND4 (894 bp) using Spinks and Shaffer (2005) primers. The number of sequences and unique haplotypes for each species are provided in Table 1.1. For *G. gibbonsi* and *G. flavimaculata* in the Pascagoula, I sequenced individuals from the three different sites in that drainage. As expected, the control region was more variable than ND4 (Table1.2). Using the CR sequence data, the uncorrected *p* distance showed the two *G. gibbonsi* populations in the Pearl and Pascagoula rivers appear to have a greater sequence divergence ($p = 0.013$) than that of the two recognized species ($p = 0.005$), *G. oculifera* and *G. flavimaculata*. The CR

Table 1.1.

Number of individuals sequenced from each species for each gene and the number of unique haplotypes detected.

	Control Region		ND4	
	# Sequenced	# Unique Haplotypes	# Sequenced	# Unique Haplotypes
G. gibbonsi				
Pearl	9	3	9	1
Pascagoula	9	2	11	1
G. oculifera	6	2	8	2
G. flavimaculata	6	2	6	1
G. pulchra	2	2	2	2
G. barbouri	2	2	2	2
G. ernsti	2	2	2	1

Table 1.2.

Pairwise uncorrected *p* distance values for CR (below the diagonal) and ND4 (above the diagonal). The bolded diagonal values represent the CR and ND4 intraspecific sequence divergence, respectively.

	1	2	3	4	5	6	7	8
1. *C. picta*	-	0.078	0.074	0.075	0.076	0.076	0.076	0.077
2. *G. barbouri*	0.078	**0.011/0.003**	0.006	0.006	0.006	0.006	0.006	0.007
3. *G. ernsti*	0.083	0.029	**0.002/0.00**	0.007	0.007	0.007	0.007	0.007
4. *G. pulchra*	0.088	0.026	0.029	**0.014/0.007**	0.007	0.007	0.007	0.007
5. *G. gibbonsi* Pascagoula	0.092	0.035	0.033	0.032	**0.002/0.00**	0	0.004	0.005
6. *G. gibbonsi* Pearl	0.14	0.04	0.04	0.035	0.013	**0.004/0.00**	0.004	0.005
7. *G. flavimaculata*	0.093	0.031	0.035	0.033	0.031	0.038	**0.003/0.00**	0.001
8.. *G. oculifera*	0.09	0.03	0.033	0.032	0.027	0.041	0.005	**0.008/0.001**

uncorrected *p* distances between the recognized "*pulchra* clade" species and *G. gibbonsi* from the Pearl River were variable and ranged from 0.040 to 0.013 with the greatest divergence seen between *G. gibbonsi* (Pearl) and two other species, *G. barbouri* and *G.ernsti* (Table 1.2). The least divergent within the clade were the Pascagoula and Pearl River *G. gibbonsi* populations. *Graptemys ernsti* was equally divergent from *G. pulchra* as it was from *G. barbouri* (Table 1.2). The ND4 sequence data showed a uncorrected *p* distance (p = 0.001) between *G. oculifera* and *G. flavimaculata* and no sequence divergence between the two *G. gibbonsi* populations. Comparing the ND4 uncorrected *p* distances among species within the "*pulchra* clade," members had very similar sequence divergences between them, ranging from 0.000 to 0.007 (Table 1.2).

In the sequence data, 95 sites were variable in the control region and 80 in ND4, of which 50 and 11 were parsimony informative, respectively. The incongruence length test found congruent phylogenetic signal ($P = 1.0$) in the two data sets, so both were combined in all phylogenetic analyses. The MP analysis identified 2 equally parsimonious trees (L = 191, CI = 0.885, RI = 0.815). The ML (–lnL = 3064.92) and Bayesian phylogenetic analyses recovered the same basic overall topology, and the strict consensus of the two most parsimonious trees was selected to represent the phylogeny (Figure 1.3). Each of the species was recovered as a moderately to strongly supported monophyletic group, but there was no resolution among the different species (Figure 1.3). Internal nodes were all weakly supported, producing a basal polytomy of the four species in the "*pulchra* clade". With *G. gibbonsi*, individuals from the Pearl River form a strongly supported clade. However, I found only weak to moderate support for the monophyly of the two haplotypes from the Pascagoula River.

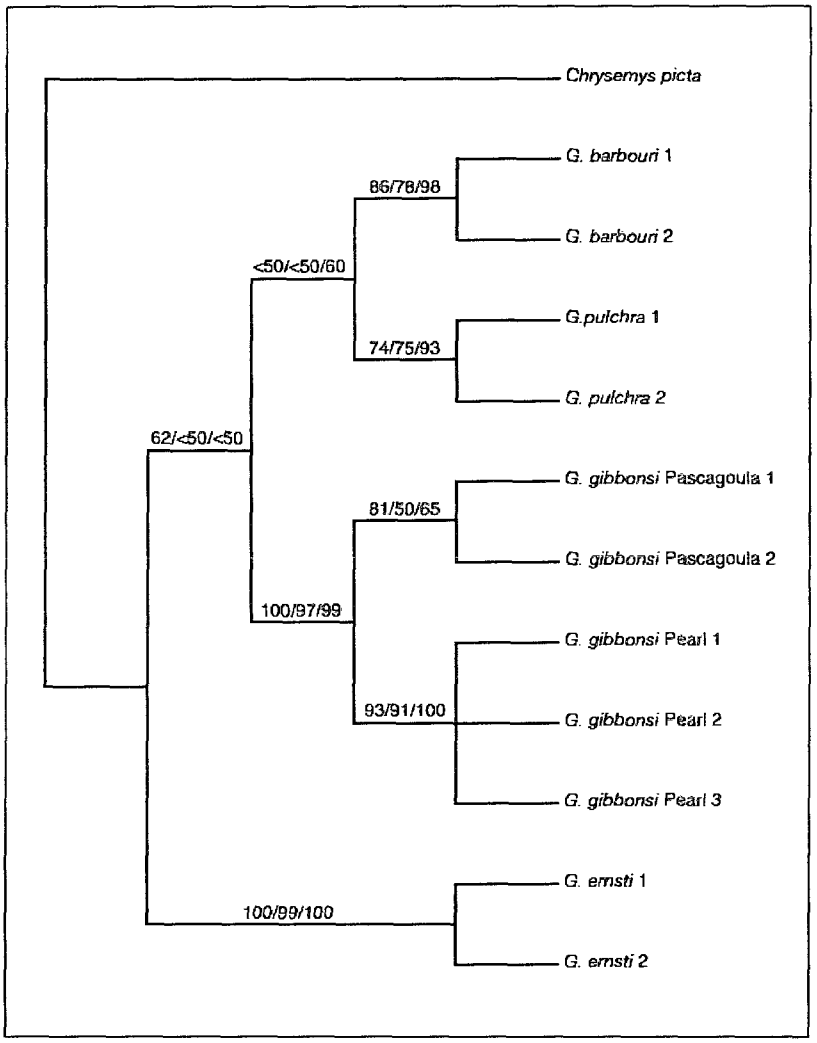

Figure 1.3. The strict consensus of the two most parsimonious trees (L = 191, CI = 0.885, RI = 0.815) recovered from the branch and bound search of the combined CR and ND4 sequence data. The support values are represented by MP and ML bootstrap and posterior probability.

Discussion

The Gulf of Mexico has experienced periodic fluctuations in sea level beginning with the Miocene (Riggs, 1984; Swift et al., 1986). Endemism patterns of Gulf Coast taxa, fishes in particular, have often been interpreted to be a consequence of the vicariance events associated with these sea level fluctuations (Wiley and Mayden, 1985; Swift et al., 1986). Recently this gulf coast allopatric speciation model has been rigorously tested in a phylogenetic framework for black basses (*Micropterus*; Near et al., 2003) and logperches (*Percina*; Near and Bernard, 2004). For *Micropterus*, the bulk of the speciation events took place during the Miocene and intraspecific diversification took place during the Pleistocene. However in *Percina*, 7 of 9 species diverged during the Pleistocene. Thus, the Gulf Coast allopatric speciation model is useful for understanding the biogeography of the region, but each taxon should be evaluated independently for how it fits within the framework of this model. The genus *Graptemys*, the "*pulchra* clade" in particular, is well known for its drainage-specific endemism (Lovich and McCoy, 1992), apparently linked to the historical fluctuations in sea level (Lamb et al., 1994; Lovich and McCoy, 1992; Wood, 1977). However, based on the comparison of the degree of genetic divergence in other chelonian genera, Walker and Avise (1998) suggested that the genus *Graptemys* is over split. They attributed this to the variety and variability of the color patterns on the heads and carapaces, which have been the focus of many species descriptions within this genus (Lovich and McCoy, 1992; Vogt, 1993; Cagle, 1954; Cagle, 1953).

The splitting of the "*pulchra* clade" resulted in all species but one, *G. gibbonsi*, having a drainage specific distribution. Lovich and McCoy (1992) noted that this exception in *G. gibbonsi* was consistent with the similarity of the fish fauna between the

Pearl and Pascagoula rivers as described by Swift et al. (1986). However, this is not to say that there are no morphological differences between *G. gibbonsi* in the two drainages. Both Lovich and McCoy (1992) and Shealy (1976) found drainage-specific colorimetric characteristics unique to populations in the Pearl and Pascagoula rivers.

However Lamb et al.'s (1994) molecular phylogeny of *Graptemys*, they were only able to recognize three distinct clades in the genus: *pulchra*, *pseudogeographica*, and *geographica*. However, their mitochondrial control region sequence data did support Lovich and McCoy's (1992) recognition of *G. pulchra* (*sensus lato*) as three distinct species, *G. pulchra*, *G. ernsti*, and *G. gibbonsi*. Even though I sequenced a different portion of the control region, my data were comparable to that of Lamb et al. (1994) in that I found similar levels of sequence divergence between species. Lamb et al.'s (1994) uncorrected *p* distances ranged from 0.020 to 0.044; while my data ranged from 0.026 to 0.040 for the same "*pulchra* clade" comparisons. However, Lamb et al. (1994) found the highest sequence divergence between *G. ernsti* and *G. barbouri* and the lowest sequence divergence between *G. pulchra* and *G. ernsti* (Table 1.3); while I found the highest sequence divergence between *G. gibbonsi* (Pearl) and both *G. barbouri* and *G. ernsti*, and the lowest sequence divergence between *G. barbouri* and *G. pulchra* (Table 1.2).

This study builds upon that of Lamb et al. (1994) in several important ways – by adding sampling of *G. gibbonsi* from the Pascagoula River and by placing the two populations of *G. gibbonsi* into the context of the rest of the "*pulchra* clade" and comparing them with the endemic species inhabiting the same drainages, *G. oculifera* and *G. flavimaculata*. *Graptemys gibbonsi* populations exhibit a much higher degree of sequence divergence in the control region compared to *G. oculifera* and *G. flavimaculata* (Table 1.3). I am not questioning the taxonomic status of *G. oculifera* and *G.*

Table 1.3.

Uncorrected *p* distance values from Lamb et al.'s (1994) data with CR (below the diagonal) and cyt *b* (above the diagonal) found within the "*pulchra* clade," *Graptemys oculifera*, and *Graptemys*

	1	2	3	4	5	6	7
1. *C. picta*	-	0.06	0.076	0.068	0.066	0.063	0.066
2. *G. barbouri*	0.124	-	0.015	0.013	0.01	0.01	0.01
3. *G. ernsti*	0.107	0.044	-	0.006.	0.003	0.003	0.003
4. *G. pulchra*	0.121	0.032	0.02	-	0.003	0.003	0.003
5. *G. gibbonsi*_Pearl	0.121	0.041	0.032	0.029	-	0	0
6. *G. flavimaculata*	0.118	0.061	0.047	0.041	0.05	-	0
7. *G. oculifera*	0.127	0.047	0.041	0.029	0.047	0.017	-

flavimaculata, since that is beyond the scope of this study, and there is ample evidence of morphological differentiation between these two species (R. Jones, Mississippi Museum of Natural Science, personal communication; Selman and Qualls, 2007; Selman and Qualls, 2006; Cagle, 1954).

The amount of control region sequence divergence between the two populations of *G. gibbonsi* was at the low end of the range exhibited among species in the "*pulchra* clade", but it was comparable to the levels of intraspecific differentiation exhibited by *G. pulchra* and *G. barbouri* (Table 1.2). This observation has several interesting biogeographic implications. The intraspecific variation within *G. gibbonsi* can easily be explained by the isolation of the two populations inhabiting two distinct drainages, the Pearl and Pascagoula Rivers. On the other hand, *G. pulchra* inhabits a single, large drainage system (the Mobile Bay Basin), but my samples were collected in two distinctive river systems within the drainage, the Tombigbee in the west and the Tallapoosa in the east. This pattern of divergence and endemism associated with portions of the Mobile Bay Basin is a common theme in freshwater fishes (e.g., several species of *Etheostoma* darters and cyprinids; Boschung and Mayden, 2004). While the divergence in *G. pulchra* and *G. gibbonsi* has an obvious biogeographic interpretation, the variation seen in my two samples of *G. barbouri* from a single site lacks a simple explanation. Overall, even with my limited data, there appears to be evidence for additional cryptic biodiversity within the "*pulchra* clade."

The distribution of *G. gibbonsi* in both the Pearl and Pascagoula Rivers is at odds with the drainage-specific endemism exhibited by the genus in Gulf Coast Rivers. Since connections between the two rivers may have occurred in a common estuary as recently as the Pleistocene (Lovich and McCoy, 1992), it is conceivable that populations of *G.*

gibbonsi in both rivers have been separated for a relatively short period of time. It is reasonable to consider the possible taxonomic significance and management implications of the genetic and morphological variation detected by my research (Fallon, 2007). The latter issue is of particular importance relative to the conservation status of *G. gibbonsi*, as currently recognized. The species has an IUCN 2007 Red List Status of "Lower Risk / Near Threatened" as assessed in 1996. In addition all *Graptemys* species are listed in Appendix III of CITES in recognition of their commercial value. Population surveys summarized in Lovich and McCoy (in press c) suggest that *G. gibbonsi* should be considered for listing as threatened under the U.S. Endangered Species Act and Endangered by the IUCN due to population declines. Likewise, recent bridge surveys in both the Pearl and Pascagoula River drainages found a lower abundance of *G. gibbonsi* relative to the two federally endangered species inhabiting the same drainages, *G. flavimaculata* and *G. oculifera* (Selman and Qualls, 2007).

In light of the putative recency of divergence for *G. gibbonsi* in the Pearl and Pascagoula Rivers, I feel more data (i.e., morphological reassessment) is needed to make a taxonomical recommendation for the Pearl River population. It is interesting that the reciprocally allopatric narrowheaded *Graptemys* that occur with *G. gibbonsi* (*G. flavimaculata* in the Pascagoula River and *G. oculifera* in the Pearl River) show lower levels of genetic differentiation than the two populations of *G. gibbonsi*. As noted by Cagle (1954), those two species likely evolved from a common ancestor as indicated by their morphological similarity, and they have been recognized as subspecies in some taxonomic treatments (Mertens and Wermuth, 1955). I do not consider the use of subspecies useful in the case of *G. gibbonsi* (Frost and Hillis, 1990), but recognize that

conservation is most effective if evolutionary units of some form are recognized and managed accordingly (Lovich and Gibbons, 1997).

My analysis suggests that *G. gibbonsi* should at least be recognized as two ESUs: one in the Pearl and the other in the Pascagoula rivers. Regardless of the below-species designations and their definitions, my data show that *G. gibbonsi* populations in the Pearl and Pascagoula rivers are distinctive genetically. Failure to recognize this diversity in conservation planning could result in loss of significant evolutionarily lineages (Lovich and Gibbons, 1997) in region well-known for its biological diversity (Lydeard and Mayden, 1995).

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CHAPTER II

A MORPHOLOGICAL AND MOLECULAR REASSESSMENT *GRAPTEMYS OCULIFERA* AND *G. FLAVIMACULATA*

Abstract

The turtle genus *Graptemys* consists of 15 recognized species and subspecies, distinguished largely on the basis of pigmentation pattern, head size, and shell morphology. However, molecular data have only resolved three clades in the genus (*pulchra*, *pseudogeographica*, and *geographica*) with many closely related species, including *G. flavimaculata* and *G. oculifera*, demonstrating low levels of sequence divergence. Both *G. oculifera* (Baur) and *G. flavimaculata* Cagle have been recognized as species since 1890 and 1954, respectively. The elevation of *G. flavimaculata* to full species status, however, was based on a limited number of characters. Several of these characters overlap between *G. flavimaculata* and *G. oculifera*, and no attempt was made to test for significant morphological differentiation. In this study I re-evaluated the morphological and genetic distinctiveness of *G. flavimaculata* and *G. oculifera* with 1) a multivariate statistical analyses of 44 morphological characters and 2) 1560 bp of sequence data from two mitochondrial genes (control region and ND4). The morphological and molecular analyses produced incongruent results. Principal components analysis (PCA) ordinations of the morphological data separated the two species along a pigmentation gradient with *G. flavimaculata* having more yellow pigmentation than *G. oculifera*. Likewise, clustering analyses separated the specimens into two distinct groups with little overlap between the species. However, the molecular data supported previous findings of limited genetic differentiation between the two species. Regardless of any taxonomic considerations, the two species should continue to

be treated as independent evolutionary units to preserve the morphological differences displayed between the two drainages.

Key words.– Conservation, *Graptemys*, Morphometrics, mtDNA, taxonomy

Introduction

The systematics and evolutionary history of the genus *Graptemys* has long been controversial (Lovich and McCoy, 1992) and remains so today. Within the North American family Emydidae, the genus *Graptemys* is the most speciose (Ernst and Lovich, 2009). Unlike other turtle genera that are usually morphologically conserved, *Graptemys* species have various shell or soft tissue patterns that often distinguish drainage-specific species (Walker and Avise, 1998). *Graptemys flavimaculata* (endemic to the Pascagoula River) and *G. ocuilfera* (endemic to the Pearl River) were described by Cagle (1954) and Baur (1890), respectively. Cagle (1954) proposed several diagnostic morphological characters to differentiate the two species, including *G. flavimaculata* having: 1) a broad orbital mark usually connected to a neck stripe, 2) broad yellow lines dominating the lower jaw, and 3) each costal scute with a large yellow blotch or crescent. However, several of the putatively diagnostic characters proposed by Cagle (1954) actually overlap between the species (e.g., shape of postorbital blotch, connection of neckline with postorbital blotches, and number of lines entering the orbit). Other diagnostic characteristics consisted of additional pattern differences (e.g., width of interorbital lines, neck lines entering orbital, markings on lower jaw, and markings of extremities), but these differences were never quantified and tested statistically. Later, and without supporting data, Mertens and Wermuth (1955) included *G. flavimaculata* as a subspecies of *G. oculifera*, but this taxonomic change was neither supported by analysis nor adopted by the scientific community.

Recent phylogenetic studies have not been particularly successful in resolving relationships among species in the genus *Graptemys*. Lamb et al. (1994) collected data on whole mitochondrial genome restriction sites and sequences for fragments of two mitochondrial genes (control region – 344 bp; cytochrome *b* – 380 bp). The combined data analysis only found support for three clades which they identified as a "*pulchra*" group, a "*pseudogeographica*" group and the basal *G. geographica*. Although the control region data were able to identify each species, there was typically little genetic differentiation among species. For example, only two bases (uncorrected *p* distance of 0.006) differed between *G. flavimaculata* and *G. oculifera*. A broader study by Stephens and Wiens (2003) for the family Emydidae combined existing molecular data with a large (300 character) data set. Analysis of the combined data found that relationships among *Graptemys* species were mostly poorly resolved with very weak bootstrap (62%) support for a monophyletic *G. flavimaculata* and *G. oculifera*. The limited degree of genetic divergence among species of *Graptemys* compared to other species of freshwater turtles led Walker and Avise (1998) to propose that the genus may be oversplit.

The low level of genetic divergence and lack of rigorous statistical tests of morphological differences raises questions as to the taxonomic validity of *G. flavimaculata* and *G. oculifera*. This is not just a question of academic interest since both species are federally listed as threatened (U.S. Fish and Wildlife Service 1986 and 1991) and listed as endangered by the state of Mississippi (Mississippi Department of Wildlife, Fisheries, and Parks, 2000). The goals of this study were to reevaluate the distinctiveness of *G. flavimaculata* and *G. oculifera* through 1) multivariate statistical analyses of a suite of morphological characters from the original species description and others used in similar studies within the genus *Graptemys* (Lovich and McCoy, 1992; Vogt, 1993) and

2) the analysis of a larger molecular data that includes different portions of the mitochondrial genome.

Materials and Methods

Morphological

Preserved specimens of *Graptemys oculifera* (55 specimens; 24 females, 31 males) and *G. flavimaculata* (93 specimens; 19 females, 74 males) were examined from the Mississippi Museum of Natural Sciences (MMNS) and the Tulane University Museum of Natural History (TU) (Appendix 1). I selected 44 characters (Table 2.1) from Cagle's (1954) description of *G. flavimaculata* and from the taxonomic literature on other *Graptemys* species (Lovich and McCoy, 1992; Vogt, 1993). All measurements were taken on the right side of each specimen.

Each sex was analyzed separately to account for sexual dimorphism (Gibbons and Lovich, 1990; Lovich and McCoy, 1992). To correct for size differences within each sex, each quantitative variable was divided by carapace or plastron length, and all ratio data were arcsine square root transformed to meet the assumptions of normality. Principal components analyses (PCA) were performed to visualize the data for males and females in multidimensional space. To test for significant differences between *G. oculifera* and *G. flavimaculata*, I used Euclidean distances to create a dissimilarity matrix of the quantitative variables and I performed a non-parametric multi-response permutation procedure (MRPP) with 50,000 permutations. MRPP is a re-sampling approach testing for a difference between groups (McCune and Grace, 2002). To determine which of the characters were driving the separation in the multidimensional space, I used the highest and lowest loading scores (i.e., absolute value of ≥ 0.20). For the qualitative variables, dissimilarity matrices were again created using Euclidean distances. These were then

used in unweighted pair group method with arithmetic means (UPGMA) cluster analyses, which when coupled with cophenetic correlation, provided a measure of how much structure was recovered from the raw data. All statistical analyses were performed using R statistical software (R Development Core Team, Version 2.8.0, 2008).

Molecular

Blood samples from a total of fourteen individuals were collected under the appropriate permits by W. Selman. The *G. flavimaculata* were either from the Chickasawhay River at Leakesville (31° 08.999 'N, 088° 32.853 'W; n = 2), Leaf River north of Hattiesburg (31°22.610 'N, 089°16.641'W; n = 2) or the lower Pascagoula River (30° 30.938 'N, 088° 36.197 'W; n = 2), while the *G. oculifera* were all from the Pearl River at Columbia (31° 17.177 'N, 089° 52.479 'W; n = 8). Total genomic DNA was extracted from the blood samples with a DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA). Lamb et al. (1994) found that the control region (CR) of the mitochondrial genome had more phylogenetic signal than cytochrome *b* (cyt *b*) within *Graptemys*. I elected to examine a separate portion of the CR as well as another mitochondrial gene (NADH dehydrogenase subunit 4 - ND4). Amplifications of the CR I performed with the primers of Spinks and Shaffer (2005). Likewise, for ND4 we used one of the primers reported by Spinks and Shaffer (2005), but I created a new primer (ND4a; 5'-TGACTACCAAAAGCACACGTAGAAGC-3') by modifying the ND4-672 primer to match the sequence of *Chrysemys picta* (GenBank Accesion AF069423) taken from GenBank. Polymerase chain reaction (PCR) amplifications were conducted in a total volume of either 25 µl or 50 µl using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 200 µM dNTPs, 2 mM $MgCl_2$, 0.5 units of *Taq* polymerase (Promega Co.), 0.3 µM of each primer, 20-150 ng of template DNA, and water to the final volume. PCR

Table 2.1.

List of potential quantitative and qualitative morphological variables and the description of the characters that were measured in this study.

Variable Category	Description of Characters
Quantitative Variables	
Shell Measurements	
Carapace	Carapace Length (CL), Carapace Height (CH), Spine Height (SH) Width of the yellow pigmentation on the first vertebral scute (WVPIG), Width of yellow and dark pigmentation on the 5th marginal scute dorsally (WPIGD and WDPD) and ventrally (WPIGV and WDPV), Length of the 5th marginal scute (MLNG)
Plastron	Plastron Width (PW), Plastron Length (PL), Gular Lengths (G), Humeral Length (H), Pectoral Length (P), Abdominal Length (AB), Femoral Length (F), Anal Length (AN), Width and Length of the yellow blotch on the axial scute (WYAP and LYAP), Width and Length of the yellow blotch on the inguinal scutes (WYIP and LYIP)
Soft Tissue	Length and Width of interorbital line (LIOL and WIOL), Width of the upper and lower neck lines enter in the orbital (NLL and NLU), Width of dark line between the upper and lower neck lines enter in the orbital (WBLO), Width of 2nd (WY2F) and 4th (WY4F) yellow line on the forelimb, Width of dark pigmentation between the 2nd and 4th lines on the forelimb (WB24), Width of 2nd (WY2H) and 4th (WY4H) hind limb lines, Width of dark pigmentation between the 2nd and 4th lines on the hind limbs (WDH)
Qualitative Variables	
Presence/Absence	Neckline extending past the interorbital line (NLIOL), 3rd digit yellow line extending through the elbow (3YFE), Ventral line connect under the chin (LLC), and a "U" shaped bar under the jaw (YUC).
Meristics	(#YHE and #YLFE), Dorsal yellow neck lines touching the postorbital blotch (#NLPOB), Number of lines entering the orbit (#NLO)
Categorical	Classification of the costal scute markings: 0 = blotch, 1 = ring, and 2 = broken ring

products were cleaned using the ExoSAP-IT system (USB Co., Cleveland, OH, USA),and then used as the template in a cycle sequencing reaction with an ABI BigDye Terminator cycle sequencing kit (Foster City, CA, USA) using the primers described above. All sequencing reactions were sephadex cleaned (Princeton Separations, Adelphia, NJ, USA) prior to gel runs at the Iowa State University DNA Sequencing and Synthesis Facility. Sequence data were edited and aligned using Sequencher v. 4.1 (GeneCodes Co., Ann Arbor, MI, USA). PAUP* 4.0b10 (Swofford, 2002) was used to calculate pairwise uncorrected *p* distances between all haplotypes.

Results

The first two axes of both PCAs (Figures 2.1 and 2.2) accounted for less than 50% of the variance in either sex (males 30.3% - Table 2.2; females 44% - Table 2.3). However, each species formed a distinct assemblage (Figures 2.1 and 2.2), and MRPPs for each sex were highly significant (females: $\Delta_o = 0.1746$, $\Delta_e = 0.2039$, $P < 0.001$; males: $\Delta_o = 0.1636$, $\Delta_e = 0.1821$, $P < 0.001$). In general, the ordinations indicated a pigmentation gradient along Axis I distinguishing the two species with *G. flavimaculata* having more yellow pigmentation and *G. oculifera* having more dark pigmentation (Figures 2.1 and 2.2). Loading scores for this axis revealed 10 variables for males and 12 variables for females, which were the most important characters differentiating the two species (Tables 2.2 and 2.3). Other than the pigmentation variables, *G. oculifera* had longer anal and shorter abdominal plastral scutes. Likewise, Table 2.4 quantitatively summarizes the variables shown to be important by the loading scores. In both PCAs, Axis II explained approximately 11% of the variance and did not differentiate between the two species as well as Axis I (Figures 2.1 and 2.2; Tables 2.2 and 2.3). Similar to the PCAs, both females and males of the two species formed distinct groups in the UPGMA

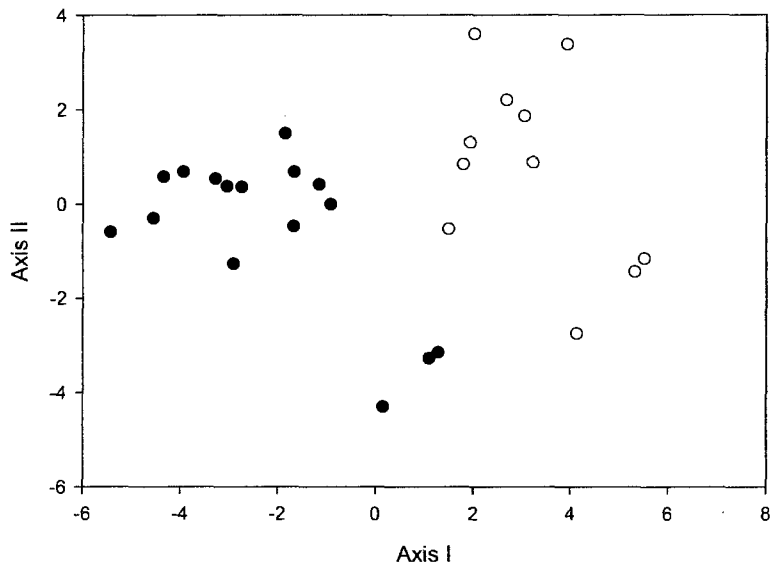

Figure 2.1. A principal components analysis (PCA) plot of female individuals of *G. oculifera* (open circles) and *G. flavimaculata* (black circles) showing a pigmentation gradient along axis I.

Table 2.2.

The PCA loading scores of male *G. oculifera* and *G. flavimaculata* showing several pigmentation characters as being important in the ordination. The first two axes explain 30.3% of variance, and axis I formed a pigmentation gradient. Percentages in the parenthesis represent variance explained by each axis, and bold characters indicate pigmentation characters.

	Axis I (21%)	Axis II (10%)
AB	-0.220	-0.176
AN	0.212	0.116
LOPB	0.284	-0.007
WBLO	-0.252	0.042
WIOL	0.230	-0.154
WY2F	0.235	-0.214
WB24	-0.210	-0.173
WPIGD	0.293	-0.021
WVPIG	0.309	0.020
WY2H	0.241	-0.070

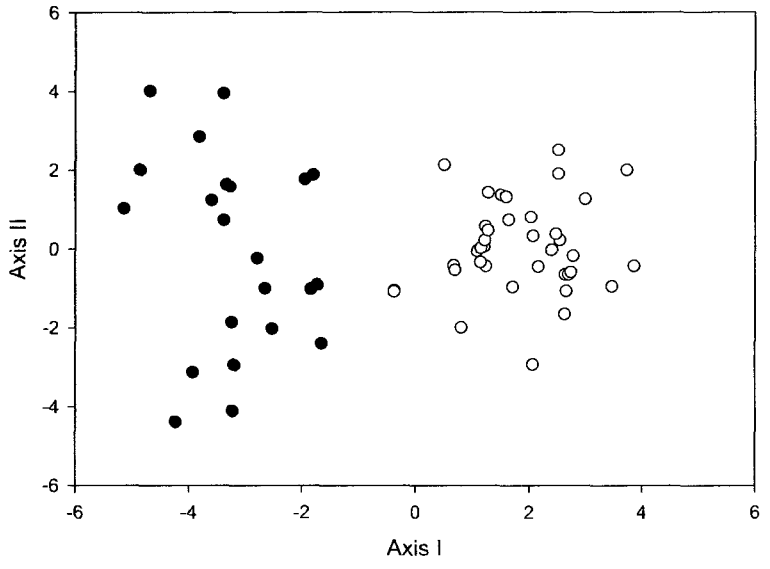

Figure 2.2. A principal components analysis (PCA) plot of male individuals of *G. oculifera* (open circles) and *G. flavimaculata* (black circles) showing a pigmentation gradient along axis I.

Table 2.3.

The PCA loading scores of female *G. oculifera* and *G. flavimaculata* showing several pigmentation characters as being important in the ordination. The first two axes explain 44% of variance, and axis I formed a pigmentation gradient. Percentages in the parenthesis represent variance explained by each axis, and bold characters indicate pigmentation characters.

	Axis I (33%)	Axis II (11%)
AB	0.208	-0.050
AN	-0.217	0.189
WOPB	-0.207	-0.051
NLU	-0.255	-0.091
NLL	-0.233	-0.133
WIOL	-0.255	-0.060
WYVLU	-0.225	-0.051
WYLL	-0.231	-0.104
WY2F	-0.220	0.101
WY4F	-0.232	0.125
WPIGD	-0.232	0.151
WVPIG	-0.242	0.198

analysis, but there was not perfect separation between the two (Figures 2.3 and 2.4). The cophenetic correlations (females, 0.8189; males, 0.8743) indicated that the clustering did not represent the structure in the data well. The clustering in both sexes was driven by head patterns and soft tissue pigmentation (Table 2.5). Besides the obvious pigmentation pattern on the coastal scutes, *G. flavimaculata* has more lines entering the orbit and post-orbital blotch (Table 2.5). Likewise, *G. flavimaculata* more frequently has a nasal trident, necklines that connect under the chin, and a "U" shaped bar under the chin (Table 2.5).

I obtained 6 sequences per species for the CR (657 bp) and 8 sequences for *G. oculifera* and 6 sequences for *G. flavimaculata* for the ND4 (894 bp). These sequences have been deposited on GenBank (accession numbers GQ253568 – GQ253573). The two unique ND4 haplotypes only had an uncorrected *p* distance of 0.0011. The most common haplotype was found in all *G. flavimaculata* and seven of the *G. oculifera*. I found four unique CR haplotypes with uncorrected *p* distances ranging from 0.0015 – 0.0091. No CR haplotypes were shared between the two species, but interestingly the two most similar haplotypes were found in *G. flavimaculata* (n=1) and *G. oculifera* (n=5), respectively.

Discussion

Some of the morphological characters Cagle (1954) used to diagnose *G. flavimaculata* and *G. oculifera* actually overlapped between the species. Although my analyses of an expanded set of characters demonstrated significant morphological differentiation between the two species, some specimens occasionally had characters that overlapped with the other species. Besides the differences in costal scute markings, which is the basis for the two species' common names, *G. flavimaculata* has more yellow

Table 2.4.

Mean ratios and standard deviation (SD) of several important pigmentation characters determined by the PCA loading scores that can differentiate between *G. flavimaculata* and *G. oculifera* in the Pascagoula and Pearl drainages.

	Meristics				Presence/Absence						1st Coastal Scute		
Species/Sex	#YHE	#NLPOB	#YLFE	#NLO	LLC	3YFE	NLIOL	DLPOB	TRIDENT	YUC	Blotch	Ring	Broken Ring
G. flavimaculata													
F	3 (1.2)	1.1 (0.97)	3.4 (1.3)	3.8 (0.38)	0.88	0.71	0.84	0.59	0.63	0.94	0.11	0.08	0.05
M	3.3 (1.1)	1.8 (0.47)	3.3 (1.0)	3.1 (0.87)	0.93	0.57	0.85	0.96	0.85	0.93	0.92	0.08	0
G. oculifera													
F	2.1 (0.51)	0.17 (0.48)	2.8 (1.6)	2.5 (0.59)	0.125	0.43	0.63	0.13	0.5	0.71	0	0.71	0.29
M	2.2 (0.65)	0.93 (0.89)	2.1 (0.81)	2.1 (0.44)	0.3	0.13	0.58	0.58	0.58	0.52	0	0.89	0.11

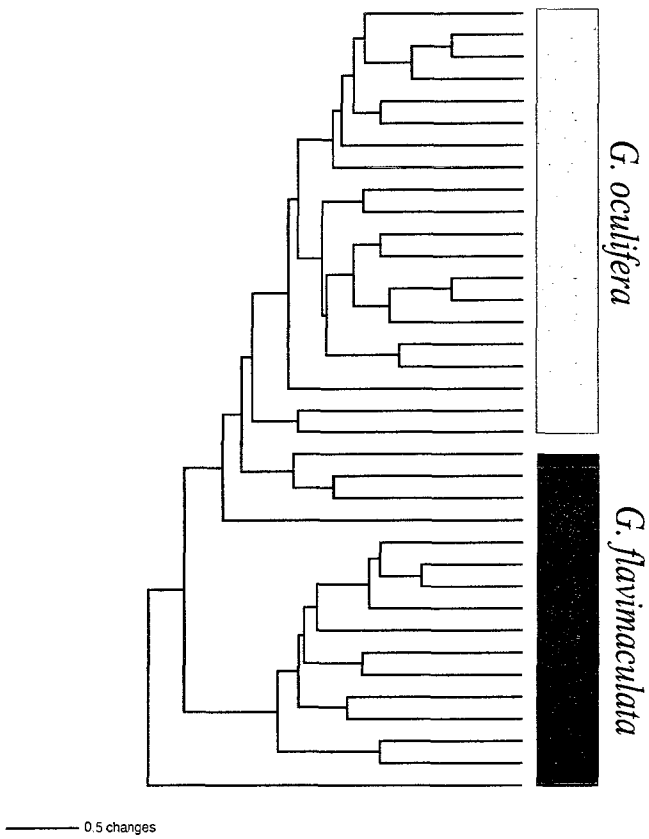

Figure 2.3. The UPGMA dendrogram showing female *G. oculifera* and *G. flavimaculata* are diagnosable using the selected qualitative characters. The cophenetic correlation (i.e., 0.8189) suggests that the clustering did not represent the structure of the raw data very well.

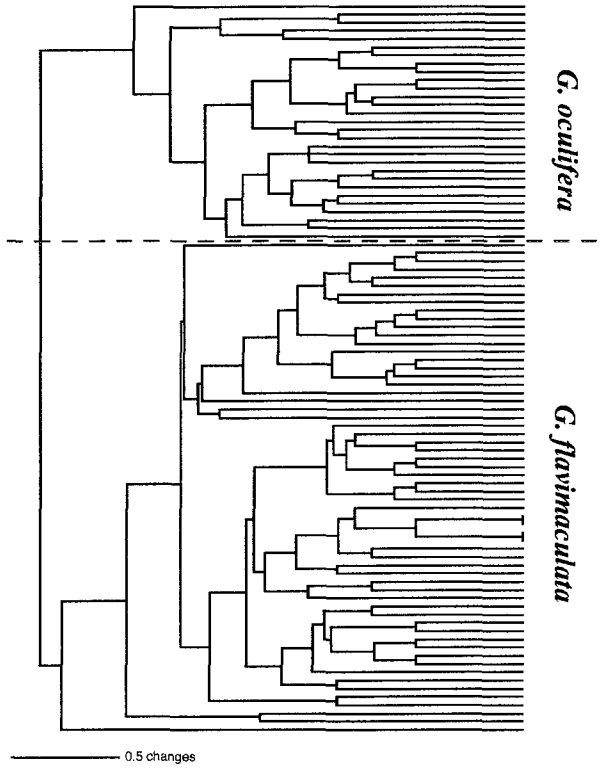

Figure 2.4.The UPGMA dendrogram showing male *G. oculifera* and *G. flavimaculata* are diagnosable using the selected qualitative characters. The cophenetic correlation (i.e., 0.8743) suggests that the clustering moderately represented the structure of the raw data very well.

Table 2.5.

Mean counts with standard deviation (SE) and frequencies of occurrence of several of the qualitative characters that differentiate between *G. flavimaculata* and *G. oculifera* in the Pascagoula and Pearl drainages.

	Meristics				Presence/Absence						1st Coastal Scute		
Species/Sex	#YHE	#NLPOB	#YLFE	#NLO	LLC	3YFE	NLIOL	DLPOB	TRIDENT	YUC	Blotch	Ring	Broken Ring
G. flavimaculata													
F	3 (1.2)	1.1 (0.97)	3.4 (1.3)	3.8 (0.38)	0.88	0.71	0.84	0.59	0.63	0.94	0.11	0.08	0.05
M	3.3 (1.1)	1.8 (0.47)	3.3 (1.0)	3.1 (0.87)	0.93	0.57	0.85	0.96	0.85	0.93	0.92	0.08	0
G. oculifera													
F	2.1 (0.51)	0.17 (0.48)	2.8 (1.6)	2.5 (0.59)	0.125	0.43	0.63	0.13	0.5	0.71	0	0.71	0.29
M	2.2 (0.65)	0.93 (0.89)	2.1 (0.81)	2.1 (0.44)	0.3	0.13	0.58	0.58	0.58	0.52	0	0.89	0.11

pigmentation on the carapace and soft tissues than *G. oculifera*. In particular, *G. flavimaculata* has more yellow pigmentation on the first vertebral and 5th marginal scutes and has a longer postorbital blotch than *G. oculifera*, which should be diagnostic in the field. Also similar to Cagle's (1954) comparison, my data showed that *G. flavimaculata* usually had yellow, dorsal necklines connecting to the post-orbital blotches and a broader yellow interorbital line than *G. oculifera*.

Distinct morphologies may not always reflect strong genetic differentiation between species. Morphological differentiation may be the product of strong selection pressure, lineage sorting of polymorphism in the ancestral population, or genotype by environment interactions (Avise, 2000; Futuyma, 1998). Interestingly, head patterns, which are diagnostic traits used in *Graptemys* taxonomy (Lovich and McCoy, 1992; Vogt, 1993 and references therein), are known to be under environmental control and exhibit clinal variation in some *Graptemys* species (Ewert, 1979; Vogt, 1993). Although I found a head pattern difference between *G. flavimaculata* and *G. oculifera*, this character has never been considered critical in distinguishing the two species. More importantly, there are no studies suggesting that the expression of other soft and hard tissue patterns that I examined are influenced by the environment.

Despite the significant morphological differentiation between *G. flavimaculata* and *G. oculifera*, like Lamb et al. (1994), I found limited genetic differentiation. The two ND4 haplotypes were different at only one base position and were shared between species while the CR haplotypes were species-specific but exhibited little divergence. The lack of strong molecular support for *G. flavimaculata* and *G. oculifera* is probably not a function of a poor choice in molecular markers. The three mitochondrial genes (i.e., control region, cyt *b*, and ND4) used in this study and by Lamb et al. (1994) are

among the most commonly employed in molecular systematic studies of turtles, and they are also among the most variable at lower taxonomic levels (FitzSimmons and Hart, 2007). Perhaps the inability of mtDNA to fully resolve the taxonomic relationships within *Graptemys* might be due to slow evolutionary rates in chelonian mitochondrial DNA (mtDNA; Avise et al., 1992), but this idea is still debated in the literature (FitzSimmons and Hart, 2007). Regardless of the rate of molecular evolution in chelonians, some species (e.g., *Sternotherus minor*, *S. odoratus*, and *Kinosternon subrubrum*) demonstrate greater intraspecific divergence than is seen between many species of *Graptemys* (Walker and Avise, 1998), even in species like *S. odoratus* that exhibit morphological homogeneity across its range (Reynolds and Seidel, 1983). The question remains as to whether or not the genus *Graptemys* may be oversplit (Walker and Avise, 1998), or if these are valid species that are the product of recent radiations associated with periodic sea level fluctuations along the Gulf of Mexico (Lovich and McCoy, 1992; Wood, 1977).

These questions about taxonomy and evolutionary history are not strictly of academic interest. *Graptemys oculifera* and *G. flavimaculata* are both federally listed as threatened (U.S. Fish and Wildlife Service, 1986, 1991) and listed as endangered by the state of Mississippi (Mississippi Department of Wildlife, Fisheries, and Parks, 2000) so their taxonomic status has important conservation implications. This study found the two species to be morphologically distinct in a variety of pigmentation characters, which may or may not be environmentally influenced, and hard characters such as plastral scute length. The lack of accompanying strong genetic differentiation is not necessarily surprising if these species are only recently diverged. In these situations, a more productive way to delimit species may be to take a population genetic approach by

defining a species as a genetically and demographically connected metapopulation rather than a genealogical one that only recognizes monophyletic groups (Shaffer and Thomson, 2007). Since *G. oculifera* and *G. flavimaculata* are restricted to different drainage systems, they most likely represent distinct metapopulations. Although, this degree of genetic isolation could be further tested through the collection of additional molecular data such as multiple microsatellite loci or single nucleotide polymorphisms. The discrepancy between the morphological and molecular aspects of this study suggests that the taxonomic status of *G. oculifera* and *G. flavimaculata* is still open to debate. However, I urge that before any formal taxonomic decisions are made that additional data be collected to better establish the degree of genetic connectivity between the two.

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CHAPTER III

LOW GENETIC DIVERSITY IN SEVERAL GOPHER TORTOISE (*GOPHERUS POLYPHEMUS*) POPULATIONS IN THE DESOTO NATIONAL FOREST, MISSISSIPPI

Abstract

Gopherus polyphemus has experienced severe population declines, especially in the western portion of its range. As a consequence, *G. polyphemus* may have experienced population bottlenecks that resulted in a decrease in genetic diversity and an accumulation of deleterious alleles. The importance of genetic diversity has been well documented for several fitness parameters (e.g., survival, disease resistance, growth and developmental rates, and developmental instability). Western populations of *G. polyphemus* in South Mississippi have demonstrated lower hatching success (e.g., 16.7 to 48%) than that found in eastern populations (e.g., 67 to 97%). Even under laboratory conditions, approximately 40% of the eggs still failed to hatch, suggesting that intrinsic (egg quality) factors may be affecting development. Using nine microsatellite loci, I genotyped individuals from four populations in South Mississippi and one eastern population and compared several genetic diversity indices (e.g., allelic richness, expected heterozygosity, and percent polymorphic loci) with published data from populations in the eastern portion of the range. I found significantly lower genetic diversity in the four Mississippi populations than in the eastern populations. However, these findings only demonstrate that these populations have low genetic diversity, and establishing any causal relationship between low genetic diversity and/or other intrinsic factor(s) (e.g., female condition) with reduced reproductive success should be further investigated.

Key Words.– Conservation genetics; Genetic diversity; *Gopherus polyphemus*

Introduction

The Gopher tortoise's (*Gopherus polyphemus*) distribution is intrinsically linked to the historical range of longleaf pine (*Pinus palustris*) (U.S. Fish and Wildlife Service, 1990). This ecosystem that once dominated the southeastern Coastal Plain has experienced approximately a 90% reduction during the last century (Croker, 1987; Kautz, 1993; Noss, 1989), which subsequently has reduced Gopher tortoise populations by 80% (Auffenberg and Franz, 1982). The loss of longleaf pine habitat has been attributed to poor forest management practices, urbanization, and agriculture. The loss of this habitat has produced fragmented *G. polyphemus* populations across its range (U.S. Fish and Wildlife Service, 1990). In particular, the western portion of the range, west of the Tombigbee and Mobile Rivers in Alabama and including south Mississippi and southeastern Louisiana, has experienced significant population reductions and fragmentation leading to a federal listing of "threatened" (U.S. Fish and Wildlife Service, 1990).

In the western portion of the range, the DeSoto National Forest (DNF) in south Mississippi contains the largest number of *G. polyphemus* and has experienced recent declines, perhaps due to low recruitment (Epperson and Heise, 2003; Noel, 2006). Several multi-year studies have reported an extremely low hatching success rate (16.7 – 48%: Epperson and Heise, 2003; Hammond, 2009; Noel, 2006; Qualls et al., unpublished data) compared to the 67-97% hatching success rate in the eastern portion of the range (Butler and Hull, 1996; Desmuth, 2001; Landers et al., 1980; Smith, 1996). In a comparison of natural and artificial incubations, Noel (2006) split clutches, incubating two eggs per clutch in the laboratory under controlled (thermal and hydric) conditions

chosen to maximize success, while leaving the remainder of each clutch in the natural (but predator protected) nest. The hatching success (58.8%) in these artificial incubations was substantially lower than typical for natural nests in eastern populations. This led Noel (2006) to suggest that approximately 40% of the eggs had some intrinsic factor(s) impeding successful hatching. The naturally incubated eggs had a hatching success of only 16.7%. Noel (2006) suggested that of the 83.3% of the eggs that failed to hatch, removing the 40% egg failure attributed to intrinsic factors would leave approximately 43% of the hatching failure attributable to some sort of extrinsic factor(s). Noel (2006) found soil clay content and temperature of the nest to only be weakly correlated with hatching success, and subsequent studies have not yet been able to causally link any specific extrinsic factors with low hatching success (Hammond, 2009; Qualls et al., unpublished data). Hatching success rates were not the only reproductive difference between tortoises in the western and eastern portions of their range. In the failed eggs, DNF populations also had a higher percentage of late-stage embryo mortality (28–53%: Epperson and Heise, 2003; Hammond 2009; Noel, 2006) when compared to an eastern population (i.e., 1%; Butler and Hull, 1996). While they have not identified any particular intrinsic factor(s) as a cause, these studies clearly demonstrate the presence of some such intrinsic problem in a disturbingly large proportion of tortoise eggs in the DNF.

Genetic diversity (i.e., heterozygosity and allelic diversity) is one intrinsic factor that has been linked to various correlates of fitness (e.g., survival, disease resistance, growth and developmental rates, and developmental instability) in wild populations (Allendorf and Leary, 1986; Crnokrak and Roff, 1999; Mitton, 1997; Ralls et al., 1988; Reed and Frankham, 2003). In oviparous species, low genetic diversity may be responsible for low reproductive success (reviewed in Keller and Waller, 2002). More specifically, low

genetic diversity seems to adversely impact hatching success in several of the more derived reptiles (i.e., avian species) such as *Parus major* (Great tit) (Kempenaers et al., 1996), *Picoides borealis* (Red-cockaded woodpecker) (Daniels and Walters, 2000) and *Gallinula chloropus* (Common moorhen) (McRae, 1996). Therefore, the goal of this study was to assess levels of genetic variation in several of the DNF populations experiencing low hatching success rates, and to compare the levels of genetic diversity between the western and eastern portions of the range. However, establishing a correlation between low genetic diversity and low hatching success does not necessarily imply a causal relationship and clearly demonstrating this is outside of the scope of this study.

Materials and Methods

From May–August 2006, I captured adult *G. polyphemus* using 13 Tomahawk Model 18 Live Traps (81.28 x 25.4 x 30.48 cm) and 25 custom-designed (71.12 x 35.56 x 27.94 cm) live traps from the four study sites on the DNF described by Noel (2006): T44 West (N 31° 04' 52", W 89° 07' 45"), T44 East (N 31° 04' 47", W 89° 06' 05"), McLaurin (N 31° 08' 47", W 89° 06' 05"), and Crossroads (N 30° 57' 24", W 89° 06' 32") (Figure 3.1). I collected a 0.5–1 mL sample of blood from each tortoise from the femoral vein using a heparinized 23-gauge needle and 1 mL syringe. Each blood sample was stored in a 1.5 mL vial with approximately 0.5 mL of tissue preservation buffer (Seutin et al., 1991). Samples from one of the eastern populations (Fort Benning, GA) were provided by Mary Mendonca and Paula Kahn of Auburn University.

I extracted total genomic DNA from the blood samples using the Qiagen DNeasy extraction kit (QIAGEN Inc., Valencia, CA), and genotyped each individual for the nine microsatellite loci reported by Schwartz et al. (2003) for *G. polyphemus*. Polymerase

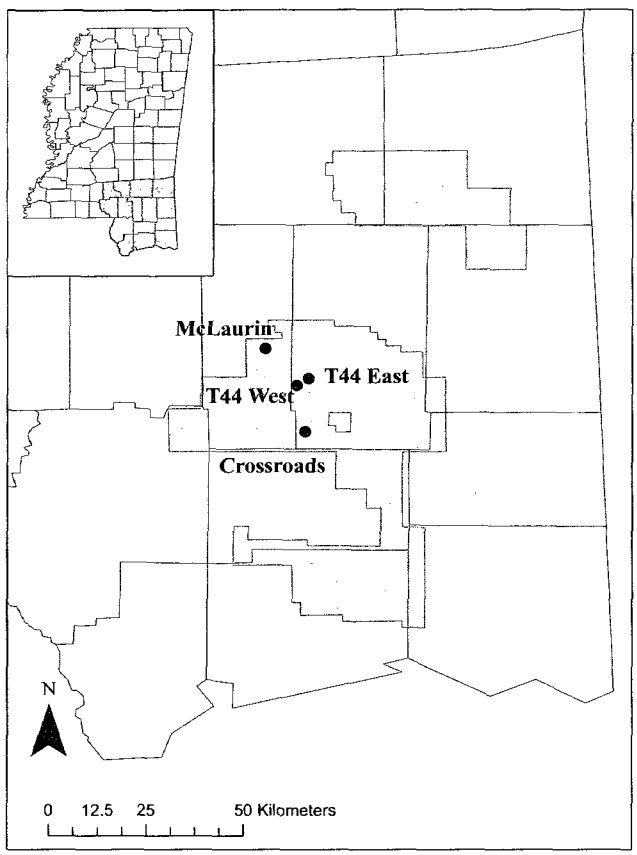

Figure 3.1. Map showing the location of our western sampling sites within the DeSoto National Forest shaded in gray. The inset map shows the location of the counties in south Mississippi relative to the entire state.

chain reaction (PCR) amplifications were conducted in a total volume of 12.5 μL using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 1.5–2.0 mM $MgCl_2$, 200 μM dNTPs, 0.1875 units of *Taq* polymerase (Promega), 0.3 μM of the M13 tailed forward primer (Boutin-Ganache et al., 2001), 0.3 μM of the reverse primer, 0.1 μM of the M13 labeled primer (LI-COR), 20–100 ng of template DNA and water to the final volume. PCR cycling conditions consisted of an initial denaturing step of 94 C for 2 min followed by 35 cycles of 30 sec at 94 C, 1 min at 56–60 C and 1 min at 72 C. A final elongation step of 10 min at 72 C ended the cycle. I visualized the microsatellite alleles using a LI-COR 4300 DNA Analysis system and scored them using Gene Image IR v. 3.55 (LI-COR).

I used GENEPOP v. 3.4 (Raymond and Rousset, 1995) to calculate the number of alleles, percentage of polymorphic loci, observed and expected heterozygosity values, and conduct exact tests for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD). Significance values for tests with multiple comparisons were adjusted with a sequential Bonferroni correction (Rice, 1989). The presence of null alleles was assessed using MICRO-CHECKER v. 2.2.3 (van Oosterhout et al., 2004). I analyzed each sampled population for bottlenecks using BOTTLENECK ver 1.2.02 (Cornuet and Luikart, 1996) and the *M* ratio of Garza and Williamson (2001). Bottleneck was run for 1000 permutations under a two-phase model of microsatellite evolution with a 30% stepwise mutation model and a 70% infinite allele model.

Ideally, when comparing genetic diversity indicies (e.g., allelic richness) among populations, one should use a rarefaction method to account for variation in sample sizes (Leberg, 2002). Therefore, I combined Schwartz and Karl's (2005) genotypic data with

our own and conducted three sets of analyses whereby we accounted for differences in sample sizes among populations. First, I conducted a rarefaction analysis using HP-RARE 1.0 (Kalinowski, 2005), and then I used a Wilcoxon Rank Sums Test to determine if there was a significant difference between western and eastern samples' adjusted allelic richness. Second, I used FSTAT v. 2.9.3.2 (Goudet, 2001) to test for significant differences among eastern and western populations in allelic richness, observed heterozygosity and expected heterozygosity using a randomization test with 15,000 permutations. Lastly, I compared number of alleles, expected heterozygosity, and percentage of polymorphic loci between my DNF samples and eastern sampled populations in a multiple response permutation procedure (MRPP). To determine if the sample size influenced the amount of genetic diversity, which could bias the statistical analyses, I used linear regression to fit a line to each of the samples' genetic diversity indices over the size of the sample. Only expected heterozygosity (H_E) demonstrated a significant relationship with sample size ($r^2 = 0.20$, $P = 0.029$), which was negative. If smaller samples underrepresented the genetic variation present in a population then one would expect a positive, not a negative relationship. Therefore, I used the three genetic diversity indices (not corrected for n) in the MRPP, which was run with 50,000 permutations using Euclidean distances to create a dissimilarity matrix. This is a re-sampling statistical technique testing for a significant difference between groups (McCune and Grace, 2002). All statistical analyses were performed using R statistical software (R Development Core Team, Version 2.8.0, 2008) and JMP 7.0.1 (SAS Institute, 2007).

Results

I genotyped a total of 129 adult individuals from my five sites (4 in DNF and Ft. Benning). Sample sizes at these sites were as follows: T44E, n = 24; T44W, n = 42; McLaurin, n = 7; Crossroads, n = 16, Ft. Benning, n = 40. All five sites showed no evidence of null alleles, deviation from HWE, or LD. Also, none of my sites demonstrated evidence of bottlenecks through heterozygosity excess or deficiency under the T.P.M. mutation-drift equilibrium, and all *M* ratios were larger than their respective critical values (M_c). The 89 individuals genotyped from my four samples of western populations possessed less genetic variation than eastern samples surveyed by Schwartz and Karl (2005). For example, the mean number of alleles per locus for the eastern populations was 3.1 (SE ± 0.16), while the mean number of alleles for Mississippi populations was 1.9 (SE ± 0.106) (Figure 3.2). Likewise, the ranges of expected heterozygosity (H_E) values and percentage of polymorphic loci (% poly loci) for the eastern population samples (H_E = 0.5 ± 0.02; % poly loci = 0.9 ± 0.03) were larger than Mississippi populations (H_E = 0.2 ± 0.01; % poly loci = 0.6 ± 0.05; Figure 3.2). Each of my analyses that accounted for differences in sample size also indicated that there was significantly less genetic variation in the western populations. The Wilcoxon Rank Sums Test comparing the adjusted allelic richness showed that the western (1.7 ± 0.06) and eastern (2.6 ± 0.082) samples were significantly different (Z = -2.69, P = 0.007). The permutation test performed by FSTAT detected significant differences between western and eastern populations in allelic richness (P = 0.0004), observed heterozygosity (P = 0.004) and expected heterozygosity (P = 0.0001). Likewise, the MRPP comparing the number of alleles, expected heterozygosity, and percent polymorphic loci between the

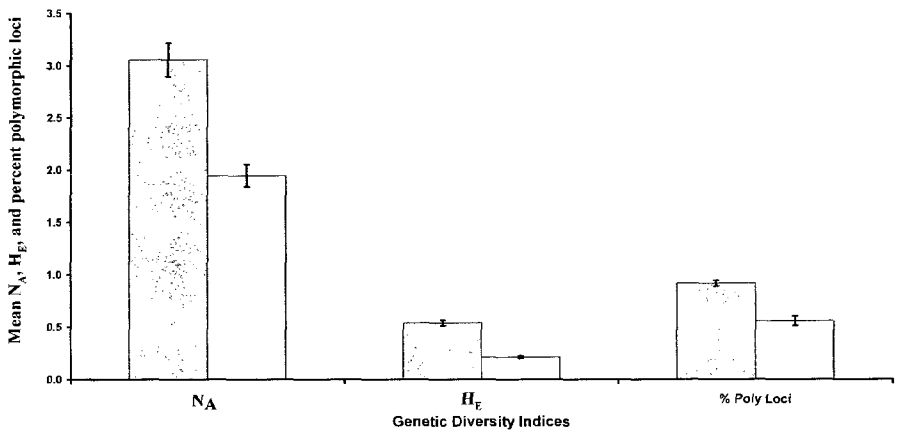

Figure 3.2. Comparison of *Gopherus polyphemus* samples' mean number of alleles (N_A), mean expected heterozygosity (H_E), and mean percentage of polymorphic loci (% poly loci) between eastern (gray) and western (white) portion of the range.

western and eastern samples also showed a significant difference ($\Delta_o = 0.08415$, $\Delta_e = 0.1456$, $P = 0.0002$).

Discussion

Compared to eastern populations, which are not federally protected and where reproductive success is higher, *G. polyphemus* has low genetic variation and a reduction heterozygosity in western populations that we sampled on the DNF. The presence of lower genetic diversity in the western portion the range could suggest prior population bottlenecks, or that historically the western populations persisted with low genetic diversity (e.g., central-marginal theory; reviewed by Eckert et al., 2008). Although bottlenecks (i.e., genetic drift) were not detected by *M* ratio and BOTTLENECK ver 1.2.02 for any of our populations, *G. polyphemus* populations have declined 80% since the late 1800s (Auffenberg and Franz, 1982). More recently in the DNF, numbers of active and inactive burrows have decreased roughly 35.7% over the past 12 years (Hammond, unpublished data). However, genetic tests can sometimes miss the signature of a bottleneck even when long-term demographic data have suggested consistent population declines (Busch et al., 2007; Kuo and Janzen, 2004). With continuously declining populations, genetic drift can precipitate loss of genetic variation by decreasing allelic richness (i.e., fixation or loss). Unlike genetic drift, inbreeding can only precipitate the decrease of heterozygosity within a population (England et al., 2003; Frankham et al., 2002; Hartl and Clark, 1997; Lande, 1988; Reed and Frankham, 2003). When compared with the eastern populations, the DNF populations have lower heterozygosity. This difference in heterozygosity could suggest inbreeding depression within the western populations. However, it may be invalid to assume a correlation

between reduced heterozygosity levels detected by a limited number of microsatellite loci (e.g., 10) and the inbreeding coefficient (Balloux et al., 2004).

The negative effects of low genetic diversity and inbreeding depression within wild populations have been well documented, including detrimental affects on several fitness parameters such as survival, disease resistance, growth and developmental rates, and developmental instability (Allendorf and Leary, 1986; Crnokrak and Roff, 1999; Ralls et al., 1988; Reed and Frankham, 2003). Low genetic diversity and inbreeding seems to affect life-history traits, such as hatching success and juvenile survival, more severely than morphological traits (e.g., body weight and scale counts) (DeRose and Roff, 1999). Notably, 14% of hatchlings in the DNF populations appear to be demonstrating elevated rates of morphological abnormalities, such as fluctuating asymmetry and scute malformations (Hammond, unpublished data). Similar to the reproductive problems found in several DNF populations of *G. polyphemus*, inbreeding depression and low genetic variability impact reproductive success in avian species (reviewed in Keller and Waller, 2002). For example, inbreeding depression lowered egg hatchability in *Parus major* (Kempenaers et al., 1996), reduced hatching rates and fledgling survival in *Picoides borealis* (Daniels and Walters, 2000), and lowered the hatching success and survival rate of offspring in *Gallinula chloropus* (McRae, 1996).

The high percentage of eggs that potentially failed due to intrinsic factors (40%; Noel, 2006) could in part be explained by low genetic variation found in our study. However, it is not out of the realm of possibility that other intrinsic factors (e.g., female condition and quality) could also contribute to the low hatching success. For example, elevated corticosterone levels in stressed avian species' females during the reproductive cycle have been shown to increase corticosterone levels in the eggs (Saino et al., 2005),

which have negative effects on embryos and hatchlings (Hayward and Wingfield, 2004). Likewise, poor body condition in females of the Snow petrel (*Pagodroma nivea*) and Baltic herring (*Clupea harengus membras*) has been shown to have negative effects on hatching success (Barbraud and Chastel, 1999; Laine and Rajasilta, 1999). However, the intrinsic factor(s) does not entirely explain the low hatching success problem. For example, 43% of the eggs could have been successful in the field experiment, but failed due to some extrinsic (nest environment) factor(s) (Noel, 2006). Temperature and soil clay content of nests were only weakly correlated to hatching success (Noel, 2006), and further experiments investigating extrinsic factor(s) have found no specific causes impeding hatch success (Qualls, unpublished data; Hammond, 2009). Artificial incubation experiments over multiple years on these same populations have produced consistent hatching success rates (~60%) while the natural nest hatching success rates have fluctuated (30–48%) between years, but never attained equal success to laboratory incubation (Qualls, unpublished data). This pattern suggests than while overall recruitment within the DNF is a combination of both extrinsic and intrinsic factors, the latter consistently contributes approximately 40% egg failure.

While my data show correlated east-west differences in genetic variation and hatching success, this study only shows that several populations of *G. polyphemus* in the western portion of the range have lower genetic diversity than the eastern portion and does not show a causal relationship between genetic diversity and hatching success. However, there is a large difference between western and eastern hatching success rates that has not been explained by extrinsic factor(s). While the consequences of this lower genetic diversity and other potential intrinsic factor(s) should be investigated in the future, they were outside the scope of this study. These findings have important

implications for *G. polyphemus* management in the DNF, because two of my sites (T44 East and West on Camp Shelby) have been aggressively managed through prescribed burns and thinning of canopy specifically for *G. polyphemus* habitat for more than a decade, but still have low hatching success. Thus, habitat protection and management alone do not appear to be sufficient to help these populations to recover. This situation leaves state and federal agencies facing a very difficult challenge in conserving and recovering viable populations of *G. polyphemus* in Mississippi.

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CHAPTER IV

A REASSESSMENT OF THE PHYLOGEOGRAPHY OF *GOPHERUS POLYPHEMUS*

Abstract

Identifying geographic barriers that partition genetic structure within a species is crucial in formulating an effective conservation plan. Since *Gopherus polyphemus* have historically been declining range wide, the identification of evolutionary significant units and management units are critical for the protection and recovery of the species. Previous molecular work, although somewhat limited in scope, does make it clear that there are distinct population assemblages across the geographic range of *G. polyphemus*. The goal of this study was to more fully sample across the western portion of the range (i.e., west of the Tombigbee and Mobile rivers) by including populations from Mississippi and western Alabama, to reassess the phylogeography of *G. polyphemus*. In particular, I wanted to more explicitly evaluate the extent of genetic isolation impacted by several proposed geographic barriers. Using a 712 bp portion of a mitochrondrial gene (NADH dehydrogenase), I found support for a modest phylogenetic break between the western and eastern portions of the range, which supported USFWS's listing of the west as a distinct population segment (DPS). However, the presence of western haplotypes in panhandle Florida and Georgia indicates that the phylogenetic break between west and east has not been impermeable to historical gene flow.

Key words.–mtDNA, ND4, Phylogeography, Conservation Genetics

Introduction

The importance of identifying geographic barriers that cause phylogenetic breaks within a species is paramount in formulating a sufficient management strategy. The failure to separately managing genetically unique populations or regions could precipitate

loss in genetic diversity and local adaptations that are essential for evolution (Hilborn et al., 2003; Luck et al., 2003). For example, managers lacked information on the phylogeography and taxonomy of the tuatara (*Sphenodon spp.*) and they treated the group as a monotypic species. This led to the extinction of unique populations and potentially an entire sub-species (Daugherty et al., 1990). In recognition of the value of genetically unique populations; in 1978 an amendment to the Endangered Species Act (ESA; 1973) allowed the United States Fish and Wildlife Service (USFWS) to protect unique populations of a species by designating them as distinct populations segment (DPS) (USFWS, 1987). The designation of a DPS could be on the basis of a "physical, physiological, morphological, ecological, behavioral, or genetic difference" (USFWS and NOAA, 1996).

Gopherus polyphemus populations have been reduced by the 80% since 1800's (Auffenberg and Franz, 1982), and the declines are continuing throughout the range (McCoy and Mushinsky, 1992; Mushinsky et al., 2006; Waddle et al., 2006; Hammond, 2009) and more disconcerting even on protected lands (McCoy et al., 2006). Under the amendment to the ESA, western populations (i.e., west of the Tombigbee and Mobile Rivers) of *Gopherus polyphemus* were considered a DPS and subsequently federally listed as threatened (USFWS, 1987). However, the distribution of *G. polyphemus* is expansive, covering 6 southeastern states (i.e., Louisiana, Mississippi, Alabama, Georgia, Florida, and South Carolina) with the federally protected populations only being a relatively small portion of this distribution. Many species with large distributions possess intraspecific genetic structuring (Avise, 2000) and *G. polyphemus* is no exception. There have been several phylogeographic studies of *G. polyphemus* including mitochondrial DNA (mtDNA; Osentoski and Lamb, 1995) and microsatellites (Schwartz and Karl,

2005) showing genetically distinct population assemblages across the geographic range (Osentoski and Lamb, 1995) and within Florida and Georgia (Schwartz and Karl, 2005). However, the largest scale study to date (Osentoski and Lamb, 1995) had limited sampling in the threatened western portion of the range (i.e., only one site in Louisiana). Thus, a complete range wide phylogeographic study has not been conducted, which could provide molecular support for the western DPS as defined by the USFWS and identifying DPS elsewhere in the range.

The goal of this study is to more fully sample across the western portion of the range by including populations from Mississippi and western Alabama, and conduct a complete phylogeographic study of *G. polyphemus*. In particular, I wanted to investigate the genetic distinctiveness of the western DPS compared to other portions of the range. These data will allow me to more explicitly evaluate the extent of genetic isolation/divergence between populations, thereby aiding federal and state agencies in making decisions on their legal protection and conservation status.

Methods

Collections and Sequencing

Samples (i.e. blood or shell pieces) were either obtained under the appropriate permits by trapping efforts by the authors or donations made by various researchers (see Acknowledgments for a complete list). This collaborative effort yielded 207 individuals from 26 sites throughout the range (Table 4.1, Figure 4.1). For the adult *G. polyphemus* captured by the authors, Tomahawk Model 18 Live Traps (81.28 × 25.4 × 30.48 cm) and custom-designed (71.12 × 35.56 × 27.94 cm) traps were used, and a 0.5 - 1 mL blood sample was collected from each tortoises' femoral or brachial vein using heparinized 23-gauge needles and 1 mL syringes. Each blood sample was stored in a 1.5 mL vial with

Table 4.1.

Coordinates, number of sequences (*n*), gene diversity (*h*), and nucleotide diversity (π) of the 26 sites sampled across *Gopherus polyphemus'* distribution. Sites are partitioned into regions based on USFWS's delineation of the western portion of the range.

Region/Site	State	Coordinates (WGS84)	*n*	*h*	Π
Western					
1. Camp Shelby	MS	N 31° 03' 52", W -89° 07' 45"	9	0.000	0.000
2. Cross Roads	MS	N 30° 57' 24", W -89° 06' 32"	10	0.000	0.000
3. Gopher Farm	MS	N 31° 27' 37", W -88° 46' 02"	12	0.000	0.000
4. McLaurin	MS	N 31° 08' 59", W -89° 12' 01"	7	0.000	0.000
5. Marion	MS	N 31° 09' 39", W -89° 43' 15"	9	0.000	0.000
6. Ward Bayou	MS	N 30° 32' 54", W -88° 38' 32"	11	0.250	0.000
7. Escatawpa	MS	N 30° 36' 13", W -88° 25' 17"	5	0.000	0.000
8. Wiggins Airport	MS	N 30° 50' 32", W -89° 09' 37"	5	0.000	0.000
9. Little Florida	MS	N 30° 40' 08", W -89° 05' 24"	1	1.000	0.000
10. Mobile	AL	N 30° 54' 29", W -88° 08' 49"	10	0.200	0.003
Eastern					
11. Ft. Benning	GA	N 32° 21' 27", W -84° 57' 22"	9	0.556	0.004
12. Walton	FL	N 30° 40' 08", W -89° 05' 24"	8	0.643	0.006
13. Gadsden	FL	N 30° 39' 51", W -84° 47' 52"	9	0.389	0.005
14. Duval	FL	N 30° 21' 57", W -81° 50' 38"	12	0.511	0.003
15. Nassau	FL	N 30° 34' 26", W -81° 33' 14"	9	0.000	0.000
16. Lake	FL	N 28° 32' 01", W -81° 44' 00"	10	0.644	0.001
17. Alachua	FL	N 29° 35' 52", W -82° 25' 09"	10	0.000	0.000
18. Hernando	FL	N 28° 33' 53", W -82° 23' 11"	12	0.000	0.000
19. Orange	FL	N 28° 43' 11", W -81° 34' 22"	8	0.000	0.000
20. Putnam	FL	N 29° 44' 02", W -81° 37' 42"	9	0.000	0.000
21. Volusia	FL	N 28° 52' 23", W -81° 09' 47"	9	0.000	0.000
22. Indian River	FL	N 27° 36' 10", W -80° 20' 08"	2	0.000	0.000
23. Okaloosa	FL	N 30° 46' 01", W -86° 33' 33"	2	1.000	0.013
24. Hillsborough	FL	N 28° 03' 51", W -82° 24' 50"	2	0.000	0.000
25. Highlands	FL	N 27° 28' 15", W -81° 30' 54"	10	0.000	0.000
26. Martin	FL	N 27° 09' 14", W -80° 40' 05"	6	0.000	0.000

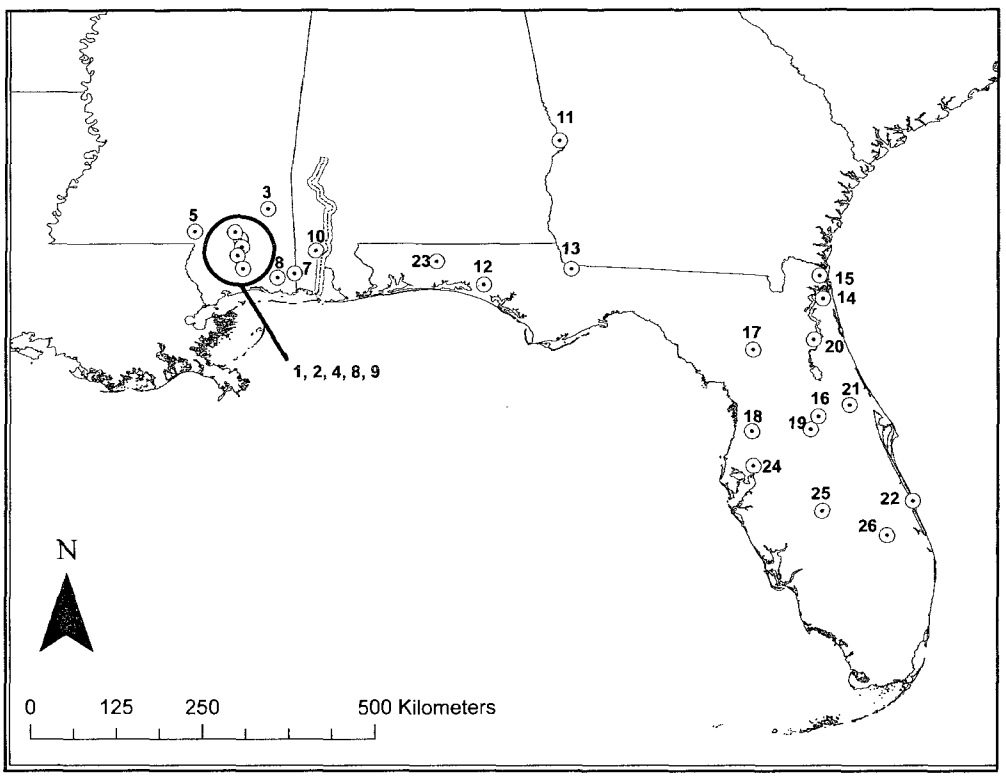

Figure 4.1. A map showing the 26 sites in four states sampled for this study. The line on the map represents USFWS's delineation of the western portion of the range. The numbers associated with each site corresponds to the site number in Table 4.1.

approximately 0.5 mL of SED tissue preservation buffer (Seutin et al., 1991). Total genomic DNA was extracted from the blood samples using the Qiagen DNeasy extraction kit (QIAGEN Inc., Valencia, CA).

I used the polymerase chain reaction (PCR) to amplify a portion of the mitochondrial, NADH dehydrogenase 4 (ND4) gene. After initial amplification were conducted using the ND4 primers designed by Spinks and Shaffer (2005). Based on sequences from these, I designed internal primers (5'-AAACTTGGAGGATA- 3' and 5'-CCCTTAAAAGTGAG-3'). In a total volume of 25 or 50 μL, PCR reaction conditions were conducted using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 1.5–3.0 mM $MgCl_2$, 200 μM dNTPs, 0.1875 units of *Taq* polymerase (Promega), 0.3 μM of the forward and reverse primer, 20–100 ng of template DNA, and water to the final volume. The cycling conditions consisted of an initial 1 min denaturing step at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 55°C and 3 min at 72°C. A final elongation step of 7 min at 72°C completed the cycle. Amplifications were Exo-Sap cleaned (USB Corp. Cleveland, Ohio), and then used as template in a cycle sequencing reaction using the ABI BigDye Terminator v 1.1 cycle sequencing kit (Foster City, CA). All sequencing reactions were sephadex cleaned (Princeton Separations, Adelphia, NJ, USA) prior to gel runs at the Iowa State University DNA Sequencing and Synthesis Facility. Sequence data were edited and aligned using Sequencher v4.1 (GeneCodes Co., Madison, WI).

Data Analyses

To visually assess how haplotypes frequencies are partitioned across the landscape, I created a haplotype network using TCS (Templeton et al., 2000). PAUP* 4.0b10 (Swofford, 2002) was used to calculate pairwise uncorrected *p* distances between

all haplotypes. Implemented in Arlequin 3.11 (Schneider et al., 2000), a mismatch distribution (MMD; Harpending et al., 1998) was used to test for a historical population expansion. Also using Arelequin 3.11 (Schneider et al., 2000), I calculated genetic diversity statistics (i.e., h–haplotype diversity, and π–nucleotide diversity).

To assess phylogeographical patterns in my data, I used Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) implemented in Arlequin 3.11 (Schneider et al., 2000). For the AMOVA, I partitioned populations on the basis of four models according to known and potential geographic barriers. Three of the models partitioned the distribution of *G. polyphemus* into two groups based on either the 1) USFWS's delineation of the Tombigbee/Mobile Rivers (USFWS, 1987), 2) literature showing genetic breaks corresponding to the Apalachicola drainage (Swift et al., 1985; Bermingham and Avise, 1986; Avise et al., 1979; Pauly et al., 2007), or 3) literature showing unique genetic structuring within peninsular Florida (Osentoski and Lamb, 1995; Clark et al., 1999; Branch et al., 2003; Schwartz and Karl, 2005). The final model was run with three groups using both Tombigbee/Mobile and peninsular Florida delineations. To remove the potential bias of the a priori group delineations used in the AMOVA, I used Spatial Analysis of Molecular Variance (SAMOVA; Dupanloup et al., 2002), which maximizes differentiation (ϕ_{CT}) among regions based on the geographical coordinates of samples. I ranged the value of K (i.e., the number of groups) from 2 – 3 with 100 simulated annealing processes to compare how SAMOVA partitioned the populations relative to the partitions tested in the AMOVA.

Results

I obtained sequences for a 712 bp portion of ND4 for 207 individuals from 26 sites throughout Mississippi, Alabama, Georgia, and Florida (Table 4.1 and Figure 4.1).

Ten unique haplotypes (Figure 4.2) were found representing two groups with a modest phylogenetic split (i.e., average uncorrected *p* distance between groups = 0.014; Table 4.2) with three haplotypes recovered from the western group and seven haplotypes recovered from the eastern. The peninsular Florida region contained the most unique haplotypes (i.e., 5) with one site (Indian River, Co., FL) having a three nucleotide substitution difference from the most common haplotype found in the eastern assemblages. The phylogenetic break did not entirely correspond to one particular geographic barrier because shared haplotypes from the eastern and western regions, as defined by USFWS, were found in the panhandle of Florida and Georgia sites (Figure 4.3). Interestingly, the federally protected region (i.e., Mississippi and west Alabama) and peninsular Florida did not share haplotypes (Figure 4.3), and only possessed western and eastern haplotypes, respectively.

There were typically few haplotypes per site with most of the sites with a relatively high genetic diversity being located in Florida and Georgia (Table 4.1). Except for sites with both eastern and western haplotypes, I typically found low nucleotide diversity (Table 4.1). My samples did not fit a sudden expansion model collectively (P = 0.029), or partitioned into eastern (P = 0.0003) and western (P = 0.039) regions. The AMOVA model testing USFWS's delineation (i.e., Tombigbee/Mobile) explained a significant portion of the molecular variance (ϕ_{CT} 74.56%; $P < 0.001$), and this delineation explained more of the variance than the Apalachicola/Flint delineation (ϕ_{CT} 72.50%; $P < 0.001$; Table 4.3). The AMOVA model using peninsular Florida as the delineation explained more of the molecular variance (ϕ_{CT} 80.46%; $P < 0.001$; Table 4.3) than either the Tombigbee/Mobile or Apalachicola/Flint models. However, the model combining the Tombigbee/Mobile and peninsular Florida delineations explained the most

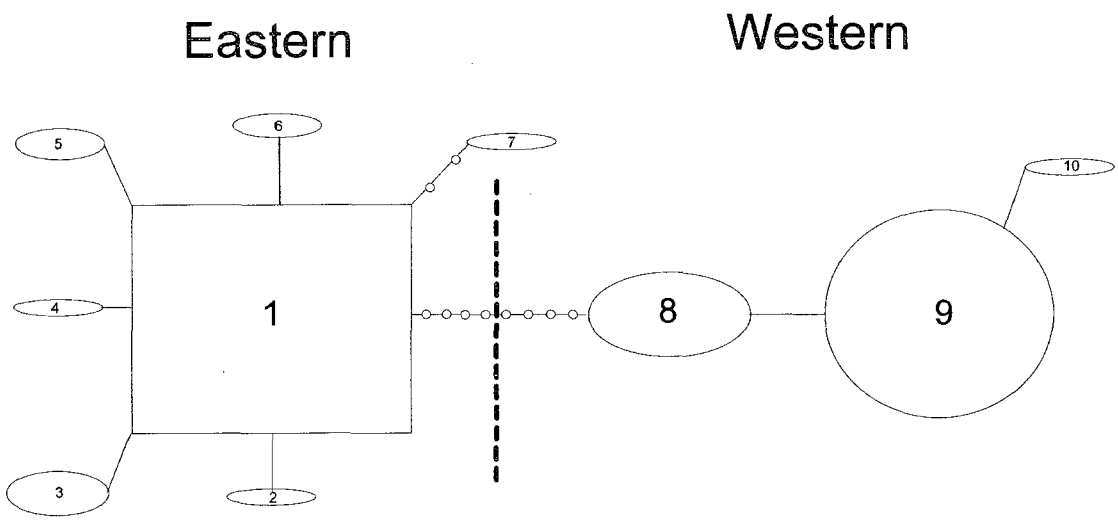

Figure 4.2. Haplotype network showing a modest phylogenetic break between the eastern and western samples. Although the particular shape of each haplotype is not relevant, the different sizes of the shapes represent how many individuals have that particular haplotype.

Table 4.2.

The pairwise uncorrected *p*-distances between each of the 10 unique haplotypes,

	1	2	3	4	5	6	7	8	9
Haplotype 1	-								
Haplotype 2	0.001	-							
Haplotype 3	0.001	0.003	-						
Haplotype 4	0.014	0.015	0.01404	-					
Haplotype 5	0.014	0.015	0.01404	0.003	-				
Haplotype 6	0.014	0.015	0.01404	0.003	0.003	-			
Haplotype 7	0.014	0.015	0.01404	0.003	0.003	0.003	-		
Haplotype 8	0.013	0.014	0.01264	0.001	0.001	0.001	0.001	-	
Haplotype 9	0.014	0.015	0.01545	0.006	0.006	0.006	0.006	0.004	-
Haplotype 10	0.014	0.015	0.01404	0.003	0.003	0.003	0.003	0.001	0.006

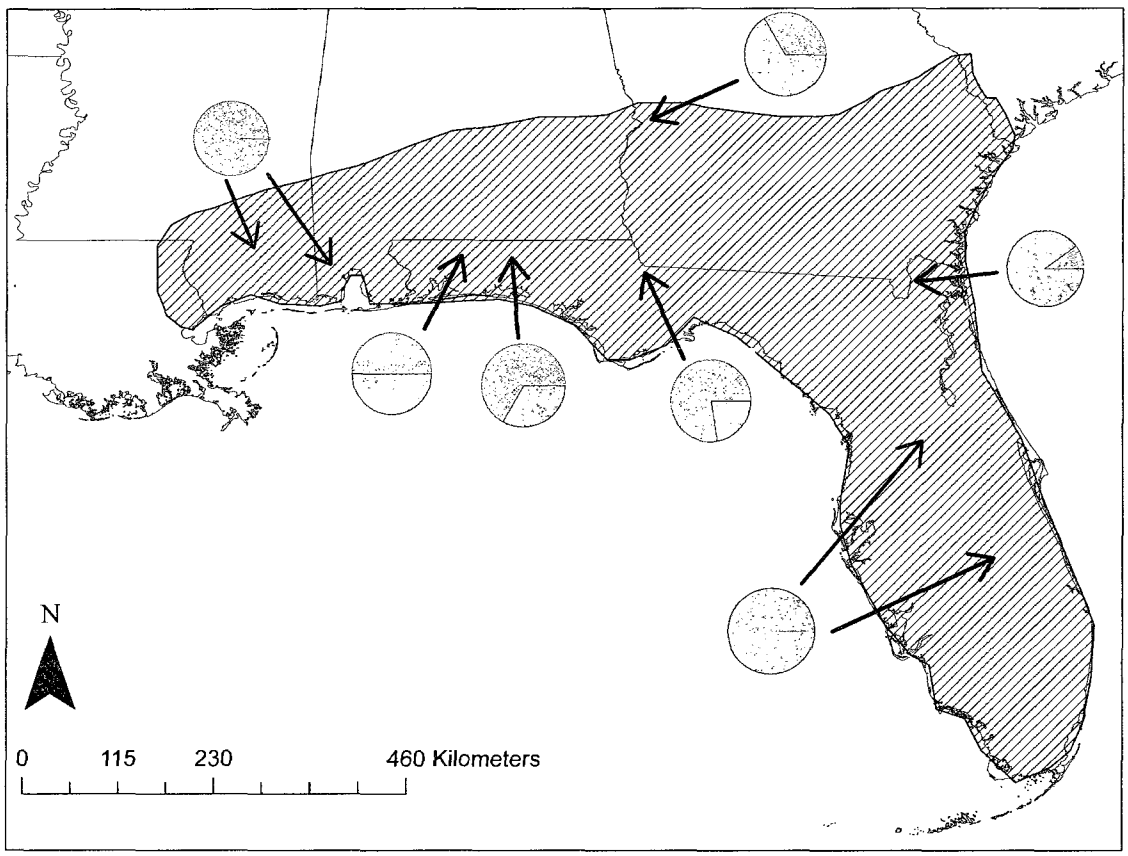

Figure 4.3. A map with the frequency (pie charts) of eastern (dark) and western (light) haplotypes at selected sites showing the western and peninsular Florida regions having only western and eastern haplotypes, respectively. The region consisting of the Florida panhandle and Georgia constitutes an area where western and peninsular Florida haplotypes were shared.

Table 4.3.

The results of the four AMOVA models and the two SAMOVA runs showing the percentage of the molecular variance being explained by among the groups (ϕ_{CT}), among populations within groups (ϕ_{SC}), and within populations (ϕ_{ST}). The letter beside the model indicates the particular portioning of samples used in that model.

Models/K		Among Groups	Among Populations within Groups	Within Populations
AMOVA				
1. Tombigbee/Mobile	a*	74.56	14.39	11.05
2. Apalachicola/Flint	b‡	72.50	15.60	11.90
3. Peninsular Florida	c†	80.46	8.24	11.30
Combined (1 & 3)		82.15	5.00	12.86
SAMOVA				
2		83.82	5.23	10.86
3		84.43	4.55	11.02

*a = west: site numbers 1–10; east = 11–26
‡b = west: site numbers 1–12, 23; east = 13–22, 24–26
†c = west: site numbers 1–13, 23; east = 14–22, 24–26

of the molecular variance (ϕ_{CT} 82.15%; $P < 0.000$; Table 4.3). Finally, the SAMOVA revealed a modest difference between a K value of 2 and 3 (Table 4.3), and this difference was caused by SAMOVA placing Indian River Co., FL into an individual assemblage using a K of 3 (Figure 4.4). For both values of K, SAMOVA partitioned the samples into east and west groupings. However, there was not a definitive geographic barrier for these groups due to overlap between the two groups that occurred in panhandle Florida and Georgia (Figure 4.4).

Discussion

Even with limited sampling in the western portion of the range, Osentoski and Lamb (1995) uncovered three assemblages (i.e., western, eastern, central Florida). Similarly, I found some support for three assemblages but with different phylogenetic breaks and delineations of the assemblages. Although Osentoski and Lamb (1995) found a phlyogenetic break between the eastern and western populations, this break was not congruent with the proposed delineation of the western DPS by USFWS. Their western assemblage included parts of western Georgia and the entire Florida panhandle, both of which extend outside of the current ESA listed area, with the Apalachicola drainage as a geographic barrier separating the two.

In contrast, my data supported the USFWS's delineation of the DPS and the Tombigbee and Mobile Rivers as the geographic barrier in both the AMOVA and SAMOVA (K= 2–3). However similar to Osentoski and Lamb (1995), western haplotypes extended east of the USFWS's DPS into the panhandle of Florida and Georgia where these samples were a collection of western and eastern haplotypes. Since *G. polyphemus* is commonly translocated (Seigel and Dodd, 2000), Osentoski and Lamb (1995) have suggested that aberrant haplotypes in a region or site are artifacts of.

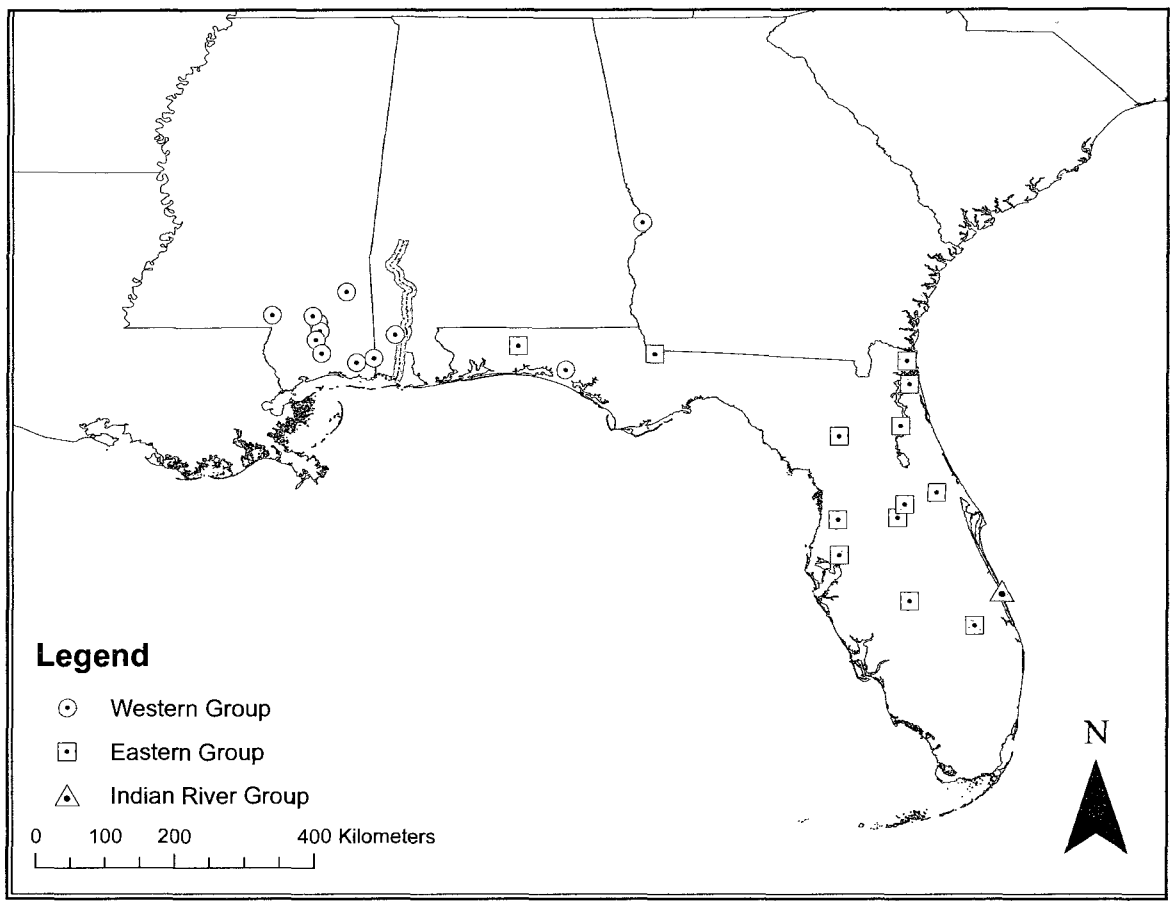

Figure 4.4. SAMOVA (K = 3) partitioned *G. polyphemus* sites into eastern and western regions with overlap in the Florida panhandle; while the third region consisted of only one site (Indian River Co., FL) in peninsular Florida.

translocations. However given the number of shared haplotypes between the assemblages found in this study, this phenomenon was highly unlikely an artifact of translocations Alternatively, this pattern of shared haplotypes could suggest that these barriers were not impermeable to historical gene flow. However, the haplotype network partitioned the samples in an east/west fashion with a modest phylogenetic break (i.e., eight nucleotide substitutions; 1.4% sequence divergence) suggesting that western and eastern regions were isolated for an extended period of time, probably during the Pleistocene, followed by mixture of haplotypes.

The AMOVA model combining both the Tombigbee/Mobile and peninsular Florida delineations explained more of the molecular variance than the other models. The peninsular Florida delineation consisted only of sites with eastern haplotypes. Interestingly, this region also explained more of the molecular variance within the groups (i.e., ϕ_{SC}; Table 4.2). Although the SAMOVA (K = 3) did not recover the peninsular Florida delineation, a site consisting of only one unique haplotype in central Florida (Indian River Co., FL) was recovered in the analysis. This structure is not unexpected since other *G. polyphemus* studies (Osentoski and Lamb, 1995; Schwartz and Karl, 2005) and other species within this region (Clark et al., 1999; Branch et al., 2003) exhibit, in some case, extensive genetic structure and genetic diversity. The fluctuation of sea levels brought on by the glacial cycle has been postulated as the mechanism for creating this structure (Osentoski and Lamb, 1995; Schwartz and Karl, 2005). Peninsular Florida consists of two systems of xeric uplands, which do not create a continuous habitat but a mosaic of isolated habitats. This unique geography, accompanied by high sea levels, could have caused multiple vicariance events (i.e., isolation) on these ridges, and the subsequent draw down could facilitate dispersal events into the surrounding region. This

recurring phenomenon throughout the Pliocene and Pleistocene could have resulted in unique and complex genetic structuring. Although other studies (Osentoski and Lamb, 1995; Schwartz and Karl, 2005; Clark et al., 1999; Branch et al., 2003) have shown a higher degree of structure within this region, I found a lesser degree of genetic structuring. For example, this region had only one distinct site (i.e., Indian River) with several other sites having unique haplotypes but also shared haplotypes with other sites.

Since populations of *G. polyphemus* continue to decline throughout its range (McCoy and Mushinsky, 1992; Mushinsky et al., 2006; Waddle et al., 2006; Hammond, 2009) and more disconcerting on protected lands (McCoy et al., 2006), effective measures must be taken to conserve the genetic integrity within the species. USFWS has taken the initial step by federally listing the genetically distinct populations west of the Tombigbee and Mobile Rivers. However the combination of this study and other molecular studies (Osentoski and Lamb, 1995; Schwartz and Karl, 2005) showing the uniqueness and high amounts of genetic diversity in peninsular Florida, indicated that any conservation strategy for *G. polyphemus* should aim to conserve the genetic integrity of this region. The failure to preserve this region's genetic diversity could result in the loss of a large portion of diversity within the species as a whole.

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APPENDIX

SPECIMENS EXAMINED

Graptemys oculifera (N = 55). — MISSISSIPPI: *Marion Co.*: Pearl River, MMNS 3280-3281, 3731-3733, 3752-3756, 4023, 7686; *Neshoba Co.*, Pearl River, MMNS 3874; *Lawrence Co.*, Pearl River, MMNS 3995, TU 14867, 21438-21450, 21645-21647, 21726-21733, 21733-21736, 21827; *Rankin Co.*, Pearl River, MMNS 4000-4003, 8393, 15816; *Hinds Co.*, Pearl River, MMNS 4005; *Madison Co.*, Pearl River, MMNS 5639, 5640; *Leake Co.*, Pearl River, MMNS 7681-7684, TU 21816; *Washington Pa.*, Pearl River, TU 21885; *St. Tammany Pa.*, Pearl River, TU 29769.

Graptemys flavimaculata (N = 93). — MISSISSIPPI: *Perry Co.*, Tallahala Creek, MMNS 1022, 1023; 1072, 1081, 1082, 1121; *Covington Co.*, Leaf River, MMNS 1026; *Greene Co.*, Chickasawhay River, MMNS 1030, 5696-5699; *George Co.*, Pascagoula River, MMNS 1039, 1040, 1043, 1045, 1052-1054, 1073-1075, 1077, 1087-1093, 1122, 4014, 4015, TU 14752, 14756-14760, 14762-14766, 14774, 14776, 14779-14785, 14799, 14804, 14806-14809, 14812, 14818, 14821, 14822, 14829, 14832, 14845, 14850, 14857, 14858, 14862, 14865, 14866, 148665, 14868-14871, 14873, 14873, 149221, 16546.1, 16546.3; *Forrest Co.*, Leaf River, MMNS 1057; *Jackson Co.*, Pascagoula River, MMNS 1066, 1105, 1114, 1117, 5641; *Jones Co.*, Eastabuchie River, MMNS 3728, MMNS 4012; *Clarke Co.*, Chickasawhay River, MMNS 10754; no specific locality, MMNS 1096; no museum voucher number or specific locality, TU.